

**EFFECTS OF DIRECT ACTIVE-SITE ANTICOAGULANTS ON THE FIBRINOLYTIC  
FUNCTION OF COAGULATION FACTOR Xa**

by

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## Abstract

Treatment of occlusive clots (thrombi) is important for proper health yet systemic bleeding remains a major complication of thrombolytic therapy. Thus, the utility of agents such as alteplase, the current thrombolytic approved to rapidly lyse thrombi, is limited. As an alternative, our laboratory has found that cleavage products of clotting factor Xa (FXa) can enhance the generation of localized plasmin, the enzyme that degrades and solubilizes clots. This function is stabilized by blocking or chemically modifying the active site of FXa such that FXa $\beta$ , the first resulting fragment, persists.

In recent years, direct oral anticoagulants (DOACs) have been approved for the treatment and prevention of venous thromboembolism and stroke in patients with atrial fibrillation. Two agents in this class of drugs, rivaroxaban and apixaban, are direct inhibitors of FXa. This dissertation addresses the effect these two DOACs have on fibrinolysis and FXa fragmentation due to their ability to block the active site of FXa. Results presented in Chapter 3 demonstrate that in normal pooled plasma, rivaroxaban and apixaban enhance clot lysis in a FX- and dose-dependent manner. Downstream effects caused by a potential reduction in thrombin generation were not the basis for the enhancement. Instead, FXa $\beta$  persisted in plasma. In purified protein experiments, FXa $\beta$ , generated in the presence of rivaroxaban and apixaban, enhanced plasmin generation by binding plasminogen.

Results in chapter 4 showed persistence of FXa $\beta$  in rivaroxaban-treated patient samples but not in samples from non-anticoagulated patients and those treated with a thrombin-specific DOAC. A strong correlation between increasing FXa $\beta$  amounts and faster fibrinolysis time was shown in rivaroxaban-treated patients with atrial fibrillation, the approved indication in which thrombosis is the least provoked. Fibrinolysis was not, however, dose-dependently enhanced.

There was also no statistical difference in fibrinolysis times between rivaroxaban-treated and non-anticoagulated atrial fibrillation patients; an observation attributed to the involvement of potential confounders. Findings in this dissertation may have implications in the use of FXa-specific DOACs as thrombolytic adjuncts and as the anticoagulants of choice in preventing post-thrombotic syndrome, a common long-term morbidity in patients with deep vein thrombosis that may result from delayed or impaired clot dissolution.

## **Lay summary**

Clearance of obstructive blood clots is crucial for good health; however, the risk of bleeding limits the use of current clot-busting drugs. As an alternative, our laboratory identified that FXa $\beta$ , a fragment of the blood clotting protein factor Xa (FXa), has the potential to safely and rapidly degrade clots. When this fragment is derived from active site-blocked FXa, it cannot be further broken down and thus its clot dissolving function is sustained. Rivaroxaban (Xarelto<sup>®</sup>) and apixaban (Eliquis<sup>®</sup>) are anticoagulants that specifically block the active site of FXa to prevent clotting. This dissertation demonstrates that they also are able to prevent further fragmentation of FXa $\beta$  and expedite the degradation of clots. In plasma from patients with an irregular heart rhythm, faster clot-busting was associated with more FXa $\beta$ . These findings have potential implications in the use of FXa-specific anticoagulants to prevent post-thrombotic syndrome, a debilitating condition linked to ineffective clot clearance.

## **Preface**

Work presented in this dissertation was approved by the University of British Columbia Ethics Board (Certificates #: H07-00624 and H16-01153-A001) and the Canadian Blood Services Research Ethics Board (Certificate #: 2007.006). The research question was originally proposed by Dr. Ed Pryzdial, clinical professor in the department of Pathology and Laboratory Medicine, UBC. A version of Chapter 3 has been submitted for publication with the title: Rivaroxaban and Apixaban Induce Clotting Factor Xa Fibrinolytic Activity. Most of the experiments within this chapter were designed by me with the feedback of my supervisor and supervisory committee members. I conducted the majority of experiments with an exception being data shown in Figure 18. The latter was done by Woosuk Hur, a PhD candidate in Dr. Christian Kastrup's research group, UBC. I wrote the draft of the manuscript and participated in its refinement along with my supervisor prior to it being submitted. Chapter 4 is composed of data that was presented orally and in poster presentations at various local, international, and national conferences and symposiums. Quantification of anticoagulant concentrations in patient plasma samples was done by Jessica Grace van der Gugten, a LC-MS/MS assay development specialist at Providence Health Services Authority. A manuscript concerning the data presented in chapter 4 is currently in preparation.

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## List of Abbreviations

%FXa $\alpha$	Percent of FXa $\alpha$ generated relative to total FXa $\alpha/\beta$
%FXa $\beta$	Percent of FXa $\beta$ generated relative to total FXa $\alpha/\beta$
Afib	Atrial fibrillation
apix	Apixaban, direct FXa inhibitor
aPL	Anionic phospholipid
AT	Antithrombin
CaCl <sub>2</sub>	Calcium chloride
C <sub>max</sub>	Maximum plasma concentration
CMK	Chloromethylketone
CVD	Cardiovascular disease
D-	Short for DEGRck
Dabi	Dabigatran, direct thrombin inhibitor
DEGRck	Dansyl-glu-gly-arg chloromethyl ketone
DMSO	Dimethyl sulfoxide
DOAC	Direct oral anticoagulant
DVT	Deep vein thrombosis
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
F	factor
f.c.	Final concentration
FIX	Factor IX
FIXa	Activated factor IX
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VIIa
FVIII	Factor VIII

FVIIIa	Activated factor VIII
FX	Factor X
FXa	Activated factor X
FXa-DOAC	DOAC targeting FXa
FXa $\beta$	FXa derivative post 1 <sup>st</sup> plasmin cleavage of FXa
FXDP	FX-deficient plasma
Gla	Gamma-carboxylated glutamic acid
HBS	HEPES-buffered saline
HBSP	HBS with 0.01% PEG
HBST	HBS with 0.1% Tween-20
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
II	Prothrombin
IIa	Thrombin
IIDP	Prothrombin deficient plasma
INR	International Normalized Ratio
K	Lysine
K330	Lysine residue 330
Lys	Lysine
Max OD	Maximum optical density
PAI-1	Plasminogen activator inhibitor 1
PCPS	Phosphatidylcholine, phosphatidylserine
PE	Pulmonary embolism
PEG	Polyethylene glycol
Pg	Plasminogen
Pn	Plasmin
PS	Phosphatidylserine
PTCI	Potato tuber carboxypeptidase inhibitor
PTS	Post-thrombotic syndrome
PVDF	Polyvinylidene difluoride
riva	Rivaroxaban; direct FXa inhibitor

RVV-X	Russell's viper venom FX activator
S.E.M	Standard error of the mean
SD	Standard deviation
SEM	Scanning Electron Microscope
TAFI	Thrombin-activatable fibrinolysis inhibitor, carboxypeptidase
TAFIa	Activated thrombin-activatable fibrinolysis inhibitor
TBS	Tris-buffered saline
TBST	TBS with 0.1% Tween-20
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Time-to-max OD	Time to reach maximum optical density, reflective of clotting time
tPA	Tissue plasminogen activation
U-PA	Urokinase plasminogen activator
VGH	Vancouver General Hospital
VTE	Venous thromboembolism
Xa33/13	FXa derivative post 2 <sup>nd</sup> plasmin cleavage:
XaAT	FXa in complex with antithrombin
Xai-K	FXa derivative in which its active site has been chemically modified

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## **Dedication**

I would like to dedicate this dissertation to my late father and brother who instilled in me the value of education and encouraged me to pursue it. I also dedicate it to my late aunt and uncle whose death, due to a heart attack and stroke respectively, triggered my interest of blood clots. To my nephew Gael, our journeys thus far have many similarities but this is a testament to you that you can make it. My final dedication is to my mother who fought as hard as I did to make this dream come through; thanks for never giving up on me.

## **Chapter 1: INTRODUCTION**

### **1.1 Hemostasis overview**

#### **1.1.1 Burden and pathology of occlusive clots**

Cardiovascular diseases (CVDs), which include heart diseases and strokes, remain the leading cause of death worldwide [1]. Of the 17.7 million deaths caused by CVDs annually, 6.7 million are due to a stroke [1]. In Canada, 25.1 % of the population who died in 2013 succumbed to heart disease and stroke [2]. The overall economic burden of CVD in Canada during 2008 was 9.4 billion dollars, a value that continues to increase with the growing number of individuals being affected by CVDs [3,4]. During 2015 in the United States, one in every three deaths was due to CVDs and one in every twenty deaths was caused by a stroke [5]. The financial burden of CVD was estimated at 316.1 billion between 2012 and 2013 in United States, with 199.6 billion for heart diseases and 33.9 billion for stroke [6].

Ischemic strokes, often caused by a thrombus blocking the flow of blood, account for more than 87 % of all strokes [6,7]. About 15 % of ischemic strokes, and up to 50 % in individuals greater than 80 years old, are due to atrial fibrillation, a condition characterized by irregular heart rhythm [8,9]. Due to the potential pooling of blood, patients with atrial fibrillation have a 5-fold increased risk of suffering from a stroke. In 2010, a global estimate of 33.5 million individuals had atrial fibrillation [10]. In Canada, 350,000 individuals are affected by atrial fibrillation annually. The average economic burden of atrial fibrillation in Canada was tabulated to be \$815 million 2010 Canadian dollars in 2007-2008 [11]. In 2005, the financial burden of the disorder in the United States was ~ 6.65 billion dollars [12] with an incremental cost range of \$6 to \$26 billion [13]. An estimate of 3 to 6 million people was affected by atrial fibrillation in the United States during 2010 [6]. Projection studies suggest that this value will increase to 12.1 -

15.9 million individuals by 2050 due to the aging of the population [14,15]. A similar increase in the prevalence of atrial fibrillation is expected globally [16,17].

Occlusive clots formed in deep veins result in a condition called deep vein thrombosis (DVT). DVT, which often originates from the deep vein of a leg, affects about 45,000 individuals in Canada annually [18]. A major complication in 20 to 50 % of DVT patients is post-thrombotic syndrome (PTS) [19,20]. A potential cause for PTS is ineffective clot dissolution [21] with residual vascular scarring [22], leading to chronic inflammation [23]. If DVT is left untreated, the clot can dislodge and travel to the lung resulting in pulmonary embolism (PE), an often life-threatening condition. An average of one in four patients with PE experience sudden death [24]. Together DVT and PE are categorized as venous thromboembolism (VTE), the third leading cause of cardiovascular disease after heart disease and stroke with 10 million cases reported worldwide [25]. In the United States, VTE contributes to more than 100,000 deaths annually and has an annual financial burden of 7 - 10 billion dollars [26].

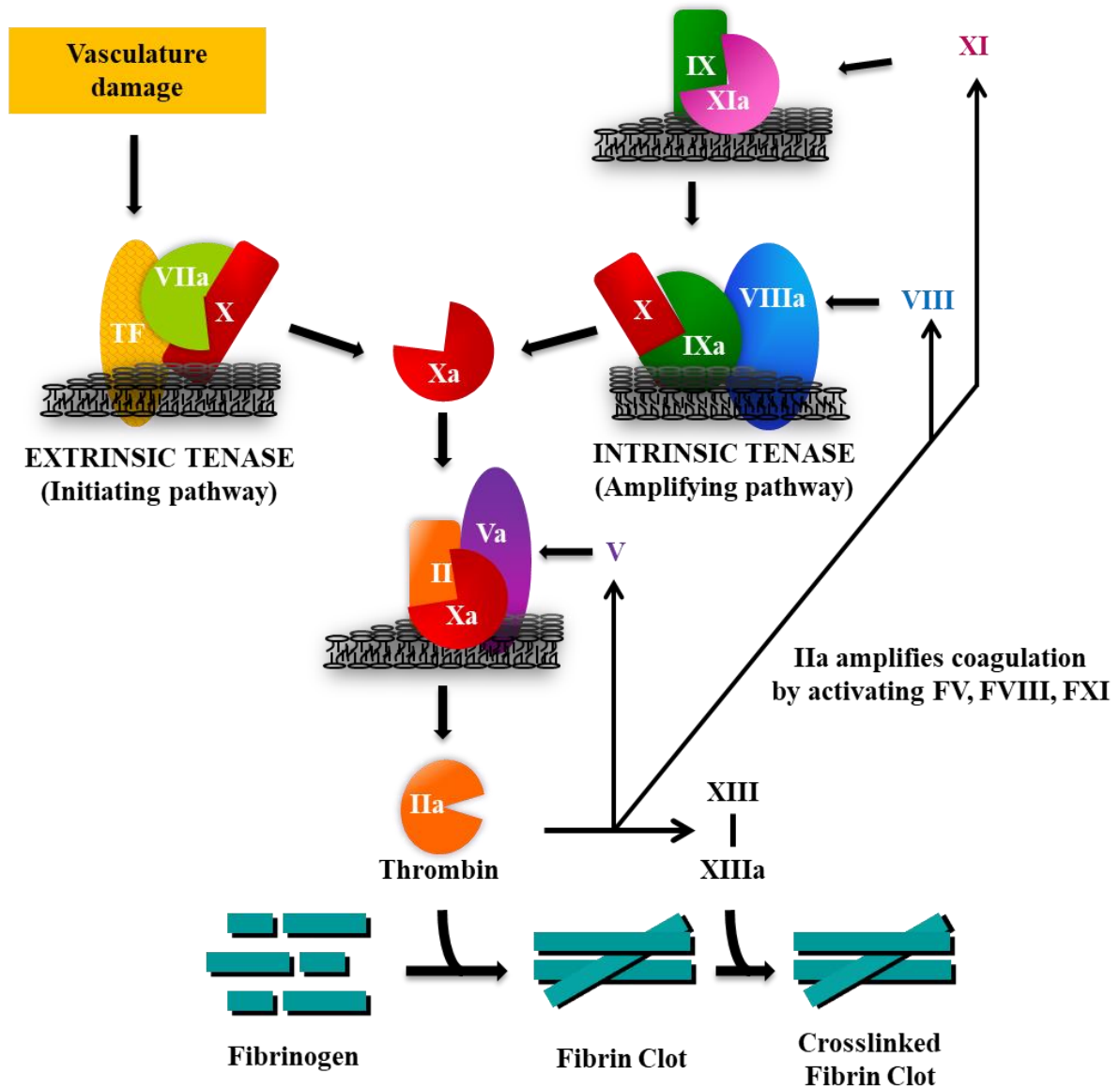
The burden of occlusive clots to an individual's health and a country's economy highlights the importance of maintaining a proper balance between coagulation and fibrinolysis. For decades, scientists have tried to understand the biochemistry of these two physiological events separately and as a whole. Coagulation can be promoted by vessel dysfunction, stasis, or a hypercoagulable state according to the Virchow's triad [27]. It often includes the formation of an initial unstable clot by the deposition of platelets followed by the incorporation of insoluble fibrin into the platelet thrombus (reviewed in [28]). The latter is achieved by a series of cascading events aimed at strengthening and stabilizing the vascular clot. In cases of VTE and atrial fibrillation, where vessel damage is not the major trigger, thrombosis is mainly initiated by

the activation of coagulation factors [29,30] although platelet activation still occurs [31]. Thus, various anticoagulant drugs were and are still being developed to prevent thrombotic episodes in individuals with an increased risk of coagulation [32-34], reviewed in [35]. For decades, warfarin, a vitamin K antagonist, has been the most widely used anticoagulant. However, this convention is now changing with the introduction of direct oral anticoagulants (DOACs) that bind to the active site of specific clotting factors, particularly thrombin or factor (F) Xa.

To maintain a hemostatic balance, once a clot is formed, it has to be degraded or it could become life threatening. Dissolution of clots or thrombus is achieved through the fibrinolytic pathway or the use of thrombolytic agents, respectively. The physiological initiator of fibrinolysis is tissue plasminogen activator (tPA). It works by generating plasmin, the enzyme that degrades and eventually solubilizes the clot. A recombinant form of tPA, known as alteplase, is the most widely used thrombolytic drug for the treatment of acute ischemic stroke [36,37]. It has also been approved for the treatment of acute massive pulmonary embolism [38]. However, its use is associated with a risk of bleeding and thus its utility is limited.

### **1.1.2 Coagulation cascade**

The cascade of events leading to the generation of a fibrin clot was first described by Drs. E. Davie [39] and R. MacFarlane [40]. It is composed of an extrinsic and intrinsic pathway, which merge into a common pathway (Fig. 1). Upon vessel damage *in vivo*, the extrinsic pathway is first activated with the functional exposure of tissue factor (TF) found beneath endothelial cells. TF then forms a complex with clotting FVIIa and together the TF-FVIIa complex cleaves the Arg-Ile bond in coagulation FX, leading to its activation [41]. Once activated, FXa interacts with FVa in the presence of anionic phospholipid and calcium to form



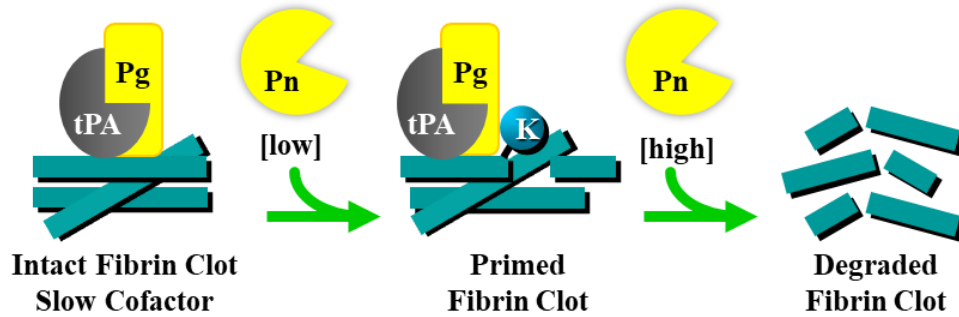
**Figure 1: Simplified coagulation cascade**

The coagulation cascade consists of a series of enzymatic reactions that ultimately form a fibrin clot. *In vivo* coagulation is initiated by vascular damage leading to the exposure of tissue factor (TF). Along with activated FVII (FVIIa), anionic phospholipid and calcium, TF makes up the extrinsic tenase complex which activates FX. This leads to minute amounts of FXa that interacts with activated FV to cleave prothrombin into thrombin in the presence of anionic phospholipid and calcium. The generated thrombin then converts fibrinogen into fibrin and activates FXIII to stabilize the clot. Thrombin also amplifies coagulation by activating FV, FVIII, and FXI such that the intrinsic tenase composed of activated FIX and its cofactor FVIIIa can activate additional FX in the presence of anionic phospholipid and calcium.

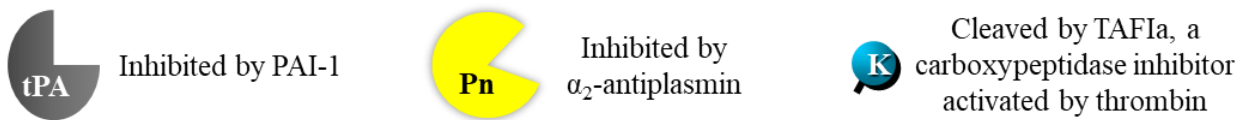
the prothrombinase complex. The latter cleaves prothrombin into thrombin [42]. Thrombin is the enzyme that is responsible for converting fibrinogen into fibrin, the molecular mesh of the clot. During this process thrombin also activates clotting factors V, VIII, and XI through a positive feedback reaction [43]. The activation of FXI leads to the downstream activation of clotting factor IX, which then complexes with FVIIIa in the presence of anionic phospholipid and calcium to form the intrinsic tenase complex. The latter is responsible for further activation of FX by cleaving the same Arg-Ile bond the TF-FVIIa complex cuts. Thus coagulation is amplified via the intrinsic pathway. The fibrin clot is stabilized through crosslinking by the transglutaminase XIIIa [44].

### **1.1.3 Fibrinolysis**

During fibrinolysis, the fibrin clot participates in its own dissolution as an accelerating cofactor [45] (Fig. 2). Fibrin contains several lysine residues that serve as weak intrinsic binding sites for tPA and its substrate plasminogen in the early phases of fibrinolysis [46]. Small amounts of plasmin are thus generated in this phase. While plasmin cleaves at arginine and lysine residues, cleavage at the latter exposes C-terminal lysine residues that serve as binding sites where additional tPA and plasminogen molecules converge and bind to fibrin with a higher affinity [47]. The resulting effect is an increase in plasmin generation that overcomes the inhibitory threshold to achieve fibrinolysis. The fibrinolytic system is controlled by inhibitors such as plasminogen activator inhibitor-1 (PAI-1) [48] and alpha-2-antiplasmin [49], which directly inhibit the activity of tPA and plasmin respectively. tPA-mediated plasmin generation is also indirectly inhibited by activated thrombin activatable fibrinolysis inhibitor (TAFIa), a carboxypeptidase inhibitor responsible for excising C-terminal lysine residues [43,50].



**Inhibitors:**



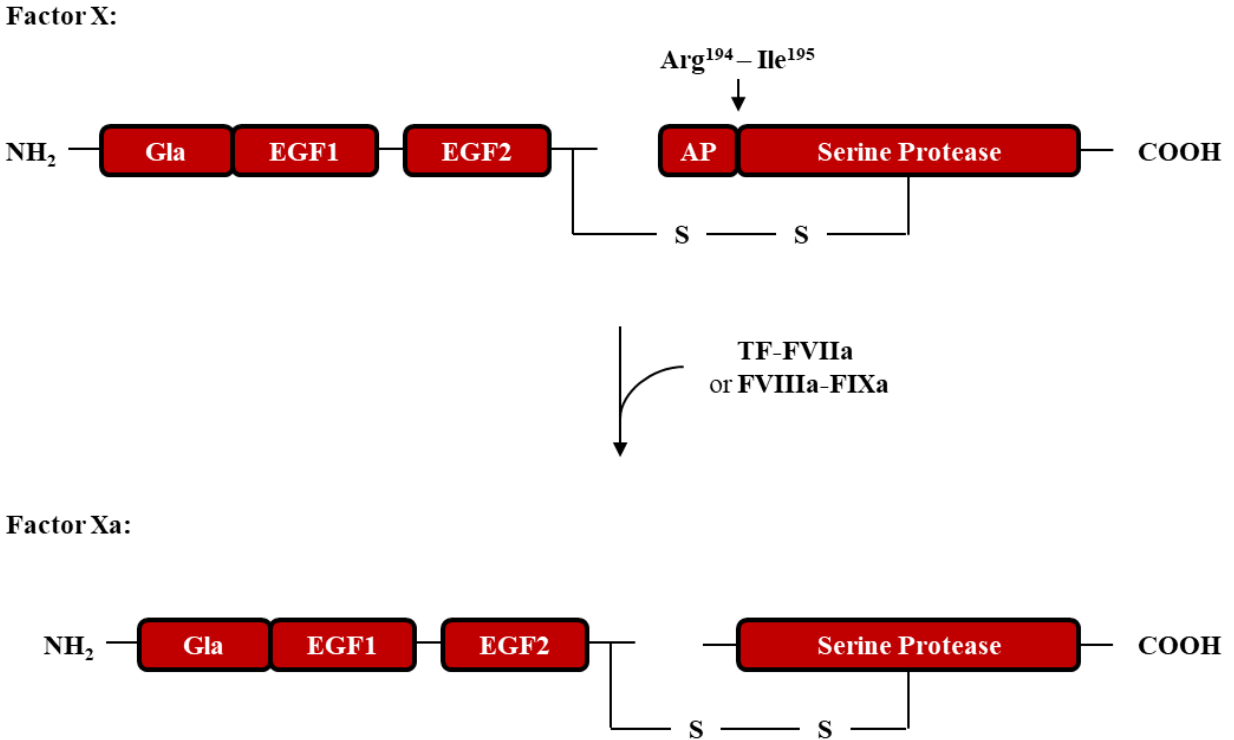
**Figure 2: The classical model of fibrinolysis and its inhibitors**

The dissolution of an intact fibrin clot is done in two steps. First the serine protease tissue plasminogen activation (tPA) and its substrate plasminogen (Pg) bind at low affinity to lysine residues in fibrin to generate low concentrations of plasmin. Plasmin then cleaves the fibrin clot to expose C-terminal lysine residues leading to stronger binding of tPA and Pg and thus a higher concentration of plasmin. The fibrin clot is consequently degraded. These reactions are modulated primarily by plasminogen activator inhibitor-1 (PAI-1) and  $\alpha_2$ -antiplasmin, which inhibit the activity of tPA and plasmin, respectively. Fibrinolysis is also controlled by activated thrombin-activatable fibrinolysis inhibitor (TAFIa) which is responsible for cleaving C-terminal lysine residues and thus reducing plasmin generation.

## 1.2 Coagulation biochemistry

### 1.2.1 Clotting factor Xa

Positioned at the convergence of the intrinsic and extrinsic branches of coagulation, the serine protease FXa is an important target for drug development [51-53]. FX, the precursor of FXa, is a 59 kDa vitamin K-dependent zymogen that is composed of a light and heavy chain held together by a disulfide bond (Fig. 3). Clotting FX, which is synthesized in the liver, circulates at a concentration of 10  $\mu\text{g/mL}$  (170 nM) and has a half-life of  $\sim 40$  hours [54,55]. The light chain of FX is composed of a  $\gamma$ -carboxyglutamic (Gla) domain and two epidermal growth factor-like domains (EGF 1 and EGF 2). The Gla domain facilitates phospholipid binding and the EGF 1 domain is involved in the recognition of FX by tissue factor and thus in FX activation [56]. The heavy chain is comprised of the protease domain with an N-terminal activation peptide (12 kDa) that is cleaved at Arg<sup>194</sup> - Ile<sup>195</sup> by the tenase complexes (extrinsic: TF-FVIIa-aPL-Ca<sup>2+</sup> or intrinsic: FVIIIa-FIXa-aPL-Ca<sup>2+</sup>) to generate the serine protease FXa [57]. *In vitro*, FX can also be activated by the enzyme Russel's viper venom-factor X activator (RVV-X) in the presence of calcium via cleavage at the Arg<sup>194</sup> - Ile<sup>195</sup> bond, as conferred by the physiological tenase complexes [57,58]. The main role of FXa within the coagulation cascade is to convert the zymogen prothrombin into its active form thrombin [59]. This function of FXa, which is localized to the site of vascular damage by a calcium-dependent association, is achieved  $\sim 3 \times 10^5$  times faster with clotting FVa and anionic phospholipid (aPL)-containing membrane [60]. Coagulation FXa can also activate other coagulation proteins including FV [61], FVII [62], and FVIII [63], as well as anticoagulant plasma protein, protein C in the presence of calcium and phospholipid [64].



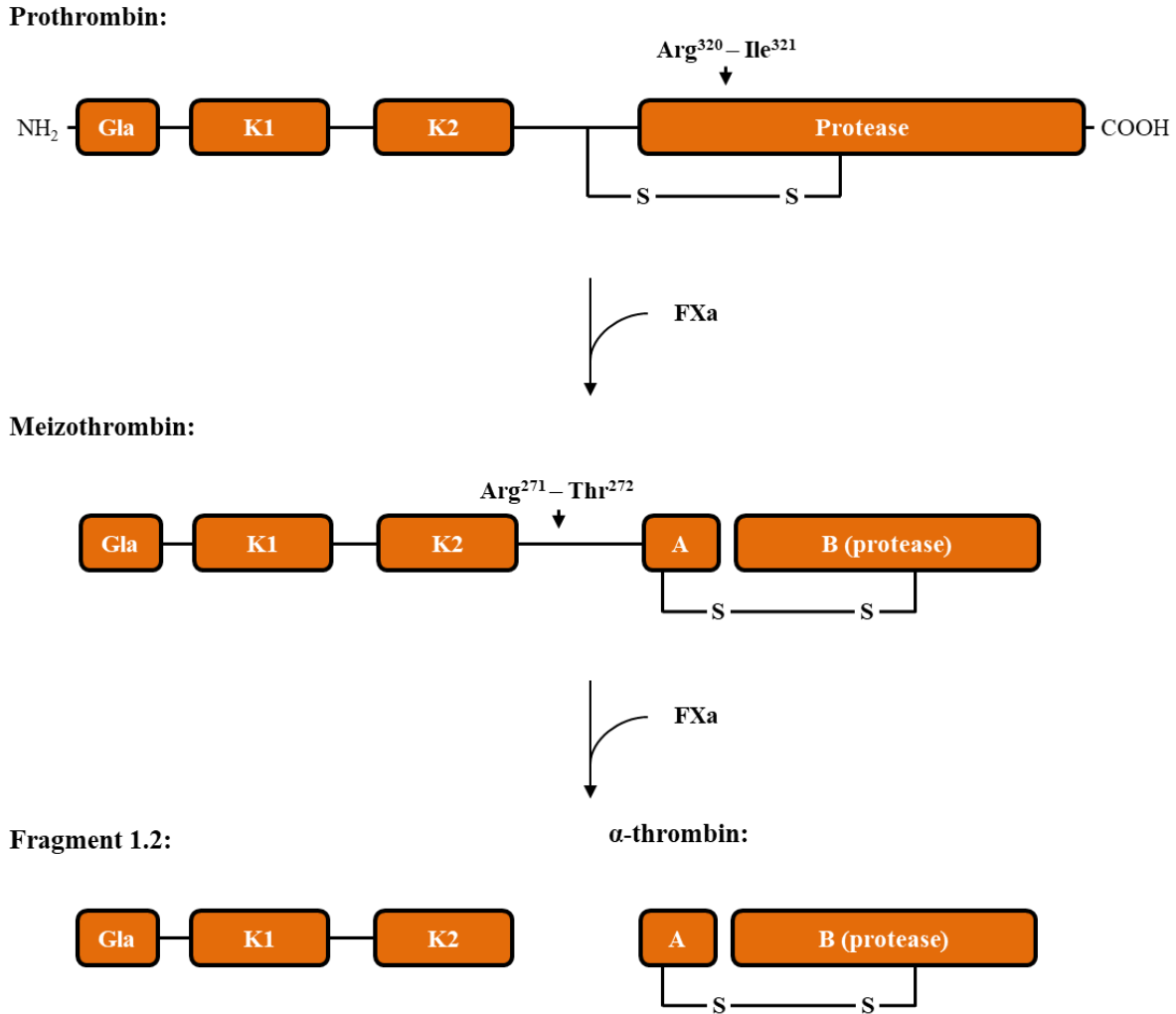
**Figure 3: Domain organization of Factor X and its conversion to FXa**

Coagulation FX plays a central role in coagulation. It is composed of a light and heavy chain. The light chain has a  $\gamma$ -carboxyglutamic acid domain (Gla) followed by two epidermal growth factor (EGF) domains. The activation peptide (AP) and serine protease domain are located in the heavy chain. Both the extrinsic and intrinsic tenase complexes activate FX into FXa by cleavage at Arg<sup>194</sup> - Ile<sup>195</sup>.

The activity of FXa is regulated physiologically by tissue factor pathway inhibitor (TFPI) and antithrombin (AT), which independently form a complex with the FXa active site. TFPI inhibits both free FXa as a binary complex and nascent FXa still associated with the extrinsic tenase by forming a FXa-TFPI-TF-FVIIa quaternary complex [65-67]. In normal coagulation, the inhibitory role of TFPI is overcome by the local accumulation of FXa generated via the intrinsic pathway [68]. Inhibition by AT occurs upon FX activation through the formation of a 1:1 irreversible complex between FXa and AT [69,70]. This reaction is enhanced ~ 3-orders of magnitude by heparin. Assembly of the FXa-AT-heparin complex, in the presence of physiological calcium, results in a 3 - 14 times even higher rate of FXa inhibition [71]. FXa can also be inhibited by synthetic inhibitors and small molecules that bind to its active site [33,34,72,73]. In the last five to six years, FXa-specific direct oral anticoagulants have been approved for the treatment and prevention of deep vein thrombosis, pulmonary embolism, venous thromboembolism post hip or knee replacement surgery, and stroke in patients with atrial fibrillation [74,75]

### **1.2.2 Prothrombin/Thrombin**

Prothrombin is a 72 kDa single chain vitamin K-dependent coagulation factor that is synthesized in the liver (Fig. 4). It circulates at a concentration of 100 µg/mL (~1.4 µM) and has a half-life of ~ 60 hours [76]. Prothrombin is composed of a Gla domain, 2 kringle domains, and a catalytic domain. Both kringles 1 and 2 have been implicated in the interaction between prothrombin and FVa within the prothrombinase complex [77,78]. The latter is responsible for converting prothrombin into thrombin through cleavage at two sites: one at Arg<sup>320</sup> - Ile<sup>321</sup> and the other at Arg<sup>271</sup> - Thr<sup>272</sup>. While the order of these reactions tend to vary based on the surface [79], in the presence of synthetic phospholipid, cleavage at Arg<sup>320</sup> - Ile<sup>321</sup> occurs first to generate a



**Figure 4: Domain organization of prothrombin showing the conversion to thrombin**

Prothrombin (II) is composed of a heavy and light chain. The light chain has a gamma-carboxyglutamic domain (Gla) followed by two kringle domains (K1 and K2) while its catalytic domain is located in its heavy chain. In the presence of synthetic phospholipid, prothrombin is cleaved by FXa or the prothrombinase complex at Arg<sup>320</sup> – Ile<sup>321</sup> to generate its intermediate meizothrombin. Further cleavage of the latter at Arg<sup>271</sup> – Thr<sup>272</sup> results in the generation of Fragment 1.2 composed of elements from the light chain and  $\alpha$ -thrombin composed of an A and B domain.

short-lived intermediate called meizothrombin [80]. This product is capable of activating coagulation factors within the extrinsic (i.e FXI [81]) and the common (i.e. FV [82]) pathway. Further proteolysis of meizothrombin leads to the generation of Fragment 1.2, composed of the Gla and two kringle domains, and  $\alpha$ -thrombin containing the catalytic domain [83]. Alpha-thrombin can also be generated by FXa activation of prethrombin-1, a prothrombin derivative in which the Gla domain and kringle 1 are missing, as well as meizothrombin desF1, the active form of prethrombin-1[84,85]. Further degradation of  $\alpha$ -thrombin result in the formation of  $\beta$ -thrombin and  $\gamma$ -thrombin; both of which can interact with FXI, XIII, antithrombin but have no ability to cleave fibrinogen or protein C [86,87]. Thus  $\alpha$ -thrombin is the form referred to when “thrombin” is used.

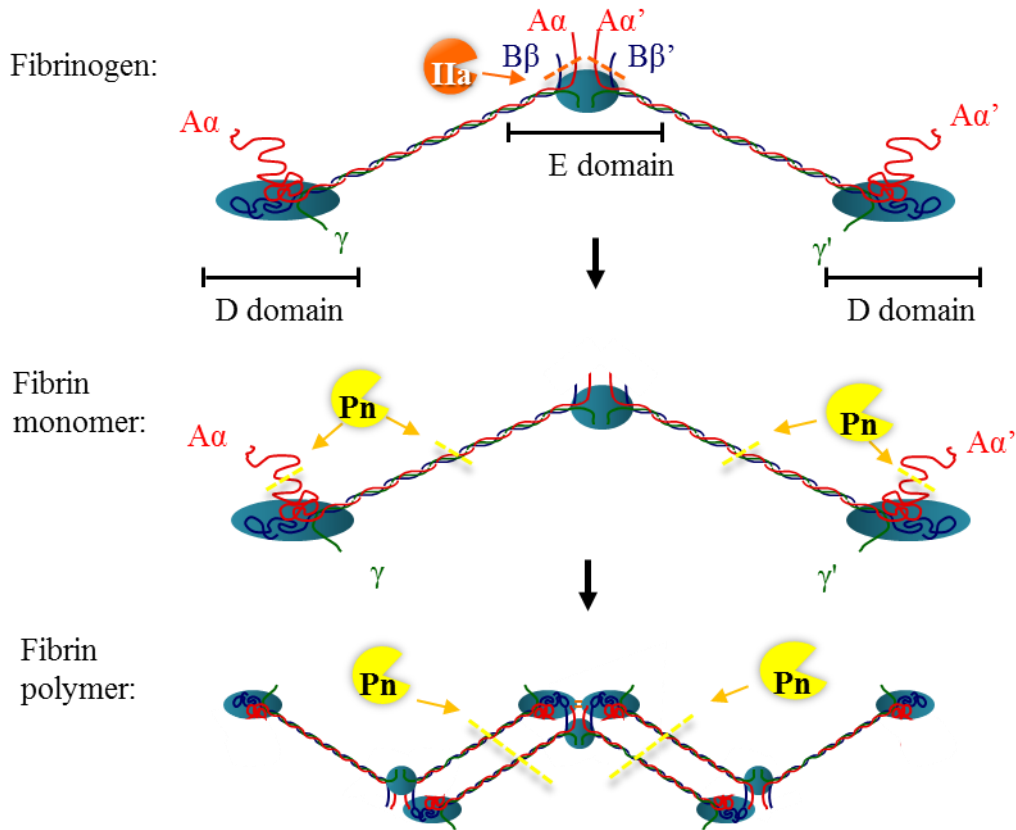
Thrombin has 3 main binding sites: an active site that interacts with its inhibitors and two anion binding exosites. Exosite 1 participates in the recognition of fibrinogen, thrombomodulin, FV, FVIII, FXI, and hirudin and exosite 2 is involved in the binding of heparin and platelet glycoprotein Ib-IX [88-92]. Thus, while the primary role of thrombin is to catalyze the conversion of soluble fibrinogen into an insoluble fibrin clot via exosite I, thrombin is pleiotropic (reviewed in [93]). Minute amounts of thrombin stabilize the clot by activating transglutaminase FXIII, which crosslinks side chains of susceptible intermolecular glutamine and lysine residues in fibrin [44]. Thrombin also feedback amplifies coagulation by activating FV [61], FVIII [63], and FXI [94], thus causing a burst in thrombin generation (Fig. 1). It is this increase of thrombin that is responsible for the activation of TAFI, a reaction that is enhanced 1250-fold in the presence of thrombomodulin [95]. Once generated, TAFIa removes C-terminal lysine residues from partially degraded clots, thus reducing or inhibiting fibrinolysis [96]. It is; however, worth noting that most procoagulant responses by thrombin take place during the initiation phase of

thrombin generation where less than 2 nM thrombin is present. The concentration of thrombin (measured as thrombin-AT) that is needed for FXIII activation is  $0.8 \pm 0.3$  nM [97]. Additionally, the thrombin concentration needed for the release of fibrinopeptide A (FPA) and B (FPB), which are initial steps in fibrin formation, is  $1.3 \pm 0.4$  nM and  $1.7 \pm 0.5$  nM respectively [97].

The multifunctional nature of thrombin makes it a key factor within the coagulation cascade. Proper balance of its generation is therefore very important for good health as an insufficient or excess amount of thrombin can result in bleeding or a thrombotic state, respectively. Within the coagulation system, there are various protein complexes that regulate the generation and activity of thrombin *in vivo* such as the thrombomodulin/protein C complex and the AT/heparin complex [98,99]. In the presence of thrombomodulin, which is released from endothelial cells, thrombin activates protein C. Activated protein C can then down-regulate the generation of thrombin by cleaving FVIIIa (intrinsic pathway) and FVa (common and prothrombinase complex) rendering them inactive [98]. The binding of thrombin to thrombomodulin also prevents it from cleaving fibrinogen and thus inhibits its participation in coagulation [100]. Antithrombin inactivates thrombin by binding to its active site to form the thrombin-AT complex, which is rapidly cleared from the body by the liver [101]. The interaction between thrombin and AT, which is increased by ~1000-fold in the presence of heparin, also restricts the participation of thrombin in coagulation [71].

### **1.2.3 Fibrinogen/Fibrin**

Fibrinogen is a 340 kDa protein that is synthesized in the liver and circulates at a concentration of 2 - 5 mg/mL (~ 6 - 15  $\mu$ M) (as reviewed in [102]) (Fig. 5). It is composed of 2



**Figure 5: Domain organization of fibrinogen and its conversion to fibrin**

Fibrinogen is composed of 2 identical sets of 3 polypeptide chains  $A\alpha$ ,  $B\beta$ ,  $\gamma$  and  $A\alpha'$ ,  $B\beta'$ ,  $\gamma'$  that are arranged such that the N-termini of each sets of chains makes up the E domain (center). The C-terminal part of the chains is contained in the D domain. Upon thrombin cleavage of the  $A\alpha/A\alpha'$  and  $B\beta/B\beta'$  chains, fibrinopeptide A and B are respectively released and fibrin monomers are formed. Staggering of fibrin fibers occur through a “knob” and “hole” interaction such that 1 knob (i.e. N terminus in E domain) interacts with a “hole” from two adjacent D domains. Dissolution of a fibrin clot is achieved via plasmin-mediated cleavages in the  $A\alpha$  chain and between the D and E domains.

copies of 3 separate gene products ( $A\alpha$ ,  $B\beta$ ,  $\gamma$  chains) arranged symmetrically such that the N-termini of all 6 polypeptide chains are linked together in the center (E domain) by noncovalent and disulfide bonds [103]. The two outer parts, termed D-domains, are each composed of the C-terminal region of the chains. Thus, fibrinogen is composed of 3 linear globules: D-E-D. The  $A\alpha$  and  $\gamma$  chains of fibrinogen possess sites for crosslinking by FXIIIa and enhancement of tPA-mediated plasmin generation while the  $B\beta$  and  $\gamma$  chains have calcium binding sites [104]. In healthy human, the  $\gamma$  chain undergoes alternative splicing to form a variant chain called  $\gamma'$  that circulates as a heterodimer with the common  $\gamma$  chain ( $\gamma A$ ) [105]. About 8 - 15 percent of circulating fibrinogen is composed of  $\gamma'/\gamma A$ . The  $\gamma'$  chain binds directly to thrombin exosite II [106,107] and clots formed with  $\gamma'/\gamma A$  show resistance to fibrinolysis [108]. Unlike elevated concentrations of fibrinogen [109], an increase in  $\gamma'$  fibrinogen has been recently shown to limit thrombin activity and thus discourage arterial thrombosis in mice [110].

The activation of soluble fibrinogen into an insoluble fibrin clot is a 3 step process composed of: the release of fibrinopeptides by thrombin; fibrin assembly; and fibrin crosslinking by FXIIIa. Polymerization is initiated by the cleavage of the  $\text{Arg}^{16} - \text{Gly}^{17}$  bond in the  $A\alpha$  chain to release fibrinopeptide A (FPA) [111]. Further proteolysis by thrombin then takes place at the  $\text{Arg}^{14} - \text{Gly}^{15}$  bond in the  $B\beta$  chain to release fibrinopeptide B (FPB) [111]. These two cleavages produce an intermediate product called fibrin monomers composed of newly exposed amino terminal residues in the  $A\alpha$  and  $B\beta$  chains (i.e “knobs”) [97,112]. Staggering of fibrin monomers is achieved by the interaction of a knob with two “holes” located on the C-terminus of two adjacent molecules [112,113]. The assembly of fibrin monomers results in the formation of protofibrils, which are laterally aggregated to form fibrin fibers. The concentration of thrombin during clot formation influences the structure of the clot [102,114]. Clots formed in the presence

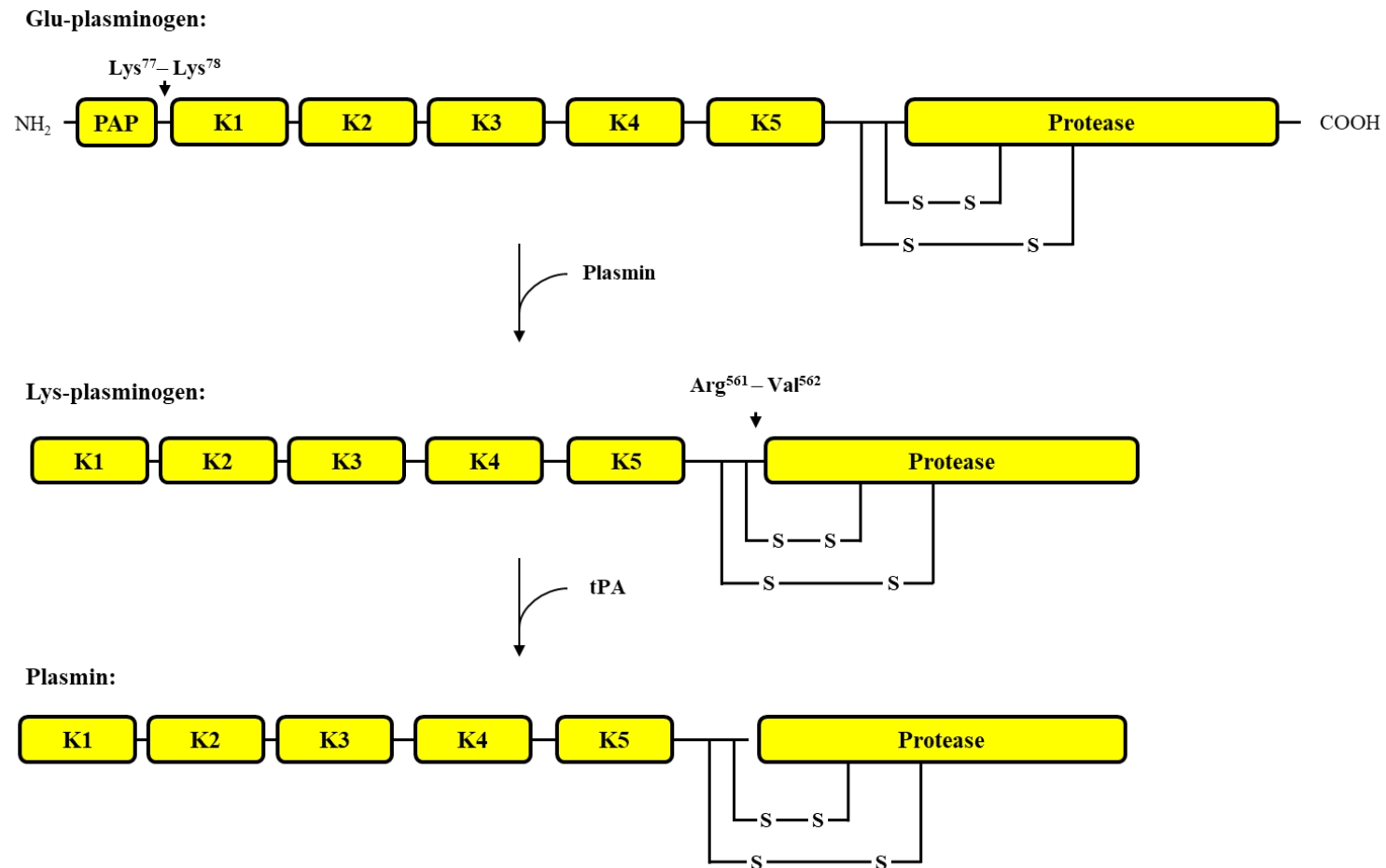
of low thrombin concentration are assembled into thick loosely woven fibers while the fibrin fibers formed in the presence of high thrombin concentrations are thin and highly branched [102,115].

The strength of the clot is attained through crosslinking of glutamine and lysine side chains within the fibrin clot [116]. This process begins in the initiation phase of thrombin generation with crosslinking of the  $\gamma$  chain occurring during fibrin assembly [97,117]. The latter is then followed by slower crosslinking involving  $A\alpha$  chains, which makes the clots stiffer and thus more resistance to clot lysis [118]. The correlation between crosslinking and reduced fibrinolysis has been explained by two reactions. One is that FXIIIa crosslinks  $\alpha_2$ -antiplasmin, the primary inhibitor of the physiological fibrinolytic enzyme, to the clot [119,120]. The second is that crosslinking reduces the binding of tPA and plasminogen, thus reducing plasmin generation [121]. The ability of tPA to bind to fibrin, therefore, gives fibrin a dual function in hemostasis: provide clot integrity and mediate its own solubilization as an accelerator of tPA [122,123].

### **1.3 Fibrinolysis**

#### **1.3.1 Plasminogen/plasmin**

Plasminogen is an 88 kDa single chain zymogen of plasmin (as reviewed in [124]) (Fig. 6). It is synthesized in the liver and the full length protein is secreted as glu-plasminogen, so named due to the glutamic acid located at its N-terminal [125]. Glu-plasminogen circulates at a concentration of 200 mg/L ( $\sim 2.3 \mu\text{M}$ ) and has a half-life of 2.2 days [126]. Its N- to C-terminal domain composition is a preactivation peptide, 5 kringle domains (K1, K2, K3, K4, and K5), and a catalytic domain [127,128]. Due to the presence of lysine binding sites, both kringles 1 and 4



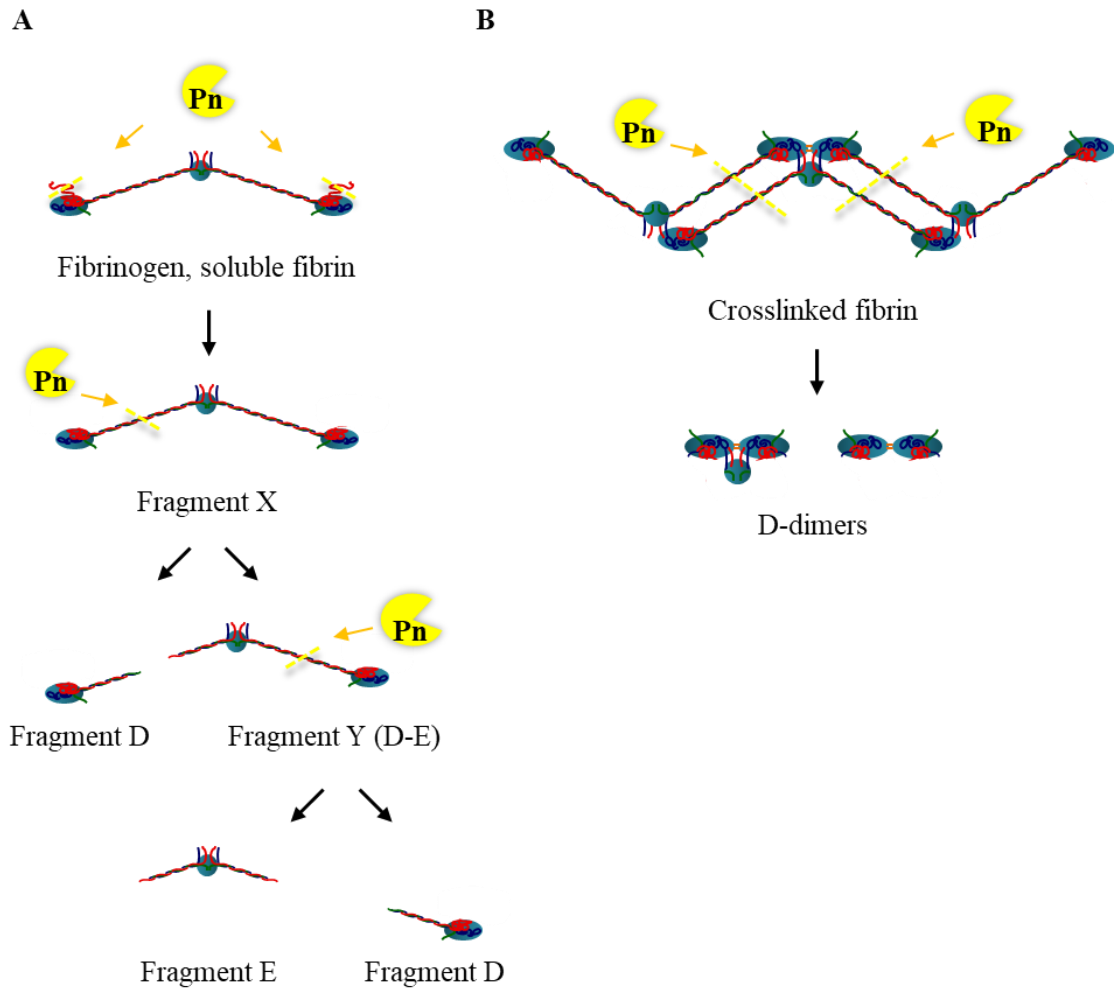
**Figure 6: Activation of glu-plasminogen into plasmin**

Glu-plasminogen is composed of a preactivation peptide (PAP), 5 kringle domains (kringles 1-5), and a protease domain. It is cleaved at Lys<sup>77</sup> – Lys<sup>78</sup> by plasmin to liberate PAP and generate Lys-plasminogen. Further cleavage at Arg<sup>561</sup> – Val<sup>562</sup> by its activator tissue plasminogen activator results in plasmin, the enzyme responsible for degrading a fibrin clot.

have been implicated in the binding of plasminogen to lysine residues in plasma constituents such as fibrin and  $\alpha_2$ -antiplasmin [129,130]. Kringle 5 has also been implicated in tPA-mediated plasminogen activation [131]. Upon plasmin cleavage at the Lys<sup>77</sup>- Lys<sup>78</sup> bond of glu-plasminogen, the preactivation peptide is lost and lys-plasminogen is formed [132]. This variant of plasminogen not only has a higher affinity for fibrinogen, but it is also a better substrate for cleavage by plasminogen activators at the Arg<sup>561</sup> - Val<sup>562</sup> bond [133,134]. The resultant species is called plasmin, a serine protease composed of a heavy chain containing the 5 homologous kringle domains linked by 2 disulfide bonds to a light chain consisting of the catalytic domain [128]. While plasmin has multiple physiological roles including the proteolytic inactivation of FXIIIa [135], its primary role in hemostasis is the degradation of fibrin fibers into fibrin degradation products [136]. Plasmin can also cleave fibrinogen (fibrinogenolysis). The first fragment formed in fibrinolysis/fibrinogenolysis is Fragment X, which retains some ability to participate in clotting [126,137] (Fig. 7). Fragment X is then further divided into Fragment Y and Fragment D. Additional Fragment D and Fragment E are then produced by cleavage of Fragment Y. Among the plasmin-induced proteolytic products of crosslinked fibrin is D-dimer (180 kDa), two fragment D molecules crosslinked together. This fragment, which is among the smallest of released fragments, is used in clinical settings to identify thrombotic disorders in human [138,139]. Localizing plasmin to the site of the clot is important as systemic plasmin activity can result in bleeding. The latter is partially controlled by the ability of  $\alpha_2$ -antiplasmin to inhibit free plasmin more effectively than clot bound plasmin [140].

### **1.3.2 Tissue plasminogen activator**

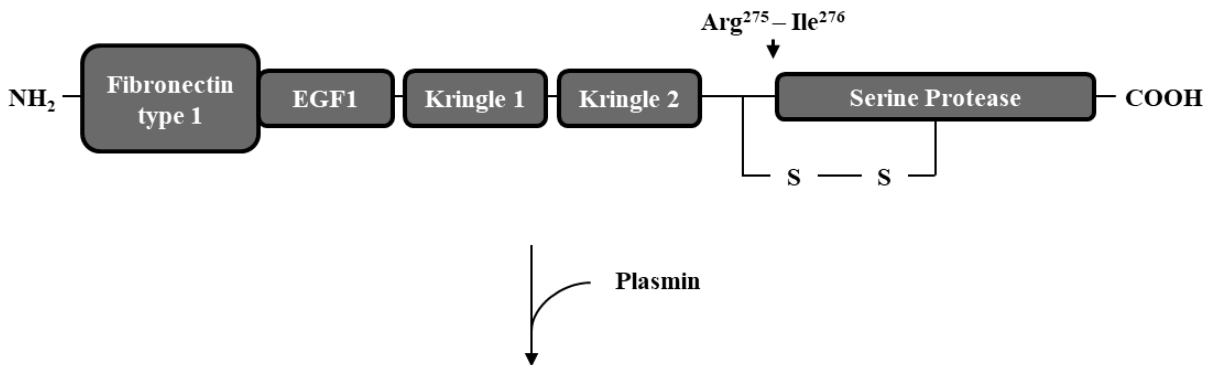
Tissue plasminogen activator (tPA) is a 70 kDa serine protease predominantly stored in vascular endothelial cells [141] (Fig. 8). It is synthesized in the liver and secreted as a single polypeptide



**Figure 7: Fragments generated from fibrinogenolysis and fibrinolysis**

Panel A: Plasmin cleaves fibrinogen and soluble fibrin at the C terminal region of A $\alpha$  chain to generate Fragment X. Further plasmin processing yields fragment D and Y (D-E). Fragment Y is then proteolysed into Fragment E and Fragment D. Panel B: Cleavage of insoluble crosslinked fibrin results in the production of various degradation products in which the smallest is D-dimer.

**Tissue Plasminogen Activator:  
(single chain)**



**Tissue Plasminogen Activator:  
(two-chain)**



**Figure 8: Tissue plasminogen activator**

Tissue plasminogen activator (tPA) is secreted as a single chain composed of a light and heavy chain. The light chain is composed of a fibronectin type 1 domain, followed by an epidermal growth factor domain (EGF) and two kringle domains. The heavy chain is the serine protease constituent of the protein. Cleavage at Arg<sup>275</sup> – Ile<sup>276</sup> renders tPA as a 2-chain molecule which has a ~ 30-fold-higher activity for plasminogen.

chain composed of 527 amino acids [142] with a normal circulating plasma concentration of ~ 70 pM [143]. This single chain precursor is composed of 5 distinct domains: a fibronectin finger-like domain, an epidermal growth factor (EGF) domain, 2 kringle domains, and the proteolytic domain (as reviewed in [141]). The fibronectin finger like domain is implicated in the binding of tPA to fibrin [144]. Both the fibronectin finger domain and the EGF domain participate in the rapid (~ 2.4 minutes) hepatic clearance of tPA [145,146]. While the function of kringle 1 is still not clearly defined, kringle 2 plays a role in the binding of tPA to fibrin [144]. Unlike the finger like domain, the interaction between tPA and fibrin via kringle 2 can be obstructed by the presence of lysine or one of its analogues; for example, epsilon-aminocaproic acid [147]. Single chain tPA has proteolytic activity and can thus trigger fibrinolysis. Plasmin cleavage of its Arg<sup>275</sup> – Ile<sup>276</sup> bond, however, renders a two chain molecule which has a ~30-fold higher enzymatic activity towards plasminogen than single chain tPA [148,149] but a similar activity in the presence of polymerized fibrin [150].

Despite the ability of tPA to convert plasminogen into plasmin, a cofactor such as fibrin is needed for optimum activity. Fibrin not only serves to locally concentrate tPA, but it also enables tPA to be appropriately positioned for interaction with plasminogen [144]. Thus, in the presence of fibrin, the ability of tPA to cleave plasminogen at the Arg<sup>561</sup> - Val<sup>562</sup> bond to generate plasmin is 600 - 1000 times higher [131].

Regulation of the activity of tPA is achieved by secretion, PAI-1inhibition and hepatic clearance. tPA follows the constitutive and regulatory (acute tPA release) pathways of secretion. A portion of tPA is consecutively release from endothelial cells while the rest is stored in endothelial storage compartments [151]. Stored tPA can then be secreted upon thrombin activation of endothelial cells. Exercise and mental stress also increase tPA secretion [152]. In

circulation, PAI-1 binds to the active site of tPA and forms an equimolar inactive complex [48]. PAI-1 is a 45 kDa serine proteinase inhibitor that is synthesized by cells such as endothelial cells, hepatocytes, and platelets [48,153]. Active PAI-1, stored in the  $\alpha$ -granules of platelets, is released upon platelet activation and aggregation [154]. Under normal hemostasis, PAI-1 circulates in plasma with antigen levels of 6-85 ng/mL and activity levels of 0.5-47 U/mL [48]. PAI-1 is diurnal with higher concentrations in the morning than in the afternoon [155]. The formation of the tPA/PAI-1 complex increases the half-life of tPA from 2.4 minutes to ~ 5 - 6 minutes [146]. Clearance of tPA and tPA/PAI-1 by the liver is directly proportional to hepatic blood flow [156]. Balance between secretion, inhibition, and clearance determine the tPA activity present in circulation and thus the rate of fibrinolysis.

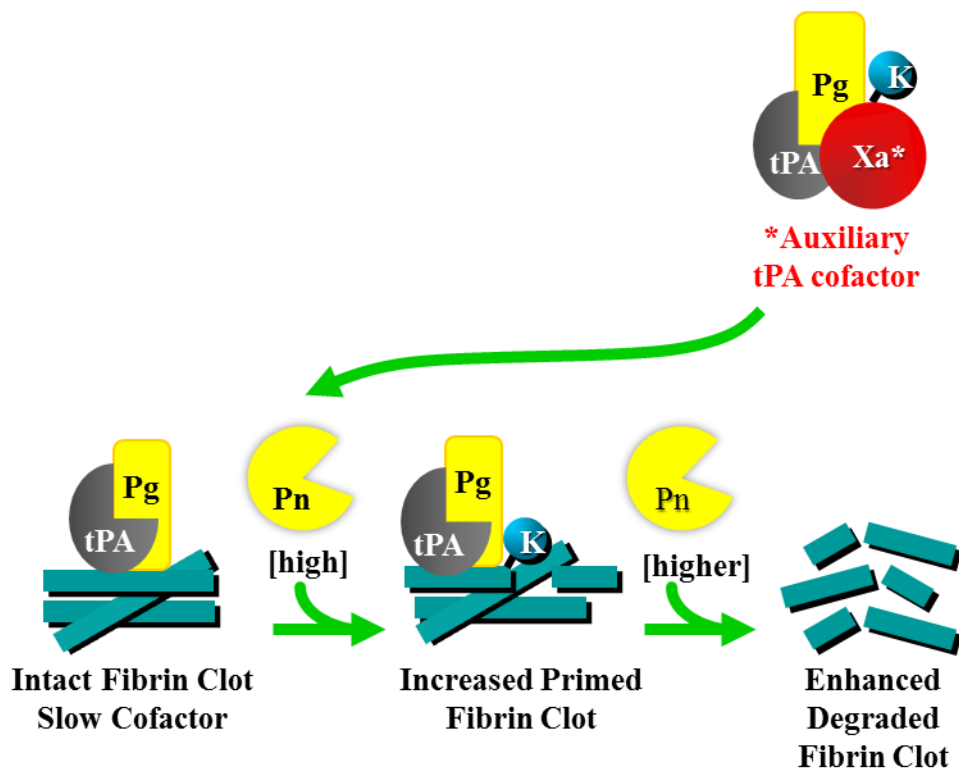
While tPA has been argued as the primary activator of plasminogen during fibrinolysis, other activators of plasminogen exist [157]. Urokinase-plasminogen activator (u-PA), which has a molecular weight of 54 kDa, is synthesized by the liver and kidney [158]. U-PA is more involved in producing plasmin for the direct degradation of extracellular matrix and indirect degradation via the activation of metalloproteases [159]. Unlike single-chain and two-chain tPA, PAI-1 forms a complex with two-chain but not single-chain u-PA [48,160]. Annexin II has also been reported as an enhancer of tPA mediated plasmin generation on the cell surface [161]. Additionally, coagulations factors such as FXa [162] and XIIa [163] contribute to fibrinolytic activity by leading to plasminogen activation.

To improve the utility and safety of tPA in thrombolytic therapy, numerous variants of tPA have been engineered based on known correlations between biochemical function and structure (as reviewed in [164]). The main differences among the variants include changes to prolong half-life, increase fibrin specificity such that bleeding complications were decreased, and

reduce inhibition by PAI-1. The most commonly used form of recombinant tPA is called alteplase, which has the wild type amino acid sequence. It has a molecular weight of 65 kDa and a half-life of only 4-5 minutes [165,166]. While alteplase has been approved for the treatment of acute pulmonary embolism, myocardial infarction, and ischemic stroke, its use is only recommended within 3 - .5 hours of symptom-onset due to a low benefit-risk ratio beyond that window [38,167-169]. More recent studies have reported some favorable outcomes with use of alteplase up to 6 hours of symptom onset [36,170,171], as reviewed in [172]; however, this time window is yet to be clinically established. Of the many tPA variants that have been produced for clinical trial, only tenecteplase has had an advantage over alteplase. Tenecteplase is a 65 kDa modified form of tPA with a 6-fold longer half-life, 15-fold greater fibrin specificity and 80-fold enhanced resistance to PAI-1 when compared to alteplase [173-175]. Studies using tenecteplase showed reduced risk in bleeding when compared to alteplase. [176,177]. The latter is an observation with potential links to the increase ability of tenecteplase to distinguish plasminogen circulating in its free form from that bound to the fibrin clot. Thus, thrombolytic agents with high fibrin specificity would more readily lead to the generation of plasmin at the site of the clot than systemically.

### **1.3.3 Auxiliary cofactor model of fibrinolysis**

In the past few years, research done in the Pryzdial laboratory identified a new role for clotting factor Xa [162,178]. In addition to its primary role in generating thrombin, aPL-bound FXa acquires fibrinolytic activity when cleaved by plasmin. The latter exposes C-terminal lysine residues that form an integral part of new binding sites for tPA and plasminogen, thus leading to enhanced plasmin generation [162] (Fig. 9). Purified FXa is a combination of FXa $\alpha$  and FXa $\beta$  due to auto-proteolysis and is therefore referred to as FXa $\alpha/\beta$ , or simply as FXa within this

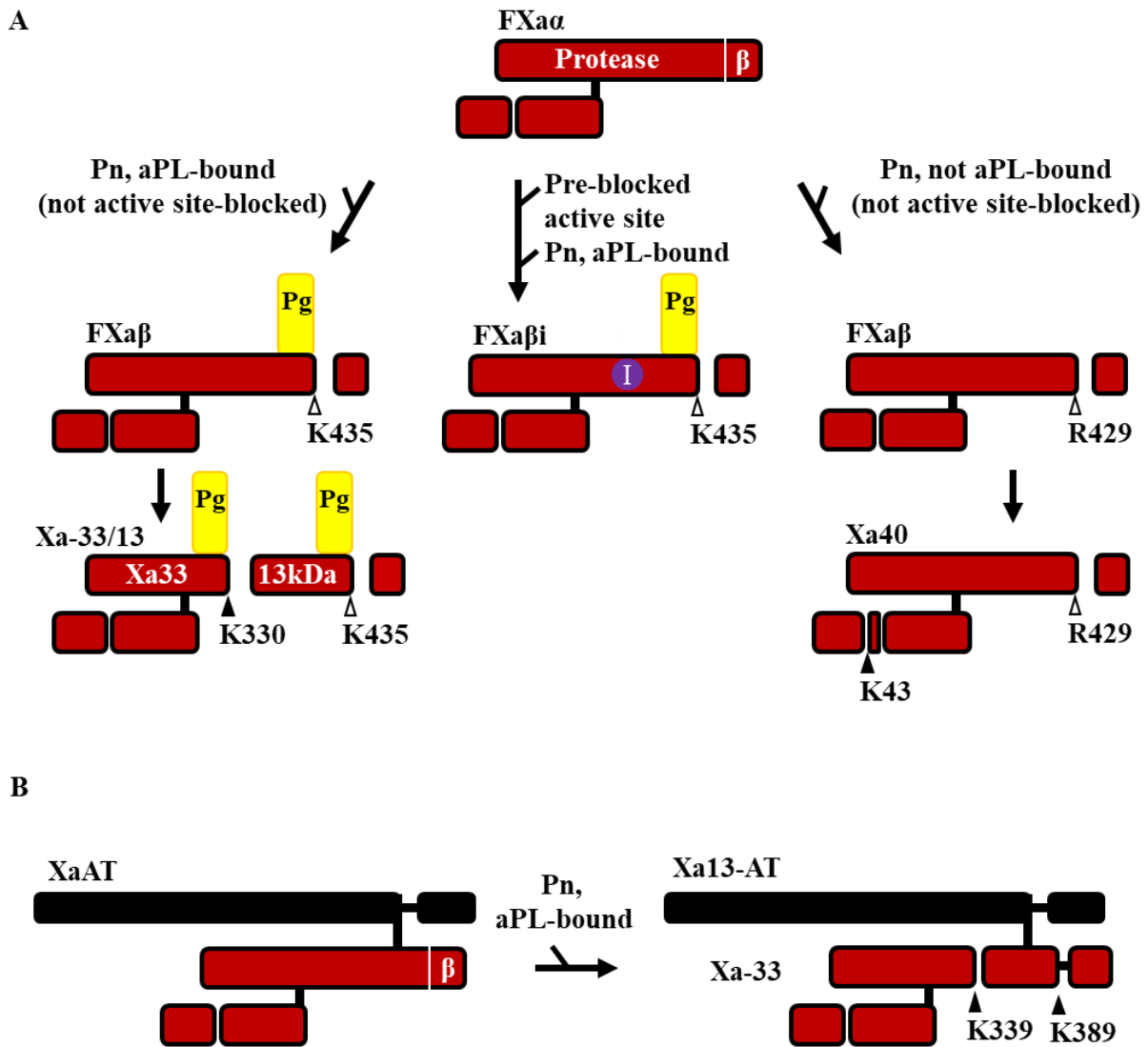


**Figure 9: Summary of auxiliary cofactor model**

Plasmin-cleaved derivatives of FXa such as FXa $\beta$ , Xa33/13, FXa $\beta$ i express auxiliary tPA cofactor activity. In the early stages of fibrinolysis, these cofactors provide c-terminal lysine (K) residues toward fibrinolysis enhancement that would otherwise be unavailable. As a result, tPA and plasminogen (Pg) bind. This leads to increased plasmin (Pn) generation and priming of the fibrin clot earlier on, thus clot degradation is enhanced.

dissertation. Sequential proteolysis of FXa most likely at lysine (Lys) 435, or at one of the proximal basic residues (Lys 427, Arg 429 or Lys 433) [179], excises a 3 kDa C-terminal peptide to convert FXa $\alpha$  to FXa $\beta$  (Fig. 10A). In the presence of aPL and calcium, a sequential cleavage occurs at Lys 330, yielding non-covalently associated fragments of ~ 33 and ~ 13 kDa, termed Xa33/13. Since the Lys 330 cleavage occurs in the protease domain of FXa $\beta$ , further involvement of this fragment in coagulation is prevented. Using purified proteins, both FXa $\beta$  and Xa33/13 bound plasminogen as well as enhanced plasmin generation and purified fibrin clot lysis [162]. Due to the additional C-terminal lysine residue on Xa33/13, however, this derivative enhances the activity of 1  $\mu$ M tPA more effectively than its precursor [162]. In the same study, a tPA concentration of 1  $\mu$ M was equivalent to a physiological ratio of tPA (~75  $\mu$ M): PAI-1 (~ 500  $\mu$ M). Additionally, it was revealed that the fibrinolytic function of FXa and its derivative is only significant at tPA concentrations below 10  $\mu$ M when using purified proteins. Above 50  $\mu$ M, the enzymatic activity of tPA converts plasminogen into high concentrations of plasmin, making the role of a cofactor obsolete [162]. The cofactor function of FXa is, therefore, more noticeable during the earlier phases of fibrinolysis when binding of tPA and plasminogen to fibrinogen, the conventional tPA cofactor, is weak. Since plasmin cleaves FXa $\alpha$  faster than fibrinogen, the former generates a higher concentration of plasmin than normal in the initial phases of fibrinolysis. Thus the clot is primed more rapidly for degradation.

It is now known that the fibrinolytic function of FXa is modulated by cleavages. Interestingly, while FXa and Xa33/13 enhance purified fibrinolysis, their ability to enhance plasma clot lysis is reduced or lost. This has been attributed to further fragmentation of Xa33/13 into a species called Xa28 and loss of plasminogen binding [52,162]. Studies carried out using Xa28 suggest that it does not bind plasminogen and therefore its fibrinolytic function is



**Figure 10: Active site-occupancy and aPL-binding of FXa alters cleavage by plasmin.**

Panel A: When bound to aPL, plasmin-mediated fragmentation of proteolytic FXa is cleaved sequentially to FXa $\beta$  and Xa33/13, which both associate with Pg, implying exposure of a respective c-terminal lysine, of which Lys 435 has been implicated. Occupancy of the active site of FXa by a chloromethyl ketone produces FXa $\beta$ i without Xa33/13 generation. These FXa derivatives enhance fibrinolysis. In the absence of aPL, plasmin cleavages sequentially yield a form of FXa $\beta$  and a unique fragment, Xa40, which do not bind plasminogen and thus have no discernable fibrinolytic function. A nearby cleavage at Arg 429 is probably the cause of lost Pg-binding with no apparent change in electrophoretic mobility compared to cleavage in the presence of aPL and calcium. Panel B: Plasmin cleavage of XaAT yields Xa13 covalently linked to AT and Xa33 (Xa33/13-AT). Determined ( $\blacktriangle$ ) and inferred ( $\triangle$ ) cleavage sites are depicted.

attenuated [52]. Additionally, when the binding of FXa to anionic phospholipid is precluded by omission of the latter or addition of EDTA, plasmin cleaves FXa at different sites. The first generates an alternate form of FXa $\beta$  and the second renders a fragment termed Xa40, both of which do not bind plasminogen and thus do not facilitate fibrinolytic activity [178,180].

To stabilize FXa and prevent further cleavage into Xa33/13, tetra-ethylene glycol was tethered to the active site of FXa along with a lysine residue to its C-terminus [52]. This form of FXa, called Xai-K, is cleaved into Xai-K $\beta$  but not into a Xa33/13 form. The stable Xai-K $\beta$  binds plasminogen, enhances tPA-mediated plasmin generation and thus participates to degrade the clot faster [52]. When used at a lower dose than therapeutic levels of tenecteplase, Xai-K $\beta$  independently restored blood flow in a carotid artery thrombotic occlusion mouse model without systemic plasmin generation [52]. This indicated a highly localized effect, likely due to aPL-binding. As an adjunct therapeutic, low concentrations of Xai-K $\beta$  induced reperfusion with sub-therapeutic levels of tenecteplase; thus eliminating the need for a high tenecteplase dose. In addition to pro-fibrinolytic abilities, Xai-K $\beta$  has anticoagulant properties as it prolongs clotting time.

In the absence of a pre-blocked active site, activated FX is rapidly complexed with AT in plasma to form XaAT, which is more susceptible to plasmin cleavage than FXa [180] (Fig. 10B). Proteolysis of XaAT at Lys 339 and Lys 389 in the presence of anionic phospholipid and calcium resulted in the generation of Xa33/13-AT, a species that binds plasminogen [180]. Studies carried using purified XaAT show that it enhances tPA mediated plasmin generation and plasma clot lysis to a greater extent than FXa. In addition to FXa, prothrombinase component FVa is also known to enhance tPA-mediated plasminogen activation [181]. This gain in function was attributed to plasmin cleavage of FVa and binding of plasminogen to these fragments [182].

Additional studies are currently being carried in our laboratory to further explore the mechanism by which FVa acquire its tPA cofactor function and to determine its effect on fibrin clot degradation.

## **1.4 Anticoagulants**

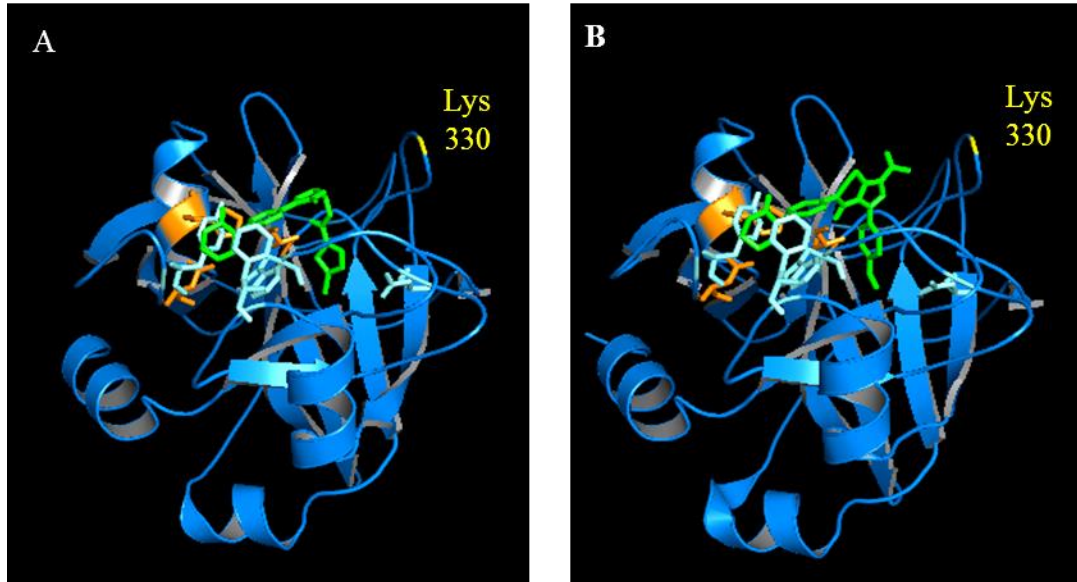
### **1.4.1 Traditional anticoagulants**

Warfarin and heparin are the most commonly used anticoagulants for the treatment and prevention of thromboembolism. Unlike heparin, which is administered intravenously or subcutaneously and has an intravenous half-life of 60 - 90 minutes, warfarin is conveniently administered orally with a half-life of 36 - 42 hours [183]. Both warfarin and heparin have a broad spectrum of targets because they act indirectly. Warfarin inhibits coagulation through reduced production of all  $\gamma$ -carboxyglutamyl-containing procoagulant factors (prothrombin, FVII, FIX, and FX) by attenuating essential vitamin-K-dependent post-translational modification [35,184]. Warfarin can also inhibit  $\gamma$ -carboxyglutamyl-containing anticoagulant factors (proteins C and S) via the same mechanism. Consequently, warfarin has both an antithrombotic and prothrombotic effect; however, its anticoagulant effect is more dominant. Unlike warfarin, heparin enhances AT-mediated suicide inhibition of numerous hemostatic proteases in the coagulation cascade [185]. Thus, these potent nonspecific and indirect anticoagulants can lead to bleeding, a major side-effect of all anticoagulants. In addition to the bleeding risk, regular blood monitoring and dose adjustments are required throughout warfarin treatment due to multiple food-drug and drug-drug interactions as well as a narrow therapeutic window [186]. These issues have been drivers for the recent development and implementation of DOACs.

### 1.4.2 Direct-oral anticoagulants

The introduction of DOACs that specifically target FXa (FXa-DOAC) or thrombin has revolutionized thrombotic therapy. Rivaroxaban and apixaban are two FXa-specific DOACs approved for the treatment and secondary prevention of VTE as well as the primary prevention of VTE in patients post hip or knee replacement surgery and stroke in patients with atrial fibrillation [74,75]. Rivaroxaban is a 435.8 Da molecule that reversibly binds to the active site of free ( $K_i = 0.4$  nM), prothrombinase-bound ( $IC_{50} = 21$  nM), and clot-bound ( $IC_{50} = 75$  nM) FXa [187] (Fig. 11A). At concentrations up to 20  $\mu$ M, rivaroxaban had a  $\sim 10,000$  fold higher affinity for FXa compared to other serine proteases including FVIIa, FIXa, thrombin, activated protein C, urokinase, and trypsin [187]. This confers its selectivity. Pharmacological studies carried out on this small molecule showed that it reaches peak concentrations  $\sim 2 - 4$  hours post administration of the drug [188]. Rivaroxaban is metabolized by cytochrome P450 dependent (oxidation) and independent (hydrolysis) mechanisms [189,190]. In patients with normal renal function to moderate renal impairment, rivaroxaban is eliminated at a half-life of  $\sim 7 - 11$  hours as both active drug (36 %) and inactive metabolites [189]. When compared to warfarin in a double-blinded randomized study (ROCKET-AF), rivaroxaban showed non-inferiority in stroke/systemic embolism, similar rates of major bleedings and less intracranial hemorrhage were seen when administered to patients with non-valvular atrial fibrillation [191].

Similar to rivaroxaban, apixaban is also a small molecule (459.5 Da); however, its specificity for FXa is higher ( $K_i = 0.08$  nM) [34] (Fig. 11B). Apixaban is  $> 30,000$ -fold more specific for FXa when compared to thrombin, activated protein C, FIXa, FVIIa, plasmin, and tPA [192]. Like rivaroxaban, apixaban binds reversibly to the active site of free FXa and is capable of inhibiting prothrombinase-bound ( $K_i = 0.62$  nM) [193] and clot-bound



**Figure 11: 3D structure of FXa complexed with rivaroxaban or apixaban**

Panel A: Ribbon structure of FXa plus rivaroxaban (PDB ID: 2W26) and Panel B: Ribbon structure of FXa plus apixaban (PDB ID: 2P16). Only the heavy chain of FXa is shown in blue with the catalytic triad residues (serine 195, histidine 57, and aspartate 102) in orange, the S1 (aspartate 189) and S4 (phenylalanine 174, tryptophan 215, and tyrosine 99) pocket residues in cyan, the lysine (Lys) 330 cleavage site in yellow, and the inhibitor in green. Images were generated using Pymol (<https://www.pymol.org/>).

( $IC_{50} = 1.3 \text{ nM}$ ) [194] FXa. It reaches its peak concentration at ~ 3 hours and is eliminated with a half-life of ~ 8 - 14 hours by hepatic and renal excretion [195]. When compared to warfarin treatment (ARISTOTLE-AF), apixaban was safer and more effective in preventing stroke, systemic embolism, major bleeding, and intracranial bleeding in patients with non-valvular atrial fibrillation [196].

While not a primary focus on this thesis, thrombin-specific inhibitors have also emerged as being effective in the treatment of venous thromboembolism [197], reviewed in [198]. The most well-known thrombin-specific DOAC is dabigatran etexilate, a prodrug that is converted into its active form, dabigatran, by serum esterases [199]. Dabigatran works by binding to the active site of thrombin reversibly at a  $K_i$  of 4.5 nM [200] and reaches its peak plasma concentration ~ 2 hours after ingestion [201]. It is then cleared predominantly by renal excretion with an elimination half-life of 14 - 17 hours after multiple doses [201]. When compared to warfarin, dabigatran was non-inferior at a dose of 110 mg twice daily and superior at a dose of 150 mg twice daily in preventing stroke and systemic embolism [202]. Major bleeding was less common in the 110 mg group and similar in the 150 mg group respectively, compared to warfarin.

### **1.4.3 Role of DOACs in fibrinolysis in *in vitro* experiment using spiked plasma**

Various studies have explored the pro-fibrinolytic function of DOACs targeting thrombin and FXa [203-205] (Table 1). A decreased in thrombin generation leading to reduced TAFI activation and altered clot structure in most cases were attributed to the DOAC-mediated enhancement of fibrinolysis. In one study, Varin et al [203] demonstrated that TF-induced plasma clots formed in the presence of therapeutic levels of rivaroxaban had thicker fibers and larger pores. This change in clot porosity results in an increased permeability and thus a greater

**Table 1: Summary of previous *in vitro* studies evaluating the effect of DOACs on fibrinolysis**

Study type	Clot initiator	Added [tPA], nM	[DOAC], nM			Impact of DOAC and observations
			Riva	Apix	Dabi	
Rivaroxaban spiked plasma ( <i>in vitro</i> ) <sup>203</sup>	TF	1.36	• 344 • 574	N/A	N/A	<ul style="list-style-type: none"> <li>• Reduced thrombin generation</li> <li>• Increased permeability</li> <li>• Enhanced D-dimer generation</li> <li>• FXIII activation not a factor</li> <li>• DOAC-mediated inhibition of TAFI activation a probably contributing factor</li> </ul>
Dabigatran spiked plasma ( <i>in vitro</i> ) <sup>204</sup>	TF (6 pM)	• 0.51 • 3.39	N/A	N/A	0-200	<ul style="list-style-type: none"> <li>• [dabigatran] positively correlated to clotting time at both [tPA]</li> <li>• Concentration-dependent reduction of clot lysis time</li> <li>• Increase clot permeability</li> <li>• Reduction of FXIII activation by dabigatran not a potential contributing factor</li> </ul>

penetration rate of tPA and plasminogen within the clot. They reported that differences in FXIII activation due to reduced thrombin generation in the presence of rivaroxaban were not a contributing factor in the enhanced degradation seen. This observation was explained by the small amount of thrombin needed to activate XIII [97]. Ammollo et al. [204] also reported a change in structure and TAFI activation when dabigatran was used. These observations, while mentioned as “not shown” in their study, were not reproduced when atroxin, a thrombin-like enzyme was used to initiate clotting [204].

#### **1.4.4 Role of DOACs in fibrinolysis in *ex vivo* experiments using patient plasma**

In addition to *in vitro* studies, *ex vivo* studies using plasma or whole blood from DOAC-treated atrial fibrillation patients have also been reported [205-207] (Table 2). These studies, which used different sources to initiate coagulation, each showed some delay in clot formation. In both whole blood and plasma experiments, apixaban enhanced endogenous fibrinolysis, while rivaroxaban showed a statistically insignificant trend towards enhanced lysis in whole blood [205,207]. When exogenous tPA was added, no difference were seen in plasma clot lysis at trough and peak concentrations of rivaroxaban and apixaban [206]. In dabigatran-treated patients; however, plasma clot lysis was significantly faster with higher drug concentration [206]. The latter observation, associated to a reduction of TAFIa-mediated inhibition of clot lysis, was not seen in rivaroxaban- and apixaban-treated patients. In the FXa-specific samples, the difference in plasma fibrinolysis time at trough and peak drug concentrations was similar in the presence and absence of potato tuber carboxypeptidase inhibitor (PTCI), a TAFIa inhibitor [201].

**Table 2: Summary of previous *ex vivo* studies on the effect of DOACs on fibrinolysis in atrial fibrillation patients**

Study type	Clot initiator	Added [tPA], nM	[DOAC], nM			Impact of DOAC and observations
			Riva	Apix	Dabi	
DOAC and warfarin treated afib patients ( <i>ex vivo</i> ) <sup>205</sup>	Kaolin Calcium	Endogenous thrombolysis	Values not specified. Therapeutic dose given and samples collect at peak time			<ul style="list-style-type: none"> <li>• Similar clot formation rates in DOACs and warfarin</li> <li>• Enhanced endogenous fibrinolysis potential in DOACs compared to warfarin</li> </ul>
	IIa (3.5 nM)	169.5				<ul style="list-style-type: none"> <li>• Dabigatran delays clotting more than FXa-DOACs</li> <li>• FXa-DOACs form stronger clots than dabigatran</li> <li>• No significant differences between DOACs in fibrinolysis</li> </ul>
DOAC treated afib patients ( <i>ex vivo</i> ) <sup>206</sup>	TF (6 pM)	0.51 (f.c.)	<ul style="list-style-type: none"> <li>• 30</li> <li>• 212</li> </ul>	<ul style="list-style-type: none"> <li>• 119</li> <li>• 234</li> </ul>	<ul style="list-style-type: none"> <li>• 60.5</li> <li>• 152</li> </ul>	<ul style="list-style-type: none"> <li>• Reduced thrombin generation for all drugs</li> <li>• Significant delay in lag time for FXa-DOACs but not dabigatran</li> <li>• No correlation between clot lysis and FXa-DOACs concentration: no difference in [peak] vs. [trough]</li> <li>• Concentration-dependent enhanced clot lysis for dabigatran.</li> <li>• No difference between [trough] and [peak] when clotting is initiated with a thrombin-like enzyme in all groups</li> <li>• TAFI dependent and independent mechanism of enhanced clot lysis proposed</li> <li>• Greater TAFIa-mediated inhibition of clot lysis in the presence of rivaroxaban and apixaban but not dabigatran</li> </ul>
DOAC and warfarin treated afib patients ( <i>ex vivo</i> ) <sup>207</sup>	High shear stress (whole blood)	Endogenous thrombolysis	Values not specified. Therapeutic dose given and samples collect at peak time			<p>Compared to baseline (prior to DOAC treatment):</p> <ul style="list-style-type: none"> <li>• DOAC and warfarin treatment delayed time to occlusion with apixaban having the least effect</li> <li>• Significant enhanced clot lysis with apixaban treatment</li> <li>• Trend towards enhanced clot lysis with rivaroxaban and dabigatran (not statistically significant) but not warfarin</li> </ul>

## 1.5 Overall thesis rationale

Thrombi block the flow of blood and contribute to cardiovascular disease, the leading cause of death worldwide. To dissolve these clots, “clot-busting” drugs have been developed. So far, the design of these drugs has been based on physiological tPA. To date, ongoing drug development aimed at reducing the life-threatening side effects and complications associated with current thrombolytic agents has been unsuccessful. Thus cerebral hemorrhage (~ 3 - 6 %) and the resistance of clots (~ 40 %) to recombinant tPA and its variants continue to be major problems. To address the need for new thrombolytic agents, our laboratory has chosen to identify a non tPA-based and non-enzymatic drug target.

The current fibrinolysis model accepts fibrin as the only cofactor required to accelerate tPA-mediated conversion of plasminogen to plasmin. By investigating the mechanism at physiological tPA concentrations, which is ~ 1000-fold lower ( $\mu\text{M}$ ; [162]) than the therapeutic levels conventionally used for *in vitro* studies, our laboratory identified that two specific plasmin-mediated cleavages of the clotting factor Xa abolish its role in coagulation and convert it into a tPA accelerator [162]. The first fragment is called FXa $\beta$  and it is further proteolyzed into Xa33/13 (Fig. 10). These cleavages expose new C-terminal lysine residues, enabling tPA and plasminogen to bind and converge [52,178]. Although FXa and Xa33/13 are outstanding tPA accelerators in purified *in vitro* systems, in plasma-based experiments FXa loses its ability to enhance clot lysis and the fibrinolytic function of Xa33/13 is rapidly inhibited with loss of antigen [52]. Working toward stabilizing the clot-dissolving function of FXa, we have found that covalently modifying the active site of FXa prevents further cleavage of FXa $\beta$  into Xa33/13 [52,162]. This blockage in fragmentation progression promoted the fibrinolytic function of FXa in plasma and in animal models [52].

Given that direct FXa anticoagulants, rivaroxaban and apixaban, specifically bind to the active site of FXa, these agents may have an impact on fibrinolysis. Therefore, the goals of this research project were to understand how the active site occupancy by these two anticoagulants may modulate the fibrinolytic function of FXa and to determine how the latter may be correlated to the FXa cleavage products generated. Through this study possible unforeseen effects of rivaroxaban and apixaban on clot dissolution as well as potential effects of these anticoagulants on FXa as a clot dissolving agent were investigated.

## **1.6 Hypothesis**

The overall hypothesis of this dissertation is that FXa-specific direct oral anticoagulants stabilize FXa $\beta$  and enhance fibrinolysis in plasma.

## **Chapter 2: MATERIAL AND METHODS**

### **2.1 Materials and proteins**

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), polyethylene glycol 8000 (PEG 8000), and dry dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc. (Missouri, USA). Tetrasodium ethylenediaminetetraacetic acid (EDTA) was obtained from Thermo Fisher Scientific (New Jersey, USA). Calcium chloride dihydrate (EMD Chemicals, Inc. New Jersey, USA), Innovin<sup>®</sup> (Siemens Healthcare Diagnostic Inc., Delaware, USA), human Lys-plasminogen (Enzyme Research Laboratories South Bend, IN, USA), Tween 20 (Amresco, Inc. Ohio, USA), single chain recombinant tissue-type plasminogen activator (tPA) (Genetech, California, USA), and plasmin (Boc-Glu-Lys-Lys-MCA) fluorogenic substrate (Peptide Institute, Inc. Osaka, JAPAN) were obtained commercially. Chromogenic substrates H-D-Val-Leu-Lys-pNA (S-2251; plasmin), Z-D-Arg-Gly-Arg-pNA (S-2765; FXa), and H-D-Phe-Pip-Arg-pNA (S-2238; thrombin) were from Chromogenix (Bedford, MA, USA). L- $\alpha$ -phosphatidylcholine and L- $\alpha$ -phosphatidylserine (Avanti Polar Lipids Inc. Alabama, USA) were used to make small unilamellar and large multilamellar vesicles in 75:25 mixture as the source of aPL [208]. Human Factor X, potato tuber carboxypeptidase inhibitor (PTCI), alpha-thrombin, and plasmin were purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Apixaban (Eliquis, Bristol-Myers Squibb, Montréal, Canada) and rivaroxaban (Xarelto; Bayer Inc. Mississauga, Canada) were obtained from a local pharmacy. Normal pooled plasma and FX-deficient plasma (FXDP) were purchased from Affinity Biologicals Inc. (Ontario, Canada). Plasmas congenitally deficient in FIX or FVIII were obtained from George King Bio-Medical, Inc. (Kansas, USA). Anti-human factor XI antibody #AHXI-5061 was purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Primary polyclonal rabbit anti-human fibrinogen antibody

#F0111 (Dako, USA), secondary horseradish peroxidase-coupled goat-anti-rabbit IgG H&L antibody #ab7090 (Abcam, UK), primary mouse anti-human FX heavy chain antibody #115-035-146 (Green Mountain Antibodies Inc., Vermont, USA) and secondary goat-anti mouse IgG H&L (HRP) antibody # GMA-508 (Jackson ImmunoResearch, Pennsylvania, USA) were purchased.

## **2.2 FXa-DOACs**

### **2.2.1 Preparation of rivaroxaban and apixaban**

Rivaroxaban and apixaban tablets were crushed and suspended in 100 % DMSO to obtain 10 mM stock solutions, then centrifuged at 13,000 rpm for 5 minutes. Prior to use, a stock of FXa-DOACs was diluted in HEPES (20 mM) buffered saline, pH 7.4, containing 0.1% polyethylene glycol (HBSP) so that the final concentration of DMSO in every assay performed was less than 0.1%. This DMSO level was found to have no effect on fibrinolytic experiments. The experimental FXa-DOAC concentration used was based on plasma concentrations after standard therapeutic doses given to atrial fibrillation patients for the prevention of stroke. For rivaroxaban, a working concentration of 0.57  $\mu\text{M}$  (250 ng/mL) was chosen as it is the maximum plasma concentration ( $C_{\text{max}}$ ) reported for patients on a typical dose of 20 mg daily [203]. Although higher plasma concentrations have been noted [204,206,209], by convention [210-212] the  $C_{\text{max}}$  used for apixaban was 0.44  $\mu\text{M}$  (200 ng/mL), which is a simple doubling of the  $C_{\text{max}}$  reported after the first of two daily 5 mg administrations [32].

### **2.2.2 Assessment of rivaroxaban and apixaban function**

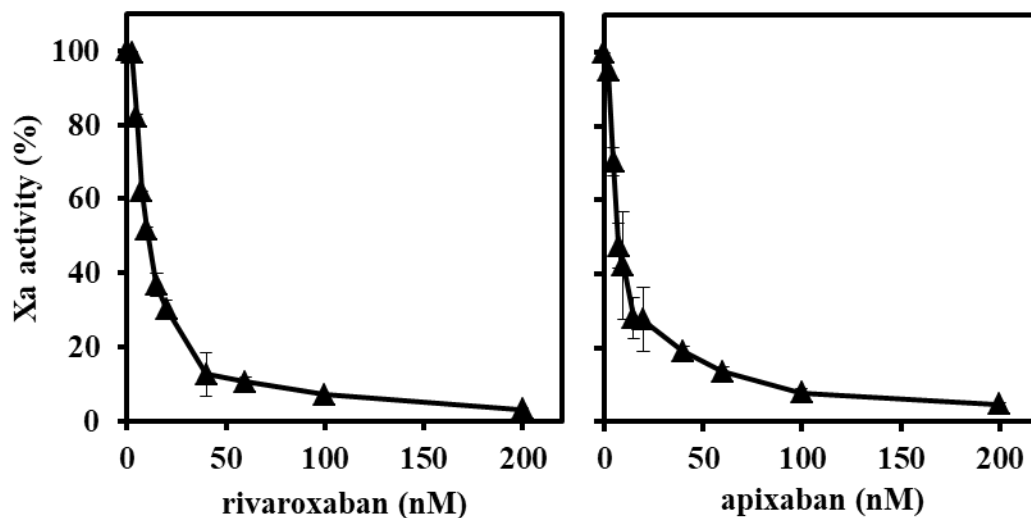
Following FXa-DOAC preparation, an active site titration of FXa was conducted to verify the specific activity of rivaroxaban and apixaban. FXa (10 nM) was incubated with increasing concentrations of rivaroxaban or apixaban (0 – 200 nM) and added to a 96-well plate.

A FXa-specific chromogenic substrate (S-2765; Z-D-Arg-Gly-Arg-pNA) diluted in 20mM EDTA/HBSP was then added to each well at a final concentration of 200  $\mu$ M. Absorbance was measured at 405 nm. Residual FXa activity, calculated from changes in optical density, was plotted against FXa-DOAC concentration (Fig. 12).

## **2.3 Assessment of fibrinolytic function**

### **2.3.1 Plasma fibrinolysis assay**

Turbidimetric assays were similar to those previously described [180], except for the addition of rivaroxaban and apixaban. FXa-DOAC or vehicle, tPA in HBS/0.01 % Tween (HBST) and a mixture containing thrombin (10 nM), CaCl<sub>2</sub> (20 mM), and Pen Strep (1 %; Thermofisher Scientific, Ontario, Canada) were added as separate droplets to a 96-well flat bottom plate. Unless described otherwise, final concentrations of 0.57  $\mu$ M rivaroxaban, 0.44  $\mu$ M apixaban and 30  $\mu$ M tPA were used. The assay was initiated by the addition of normal pooled plasma, FX-deficient plasma  $\pm$  purified human FX (170 nM), congenital FIX-deficient plasma, congenital FVIII-deficient plasma in the presence or absence of a FIX inhibitory antibody (160 nM), or normal pooled plasma supplemented with 2  $\mu$ M PTCI to eliminate TAFIa activity. All reactions had a final plasma concentration of 35 %. The extent of clot formation and dissolution was monitored at 405 nm with readings at 5 minute intervals. A fibrinolysis end point was chosen where clot lysis was near or at completion for the experimental conditions being evaluated and used to calculate the area under the curve (absorbance 405 nm x time) using GraphPad Prism (California, USA). This experimental end point was consistently used for the respective controls conducted in the absence of FXa-DOACs, which did not approach complete lysis. In the measurement of area under the curve, a baseline of 0.2 was chosen as it is the minimum absorbance value reported for clots that have completely lysed.



**Figure 12. Active site titration of FXa with rivaroxaban or apixaban**

Panel A: FXa (10 nM) was incubated with increasing concentrations of rivaroxaban (0-200 nM). Chromogenic substrate (S2267) was then added. Readings for FXa in the absence of rivaroxaban was assigned 100% FXa activity and subsequent reading were relativized to that to determine percent of Xa activity for every given rivaroxaban concentration. Xa activity was then plotted against the corresponding rivaroxaban concentration. Panel B: Similar to Panel A with except that apixaban (0- 200 nM) was titrated.

### **2.3.2 Plasmin generation during fibrinolysis**

Plasma clot lysis assays were conducted as described above and cleavage of a plasmin-selective fluorogenic substrate was monitored during the experiment according to previously described protocols with slight modifications [213,214]. A 15  $\mu\text{L}$  sample of the reactant solution (consisting of FXa-DOAC or buffer, 30  $\mu\text{M}$  tPA, 10 nM thrombin, 20 mM calcium, and 1 % Pen Strep) was added to a black polystyrene 96-well flat bottom plate (Corning Inc., New York, USA) followed by 5  $\mu\text{L}$  of the plasmin-selective fluorogenic substrate (Boc-Glu-Lys-Lys-MCA). The plate was then placed in a POLARstar OPTIMA plate reader for 3 mins at 37°C while the plasma was warmed in a 37 °C water bath. After 3 minutes, 80  $\mu\text{L}$  of plasma was added to the plate and the amount of plasmin generated during the course of the experiment was measured (excitation, 390 nm; emission, 460 nm). Readings were taken every 30 seconds for the first 120 minutes, with a final measurement at 1600 minutes to further highlight potential differences in plasmin generation.

### **2.3.3 Purified tPA-mediated plasmin generation**

FX (0.1  $\mu\text{M}$ ), FXa (0.1  $\mu\text{M}$ ), or RVV-X (21 nM)-activated FX (0.1  $\mu\text{M}$ ) with or without rivaroxaban or apixaban, plasminogen (0.5  $\mu\text{M}$ ), PCPS (50  $\mu\text{M}$ ), and  $\text{CaCl}_2$  (5 mM) were incubated in a 96-well flat bottom microtitre plate. A sample was taken at time 0 and tPA was then added to the mixture. The amount of plasmin generated during the experiment was measured by mixing the content of the reaction well to a well containing S-2251 (D-Val-Leu-Lys-pNA; 200  $\mu\text{M}$ ), a plasmin-specific chromogenic substrate. For each time point, a total of 6 reads were taken over 1 minute at 405 nm. Samples from the reaction mix of an identical experiment were removed for anti-FX western and iodine-radiolabelled plasminogen ligand blot analyses. The latter was done by first incubating the polyvinylidene fluoride (PVDF) membrane

containing the FXa fragments with iodine-radiolabelled plasminogen for 1 hour. The membrane was then washed with 10 mg/mL bovine serum albumin in Tris-buffered saline. Using an x-ray film cassette and intensifying screen, the PVDF was exposed to clear blue x-ray film (Thermo Fisher Scientific, IL, USA) at  $-80\text{ }^{\circ}\text{C}$  and developed. Anti-FX western blot analyses were carried out on the same blots as previously described [162].

## **2.4 Assessment of clot integrity in the presence of rivaroxaban and apixaban**

### **2.4.1 Fibrin generation and crosslinking**

Clots (40  $\mu\text{L}$ ) were formed using the same final concentrations as those used for plasma clot lysis assays with the exception that tPA was omitted. To each sample, 100  $\mu\text{L}$  of quench buffer (50 mM dithiothreitol, 12.5 mM EDTA, 8 M Urea) was added and samples were incubated at  $60\text{ }^{\circ}\text{C}$  until the clots dissolved. The samples were then reduced, boiled, separated on 10% Tris-glycine gels, transferred to nitrocellulose membranes, and probed for fibrinogen with a polyclonal rabbit anti-human fibrinogen antibody. After 3 washes with phosphate buffered saline containing tween 20, a secondary antibody (product # ab7090, goat anti-rabbit IgH H&L HRP, Abcam) was applied, followed by additional washes. The membranes were treated with Clarity Western ECL substrate (Bio-Rad; Rutherford, NJ, USA) and the luminescence was detected on x-ray films (Mandel, ON, Canada).

### **2.4.2 Scanning electron microscopy**

Preparation, processing and imaging techniques were done according to training instructions received at the UBC Bio-imaging facility. Plasma clots (100  $\mu\text{L}$ ) were formed in 12x75 mm polypropylene tubes using similar conditions as above. After 1 hour, polymerized clots were washed and fixed in a mixture of 2.5 % glutaraldehyde and 4 % formaldehyde. They were then carefully dehydrated to ensure no changes to their morphology in a two-step process.

First, increasing concentrations (30 % - 100 %) of ethanol were used as an intermediate fluid to replace water molecules within clots. Next, a critical point dryer was used to replace the ethanol fluid with liquid carbon dioxide, which was then converted into gaseous carbon dioxide at its critical point (i.e., the point where its liquid and gas phases are in equilibrium) [215]. After clots were sputter-coated with platinum/palladium (5 nm), they were imaged using a Hitachi S-4700 Field Emission Scanning Electron Microscope. For each condition, an image was taken at 3 random locations to give an overall perspective of the clot. A grid was added to each image using ImageJ software and the diameter of 10 non-superimposed fibers per image was measured, enabling 30 measures per condition. Clot porosity was determined by converting scanning electron microscopy (SEM) images into binary images using the automatic thresholding feature in ImageJ to differentiate between fibers (pixels less than threshold value) and pores (pixels greater than threshold value). Black pixels representing the pores were then quantified.

## **2.5 FXa fragmentation**

### **2.5.1 Innovin<sup>®</sup>-initiated Xa fragmentation in plasma**

Innovin<sup>®</sup> (8  $\mu$ L) as a source of TF, CaCl<sub>2</sub> (5 mM; 1.5  $\mu$ L), tPA in 0.01% HBST (50 nM; 1.5  $\mu$ L) and HBS (9  $\mu$ L) were incubated with or without rivaroxaban or apixaban. The reaction was started with plasma (10  $\mu$ L) for a total reaction volume of 30  $\mu$ L. Separate reactions were made for 5 time points in to obtain a time course. At the desired reaction time, 10  $\mu$ L of denaturing Laemmli sample buffer containing sodium dodecylsulfate (SDS) was added to stop the reaction and prepare the sample for electrophoresis and western blot analysis. Samples were either further diluted 1:10 in Laemmli sample buffer or not, such as the case for DVT/PE and post-surgery patient plasma samples. Western blots were probed with mouse anti-human FX heavy chain antibody, as previously described [162]. Molecular weight markers (Bio-Rad;

Rutherford, NJ, USA) and purified XaAT [180], FX, FXa, and Xa33/13 [162] were used to identify bands.

## **2.5.2 Plasmin-mediated fragmentation of FXa using purified proteins**

Purified FXa (2  $\mu$ M), PCPS (300  $\mu$ M), and CaCl<sub>2</sub> (5 mM) were combined with or without rivaroxaban or apixaban. To maintain a ratio of 1:5.7 and 1:4.4 between FXa and the FXa-DOACs, as per the other assays, rivaroxaban and apixaban were used at final concentrations of 11.4  $\mu$ M and 8.8  $\mu$ M, respectively. Plasmin (0.2  $\mu$ M) was then added to the mixture. At various times, samples were taken and added to Laemmli electrophoresis sample buffer for anti-FX western blot analysis, as previously described [162].

## **2.6 Patient study**

### **2.6.1 Study sample**

Samples used in this exploratory study were residual plasma specimens collected from either a previous study investigating DOAC concentration in plasma (rivaroxaban and dabigatran samples; ethics certificate #: H11-00084) or from preoperative international normalized ratio (INR) assessment (non-anticoagulated samples; ethics certificate #: H16-0115-A001). Samples from patients who had been treated with rivaroxaban were used to investigate its effect on FXa fragmentation and plasma fibrinolysis. While the FXa fragmentation in patients treated with rivaroxaban for DVT/PE and the prevention of venous thromboembolism after hip or knee replacement surgery was noted, it was not used for further analysis. Instead, a primary focus was placed on those treated for the prevention of stroke in atrial fibrillation. The latter group was chosen as thrombosis is the least provoked in patients with atrial fibrillation compared to the other indications for which the FXa-DOACs are administered [216,217]. Consequently, plasma samples from rivaroxaban-treated atrial fibrillation patients were compared to those from atrial

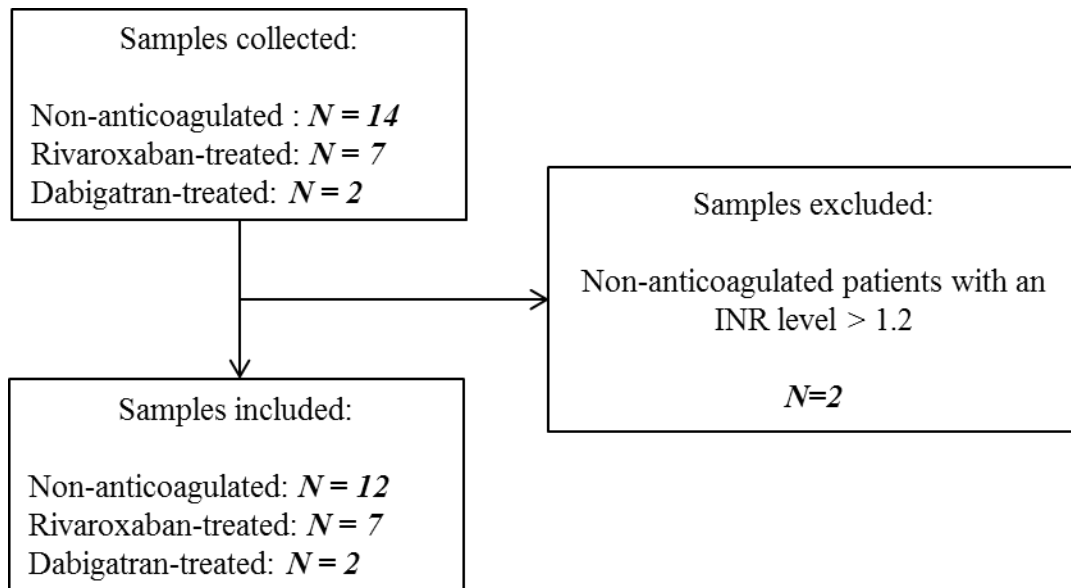
fibrillation patients who were previously treated with warfarin but were taken off of their treatment for 5 - 6 days [218] to obtain an INR level of 0.9-1.2 prior to their surgical procedure (non-anticoagulated). Plasma samples from patients treated with the thrombin-specific anticoagulant, dabigatran, were used as a negative control (Fig. 13). Since dabigatran inhibits thrombin and plasma clot lysis assays in this study are initiated with thrombin, samples from patients treated with it were only included in FXa-fragmentation but not in plasma fibrinolysis studies. Baseline characteristics of study groups are found in Table 3.

### **2.6.2 Sample size calculation**

The number of non-anticoagulated patient donors needed to carry out this study was decided using preliminary data comparing the mean complete lysis time of commercial normal pooled plasma (7063.3 minutes) to that of rivaroxaban-treated patient plasma samples (5590.8 minutes). The common standard deviation between the two groups (sigma) was 943.3 minutes. Based on preliminary data, it was determined that 7 patients per group were needed to provide a power of 0.80 and an  $\alpha$  value of 0.05. Since 7 rivaroxaban-treated samples were available from our collaborator, a total of 14 age-matched non-anticoagulated patients' residual samples were obtained under ethics.

### **2.6.3 Blood collection and analysis details obtained with residual samples**

Blood was drawn from consenting patients at the Vancouver General Hospital (VGH) outpatient laboratory by trained laboratory personnel using a protocol jointly approved by the University of British Columbia and Canadian Blood Services Research Ethics Boards (certificate #: H11-00084). Each patient's rivaroxaban/dabigatran dose and the time of the last dose were recorded at the phlebotomy clinic. Blood was collected into 0.109 M sodium citrate tubes (BD



**Figure 13: Flow diagram of study sample**

A total of 14, 7, and 2 patient plasma samples were collected from non-anticoagulated, rivaroxaban-treated, and dabigatran-treated atrial fibrillation patients respectively. From these, 2 were excluded in the non-anticoagulated group because their international normalized ratio (INR) levels were above 1.2. Thus, plasma samples from 12 non-anticoagulated, 7 rivaroxaban-treated, and 2 dabigatran-treated atrial fibrillation patients were included in the study.

**Table 3: Baseline characteristics of atrial fibrillation patients and normal pooled plasma**

	Sex		Age (yrs)	DOAC (nM)	Fibrinogen (g/L)
	M	F			
Rivaroxaban-treated (n=7)	5	2	50 - 90	149.4 - 504.7	1.02 - 5.20
Dabigatran-treated (n=2)	2	0	55 - 67	109.6 - 934.2	3.76
Non-anticoagulated (n=12)	10	2	62 - 92	n/a	n/a
Normal <u>pooled</u> plasma (n=25)	21	4	25 - 63	n/a	3.06

Biosciences®) then centrifuged at 1,500 g for 15 minutes at room temperature to generate platelet-poor plasma. The plasma samples were then aliquoted, frozen and stored at  $-70^{\circ}$  for future use. Fibrinogen levels for each plasma sample obtained from treated patients were quantified at VGH using a Clauss fibrinogen assay (Stago STA-Fibrinogen). All assays were performed using the STA-R Evolution coagulation analyzer (Diagnostica Stago, Ansieres, France). Residuals samples were stored and later transferred to the Pryzdial laboratory for this current project.

#### **2.6.4 Quantification of rivaroxaban and dabigatran in patient plasma by LC-MS/MS**

Plasma concentration of rivaroxaban and dabigatran were independently evaluated at Providence Health Services Authority by unpublished methods and are thus detailed. Internal DOAC standards used for LC-MS/MS quantification, rivaroxaban-d4 and dabigatran-d3, were obtained from Toronto Research Chemicals Inc. (Ontario, CA). Stock calibrator solutions were prepared by dissolving 2.27 mg rivaroxaban (Toronto Research Chemicals Inc., Ontario, CA) in 1 mL DMSO and 0.96 mg dabigatran (Toronto Research Chemicals Inc., Ontario, CA) in 1 mL acidified dimercaptosuccinic acid. Working solutions of standards and calibrators were made up in 1:1 methanol:water.

##### **2.6.4.1 Sample preparation**

50  $\mu$ L of each sample (calibrators, quality control or patient plasma) was pipetted into a 2 mL deep 96-well plate followed by 25  $\mu$ L of the rivaroxaban-d4 or dabigatran-d3 working solution. These were then mixed. Base hydrolysis of the dabigatran metabolite was performed by adding 0.2 N sodium hydroxide (25 $\mu$ L) to each sample, mixing the samples, and incubating them in a water bath maintained at 37  $^{\circ}$ C for 2 hours. Precipitation of DOACs from samples was achieved with the addition of 10 g/L (400  $\mu$ L) zinc sulfate in 70:30 methanol:water to each well.

The 96-well plate was sealed and vortex-mixed for 1 minute at high speed followed by centrifugation at ambient temperature for 10 minutes. The supernatant (10 $\mu$ L) was injected to the LC-MS/MS system (SCIEX 5500 QTrap MS with Shimadzu 20AD LC).

#### **2.6.4.2 High-performance liquid chromatography conditions**

High-performance liquid chromatography was done using 2 M ammonium acetate with 0.1% formic acid in H<sub>2</sub>O (A) as the aqueous mobile phase and 2 M ammonium acetate with 0.1% formic acid in methanol as the organic mobile phase (B). Separation was performed on a Shimadzu LC-20AD/CT-20A/SIL-20ACHT ultra-fast liquid chromatograph with the column oven heated to 60 °C. Sample extracts were injected at 2 % B, held for 0.25 minutes, followed by a gradient ramp to 100 % B over 0.75 minutes, held 1 minute then returned to starting conditions for 1 minute. The elution time for rivaroxaban was approximately 1.5 minutes with a sample run time of 3.1 minutes per specimen.

#### **2.6.4.3 Mass spectrometry conditions**

Mass spectrometric detection was performed using the SCIEX 5500 instrument in electrospray ionization positive ion mode. Ionization conditions and multiple reaction monitoring transitions were reported. The latter were chosen by infusing pure rivaroxaban or dabigatran into the mass spectrometer. Source and compound dependent conditions were optimized for sensitivity using SCIEX Analyst® software (v 1.6.1) compound optimization protocols.

#### **2.6.5 Analysis of plasma clot lysis in patient samples**

Plasma clot lysis was carried out according to methods stated in section 2.3.1 with the exception that plasma was used from patients treated with rivaroxaban and those previously on warfarin with an INR level of < 1.2 at the time of blood collection. Clots were allowed to

completely lyse and lysis time was defined as the time it took for maximum turbidity to be reduced by half [219].

#### **2.6.6 Analysis of FXa fragmentation bands in atrial fibrillation patient samples**

Experiments were conducted as stated in section 2.5.1. Molecular weight markers and known amounts of purified FX, FXa, XaAT [180], and Xa33/13 [162] were included in each gel as mobility controls. The band intensity in patient samples was quantified according to that of purified FX control band. In rivaroxaban-treated patient samples, the percent of FXa $\beta$  relative to total FXa was obtained by calculating the ratio of band intensity according to the equation:  $(FXa\beta / (FXa\alpha + FXa\beta)) * 100$ . Similarly, percent FXa $\alpha$  relative to total FXa was determined by computing  $(FXa\alpha / (FXa\alpha + FXa\beta)) * 100$ . Since all bands on a given gel were quantified based on the same FX control band on that gel, differences in the proportion of FXa $\alpha/\beta$  at 3 - 60 minutes to the starting amount of FX at time 0 was accounted for in every patient sample. Calculations were done for each time point separately.

#### **2.6.7 D-dimer quantification**

ZYMUTEST D-dimer ELISA (Anaira, USA) was used to measure D-dimer levels at baseline according to the manufacturer's guidelines.

#### **2.6.8 Statistics for patient study**

The distribution of all variables was analyzed. Variables that were not normally distributed initially were log transformed in an effort to gain normality. The means computed for half-clot lysis times, maximum optical density (max OD), time-to-max OD, and D-dimer levels between patient groups were compared using a 2 tailed unpaired t-test. The mean half-clot lysis time and D-dimer level in normal pooled plasma were considered a hypothetical value for comparison with mean values of rivaroxaban-treated atrial fibrillation samples. The latter was

done using a one sample t-test. Pearson correlation and linear regressions were carried out on two variables of interest at a time to identify potential relationships. All analyses were done using the statistical software, XLSTAT (Addinsoft, New York, NY).

## **Chapter 3: EFFECTS OF DIRECT ORAL ANTICOAGULANTS ON FXa FIBRINOLYTIC FUNCTION IN PLASMA AND PURIFIED PROTEINS**

### **3.1 Overview and specific goals**

Within the auxiliary model of fibrinolysis, plasmin-cleaved clotting FXa can be modulated to enhance plasma clot lysis [52,162]. In the presence of calcium and aPL, FXa is converted to FXa $\beta$  and then to Xa33/13 upon further plasmin cleavage. In plasma, this results in a loss of the pro-fibrinolytic function conferred by Xa33/13 [52]. Previous studies within our laboratory have shown that by chemically modifying the active site of FXa with a chloromethylketone (CMK), Xa33/13 generation is prevented and the pro-fibrinolytic function of FXa $\beta$  is stabilized [52]. This thesis chapter focuses on rivaroxaban and apixaban, two FXa-specific direct oral anticoagulants that bind with high affinity to nearly identical locations within the FXa catalytic site. These sites are proximal to, if not overlapping with, the area affected by CMKs. Here the hypothesis is addressed that rivaroxaban and apixaban may enhance fibrinolysis by stabilizing FXa $\beta$  in a similar manner as CMKs, which irreversibly block the active site of FXa [52]. Interesting previous studies have investigated the effects of DOACs on fibrinolysis [203,204,206]. The data showed that reduced thrombin-mediated feedback, caused by the anticoagulant effect of the drugs, altered clotting parameters and prevented TAFI activation. Thus, clots were more susceptible to fibrinolysis [203]. However, experiments were conducted at tPA concentrations that could potentially mask a contribution of the specific participation of FXa as a fibrinolytic component. Furthermore tissue factor was used to initiate clotting, which causes differential thrombin generation in the presence of DOACs [220]. Consequently, the effects of FXa-DOACs on FXa in fibrinolysis were investigated in this chapter using tPA levels within the physiological range. Furthermore, thrombin, instead of the more typical TF, was used to initiate

clotting and overcome the anticoagulant effect of the FXa-DOACs. The goals of this study were to:

- 1) Investigate the effects of FXa-DOACs in plasma fibrinolysis under described experimental conditions
- 2) Evaluate potential confounding effects due to differential thrombin generation
- 3) Determine the effect of FXa-DOACs on FXa fragmentation in normal pooled plasma
- 4) Dissect a potential mechanism for FXa plus FXa-DOAC-enhanced clot lysis using purified proteins

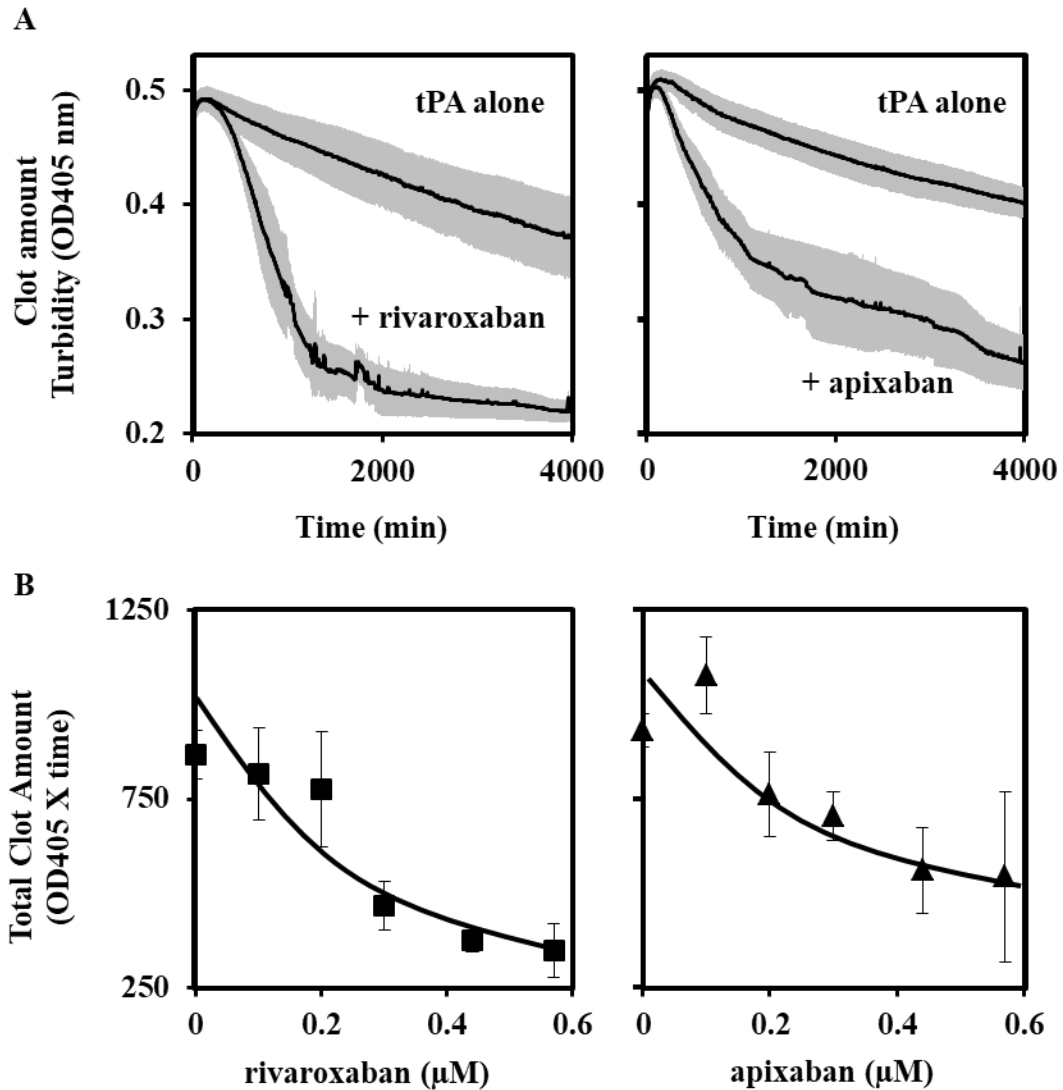
## **3.2 Results**

### **3.2.1 Plasma fibrinolysis is enhanced by FXa-DOACs in a dose-dependent manner**

To overcome the anticoagulant effects of FXa-DOACs, other laboratories have adjusted the initiating TF concentration to equalize clotting times between samples in the presence of anticoagulant [221,222]. These findings were taken into account when designing experiments in the current work and clot formation was initiated with 10 nM purified thrombin to bypass the anticoagulant effects of the FXa-DOACs. The extent of clot formation and dissolution were then monitored by changes in turbidity. When rivaroxaban and apixaban were added at their respective average C<sub>max</sub>, clot degradation was enhanced (Fig. 14A). Titrating rivaroxaban and apixaban at concentrations below the average C<sub>max</sub> demonstrated a dose response to approximately 0.1 μM (Fig. 14B).

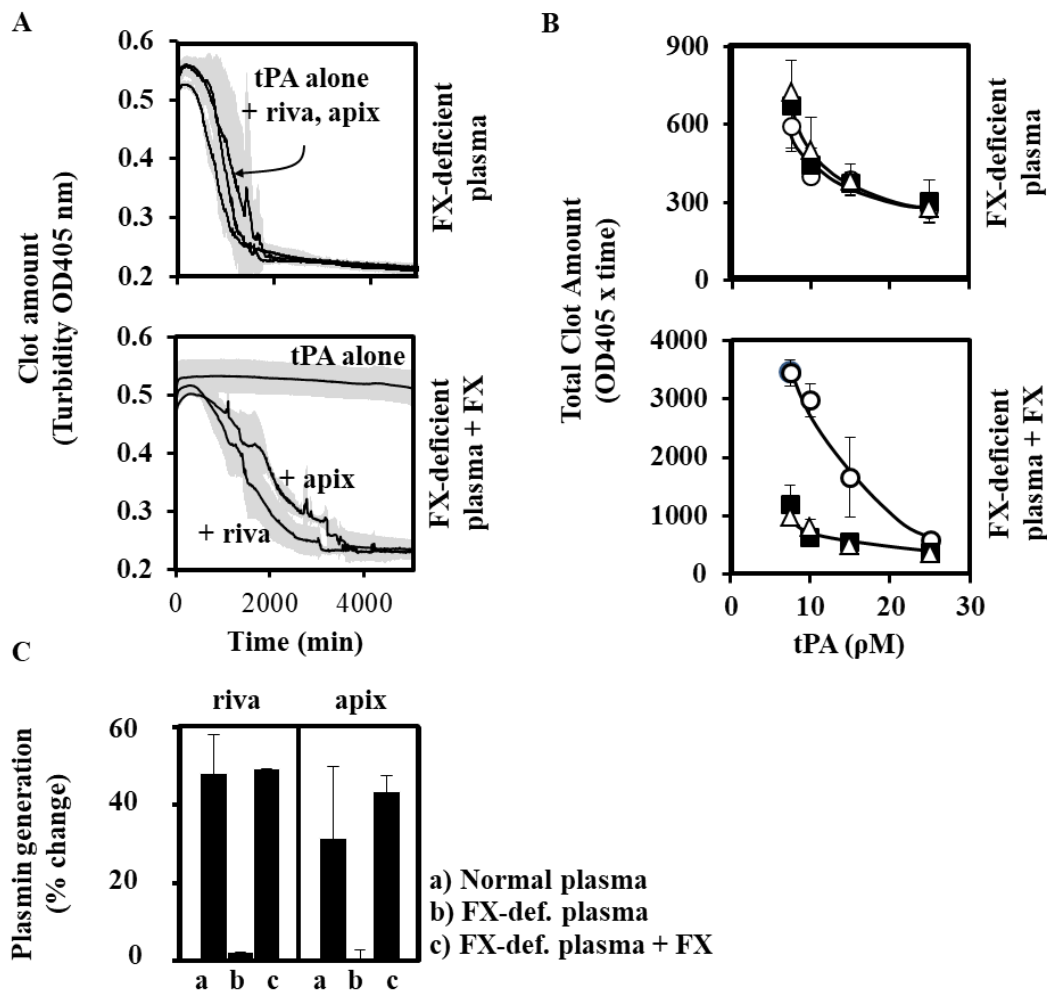
### **3.2.2 Importance of FX and FXa in FXa-DOAC-mediated enhancement of fibrinolysis**

To confirm the involvement of FX in the enhancement of fibrinolysis by rivaroxaban and apixaban, plasma clot lysis was performed using FXDP either with or without the physiological concentration of purified FX. Fig. 15A clearly depicts that fibrinolysis is only enhanced in the



**Figure 14: Plasmin generation in the presence of FXa-DOACs correlates to altered FXa fragmentation**

Panel A: Thrombin (10 nM)-induced clots were formed in normal pooled plasma using 30  $\mu$ M tPA. The extent of clot formation and dissolution were followed by turbidity in clots containing rivaroxaban (0.57  $\mu$ M), apixaban (0.44  $\mu$ M), or buffer (DMSO in HBPS noted as tPA alone). Solid line: n = 3 averaged data  $\pm$  standard error of the mean indicated by grey shade. Panel B: Under the same conditions as Panel A, FXa-DOACs were titrated. Each point represents the mean of total clot amount obtained by area under the curve measurements for three separate experiments  $\pm$  standard error of the mean (arbitrary fit of data).



**Figure 15: FX is required for FXa-DOACs to enhance plasma lysis.**

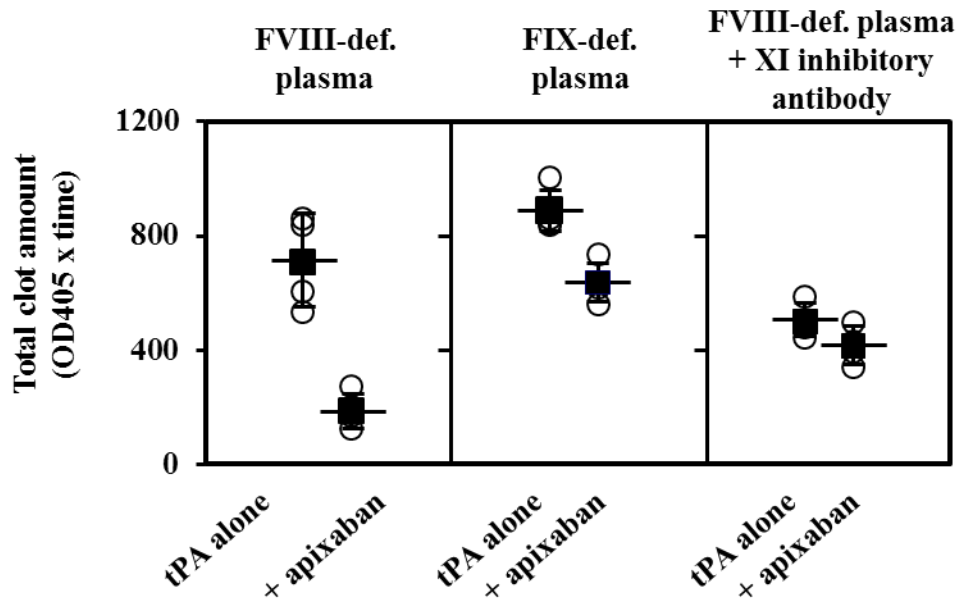
Panel A: Thrombin (10 nM)-induced clots were formed in FX-deficient plasma  $\pm$  purified FX (170 nM) supplemented with 10  $\mu$ M tPA. The extent of clot formation and dissolution was followed by turbidity as in Fig. 14. Graphs are a representation of the first 5000 minutes. Panel B: Area under the curve was derived from plasma fibrinolysis profiles obtained in the absence ( $\circ$ ), or presence of rivaroxaban ( $\blacksquare$ ) or apixaban ( $\triangle$ ) in FX-deficient plasma  $\pm$  FX. Panel C: Plasmin generation was monitored under conditions identical to the plasma turbidimetric assay using a plasmin fluorogenic substrate in normal plasma (NP), FX-deficient plasma (FXDP) and FX-deficient plasma supplemented with 170nM FX (FXDP + FX). Bar graphs show the relative percent change in plasmin generation at  $\sim$  1600 minutes in the presence of rivaroxaban or apixaban.

presence of FXa-DOACs when FX is also present. Notably, premature fibrinolysis has been previously reported when using FXDP [223]. To determine if the lack of enhancement by the FXa-DOACs in FXDP was primarily due to premature lysis, tPA was titrated. The area under the fibrinolysis profiles was quantified (Fig. 15B) and showed that at concentrations below 25  $\mu$ M tPA, rivaroxaban and apixaban enhanced fibrinolysis of FXDP, but only when FX was present at a physiological concentration. To further understand the mechanism, a specific fluorogenic substrate was used to measure the amount of plasmin generated during the course of fibrinolysis in FXDP with and without physiological FX, and normal plasma. The percentage difference in plasmin generated under each condition was derived for comparison. Results show that plasmin generation in the presence of FXa-DOACs was only enhanced in plasmas containing FX, thus accounting for the enhanced fibrinolysis in those plasma samples(Fig. 15C).

In the intrinsic pathway of coagulation, FX is activated in the presence of anionic phospholipid and calcium by FVIIIa and FXIa-activated FIX. The need for FX activation and not just inactive FX was therefore investigated in plasma clot lysis using congenital FVIII-deficient plasma  $\pm$  a FXI inhibitory antibody and congenital FIX-deficient plasma. As shown in Fig. 16, apixaban enhanced plasma clot lysis in FVIII-deficient plasma, but did so minimally in FIX-deficient plasma. When an inhibitory FXI-antibody was added to FVIII-deficient plasma to block FX activation through the intrinsic pathway, enhancement of plasma clot lysis was even further reduced. This suggests that activation of FX is needed for FXa-DOAC enhancement.

### **3.2.3 Impact of thrombin in FXa-DOAC-mediated enhancement of fibrinolysis**

Most anticoagulants enhance the rate of fibrinolysis through mechanisms involving reduced activation of TAFI because of restricted thrombin production [203,204]. Previous work



**Figure 16: FX activation is needed for apixaban to enhance plasma clot lysis**

Thrombin (10 nM)-induced plasma fibrinolysis assays were carried out in FVIII-deficient plasma  $\pm$  a XI inhibitory antibody (160 nM), and FIX-deficient plasma. Each point (o) represents the area under the curve (absorbance X time) of fibrinolysis assays. These were conducted in quadruplicate and the mean (■) is shown with error bars representing technical error of the mean.

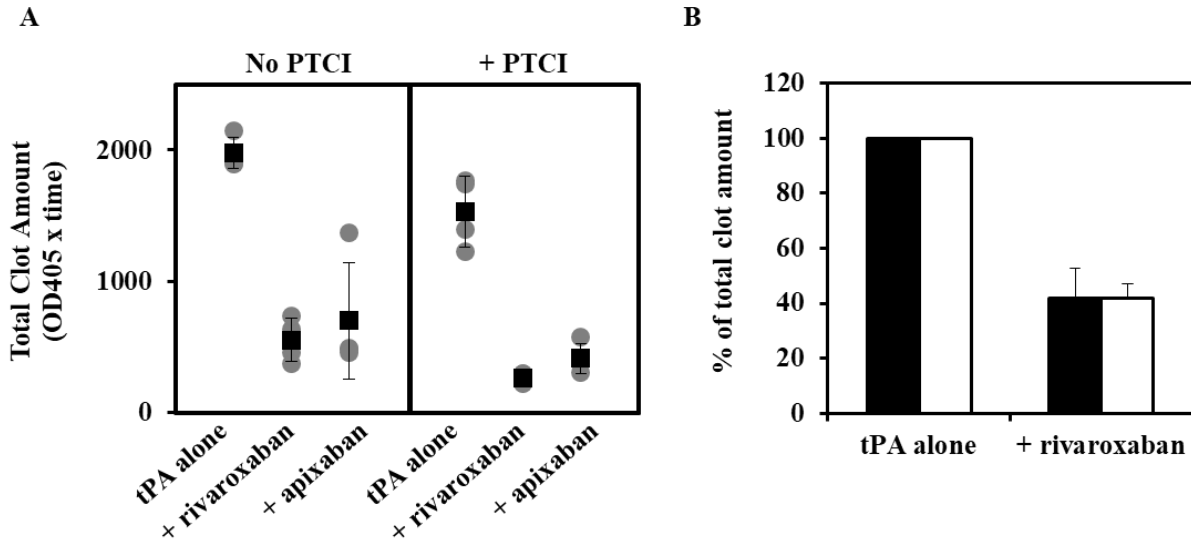
has demonstrated that 2  $\mu$ M PTCI completely inhibits the activity of TAFIa during thrombin-induced clot formation in normal plasma [224]. In the current study, rivaroxaban and apixaban enhanced fibrinolysis irrespective of the presence of 2  $\mu$ M PTCI (Fig. 17).

To further exclude thrombin-mediated effects as a contributing factor to the observation that FXa-directed DOACs enhance fibrinolysis, a western blot was conducted to examine fibrin generation and crosslinking as potential thrombin-mediated variables. The addition of rivaroxaban or apixaban to either normal pooled plasma or FXDP made no difference to the conversion of fibrinogen to fibrin and its intermolecular crosslinking (Fig. 18). This included the extent of covalent  $\gamma$ - $\gamma$  bond formation due to FXIIIa activity.

Complementing the previous results, SEM imaging of clots was conducted to rule out additional thrombin-related variables. Clots were formed in normal plasma as in Fig. 14 except without tPA to study clot ultrastructure without dissolution. Representative SEM images shown in Fig. 19A further confirm a lack of significant qualitative differences in fibrin structure that account for the effects of rivaroxaban and apixaban on enhanced fibrinolysis. Using a grid, the diameter (Fig. 19B) and porosity (Fig. 19C) of fibrin fibers was quantified. No significant ultrastructural differences were revealed under the conditions used here to trigger fibrin polymerization.

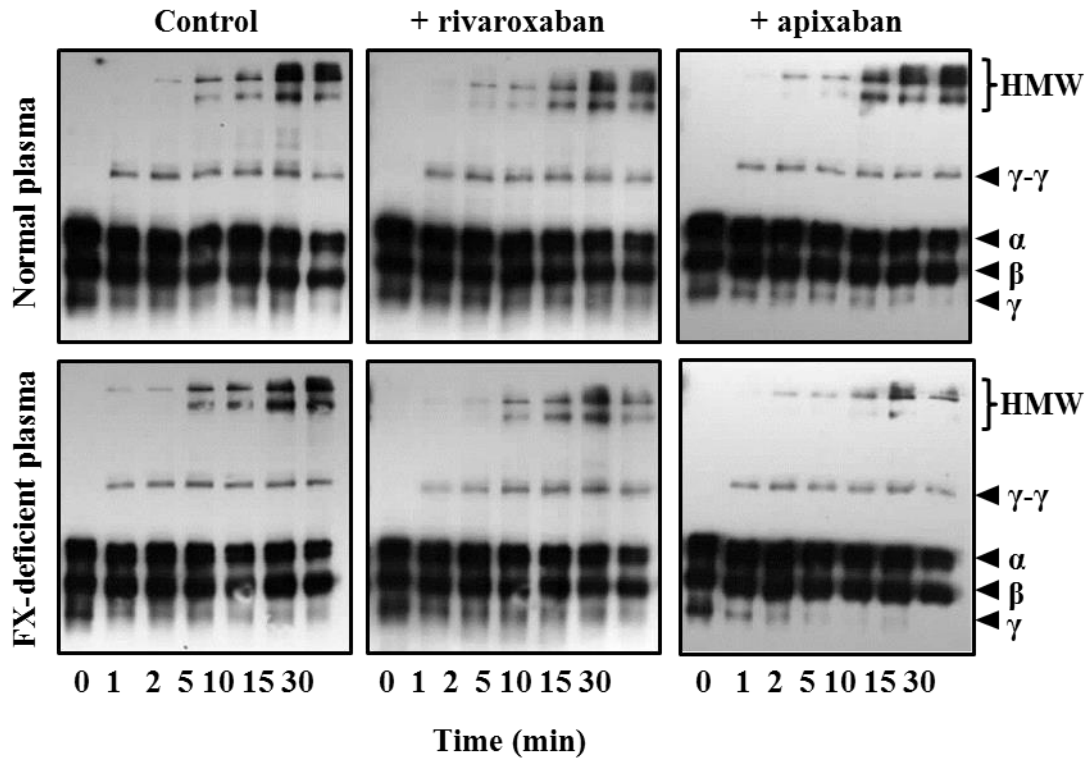
#### **3.2.4 Rivaroxaban and apixaban promote the accumulation of FXa $\beta$ by preventing the generation of Xa<sub>33/13</sub>**

To investigate the relationship between FXa fragmentation in plasma and the expression of fibrinolytic activity, FX-specific western blots were conducted at various stages of plasma clot dissolution (Fig. 20A). Using Innovin<sup>®</sup> as a source of TF to activate FX and initiate upstream



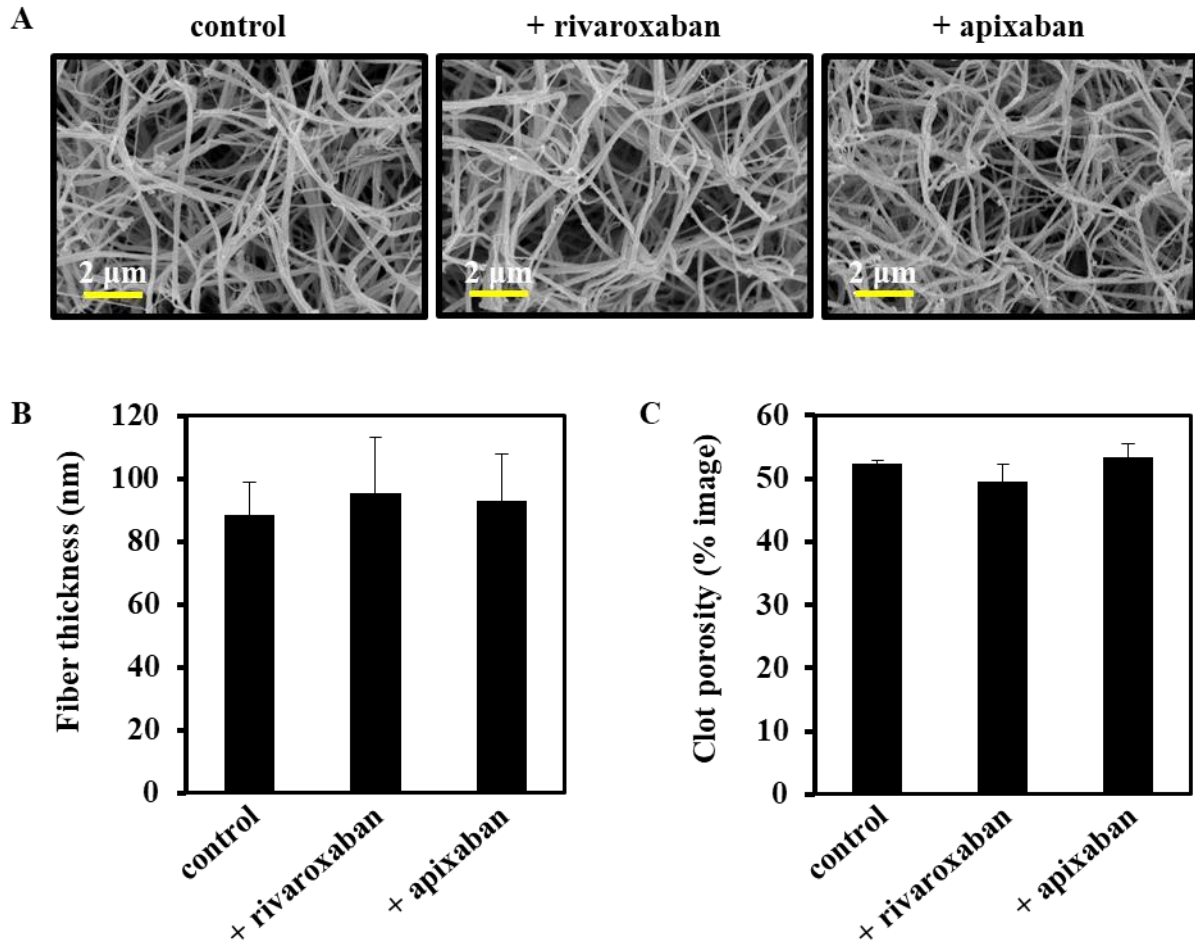
**Figure 17: Inhibition of TAFIa does not affect enhanced fibrinolysis by FXa-DOACS.**

Panel A: Thrombin (10 nM)-induced plasma fibrinolysis assays as in Fig. 14 were carried out in the absence or presence of PTCl (2  $\mu$ M). Each point ( $\bullet$ , gray) represents the area under the curve (absorbance X time) of fibrinolysis assays. These were conducted in quadruplicate and the mean ( $\blacksquare$ ) is shown with error bars representing technical error of the mean. Panel B: Separate experiment carried out to further demonstrate that enhanced clot lysis by rivaroxaban does not differ in the absence (black bar) or presence (white bar) of 2  $\mu$ M PTCl. AUC of each independent “tPA alone” and “+ rivaroxaban” clot lysis curve was quantified. A value of 100 % was allocated to the AUC of tPA alone at 0  $\mu$ M PTCl and 2  $\mu$ M PTCl. AUC of “+ rivaroxaban” group was subsequently computed relative to that of tPA alone. Shown is the average and standard deviation of the 3 independent experiments done in quadruplicates.



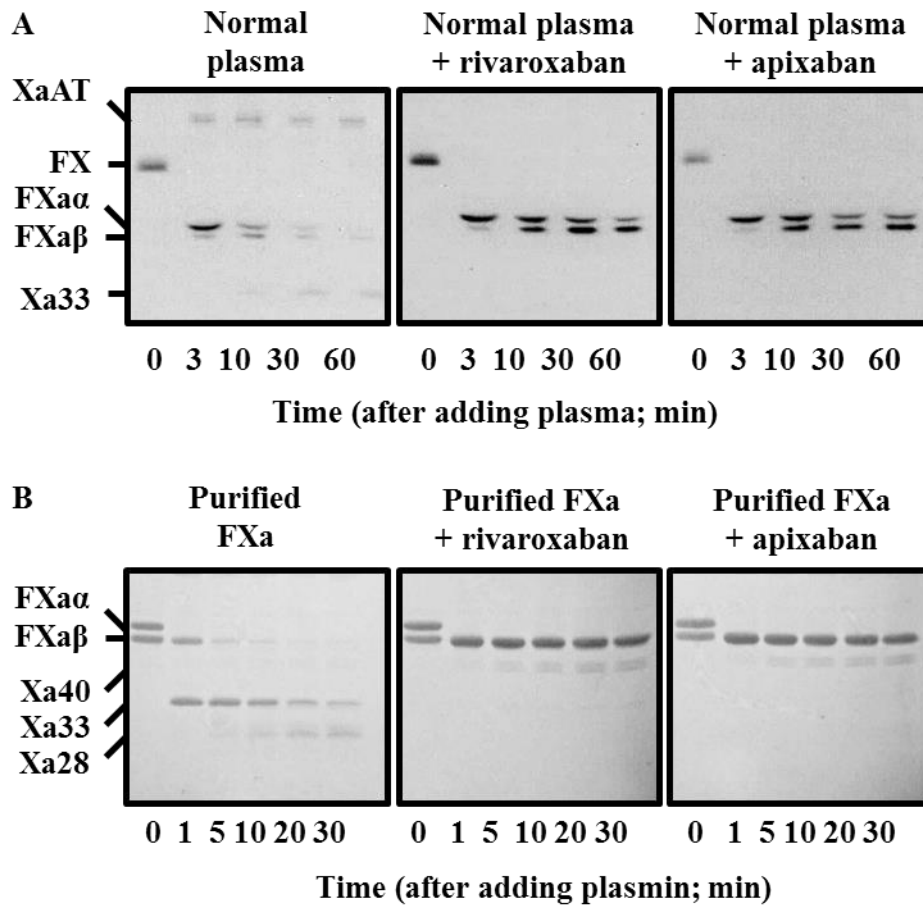
**Figure 18: Fibrin crosslinking is not affected by FXa-DOACs.**

Clots were formed using the same final concentrations as Fig. 14 with the exception that tPA was omitted. In the presence or absence of FXa-DOACs, fibrin clot was formed within 1 minute. A band appeared having an apparent molecular weight consistent with crosslinked  $\gamma$ -chain dimer ( $\gamma$ - $\gamma$ ).



**Figure 19: Fibrin fiber diameter and porosity are not altered by FXa-DOACs.**

Clots were formed as in Fig. 14 and then imaged by scanning electron microscopy at three random locations within each clot. Panel A: Platinum/palladium SEM images from one location. Panel B: Average diameter of non-superimposed fibers measured with ImageJ software. Standard deviation was calculated from the average of 30 measurements (10 fibers measured per location). Panel C: Porosity was measured by converting micrographs into a binary image and determining the percentage of black pixels using ImageJ. Standard deviation is shown for 3 measurements.



**Figure 20: FXa-DOACs prevent the generation of Xa33/13.**

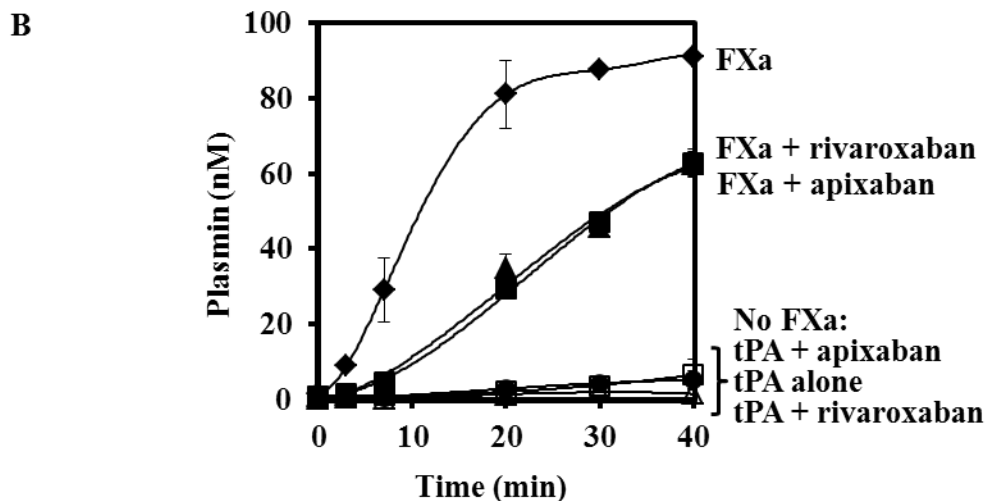
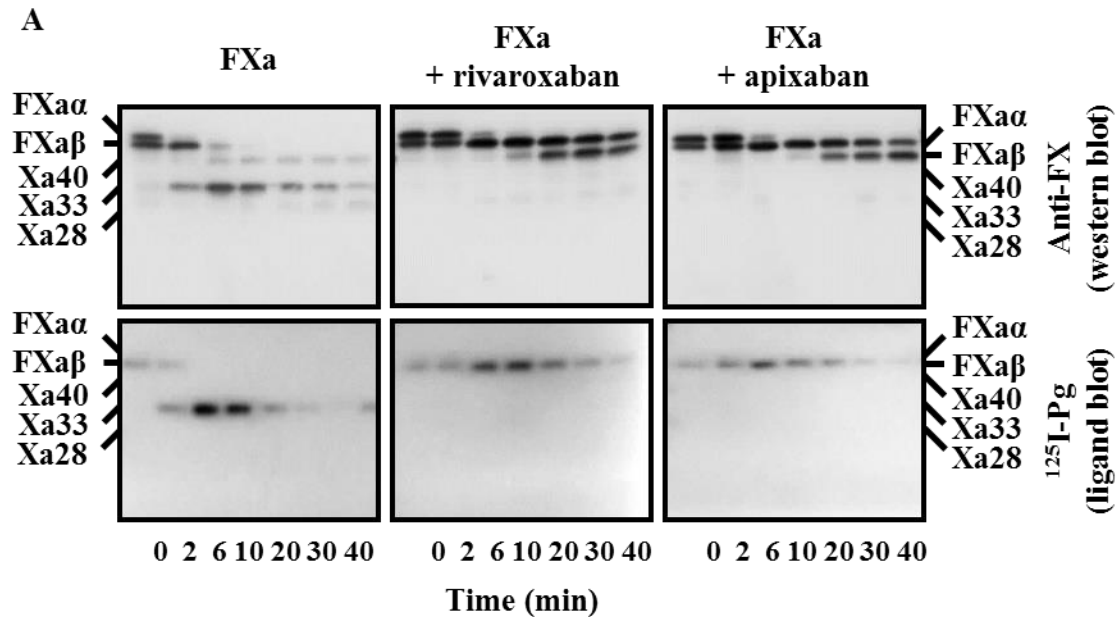
Panel A: The FXa cleavage profile was evaluated during fibrinolysis by western blot in normal plasma with and without rivaroxaban (0.57  $\mu$ M) or apixaban (0.44  $\mu$ M). Innovin® was used to activate FX and initiate clot formation in plasma supplemented with therapeutic levels of tPA. At the indicated times, denaturing Laemmli sample buffer was added to aliquots for western blot analysis using an anti-human FX heavy chain antibody. Panel B: Purified FXa, anionic phospholipid (300  $\mu$ M) and calcium (5 mM) were incubated with or without FXa-DOACs. The reaction was started with the addition of plasmin. During the course of the experiment samples were withdrawn, subjected to analysis as in Panel A.

clotting, rivaroxaban and apixaban prevented the generation of Xa33/13, allowing FXa $\beta$  to persist. The same fragmentation profile was observed when purified FXa was treated with plasmin in the presence of FXa-DOACs (Fig. 20B). Thus, rivaroxaban and apixaban block the proteolytic transition of FXa $\beta$  to Xa33/13.

### **3.2.5 Plasmin generation in the presence of FXa-DOACs is correlated to accumulation of FXa $\beta$**

To unambiguously correlate enhanced plasmin generation to the effects of FXa-DOACs on FXa fragmentation, purified tPA and plasminogen were mixed with combinations of FXa, rivaroxaban or apixaban. Identical samples were taken for fragmentation and plasmin generation analyses. Western blots (Fig. 21A) confirmed that Xa33/13 generation with purified proteins is prevented in the presence of FXa-DOACs, as in plasma (Fig. 20A). Binding of  $^{125}\text{I}$ -plasminogen to the various FXa fragments confirmed a strong interaction with Xa33/13. In the presence of FXa-DOACs; however,  $^{125}\text{I}$ -plasminogen was predominantly associated with FXa $\beta$ . Further cleavage of FXa $\beta$  into Xa33/13 is known to enhance tPA activity more effectively than CMK-modified FXa $\beta$  in a purified system, but not in plasma where it is rapidly degraded. Since Xa33/13 was not generated, the production of plasmin-dependent chromogenic activity in the presence of FXa-DOACs was consequently reduced (Fig. 21B). Consistent with plasma experiments unless FXa was present, neither rivaroxaban nor apixaban increased tPA-mediated plasmin generation. In both the presence and absence of FXa-DOACs, appearance of the most intense  $^{125}\text{I}$ -plasminogen-binding species correlated to the acquisition of chromogenic activity (~ 6 minutes).

In plasma experiments, FXa-DOACs are present during FX activation. While previous work has shown that FX on its own is able to enhance plasmin generation [225], rivaroxaban is



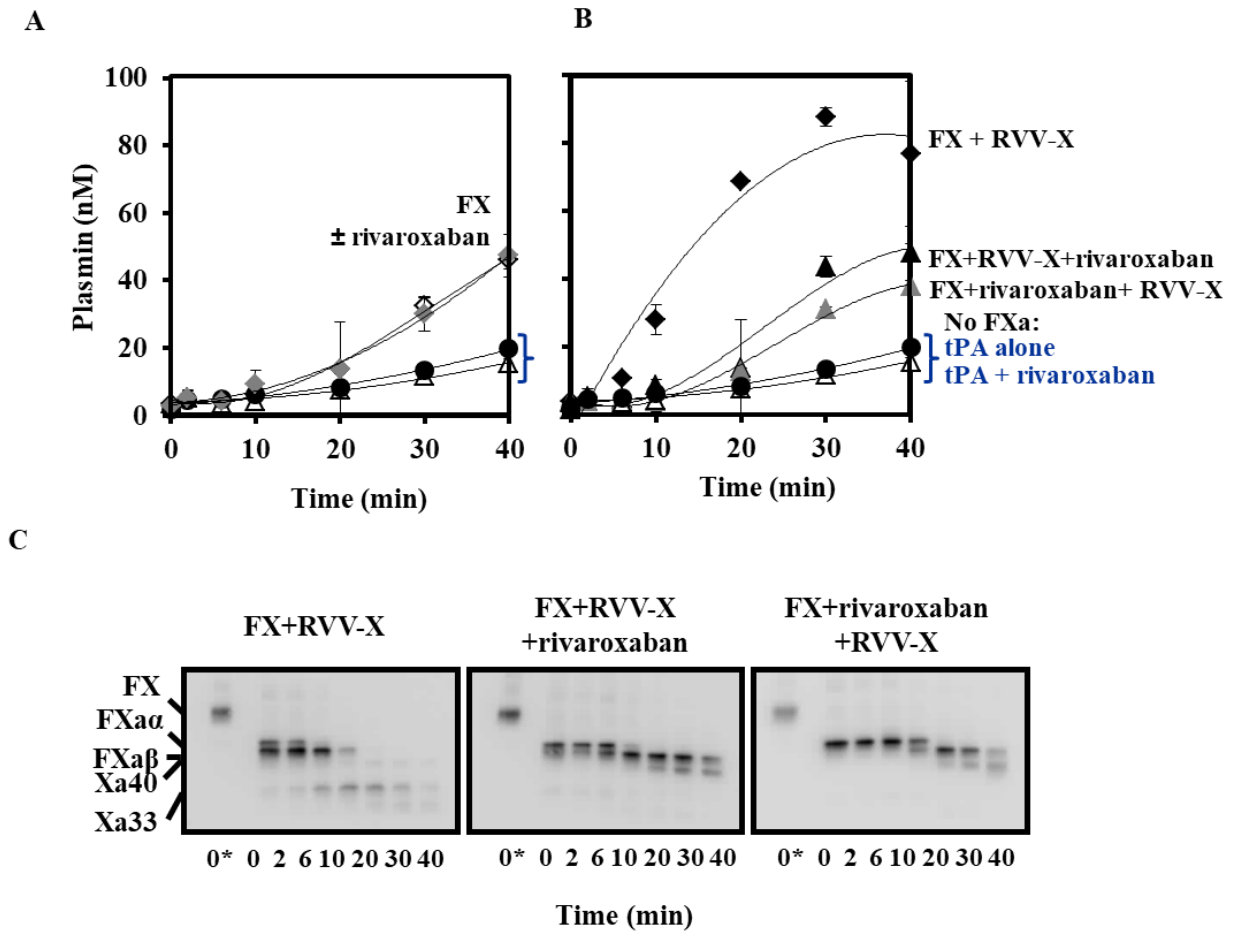
**Figure 21: Plasmin generation in the presence of FXa-DOACs correlates to altered FXa fragmentation.**

Activation of Pg (0.5  $\mu$ M) by tPA (10 nM) was initiated in the presence of constant aPL (50  $\mu$ M) and calcium (2 mM), with or without FXa (0.1  $\mu$ M), rivaroxaban or apixaban. Panel A: Non-reduced anti-human FX western blot and <sup>125</sup>I-labelled plasminogen ligand blots. Markers were positioned using simultaneously run purified FXa fragments and molecular weight markers (not shown). Panel B: Plasmin production was monitored by S2251 chromogenic substrate hydrolysis in a similar experiment as Panel A: FXa (◆); FXa + rivaroxaban (▲); FXa + apixaban (■); rivaroxaban (Δ); apixaban (□); or tPA alone (●).

known to be specific for FXa and not FX. Fig 22A confirms that rivaroxaban has no effect of the tPA cofactor function of FX and thus supports the need for FX activation in the enhancement of fibrinolysis by FXa-DOACs. To determine if plasmin generation differs when FXa-DOACs are added prior to (similar to plasma) or after FX activation (similar to using purified FXa), tPA-mediated plasmin generation was evaluated using the enzyme RVV-X to activate FX. Fig. 22B shows that similar to purified FXa (Fig. 21B), RVV-X-activated FX enhances plasmin generation in the presence of FXa-DOACs. Compared to FX+RVV-X+rivaroxaban, a slight decrease in plasmin generation was noted when rivaroxaban was present during FX activation (FX + rivaroxaban + RVV-X). According to western blot analysis (Fig. 22C), this reduction in plasmin generation is due to a delay in FXa $\beta$  formation, further highlighting the importance of FXa $\alpha/\beta$  cleavage into FXa $\beta$  for the acquisition of tPA cofactor function.

### **3.3 Discussion**

The availability of DOACs has revolutionized antithrombotic therapy over the past decade. While this class of drugs has been among the most extensively tested in history, evidence is presented in the current study that reveals an unforeseen effect of the FXa-directed DOACs in fibrinolysis due to the profibrinolytic function of FXa. Similar to the finding that covalent modification of the FXa active site conferred plasma fibrinolytic activity [52,162,180], the reversible small molecule FXa active site inhibitors, rivaroxaban and apixaban, also impart this novel FXa function in plasma. Complementary purified protein and plasma studies demonstrated that these FXa-DOACs enhanced fibrinolysis and stabilized FXa $\beta$  (Fig. 20), a FXa proteolytic derivative that has tPA accelerating activity. Thus, sequential production of Xa<sub>33/13</sub>



**Figure 22: Plasmin generation by FXa+rivaroxaban is modestly reduced when FX is activated in the presence of rivaroxaban.**

Panel A: Activation of Pg (0.5  $\mu$ M) by tPA (10 nM) was initiated in the presence of constant aPL (50  $\mu$ M) and calcium (2 mM), with or without FX (0.1  $\mu$ M), rivaroxaban (0.57  $\mu$ M), FX + rivaroxaban. Plasmin production was monitored by S2251 chromogenic substrate hydrolysis: FX ( $\diamond$ ); FX+rivaroxaban ( $\blacklozenge$ , gray); rivaroxaban ( $\Delta$ ); or tPA alone ( $\bullet$ ). Panel B: Same as panel A with the exception that Russel's viper venom (RVV-X) was used to activate FX. Rivaroxaban was either added after or prior to RVV-X activation of FX (i.e. FX+RVV-X+rivaroxaban or FX+rivaroxaban+RVV-X respectively). Plasmin production was monitored by S2251 chromogenic substrate hydrolysis: FX+ RVV-X ( $\blacklozenge$ ); FX + RVV-X+rivaroxaban ( $\blacktriangle$ ); FX+rivaroxaban+RVV-X ( $\blacktriangle$ , gray); rivaroxaban ( $\Delta$ ); or tPA alone ( $\bullet$ ). Panel C: Non-reduced anti-human FX western blot and  $^{125}$ I-labelled plasminogen ligand blots. Markers were positioned using simultaneously run purified FXa fragments and molecular weight markers (not shown).

which would otherwise enable a lost in fibrinolytic activity in plasma was prevented. Results in this study indicate that both rivaroxaban and apixaban enhance plasma fibrinolysis in a dose- and FX-dependent manner without significantly affecting other parameters known to be feedback modulated by thrombin production.

The effects of anticoagulants on clot dissolution have been previously investigated [203,204,206,226] with the conclusion that fibrinolysis was enhanced by reducing thrombin generation and its consequent feedback pathways. By inducing coagulation with sufficient purified thrombin to overcome its anticoagulant effect, this variable was eliminated to investigate the involvement of up-stream FX. Furthermore, the tPA concentration used in this study was within physiological range compared to typically higher therapeutic levels [203]. These conditions minimized the known cofactor-independent enzymatic activity of tPA [162] and permitted an evaluation of FX in fibrinolysis.

The involvement of FX in rivaroxaban- and apixaban-enhanced fibrinolysis was established by using FX-deficient plasma, where fibrinolysis was not affected by FXa-DOACs unless purified FX was added. This observation was masked at tPA concentrations above ~ 20  $\mu$ M, suggesting that intrinsic tPA-mediated plasmin generation overshadowed the effect of FXa-DOACs. In studies, the latter is an observation typically implying much higher tPA levels (e.g. ~50-fold [203]). The basis for FXa-DOAC-facilitated enhancement of plasma fibrinolysis by FX was attributed to an increase in plasmin generation by rivaroxaban and apixaban only when a source of FX was available. Both rivaroxaban and apixaban are known to have high specificity for FXa and no known effect on FX [187,192]. The use of congenital deficient plasma samples and inhibitory antibodies to disrupt the intrinsic pathway, confirmed that FX activation and not just FX is needed for enhanced plasma clot lysis.

To exclude potential differences in thrombin generation due to the presence or absence of FXa-DOACs, TAFIa function and clot stabilization were analyzed. Anticoagulants have been previously shown to impair TAFI activation by reducing new thrombin production. This hastened clot lysis [203,204,206]. Under the conditions employed here, a contribution of TAFI activation was eliminated by demonstrating that even in the presence of the potent TAFIa inhibitor PTCL, FXa-DOACs enhanced fibrinolysis [206,224]. Additionally, FXa-DOACs had no effect on the extent of crosslinking between the fibrin subunits or the extent of fibrin  $\gamma$ - $\gamma$  dimers that formed during fibrinolysis. The lack of difference in fibrin crosslinking is consistent with a study showing that FXIII activation was not a contributor to the enhanced degradation of tissue-factor induced clots in the presence of rivaroxaban [203]. This is because only a small amount of thrombin ( $< 2$  nM) is needed to initiate the crosslinking of fibrin monomers [203,227]. In this study, it made no difference whether clots were produced in normal plasma or FX-deficient plasma (with or without FXa-DOAC). This further suggests that differences in initial thrombin generation are not the basis for the enhancement by FXa-DOACs. These results are further supported by studies on fibrin clot structure, which showed similar fiber diameter and relative porosity with or without rivaroxaban or apixaban. The latter observation differs from other studies that reported architectural changes in the clot due to FXa-specific DOACs [203,226], because in the current study ample thrombin was used to over-ride these confounding variables.

Plasmin cleavage of active site blocked FXa using purified proteins and plasma, showed persistence of FXa $\beta$ . These results are consistent with those previously reported when a CMK was used to irreversibly modify the FXa active site [52]. Chromogenic assays revealed no difference in enhanced plasmin generation by FX in the presence or absence of rivaroxaban or

apixaban. However, tPA-mediated plasmin generation by rivaroxaban and apixaban was enhanced in the presence of added FXa or RVV-X activated FX. In the absence of FXa, neither rivaroxaban nor apixaban enhanced plasmin generation. These results further highlight the need for activated FX in the enhancement of plasma clot lysis. Note that while FXa enhances plasmin generation to a greater extent than FXa complexed with FXa-DOACs when purified proteins are used, the primary contributor of this enhancement is further cleavage into Xa<sub>33/13</sub>, a species that is rapidly turned off in plasma [52]. Thus, FXa $\beta$  is the primary contributor of plasmin generation in the presence of FXa-DOACs in plasma.

Currently, thrombi are predominantly treated with the combination of a recombinant form of tPA followed by an anticoagulant to prevent re-occlusion [157]. The use of tPA, however, is associated with possible life-threatening hemorrhage (~ 3 - 6 %), potentially due to systemic plasmin generation caused by the high dose it is administered for efficacy [157,228]. Identifying a role for rivaroxaban and apixaban in fibrinolysis at low tPA concentration may suggest new clinical applications such as in the case of PTS. Quality of anticoagulation therapy in the early stages of DVT treatment is an important determinant in the risk of developing PTS [229], with subtherapeutic warfarin anticoagulation increasing a patients' likelihood for this chronic disorder [230]. DOACs have predictable pharmacokinetics and pharmacodynamics, and thus do not have the same challenges warfarin has in achieving and maintaining therapeutic levels [231]. Studies comparing traditional anticoagulants to FXa-DOACs demonstrated that the latter is non-inferior in preventing recurrent DVT, which is a risk factor for PTS [232,233]. While additional studies are still needed to determine the effects of FXa-DOACs versus heparin/warfarin in preventing PTS, a post-hoc analysis indicated a trend towards better clot resolution and thus a reduced risk in PTS patients treated with rivaroxaban [234]. A potential reason for the latter may be the dual

anticoagulant and fibrinolytic role of rivaroxaban, which would indicate an added benefit for the utilization of FXa-specific DOACs.

## **Chapter 4: EFFECTS OF DIRECT ORAL ANTICOAGULANTS ON FIBRINOLYSIS IN PATIENTS**

### **4.1 Overview and specific goals**

Limited plasmin-mediated cleavage in the presence of anionic phospholipid and calcium converts FXa into a pro-fibrinolytic agent [162]. This function is further stabilized in plasma by chemically modifying the active site of FXa such that the first cleavage product (FXa $\beta$ ), but not the second (Xa<sub>33/13</sub>) is generated [52]. Using normal pooled plasma and purified proteins, the work presented in Chapter 3 of this dissertation showed that FXa-specific DOACs, rivaroxaban and apixaban, also alter the fragmentation of FXa such that the generation of Xa<sub>33/13</sub> is prevented and FXa $\beta$  persists. Consistently, turbidity assays demonstrated that plasma clot lysis is enhanced in the presence of FXa-specific DOACs even when coagulation variables are carefully controlled to emphasize the role of FXa. It is not yet known if similar observations exist in patients treated with these FXa-DOACs.

The FXa-specific DOAC rivaroxaban is approved for the treatment of DVT and the prevention of PE, venous thromboembolism post hip or knee replacement surgery and stroke in atrial fibrillation patients [235]. Incompletely cleared fibrinolysis and remnant scar tissue after deep vein thrombosis are causes of PTS. Interestingly, a trend towards a reduced risk of PTS has been reported in rivaroxaban-treated patients compared to enoxaparin/vitamin K antagonist-treated patients [234]. While clinical trials are still under way to confirm this trend, the persistence of FXa $\beta$  due to the presence of rivaroxaban and its role in fibrinolysis has not been previously considered as a contributor to better clot resolution and reduced clot reoccurrence in rivaroxaban-treated patients. Moreover, it has been reported in a case study that thrombus clearance in a dabigatran-treated patient was only achieved after the patient was switched to a

FXa-specific DOAC [236]. As a direct thrombin inhibitor, dabigatran is not expected to affect FXa fragmentation in a similar manner as FXa-specific DOACs. Therefore, this dissertation chapter is aimed at investigating the fragmentation of FXa and fibrinolysis in plasma samples from atrial fibrillation patients. This group of patients were chosen over DVT/PE and post hip or knee replacement surgery patients because, although all three groups of patients have abnormal hemostasis, thrombosis is the least provoked in atrial fibrillation patients. Namely, while it is now known that thrombosis in atrial fibrillation can be induced by stasis, endothelial damage or dysfunction, and hypercoagulability (as reviewed in [27]), soft-tissue trauma at site of surgery and potential bone marrow destruction promote the hypercoagulable state in post-surgery patients [237], reviewed in [238]. Thus, the risk of thrombosis without anticoagulation in atrial fibrillation is lower (1.9 - 18.2 % per year) [239] than that in patients post hip or knee replacement surgery (40 - 60 %) [217]. In DVT/PE, compared to atrial fibrillation, thrombosis is further stimulated by the cause for the initial thrombus (i.e. thrombophilia) or the presence of residual thrombus [240]. Using atrial fibrillation patients, it was therefore hypothesized that Xa33/13 generation will be prevented and plasma clot lysis enhanced in samples from patients treated with rivaroxaban. Conversely, patients treated with the thrombin-specific anticoagulant, dabigatran, or no anticoagulant, would not have the same effect. To test this hypothesis, the specific goals of the current dissertation chapter were to:

- 1) Compare FXa fragmentation in non-anticoagulated, rivaroxaban-treated, and dabigatran-treated atrial fibrillation patients
- 2) Investigate fibrinolysis in non-anticoagulated vs. rivaroxaban-treated atrial fibrillation patients

- 3) Explore potential trends or correlations between densitometry of FXa fragments and fibrinolysis parameters in treated vs. non-anticoagulated patients.

## **4.2 Results**

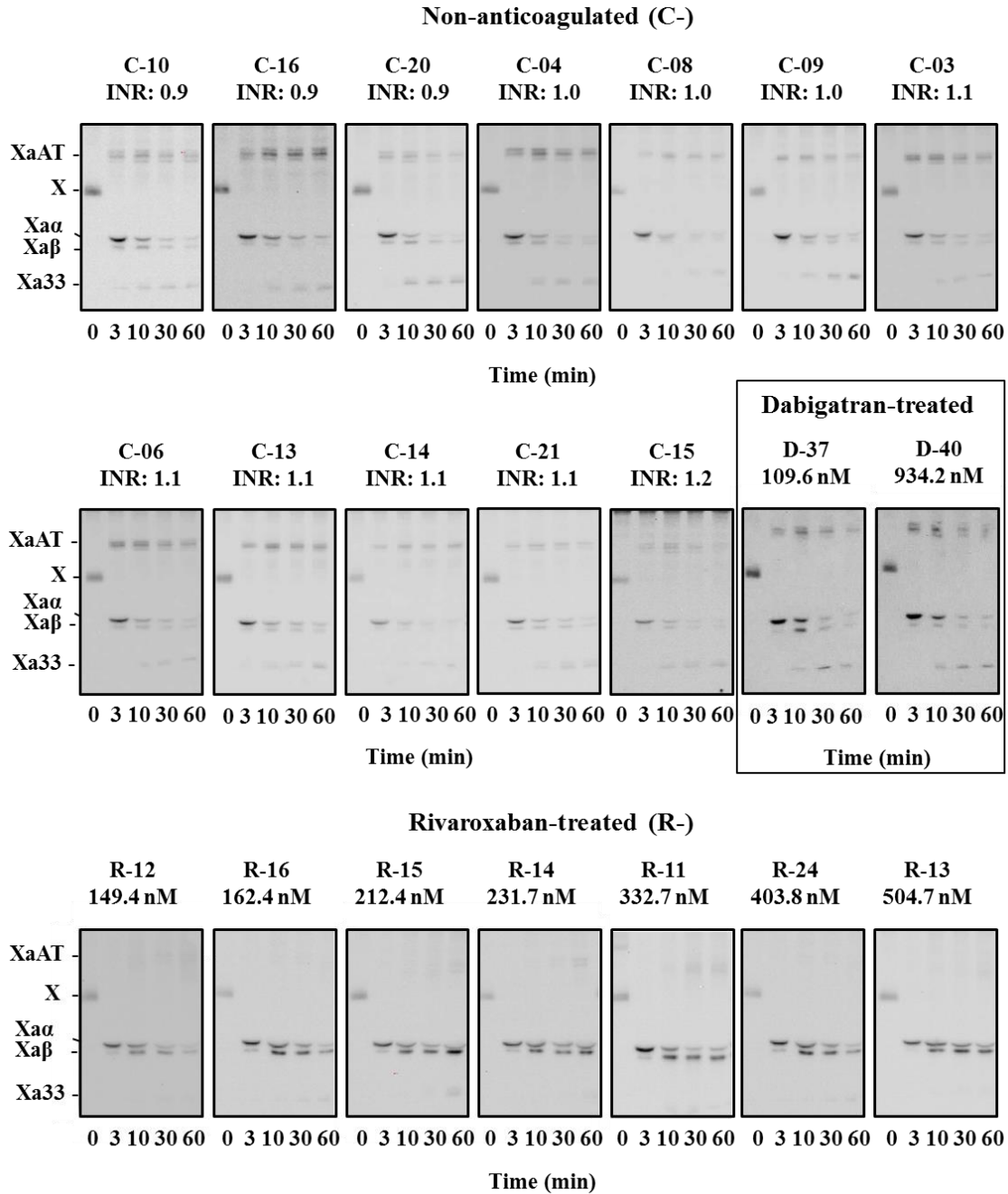
### **4.2.1 Assessment of FXa fragmentation**

To confirm that persistence of FXa $\beta$  is indeed DOAC-specific and to determine if this rivaroxaban-mediated pattern is seen in patients, the fragmentation of FXa was analyzed in atrial fibrillation patients. Using Innovin<sup>®</sup> as a source of TF, patient plasma FX was activated in the presence of anionic phospholipid and calcium. Fig. 23 clearly shows that FXa $\beta$  persisted, without conversion to Xa33/13, only in samples from rivaroxaban-treated patients. In samples from non-anticoagulated patients or those treated with dabigatran, FXa $\beta$  was further cleaved into Xa33/13.

### **4.2.2 Assessment of fibrinolytic properties in patients**

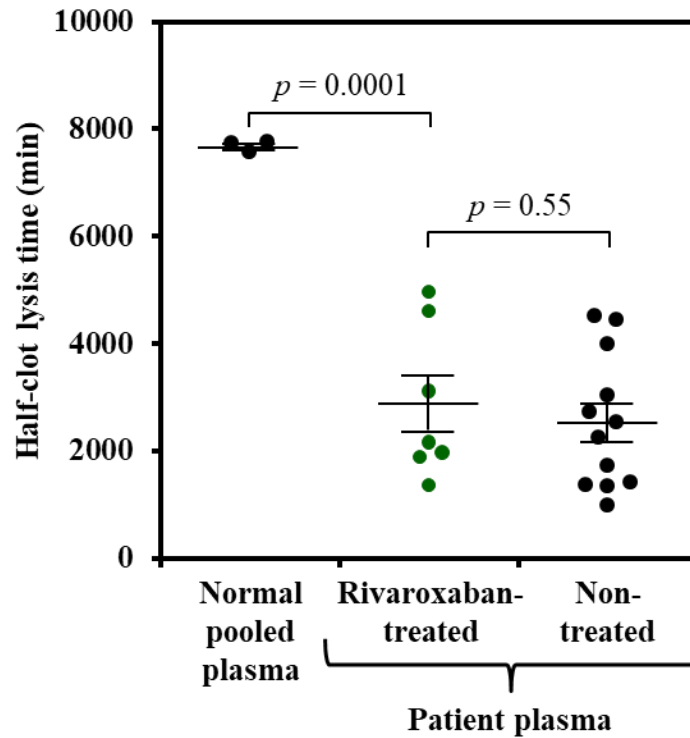
Since persistence of FXa $\beta$  is known to be associated with enhanced fibrinolysis, turbidimetric assays were carried out using patient plasma samples. In these experiments, the clot lysis time was reported as the time for max OD to be reduced by 50 percent (i.e. half-clot lysis time). In doing so, the inter-variable patient fibrinogen concentration and its consequent effect on max OD are avoided [219]. As demonstrated in Fig. 24, rivaroxaban-treated patients had a faster clot lysis time than commercial normal pooled plasma from healthy donors ( $p = 0.0001$ ). Interestingly; however, there was no difference between rivaroxaban-treated and non-anticoagulated atrial fibrillation patients ( $p = 0.55$ ).

D-dimer is specific to plasmin degradation of a crosslinked clot and is used as a surrogate for thrombosis, which triggers fibrinolysis. Since the patient plasma samples used were residual



**Figure 23: Rivaroxaban is the cause FXa $\beta$  persistence in patient plasma.**

FXa fragmentation was followed by western blot in Innovin<sup>®</sup>-treated clots from non-anticoagulated patients and those treated with rivaroxaban or dabigatran. Since enhancement of plasmin generation is correlated to the conversion of FXa $\alpha/\beta$  into FXa $\beta$ , the percent of FXa $\alpha$  and FXa $\beta$  relative to total FXa (FXa $\alpha/\beta$ ) was tabulated using densitometry for each time point. Values obtained for rivaroxaban-treated plasma samples at the 60 minute time point were used and depicted in correlation studies.



**Figure 24: Rivaroxaban-treated patient plasma clots lyse faster than clots in normal pooled plasma but not clots formed in non-anticoagulated patient plasma.**

Plasma clot lysis was carried out as in Fig. 14 with the exception that patient plasma samples were also used. Half-clot lysis times were derived for clots formed with normal pooled plasma from healthy individuals and those formed with plasma from atrial fibrillation patients treated with rivaroxaban or non-anticoagulated. Circles represent the mean of 3 individual experiments conducted in triplicate. In patient groups, each circle is a different patient. Error bar is standard error of the mean.

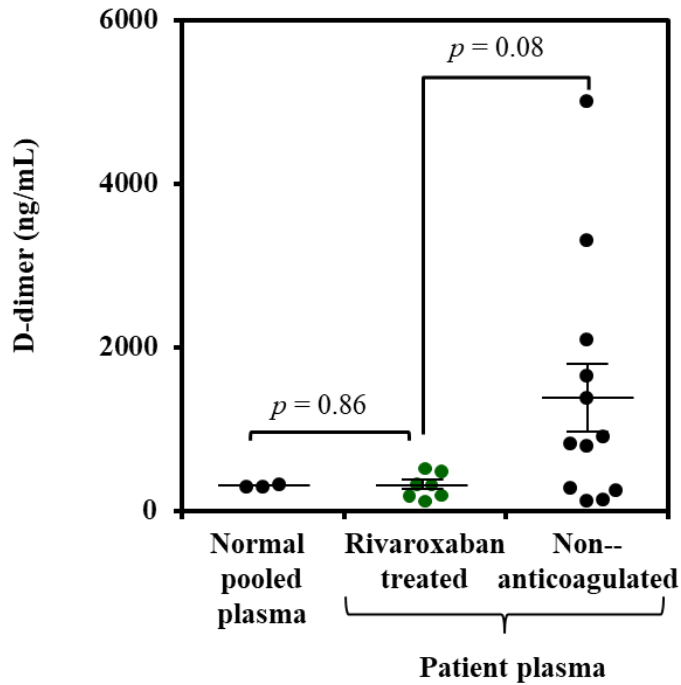
from other studies, the D-dimer level of each patient plasma sample was measured. According to the data presented in Fig. 25A, rivaroxaban-treated patients had similar D-dimer levels as commercial normal pooled plasma ( $p = 0.86$ ); however, non-anticoagulated patients had a higher D-dimer level on average ( $p = 0.08$ ). D-dimer levels did not, however, correlate with clot lysis times in either rivaroxaban-treated (Fig. 25B) or non-anticoagulated patient plasma sample (Fig. 25C).

### **4.2.3 Statistical analysis to determine trends in rivaroxaban-treated patient samples**

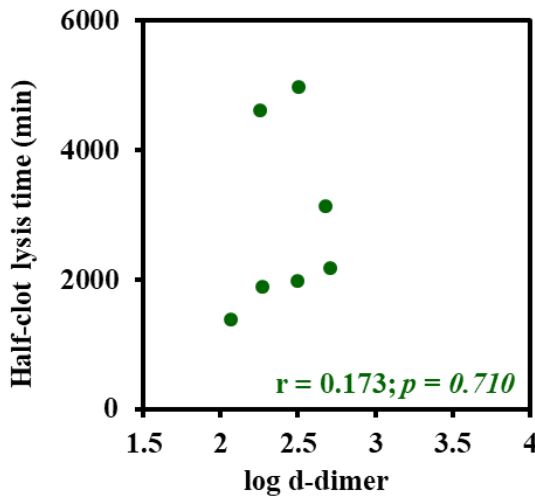
#### **4.2.3.1 Percent of FXa $\beta$ and FXa $\alpha$ are strongly correlated to clot lysis time at 60 minutes**

To determine if there was a direct relationship between the amount of FXa $\beta$  produced relative to total FXa $\alpha/\beta$  and half-clot lysis time, a correlation analysis between these two variables was conducted. Fig. 26A shows a strong negative correlation ( $r = -0.928$ ;  $p = 0.003$ ) between clot lysis time and percent of FXa $\beta$  at 60 minutes, the last point in the time course in which most of the FXa $\alpha$  had been converted into FXa $\beta$  for each patient. Graphical representation indicated that the higher the amount of FXa $\beta$  generated relative to total FXa $\alpha/\beta$  (i.e. percent FXa $\beta$ ), the faster the clot was degraded. Since only FXa $\alpha$  and FXa $\beta$  but no Xa $33/13$  were observed in these western blots, there was a direct inverse effect such that the higher the amount of FXa $\alpha$  relative to total FXa $\alpha/\beta$  (i.e. percent FXa $\alpha$ ), the slower the clot lysis (Fig. 26B). These results take into context the starting amount of FX as measured by western blot analysis. Since the correlation between half-clot lysis time and FXa $\beta$  was the strongest and most significant at 60 minutes, band intensity values were chosen at this time point for further analysis.

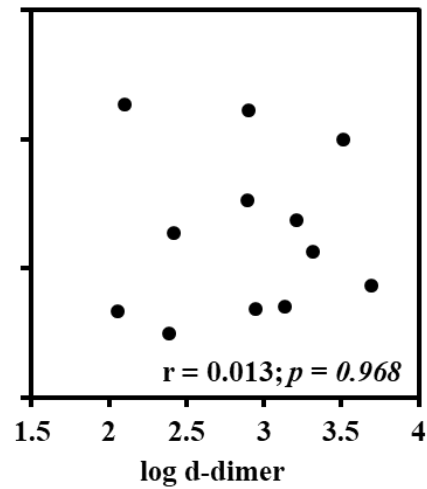
A



B

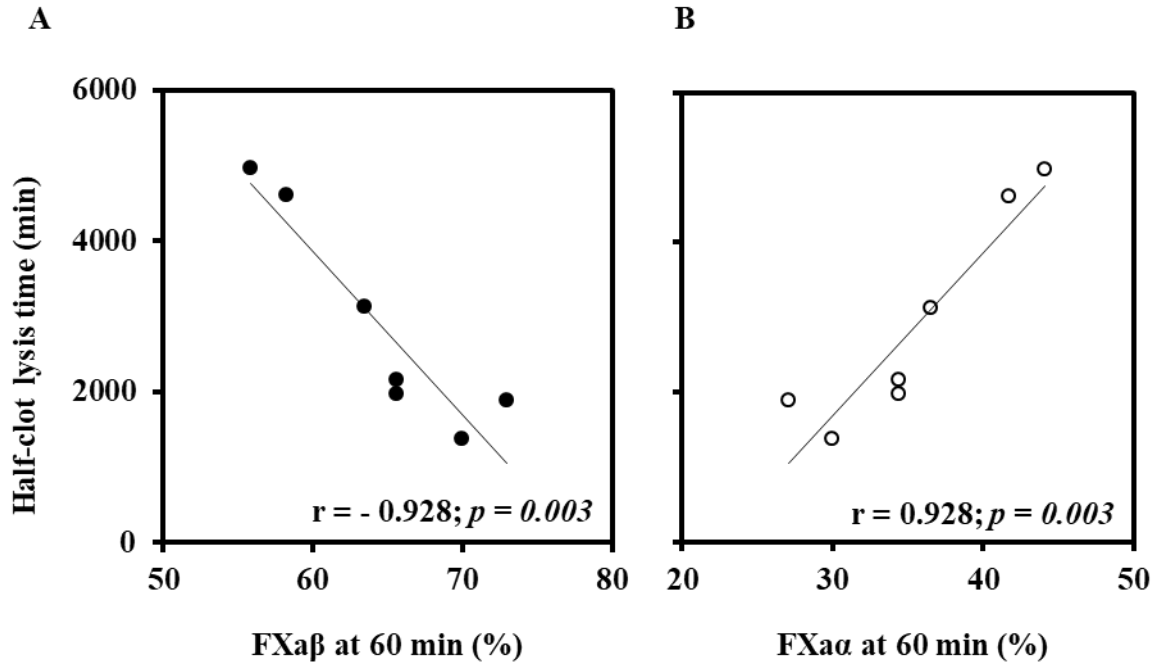


C



**Figure 25: Non-anticoagulated patient plasma has high D-dimer levels, but it is not correlated to half-clot lysis times.**

Panel A: The mean D-dimer levels for each patient (filled circles) are represented by a vertical bar  $\pm$  standard error of the mean for 3 independent experiments. Panel B: Correlation between D-dimer levels and half-clot lysis times in rivaroxaban-treated patients. Correlation and  $p$ -value are shown at the bottom right of the graph. Panel C: Similar to Panel B with the exception that plasma samples from non-anticoagulated patients were used.



**Figure 26: Higher percent FXaβ is strongly correlated to faster fibrinolysis times.**

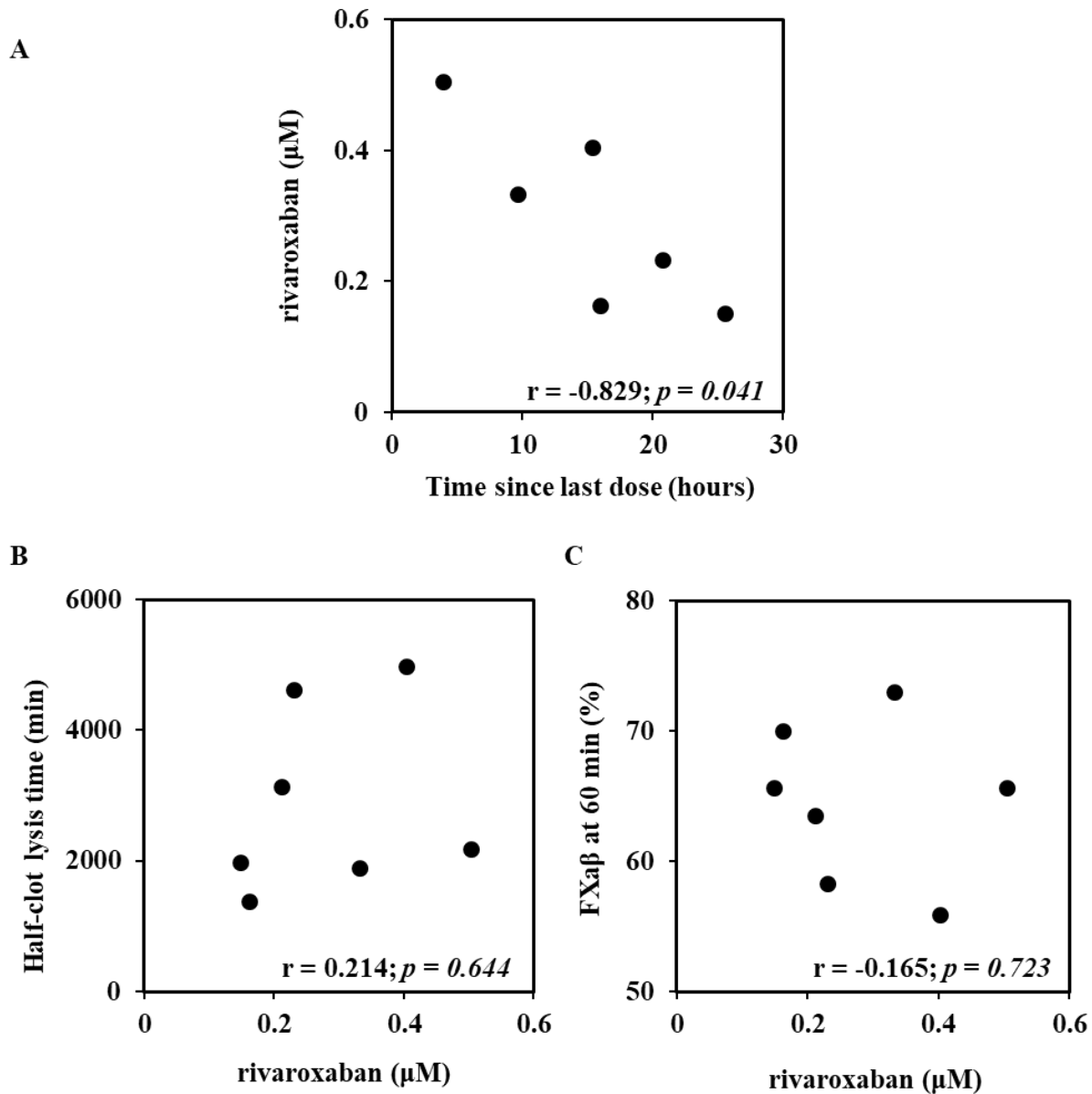
Panel A: Percent of FXaβ generated in patient plasma where clots were initiated with Innovin<sup>®</sup> was measured by densitometry of western blots and relativized to total FXa. Panel B: Similar to panel A, but percent of FXaα is plotted instead. Correlation and *p*-value are shown at the bottom right. Each point represents the mean of 3 independent experiments for rivaroxaban-treated patient sample. A best-fit linear regression is included for better visualization of the correlation between variables.

#### **4.2.3.2 Correlation between rivaroxaban concentration and time since last dose, half-clot lysis time, or percent FXa $\beta$**

As expected, time between patients' last dose and blood collection was strongly correlated with the rivaroxaban concentration measured within the plasma samples ( $r = -0.829$ ;  $p = 0.041$ ) (Fig. 27A). Since rivaroxaban dose-dependently enhanced the degradation of clots formed with commercial normal pooled plasma (Fig. 14), a correlation analysis between half-clot lysis time and rivaroxaban concentration in treated patient samples was conducted. Unlike in normal pooled plasma, Fig. 27B shows that the two variables were not significantly correlated ( $r = 0.214$ ;  $p = 0.644$ ). There was also no correlation ( $r = -0.165$ ;  $p = 0.723$ ) between rivaroxaban concentration and percent FXa $\beta$  under these experimental conditions (Fig. 27C).

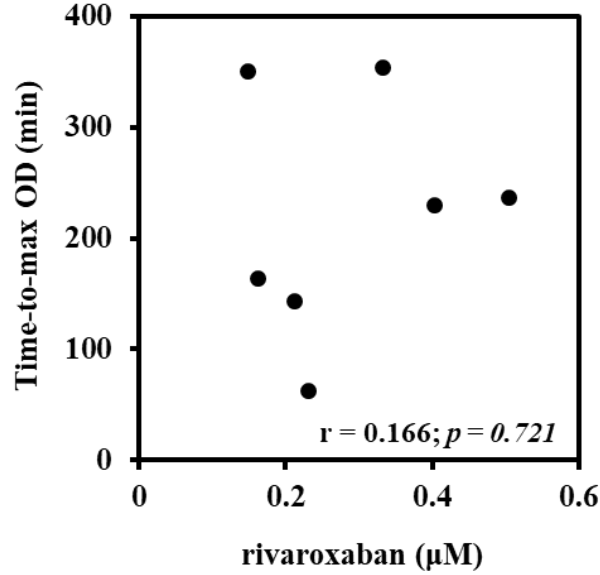
#### **4.2.3.3 Rivaroxaban concentration is not correlated to clotting time when clots are initiated with thrombin**

Rivaroxaban prolongs coagulation by inhibiting thrombin generation in tissue factor-induced clots. In this study, clots were initiated with 10 nM thrombin to bypass differences in thrombin generation in the presence versus absence of FXa-DOACs. To confirm that clotting time, measured as time-to-max OD, was insignificantly altered over the residual circulating concentration range of rivaroxaban in patient plasma samples, a correlation analysis was conducted. Fig. 28 indicates that both variables were insignificantly related ( $r = 0.166$ ;  $p = 0.721$ ). Thus, the lack of significant influence of rivaroxaban on clotting time due to initiation by thrombin was confirmed. This observation is further supported by no difference in max OD and time-to-max OD between the non-anticoagulated and rivaroxaban-treated patient groups (Table 4).



**Figure 27: Rivaroxaban concentration is correlated to time since last dose but not half-clot lysis time or percent FXaβ**

Panel A: Correlation between time since last dose and rivaroxaban concentration. Note that the corresponding symbol for patient code R-15 I missing in Panel A because ‘time since last dose’ is was unknown at time of blood draw. Panel B and C show the correlations between rivaroxaban concentration and half-clot lysis time or percent FXaβ respectively. Each symbol represents the mean of 3 independent experiments for each rivaroxaban-treated patient. Correlation and significance is included in the bottom left of the each plot.



**Figure 28: Time-to-max OD is not correlated to rivaroxaban concentration in patient plasma**

Rivaroxaban concentration is plotted against clotting time such that every symbol represents the mean of 3 independent experiments for each rivaroxaban-treated patient. Correlation and significance are stated on the bottom right corner of the graph.

**Table 4: No statistical difference in clotting parameters between rivaroxaban-treated and non-anticoagulated.**

	Rivaroxaban-treated (n=7)		Non-anticoagulated (n=12)		Unpaired t test <i>p</i> value*
	Mean ± SD	S.E.M	Mean ± SD	S.E.M	
Max OD (A405 nm)	0.71 ± 0.14	0.05	0.64 ± 0.18	0.05	0.44
Time-to-max OD (min)	220.04 ± 107.27	40.54	396.77 ± 426.97	123.25	0.35

\*Time to max OD was log transformed to acquire a normal distribution prior to t-test.

#### **4.2.3.4 Percent FXa $\beta$ is not significantly correlated to time-to-max OD**

We previously published that purified Xai-K acts as both a pro-fibrinolytic and an anticoagulant agent [52]. To determine if rivaroxaban-stabilized FXa $\beta$  has a dual function as well, percent FXa $\beta$  was correlated to time-to-max OD. While there was a moderate positive correlation ( $r = 0.505$ ) between percent FXa $\beta$  at 60 minutes and clotting time, suggesting that FXa $\beta$  lengthens clotting time, the  $p$ -value was relatively high at 0.248 (Table 5). This observation was also made at the 10 and 30 minute time points where a higher amount of FXa $\beta$  versus FXa $\alpha$  was present.

#### **4.2.4 FXa fragmentation in rivaroxaban-treated DVT/PE and post-surgery patients**

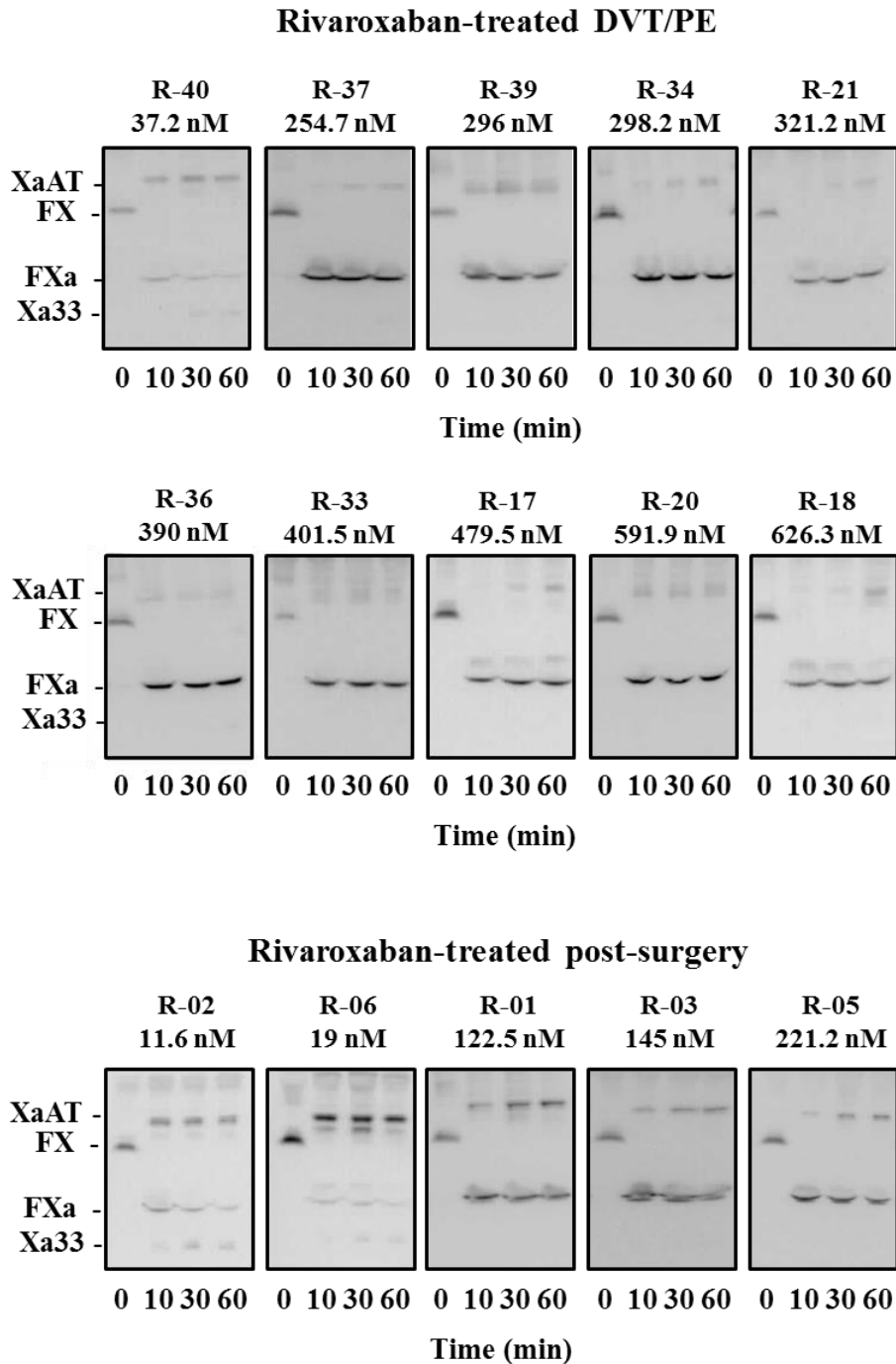
In addition to atrial fibrillation, rivaroxaban is also approved for the treatment of DVT/PE and the prevention of venous thromboembolism after hip and knee replacement surgery. To determine if rivaroxaban-treatment alters the fragmentation of FXa in a similar manner regardless of the condition being treated, plasma samples from 11 DVT/PE patients and 5 post-surgery patients were tested. In the absence of FXa-DOACs, XaAT is generated shortly after FX activation and has been reported as the probable source of Xa33/13 in plasma [180]. In the presence of FXa-DOACs, the active site of FXa is already occupied and AT cannot bind, thus preventing XaAT generation. Fig. 29 shows that at lower concentrations of rivaroxaban (< 40nM), when most of the drug has been cleared, XaAT and Xa33/13 are generated. At concentrations similar to those seen in atrial fibrillation patient samples (i.e. greater than 100 nM), however, Xa33/13 generation was prevented. Note that compared to atrial fibrillation patients, the generation of higher molecular weight complexes are more readily seen in these patients, an observation even more pronounced in post-surgery patient samples.

**Table 5: Summary of correlations (r) and p values (p) carried out on rivaroxaban-treated atrial fibrillation patients' normally distributed variables.**

Variables	AGE (Y)		Rivaroxaban (µM)		Hours since last dose		Fibrinogen (g/L)		D-dimer (ng/mL)		Time to maxOD		MaxOD		1/2 lysis time (mins)	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
AGE (Y)	<b>1</b>	<b>0</b>	-0.162	0.728	0.306	0.556	-0.377	0.404	0.680	0.093	0.037	0.937	0.166	0.722	-0.107	0.942
Rivaroxaban (µM)	-0.162	0.728	<b>1</b>	<b>0</b>	<b>-0.829</b>	<b>0.041</b>	0.248	0.592	0.480	0.276	0.166	0.721	-0.625	0.133	0.214	0.644
Hours since last dose	0.306	0.556	<b>-0.829</b>	<b>0.041</b>	<b>1</b>	<b>0</b>	-0.296	0.569	-0.449	0.371	-0.115	0.829	0.644	0.168	0.219	0.677
Fibrinogen (g/L)	-0.377	0.404	0.248	0.592	-0.296	0.569	<b>1</b>	<b>0</b>	-0.414	0.356	0.733	0.061	0.346	0.447	-0.406	0.366
D-dimer (ng/mL)	0.680	0.093	0.480	0.276	-0.449	0.371	-0.414	0.356	<b>1</b>	<b>0</b>	0.062	0.895	-0.318	0.487	0.073	0.876
Time to maxOD	0.037	0.937	0.166	0.721	-0.115	0.829	0.733	0.061	0.062	0.895	<b>1</b>	<b>0</b>	0.620	0.137	-0.493	0.261
MaxOD	0.166	0.722	-0.625	0.133	0.644	0.168	0.346	0.447	-0.318	0.487	0.620	0.137	<b>1</b>	<b>0</b>	-0.344	0.450
1/2 lysis time (mins)	-0.107	0.819	0.214	0.644	0.219	0.677	-0.406	0.366	0.073	0.876	-0.493	0.261	-0.344	0.450	<b>1</b>	<b>0</b>
Complete lysis time (min)	-0.058	0.901	0.608	0.147	-0.202	0.701	-0.291	0.526	0.332	0.467	-0.425	0.341	-0.622	0.136	<b>0.869</b>	<b>0.011</b>

Variables	Log FX		%FXaα, 10		%FXaα, 30		%FXaα, 60		%FXaβ, 3		%FXaβ, 10		%FXaβ, 30		%FXaβ, 60	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
AGE (Y)	-0.111	0.812	0.254	0.583	0.109	0.816	0.200	0.667	-0.171	0.746	-0.254	0.583	-0.109	0.816	-0.200	0.667
Rivaroxaban (µM)	-0.525	0.226	0.470	0.288	0.120	0.798	0.165	0.723	-0.396	0.437	-0.470	0.288	-0.120	0.798	-0.165	0.723
Hours since last dose	0.441	0.381	-0.059	0.912	0.368	0.473	0.315	0.544	-0.041	0.947	0.059	0.912	-0.368	0.473	-0.315	0.544
Fibrinogen (g/L)	-0.030	0.949	-0.270	0.558	-0.127	0.786	-0.468	0.289	-0.537	0.272	0.270	0.558	0.127	0.786	0.468	0.289
d-dimer	-0.630	0.129	0.395	0.380	-0.063	0.892	0.233	0.614	-0.290	0.577	-0.395	0.380	0.063	0.892	-0.233	0.614
Time to maxOD	-0.551	0.200	-0.408	0.364	-0.468	0.290	-0.505	0.248	<b>-0.861</b>	<b>0.028</b>	0.408	0.364	0.468	0.290	0.505	0.248
MaxOD	-0.072	0.878	-0.616	0.141	-0.346	0.447	-0.343	0.452	-0.639	0.172	0.616	0.141	0.346	0.447	0.343	0.452
1/2 lysis time (mins)	-0.084	0.858	0.642	0.120	0.688	0.088	<b>0.928</b>	<b>0.003</b>	0.025	0.962	-0.642	0.120	-0.688	0.088	<b>-0.928</b>	<b>0.003</b>
Complete lysis time (min)	-0.218	0.638	0.723	0.066	0.605	0.150	<b>0.810</b>	<b>0.027</b>	0.037	0.945	-0.723	0.066	-0.605	0.150	<b>-0.810</b>	<b>0.027</b>

Bold values:  $p \leq 0.05$



**Figure 29: Rivaroxaban prevents Xa33/13 in patients treated for DVT/PE and post-surgery**

FXa fragmentation was followed by western blot in Innovin<sup>®</sup>-treated clots formed with rivaroxaban-treated patient plasma. Note that densitometry of bands and potential correlations were not measured for these fragments as these samples were complicated by increased activation of hemostasis and were therefore not a focus of the study.

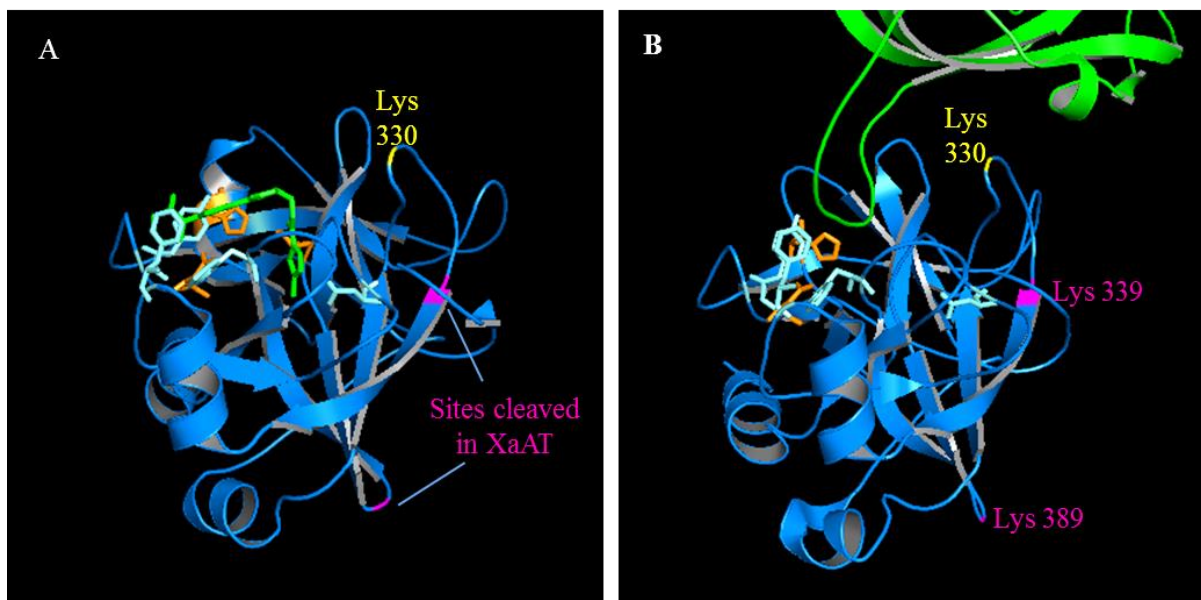
### 4.3 Discussion

Enhanced dissolution of a tissue-factor induced plasma clot in the presence of a DOAC has often been attributed to the drug's anticoagulant effect. However, this dissertation chapter demonstrates that when the anticoagulant effects are bypassed by starting clot formation with thrombin, enhancement of clot dissolution is strongly correlated with an increase of FXa $\beta$  generation ( $p = 0.003$ ). Over the last few years, derivatives of FXa, such as FXa $\beta$  have emerged as thrombolytic drug targets [52,162,180]. Of these, FXa $\beta$  has been shown to be the most effective in enhancing clot lysis in both *in vivo* and *in vitro* plasma studies [52,180]. The latter fragment is stabilized when the FXa active site is blocked or modified such that further cleavage of FXa $\beta$  into Xa33/13 is prevented. While Xa33/13 was originally targeted by our laboratory as a plausible thrombolytic therapeutic, it was found to have limited pro-fibrinolytic function in plasma. Instead, it was identified to be a rapidly degraded transient intermediate leading to an as yet unidentified cleavage product, possibly appearing as a 28 kDa fragment [52]. The objective of the current study was to investigate the physiological applicability of results obtained in the preceding chapter where commercial normal pooled plasma was used. In the latter, the addition of rivaroxaban or apixaban led to the persistence of FXa $\beta$  and enhanced clot lysis.

Comparing non-anticoagulated and DOAC-treated atrial fibrillation patients, this study confirmed that persistence of FXa $\beta$  occurs in patients treated with the FXa-specific DOAC, rivaroxaban. Unlike patients taking rivaroxaban, XaAT and Xa33/13 were generated in plasma samples from non-anticoagulated and dabigatran-treated patients. Since autoproteolysis was excluded in the generation of Xa33/13 by treating CMK-altered FXa $\beta$  with proteolytic FXa [162], the probable basis for altered fragmentation by plasmin is substrate recognition. An explanation for the lack of Xa33/13 generation in the presence of rivaroxaban may be that Lys

330, the plasmin-mediated cleavage site that transitions FXa $\beta$  to Xa33/13 (Fig. 10), is protected when the active site of FXa is occupied. The latter is further highlighted by previous work from our laboratory showing that XaAT is cleaved at Lys 339 and not Lys 330 to generate the probable source of Xa33/13 in plasma [180]. Fig. 30 depicts a three dimensional rendering of the atomic coordinates deposited in the Brookhaven Protein Data Bank for the heavy chain of FXa complexed with rivaroxaban or antithrombin. Unlike in the case of XaAT, an alternate proteolytic site may not be afforded in the case of FXa active site-bound DOAC. Regardless, FXa $\beta$  is stabilized in plasma by FXa-specific DOACs but not by the thrombin-specific DOAC dabigatran, which was used as a negative control to confirm specificity as it does not bind to FXa. Persistence of FXa $\beta$  and thus plasmin generation in the presence of FXa-specific DOACs may help to understand why a thrombus from a patient previously treated by dabigatran was only cleared after being switched to a FXa-specific DOAC [236]. A mutation study is underway in our laboratory (initiated by Dr. Amanda Vanden Hoek [241]), where preliminary results suggest that altering Lys 330 to a glutamine also prevents Xa33/13 generation and enhances fibrinolysis in plasma, similar to FXa-DOACs.

Interestingly, despite differences in FXa fragmentation between rivaroxaban-treated and non-anticoagulated patient plasma samples, the half-clot lysis time between the two groups were similar. The latter indicates the involvement of other pro-fibrinolytic elements in non-anticoagulated patients or differences in the level of regulatory proteins that were not accounted for. In support of this possible confounder, non-anticoagulated patient samples were found to have a higher D-dimer level compared to normal pooled and rivaroxaban-treated plasma ( $p = 0.08$ ), suggesting that active coagulation and fibrinolysis processes were taking place at the time of blood collection in these patients. The data are in accordance with other studies showing



**Figure 30: 3D structure of FXa complexed with rivaroxaban or antithrombin**

Panel A: Ribbon structure of FXa plus rivaroxaban (PDB: 2W26) and Panel B: Ribbon structure of FXa plus antithrombin (PDB: 2GDA). Only the heavy chain of FXa is shown (blue) along with the catalytic triad residues (serine 195, histidine 57, aspartate 102) in orange, the S1 (aspartate 189) and S4 (phenylalanine 174, tryptophan 215, and tyrosine 99) pocket residues in cyan, the Lys330 cleavage site (yellow), and the inhibitor (green). Also depicted is the two cleavage sites of XaAT (magenta) predicted to render Xa33/13. The reaction loop in antithrombin is the focus and thus the entire molecule is not shown. Images were visualized using Pymol (<https://www.pymol.org/>).

an increased D-dimer level in atrial fibrillation patients [242] and a reduced D-dimer level in anticoagulated patients [242,243]. Elevated D-dimer levels are associated with an increased risk of bleeding [242], mainly due to consumption of coagulation factors or enhanced fibrinolysis. Correlation studies however showed no relationship between D-dimer levels and half-clot lysis times, thus eliminating the former as a direct explanation for the lack of difference between the two patients groups tested. Despite the lack of relationship between D-dimer levels and fibrinolysis, patients in the non-anticoagulant atrial fibrillation group may still have other confounders that remain unaccounted for, as per their need for surgery. Thus, collection of plasma from non-anticoagulated age-matched healthy donor is near completion and the experiments to obtain final data for this new control group will be the subject of future studies.

For rivaroxaban-treated patients, there was no correlation between rivaroxaban concentration and half-clot lysis time. While this observation contradicts results obtained for normal pooled plasma titrated with rivaroxaban (Fig. 14), it is in agreement with those reported by Semeraro et al. [206] in atrial fibrillation patients. This discrepancy highlights likely differences when using commercial pooled plasma vs. individual patient plasma samples. For example, it is important to note that in plasma, to which a DOAC had been added *ex vivo*, various concentrations of DOAC can be added at the same time and the assay constantly initiated within an hour. In patients; however, the concentration of rivaroxaban is a direct reflection of the amount of anticoagulant eliminated from the body over time ( $r = -0.829$ ;  $p = 0.041$ ). Moreover, unlike spiked plasma, 2/3 of the rivaroxaban in patients undergoes oxidative degradation and hydrolysis prior to clearance [189]. Although unchanged rivaroxaban has been classified as the major circulating compound in plasma analyzed from human, low levels of metabolites are still detectable [189]. The effect these metabolites may have on fibrinolysis despite being inactive as

an anticoagulant is unknown. In addition, patients were significantly older than donors for the normal pooled plasma. Thus, it is likely that levels of regulatory proteins due to age and condition were different between the two groups and would contribute the results seen.

The absence of statistical relationship between rivaroxaban concentration and percentage of FXa $\beta$  at 60 minutes could be due to the saturation of drug. It is probable that circulating concentrations of rivaroxaban are sufficient to fully block the active site of activated FX. This observation could be further explained by the FX/Xa banding pattern of DVT/PE and post-surgery patient samples western blots in which concentrations of rivaroxaban > 40 nM were needed for FXa $\beta$  persistence. Below this amount, XaAT was generated and further cleavage into Xa33/13 occurred. The concentration range in atrial fibrillation rivaroxaban-treated samples was all above 40 nM (i.e 149 – 504 nM).

Previous studies using tissue factor to initiate clotting have indicated that rivaroxaban enhances plasma clot lysis through its anticoagulant properties [203]. The use of thrombin, instead of tissue factor, in this study was done to bypass the anticoagulant effect of rivaroxaban and thus emphasize the pro-fibrinolytic function of FXa $\beta$ . The lack of correlation between rivaroxaban concentrations and time to achieve complete clot formation (i.e. max OD) supports the concept that adequate thrombin was used to overcome the anticoagulant effects of rivaroxaban. The latter is complemented by the lack of significance in max OD and time-to-max OD between rivaroxaban-treated and non-anticoagulated patient samples. Thus, clotting was not altered by the presence of rivaroxaban.

Xai-K, a version of FXa that has a chemically modified active site, acts as both a pro-fibrinolytic agent and competitive anticoagulant [52]. Despite a moderately positive correlation between the percent of FXa $\beta$  produced relative to total FXa in patient plasma and clotting time

( $p = 0.505$ ), statistical significance with  $p < 0.05$  was not reached. Although power calculations suggested that an adequate number of patients had been recruited for this study, it is possible that with a larger sample a significant difference would be established to verify the observed trend.

It must be noted, that this study is an initial exploration into a completely unforeseen side effect of rivaroxaban. Presumably other FXa-directed DOACs will have a similar effect as indicated by apixaban having nearly identical fibrinolytic characteristics *in vitro* (Chapter 3). As an initial clinical study there are several limitations. Plasma samples used in this chapter were originally collected for an alternative study aimed at measuring DOAC concentrations in patients who were treated with rivaroxaban or dabigatran for atrial fibrillation or for preoperative INR monitoring (non-anticoagulated samples). Consequently, parameters that could affect fibrinolysis such as (i.e. diabetes, cancer, alcohol consumption, smoking, and time of phlebotomy) were not taken into account during sample collection. Moreover, samples from rivaroxaban-treated patients prior to starting their FXa-DOAC treatment were not available to establish an accurate baseline. To substitute for the latter, commercial normal pooled and preoperative non-anticoagulated plasma samples were used as controls. As indicated by aberrant D-dimer levels in the latter, a more appropriate control group may be healthy age-matched donors, which will be evaluated in our laboratory for comparison.

While the number of samples available were relatively small, power calculations suggested that there would be an adequate number of samples to establish a significant difference between rivaroxaban-treated and non-anticoagulated patients. Nevertheless, extensive statistical analyses to identify potential confounders could not be fully satisfied without a larger population number. Power calculations to estimate the sample number required were conducted with values from a preliminary study using commercial normal plasma clot lysis data compared to

rivaroxaban-treated patients. Findings in clot lysis assays suggest that additional confounding factors existed that may not be taken into account in the preliminary experiments that were used to estimate power.

Rivaroxaban is a FXa-specific anticoagulant approved for the treatment of DVT/PE, the prevention of venous thromboembolism after hip and knee replacement surgery, and the prevention of stroke in patients with atrial fibrillation. This study for the first time identifies FXa $\beta$  as a fibrinolysis marker in rivaroxaban-treated atrial fibrillation patients. With the lack of correlation between rivaroxaban concentration and half-clot lysis time or percent FXa $\beta$ , a greater emphasis can be placed on the conversion of FXa $\alpha/\beta$  into FXa $\beta$  and thus plasmin generation as a marker. Although not quantitative, through the absence of Xa33/13 and therefore the persistence of FXa $\beta$ , the study also provides an additional method for evaluating the clearance of rivaroxaban to subtherapeutic levels.

## Chapter 5: SUMMARY

The discovery that clotting FXa can participate in fibrinolysis has opened a new avenue in thrombolysis research. Previous work carried out in our laboratory indicates that the novel pro-fibrinolytic function of FXa is acquired when it is cleaved by plasmin in the presence of aPL and calcium to generate FXa $\beta$ . In plasma, further cleavage of FXa $\beta$  into Xa33/13 results in a loss of tPA-cofactor function. The latter can be prevented by irreversibly modifying the active site of FXa such that FXa $\beta$  is stabilized. With the induction of FXa-specific DOACs, a new opportunity to study FXa cofactor function was created. The objective of this dissertation was to investigate the effect that rivaroxaban and apixaban, reversible FXa active site inhibitors, would have on the function of FXa as an auxiliary tPA cofactor.

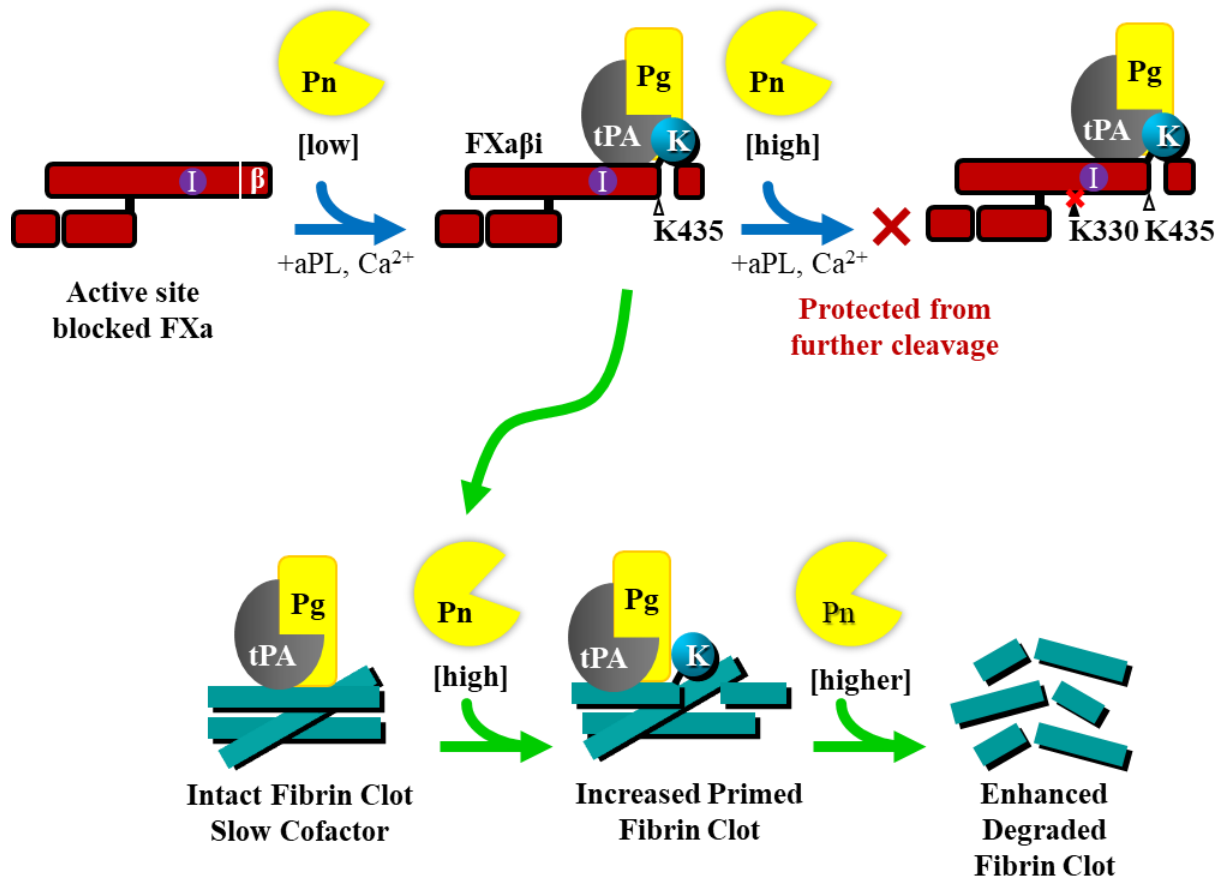
Using commercially available normal pooled plasma, studies in this dissertation demonstrated that both rivaroxaban and apixaban enhance the degradation of thrombin-induced plasma clots in a dose- and FX- dependent manner. Since DOACs are known to enhance clot lysis through downstream effects on thrombin generation, feasible differences in TAFIa activity, clot ultrastructure, and clot stabilization by intra-chain cross-links were evaluated and found to not be affected by FXa-DOACs under the experimental conditions developed here. As a molecular explanation, both rivaroxaban and apixaban altered the cleavage of FXa such that Xa33/13 was not generated in plasma, FXa $\beta$  persisted and enhanced fibrinolysis was proportional to the latter in plasma. The use of purified proteins to dissect the mechanism demonstrated that FXa $\beta$  generated in the presence of rivaroxaban and apixaban bind plasminogen and thus enhance plasmin generation.

In patients treated with DOACs for atrial fibrillation, a prominent role for FXa $\beta$  was observed. Unlike non anticoagulated and dabigatran-treated patients, FXa $\beta$  only persisted in

plasma from rivaroxaban-treated patients. Moreover, the rate of fibrinolysis was strongly correlated with the amount of FXa $\beta$  generated, as measured by band intensity relative to starting FX and total FXa amount. In plasma of patients treated for the prevention of DVT/PE and venous thromboembolism post hip or knee replacement surgery, there was also a lack of Xa33/13 production and persistence of the FXa as determined by western blots. This is likely attributed to protection of FXa $\beta$  from further cleavage. Interestingly, generation of Xa33/13 was only achieved at concentrations less than 40 nM, suggesting its application as a surrogate marker for subtherapeutic concentrations of circulating FXa-DOACs.

Overall this dissertation identifies a new role for FXa-specific anticoagulants in their ability to prevent further cleavage of FXa $\beta$  and accelerate fibrinolysis (Fig. 31). The latter is of particular interest in the treatment of DVT as ineffective clot dissolution is a potential cause for PTS. While large clinical trials are ongoing, studies in this dissertation support preliminary findings showing a trend towards a reduced risk of developing PTS in patients treated with a FXa-specific DOAC compared to traditional treatment [234]. This reduction could be attributed to a dual anticoagulant and fibrinolytic role.

Moreover, while FXa-DOACs have predictable pharmacological profiles, measurement of the degree of anticoagulation may be needed in critical situations (reviewed in [244]). Assays such as activate partial thromboplastin time and prothrombin time/international normalized ratio have been deemed ineffective to accurately measure anticoagulant effects [245]. Thus, physicians have to rely more on the measurement of DOAC plasma concentration by chromogenic anti-FXa assay. Through this dissertation, a unique method involving detection of Xa33/13 is offered to ensure clearance of anticoagulants in patients post-treatment.



**Figure 31: Role of FXa-specific DOACs in fibrinolysis**

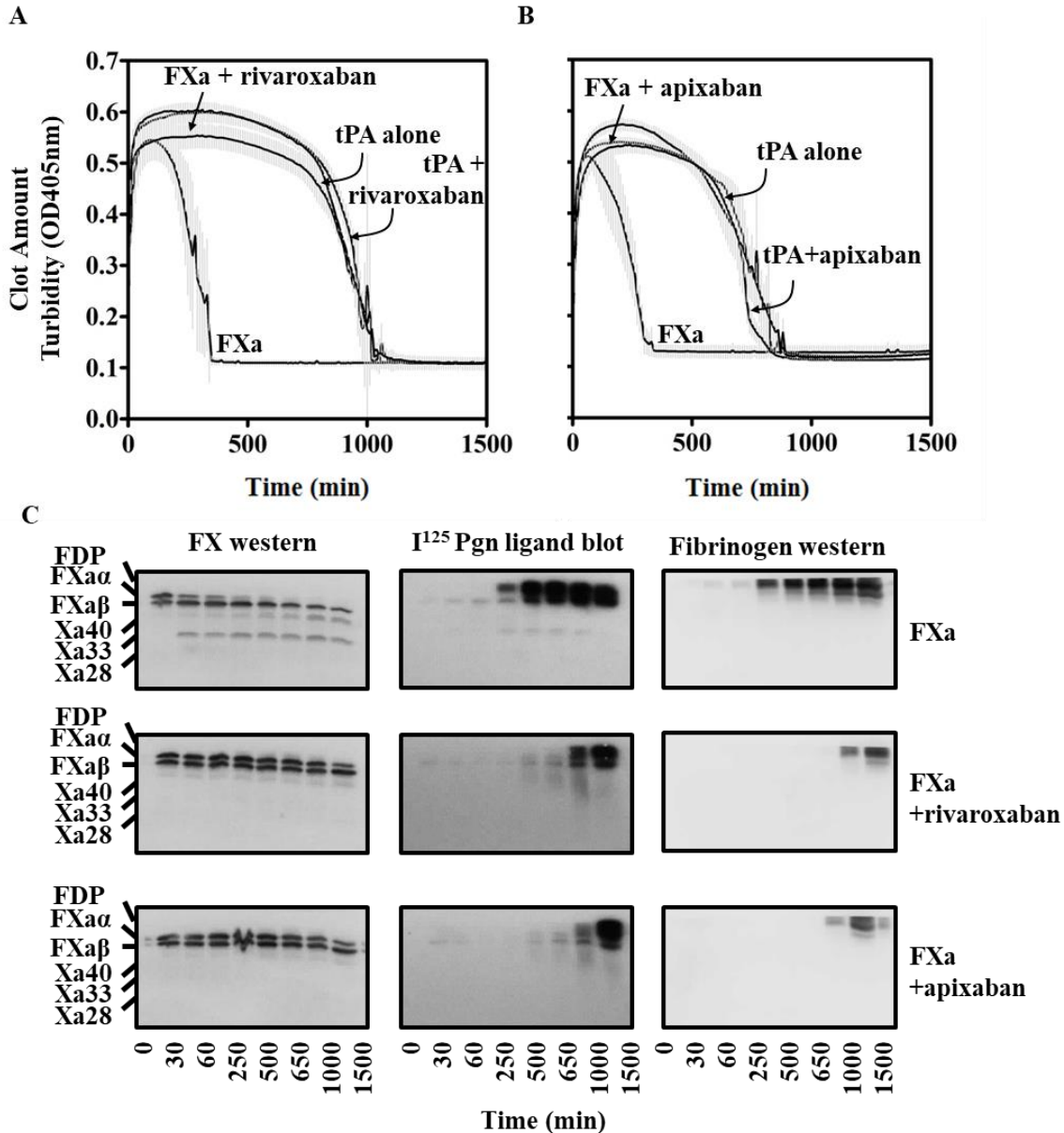
In the presence of anionic phospholipid and calcium, active site blocked (I)-FXa is cleaved by plasmin into FXaβI. This fragment is protected from further cleavage into Xa33/13, the species that loses its fibrinolytic function in plasma. Since FXaβI possesses a C-terminal lysine residue, it is able to bind to plasminogen and enhance plasmin generation. The resulting plasmin can then participate in the early phase of fibrinolysis to dissolve the fibrin clot faster.

## Chapter 6: FUTURE STUDIES

### 6.1 Reconciling differences between purified fibrinogen and plasma clot lysis

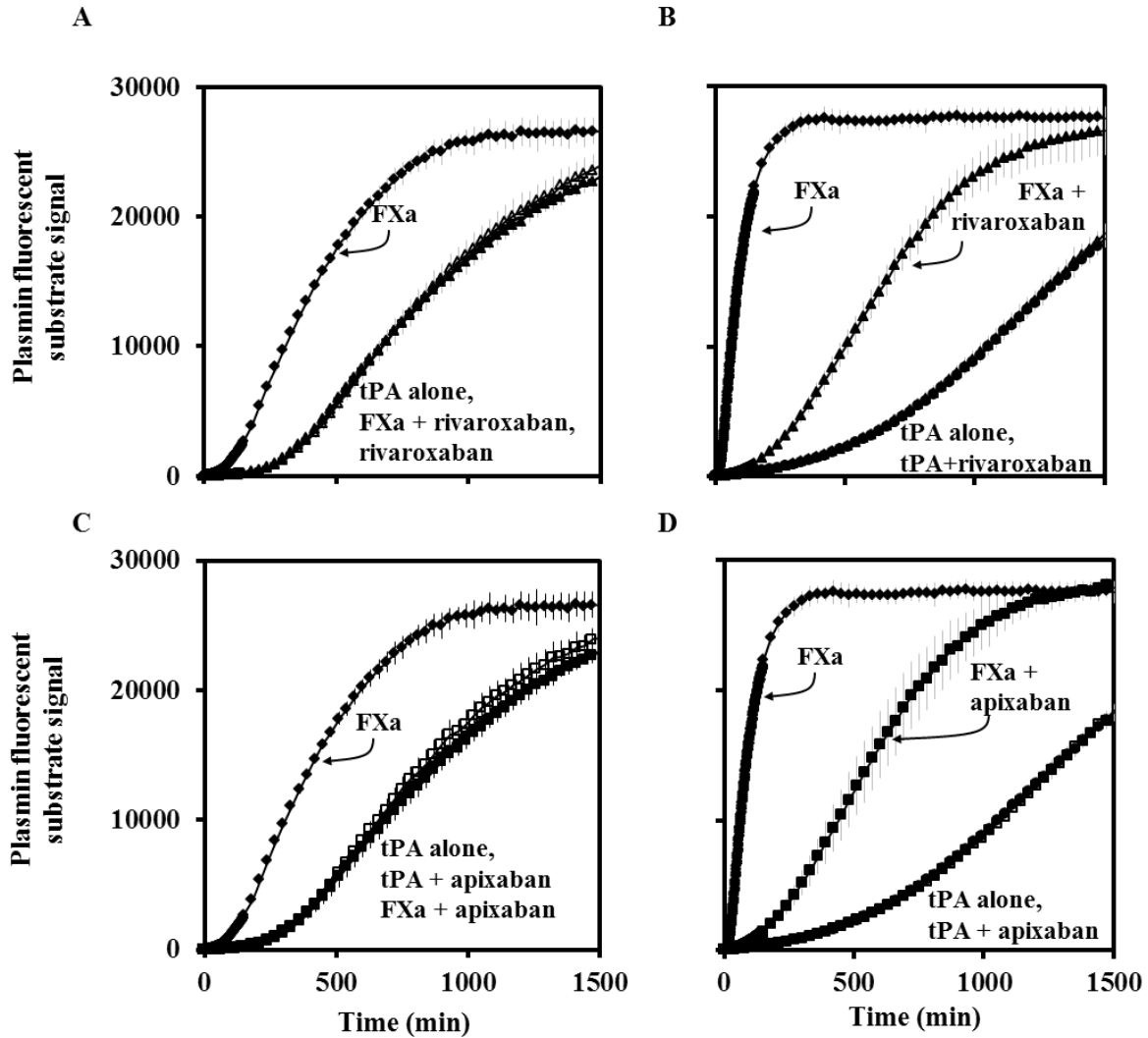
While FXa plus rivaroxaban or apixaban prevent the further cleavage of FXa $\beta$  to Xa<sub>33/13</sub> and enhance tPA-mediated plasmin generation in plasma and purified experiments, surprisingly neither of the two FXa-DOACs have an effect on the clot lysis of purified fibrin (Fig 32A and B). Western blot analysis during the course of purified fibrin clot lysis revealed limited to no cleavage of FXa $\alpha$  to FXa $\beta$  in the presence of rivaroxaban and apixaban (Fig. 32C). Since the latter conversion is necessary for gain of pro-fibrinolytic function, plasmin generation during the course of clot lysis was analyzed with a plasmin-specific fluorogenic substrate (Fig. 33A and 33C) and compared to that in the absence of added fibrinogen and thrombin (Fig. 33B and 33D). Results showed that enhanced plasmin generation by FXa with rivaroxaban or apixaban is lost when thrombin and fibrinogen is present to form a purified fibrin clot. In a similar experiment, the latter increase in plasmin generation, relative to controls (tPA alone and tPA + rivaroxaban or apixaban), was seen in the presence of thrombin or fibrinogen alone. This suggests that cleavage of FXa in the presence of rivaroxaban or apixaban is affected by the fibrin during purified protein clot lysis.

The absence of enhancement in clot lysis in the presence of FXaDOACs when purified proteins are used differs from the results obtained in plasma in which clot lysis was enhanced (Fig. 14). Thus to determine if similar amounts of FX as would be present in 35 % diluted plasma may be the reason for the discrepancy, a purified fibrin clot was formed as in Fig. 32 with an addition of ~ 60 nM FX. Fig. 34 shows that added purified FX does not explain this difference. These observations suggest that yet unknown mechanisms or elements in plasma help



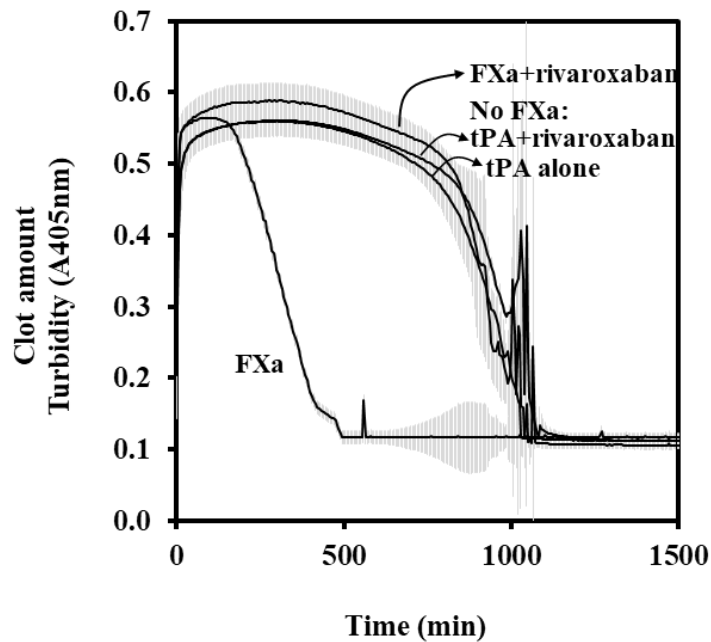
**Figure 32: In purified fibrin clot lysis, the lack of enhancement by FXa plus FXa-DOAC is linked to restricted FXaβ production**

Panel A: Fibrinogen (3  $\mu$ M), plasminogen (0.6  $\mu$ M), aPL (50  $\mu$ M), Ca<sup>2+</sup> (5 mM) and tPA (0.4  $\mu$ M) were combined in the absence or presence of FXa (0.1  $\mu$ M) with or without rivaroxaban (0.57  $\mu$ M), or rivaroxaban. Clotting was induced with thrombin (3 nM) and the extent of clot formation and dissolution were followed by turbidity. Solid line: averaged data  $\pm$  SD indicated by grey shade (n = 3). Panel B: Similar to Panel A, with the exception that 0.44  $\mu$ M apixaban was used. Panels C: Non-reduced anti-human FX western blot, iodine-125 radiolabelled plasminogen ligand blots, and fibrinogen western blot (FDP bands) of samples from FXa, FXa+rivaroxaban, and FXa+ apixaban taken at various times during clot lysis.



**Figure 33: The ability of FXa+rivaroxaban and FXa+apixaban to enhance plasmin generation is prevented in the presence of fibrin clot.**

Panels A and C: Fibrinogen (3  $\mu\text{M}$ ), plasminogen (0.6  $\mu\text{M}$ ), aPL (50  $\mu\text{M}$ ),  $\text{Ca}^{2+}$  (5 mM), tPA (0.4  $\mu\text{M}$ ), and the plasmin fluorogenic substrate (200  $\mu\text{M}$ ) were combined in the absence or presence of FXa (0.1  $\mu\text{M}$ ) with or without rivaroxaban (0.57  $\mu\text{M}$ ), apixaban (0.44  $\mu\text{M}$ ) or rivaroxaban. Clotting was induced with thrombin (3 nM). Fluorogenic signal was graphed. Shown is average  $\pm$  SD. Panels B and D: In the same plate as Panels A and C, activation of Pg (0.5  $\mu\text{M}$ ) by tPA (0.4  $\mu\text{M}$ ) was initiated in the presence of constant aPL (50  $\mu\text{M}$ ) and calcium (2 mM), with or without FXa (0.1  $\mu\text{M}$ ) and rivaroxaban or apixaban. A plasmin specific fluorogenic substrate (200  $\mu\text{M}$ ) was added and its signal was graphed. Shown is average  $\pm$  SD.



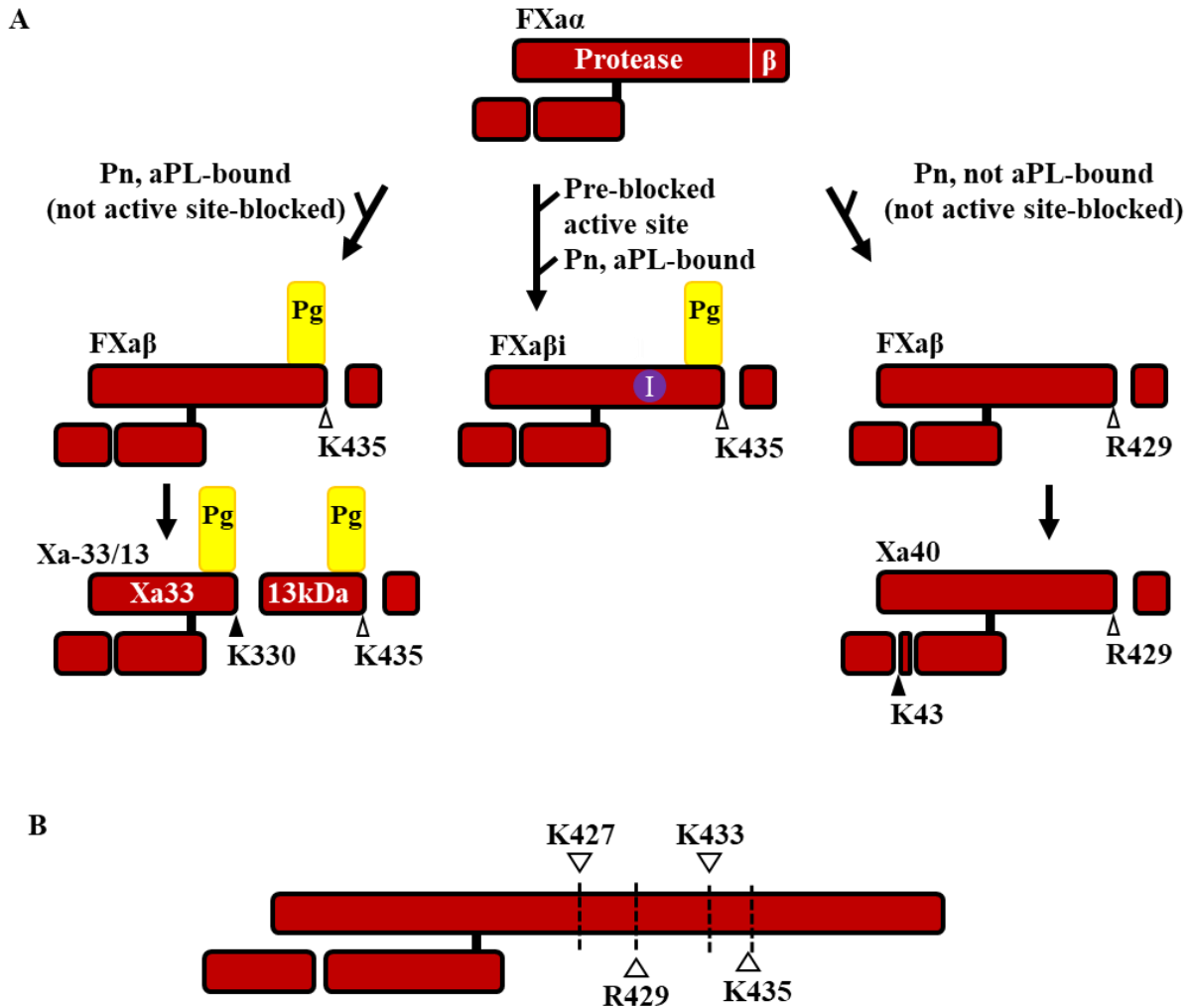
**Figure 34: Trace amount of FX has no effect on purified fibrin clot lysis and thus is not the cause for the lack of enhancement seen by FXa±rivaroxaban**

Fibrinogen (3  $\mu\text{M}$ ), Pg (0.6  $\mu\text{M}$ ), aPL (50  $\mu\text{M}$ ), tPA (0.4  $\rho\text{M}$ ), and FX (59.42 nM) were combined in the absence or presence of FXa (0.1  $\mu\text{M}$ ) with or without rivaroxaban (0.57  $\mu\text{M}$ ). Clotting was induced with thrombin (3 nM) and  $\text{Ca}^{2+}$  (5 mM), and the extent of clot formation and dissolution were followed by turbidity. Solid line: averaged data  $\pm$  SD indicated by grey shade (n = 3).

to support the pro-fibrinolytic function of FXa in the presence of rivaroxaban or apixaban. These additional constituents, which may include regulatory proteins of fibrinolysis in plasma, would need to be identified. Furthermore, while the same concentration of FXa-DOAC was used in purified protein and plasma experiments, 92 – 95 % of rivaroxaban and 87 % of apixaban have been reported to associate with plasma proteins other than FXa in which serum albumin is the main contributor [246]. Therefore the concentration of available DOACs in plasma-based experiments is less than that in the presence of purified proteins when rivaroxaban and apixaban are used at the same concentration in both experimental systems. When the FXa-specific DOACs are used in a 1:1 molar ratio with FXa in purified systems, enhancement of lysis is found. The latter suggests that excess rivaroxaban or apixaban may be having an effect on fibrinolysis when purified proteins are used. Xa-D, an irreversible CMK active site-blocked FXa in which excess CMK has been removed, has similar tPA-mediate plasmin generation abilities as FXa plus rivaroxaban or apixaban [7]. However, unlike reversible DOAC-treated FXa, Xa-D enhances purified fibrinogen clot lysis, an observation that further supports a role for unbound FXa-DOACs. As another potential explanation, it is possible that the cleavage site of FXa is different in the presence of a purified fibrin clot. This is because previous mutagenesis studies in our laboratory [179] showed that while the lysine residue 435 is the preferred plasmin cleavage site on FXa other sites do exist (Fig. 35). Identifying what causes this loss of function during purified fibrinogen clot lysis can further our understanding of how the pro-fibrinolytic function of active site blocked FXa is modulated between experiment systems.

## **6.2 Directly linking FXa fragmentation to plasma clot lysis times using prothrombin deficient plasma**

While carefully controlled thrombin-induced plasma experiments conducted in Chapter 3



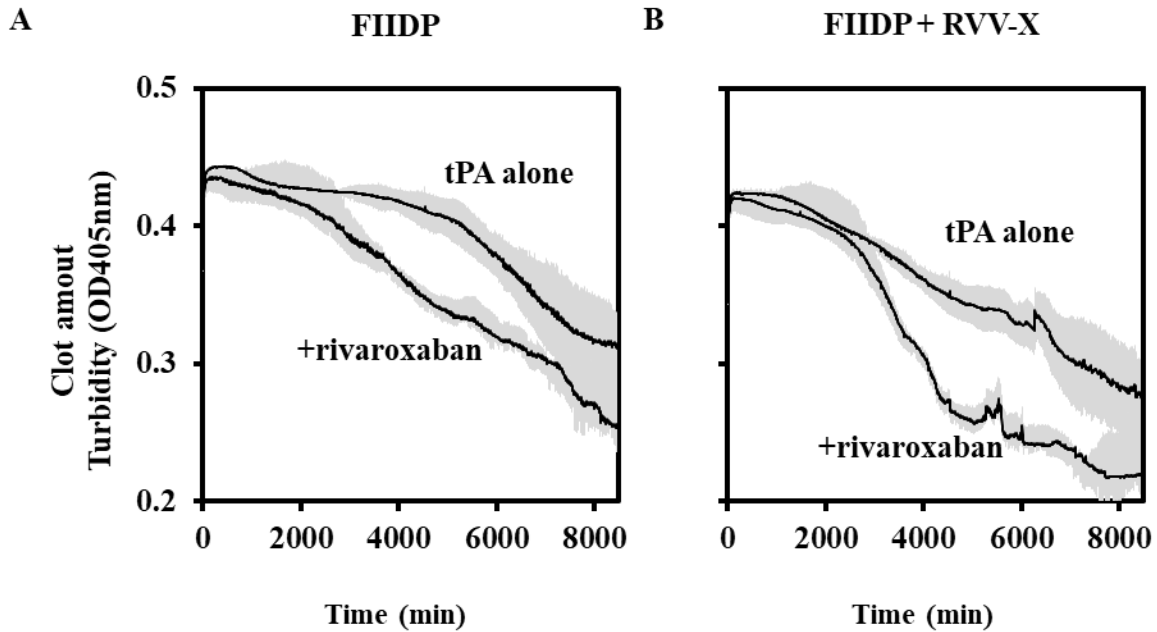
**Figure 35: Multiple cleavage sites exist to generate the FXa $\beta$  fragment**

Panel A: FXa fragmentation as depicted in Figure 10. Panel B: Potential plasmin cleavage sites in the FXa $\beta$  region. While Lys (K) 435 is the probable preferred site of cleavage for FXa $\beta$  generation, mutagenesis studies previously done in our laboratory show that other sites are cleaved by plasmin (i.e. Lys 427, Arg 429, and Lys 433). Thus cleavage at sites other than Lys 435 may differentially affect the auxiliary cofactor function of FXa. Determined ( $\blacktriangle$ ) and inferred ( $\triangle$ ) cleavage sites are depicted.

showed that FX activation is needed for the enhancement of plasma fibrinolysis by FXa-DOACs, levels of FXa capable of being detected by western blot were only achieved with the use of tissue factor as Innovin<sup>®</sup>. Thus, to further monitor FXa fragmentation during the degradation of thrombin-induced clot, purified FXa or RVV- activated FX in the presence of FXa-DOACs could be evaluated in prothrombin deficient plasma (IIDP). The use of IIDP would ensure that consistent thrombin concentration is maintained despite the addition of FXa. Samples could then be taken during the course of clot degradation for western blot analysis to directly correlate FXa fragmentation to clot lysis times. In a study by von dem Borne *et al.* thrombin concentrations greater than 20 nM began to correct the premature clot lysis seen in IIDP [247]. Using 30nM thrombin to initiate clotting in IIDP, preliminary investigations have thus far confirmed that the addition of rivaroxaban enhanced the lysis of IIDP clot in which activation of FX via the intrinsic pathway is unaffected (Fig. 36). This effect was further increased when RVV-X was added to activate endogenous X. A similar observation was seen when 100 nM thrombin and apixaban were used. While further optimization of the assay is still needed, these data complement those in Chapter 3 demonstrating that enhancement of plasma clot lysis in the presence of rivaroxaban and apixaban is dependent on FX activation when their anticoagulant effect is bypassed.

### **6.3 Rivaroxaban-treated atrial fibrillation patients vs. non-anticoagulated healthy age-match donors**

As expected, FXa $\beta$  persisted in rivaroxaban-treated patients while further cleavage into Xa33/13 occurred in normal pooled and non-anticoagulated patient plasma samples (Fig. 22). From the fragmentation profile it was therefore anticipated that plasma fibrinolysis would be enhanced in the treated group compared to the two untreated groups. However, this was not the case. While enhanced fibrinolysis was seen in the rivaroxaban-treated group relative to normal



**Figure 36: Enhanced plasma clot lysis by rivaroxaban in prothrombin deficient plasma is further increased with Russell Viper venom-FX activator**

Panel A: Thrombin (30 nM)-induced clots were formed in prothrombin deficient plasma using 30  $\mu$ M tPA. The extent of clot formation and dissolution were followed by turbidity in clots containing rivaroxaban or buffer (tPA alone). Solid line: n = 1 averaged data  $\pm$  SD indicated by grey shade. Panel B: Similar to Panel A with the exception that RVV-X (21 nM) was added to activate FX. Solid line: n = 1 averaged data  $\pm$  SD indicated by grey shade

pooled plasma, there was no statistical difference between the former and non-anticoagulated patient plasma ( $p = 0.55$ ) (Fig. 23). Note that donors who made up the commercial normal pooled plasma were both healthy and significantly younger ( $p < 0.0001$ ) than the patient groups. If age was the only factor to consider, it would be expected that clots would have lysed slower in the patient group than in the healthy group due to changes in fibrinolytic properties with age (reviewed in [248]). However, it is known that atrial fibrillation patients have elevated levels of D-dimer and endogenous tPA as well as reduced plasmin- $\alpha_2$ antiplasmin complex levels [249], reviewed in [27]. Potential differences in age and medical condition were the reason non-anticoagulant atrial fibrillation patients were chosen as a control in addition to normal pooled plasma since samples prior to treatment in the rivaroxaban-treated group were unavailable. Nevertheless, it is possible that hemostasis in the non-anticoagulated group might have been affected by factors related to prior warfarin treatment or the need for surgery. Thus, rivaroxaban-treated patients will be compared to age-matched healthy donors in an effort to determine the reason for the lack of difference between the two groups. The latter patient group is expected to have similar D-dimer levels as treated atrial fibrillation patients. Enhancement of plasma clot lysis in the age-matched healthy patient group relative to the rivaroxaban-treated patient group would help to substantiate the observation and lead to more investigation on FXa $\beta$  as both a marker of enhanced fibrinolysis and anticoagulant presence in rivaroxaban-treated patients. A similar analysis can be done in apixaban-treated patients. A protocol has already been approved by the ethics board and collection of age-matched healthy donor plasma samples has already begun.

#### **6.4 *In vivo* studies using ferric chloride carotid model or a deep vein thrombosis model**

Post-thrombotic syndrome, potentially due to ineffective clot dissolution, continues to be

a major issue in patients treated for DVT. Despite signs that FXa-DOAC treatment may reduce the risk of developing PTS [234], clinical trials are on-going to confirm this trend. Work carried out on chemically-modified FXa, Xai-K, showed that a stable form of FXa $\beta$  is able to not only act as a thrombolytic to enhance the restoration of blood flow in a carotid artery mouse model, but it can also act as an anticoagulant [52]. With the observation that FXa-specific anticoagulants can stabilize FXa $\beta$  and enhance plasma clot lysis, our readily available carotid artery thrombosis mouse model or a DVT mouse model can be used to investigate the effects of these DOACs *in vivo*.

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