UNIVERSITY OF
BIRMINGHAM

INHIBITION OF THE PLATELET GLYCOPROTEIN VI RECEPTOR AS A POTENTIAL TREATMENT FOR ACUTE CORONARY SYNDROMES

by

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A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

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MAY 2021

Abstract

Aspirin and a $P2Y_{12}$ inhibitor, such as ticagrelor, are routine treatments for myocardial infarction. However, these drugs are not always sufficient for heavy coronary thrombus burden during ST-elevation myocardial infarction (STEMI). More potent antiplatelet drugs (glycoprotein IIb/IIIa inhibitors) may help in this setting but are limited by excessive bleeding. As glycoprotein VI (GPVI) plays major roles in thrombosis, this thesis aimed to investigate whether a novel platelet GPVI inhibitor, glenzocimab (Acticor Biotech), provides additional antithrombotic effects when combined with aspirin and ticagrelor. Glenzocimab showed a amplified antiplatelet effect on collagen and atherosclerotic plaque-induced platelet aggregation without showing off-target effects on platelet activation mediated by non-GPVI agonists. Glenzocimab and eptifibatide (a glycoprotein IIb/IIIa antagonist) both exhibited similar inhibitory effects on collagen- and atherosclerotic plaque-induced platelet aggregation when used in combination with aspirin and ticagrelor. Glenzocimab also reduced fibrin-stimulated platelet aggregation more than aspirin and ticagrelor. Glenzocimab provided additional antiplatelet effects on platelet aggregation and adhesion and thrombus formation in blood sampled from patients with acute coronary syndromes treated with aspirin and ticagrelor. Glenzocimab blocked platelet procoagulant activity and reduced tissue factor-mediated peak thrombin generation. Glenzocimab did not affect early phases of coagulation (initiation, formation, and strength), which were greatly affected by eptifibatide, but it exerted some effects on clot lysis (late phases of coagulation) as assessed by ROTEM. This is the first work to show that the addition of a novel GPVI inhibitor, glenzocimab, to aspirin and ticagrelor provides greater inhibition of multiple critical mechanisms of arterial thrombosis. This is a promising strategy for the further development of treatment for STEMI due to the minimal role of GPVI in haemostasis.

PUBLICATIONS ARISING FROM THIS WORK

Fawaz O. Alenazy, Mark R. Thomas. Novel antiplatelet targets in the treatment of acute coronary syndromes. Platelets. 2021 Jan 2;32(1):15-28. doi: 10.1080/09537104.2020.1763731. Epub 2020 Jun 12.

Fawaz O. Alenazy[1], Maan H. Harbi[1], Dean P. Kavanagh[1], Joshua Price[2], Paul Brady[1,3], Oscar Hargreaves[1], Paul Harrison[2], Alex Slater[2], Alok Tiwari[4], Phillip L. R. Nicolson[1], Derek L. Connolly[3], Paulus Kirchhof[1], Neena Kalia[1], Martine Jandrot-Perrus[5], Pierre H. Mangin[6], Steve P. Watson[1,7], Mark R. Thomas[1,3,8]. GPVI inhibition by glenzocimab synergistically inhibits atherosclerotic plaque-induced platelet activation when combined with conventional dual antiplatelet therapy. In preparation.

PUBLICATIONS ARISING FROM OTHER WORK

Green, HLH, Zuidscherwoude, M, **Fawaz O. Alenazy**, Smith, CW, Bender, M, Thomas, SG. SMIFH2 inhibition of platelets demonstrates a critical role for formin proteins in platelet cytoskeletal dynamics. J Thromb Haemost. 2020; 18: 955– 967.

Alexandre Slater, Ying Di, Joanne C Clark, Natalie Jasmin Jooss, Eleyna M Martin, **Fawaz O. Alenazy**, Mark R Thomas, Robert AS Ariëns, Andrew B. Herr, Natalie S Poulter, Jonas Emsley, Stephen P. Watson; Structural characterisation of a novel GPVI nanobody-complex reveals a biologically active domain-swapped GPVI dimer. *Blood* 2021; blood.2020009440.

Acknowledgements

In the name of Allah, Most gracious, Most merciful. I thank God Almighty, and I praise Him for being able to complete this work. I would like to thank my principal supervisor Dr Mark Thomas for his help, advice and support during weekly meetings, planning for lab experiments, and statistical analysis. I want to thank my secondary supervisor Prof Steve Watson for his meaningful directions that helped to guide the project.

Also, I would like to extend my thanks to all Birmingham platelet group members especially those who taught me techniques in the lab. Special thanks to Stephanie Watson and Lourdes Quintanilla for teaching me some lab techniques. Thanks to Maan Harbi and Beata for ordering reagents and other materials. Also, I would like to thank Joshu Price from the research laboratory at the Queen Elizabeth Hospital for teaching me how to use ROTEM and CAT instruments. I would also like to thank Paul Brady, Oscar Hargreaves, and City Hospital nurses who have helped in patient requirement. Thanks to Maan again for helping me in patients' samples processing. Thanks to John from Scribbr's professional proofreading services for proofreading spelling and grammar in my thesis.

I would like to extend my gratitude to my sponsor, Aljouf university, for granting me a PhD scholarship and the Saudi cultural attaché in London for facilitating all my academic and financial issues during studying in the UK.

Finally, Special thanks also to my parents for their endless support and encouragement. Deepest thanks must go to my dearest wife for her care, support, standing alone and taking care of our children during the past 4 years.

Dedication

This thesis work is dedicated to my parents, to my wife, and to our children.

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List of Abbreviations

AA	Arachidonic acid
ADP	Adenosine diphosphate
ACD	Acid citrate dextrose
ACS	Acute coronary syndrome
ATP	Adenosine triphosphate
ASA	Acetylsalicylic acid
Btk	Bruton's tyrosine kinase
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAT	Calibrated automated thrombogram
CFT	Clot formation time
CRP	Collagen-related peptide
CT	Clotting time
Cvx	Convulxin
DAG	Diacylglycerol-1,2
DAPT	Dual antiplatelet therapy
DMSO	Dimethyl sulfoxide
$DiOC_6$	3,3′-Dihexyloxacarbocyanine,iodide
ELISA	Enzyme-linked immunosorbent assay
Epti	Eptifibatide
ETP	Endogenous thrombin potential
Fab	Fragment antigen binding
FcRγ-chain	Fragment crystallizable receptor γ-chain
Glenz	Glenzocimab
GPO	Glycine-Proline-Hydroxyproline
GPIa/IIa	Glycoprotein Ia/IIa
GPIIb /IIIa	Glycoprotein IIb/IIIa
GPVI	Glycoprotein VI

HRP	Horseradish peroxidase
IgE	Immunoglobulin E
IgSF	Immunoglobulin superfamily
ITAM	Immunoreceptor tyrosine-based activation motif
ITP	Idiopathic thrombocytopenia purpura
kDa	Kilodalton
LAT	Linker for activation of T lymphocytes
LI	Lysis index
LTA	light transmission aggregometry
mAb	Monoclonal antibody
MCF	Maximum clot firmness
MEA	Multiple electrode aggregometry
ML	Maximum lysis
PAGE	Polyacrylamide gel electrophoresis
PAR1	Protease-activated receptor 1
PAR4	Protease-activated receptor 4
PBS	Phosphate-buffered saline
P62	Polypeptide 62 kDa
PCI	Percutaneous coronary intervention
PDE3	Phosphodiesterase 3
PDI	Protein disulfide isomerase
PI-3 kinase	Phosphatidylinositol 3–kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKD	Protein Kinase D
PLCγ2	Phospholipase C-gamma2
PRD	Proline-rich domain
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PTP	Protein tyrosine phosphatase
pY	Phosphotyrosine
ROTEM	Rotational thromboelastometry
scFvs	Single Chain variable fragments

sdAbs	Single Domain Antibodies
SEM	Standard error of the mean
SFKs	Src family kinases
SLP-76	Src homology 2 domain-containing leucocyte protein of 76 kDa
Syk	Spleen tyrosine kinase
TCR	T cell receptors
Tic	Ticagrelor
TP	Thromboxane prostanoid
TxA_2	Thromboxane A2
vWf	Von Willebrand factor
WB	Western blot
WP	Washed platelets
Y	Tyrosine

CHAPTER 1 : GENERAL INTRODUCTION

1.1 Overview of cardiovascular diseases

Cardiovascular diseases (CVDs) encompass a variety of diseases that affect the blood circularity system particularly heart and the vasculature. CVDs are the leading cause of death for non-infectious diseases in Europe, accounting for 50% of mortality (Nichols et al., 2014). CVDs account for ≈ 32% of deaths worldwide (Benjamin et al., 2017). Despite the recent improvements in the diagnosis and management of cardiovascular diseases, CVDs are still the leading cause of premature mortality and a major contributor of disability. CVDs represent a major challenge for public health sectors due to high economic burden associated with health care and clinical programs that established for treatment and prevention of CVDs (Roth et al., 2020).

CVDs are different in the terms of aetiology, incidence, pathogenesis, clinical presentations, and treatment. CVDs include atherosclerosis with its subtypes (coronary and cerebral) and its complications myocardial infarction and ischemic stroke, peripheral artery disease, heart failure, atrial fibrillation, arrhythmia, rheumatic heart diseases, congenital heart diseases, deep vein thrombosis, and venous thromboembolism (Thiriet, 2019).

1.2 Thrombotic cardiovascular diseases

Thrombotic cardiovascular diseases are the most common form of cardiovascular diseases. The most prevalent forms of thrombotic cardiovascular diseases are ischemic heart diseases, ischemic stroke, and deep vein thrombosis (Raskob et al., 2014). Thrombotic cardiovascular diseases result from an obstruction or a narrowing in circulatory system due to either atherosclerotic plaque build-up inside the wall of blood vessel or thrombus formation that results from plaque rupture or erosion, leading obstruction of blood flow inside the lumen of blood vessels. Thrombotic cardiovascular diseases can develop in the absence of plaque

formation especially in venous thrombosis which largely relies on coagulation activation (Mackman et al., 2007).

1.3 Haemostasis versus thrombosis

The term of thrombosis refers to unwanted activation of platelets and coagulation systems which result in serous pathological conditions that need treatment, close medical observation, and lifestyle modifications. In other term, thrombosis develops in pathological conditions when haemostasis is tremendously activated in the absence of bleeding (Rasche, 2001). On the other hand, haemostasis refers to a physiological process that keep blood smoothly flowing inside in the vasculature system and prevents any blood leakage when the integrity of blood vessel is breached. Haemostasis prevents blood loss from blood vessel by vasoconstriction, platelet activation (primary haemostasis), coagulation activation (secondary haemostasis), and fibrinolysis (Verhamme and Hoylaerts, 2009). Thrombosis is termed an arterial thrombosis if thrombi developed in arteries and termed venous thrombosis if thrombi developed in the veins. The arterial thrombosis is different from venous thrombosis in several aspects (Chernysh et al., 2020, Koupenova et al., 2017).

1.4 Arterial thrombosis versus venous thrombosis

Arterial thrombosis is highly dependent on platelets aggregation and platelets thrombus formation that are mediated by collagen other thrombogenic materials exposed from plaque rupture or erosion (Fernández-Ortiz et al., 1994). Arterial thrombosis develops at high shear rates that increase platelet stimulation as platelet integrin receptors and vWF are fully functional at high flow forces (Nesbitt et al., 2009, Maxwell et al., 2007). The clots result from arterial thrombosis are white in colour and consist of platelets (31%), fibrin (43%), and red blood cells (17%) (Chernysh et al., 2020). Arterial thrombosis causes myocardial infarction and ischemic stroke. Because of the main role of platelets in arterial thrombosis, antiplatelet therapy is the

corner stone treatment in arteriothrombotic diseases such as acute coronary syndromes and ischemic stroke. On the other hand, venous thrombosis is highly dependent on coagulation systems activation mainly on tissue factor induced thrombin generation (Manly et al., 2011). Venous thrombosis develops at low shear rates (Sakariassen et al., 2015). The main risk factors for vein thrombosis are immobility, cancer, inflammation, obesity, hormone therapy ,and homocysteine elevation (Cushman, 2007). The clots that result from venous thrombosis are red in colour and consist mainly of red blood cells (63%), and fibrin (35%) with few platelets (0.4%) (Chernysh et al., 2020). Venous thrombosis causes deep vein thrombosis and pulmonary embolism (Cushman, 2007). As coagulation systems have a major role in the development of venous thrombosis, anticoagulant therapy is the mainstay treatment of deep vein thrombosis (Kearon and Akl, 2014).

1.5 Overview of platelets

1.5.1 Platelet production

Platelets, the smallest anucleate discoid-shaped blood cells (2–4 μm), are produced from megakaryocytes in the bone marrow in a process known as thrombopoiesis, which is regulated by thrombopoietin (TPO). TPO is a glycoprotein hormone that is mainly produced in the liver with a small amount produced in the kidney and bone marrow; its deficiency leads to thrombocytopenia ;therefore, TPO mimetics are clinically used to correct thrombocytopenia caused by TPO deficiency (Hoffmeister et al., 2014). Megakaryocytes undergo endomitosis that is promoted by TPO and form multilobed nucleus and polyploids in the cytoplasm (Nagata et al., 1997). The cytoplasmic remodelling process in the megakaryocytes results proplatelet formation (Richardson et al., 2005). Proplatelets elongate and undergo continuous fission associated with cytoskeletal changes that eventually develop into platelets (Thon et al., 2010). The formed platelets are released into the blood circulatory system via bone marrow sinuses,

have a life-span of 7–10 days, and circulate at a concentration of 150–450 × 10^9 per litre of blood (Harker et al., 2000, Ross et al., 1988). Aged platelets are characterised by structural changes in their surfaces such as phosphatidylserine upregulation and deglycosylation of membrane glycoproteins that signal their clearance from the circulation by the splenic macrophages and liver Kupffer cells (Hartwig, 2017).

1.5.2 Platelet structure

Platelets consist of four structural zones: peripheral zone, sol-gel zone, organelle zone, and membrane systems (White, 1979). The peripheral zone is formed from a covering (glycocalyx) and cytoplasmic membrane, and it harbours surface receptors involved in adhesion and aggregation. The sol-gel zone contains microtubules, microfilaments, and a cytoskeletal network that support the platelet structure and facilitate shape change during platelet activation. The organelle zone contains secretory granules (α-granules and dense granules) and lysosomes that play essential roles in haemostasis and thrombosis and additional roles in inflammation. In addition to secretory vesicles, the organelle zone contains mitochondria, glycosomes, and tubular inclusions. Two membrane systems are present in platelets, the open canalicular system and the dense tubular system. The open canalicular system forms a series of conduits that extend from the platelet surface membrane to its interior, providing an entry route for external substances and passage for the release of granule contents. The dense tubular system in the platelet cytoplasm serves as a storage site for ionised calcium (White, 1979, Gremmel et al., 2016). The principal role of platelets is to maintain haemostasis by preventing blood leakage when blood vessel walls are breached. Platelets also play versatile roles beyond haemostasis; for example, in thrombosis, cancer metastasis, angiogenesis, inflammation, and host defence (Ghoshal and Bhattacharyya, 2014).

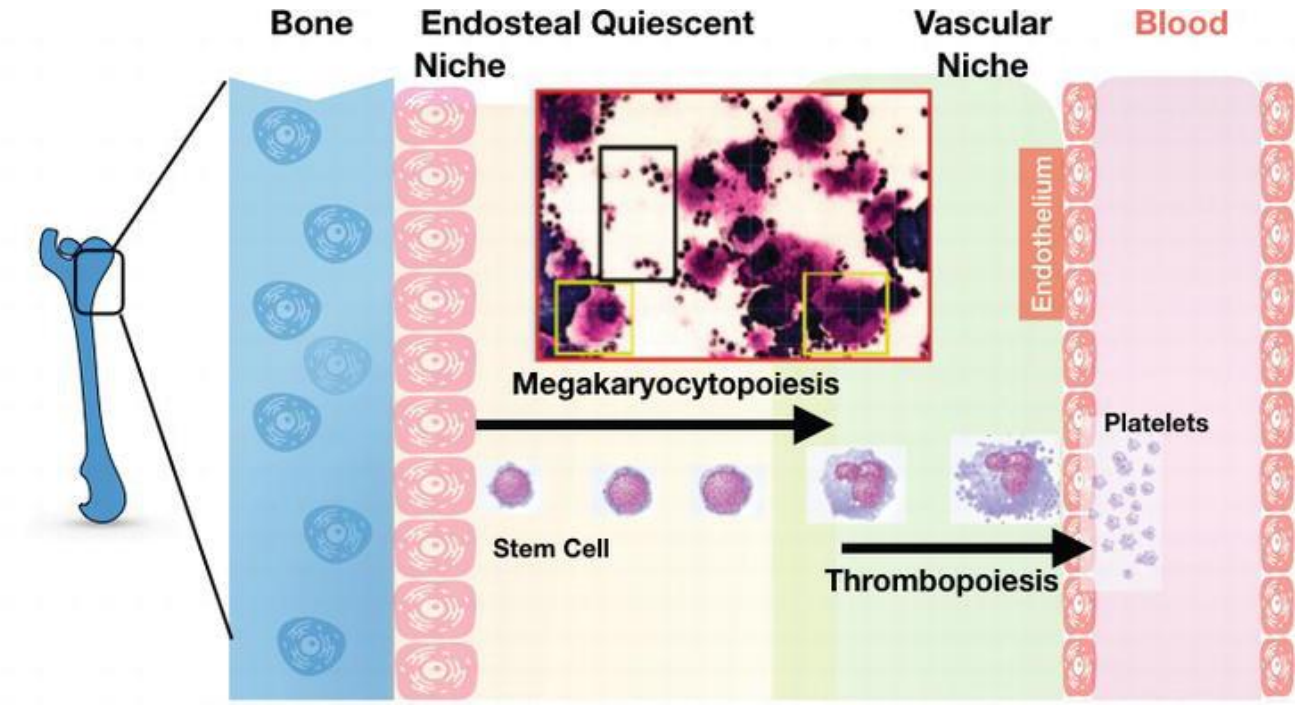


Figure 1.1 Platelets formation process. Platelets are produced from megakaryocytes in the bone marrow in process called thrombopoiesis which is controlled by thrombopoietin (Twomey et al., 2018).

1.6 Haemostatic function of platelets

Haemostasis is a multifactorial process that can be divided into three steps: primary haemostasis, in which platelets play their principal role; secondary haemostasis, during which coagulation factors form a dense fibrin clot; and fibrinolysis, which refers to the removal of the clot after the injured blood vessel is healed. Platelets achieve their haemostatic function by the formation of a platelet plug at the site of a blood vessel injury. The plug is usually fragile and easily dislodged but is strengthened during secondary haemostasis when it is crosslinked with fibrin, forming a more stable plug (Stassen et al., 2004). Platelets have multiple functions during the formation of a stable clot, including adhesion, activation, shape change, secretion, and aggregation.

1.6.1 Tethering, rolling, and initial adhesion

When a blood vessel is injured, many physiological events are initiated to arrest blood loss via the injury site. Vasoconstriction reduces blood flow to the damaged vasculature. The damaged vascular sub-endothelium exposes thrombogenic matrix proteins such as collagen fibrils, von Willebrand factor (vWF), and tissue factor. Under high shear rates, platelets start rolling and tethering on multimeric vWF immobilised on the exposed collagen via the membrane adhesion receptor glycoprotein (GP) Ibα (Sakariassen et al., 1979, Coller et al., 1983). The association between GPIbα and vWF is weak and characterised by fast association and dissociation rates, causing reversible platelet adhesion so that platelets continue rolling the vascular endothelium in the direction of the blood flow (Miura et al., 2000, Donadelli et al., 2006).

1.6.2 Firm adhesion

Despite the association between vWF and GPIbα being rapid and unstable, it is sufficient to stimulate actin polymerisation and cytoskeletal reorganisation, generating procoagulant microparticles and inducing sustained platelet activation that eventually results in irreversible

adhesion (Yuan et al., 1999, Reininger et al., 2006). The vWF–GPIbα association reduces platelet velocity and allows proximal interaction between collagen and its receptors, GPIa/IIa (integrin α2β1) and GPVI. Signal transduction via vWF–GPIbα interaction activates GPIa/IIa that binds directly to exposed collagen and GPIIb/IIIa (integrin αIIbβ3) that binds to vWF itself and fibrinogen (Kasirer-Friede et al., 2004, Cruz et al., 2005). As a central signalling platelet receptor for collagen, GPVI interaction with collagen fibrils causes extensive inside-out activation of GPIa/IIa and GPIIb/IIIa that arrest platelets, leading to firm platelet adhesion (Pugh et al., 2017).

1.6.3 Spreading and degranulation

Once stable platelet adhesion on its ligand takes place, the platelet transforms from a small discoid shape into a large fully spread platelet. Platelet spreading is mediated by actin polymerisation and tubulin cytoskeleton reorganisations. These processes lead to the emergence of cellular protrusions, mainly filopodia, which make a connection between platelets, and lamellipodia, which aid platelets to cover the wound (Sandmann and Köster, 2016). Degranulation of platelet granules leads to the release prothrombotic substances such as adenosine diphosphate (ADP) and serotonin, adhesion molecules such as vWF, and fibrinogen and exposes P-selectin, which mediates the interaction between leukocytes and platelets. Moreover, degranulation liberates procoagulant substances such as ionised calcium and coagulation factors including FV and FXI and polyphosphate (Golebiewska and Poole, 2015a). GPIIb/IIIa is expressed on the granules and believed to further boost platelet aggregation (Blair and Flaumenhaft, 2009a). α-granules are the largest and the most abundant platelet granules; it is estimated there are 50–80 α-granules per platelet (Fitch-Tewfik and Flaumenhaft, 2013). α-granules store and contain proteins membrane (P-selectin, GPIIb/IIIa, and GPIα), and adhesion proteins (fibrinogen and vWF), and coagulation factors and fibrinolytic proteins (FV, FXI, and

plasminogen) (Golebiewska and Poole, 2015b, Fitch-Tewfik and Flaumenhaft, 2013). Dense granules are smaller in size and fewer in number than α-granules (150nm; 3-8/platelet) (Fitch-Tewfik and Flaumenhaft, 2013). Dense granules contain ionised cations (Ca^{+2} and Mg^{+2}) and active amines (histamine and serotonin), nucleotides (ADP and ATP) and polyphosphate (Fitch-Tewfik and Flaumenhaft, 2013, Golebiewska and Poole, 2015b).

1.6.4 Aggregation and thrombus formation

In blood flowing under high shear rates, vWF mediates platelets adhesion on exposed collagen fibres via GPIb and GPIIb/IIIa (Wu et al., 2000). Activated GPIIb/IIIa binds to fibrinogen circulating in plasma in a process that forms bridges between platelets and leads to the formation of a monolayer of platelet aggregate at the site of vascular injury. The secondary soluble and potent platelet mediators ADP and thromboxane A2 (TxA_2) activate and attract circulating platelets to the newly formed platelet aggregate, thereby significantly contributing to platelet thrombus development. GPVI is principally involved in thrombus formation by functioning a central signalling receptor for collagen (Dubois et al., 2004). GPVI further propagates thrombus growth and stability by interacting with fibrin (Alshehri et al., 2015). vWF enhances thrombus formation by mediating platelets and fibrin interaction via GPIb and GPIIb/IIIa (Hantgan et al., 1990). Thrombin also increases thrombus formation and growth by activating protease-activated receptor1 (PAR1) and protease-activated receptor4 (PAR4).

1.6.5 Priming coagulation and amplifying thrombin generation

The role of Platelets extends beyond formation platelet plugs (primary haemostasis) to priming blood coagulation and amplifying thrombin generation (secondary haemostasis). A special population of platelets involved in this process is known as procoagulant platelets. Procoagulant platelets expose phosphatidylserine, providing a negative surface that enhances coagulation and thrombin generation (Agbani and Poole, 2017). Phosphatidylserine is exposed from the inner

leaflet of the plasma membrane to the outer leaflet of the plasma membrane under the influence of intracellular Ca^{2+} elevation and activation of phospholipid scramblase (Keuren et al., 2005, Stout et al., 1997). Exposed phosphatidylserine provides assembly site for the intrinsic tenase complex (FVIIIa, FIXa, and FX) and prothrombinase complex (FVa, FXa, and prothrombin), thereby leading to the initiation of thrombin generation and propagation of coagulation process (Reddy and Rand, 2020a). Thrombin, a serine proteinase produced from the coagulation pathway, further amplifies thrombus formation by activating more platelets via PAR1 and PAR4 receptors. Furthermore, thrombin cleaves fibrinogen into fibrin. FXIII-crosslinked polymerised fibrin reinforces and stabilises the platelet thrombus by surrounding it with meshwork (Crawley et al., 2007).

1.6.6 Clot retraction

Clot retraction is an important haemostatic platelet function in which fibrin meshes undergo a contraction process that results in a smaller clot volume and extrusion of fluid. The contracted fibrin clot brings the edges of the damaged tissue together and secures the haemostatic plug. Platelets mediate fibrin clot retraction via GPIIb/IIIa outside-in signalling (Law et al., 1999). This GPIIb/IIIa signalling mediates platelet contractile activity via actin–myosin interaction that mediates platelet cytoskeleton reorganisation and contracts platelet pseudopodia attached to the fibrin (Cohen et al., 1982). Actin is attached and anchored to platelet membrane, facing towards myosin filaments that are localised in the centre of activated platelet (Cohen, 1979). Long pseudopodia from different platelets interlock with each others and compress polymerised fibrin clot; this contractile platelet activity is mediated by actin–myosin association (Cohen, 1979). Actin–myosin interaction is dependent on the phosphorylation of the myosin II light chain kinase (MLCK) induced by outside-in signalling of GPIIb/IIIa (Johnson et al., 2007). Ras homolog family member A (RhoA)/ Rho kinase (ROCK), and rac family small GTPase 1 (Rac-

1) are largely involved in mediating the myosin II light chain activation (Egot et al., 2013). Sarcoma (Src) family kinases are partially involved in mediating clot retraction by activating phospholipase C gamma 2 (PLCγ2) downstream of GPIIb/IIIa during outside-in signalling (Suzuki-Inoue et al., 2007).

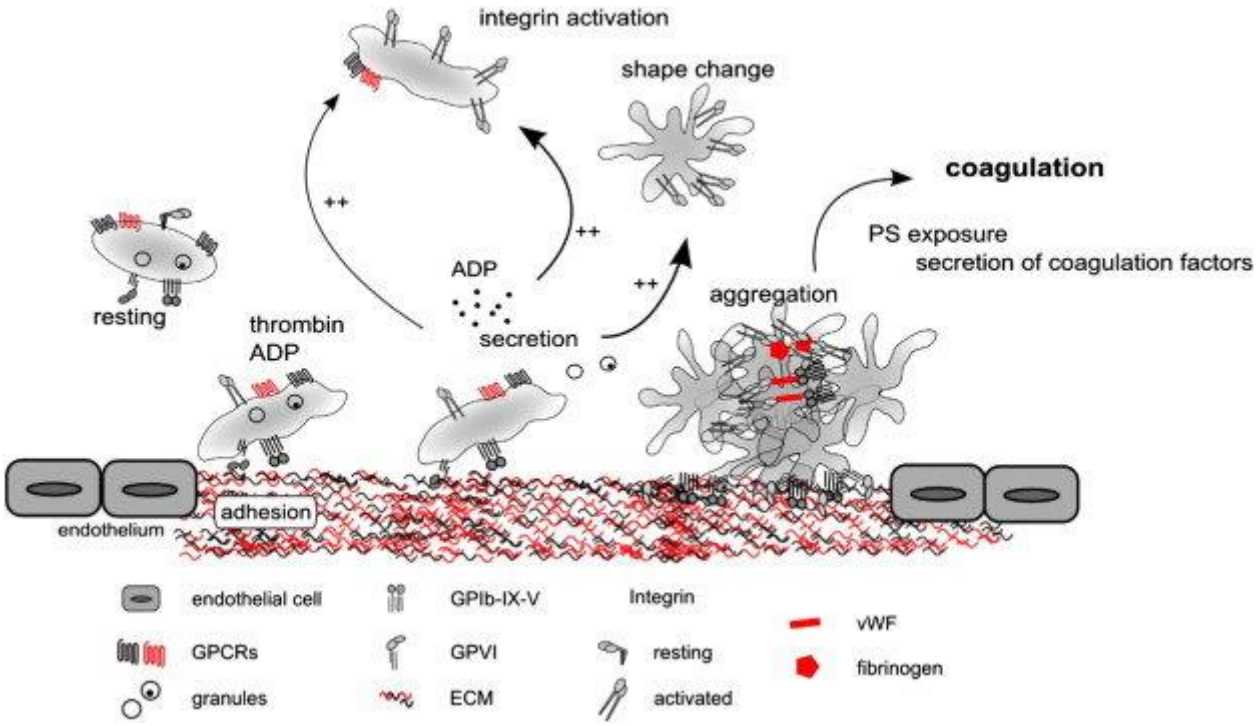


Figure 1.2 Platelet activation process. Platelets undergo multiple activation phases during the formation of a stable clot, including adhesion, shape change, secretion, aggregation, and procoagulant platelet formation (Golebiewska and Poole, 2015b).

1.7 Inflammatory role of platelets

The role of platelets in inflammation and immunity has been well established using animal models of inflammation and clinical observations in patients with thromboinflammatory conditions. Platelets modulate immune responses via secretion of cytokines, chemokines, and adhesion molecules that allow platelets to directly interact with leukocytes. Furthermore, some platelet receptors are directly involved in immune responses. To clarify concepts of immunity and inflammation, immunity refers to a complex process of physical, cellular, and humoral mechanisms that keeps the body protected against pathogens and infections. The immune system is classified into the innate immune system (skin and mucous membranes, phagocytic cells, and complement proteins) and the adaptive immune system (T-lymphocytes, B-lymphocytes, and antibodies) (Vivier and Malissen, 2005). On the other hand, inflammation develops as a result of the immune response and refers to a complex plethora of responses of the innate immune system and the adaptive immune system to microbial stimuli such as bacteria, viruses, parasite, and fungi or pathological stimuli like damage-associated molecular patterns (DAMPs). Inflammation can be either a local inflammation or a systemic inflammation (Mònica Arman, 2015).

1.7.1 Platelet-derived soluble immune mediators

Platelets secrete proinflammatory and immune mediators that play various roles in mediating immune responses (innate immunity) and regulating inflammation (Ali et al., 2015). Several chemokines are released from platelet α-granules exocytosis, including platelet factor 4, microphage inflammatory protein-1α (MIP-1α), and regulated upon activation, normal T-cell expressed and secreted (RANTES) chemokine. These chemokines cause chemotactic activity by attracting neutrophil, monocytes, eosinophil, and macrophages to the site of vascular injury. This chemotactic activity causes pro-inflammatory reactions, atherogenesis, and further

regulates leukocyte proliferation and differentiation (Gleissner et al., 2008, Gerdes et al., 2011). Platelets are a source of interleukin 1 (IL-1), which is an inflammatory cytokine that has a central role in innate immunity. Platelet-derived IL-1 stimulates cytokine production from smooth muscle cells in blood vessels, and platelets also release IL-33, a member of the interleukin-1 family, which has been recently shown to regulate allergic reactions (Loppnow et al., 1998). Platelet CD40L, a member of the tumour necrosis factor receptor family, stimulates endothelial cells to produce chemokines and to express adhesion molecules (Henn et al., 1998). Platelet microbicidal proteins disrupt the cytoplasmic membrane of pathogenic micro-organisms (Yeaman et al., 1998).

1.7.2 Direct platelet–leukocyte interaction

P-selectin, an adhesion molecule translocated on the surface of activated platelets, mediates direct platelet interaction with neutrophils, monocytes, and eosinophils via P-selectin glycoprotein ligand-1 (Palabrica et al., 1992). This cellular interaction produces what is known as a platelet–leukocyte aggregate that is implicated in thrombosis, pro-inflammatory process, and vascular wall changes (Koyama et al., 2003). Platelet CD40L is also involved in mediating platelet–leukocyte interaction and its subsequent inflammatory responses (Lievens et al., 2010). Soluble CD40 ligands have been shown to induce leukocyte activation and facilitate leukocyte adhesion via Mac-1 (Zirlik et al., 2007).

1.7.3 Platelet surface receptors involved in immunoinflammatory reactions

Many platelet receptors that have principal roles in haemostasis and thrombosis are reported to play additional roles in the context of immunity-inflammation interaction. Toll-like receptors (TLR1-9) are group of receptors that recognise pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide and play an essential role in innate immunity (Chen et al., 2020). Platelet TLR-4 mediates platelets -neutrophils interaction during sepsis, forming

neutrophil extracellular traps (NETs) formation and bacteria capturing (Clark et al., 2007). Platelet TLR-2 mediates platelet aggregation by Streptococcus pneumoniae and mediates platelet activation and secretion by Human cytomegalovirus (Keane et al., 2010, Assinger et al., 2014). Formyl peptide receptors (FPR1-3) are G protein-coupled receptors that play a major role in innate immunity and anti-microbial host defence by inducing chemotactic response and recognising PAMPs (Jeong and Bae, 2020). Platelet FPR1 induces platelet chemotaxis and migration in response to towards a gradient of bacterially derived formyl peptides, indicating an essential role for platelets in innate and acquired immunity (Czapiga et al., 2005). Platelet $P2Y_{12}$ receptor contributes to chronic inflammatory pain in the Complete Freund's adjuvant (CFA) model of induced inflammation in mice (Bekő et al., 2017). $P2Y_{12}$ inhibitors decrease systemic inflammation in the human model of lipopolysaccharide-induced inflammation (Thomas et al., 2015). GPVI and thromboxane prostanoid (TP) receptors enhance pro-inflammatory macrophage phenotypes in cutaneous inflammation, whereas deletion of GPVI and TP promotes the production of macrophage phenotypes with high-activity anti-inflammatory cytokines (Pierre et al., 2017). GPVI secures and maintains the vascular integrity of inflamed blood vessels by sealing the vascular breaches induced by leukocyte migration and providing a local haemostatic function (Boulaftali et al., 2018). The activated GPIIb/IIIa directly binds to neutrophil and mediates NETosis under flow conditions (Constantinescu-Bercu et al., 2018).

1.8 Overview of coagulation systems

Coagulation systems secure haemostasis by forming a fibrin mesh that reinforces and stabilises platelet plug at the site of vascular injury. There are three coagulation pathways which are extrinsic pathway, intrinsic pathway, and common pathway. Extrinsic pathway includes Factor VII (FVII) and is activated by tissue factor (TF) that is released from the damaged tissue

(Mackman et al., 2007). TF/FVIIa complex binds and activates factor X (FX). In the presence of factor V(FVa), activated factor X (FXa) converts prothrombin (FII) to thrombin (FIIa) which catalyses the conversion of fibrinogen into fibrin (Mackman et al., 2007). The extrinsic coagulation pathway is controlled by tissue factor pathway Inhibitor (TFPI) that protects against hypercoagulation (Mast, 2016). The extrinsic coagulation pathway is monitored by the traditional prothrombin time (PT) with INR or by EXTEM in the Rotational thromboelastometry (ROTEM) in a more sophisticated way (Balendran et al., 2017). Despite the fact that the extrinsic coagulation pathway is quicker than intrinsic coagulation pathway in response to trauma, TFPI can inhibit TF/FVIIa; therefore, intrinsic pathway is required to boost coagulation process.

The intrinsic coagulation pathway involves FXII, FXI, FIX, and FVIII (Tillman et al., 2018). The first step in the intrinsic coagulation pathway is activation of FXII via contact with a negatively charged surface in the presence of high molecular weight kininogen and prekallikrein (España and Ratnoff, 1983). The activation cascade continues from FXII to FIX, leading to activation of FX and subsequently initiation of the common coagulation cascade. The intrinsic coagulation pathway is monitored by partial thromboplastin time (PTT) or by INTEM in ROTEM (Kim et al., 2013a). The common coagulation pathway includes FI, FII, FV, FX, and FXIII. Activation of FX through either extrinsic or intrinsic pathway signals the start of common coagulation cascade, leading to formation fibrin clot that is crosslinked by FXIII (Palta et al., 2014). In the context of anticoagulation therapy for cardiovascular thrombotic diseases, the coagulation pathways can be targeted by several inhibitors such as warfarin, enoxaparin heparin, dabigtran, apixaban, endoxaban, and rivaroxaban that work on different mechanisms (Paulus et al., 2016).

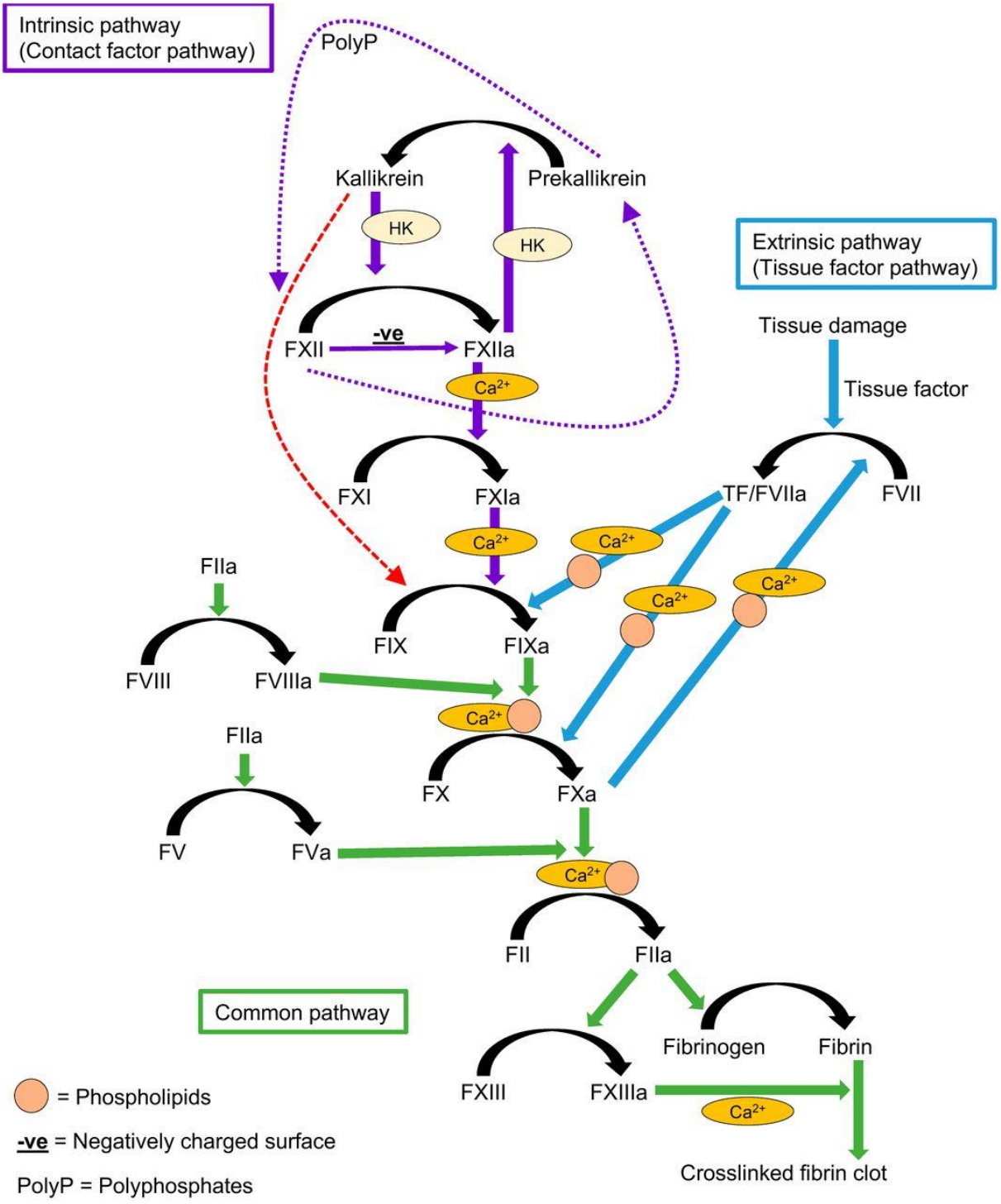


Figure 1.3. Coagulation pathways and their clotting factors. This figure illustrates main coagulation pathways which are extrinsic, intrinsic, and common pathways as well as coagulation factors involved in these pathways (Kearney et al., 2021).

1.9 Overview of acute coronary syndromes

1.9.1 Epidemiology

Coronary artery disease continues to be a leading cause of mortality and morbidity across the world (Roth et al., 2017). The 2019 update on heart disease and stroke statistics form the American heart association (AHA) reported that the overall prevalence of total coronary heart disease (CHD) is 6.7 % in American adults and the prevalence of myocardial infarction (MI) is 3% in the same population, and CHD is the main cause of CVD deaths (43.2%) in the United States. The report also estimated 18.2 million Americans above 20 years of age suffered from coronary heart disease (Benjamin et al., 2019). In the UK, the prevalence of CHD is 3-4% in the UK general population according to the British Heart Foundation epidemiological report released in 2015 (Bhatnagar et al., 2016). CHD presents with different epidemiological trends regarding gender, age, and ethnicity. From gender perspective, CHD has a higher incidence rate in men than women, yet mortality and worst outcomes associated with this disease are more common in women (Maas and Appelman, 2010). CHD has a huge direct economic burden in term of diagnosis and treatment. It is estimated that treatment of CHD particularly prescriptions and operations cost 6.8 billionin in England (Giedrimiene and King, 2017). CHD also indirectly causes financial loss as some patients with CHD lose their ability to work and subsequently reduce their productivity.

1.9.2 Nomenclatures and classifications

Acute coronary syndrome (ACS) encompasses a group of interrelated pathological conditions that are associated with a partial or a complete acute reduction in blood flow to heart muscles via coronary arteries due to an intra-lumen narrowing or blockage that results from a chronic inflammatory condition known as atherosclerosis (Ambrose and Singh, 2015, Torpy et al., 2010). Based on electrocardiogram patterns, ACS is divided into two main categories, ST-

segment elevation myocardial infarction (STEMI) and non-ST-segment elevation ACS (Nikus et al., 2014). In STEMI, blood flow is entirely blocked, and so patients presenting with this form of ACS need immediate treatment. NSTE-ACS is further classified into NSTEMI and unstable angina (US) (Amsterdam et al., 2014b).

1.9.3 Pathophysiology

Coronary arteries provide cardiac muscles with oxygen and nutrients for heart to pump blood constantly and efficiently to the body organs, yet this supply can be deteriorated when coronary arteries reduce or imped blood flowing to the heart due to intra lumen blockage. Blood flow can be partially or completely obstructed due to either development or rupture of the atherosclerotic plaque. This reduction in blood flow can causes myocardial ischemia and myocardial infarction (Ross, 1995).

1.9.4 Atherosclerosis as a leading cause of acute coronary syndromes

Atherosclerotic Plaque gains its name from a chronic pathological condition termed atherosclerosis. Atherosclerosis is the most prevalent form of arteriosclerosis; it is defined as the disease of artery wall, and it is initiated when lipid is retained, oxidized, and modified deep inside the layers of artery's wall by a chronic inflammatory process (Ross, 1999). Development of atherosclerotic plaque is initiated when low density lipoprotein is deposited in the artery wall (the tunica intima) and extensively oxidized by lipid peroxidation process involving the phospholipid molecules (Khatana et al., 2020). Oxidized low density lipoprotein (Ox-LDL) elicits a chronic inflammatory process in the artery wall. Ox-LDL attracts circulating monocytes to the tunica intima where monocytes differentiate to macrophages. Monocyte's recruitment is mediated by monocyte chemoattractant protein-1 MCP-1 which is upregulated by endothelial cells and smooth muscle cell in the arterial intima (Harrington, 2000). Macrophages engulfs Ox-LDL and transform into foam cells that form fatty streaks (Haberland

et al., 2001). Fatty streaks become the precursors of more advanced pathological lesions that are characterized by formation of lipid-rich necrotic debris and accumulation of the smooth muscle cells (Lusis, 2000). Dying foam cells, smooth muscle cells form the necrotic center of atherosclerotic plaque which is considered the most thrombogenic constitutes of the plaque. Atherosclerotic plaque development is accompanied by calcium deposition in the coronary artery, leading to arterial calcification which is considered as an excellent indicator of atherosclerosis (Sangiorgi et al., 1998, Burke et al., 2001). The flow adhesion assays revealed that the atheromatous core is the most thrombogenic of human atherosclerotic plaque lesion (Fernández-Ortiz et al., 1994). Collagen is largely involved in the progression and complications of atherosclerotic plaque lesion (Adiguzel et al., 2009). The rate of collagen synthesis and degradation is increased atherosclerotic plaque lesion. Histological studies of animal models of atherosclerosis showed overexpression of the matrix metalloproteinase-13 and collagenase-3 that weaken the structural support of atherosclerotic plaque by diminishing the collagen content in the fibrous cap, leading to atherosclerotic plaque erosion and rupture (Deguchi et al., 2005). Human macrophages produce matrix-degrading metalloproteinases that breakdown collagen fibres in the fibrous cap (Shah et al., 1995). Collagen type I and III are localized and accumulated in the thickened intima of atherosclerotic plaque lesion; collagen type I and III are the main collagen that are involved in platelet aggregation (Katsuda et al., 1992).

Development of atherosclerotic plaque in the artery wall can reduce the artery diameter, causing stenosis and reducing blood flow. Atherosclerotic plaque without thrombus formation can cause stenosis and limit blood flow which can clinically corrected by PCI (Stone et al., 2020).

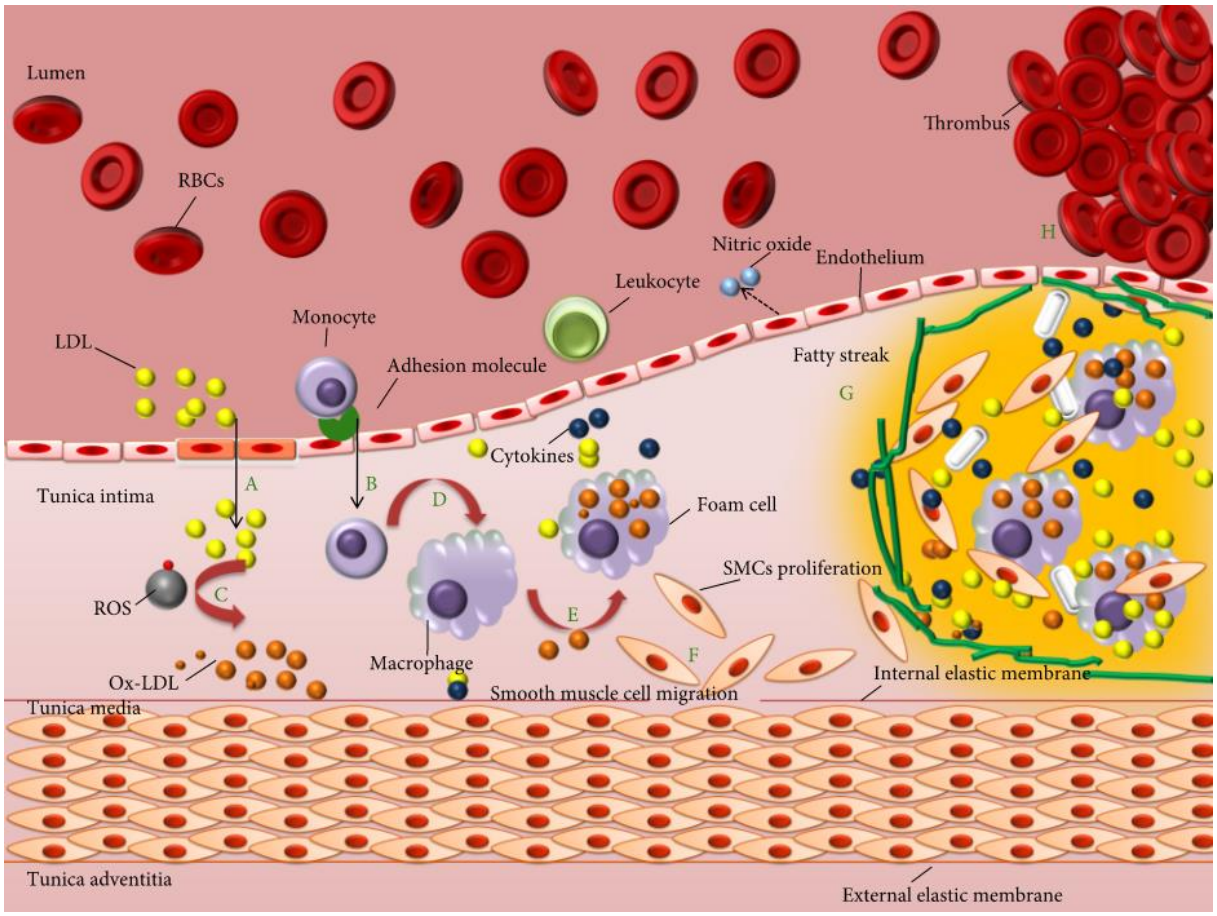


Figure 1.4. Development of atherosclerotic plaque. Atherosclerotic plaque formation is initiated when LDL is deposited and oxidised in the artery wall, leading to migration of monocytes that develop into macrophages and start engulfing ox-LDL and transform into foam cells, forming the necrotic core of atherosclerotic plaque. Atherosclerotic plaque formation also is associated with smooth muscle cells proliferation and increased collagen synthesis and degradation (Khatana et al., 2020).

1.9.5 Diagnosis

Diagnosis and classification of ACS are based on the combination of clinical presentations, electrocardiogram, and cardiac enzymes and biomarkers.

Clinical presentations

Clinical presentations in the term of signs and symptoms are important in diagnosing and identifying patients with ACS. Patients with STEMI usually present a sharp chest pain (a crushing and burning feeling) that develops without an exertion and does not resolve with the rest. The pain usually radiates to the left shoulder, arm or neck (Malik et al., 2013). Patients with NSTEMI usually present with a pressure-type chest pain that radiates to either jaw, neck or arm, develops with minimal exertion and lasts for 10 minutes (Antman et al., 2000). Patients with NSTEMI may suffer from unexplained dyspnea, abdominal pain, nausea, and syncope (Amsterdam et al., 2014a). Patients with US may experience chest pain that develops at rest or during physical exercise and lasts for more than 20 minutes (Braunwald et al., 2000).

Electrocardiogram

A 12-lead electrocardiogram (ECG) is most commonly used medical tool in diagnosing and classifying patients with ACS, and it should be carried out within 10 minutes of the patient's arrival at the emergency department in patients with suspected heart attack or those suffering from unexplained chest pain (Thygesen et al., 2012). A 12-lead ECG involves using sensors that are attached to the skin on the chest (electrodes) to record the electrical activity of the heart. ECG traces are made from different segments that represents electrical events generated during the cardiac cycle (Vogel et al., 2019). Distinctive ECG changes in patients with STEMI include persistent ST-elevation and anterior ST depression (O'Gara et al., 2013). Patients with NSTEMI usually show transient ST-elevation, ST depression, or new T-wave inversion in their ECG (Amsterdam et al., 2014a). Patients with US may show pathological T-wave changes such as

hyperacute T-wave and flattening of the T-waves, and also they may show ST depression (Braunwald et al., 2000).

Cardiac biomarkers

Cardiac troponin T (cTnT) and troponin I (cTnI) are exclusively expressed in the myocardium; therefore, they are the mainstay for diagnosis and classification of ACS. Creatine kinase-MB (CK-MB) is found in the myocardium, and it was previously used for detection of acute myocardial injury (Amsterdam et al., 2014a). Levels of cardiac troponins are elevated within 2-3 hours after the onset of symptoms of acute myocardial infarction and stay at high levels for longer periods (Mahajan and Jarolim, 2011). Measurement of CK-MB mass is preferred over measurement of CK-MB activity, as CK-MB mass is more sensitive for early detection of STEMI and less effected by experimental manipulation or haemolysis (Al-Hadi and Fox, 2009). CK-MB is released within 6-9 hours after symptom onset of acute myocardial infarction and remained elevated for shorter periods (Achar et al., 2005). CK-MB is less sensitive and specific compared to cardiac troponin in detection of myocardial necrosis; as a result, CK-MB is widely replaced by high sensitivity troponin test as diagnostic tool of ACS. Combining ECG with cardiac biomarkers is the main approach for classification of ACS. Patients with STEMI showed persistent ST-elevation in their ECG graph with an elevated level of cardiac troponin, indicating myocardial injury. Patients with NSTEMI lack persistent ST-elevation on their graph, but they still show other abnormal ECG changes with elevated levels of troponin or CK-MB. Patients with US may have abnormal ECG changes without elevations in cardiac troponin and CK-MB levels (Braunwald et al., 2000).

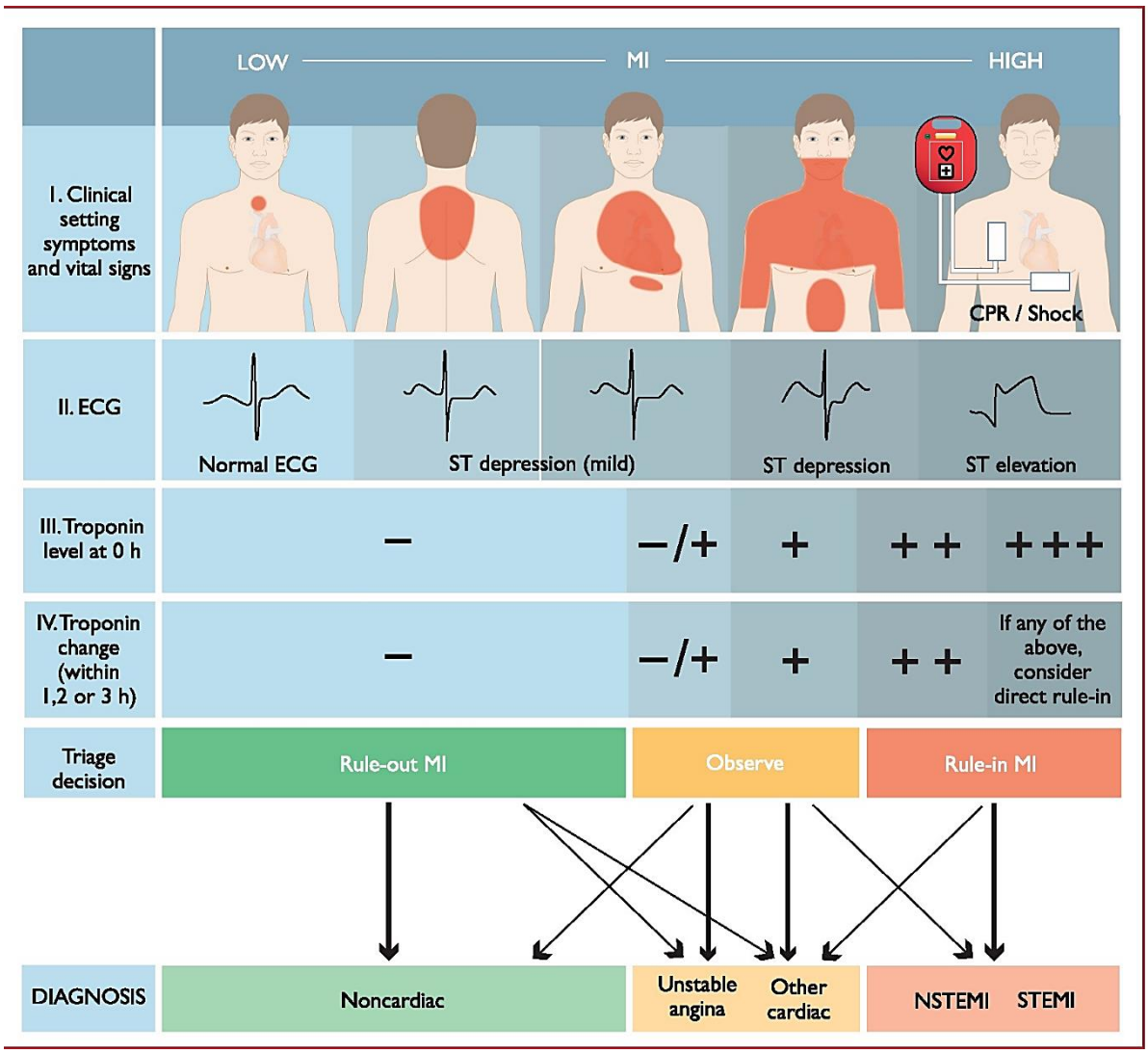


Figure 1.5. Diagnostic and triage algorithm for patients with suspected ACS. Combination of ECG with high sensitivity troponin is an essential approach for identification and classification of patients with ACS (ESC, 2021).

1.9.6 Treatment

Treatments that are used for patients with ACS include reperfusion therapy, antiplatelet therapy, anticoagulant therapy, beta-adrenergic blockers, angiotensin-converting enzyme (ACE) inhibitors, and statin therapy. Treatment protocol depends on the ACS class; patients with SETMI need an immediate and vigorous treatment that involve using the emergency primary percutaneous intervention (pPCI) in combination with antiplatelet and anticoagulant therapy.

Reperfusion therapy

Myocardial revascularization is an essential step in treating patients with ACS and any delay in the reperfusion therapy is associated with increased the risk of morbidity and mortality (Lemkes et al., 2019). The emergency primary percutaneous coronary intervention (pPCI) is an interventional, non-invasive procedure that is aimed to restore blood flow in the blocked artery and prevent myocardial infarction and myocardial ischemia in patients with STEMI. pPCI is accompanied by stent implantation either bare metal stent or drug eluting stent to prevent occlusion of blood vessel and restenosis (Williams and Awan, 2017). pPCI can be an elective procedure for patients with NSTEMI and US especially if the patient has unstable atherosclerotic plaque or suffer from recurrent angina (Braunwald, 2012). Coronary artery bypass graft surgery (CABGS) is more invasive procedure than pPCI, and it is used when pPCI cannot be carried out due to coronary anatomy or in patients who suffer from recurrent ischemia, cardiogenic shock, or severe HF (O'Gara et al., 2013).

Antiplatelet therapy

Patients with ACS will receive a dual combination of antiplatelet therapy that consists of aspirin and a $P2Y_{12}$ inhibitor such as clopidogrel, prasugrel or ticagrelor. GPIIb/IIIa inhibitors are only used if the patient is experiencing a heavy thrombosis or to bail-out thrombosis during pPCI. Antiplatelet therapy will be explained in more details in **section 1.11.**

Anticoagulant therapy

In addition to antiplatelet therapy, patients with ACS will receive anticoagulant therapy to increase the scope of antithrombotic treatment beyond platelet inhibition by targeting thrombin production and action in order provide maximal protection against myocardial infarction and stent thrombosis. There are several anticoagulant agents that are used to treat patients with ACS such as unfractionated heparin, low-molecular-weight heparin, bivalirudin, argatroban, and fondaparinux which vary in the mechanism of action and route of administration (Amsterdam et al., 2014a, O'Gara et al., 2013). Enoxaparin (low-molecular-weight heparin) is one of most used commonly anticoagulant agents for ACS treatment, and it has a balanced anti-Xa and anti-thrombin effect (de Lemos et al., 2004).

Beta-adrenergic blockers

Beta-1 receptors are expressed in the heart and mediate heart muscles activity (Gabriel et al., 2019). Beta-1 receptors are activated by noradrenaline secreted by sympathetic nerve terminal and by circulating catecholamines (Gabriel et al., 2019). Metoprolol succinate, bisoprolol, and carvedilol are the most common beta-1 blockers that are used for ACS treatment (Amsterdam et al., 2014a**).** Patients with ACS especially STEMI usually receive oral or intravenous beta blockers during the first 24 hours of hospital admission. Use of beta blockers in patients with ACS is associated with a reduction in the mortality and adverse ischemic events (de Matos Soeiro et al., 2016). Beta-1 blockers reduce oxygen demand by reducing heartbeat, reduce infarct size, and also reduce blood pressure.

Angiotensin-converting enzyme inhibitors

Angiotensin II causes vasoconstriction of precapillary arterioles and postcapillary venules, increasing blood pressure (Ruddy and Kostis, 2005). Angiotensin II is a peptide that is produced from angiotensinogen (inactive precursor). Angiotensinogen is converted into angiotensin I by renin, and angiotensin I is further converted to angiotensin II (active form) by angiotensin-converting enzyme (ACE) (zhu, 1997). As a result, Angiotensin-converting enzyme (ACE) inhibitors are used in clinical practice to inhibit angiotensin II in patients with hypertension. Administration of ACE inhibitors in patients with recent onset of myocardial infarction associated with left ventricular dysfunction has been shown to mortality (Amsterdam et al., 2014a). ACE inhibitors have been shown to reduce the incidence of nonfatal cardiac failure in patients with ACS. Captopril and enalapril, short-acting ACE inhibitors, are as an early treatment for patients with acute myocardial infarction (Amsterdam et al., 2014a).

Statin therapy

Statins such as lovastatin, pravastatin, fluvastatin and atorvastatin are used to reduce total-cholesterol and LDL-cholesterol by inhibiting 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase that is involved in synthesis of cholesterol precursor (mevalonate), as a primary mean to reduce hyperlipidaemia and subsequently prevent cardiovascular diseases (Faergeman et al., 2008, Stancu and Sima, 2001). The early initiation of statin therapy in patients with ACS has been shown to decrease lipid parameters , reduce inflammation markers, and modulate blood coagulation (Ostadal, 2012). Statin therapy also improves endothelium-dependent vasodilation; therefore, statins can be effective in patients suffering from vasospastic angina (Amsterdam et al., 2014a).

1.10 Platelets in the pathogenesis of acute coronary syndromes

ACS is frequently caused by rupture of atherosclerotic plaque, leading to an occlusive thrombus forming inside a coronary artery, which causes myocardial ischaemia (Ambrose and Singh, 2015, Torpy et al., 2010). Platelets play major roles in the pathogenesis of ACS and its recurrence. The formation of platelet-rich thrombi is the key pathological event following atherosclerotic plaque rupture that exposes potent thrombogenic endothelial substrates and initiators of coagulation, such as collagen, vWF, fibronectin, laminin, and tissue factor (Gawaz, 2004). Platelets are strongly activated by the interaction of their surface receptors with mediators that are exposed and released from the ruptured atheromatous plaque. This results in the formation of a platelet-rich thrombus, which may subsequently impede blood flow and cause myocardial ischaemia and infarction (Ruggeri, 2002, Gawaz, 2004).

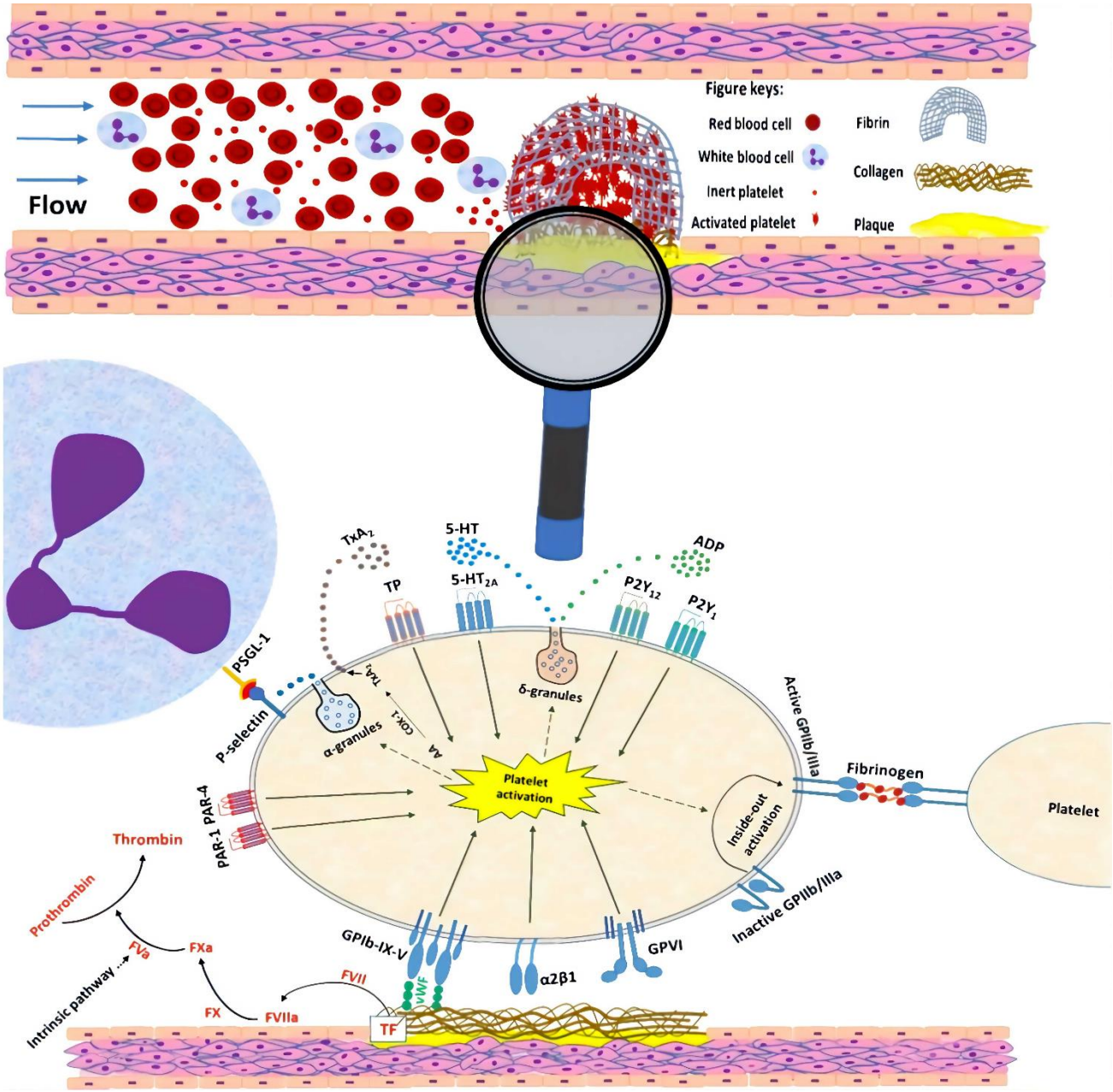


Figure 1.6. Platelet thrombus formation.

Platelets are tethered at the site of vascular injury by GPIb-IX-V-vWF interaction. This interaction allows close contact that forms between collagen and its direct receptors, GPIa/IIa and GPVI. Platelet activation by GPVI triggers release of secondary mediators and causes inside-out activation of GPIIb/IIIa that links platelets together, forming the platelet aggregate. Tissue factor released from the site of injury also triggers the coagulation cascade that generates thrombin that in turn stimulates platelets via PARs (Alenazy and Thomas, 2021).

1.11 Current antiplatelet therapy in acute coronary syndromes management

Following atherosclerotic plaque rupture, platelet activation and aggregation are the two major elements that initiate thrombus formation inside a coronary artery, which can obstruct blood flow and cause myocardial ischaemia. Thus, antiplatelet therapy forms a major part of the treatment strategy for ACS. Patients with ACS routinely receive dual antiplatelet therapy (DAPT), which consists of aspirin and a platelet $P2Y_{12}$ inhibitor to inhibit multiple pathways to provide a strong amplified antiplatelet effect that can treat and prevent atherothrombosis. The amplified effect of combining two drugs can be either additive or synergistic. Additive effect of combining two drugs can be defined as the sum of the effect of each inhibitor administered alone; an effect that is more than the effect of either inhibitor giving alone (Foucquier and Guedj, 2015). On the other hand, the effect of combination of two inhibitors is considered a synergistic when the produced effect is greater than the additive effect of each inhibitor (Foucquier and Guedj, 2015, García-Fuente et al., 2018). $P2Y_{12}$ inhibition provided a synergistic antiplatelet effect when used in combination with aspirin in patients with ACS (Zhang et al., 2015, Payne et al., 2002). Use of platelet GPIIb/IIIa inhibitors is now limited due to the risk of severe bleeding and thrombocytopenia. Thus, administration of GPIIb/IIIa inhibitors is generally restricted to bail out thrombotic events during percutaneous coronary intervention (PCI).

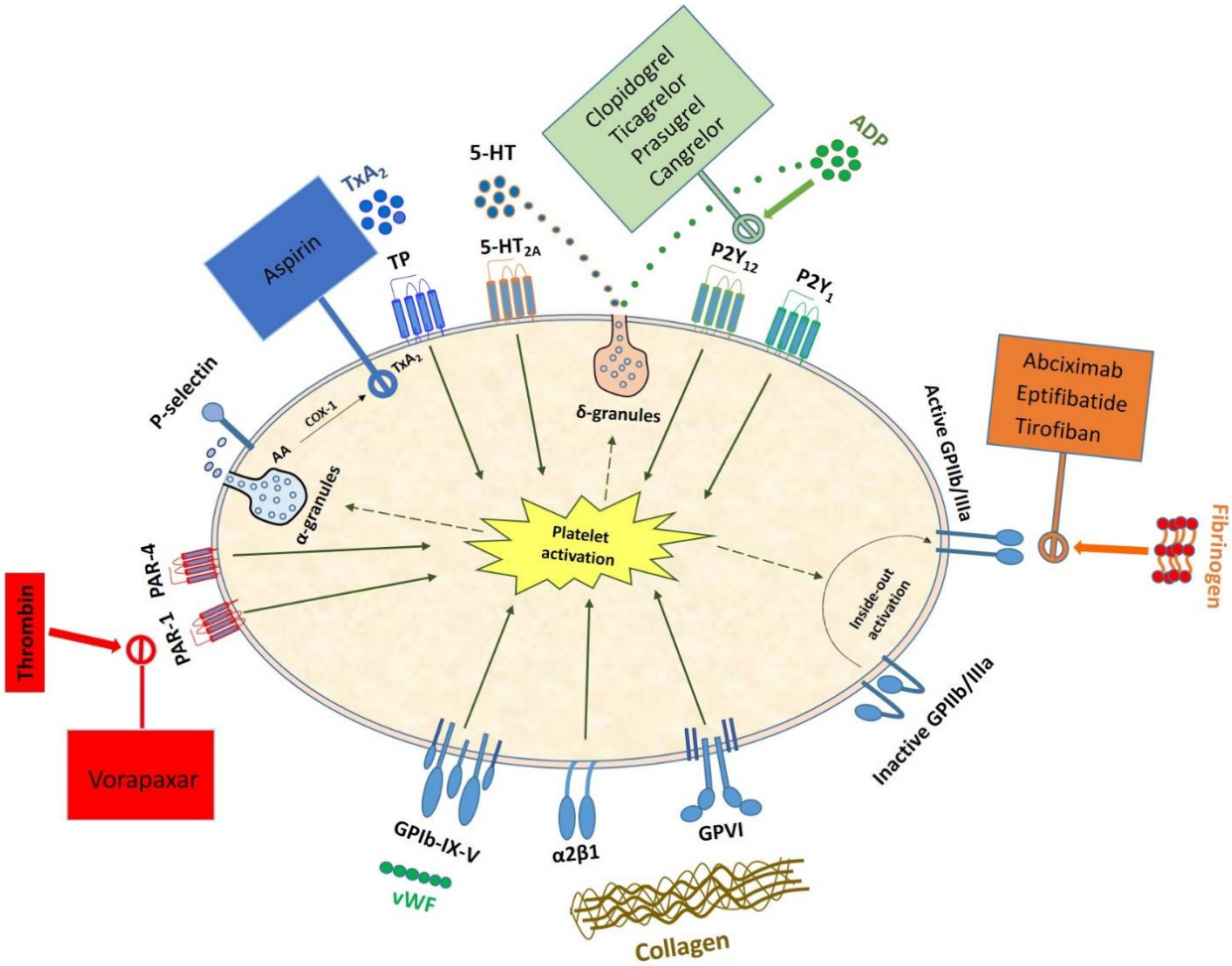


Figure 1.7. Current antiplatelet therapy used for acute coronary syndromes treatment. Dual antiplatelet therapy based on aspirin and $P2Y_{12}$ inhibitors is used routinely to treat patients with ACS. Use of GPIIb/IIIa inhibitors is generally limited to patients presenting with a heavy thrombus burden or to inhibit thrombosis during pPCI (Alenazy and Thomas, 2021).

1.11.1 Thromboxane A_2 blockade

1.11.1.1 Thromboxane A_2 biosynthesis

Thromboxane A_2 (TxA_2) is biosynthesised from arachidonic acid by the sequential action of multiple enzymes. Arachidonic acid is first released from platelet membrane phospholipids following activation via phospholipase A_2 (Yoshida and Aoki, 1978). Once liberated from the membrane phospholipid, arachidonic acid is metabolised into prostaglandin endoperoxide H_2 by cyclooxygenase-1 (COX-1). Prostaglandin endoperoxide H_2 is further metabolised into the active metabolite, TxA_2, by TxA_2 synthase. TxA_2 has a very short half-life of 30 seconds, which makes it difficult to measure in biological samples. Therefore, TxB_2 and 11-dhTxB2, inactive metabolites of TxA_2, are commonly used as indicators of it (Lefer et al., 1981).

1.11.1.2 Thromboxane A_2 function

TxA_2 is a potent positive feedback mediator that plays an essential role in amplifying platelet activation and platelet recruitment during vascular injury. TxA_2 stimulates platelet shape change, secretion, and aggregation via a G-coupled signalling pathway. TxA_2 is also considered a potent vasoconstrictor of smooth muscle.

1.11.1.3 Thromboxane A_2 signalling pathway

TxA_2 acts in an autocrine and paracrine manner and activates platelets via the TP receptor. The TP receptor belongs to the G-protein-coupled receptor superfamily. There are two isoforms of the TP receptor expressed on the platelet membrane, TPα being the most predominant and TPβ the least expressed (Hirata et al., 1996). TP isoforms arise from alternative splicing of their common TP gene, which leads to the generation of isoforms that differ mainly in the length of their C-terminal tail (Raychowdhury et al., 1994). Upon stimulation with TxA_2, TP couples with a group of heterotrimeric G proteins, mainly G_q and $G_{12/13}$, and initiates a cascade of activation mechanisms that induce shape change, calcium rise, inside-out activation, and

aggregation (Huang et al., 2004). Once G_q activated, it is dissociated to Gαq and Gβγ subunits, exposing their surfaces to enable interaction with their effector proteins. Gαq subunits activate PLCβ, which breakdowns phosphatidylinositol-(4,5)-bisphosphate (PIP2) and gives rise to the generation of diacylglycerol (DAG) and inositol tris-phosphate (IP_3). DAG and IP_3 activate protein kinase C (PKC) and induce the release of intraplatelet calcium from the dense tubular system. Activation of the G12/13 stimulates the Rho/Rho-kinase pathway, which in turn causes phosphorylation of MLCK and subsequent actin cytoskeleton organisation and shape change (Nakahata, 2008).

1.11.1.4 Thromboxane A_2 inhibitors

Aspirin at low dose inhibits TxA_2 synthesis by inhibiting cyclooxygenase-1 **(**COX-1) (Tohgi et al., 1992). Aspirin is the only TxA_2 inhibitor that is routinely used as an antiplatelet drug in ACS management. Indeed, aspirin was the first antiplatelet drug used to treat arterial thrombosis in the context of cardiovascular diseases, and it is still the most commonly used antiplatelet agent. In the ISIS-2 study, aspirin monotherapy provided a 23% risk reduction in cardiovascular death compared with placebo in patients with acute myocardial infarction. Aspirin is also known as acetylsalicylic acid and blocks TxA_2 synthesis by inhibiting COX-1 activity in platelets and megakaryocytes. Aspirin irreversibly acetylates serine 529 at the active site within the COX-1 enzyme, causing a long-lasting antiplatelet effect in the circulating platelets, since the platelets lack de novo synthesis of enzymes until they are cleared from circulation (Funk et al., 1991).

1.11.2 $P2Y_{12}$ blockade

1.11.2.1 $P2Y_{12}$ function

Platelets express three types of purinergic receptors: $P2X_1$, $P2Y_1$, and $P2Y_{12}$. With regard to their extracellular nucleotide stimulants, $P2X_1$ is activated by adenosine triphosphate (ATP)

and $P2Y_1$ and $P2Y_{12}$ are activated by ADP. $P2Y_{12}$ is also known as P2T, P2Yac, and SP1999, and it is estimated that there are around 400 $P2Y_{12}$ copies per platelet (Ohlmann et al., 2013). As well as its expression on platelets, $P2Y_{12}$ is expressed on lymphocytes, monocytes, macrophages, osteoclasts, and microglial cells. ADP is a secondary mediator that plays an important role in amplifying and sustaining platelet activation (Gachet, 2012). Because ADP is a weak platelet stimulus, concomitant activation of $P2Y_1$ and $P2Y_{12}$ is required to induce platelet aggregation and secretion (Jin and Kunapuli, 1998). $P2Y_{12}$ plays its central role in platelet activation, aggregation, secretion, and procoagulant activity when stimulated by ADP or non-ADP-induced platelet stimulation by other agonists (Storey et al., 2000b). These $P2Y_{12}$-mediated platelet events are required to reinforce thrombus growth and stability during arterial thrombosis. $P2Y_{12}$ antagonism is therefore routinely applied in ACS treatment and prophylaxis (Andre et al., 2003, Hacke, 2002).

1.11.2.2 $P2Y_{12}$ signalling pathway

Molecular modelling of $P2Y_{12}$ revealed 3 different binding sites for ADP (Zhan et al., 2007). ADP (secreted from platelets or released other cells) binds to into its active binding sites on $P2Y_{12}$, triggering $P2Y_{12}$ signalling pathway (Zhan et al., 2007). Upon stimulation of $P2Y_{12}$ with ADP, $P2Y_{12}$ is coupled to the heterodimeric G_{i2} protein, leading to the initiation of a cascade of signalling events downstream of the receptor mediated by activation of PI 3-kinase (PI3K) and inhibition of adenylate cyclase (Hollopeter et al., 2001). $P2Y_{12}$ signalling causes dissociation of the G_{i2}-coupled protein into $G\alpha_{i2}$, $G\beta_{i2}$, and $G\gamma_{i2}$ subunits. The $G\alpha_{i2}$ subunit mediates the inhibition of adenylate cyclase, thereby decreasing the level of cAMP, which in turn leads to the impairment of protein kinase A activation (Jantzen et al., 2001, Ohlmann et al., 1995). $G\beta\gamma_{i2}$ subunits induce activation of various effector proteins such as PI3K, Protein kinase B (PKB) also known as Akt, extracellular signal-regulated kinase 2 (ERK2), the Src

family of tyrosine kinases, Rap1b, and G protein-gated inwardly rectifying potassium channels (GIRK) that regulate ADP-$P2Y_{12}$-mediated platelet responses (Kim and Kunapuli, 2011). PI3K is the main upstream effector that activates Akt and ERK2 in the $P2Y_{12}$ downstream signalling pathway and mediates platelet secretion, aggregation, and thrombus formation (Maier et al., 1999, Kim et al., 2004). PI3K also stimulates Rap1b activation, which causes inside-out signalling of GPIIb/IIIa and subsequently platelet aggregation (Woulfe et al., 2002). Src family kinases (SFKs) are also reported to be activated in the $P2Y_{12}$ pathway, leading to $cPLA_2$ phosphorylation and GPIIb/IIIa stimulation (Dorsam et al., 2005). GIRKs regulate the irreversible platelet aggregation, amplify granule secretion, enhance Akt phosphorylation, and contribute to Src-mediated signalling events in the $P2Y_{12}$ pathway (Shankar et al., 2004, Shankar et al., 2006).

1.11.2.3 $P2Y_{12}$ inhibitors

Different classes of $P2Y_{12}$ antagonists have been introduced into clinical practice. They vary in their structure (thienopyridines vs. non-thienopyridines), mode of platelet inhibition (reversible vs. irreversible), and onset of the effect (drug vs. prodrug).

1.11.2.3.1 Thienopyridine $P2Y_{12}$ inhibitors

Ticlopidine, clopidogrel, and prasugrel are thienopyridines that are absorbed in the gut and need metabolic activation to induce their antiplatelet effect. Once they are metabolised in the liver by the cytochrome P450 system (CYP450) into active metabolites, the active metabolites irreversibly bind to ADP-binding sites in the $P2Y_{12}$ and cause long-lasting platelet inhibition.

Ticlopidine

Ticlopidine was the first $P2Y_{12}$ inhibitor, but it was shortly discontinued and replaced by clopidogrel due to the incidence of thrombocytopenia, thrombotic thrombocytopenic purpura, aplastic anaemia, and neutropenia associated with its use (Love et al., 1998).

Clopidogrel

Clopidogrel is an indirect $P2Y_{12}$ inhibitor that is metabolised by two pathways that slow its antiplatelet onset. In the first pathway, clopidogrel is converted to inactive clopidogrel carboxylic acid by de-esterification (Caplain et al., 1999). In the second pathway, a portion of inactive clopidogrel carboxylate is hydrolysed by two CYP450-dependent steps into the active thiol metabolite that irreversibly binds to extracellular Cys17 and Cys270 residues of the $P2Y_{12}$ receptor (Ding et al., 2003, Clarke and Waskell, 2003). Platelet inhibition is achieved within 1–2 hours after the loading dose of clopidogrel. One of the main limitations of clopidogrel use is the CYP genetic variants that undermined its clinical value. CYP genetic polymorphisms, for example in CYP2C19, CYP2C9, and CYP2B6, can reduce or increase its metabolism and subsequently its plasma concentration and therapeutic efficacy, causing interindividual variability (Wallentin, 2009). Combining clopidogrel with aspirin achieved a further reduction in major adverse cardiovascular events of 20% compared with aspirin alone (Jneid et al., 2003). Accordingly, this combination became the routine antiplatelet regimen for patients diagnosed with ACS, although it has since been superseded by the use of the potent $P2Y_{12}$ inhibitors ticagrelor and prasugrel.

Prasugrel

Prasugrel is third-generation thienopyridine $P2Y_{12}$ antagonist that irreversibly antagonises ADP-$P2Y_{12}$-mediated platelet responses. Prasugrel is metabolised by carboxylesterases in the intestine into thiolactone metabolite, which is further biotransformed in the liver into prasugrel

active metabolite by one CYP450-dependent step (Dobesh, 2009). Prasugrel has a fast antiplatelet effect compared to clopidogrel, with platelet inhibition being achieved within 15–30 min after the first oral intake. This superiority is attributed to fast metabolism, since it requires only one CYP450 step. Furthermore, the biotransformation of prasugrel active metabolite is not affected by the CYP genetic polymorphisms that cause the variable platelet responses seen in patients on clopidogrel (Varenhorst et al., 2009). In the TRITON-TIMI 38 trial, which compared prasugrel and clopidogrel in patients with ACS undergoing PCI, the incidence of adverse cardiovascular events was lower in the prasugrel arm with a higher risk of bleeding (Wiviott et al., 2007).

1.11.2.3.2 Non-thienopyridine $P2Y_{12}$ inhibitors

Non-thienopyridines $P2Y_{12}$ antagonists have been pharmacologically developed to prevent and treat atherothrombosis. These antagonists include ticagrelor and cangrelor.

Ticagrelor

Ticagrelor is the first oral reversible, direct-acting $P2Y_{12}$ inhibitor that belongs to the chemical class of cyclopentyl-triazolopyrimidine (van Giezen and Humphries, 2005). Ticagrelor is absorbed in the intestine and rapidly metabolised in the liver by the CYP3A4 enzyme into its active metabolite, known as AR-C124910XX, without the need for metabolic activation (Teng et al., 2010). In contrast to thienopyridine $P2Y_{12}$ inhibitors, ticagrelor reversibly binds to different binding sites on $P2Y_{12}$ and antagonises ADP in a non-competitive manner (Giezen et al., 2009) . In addition to direct blocking of ADP, ticagrelor inhibits agonist-independent $P2Y_{12}$ activity by acting as an inverse agonist and increases platelet cAMP levels via inhibition of platelet equilibrative nucleoside transporter-1(ENT1). This leads to accumulation of extracellular adenosine, which in turn stimulates platelet A_{2A} adenosine receptors (Aungraheeta et al., 2016). The antiplatelet effect of ticagrelor has a rapid onset, with significant platelet

inhibition being observed within 30 min and the maximum inhibition being reached at 2 hours after administration of the loading dose (Gurbel et al., 2009). Ticagrelor showed superiority over clopidogrel in reducing myocardial infarction and death from thrombotic vascular events in patients with ACS in the PLATO trial, which has generally led to replacement of clopidogrel with ticagrelor in the treatment of ACS (Wallentin et al., 2009).

Cangrelor

Cangrelor is the latest direct $P2Y_{12}$ blocker that has been approved by the United States Food and Drug Administration as an adjunct to PCI for treatment of thrombotic cardiovascular diseases. Cangrelor is an intravenous ATP analogue (2-trifluoropropylthio, N-(2-(methylthio) ethyl)-β,γ-dichloromethylene ATP) that reversibly inhibits ADP-mediated platelet activation in a competitive fashion (Norgard, 2009, Ingall et al., 1999). In contrast to ATP that has a low affinity for $P2Y_{12}$ receptors and is rapidly hydrolysed by ectonucleotidases, this modified ATP analogue resists hydrolysis by ectonucleotidases and has a high affinity for $P2Y_{12}$ receptors (Ingall et al., 1999). Cangrelor has the fastest onset and offset of platelet inhibition among the $P2Y_{12}$ inhibitors. It immediately blocks ADP-stimulated platelet aggregation within 1–2 min (Bulluck et al., 2019). Since cangrelor has a very short half-life (3–6 min), its applications are limited to a narrow range of medical settings. One possible use of cangrelor is as a bridging antiplatelet agent to maintain platelet inhibition in patients undergoing a coronary artery bypass graft (CABG) who need to discontinue oral $P2Y_{12}$ antagonists to reduce bleeding risk (Angiolillo et al., 2012).

1.11.3 GPIIb/IIIa blockade

1.11.3.1 GPIIb/IIIa function

GPIIb/IIIa, also known as integrin αIIbβ3, is the most abundant glycoprotein (GP) receptor expressed on the platelet surface with an estimated 80,000–100,000 copies in a low-affinity

status per resting platelet. GPIIb/IIIa is also stored inside platelets in α-granules and dense granules, with an estimated 20,000–40,000 molecules per platelet (Saboor et al., 2013). GPIIb/IIIa consists of αIIb and β3 subunits that form the heterodimeric extracellular domains of the receptor with an extended transmembrane helix and cytoplasmic tail. The αIIb subunit consists of a heavy and a light chain, whereas the β3 subunit consists of only a single polypeptide chain (Joo, 2012). As there are many copies, GPIIb/IIIa interacts with different ligands, including fibrinogen, fibrin, vWF, vitronectin, and fibronectin, which enables it to play various functional roles (Varga-Szabo et al., 2008). GPIIb/IIIa functions as the main platelet receptor for fibrinogen, where it interacts with the arginine-glycine-aspartic acid (RGD) motif and the the Lys-Gln-Ala-Gly-Asp-Val (KQAGDV)-containing sequence in the γ-chain C terminus of the fibrinogen and bridges platelets together, forming a platelet aggregate. This step is required to arrest bleeding at the wounded area and has a significant implication in intravascular thrombosis (Hawiger et al., 1982). GPIIb/IIIa is a receptor for vWF, which supports platelet aggregation under low shear rates and shear resistant platelet adhesion to collagen (Gr ner et al., 2003, Naimushin and Mazurov, 2004). GPIIb/IIIa also mediates platelet spreading and clot retraction (Weiss et al., 1991, Seiffert et al., 2002). GPIIb/IIIa deficiency leads to a pathological condition known as Glanzmann thrombasthenia, which is characterised by loss of platelet aggregation and absence or reduction in the receptor expression with severe bleeding phenotypes (Botero et al., 2020).

1.11.3.2 GPIIb/IIIa signalling pathway

GPIIb/IIIa exists in a low-affinity state on resting platelets where extracellular subunits are present in a bent conformation to hide RGD binding sites, thus preventing platelet activation by adhesive ligands containing RGD sequences. On activated platelets, GPIIb/IIIa changes into a high-affinity state where extracellular domains are present in an open conformation, exposing

RGD binding sites and allowing interaction with its ligand. GPIIb/IIIa is considered a bidirectional receptor in terms of its signalling pathway and signal transduction (Ginsberg et al., 2005). Platelet activation via G-protein-coupled receptors (ADP, TxA_2, PAR1, and PAR4), collagen, and vWF leads to inside-out activation of GPIIb/IIIa (Smyth et al., 2009). Direct interaction of GPIIb/IIIa with its ligands results in outside-in signalling. Many proteins are reported to mediate inside-out and outside-in signalling of GPIIb/IIIa by transmitting signals between extracellular domains and cytoplasmic tails. Talin-1 and Kindlin-3 are among the proteins that regulate inside-out activation of GPIIb/IIIa. The low-affinity state is believed to be maintained by a helical interface formed between α and β cytoplasmic membrane proximal regions via a salt bridge, and any separation, twisting, pistoning, or hinging that rearrange this helical interface can lead to integrin activation (Banno and Ginsberg, 2008). Talin-1 (a cytoskeletal protein) binds into the β cytoplasmic domain via a phosphotyrosine-binding (PTB) domain-like interaction linking it to the actin. The head region of talin-1 contains four subdomains (F1, F2, F3, and F4). F3 subdomain of PTB domain interacts with the Asn-Pro-x-Tyr (NPXY) motif in β cytoplasmic tail leading to disruption of the salt bridge between the α and β cytoplasmic domains, which further leads to tail separation and receptor activation. For the F3 subdomain of the talin head to bind to the β cytoplasmic tail, the binding site must be unmasked by the interaction between Rap1-GTP and talin-1 by RAIM, a Rap1 effector molecule. For this interaction to take place, Rap1-GTP is activated first and translocated into the membrane by PKC and calcium diacylglycerol-guanine nucleotide exchange factor I (CalDAG-GEFI) that are activated by DAG and Ca^{2+} resulting from the outside-in activation of platelets by an agonist (Nieswandt et al., 2009). Kindlin-3 contributes to the inside-out signalling process by binding to the β cytoplasmic domain via its F3 subdomain (Moser et al., 2008).

Inside-out activation of GPIIb/IIIa changes it to a high-affinity state with an open structural conformation, allowing direct contact with its ligands, which induces the outside-in signal-transduction process (Shattil et al., 2010). The outside-in signalling is mediated by a panel of effector enzymes and adapters, some of which are shared by other platelet receptors, including SFK (c-Src, Src, Fyn), Syk, PLCγ2, FAK, degranulation-promoting adapter protein (ADAP), Ras, Rho GTPases, Cas/Crk adapter protein. Following binding to its ligand, GPIIb/IIIa undergoes clustering and oligomerisation that enhances signal transduction through activation of c-Src that is associated with the β_3 cytoplasmic tail via an RGT motif. c-Src activation leads to the activation and recruitment of Syk to the β_3 cytoplasmic domain. This signalling cascade will continue to downstream effectors such as PLCγ2, ADAP, and FAK (Durrant et al., 2017). PLCγ2 stimulates formation of DAG and IP3 from membrane PtdIns (4,5)P2, which results in PKC activation and calcium mobilisation. Calcium- and integrin-binding protein 1 (CIB1) is involved in outside-in signalling by binding to αIIb cytoplasmic tail (Naik et al., 2017).

1.11.3.3 GPIIb/IIIa inhibitors

As GPIIb/IIIa has a great involvement in platelet mechanisms that are critical in platelet thrombosis; therefore, several anti-GPIIb/IIIa inhibitors have been introduced in clinical settings in the context of thrombotic cardiovascular disease treatment, including abciximab, eptifibatide, and tirofiban. However, the use of GPIIb/IIIa inhibitors has been limited to the treatment of heavy thrombosis during PCI due to the associated risk of severe bleeding and the availability of $P2Y_{12}$ inhibitors.

Abciximab

Abciximab (ReoPro®) is a chimeric monoclonal Fab fragment that binds to GPIIb/IIIa on activated and non-activated platelets with a high affinity and a dissociation constant of 5 nM (Schrör and Weber, 2003). It also binds to integrin αMβ2 and inhibits integrin αMβ2 activation

by vitronectin, but this cross-reactivity is considered benign and may provide an extra anti-thromboinflammatory effect, since vitronectin is a ligand for GPIIb/IIIa and integrin αMβ2 that mediates monocyte–extracellular matrix interaction (Simon et al., 1997, Kanse et al., 2004). Furthermore, abciximab binds to integrin αvβ3 which is another receptor for vitronectin (Kintscher et al., 2000). Abciximab was the first GPIIb/IIIa blocker used to treat myocardial infarction and ACS, and it has been shown to reduce ischemic complications, myocardial infarction, and mortality (Topol et al., 1994). Within 15 min after 0.25 mg/kg intravenous bolus, abciximab achieves an 80% blockade of GPIIb/IIIa that can last up to 24 hours (Tcheng et al., 1994). Despite the beneficial antithrombotic properties of abciximab administration, bleeding is the most common toxicity profile of abciximab. Due to its chimeric nature (human-marine), it is reported that 6% of its recipients develop human anti-chimeric antibodies without allergic reaction (Faulds and Sorkin, 1994). The use of abciximab is associated with thrombocytopenia in some cases, which may be due to immunologic reactions against the inhibitor (Curtis et al., 2004).

Eptifibatide

Eptifibatide (Integrilin®) is a small-molecule, cyclic heptapeptide derived from a snake venom disintegrin that structurally mimics the KGD sequence (Scarborough et al., 1993). Eptifibatide reversibly and specifically binds only to GPIIb/IIIa on both activated and resting platelets, thus preventing binding of fibrinogen, vWF and other RGD-containing adhesive proteins. In contrast to abciximab, eptifibatide competitively binds to GPIIb/IIIa with a low affinity (dissociation constant = 120 nM), allowing it to be rapidly dissociated from its target (Schrör and Weber, 2003). Eptifibatide has a rapid onset and offset of platelet inhibition. It reaches peak plasma concentration within 5 min and achieves maximal platelet inhibition within 15 min, which lasts for 2 hours, with full platelet recovery at 4 hours (Harrington et al., 1995). Eptifibatide reduces

death and non-fatal myocardial infarction in patients with ACS, as reported in the PURSUIT trial (PURSUIT Trial Investigators, 1998). The most significant adverse effect of eptifibatide is major bleeding (PURSUIT Trial Investigators, 1998). Cases of profound thrombocytopenia have also been reported with the use of eptifibatide in clinical practice (Bhatia et al., 2017).

Tirofiban

Tirofiban (Aggrastat®) is a non-peptide tyrosine derivative with a low molecular weight that contains the RGD sequence and specifically binds to the ligand-binding site of GPIIb/IIIa on platelets in a competitive manner with a dissociation constant of 15 nM (Hartman et al., 1992, Schrör and Weber, 2003). Tirofiban has a rapid onset and offset of action. It blocks GPIIb/IIIa within 30 min of administration with a platelet-bound half-life of 11 s, plasma half-life of 1.2–2 hours and restoration of platelet function at 4–6 hours after discontinuation (McClellan and Goa, 1998). Tirofiban reduces ischemic events in patients with ACS, but it is less effective than abciximab (Topol et al., 2001). Like eptifibatide, tirofiban is cleared by the kidneys, and so the dose should be adjusted in patients with renal insufficiency or on haemodialysis (Anderson and Riding, 2008).

Table 1.1 Current antiplatelet therapy in ACS treatment

Drug	Time to Peak	Mode of platelet inhibition	Dose	Frequency	Route
Aspirin	1-2 hours	Irreversible	Loading dose: 300 mg Maintenance dose: 75 mg	Once per day	Oral
Clopidogrel	45 min	Irreversible	Loading dose: 300–600 mg Maintenance dose: 75 mg	Once per day	Oral
Prasugrel	30 min	Irreversible	Loading dose: 60 mg maintenance dose: 10 mg	Once per day	Oral
Ticagrelor	1.5 hours	Reversible	Loading dose: 180 mg Maintenance dose: 90 mg	Twice per day	Oral
Cangrelor	2 min	Reversible	Bolus: 30 µg/kg Infusion: 4 µg/kg/min infusion for 2-4 hours	One-off treatment	IV
Abciximab	30 min	Reversible	Bolus: 0.25 mg/kg over 1 min Infusion:0.125 µg/kg/min for 12 hours	One-off treatment	IV
Eptifibatide	5 min	Reversible	Bolus: 180 µg/kg over 1min After 10 mins: 180µg/kg over 1min Infusion: 2 µg/kg/min for 18-24 hours	One-off treatment	IV
Tirofiban	5 min	Reversible	Bolus: 0.4 µg/kg/min for 30 min Infusion: 0.1 µg/kg/min for 12-24 hours	One-off treatment	IV

1.12 Could antiplatelet therapy be more targeted?

The key to future antiplatelet drug development is to identify mechanisms that have a disproportionate role in thrombosis compared to their role in haemostasis. This section focuses on platelet pathways that are less physiologically required to maintain haemostasis than existing drug targets but also have a major role in atherothrombosis and can be used as potential antiplatelet targets. As an example, the GPVI pathway appears to have little role in haemostasis. Therefore, combining a GPVI inhibitor with DAPT for triple antiplatelet therapy might lead to additional antithrombotic effects while only minimally affecting the risk of bleeding. The novel targets that are detailed in the following pages play diverse roles and are present at different locations in platelets. Some are either expressed on the platelet membrane, such as GPVI, PAR4, GPIbα, and 5-hydroxytryptamine receptor subtype 2A, or stored inside the platelets and translocated to the surface upon activation, such as P-selectin and protein disulphide isomerases. Others are found inside platelets and function as the main intracellular effectors in downstream signalling pathways, such as phosphoinositide 3-kinase β and phosphodiesterase 3. As well as their role in platelets, 5-hydroxytryptamine receptor subtype 2A and phosphodiesterase 3 are expressed in endothelial cells of blood vessels and are also involved in vasoconstriction. These receptors have therefore also been investigated in peripheral vascular disease.

1.12.1 PAR4 receptor

Thrombin activates human platelets via two distinct protease-activated receptors, PAR1 (high-affinity thrombin receptor) and PAR4 (low-affinity thrombin receptor), that signal through heterotrimeric G proteins (Rasmussen et al., 1991, Xu et al., 1998a). Due to its high affinity for thrombin, PAR1 was seen as a promising antithrombotic target. However, in phase III studies of the PAR1 antagonist vorapaxar, it was associated with significant increase in risk of major

bleeding, including intracranial haemorrhage, when used in combination with aspirin and/or a $P2Y_{12}$ inhibitor, which limited its clinical use (Ungar et al., 2018, Tricoci et al., 2012). As a result, the interest shifted towards PAR4 as a new therapeutic target for thrombin-induced platelet mechanisms. Thrombin cleaves PAR4 through its N-terminus and induces a low level of sustained calcium signalling required for thrombus stabilisation and ADP release, while synergistically activating $P2Y_{12}$ and mediating platelet–leukocyte interactions (Xu et al., 1998b, Shapiro et al., 2000, Covic et al., 2000, Holinstat et al., 2006, Rigg et al., 2019). The potential therapeutic benefit of PAR4 antagonism might be affected by genetic variants of the receptor, such as the single nucleotide polymorphisms Ala120, Thr120, p.Tyr157Cys, and rs773902. These variants alter receptor reactivity, which is important to take into account during pharmacological development and application of PAR4 inhibitors (Edelstein et al., 2014, Morikawa et al., 2018, Norman et al., 2016). Indeed, a new antibody-based PAR4 inhibitor has been developed to target some of these variants (French et al., 2018).

1.12.2 P-selectin

P-selectin, also known as CD62P, is a protein that functions as an adhesion molecule on the surface of stimulated platelets and epithelial cells (McEver and Martin, 1984). It is stored in the alpha granules of platelets and the Weibel–Palade bodies in endothelial cells. It is expressed on the cell surface once the platelet is activated and is therefore used as a marker of platelet activation (McEver and Martin, 1984, Vischer and Wagner, 1993). P-selectin binds to P-selectin glycoprotein ligand-1 that is present on leukocytes (neutrophils, monocytes, eosinophils, and lymphocytes) and mediates platelet interactions with these cells and leukocyte–endothelial interactions (Furie and Furie, 2004, Burns et al., 1999). P-selectin may also facilitate platelet–platelet interactions, thereby stabilising platelet aggregates (Merten and Thiagarajan, 2000). P-selectin has an established role in vaso-occlusion in cardiovascular diseases through reinforcing

platelet thrombus formation and mediating platelet–leukocyte interactions and subsequent inflammatory responses (Sarma et al., 2002, Huo et al., 2003). Platelet–leukocyte–endothelial cell interaction is involved in the pathogenesis of atherosclerotic plaque formation and thrombi amplification that arise after rupture of the plaque. Blocking platelet-leukocyte aggregate formation by inhibiting P-selectin appears to be an attractive antiplatelet strategy with a promising therapeutic value, given that the current antiplatelet approach does not fully block platelet-leukocyte aggregate formation, although the formation is partially diminished by $P2Y_{12}$ inhibitors (Storey et al., 2002, Xiao and Théroux, 2004).

1.12.3 5-hydroxytryptamine subtype 2A receptor

The 5-hydroxytryptamine subtype 2A receptor ($5\text{-}HT_{2A}$) is a G protein-coupled receptor expressed in the central nervous system, platelets, and vascular smooth muscle. Serotonin (5-HT) is stored in platelet dense granules, and it is thought to amplify platelet activation through $5\text{-}HT_{2A}$ (Ashton et al., 1986). 5-HT is a weak platelet stimulus that potentiates platelet aggregation induced by major platelet ligands, such as ADP, thrombin, and epinephrine, but not on its own (Li et al., 1997). Use of selective serotonin reuptake inhibitors (SSRIs), clinically used as antidepressants, inhibits the serotonin transporter (SERT) that prevents the reuptake of 5-HT by platelets. This approach depletes 5-HT from platelet dense granules and reduces serotonin potentiated platelet activation (Bismuth-Evenzal et al., 2012). In fact, a recent study showed that patients with depression and cardiovascular disease have higher $5\text{-}HT_{2A}$ receptor density with upregulated signalling, as well as a higher incidence of cardiac events (Williams et al., 2019). Correspondingly, long-term use of selective serotonin reuptake inhibitors (SSRIs) is associated with a reduced risk of myocardial infarction (Sauer et al., 2001, Noordam et al., 2016).

1.12.4 Protein disulfide isomerase

PDI is expressed on the extracellular surface of platelets in a catalytically active form and is secreted by activated platelets from the dense tubular system where it is stored (Essex et al., 1995, Chen et al., 1992). PDI supports platelet aggregation and secretion, stimulates GPIIb/IIIa activation, and supports thrombus formation without affecting haemostasis (Kim et al., 2013b, Essex and Li, 1999). PDI also enhances platelet-neutrophil interactions, dependant on GPIbα under thrombo-inflammatory conditions (Li et al., 2019a), and is upregulated in the area of the myocardial infarct (Severino et al., 2007). PDI inhibition offers a unique pharmacological property in that it has both antiplatelet and anticoagulant effects, which may lead to a new form of antithrombotic therapy. Most therapeutic agents used in ACS treatment are either solely antiplatelet or solely anticoagulant in their inhibitory mechanisms, and so they are given in combination. PDI would be considered an antithrombotic target when there is a need to suppress both platelet and coagulation functions in one compound.

1.12.5 Phosphoinositide 3-kinase β

Phosphoinositide 3-kinases (PI3Ks; class I, II, and III) are lipid kinases that activate and modulate several effector proteins involved in ubiquitous intracellular signalling pathways that are triggered following phosphorylation of the 3-OH group of membrane phosphoinositide (PIs) by PI3Ks (Vanhaesebroeck et al., 2012). All class I PI3K isoforms (α, β, γ, and δ) are present in platelets, but the major focus of investigation regarding their antithrombotic effect has been on PI3Kβ, as this is the main isoform that contributes to platelet responses (Jackson et al., 2005). PI3Kβ regulates GPIIb/IIIa-dependent adhesion, Akt activation mediated by GPVI and ADP, and ERK activation, including subsequent TxA_2 synthesis (Jackson et al., 2005, Kim et al., 2009, Garcia et al., 2010). Depletion of PI3Kβ prevents occlusive thrombosis development in animal models while preserving primary haemostasis (Jackson et al., 2005, Martin et al., 2010).

PI3Kβ could potentially be utilised as an antiplatelet target to provide antithrombotic protection in instances where combining aspirin with $P2Y_{12}$ antagonists presents too high a risk of bleeding, attributed to the fact that AZD6482 produced more significant inhibition of platelet activation when used with aspirin than that achieved with aspirin plus clopidogrel with less bleeding tendency. The use of PI3Kβ as a substitute for $P2Y_{12}$ inhibitors in settings involving ACS could next be validated in a phase II clinical trial, because the phase I trial demonstrated encouraging antiplatelet effects when COX-1 and PI3Kβ inhibitors were combined in healthy volunteers.

1.12.6 Phosphodiesterase 3

Phosphodiesterases (PDEs) catalyse the breakdown of the intracellular second messengers cAMP and cGMP to inactive 5′-AMP and 5′-GMP, which are powerful inhibitors of signal transduction during cellular activation (Azevedo et al., 2014). Human platelets possess three PDE isoenzymes: PDE2, PDE3, and PDE5 (Gresele et al., 2011, Hidaka and Asano, 1976, Grant and Colman, 1988). In addition to platelets, PDEs are present in the vascular smooth muscle, heart, and corpus cavernosum (Tilley and Maurice, 2002, Movsesian et al., 2018, Wallis et al., 1999). Targeting PDE3 is believed to provide more effective antiplatelet action than PDE2 and PDE5. The antiplatelet effect of PDE3 has been shown in several PDE3 inhibitors that have been introduced in clinical practice, such as cilostazol and milrinone (Manns et al., 2002). PDE3 inhibitors deliver their antiplatelet effect with a vasodilatory effect that may reduce restenosis and improve blood flow after PCI. As an example, in the CREST trial, cilostazol did not increase bleeding when used in combination with a GPIIb/IIIa inhibitor plus DAPT in patients undergoing PCI (Douglas et al., 2005, Bangalore et al., 2014). As a result, use of PDE3 inhibitors concomitantly with aspirin and $P2Y_{12}$ antagonists in patients with ACS undergoing PCI could lead to better outcomes regarding revascularisation.

Table 1.2. Overview of key PAR4 and P-selectin inhibitors and their effects on thrombosis and haemostasis

Platelet targets & their inhibitors	Class of inhibitor	Mode of action	Antiplatelet properties	Risk of Bleeding	Route	The level of development	Reference
PAR4							
BMS-986120	Small molecule	Directly binds to PAR4	Reduced thrombin-induced platelet aggregation in humans Inhibited PAR4-AP-induced platelet aggregation and prevents occlusive thrombus formation in an animal model	Safe and did not cause bleeding or affect coagulation No bleeding reported in phase I	Oral	Clinical trials: Phase I completed	(Wilson Simon et al., 2018)
BMS-986141	Small molecule	Directly binds to PAR4	Blocked PAR4- mediated platelet mechanisms and prevented occlusion of blood vessels in a non-human primate model of arterial thrombosis	Slightly increased mesenteric artery bleeding time (1.2-fold) but less than aspirin (2.2-fold) and clopidogrel (8.1-fold) in monkeys	Oral	Clinical trials: Phase I completed, phase II in process	(Wong Pancras et al., 2017)
P-selectin							
Inclacumab	Monoclonal antibody	Binds to P-selectin	Reduced soluble P-selectin levels and total P-selectin activity and reduced platelet-leukocyte aggregate formation	Safe, it did affect bleeding time or coagulation in healthy subjects during phase I trial No bleeding events reported in CABG patients following inclacumab infusion	IV	Clinical trials: Phase I completed Phase II completed	(Schmitt et al., 2015, Kling et al., 2013)
Crizanlizumab	Monoclonal antibody	Binds to P-selectin	Blocked P-selectin function	Safe, no severe bleeding reported in phase I and II trials	IV	Clinical trials: Phase I completed Phase II completed	(Stocker et al., 2013, Kutlar et al., 2019)

Table 1.3 Overview of key 5-HT_{2A} inhibitors and their effects on thrombosis and haemostasis

Platelet targets & their inhibitors	Class of inhibitor	Mode of action	Antiplatelet properties	Risk of Bleeding	Route	The current level of development	Reference
5-HT_{2A}							
Sarpogrelete	Small molecule	Binds to 5-HT_{2A}	Reduced platelet aggregation-stimulated by collagen and epinephrine Prolonged artery occlusion time and reduced thrombus formation in an animal model	Safe and did not increase bleeding when used on the top of DAPT in STEMI patients Bleeding was decreased significantly in sarpogrelete arm than aspirin arm when evaluated in stroke patients	Oral	Already in clinical use	(Uchiyama et al., 2007, Choi et al., 2017, Hara et al., 1991, Shinohara et al., 2008)
APD791	Small molecule	Binds to 5-HT_{2A}	Inhibited 5-HT-mediated amplification of platelet aggregation-induced by ADP and collagen Diminished recurrent thrombosis and improved coronary patency in an animal model of thrombosis	Did not increase template bleeding time in dogs Safety needed to be established in humans	Oral	Clinical trials: Phase I terminated	(Adams et al., 2009, Przyklenk et al., 2010)
Cyproheptadine and pizotifen	Small molecules	Bind to 5-HT_{2A}	Blocked serotonin enhancement of ADP and TxA_2 mediated platelet responses Delayed carotid artery occlusion time in animal models of thrombosis	Prolonged tail bleeding time in mice	Oral	Already in clinical use	(Lin et al., 2014)

Table 1.4. Overview of key PDI inhibitors and their effects on thrombosis and haemostasis

Platelet targets & their inhibitors	Class of inhibitor	Mode of action	Antiplatelet properties	Risk of Bleeding	Route	The current level of development	Reference
PDI							
Quercetin-3-rutinoside	Small molecule	Binds to the substrate-binding b'x domain of PDI	Inhibited human and mouse platelet aggregation Inhibited thrombus formation in an animal model	Safe, it is a dietary flavonoid	Oral	Pre-clinical	(Lin et al., 2015, Jasuja et al., 2012)
Isoquercetin	Small molecule	Binds to the substrate-binding b'x domain of PDI	Decreased infarct size in the rat model of ischemic stroke Reduced thrombin generation, D-dimer formation, and soluble P-selectin level in healthy volunteers and patients	Safe, no bleeding events reported in cancer patients during phase II trial	Oral	Clinical trials: Phase I completed Phase II completed	(Stopa et al., 2017, Zwicker et al., 2019)
ML359	Small molecule	Binds to the substrate-binding b'x domain of PDI	Inhibited platelet -thrombus formation in a murine model of arterial thrombosis	Unknown; has not been assessed yet	Given intravenously to mice during the pre-clinical study	Pre-clinical	(Bendapudi et al., 2014)
HPW-RX40	Small molecule	Binds to catalytic a′ domain of PDI	Inhibited platelet aggregation, GPIIb/IIIa stimulation, diminished thrombus formation in human blood under flow conditions and protected mice from arterial occlusion	Unknown; has not been assessed yet	Given via intraperitoneal injection to mice during the pre-clinical study	Pre-clinical	(Kung et al., 2017)

Table 1.5 Overview of key PI3Kβ and PDE3 inhibitors and their effects on thrombosis and haemostasis

Platelet targets & their inhibitors	Class of inhibitor	Mode of action	Antiplatelet properties	Risk of Bleeding	Route	The current level of development	Reference
PI3Kβ							
AZD6482	Small molecule	Binds to ATP binding site of PI3Kβ	Blocked platelet aggregation-stimulated by low concentrations of multiple agonists and inhibited platelet aggregation and adhesion When combined aspirin, it provided more significant inhibition of platelet activation than that observed with aspirin and clopidogrel	Safe, it did not increase bleeding time in healthy volunteers during phase I Less impact on bleeding when combined with aspirin than aspirin plus clopidogrel	IV	Clinical trials: Phase I completed	(Nylander et al., 2012, Nylander et al., 2015)
PDE3							
Cilostazol	Small molecule	Binds to PDE3 and rises cAMP	Inhibited shear stress-stimulated platelet aggregation and collagen, arachidonic acid ADP-induced platelet aggregation *in vitro* and *ex vivo*	Safe, haemorrhagic stroke and GI bleeding were less frequent in patients on cilostazol than those on aspirin	Oral	Already in clinical use	(Minami et al., 1997, Ikeda et al., 1987)
Milrinone	Small molecule	Binds to PDE3 and rises cAMP	Reduced monocyte tissue factor expression Inhibited ADP and AA-stimulated platelet aggregation *in vitro*	Safe, it did not cause intraoperative bleeding in CABG patients	IV	Already in clinical use	(Wesley et al., 2009, Beppu et al., 2009, Kikura and Sato, 2003)

1.13 GPVI as novel antiplatelet target in acute coronary syndromes

1.13.1 Identification of GPVI as a collagen receptor

GPVI was first described by David et al. in 1977 by gel electrophoresis with a molecular weight around 60 kDa (Moroi and Jung, 2004). The notion of GPVI as a possible receptor for collagen was proposed by Sugiyama et al. in 1987 when they reported a case of idiopathic thrombocytopenia purpura (ITP) in which the patient's platelets were not aggregating in response to stimulation by collagen (Sugiyama et al., 1987). The role of GPVI as a platelet receptor for collagen was confirmed in 1989 when Mori et al. reported a case of ITP in which the patient serum contained antibodies against GPVI. Mori and his colleagues found that the platelets from the patient normally responded to stimulation induced by multiple agonists, and collagen was the only exception. Moreover, they revealed that the autoantibodies from the patient's serum did not react with GPVI-deficient platelets isolated from the same patient. Upon this observation Mori et al. proved that P62 and GPVI are the same compound (Moroi and Jung, 2004, Moroi et al., 1989).

Isolation and cloning of the GPVI gene in 1999 helped to reveal the function of GPVI and its genetics and molecular structure and to identify its agonists and antagonists (Clemetson et al., 1999). GPVI was confirmed as a central signalling platelet receptor for collagen in 2003 by Kato et al. through the generation of a $GPVI^{null}$ mice model via deletion of the GPVI gene. Platelets from the $GPVI^{null}$ mice failed to aggregate in the absence of the thrombus formation (Kato et al., 2003). The crystal structure of GPVI and the binding sites for interaction with collagen were identified by the molecular replacement technique in 2006 (Horii et al., 2006).

1.13.2 Structure of the GPVI/FcRγ complex

GPVI is a type I membrane protein and a member of the immunoglobulin superfamily of cell surface receptors with two external immunoglobulin-like domains known as D1 and D2 with an O-glycosylation site and an N-linked glycosylation site, a transmembrane domain and one cytoplasmic tail (Figure 1; (Gardiner et al., 2008, Horii et al., 2006). The two external loops are aligned at an angle of 90 degrees and kept at a distance from the platelet surface membrane via a rigid mucin-type link (Horii et al., 2006). Regarding its molecular structure, human GPVI consists of 339 amino acids; it is encoded by the GP6 gene, which consists of 8 exons and is located on the chromosome 19q13.4 (Ezumi et al., 2000). The hinge region between D1 and D2 along with the uppermost portion of the D1 loop contains a group of basic residues (K41, R46, K59, R166), hydrophobic residues (L53, F54, P56, L62, and Y66), and several polar residues (S43, S44, Q48, Q50, and S61). Those basic residues and hydrophobic sites are involved in the interaction of GPVI with collagen (Horii et al., 2006). The D2 loop also contributes to GPVI binding with collagen through the formation of back-to-back dimers (Horii et al., 2006). The N-glycosylation site present at the far end of external domains is important for the maximal binding of the receptor to collagen and convulxin (Kunicki et al., 2005). The O-glycosylation site found at the end of the external domains immediately above the cell membrane is believed to have a role in the elongation of the external domains and their protection against proteolysis (Clemetson et al., 1999). The cytoplasmic tail contains one juxta-membrane calmodulin-binding basic motif and one proline-rich domain (PxxP) to which Lyn and Fyn (the Src-family kinases) are attached in a pre-active configuration that changes the receptor to a ‘ready to work’ state (Schmaier et al., 2009).

GPVI is expressed with the fragment crystallisable receptor γ-chain (FcRγ-chain) in a noncovalent association via a salt bridge, forming a GPVI/FcRγ complex (Tsuji et al., 1997).

The salt bridge is formed through conjugation of the arginine 252 residue in the transmembrane domain of GPVI with an aspartate residue in the transmembrane domain of FcRγ (Berlanga et al., 2002). The FcRγ-chain, which was first recognised as a subunit of the specific receptors for immunoglobulin E, is expressed on multiple types of immune cells, such as mast cells, basophil cells, and NK cells (Park et al., 1995). The FcRγ-chain itself is composed of two monomers (homodimers) linked by a disulfide bond (Ernst et al., 1993). The FcRγ-chain is a critical constituent for the expression and function of GPVI, as has been shown in FcRγ chain-immunodepleted mice, which lost the response to collagen and lost the GPVI bands obtained by immunoprecipitation (Nieswandt et al., 2000). The FcRγ-chain contains two immunoreceptor tyrosine-based activation motifs (ITAMs) in its intracellular domain that are a necessary component for the GPVI signalling process to lead to platelet activation. The GPVI/FcRγ complex exists in monomeric and dimeric forms. Compared with the monomeric form, the dimeric form has a high affinity for collagen, and so dimerisation and clustering of GPVI significantly affect its signalling and function (Miura et al., 2002). GPVI is solely expressed on megakaryocytes (platelet precursors) and platelets, and it is estimated that there are approximately 3,000 copies of GPVI expressed on each platelet (Ozaki et al., 2013).

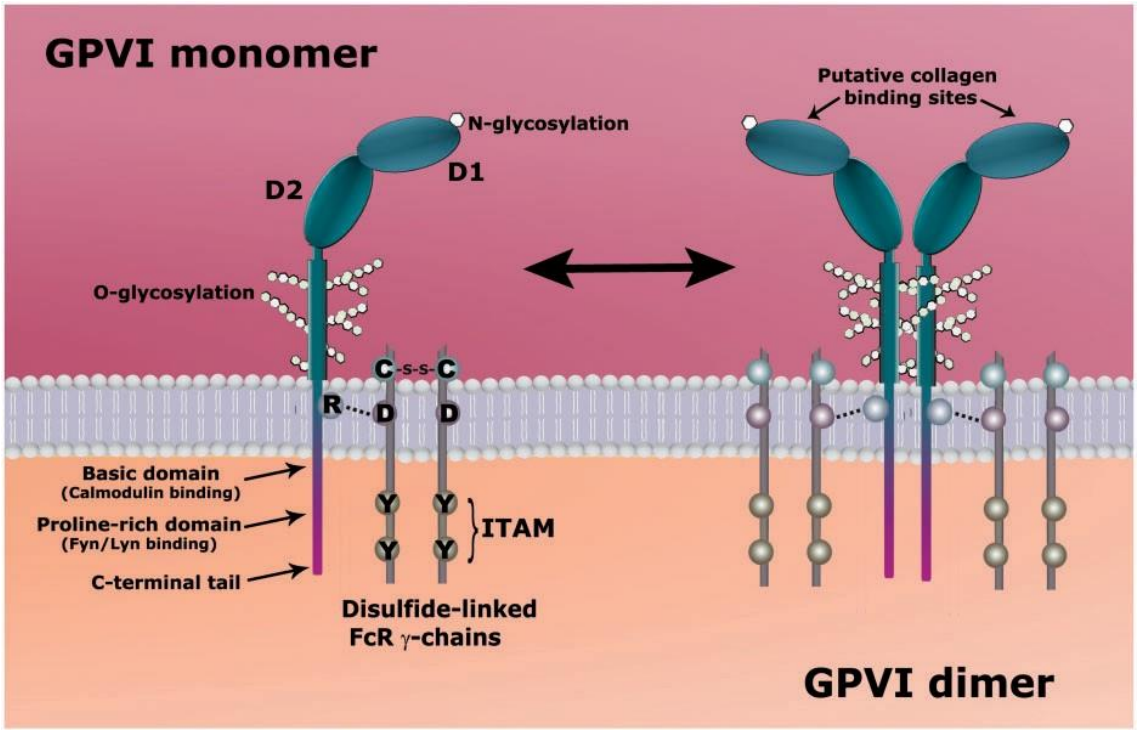


Figure 1.8. Schematic structure of monomeric and dimeric GPVI molecules.

Each GPVI monomer consists of 2 extracellular Ig-like domains (D1 and D2) with an N-glycosylation site at the terminal end of D domains and O-glycosylation at their basal ends on the membrane surface, one transmembrane domain, and a cytoplasmic tail. Each GPVI monomer through its transmembrane domain is a non-covalently associated FcRγ-chain that is itself made of two monomers linked together by a disulfide bond. FcRγ-chain contains signalling ITAM (Induruwa et al., 2016).

1.13.3 GPVI signalling pathway

The GPVI signalling pathway starts with the crosslinking of the GPVI/FcRγ complex by one of its ligands. The presence of GPVI in dimeric form is not sufficient to induce platelet activation unless it is crosslinked by one of its multivalent ligands (Poulter et al., 2017b). It has been proposed that GPVI dimerisation and GPVI clustering (oligomerisation) are the two main conformational changes that GPVI undergoes prior to platelet activation (Poulter et al., 2017b). In basal platelets, homodimers of the FcRγ chain are maintained away from each other to prevent platelet activation, and disruption of this orientation may induce platelet signalling (Berlanga et al., 2007). It has been suggested that the signalling chain homo-oligomerisation model applies to GPVI and FcRγ chain interaction that initiates intraplatelet signalling (Sigalov, 2008). This model states that both the nearness of the signalling chain to the receptor and the optimal relative orientation to each other are required to stimulate the phosphorylation of cytoplasmic tyrosine (Sigalov, 2008). Clustering of GPVI dimers induces upstream signalling in the lipid raft through sequential phosphorylation of tyrosine residues in ITAM in the FcRγ immunoreceptor by binding with the tandem SH2 domains of Fyn and Lyn (SFKs) (Ezumi et al., 1998a, Briddon and Watson, 1999). The proline-rich domain of GPVI's cytoplasmic tail mediates the interaction between ITAMs and Src kinases; through their SH3 domain, Lyn and Fyn are associated with the proline-rich domain (Suzuki-Inoue et al., 2002). The receptor-like protein tyrosine phosphatase CD148 positively regulates the signal transmission between ITAMs and SFKs by dephosphorylation of the inhibitory sites in SFKs (Senis et al., 2009). Furthermore, the tyrosine phosphatase CD148 promotes GPVI signalling and enhances platelet response to collagen stimulation by maintaining an active pool of SFKs (Ellison et al., 2010).

Phosphorylated ITAMs recruit and bind to an SH2 domain of the spleen tyrosine kinase (Syk), which in turn leads to initiation of further downstream signalling cascades (Asselin et al., 1997).

Phosphorylated Syk triggers downstream platelet signalling through the involvement and phosphorylation of a complex mixture of protein adapters and protein kinases: LAT, Tec family tyrosine kinases (Tec and Btk), phosphatidylinositol 3-kinase, the GTP exchange factors (Vav1 and Vav3), and small G proteins known as LAT signalosome. LAT signalosome passes signal transduction down to phospholipase C-gamma-2 (PLCγ2), and its activation, in turn, leads to mobilisation of intracellular calcium (Atkinson et al., 2003a, Quek et al., 1998, Gross et al., 1999, Nieswandt et al., 2001a, Watson et al., 1985, Pearce et al., 2004). Among the adaptors involved in the signalling are two cytosolic adapters, known as the Src homology 2 domain-containing leucocyte protein of 76 kDa (SLP-76) and Gads, and one transmembrane adapter, linker for activation of T lymphocytes (LAT; (Gross et al., 1999, Pasquet et al., 1999). Gads are needed to create a link between SPL-76 and LAT to form the LAT signalosome. Activation of LAT is achieved by phosphorylating the Y195, Y175, and Y235 C-terminal tyrosine residues of LAT. Engineered mutations in these residues can cause inhibition of PI 3-kinase activation, but not its ability to translocate PLCγ2 to the plasma membrane (Ragab et al., 2007). Tec kinase can compensate for the absence of Btk kinase in the tyrosine phosphorylation of PLCγ2 in murine platelet activation related to GPVI (Atkinson et al., 2003a). Btk-null platelets from a human patient with X-linked agammaglobulinemia showed a reduction in aggregatory and secretory functions caused by diminished phosphorylation of PLCγ2 in response to GPVI agonists (Quek et al., 1998). Activation of PLCγ2 breaks down its substrate, phosphatidylinositol-4,5-bisphosphate (PIP2), to generate the second messengers, DAG and IP_3 (Watson et al., 1985, Rittenhouse and Allen, 1982). The produced IP_3 causes the release of the calcium from the dense tubular system (Authi and Crawford, 1985, Adunyah and Dean, 1985). Moreover, by releasing intracellular calcium, IP_3 stimulates the activation of phospholipase A_2 and subsequently the production of thromboxane A_2 from arachidonic acid breakdown by

thromboxane synthase and cyclooxygenase-1 (Watson et al., 1986, McNicol and Shibou, 1998). Thromboxane A_2 serves as a positive mediator during platelet activation, and it recruits and aggregates platelets near the formed plug via activation of G_q-coupled receptors (Kinsella et al., 1997). DAG activates the PKC family, including the conventional PKC isomers (PKCα and PKCβ) and the novel isoforms (δ and θ; (Huang, 1989, Gilio et al., 2010). The conventional PKCα and PKCβ isoforms mediate α-granule secretion and thrombus growth. PKC stimulates the release of the contents of dense granules via activation of protein kinase D (Konopatskaya et al., 2011). Dense granules contain ADP, ionised calcium, and serotonin, while α-granules encompass fibrinogen, vWF, P-selectin, and coagulation factors V and XI. The liberation of these vesicles' content therefore potentiates the coagulation process, as the released elements have central roles in both primary and secondary haemostasis (Blair and Flaumenhaft, 2009b).

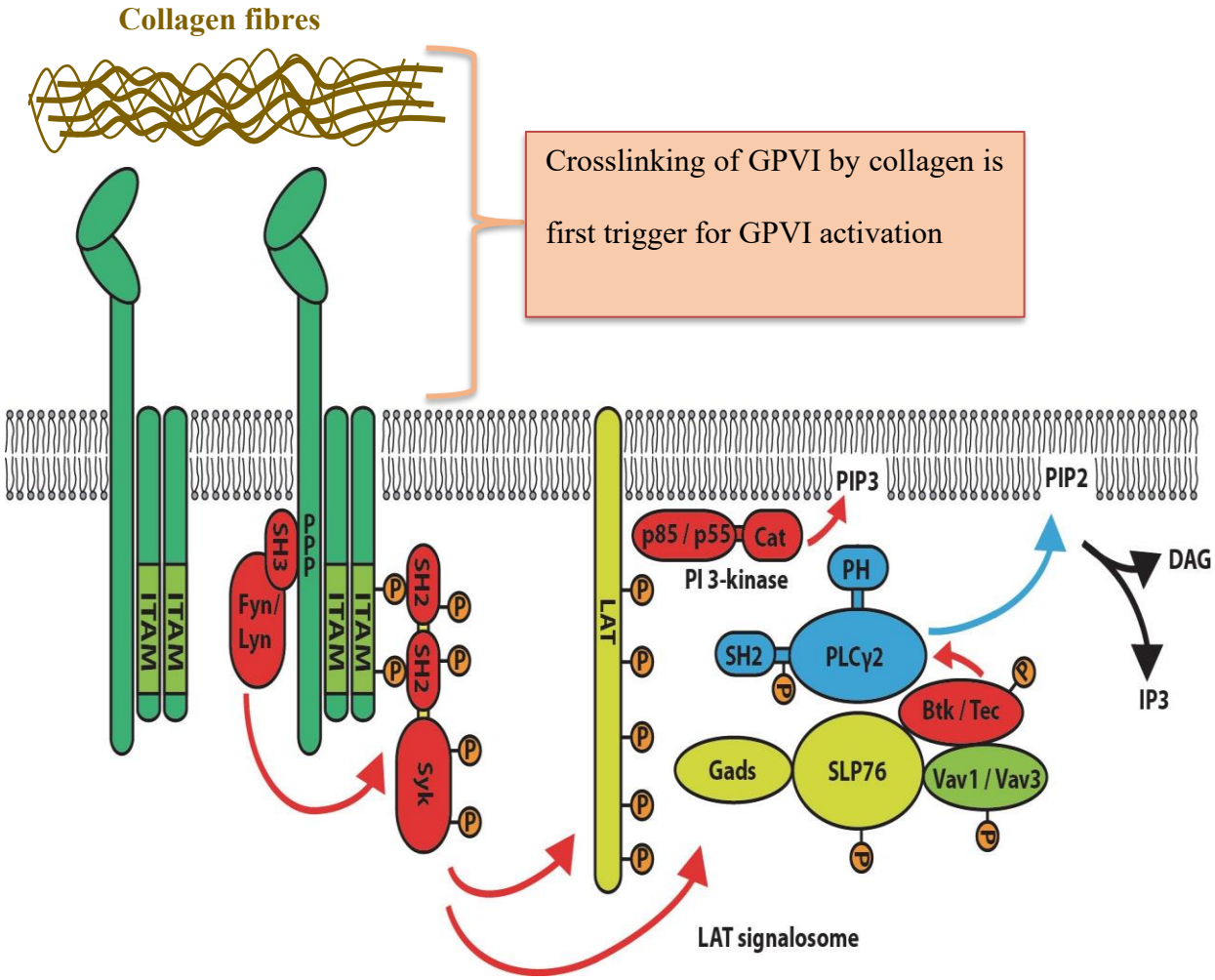


Figure 1.9. GPVI signalling pathway.

GPVI signalling starts with clustering GPVI dimers, which causes phosphorylation of tyrosine residues in the ITAM signalling motif in FcRγ-chain by Src kinases (Fyn and Flyn). Phosphorylated ITAM recruits and activates Syk, which in turn triggers phosphorylation of a complex of adapter proteins such as LAT, Gads, SLP76 protein kinases, Tec family tyrosine kinases (Tec and Btk), phosphatidylinositol 3–kinase (PI-3 kinase) and GTP exchange factors (Vav1 and Vav3). The Lat signalosome eventually activates PLCγ2 leading to the mobilisation of intracellular calcium, the release of potent mediators and inside-out signalling. The figure was adapted from (Bleijerveld et al., 2013).

1.13.4 GPVI function

GPVI appears to have a minor role in preventing bleeding after an injury by activating platelets that have come into contact with collagen that has been exposed in the subendothelial part of the injured vessel. GPVI is involved in the mediation of the firm adhesion of platelets to collagen (Watson et al., 2010). It facilitates platelet adhesion under high shear rate along with GPIa/IIa to collagen (Chen et al., 2002). Platelets from GPVI/FcRγ complex-deficient mice failed to form a thrombus at shear rates of 800 s^{-1} and 1,500 s^{-1} (Best et al., 2003a). Not only does GPVI promote platelet adhesion to collagen, but more importantly it also induces collagen signalling cascades that initiate platelet activation. When circulating platelets bind to immobilised collagen fibres via adhesive receptors such as GPIa/IIa, GPVI triggers platelet activation pathways, in which the platelets undergo a series of biochemical events and structural changes that eventually lead to platelet thrombus formation (Carrim et al., 2014). Dimerisation of GPVI has a significant impact on the function of the receptor in platelet adhesion and signalling, because the dimeric form of GPVI has a higher affinity for collagen fibres than the monomeric form (Jung et al., 2012). Although measurement is problematic, it is estimated that around 29% of GPVI receptors on resting platelets are present in dimeric form, giving platelets the ability to respond quickly to exposed collagen in the injured vessel (Jung et al., 2012). As well as its dispensable role in haemostasis and critical role in thrombosis, GPVI plays additional roles in inflammation and immune responses (Claushuis et al., 2018). It has been found that GPVI helps in local immunity against gram-negative pneumonia-derived sepsis by enhancing leukocyte–platelet interaction (Claushuis et al., 2018).

1.13.5 GPVI ligands

The GPVI receptor can be activated by different ligands, including physiological agonists such as collagen, fibrin, laminin, and naturally occurring ligands like convulxin and synthetic agonists such as collagen-related peptide (CRP).

1.13.5.1 Collagen

Collagen is one of the most abundant proteins in the human body, comprising a third of the total protein content, with a vast range of functions and roles related to the structure of body tissues, cellular adhesion, and cellular migration (Shoulders and Raines, 2009, Kadler et al., 2007). There are 28 types of collagen reported in vertebrates, which are assigned Roman numerals (I to XXVIII) (Kadler et al., 2007). Structurally, collagens are triple-helical proteins, and the most abundant types of collagen in the human body are collagen I and II fibrils that are present as fundamental *D*-periodic repeats of 67 nm (Kadler et al., 2008). The extracellular matrix of collagen's triple helix consists of a unique amino acid sequence of (Gly-X-Y)(n) repeats, which gives the triple-helical collagen an essential motif that is adaptable and applicable to different functions (Brodsky and Persikov, 2005). Collagen types I (the most predominant), III, and V are involved in the construction of the extracellular matrix of blood vessels, while collagen IV is more common in the basement membrane (Morton and Barnes, 1982). In addition to its abundance in the vessel wall, collagen is one of the most thrombogenic constituents of the exposed subendothelial area of the injured vessel. Interaction of collagen fibrils with platelets results in platelet activation and aggregation. Collagen binds platelets directly via GPVI and GPIa/IIa receptors and indirectly via the GPIb-V-IX receptor complex with the aid of vWF, which is needed to maintain platelet adhesion under high shear (Nuyttens et al., 2011). Collagen fibrils bind GPVI through a specific motif of glycine-proline-hydroxyproline (GPO) triplets (Knight et al., 1999b). As mentioned before, only dimeric GPVI binds collagen with high

affinity, making GPVI dimerisation a prerequisite for collagen-induced platelet activation. Moreover, GPVI possesses variable affinity for different collagen fibril types. The collagen types it binds with the highest affinity are types I and III. (Semeniak et al., 2016).

1.13.5.2 Laminin

Laminin is another physiological ligand that activates GPVI (Ozaki et al., 2009). It is the most abundant non-collagenous protein in the basement membrane (Aumailley and Smyth, 1998). Laminin is a heterodimer glycoprotein that consists of α, β, and γ chain subunits (Colognato and Yurchenco, 2000). As well as its main structural function in the basal lamina, laminin isoforms interact with intracellular signalling pathways through their surface receptors, which mainly consist of integrins (Mercurio, 1995). Laminin promotes cancer dissemination, growth, and metastasis due to its anatomical position and its ability to induce signals via different signal transduction pathways that include various elements such as G-proteins, phospholipase D, intracellular Ca^{2+}, focal adhesion kinase, and mitogen-activated protein kinases (Givant-Horwitz et al., 2005). Laminin is generally considered a weak platelet agonist, and its effect can be masked by collagen (Bergmeier and Hynes, 2012). In 1984, laminin was identified an adhesive protein for platelets, contributing to platelet adhesion to the endothelial matrix (Ill et al., 1984). Laminin-induced platelet activation was first demonstrated in 1994 when Willette et al. showed that laminin stimulates platelet aggregation through the integrin α6β1 receptor (Willette et al., 1994). Laminins also cause platelet activation through interaction with GPVI, and the binding of laminin to the α6β1 receptor is prerequisite to GPVI–laminin binding (Inoue et al., 2006).

1.13.5.3 Fibrin

After collagen, fibrin may be the second most important endogenous GPVI ligand. It is generated from fibrinogen under the influence of thrombin during coagulation. Subsequently,

generation of the crosslinked fibrin clot strengthens and stabilises the platelet plug. Alongside its contribution to the haemostatic plug stabilisation, fibrin promotes wound repair and regulates fibrinolysis (Thompson et al., 1991, Aoki et al., 1983). Fibrinogen is a fundamental glycoprotein in blood coagulation, consisting of three pairs of polypeptide chains (Aα, Bβ, and γ_2) bound together by disulfide linkages (Weisel, 1986). Thrombin cleaves fibrinopeptide A and B from Aα- and Bβ-chains of soluble fibrinogen at two specific sites (Blomback et al., 1978). It rapidly releases fibrinopeptide A from the Aα-chain at 16Arg-17 Gly, exposing the N-terminus of the α-chain motif Gly-Pro-Arg, known as knob A. At a lower rate, thrombin liberates fibrinopeptide B from the Bβ-chain at 14Arg-15Gly, exposing the N-terminus of the β-chain motif Gly-His-Arg-Pro, known as knob B (Zucker et al., 1979). Knob A is complementary to hole 'a' located in the γ-nodules of another fibrin molecule, and knob B is complementary to hole 'b' based in the globular β-nodule of another fibrin molecule (Kostelansky et al., 2002). The knob–hole interactions take place after the release of fibrinopeptides that form fibrin monomers, during the earliest process of fibrinogen cleavage by thrombin. The knob–hole engagements convert fibrin monomers to fibrin oligomers, which are transformed to fibrin protofibrils under the action of lateral aggregation. This is enhanced by the homophilic attractions between α-C-αC- regions, which arise within and between protofibrils. Protofibrils are then packaged into fibrin fibres (Weisel, 1986). Calcium ions are required in the fibrin polymerisation process, promoting the lateral aggregation of fibrin oligomers into fibrin protofibrils and supporting the crosslinking of fibrin with factor XIIIa (Brass et al., 1978, Siebenlist et al., 2001). Alshehri et al. reported that fibrin is an endogenous agonist for the GPVI receptor in human and mice platelets and that fibrin induces tyrosine phosphorylation following GPVI binding in a pattern similar to that caused by collagen binding (Alshehri et al., 2015). Mammadova-Bach et al. also showed that polymerised fibrin is a ligand

for human GPVI and that the fibrin–GPVI interaction increases thrombin generation. Furthermore, this interaction attracts more circulating platelets on to thrombi that have been crosslinked by fibrin, ultimately stimulating thrombus propagation and growth (Mammadova-Bach et al., 2015). The exact site of recognition and the forms of GPVI involved in the GPVI–fibrin interaction is a matter of dispute. Onselaer et al. showed that monomeric GPVI binds to fibrin at its D-domain (D-dimer), in contrast to the collagen–GPVI association, in which collagen binds mainly to dimeric GPVI (Onselaer et al., 2017). However, Induruwa I et al. reported that only dimeric GPVI recognises fibrin at its D-domain (Induruwa et al., 2018).

1.13.5.4 Collagen-related peptide

Collagen-related peptide (CRP) is a prominent synthetic stimulus of GPVI widely used to study GPVI by unveiling events underlying the GPVI signalling pathway and to evaluate the inhibitory effects of GPVI antagonists. CRP was first used in 1995 by Morton et al. to test the effect of collagen-like synthetic peptides made of repeated sequences of Gly-Pro-Hyp on the reactivity of platelets. The researchers concentrated on adhesion, aggregation, and secretion and found that CRP fully activates platelets in a collagen-like fashion (Morton et al., 1995). The synthetic triple-helical structure of CRP mimics collagen fibres and stimulates platelet response via GPVI receptors in the same way that collagen does. CRP activates platelets more potently than collagen: it can activate platelets even when ADP and TxA_2 inhibitors are used, whereas these inhibitors can block the stimulatory effect of collagen (Jarvis et al., 2002b). CRP stimulates GPVI activation via a motif composed of GPO triplets, and a change in the structural expression or quantity of the GPO content of CRP affects the potency of the agonist. For instance, monomer CRP is weaker than crosslinked CRP (CRP-XL), and the increase in GPO quantity within CRP increases the potency of its platelet activation (Smethurst et al., 2007).

1.13.5.5 Convulxin

Convulxin (Cvx) is a C-type lectin-like reptile-derived ligand that activates GPVI. It is a non-enzymatic glycoprotein isolated and purified from the venom of three subspecies of a tropical rattlesnake: *Crotalus durissus cascavella*, *Crotalus durissus terrificus*, and *Crotalus durissus collilineatus* (Prado-Franceschi et al., 1981). It is a heterodimeric glycoprotein (72 kDa) in nature, consisting of Mr 72,000 constructed of α and β subunits linked by disulphide bridges. The crystal structure of Cvx revealed that the α-β heterodimers could link together via disulfide bonds, creating a cyclic tetrameric structure (Marlas et al., 1984, Murakami et al., 2003, Leduc and Bon, 1998). Identification of Cvx as a platelet agonist preceded the identification of GPVI's function in platelet physiology. The first attempt to understand the mechanism behind Cvx-induced platelet activation and aggregation was made by B.B. Vargaftig et al. in 1980, who isolated the protein from the venom using a gel filter. The investigators observed that Cvx caused platelet aggregation in a concentration-dependent manner, accompanied by the release of ATP, the formation of thromboxane, the activation of phospholipase A_2, and a rise in intracellular calcium (Vargafting et al., 1980). Two years later, B.B. Vargaftig et al. carried out another study in which they concluded that Cvx stimulates platelet aggregation in a manner that resembles collagen-dependent platelet activation, independently of ADP and TxA_2 (Vargaftig, 1982). Cvx robustly activates platelets via the GPVI/FcRγ signalling pathway. It is the most potent GPVI stimulus, and its extreme potency can be attributed to the agonist's multimeric structure, which makes it able to cluster eight GPVI dimers (Horii et al., 2009).

1.13.6 Role of GPVI in thrombosis and acute coronary syndromes

As the central signalling platelet receptor for collagen, GPVI plays a major part in the initiation of atherothrombotic events (Cosemans et al., 2005). Upon contact with collagen fibres exposed from a damaged endothelium, clustering of dimeric GPVI takes place (Poulter et al., 2017a).

GPVI becomes activated and signals by a serial tyrosine phosphorylation cascade, resulting in inside-out signalling of integrin receptors and release of secondary mediators (Watson et al., 2005). Agents released from adherent platelets recruit more circulating platelets to the newly formed platelet monolayer at the site of the injured vessel wall. The developing clot is stabilised by crosslinked fibrin, which is generated by the action of thrombin on fibrinogen (Ariens et al., 2002). GPVI therefore acts as a primary thrombotic trigger following exposure of subendothelial collagen by facilitating platelet adhesion and more significantly initiating platelet activation. In an *in-vivo* model of arterial thrombosis, genetic depletion or inhibition of GPVI decreased platelet tethering and adhesion to the vessel wall by 89%, and the platelets failed to form a stable aggregation (Massberg et al., 2003). In addition to the essential role that GPVI plays in the first steps of adhesion and signalling, it makes another vital contribution to the development and growth of the recently created thrombus by functioning as a signalling receptor for polymerised fibrin that stabilises the thrombus (Mammadova-Bach et al., 2015). Fibrin polymers attract more platelets to the recently formed thrombus, increasing the size of the platelet aggregate and potentially leading to occlusion of the blood vessel. This can reduce blood flow, resulting in mortality and morbidity dependent on the location, duration, size, and type of occlusion and whether the vessel is arterial or venous. Therapeutic inhibition of collagen- and fibrin-induced GPVI signalling could be a useful and safe tool to prevent thrombosis in ACS.

1.13.7 GPVI as a therapeutic target in acute coronary syndromes

Since the identification of GPVI as a major signalling receptor for collagen, there has been interest in it as an antithrombotic target. This interest is based on the concept that GPVI may offer a favourable balance of efficacy and safety in patients at risk of thrombotic cardiovascular disease or patients who have already developed the disease. GPVI seems to be a promising

therapeutic target for ACS despite its low copy number for several reasons. First, GPVI plays a critical and extended role in arterial thrombosis, starting form platelet adhesion and activation, which initiates thrombus formation, and followed by fibrin-induced platelet signalling that supports propagation of the blood clot. Numerous studies have provided evidence that genetic depletion or inhibition of GPVI in animal models of arterial thrombosis reduces thrombus formation (Cosemans et al., 2005, Massberg et al., 2004). The second reason relates to the stimulatory effect. Collagen is one of the most thrombogenic components of the subendothelial matrix that is exposed following plaque rupture; in particular, collagen I and III are the main types of collagen present in the subendothelial layer of blood vessels (Morton and Barnes, 1982). Inhibition of GPVI reduces platelet aggregation stimulated by human atherosclerotic plaque under static and *ex vivo* arterial flow conditions (Jamasbi et al., 2015). As GPVI is a central signalling collagen receptor with high affinity to collagen types I and III, reducing its ability to bind to collagen can protect against the development of occlusive thrombi. Another advantageous feature that makes GPVI a promising safe therapeutic target is that cases of GPVI deficiency (either immunodeficiency or genetic deficiency) have shown that lack of GPVI can be tolerated, and patients usually have slightly prolonged mild bleeding defects and slightly or moderately low platelet counts (Kojima et al., 2006, Rabbolini et al., 2017, Hermans et al., 2009).

The current antiplatelet regimen prescribed for patients with ACS consists mainly of a COX-1 inhibitor (aspirin) and one of the $P2Y_{12}$ inhibitors (clopidogrel, prasugrel or ticagrelor). Aspirin irreversibly inhibits platelet aggregation by acetylating the COX-1 enzyme which is required to produce the potent platelet aggregation potentiator thromboxane A_2 from arachidonic acid (Schror, 1997). $P2Y_{12}$ inhibitors bind to ADP receptors ($P2Y_{12}$) and prevent ADP from inducing platelet shape changes, granule secretion and platelet aggregation (Storey et al., 2000a, Kerneis

et al., 2015). Both thromboxane A_2 and ADP are required for normal haemostasis, and so blocking these pathways with aspirin and $P2Y_{12}$ inhibitors is associated with increased risk of major bleeding (Wilson et al., 2017). In addition to risk of haemorrhage, aspirin and clopidogrel resistance among patients with ACS decreases the efficacy of the current antiplatelet therapy (Weber et al., 2002, Gurbel et al., 2003). Therefore, therapeutic targeting of GPVI in ACS treatment might provide a level of safety that is not available from other antiplatelet agents. It is likely that inhibition of GPVI will be a safe approach in patients with ACS, because the absence of GPVI's activating role in haemostasis can be compensated for by other G-protein-coupled receptors and their agonists, ADP and thrombin, which stimulate platelet aggregation independently of the activation of GPVI downstream Syk and Src kinases (Hughan et al., 2007). Lastly, expression of GPVI only on platelets and their precursor might make anti-GPVI agents more efficacious in action and safer in drug delivery.

1.13.8 Mechanisms for antagonising GPVI

Several approaches have been established to inhibit GPVI–collagen interaction, including immune depletion of GPVI, immune blockade of GPVI, competitive binding of collagen, and inhibition of the downstream signalling of GPVI.

1.13.8.1 GPVI inhibitors based on immune depletion of GPVI

Approaches based on the immune blockade of GPVI involve the removal of GPVI from the platelet surface by targeting the receptor with specific antibody-producing GPVI-null platelets. Multiple antibodies have been developed to dampen GPVI activity *in vivo* and *ex vivo*. These antibodies are variable in their specificity, their binding affinity, the extent of their effect (inhibitory or depleting), and whether they induce platelet aggregation (Zahid et al., 2012b). Autoantibodies in ITP patients, JAQ-1, mF1232, cF1232, mF1201 ,11A12, and 6B112, are the main examples of antibodies that deplete GPVI (Zahid et al., 2012b, Takayama et al., 2008,

Boylan et al., 2006, Nieswandt et al., 2001c). In animal models of thrombosis, these antibodies cause long-lasting GPVI depletion, but use of the intact whole antibody (IgG) or divalent $F(ab)_2$ fragments might lead to thrombocytopenia due to platelet activation and aggregation as a result of GPVI crosslinking by the antibody (Takayama et al., 2008). For example, Matsumoto et al. showed that the use of the monoclonal OM1 antibody induced platelet activation and aggregation, presumably by crosslinking of GPVI, whereas OM1 antigen-binding fragments did not activate platelets (Matsumoto et al., 2007). This model has been tested *in vivo* in animals only, but autoantibodies in ITP patients*- can give valuable insights into the safety and efficacy of this approach and whether immune depletion of the GPVI receptor in humans may be an appropriate therapeutic approach.

1.13.8.2 GPVI inhibitors based on immune blocking of GPVI

Approaches based on immune blocking of GPVI involve the direct binding of two extracellular Ig domains (D1, D2) of GPVI with specific monoclonal antibody fragments that block the receptor from getting closer to its stimulating agonist, collagen. Monoclonal antibodies are produced from a single B-cell clone; therefore, monoclonal antibodies are monovalent in the term of affinity and recognise the same epitope of the antigen (Lipman et al., 2005). On the other hand, polyclonal antibodies are produced from mixed and multiple B-cell clones; as a result, polyclonal antibodies are polyvalent in the term of affinity and recognise and bind to different epitopes of the antigen (Lipman et al., 2005). Furthermore, there is a difference between monoclonal antibodies and polyclonal antibodies in the way of production. Monoclonal antibodies are produced in cell lines *ex vivo* whereas polyclonal antibodies are produced *in vivo* by injecting animals with an immunogen. Fragment antigen binding (Fab) fragments, single-chain variable fragments (scFvs), and human single-domain antibodies

(sdAbs) represent the main forms of antibody fragments that have been used to block the GPVI pathway.

Fragment antigen-binding fragments of monoclonal antibodies

The anti-GPVI Fab fragment is the most commonly used type of antibody fragment to block GPVI activity for therapeutic purposes. To avoid undesired platelet activation that might be caused by GPVI clustering, Fab fragments should be monovalent and monospecific in their inhibitory intervention (Zahid et al., 2012b). The injection of anti-GPVI Fabs known as 9O12.2 and OM2 in non-human primates (cynomolgus monkeys**)** prevents *ex-vivo* thrombus formation and collagen-induced platelet aggregation without causing thrombocytopenia or significant bleeding risk (Matsumoto et al., 2006, Ohlmann et al., 2008).

Single-chain variable fragments

Single-chain variable fragments (scFvs) are engineered recombinant molecules consisting of variable regions of heavy and light chains linked collectively by a flexible bond (Nelson, 2010). A10, humanised scFv 9O12, and 10B12 are the most potent anti-GPVI scFvs that have been reported. They effectively prevent collagen-induced platelet activation, especially A10, which also inhibits convulxin binding to GPVI (Muzard et al., 2009, Qian et al., 2002, Smethurst et al., 2004). Qinghong Liu and his co-workers also produced humanised scFv fragments from autoantibodies in ITP patients that prevent collagen-stimulated platelet activation and aggregation (Liu et al., 2016).

Human single-domain antibodies

Human single-domain antibodies (sdAbs), also called nanobodies, are another type of antibody fragment that have been used to inhibit GPVI activity. They are considered the smallest (11–13 kDa) active functional fragment of Ig and consist of only a single light chain or heavy

variable domain (Walker et al., 2009). They have multiple advantages over other kinds of antibody fragments, being more stable, less immunogenic, and protected against proteolysis. BLO8-1 is an example of a sdAb that has been specifically developed to block collagen-induced platelet aggregation and thrombus formation in anticoagulated whole blood under arterial shear rate (Walker et al., 2009).

1.13.8.3 GPVI inhibitors based on competitive binding of collagen

Occupation of GPO motifs on exposed collagen fibres by a competitive GPVI mimic is an alternative way to prevent the major collagen signalling receptor from becoming activated. In other words, GPVI mimetics play a localised antithrombotic effect at the site of the injured vessel, and they act only at sites where collagen fibres are exposed. By binding to exposed collagen, GPVI mimetics competitively occupy GPVI binding sites and prevent GPVI contact with collagen. Massberg et al. cloned extracellular domains of human GPVI and then fused them onto the Fc domain of immunoglobulin with a specific hinge region. The resultant recombinant soluble dimeric -Fc exclusively binds to immobilised collagen, preventing arterial thrombosis *in vivo* following rupture of atherosclerotic plaque in mice. It caused only moderately prolonged tail bleeding but had no effect on peripheral platelet count (Massberg et al., 2004). Grüner et al. compared the antithrombotic effect of soluble GPVI-Fc with the anti-GPVI antibody JAQ-1 *in vitro* and *in vivo*. They concluded that GPVI-Fc significantly inhibits platelet aggregation *in vitro* but offers no apparent protection against arterial thrombosis *in vivo*, which is inconsistent with the results of Massberg et al. This inconsistency may be due to differences in GPVI-Fc preparation between the two studies. In mice, the GPVI depleting antibody JAQ-1 strongly inhibited platelet aggregation *in vitro* and completely protected against arterial thrombosis *in vivo* (Grüner et al., 2005). Another group demonstrated that a GPVI-Fc fusion protein significantly reduced atherosclerotic plaque-induced thrombus size in

an *in vivo* model of atherothrombosis (Ungerer et al., 2013). Soluble GPVI-Fc has tissue remodelling properties. It has been shown in mouse models of induced carotid artery injury that GPVI-Fc binds to exposed subendothelial collagen, reduces scar (neointima) formation, and prevents atherogenesis development (Schönberger et al., 2008).

1.13.8.4 GPVI downstream signalling inhibitors

Targeting the key regulator enzymes and protein adapters involved in the GPVI activation pathway could inhibit downstream signalling of GPVI, thereby reducing subsequent platelet stimulation. Inhibition of Syk, which is a principal mediator of the tyrosine phosphorylation cascade, abrogates GPVI-mediated platelet activation (Spalton et al., 2009a). The small molecule R406 (an active metabolite of fostamatinib) is an ATP-competitive antagonist of Syk that inhibits GPVI-dependent platelet activation (Spalton et al., 2009b). PRT060318 is another highly specific Syk inhibitor that has been investigated in animal models of thrombosis initiation and arterial vascular injury. Injection of PRT060318 into mice, rabbits, and pigs granted significant protection against atherosclerosis and arterial thrombosis, prevented neointima formation, and promoted vascular remodelling (Andre et al., 2011). In addition to Syk inhibitors, the use of Bruton's tyrosine kinase (Btk) inhibitors is believed to impair the ability of GPVI to exert its stimulatory effect on platelet activation. Btk kinases are thought to be activated downstream of Src and Syk kinases in GPVI signalling (Atkinson et al., 2003a). BTKI-43607 and BTKI-43761 are analogues of ibrutinib, which is an irreversible Btk inhibitor licensed for treating haematological malignancy that inhibits GPVI activation and GPVI-dependent platelet stimulation (Rigg et al., 2016). Kamel et al showed that use of ibrutinib is associated with reduced collagen-induced platelet aggregation in 23 patients with chronic lymphocytic leukaemia. The inhibitory effect of ibrutinib faded when inhibitor administration was ceased (Kamel et al., 2014). Contrary to previous knowledge of the role of Btk in the GPVI

signalling pathway, Nicolson et al. found that inhibition of Btk phosphorylation by ibrutinib delays but does not block GPVI-mediated platelet activation and that ibrutinib likely blocks GPVI-dependent platelet activation by targeting an unidentified required element for receptor activation (Nicolson et al., 2017).

Dasatinib is a tyrosine kinase (Src-kinase) inhibitor that is used to treat patients with imatinib-resistant chronic myeloid leukaemia and could also be used to modulate GPVI function through inhibition of tyrosine phosphorylation downstream of GPVI (Li et al., 2009). Although antithrombotic efficacy could potentially be achieved from the inactivation of the chief enzymes and protein adapters needed for the GPVI signalling pathway, targeting these elements might give rise to safety issues. Many of these enzymes and protein effectors are universal in function, with signalling roles in platelet receptors and further roles in other body cells such as leukocytes. In three phase III trials of ibrutinib, the incidence of atrial fibrillation was 7% in the treatment group compared to 2% in the control group (Thorp and Badoux, 2018). In general, dasatinib is considered a safe and well-tolerated drug, although it has some mild to moderate side effects that include cytopenia, anaemia, and fluid retention (Conchon et al., 2011).

1.13.9 Clinical grade GPVI inhibitors

1.13.9.1 Glenzocimab

Glenzocimab (previously known as ACT017) is a humanised monovalent Fab fragment that was developed specifically to block GPVI. Production of glenzocimab involved several cycles of structural modulation, humanisation, and purification. Glenzocimab was chosen and produced with regard to its stability, specificity, inhibitory potential, and affinity to GPVI in comparison to those qualities in 15 other humanised variants of Fab 9O12, which was itself proteolytically cleaved from the rodent monoclonal 9O12 IgG (Lebozec et al., 2017). For humanisation process, rodent Fab 9012 V-domains were substituted with humanised V-

domains using a template with following criteria: (1) human germline most similar to rodent fab 9O12 ; (2) high sequence identity and identical canonical subclasses; (3) same antibody template for both V-domains (Lebozec et al., 2017). Glenzocimab fits within the range of licensed non-immunogenic fabs as assessed by EpiMatrix immunogenicity scale, an algorithm approved by the Food and Drug Administration (FDA) to predict the immunogenicity (Lebozec et al., 2017). Glenzocimab was expressed and produced in CHO cells using the GPEx® technology (Lebozec et al., 2017). *In-vitro* platelet studies have shown that glenzocimab inhibits platelet aggregation induced by collagen. Reversible collagen-induced platelet aggregation occurred without thrombocytopenia or GPVI depletion in ex vivo samples observed in a pharmacological assessment of glenzocimab in cynomolgus monkeys (Lebozec et al., 2017).

In a first in man dose-escalation phase I randomised controlled trial (RCT), glenzocimab (62.5-2000 mg) was investigated in 48 healthy subjects. Glenzocimab dose-dependently and reversibly inhibited collagen-induced platelet aggregation with a Cmax of ~5-100 µg/ml depending on the dose. The magnitude and duration of platelet inhibition of glenzocimab are dose-dependent with full platelet recovery after 48 hours. Glenzocimab was safe with consistent pharmacodynamics and pharmacokinetics without showing any effect on platelet count and bleeding time (Voors-Pette et al., 2019). The trial's results warrant further investigation of glenzocimab in patients with atherothrombotic disease, such as patients with ACS.

1.13.9.2 Revacept

Revacept (GPVI-Fc) is the first anti-GPVI agent that has been granted approval to be evaluated in human subjects. An open-labelled dose-escalation phase I randomised clinical trial of Revacept started in March 2006 and concluded in October 2007 to assess the safety and pharmacokinetics of the agent in 30 healthy human subjects. The phase I trial demonstrated that

Revacept is a safe, tolerable antiplatelet agent that dose-dependently inhibited platelet aggregation without affecting general haemostasis (Ungerer et al., 2011). A phase II randomised double-blind placebo-controlled clinical trial of Revacept was launched in November 2017 in multiple locations in Germany to assess the efficacy and tolerability of two doses of Revacept (80 mg and 160 mg) versus a placebo in patients with stable coronary artery disease undergoing elective PCI. The trial showed that revacept did not provide any extra benefits when combined with standard antiplatelet therapy compared to standard antiplatelet therapy alone (Mayer et al., 2021).

1.14 Rationale for investigating glenzocimab as potential treatment for ACS

Glenzocimab is a novel humanized antibody fragment that blocks platelet GPVI and therefore potently inhibits collagen-induced platelet aggregation (Lebozec et al., 2017). Emerging evidence also shows that glenzocimab is able to cause platelets in a pre-formed thrombus to disaggregate (Ahmed et al., 2020). A phase I clinical trial that was published in 2019 demonstrated that a single dose of glenzocimab has a half-life of approximately 12-24 hours and was safe, well-tolerated and had no effect on hemostasis, as assessed by bleeding time (Voors-Pette et al., 2019). In a phase Ib study of fibrinolysis-treated patients with stroke, glenzocimab had a favorable safety profile with no overt effect on risk of intracranial haemorrhage or other forms of major bleeding (Mazighi M, 2021). GPVI is therefore a promising target for the treatment of STEMI as it appears to have several major roles in thrombosis without adversely affecting general haemostasis (Borst and Gawaz, 2021). Therefore, glenzocimab could provide additional antithrombotic effects compared to contemporary dual antiplatelet therapy that is used routinely for ACS particularly STEMI.

1.15 Hypothesis

Aspirin and ticagrelor are not sufficient to treat heavy thrombosis during emergency PCI that requires the use of GPIIb/IIIa inhibitors such as eptifibatide as a bailout; however, the use of GPIIb/IIIa inhibitors is associated with increased risk of bleeding. Combining GPVI inhibition by glenzocimab with aspirin and ticagrelor blocks a wide range of collagen- and fibrin-mediated platelet mechanisms that are critical in thrombosis, potentially offering an effective and safe treatment for patients with ACS.

1.16 Aims

Aim 1: Determine the effect of glenzocimab in combination with aspirin and ticagrelor on collagen- and CRP-XL-mediated platelet mechanisms.

Aim 2: Determine the effect of glenzocimab in combination with aspirin and ticagrelor on atherosclerotic plaque-mediated platelet mechanisms.

Aim 3: Determine the effect of glenzocimab in combination with aspirin and ticagrelor on fibrin-mediated platelet mechanisms, thrombin generation, and haemostasis.

Aim 4: Determine the additive effect of glenzocimab on different platelet mechanisms, thrombin generation, and haemostatic function in samples from patients with ACS treated with aspirin and ticagrelor.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Materials

2.1.1 Agonists, antagonists, detection antibodies, and detection stains

The details for platelet agonists used in this thesis are stated in **Table 2.1**; the agonists were used at concentrations that were recommended by the commercial suppliers or platelet function testing panels (Koltai et al., 2017, Vlot et al., 2016). The information about the coagulation and thrombin generation agonists and reagents is presented in **Table 2.2**. The inhibitors and antagonists used in the study are listed in **Table 2.3**, and the detection antibodies and stains are detailed in **Tables 2.4** and **2.5**, respectively. If unstated, items used were from Sigma-Aldrich (Poole, UK) or described sources.

Table 2.1 Targets and sources of platelet agonists

Agonist	Receptor target	Description	Concentration for aggregation	Source
Horm collagen	GPVI and GPIa/IIa	Fibrillar type I collagen (Horm)	LTA: 15, and 10 µg/ml MEA: 3.2 µg/ml	Nycomed (Munich, Germany)
CRP-XL	GPVI	Cross-linked collagen-related peptides	LTA: 3 and 10 µg/l MEA: 3 and 10 µg/ml	Prof. Richard Farndale (University of Cambridge, UK)
Fibrinogen*	GPIIb/IIIa	Human fibrinogen (purified)	See footnotes	Enzyme Research Laboratories (Swansea, UK)
ADP	$P2Y_{12}$ and $P2Y_1$	A lyophilised powder of adenosine-5'-diphosphate (ADP)	LTA: 5 µM MEA: 6.5 µM	Roche Diagnostics Limited (West Sussex, UK)

AA	TxA_2	A lyophilised preparation of arachidonic acid (AA)	LTA: 1 mM MEA: 0.5 µM	Roche Diagnostics Limited (West Sussex, UK)
TRAP-6	PAR1	A lyophilised powder of thrombin receptor activating peptide-6 (TRAP-6)	MEA: 32 µM	Roche Diagnostics Limited (West Sussex, UK)
Thrombin*	PAR1 and PAR4	A lyophilised powder of thrombin	See footnotes	Sigma-Aldrich (Poole, UK)
Polymerised fibrin	GPVI	See section 2.2	LTA: 1/100 dilution	In-house preparation
Atherosclerotic plaque	GPVI	See section 2.2	LTA: 70 µg/ml MEA: 140 µg/ml	In-house preparation

*Fibrinogen used as source to generate fibrin.

*Thrombin was used to cleave fibrinogen into fibrin and not to activate platelets.

Table 2.2 Uses and sources of coagulation and thrombin generation reagents

Reagent	Intended use	Description	Source
*ROTEM® EXTEM	Extrinsic coagulation pathway activation	Contains TF (thromboplastin) and phospholipids	Werfen Limited (Warrington, UK)
*ROTEM® INTEM	Intrinsic coagulation pathway activation	Contains ellagic acid (contact activator)	Werfen Limited (Warrington, UK)
ROTEM® STAR-TEM	Recalcification solution	Contains 0.2 mol/L $CaCl_2$	Werfen Limited (Warrington, UK)
CAT PRP	Stimulation of thrombin generation in the PRP sample	Contains 0.5 pM TF and a minimal amount of phospholipids	Diagnostica Stago UK Ltd (Reading, UK)
CAT Thrombin calibrator	Calibration of inner filter effect and substrate consumption	Contains human α_2M-thrombin complex	Diagnostica Stago UK Ltd (Reading, UK)
CAT FluCa-kit	Initiation and detection of thrombin generation	Fluo-substrate contains 417 µM fluorogenic substrate Z-Gly-Gly-Arg-AMC; Fluo-buffer contains 16.7 mM $CaCl_2$	Diagnostica Stago UK Ltd (Reading, UK)

*Exact concentrations were not given by the manufacturer for ROTEM EXTEM and INTEM. Instead, the manufacturer recommends using certain volumes of EXTEM (20 µl) and INTEM (20 µl) to stimulate blood coagulation in whole blood samples (300 µl).

Table 2.3 Antagonists and inhibitors

Inhibitor	Class	Application	Source
Glenzocimab	Humanised monoclonal Fab	GPVI blockade	Acticor Biotech (Paris, France)
6F1	Mouse monoclonal IgG	GPIa/IIa blockade	Prof. Barry Coller (The Rockefeller University, New York, NY, USA)
Aspirin	Acetylsalicylic acid	COX-1 inhibition	Sigma-Aldrich (Poole, UK)
Ticagrelor	Cyclopentyl triazolopyrimidine	$P2Y_{12}$ inhibition	Sigma-Aldrich (Poole, UK)
Eptifibatide	Cyclic heptapeptide	GPIIb/IIIa blockade	Sigma-Aldrich (Poole, UK)
Dasatinib	N-(2-Chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl] amino]-5-thiazolecarboxamide	Src and BCR-ABL kinase inhibition	LC Laboratories (Woburn, MA)
PP2	4-amino-5-(4-chlorophenyl)-7-(t-butyl)-pyrazolo[3,4-d] pyrimidine	Src inhibition	Sigma-Aldrich (Poole, UK)
Prostacyclin (PGI_2)	6,9α-epoxy-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid, monosodium salt	TxA_2 inhibition	Cayman Chemicals (Cambridge, UK)
PPACK	D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone	Thrombin neutralisation	Sigma-Aldrich (Poole, UK)
Citrate-dextrose solution (ACD)	D-Glucose-2-hydroxy-1,2,3-propanetricarboxylic acid	Ca^{2+} chelator	Sigma-Aldrich (Poole, UK)

Table 2.4 Source, application, and dilution of the detection antibodies

Antibody	Type/Biological source	Application/ Dilution*	Source
Primary antibody			
p-Tyrosine (4G10)	Monoclonal, mouse	WB: 1:1000	Millipore (Bucks, UK)
p-Src (Tyr-418)	Polyclonal, rabbit	WB: 1:1000	Life Technologies (Carlsbad, CA, USA)
p-Syk (Tyr525/6) (C87C1)	Monoclonal, rabbit	WB: 1:500	Cell Signalling Technology (Hitchin, UK)
P-LAT (Tyr200)	Polyclonal, rabbit	WB: 1:500	Abcam (Cambridge, UK)
P-PLCγ2 (Y1217)	Polyclonal, rabbit	WB: 1:250	Abcam (Cambridge, UK)
GPVI tail	Polyclonal, rabbit	WB: 1 µg/ml	Dr Elizabeth Gardiner (Canberra, Australia)
Syk (4D10)	Monoclonal, mouse	WB: 1:200	Santa Cruz Biotechnology (Heidelberg, Germany)
LAT	Polyclonal, rabbit	WB: 1:500	Millipore (Bucks, UK)
PLCγ2 (SC 407)	Polyclonal, rabbit	WB: 1:200	Santa Cruz Biotechnology (Heidelberg, Germany)
αTubulin	Monoclonal, mouse	WB: 1:1000	Millipore (Bucks, UK)
AF647 anti-human CD62P (P-selectin)	Monoclonal, mouse	FA: 1:80	BioLegend (London, UK)
Anti-6-His Tag antibody HRP conjugated	Polyclonal, rabbit	ELISA: 1:10,000	Cambridge Bioscience (Cambridge, UK)

Secondary antibody			
Rabbit IgG HRP conjugate	Polyclonal, donkey	WB: 1:10,000	Amersham Bioscience (Buckinghamshire, UK)
Mouse IgG HRP conjugate	Monoclonal, sheep	WB: 1:10,000	Amersham Bioscience (Buckinghamshire, UK)

HRP: horseradish peroxidase; WB: western blot; FA: flow adhesion; AF: Alexa Fluor®; ELISA: enzyme-linked immunosorbent assay.

*The antibodies stated in **table 2.4** were used at dilutions that were recommended by their suppliers or optimised by some publications from Birmingham platelet group. The chosen specific phosphorylation sites for kinases and adapters involved in GPVI signalling pathway represent the most common phosphorylation sites that were used to study GPVI function and GPVI signalling in multiple publications (Nicolson et al., 2017, Alshehri et al., 2015).

Table 2.5 Dilution and source of the detection stains

Stain	**Use**	**Application and Dilution**	**Source**
AF568 conjugated annexin V	To detect phosphatidylserine exposure	FA: 1:100	Fisher Scientific (Loughborough, UK)
AF488-conjugated Phalloidin	To visualise platelets (Actin stain)	Spreading: 1:1000	Fisher Scientific (Loughborough, UK)
$DiOC_6(3)$	To visualise platelets (mitochondrial stain)	FA: 2μM	Thermo Fisher Scientific (Gloucester, UK)

FA: flow adhesion; AF: Alexa Fluor®

2.2 Methods

2.2.1 In-house agonist preparation

2.2.1.1 Preparation of polymerised fibrin

In a 15-ml Falcon tube, 500 µl of $CaCl_2$ (10 mM), 200 µl of depleted fibrinogen (50 mg/ml), 10 µl of FXIIIa (700 µg/ml; Zedira, Darmstadt, Germany) and 9.3 ml of phosphate-buffered saline (PBS) were added, and the contents mixed well. Then, 10 µl of thrombin (1000 U/ml) was added to convert fibrinogen into polymerised fibrin, which was cross-linked in the presence of calcium ions and FXIIIa. Following the addition of thrombin, the tube was mixed, rotated and inverted until fibrin started to form. The tube was incubated at room temperature (RT) for 1 hour, after which 10 µl of PPACK (20 mM) was added to neutralise thrombin residues, and the tube was mixed well and incubated for another 15 min at RT. The tube contents were then sonicated at an amplitude between 80 and 100 % to disperse the fibrin fibres. The tube was centrifugated at 1000 *g* for 5 min, the supernatant was discarded, and the contents were resuspended in 2 ml of Tyrode's buffer**.**

2.2.1.2 Preparation of atherosclerotic plaque homogenate

Ten atherosclerotic plaque samples were collected from 10 patients with transient ischaemic attacks (TIAs) who underwent carotid endarterectomy for high-grade symptomatic carotid artery stenosis and stored at -80°C after sampling. Cryosections were taken from each atherosclerotic plaque specimen for histological analysis purposes. Subsequently, all atherosclerotic plaque specimens were placed in one 500-ml borosilicate glass laboratory reagent bottle immersed in liquid nitrogen (-196°C) and manually crushed with a glass mortar until a fine powder was obtained. Then, 20 ml of PBS was added to the glass bottle containing the atherosclerotic plaque homogenate, thawed at 37°C for 3 min and transferred into a 50-ml Falcon tube. The atherosclerotic plaque homogenate was sonicated (10 times for 5 seconds at

12% amplitude) on ice for tissue dispersal, followed by centrifugation (1200 rpm for 1 min). The supernatant was retrieved, and the protein concentration was measured with a standard bicinchoninic acid protein assay.

2.2.2 Whole-blood sampling from healthy donors and patients with acute coronary syndromes

All experiments involving human subjects were performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Ethical approval for blood sampling from healthy donors was granted by the Birmingham University Internal Ethical Review (ERN_11-480175). Ethical approval for blood collection from patients with acute coronary syndromes (ACS) was granted by NHS research and ethics committees (North West: Haydock Research Ethics Committee 20/NW/0001; West Midlands: South Birmingham Research Ethics Committee 18/WM/0386). Venous blood was collected from drug-free, healthy volunteers and from patients with ACS receiving aspirin and ticagrelor, who had been provided with consent forms before blood withdrawal. Blood was taken by antecubital venepuncture into evacuated S-Monovette® Citrate and Hirudin vacutainers (Sarstedt, Germany).

2.2.3 Study design and inclusion criteria for patients with acute coronary syndromes

2.2.3.1 Study design

The additive effect of glenzocimab and eptifibatide was investigated *ex vivo* in samples from patients with ACS who were taking aspirin and ticagrelor. This was conducted as a sub-study of the CASCADE study, a large clinical study that aims to predict bleeding via utilising a panel of laboratory tests that consume a large portion of the sample. For this sub-study, only a small volume of the sample could be used, which limited the capacity to conduct a wide range of tests, as done for the healthy donors. Therefore, this sub-study investigated the effect of

glenzocimab and eptifibatide on platelet aggregation in MEA, on platelet thrombus formation and adhesion on collagen under flow in a microfluidic chamber, and on thrombin generation and haemostatic function in CAT and ROTEM respectively. However, some patients' samples were not large enough to run all the planned tests, so multiplate and flow adhesion were prioritised in this scenario.

2.2.3.2 Inclusion criteria

The inclusion criteria for patients with acute coronary syndromes included in this study were the following: (i) patients are diagnosed with acute coronary syndromes which include ST segment elevation myocardial infarction (STEMI), Non-ST segment elevation myocardial infarction (NSTEMI), and Unstable angina (US), (ii) patients are treated with antiplatelet therapy that consists of aspirin and ticagrelor only, (iii) Patients are receiving the antiplatelet therapy for more than one week at least to allow enough time to for platelet inhibition and also to ensure complete clearance of anticoagulant therapy that patients may receive during pPCI.

2.2.4 Preparation of platelet-rich plasma

Tubes containing blood were centrifuged at 200 *g* for 20 min. Once the centrifugation was complete, the platelet-rich plasma (PRP) was transferred into a 50-ml Falcon tube. The remaining blood was centrifuged at 1000 *g* for 10 min. Then, the platelet-poor plasma (PPP) was isolated with a Pasteur pipette into a 15-ml Falcon tube to be used as a blank.

2.2.5 Preparation of human washed platelets

The anticoagulated blood was poured into a 50-ml Falcon tube containing 10% of prewarmed acid citrate dextrose (ACD) and mixed and aliquoted into plastic test tubes, balanced and centrifuged at 200 *g* for 20 min. PRP was transferred with Pasteur pipettes into a 50-ml Falcon tube, with the addition of 10 µl of prostaglandin I (PGI_2), and then centrifuged at 1000 *g* for 10

min. Once centrifugation was complete, the supernatant was discarded, and the platelets were suspended in 2 ml of modified Tyrode's buffer (with 5 mM glucose; pH 7.3), ACD and PGI_2 (1 μg/ml). This was then centrifuged again at 1000 *g* for 10 mins. The supernatant was discarded, and the platelets were resuspended in 4 ml of modified Tyrode's buffer. The platelet count was performed using a Z1 S Coulter Particle Counter (Beckman Coulter Ltd, High Wycombe, UK) and adjusted to 2.0×10^8/mL for aggregation and to 5.0×10^8/ml for phosphorylation and shedding. The washed platelets were allowed to rest for 30 mins before the platelet function and aggregation tests to ensure complete PGI_2 degradation.

2.2.6 Platelet function testing

2.2.6.1 Platelet aggregation

2.2.6.1.1 Light transmittance aggregometry

Platelet aggregation studies were performed using a Platelet Aggregation Profiler (PAP-8E) aggregometer (Bio/Data Corporation, Horsham, PA, USA). Light transmittance aggregometry (LTA) measures changes in light transmission in stirred platelets in a test tube following stimulation with an agonist against a calibration made by poor platelets plasma or Tyrode's buffer to set a baseline of 100% light transmission (Frontroth, 2013). To carry out LTA, platelets rich plasma or washed platelets was preincubated with control or inhibitors at 37°C for 10 min in the incubation wells. The tubes were then transferred into stirred incubation wells and incubated under stirring conditions for 2 min, after which the tubes were moved to activation wells. Aggregation was stimulated by adding the agonist of interest.

2.2.6.1.2 Multiple electrode aggregometry

Platelet aggregation studies were performed using a Multiplate® aggregometer (Roche Diagnostics Munich, Germany) in whole-blood samples. Multiple electrode aggregometry (MEA) measures the increase in the electrical impedance that is caused by sticking of platelets

to electrodes in response to stimulation with platelets aggregating agents (Toth et al., 2006). Blood was collected in hirudin-containing tubes, as the hirudin is the anticoagulant of choice to perform platelet aggregation in the Multiplate® aggregometer. To carry out MEA, hirudin-anticoagulated blood was diluted in half with saline and preincubated with the control and different combinations of inhibitors at 37°C for 10 min in the incubation wells. Aggregation was stimulated by adding an agonist, and platelet aggregation was assessed over 6 min as per the manufacturer's instructions (Toth et al., 2006).

2.2.6.2 Adenosine triphosphate release in platelet-rich plasma in lumi-aggregometry

Adenosine triphosphate (ATP) secretion in PRP was determined using the Chrono-log Model 700 aggregometer (Havertown, PA, USA). Lumi-aggregometry measures the released ATP from platelets that reacts with the firefly luciferase system and generates luminescence that is calculated by a Lumi-aggregometer against an ATP standard that accounts for free ATP in the sample (Feinman et al., 1977, Pai et al., 2011). To carry out lumi-aggregometry, PRP was incubated with 10 µl of Chrono-Lume® reagent which contains a mixture of D-luciferin and luciferase for 1 min to detect ATP release by the luminescence method in a closed chamber. After 1 min of incubation, platelet aggregation was stimulated, and ATP secretion was continuously measured for 6 min. Calibration was performed at the beginning of the experiment by adding 5 µl of ATP standard (2 nmol) for each channel, so that ATP release parameters could be calculated by AGGRO/LIK8 software (Trampuš-Bakija et al., 2020).

2.2.6.3 Platelet spreading

Round, glass coverslips were coated with collagen, atherosclerotic plaque homogenate, fibrinogen or fibrin and placed in a 24-well plate for 1 hour at RT. After 1 hour, coverslips were washed once with PBS and blocked with bovine serum albumin (BSA 5 mg/mL) in PBS for 60

min at RT. In the fibrin wells, thrombin was neutralised by adding PPACK. Washed human platelets (2×10^7 mL prepared in modified Tyrode's buffer) were preincubated with vehicle control and different combinations of inhibitors at 37°C for 30 min. The platelets were then allowed to spread on coverslips for 30 min at 37°C. Subsequently, coverslips were washed once with PBS to remove nonadherent platelets, fixed with 4% neutralised formalin buffer for 10 min and washed three times with PBS. To stain the actin, platelets were permeabilised with 0.1% Triton X-100 in PBS for 5 min, washed three times with PBS, stained with Alex-488-phalloidin for 45 min in the dark and washed three times with PBS, and the coverslips were then mounted on glass slides and imaged using the Zeiss Axiovert 200M microscope (Carl Zeiss AG, Oberkocken, Germany). To calculate platelet surface area, perimeter and number, acquired images were analysed using a workflow published by Pike et al., which employs the KNIME 4.1.3 analytics platform (KNIME.com AG, Konstanz, Germany) with Ilastik 1.1.2 machine learning software (University of Heidelberg, Germany) (Pike et al., 2020).

2.2.6.4 Platelet adhesion and thrombus formation under flow conditions

An ibidi® u-Slide VI 0.1 Uncoated chamber (ibidi®, Martinsried, Germany) was coated with either 50–200 µg/ml Horm collagen or 1 mg/ml atherosclerotic plaque homogenate overnight and kept in the fridge at 4°C. Subsequently, 1 hour before carrying out the flow adhesion experiment, excess collagen was removed from the chamber, and the chamber was blocked with 4 mg/ml BSA made in PBS and kept again at 4°C. The citrate-anticoagulated whole-blood sample was stained with 2 µM $DiOC_6$ (for platelet visualisation) and incubated with vehicle or inhibitors for 15 min at 37°C. The chamber was then flushed with PBS to remove BSA, and the blood was perfused at 1000 s^{-1} shear rate over immobilised collagen for 10 min using a PHD 2000 Syringe Pump (Harvard Apparatus, Holliston, MA, USA). Platelet adhesion and thrombus formation were continuously captured by recording a z-stack (41 planes/step size 0.5 µm) for

every 30 seconds with an Evos® FL Auto imaging system (Life Technologies, Paisley, UK) using a 20× objective for the entire flow period. Images were processed and analysed to measure fluorescence intensity and surface coverage area (%) using semi-automated scripts in Image J software (NIH, Bethesda, MD, USA).

2.2.6.5 P-selectin expression and phosphatidylserine exposure under flow conditions

An ibidi® u-Slide VI 0.1 Uncoated chamber was coated with either 200 µg/ml Horm collagen or 1 mg/ml atherosclerotic plaque homogenate and kept overnight at 4°C. Subsequently, 1 hour before carrying out the flow adhesion experiment, excess collagen was removed from the chamber, and the chamber was blocked with 4 mg/ml BSA made in PBS and kept again at 4°C. The presence of normal levels of Ca^{2+} and Mg^{2+} is necessary for phosphatidylserine exposure, procoagulant platelet formation, and platelet activation. To avoid any issue that may be caused by the recalcification of citrated blood such as prompt clotting, the hirudin -anticoagulated whole blood was used to assess the effect of antiplatelet agents on P-selectin expression and phosphatidylserine exposure in platelets flowing on thrombogenic surfaces. The hirudin-anticoagulated whole-blood sample was stained with AF647-labelled anti-human CD62P monoclonal antibody for P-selection visualisation or with AF568-labelled annexin A5 for phosphatidylserine visualisation and incubated with the vehicle or inhibitors for 15 min at 37°C. The chamber was then flushed with PBS to remove the BSA, and the blood was perfused at 1000 s^{-1} shear rate over immobilised collagen for 10 min using a PHD 2000 Syringe Pump. P-selectin expression and phosphatidylserine exposure were continuously captured by recording a z-stack (41 planes/step size 0.5 µm) for every 35 seconds by an Evos ® FL Auto imaging system using a 20× objective for the entire flow period. Images were processed and analysed

to measure fluorescence intensity and surface coverage area (%) using semi-automated scripts in Image J software.

2.2.7 Tyrosine phosphorylation

2.2.7.1 Washed platelet lysate preparation

Washed human platelets (5.0×10^8 mL prepared in modified Tyrode's buffer) treated with 9 μM eptifibatide were incubated with vehicle control or inhibitors for 10 min at 37°C. The platelets were then stimulated with collagen, CRP-XL or atherosclerotic plaque homogenate for 180 seconds under stirred conditions. For sustained tyrosine signalling, platelets were stimulated with plaque at different time points (1, 3, 10, 20, 30 and 60 min) under stirred conditions at 37°C. Platelet activation was stopped by adding an equal volume of 5X SDS reducing buffer and boiled for 5 min in order to prepare platelet lysates.

2.2.7.2 SDS-PAGE and western blotting

Chapter 1 Platelet lysate and stained protein ladders were loaded into a 4-12% gradient Invitrogen™ NuPAGE™ gels (Fisher Scientific, Loughborough, UK) and electrophoresis run first at 60 V for 15 min, followed by 120 V for 1 hour. Proteins were transferred from gel casts into polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hemel Hempstead, UK) for 10 min using the Trans-Blot Turbo Transfer System. After completing the transfer process, membranes were blocked in a blocking buffer made of 3% BSA in TBS-T (Tris-buffered saline [200 μM Trizma base, 1.37 M sodium chloride; pH 7.6] with 0.2% Tween-20 and 0.1% sodium azide) for 1 hour at RT with constant mixing. To detect phosphorylated proteins, membranes were then incubated with the indicated primary antibodies prepared in blocking buffer overnight at 4°C. Following overnight incubation, the membranes were washed three times in TBS-T buffer and incubated with the indicated horseradish peroxidase (HRP)-conjugated secondary antibodies prepared in TBS-T for 1 hour at RT followed by three cycles of washes in TBS-T

(10 min/wash). Phosphorylated proteins were detected using the Enhanced Chemiluminescence detection system (Life Technologies), and chemiluminescence signals were visualised with autoradiography film (GE Healthcare, Buckinghamshire, UK). To detect total proteins, bound primary and secondary antibodies against phosphorylated proteins were removed by two cycles of membrane incubation, using only stripping buffer in the first cycle and stripping buffer with 1% β-mercaptoethanol in the second cycle. Then, the same previous steps were conducted to detect phosphorylated protein, but with the indicated primary antibodies against total proteins.

2.2.8 GPVI shedding as detected by western blot

Washed human platelets (5.0×10^8 mL prepared in modified Tyrode's buffer) treated with 9 μM eptifibatide were stimulated with 600 μg/ml atherosclerotic plaque homogenate or 10 μM A23187 (positive control) for 2 hours under stirred conditions at 37°C in the presence of 1 mM $CaCl_2$. Platelet activation was stopped by adding an equal volume of 5X SDS reducing buffer and boiled for 5 min in order to prepare platelet lysates. The platelet lysates and stained protein ladders were loaded into a 4-12% gradient Invitrogen™ NuPAGE™ gels and electrophoresis run first at 60 V for 15 min, followed by 120 V for 1 hour. Proteins were transferred from the gel casts into PVDF membrane (Bio-Rad) for 10 min using the Trans-Blot Turbo Transfer System. After completing the transfer process, the membranes were blocked in 3% BSA in TBS-T blocking buffer for 1 hour at RT under constant mixing. To detect the GPVI band, membranes were then incubated with anti-GPVI tail antibody prepared in blocking buffer overnight at 4°C under continuous mixing. Following this overnight incubation, membranes were washed three times in TBS-T buffer and incubated with rabbit IgG HRP-conjugated secondary antibodies prepared in TBS-T for 1 hour at RT, followed by three cycles of washes in TBS-T. GPVI bands were detected using the Enhanced Chemiluminescence detection system

(Life Technologies), and chemiluminescence signals were visualised with autoradiography film (GE Healthcare).

2.2.9 ELISA

2.2.9.1 Direct ELISA for monomeric GPVI

A Nunc™ MaxiSorp™ flat-bottom 96-well plate (Fisher Scientific, Loughborough, UK) was coated with 140 µg/ml atherosclerotic plaque homogenate overnight at 4°C with constant shaking, then the plate was washed three times with PBS-T (PBS with 0.05% Tween-20) and blocked in 3% BSA made in PBS for 1 hour at RT, followed by three cycles of washing with PBS-T. This was followed by adding increasing concentrations of monomeric GPVI (0.2–10 µg/ml) for 1 hour at RT. The plate was then washed five times with PBS-T to remove unbound monomeric GPVI, followed by adding rabbit anti-6-His HRP-conjugated antibody (Bethyl A190-114P) for 1 hour at RT to detect monomeric GPVI. The plate was washed five times with PBS-T to remove unbound antibodies and incubated with 3.3', 5.5'-tetramethylbenzidine (TMB) Substrate Solution (Fisher Scientific, Loughborough, UK), waiting 5–30 min for the colour to develop. Then, the reaction was stopped by adding 1 M H_2SO_4, and the absorbance was read at 450 nm using the SpectraMax Plus 384 Microplate reader (Molecular Devices, San Jose, CA, USA). To determine the effect of glenzocimab on GPVI binding, the same previous steps were followed, with the exception that one fixed concentration of monomeric GPVI (2.5 µg/ml) was incubated with the vehicle or rising concentrations of glenzocimab (1–50 µg/ml) for 15 min at 37°C, before adding the mixture into the atherosclerotic plaque.

2.2.9.2 Indirect ELISA for dimeric GPVI

A Nunc™ MaxiSorp™ flat-bottom 96-well plate was coated with 140 µg/ml atherosclerotic plaque homogenate overnight at 4°C with continuous shaking, then the plate was washed three times with PBS-T and blocked in 3% BSA made in PBS for 1 hour at RT, followed by three

cycles of washing with PBS-T. This was followed by adding increasing concentrations of dimeric GPVI (0.2–10 μg/ml) for 1 hour at RT. The plate was then washed five times with PBS-T to remove unbound dimeric GPVI, followed by adding NB35 (anti-GPVI nanobody tagged with a 6-His tag developed by the Birmingham Platelet Group) for 1 hour at RT to bind to dimeric GPVI. The plate was washed five times with PBS-T to remove unbound NB35, followed by incubation with rabbit anti-6-His HRP-conjugated antibody (Bethyl A190-114P) for 1 hour at RT to indirectly detect dimeric GPVI by detecting bound NB35. The plate was washed five times with PBS-T to remove unbound antibodies and incubated with TMB Substrate Solution, waiting for 5–30 min for the colour to develop. Then, the reaction was stopped by adding 1 M H_2SO_4, and the absorbance was read at 450 nm using the SpectraMax Plus 384 Microplate reader. To determine the effect of glenzocimab on GPVI binding, the same previous steps were followed, with the exception that one fixed concentration of dimeric GPVI (2.5 μg/ml) was incubated with the vehicle or rising concentrations of glenzocimab (1–50 μg/ml) for 15 min at 37°C, before adding the mixture into the atherosclerotic plaque.

2.2.10 Coagulation testing

2.2.10.1 Clot retraction

Citrate-anticoagulated PRP was allowed to rest after isolation for 10 min at RT and was then incubated with the vehicle and inhibitors for 10 min at 37°C. After incubation, the PRP was recalcified with 5 mM $CaCl_2$, coagulation was stimulated by 1 U/ml thrombin and the tubes containing PRP were kept in a water bath for 2 hours at 37°C. After 2 hours, the clots in the tubes were imaged and the clots weighed.

2.2.10.2 Whole-blood coagulation based on rotational thromboelastometry

Rotational thromboelastometry (ROTEM®), a viscoelastic-based method for the assessment of whole-blood coagulation, was used to assess the effect of antiplatelet agents on blood

coagulation (Mauch et al., 2011). ROTEM® involves using a disposable cup that is attached to a moving pin that rotates back and forth at 4.75°. When coagulation is stimulated in the recalcified citrated whole blood, the fibrin fibres start forming between the cup's wall and pin which causes a resistance in the pin rotation. This resistance is directly proportional to the viscoelastic clot strength (Görlinger et al., 2016). Citrate-anticoagulated whole blood was incubated with inhibitors at 37°C for 10 min then added to a cup containing $CaCl_2$ and a coagulation trigger, as per the manufacturer's instructions for the ROTEM® delta thromboelastometry system (Tem Systems Inc., Munich, Germany). EXTEM was used to monitor the antiplatelet effects of tested inhibitors on the extrinsic coagulation pathway, and INTEM was used to monitor antiplatelet agents' impact on the intrinsic coagulation pathway. Blood coagulation measurement was monitored until all the standard ROTEM® parameters had been acquired.

2.2.10.3 Thrombin generation based on the Calibrated Automated Thrombogram

The Calibrated Automated Thrombogram (CAT) is a fluorogenic-based method developed by Hakmer et al. to measure thrombin generation in RPR and PPP samples (Hemker et al., 2002). CAT measures thrombin generation in a recalcified sample (PRP or PPP) following stimulation with tissue factor by measuring the intensity of the fluorescence that is emitted from fluorogenic substrate that is hydrolysed by the generated thrombin in the sample. CAT involves using a thrombin calibrator which contains α2M-thrombin and mathematical calculations that are used to account for inner filter effect known as fluorescence quenching (Hemker et al., 2002, Hemker and Kremers, 2013). To carry out CAT, PRP was incubated with inhibitors at 37°C for 10 min and added to an Immulon 2 HB 96-well round-bottom plate (Fisher Scientific, Loughborough, UK). Then, thrombin generation was stimulated by adding PRP reagent, which contains a recombinant tissue factor with a minimal amount of phospholipids and FluCa

reagent, which contains the fluorogenic substrate $CaCl_2$, in the duplicate test wells and thrombin calibrator in the duplicate calibration wells, as per the manufacturer's instructions. Thrombin generation was read and monitored in a Fluoroskan Ascent® fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Data were acquired and calculated using Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands).

2.2.11 Statistical analysis

All results are presented as a mean ± standard error of the mean (SEM). The effect of different treatments on the inhibition of responses to different agonists was assessed using one- or two-way ANOVA, as appropriate. Dunnett's correction was used to adjust for multiple comparisons. If ANOVA was not computable due to one or more missing values, then a mixed-model analysis was utilised in its place. A two-tailed *P*-value of <0.05 was considered significant. All statistical analyses were performed using GraphPad Prism software version 8.03 (GraphPad, San Diego, CA, USA).

CHAPTER 3 : EFFECT OF GLENZOCIMAB ON COLLAGEN AND CRP-XL-MEDIATED PLATELET RESPONSES

3.1. Introduction

Collagen is the most abundant protein in the extracellular matrix and the most thrombogenic component of the vascular subendothelium that is exposed following vascular injury or atherosclerotic plaque rupture. Collagen type I and type III are the main types of collagen in the media and adventitia layers of human blood vessels (Xu and Shi, 2014). Collagen types VI, IV, XV, and XVIII, along with laminin, are present predominantly in the basement membrane (Manon-Jensen et al., 2016). Collagen directly interacts with platelets via GPVI and GPIa/IIa and indirectly with GPIb and GPIIb/IIIa via vWF (Nieswandt and Watson, 2003).

GPVI is the main signalling platelet receptor for fibrillar collagen, and it is strictly expressed only on platelets and platelet precursors. GPVI is a member of the Ig superfamily and is co-expressed with the Fc receptor γ-chain (FcRγ) that harbours the immunoreceptor tyrosine-based activation motif, (ITAM) (Gibbins et al., 1996). GPVI is present in monomeric and dimeric forms on the platelet membrane. Dimeric GPVI has a greater affinity for collagen, and GPVI dimerisation is increased upon stimulation with collagen. Furthermore, soluble GPVI that is cleaved during GPVI shedding is reported to be in a dimeric conformation (Loyau et al., 2012, Miura et al., 2002). GPVI has two external Ig-like domains (D1 and D2) that contain a groove region with binding sites that associate with collagen (Horii et al., 2006). GPVI binding to GPO motif (recognition motif) in collagen initiates a cascade of tyrosine phosphorylation in the Src and Syk pathways, which eventually leads to platelet activation (Ezumi et al., 1998b).

GPIa/IIa, also known as integrin α2β1, is the second most expressed integrin on the platelet membrane after GPIIb/IIIa. Apart from its expression on platelets and megakaryocytes, GPIa/IIa is expressed on epithelial cells, endothelial cells, fibroblasts, T cells, and myeloid cells (Kirchhofer et al., 1990). GPIa/IIa is the main platelet receptor for collagen and is believed to be involved mainly in mediating platelet adhesion to collagen (Saelman et al., 1994). GPIa/IIa

is a heterodimer consisting of transmembrane α and β subunits that are non-covalently associated, maintained in a low-affinity state for its ligands on resting platelets. The α2 subunit contains an α2-I domain which serves as a binding site for collagen and contains a metal ion-dependent adhesion site (MIDAS) (Emsley et al., 1997). Binding of GPIa/IIa to collagen is dependent on the presence of metal ions, mainly Mn^{2+} and Mg^{2+} (Calderwood et al., 1997). GPIa/IIa has more affinity for monomeric collagen, and it recognises collagen via the GFOGER motif (Emsley et al., 2000, Jung and Moroi, 1998). Inside-out activation of GPIa/IIa is mediated by activation of platelets by GPVI agonists, thrombin, or soluble mediators ADP and TxA_2, which transform GPIa/IIa into a high-affinity state for collagen, and the subsequent collagen binding initiates outside-in activation of GPIa/IIa (Jung and Moroi, 2000).

Table 3.1 Characteristics of GPVI and GPIa/IIa as collagen receptors

Receptor	GPVI	GPIa/IIa
Type	Immunoglobulin superfamily	Integrin family
Distribution	Megakaryocytes and platelets	Epithelial cells, endothelial cells, fibroblasts, T cells, myeloid cells, and megakaryocytes and/or platelets (Kirchhofer et al., 1990, Carter et al., 1990)
Copies per platelet	~3000–5000 molecules per platelet (Best et al., 2003a).	~1000–3000 molecules per platelet (Best et al., 2003a).
Inter-individual variability	Less variable; ~12% (Best et al., 2003b)	More variable; ~25% (Best et al., 2003b)
Structural conformation on resting platelets	Monomeric form (low affinity) and dimeric form (high affinity) (Miura et al., 2002)	Inactive heterodimer with low affinity (Jung and Moroi, 2000).

Structural conformation on activated platelets	Dimerization and clustering (Poulter et al., 2017b)	Active form with high affinity (Jung and Moroi, 1998).
Binding site for collagen	Extracellular Ig domains (D1 and D2)	α2-I domain
Recognition motifs in collagen	GPO sequence (Knight et al., 1999a)	GFOGER sequence (Knight et al., 2000)
Cation requirement for binding	Ion-independent	Mg^{2+}, Mn^{2+} (Jung and Moroi, 1998, Luque et al., 1994)
Dependency on the enzymatically catalysed disulfide exchange in adhesion to collagen	Independent (Lahav et al., 2003)	Dependent (Lahav et al., 2003)
Signal transduction	Outside-in (Chen and Kahn, 2003)	Inside-out (Jung and Moroi, 2000, Chen and Kahn, 2003)
Affinity for collagen type	Fibrous collagen type I and type III (Jung and Moroi, 1998)	Acid-soluble collagen type I and type III (Monnet et al., 2000, Mazzucato et al., 2009, Jung and Moroi, 1998)
Physiological role	Key platelet signalling receptor for collagen (Kehrel et al., 1998)	Mediates firm platelet adhesion following the interaction of GPIb with vWF (Cruz et al., 2005)

3.2. Aims

This chapter aims to investigate the effect of GPVI blockade with glenzocimab (in combination with aspirin and ticagrelor) on collagen-mediated platelet responses. The following aspects of platelet function were investigated: aggregation, spreading, platelet thrombus formation, and P-selectin expression under flow as well as tyrosine phosphorylation. A specific GPVI agonist, collagen-related peptide (CRP-XL), was also used to investigate GPVI-mediated platelet responses.

3.3. Results

3.3.1. Effect of glenzocimab with aspirin and ticagrelor on collagen-mediated platelet responses

Collagen is considered to be the main physiological ligand for GPVI. The effect of glenzocimab on different platelet responses mediated by Horm collagen was investigated either in the presence or absence of aspirin and ticagrelor, and these responses were also compared with those observed with eptifibatide. Furthermore, the effect of glenzocimab on different platelet responses mediated by non-GPVI agonists was also investigated to exclude off-target effects of glenzocimab.

3.3.1.1 Glenzocimab dose-dependently inhibited collagen-induced platelet aggregation

Glenzocimab blocked platelet aggregation induced by 1, 5, and 10 µg/ml collagen in platelet-rich plasma (PRP) in a dose-dependent fashion (Figure 3.1). The inhibitory effect of glenzocimab on collagen-stimulated platelet aggregation was inversely related to the collagen concentration; if more collagen was used, then less inhibition was achieved.

In platelet aggregation induced by 1 µg/ml collagen, final platelet aggregation at 10 min was 67 ± 10% in the control samples. Significant inhibition of platelet aggregation was achieved by 2.5 µg/ml glenzocimab (final aggregation: 34 ± 14%; P=0.007), and near-complete inhibition of platelet aggregation was achieved with 50 µg/ml glenzocimab (final aggregation: 4 ±1%; P<0.001). In platelet aggregation induced by 5 µg/ml collagen, final aggregation was 82 ± 2% in the uninhibited controls. At a 10 µg/ml concentration, glenzocimab significantly reduced platelet aggregation (final aggregation: 41 ± 10%; P<0.001). Potent inhibition of platelet aggregation was achieved by 50 µg/ml glenzocimab (final aggregation: 14 ± 2%; P<0.001). In platelet aggregation induced by 10 µg/ml collagen, which was selected as the maximum

concentration, final aggregation in the control samples was 82 ± 2%. Again, 10 μg/ml glenzocimab significantly reduced platelet aggregation than that in control (final aggregation: 56 ± 10%; P=0.03). Potent inhibition of platelet aggregation was achieved by 25 μg/ml glenzocimab (final aggregation: 34 ± 11%; P=0.0001). In summary, these results showed that glenzocimab dose-dependently inhibited collagen-induced platelet aggregation. However, using a higher concentration of collagen to stimulate platelet aggregation led to a decrease in the inhibitory effect of glenzocimab.

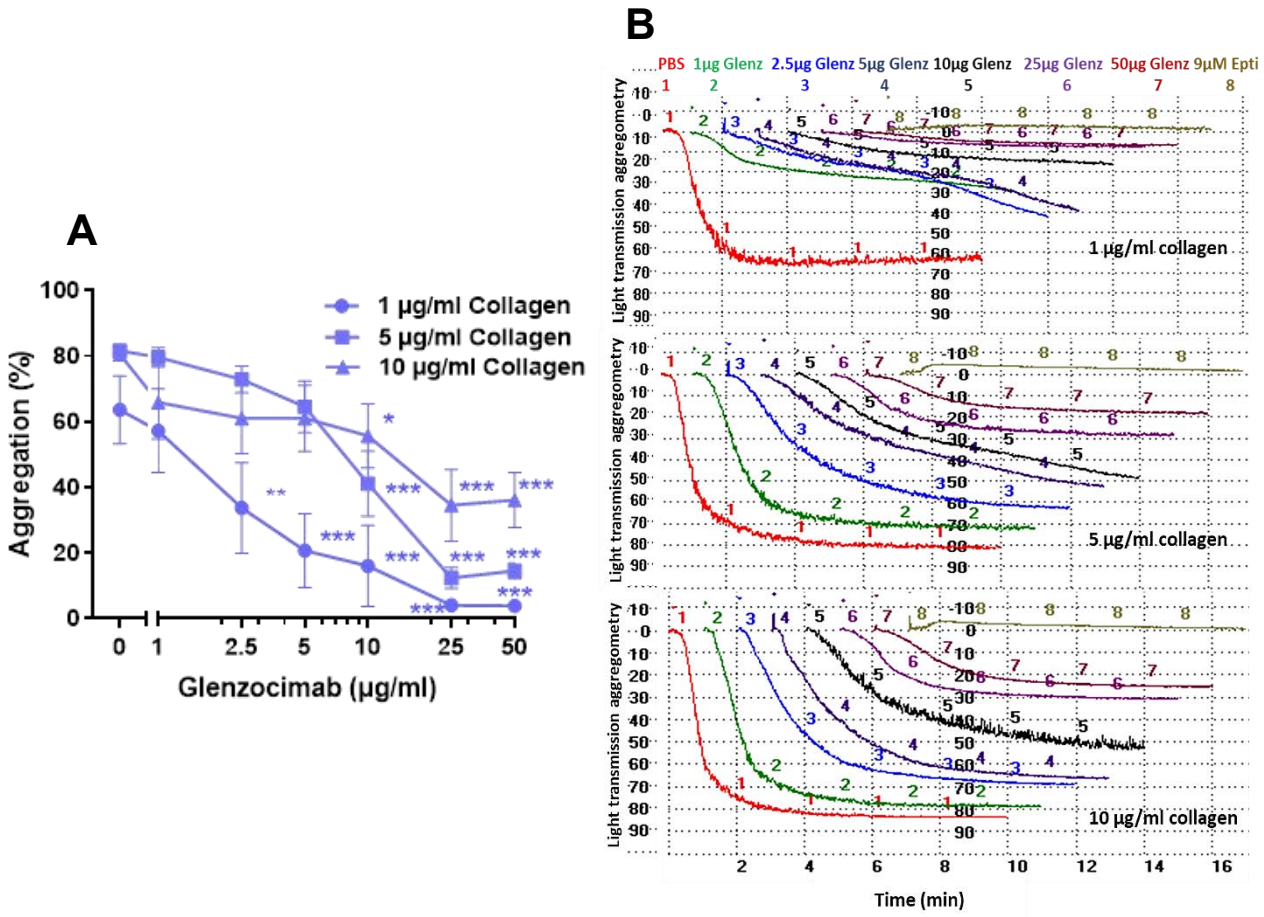


Figure 3.1. Effect of glenzocimab on collagen-stimulated platelet aggregation in PRP.

A) Dose-response curve of glenzocimab (1-50 µg/ml) on 1, 5, and 10 µg/ml collagen-induced platelet aggregation. **B)** Representative traces from a single LTA experiment, where human PRP was preincubated with different concentrations of glenzocimab or (PBS) control for 10 min at 37 °C and stimulated with 1, 5, and 10 µg/ml collagen respectively, then aggregation was monitored for 10 min in PAP-8E aggregometer. The effect of different concentrations of glenzocimab were compared to the control samples and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=6, *P<0.05, **P<0.01, and ***P<0.001).

3.3.1.2 Glenzocimab showed amplified antiplatelet effects on collagen-induced platelet aggregation when combined with aspirin and/or ticagrelor

Conventional antiplatelet therapy is usually based on a combination of two antiplatelet agents that work on different activation pathways to increase antithrombotic potency. The inhibitory effect of ascending concentrations of aspirin and ticagrelor was investigated on platelet aggregation stimulated by 1, 5, and 10 µg/ml Horm collagen as presented in the supplementary Figure 1 and Figure 2. The magnitude of inhibitory effect of aspirin and ticagrelor on collagen-induced platelet aggregation was dependent on the concentration of collagen; aspirin and ticagrelor showed a stronger inhibitory effect on platelet aggregation stimulated by low collagen (1µg/ml).

Aspirin at concentration of 30 µM and/or ticagrelor at 1µM were combined with a range concentration of glenzocimab were investigated on collagen-stimulated platelet aggregation as aspirin at 30 µM and ticagrelor at 1 µM effectively blocked their specific targets as shown in Figure 3.2B and C. Furthermore, spiking PRP samples with 30 µM ASA in LTA *in vitro* was produced a similar effect of 325 mg aspirin which is used as maintenance dose for treating patients with ACS as shown in a previous study (Khan et al., 2020, Zeymer, 2013). In addition, ticagrelor at 1µM was chosen and investigated as an optimal concentration to study the effect of ticagrelor in combination with GPVI inhibitor (revacept) on platelet thrombus formation on atherosclerotic plaque *in vitro* in a previous study (Mojica Munoz et al., 2017).

The results demonstrated that glenzocimab potently inhibited collagen-induced platelet aggregation in combination with aspirin and ticagrelor whereas the dual combination of aspirin and ticagrelor did not cause significant inhibition of platelet aggregation mediated by collagen (Figure 3.2A and D).

Aspirin had no significant effect on platelet aggregation (final aggregation: 61 ± 8% compared to 80 ± 2% in uninhibited samples; P=0.32), and ticagrelor did not reduce platelet aggregation (76 ± 2%; P=0.66). The combined use of aspirin and ticagrelor had no significant effect on platelet aggregation (final aggregation: 44 ± 9%; P=0.10), and 5 μg/ml glenzocimab also showed no significant effect on platelet aggregation (final aggregation: 51 ± 12%; P=0.42). However, 5 μg/ml glenzocimab in combination with aspirin reduced platelet aggregation by 80% (final aggregation: 16 ± 8%; P<0.0001). In combination with ticagrelor, glenzocimab also significantly reduced platelet aggregation by 60% (final aggregation: 32 ± 13%; P<0.001). The combination of 5 μg/ml glenzocimab with aspirin and ticagrelor reduced platelet aggregation by 86% (final aggregation: 11 ± 6%; P<0.001). Concurrent use of high concentrations (50 μg/ml) of glenzocimab with aspirin and ticagrelor caused the most potent reduction in platelet aggregation (final aggregation: 6 ± 3%; P<0.001).

In summary, combining glenzocimab with aspirin and ticagrelor produced amplified inhibitory effect on collagen induced platelet aggregation in PRP in LTA. On the other hand, dual combination of aspirin and ticagrelor did not show any significant inhibitory effect on platelet aggregation stimulated by collagen.

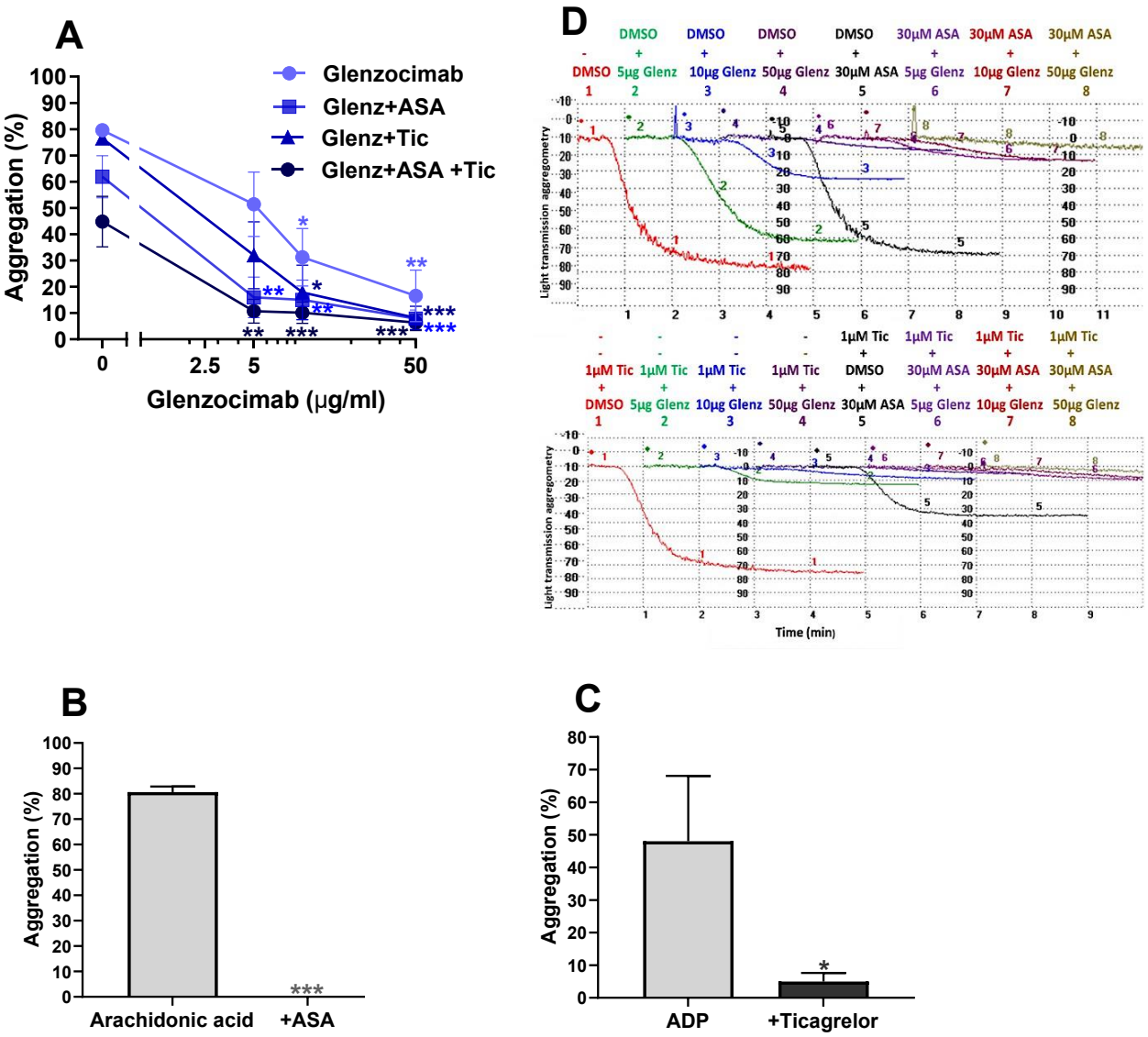


Figure 3.2. Dose-response curve of combined use of glenzocimab with aspirin and ticagrelor on collagen-induced platelet aggregation in PRP.

A) Glenzocimab in combination with the other antiplatelet agents on platelet aggregation induced by 5 µg/ml collagen. **B)** Inhibition of 1 mM AA-stimulated platelet aggregation by 30 µM aspirin. **C)** Inhibition of 5 µM ADP-stimulated platelet aggregation by 1 µM ticagrelor. **D)** Representative traces from a single experiment for glenzocimab in combination with aspirin and ticagrelor on 5 µg/ml collagen induced platelet aggregation. The effect of combinations of different concentrations of glenzocimab in combination with other antiplatelet agents was compared to vehicle control and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=6, *P<0.05, **P<0.01, and ***P<0.001).

3.3.1.3 Glenzocimab combined with aspirin and ticagrelor showed a pattern of inhibition of collagen-induced platelet aggregation similar to eptifibatide

Multiple electrode aggregometry (MEA) assesses platelet aggregation in whole blood, and its results are predictive of adverse clinical outcomes in patients with acute coronary syndromes (Aradi et al., 2014). Therefore, initial amplified results observed with light transmission aggregometry (LTA) were further confirmed using MEA by investigating the inhibitory effect of glenzocimab (50 μg/ml), aspirin (30 μM), and ticagrelor (1 μM) ± eptifibatide (9 μM) as these are currently frequently used for patients with STEMI. Eptifibatide at a concentration of 9 μM was selected to study the effect of GPIIb/IIIa inhibition versus GPVI inhibition with glenzocimab, as 9 μM is the most commonly used concentration of eptifibatide in the publications that studied effect of GPIIb/IIIa blockade on platelet activation.

Glenzocimab significantly inhibited collagen-stimulated platelet aggregation assessed by MEA, and glenzocimab combined with aspirin and ticagrelor yielded a pattern of inhibition similar to that achieved by a combination of eptifibatide with aspirin and ticagrelor (Figure 3.3). Glenzocimab reduced collagen-induced platelet aggregation by more than 50% (39 ± 2 U in comparison with 89 ± 6 U in the uninhibited control samples; P=0.002) (Figure 3.3). Glenzocimab in combination with aspirin and ticagrelor caused a further significant reduction in platelet aggregation (21 ± 2 U; P<0.001), which was greater than the inhibition achieved by aspirin and ticagrelor alone (57 ± 9 U; P=0.01). Eptifibatide caused the most profound inhibition of platelet aggregation compared with the other antiplatelet agents (18 ± 3 U; P<0.001). With the addition of aspirin and ticagrelor, there was an additional slight decrease in platelet aggregation (13 ± 3 U; P<0.001). More importantly, adding glenzocimab to aspirin and ticagrelor provided a pattern of inhibition of collagen-induced platelet aggregation similar to that observed with eptifibatide.

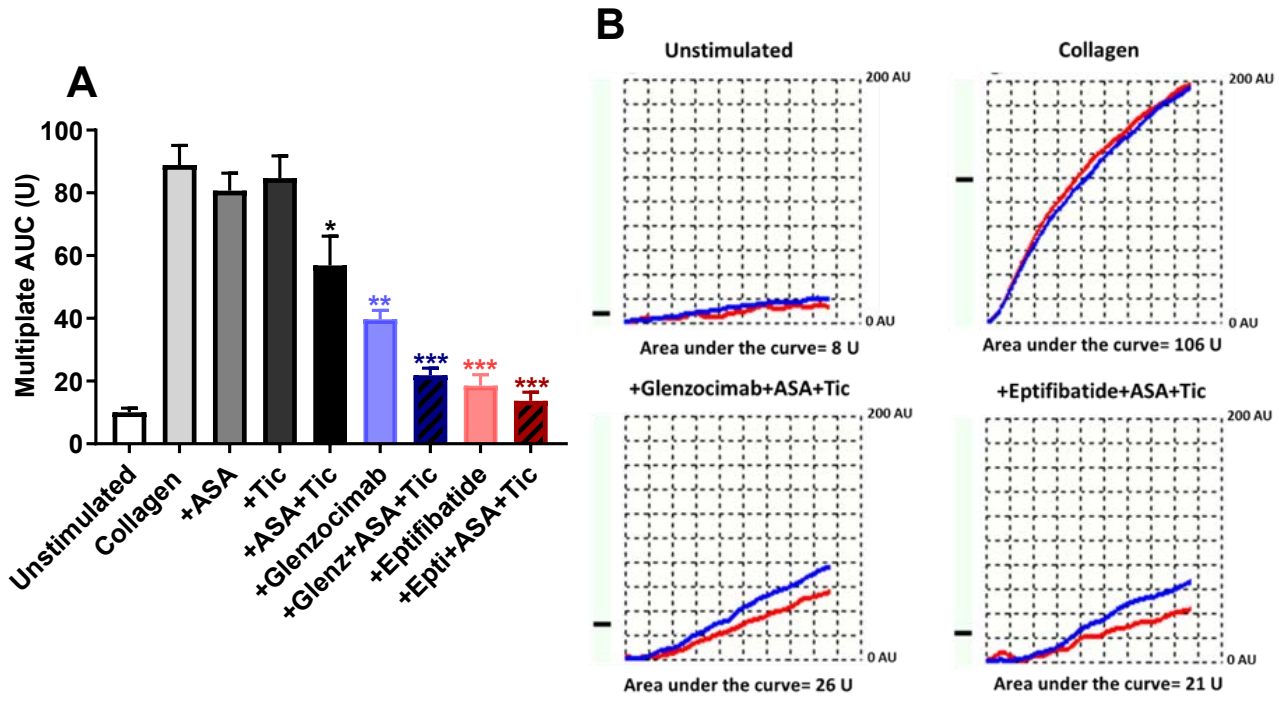


Figure 3.3. Effect of glenzocimab or eptifibatide in combination with aspirin and ticagrelor on collagen-induced platelet aggregation in whole blood samples assessed by multiple electrode aggregometry.

A) Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) combined with aspirin (30 µM) and ticagrelor (1 µM) on platelet aggregation induced by 3.2 µg/ml collagen in multiple electrode aggregometry. **B)** Representative aggregation traces from a single experiment that was performed in the Multiplate® analyser as per the manufacturer's instructions. The effect of treatment groups was compared to the control samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=6, *P<0.05, **P<0.01, and ***P<0.001).

3.3.1.4 Glenzocimab did not demonstrate off-target effects on platelet aggregation stimulated by non-GPVI agonists in platelet-rich plasma and whole blood samples

To investigate and exclude any off-target antiplatelet effects of GPVI inhibition, the effect of glenzocimab was examined on platelet aggregation mediated by non-GPVI agonists, particularly the main activation mechanisms that are blocked by conventional antiplatelet therapy.

Aggregation results showed that glenzocimab did not have any inhibitory effects on the main platelet activation pathways mediated by TxA_2, $P2Y_{12}$, and PAR1, which are stimulated by arachidonic acid (AA, also referred to as the ASPI test when used in MEA), ADP, and thrombin receptor-activating peptide (TRAP), respectively, whether in whole blood or PRP (Figure 3.4). Platelet aggregation values in glenzocimab (50 μg/ml)-treated samples were similar to uninhibited vehicle-control samples confirming that GPVI blockade does not have additional effects on platelet receptors targeted by current antiplatelet therapy. As expected, aspirin (30 μM) and ticagrelor (1 μM) exerted their antiplatelet effect on their correspondent targets effectively. Furthermore, aspirin and ticagrelor also significantly reduced platelet aggregation mediated by non-TxA_2 and $P2Y_{12}$ agonists. Eptifibatide (9 μM) entirely blocked platelet aggregation in both PRP and whole blood regardless of the platelet receptor and agonist involved.

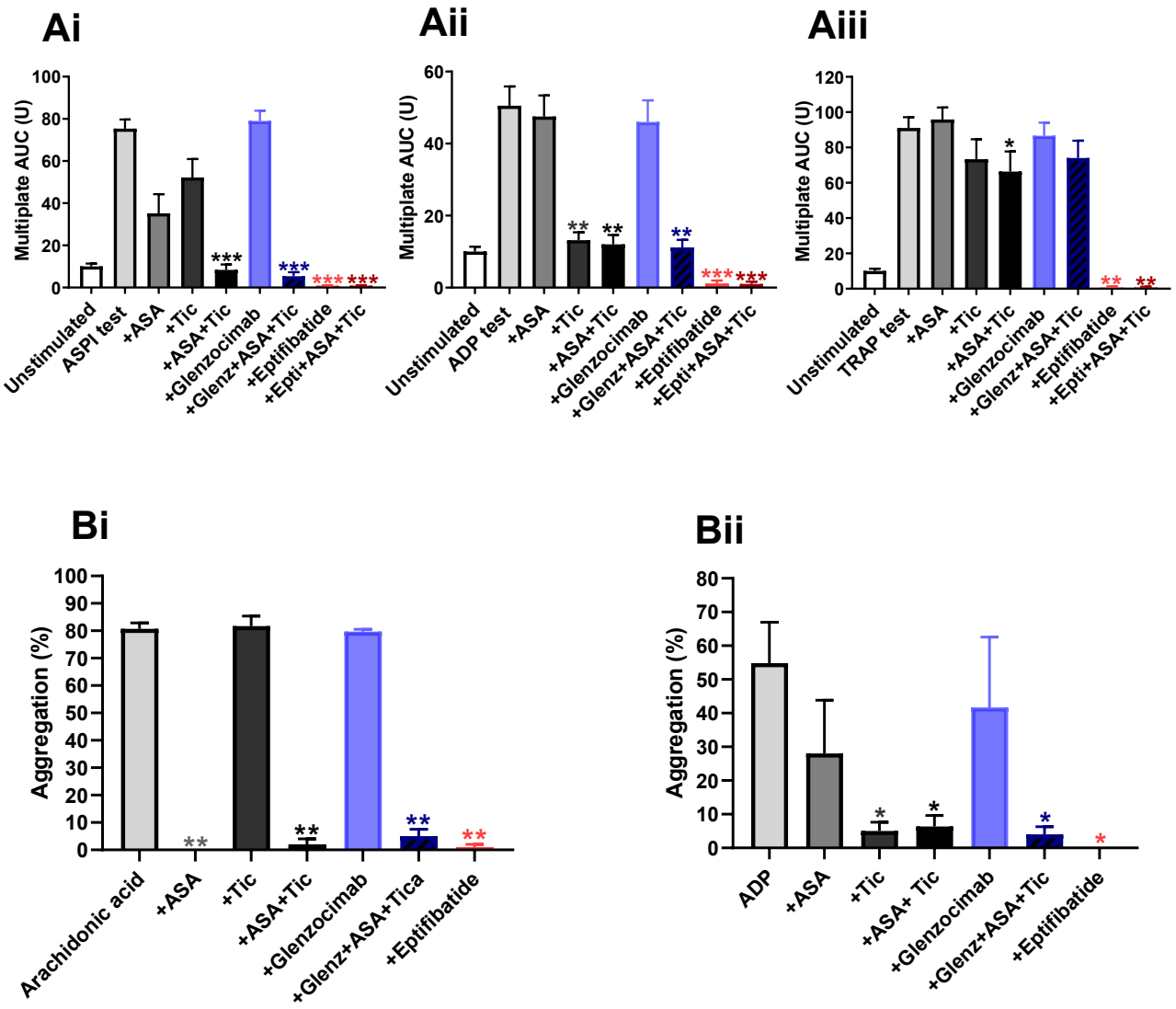


Figure 3.4. Effect of glenzocimab on platelet aggregation stimulated by non-GPVI agonists in whole blood and PRP.

Ai-iii) Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on platelet aggregation in whole blood stimulated by 0.5 µM AA (Ai), 6.5 µM ADP (Aii) and 32 µM TRAP (Aiii) respectively. **Bi-ii)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on platelet aggregation in PRP stimulated by 1 mM AA (Bi) and 5 µM ADP (Bii) respectively. Effect of treatment groups was compared to the control sample and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=6 for MEA, n=3 for LTA, *P<0.05, **P<0.01, and ***P<0.001).

3.3.1.5 Glenzocimab inhibited platelet adhesion and thrombus formation on collagen under arterial flow conditions in a microfluidic chamber

The antiplatelet effects of glenzocimab or eptifibatide (± aspirin and ticagrelor) were investigated on platelet thrombus formation and platelet adhesion on a surface coated with a low concentration of Horm collagen in a microfluidic chamber under an arterial flow rate. Fluorescence intensity was used as an indicator of platelet-rich thrombus formation; more platelet deposition was associated with more intense fluorescence. The percentage of platelet surface coverage was used to calculate the effect of the inhibitor on platelet adhesion. The results showed that glenzocimab, aspirin and ticagrelor, and eptifibatide potently blocked platelet thrombus formation (Figure 3.5A). On the other hand, eptifibatide completely blocked platelet adhesion followed by glenzocimab in the magnitude of inhibition, without a significant effect for aspirin and ticagrelor on platelet adhesion on collagen (Figure 3.5B).

Glenzocimab blocked thrombus formation by 90% (3 ± 0 AU in comparison with 34 ± 9 AU at 10 min in the control sample; $P<0.001$). Glenzocimab inhibited thrombus formation by 91% when combined with aspirin and ticagrelor (2 ± 0 AU; $P<0.001$), whereas the use of only aspirin and ticagrelor led to an 85% reduction in thrombus formation on collagen (5 ± 1 AU; $P<0.001$). Eptifibatide alone and eptifibatide combined with aspirin and ticagrelor reduced thrombus formation by 96% (1 ± 0 AU; $P<0.001$). Aspirin and ticagrelor did not significantly reduce the platelet surface coverage (25 ± 6% compared to 48 ± 6% in the control samples; $P=0.16$). Glenzocimab significantly reduced surface coverage by 72% (13 ± 2%; $P=0.002$). When added with aspirin and ticagrelor, glenzocimab further decreased the surface coverage by 76% (11 ± 2%; $P=0.02$). Eptifibatide alone almost entirely blocked platelet adhesion as it caused a 95% reduction in the surface coverage (2 ± 1%; $P=0.004$). Combining eptifibatide with aspirin and ticagrelor further reduced the surface coverage by 97% (1 ± 1%; $P=0.003$).

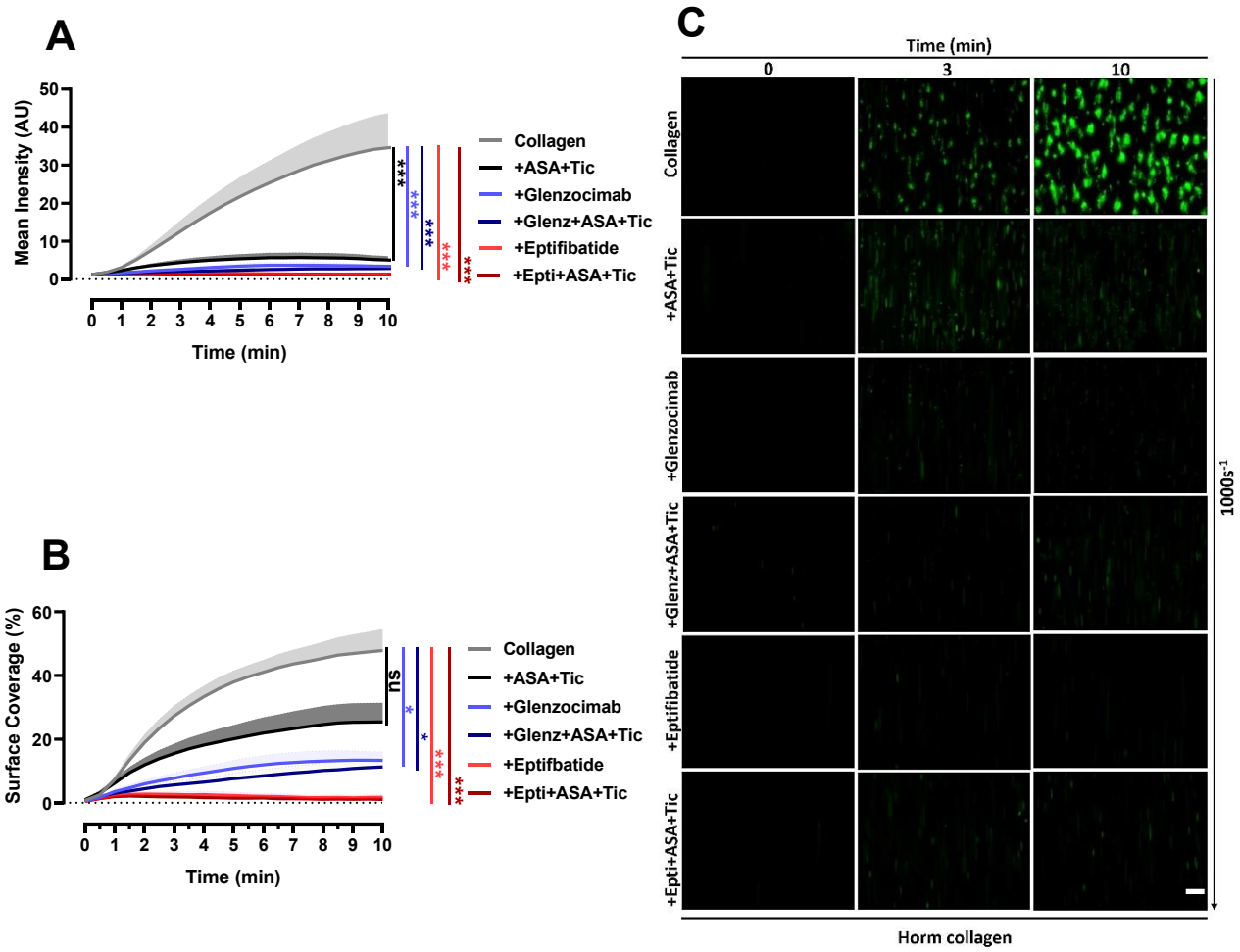


Figure 3.5. Effect of glenzocimab on platelet adhesion and thrombus formation on low concentrations of Horm collagen under flow conditions.

A) Glenzocimab potently inhibited platelet thrombus formation as reflected by fluorescence intensity. **B)** Glenzocimab significantly reduced platelet adhesion as reflected by platelet surface coverage %. **C)** Representative images from a single flow adhesion experiment, where $DiOC_6$-labelled blood was incubated with 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide or 0.01% DMSO for 15 min at 37°C then flowed over 50 µg/ml Horm collagen at a shear rate of 1000 s^{-1} for 10 min. Images were taken every 30 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by ImageJ software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of antiplatelet agents was compared to the control (DMSO) samples and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=6, ns=non-significant, *P<0.05, **P<0.01, and ***P <0.001).

3.3.1.6 Glenzocimab reduced platelet thrombus formation but not adhesion on high concentrations of collagen under arterial flow conditions

When assessing adhesion under flow conditions in a microfluidic chamber coated with 50 µg/ml collagen, glenzocimab had a potent effect on both platelet thrombus formation and platelet adhesion; therefore, glenzocimab with the same combination of antiplatelet agents and concentration was tested against 200 µg/ml collagen to investigate whether glenzocimab can maintain its antiplatelet potency on a higher collagen concentration. The results demonstrated that all investigated antiplatelet agents including glenzocimab maintained their potency on inhibiting platelet thrombus formation, but only eptifibatide was still able to block platelet adhesion on a higher concentration of collagen (Figure 3.6).

Aspirin and ticagrelor reduced thrombus formation by 91% (17 ± 1 AU compared to 210 ± 17 AU in the uninhibited samples; $P<0.001$). Glenzocimab alone decreased platelet thrombus formation by 77% (47 ± 11 AU; $P=0.003$). When combined with aspirin and ticagrelor, glenzocimab showed a slightly increased antithrombotic effect by reducing thrombus development by 92% (15 ± 2 AU; $P<0.001$). Eptifibatide with/without aspirin and ticagrelor nearly completely blocked thrombus formation (by 99%; 2 ± 1 AU; $P<0.001$).

Aspirin and ticagrelor had no significant effect on the platelet surface coverage (60 ± 6% compared to 81 ± 1% in the untreated samples; $P=0.11$). Glenzocimab also did not reduce the surface coverage (72 ± 4%; $P=0.25$). However, adding glenzocimab in combination with aspirin and ticagrelor partially decreased the surface coverage (58 ± 3%; $P=0.005$). Eptifibatide alone potently decreased the surface coverage (4 ± 1%; $P<0.001$). Combination of eptifibatide with aspirin and ticagrelor provided no additional effect on the surface coverage (4 ± 1%; $P<0.001$).

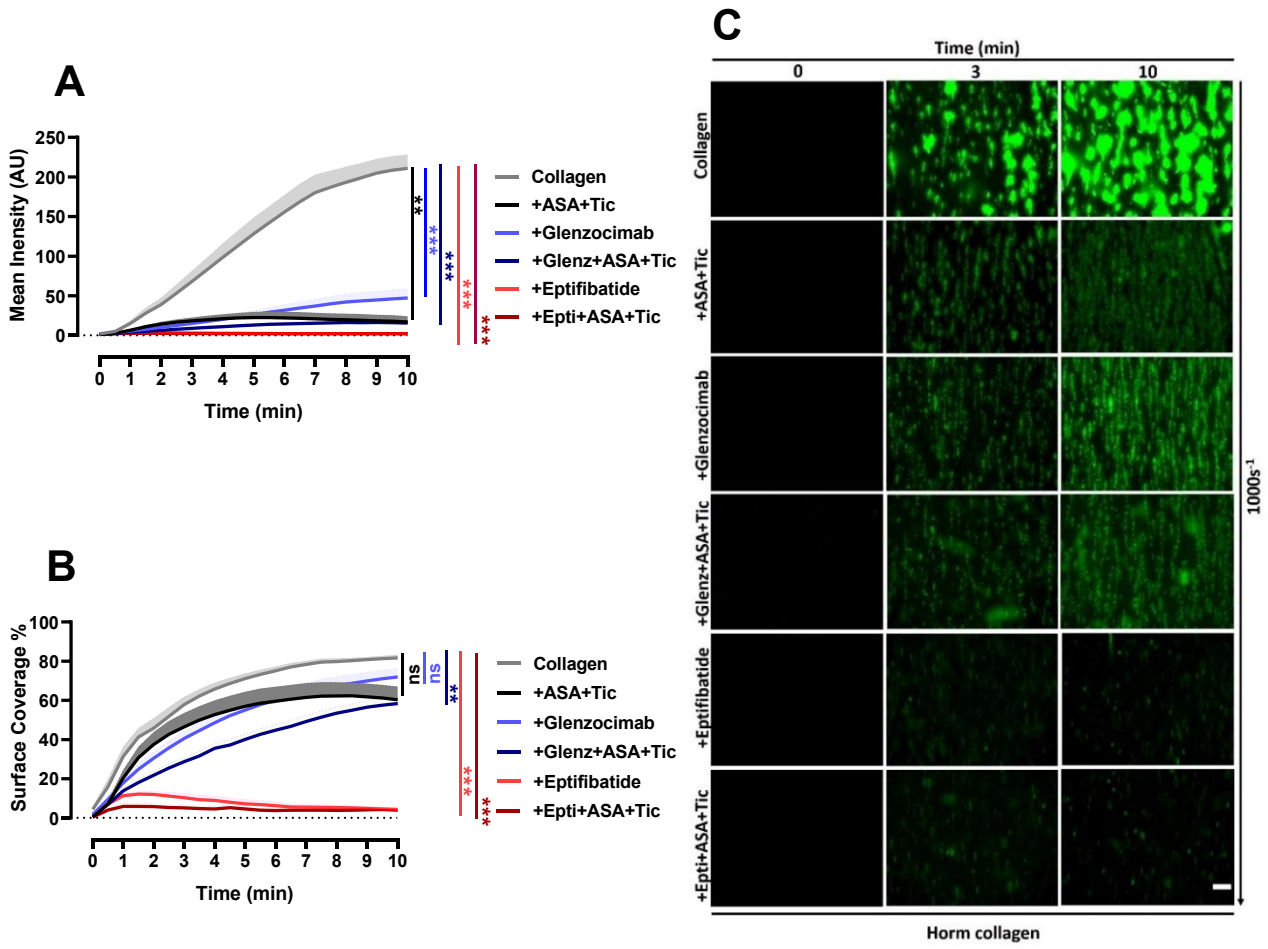


Figure 3.6. Effect of glenzocimab on platelet adhesion and thrombus formation on high concentrations of Horm collagen under flow conditions.

A) Glenzocimab inhibited platelet thrombus formation as reflected by fluorescence intensity. **B)** Glenzocimab did not affect platelet adhesion as reflected by platelet surface coverage %. **C)** Representative images from a single flow adhesion experiment, where $DiOC_6$-labelled blood was incubated with 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide or 0.01% DMSO for 15 min at 37°C then flowed over 200 µg/ml Horm collagen at a shear rate of 1000 s^{-1} for 10 min. Images were taken every 30 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by ImageJ software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of antiplatelet agents was compared to untreated (DMSO control) samples and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=6, ns=non-significant, **P<0.01, and ***P<0.001).

3.3.1.7 Glenzocimab exerted a greater inhibitory effect on P-selectin expression than conventional antiplatelet agents in whole blood flowing on collagen under an arterial shear rate

The investigation of the effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) was extended to P-selectin expression in hirudin-anticoagulated whole blood flowing at 1000 s^{-1} in a microfluidic chamber coated with 200 µg/ml Horm collagen. The results showed that glenzocimab exerted a greater effect on P-selectin expression than eptifibatide and aspirin and ticagrelor particularly in the first 5 min of flow despite its partial effect on the surface coverage and marginal effect on the thrombus fluorescence intensity (the development of thrombi expressing P-selectin) observed at the end of flow (Figure 3.7).

Aspirin and ticagrelor reduced thrombus fluorescence intensity by 65% (20 ± 4 AU compared to 58 ± 8 AU in the control samples; P=0.03). Glenzocimab decreased thrombus fluorescence intensity by 73% (15 ± 1 AU; P=0.03) and by 75% (14 ± 2 AU; P=0.03) when used concomitantly with aspirin and ticagrelor, respectively. Eptifibatide decreased thrombus fluorescence intensity by 76% (13 ± 3 AU; P=0.03) when used alone and by 79% (12 ± 3 AU; P=0.02) when used in combination with aspirin and ticagrelor.

Aspirin and ticagrelor did not affect the surface coverage (79 ± 4% compared to 80 ± 1% in the control samples; P>0.99). Glenzocimab marginally reduced the surface coverage (71 ± 1%; P=0.008) with no additional effect observed upon combination with aspirin and ticagrelor (74 ± 3%; P=0.39). Eptifibatide showed no significant effect on the surface coverage (66 ± 10%; P=0.58) even when used in combination with aspirin and ticagrelor (63 ± 10%; P=0.45).

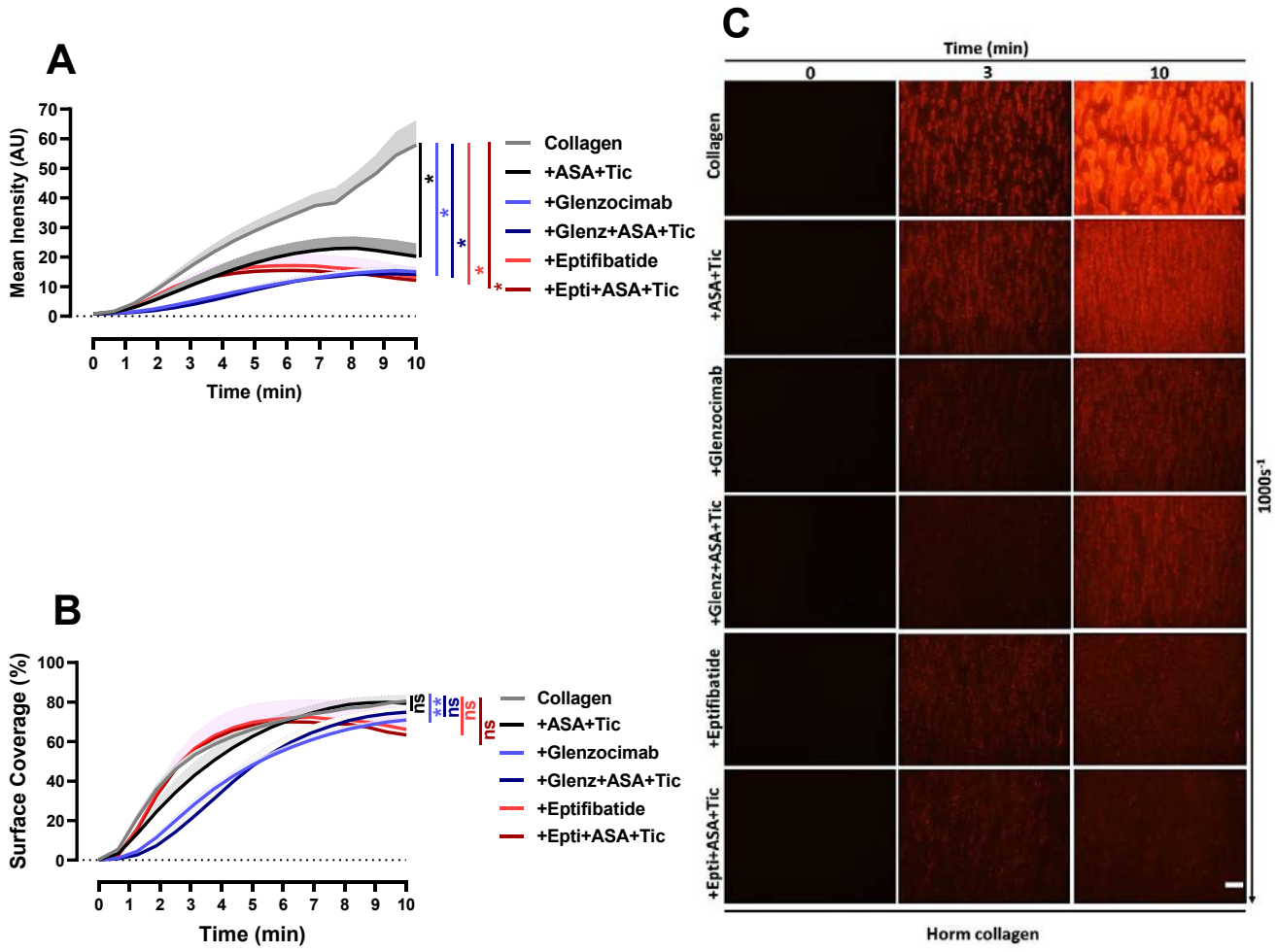


Figure 3.7. Effect of glenzocimab and other antiplatelet agents on P-selectin expression on platelets that have adhered to Horm collagen under flow conditions.

A) Glenzocimab significantly reduced accumulation of P-selectin expressing platelets as reflected by fluorescence intensity. **B)** Glenzocimab reduced adhesion of P-selectin expressing platelets as reflected by platelet surface coverage %. **C)** Representative images from a single flow adhesion experiment where hirudin-anticoagulated blood was incubated with AF647-labelled anti-human CD62P mAb, 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide or 0.01% DMSO for 15 min at 37°C then flowed over 200 µg/ml Horm collagen at a shear rate of 1000 s^{-1} for 10 min. Images were taken every 35 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by Image J software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of antiplatelet agents compared to (DMSO) control were determined using two-way ANOVA with Dunnett correction for multiple comparisons (n=4, ns=non-significant, *P<0.05, and **P<0.01).

3.3.1.8 Glenzocimab in combination with aspirin and ticagrelor reduced the surface area of spread platelets but not the number of adherent platelets on immobilised collagen under static conditions

The effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on platelet spreading on immobilised collagen was determined in washed platelets, where plasma proteins such as fibrinogen are removed to avoid their effect on spreading. The effect of antiplatelet agents on platelet spreading was assessed by measuring the platelet surface area, platelet perimeter, and counting the number of adherent platelets. Dasatinib, a potent inhibitor of platelet spreading, was used as a positive control.

The results showed that glenzocimab marginally reduced platelet spreading without affecting the static adhesion of platelets on collagen, whereas aspirin and ticagrelor partially reduced static adhesion but not spreading; however, their addition intensified the effect of glenzocimab on platelet spreading (Figure 3.8). Eptifibatide reduced platelet spreading but not adhesion; however, dasatinib reduced both platelet spreading and adhesion.

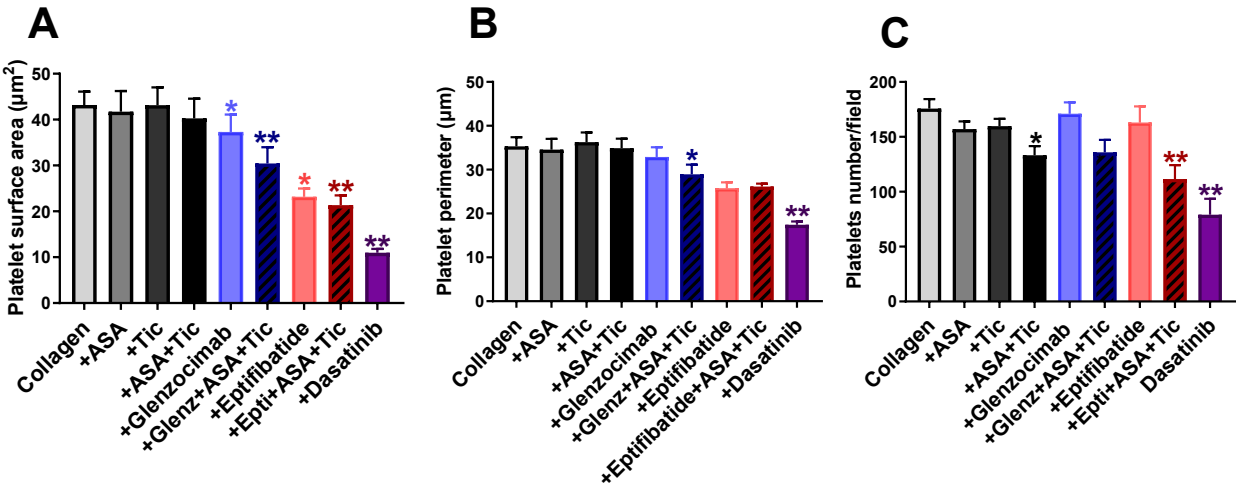
Aspirin and ticagrelor showed no effect on the platelet surface area (40 ± 4 μm^2 compared to 43 ± 3 μm^2 in the DMSO control; P=0.56). Glenzocimab alone marginally reduced the surface area by 13% (37 ± 3 μm^2; P=0.04); however, when combined with aspirin and ticagrelor, glenzocimab further decreased the surface area by 29% (30 ± 3 μm^2; P=0.002). Eptifibatide substantially decreased the surface area by 46% (23 ± 1 μm^2; P=0.01) when used alone and by 50% (21 ± 2 μm^2; P=0.006) with the addition of aspirin and ticagrelor. Dasatinib, an Src inhibitor, markedly reduced the surface area (by 74%; 11 ± 1 μm^2; P=0.001).

Aspirin and ticagrelor did not decrease the platelet perimeter (34 ± 2 μm compared to 35 ± 2 μm in the DMSO control; P=0.84). Glenzocimab had no significant effect on the perimeter (32

± 2 μm; P=0.31). However, the combination of glenzocimab with aspirin and ticagrelor marginally reduced the perimeter by 18% (29 ± 2 μm; P=0.02). There was a non-significant trend for eptifibatide of lowering the perimeter by 27% (25 ± 1 μm; P=0.09); the combination of aspirin and ticagrelor with eptifibatide did not additionally affect the perimeter (26 ± 1 μm; P=0.12). Similar to the extent of its potency on the surface area, dasatinib reduced the perimeter by 50% (17 ± 1 μm; P=0.02).

Counting the number of adherent platelets that remained attached to the collagen after one wash to remove non-adherent platelets revealed no effect of glenzocimab treatment on platelet adhesion on collagen under static conditions when platelets were allowed to spread on collagen for 30 min. Glenzocimab did not reduce platelet adhesion (171 ± 10 platelets compared to 175 ± 8 platelets in the untreated samples; P>0.99). The combined use of aspirin and ticagrelor reduced platelet adhesion by 24% (133 ± 8 platelets; P=0.01). The addition of glenzocimab plus aspirin and ticagrelor did not have an additional effect (135 ± 11 platelets; P=0.12). Eptifibatide combined with aspirin and ticagrelor decreased platelet adhesion by 37% (111 ± 12 platelets; P=0.006). Dasatinib greatly reduced platelet adhesion by 55% (79 ± 14 platelets; P=0.006).

In summary, these results indicated that glenzocimab marginally reduced the surface area of platelet spread on collagen coated surfaces without an effect on platelet adhesion. Addition of aspirin and ticagrelor amplified the effect of glenzocimab on platelet spreading but not adhesion. Eptifibatide significantly reduced platelet spreading and exerted a significant effect on platelet adhesion on collagen in the presence of aspirin and ticagrelor.


A
Platelet surface area (μm²)
0
10
20
30
40
50
Collagen
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
B
Platelet perimeter (μm)
0
10
20
30
40
50
Collagen
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Eptifibatide+ASA+Tic
+Dasatinib
C
Platelets number/field
0
50
100
150
200
Collagen
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
Dasatinib

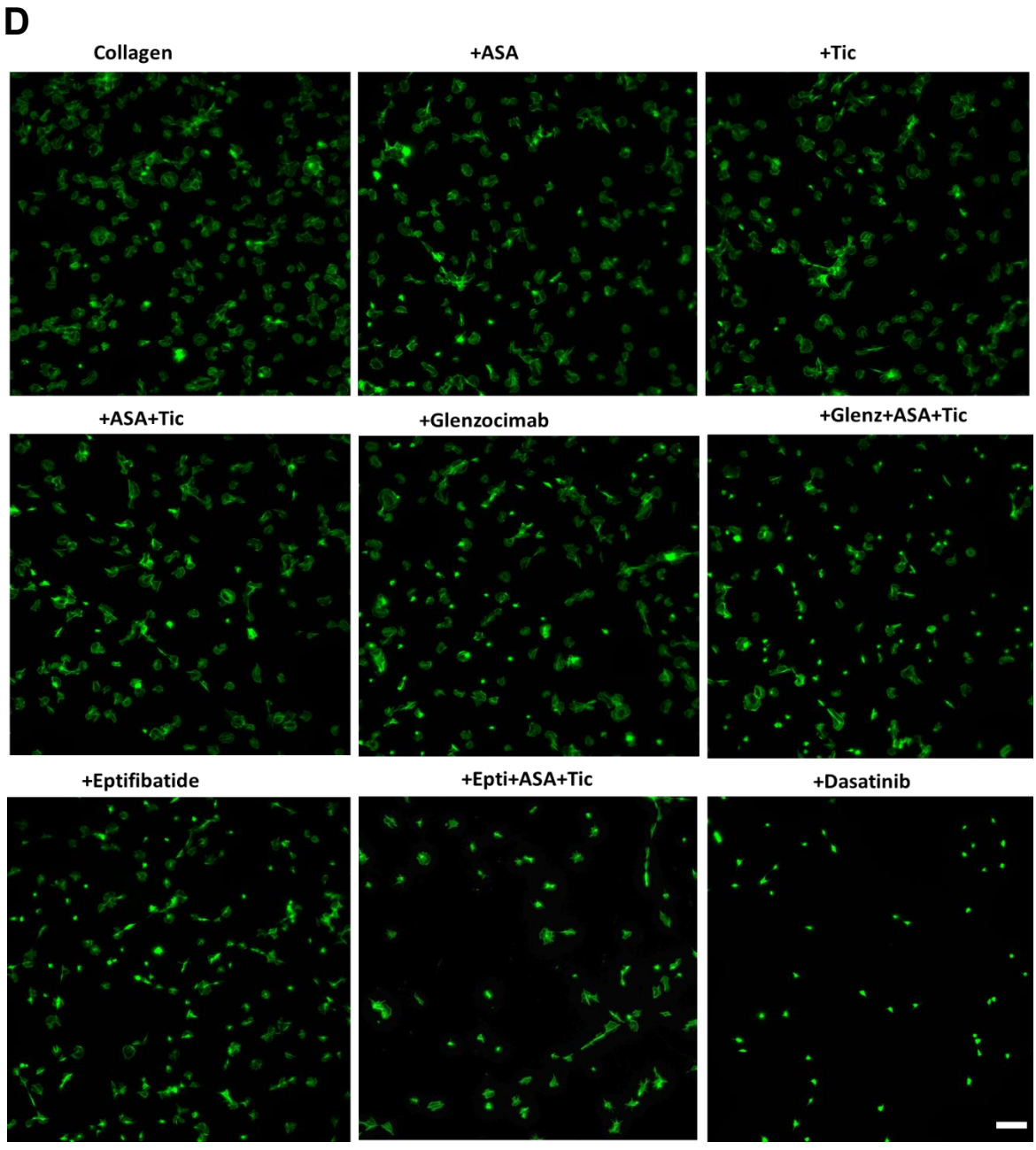

D
Collagen
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib

Figure 3.8. Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on platelet spreading on collagen under static conditions.

A) Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet surface area. **B)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet perimeter. **C)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the number of adherent platelets. **D)** Representative images from a single spreading experiment, where human WP (2.0×10^7 mL) was preincubated with 0.01% DMSO, glenzocimab (50 µg/ml) or eptifibatide (9 µM) ± aspirin (30 µM) and ticagrelor (1 µM) or dasatinib (10 µM) for 30 min at 37ºC. Then, pre-treated platelets were allowed to spread on immobilized collagen (10 µg/ml) on coverslips for 30 min at 37ºC. Platelets were fixed with 4% neutralized formalin buffer for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min and stained with Alex-488-phalloidin for 45 min in the dark. The coverslips were mounted on glass slides and imaged under the Zeiss Axiovert 200M microscope. 5 random images were analysed for each experiment. Scale bar 20 µm. The effect of treatment groups was compared to the control and assessed used one-way ANOVA with Dunnett correction for multiple comparisons (n=6, * $P<0.05$ and** $P<0.01$).

3.3.1.9 Glenzocimab significantly reduced phosphorylation of the main tyrosine kinases in GPVI signalling induced by collagen, and its effect was amplified in the presence of aspirin and ticagrelor

This study also included the molecular investigation of the main tyrosine kinases (Syk, LAT, and PLCγ2) involved in the GPVI/FcRγ2 signalling pathway. This provided more mechanistic insights into the effect of glenzocimab alone or in combination with aspirin and ticagrelor on GPVI activation induced by collagen. Washed human platelets were pre-treated with eptifibatide and incubated for 10 min to block GPIIb/IIIa outside-in signalling and platelet aggregation prior to stimulation with collagen in the presence of antiplatelet agents. Protein phosphorylation analysis showed that glenzocimab significantly reduced Syk, LAT, and PLCγ2 phosphorylation, and the effect of glenzocimab on collagen-induced tyrosine phosphorylation was augmented by aspirin and ticagrelor, which on their own did not inhibit tyrosine phosphorylation.

For the effect on Syk phosphorylation as highlighted in Figure 3.9, glenzocimab alone caused a significant reduction in Syk phosphorylation (34 ± 23% of control; P=0.02). When combined with aspirin and ticagrelor, glenzocimab led to a further decrease in Syk phosphorylation (14 ± 7% of control; P=0.003). Aspirin and ticagrelor together did not cause a significant decrease in Syk phosphorylation (87 ± 16% of control; P=0.94).

LAT adapter plays an essential role in the GPVI downstream activation cascade by forming the LAT signalosome that recruits PLCγ2 to the cytoplasmic membrane. Therefore, the effect of glenzocimab ± aspirin and ticagrelor was assessed in blocking LAT phosphorylation following GPVI stimulation with collagen. As presented in Figure 3.10, glenzocimab significantly reduced LAT phosphorylation (34 ± 9% of control; P<0.001). When combined with aspirin and ticagrelor, glenzocimab markedly decreased LAT phosphorylation (5 ± 3% of control;

P<0.001). The combined use of aspirin and ticagrelor had no apparent effect on LAT phosphorylation with (88 ± 7% of control; P=0.47).

With respect to PLCγ2 phosphorylation as shown in Figure 3.11, glenzocimab produced a pattern of inhibition similar to that observed with Syk phosphorylation. Glenzocimab significantly decreased PLCγ2 phosphorylation (29 ± 6% of control; P=0.02). When aspirin and ticagrelor were combined with glenzocimab, PLCγ2 phosphorylation was further inhibited (9 ± 8% of control; P=0.02). Aspirin and ticagrelor combined had no significant effect on PLCγ2 phosphorylation (86 ± 43% of control; P>0.99).

In summary, these results indicated GPVI blockade with glenzocimab significantly reduced collagen-mediated tyrosine phosphorylation of Syk, LAT, and PLCγ2. On the other hand, the combined use of aspirin and ticagrelor did not affect collagen-induced tyrosine phosphorylation of Syk, LAT, and PLCγ2, but they intensified the inhibitory effect of glenzocimab on these proteins.

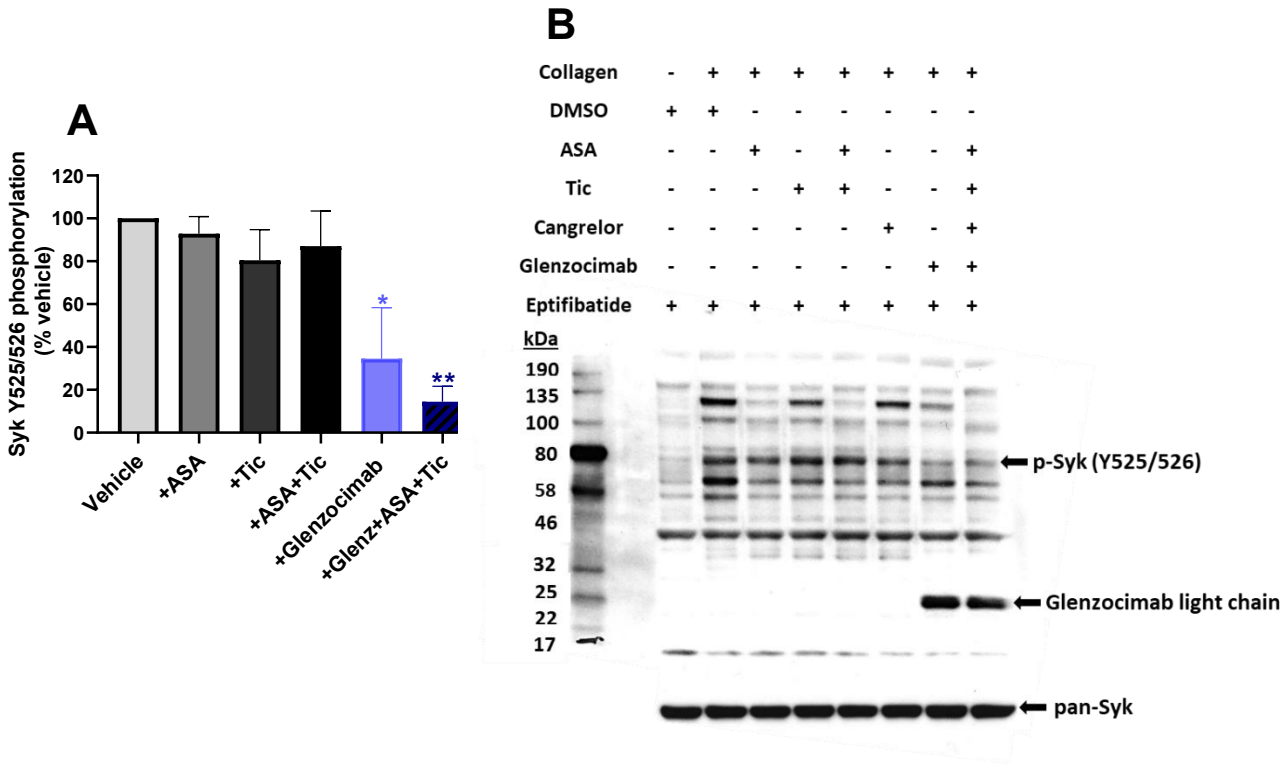


Figure 3.9. Effect of glenzocimab with/without aspirin and ticagrelor on Syk phosphorylation induced by collagen.

A) Effect of glenzocimab alone and combined with aspirin and ticagrelor on Syk phosphorylation induced by Horm collagen. **B)** A representative western blot, where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with glenzocimab (50 µg/ml) ± aspirin (30 µM) and ticagrelor (1 µM) or 0.01% DMSO for 10 min, and platelet activation was stimulated by 5 µg/ml collagen for 180 seconds. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated Syk (Y525/526) with anti-phospho-Syk (Y525/526) ≈ 72 kDa. The effect of antiplatelet agents was compared to (DMSO control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, *P<0.05, **P<0.01).

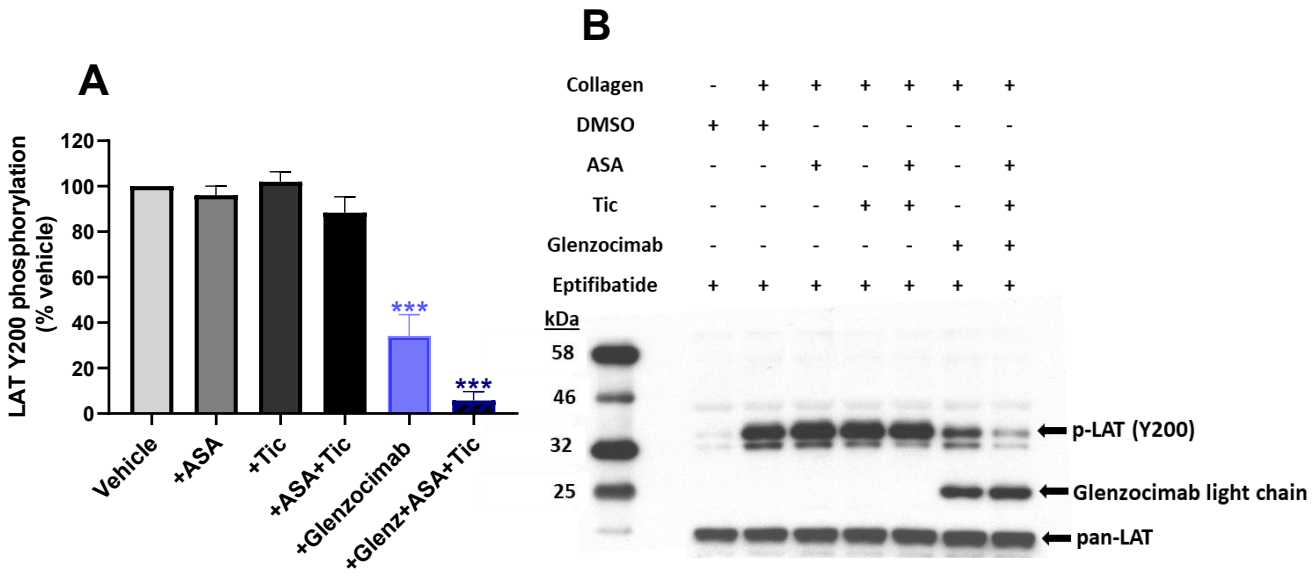


Figure 3.10. Effect of glenzocimab with/without aspirin and ticagrelor on LAT phosphorylation induced by Horm collagen.

A) Effect of glenzocimab alone and combined with aspirin and ticagrelor on LAT phosphorylation induced by Horm collagen. **B)** A representative blot, where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with glenzocimab (50 µg/ml) ± aspirin (30 µM) and ticagrelor (1 µM) or 0.01% DMSO for 10 min, and platelet activation was stimulated by 5 µg/ml collagen for 180 seconds. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated-LAT (Y200) with anti-phospho-LAT (Y200) ≈ 37 kDa. The effect of antiplatelet agents was compared to untreated (DMSO control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, ***P<0.001).

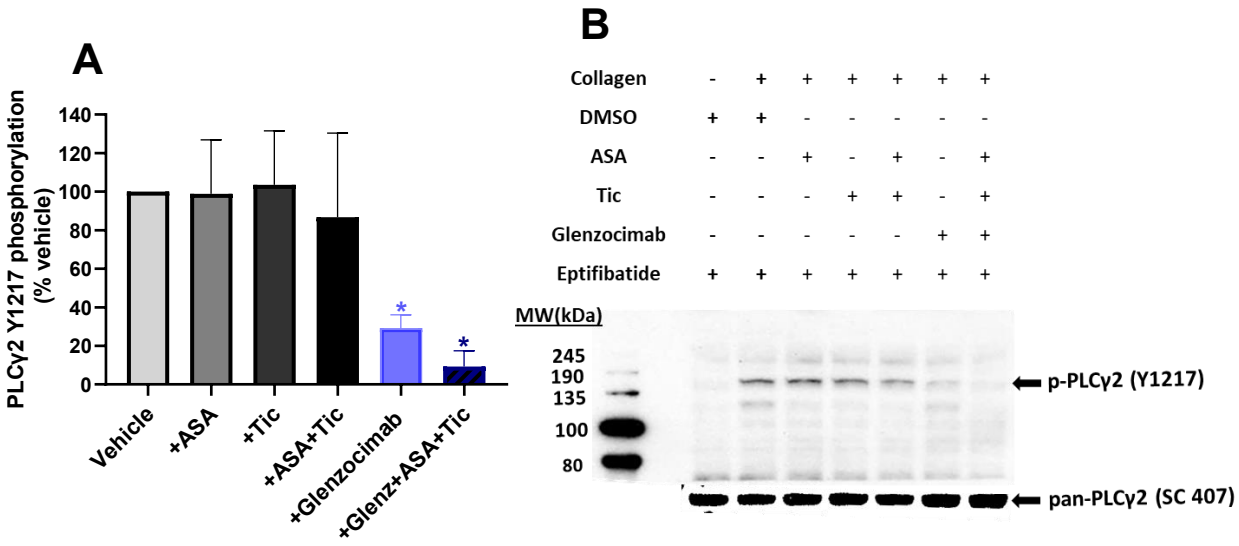


Figure 3.11. Effect of glenzocimab with/without aspirin and ticagrelor on PLCγ2 phosphorylation induced by Horm collagen.

A) Effect of glenzocimab alone and combined with aspirin and ticagrelor on PLCγ2 phosphorylation induced by Horm collagen. **B)** A representative blot, where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with glenzocimab (50 µg/ml) ± aspirin (30 µM) and ticagrelor (1 µM) or 0.01% DMSO for 10 min, and platelet activation was stimulated by 5 µg/ml collagen for 180 seconds. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated PLCγ2 (Y1217) with anti-phospho-PLCγ2 (Y1217) ≈ 140 kDa. The effect of antiplatelet agents was compared to the DMSO control and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, *P<0.05).

3.3.2. Effect of glenzocimab and 6F1 on Horm collagen-mediated platelet aggregation and platelet thrombus formation

Apart from GPVI, GPIa/IIa is the other main platelet receptor that directly interacts with collagen and mediates platelet responses. Therefore, the extent of GPIa/IIa involvement in Horm collagen-mediated platelet aggregation and thrombus formation was also investigated because residual platelet aggregation, adhesion, and thrombus formation was observed after complete GPVI blockade with glenzocimab. 6F1 is a well-known GPIa/IIa inhibitor that is used extensively in research to study function and involvement of GPIa/IIa in collagen mediated platelet mechanisms; therefore, 6F1 was chosen in this study to investigate the role of GPIa/IIa in mediating the residual platelet aggregation and thrombus formation upon stimulation with Horm collagen observed after GPVI inhibition with glenzocimab.

3.3.2.1 6F1 alone did not show an inhibitory effect on Horm collagen-induced platelet aggregation in citrate-anticoagulated platelet-rich plasma, but it blocked the residual platelet aggregation that remained after treatment with glenzocimab

A previous study showed that GPIa/IIa blockade with 6F1 only slightly delayed shape change but did not inhibit platelet aggregation upon platelet stimulation with Horm collagen in LTA in heparin-anticoagulated PRP, which preserves divalent cations such as Mg^{2+} and Mn^{2+}, which are required for binding of GPIa/IIa to collagen. It was therefore expected that there would be no effect or at least minimal effect of 6F1 on Horm collagen-induced platelet aggregation in citrate-anticoagulated PRP because sodium citrate chelates cations, such as Ca^{+2}, Mg^{2+}, and Mn^{2+}, that facilitate the receptor–ligand interaction. In addition, Horm collagen is not the optimal collagen type to effectively stimulate GPIa/IIa.

To investigate whether 6F1 inhibits the residual platelet aggregation observed after GPVI blockade, increasing concentrations (1–50 µg/ml) of 6F1 antibody were tested against 5 µg/ml

Horm collagen. Furthermore, a concentration (50 μg/ml) of 6F1 were compared with an equal concentration of glenzocimab, and a low concentration of 6F1 (5 μg/ml) was added in combination with glenzocimab to test whether this combination eliminates the residual platelet aggregation observed after GPVI antagonism with glenzocimab.

The LTA results showed that the use of increasing concentrations of 6F1 did not have a significant inhibitory effect on Horm collagen in citrate-anticoagulated PRP, but the combination of a low concentration of 6F1 with glenzocimab completely blocked platelet aggregation (Figure 3.12). At the highest concentration (50 μg/ml) in the dose-response curve, 6F1 did not affect platelet aggregation (81 ± 3% compared to 82 ± 7% in the uninhibited sample; P=0.44). In contrast, glenzocimab significantly reduced platelet aggregation (3 ± 3%; P<0.001). More interestingly, the combined use of 6F1 (5 μg/ml) with glenzocimab completely inhibited platelet aggregation (0 ± 0%; P<0.001). These results confirmed that the residual platelet aggregation observed following GPVI inhibition was due to GPIa/IIa.

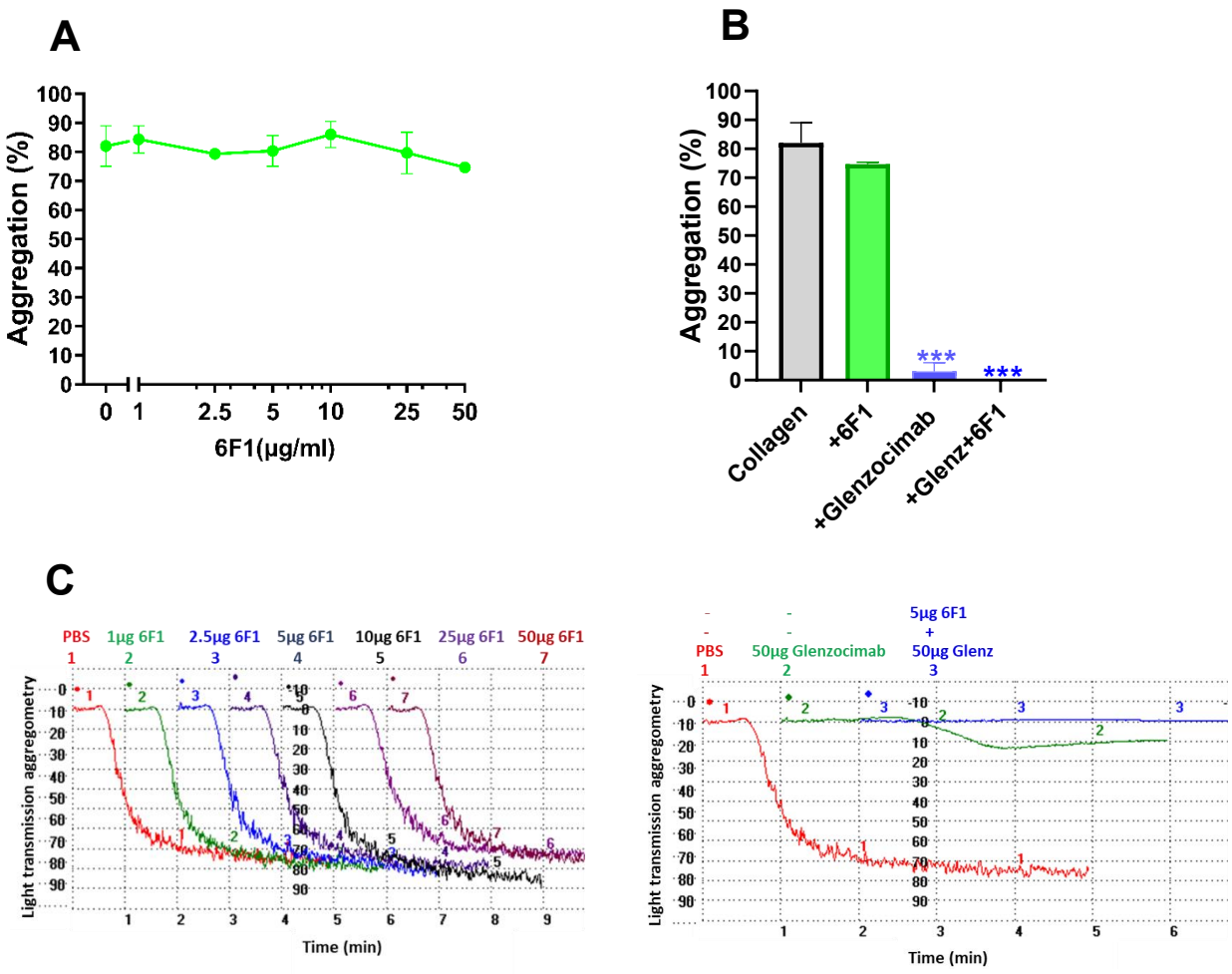


Figure 3.12. Effect of 6F1 antibody and glenzocimab on Horm collagen-induced platelet aggregation in PRP as assessed by LTA.

A) Dose-response curve of 6F1 on 5 µg/ml collagen-induced platelet aggregation. **B)** Effect of the combined use of 6F1 and glenzocimab on 5 µg/ml collagen-induced platelet aggregation. **C)** A representative image of a single LTA aggregation experiment where, human PRP was preincubated with different concentrations of 6F1 (1, 2.5, 5, 10, 25, and 50 µg/ml), glenzocimab (50 µg/ml) or (PBS) vehicle for 10 min, stimulated with 5 µg/ml, then aggregation was monitored for 5 min in PAP-8E aggregometer. The effect of antiplatelet agents was compared to untreated (PBS control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=5, ***P<0.001).

3.3.2.2 Multiple electrode aggregometry showed that 6F1 dose-dependently reduced collagen-induced platelet aggregation in hirudin-anticoagulated whole blood

As demonstrated in section 3.3.1.3, MEA showed that glenzocimab significantly reduced platelet aggregation induced by collagen in whole blood. However, this inhibition was to a lesser extent than the inhibition in PRP observed using LTA, where glenzocimab potently inhibited platelet aggregation with minimal residual platelet aggregation. Therefore, the discrepancy between the impact of GPVI inhibition on platelet aggregation assessed using LTA and MEA was investigated. Notably, there are differences between LTA and MEA in terms of the sample type, anticoagulant type, and aspects of platelet function that are assessed. Light transmission aggregometry is based on the principle that light transmission through a sample increases as platelets start to aggregate; thus, LTA measures only platelet aggregation. In addition, citrate-anticoagulated PRP is the sample of choice for LTA studies. In contrast, MEA assesses both platelet aggregation and adhesion, and hirudin-anticoagulated whole blood is the sample of choice.

The MEA results indicated that GPIa/IIa is principally involved in collagen-induced platelet aggregation (Figure 3.13). 6F1 dose-dependently inhibited collagen-induced platelet aggregation, showing a significant inhibitory effect at a concentration of 5 μg/ml, leading to a more than 50% reduction in platelet aggregation (42 ± 13 U compared to 92 ± 8 U in the control sample; P=0.004). More importantly, 20 μg/ml 6F1 produced a pattern of inhibition similar to that produced by 50 μg/ml glenzocimab (33 ± 11 U compared to 32 ± 4 U; P<0.001). Additionally, combining glenzocimab with 6F1 (5 μg/ml) led to more potent inhibition of collagen-induced platelet aggregation (8 ± 2 U; P<0.001).

In summary, these results indicated that GPIa/IIa is significantly involved in collagen-mediated platelet aggregation in hirudin-anticoagulated whole blood in MEA. Furthermore, GPIa/IIa blockade with 6F1 intensified the inhibitory effect of glenzocimab on collagen-induced platelet aggregation in MEA.

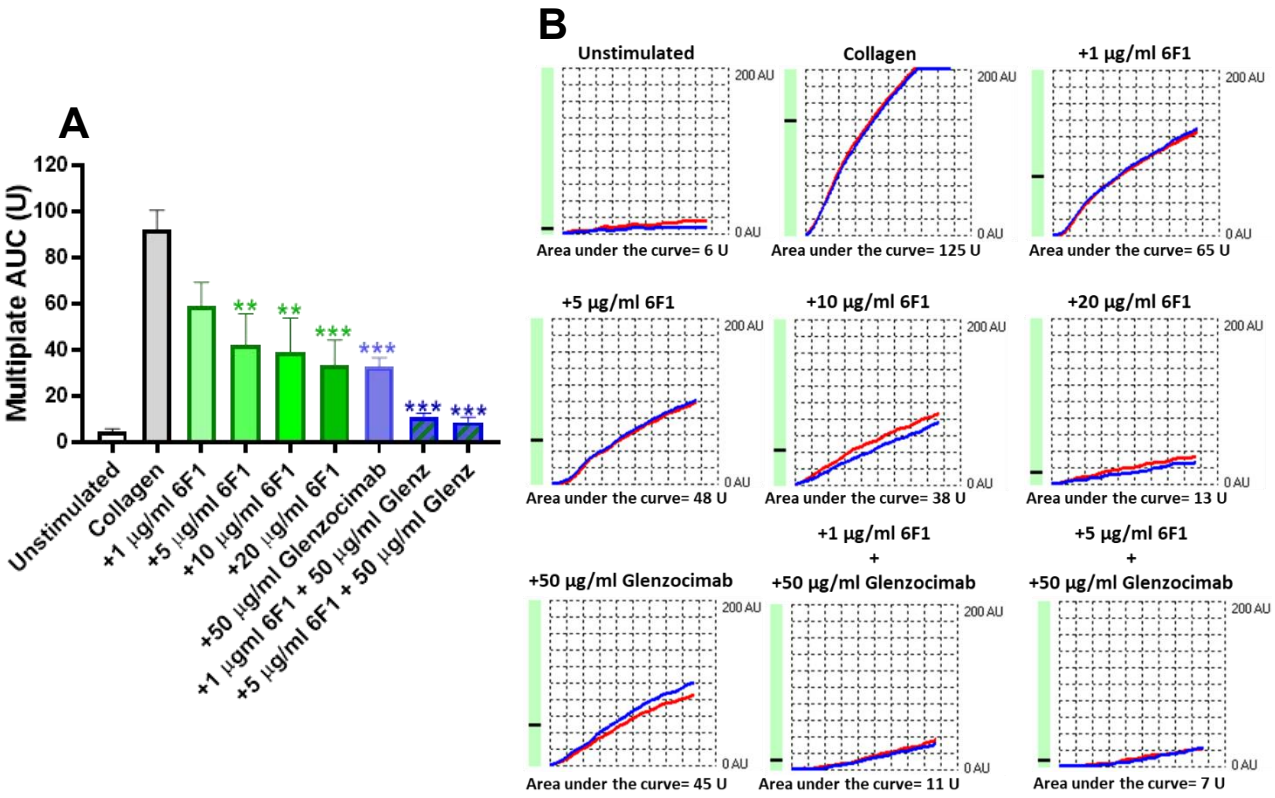


Figure 3.13. Effect of 6F1 and glenzocimab on Horm collagen-induced platelet aggregation in whole blood assessed by multiple electrode aggregometry.

A) Effect of 6F1 and glenzocimab on platelet aggregation stimulated by collagen in whole blood sample in MEA. **B)** Representative aggregation traces from a single MEA experiment, where human whole blood was preincubated with different concentrations of 6F1 (1, 5, 10, and 20 µg/ml), glenzocimab (50 µg/ml) or (PBS) vehicle for 10 min, stimulated with 3.2 µg/ml, then aggregation was monitored for 6 min in the Multiplate® analyser. The effect of antiplatelet agents was compared to untreated (PBS control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=5, **P<0.01, and ***P<0.001).

3.3.2.3 6F1 partially reduced platelet thrombus formation but not adhesion on Horm collagen under flow conditions in citrate-anticoagulated whole blood

In the previous flow adhesion experiments, the effect of glenzocimab on platelet adhesion was dependant on collagen concentration. Glenzocimab significantly reduced platelet adhesion on low Horm collagen under flow, but it lost its potency in the blood flowing on a high concentration of collagen as assessed by percent platelet surface coverage. To investigate whether the GPIa/IIa contributes to mediating platelet adhesion on Horm collagen, the effect of GPIa/IIa blockade by 6F1 was investigated. The results revealed no significant effect of 6F1 on platelet adhesion on Horm collagen, but it partially inhibited platelet thrombus formation. Furthermore, 6F1 combined with glenzocimab led to further inhibition in platelet thrombus formation (Figure 3.14).

6F1 did not significantly reduce the platelet surface coverage (60 ± 8% compared to 80 ± 2% in the control sample; P=0.99). Similarly, glenzocimab did not exert a significant effect on the surface coverage (73 ± 6%; P>0.99). Concomitant use of 6F1 and glenzocimab did not show an additive effect (60 ± 6%; P=0.99). Eptifibatide, which was the positive control, reduced the surface coverage by 94% (4 ± 1%; P<0.001).

Contrary to expectations, 6F1 reduced platelet thrombus formation by 51% (99 ± 36 AU compared to 203 ± 16 AU in the control sample; P=0.008). Glenzocimab reduced platelet thrombus formation by 71% (58 ± 16 AU; P<0.001). The combination of glenzocimab and 6F1 further reduced platelet thrombus formation by 87% (25 ± 9 AU; P<0.001). Eptifibatide markedly reduced platelet thrombus formation by 98% (2 ± 1 AU; P<0.001).

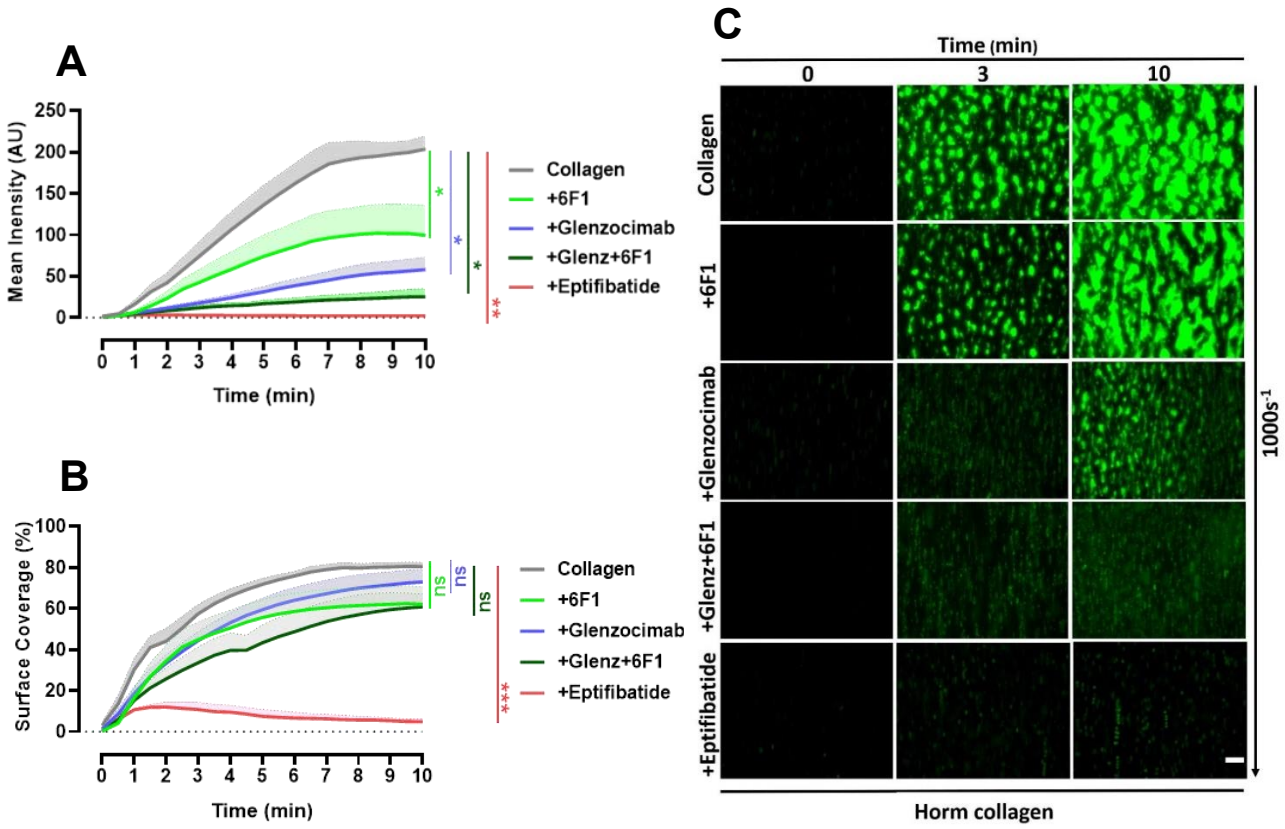


Figure 3.14. Effect of 6F1 and glenzocimab on platelet adhesion and thrombus formation on Horm collagen under flow conditions.

A) Effect of GPVI and GPIa/IIa blockade on platelet thrombus formation as reflected by fluorescence intensity. **B)** Effect of GPVI and GPIa/IIa blockade on platelet adhesion as reflected by platelet surface coverage %. **C)** Representative images from a single flow adhesion assay, where $DiOC_6$-labelled blood was incubated with 50 µg/ml glenzocimab, 50 µg/ml 6F1 and 9 µM eptifibatide or PBS for 15 min at 37°C then flowed over 200 µg/ml Horm collagen at a shear rate of 1000 s^{-1} for 10 min. Images were taken every 30 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by ImageJ software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of antiplatelet agents was compared to untreated (PBS control) and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=5, *P<0.05, **P<0.01, and ***P<0.001).

3.3.3. Effect of glenzocimab with aspirin and ticagrelor on CRP-XL-mediated platelet responses

Unlike collagen that potentially activates multiple platelet receptors, CRP-XL only activates GPVI; therefore, it can be used to obtain more specific information on the effect of glenzocimab on GPVI-mediated platelet function.

3.3.3.1 Glenzocimab entirely inhibited CRP-XL-induced platelet aggregation while aspirin and ticagrelor showed no antiplatelet effect on CRP-XL-induced platelet aggregation in platelet-rich plasma

The effect of glenzocimab ± aspirin and ticagrelor on platelet aggregation following GPVI activation was determined by stimulating platelets with 3 and 10 µg/ml CRP-XL. The LTA results demonstrated that glenzocimab at a low concentration (5 µg/ml) completely inhibited CRP-XL-induced platelet aggregation without any effect of aspirin and ticagrelor (Figure 3.15). Platelet aggregation stimulated by 3 µg/ml CRP-XL was completely inhibited by 5 µg/ml glenzocimab (0 ± 0% in comparison to 78 ± 1% in the control sample; $P<0.001$). In contrast, the combination of aspirin and ticagrelor did not inhibit CRP-XL-induced platelet aggregation (71 ± 3%; $P=0.31$). Because 5 µg/ml glenzocimab completely inhibited platelet aggregation and may mask any amplified effect of aspirin and ticagrelor, the effect of aspirin and ticagrelor in combination with a very low concentration of glenzocimab was further investigated. Aspirin and ticagrelor when combined with 0.2 µg/ml glenzocimab still showed no additional antiplatelet effect (73 ± 4% compared to 76 ± 1% in sample treated with 0.2 µg/ml glenzocimab; $P=0.70$). When platelet aggregation was stimulated by 10 µg/ml CRP-XL, glenzocimab continued to demonstrate complete inhibition as previously observed with 3 µg/ml CRP-XL. At 5–50 µg/ml, glenzocimab completely inhibited platelet aggregation (0 ± 0%; $P=0.002$

compared to 76 ± 1% in the control sample). There was not even a marginal effect of aspirin and ticagrelor when platelets were stimulated with 10 μg/ml CRP-XL (77 ± 1%; P>0.99).

In summary, these results indicated that glenzocimab is a potent GPVI antagonist as a low concentration of glenzocimab completely blocked CRP-XL-induced platelet aggregation. On the other hand, the combined use of aspirin and ticagrelor showed no effect on CRP-XL-induced platelet aggregation.

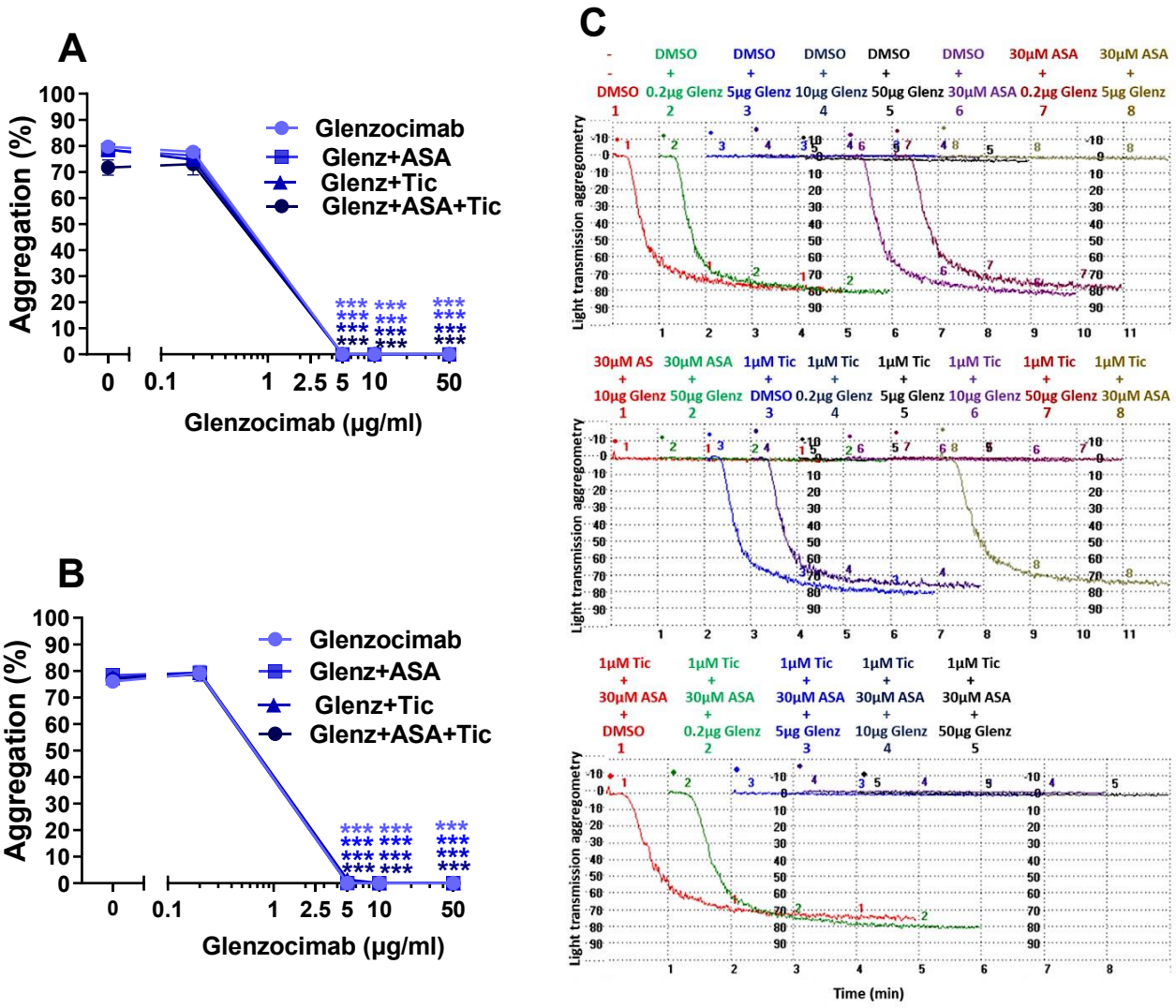


Figure 3.15. Dose-response curve of the combined use of glenzocimab with aspirin and ticagrelor on CRP-XL-induced platelet aggregation in PRP.

A) Effect of glenzocimab ± aspirin and ticagrelor on platelet aggregation stimulated by 3 µg/ml CRP-XL. **B)** Effect of glenzocimab ± aspirin and ticagrelor on platelet aggregation stimulated by 10 µg/ml CRP-XL. **C)** Representative aggregation traces, where human PRP was preincubated (0.01% DMSO) control or different concentrations of glenzocimab with/without aspirin (30 µM) and ticagrelor (1 µM) for 10 min at 37 °C and platelet aggregation stimulated with 3 and 10 µg/ml CRP-XL. Then aggregation was monitored for 5 min in PAP-8E aggregometer. The effect of combinations of different concentrations of glenzocimab with other antiplatelet agents was compared to untreated (control) samples and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=3, ***P<0.001).

3.3.3.2 Glenzocimab potently blocked CRP-XL-induced platelet aggregation and provided an amplified effect when combined with aspirin and ticagrelor in whole blood

The effect of glenzocimab ± aspirin and ticagrelor was further investigated in CRP-XL-induced platelet aggregation in whole blood samples using MEA. This further investigation sought to characterise how GPVI blockade with glenzocimab alone or combined with aspirin and ticagrelor affect platelet aggregation triggered by 3 µg/ml CRP-XL in hirudin-anticoagulated whole blood samples with a method that relies on both platelet aggregation and adhesion. The other purpose of this experiment was to determine whether GPVI inhibition would show a differential effect when assessed by LTA and MEA, as observed in platelets stimulated with Horm collagen.

The MEA results showed that glenzocimab potently blocked CRP-XL-mediated platelet aggregation up to the level of the spontaneous platelet aggregation (SPA) observed in unstimulated samples, and the addition of aspirin and ticagrelor with glenzocimab caused a near complete inhibition of platelet aggregation (Figure 3.16). The combined use of aspirin and ticagrelor did not significantly reduce platelet aggregation (56 + 11 U; P=0.62 in comparison to 71 ± 9 U in the control sample; P=0.62). Glenzocimab potently reduced platelet aggregation by 81% (13 ± 1 U; P<0.001) when used alone and by 97% (2 ± 1 U; P<0.001) when used in combination with aspirin and ticagrelor. Eptifibatide inhibited platelet aggregation by 98% (1 ± 1 U; P<0.001). In the presence of aspirin and ticagrelor, eptifibatide completely blocked platelet aggregation (0 ± 0 U; P<0.001).

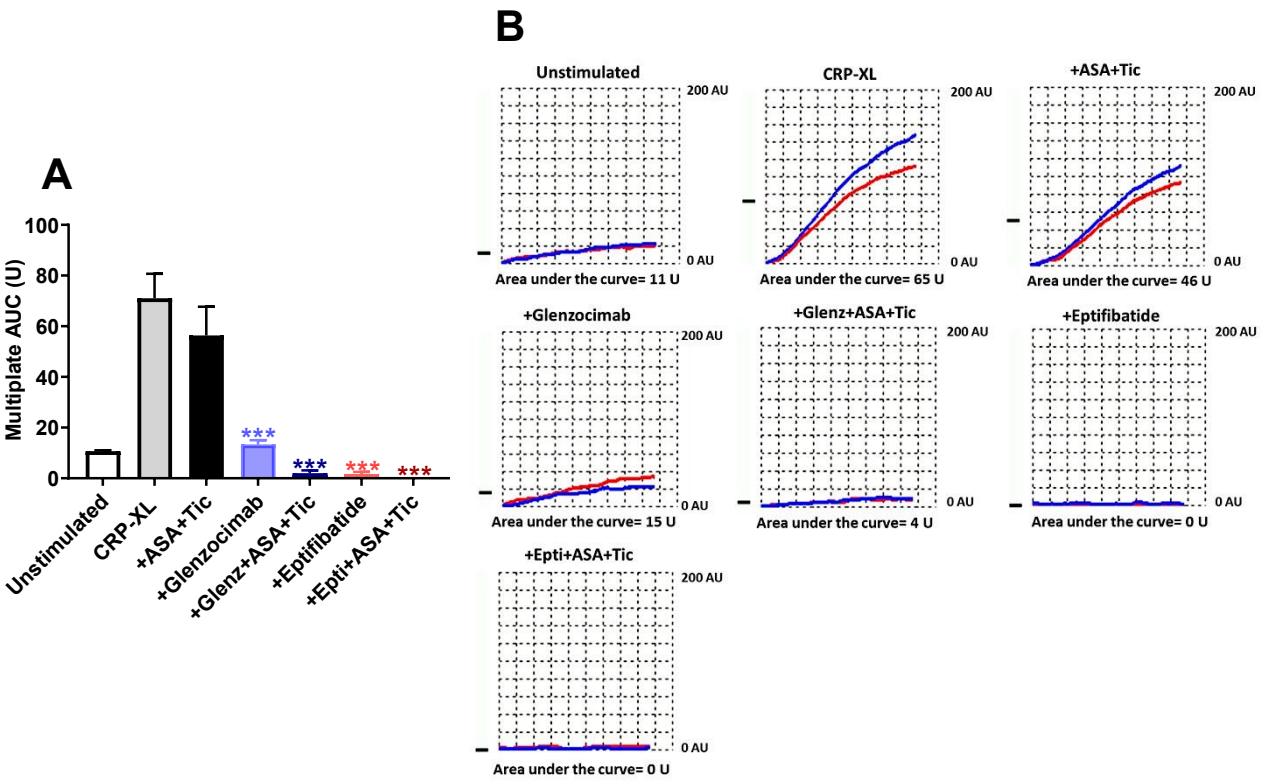


Figure 3.16. Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on CRP-XL-stimulated platelet aggregation in whole blood sample assessed by multiple electrode aggregometry.

A) Effect of glenzocimab or eptifibatide combined with aspirin and ticagrelor on platelet aggregation induced by 3 µg/ml CRP-XL. **B)** Representative aggregation traces from a single experiment, where hirudin-anticoagulated whole blood was diluted in half with normal saline and incubated with the vehicle (0.01% DMSO), glenzocimab (50 µg/ml) or eptifibatide (9 µM) ± aspirin (30 µM) and ticagrelor (1 µM) for 10 min at 37°C under stirring conditions, then platelet aggregation was stimulated by 3 µg/ml CRP-XL, and aggregation was monitored for 6 min in the Multiplate® analyser. The effect of treatment groups was compared to the control and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, ***P<0.001).

3.3.3.3 Glenzocimab marginally reduced the surface area of spread platelets but greatly reduced the number of adherent platelets on immobilised CRP-XL under static conditions

Glenzocimab only showed a marginal effect on platelet spreading on immobilised collagen, and this limited effect could be due either to the presence of the other main collagen receptor (GPIa/IIa) or to the important role of GPIIb/IIIa in platelet spreading. To further analyse this mechanism and eliminate the possible involvement of GPIa/IIa and GPIIb/IIIa, the effect of glenzocimab with/without other antiplatelet agents on platelet spreading on the specific GPVI ligand (CRP-XL) was evaluated. Analysis of platelet spreading and adhesion on CRP-XL showed that glenzocimab partially reduced platelet spreading as indicated by the platelet surface area and perimeter but markedly decreased platelet adhesion as estimated by the number of adherent platelets, whereas aspirin and ticagrelor showed no effect on platelet spreading and adhesion. In contrast, GPIIb/IIIa blockade with eptifibatide significantly reduced platelet spreading but not platelet adhesion on CRP-XL (Figure 3.17).

The combined use of aspirin and ticagrelor did not have a significant effect on the spread platelet surface area (37 ± 3 μm^2; compared to 41 ± 1 μm^2 in the control sample; P=0.74). Glenzocimab reduced the surface area by 22% (32 ± 2 μm^2; P=0.04) when used alone and by 34% (27 ± 4 μm^2; P=0.10) when combined with aspirin and ticagrelor. Eptifibatide reduced the surface area by 43% (23 ± 2 μm^2; P=0.03). The combination of aspirin and ticagrelor with eptifibatide did not additionally affect the surface area (24 ± 3 μm^2; P=0.05). Dasatinib reduced the surface area by 56% (18 ± 3 μm^2; P=0.009).

The combination of aspirin and ticagrelor did not affect the platelet perimeter (29 ± 1 μm compared to 29 ± 1 μm in the samples spiked with the vehicle; P>0.99). Glenzocimab alone reduced platelet perimeter by 11% (26 ± 1 μm; P=0.04). Eptifibatide did not significantly affect the perimeter (22 ± 1 μm; P=0.12) even when combined with aspirin and ticagrelor (24 ± 2 μm; P=0.29). Dasatinib did not significantly reduce the perimeter (21 ± 2 μm; P=0.12).

With respect to platelet adhesion to CRP-XL, the combination of aspirin and ticagrelor did not significantly reduce the number of adherent platelets (130 ± 17 platelets compared to 155 ± 8 platelets in the control samples; P=0.58). Glenzocimab alone produced the most significant inhibition of platelet adhesion by reducing the number of adherent platelets by 74% (40 ± 7 platelets; P<0.001). The combination of glenzocimab with aspirin and ticagrelor showed no additional effect on platelet adhesion (48 ± 8 platelets; P<0.001). Eptifibatide showed no effect on platelet adhesion (160 ± 15 platelets; P=0.96); the combination of eptifibatide with aspirin and ticagrelor also did not significantly reduce platelet adhesion (110 ± 20 platelets; P=0.12). Dasatinib had no significant effect on platelet adhesion (99 ± 24 platelets; P=0.07).

These results indicated glenzocimab potently inhibited static adhesion of platelet on CRP-XL coated surfaces, but it marginally reduced platelet spreading. In addition, aspirin and ticagrelor showed no effect on platelet spreading or adhesion on CRP-XL, without amplifying the effect of glenzocimab on spreading and adhesion. On the other hand, eptifibatide significantly reduced platelet spreading only.

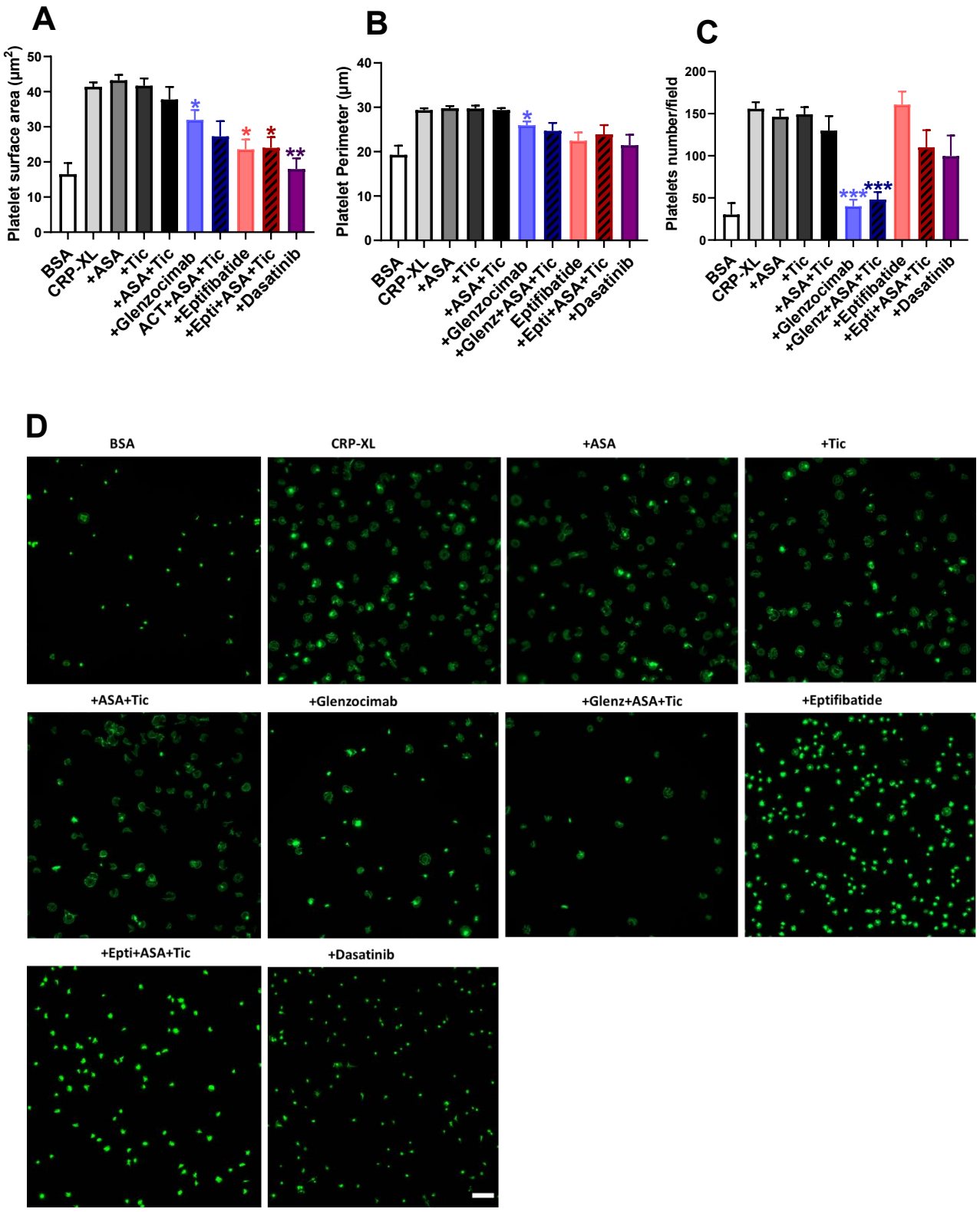

A
Platelet surface area (μm²)
50
40
30
20
10
0
BSA
CRP-XL
+ASA
+Tic
+ASA+Tic
+Glenzocimab
ACT+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
B
Platelet Perimeter (μm)
40
30
20
10
0
BSA
CRP-XL
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
Eptifibatide
+Epti+ASA+Tic
+Dasatinib
C
Platelets number/field
200
150
100
50
0
BSA
CRP-XL
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
D
BSA
CRP-XL
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib

Figure 3.17. Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on platelet spreading on CRP-XL under static conditions.

A) Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet surface area. **B)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet perimeter. **C)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the number of adherent platelets. **D)** Representative images from a single spreading experiment, where human WP (2.0×10^7 mL) was preincubated with 0.01% DMSO, glenzocimab (50 µg/ml) or eptifibatide (9 µM) ± aspirin (30 µM) and ticagrelor (1 µM) or dasatinib (10 µM) for 30 min at 37ºC. Then, pre-treated platelets were allowed to spread on CRP-XL (10 µg/ml) on coverslips for 30 min at 37ºC. Platelets were fixed with 4% neutralized formalin buffer for 10 min followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min and stained with Alex-488-phalloidin for 45 min in the dark. The coverslips were then mounted on glass slides and imaged under the Zeiss an Axio Observer 7 inverted epifluorescence microscope. 5 random images were analysed for each experiment. Scale bar 20 µm. The effect of treatment groups was compared to 0.01% DMSO and assessed using a linear mixed model effect with Dunnett correction for multiple comparisons (n=4, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

3.3.3.4 Glenzocimab completely blocked tyrosine phosphorylation downstream of GPVI upon stimulation with CRP-XL

When tyrosine phosphorylation in platelets was stimulated by collagen, glenzocimab significantly reduced the phosphorylation of the main effector proteins in the GPVI signalling pathway, which are Syk, LAT, and PLCγ2. Aspirin and ticagrelor amplified the inhibitory effect of glenzocimab, leading to a further decrease in the intensity of tyrosine phosphorylation, but a residual minimal activity of phosphorylation was observed. Therefore, the effect of glenzocimab ± aspirin and ticagrelor on the major signalling proteins upon platelet stimulation with the specific ligand CRP-XL was further investigated in order to exclude the contribution of another main collagen receptor, GPIa/IIa, which may be responsible for the residual tyrosine phosphorylation observed with Horm collagen. Furthermore, this study aimed to determine whether aspirin and ticagrelor directly inhibit signalling induced by specific GPVI activation and to determine whether the concentration of glenzocimab (50 µg/ml) that was used previously to inhibit Horm collagen-induced platelet activation is enough to fully neutralise the effect of GPVI.

The protein phosphorylation results showed that glenzocimab completely blocked CRP-XL-induced phosphorylation of Syk, LTA, and PLCγ2, whereas aspirin and ticagrelor partially inhibited PLCγ2 phosphorylation only.

As shown in Figure 3.18, the combined use of aspirin and ticagrelor did not significantly reduce Syk phosphorylation (88 ± 21% of control; P=0.96). Glenzocimab almost completely inhibited Syk phosphorylation (1 ± 1% of control; P<0.001). The combination of aspirin and ticagrelor with glenzocimab did not further inhibit Syk phosphorylation (1 ± 1% of control; P<0.001).

As presented in Figure 3.19, aspirin and ticagrelor did not cause any reduction in LAT phosphorylation (105 ± 6% of control; P=0.86). Glenzocimab markedly reduced LAT phosphorylation (1 ± 2% of control; P=0.001). The concomitant use of aspirin and ticagrelor plus glenzocimab entirely blocked LAT phosphorylation (0 ± 0% of control; P<0.001).

As depicted in Figure 3.20, the combined use of aspirin and ticagrelor partially reduced PLCγ2 phosphorylation induced by CRP-XL (67 ± 6% of control; P=0.02). Glenzocimab potently decreased PLCγ2 phosphorylation (4 ± 2% of control; P=0.001). The addition of aspirin and ticagrelor augmented the effect of glenzocimab in blocking PLCγ2 phosphorylation (3 ± 1% of control; P<0.001).

In summary, these results indicated that glenzocimab is a potent GPVI inhibitor as it abolished CRP-XL-induced phosphorylation of Syk, LAT, and PLCγ2. In addition, these results showed no effect for aspirin and ticagrelor on GPVI-mediated tyrosine phosphorylation except the partial inhibition of PLCγ2.

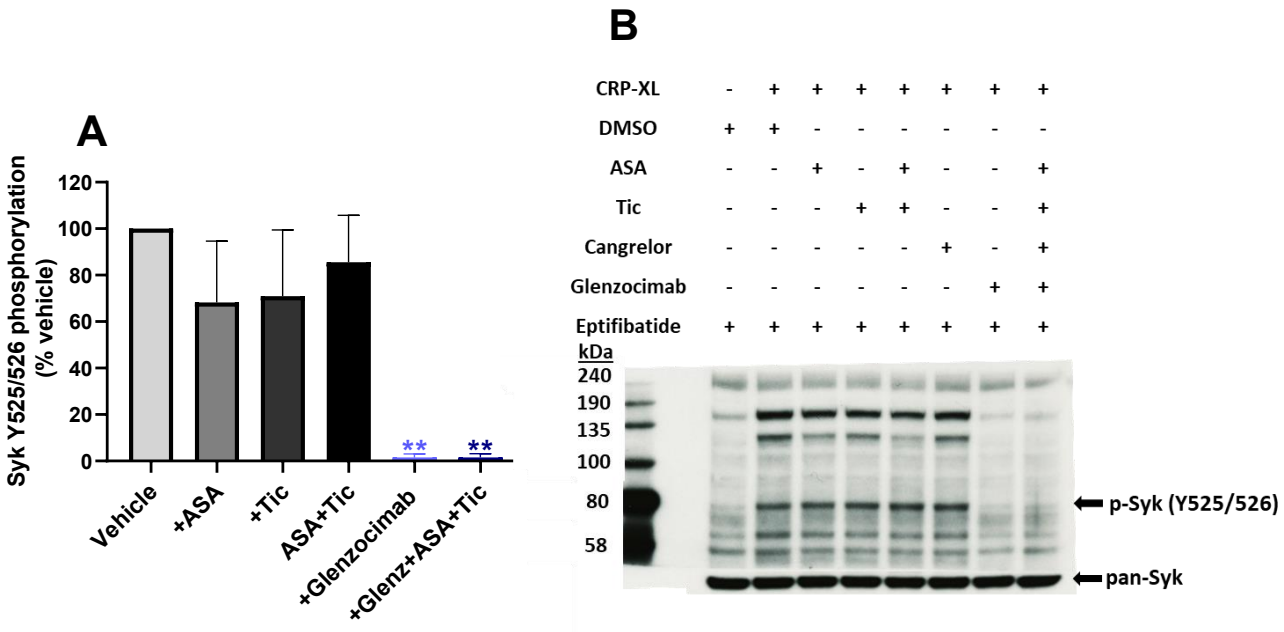


Figure 3.18. Effect of glenzocimab with/without aspirin and ticagrelor on Syk phosphorylation induced by CRP-XL.

A) Effect of glenzocimab alone and combined with aspirin and ticagrelor on Syk phosphorylation induced by 3 µg/ml CRP-XL. **B)** A representative blot, where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with glenzocimab (50 µg/ml) ± aspirin (30 µM) and ticagrelor (1µM) for 10 min, and platelet activation was stimulated by 3 µg/ml CRP-XL for 180 seconds. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated Syk (Y525/526) with anti-phospho-Syk (Y525/526) ≈ 72 kDa. The effect of antiplatelet agents was compared to control samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, **P<0.001).

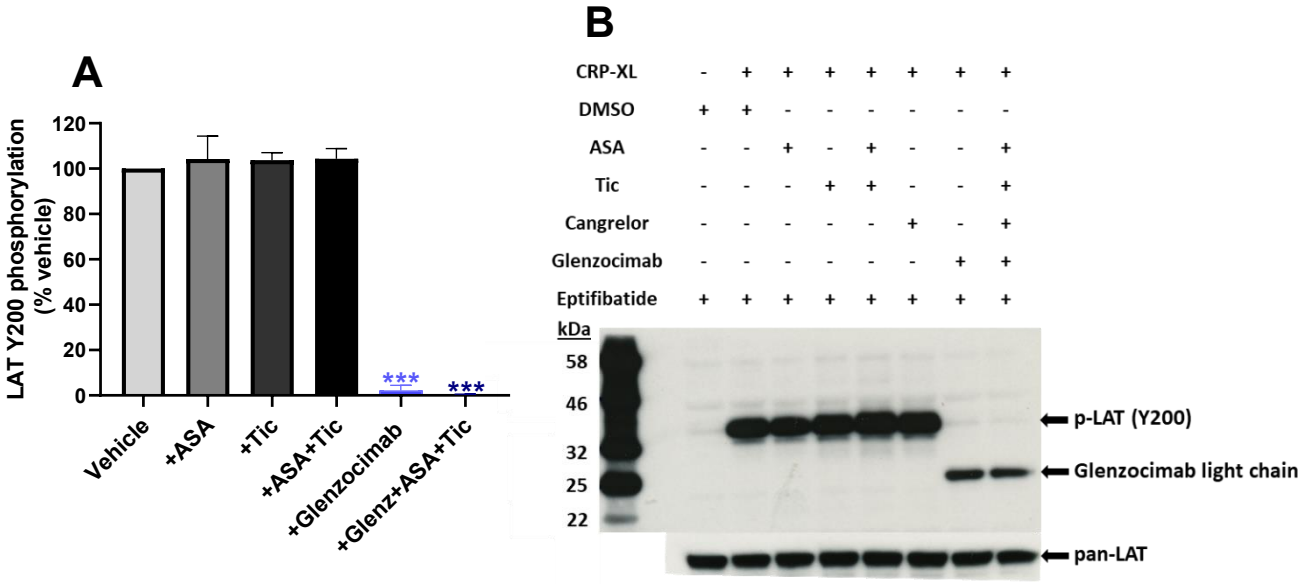


Figure 3.19. Effect of glenzocimab with/without aspirin and ticagrelor on LAT phosphorylation induced by CRP-XL.

A) Effect of glenzocimab alone and combined with aspirin and ticagrelor on LAT phosphorylation stimulated by 3 µg/ml CRP-XL. **B)** A representative blot, where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with glenzocimab (50 µg/ml) ± aspirin (30 µM) and ticagrelor (1 µM) for 10 min, and platelet activation was stimulated by 3 µg/ml CRP-XL for 180 seconds. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated-LAT (Y200) with anti-phospho-LAT (Y200) ≈ 37 kDa. The effect of antiplatelet agents was compared to the control samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, ***P<0.001).

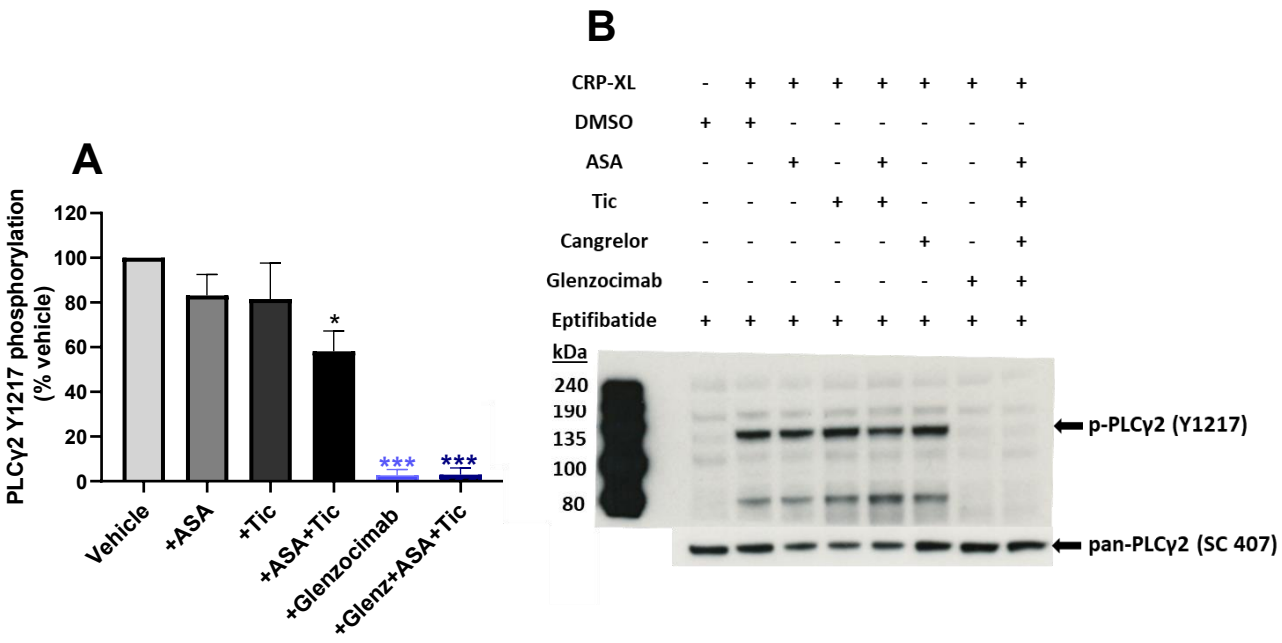


Figure 3.20. Effect of glenzocimab with/without aspirin and ticagrelor on PLCγ2 phosphorylation induced by CRP-XL.

A) Effect of glenzocimab alone and combined with aspirin and ticagrelor on PLCγ2 phosphorylation stimulated by 3 µg/ml CRP-XL. **B)** A representative blot, where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with glenzocimab (50 µg/ml) ± aspirin (30 µM) and ticagrelor (1 µM) for 10 min, and platelet activation was stimulated by 3 µg/ml CRP-XL for 180 seconds. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated PLCγ2 (Y1217) with anti-phospho-PLCγ2 (Y1217) ≈ 140 kDa. The effect of antiplatelet agents was compared to control samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, *P<0.05, ***P<0.001).

3.4 Discussion

The main findings of this chapter are as follows: (i) glenzocimab inhibited collagen-mediated platelet responses more than aspirin and ticagrelor; (ii) glenzocimab produced an amplified effect on collagen-induced platelet responses when combined with aspirin and ticagrelor; (iii) glenzocimab did not have off-target effects on GPVI-independent platelet activation pathways (iv); GPIa/IIa activation mediates residual platelet aggregation induced by Horm collagen that occurs despite potent GPVI inhibition; (v) glenzocimab completely blocked CRP-XL-mediated platelet responses without an effect of aspirin and ticagrelor.

Collagen is one of the most thrombogenic parts of the blood vessel wall, which is exposed following vascular injury or plaque rupture and activates platelets via GPVI and GPIa/IIa. Collagen also interacts indirectly with platelets by GPIb-V-IX and GPIIb/IIIa via vWF (Savage et al., 1998). The collagen used in the experiments in this thesis is Horm collagen, which is extracted from equine tendons and contains collagen type I (95%) and collagen type III (5%), according to the manufacturer's description. Glenzocimab caused a dose-dependent inhibition of collagen-mediated platelet aggregation in citrate-anticoagulated PRP.

The dose-dependent pattern of inhibition of platelet aggregation is consistent with the results of the phase I trial of glenzocimab, in which intravenous administration of increasing concentrations of glenzocimab caused a dose-dependent inhibition of collagen-induced platelet aggregation in healthy volunteers (Voors-Pette et al., 2019). At high concentrations of the inhibitor, platelet aggregation in response to collagen was nearly eliminated but not completely blocked. More remarkably, stimulation of platelet aggregation with a higher concentration of collagen (10 μg/ml) decreased the inhibition of platelet aggregation by glenzocimab. The inability of glenzocimab to inhibit platelet responses that are induced by higher concentrations of collagen suggests the presence of another interaction site on GPVI for collagen. Schulte et

al. suggested the existence of two distinct activation sites on GPVI for collagen. Schulte et al. showed that platelet pre-treatment with JAQ1, anti-GPVI IgG completely blocked platelet aggregation and tyrosine phosphorylation induced by CRP-XL and low concentrations of collagen but not high concentrations of collagen. These results indicate the existence of a second activation site for collagen that signals via the FcRγ-chain but is distinct from the site occupied by CRP (Schulte et al., 2001). Resolving the crystal structure of GPVI/CRP association by Horii et al. revealed two binding sites for CRP/collagen on GPVI (Horii et al., 2006). Furthermore, collagen type III, which represents approximately 5% of Horm collagen, contains a major binding locus for GPVI (Jarvis et al., 2008). Therefore, using high concentrations of Horm collagen may provide adequate levels of collagen type III in the suspension to cause greater platelet activation and aggregation. Alternatively, this could be due to the involvement of the other major collagen receptor, GPIa/IIa.

One of the main aims of this chapter was to determine whether aspirin and ticagrelor, which are used commonly in the treatment of ACS, amplify the inhibitory effect of glenzocimab on collagen-mediated platelet responses. Glenzocimab yielded an amplified effect with aspirin and ticagrelor in inhibiting collagen-induced platelet aggregation in PRP and whole blood. The LTA results indicated that a low concentration of glenzocimab (5 µg/ml) did not significantly reduced platelet aggregation. However, when glenzocimab was combined with aspirin and ticagrelor at this concentration, it potently inhibited platelet aggregation. The MEA results showed that glenzocimab in combination with aspirin and ticagrelor reproduced the antiplatelet synergism observed in PRP, providing a pattern of platelet aggregation inhibition similar to that achieved by the triple combination of aspirin, ticagrelor, and eptifibatide. However, MEA showed that the combined use of aspirin and ticagrelor partially decreased platelet aggregation.

In patients treated with aspirin and ticagrelor, platelet aggregation in response to collagen is partially inhibited (Franchi et al., 2016).

There is only one study that has examined the additive effect of immunological blockade of GPVI with dual antiplatelet therapy agents on collagen-mediated platelet responses. Florian et al. investigated the additive effect of SAR264565, anti-GPVI antibody, on platelet aggregation and thrombus formation *ex vivo* in blood collected from healthy volunteers who received standard doses of either aspirin alone or aspirin with clopidogrel. SAR264565 significantly inhibited collagen-mediated platelet aggregation without an additive effect when combined with aspirin or aspirin plus clopidogrel (Florian et al., 2017). The findings of Florian et al. show a lack of additive effect of GPVI inhibition with aspirin and aspirin with a $P2Y_{12}$ inhibitor, which contradicts the aggregation results of the present study. This discrepancy may be explained by the fact that, in addition to the different GPVI inhibitor (SAR264565) that was used, they used a low concentration of collagen (1 μg/ml) to stimulate platelet aggregation, which was already blocked by aspirin and clopidogrel. Clopidogrel is also known for its variable effects on $P2Y_{12}$ antagonism, whereas ticagrelor is a more potent $P2Y_{12}$ inhibitor with less variable responses (Ganesan et al., 2013).

GPVI inhibition with glenzocimab showed no off-target effects on platelet aggregation mediated by non-GPVI agonists even when it was added in combination with aspirin and ticagrelor. Glenzocimab did not inhibit platelet aggregation induced by TRAP, AA, and ADP in MEA or that induced by AA and ADP in LTA. In contrast, aspirin and ticagrelor inhibited TRAP-induced platelet aggregation, and ticagrelor amplified the effect of aspirin on AA-induced platelet aggregation, which would be expected considering the broad-acting mechanisms of these drugs. GPIIb/IIIa blockade with eptifibatide inhibited platelet aggregation regardless of which agonist was used. These results confirmed the findings of the phase I trial

of glenzocimab, which showed no effect on platelet aggregation induced by ADP, TRAP, U46619, and ristocetin (Voors-Pette et al., 2019). Furthermore, GPVI-deficient patients also respond normally to platelet activation by non-GPVI stimuli (Arthur et al., 2007).

GPVI has a principal role in platelet thrombus formation and growth, but its role in mediating platelet adhesion is debatable (Dubois et al., 2006, Ruggeri and Mendolicchio, 2007, Goto et al., 2002). In the present study, GPVI blockade with glenzocimab inhibited platelet thrombus formation, but the degree of its effect on platelet adhesion was related to collagen concentration. Combining aspirin and ticagrelor with glenzocimab did not produce additional antithrombotic effects on platelet adhesion, except a delay in surface coverage observed in the first 5 min of flow. There are no published studies about the additive effect of direct GPVI inhibition with TxA_2 and $P2Y_{12}$ blockade, except the study by Florian et al., which showed that the addition of GPVI inhibitor *ex vivo* to blood from healthy volunteers pre-dosed with aspirin only or aspirin and clopidogrel further reduced platelet deposition on collagen under flow in blood from volunteers treated with aspirin but not in blood from those treated with aspirin and clopidogrel. They incubated the blood samples with the antibody for only 1 min, which may have not been sufficient for full inhibition by the antibody (Florian et al., 2017).

Combined use of aspirin and ticagrelor inhibited platelet thrombus formation but not platelet adhesion, whereas eptifibatide inhibited both thrombus formation and platelet adhesion. Aspirin and ticagrelor prevent soluble mediators TxA_2 and ADP—which act by providing positive feedback during the second wave of platelet activation that plays an essential role in amplifying platelet activation and platelet recruitment to the site of injury—from activating platelets via the TP and $P2Y_{12}$ pathways, respectively (Offermanns, 2006). Mendolicchio et al. showed that *in-vitro* treatment with aspirin and 2-MeSAMP, a $P2Y_{12}$ inhibitor, moderately reduced surface coverage but significantly lowered thrombus volume on collagen under flow when using blood

from healthy donors, and they further showed that blood from patients with ACS treated with aspirin and clopidogrel exhibits smaller thrombi on collagen *ex vivo* than blood from healthy controls (Mendolicchio et al., 2011).

Eptifibatide has been shown to inhibit thrombus formation on collagen in a dose-dependent manner (Stephens et al., 2012). Furthermore, Molloy et al. showed that eptifibatide inhibited platelet adhesion and platelet aggregate formation on collagen under flow (Molloy et al., 2017). The potent inhibition of thrombus formation and platelet adhesion by eptifibatide is attributed to different factors. Eptifibatide blocks platelet aggregation, which is a critical step in thrombus formation, prevents platelet spreading, and blocks vWF association with GPIIb/IIIa, which mediates indirect interaction with collagen (Jackson et al., 2009, Constantinescu-Bercu et al., 2020). Moreover, eptifibatide is able to disaggregate preformed platelet thrombi on collagen under flow (Goto et al., 2004).

P-selectin is a universal indicator of platelet activation because it is stored in platelet α-granules and exposed on the platelet surface during platelet activation (McEver and Martin, 1984). P-selectin functions as an adhesion molecule to mediate interaction between platelets, leukocytes, and epithelial cells (Burns et al., 1999). Glenzocimab, aspirin and ticagrelor, and eptifibatide reduced formation of platelet thrombi expressing P-selectin (fluorescence intensity) on collagen under flow to a similar extent. However, these antiplatelet agents did not decrease P-selectin expression in platelets monolayer formed over collagen (surface coverage %). Furthermore, glenzocimab delayed P-selectin expression during the first 5 min of blood flow compared with aspirin and ticagrelor or eptifibatide. In most studies that assessed the effect of aspirin and $P2Y_{12}$ inhibitors, the P-selectin expression were induced by ADP or AA and measured by flow cytometry, which is different from measuring P-selectin in platelet thrombi under flow as presented here (Bernlochner et al., 2015, Fox et al., 2019, Lev et al., 2006). It has been shown

that ADP-induced P-selectin expression is more reduced in patients treated with aspirin and ticagrelor than in patients treated with aspirin and prasugrel (Bernlochner et al., 2015). Blockade of TxA_2 and $P2Y_{12}$ is expected to reduce P-selectin expression in platelets flowing on collagen as they are important in amplifying platelet thrombus growth as observed in the flow adhesion assay over collagen. Glenzocimab reduced the formation of thrombi expressing P-selectin but not surface coverage, and this may be also attributed to its ability to inhibit thrombus formation rather than adhesion when high concentrations of collage are used. In contrast to its potent effect on thrombus formation, eptifibatide exerted a lower effect on P-selectin expression. Eptifibatide has been demonstrated to exert a weak inhibitory effect on shear-induced P-selection expression and potentiate collagen-induced platelet–leukocyte interaction that is mediated by P-selectin (Goto et al., 2003, Scholz et al., 2002).

Platelet spreading is a process driven by cytoskeletal arrangements mediated by actin and myosin in a GPIIb/IIIa-dependant pathway (Weiss et al., 1991). GPVI is involved in regulating GPIIb/IIIa-mediated platelet spreading on a collagen surface (Nakamura et al., 1999). Glenzocimab marginally reduced platelet spreading without affecting static platelet adhesion on immobilised collagen. Conversely, aspirin and ticagrelor partially reduced platelet adhesion but not spreading on collagen. The addition of aspirin and ticagrelor with glenzocimab further reduced platelet spreading without affecting adhesion. The insignificant effect of glenzocimab on static platelet adhesion on collagen is unexpected. Lecut et al. showed that GPVI blockade with 9O12—rodent origin of glenzocimab—reduced static platelet adhesion on collagen; however, the incubation time on collagen was 15 min, which is different from the incubation time used in the present study (30 min) (Lecut et al., 2003). Another possible explanation of the weak effect of glenzocimab on platelet spreading and adhesion is the role of GPIa/IIa in mediating platelet adhesion and spreading on collagen. Previous studies showed that inhibition

of GPIa/IIa augmented the inhibitory effect of GPVI blockade on static platelet adhesion on collagen (Lecut et al., 2003, Nakamura et al., 1998). Inoue et al. demonstrated that outside-in signalling of GPIa/IIa mediates platelet spreading and adhesion on collagen, and it supports platelet spreading and adhesion in the absence of GPVI (Inoue et al., 2003). The potent inhibition of platelet spreading by eptifibatide is consistent with the role of its target, GPIIb/IIIa, in the regulation of platelet spreading rather than adhesion under static conditions (Induruwa et al., 2018).

GPVI signalling is regulated by tyrosine phosphorylation of the ITAM motif by Src that in turn phosphorylates Syk, LAT, and PLCγ2, leading to platelet activation (Dunster et al., 2015). Glenzocimab significantly reduced but did not completely block tyrosine phosphorylation of Syk (Y525/526), LAT (Y200), and PLCγ2 (Y1217) upon platelet stimulation with collagen. In contrast, aspirin and ticagrelor showed no apparent effect on tyrosine phosphorylation of these effectors, but they amplified the effect of glenzocimab, leading to further inhibition of tyrosine phosphorylation. The residual tyrosine phosphorylation observed with the triple combination of aspirin, ticagrelor, and glenzocimab may be mediated by GPIa/IIa signalling. Atkinson et al. showed that GPIa/IIa is involved in the early phases of GPVI activation, and its blockade delayed tyrosine phosphorylation. Furthermore, Atkinson et al. demonstrated that $P2Y_1$, $P2Y_{12}$, and TP inhibition delayed tyrosine phosphorylation and strengthened the effect of GPIa/IIa blockade on tyrosine phosphorylation of PLC (Atkinson et al., 2003b). However, Vélez et al. showed that GPVI signalling is elevated in patients with STEMI despite treatment with aspirin and $P2Y_{12}$ antagonism (Vélez et al., 2016).

Glenzocimab blocked Horm collagen-induced platelet aggregation in citrate-anticoagulated PRP, whereas no apparent antiplatelet effect was observed with the GPIa/IIa inhibitor 6F1 when it was used on its own. However, 6F1 blocked the low residual platelet aggregation after the GPVI blockade. Results of MEA suggest that 6F1 dose-dependently inhibited collagen-induced platelet aggregation. Additionally, combining glenzocimab with 6F1 led to more potent inhibition of platelet aggregation induced by Horm collagen. There are many factors underlying this significant effect of GPIa/IIa inhibition on platelet aggregation induced by Horm collagen in hirudin-anticoagulated whole blood in MEA, ranging from the anticoagulant and sample type to the principle of measurement and platelet mechanisms that are assessed by MEA.

First, hirudin, an anticoagulant used to collect blood samples for MEA measurement, prevents blood coagulation by binding to thrombin and preserving metal ions and cations (Mannuß, 2020). A divalent cation environment is required for GPIa/IIa and collagen interaction; while this may partially contribute to the discrepancy between MEA and LTA findings, it should not account for a major effect because it has been shown before that 6F1 had a very little inhibitory effect on Horm collagen-induced platelet aggregation by delaying only the onset of aggregation in heparinised PRP, using LTA, which also provides an optimal medium for GPIa/IIa activation (Jarvis et al., 2002a). Second, spontaneous platelet aggregation is one of the limitations of MEA, and it may significantly contribute to the remarkable effect of 6F1 observed using MEA. Spontaneous platelet aggregation results from stirring conditions that lyse old and fragile red blood cells, leading to the release of the platelet agonist, ADP (Saniabadi et al., 1984). In the present study, samples were incubated with inhibitors for 10 min under the standardised stirring condition (900 rpm), which can induce significant spontaneous platelet aggregation; Bampalis et al. showed that stirring at 800 rpm caused a significant release of ATP and subsequently increased spontaneous platelet aggregation, which could be prevented by ADP scavenger,

apyrase (Bampalis et al., 2012). It is known that ADP at a low concentration is considered a major stimulus that activates GPIa/IIa and increases its affinity for collagen, and this GPIa/IIa dependency on ADP was blocked by apyrase (Jung and Moroi, 2000, Jung and Moroi, 2001). Third, MEA measures two main platelet activation events—adhesion and aggregation (Toth et al., 2006). GPIa/IIa is principally involved in stabilizing platelet adhesion, which allows close contact between collagen and GPVI, which leads to more platelet activation and signalling (Kuijpers et al., 2003).

Because MEA captures platelet adhesion and provides the optimal environment for activation of GPIa/IIa (divalent cation and released ADP), GPIa/IIa blockade with 6F1 led to a major reduction in platelet adhesion and subsequent aggregation in MEA. Furthermore, 6F1 significantly reduced thrombus formation on Horm collagen and produced an additive effect on thrombus formation when combined with glenzocimab in citrate-anticoagulated whole blood without compensation for Ca^{2+} or Mg^{2+}.

CRP-XL is a potent, selective agonist of GPVI activation, which was used to obtain an exclusive overview of GPVI blockade regarding the optimal concentration of its inhibitor or the effect of conventional antiplatelet therapy agents on GPVI function. Glenzocimab fully inhibited CRP-XL-mediated platelet aggregation in PRP at 5 µg/ml, whereas with collagen-mediated platelet aggregation, 50 µg/ml of glenzocimab was required to achieve maximal inhibition. In CRP-XL-induced whole blood aggregation, glenzocimab significantly inhibited platelet aggregation to the level of the unstimulated sample. Despite partial inhibition of collagen-induced platelet aggregation, aspirin and ticagrelor showed no effect on CRP-XL-mediated platelet aggregation except a small additive effect on glenzocimab observed in MEA. Gavin et al. showed that concurrent antagonism of TxA_2 and ADP ($P2Y_1$ and $P2Y_{12}$) exerted a

higher inhibitory effect on collagen-induced platelet aggregation than on CRP-XL-induced platelet aggregation (Jarvis et al., 2004).

Regarding platelet spreading and static adhesion on CRP-XL, glenzocimab markedly reduced platelet adhesion. The potent inhibition of static platelet adhesion on CRP-XL by glenzocimab confirms the GPVI-dependant adhesion on CRP (Knight et al., 1999). Eptifibatide inhibited platelet spreading but not adhesion on GPVI ligand, again indicating that spreading is primarily mediated by GPIIb/IIIa regardless of the specific platelet agonist that is used. Glenzocimab completely blocked CRP-XL-induced phosphorylation of Syk, LAT, and PLCγ2. Aspirin and ticagrelor showed no effect on platelet spreading and adhesion on immobilised CRP-XL or CRP-XL-induced tyrosine phosphorylation except the partial inhibition of PLCγ2. The lack of effect of aspirin and ticagrelor, which are used as a routine treatment for patients with ACS, on GPVI-mediated platelet activation may explain the recurrence of thrombotic events in patients with ACS regardless of treatment with dual antiplatelet therapy and the need to therapeutically target GPVI.

3.5 Limitations

Some limitations in this chapter could affect the results and their interpretations should be highlighted. Optimisation for the inhibitors including glenzocimab regarding the length of incubation period was not performed; extending the incubation time for longer periods might increase the inhibitory effect of investigated antiplatelet agents. Blood flow adhesion assays for thrombus formation and platelet adhesion were carried out in citrated whole blood without recalcification. As it is known that citrate prevents clotting by chelating Calcium; calcium is necessary for coagulation and removing calcium changes the natural process of blood coagulation and platelet activation (Mann et al., 2007, Fujimura and Phillips, 1983). Citrate also

chelates magnesium which is important for activation of platelets integrin receptors (Onley et al., 2000).

3.6 Summary

In conclusion, results presented in this chapter confirm the unmet need to target GPVI as the main receptor for collagen because routine antiplatelet therapy provides only partial inhibition of collagen-mediated and not CRP-XL-mediated platelet activation. More importantly, dual antiplatelet therapy agents amplified the inhibitory effect of glenzocimab on collagen-mediated platelet responses. Residual platelet aggregation observed in Multiplate® analyser after GPVI blockade with glenzocimab is mediated by GPIa/IIa, which should be taken into account during monitoring or assessment of GPVI inhibition in MEA.

CHAPTER 4 : EFFECT OF GLENZOCIMAB IN COMBINATION WITH ASPIRIN AND TICAGRELOR ON ATHEROSCLEROTIC PLAQUE-MEDIATED PLATELET RESPONSES

4.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide in the report of the world health organisation. Ischemic coronary heart disease is the most common form of CVD, followed by ischemic stroke. In ischemic heart disease, the blood supply to the heart is diminished due to either chronic narrowing of the coronary arteries by atherosclerotic plaque or occlusion of the coronary arteries by platelet thrombus that result from rupture of atherosclerotic plaque (van der Wal and Becker, 1999).

Atherosclerosis is initiated when lipid is retained, oxidised, and modified inside the deep layers of an artery's wall by a chronic inflammatory process (Ross, 1999). The development of atherosclerosis involves different pathological stages, which start from fatty streak formation and evolve into atherosclerotic plaque formation (Rafieian-Kopaei et al., 2014). The formation of fatty streaks is initiated when atherogenic particles such as low-density lipoprotein (LDL) are deposited and oxidised in the arterial wall from the circulation. Oxidised LDL (Ox-LDL) trigger an inflammatory response in the vascular wall that leads to leukocytes transmigration and infiltration. Monocyte chemoattractant protein-1 (MCP-1) which is upregulated by endothelial cells and smooth muscle cells in the arterial intima attract more monocytes from circulation that develop to macrophage in the vascular wall and start engulfing Ox-LDL and transform into foam cells (Erl et al., 1998, Harrington, 2000). Dying foam cells transform into a lipid-rich, necrotic core which is enclosed by a fibrous cap made of smooth muscle cells and extracellular matrix protein (Lusis, 2000). Collagen synthesis is increased in atherosclerotic arteries and represents the most thrombogenic part (Opsahl et al., 1987). In addition to collagen, atherosclerotic plaque tissues contain many other thrombogenic substances that activates platelets or coagulation such as vWf, fibronectin, tissue factor, and fibrin (Bentzon et al., 2014, Dutta et al., 2017, Annex et al., 1995, Ząbczyk et al., 2021). Atherosclerotic plaque tissues

contain high amounts of FVIII, implying an important contribution for intrinsic coagulation pathway in thrombus formation that is caused by atherosclerotic plaque rupture (Ananyeva et al., 2002). Atherosclerotic plaque tissues also contain thrombogenic microparticles that are sourced from different cells such as platelets, red blood cells, smooth muscle cells, and white blood cells (Leroyer et al., 2007). In short, thrombogenic atherosclerotic plaque is characterized by a large necrotic core with few smooth muscle cells and high macrophage density covered by a thin fibrous cap and spotty calcification (Bentzon et al., 2014).

Platelets also contribute to the formation and progression of atherosclerosis plaque. Hypercholesteremia has been shown to promote platelet interaction with endothelial cells in postcapillary venules in a P-selectin dependant pathway (Tailor and Granger, 2003). Activated platelets interact with vascular endothelium via P-selectin, leading to platelet release of pro-inflammatory mediators which recruit monocytes to the inflamed endothelium, thereby promoting atherosclerotic plaque formation (Huo et al., 2003). Besides P-selectin, GPIbα and GPIIa/IIIb are reported to be involved in platelet-endothelial interaction events that precede initiation of atherosclerotic plaque formation (Massberg et al., 2002). Platelet CD40 is reported to exacerbate atherosclerosis by facilitating platelets-leukocyte aggregate formation and platelet-endothelial interactions (Gerdes et al., 2016).

GPVI is largely involved in atherosclerotic plaque-mediated platelet responses (Penz et al., 2005). GPVI binds *in-vitro* to collagen present in carotid atherosclerotic plaque that has been sampled from patients with high-grade carotid artery stenosis (Schulz et al., 2008). Furthermore, GPVI mediates platelet recruitment and adhesion to atherosclerotic vascular wall in animal models of atherosclerosis (Schulz et al., 2008). GPVI triggers the release of CD40 ligand and induces endothelial activation, which could contribute to inflammation-mediated atherosclerosis (Cabeza et al., 2004).

GPVI expression is increased in patients with acute coronary syndromes (Bigalke et al., 2006). GPVI signalling is upregulated in patients with STEMI compared to patients with stable coronary artery disease (Vélez et al., 2016) and soluble GPVI levels are reported to be elevated in patients with ACS. Soluble GPVI levels have therefore been suggested as a possible diagnostic or prognostic marker (Smith et al.). Soluble GPVI results from shedding of GPVI ectodomain from platelet surface by metalloproteinase ADAM10 and ADAM17 following platelet activation by a GPVI ligand (Gardiner et al., 2007).

4.2 Aims

This chapter aims to investigate the effect of GPVI blockade with glenzocimab (in combination with aspirin and ticagrelor) on atherosclerotic plaque-mediated platelet responses.

4.3 Results

4.3.1. Effect of glenzocimab with aspirin and ticagrelor on platelet aggregation induced by atherosclerotic plaque homogenate

The effect of increasing concentrations of glenzocimab (1–50 µg/ml) on platelet aggregation stimulated by atherosclerotic plaque homogenate was investigated in PRP. The effect of aspirin and ticagrelor on their own or in combination with glenzocimab was determined to demonstrate whether they have an amplified effect on platelet responses induced by atherosclerotic plaque. Platelet aggregation was determined using light transmission aggregometry (LTA) and multiple electrode platelet aggregometry (MEA).

4.3.1.1 Glenzocimab dose-dependently inhibited atherosclerotic plaque-induced platelet aggregation in platelet-rich plasma

Different concentrations of atherosclerotic plaque were investigated on platelet aggregation in PRP in LTA as shown in the supplementary Figure 3. The concentration of 70 µg/ml was chosen

to study the effect of antiplatelet agents on atherosclerotic plaque mediated platelet aggregation, as it strongly induced platelet aggregation compared to other concentrations. The results showed that atherosclerotic plaque (70 µg/ml) potently induced platelet aggregation in citrate-anticoagulated PRP, which was dose-dependently inhibited by glenzocimab (Figure 4.1). Final aggregation in the vehicle-treated sample (control) was 65 ± 2%. At a concentration of 5 µg/ml, glenzocimab significantly reduced platelet aggregation by 50% (final aggregation: 32 ± 12%; P=0.001). At a two-fold higher concentration of glenzocimab (10 µg/ml), platelet aggregation was further reduced by 92% (final aggregation: 4 ± 3%; P<0.001). Near complete inhibition of atherosclerotic plaque-induced platelet aggregation was achieved with 25 µg/ml glenzocimab where final platelet aggregation was reduced by 98% (final aggregation: 1 ± 1%; P<0.001).

A GPIIb/IIIa inhibitor, eptifibatide, was also investigated as it blocks the final common pathway of platelet aggregation. In response to most agonists, eptifibatide (9 µM) generally caused greater inhibition of platelet aggregation than glenzocimab. However, when platelets were stimulated by atherosclerotic plaque in platelet-rich plasma (PRP), glenzocimab appeared to be more potent than eptifibatide in blocking platelet aggregation. Glenzocimab (50 µg/ml) inhibited platelet aggregation by 98% (final aggregation: 1 ± 1% compared to 64 ± 2% in the control samples; P<0.001). In contrast, 9 µM eptifibatide decreased platelet aggregation by 87% (final aggregation: 8 ± 1; P<0.001), leaving around 12% residual platelet aggregation. This difference in the final platelet aggregation endpoints between glenzocimab and eptifibatide was statistically significant (P=0.03).

In Summary, these results demonstrated that glenzocimab is a potent inhibitor of atherosclerotic plaque-mediated platelet aggregation. In addition, glenzocimab appeared to me potent than eptifibatide in inhibiting atherosclerotic plaque-induced platelet aggregation in PRP.

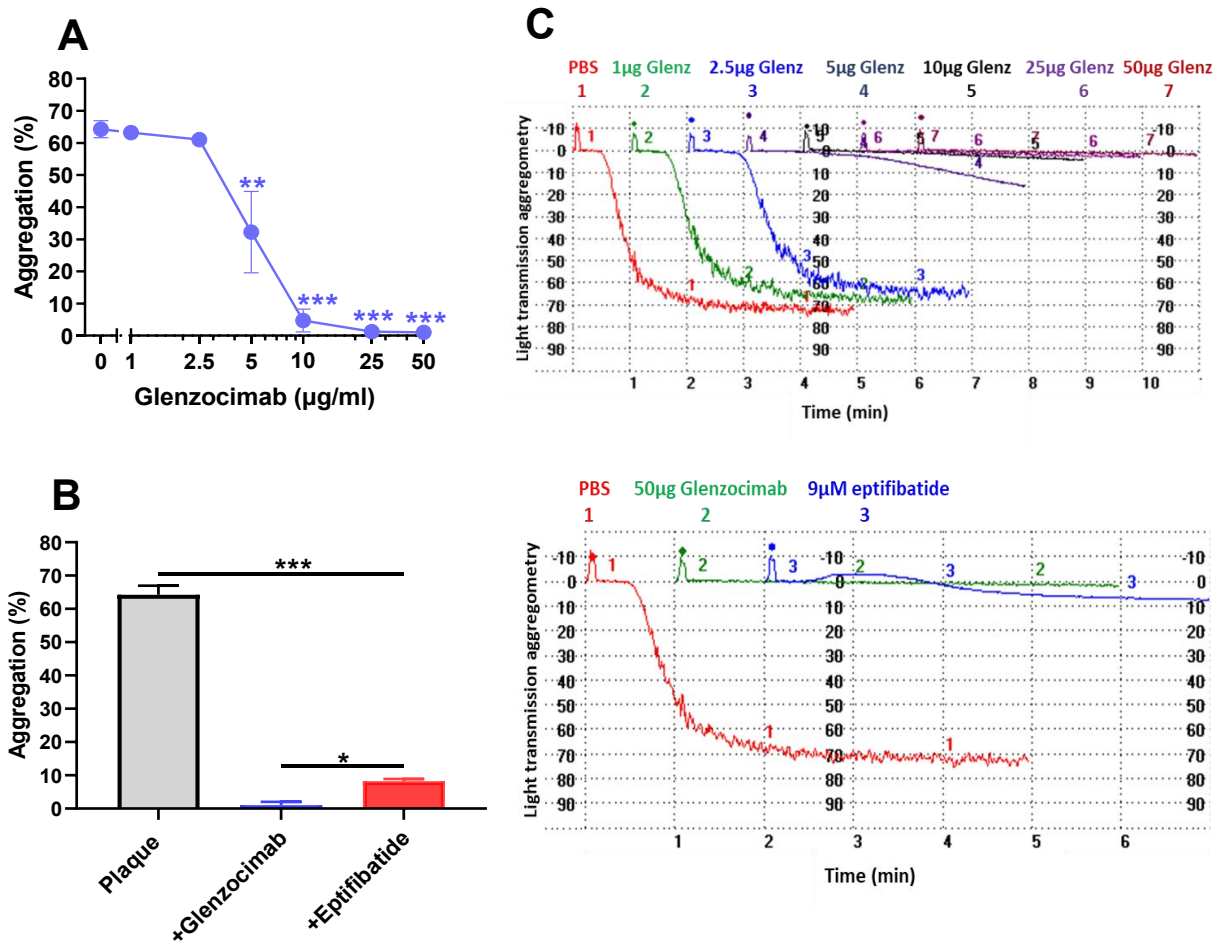


Figure 4.1. Glenzocimab blocked atherosclerotic plaque-induced platelet aggregation in a dose- dependent manner.

A) Dose-dependent inhibition of atherosclerotic plaque stimulated platelet aggregation by glenzocimab. **B)** Glenzocimab is superior to eptifibatide regarding inhibition of platelet aggregation stimulated by atherosclerotic plaque. **C)** Representative aggregation traces for a single LTA experiment, where human PRP was incubated with increasing concentrations of glenzocimab (1, 2.5, 5, 10, 25, and 50 µg/ml), 9 µM eptifibatide or PBS (control) for 10 min at 37°C, then platelet aggregation was stimulated by 70 µg/ml atherosclerotic plaque homogenate and monitored for 5 min. The effect of different concentrations of glenzocimab was compared to untreated (PBS control) samples or glenzocimab versus eptifibatide were assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=4, *P<0.01, **P<0.01, and ***P<0.001).

4.3.1.2 Glenzocimab showed a strong amplified inhibitory effect when combined with aspirin and ticagrelor on atherosclerotic plaque-stimulated platelet aggregation in platelet-rich plasma

The next step after establishing the dose-response curve of glenzocimab on atherosclerotic plaque-induced platelet aggregation was to determine the effect of aspirin and ticagrelor on their own and to see if these agents provide an amplified effect when combined with glenzocimab. Glenzocimab (1-50 µg/ml) was investigated in the presence of aspirin (30 µM), ticagrelor (1 µM) or both.

The results showed Glenzocimab provided amplified effects with aspirin and ticagrelor on inhibiting atherosclerotic plaque-induced platelet aggregation. On the other hand, the independent use or combined use of aspirin and ticagrelor showed no significant effect on atherosclerotic plaque-induced platelet aggregation (Figure 4.2).

Atherosclerotic plaque homogenate at a concentration of 70 µg/ml strongly induced platelet aggregation (final aggregation: 65 ± 2%). There was no significant effect for dual combination of aspirin and ticagrelor on platelet aggregation (final aggregation: 40 ± 4%; P=0.21). However, when aspirin and ticagrelor were combined with glenzocimab, they had a potent amplified blocking effect on platelet aggregation. Glenzocimab at a concentration of 2.5 µg/ml did not significantly reduce platelet aggregation (final aggregation: 59 ± 2%; P=0.46) when used alone; however, it reduced platelet aggregation by 87% (final aggregation: 8 ± 2%; P=0.002) in the presence of aspirin and ticagrelor. This pattern of amplified inhibition of atherosclerotic plaque-stimulated platelet aggregation continued to increase with increasing concentrations of glenzocimab (5, 10, and 50 µg/ml) in combination with aspirin and ticagrelor, leading to a complete inhibition of platelet aggregation.

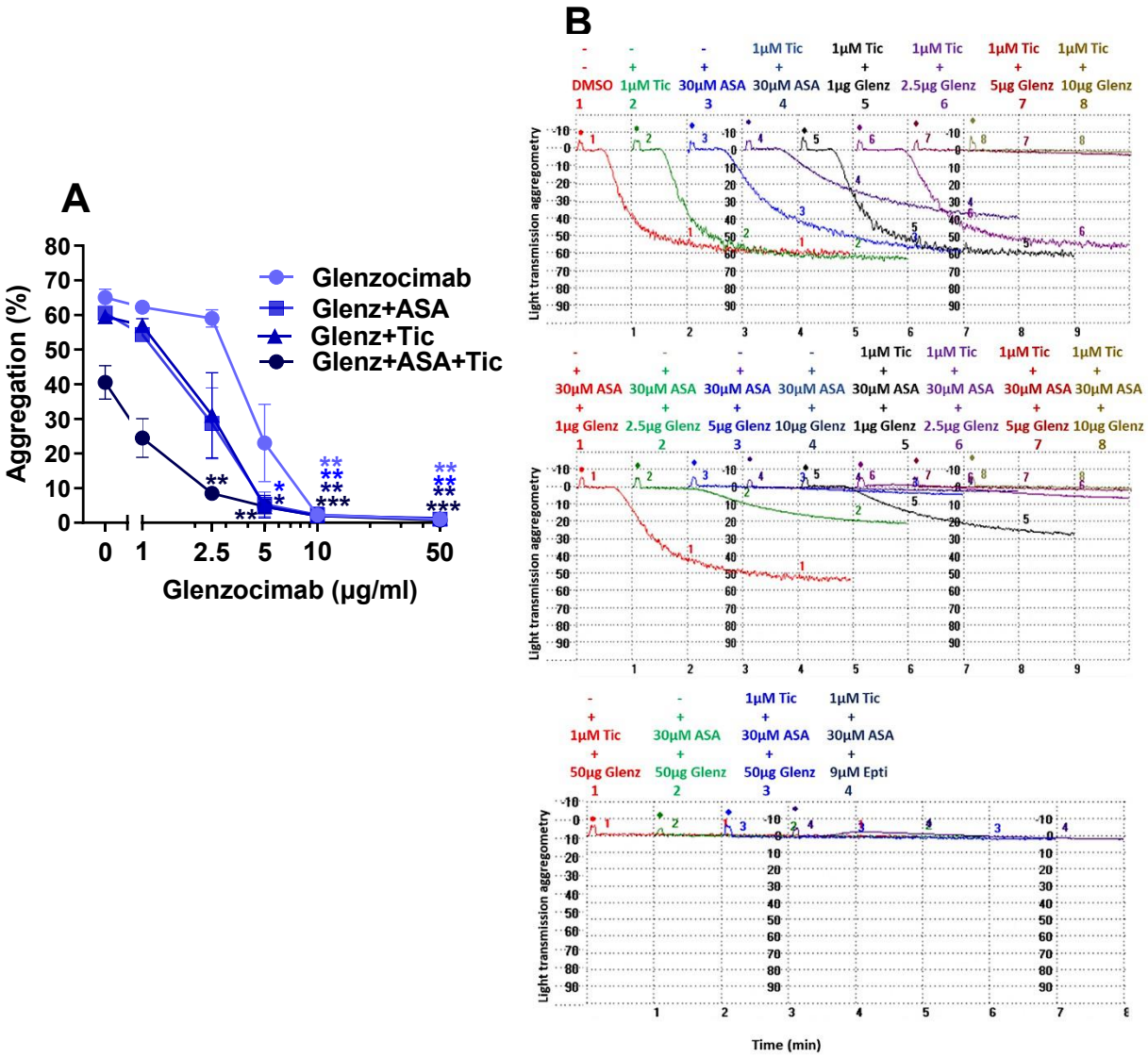


Figure 4.2. Amplified antiplatelet effects of glenzocimab with aspirin and ticagrelor on atherosclerotic plaque-induced platelet aggregation.

A) Dose-response curve of the amplified effect of glenzocimab with aspirin and ticagrelor on platelet aggregation stimulated by atherosclerotic plaque. **B)** Representative aggregation traces from a single LTA experiment, where PRP was incubated with vehicle (DMSO), 1-50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor for 10 min at 37°C, then platelet aggregation was stimulated by adding atherosclerotic plaque homogenate (70 µg/ml), and aggregation was monitored for 5 min in PAP8-aggregometer. The effect of different antiplatelet agents was compared to untreated (DMSO control) samples and assessed using a mixed effects model with Dunnett correction for multiple comparisons (n=4, **P<0.01, and ***P<0.001).

4.3.1.3 Glenzocimab combined with aspirin and ticagrelor inhibited atherosclerotic plaque-induced platelet aggregation in whole blood to the same extent as eptifibatide

Glenzocimab continued to exert a potent inhibitory effect on platelet aggregation and in combination with aspirin and ticagrelor potently inhibited platelet aggregation in whole blood in MEA (Figure 4.3). A higher concentration of atherosclerotic plaque (140 μg/ml) was used to stimulate platelet aggregation in MEA as MEA is not sensitive to light transmission as it in LTA in order to see if glenzocimab can still inhibit platelet aggregation stimulated by a higher concentration of atherosclerotic plaque than the one used in LTA. Platelet aggregation in samples treated with DMSO was 65 ± 4 U. Ticagrelor did not significantly inhibit platelet aggregation. In contrast, aspirin reduced platelet aggregation by 21% (51 ± 1 U; P=0.003) when used alone and by 47% (34 ± 3 U; P<0.001) in the presence of ticagrelor. Glenzocimab inhibited platelet aggregation by 74% (16 ± 4 U; P<0.001) when used alone and by 95% (3 ± 1 U; P<0.001) when in combination with aspirin and ticagrelor. GPIIb/IIIa blockade with eptifibatide in whole blood sample was superior to glenzocimab as it reduced platelet aggregation by 99% (1 ± 1 U; P<0.001).

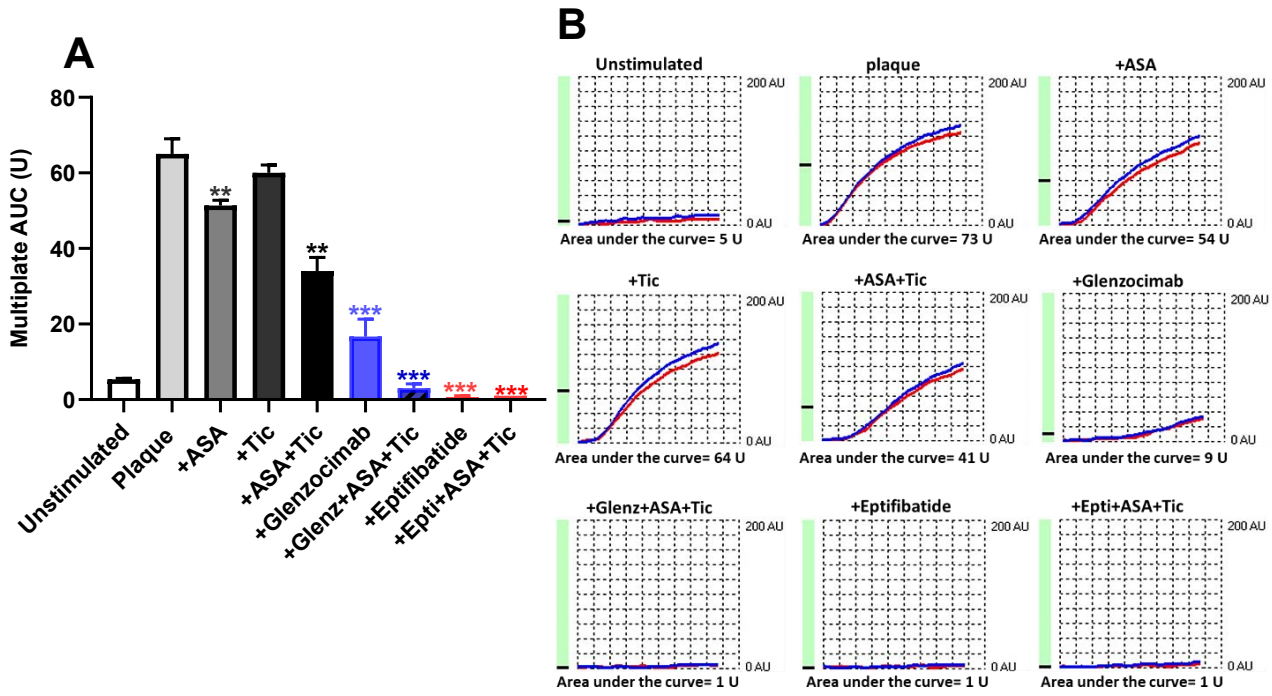


Figure 4.3. Combining glenzocimab with aspirin and ticagrelor provided a similar inhibition of atherosclerotic plaque-induced platelet aggregation as eptifibatide.

A) Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on whole blood platelet aggregation stimulated by atherosclerotic plaque. **B)** Representative aggregation traces from a single MEA experiment, where hirudin anticoagulated whole blood was diluted in half with normal saline and incubated with vehicle (DMSO), 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, 9 µM eptifibatide for 10 min at 37°C under stirring conditions, then platelet aggregation was stimulated by adding atherosclerotic plaque homogenate (140 µg/ml), and aggregation was monitored for 6 min in the Multiplate® analyser. The effect of different antiplatelet agents was compared to untreated (DMSO control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, **P<0.01, and ***P<0.001).

4.3.2 Acute effect of glenzocimab and conventional antiplatelet agents on platelet aggregation induced by atherosclerotic plaque in platelet-rich plasma assessed by light transmission aggregometry

After showing platelets pre-treatment (incubation) with glenzocimab completely inhibited platelet aggregation induced by atherosclerotic plaque homogenate in PRP, the next step was to determine the acute effect of glenzocimab, aspirin, ticagrelor, and eptifibatide on atherosclerotic plaque-induced platelet aggregation. Furthermore, it was determined whether glenzocimab and eptifibatide can exert an immediate inhibitory effect on platelet aggregation in samples that were pre-treated with dual antiplatelet agents. This was investigated as aspirin and ticagrelor are often administered immediately and eptifibatide is later used in patients with ACS with heavy thrombus burden.

4.3.2.1 Only eptifibatide exerted an immediate inhibitory effect on atherosclerotic plaque-stimulated aggregation of untreated platelets in light transmission aggregometry

To test the acute effect of antiplatelet agents on atherosclerotic plaque-induced platelet aggregation, platelet aggregation was stimulated by adding 70 µg/ml atherosclerotic plaque homogenate and monitored for 1 min. When platelet aggregation reached half of the maximum platelet aggregation, DMSO, aspirin (30 µM), ticagrelor (1 µM), glenzocimab (50 µg/ml), and eptifibatide (9 µM) were added to designated test tubes, and platelet aggregation was further monitored for 4 min. To calculate the immediate inhibition potency of antiplatelet agents on platelet aggregation, the following equation was used: 100 − (final aggregation after addition of platelet inhibitor/maximum aggregation of the vehicle) × 100. This equation was adapted from Martin et al. who used it to calculate the ability of GPIIb/IIIa inhibitors to reverse ADP-stimulated platelet aggregation (Moser et al., 2003).

As shown in Figure 4.4, eptifibatide on its own demonstrated the ability to exert an immediate inhibition of platelets aggregation after stimulation with atherosclerotic plaque homogenate. The final aggregation in the sample-treated with eptifibatide was 19 ± 9% compared to 57 ± 9% in the vehicle treated sample; P=0.05. In contrast, dual treatment with aspirin and ticagrelor did not show any sign of immediate inhibition of platelet aggregation (55 ± 11%; P>0.99). Glenzocimab also did not exert an immediate inhibition of platelet aggregation (53 ± 6%; P=0.98). The immediate inhibition potency of eptifibatide and glenzocimab correlates well with their effect on the final aggregation of atherosclerotic plaque-induced platelets. Eptifibatide acutely inhibited platelet aggregation with an immediate effect potency of 68 ± 14%, compared with 10 ± 2% of glenzocimab; P=0.02. These results indicated only eptifibatide had the ability to exert an immediate inhibition of atherosclerotic plaque-stimulated platelet aggregation (untreated platelets).

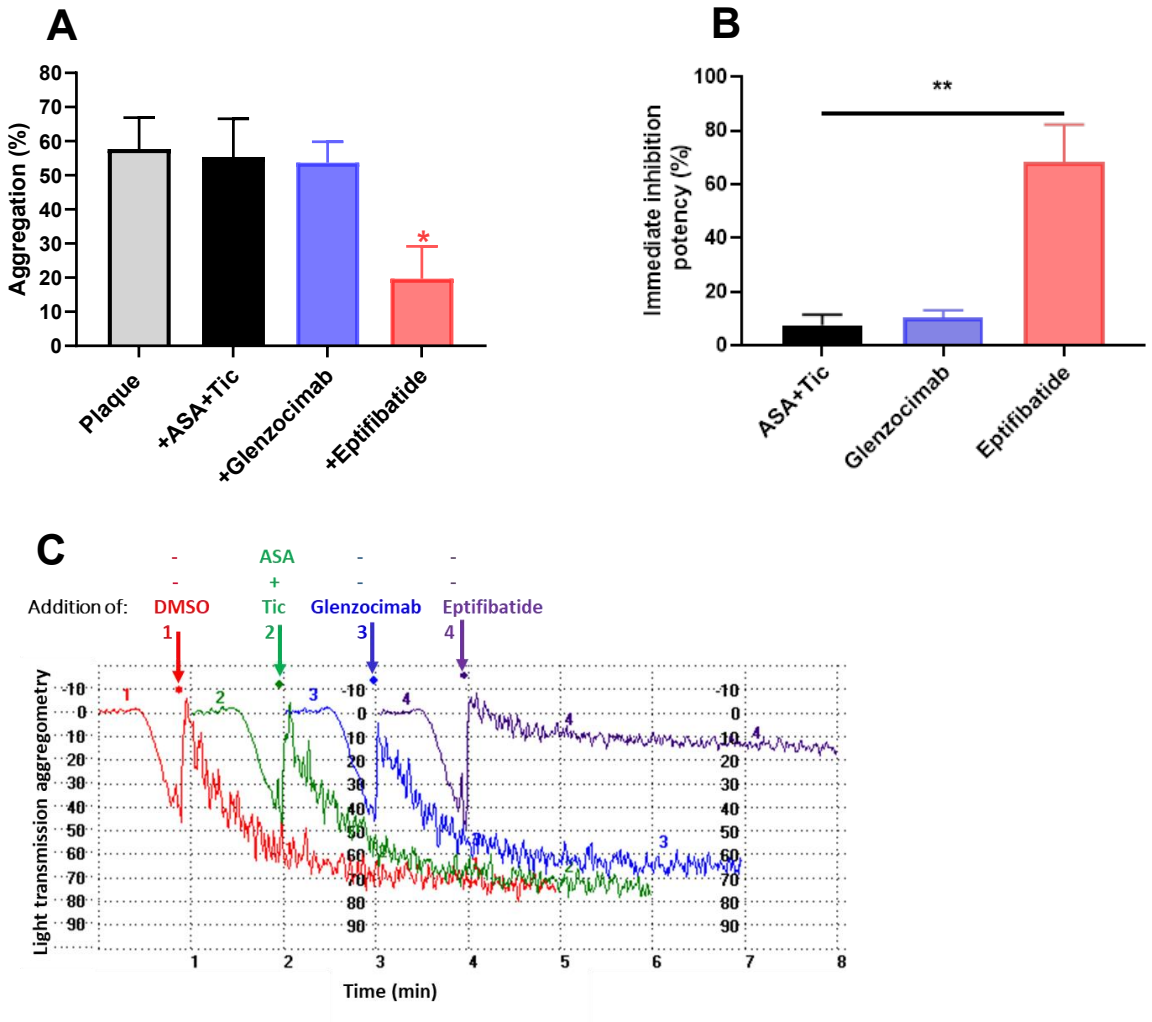


Figure 4.4. Acute effect of glenzocimab, aspirin and ticagrelor, and eptifibatide on atherosclerotic plaque-induced platelet aggregation.

A) Only eptifibatide provided an immediate inhibitory effect on aggregation of platelets after stimulation with atherosclerotic plaque. **B)** Immediate inhibition potency of antiplatelet agents on atherosclerotic plaque-stimulated platelet aggregation. **C)** A representative aggregation trace, where untreated human PRP was stimulated with 70 µg/ml atherosclerotic plaque; after 1 min of stimulation, vehicle (DMSO), 30 µM aspirin or 1µM ticagrelor, 50 µg/ml glenzocimab, and 9 µM eptifibatide were added to the actively aggregating platelets as indicated by traces jump up (arrowhead). Then platelet aggregation was continuously monitored for an additional 4 min in the PAP-8E aggregometer. The ability of antiplatelet agents to exert an immediate inhibition of platelet aggregation was compared to vehicle (DMSO) samples and the immediate inhibition potency between platelet inhibitors was assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, *P<0.01, and **P<0.001).

4.3.2.2 Glenzocimab provided an immediate inhibitory effect on atherosclerotic plaque-induced aggregation of aspirin and ticagrelor pre-treated platelets

Following the previously described experiment, the investigation was extended into see the acute effect of glenzocimab and eptifibatide on aggregation of platelets that were pre-treated with aspirin and ticagrelor as these are commonly administered to patients prior to primary percutaneous coronary intervention for patients with STEMI. PRP was preincubated with both ticagrelor and aspirin for 10 minutes at 37°C before stimulation with atherosclerotic plaque homogenate and reversal of platelet aggregation by adding glenzocimab or eptifibatide. As shown in Figure 4.5, both glenzocimab and eptifibatide exerted an immediate inhibition of aggregation of aspirin and ticagrelor-treated platelets to a similar extent.

Final aggregation after adding glenzocimab and eptifibatide was 8 ± 2% (P=0.005) and 9 ± 4% (P=0.006), respectively, which was significantly different from the final aggregation of 53 ± 9% in the control sample where the vehicle was added. As glenzocimab and eptifibatide produced a similar immediate inhibitory effect on aggregation of platelets that were pre-treated with aspirin and ticagrelor, their immediate inhibition potency was very similar. Glenzocimab and eptifibatide inhibited platelet aggregation with immediate effect potency of 83 ± 3% and 84 ± 5%, respectively, with no statistically significant difference. The findings suggest that blockade of the secondary mediators ($P2Y_{12}$ and TxA_2) is required for glenzocimab to exert an immediate inhibitory effect on atherosclerotic plaque-induced platelet aggregation. Moreover, inhibition of $P2Y_{12}$ and TxA_2 amplified the immediate inhibition potency of eptifibatide from 68 ± 14% in untreated platelets to 84 ± 5% in platelets pre-treated with aspirin and ticagrelor.

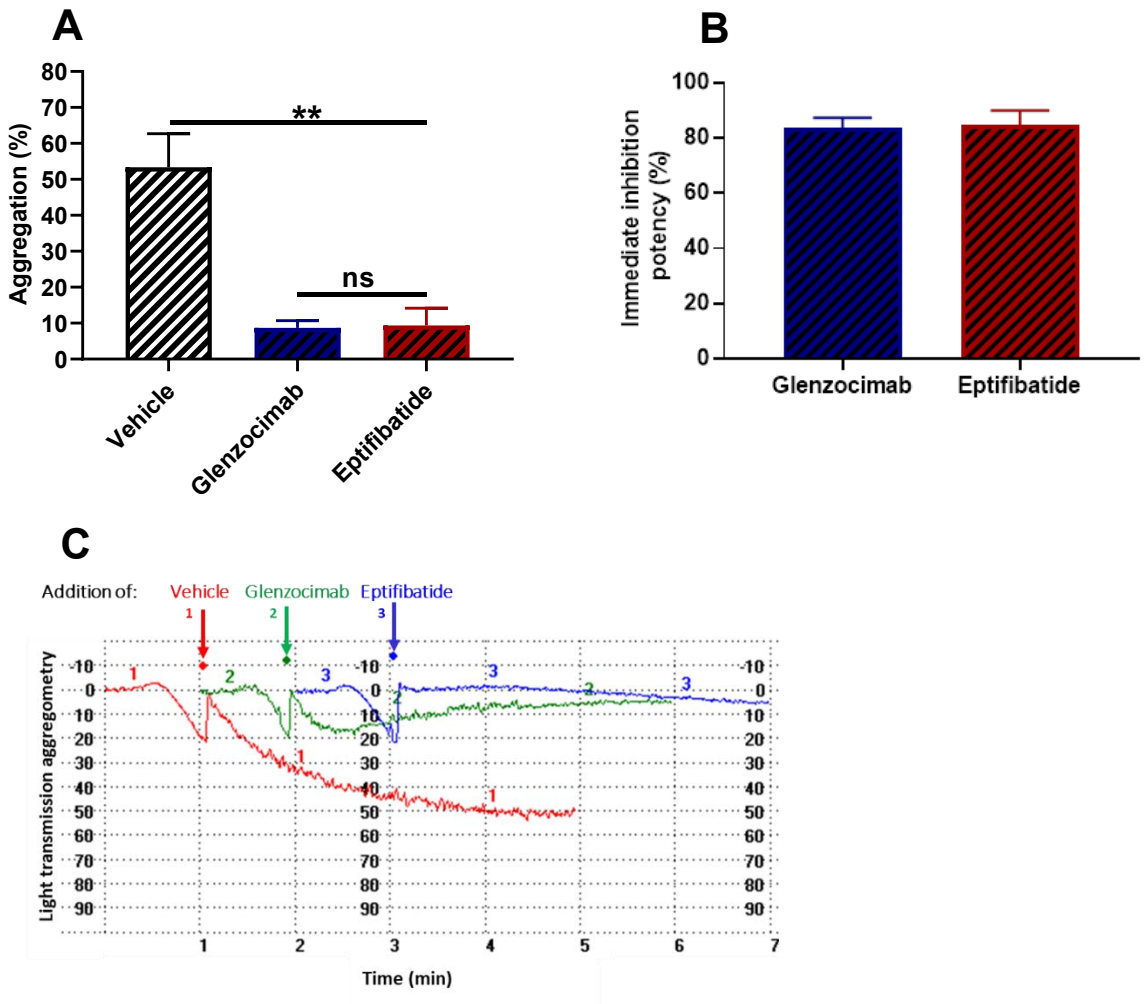


Figure 4.5. Acute effect of glenzocimab and eptifibatide on atherosclerotic plaque-stimulated aggregation in platelets that were pre-treated with aspirin and ticagrelor.

A) Glenzocimab and eptifibatide provided an immediate inhibitory effect on platelet aggregation in aspirin and ticagrelor pre-treated platelets after stimulation with atherosclerotic plaque. **B)** Immediate inhibition potency of glenzocimab and eptifibatide on atherosclerotic plaque-stimulated aggregation of aspirin and ticagrelor pre-treated platelets. **C)** Aggregation traces from a single LTA experiment, where human PRP was incubated with 30 µM aspirin and 1 µM ticagrelor for 10 min at 37°C, then platelet aggregation-stimulated by 70 µg/ml atherosclerotic plaque; after 1 min of stimulation, vehicle (PBS), 50 µg/ml glenzocimab, and 9 µM eptifibatide were added into the aggregating platelets as indicated by traces jump up (arrowhead). Then aggregation was further monitored for 4 min in the PAP-8E aggregometer. The ability of the antiplatelet agent to exert an immediate inhibitory effect on platelet aggregation was compared to vehicle (PBS) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, **P<0.01).

4.3.3 Effect of glenzocimab combined with aspirin and ticagrelor on atherosclerotic plaque-induced platelet secretion

ATP release from dense granules following platelet activation potentiates platelet responses in haemostasis and thrombosis by regulating calcium mobilisation via its purinoceptor, $P2X_1$ (Fung et al., 2007). The effect of increasing concentrations of glenzocimab on platelet secretory function as measured by ATP secretion was investigated. Increasing concentrations of glenzocimab were also combined with aspirin and ticagrelor to investigate whether the presence of conventional antiplatelet medications amplify the effect of glenzocimab on ATP secretion. Furthermore, the effect of glenzocimab with/without aspirin and ticagrelor was further investigated on α-granule secretion as assessed by P-selectin expression on atherosclerotic plaque homogenate under flow.

4.3.3.1 Glenzocimab dose-dependently blocked atherosclerotic plaque-induced dense granule secretion as assessed by ATP release, and its effect was amplified in the presence of aspirin and ticagrelor

The results demonstrated that glenzocimab dose-dependently inhibited ATP release stimulated by atherosclerotic plaque, and the addition of aspirin and ticagrelor strengthened the effect of glenzocimab, whereas the triple combination of aspirin, ticagrelor, and eptifibatide only partially inhibited ATP secretion (Figure 4.6).

As shown in Figure 4.6A, platelet stimulation with atherosclerotic plaque homogenate induced considerable ATP secretion in the vehicle-treated sample (4.36 ± 0.66 AUC). Glenzocimab at 5 µg/ml significantly inhibited ATP secretion by 52% (2 .10 ± 0.85 AUC; P =0.02), whereas at 50 µg/ml, it greatly inhibited ATP secretion by 91% (0.40 ± 0.20 AUC; P<0.001). The combination of aspirin and ticagrelor with glenzocimab intensified its effect on the blockade of ATP secretion (Figure 4.6B). For example, 1 µg/ml glenzocimab alone did not significantly

reduce ATP secretion (3.86 ± 1.12 AUC; P=0.92); however, when it was combined with aspirin and ticagrelor, the ATP secretion was reduced by 79% (0.90 ± 0.25 AUC; P<0.001). The presence of aspirin and ticagrelor continued to enhance the effect of the remaining concentrations of glenzocimab; addition of aspirin and ticagrelor to 50 μg/ml glenzocimab amplified its inhibitory effect from 91% (0.40 ± 0.20 AUC; P<0.001) to 98% (0.10 ± 0.05 AUC; P<0.001).

Conventional antiplatelet therapy agents had no significant effect on atherosclerotic plaque-stimulated ATP release apart from the combination of aspirin, ticagrelor and eptifibatide, where they partially reduced ATP secretion (Figure 4.6C). Aspirin at 30 μM had no significant effect on ATP release (3.33 ± 0.66 AUC; P=0.95), and 1 μM ticagrelor had no effect on ATP (3.90 ± 1.35 AUC; P>0.99). Remarkably, the combined use of aspirin and ticagrelor produced no additional effect on ATP secretion compared with aspirin alone (3.33 ± 0.56 AUC; P>0.99). Similar to aspirin, 9 μM eptifibatide showed no significant effect on ATP release (3.53 ± 1.36 AUC; P=0.95). However, the triple combination of aspirin, ticagrelor, and eptifibatide significantly reduced ATP secretion by 66% (1.46 ± 0.46 AUC; P=0.02). Despite the significant reduction in ATP secretion achieved by combination of aspirin, ticagrelor and eptifibatide, it was still not as large as the reduction achieved with an intermediate concentration of glenzocimab (10 μg/ml), which potently reduced ATP release by 84% (0.70 ± 0.40 AUC; P<0.001).

In summary, these results showed that glenzocimab alone blocked atherosclerotic plaque-induced ATP secretion. In contrast, the combined use of aspirin and ticagrelor or eptifibatide did not affect ATP secretion unless all three antiplatelet agents were added together, where they caused a partial inhibition of atherosclerotic plaque-stimulated ATP release.

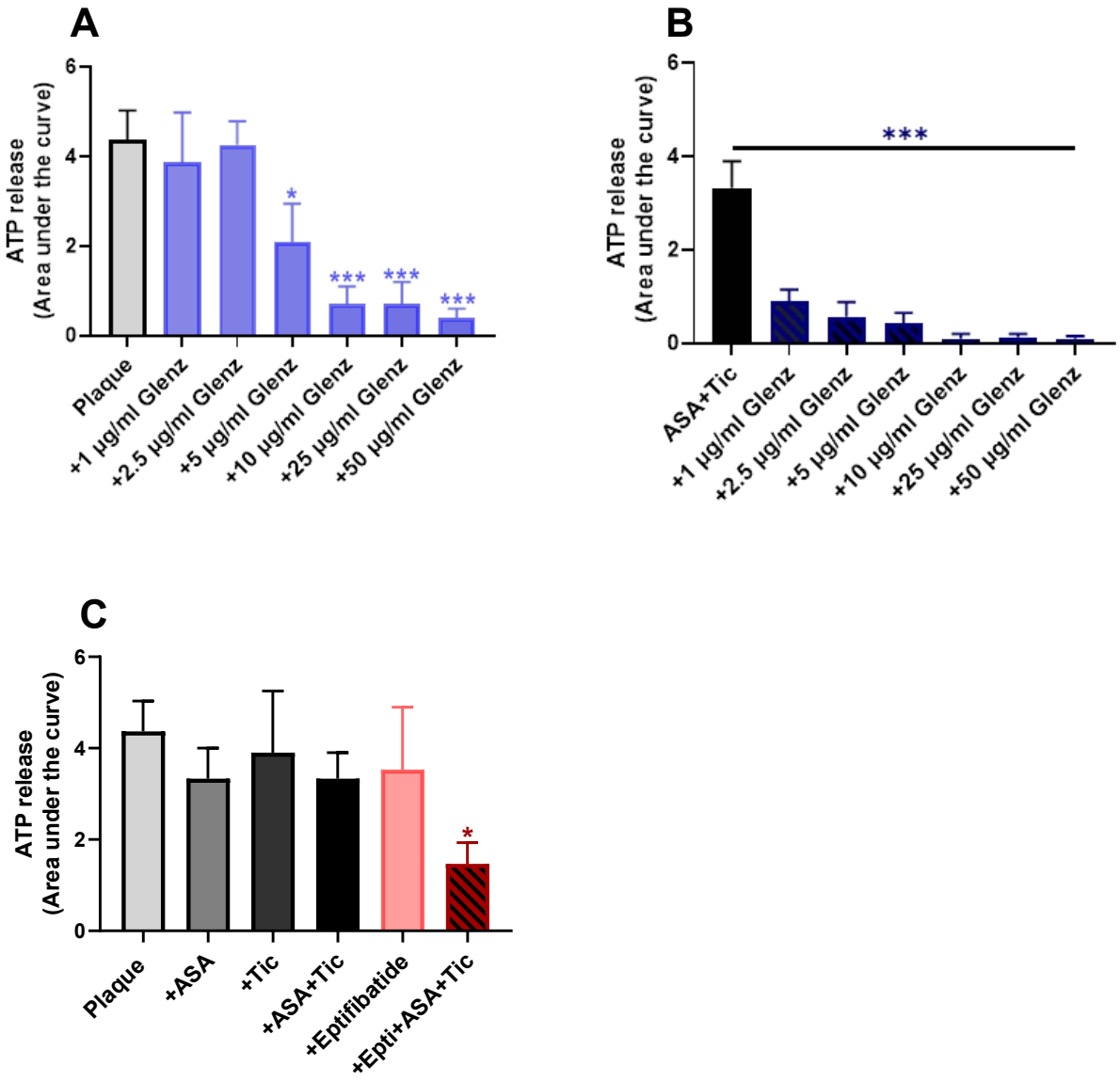


Figure 4.6. Glenzocimab potently blocked atherosclerotic plaque-stimulated ATP secretion in platelets under aggregating conditions, while conventional antiplatelet agents only produced significant effect when all combined with eptifibatide.

A) Dose-dependent inhibition of ATP secretion by 1-50 µg/ml glenzocimab. **B)** The amplified inhibition of ATP secretion by combining glenzocimab with aspirin and ticagrelor. **C)** Effect of 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide on ATP secretion, where they caused a significant inhibition of ATP release when they all combined. Data was analysed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, *P<0.05, and **P<0.001).

4.3.3.2 Glenzocimab blocked α-granule secretion as assessed by P-selectin expression on atherosclerotic plaque homogenate under an arterial shear rate in a microfluidic chamber

The effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) was investigated on P-selectin expression in hirudin-anticoagulated whole blood flowing over atherosclerotic plaque homogenate under a shear rate of 1000 s^{-1}. The results show that glenzocimab and eptifibatide completely inhibited P-selectin expression in whole blood flowing on atherosclerotic plaque under arterial shear rate as measured by the surface coverage %. In contrast, aspirin and ticagrelor did not inhibit P-selection expression and showed no additional effect when combined with glenzocimab or eptifibatide (Figure 4.7).

As highlighted in Figure 4.7A, aspirin and ticagrelor did not affect thrombus fluorescence intensity (8.00 ± 3.28 AU compared to 9.03 ± 2.02 AU in the control samples; P=0.98). Glenzocimab showed a non-significant trend of reducing thrombus fluorescence intensity by 89% (1.02 ± 0.08 AU; P=0.08) without showing additional effect in the presence of aspirin and ticagrelor (1.04 ± 0.08 AU; P=0.09). Eptifibatide showed a non-significant trend of decreasing thrombus fluorescence intensity by 87% (1.20 ± 0.25 AU; P=0.07) without an additional effect of aspirin and ticagrelor (1.52 ± 0.42 AU; P=0.09).

Treatment with aspirin and ticagrelor had no significant effect on the surface coverage (25.36 ± 13.13% compared to 44.30 ± 7.50% in the control samples; P=0.34; Figure 4.7B). Glenzocimab alone reduced the surface coverage by 99% (0.31 ± 0.15%; P=0.03). In contrast, eptifibatide reduced the surface coverage by 95% (2.22 ± 0.91%; P=0.04) without showing an additive effect when combined with aspirin and ticagrelor (4.23 ± 2.38%; P=0.06).

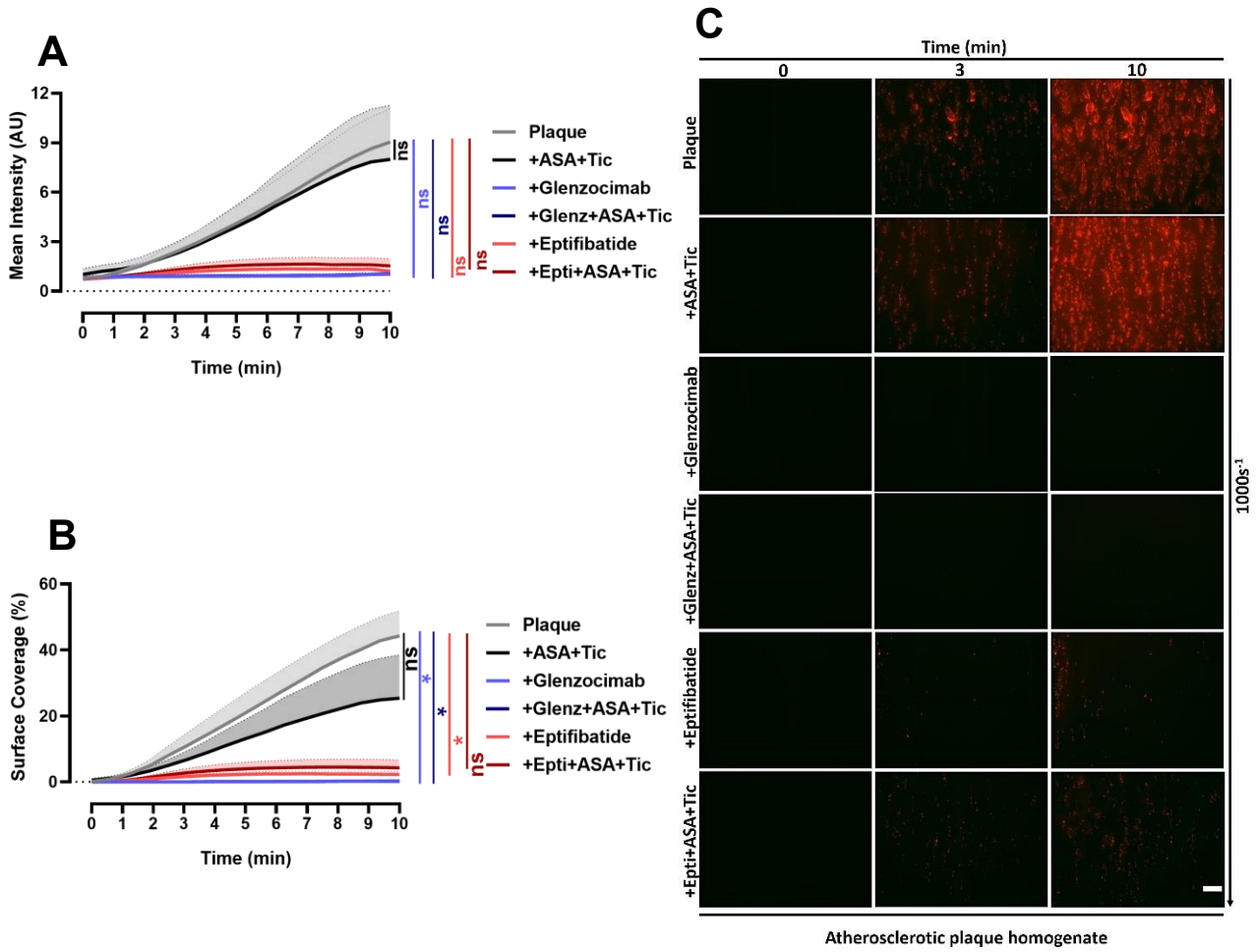


Figure 4.7. Effect of glenzocimab and other antiplatelet agents on P-selectin expression on platelets that have adhered to atherosclerotic plaque under flow conditions.

A) Glenzocimab showed a non-significant trend of reducing formation of P-selectin expressing thrombi as reflected by fluorescence intensity. **B)** Glenzocimab reduced adhesion of P-selectin expressing platelets as reflected by mean of surface coverage %. **C)** Representative images from a single flow adhesion experiment, where hirudin-anticoagulated blood was incubated with AF647-labelled anti-human CD62P mAb, 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide for 15 min at 37°C then flowed over 1 mg/ml atherosclerotic plaque at a shear rate of 1000 s^{-1} for 10 min. Images were taken every 35 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by Image J software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of antiplatelet agents was compared to untreated samples and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=4, ns=non-significant, *P<0.05, and **P<0.01).

4.3.4 Effect of glenzocimab with aspirin and ticagrelor on platelet adhesion and thrombus formation on atherosclerotic plaque homogenate under flow

The effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) was investigated on platelet thrombus formation and adhesion on 1 mg/ml atherosclerotic plaque coated surfaces in a microfluidic chamber under arterial shear rate of 1000 s^{-1}. The same combinations and concentrations of the inhibitors were further investigated on platelet thrombus formation and platelet adhesion on 1 mg/ml atherosclerotic plaque homogenate but at a lower shear rate (300 s^{-1}) to examine whether the investigated inhibitors, including glenzocimab, can maintain the antiplatelet effect observed with a shear rate of 1000 s^{-1}.

4.3.4.1 Glenzocimab abrogated platelet adhesion and thrombus formation on atherosclerotic plaque homogenate under arterial shear rate in a microfluidic chamber

The results demonstrated that glenzocimab and eptifibatide blocked platelet thrombus formation and platelet adhesion on atherosclerotic plaque, whereas aspirin and ticagrelor potently inhibited platelet thrombus formation but blocked platelet adhesion to a lesser extent (Figure 4.8).

Aspirin and ticagrelor significantly reduced thrombus formation by 89% (4 ± 1 AU compared to 41 ± 10 AU in the DMSO-treated samples; P=0.04). Glenzocimab on its own reduced thrombus formation by 93% (2 ± 1 AU; P=0.04) without showing additional effect in the presence of aspirin and ticagrelor (2 ± 1 AU; P=0.04). In contrast, eptifibatide alone delivered the most potent inhibition of thrombus development by 96% (1 ± 1 AU; P=0.04) and did not show any significant additional effect when it was combined with aspirin and ticagrelor (1 ± 1 AU; P=0.05).

The combination of aspirin and ticagrelor reduced the surface coverage to a lesser extent, by 64% (11 ± 1% compared to 32 ± 4% in the control; P=0.006). Glenzocimab greatly reduced the surface coverage by 85% (5 ± 1%; P=0.002) and by 89% (3 ± 1%; P=0.003) when combined with aspirin and ticagrelor. Eptifibatide as a single antiplatelet agent produced the most potent reduction in the surface coverage of 93% (1 ± 1%; P=0.002). The use of aspirin and ticagrelor with eptifibatide did not provide an additional effect (2 ± 1%; P=0.009).

In summary, these results showed that glenzocimab and eptifibatide greatly inhibited platelet thrombus formation and adhesion on atherosclerotic plaque in blood flowing at 1000 s^{-1}. On the other hand, the combined use of aspirin and ticagrelor greatly inhibited platelet thrombus formation but exerted a lesser effect on platelet adhesion on atherosclerotic plaque.

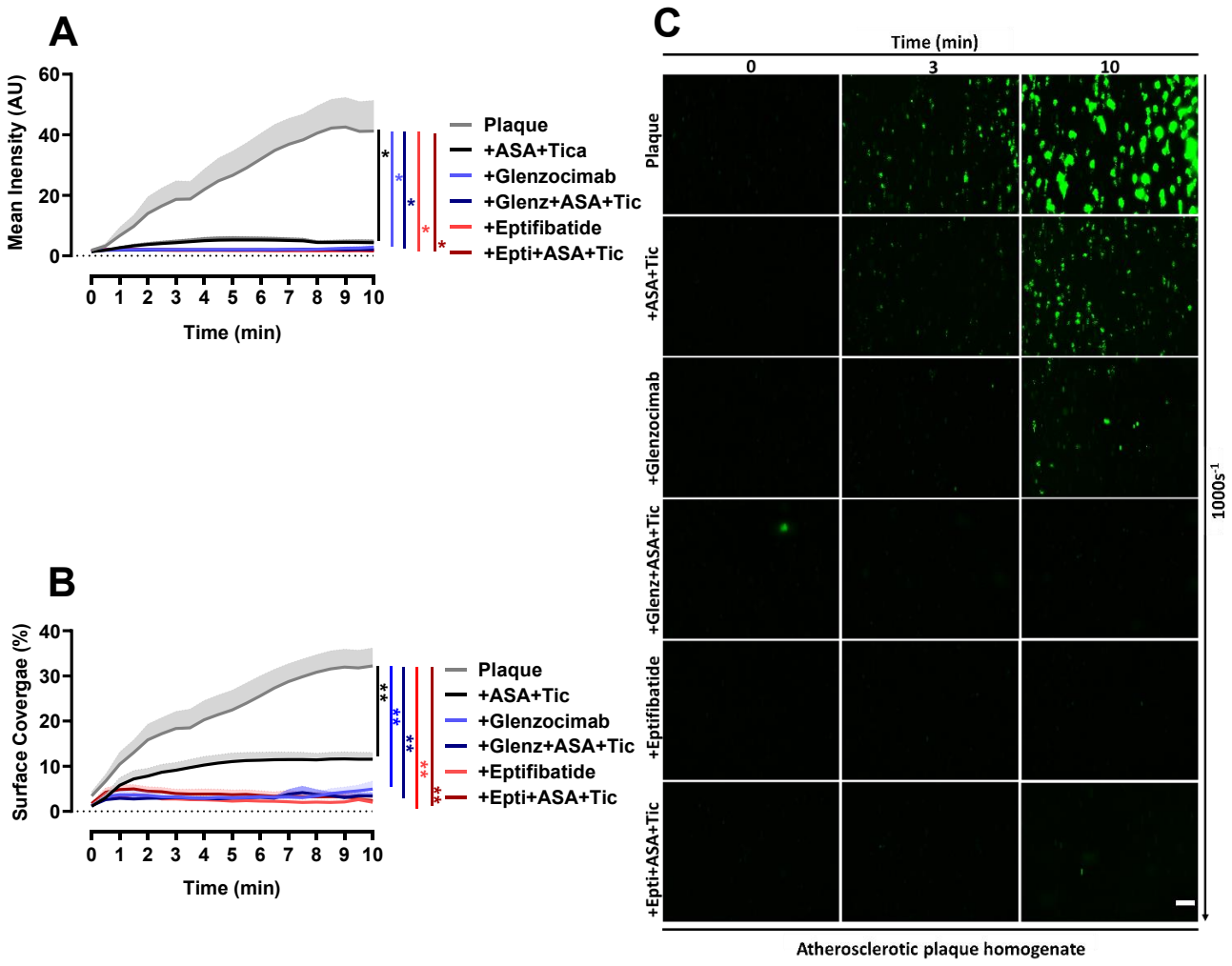


Figure 4.8. Effect of glenzocimab and other antiplatelet agents on platelet adhesion and thrombus formation on atherosclerotic plaque homogenate under an arterial shear rate. **A)** Glenzocimab potently inhibited platelet thrombus formation as reflected by fluorescence intensity. **B)** Glenzocimab inhibited platelet adhesion as reflected by platelet surface coverage %. **C)** Representative images from a single flow adhesion experiment, where $DiOC_6$-labelled citrated blood was incubated with 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide for 15 min at 37°C then flowed over 1mg/ml atherosclerotic plaque homogenate at a shear rate of 1000 s^{-1} for 10 min. Images were taken every 30 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by ImageJ software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of antiplatelet agents was compared to untreated (DMSO control) samples and assessed using a mixed effects model with Dunnett correction for multiple comparisons (n=6, *P<0.05 and **P<0.01).

4.3.4.2 Glenzocimab maintained its ability to abrogate platelet adhesion and thrombus formation on atherosclerotic plaque homogenate under a lower shear rate in a microfluidic chamber

The same combinations and concentrations of the inhibitors were further investigated on platelet thrombus formation and platelet adhesion on 1mg/ml atherosclerotic plaque homogenate but at a lower shear rate (300 s^{-1}). The reason to study the effect of the inhibitors at shear rate of 300 s^{-1} to see if the investigated inhibitors including glenzocimab can maintain their antiplatelet effect seen with shear rate of 1000 s^{-1} since flowing blood at a low shear rate will allow more interaction between platelets and atherosclerotic plaque homogenate thereby increase platelet disposition and adhesion. Also, flushing the chamber with PBS at a shear rate of 1000s^{-1} removes some atherosclerotic plaque coatings as noticed in the previous experiment which might reduce platelet adhesion and thrombus formation during blood flow. As expected, both platelet accumulation and platelet adhesion were higher at a shear rate of 300 s^{-1} than at 1000 s^{-1}; nevertheless, glenzocimab and eptifibatide retained their efficacy in reducing both platelet thrombus growth and platelet adhesion in blood flowing on atherosclerotic plaque homogenate at a lower shear rate. In contrast, aspirin and ticagrelor had no effect on platelet adhesion at a lower shear rate, but they still showing a non-significant trend of reducing platelet thrombus formation (Figure 4.9).

As depicted in Figure 4.9A, there was a non-significant trend for the combined use of aspirin and ticagrelor of reducing thrombus formation by 84% (10 ± 3 AU compared to 65 ± 9 AU in the control; P=0.09). Glenzocimab showed a non-significant trend of decreasing thrombus formation by 96% (2 ± 1 AU; P=0.06). Combining aspirin and ticagrelor with glenzocimab did not cause further inhibition (2 ± 1 AU; P=0.06). Eptifibatide showed a non-significant trend of

reducing thrombus formation by 96% (2 ± 1 AU; P=0.05), without additional effect in the presence of aspirin and ticagrelor (1 ± 1%; P=0.05).

As shown in Figure 4.9B, there was no significant effect for the combined use of aspirin and ticagrelor on the surface coverage (41 ± 8% compared to 66 ± 2% in the control; P=0.30). Glenzocimab reduced the surface coverage by 87% (8 ± 4; P=0.01) when used alone and by 92% (5 ± 3%; P=0.009) in combination with aspirin and ticagrelor. Eptifibatide, however, decreased platelet adhesion by 91% (5 ± 1%; P=0.01). The addition of aspirin and ticagrelor to eptifibatide further reduced platelet adhesion by 95% (3 ± 1%; P=0.006).

In summary, glenzocimab maintained its ability in blocking thrombus formation and platelet adhesion at a lower shear rate while the combined use of aspirin and ticagrelor reduced thrombus formation but not platelet adhesion at the same shear rate.

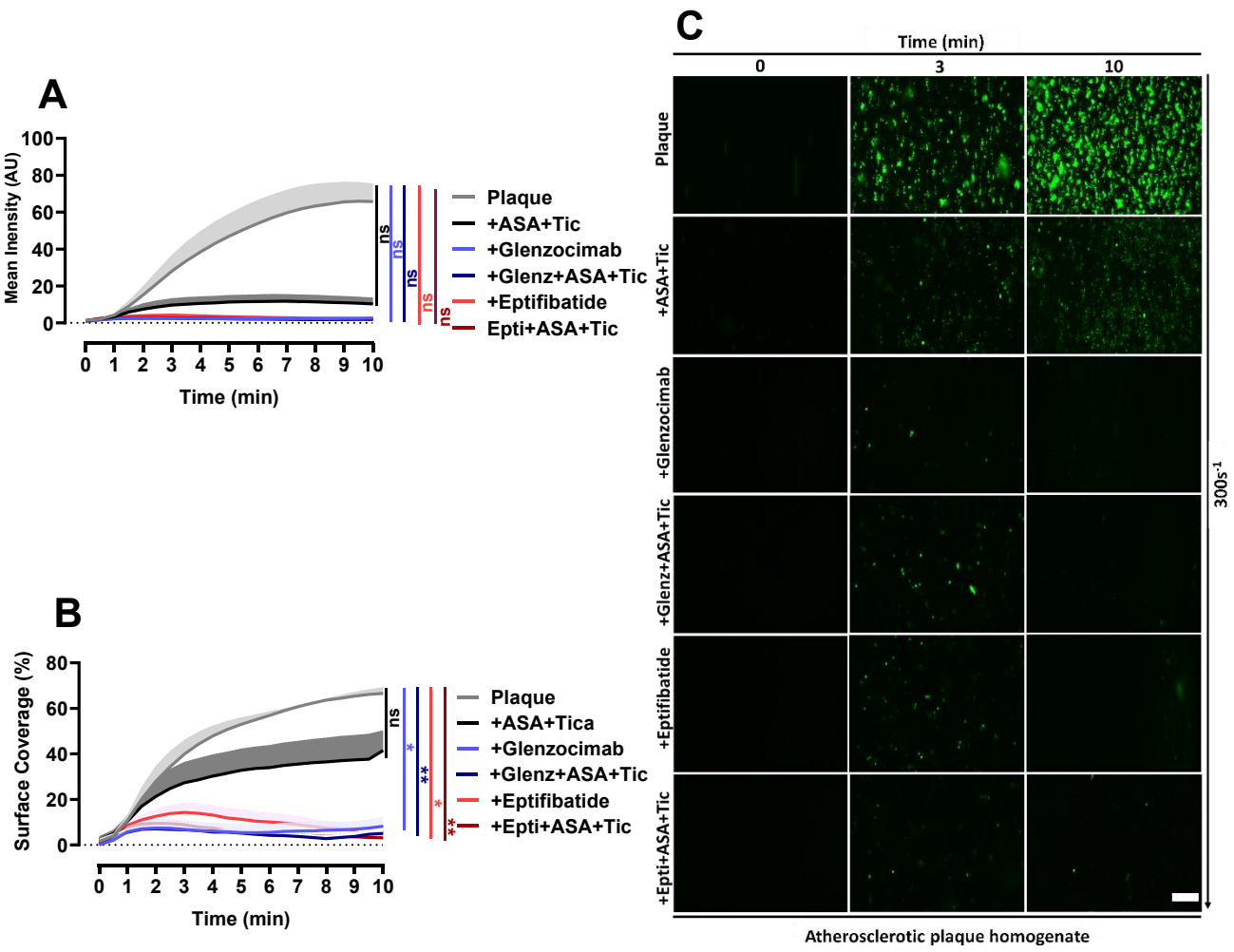


Figure 4.9. Effect of glenzocimab and other antiplatelet agents on platelet adhesion and thrombus formation on atherosclerotic plaque homogenate under low shear rate.

A) Glenzocimab showed a non-significant trend of blocking platelet thrombus formation as reflected by fluorescence intensity. **B)** Glenzocimab inhibited platelet adhesion as reflected by platelet surface coverage %. **C)** Representative images from a single flow adhesion experiment, where $DiOC_6$-labelled citrated blood was incubated with 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide for 15 min at 37°C then flowed over 1mg/ml atherosclerotic plaque homogenate at a shear rate of 300 s^{-1} for 10 min. Images were taken every 30 seconds during flow using EVOS FL Auto 2 Imaging System. Images were analysed by ImageJ software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of antiplatelet agents was compared to untreated (DMSO control) samples and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=3, ns=non-significant, *P<0.05, and**P<0.01).

4.3.5 Glenzocimab partially decreased the number of adherent platelets on immobilised atherosclerotic plaque homogenate under static conditions with the addition of aspirin and ticagrelor

As glenzocimab potently reduced both platelet thrombus formation and platelet adhesion on atherosclerotic plaque homogenate under flow conditions in whole blood, the effect of glenzocimab with/without other antiplatelet agents on platelet spreading and adhesion under a static condition in washed platelets was further characterised.

The results revealed that glenzocimab did not significantly reduce platelet spreading and adhesion on the immobilised atherosclerotic plaque homogenate (Figure 4.10). However, glenzocimab showed an additional effect on platelet perimeter when combined with 6F1 and on the number of adherent platelets when combined with aspirin and ticagrelor or 6F1. Eptifibatide alone decreased platelet surface area and perimeter, but it failed to reduce platelet adhesion; the presence of aspirin and ticagrelor was required to reduce the number of adherent platelets. Dasatinib exerted the most significant inhibition on platelet spreading among all used platelet inhibitors.

As shown in Figure 4.10A, aspirin and ticagrelor did not reduce the platelet surface area (42 ± 2 μm^2 compared to 44 ± 1 μm^2 in the control; P>0.99). Glenzocimab had no significant effect on the surface area (34 ± 5 μm^2; P= 0.43) even when used with aspirin and ticagrelor (36 ± 2 μm^2; P=0.12) or used with 6F1 (31 ± 1 μm^2; P=0.06). Eptifibatide significantly reduced the surface area by 42% (26 ± 2 μm^2; P=0.007) when used alone and by 44% (25 ± 1 μm^2; P=0.002) when combined with aspirin and ticagrelor. Dasatinib markedly decreased the surface area by 65% (15 ± 2 μm^2; P=0.003).

Aspirin and ticagrelor also showed no effect on the platelet perimeter (30 ± 1 μm in comparison to 31 ± 1 μm in the control; P>0.99). Glenzocimab alone did not affect the perimeter (26 ± 2 μm; P=0.43), and the combination of aspirin and ticagrelor with glenzocimab did not cause additional reduction in the perimeter (28 ± 1 μm; P=0.50). However, combining 6F1 with glenzocimab further decreased the perimeter by 22% (24 ± 1 μm; P=0.02). Eptifibatide showed a non-significant trend of reducing the perimeter by 25% (23 ± 1 μm; P=0.06) and provided no additional effect when it was combined with aspirin and ticagrelor (26 ± 1 μm; P=0.05). There was a non-significant trend for dasatinib of reducing the perimeter by 37% (19 ± 2 μm; P=0.05).

The combined use of aspirin and ticagrelor had no significant effect on platelet adhesion (101 ± 8 platelets compared to 142 ± 2 platelets in the control samples; P=0.06; Figure 4.10C). Glenzocimab alone did not significantly reduce platelet adhesion (80 ± 14 platelets; P=0.06). Glenzocimab significantly reduced platelet adhesion by 58% (59 ± 10 platelets; P=0.04) when combined with aspirin and ticagrelor and by 65% (50 ± 6 platelets; P=0.04) when combined with 6F1. Remarkably, in contrast to its effect on surface area and perimeter, eptifibatide had no significant effect on platelet adhesion (126 ± 5 platelets; P=0.15). Nevertheless, the addition of aspirin and ticagrelor to eptifibatide intensified its effect on platelet adhesion by 44% (79 ± 6 platelets; P=0.007). Dasatinib potently reduced platelet adhesion by 85% (21 ± 3 platelets; P=0.002).

In summary, these results indicated that glenzocimab alone did not significantly reduce platelet spreading and static adhesion on atherosclerotic plaque coated surfaces; however, the addition of aspirin and ticagrelor with glenzocimab led to a significant decrease in platelet adhesion. In contrast, eptifibatide significantly reduced platelet spreading, but it needed the addition of aspirin and ticagrelor to cause a significant decrease in platelet adhesion.

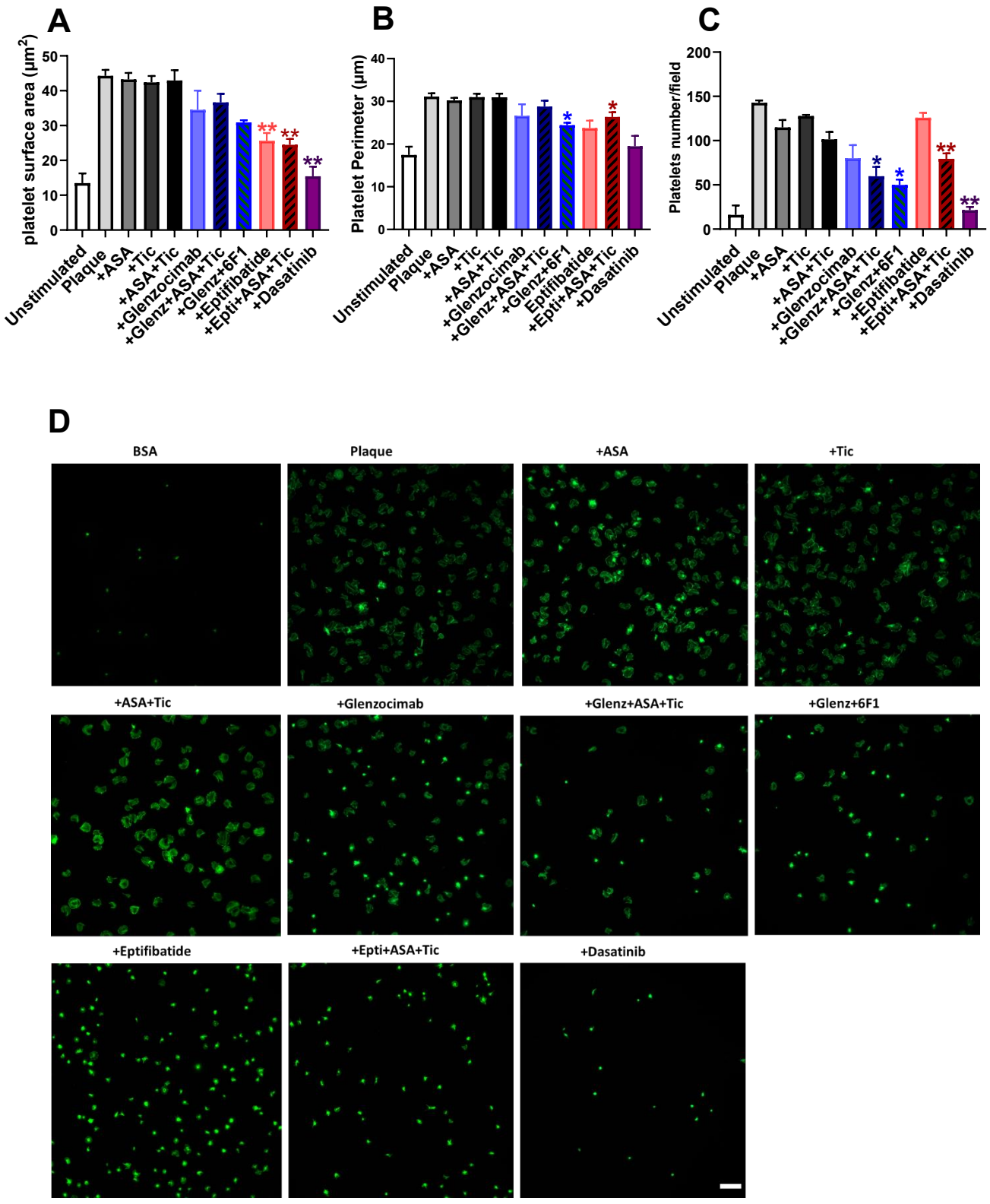

A
platelet surface area (μm²)
0
10
20
30
40
50
Unstimulated
Plaque
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Glenz+6F1
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
B
Platelet Perimeter (μm)
0
10
20
30
40
C
Platelets number/field
0
50
100
150
200
D
BSA
Plaque
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Glenz+6F1
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib

Figure 4.10. Effect of glenzocimab and other antiplatelet agents on platelet spreading on atherosclerotic plaque homogenate under static conditions.

A) Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet surface area. **B)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet perimeter. **C)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the number of adherent platelets. **D)** Representative images, where human WP (2.0×10^7 mL) was preincubated with 0.01% DMSO, glenzocimab (50 µg/ml) or eptifibatide (9 µM) ± aspirin (30 µM) and ticagrelor (1 µM), dasatinib (10 µM) or glenzocimab (50 µg/ml) + 6F1 (10 µg/ml) for 30 min at 37ºC. Then, pre-treated platelets were allowed to spread on immobilized atherosclerotic plaque homogenate (70 µg/ml) on coverslips for 30 min at 37ºC. Platelets fixed with 4% neutralized formalin buffer for 10 min. Platelets permeabilized with 0.1% Triton X-100 in PBS for 5 min and stained with Alex-488-phalloidin for 45 min in the dark. The coverslips were then mounted on glass slides and imaged under the Zeiss Axiovert 200M microscope. 5 random images were analysed for each experiment. Scale bar 20 µm. Effect of treatment group was compared to control and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=4, * $P<0.05$ and ** $P<0.01$).

4.3.6 Glenzocimab significantly reduced phosphorylation of main tyrosine kinases in the GPVI signalling pathway upon stimulation with atherosclerotic plaque homogenate, and its effect was amplified in the presence of aspirin and ticagrelor

GPVI blockade with glenzocimab inhibited platelet aggregation and ATP secretion stimulated by atherosclerotic plaque homogenate and platelet thrombus formation and platelet adhesion on atherosclerotic plaque under flow, whereas conventional antiplatelet therapy agents exerted the inhibitory effect to a lesser extent. To understand this selective inhibition of atherosclerotic plaque-mediated platelet responses by glenzocimab, the molecular-level effect of glenzocimab and other antiplatelet agents was investigated on the main tyrosine kinases in GPVI signalling pathway (Syk, LAT, and PLCγ2) following platelet stimulation with atherosclerotic plaque homogenate.

The western blotting results show that atherosclerotic plaque strongly induced tyrosine phosphorylation of Syk, LAT, and PLCγ2, which was potently blocked by glenzocimab, leaving a minimal residual tyrosine phosphorylation, which was further inhibited by combining glenzocimab with aspirin and ticagrelor. In contrast, elements of DAPT (aspirin and ticagrelor) showed no effect on tyrosine phosphorylation when used alone or in combination, except on PLCγ2 phosphorylation, which was partially reduced by the combination of aspirin and ticagrelor. Blockade of the other collagen receptor, GPIa/IIa, with 6F1 did not inhibit tyrosine phosphorylation, suggesting that atherosclerotic plaque activates platelets mainly via GPVI. GPIIb/IIIa inhibition with eptifibatide did not block tyrosine phosphorylation as platelets were pre-treated with 9 μM eptifibatide to prevent platelet aggregation and to ensure that signalling was only induced by the ligand by preventing platelet–platelet interaction and its subsequent signalling.

Regarding Syk phosphorylation as highlighted in Figure 4.11, the combined use of aspirin and ticagrelor did not inhibit its phosphorylation (64 ± 13% of control; P=0.24). Platelet pre-treatment with 6F1 had no significant effect on Syk phosphorylation (61 ± 13% of control; P=0.19). Glenzocimab alone significantly reduced Syk phosphorylation (14 ± 7% of control; P=0.005). More importantly, glenzocimab completely inhibited Syk phosphorylation in the presence of aspirin and ticagrelor (0 ± 0% of control; P<0.001) and nearly inhibited it in the presence of 6F1 (1 ± 1% of control; P<0.001).

As presented in Figure 4.12, the combined use of aspirin and ticagrelor did not reduce LAT phosphorylation (95 ± 10% of control; P>0.99). GPIa/IIa antagonism with 6F1 did not affect LAT phosphorylation (96 ± 7% of control; P>0.99). Glenzocimab greatly reduced LAT phosphorylation (21 ± 4% of control; P=0.001). Furthermore, the addition of aspirin and ticagrelor on top of glenzocimab maximised its inhibitory effect on LAT phosphorylation (5 ± 1% of control; P<0.001). Moreover, 6F1 strengthened the inhibition of LAT phosphorylation by glenzocimab (9 ± 5% of control; P=0.001).

As shown in Figure 4.13, the dual use of aspirin and ticagrelor partially reduced PLCγ2 phosphorylation (43 ± 8% of control; P=0.03). 6F1 did not inhibit PLCγ2 phosphorylation (83 ± 9% of control; P=0.47), whereas glenzocimab greatly reduced it (7 ± 4% of control; P<0.001). Combining aspirin and ticagrelor with glenzocimab almost completely inhibited PLCγ2 phosphorylation (1 ± 1% of control; P<0.001). The dual combination of glenzocimab and 6F1 further inhibited PLCγ2 phosphorylation (4 ± 2% of control; P<0.001). In summary, these results indicated that atherosclerotic plaque homogenate stimulated platelets via GPVI pathway as it strongly phosphorylated Syk, LAT, and PLCγ2. Furthermore, glenzocimab alone significantly blocked tyrosine phosphorylation of these proteins and caused a near complete inhibition in the presence of aspirin and ticagrelor.

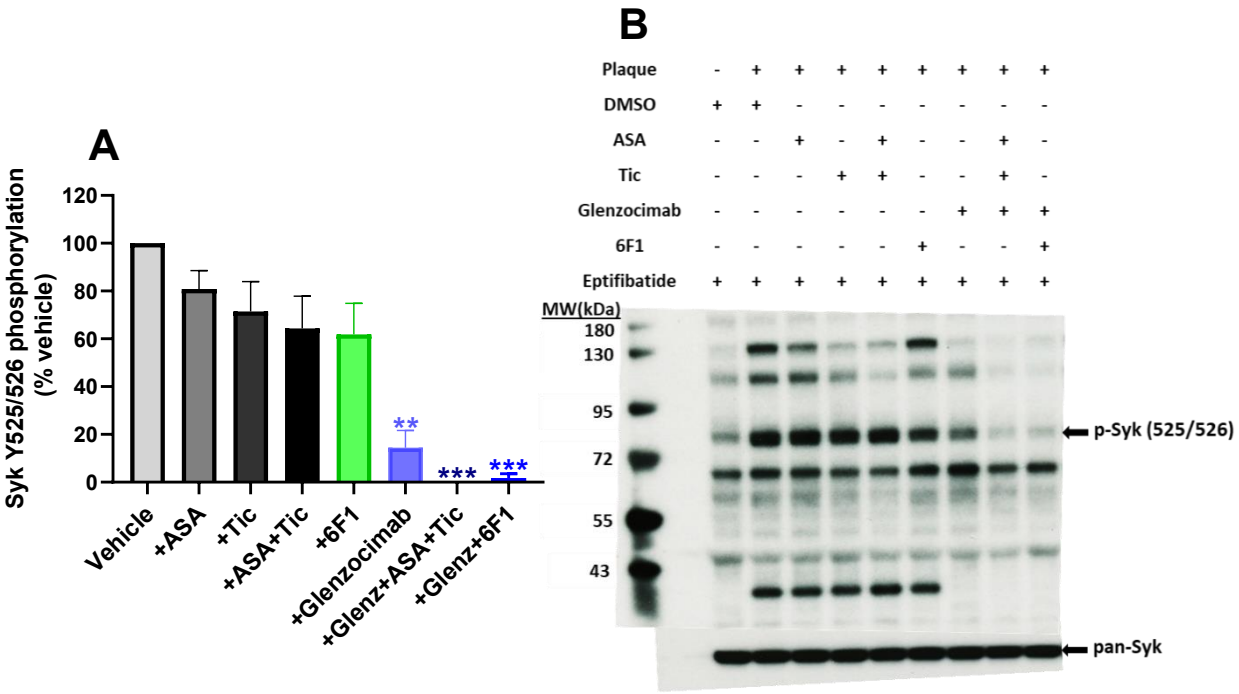


Figure 4.11. Effect of glenzocimab with/without other antiplatelet agents on Syk phosphorylation induced by atherosclerotic plaque homogenate.

A) Effect of glenzocimab alone and combined with aspirin and ticagrelor on Syk phosphorylation stimulated by atherosclerotic plaque homogenate. **B)** A representative phosphorylation blot where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with (50 µg/ml) glenzocimab ± aspirin (30 µM), ticagrelor (1 µM), and 6F1 (10 µg/ml) for 10 min and platelet activation was stimulated by 70 µg/ml atherosclerotic plaque homogenate for 180 seconds under stirring conditions. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated syk (Y525/526) with anti-phospho-Syk (Y525/526) ≈ 72 kDa. The effect of antiplatelet agents was compared to untreated (DMSO control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=4, **P<0.01, ***P<0.001).

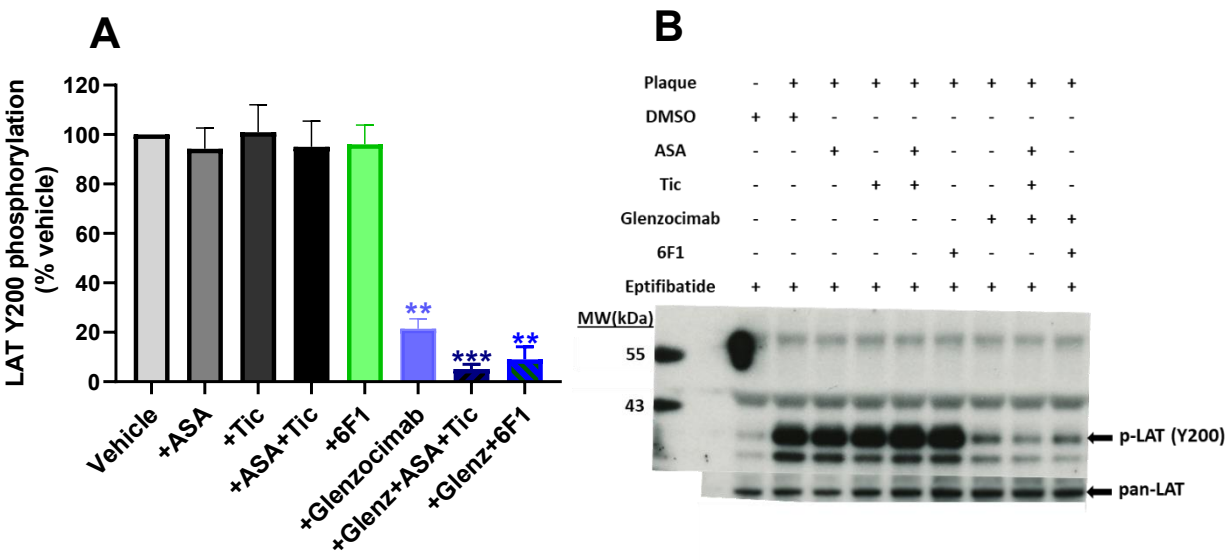


Figure 4.12. Effect of glenzocimab with/without other antiplatelet agents on LAT phosphorylation induced by atherosclerotic plaque homogenate.

A) Effect of glenzocimab alone or combined with aspirin and ticagrelor on LAT phosphorylation stimulated by atherosclerotic plaque homogenate. **B)** A representative phosphorylation blot, where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with glenzocimab (50 µg/ml) ± aspirin (30 µM), ticagrelor (1 µM), and 10 µg/ml 6F1 for 10 min and platelet activation stimulated by 70 µg/ml atherosclerotic plaque homogenate for 180 seconds. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated LAT (Y200) with anti-phospho-LAT (Y200) ≈ 37 kDa. The effect of antiplatelet agents was compared to untreated (DMSO control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=4, **P<0.01, ***P<0.001).

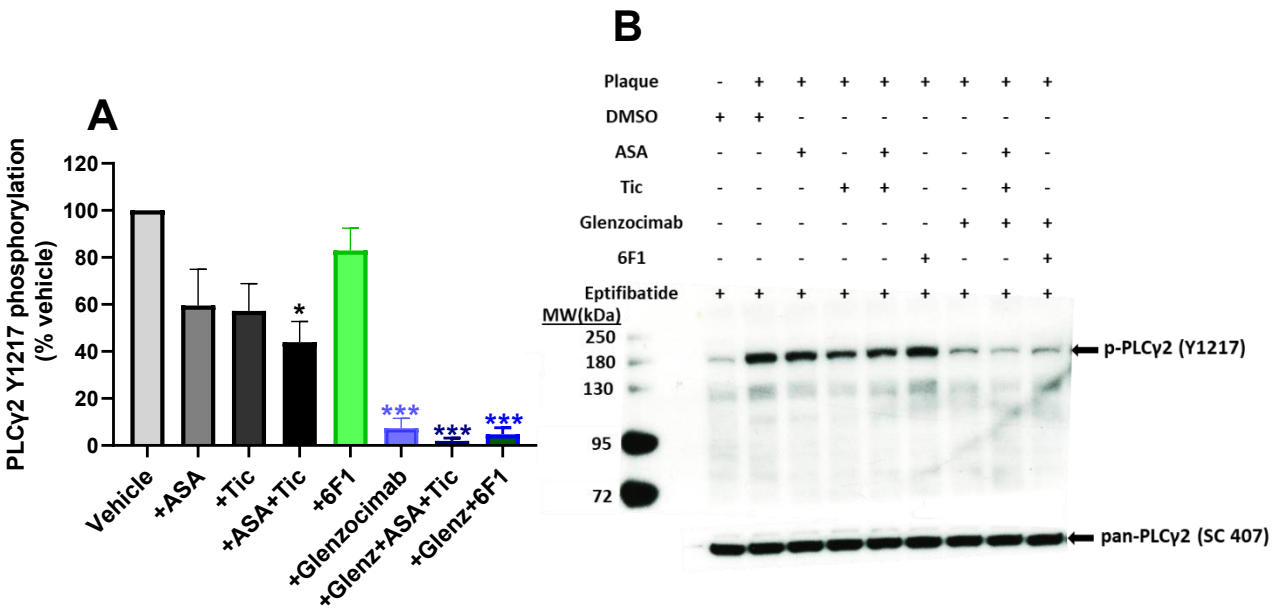


Figure 4.13. Effect of glenzocimab with/without other antiplatelet agents on PLCγ2 phosphorylation induced by atherosclerotic plaque homogenate.

A) Effect of glenzocimab alone and combined with aspirin and ticagrelor on PLCγ2 phosphorylation stimulated by atherosclerotic plaque homogenate. **B)** A representative phosphorylation blot, where washed human platelets were pre-treated with eptifibatide (9 µM) and incubated with glenzocimab (50 µg/ml) ± aspirin (30 µM), ticagrelor (1 µM), and 6F1 (10 µg/ml) for 10 min and platelet activation stimulated by 70 µg/ml atherosclerotic plaque homogenate for 180 seconds. Then, platelet activation was stopped by adding lysis buffer and boiling for 5 min. Whole cell lysates were separated by gel electrophoresis (SDS-PAGE), transferred into PVDF membrane, and western blotted for phosphorylated PLCγ2 (Y1217) with anti-phospho-PLCγ2 (Y1217) ≈ 140 kDa. The effect of antiplatelet agents was compared to untreated (DMSO control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=4, *P<0.05, and ***P<0.001).

4.3.7 Dimeric and monomeric GPVI bound to atherosclerotic plaque, and their binding was prevented by GPVI blockade with glenzocimab

In atherosclerotic plaque-induced platelet aggregation, glenzocimab fully inhibited platelet aggregation, which suggests that atherosclerotic plaque homogenate activates platelets mainly via GPVI. To further analyse this mechanism, enzyme-linked immunosorbent assay (ELISA) was utilised to investigate the binding of human dimeric GPVI (GPVI-Fc) and monomeric GPVI (GPVI-ex) to adsorbed atherosclerotic plaque homogenate by incubating increasing concentrations (0.1, 0.5, 1, 2.5, 5, and 10 μg/ml) of both dimeric and monomeric GPVI constructs. The direct ELISA method was used first, and it showed that monomeric GPVI binds to atherosclerotic plaque with high affinity in a dose-dependent manner. However, the direct ELISA did not work for dimeric GPVI as the anti-IgG-Fc antibody that was used to detect GPVI-Fc showed non-specific binding, probably because it bound to other proteins in the atherosclerotic plaque that contain the Fc domain, thus generating random OD values. Therefore, indirect ELISA was used to assess the binding of GPVI-Fc by using an anti-GPVI nanobody tagged with histidine as a primary antibody, followed by anti-His6 antibody as a secondary antibody used to detect GPVI-ex, which produced clear results without showing a non-specific binding pattern. The indirect ELISA revealed that GPVI-Fc dose-dependently binds to atherosclerotic plaque homogenate, but it is difficult to identify which GPVI form has more affinity to atherosclerotic plaque because the method of detection was not the same for the two GPVI constructs; the indirect ELISA involved using two antibodies for detection with more washing steps, which may have removed some bound GPVI-Fc.

After establishing the dose-binding curve of GPVI to atherosclerotic plaque, the inhibitory effect of glenzocimab on GPVI binding to atherosclerotic plaque was investigated by incubating 2.5 μg/ml of GPV-Fc and GPVI-ex with increasing concentrations of glenzocimab (1–50

μg/ml) before adding them to adsorbed atherosclerotic plaque. Glenzocimab dose-dependently inhibited the binding of both GPVI forms to atherosclerotic plaque, potently inducing the blockade of GPVI binding at 10 μg/ml.

In summary, these results indicated that both dimeric and monomeric forms of GPVI bind into atherosclerotic plaque homogenate coated surfaces with a great affinity. More importantly, glenzocimab inhibited their binding into atherosclerotic plaque homogenate.

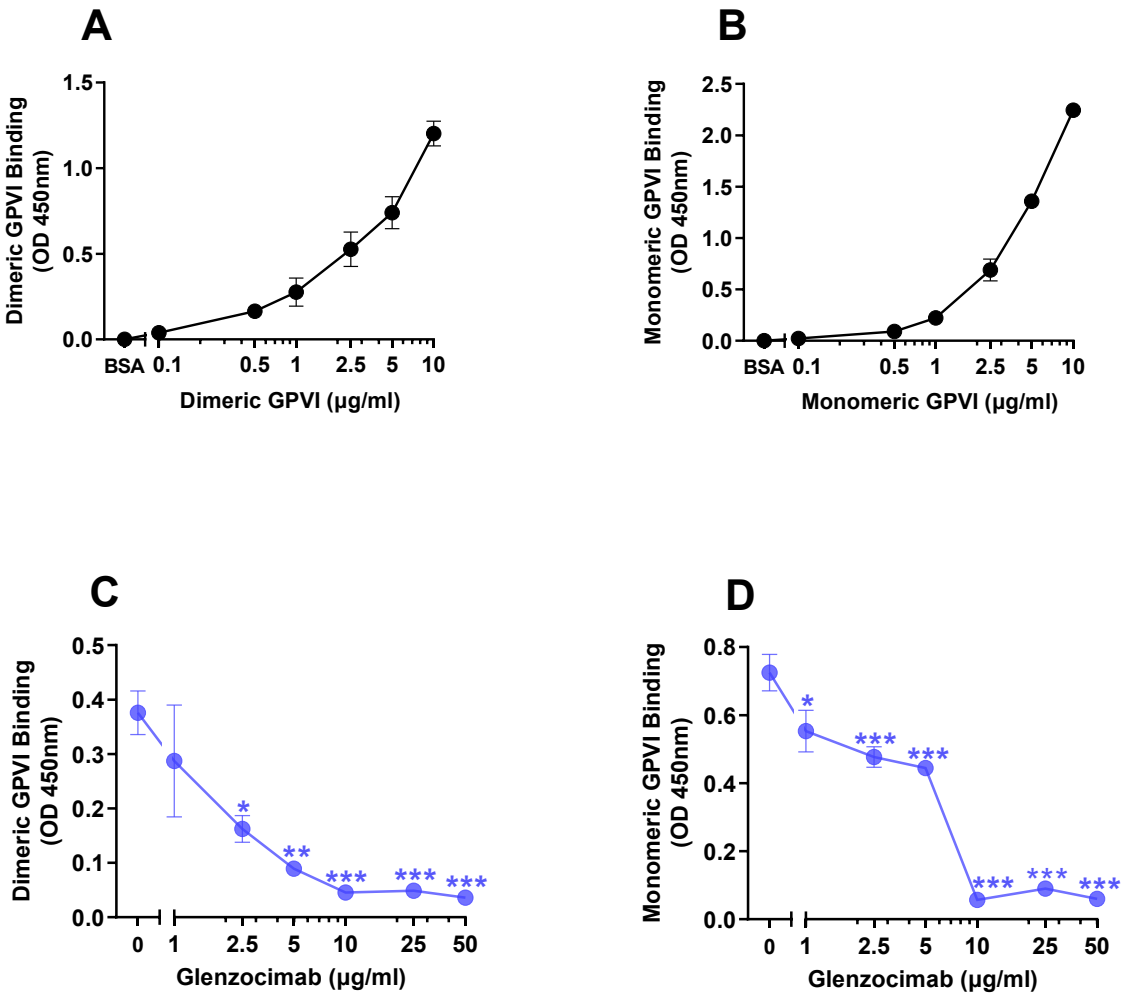


Figure 4.14. Dimeric and monomeric GPVI bind to atherosclerotic plaque homogenate and their binding is blocked by glenzocimab in dose-dependent manner.

A) Dose-response curve for binding of monomeric GPVI into atherosclerotic plaque homogenate. **B)** Dose-response curve for binding of dimeric GPVI into atherosclerotic plaque homogenate. **C)** Dose-dependent blockade of monomeric GPVI (2.5 µg/ml) binding into atherosclerotic plaque by glenzocimab. **D)** Dose-dependent inhibition of the dimeric GPVI (2.5 µg/ml) binding into atherosclerotic plaque by glenzocimab. Data analysed using one-way ANOVA with Dunnett correction for multiple comparisons (n=4, *P<0.01, **P<0.01, ***P<0.001).

4.3.8 Atherosclerotic plaque homogenate induced tyrosine signalling in via Src-kinases

After showing that glenzocimab largely inhibited atherosclerotic plaque-mediated tyrosine phosphorylation by inhibition of Syk, LAT, and PLCγ2, the molecular investigation was further extended to Src phosphorylation using specific Src inhibitors, dasatinib and PP2, to examine whether atherosclerotic plaque stimulates tyrosine phosphorylation through Src.

The results showed that atherosclerotic plaque homogenate activated platelets in the Src-dependent pathway, as shown in Figure 4.15. Treatment with dasatinib completely abolished phosphorylation of Src, Syk, LAT, PLCγ2, and other proteins. Dasatinib blocked phosphorylation of Src (2 ± 1% of control; P<0.001), Syk (4 ± 3% of control; P<0.001), LAT (6 ± 2% of control; P<0.001), and PLCγ2 (2 ± 1% of control; P<0.001). Platelet treatment with PP2 also inhibited phosphorylation of Src, Syk, LAT, and PLCγ2, but the inhibitory effect on LAT phosphorylation was lower than that of dasatinib. PP2 blocked phosphorylation of Src (46 ± 5% of control; P=0.02), Syk (6 ± 3% of control; P<0.001), LAT (24 ± 8% of control; P=0.02), and PLCγ2 (2 ± 1% of control; P<0.001). Atherosclerotic plaque might activate platelets in other pathways other than Src kinases; therefore, the effect of atherosclerotic plaque on the main proteins involved in the upstream signalling pathways in other platelet receptors should be explored.

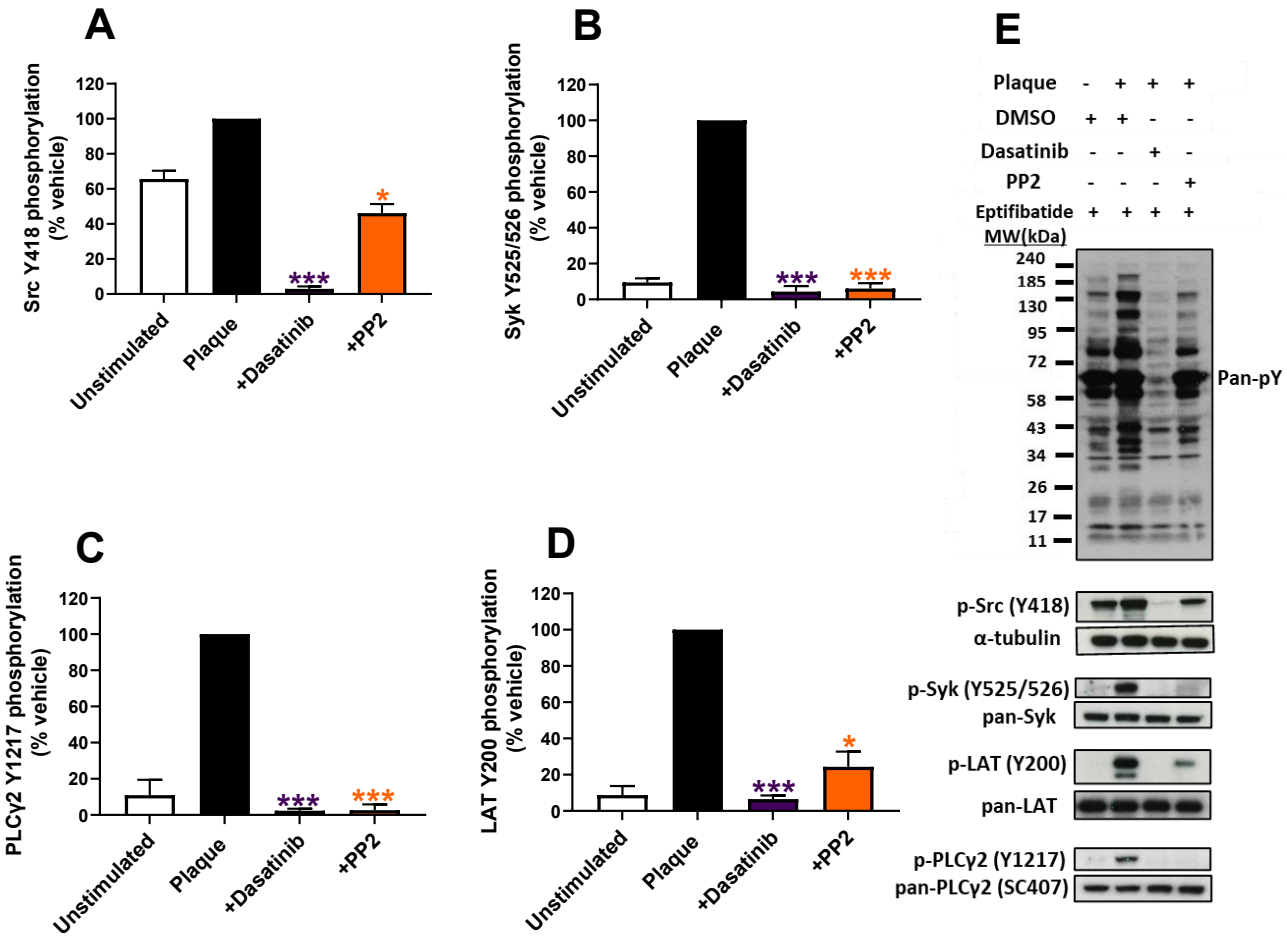


Figure 4.15. Atherosclerotic plaque induces tyrosine phosphorylation via Src kinases, but other kinases and signalling proteins might be involved in atherosclerotic plaque induced tyrosine phosphorylation.

A) Complete Inhibition of Src (Y418) phosphorylation upon stimulation with 70 µg/ml atherosclerotic plaque by Dasatinib and PP2. **B)** Dasatinib and PP2 completely blocked Syk (Y525/526) phosphorylation by 70 µg/ml atherosclerotic plaque. **C)** Complete blockade of atherosclerotic plaque-induced LAT (Y200) phosphorylation was achieved by dasatinib, whilst PP2 partially inhibited it. **D)** Complete inhibition of atherosclerotic plaque stimulated PLCγ2 (Y1217) phosphorylation by Dasatinib 10 µM and PP2 10 µM. **E)** Representative blot showing atherosclerotic plaque activates platelets via Src kinases. The effect of treatment with Dasatinib and PP2 was compared to the uninhibited sample and assessed using one-way ANOVA followed by Dunnett correction for multiple comparisons (n=3, *P<0.05 and ***P<0.001).

4.3.9 Atherosclerotic plaque homogenate induced sustained signalling of the main tyrosine kinases and adapters involved in the GPVI signalling pathway

To examine whether atherosclerotic plaque homogenate induces sustained signalling of the main tyrosine kinases such as Src, Syk, LAT, and PLCγ2 that mediate the GPVI signalling pathway, washed platelets were stimulated with 70 μg/ml atherosclerotic plaque homogenate for different time points (0, 1, 3, 10, 20, 30, and 60 min) under stirring conditions at 37°C in the presence of eptifibatide (9 μM) to prevent platelet–platelet activation through GPIIb/IIIa and to ensure that platelet activation is induced only via plaque.

As shown in Figure 4.16, platelets stimulated with atherosclerotic plaque triggered as sustained tyrosine signalling of Src, Syk, LAT, and PLCγ2, and other proteins as indicated by pan-tyrosine phosphorylation. In all studied effectors, Src (Figure 4.16 A), Syk (Figure 4.16 B), LAT (Figure 4.16 C), PLCγ2 (Figure 4.16 D), and other tyrosine-containing proteins (Figure 4.16 E), signalling was statistically significant for Src (P=0.01) and PLCγ2 (P=0.003) at 3 min following plaque-induced activation of platelets and sustained over the observation period (1–60 min), implying sustained activation of the GPVI pathway. Sustained Signalling was statistically significant for LAT (P=0.05) at 20 min and for Syk (P=0.01) at 30 min.

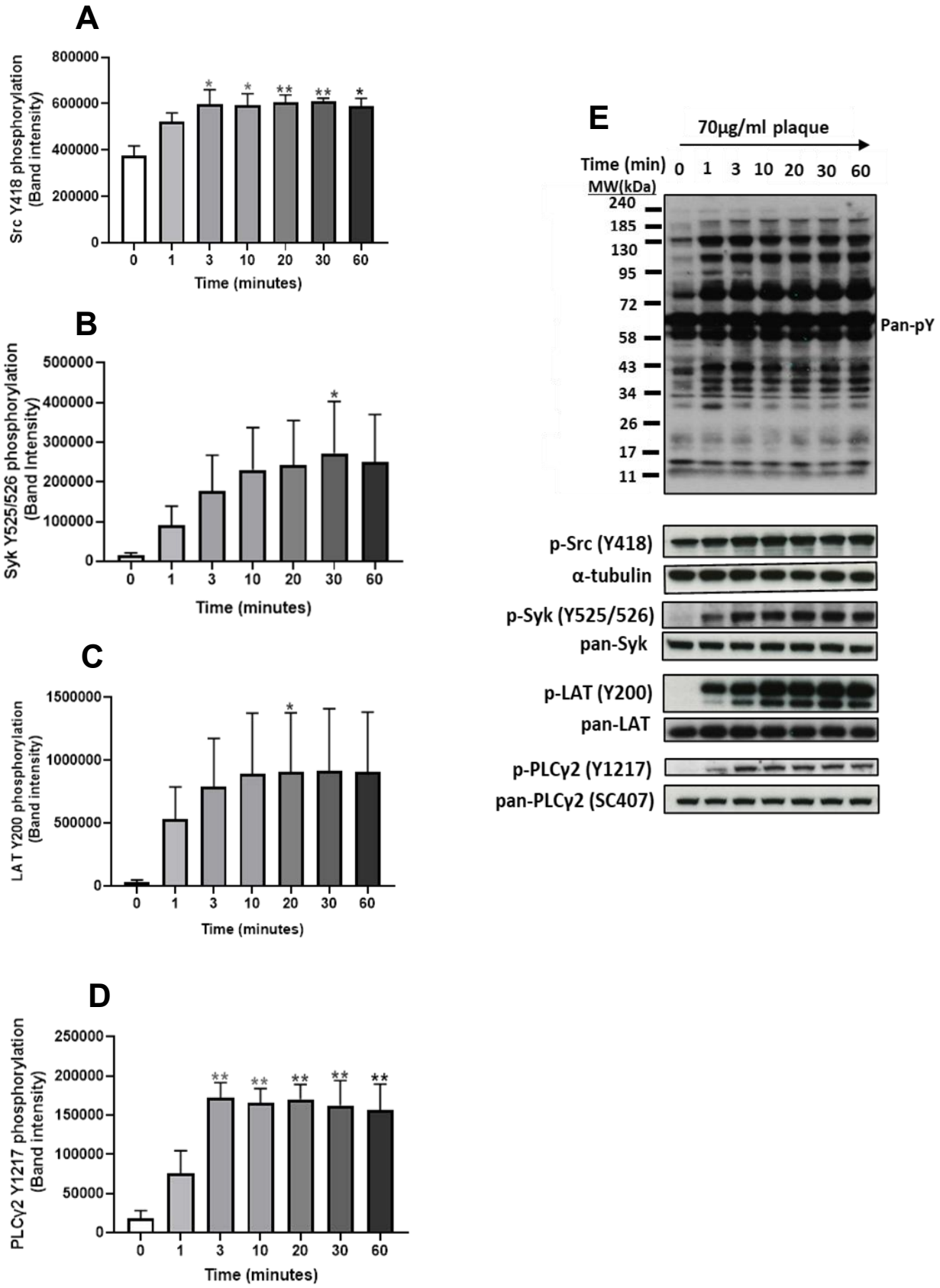

A
Src Y418 phosphorylation (Band intensity)
800000
600000
400000
200000
0
0 1 3 10 20 30 60
Time (minutes)
B
Syk Y525/526 phosphorylation (Band Intensity)
500000
400000
300000
200000
100000
0
0 1 3 10 20 30 60
Time (minutes)
C
LAT Y200 phosphorylation (Band intensity)
1500000
1000000
500000
0
0 1 3 10 20 30 60
Time (minutes)
D
PLCγ2 Y1217 phosphorylation (Band intensity)
250000
200000
150000
100000
50000
0
0 1 3 10 20 30 60
Time (minutes)
E
70μg/ml plaque
Time (min) 0 1 3 10 20 30 60
MW(kDa)
240
185
130
95
72
58
43
34
26
17
11
Pan-pY
p-Src (Y418)
α-tubulin
p-Syk (Y525/526)
pan-Syk
p-LAT (Y200)
pan-LAT
p-PLCγ2 (Y1217)
pan-PLCγ2 (SC407)

Figure 4.16. Atherosclerotic plaque induced sustained tyrosine signalling of the main tyrosine kinases and adapters involved in the GPVI pathway.

A) Sustained signalling of Src (Y418) following stimulation of washed platelets with 70 µg/ml plaque for 1 hour. **B)** Sustained signalling of Syk (Y525/526) following washed platelet stimulation with 70 µg/ml plaque for 1 hour. **C)** Sustained signalling of LAT (Y200) following WP stimulation with 70 µg/ml plaque for 1 hour. **D)** Sustained signalling of PLCY2 (Y1217) following platelet activation with 70 µg/ml plaque up to 1 hour. **E)** Representative blots Data analysed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, *P<0.01, and **P<0.01).

4.3.10 Atherosclerotic plaque homogenate induced GPVI shedding

It has been established that platelet activation with GPVI agonists such as collagen and CRP-XL induce GPVI shedding. Moreover, the level of soluble GPVI, which results from GPVI shedding, is elevated in patients with ACS. Therefore, platelets were stimulated with atherosclerotic plaque homogenate to examine whether it induces GPVI shedding because atherosclerotic plaque seems to activate platelets via GPVI. Platelets were incubated with 600 μg/ml atherosclerotic plaque homogenate or calcium ionophore, A23187, which is a potent mediator of shedding, for 2 hours at 37°C. A higher concentration of atherosclerotic plaque was used to induce GPVI shedding because shedding experiments require using a high concentration of the agonist as highlighted in previous studies (Montague et al., 2018).

The results showed that atherosclerotic plaque causes GPVI shedding, as detected by anti-GPVI tail antibody (Figure 4.17). Atherosclerotic plaque induced GPVI ectodomain shedding from platelet membrane (52 ± 3% of the basal sample; $P<0.001$). A23187 potently induced GPVI shedding (8 ± 1% of the basal sample; $P<0.001$).

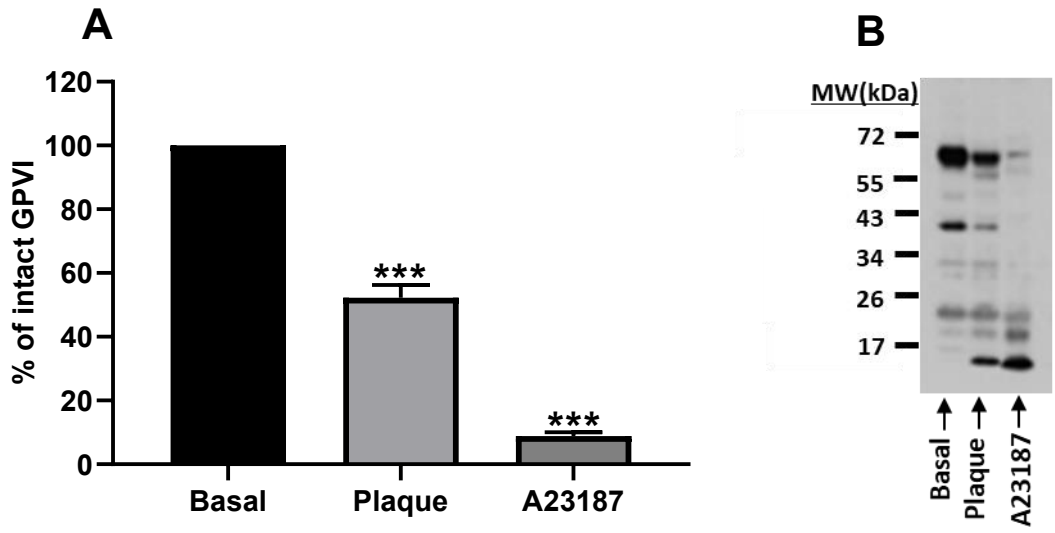


Figure 4.17. Atherosclerotic plaque induced GPVI shedding in suspension.

A) Atherosclerotic plaque induced GPVI shedding following washed platelet stimulation with 600µg/ml atherosclerotic plaque and 10 µM A23187 (a positive control for GPVI shedding) for 2 hours under stirring conditions at 37°C in the presences of 1 mM $CaCl_2$ and 9 µM eptifibatide. **B)** A representative blot for GPVI shedding. The effect of atherosclerotic plaque and A23187 on GPVI shedding was compared to the basal sample and assessed using one-way ANOVA followed by Dunnett correction (n=3, ***P<0.001)

4.4 Discussion

The main findings of this chapter are as follows: (i) glenzocimab potently blocked atherosclerotic plaque-mediated platelet responses, whereas the combined use of aspirin and ticagrelor partially inhibited platelet aggregation and platelet thrombus formation and platelet adhesion under flow; (ii) eptifibatide also potently blocked some atherosclerotic plaque-mediated platelet response and showed superiority over glenzocimab in GPIIb/IIIa-mediated functions such as the immediate inhibition of platelet aggregation and reduction of platelet spreading; (iii) dimeric and monomeric forms of GPVI are involved in atherosclerotic plaque-mediated platelet activation; (iv) atherosclerotic plaque stimulated platelet activation in the GPVI-dependent pathway because tyrosine phosphorylation was largely blocked by glenzocimab without an apparent role of GPIa/IIa; (v) atherosclerotic plaque caused GPVI shedding, which may provide an explanation for the elevated level of soluble GPVI levels in patients with STEMI.

Atherosclerotic plaque potently induced platelet aggregation, which was observed to be completely blocked by glenzocimab in LTA and potently blocked in MEA. Aspirin and ticagrelor were observed to partially inhibit platelet aggregation in LTA and MEA; however, the addition of aspirin and ticagrelor were observed to intensify the effect of low concentrations of glenzocimab in LTA and to provide a near complete inhibition of platelet aggregation upon combination with a high concentration of glenzocimab in MEA. Conversely, eptifibatide were observed to entirely block platelet aggregation in MEA and block it to a lesser extent in LTA. Overall, atherosclerotic plaque-stimulated platelet aggregation showed a large dependency on GPVI as it was blocked by glenzocimab. Penz et al. were the first to show that atherosclerotic plaque-induced aggregation of mouse and human washed platelets occurs mainly via GPVI. Penz et al. further showed that collagenous structures present in atherosclerotic plaque are

responsible for platelet activation, as they demonstrated that incubating plaque with either anti-collagen type II and III antibodies or collagenase prevented platelet aggregation (Penz et al., 2005). Another study by the same authors showed that a high concentration of aspirin (1 mM) along with inhibition of $P2Y_{12}$ and $P2Y_1$ effectively blocked atherosclerotic plaque-induced platelet aggregation in LTA and MEA (Penz et al., 2007). In the present study, dual treatment with aspirin and ticagrelor only partially inhibited platelet aggregation. To clarify, at the chosen concentrations, aspirin (30 μM) and ticagrelor (1 μM) effectively blocked their targets as described in chapter 3, and $P2Y_1$ was not blocked in the present work as it is not targeted by clinically-used medications.

GPIIb/IIIa inhibitors are able to disaggregate platelet-rich thrombi formed in vitro under flow conditions (Speich et al., 2009). Moreover, GPIIb/IIIa inhibitors, including eptifibatide, have been shown to reverse platelet aggregation in response to low ADP stimulation (Moser et al., 2003). Eptifibatide produced an immediate inhibitory effect on atherosclerotic plaque-stimulated platelet aggregation. In contrast, glenzocimab failed to induce an immediate inhibitory effect on atherosclerotic plaque-induced platelet aggregation despite the complete inhibition exerted on platelet aggregation. However, in platelets pre-treated with aspirin and ticagrelor, glenzocimab exerted an immediate inhibition of platelet aggregation as eptifibatide did (Figure 4.5). It is clear from these results that eptifibatide, which blocks the final pathway of platelet aggregation, can exert an immediate inhibition of atherosclerotic plaque-stimulated platelet aggregation independent of the blockade of positive feedback mediators, whereas glenzocimab requires TxA_2 and $P2Y_{12}$ inhibition to deliver an immediate inhibitory effect on platelet aggregation.

Platelet activation leads to platelet secretion, which is a fundamental process for haemostasis, coagulation, and inflammation, where activated platelets release around 300 substances from

granules, which play various roles (Golebiewska and Poole, 2015b). Atherosclerotic plaque caused platelet secretion as assessed by ATP release in LTA as a marker of dense granule secretion and P-selectin expression under flow as a marker of α-granule exocytosis. Glenzocimab potently blocked ATP release and P-selectin expression, which indicates a profound inhibition of platelet secretion. In contrast, eptifibatide strongly inhibited P-selectin expression but showed no effect on ATP release. The potent effect of eptifibatide on P-selectin expression may be attributed to its ability to block platelet adhesion which may omit the real effect on P-selectin expression. Aspirin and ticagrelor partially blocked P-selectin expression and demonstrated no effect on ATP secretion unless they were combined with eptifibatide, where a partial inhibition of ATP release was achieved. Glenzocimab showed superiority over aspirin, ticagrelor, eptifibatide in blocking ATP release and P-selectin expression, and this has a therapeutic potential as platelet exocytosis releases several inflammatory mediators including P-selectin, CD40L, and chemokines that have been linked to atherosclerosis (Weber, 2005). Furthermore, it has been demonstrated that collagen via GPVI activation can generate a secretory platelet phenotype independent of platelet aggregation, which could lead to thrombo-inflammatory responses (Ollivier et al., 2014).

Glenzocimab profoundly blocked thrombus formation and platelet adhesion on atherosclerotic plaque under flow under shear rates of 1000 s^{-1} and 300 s^{-1}. Eptifibatide also blocked platelet thrombus formation under both shear rates. Aspirin and ticagrelor greatly inhibited platelet thrombus formation but partially inhibited platelet adhesion at 1000 s^{-1}; however, the partial inhibition of platelet adhesion achieved by aspirin and ticagrelor was lost in blood flowing at 300 s^{-1}. The complete inhibition of platelet thrombus formation and adhesion by glenzocimab clearly indicates that mainly GPVI mediates platelet adhesion and thrombus formation on atherosclerotic plaque, which differs from the case of platelet thrombus formation and adhesion

on Horm collagen where both GPVI and GPIa/IIa are involved. These results reflect those of Penz et al. who also showed that only GPVI blockade but not GPIa/IIa inhibited platelet thrombus formation on atherosclerotic plaque under flow (Penz et al., 2005).

Furthermore, Jamasbi et al. showed that direct targeting of GPVI by antibodies achieved almost complete inhibition of platelet thrombus formation on atherosclerotic plaque, whereas the competitive inhibition of GPVI by revacept only provided a partial blockade (Jamasbi et al., 2015). The partial inhibition of platelet adhesion on atherosclerotic plaque by aspirin and ticagrelor in the present study is consistent with the findings by Muñoz et al., who reported that aspirin and ticagrelor partially inhibited platelet adhesion. Muñoz et al. also reported that GPIIb/IIIa antagonism with abciximab potently blocked platelet adhesion under flow (Mojica Muñoz et al., 2017).

Glenzocimab alone or with aspirin and ticagrelor or 6F1 did not affect platelet spreading on atherosclerotic plaque. In contrast to its potent effect on platelet adhesion on atherosclerotic plaque under flow, glenzocimab did not significantly platelet adhesion on atherosclerotic plaque under static conditions; however, in combination with aspirin and ticagrelor or 6F1, it exerted additional but incomplete inhibition. Eptifibatide reduced platelet spreading but not adhesion except in the case of triple combination with aspirin and ticagrelor, which partially inhibited platelet adhesion. This incomplete inhibition of platelet adhesion under static conditions obtained with combined blockade of GPVI and GPIa/IIa inconsistent with the findings by Penz et al. suggests that blockade of both collagen receptors abolished platelet adhesion on atherosclerotic plaque. They referred to spreading results without publishing the data and without mentioning details regarding incubation time or inhibitors. However, the effect of eptifibatide on platelet spreading and static adhesion on atherosclerotic plaque in the present study is consistent with Penz et al. findings. Penz et al. mentioned that GPIIb/IIIa inhibition

with RGDS blocked platelet spreading on atherosclerotic plaque but not adhesion under static conditions (Penz et al., 2005).

It is reported that dimeric GPVI has a higher affinity for collagen, whereas monomeric GPVI has a higher affinity for fibrin (Onselaer et al., 2017). Based on the notion that dimeric GPVI is the main form of GPVI that interacts with collagen, revacept (a recombinant dimeric GPVI) has been developed to therapeutically target GPVI-mediated platelet activation by competitive binding to exposed collagen at the site of vascular injury (Ungerer et al., 2011). However, atherosclerotic plaque possesses an affinity for both dimeric and monomeric GPVI. Both dimeric and monomeric forms of GPVI bound to atherosclerotic plaque with great affinity, and their binding was blocked by glenzocimab. The GPVI binding results further strengthen the findings of Jamasbi et al. that showed that antibodies raised against dimeric and monomeric GPVI inhibited atherosclerotic plaque-mediated platelet aggregation more effectively than antibody raised against dimeric GPVI only, indicating a functional role for both monomeric and dimeric GPVI in atherosclerotic plaque-induced platelet activation (Jamasbi et al., 2015).

As glenzocimab inhibited GPVI binding to atherosclerotic plaque, it blocked GPVI signalling as assessed by the inhibition of tyrosine phosphorylation of main signalling proteins that process signal transduction in the GPVI pathway, ultimately leading to platelet activation. Glenzocimab inhibited phosphorylation of Syk, LAT, and PLCγ2 following platelet stimulation with atherosclerotic plaque. The results indicated that GPIa/IIa is not involved in atherosclerotic plaque-mediated tyrosine phosphorylation as pre-treatment with 6F1 did not prevent tyrosine phosphorylation. Aspirin and ticagrelor showed no effect on tyrosine phosphorylation except partial inhibition of PLCγ2. It has been shown that GPVI signalling is upregulated in patients with STEMI despite treatment with aspirin and $P2Y_{12}$ inhibitors (Vélez et al., 2016).

Furthermore, GPVI expression on the platelet surface is elevated in patients with ACS (Bigalke et al., 2006).

Atherosclerotic plaque stimulated tyrosine phosphorylation in the Src signalling pathway. Platelet pre-treatment with Src inhibitors (Dasatinib and PP2) abolished Src phosphorylation and downstream tyrosine phosphorylation following platelet stimulation with atherosclerotic plaque. Src is associated with the cytoplasmic domain of GPVI, where it quickly transduces GPVI upstream signalling in response to GPVI activation. In addition to its main role in GPVI-FcR γ-chain signalling, Src also regulates GPIb-IX-V, GPIa/IIa, GPIIb/IIIa, and G_i-coupled receptor signalling (Senis et al., 2014). Parguiña et al. showed that Src and GPVI are upregulated in patients with STEMI compared with patients with stable coronary artery diseases or healthy controls (Parguiña Andrés et al., 2011).

Atherosclerotic plaque caused sustained signalling of key proteins involved in GPVI activation pathway. The sustained signalling of GPVI by atherosclerotic plaque could maintain continuous platelet activation. Collagen has been shown to induce sustained GPVI signalling in platelets in suspension and cell lines, and the sustained signalling via GPVI, supports platelet activation required to maintain platelet functions such as spreading (Tomlinson et al., 2007). Pallini et al. recently showed that immobilised collagen triggered a sustained GPVI signalling associated with sustained calcium signalling, cytoskeletal rearrangement, and GPIa/IIa interaction with collagen (Pallini et al., 2021). Sustained platelet activation is critical for thrombus stability. Ahmed et al. showed that sustained platelet activation by GPVI is necessary to maintain thrombus stability, and GPVI blockade causes platelet thrombi to disaggregate (Ahmed et al., 2020).

GPVI shedding leads to the release of GPVI ectodomain from the platelet membrane by ADAM10/17 sheddases after platelets are activated by a specific GPVI agonist (Gardiner et al., 2007). It is suggested that soluble GPVI could be used as a diagnostic and prognostic marker in patients with ACS because these patients have elevated levels of soluble GPVI (Chatterjee and Gawaz, 2017). The present study shows, for the first time, that atherosclerotic plaque causes GPVI shedding from platelets that have been exposed to atherosclerotic plaque in suspension and under a stirring condition *in vitro*. This finding may explain the mechanism behind GPVI shedding and elevated levels of soluble GPVI reported in patients with ACS.

4.5 Limitations

The number of repeats for some experiments in this chapter including thrombus formation and platelet adhesion at 300 s^{-1} and P-selectin expression under flow were less than 6 repeat (donors) which could weaken the statistical analysis and the interpretation of the results. No specific Syk inhibitor was used in signalling experiments which could give more insight on the role of Syk in atherosclerotic plaque-induced tyrosine signalling. Homogenised atherosclerotic plaques contain a variety of agonists other than collagen such as vWf, laminin, and fibronectin that activate platelet and tissue factor that activates coagulation, therebefore; using specific inhibitors against those agonists may help in exploring their roles in atherosclerotic plaque mediated platelet activation. Finally, integrating histological analysis with the presented results for atherosclerotic plaque-induced platelet responses in this chapter could give a comprehensive insight on atherosclerotic plaque-mediated platelet mechanisms.

4.6 Summary

Taken all together, atherosclerotic plaque stimulates platelet activation predominantly via GPVI as demonstrated by the ability of glenzocimab to potently block atherosclerotic plaque-mediated platelet responses. In contrast, aspirin and ticagrelor, which are considered the

essential treatment for patients with ACS, only exerted a partial inhibition of atherosclerotic plaque-mediated platelet mechanisms; however, they amplified the inhibitory effect of glenzocimab on plaque-induced platelet activation. Mechanistic investigation of atherosclerotic plaque–GPVI interaction showed that both dimeric and monomeric forms of GPVI bind to atherosclerotic plaque and that plaque causes sustained GPVI signalling and GPVI shedding.

CHAPTER 5 : EFFECT OF GLENZOCIMAB WITH ASPIRIN AND TICAGRELOR ON FIBRIN-MEDIATED PLATELET ACTIVATION, PLATELET PROCOAGULANT ACTIVITY, THROMBIN GENERATION, AND HAEMOSTATIC FUNCTION

5.1 Introduction

Beyond securing primary haemostasis, platelets play an additional role in priming blood coagulation (Brouns et al., 2020). Platelets facilitate thrombin generation, fibrin formation, and clot retraction (Swieringa et al., 2018). Fibrin was identified as a physiological ligand for glycoprotein VI (GPVI) in two independent studies in 2015, amplifying the role of GPVI in thrombosis. They also showed that platelets adhere on fibrin-coated surfaces under flow, and fibrin-rich thrombi attract platelets in a GPVI-dependent manner (Mammadova-Bach et al., 2015). Alshehri et al. reported that fibrin stimulates tyrosine phosphorylation via a GPVI-dependent pathway, enhances platelet procoagulant activity, and increases thrombus growth and stability (Alshehri et al., 2015). Monomeric GPVI mainly binds to fibrin through its D-domain, resulting in platelet activation (Onselaer et al., 2017). Platelet pre-treatment with D-dimer, the end product of fibrinolysis, blocks fibrin-stimulated platelet activation (Onselaer et al., 2017). In addition to GPVI, fibrin has been recognised as a ligand for GPIIb/IIIa (Litvinov et al., 2016a).

Fibrin is a heterogenous in its nature which makes it difficult from an experimental aspect, especially in the term of reproducibility. The heterogenicity of the fibrin is attributed to the differences in spatial proteolysis of the protein, polymerisation of amino acids, alternative splicing and genetic polymorphisms (Undas and Ariëns, 2011). FXIII polymorphisms have been reported to cause structural changes in the fibrin since FXIII cross-links fibrin (Ariëns et al., 2000). GPVI and fibrin interaction is still debatable matter particularly which GPVI form interacts with fibrin; the difference in the fibrin preparation between different studies that evaluated GPVI-fibrin association could be one of the factors that led to different conclusions and contradictions (Slater et al., 2019).

Fibrinogen, a precursor of fibrin, is also reported to interact with GPVI. Dimeric GPVI recognises fibrinogen via its D-domain, and this interaction mediates platelet activation and adhesion that support thrombus formation (Induruwa et al., 2018). Immobilised fibrinogen has been shown to stimulate platelet activation by GPVI (Mangin et al., 2018). A recent study demonstrated that the high affinity shown by fibrinogen for dimeric GPVI is governed by avidity and that the αC region in fibrinogen is the main binding site for GPVI (Xu et al., 2021).

Platelet procoagulant activity extends the role of platelets from simply securing primary haemostasis to also regulating the coagulation process. Procoagulant platelets are structurally characterised by membrane blebbing and balloon-like structures that express phosphatidylserine on their surfaces (Reddy and Rand, 2020b). Phosphatidylserine is translocated from the inner leaflet of the platelet membrane to the outer surface following platelet stimulation with a ligand such as collagen and thrombin (Bevers et al., 1982). Phosphatidylserine amplifies coagulation by providing sites for assembly and activation of prothrombinase complexes (Heemskerk et al., 2002). Procoagulant platelets have been shown to increase microvascular thrombus growth in arterioles and venules (Kuijpers et al., 2008).

As part of their roles in coagulation, platelets propagate thrombin generation via the formation of procoagulant platelet populations that are characterised by phosphatidylserine exposure. Thrombin is generated on the ballooned surfaces of platelets spread on collagen (Agbani Ejaife et al., 2015). GPVI plays an essential role in the amplification of platelet-dependent thrombin generation, and it has been shown that platelet stimulation with collagen enhances tissue factor-induced thrombin generation via GPVI but not GPIa/IIa (Lecut et al., 2005). Recent evidence shows that collagen can stimulate thrombin generation via GPVI independent of tissue factor and FXII (Li et al., 2019b). GPVI also enhances thrombin generation signalling via another ligand, polymerised fibrin (Mammadova-Bach et al., 2015).

5.2 Aims

This chapter aims to investigate the effect of glenzocimab and eptifibatide (in combination with aspirin and ticagrelor) on fibrin-mediated platelet responses, platelet procoagulant activity, thrombin generation, and whole blood coagulation.

5.3 Results

5.3.1 Effect of glenzocimab or eptifibatide with and without aspirin and ticagrelor on polymerised fibrin-induced platelet activation

Polymerised fibrin is another GPVI agonist that has recently been shown to be involved in platelet activation via GPVI. It was necessary to establish the right sample type and the right platelet aggregation methodology for fibrin-induced platelet aggregation. Polymerised fibrin-stimulated platelet aggregation was therefore characterised using different samples and methods. After establishing a method to stimulate platelet aggregation by fibrin, ascending concentrations of glenzocimab, glenzocimab with aspirin and ticagrelor, and eptifibatide were assessed on polymerised fibrin-stimulated platelet aggregation. In addition, the effect of glenzocimab and other antiplatelet agents was examined on platelet spreading and static adhesion on fibrin coated surfaces.

5.3.1.1 Polymerised fibrin induced faster and stronger platelet aggregation in platelet-rich plasma than washed platelets using light transmission aggregometry and failed to induce reliable platelet aggregation when using multiple electrode aggregometry

No previous studies have determined which is the optimal medium (PRP or WP) to carry out platelet aggregation assays based on light transmission aggregometry (LTA) with polymerised fibrin, and so the potency of cross-linked polymerised fibrin in stimulating platelet aggregation was assessed in both PRP and WP prepared from the same donor in three independent

experiments using a PAP-8E aggregometer. Fibrin-stimulated platelet aggregation was further tested in whole blood using multiple electrode aggregometry (MEA) using Multiplate® analyser.

The results showed that polymerised fibrin induces stronger and faster platelet aggregation in PRP than in WP (Figure 5.1). In the PRP sample, the polymerised fibrin produced 10% platelet aggregation within the first minute after its addition (10 ± 1%), reached maximum platelet aggregation of 57 ± 4% at 14 min, and ended with a platelet aggregation of 53 ± 7% at 30 min. In the WP sample, in contrast, the polymerised fibrin caused only 2 ± 0.73% platelet aggregation by the end of the first minute, reached 10 ± 2% platelet aggregation at 14 min, and ended with 24 ± 5% platelet aggregation at 30 min.

The results also showed that MEA is not a suitable technique to carry out polymerised fibrin-induced platelet aggregation in whole blood. Polymerised fibrin induced false aggregation even when added to a platelet-free sample of 0.9% NaCl used to dilute whole blood before stimulating the blood with an agonist. After adding polymerised fibrin into a test cell containing only 0.9% NaCl, aggregation values were 76 ± 13 U, which was higher than the aggregation values for the whole blood test cell (56 ± 5 U). This result indicates that polymerised fibrin fibres bind into the electrodes in the test cell, generating a false reading.

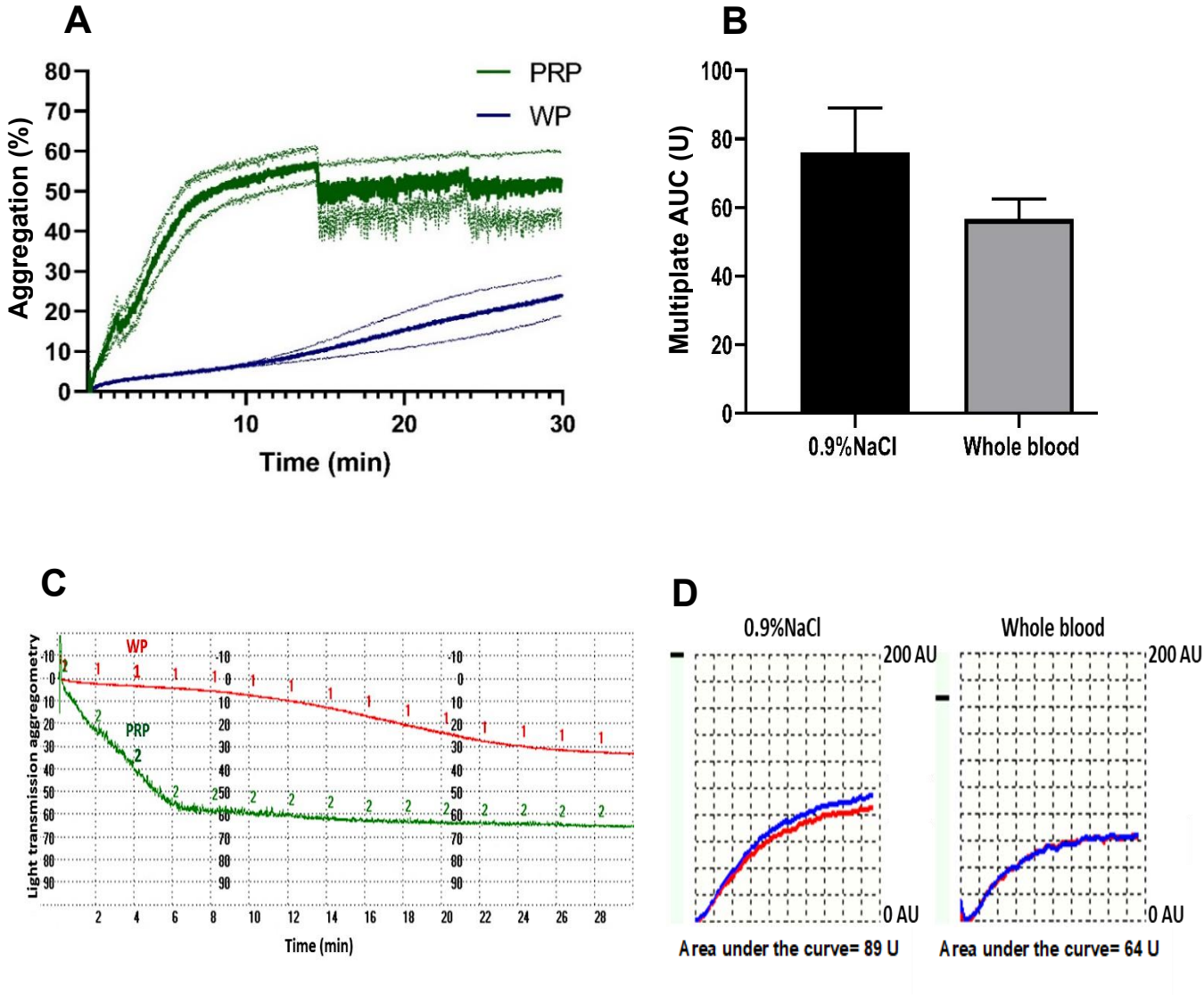


Figure 5.1 Polymerised fibrin induced platelet aggregation.

A) Polymerized fibrin-stimulated platelet aggregation in PRP and WP showing that PRP is optimal sample for fibrin-stimulated platelet aggregation in LTA. **B)** Polymerised fibrin-induced platelet aggregation in platelet free-normal saline and diluted whole blood sample in MEA. **C)** A representative aggregation trace from a single LTA experiment, where PRP and WP (2.0×10^8 mL) from the same donor were stimulated with polymerised fibrin, and platelet aggregation was monitored for 30 min. **D)** A representative aggregation trace from a single MEA experiment, where diluted whole blood and 0.9% NaCl solution were stimulated with polymerised fibrin, and the aggregation was monitored for 6 min. The results are expressed as mean ± SEM and generated from 3 independent experiments for LTA and 3 replicates for MEA.

5.3.1.2 Glenzocimab significantly reduced polymerised-fibrin induced platelet aggregation

The effect of glenzocimab on fibrin-stimulated platelet aggregation was tested in PRP, because, as described above, it was identified as the optimal medium in which to study polymerised fibrin-induced platelet aggregation. At a low concentration, glenzocimab reduced platelet aggregation triggered by the polymerised fibrin (Figure 5.2).

The polymerised fibrin induced platelet aggregation at a slow rate (58 ± 5%). A glenzocimab concentration of 1 µg/ml significantly reduced polymerised fibrin-stimulated platelet aggregation by 65% (final aggregation: 20 ± 9%; P=0.04). A glenzocimab concentration of 2.5 µg/ml further reduced platelet aggregation by 79% (final aggregation: 12 ± 5%; P<0.001). At 5 µg/ml, 10 µg/ml, 25 µg/ml, and 50 µg/ml, glenzocimab still significantly inhibited platelet aggregation but with minor fluctuations. These fluctuations may have been due to variations in the number of polymerised fibrin fibres, although a fixed volume of fibrin was used in all the aggregation experiments. Despite the fluctuations in this assay, it can be concluded from these results that glenzocimab is a novel inhibitor of platelet aggregation stimulated by polymerised fibrin.

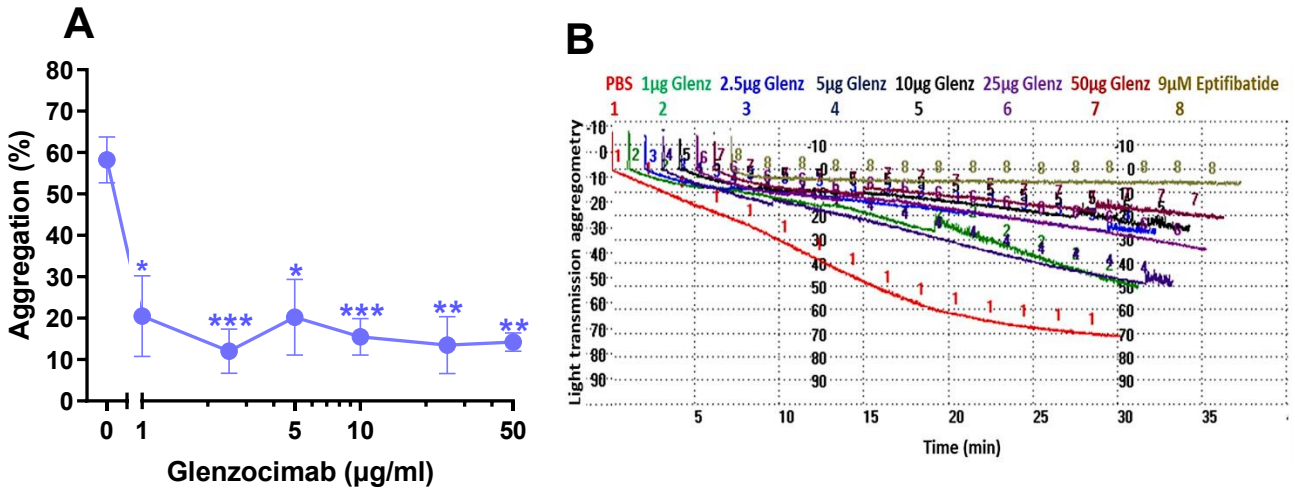


Figure 5.2 Effect of glenzocimab on fibrin-induced platelet aggregation.

A) Glenzocimab significantly inhibited fibrin-induced platelet aggregation in PRP. **B)** Representative traces from a single PRP aggregation experiment, where PRP was preincubated with different concentrations of glenzocimab or vehicle (PBS) for 10 min, then platelets stimulated with polymerized fibrin, and the aggregation was monitored in PAP-E8 aggregometer for 30 min. The effect of different concentrations of glenzocimab was compared to untreated (PBS control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=4, *P<0.05, **P<0.01, and ***P<0.001).

5.3.1.3 Glenzocimab inhibited polymerised fibrin-stimulated platelet aggregation more potently than aspirin and ticagrelor but not eptifibatide

The next step after establishing that glenzocimab significantly inhibited platelet aggregation stimulated by polymerised fibrin was to examine the effect of aspirin (30 μM) and ticagrelor (1 μM) on platelet aggregation-induced by polymerised fibrin to determine whether their antiplatelet potency increases when combined with glenzocimab. Another aim of this assay was to carry out a head-to-head comparison between the inhibitory effects of glenzocimab and eptifibatide on polymerised fibrin-stimulated platelet aggregation. The results showed that aspirin and ticagrelor partially blocked polymerised fibrin-induced platelet aggregation and showed no additional effect when combined with glenzocimab. Moreover, eptifibatide inhibitory effect on polymerised fibrin-induced platelet aggregation was more potent than that of glenzocimab (Figure 5.3).

Even when used concomitantly, aspirin and ticagrelor still exhibited a lower antiplatelet effect than what was achieved with a low concentration of glenzocimab (5 μg/ml), as shown in Figure 5.3A. There was no significant effect for ticagrelor on platelet aggregation (final aggregation: 23 ± 6% compared to 44 ± 2% in the control; P=0.13). Aspirin alone reduced platelet aggregation by 57% (final aggregation: 19 ± 2%; P=0.03) and by 55% when combined with ticagrelor (final aggregation: 20 ± 3%; P=0.04). In comparison, 5 μg/ml glenzocimab reduced platelet aggregation by 83% (final aggregation: 7 ± 1%; P=0.01). Eptifibatide was superior to glenzocimab in the inhibition of polymerised fibrin-induced platelet aggregation (Figure 5.3B). Eptifibatide reduced platelet aggregation by 94% (final aggregation: 3 ± 1% compared to 57 ± 4% in the control; P=0.004), whereas glenzocimab reduced platelet aggregation by 75% (final aggregation: 14 ± 2; P=0.006).

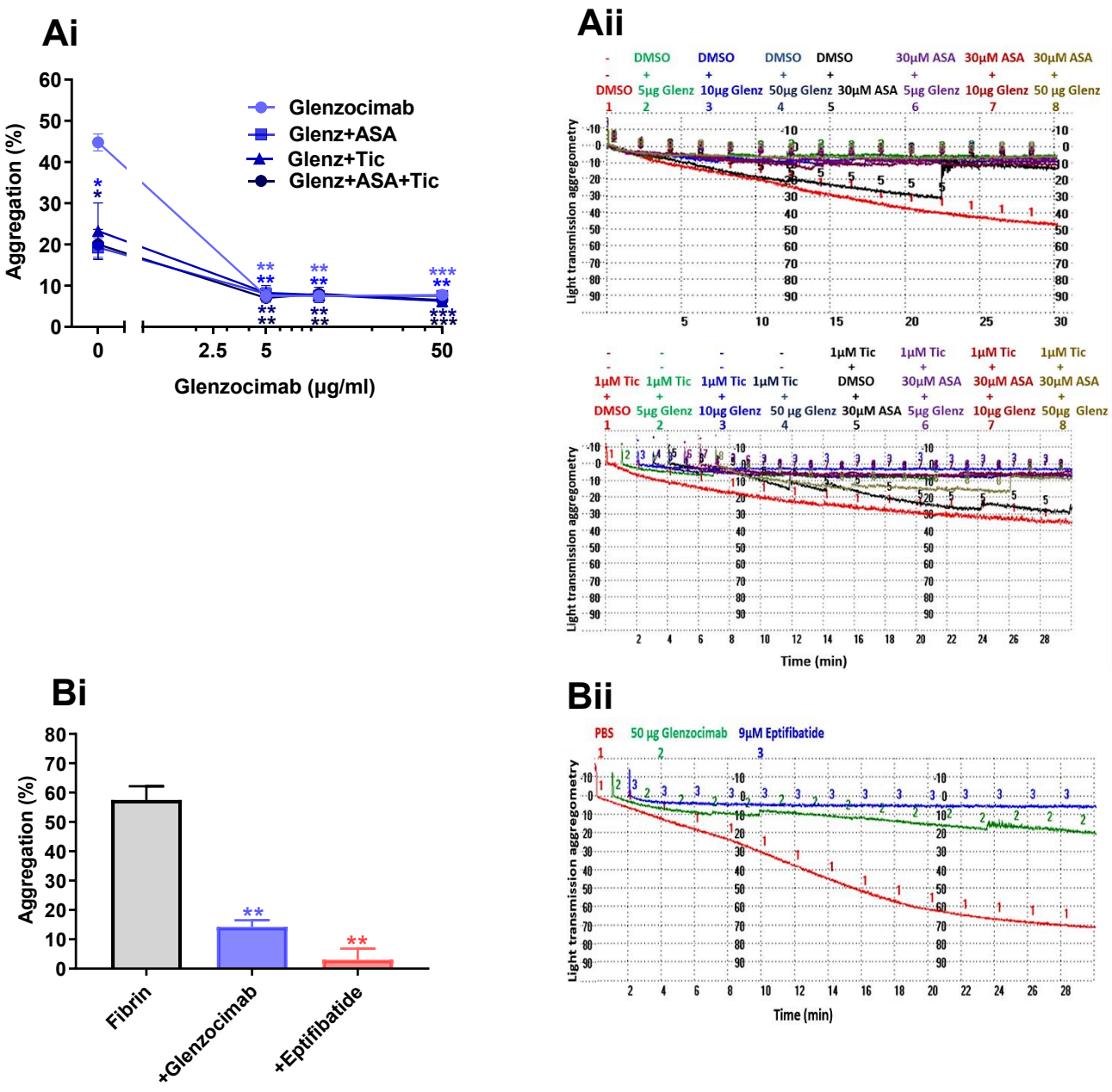


Figure 5.3. Effect of glenzocimab (± aspirin and ticagrelor) and eptifibatide on fibrin-stimulated platelet aggregation in PRP.

Ai-ii) Inhibition of polymerised fibrin-induced platelet aggregation by glenzocimab (5-50 µg/ml) ± aspirin (30 µM) and ticagrelor (1 µM). **Bi-ii)** Inhibition of polymerised-stimulated platelet aggregation by glenzocimab (50 µg/ml) and eptifibatide (9 µM). The effect of combinations of different concentrations of glenzocimab with aspirin and ticagrelor was compared to untreated samples and assessed using two-way ANOVA and one-way ANOVA for comparison between glenzocimab and eptifibatide with Dunnett correction for multiple comparisons (n=4, *P<0.05, **P<0.01, and ***P<0.001).

5.3.1.4 Platelets showed a similar spreading and adhesion characteristics on fibrinogen- and polymerised fibrin-coated surfaces under static conditions

Fibrin is formed from fibrinogen under the action of thrombin. Both fibrin and fibrinogen are reported to interact with GPVI, fibrin as a full receptor for monomeric GPVI and fibrinogen as a partial receptor for GPVI (Mangin et al., 2018). The characteristics of platelet spreading on surfaces coated with fibrin and fibrinogen were compared. The results showed no significant differences in terms of total surface area, perimeter, or number of adherent platelets (Figure 5.4).

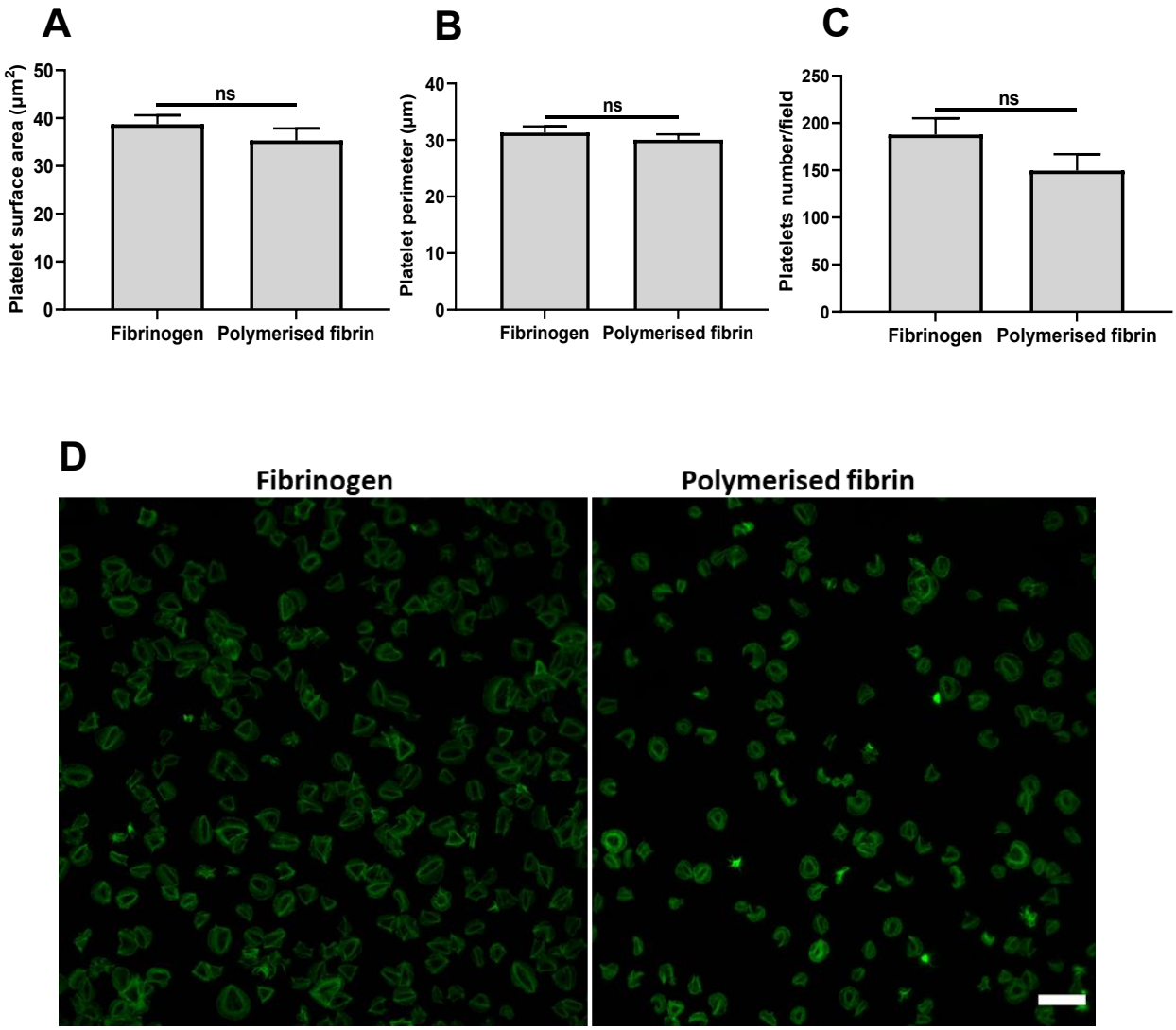


Figure 5.4. Characteristics of platelet spreading and adhesion on immobilized fibrinogen and polymerised fibrin.

A) Surface area of platelets spread on fibrinogen and polymerized fibrin. **B)** Perimeter of platelets spread on fibrinogen and polymerized fibrin. **C)** Number of adherent platelets on fibrinogen and fibrin. **D)** Representative figures from a single platelet spreading experiment. 10 µg/ml. 5 random images were analysed for each experiment. Scale bar 20 µm. Characteristics of platelet spreading on fibrinogen and polymerised fibrin were analysed using unpaired t-test (n=8, ns=non-significant).

5.3.1.5 Glenzocimab did not affect platelet spreading on polymerised fibrin, which was partially affected by the combination of aspirin and ticagrelor and significantly reduced by eptifibatide and dasatinib

As GPVI has been identified as the main signalling receptor for polymerised fibrin, one of the main characteristics of the platelets from GPVI-deficient patients is reduced spreading on the immobilised fibrin under static conditions. This part of the study had three aims: (1) to determine whether glenzocimab can block platelet spreading on polymerised fibrin, (2) to determine whether glenzocimab has more effect on platelet spreading than aspirin and ticagrelor, and (3) to examine the effect of eptifibatide and dasatinib on platelet spreading on polymerised fibrin that was generated from fibrinogen under the action of thrombin and then neutralised by PPACK, under static conditions. The results showed that glenzocimab did not exert any effect on platelet spreading and did not gain any potency upon combination with aspirin and ticagrelor, as assessed by the platelet surface area, the platelet perimeter, and the number of adherent platelets. In contrast, the combined use of aspirin and ticagrelor partially reduced platelet spreading, and eptifibatide and dasatinib greatly reduced platelet spreading on fibrin (Figure 5.5), as described below.

Aspirin and ticagrelor partially reduced the surface area by 37% (22 ± 3 μm^2 compared to 35 ± 2 μm^2 in the control; P=0.01). Glenzocimab did not significantly decrease the platelet surface area (29 ± 3 μm^2; P=0.59); the combination of glenzocimab with aspirin and ticagrelor did not provide additional effect on the surface area (23 ± 3 μm^2; P= 0.03). Eptifibatide on its own significantly reduced the surface area by 63% (13 ± 1 μm^2; P<0.001) and by 68% (11 ± 1 μm^2; P<0.001) with the addition of aspirin and ticagrelor. Dasatinib also significantly decreased the surface area by 65% (12 ± 1 μm^2; P<0.001).

With respect to the effect on the platelet perimeter, the tested antiplatelet agents reproduced the effect observed on the total surface area. Dual use of aspirin and ticagrelor partially reduced the perimeter by 24% (22 ± 1 µm in comparison to 30 ± 1 µm in the control; P=0.004). Glenzocimab had no significant effect on the perimeter (27 ± 1 µm; P=0.79) on its own, and it did not amplify the effect of aspirin and ticagrelor on the perimeter (23 ± 1 µm; P=0.01). Eptifibatide alone reduced the perimeter by 38% (18 ± 1 µm; P<0.001) and by 41% (17 ± 1 µm; P<0.001) when combined with aspirin and ticagrelor. Dasatinib significantly decreased the perimeter by 34% (19 ± 1 µm; P<0.001).

In regard to the effect on platelet adhesion, the combined use of aspirin and ticagrelor did not significantly reduce platelet adhesion (106 ± 19 platelets compared to 149 ± 17 platelets in the control; P= 0.45). Glenzocimab did not affect platelet adhesion even when used with aspirin and ticagrelor (114 ± 18 platelets; P=0.69). Eptifibatide alone significantly decreased platelet adhesion by 54% (68 ± 15 platelets; P=0.02) and by 61% (58 ± 15 platelets; P= 0.007) upon combination with aspirin and ticagrelor. Dasatinib produced the strongest effect on platelet adhesion among the investigated antiplatelet agents, reducing adhesion by 67% (48 ± 19 platelets; P= 0.002).

In summary, these results showed on effect for glenzocimab on platelet spreading and static adhesion on fibrin coated surfaces, whereas the combined use of aspirin and ticagrelor partially reduced platelet spreading but not adhesion. In contrast, eptifibatide potently reduced platelet spreading and adhesion on fibrin.

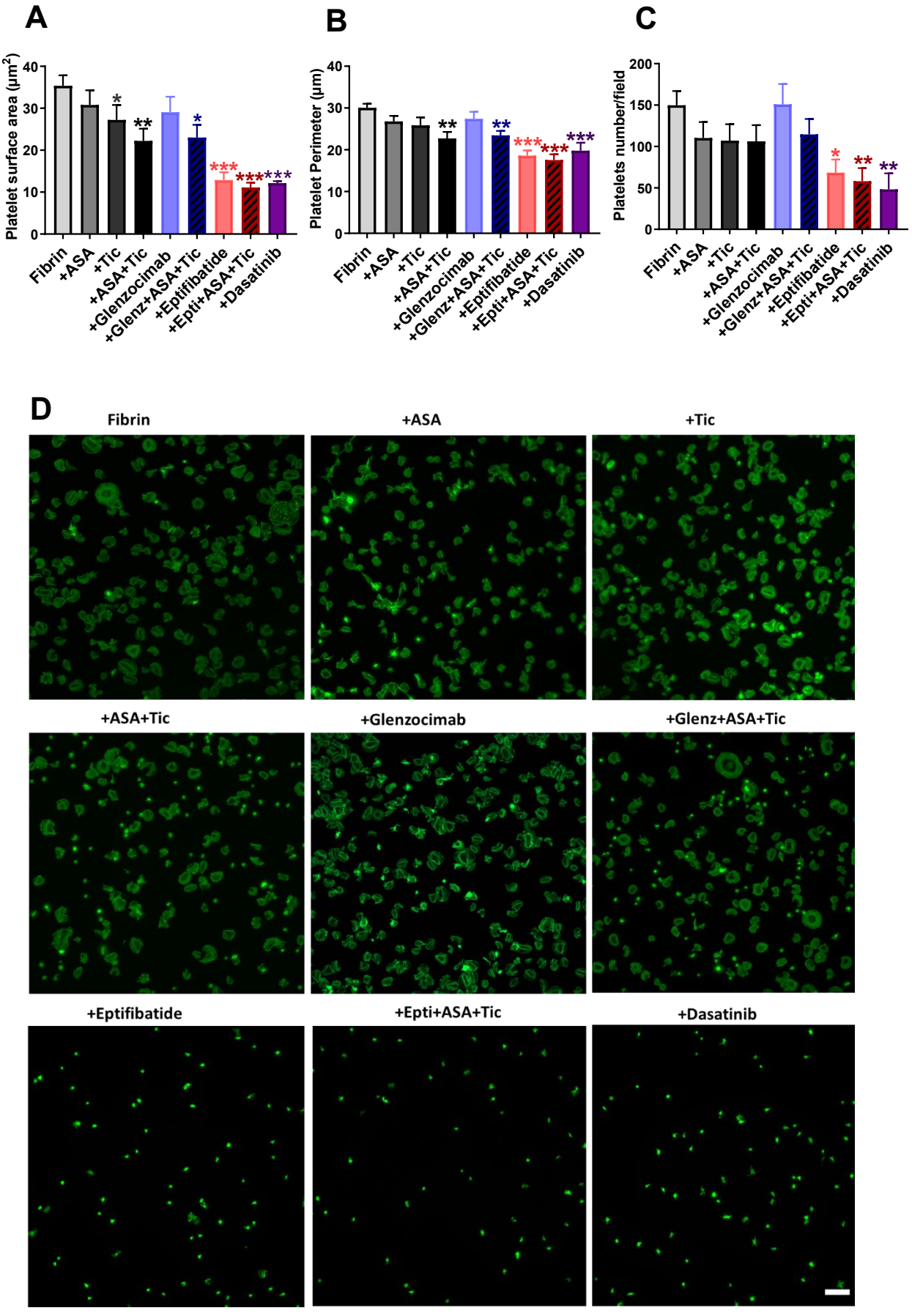

A
Platelet surface area (μm²)
40
30
20
10
0
*
**
*
***
***
***
Fibrin
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
B
Platelet Perimeter (μm)
40
30
20
10
0
**
**
***
***
***
Fibrin
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
C
Platelets number/field
200
150
100
50
0
*
**
**
Fibrin
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
D
Fibrin
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib

Figure 5.5. Effect of glenzocimab or eptifibatide with/without aspirin and ticagrelor on platelet spreading on polymerised fibrin under static conditions.

A) Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet surface area. **B)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet perimeter. **C)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the number of adherent platelets. **D)** Representative images from a single spreading experiment, where human WP (2.0×10^7 mL) was preincubated with 0.01% DMSO, glenzocimab (50 µg/ml) or eptifibatide (9 µM) ± aspirin (30 µM) and ticagrelor (1 µM) or dasatinib (10 µM) for 30 min at 37ºC.Then, pre-treated platelets were allowed to spread on freshly prepared polymerised fibrin (10 µg/ml fibrinogen) on coverslips for 30 min at 37ºC. Platelets were fixed with 4% neutralized formalin buffer for 10 minutes followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min and stained with Alex-488-phalloidin for 45 min in the dark. The coverslips were then mounted on glass slides and imaged under the Zeiss an Axio Observer 7 inverted epifluorescence microscope. 5 random images were analysed for each experiment. Scale bar represents 20 µm. The effect of treatment groups was compared to 0.01% DMSO and assessed using a linear mixed model effect with Dunnett correction for multiple comparisons (n=6, * $P<0.05$, **$P<0.01$, and ***$P<0.001$).

5.3.1.6 Glenzocimab did not affect platelet spreading either on fibrinogen that was marginally affected by the combination of aspirin and ticagrelor and significantly reduced by eptifibatide and dasatinib

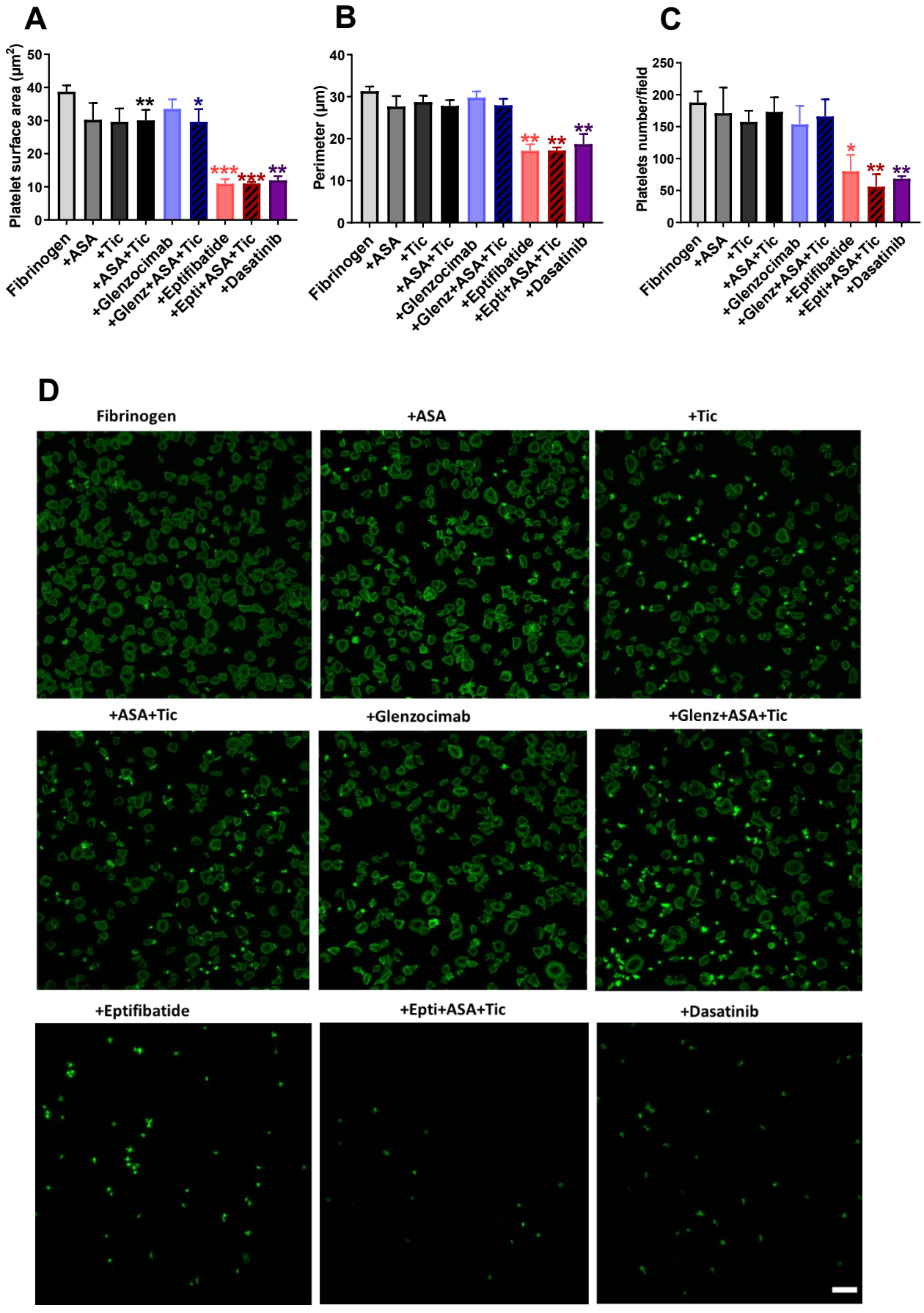
Two years after the identification of GPVI as a platelet receptor for polymerised fibrin, Induruwa et al. and Mangin et al. in two independent studies, identified GPVI as a signalling platelet receptor for the fibrin precursor, fibrinogen (Induruwa et al., 2018, Mangin et al., 2018). Therefore, the blockade of GPVI by glenzocimab with and without aspirin and ticagrelor was evaluated on immobilised fibrinogen and compared with the blockade of GPIIb/IIIa by eptifibatide with and without aspirin and ticagrelor, as GPIIb/IIIa is the main platelet functional receptor for fibrinogen. The platelets that were pre-treated with different inhibitors or vehicle and allowed to spread on surfaces coated with 10 μg/ml fibrinogen of the type used previously to generate the polymerised fibrin. The results generally revealed similar inhibitory effects to those observed on polymerised fibrin. Aspirin and ticagrelor had less effect, however, appearing to lose the partial effect on platelet spreading on polymerised fibrin that they exhibited upon combination; they exerted only a marginal effect on platelet spreading on fibrinogen. In contrast, eptifibatide and dasatinib greatly reduced platelet spreading and adhesion on fibrinogen (Figure 5.6).

The combined use of aspirin and ticagrelor reduced the surface area by 22% (30 ± 2 μm^2 compared to 38 ± 1 μm^2 in the control; P=0.02). Glenzocimab showed no effect on the surface area (33 ± 2 μm^2; P=0.07); the combination of glenzocimab with aspirin and ticagrelor also provided no additional effect on the surface area (29 ± 4 μm^2; P=0.03). Eptifibatide greatly reduced the surface area by 71% (11 ± 1 μm^2; P<0.001), and the addition of aspirin and ticagrelor along with eptifibatide did not provide any additional effect (11 ± 1 $\mu m^{2;}$ P<0.001). Dasatinib greatly reduced the surface area by 69% (12 ± 1 μm^2; P=0.001).

With regard to the effect on the platelet perimeter, dual treatment with aspirin and ticagrelor did not significantly reduce the perimeter (27 ± 1 μm in comparison to 31 ± 1 1 μm in the control; P=0.14). Glenzocimab on its own did not affect the perimeter (29 ± 1 μm; P=0.52); the combination of glenzocimab with aspirin and ticagrelor did not provide additional effect on the perimeter (28 ± 1 μm; P=0.11). Eptifibatide alone significantly reduced the perimeter by 45% (17 ± 1 μm; P=0.008), and the addition of aspirin and ticagrelor with eptifibatide did not lead to a further reduction in the perimeter (17 ± 1 μm; P=0.002). Dasatinib also significantly diminished the perimeter by 40% (18 ± 2 μm; P=0.003).

Glenzocimab did not reduce platelet adhesion (153 ± 29 platelets compared to 187 ± 17 platelets in the control; P=0.93). The combined use of aspirin and ticagrelor also did not reduce platelet adhesion (172 ± 23 platelets; P>0.99) and showed no additional effect when combined with glenzocimab (166 ± 26 platelets; P>0.99). Eptifibatide alone reduced platelet adhesion by 57% (80 ± 25 platelets; P=0.02) and further reduced platelet adhesion by 70% (55 ± 19 platelets; P=0.003). Dasatinib reduced platelet adhesion by 63% (68 ± 4 platelets; P=0.008).

In summary, these results indicated no effect for glenzocimab on platelet spreading and static adhesion on fibrinogen coated surfaces. The combined use of aspirin and ticagrelor marginally reduced platelet spreading but not adhesion, whereas eptifibatide potently reduced both platelet spreading and static adhesion on fibrinogen.


A
Platelet surface area (μm²)
50
40
30
20
10
0
**
*
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***
**
Fibrinogen
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
B
Perimeter (μm)
40
30
20
10
0
**
**
**
Fibrinogen
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
C
Platelets number/field
250
200
150
100
50
0
*
**
**
Fibrinogen
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib
D
Fibrinogen
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
+Dasatinib

Figure 5.6. Effect of glenzocimab or eptifibatide with/without aspirin and ticagrelor on platelet spreading on fibrinogen under static conditions.

A) Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet surface area. **B)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the platelet perimeter. **C)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the number of adherent platelets. **D)** Representative images from a single spreading experiment, where human WP (2.0×10^7mL) was preincubated with 0.01% DMSO, glenzocimab (50 µg/ml) or eptifibatide (9 µM) ± aspirin (30 µM) and ticagrelor (1 µM) or dasatinib (10 µM) for 30 min at 37ºC. Then, pre-treated platelets were allowed to spread on immobilised fibrinogen (10 µg/ml) on coverslips for 30 min at 37ºC. Platelets were fixed with 4% neutralized formalin buffer for 10 min followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min and stained with Alex-488-phalloidin for 45 min in the dark. The coverslips were then mounted on glass slides and imaged under the Zeiss an Axio Observer 7 inverted epifluorescence microscope. 5 random images were analysed for each experiment. Scale bar 20 µm. The effect of treatment groups was compared to the control (0.01% DMSO) and assessed via a linear mixed model effect with Dunnett correction for multiple comparisons (n=6, * P<0.05, **P<0.01, and ***P<0.001).

5.3.2 Effect of glenzocimab and eptifibatide with aspirin and ticagrelor on fibrin clot retraction

The effect of glenzocimab and eptifibatide with aspirin and ticagrelor was assessed on fibrin clot retraction that was formed in recalcified PRP following stimulation with thrombin. The results showed that eptifibatide with aspirin and ticagrelor caused a significant delay in clot retraction as reflected by measured clot weight (Figure 5.7).

The weight of the fibrin clot in the vehicle-treated samples was 10 ± 2 mg. The combined use of aspirin and ticagrelor did not significantly delay clot retraction (71 ± 19 mg; P=0.10). Glenzocimab had no significant effect on clot retraction (50 ± 23 mg; P=0.63), and no additional impairment was achieved upon combination with aspirin and ticagrelor (72 ± 13 mg; P=0.05). Mono treatment with eptifibatide had no significant effect on clot retraction (73 ± 27 mg; P=0.11). The addition of aspirin and ticagrelor along with eptifibatide led to a significant delay in clot retraction (82 ± 20 mg; P=0.03).

In summary, antiplatelet agents especially dual combination of aspirin and ticagrelor or eptifibatide alone tend to cause a delay in clot retraction, but their effect was not statistically significant. More experiments (n) are required to get a definitive result for the effect of these antiplatelet agents on clot retraction. Nevertheless, the triple combination of eptifibatide with aspirin and ticagrelor caused a significant delay in fibrin clot retraction.

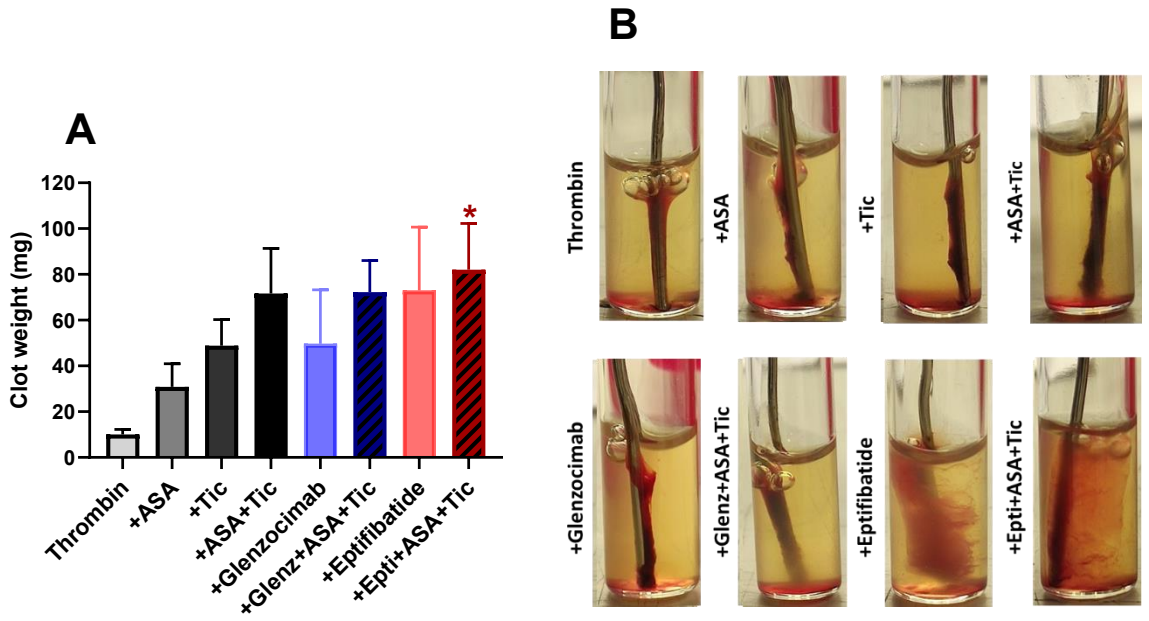

Figure 5.7. Effect of glenzocimab or eptifibatide with/without aspirin and ticagrelor on fibrin clot retraction.

A) Effect of glenzocimab and other antiplatelet agents on the fibrin clot retraction that was formed by thrombin. **B)** A representative image for a fibrin clot retraction experiments where PRP was incubated with glenzocimab (50 µg/ml), aspirin (30 µM), ticagrelor (1 µM), and eptifibatide (9 µM) for 10 min at 37ºC. Then, PRP was recalcified with $CaCl_2$ (5 mM), stimulated with thrombin (1 U) and kept for 120 min at 37ºC. After 2 hours of incubation, tubes were removed from water bath and clot weighed. Effect of treatment groups was compared to the control and assessed via one-way ANOVA with Dunnett correction for multiple comparisons (n=4, * $P<0.05$).

5.3.3 Effect of glenzocimab and eptifibatide with and without aspirin and ticagrelor on platelet procoagulant activity

The effect of glenzocimab or eptifibatide (with and without aspirin and ticagrelor) was evaluated on procoagulant platelet formation in hirudin-anticoagulated blood flowing on Horm collagen and atherosclerotic plaque homogenate at a shear rate of 1000 s^{-1}. The platelet procoagulant activity was measured by phosphatidylserine exposure as detected by Annexin V.

5.3.3.1 Glenzocimab inhibited platelet procoagulant activity in whole blood flowing on Horm collagen, while conventional antiplatelet agents showed no significant inhibition on platelet procoagulant formation

The results showed that only glenzocimab potently blocked phosphatidylserine exposure, indicating inhibition of procoagulant platelet formation on Horm collagen under flow. Aspirin and ticagrelor partially inhibited the development of phosphatidylserine-exposing platelet thrombi but not the adhesion of procoagulant platelets. Eptifibatide showed a non-statistically significant trend in lowering platelet procoagulant formation (Figure 5.8).

Regarding the formation of the procoagulant platelets, as reflected in the fluorescence intensity of phosphatidylserine exposure (Figure 5.8A), aspirin and ticagrelor reduced it by 60% (18 ± 10 AU compared to 46 ± 9 AU in the control; P=0.002). Glenzocimab alone reduced procoagulant platelet formation by 90% (4 ± 1 AU; P=0.05) and further reduced it by 94% (2 ± 1 AU; P=0.05) with the addition of aspirin and ticagrelor. Eptifibatide showed a non-significant trend of reducing procoagulant platelet formation by 63% (17 ± 2 AU; P=0.06) and additionally reduced procoagulant platelet formation by 73% (12 ± 2 AU; P=0.05) in the presence of aspirin and ticagrelor.

With respect to the surface coverage of adherent procoagulant platelets, aspirin and ticagrelor had no significant effect on it (43 ± 13% in comparison to 76 ± 1% in the control; P=0.21). Glenzocimab alone reduced the surface coverage by 79% (15 ± 4%; P=0.003) and further inhibited it by 91% (6 ± 1%; P<0.001) with the addition of aspirin and ticagrelor. In contrast, eptifibatide had no significant effect on the surface coverage (65 ± 6%; P=0.28). Combining eptifibatide with aspirin and ticagrelor showed no additional effect on the surface coverage (52 ± 8%; P=0.11).

In summary, these results showed that glenzocimab potently inhibited procoagulant platelet formation on collagen under flow conditions. Dual combination of aspirin and ticagrelor partially reduced procoagulant platelet formation, and they amplified the inhibitory effect of glenzocimab on procoagulant platelet formation on collagen. In contrast, eptifibatide showed no significant effect on procoagulant platelet formation on collagen.

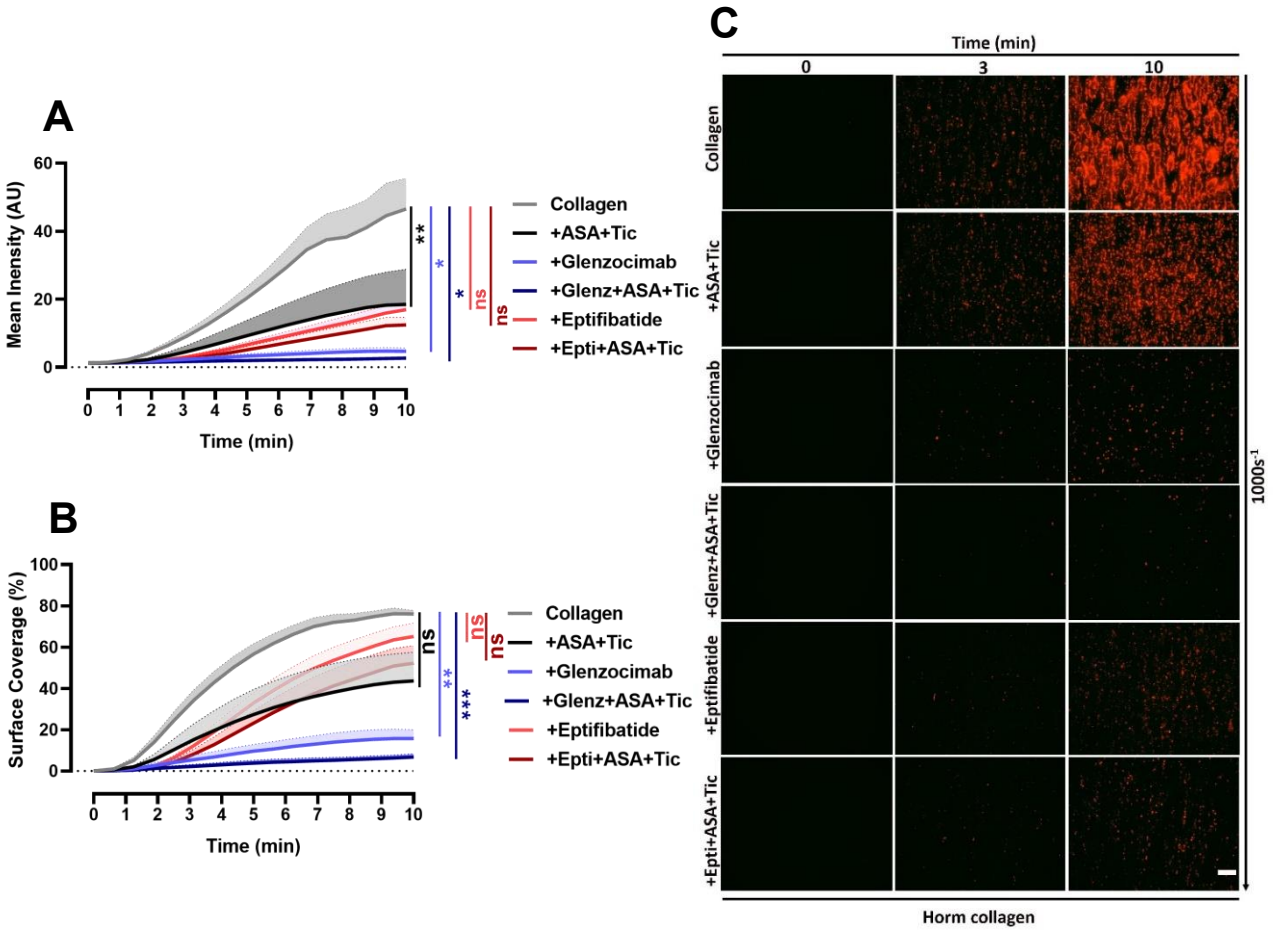

Figure 5.8 Effect of glenzocimab and other antiplatelet agents on phosphatidylserine exposure on platelets that have adhered to Horm collagen under flow conditions.

A) Glenzocimab significantly blocked formation of phosphatidylserine exposing thrombi as reflected by the fluorescence intensity. **B)** Glenzocimab reduced adhesion of phosphatidylserine exposing platelets as reflected by the surface coverage %. **C)** Representative images from a single flow adhesion experiment, where hirudin-anticoagulated blood was incubated with AF568-labelled annexin A5, 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide for 15 min at 37°C then flowed over 200 µg/ml Horm collagen at a shear rate of 1000 s^{-1} for 10 min. Images were taken every 35 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by Image J software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of anti-platelet agents was compared to untreated samples and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=4, ns= non-significant, *P<0.05, **P<0.01, and ***P<0.001).

5.3.3.2 Glenzocimab completely blocked phosphatidylserine exposure on atherosclerotic plaque homogenate under flow, whereas other antiplatelet agents caused significant but not complete inhibition

The results demonstrated that glenzocimab entirely blocked platelet procoagulant activity on atherosclerotic plaque, as shown by the complete inhibition of adhesion of phosphatidylserine exposing platelets (surface coverage %). Eptifibatide and aspirin and ticagrelor exhibited a lower inhibitory effect on platelet procoagulant activity (Figure 5.9).

As shown in Figure 5.9A, spiking samples with aspirin and ticagrelor had no significant effect on procoagulant platelet formation (2.68 ± 0.66 AU compared to 6.40 ± 1.36 AU in the control; P=0.16). Glenzocimab alone also showed a non-significant trend of reducing procoagulant platelet formation by 75% (1.60 ± 0.22 AU; P=0.07) without showing additional effect in combination with aspirin and ticagrelor (1.40 ± 0.05 AU; P=0.10). Eptifibatide alone showed a non-significant trend of decreasing procoagulant platelet formation by 70% (1.90 ± 0.38 AU; P=0.06), and on extra effect was observed with the addition of aspirin and ticagrelor (1.75 ± 0.36 AU; P=0.06).

In regard to the effect on the surface occupied by phosphatidylserine-exposing platelets, as presented in Figure 5.9B, aspirin and ticagrelor reduced it by 73% (5.00 ± 2.32% compared to 18.50 ± 2.90% in the control; P=0.03). Glenzocimab reduced the surface coverage by 99% (0.18 ± 0.08%; P=0.02) on its own and by 99% (0.17 ± 0.09%; P=0.02) upon combination with aspirin and ticagrelor. In contrast, eptifibatide alone reduced the surface coverage by 90% (1.77 ± 0.98%; P=0.02), and no additional effect was observed in the presence of aspirin and ticagrelor (2.17 ± 1.51%; P=0.03).

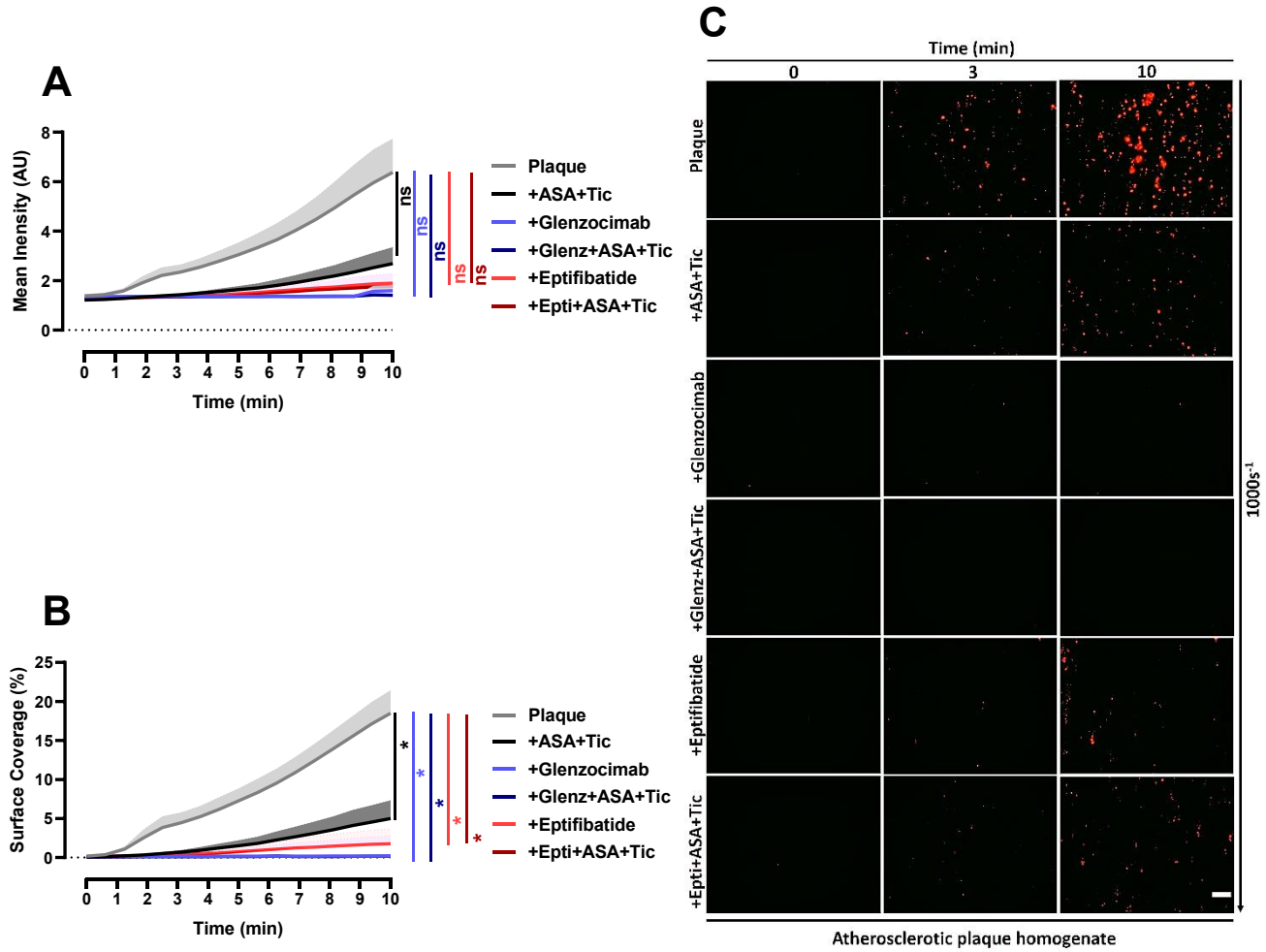

Figure 5.9 Effect of glenzocimab and other antiplatelet agents on phosphatidylserine exposure on platelets that have adhered to atherosclerotic plaque under flow conditions. **A)** Glenzocimab showed a non-significant trend of reducing formation of phosphatidylserine exposing thrombi as reflected by the fluorescence intensity. **B)** Glenzocimab blocked adhesion of phosphatidylserine exposing platelets as reflected by the surface coverage %. **C)** Representative images from a single flow adhesion experiment, where hirudin-anticoagulated blood was incubated with AF568-labelled annexin A5, 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide for 15 min at 37°C then flowed over 1 mg/ml atherosclerotic plaque at a shear rate of 1000 s^{-1} for 10 min. Images were taken every 35 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by Image J software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of antiplatelet agents was compared to untreated samples and assessed using two-way ANOVA with Dunnett correction for multiple comparisons (n=4, ns= non-significant, and *P<0.05).

5.3.4 Glenzocimab significantly reduced some thrombin generation parameters more than existing antiplatelet agents including eptifibatide as evaluated by the calibrated automated thrombogram

The effect of glenzocimab or eptifibatide (with and without aspirin and ticagrelor) was investigated on thrombin generation in platelet-rich plasma induced by tissue factor, which does not directly activate GPVI or platelets. Thrombin generation was stimulated by PRP reagent, which contains tissue factor and a minimal amount of phospholipid, so that platelet phospholipids (phosphatidylserines and phosphatidylethanolamines) augment thrombin generation. The effect of antiplatelet treatment on thrombin generation was evaluated by analysing a range of calibrated automated thrombogram (CAT) variables such as peak thrombin, the velocity index, and the endogenous thrombin potential (ETP) that describe thrombin generation kinetics and the amount of thrombin generated.

The results showed that both GPVI inhibition with glenzocimab alone and GPIIb/IIIa blockade with eptifibatide in the presence of aspirin and ticagrelor significantly reduced a panel of thrombin generation kinetics variables, including peak thrombin, velocity index, and start tail. Aspirin and ticagrelor had no effect on these variables. All evaluated antiplatelet agents, including glenzocimab and eptifibatide, showed no effect on the amount of total thrombin generated, as reflected by the ETP (Figure 5.10).

Peak thrombin represents the maximum concentration of thrombin that can be generated during the measurement. Among the investigated antiplatelet agents, only glenzocimab alone or eptifibatide with aspirin and ticagrelor significantly reduced peak thrombin. Aspirin and ticagrelor did not show any effect on peak thrombin (77 ± 21 nM compared to 85 ± 23 nM in the control; $P>0.99$). Glenzocimab alone reduced peak thrombin more than the other antiplatelet agents, reducing it by 50% (41 ± 7 nM; $P=0.002$), and the addition of aspirin and ticagrelor

along with glenzocimab reduced the peak by 53% (39 ± 7 nM; P=0.003). Eptifibatide did not significantly reduce the peak (50 ± 7 nM; P=0.11). The presence of aspirin and ticagrelor amplified the effect of eptifibatide, reducing the peak by 54% (39 ± 6 nM; P=0.02).

The velocity index represents the slope of the thrombin generation curve. Glenzocimab alone and eptifibatide only in the presence of aspirin and ticagrelor significantly reduced the velocity index. Aspirin and ticagrelor did not affect the velocity index (5.66 ± 1.51 nM/min compared to 7.16 ± 1.97 nM/min in the control; P>0.99). Glenzocimab significantly reduced the velocity index by 63% (2.61 ± 0.33 nM/min; P=0.007). The combination of glenzocimab, aspirin, and ticagrelor decreased the velocity index by 66% (2.40 ± 0.37 nM/min; P=0.005). Eptifibatide showed a non-significant trend of reducing the velocity index by 56% (3.11 ± 0.40 nM/min; P=0.06) and additionally by 65% (2.50 ± 0.52 nM/min; P=0.02) in the presence of aspirin and ticagrelor.

The ETP represents the total amount of thrombin generated throughout the test (i.e., the area under the curve). None of the tested antiplatelet agents showed a significant effect on the ETP. Lag time is the time from the initiation with tissue factor to the start of the thrombin burst. None of the examined antiplatelet agents demonstrated any effect on the lag time. Time to peak (ttPeak) is the time from the start of the measurement to the time when peak thrombin is reached. The results showed that none of the investigated antiplatelet drugs exerted any significant delay on ttPeak.

Start tail (min) is the time from the beginning of the measurement to the point when the thrombin generation curve reaches zero. Both glenzocimab and eptifibatide in the presence of aspirin and ticagrelor significantly prolonged the start tail, whereas aspirin and ticagrelor did not extend the start tail. Dual use of aspirin and ticagrelor did not significantly delay the start

tail (57 ± 2 min in comparison to 55 ± 1 min in the control; P>0.99). Glenzocimab alone significantly delayed the start tail by 20 min (75 ± 6 min; P=0.009) and by 25 min (80 ± 5 min; P<0.001) in the presence of aspirin and ticagrelor. Eptifibatide did not significantly prolong the start tail (68 ± 5 min; P=0.17); however, the addition of aspirin and ticagrelor significantly intensified the effect of eptifibatide, delaying the start tail by 17 min (72 ± 3 min; P=0.04).

In summary, these results showed that glenzocimab significantly affected some thrombin generation parameters such as peak thrombin, the velocity index, and start tail. The combined use of aspirin and ticagrelor did not affect any of thrombin generation parameters. Eptifibatide caused a significant effect on peak thrombin, the velocity index, and start tail in the presence of aspirin and ticagrelor only.

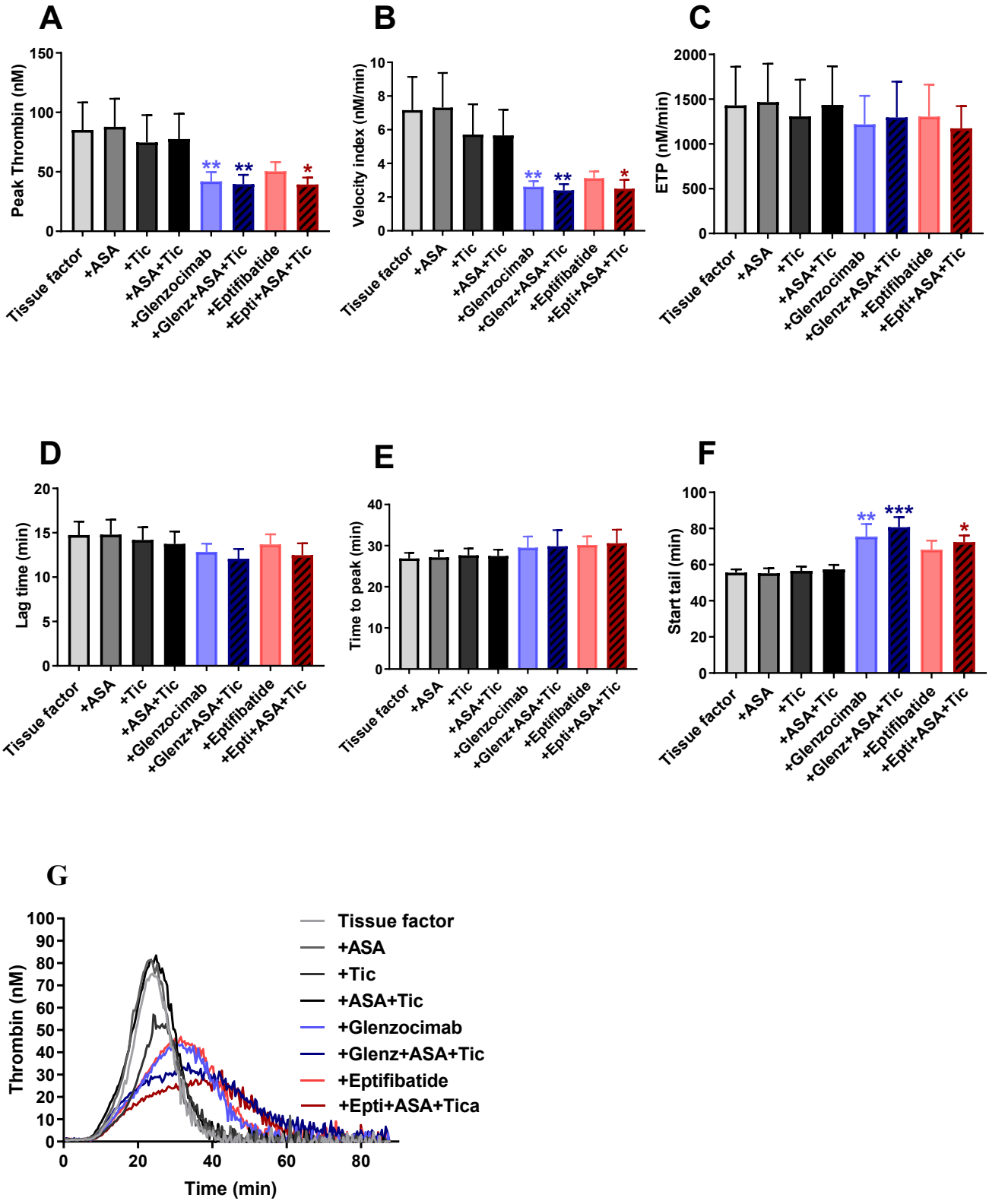
A
Peak Thrombin (nM)
150
100
50
0
**
**
*
Tissue factor
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
B
Velocity index (nM/min)
10
8
6
4
2
0
**
**
*
Tissue factor
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
C
ETP (nM/min)
2000
1500
1000
500
0
Tissue factor
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
D
Lag time (min)
20
15
10
5
0
Tissue factor
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
E
Time to peak (min)
40
30
20
10
0
Tissue factor
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
F
Start tail (min)
100
80
60
40
20
0
**
***
*
Tissue factor
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
G
Thrombin (nM)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
Time (min)
Tissue factor
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tica

Figure 5.10. Effect of glenzocimab and other antiplatelet agents on the TF-stimulated thrombin generation in the CAT.

A) Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the thrombin generation peak. **B)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the velocity index of the thrombin generation. **C)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the endogenous thrombin potential (ETP). **D)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the lag time. **E)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the time to peak. **F)** Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the start tail. **G)** A representative thrombogram from a single CAT experiment, where human PRP was pre-treated with 50 µg/ml glenzocimab, 30 µM aspirin, 1 µM ticagrelor, and 9 µM eptifibatide or 0.01%DMSO, then platelets were incubated with PRP reagent that contains 0.5 pM TF with a minimal amount of phospholipids in a 96-well plate for 10 min. Thrombin generation was initiated by automatic dispensing of the FluCa reagent which contains a mixture of fluorogenic substrate and $CaCl_2$, thrombin generation was monitored with a Fluoroscan Ascent plate reader, and the thrombin generation data were calculated against thrombin calibrator using Thrombinoscope® software. The effect of treatment groups was compared to 0.01% DMSO and assessed via one-way ANOVA with Dunnett correction for multiple comparisons (n=7, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

5.3.5 Effect of glenzocimab and eptifibatide with and without aspirin and ticagrelor on whole blood coagulation stimulated via extrinsic and intrinsic coagulation pathways in rotational thromboelastometry as reflected by EXTEM and INTEM parameters

Rotational thromboelastometry (ROTEM) is used to assess the viscoelastic properties of blood clots under low shear conditions and is an indicator of general haemostasis because it is mediated by the coagulation cascade as well as platelets (Korpallová et al., 2018). ROTEM provides a comprehensive overview of clot kinetics and properties, ranging from clot initiation, clot formation kinetics, clot strength, and clot lysis.

5.3.5.1 Glenzocimab showed no effect on clot initiation as reflected by clotting time

Clotting time (CT) is the first recorded ROTEM parameter that reflects the clot initiation phase. CT mainly reflects the contribution of coagulation factors in the whole blood coagulation process. The effect of GPVI antagonism with glenzocimab in the presence or absence of aspirin and ticagrelor was compared with that of GPIIb/IIIa blockade with eptifibatide with and without aspirin and ticagrelor on the CT upon stimulation of whole blood coagulation via extrinsic and intrinsic coagulation pathways. The results showed no effect on EXTEM-CT or INTEM-CT for all antiplatelet agents, including glenzocimab and eptifibatide, either alone or combined with aspirin and ticagrelor (Figure 5.11).

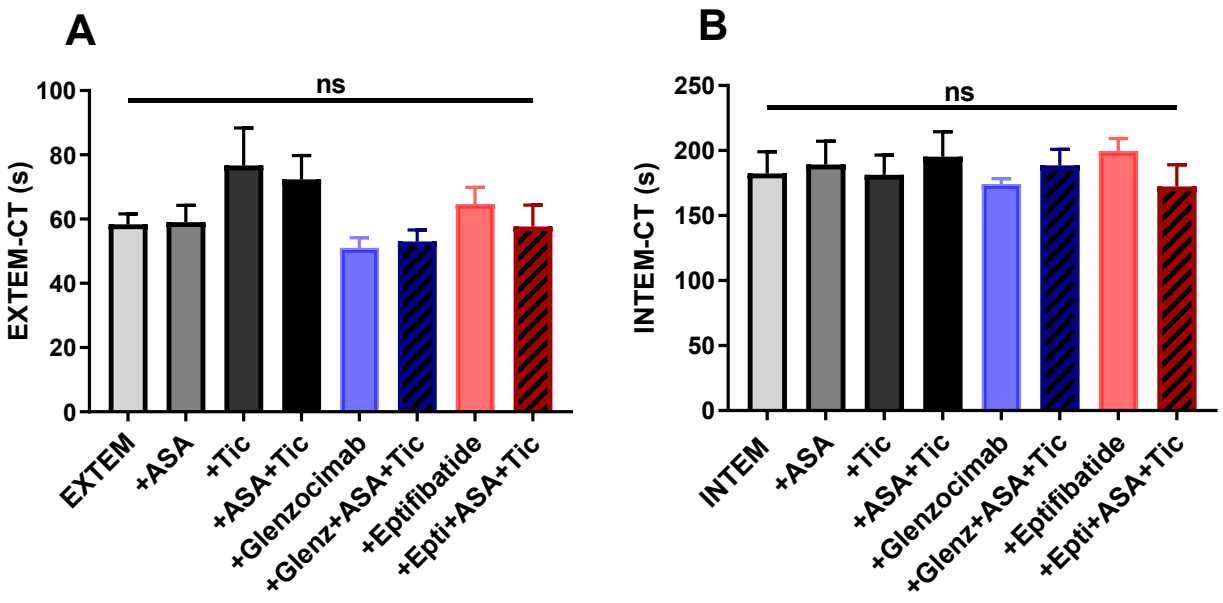

Figure 5.11. Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on clotting time parameter in the ROTEM.

A) Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the EXTEM-CT. **B)** Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the INTEM-CT. The effect of different treatment groups was compared to untreated (DMSO control) samples and assessed using one-way ANOVA with Dunnett correction for multiple comparisons (n=3, ns=non-statistically significant).

5.3.5.2 Glenzocimab did not affect clot formation kinetics as reflected by clot formation time, while the GPIIb/IIIa inhibitor eptifibatide markedly reduced them

Clot formation time (CFT) and α-angle are the main ROTEM parameters that provide useful information on clot formation kinetics. CFT is the time lapse between 2 mm amplitude and 20 mm amplitude of the clotting signal. The results demonstrated that, of all the tested antiplatelet agents, only eptifibatide affected the CFT upon stimulation of extrinsic and intrinsic coagulation pathways by EXTEM and INTEM. Aspirin and ticagrelor, glenzocimab alone, and glenzocimab combined with aspirin and ticagrelor did not exert any effect on the CFT in the whole blood coagulation stimulated by EXTEM and INTEM (Figure 5.12).

Eptifibatide alone greatly increased EXTEM-CFT (338 ± 50 s compared to 67 ± 2 s in the control; $P<0.001$). Interestingly, the addition of aspirin and ticagrelor did not further augment eptifibatide's influence on EXTEM-CFT (351 ± 27 s; $P<0.001$). A similar pattern for the effect of eptifibatide on the CFT was obtained with INTEM. Eptifibatide markedly increased INTEM-CFT (332 ± 88 s in comparison to 66 ± 6 s in the control; $P=0.001$). Combining eptifibatide with aspirin and ticagrelor did not exert a further increase in INTEM-CFT (226 ± 66 s; $P=0.06$).

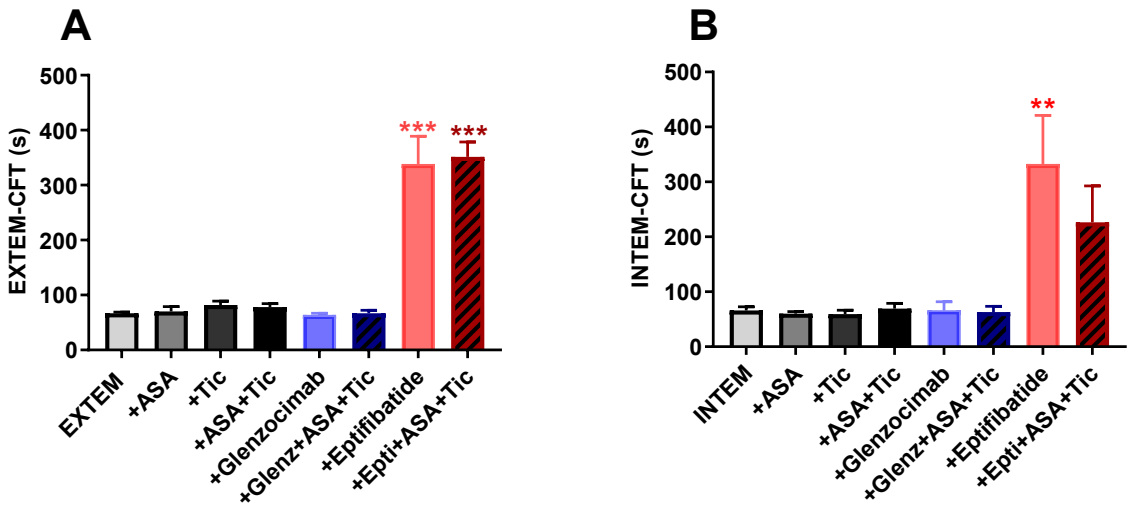

Figure 5.12. Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the clot formation kinetics in the ROTEM as reflected by CFT.

A) Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the EXTEM-CFT. **B)** Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the INTEM-CFT. The effect of different treatment groups was compared the vehicle and assessed using one-way ANOVA with Dunnett's correction for multiple comparisons (n=3, **P<0.01, and ***P<0.001).

5.3.5.3 Glenzocimab did not affect clot firmness, while the GPIIb/IIIa inhibitor eptifibatide significantly reduced them

In ROTEM, clot firmness is assessed by maximum clot firmness (MCF), Maximum clot firmness-time (MCF-t), and amplitude at 5, 10, 15, 20, 25, and 30 min. MCF represents the maximum firmness and strength that the clot exhibits during the measurement. MCF-t is the time from CT until the MCF is reached. Amplitude (A5–A30, mm) reflects the clot firmness at specified time points: 5, 10, 15, 20, 25, and 30 min during the measurement. The effect of glenzocimab and eptifibatide (with and without aspirin and ticagrelor) was assessed on the clot firmness in whole blood coagulation stimulated via extrinsic (EXTEM) and intrinsic (INTEM) coagulation pathways.

The results demonstrated that only eptifibatide significantly decreased blood clot firmness, as revealed by the significant reduction in the MCF and amplitude at every time point from 5 min to 30 min as well as the significant increase in the MCF-t in both EXTEM and INTEM-induced blood coagulation. The combination of eptifibatide with aspirin and ticagrelor did not cause any extra reduction in the clot firmness parameters compared to the effect obtained with eptifibatide alone. In contrast, aspirin and ticagrelor, glenzocimab alone, and glenzocimab with aspirin and ticagrelor had no impact on the previously described EXTEM and INTEM clot firmness parameters (Figure 5.13).

Eptifibatide significantly reduced EXTEM-MCF (48 ± 4 mm in comparison to 65 ± 1 mm in the control; $P<0.001$). No additional effect observed on the EXTEM-MCF with the combination of eptifibatide with aspirin and ticagrelor (48 ± 1 mm; $P<0.001$). Eptifibatide significantly increased EXTEM-MCF-t (2,483 ± 243 s compared to 1,439 ± 108 s in the control; $P=0.01$), indicating a significant delay in reaching the MCF. Combining aspirin and ticagrelor with eptifibatide did produce an important additive effect on the MCF-t-EXTEM (2,533 ± 265 s;

P=0.010). Eptifibatide also significantly reduced EXTEM-amplitude at every time point from 5 min to 30 min throughout the measurement. Eptifibatide reduced EXTEM-A5 (18 ± 1 mm compared to 49 ± 1 mm in the control; P<0.001). Eptifibatide decreased EXTEM-A30 (46 ± 4 mm in comparison to 66 ± 1 mm in the control; P=0.01). No additional effects were observed upon the combination of eptifibatide with aspirin and ticagrelor on the EXTEM-amplitude.

Identical results were also obtained for the effect of the antiplatelet agents on the clot firmness variables on whole blood coagulation initiated through the intrinsic coagulation pathway by INTEM. Only eptifibatide significantly reduced the clot firmness in whole blood clots formed via activation of the intrinsic coagulation pathway. Eptifibatide reduced INTEM-MCF (48 ± 4 mm compared to 65 ± 1 mm in the control; P<0.001). No further decrease was observed in the INTEM-MCF in samples spiked with eptifibatide, aspirin, and ticagrelor (48 ± 1 mm; P=0.005). Regarding the effect on the time to reach the MCF, eptifibatide significantly increased INTEM-MCF-t (2,226 ± 43 s compared to 1,398 ± 113 s in the control; P=0.003). The addition of aspirin and ticagrelor along with eptifibatide did yield an extra increase in the INTEM-MCF-t (2,200 ± 75 s; P=0.004). Eptifibatide's impairment of INTEM-amplitude (A5–A30) was unaffected by the presence of aspirin and ticagrelor. Eptifibatide significantly reduced INTEM-A5 (20 ± 3 mm in comparison to 48 ± 1 mm in the control; P=0.02). Eptifibatide did not significantly decrease INTEM-A30 (45 ± 3 mm compared to 63 ± 1 mm in the control; P=0.13). No extra effects were observed upon the combination of eptifibatide with aspirin and ticagrelor on the INTEM-amplitude.

In summary, these results showed no effect for glenzocimab alone or combined with aspirin and ticagrelor on the clot firmness. In contrast, eptifibatide significantly reduced clot firmness.

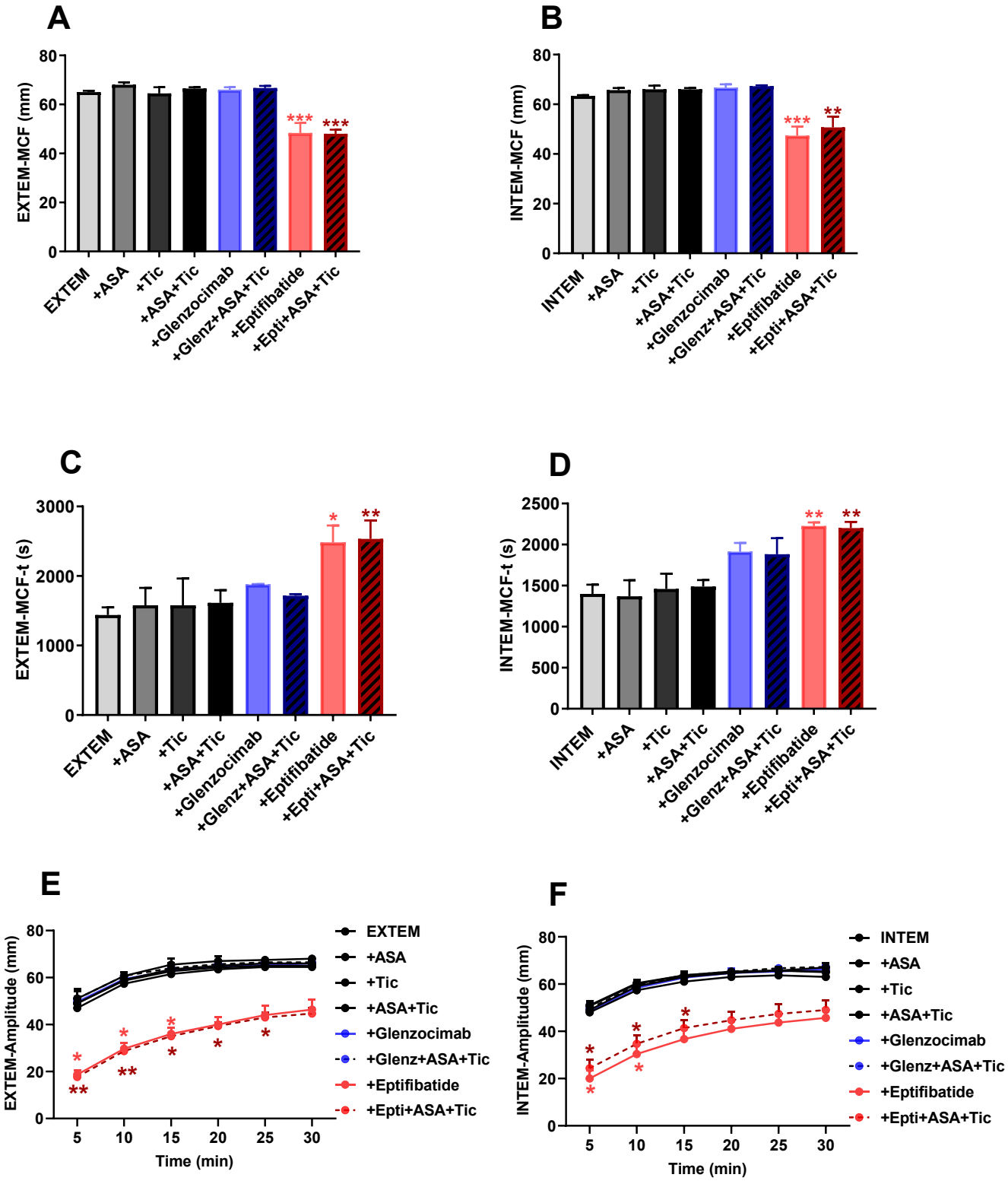
A
EXTEM-MCF (mm)
EXTEM
+ASA
+Tic
+ASA+Tic
+Glenzocimab
+Glenz+ASA+Tic
+Eptifibatide
+Epti+ASA+Tic
B
INTEM-MCF (mm)
INTEM
C
EXTEM-MCF-t (s)
D
INTEM-MCF-t (s)
E
EXTEM-Amplitude (mm)
Time (min)
F
INTEM-Amplitude (mm)

Figure 5.13. Effect of glenzocimab or eptifibatide (± aspirin and ticagrelor) on the whole blood clot firmness stimulated by EXTEM and INTEM.

A) Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the EXTEM-MCF. **B)** Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the INTEM-MCF. **C)** Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the EXTEM-MCF-t. **D)** Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the INTEM-MCF-t. **E)** Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the EXTEM-amplitude (A5-A30). **F)** Effect of 50 µg/ml glenzocimab or 9 µM eptifibatide (± 30 µM aspirin and 1µM ticagrelor) on the INTEM-amplitude (A5-A30). The effect of different treatment groups was compared the vehicle (0.01%DMSO) and assessed using one-way ANOVA with Dunnett's correction for multiple comparisons (n=3, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

5.4 Discussion

The main results for this chapter are as follows: (i) glenzocimab significantly but not completely inhibited polymerised fibrin-induced platelet aggregation; (ii) aspirin and ticagrelor exerted a lesser inhibitory effect on fibrin-induced platelet aggregation than glenzocimab, and eptifibatide exhibited the most potent blockade of platelet aggregation; (iii) glenzocimab showed no effect on platelet spreading and static adhesion on fibrin and fibrinogen, whereas aspirin and ticagrelor exerted partial inhibition of platelet spreading on fibrin but not fibrinogen. Eptifibatide, however, significantly reduced platelet spreading and adhesion on both fibrin and fibrinogen; (iv) the combined use of aspirin and ticagrelor with eptifibatide but not glenzocimab impaired clot retraction; (v) glenzocimab potently blocked platelet procoagulant formation on Horm collagen and atherosclerotic plaque under flow; (vi) glenzocimab as well as eptifibatide in the presence of aspirin and ticagrelor reduced peak thrombin and other parameters in tissue factor-stimulated thrombin generation; (vii) glenzocimab did not affect haemostatic functions measured by ROTEM, which was dramatically affected by eptifibatide.

Glenzocimab delivered a significant but incomplete inhibition of fibrin-stimulated platelet aggregation at the lowest concentration (1 μg/ml) without showing a clear pattern of dose-dependent blockade. The lack of a dose-dependent pattern of inhibition could be attributed to the variations in fibrin amount, as fibrin fibres were observed to be stuck to the wall of the pipette while dispensing it into the samples. Onselaer et al. showed that platelets in GPVI-deficient patients failed to aggregate in response to stimulation with fibrin (Onselaer et al., 2017). In the present work, a portion of platelets still aggregated in response to fibrin despite GPVI blockade with glenzocimab, leading to incomplete inhibition. This finding is consistent with recent work published by Perrella et al. in which they showed that GPVI blockade with

9012 antibody led to substantial inhibition of fibrin-stimulated platelet aggregation (Perrella et al., 2021).

Aspirin and ticagrelor resulted in weaker inhibition of fibrin-induced platelet aggregation than glenzocimab without showing an amplified effect, and eptifibatide exerted the most potent inhibition of fibrin-triggered platelet aggregation. It is striking that aspirin and ticagrelor produced an amplified effect when combined with each other but not with glenzocimab. The lack of synergism between aspirin, ticagrelor, and glenzocimab is at variance with what was achieved with collagen and atherosclerotic plaque, where aspirin and ticagrelor produced a strong amplified effect with glenzocimab on platelet aggregation. Fibrin interacts with GPVI in a different way than with collagen and atherosclerotic plaque. Fibrin is reported to associate with GPVI in a charge-dependant way, and this association can be disrupted by polyanionic molecules (Montague et al., 2020). Monomeric GPVI is the main form of GPVI that contributes to fibrin-mediated platelet activation (Onselaer et al., 2017). GPIIb/IIIa blockade with eptifibatide inhibited fibrin-stimulated platelet aggregation, indicating that platelet aggregation is mediated by GPIIb/IIIa. Montague et al. showed that fibrin causes platelet aggregation independent of GPIIb/IIIa involvement, but they used a different method to stimulate platelet aggregation than the one used in this study (Montague et al., 2020). Consistent with the present result for eptifibatide, Perrella et al. have demonstrated that GPIIb/IIIa antagonism with tirofiban inhibited fibrin-induced platelet aggregation (Perrella et al., 2021).

Platelets showed similar spreading and static adhesion characteristics on polymerised fibrin and fibrinogen. Glenzocimab failed to block platelet spreading and adhesion on both fibrin and fibrinogen under static conditions and showed no additional effect upon combination with aspirin and ticagrelor. Onselaer et al. showed that platelets from homozygous patients (complete GPVI deficiency) did not spread on fibrin and their adhesion markedly decreased (Onselaer et

al., 2017). Mangin et al. showed that platelets from homozygous patients who lack GPVI expression but not from heterozygous patients (50% GPVI expression) failed to spread on immobilised fibrinogen with a partial reduction in static adhesion, which led to the conclusion that fibrinogen is a ligand for GPVI (Mangin et al., 2018). In both studies, no GPVI inhibitors were used to study the effect of GPVI blockade on platelet spreading on fibrin or fibrinogen; only platelets from GPVI-deficient patients were used, which makes it difficult to compare with glenzocimab.

Dual use of aspirin and ticagrelor exerted a greater effect, although still partial, on platelet spreading on fibrin than on fibrinogen. Goncalves et al. reported that ADP and TxA_2 blockade did not block platelet spreading on immobilised fibrinogen and that GPIIb/IIIa mediates platelet spreading and calcium signalling on fibrinogen independently of the positive feedback mediators (Goncalves et al., 2003). Eptifibatide blocked platelet spreading and static adhesion on fibrin and fibrinogen, with a slightly more pronounced effect on fibrinogen. Litvinov et al. showed that fibrin is more reactive to GPIIb/IIIa than fibrinogen, with less sensitivity to GPIIb/IIIa inhibitors, which could explain why eptifibatide exerted a greater inhibitory effect on fibrinogen than on fibrin (Litvinov et al., 2016b).

Fibrin clot retraction is mediated and regulated by FXIII and the outside-in signalling of GPIIb/IIIa (Kasahara et al., 2013). Clot retraction of intravascular thrombi *in vivo* is a critical step to restore blood flow past fibrin-rich thrombus or embolus (Tutwiler et al., 2017). Moreover, it has been shown that clot retraction translocates procoagulant platelets on the thrombus surface (Nechipurenko et al., 2019). The combined use of aspirin and ticagrelor or eptifibatide alone appeared to cause a delay in clot retraction but it was not statistically significant. However, the combined use of aspirin and ticagrelor with eptifibatide produced the strongest impairment of clot retraction, reaching the degree of statistical significance. It has

been reported that both ADP and TxA_2 antagonism inhibited clot retraction in fibrin-rich thrombi after arresting blood flow in a microfluidic chamber coated with collagen (Muthard and Diamond, 2012). Eptifibatide also has been shown before to inhibit fibrin clot retraction (Osdoit and Rosa, 2001). Interestingly, glenzocimab alone produced no significant effect clot retraction, and it did not lead to an additional delay in clot retraction in the presence of aspirin and ticagrelor.

Glenzocimab potently inhibited platelet procoagulant activity as measured by phosphatidylserine exposure in blood flowing on Horm collagen and atherosclerotic plaque at an arterial shear rate. Aspirin and ticagrelor together and eptifibatide alone appeared to have a little effect on procoagulant platelet formation on Horm collagen. These antiplatelet agents only partially reduced fluorescence intensity that reflects procoagulant platelet thrombi formation without an effect on the percentage of platelet surface coverage that reflects adherent procoagulant platelets on collagen. In contrast, on atherosclerotic plaque, aspirin and ticagrelor together and eptifibatide alone greatly reduced platelet procoagulant activity; however, that this effect was observed on atherosclerotic plaque but not on Horm collagen could be due to the difference in the magnitude of platelet procoagulant activity observed on collagen and plaque. Untreated platelets in blood flowing on Horm collagen showed stronger phosphatidylserine exposure than platelets flowing on atherosclerotic plaque. Collagen is the most prominent adhesive ligand for platelet procoagulant generation, accounting for the generation of 40%–80% of adherent procoagulant platelets (Agbani and Poole, 2017). GPVI, as a major receptor for collagen, mediates the platelet procoagulant response (Heemskerk et al., 1999). Phosphatidylserine exposure is completely eliminated in blood flowing on collagen under coagulating conditions from GPVI-deficient patients (Nagy et al., 2020). The potent inhibition of platelet procoagulant formation achieved by glenzocimab is consistent with the principal role

of GPVI in mediating platelet procoagulant formation. GPVI inhibition has been shown to inhibit phosphatidylserine exposure and subsequently attenuate thrombin generation and fibrin formation (Munnix Imke et al., 2005, Lecut et al., 2003). Aspirin and ticagrelor augmented the effect of glenzocimab on phosphatidylserine exposure on collagen. The partial effect of combined aspirin and ticagrelor is in agreement with some clinical studies. Platelet procoagulant formations are partially suppressed in patients on dual antiplatelet therapy, which could pose a risk for thrombotic events (Pasalic et al., 2018). The inability of eptifibatide to block phosphatidylserine exposure is consistent with previous studies that showed the lack of the effect of platelet pre-treatment with eptifibatide on collagen-mediated procoagulant responses (Furman et al., 2000, Lages and Weiss, 2001).

Glenzocimab significantly reduced some tissue factor-stimulated thrombin generation parameters in CAT, mainly peak thrombin, velocity index, and start tail, without an additional effect for aspirin and ticagrelor. Among all the tested antiplatelet agents, glenzocimab exerted the largest reduction on peak thrombin. Increased peak thrombin has been shown to be associated with the risk of thrombosis, and it is increased in patients with acute myocardial infarction (Lutsey et al., 2009, Loeffen et al., 2016). The role of GPVI in the amplification of tissue factor-induced thrombin generation (collagen-independent thrombin generation) is attributed to mediating platelet procoagulant activity via fibrin (Mammadova-Bach et al., 2015). Dual antiplatelet therapy does not affect thrombin generation, which poses a risk for recurrent thrombosis. It is therefore suggested to use anticoagulant therapy jointly with antiplatelet therapy (Yano et al., 2008). Furthermore, thrombin generation as assessed by peak thrombin and velocity index has been shown to be persistently elevated for a long time in patients with acute coronary syndrome (Yip et al., 2020). Eptifibatide reduced peak thrombin and velocity index with the addition of aspirin and ticagrelor. It has been shown that GPIIb/IIIa

blockade suppressed tissue factor-induced phosphatidylserine exposure and subsequent procoagulant responses (van der Meijden et al., 2012).

Glenzocimab alone or combined with aspirin and ticagrelor did not affect haemostasis as evaluated by ROTEM, which is used to assess clot kinetics including clot initiation, clot formation, clot strength, and clot lysis. In contrast, eptifibatide dramatically affected clot formation and clot strength. None of the investigated antiplatelet agents affected CT (clot initiation). CT, similar to traditional coagulation tests prothrombin time (PT) and activated partial thromboplastin time (APTT) , is a sensitive measurement for coagulation cascades, and so it was not influenced by antiplatelet agents (Vedovati et al., 2020). Eptifibatide markedly reduced CFT (clot formation time), which measure the speed at which a clot is formed and the rapidity of fibrin polymerisation, which is affected by platelet function, fibrinogen, and other coagulation factors (Whiting et al., 2015). Eptifibatide has been shown to prolong the time required for clot formation in blood coagulation stimulated by tissue factor (Butenas et al., 2001). Eptifibatide reduced clot strength as reflected by amplitude (A5–A30 min), MCF and MCF-t. The Society of Cardiovascular Anaesthesiologists' guidelines on the management of perioperative bleeding and haemostasis in cardiac surgery patients recommended transfusing platelet concentrate in patients with EXTEM-A10 < 40 mm (Raphael et al., 2019). Eptifibatide reduced EXTEM-A10 by 30 mm. MCF is largely dependent on platelet count and platelet function (Ranucci and Baryshnikova, 2020). GPIIa/IIIb inhibitors reduce clot firmness and stiffness by blocking platelets and fibrin mesh interaction (Khurana et al., 1997). Eptifibatide has been used to produce an *in vitro* model of Glanzmann thrombasthenia in ROTEM (Shenkman et al., 2012). Overall, eptifibatide prolonged CFT and reduced MCF, and these parameters are recommended to be used in treatment algorithms as a guide for the management of severe bleeding (Lier et al., 2013).

5.5 Limitations

Some practical limitations in the fibrin preparation should be pointed out. Although PPACK was added after the fibrin was formed to neutralise thrombin residuals, thrombin was not measured in the fibrin suspension that was used to stimulate platelets (aggregation and spreading) which could overcome the inhibitory effect of glenzocimab. Nevertheless, PPACK has the ability to inhibit thrombin through its exosite, and it has been shown to neutralise thrombin bound to fibrin (Kaminski et al., 1991, Weitz et al., 1990). FXIII which was used to cross-link fibrin has been reported to activate platelets via GPIIb/IIIa and integrin $\alpha_v\beta_3$ which could undermine the inhibitory effect of glenzocimab (Magwenzi et al., 2011). Finally, the number of experiments (replicates) for phosphatidylserine exposure, fibrin clot retraction and ROTEM was relatively low which could undermine the statistical power and ultimately change the interpretation of the results.

5.6 Summary

In summary, glenzocimab reduced fibrin-induced platelet aggregation more than aspirin and ticagrelor without changing the effect of aspirin and ticagrelor on platelet spreading and static adhesion on fibrin or fibrinogen and fibrin clot retraction. Glenzocimab potently blocked platelet procoagulant activity and reduced some tissue factor-stimulated thrombin generation parameters amplified by procoagulant platelet formation. Furthermore, glenzocimab either alone or combined with aspirin and ticagrelor did not affect general haemostasis, as measured using ROTEM, which was affected by eptifibatide.

CHAPTER 6 : THE ADDITIVE EFFECT OF GLENZOCIMAB ON DIFFERENT PLATELET ACTIVATION MECHANISMS *EX VIVO* ON SAMPLES FROM PATIENTS WITH ACS-TREATED WITH ASPIRIN AND TICAGRELOR

6.1 Introduction

Patients with ST-segment elevation myocardial infarction (STEMI), the most severe form of acute coronary syndrome, need immediate and definitive treatment. Patients presenting with STEMI should undergo primary PCI in the cardiac catheterisation laboratory to restore blood flow no later than 120 minutes after diagnosis. In addition to the revascularisation procedure, dual antiplatelet therapy with aspirin and $P2Y_{12}$ inhibitor is initiated. Patients also receive anticoagulant therapy, mostly unfractionated heparin as an adjunct to PCI. Fibrinolysis is considered in patients within 12 hours of symptoms if there is a delay in PCI (i.e., it cannot be completed within 120 minutes) (Neumann et al., 2019). GPIIb/IIIa inhibitors are not routinely used in STEMI due to the risk of bleeding associated with their administration (Ali et al., 2004). Their use is limited to patients with a heavy thrombus burden, particularly during PCI intervention, where dual antiplatelet therapy is not enough to completely block thrombosis (Neumann et al., 2019).

Many improvements in antiplatelet therapy for patients with STEMI are still required. Dual antiplatelet therapy agents are taken orally, which delays their antithrombotic effects. In part, this undermines their benefits, especially for patients with active platelet thrombosis such as STEMI. Furthermore, dual antiplatelet therapy is not sufficient in patients with heavy a thrombus burden as it requires a more potent antiplatelet therapy such as GPIIb/IIIa inhibitors, which prevent platelet thrombosis but at the expense of bleeding. Recurrent thrombosis and stent thrombosis are also risks for a patient with ACS. The incidence of stent thrombosis is estimated to occur in 2% of patients in the first year following stent implantation and is associated with up to 45% risk of increased mortality and morbidity (Barra et al., 2016). Prolonged use of DAPT has been shown to reduce stent thrombosis, but it increases the risk of bleeding (Stefanescu Schmidt Ada et al., 2017). The limitations of current antiplatelet therapy

represent an unmet need to explore the therapeutic benefits of potential and novel antiplatelet targets such as GPVI.

6.2 Aims

This chapter aims to investigate the additive effect of glenzocimab and eptifibatide on platelet-mediated mechanisms and haemostatic function *ex vivo* on blood sampled from patients with ACS (STEMI) who are treated with aspirin and ticagrelor.

6.3 Results

6.3.1 Demographic data and baseline characteristics of patients with ACS included in this study

All ACS patients were recruited from Birmingham city hospital in the period between 07/2019 and 02/2020. The details for the demographic data and baseline characteristics of patients included in the study are summarised in **table 6.1.** The total number of enrolled patients is 20. The age mean for patients is 60 years and 85% of them were men. In the term of ethnicity, 75% of the patients were Caucasians. In the term of ACS class, 70% of the patients were STEMI patients , 25% were NSTEMI patients, and 5% were US patients. All the patients on aspirin and ticagrelor. 85% of the patients were on beta blockers and 65% of the patients were on ACE inhibitors. All the patients were treated with statin.

Table 6.1 Baseline characteristics of patients with acute coronary syndromes

Characteristic	
Age – median (IQR)	60.5 (55.75, 65)
Male gender – no. (%)	17/20 (85%)
Ethnicity	
Caucasian – no. (%)	15/20 (75%)
Black – no. (%)	1/20 (5%)
Asian – no. (%)	4/20 (20%)
Current smoker – no. (%)	6/20 (30%)
Hypertension – no. (%)	6/20 (30%)
Diabetes mellitus – no. (%)	3/20 (15%)
Current presentation STEMI – no. (%)	14/20 (70%)
Current presentation NSTEMI – no. (%)	5/20 (25%)
Current presentation unstable angina– no. (%)	1/20 (5%)
Prior history of MI – no. (%)	5/20 (25%)
Prior history of PCI – no. (%)	2/20 (10%)
Previous coronary bypass grafting – no. (%)	0/20 (0%)
Heart failure – no. (%)	0/20 (0%)

Stroke – no. (%)	1/20 (5%)
Concomittant medication	
Aspirin	20/20 (100%)
Ticagrelor	20/20 (100%)
Beta blocker	17/20 (85%)
ACE inhibitor	13/20 (65%)
Statin	20/20 (100%)

6.3.2 The additive effect of glenzocimab and eptifibatide on platelet aggregation stimulated by GPVI and non-GPVI agonists

The effect of glenzocimab and eptifibatide was investigated on platelet aggregation induced by GPVI ligands, collagen and CRP-XL. The effect of glenzocimab and eptifibatide was further investigated on platelet aggregation induced by non-GPVI agonists (ADP, AA, and TRAP) to rule out off-targets for GPVI inhibition with glenzocimab.

6.3.2.1 Glenzocimab significantly inhibited platelet aggregation mediated by Horm collagen in MEA

Glenzocimab previously indicated an amplified effect with empirical concentrations of aspirin and ticagrelor on collagen-mediated platelet aggregation in samples collected from healthy donors, as presented in Chapter 3. This observation was extended into collagen-stimulated platelet aggregation from patients with ACS receiving the recommended doses of aspirin and

ticagrelor to observe the magnitude of the effect of standard treatment with aspirin and ticagrelor on collagen-induced platelet aggregation before and after the addition of glenzocimab or eptifibatide *ex vivo*. The MEA results demonstrate that routine dual antiplatelet therapy moderately inhibits collagen-mediated platelet aggregation. The addition of glenzocimab provided significant inhibition of platelet aggregation, and the addition of eptifibatide completely inhibited platelet aggregation, as presented in Figure 6.1.

Spontaneous platelet aggregation (unstimulated samples) was 4 ± 1 U. Platelets stimulated with 3.2 µg/ml collagen induced a residual platelet aggregation of 26 ± 1 U, which was used as the baseline to check for the additive effect of glenzocimab and eptifibatide. Spiking the samples with glenzocimab reduced platelet aggregation by 49% (13 ± 1 U; $P<0.001$), while eptifibatide reduced platelet aggregation by 82% (4 +1 U; $P<0.001$), which was equivalent to spontaneous aggregation in the unstimulated sample. The incomplete inhibition of collagen induced platelet aggregation by glenzocimab could be due to the involvement of other collagen receptor GPIa/IIa as highlighted in Chapter 3.

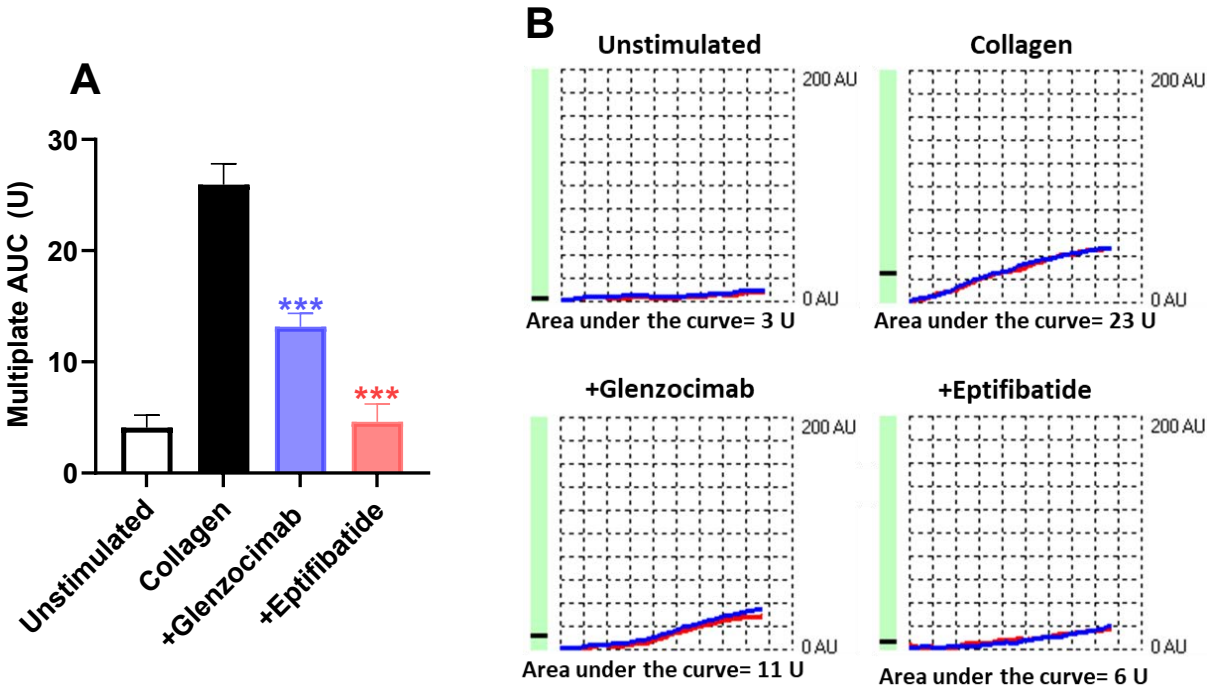

Figure 6.1. Additive effect of glenzocimab or eptifibatide on collagen-induced platelet aggregation in whole blood samples form patients with ACS on aspirin and ticagrelor assessed by MEA.

A) Effect of glenzocimab or eptifibatide as well as the baseline samples for platelet aggregation induced by 3.2 µg/ml Horm collagen. **B)** Representative aggregation traces for a single MEA experiment, where hirudin-anticoagulated whole blood was diluted in half with physiological normal saline then incubated with nothing (control or baseline), 50 µg/ml glenzocimab or 9 µM eptifibatide for 10 min at 37C° under stirring conditions, then platelet aggregation was stimulated by 3.2 µg/ml Horm collagen and monitored for 6 min in the the Multiplate® analyser. The effect of glenzocimab and eptifibatide was compared to the baseline and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=20, ***P<0.001).

6.3.2.2 Glenzocimab completely inhibited residual GPVI-mediated platelet aggregation as assessed by CRP-XL in MEA

Two concentrations (3 and 10 µg/ml) of the specific GPVI agonist, CRP-XL, were used to identify the effect of standard dual antiplatelet therapy (aspirin and ticagrelor) on GPVI activity and to see if glenzocimab can block platelet aggregation upon the activation of GPVI by CRP-XL. The results demonstrate significant residual GPVI activity that induced platelet aggregation despite treatment with standard doses of aspirin and ticagrelor, as illustrated in Figure 6.2. Furthermore, glenzocimab was shown to be able to completely block the residual GPVI activity.

Platelet aggregation was 21 ± 2 U in the unspiked samples that were stimulated by 3 µg/ml CRP-XL. Glenzocimab blocked platelet aggregation by 88% (2 ± 1 U; $P<0.001$), which was lower than spontaneous platelet aggregation (4 ± 1 U). Eptifibatide inhibited platelet aggregation by 90% (2 ± 1 U; $P<0.001$).

In contrast to 3 µg/ml CRP-XL, platelet activation with 10 µg/ml CRP-XL induced stronger platelet aggregation (33 ± 3 U) (see Figure 6.2A). Despite the important increase in platelet aggregation obtained with 10 µg/ml CRP-XL, glenzocimab still profoundly inhibited platelet aggregation by 93% (2 ± 1 U; $P<0.001$) and eptifibatide inhibited platelet aggregation by 96% (1 ± 1 U; $P<0.001$).

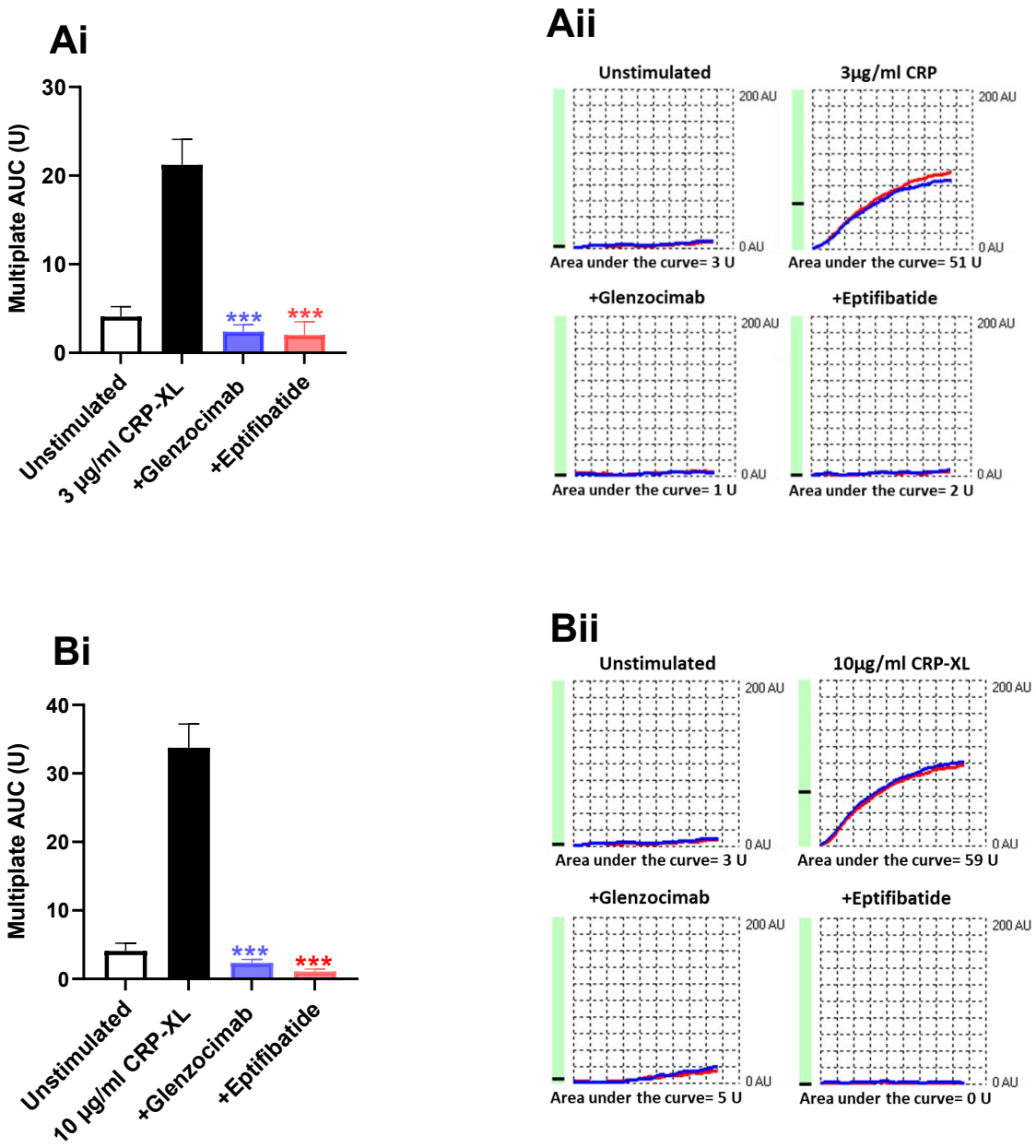

Figure 6.2. Blockade of CRP-XL-induced platelet aggregation by glenzocimab and eptifibatide in whole blood samples from patients with ACS on aspirin and ticagrelor assessed by MEA.

Ai-ii) Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) as well as the baseline samples for platelet aggregation induced by 3 µg/ml CRP-XL. **Bi-ii)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) as well as the baseline samples for platelet aggregation stimulated by 10 µg/ml CRP-XL. The effect of glenzocimab and eptifibatide was compared to baseline and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=20, ***P<0.001).

6.3.2.3 Glenzocimab did not indicate off-target effects on platelet aggregation mediated by non-GPVI agonists, as assessed in MEA

To discover whether glenzocimab has off-target inhibition outside the scope of GPVI-mediated platelet activation and to explore the effect of standard antiplatelet therapy on its targets, the effect of glenzocimab and eptifibatide was investigated on platelet aggregation triggered via non-GPVI agonists, mainly those platelet receptors or pathways targeted by aspirin and ticagrelor, respectively. In addition, the effect of glenzocimab and eptifibatide was further evaluated on TRAP-induced platelet aggregation, which has been used in clinical settings to monitor antiplatelet therapy with GPIIb/IIIa inhibitors such as eptifibatide. The results indicated that GPVI blockade with glenzocimab did not exert any off-target inhibitory effects, whereas eptifibatide completely blocked TRAP-induced platelet aggregation and the residual ADP-induced platelet aggregation (Figure 6.3). Furthermore, treatment with standard doses of aspirin and ticagrelor blocked their targets effectively as expected. The agonists (ADP, AA, and TRAP) were used at concentrations that were recommended in clinical settings to monitor antiplatelet therapy with aspirin , $P2Y_{12}$ inhibitors, and GPIIb/IIIa inhibitors.

In ADP-stimulated platelet aggregation, as illustrated in Figure 6.3A, the platelet aggregation in baseline samples was 8 ± 1 U compared to 3 ± 1 U in the unstimulated samples, indicating that antiplatelet therapy with ticagrelor achieved a significant inhibition of $P2Y_{12}$. Glenzocimab did not affect the residual platelet aggregation (5 ± 1 U; P=0.17), whilst eptifibatide significantly inhibited platelet aggregation (1 ± 1 U; P=0.004). As shown in Figure 6.3B, routine treatment with aspirin potently inhibited arachidonic acid-induced platelet aggregation (3 ± 1 U). Treatment with aspirin and ticagrelor exerted a partial inhibition of PAR1-mediated platelet aggregation, as reflected by TRAP-stimulated platelet aggregation (37 ± 4 U in the baseline samples), as presented in Figure 6.3C. Glenzocimab did not reduceplatelet aggregation

(36 ± 6 U; P>0.99). However, eptifibatide blocked TRAP-induced platelet aggregation by 96% (1 ± 1 U; P<0.001).

In summary, dual antiplatelet therapy with aspirin and ticagrelor blocked their targets effectively. Dual antiplatelet therapy marginally reduced TRAP-induced platelet aggregation. Addition of glenzocimab did exert any off-taret inhibitory effect on ADP and TRAP-induced platelet aggregation, whereas eptifibatide entirely blocked ADP and TRAP-induced platelet aggregation.

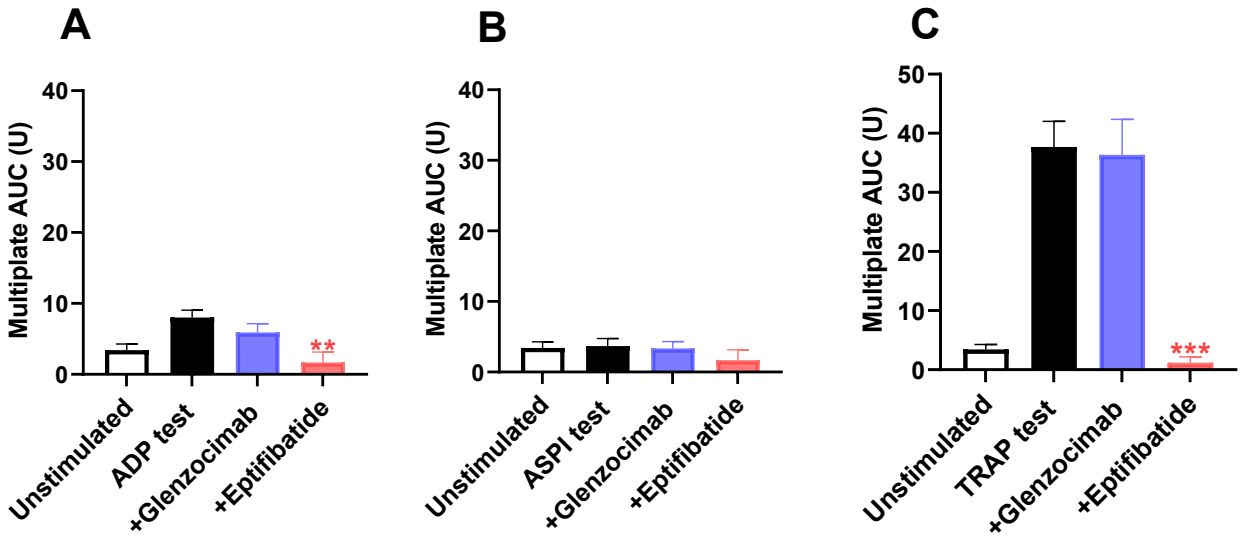

Figure 6.3. Glenzocimab did not show off-target effects on platelet aggregation mediated by non-GPVI agonists in whole blood samples from patients with ACS on aspirin and ticagrelor assessed by MEA.

A) Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) as well as the baseline samples for platelet aggregation stimulated by 6.5 µM ADP. **B)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) as well as the baseline samples for platelet aggregation induced by 0.5 µM AA. **C)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) as well as the baseline samples for platelet aggregation induced by 32 µM TRAP. The effect of glenzocimab and eptifibatide was compared to the baseline and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=15, **P<0.01, and ***P<0.001).

6.3.3 Glenzocimab further inhibited platelet thrombus formation and adhesion in a microfluidic chamber coated with Horm collagen under an arterial shear rate

As outlined in Chapter 3, spiking healthy donor samples with glenzocimab as well as combined concentration of aspirin and ticagrelor significantly inhibits platelet thrombus formation, but not platelet adhesion in a microfluidic chamber coated with 200 µg/ml Horm collagen. It is surprising that aspirin and ticagrelor, at the experimental concentrations used, provided a similar effect to that achieved with glenzocimab alone without showing a clear amplified effect upon the combination. To further understand the exact effect of routine treatment of aspirin and ticagrelor in patients with ACS on platelet adhesion and thrombus formation on Horm collagen under flow conditions, and to determine if spiking the samples with glenzocimab can yield an additional antiplatelet effect, unspiked and spiked citrate-anticoagulated whole blood with glenzocimab or eptifibatide were flowed in a microfluidic chamber coated with 200 µg/ml collagen at a shear rate of 1000 s^{-1}.

As highlighted in Figure 6.4, dual treatment with aspirin and ticagrelor reduced, but did not prevent, thrombus formation and platelet adhesion on Horm collagen, as evaluated by fluorescence intensity and surface coverage percentage. However, the addition of glenzocimab and eptifibatide provided additional inhibition. Glenzocimab reduced thrombus formation by 47% (11 ±1 AU compared to 21 ± 2 AU in the baseline samples; $P<0.001$). Eptifibatide further reduced thrombus formation by 89% (2 ± 1 AU; $P<0.001$). With regard to platelet adhesion, glenzocimab decreased the surface coverage by 28% (41 ± 4% compared to 58 ± 3% in the baseline samples; $P<0.001$). On the other hand, eptifibatide reduced surface coverage by 89% (6 ± 1%; $P<0.001$).

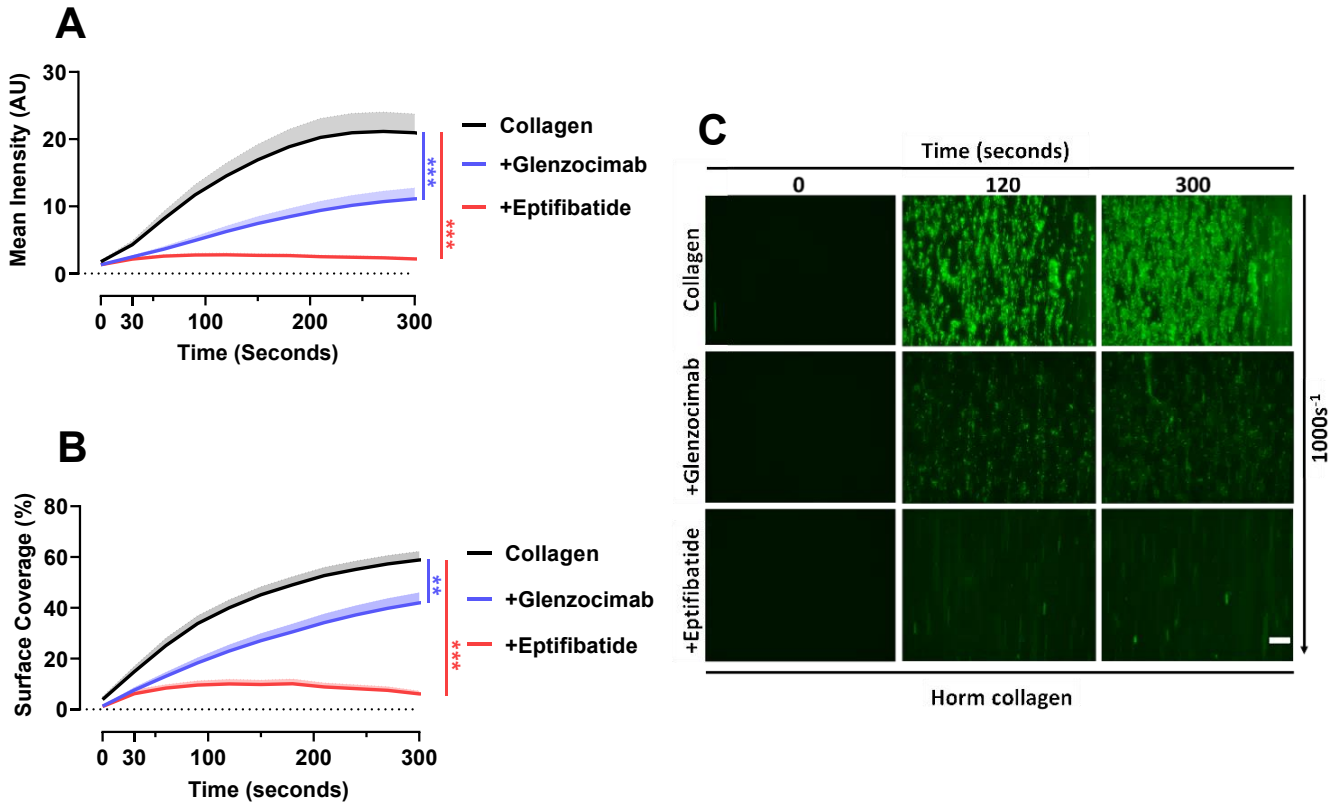

Figure 6.4. Effect of glenzocimab or eptifibatide on platelet adhesion and platelet-thrombus formation on Horm collagen in a microfluidic chamber under an arterial shear rate in whole blood samples from patients with ACS-treated with aspirin and ticagrelor.

A) Effect of glenzocimab or eptifibatide on platelet thrombus formation as reflected by flouroscence intensity. **B)** Effect of glenzocimab or eptifibatide on platelet adhesion as reflected by platelet surface coverage %. **C)** Representative images from a single flow adhesion experiment, where $DiOC_6$-labelled citrated blood was incubated with 50 µg/ml glenzocimab or 9 µM eptifibatide for 15 min at 37°C then flowed over 200 µg/ml Horm collagen at a shear rate of 1000 s^{-1} for 5 min. Images were taken every 30 seconds during flow using evos FL Auto 2 Imaging System. Images were analysed by ImageJ software (NIH ImageJ, Version 1.42-l). Scale bar 50 µm. The effect of glenzocimab and eptifibatide was compared to baseline samples and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=18, **P<0.01, and ***P<0.001).

6.3.4 Glenzocimab and eptifibatide reduced platelet-dependent thrombin generation peak upon stimulation with tissue factor in CAT in samples from patients with ACS on aspirin and ticagrelor

The effect of glenzocimab and eptifibatide was investigated *ex vivo* on tissue factor-induced thrombin generation in samples from patients with ACS-treated with aspirin and ticagrelor. CAT results indicate that both glenzocimab and eptifibatide provide a significant reduction in peak thrombin only.

Glenzocimab reduced peak thrombin by 26% (110 ± 26 nM compared to 149 ± 28 nM in the baseline samples; P=0.03) while eptifibatide decreased peak thrombin by 30% (103 ± 28 nM; P=0.04), as presented in Figure 6.5A. Glenzocimab and eptifibatide did not show effects on other CAT parameters such as velocity index, lag time, time to peak, and ETP.

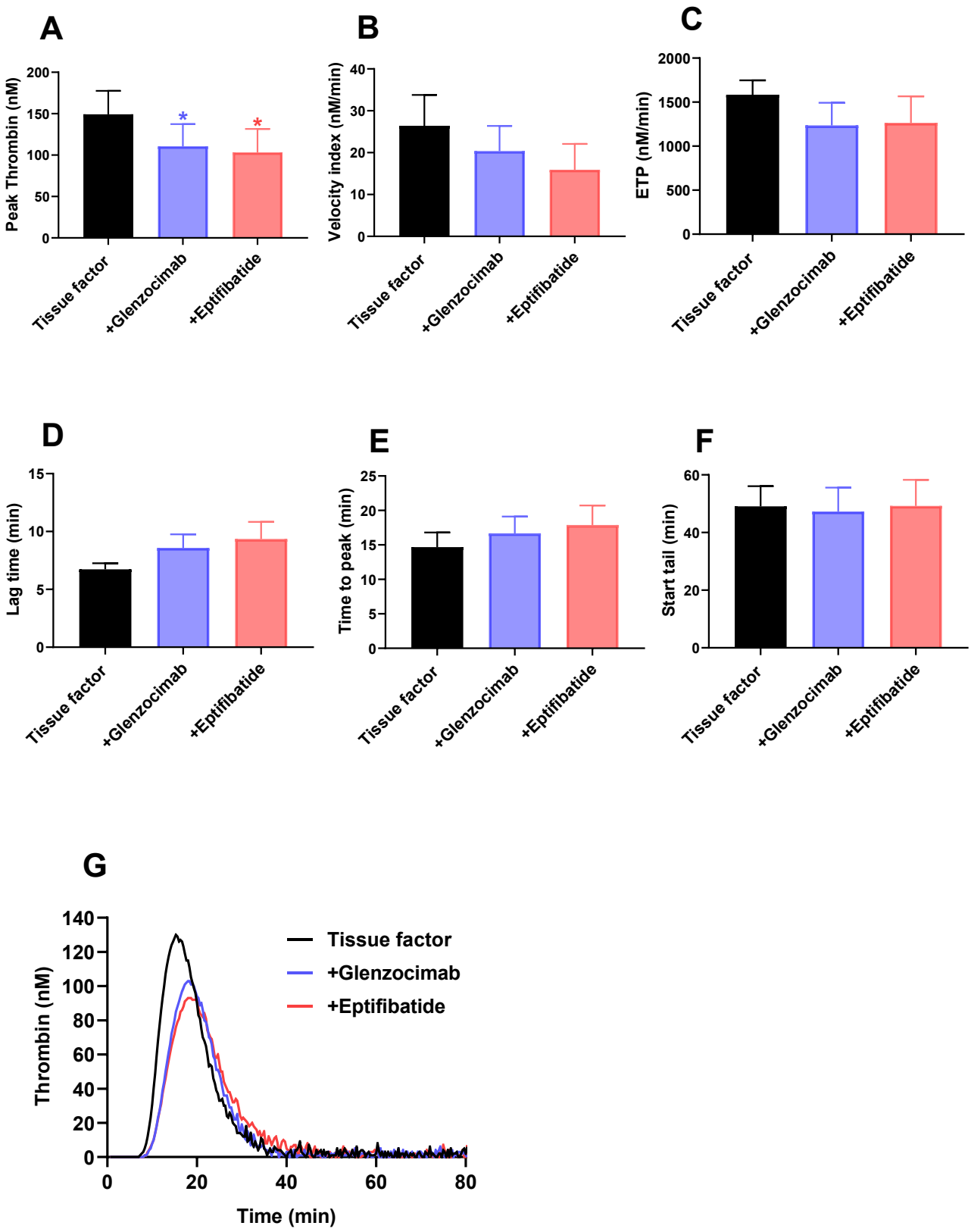
A
Peak Thrombin (nM)
200
150
100
50
0
*
*
Tissue factor
+Glenzocimab
+Eptifibatide
B
Velocity index (nM/min)
40
30
20
10
0
Tissue factor
+Glenzocimab
+Eptifibatide
C
ETP (nM/min)
2000
1500
1000
500
0
Tissue factor
+Glenzocimab
+Eptifibatide
D
Lag time (min)
15
10
5
0
Tissue factor
+Glenzocimab
+Eptifibatide
E
Time to peak (min)
25
20
15
10
5
0
Tissue factor
+Glenzocimab
+Eptifibatide
F
Start tail (min)
60
40
20
0
Tissue factor
+Glenzocimab
+Eptifibatide
G
Thrombin (nM)
140
120
100
80
60
40
20
0
0
20
40
60
80
Time (min)
Tissue factor
+Glenzocimab
+Eptifibatide

Figure 6.5. Effect of glenzocimab or eptifibatide on tissue factor-stimulated thrombin generation in PRP samples from patients with ACS-treated with aspirin and ticagrelor assessed using CAT.

A) Effect of glenzocimab or eptifibatide on the peak thrombin. **B)** Effect of glenzocimab or eptifibatide on the velocity index. **C)** Effect of glenzocimab or eptifibatide on the endogenous thrombin potential (ETP). **D)** Effect of glenzocimab or eptifibatide on the lag time. **E)** Effect of glenzocimab or eptifibatide on the time to peak. **F)** Effect of glenzocimab or eptifibatide on the start tail. **G)** a representative thrombogram from a single CAT experiment, where PRP was treated with 50 μg/ml glenzocimab and 9 μM eptifibatide, or nothing (baseline) then platelets were incubated with PRP reagent that contains 0.5 pM TF with a minimal amount of phospholipids in 96-well plate for 10 min. The thrombin generation was initiated by automatic dispensing of the FluCa reagent which contains a mixture of fluorogenic substrate and $CaCl_2$, thrombin generation was monitored with a Fluoroscan Ascent plate reader, and the thrombin generation data were calculated against thrombin calibrator using Thrombinoscope® software. Effect of glenzocimab and eptifibatide was compared to the baseline samples and and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=7, *P<0.05).

6.3.5 Effect of glenzocimab and eptifibatide on haemostatic function and clot properties during assessment of blood coagulation in ROTEM in samples from patients with ACS on aspirin and ticagrelor

The effect of glenzocimab and eptifibatide was investigated *ex vivo* on whole blood coagulation stimulated by extrinsic and intrinsic coagulation pathways in samples collected from patients with ACS on aspirin and ticagrelor. Clot initiation, clot formation, clot firmness and strength, and clot lysis parameters were analysed for glenzocimab and eptifibatide.

6.3.5.1 Glenzocimab showed no effect on the clot initiation parameter, as reflected by clotting time

Glenzocimab and eptifibatide did not affect clot initiation in whole blood coagulation, as assessed by clotting time (CT) by EXTEM and INTEM, as illustrated in Figure 6.6. In baseline (unspiked) samples, EXTEM- CT was 61 ± 3 s. Glenzocimab exerted no effect on the EXTEM-CT (64 ± 3 s; P=0.77), and eptifibatide also had no impact on the EXTEM-CT (56 ± 3 s; P=0.52). INTEM-CT was 178 ± 7 s in baseline samples. In addition, glenzocimab did not significantly affect INTEM-CT (194 ± 11 s; P=0.42), and eptifibatide also demonstrated no effect on the INTEM-CT (179 ± 13 s; P>0.99).

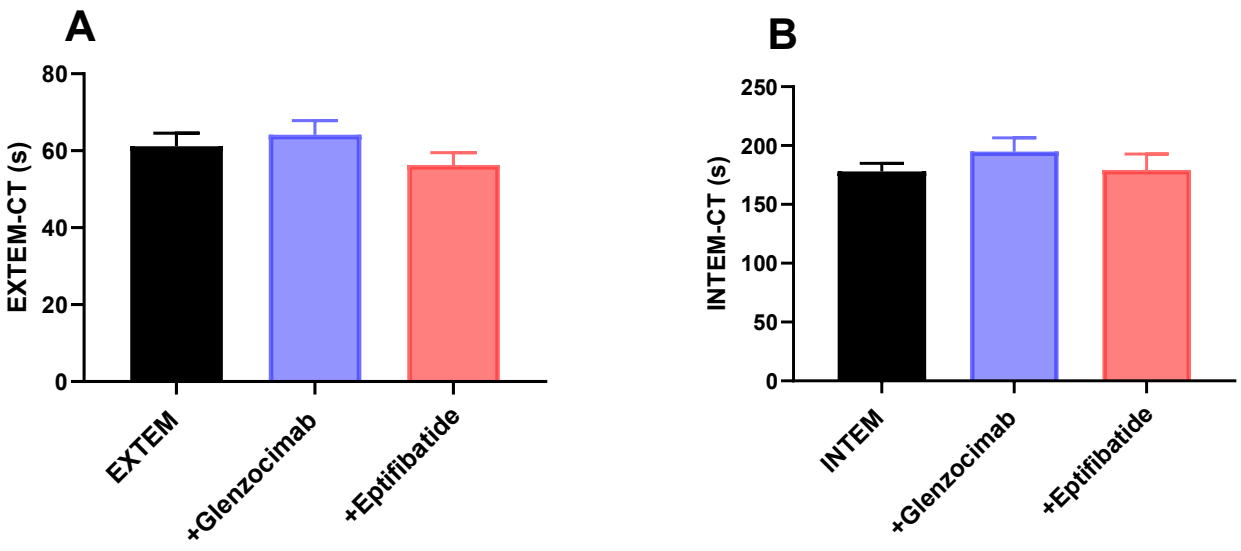

Figure 6.6. Effect of glenzocimab or eptifibatide on the clotting time in whole blood samples from patients with ACS-treated with aspirin and ticagrelor assessed by ROTEM. **A)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM-CT. **B)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the INTEM-CT. The effect of gelenzocimab and eptifibatide was compared to baseline samples and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=7, ns=non-statistically significant).

6.3.5.2 Glenzocimab did not affect clot formation time, while the GPIIb/IIIa inhibitor, eptifibatide, significantly reduced clot formation time

Eptifibatide, but not glenzocimab, significantly affected clot formation time (CFT) *ex vivo* in whole blood coagulation stimulated by EXTEM and INTEM in samples from patients with ACS on aspirin and ticagrelor as depicted in Figure 6.7.

Glenzocimab did not affect EXTEM-CFT (67 ± 5 s compared to 62 ± 5 s in the baseline samples; P>0.99). Conversely, eptifibatide significantly prolonged EXTEM-CFT (259 ± 86 s; P=0.02). Glenzocimab did not impact INTEM-CFT (66 ± 8 s compared to 67 ± 12 s in the baseline samples; P>0.99), while eptifibatide significantly extended INTEM-CFT (246 ± 50 s; P<0.001)

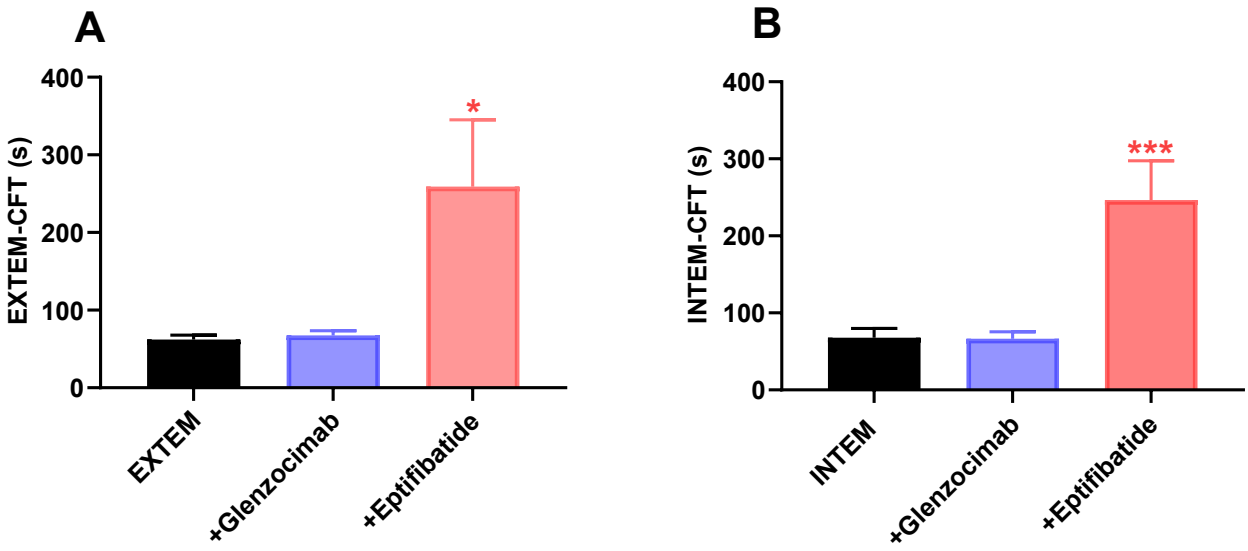

Figure 6.7. Effect of glenzocimab or eptifibatide on the clot formation time in whole blood samples from patients with ACS-treated with aspirin and ticagrelor assessed by ROTEM

A) Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM-CFT. **B)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the INTEM-CFT. The effect of gelenzocimab and eptifibatide was compared to baseline samples and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=7, *P<0.05, and ***P<0.001).

6.3.5.3 Glenzocimab did not influence the clot strength parameters, as reflected by maximum clot firmness (MCF) and amplitude (A5–A30), while eptifibatide significantly affected them

Eptifibatide, but not glenzocimab, decreased clot strength *ex vivo* in whole blood coagulation stimulated by EXTEM and INTEM in samples collected from patients with ACS on aspirin and ticagrelor, as presented in Figure 6.8.

Glenzocimab did not affect EXTEM-MCF (70 ± 1 mm compared to 67 ± 1 mm in the baseline samples; P= 0.69), but eptifibatide significantly reduced EXTEM-MCF (53 ± 4 mm; P=0.01). With regard to INTEM-MCF, neither glenzocimab nor eptifibatide had an impact on it (69 ± 2 mm; P=0.22 and 57 ± 5 mm; P=0.22 respectively, compared to 66 ± 1 mm in the baseline samples). However, glenzocimab and eptifibatide increased the time to reach MCF in EXTEM and INTEM. Glenzocimab and eptifibatide increased EXTEM-MCF-t (1,885 ± 123 s; P=0.006 and 2,860 ± 172 s; P<0.001 compared to 1196 ± 133 s in the baseline samples). In addition, glenzocimab and eptifibatide increased INTEM-MCF-t (1,754 ± 155 s; P=0.04 and 2,367 ± 142 s; P<0.001 compared to 1,271 ± 119 s in the baseline samples).

Eptifibatide reduced amplitude (A5–A30), which represents clot strength at specific time points. A10 is widely used in the treatment logarithms to guide blood transfusion. Glenzocimab did not affect EXTEM-A10 (63 ± 1 mm compared to 62 ± 2 mm in the baseline samples; P=0.96), whereas eptifibatide significantly reduced EXTEM-A10 (33 ± 3 mm; P<0.001). In addition, glenzocimab did not affect INTEM-A10 (60 ± 1 mm compared to 59 ± 2 mm in the baseline samples; P=0.91), whilst eptifibatide greatly reduced INTEM-A10 (35 ± 3 mm; P<0.001).

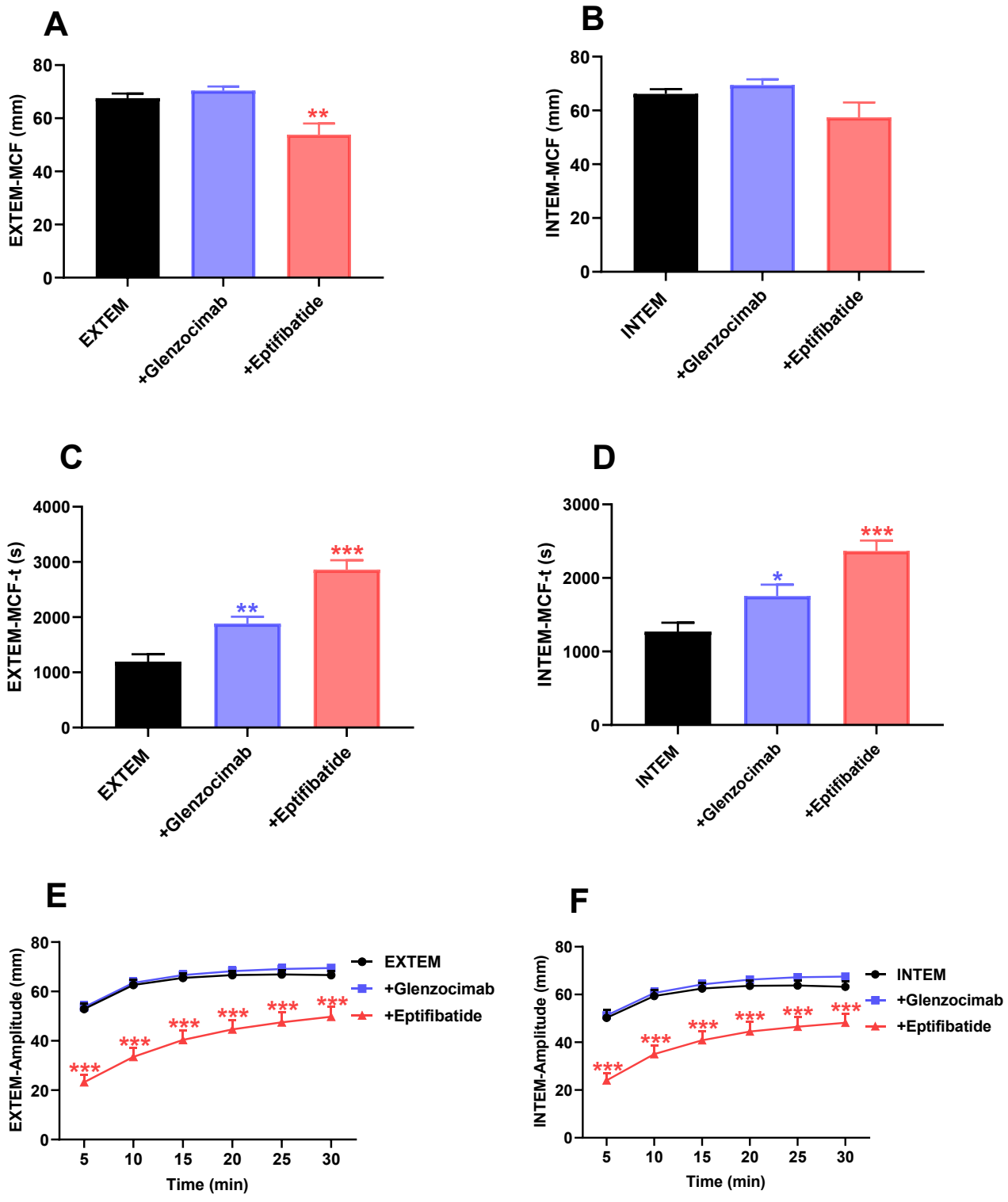
A
EXTEM-MCF (mm)
80
60
40
20
0
**
EXTEM
+Glenzocimab
+Eptifibatide
B
INTEM-MCF (mm)
80
60
40
20
0
INTEM
+Glenzocimab
+Eptifibatide
C
EXTEM-MCF-t (s)
4000
3000
2000
1000
0
**
***
EXTEM
+Glenzocimab
+Eptifibatide
D
INTEM-MCF-t (s)
3000
2000
1000
0
*
***
INTEM
+Glenzocimab
+Eptifibatide
E
EXTEM-Amplitude (mm)
80
60
40
20
0
***
5
10
15
20
25
30
Time (min)
EXTEM
+Glenzocimab
+Eptifibatide
F
INTEM-Amplitude (mm)
80
60
40
20
0
***
5
10
15
20
25
30
Time (min)
INTEM
+Glenzocimab
+Eptifibatide

Figure 6.8. Effect of glenzocimab or eptifibatide on the clot firmness in whole in blood samples from patients with ACS-treated with aspirin and ticagrelor assessed by ROTEM.

A) Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM-MCF. **B)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the INTEM-MCF. **C)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM-MCF-t. **D)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the INTEM-MCF-t. **E)** Time-dependent curves show the effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM-amplitude (A5-A30). **F)** Time-dependent curves show the effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the the INTEM-amplitude (A5-A30). The effect of glenzocimab and eptifibatide was compared to baseline samples and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=7, *P<0.05, **P<0.01, and ***P<0.001).

6.3.5.4 Glenzocimab did not influence clot rigidity and elasticity parameters, as reflected by G and MCE, while eptifibatide significantly affected them

As research parameters, G and MCE are not widely used but they can provide additional insight into clot strength. G stands for shear modulus strength (dyn/cm^2) and is used to measure clot rigidity and calculated using the formula: 500 x MCF/(100 - MCF). Maximum clot elasticity (MCE) is calculated as: 100 x MCF / (100 - MCF) (Hochleitner et al., 2017). Eptifibatide, but not glenzocimab, reduced clot rigidity and elasticity *ex vivo* in whole blood coagulation stimulated by EXTEM and INTEM in samples collected from patients with ACS on aspirin and ticagrelor, as presented in Figure 6.9.

Glenzocimab did not impact EXTEM-G (12,083 ± 1,008 dyn/cm^2 compared to 10,776 ± 1,057 dyn/cm^2 in the baseline samples; P=0.06), whereas eptifibatide reduced EXTEM-G (6,321 ± 959 dyn/cm^2; P=0.001). Furthermore, glenzocimab did not affect INTEM-G (10,839 ± 884 dyn/cm^2 compared to 9,384 ± 1,069 dyn/cm^2 in the baseline samples; P=0.19), whilst eptifibatide decreased INTEM-G (5,205 ± 938 dyn/cm^2; P=0.003).

Glenzocimab did not affect EXTEM-MCE (241 ± 20 compared to 215 ± 21 in the baseline samples; P=0.10), whilst eptifibatide reduced EXTEM-MCE (126 ± 19; P=0.001). Glenzocimab also did not impact INTEM-MCE (216 ± 17 compared to 187 ± 21 in the baseline samples; P=0.19), whereas eptifibatide decreased INTEM-MCE (104 ± 18; P=0.003).

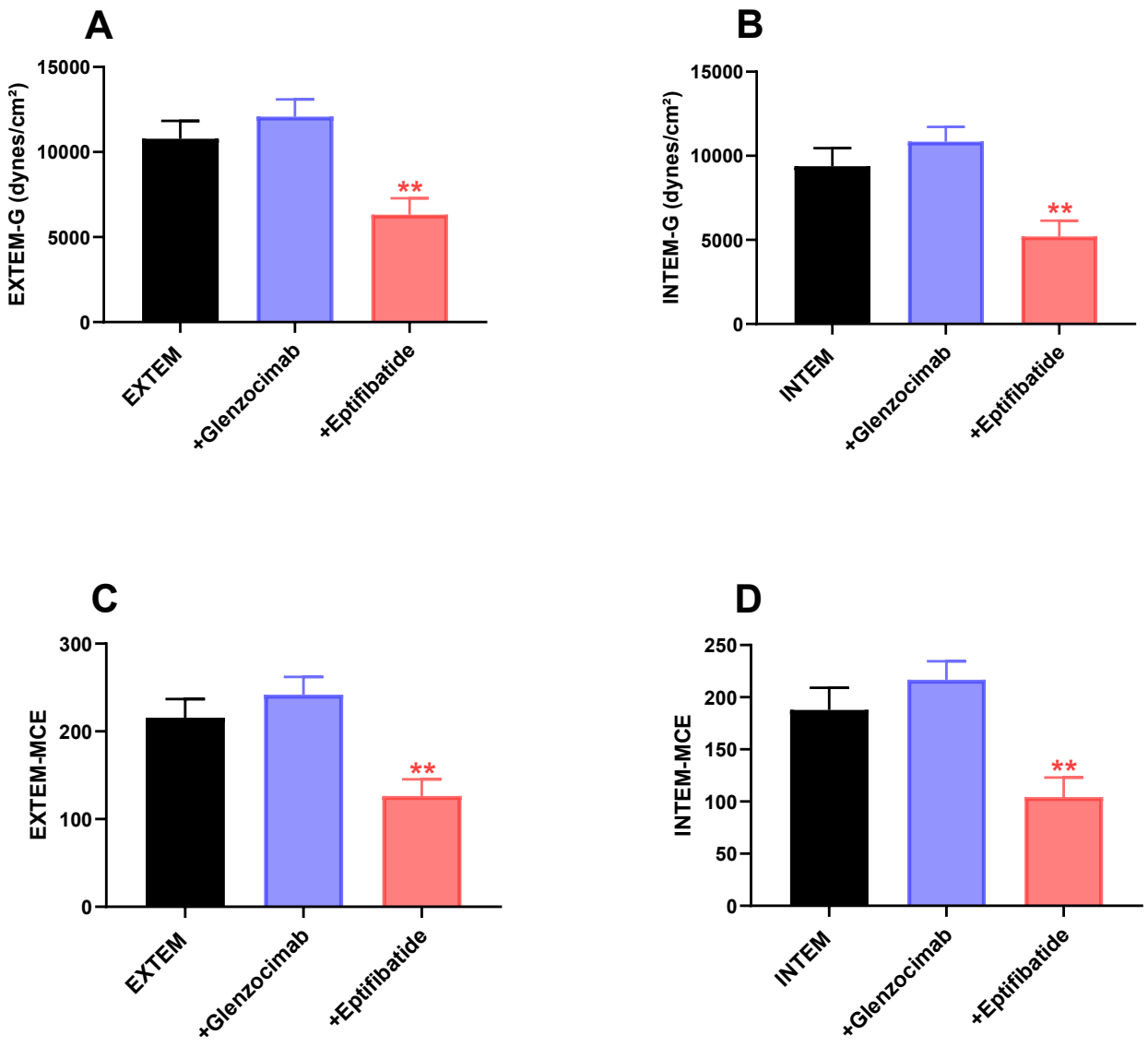

Figure 6.9. Effect of glenzocimab or eptifibatide on clot rigidity and elasticity in whole blood samples from patients with ACS-treated with aspirin and ticagrelor assessed by ROTEM.

A) Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM- shear elastic modulus strength. **B)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the INTEM-shear elastic modulus strength. **C)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM-maximum clot elasticity. **D)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the INTEM-maximum clot elasticity. The effect of glenzocimab and eptifibatide was compared to baseline samples and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=7, **P<0.01).

6.3.5.5 Glenzocimab and eptifibatide significantly delayed clot lysis parameters, as reflected by maximum lysis and lysis index

One of the clot characteristics that can be analysed in ROTEM is clot lysis (stability). Clot lysis variables include maximum lysis (ML), lysis index at 30 min (LI30), 45 min (LI450), and 60 min (LI60). Unexpectedly, the results indicated that both glenzocimab and eptifibatide reduced clot lysis triggered by EXTEM and INTEM, as illustrated in Figure 6.10.

Glenzocimab and eptifibatide reduced EXTEM-ML (2 ± 0.77%; P<0.001 and 5 ± 1%; P=0.005 compared to 11 ± 1% in the baseline samples). Glenzocimab and eptifibatide also reduced INTEM-ML (3 ± 1%; P=0.001 and 6 ± 1%; P=0.004 compared to 11 ± 1% in the baseline samples).

Both glenzocimab and eptifibatide reduced EXTEM-LI45 (99 ± 0.42%; P=0.01 and 99 ± 0.14%; P=0.02 compared to 95 ± 1% in the baseline samples), and reduced EXTEM-LI60 (98 ± 0.78%; P<0.001 and 98 ± 0.80%; P<0.001 compared to 91 ± 1% in the baseline samples). Glenzocimab and eptifibatide also decreased INTEM-LI45 (99 ± 0.59%; P=0.003 and 99 ± 0.24%; P=0.002 compared to 94 ± 1% in the baseline samples), and reduced INTEM-LI60 (98 ± 0.96%; P<0.001 and 97 ± 1%; P<0.001 compared to 91 ± 1% in the baseline samples).

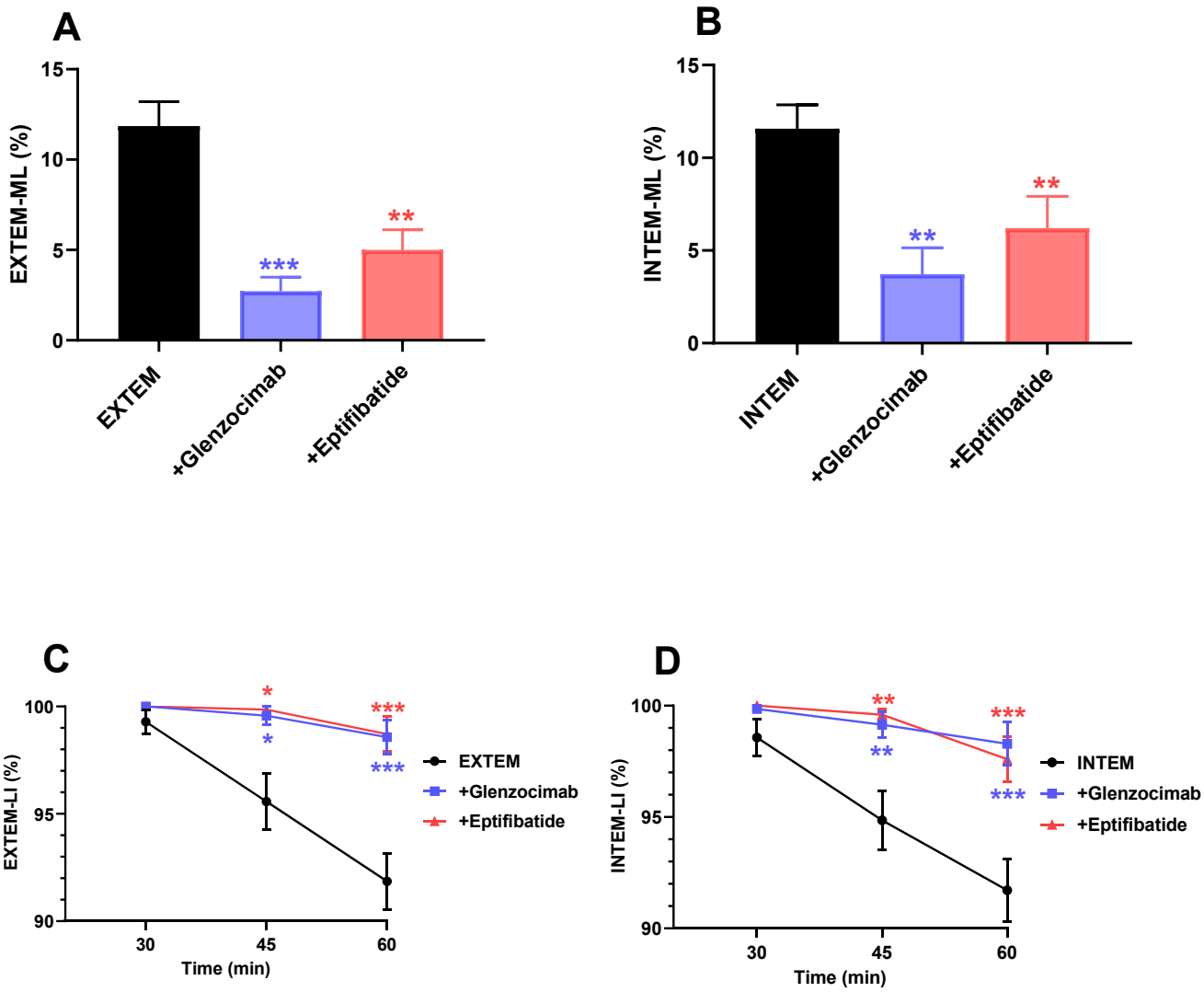

Figure 6.10. Effect of glenzocimab or eptifibatide on clot lysis in whole blood samples from patients with ACS-treated with aspirin and ticagrelor assessed by ROTEM.

A) Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM-maximum lysis. **B)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the INTEM-maximum lysis, **C)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the EXTEM-lysis index. **D)** Effect of glenzocimab (50 µg/ml) or eptifibatide (9 µM) on the INTEM-lysis index. The effect of glenzocimab and eptifibatide was compared to baseline samples and assessed using a mixed-effects model with Dunnett correction for multiple comparisons (n=7, *P<0.05, **P<0.01, and ***P<0.001).

6.4 Discussion

The main results presented in this chapter are: (i) glenzocimab inhibited *ex-vivo* residual platelet aggregation mediated by GPVI, which was only partially inhibited by routine treatment with dual antiplatelet therapy, without showing off-target effects on non-GPVI-mediated mechnaisms; (ii) glenzocimab significantly reduced *ex-vivo* thrombus formation and adhesion on Horm collagen under flow in samples from patients with ACS treated with aspirin and ticagrelor; (iii) glenzocimab reduced *ex-vivo* peak thrombin in thrombin generation stimulated by tissue factor in samples from patients with ACS who received aspirin and ticagrelor; (iv) glenzocimab did not affect the haemostatic properties of whole blood clots formed *ex vivo* from patients with ACS on aspirin and ticagrelor, whilst eptifibatide impacted blood clot properties.

Dual treatment with aspirin and ticagrelor partially inhibited collagen-stimulated platelet aggregation, as illustrated in the baseline (unspiked) samples in MEA. Wadowski et al. demonstrated that treatment with aspirin and ticagrelor partially inhibits collagen-mediated platelet aggregation but does weaken the stability of platelet aggregates; nevertheless, they used a high concentration of collagen (190 µg/ml) and a different method to assess platelet aggregation (LTA) (Wadowski et al., 2017). Vélez et al. found that platelet aggregation in response to collagen is higher in STEMI patients on aspirin and clopidogrel than in patients with stable coronary artery disease receiving the same combination of inhibitors (Vélez et al., 2016). Hennigan et al., who used the same method and collagen concentration (Multiplate, 3.2 µg/ml collagen), had similar results in the GLOBAL LEADERS sub-study to the results presented in this study. They reported that dual treatment with aspirin and ticagrelor partially inhibited collagen-stimulated platelet aggregation in patients with ACS treated with aspirin and ticagrelor (37 ± 22 U) (Hennigan et al., 2020). Glenzocimab significantly, but not completely, inhibited Horm collagen-induced platelet aggregation. The residual platelet aggregation

induced by collagen observed after GPVI blockade with glenzocimab could be mediated by the other collagen receptor, GPIa/IIIa. As noted in Chapter 3, 6F1, GPIa/IIIa inhibitor greatly inhibited collagen-stimulated platelet aggregation in MEA.

A significant GPVI-mediated platelet aggregation still exists despite dual treatment with aspirin and ticagrelor, as assessed by the specific GPVI agonist CRP-XL. However, spiking samples with glenzocimab completely inhibited GPVI-mediated platelet aggregation. As there are no clinical GPVI inhibitors in use, CRP-XL is not widely used in the assessment of platelet response to antiplatelet treatment. Vélez et al. used CRP-XL to examine GPVI reactivity in different sets of patients with ACS treated with aspirin and $P2Y_{12}$ inhibitors. They found that CRP-stimulated platelet aggregation is higher in patients with STEMI than in patients with stable coronary artery disease (Vélez et al., 2016).

GPVI inhibition had no off-target effects on either platelet pathways targeted by dual antiplatelet therapy (TxA_2 and $P2Y_{12}$) or TRAP-induced platelet aggregation. Dual treatment with aspirin and ticagrelor inhibited their targets effectively, which is consistent with the results reported by Hennigan et al. and others (Hennigan et al., 2020, Baber et al., 2020). The absence of off-target effects of glenzocimab on non-GPVI-mediated platelet activation *ex vivo* in samples from patients with ACS treated with aspirin and ticagrelor is in agreement with the results presented in Chapter 3, where glenzocimab did not affect platelet aggregation stimulated by non-GPVI stimuli in samples from healthy donors even when combined with aspirin and ticagrelor. However, as expected, eptifibatide inhibited collagen, CRP-XL, ADP, and TRAP-induced platelet aggregation. Eptifibatide bolus, even when combined with clopidogrel, a $P2Y_{12}$ inhibitor with variable platelet responses, completely inhibited ADP and TRAP-induced platelet aggregation (Marian et al., 2019).

Platelet thrombus formation, but not platelet adhesion under flow *ex vivo* on Horm collagen, were reduced in samples from patients with ACS treated with aspirin and ticagrelor. Tsoumani et al. compared platelet adhesion and platelet thrombi formation on 200 µg/ml collagen under flow between healthy donors and patients with ACS on aspirin and ticagrelor, and found that treatment with aspirin and ticagrelor significantly inhibited platelet aggregate formation without an effect on platelet adhesion (surface coverage %), which was similar to the surface coverage of adherent platelets formed on collagen from healthy donors (Tsoumani et al., 2016). Interestingly, the percentage of platelet surface coverage values obtained from spiking samples from healthy donors with aspirin and ticagrelor, which did not affect platelet adhesion on 200 µg/ml collagen (as presented in Chapter 3), were similar to values achieved in this study from patients' samples. Glenzocimab provided significant additional effects on platelet thrombus formation and platelet adhesion, which indicates the unmet need to target GPVI in patients with STEMI.

The incomplete inhibition of platelet adhesion and platelet thrombi by glenzocimab is not surprising as the other collagen receptor, GPIb/IIIa, was not antagonised. As demonstrated in Chapter 3, 6F1 partially reduces platelet thrombus formation and aguments the inhibitory effect of glenzocimab on platelet thrombi formation. Nagy et al. recently found that platelet aggregate formation is reduced but not adhesion in blood flowing on collagen from GPVI-deficient patients. Furthermore, treating blood from GPVI-deficient patients with 6F1 blocked platelet adhesion on collagen (Nagy et al., 2020). Eptifibatide yielded more potent inhibition of both platelet thrombus formation and platelet adhesion, leaving small patches of a monolayer of adherent platelets.

It is difficult to conclude that treatment with aspirin and ticagrelor did not affect tissue factor stimulated thrombin generation in PRP *ex vivo* from patients with ACS since there were no

results for thrombin generation before initiation of dual antiplatelet therapy that could be used as a base line control for comparison. Berezovskaya et al. found that thrombin generation (peak thrombin and ETP) was reduced in PRP from patients treated with aspirin and clopidogrel, indicating an inhibitory role for DAPT on thrombin generation (Berezovskaya et al., 2018). Their findings contradict a previous study that reported dual treatment with aspirin and clopidogrel did not affect platelet-dependant thrombin generation (Müller et al., 2002). Unlike Berezovskaya et al.'s study, Yip et al. found thrombin generation (CAT method) was higher in a large set of patients with ACS on dual antiplatelet therapy than in patients with chronic coronary artery disease and healthy controls (Yip et al., 2020). Glenzocimab provided a significant reduction in peak thrombin wihtout affecting other CAT parameters. Eptifibatide also reduced peak thrombin. Overall, more samples are required to explore the additive effect of glenzocimab and eptifibatide on thrombin generation.

Glenzocimab did not affect ROTEM parameters that are associated with preservation of haemostatic functions (clot initiation, clot formation, and clot strength), whilst eptifibatide impacted clot formation and clot strength. No effect for glenzocimab and eptifibatide was observed on clot initiation as measured by CT. CT is sensitive to defects in extrinsic and intrinsic coagulation pathways (coagulation factor deficiency) rather than platelets (Theusinger et al., 2013). Therefore, the potent inhibition of platelet function with eptifibatide did not affect CT. Eptifibatide significantly prolonged CFT, which is closely related to clot amplification and propagation phases (Theusinger et al., 2013).

Eptifibatide reduced clot strength, as evaluated by MCF, MCF-t, and amplitude (A5–A30). Dötsch et al. and others reported that INTEM-MCF is predictive of postoperative non-surgical bleeding (Dötsch et al., 2017, Theusinger et al., 2013). The reduced MCF is correlated with either a defect in platelet function or a low platelet count and low fibrinogen (Ogawa et al.,

2012). As a result, MCF, especially early predictors of MCF, A5 and A10, are used in the treatment protocols for guided blood transfusion (Dirkmann et al., 2013). Eptifibatide also decreased other ROTEM parameters that provide additional insights on the clot strength in terms of elasticity (MCE) and rigidity (G). A recent study suggests that MCE could reflect platelet contribution (count and function) to clot strength better than MCF (Ranucci et al., 2020). Eptifibatide reduced shear elastic modulus strength (G), indicating the formation of a weak clot that is easily deformed by applying a low shear. Mousa et al. found that a GPIIb/IIIa inhibitor reduced shear elastic modulus strength of tissue factor-stimulated clots in PRP in thromboelastography (TEG) (Mousa Shaker et al., 2000).

Glenzocimab and eptifibatide reduced ROTEM parameters for clot lysis. GPIIb/IIIa inhibitors have been demonstrated to decrease clot lysis in viscoelastic based assays. Katori et al. elucidated the mechanisms by which a GPIIb/IIIa blockade affected clot lysis parameters in TEG. They found that GPIIb/IIIa inhibitors reduced clot lysis by blocking clot retraction and that the clot retraction process produced similar phenotypes to fibrinolytic patterns on clot lysis parameters (Katori et al., 2005). However, it is not clear how glenzocimab reduced clot lysis parameters. Glenzocimab decreased clot lysis parameters even more than eptifibatide, which excluded the possibility of inhibition of clot retraction as an underlying mechanism in the case of glenzocimab. In addition, glenzocimab with aspirin and ticagrelor did not significantly affect clot retraction compared to eptifibatide combined with aspirin and ticagrelor, as demonstrated in Chapter 5. It is unlikely that glenzocimab affected clot lysis parameters by inhibiting the fibrinolysis. Ahmad et al. showed that glenzocimab facilitated fibrinolytic activity of recombinant tissue plasminogen activator on the lysis of fibrin-rich clots (Ahmed et al., 2020).

6.5 Limitations

Many limitations in this chapter should be highlighted. Not all the included ACS patients were STEMI patients; some of them were NSTEMI patients. The main aim for this thesis is to investigate the use of glenzocimab as a potential antiplatelet therapy for patients with STEMI; however, due to some difficulties in the STEMI patients' recruitment, patients with other ACS classes were recruited. Nevertheless, glenzocimab could be used during PCI even in NSTEMI and US patients if heavy thrombosis is experienced during the elective PCI. The volume of the sample from some patients were not enough to run all planned assays; as a result, the power of statistical analysis for some results was affected. Finally, there was a delay in the transportation of some samples from the recruitment site to our laboratory which could affect the quality of the results.

6.6 Summary

In summary, despite treatment with aspirin and ticagrelor, a potent $P2Y_{12}$ inhibitor, GPVI can mediate significant platelet responses including platelet aggregation, platelet thrombus formation, and platelet-dependent thrombin generation. Glenzocimab completely inhibited GPVI-mediated platelet activity, as reflected by the complete inhibition of CRP-XL-induced platelet aggregation. Furthermore, glenzocimab additionally decreased collagen-induced platelet aggregation and platelet thrombus formation. Glenzocimab also further reduced peak thrombin in platelet-amplified thrombin generation without an impact on haemostatic functions assessed by ROTEM in blood samples from patients with ACS treated with aspirin and ticagrelor.

CHAPTER 7 : GENERAL DISCUSSION

7.1 Summary of results

GPVI is a promising antiplatelet target for thrombotic cardiovascular diseases. Current antiplatelet therapy, which represents the mainstay for ACS treatment, requires improvement in terms of efficacy and safety. This interest in targeting GPVI has been translated into the pharmacological development of two clinical-grade GPVI inhibitors, glenzocimab and revacept, which have different mechanisms of GPVI inhibition. More attention is being paid to glenzocimab, however, after revacept failed to provide any additional protective effects when combined with standard antiplatelet therapy compared to standard antiplatelet therapy alone (Mayer et al., 2021).

This thesis investigated the effect of glenzocimab on platelet inhibition and compared it with antiplatelet medications (aspirin, ticagrelor, and eptifibatide) that are used to treat patients with STEMI. A wide range of platelet mechanisms and aspects of coagulation were investigated in *in-vitro* models of thrombosis and haemostasis that utilised blood from healthy donors and patients with STEMI that were treated with aspirin and ticagrelor. The main results of this work are as follows: (i) glenzocimab provided an amplified effect with aspirin and ticagrelor on collagen-mediated platelet responses; (ii) GPIa/IIa mediates Horm collagen-stimulated platelet aggregation and thrombus formation; (iii) glenzocimab completely blocked platelet activation mediated by GPVI, whereas current antiplatelet therapy agents showed no significant effect on GPVI-dependent activation as assessed by CRP-XL; (iv) glenzocimab inhibited atherosclerotic plaque homogenate-induced platelet activation, indicating a strong dependency on GPVI for atherosclerotic plaque in platelet activation; (v) glenzocimab inhibited fibrin-induced platelet aggregation, abrogated platelet procoagulant activity, and reduced peak thrombin in tissue factor-stimulated thrombin generation; (vi) *ex-vivo* platelet investigation from aspirin- and ticagrelor-treated patients with STEMI showed that residual GPVI activity can mediate

different platelet mechanisms that were additionally inhibited by glenzocimab; (vii) glenzocimab did not show off-target effects on platelet activation triggered by non-GPVI pathways; and (viii) unlike eptifibatide, glenzocimab did not affect haemostatic function as measured by ROTEM .

7.1.1 Glenzocimab provided amplified antiplatelet effects with aspirin and ticagrelor on collagen-mediated platelet responses

Collagen is the most thrombogenic part of the vascular subendothelium and acts as an adhesive ligand for circulating platelets, causing platelet adhesion and activation that are essential for thrombus formation at the site of the vascular injury (Roberts et al., 2004). Despite its critical role in the initiation of platelet thrombosis, current antiplatelet therapy targets only secondary soluble mediators, TxA_2 and ADP, that provide positive feedback loops for the amplification of platelet activation rather than triggering platelet thrombus formation (Fälker et al., 2004). GPVI, which functions as a direct receptor for collagen, has a major role in the initiating of collagen-triggered platelet thrombotic responses; the other direct collagen receptor, GPIa/IIa, aids in the platelet adhesion without being as essential as GPVI. As a result, GPVI has emerged as an attractive antiplatelet target for inhibiting collagen-mediated platelet responses in arterial thrombosis (Nieswandt et al., 2001b, Zahid et al., 2012a).

Glenzocimab significantly inhibited collagen-induced platelet aggregation in PRP and whole blood, as presented in Chapter 3. The dual antiplatelet therapy agents aspirin and ticagrelor partially inhibited collagen-stimulated platelet aggregation; however, this partial inhibition is greatly maximised in the presence of the GPVI inhibitor glenzocimab. Under an arterial shear rate, glenzocimab inhibited platelet-rich thrombus formation and platelet adhesion on immobilised collagen. A higher concentration of collagen reduced the ability of glenzocimab to block platelet adhesion, but glenzocimab still significantly inhibited platelet thrombus

formation. Glenzocimab showed no effect on platelet spreading on immobilised collagen, which was an expected result, as platelet spreading is a unique functional aspect of GPIIb/IIIa. Glenzocimab significantly reduced phosphorylation of the main effector proteins (Syk, LAT, and PLCγ2) in the GPVI signalling pathway in collagen-stimulated platelet activation. Aspirin and ticagrelor did not affect the tyrosine phosphorylation, but they provided an additional effect when combined with glenzocimab, causing a further reduction in phosphorylation of Syk, LAT, and PLCγ2. The failure of aspirin and ticagrelor to block collagen-stimulated GPVI signalling could explain the increased GPVI expression, increased collagen-induced platelet aggregation, platelet hyperreactivity, and subsequent atherothrombotic events in patients with cardiovascular diseases treated with dual antiplatelet therapy (Guha et al., 2011, Vélez et al., 2016, Bigalke et al., 2008). Furthermore, GPVI signalling is upregulated in diabetic patients, which might provide an explanation for the reduced responsiveness to dual antiplatelet therapy in this category of patients (Angiolillo et al., 2005, Arthur et al., 2017).

7.1.2 GPIa/IIa has a major role in collagen-stimulated platelet aggregation in MEA

GPVI is considered the main collagen receptor that mediates platelet aggregation in response to stimulation with fibrous (Horm) collagen, as GPVI-deficient platelets fail to aggregate in response to Horm collagen (Jandrot-Perrus et al., 2019). GPVI blockade with glenzocimab leads to a more potent inhibition of collagen-stimulated platelet aggregation in citrate-anticoagulated PRP in LTA than hirudin-anticoagulated whole blood in MEA. The contribution of GPIa/IIa in collagen-induced platelet aggregation in LTA and MEA was investigated using its well-known inhibitor, 6F1. Investigating the difference between LTA and MEA regarding collagen-induced platelet aggregation is motivated by the fact that there are GPVI inhibitors under clinical development, and so it is necessary to know to what extent GPIa/IIa contributes to platelet aggregation in these methods. In LTA, residual platelet aggregation after GPVI

blockade with glenzocimab was mediated by GPIa/IIa, as it was blocked by 6F1. Rising concentrations of 6F1 failed to show any effect on collagen-induced platelet aggregation. However, when 6F1 combined with glenzocimab, collagen-stimulated platelet aggregation inhibited completely.

In MEA, however, GPIa/IIa was significantly involved in collagen-stimulated platelet aggregation, as demonstrated by the significant inhibition of platelet aggregation by 6F1. Different factors may contribute to GPIa/IIa's involvement. Spontaneous platelet aggregation in MEA results from stirring force-induced lysis of old and fragile RBCs that release the platelet stimuli ADP and ATP (Bampalis et al., 2012). By activating $P2Y_{12}$ and $P2Y_1$, ADP primes and mediates the inside-out activation of GPIa/IIa, moving the receptor to a high-affinity state for interaction with collagen (Jung and Moroi, 2001). The anticoagulant hirudin provides an optimal condition for GPIa/IIa by maintaining cations that are required for GPIa/IIa to bind to and interact with collagen (Mannuß, 2020, Nakamura et al., 1998). MEA measures both platelet adhesion and aggregation, and it is known that GPIa/IIa mediates platelet adhesion more than GPVI does (Toth et al., 2006, Moroi et al., 2000).

7.1.3 Glenzocimab completely blocked GPVI-mediated platelet activation, whereas current antiplatelet therapy agents showed no effect on GPVI-dependent activation as assessed by CRP-XL

Glenzocimab is a potent GPVI inhibitor, as demonstrated by its ability to fully block GPVI-mediated platelet aggregation at a low concentration (5 µg/ml). This potency is in accordance with its high affinity for GPVI (K_D = 4 nM), as reported by Martine Jandrot-Perrus's research group (Lebozec et al., 2017). Interestingly, glenzocimab inhibited platelet adhesion on CRP-XL and partially reduced platelet spreading. It is not possible to carry out flow adhesion assays on CRP-XL, as it was shown to support only a rapid and transient platelet adhesion.

Consequently, the effect of glenzocimab and other antiplatelet agents was not investigated on platelet thrombus formation and adhesion on CRP-XL under shear rate (Polanowska-Grabowska et al., 2003). Consistent with its potent inhibition of CRP-XL-induced platelet aggregation, glenzocimab completely prevented GPVI signalling, as indicated by inhibition of tyrosine phosphorylation of Syk, LAT, and PLCγ2-stimulated by CRP-XL. At the investigated concentrations, aspirin and ticagrelor did not affect specific GPVI-mediated platelet mechanisms such as aggregation, adhesion and spreading, and tyrosine signalling. The potent inhibition of platelet activation by glenzocimab could omit the additive effect of aspirin and ticagrelor on CRP-XL-induced platelet activation. In contrast, eptifibatide blocked GPVI-triggered platelet aggregation and reduced platelet spreading, as these platelet responses are functional aspects of GPIIb/IIIa.

7.1.4 Glenzocimab inhibited atherosclerotic plaque homogenate-induced platelet activation, indicating a strong dependency on GPVI for atherosclerotic plaque in platelet activation as confirmed by protein binding and molecular investigations

Glenzocimab blocked atherosclerotic plaque-induced platelet aggregation and secretion more effectively than agents of the routine dual antiplatelet therapy, aspirin and ticagrelor, which are key prophylaxis agents for atherosclerotic cardiovascular diseases. The combination of aspirin and ticagrelor partially inhibited platelet aggregation and but not platelet secretion upon stimulation with atherosclerotic plaque homogenate. Eptifibatide inhibited platelet aggregation but not activation, as reflected by ATP release. The dependency of human atherosclerotic plaque on GPVI in activating platelets and their subsequent aggregation and secretion found in the present study is consistent with the findings of Penz et al. (Penz et al., 2005). Glenzocimab blocks both platelet aggregation and activation, as platelet activation mediates inflammatory

responses by releasing inflammatory mediators and cytokines from platelet granules. Eptifibatide does not prevent inflammatory responses in patients with ACS (Mazaev et al., 2009).

Glenzocimab exerted an immediate inhibiton of aspirin- and ticagrelor-treated platelet aggregation that had been stimulated with atherosclerotic plaque material, providing a similar degree of immediate inhibition potency to that achieved by eptifibatide. GPIIb/IIIa inhibitors are used to disperse platelet aggregate and platelet-rich thrombi during emergency PCI (Speich et al., 2009). Dispersal of platelet aggregate by glenzocimab supports the recent evidence that shows that glenzocimab can disaggregate preformed thrombi in the absence of thrombin under flow (Ahmed et al., 2020).

Platelet thrombus formation following rupture of atherosclerotic plaque is a critical event that can lead to the obstruction of blood flow in a coronary artery, which can result in myocardial infarction and ischemia. Glenzocimab completely blocked platelet thrombus formation on atherosclerotic plaque in a microfluidic chamber under arterial shear rate, providing a similar degree of inhibition to eptifibatide. In contrast, aspirin and ticagrelor partially inhibited platelet thrombus on atherosclerotic plaque. The complete inhibition of platelet thrombus formation by glenzocimab demonstrates that the GPVI blockade is sufficient to prevent platelet thrombus formation (Cosemans et al., 2005, Jamasbi et al., 2015). Furthermore, glenzocimab completely blocked P-selectin expression on plaque under flow, whereas aspirin and ticagrelor did not inhibit P-selectin expression. P-selectin expression is reported to be persistently higher in patients on dual antiplatelet therapy than in those on triple antiplatelet therapy (Lee et al., 2007).

Dimeric GPVI exhibits more affinity for collagen, whereas monomeric GPVI exhibits more affinity for fibrin (Onselaer et al., 2017). Accordingly, revacept (a recombinant dimeric GPVI)

has been developed to therapeutically target GPVI-mediated platelet activation by competitively binding to exposed collagen at the site of the vascular injury. By contrast, atherosclerotic plaque material revealed an affinity for both dimeric and monomeric GPVI, which was blocked by glenzocimab. The results of the dimeric and monomeric GPVI binding support an earlier study that showed that antibodies raised against both dimeric and monomeric GPVI inhibited atherosclerotic plaque-mediated platelet aggregation more effectively than antibodies raised against dimeric GPVI only (Jamasbi et al., 2015).

Atherosclerotic plaque induced sustained GPVI signalling, which maintains platelet activation for longer periods, as assessed by Syk, LAT, and PLCγ2. Sustained GPVI signalling promotes sustained platelet activation processes such as continous platelet adhesion and thrombus stabilisation (Pallini et al., 2021, Ahmed et al., 2020). Glenzocimab inhibited tyrosine phosphorylation of Syk, LAT, and PLCγ2 following platelet stimulation with atherosclerotic plaque material, whereas aspirin and ticagrelor showed no effect on tyrosine phosphorylation, although they amplified the effect of glenzocimab, achieving a near complete inhibition of tyrosine phosphorylation.

Atherosclerotic plaque material induced GPVI shedding from platelets *in vitro*. GPVI shedding occurs after platelets are activated by a specific GPVI agonist or thrombin, forming soluble GPVI. Soluble GPVI is elevated in patients with STEMI, indicating an active GPVI shedding due to platelet activation following atherosclerotic plaque rupture (Chatterjee and Gawaz, 2017).

7.1.5 Glenzocimab inhibited fibrin-induced platelet aggregation, abrogated platelet procoagulant activity, and reduced peak thrombin in tissue factor-stimulated thrombin generation

Glenzocimab blocked fibrin-induced platelet aggregation more effectively than aspirin and ticagrelor. GPVI interaction with fibrin is believed to propagate thrombus growth and generate a positive feedback for thrombin generation (Mammadova-Bach et al., 2015, Alshehri et al., 2015). Platelets from GPVI-deficient patients failed to aggregate in response to fibrin stimulation, indicating major involvement of GPVI in fibrin-triggered platelet responses (Onselaer et al., 2017).

Glenzocimab reduced peak thrombin generation mediated by tissue factor and inhibited platelet procoagulant activity more than existing antiplatelet therapy agents. Samples from healthy donors spiked with aspirin and ticagrelor and samples from patients with ACS on aspirin and ticagrelor showed that those agents had no effect on reducing thrombin generation peak. It has been shown that thrombin generation is increased and persists a long time in patients with ACS regardless of continuous dual antiplatelet therapy (Seneviratna et al., 2016, Yip et al., 2020). In the event of immunological GPVI blockade or in GPVI-deficient patients, thrombin generation peak is reduced, which give glenzocimab an additional antiplatelet property over aspirin and ticagrelor (Mammadova-Bach et al., 2015).

GPVI amplifies platelet-dependent thrombin generation by increasing platelet procoagulant activity, which it achieves by exposing phosphatidylserine following the interaction with the fibrin generated during thrombin generation (Mammadova-Bach et al., 2015). Only glenzocimab blocked both collagen- and atherosclerotic plaque-mediated platelet procoagulant activity. Eptifibatide also reduced thrombin generation peak in healthy donors and patients *ex vivo*. Outside-in activation of GPIIb/IIIa by fibrinogen enhances platelet procoagulant

activity by stimulating phosphatidylserine exposure, eventually increasing platelet-dependent thrombin generation, and hence GPIIb/IIIa blockade reduces thrombin generation (van der Meijden et al., 2012).

7.1.6 *Ex-vivo* investigation of platelets from aspirin and ticagrelor-treated patients with STEMI showed that residual GPVI activity could mediate different platelet mechanisms that were additionally inhibited by glenzocimab

Dual antiplatelet therapy with aspirin and ticagrelor blocked their targets effectively but exerted less potent inhibition of GPVI-mediated platelet aggregation, as measured by collagen and CRP-XL. This GPVI-mediated platelet activation induced significant platelet aggregation and achieved platelet thrombus formation on collagen-coated surfaces under arterial flow rate. It has been shown that collagen-mediated platelet aggregation is less inhibited by dual treatment with aspirin and $P2Y_{12}$ inhibitors (Wadowski et al., 2017). Moreover, platelets from patients with ACS on aspirin and $P2Y_{12}$ have the capacity to adhere to collagen and form thrombi in blood flowing at an arterial shear rate (Tsoumani et al., 2016). Glenzocimab provided additional antiplatelet effects when added to samples from patients with ACS on aspirin and ticagrelor. It completely blocked GPVI-mediated platelet aggregation (CRP-XL) and significantly decreased, but not completely blocked, collagen-mediated aggregation, as there is another collagen receptor, GPIa/IIa, that can contribute to platelet aggregation. Under flow conditions and on collagen-coated surfaces, glenzocimab further reduced platelet adhesion and thrombus formation. The inhibitory effect of glenzocimab was more pronounced in platelet thrombus formation than in platelet adhesion on collagen, implying that GPIa/IIa plays a role in mediating platelet adhesion (Lecut et al., 2004).

7.1.7 Glenzocimab did not show off-target effects on platelet activation induced by non-GPVI pathways

The antiplatelet effect of glenzocimab is limited to the GPVI-triggered platelet mechanisms (collagen, CRP-XL, and atherosclerotic plaque materials). The addition of glenzocimab to blood spiked with aspirin and ticagrelor from healthy donors or to blood from aspirin and ticagrelor-treated patients did not augment their effect on platelet activation mediated by non-GPVI agonists, even on their targets. GPVI is present only on platelets, which reduces the risk of off-target effects of GPVI inhibitors. In contrast, current antiplatelet therapy agents exerted off-target antiplatelet effects. Aspirin and ticagrelor partially affected collagen-mediated platelet responses and marginally inhibited TRAP-induced platelet activation. Ticagrelor augmented the effect of aspirin on AA-induced platelet aggregation. $P2Y_{12}$ blockade also has been recently shown to significantly inhibit PAR4 mediated platelet aggregation in patients-treated with clopidogrel (Kamada et al., 2021). Furthermore, current antiplatelet targets, TP and $P2Y_{12}$, are expressed on various cells as well as platelets and play functional roles beyond the scope of platelets; therefore, inhibiting these pathways demonstrated off-target pharmacological activities (Nylander and Schulz, 2016, Ornelas et al., 2017).

7.1.8 Unlike eptifibatide, glenzocimab did not affect haemostatic function as measured by ROTEM

ROTEM provides a comprehensive overview of the blood clotting process stimulated by extrinsic or intrinsic coagulation pathways. Glenzocimab showed no effect on ROTEM parameters by itself, in healthy samples spiked with aspirin and ticagrelor, or in patients with ACS treated with aspirin and ticagrelor. Glenzocimab with and without aspirin and ticagrelor did not affect clot formation or clot strength. The results of this thesis are in accordance with those of a phase I clinical trial published in 2019, which showed that glenzocimab is safe, is

well-tolerated, and has no effect on haemostasis, as assessed by bleeding time (Voors-Pette et al., 2019). One of the major limitations of GPIIb/IIIa blockade is severe bleeding that can undermine its protective antiplatelet benefits. Eptifibatide affected clot formation kinetics and clot strength parameters. Many of these parameters are incorporated into treatment protocols and algorithms that are used to monitor bleeding and haemostatic function and guide blood transfusion in intraoperative and postoperative surgical patients (Raphael et al., 2019, Dötsch et al., 2017, Dirkmann et al., 2013).

7.2 Limitations of using glenzocimab as potential treatment for ACS

Glenzocimab could be a game changing treatment for patients with ACS, yet some limitations especially in the way that glenzocimab is administered could undermine its therapeutic value. Glenzocimab is designed to be administered via intravenous way, so it is only administered in clinical settings. As a result, use of glenzocimab is limited only in clinical settings which could prevent extending duration of GPVI inhibition and deprive patients with ACS from its protective antithrombotic effect after they discharged from hospitals, putting them at the risk of recurrent thrombosis and heart attacks. Glenzocimab appears to be a safe antiplatelet agent, yet the safety of using of glenzocimab with dual antiplatelet therapy in patients with STEMI undergoing pPCI or patients with high risk of bleeding should be established and thoroughly investigated in a clinical trial.

7.3 Use of glenzocimab for other forms of thrombotic diseases

Glenzocimab could be used out scope of ACS in other forms of thrombotic diseases. Acute ischemic stroke is one of these diseases that could be targeted by glenzocimab. *In vitro* studies showed that addition of glenzocimab with standard thrombolytic agent which is used to treat patients with stroke facilitated its thrombolytic effect in dissolving fibrin clots (Ahmed et al., 2020). In fact, phase IIb clinical trial named Acute Ischemic Stroke Interventional Study

(ACTIMIS) recruited patients with acute ischemic stroke to in two treatment arms; one arm received standard therapy alone and the other arm received standard therapy plus escalating doses of glenzocimab that range from 125 mg to 1000 mg (Mazighi M, 2021). The ACTIMIS trial showed that administration of glenzocimab is safe in patients with acute ischemic stroke receiving standard therapy without causing bleeding even when it was used at the highest concentration (1000 mg) (Mazighi M, 2021).

7.4 Study limitations

This study has some limitations that should be pointed out. The combination of glenzocimab with aspirin and ticagrelor was performed in *in vitro* models of thrombosis. Using a mouse model of arterial thrombosis to examine the effect of glenzocimab with and without aspirin and ticagrelor on thrombosis *in vivo* is perhaps not the best approach, because there are some functional differences between human and mouse GPVI. Human GPVI interacts with fibrinogen, whereas mouse GPVI does not (Mangin et al., 2018). Moreover, human GPVI has a major role in thrombus stability, whereas mouse GPVI is not involved in thrombus progression and stabilisation (Janus-Bell et al., 2021). The atherosclerotic plaque was not evaluated in patients with ACS *ex vivo* due to disruption of participant recruitment by COVID-19. Furthermore, the number of samples from patients with ACS used in the investigation of the effect of glenzocimab on thrombin generation and haemostatic function was relatively low. In addition, there was a delay in the transportation of some samples from the site of recruitment to the lab where the samples were processed. More advanced data analysis is required to determine the nature of the amplified effect that was obtained by combining glenzocimab with aspirin and ticagrelor whether its synergistic effect or additive effect.

7.5 Conclusion and future directions

The addition of the novel GPVI inhibitor glenzocimab to aspirin and ticagrelor provides amplified inhibition of multiple critical mechanisms of atherothrombosis. Glenzocimab and the GPIIb/IIIa inhibitor eptifibatide have many similar antithrombotic effects, although glenzocimab has less impact than eptifibatide on mechanisms involved in haemostasis. Further studies are needed to demonstrate whether glenzocimab provides amplified antithrombotic effects in patients with STEMI in a clinical trial.

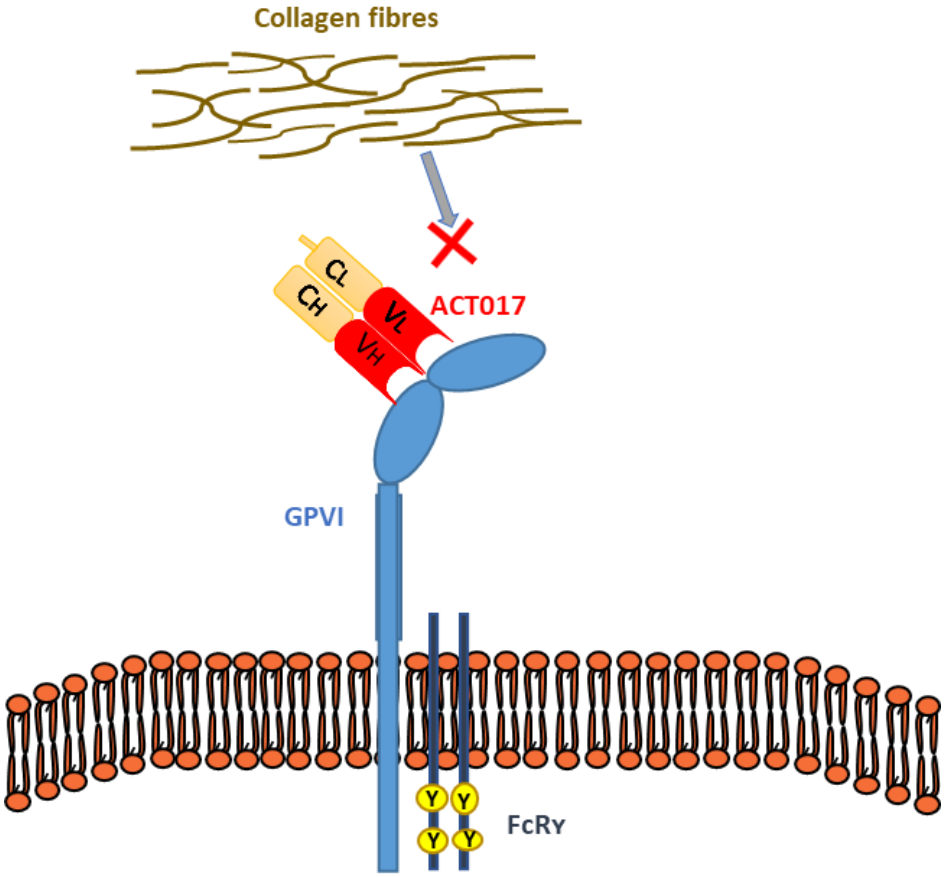

Figure 7.1. Glenzocimab is a novel GPVI inhibitor that inhibits GPVI-mediated platelet mechanisms that are critical in thrombosis and have minimal involvement in haemostasis.

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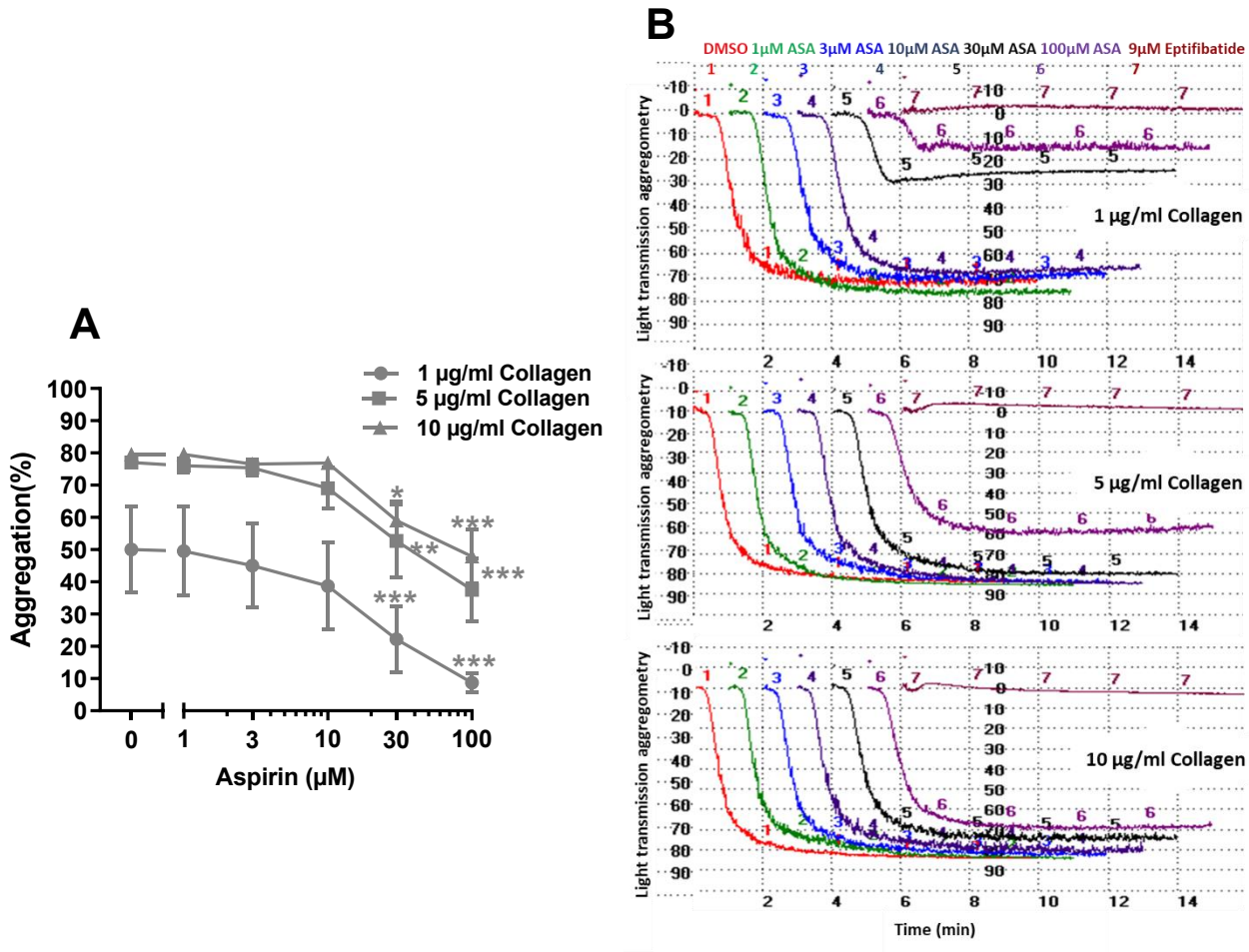
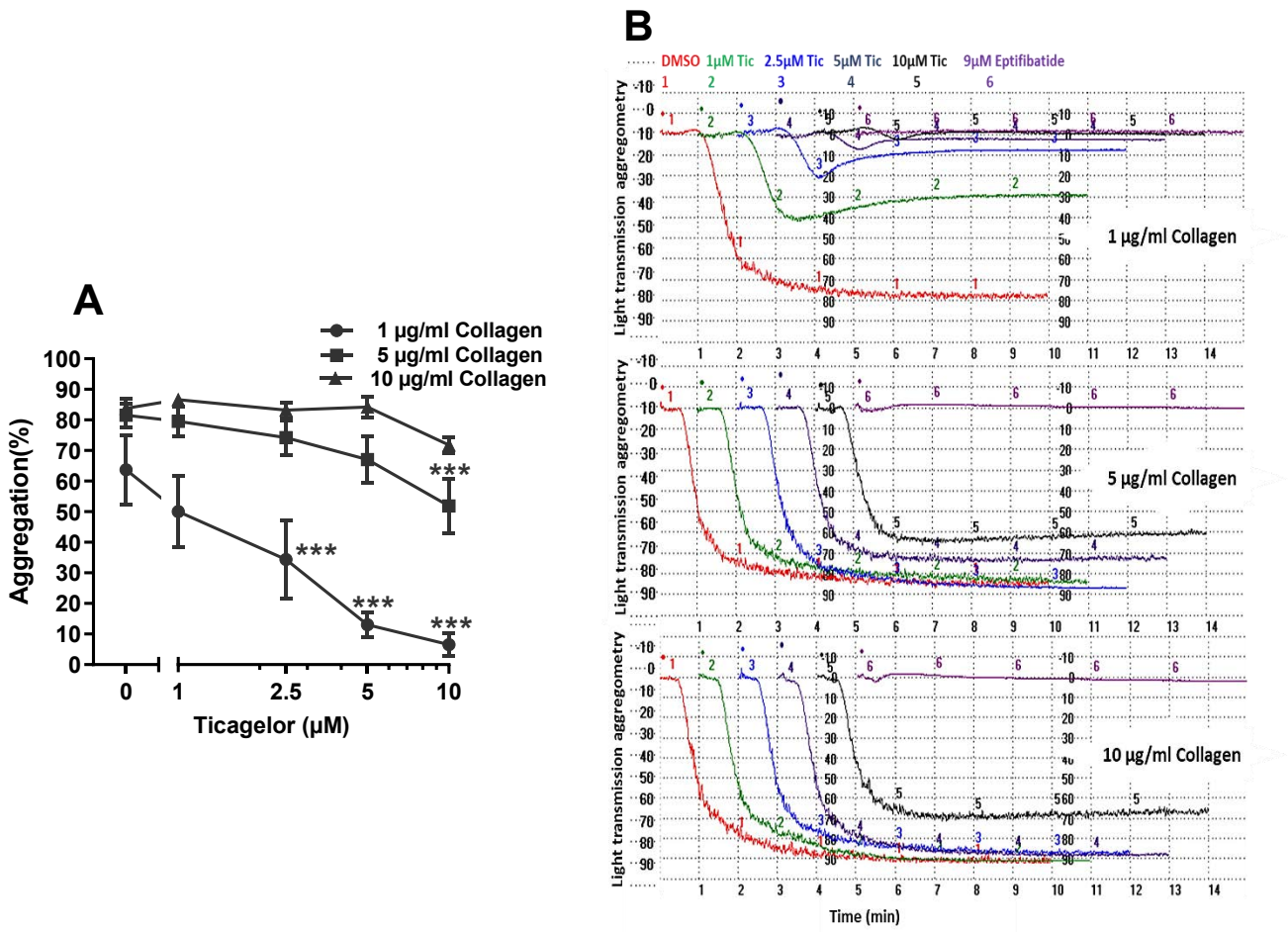
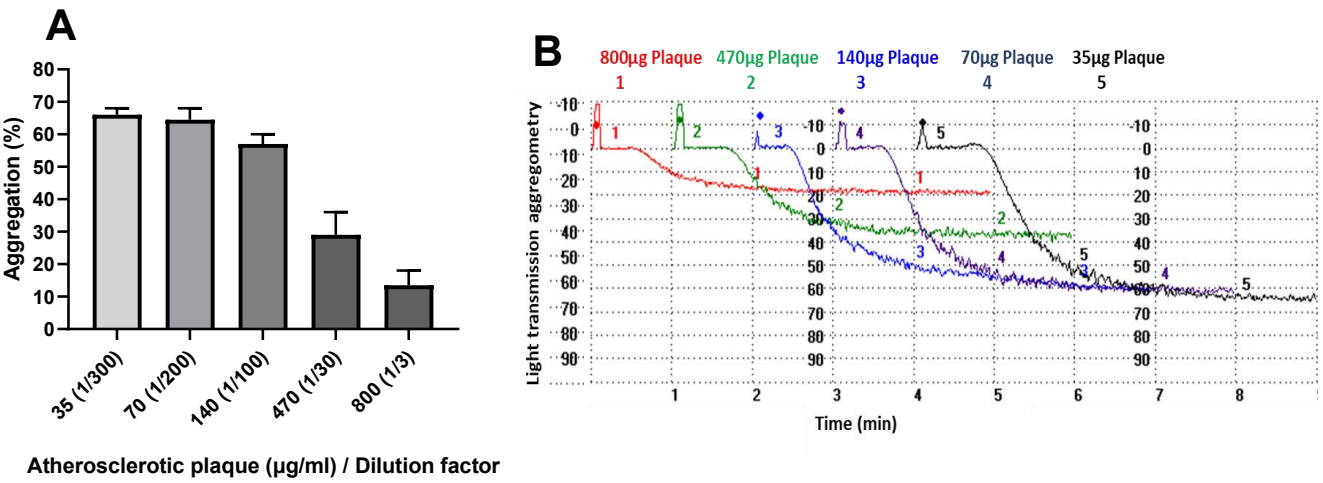
APPENDIX



Supplementary Figure 1. Effect of aspirin on collagen stimulated platelet aggregation in PPR. A) Dose-response curve of aspirin on 1, 5, and 10 µg/ml collagen-stimulated platelet aggregation, **B)** A representative image of a single aspirin experiment where human platelets incubated with different concentrations of aspirin (1-100 µM) for 10 minutes, then pretreated platelets stimulated with 1,5, and 10 µg/ml respectively, and aggregation was monitored in PAP-E8 aggregometer for 10 minutes. These results are expressed as mean ± SEM. The effect of different concentrations of anti-platelet agents compared to untreated (DMSO control) samples were determined using 2-way ANOVA with Dunnett correction for multiple comparisons (n=6, *P<0.05, **P<0.01, and ***P<0.001).



Supplementary Figure 2. Effect of ticagrelor on collagen stimulated platelet aggregation in PPR. A) Dose-response curve of Ticagrelor on 1, 5, and10 µg/ml collagen-induced platelet aggregation, **B)** A representative image of a single Ticagrelor experiment where human platelets preincubated different concentrations of ticagrelor (1-10 µM) for 10 minutes, then platelets stimulated with 1, 5, and 10 µg/ml, and aggregation was monitored in PAP-E8 aggregometer for 10 minutes. These results are expressed as mean ± SEM. The effect of different concentrations of anti-platelet agents compared to untreated (DMSO control) samples were determined using 2-way ANOVA with Dunnett correction for multiple comparisons (n=6, *P<0.05, **P<0.01, and ***P<0.001).



Supplementary Figure 3. Atherosclerotic plaque stimulates platelet aggregation in PRP samples in LTA. **A)** Atherosclerotic plaque homogenate stimulated platelet aggregation in PRP in LTA, inducing a stronger platelet aggregation at lower dilutions that higher dilutions which could be due to light transmission principle of LTA; high concentrations of plaque might obstruct light transmission, **B)** Representative aggregation traces for different concentration of atherosclerotic plaque homogenate (35-800 µg/ml). N=2.