Platelet function and interaction with the contact system of coagulation

By

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CERTIFICATE OF APPROVAL

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Table of contents

List of Figures

List of tables

List of abbreviations

ADAMTS13	a disintegrin like and metalloprotease with thrombospondin type I repeats-13
ADP	adenosine diphosphate
ApoER2	apolipoprotein E receptor 2
BK	Bradykinin
BSA	Bovine serum albumin
BSS	Bernard-Soulier syndrome
BTK	Bruton's tyrosine kinase
C1r	component 1r
C1s	component 1s
CBR	Cannabinoid receptor
COX-1	cyclooxygenase-1
CRP-XL	Collagen related peptide XL
CVD	Cardiovascular disease
DAMPs	damage-associated molecular patterns
DNA	Deoxyribonucleic acid
DOACs	Direct oral anticoagulants
ECM	Extracellular matrix
Factor II	Prothrombin
FDP	fibrin degradation products
FIIa	activated factor VII

FITC	Fluorescein isothiocyanate
FIX	factor IX
FV	factor V
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
FXIa	Activated factor XI
FXIII	Factor XIII
Gla	gamma-carboxyglutamate
Glu	glutamate
GPCR	G-protein coupled receptor
GPIb	Glycoprotein Ib
GPO	glycin-proline-hydroxyproline
GPVI	Glycoprotein VI
HAE	hereditary angioedema
HC	Heavy chain
HK	high molecular weight kininogen
ITAM	immunoreceptor, tyrosine-based activation motif
kDa	Killo dalton
KKS	kallikrein-kinin system
LAT	linker for activation of T cells
LC	Light chain
MAPK	mitogen-activated protein kinases
MI	Myocardial infarction

NETs	neutrophil extracellular traps
NHP	non-human primate
NO	Nitric Oxide
PAI	plasminogen activator inhibitor
PAMPs	pathogen-associated molecular patterns
PAR	Protease activated receptor
PGI2	prostaglandin I2
PI3K	phosphoinositide 3-kinase
PK	Prekallikrein
PLC-ɣ2	phospholipase C-ɣ2
PN-II	Protease nexin-II
PS	phosphatidylserine
RAS	renin-angiotensin system
RGD	arginine-glycine-asparagine
RNA	Ribonucleic acid
SFK	Src family kinases
TF	Tissue factor
TFPI	tissue factor pathway inhibitor
THC	Δ9-tetrahydrocannabinol
TLR	Toll like receptors
TM	Thrombomodulin
TP	Thromboxane receptor

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Abstract

Platelet function and interaction with the contact system of coagulation

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The prevention of bleeding and protection of the vascular wall integrity is mediated by the blood cells platelets and enzymatic coagulation reactions in a process called hemostasis. An imbalance in this intricate hemostatic interplay of platelets and coagulation enzymes will cause pathological thrombus formation and this may lead to cardiovascular diseases such as myocardial infarction, stroke, and venous thrombosis. The present research focuses on the role of platelets in providing a surface that support coagulation and function and how platelets activation states can be affected by interference of their signaling cascades by environmental and dietary components.

In our first study, we aimed to investigate the secretory and binding mechanisms by which platelets could support or inhibit coagulation enzymes, specifically the intrinsic pathway enzyme, activated factor FXI (FXIa). Different platelet activation phenotypes are able to protect the enzymatic activity of FXIa. Intrinsic pathway members like FXIa are able to bind to platelet

adhesion receptors, the role of this interaction remains unknown. We observed that FXIa binding to the platelet membrane increases its capacity to activate its downstream substrate, FIX, by likely protecting it from inhibition by several inhibitors secreted by activated platelets. This study provides mechanistic insights in how platelets protect and support coagulation by localizing it to the platelets surface.

Next, our studies were focused on the hemostatic function of platelets, which is partially dependent on platelet recruitment to damaged endothelial surfaces. Platelet recruitment to sites of vascular injury is mediated by the adhesive protein von Willebrand factor (VWF). As such the binding and conformational changes in shear-induced unraveling of ultra-large VWF multimers causes the formation of a “stringlike” conformation, which rapidly recruits platelets from the bloodstream. A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), regulates this process by cleaving VWF to prevent aberrant platelet adhesion; but, it is unclear how the activity of ADAMTS13 itself is regulated. We demonstrate a novel mechanism for the coagulation proteinase FXIa in regulating ADAMTS13 activity and function. This may represent an additional hemostatic function by which FXIa promotes local platelet deposition at sites of vessel injury.

Finally, we studied the effects of environmental and dietary components on systemic platelet functions, as this is ill-defined. One dietary component is cannabis; its usage has steadily increased nationally as acceptance is growing for both medical and recreational reasons. Medical cannabis is administered for treatment of chronic pain based on the premise that the endocannabinoid system signals desensitize pain sensor neurons and produce anti-inflammatory effects. The major psychoactive ingredient of cannabis is Δ9-tetrahydrocannabinol (THC) that signals mainly through cannabinoid receptor-1 (CBR1), located primarily on neurons

and also present on non-neuron cells including circulating platelets. This study investigates the effects of chronic THC administration on platelet function using a non-human primate (NHP) model. We observed a reduction in platelet adhesion, aggregation and granule secretion in response to select platelet agonists in animals fed a daily THC edible. These results indicate that chronic THC edible administration desensitized platelet activity and function in response to platelet receptors stimulation by interfering with the platelet primary and secondary feedback signaling pathways. This study provides insights in the autologous mechanisms that cannabis has on platelet function, and this can lead to improvements of health policies for medical management of patients who use THC derived medicine.

Overall, these studies define new insights in platelets function in supporting coagulation and the interference with platelet signaling. Future studies, will investigate how thrombin platelet receptor signaling works and identify novel targets for drug interference in platelet functionality that may help to predict hematological outcomes of clinical treatments.

Chapter 1 Introduction to platelet biology and coagulation cascade involvement

1.1 Overview

In the human circulatory system, cells and enzymes are tremendously important to sustain a healthy organ function. Platelets are the smallest circulating cells, which are single anuclear cells derived from bone marrow located megakaryocytes. Platelets circulate for 7-10 days at 150,000-450,000 per microliter of blood and are cleared from the circulation by the spleen. Their main role is to patrol endothelial vessel walls to maintain healthy endothelial barrier function and prevent vascular leaks, which is part of a process called hemostasis. The endothelial barrier can be damaged and injured, which leads to the expression of activating components by the endothelial cells, like tissue factor (TF), and exposure of extracellular matrix proteins (ECM), to which platelets will rapidly attach. Platelet attachment to ECM leads to platelet activation, including shape change, releasing chemical messengers and recruiting more platelet aggregates to the site of injury.[1] In parallel, and in response to platelet activation, is the initiation of blood coagulation enzymes that normally circulate as inactive zymogens. Activation of the coagulation enzymes commence the production of thrombin, which is a potent platelet activator as well as the main protease to convert fibrinogen into fibrin for the formation of a tightly packed clot of platelet aggregates. In the following sections, I will dissect the functional roles of platelets and the importance of their interplay with coagulation enzymes leading to clot formation in a healthy setting, like hemostasis and in pathological disease states like thrombosis.

1.2 Platelets and coagulation in cardiovascular disease

Cardiovascular disorders like ischemic stroke, myocardial infarction (MI) and venous thromboembolism (VTE), result primarily from the formation of a pathological thrombus. Globally, coronary heart disease and stroke are the main leading causes for disease burden, with thrombosis as underlying cause.[2] This high percentage of the American population affected

by cardiovascular diseases (CVD) brings a tremendous burden on the healthcare system as the number of people dying from cardiovascular disease has progressively increased over time to 15%; reaching as high as 17.6 million deaths per year in the United States.[3]

A thrombus consists of multiple components, and primarily comprised of red blood cells and platelets as well as enzymatic coagulation reactions leading to fibrin generation. Current anti-thrombotic therapies target both platelet activation and responsiveness or coagulation enzymes. These therapies are successful in the treatment of thrombosis, however, the risk of bleeding is dangerously high.[4,5] Therefore, there is an unmet clinical need for new anti-thrombotic therapeutic targets that uncouple the bleeding side effects while still being effective against thrombosis.

1.3 Endothelial cells

Part of the circulatory system in our body are endothelial cells that align the vessel wall, with the main function of separating circulating blood from tissue.[6] The endothelium is a monolayer of cobblestone-shaped cells, which covers the inner wall of blood vessels separating the lumen from the surrounding tissue. Endothelial cells influence the microenvironment by expressing membrane-bound receptors for a variety of molecules like proteins, hormones, lipid-transporting particles, metabolites and junctional proteins and receptors that regulate cell-cell and cell-ECM interactions.[7] Physiologically, the endothelial cells play a role in regulating platelet function by mechanisms including secretion of nitric oxide (NO) and prostaglandin I2 (PGI2), which are potent platelet inhibitors and keep the platelets quiescent.[8] Other mediators expressed by the endothelium are thrombomodulin (TM), which regulates coagulation activation and indirectly platelet reactivity. Furthermore, the endothelial cells are a source of bioactive lipid mediators

called oxylipins that can in turn affect platelet function by either acting as inhibitors or activators of platelets.[9,10]

1.4 Platelet adhesion to endothelial cells and extracellular matrix proteins

At sites of vascular injury, the endothelium shifts from an anticoagulant to a procoagulant phenotype to prevent excessive blood loss. Upon damage or inflammation, the endothelium will express inflammatory receptors and increase von Willebrand factor (VWF) secretion that will lead to increased platelet attachment and reactivity in inflammatory settings. Upon vascular injury the endothelial barrier is disrupted and exposes underlying ECM proteins, which are the surrounding connective tissue adjacent to the vessel wall. Subjection of ECM proteins to the blood will induce rapid recruitment of attachment proteins and platelets to the site of injury via

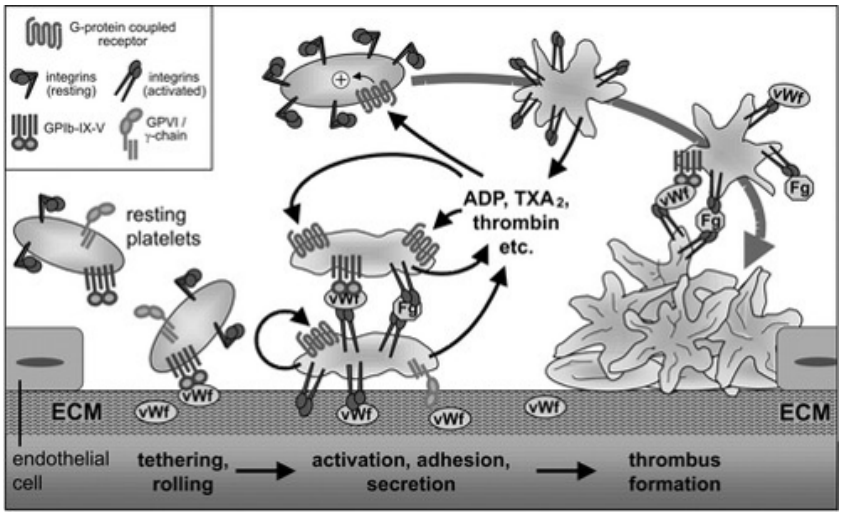

Figure 1.1 Activation of platelets at sites of vascular injury.
The initial contact of platelets with the extracellular matrix (ECM) is mediated by plasma von willebrand factor (VWF) binding to exposed collagen and platelet GPIb, followed by the activation of platelets by collagen via the collagen receptor GPVI. Leading to tethered adhesion to VWF. More platelets are activated and attracted into the growing platelet plug via integrin αIIbβ3 interaction between fibrinogen and a secondary platelet. Fg indicates fibrinogen; ADP, adenosine di phosphate; TXA2, thromboxane. Figure adapted from Offermanns et al., Circulation Research. 2006;99:1293–1304. Reprinted with permission from Wolters Kluwer Health, Inc.

VWF attachment to the ECM. This recruitment is tightened with the interaction of platelet cell surface receptor binding with ECM proteins (Figure 1.1). The composition of the ECM are a large variety of adhesive macromolecules, such as laminin, fibronectin and collagens.[11]

1.4.1 Von Willebrand factor

The initial and rapid recruitment of platelets to sites of vascular damage is mediated by the VWF at the endothelial site of injury or surface. Von Willebrand factor has a large multimeric glycoprotein structure that is present in storage granules of endothelial cells called Weibel-Palade bodies, in platelets granules and shorter forms present in the circulation.[12] Megakaryocytes, platelets precursor cells, and endothelial cells produce VWF as a propeptide of 2813 amino acids and the various cysteine bonds are important for generating VWFs multimeric structure and formation of the 2050 amino acid mature VWF protein (Figure 1.2) after secretion. The VWF domains are important for its structural function as well as interaction sites such as: (I) the D'-D3 domains interact with coagulation factor VIII that is important for functional coagulation processes, (II) the A1 domain interacts with platelet receptor glycoprotein Ib (GPIb) and collagen type IV, (III) A2 domain is the cleaving site for VWF length regulation, (IV) A3 domain interacts with collagen types I and III, and (V) the C1 domain for platelet integrin receptors.[13] VWF secretion from granules depends on the cell type. Platelets release VWF

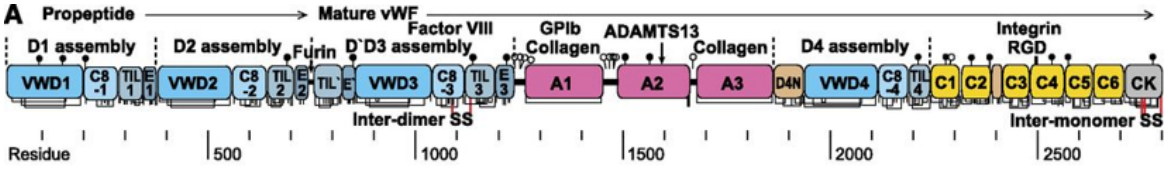

Figure 1.2 Von Willebrand factor (VWF) structure and important structural domains. VWF is a 2813 amino acid long protein with the main function of carrying coagulation factor VIII via its D3 domain and platelet receptor Glycoprotein (GP) Ib binding via the A1 domain. Figure adapted from Springer et al., Blood, 2014 124 (9): 1412–1425. Reprinted with permission from American Society of Hematology.

predominantly upon platelet activation, while endothelial cells have a basal and regulated release of VWF from the Weibel-Palade bodies.[14] The plasma circulating VWF will be mostly originated from the basal release of endothelial cells. Under static conditions, VWF is irregularly coiled and under flow conditions it extends and exposes important domains.[15] The main regulator of VWF is a metalloprotease named ADAMTS13 (a disintegrin like and metalloprotease with thrombospondin type I repeats-13), which is a 200kDa protein that circulates in a constitutively active form and primarily mainly functions to proteolyze VWF in its A2 domain.[16] Secreted VWF threads bundle into 100- to 1000-μm-long strings bound to the endothelial cell surface. The cleavage by ADAMTS13 facilitates VWF release into the plasma, where further proteolytic cleavage by ADAMTS13 occurs, reaching lengths up to 40 to 200 monomers (3 to 15 μm lengths).[15,17] The proteolytic processing of VWF multimers is important to prevent microvascular thrombi formation.[18] Upon vessel injury, or pathological conditions, the flow conditions will change and plasma VWF binds with its A3 domain to the exposed ECM collagen and participates in the recruitment of platelets to the endothelial wall, by providing a "catch and release" tethering concept with platelets prior to firmly adhering to the ECM. With the A1 domain exposed, platelets interact with VWF via GPIb-V-IX.[19] Deficiencies or malfunctions in the VWF protein can lead to bleeding tendencies, as this is called von Willebrand disease.[20]

1.4.2 Collagen

Collagen is the most abundant fibrous protein in the extracellular matrix.[21] In the body there are different types of collagens, including, fibrillar collagens type I, III, and V, which are found in the deeper layers of the ECM and are thus not accessible after superficial injury. Type IV, VIII, and XVIII collagen are expressed in the basement membrane, i.e., closer to the endothelial layer.[22] When collagen is exposed to the blood, platelets from the circulation firmly adhere to collagen via receptor glycoprotein VI (GPVI), after being tethered rolling on VWF structures bound with

the A1 (collagen type IV) and A3 (collagen types I and III) domains to collagen.[22] The interaction of GPVI with collagen is mediated by glycine-proline-hydroxyproline (GPO) sequences on collagen. Firm attachment of the platelets to collagen is also partially mediated via integrin α2ß1, reinforcing collagen binding and enhancing GPVI signaling. This induces signaling cascades that lead to a secondary activation wave of platelets and recruitment of platelet-platelet aggregate formation from the circulation.

1.4.3 Other ECM basement membrane proteins

In addition to collagen and VWF, platelets are exposed to a wide variety of ECM proteins upon vessel injury. On the platelet surface are numerous integrin receptors expressed that bind ECM proteins via arginine-glycine-asparagine (RGD) motifs. Interactions can include fibronectin, laminins, nidogens and perlecans that are able to induce mild platelet adhesion and also enforce potent signaling for GPVI via collagen.[22]

1.5 Platelet adhesion receptor mediated signaling

The initial stage of hemostasis is the recruitment and firm adhesion of platelets to site of injury, where platelet attachment receptors are activated. The adhesion of platelets lead to internal platelet signaling and results in platelet shape changes by a process of actin skeleton rearrangement. This includes the VWF receptor GPIb, collagen receptor GPVI and fibrinogen receptor integrin αIIßIII. GPVI propagates signaling through an immunoreceptor, tyrosine-based activation motif (ITAM). Together, this leads to downstream activation of kinases and phosphorylation events and recruitment of internal phospholipases and adaptor proteins (Figure 1.3).[23]

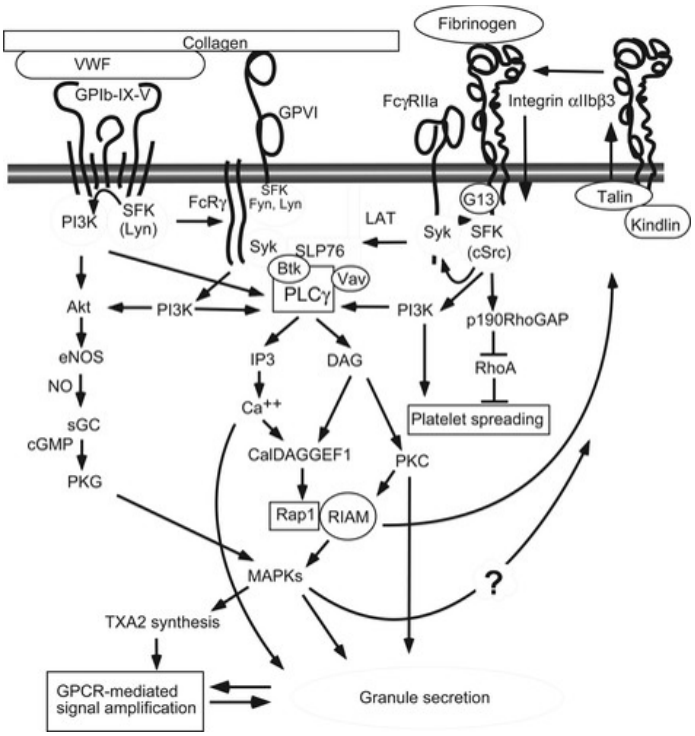

Figure 1.3 The main signaling pathways of platelet adhesion receptors.
Schematic representation of signaling pathways of the three major platelet adhesion receptors; GPVI, VWF and integrin receptor αIIbβ3. Activation of adhesion receptors by binding to collagen VWF or fibrinogen lead to signaling and granule secretion. Figure adapted from Li et al., Arteriosclerosis, Thrombosis, and Vascular Biology. 2010;30:2341–2349. Reprinted with permission.

1.5.1 GP1b- IX-V

GPIb-IX-V is a glycoprotein receptor complex and highly abundant, with ~25,000 copies per platelet, which is exclusively expressed on megakaryocytes and platelets. The GPIb-IX-V complex belongs to the leucine-rich repeat protein superfamily and is comprised of 9 transmembrane units: one outer GPIX unit, two GPIbß units, one GPIbα, one central GPV, followed by another GPIbα, two GPIbß units, and a GPIX.[24] The major functional subunit of the receptor complex is GPIbα with a molecular weight of 135kDa, and is linked via disulphide bonds to the ß units and non-covalently associated to the GPIX and GPV.[25] GPIbα interaction partners are VWF A2 domain, platelet granule P-selectin, thrombin, high molecular weight

kininogen (HK), and factor XII.[26] Signaling downstream of the cytoplasmic domain of GPIbα involves Src family kinases (SFK), Lyn, phosphoinositide 3-kinase (PI3K) and Akt leading to calcium changes inside the platelet and integrin receptor activation.[27] A lack or dysfunction of GPIb-V-IX complex has been associated with the Bernard-Soulier syndrome (BSS), a congenital bleeding disorder characterized by mild thrombocytopenia and giant platelets.[28]

1.5.2 GPVI

Human GPVI platelet surface receptor (62kDa) consists of two Ig domains, a mucin-rich stalk and a cytosolic sequence of 51 amino acids that are coupled to a Fc Receptor ɣ chain homodimer via disulfide linking. The cytosolic proline rich region of GPVI serves after stimulation with collagen as a recruitment site for SFK that phosphorylates the ITAM of FcRy, inducing signaling events via tyrosine kinase Syk and the initiation of downstream signaling events via the transmembrane adapter, linker for activation of T cells (LAT) signalosome consisting of Bruton's tyrosine kinase (BTK) and phospholipase C (PLC)-ɣ2 and activation of mitogen-activated protein kinases (MAPKs) (Figure 1.3).[29,30] Human patients with defects associated with GPVI are rare and usually associated with a mild bleeding disorder.[31]

1.5.3 Integrin receptors αIIbβ3

The dominant platelet integrin receptor αIIbβ3 is part of the family of transmembrane glycoprotein signaling receptors and is comprised of α and β noncovalently associated subunits. These subunits have a large extracellular domain for ligand binding, a transmembrane domain and a smaller cytoplasmic tail for the recruitment of adaptor proteins, signaling proteins, and cytoskeleton-associated proteins, which play an essential role in integrin bidirectional signaling (inside-out signaling and outside-in signaling). Integrin receptor is continually expressed on the platelet surface in an inactive form, upon platelet activation and signaling pathways and rising

intracellular calcium and recruitment of talin and kindlins,[27] the integrin receptor changes conformation to an open form. The activated αIIbβ3 receptor is now receptive for ligand binding with RGD containing ligands, such as fibrinogen which mediates platelet-platelet interaction and aggregate formation. Other ligands are fibrin, VWF and fibronectin. The outside-in signaling is triggered by the binding of soluble fibrinogen to activated integrin αIIbβ3 and leads to intracellular signaling events important for platelet irreversible adhesion, spreading, cytoskeletal reorganization and irreversible aggregation.[32,33] Patients with the congenital bleeding disorder Glanzmann thrombasthenia demonstrate a the lack of a functional platelet integrin receptor.[34]

1.6 Platelet aggregation

To form a hemostatic clot, a multistep process of platelet-platelet interaction is needed at a site of vascular injury. A main component in platelet-platelet interactions is the plasma protein fibrinogen.[1] Fibrinogen is produced by the liver, and is a 340kDa large hexameric homodimer that plays a massive role between platelets and coagulation in hemostasis.[35] Without the presence of fibrinogen, initial platelet adhesion can occur, however the formed clot does not grow and is considered unstable.[36]

1.7 Platelet GPCR signaling

Other than the signaling induced by the adhesion to adhesive proteins, soluble platelet agonists mostly signal to a receptor class called G-protein coupled receptor (GPCR) as shown in Figure 1.4. While primary adhesion of platelets to the vessel wall is largely independent of G protein–mediated signaling, the subsequent recruitment of additional platelets into a growing platelet thrombus requires diffusible mediators such as adenosine diphosphate (ADP), thromboxane A2 (TXA_2), or thrombin, which act through GPCRs. Platelet activation via GPCRs involves 3 major G protein–mediated signaling pathways that are initiated by the activation of the G proteins G_q,

G_s, G_{13}, and G_i that forms a complex. GPCRs are 7-transmembrane domain receptors that signal through heterotrimeric G proteins consisting of 3 subunits (α, β and γ) that bind to GPCRs to form a complex. The α subunit is converted from GDP- to GTP-bound active form upon receptor ligation, dissociating from the receptor and interact with downstream targets for signal transduction.[37] The β and γ complex can also interact with effectors downstream.[38] G proteins are coupled to agonist receptors (GPCRs) that stimulate platelet activation, except for Gs that are coupled to platelet inhibitors (like prostacyclin and adenosine).

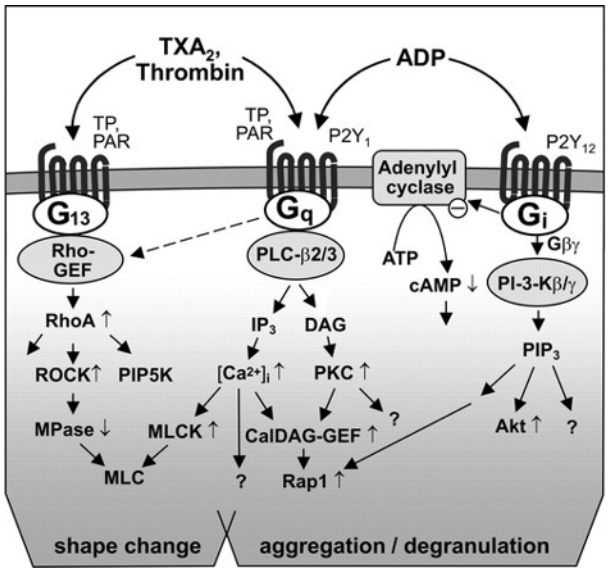

Figure 1.4 Signaling pathways induced via GPCRs in platelets. Schematic representation of the upstream signaling mechanisms linking the activation of GPCRs by ADP, TxA2, and thrombin via the G proteins G13, Gq, and Gi to the induction of platelet-shape change, inside-out activation of integrins, and degranulation. RhoGEF indicates Rho-guanine nucleotide exchange factor; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; MPase, myosin phosphatase; MLCK, MLC kinase; DAG, diacyl glycerol; CalDAG-GEF, calcium and diacyl glycerol-regulated guanine nucleotide exchange factor; PIP3, phosphatidylinositol-3,4,5-trisphosphate. Figure adapted from Offermanns et al., Circulation Research. 2006;99:1293–1304. Reprinted with permission from Wolters Kluwer Health, Inc.

1.7.1 P2Y1 and P2Y12 receptor

Secondary activation of platelets by soluble components is mediated via GPCRs. ADP released from granules binds to the P2Y receptors that bind purine and pyrimidine nucleotides, with platelets expressing two types, P2Y1 and P2Y12. The P2Y1/12 receptor stimulation on the platelet is necessary for integrin activation, shape change and membrane flipping of phosphatidylserine (PS). P2Y1 is coupled to G_q, while P2Y12 is coupled to G_i. P2Y12 expression is mostly limited to platelets, vascular smooth muscle cells, and brain microglial cells. In contrast, P2Y1 is widely distributed on multiple cells and tissues.[27,39]

1.7.2 Protease activated receptor 1 & 4 (PAR1/4)

Human platelets express a GPCR class of proteins called protease activated receptors (PAR) that consists of 7 transmembrane domains with 3 subdomains (α,ß,ɣ).[40] Human and non-human primate (NHP) platelets express protease-activated receptors 1 and 4 (PAR1/4), GPCRs that are activated through N-terminal cleavage by proteases such as thrombin, cathepsin G, plasmin, and trypsin.[41] The cleavage of the N-terminal domain generates a tethered peptide ligand that binds back to the PAR receptor (Figure 1.5) and initiates signaling through G_q, $G_{12/13}$, and G_i as seen in Figure 1.4. Additional downstream signaling leads to platelet shape change, granule secretion and membrane flipping.

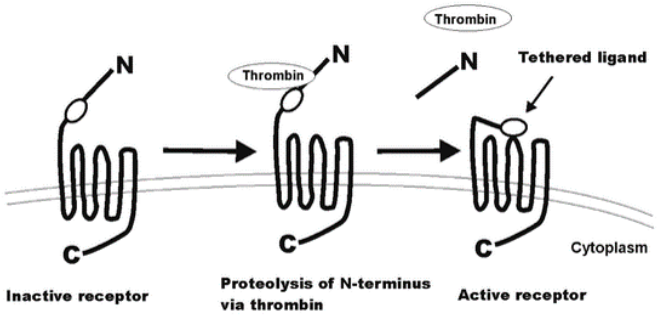

Figure 1.5 Protease Activated Receptor (PAR) activation by thrombin. The N terminal cleavage of thrombin will lead to a tethered ligand that activates the PAR receptor and downstream signaling events. Figure adapted from Wojtukiewicz, M.Z., Hempel, D., Sierko, E. et al. Protease-activated receptors (PARs)—biology and role in cancer invasion and metastasis. Cancer Metastasis Rev 34, 775–796 (2015). Figure reprinted with permission.

1.7.3 Thromboxane receptor (TP)

Part of the secondary positive-feedback during platelet activation is the production of TXA2, which is derived from the platelet membrane component arachidonic acid. The conversion of arachidonic acid to TXA2 is mediated by cyclooxygenase-1 (COX-1)-dependent metabolism.[42] Aspirin targets COX-1 activity and prevents TXA2 generation. The GPCR receptor for TXA2 is TP that is leading to G-protein signaling via G_q and $G_{12/13}$ resulting in PLC-ß upregulation.[43] The role for TXA2 signaling is as an amplifier of platelet aggregation.[44] TxA2 can also act as a vasoconstrictor during times of tissue injury and inflammation.[45]

1.7.4 Cannabinoid (CB) receptor 1 and 2

Platelets express a class of GPCRs that are part of an immunomodulating system, the endocannabinoid system, expressed in the central nervous system and on immune cells in the periphery. It is composed of endogenous secreted cannabinoids (anandamide, 2-arachidonoylglycerol) that portrays an important role in maintaining immune system homeostasis as well as in modulating inflammatory processes and pain management.[46] It has primarily expressed GPCR receptors CB1 and CB2 that are also present on platelets with an

unknown function. Potentially, they regulate platelet immune function and interaction with other immune cells and the endothelial cells.[47] The most known exogenous ligand of CB1 is Δ9-tetrahydrocannabinol (THC). Both CB1 and CB2 receptors (CB1R and CB2R) primarily signal through the inhibitory G proteins G_i and G_0.[48] CB1R is encoded by the gene *CNR1* and consists of 472 amino acids in humans. CB2R is encoded by the gene *CNR2*, which consists of 360 amino acid in humans.[49] The implications of inhibitory G protein activation is that stimulation of CB1R leads to the inhibition of adenylyl cyclase, the activation of MAPK, the inhibition of certain voltage-gated calcium channels and the activation of G protein-linked potassium channels. Stimulation of CB2R has similar signaling pathways, except the modulation of ion channels by CB2R is more variable. However, CB1R, but not the CB2R, has been reported to activate other G proteins in certain circumstances in a cell type- and ligand-dependent manner.[48]

1.8 Platelet granule secretion

Part of platelets distinct function in regulating its own activity and its microenvironment is their ability of releasing granules at distinct times. To date, more than 300 distinct molecules have been detected in platelet supernatants.[50] Upon activation platelets have granules stored away and increasing calcium concentrations drive membrane trafficking of vesicles and the secretion of components that are anti- and pro-coagulant.[51,52] Platelets contain three types of granules: alpha (α) granules, dense (δ) granules and lysosomes. Each platelet contains ~3–6 dense granules, with a diameter of 150nm[53] that store small molecules such as ADP, ATP, GDP, 5-HT, pyrophosphate, magnesium, polyphosphate and calcium. Dense granules are the first to be secreted after platelet activation. The number of α-granules are preserved to 50-80 per platelet with a 200-500nm diameter and contain a multitude of proteins that mostly make up the platelet secretome.[52] The α-granule contents includes hemostatic factors Factor V, VWF, fibrinogen, angiogenic factors like vascular endothelial growth factor (VEGF), anti-angiogenic factors like

platelet factor-4, growth factors, membrane proteases, and other cytokines.[54] Most granule elements are produced by the megakaryocyte, however, platelets also take up proteins from the circulation via endocytosis. The third class of platelet granules besides alpha- and delta-granules, are lysosomes, which store glycohydrolases able to degrade glycoproteins, glycolipids and glycosaminoglycans.[55]

1.9 Procoagulant platelets

Platelet adhesion to VWF leads to the initial platelet rolling and adhesion to the site of injury. Granule secretion and soluble factors can provide a secondary platelet signaling activation cascade leading to more platelet recruitment from the circulation. However, to form a stable thrombus, platelets need to be attached and incorporated by a fibrin network. In a growing thrombus, distinct platelet populations can be found in different layers of the thrombus. Most platelets are aggregatory platelets, and only 8-10% of the platelet population in the thrombus experiences ultra-intracellular calcium rises.[56] This increase in calcium levels will inhibit membrane specific flippase and stimulate the nonselective lipid transporter scramblase, resulting in PS membrane flipping and exposure to the outside, creating of a negatively charged surface that functions as a coagulation activating platform.[57] The intracellular increase in calcium levels will only subdue after a dual stimulation via GPVI and PAR stimulation.[58]

1.10 Coagulation

Hemostasis, a highly preserved process among species for survival, enables repair of damaged vasculature to prevent blood loss. The blood coagulation system represents the first line of defense for many organisms.[1] This system consists of a cascade of circulating inactive serine proteases, zymogens, as well as regulatory cofactors and inhibitors. Thrombin is the main end product, which is important for the propagation of the coagulation cascade. This basic clotting system was historically present in jawless fish with evidence starting 450 million years ago. The initial system of thrombin generation was based on vitamin K-dependent proteases that are part of the extrinsic pathway of coagulation (Figure 1.6).[59] In some vertebrates however, another system can trigger enzymatic blood clotting, via contact activation known as the intrinsic pathway of coagulation (Figure 1.7).[60]

1.10.1 Extrinsic pathway of coagulation

The extrinsic pathway of coagulation is initiated by a disruption in the endothelial cell barrier. This leads to the exposure of TF that forms a complex with activated coagulation factor VIIa (FVIIa). 1%-4% of FVII already circulates in plasma in the active form FVIIa ready to serve coagulation when TF is exposed.[61] This will set off a cascade of enzymatic reactions leading to the generation of thrombin and formation of a fibrin clot. A hallmark of the extrinsic pathway coagulation enzymes is that they are vitamin K-dependent factors. Vitamin K is necessary for the post translational carboxylation modification that converts specific glutamate residues (Glu) to gamma-carboxyglutamate residues (Gla) in vitamin K dependent clotting factors.[62] The Gla domain of the vitamin K-dependent factors is essential for the binding to negatively charged phospholipids in a calcium dependent manner, such as PS, which mediates their optimal activation. The vitamin K-dependent coagulation factors are factor II (prothrombin), factor VII, factor IX (FIX), factor X (FX) and proteins C and S. A deficiency in vitamin K can result in

bleeding, as observed with warfarin, which inhibits the function of the vitamin K epoxide reductase complex in the liver resulting in depletion of the reduced form of vitamin K and prevents thereby the liver from producing essential extrinsic pathway coagulation factors.[63]

With the TF/VIIa complex formed after endothelial cell injury, it subsequently activates coagulation FX and coagulation FIX, which leads to trace amount of thrombin. Thrombin will then activate the co factors factor V (FV) and factor VIII (FVIII) amplifying the reaction by ~300,000 fold.[64] In a calcium and phospholipid-dependent manner, the tenase and prothrombinase complex will be formed. The tenase complex consisting of activated FIX (FIXa), FX, and the cofactor activated factor VIII (FVIIIa), to promote further activation of FX. Subsequently, the prothrombinase complex will be formed, consisting of activated factor X (FXa), prothrombin, and the cofactor activated factor V (FVa), converting prothrombin into thrombin.

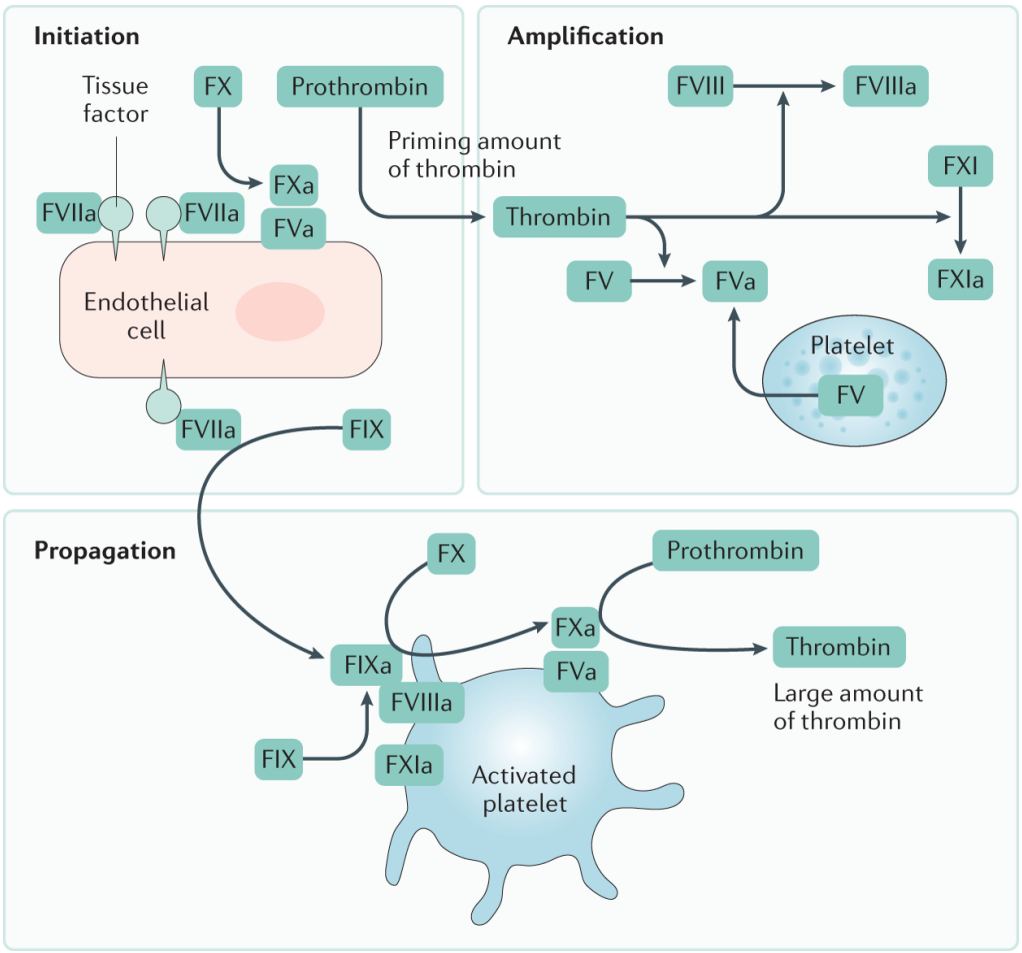

Figure 1.6 Enzymatic coagulation reactions of the extrinsic pathway of coagulation. Upon vascular damage, tissue factor (TF) is exposed to the blood leading to complex formation with factor (F)VIIa and initiation of thrombin generation via activation and formation of the prothrombinase complex. Free thrombin mediates its own amplification by activation of the cofactors factor V and VIII into their active forms, FVa and FVIIIa respectively. Furthermore, thrombin will activate the intrinsic pathway member, factor XI (FXI) into FXIa that propagates the enzymatic signal by activation of FIX and tenase complex formation on the platelet surface leading to a large amount of thrombin. To stabilize a thrombus, thrombin converts soluble fibrinogen into insoluble fibrin strands to tighten the formed clot. Subsequently, thrombin generated in the initial stages will lead to platelet activation via protease activated receptor (PAR) signaling and the membrane surface exposure of phosphatidylserine (PS). Essential for the phospholipid interaction with the tenase and prothrombinase formation are calcium ions released from activated platelet granules. Figure adapted from E. Moore et al., Trauma-induced coagulopathy. Nat Rev Dis Primers (2021). Figure reprinted with permission.

In the amplification phase of thrombin generation, thrombin also activates factor FXI, one of the members of the intrinsic pathway of coagulation. Activated factor XI (FXIa) can also activate FIX. But in contrasts to the tenase and the prothrombinase complex, the activation of FIX by FXIa does not depend on a phospholipid surface.[65,66] FIX is a 55kDa protein consisting of a heavy chain and a light chain. FIX deficiency is associated with the bleeding disease Hemophilia B, whereas a deficiency in the cofactor FVIII from the tenase complex causes the bleeding disorder hemophilia A. Regulation of the extrinsic pathway is performed mainly by tissue factor pathway inhibitor (TFPI), a kunitz type inhibitor that inhibits the TF/FVIIa complex and FXa.[67] The cofactors FVa and FVIIIa are inhibited by activated protein C, which is activated by the thrombin-TM complex on endothelial cells.[68]

The ultimate goal of the extrinsic pathway is the formation of a hemostatic clot to control blood loss after endothelial cell barrier injury. Thrombin, the free enzymatic end product of the extrinsic and intrinsic pathway of coagulation will convert fibrinogen into fibrin fibers that are needed for a stabilized clot.[1] Fibrinogen is a large hexameric homodimer of 340kDa that circulates at a concentration of 2-4 mg/ml in plasma. The fibrinogen structure consists of two sets of three distinct disulfide-linked polypeptide chains (Aα, Bβ, and γ). Thrombin mediated proteolysis converts fibrinogen into fibrin fibers that are crosslinked by coagulation factor XIII (FXIII), which is also activated by thrombin and is present in plasma and platelets.[69,70]

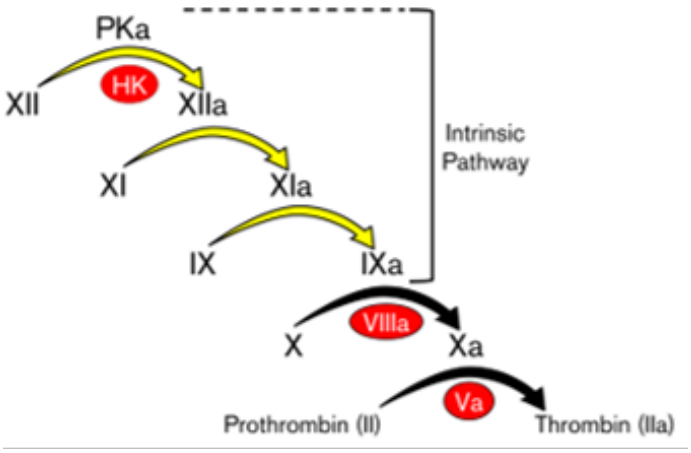

Figure 1.7 Intrinsic pathway of coagulation. Negative charged surface activation of factor XII (FXII) and prekallikrein (PK) will lead to downstream enzymatic conversions and activation of proteases leading to thrombin generation. Figure adapted from Ponczek et al., The evolution of factor XI and the kallikrein-kinin system. Blood advances (2020). Figure reprinted with permission.

1.10.2 Intrinsic pathway of coagulation

Besides the activation of the extrinsic pathway of coagulation that leads to a rapid response and fibrin formation, another pathway can be activated that lead to FXa formation and thrombin. The intrinsic pathway of coagulation, also called the contact system, is activated mostly on negatively charged artificial surfaces, such as biomaterials used to construct heart valves and stents, or biological molecules, such as DNA, RNA, denatured proteins, collagen, polyphosphate, and neutrophil extracellular traps (NETs).[71] The initiation of the intrinsic pathway is started by auto activation of coagulation factor XII (FXII) on a surface into activated FXII(a), which is greatly enhanced in the presence of other contact pathway members, prekallikrein (PK), co-factor High molecular weight kininogen (HK) and factor XI (FXI). FXIIa cleaves PK to generate kallikrein (PKa) that in turn activates FXIIa via reciprocal activation. FXIIa initiates the activation of FXI to FXIa, which in turn activates FIX to FIXa leading to the generation of thrombin and fibrin formation (Figure 1.7). The activation of FXII and PK can also contribute to inflammatory responses via the kallikrein-kinin system (KKS) (Figure 1.10).[72,73] One of the main

differences between the intrinsic and the extrinsic pathway of coagulation is that the activation of the intrinsic pathway is independent of a phospholipid surface and calcium.

Coagulation Factor XII (FXII)

FXII is a 80kDa glycoprotein produced by the liver and circulates in plasma at a concentration of ~375nM (40ug/ml).[72] The main function of FXII as a coagulation protein is the activation of coagulation FXI. Other FXIIa substrates as a serine protease include PK, plasminogen, complement component 1r (C1r), and C1s. FXII function and substrate interaction mainly derives from its different domains (Figure 1.8), which are homologous to serine protease domains except for the FXII proline-rich region.[71] The single chain activity of FXII can lead to

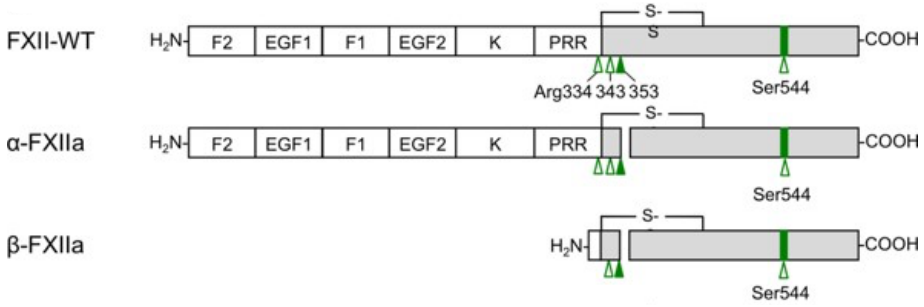

Figure 1.8 FXII protein domain structure and activation sites. The FXII protein consisting of a heavy chain and a light chain that give rise to 2 activated forms of FXII. Figure adapted from Shamanaev et al., Proteolytic activity of contact factor zymogens. Journal of Thrombosis and Haemostasis, Volume: 19, Issue: 2, Pages: 330-341 (2020). Figure reprinted with permission.

autoactivation in the presence of negatively charged surfaces.[74] Proteolytic cleavage of FXII after Arginine 353 by either autoactivation or PKa generates α-FXIIa, consisting of a heavy and a light chain held together by disulfide bonds. Extra proteolytic cleaving by PKa after the Arginine 334 region generates ß-FXIIa. This version of FXIIa eliminates its potential to bind to negatively charged surfaces. While ß-FXIIa retains proteolytic activity, it does not support coagulation via activation of FXI, but it promotes PKa generation. The main regulator in plasma for FXIIa is C1-inhibitor and anti-thrombin.[75] Interestingly, deficiency in FXII does not exhibit a

bleeding tendency. However, some mutations in the FXII protein cause robust inflammatory responses such as hereditary angioedema (HAE).[76]

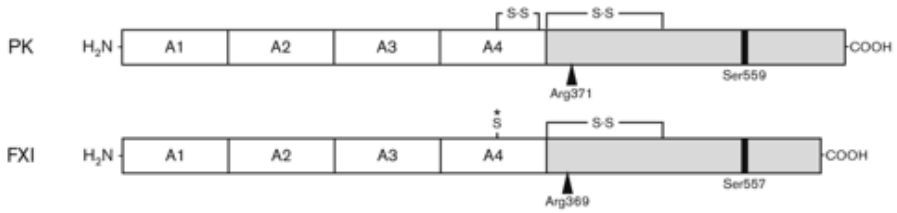

Figure 1.9 Structure of contact pathway proteins prekallikrein (PK) and factor XI (FXI). There is 60% sequence homology between FXI and PK. Both consists of 4 apple (A) domains combined with a catalytic domain. However FXI zymogen circulates in circulation as a dimer. Figure adapted from Ponczek et al., The evolution of factor XI and the kallikrein-kinin system. Blood advances (2020). Figure reprinted with permission.

Prekallikrein (PK)

PK is the precursor for the plasma protease PKa with a monomeric structure of 88kDa. In the circulation PK is mostly complexed with HK at a concentration of 490nM (50µg/ml).[77,78] The initiation of contact system activation starts with reciprocal activation of PKa and FXIIa.[79] Activation of PK by FXIIa is stabilized by HK for optimal activation,[80] and results into a heavy chain and a light chain, which contain the catalytic domain and held together by disulfide bonds (Figure 1.9). Recently, it has been shown that PK in its single-chain form can express proteolytic activity,[77] which might explain the initiation start of reciprocal activation. Another substrate for PKa besides FXII was recently identified, as PKa can activate FIX during contact activation in plasma in certain conditions independent of FXI.[81,82] However, this reaction is slow compared to FXIa standards. The participation of PK in inflammatory events is mediated by its ability to generate a vasoactive peptide, bradykinin (BK), derived from HK that causes inflammation, vasodilatation and an increase in vessel permeability.[83]

Coagulation Factor XI (FXI)

The serine protease coagulation factor XI (FXI) has a unique dimeric structure of 160kDa and has a 60% sequence homology with PK as a result of gene duplication in mammalian evolution. Hepatocytes are the main source of FXI secretion into the bloodstream, where it circulates at a concentration of ~30nM (2-7μg/ml). The dimeric structure of FXI contains of 4 apple domains and a catalytic domain attached via a disulfide bond.[84] FXI activation to the activate protease FXIa is mediated by cleaving after the Arg369 site.[85] In solution, most FXI circulates complexed to HK, potentially for the mediation of optimal binding to surfaces. Once FXIa is activated, it is dissociated from HK. (Figure 1.10). FXI role is important for the amplification of thrombin

Figure 1.10 FXI zymogen dimeric structure. FXI structural characteristics for the first, second, third, and fourth apple domains coupled to a catalytic domain (CD) via disulfide bonds. The dimeric structure consists of two FXI monomers. Figure adapted from Mohammed et al., An update on factor XI structure and function. Thrombosis research (2017). Figure reprinted with permission.

generation, as can be activated by thrombin generated by the extrinsic pathway of coagulation, or via FXIIa activation by intrinsic pathway of coagulation. The main substrate for FXIa is FIX, and more recently FXIa has also been shown to activate FX *in vitro* and to promote thrombin generation by activating the cofactors FVIII and FV,[84] and by cleaving TFPI.[86] Hemophilia C is a bleeding disorder that is seen in patients partially or completely deficient in FXI protein. Symptoms are associated with postoperative or posttraumatic bleeding, specifically in tissue of high fibrinolytic activity such as the nose, oral cavity, and urinary tract.[87]

1.10.3 The Kallikrein Kinin System (KKS)

The end product of the KKS, bradykinin (BK), is generated via reciprocal activation of FXIIa and PKa and PKa cleaving HK to derive the vasoactive peptide (Figure 1.11). The bradykinin B1/2 receptor (B1R and B2R) is expressed on the endothelial surface. The B2R is a constitutively expressed GPCR that can interact with the renin-angiotensin system (RAS), which is important for stimulation of NO and PGI2 production and tissue plasminogen activator (tPA) release. In inflammatory states, a second BK receptor, the B1R, is up-regulated to mediate additional activities such as vascular permeability, vasodilation edema and hypotension.[88] Uncontrolled BK generation is associated with recurrent local cutaneous or mucosal edema episodes in hereditary angioedema (HAE) patients.[83] The participation of FXI in immune processes, has until recently mostly been overlooked. However, FXI has been studied recently to activate FXII and can have inflammatory properties inducing vascular leakage.[89] Based on these recent findings and observations in our laboratories, FXIa could be also incorporated into the KKS.

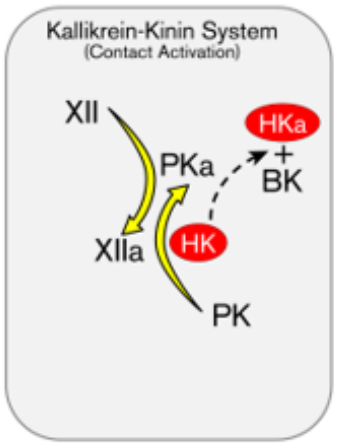

Figure 1.11 Kallikrein kinin system (KKS). Generation of the vasoactive peptide bradykinin (BK) is mediated by the proteolytic conversion of high molecular weight kininogen (HK) by kallikrein (PKa). The reciprocal activation between FXII and PK will lead to PKa. Figure adapted from Ponczek et al., The evolution of factor XI and the kallikrein-kinin system. Blood advances (2020). Figure reprinted with permission.

1.10.4 Platelet surface catalysis and cofactors

Platelets are important mediators in the interaction with coagulation factors and are present in different stages of a developing thrombus, as platelets endorse both pro- and anti-coagulant components as seen in Figure 1.12.[90] Platelets and coagulation intertwine by regulating thrombin generation.[91] Activated platelets can regulate thrombin formation by supporting the tenase and prothrombinase complex through the interaction with PS exposed on the platelet membrane or plasma vesicles. The members of the contact pathway, FXII, FXI and HK can bind to the platelet receptor GPIb,[78] although the effect of this interaction in thrombin generation is still unknown. Also, the coagulation factor thrombin induces platelet activation through the cleavage of PAR receptors for platelet internal signaling. Platelet granule secretory content are also potent regulators of the coagulation cascade. Dense granule contain polyphosphates (70-80 monomer length), which are cofactors of the thrombin amplification loop by enhancing FV

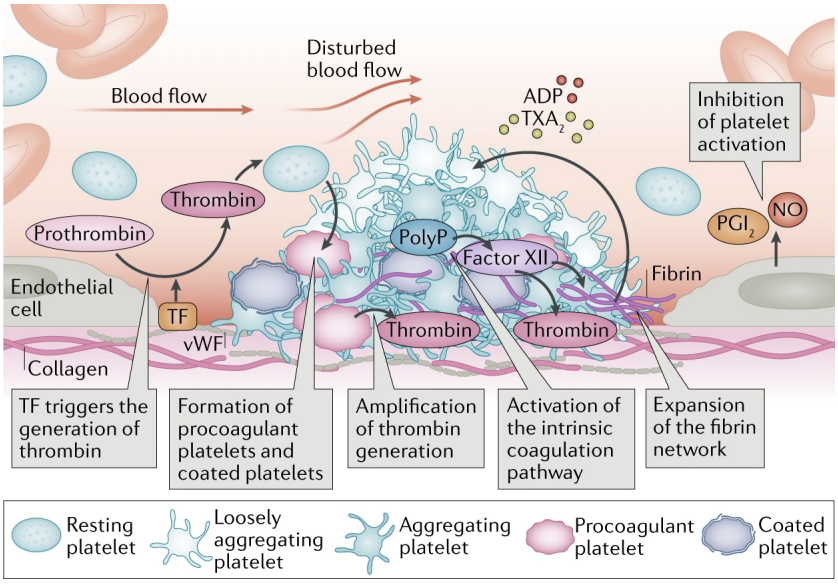

Figure 1.12 Interplay of coagulation and platelets in hemostasis and thrombosis. The activation of platelets and initiation of coagulation pathways is tightly intertwined. Distinct layers of platelet activation are present in a thrombus, with thrombus formation intitially triggered by tissue factor (TF). Thrombin amplifies itself via FXI activation and in parallel activates more procoagulant platelets. More aggregatory platelets are recruited from the circulation and the clot is growing with the expansion of the fibrin network. Figure adapted from van der Meijden et al., Nature Reviews Cardiology ISSN 1759-5010. Reprinted with permission from the Springer Nature publishing group.

activation and the activation of FXI by thrombin, making the reaction ~3000 fold more favorable.[92,93] Also, the secreted calcium from dense granules support Vitamin-K coagulation factors interaction with the PS exposed on platelet surface.[54] The potent prothrombinase regulator, TFPI, also resides in platelets; although, it is unknown where it is located, as it is not found in lysosomes or α-granules. The release of TFPIα after platelet activation increases local TFPIα concentrations by 2-3 fold and help dampen thrombin generation.[94] Platelets α-granules store fibrinogen and FV, which will further promote localized clot formation. Furthermore, platelet α-granules has protease nexin-II (PN-II), which is a potent FXIa inhibitor.[95,96]

1.11 Fibrinolysis

Once a hemostatic plug has formed, it is important to dissolve the clot to initiate the wound healing process.[97] The fibrinolytic system is crucial for clot remodeling and lysis. The main enzyme of the fibrinolytic system and initiator is plasmin, which is converted from plasminogen by tPA, urokinase plasminogen activator (uPA) or PK/FXIIa. The main role of plasmin is to degrade fibrin to maintain vascular fluidity and to degrade ECM and BM to facilitate tissue remodeling. The degradation of cross-linked fibrin, give rise to soluble fibrin degradation

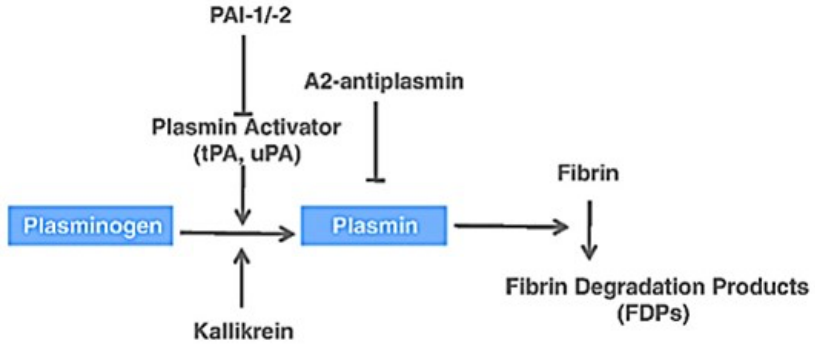

Figure 1.13 Fibrinolytic system.
The activation of plasminogen into plasmin leads to fibrin clot degradation. This process is regulated by anti-plasmin or PAI1/2. Figure adapted from Heissig et al., Cancer therapy targeting the fibrinolytic system. Advanced Drug Delivery Reviews 2016 Pages 172-179 doi.org/10.1016/j.addr.2015.11.010. Figure reprinted with permission.

products (FDPs) that can have immunomodulatory and chemotactic functions. Plasmin is inhibited and cleared from the circulation by α2-antiplasmin or α2-macroglobulin. tPA and uPA are inhibited by plasminogen activator inhibitor-1 and −2 (PAI-1/2) as seen in Figure 1.13. [98]

1.12 Inflammation and platelets

Beyond their role in hemostasis, platelets are also involved in other aspects of host defense such as inflammation, which is a complex response of the innate immune system to pathological stimuli.[54,99] Platelets can participate by the secretion of complement factors that can be regulated by coagulation.[100] Furthermore, platelets express Toll like receptors (TLRs) that are important for the innate immune system. TLR is part of the family of pattern recognition receptors. They detect conserved molecules from pathogen-associated molecular patterns (PAMPs) and host-derived damage-associated molecular patterns (DAMPs). Platelets are able to drive thrombo-inflammatory responses like infections and sepsis, with the release of inflammatory mediators.[101]

1.13 Thrombosis

Previous sections have introduced both platelets and coagulation enzymes in a healthy system and their role in hemostasis. Thrombosis is the process in which a pathological thrombus has formed causing obstruction of normal blood flow leading to ischemic events. The concept of disturbances in blood components was postulated in 1856 by Rudolf Virchow, who observed that abnormalities in blood flow, changes in blood components, and vessel wall dysfunction are all related to thrombus formation in venous or arterial settings.[102] This concept, Virchow's triad, is highly applicable in the understanding of thrombosis where a pathological thrombus is formed. Two classes of thrombi are observed in the clinic, primarily white thrombus versus red thrombus, and are composed of fibrin, red blood cells, platelets, leukocytes, and NETs. A thrombus is highly heterogeneous and can be influenced by the ischemic time in MI patients.

Overall, thrombi in MI patients consists of platelets, red blood cells, leukocytes and cholesterol crystals, whereas in ischemic stroke patients the thrombi are highly consisted of platelets and red blood cell rich regions. Venous thromboembolism thrombi contain mainly red blood cells and fibrin fibers with occasional platelets and leukocytes.[103] Venous thrombosis is triggered by expression of procoagulant activity on intact inflamed endothelium via TF and blood stasis from prolonged immobility leading to fibrin formation mostly at vascular valve places.[104,105] Foremost, the intrinsic pathway of coagulation does appear to play a limited role in hemostasis through FXIa.[4] However, it has a significant role in the contribution of pathological thrombus formation, such as venous thrombosis, ischemic stroke, and MI.[72,106] Supporting this idea, elevated levels of FXI have been proposed as risk factor for deep vein thrombosis, ischemic stroke and MI.[107,108]

1.14 Anti-Platelet and anti-coagulant drug therapies

Anti-platelet drugs have proven to be successful in the prevention and reducing mortality related to arterial thrombosis. The paradox of anti-thrombotic drug therapies remains that in order to treat excessive clot formation, the side effects increase such as bleeding.[109] Current anti-platelet therapies are directed to target specific platelet receptors or interfere with platelet normal functionality, such as cyclooxygenase 1 inhibitors, P2Y12 antagonists, PAR1 antagonists and αIIbβ3 antagonists.[110] Aspirin is well known to target platelet cyclooxygenases to prevent arachidonic acid oxidation, thromboxane A2 generation and feedback activation. P2Y12 receptor antagonists like clopidogrel and ticagrelorare are also well-established antiplatelet agents that prevent secondary activation of platelets by ADP.[111,112] In addition, drugs targeting the integrin receptor αIIbβ3 like abciximab, eptifibatide, and tirofiban are effective in preventing excessive thrombus growth.[113] Development of a monoclonal antibody against the GPVI receptor showed reduction in GPVI mediated thrombosis without the anticipated side

effects.[114,115] Varoxapar, a PAR1 antagonist was approved for treatments of cardiovascular events and atherosclerotic events[116,117], however excessive bleeding was observed in patients treated with the drug. This was due to the fact PAR1 is also expressed on endothelial cells, causing endothelial barrier dysfunction and reduced platelet activity. Therefore research is now studying PAR4 receptor targeting. An antagonist called BMS-986120 inhibits human *ex vivo* thrombus formation in a platelet PAR4 dependent manner.[118]

Current treatments for thrombotic disorders with anti-coagulant drugs are directed to active-site inhibitors of thrombin and FXa, which can have the clinically significant side effect of bleeding. Direct oral anticoagulants (DOACs) like dabigatran, rivaroxaban, apixaban, and edoxaban are approved for lowering the risk of stroke and embolism as well as deep vein thrombosis and pulmonary embolism treatment.[119,120] With bleeding as a major side effect of DOACs and vitamin K inhibitors (like warfarin), it is important to find new targets for the development of novel anticoagulants that are uncoupled from these side effects. A potential candidate is the targeting of intrinsic pathway FXI, as abnormal bleeding in FXI deficient patients is seldom severe, and usually accompanies trauma or surgery to tissues rich in fibrinolytic activity such as the mouth, nose and urinary tract.[121] Also, elevated levels of FXI is associated with increased risk of deep vein thrombosis, ischemic stroke and MI. Targeting FXI may reduce bleeding symptoms while still being effective against thrombosis. Numerous antibodies and small molecules targeting FXI have been developed and are being tested in clinical trials. Preliminary data shows that FXI is a safe drug therapy in the prevention of thrombosis.[122]

1.15 Thesis overview

In this thesis, we examine the mechanistic roles of platelets and the intrinsic pathway of coagulation in hemostatic and thrombotic processes. The following studies look more closely

into the interaction and regulation of intrinsic pathway members with different platelet phenotypes, the identification of a novel platelet-VWF regulator, and the impact of dietary THC administration on platelet physiology.

In Chapter 3, we study the effects of different platelet phenotypes on providing and supporting coagulation enzymes, specifically the coagulation factor of the intrinsic pathway, FXI. We study the role of FXI interaction and activity with the platelet surface by utilizing biochemistry assays and chromogenic substrates to test for enzyme activity. We found that platelet interaction supports FXIa activity and protects it from inhibition by platelet granule secreted content, in the end enhancing FXIa ability to promote thrombin generation. We explored the importance of FXIa anion binding sites in the interaction with the platelet surface. Overall, the data presented in this chapter highlights the function of platelet in providing an interactive and protective surface for the intrinsic pathway member, FXI. This knowledge might be helpful in the identification of the bleeding tendency in FXI-deficient patients, and in the identification of specific anti-coagulant drug targets.

Next in Chapter 4, we dive deeper into platelet functionality in hemostasis and thrombosis by studying the platelet endothelial cell interaction. We specifically were interested in the regulation of platelet and endothelial cell interactions, mediated by VWF. The main regulator of VWF is ADAMTS13; however, no known physiological regulators of ADAM13 activity have been identified. We utilized biochemistry assays to study the proteolytic effects of intrinsic pathway involvement in regulating ADAMTS13 activity on endothelial cells. We found that at the site of a local thrombi, where platelets are attached to VWF and intrinsic pathway activation occurs, ADAMTS13 activity was inhibited by FXIa. In a flow based system we observed VWF functional effects on endothelial cell surface and how FXIa was enhancing platelet string formation on

VWF. These results identify a previously undiscovered substrate for FXIa and this may represent an additional hemostatic function by which FXIa promotes local platelet deposition at sites of vessel injury.

Studies in Chapter 5 investigates the systemic effects of cannabis, specifically the main psychoactive ingredient THC, on platelets reactivity and function. We utilized a NHP model to administer THC edibles and study platelet function. Understanding the health effects of marijuana is under great interest with the current legalization wave across America, studies conducted to determine THC function are important for public health assessments. We demonstrate that chronic THC exposure had a significantly desensitizing effect on platelet functions such as adhesion, aggregation and α-granule secretion. We also observed changes in plasma oxylipin levels with chronic THC edible administration. Together, our observations may lead to more research knowledge and insights for clinical health policies regarding the use of medical marijuana and for health professionals caring for patients using marijuana in the future.

Finally, Chapter 6 presents conclusions from the studies presented in Chapter 3-5 and proposes future work to extend the results we observed in platelet activation phenotypes. We will dive deeper into the downstream signaling of GPCR activation by using a systems biology approach. We will study downstream phosphorylation events of platelet PAR signaling with a mass spectrometry-driven phosphoproteomics approach. The overall goal of the presented work in this thesis is to elucidate mechanisms of platelet activation and function in supporting thrombus formation in an intrinsic pathway-dependent manner. This work will gain mechanistic knowledge in the understanding of platelet-intrinsic pathway interactions and platelet activation signaling states, as well as the risk factors associated with thrombotic complications for optimal drug development.

Chapter 2 General material and methods

2.1 Ethics Statement

Human studies were carried out in accordance with the recommendations of Oregon Health & Science University Institutional Review Board committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Oregon Health & Science University Institutional Review Board committee. For all studies with Rhesus macaques (*Macaca mulatta*), procedures and experimental THC administration has been approved by the Oregon Health & Science University, Oregon National Primate Research Center Institutional Animal Care and Use Committee (IACUC) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for humane animal care were followed (IP0001389). The THC used in our study was supplied directly from the National Institute of Drug Abuse (NIDA) Drug Supply Program. Permission was obtained for all experimental protocols involving THC administration prior to the initiation of the study and the study was conducted in accordance with institutional and national guidelines.

2.2 Common reagents

Human plasma-derived FXIa, FXI, FIX, FXa and α-thrombin were from Haematologic Technologies (Essex Junction, VT, USA). Plasma kallikrein (PKa), α-FXIIa, high molecular weight kininogen (HK) and fibrinogen were from Enzyme Research Laboratories, Inc. (South Bend, IN, USA). Recombinant ADAMTS13 (rADAMTS13) was donated from Shire (Benatzkygasse, Austria). Bovine serum albumin (BSA), Phe-Pro-Arg chloromethylketone (PPACK) and hirudin were from Sigma-Aldrich (St. Louis, MO, USA). Prostacyclin (PGI_2) was from Cayman Chemical (Ann Arbor, MI). Integrilin (eptifibatide) was from Merck & Co. (Whitehouse Station, NJ). Thrombin receptor activator peptide 6 (TRAP-6; SFLLRN) was

obtained from Tocris (Bristol, UK). Collagen-related peptide (CRP-XL) was from R. Farndale (Cambridge University, Cambridge, UK). Chromogenic substrates S-2366, S-2302 were from Diapharma Group, Inc (West Chester, OH, USA). Spectrozyme FXa was from American Diagnostica, Inc (Stamford, CT, USA).

2.3 Platelet preparation

Whole blood was drawn by venipuncture from healthy adult donors into sodium citrate (0.38% w/v). Platelets were prepared by the addition of warmed acid-citrate-dextrose (85 mM sodium citrate, 100 mM glucose, 71 mM citric acid, 30°C) to the anticoagulated whole blood and then centrifuged at 200 × g for 20 min to separate platelet-rich plasma (PRP). The PRP was further purified by centrifugation in the presence of PGI2 (0.1 μg/ml) at 1,000 × g for 10 min. Purified platelets were resuspended in modified HEPES/Tyrode buffer (129 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM HEPES, 1 mM MgCl2, pH 7.3; supplemented with 5 mM glucose) containing 0.1 μg/ml PGI2. Platelets were washed once by centrifugation at 1,000 × g for 10 min and resuspended in HEPES/Tyrode buffer at indicated concentrations.

2.4 Plasma preparation

Plasma was isolated by centrifuging whole blood (mixed with sodium citrate, 0.32% w/v) at 2,000 × g for 10min at RT and stored at -80°C.

2.5 Platelet aggregation

Platelet rich plasma (PRP; 300 μl per sample) were pre-incubated in glass cuvettes and warmed to 37°C. Platelet aggregation under stirring conditions was initiated by select agonists (1:100), and changes in light transmission were monitored for 5 minutes using a PAP-4 aggregometer (Chrono-Log Corporation).

2.6 Flow cytometry

P-selectin and PAC-1 expression on the platelet surface were detected using flow cytometry. Adult citrated whole blood was diluted with modified HEPES/Tyrode's buffer. Isolated and washed platelets (2×10^8 platelet/mL) were incubated with antibodies against P-selectin (APC-CD62P) and activated integrin GPIIb/IIIa (FITC-PAC1), with a dilution of 1:25, in the presence of select agonists or vehicle control (HEPES/Tyrode's buffer) for 20 min at room temperature (RT). Reactions were stopped by the addition of 2% paraformaldehyde (PFA) for 10min. Finally, samples were diluted with PBS, measured by flow cytometery, and analyzed using FlowJo software.

2.7 Western Blotting

Protein samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes and blotted with indicated antibodies and horseradish peroxidase-conjugated secondary antibodies. Protein was detected using enhanced chemiluminescence.

2.8 Chromogenic substrate assays

96-well plate was blocked using phosphate-buffered saline with 0.5% BSA, select concentrations of either FXIa (0.25–1nM), FXIIa (0.25–2nM), PKa (0.25–1nM) or FXa (0.25–1nM) were incubated with vehicle, resting, activated, exhausted platelets or platelet secretome (0.5×108 platelets/ml) for 1 hour at RT in the presence of 10μM ZnCl2 in Hepes-Tyrode's buffer. Enzyme activity was measured at 405nm with a spectrophotometer using chromogenic substrates selective for FXIa (S-2366), FXIIa and PKa (S-2302), FXa (Spectrozyme Xa) at a final concentration of 0.8mM.

2.9 Microscopy imaging

Purified platelets (2×10^7 platelets/ml) were incubated over glass coverslips coated with either 50µg/ml fibrinogen, purified or recombinant FXI and FXIa, or recombinant FXIa-ABS1 or FXIa-ABS2 (50µg/ml) and blocked with 0.5 % BSA, for 45min at 37°C. Slides were washed and fixed with 4% PFA. Platelet adhesion and spreading was detected using differential interference contrast (DIC) optics and quantified using Image J software as described.[123]

2.10 Statistical analysis

Data were analyzed using GraphPad PRISM 8.3 software (San Diego, CA, USA). Data are represented as mean ± standard error of at least three independent experiments. For all comparisons, $p \leq 0.05$ was used to establish significance. For *in vivo* experiments, samples were analyzed using a 2-way ANOVA with Dunnet's multiple comparison test. Data are presented as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to compare between baseline and dosage groups. Kruskal-Wallis with Dunn post hoc test was used to compare between groups when the data did not qualify for parametric statistics. $p < 0.05$ was considered significant. All statistical analyses were conducted using GraphPad Prism 9.

Chapter 3 Role of platelets in regulating activated coagulation factor XI activity

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3.1 Abstract

Factor XI has been shown to bind to platelets, ex vivo, but the functional significance of this observation remains unknown. Platelets are essential for hemostasis and play a critical role in thrombosis, while FXI is not essential for hemostasis, but promotes thrombosis. An apparent functional contradiction, platelets are known to support thrombin generation, yet, platelet granules release protease inhibitors, including those of FXIa. We aim to investigate the secretory and binding mechanisms by which platelets could support or inhibit FXIa activity. The presence of platelets enhanced FXIa activity towards a chromogenic substrate as well as increased FIX activation by FXIa. In contrast, platelets reduced the activation of FXI by FXIIa and the activation of FXII by PKa. Incubation of FXIa with the platelet secretome, which contains FXIa inhibitors, such as PN-II, abolished FXIa activity, yet in the presence of activated platelets the secretome was not able to block the activity of FXIa. FXIa variants lacking the anion-binding sites did not alter the effect of platelets on FXIa activity or interaction. Western blot analysis of bound FXIa (by FXI(a)-platelet membrane immunoprecipitation) showed that the interaction with platelets is zinc-dependent and, unlike FXI binding to platelets, not dependent on GPIb. FXIa binding to the platelet membrane increases its capacity to activate FIX likely by protecting it from inhibition by several inhibitors secreted by activated platelets. Our findings suggest that an interaction of FXIa with the platelet surface may induce an allosteric modulation of FXIa.

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3.2 Introduction

Studies conducted in this thesis were designed to study platelet functions and interactions with the endothelial cell surface as well as regulating coagulation in thrombus formation. Platelets are important mediators in hemostatic processes by providing the catalytic surfaces and cofactors to support coagulation and platelet feedback activation. The intrinsic pathway of coagulation is known to interact with platelet surface receptor GPIb. The role of this interaction however remains unknown. Therefore we first aimed to characterize the effects of platelet activation states on regulating coagulation enzymes, specifically contact system serine protease FXI. Here, we demonstrate that the platelet surface interacts, promotes and protects FXIa from inhibition by platelet granule secreted components. This work will help contribute to the better understanding of the mechanistic functions of platelets display on FXI which is a novel target for anticoagulant drug therapies.

3.3 Background

The blood plasma protein FXI is a member of the contact activation complex. FXI is a dimer with two identical 80kDa subunits, each consisting of four apple (A) domains and a catalytic domain.[85] The contact system is initiated by the conversion of FXII into its active form FXIIa on a “surface” that often carries a negative charge. The generation of FXIIa drives a feedback activation loop of PK activation into PKa, which in turn activates additional FXII in the presence of the cofactor HK.[124] FXIIa converts its downstream substrate FXI into the active serine protease FXIa, which, among other enzymatic activities, propagates coagulation by activating

FIX, which subsequently contributes to generation of thrombin.[84] Thrombin, in turn, may amplify the coagulation response by feed-back activation of FXI.[125]

Platelets have been shown to play balanced roles in propagating and sequestering thrombin generation to preserve vascular integrity.[1] Some members of the contact system including FXI have been shown to interact with receptors and cofactors on the platelet surface[126], yet whether or how FXI/FXIa-platelet interactions regulate the activity of FXIa or activation of FXI remains elusive. FXI is known to bind to the platelet receptors GPIb-IX-V[127] and apolipoprotein E receptor 2 (ApoER2).[123] Furthermore, binding competition studies found that FXI did not compete with FXIa for binding to platelets, suggesting that FXIa as an active enzyme binds to platelets at a different site than the zymogen FXI.[128] Moreover, the interaction of FXIa with platelets was shown to prevent FXIa inhibition by one of FXIa's major inhibitors, PN-II, [96,129] an abundant platelet α-granule protein which is secreted upon platelet activation.[130]

Even with the observation that FXI interacts with the platelet surface, the implications of this interaction remain uncertain, particularly regarding whether FXI activation by thrombin is enhanced by specific platelet receptors.[131-133] The platelet receptor for FXIa has remained elusive, and the relevance of the interaction of FXIa with platelets has not been determined. Herein, we demonstrate that the platelet surface is a potent regulator of contact system activity, specifically enhancing FXIa activity towards FIX and protecting it from inhibition by the inhibitors secreted by activated platelets. Together, our findings suggest that an interaction of FXIa with the platelet surface may induce an allosteric modulation of FXIa.

3.4 Methods

3.4.1 Reagents

Human plasma-derived FXIa, FXI, FIX, FXa and α-thrombin were from Haematologic Technologies (Essex Junction, VT, USA). Plasma kallikrein (PKa), α-FXIIa, high molecular weight kininogen (HK) and fibrinogen were from Enzyme Research Laboratories, Inc. (South Bend, IN, USA). Bovine serum albumin (BSA), Phe-Pro-Arg chloromethylketone (PPACK) and hirudin were from Sigma-Aldrich (St. Louis, MO, USA). Prostacyclin (PGI_2) was from Cayman Chemical (Ann Arbor, MI). Integrilin (eptifibatide) was from Merck & Co. (Whitehouse Station, NJ). Thrombin receptor activator peptide 6 (TRAP-6; SFLLRN) was obtained from Tocris (Bristol, UK). Collagen-related peptide (CRP) was from R. Farndale (Cambridge University, Cambridge, UK). Protein A/G PLUS-agarose beads were from Santa Cruz Biotechnology (Dallas, TX). Chromogenic substrates S-2366, S-2302 were from Diapharma Group, Inc (West Chester, OH, USA). Spectrozyme FXa was from American Diagnostica, Inc (Stamford, CT, USA). Short (~70–85 phosphate units in length) and long (>1000 phosphate units in length) polyphosphates were prepared as described previously [134]. FXIa species with alanine substitutions for Lys529, Arg530 and Arg532 (FXIa-ABS-2) and substitution Arg250-255 (FXIa-ABS-1) were prepared as described [135].

3.4.2 Antibodies

The anti-FXI/XIa light chain (LC) antibody (10C9) used for Western blot was generated as previously described.[86] The anti-FXI antibody (1A6) used for immunoprecipitation assays was generated as previously described.[136,137] The anti-FIX heavy chain antibody was from Haematologic Technologies (Essex Junction, VT, USA). The polyclonal antibody against FXI was from LSBio (WA, USA). The anti-FXII heavy chain was from Santa Cruz Biotechnology

(Dallas, TX). The anti-CD42b was from Santa Cruz Biotechnology (Dallas, TX). The anti-CD62P-FITC and Annexin-V FITC antibodies were from Biolegend (San Diego, CA, USA).

3.4.3 Preparation of activated, exhausted platelets and platelet secretome

Blood was drawn by venipuncture from healthy human donors in accordance with an Oregon Health & Science University IRB-approved protocol into a final concentration of 0.38% sodium citrate and platelets were purified as previously described.[123] Isolated and washed platelets (0.5×10^8 platelets/mL) were incubated with 20μg/ml Integrilin for 20 min at 37ºC prior to stimulation with 1nM α-thrombin and 2mM $CaCl_2$ for 15 min at 37ºC. Thrombin activity was stopped with 20μg/ml hirudin. To prepare exhausted platelets and platelet secretome, activated platelets were centrifuged for 5min at 1000×g, the supernatant was collected (platelet secretome), and the pellet (exhausted platelets) was resuspended in Hepes-Tyrode's buffer.

3.4.4 Flow cytometry analysis

Washed human platelets (2×10^8 platelets/ml) were incubated for 30 min with anti-CD62P-FITC (1:20) or Annexin-V FITC (1:20) in Hepes-Tyrode's buffer with 5mM $CaCl_2$. Samples were diluted in Hepes-Tyrode's buffer and analyzed by flow cytometry (BD FACSCanto II, Becton Dickinson). Platelets were identified by logarithmic signal amplification for forward and side scatter, and the geometric mean fluorescence of each sample was recorded, as described before.[138]

3.4.5 Chromogenic substrate assay for FXIa, FXIIa, PKa and FXa activity in solution

96-well plate was blocked using phosphate-buffered saline with 0.5% BSA, select concentrations of either FXIa (0.25–1nM), FXIIa (0.25–2nM), PKa (0.25–1nM) or FXa (0.25–1nM) were incubated with vehicle, resting, activated, exhausted platelets or platelet secretome

(0.5×10^8 platelets/ml) for 1 hour at RT in the presence of 10μM $ZnCl_2$ in Hepes-Tyrode's buffer. Enzyme activity was measured at 405nm with a spectrophotometer using chromogenic substrates selective for FXIa (S-2366), FXIIa and PKa (S-2302), FXa (Spectrozyme Xa) at a final concentration of 0.8mM.

3.4.6 Chromogenic substrate assay for FXIa activity on a fibrinogen surface

In a 96-well plate, the surface was coated with 50μg/ml fibrinogen and blocked with 0.5% denatured BSA. 5×10^8 platelets/ml were seeded on fibrinogen surface for 45min at 37ºC. After washing, platelets or fibrinogen alone were incubated with increasing concentrations of FXIa (0.25–1nM) for 1 hr at RT in the presence of 10μM $ZnCl_2$ in modified Hepes-Tyrode's buffer. Enzyme activity was measured using a chromogenic substrate selective for FXIa (S-2366, final concentration 0.8mM). In selected experiments, platelets were activated with thrombin, and thrombin activity was quenched with hirudin as described above, prior to seeding on fibrinogen surface.

3.4.7 FIX activation by FXIa

FIX (100nM) was incubated with FXIa (0.75nM) for 0–30min in the absence or presence of platelets (0.5×10^8 platelets/ml) in modified Hepes-Tyrode's buffer with 5mM Ca^{2+} and 10μM $ZnCl_2$. 30nM HK was included in some experiments. At select time points, aliquots were taken and Laemmli sample buffer (Bio Rad, Hercules, CA) containing 200 mM DTT was added. FIX activation was detected via Western blot with an anti-FIX antibody.

3.4.8 FXI activation by FXIIa

FXI (100nM) was incubated with FXIIa (60nM) for 0–180min in the absence or presence of platelets or platelet secretome (0.5×10^8 platelets/ml) in modified Hepes-Tyrode's buffer with

10μM $ZnCl_2$. At select time points, aliquots were taken and Laemmli sample buffer containing 200 mM DTT was added. FXI activation was detected by Western blot with an anti-FXI antibody.

3.4.9 FXII activation by PKa

FXII (200nM) was incubated with PKa (25nM) for 0–180min in the absence or presence of platelets or platelet secretome (0.5×10^8 platelets/ml) in modified Hepes-Tyrode's buffer with 10μM $ZnCl_2$. At select time points, aliquots were taken and Laemmli sample buffer containing 200 mM DTT was added. FXII activation was detected by Western blot with an anti-FXII HC antibody.

3.4.10 Static adhesion assay

Purified platelets (2×10^7 platelets/ml) were incubated over glass coverslips coated with either 50μg/ml fibrinogen, purified or recombinant FXI and FXIa, or recombinant FXIa-ABS1 or FXIa-ABS2 (50μg/ml) and blocked with 0.5 % BSA, for 45min at 37°C. Slides were washed and fixed with 4% PFA. Platelet adhesion and spreading was detected using differential interference contrast (DIC) optics and quantified using Image J software as described.[123]

3.4.11 Immunoprecipitation and Western blotting.

Isolated and washed platelets (5×10^8 platelets per ml) were seeded on a fibrinogen surface in 6-well plates and incubated with vehicle, FXI (50nM) or FXIa (50nM) in Hepes-Tyrode's buffer for 2 hrs at RT. Platelets were washed 3× in modified Hepes-Tyrode's buffer followed by the addition of a lysis/IP buffer (10 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100). Platelet lysates were pre-cleaned with Protein A/G Sepharose and then incubated with 1μg of an anti-FXI antibody (1A6) for 16 hrs at 4°C. Antibody-protein complexes were then captured with Protein A/G PLUS-Agarose beads for 4 hr at 4 °C, and washed 3× in an IP buffer.

FXI/FXIa precipitates were then eluted with 40 µl of Laemmli sample buffer (Bio Rad, Hercules, CA) containing 200 mM DTT. Protein samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes, blotted with an anti-FXI/FXIa light chain (LC) antibody or an anti-GPIb antibody. Proteins were detected using ECL (Thermo Scientific).

3.4.12 Statistical analysis

Data were analyzed using GraphPad PRISM 8.3 software (San Diego, CA, USA). Data are represented as mean ± standard error of at least three independent experiments. For all comparisons, $p \leq 0.05$ was used to establish significance.

Samples were analyzed using a 2-way ANOVA with Dunnet's multiple comparison test.

3.5 Results

3.5.1 Evaluation of FXIa activity and FIX activation in the presence of platelets

Previous studies have shown that FXI/FXIa binds to the platelet surface [123,127], yet whether or how FXI/ FXIa-platelet interactions regulate its activation or activity remains unclear. We designed experiments to evaluate whether the resting or activated platelets or supernatant from activated platelets regulates FXIa activity. Platelet activation by thrombin was confirmed by flow cytometry using antibodies against P-selectin and phosphatidylserine (Figure 3.1). We found that the addition of resting platelets to FXIa nearly tripled FXIa activity toward a chromogenic substrate as compared to vehicle (Figure 3.4A). A similar increase in FXIa activity was observed in the presence of platelets activated with thrombin (activated platelets including their secretory supernatant) or thrombin-activated platelets that had been washed (exhausted platelets) as compared to vehicle alone. Resting, activated or exhausted platelets did not promote the cleavage of the chromogenic substrate in the absence of FXIa, Platelets that were stimulated with the GPVI-agonist CRP alone or in combination with thrombin also enhanced the activity of FXIa (Figure 3.2). The presence of either zinc ions or HK did not influence the effects of platelets on FXIa activity (Figure 3.3). Interestingly, when the supernatant from activated platelets (platelet secretome) was added to FXIa, a decrease in activity was observed (Figure 3.4A). In contrast, the presence of platelets, whether activated or not, or activated platelet supernatant alone all enhanced the activity of the serine proteases FXIIa and PKa towards their chromogenic substrates, while the activity of FXa was insensitive to the presence of platelets (Figure 3.4B-D).

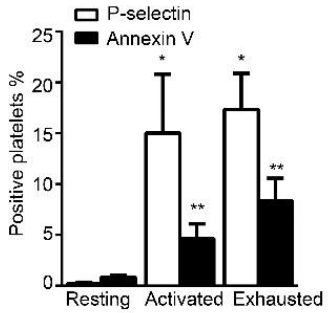

Figure 3.1 Measurement of P-selectin and phosphatidylserine exposure on platelets after activation by α-thrombin. 2×10^8 platelets/mL were either left resting, activated with α-thrombin or activated and washed with Hepes-Tyrodes buffer (exhauted platelets). P-selectin and phosphatidylserine exposure were determined by flow cytometry using anti-CD62P FITC and Annexin-V FITC, respectively. Data are mean ± SD (n = 3). *P<.05 with respect to resting. **P<.05 with respect to resting. Tukey's multiple comparisons test was used for statistical comparisons.

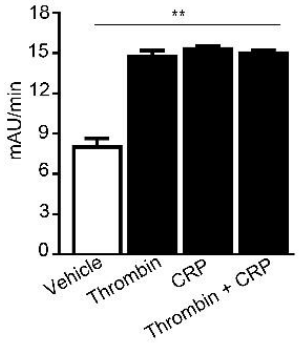

Figure 3.2 Effect of select platelet agonists on FXIa activity. FXIa (500pM) was incubated with either vehicle or platelets stimulated with thrombin, the GPVI-agonist CRP or combination of thrombin with CRP for 1hr. The rate of substrate hydrolysis by FXIa was measured at 405 nm. Data are mean ± SEM (n = 3). **P<.05 with respect to vehicle. Tukey's multiple comparisons test was used for statistical comparisons.

We next confirmed the findings from our chromogenic studies by investigating the cleavage products of the members of the contact system and downstream substrates by Western blot. Our results show that the addition of platelets accelerates the cleavage of FIX by FXIa (Figure 3.4E). In contrast, the cleavage of FXI by FXIIa was slowed by the presence of platelets and nearly halted by the presence of the platelet secretome (Figure 3.4F). A similar observation was

made for the cleavage FXII by PKa, in which platelets or the secretome alone almost completely inhibited the generation of FXIIa (Figure 3.4G).

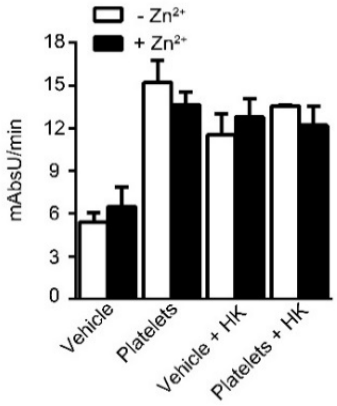

Figure 3.3 Role of HK and Zinc ions on FXIa activity in the presence of platelets. FXIa (500pM) was incubated with platelets in the presence or absence of either Zn2+ (10μM) or/and HK (30 nM). The rate of substrate hydrolysis by FXIa was measured at 405 nm. Data are mean±SEM (n=3).

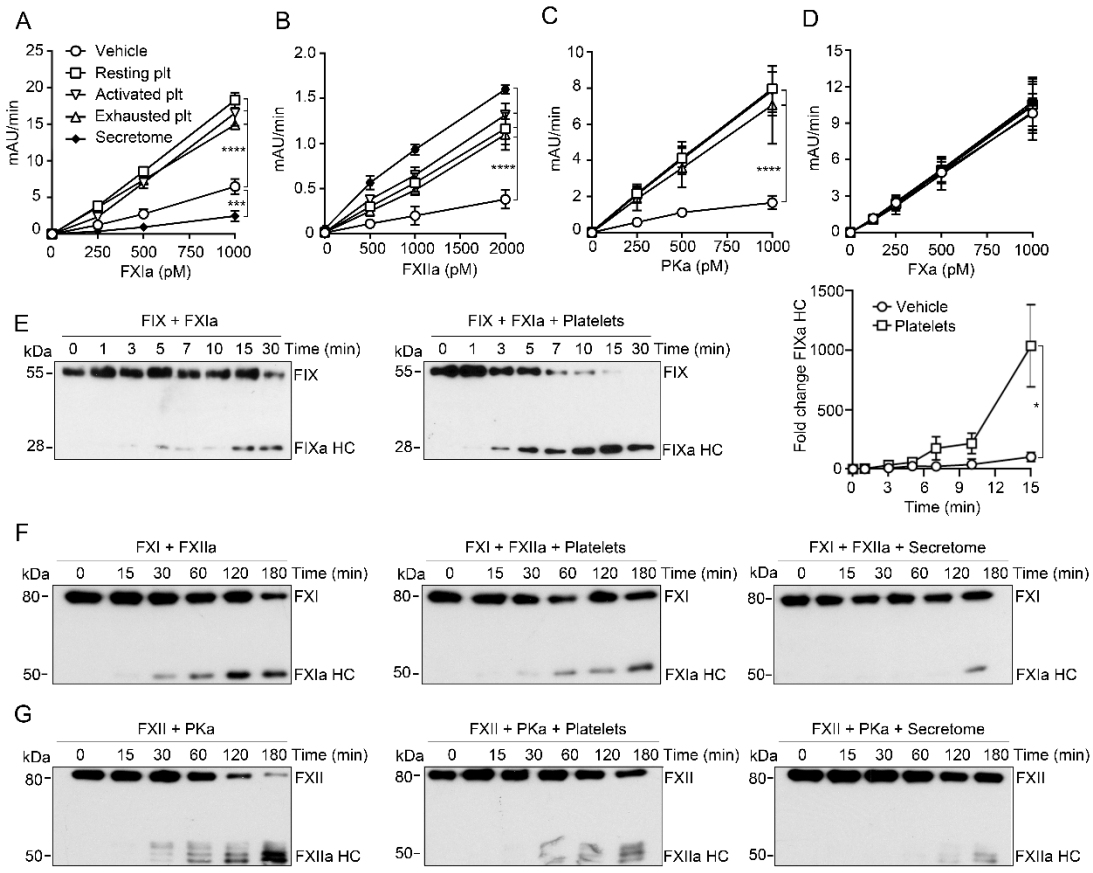

Figure 3.4 The effect of platelets on the activity of FXIa, FXIIa, PKa and FXa. Select concentrations of either FXIa (A), FXIIa (B), PKa (C) or FXa (D) were incubated for 1 hr at 25ºC with vehicle (○), resting platelets (□), activated platelets (▽), exhausted platelets (△) or platelet secretome (♦).FXIa, FXIIa, PKa and FXa activity was determined by adding S-2366 for FXIa, S-2302 for FXIIa or PKa and FXa spectrozyme for FXa (0.8mmol/L).The rate of substrate hydrolysis was measured at 405 nm. (E) 100nM FIX was incubated with 0.75nM FXIa in the absence (vehicle) or presence of platelets for selected times (0-30min). FIX activation was detected by Western blot using an anti-FIX heavy chain antibody. (F) 100nM FXI was incubated with 65nM FXIIa in the absence (vehicle) or presence of platelets or platelet secretome for selected times (0-180 min). FXI activation was determined by Western blotting using an anti-FXI antibody. (G) 200nM FXII was incubated with 25nM PKa in the absence (vehicle) or presence of platelets or platelet secretome for selected times (0-180min). FXII activation was determined by Western blotting using an anti-FXII antibody. Data are mean±SEM from at least n>3.

3.5.2 Role of the platelet surface and secretome in regulating FXIa activity

We next performed a time course experiment to study whether the platelet surface and secretome differentially regulate FXIa activity. The presence of resting platelets increased FXIa activity at baseline (time=0 min); a steady increase in FXIa activity was observed when combined with resting platelets over a 2 hr time course (Figure 3.5A). Likewise, an increase at both at baseline and over time was observed for FXIa activity in the presence of activated platelets, which would include both platelets as well as their secreted supernatants. While an increase at baseline was observed for exhausted platelets (activated platelets that had been washed) relative to vehicle, the level of FXIa activity remained constant over time. Of note, a sustained decrease in FXIa activity was observed in the presence of platelet secretome alone over the entire time course.

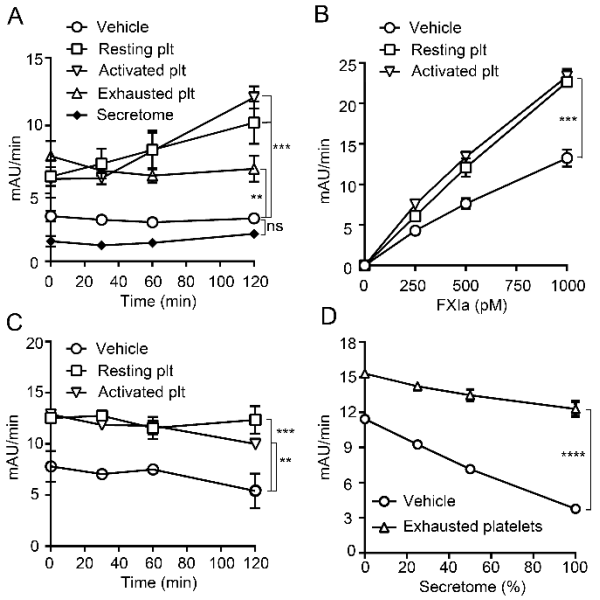

Figure 3.5 The effect of platelets on FXIa activity. (A) 500pM FXIa was incubated with vehicle (○), resting platelets (□), activated platelets (▽), exhausted platelets (△) or platelet secretome (♦) for selected time points (0-120min). The rate of substrate hydrolysis by FXIa was measured at 405 nm. (B) Select concentrations of FXIa (0-1nM) were incubated for 1 hr in the absence or presence of resting or activated platelets seeded on fibrinogen. (C) 500pM of FXIa was incubated with resting or activated platelets seeded on fibrinogen for selected time points (0-120min). The rate of substrate hydrolysis by FXIa was measured at 405 nm. (D) FXIa (1nM) was incubated with either vehicle or exhausted platelets for 1 hr in the presence or absence of select dilutions of platelet secretome (5x108 platelets/ml). The rate of FXIa substrate (S-2366; 0.8mmol/L) hydrolysis was measured at 405 nm. Data are mean±SEM (n=3).

We next tested the ability of immobilized platelets to regulate FXIa activity. We quantified the amidolytic activity of FXIa that had been added to wells coated with either resting or thrombin-stimulated platelets on fibrinogen or fibrinogen alone. We found that fibrinogen-immobilized platelets promoted FXIa amidolytic activity regardless of whether the platelets were stimulated with thrombin or not (Figure 3.5B). The increase in FXIa amidolytic activity above baseline remained constant over a 2hr time course (Figure 3.5C). The fact that we didn't observe a difference between resting and activated platelets, which would have secreted their contents, in this assay led us to ponder whether the platelet surface was protecting FXIa from being inhibited by the platelet secretome. Consistent with our findings above, a dose-dependent decrease in FXIa amidolytic activity for a single concentration of FXIa was observed as a function of increasing dilutions of exogenously added platelet secretome. Strikingly, the presence of exhausted platelet membranes both increased the baseline level of FXIa amidolytic activity as well as protected FXIa from inhibition by the platelet secretome (Figure 3.5D).

3.5.3 Assessment of the role of platelets in the procoagulant activity of FXIa.

Our data in purified systems suggest that FXIa activity was enhanced towards FIX in the presence of platelets. We next sought to test whether platelets played a functional role in stimulating the procoagulant activity of FXIa to generate fibrin in plasma. To eliminate any potential role for contact activation, citrated platelet-poor plasma (PPP) was pre-treated with CTI and the antibody 14E11, which block FXIIa activity toward FXI. Next, we performed clotting time assays in PPP in which fibrin generation was initiated by the addition of FXIa in order to determine whether the presence of platelets in plasma enhanced the activity of FXIa. Our data show that the addition of resting or activated platelets shortened the clotting time initiated by 0.25nM FXIa by nearly 100 sec (Figure 3.6A). Next, we investigated whether platelets

immobilized on a fibrinogen surface enhanced the procoagulant activity of FXIa. As shown in Figure 3.6B, we observed that the presence of immobilized resting or activated platelets decreased the time required for FXIa to initiate fibrin generation by more than 300 sec compared to vehicle condition. In the presence of activated platelets, the time to initiate fibrin was significantly decreased compared both to resting platelets and to the vehicle condition.

We next tested if the observed shortening of clotting times in the presence of platelets on FXIa-induced fibrin formation was due to the presence of phosphatidylserine (PS). Increasing concentrations of the (PS) inhibitory agent, lactadherin, prolonged clotting times initiated by FXIa in solution for both the vehicle condition and activated platelets (Figure 3.6C). We observed that even in the presence of lactadherin, activated platelets shortened the clotting time initiated by FXIa (Figure 3.6C). Furthermore, pretreating platelets with 500 nM lactadherin did not modify the effect of platelets on the initiation of fibrin generation by FXIa or the time to reach ½ maximal turbidity, suggesting these observations were not simply due to the presence of platelet surface-exposed PS.

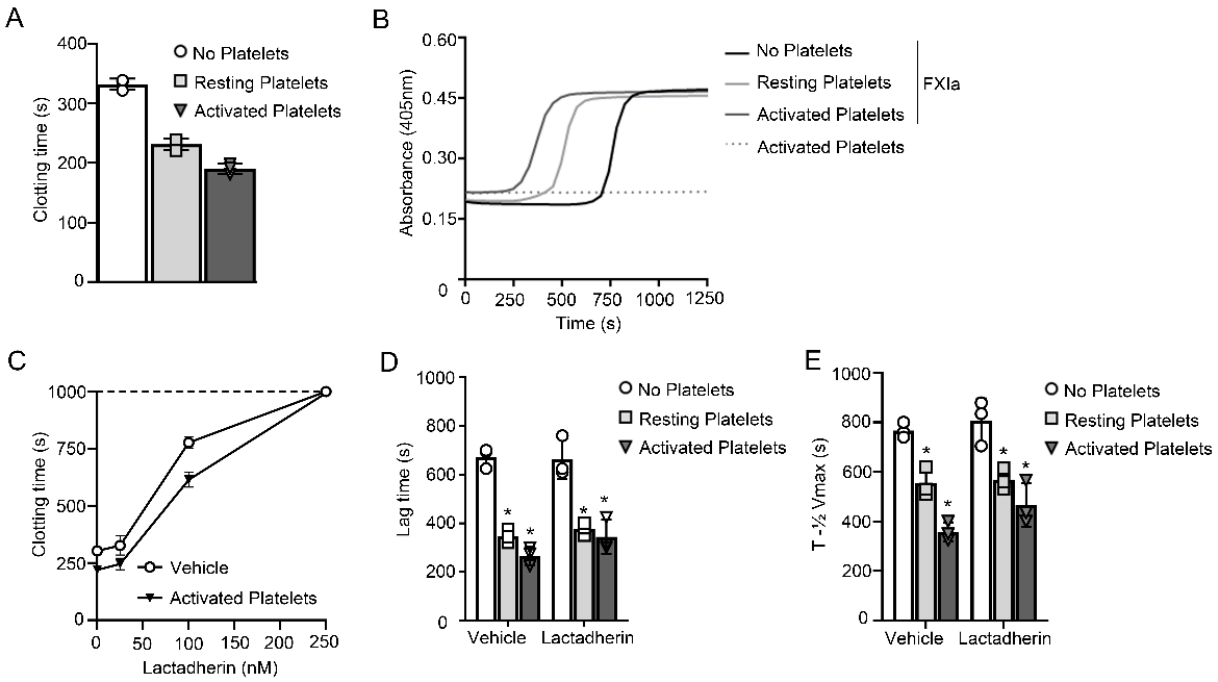

Figure 3.6 Role of HK and buffer conditions on FXIa activity in the presence of platelets. (A) FXIa (500pM) was incubated with platelets in the presence or absence of either Zn2+ (10μM) or/and HK (30 nM). The rate of substrate hydrolysis by FXIa was measured at 405 nm. (B) FXIa (500pM) was incubated with platelets in the presence of either Hepes-Tyrode (HT), Hepes-buffered saline (HBS), or 25mM or 50 mM Tris buffers in the absence or (C) presence of 0.1% BSA. The rate of substrate hydrolysis by FXIa was measured at 405 nm. Data are mean±SEM (n=3).

3.5.4 Assessment of FXIa anion binding sites on FXIa activity and binding to platelets

FXIa synthetic peptides of the anion binding sites of FXIa have been shown in the past to interfere with FXIa binding to platelets. FXIa has two anion binding regions; one located on its apple 3 domain (ABS-1) and one located in the catalytic domain (ABS-2) [135]. Using recombinant forms of FXIa that were mutated in either the ABS-1 (located in the apple 3 domain) or ABS-2 (located in the catalytic domain) region, we next explored whether these domains play a role in mediating FXIa-platelet interactions. Our results show that mutating either the ABS-1 or ABS-2 domains did not reduce the ability of platelets to potentiate FXIa activity either in solution or

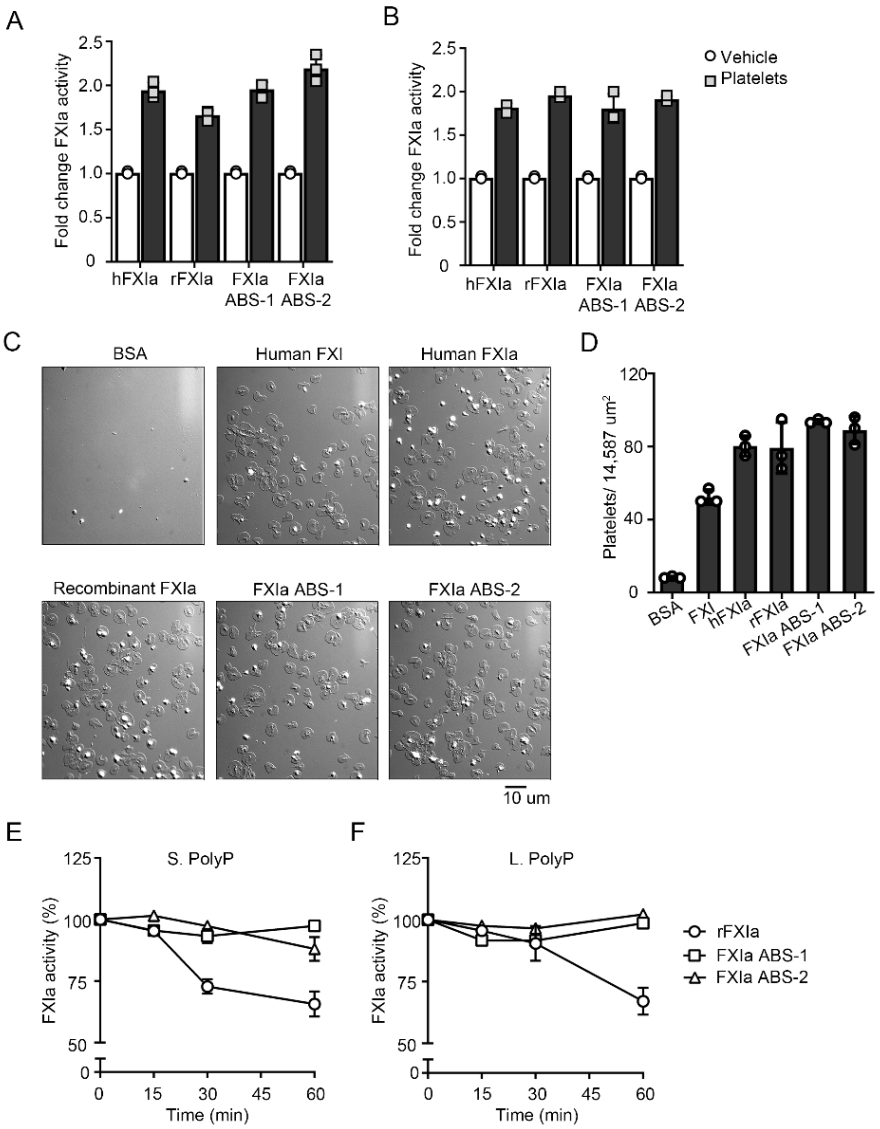

Figure 3.7 The role of FXI anion binding sites (ABS)-1 and -2 on the effect of platelets on FXIa activity. rFXIa, FXIa ABS-1or FXIa ABS-2 (500pM) was incubated with either (A) platelets in solution or (B) platelets immobilized on a fibrinogen surface. (C) FXI, rFXIa, FXIa ABS-1 or FXIa ABS-2 (50µg/ml) was coated on glass coverslip surfaces prior to the addition of platelets (2×107 platelets/ml). DIC microscopy images were taken and (D) quantified. (E) The activity of rFXIa (□), FXIa ABS-1 (□) or FXIa ABS-2 (□) was measured in the presence of short or (F) long chain sized polyphosphates (PolyP; 10µM) to induce autolysis of FXIa in a BSA pre-blocked 96-well plate. Data are mean±SEM (n=3).

when platelets were immobilized on fibrinogen (Figure 3.7A and B, respectively). Moreover, mutating either the ABS-1 or ABS-2 domain of FXIa did not reduce the ability of immobilized FXIa to support platelet adhesion or spreading. To validate a known role for the ABS-1 and

ABS-2 domains of FXIa, we show that the ability of either short- or long-chain polyphosphate to reduce the activity of FXIa over time was abrogated by the mutation of either the ABS-1 or ABS-2 domains in FXIa (Figure 3.7E and F).

3.5.5 Assessment of FXIa binding sites on platelet interaction

Previous studies have shown that FXI is able to bind to the platelet receptor GPIb,[127] and that FXIa does not compete for FXI binding to platelets, together suggesting that FXI and FXIa bind to disparate sites on the platelet surface.[128] To investigate this in our system, a suspension of platelets was incubated with FXI or FXIa for 2 hours to allow binding. Platelets were then washed to remove any unbound FXI(a). Washed platelets were then lysed and immunoprecipitated for FXI(a). As shown in Figure 3.8A, activation of platelets with TRAP induced a dramatic increase in binding of FXI but not FXIa to GPIb. Subsequently we studied FXI(a) binding to platelets that had been immobilized on fibrinogen. Our results show that the addition of zinc to this system induces a robust binding of FXI and FXIa to platelets, as recorded by ~80 kDa and ~30 kDa bands on the Western blot detected by an antibody that recognizes the light chain of FXI(a), respectively (Figure 3.8B). This binding pattern was also observed when platelets were activated with thrombin. Taken together, these results suggest that FXIa interacts with platelets on a different site as compared to FXI and that this binding is zinc dependent.

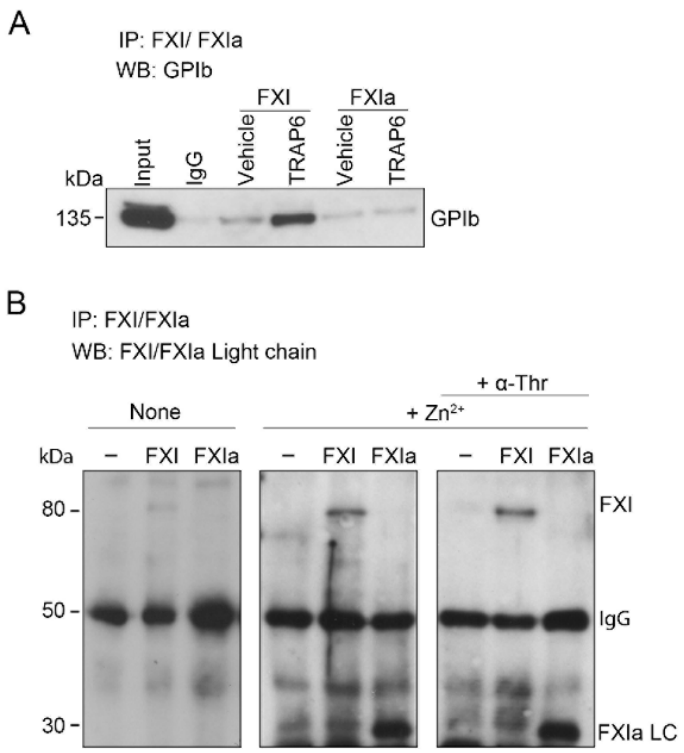

Figure 3.8 Characterization of FXI/FXIa-platelet interactions as detected by Western blotting. (A) Resting or platelets stimulated with the thrombin receptor activator peptide 6 (TRAP6) were incubated with FXI (30nM) or FXIa (30nM) and immunoprecipated (IP) with the anti-FXI/FXIa antibody 1A6 for 16h at 4°C. Protein A/G Plus Agarose beads were added and beads were washed and followed by Western blotting with an anti-GPIb antibody. (B) Resting or platelets activated by thrombin (α-thr, 1nM) were immobilized on fibrinogen surfaces and incubated with vehicle, FXI (50nM) or FXIa (50nM) at room temperature for 2hr, either in the absence or presence of 10μM Zn2+. Platelets were washed and lysed, and incubated with the anti-FXI/FXIa antibody 1A6 for 16h at 4°C. Protein A/G Plus Agarose beads were added and beads were washed and followed by western blotting with an anti-FXI light chain (LC) antibody 10C9. Data are representative of at least 3 independent experiments.

3.6 Discussion

Platelet activation is triggered following binding to exposed extracellular matrix proteins such as those that are exposed to circulating blood upon loss of blood vessel integrity. This incites platelets to rapidly reorganize their cytoskeleton, flip their membrane and expose phosphatidylserine (PS), and secrete their granule content, accelerating and localizing coagulation at the wound site [139]. The proteins of the contact pathway have been shown to interact with the surface of platelets, yet their activity is not influenced by lipid membrane containing PS, which is unlike the rest of the members of the coagulation cascade. Since the role of platelets in regulating contact pathway activity or activation is not well understood, the current study was designed to investigate whether the platelet surface regulates activity of the contact system of coagulation. Here, we demonstrate that the platelet surface is a potent regulator of contact system amiodolytic activity, specifically enhancing FXIa activity towards FIX, protecting it from inhibition by the inhibitors secreted from activated platelets and enhancing fibrin generation in a PS-independent manner. The platelet surface was also able to reduce the procoagulant activity of both FXIIa and PKa. Together, our findings suggest that an interaction of FXIa with the platelet surface may induce an allosteric modulation of FXIa.

Previous studies show that while soluble FXIa is rapidly inactivated by PN-II, platelet-bound FXIa is resistant to PN-II inhibition, suggesting that platelets provide a protective surface and may sustain FXIa activity and local FIX activation [129]. The binding of HK to FXIa also protects FXIa from inhibition by PN-II [96] and anti-thrombin [140]. Platelet activation induces the secretion of FXIa inhibitors [141] including PN-II [130], which are largely stored in the platelet alpha granules. In this study we observed that the activated platelet surface prevents the inhibition of FXIa activity by the platelet granule content. This result confirms the observation by Baird et al, that platelet-bound FXIa was resistant to inhibition by PN-II [129]. Moreover, we found that the platelets not

only amplify FXIa activity through a protective mechanism, but also enhance the amidolytic activity of FXIa towards FIX and enhance fibrin generation in a platelet surface exposed PS-independent manner by an unknown mechanism. FIXa, FX, FXa and prothrombin bind to activated platelets by their PS-binding Gla domains; thus the activity of FXase and prothrombinase is dependent upon the presence of PS on the activated platelet surface [1]. In contrast, FXIa does not have a PS-binding Gla domain and its activity is not influenced by PS-containing lipids. Perhaps the reason why PS is not required for FIX activation by FXIa on activated platelets is due to the fact that the binding of the FIX Gla domain to the FXIa A3 domain is sufficient to orient the activation peptide of FIX to be cleaved by FXIa [65,142]. Our findings in plasma suggest that platelets play a favorable role in FXI-dependent fibrin formation, and in a manner independent of platelet PS exposure. This is perhaps analogous to studies showing that thrombin generation mediated by the platelet receptor CD36 was dependent on FXI rather than PS exposure alone on the platelets [143].

FXI binds through its A3 domain to the receptor GPIb on activated but not resting platelets.[127] Moreover it has been suggested that FXIa and FXI bind to different receptor(s) on platelets.[128] In our study we observed that while FXI and FXIa were able to bind to the platelet surface following platelet activation with a protease-activated receptor-1 (PAR-1) agonist, only FXI was able to bind GPIb, confirming the observation by Miller and coworkers that FXIa and FXI seems to bind to different receptors on the platelet.[128] It has been proposed that upon FXI activation, sites within the A3 domain become concealed and a cryptic site within the catalytic domain is exposed which then allows for FXIa binding.[128] We explored whether mutations at Lys529, Arg530 and Arg532 in the FXIa anion-binding site (ABS) of the catalytic domain, which mediates interactions with polyphosphate and heparin [135], would effect FXIa-platelet binding, yet were unable to detect any differences in binding or the enhancement of FXIa activity, suggesting that

a region outside of the ABS of the catalytic domain is likely responsible for mediating this interaction.

Our data suggests that platelets enhance the activity of FXIa. In contrast, one of the surprising findings from our study was the observation that platelets alone or even just their granule content nearly abrogated the procoagulant activity of FXIIa and PKa, suggesting the presence of contact system inhibitors on platelets or their secretome. Platelets contain the known contact pathway inhibitor histidine-rich glycoprotein (HRG) in the α-granules and on the platelet surface [144]. HRG binds to FXIIa and PKa with high affinity and inhibits their capacity to activate FXI and FXII, respectively. HRG does not bind to the catalytic domain of FXIIa; rather the activity of FXIIa is modified allosterically upon HRG binding. This results in the increased amidolytic activity of FXIIa toward a chromogenic substrate, yet decreased activation of FXI as a result of HRG-FXIIa binding. We observed that the content of activated platelets enhanced the cleavage of a chromogenic substrate by FXIIa and PKa, yet reduced both FXI activation by FXIIa and FXII activation by PKa, consistent with the observation that HRG modifies FXIIa activity allosterically in a purified system [140]. We posit that this is attributed to the presence of HRG on the platelets surface and in the platelets secretome. This hypothesis is further supported by our observation that heat-inactivating the platelet secretome abolished its ability to promote FXIIa

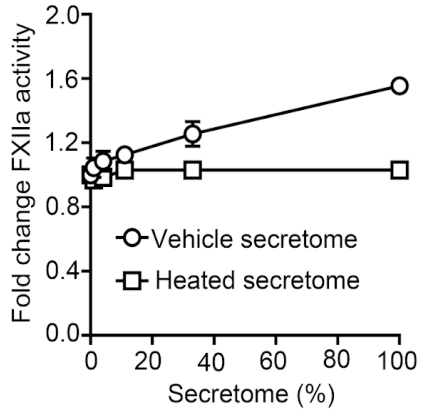

Figure 3.9 Effect of denatured secretome on FXIIa activity.
FXIIa (2nM) was incubated with select concentrations (0-100%) of either basal secretome or denatured secretome (heat-inactivated for 10min at 100ºC) for 1hr. The rate of substrate hydrolysis by FXIIa was measured at 405 nm and. Data are mean ± SD (n = 3).

activity towards a chromogenic substrate (Figure 3.9), indicating that a protein rather than an inorganic molecule such as polyphosphate is responsible for this effect.

Our finding that interactions between the platelet surface and FXIa promotes contact pathway activity in purified system and in plasma has notable clinical implications. Individuals with FXI deficiency are known to exhibit a mild and variable bleeding phenotype, with bleeding typically occurring in the setting of surgical hemostatic challenges rather than occurring spontaneously, unlike the more severe bleeding diathesis seen in other coagulation factor deficiencies [84]. Moreover, the bleeding tendency in FXI deficiency does not seem to directly correlate with FXI levels or genotype, and a bleeding phenotype is not seen in individuals deficient in FXII, PK, and HK [145]. Interestingly, despite its apparent minor role in normal hemostasis, FXI does play a clinically relevant role in thrombus formation, and FXI deficiency is associated with a reduced risk of pathologic thrombosis [146]. The variability in phenotype amongst individuals with FXI deficiency has complicated efforts to standardize management of these patients, with bleeding tendency difficult to predict in advance of a hemostatic challenge. Prior work has shown that under specific conditions designed to replicate various pathways of contact system activation, the thrombin generation assay can help identify elevated bleeding risk in FXI deficiency, especially when platelet rich plasma was used independent of contact pathway activation and dependent on the extrinsic pathway [147]. These studies may even suggest that FXI-platelet interactions are important for hemostatic plug formation when anionic binding sites are limited. The fact that the thrombin generation assay measured in the presence of platelets differentiated between bleeders and non-bleeders suggests that platelet-FXIa interactions and amplification of FIX activation may play an important role in supporting hemostasis in tissues that are prone to traumatic bleeding in FXI deficiency. Based on this work, we propose further investigation into the role of platelet membrane interactions in modulating FXIa, including the effects of the

fibrinolytic system, may allow for clarification of the anatomical, environmental, and physiological conditions regulating local FXIa activity, which could ultimately allow for preemptive identification of individuals with FXI deficiency who are at risk for bleeding in certain types of surgeries.

Chapter 4 Removal of the C-Terminal Domains of ADAMTS13 by Activated Coagulation Factor XI induces Platelet Adhesion on Endothelial Cells

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†Authors contributed equally to manuscript.

4.1 Abstract

Platelet recruitment to sites of vascular injury is mediated by von Willebrand factor (VWF). The shear-induced unravelling of ultra-large VWF multimers causes the formation of a “stringlike” conformation, which rapidly recruits platelets from the bloodstream. A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) regulates this process by cleaving VWF to prevent aberrant platelet adhesion; it is unclear whether the activity of ADAMTS13 itself is regulated. The serine proteases α-thrombin and plasmin have been shown to cleave ADAMTS13. Based on sequence homology, we hypothesized that activated coagulation factor XI (FXIa) would likewise cleave ADAMTS13. Our results show that FXIa cleaves ADAMTS13 at the C-terminal domains, generating a truncated ADAMTS13 with a deletion of part of the thrombospondin type-1 domain and the CUB1-2 domains, while α-thrombin cleaves ADAMTS13 near the CUB1-2 domains and plasmin cleaves ADAMTS13 at the metalloprotease domain and at the C-terminal domain. Using a cell surface immunoassay, we observed that FXIa induced the deletion of the CUB1-2 domains from ADAMTS13 on the surface of endothelial cells. Removal of the C-terminal domain of ADAMTS13 by FXIa or α-thrombin caused an increase in ADAMTS13 activity as measured by a fluorogenic substrate (FRETS) and blocked the ability of ADAMTS13 to cleave VWF on the endothelial cell surface, resulting in persistence of VWF strands and causing an increase in platelet adhesion under flow conditions. We have demonstrated a novel mechanism for coagulation proteinases including

FXIa in regulating ADAMTS13 activity and function. This may represent an additional hemostatic function by which FXIa promotes local platelet deposition at sites of vessel injury.

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4.2 Introduction

Platelets play an important role in regulating coagulation enzymes as we described in Chapter 3, where we found that different platelet activation states have regulatory effects on coagulation and thrombin generation. However, what the effects of regulating coagulation on a more complex setting, such as thrombus formation needs to be further studied. The functional roles of platelet activation and the interaction to the endothelial wall is mediated via VWF interaction with the ECM protein collagen. In Chapter 4 we will study the functional platelet effects of interaction with the endothelial cells via vWF and how this interaction is regulated by ADAMTS13 and specifically how ADAMTS13 activity is inhibited by a physiological inhibitor. We demonstrate that the platelet endothelial cell interaction is facilitated by vWF and its interaction can indirectly be regulated by FXIa. This work will generate more knowledge of the interplay between contact system of coagulation and platelets.

4.3 Background

After vascular injury, the large glycoprotein (GP) von Willebrand factor (VWF) binds to exposed collagen through its A3 domain. VWF multimers circulate in plasma in a globular form under normal flowing conditions. However, VWF unravels into a “string-like” conformation when it is exposed to increased shear forces.[148] This reveals the VWF-binding site located within the A1 domain for the platelet receptor GPIbα, resulting in the rapid recruitment and adhesion of

platelets to VWF.[149] Endothelial cells constitutively secrete VWF as multimers of varying size into the blood. VWF is also stored in endothelial cells within Weibel–Palade bodies, predominantly as “ultra-large” multimers (UL-VWF).[150] The presence of increased levels of UL-VWF multimers in plasma has been shown to initiate the formation of VWF-platelet microthrombi, resulting in debilitating thrombotic complications such as thrombotic thrombocytopenic purpura (TTP).[151] The cause of increased UL-VWF levels in the majority of patients with TTP has been attributed to either congenital defects in a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) or due to the presence of autoantibody inhibitors that compromise the function of ADAMTS13.[151]

ADAMTS13 has a molecular weight of 200 kDa, consisting of a metalloprotease (MET) domain, a disintegrin-like domain, a first thrombospondin type-1 repeat (TSP1) domain, a Cys-rich domain, and a spacer domain.[152] Moreover, the C-terminal domain of ADAMTS13 contains an additional seven TSP1 repeats and two CUB domains.[153] The hemostatic potential of VWF is regulated through a multistep mechanism of proteolysis of its A2 domain by ADAMTS13. VWF is resistant to cleavage by ADAMTS13 until subjected to fluid shear stress,[154] adsorbed on a surface,[153,154] or treated with reagents that cause denaturation,[153,155] all of which “unravel” VWF and permit ADAMTS13 access to the scissile bond within the A2 domain of VWF. ADAMTS13 has been shown to adopt a natural folded conformation, allowing the CUB-1 domains to interact with its spacer domain.[156] This generally closed conformation prohibits the functional exosite on the spacer domain from interacting with the proteolytic site on the A2 domain of VWF.[156,157] The binding of the C-terminal TS8-CUB2 domain of ADAMTS13 with the C-terminal D4-CK domain of VWF mediates a conformational activation of ADAMTS13, leading to the exposure of the spacer, cysteine-rich, disintegrin-like, and MET domains exosite, and ultimately increased proteolysis of VWF by ADAMTS13.[158-160] Deletion of the C-terminal TSP1 and CUB domains of ADAMTS13 impairs the cleavage of large VWF multimers in vitro,[158,161] increases vascular

thrombosis in vivo,[161] and yet increases the cleavage of peptide substrates such as FRETS-VWF73 (10).[157] These findings suggest that the CUB domains regulate ADAMTS13 activity.

ADAMTS13 is constitutively active and has no known inhibitors in vivo. To date, it is still uncertain how ADAMTS13 activity is regulated, and what impact this has on the inactivation of VWF. The serine proteases α-thrombin, activated FX (FXa), and plasmin have been shown to cleave the C-terminal of ADAMTS13 in vitro under static conditions.[162] Yet, it is still unknown whether the deletion of the C-terminal of ADAMTS13 by α-thrombin or plasmin inhibits the functional activity of ADAMTS13 on the processing of endothelial VWF to affect platelet recruitment and aggregation under flow conditions.

FXI is a contact pathway serine protease that has been shown to play an increasingly relevant role in hemostasis.[163] Congenital factor XI deficiencies were reported to exhibit protection from ischemic stroke and to exhibit a lower incidence of venous thromboembolism (VTE),[164,165] while elevated levels of FXI are an independent risk factor for VTE and ischemic stroke.[107,108] The primary substrate of the serine protease activated FXI (FXIa) in the classic coagulation model is FIX; however, increasing evidence has shown that FXIa promotes thrombin generation by enzymatic activation of FXI, FX, FVIII, FV,[82,166,167] and inactivation of tissue factor pathway inhibitor (TFPI), in vitro.[86]

Based on sequence homology, we hypothesized that FXIa cleaves and inactivates ADAMTS13 leading to VWF string formation, platelet aggregation, and thrombus formation. In the present study, we developed an endothelialized flow chamber that allowed us to study whether the components of the coagulation cascade can regulate ADAMTS13 activity under flow conditions.

4.4 Materials and Methods

4.4.1 Reagents

Recombinant ADAMTS13 (rADAMTS13) was donated from Shire (Benatzkygasse, Austria). Plasma-derived FXIa, α-thrombin, plasmin, and FXa were purchased from Haematologic Technologies (Essex Junction, VT, USA). α-FXIIa and kallikrein were from Enzyme Research Laboratories, Inc. (South, IN, USA). Rabbit polyclonal anti-ADAMTS13 antibody, specific for the MET domain, was from Abcam (Cambridge, MA, USA). Rabbit polyclonal anti-ADAMTS13 antibody, specific for the CUB1-2 domains, was from Santa Cruz Biotechnology (Dallas, TX, USA). Goat polyclonal anti-ADAMTS13 antibody, specific for the TSP4 domains, was from Bethyl Laboratories (Montgomery, TX, USA). Hirudin, O-phenylenediamine (OPD) substrate, and aprotinin were from Sigma-Aldrich (St. Louis, MO, USA). FRETS-VWF73 (VWF residues 1,596–1,668) was purchased from PeptaNova (Louisville, KY, USA).

4.4.2 Coomassie Blue Staining and Western Blot

rADAMSTS13 (250 nM) was incubated with FXIa (50 nM) with or without aprotinin (50 μM), and with plasmin, α-thrombin, FXa, FXIIa, or kallikrein at 37°C over a time interval of 0–4 h in 25 mM Hepes, 150 mM NaCl (HBS) pH 7.4. The addition of Ca2+ (5 mM) was necessary for proteolytic activity of α-thrombin, plasmin, and FXa. Samples were separated by SDS-PAGE under reducing conditions and analyzed by Coomassie blue staining, or they were transferred to PVDF membrane and immunoblotted with an anti-MET domain antibody, anti-ADAMTS13 CUB1-2 domain antibody, or an anti-TSP4 domain antibody, followed by HRP-conjugated secondary antibodies. Proteins were detected using ECL (GE Healthcare, Piscataway, NJ, USA).

4.4.3 Cell Surface Immunoassays

Human umbilical vein endothelial cells (HUVECs, ATCC, Manassas, VA, USA) were grown to confluence in a 96-well plate using an endothelial cell basal medium-2 enriched with supplements (Lonza, Walkersville, MD, USA) and incubated with rADAMTS13 (50 nM) for 1 h at 37°C in serum-free medium (SFM, Thermo Fisher Scientific) with BSA (0.1%) and ZnCl2 (10 μM). HUVECs were then washed and incubated with FXIa (30 nM), α-thrombin (30 nM), or plasmin (30 nM) for 0, 1, or 2 h at 37°C in SFM with BSA (0.1%) and ZnCl2 (10 μM), followed by incubation with 50 μM aprotinin and 10 μg/mL hirudin for 10 min at 37°C. HUVECs were then washed with HBS, fixed with paraformaldehyde (2%), blocked in PBS with tween (0.05%) and BSA (3%), and probed with either the anti-ADAMTS13 CUB antibody (5 μg/mL) or an anti-ADAMTS13 TSP4 antibody (1 μg/mL) for 1 h. This was followed by incubation with an HRP-coupled secondary anti-rabbit antibody (1:2,000) or anti-goat antibody (1:2,000), and then measured with an OPD substrate at an absorbance of 450 nm.

4.4.4 ADAMTS13 Activity Assay

rADAMSTS13 (30 nM) was incubated with either FXIa (5 nM), plasmin (5 nM), α-thrombin (5 nM), or vehicle for 3 h at 37°C in HBS. Aprotinin (50 μM) and hirudin (10 μg/mL) was added to each sample and allowed to sit at RT for 10 min. rADAMTS13 was diluted to 4 nM in reaction buffer (5 mM Bis-Tris, 25 mM CaCl2, 0.005% Tween; pH 6.0). The reaction was initiated by the addition of an equal volume of 4 μM FRETS-VWF73 substrate. Fluorescence (excitation, 340 nm; emission, 460 nm) was measured at 1-min intervals for 1 h using a fluorescence spectrometer.

4.4.5 Flow Experiment and Staining

HUVECs were cultured in the parallel-plate flow chamber (ibidi μ-slide VI0.1) under shear conditions beginning 48 h before the flow experiment. Devices were incubated with cells for 30

min prior to induction of passive flow by adding growth medium to the inflow port of each channel. Passive flow was maintained by emptying the outflow and refilling the inflow for 1 h or until a morphology changes in HUVECs from round to elongated was noted. Inflow ports of the flow devices were then connected to a growth medium well and the outflow ports were connected to a waste well. All six channels were connected to the same growth medium well and the outflowing growth medium was recirculated. The height of the medium well was maintained to achieve an initial shear stress of 1.54 dyne/cm2 for 48 h or until confluence.

Cells were starved in SFM for 2 h, and then stimulated with 10 ng/mL TNFα in SFM supplemented with 3% fatty acid-free BSA for 4 h. Human whole blood was drawn into 3.8% sodium citrate from healthy donors per institutional IRB protocol and washed platelets were purified as previously described.[168] Washed platelets (3 × 108/mL) in the presence or absence of rADAMTS13 (2.5 nM) were perfused through the flow chamber for 10 min at 2.5 dyne/cm2. Samples were fixed with 4% PFA overnight at 4°C. Platelet-VWF string formation was visualized by labeling with mouse anti-human CD41-FITC (ThermoFisher Scientific, Grand Island, NY, USA) and rabbit anti-human VWF antibody, respectively, and AlexaFluor-labeled secondary antibodies (Thermo Fisher Scientific, Grand Island, NY, USA). Five random fields at 20× magnification were imaged for each chamber by using a Zeiss Axiovert 200M microscope and SlideBook 6 software. The number of platelet-VWF string formations were manually counted. UL-VWF length was analyzed by using ImageJ software. Statistical significance was evaluated using the Dunnett's multiple comparison test, and p-values <0.05 were considered to be statistically significant.

4.5 Results

4.5.1 Proteolytic Cleavage of ADAMTS13 by FXIa

Previous studies have shown proteolytic cleavage and inactivation of ADAMTS13 by the known serine proteases plasmin and α-thrombin.[162] Herein, we investigated whether FXIa was able to proteolyze ADAMTS13. We observed via SDS-PAGE analysis that incubation of rADAMSTS13 with FXIa for 2 h led to the disappearance of the ~200 kDa ADAMTS13 band and the appearance of lower molecular weight bands under reducing conditions (Figure 4.1A). The presence of aprotinin, which inhibits FXIa activity, blocked the degradation of ADAMTS13 by FXIa (Figure 4.1A). Then, we compared the capacity of FXIa to proteolyze ADAMTS13 relative to plasmin and α-thrombin. In accordance with previous studies (16), we observed that plasmin rapidly degraded ADAMTS13; in contrast, 15 nM α-thrombin barely cleaved ADAMTS13 after 4 h of incubation (Figure 4.1B). FXa, Kallikrein, or FXIIa were not able to cleave ADAMTS13 under the conditions tested here (Figure 4.1C). The addition of Ca2+ was necessary for proteolytic activity of α-thrombin and plasmin; in contrast, neither Ca2+ nor Zn2+ were required for the proteolytic cleavage of ADAMTS13 by FXIa (Figure 4.1D).

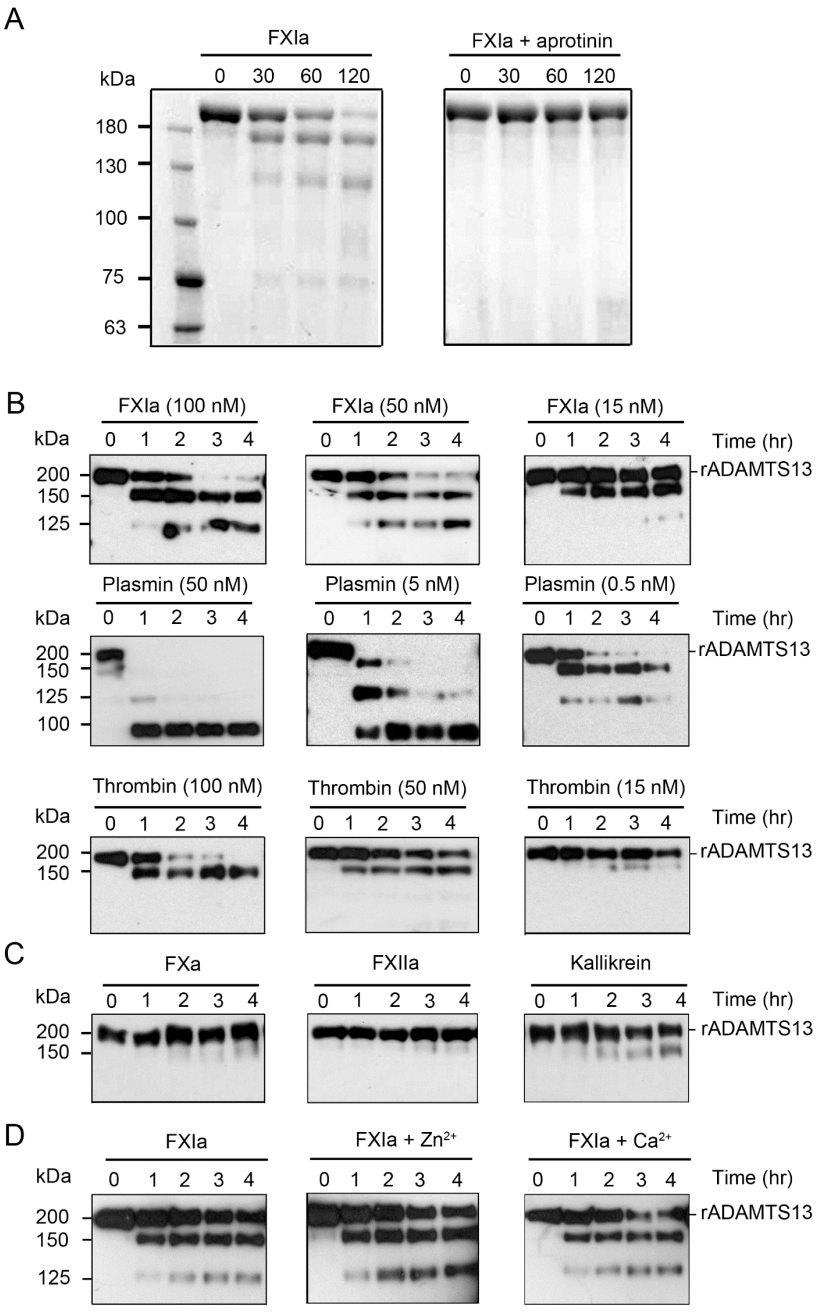

Figure 4.1 Proteolysis of ADAMTS13 by FXIa. (A) rADAMTS13 (250 nM) was incubated with FXIa (50 nM), in the absence or presence of aprotinin (50 μM) for selected times (0–120 min) at 37°C before being separated by SDS-PAGE under reduced conditions and analyzed by Coomassie blue staining. rADAMTS13 fragment size (kDa) is shown following proteolysis by FXIa (B) rADAMTS13 (250 nM) was incubated with FXIa (100–15 nM), plasmin (50–0.5 nM), or α-thrombin (100–15 nM) for selected times (0–4 h) at 37°C. rADAMTS13 was analyzed by western blotting using an anti-ADAMTS13 MET domain antibody (C) rADAMTS13 (250 nM) was incubated with FXa (50 nM), FXIIa (50 nM), and kallikrein (50 nM) for selected times (0–4 h) at 37°C. rADAMTS13 was analyzed by western blotting using an anti-ADAMTS13 MET domain antibody (D) rADAMTS13 (250 nM) was incubated with FXIa (50 nM) for selected times (0–4 h) at 37°C before being analyzed by western blotting using an anti-ADAMTS13 MET domain antibody in the absence or presence of CaCl2 or ZnCl2 (n = 3).

4.5.2 FXIa deletes the TSP6-8 Domains and the C-Terminal CUB1-2 Domains of ADAMTS13

Experiments were next designed to identify the fragments of rADAMTS13 generated by FXIa, α-thrombin, and plasmin. rADAMTS13 (250 nM) was incubated with FXIa (50 nM), α-thrombin (50 nM), or plasmin (5 nM) for increasing times (0–4 h) at 37°C before being analyzed by western blot using an anti-ADAMTS13 antibody against the MET domain (N-terminal), the TSP4 domains, or the two CUB domains (C-terminal). We observed that FXIa caused the disappearance of the ~200 kDa ADAMTS13 band and the appearance of first a band at ~150 kDa (Fragment 1) and then a second band at ~125 kDa (Fragment 2) when the samples were analyzed with the anti-MET domain antibody or an anti-TSP4 domain antibody (Figure 4.2A). A band at ~50 kDa (Fragment B) and a fragment at ~75 kDa (Fragment A) appeared when the samples were analyzed with an anti-CUB1-2 domain antibody (Figure 4.2A). These data suggest that FXIa cleaves ADAMTS13 at two sites, first near the two CUB domains, generating a band ~150 kDa and a band ~50 kDa, and then near the TSP6-8 domain, generating a band ~125 kDa (Figure 4.2A). The location of these fragments is predicted in Figure 4.2A. We observed that α-thrombin was only able to cleave ADAMTS13 near the CUB1-2 domain after 4 h of incubation, generating one band at ~150 kDa when the sample was analyzed with the anti-MET domain antibody (Figure 4.2B), suggesting that α-thrombin can cleave ADAMTS13 at only one site near the two CUB domains.

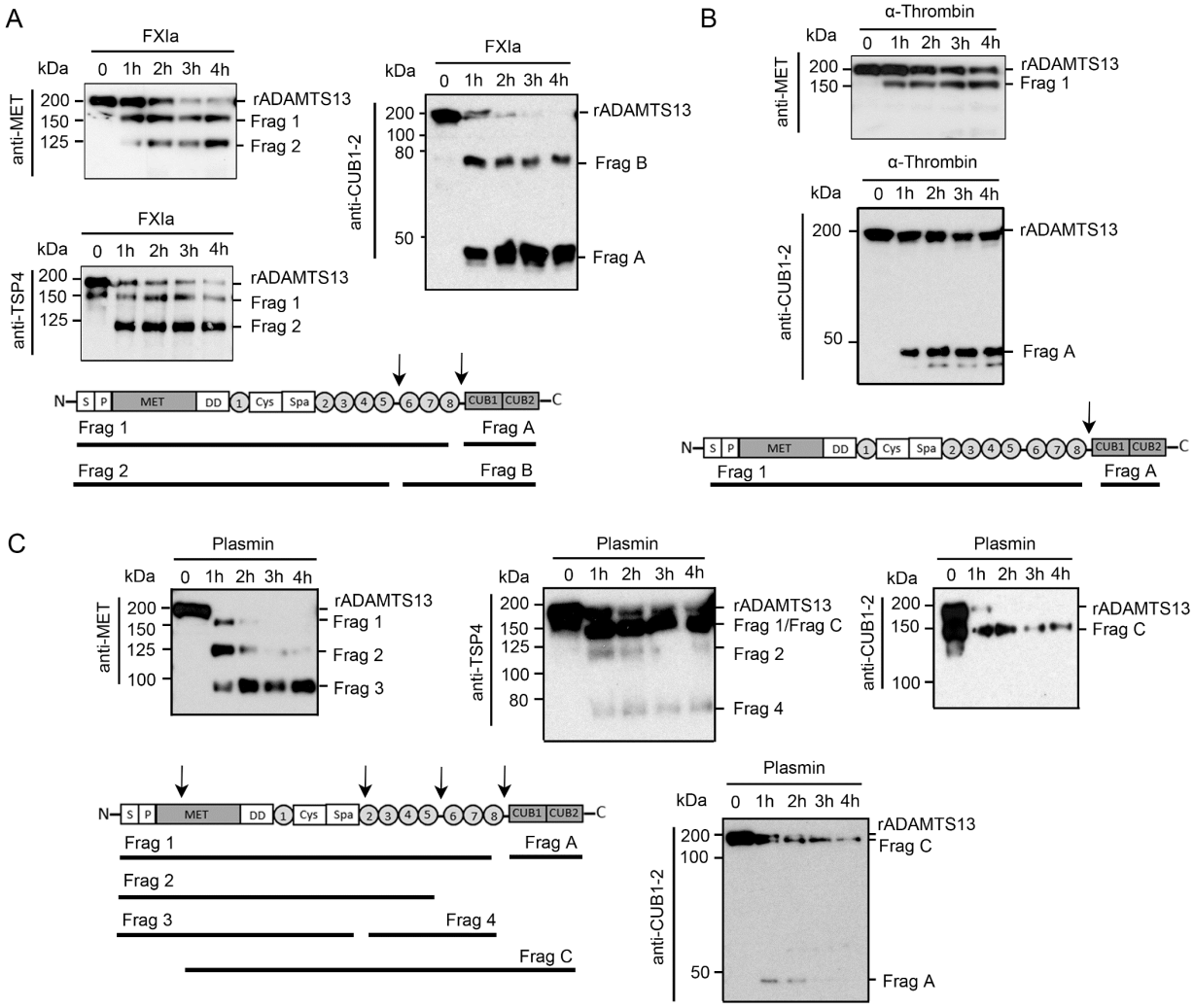

Figure 4.2 Characterization of ADAMTS13 proteolysis by FXIa, α-thrombin, and plasmin. rADAMTS13 (250 nM) was incubated with (A) FXIa (50 nM), (B) α-thrombin (50 nM), or (C) plasmin (5 nM) for selected times (0–4 h) at 37°C before being analyzed with three different antibodies: anti-ADAMTS13 MET domain antibody, anti-ADAMTS13 CUB1-2 domain antibody, and anti-TSP4 domain antibody. The predicted identities of the formed fragments upon cleavage of rADAMTS13 by FXIa in (A), or by α-thrombin in (B) or by plasmin in (C) are depicted in a schematic overview (n = 3).

In contrast to FXIa, plasmin was able to generate a third band at ~100 kDa (Fragment 3) when the samples were analyzed with the anti-MET domain antibody. This band around 100 kDa was not detected when the samples were analyzed with the anti-TSP4 domain antibody; instead, an extra band at ~70 kDa (Fragment 4) was detected (Figure 4.2C). These results indicate that plasmin cleaves ADAMTS13 at multiple sites, not only at the C-terminal domain but also after the spacer domain. When the samples were analyzed with an anti-CUB1-2 domain antibody, plasmin generated a band at ~50 kDa at 1 h of incubation, which disappeared after 4 h of incubation, suggesting that plasmin cleaves at the CUB domain, destroying the binding of the anti-CUB domain antibody. Also, the anti-CUB domain antibody detected a band at 150 kDa, indicating that plasmin may cleave ADAMTS13 at the MET domain located near the N-terminal domain of ADAMTS13 (Figure 4.2C). The location of these fragments is predicted in Figure 4.2C.

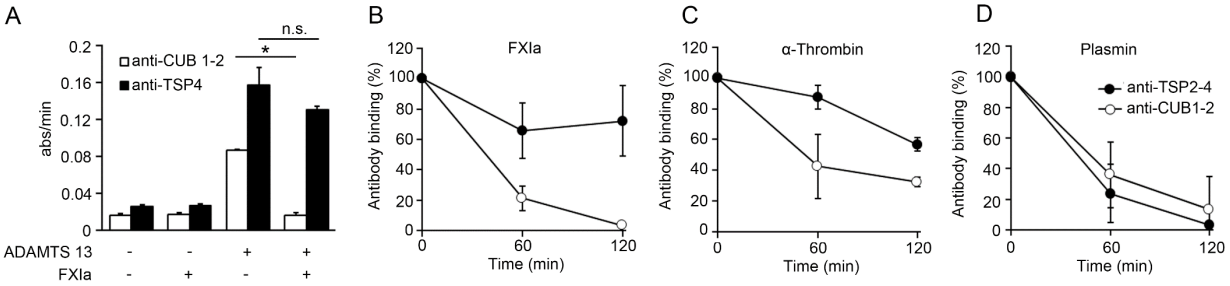

Figure 4.3 FXIa cleaves the CUB1-2 domain of ADAMTS13 on the endothelial surface. (A) HUVECs were incubated with or without rADAMTS13 (50 nM) for 1 h at 37°C, washed, and treated with or without FXIa (30 nM) for 2 h. Reactions were stopped with aprotinin (50 μM), followed by cell surface detection of ADAMTS13 by using either an anti-ADAMTS13 CUB1-2 domain antibody or an anti-ADAMTS13 TSP4 domain antibody. (B) HUVECs incubated with rADAMTS13 (50 nM) treated with FXIa (30 nM), (C) α-thrombin (30 nM), or (D) plasmin (30 nM) for 0–2 h at 37°C. Reactions were stopped with aprotinin (50 μM) and hirudin (10 μg/mL), followed by cell surface detection of ADAMTS13 by using either an anti-ADAMTS13 CUB1-2 domain antibody (○) or an anti-ADAMTS13 TSP4 domain antibody (●). Data are mean ± SE (n = 3).

4.5.3 FXIa Cleaves the CUB1-2 Domain of ADAMTS13 on the Endothelial Surface

Prior studies have shown that ADAMTS13 binds endothelial cells in a specific manner and that the cleavage of VWF by ADAMTS13 occurs mainly on the EC surface.[17] In order to determine if the deletion of the ADAMTS13 C-terminal domain by FXIa or α-thrombin could take place on the surface of ECs, we performed a cell surface immunoassay using an anti-CUB1-2 domain antibody and an anti-TSP4 domain antibody. We incubated HUVECs with rADAMTS13 and measured the binding of both antibodies following incubation of the cells with the serine protease FXIa. We observed that both antibodies only bound to the endothelial cell surface in the presence of rADAMTS13, indicating that both antibodies are specific for ADAMTS13 (Figure 4.3A). The detection of ADAMTS13 CUB1-2 domains on HUVECs was lost over a period of 2 h following incubation with FXIa (Figures 4.3A,B). In contrast, FXIa treatment induced a slight decrease in binding of the anti-TSP4 domain antibody (Figures 4.3A,B). Incubation with α-thrombin only slightly decreased the binding of either the anti-CUB1-2 domain antibody or the anti-TSP4 domain antibody (Figure 4.3C). In contrast, incubation with plasmin dramatically decreased the binding of antibodies to the ADAMTS13 CUB1-2 domain or the ADAMTS13 TSP4 domain (Figure 4.3D). These results suggest that FXIa is able to remove the CUB1-2 domains of ADAMTS13 on the surface of ECs. α-thrombin weakly cleaved ADAMTS13 on the endothelial surface, whereas plasmin was able to cleave ADAMTS13 at multiple sites, including the N-terminal domain of ADAMTS13.

4.5.4 Deletion of the TSP6-8 and the C-Terminal CUB1-2 Domains of ADAMTS13 by FXIa Induces an Allosteric Conformation of ADAMTS13

It has been shown that truncation of ADAMTS13 after the TSP2-8 domain or antibodies against the CUB1-2 domains induces a conformational change of ADAMTS13, causing ADAMTS13 to unfold fully and expose the spacer domain, resulting in enhanced cleavage of the peptide substrate FRETS-VWF73.[157] Based on the fact that both FXIa and α-thrombin cleave ADAMTS13 near the CUB1-2 domains as described above, experiments were designed to determine whether FXIa or α-thrombin induced an allosteric conformation of ADAMTS13. We compared this with the serine proteases plasmin, which can also cleave ADAMTS13 at the MET domain.[162] We observed that after incubation of rADAMTS13 with FXIa at 37°C for 3 h, the activity of ADAMTS13 was increased as measured by the FRETS-VWF73 assay (Figure 4.4). Analysis of the samples by western blot using an anti-ADAMTS13 MET domain antibody confirmed the generation of 150 and 125 kDa fragments following incubation with FXIa. α-thrombin also was able to increase ADAMTS13 activity toward the peptide substrate FRETS-VWF73. In contrast, plasmin reduced ADAMTS13 activity toward the peptide substrate FRETS-VWF73 due to its capacity to cleave ADAMTS13 at multiple places. These data suggest preferential proteolytic cleavage near the CUB domains of ADAMTS13 by FXIa or α-thrombin, allowing ADATMS13 to adopt a more open configuration and exposing the proteolytic site on ADAMTS13 responsible for cleaving FRETS-VWF73.

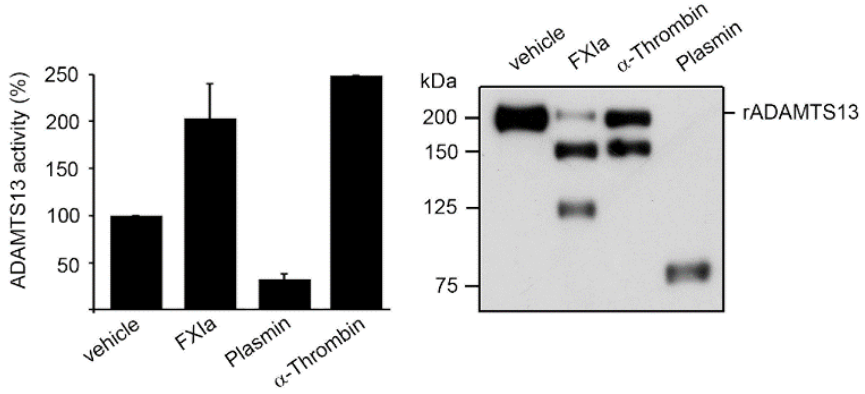

Figure 4.4 ADAMTS13 activity following proteolysis by FXIa. (**A**) rADAMTS13 (30 nM) was incubated at 37°C for 4 h with the following: FXIa (5 nM), plasmin (5 nM), or α-thrombin (5 nM) in HBS with 5 mM CaCl2. Reactions were stopped with aprotinin (50 μM) and hirudin (10 μg/mL). rADAMTS13 was diluted to 4 nM in reaction buffer (5 mM Bis-Tris pH 6.0, 25 mM CaCl2, and 0.005% Tween-20) and the reaction was initiated by the addition of an equal volume of FRETS-VWF73 substrate (4 μM). Data are mean ± SE (n = 3). (**B**) Western blot of the samples using an anti-ADAMTS13 MET domain antibody to confirm proteolytic cleavage of ADAMTS13 by the proteases.

4.5.5 Deletion of the TSP6-8 and the Two C-Terminal CUB Domains of ADAMTS13 by FXIa Inhibits ADAMTS13 Activity Resulting in Increased Platelet Adhesion under Flow

It has been proposed that the binding of the ADAMTS13 TSP7-CUB2 domain to the VWF D4CK domains induces a conformational activation of ADAMTS13, causing ADAMTS13 to unfold fully and expose the spacer domain.[169] The spacer domain can then directly interact with the VWF A2 domain, enhancing the cleavage of VWF by ADAMTS13 under flow conditions. Besides the fact that removal of the C-terminal of ADAMTS13 domains or antibodies against the CUB1-2 domains enhance the cleavage of the peptide substrate FRETS-VWF73,[157] removal of the C-terminal of ADAMTS13 abrogate the capacity of ADAMTS13 to bind and cleave VWF under flow conditions ex vivo[159] and in vivo.[170] Thus we next determined whether cleavage of the TSP6-8 and the two C-terminal CUB domains of ADAMTS13 by FXIa or the cleavage of the CUB 1-2 domains of ADAMTS13 alone by α-thrombin might abrogate ADAMTS13 activity under flow by

assessing the length of VWF that had been released by activated endothelial cells under shear. HUVECs were preincubated with TNFα to induce VWF release and the formation of platelet-VWF strings, the number and the length of UL-VWF were then quantified. We observed that the addition of 2.5 nM full-length rADAMTS13 abrogated platelet-VWF string formation (Figure 4.5A,B). In contrast, the incubation of rADAMTS13 with FXIa reversed the ability of ADAMTS13 to cleave VWF, resulting in an increase in the formation of platelet-VWF strings. A similar effect was observed when rADAMTS13 was incubated with either α-thrombin or plasmin. Interestingly, the addition of an anti-ADAMTS13 CUB1-2 domain antibody also blocked ADAMTS13 activity under flow (Figures 4.5A,B).

Utilizing this in vitro endothelialized flow chamber technique, our data suggest a novel mechanism by which the activity of ADAMTS13 is regulated: proteolysis by the serine proteases FXIa, α-thrombin, and plasmin inhibits the ability of ADAMTS13 to cleave VWF, promoting platelet-VWF string formation on inflamed endothelial cells under shear flow.

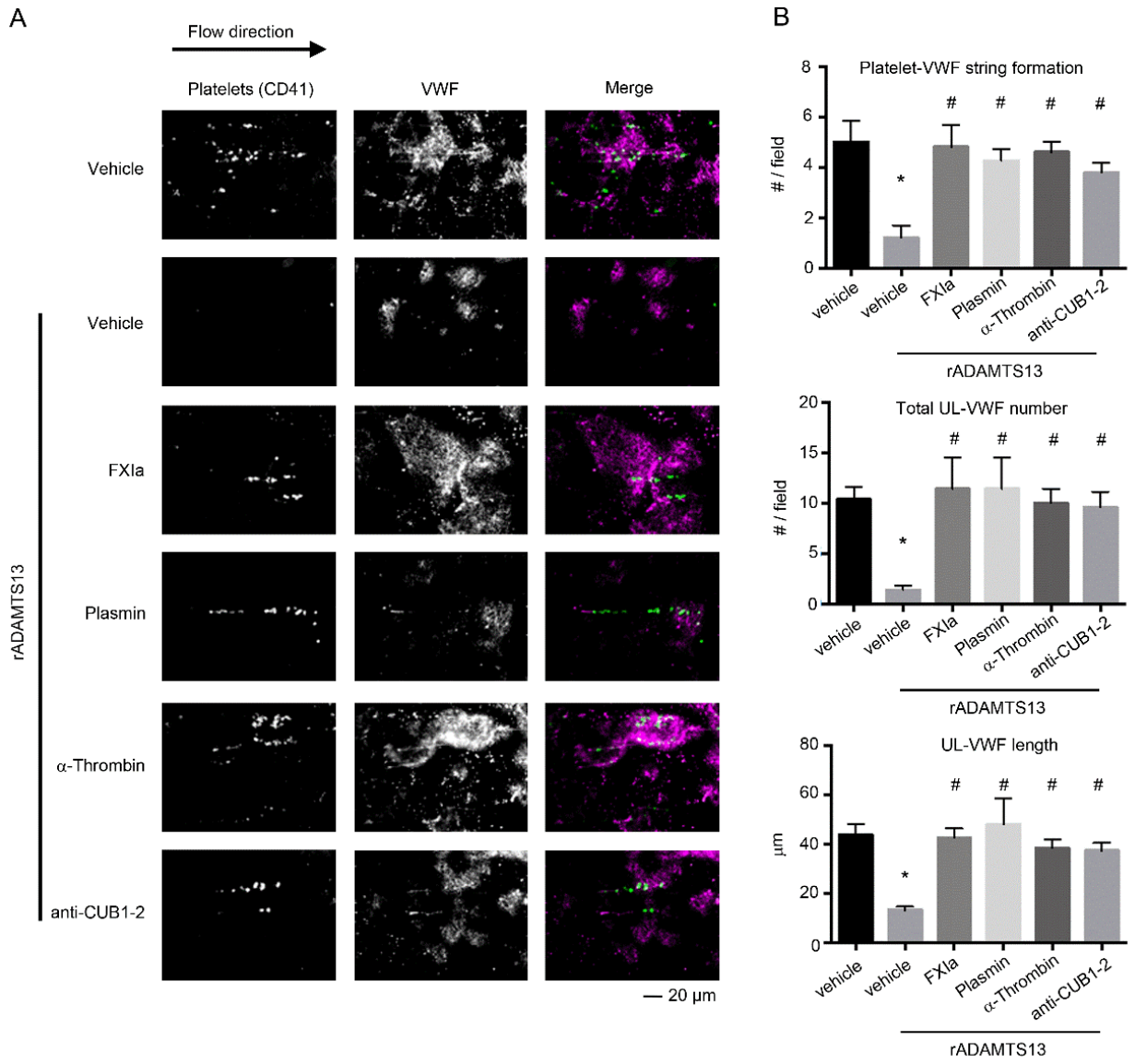

Figure 4.5 FXIa inhibits ADAMTS13 cleavage of VWF. rADAMTS13 (250 nM) was incubated at 37°C for 4 h with the following: FXIa (50 nM), α-thrombin (50 nM), and plasmin (50 nM) in HBS with 5 mM CaCl2. Reactions were stopped with aprotinin (50 μM) and hirudin (10 μg/mL). Endothelialized parallel-plate flow chambers were prepared and EC's were stimulated with TNFα to release VWF as described above. (**A**) VWF string formation and platelet adhesion depicted with fluorescence following perfusion of washed platelets at a venous flow rate of 2.5 dyne/cm2 in the absence or presence of rADAMTS13 (2.5 nM) incubated with either vehicle, FXIa, α-thrombin, plasmin, or an anti-ADAMTS13 CUB domain antibody (20 ng/mL). (**B**) Quantification of platelet string formation, total VWF number, and VWF length compared between noted substrates. Using Dunnett's multiple comparison test, * and # indicate statistical significance ($p < 0.05$). Data are mean ± SE ($n = 3$).

4.6 Discussion

In this study, we have shown a novel mechanistic role by which the serine protease FXIa may regulate platelet deposition at sites of endothelial cell damage by inactivating ADAMTS13. This finding further expands the classical pathway by which activation of the contact pathway of coagulation promotes thrombus formation. FXIa is known to promote thrombin generation through direct activation of FIX, FX, FV, and FVIII[82,166,167] and inactivation of TFPI, in vitro.[86] These alternative pathways explain in part why mice lacking both FIX and FXI are more resistant to chemical injury-induced arterial thrombosis than are mice deficient in FIX alone.[171] These observations suggest that the role of FXIa in hemostasis and thrombus formation may include activities that bypass the FIX-mediated intrinsic pathway of thrombin generation. Here, we show not only that FXIa cleaves and inactivates ADAMTS13 leading to platelet aggregation along persistent VWF strands under flow but that this interaction can occur on the endothelial surface.

ADAMTS13 has been shown to adopt a closed conformation due to an interaction between its CUB-1 domain and spacer domain.[157] Following binding to VWF and under shear flow, ADAMTS13 unfolds and the TS8-CUB2 domain binds to the D4-CK domain on the elongated VWF strand.[158,160] The spacer domain of ADAMTS13 can then bind to the VWF A2 domain leading to the cleavage of VWF. To date, there are no known endogenous inhibitors of ADAMTS13 activity, which would in turn promote persistence of ultra-long VWF strands and resultant platelet aggregation at sites of endothelial injury. Prior studies have demonstrated that the serine proteases α-thrombin and plasmin are able to cleave ADAMTS13, abolishing its enzymatic activity toward purified human VWF in vitro.[162] Here, we observed that plasmin cleaves ADAMTS13 at multiple sites and more rapidly than α-thrombin, confirming findings from

previous reports.[162] Previous work has shown that 9 nM α-thrombin is capable of rapid proteolysis of full-length ADAMTS13.[162] Under the conditions used in our study, we found that the proteolysis of ADAMTS13 by α-thrombin was slow at higher concentrations, and almost non-existent at 15 nM. While we observed that α-thrombin preferentially cleaves ADAMTS13 just before the CUB1-2 domains, FXIa appears to cleave ADAMTS13 at the C-terminal domain at two sites. The first cleavage site appears to be located near to the start of the first CUB domain, producing a fragment of ~150 kDa, while the second cleavage site occurs near to the start the TSP6 domain, producing a fragment of ~125 kDa. This suggests that FXIa removes the TSP6-8-CUB1-2 domain of ADAMTS13.

Plasmin shows a preference for lysine at the P1-position for substrate cleavage; in contrast, thrombin and FXIa have a P1 preference for arginine. While FXa also has a P1 preference for arginine, it shows preference for glycine at the P2-position. However, thrombin has a strict preference for proline at the P2-position, while FXIa has preference for either proline or threonine at the P2-position.[172] This could explain the difference in the ADAMTS13 cleavage site selectively between the serine proteases. Also, unlike plasmin or α-thrombin, the cleavage of ADAMTS13 by FXIa does not require the presence of Ca2+. In contrast, activation of FIX by FXIa is a calcium-dependent process. The FIX gamma-carboxyglutamic (Gla) domain requires the binding of calcium ions in order to bind to the apple 3 domain of FXIa.[173] Previously, we demonstrated that FXIa also can activate FX and FV and cleave TFPI, albeit that these reactions are less efficient as compared to the activation of FIX by FXIa. However, Ca2+ was not a requirement for any of these reactions.[82,86,166] These results suggest that only FIX requires the binding of Ca2+ to the Gla domain for the interaction with FXIa, while the other FXIa substrates do not need the binding of calcium ions to interact with FXIa.

UL-VWF multimers released by endothelial cells and elongated under high shear flow conditions typically undergo rapid proteolysis by ADAMTS13. In vitro studies have suggested that the endothelial cell surface accelerates the cleavage of VWF strands, as the endothelium serves as an anchor for ADAMTS13.[17] We sought to determine whether FXIa, α-thrombin, or plasmin cleaves ADAMTS13 on the endothelial surface. We showed the C-terminus of ADAMTS13 was removed following proteolysis by all three serine proteases, most notably FXIa. Importantly, this work shows a role for the endothelium in facilitating the inactivation of ADAMTS13 by FXIa.

It has been shown that a rADAMTS13 mutant lacking the C-terminal TSP1 and CUB domains maintains the same VWF-cleaving activity as wild-type rADAMTS13, yet, cleaves VWF even more efficiently under static conditions. This indicates that the C-terminal domains are dispensable for ADAMTS13 activity under these conditions.[159] Interestingly, the same study shows that removal of the C-terminal TSP1 and CUB domains results in a marked decrease in VWF-ADAMTS13 binding and cleavage of VWF by ADAMTS13 when subjected to shear flow.[159] Synthetic peptides derived from the first CUB domain have been shown to inhibit the cleavage of VWF multimers by ADAMTS13 on endothelial cells under flow,[160] while removal of the TSP7-CUB1-2 domains has been shown to accelerate thrombus growth in vivo.[170] These results indicate that the C-terminal domain of ADAMTS13 plays a crucial role in the recognition and cleavage of VWF. However, deletion of the distal ADAMTS13 domain or use of monoclonal antibodies against the C-terminal domains showed accelerated cleavage of the peptide substrate FRETS-VWF73 due to a conformational change in ADAMTS13.[157] Here, we have shown that the removal of the ADAMTS13-CUB domains by the serine proteases FXIa and α-thrombin enhances the capacity of ADAMTS13 to cleave the peptide substrate FRETS and

blocks its ability to cleave VWF strings on endothelial cells (Figure 4.6). This resulted in increased platelet adhesion to endothelial cells under flow conditions, suggesting that cleavage of ADAMTS13 by either FXIa or α-thrombin likely reduces ADAMTS13 binding to VWF under flow conditions. We show that plasmin cleaves ADAMTS13 at multiple sites, confirming that this induces a decrease in the cleavage of FRETS-VWF73 as previously reported[174] and the cleavage of VWF under flow conditions. Our results suggest that removal of the C-terminal TSP1 and CUB domains by FXIa and α-thrombin may limit ADAMTS13-mediated VWF inactivation in vivo.

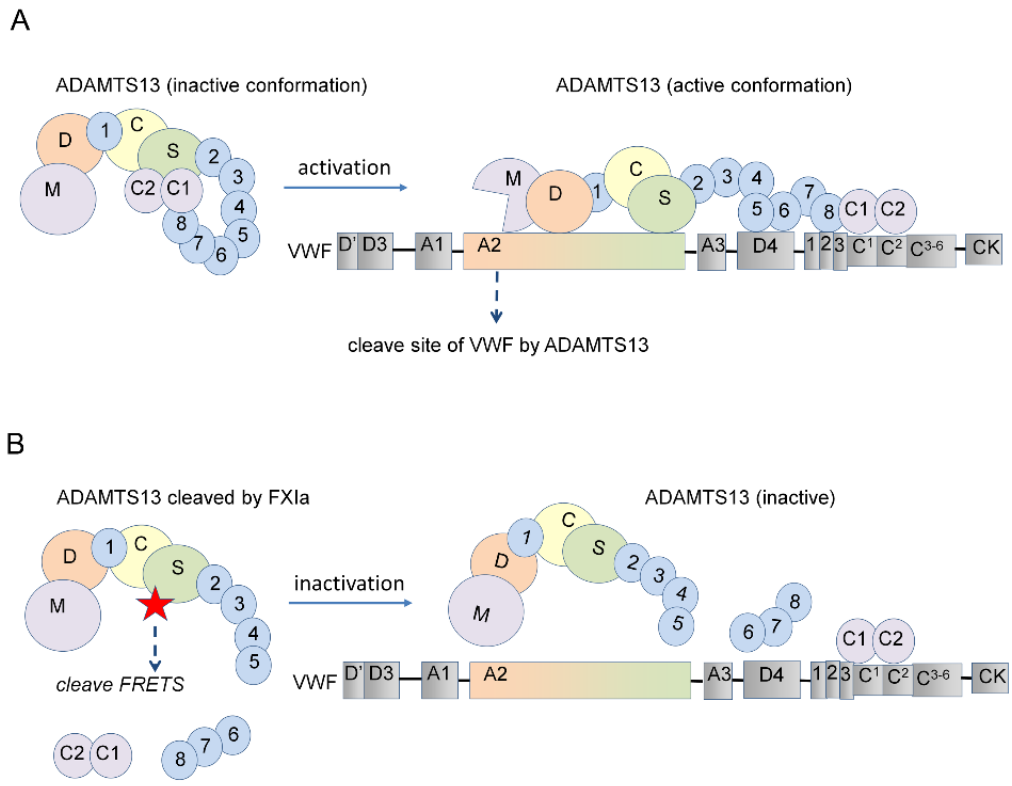

Figure 4.6 Schematic representation of the proposed model for ADAMTS13 substrate cleavage. (**A**) ADAMTS13 circulates in plasma in a closed conformation due to an interaction between its CUB-1 domain and the spacer domain. Following the binding of the ADAMTS13 TSP7-CUB2 domain to the VWF D4CK domains under shear flow, ADAMTS13 unfolds and the spacer domain of ADAMTS13 becomes available for binding to the A2 domain of VWF, leading to the efficient cleavage of VWF. (**B**) However, deletion of the C-terminal domains of ADAMTS13 induce a conformational change, increasing the cleavage of the FRETS-VWF73 peptide substrate under static conditions. In contrast, the removal of the C-terminal TSP1 and CUB domains from ADAMTS13 decreases the binding of ADAMTS13 to VWF and also decreases its capacity to cleave VWF when subjected to shear flow. In this study, we show that the removal of the C-terminal domains of ADAMTS13 by the serine proteases FXIa and α-thrombin enhance the capacity of ADAMTS13 to cleave the FRETS peptide substrate under static conditions and abolished the ability to cleave VWF strings on endothelial cells under flow conditions.

Circulating plasma proteins play an important role in the delicate balance between hemostasis and thrombosis when damage to the endothelium occurs. Congenital deficiency of FXI, also known as Hemophilia C, typically causes mucosal bleeding symptoms or presents as increased bleeding in patients who have undergone a surgical procedure.[163] There is poor correlation between measured FXI plasma levels and bleeding symptoms. Interestingly, studies have observed an association between bleeding tendency in partial FXI deficiency and VWF plasma levels.[175,176] The only known mechanism of action for ADAMTS13 is regulating VWF length. Our results lead us to hypothesize an additional hemostatic mechanism of action for FXIa, with regards to regulation of ADAMTS13 activity. It has been shown that ADAMTS13 and FXI complexes are not found in plasma, suggesting that a circulating complex between these two proteins is unlikely to play a role in normal hemostasis or in the pathophysiology of TTP.[177] However, FIX, the main substrate for FXIa does not bind to zymogen FXI, either. FIX binds to an exosite on the apple 3 domain of FXIa, indicating that binding sites are unmasked by a conformational change when FXI is activated.[178] Along these lines, we recently found that FXIa is able to cleave TFPI despite the fact that the zymogen FXI is not able to bind to TFPI; rather, only the active form of FXI is able to bind to TFPI.[86]

Several studies have shown that elevated levels of FXI promote thrombosis, where patients are at increased risk for VTE and ischemic stroke.[107,108] Experimental thrombosis is reduced in mice lacking the contact pathway factors FXII, HK, PK, or FXI.[171] A recent clinical study demonstrated that reducing FXI levels served as an effective anticoagulation regimen for preventing postoperative VTE.[179] Thrombus formation can have morbid consequences when ADAMTS13 activity is inhibited by the presence of autoantibodies, as in TTP, or when ADAMTS13 is absent, as in congenital TTP.[151] Moreover, reduced activity of ADAMTS13 toward VWF has been

suggested to promote both ischemic stroke and myocardial infarction.[180,181] Herein, we show a possible procoagulant mechanism through which FXIa, like the other serine proteases, downregulates ADAMTS13 activity to promote platelet aggregation along persistent VWF strands. Specifically, we show that FXIa-mediated deletion of the ADAMTS13-CUB domains leads to inactivation of ADAMTS13 and resultant persistence of VWF-platelet strings under flow conditions. This interaction suggests a mechanism by which elevated levels of FXI may put patients at a higher risk for VTE events and provides a basis for directing targeted therapeutics in debilitating thrombotic diseases.

Chapter 5 Chronic edible dosing of Δ9-tetrahydrocannabinol (THC) in non-human primates reduces systemic platelet activity and function

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5.1 Abstract

Cannabis usage has steadily increased as acceptance is growing for both medical and recreational reasons. Medical cannabis is administered for treatment of chronic pain based on the premise that the endocannabinoid system signals desensitize pain sensor neurons and produce anti-inflammatory effects. The major psychoactive ingredient of cannabis is Δ9-tetrahydrocannabinol (THC) that signals mainly through cannabinoid receptor-1 (CBr), which is also present on non-neuron cells including blood platelets of the circulatory system. In vitro, CBr-mediated signaling has been shown to acutely inhibit platelet activation downstream of the platelet collagen receptor glycoprotein (GP)VI. The systemic effects of chronic THC administration on platelet activity and function remain unclear. This study investigates the effects of chronic THC administration on platelet function using a non-human primate (NHP) model. Our results show that female and male NHPs fed daily THC edible had reduced platelet adhesion, aggregation and granule secretion in response to select platelet agonists. Furthermore, a change in bioactive lipids (oxylipins) was observed in the female cohort. Indicating that chronic THC edible administration desensitized platelet activity and function in response to GPVI- and G-protein coupled receptor-based activation by interfering with primary and secondary feedback signaling pathways. These observations may have important clinical implications for patients who use medical marijuana and for providers caring for these patients.

This work is under review by *American journal of Cell physiology January* 2021;

5.2 Introduction

Studies in Chapter 3 and 4 have described how platelet coagulation interaction and regulation is important for platelet functional role in thrombus formation. Platelet activation is mediated by several pathways like ITAM mediated signaling and GPCR. While this is known and well defined the studies regarding the environmental and dietary changes and effects on platelets function is ill defined. In our study in Chapter 5, we studied THC, as it can modulate functions via GPCRs on neurological cells, for its effects on platelets. We will study the effects of activating the endocannabinoid system via orally administrating THC in non-human primates and how this affects platelet reactivity and function in Chapter 5. Here, we demonstrate that chronic THC exposure had desensitizing effect on platelet functions in adhesion, aggregation and granule secretion. Our observations may lead to more research knowledge and insights for clinical health policies regarding the use of medical marijuana and for health professionals caring for patients using marijuana in the future.

5.3 Background

Marijuana is the most commonly used federally illegal drug in the United States and worldwide with increasing popularity as both a recreational and medicinal drug.[182,183] The prevalence of use is on the rise with approximately 192 million people (3.9%) worldwide using marijuana in 2016, a 16% increase from 2006.[183] In the United States, there are approximately 22.2 million marijuana users each month.[184] This high prevalence is due in part to the recent legalization and decriminalization at the state level, which promotes the availability of marijuana and its perceived safety. Marijuana use can lead to the development of problem use and in severe

cases, it takes the form of addiction. Approximately a third of marijuana users have some degree of marijuana use disorder[185] and 4 million people in the United States met diagnostic criteria in 2015.[186] Of equal concern is that the content of delta-9-tetrahydrocannabinol, marijuana's main psychoactive component, has increased from less than 4% in the early 1990s to more than 15% in 2018 in confiscated marijuana samples.[183]

THC acts through the endocannabinoid system that is composed of endogenously produced cannabinoids, cannabinoid receptors and regulating enzymes. Both cannabinoid 1 (CB1) and CB2 receptors are G protein-coupled receptors (GPCRs),[187,188] and facilitate specific downstream signaling resulting in regulation in neurological processes and immune modulation.[189] Although marijuana has been used for the treatment of chronic pain, insomnia, nausea and vomiting, studies have also suggested that marijuana use is associated with cardiovascular system dysfunction including both thrombosis and comprised vascular integrity in select cases.[190] Blood platelets, which occupy essential physiological and pathological cardiovascular roles, have been shown to express CB1 and CB2 receptors on their cell membrane, suggesting a possible direct effect of THC on platelet function.[191,192]

A potential mechanistic role of cannabinoids (CBs) in regulating platelet function is still being explored. Earlier studies found that *in vitro* addition of the endocannabinoids 2-arachidonoylglycerol (2-AG) promoted platelet aggregation, demonstrating a prohemostatic or prothrombotic role for CB receptor signaling in platelet function, and concluded its role in prothrombotic events.[193] Yet, later studies showed a reduced platelet response to platelet glycoprotein (GP)VI immunoreceptor tyrosine-based activation motif (ITAM) stimulation under static and flow conditions, suggesting that CB receptor-dependent signaling would dampen essential platelet function.[194] Thus, it still remains to be seen how chronic exposure of marijuana

components affect systemic platelet function *in vivo*. Therefore, our aim was to study the effects of chronic controlled THC edible exposure on platelet function in non-human primates (NHPs). This study builds upon previous work by De Angelis et al.,[194] where acute endocannabinoid exposure as self-reported by people induced a reduction in platelet surface adhesion. In the current study, we demonstrate that the administration of chronic THC edibles in NHPs had desensitizing effects on platelet adhesion, aggregation and granule secretion functions. Together, our observations may have important implications for clinical health policies regarding the use of medical marijuana.

5.4 Methods

5.4.1 Reagents

Crosslinked collagen-related peptide (CRP-XL) was from R. Farndale (CambCol Laboratories, Cambridge University, UK). Fibrillar collagen from Chrono-Log Corporation (Havertown, PA, USA). Adenosine diphosphate (ADP) and bovine serum albumin (BSA) were obtained from Sigma Aldrich (St. Louis, MO, USA). Thrombin receptor activator peptide 6 (TRAP6; SFLLRN) was obtained from Tocris (Bristol, UK). Protease-activated receptor 4 agonist (GFPKF-NH_2) was obtained from Genescript (Piscataway, NJ, USA). Pam2CSK4 was from Invivogen (San Diego, CA, USA). Prothrombin time (PT) Dade® Innovin® obtained from Siemens Healthcare Diagnostics (Germany) and activated partial thromboplastin time (aPTT) reagent from Thermo Fisher Scientific (Middletown, VA, USA).

5.4.2 Antibodies

Flow cytometry antibodies CD62-FITC (Cat#: 550866) and PAC1-FITC (Cat#:340507) were from BD biosciences (San Diego, CA, USA).

5.4.3 Ethical statement

All animal procedures and experimental THC administration has been approved by the Oregon Health & Science University, Oregon National Primate Research Center Institutional Animal Care and Use Committee (IACUC) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for humane animal care were followed (IP0001389). The THC used in our study was supplied directly from the National Institute of Drug Abuse (NIDA) Drug Supply Program. Permission was obtained for all experimental protocols involving THC administration prior to the initiation of the study and the study was conducted in accordance with institutional and national guidelines.

5.4.4 Experimental design

A cohort of 10 (n=7 female, n=3 male) young, sexually mature Rhesus macaques (*Macaca mulatta)* were indoor-housed under controlled conditions. Animal ages ranged from 6.7 years to 12.7 years, with a mean age of approximately 10 years old. Animals were maintained on a standard chow diet (TestDiet, St. Louis, Missouri). To minimize potential confounders and inter-individual variability, each individual animal served as its own control during the evaluation of platelet function obtained at the different time points of THC induction.

5.4.5 THC induction and measurements

In addition to a standard chow diet, animals were given one cookie containing THC (THC edible) per day that were made using research-grade THC obtained directly from the National Institute of Drug Administration (NIDA). All cookies were administered in the morning prior to the animal's daily chow so that they were consumed on an empty stomach and to confirm they were completely ingested. Animals were slowly titrated up to 2.5 mg/7kg/day of THC over approximately a 3 month time period to model Colorado state's medical marijuana acclimation

recommendations for the female cohort. Male animals were titrated up to 2.5 mg/7kg/day of THC over approximately a 7 month time period. Animals were maintained on a dose for 21 days for females and 70 days for males prior to THC dosage increase. Dosing regimen of the animal cohorts were based on the menstrual cycle for female animals and the renewal rate of semen production for the male animals. The THC dosage was calculated from the recommended THC starting dose of 5mg (standard research unit of THC per NIDA) for a 68 kg adult (the average rhesus macaque weighs approximately 6-7.5 kg), followed by titration to 10mg for moderate users (standardized serving size for edible retail marijuana products in Colorado), and 20-30 mg for heavy users, as described earlier.[188] Serum was drawn at each dose adjustment time point during THC induction, 3 hours following edible consumption, to determine THC concentrations with each increase in dosage and analyzed as described before.[188]

5.4.6 Blood collection

Blood (~12ml) was collected from female and male Rhesus macaques by venipuncture and anticoagulated with 3.2% sodium citrate. To obtain platelet rich plasma (PRP), whole blood was centrifuged for 8 min at 1000 rpm. Platelet poor plasma (PPP) was obtained and by spinning down remaining red blood cells at 10,000 rpm for 3min.

5.4.7 Platelet aggregation under flow

Channels of Ibidi µ-slide VI0.1 chamber were coated with 100 µg/ml of fibrillar type I collagen (Chrono-Log Corp, Havertown, PA, USA) for 1 hour at room temperature (RT). Channels were washed with Hepes Tyrodes buffer and then blocked with 5 mg/ml denatured bovine serum albumin for 1 hour at RT before connecting the outflow ports of the chamber to a syringe pump. PRP from non-human primates were perfused into the chambers at a shear rate of 300 s^{-1} for 5 minutes. Channels were fixed with 4% paraformaldehyde (PFA) and washed with HT post

perfusion. Three random fields were imaged for each channel at 40× magnification using a Zeiss Axiovert 200M microscope and SlideBook 6 software. Surface area and number of platelet aggregates on each image were measured using ImageJ software as previously described.[195]

5.4.8 Platelet aggregation and thromboxane generation

Platelet rich plasma (PRP; 300 µl per sample) were pre-incubated in glass cuvettes and warmed to 37°C. Platelet aggregation under stirring conditions was initiated by CRP-XL (1µg/ml) or ADP (3µM), and changes in light transmission were monitored for 5 minutes using a PAP-4 aggregometer (Chrono-Log Corporation). After 5 min, solutions were removed from cuvettes and cleared by centrifugation prior to analysis for thromboxane B2 (TXB2) content by ELISA assay (Enzo life sciences). Fold change of platelet aggregation relative to baseline control and measured TXB2 concentrations were analyzed for statistical significance by one-way ANOVA testing for multiple comparisons using GraphPad PRISM 8 software.

5.4.9 Platelet flow cytometry

PRP was diluted 1:4 in Hepes-Tyrode's buffer prior to stimulation with the agonists CRP-XL (1 µg/ml), TRAP-6 (30µM) and GFPKF-NH_2 (200 µM), ADP (30 µM) and Pam-2CSK4 (10 µg/ml) in the presence of either anti-CD62P-FITC antibody or human PAC-1-FITC antibody for 20min at 37ºC. Reactions were stopped by adding 2% PFA to samples and platelet activation was determined by flow cytometry analysis using Canto II machine. Platelet activation upon agonist stimulation was analyzed relative to unstimulated platelets and compared to baseline control for statistical significance.

5.4.10 Clotting times

Clotting times of PPP (33% final volume) were measured with a KC4 Coagulation Analyzer at 37°C for 3 minutes with activated partial thromboplastin time (aPTT) reagent. Clotting was initiated with addition of 25 mM $CaCl_2$ and clotting time was recorded. For prothrombin time (PT), clotting was initiated with the addition of Dade® Innovin® reagent to 50% PPP.

5.4.11 Sample preparation for oxylipin analysis

Oxylipins were extracted from PPP using the approach described by Pedersen et al.[196] with minor modifications by García-Jaramillo et al.[197] In short, 100 µl PPP was transferred to 2 ml polypropylene tubes. Cold LC–MS-grade methanol (35 µl) and an anti-oxidant solution (0.2 mg/ml solution BHT (butylated hydroxytoluene) in 1:1 methanol:water) (5 µl) was added to each sample. Each sample also received 10 µl of a deuterated oxylipin recovery standard solution; the standards included 22 deuterated oxylipins in methanol at a concentration of 5 ng/µl. PPP were transferred to a 96-well Ostro Pass Through Sample Preparation Plate (Waters Corp, Milford, MA, USA) and eluted into glass inserts containing 10 µl 20% glycerol in methanol by applying a vacuum for 10 min. Eluents were dried by vacuum centrifugation in a Labconco centrivap vacuum concentrator for 2 hours at RT. Once dry, samples were reconstituted with 100 µl of methanol: acetonitrile (50:50), containing the internal standard (CUDA at 50 ng/ml). Samples were transferred to a spin filter (0.22 µm PVDF membrane, Millipore-Sigma, Burlington, MA, USA) and centrifuged (3 min at 6°C at 9000 rpm) before transferred to 2 ml amber LC–MS vials. Extracts were analyzed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS). The internal oxylipin standards added to the samples were used to correct the recovery of the quantified oxylipins.

5.4.12 LC-MS/MS analysis

High Performance Liquid Chromatography (HPLC) was performed using a Shimadzu system (Shimadzu, Columbia, MD, USA) coupled to a QTRAP 4000 (AB SCIEX, Framingham, MA, USA). Chromatographic separation of lipids was achieved on a Waters Acquity UPLC CSH C18 column (100 mm length × 2.1 mm id; 1.7 µm particle size) with an additional Waters Acquity VanGuard CSH C18 pre-column (5 mm × 2.1 mm id; 1.7 µm particle size) held at 60°C. The mobile phase consisted of (A) water containing 0.1% acetic acid, and (B) acetonitrile/isopropanol (ACN/IPA) (90/10, *v*/*v*) containing 0.1% acetic acid. Gradient elution conditions were: 0–1.0 min, 25-40% B; 1.0–2.5 min, 40–42% B; 2.5–4.5 min, 42–50% B; 4.5–10.5 min, 50–65% B; 10.5–12.5 min, 65–75% B; 12.5–14 min, 75–85% B; 14–14.5 min, 85–95% B; 14.5–21 min, 95–95% B; 21–22.5 min, 95–25% B; 22.5–27 min, 25–25% B. A 5-µl aliquot of each sample was injected onto the column. The column effluent was introduced via an electrospray ion source. The flow rate was 0.15 ml/min. All samples were kept 10^{o}C throughout the analysis. The MS/MS was performed on an Applied Biosystems 4000 QTRAP hybrid linear ion trap-triple quadrupole instrument (AB Sciex, Concord, ON, Canada) operated at a source temperature of 525^{o}C with a needle voltage of -4500 kV. The operation parameters of the MS/MS detector was CAD -2 psi, CUR 30 psi, GS1 40 psi, GS2 40 psi, CXP -15 V, and EP -10 V. Nitrogen was used as the source gas, curtain gas, and collision gas. Dynamic multiple reaction monitoring (dMRM) experiments were conducted at collision energies ranging from -15 to -50 eV (supplemental Table 2). Concentrations were calculated using the internal calibration method and MultiQuant (v. 3.0.2, Sciex) software.

5.4.13 Statistical analysis

Data are presented as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used to compare between baseline and dosage groups.

Kruskal-Wallis with Dunn *post hoc* test was used to compare between groups when the data did not qualify for parametric statistics. $p < 0.05$ was considered significant. All statistical analyses were conducted using GraphPad Prism 9.

5.5 Results

5.5.1 Chronic edible administration of THC to non-human primates reduces platelet adhesion and aggregate formation under flow.

Endocannabinoid receptor agonists have been shown to reduce *ex vivo* platelet activation and aggregate formation on collagen under flow.[194] We therefore first sought to examine whether chronic THC edible administration in non-human primates (NHPs) affected *ex vivo* platelet adhesion on collagen under flow. As shown in Figure 5.1A, our model consisted of two cohorts: 7 female NHPs fed a daily THC edible for a total of 84 days, and 3 male NHPs fed a daily THC edible for a total of 210 days. In the female cohort, the THC dose was increased every 21 days until a final concentration of 2.5 mg/7kg/day was achieved, while in the male cohort, the THC dose was increased every 70 days until a final concentration of 2.5 mg/7kg/day was achieved. An increasing concentration of plasma THC levels and corresponding metabolites were observed in both cohorts as a function of dose (Figure 5.1A). Complete blood counts including platelets remained largely constant for both cohorts (Table 1 & 2), although a slight increase was observed for neutrophils in the female cohort, perhaps consistent with prior observations related to the presence of CB2 on neutrophils.

Table 1 Female NHP complete blood count. Mean ±SEM

THC (mg/7kg/day)	0	0.5	1	2	2.5
WBC (k/μL)	4.35 (±1.0)	4.76 (±1.79)	5.33 (±1.22)	4.81 (±1.05)	5.49 (±1.14)
Neutrophils (k/μL)	0.38 (±0.52)	1.43 (±0.53)	1.84 (±0.86)	1.82 (±0.81)	2.37 (±0.91)
Hb (g/dL)	8.90 (±0.60)	9.53 (±0.66)	9.97 (±0.73)	10.43(±1.17)	10.64 (±0.76)
HCT (%)	28.21 (±3.03)	31.20 (±2.73)	32.09 (±1.32)	31.50 (±2.80)	30.74 (±1.41)
RBC (M/μL)	4.21 (±0.44)	4.56 (±0.41)	4.71 (± 0.27)	4.66 (±0.48)	4.54 (±0.30)
Lymphocyte (k/μL)	10.46 (±1.41)	11.73 (±1.05)	12.31 (±0.51)	12.14 (±0.60)	12.31 (±0.32)
Monocyte (k/μL)	0.17 (±0.13)	0.28 (±0.18)	0.26 (±0.13)	0.25 (±0.11)	0.38 (±0.26)
Eosinophils (k/μL)	0.12 (±0.11)	0.33 (±0.16)	0.31 (±0.18)	0.32 (±0.13)	0.40 (±0.22)
PLT (k/μL)	374.3 (±38.2)	304.9 (±55.2)	308.3 (±50.4)	344.6 (±36.6)	318.4 (±76.2)
MCV (fl)	67.11 (±2.56)	68.19 (±2.00)	69.74 (±3.03)	67.81 (±3.34)	66.31 (±3.04)
MCH (pg)	21.36 (±2.18)	21.67 (±1.95)	21.40 (±1.26)	22.40 (±1.32)	23.17 (±1.97)
MCHC (g/dl)	31.79 (±2.92)	31.81 (±2.94)	30.73 (±1.63)	33.10 (±2.26)	34.86 (±1.72)
RDW (%)	13.74 (±0.43)	13.44 (±0.53)	13.31 (±0.63)	13.31 (±0.74)	13.30 (±0.74)
MPV (fl)	8.87 (±1.80)	8.97 (±1.29)	9.43 (±2.42)	8.40 (±1.79)	8.87 (±2.70)

Table 2 Male NHP complete blood count. Mean ±SEM

THC (mg/7kg/day)	0	0.5	1	2.5
WBC (k/μL)	7.66 (±3.10)	5.58 (±2.51)	6.51 (±2.25)	7.14 (±1.82)
Neutrophils (k/μL)	4.37 (±2.63)	2.67 (±1.70)	2.54 (±1.37)	3.16 (±1.39)
Hb (g/dL)	11.70 (±0.64)	10.37 (±0.34)	11.63 (±0.42)	9.83 (±0.26)
HCT (%)	37.43 (±1.04)	34.53 (±1.70)	40.63 (±1.22)	41.77 (±0.21)
RBC (M/μL)	5.16 (±0.20)	4.78 (±0.22)	5.19 (±0.14)	5.61 (±0.11)
Lymphocyte (k/μL)	2.54 (±0.30)	2.33 (±0.56)	3.17 (±0.56)	2.51 (±0.27)
Monocyte (k/μL)	0.31 (±0.14)	0.25 (±0.13)	0.35 (±0.18)	0.68 (±0.30)
Eosinophils (k/μL)	0.42 (±0.24)	0.33 (±0.15)	0.44 (±0.16)	0.76 (±0.21)
PLT (k/μL)	404.33 (±31)	339.33 (±45)	381.33 (±24)	330.67 (±43)
MCV (fl)	72.63 (±3.07)	72.23 (±1.15)	78.27 (±0.31)	74.40 (±1.19)
MCH (pg)	22.73 (±1.99)	21.73 (±0.41)	22.40 (±0.22)	17.53 (±0.65)
MCHC (g/dl)	31.27 (±1.55)	30.03 (±0.48)	28.60 (±0.16)	23.57 (±0.74)
RDW (%)	13.07 (±0.50)	13.27 (±0.39)	13.23 (±0.33)	13.77 (±0.56)
MPV (fl)	6.87 (±0.95)	7.20 (±1.36)	7.40 (±0.62)	9.03 (±2.37)

Blood drawn at select time points was perfused over a collagen-coated surface and assessed for platelet adhesion and aggregate formation (Figure 5.1B). Consistent with the studies using endocannabinoid receptor agonists,[194] we show that chronic edible administration of THC

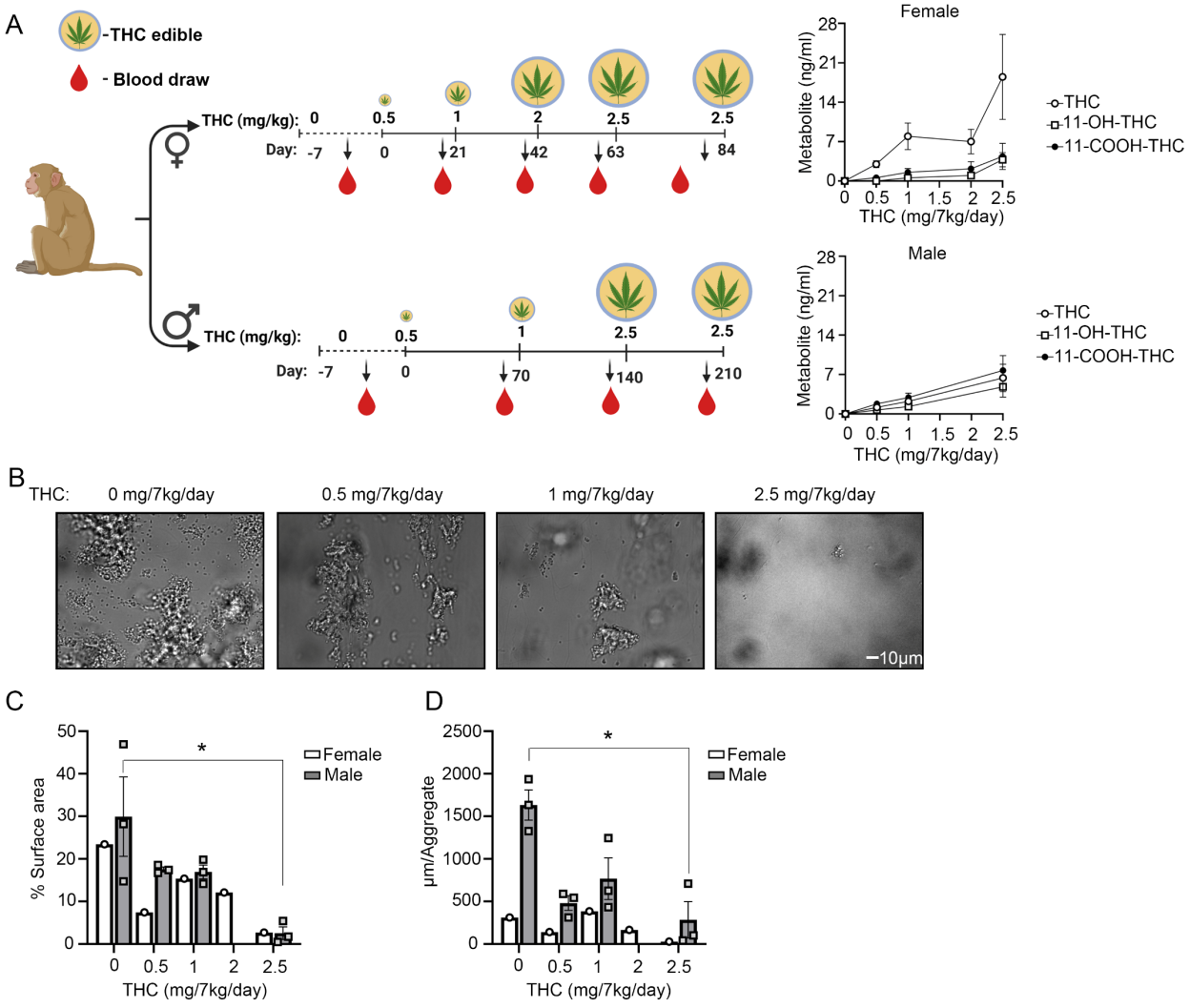

Figure 5.1 Assessment of platelet adhesion on a collagen surface under flow after chronic THC edible administration. (A) Timeline of THC edible administration in females (n=7) and males (n=3) and measured plasma THC levels. (B) Platelet rich plasma (PRP) from n=3 male animals were perfused at 300s-1 over collagen coated surface for 5min prior to fixating. (C) Representative images were taken and quantified for platelet surface coverage per time point and (D) aggregate size on collagen surface for female (n=1) and male (n=3). Data is mean±SEM. Data were analyzed using ANOVA with repeated measures and Kruskal-Wallis with Dunn post hoc test. *p < .05 vs. baseline. Figure made with biorender.com

significantly reduced platelet surface area coverage and aggregate size on collagen under physiologically relevant levels of shear flow (Figure 5.1C&D). This data suggests that chronic THC edibles may impair select platelet functions including thrombus formation under flow.

5.5.2 Chronic edible administration of THC to non-human primates reduces agonist-induced platelet aggregation and thromboxane production.

Next, we performed studies to determine the effects of chronic THC administration on platelet functions including aggregation and granule secretion in solution. Our data demonstrates robust aggregation of NHP platelets in response to the GPVI-agonist CRP-XL prior to chronic THC administration (Fig. 5.2A, baseline). A dose-dependent inhibition of aggregation to CRP-XL was observed following chronic THC administration, as quantified by fold change in aggregation per animal for both female (at 2.5 mg/kg/day dose) and male (at both 1 and 2.5 mg/7kg/day doses) cohorts (Figure 5.2B). Aligned with the fact that GPVI-mediated platelet activation is largely dependent on granule secretion of the secondary mediators including thromboxane and ADP, we found that chronic THC administration reduced GPVI-mediated thromboxane secretion in the female cohort (Figure 5.2C). Moreover, a direct effect on ADP-mediated platelet activation was observed as a dose-dependent inhibition of ADP-induced platelet aggregation for both female (at both 2 and 2.5 mg/7kg/day doses) and male (at 2.5 mg/7kg/day dose) cohorts following THC administration (Figure 5.2D-E).

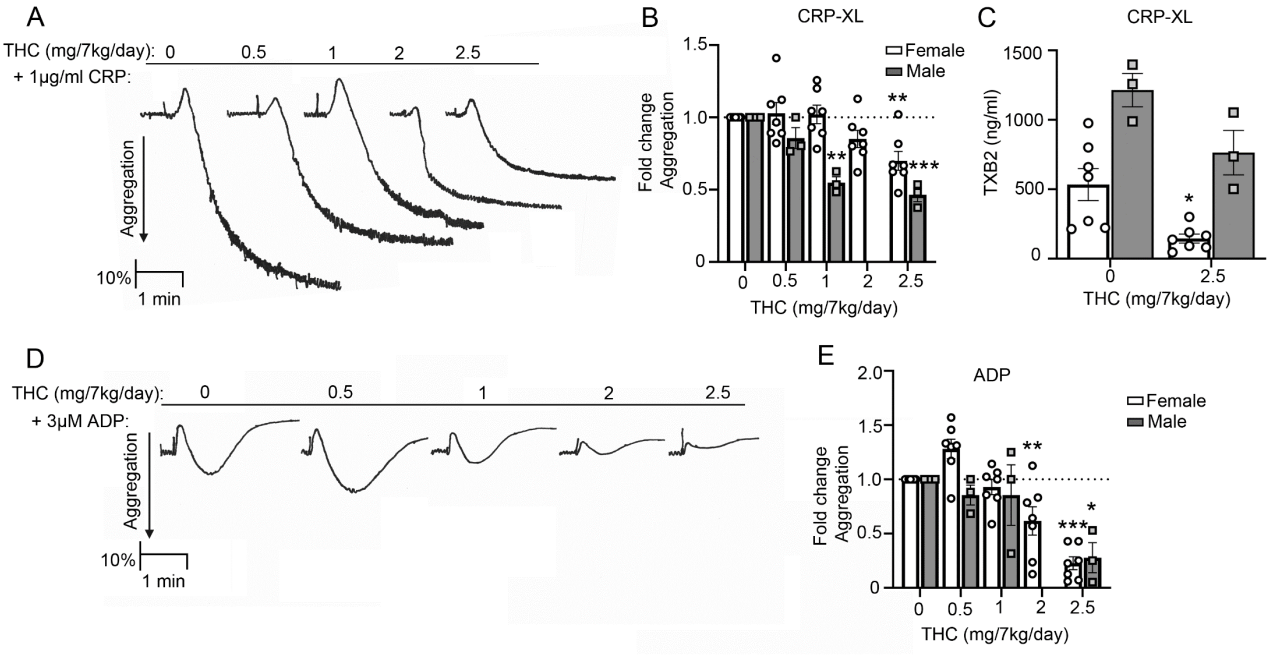

Figure 5.2 THC edibles effect on platelet aggregation and thromboxane production after CRP-XL and ADP stimulation. (A) Representative PRP aggregation traces over time from 1 animal after CRP-XL stimulation. (B) Quantification of aggregation fold change compared to baseline for 1µg/ml CRP-XL for 7 females and 3 males. (C) Thromboxane production measured by ELISA for 1µg/ml CRP-XL. (D) Representative aggregation traces from 1 animal after ADP stimulation. (E) Quantification of fold change aggregation in response to 3 µM ADP for 7 females and 3 males. Data is mean±SEM with every animal representing 1 data point. Data were analyzed using ANOVA with repeated measures and Kruskal-Wallis with Dunn post hoc test. *p < .05; **p <.001; ***p < .0001 vs. baseline.

5.5.3 Chronic edible administration of THC to non-human primates reduces agonist-induced platelet α-granule secretion.

Platelet granule secretion and integrin activation are hallmarks of platelet activation and are essential hemostatic functions. In light of the inhibitory effects observed for THC on platelet aggregation, we next sought to determine if THC edibles were able to inhibit platelet α-granule secretion as measured by P-selectin expression in response to select platelet agonists. As shown in Figure 5.3A-D, we found that platelet P-selectin expression in response to the GPVI-agonist CRP-XL was significantly reduced at the highest THC dose for both the female and male cohorts (Figure 5.3A). A similar trend in reduced P-selectin expression in the platelets from

THC-treated animals was observed for the P2Y12/P2Y1 agonist ADP (Figure 3B), while a reduced P-selectin expression in response to the PAR-agonists TRAP6 and GFPKF-NH_2 was only observed for the female cohort (Figure 5.3C). Platelet P-selectin expression in response to the TLR2/6 ligand Pam2CSK4 was insensitive to chronic administration of THC (Figure 5.3D). Similarly, chronic edible administration of THC did not affect agonist-induced activation of the platelet integrin αIIbßIII receptor under the conditions tested herein as measured by PAC-1 binding (Figure 5.3E-H). Taken together, these results suggest that chronic edible administration of THC inhibits platelet α-granule secretion to select agonists that signal via either the ITAM or GPCR-signaling pathways.

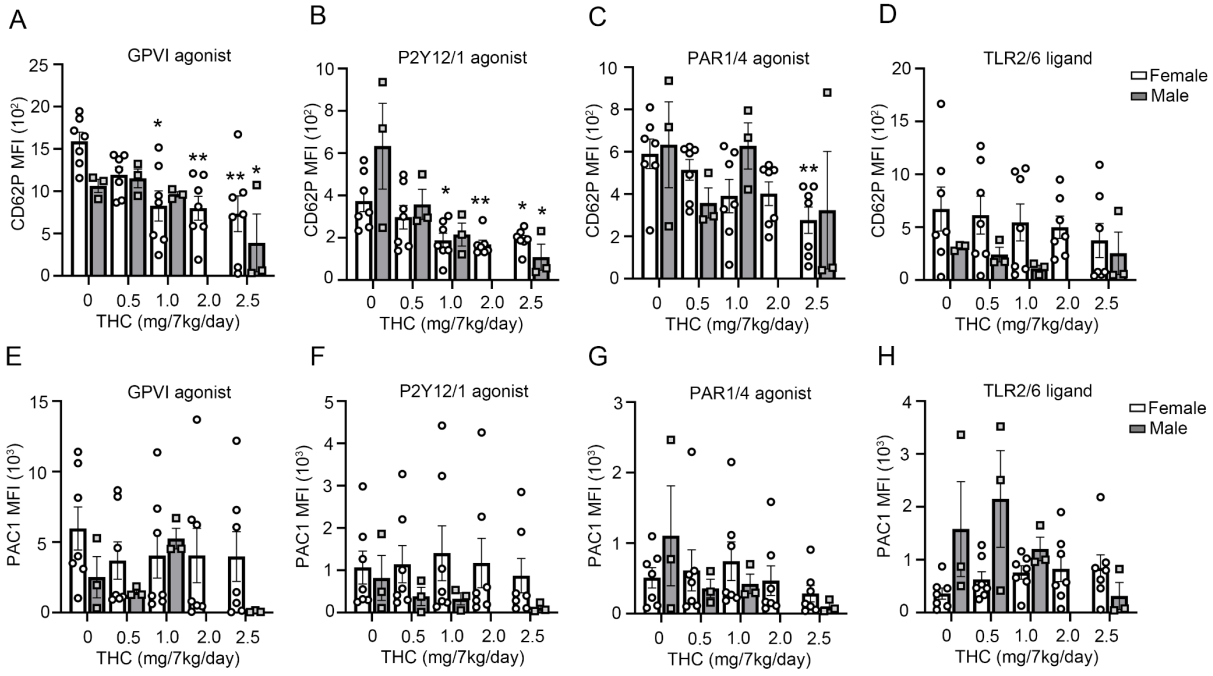

Figure 5.3 THC edibles effect on platelet α-granule secretion and integrin receptor activation measured by flow cytometry. PRP was analyzed by flow cytometry for P-selectin (CD62P) expression after stimulation for 20min with the (A) GPVI receptor agonists CRP-XL, (B) P2Y12/Y1 agonist ADP, (C) PAR1 agonist TRAP-6 and PAR4 agonist GFPKF-NH2 and (D) TLR2/6 ligand Pam-2. PRP was analyzed by flow cytometry for integrin activation (PAC1) after stimulation for 20min with the agonists (E) CRP-XL, (F) P2Y12/Y1 agonist ADP, (G) PAR1 agonist TRAP-6 and PAR4 agonist GFPKF-NH2 and (H) TLR2/6 ligand Pam-2CSK4. Data is mean±SEM for female (n=7) and male (n=3) representing each data point per animal per time point. Data were analyzed using ANOVA with repeated measures and Kruskal-Wallis with Dunn post hoc test. *p < .05; **p <.001 vs. baseline.

5.5.4 Effect of chronic THC edibles on plasma clotting times.

As platelet activity and activation of the coagulation cascade are both requisite for normal hemostasis, we next assessed whether THC edibles affected plasma clotting times. We therefore measured the clotting times of platelet poor plasma (PPP) from the female or male cohorts initiated by either lipidated tissue factor (Innovin®) or aPTT reagent. We did not observe any differences in either clotting times for animals on chronic edible THC (Figure 5.4).

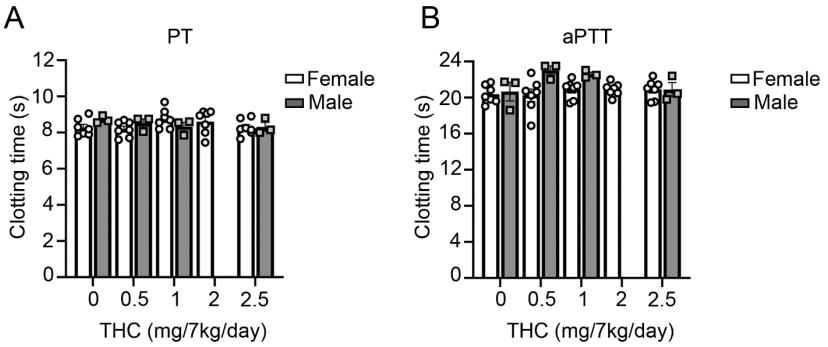

Figure 5.4 THC edibles effect on plasma clotting times. (A) Platelet poor plasma (PPP) was stimulated by TF Innovin® to measure prothrombin time (PT). (B) Clotting times were measured by the addition of aPTT reagent for 3min at 37°C and clotting was initiated with CaCl2 and clotting time was recorded. Data is mean±SEM for female (n=7) and male (n=3) representing each data point per animal per time point.

5.5.5 Effect of chronic THC edibles on oxylipin levels in plasma.

Endocannabinoids are derived from polyunsaturated fatty acids (PUFAs), including arachidonic acid (ARA), and these lipid mediators can signal through CB receptors.[198]

Another class related to endocannabinoids are oxylipins that are derived also from PUFAs, including ARA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These unique lipid mediators are generated by the three major oxygenases, cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP450).[9] Platelet activation is associated with significant changes of membrane lipids and formation of oxylipins, which are potent bioactive lipid mediators amplifying platelet activation in a paracrine and autocrine manner. To study more

broadly the potential effects of chronic THC edible dosing on the blood environment, we analyzed the plasma from the female cohort for metabolic changes by mass spectrometry. Employing dynamic multiple reaction monitoring (dMRM), we evaluated 66 oxylipins, 22 deuterated oxylipins, CUDA, and the deuterated surrogates eicosapentaenoic acid-d5 (EPA-d5), docosahexaenoic acid-d5 (DHA-d5), linolenic acid-d5 (ALA-d5), linoleic acid-d4 (LA-d4) and arachidonic acid-d8 (ARA-d8) in a 27 min LC-run in a targeted approach (Appendix I Table 3). Twenty-nine oxylipins were detected. The detailed list of MRM transitions can be found in the Appendix I, Table 4. Our study focused on quantifying 29 detectable oxylipin metabolites found in plasma (Figure 5A) and Z scores were determined as shown in the heatmap. The major precursors for oxylipin generation, DHA, ARA and EPA showed reduced levels with increased THC concentrations (Figure 5B). In particular, the administration of THC edibles showed an increased trend of 12(S)-HETE, 9(R)-HETE, 18-HEPE and 8-HODE (Figure 5C). These results give an insight in plasma changes of oxylipin levels in response to chronic THC concentrations *in vivo*.

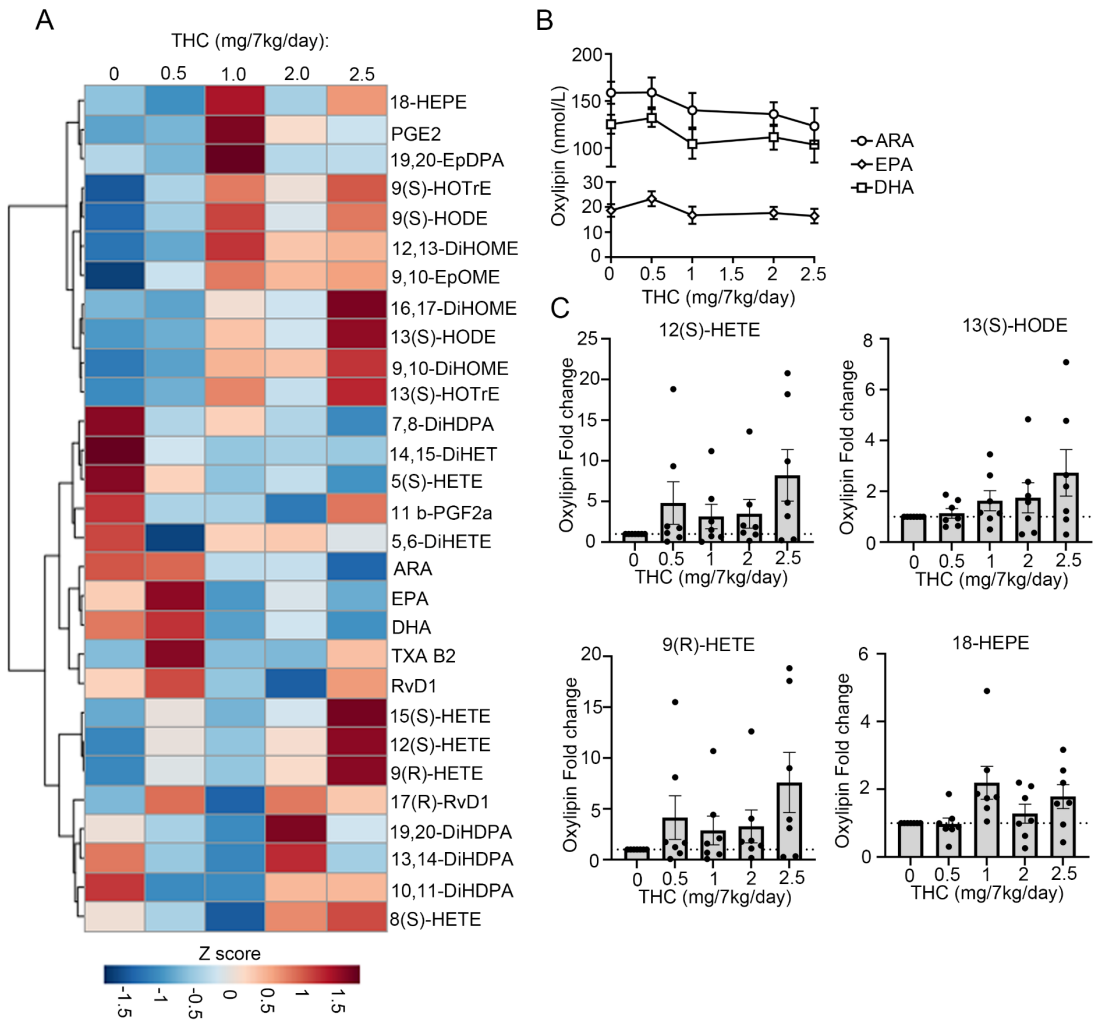

Figure 5.5 THC edibles effect on oxylipin metabolome in plasma from female animals. (A) Platelet poor plasma (PPP) was obtained from n=7 females, and lipid mediators were extracted, identified and quantified using LC-MS/MS based lipid profiling. (B) Oxylipin concentrations from the precursors arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). (C) Fold change of the oxylipins 18-HEPE, 12S-HETE, 13S-HODE and 9R-HETE. Data are mean±SEM.

5.6 Discussion

Medical cannabis is administered for chronic pain treatment based on the premise that the endocannabinoid system both signals and desensitizes pain sensor neurons and has anti-inflammatory effects.[188] The chronic effects of marijuana have not been extensively studied and the effects on physiology remains unclear. Our study used controlled administration of chronic THC edibles in both a female and male NHP cohort to study the effects of chronic THC edibles on platelet function. Our results show a reduction of platelet adhesion on collagen under flow. Also, we observed significant reduction in platelet aggregation in response to the GPVI-agonist CRP-XL in combination with a reduction in platelet thromboxane production. Platelet aggregation in response to the P2Y1/Y12 receptor, ADP, was completely abolished in both cohorts with the highest dose of THC edible administration. Similar effects were observed in a reduction in platelet P-selectin expression. Additionally, chronic THC administration resulted in changes in oxylipin production in plasma. The underlying mechanism of desensitized platelet activation states associated with chronic THC edible administration is potentially by interference of the secondary feedback loop of platelet activation.

The policy changes within individual states and provinces in the United States and Canada have led to significant increases in the use of recreational and medical marijuana.[199] Associated with this overall increase, is a trend of substance abuse greater in males and an increase in medical marijuana use largely in females.[200] Several animal model studies suggest that females may be more sensitive than males to the reinforcing and discriminative effects of cannabinoids.[200] Differences between sexes have also been observed in the metabolic processing of THC or the levels of endocannabinoids in the brain and periphery.[201] Consideration of the metabolism of THC is also important in this context and is often not measured in most analyses despite the fact that the metabolites of THC are highly bioactive, influenced by type of administration and

impacted by sex, as females metabolize THC at a faster rate than males.[202] Differences in cannabinoid sensitivity could occur from the direct influence of sex chromosomes that control cannabinoid receptor expression, as receptor density and efficacy for GPCR activation differences were observed in the brains of both male and female rats.[203] Female rodents were also more sensitive to the effects of THC,[204] with a potential role for the estrous cycle on THC sensitivity as fluctuations across the estrous cycle changes THC sensitivity.[188,205] In our study we studied the effects of chronic THC edibles in both a female and a male NHP cohort. Both cohorts showed a significant reduction in platelet activation effect. However, we observed differences in THC metabolites. THC is metabolized mainly by the liver, resulting in the 2 main metabolites 11-OH-THC and 11-COOH-THC. In our female cohort, the plasma THC levels were higher compared to the males. However, the males had increased levels of THC metabolites. Our study utilizes sex different cohorts based on select timelines, 3 months for females versus 7months for males. This therefore limited our ability to directly compare the results from the female and male cohorts; this was further compounded by a low number of males available for this study. Still, interestingly, while the males were on a longer timeline of THC administration, the impairment of their platelet function was somewhat diminished as compared to the female cohort, perhaps as a result from this differing THC metabolism between males and females. These differences could also be reflective of inherent sex-differences in platelet function. Future studies are required to elucidate the chronic effects of cannabis use and sex differences on platelet and immune cell biological functions.

The endocannabinoid system is known to be expressed by the central nervous system, wherein THC signals through CB1 in neuronal and non-neuronal cells alike.[46,187] THC can act as an agonist and antagonist at CB receptors depending on receptor density.[188] Studies have shown that CB2 signaling in cells drives the secretion of interleukin (IL)6 and IL10.[206] However, in our

study we did not see any detectable changes in IL6 or IL10 expression in serum in either of our female and male cohort at baseline or at highest dose of THC administered (data not shown). Platelets have been reported to express cannabinoid receptors;[192] however, some studies contradict these findings and failed to detect either CB1 or CB2 proteins or their mRNAs in platelets.[207,208] An additional hypothesis could be that platelet function is effected by the uptake and metabolization of the endocannabinoid components and that this can lead to changes in platelet functional effects.

Platelet function is mainly driven by the receptor-mediated signaling leading to secondary feedback activation via secretion of ADP and production of thromboxane by the platelet membrane. Oxylipins are known to be important bioactive lipids that regulate platelet activation and function.[9] We observed THC-dependent changes in plasma lipid metabolites in our female cohort. An important change was observed in 12(S)-HETE, 9(R)-HETE, 18-HEPE, 13(S)-HODE, 9(S)-HOTrE and 13(S)-HOTrE. Studies have demonstrated a role for THC in affecting arachidonic acid metabolism and changes in oxylipin secretion by cells *in vitro*.[209,210] Oxylipin metabolites are involved in modulating immune responses such as 15(S)-HETE inhibiting polymorphonuclear leukocytes, and increasing levels of 18-HEPE and 13(S)-HOTrE has been shown to exhibit anti-inflammatory effects in modulating macrophage function.[211-213] Moreover, 11β-prostaglandin $F_{2\alpha}$ (11β-$PGF_{2\alpha}$) is the primary metabolite of PGD_2 and has pro- and anti-coagulant effects.[214] Further, THC has also been proposed to be a potential COX-2 inhibitor;[215] this might have a resemblance to aspirin treatment, where a reduction in oxylipin profile in platelets was observed in a patient cohort on aspirin.[216] Levels of 13(S)-HODE limits platelet aggregation and endothelial cell interaction.[217] The production of 12S-HETE by platelets can both exhibit platelet activation and platelet aggregate inhibition depending on agonists used and concentrations *in vitro*.[9,218] Patients who have a low platelet 12-HETE production are at an

increased risk to present with bleeding;[219] this may be a predictive outcome of chronic THC use based on our study in which we observed increased levels of 12(S)-HETE in plasma in addition to diminished agonist-induced platelet activation. We demonstrate for the first-time changes in oxylipin metabolite profile in the presence of chronic THC administration. The observed changes in oxylipin profile might be related to our observed reduced platelet functions (Figure 5.6). However, further studies need to be conducted in order to locate the source of oxylipin production in the presence of chronic THC administration.

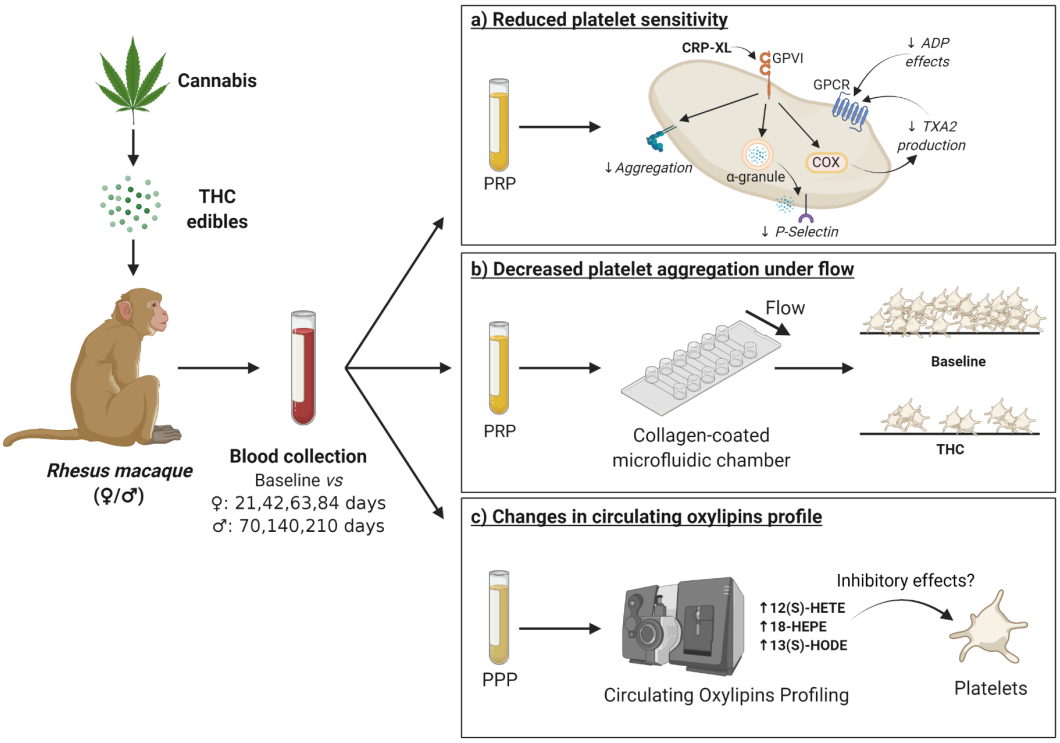

Figure 5.6 Schematic representation of THC edibles effects on platelet function. Schematic representation of THC edibles effects on platelet function. Administration of THC edible dosing in a female and male cohort was studied by collecting blood samples and analyzed for platelet function. In panel A the systemic THC exposure resulted in a reduction of platelet sensitivity, which can be seen as a reduction in CRP-XL and ADP induced platelet aggregation, a reduction in integrin receptor activation and thromboxane production, as well as a reduction in P-selectin exposure from the alpha granules. In panel B, we observed a reduction in platelet aggregate formation under shear flow on a collagen coated surface with THC edible administration. In panel C we observed changes in plasma oxylipin levels between baseline and THC edible administration that may have inhibitory effects on platelets. Figure created with BioRender.com

The clinical implications of chronic cannabis use in the population has not only effects on behavioral sciences but also impacts the cardiovascular system.[190] Studies have shown that endocannabinoids such as anandamide and 2-arachidonoylglycerol (2-AG), were able to promote platelet aggregation.[220,221] Yet, anandamide was also shown to inhibit platelet activation by the collagen receptor GPVI under static and flow conditions.[194] The early studies were limited by reliance on the sole use of synthetic agonists, whereas the later work included a pilot study of self-reported cannabis users without control of dosage. Platelets also have additional functions beyond maintaining vessel integrity, such as in wound healing and angiogenesis. As cannabis is the most commonly used illicit drug in pregnancy,[222] this is concerning because during pregnancy and placental development, platelets play an important role in providing necessary growth factors and support for placental angiogenesis to occur.[223] The underlying mechanism for this is not well understood, but these findings suggests the importance of taking a thorough patient drug history for those undergoing surgery or are pregnant. As the effect of THC use on platelets may impair its role in normal placental development, it is important that healthcare providers appropriately counsel women that use marijuana who are planning to conceive or pregnant. In conclusion, our study expands our current knowledge regarding the effects of cannabinoids on platelet reactivity by studying the direct effects of chronic THC use in both female and males NHPs. Our observations may have important implications for clinical health policies regarding the use of medical marijuana in the future.

Chapter 6 Conclusions and Future Directions

6.1 Conclusions

The prevention of bleeding and protection of the vascular wall integrity is mediated by the blood cells platelets and enzymatic coagulation reactions in a process called hemostasis. The studies outlined in this thesis give an insight in the importance of the complex interplay of platelets and coagulation factors. The gained mechanistic knowledge of the roles they play in regulating both pathways will improve therapeutic approaches to treat thrombotic diseases. As the intrinsic pathway of coagulation is considered a promising target for anti-coagulant drug therapy for its minor role in hemostasis but contributes to pathological thrombus formation. We aimed to study the mechanistic processes of the intrinsic pathway and the interaction with platelets.

In our first study, we elucidated the importance of contact system interaction with the platelet surface, which is still ill understood. With our results we determined that platelets activation states are important for the interaction with the intrinsic pathway of coagulation. This interaction was favorable in the protection of FXIa activity and localize the amplification reaction to the local platelet clot formation.

In our second study, we observed a novel substrate for intrinsic pathway member FXIa that indirectly was able to regulate platelet-VWF interaction on an endothelial cell surface. We have demonstrated a novel mechanism for coagulation proteinases including FXIa in regulating ADAMTS13 activity and function on cleaving VWF ultra long strings. These results may represent an additional hemostatic function by which FXIa promotes local platelet deposition at sites of vessel injury.

In the final study we examined the chronic and systemic dietary effects of cannabis main psychoactive ingredient, THC, on platelet function. The daily administration of THC edibles in a non-human primate study had significant effects on platelet sensitivity. We observed a reduction in platelet activation states to select GPVI and GPCR agonists as well as a reduced ability to form platelet aggregates under shear flow. Our study expands the current knowledge regarding the effects of cannabinoids on platelet reactivity by studying the direct effects of chronic THC. Our observations in this study may have important implications for clinical health policies regarding the use of medical marijuana in the future.

Overall, these studies provide new insights in platelet function and will help support new strategies to regulate coagulation by altering platelet signaling pathways. Future studies, as described in Chapter 6, will define downstream signaling events of thrombin mediated platelet GPCR activation to identify novel targets for drug interference in platelet functionality that may help to predict hematological outcomes of clinical treatments.

6.2 Future directions

Antiplatelet therapies have been successful in reducing mortality rates in CVD. However, current limitations of antiplatelet therapies can include a slow or weak inhibition of platelet function, as well as increasing bleeding side effects.[5] Antiplatelet therapies are partially focused on platelet PAR receptors. However, most signaling pathways downstream of PARs still remain unknown and there is still an unmet clinical need for new anti- thrombotic therapies that are effective but uncoupled from the bleeding risks. With the development of improved and new techniques, it is possible to study more broadly the phosphorylation sites that drive platelet activation and functionality in hemostatic and thrombotic processes, and to identify potential new anti-platelet drug targets.

6.2.1 Platelet PAR signaling implications for platelet phenotype

Platelet activation is essential for optimal thrombus formation in hemostatic processes as well under pathological conditions like thrombosis. Thrombin, the coagulation end protease, is a potent platelet activator as well as important in the generation of fibrin fibers. Thrombin mediated activation of platelets is facilitated through the activation of the GPCRs PAR1 and PAR4. The first to be discovered on platelets was PAR1, soon PAR4 was discovered on platelets, and that PAR1 is not conserved by mouse platelets complicating *in vivo* PAR functional studies.[40] The PAR signaling occurs by the proteolysis of the N-terminal exodomain of the receptor by proteases like thrombin, and the formed N-terminus tethered ligand activates the receptor and initiates signaling via G-proteins. However, the structural differences between PAR1 and PAR4 result in differing outputs in platelet function.[224-226] PAR1 has two binding sites, the active sites and a hirudin-like sequence for exosite I interaction that induced allosteric effects on thrombin to make the receptor cleavage more efficient. PAR4 lacks this hirudin-like sequence, interacting only via the active site and consequently needs more protease to activate the receptor and is therefore a low affinity receptor. PAR4 has an important anionic sequence downstream of the thrombin cleavage site that allowed for a more sustained thrombin signal, compared to PAR1 which is regulated quickly. In order to study both platelet PARs independently agonists has been developed, the peptide SFLLRN, which mimics the first six amino acids of the new N-terminus that are unmasked by receptor cleavage, functions as a PAR1 agonist and activates the receptor independent of proteolysis by thrombin.[224] The sequence for PAR4 is the peptide sequence GYPGKF, however, a mutation of the first G into A increased the potency of the PAR4 agonist in inducing receptor signaling.[227]

Platelet activation is dependent on secondary feedback activation of ADP and thromboxane generation. PAR signaling is mostly dependent on this amplification signal, especially PAR4, which generates a sustained but less potent platelet signaling response.[228] PAR4 and P2Y12 are able to dimerize and co-internalize to induce activation of Akt signaling pathways via the endosomal recruitment of β-arrestin, which terminate further G protein activation by steric inhibition.[229]

Clinical studies have shown that thrombin stimulation of PARs has increased risk for cardiovascular events. Almost all thrombin signaling in platelets is mediated by PAR1 and PAR4, so antagonists that block these receptors might be useful antithrombotic agents.[224] The PAR1 antagonist vorapaxar has been shown to be effective in the treatment of thrombosis, however there is an unacceptably high bleeding risk. This shows the importance of a functional PAR1, and therefore targeting PAR4 might be a better option as the concentrations of thrombin needed to activate are higher. Therefore, the targeting of PAR4 may be as effective in thrombosis treatment without the bleeding risks.[230,231] With the knowledge that the intracellular signaling pathways downstream of platelet PARs both mediate hemostasis and also contribute to thrombosis, the mechanisms of these specific signaling events remain unspecified. We assess the hypothesis that platelet PAR1 and PAR4 each activate specific, as well as overlapping signaling systems to drive platelet responses underlying hemostasis and thrombosis. Overall, I propose to utilize platelet biochemical assays as well as phosphoproteomic approaches to characterize the platelet PAR signaling profile.

To study the platelet phenotype after stimulation by either PAR1, PAR4 or the combination of both PARs, we conducted platelet functional response assays. We isolated and washed human platelets that we stimulated to look for platelet aggregate formation using aggregometry

methods (Figure 6.1A). We observed that platelets rapidly aggregate in response of PAR stimulation, differences in initial shape change can be observed for PAR4 stimulation (Figure 6.1A). The effects on platelet secondary feedback loop activation via ADP and thromboxane were studied using specific inhibitors, apyrase for ADP chelation and indomethacin for thromboxane production blockage (Figure 6.1B). We observed for PAR1 that the activation of the integrin receptor relied on the feedback activation of ADP and thromboxane, however, PAR4 and thrombin did not seem to have the same profound effect. The same can be observed by flow cytometry (Figure 6.1C) where we tested the PAR agonists on integrin receptor activation by the fluorescent adhesion of PAC1 by flow cytometry. Furthermore, in order for platelets to become activated, they undergo cytoskeletal rearrangement resulting in a shape change and therefore release of granule content. We observed increased cytoskeleton rearrangement via actin polymerization, and dense and alpha granule release (Figure 6.1D-F). Platelets procoagulant phenotype with the expression of PS was mostly mediated by PAR4 stimulation as measured by lactadherin interaction (Figure 6.1G).

The observed functional effects from platelets after activation of PAR receptors are mediated by intracellular signaling pathways of kinases, lipases and scavenger proteins. In order to study these signaling events we prepared platelet lysates for western blot analyses with antisera against generalized phosphorylated protein kinase substrate consensus motifs. Interestingly, we observed that thrombin mediated platelet activation can induce tyrosine phosphorylation (4G10) however, this is weaker compared to GPVI platelet activation.[30] PAR stimulation is prominent in activating PKC substrates, AKT substrates and MAPK substrates (Figure 6.1H).

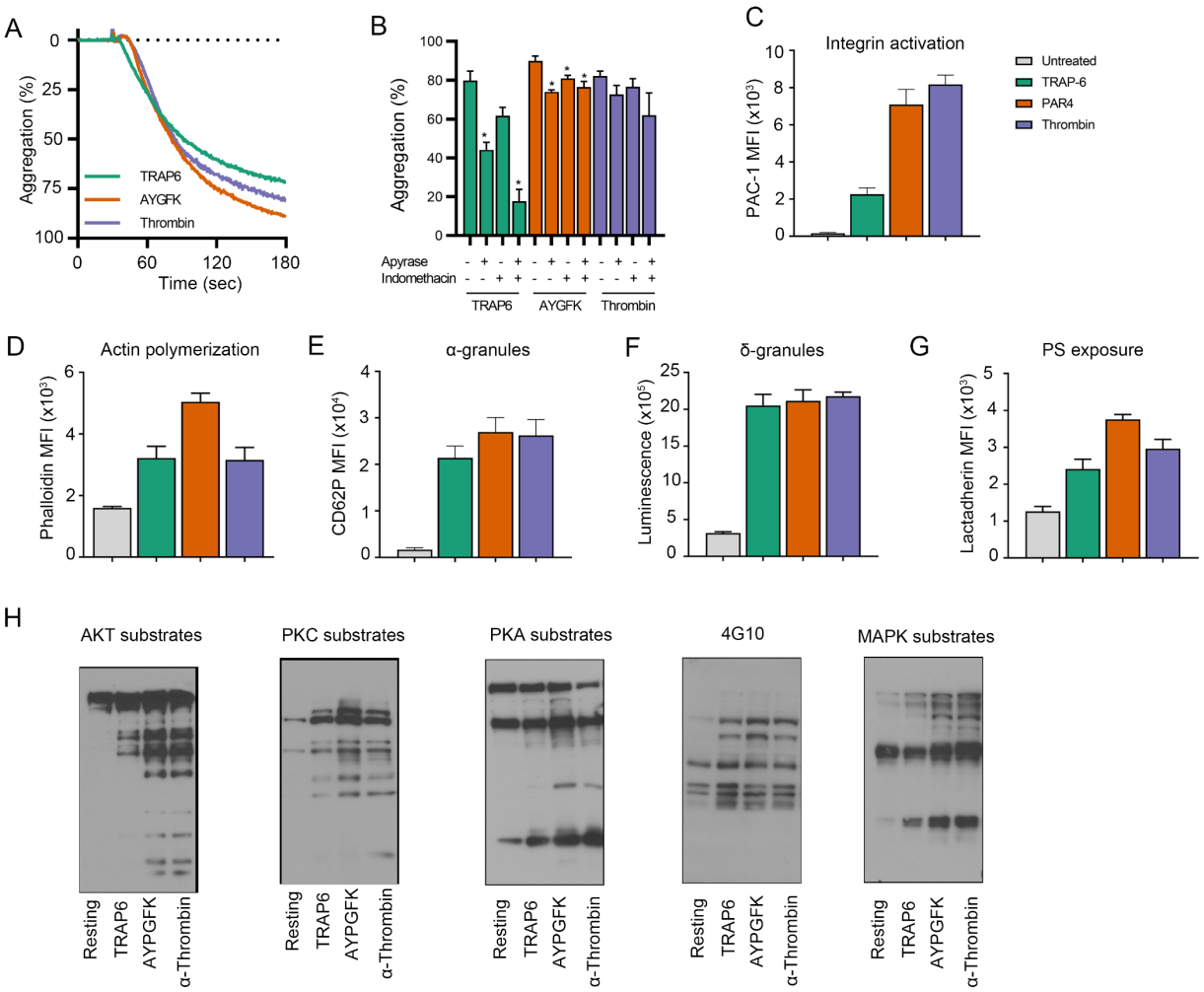

Figure 6.1 Functional platelet phenotypes after PAR stimulation.
(A) Aggregation traces of 3min stimulation with indicated PAR agonists and changes in light transmission rates were measured. (B) Quantification of aggregation in the presence of platelet secondary feedback inhibitors, apyrase or indomethacin. (C) Integrin receptor activation by flow cytometry measured by PAC1-FITC staining. (D). Cytoskeletal rearangement via actin polymerization measured as positive phalloidin staining. (E) Dense granule, (F) alpha granule, (G) and phosphatidylserine (PS) exposure measured as bound lactadherin. (H) Phosophorylation events after PAR stimulation as observed by western blot analysis of generalized phosphorylated protein kinase substrate consensus motifs for AKT substrates, PKC substrates, PKA substrates, 4G10 (tyrosine phosphorylations) and MAPK substrates. Data are mean SD. N=4

6.2.2 Phosphoproteomics approach and quantification of PAR signaling

To study phosphorylation events on a peptide level, we utilized a multiplexed mass spectrometry approach, to perform quantitative measurements of the complete platelet phosphopeptide proteome. To characterize the different downstream events leading up to platelet functional outcomes, we studied the phosphorylation events occurring by quantitative tandem-mass-tag (TMT) labeling for phosphoproteomics. In Figure 6.2 we show a general workflow of sample preparation and collection. In short, we incorporated four healthy donors, two male and two female, of which we isolated and obtained washed platelets. We pre-treated the samples with inhibitors against the integrin receptor, (Integrilin; 20µg/ml) to prevent aggregate formation, followed by PAR stimulation with either 30µM TRAP6 (PAR1), 500µM AYPGFK-NH3 (PAR4) or 5nM alpha-thrombin (PAR1+PAR4) for 3min at 37ºC 200rpm shaking. After stimulation we lysed the samples and snap freeze them to stop the reaction and store them for later proteomic analysis. We isolate the peptides and process them for MS analysis. The use of 16plex TMT labelling increased our probability of measuring all samples at the same

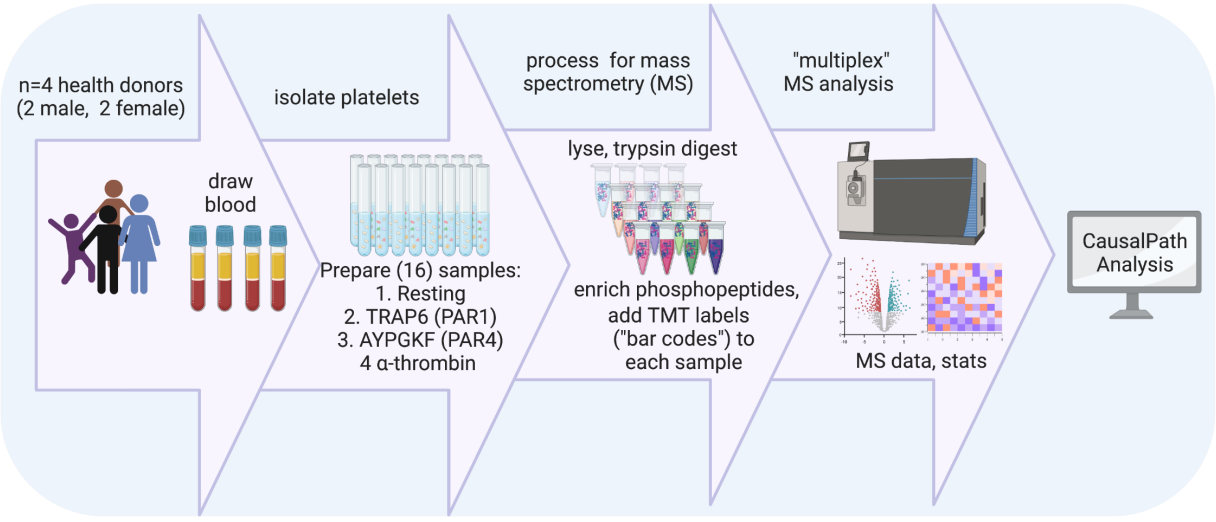

Figure 6.2 PAR proteomics workflow of sample preparation and proteomics analysis. Platelet preparation from 4 healthy donors were collected and samples were prepared by stimulation for 3min with either HT (Vehicle), TRAP6 (PAR1), AYPGKF (PAR4) or α-thrombin. Samples are then lysed trypsin digested and enriched with phsophopeptides and TMT labels for multiplex MS analysis. Causalpath anaylsis of the obtained data set will be made for the study of protein-protein relations.

time via orbitrap fusion MS. Relative to resting platelets, we measured more than 1,000 significant phosphorylation events in response to PAR agonists with a fold-change more than 1.5, and a false discovery rate of less than 0.01. Figure 6.3 shows the volcano plots showing the differences between PAR stimulation and unstimulated platelets and its significance of the detected targets. To validate our data set, we further analyzed the significantly changed targets compared to resting conditions by western blot in Figure 6.4. Boxplots derived from the reporter ion intensity were compared with the validating western blots of the samples. We observed validation for most increasing targets for our different conditions, however, it remained harder to observe the expected decrease in phosphorylation sites by western blot. Potentially due to the limiting step in detecting a slight decreasing amount of protein in an already high rate of phosphorylation.

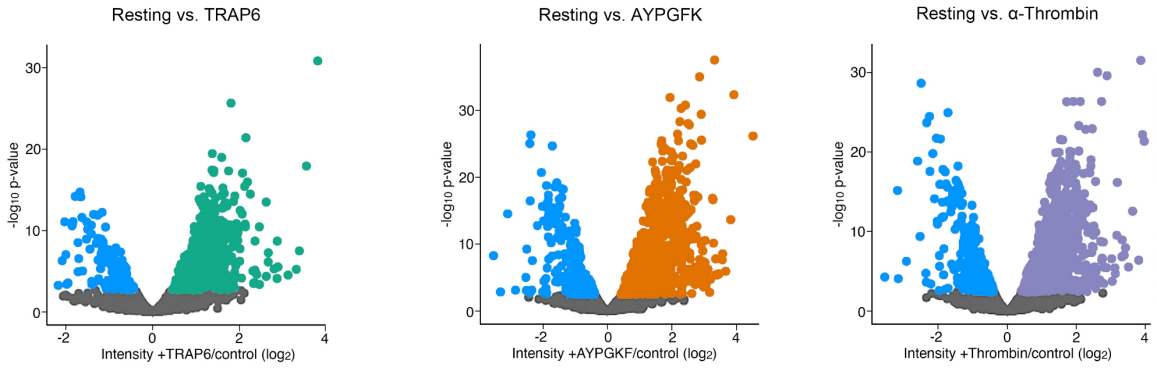

Figure 6.3 Volcano plots of phosphopeptide reporter ion intensity ratios of platelet PAR signaling. Resting, unstimulated platelets (indicated in blue) vs p-values of PAR1 stimulating agonist (TRAP6; indicated in green), PAR4 agonist (AYPGFK; indicated in orange) or α-thrombin (indicated in purple) stimulated platelets.

Our results showed over 600 phosphorylation events were common to TRAP6, AYPGFK and α-thrombin stimulation. This included phosphorylation of well-established mediators like GSK3α, PAK2 and more novel and emerging effectors in platelet activation pathways (BIN2, NKX3-2). Specific PAR1 and PAR4 agonist responses of mechanistic and translational interest were also noted, including phosphorylation of PAR1 T410 or PAR4 S369; thrombin uniquely activated

tyrosine kinase Fer Y714 phosphorylation, in a manner that may integrate PAR1 and PAR4 signaling.

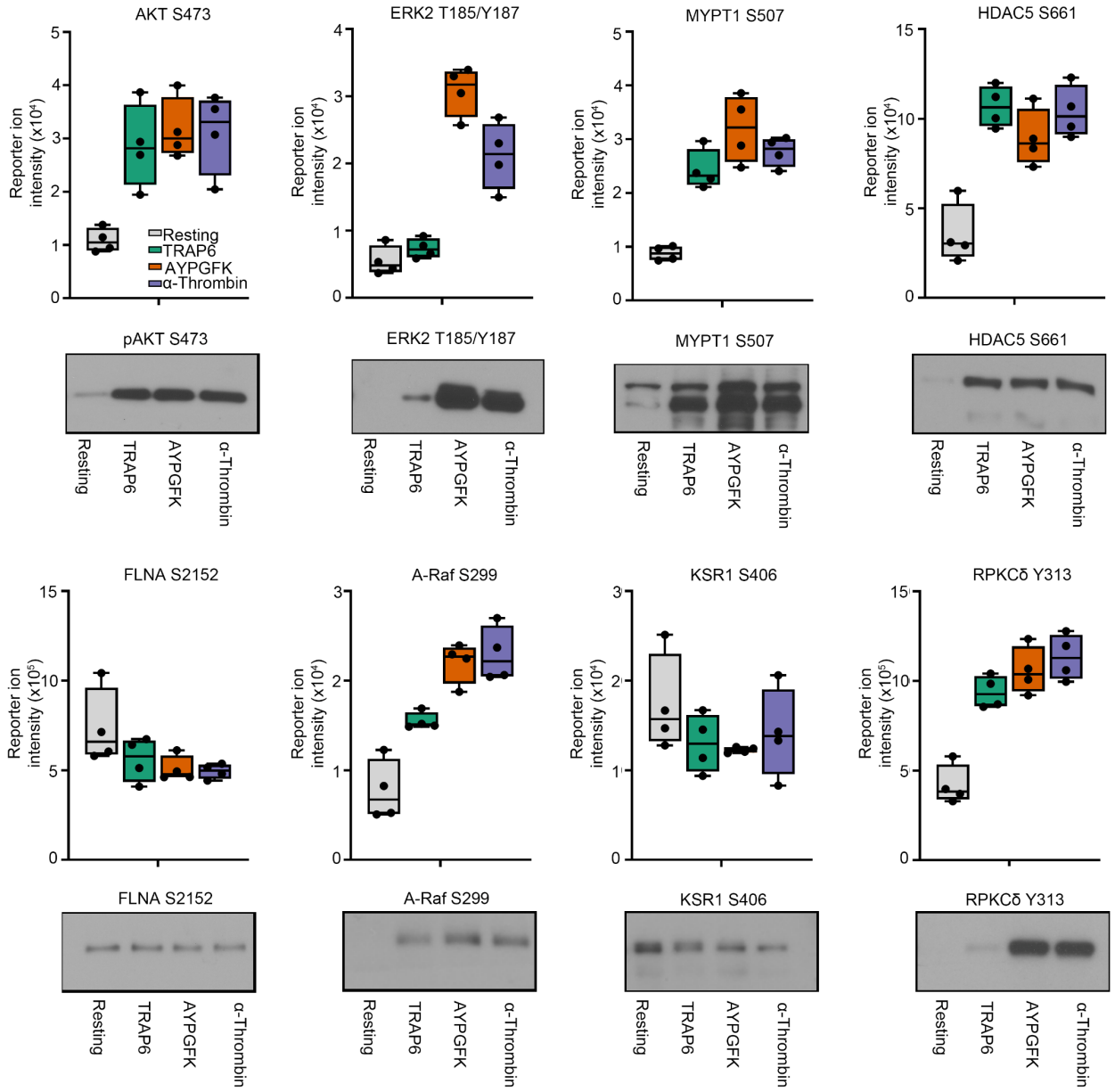

Figure 6.4 Data set validation via western blot analysis. Reporter ion intensity measurements of representative phosphopeptides corresponding to specific protein phosphorylation sites for (n = 4) control (grey), PAR1 (TRAP6; green), PAR4 (AYPGFK; orange) and thrombin (purple). Lysates of control and PAR agonist stimulated platelet samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and examined by western blot with phosphorylation site-specific antisera (phos.) against AKT S473, ERK2 T185/Y187, MYPT1 S507, HDAC5 S661, A-Raf S299, KSR1 S406, FLNA S2152 and RPKCdelta Y313.

6.2.3 Causal relations in platelet PAR signaling

We next analyzed different platelet protein modifications measured with CausalPath. This program computationally identifies pairs of protein phosphorylation changes in relation to protein-protein interactions. As can be observed in Figure 6.5, for thrombin, the CausalPath analysis identified over 100 signaling relations among site-specific phosphorylation changes downstream of thrombin PAR activation, around MAPK, PI3K/Akt, mTOR/S6K and other pathways that are important for shape change, vesicle transport and integrin activation.

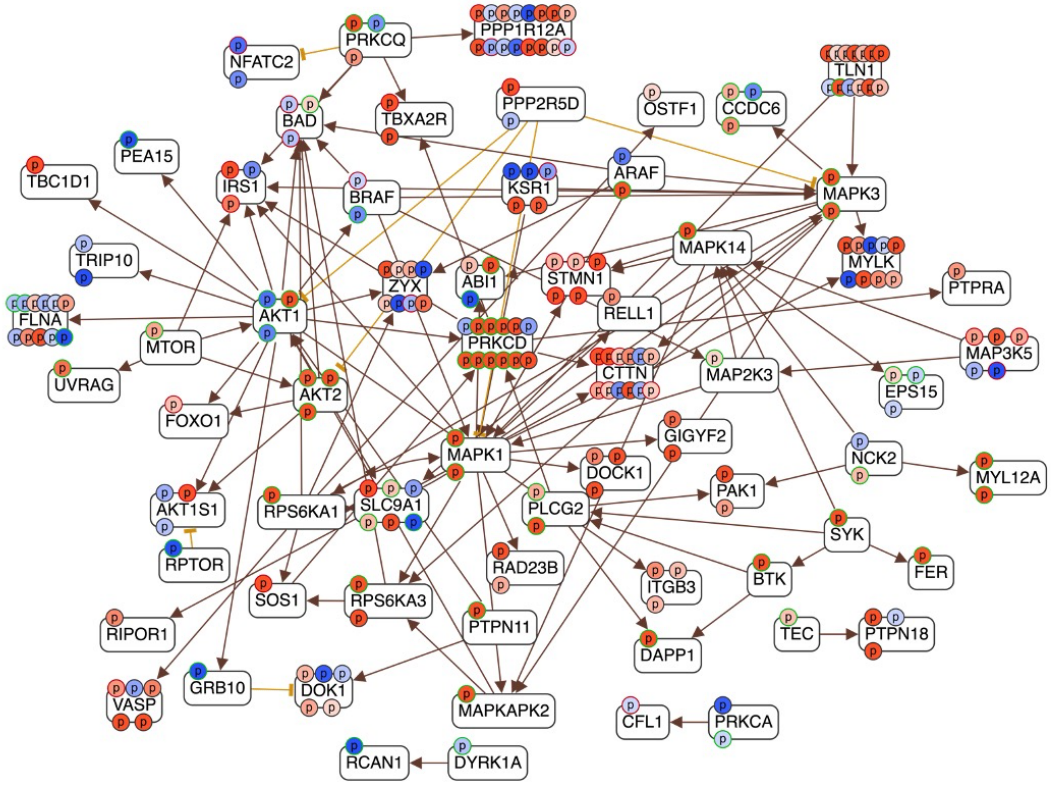

Figure 6.5 Causalpath analysis of thrombin stimulated platelets compared to resting platelets. Nodes (conventionally labeled with gene names) represent significantly modified phosphoproteins. Edges represent causal phosphorylation (green arrow) or dephosphorylation (red arrow) processes. Protein phosphorylation sites are shown with smaller “p” circles, where a green border indicates an activating site and red border indicates inactivating site. In general, more kinases than phosphatases place directly into CausalPath models because kinase-substrate relations are more completely detailed in literature, and phosphorylation site-specific mechanisms of phosphatase regulation are less prevalent.

6.2.4 Implications of mapping the PAR signaling proteome

In conclusion of our results, we provide a quantitative omics study and causal analysis of platelet PAR signaling, including specific PAR1 and PAR4 agonist responses. Finally, physiological assays of platelet adhesion, secretion and aggregation, as well as biochemical assays of platelet signaling will validate roles for several effectors and pathways in platelet PAR responses. As thrombin generation is the result of the activation of the extrinsic pathway of coagulation via TF-FVIIa complex formation and the intrinsic pathway of coagulation via negative surface FXII activation, it would be interesting to study the effects of thrombin concentration on platelet activation in pathological settings. The amount of thrombin generated via both pathways is estimated and computationally modeled, however, the exact thrombin amounts and the concentration gradient on platelet activation phenotypes is not well defined. Studies indicating a difference in the activation mechanism of PAR1 and PAR4 indicate the importance of local thrombin concentrations and platelet responses. To study the effects of extrinsic or intrinsic pathway generated thrombin on platelet activation and its favorable effect on either PAR1 or PAR4, we will utilize a thrombin generation assay (TGA) where we can study the amount of thrombin generated by either pathway. The effects on platelets can then subsequently be tested via flow cytometry for platelet activation markers (P-selectin, integrin activation, PS exposure). Platelet PAR specific inhibitors as well as signaling cascade interference can be identified based on our newly generated data set for studying the involvement in platelet supporting thrombus formation.

An example of utilizing our PAR proteomics data set, is the tyrosine kinase Fer. We identified Fer as a highly significant target specifically for thrombin, in a manner that may integrate PAR1 and PAR4 signaling. A study in 2003 discovered the presence of the Fer tyrosine kinase in platelets after activation with collagen via the GPVI receptor and in a less extent in thrombin

stimulated platelets.[232] Fer kinase is activated by Syk phosphorylation, however, the downstream targets for Fer tyrosine kinase are unknown. Therefore, we will utilize a Fer kinase specific inhibitor (E260) and look for Fer involvement in platelet functions by flow cytometry, aggregation and western blot. These studies can give more insight in the role of the Fer kinase in platelet function. In different cell types Fer tyrosine kinase have been detected around vesicular structures. This raised the possibility that Fer may have a regulatory function in cytoskeletal rearrangement and α-granule trafficking.

As platelets are important for providing a supportive and regulatory environment for sufficient coagulation reactions to happen, their secreted cargo could be an interesting field to study. How platelet signaling events downstream of platelet receptors are involved in platelet functional processes is an important step for anti-platelet drug therapies. Ultimately, this work will help to specify essential effectors, as well as biomarkers and therapeutic targets in platelet dysregulation, hyperactivity and thrombotic diseases.

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Biographical sketch

Stéphanie Elena Reitsma was born on July 16, 1991 in Zwolle, The Netherlands to Leo and Bianca Reitsma. Stéphanie attended Stad en Esch Zuideinde high school where she learned the curiosity of the biomedical sciences.

Stéphanie attended Utrecht University and obtained a B.A. in Biomedical sciences in 2015. Her master degree was with a focus in biology of disease and resulted in a M.S.in Biomedical sciences.

In the Fall of 2017 Stéphanie moved to Portland, Oregon to pursue a graduate career in the Biomedical Engineering Department with Dr. Owen McCarty at Oregon Health & Science University (OHSU). Stéphanie's research has been focused on platelet- contact pathway interactions. During her graduate studies at OHSU she was awarded the Young Investigator award from the International Society on Thrombosis and Haemostasis (ISTH) in (2018) and (2019). Stéphanie has presented her research orally at the Kinin conference (2018) and Earl Davis Symposium (2020), as well as numerous poster presentations at the ISTH conference in 2018 and 2019. For her work on FXIa platelet interaction she was awarded an American Heart Association (AHA) pre doctoral grant in 2020.

Following graduation from OHSU, Stéphanie plans to pursue her academic career as a postdoctoral fellow at University of North Carolina (UNC) in Dr. Alisa Wolberg lab. Stéphanie's current publications, presentations are listed below.

Publications

Peer-reviewed

1. Meyer S, Evers M, Jansen JHM, Buijs J, Broek B, **Reitsma SE**, Moerer P, Amini M, Kretschmer A, Ten Broeke T, den Hartog MT, Rijke M, Klein C, Valerius T, Boross P, Leusen JHW. New insights in Type I and II CD20 antibody mechanisms-of-action with a panel of novel CD20 antibodies. Br J Haematol. 2018 Mar;180(6):808-820. doi: 10.1111/bjh.15132

2. ***Reitsma SE**, Garland KS, Shirai T, Zilberman-Rudenko J, Tucker EI, Gailani D, Gruber A, McCarty OJT and Puy C*. Removal of the C-Terminal Domains of ADAMTS13 by Activated Coagulation Factor XI induces Platelet Adhesion on Endothelial Cells under Flow Conditions. *(2017) Front. Med. 4:232. doi: 10.3389/fmed.2017.0023*

3. Zilberman-Rudenko J, Zhao FZ, **Reitsma SE**, Mitrugno A, Pang J, Shatzel JJ, Rick B, Tyrrell C, Hasan W, McCarty OJT, Schreiber MA. Effect of Pneumatic Tubing System Transport on Platelet Apheresis Units. *Cardiovasc Eng Technol*. 2018;9(3):515-527. doi:10.1007/s13239-018-0361-2

4. Zilberman-Rudenko J, **Reitsma SE**, Puy C, Rigg RA, Smith SA, Tucker EI, Silasi R, Merkulova A, McCrae K, Maas C, Urbanus RT, Gailani D, Morrissey JH, Gruber A, Lupu F, Schmaier AH, McCarty OJT. Factor XII Activation Promotes Platelet Consumption in the Presence of Bacterial-Type Long-Chain Polyphosphate In Vitro and In Vivo Arteriosclerosis, Thrombosis, and Vascular Biology. 2018;38:1748-1760

5. Ngo ATP, Jordan KR, Mueller PA, Puy C, **Reitsma SE**, Hagen MW, Revenko AS, Lorentz CU, Tucker EI, Hinds MT, Fazio S, Monia BP, Gailani D, Gruber A, Tavori H, McCarty OJ. Pharmacological targeting of coagulation factor XI mitigates the development of experimental atherosclerosis in low-density lipoprotein receptor-deficient mice. *Journal of Thrombosis and Haemostasis : JTH. 2021 Apr;19(4):1001-1017. DOI: 10.1111/jth.15236.*

6. **Reitsma SE**, Pang J, Raghunathan V, Shatzel JJ, Lorentz CU, Tucker EI, Gruber A, Gailani D, McCarty OJ, Puy C. Role of platelets in regulating activated coagulation factor XI activity. American Journal of Physiology-Cell Physiology 2021 320:3, C365-C374

7. Puy C, Pang J, **Reitsma SE**, Lorentz CU Tucker EI , Gailani D, Gruber A, Lupu F, McCarty OJT. Cross-Talk between the Complement Pathway and the Contact Activation System of Coagulation: Activated Factor XI Neutralizes Complement Factor H. *The Journal of Immunology April 2021, ji2000398; DOI: 10.4049/jimmunol.2000398*

8. Parra-Izquierdo I, Melrose AR, Pang J, Lakshmanan HHS, **Reitsma SE**, Vavilapalli SH, Larson MK, Shatzel JJ, McCarty OJT, Aslan JE. Janus kinase inhibitors ruxolitinib and baricitinib impair glycoprotein-VI mediated platelet function. Platelets. 2021 Jun 7:1-12. doi: 10.1080/09537104.2021.1934665. PMID: 34097573.

9. Parra-Izquierdo I, Lakshmanan HHS, Melrose AR, Pang J, Zheng TJ, Jordan KR, **Reitsma SE**, McCarty OJT, Aslan JE. *The Toll-Like Receptor 2 Ligand Pam2CSK4 Activates Platelet Nuclear Factor-κB and Bruton's Tyrosine Kinase Signaling to Promote Platelet-Endothelial Cell Interactions.* Front Immunol. 2021 Aug 30;12:729951. doi: 10.3389/fimmu.2021.729951.

PMID: 34527000; PMCID: PMC8435771.

Under review

1. **S.E. Reitsma**, J.Johnson, J.Pang, I. Parra-Izquierdo, H. Lakshmanan, A.R. Melrose, M.T. Hinds, J.E. Aslan, O.J.T. McCarty, J.O. Lo. *Chronic edible dosing of Δ9-tetrahydrocannabinol (THC) in non-human primates reduces systemic platelet activity and function.* Under submission at AJP-Cell November 2021

2. H. Lakshmanan, A. Estonilo, **SE. Reitsma**, T.J. Zheng1, J. Subramanian, AR. Melrose, J. Maddala, A. Gruber, DE Gailani, O.J.T. McCarty, P.L. Jurney and C. Puy. *Revised model of the tissue factor pathway of thrombin generation: role of the feedback activation of FXI.* Under Review JBC November 2021

Research abstracts and presentations

1. **Reitsma SE**, Puy C, Urbanus RT, Gailani D, Tucker EI, Gruber A, McCarty OJ. "Role of the Platelet Surface in the Activation of FXI by FXIIa", *XXVI Congress of the International Society on Thrombosis and Haemostasis*, Berlin, Germany (July, 2017). Poster presentation
2. **Reitsma S**, Puy C, Gailani D, Tucker EI, Gruber A, McCarty OJ. "The surface of activated platelets enhances the activity of FXIa towards FIX", KININ2018, Cleveland, OH (Jun, 2018). Oral communication
3. **Reitsma S**, Puy C, Gailani D, Tucker EI, Gruber A, McCarty OJ. "The surface of activated platelets enhance the activity of FXIa", *60th Annual Scientific & Standardization Committee of the International Society on Thrombosis and Haemostasis*, Dublin, IR (July, 2018). Poster presentation
4. **Reitsma S**, Puy C, Gailani D, Tucker EI, Gruber A, McCarty OJ. "The surface of activated platelets enhance the activity of FXIa", *Annual International Society on Thrombosis and Haemostasis* conference, Melbourne, Australia (July, 2019). Poster presentation.
5. **Reitsma S**, Puy C, Gailani D, Tucker EI, Gruber A, McCarty OJ. "The role of platelets in regulating FXIa activity towards FIX", *Earl Davis symposia, Vancouver,BC Canada (virtual)* (Oct, 2020). Oral presentation.
6. **Reitsma S**, Jennifer Johnson Jiaqing Pang, Ivàn Parra-Izquierdo, Hari Hara Sudhan Lakshmanan, Alex R. Melrose, Monica T. Hinds, Joseph E. Aslan, Owen J.T. McCarty, Jamie O. Lo. "Chronic edible dosing of Δ9-tetrahydrocannabinol (THC) in non-human primates reduces systemic platelet activity and function." *XXIX Congress of the International Society on Thrombosis and Haemostasis*, Philadelphia, PA USA (July, 2021). Live ePoster presentation

Invited lectures

Lecturer. Biomedical Engineering Course 608 - Grant Writing. Oregon Health & Science University. 2021.

Undergraduate mentoring

Student. Katie Trese (2017), current undergraduate, Oregon State University

Appendix I.

Table 3 Oxylipin ID panel.

	nM		
No	**Peak Name**	**ChEBI ID**	**InChi Key**
1	11 b-PGF2a	27595	PXGPLTODNUVGFL-ZWAKLXPCSA-N
2	Thromboxane B2	28728	XNRNNGPBEPRNAR-JQBLCGNGSA-N
3	PGE2	15551	XEYBRNLFEZDVAW-ARSRFYASSA-N
4	17(R)-Resolvin D1	138179	OIWTWACQMDFHJG-BJEBZIPWSA-N
5	Resolvin D1	81564	OIWTWACQMDFHJG-CCFUIAGSSA-N
6	12,13-DiHOME	72665	CQSLTKIXAJTQGA-GJGKEFFFSA-N
7	9,10-DiHOME	72663	XEBKSQSGNGRGDW-YFHOEESVSA-N
8	19,20-DiHDPA	72657	FFXKPSNQCPNORO-MBYQGORISA-N
9	5,6-DiHETE	165283	VPXVODYVPILPRC-LTKCOYKYSA-N
10	14,15-DiHET	138591	SYAWGTIVOGUZMM-JHIAIUNDSA-N
11	16,17-DiHDPA	N/A	YXQCSWUATWXVGK-QCAYAECISA-N
12	13,14-DiHDPA	165248	LINXWSBRRJSWHL-UQZHZJRSSA-N
13	9(S)-HOTrE	80447	RIGGEAZDTKMXSI-MEBVTJQTSA-N
14	10,11-DiHDPA	N/A	OAZUCYZBXHOCES-VABGYXHOSA-N
15	13(S)-HOTrE	84441	KLLGGGQNRTVBSU-FQSPHKRJSA-N
16	18-HEPE	72802	LRWYBGFSVUBWMO-UXNZXXPISA-N
17	7,8-DiHDPA	N/A	DPZIOENSPXELQY-JYFGGXQQSA-N
18	13(S)-HODE	34154	HNICUWMFWZBIFP-IRQZEAMPSA-N
19	9(S)-HODE	34496	NPDSHTNEKLQQIJ-UINYOVNOSA-N
20	15(S)-HETE	15558	JSFATNQSLKRBCI-VAEKSGALSA-N
21	12(S)-HETE	34146	ZNHVWPKMFKADKW-LQWMCKPYSA-N
22	8(S)-HETE	34486	NLUNAYAEIJYXRB-VYOQERLCSA-N
23	9(R)-HETE	165288	KATOYYZUTNAWSA-AZFZJQGOSA-N
24	5(S)-HETE	28209	KGIJOOYOSFUGPC-JGKLHWIESA-N
25	19,20-EpDPA	137183	OSXOPUBJJDUAOJ-MWEXLPNRSA-N
26	9,10-EpOME	N/A	FBUKMFOXMZRGRB-XKJZPFPASA-N
27	EPA	28364	JAZBEHYOTPTENJ-JLNKQSITSA-N
28	DHA	28125	MBMBGCFOFBJSGT-KUBAVDMBSA-N
29	ARA	15843	YZXBAPSDXZZRGB-DOFZRALJSA-N

Table 4 Raw data of measured oxylipin panel. List of dynamic mulitple reaction mornitoring (dMRM) transitions of the oxylipins and deuterated-oxylipins and CUDA (12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid) used as internal standards for our analysis.

No	Name	Precursor Ion	Product Ion	Expected RT (min)	Start RT (min)	End RT (min)	CE	DP	Internal standard
1	6-keto PGF1a - D4	373	167	5.3	0	27	-40	-70	
2	6-keto PGF1a	369	163	5.4	4.5	6.2	-40	-70	6-keto PGF1a - D4
3	Resolvin E1	349	107	5.4	4.6	6.2	-30	-50	Resolvin E1 - D4
4	Resolvin E1 - D4	353	197	5.5	4.6	6.3	-20	-40	
5	8-iso PGF3a	351	307	5.7	4.8	6.5	-26	-80	PGF2a - D4
6	8-iso PGF2a	353	193	6.3	5.5	7.1	-33	-70	PGF2a - D4
7	11 b-PGF2a	353	193	6.5	5.7	7.3	-40	-60	PGF2a - D4
8	Thromboxane B2 - D4	373	173	6.7	5.8	7.5	-21	-50	
9	Thromboxane B2	369	169	6.7	5.8	7.5	-21	-50	Thromboxane B2 - D4
10	PGF2a - D4	357	197	7	6.2	7.8	-33	-70	

11	PGE2	351	271	7.3	6.4	8.1	-25	-35	PGE2 - D4
12	PGE2 - D4	355	275	7.4	6.5	8.2	-25	-35	
13	Resolvin D3	375	147	7.4	6.6	8.2	-30	-50	Resolvin D1 - D5
14	Resolvin D2	375	175	7.5	6.7	8.3	-30	-60	Resolvin D1 - D5
15	15-keto PGF2a	351	315	7.5	6.7	8.3	-20	-40	6 keto PGF1a - D4
16	PGD2	351	271	7.6	6.7	8.4	-25	-35	PGD2 - D4
17	PGD2 - D4	355	275	7.4	6.6	8.3	-25	-35	
18	15-keto PGE2	349	331	7.6	6.8	8.4	-20	-40	6 keto PGF1a - D4
19	17(R)-Resolvin D1	375	215	8.2	7.4	9	-30	-60	Resolvin D1 - D5
20	Resolvin D1	375	141	8.2	7.4	9	-20	-60	Resolvin D1 - D5
21	Resolvin D1 - D5	380	141	8	7.2	8.8	-20	-40	
22	13,14-dihydro-15-keto PGF2a	353	113	8.2	7.4	9	-40	-40	6 keto PGF1a - D4
23	13,14-dihydro-15-keto PGE2	351	175	8.7	7.9	9.5	-30	-30	PGE2 - D4

24	13,14-dihydro-15-keto PGD2	351	207	8.7	7.9	9.5	-30	-40	PGD2 - D4
25	PGJ2	333	189	9.3	8.4	10.1	-20	-30	PGD2 - D4
26	Leukotriene B5	333	195	9.3	8.5	10.1	-20	-50	Leukotriene B4 - D4
27	PDX	359	153	10.3	9.4	11.1	-20	-20	Resolvin E1 - D4
28	Resolvin D5	359	199	10.4	9.6	11.3	-20	-50	Resolvin D1 - D5
29	17,18-DiHETE	335	203	10.4	9.6	11.3	-22	-60	15(S)-HETE - D8
30	Leukotriene B4	335	195	10.7	9.8	11.5	-21	-70	Leukotriene B4 - D4
31	Leukotriene B4 - D4	339	197	10.6	9.8	11.5	-21	-70	
32	11,12-DiHETE	335	167	11	10.1	11.8	-22	-55	15(S)-HETE - D8
33	CUDA	339	214	11	10.2	11.8	-35	-65	
34	12,13-DiHOME	313	183	11.3	10.4	12.1	-30	-70	12,13-DiHOME - D4
35	12,13-DiHOME - D4	317	185	11.2	10.4	12	-30	-70	
36	14,15-DiHETE	335	111	11.3	10.4	12.1	-22	-55	12(S)-HETE - D8

37	8,9-DiHETE	335	185	11.3	10.4	12.1	-22	-55	15(S)-HETE - D8
38	9,10-DiHOME	313	201	11.6	10.7	12.4	-30	-50	12,13-DiHOME - D4
39	19,20-DiHDPA	361	229	11.8	11	12.7	-24	-74	15(S)-HETE - D8
40	5,6-DiHETE	335	145	11.8	11	12.7	-22	-55	15(S)-HETE - D8
41	14,15-DiHET	337	207	11.9	11	12.7	-25	-65	12(S)-HETE - D8
42	16,17-DiHDPA	361	233	12.2	11.4	13	-24	-80	15(S)-HETE - D8
43	11,12-DiHET	337	319	12.4	11.5	13.2	-20	-50	15(S)-HETE - D8
44	13,14-DiHDPA	361	193	12.4	11.5	13.2	-24	-80	15(S)-HETE - D8
45	9(S)-HOTrE	293	171	12.5	11.7	13.4	-20	-60	9(S)-HODE - D4
46	10,11-DiHDPA	361	153	12.6	11.8	13.5	-24	-80	15(S)-HETE - D8
47	13(S)-HOTrE	293	195	12.7	11.9	13.5	-20	-20	9(S)-HODE - D4
48	13(S)-HOTrE(gamma)	293	193	12.9	12	13.7	-20	-20	9(S)-HODE - D4
49	18-HEPE	317	215	12.9	12.1	13.7	-20	-50	15(S)-HETE - D8

50	15-deoxy-delta12,14-PGJ2	315	203	13.1	12.3	13.9	-30	-50	PGF2a - D4
51	7,8-DiHDPA	361	127	13.2	12.4	14	-24	-80	15(S)-HETE - D8
52	20-HETE - D6	325	281	13.3	12.4	14.1	-24	-65	
53	20-HETE	319	245	13.3	12.5	14.1	-24	-65	20-HETE - D6
54	13(S)-HODE	295	195	14.2	13.3	15	-25	-65	13(S)-HODE - D4
55	13(S)-HODE - D4	299	198	14.1	13.3	15	-25	-65	
56	9(S)-HODE	295	171	14.3	13.5	15.2	-25	-60	9(S)-HODE - D4
57	9(S)-HODE - D4	299	172	14.3	13.4	15.1	-25	-60	
58	15(S)-HETE	319	219	14.5	13.7	15.3	-20	-40	15(S)-HETE - D8
59	15(S)-HETE - D8	327	226	14.4	13.5	15.2	-16	-70	
60	17,18-EpETE	317	215	14.6	13.7	15.4	-15	-55	15(S)-HETE - D8
61	11(S)-HETE	319	195	14.8	14	15.6	-20	-50	12(S)-HETE - D8
62	15-OxoETE	317	113	14.9	14.1	15.7	-30	-60	15(S)-HETE - D8

63	12(S)-HETE	319	179	15.1	14.2	15.9	-20	-50	12(S)-HETE - D8
64	12(S)-HETE - D8	327	184	14.9	14.1	15.8	-21	-60	
65	14,15-EpETE	317	248	14.9	14.1	15.8	-15	-45	12(S)-HETE - D8
66	11,12-EpETE	317	195	15.1	14.2	15.9	-16	-70	12(S)-HETE - D8
67	8(S)-HETE	319	155	15.1	14.3	15.9	-20	-40	12(S)-HETE - D8
68	8,9-EpETE	317	155	15.2	14.4	16	-16	-75	12(S)-HETE - D8
69	9(R)-HETE	319	139	15.3	14.4	16.1	-20	-60	5(S)-HETE - D8
70	12-OxoETE	317	153	15.4	14.5	16.2	-20	-60	15(S)-HETE - D8
71	5(S)-HETE	319	115	15.5	14.7	16.3	-20	-60	5(S)-HETE - D8
72	5(S)-HETE - D8	327	116	15.4	14.6	16.2	-20	-50	
73	19,20-EpDPA	343	241	15.7	14.9	16.5	-20	-45	15(S)-HETE - D8
74	14,15-EET	319	219	15.9	15.1	16.7	-16	-70	14,15 - EET(EpETrE) - D11

75	14,15 - EET(EpETrE) - D11	330	175	15.8	15	16.7	-16	-70	
76	12,13-EpOME	295	251	15.9	15	16.7	-20	-50	12,13-DiHOME - D4
77	9,10-EpOME	295	277	16	15.1	16.8	-20	-50	12,13-DiHOME - D4
78	16,17-EpDPA	343	274	16	15.2	16.8	-15	-55	12(S)-HETE - D8
79	13,14-EpDPA	343	161	16.1	15.3	17	-15	-55	12(S)-HETE - D8
80	5-OxoETE	317	203	16.1	15.3	17	-30	-60	15(S)-HETE - D8
81	10,11-EpDPA	343	153	16.1	15.3	17	-15	-55	12(S)-HETE - D8
82	11,12-EET	319	167	16.3	15.4	17.1	-15	-55	11,12-EET(EpETrE) - D11
83	11,12-EET(EpETrE) - D11	330	167	16.2	15.3	17	-15	-55	
84	7,8-EpDPA	343	113	16.3	15.5	17.1	-15	-55	12(S)-HETE - D8
85	EPA	301	147	17.4	16.6	18.2	-20	-30	EPA - D5
86	EPA - D5	306	262	17.4	16.6	18.2	-20	-55	

87	a-Linolenic Acid	277	113	17.5	16.7	18.3	-20	-30	a-Linolenic Acid - D5
88	a-Linolenic Acid - D5	282	238	17.5	16.7	18.3	-20	-20	
89	DHA	327	229	17.9	17.1	18.7	-20	-60	DHA - D5
90	DHA - D5	332	234	17.8	17	18.7	-20	-55	
91	ARA	303	259	18	17.2	18.8	-20	-50	ARA - D8
92	ARA - D8	311	183	18	17.2	18.8	-50	-60	
93	Linoleic Acid	279	117	18.1	17.3	19	-20	-40	Linoleic Acid - D4
94	Linoleic Acid - D4	283	201	18.1	17.3	19	-20	-30	

RT: retention time
CE: Collision energy
DP: Declustering potential
66 oxylipins
22 deuteriated oxylipins as internal standard
CUDA
5 deuterated surrogates:
(EPA-d5, DHA-d5, ARA-d8, ALA-d5, LA-d4)