The roles of fibrinolysis in regulating coagulation factor XIII

by

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Abstract

Coagulation factor XIII (FXIII) is a protransglutaminase enzyme that is activated at the end of the coagulation cascade. Activated FXIII (FXIIIa) stabilizes the blood clot from premature lysis by covalently crosslinking fibrin molecules to itself and to other anti-fibrinolytic proteins. Although the role of FXIIIa as an antifibrinolytic protein has been well characterized, the role of fibrinolysis in regulating FXIII and FXIIIa has not been characterized.

FXIIIa lies in close proximity to fibrin during the hemostatic processes; therefore, the effect of plasmin on FXIIIa activity was examined. This thesis shows that plasmin preferentially cleaves the active enzyme FXIIIa, but not the zymogen FXIII. The primary cleavage site identified by mass spectrometry was between K468-Q469. Inactivation of FXIIIa occurred during clot lysis, but not during clot formation. These results indicate FXIIIa activity can be modulated by fibrinolytic enzymes, and suggest that changes in fibrinolytic activity may influence cross-linking of blood proteins.

Thrombosis patients who are treated with thrombolytic therapy receive high doses of fibrinolytic enzymes. Since FXIIIa is inactivated by plasmin, the stability of FXIII and FXIIIa during thrombolytic therapy was examined using purified proteins and blood collected from nine DVT patients undergoing CDT. During CDT, FXIII levels were decreased by more than 40% in 5 of 9 patients and FXIIIa levels were decreased by more than 85% in 2 patients when it was activated. FXIII and FXIIIa can decrease during CDT in some patients, warranting further research into the role of FXIIIa in bleeding from thrombolysis.

Amyloid beta (Aβ) peptide inhibits fibrinolysis and can form complexes with FXIIIa. Although Aβ can be crosslinked by tissue transglutaminase, the ability of FXIIIa to crosslink Aβ

has not been demonstrated. The thesis shows that FXIIIa covalently crosslinked Aβ40 into dimers and oligomers, as well as to fibrin, and to blood clots under flow *in vitro*. Aβ40 also increased the stiffness of platelet-rich plasma clots in the presence of FXIIIa. These results suggest that crosslinking of Aβ40 by FXIIIa may contribute to the formation of vascular deposits in cerebral amyloid angiopathy.

Lay Summary

Coagulation factor XIIIa (FXIIIa) stabilizes blood clots from premature clearance and reduces blood loss after vascular damage. The role of FXIIIa on fibrinolysis, the process of clearing blood clots after wound healing, has been well characterized; however, the effect of fibrinolysis on FXIIIa has not been clear. Here this thesis shows that fibrinolytic enzymes can inactivate FXIIIa. This reaction also occurs in some deep vein thrombosis patients treated with thrombolytic therapy, potentially explaining the cause of major bleeds observed in thrombolytic therapy. This thesis found another link between FXIIIa and fibrinolysis; amyloid beta (Aβ) inhibits fibrinolysis and I found that Aβ can be crosslinked by FXIIIa.

Preface

Approvals for the study were given by the research ethics boards of the University of British Columbia and Vancouver Coastal Health Research Institute. The UBC Ethics Certificate is H14-01581for human blood collection and A16-0176 for mouse blood collection. The VCHRI Ethics Certificate is V14-01581 for collecting blood from patients with deep vein thrombosis. The reuse and reprint of all published work is with permission from all journals referenced.

A modified version of chapter 2 has been published in Blood: W.S. Hur, N. Mazinani, X.J. Lu, H.M. Britton, J.R. Byrnes, A.S. Wolberg, C.J. Kastrup (2015). Coagulation factor XIIIa is inactivated by plasmin (paper attached in the appendix). W.S.H. conceived the idea, designed all experiments and performed experiments for Figures 2.1, 2.2A-E, 2.3, 2.4, 2.5, 2.7, 2.8, 2.9, 2.10, 2.10A-B and 2.11, analyzed and interpreted all data and wrote the paper. N.M. designed and performed experiments for Figure 2.2F, 2.6, 2.10C-E, analyzed and interpreted data, and wrote the paper. X.J.L and H.M.B. performed experiments. J.R.B. and A.S.W. helped develop methods for detecting FXIII on Western blots and helped interpret data. C.J.K. helped design experiments, interpret data and write the paper.

A modified version of chapter 3 has been accepted to *Blood Coagulation and Fibrinolysis* (*BCF*): W.S. Hur, H. Warner, L. Machan, C.J. Kastrup (2018). Coagulation Factors XIII-A and XIII-A* Decrease in Some DVT Patients Following Catheter-directed Thrombolysis. W.S.H. designed, performed and analyzed experiments, and wrote the paper. H.W. and L.M. recruited participants and designed experiments. C.J.K. designed and analyzed experiments and wrote the paper.

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List of Symbols

α	alpha
β	beta
γ	gamma
ε	epsilon

List of Abbreviations

Aβ	Amyloid-β
ADP	Adenosine diphosphate
AICD	Amyloid precursor protein intracellular domain
AP	α2-antiplasmin
APP	Amyloid precursor protein
CAA	Cerebral amyloid angiopathy
CDT	Catheter-directed thrombolysis
COL	Collagen
CSF	Cerebrospinal fluid
DVT	Deep vein thrombosis
EACA	ε-aminocaproic acid
FXI(a)	(Activated) coagulation factor IX
FVII(a)	(Activated) coagulation factor FVII
FVIII(a)	(Activated) Coagulation factor FVIII
FX(a)	(Activated) coagulation factor FX
FXI(a)	(Activated) coagulation factor FXI
FXII(a)	(Activated) coagulation factor FXII
FXIII(a)	(Activated) coagulation factor FXIII
FXIIID	Coagulation factor FXIII deficiency
HMWP	High molecular weight proteins
ICH	Intracranial hemorrhage

KO	Knock-out
KPI	Kunitz protease inhibitor
MI	Myocardial infarction
MMPs	Matrix metalloproteinase
NFT	Neurofibrillary tangles
PAI	Plasminogen activator inhibitor
PE	Pulmonary embolism
PS	Phosphatidylserine
RBC	Red blood cells
SAH	Subarachnoid hemorrhage
Sc-tpA	Single-chain tissue plasminogen activator
SK	Streptokinase
TAFI	Thrombin activatable fibrinolysis inhibitor
Tc-tPA	Two-chain tissue plasminogen activator
TEG	Thromboelastography
THR	Thrombin
tPA	Tissue plasminogen activator
TXA	Tranexamic acid
uPA	Urokinase plasminogen activator
VTE	Venous thromboembolism
WT	Wild type

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Dedication

To everyone who has supported me throughout my Ph.D. career.

Chapter 1: Introduction

1.1 Overview of motivation and significance

Activated coagulation factor XIII (FXIIIa or FXIII-A*) is a transglutaminase that is generated at the end of the coagulation cascade in response to vascular damage. Coagulation factor XIII (FXIIII) contributes to a variety of physiological processes such as wound healing, angiogenesis and pregnancy[1-3]. However, a primary role of FXIIIa is to stabilize blood clots from premature lysis by covalently crosslinking fibrin molecules to itself and to other anti-fibrinolytic proteins such as antiplasmin[4]. Although the role of FXIIIa in regulating fibrinolysis has been well characterized, the effect of the fibrinolytic system on FXIIIa regulation has not been examined in detail.

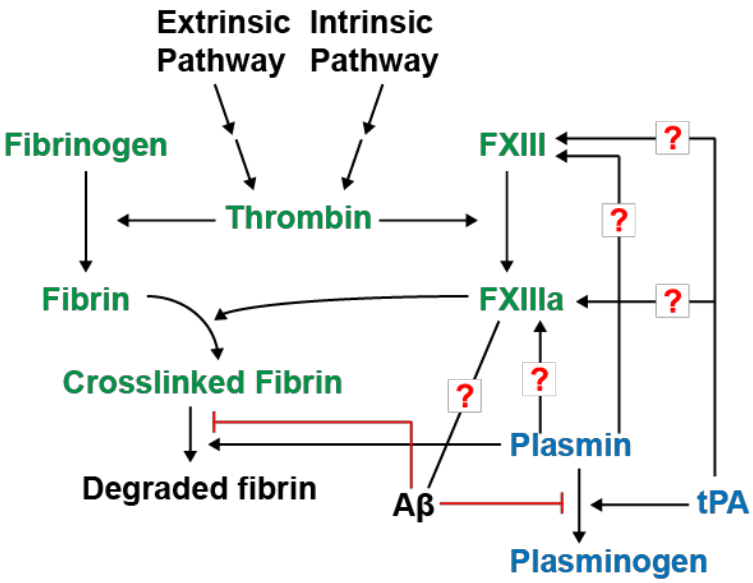


Figure 1.1 The interaction between coagulation cascade and the fibrinolytic system

Coagulation factors are written in green. Fibrinolytic proteins are written in blue. Red lines indicate inhibition. Question marks denote the question the thesis will address.

1.2 Specific thesis objectives

1.2.1 Identify the mechanism of inactivation of FXIII.

1.2.1.1 Biological question

FXIII is a key regulator of blood clot stability. When FXIII is activated by thrombin at the end of the coagulation cascade, it covalently crosslinks fibrin to itself and to other proteins to make the blood clot more resistant to premature lysis[5]. The synthesis, localization, activation of FXIII has been well characterized; however, the physiological mechanism of inactivation has remained elusive. The objective was to test if plasmin can inactivate FXIII. Plasmin is the active protease that is responsible for the clearance of blood clots. Since plasmin, fibrin and FXIII are in close proximity during vascular damage, the ability of plasmin to inactivate FXIII was tested[5]. The rate of proteolytic cleavage and the cleavage sites were measured to further characterize the reaction. The situations where this reaction might be relevant was examined: during clot formation, during clot lysis and under thrombolytic conditions.

1.2.1.2 Significance

Presumably, uncontrolled polymerization of blood proteins can lead to reduced blood flow and subsequent death; consequently, FXIIIa activity must be regulated and inhibited. Although we don't know what happens when there's overexpression of FXIIIA, the size of the blood clot was significantly reduced when the activity of FXIIIa inhibited pharmacologically or genetically[6]. Since FXIIIa, and the fibrinolytic enzyme such as plasmin and tissue plasminogen activator (tPA) all localize in close proximity near fibrin during clotting, it was logical that fibrinolytic enzymes may regulate FXIIIa activity. Identifying a mechanism to inactivate FXIIIa

in vivo may provide an alternative method to regulate FXIIIa activity, as there are no small molecule inhibitors available to modify FXIIIa activity.

1.2.2 Determine the mechanisms responsible for bleeding during thrombolysis.

1.2.2.1 Biological Question

Thrombosis is a major health concern as one of the major causes of death in Canada. In patients with the appropriate anatomy, tPA is administered to degrade the thrombus and restore blood flow. Although thrombolysis is an effective treatment option, CDT carries a 1-2% risk of major bleeding, with high mortality and morbidity[7,8]. High levels of plasmin are generated during CDT to clear the blood clot. Since FXIIIa can be degraded by plasmin and congenital FXIII deficiency is associated with bleeding, the objective was to test if CDT alters FXIII and FXIIIa stability in DVT patients receiving CDT[9]. The concentrations of FXIII zymogen before and after thrombolytic therapy were measured to test if CDT leads to decreased FXIII levels. The stability of FXIIIa was also tested, comparing before and after CDT.

1.2.2.2 Significance

Although thrombolytic therapy carries significant risks, such as life-threatening bleeds, the mechanisms responsible for major bleeds and intracerebral hemorrhages during thrombolysis are not fully understood[10]. The side effects associated with thrombolysis are similar to symptoms observed in FXIII deficiency (FXIIID), providing sufficient rationale to examine the fate of FXIII(a) in patients treated with thrombolysis[11]. Identifying causes that lead to bleeding during thrombolysis will improve the treatment of thrombosis patients by reducing the side effects associated with thrombolysis.

1.2.3 Examine the role of FXIIIa in cerebral amyloid angiopathy

1.2.3.1 Biological question

In cerebral amyloid angiopathy (CAA), Aβ accumulates along the cerebral vasculature and is associated with degeneration of smooth muscle cells and microhemorrhages[12]. Aβ can bind with fibrin to alter clot structure and impede fibrinolysis[13]. Although FXIIIa colocalizes with Aβ in CAA and can crosslink fibrin, the ability of FXIIIa to crosslink Aβ has not been demonstrated[14]. The objective of this study was to determine if FXIIIa crosslinks Aβ to itself and to other proteins. The kinetic parameters of this reaction were determined using spectrometric analyses. Since platelets also contain FXIII and have been implicated in Alzheimer's disease (AD) development, the ability of platelets to induce Aβ oligomerization was tested as well. The effect of FXIIIa on inducing oligomerization of mutant forms of Aβ, which leads to AD development, was examined as well.

1.2.3.2 Significance

The accumulation and deposition of Aβ along the vasculature leads to degeneration and dysfunction of surrounding tissues. Although it is highly prevalent in patients with AD, the mechanism underlying Aβ deposition and accumulations has remained elusive[15]. Transglutaminase 2, another transglutaminase enzyme, induces accumulation and deposition of Aβ in the brain and leads to AD symptoms[16]. Understanding the mechanisms that lead to Aβ deposition along the vasculature will provide insights to prevent CAA and AD development.

1.3 Background information

1.3.1 Hemostasis

The process of preventing loss of blood during vascular damage is called hemostasis. At the first signal of vascular damage, platelets are the first cells to respond and initiate primary hemostasis. Platelets will aggregate at the sites of damaged vasculature to form the platelet plug and become irreversibly activated by the various signals to release biomolecules to proceed to secondary hemostasis, the generation of insoluble fibrin clots. The coagulation cascade can be initiated through two pathways: the extrinsic pathway and the intrinsic pathway, which act subsequently on the common pathway[17].

1.3.1.1 Extrinsic pathway

The extrinsic pathway is activated when the body recognizes the stress signal via tissue factor to rapidly make a blood clot. Tissue factor, in its activated form, binds and activates coagulation factor VII (FVII) to form the activated complex TF-FVIIa. This activated complex in turn activates coagulation factor IX (FIX) and when the concentration of TF-FVIIa is high, coagulation factor X (FX). TF-VIIa can also activate FX to FXa. Once FIX is activated, it forms another complex with activated coagulation factor VIII (FVIIIa) and activate FX. Activated FX (FXa) forms another complex with activated factor V (FVa) on phosphatidyl serine (PS) surfaces of platelets and activate prothrombin to thrombin, the key effector enzyme of the coagulation cascade[18].

1.3.1.2 Common pathway

Once thrombin is activated, it has many roles within the coagulation cascade: to form fibrin, to amplify the signal to generate more thrombin, the main constituent of blood clots, and to turn off the coagulation cascade in time. Thrombin cleaves fibrinogen into fibrin, which aggregates to form insoluble fibrils, preventing the flow of blood. Thrombin activates coagulation factor XIII (FXIII), which will covalently crosslink fibrin to itself and to other proteins to make the blood clot more resistant to degradation. Thrombin, together with thrombomodulin, also activates protein C, which leads to inhibition and clearance of FVa and FVIIIa. However, thrombin positively feeds back on its own activation via the intrinsic pathway, by activating factor XI (FXI), factor V (FV) and factor VIII (FVIII), which will lead to greater thrombin generation.[19]

1.3.1.3 Intrinsic pathway

The intrinsic pathway can be initiated by various signals such as nucleic acids, polyphosphate or clay to activate factor XII (FXII). Activated FXII (FXIIa) then activates FXI to activated FXI (FXIa), which cleaves FIX to activated FIX (FIXa). FIXa then binds with FVIIIa to form the tenase complex, activating factor FX and thus the common pathway. The intrinsic pathway serves as an amplification loop to generate more thrombin such that the "thrombin burst" will be achieved faster to effectively minimize blood loss[19].

1.3.2 Fibrinolysis

Once a blood clot has sealed the vascular damage to prevent blood loss, the body initiates a series of processes to heal the wound. Once the wound has been healed, the blood clot becomes degraded to restore normal blood flow through the vasculature. This process of clearing the blood clot after wound healing is called fibrinolysis. Fibrinolysis can be initiated either by tPA, which is released by the endothelial cells surrounding the wound site, or by urokinase plasminogen activator (uPA), which is released by platelets. These two enzymes cleave plasminogen to plasmin, the main effector of blood dissolution. Plasmin binds to and cleaves fibrin fibrils. There are innate inhibitors of fibrinolysis; plasminogen activator inhibitor (PAI) 1 prevent the protease functions of tPA and uPA. Plasmin can be inhibited by α2-antiplasmin (AP), by covalently attaching to the catalytic site[20].

1.4 Literature review

1.4.1 Coagulation factor XIII

FXIII is a 320 kDa tetrameric enzyme consisting of two catalytic A subunits (83 kDa) and two regulatory B subunits (80 kDa)[21]. FXIII is a protransglutaminase that, upon activation, covalently crosslinks fibrin to itself and to other proteins to make the blood clot resistant to fibrinolysis[22]. The catalytic A subunit is synthesized by cells of the bone marrow origin and released into the bloodstream or is expressed and maintained within platelets within the cytoplasm[23-25]. The regulatory B subunit is synthesized by hepatocytes and released into the blood stream and binds to the catalytic A subunit to increase its half-life[26,27]. Most plasmatic FXIII circulates in blood bound to fibrinogen, mediated by the FXIII B subunit[28,29].

FXIII is activated primarily by thrombin, which cleaves the first 37 residues of FXIII-A, the activation peptide[30]. In the presence of calcium, FXIII undergoes a conformational change and the two subunits dissociate into two homodimers[31]. The catalytically active A subunit, with its activation peptide cleaved, called FXIIIa or FXIII-A*.

The acyl transfer activity of FXIIIa is a two-step process through the catalytic triad Cys314, His373 and Asp396 of the A subunit[32]. The initial reaction is the formation of a thioester bond via a nucleophilic attack by the sulfhydryl group of cysteine and the release of ammonia from glutamine. The second reaction is the deacylation of the enzyme via transfer of the acyl group to a primary amine to form the isopeptide bond[32]. The most common primary amine donors are protein-bound lysine residues, facilitating the covalent linkage between two proteins such as fibrin and AP[33].

FXIIIa has over 140 substrates that it can crosslink, such as fibrin, fibronectin, collagen and vitronectin[34]. Fibrin is the most prominent and well-studied substrate of FXIIIa; crosslinking of fibrin by FXIIIa increases the stiffness, elasticity and branching of the fibrin fibers (Figure 1.2). Although the B subunit does not contribute directly to the catalytic activity of FXIIIa, its presence accelerates the crosslinking rate of fibrin[29]. FXIIIa also crosslinks AP to fibrin and increases the local concentration of AP and thus increase its efficacy to inhibit plasmin[35]. Other blood proteins that can be covalently crosslinked include PAI-1, plasminogen and complement proteins, but their significance has not been fully investigated[34]. FXIIIa within the cytoplasm of platelets can crosslink actin cytoskeleton and modulate the morphology of platelets[36].

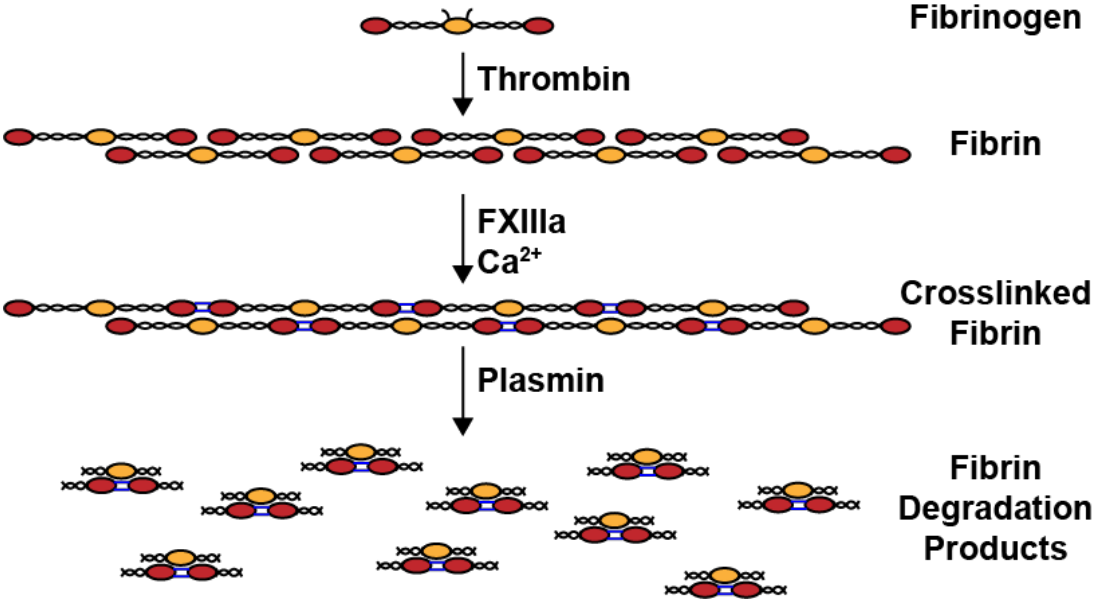


Figure 1.2 FXIIIa covalently crosslinks fibrin.

Since excessive FXIIIa activity leads to increased resistance to clot dissolution, there must be a mechanism to inactivate the enzyme[37]. However, the mechanism of inactivation of FXIIIa has remained elusive for decades. FXIII is degraded by several promiscuous proteases such as trypsin and chymotrypsin when incubated together[38]. Both subunits of FXIII can be degraded by proteases that are released by activated polymorphonuclear granulocytes, such as elastase, cathepsin G and metalloproteinase 9[39]. There are conflicting reports on whether enzymes of the fibrinolytic system can degrade FXIII. Henriksson and Nilsson showed that FXIII A could be degraded by leukocyte extracts that resembles plasmin[40]. Baggett et al. published a case study in which a patient with hyperfibrinolysis had reduced FXIII activity and treatment with aminocaproic acid to reduce fibrinolytic activity lead to restoration of FXIII activity in the patient[41]. Miloszewski et al. showed that FXIII levels remained normal when streptokinase, an activator of plasminogen, was incubated in plasma[5]. Rider and McDonagh

published a paper in 1981 that showed that FXIII is resistant to degradation or activation by plasmin[42].

FXIII has many roles in physiology, apart from the main role of hemostasis. Congenital FXIII deficiency (FXIIID) is a rare bleeding disorder occurring 1 in 2 million people[43]. FXIII deficiency often remains undiagnosed since most routine clinical assays such as platelet count, activated partial thromboplastin time (aPTT) and prothrombin time (PT) are normal in FXIIID[44]. Patients with FXIIID suffer from gastrointestinal bleeds and recurrent miscarriages. However, the most life-threatening condition for patients with FXIIID is intracranial hemorrhages (ICH). The most prominent bleed sites within the brain is intraparenchymal (accounting for >90%), though some reports of subdural and epidural bleeds have been reported[11]. Although the frequency of ICH is the highest among patients with FXIIID compared to other congenital bleeding disorders, the mechanism remains unknown.

1.4.2 Fibrinogen

Fibrinogen is a 340 kDa protein consisting of two Aα, two Bβ and two γ gamma chains. The concentration of fibrinogen in blood is 2-4 mg/ml, one of the most abundant protein in blood. Upon inflammation, its concentration can be acutely increased to as high as 7 mg/ml[45]. It consists of two outer D domains that connect to the central E domain via coiled coils[46]. The Aα, Bβ and γ chains dimerize within the E domain by 5 symmetrical disulfide bridges. Fibrinogen is synthesized within the hepatocytes before being released into the blood stream[47].

Fibrinogen circulates throughout blood until it reaches vascular damage and is cleaved by thrombin. Thrombin initially cleaves the fibrinopeptide A of the Aα chain, exposing Knob A to

which the binding socket of the γ binds; this interaction leads to the formation of half-staggered fibrin oligomers. Subsequently, fibrinopeptide B is cleaved from the Bβ chain, exposing Knob B, which binds to a corresponding pocket within another βchain, facilitating lateral aggregation of the fibrin fibers[48]. The cleavage of fibrinopeptide B occurs at the same time scale as the activation of FXIII, whose binding is regulated by the γ chain residues 390-396 and the B subunit[49,50]. Thus, the crosslinking of fibrin molecules to itself and to other proteins occurs after fibrin aggregate formation[51].

The primary function of fibrin is to create a mesh to prevent bleeding. The fibrin molecules will aggregate with itself to protofibrils which will further aggregate to form an intertwined network of fibrin[52]. Platelets will further bind to the C-terminus of fibrinogen γ-chain through the $\alpha_{IIb}\beta_{III}$ integrin[53]. White blood cells and red blood cells are trapped mechanically by the fibrin network to minimize the loss of blood cells[6,54]. Fibrin also plays a significant role in inflammation, interacting with leukocytes in many ways. Fibrinopeptide B is a chemoattractant for leukocytes and fibrinogen can bind with and activate leukocytes through receptors such as Mac-1 complement receptor[55]. Fibrinogen deficient mice have reduced inflammation in many diseases such as multiple sclerosis and arthritis[56,57].

The stability of fibrinogen is regulated by multiple mechanisms. FXIIIa crosslinks fibrin molecules to itself and antifibrinolytic proteins such as AP to the fibrin clot, to increase its resistance to fibrinolysis. The initial residues that are crosslinked by FXIIIa are Lys406 of the γ chain and GlN398 or Gln399 of another γ chain, forming the γ-γ dimers. FXIIIa can also form α chain oligomers, though much slower than γ-γ dimers, via Gln221, Gln237, Gln328 and Gln366

and many potential lysine residues[58]. The crosslinking of the fibrin chains increases the branching of the fibrin fibers as well as the stiffness and elasticity, making the fibrin fibrils more stable to physical detachment by the blood flow[37]. The density of the fibrin clot also regulates the stability of the blood clots. Denser clots have thinner fibrin fibrils, making it easier for the fibrinolytic enzymes to degrade through the clot. However, if the clot is denser, fibrinolytic enzymes have less access to fibrin strands, leading to increased stability[52]. One of the key factors that regulate fibrin density is the "thrombin burst"; higher the thrombin generation during coagulation, denser the clot will become[59].

Because fibrinogen contributes to a host of physiological reactions, fibrinogen deficiency is associated with numerous symptoms. The most prominent symptom is bleeding, which can be identified from bleeding within the umbilical cord[60]. Intracerebral hemorrhages are also common causes of death among patients with fibrinogen deficiency[61,62]. Depending on the severity of fibrinogen deficiency, patients may suffer from impaired wound healing, spontaneous splenic rupture, bone cysts and complications during menorrhagia and pregnancy[63-65].

1.4.3 Plasminogen

Plasminogen consists of a pre-activation peptide, 5 Kringle domains and the catalytic protease domain with a plasma concentration of 200 μg/ml[66]. The circulating form of plasminogen is the full-length peptide containing the pre-activation peptide (Glu-plasminogen); Glu-plasminogen can be cleaved by plasmin between Lys77 and Lys78 to form the Lys-plasminogen, which is more readily activated to plasmin than Glu-plasminogen due to its open configuration[67]. There are two glycoforms of plasminogen: type I containing both the N-linked

glycosylation to N289 and the O-linked glycosylation to T346 and type II containing only the O-linked glycosylation. The latter is found more prominently within blood clots[68,69].

Plasminogen circulates within the bloodstream in its closed conformation; when it binds to the C-terminal lysines of fibrin via its Kringle domains, it undergoes a conformational change to expose its cleavage site[70,71]. Its main activator, tPA, also binds to fibrin via its C-terminal lysines[72]. The tissue plasminogen activator will cleave the peptide bond between Arg561 and Val562 of plasminogen to form plasmin which consists of the N-terminal heavy chain and the C-terminal light chain bound together through disulfide links[66].

Plasmin is a serine protease that is mostly responsible for the dissolution of blood clots. Plasmin is able to cleave both fibrinogen and fibrin, although the catalytic efficacy is higher for fibrin than fibrinogen. It cleaves fibrin within the coiled-coil region, releasing Fragment E, Fragment D and D-dimers if fibrin is covalently crosslinked by FXIIIa[73]. D-dimers are frequently used clinically to assess the extent of thrombosis and/or fibrinolysis[74]. Although the main substrate of plasmin is fibrin, it has many other substrates, including FV, FIX, fibronectin, matrix metalloproteases (MMPs) and vitronectin[75].

Its activity in hemostasis is primarily regulated by serpins such as α2-macroglobulin and AP. The serpins contain a substrate-like domain to which the catalytic domain of plasmin reacts. The reaction leads to a covalent linkage of plasmin catalytic site with the serpin, generating a non-functional protease[76]. Thrombin activatable fibrinolysis inhibitor (TAFI) can also inhibit plasmin activity, not by directly inhibiting the protease function of plasmin, but by removing the terminal lysines to which plasmin binds[77]. Plasmin can also be inhibited with lysine-analogs such

as tranexamic acid (TXA) or ε-aminoprocaic acid (EACA); through their primary amines, they prevent the binding of plasmin to the substrate through its Kringle domain[78]. The function of plasmin is not limited to hemostasis but has many roles in complement system, wound healing and neuronal development[35].

Although a plasminogen deficiency occurs systemically, patients with plasminogen deficiency are often identified with ligneous conjunctivitis. It occurs in 80% of patients with plasminogen deficiency and is associated with the formation of a thick fibrin-rich pseudomembrane covering the upper eyelid or the cornea[79,80]. Patients with plasminogen deficiency also experience problems with pregnancy and breathing[80-82]. Surprisingly, deficiency in plasminogen is not associated with thrombosis, although there are issues with fibrinolysis[83]. However, there are many conditions in which plasmin is hyperactive such as during trauma-induced coagulopathy (TIC) and in patients with Quebec platelet disorder (QPD)[84,85].

1.4.4 Tissue plasminogen activator

Tissue plasminogen activator is a serine protease that is mostly responsible for the activation of plasminogen to plasmin. It plays a significant role in fibrinolysis, cell migration and tissue remodeling, as well as neuronal development[86]. It is 70 kDa and contains two Kringle domains that are involved with the binding of tPA and fibrin. TPA is synthesized as a single chain (sc-tPA) protein, which has near full activity. It can be cleaved by plasmin, creating two chains (tc-tPA) which remain bound with a disulfide bond[72]. The regulation of the tPA activity is crucial for proper clot lysis. Upon vascular damage, the endothelial cells surrounding the site of

damage will synthesize and release tPA to significantly increase the local concentration of tPA at the site of damage[87].

TPA cleaves plasminogen to plasmin through the catalytic triad involving His-322, Asp-371 and Ser-478[72]. Both sc-tPA and tc-tPA are proteolytically active, although the tc-tPA is more active than sc-tPA in the absence of allosteric regulators. However, activity of sc-tPA and tc-tPA are comparable in the presence of fibrin, where tPA and plasminogen are optimally oriented for the reaction to occur 1000-fold more efficiently compared to in the absence of fibrin[88].

TPA activity is highly regulated to prevent aberrant clot lysis. TPA can be inhibited by plasminogen activator inhibitor 1 or 2 (PAI-1 or PAI-2, respectively), although PAI-2 plays a significant role only during pregnancy[89]. PAI-1 is synthesized from the endothelial cells, as well as adipocytes. However, the majority of PAI-1 is found within platelets, which are released upon platelet activation[90]. PAI-1 is a serpin that will interact with the catalytic site of tPA to irreversibly inhibit protease function. The half-life of free tPA is approximately 4 minutes in plasma although the half-life tPA-PAI-1 complex are cleared twice as fast in the liver[20,91].

Dysregulation of tPA has many consequences, particularly in fibrinolysis and brain development. In fibrinolysis, a deficiency of tPA can lead to increased thrombosis, particularly along the cerebrovasculature, compared to wild type (WT) mice. Mice deficient in tPA have reduced long-term potentiation, suggesting that tPA is involved in memory formation[92]. Increased tPA activity, either by administration of tPA during thrombolytic therapy or released during TIC can lead to disruptions in the blood-brain barrier, increasing probability of

intracerebral hemorrhage (ICH)[93,94]. Deficiency in tPA is also associated with rearrangement of the cerebrovasculature and ventricular malformations[95].

1.4.5 Thrombosis

Thrombosis is the formation of an aberrant blood clot that prevents blood flow. Thrombosis can be divided into two types: arterial and venous thrombosis. Arterial thrombosis is the formation of blood clots within the arteries, mostly caused by the rupturing of a fat-rich deposit within the blood vessels, an atheroma. Arterial thrombosis is often considered "white"; the blood clot contains fewer red blood cells (RBC) and fibrin and more platelets in comparison to clots found in venous thrombosis. Venous thromboembolism (VTE) is the formation of blood clots within the veins, often caused by disturbed blood flow near the valves of deep veins. Venous blood clots are considered "red" as they have a more RBC and fibrin content and less platelets in their composition[96].

Stroke is one of the leading causes of death worldwide. Each year, approximately 15 million people suffer from stroke; of these, 5 million die and another 5 million people become permanently disabled[97]. There are two types of strokes: ischemic and hemorrhagic. Hemorrhagic stroke occurs when a blood vessel of the brain bursts and blood leaks into the brain; the decreased transport of nutrients to the brain as well as increased pressure causes damage to the surrounding brain cells (further discussed in Section 1.1.7.) Ischemic stroke accounts for over 85% of strokes and occurs when the blood vessels in the brain are blocked and prevent blood flow and consequently transport of nutrients and oxygen[98]. The most prominent cause of stroke is atherosclerosis, narrowing of the blood vessel. When the atherosclerotic plaque ruptures, blood

clots form at the site of the plaque rupture to prevent blood flow, or can be subsequently dislodged to be lodged somewhere else in near the brain[99].

Myocardial Infarction (MI), also known as heart attack, occurs when the blood flow to the heart is insufficient, usually blocked by a blood clot. It is one of the leading causes of death worldwide, accounting for 12.7% of all deaths, with 7.3 million deaths, although up to 20-fold variation was observed among different countries[100]. MI can be either symptomatic or asymptomatic; about 1 in 5 cases of MI are asymptomatic such that patients may not know of their own conditions. There are many risk factors associated with MI such as abnormal lipids, smoking, hypertension, obesity and lack of physical exercise[101].

Deep vein thrombosis (DVT) occurs when blood from returning to the heart is blocked by a blood clot deep in the body, usually in the lower leg or pelvis. There are several factors that lead to DVT, including reduced blood flow around the valves of the veins, endothelial damage and genetics[102]. It affects 1 to 2 in 1000 people in US, with mortality as high as 100 000 per year[103]. The reduced blood flow near the valves facilitates the accumulation of clotting signals that results activation of the coagulation cascade and subsequent clot formation. The blood clot may remain deep in the body, or may break off and embolize.

When the blood clots from DVT embolize, they ultimately end up in the pulmonary arteries to block blood flow to the lungs, a condition known as pulmonary embolism (PE)[104]. It affects up to 60-70 per 100 000 people; however, silent PE can develop in 40-50% of patients with DVT [105]. Untreated PE can be a fatal condition with mortality rate as high as 30%, which can decrease to 8% following treatment[106]. PE and DVT share the same risk factors; age,

genetics, hospitalization and comorbidities such as cancer can increase the risk of PE. In cancer patients, the incidence of PE was higher at 3%[107].

There are many options to treat various types of thromboses such as stroke and DVT. The most common form of treatment is prophylaxis. Some of the risk factors of thrombosis can be modified by losing weight, increasing exercise and ceasing to take contraceptives[108,109]. Non-modifiable risk factors such as Factor V Leiden mutation can be treated with medications; anti-coagulants such as warfarin or rivaroxaban and anti-platelets such as aspirin are often used for prophylaxis of thrombosis[110]. Both anti-coagulants and anti-platelets are used to treat arterial thrombosis, but anti-platelet drugs are not as effective as anti-coagulants in preventing incidences of VTE[111,112]. A major side effect of the anti-coagulant and anti-platelet therapy is the risk of bleeds. Although there are some reversal agents for these drugs, bleeding remains a major complication to consider in determining treatment options.

Once the patients are admitted to the hospital with deep vein thrombosis, they are treated with anti-coagulants such as low molecular weight heparin and direct oral anti coagulants. They will prevent further expansion of the thrombus and allow the physiological fibrinolytic system to degrade the clot. Although anticoagulation is the primary treatment, two other options should be mentioned. One complementary treatment is to place inferior vena cava filters upstream of the blood clot towards the heart. Although the incidences of PE decrease with filter placements, the risk of recurrent DVT increases[113].

1.4.6 Thrombolytic therapy

The other treatment is thrombolysis; thrombolytic therapy involves the injection of "clot busting" enzymes into the blood to degrade the blood clot. Once these thrombolytic agents are injected, the concentration of plasmin generated will overcome the anti-fibrinolytic effect in blood and degrade the thrombus.

There are several Food and Drug Agency approved products as thrombolytic agents: streptokinase (SK), urokinase plasminogen activator (uPA) and tPA[114]. Both SK and uPA, unlike tPA, can activate plasminogen efficiently with or without fibrin and therefore has a higher probability of systemic fibrinolysis. Several variants of tPA are approved as thrombolytics. Altepase (recombinant tPA), Retaplase (smaller derivative of tPA with higher potency and faster acting) and Tenecteplase (tPA derivative with longer half-life and greater binding affinity for fibrin) are all available options[115]. In DVT treatment, thrombolytic agents are injected at the site of the blood clots using a catheter, a technique called catheter-directed thrombolysis (CDT). Alteplase is injected directly to the thrombus at a rate of 1 to 2 mg/hr up to 4 days until the blood flow is restored[116].

Although CDT reduces the duration of hospital stay and the probability of mortality, recurrent VTE and post thrombotic syndrome, it increases the probability of major bleeds as a side effect. Also, only 26% of patients with DVT are eligible for thrombolytic therapy[117]. There are many factors to consider when selecting patients for CDT; the experienced vascular interventionalists must consider the DVT characteristics, such as time of onset, and patient characteristics, such as age and comorbidities and risk for post-thrombotic syndrome to minimize

the risk of undesired bleeding. One of the most feared side effects of thrombolytic therapy is intracranial hemorrhages. In DVT patients, about 1% of all patients receiving CDT suffer from ICH. However, the causes of ICH during thrombolytic therapy remains largely unknown[7,8].

Many factors contribute to the frequency of ICH during thrombolysis. Low concentration of fibrinogen, the main constituent of the clot, has been associated with increased risk of major bleeds. The rate of major bleeds was significantly higher in patients whose fibrinogen level at the end of the treatment was lower than 1.5mg/ml[118]. Other risk factors that lead to major bleeds during thrombolysis are longer duration of therapy, hypertension, diabetes, obesity and pregnancy[119].

1.4.7 Intracranial hemorrhage

ICH is a life-threatening condition that occurs spontaneously to 25 per 100 000 people worldwide[120]. The mortality rate is 35% to 52% within 30 days and only 20% of the patients are expected to make full recovery within 6 months[121]. There are several risk factors of ICH: male sex, older age, ethnicity, alcohol intake, hypertension and cerebral amyloid angiopathy (CAA), a condition in which protein aggregates are deposited along the cerebral vasculature[122]. Chronic hypertension causes degeneration along the walls of the small-to-medium penetrating vessels. CAA leads to degeneration of surrounding smooth muscle cells, thickening of the vessel wall and narrowing of the lumen and subsequent microhemorrhages. Once the vessel ruptures, the increased pressure from the hematoma causes brain damage. The blood components have their subsequent damage through inflammation and coagulation[10].

There are several types of ICH, categorized by the location where the bleeds occur. Epidural hematoma occurs when a blood clot forms between the skull and the tough outer most covering of the brain, dura mater. It often occurs following a head injury, as the brain bounces against the skull and the internal lining and the tissues within the brain are torn. Subdural hematoma is the accumulation of blood between the dura mater and the underlying arachnoid mater. The mortality rate ranges from 50-90%, with only 20-30% of the treated patients expected to make a full recovery. Subdural hematomas are common in patients undergoing anticoagulant or antiplatelet therapy[10]. Subarachnoid hemorrhages (SAH) occur when blood leaks into the space between the arachnoid membrane and the pia mater surrounding the brain. SAH may occur following a head injury or spontaneously, often following a cerebral aneurysm. Spontaneous SAH occurs in about 1 in 10 000 people each year[123]. Intracerebral hemorrhage occurs within the brain tissues, often due to hypertension, trauma, aneurysms and tumours. Spontaneous intracerebral hemorrhages occur in approximately 3 per 10 000 people every year[124]. FXIII deficient patients, thrombosis patients treated with thrombolysis and patients with CAA deposits all have increased risk of developing intracerebral hemorrhages, particularly within the intraparenchymal spaces[11,125-127].

1.4.8 Alzheimer's disease and cerebral amyloid angiopathy

Alzheimer's disease (AD) is a neurodegenerative disease that affect more than 50 million people worldwide. It accounts for 60-70% of all types of dementia. Patients with AD suffer from short-term memory loss as well as mood swings, cognitive impairment and disorientation as the disease progresses. The frontal cortex and the hippocampus slowly degenerate over time, leading to a decreased brain mass and loss of neuronal connectivity. Currently, there are no treatment

options for AD, although drugs have been approved to reduce the rate of cognitive decline. Although the cause of AD has not been fully elucidated, AD is strongly associated with aggregates of amyloid beta (Aβ) and tau proteins. Senile plaques are large protein aggregates within the brain parenchyma, consisting mostly of Aβ. CAA is the accumulation of Aβ and other proteins along the cerebral vasculature. Neurofibrillary tangles (NFT) are intracellular deposits of tau proteins that have misfolded and aggregated. In all of these cases, the accumulation of protein aggregates has toxic effects on surrounding cells, leading to death of surrounding cells, microhemorrhages and synaptic disfunction[128].

CAA is a disease of the vessel wall that is characterized by the amyloid deposits around the vessel wall in the cerebral nervous system. The deposits form within the cerebral cortex and the leptomeninges; it usually occurs within the posterior cortical regions, frontal temporal and parietal lobes as well as the cerebellar regions[122]. Aβ is deposited within the tunica media, smooth muscle cells and adventitia, leading to smooth muscle cell loss, microaneurysms and lobar intracerebral hemorrhages[15]. Although Aβ is the major constituent of CAA deposits, many coagulation factors, such as fibrinogen, FXIII and thrombin have been detected within CAA deposits[14].

There are two types of CAA: type 1 and 2. Type 1 CAA has accumulation of Aβ within the cortical capillaries and other vessels. Type 1 a has higher association with AD development. Type 2 CAA are detected in the leptomeningeal and cortical arteries, arterioles and veins only[129]. Post-mortem studies show that CAA is detected in 10-40% of non-demented people and in over 80% of AD patients, although only 25% showed severe CAA pathology[130,131]. There are few risk

factors of CAA, such as increased age and genetics. Apolipoprotein E alleles increase the risk for sporadic CAA as well as mutations within the amyloid beta precursor protein (APP). The Dutch-type mutation (E693Q), Italian mutation (E693K), Arctic mutation (E693G), Flemish mutation (A692Q) and Iowa mutation (E694N) all lead to increased CAA pathology[132-136]. Gene duplication or extra chromosome 23, where the *APP* gene is located, also leads to increased CAA phenotype[137].

1.4.9 Amyloid beta and its precursor protein

APP is a type 1 transmembrane protein that is expressed in a wide range of cells including neurons, glial cells, astrocytes and platelets. There are 3 major isoforms of APP that are 695, 751 and 770 amino acids long; the 696 amino acid isoform is the most prominent in the central nervous system, while the other two isoforms are expressed ubiquitously. APP consists of several domains, including the Kunitz protease inhibitor (KPI) domain, heparin binding domain, zinc binding domain and collagen binding domain[138].

The physiological function of APP remains unclear. Transgenic mice overexpressing WT APP had increased neuron size, while the cell lines with APP transfected showed increased cell-cell adhesion, motility, neuronal outgrowth and survival[139]. The extracellular domain, when injected into adult AD animals, can improve cognitive function and synaptic density[140]. The KPI domain of APP has been implicated to play a role in regulating coagulation in the cerebrovasculature[141].

On the contrary, genetic deletion of APP in mice produced very minor phenotype. The knock out (KO) mice have about 20% less body mass compared to wild type (WT) mice. They

have reduced locomotor activity, weakened grip strength and compromised performance during in a Morris water-maze spatial memory task[142]. However, the triple KO of APP and amyloid beta precursor protein like protein (APLP) 1 and 2 are embryonic lethal, suggesting that there is redundancy of function in the protein family[143].

APP is localized to either the cell surface or endosome. On the cell surface, APP can be cleaved by α-secretase to generate the sAPPα, and C83 peptide. The C83 peptide is further cleaved by γ-secretase to make APP intracellular fragment (AICD) and p3 fragment. This is the non-amyloidogenic pathway and occurs under normal conditions. In the endosomes, APP undergoes the amyloidogenic pathway within the lipid rafts. It is first cleaved by β-secretase to generate sAPPβ and C99 fragment, which is cleaved by γ-secretase to generate AICD and Aβ. The γ-secretase is a promiscuous enzyme and cleaves C99 at one of several sites to generate Aβ that vary in size. The most common forms of Aβ are 40 and 42 amino acids long[138].

Aβ is a 4 kDa protein that is intrinsically disordered. It can be detected in blood and in the cerebrospinal fluid (CSF) at concentrations near 250 pg/ml and 5 ng/ml, respectively[144]. Due to its hydrophobic nature, Aβ aggregates into beta-sheet fibrillar structures[145]. The aggregation of Aβ is a two-step process with nucleation and elongation of the fibrils. The aggregation is accelerated in the presence of divalent ions such as Ca^{2+}, Zn^{2+} and Cu^{2+}[146,147]. Another factor that regulates the rate of Aβ aggregation is the concentration of Aβ; the rate of Aβ synthesis and clearance play significant roles in Aβ aggregation and accumulation in the brain and in blood[148].

1.4.10 Aβ, APP and AD in hemostasis

Over the last decade, there has been increasing research on the relationship between hemostasis and AD. Platelets have been widely studied as an *ex vivo* model of AD and has been considered as a potential biomarker for AD. There are two forms of platelet APP detected on Western blots at 110 kDa and 130 kDa[149]. Although one study showed that patients with AD had increased ratio of 130 kDa isoform to the 110 kDa isoform compared to people without AD, the results could not be repeated and is still under investigation[150]. Platelets account for over 95%, with 9300 copies/platelet, of all circulating APP and express all the secretases to cleave APP into its metabolites[151]. Platelets store APP and Aβ at the surface membrane and the α-granules; upon activation, platelets process APP to synthesize Aβ and also release Aβ from the α-granules[152]. Platelets have also been implicated in CAA development; in a mouse model of AD, anti-platelet drugs were injected and the mice had reduced CAA burden and had improved cognitive function compared to mice without anti-platelet drugs[153].

APP interact with hemostasis in multiple ways. Platelet APP, through its KPI domain, can inhibit FXa, FIXa, FXIa and FVIIA:TF complex[154,155]. APP KO mice had increased clot size in a stasis model of VTE compared to WT mice, suggesting platelet APP has anti-coagulant properties[156]. Platelet APP did not play a role in platelet aggregation, activation, secretion of granule content, tail bleed times or arterial clot formation.

Aβ binds with multiple coagulation factors. Fibrillar Aβ42 can interact with FXII to activate it and promotes thrombin generation[157]. Aβ42 aggregates interact with fibrinogen with a K_d of 26.3 ± 6.7 nM, binding to the C-terminus of the β chain[13]. This interaction leads to

abnormal clot formation with delayed fibrinolysis, where clusters of Aβ42 aggregates prevented non-homogenous fibrin arrangement[158]. Moreover, when Aβ42 binds to fibrin, it inhibits the binding of both plasminogen and tPA, leading to reduced rate of fibrinolysis. Preventing the interaction between Aβ and fibrin reduces the accumulation of Aβ in the cerebral cortex and improved the cognitive function in a mouse model of AD[159]. Cerebral vascular cells react to Aβ by increasing the expression of uPA and its receptor[160]. Aβ, FXIII and thrombin are all present and colocalize within CAA[14]. Aβ and FXIII can form stable complexes, but whether FXIIIa can covalently crosslink Aβ has not been demonstrated. Aβ peptide, particularly the $A\beta_{25\text{-}35}$, can activate platelets through the RhoA-mediated platelet cytoskeleton remodeling[161].

Chapter 2: Coagulation factor XIIIa is inactivated by plasmin

2.1 Contribution

This project was a collaborative research and published in Blood (paper attached in the appendix). W.S.H. conceived the idea, designed all experiments and performed experiments, analyzed and interpreted all data and wrote the paper. I performed experiments to collect data specifically for Figures 2.1, 2.2A-E, 2.3, 2.4, 2.5, 2.7, 2.8, 2.9, 2.10, 2.10A-B and 2.11. I contributed to 75% of this paper. N.M. designed and performed experiments for Figure 2.2F, 2.6, 2.10C-E, analyzed and interpreted data, and wrote the paper. C.J.K. helped design experiments, interpret data and write the paper. Collaborators and undergraduate thesis students contributed to different aspects of the paper, such as methods development (J.R.B.), performing preliminary experiments (X.J.L and H.M.B.) and data analysis (A.S.W.).

2.2 Introduction

The coagulation system controls a polymerization process that is required to seal vascular damage. Fibrin is quickly generated and cross-linked into a mesh of insoluble fibers by the transglutaminase factor XIIIa (FXIIIa). Mechanisms of synthesis and activation of FXIIIa are known[4], however the physiological mechanism of inactivation remains unclear.

Plasma FXIII (pFXIII-A_2B_2) consists of two catalytic A subunits and two regulatory B subunits arranged as a heterotetramer, whereas platelets and monocytes express intracellular FXIII-A_2 without the B subunits (cFXIII-A_2)[4]. During clotting, thrombin cleaves a 37-residue activation peptide from the A subunit of pFXIII-A_2B_2. Then, in the presence of calcium, the A subunits disassociate from the B subunits and undergo a conformational change to become

catalytically active. The active FXIII-A_2*, also referred to as FXIIIa, catalyzes the formation of a pseudo-peptide bond between a glutamine residue of one protein and a lysine residue of another protein, releasing ammmonia[4]. Fibrin is the major substrate of FXIII-A_2*. Self-assembled fibrin becomes stabilized when it is covalently cross-linked to itself, the vessel wall, and to anti-fibrinolytic proteins by FXIII-A_2*. In plasma, pFXIII-A_2B_2 circulates bound to fibrinogen[162]. Although most FXIIIa remains bound to fibrin during clot formation[163], active FXIIIa released from the clot, such as during clot lysis, would presumably circulate systemically. While it has been reported that FXIIIa can be inactivated by thrombin[164] or proteolytic enzymes released by granulocytes[39], it remains unclear whether these mechanisms extend to all the intracellular, intravascular and extravascular FXIIIa. These reports have led to the question: Are there other mechanisms of inactivation of FXIIIa?

A long-standing hypothesis is that FXIIIa is inactivated by the fibrinolytic system, which normally degrades the fibrin clot and associated proteins[165]. The fibrinolytic enzyme plasmin is generated from plasminogen by activators such as tissue plasminogen activator (tPA). Previous reports found that cFXIII-A_2, cFXIII-A_2*, and pFXIII-A_2B_2 were resistant to plasmin degradation[42]. Here, we show that zymogen FXIII is resistant to degradation by fibrinolytic enzymes at physiological concentrations. However, contrary to previous reports, the enzymatically active FXIII-A_2* is readily degraded by endogenous plasmin during clot lysis.

2.3 Methods

2.3.1 Activating and degrading FXIII A and B subunits in purified systems and in blood

To generate FXIII-A_2*, human pFXIII-A_2B_2 (Haematologic Technologies Inc.) was incubated with bovine thrombin (Sigma, 400 nM, 2 U/mL) and calcium chloride (4 mM) for 30 min in HEPES-buffered saline (HBS) (10 mM HEPES, 140 mM NaCl, pH 7). Thrombin was then inhibited by hirudin (EMD Millipore, 400 ATU/mL), and plasmin (Haematologic Technologies Inc.) was added to pFXIII-A_2B_2 or pFXIII-A_2* (30 nM, 0.6 U/mL). For platelet experiments, platelets (1×10^{11}/L) were activated with bovine thrombin (400 nM, 2 U/mL) before incubation with plasmin or tPA (Genentech). For plasma-based experiments, citrated plasma was recalcified (10 mM $CaCl_2$) and then activated with diluted Innovin (Dade Behring, 1 pM) for 30 min. Fibrinogen-deficient plasma was activated with thrombin (200 nM, 1 U/mL) to fully activate FXIII. In control samples that were not activated, hirudin (100 ATU/mL) was added to inhibit endogenous thrombin and thus FXIIIa generation. Tranexamic acid (TXA) (Sigma, 7.5 mM), an inhibitor of plasmin and tPA[166], was added to specified samples before plasmin or tPA was added. All samples were incubated at 37 °C. Each experiment was repeated at least three times. All *p* values were calculated by two-tailed Student's *t*-test.

2.3.2 Preparing platelets

This study was approved by the research ethics boards of the University of British Columbia, and informed consent was obtained from all healthy volunteers before whole blood donation. Whole blood was collected into tubes containing sodium citrate (12 mM) and was centrifuged at 100 × g for 20 min. The top 75% of the PRP fraction was collected. Platelets were

pelleted by centrifugation at 250 × g for 10 min, and washed in CGS buffer (120 mM NaCl, 30 mM D-glucose, 11 mM trisodium citrate, pH 6.5) and then in Tyrode's buffer (1.8 mM $CaCl_2$,137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 10 mM HEPES, 5.6 mM monohydrate D-glucose, 1.1 mM $MgCl_2$, pH 6.5). Platelets were then resuspended in Tyrode's buffer at a final concentration of 2×10^{11} platelets/L.

2.3.3 Western blotting

Samples were prepared as described previously.[6] All samples were reduced and boiled prior to electrophoresis on 4-15% Mini-PROTEAN TGX gel (Bio-Rad) and transferred to a nitrocellulose membrane (Pall Life Sciences). Boiling the samples did not induce auto-degradation of FXIII-A* (Figure 2.1). After blocking with Odyssey Blocking Buffer (Li-Cor), the membrane was incubated overnight at 4 °C with the primary antibody; sheep or rabbit anti-human FXIII A (Affinity Biologicals or Thermo, respectively, diluted 1:1000), rabbit anti-human FXIII B (Sigma, diluted 1:1000) or rabbit anti-human fibrinogen (Dako, diluted 1:50 000). The membrane was washed with phosphate buffered saline with Tween 20 (8 mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl, 0.05% Tween 20, pH 7.4). After incubation with the pre-adsorbed secondary antibody (Abcam, diluted 1:10,000), the membrane was washed and antibody detected with ECL substrates (Bio-Rad). Membranes were stripped with a solution of 62.5 mM Tris pH 6.8, 2% SDS, and 0.7% beta-mercaptoethanol for 1 hr at 60 °C with occasional agitation and then reprobed. The statistical significance was calculated using a two-tailed Student's *t* test.

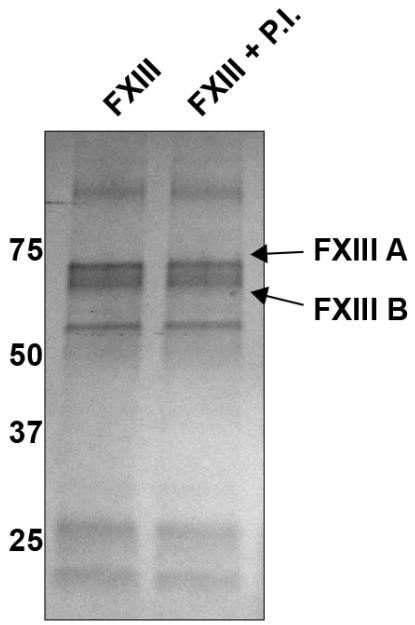


Figure 2.1 FXIII did not degrade when heat-inactivated at 95°C.

FXIII was incubated with or without a protease inhibitor cocktail (P.I.) and heated for 5 min at 95°C.

2.3.4 Identifying the plasmin-mediated cleavage site of FXIIIa

FXIII-A_2* (10 μM) was incubated with plasmin (2.7 μM) at 37 ºC for 2 hr. The reaction was stopped by heat-inactivating the sample at 95 ºC for 5 minutes. The samples were labeled on the newly exposed N-termini using reductive methylation, trypsin-digested and cleaned[167-169]. Peptide samples were purified by solid phase extraction and analyzed using a linear-trapping quadrupole-Orbitrap mass spectrometer on-line coupled to an Agilent 1290 Series HPLC using a

nanospray ionization source. Centroided fragment peak lists were processed with Proteome Discoverer v.1.2 and searched with Mascot algorithm. The peptides identified as cleavage products were those that had IonScores over the 99% confidence limit. This experiment was repeated twice with similar results.

2.3.5 Measuring the kinetics of inactivation of FXIII-A_2* by plasmin

Plasmin-mediated inactivation of FXIII-A_2* was evaluated using steady-state kinetics at 37 ºC. The rate of inactivation of FXIIIa was determined by measuring the transglutaminase activity of FXIII-A_2* via the production of ammonia. First, a calibration curve was generated that related the concentrations of ammonia to A_{570} using a colorimetric detection assay for ammonia (BioVision Inc.), monitored with a Tecan M200 plate reader. A second calibration curve was generated that related the concentration of FXIII-A_2* (1.6 to 78.1 nM) to the velocity of ammonia generation. This transglutaminase assay was performed by mixing FXIII-A_2* with an amine donor (glycine ethyl ester, GOE, 2.5 mM), a glutamine-containing peptide (NQEQVSPLTLLK, 1 mM), DTT (40 μM), and $CaCl_2$ (3 mM). Aliquots from the transglutaminase reaction mixture were removed and quenched every 15 min with EDTA (15 mM). Plasma FXIII-A_2B_2 (7.8 to 62.5 nM) was converted to FXIII-A_2* by pretreating it with thrombin (4 U/mL) and $CaCl_2$ (3 mM) for 1 hr to distinguish the rate of degradation by plasmin from the rate of activation by thrombin. Thrombin was then inhibited with excess hirudin (13 ATU/mL). To this mixture, plasmin (300 nM) was added. At various time-points, aliquots were removed and plasmin activity was quenched with a cocktail of protease inhibitors (Sigma). The velocity of inactivation of FXIII-A_2* was determined

by measuring the residual amount of FXIII-A_2* activity at each time point using the transglutaminase assay with the two calibration curves. Kinetic parameters were calculated using graphing software (OriginPro 9.1). This full experiment was repeated three times with similar results.

2.3.6 Measuring elastic moduli by thromboelastography (TEG)

Studies were performed using normal, plasminogen-deficient, α2-antiplasmin-deficient or fibrinogen-deficient plasma (Haematologic Technologies Inc.) and shear elastic moduli were evaluated at 37 ºC using a TEG Hemostasis Analyzer System 5000 (Haemoscope Corporation). Inactivation of FXIII-A_2* was evaluated during clot formation by combining 230 μL of with $CaCl_2$ (10 mM), Innovin (2 pM), and tPA (200 pM), with or without T101 (0.8 mM). FXIIIa inactivation was evaluated during clot lysis by initiating clotting of plasma with $CaCl_2$ (10 mM) and bovine thrombin (400 nM, 2 U/mL), allowing the clot to form for 30 min and then adding tPA (800 nM). Fibrinolysis was inhibited by TXA (10 mM) at either 1 or 3 hr after addition of tPA and the resulting plasma was added to a TEG cup containing fibrinogen (FXIII-free 1.4 mg/mL, Enzyme Research Laboratories), thrombin (2 U/mL), and either HBS, T101 (0.8 mM), or FXIII (200 nM), upon which the TEG analysis began. FXIII-A_2* inactivation was evaluated during clot formation under conditions mimicking thrombolysis by combining $CaCl_2$ (10 mM), Innovin (2 pM), and each plasma containing either 5 nM tPA (α_2-antiplasmin-deficient plasma) or 50 nM tPA (normal and plasminogen-deficient plasma). Activation of clotting progressed for 4 minutes before adding

TXA (10 mM), followed by adding HBS, T101 (0.8 mM), or FXIII (200 nM), then fibrinogen (1.4 mg/mL, Haematologic Technologies Inc.), before commencing TEG analysis.

2.4 Results

2.4.1 Activated FXIII-A_2*, but not zymogen pFXIII-A_2B_2, is proteolytically inactivated by plasmin

To evaluate the degradation of plasma-derived FXIII by plasmin, pFXIII-A_2B_2 and FXIII-A_2* were each treated with varying concentrations (100 pM to 100 nM) of plasmin for 3 hr, and analyzed by Western blot. During the 3 hr, FXIII-A_2* was cleaved by concentrations as low as 1 nM of plasmin (Figure 2.2A). Cleavage products of ~50 and 25 kDa were visible, but were transient. However, the A subunit of the zymogen was more resistant to cleavage than the enzyme (Figure 2.2B, Figure 2.3). Degradation of the B subunit from both activated and unactivated pFXIII-A_2B_2 also occurred (Figure 2.2C and 2.2D). Activation of FXIII by plasmin was not observed. Cleavage products of the B subunit, and possibly other cleavage products of the A subunit, may not have been detected due to a faster rate of degradation relative to that of intact FXIII, or a low abundance or absence of antigens identified by the antibody (Figure 2.4). To evaluate if the cleavage products were enzymatically active, the cleavage and activity of FXIII-A_2* were monitored over time. Degradation of FXIII-A_2* by plasmin (90 nM) was evident within 5 min and the transglutaminase activity of FXIII-A_2* decreased rapidly over 30 minutes (Figure 2.2E and 2.2F). The loss of FXIII-A_2* activity correlated with the loss of fully intact FXIII-A_2*, indicating that cleavage products of FXIII-A_2* were enzymatically inactive.

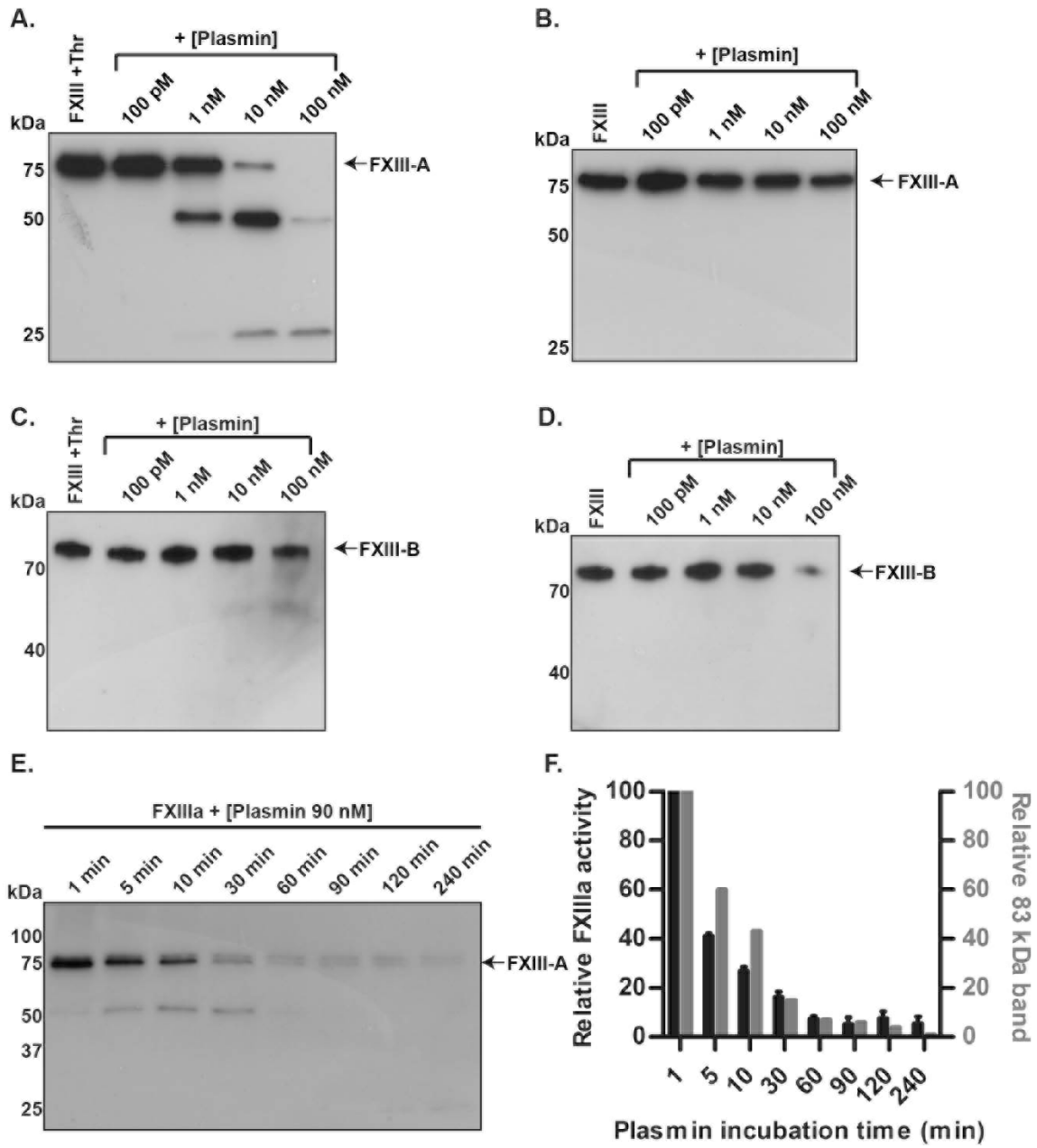


Figure 2.2 FXIII-A_2* is cleaved and inactivated by plasmin.

FXIII (100 nM), with or without prior activation by thrombin (400 nM), was mixed with varying concentrations of plasmin (100 pM to 100 nM) for 3 hr, and analyzed by Western blot against FXIII A and B subunits. (A) Blot against the A subunit of pFXIII-A_2*, with thrombin activation.

(B) Blot against the A subunit of pFXIII-A_2B_2. (C) Blot against the B subunit of pFXIII-A_2*, with thrombin activation. (D) Blot against the B subunit of pFXIII-A_2B_2. (E) Time course of cleavage of FXIII-A_2* by plasmin (90 nM). (F) Transglutaminase activity (left axis) of FXIII-A_2* after incubation with plasmin (90 nM) and the relative amount of intact FXIII-A_2* (right axis), determined by quantifying the intensity of the band at 83 kDa. FXIII-A* was calculated as % of total signal in the lane using densitometry. The error bars represent S.E.M. n = 3 for all experiments

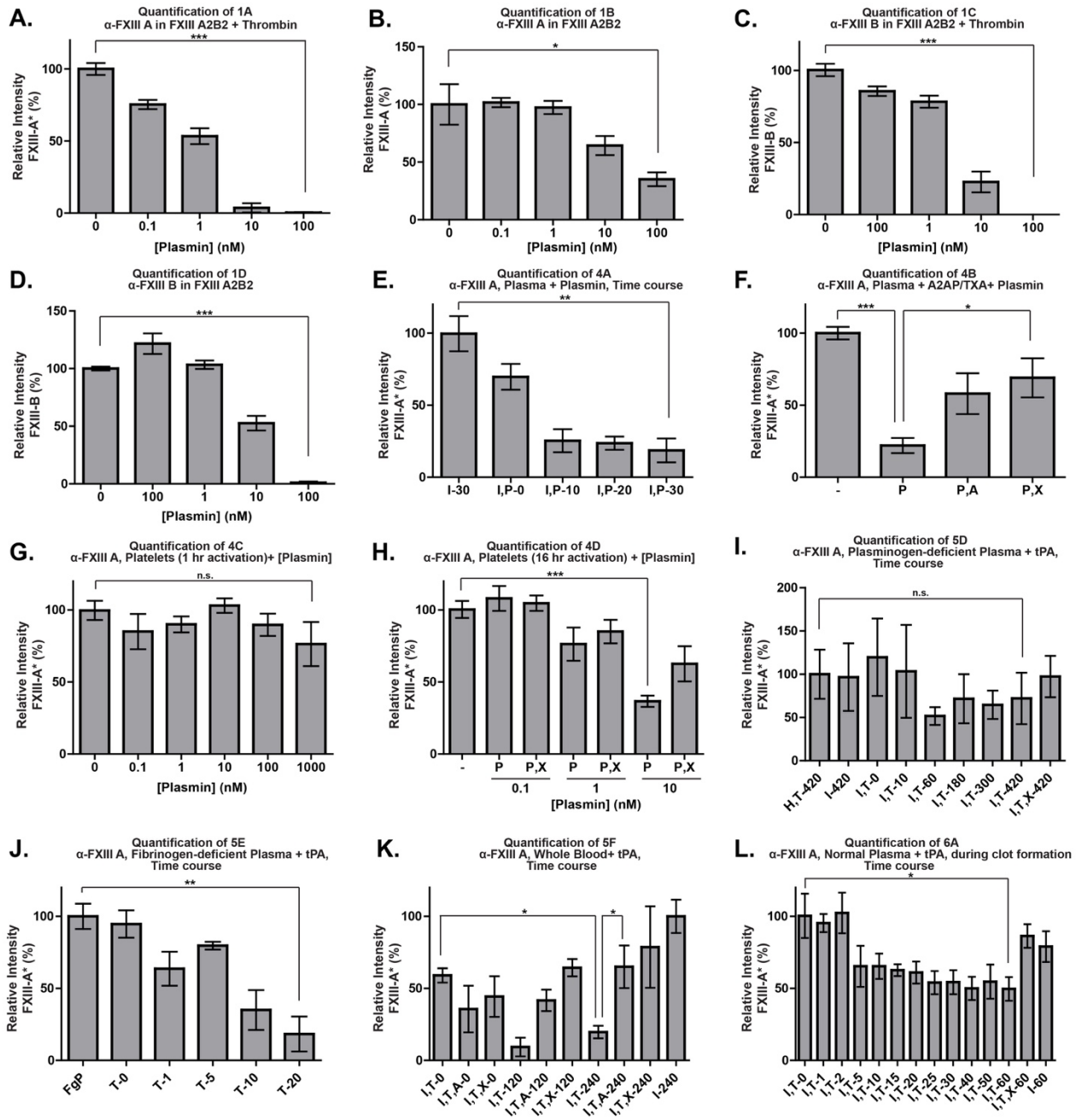


Figure 2.3 Quantification of Western Blots from figures in the main text.

FXIII-A* was calculated as % of total signal in the lane using densitometry. *P < 0.05, **P < 0.01, ***P < 0.001; n.s, not significant. The error bars represent SEM.

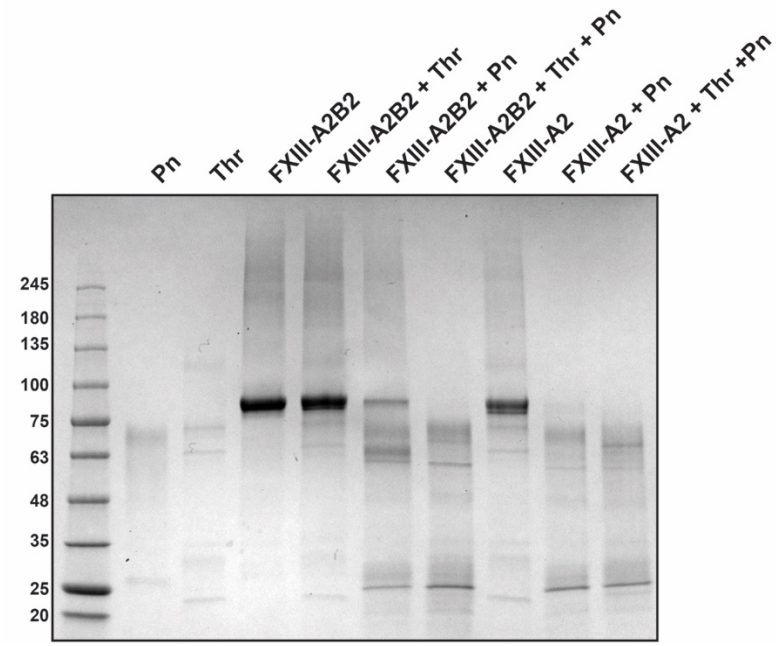


Figure 2.4 Blue-silver stained gel of FXIII(a) treated with plasmin

Blue silver stained gel containing FXIII-A_2B_2 (600 nM), FXIII-A_2 (600 nM), and thrombin (Thr, 400nM), incubated with plasmin (Pn, 600 nM) for 3 hr.

2.4.2 FXIII-A_2* is cleaved by plasmin at multiple sites

To identify which sites of FXIII-A_2* were cleaved by plasmin, purified FXIII-A_2* was incubated with plasmin and analyzed by mass spectrometry. Nineteen cleavage sites were identified, 8 at arginines and 11 at lysines, out of the 43 arginines and 34 lysines found in FXIII-A, with detection frequencies between 0.3 – 26% (Figure 2.5A). The most prominent cut-site was between K468 and Q469, the product of which corresponds in size to the 25 and 50 kDa cleavage products observed in figure 2.2A. To determine the spatial location of cleavage, the site was

mapped onto 3-dimensional structures of FXIII-A_2* and FXIII-A_2[170,171]. In the FXIII-A_2* structure, the K468-Q469 cleavage site is near the surface, consistent with accessibility to plasmin-mediated cleavage (Figure 2.5B). Interestingly, this K468-Q469 bond also appears surface-accessible in the FXIII-A_2 (unactivated) structure (Figure 2.5C). Since FXIII-A_2 is not readily cleaved by plasmin, as shown in Figure 1B, this bond may be protected in the zymogen conformation by the B subunit, which has not been co-crystalized with the A subunit. Five other surface-exposed cleavage sites were identified, but detected 8 to 38-fold less frequently.

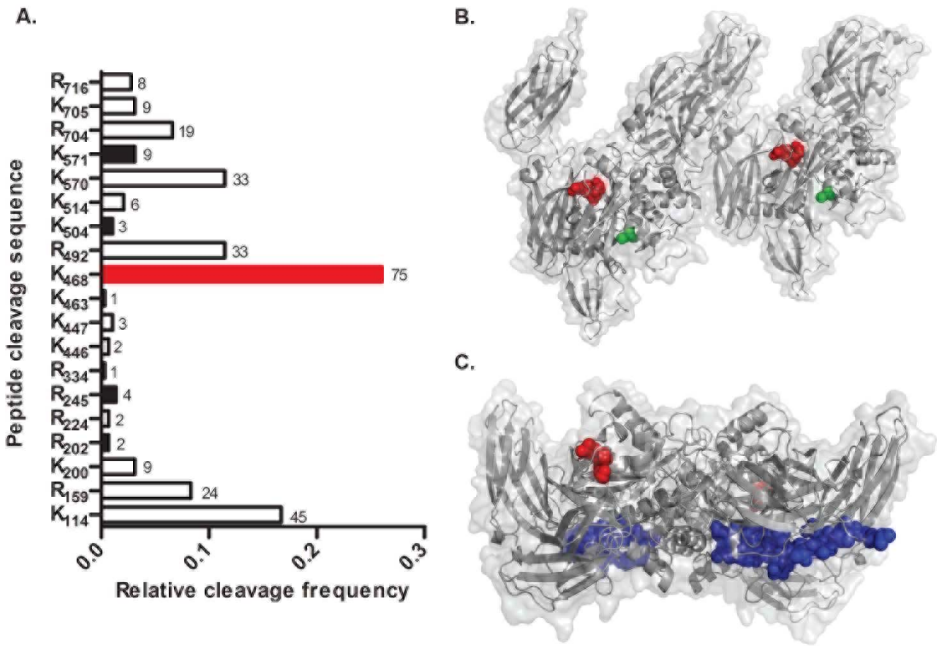


Figure 2.5 Plasmin cleaves FXIII-A_2* at multiple sites.

(A) FXIII-A_2* cleavage sites were identified by MALDI/TOF mass spectrometry after purified FXIIIa (10 μM) was incubated with plasmin (2.7 μM) for 2 hr. Surface exposed sites are

represented with black bars, and the primary cleavage site with a red bar. The frequency of detection of cut sites is indicated beside the respective bars (n = 2). (B) A reported structure of FXIII-A_2*[171], showing the surface-exposed K468-Q469 cleavage site (red) and the catalytic cysteine (green). The distance between the cleavage site and the catalytic cysteine is 18 Å. (C) A reported structure of FXIII-A_2[170], showing K468-Q469 (red), and the activation peptide (blue).

2.4.3 The rate of inactivation of FXIII-A_2* can occur on a physiologically-relevant timescale

To determine the kinetic parameters of plasmin-mediated inactivation of purified FXIII-A_2*, the loss of FXIII-A_2* activity with plasmin treatment was measured. Plasmin-mediated inactivation of FXIII-A_2* had an apparent K_m of 0.49 ± 0.02 μM and k_{cat} of 4.2 ± 1.1 × 10^{-3} s^{-1}, resulting in a catalytic efficiency of 8.3 ± 1.7 × 10^3 $M^{-1}s^{-1}$. The K_m was ~10-fold higher and the k_{cat} was ~10-fold lower than reported parameters for fibrin[172]. The half-life of FXIIIa degradation by plasmin, in the absence of fibrin, is estimated to be 34 sec, using these experimentally-determined kinetic parameters and the physiological concentrations of the zymogens (200 nM for plasma- and platelet-derived FXIII[a], and 2.4 μM for plasmin[ogen]) in circulating blood. V_{max} and K_m were calculated by nonlinear fitting of the measured initial velocities to the Michaelis-Menten equation, which produced similar values to those obtained from a Lineweaver-Burke plot (Figure 2.6A and 2.6B). The rate of inactivation was calculated from the loss of FXIII-A_2* activity, determined using an ammonia production assay (Figure 2.6C). The activity of FXIII-A_2* decreased as it was

cleaved by plasmin, resulting in a lower rate of ammonia generation (Figure 2.6D). These data indicate that the rate of FXIII-A_2* degradation can occur on a physiologically-relevant timescale.

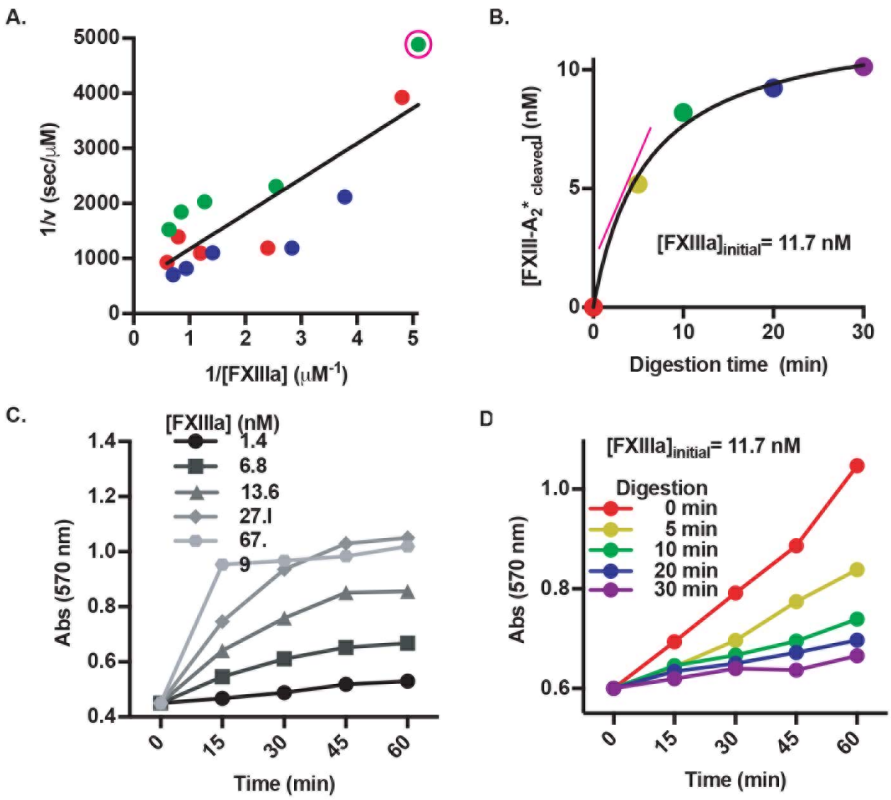


Figure 2.6 The rate of inactivation of FXIII-A_2* can occur on a physiologically-relevant timescale.

(A) Lineweaver-Burk plot used to generate kinetic parameters for FXIII-A_2*, with data sets represented by different colors. The data point circled in pink is derived from the slope of the initial velocity in panel B (pink line). (B) The generation of FXIII-A_2* cleavage products by plasmin (300 nM) at a representative FXIII-A_2* concentration, determined using an ammonia

release assay. (C) Standard curves of ammonia generation over time using various [FXIII-A_2*]. (D) Ammonia generation over time, with varying times of FXIII-A_2* digestion with plasmin. n = 3 for all experiments.

2.4.4 Plasmin inactivates both plasma-derived and platelet-derived FXIII-A_2*

To test if FXIII-A_2* is sensitive to degradation by plasmin in plasma, plasmin was added to fibrin clots in platelet-poor plasma. FXIII-A_2* was mostly degraded within 30 min by plasmin (3 μM, Figure 2.7A). Degradation of FXIII-A_2* was inhibited when exogenous α2-antiplasmin or TXA was added (Figure 2.7B). These data show that plasma-derived FXIII-A_2* can be degraded by plasmin in the presence of fibrin. To test if cFXIII-A_2 (from platelets) was sensitive to degradation by plasmin, platelets were initially activated by thrombin, followed by the addition of plasmin (1 nM to 1 μM). Platelet cFXIII-A_2 was degraded, and transient degradation products were observed when concentrations of plasmin as low as 10 nM were added 16 hr after platelet activation, but not when plasmin was added 1 hr after platelet activation (Figure 2.7C and 2.7D). The degradation products were also observed in the presence of TXA, likely because reduced plasmin activity slowed their rate of cleavage. It is known that cFXIII-A_2 translocates from the cytoplasm to the membrane upon platelet activation[33]; however, the results here suggest that cFXIII-A_2 was not exposed to plasmin and thus not degraded shortly after platelet activation. To test if cFXIII-A_2 was shielded from extracellular proteases, trypsin, which can cleave zymogen FXIII-A_2B_2[173], was added to platelets at 1 and 16 hr after activation. Platelet cFXIII-A_2 was not degraded by trypsin at 1 hr, but was degraded by trypsin at 16 hr (Figure 2.8), indicating that

exposure of cFXIII-A_2 to extracellular proteases was delayed relative to platelet activation. Thus, platelet-derived FXIII-A_2* can be degraded by plasmin following platelet activation.

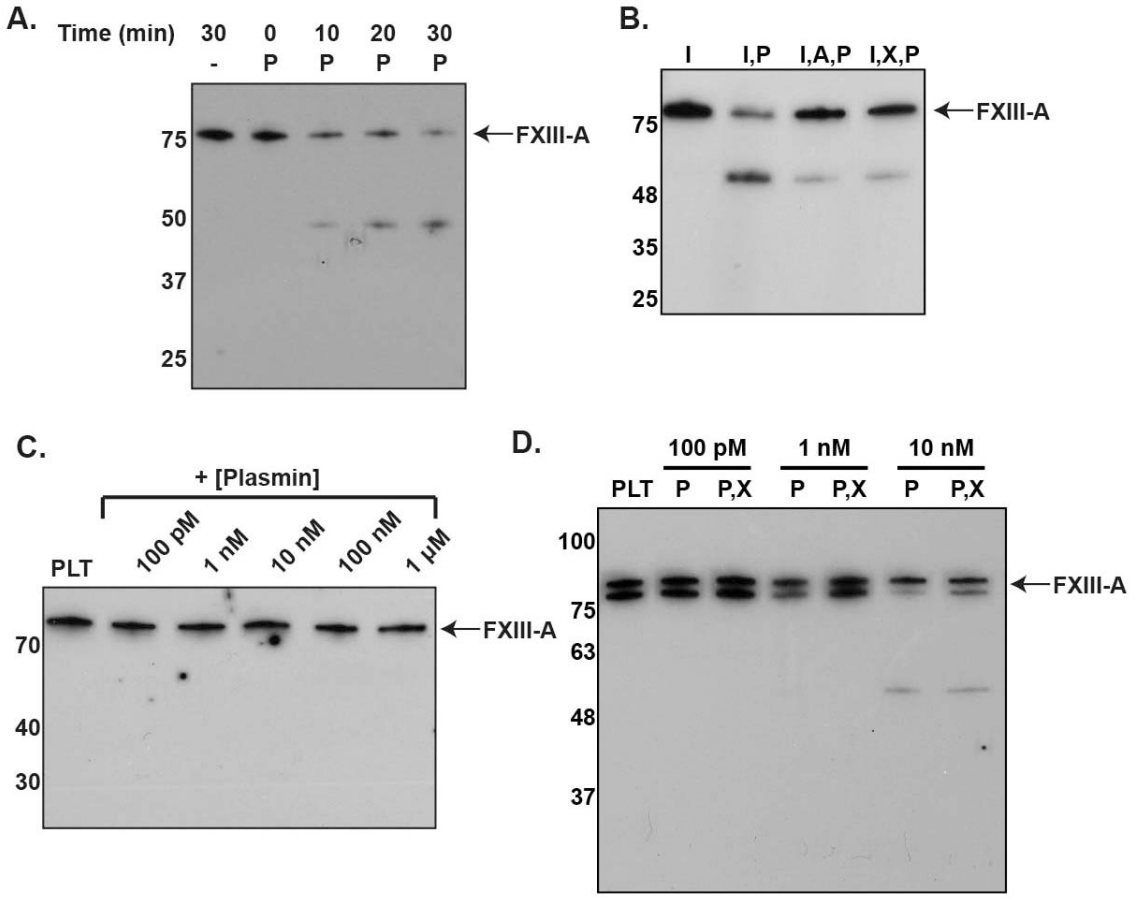


Figure 2.7 Plasmin degrades plasma- and platelet-derived FXIII-A*.

Western blots against the FXIII A subunit. (A) Endogenous FXIII-A_2* from human plasma after adding plasmin (3 μM) for various times. (B) FXIII-A_2* from plasma with plasmin (3 μM) and α2-antiplasmin (5 μM) or TXA (7.5 mM). (C-D) Endogenous FXIII-A_2/FXIII-A_2* from platelets (PLT) 1 hr (C) and 16 hr (D) after exposure to thrombin, and incubating with various

concentrations of plasmin. Samples contain combinations of Innovin (I), plasmin (P), α2-antiplasmin (A), and TXA (X). n = 3 for all experiments.

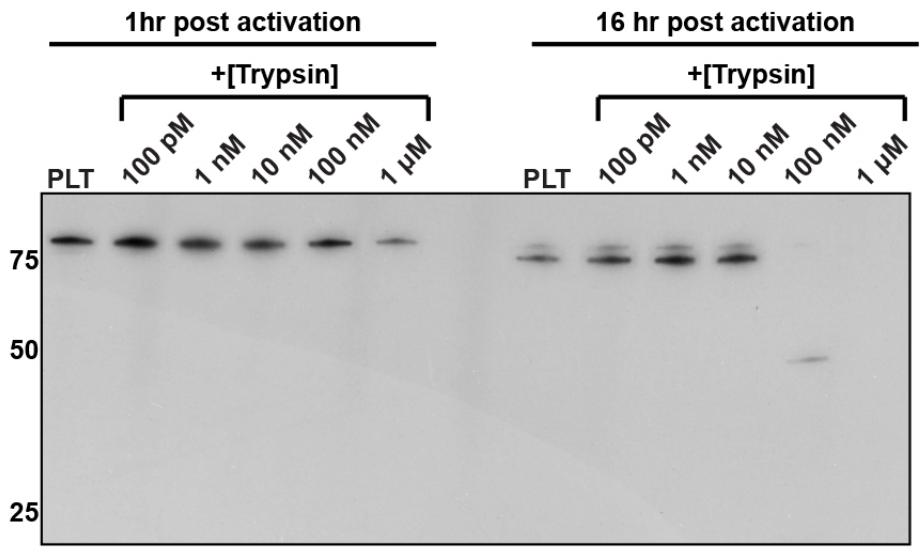


Figure 2.8 Trypsin degrades platelet derived FXIII-A

Endogenous FXIII-A_2/FXIII-A_2* from platelets (PLT) 1 hr and 16 hr after exposure to thrombin, incubated with various concentrations of trypsin.

2.4.4.1 Addition of tPA to plasma leads to degradation of pFXIII-A_2* by endogenous plasmin

Addition of tPA (2 μM) to clotted normal plasma led to the degradation of FXIIIa and fibrin within 3 hr (Figure 2.9A-C). Degradation was inhibited by TXA. The degradation of pFXIII-A_2* and fibrin occurred within a similar time-frame, beginning within 10 min, and continuing to

180 min. To determine whether tPA degraded FXIIIa directly or by generating plasmin, plasminogen-deficient plasma was treated with tPA. Degradation of FXIIIa did not occur in plasminogen-deficient plasma, indicating that FXIIIa was degraded by tPA-mediated plasmin activity (Figure 2.9D). In fibrinogen-deficient plasma, FXIIIa was degraded more rapidly than in normal plasma when treated with tPA (comparing Figure 2.9E with Figure 2.9A), and was almost completely degraded within 10-20 min. In whole blood, FXIII-A_2* was degraded when tPA was added, except in the presence of α2-antiplasmin or TXA (Figure 2.9F). Together, these data demonstrate that pFXIII-A_2* degradation can occur by endogenous plasmin.

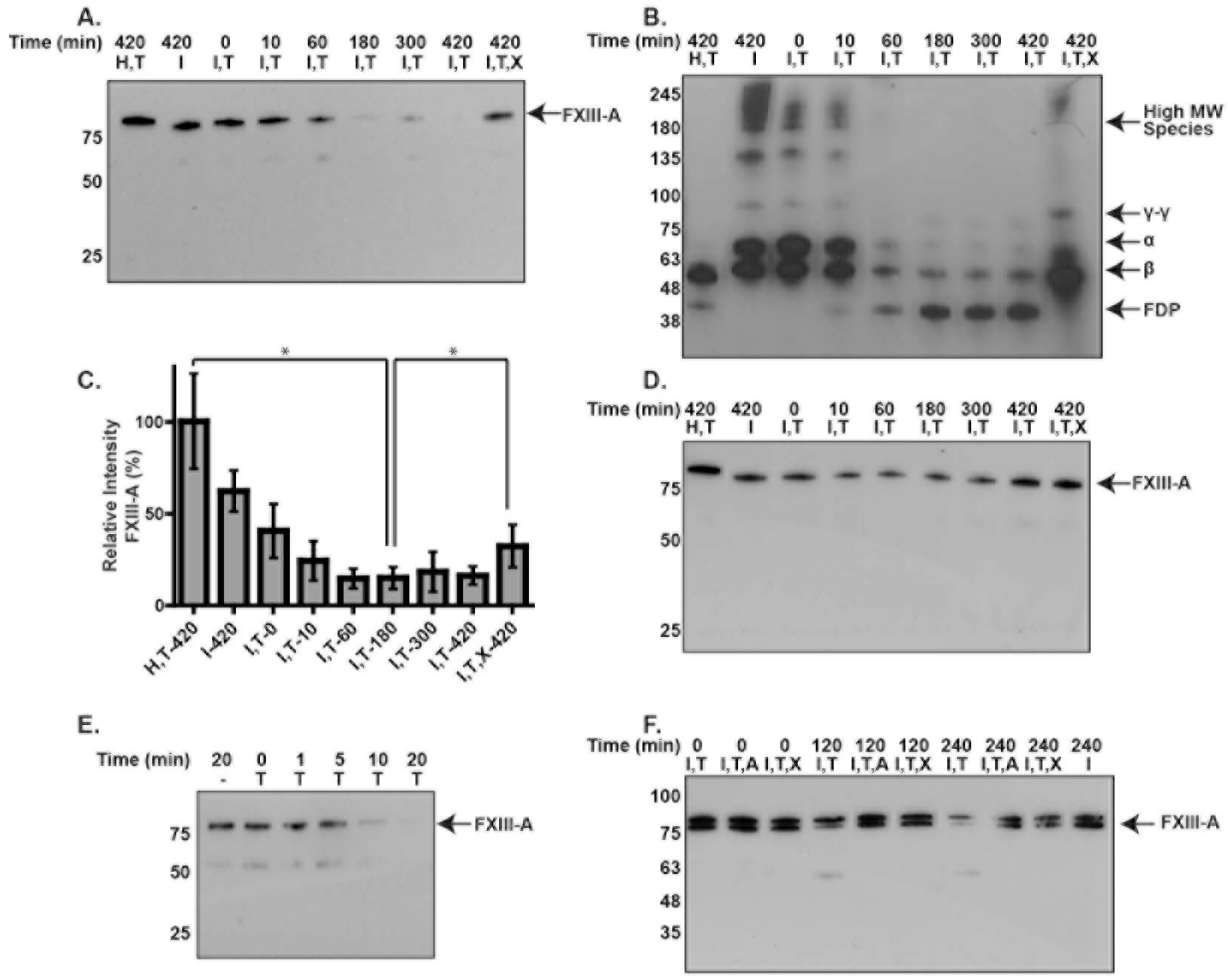


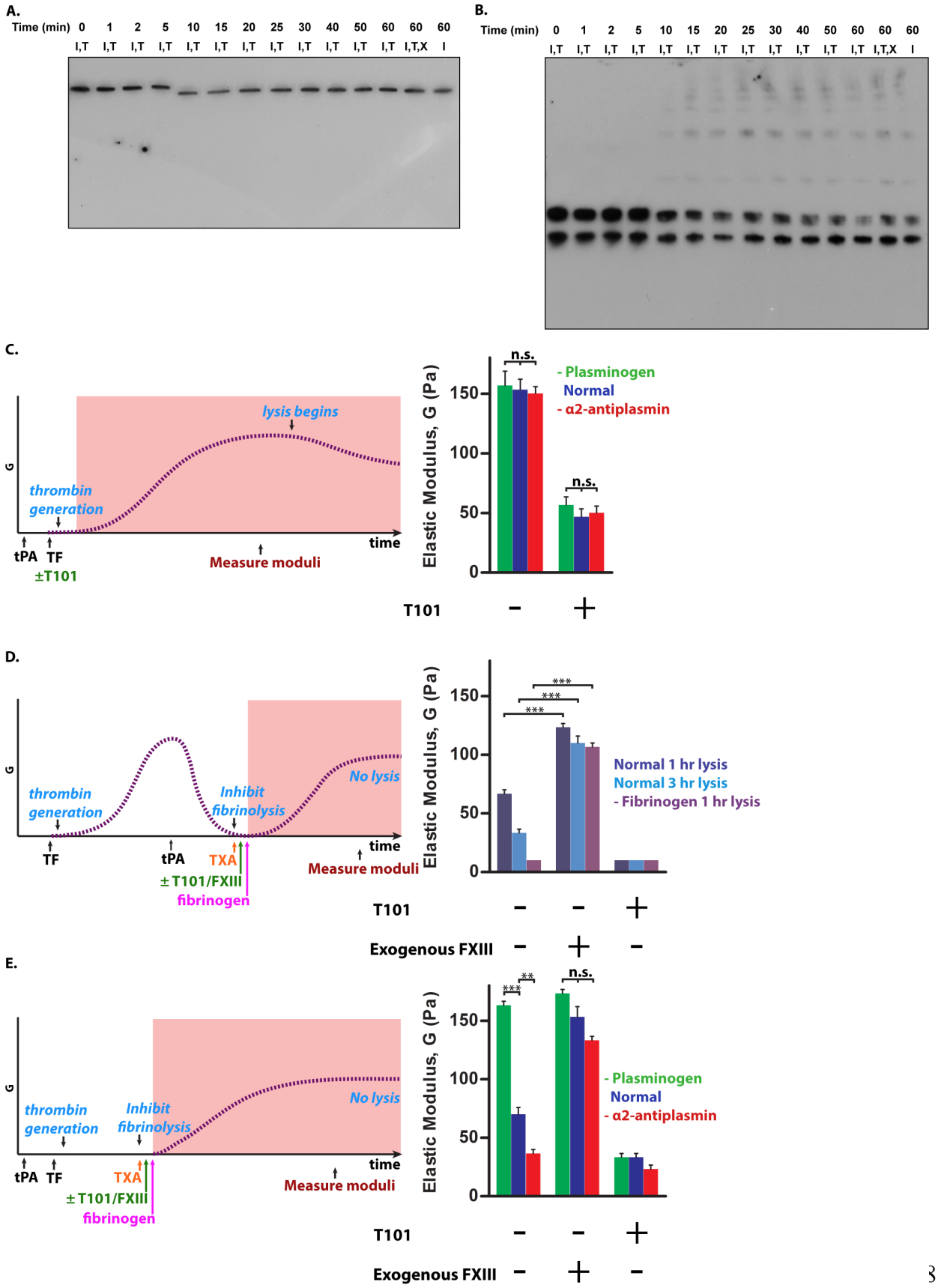
Figure 2.9 Endogenous plasminogen is activated by tPA to degrade endogenous FXIIIa.

Plasma was analyzed by Western blot against FXIII-A, after addition of tPA and TXA. (A-B) Time-dependent degradation of endogenous (A) FXIII-A_2* and (B) fibrin(ogen) in normal plasma. (C) The relative amount of intact FXIII-A* from panel A using densitometry, calculated as % of total signal in the lane. *$p < 0.05$ (n = 3). (D) Time-course for plasminogen-deficient plasma (n = 3). (E) Time-dependent degradation of FXIIIa in fibrinogen-deficient plasma following addition of tPA (n = 3). (F) Degradation of endogenous FXIII-A_2*, but not FXIII-A_2,

in whole blood clots with tPA (n = 3). Samples contain combinations of hirudin (H), Innovin (I), tPA (2 μM) (T), TXA (7.5 mM) (X), and α2-antiplasmin (4 μM) (A).

2.4.5 Plasmin-mediated FXIIIa inactivation occurs following fibrinolysis

To further characterize the pFXIII-A_2* degradation by plasmin, we examined if degradation occurred during or after clot formation, and if there were downstream effects on fibrin cross-linking. We first used Western blotting to monitor pFXIII-A_2* and fibrin during clot formation. TPA (200 pM) was added to plasma, and clotting was immediately initiated. Only a portion of pFXIII-A_2* was degraded during fibrin formation and cross-linking, indicating that pFXIII-A_2* remained active during clot formation in normal plasma (Figure 2.10A and 2.10B). We then evaluated pFXIII-A_2* activity and its downstream effects using TEG because the mechanical strength (shear elastic modulus, G) of fibrin is closely tied to the activity of pFXIII-A_2*. For these experiments, we utilized plasminogen-deficient plasma (reduced plasmin activity), normal plasma, and α_2-antiplasmin-deficient plasma (increased plasmin activity), all containing tPA (200 pM). No differences in the moduli were observed between clots from the three types of plasma prior to fibrinolysis (Figure 2.10C). The moduli were all ~3-fold higher compared to samples containing an inhibitor of FXIIIa (T101), which does not inhibit the plasmin-mediated degradation of FXIII-A_2* (Figure 2.11). These data indicate that pFXIII-A_2* was not inactivated prior to fibrinolysis.


A.
Time (min) 0 1 2 5 10 15 20 25 30 40 50 60 60 60
I,T I,T I,T I,T I,T I,T I,T I,T I,T I,T I,T I,T I,T,X I
B.
Time (min) 0 1 2 5 10 15 20 25 30 40 50 60 60 60
I,T I,T I,T I,T I,T I,T I,T I,T I,T I,T I,T I,T I,T,X I
C.
G
thrombin generation
lysis begins
tPA TF
±T101
Measure moduli
time
Elastic Modulus, G (Pa)
n.s.
n.s.
- Plasminogen
Normal
- α2-antiplasmin
T101 − +
D.
G
thrombin generation
Inhibit fibrinolysis
No lysis
TF
tPA
TXA
±T101/FXIII
fibrinogen
Measure moduli
time
Elastic Modulus, G (Pa)
***
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Normal 1 hr lysis
Normal 3 hr lysis
- Fibrinogen 1 hr lysis
T101 − − +
Exogenous FXIII − + −
E.
G
thrombin generation
Inhibit fibrinolysis
No lysis
tPA TF
TXA
±T101/FXIII
fibrinogen
Measure moduli
time
Elastic Modulus, G (Pa)
***
**
n.s.
- Plasminogen
Normal
- α2-antiplasmin
T101 − − +
Exogenous FXIII − + −

Figure 2.10 Plasmin-mediated inactivation of FXIII-A_2* does not occur during normal clot formation, but does occur during fibrinolysis and thrombolytic conditions.

(A-B) Clot formation in normal plasma with tPA (200 pM) and, in some cases, TXA (7.5 mM). Western blots against (A) FXIII-A and (B) fibrin(ogen) (n = 3). (C-E) TEG analyses of clot formation and cross-linking of exogenous (purified) fibrin in plasma. Schematics on the left show timelines of the procedures and characteristic shear elastic moduli (G, dashed lines), with shaded areas indicating time-periods analyzed with TEG. Charts on the right side show measured moduli of fibrin clots, a direct indicator of FXIII-A_2* activity and fibrin structure. Control samples contain exogenous FXIIIa or T101. (C) Moduli of clots from plasminogen deficient, normal, and α2-antiplasmin deficient plasma formed in the presence of tPA (200 pM). (D) Moduli of exogenous fibrin (indicator of residual FXIIIa activity), added following clot lysis. Exogenous fibrinogen (1.4 mg/mL) and TXA were added 1 or 3 hr after clot lysis by tPA (800 nM). (E) Moduli of exogenous fibrin that was added during clot formation under thrombolytic conditions. TXA and then fibrinogen were added 4 min after clotting was initiated in the presence of tPA (50 nM or for α2-antiplasmin deficient plasma, 5 nM). Samples contain combinations of Innovin (I), tPA (T), and TXA (X). $^{**}p < 0.01$ $^{***}p < 0.001$, n = 3 for all experiments.

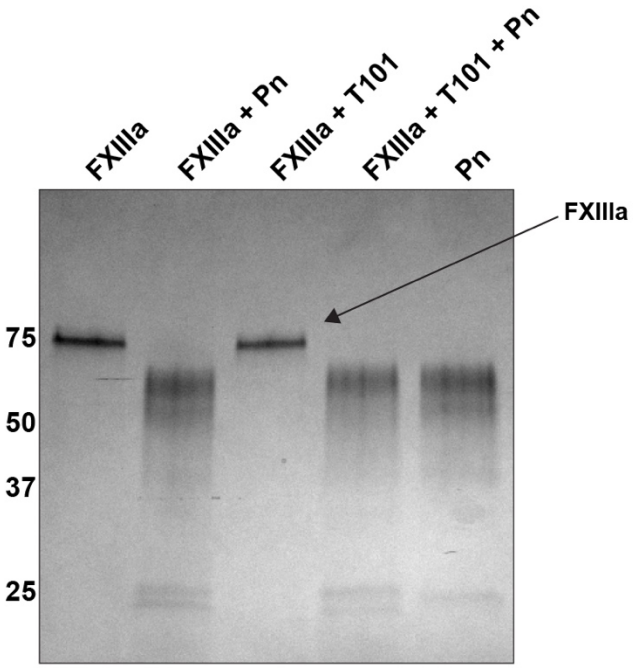


Figure 2.11 T101 does not affect FXIIIa stability

Blue-silver stained gel containing FXIIIa (1.6 μM) and T101 (5 μM), incubated with plasmin (Pn, 1 μM) for 3 hr.

To determine if inactivation occurred during fibrinolysis, normal plasma clots were fully formed and tPA (800 nM) was added to facilitate rapid lysis. The fibrinolytic system was inhibited by TXA at either 1 or 3 hr after addition of tPA and the contribution of residual pFXIII-A_2* to the modulus of exogenous (purified) fibrin was determined. There was 42% and 60% decrease in the moduli at 1 hr and 3 hr, respectively, compared to control samples where fibrin was fully cross-linked with exogenous pFXIII-A_2* (Figure 2.10D). These decreases in moduli indicate that FXIIIa

became inactivated during fibrinolysis, and the downstream effect of this inactivation was reduced cross-linking of fibrin. This result is consistent with the Western blots of degradation of pFXIII-A_2* and fibrin shown in Figure 2.9A-C. Furthermore, in fibrinogen-deficient plasma, at 1 hr into lysis, the modulus of exogenous fibrin was 90% less than when fibrin was fully cross-linked, consistent with Figure 5E. The moduli of normal and fibrinogen-deficient plasma were similar when exogenous pFXIII-A_2* was added; this indicates the moduli were dependent primarily on exogenous fibrin rather than residual endogenous fibrin.

2.4.6 Inactivation of pFXIII-A_2* occurs during clot formation under thrombolytic conditions in plasma

Finally, we probed pFXIII-A_2* activity and its downstream effects on fibrin during clot formation under thrombolytic conditions. Prior to clot initiation, thrombolytic levels of tPA were added to plasma with varying plasmin activities. The fibrinolytic system was inhibited 4 min into clot formation by adding TXA. Exogenous fibrinogen was then added and moduli were measured and compared to samples also containing exogenous pFXIII-A_2* or T101. The resulting elastic modulus was 56% less in normal plasma than in plasminogen-deficient plasma (Figure 2.10E). Exogenous pFXIII-A_2* rescued the modulus of normal plasma (bringing it to a similar value as plasminogen-deficient plasma) while it had little effect on plasminogen-deficient plasma. The loss of FXIIIa activity at 4 min was exacerbated in α_2-antiplasmin-deficient plasma, where the modulus was 43% lower than normal plasma and 75% lower than plasminogen-deficient plasma, but could

again be rescued by adding exogenous pFXIII-A_2*. Thus, high fibrinolytic activity rapidly prevented pFXIII-A_2* from cross-linking fibrin.

2.5 Discussion

The results show that FXIIIa can be inactivated by plasmin, and that plasmin is selective for FXIII-A_2*, the active enzyme, over FXIII-A_2B_2, the zymogen. The specificity of plasmin for pFXIII-A_2* over pFXIII-A_2B_2 may have confounded earlier reports, which only analyzed pFXIII-A_2B_2 and cFXIII-A_2* to conclude that FXIII was resistant to degradation[42]. Importantly, degradation of FXIII-A_2* and resistance of FXIII-A_2B_2 were observed in both purified systems and in plasma. Thus, these findings reveal a newly-recognized mechanism that may regulate cross-linking in physiologically-relevant circumstances.

Degradation of FXIII-A_2* in clots occurred within the same time-period as degradation of fibrin. However, the rate of cleavage of FXIII-A_2* in plasma was slower than the estimated half-life of FXIII-A_2*, likely due to the presence of competing substrates such as fibrin[174] and inhibitors of plasmin such as α_2-antiplasmin[35]. Notably, however, during normal clot formation, FXIII-A_2* appeared to reach its full potential in cross-linking fibrin before it was inactivated, but fibrin was not cross-linked when added to reactions following FXIII-A_2* inactivation. Thus, this mechanism may prevent FXIII-A_2* from aberrantly cross-linking fibrin and other proteins in blood vessels.

Interestingly, degradation of platelet-derived cFXIII-A_2* only occurred when plasmin was added 16 hr after platelet activation, but not 1 hr after activation. The slow availability of degradable cFXIII-A_2* may be due to intracellular localization of cFXIII-A_2 and thus inaccessibility by thrombin and plasmin. The initial resistance of cFXIII-A_2 to degradation is

consistent with the degradation of plasma FXIII-A_2, which occurred primarily during fibrinolysis. Likewise, a subpopulation of FXIII-A_2 was not readily activated in whole blood, as seen in the blot of Figure 5F; this initially spared a portion of the FXIII-A_2, likely cFXIII-A_2, from degradation.

Overall, these data support the notion that FXIII-A_2* is inactivated during fibrinolysis, but not during clot formation. However, we note that these *in vitro* experiments do not rule out the possibility that plasmin may inactivate FXIIIa during clot formation *in vivo.* Moreover, plasmin may not be the sole inhibitor of FXIIIa *in vivo*. FXIIIa can also be cleaved and inhibited by thrombin and polymorphonuclear granulocyte proteases[39,164]. In our experiments in plasma, FXIIIa was only degraded in the presence of plasmin, suggesting cleavage of FXIIIa by thrombin may be secondary to that of plasmin. Questions remain regarding whether plasmin and polymorphonuclear granulocyte proteases work in concert or if they inactivate FXIIIa under distinct circumstances. Future studies may reveal additional points of FXIII-A_2* inactivation in certain circumstances *in vivo*.

FXIII-A_2* is at the interface between coagulation and fibrinolysis, and its ability to inhibit fibrinolysis is well-established. The results presented here indicate fibrinolytic enzymes can, in turn, down-regulate FXIII-A_2*. Thus, our findings reveal cross-talk between these pathways that may provide critical information for managing thrombosis and hemostasis[175,176]. For example, the therapeutic use of plasminogen activators to treat embolic stroke[177] and heart attack[178] may be complicated by the novel discovery of plasmin-mediated inhibition of FXIII-A_2*. Physiologically, tPA is present in the blood at ~70 pM; however, therapeutic tPA is typically administered either intravenously, leading to systemic blood concentrations of ~50 nM[179], or locally into clots from intravascular catheters at ~400 nM[35]. In our experiments, FXIII-A_2* was degraded during clot

formation under thrombolytic conditions, at 50 nM of tPA. This mechanism may contribute to hemorrhaging associated with thrombolytic therapy[177]. Notably, side effects of tPA administration resemble the phenotype of patients with FXIII deficiency, where in both cases there is a higher incidence of intracranial hemorrhage than would be expected when compared to other types of hemorrhage[175,177]. Further research into the relevance of this mechanism in thrombolytic therapy is warranted.

Inhibition of FXIII-A_2* by plasmin may also have implications in diseases where plasmin activity is abnormal, although this still needs to be verified *in vivo*. For example, patients with Quebec Platelet Disorder have elevated urokinase activity and thus are hyperfibrinolytic[180], suggesting that there may be less FXIII-A_2* activity. In plasminogen deficiency, there may be higher FXIII-A_2* activity, and increased aberrant cross-linking of proteins. In fibrinogen deficiency, degradation of FXIII-A_2* could be enhanced since fibrin is a competing substrate for plasmin[174], or the degradation could be slower since fibrin is a cofactor for fibrinolytic enzymes[181]. We observed the former in both Western blots and TEG, and this rapid inactivation may have implications in congenital fibrinogen deficiency. Thus, these results provide insight into several physiological and pathophysiological scenarios that warrant further investigation.

In conclusion, the experiments show that plasmin preferentially inactivates FXIII-A_2* over FXIII-A_2B_2, and that this mechanism occurs in a wide range of experimental conditions. The downstream effect of fibrinolytic inactivation of FXIII-A_2* is that FXIII-A_2* is no longer able to contribute to cross-linking and maintaining the mechanical strength of fibrin. However, to confirm that plasmin-mediated inactivation of FXIII-A_2* plays a major role in thrombosis or hemostasis, additional data from human samples are necessary.

Chapter 3: Coagulation factors XIII and XIII-A* decrease in some DVT patients following catheter-directed thrombolysis

3.1 Contributions

This research was done in collaboration with interventional radiologist at Vancouver General Hospital. W.S.H. designed, performed and analyzed all the experiments, and wrote the paper. H.W. and L.M. recruited participants, designed experiments and edited the paper. C.J.K. helped design and analyze experiments and write the paper.

3.2 Introduction

Deep vein thrombosis (DVT) affects approximately 1 in 1000 people and which can lead to 50 000 to 200 000 deaths annually in the United States[182,183]. In patients with the appropriate blood vessel anatomy, thrombolytic therapy can be an effective treatment to degrade these blood clots, restore blood flow and reduce the acute symptoms of DVT and the longer-term outcomes, though with variable efficacy[7,8,184]. Eligible patients receive local administration of tissue plasminogen activator (tPA) via a catheter introduced into the venous thrombus. tPA is a serine protease that binds to the thrombus and activates plasminogen to plasmin to degrade fibrin[20]. Typically, 1 mg/hr (16 μM) of tPA is instilled into the thrombosed vein for up to 4 days[116]. To prevent further clot formation, most patients also receive anticoagulant therapy such as heparin, warfarin, or direct oral anticoagulants. Intracranial hemorrhage (ICH) is a devastating potential complication of thrombolysis and catheter-directed thrombolysis (CDT) is contraindicated in those patients at increased risk of ICH[117]. Despite the strict guidelines to exclude patients at increased risk, CDT carries a 1-2% risk of major bleeding, such as ICH[7,8].

Inherited deficiency of coagulation factor XIII (FXIII or FXIII-A_2B_2) is associated with a higher incidence of ICH compared to deficiencies in other coagulation factors[125]. Activated FXIII (FXIII-A*) is formed from when thrombin cleaves the activation peptide of the A subunit of FXIII, leading to dissociation of the catalytic A subunits and the regulatory B subunits. FXIII lies at the intersection of coagulation and fibrinolysis; FXIII-A* crosslinks fibrin to itself and to anti-fibrinolytic proteins to stabilize the blood clot from premature clot lysis[4]. To maintain that balance, the fibrinolytic protein plasmin degrades FXIII-A*, but not FXIII, *in vitro*[9]. In acute ischemic stroke (AIS) patients treated with tPA, FXIII levels were decreased by 12% when measured 24 hrs after the end of thrombolytic therapy[9]. Here, we tested if CDT alters FXIII levels and FXIII-A* stability in 9 DVT patients. We found that FXIII was reduced and FXIII-A* was degraded in some, but not all, of the patients' blood following treatment.

3.3 Methods

3.3.1 Blood collection and isolation

This study was approved by the research ethics boards of the University of British Columbia (H14-01581) and the Vancouver Coastal Health Research Institute (V14-01581), and informed consent was obtained before participation in the study, in accordance with the Declaration of Helsinki. Whole blood was collected from the DVT patients immediately before tPA was administered, immediately after the therapy had ended, and during the recovery period within 6-24 hours after the therapy. Whole blood was collected into tubes containing sodium citrate (12 mM) and centrifuged at 1000 x g for 10 min twice to collect platelet poor plasma (PPP).

3.3.2 Degrading FXIII(a) in purified systems and in blood

To test if FXIII was degraded by tPA in a purified system, FXIII (Haematologic Technologies Inc., 30 nM) was incubated with Alteplase (Genetech) and $CaCl_2$ (4 mM) with or without human thrombin (Haematologic Technologies Inc, 70 nM) for 3 hr at 37 °C in HEPES-buffered saline (10 mM HEPES, 140 mM NaCl, pH 7). To test if tPA can degrade FXIII in plasma, citrated plasma (Affinity Biologicals) was incubated with hirudin (EMD Millipore, 200U/mL) and Alteplase for 1 hr or 16 hr. To test if CDT can lead to FXIII-A* degradation, citrated plasma from patients was incubated with $CaCl_2$ (10 mM) and diluted tissue factor (TF, Innovin, Dade Behring, 1 pM) or human thrombin (Haematologic Technologies Inc., 70 nM) at 37 °C for up to 4 hr.

3.3.3 Quantifying the concentration of FXIII

The FXIII concentration in plasma of DVT patients was measured in triplicate using commercially-available sandwich enzyme-linked immunoabsorbent assay (ELISA) kit (Ab108836, Abcam), following the manufacturer's instructions. For Western blotting, samples were prepared as described previously[9]. Reactions containing plasma were quenched by adding quench buffer (50 mM dithiothreitol, 12.5 mM EDTA, 8 M urea) and incubated at 60°C with occasional agitation until the clot was dissolved. All samples were reduced and boiled prior to electrophoresis on 4-15% Mini-PROTEAN TGX gel (Bio-Rad) and transferred to a nitrocellulose membrane (Pall Life Sciences). After blocking with Odyssey Blocking Buffer (Li-Cor), the membranes were incubated with sheep anti-human FXIII A antibody (SAF13A-AP, Affinity Biologicals, diluted 1:1000). The membranes were washed with phosphate buffered

saline with Tween 20 (PBST, 8 mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl, 0.05% Tween 20, pH 7.4). After incubating the membrane with the rabbit anti-sheep IgG antibody (ab7111, Abcam, diluted 1:10,000), it was washed with PBST and detected with ECL substrates (Bio-Rad). Images of Western blots were scanned and quantified using ImageJ.

3.3.4 Statistics

A Wilcoxon matched pair test was used to test if FXIII levels were decreased following CDT.

3.4 Results

3.4.1 FXIII decreases after CDT in some patients.

To determine if the FXIII levels are altered after CDT in DVT patients, FXIII levels were quantified with ELISA in plasma samples collected before and immediately after CDT. The concentrations of FXIII were variable among the patients even before the therapy began (Figure 3.1). It ranged from 7.81 μg/mL to 25.69 μg/mL (mean: 17.71 μg/mL, standard deviation: 6.33 μg/mL), compared to 6 healthy donors that had a range of 9.01 μg/mL to 15.35 μg/mL (mean: 11.82 μg/mL, standard deviation: 2.48 μg/mL)[185-187]. Although FXIII levels decreased by an average of 18% following CDT after therapy, this difference was not statistically significant for this number of patients (p value: 0.10). However, FXIII levels were decreased by 40% or more following CDT in 4 of 9 patients.

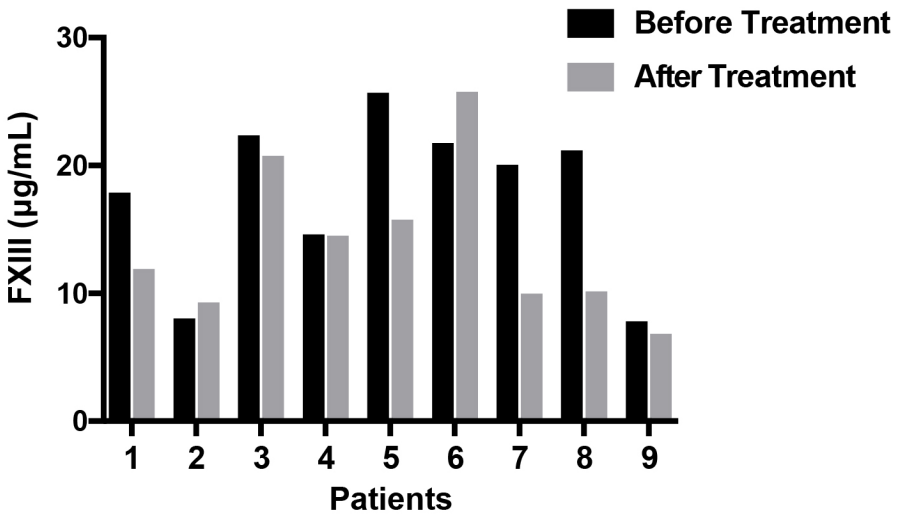


Figure 3.1 FXIII-A levels become reduced in some patients after receiving thrombolytic therapy.

FXIII levels were quantified using ELISA in 9 DVT patients receiving thrombolytic therapy.

3.4.2 FXIII is degraded by tPA *in vitro*.

To investigate if the decrease in FXIII was caused by degradation due to the thrombolytic therapy, we tested if tPA can degrade FXIII. We have previously shown that 100 nM of plasmin can degrade only FXIII-A*, but not FXIII; therefore, we tested if tPA can directly degrade FXIII and FXIII-A*. In a purified system, tPA degraded the A subunit of FXIII (FXIII-A) both in the zymogen and the active enzyme state at concentrations as low as 400 nM of tPA (Figure 3.2A). The degradation of FXIII-A by 800 nM of tPA occurred within 10 min (Figure 3.2B). Degradation products were not observed by Western blot, consistent with what we previously reported[9], where the cleaved products were degraded quickly and possibly had weaker binding to

the FXIII-A antibody. In plasma, 92% of FXIII-A was degraded by 10 μM of tPA within 1 hr (Figure 3.2C). With tPA concentrations of 1 μM and 100 nM, 39% and 28% of FXIII-A was degraded, respectively within 1 hr.

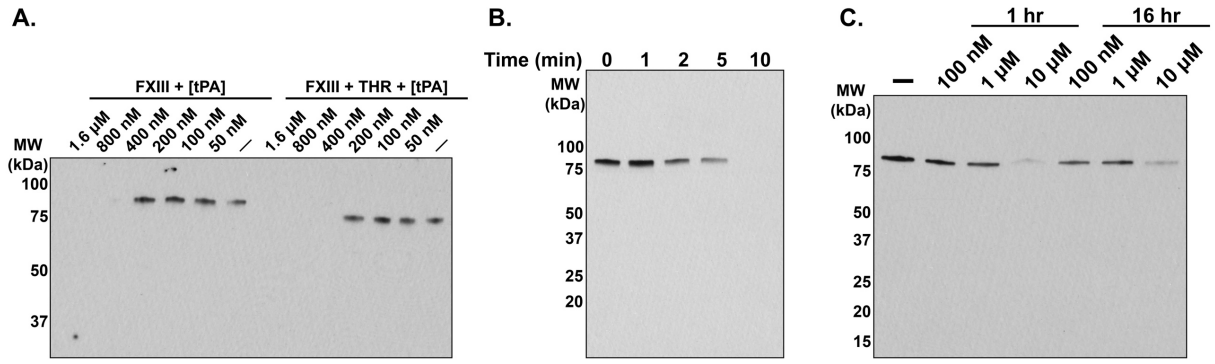


Figure 3.2 Purified tPA directly degrades FXIII-A *in vitro*.

Western blots were stained with an antibody against FXIII-A. (A) Purified FXIII with or without thrombin was incubated with various concentrations of tPA for 180 min. (B) Purified FXIII was incubated with 800 nM of tPA for various times. (C) Plasma was incubated with hirudin and various concentrations of tPA. THR: Thrombin.

3.4.3 FXIII-A* is degraded after CDT in some patients.

Although physiological concentrations of plasmin cannot degrade FXIII, plasmin could potentially degrade FXIII-A* when plasmin is generated by tPA during thrombolytic therapy. In blood collected before CDT, FXIII-A remained stable even when activated to FXIII-A* in all of

the 9 patients' samples (Figure 3.3A and 3.3B). In blood collected immediately after CDT, FXIII was not activated in 7 patients when TF (1 pM) was added. However, when a high concentration of thrombin (70 nM) was added, FXIII-A* was generated; this high concentration of thrombin was likely required to activate FXIII since patients with DVT are initially treated with anticoagulants. In 7 of 9 samples, the concentration of FXIII-A* did not change by more than 50%. In 2 of these 9 samples, 85% of FXIII-A* was degraded (Figure 3.3C). In blood collected during the recovery period 6 to 24 hr after the CDT was terminated, FXIII-A* was stable over 4 hr.

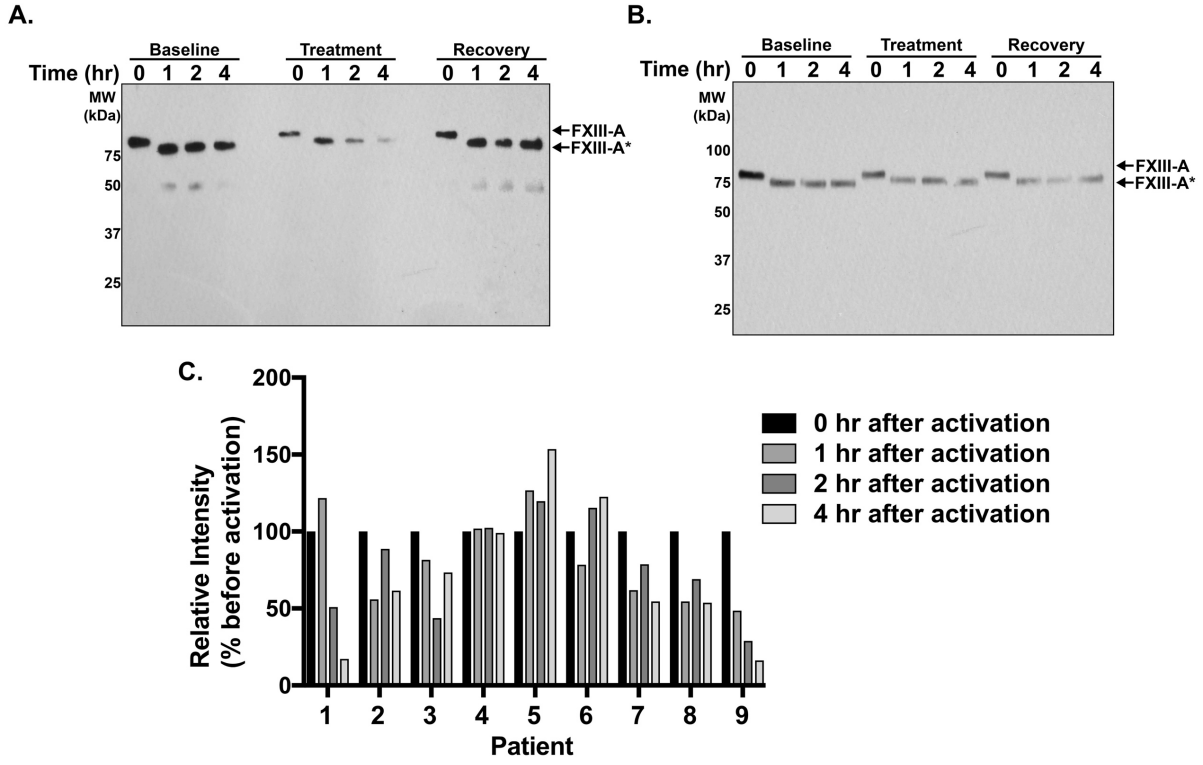


Figure 3.3 FXIII-A* is degraded after thrombolytic therapy in some patients.

(A-B) Western blot against FXIII-A in blood from patients before treatment (baseline), immediately after thrombolytic treatment (treatment) and 6 to 24 hr after treatment ended (recovery). Each blood sample was recalcified and treated with tissue factor (1 pM) or thrombin (70 nM) *ex vivo* to generate FXIII-A*. Images of the Western blots are from patient 1 (A) and patient 8 (B). (C) Quantification of FXIII-A or FXIII-A* intensity in the Western blots of blood from each patient.

3.5 Discussion

Here we show that thrombolytic therapy leads to a decrease in the plasma concentration of FXIII in some patients and a decrease in FXIII-A* stability in others. Although the changes were not statistically significant, FXIII levels decreased by more than 40% in 4 patients and FXIII-A* was decreased by 85% in 2 patients. The decrease in FXIII is consistent with the ability of purified tPA to directly degrade FXIII-A in buffer and in plasma.

Although FXIII level varies slightly within the population, there was substantial variation in the DVT patients before thrombolytic therapy was administered. Similar variation was previously observed in patients with AIS, in which the standard deviation of FXIII levels was 7.6 μg/mL[188]. The greater variation among thrombosis patients may be caused by consumption of FXIII into the thrombus or compensatory synthesis of FXIII following consumption. There are

mechanisms to induce fibrinogen synthesis after fibrinogen levels decrease[189,190], but it is unknown if this occurs with FXIII.

The decrease in FXIII in some patients following CDT may be due either to its consumption into the thrombus or its degradation by thrombolytic proteases. Consumption into the thrombosis is a less likely mechanism, because the patients were on anticoagulant therapy. The plasma collected from the DVT patients required a high concentration of thrombin for clotting to occur, suggesting that the anticoagulation therapy was successful and FXIII was not consumed, but degraded by fibrinolytic enzymes. Although plasmin does not degrade FXIII at physiological concentrations of plasminogen (less than 3 μM), the results here show that 10 μM of tPA markedly reduced FXIII-A levels in plasma; this is within the tPA concentrations used during CDT, where approximately 16 μM of tPA is injected. The degradation of FXIII-A by tPA was likely highest near the catheter, and much lower away from the catheter where tPA becomes diluted.

Others have reported changes in FXIII levels following thrombolytic therapy for AIS patients[188]. The FXIII concentration and FXIII activity of AIS patients decreased by 12% and 8%, respectively, 24 hr following the end of thrombolytic therapy. This is consistent with our findings where FXIII decreased by an average of 18% following thrombolytic therapy, although statistical significance was not achieved with our sample size of 9 patients. Further studies involving more patients should be performed to determine the factors that contribute to the decrease in FXIII following thrombolytic therapy.

In 2 of 9 patients, 85% or more of FXIII-A* was degraded following thrombolytic therapy once FXIII was activated by thrombin. This is consistent with previous findings that plasmin can degrade FXIII-A*. Several factors may explain why FXIII-A* was degraded in only a subset of patients. Longer duration of the therapy leads to higher amounts of tPA administered. Since DVT patients continue receiving thrombolytics until the blood flow is restored, the patients with the longest treatment duration may be most susceptible to FXIII-A* degradation. Variation in anti-fibrinolytic activity may also contribute to variability in FXIII-A* degradation. DVT patients have highly variable levels of antifibrinolytic proteins, leading to variation in active plasmin levels during CDT[191]. However, a larger sample size would be required determine the factors that contribute to FXIII-A* stability.

In conclusion, FXIII levels are highly variable in DVT patients who will receive CDT. FXIII-A_2B_2 levels can decrease in some patients following CDT, likely due to tPA directly degrading FXIII-A. Furthermore, FXIII-A* is degraded when it is formed in some patients, likely by plasmin. Future studies are needed to identify the factors that regulate FXIII levels and FXIII-A* stability and to explore if there is a relationship between FXIII and major bleeds following thrombolytic therapy.

Chapter 4: Coagulation factor XIIIa crosslinks amyloid β into dimers and oligomers and to blood proteins

4.1 Contributions

This publication was a collaborative work and published in the Journal of Biological Chemistry (pre-published version attached in the appendix). W.S.H. designed, performed experiments, analyzed and interpreted all the data and wrote the paper. W.S.H. performed experiments to collect data for Figure 4.1A-B, 4.2C-D, 4.3, 4.4, 4.5A, 4.7A and 4.7C-D. The overall contribution to this paper was 60%. N.M. performed experiments for Figure 4.1C, 4.5B-C, 4.6 and 4.7B and wrote 10% of the paper. J.B. performed experiments for Figure 4.2A-B. C.J.K. helped design and analyze experiments and write the paper. Collaborators and undergraduate thesis students contributed to other aspects of the paper such as preliminary data collection (D.L., L.S.Y., L.H., J.H.Y, S.F.), and data analysis and editing of the paper (A.S.W. and W.A.J.).

4.2 Introduction

Amyloid-beta (Aβ) is a 4 kDa intrinsically disordered protein that accumulates along the cerebral vasculature during cerebral amyloid angiopathy (CAA). The accumulation of Aβ leads to the degeneration of surrounding cells, and is associated with microhemorrhages [12]. Although CAA is present in over 90% of patients with Alzheimer's disease (AD) [192], the mechanisms underlying Aβ deposition on blood vessels remains unclear.

There are many links between hemostasis and cerebrovascular pathology in AD. Aβ can activate platelets, induce microhemorrhages in the brain, and interact with several coagulation factors [13,14,157,161]. Aggregates of Aβ can activate coagulation factor XII (FXII) to initiate blood clotting, and can increase fibrin density and resistance to fibrinolysis [157,158]. CAA deposits contain several coagulation factors, and antiplatelet therapy reduces accumulation of CAA deposits and improves cognitive function in mice [153]. Currently, the biochemical mechanisms that connect intravascular CAA deposition and hemostasis are not clear.

Aβ is formed from the amyloid beta precursor protein (APP), which is expressed by several cells, including platelets, neurons, glial cells and astrocytes. Platelets account for 95% of circulating APP [151]. APP can be cleaved to generate Aβ peptides of typically 40 or 42 residues long, Aβ40 and Aβ42, respectively. Platelets cleave APP and release both Aβ40 and Aβ42 when they are activated [141]. In both blood and CAA deposits, Aβ40 is more abundant than Aβ42, whereas Aβ42 is more abundant in senile plaques within the brain parenchyma, which is a hallmark of AD [193]. Mutations within the Aβ sequence can alter the pathogenicity of the peptide; for example, patients with the Flemish or Italian mutation (A21G and E22K, respectively) have increased CAA deposits, while patients with the Arctic mutation (E22G) have more plaque burden [132-134,136,194]. Aβ40 and Aβ42 can spontaneously aggregate into small, non-covalent oligomers and subsequently large aggregates, both of which are toxic to surrounding cells [135].

The formation of protein aggregates in CAA and AD may be regulated in part by transglutaminases (TG). TGs are a family of enzymes that form ε-(γ-glutamyl) lysyl isopeptide bonds between their substrates, creating irreversible bonds. TG activity colocalizes with plaques in brains in AD [14]. Tissue transglutaminae 2 (TG2) can induce Aβ oligomerization and aggregation

in vitro and reduce its clearance [16]. However, it is unknown if activated coagulation factor XIIIa (FXIIIa), a transglutaminase in blood plasma and on platelets, can crosslink Aβ in blood. FXIIIa is formed from coagulation factor XIII (FXIII), a protransglutaminase, when it is activated by thrombin in the presence of calcium during blood clotting [4]. The primary function of FXIIIa is crosslinking fibrin to itself and to other proteins to stabilize blood clots from premature fibrinolysis. FXIIIa also increases clot stiffness and platelet adhesion to further reduce blood loss.

Aβ forms stable complexes with FXIIIa and colocalizes with FXIIIa and fibrin in CAA deposits of AD patients [14]. Because Aβ is a substrate for TG2 and influences hemostasis in several ways [16], we tested if Aβ is a substrate for FXIIIa, and found that it is.

4.3 Experimental procedures

4.3.1 Platelet Preparation

This study was approved by the research ethics board of the University of British Columbia (H12-01516), and written informed consent was obtained from all healthy volunteers in accordance with the Declaration of Helsinki. Platelets and PRP were isolated as previously described [9]. Platelets were resuspended in Tyrode's buffer (119 mM NaCl, 5 mM KCl, 25 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 6 g/L glucose, pH 6.5) or in plasma at a final concentration of 2 x 10^9 platelets/mL or as otherwise specified.

4.3.2 Crosslinking of Aβ

Aβ peptides (Anaspec, California, USA) were initially dissolved in dimethylsulphoxide at 20 mg/ml and diluted with 25 mM HEPES buffer to 1 mg/ml. To test if Aβ is crosslinked by

purified FXIIIa, Aβ (25 μM) was incubated with purified FXIIIa (200 nM, Haematologic Technologies, Vermont, USA), $CaCl_2$ (4 mM), dithiothreitol (DDT, 200 μM), human thrombin (70 nM, Haematologic Technologies Vermont, USA) and T101 (2.5 mM, Zedira GambH, Germany) or Z006 (2.5 mM, Zedira GambH, Germany) at 37 °C for 16 hr. To test if Aβ is crosslinked to other proteins, human normal plasma (Affinity Biologicals, Ontario, Canada) or purified fibrinogen (6 mM, Haematologic Technologies, Vermont, USA) was incubated with Aβ (25 μM), $CaCl_2$ (4 mM) and tissue factor (TF, 1 pM, Dade Behring, Illinois, USA) or human thrombin (70 nM) at 37°C. The samples were treated with a reaction-quenching buffer (8 M Urea, 50 mM DTT, 12.5 EDTA) for at least 1 hr at 60 °C to dissolve the clot. To test if Aβ was crosslinked to platelet proteins, platelets were incubated with Aβ40 (25 μM), $CaCl_2$ (4 mM), and human thrombin (70 nM), ADP (50 μM), rat tail collagen (10 nM, Sigma, Germany), EDTA (12.5 mM) or T101 (2.5 mM).

4.3.3 Western Blotting

Samples were reduced, boiled, and separated on 10% or 4-15% Tris-glycine gradient gels (Bio-Rad, California, USA). After electrophoresis, the samples were transferred to a nitrocellulose membrane (GE Healthcare, Illinois, USA) and blocked with Odyssey Blocking Buffer (Li-Cor, Nebraska, USA). The membranes were treated with a primary antibody against Aβ (1:1000; 6E10, Covance, New Jersey, USA), FXIII-A (1:1000; SAF13A-AP, Affinity Biologicals, Ontario, Canada) or fibrin (1:50 000; A0080, Dako, California, USA), washed, and treated with HRP-labeled anti-host secondary antibody (1:15 000; Abcam, Cambridge, UK).

4.3.4 Kinetics assay

The rate of crosslinking of Aβ was determined by measuring the rate at which ammonia was produced during the TG reaction, using steady state kinetics at 37°C. A calibration curve of ammonia concentration and absorbance at 570 nm was determined with a Tecan M200 plate reader (BioVision Inc., California, USA). FXIIIa was mixed with Aβ peptides or GOE (5 – 50 μM) as amine donors, a glutamine-containing peptide (NQEQVSPLTLLK, 1 mM), DTT (40 μM), and $CaCl_2$ (3 mM). Aliquots from the transglutaminase reaction mixture were removed and quenched every 15 min with EDTA (15 mM). Kinetic parameters were calculated using graphing software (OriginPro 9.1).

4.3.5 Microfluidic analysis

Microfluidic devices were prepared from polydimethylsiloxane (PDMS) as previously described [195]. The channels were coated with lipid vesicles containing TF, phosphatidylserine (PS) and phosphatidylcholine (PC), while the rest of the device was coated with inert PC vesicles. The vesicles were prepared and devices were coated as previously described.[196] Citrated PRP containing fluorescent Aβ40-TAMRA (100 μg/mL, Anaspec) and fluorescent α-CD42b-FITC antibodies (1:100, eBioscience, California, USA) with or without T101 (500 μM) was flowed into the device at 1 μL/min along with a calcium-saline solution (40 mM $CaCl_2$, 90 mM NaCl) at a rate of 0.33 μL/min, which corresponds to venous shear rates (20 s^{-1}). Clotting was monitored using an epifluorescence microscope (Leica DMI6000B). The clots were then washed with calcium-saline solution (40 mM $CaCl_2$, 90 mM NaCl) at a rate of 5 μL/min for 10 min and imaged. For statistical

analysis, fluorescence intensities were measured at five equally distributed locations along the length of the channel.

4.3.6 Thromboelastography

The shear elastic moduli were evaluated at 37°C using a TEG Hemostasis Analyzer System 5000 (Haemoscope Corporation, Massachusetts, USA). Citrated whole blood, PRP, or PPP (Affinity Biologicals, Ontario, Canada) was combined with $CaCl_2$ (10 mM) and thrombin (200 nM), with or without Aβ40 (15 μM), T101 (800 μM), or eptifibatide (1.4 mM, Sigma, Germany) over 3 hr.

4.3.7 Statistical evaluation

Statistical analyses were performed using GraphPad Prism 7.0. All results presented in graphs are the mean ± standard error of the mean (SEM). N indicates number of independent experiments, performed on separate days. A 2-tailed unpaired Student's *t* test was used for all analyses. Significance was designated at p values <0.05.

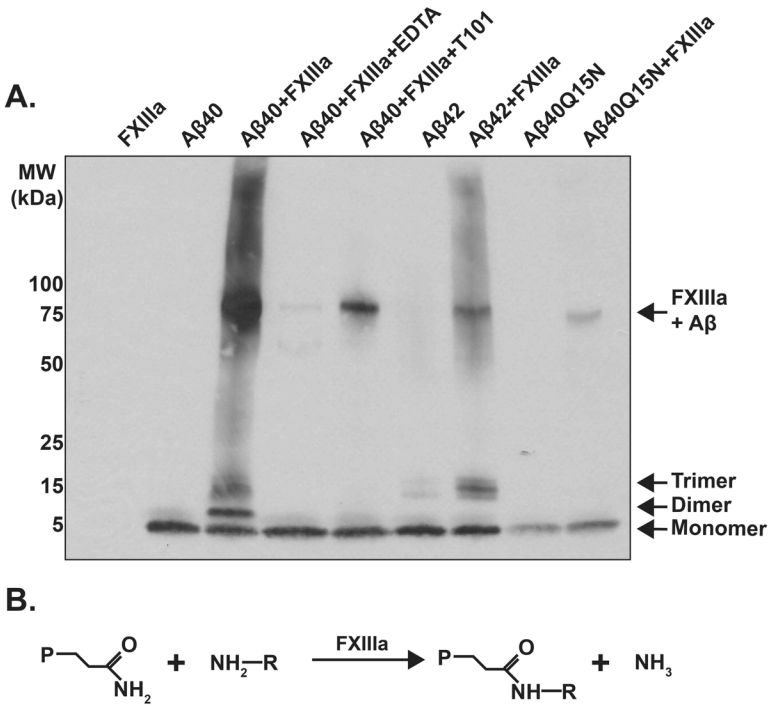
4.4 Results

4.4.1 Aβ40 is a substrate of FXIIIa.

To test if Aβ could be covalently crosslinked by FXIIIa, Aβ was incubated with FXIIIa and changes in molecular weights were detected using Western blot. FXIIIa crosslinked monomeric Aβ40 into dimers and oligomers, and to FXIIIa itself (Figure 4.1A). Crosslinked Aβ oligomers did not form when FXIIIa was inhibited by chelating calcium ions with EDTA or with T101, an irreversible inhibitor of FXIIIa transglutaminase activity. However, with T101, there was a distinct

band near 85 kDa, corresponding to the molecular weight of FXIIIa attached to Aβ40, which has been previously reported [14]. Similar trends were observed with Aβ42, although low concentrations of SDS-resistant oligomers formed without FXIIIa. When the only glutamine of Aβ40 was mutated to asparagine (Aβ40 Q15N), FXIIIa did not generate Aβ oligomers, indicating that the oligomerization was dependent on the glutamine residue of Aβ40.

To determine the kinetic constants of FXIIIa-mediated Aβ crosslinking, the release of ammonia, a product of the transglutaminase reaction (Figure 4.1B), was measured using a photometric assay. FXIIIa-mediated crosslinking of Aβ40 had K_m of 8.5 ±1.2 μM and k_{cat} of 1.3 ± 0.5 s^{-1}, resulting in a catalytic efficiency of 1.5 ± 0.5 x10^5 $M^{-1}s^{-1}$. A small molecule substrate of FXIIIa, glycine ethyl ester (GOE), had a similar catalytic efficiency of 2.0 ± 0.7 x10^5 $M^{-1}s^{-1}$ (Figure 4.1C). The catalytic efficiency of a peptide with the Aβ40 residues scrambled had 10-fold lower catalytic efficiency, indicating that the sequence of Aβ40 is important for FXIIIa activity. We were not able to calculate the catalytic efficiency of Aβ42 because Aβ42 precipitated at the concentrations necessary to perform the assay.



C.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
GOE	4.3±2.4	0.8±0.2	2.0±0.7 x 10^5
Aβ40	8.5±1.2	1.3±0.5	1.5±0.5 x 10^5
Aβ40 Scrambled	10.9±0.3	0.23±0.1	2.1±0.1 x 10^4
Aβ42	N.D.	N.D.	N.D.

Figure 4.1 Factor XIIIa can covalently crosslink Aβ.

(A) Western Blot against Aβ, after Aβ40, 42 or Q15N with or without EDTA or T101 were incubated with FXIIIa. (B) The transglutaminase reaction by FXIIIa. P indicates protein or polypeptide containing appropriate glutamine donor. R indicates a source of primary amine, such as a protein containing the appropriate lysine residue. (C) Table of kinetic parameters for FXIIIa crosslinking Aβ40 and 42. GOE: glycyl ethyl ester. N.D.: Not determined. n = 3

4.4.2 FXIIIa covalently crosslinked Aβ40 to fibrin.

Aβ can bind to many proteins in blood, such as FXII, FXIII, and fibrinogen [13,157,158]. To test if Aβ40 could be covalently crosslinked to other blood proteins, plasma containing Aβ40 was clotted, separated by SDS-PAGE, and immunoblotted against Aβ. Within 10 min, distinct Aβ bands were visible around 50 kDa, 70 kDa, and 100k kDa, and much higher molecular weights (Figure 4.2A). The molecular weights of these bands were similar to those of the α and γ chains of fibrin, the main substrates of FXIIIa. Bands with similar molecular weights as fibrin were visible after the γ-γ dimers were formed (Figure 4.2B). Aβ was still crosslinked when an inhibitor of TG2 (Z006) [197] was added to plasma, but not when T101 was added, indicating that FXIIIa, not TG2, crosslinked Aβ (Figure 4.3). To confirm that Aβ40 was crosslinked directly to fibrin, Aβ40 was incubated with purified fibrinogen, FXIIIa, and thrombin. Bands of Aβ were visible around 50 kDa, 70 kDa and 100 kDa, correlating to the molecular weights of the α and γ chains of fibrin and crosslinked γ-γ dimers (Figure 4.2C and 4.2D). Aβ40 was crosslinked to fibrin chains faster than to itself. Aβ40 was not crosslinked to fibrin chains when FXIIIa was inhibited with T101. Lower concentrations of Aβ40 (1 μM) also crosslinked to both purified and plasmatic fibrin by FXIIIa (Figure 4.4).

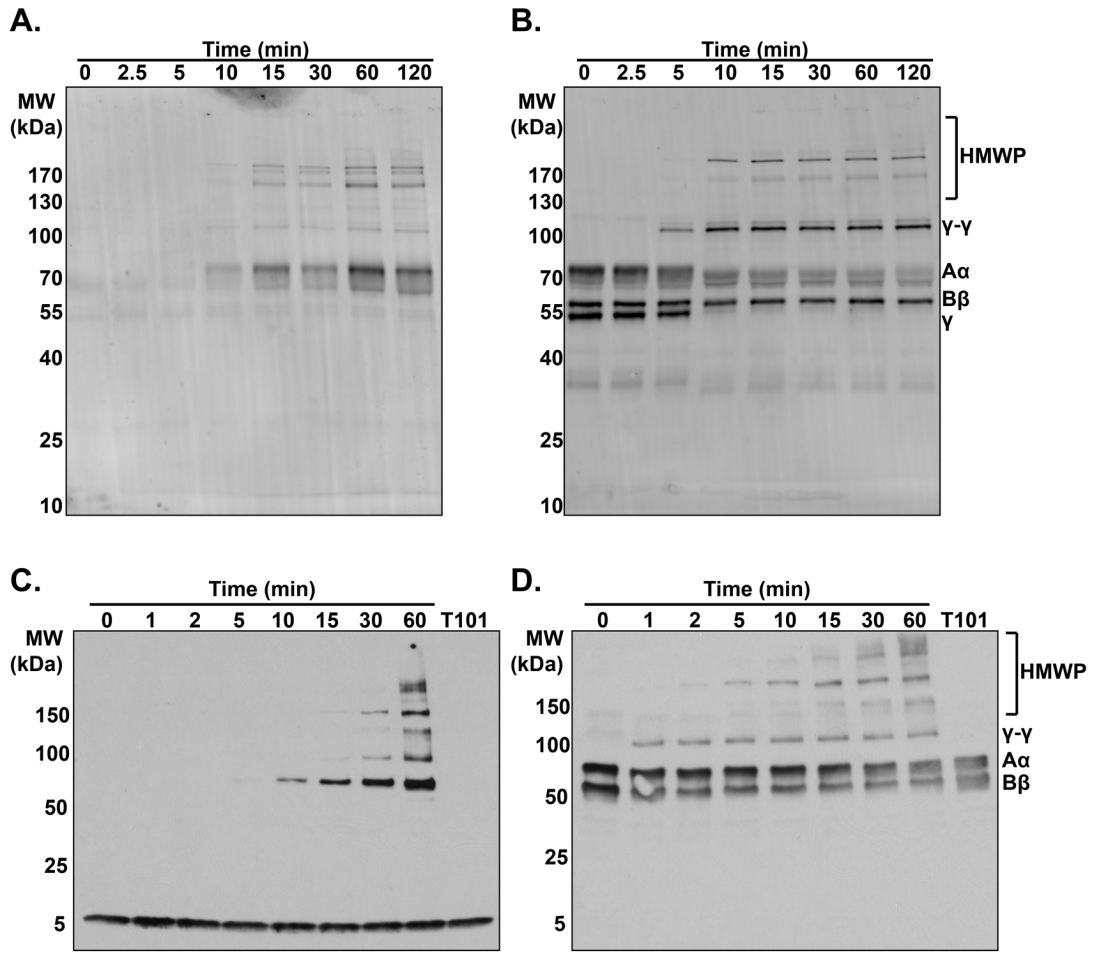


Figure 4.2 Aβ40 is crosslinked to fibrin during clotting.

(A-B) Western blot against Aβ (A) and fibrin(ogen) (B) after Aβ40 and tissue factor were added to recalcified plasma. (C-D) Western blot against Aβ40 (C) and fibrin(ogen) (D) after Aβ40 were incubated with fibrinogen, FXIIIa, thrombin, and $CaCl_2$. HMWP: High molecular weight polymers. Aα: α-chain of fibrinogen. Bβ: β-chain of fibrinogen, γ: γ-chain of fibrinogen, γ-γ: γ-dimers of fibrinogen.

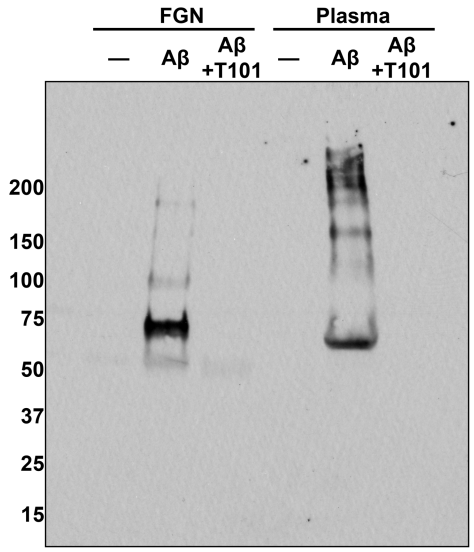


Figure 4.3 Aβ40 is crosslinked in plasma.

(A-B) Western blot against Aβ (A) and fibrin(ogen) (B) after Aβ40 and tissue factor were added to plasma containing T101 (inhibitor of FXIIIa and TG2) or Z006 (inhibitor of TG2). HMWP: High molecular weight polymers; FGN, fibrinogen

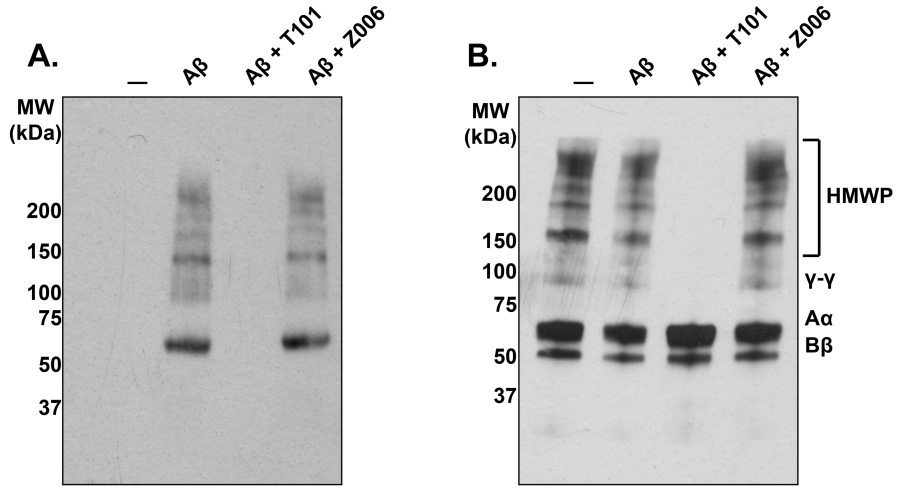


Figure 4.4 Aβ40 is crosslinked to fibrin.

Western blot against Aβ after 1 μM of Aβ40 was incubated with fibrinogen, FXIIIa, thrombin, and $CaCl_2$, or in plasma activated with tissue factor.

4.4.3 FXIIIa crosslinked Aβ40 to platelet proteins under flow.

Since platelets contain the FXIII-A subunits, which can be activated by high concentrations of Ca^{2+}, Aβ40 was incubated with platelets to test if platelet-derived FXIIIa could crosslink Aβ40 to itself or to other proteins. When platelets were activated with adenosine diphosphate (ADP), collagen, or thrombin, different patterns of Aβ crosslinking were detected compared to when platelets were not activated (Figure 4.5A). The Aβ bands formed with platelets had higher molecular weights than Aβ dimers and trimers, suggesting Aβ was crosslinked to platelet proteins. Both EDTA, which chelates the Ca^{2+} required for FXIIIa activity and platelet activation, and T101 prevented Aβ oligomers from forming. In contrast, Z006 did not prevent Aβ oligomers from forming, indicating that FXIIIa, not TG2, is responsible for crosslinking Aβ in platelets.

To test whether FXIIIa crosslinks Aβ40 to blood clots formed under flow, plasma containing platelets and fluorescently-tagged Aβ40 were flowed through a microfluidic device. Aβ40 accumulated on the clot, and colocalized directly on platelet aggregates and fibrin fibers. The co-localization of Aβ40 with platelets, measured by the ratio of Aβ40 fluorescence to platelet fluorescence, was significantly decreased when T101 was added, indicating that FXIIIa can crosslink Aβ40 to blood clots under flow (Figure 4.5B and 4.5C).

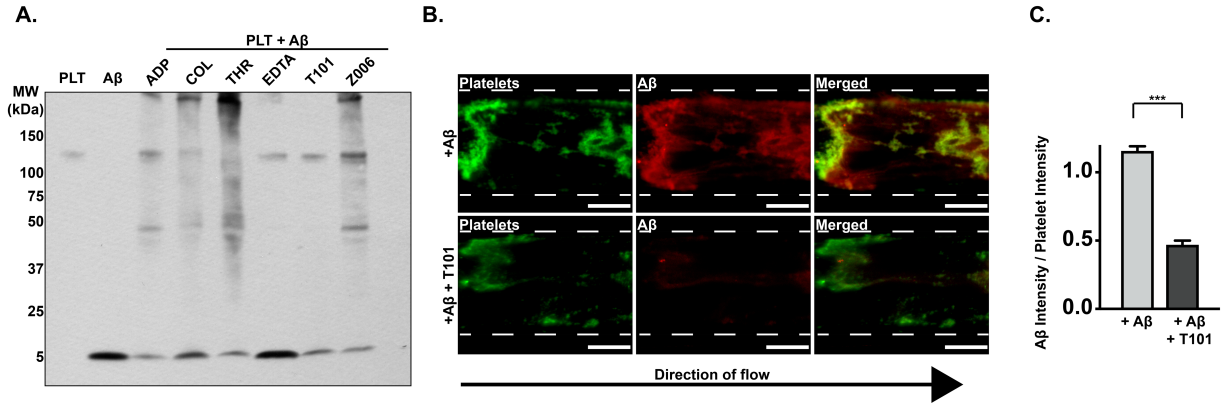


Figure 4.5 Platelet FXIIIa crosslinks Aβ40 to platelet proteins and localizes Aβ to blood clots under flow.

(A) Western blot against Aβ, after Aβ40 was incubated with platelets. PLT: platelets, ADP: adenosine diphosphate, COL: collagen, THR: thrombin. (B) Platelet-rich plasma (platelets stained green with α-CD42b-FITC antibody) containing Aβ40-TAMRA (red) with or without T101 was flowed through a channel containing a tissue factor-coated region. Scale bar: 200 μm. (C) Graph quantifying the colocalization of Aβ40 and platelets in images in panel B. ***$P < 0.001$. Error bars indicate ± SEM. n = 3.

4.4.4 Aβ40 increases clot stiffness of PRP and PPP via FXIIIa.

Since crosslinking increases fibrin stiffness [4], we tested the effect of Aβ40 on fibrin clot stiffness using thromboelastography (TEG). When whole blood was clotted in the presence of Aβ40, no significant difference in clot stiffness was observed (Figure 4.6A). Since the contribution of red blood cells may have masked subtle differences of Aβ on clot stiffness, we tested if the

influence of Aβ40 on fibrin could be detected when red blood cells were removed [198]. Aβ40 increased the stiffness of clots formed in platelet-rich plasma (PRP) and platelet-poor plasma (PPP) by 27% and 39%, respectively (Figure 4.6B and 4.6C). The increase in clot stiffness was dependent on both crosslinking by FXIIIa and platelet-platelet interactions, since inhibitors of FXIIIa (T101) or integrin $\alpha II_b\beta_3$ (eptifibatide) abrogated the increase of clot stiffness induced by Aβ40.

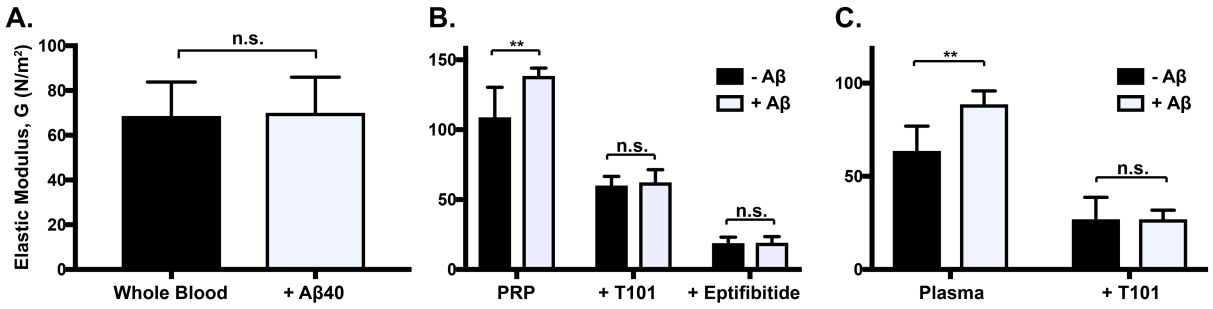
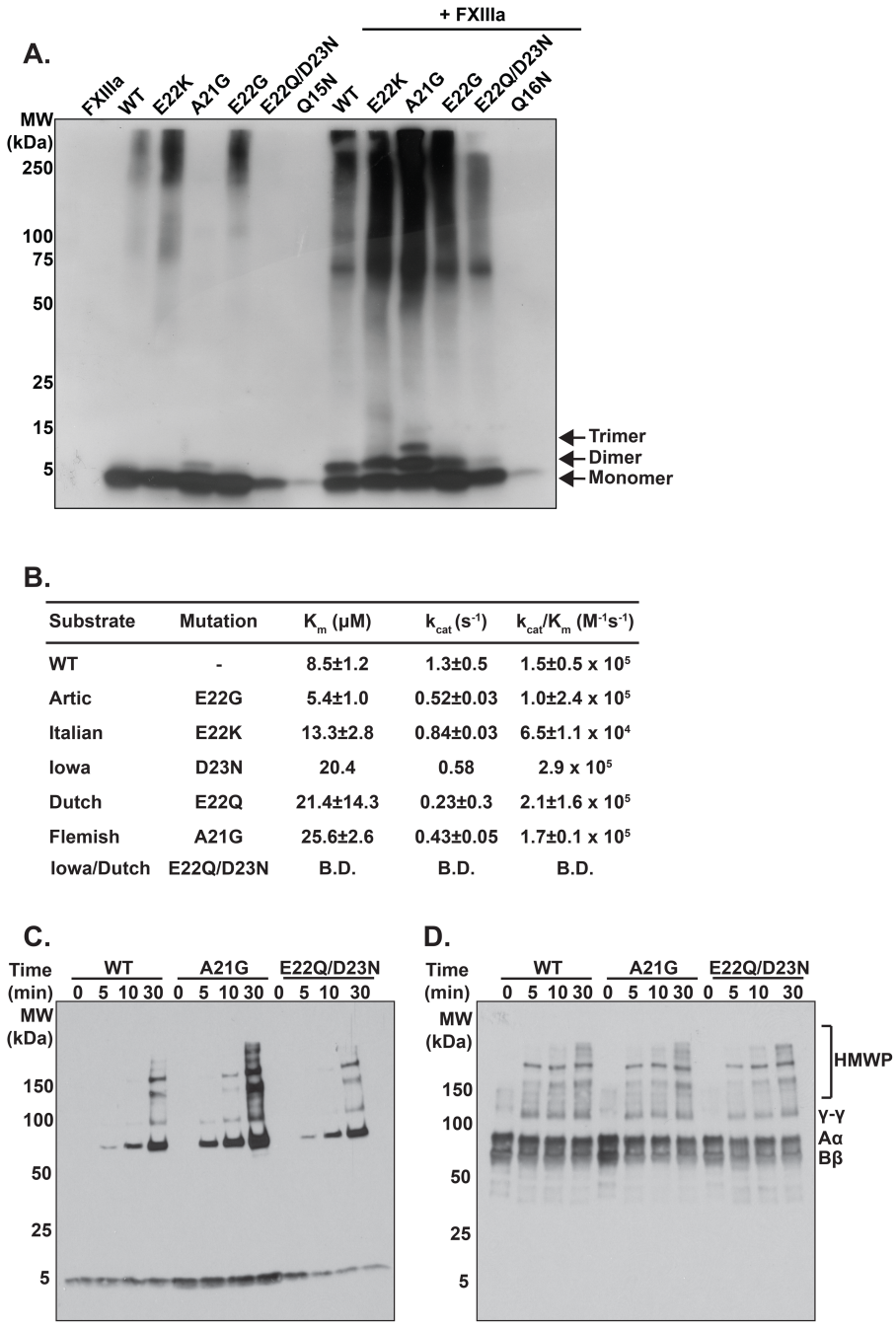


Figure 4.6 Aβ40 increases stiffness of platelet-rich plasma clots and platelet-poor plasma clots in a FXIIIa-dependent manner.

Thromboelastography analysis of blood and blood fractions with and without Aβ40. (A) Moduli of whole blood clots. (B) Moduli of platelet-rich plasma clots with or without T101, or eptifibatide (platelet aggregation inhibitor). (C) Moduli of platelet-poor plasma clots with and without T101. $^{**}P < 0.01$. n.s. indicates no significance. Error bars indicate ± SEM. PRP: platelet-rich plasma. n = 5-9 for all experiments.

4.4.5 Aβ40 mutants are differentially crosslinked by FXIIIa.

Certain point mutations of Aβ increase the probability of developing CAA [132-134,136,194]. To test whether FXIIIa crosslinks mutants of Aβ40, FXIIIa was incubated with Aβ40 containing Arctic (E22G), Italian (E22K), Iowa (D23N), Dutch (E22Q), Flemish (A21G) or Iowa/Dutch (E22Q/D23N) mutations. Oligomerization occurred in some mutants even in the absence of FXIIIa, which is consistent with the high propensity of these mutants to aggregate. However, when the mutants were incubated with FXIIIa, different species of Aβ oligomers were formed in varying concentrations. For example, the Flemish mutation (A21G) was crosslinked to a greater extent than WT, while the Iowa/Dutch mutation (E22Q/D23N) was crosslinked to a lesser extent (Figure 4.7A). The catalytic efficiencies of these mutant Aβ40 sequences were lower than, or comparable to, the catalytic efficiency of WT Aβ40 (Figure 4.7B). In crosslinking to fibrin chains, the Flemish mutation was crosslinked to a greater extent than WT (Figure 4.7C and 4.7D).



Substrate	Mutation	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
WT	-	8.5±1.2	1.3±0.5	1.5±0.5 x 10^5
Artic	E22G	5.4±1.0	0.52±0.03	1.0±2.4 x 10^5
Italian	E22K	13.3±2.8	0.84±0.03	6.5±1.1 x 10^4
Iowa	D23N	20.4	0.58	2.9 x 10^5
Dutch	E22Q	21.4±14.3	0.23±0.3	2.1±1.6 x 10^5
Flemish	A21G	25.6±2.6	0.43±0.05	1.7±0.1 x 10^5
Iowa/Dutch	E22Q/D23N	B.D.	B.D.	B.D.

Figure 4.7 FXIIIa differentially crosslinks mutant Aβ40 peptides.

(A) Western blot against Aβ, after Aβ40 and mutants of Aβ40 were incubated with FXIIIa. (B) Table of kinetic parameters for FXIIIa crosslinking Aβ40 mutants, measured by the rate of

ammonia release. WT: Wild type, B.D.: Below detection n = 3. (C-D) Western blot against Aβ40 (C) and fibrin(ogen) (D) after Aβ40 WT, Aβ40 A21G (Flemish) or Aβ40 E22Q/D23N (Iowa/Dutch) were incubated with fibrinogen, FXIIIa, thrombin, and $CaCl_2$. HMWP: High molecular weight polymers. Aα: α-chain of fibrinogen. Bβ: β-chain of fibrinogen, γ: γ-chain of fibrinogen, γ-γ: γ-dimers of fibrinogen.

4.5 Discussion

The results show that Aβ40 is covalently crosslinked by FXIIIa, both to itself to form dimers and oligomers, and also to other blood proteins in plasma, such as fibrin. FXIIIa also crosslinked Aβ40 to clots under flow, and the crosslinking of Aβ40 increased clot stiffnesses in PRP and PPP, but not in whole blood. Although the reaction occurs *in vitro*, the physiological relevance and significance of these reactions *in vivo* must be further investigated.

The crosslinking of Aβ40 to fibrin chains was visible only after the γ-γ dimers were formed. This is consistent with the kinetic data, where the catalytic efficiency of FXIIIa and Aβ (k_{cat}/K_m = $1.5 \pm 0.5 \times 10^5$ $M^{-1}s^{-1}$) was lower than that of fibrin γ-chains (5.1×10^7 $M^{-1}s^{-1}$) [199]. Aβ was crosslinked to fibrin at an Aβ concentration of 1 μM, which is a concentration that may occur at sites of cerebrovascular damage [200].

Aβ and FXIIIa can form stable complexes *in vitro*, and FXIIIa is catalytically active in vessels with CAA [14]. Although isopeptide bonds formed by transglutaminases have been discovered in CAA, covalent crosslinking of Aβ by FXIIIa was not previously detected *ex vivo* [14].

The discrepancy with the data here may be due to the higher, though physiological, FXIIIa concentrations and longer reaction times used here, and potentially higher specific activity of FXIIIa. Although the crosslinking of Aβ to itself was visible only after 3 hr, crosslinking to fibrin occurred within minutes.

The crosslinking of Aβ may potentially be influenced by Aβ-albumin interactions. Albumin sequesters approximately 90% of Aβ in plasma and preferentially binds oligomeric Aβ to monomeric Aβ [201,202]. FXIIIa crosslinked Aβ to fibrin both in buffer and in plasma at similar rates, suggesting that albumin does not play a significant role in influencing the rate of crosslinking in these conditions. However, how albumin affects the clearance of crosslinked oligmeric Aβ requires further examination.

Crosslinking of Aβ may have implications in at least two scenarios. First, Aβ may modify clot structure at sites of damage in the cerebral vasculature or at platelet aggregates. It remains to be determined what the effect on clotting might be, but it may contribute to fibrinolysis since non-crosslinked aggregates of Aβ increase resistance to fibrinolysis and activate the coagulation cascade through FXII [157,159]. Crosslinking Aβ to fibrin could enhance clotting by localizing the platelet activating sequence (Aβ25-35) to fibrin [161]. Crosslinking of Aβ may have a greater significance in arteries than veins, as arterial clots have fewer red blood cells, since Aβ increased the stiffness of PRP, but not whole blood clots.

Second, FXIIIa-mediated activity may contribute to CAA and AD pathology. Given that blocking the binding between Aβ and fibrin with a small molecule can improve cognitive impairment in mouse models of AD, covalent crosslinking of Aβ and fibrin may exacerbate CAA

pathology [159]. In patients with AD, there is a higher frequency of a FXIII allele (Val-34-Leu) that undergoes faster activation, suggesting that accelerated crosslinking can aggravate AD development [203]. The differences in crosslinking between the Aβ variants and mutants provides further insight to the potential significance of the interaction between FXIIIa and Aβ. FXIIIa crosslinked Aβ40 to a higher extent than Aβ42, providing a potential explanation as to why Aβ40 is the more prominent form of Aβ within CAA. AD patients with the Flemish mutation (Aβ40 A21G) have increased CAA phenotype and Aβ40 with the Flemish mutation was crosslinked to a higher extent compared to wild type Aβ40 [135]. An alternative hypothesis is that crosslinking of Aβ by FXIIIa is a physiological process that is separate from aggregation and amyloid accumulation.

In conclusion, synthetic Aβ40 can be covalently crosslinked to itself, and to fibrin and platelet proteins by FXIIIa under flow. Given that Aβ and FXIIIa colocalize within CAA, these results provide motivation to test if FXIIIa contributes to the accumulation of intravascular deposits of Aβ in CAA.

Chapter 5: Conclusion

This thesis describes novel mechanisms of how FXIIIa interacts with the fibrinolytic system. Previously, the anti-fibrinolytic function of FXIIIa, by crosslinking fibrin to itself and to AP has been well characterized. Here in this thesis, the role of fibrinolytic system on FXIII(a) inactivation was examined. Plasmin can inactivate FXIIIa preferentially to FXIII (Chapter 2). The significance of this reaction in DVT patients being treated for thrombolysis was examined (Chapter 3). Finally, a novel substrate of FXIIIa, Aβ, which can inhibit fibrinolysis, was examined (Chapter 4). The studies here show that the coagulation factor XIIIa lies at the interface of coagulation and fibrinolysis in more ways than previously determined.

5.1 Coagulation factor XIIIa is inactivated by plasmin

In Chapter 2, whether the fibrinolytic enzymes can inactivate FXIIIa was investigated. Plasmin can proteolytically inactivate FXIIIa, but not FXIII, at concentrations as low as 100 nM in buffer. The primary cleavage site of FXIIIa by plasmin was determined to be between K468 and Q 469. The reaction had an apparent K_m of 0.49 ± 0.02 μM and k_{cat} of $4.2 \pm 1.1 \times 10^{-3}$ s^{-1}, resulting in a catalytic efficiency of $8.3 \pm 1.7 \times 10^3$ $M^{-1}s^{-1}$. Plasmin degraded FXIIIa in plasma as well as FXIIIa from activated platelets. Plasmin did not inactivate FXIIIa during clot formation, but FXIIIa was inactivated and degraded during fibrinolysis and at tPA conditions resembling thrombolytic therapy. The significance of this reaction should be further investigated in situations where plasmin activity is abnormal; patients with Quebec platelet disorder, patients undergoing trauma induced coagulopathy (TIC), or patients with plasminogen deficiency would provide insights to the significance of plasmin-mediated inactivation of FXIIIa in pathophysiology.

5.2 Coagulation factors XIII and XIII-A* decrease in some DVT patients following catheter-directed thrombolysis

In Chapter 3, the significance of plasmin-mediated inactivation of FXIIIa in patients undergoing thrombolysis was examined. Among 9 DVT patients undergoing CDT, FXIII levels were highly variable, even before the start of therapy. After the administration of tPA, FXIII zymogen levels were decreased by more than 40% in 5 of 9 patients. Since plasmin does not degrade the zymogen, the decrease may due to tPA degrading FXIII directly. *In vitro*, tPA directly cleaved FXIII zymogen at concentrations of 200 nM in buffer and 10 μM in plasma. With respect to FXIIIa, it remained stable in blood samples collected before the administration of tPA. However, in 2 of 9 patients, more than 85% of FXIIIa was degraded 4 hours after being activated in blood samples collected after the therapy. Given the small cohort of patients recruited in this study, further studies are required to identify what regulates FXIII levels and FXIIIa stability in these patients and if the decrease in FXIIIa contributes to the major bleeding events seen during thrombolysis.

5.3 Coagulation factor XIIIa crosslinks amyloid β into dimers and oligomers and to blood proteins.

In Chapter 4, Aβ, which can inhibit fibrinolysis, was identified as a novel substrate of FXIIIa. FXIIIa covalently crosslinked Aβ to itself with a catalytic efficiency of $k_{cat}/K_m = 1.5 \times 10^5\ M^{-1}s^{-1}$. FXIIIa also crosslinked Aβ to fibrin in buffer and in plasma; the crosslinking of Aβ to fibrin occurred much faster than to itself. Aβ was crosslinked to platelet proteins and could be covalently attached to platelets under flow in plasma. Furthermore, Aβ increase the clot strength of PRP and PPP, but not in whole blood. Aβ mutants were also crosslinked by FXIIIa to itself and to fibrin, with variable efficacy depending on the

mutation. The oligomerization of Aβ, caused by crosslinking activity of FXIIIa may be responsible for creating the nucleation sites for Aβ accumulation, leading to Aβ deposition along the cerebrovasulature, and hence CAA development. This provides motivation to further characterize how FXIIIa may be involved in CAA and how inhibition or downregulation of FXIIIa affects CAA development in patients.

Chapter 6: Future Directions

6.1 Exploring the impact of plasmin-mediated inactivation of FXIIIa in pathophysiology

6.1.1 Patients receiving thrombolytic therapy

Future work will examine the significance of plasmin-mediated inactivation of FXIIIa in pathophysiology. Although Chapter 3 describes how FXIIIa stability is altered in patients receiving CDT, further studies should be performed with a larger number of patients. Increasing the patient population will provide insight into the factors that regulate FXIII levels in patients before the treatment, and the factors that regulate FXIIIa stability such as dosage and duration of thrombolytic therapy, as well as the concentrations of fibrinolytic proteins such as plasminogen, tPA and/or PAI.

Patients suffering from ischemic strokes are also treated with thrombolytic therapy, if eligible. Unlike CDT where the patients are administered 1mg/hr of tPA until clot dissolution, stroke patients receive a bolus injection of 0.1mg of tPA followed by continuous injection of 0.9mg of tPA for 1 hr. In collaboration with neurologists, investigating the stability of FXIIIa in stroke patients treated with thrombolytics may help predict patients who can benefit from thrombolysis without the bleeding. Although a slight decrease in FXIII levels following stroke treatment has been reported, the stability of FXIIIa, once activated, has not been characterized[188].

6.1.2 Patients with Quebec platelet disorder

Patients with QPD have bleeding tendency due to hyperfibrinolysis. They have a genetic mutation leading to a duplication of the uPA gene, leading to increased uPA concentration in the platelet α-granules[180]. It would be interesting to examine how QPD influences FXIIIa stability

and activity. If FXIIIa activity is reduced in QPD patients, the increase in fibrinolytic potential will be two-fold: increasing the activation of plasminogen and inhibiting the anti-fibrinolytic protein FXIIIa. The findings may lead to novel mechanisms to treat QPD patients.

6.1.3 Patients with trauma induced coagulopathy

Patients who are suffering from trauma-induced coagulopathy (TIC) have hyperfibrinolysis. TIC patients often suffer from hyperfibrinolysis and depletion of their coagulation factors, leading to impaired thrombin generation and subsequent premature clot clearance[84]. It would be useful if future work examines whether FXIII(a) stability and activity is altered in TIC patients and whether FXIIIa levels contribute to the mortality and morbidity of TIC patients. The insights gathered from these studies will provide helpful information in the management of hyperfibrinolysis in patients suffering from TIC.

6.2 Exploring the significance of FXIIIa-crosslinking of Aβ

6.2.1 Examining the contribution of FXIIIa in CAA progression

The accumulation and deposition of Aβ along the cerebrovasculature causes degeneration of the blood brain barrier, leading to ICH and subsequent neuronal degeneration[144]. Although FXIIIa and Aβ colocalize along the cerebrovasculature in patients with CAA, whether FXIIIa contributes to Aβ deposition has not been elucidated[14]. Interestingly, there is a higher prevalence of the FXIII V34L mutation, which is activated more readily by thrombin, in AD patients compared to WT FXIII[203]. It would be useful if future work examines whether the FXIIIa-mediated crosslinking of Aβ leads to increased aggregation and subsequent deposition along the

vasculature. If FXIIIa contributes to the development of CAA, regulating FXIII may be a viable adjunct therapy in AD treatment.

6.2.2 Identifying the significance of interaction of platelet FXIII and Aβ/APP

Despite the wealth of literature on Aβ and APP, the physiological function of Aβ and APP remains unclear, especially in platelets. Platelets account for over 90% of circulating APP and can be processed to generate Aβ and be released upon platelet activation[141,151]. Aβ/PP and FXIII, which are both released from platelets, can form stable complexes[14]. It would be interesting to examine whether platelet Aβ/APP modulate FXIIIa biochemistry, such as its activation, synthesis, localization, inactivation and activity. Identifying the physiological function of Aβ/APP in platelets will provide insight to the role of Aβ/APP in the brain and help develop future treatments of AD.

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Appendices

Appendix A Coagulation factor XIIIa is inactivated by plasmin

This paper was the basis for Chapter 2 of the dissertation and can be found here:

http://www.bloodjournal.org/content/126/20/2329.long?sso-checked=true

Appendix B Coagulation factors XIII and XIII-A* decrease in some D VT patients following catheter-directed thrombolysis

This paper was the basis for Chapter 3 of the dissertation and can be found here:

https://journals.lww.com/bloodcoagulation/Abstract/publishahead/Coagulation_factor_XIII_A_and_activated_XIII_A.98579.aspx

Appendix C Coagulation factor XIIIa crosslinks amyloid β into dimers and oligomers and to blood proteins

This paper was the basis for Chapter 4 of the dissertation and can be found here:

http://www.jbc.org/content/294/2/390.long

Appendix D Rivaroxaban and apixaban induce clotting factor Xa fibrinolytic activity.

I was a co-author on this publication in the Journal of Thrombosis and Haemostasis: R.L.R. Carter, K. Talbot, W.S. Hur, S.C. Meixner, J.G. Van der Gugten, D.T. Holmes, H.C.F. Cote, C.J. Kastrup, T.W. Smith, A.Y.Y. Lee, E.L.G. Pryzdial (2018). Rivaroxaban and apixaban induce clotting factor Xa fibrinolytic activity. I obtained the data for Figure 5 and helped analyze the data and revise the manuscript. This paper can be found here:

https://onlinelibrary.wiley.com/doi/full/10.1111/jth.14281