FACTOR XI ACTIVATION AND INHIBITION IN CATHETER-INDUCED CLOTTING

FACTOR XI ACTIVATION AND INHIBITION IN CATHETER-INDUCED CLOTTING

BY

RUIQI YIN, Honours B.Sc.

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LAY ABSTRACT

Thrombosis, or blood clot formation in vessels, is responsible for 1 in 4 deaths in Canada and worldwide. Patients with acute coronary syndrome (ACS), atrial fibrillation (AF), or cancer often require central venous catheters (CVCs) for medical access, such as receiving chemotherapy, drugs, and nutrition. These catheters, like peripherally inserted central catheters (PICC) and implanted ports, are associated with an increased risk of thrombosis, as clots tend to form inside the catheters and block the vessels. Vascular blockages cause severe complications like deep vein thrombosis (DVT) and pulmonary embolism (PE). Therefore, effective and safe anticoagulants are essential to manage and prevent catheter-induced clotting. Factor (F) XI in the contact pathway of coagulation has emerged as a promising target for anticoagulant therapy because FXI is key for blood clot formation but is not essential in normal blood clotting. Consequently, FXI inhibitors have become a safer alternative to currently available anticoagulants. FXI can be autoactivated by negatively charged surfaces like polyphosphate (polyP), released from activated platelets, or synthetic surfaces like catheters. Thus, FXI is a root cause of catheter- and polyP-induced clotting. Milvexian and abelacimab, both FXI inhibitors, are currently undergoing large phase 3 trials in patients with ACS, AF, or cancer. However, the effect of these FXI inhibitors on catheter-induced clotting has not been explored. We previously demonstrated that inhibiting FXI reduces catheter-induced clotting in rabbits. This thesis aims to investigate the role of catheters and polyP in FXI activation and to explore the anticoagulant effects of FXI inhibitors in catheter-induced thrombosis using our established plate-based assays. We found that catheters and polyP activate clotting at the level of FXI, bypassing FXII. Both milvexian and abelacimab attenuate catheter-induced clotting. These findings suggest that FXI inhibitors could be a potential therapeutic option for catheter- and polyP-induced thrombosis without increasing bleeding risk.

ABSTRACT

Factor (F) XI has emerged as a promising target for novel anticoagulants that could be safer than the currently available anticoagulants. This is because of mounting evidence that FXI is essential for thrombus growth but mostly dispensable for hemostasis. Milvexian, a small-molecule inhibitor of FXIa, is under evaluation in a FDA fast-track Phase 3 clinical trial program enrolling over 46,000 patients. The LIBREXIA AF trial (NCT05757869) compares milvexian to apixaban, a FXa inhibitor, for stroke prevention in atrial fibrillation (AF) patients. In contrast, the LIBREXIA Stroke (NCT05702034) and ACS (NCT05754957) trials evaluate milvexian versus placebo on top of antiplatelet therapy in patients with non-cardioembolic stroke and acute coronary syndrome (ACS). Abelacimab, an antibody that binds FXI and blocks its activation, is also in Phase 3 trials for treating cancer-related venous thromboembolism (VTE). The ASTER trial (NCT05171049) compares abelacimab with apixaban in patients eligible for direct oral anticoagulants (DOACs), while the MAGNOLIA trial (NCT05171075) compares abelacimab with dalteparin in cancer patients at high bleeding risk.

Such patients with AF, ACS, or cancer often require central venous catheters (CVC), which include peripherally inserted central catheters, tunneled catheters, and implanted ports, to facilitate venous access for administering chemotherapy, blood products, and parenteral nutrition. However, CVCs carry a risk of symptomatic deep vein thrombosis (DVT), with reported incidence rates from 1-5% over duration of catheter use for a few weeks to several months. Patients with CVC-associated DVT often experience arm pain, swelling, and impaired catheter function. Upper extremity DVT can lead to life-threatening

pulmonary embolism (PE) if the clot breaks off and travels to the lungs. In addition, blood clots inside the catheter can disrupt its function, making it difficult to deliver nutrition and drugs or draw blood and necessitating removal and replacement. Consequently, there is a need for effective anticoagulant regimens to prevent and treat CVC-induced thrombosis.

Previously, we showed that catheters adsorb FXII and trigger its autoactivation. FXIIa then activates FXI to FXIa, contributing to thrombin generation and fibrin clot formation. Heparin inhibits clotting by binding to antithrombin and enhancing its capacity to inhibit FXIIa, FXIa, FIXa, thrombin, and FXa. The rabbit model of catheter thrombosis demonstrated that knockdown of either FXII or FXI by antisense oligonucleotides (ASO) attenuates catheter-induced clotting and thrombin generation, suggesting that both FXII and FXI are attractive targets in catheter thrombosis. Despite evidence that FXII inhibition attenuates arterial and venous thrombosis, more attention has focused on FXI inhibition due to the possibility that feedback activation of FXI by thrombin amplifies coagulation, which may overcome FXIIa inhibition. These insights emphasize FXI as an attractive target for anticoagulation in patients requiring CVCs. However, the extent of FXIa inhibition required to prevent catheter thrombosis remains unknown.

To address this question, our thesis explores the effect of FXI inhibition on catheter thrombosis. Building on ASTER and MAGNOLIA trials, we strive to examine the impact of abelacimab on catheter-induced clotting and thrombin generation, comparing the results to that of apixaban and dalteparin using an established plate-based model of catheter thrombosis. Based on the LIBREXIA trials, we aim to compare the effects of milvexian versus heparin, given routinely in patients undergoing percutaneous coronary intervention (PCI) to prevent thrombotic complications.

Although the rate of FXI activation by thrombin is slow *in vitro*, polyphosphate (polyP) released by activated platelets significantly enhances this reaction by providing a template that binds FXI and thrombin. PolyP in platelets is an anionic linear polymer of 60-100 phosphate units. It is a potent procoagulant, specifically initiating coagulation via FXII in the contact pathway, which in turn triggers activation of FXI, leading to thrombin generation. The activity of thrombin is primarily mediated by its two exosites: exosite 1 and 2. Evidence suggests that exosite 2 binds to polyP and activates FXI. However, the role of exosite 1, which is the substrate-binding site, remains less clear.

In this thesis, we examine the role of catheters and polyP in feedback activation of FXI by thrombin and the effects of FXI inhibitors, such as milvexian and abelacimab, on catheter-induced clotting. First, we show that FXI binds to polyP and catheters. Such binding interaction can promote FXI autoactivation and activation by thrombin, allowing coagulation to proceed without FXII. These results help to explain our observations as to why catheter-induced clotting was partially decreased in FXII-depleted plasma but was completely abolished in FXI-depleted plasma. Second, we demonstrate that FXI activation by thrombin is mediated via an exosite-dependent mechanism. Specifically, exosite 2 ligands reduce FXI activation by blocking polyP from binding to thrombin, whereas exosite 1 ligands attenuate FXI activation through allosteric modulation. Next, we identify that milvexian and abelacimab attenuate catheter-induced clotting, with adjunctive heparin further enhancing the inhibitory effect of milvexian. These findings promote the FXI inhibitors as a novel antithrombotic strategy in patients with ACS and AF enrolled in the LIBREXIA trials undergoing PCI.

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P.S. I apologize in advance if I missed anyone.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ADP	Adenosine diphosphate
AF	Atrial fibrillation
ACS	Acute coronary syndrome(s)
ANOVA	Analysis of Variance
APC	Activated Protein C
aPTT	Activated partial thromboplastin time
ASO	Antisense oligonucleotide
AT	Antithrombin
AUC	Area under the curve
CaCl2	Calcium chloride
CIHR	Canadian Institute for Health Research
CTI	Corn trypsin inhibitor
CVC	Central venous catheter
CD	Catalytic domain
Da	Dalton(s)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DOAC	Direct oral anticoagulant
DVT	Deep vein thrombosis
ECMO	Extracorporeal membrane oxygenation
ETP	Endogenous thrombin potential
F	Factor
FeCl3	Ferric chloride

GAGs	Glycosaminoglycans
HBS	Hepes buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK	High-molecular-weight kininogen
HRG	Histidine-rich glycoprotein
IU	International unit
IV	Intravenously
Ka	α-Kallikrein
KO	Knockout
Kd	Dissociation constant
Ki	Inhibition constant
LMWH	Low molecular weight heparin
mRNA	Messenger ribonucleic acid
MHV	Mechanical heart valve
NaCl	Sodium chloride
NETs	Neutrophil extracellular traps
PAR	Protease-activated receptor
PC	Protein C
PCI	Percutaneous coronary intervention
PE	Pulmonary embolism
PEG	Polyethylene glycol
PEO	Polyethyleneoxide
PICC	Peripherally inserted central catheter
PKa	Plasma kallikrein (activated)
PolyP	Polyphosphate
PPP	Platelet-poor plasma

PS	Phosphatidylserine
PT	Prothrombin time
RNA	Ribonucleic acid
RU	Response units
SA	Streptavidin
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SC	Subcutaneously
SPR	Surface plasmon resonance
TAFI	Thrombin activatable fibronlysis inhibitor
TBS	Tris-buffered saline
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
TM	Thrombomodulin
tPA	Tissue plasminogen activator
UFH	Unfractionated heparin
UPA	Urokinase plasminogen activator
VKA	Vitamin K-antagonist
VTE	Venous thromboembolism
vWF	Von Willebrand factor
WT	Wild-type

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Ruiqi Yin, declare that this thesis, entitled "Factor XI Activation and Inhibition in Catheter-induced Clotting," is a record of my independent research under the supervision of Dr. Jeffrey Weitz. No part of this thesis has been submitted or published for the award of any other degree.

My supervisor, Dr. Jeffrey Weitz, and the members of my supervisory committee, Dr. Patricia C. Liaw and Dr. Peter L. Gross, have provided guidance and support at all stages of this project.

All research work reported in this thesis is presented as three manuscripts in Chapters 3 to 5. Chapter 3 has been published in the *Journal of Thrombosis and Haemostasis*. Chapter 4 is currently being peer-reviewed by the Janssen Pharmaceutical Company. Chapters 4 and 5 are being prepared to be submitted to the *Journal of Thrombosis and Haemostasis.*

CHAPTER 1: INTRODUCTION

1.1 Overview of Hemostasis

Hemostasis is a physiological process that stops bleeding while maintaining blood in a fluid state within the vascular system. Upon vascular injury, the body reacts by constricting the blood vessels, forming a platelet plug, activating the coagulation cascade, and eventually leading to the formation of a fibrin clot. Excessive formation obstructs the blood vessels and disrupts the normal blood flow. Therefore, hemostasis requires a delicate balance between clot formation and clot lysis (Gale, 2011) (Periayah et al., 2017).

Hemostasis has three important stages: primary, secondary, and tertiary (Periayah et al., 2017). Primary hemostasis begins with narrowing the blood vessels to limit bleeding at damaged endothelial cells rapidly. In addition, collagen from the sub-endothelial cells is exposed to the blood, mediating platelet adhesion and forming a primary platelet plug. Secondary hemostasis involves the activation of clotting factors, leading to the formation of insoluble fibrin and cross-linked fibrin mesh by thrombin cleavage, which stabilizes the platelet plug (Gale, 2011) (Periayah et al., 2017). The intrinsic and extrinsic coagulation pathways mediate this process, both merging at the level of factor (F) X activation in the common pathway, resulting in the formation of fibrin clots (Versteeg et al., 2013) (Periayah et al., 2017). Tertiary hemostasis, or clot lysis, involves the breakdown of the fibrin clots. This step is important in preventing excessive fibrin clot formation and ensuring healthy blood flow (Periayah et al., 2017). Any disruption in the balance between thrombus clot formation and clot lysis can eventually lead to bleeding or thrombotic diseases, which are detrimental to health.

1.2 Thrombosis

Thrombosis is a pathological condition that refers to the formation of an occlusive blood clot inside the lumen of a blood vessel (Raskob et al., 2014), blocking normal blood flow and potentially leading to ischemia and death (Smith et al., 2015). Statistics show that thrombosis-associated diseases are the leading cause of mortality and are directly correlated with 1 in 4 deaths worldwide (Raskob et al., 2014). The underlying risk factors of thrombosis can be varied, including immobility, inflammation, obesity, surgery, and cancer (Engbers et al., 2010). Therefore, an improved understanding of hemostasis and thrombosis continues to benefit patients with thrombosis disorders.

There are two main classifications of thrombosis: arterial and venous thrombosis. Although both are associated with occlusion in the blood vessels, their formation processes have major differences. Arterial thrombosis occurs in the arteries under high shear flow and forms when platelet-rich thrombi localize around damaged endothelium or ruptured atherosclerotic plaques. As the atherosclerotic plaque ruptures, the prothrombotic materials from the necrotic core are exposed and induce platelet aggregation and activation (Koupenova et al., 2016). Typical arterial thrombosis occurs in the coronary arteries in patients with myocardial infarction or the cerebral arteries in patients with stroke. Venous thrombosis often occurs under low shear flow and mostly forms around intact endothelial walls of deep veins in the lower legs, a condition known as deep vein thrombosis (DVT) (Koupenova et al., 2016) (Wall et al., 2016). DVT is often influenced by hypercoagulability, stasis, and endothelial injury, known as Virchow's triad (Figure 1) (Wall et al., 2016). These thrombus clots sometimes cause fatal conditions, for instance, when the clots break off, travel through the circulation, and create a blockage in the lungs, a condition known as pulmonary embolism (PE). Although both arterial and venous clots are composed of platelets and fibrin, arterial blood clots are associated with higher levels of platelets, whereas venous blood clots contain higher levels of fibrin (Koupenova et al., 2016). Therefore, antiplatelet therapy is often used to treat arterial thrombosis, while anticoagulant therapy treats venous thrombosis.

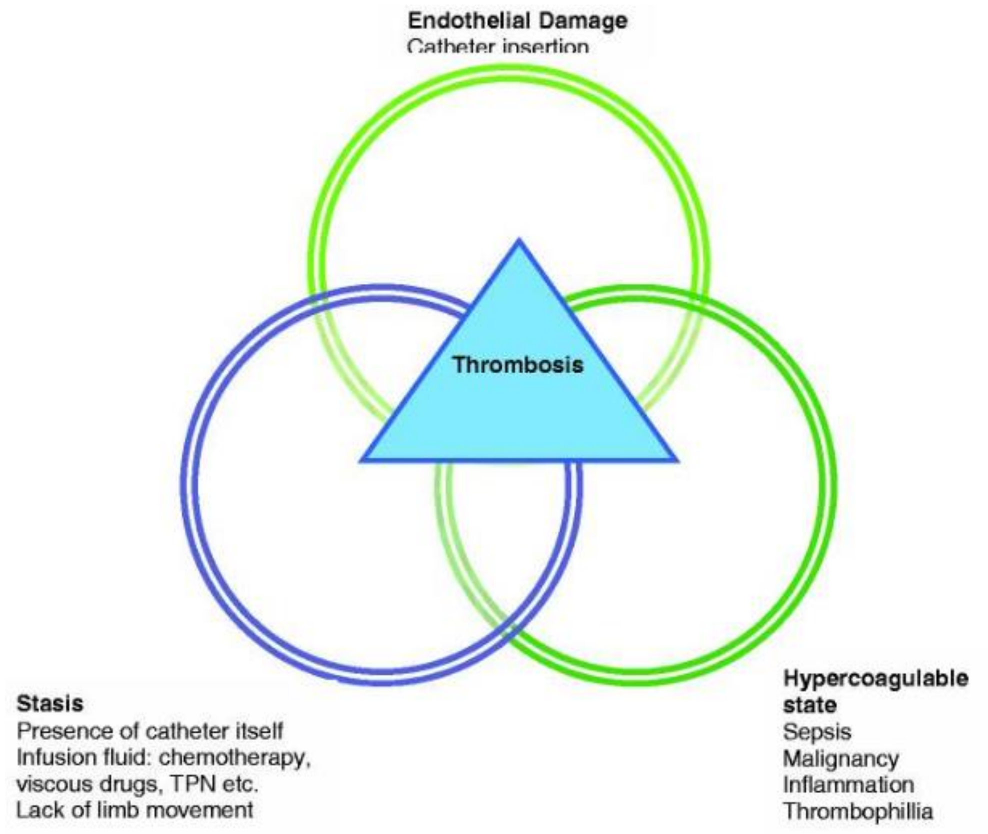
Endothelial Damage
Catheter insertion
Thrombosis
Stasis
Presence of catheter itself
Infusion fluid: chemotherapy,
viscous drugs, TPN etc.
Lack of limb movement
Hypercoagulable
state
Sepsis
Malignancy
Inflammation
Thrombophillia

Figure 1. Virchow's triad contributes to the development of thrombosis.

Thrombosis is initiated through endothelial damage or vascular injury at the catheter insertion site, which exposes tissue factors and induces the hypercoagulable state. Patients with certain underlying diseases have increased coagulability. The formation of a thrombus around or inside the catheter obstructs blood flow (stasis). Stasis and hypercoagulable state play a critical role in device-associated thrombosis. Figure is obtained from (Wall et al., 2016).

1.3 Coagulation System

The coagulation system is a dynamic process consisting of a series of proteolytic reactions in which clotting factors are activated, forming a fibrin clot to stop bleeding. In 1964, the Waterfall Model of coagulation cascade was proposed (Davie & Ratnoff, 1964). This model describes the coagulation process as occurring via two pathways, extrinsic and intrinsic pathways, which converge downstream to form the common pathway (Figure 2) (Grover & Mackman, 2019).

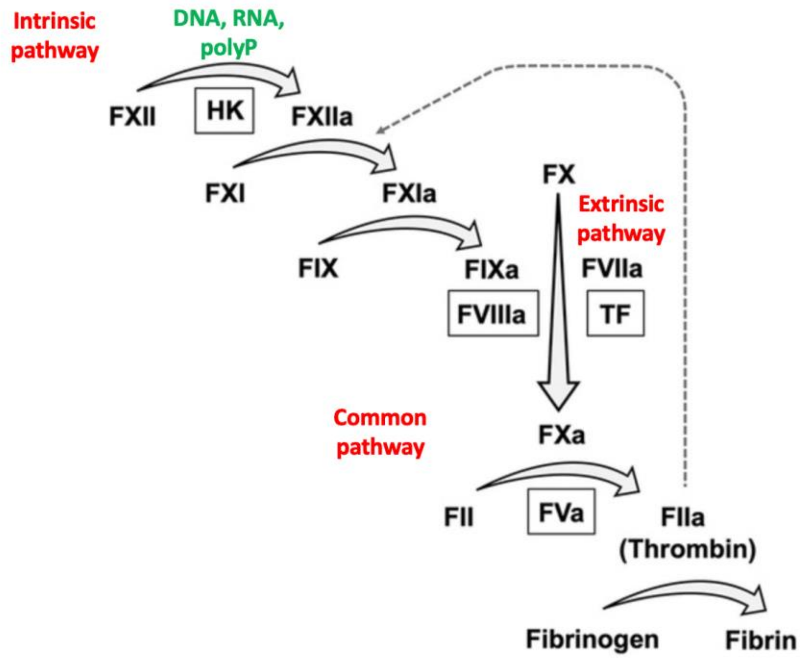
Intrinsic pathway
DNA, RNA, polyP
FXII
HK
FXIIa
FXI
FXIa
FX
Extrinsic pathway
FIX
FIXa
FVIIa
FVIIIa
TF
Common pathway
FXa
FII
FVa
FIIa (Thrombin)
Fibrinogen
Fibrin

Figure 2. The waterfall model of the coagulation cascade.

Coagulation occurs when the intrinsic and extrinsic pathways unite downstream to activate FX in the common pathway. The extrinsic pathway is activated when tissue factor (TF) binds to circulating FVII, forming a TF-FVIIa complex (extrinsic tenase). Extrinsic tenase initiates the downstream activation of coagulation factors and ultimately leads to the initial generation of thrombin. The intrinsic pathway is triggered when FXII interacts with negatively charged surfaces or polyanions, such as DNA, RNA, and polyphosphate (polyP), forming FXIIa. FXIIa then activates FXI to FXIa, which promotes thrombin generation and fibrin clot formation through activation of FIX. The generated thrombin can also provide feedback to activate FV, FVIII, and FXI, upregulating its generation. Figure is adapted from (Grover & Mackman, 2019). *Abbreviations*: DNA, deoxyribonucleic Acid; RNA, ribonucleic Acid ; polyP, polyphosphate; FXII, factor XII; FXI, factor XI; FIX, factor IX; FVIII, factor VIII; FII, thrombin; FV, factor V; TF, tissue factor; FX, factor X; FVII; factor VII; PARs, protease-activated receptors. Figure is adapted from (Hungtinton, 2005).

1.3.1 Extrinsic Pathway

The extrinsic pathway begins with factors external to the blood. It is also known as the tissue factor (TF) pathway. This pathway is activated when plasma comes into contact with TF, which is expressed by sub-endothelial cells after vascular injury or by either TF-bearing microparticles or TF-positive monocytes in the blood (Mackman, 2006) (Smith 2009). TF is an integral membrane protein which contains three domains: extracellular, transmembrane, and intracellular portion (Vojacek, 2017). The extracellular portion of TF binds to FVIIa, forming the extrinsic tenase complex (TF-FVIIa) through strong protein-protein interactions. In addition, the formation of this complex is stabilized and positioned on the TF-bearing cell membrane via protein interactions, and protein-phospholipid interactions in the presence of calcium (Ca^{2+}) ions enhance the activity. The intracellular portion is responsible for mediating the signaling function of TF. The TF-FVIIa complex activates FX to FXa in the presence of Ca^{2+} (Vojacek, 2017). In the absence of TF, extrinsic pathway activation does not occur. To date, there are no reports of humans deficient in TF (Grover & Mackman, 2018), which may be because such a genetic defect results in non-viable embryos (Toomey et al., 1996). In addition, previous studies have shown that mice deficient in TF and FVII die during embryonic development or shortly after birth, suggesting a significant role of TF in hemostasis (Bugge et al., 1996) (Carmeliet et al., 1996).

1.3.2 Intrinsic (or Contact) Pathway

The intrinsic pathway is called ‘intrinsic’ because it responds to spontaneous and internal vascular endothelium damage. It is initiated by the activation of FXII to FXIIa

(Smith et al., 2015). FXII can be activated by binding to negatively charged molecules like naturally occurring polyanions (Smith et al., 2006) (Kannemeier et al., 2007) (Patri et al., 2016) (Noubouossie et al., 2017). Once activated, FXIIa activates prekallikrein (PK) to kallikrein in the presence of high molecular weight kininogen (HK). Kallikrein further catalyzes the conversion of FXII to FXIIa, forming a positive feedback loop that significantly amplifies FXII activation (Smith et al., 2015) (Engel et al., 2014). FXIIa converts FXI to FXIa, which then activates FIX to FIXa. FXI circulates with HK, a non-enzymatic cofactor (Monroe & Hoffman, 2006). HK brings FXI and PK closer to the activating surface, allowing FXIIa to activate FXI more efficiently (Engel et al., 2014) (de Maat & Maas, 2016). Together with Ca^{2+} ions and phospholipids, FIXa forms an intrinsic tenase with its regulatory cofactor FVIIIa and catalyzes the activation of FX to FXa in the common pathway, leading to the formation of thrombin (van der Meijden & Heemskerk, 2010) (Smith et al., 2015). In addition, thrombin can feedback and activate FV, FVIII, and FXI, ensuring a continuous amplification of thrombin generation (Geng et al., 2013). Furthermore, the intrinsic pathway is connected to the inflammatory response by activating kallikrein, which cleaves HK and releases bradykinin (BK), a mediator associated with inflammation (Schmaier, 2016).

The contact system is often merged with the intrinsic pathway because they are both activated in the presence of negatively charged polyanions, contributing to the overall coagulation process. The contact system involves autoactivation of FXII upon interaction with surfaces like kaolin or other polyanions, leading to the activation of prekallikrein and high-molecular-weight kininogen (HK). This activation ultimately leads to activation of the intrinsic pathway, which begins via the activation of FXI. Understanding how naturally occurring negatively charged polyanions activate the contact system is important in studying the formation of medical device-induced thrombosis, as device materials mimic these polyanions (Jaffer et al., 2015). Despite overlap between contact system and instrinsic pathway, the contact system is primarily associated with clotting factors, such as FXII, PK, and HK, which are responsible for coagulation as well as inflammation (Long et al., 2016). In contrast, the intrinsic pathway involves FXI and FIX, which aim to form a fibrin clot (Engel et al., 2014) (de Maat & Maas, 2016).

1.3.3 Common Pathwayx

As mentioned earlier, the end products of extrinsic and intrinsic pathways converge at the level of FX activation in the common pathway (Smith et al., 2015) (Vojacek, 2017). The common pathway occurs includes the prothrombinase complex (FXa-FVa) which cleaves prothrombin into its activated serine protease form, thrombin, in the presence of Ca^{2+}. Thrombin mediates fibrin clot formation by converting soluble plasma fibrinogen to insoluble fibrin strands (Patri et al., 2016). Thrombin-activated FXIIIa further stabilizes the clot by catalyzing the cross-linking of fibrin molecules, rendering it resistant to plasmin degradation.

1.3.4 Cell-based Model of Coagulation

The waterfall cascade model focuses on a sequential series of biochemical reactions but is less comprehensive in explaining physiological conditions (Rana & Neeves, 2016). Currently, the cell-based coagulation model is widely used because it demonstrates the complexity of coagulation *in vivo*, emphasizing the interactions of cellular elements of blood, including platelets, endothelial cells, and coagulation factors (Monroe & Hoffman, 2006) (Smith 2009). In the cell-based model, thrombin production is highlighted in three phases: initiation, amplification, and propagation (Figure 3).

During the initiation phase, the extrinsic tenase (TF-FVIIa) complex activates FX to FXa, marking the start of the common pathway. In particular, it occurs when cells expressed TF are exposed to blood at the injury site. The TF binds to FVIIa, forming TF-FVIIa complex (Vojacek, 2017) responsible for the activation of small amounts of FIX and FX, thereby generating small amounts of thrombin (Smith 2009) (Vojacek 2017). In this phase, platelets bind to collagen exposed by subendothelial cells and von Willebrand factor (vWF) to mediate platelet adhesion and aggregation. In addition, thrombin activates cofactors FVIII and FV on the platelet surface, further amplifying the coagulation system.

The amplification phase occurs when small amounts of thrombin on the TF-bearing cells activate nearby platelets, exposing receptors and binding sites for various clotting factors, such as FV and FVIII, in their activated forms, FVa and FVIIIa, respectively (Smith 2009) (Swieringa et al. 2018). This phase allows the recruitment of more platelets to the site of injury and the formation of prothrombinase complex on activated platelets (Mutch et al., 2010). This complex comprises FXa and its cofactor FVa in the presence of Ca^{2+} ions and phospholipids. Phospholipids on the surface of activated platelets provide a negatively charged surface essential for the assembly and activity of the prothrombinase complex.

Small amounts of thrombin from the initiation phase also activate FXI to FXIa on the platelet surface. In the cell-based model, the mechanism that thrombin back activates FXI plays an important role in sustaining thrombin generation and consolidating coagulation (Emsley et al., 2010). Observations that people with FXI deficiency (hemophilia C) generally experience only mild or no bleeding support this, as the deficiency indicates other processes exist to compensate for the lack of FXI in maintaining hemostasis. This observation is further supported by findings regarding the absence of bleeding in individuals with FXII deficiency (Asakai et al., 1991) (Gailani & Broze, 1991) (Seligsohn, 2007) (Gailani and Smith 2009).

In the propagation phase, thrombin generation is maximized by forming intrinsic tenase on activated platelets. FIXa generated by extrinsic tenase from the initiation phase binds to FVIIIa to form the intrinsic tenase (FIXa-FVIIIa) complex in the presence of Ca^{2+} ions and phospholipids. The intrinsic tenase cleaves FX to FXa, which binds to cofactor FVa and accelerates the cleavage of prothrombin to thrombin (Smith, 2009) (Swieringa et al. 2018). Hence, the formation of intrinsic tenase results in a robust thrombin generation necessary to produce a stable fibrin clot (Smith, 2009). Therefore, the extrinsic and intrinsic tenase play critical roles in the amplification and propagation phase in a cell-based model of coagulation.

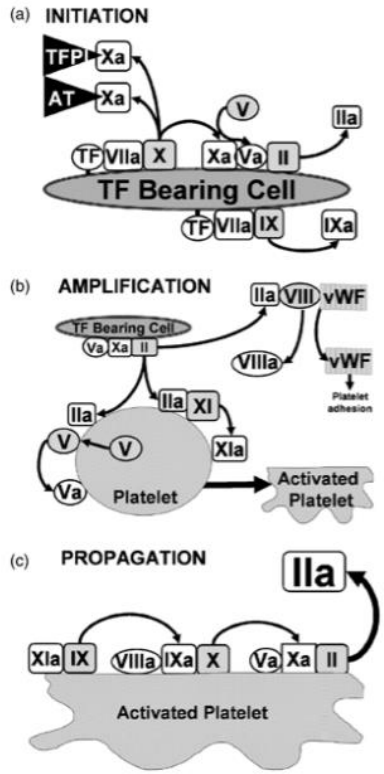
(a) INITIATION
TFPI
Xa
AT
Xa
V
IIa
TF
VIIa
X
Xa
Va
II
TF Bearing Cell
TF
VIIa
IX
IXa
(b) AMPLIFICATION
IIa
VIII
vWF
TF Bearing Cell
Va
Xa
II
VIIIa
vWF
Platelet adhesion
IIa
XI
IIa
V
V
XIa
Va
Platelet
Activated Platelet
(c) PROPAGATION
IIa
XIa
IX
VIIIa
IXa
X
Va
Xa
II
Activated Platelet

Figure 3. Cell-based model coagulation.

(a) The initiating phase occurs when circulating FVIIa in the blood binds to the TF-bearing cell, forming the extrinsic tenase, activating FIX and FX. FXa and its cofactor FVa generate the first trace of thrombin from prothrombin. (b) During the amplification phase, thrombin activates FXI, FVIII, and FV, ultimately forming a prothrombinase complex, FXa-FVa, to allow a significant thrombin production. (c) The propagation step occurs when FXIa generated from the initiation phase activates FIX to FIXa, which binds to FVIIIa to form the intrinsic tenase complex FIXa-FVIIIa. This intrinsic tenase complex activates FX to FXa, resulting in a burst production of thrombin in cofactor FVa. *Abbreviations*: TFPI, Tissue factor pathway inhibitor; AT, anti-thrombin; polyP, polyphosphate; FXII, factor XII; FXI, factor XI; FIX, factor IX; FVIII, factor VIII; FII, thrombin; FV, factor V; TF, tissue factor; FX, factor X; FVII; factor VII; vWF, von Willebrand Factor. Figure is obtained from (Patri et al., 2016).

1.4 Activators of Contact System

Activation of the contact pathway typically involves the interaction of contact factors, such as prekallikrein, HMWK, and FXII, with exposed collagen or negatively charged surfaces that promote activation of zymogen FXII (Figure 4). Upon surface binding, zymogen FXII undergoes a conformational change, leading to autoactivation and the formation of FXIIa (Engel et al., 2014). Autoactivation of FXII occurs at the Arg353-Val 354 bond, converting the single chain to two chains of FXIIa linked by a disulfide bond (Naudin et al., 2017). Activators that trigger FXII autoactivation *in vivo* include physiological surfaces, such as polyphosphate (polyP), collagen, nucleic acids (DNA and RNA), as well as synthetic surfaces (glass, kaolin, ellagic acid, dextran sulfate, and silica) (Müller et al., 2009) (Smith et al., 2015) (Schmaier, 2016). Medical components found in mechanical heart valves and guide catheters can also act as activators (Yau et al., 2011) (Eikelboom et al., 2013). FXIIa promotes the intrinsic coagulation system via FXI activation, which leads to thrombin and fibrin formation. Alternatively, FXIIa triggers the kallikrein-kinin system via PK, which releases the proinflammatory marker bradykinin (Naudin et al., 2017). Hence, the contact system is critical to proinflammatory and procoagulant disorders.

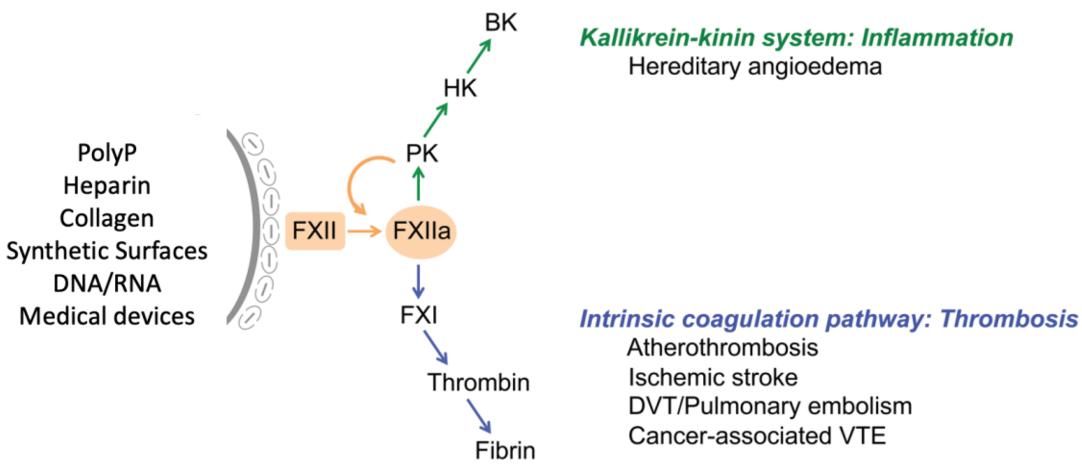
BK
HK
PK
PolyP
Heparin
Collagen
Synthetic Surfaces
DNA/RNA
Medical devices
FXII
FXIIa
FXI
Thrombin
Fibrin
Kallikrein-kinin system: Inflammation
Hereditary angioedema
Intrinsic coagulation pathway: Thrombosis
Atherothrombosis
Ischemic stroke
DVT/Pulmonary embolism
Cancer-associated VTE

Figure 4. Naturally occurring and synthetic activators of the contact system.

Negatively charged surfaces, including naturally occurring polyanions (polyP, DNA, and RNA), synthetic surfaces (glass, kaolin, ellagic acid, dextran sulfate, and silica), and medical devices (catheters and mechanical heart valve) induce FXII autoactivation. FXIIa activates intrinsic coagulation via FXI, resulting in thrombin and fibrin formation and contributing to various thrombotic disorders. FXIIa also activates the kallikrein-kinin system via PK, forming BK and ultimately contributing to proinflammatory diseases. *Abbreviations*: BK, bradykinin; HK, high-molecular-weight kininogen; polyP, polyphosphate; PK, plasma kallikrein ; FXII, factor XII; FXI, factor XI. Figure is adpated from (Naudin et al., 2017).

1.4.1 Nucleic acids

Nucleic acids, such as DNA and RNA, are negatively charged polymers and exhibit extracellular roles in inflammation and coagulation. DNA and RNA can be derived from cells undergoing apoptosis or necrosis. DNA and RNA are associated with enhancing FXII activation by PK and HK as well as accelerating FXI activation by thrombin up to 12-fold (Kannemeier et al., 2007) (Vu et al., 2016). In addition, both DNA and RNA are potent activators of FXII autoactivation in plasma, indicating their contributions to chronic contact system-induced diseases (Kannemeier et al., 2007). Single-stranded DNA from neutrophil extracellular traps (NETs), which are activated neutrophils that release de-condense chromatin, have been recognized as a critical activator of the coagulation system, promoting the assembly of FXII zymogen and leading to thrombus propagation of in murine model of DVT (von Brühl et al., 2012). In sepsis, nucleic acids from bacteria may activate FXII, which ultimately promotes coagulation and inflammation (Kannemeier et al., 2007) (Brill et al., 2012). Therefore, nucleic acids are procoagulant and proinflammatory mediators, and targeting DNA/RNA may effectively combat thrombotic disorders and sepsis.

1.4.2 Polyphosphate

For almost half a century, it has been known that platelet-rich plasma activates FXII and promotes coagulation in an FXII-dependent manner independently of the TF-induced extrinsic pathway (Naudin et al., 2017). What exactly triggers FXII contact activation in the procoagulant platelets? Multiple experiments using human and murine plasma identified platelet polyP as an important FXII contact activator *in vivo* (Ruiz et al., 2004).

PolyP is an anionic linear polymer consisting of inorganic phosphate groups that are linked by high-energy phosphoanhydride bonds (Figure 5) (Müller et al., 2009) (Morrissey, 2012). Previous studies have identified that polyP has a half-life of 90 minutes in plasma due to degradation by phosphatase (an enzyme that hydrolyzes polyphosphate) (Smith et al., 2010). It is reported that when polyP is stored in a dense granule, it has a concentration of approximately 130 mM (Ruiz et al., 2004). Upon platelet activation, polyP is secreted into the blood circulation and has a concentration of about 1-3 μM (Ruiz et al., 2004) (Morrissey et al., 2012). The chain length of polyP varies among different organisms. Microorganisms or bacteria release longer polymers ranging from 100 to more than 1000 phosphate units. In contrast, platelet-dense granules, lysosomes, and mitochondria release shorter polymers ranging from 60 to 100 phosphate units upon platelet activation (Müller et al., 2009) (Smith et al., 2010). To date, the function, biosynthesis, and storage of prokaryotic polyP are well studied, whereas mammalian polyP requires further study.

PolyP is proposed as a potent modulator of hemostasis, as it initiates the human blood-clotting system via the activation of contact pathway through activation of FXII (Smith et al., 2006), accelerates thrombin-mediated FXI activation (Choi et al., 2011), promotes FV activation by thrombin and FXa, and enhances the thickness of the fibrin clot structure (Smith et al., 2006) (Smith and Morrissey, 2008) (Figure 6). While polyP has been shown *ex vivo* to activate pathways not only through FXII, its *in vivo* roles in FXII-independent mechanisms remain to be elucidated.

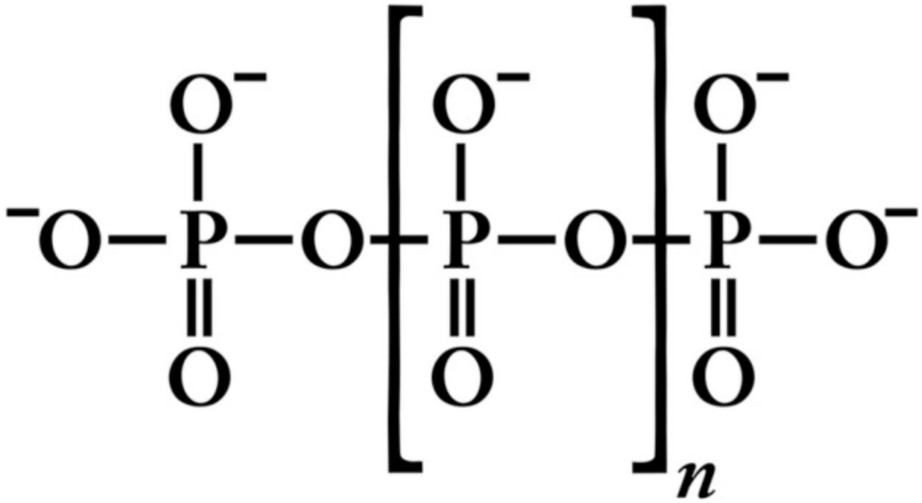

Figure 5. Structure of inorganic polyP.

PolyP varies in length from just a few phosphates to thousands of phosphate units long, depending on the source of a biological system. Human-activated platelets release approximately 60-100 phosphate units long, and bacteria can release hundreds or even thousands of phosphate units long. Figure is obtained from (Morrissey, 2012).

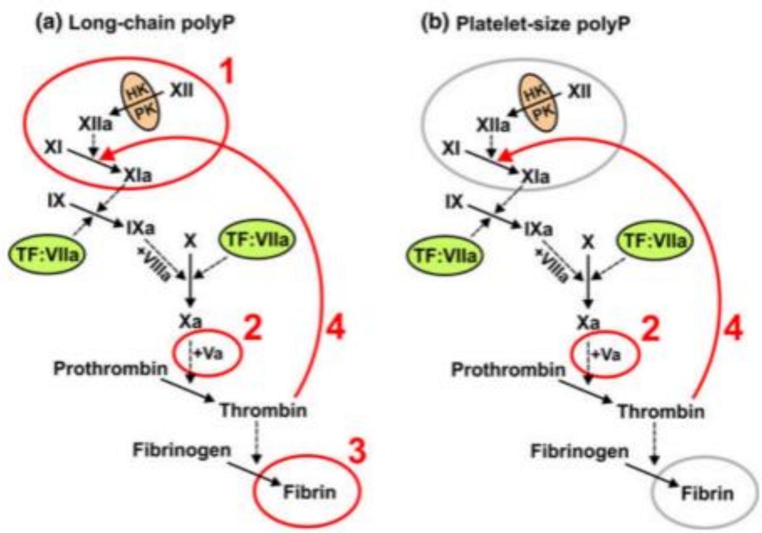
(a) Long-chain polyP
(b) Platelet-size polyP
HK
PK
XII
XIIa
XI
XIa
IX
IXa
X
Xa
TF:VIIa
+VIIIa
+Va
Prothrombin
Thrombin
Fibrinogen
Fibrin
1
2
3
4

Figure 6. Differential roles of long-chain polyP and platelet-size polyP in coagulation cascade.

(a) Long-chain polyP primarily promotes blood clotting at 4 points of the coagulation cascade: 1. initiates contact activation by autoactivating FXII to FXIIa, 2. promotes FV activation by thrombin and FXa, 3. enhances fibrin thickness, and 4. Promotes thrombin-mediated feedback activation of FXI. (b) Platelet-size polyP primarily accelerates blood clotting via 3 points: FXII autoactivation, FV to FVa activation, and thrombin-mediated feedback activation of FXI. *Abbreviations*: PolyP, polyphosphate; FXII, factor XII; FXI, factor XI; FIX, factor IX; FVIII, factor VIII; FV, factor V; FX, factor X. Figure is obtained from (Morrissey, 2012).

1.5 Natural Anticoagulant Pathways

The anticoagulant systems are critical in limiting excessive clot formation, and maintaining the blood in a fluid state. There are three major anticoagulant systems: antithrombin (AT), the protein C pathway, and tissue factor pathway inhibitor (TFPI) (Palta et al., 2014).

1.5.1 Antithrombin

AT (also known as antithrombin III) is a serine protease inhibitor (serpin) produced in the liver. It primarily inhibits thrombin and FXa, as well as FIXa and FVIIa (Ezihe-Ejiofor & Hutchinson, 2013). The inhibition of thrombin by AT alone is relatively slow, however, it is greatly increased by about 2000-fold in the presence of heparan sulfate (Blajchman, 1994) (Frebelius et al., 1996). Heparan sulfate is a polysaccharide found on vascular endothelial cells, which binds to AT, inducing a conformational change. This action allows AT forms a stable covalent complex, with the protease, resulting in a potent inhibitory effect on the coagulation process (Ezihe-Ejiofor & Hutchinson, 2013).

1.5.2 Protein C

Protein C is a vitamin-K-dependent serine protease zymogen produced in the liver and circulates in the blood (Dahlbäck & Villoutreix, 2005) (Ezihe-Ejiofor & Hutchinson, 2013). Vitamin-K dependent proteins include clotting factors such as prothrombin, FVII, FIX, and FX, as well as natural anticoagulants protein C and S. These proteins require vitamin K as a cofactor to undergo carboxylation of select glutamic acid residues, enabling their activation. Protein C becomes activated to activated protein C (APC) by binding to endothelial protein C receptor (EPCR) and thrombin-thrombomodulin (TM) complex in the presence of protein S (a cofactor of APC). APC serves as a potent anticoagulant as it mainly cleaves and limits the activities of FVa and FVIIIa in the presence of protein S and phospholipids (Dahlbäck & Villoutreix, 2005) (Ezihe-Ejiofor & Hutchinson, 2013).

1.5.3 Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is a polypeptide produced by endothelial cells and circulates in the plasma (Lindahl et al., 1991) (Ezihe-Ejiofor & Hutchinson, 2013). It is also the primary inhibitor of the tissue factor pathway. It constrains the function of FVIIa and FXa via the formation of TFPI-FXa complex and TFPI-FXa-TFPI-FVIIa quaternary complex in the presence of Ca^{2+} ions and phospholipids (Lindahl et al., 1991) (Ezihe-Ejiofor & Hutchinson, 2013).

1.6 Fibrinolysis

Fibrinolysis is a process of breaking down fibrin mesh to reduce blood clots from growing inside the vasculature (Cesarman‐Maus & Hajjar, 2005) (Thrall et al., 2007) (Mutch & Medcalf, 2023). The main fibrinolytic protease is plasmin, which cleaves fibrin (insoluble form) into soluble fibrin degradation products. Plasminogen is an inactive precursor of plasmin, that is produced by the liver and that circulates in the bloodstream (Cesarman‐Maus & Hajjar, 2005). Plasminogen is activated by tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Plasmin activity is limited by inhibitors including alpha-2-antiplasmin and alpha-2-macroglobulin (Cesarman‐Maus & Hajjar, 2005) (Thrall et al., 2007). The balance between the procoagulant, anticoagulant pathways, and fibrinolytic system is essential to maintain normal hemostasis. This not only ensures cessation of bleeding from a damaged blood vessel but also prevents the formation of excessive clots that obstruct normal blood flow.

1.7 Thrombin

Thrombin is a central player in the intricate balance between hemostasis and thrombosis within the coagulation system (Lane et al., 2005). It is an important procoagulant factor, which has various functions in coagulation, such as mediating platelet activation, cleavage of fibrinogen to fibrin, and feedback activation of many clotting factors necessary for the amplification of coagulation process (Di Cera, 2003) (Lane et al., 2005). However, thrombin activation is a highly regulated process. Thrombin simultaneously serves as an anticoagulant with multiple checkpoints to prevent excessive clot formation. Therefore, thrombin formation is a central target for preventing and treating thrombotic disorders.

1.7.1 Thrombin Structure and Regulation

Thrombin is a serine protease derived from the circulating zymogen prothrombin, with a molecular weight of approximately 37 kDa. Thrombin is composed of two chains covalently linked by a single disulfide chain: a 49 amino acid light chain (A-chain) and a 259 amino acid heavy chain (B-chain) (Carter et al., 2010).

Thrombin is a central mediator of thrombosis and hemostasis. It acts as a procoagulant mediator when converting fibrinogen to fibrin, and inducing platelet activation. In addition, thrombin amplifies its generation through feedback activation of FV, FVIII, and FXI (Adams & Huntington, 2006). However, interaction with other cofactors changes the specificity of thrombin to an anticoagulant mediator. When thrombin binds to TM, it mediates the conversion of protein C to APC on the endothelial cell surface, which limits thrombin formation by inhibiting activation of FV and FVIII (Esmon & Owen, 1981) (Adams & Huntington, 2006). Moreover, thrombin attenuates fibrinolysis through activation of thrombin-activatable fibrinolysis inhibitor (TAFI) by reducing the binding of plasminogen and tPA for fibrin, removing the C-terminal lysine and arginine residues from fibrin (Morser, 2012). Hence, the dual functions of thrombin in the coagulation process make it a key regulator of hemostasis.

1.7.2 Thrombin Exosites

Thrombin contains two critical domains, known as exosite 1 and exosite 2, that regulate substrate recognition and modulate its diverse function (Lane et al., 2005). Previous studies have shown that most of the thrombin substrates and cofactors interact with at least one exosite (Davie & Kulman, 2006). These two positively charged exosites are positioned at the opposite sides of the active site cleft (Figure 7). The active site of thrombin contains a serine residue (S195) where its substrate must align along this groove. The loops and charged patches surrounding the active site pocket control the thrombin's specificity (Lane et al., 2005).

Exosite 1 serves as the fibrinogen recognition site and is also important for interaction with FV, FVIII, TM, protease-activated receptors (PARs), and thrombin inhibitors, such as heparin cofactor II, and hirudin (Lane et al., 2005). Interaction of exosite 1 with fibrin facilitates the cleavage of FXIII to FXIIIa, strengthening the fibrin clot and ensuring further coagulation in the platelet plug (Janus et al., 1983) (Hall et al., 2001). All these ligands bind to exosite 1 and orient them into the active site cleft. On the other hand, exosite 2 plays an important role in thrombin localization and regulation. It is initially identified as the binding site for heparin (Fenton et al., 1988). By binding to exosite 2, heparin bridges thrombin and inhibitors, such as AT, to reduce thrombin activity and attenuate thrombus formation (Olson & Björk, 1994). In addition, exosite 2 binds to platelet surface receptor (Gp1bα), localizing thrombin on the platelet surface, thereby promoting cleavage of PARs and platelet activation (Mutch et al., 2010). Furthermore, the interaction of exosite 2 and γ' chain of fibrin protects against inhibition by AT and heparin cofactor II (Lovely et al., 2003) (Petrera et al., 2009). Therefore, exosite interactions are important for substrate recognition and regulation.

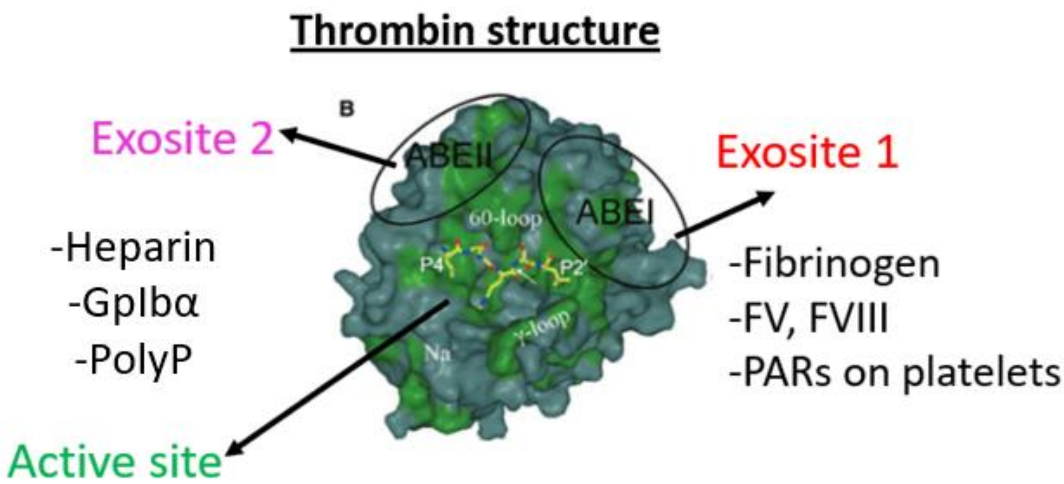
Thrombin structure
B
Exosite 2
ABEII
Exosite 1
ABEI
60-loop
P4
P2'
Na
γ-loop
-Heparin
-GpIbα
-PolyP
-Fibrinogen
-FV, FVIII
-PARs on platelets
Active site

Figure 7. Schematic figure of thrombin structure.

Thrombin is a serine protease with a molecular weight of 37,000. Exosite 1 and 2 are located on the opposite sides of the thrombin active site. Exosite 1 is the fibrinogen recognition site and is important for interacting with FV, FVIII, and protease-activated receptors (PARs) on platelets. Exosite 2 mediates interaction with heparin, polyP, and GPIbα on platelets. *Abbreviations:* ABE I, anion-binding exosite 1; ABE II, anion-binding exosite 2; GpIbα, platelet glycoprotein 1b alpha; polyP, polyphosphate; FV, factor V; FVIII, factor VIII; PARs, protease-activated receptors. Figure is adapted from (Hungtinton, 2005).

1.7.3 Allosteric Regulation

Thrombin regulation is not limited to substrate recognition, localization, and competition for exosite binding but also includes allosteric modulation (Adams & Huntington, 2006). Previous studies have demonstrated that TM binding to exosite 1 or prothrombin fragment 2 binding to exosite 2 induces a conformational change in the active site, allowing the thrombin substrate to interact more efficiently (Koeppe et al., 2005). In addition, inter-exosite allostery also exists, where fibrin binding to exosite 1 is attenuated by the addition of exosite 2 ligands. Our previous findings have shown that exosite 2 ligand, HD22, reduces the binding affinity of thrombin for TM and γA/γA-fibrin isoform of fibrin(ogen), an interaction specifically mediated by exosite 1 (Petrera et al., 2009) . Similar observations are made with addition of another exosite 2 ligands, such as γ′-peptide, GpIbα peptide analog (Chen et al., 2017). It is important to note that these allosteric effects also apply reciprocally, where the addition of exosite 1 ligands, such as HD1 or hirudin, attenuates exosite 2 interactions with immobilized γ′-peptide (Petrera et al., 2009). Therefore, these findings suggest that there is a bidirectional communication between the two exosites.

1.8 Traditional Anticoagulants

For decades, anticoagulants have been the primary strategy for preventing and treating thrombosis, demonstrating favorable efficacy and tolerability in patients with thrombotic diseases. Since the discovery of heparin in 1916, there have been great strides in the development of anticoagulants for the treatment of thrombosis (Figure 8) (Basaran et al., 2016).

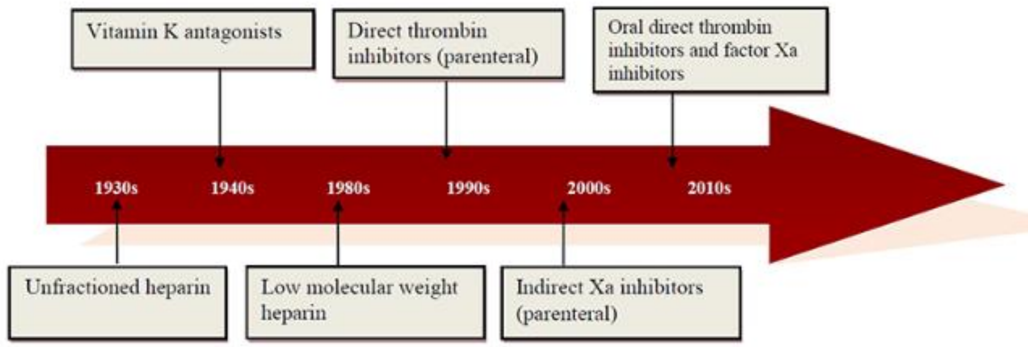
Vitamin K antagonists
Direct thrombin inhibitors (parenteral)
Oral direct thrombin inhibitors and factor Xa inhibitors
1930s
1940s
1980s
1990s
2000s
2010s
Unfractioned heparin
Low molecular weight heparin
Indirect Xa inhibitors (parenteral)

Figure 8. Historical development of anticoagulants.

In the 1930s, purified unfractionated heparin was produced for clinical use. Although not investigated in clinical trials as we understand today, patients' effectiveness and safety were evaluated. In the 1940s, vitamin K antagonist (VKA) was given to patients at the Mayo Clinic. For decades, VKAs were the only oral anticoagulants for antithrombic treatment. In the mid-1980s, researchers found that low molecular weight heparin (LMWH) effectively prevented venous thromboembolism (VTE). From the 1990s to the 2000s, scientists developed parenteral direct thrombin inhibitors, such as argatroban and bivalirudin, and parenteral FXa inhibitors, fondaparinux. By the 2010s, novel oral direct thrombin and FXa inhibitors had been approved in North America and Europe. The International Society on Thrombosis and Haemostasis (ISTH) had recommended them for anticoagulation control. Figure is obtained from (Basaran, 2015).

1.8.1 Vitamin K antagonists (VKAs)

Warfarin is a vitamin K antagonist that produces an anticoagulant effect by inhibiting the vitamin K epoxide reductase (VKOR), an essential enzyme for activating vitamin K (Wallin & Martin, 1985) (Hao et al., 2020). Vitamin K is responsible for vitamin K-dependent clotting factors undergoing γ-carboxylation, a process necessary for their functional activities (Hao et al., 2020). These proteins include clotting factors, such as prothrombin and FVII, FIX, and FX, as well as coagulation regulatory proteins C and S (Wallin & Martin, 1985). During γ-carboxylation, VKOR converts glutamate residues (Glu) to carboxyglutamates (Gla) on their N-terminus of vitamin-K dependent proteins. This conversion is critical for vitamin K-dependent clotting factors binding to the negatively charged phospholipid membranes (Hao et al., 2020). Thus, warfarin inhibits VKOR, blocking the recycling of vitamin K and impairing the biosynthesis of those clotting factors. It also prevents their interaction with the negatively charged endothelial surfaces or phospholipid membranes, resulting in reducing their procoagulant activity.

Warfarin has a half-life of about 36 to 42 hours. Its slow onset of action requires several days to reach an adequate antithrombotic effect (Pirmohamed, 2006). Given this limitation, patients who were prescribed warfarin may be at an even higher risk of developing thrombosis (Pirmohamed, 2006) (Weitz & Gross, 2012). Therefore, patients on warfarin must be paired with a rapidly acting anticoagulant such as heparin for the first five days (Pirmohamed, 2006).

1.8.2 Heparins

For decades, heparin has been used as an anticoagulant for the prevention and treatment of various thrombotic disorders, including DVT, and PE, and during surgical procedures to prevent mechanical and catheter-induced thrombosis (Weitz & Gross, 2012). Heparin is usually extracted from porcine intestinal mucosa, followed by purification (Lee et al., 2020). The currently available parenteral heparins, unfractionated and low molecular heparin, are discussed (Table 1).

1.8.2.1 Unfractionated heparin

Heparin, or unfractionated heparin (UFH), is a heterogeneous mixture of partially sulfated glycosaminoglycans (GAGs). The molecular weight of heparin ranges from 6000 to 30000 , with an average of 15 kDa (Hetzel & Sucker, 2005). Some heparin chains contain a specific pentasaccharide sequence that binds AT. This binding triggers a conformational change in the reactive center of AT, accelerating the inhibition of FXa, thrombin, FXIIa, FXIa, and FIXa, with thrombin and FXa being the most responsive to inhibition (Figure 9) (Hetzel & Sucker, 2005) (Grover & Mackman, 2022). For thrombin inhibition, heparin requires at least 18 saccharide units (> 5.4 kDa) to be able to form a stable ternary complex with AT and thrombin (Oduah et al., 2016). In contrast, anti-FXa activity only requires binding to the pentasaccharide sequence, which is found in one-third of heparin and low molecular weight heparin (LMWH) (Hetzel & Sucker, 2005). The heparin molecules of less than 18 saccharide units are too short to bridge AT and thrombin and, therefore, cannot inhibit thrombin. Thus, heparin induces a conformational change of AT, resulting in an anti-FXa/anti-thrombin ratio 1:1.

Heparin exhibits distinctive pharmacokinetics. It is administered via an intravenous (IV) infusion or subcutaneous (SC) injection, with bioavailability varying from 30 to 70% (Nutescu et al., 2016). When administered intravenously, heparin rapidly achieves its peak plasma concentration, but its pharmacokinetics do not exhibit a sustained peak level because it is quickly distributed and metabolized in the body. This underscores the need for careful monitoring using activated partial thromboplastin time (aPTT) or anti-FXa activity and adjustment of dosages to ensure therapeutic effectiveness while minimizing the risk of bleeding complications (Nutescu et al., 2016) (Lardinois et al., 2022).

The clinically relevant therapeutic concentration of heparin for therapeutic anticoagulation is about 0.3 to 0.7 IU/ml (Ratano et al., 2019). Because heparin has a short half-life, typically lasting 1 to 2 hours, continuous intravenous infusion or frequent subcutaneous injections for therapeutic anticoagulation are required (Nutescu et al., 2016). Its high volume of distribution allows it to distribute widely within the bloodstream, influencing its overall pharmacodynamic effects. Heparin undergoes clearance primarily in the liver, with renal clearance also playing a significant role in the elimination of UFH (Musalem et al., 2023).

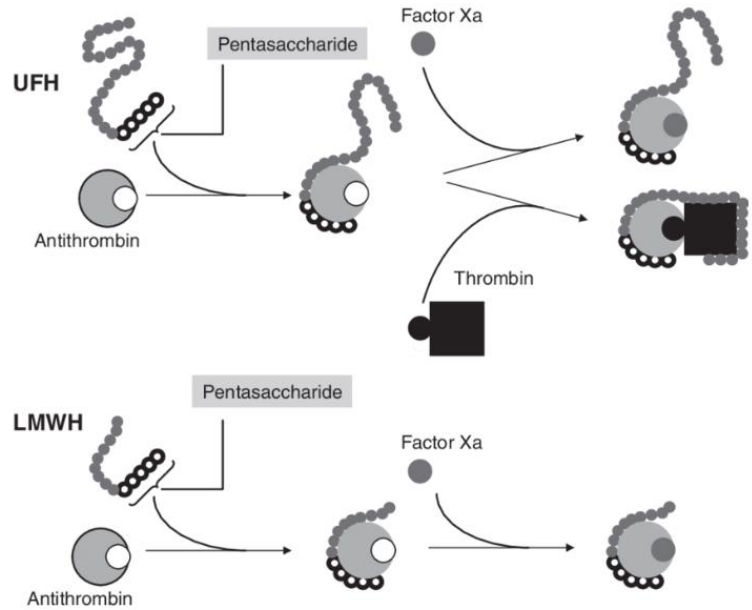
UFH
Pentasaccharide
Factor Xa
Antithrombin
Thrombin
LMWH
Pentasaccharide
Factor Xa
Antithrombin

Figure 9. Mechanism of action of UFH and LMWH.

UFH and LMWH bind to AT via their pentasaccharide sequence, inducing a conformational change in the reactive center of AT and allowing inhibition of FXa and thrombin. LMWH accelerates the inhibition of both FXa and thrombin, but its inhibitory effect is reduced because of its size limitation, which limits it to bridging AT and thrombin. *Abbreviations:* UFH, unfractionated heparin; LMWH, low molecular weight heparin; FXa, activated factor X. Figure is obtained from (Hetzel & Sucker, 2005).

1.8.2.2 Low molecular weight heparin

LMWH, such as dalteparin, is prepared from UFH through controlled enzymatic or chemical depolymerization. The depolymerization process yields heparin chains of an average of 5 kDa (corresponding to 17 saccharide units). Although the short heparin chains in LMWH are inadequate to bridge AT and thrombin simultaneously, the pentasaccharide sequence still exists and is sufficient to accelerate FXa inhibition, similarly to UFH (Oduah et al., 2016). The shorter chain of LMWH favours inhibition of FXa over thrombin, resulting in an anti-FXa/anti-thrombin ratio ranging from 2:1 to 4:1 (Hirsh, 1998) (Oduah et al., 2016).

LMWH are administered subcutaneously and gradually absorbed, reaching their maximum plasma concentration over time. The clinically relevant therapeutic range for anti-FXa levels of dalteparin is about 0.6-1.0 IU/ml (Barbour et al., 2004), whereas for prophylactic anticoagulation, it is, on average, about 0.3 IU/ml (Hellgren & Mistafa, 2019). LMWH has high bioavailability, approaching 100%, which allows for a predictable anticoagulant effect. One of the advantages of LMWH compared with UFH is the lack of pronounced peak concentration, allowing for a predictable distribution in the bloodstream (Nutescu et al. 2016). Therefore, unlike UFH, LMWH does not need routine aPTT monitoring. After administration, LMWH is primarily excreted through the kidneys (Hirsh, 1998) (Nutescu et al., 2016), with a half-life of approximately 4 hours, which is longer than that of UFH. The peak plasma level is generally within a few hours after administration, typically around 3 to 4 hours. Therefore, LMWH enables less frequent dosing (Nutescu et al. 2016).

To prevent excessive anticoagulation and reduce the risk bleeding with the use of heparin, careful dosing and availability of reversible agents are necessary. Protamine sulfate neutralizes the effect of heparins and is the currently used antidote. (Chawla et al., 2004). Protamine sulfate forms a stable complex with the negatively charged heparin molecules, effectively neutralizing their anticoagulant effects. While protamine is the specific antidote for heparins, its reversal effect may not be immediate (Ruseska et al., 2021). The reversal effect of LMWH with protamine is less effective compared with UFH (Crowther & Warkentin, 2008). Therefore, using LMWH should be guided and carefully considered based on the increased bleeding risk.

Drugs	**UFH**	**LMWH**
Size (kDa)	5-30	4.5-5
Catalysis of FXa inhibition	Yes	Yes
Catalysis of thrombin inhibition	Yes	Yes (less effective)
Anti-FXa/anti-thrombin ratio	1:1	2:1 to 4:1
Plasma half-life (h)	1.5	4
Routine coagulation monitoring	Yes	Generally, no
Antidote	Protamine sulfate	Protamine sulfate (partially)

Table 1. Comparisons of heparins.

UFH has a wider molecular weight range (5000-30000) than LMWH (4500-6000) and requires routine coagulation monitoring. LMWH has a longer plasma half-life (4 hours) than UFH. The differences between UFH and LMWH in terms of pharmacokinetics are because of the binding properties of LMWH. LMWH is less effective at inhibiting thrombin than UFH due to its smaller size but remains the same inhibitory effect against FXa because they have pentasaccharides needed for FXa inhibition. UFH shows an anti-FXa/anti-thrombin ratio of 1:1, while LMWHs have a ratio ranging from 2:1 to 4:1. *Abbreviations:* UFH, unfractionated heparin; LMWH, low molecular weight heparin; FXa, activated factor X;

1.8.3 Direct Oral Anticoagulants

Direct oral anticoagulants (DOACs) were developed to combat limitations with traditional oral anticoagulants (Table 2), such as vitamin K antagonists. The two classes of DOACs, direct thrombin inhibitors like dabigatran and direct FXa inhibitors like apixaban, provide a rapid onset of action, exhibit minimal food/drug interactions, require less routine monitoring due to fixed dosing, and have short offset action.

	Heparins	**VKAs (warfarin)**	**DOACs**
Advantages	Rapid onset and offset of actions Few food/drug interactions Long history of clinical experience	Oral agent High bioavailability Low-priced	Fast onset and offset of actions No need for bridging AT Less requirement for routine monitoring of fixed dosing
Mechanism limitations	Parenteral agent Poor availability at low doses	Slow onset and offset of actions Many food and drug interactions Extensive half-life (cause bleeding in some cases)	Lack of reliable laboratory measure of anticoagulant effect Clearance in some cases
Clinical complications	Bleeding HIT (UFH, LMWH) Osteoporosis (UFH, LMWH)	Bleeding Fetal abnormalities during pregnancies Requires routine and frequent dose monitoring	Bleeding High-cost Developed gastric intolerance

Table 2. Comparisons of traditional and new anticoagulants.

The advantages and limitations of anticoagulants. *Abbreviation:* UFH, unfractionated heparin; LMWH, low molecular weight heparin; VKA, vitamin K antagonist; HIT, heparin-induced thrombocytopenia; AT, antithrombin.

1.8.3.1 Dabigatran etexilate

Dabigatran etexilate, an oral thrombin inhibitor, inhibits both free and fibrin-bound thrombin (Samama, 2011) (Hankey & Eikelboom, 2011). After oral administration, dabigatran etexilate is absorbed by the gastrointestinal tract and converted by the liver from its inactive precursor into the active form, dabigatran. Dabigatran forms an ionic interaction between its basic functional group and the base of the S1 pocket in the active site of thrombin (Samama, 2011). By binding to the active site of thrombin, dabigatran inhibits thrombin and prevents it from converting fibrinogen to fibrin. Dabigatran has a predictable pharmacokinetic profile, so fixed doses can be given without routine monitoring. After oral administration, dabigatran has a mean half-life of 12 hours and reaches peak plasma concentration 1 to 3 hours post administration.

Dabigatran has received approval for stroke prevention in patients with AF at a fixed dose of 150 mg twice daily, where it is superior to warfarin for reduction in the risk of stroke and systemic embolism and demonstrates significantly less intracranial bleeding (Connolly et al., 2009). Dabigatran at 150 mg or 220 mg twice a day is as effective as enoxaparin once daily in preventing VTE in patients undergoing hip or knee replacement surgery (Eriksson et al., 2007). However, these results are not aligned with the RE-ALIGN trial. At doses between 150 mg and 300 mg twice daily, dabigatran is less effective for stroke prevention in patients undergoing mechanical heart valve replacement compared with warfarin (Eikelboom et al., 2013). Hence, the study was stopped early because there was an increased risk of stroke in the dabigatran group compared with the warfarin group. We have previously shown that concentrations of dabigatran at clinically relevant doses, 90-180 ng/ml (0.15-0.3 μM), have little effect on inhibiting MHV-induced thrombin generation (Jaffer et al., 2015).

Approximately 80% of dabigatran is cleared by the kidneys, and thus renal function significantly influences its anticoagulant effect (Hankey & Eikelboom, 2011). Several studies investigated the association between dabigatran concentration and bleeding risks. The risk of bleeding increases when the concentration of dabigatran is too high, while the occurrence of thromboembolism increases when the concentration is too low (Ng et al., 2022). These findings suggest that dabigatran concentration should be carefully administered to improve safety and efficacy.

1.8.3.2 Apixaban

Apixaban, an oral direct FXa inhibitor, is effective and safe for the prevention of stroke in patients with AF, and for the prevention of DVT in patients undergoing knee or hip replacement surgery (Patel et al., 2011) (Turpie et al., 2011) (Mega et al., 2012). Apixaban binds the active site of FXa and inhibits free and bound FXa, which ultimately reduces thrombin generation. Given that apixaban has a half-life of 12 hours, a twice-daily dosing regimen can maintain steady therapeutic levels and ensure effectiveness. (Byon et al., 2019). In general, the therapeutic range for apixaban is approximately 20 -100 ng/ml (0.04-0.2 μM) for 2.5 mg twice daily dosing and 30-412 ng/ml (0.07-0.9 μM) for 5 mg twice daily dosing (Gosselin & Adcock, 2016) (Dale et al., 2016). Apixaban reaches its peak plasma concentration approximately 3.5 hours after oral administration and exhibits a bioavailability of about 50% (Grześk et al., 2021). This high bioavailability underscores its effectiveness as an oral anticoagulant.

In the ARISTOTLE trial, patients taking apixaban show a significantly lower risk of stroke and bleeding than those taking warfarin (Granger et al., 2011) . Recently, meta-analyses show that the use of apixaban significantly lowers systemic embolism better than dabigatran (Buckley et al., 2022). Apixaban exhibits better efficacy and safety profiles than LMWH in the prevention and treatment of DVT after orthopedic surgery in older adult patients (Jiang et al., 2019).

1.9 Targeting Contact Pathway for Anticoagulation

FXII and FXI have emerged as attractive targets for developing antithrombotic therapy to reduce the risk of bleeding associated with traditional anticoagulants and DOACs (Weitz & Fredenburgh, 2017) (Cohen & Ageno, 2022). This novel antithrombotic strategy is primarily based on epidemiological studies showing that people with FXII deficiency do not have a bleeding tendency (Lämmle et al., 1991). Similarly, patients with FXI deficiency experience no or minor spontaneous bleeding (Asakai et al., 1991). In addition, animal models deficient in either FXII or FXI demonstrate a reduction in arterial and venous thrombosis (Grover et al., 2020).

Although FXII seems to be a safer target than FXI, current novel anticoagulant development has focused on FXI rather than FXII (Table 3). This is because there is weak clinical evidence linking FXII with thrombosis in humans, and the feedback activation of FXI by thrombin can bypass FXIIa inhibition, making the role of FXII in TF-initiated thrombosis uncertain (Chan & Weitz, 2023). However, there is no evidence that having a deficiency or reduction of FXII is protective against VTE, whereas low FXI does (Yuan et al., 2021). This suggests that FXI could be a better target than FXII for antithrombotic management. Hence, FXI inhibition could provide antithrombotic protection with minimal bleeding risk (Fredenburgh & Weitz, 2023).

	FXI directed	FXII directed
Evidence	Strong (preclinical, epidemiological, and phase II clinical trials)	Weak (preclinical, inconsistent epidemiological data)
Impact on hemostasis	Minimal	Dispensable
Potential for bypassing inhibition	None	Feedback activation of FXI by thrombin can bypass FXIIa inhibition
Efficacy against inflammation-mediated thrombosis	Likely	Less likely because thrombin can bypass FXIIa inhibition

Table 3. Comparisons of FXI and FXII as Novel Targets.

The advantages and disadvantages of FXII and FXI as therapeutic targets for the prevention and treatment of venous thromboembolism. *Abbreviation:* FXI, factor XI; FXII, factor XII; Table is obtained from (Chan & Weitz, 2023).

1.9.1 Factor XI Structure

FXI is a 160 kDa disulfide-linked homodimer that is synthesized in the liver. Each monomer contains four 90- or 91-amino acid repeats, so-called the apple domains (A1, A2, A3, and A4), and a C-terminal trypsin-like catalytic domain (CD) (Figure 10). The A1 domain facilitates interaction with HK and thrombin, promoting activation of FXI (Baglia & Walsh, 1996). The A2 domain is required for HK binding, with supplementary contributions from the A1 and A4 domains (Emsley et al., 2010). The A3 domain is essential in mediating the interaction of FXI with platelet GPIbα. It also facilitates binding to negatively charged surfaces, such as polyP and heparin, during contact activation and inhibition. Moreover, it serves as the most important binding site for FIX (Gailani & Smith, 2009) (Emsley et al., 2010). The A4 domain is responsible for the stabilization of the FXI homodimer. It enhances the binding of FXI to negatively-charged surfaces, promoting its role in the coagulation cascade (Samuel et al., 2007). In human plasma, FXI circulates at a concentration of approximately 30 nM, primarily forming a non-covalent complex with the high molecular weight kininogen (HK) (Mohammed et al., 2018). Prekallikrein (PK) is a monomeric homolog of FXI with the same domain structure that also circulates in complex with HK (Emsley et al., 2010).

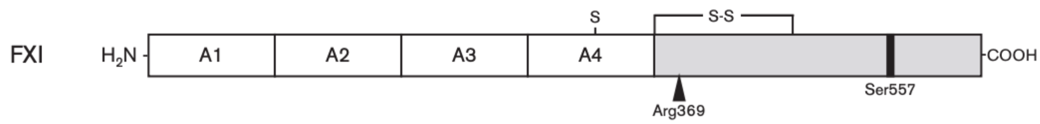
FXI
H2N
A1
A2
A3
A4
S
S-S
COOH
Arg369
Ser557

Figure 10. Domain structure of FXI.

Zymogen FXI is a 160 kDa disulfide-linked dimer, consisting of two identical 80 kDa monomers. Each monomer contains of 4 apple domains indicated as A1-A4 (white boxes) in the heavy chain and catalytic domain (grey box) in the light chain. Activation of FXI to FXIa is mediated by the cleavage after Arg-369 located in the catalytic triad. Ser-557 is required for proteolytic activity. The disulfide bond (S-S) in the catalytic domain maintains the structural conformation which is important for its ability to activate FIX. *Abbreviation:* A1, apple 1 domain; A2, apple 2 domain; A3, apple 3 domain; A4, apple 4 domain; Arg, arginine; Ser, serine; Table is obtained from (Chan & Weitz, 2023). Figure is obtained from (Ponczek et al., 2020).

1.9.2 Activation and Regulation of FXI

FXI activation involves the conversion of the zymogen FXI into the active protease of FXIa through the cleavage of Arg 369-IIe 370 bond (Gailani & Smith 2009). In the coagulation cascade, FXI undergoes activation by FXIIa, thrombin, or FXIa (autoactivation). However, FXIIa is not necessary for FXI activation because people with FXII deficiency do not experience bleeding, indicating there is an alternative mechanism for FXI activation. FXI plays an important role in a feedback activation by thrombin that not only sustains early fibrin formation but also contributes to hemostasis by activating FIX (Emsley et al., 2010). This is shown when low-TF mice with deficiency of FIX or FXI show a fatal bleeding tendency in utero. In contrast, mice with deficiency of FXII survive and demonstrate similar phenotypes to low TF mice with normal FXII levels (Emsley et al., 2010). FXI autoactivation occurs in the presence of negatively charged surfaces, such as polyP and dextran sulfate (Choi et al., 2011). It is commonly believed that A3 domain mediates this activation process because it contains an anion binding site (Ho et al., 1998) (Geng et al., 2013).

FXIa is primarily regulated by plasma serpins like antithrombin, C1-inhibitor, and others. FXI inhibition reduces platelet accumulation in the developing thrombi. The inhibition of FXIa by antithrombin involves a template mechanism with heparin binding, where it enhances the binding of the A3 domain and serpin (Emsley et al., 2010). In addition, heparin interacts with the FXIa CD and inhibits FXIa through mechanisms likely linked to charge neutralization or allosteric effects, thereby reducing repulsion between FXI and serpin (He et al., 2012).

1.9.3 Novel anticoagulants: Factor XI inhibitors

To date, several FXI inhibitors are in various stages of development, reflecting the growing interest in targeting these factors for anticoagulation with a reduced risk of bleeding. FXI inhibitors are classified into three main types based on their chemical structures: (a) antisense oligonucleotides (ASOs), (b) monoclonal antibodies (mAbs), and (c) small molecules (Table 4).

Class	Compounds	Mechanisms of action	Administration	Onset-offset
Antisense oligonucleotides	IONIS FXI Rx, fesomersen	Reduce hepatic synthesis of FXI by inducing catalytic degradation of FXI mRNA	Parenteral	Slow onset and offset
Monoclonal antibodies	Osocimab, abelacimab, gruticibart (*Xisomab/AB023*)	Suppress FXIa generation or inhibit FXIa activity	Parenteral	Rapid onset and slow offset
Small molecule inhibitors	Milvexian, asundexian	Block the active site of FXIa or induce allosteric modulation	Oral	Rapid onset and relatively rapid offset

Table 4. Different strategies to inhibit FXI and their features.

Antisense oligonucleotides binds to FXI mRNA and reduce the hepatic synthesis of FXI. Monoclonal antibodies, such as osocimab, gruticibart, and abelacimab block both FXI activation and FXIa activity. Small molecules, such as asundexian and milvexian, block the active site of FXIa. Table is obtained from (Chan & Weitz, 2023).

1.9.3.1 Abelacimab

Abelacimab, a monoclonal antibody (MAA868), binds to catalytic domain of FXI and locks it in a zymogen form. The fragment antigen-binding region (Fab) of abelacimab exhibits high binding affinity to FXI and FXIa, locking FXI in the zymogen conformation and inhibiting 50% of FXIa chromogenic activity with an IC_{50} of 2.8 nM (Campello et al., 2022). Abelacimab binds the catalytic domain of FXI and FXIa, with Kd values of approximately 1.3 and 4.7 pM, respectively. This binding is very specific because abelacimab does not inhibit other coagulation factors, such as FVIIa, FIXa, FXa, FXIIa, thrombin, and PKa. Additionally, abelacimab inhibits FXIa activated by FXIIa in the contact pathway and by thrombin. In contrast, it does not inhibit thrombin generation from the extrinsic pathway (Campello et al., 2022).

Abelacimab is administered through intravenous (IV) infusion for rapid FXI inhibition, followed by subcutaneous injections monthly to sustain almost complete inhibition in long-term management (Verhamme et al., 2021) (Yi et al., 2022). The plasma concentration of abelacimab ranges from 20-50 μg/ml (0.13-0.33 μM) (Yi et al. 2022). Some studies show that administration of abelacimab through both IV and repeated SC routes is found to be safe and well-tolerated by patients (Verhamme et al., 2021) (Yi et al., 2022). Research indicates that IV infusion of abelacimab significantly doubles aPTT ratio by one hour of treatment. Furthermore, a phase 2 study reveals that a single IV dose of abelacimab in patients undergoing knee replacement surgery is effective for the prevention of VTE compared to enoxaparin with a low risk of bleeding (Verhamme et al., 2021).

Abelacimab is currently being explored in several phase 3 clinical trials, aiming to redefine the management of patients predisposed to thrombosis. One of the key studies includes the *LILAC-TIMI 76* trial (NCT05712200) which is initiated by Anthos Therapeutics. It is a randomized, double-blinded, placebo-controlled trial, investigating the efficacy and safety in patients with AF who are unsuitable for existing oral anticoagulant therapies. Participants are administered abelacimab 150 mg subcutaneously (SC) once monthly, with the study spanning over 300 sites globally. This trial aims to enroll around 1900 participants and is designed to assess the efficacy and safety of abelacimab in preventing ischemic stroke or systemic embolism compared to placebo.

Abelacimab is currently being tested in cancer-associated thrombosis, which consists of two important studies: the ASTER (NCT05171049) and MAGNOLIA (NCT05171075) trial. These studies have enrolled approximately 2700 patients across the world, making it the most extensive program for any anticoagulant in this specific patient population. In the MAGNOLIA trial, abelacimab is compared with dalteparin in patients with cancer-associated VTE, specifically those with cancers of gastrointestinal system. On the other hand, the ASTER trial compares abelacimab with apixaban for cancer-associated VTE in patients.

1.9.3.2 Milvexian

Milvexian (BMS-986177/JNJ-70033093) is an oral small molecule that inhibits FXIa with high affinity and has a half-life of approximately 12 hours (Perera et al., 2022). It binds rapidly and reversibly to the active site of FXIa with a Ki of 0.11 nM. This effect is shown to be specific with exception being chymotrypsin and plasma kallikrein, which have Ki values of 35 and 44 nM, respectively (Dilger et al., 2022). The expected therapeutic range for milvexian is from 3-5 μM with 25 mg and 100 mg twice daily doses, respectively (Perera et al., 2022).

Milvexian has been evaluated for various indications in Fast Tract Designation by U.S. FDA under phase 3 evaluation in the three-part LIBREXIA program involving more than 46, 000 patients. This series of trials features the LIBREXIA AF trial (NCT05757869), which aims to assess milvexian against apixaban in the context of stroke prevention among AF patients. Additionally, the LIBREXIA Stroke and ACS trials (NCT05702034 and NCT05754957) are set to evaluate milvexian alongside antiplatelet therapy compared with placebo, targeting patients with non-cardioembolic stroke and acute coronary syndrome (ACS), respectively. In the AXIOMATIC-TKR trial, milvexian is shown to be effective for the prevention of VTE in patients undergoing knee replacement surgery without increasing the risk of bleeding, highlighting its importance in the field of anticoagulation therapy (Weitz et al., 2021) .

1.10 Medical Device-Induced Thrombosis

Because foreign surfaces are thrombogenic, thrombosis is a common cause of failure in blood-contacting medical devices. Thrombus formation occurs in patients with stents, vascular grafts, heart valves, and guide catheters, which are often used to treat cardiovascular diseases (Jaffer et al., 2015). Medical device-associated thrombosis leads to a significant impact on patient treatment and outcomes, eventually reducing the quality of life (Table 5). The overall incidence of device-induced thrombosis varies between 0-17.6% and is associated with a more than 3-fold higher risk of stroke and thromboembolism (Dukkipati et al., 2018).

Medical Device Type	**Reported Thrombosis Rate Per Year**	**Impact on Patient Outcomes**	**Reference**
Stents	0.5 – 2.2%	Critical; death or MI	(Saleh et al., 2016)
Vascular Grafts	13%	Significant; acute limb ischemia	(Ichihashi et al., 2021)
Mechanical Heart Valves	0.1- 5.7%	Acute heart failure	(Salamon et al., 2015)
Guide Catheters	5% symptomatic	Require management with anticoagulation therapy	(Wall et al., 2016)

Table 5. Overview of the influence of medical device-associated thrombosis.

Thrombosis continues to be a serious complication in patients with implanted medical devices that can be detrimental. Patients with symptomatic catheter-related thrombosis require management with anticoagulation therapy. Sources: mentioned in the table.

1.10.1 Catheter Thrombosis

Thrombosis on devices, such as MHV and catheters, result from an interplay of various complex mechanisms. Typically, thrombosis arises from a combination of endothelial damage, reduced blood flow, and hypercoagulability (Virchow's triad). This is a complex mechanism, involving interplay of platelet proteins, contact pathway, and the complement system (Figure 11). Protein adsorption is the initiation process in catheter-related thrombosis (Jaffer et al., 2015). Briefly, charged groups of proteins interact with the surface, leading to a conformational change in protein structure and forming a protein monolayer. This process is dependent on three factors: hydrophilicity, protein, and surface charge. There is more protein absorption on negatively charged hydrophobic surfaces than on hydrophilic surfaces because the hydrophobic residues of proteins can better interact with hydrophobic artificial surfaces (Turbill et al., 1996). The first plasma protein deposited on catheter is fibrinogen, followed by fibronectin and vWF (Jaffer et al., 2015). Absorbed fibrinogen is the most important in mediating the attachment of platelets, resulting in various platelet interaction on the catheter surface, followed by fibronectin and vWF (Savage & Ruggeri, 1991). When platelets become activated, this induces the release of ADP, thromboxane A2, and other agonists that eventually trigger platelet adhesion, activation, and aggregation, leading to thrombus formation. However, the circulating platelets count reduces when there is a prolonged exposure of catheter surface (Jaffer et al., 2015). Then, activated platelets mediate leukocyte (or neutrophils) adhesion via the interaction of platelet p-selectin with leukocyte p-selectin glycoprotein ligand-1 (Morley & Feuerstein, 1989). This process is also supported by activated complement proteins of the immune system, followed by red blood cell adhesion (Bowers et al., 1989). Hence, protein absorption on artificial surfaces plays a critical role in the pathogenesis of thrombosis.

Absorbed fibrinogen is quickly replaced by clotting factors in the contact pathway, such as FXII and FXI (Alibeik et al., 2012). Absorbed FXII undergoes autoactivation to become FXIIa, followed by activation of FXI, leading to thrombin generation as previously described (Jaffer et al., 2015). Our previous research has shown the critical role of the contact pathway in catheter-related clotting (Yau et al., 2011). For instance, catheters induce plasma clotting time 3-fold faster than in its absence. In addition, the clotting time is reduced by 4.6-fold and 7-fold when plasma is depleted of FXII or FXI, respectively. However, there is no change in clotting using plasma depleted of FVII. These findings suggest that FXI and FXII are promising targets for managing catheter-related thrombosis.

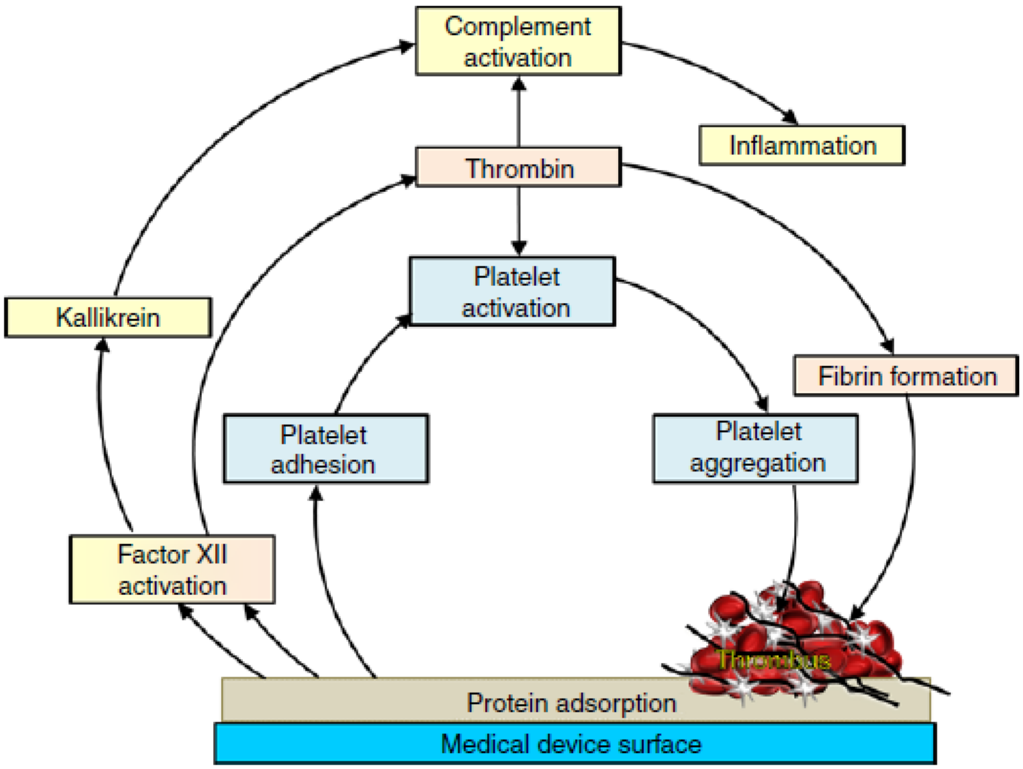
Complement activation
Inflammation
Thrombin
Platelet activation
Kallikrein
Fibrin formation
Platelet adhesion
Platelet aggregation
Factor XII activation
Thrombus
Protein adsorption
Medical device surface

Figure 11. Blood contacting catheter-induced thrombosis.

Protein absorption on medical device surface induces platelet adhesion, activation, and aggregation, followed by the formation of a thrombus. Protein absorption also initiates FXII autoactivation, forming a fibrin monomer that stabilizes the thrombus clot and activates PK to kallikrein, which triggers an inflammatory response. *Abbreviation:* FXII, factor XII; Figure is obtained from (Jaffer et al., 2015).

1.10.2 Prevention and Treatment

To prevent or treat device-induced thrombosis, it is important to reduce platelet adhesion, fibrin formation, and thrombin generation on the artificial surfaces. These strategies can be implemented via surface modification, involving the synthesis of less thrombogenic biomaterials, or long-term treatment with anticoagulant/antiplatelet drugs, or both.

1.10.2.1 Surface modification

Surface modification primarily aims to interfere electrostatic and hydrophobic interaction between plasma proteins and cell binding. Polyethylene oxide (PEO), chemical structure (CH_2-CH_2-O), is a good candidate for modifying the artificial surfaces because of the hydrophilic nature and the presence of ether oxygen show significant resistance to protein and cell adhesion *in vitro* (Jaffer et al., 2015). However, this effect has not been shown to be effective *in vivo.* Another popular surface modification is the passive adsorption of elastin polypeptides onto artificial surfaces. It enables a reduction of fibrinogen, protein adsorption, and cytokine release from monocytes (DeFife et al., 1999). Coating elastin polypeptides on artificial surface demonstrates a marked decrease of platelet activation in platelet rich plasma with noncoated controls (Woodhouse et al., 2004). Another popular surface modification is heparin coating. Heparin-coated medical devices, such as coronary stents, catheters, and vascular grafts have been in clinical trials for three decades and are available in the commercial market. Surface-bound heparin binds to AT and inactivates coagulation factors, like FXa and thrombin. Results have shown that heparin coating improves the biocompatibility of medical devices by reducing formation of thrombus clots on the surfaces, while minimizing protein and cell adhesion (Biran & Pond, 2017).

Although these methods seem promising, they require more studies due to insufficient clinical data in animal and human trials or inconsistent results showing no significant differences (Jaffer et al., 2015). Therefore, synthesizing less thrombogenic artificial surfaces through the inhibition of protein and cell binding alone may not be sufficient for preventing thrombosis on surfaces. These findings highlight the importance of targeting not only protein deposition but also the root cause of catheter thrombosis, which is the activation of the contact pathway.

We have previously accomplished this goal by attaching polyethylene glycol (PEG) followed by corn trypsin inhibitor (CTI), a specific FXIIa inhibitor, on polyurethane catheters. When the PEG-CTI-coated catheters are implanted into the jugular veins of rabbits, catheter occlusion time is approximately 2.5-fold longer compared with unmodified catheters. In addition, PEG alone reduces fibrinogen adsorption from plasma, and CTI inhibits FXII autoactivation on the catheters, inducing less FXI activation (Yau et al., 2012). Moreover, our recent study demonstrates that knocking down the levels of FXII attenuates catheter thrombosis in rabbits (Malik et al., 2023). In addition, depleting histidine-rich glycoprotein (HRG), a protein that binds FXII and modulates its activity, with a specific antisense oligonucleotide (ASO) results in a shorter occlusion time than saline controls. Our findings confirm that attenuating activation of the contact pathway and protein deposition are essential in effectively preventing or treating catheter-induced clotting.

1.10.2.2 Anticoagulation

Patients with inserted medical devices still require short- or long-term antiplatelet drugs or anticoagulants to prevent clotting (Jaffer et al., 2015) to prevent thromboembolism. Heparin is the standard for preventing catheter-induced thrombosis in patients undergoing cardiopulmonary bypass, extracorporeal membrane oxygenation (ECMO), and percutaneous coronary intervention (PCI) (Jaffer et al., 2015). However, the inhibitory effect of heparin on catheter-induced clotting is polymer chain length-dependent (Yau et al., 2011). At the same anti-Xa activity, the effect of heparin on catheter-induced clotting is superior to enoxaparin. This observation is further confirmed by using fondaparinux alone, where the inhibition of plasma clotting does not involve upstream clotting factors in the contact pathway or those above the level of FXa. In ACS patients undergoing PCI, there is less thrombus formation with enoxaparin than with fondaparinux (Jolly et al., 2009). One explanation of this observation is that longer polymer chains are more capable of performing bridging function of binding multiple plasma proteins simultaneously, such as AT, FIX, FXI, or FXII, whereas shorter polymers cannot. What happens if we combine heparin and fondaparinux? Previously, we have shown that supplemental heparin to fondaparinux results in a more than additive manner that significantly prolongs catheter occlusion time in rabbit model compared to when either drug is used alone (Yau et al., 2011). These findings suggest that targeting multiple clotting factors may be the better therapeutic strategy to prevent catheter-induced thrombosis. This effect is aligned with the RE-ALIGN trial where warfarin is more effective for stroke prevention in patients with mechanical heart valve compared with dabigatran etexilate, although it is associated with elevated bleeding risks (Eikelboom et al., 2013).

1.11 Novel Approaches of Catheter Thrombosis

Targeting FXII or FXI may be particularly effective in catheter-induced thrombosis (Jaffer et al., 2015) because it could directly inhibit the root cause of device-related thrombosis. For example, a FXI-directed antibody attenuates clotting on vascular graft in a baboon model of arterio-venous shunt (Tucker et al., 2009). Moreover, a FXII-directed antibody reduces platelet and fibrin accumulation on the vascular graft in this model (Matafonov et al., 2014). Both FXII and FXI are attractive targets for the prevention of thrombosis. However, FXII inhibition may influence PK activation and impact inflammation. Furthermore, some epidemiological studies proposed that FXI plays a more important role than FXII in thrombotic disorders, such as MI, stroke, and VTE (Tillman & Gailani, 2018). Hypothetically, inhibition of FXI would produce a greater antithrombotic effect than FXII because it reduces thrombin generation from the contact pathway and feedback activation by thrombin, which sustains FIX activation (Tillman & Gailani, 2018).

A major advantage of targeting FXI for the prevention of catheter thrombosis is safety. Most people with congenital FXI deficiency do not have spontaneous bleeding unless they undergo trauma or surgery (Asakai et al., 1991). In comparison, few studies have been published on the association of bleeding and FXII deficiency because congenital deficiency of FXII is rare. In addition, results from phase 2 clinical trials suggest that decreasing FXI level by FXI-ASO (ISIS 416858) is more effective for the prevention of venous thrombosis in patients undergoing knee replacement surgery than the use of enoxaparin without increased bleeding risks (Büller et al., 2015). This suggests that FXI is an attractive target for novel strategies to prevent device-induced thrombosis. However, it is currently unknown whether FXI inhibitors can be combined with standard anticoagulants can effectively attenuated thrombosis while leave hemostasis intact.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1 Aims and Hypotheses

FXI can be activated by thrombin in a positive feedback reaction (Gailani & Broze, 1991) (Gailani & Smith, 2009). Although thrombin feedback activation of FXI sustains thrombus growth, this feedback mechanism appears to be generally slow (Bouma & Meijers, 2000) (Gailani & Gruber, 2016). However, polyP released from activated platelets enhances this activation by providing a template that binds FXI A3 domains and thrombin exosite 2 and increases their proximity. (Gailani & Broze, 1991) (Müller et al., 2009) (Choi et al., 2011) (Geng et al., 2013). Evidence also suggests that allosteric communication between the 2 thrombin exosites, where ligands binding to one exosite influences the other (Petrera et al., 2009) (Chen et al., 2017).

The role of exosite 1 in such activation remains contradictory. One study suggested that exosite 1 is required for sufficient FXI activation by thrombin (Yun et al., 2003). While, another study showed that thrombin exosite 1 mutant can still activate FXI (Matafonov et al., 2011). Given that thrombin exosite 1 has numerous roles in coagulation and anticoagulation, we hypothesized that the interaction of polyP with exosite 2 is the primary driver of FXI activation by thrombin. We also hypothesized that allosteric modulation between thrombin exosites modulates FXI activation.

To test these hypotheses, we evaluated the role of exosite 1 in polyP-catalyzed activation of FXI by thrombin by using thrombin and exosite variants to examine their affinities for polyP and abilities to activate FXI in the absence or presence of polyP. We also utilized exosite 1 and 2 ligands to study their effects on thrombin binding to polyP and its capacity to activate FXI.

Apart from natural polyanions, catheters often used in patients with AF and ACS can also activate the contact pathways (Yau et al., 2011) (Yau et al., 2012) (Jaffer et al., 2015) (Lawton et al., 2022). Catheters trigger clotting by activating FXII, and the resultant FXIIa then activates FXI to induce clot formation (Jaffer et al., 2015). Heparin is routinely given during PCI to prevent catheter thrombosis (Niccoli, 2002). However, traditional anticoagulants are often ineffective. Since FXIa plays an important role in device-induced thrombosis (Yau et al., 2012) (Yau et al., 2014) (Carvalho Silva et al., 2019), we hypothesized that FXIa inhibitors like milvexian and abelacimab may prevent catheter thrombosis, thereby rendering heparin redundant for patients taking milvexian who require PCI.

To address these questions, we first determined the minimum level of FXI and FXII needed to trigger clotting. In addition, we examined the capacity of catheters to activate FXI using purified proteins. Finally, we investigated the effect of milvexian on catheter-induced clotting and thrombin generation on its own and in the presence of heparin to determine the extent to which milvexian inhibits clotting and whether heparin potentiates its inhibitory effects. Dabigatran, a potent and specific thrombin inhibitor, was used as a positive control in these studies because we have previously shown that, at high concentrations, dabigatran attenuates catheter-induced clotting in vitro and a catheter thrombosis model in rabbits.

We futher hypothesized that abelacimab, which inhibits both FXI and FXIa, will be more effective than apixaban, which targets downstream at the level of FXa. Previous findings showed that even small amounts of FXI, as little as 2%, are sufficient to trigger thrombin activation through the feedback activation of FXI. We also hypothesized that dalteparin, which works downstream to abelacimab by inhibiting FXa and thrombin, will be more effective than abelacimab.

Using our plate-based catheter thrombosis model, we examined the effect of abelacimab on aPTT, catheter-induced clotting, and thrombin generation, comparing them to dalteparin and apixaban at therapeutically relevant concentrations. Our approch ensures that the observed effects are applicable to clinical trials and aims to identify the most effective anticoagulants in catheter thrombosis that are currently under phase 3 clinical trials (ASTER and MAGNOLIA).

2.2 Objectives

Our objectives were as follows:

Objective 1: To determine the contribution of thrombin exosites to polyP-induced potentiation of FXI activation by thrombin.

Specific aims:

1. Investigate the role of polyP on FXI activation by thrombin and FXI autoactivation *in vitro* using a chromogenic assay.
2. Examine the activation of FXI by thrombin in the presence of polyP and exosite ligands.

Objective 2: To determine the capacity of FXIa inhibition required to prevent catheter thrombosis.

Specific aims:

3. Compare the effect of FXII or XI depletion on catheter-induced clotting.
4. Examine the capacity of catheters to activate FXI.
5. Determine the effect of milvexian alone or with heparin on catheter-induced clotting and thrombin generation.

Objective 3: Examine the effect of abelacimab, apixaban, and dalteparin in preventing catheter-induced clotting and thrombin generation *in vitro* at clinically relevant concentrations

Specific aims:

6. Examine the effects of abelacimab, apixaban, and dalteparin on prolongation of aPTT and catheter-induced clotting, as well as reduction of catheter-induced thrombin generation.

   7. Determine the relative effect of abelacimab, apixaban, and dalteparin for the prevention of catheter thrombosis in patients relevant to the clinical context of ongoing Phase 3 ASTER and MAGNOLIA clinical trials.

CHAPTER 3 - ALLOSTERIC MODULATION OF EXOSITE 1 ATTENUATES POLYPHOSPHATE-CATALYZED ACTIVATION OF FACTOR XI BY THROMBIN

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Foreword: The role of thrombin exosite 1 and 2 in FXI activation in the presence of polyP is described in Chapter 3.0. We demonstrate that exosite 2 binds to polyP and mediates FXI activation. Also, we show that exosite 1 ligands attenuate FXI activation by allosterically modulating thrombin exosite 2 and active site.

Running title: Ligands binding to exosite 1 attenuates FXI activation by thrombin

Author Contributions: R.Y. designed the research, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. V.P. designed and performed the experiments. R.A.M. performed the experiments. J.C.F. and J.I.W. designed the experiments, analyzed and interpreted the data, and wrote the manuscript.

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References for this manuscript have been incorporated into the Bibliography at the end of this thesis.

3.1 KEY POINTS

- How polyphosphate enhances factor (F) XI activation by thrombin is poorly understood.
- The role of thrombin exosites was investigated using exosite variants and ligands.
- Thrombin binds polyphosphate via exosite 2; however, exosite 1 ligands attenuate FXI activation.
- Exosite 1 ligands inhibit FXI activation by allosterically modulating exosite 2 and the active site.

3.2 ABSTRACT

Polyphosphate (polyP) promotes feedback activation of factor (F) XI by thrombin by serving as a template. The contribution of thrombin's exosites to these interactions is unclear. To determine the contribution of thrombin exosites 1 and 2 to polyP-induced potentiation of FXI activation by thrombin. The affinities of α-thrombin; K109E/110E-thrombin, an exosite 1 variant, or R93E-thrombin, an exosite 2 variant; FXI; and FXIa for polyP-70 were quantified using surface plasmon resonance in the absence or presence of exosite ligands. FXI was activated with α-thrombin or thrombin variants in the absence or presence of polyP-70 and exosite ligands. α-Thrombin, K109/110E-thrombin, FXI, and FXIa bound polyP-70, whereas R93E-thrombin exhibited minimal binding. Exosite 1 and exosite 2 ligands attenuated thrombin binding to polyP-70. PolyP-70 accelerated the rate of FXI activation by α-thrombin and K109E/110E-thrombin but not R93E-thrombin up to 1500-fold in a bell-shaped, concentration-responsive manner. Exosite 1 and exosite 2 ligands had no impact on FXI activation by thrombin in the absence of polyP-70; however, in its presence, they attenuated activation by 40% to 65%. PolyP-70 binds FXI and thrombin and promotes their interaction. Exosite 2 ligands attenuate activation because thrombin binds polyP-70 via exosite 2. Attenuation of FXI activation by exosite 1 ligands likely reflects allosteric modulation of exosite 2 and/or the active site of thrombin because exosite 1 is not directly involved in FXI activation. Therefore, allosteric modulation of thrombin's exosites may represent a novel strategy for downregulating FXI activation.

3.3 INTRODUCTION

Factor (F) XI is a key component of the contact system (Key, 2014) (Srivastava & Gailani, 2020) (Hsu et al., 2021). FXI can be activated by FXIIa or thrombin in a positive feedback reaction (Gailani & Broze, 1991)(Naito & Fujikawa, 1991)(Gailani & Smith, 2009)(Cheng et al., 2010). Thrombin activation of FXI sustains thrombin generation through the activation of FIX, which triggers thrombus growth and prevents premature fibrinolysis (Bouma & Meijers, 2000) (Gailani & Gruber, 2016). Mice deficient in FXII or FXI exhibit attenuated thrombosis in response to arterial or venous injury without perturbation of hemostasis (Renné et al., 2005). In phase 2 clinical trials, knockdown of FXI with an antisense oligonucleotide; FXI inhibition with abelacimab, an antibody that binds FXI and prevents its activation; or FXIa inhibition with milvexian, a small molecule that binds to the active site of FXIa, was superior to enoxaparin for prevention of venous thromboembolism after elective knee arthroplasty and did not increase the risk of bleeding (Büller et al., 2015) (Weitz et al., 2021). These findings identify FXI as a promising target for the development of potentially safer anticoagulants (Hsu et al., 2021) (Fredenburgh & Weitz, 2021).

Although the rate of FXI activation by thrombin is slow, polyphosphate (polyP) released from activated platelets enhances this reaction by providing a template that binds FXI and thrombin (Gailani & Broze, 1991) (Müller et al., 2009) (van der Meijden & Heemskerk, 2010) (Choi et al., 2011). PolyP is an anionic linear polymer consisting of inorganic phosphate groups linked by high-energy phosphoanhydride bonds (Rao et al., 2009) (Morrissey et al., 2012). PolyP in platelets consists of 60 to 100 phosphate units and is released upon platelet activation (Ruiz et al., 2004) (Smith et al., 2010). In addition to promoting FXI activation by thrombin, platelet polyP promotes FXII activation, enhances FV activation by thrombin, alters the thickness of fibrin fibers, and modulates fibrinolysis (Morrissey & Smith, 2015). Therefore, polyP has multiple effects on coagulation.

Both FXI and thrombin bind to polyP. The interaction of FXI with polyP is mediated by its apple 3 and catalytic subdomains, whereas the interaction of thrombin with polyP is mediated by exosite 2, the polyanion-binding site on thrombin that flanks the active site (Mutch et al., 2010) (Geng et al., 2013). However, the role of exosite 1, the substrate-binding site on thrombin, is less clear. This study was undertaken to evaluate the role of exosite 1 in polyP-catalyzed activation of FXI by thrombin. We used thrombin and exosite variants to examine their affinities for polyP and abilities to activate FXI in the absence or presence of polyP. Exosite 1 and exosite 2 ligands were used to examine their effects on thrombin binding to polyP and thrombin's capacity to activate FXI.

3.4 METHODS

3.4.1 Reagents

Chromogenic substrates S-2366 and S-2238 were obtained from Hyphen BioMed. PolyP-6 and polyP-20 consisting of approximately 6 and 20 phosphate units, respectively, were generously provided by Dr. James Morrissey, University of Michigan. Short-chain polyP (polyP- 70), which was a gift from Dr. Thomas Staffel (70.5 CAREPHOS 244, BK Giulini, GmbH), was reconstituted in 20-mM tris(hydroxymethyl) aminomethane, 150 mM NaCl, pH 7.4 (Tris-buffered saline [TBS]) to 1 mg/mL, and after mixing overnight at 4 °C, it was frozen in aliquots at −20 °C. PolyP-70 is polydisperse and contains phosphate chains consisting of 20 to 280 phosphate units (Malik et al., 2021). Platelet-derived polyP was isolated using a previously published method (Müller et al., 2009). Briefly, washed human platelets were activated with 3 U/mL thrombin, and the polyP in the releasate was isolated using phenol/chloroform extraction and ethanol precipitation. Human plasma FXI, FXIa, and thrombin were purchased from Enzyme Research Laboratories. Recombinant wild-type (WT) thrombin and exosite 1 (K109/110E; chymotrypsin numbering) and exosite 2 variant (R93E) forms of thrombin expressed in Escherichia coli were purchased from Cambridge Protein Works. HD1, an exosite 1–directed DNA aptamer (5′-GGTTGGTGTGGTTGG-3′); HD22, an exosite 2–directed DNA aptamer (5′-AGTCCGTGG- TAGGGCAGGTTGGGGTGACT-3′); and HD23, a nonspecific DNA aptamer (5′-AGTCCGTAATAAAGCAGGTTAAAATGACT-3′) were synthesized by the Molecular Biology and Biotechnology Institute at McMaster University (Griffin et al., 1993) (Tasset et al., 1997) (Kretz et al., 2006). A peptide corresponding to GpIbα residues 269 to 286 with 3 phosphorylated Tyr residues (GPIbα269ppp), [Asp-Glu-Gly-Asp-Thr-Asp-Leu-Tyr(PO_3H_2)-Asp-Tyr(-PO_3H_2)-Tyr(PO_3H_2)-Pro-Glu-Glu-Asp-Thr-Glu-Gly], was prepared by Biomatik (Lechtenberg et al., 2014). Hirudin was obtained from Dade-Behring, and hirugen, a synthetic 12 amino acid peptide analog of residues 54 to 65 in the carboxy-terminal domain of hirudin, was obtained from Bachem. Thrombomodulin peptide consisting of the fourth, fifth, and sixth epidermal-like growth factor–like domains (TM-456) was a generous gift from Dr. T. Mather (University of Oklahoma) (Rezaie & Esmon, 1992). Polybrene was purchased from Sigma–Aldrich. BIAcore sensor chips (CM5) and an amine coupling kit were obtained from Cytiva Life Sciences.

3.4.2 Affinities of FXI, FXIa, and thrombin for polyP-70

The affinities of FXI, FXIa, and thrombin for polyP-70 were quantified in the absence or presence of 6 μM $ZnCl_2$, aptamers, hirugen, Gp1bα, or TM456 using surface plasmon resonance (SPR) on the BIAcore T200 (Cytiva Life Sciences). PolyP-70 was attached to a CM5 chip via a diamine linker as described previously (Truong et al., 2021). FXI or FXIa (in concentrations up to 400 nM), or thrombin or its variants (in concentrations up to 2 μM), was injected at a flow rate of 30 μL/min for 90 seconds in TBS containing 0.01% Tween 20 (TBS–Tw). After monitoring dissociation for 300 seconds, flow cells were regenerated with 1M NaCl. Competition experiments were performed to examine the effect of exosite ligands on the interaction of thrombin with polyP-70. Samples containing 250-nM plasma-, recombinant WT–, K109/110E-, or R93E-thrombin were injected in the presence of 0 to 2000 nM of HD1, HD22, or HD23. Response units values were normalized by dividing them by the response units values determined in the absence of a competitor. Sensorgrams were analyzed using the BIAevaluation software version 1.0 (BIAcore). Dissociation constants (K_D) and half maximal effective concentrations (EC_{50}) were determined by steady- state analysis of equilibrium values using the instrument software (BIAcore). Each experiment was repeated 2 times in duplicate.

3.4.3 Effect of polyP-70 on thrombin-mediated FXI activation

Thrombin-mediated FXI activation was determined in the absence or presence of polyP. Assays were performed in 100-μL reactions in TBS. Concentrations of polyP-6, polyP-20, polyP-70, or platelet-derived polyP up to 100 μg/mL were added to individual wells of a 96-well microplate (VWR) containing 40 nM FXI and 10-nM plasma-, WT-, K109/110E-, or R93E-thrombin. Reactants were incubated at 37 °C for 15 minutes before the addition of 100-nM hirudin and 25-μg/mL polybrene to inhibit thrombin and neutralize the polyP-70, respectively. After an additional 5 minutes of incubation at 37 °C, 400-μM S- 2366 was added, and absorbance was monitored at 405 nm every 5 seconds for 30 minutes at 37 °C using a SpectraMax plate reader (Molecular Devices). FXIa generation was calculated by dividing rates of absorbance change over time by the specific activity of FXIa (11.1 mOD/s/nM), which was determined separately. Each experiment was performed 3 times in duplicate.

3.4.4 Effect of exosite ligands on thrombin-mediated FXI activation

In 100-μL reactions, HD1, HD22, or HD23 in individual concentrations up to 10 μM, GPIbα269ppp in concentrations up to 25 μM, TM-456 in concentrations up to 100 nM, or hirugen in concentrations up to 2 μM was added to a solution containing 40 nM FXI and 2 nM plasma- thrombin in aptamer buffer (20 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 0.1% polyethylene glycol, and pH 7.4) in the absence or presence of 10-μg/mL polyP-70. After 15 minutes of incubation at 37 °C, 100 nM hirudin and 25 μg/mL polybrene were added, and after an additional 5 minutes of incubation, 400 μM S-2366 was added and absorbance was monitored at 405 nm as described in Section 3.4.3. To compensate for the slow rate of FXI activation in the absence of polyP-70, 100 nM FXI and 40 nM thrombin were used. The rates of FXI activation normalized relative to that obtained in the absence of aptamers were plotted against aptamer concentrations, and EC_{50} values were determined. The experiment was repeated 3 times in duplicate.

3.4.5 Effect of exosite ligands on thrombin activity

The effect of the exosite ligands on thrombin activity was examined using a chromogenic assay. In 100-μL reactions, TM-456 and HD1 or HD22 in concentrations up to 100 nM were added to wells containing 10 nM plasma-thrombin in TBS and incubated for 15 minutes at 37 °C. After the addition of 400 μM S-2366, absorbance was monitored as described earlier in the article. The rates of substrate hydrolysis as a function of ligand concentration were plotted, and EC_{50} values were calculated by nonlinear regression analysis of a rectangular hyperbola using TableCurve 2D Version 4 (Jandel Scientific Software, SPSS Inc). Experiments were performed 2 times in duplicate.

3.4.6 Statistical analysis

Results are presented as means ± SD. The significance of differences was determined using 1- or 2-way analysis of variance, followed by the Holm–Sidak test for multiple comparisons. SigmaPlot (Systat Software) and IBM SPSS statistics software were used to create graphs and perform the statistical analyses, respectively. For all analyses, P values of $P < 0.05$ were considered statistically significant.

3.5 RESULTS

3.5.1 Binding of thrombin, its variants, FXI, and FXIa to immobilized polyP-70

To verify whether polyP binds FXI and thrombin, the affinities of thrombin (plasma and WT), thrombin exosite variants, FXI, and FXIa for polyP-70 were quantified using SPR. Plasma-, WT-, and K109/ 110E-thrombin bound to polyP-70 with K_D values of 1304 ± 54 nM, 825 ± 4 nM, and 792 ± 2 nM, respectively (Figure 3.1 A, B). In contrast, R93E-thrombin exhibited minimal binding, suggesting that thrombin binds to polyP-70 via exosite 2 (Mutch et al., 2010).

To confirm this concept, we examined the effect of HD1, HD22, and HD23 on thrombin binding to polyP-70. HD22, which binds thrombin exosite 2, abrogated the binding of plasma- and WT- thrombin to polyP-70 with EC_{50} values of 275 ± 62 nM and 213 ± 37 nM, respectively (Figure 3.2 A, B), whereas the nonspecific aptamer HD23 had no effect. HD1, which binds thrombin exosite 1, attenuated the binding of plasma- and WT-thrombin to polyP-70 by 60% to 80% with EC_{50} values of 473 ± 71 nM and 402 ± 71 nM, respectively. Likewise, TM-456 and hirugen, which, similar to HD1, also bind to exosite 1 on thrombin, reduced thrombin binding to immobilized polyP-70, with EC_{50} values similar to those of HD1 (data not shown). Because exosite 2 mediates binding to polyP-70, these findings raise the possibility that HD1 binding to exosite 1 allosterically modulates the exosite 2–mediated interaction of thrombin with polyP.

With K109/110E-thrombin, HD22 attenuated the binding to polyP- 70 with an EC_{50} of 237 ± 36 nM (Figure 3.2 C), a value comparable with that for plasma- and WT-thrombin. Although its exosite 1 is mutated, K109/110E-thrombin binding to polyP-70 was attenuated by HD1 with an EC_{50} value of 597 ± 41 nM. This is consistent with our previous finding that γ-thrombin lacking exosite 1 retains an HD1-binding subdomain (Petrera et al., 2009). Although R93E-thrombin exhibited weak binding to polyP- 70 (Figure 3.1 B), HD1 attenuated this interaction with an EC_{50} value of 214 ± 16 nM. HD23 had minimal effects on the binding of K109/110E- thrombin or R93E-thrombin to polyP-70 (Figure 3.2 D).

FXI and FXIa bound to polyP-70 with K_D values of 127 ± 13 nM and 110 ± 7 nM, respectively (Figure 3.1 C, D), indicating that both the zymogen and activated forms of FXI bind to polyP-70 with similar affinity. Neither HD1, HD23, hirugen, Gp1bα, nor TM456 had any effect on FXI binding to polyP-70, whereas HD22 attenuated FXI binding to polyP-70 by 30% at 2 μM (data not shown). The divergent findings with HD1 and HD23 compared with that with HD22 render it unlikely that HD22 influences the binding of FXI to polyP in a nonspecific manner because the aptamer's phosphate backbone resembles that of polyP. The addition of zinc did not promote FXI binding to polyP (data not shown). Therefore, by binding thrombin and FXI, polyP serves as a template to promote their interaction.

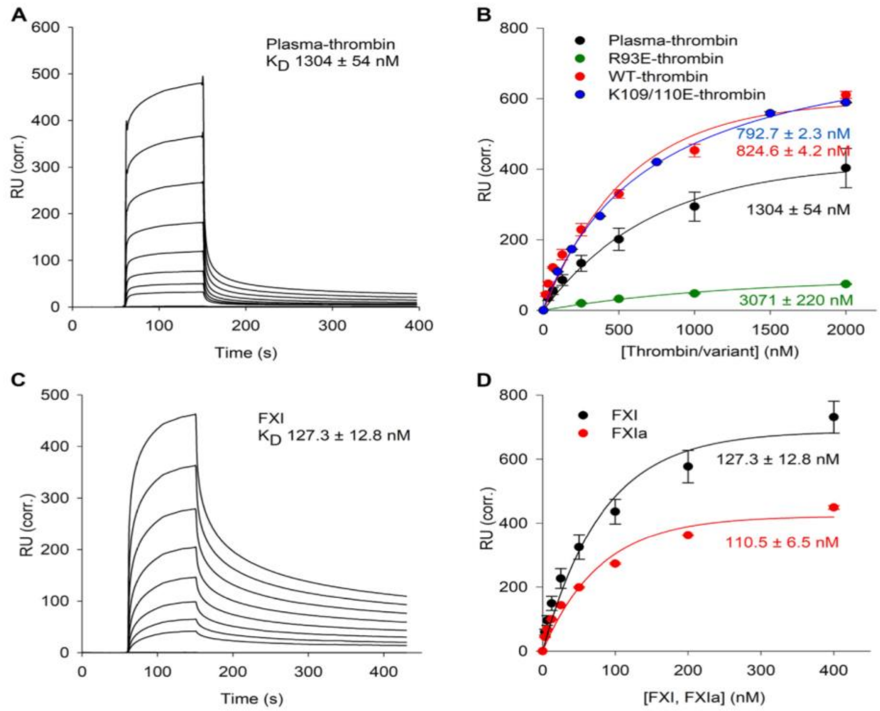
A
Plasma-thrombin
K_D 1304 ± 54 nM
RU (corr.)
Time (s)
B
Plasma-thrombin
R93E-thrombin
WT-thrombin
K109/110E-thrombin
792.7 ± 2.3 nM
824.6 ± 4.2 nM
1304 ± 54 nM
3071 ± 220 nM
RU (corr.)
[Thrombin/variant] (nM)
C
FXI
K_D 127.3 ± 12.8 nM
RU (corr.)
Time (s)
D
FXI
FXIa
127.3 ± 12.8 nM
110.5 ± 6.5 nM
RU (corr.)
[FXI, FXIa] (nM)

Figure 3.1. The binding of proteins to polyphosphate 70 monitored by surface plasmon resonance.

Polyphosphate 70 was immobilized onto a CM5 sensor chip to 140 response units (RU) by diamine coupling. (A, C) Plasma-thrombin (0-4000 nM) or factor XI (FXI [0-400 nM]) were injected at a flow rate of 30 μL/min, and the association was monitored for 90 seconds before injection of running buffer to monitor dissociation. RU values were corrected for blank control (baseline) and plotted against time. (B, D) The corrected RU values at equilibrium are plotted against the concentrations of thrombin (plasma, wild-type [WT] recombinant), K109/110E-thrombin, R93E-thrombin, FXI, and factor XIa (FXIa). Binding data were subjected to steady-state analysis (lines) to determine dissociation constants (K_D). Symbols represent the means of 2 independent experiments, each performed in duplicate, with error bars indicating standard deviation (SD).

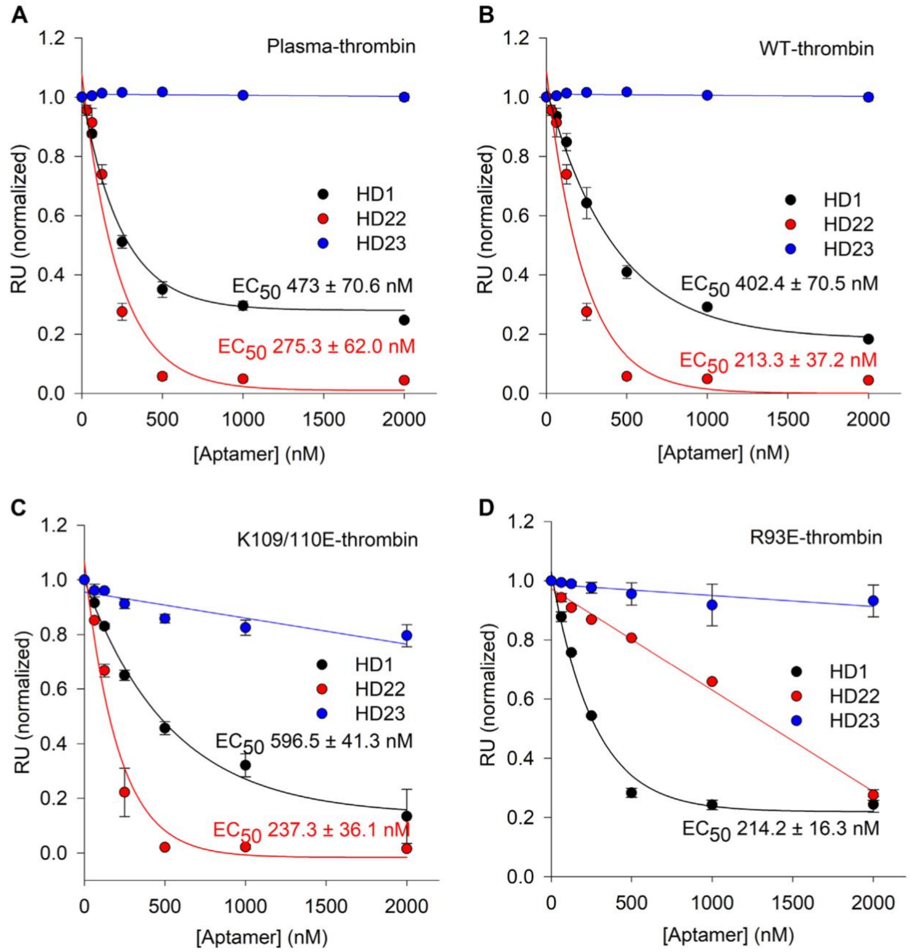
A
Plasma-thrombin
HD1
HD22
HD23
EC50 473 ± 70.6 nM
EC50 275.3 ± 62.0 nM
RU (normalized)
[Aptamer] (nM)
B
WT-thrombin
HD1
HD22
HD23
EC50 402.4 ± 70.5 nM
EC50 213.3 ± 37.2 nM
RU (normalized)
[Aptamer] (nM)
C
K109/110E-thrombin
HD1
HD22
HD23
EC50 596.5 ± 41.3 nM
EC50 237.3 ± 36.1 nM
RU (normalized)
[Aptamer] (nM)
D
R93E-thrombin
HD1
HD22
HD23
EC50 214.2 ± 16.3 nM
RU (normalized)
[Aptamer] (nM)

Figure 3.2. Impact of exosite aptamers on thrombin and variants binding to immobilized polyphosphate 70.

(A) plasma-, (B) wild-type (WT), (C) K109/110E-, or (D) R93E-thrombin (250 nM) was injected over polyphosphate 70–immobilized flow cell in the presence of HD1, HD22, and HD23 aptamers (0-2000 nM). Aptamer concentrations were plotted against normalized response units (RU) values. The data were analyzed by rectangular hyperbola to obtain half maximal effective concentration (EC_{50}) values (lines). Symbols represent the means of 2 independent experiments, each performed in duplicate, with error bars indicating standard deviation (SD).

3.5.2 PolyP-70 and platelet-derived polyP potentiate FXI activation by thrombin

To investigate the chain length of polyP required to promote FXI activation by thrombin, FXI was incubated with plasma-thrombin in the presence of 0 to 100 μg/mL of polyP-6, polyP-20, polyP-70, or platelet-derived polyP, and FXIa generation was quantified. PolyP-70 and platelet-derived polyP at 10 μg/mL promoted FXI activation by 10-nM thrombin to a similar extent, generating FXIa at rates of 1.3 and 1.4 nM/min, respectively. PolyP-70 accelerated FXI activation by thrombin in a bell-shaped and concentration-dependent manner (Figure 3.3). At 10 μg/mL, polyP-70 significantly ($p < .001$) enhanced FXI activation by thrombin by 1500-fold over that determined in its absence. In contrast, neither polyP-6 nor polyP-20 promoted FXI activation, consistent with a previous report that the enhancement of FXI activation by thrombin is dependent on the size of the phosphate chains (Choi et al., 2011). These findings confirm that polyP enhances the activation of FXI by thrombin by serving as a template (Smith et al., 2010)(Choi et al., 2011).

To assess the contribution of exosites 1 and 2 to FXI activation, WT-, K109/110E- and R93E-thrombin were used in place of plasma- thrombin. Peak FXIa generations with plasma- and WT-thrombin were similar (Figure 3.4), indicating that the activity of WT-thrombin is comparable with that of plasma-thrombin. Activation of FXI by the exosite 1 variant K109/110E-thrombin was 30% lower than that by plasma- or WT-thrombin ($p = 0.04$); however, the bell-shaped concentration-dependent response was maintained, suggesting that the template mechanism is mediated by exosite 2 rather than exosite 1. In contrast, there was almost no FXI activation by R93E-thrombin, suggesting that exosite 2 is essential for the enhancement of FXI activation by thrombin in the presence of polyP-70. These results indicate that exosite 2 plays a more important part than exosite 1 in polyP-induced promotion of FXI activation by thrombin (Matafonov et al., 2011).

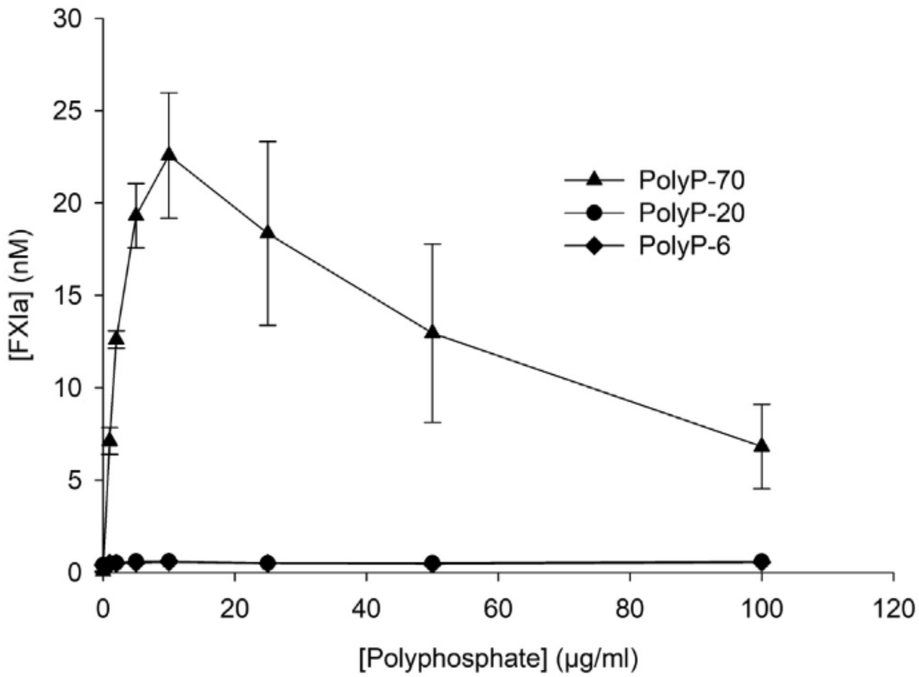
30
25
20
15
10
5
0
[FXIa] (nM)
0
20
40
60
80
100
120
[Polyphosphate] (µg/ml)
PolyP-70
PolyP-20
PolyP-6

Figure 3.3. Effect of polyphosphate (polyP) chain length on thrombin-mediated factor XI activation.

Factor XI (40 nM) was activated with 10-nM plasma-thrombin in the absence or presence of varying concentrations of polyP-6 (◆), polyP-20 (●), or polyP- 70 (▲). Thrombin activity was inhibited with 100 nM hirudin, and polyP was neutralized with 25 μg/mL polybrene before monitoring the hydrolysis of S-2366 (400 μM) to quantify factor XIa (FXIa) generation. The concentrations of FXIa generated are plotted against polyP concentrations. Data represent the mean of 2 experiments, each done in duplicate. Symbols represent the means of 2 independent experiments, each performed in duplicate, with error bars indicating standard deviation (SD).

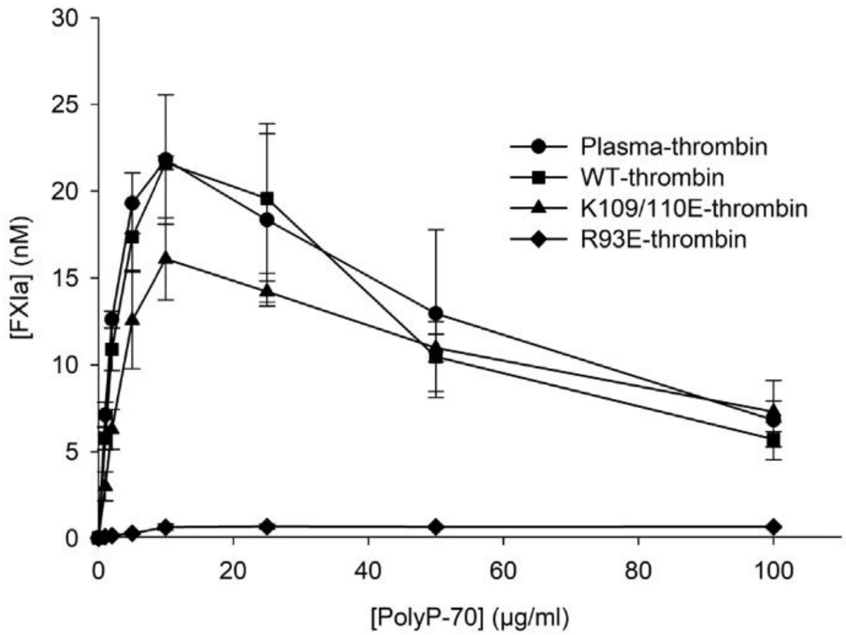
30
25
20
15
10
5
0
[FXIa] (nM)
0
20
40
60
80
100
[PolyP-70] (µg/ml)
Plasma-thrombin
WT-thrombin
K109/110E-thrombin
R93E-thrombin

Figure 3.4. Factor XI activation by thrombin in the presence of polyphosphate (polyP)-70.

Factor XI (40 nM) was incubated with 10 nM plasma- (●), wild-type (WT)- (▪), K109/110E- (▲), or R93E- thrombin (◆) in the presence of polyP (0–100 μg/mL). Thrombin activity was inhibited by the addition of 100-nM hirudin, and polyP was neutralized with 25 μg/mL polybrene. Factor XIa (FXIa) generation was then quantified by monitoring the hydrolysis of S-2366 (400 μM). Data represent the mean of 3 experiments each done in duplicate. Symbols reflect the mean, and the bars reflect the SD.

3.5.3 Exosite 1 ligands attenuate FXI activation

FXI activation by thrombin in the presence of 10 μg/mL polyP-70 was quantified in the absence or presence of HD1, HD22, or HD23. HD1 attenuated FXI activation by plasma-thrombin by up to 40%, with an EC_{50} of 63 ± 3 nM ($p < 0.001$), whereas HD22 produced up to 60% inhibition, with an EC_{50} of 40 ± 9 nM (Figure 3.5 A). In contrast, HD23 had no effect. The reductions in FXI activation by thrombin observed with both HD1 and HD22 suggest that ligand binding to either exosite modulates FXI activation.

To provide further insight, we examined the effect of other exosite-specific ligands on FXI activation by thrombin in the presence of polyP-70. GPIbα269ppp peptide, which binds exosite 2 (Lechtenberg et al., 2014), attenuated FXI activation by plasma-thrombin in a concentration- dependent manner with 60% inhibition at 25 μM (Figure 3.5 B). Furthermore, the peptide ligands hirugen and TM-456, which bind to exosite 1 (Hortin & Benutto, 1990), attenuated thrombin-mediated FXI activation by 35% and 45%, respectively, with EC_{50} values of 481 ± 22 nM and 4 ± 0.5 nM, respectively, values that were 7-fold higher ($p < 0.001$) and 24-fold lower ($p = 0.05$), respectively, than those for HD1 (Figure 3.5 C, D). These results suggest that by modulating the active site or exosite 2, exosite 1 ligands attenuate FXI activation by thrombin.

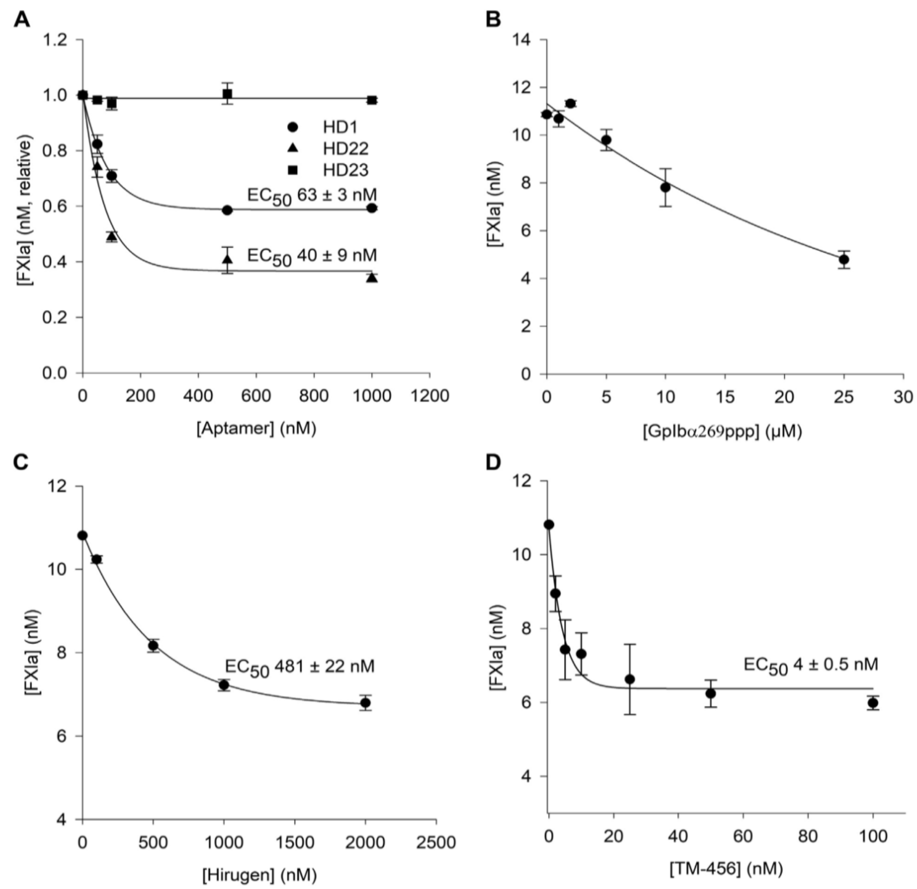
A
B
C
D
HD1
HD22
HD23
EC50 63 ± 3 nM
EC50 40 ± 9 nM
[FXIa] (nM, relative)
[Aptamer] (nM)
[FXIa] (nM)
[GpIbα269ppp] (µM)
EC50 481 ± 22 nM
[Hirugen] (nM)
EC50 4 ± 0.5 nM
[TM-456] (nM)

Figure 3.5 Influence of DNA aptamers and exosite ligands on thrombin-mediated factor XI (FXI) activation in the presence of polyphosphate 70.

Reaction mixtures containing 40-nM FXI, 10 μg/mL polyphosphate 70, and 10-nM plasma-thrombin were incubated with increasing concentrations of (A) HD1 (●), HD22 (▲), or HD23 (■), (B) GpIbα269ppp peptide, (C) hirugen, or (D) thrombomodulin (TM) 456. FXI activation was determined and normalized compared with that determined in the absence of exosite ligands in (A). Half maximal effective concentration (EC_{50}) values were calculated by nonlinear regression (lines). Symbols represent the means of 3 independent experiments, each performed in duplicate, with error bars indicating 1 standard deviation (SD).

3.5.4 Exosite 1 modulates FXI activation through allosteric regulation

Next, we investigated the effect of exosite 1 ligands on thrombin activation of FXI in the absence of polyP-70. Higher concentrations of plasma-thrombin and FXI were used in these experiments to overcome the slow rate of FXI activation in the absence of a polyanion. Compared with the control, HD1, TM-456, and HD22 significantly promoted thrombin-mediated activation of FXI by > 50% ($p < 0.001$), 20% ($p < 0.05$), and 120% ($p < 0.001$), respectively (Figure 3.6 A–C). The enhanced rates of activation observed in the presence of exosite 1– directed ligands render it unlikely that their effects reflect steric hindrance of the active site of thrombin.

To examine a potential allosteric effect of the ligands on the active site of thrombin, we tested their influence on plasma-thrombin chromogenic activity. HD1 increased thrombin chromogenic activity by 10% with an EC_{50} of approximately 6 nM (Figure 3.7 A), which is lower than the EC_{50} of 134 nM required for HD1 attenuation of prothrombin activation or the EC_{50} of 63 nM required for HD1 modulation of thrombin-mediated activation of FXI in the presence of polyP-70. TM-456 promoted thrombin chromogenic activity by approximately 70% in a saturable manner, yielding an EC_{50} value of 33 ± 0.2 nM (Figure 3.7 B). This value is higher than the EC_{50} values of 2 nM and 6 nM reported previously based on chromogenic and fluorescent activity, respectively (Chen et al., 2017). These results suggest that ligand binding to exosite 1 allosterically modulates both the active site and exosite 2. HD22 increased thrombin chromogenic activity by 17%, with an EC_{50} of approximately 16 nM (Figure 3.7 C), a value similar to the reported K_D value of 29 nM (Kretz et al., 2006). The observation that exosite ligands increase thrombin chromogenic activity renders it unlikely that their capacity to attenuate FXI activation by thrombin in the presence of polyP reflects steric hindrance at the active site of thrombin.

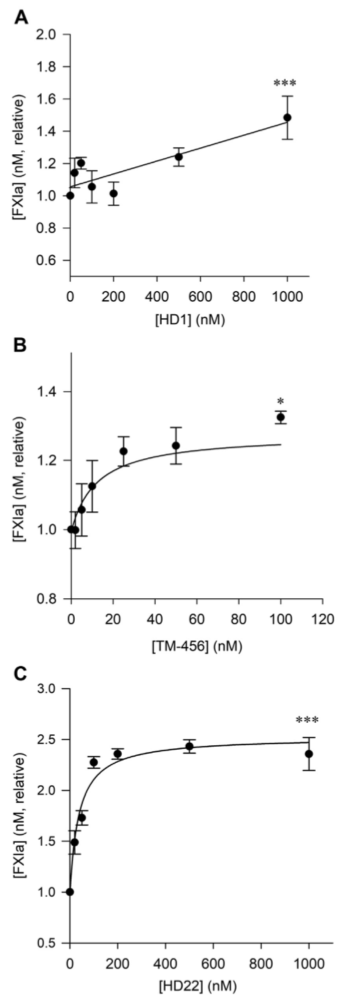
A
[FXIa] (nM, relative)
[HD1] (nM)
***
B
[FXIa] (nM, relative)
[TM-456] (nM)
*
C
[FXIa] (nM, relative)
[HD22] (nM)
***

Figure 3.6. The impact of HD1, TM-456, or HD22 on thrombin- mediated factor XI activation in the absence of polyphosphate 70.

Reactions containing 40 nM factor XI and 10-nM plasma-thrombin were incubated in the absence or presence of (A) HD1, (B) TM-456, and (C) HD22 at the concentrations shown. The generated factor XIa (FXIa) was normalized relative to that determined in the absence of a ligand. The data were analyzed by rectangular hyperbola (lines). Symbols represent the means of 2 independent experiments, each performed in duplicate, with error bars indicating standard deviation (SD). $^*P < 0.05$; $^{***} P < 0.001$ compared with control in the absence of exosite 1 ligands.

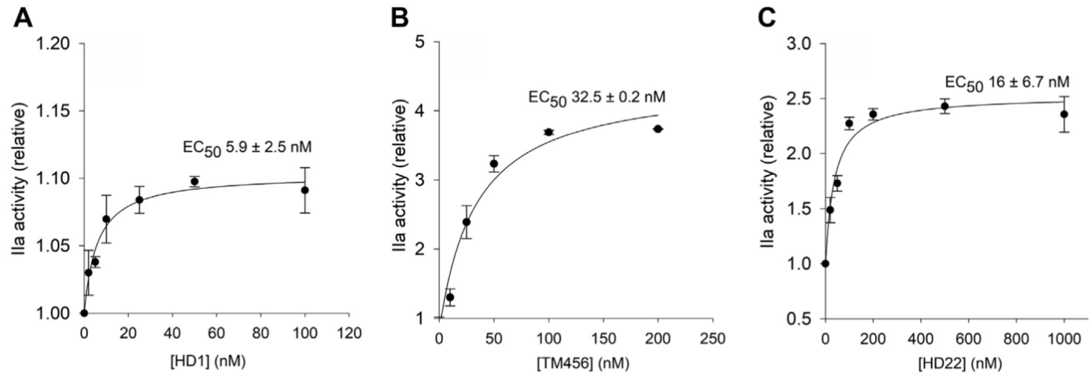
A
B
C
IIa activity (relative)
EC50 5.9 ± 2.5 nM
EC50 32.5 ± 0.2 nM
EC50 16 ± 6.7 nM
[HD1] (nM)
[TM456] (nM)
[HD22] (nM)

Figure 3.7. The impact of exosite 1 or exosite 2 ligands on plasma-thrombin(IIa) chromogenic activity.

Plasma-thrombin (10nM) was incubated with the indicated concentrations of (A) HD1, (B) TM-456, or (C) HD22. The hydrolysis of 400 μM S-2366 was monitored at 405 nm. The rates of cleavage were normalized and plotted against the concentrations of exosite ligands. Data were analyzed by nonlinear regression (lines) to calculate the half maximal effective concentration (EC_{50}) values. Symbols represent the means of 3 independent experiments, each performed in duplicate, with error bars indicating standard deviation (SD).

3.6 DISCUSSION

There is mounting evidence in animals and humans that feedback activation of FXI by thrombin is important for thrombosis (Hsu et al., 2021) (Srivastava & Gailani, 2020). PolyP released from activated platelets promotes this reaction (Choi et al., 2011). In this study, we show the following: (a) that platelet-derived polyP and polyP-70 promote FXI activation by thrombin to a similar extent; (b) that thrombin, FXI, and FXIa bind to polyP-70 with high affinity; (c) that thrombin binds to polyP-70 via exosite 2, an interaction abrogated with exosite 2 ligands; (d) that polyP-70 enhances FXI activation by thrombin by serving as a template; and (e) that exosite 1 ligands attenuate thrombin activation of FXI by allosterically modulating exosite 2 and the active site.

By binding FXI and thrombin, polyP-70 serves as a template and accelerates FXI activation by thrombin up to 1500-fold compared with the control. These findings are consistent with previous reports that polyP stimulates this reaction by 3000-fold (Choi et al., 2011) (Geng et al., 2013). We also confirmed that thrombin, FXI, and FXIa bind to polyP-70 with high affinity, albeit with K_D values higher than those reported previously (Mutch et al., 2010) (Geng et al., 2013). These differences could result from differences in the source of polyP or the method used for its immobilization in the SPR studies. Similar to their interaction with polyP-70, we have previously shown that thrombin and FXI bind to other naturally occurring polyPs, such as DNA and RNA (Vu et al., 2016). Therefore, the results reported here are consistent with the concept that polyP and other polyanions serve as templates that bind thrombin and FXI and promote FXI activation.

Because the activity of thrombin is mediated largely by its exosites, variants with mutated exosites were examined for effects on binding and activation. Exosite 1 variant K109/110E-thrombin exhibited 60% activity of that of thrombin for activating FXI. These results are at odds with a report that relative to thrombin, β-thrombin is a poor activator of FXI in the presence of polyP (Geng et al., 2013). This could relate to the fact that β-thrombin is a proteolytic derivative of α-thrombin, whereas K109/110E-thrombin has an intact B chain. In contrast to the exosite 1 variant of thrombin, FXI activation by the exosite 2 variant R93E-thrombin was only 2% of that produced by thrombin despite the fact that R93E-thrombin and thrombin have similar fibrinogen-clotting activity. The inability of R93E-thrombin to activate FXI in the presence of polyP-70 is consistent with the concept that exosite 2, but not exosite 1, is required to support the interaction of thrombin with polyP and to promote the formation of a ternary polyP-FXI-thrombin complex (Choi et al., 2011) (Geng et al., 2013) (Matafonov et al., 2011).

There is mounting evidence of bidirectional communication between the exosites of thrombin where ligand binding to either exosite influences the function of the other (Fredenburgh et al., 1997) (Petrera et al., 2009) (Malovichko et al., 2013) (Chen et al., 2017) (Troisi et al., 2021). Here, we demonstrate that ligation of exosite 1 attenuates exosite 2 binding to polyP, thus inhibiting ternary complex formation and FXI activation.

This is consistent with a recent observation that HD1 attenuates FXI activation by thrombin stimulated by phospholipid vesicles (Omarova et al., 2021). Although a previous study reported that hirugen does not attenuate FXI activation by thrombin, those experiments were performed with dextran sulfate and not with polyP (Matafonov et al., 2011). Thus, this study suggests that the numerous ligands that bind exosite 1, such as fibrinogen, protease- activated receptor (PAR)-1, and thrombomodulin, may attenuate FXI activation. The interexosite 1→2 allostery observed here parallels previous work showing that exosite 2 ligands alter exosite 1 function in terms of the binding of thrombin to fibrin or thrombomodulin (Petrera et al., 2009) (Chen et al., 2017).

The fact that FXI does not use exosite 1 for activation by thrombin diverges from the dogma that substrates dock to thrombin via exosite 1 before occupying the active site for catalysis. Although this is true for fibrinogen and PAR-1, there are alternative mechanisms. FXIII and protein C use exosite 1 binding to cofactors fibrin and thrombomodulin, respectively, to bring substrate and thrombin into proximity (Huntington, 2005). FXI activation resembles inhibition of thrombin by antithrombin in the presence of heparin, where exosite 2 tethers thrombin to a polyanion to promote binding to the target protein. PAR4 is another outlier because it engages exosite 2 directly to promote interaction before cleavage (Han & Nieman, 2020). These findings underscore the versatility by which thrombin engages substrates and inhibitors.

Because numerous interactions are competing for exosite involvement, more studies are needed to demonstrate whether the allosteric modulation of exosites has any physiological relevance in FXI activation. However, occupation of both exosites can induce unique modulation of the active site of thrombin to ensure optimal activation of some clotting factors. The fact that thrombin-mediated activation of cofactors FV and FVIII involves both exosites and residues outside of the exosites may explain why efficient FXI activation is modulated by both exosites. Because numerous ligands of thrombin exist in the confines of a thrombus, it is difficult to evaluate the net effects of various substrates, cofactors, and inhibitors on thrombin activity. The complexity suggests that environmental and temporal factors may have evolving effects on thrombin activity (Adams & Huntington, 2006) (Crawley et al., 2007).

In summary, these findings build on the proposed mechanism through which the exosites contribute to the procoagulant activity of thrombin as it feeds back to activate FXI in the presence of polyP. We show that the interaction of polyP-70 with exosite 2 is the main driver of FXI activation by thrombin. Although exosite 1 does not contribute to FXI activation, ligand binding to this exosite allosterically modulates FXI activation via exosite 2 and the active site. The identification of a regulatory role for exosite 1 in the feedback activation of FXI reveals a novel strategy for attenuating thrombosis by inhibition of FXI activation.

CHAPTER 4 - COMPARISON OF THE EFFECTS OF MILVEXIAN AND DABIGATRAN ON CATHETER-INDUCED CLOTTING AND THROMBIN GENERATION

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Foreword: The effects of milvexian, dabigatran, and heparins are described in Chapter 4.0. We demonstrate that catheters induce FXI autoactivation, bypassing FXII. Milvexian inhibits catheter-induced clotting and thrombin generation, with its effect being enhanced when used in conjuction with heparin.

Running title: Milvexian inhibits catheter thrombosis.

Author Contributions: R.Y. designed the research, performed the experiments, analyzed, and interpreted the data, and wrote the manuscript. J.C.F. and J.I.W. designed the experiments, analyzed, and interpreted the data, and wrote the manuscript.

Footnotes: J.I.W. holds the Heart and Stroke Foundation J. Fraser Mustard Chair in Cardiovascular Research and the Canada Research Chair (Tier 1) in Thrombosis. This work was supported, in part, by grants-in-aid from the Canadian Institutes of Health Research (FDN-159928) and the Heart and Stroke Foundation of Canada (G-16-00013163).

Conflict-of-interest statements: J.I.W. has served as a consultant and has received honoraria from Bristol-Myers Squibb, Johnson and Johnson, and Boehringer Ingelheim. The other authors declare no competing financial interests.

This manuscript is currently under peer-review by the research and development department at Janssen Pharmaceuticals and will be submitted to the *Journal of Thrombosis and Haemostasis.*

4.1 KEY POINTS

- FXII depletion attenuates catheter-induced clotting, whereas FXI depletion abolishes it.
- Catheter-induced FXI autoactivation may bypass FXII.
- Neither milvexian nor dabigatran efficiently attenuates catheter-induced clotting at clinically relevant concentrations.
- Heparin enhances the inhibitory effects of milvexian on catheter-induced clotting.

4.2 ABSTRACT

Background: Milvexian, an oral factor (F) XIa inhibitor, is in phase 3 evaluation for atrial fibrillation and acute coronary syndrome. Patients in these trials may require percutaneous coronary intervention (PCI). PCI catheters trigger clotting by inducing FXII activation, and it is unknown whether milvexian blocks this process or if adjunctive heparin is needed.

Aim: The aims were to (a) compare the effect of FXII or XI depletion on catheter clotting, and (b) determine the effect of milvexian alone or with heparin on catheter-induced clotting and thrombin generation. Dabigatran was used as a positive control because we have previously shown that it attenuates catheter clotting at high doses and because, like milvexian, it prolongs the activated partial thromboplastin time (aPTT).

Methods: In a 96-well plate assay, catheter-induced clotting and thrombin generation were assessed in recalcified control, FXII-, or FXI-depleted plasma by monitoring absorbance and thrombin-directed substrate hydrolysis. Depleted plasmas were supplemented with various levels of control plasma. Milvexian, dabigatran, heparin, or milvexian plus heparin were added to control plasma, and their effects on catheter-induced clotting and thrombin generation were determined.

Results: Catheter-induced clotting was attenuated in FXII-depleted plasma and abolished in FXI-depleted plasma. Even small amounts of FXI triggered clotting. At clinically relevant concentrations, neither milvexian nor dabigatran prevented catheter-induced clotting and thrombin generation. Heparin potentiated the effect of milvexian on catheter-induced clotting.

Conclusion: The limited capacity of milvexian to inhibit catheter-induced clotting suggests that milvexian-treated patients undergoing PCI should receive adjunctive heparin.

4.3 INTRODUCTION

Factor (F) XI has emerged as a promising target for new anticoagulants that may be safer than currently available agents, including direct oral anticoagulants (DOACs) (Fredenburgh & Weitz, 2023) (Kumar & Ilkhanoff, 2024). Milvexian is an oral FXIa inhibitor that is currently undergoing phase 3 evaluation in the three-part LIBREXIA program (Fredenburgh & Weitz, 2023). This program includes the LIBREXIA AF trial (NCT05757869), which will compare milvexian with apixaban for stroke prevention in patients with atrial fibrillation (AF), and the LIBREXIA Stroke and ACS trials (NCT05702034 and NCT05754957), which will compare milvexian with placebo on top of antiplatelet therapy in patients with non-cardioembolic stroke and acute coronary syndrome (ACS), respectively. Therefore, milvexian is being evaluated in a comprehensive clinical trial program that will enroll over 46,000 patients.

Patients with AF or ACS often require percutaneous coronary intervention (PCI). Heparin is given during PCI to attenuate thrombosis at sites of plaque disruption and to prevent clotting on the angioplasty hardware (Lawton et al., 2022). The guidewires and catheters used for PCI promote clotting by binding FXII and inducing its autoactivation (Yau et al., 2011) (Yau et al., 2012) (Jaffer et al., 2015). FXIIa then activates FXI and induces thrombin generation and fibrin formation via the intrinsic and common pathways of coagulation (Jaffer et al., 2015). Heparin blocks this process by binding to antithrombin and enhancing its capacity to inhibit thrombin and FXa, as well as FIXa, FXIa, and FXIIa (De Caterina et al., 2013). Therefore, heparin is a potent inhibitor of catheter thrombosis.

Because the propagation of coagulation by FXIa is important in the pathogenesis of catheter thrombosis (Yau et al., 2011), milvexian may block this process, thereby rendering heparin redundant for patients taking milvexian who require PCI. However, the extent of FXIa inhibition required for the prevention of catheter thrombosis is unknown, and even small amounts of residual FXIa may be sufficient to propagate coagulation and induce catheter thrombosis. To address these questions, we used an established 96-well microtiter plate-based assay wherein clotting and thrombin generation in recalcified human plasma is induced by PCI catheter segments (Yau et al., 2011). Catheter-induced clotting was first examined in FXI- or FXII-depleted plasma reconstituted with various concentrations of control plasma to identify the minimum levels of FXI and FXII needed to trigger clotting. Next, using purified proteins, the capacity of catheters to activate FXI was examined. Finally, the effect of milvexian on catheter-induced clotting and thrombin generation was examined on its own and in the presence of heparin to determine the extent to which milvexian inhibits clotting and to examine whether heparin potentiates its inhibitory effects. Dabigatran, a potent and specific thrombin inhibitor, was used as a positive control in these studies because, like milvexian, dabigatran prolongs the activated partial thromboplastin time (aPTT), thereby enabling comparison of dabigatran and milvexian at concentrations that produce similar prolongations of the aPTT and because we have previously shown that concentrations of dabigatran far exceeding the therapeutic range of 0.15 to 0.3 µM (90 and 180 ng/ml) obtained at peak and trough, respectively, with the 150 mg twice daily dose of dabigatran (Ng et al., 2022) attenuate catheter-induced clotting in vitro and in rabbits (Yau et al., 2014).

4.4 MATERIALS AND METHODS

4.4.1 Materials

Following approval from the Research Ethics Board (REB) and obtaining signed informed consent, blood was collected into citrate from 15 healthy volunteers, and platelet-poor plasma was obtained and pooled into aliquots and stored at -80°C, as previously described (Guan et al., 2023). Human plasma depleted of FXI or FXII was purchased from Affinity Biologicals (Ancaster, ON). Human FXI, FXII, FXIIa, thrombin, and corn trypsin inhibitor (CTI) were purchased from Enzyme Research Laboratories (South Bend, IN). Milvexian was provided by Dr. M. Chintala (Janssen Research and Development, Spring House, PA) and dabigatran was provided by Dr. J. van Ryn (Boehringer-Ingelheim, Biberach, Germany). Milvexian and dabigatran were dissolved in 100% dimethyl sulfoxide (DMSO) to 10 mg/ml and diluted with 20 mM *N*-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 150 mM NaCl, pH 7.4 (HBS) to 10 mM (final DMSO concentration of 2%, an amount that had no effect on thrombin generation in control experiments (Jaffer et al., 2015)). Unfractionated heparin and polybrene (hexadimethrine bromide) were purchased from Sigma (St. Louis, MO) and reconstituted in Milli-Q water. APTT-SP HemosIL was purchased from Instrumentation Laboratory Co. (Bedford, MA). Hirudin, a potent and specific thrombin inhibitor, was obtained from Dade-Behring (Marburg, Germany). The FXI-directed chromogenic substrate S-2366 was from Hyphen BioMed (Neuville-sur-Oise, France), whereas the thrombin-directed fluorescent substrate, Z-Gly-Gly-Arg-AMC, was from Bachem (Bubendorf, Switzerland). Cyber guide catheters (6 Fr) with an outer diameter of 2 mm and composed of polyether block amide and polytetrafluoroethylene (Berg and Galdonik, 19 A.D.) were a generous gift from Boston Scientific (Marlborough, MA).

4.4.2 Dependence of catheter-induced clotting on FXII and FXI

Catheters were pressed flat with a hand roller and cut into 1.7 cm segments that were rolled into rings and placed in the periphery of wells of a black flat-bottom polystyrene 96-well microtiter plate (Costar Corning, NY) as previously described (Yau et al., 2011) (Jaffer et al., 2015). To obtain plasmas with varying levels of FXI and FXII, FXI- or FXII-depleted plasmas were mixed with varying amounts of control plasma. In wells, both with or without catheter segments, 100 µl aliquots of control, FXI- or FXII-depleted plasma were mixed with 80 µl of 20 mM HEPES pH 7.4. In some experiments, 1 µM CTI was added to eliminate any potential contribution of FXIIa. After 15-minute incubation at 37 ºC, clotting was initiated by the addition of 20 µl of a 260 mM solution of $CaCl_2$, yielding a final calcium concentration of 26 mM, and absorbance was monitored at 405 nm every 10 seconds for 1 hour using an M3 plate reader (Molecular Devices, Sunnyvale, CA). The clotting time was calculated as the time to reach half-maximal absorbance as determined by the instrument software. Each experiment was performed three times in duplicate.

4.4.3 Catheter-induced FXI activation

To explore the mechanism responsible for catheter-induced clotting in FXII-depleted plasma, FXI was incubated alone or with FXIIa or thrombin in the absence or presence of catheter segments. To the wells was added a 200-ul aliquot of 200 nM FXI in HBS containing 40 nM FXIIa, 50 nM thrombin, or no enzyme. After incubation at 37 °C for 90 minutes, 100 nM hirudin was added to inhibit thrombin activity, and FXIa generation was quantified by monitoring the hydrolysis of 400 µM S2366 at 405 nm every 5 seconds for 30 minutes at 37 °C using a SpectraMax plate reader (Molecular Devices). The FXI activation rate (nM/min) was calculated by dividing the change in absorbance change over time by the specific activity of FXIa (11.1 mOD/s/nM), which was determined separately. Each experiment was performed three times in duplicate.

4.4.4 Effect of milvexian or dabigatran on the dilute aPTT

To wells of a 96-well plate, 100 µl aliquots of control plasma were added to 80 µl of 20 mM HEPES pH 7.4 containing up to 10 µM milvexian or dabigatran, and 20 µl of a 1:5 dilution of aPTT-SP. After 15-minute incubation at 37 ºC, clotting was initiated by the addition of 20 µl of a 260 mM solution of $CaCl_2$, yielding a final calcium concentration of 26 mM, and absorbance was monitored at 405 nm every 10 seconds for 1 hour using an M3 plate reader (Molecular Devices). The dilute aPTT was determined as the time to reach half-maximal absorbance as calculated by the instrument software. Experiments were repeated three times in duplicate.

4.4.5 Effect of milvexian, dabigatran, and heparin on catheter-induced clotting

To compare the effects of milvexian, dabigatran, and heparin on catheter-induced clotting, experiments were performed using control plasma containing these anticoagulants. In each setup, to wells containing catheter segments was added 100 µl of control plasma containing up to 10 µM milvexian or dabigatran, or a combination of milvexian up to 5 µM and heparin up to 0.05 U/ml and 80 µl of 20 mM HEPES pH 7.4. After 15 minutes of incubation at 37 ºC, clotting was initiated by the addition of 20 µl of a 260 mM solution of $CaCl_2$, and the clotting time was determined as described above. Each experiment was performed three times in duplicate.

4.4.6 Effect of milvexian, dabigatran, and heparin on catheter-induced thrombin generation

To compare the effects of milvexian, dabigatran, and heparin on catheter-induced thrombin generation, 100 µl aliquots of control plasma containing milvexian up to 5 µM, dabigatran up to 2 µM, or a combination of milvexian up to 5 µM and heparin up to 0.05 U/ml. After 15 min of incubation at 37 ºC, 100 µl aliquots of 20 mM HEPES pH 7.4 containing 15 mM $CaCl_2$ and 1 mM Z-Gly-Gly-Arg-AMC were added to the wells, and substrate hydrolysis was monitored at 1-minute intervals at 37 ºC for 90 minutes in an M3 plate reader at excitation and emission wavelengths of 360 nm and 460 nm, respectively, with a cutoff filter set at 455 nm. Thrombin generation profiles were analyzed using Technothrombin TGA software and the assay was calibrated with the Technothrombin TGA CAL SET according to manufacturer instructions (Technoclone, Vienna, Austria) as previously described (Vu et al., 2015). Experiments were repeated three times in duplicate.

4.4.7 Statistical Analysis

Results are presented as mean ± standard deviation. The significance of differences was determined using one or two-way analysis of variance (ANOVA) followed by the Holm-Sidak or Tukey's comparison test. SigmaPlot (Systat Software, San Jose, CA) and IBM PSS statistics software programs (Armonk, NY) were used to create graphs and perform the statistical analyses, respectively. For all analyses, P values less than 0.05 were considered statistically significant.

4.5 RESULTS

4.5.1 Catheter segments shorten the clotting time and promote thrombin generation

Consistent with our previous results (Yau et al., 2011), the mean clotting time in the presence of catheter segments was 2.7-fold shorter than that measured in their absence (681 ± 72 s and 1862 ± 147 s, respectively; $P< 0.001$, data not shown), and the mean peak thrombin was 3.6-fold higher with catheter segments than without (134 ± 4 nM and 37 ± 10 nM, respectively; $P < 0.001$, data not shown). Therefore, catheter segments are prothrombotic.

4.5.2 FXI is more important than FXII for catheter-induced clotting

Compared with the catheter-induced clotting time of 681 ± 72 s in control plasma, the clotting time was significantly prolonged ($P < 0.001$) to 2304 ± 75 s in FXII-depleted plasma and was unmeasurable in FXI-depleted plasma (Figure 4.1). These observations suggest that although FXII is an essential trigger of catheter-induced clotting, FXI also is important.

Reconstitution of FXI or FXII-depleted plasma with control plasma to achieve varying levels of FXII or FXI shortened the catheter-induced clotting times in a concentration-dependent manner. Clotting times with 100% FXI and FXII were 457 ± 70 and 561 ± 15 s. respectively. Compared with the clotting times in depleted plasma, repletion with less than 2% FXI or 5% FXII restored the clotting times halfway back to those measured with 100% FXI or FXII, and the clotting times were comparable with those in control plasma with 50% FXI or FXII.

CTI was added to FXII-depleted plasma to ensure that the difference in catheter-induced clotting times between FXII and FXI-depleted plasma was not the result of residual FXII. The effect of CTI on catheter-induced clotting in control and FXI-depleted plasma also was investigated. The addition of CTI to control plasma significantly prolonged the catheter clotting time from 821 ± 20 s to 1899 ± 22 s ($P < 0.001$), consistent with the importance of FXIIa in this process (Figure 4.2). In contrast, CTI had a minimal effect on the clotting time in plasma depleted of FXII, suggesting that residual FXII was not responsible for catheter-induced clotting. With or without CTI, there was no catheter-induced clotting in FXI-depleted plasma. These results raise the possibility that catheters may activate clotting at the level of FXI, thereby bypassing FXII.

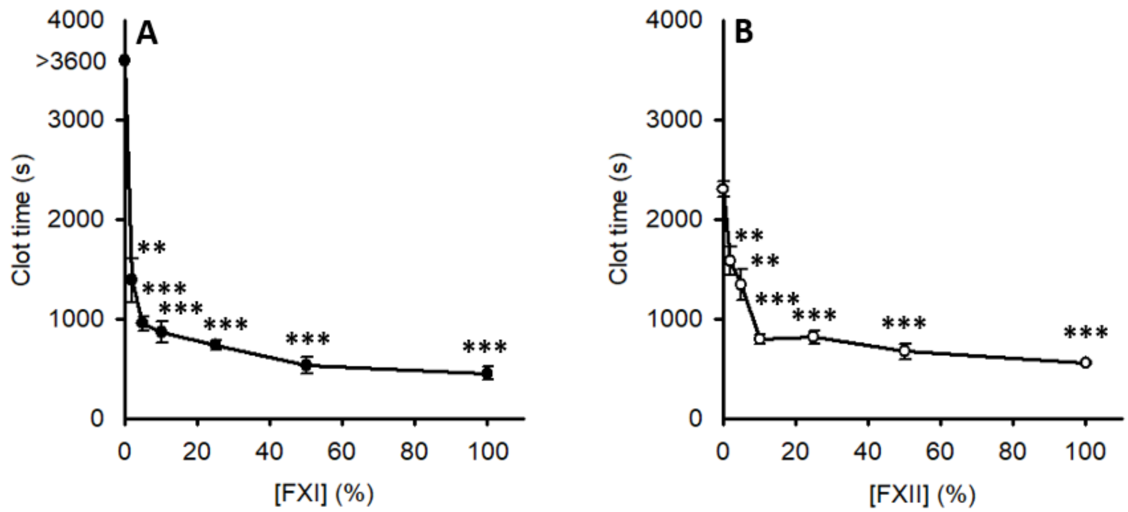

A
4000
>3600
3000
2000
1000
0
Clot time (s)
0 20 40 60 80 100
[FXI] (%)
**
***
***
***
***
***
B
4000
3000
2000
1000
0
Clot time (s)
0 20 40 60 80 100
[FXII] (%)
**
**
***
***
***
***

Figure 4.1. Effect of varying FXI or FXII levels on catheter-induced clotting.

Catheter segments were incubated for 15 minutes at 37 ºC in (A) FXI- or (B) FXII-depleted human plasma reconstituted with control plasma to the specified percentages. Clotting was initiated by adding 26 mM of $CaCl_2$ and clot times were measured. The symbols represent the mean of 2 separate experiments each done in duplicate, whereas the bars reflect the SD (two-way ANOVA, ** $p<0.01$, *** $p<0.001$ compared with 0% FXI or FXII).

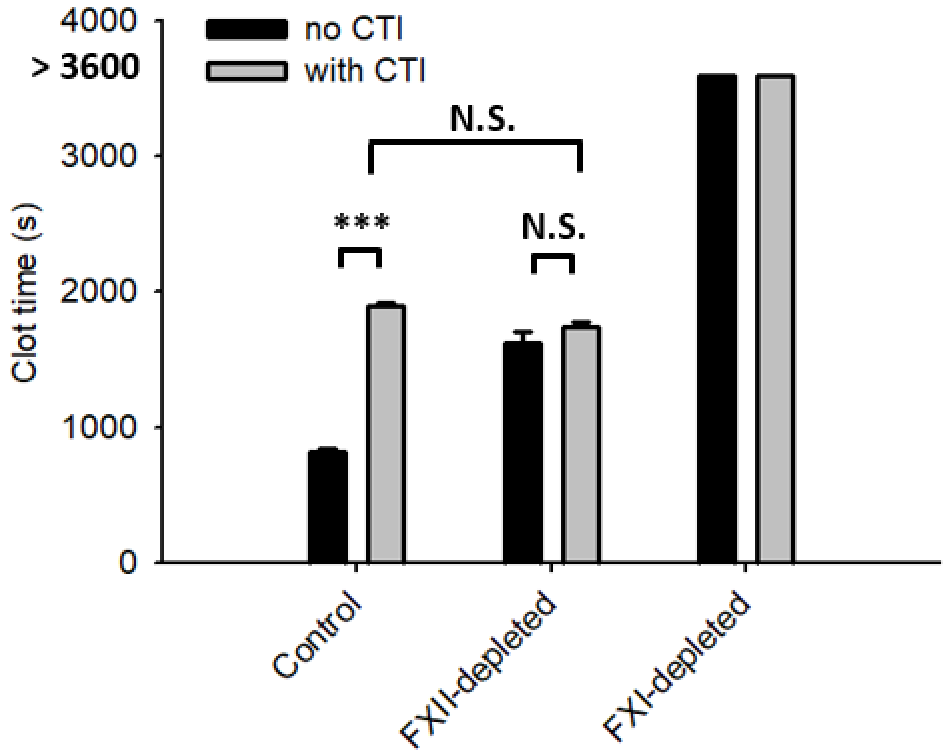

no CTI
with CTI
4000
> 3600
3000
2000
1000
0
Clot time (s)
N.S.
***
N.S.
Control
FXII-depleted
FXI-depleted

Figure 4.2. Effect of CTI on catheter-induced clotting times in control, FXII-depleted, or FXI-depleted plasma.

Control, FXII-depleted, or FXI-depleted plasma was incubated with catheter segments for 15 minutes at 37 ºC in the absence or presence of 1 µM CTI before the addition of $CaCl_2$ to 26 mM. Clot times were determined. The bars represent the mean of 3 experiments, each one in duplicate, whereas the bars represent the SD (one-way ANOVA, *** $p<0.001$). N.S., not significant.

4.5.3 Catheters induce FXI autoactivation

To explain how catheters induce clotting in FXII-depleted plasma, we investigated whether catheters induce FXI autoactivation or promote FXI activation by thrombin or FXIIa. Compared with the control without catheter segments, catheter segments significantly promoted FXIa generation by 12-fold ($P < 0.001$), suggesting that catheters induce FXI autoactivation (Figure 4.3), a finding consistent with previous reports that negatively charged surfaces induce FXI autoactivation (Gailani & Broze, 1991). Furthermore, in the presence of catheter segments, FXI activation by FXIIa or thrombin was 4- and 3-fold greater, respectively, than in their absence ($p < 0.001$). These findings suggest that catheters induce FXI autoactivation and promote FXI activation by FXIIa or thrombin. The capacity of catheters to induce FXI autoactivation provides a plausible explanation for the catheter-induced clotting observed in FXII-depleted plasma. Furthermore, catheters act as a cofactor for FXI activation by FXIIa and thrombin, a role typically attributed to polyanions such as polyphosphate or nucleic acids.

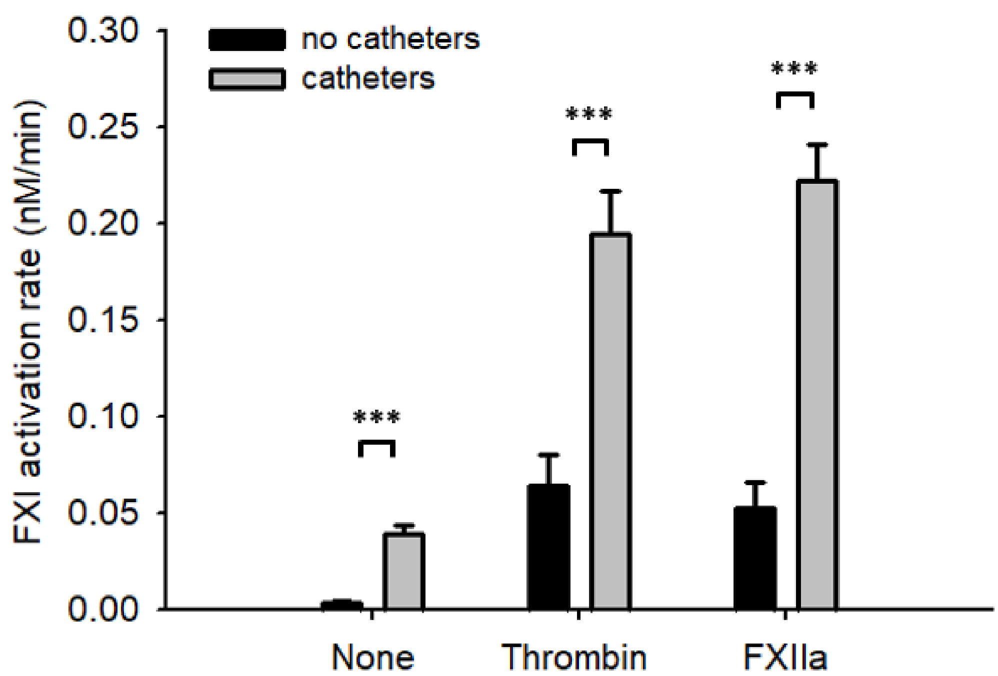

no catheters
catheters
FXI activation rate (nM/min)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
***
***
***
None
Thrombin
FXIIa

Figure 4.3. FXI activation in the absence or presence of catheter segments.

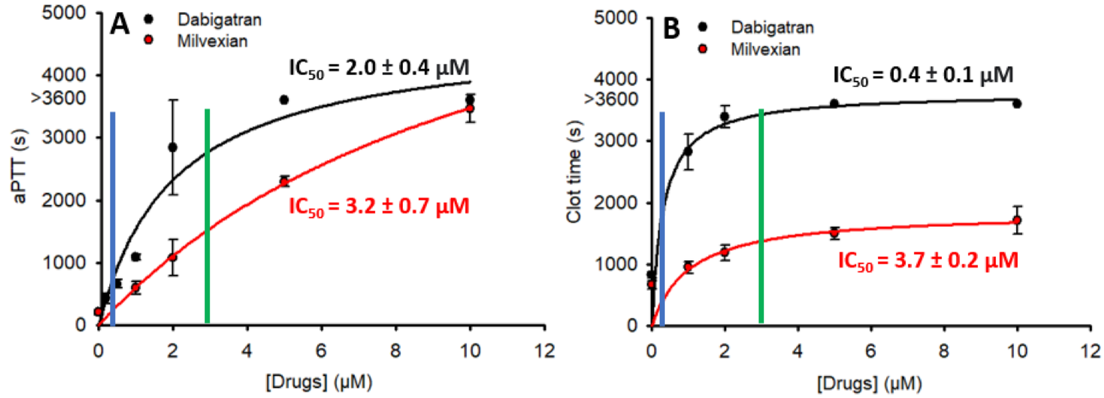
FXI (200 nM) was incubated for 90 min alone or with 50 nM thrombin or 40 nM FXIIa in the absence or presence of catheter segments. After inhibiting thrombin with 100 nM hirudin, FXI activation was quantified by monitoring the hydrolysis of 400 µM S-2366. The bars represent the mean of 3 experiments each done in duplicate, whereas the bars represent the SD (one-way ANOVA, *** $p<0.001$).

4.5.4 Milvexian has less of an effect on the aPTT and catheter-induced clotting and thrombin generation than dabigatran

The dilute aPTT was used to compare the anticoagulant effects of milvexian and dabigatran (Figure 4.4 A). Both milvexian and dabigatran prolonged the aPTT in a concentration-dependent manner with plateaus achieved at 10 µM and 5 µM, respectively, and with IC_{50} values of 3.2 ± 0.7 µM and 2.0 ± 0.4 µM (P = 0.06), respectively. These results need to be considered in the context of the aPTT values expected with therapeutic concentrations of milvexian and dabigatran. The expected therapeutic range for milvexian is from 3 to 5 µM with the 25 mg and 100 mg twice daily doses, respectively (Perera et al., 2022), whereas the therapeutic concentrations of dabigatran range from 0.15 to 0.3 µM (90 to 180 ng/ml) at trough and peak, respectively, for the 150 mg twice-daily dose (Ng et al., 2022). Although dabigatran had a more potent effect on the aPTT than milvexian, the aPTT was less prolonged by clinically relevant concentrations of dabigatran than by those of milvexian.

Both milvexian and dabigatran prolonged the catheter-induced clotting times in a concentration-dependent manner, but milvexian was less potent than dabigatran (Figure 4.4 B). Thus, milvexian prolonged the clotting time up to 3-fold at 10 µM with an IC_{50} value of 3.7 ± 0.2 µM. In contrast, dabigatran abrogated catheter-induced clotting at 2 µM and the IC_{50} value was 0.4 ± 0.1 µM, a value 9.3-fold lower than that of milvexian ($P < 0.001$). At 1 µM dabigatran and 2 µM milvexian, concentrations that produced a similar prolongation of the aPTT, the catheter-induced clotting time was significantly longer with dabigatran than with milvexian (2822 ± 288 and 1186 ± 128 s, respectively; $P < 0.001$).

Overall, milvexian had less of an effect on catheter-induced thrombin generation than dabigatran. Thus, with 1 µM dabigatran, thrombin generation was abrogated and the peak thrombin was reduced to unmeasurable levels (Figure 4.5 A, B). In contrast, even with a milvexian concentration of 5 µM, peak thrombin was only reduced to background levels (Figure 4.5 D, E). Endogenous thrombin potential (ETP) followed a similar trend as peak thrombin (Figure 4.5 C, F). However, at clinically relevant dabigatran concentrations, its effects on thrombin generation were similar to those of milvexian.



A
Dabigatran
Milvexian
IC50 = 2.0 ± 0.4 µM
IC50 = 3.2 ± 0.7 µM
aPTT (s)
5000
4000
>3600
3000
2000
1000
0
0
2
4
6
8
10
12
[Drugs] (µM)
B
Dabigatran
Milvexian
IC50 = 0.4 ± 0.1 µM
IC50 = 3.7 ± 0.2 µM
Clot time (s)
5000
4000
>3600
3000
2000
1000
0
0
2
4
6
8
10
12
[Drugs] (µM)

Figure 4.4. Effect of milvexian or dabigatran on the aPTT and catheter-induced clotting times.

After incubating a 1/50-dilution of aPTT reagent (A) or catheter segments (B) with plasma containing dabigatran or milvexian at the indicated concentrations for 15 minutes at 37 ºC, clotting was initiated by addition of $CaCl_2$ to 26 mM and the clotting times were determined. The clinically relevant concentrations of dabigatran and milvexian of approximately 0.2 and 3 μM, respectively, are shown with the blue and green lines, respectively. The data were analyzed by fitting to a rectangular hyperbola (lines) to obtain the half maximal inhibitory concentration (IC_{50}) values. The symbols represent the mean of 3 experiments, each done in duplicate, whereas the bars reflect the SD.

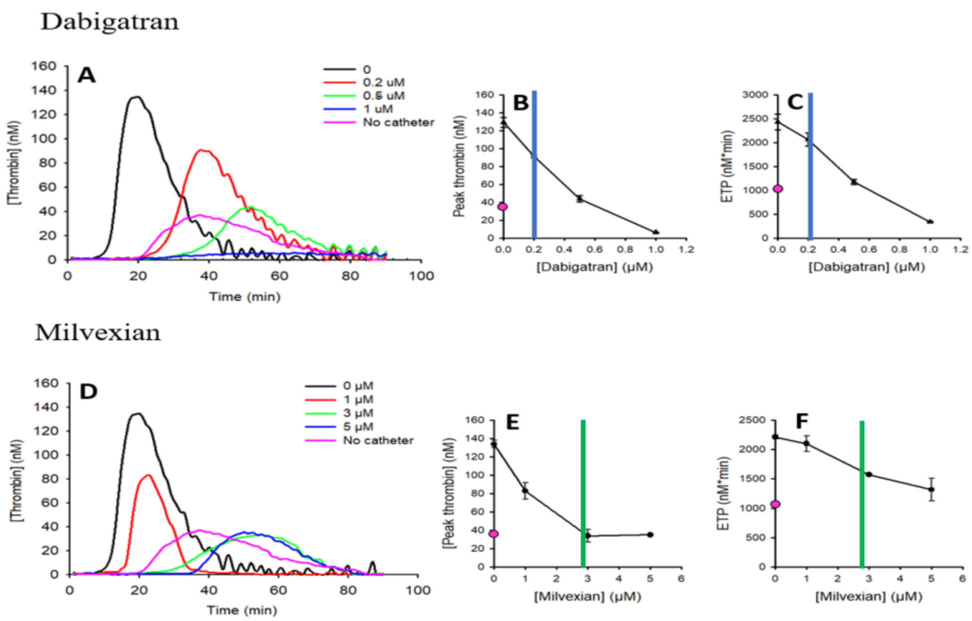

Dabigatran
A
0
0.2 uM
0.5 uM
1 uM
No catheter
[Thrombin] (nM)
Time (min)
B
Peak thrombin (nM)
[Dabigatran] (μM)
C
ETP (nM*min)
[Dabigatran] (μM)
Milvexian
D
0 μM
1 μM
3 μM
5 μM
No catheter
[Thrombin] (nM)
Time (min)
E
[Peak thrombin] (nM)
[Milvexian] (μM)
F
ETP (nM*min)
[Milvexian] (μM)

Figure 4.5. Effect of milvexian or dabigatran on catheter-induced thrombin generation.

Catheter segments were incubated with plasma containing varying concentrations of dabigatran or milvexian for 15 min at 37 °C. Thrombin generation was initiated by the addition of 7.5 mM $CaCl_2$, and the level of thrombin generation was quantified by monitoring the hydrolysis of 0.5 mM Z-Gly-Gly-Arg-AMC. A and D illustrate the effect of dabigatran up to 1 µM and milvexian up to 5 µM on representative thrombin generation profiles. The clinically relevant concentrations of dabigatran and milvexian of approximately 0.2 and 3 µM, respectively, are shown with the blue and green lines, respectively. Values for peak thrombin and endogenous thrombin potential (ETP) are plotted in panels B and E and C and F, respectively. Thrombin generation values without catheter segments (control) are shown on the y-axis (●). The symbols represent the mean of 3 separate experiments, each done in duplicate, and the bars reflect the SD.

4.5.5 Heparin enhances the effect of milvexian on catheter-induced clotting and thrombin generation

Because therapeutic concentrations of milvexian had suboptimal effects on catheter-induced clotting and thrombin generation, we set out to determine whether supplemental heparin augments the effect of milvexian. Accordingly, we compared the effect of heparin or milvexian alone or in combination on catheter-induced clotting and thrombin generation. Heparin concentrations up to 0.05 U/ml were chosen for these studies because there was no clotting with higher concentrations (data not shown). Milvexian concentrations up to 5 μM were examined because higher concentrations are unlikely to be achieved with the doses of milvexian that are being evaluated in the phase 3 LIBREXIA program (Perera et al., 2022). Individually, milvexian and heparin prolonged catheter-induced clotting times in a dose-dependent manner (Table 4.1). When the two agents were combined, the clotting time was prolonged in a more than additive manner. Thus, on their own, 0.025 U/ml heparin and 1 μM milvexian prolonged the clotting time from 673 ± 41 s to 2297 ± 247 and 945 ± 58 s, respectively, whereas the clotting time was prolonged to 4430 ± 126 s when they were combined. The addition of heparin also enhanced the inhibitory effect of milvexian on thrombin generation (Table 4.2). These results suggest that when used together, milvexian and heparin have more than additive effects on the inhibition of catheter-induced clotting and thrombin generation.

Clot time (min)

	Milvexian (µM)			
Heparin (U/ml)	**0**	**1**	**3**	**5**
0	673 ± 41	945 ± 58	1483 ± 72	1713 ± 104
0.025	2297 ± 247	4430 ± 126	>5400	>5400
0.05	3358 ± 135	>5400	>5400	>5400

Table 4.1. Effect of heparin, milvexian, or both on catheter-induced clotting.

Catheter segments were incubated for 15 minutes at 37 ºC in plasma containing heparin, milvexian, or both at the indicated concentrations prior to initiating clotting by the addition of $CaCl_2$ to 26 mM and determining the clotting time. The symbols represent the mean of 3 experiments each done in duplicate, whereas the bars reflect the SD.

Lag time (min)

	Milvexian (µM)			
Heparin (U/ml)	**0**	**1**	**3**	**5**
0	9 ± 3	15 ± 1	29 ± 1	36 ± 1
0.025	11 ± 2	22 ± 0	29 ± 2	33 ± 2
0.05	12 ± 2	24 ± 1	28 ± 1	36 ± 2

Peak thrombin (nM)

	Milvexian (µM)			
Heparin (U/ml)	**0**	**1**	**3**	**5**
0	134 ± 4	83 ± 9	34 ± 7	35 ± 1
0.025	89 ± 16	24 ± 3	8 ± 1	4 ± 1
0.05	78 ± 13	18 ± 4	6 ± 1	3 ± 1

Time to peak (min)

	Milvexian (µM)			
Heparin (U/ml)	0	1	3	5
0	17 ± 5	23 ± 3	53 ± 9	52 ± 3
0.025	27 ± 6	62 ± 2	87 ± 1	87 ± 1
0.05	30 ± 1	70 ± 4	91 ± 2	96 ± 3

ETP (nM*min)

	Milvexian (µM)			
Heparin (U/ml)	**0**	**1**	**3**	**5**
0	2213 ± 35	2102 ± 134	1574 ± 22	1315 ± 194
0.025	2254 ± 76	1558 ± 43	303 ± 57	483 ± 139
0.05	2071 ± 165	916 ± 133	138 ± 20	287 ± 37

Table 4.2. Effect of heparin, milvexian or both on catheter-induced thrombin generation.

Catheter segments were incubated for 15 minutes at 37 ºC in plasma containing heparin, milvexian, or both at the indicated concentrations prior to initiating thrombin generation by the addition of $CaCl_2$ to 15 mM and monitoring the hydrolysis of 1 mM Z-GGR-AMC. The lag time, peak thrombin, time to peak, and ETP values represent the mean of 3 experiments, each done in duplicate, and the SD.

4.6 DISCUSSION

This study was undertaken to determine the capacity of milvexian, an oral FXIa inhibitor, to inhibit catheter-induced clotting. We show that (a) FXII depletion attenuates catheter-induced clotting whereas FXI depletion abrogates it, possibly because catheter-induced FXI autoactivation bypasses FXII deficiency, (b) even small amounts of FXI are sufficient for catheter-induced clotting, (c) although milvexian is less potent than dabigatran at prolonging the aPTT and attenuating catheter-induced clotting and thrombin generation, the drugs have similar effects at clinically relevant concentrations, and (d) small amounts of heparin potentiate the effect of milvexian on catheter-induced clotting and thrombin generation. The limited capacity of therapeutic concentrations of milvexian to attenuate catheter-induced clotting and thrombin generation and the more than additive effect of supplemental heparin on these activities may help to inform the approach to the prevention of catheter-induced thrombosis in patients receiving milvexian who require PCI.

FXII depletion or the addition of CTI to control plasma prolongs the catheter-induced clotting time about 3.4-fold, consistent with our previous studies indicating that catheters initiate clotting by activating FXII (Malik et al., 2023) and that FXII depletion with an antisense oligonucleotide attenuates catheter thrombosis in rabbits (Malik et al., 2023) (Yau et al., 2014). Whereas FXII depletion attenuates catheter-induced clotting, FXI depletion abolishes it. We reported similar results when mechanical heart valve components were used to induce thrombin generation. Thus, thrombin generation induced by valve leaflets or sewing ring segments was attenuated to background levels in FXII-depleted plasma and abolished in FXI-depleted plasma (Jaffer et al., 2015). Therefore, like

FXII, FXI is an important contributor to medical device-induced clotting, which is consistent with our previous report that FXI or FXII depletion attenuates catheter thrombosis in rabbits (Yau et al., 2014).

Catheter-induced clotting in FXII-depleted plasma is unlikely to be triggered by residual FXII because it persists after CTI addition. Instead, catheter-induced FXI autoactivation may provide a mechanism for bypassing FXII. Previous studies have shown that FXI autoactivation is induced by polyanions such as dextran sulfate or sulfatides (Gailani & Broze, 1993) (Yau et al., 2014). Our data suggest that negatively charged surfaces such as catheters have a similar effect. Such a FXIIa-bypassing mechanism may limit the utility of FXIIa-directed inhibitors for the prevention of medical device-induced clotting (Fredenburgh & Weitz, 2023).

Supplementation of FXI-depleted plasma with as little as 2% control plasma restores the capacity of catheters to induce clotting and indicates that even small amounts of FXI are sufficient to trigger this process. This poses a challenge for milvexian or other small molecule, reversible inhibitors of FXIa because without complete inhibition of FXIa, thrombin is generated, which can feed back and activate FXI, thereby generating more FXIa. This phenomenon may explain why at high concentrations, dabigatran, which works downstream to milvexian by inhibiting thrombin, attenuates catheter-induced clotting and thrombin generation to a greater extent than milvexian. At clinically relevant concentrations, however, dabigatran is limited in its capacity to attenuate catheter-induced clotting. This finding is consistent with our previous report that therapeutic concentrations of dabigatran had limited effects on thrombin generations induced by mechanical heart valves (Vu et al., 2015).

Heparin is given routinely during PCI at least in part to prevent clotting on the PCI hardware. Because milvexian is limited in its capacity to attenuate catheter-induced clotting, we examined whether heparin would potentiate the effect of milvexian on this process. Low concentrations of heparin were evaluated because higher concentrations abrogated catheter-induced clotting. However, even with low heparin concentrations, its addition to milvexian promotes the effect of milvexian on catheter-induced clotting and thrombin generation. These findings suggest that if patients receiving milvexian require PCI, adjunctive heparin administration will prevent catheter-induced clotting.

The findings in a static, plate-based catheter thrombosis model are unlikely to recapitulate the complex events that occur in humans. However, our previous demonstration that fondaparinux has a limited capacity to inhibit catheter thrombosis in this plate-based assay (Yau et al., 2012) likely explained why catheter thrombosis was observed in patients undergoing PCI who were randomized to fondaparinux in the Fifth and Sixth Organization to Assess Strategies in Ischemic Syndromes (OASIS-5 and OASIS-6 trials) (Mehta et al., 2008). Like our findings with milvexian, heparin augmented the capacity of fondaparinux to inhibit catheter-induced clotting (Jaffer et al., 2020), and this finding prompted the recommendation to administer adjunctive heparin to patients enrolled in the OASIS-5 and 6 trials who underwent PCI, thereby reducing the risk of catheter thrombosis. Furthermore, using a similar plate-based assay, we demonstrated that, at clinically relevant concentrations, dabigatran and apixaban were less effective than warfarin at attenuating thrombin generation induced by mechanical heart valves (Jaffer et al., 2015) (Jaffer et al., 2020), which explained why there were more strokes with dabigatran and apixaban than with warfarin in the Randomized, Phase II Study to Evaluate the Safety and Pharmacokinetics of Oral Dabigatran Etexilate in Patients after Heart Valve Replacement (RE-ALIGN) and A Trial to Determine if Participants With an On-X Aortic Valve Can be Maintained Safely on Apixaban (PROACT Xa) **t**rials, respectively (Eikelboom et al., 2013) (Wang et al., 2023). Therefore, our in vitro findings in plate-based assays may well be of clinical importance.

In summary, the limited capacity of milvexian to attenuate catheter-induced clotting is overcome with adjunctive heparin. These findings suggest that the administration of adjunctive heparin to milvexian-treated patients undergoing PCI will be needed to prevent catheter-induced clotting.

CHAPTER 5 - COMPARISON OF THE EFFECTS OF ABELACIMAB, DALTEPARIN, AND APIXABAN ON CATHETER-INDUCED CLOTTING AND THROMBIN GENERATION

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Foreword: Chapter 5 examines the effects of the Phase 3 clinical FXI inhibitor abelacimab, compared to apixaban and dalteparin, on catheter thrombosis. We show that abelacimab and dalteparin are more effective than apixaban in reducing catheter-induced clotting and thrombin generation.

Running title: Abelacimab attenuates catheter thrombosis

Author Contributions: R.Y. designed the research, performed the experiments, analyzed, and interpreted the data, and wrote the manuscript. J.C.F. and J.I.W. designed the experiments, analyzed, and interpreted the data, and wrote the manuscript.

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This manuscript is prepared to be submitted to the *Journal of Thrombosis and Haemostasis.*

5.1 KEY POINTS

- At clinically relevant concentrations, dalteparin demonstrates the greatest prolongation of aPTT and catheter-induced clotting, as well as the most pronounced reduction on thrombin generation compared to abelacimab and apixaban.
- Abelacimab and dalteparin are more effective than apixaban in reducing catheter-induced clotting and thrombin generation.
- Ongoing ASTER and MAGNOLIA trials will determine if these in vitro results translate to better prevention of catheter-induced thrombosis in cancer patients

5.2 ABSTRACT

Background: In ongoing Phase 3 trials, abelacimab, an antibody that binds factor XI and blocks its activation, is being compared with apixaban and dalteparin in patients with cancer-associated venous thromboembolism. Patients with cancer often have central venous catheters, which are prone to clotting. How abelacimab compares with apixaban and dalteparin for the prevention of catheter clotting is unknown.

Aim: To compare the effects of abelacimab, apixaban, and dalteparin on the activated partial-thromboplastin time (aPTT) and catheter-induced clotting and thrombin generation using an established plate-based catheter thrombosis model.

Methods: The effect of abelacimab, apixaban, and dalteparin on aPTT was determined. Human plasma containing abelacimab, apixaban, or dalteparin was added to wells of 96-well plates with or without catheter segments, and after incubation and recalcification, the time to clot formation and thrombin generation were quantified.

Results: All three anticoagulants prolonged the aPTT and suppressed catheter-induced clotting and thrombin generation in a concentration-dependent manner. At clinically relevant concentrations, dalteparin and abelacimab had more potent effects on the aPTT and catheter-induced clotting and thrombin generation than apixaban.

Conclusion: The findings suggest that abelacimab and dalteparin suppress catheter-induced clotting more effectively than apixaban. The results of the ASTER and MAGNOLIA trials will determine whether these results will translate into a lower incidence of central venous catheter thrombosis with abelacimab than with apixaban and a similar incidence with dalteparin in cancer patients.

5.3 INTRODUCTION

Factor (F) XI has emerged as a target for new anticoagulants that have the potential to be safer than the currently available agents because FXI is essential for thrombosis but mostly dispensable for hemostasis (Hsu et al., 2021). Abelacimab is a monoclonal antibody that binds to the catalytic domain of FXI and has a dual mechanism of action. Thus, it locks FXI in the zymogen conformation thereby preventing its activation by FXIIa or thrombin and it blocks FXIa activity (Koch et al., 2019). Therefore, abelacimab attenuates FXIa generation and inhibits FXIa activity.

Abelacimab is currently undergoing Phase 3 evaluation for the treatment of patients with cancer-associated venous thromboembolism (VTE) in two trials. The ASTER trial (NCT05171049) compares the efficacy and safety of abelacimab with apixaban in patients eligible for a direct oral anticoagulant (DOAC), whereas the MAGNOLIA trial (NCT05171075) compares the efficacy and safety of abelacimab with dalteparin in patients at high risk for bleeding such as those with gastroesophageal cancer, unresected colon cancer, or genitourinary cancer. In both trials, the first dose of abelacimab is given intravenously to ensure a rapid onset of action, and subsequent abelacimab doses are given subcutaneously on a once-monthly basis. Patients with VTE on the background of cancer are at higher risk for recurrence and for bleeding than those without cancer (Prandoni et al., 2002). Consequently, the ASTER and MAGNOLIA trials will be the first to establish the role of abelacimab in the treatment of high-risk patients with VTE.

Patients with cancer often have central venous catheters (CVCs), which include peripherally inserted central catheters, tunneled catheters, and implanted ports, for venous access and the delivery of chemotherapy, blood products, and parenteral nutrition. However, CVCs are associated with a risk of symptomatic upper-extremity deep vein thrombosis (DVT), which occurs with a reported incidence ranging from 1% to 5% (Geerts, 2014). Patients with CVC-related DVT often present with arm pain and swelling, loss of line function, and risk for pulmonary embolism (Verso & Agnelli, 2003) (Jaffer & Weitz, 2019) (Citla Sridhar et al., 2020). CVC thrombosis is associated with significant morbidity because it can delay cancer treatments, necessitate CVC removal, and lead to post-thrombotic syndrome (Baumann Kreuziger et al., 2017). Consequently, there is a need for effective anticoagulant regimens for the prevention and treatment of CVC thrombosis (Debourdeau et al., 2021).

CVCs trigger clotting by binding FXII and inducing its autoactivation (Jaffer et al., 2015). Using a microplate-based catheter thrombosis model, we previously showed that catheter-induced clotting was attenuated in plasma deficient in FXII or FXI, but not in plasma deficient in FVII, and that corn trypsin inhibitor (CTI), a potent and specific inhibitor of FXIIa, prevented catheter-induced clotting (Yau et al., 2011). The importance of FXII as the initiator of catheter thrombosis was confirmed in a rabbit jugular vein catheter thrombosis model by demonstrating that (a) compared with uncoated catheters, those coated with CTI exhibited attenuated thrombosis, and (b) knockdown of FXII or FXI levels with factor-specific antisense oligonucleotides prolonged the time to catheter occlusion compared with a control, whereas knockdown of FVII did not (Yau et al., 2012) (Yau et al., 2014) (Malik et al., 2023). Therefore, because catheter thrombosis is driven via the contact system, by inhibiting FXIa generation and FXIa activity, abelacimab has the potential to block this process. By contrast, using the same plate-based assay system, we previously demonstrated that direct oral anticoagulants such as apixaban and rivaroxaban were less efficacious than enoxaparin for the prevention of catheter clotting (Guan et al., 2023).

The purpose of this study was to use the established plate-base assay to compare the efficacy of abelacimab, apixaban, and dalteparin, the anticoagulants that are being evaluated in the ASTER and MAGNOLIA trials, for the prevention of catheter-induced clotting and thrombin generation. Based on our previous studies, we hypothesized that abelacimab and dalteparin would be more efficacious than apixaban.

5.4 MATERIALS AND METHODS

5.4.1 Materials

Abelacimab was provided by Anthos Therapeutics (Cambridge, MA). Apixaban and dalteparin were obtained from Pfizer Canada (Kirkland, QC). For comparison purposes, dalteparin concentrations were expressed in molar terms using the conversion 1 IU/ml = 1.28 μM. Apixaban was dissolved in 100% dimethyl sulfoxide (DMSO) to 10 mg/ml and further diluted with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 150 mM NaCl, pH 7.4 (HBS) to 10 mM (final DMSO concentration of 1%, an amount that had no effect on thrombin generation in control experiments (Jaffer et al., 2015)). The activated partial-thromboplastin time (aPTT) reagent, APTT-SP HemosIL, was purchased from Instrumentation Laboratory Co. (Bedford, MA). The thrombin-directed fluorescent substrate, Z-Gly-Gly-Arg-AMC, was from Bachem (Bubendorf, Switzerland). Cyber guide catheters (6 Fr) with an outer diameter of 2 mm and composed of polyether block amide and polytetrafluoroethylene (Berg and Galdonik, 19 A.D.) were a generous gift from Boston Scientific (Marlborough, MA).

5.4.2 Preparation of pooled human platelet-poor plasma

Following approval from the Research Ethics Board (REB) and obtaining signed informed consent, using a butterfly needle, blood was collected from the antecubital veins of at least 17 healthy volunteers into 3.5% citrate (9:1 vol/vol), and platelet-poor plasma was obtained, pooled, and stored in aliquots at -80°C, as previously described (Guan et al., 2023).

5.4.3 Effect of abelacimab apixaban, and dalteparin on the dilute aPTT

To wells of a 96-well plate were added 80 µl aliquots of 20 mM HEPES, pH 7.4, containing up to 1 µM abelacimab, 1.3 µM apixaban, or 2 µM dalteparin and 100-µl aliquots of control plasma. These drug concentrations were chosen to span the upper limits of the plasma concentration of abelacimab, apixaban, and dalteparin achieved with doses of 150 mg subcutaneously once monthly, 5 mg orally twice daily, and 200 IU/kg subcutaneously once daily, respectively. After the addition of 20 µl of a 1:5 dilution of aPTT-SP to each well and incubation for 15 minutes at 37 ºC, clotting was initiated by the addition of 20 µl of a 260 mM solution of $CaCl_2$ (final calcium concentration, 26 mM), and absorbance was monitored at 405 nm every 10 seconds for 1 hour using an M3 plate reader (Molecular Devices). The aPTT was determined as the time to reach half-maximal absorbance as calculated by the instrument software. Experiments were repeated three times in duplicate.

5.4.4 Effect of abelacimab, apixaban, and dalteparin on catheter-induced clotting

To wells containing catheter segments was added 100 µl of control plasma containing up to 1 µM abelacimab, 1.3 µM apixaban, or 0.5 µM dalteparin, and 80 µl of 20 mM HEPES, pH 7.4. After 15 minutes of incubation at 37 ºC, clotting was initiated by the addition of 20 µl of a 260 mM solution of $CaCl_2$, and the clotting time was determined as described above. Each experiment was performed three times in duplicate.

5.4.5 Effect of abelacimab, apixaban, and dalteparin on catheter-induced thrombin generation

To wells containing catheter segments were added 100-µl aliquots of control plasma containing apixaban up to 8.5 µM, abelacimab up to 5 µM, and dalteparin up to 1.3 µM. These higher drug concentration ranges were selected to ensure that the thrombin generation values would be lower than the controls and to include the clinically relevant concentrations of the drugs. After 15-minutes of incubation at 37 ºC, 100-µl aliquots of 20 mM HEPES, pH 7.4, containing 15 mM $CaCl_2$ and 1 mM Z-Gly-Gly-Arg-AMC were added to the wells, and substrate hydrolysis was monitored at 1-minute intervals for 90 minutes at 37 ºC in an M3 plate reader at excitation and emission wavelengths of 360 nm and 460 nm, respectively, with a cutoff filter set at 455 nm. Thrombin generation profiles were analyzed using Technothrombin TGA software and the assay was calibrated with the Technothrombin TGA CAL SET according to manufacturer instructions (Technoclone, Vienna, Austria) as previously described (Vu et al., 2015). Experiments were repeated three times in duplicate.

5.4.6 Statistical Analysis

Results are presented as mean ± standard deviation. The significance of differences was determined using a one-way analysis of variance (ANOVA) followed by the Holm-Sidak or Tukey's comparison test. SigmaPlot (Systat Software, San Jose, CA) and IBM PSS statistics software (Armonk, NY) were used to create graphs and to perform the statistical analyses, respectively. For all analyses, P values less than 0.05 were considered statistically significant.

5.5 RESULTS

5.5.1 Catheter segments promote clotting and thrombin generation

The mean clotting time in the presence of catheter segments was 2.7-fold shorter than that measured in their absence (681 ± 72 s and 1862 ± 147 s, respectively; $P < 0.001$, data not shown). The mean peak thrombin was 3.4-fold higher with catheter segments than without (146 ± 6 nM and 43 ± 2 nM, respectively; $P < 0.001$, data not shown) and the lag time was 2.8-fold longer with catheter segments than without (25 ± 8 min and 9 ± 2 min, respectively; $P < 0.001$, data not shown). Therefore, catheter segments are prothrombotic.

5.5.2 Abelacimab prolongs the aPTT more than apixaban but less than dalteparin

The dilute aPTT was utilized to compare the anticoagulant effect of abelacimab, apixaban, and dalteparin. All three anticoagulants prolonged the dilute aPTT in a concentration-dependent manner (Figure 5.1). At a therapeutically relevant concentration of dalteparin of 1.3 μM, the aPTT was approximately 2- and 4-fold longer than with clinically relevant concentrations of abelacimab and apixaban of 0.3 μM and 1 μM, respectively. With abelacimab, the aPTT reached a plateau at 1 μM, and the IC_{50} value was 0.2 ± 0.05 μM.

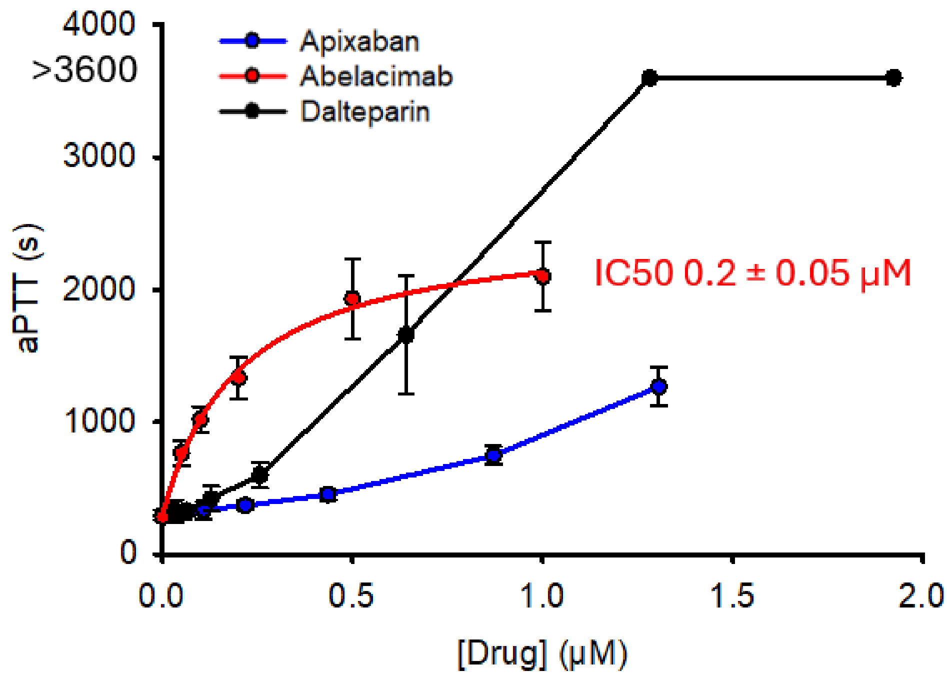
Apixaban
Abelacimab
Dalteparin
IC50 0.2 ± 0.05 μM
aPTT (s)
4000
>3600
3000
2000
1000
0
0.0
0.5
1.0
1.5
2.0
[Drug] (μM)

Figure 5.1. Effect of abelacimab, apixaban, or dalteparin on the aPTT.

After incubating a 1/50-dilution of aPTT reagent with plasma containing apixaban (blue), abelacimab (red) or dalteparin (black) at the indicated concentrations for 15 minutes at 37 °C, clotting was initiated by addition of $CaCl_2$ to 26 mM and the clotting times were analyzed by fitting to a rectangular hyperbola (lines) to obtain the half maximal inhibitory concentrations (IC_{50}) values for abelacimab. The symbols represent the mean of 3 experiments, each done in duplicate, whereas the bars reflect the SD.

5.5.3 Abelacimab and dalteparin attenuate catheter-induced clotting more effectively than apixaban

In the presence of catheter segments, apixaban, abelacimab, and dalteparin attenuated clotting in a concentration-dependent manner (Figure 5.2). With therapeutic concentrations of abelacimab and dalteparin (0.3 μM and 1.3 μM, respectively), the clotting time was about 2500 s. In contrast, with a therapeutic concentration of apixaban of 1 μM, the maximal clotting time was approximately 1000 s. The IC_{50} values with dalteparin was 0.04 ± 0.03 μM, which was significantly lower than those of abelacimab (0.12 ± 0.04 μM) and apixaban (0.3 ± 0.01 μM) ($P< 0.001$). No clotting was observed with higher concentrations of dalteparin and abelacimab, whereas there was clotting with the highest concentration of apixaban that was evaluated. These findings suggest that dalteparin has the most potent inhibitory effect on catheter-induced clotting followed by abelacimab. Apixaban has the least potent effect on catheter-induced clotting.

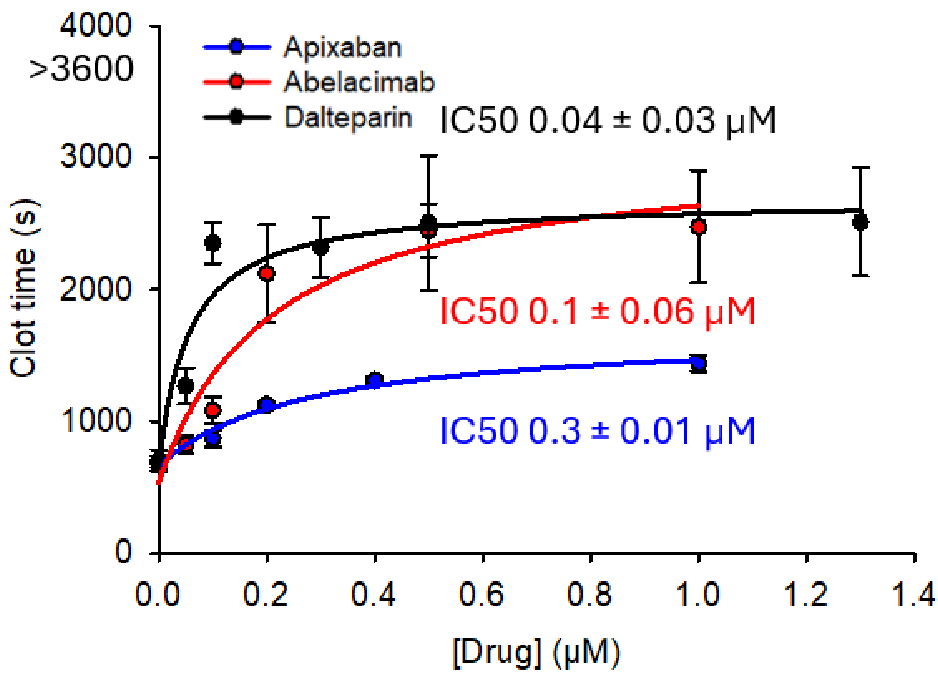
4000
>3600
3000
2000
1000
0
Clot time (s)
Apixaban
Abelacimab
Dalteparin
IC50 0.04 ± 0.03 μM
IC50 0.1 ± 0.06 μM
IC50 0.3 ± 0.01 μM
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
[Drug] (μM)

Figure 5.2. Effect of abelacimab, apixaban, or dalteparin on catheter-induced clotting times.

After incubating catheter segments with plasma containing apixaban (blue), abelacimab (red) or (B) dalteparin (black) at the indicated concentrations for 15 minutes at 37 °C, clotting was initiated by addition of $CaCl_2$ to 26 mM and the clotting times were analyzed by fitting to a rectangular hyperbola (lines) to obtain the half maximal inhibitory concentrations (IC_{50}) values for dalteparin and abelacimab. The symbols represent the mean of 3 experiments, each done in duplicate, whereas the bars reflect the SD.

5.5.4 Abelacimab and dalteparin attenuate catheter-induced thrombin generation more effectively than apixaban

All three anticoagulants attenuated catheter-induced thrombin generation in a concentration-dependent manner (Figure 5.3 A, B, and C). The thrombograms demonstrate that at therapeutic concentrations, dalteparin is the most effective in inhibiting peak thrombin generation to levels less than the no catheter control (Figure 5.3 D). Abelacimab suppresses thrombin generation to levels like no catheter control and more than that with apixaban. The relative effects of these agents on endogenous thrombin potential (ETP) followed a similar trend as peak thrombin (Figure 5.3 E) with dalteparin having the greatest effect followed by abelacimab and apixaban (Table 5.1).

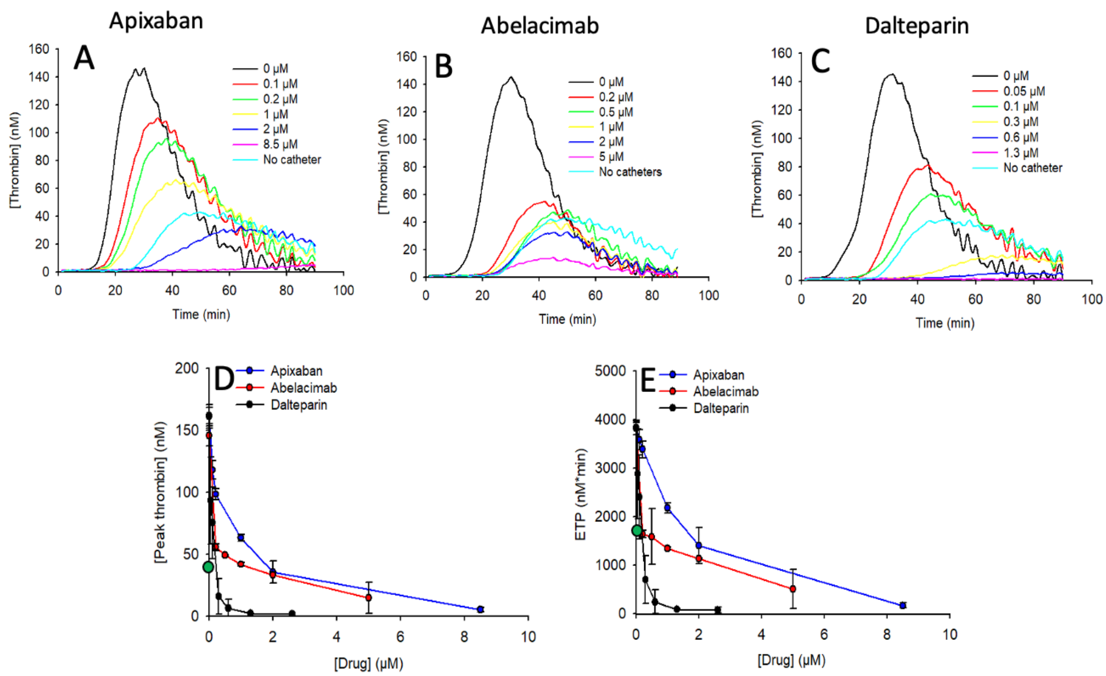
Apixaban
A
0 μM
0.1 μM
0.2 μM
1 μM
2 μM
8.5 μM
No catheter
[Thrombin] (nM)
Time (min)
Abelacimab
B
0 μM
0.2 μM
0.5 μM
1 μM
2 μM
5 μM
No catheters
Dalteparin
C
0 μM
0.05 μM
0.1 μM
0.3 μM
0.6 μM
1.3 μM
No catheter
D
Apixaban
Abelacimab
Dalteparin
[Peak thrombin] (nM)
[Drug] (μM)
E
ETP (nM*min)

Figure 5.3. Effect of apixaban, abelacimab, or dalteparin on catheter-induced thrombin generation.

Catheter segments were incubated with plasma containing varying concentrations of abelacimab for 15 min at 37°C. Thrombin generation was initiated by the addition of 7.5 mM $CaCl_2$, and the level of thrombin generation was quantified by monitoring the hydrolysis of 0.5 mM Z-Gly-Gly-Arg-AMC. Representative thrombin generation profiles of (A) apixaban up to 8.5 µM, (B) abelacimab up to 5 µM or (C) dalteparin up to 1.3 µM are illustrated. Values for peak thrombin and endogenous thrombin potential (ETP) of apixaban and abelacimab are illustrated in (D) and (E). Thrombin generation values without catheter segments (control) are shown on y-axis (green). The lines represent the mean of 3 separate experiments, each done in duplicate, and the bars reflect the SD.

	IC 50 (μM; mean ± SEM; n= 3)	
Drugs	Peak thrombin	ETP
Apixaban	0.5 ± 0.1	1.1 ± 0.1
Abelacimab	0.2 ± 0.1	0.4 ± 0.2
Dalteparin	0.06 ± 0.01	0.1 ± 0.02

Table 5.1. Parameters of catheter-induced thrombin generation.

Peak thrombin values and ETP were analyzed by fitting to a rectangular hyperbola (lines) to obtain the half maximal inhibitory concentrations (IC_{50}) values. The symbols represent the mean of 3 separate experiments, each done in duplicate, and the bars reflect the SD.

5.6 DISCUSSION

In this study, we used plate-based assays to compare the effects of abelacimab, apixaban, and dalteparin on catheter-induced clotting and thrombin generation. At clinically relevant concentrations, dalteparin prolongs the aPTT the most and has the most potent effect on the suppression of catheter-induced clotting and thrombin generation, whereas abelacimab has a greater effect on the aPTT and catheter-induced clotting and thrombin generation than apixaban.

Dalteparin is an indirect anticoagulant consisting of polydisperse heparin chains with a mean molecular weight of about 5000 that potentiates antithrombin-mediated inhibition of FXa and thrombin, as well as other clotting enzymes in the intrinsic pathway of coagulation (Elit et al., 2012). In contrast, abelacimab and apixaban are direct anticoagulants that target FXI and FXa, respectively. Because of its multiple targets in the intrinsic and common pathways of coagulation, it is not surprising that dalteparin has the most potent effect on the aPTT and catheter-induced clotting and thrombin generation. By blocking FXI activation and FXIa activity, which propagate coagulation after catheters induce FXII autoactivation (Jaffer et al., 2015), abelacimab also prolongs that aPTT and attenuates catheter-induced clotting and thrombin generation. Unlike dalteparin, however, the effect of abelacimab on these measures reaches a plateau, likely reflecting FXI saturation with the antibody. Catheter-induced clotting and thrombin generation are almost completely suppressed with an abelacimab concentration of about 0.3 µM, which is the concentration achieved with a monthly abelacimab dose of 150 mg (Yi et al. 2022). Consistent with our previous observations that apixaban has less of an effect on the aPTT and thrombin generation than rivaroxaban (Kim et al., 2018), and attenuates catheter-induced clotting less effectively than rivaroxaban and enoxaparin (Guan et al., 2023), apixaban is the least effective of the three anticoagulants for prolonging the aPTT and attenuating catheter-induced clotting and thrombin generation. Therefore, the results of this study suggest that in patients with cancer, dalteparin and abelacimab may prevent CVC clotting more effectively than apixaban.

The findings in a static, plate-based catheter thrombosis model may not recapitulate the complex events that occur in humans. However, our previous demonstration that fondaparinux has a limited capacity to inhibit catheter thrombosis in this plate-based assay (Yau et al., 2011) was consistent with the catheter thrombosis observed during percutaneous coronary intervention in patients randomized to fondaparinux in the Fifth and Sixth Organization to Assess Strategies in Ischemic Syndromes (OASIS-5 and OASIS-6 trials) (Mehta et al., 2008). In addition, we previously demonstrated that high concentrations of dabigatran were needed to suppress catheter clotting in this plate-based model system and a jugular vein catheter thrombosis model in rabbits (Yau et al., 2014). Finally, our findings in the plate-based assay with mechanical heart valve components that dabigatran and apixaban were less effective than warfarin at attenuating valve-induced thrombin generation (Jaffer et al., 2015) explained why there were more strokes with dabigatran and apixaban than there were with warfarin in the RE-ALIGN (Randomized, Phase II Study to Evaluate the Safety and Pharmacokinetics of Oral Dabigatran Etexilate in Patients after Heart Valve Replacement) and PROACT Xa (A Trial to Determine if Participants With an On-X Aortic Valve Can be Maintained Safely on Apixaban) trials, respectively (Eikelboom et al., 2013) (Wang et al., 2023). Therefore, our in vitro findings in these plate-based assays may well be of clinical importance.

In summary, abelacimab and dalteparin are more effective than apixaban in attenuating catheter-induced clotting and thrombin generation in vitro. The incidence of CVC thrombosis in patients enrolled in the ASTER and MAGNOLIA trials will determine whether the same is true in patients with cancer.

CHAPTER 6-GENERAL DISCUSSION AND CONCLUDING REMARKS

6.1 GENERAL DISCUSSION

Thrombosis accounts for 1 in 4 deaths in Canada and worldwide, rendering it a significant public health priority (Wendelboe & Weitz, 2024). Traditional anticoagulants for these diseases, including heparin and vitamin K antagonists like warfarin, are primarily used for VTE prevention, whereas antiplatelet therapy is responsible for the treatment and prevention of myocardial infarction and non-cardioembolic stroke (Chan et al., 2020). Despite their use for over 70 years, there are still major weaknesses associated with these agents, which DOACs have tried to address. Although DOACs are widely used for VTE treatment as well as stroke prevention in atrial fibrillation, bleeding remains a major side effect (Carnicelli et al., 2022). Therefore, developing safer anticoagulants with minimized bleeding risk is a top priority.

FXI has emerged as a promising target for new anticoagulants that can potentially be safer than those currently used. Patients with severe congenital FXI deficiency (1 in 450 Ashkenazi Jews) have a lower risk of thrombosis and rarely experience spontaneous bleeding (Fredenburgh & Weitz, 2023). In extensive cohort studies, the risk of thrombosis is 2-fold higher in populations with elevated FXI compared with those with normal levels and 40-90% lower in those with reduced FXI (Meijers et al., 2000). Animal studies in mice, rabbits, monkeys, and baboons align with human studies, demonstrating a lower risk of thrombosis with FXI inhibition without increased bleeding (Gailani et al., 2015). Therefore, there is a great interest in developing agents for FXI inhibition (Weitz et al., 2021) (Verhamme et al., 2021).

The future of novel anticoagulants targeting FXII and FXI in the contact pathway appears promising (Weitz & Chan, 2020) (Fredenburgh & Weitz, 2023). Despite robust evidence in animal models that FXII or FXI inhibition is protective against arterial and venous thrombosis without an increase in major bleeding (Grover et al., 2020), the question remains: which of these two factors is a better target for therapeutic intervention? Given that there is limited epidemiological evidence linking FXII levels with thrombosis, FXI has gained the most attention as a target for novel anticoagulant therapy. Moreover, FXI is important in amplifying thrombus expansion activated through feedback activation by thrombin, a process that may bypass the inhibition of FXIIa (Chan & Weitz, 2023). While people with FXI deficiency demonstrate a lower risk of VTE, those with FXII deficiency do not. These differences could explain why anticoagulants targeting FXI, such as abelacimab (phase 3) and milvexian (phase 3), are in the most advanced stages of clinical evaluation, while FXII inhibitors are still in the early stages of development. The focus on FXI is especially relevant in catheter-induced thrombosis, a severe complication in patients undergoing PCI, CVCs, and PICCs (Jaffer et al., 2015). Catheters can induce FXII autoactivation, leading to FXI activation and thrombin generation. Since FXI is critical in the pathogenesis of catheter thrombosis (Yau et al., 2011), the effects of FXI inhibitors are of great interest.

Evidence from a randomized Phase 2 clinical trial (AXIOMATIC-TKR) suggests that milvexian, an oral FXIa inhibitor, compared with enoxaparin, is effective in preventing VTE in patients undergoing TKR surgery. The primary outcome includes asymptomatic DVT, symptomatic VTE, or death. The results also show that milvexian does not cause a dose-dependent increase in bleeding (Weitz et al., 2021). Findings from another phase 2 clinical trial demonstrate that FXI ASO, IONIS-FXI Rx, effectively reduces FXI levels in TKA patients and lowers the risk of developing postoperative VTE (Büller et al., 2015). In the ongoing phase 3 trials, abelacimab is compared with apixaban in the ASTER trial (NCT05171049) and dalteparin in the MAGNOLIA trial (NCT05171075) for the treatment of cancer-associated VTE. However, cancer patients often require CVC for administering chemotherapy, blood products, and nutrition and are at higher risk of developing CVC-induced thrombosis, such as DVT. Therefore, we aim to address whether FXI inhibition effectively reduces catheter thrombosis in the patients enrolled in these clinical trials using our previously established plate-based assays.

The primary aim of this thesis was to examine the biochemical mechanisms underlying the regulation of FXI, with a particular focus on its prothrombotic effects interacting with natural procoagulants and antithrombotic effects in the presence of novel anticoagulants. We report that: (1) polyP enhances the activation of FXI by thrombin by serving as a template (Chapter 3.0); (2) milvexian attenuates catheter-induced clotting, and its inhibitory effect is enhanced with the addition of heparin (Chapter 4.0), and (3) abelacimab is more effective than apixaban in attenuating catheter-induced clotting and thrombin generation (Chapter 5.0). These results suggest that FXI is a novel target for preventing physiological (platelet-sized polyP) or artificial surface (catheter)-induced thrombosis.

6.2 ROLE OF POLYPHOSPHATE IN COAGULATION AND POTENTIAL THERAPEUTIC INHIBITORS

Synthetic and platelet polyP is polydisperse, containing phosphate chains consisting of 20 to 280 phosphate units. (Malik et al., 2021). PolyP contributes to coagulation via the contact pathway by triggering FXII autoactivation, where polyP serves as a template during this progress (Malik et al., 2021) (Yin et al., 2023). Our observation that synthetic polyP can mimic the prothrombotic effects of platelet polyP indicates that the structure and function of this synthetic analog closely aligns with those of natural platelet-derived polyP. For instance, in Chapter 3.0, we found that both platelet-derived polyP and synthetic polyP promote FXI activation by thrombin to a similar extent of 1500-fold, emphasizing the important role of polyP for promoting thrombosis (Choi et al., 2011) (Geng et al., 2013). Therefore, synthetic polyP can be used as a surrogate to study the effects of platelet polyP *in vitro* or *in vivo* systems (Malik et al., 2024).

Our current and previous studies show that polyP released from activated platelets has the potential to induce FXI activation in 3 ways: a) inducing FXII autoactivation, b) promoting FXI activation by thrombin, and c) inducing FXI autoactivation (Choi et al., 2011) (Truong et al., 2021). These findings indicate that polyP is an essential amplifier of coagulation, providing multiple ways to ensure FXI activation and thrombin generation, enabling the body to respond immediately to injuries. If one pathway is compromised, polyP allows FXI activation by the other two. Hence, this significant feature of polyP on FXI activation makes both potential therapeutic targets for preventing and treating thrombosis.

Platelet activation leads to the release of polyP. Previously, others have demonstrated that platelets affect clotting not only by releasing polyP when activated but also by releasing other procoagulants (such as fibrinogen and partially activated FV) and by providing a phospholipid surface onto which clotting factors assemble to form tenase and prothrombinase (Morrissey et al., 2012). The involvement of platelet polyP in FXI activation highlights the dual role of platelets in primary hemostasis, where they form a platelet plug to stop bleeding and contribute to secondary hemostasis via fibrin formation through activation of coagulation factors. Therefore, polyP bridges primary and secondary hemostasis, promoting the transition from a temporary platelet plug to a stable fibrin clot.

We previously developed a non-lethal mouse model demonstrating that intraperitoneal (IP) injection of synthetic polyP induces pulmonary thrombosis (Malik et al., 2024). Although polyP is recognized as a viable target for anticoagulation therapy (Smith et al., 2012), our findings contribute to a more comprehensive understanding of how polyP facilitates FXI/thrombin activation, providing insights into potential polyP-specific interventions. In Chapter 3.0, knowing that polyP is an important template for the binding of exosite 2, allowing thrombin to activate FXI effectively, suggests that targeting the interaction of thrombin and polyP can reduce FXI activation without widely influencing other physiological functions of thrombin.

Our findings that allosteric modulation of thrombin alters FXI activation could lead to the development of novel thrombin exosite inhibitors that selectively block the interaction between thrombin and FXI rather than inhibiting thrombin’s active site like traditional anticoagulants, such as dabigatran. Such exosite inhibitors could maintain the critical role of thrombin in normal hemostasis and attenuate its role in thrombosis. One example is bivalirudin, a direct thrombin inhibitor that blocks exosite 1 and the active site, reducing the interaction with substrates like fibrinogen. Blocking this interaction would usually prevent thrombin from efficiently cleaving fibrinogen to fibrin and impair clot formation. However, this is not technically the case for bivalirudin. After binding to thrombin, however, bivalirudin gets cleaved, leaving exosite 1 available to bind fibrinogen and other substrates, which brings back partial thrombin activity (Coppens et al., 2012). In a meta-analysis of patients with MI undergoing PCI, the incidence of stent thrombosis with bivalirudin is not significantly different from that of heparin, but bivalirudin shows significantly lower rates of serious bleeding (3.3%) compared with heparin (5.5%) (Bikdeli et al., 2023). Hence, the allosteric modulation of thrombin may become an alternative strategy to attenuate FXI activation and reduce thrombosis without increasing bleeding. It is debatable whether this mechanism is truly allosteric because hirugen acts as a competitive inhibitor for fibrinogen. However, this remains an exosite-directed approach.

Previous studies have identified some polyP inhibitors, including cationic proteins and small molecules, that have shown promise in reducing thrombin generation both *in vitro* and *in vivo* (Smith et al., 2012). For instance, the cationic polyP-binding proteins, such as the recombinant polyP-binding domain isolated from *Escherichia coli* exopolyphosphatase (PPXbd), significantly interfere with thrombin-polyP interaction and thereby potently block polyP-mediated FXI activation by thrombin (Choi et al., 2011). Although phosphatases can degrade polyP, they can digest other biological molecules containing phosphate groups, such as DNA and RNA. On the other hand, intravenous injection of cationic small molecules such as polymyxin B (an antibiotic that targets bacterial lipopolysaccharide) significantly prolongs clot formation and reduces vessel occlusion in arterial and venous $FeCl_3$-induced mouse models without increasing bleeding (Smith et al., 2012). However, the use of polymyxin B is associated with an increased risk of toxicity, such as nephrotoxicity in a dose-dependent manner, emphasizing the need for careful dosing to minimize these risks. We previously reported that histidine-rich glycoprotein (HRG), which inhibits FXIIa, is also a promising polyP inhibitor. HRG binds to polyP with high affinity, neutralizing its procoagulant activity and reducing IP polyP-induced thrombosis in a murine model (Malik et al., 2024). We also demonstrated that HRG inhibits FXII autoactivation and FXIIa-mediated FXI activation in the presence of polyP and $ZnCl_2$ (Truong et al., 2021). Therefore, HRG may also serve as a potent inhibitor for polyP-mediated FXI activation by thrombin.

6.3 ROLE OF EXOSITES IN POLYPHOSPHATE AND CATHETER-MEDIATED FXI ACTIVATION AND REGULATION

The most common materials of catheters include polyurethane, polytetrafluoroethylene, and silicone. Such materials are also valued for their biocompatibility and versatility in producing central venous catheters (Jaffer et al., 2015). While designed to improve medical access, they unavoidably contribute to thrombus formation by providing a surface that triggers the clotting factors in the contact pathway, thus sharing some functional similarities with polyP. In Chapters 4.0 and 5.0, we show that catheters promote FXI autoactivation and feedback activation of FXI, facilitating coagulation like polyP. This response is because catheters and polyP share the ability to provide a charged binding surface to adsorb charged coagulation proteins, allowing them to act as templates and bind to clotting factors, ultimately leading to thrombus formation via activation of the contact pathway.

Thrombin contains an active site and two exosites (exosite 1 and 2), which are critical in thrombin localization and substrate recognition (Troisi et al., 2021). In Chapter 3.0, our results confirm previous findings that exosite 2 binds to polyP with high affinity. Ligands binding to exosite 2 reduce FXI activation because they interfere with polyP and thrombin interaction, decreasing the capacity of thrombin to activate FXI (Figure 12) (Mutch et al., 2010) (Geng et al., 2013). In this thesis, we confirmed the role of polyP as a cofactor in the feedback activation of FXI by thrombin, a process similar to that performed by catheters. Such a comparison would be meaningful in determining whether catheter-induced clot formation mimics the natural clotting processes promoted by polyP, which could support the development of future anticoagulation strategies. However, whether catheters bind to thrombin exosite 2 and modulate FXI activation like polyP remains unknown.

To answer this question, we can utilize thrombin variants and examine whether catheters promote FXI activation by thrombin in an exosite-2 dependent manner. Specifically, we can incubate FXI and catheter segments with either α-thrombin or R93E-thrombin (an exosite 2 mutant) and measure FXI activation by hydrolysis of a FXIa chromogenic substrate (S2366). Synthetic polyP will be used as a control. Next, we will perform the same experiment by adding exosite 2-directed ligands, such as HD22 aptamer and GPIbα269 peptide, and quantify FXI activation by thrombin in the presence and absence of these ligands. We hypothesize that adding R93E-thrombin, HD22 aptamer, and GP1bα269 peptide will demonstrate a reduced catheter-induced FXI activation by thrombin compared to the addition of α-thrombin.

Exosite 1 is responsible for cofactor recognition, such as fibrin and TM, to accelerate FXIII activation and to enhance protein C activation, respectively (Huntington, 2005). On the platelet surface, exosite 1 interacts with PAR-1 and glycoprotein V (GPV). These interactions promote thrombin binding to the GPIbα receptor, where FXI binds on platelets, bringing FXIa and thrombin into proximity. This proximity then supports a sequential activation of FIX, FX, and prothrombin, resulting in normal hemostasis (Al-Amer, 2022). However, our findings suggest that while exosite 1 does not directly contribute to FXI activation, ligands binding to exosite 1 allosterically modulate FXI activation by influencing exosite 2 binding to polyP. This is consistent with previous studies that there is reciprocal communication between the two exosites, where ligand binding to either exosite influences the other (Petrera et al., 2009). Therefore, exosite inhibitors that directly inhibit thrombin and may have an off-target effect on inhibiting FXI activation.

Although our *in-vitro* model demonstrates that optimal FXI activation is regulated by both exosites, investigating whether the effect of exosite ligands on FXI activation is physiologically relevant remains challenging. This is primarily because countless thrombin ligands in the vascular system constantly compete for exosite binding. For example, heparan sulfate and platelet-derived polyP both bind to exosite 2. In contrast, TM and fibrinogen bind to exosite 1, raising the question of which one binds first or if they bind simultaneously? While various *in vitro* studies suggest that allosteric modulation regulates thrombin activity, translating these findings to *in vivo* models is difficult. Since thrombin plays a central role in hemostasis, any depletion of thrombin may lead to bleeding. Currently, many direct thrombin inhibitors are effective in the prevention of thrombosis. For example, bivalirudin blocks exosite 1 and is approved and used for patients undergoing PCI for acute MI (Anantha-Narayanan et al., 2018). Another well-known thrombin inhibitor, dabigatran, which binds to the active site, was approved over a decade ago for the treatment of stroke and systemic embolism in patients with non-valvular AF (Alberts et al., 2012). In Chapter 3.0, we examined the effect of adding hirugen (a precursor of bivalirudin), which binds to exosite 1, on FXI activation. We showed that adding hirugen reduces the feedback activation of FXI by thrombin in the presence of polyP. Although exosite 1 inhibitors can effectively attenuate FXI activation, their mechanism of action mimics the binding of fibrinogen and PAR-1, which constantly compete for binding with physiological exosite 1 substrates that interfere with the procoagulant function of thrombin. Therefore, exosite 2 inhibitors, like HD22 aptamer, should be considered an alternative strategy because they specifically target the binding between thrombin and polyP, potentially reduce the risk of bleeding (Di Cera, 2003) (Petrera et al., 2009). While inhibition of the active site is expected to attenuate FXI activation, further investigations are needed to examine the inhibition of exosite 1 or 2 alone on FXI activation.

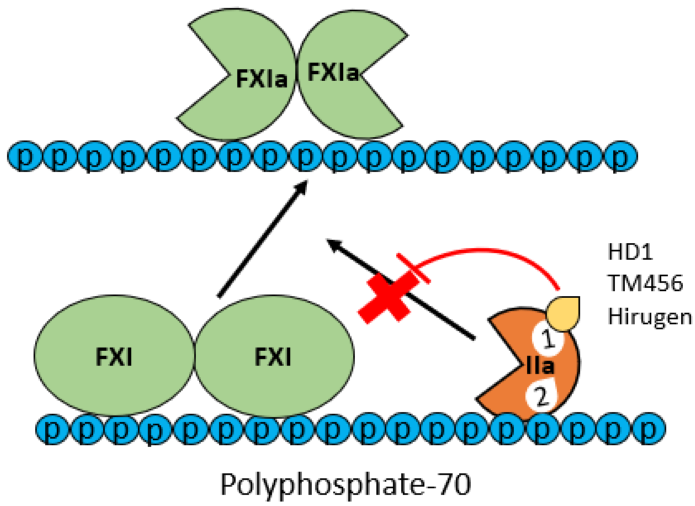
FXIa
FXIa
FXI
FXI
HD1
TM456
Hirugen
1
IIa
2
Polyphosphate-70

Figure 12: Proposed mechanism of thrombin-mediated factor XI (FXI) activation. Polyphosphate (PolyP)-70 serves as a template that binds thrombin and dimeric FXI and promotes their interaction. Thrombin binds to polyP via exosite 2. Exosite 1 ligands, such as HD-1, TM-456, and hirugen, attenuate FXI activation by thrombin by allosterically regulating exosite 2 and the active site. This figure is obtained from (Yin et al., 2023).

6.4 IMPORTANCE OF TARGETING FXI IN CATHETER-INDUCED THROMBOSIS

Anticoagulation therapy is the mainstay for prevention and treatment of thrombosis (Chan & Weitz, 2023). Historically, VKAs such as warfarin were the only available oral anticoagulants (Hsu et al., 2021). However, over the past 15 years, DOACs targeting thrombin (i.e. dabigatran) or FXa (i.e. apixaban, rivaroxaban, and edoxaban) have proven to be effective anticoagulants. Previous study has shown that DOACs are more effective and safer than warfarin in reducing the risk of stroke and systemic embolism by 19% and intracranial bleeding by 50% in patients with nonvalvular AF (Ruff et al., 2014).

Despite DOACs offering a safer profile compared to warfarin, their efficacy and safety are limited in certain types of thrombosis. For instance, the use of DOACs is not effective for device-induced thrombosis, and the fear of bleeding persists. In patients with MHVs, dabigatran, compared with warfarin, has been associated with an increased risk of ischemic strokes and higher bleeding rates (Eikelboom et al., 2013). In addition, apixaban is also problematic in patients with mechanical heart valves as it is less effective than warfarin for the prevention of valve thrombosis (Wang et al., 2023). Nonetheless, our recent work using a plate-based catheter thrombosis model found that apixaban or rivaroxaban is less effective in attenuating catheter-induced clotting and thrombin generation when compared with enoxaparin (Guan et al., 2023). The findings that DOACs are less effective in device-related thrombosis promoted a black box warning against the use of DOACs in such patients (Weitz & Fredenburgh, 2017), suggesting the need for new anticoagulants that go beyond FXa or thrombin inhibition to advance the antithrombotic treatment of device-induced thrombosis.

Catheters are thrombogenic primarily because they adsorb and activate FXII, which undergoes autoactivation and initiates thrombin generation and fibrin formation (Jaffer & Weitz, 2019). In support of this concept, central venous catheter clotting is attenuated in a rabbit model when catheters are coated with CTI, a potent and specific inhibitor of FXIIa (Yau et al., 2012). The knockdown of FXII or FXI also attenuate catheter thrombosis in the same rabbit model. However, the knockdown of FVII, the initiator of the extrinsic pathway, did not. Although these findings suggest that FXII and FXI would be good targets for preventing and treating catheter-induced thrombosis, our findings in Chapter 4.0 have revealed that catheters may activate FXI independently of FXII. Consequently, FXI appears to be a better target to prevent catheter thrombosis than FXII.

In Chapter 4.0, the importance of FXI over FXII is also demonstrated by our observation of other medical devices, such as mechanical heart valves and sewing ring segments. We used mechanical heart valve or sewing ring segments to induce clotting and found that thrombin generation is reduced in FXII-depleted plasma but abolished in FXI-depleted plasma (Jaffer et al., 2015). Given the role of FXIa in propagating coagulation and the pathogenesis of catheter thrombosis, targeting FXI could be more effective than targeting FXII in regulating catheter-induced thrombosis

We used an established plate-based catheter thrombosis model to compare the effects of FXI activation in the absence and presence of catheters and found that catheters enhance FXI and FXII autoactivation by 12- and 20-fold, respectively ($P < 0.001$) (Malik et al., 2023), raising the possibility that FXI contributes to catheter thrombosis even without

FXII. This finding aligns with a previous study that synthetic negatively charged polyanions, such as dextran sulfate and sulfatides, induce FXI autoactivation (Naito & Fujikawa, 1991). Also, these results explain our previous observation that catheters can induce clotting in rabbits even after FXII depletion with ASO treatment, potentially due to the catheter's ability to autoactivate FXI and contribute to thrombin generation (Yau et al., 2014).

Based on results from Chapter 5.0, we show that abelacimab demonstrates superior efficacy over apixaban for inhibiting catheter-induced clotting and thrombin generation at therapeutically relevant concentrations. In Chapter 4.0, milvexian shows a suboptimal effect alone but is more effective with adjunctive heparin. This finding supports the idea that patients on milvexian undergoing PCI require adjunctive heparin. The results emphasize that FXI inhibition by milvexian alone may not be comprehensive enough to prevent device-induced thrombotic complications, reinforcing the concept that FXI inhibition, possibly in combination with heparin, could offer a targeted approach in novel anticoagulation therapy. Despite effectiveness, clinical trials are needed to examine whether patients on milvexian are at higher risk of bleeding when they receive heparin for PCI.

Cancer patients are at approximately nine times higher risk of developing VTE compared with those without cancer (Abdel-Razeq & Al-Jaghbeer, 2024). Consequently, DVT is associated with delayed anti-cancer treatments and increased morbidity and mortality in cancer patients. These patients also face an increased risk of bleeding when treated with anticoagulants due to the inflammatory nature of the disease, chemotherapy, and prolonged immobility. Although CVCs support essential access to treatment chemotherapy and nutrition, they simultaneously increase the risk of VTE, which complicates treatment outcomes (Jaffer et al., 2015). Our results on milvexian and abelacimab indicate their potential to attenuate catheter thrombosis in this vulnerable population. Comparing results from Chapters 4.0 and 5.0, abelacimab is better than milvexian for preventing catheter thrombosis, suggesting that inhibiting FXI generation may be a more effective strategy than inhibiting FXIa activity. From a mechanistic perspective, abelacimab binds to the catalytic domain of FXI and locks it in a zymogen or an inactive precursor conformation. By locking FXI in this inactive stage, abelacimab prevents FXI activation by FXIIa or thrombin, which will potentially stop FXI activation. In contrast, milvexian only blocks the active site, but this is after FXI has been activated. Therefore, it may allow some residual FXIa activity. Because milvexian is a reversible inhibitor, there is also a possibilty for some FXIa to resume their activity when the inhibitor dissociates from the active site. The distinction of inhibiting both FXI and FXIa ensures that abelacimab inhibits the entire FXI activation, making it more effective than milvexian in preventing FXI activation. Chapter 4.0 shows that only 2% FXI is necessary to permit catheter clotting and restore the clotting times halfway back to those measured in control plasma. Hence, even small amounts of FXI can initiate coagulation via the contact pathway and potentially sustain coagulation in catheter-induced thrombus formation. Therefore, targeting FXI and locking it in the zymogen conformation with abelacimab appears to be a more effective strategy than using milvexian for preventing catheter-induced thrombosis after FXIa has already been generated.

Although FXI inhibition with small molecules holds promise, our data suggest that they still need to be combined with another anticoagulant, such as heparin, to achieve optimal anticoagulation in device-induced thrombosis. For instance, adjunctive use of anticoagulant agents like heparin, which can directly inhibit thrombin and other coagulation factors, may effectively inhibit the feedback activation of FXI and entirely prevent catheter-induced thrombosis. The effect of adjunctive therapy combining FXI inhibitors and heparin in different types of thrombosis requires further investigation. While anticoagulation with heparin effectively reduces thrombosis, it is associated with higher bleeding risk, mainly when used in a higher-dosing regimen (Krishnaswamy et al., 2011). The bleeding risk with anticoagulation therapy remains a significant limitation in many clinical settings, especially in patients with cancer who are already at high risk for bleeding due to chemotherapy (Johnstone & Rich, 2018). FXI inhibitors offer an advantage over current anticoagulants because they reduce the risk of bleeding in some high-risk patients.

6.5 SURFACE MODIFICATION ON MEDICAL DEVICES TO TARGET FXI

Catheters and blood-contacting devices, such as vascular stents, mechanical heart valves, or membrane oxygenators, are widely used for various clinical implications. However, catheters are associated with an increased risk for thrombosis unless anticoagulants are administered. Although prophylactic heparin or enoxaparin are commonly used, their abilities in preventing catheter thrombosis remain uncertain. Traditionally, surface modification has seen commercial success in reducing thrombus formation by immobilizing anticoagulants onto surfaces of medical devices like heparin and hirudin (Jaffer et al., 2015) (Biran & Pond, 2017). These modifications primarily target key enzymes downstream of the coagulation system, such as FXa and thrombin, which play a critical role in the most crucial stage of clot formation (Jaffer et al., 2015).

Heparin is a commonly used anticoagulant in patients with medical devices to enhance the activity of AT. The heparin-AT complex inhibits FXa and thrombin, preventing the conversion of fibrinogen to fibrin and inhibiting clot formation. In Chapters 4.0 and 5.0, we found that adding LMWH alone effectively reduces catheter-induced clotting. The efficacy of LMWH as prophylaxis for VTE prevention also has been evaluated in clinical trials. An older meta-analysis from 2014 demonstrated that prophylactic LMWH reduces symptomatic VTE in cancer patients with CVCs by 50% compared with prophylaxis (Akl et al., 2014). A recent meta-analysis from 2018 showed similar results in terms of the reduction of symptomatic VTE, but its effect on major bleeding and mortality is less specific (Kahale et al., 2018). In addition, the use of heparin is associated with other side effects. Previous studies suggest that heparin-coated extracorporeal membrane oxygenation (ECMO) circuit is associated with the potential of developing heparin-induced thrombocytopenia (HIT). This serious immune condition further complicates treatment outcomes (Natt et al., 2017). Despite low platelet count, nearly half of the patients with HIT are at higher risk of developing arterial and venous thrombosis compared with those without HIT (Ahmed et al., 2007). Hence, continuous heparin infusion or coating of medical devices may be problematic as it potentially complicates the clinical management of catheter thrombosis.

Our previous research has focused on surface modifications to reduce the thrombotic potential of catheters to explore other possible strategies to prevent catheter thrombosis. Because catheters initiate coagulation clotting by activating FXII, we previously investigated the anticoagulation effect of CTI immobilization on catheters. We demonstrate that CTI-coated catheters reduce the adsorption of FXII and fibrinogen and showed a 43-fold reduction in FXIIa activity after catheter-induced autoactivation and a 13-fold reduction in FXIIa-mediated FXI activation (Yau et al., 2012). This finding indicates that FXII is a viable target for strategies to attenuate catheter thrombosis. It highlights the potential to utilize specific protease inhibitors and offers a novel approach to enhancing the biocompatibility of medical devices.

Building on these findings, our study aims to explore the role of FXI in the coagulation process related to catheter thrombosis. We are the first group to report that FXI undergoes autoactivation in the presence of catheters, even in the absence of FXII, indicating that targeting FXI could be more effective than targeting FXII. Consequently, anticoagulation or surface modification targeting FXI may offer comprehensive protection against catheter thrombosis. The prospect of coating catheters with abelacimab appears to be a promising strategy. Since abelacimab offers a dual inhibition towards FXI and FXIa, abelacimab coating should significantly reduce thrombosis on catheters. The methodology for attaching abelacimab to PCI catheters will be performed as described previously (Yau et al., 2012). The attachment primarily undergoes two significant steps: (1) abelacimab conjugation with PEG and (2) catheter coating. The activity of FXIa generated in situ will be determined using the method described above. In vitro assays often fail to replicate biological process conditions because they may not account for blood flow, platelet interaction with other proteins, and natural clearance responses. Compared with control catheters, we hypothesize that abelacimab-coated catheters will reduce adsorption of FXI and fibrinogen and show a greater inhibition of FXIa generated by catheter-induced autoactivation. Therefore, future research should focus on testing abelacimab-coated catheters in animal models that mimic vascular environments and evaluating their thrombosis prevention under hemodynamic conditions.

CHAPTER 7 - CONCLUSION

Emerging evidence suggests that FXIa inhibitors are a promising class of anticoagulants because FXI and FXIa are involved in thrombosis but dispensable in hemostasis. Despite a minor bleeding risk, individuals with FXI deficiency are protected against thrombosis, such as VTE and ischemic stroke, compared with those with normal FXI levels. In this thesis, we showed that charged surfaces, such as catheters and polyP, trigger FXI autoactivation and feedback activation of FXI by thrombin, a process independent of FXII. These results suggest that FXI is at least as crucial as FXII for catheter thrombosis. These findings complement ongoing phase 3 trials like LIBREXIA AF and ACS, along with ASTER and MAGNOLIA, allowing future clinical research to focus on FXI inhibitors, such as milvexian and abelacimab, to optimize patient outcomes, especially for high-risk patient populations, including those with cancer or undergoing PCI. We also identified that FXI activation is attenuated via ligand binding to either exosite 1 or 2 of thrombin, emphasizing allosteric modulation as a potential therapeutic strategy to downregulate FXI activation. In summary, our thesis addresses the greater effectiveness of FXI inhibitors over current anticoagulants, paving the way for safer and more targeted therapies for preventing and treating catheter thrombosis that enhances patient care and outcomes.

CHAPTER 8 - FUTURE DIRECTIONS

Feedback activation of FXI by thrombin pathway is an important mechanism that ensures thrombus stabilization and growth. This contributes to a positive feedback loop that amplifies thrombin generation and stabilizes thrombus formation. In this thesis, we have shown that polyP and catheters are important mediators of FXI autoactivation and activation by thrombin. While polyP and catheters trigger FXII autoactivation, they also mediate FXI activation by thrombin and FXI autoactivation (Chapters 3.0 and 4.0), emphasizing the potential of FXI inhibitors in attenuating catheter thrombosis. While both milvexian and abelacimab have shown efficacy in prolonging catheter-induced clotting and thrombin generation (Chapters 4.0 and 5.0), these findings are based on an in vitro model. Such models may not fully recapitulate the complex events that occur in human systems. Building on this, this chapter proposes 3 projects: (i) to examine whether polyP mediates FXI autoactivation and FXI activation by thrombin *in vivo*, (ii) to compare the anticoagulant effect of different FXI inhibitors on low TF-induced IP polyP-mediated thrombosis, and (iii) to investigate the antithrombotic effect of milvexian with conjunctive therapy on a rabbit model of catheter-induced thrombosis. These studies aim to provide a comprehensive understanding of the mechanistic roles of feedback mechanism of FXI in thrombus growth to examine the therapeutic potential of FXI inhibitors in more clinically relevant settings.

8.1 Project #1: To examine whether polyP mediates FXI autoactivation and FXI activation by thrombin independent of FXIIa *in vivo*.

The objective of this study will be to evaluate whether polyP activates FXI autoactivation and activation by thrombin in the absence of FXIIa *in vivo*. TF-FVIIa and feedback loop in which thrombin activates FXI from the contact pathway are thought to play important roles in thrombin generation (Grover et al., 2020). Previous studies have shown that FXI contributes to thrombin generation in human plasma initiated by low, but not high, concentrations of TF (Zhu et al., 2015) (Leiderman et al., 2016). To further explore this, we will use low TF concentrations in our experimental models to better understand thrombus growth under the feedback activation of FXI by thrombin.

Proposed experiment:

A preliminary study will be performed in mice treated with FXII ASO to examine the prothrombotic effect after IP polyP and low TF injections (Figure 13). The objective of this study is to examine thrombus formation via low TF-induced, polyP-mediated thrombin/FXI pathway in FXII-depleted condition. In the *in vitro* study, FXII-deficient plasma with be treated with either 10 ug/ml polyP or 6 pM low TF, or both. A buffer will serve as a negative control. The results of these treatments will be evaluated using aPTT, plasma recalcification, and thrombin generation assays across these three conditions. Mice will be injected with saline (control) or FXII-directed ASO (20 mg/ C57BL/6 mice (The Jackson laboratory) subcutaneously twice weekly for 4 weeks to knockdown FXII, as previously described (Malik et al., 2021). Then, blood samples will be collected and the level of FXII will be measured by comparing hepatic FXII messenger RNA and plasma FXII levels before and after ASO administration. These mice will be injected with Thromborel S relipidated recombinant TF via the jugular vein (5 μl/g, Siemens Healthineers, Germany), polyP (100 mg/kg in saline) by intraperitoneal (IP) injection, or both (Grover, Schmedes, et al., 2020) (Malik et al., 2021). After 30 min, mice will be anesthetized and their blood will be collected via inferior vena cava before euthanasia via cervical dislocation and thrombin generation will be assessed. We will be monitoring thrombus formation in mice injected with (a) low TF, (b) polyP, or (c) both. Also, we will be comparing FXIa-AT and TAT levels using ELISA in these three conditions to select the optimal thrombogenic trigger that only stimulates minimal thrombin that is enough to back activate FXI. Then, we will be assessing fibrin deposition in lungs, liver, and kidneys and examining occlusion in arteries, veins, and microvessels. Based on our preliminary data, we expect that (a) FXIa-AT and TAT complexes in plasma will be elevated and (b) addition of polyP will promote FXI activation.

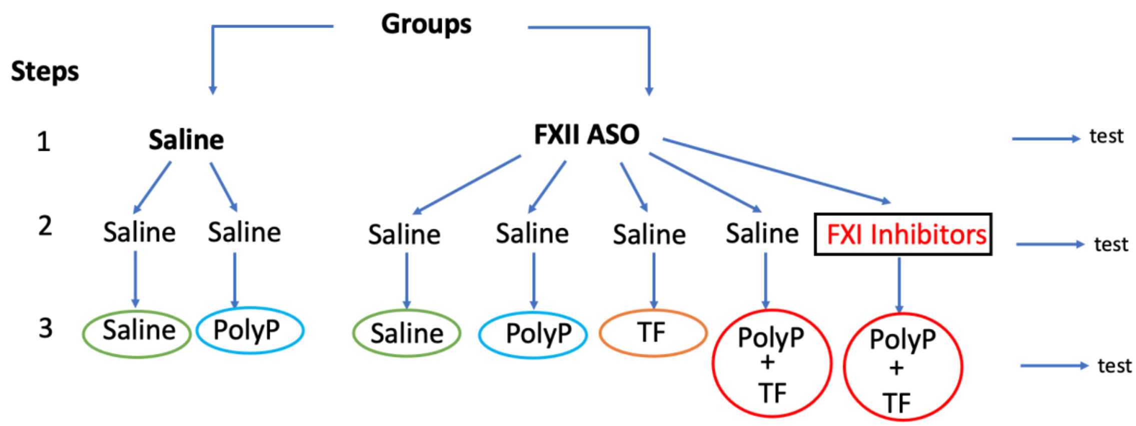
Groups
Steps
1
Saline
FXII ASO
test
2
Saline
Saline
Saline
Saline
Saline
Saline
FXI Inhibitors
test
3
Saline
PolyP
Saline
PolyP
TF
PolyP + TF
PolyP + TF
test

Figure 13. Experimental design to assess the effect of FXI autoactivation and activation by thrombin in a mouse model.

The effect of injecting IP polyP alone and in combination with low TF will be compared. If polyP mediates FXI autoactivation and activation by thrombin, then the anticoagulant effects of different FXI inhibitors will be tested. Step 1: C57BL/6 mice will be divided into two groups: one group will receive FXII ASO (20 mg/kg) for 4 weeks to prevent FXIIa activity and FXI activation by FXIIa, while the other group will be treated with saline (control). Step 2: all groups will receive saline to standardize the treatment volume. Step 3: Mice in saline- and FXII ASO-treated groups will be further divided. One branch of mice will receive either IP polyP, low-TF, or both to trigger coagulation. The other receives a third saline dose (control).

8.2 Project #2: To compare the anticoagulant effect of different FXI inhibitors on low TF-induced IP polyP-mediated thrombosis

We hypothesize that polyP activates FXI autoactivation and activation by thrombin in the presence of low TF independtly of FXII (Project #1). Then, we will compare the effect of different FXI inhibitors, including abelacimab and milvexian, in the same model (Figure 13). The purpose of this study is to examine the relative efficacy of different FXI inhibitors in attenuating low TF-induced polyP-mediated thrombosis.

Proposed experiment:

Since milvexian has a weaker potency in mice and rats (Wong et al., 2022), a pilot study using higher doses of milvexian will be necessary. In this pilot study, milvexian (0.42-1.67 mg/kg) will be administered intravenously via the retro-orbital route (Malik et al., 2021) (Wang et al., 2023), while abelacimab will be given intravenously at a dose of 0.6 mg/kg before IP polyP injections (Koch et al., 2019). After 30 and 60 min, blood samples will be collected via the inferior vena cava and thrombus formation, thrombin generation, and aPTT assays will be evaluated in the presence of milvexian and compared with abelacimab. Based on our previous observation, we hypothesize that abelacimab may have a greater potential than milvexian to significantly reduce thrombus formation.

8.3 Project #3: To investigate the antithrombotic effect of milvexian with heparin as conjunctive therapy on a rabbit model of catheter-induced thrombosis.

Anticoagulation is essential for patients who have implanted catheters to prevent catheter-related thrombosis. Current anticoagulant treatments, including heparin, target multiple clotting factors of the coagulation system, which can potentially increase the risk of bleeding. In Chapter 4.0, we identified that FXI plays a critical role in catheter-induced clotting, emphasizing the importance of FXI inhibitors as a promising strategy to attenuate catheter thrombosis. In addition, our findings showed that while milvexian inhibits catheter-induced clotting and thrombin generation in a concentration-dependent manner, its optimal effect requires conjunctive therapy with heparin. These findings are based on plate-based experiments, further in vivo studies are needed to validate the observed findings.

Proposed experiments

The aim of this study will be to examine the antithrombotic effect of milvexian in rabbit model of catheter thrombosis and to compare it with conjunctive therapy involving heparin and the direct thrombin inhibitor dabigatran. The rabbit catheter thrombosis model was established as previously described (Yau et al., 2014). Briefly, approximately 7 cm length of catheter segments will be implanted into their right external jugular vein and advanced into superior vena cava. The occlusion time will be assessed daily over a period of 35 days. Rabbits will be randomized into 4 groups receiving: a) milvexian, b) heparin, c) both milvexian and heparin, and d) saline via intravenous infusion prior and after the catheter insertion. The dosing of milvexian will be selected based on a previous rabbit AV shunt model (Wang et al., 2023). Milvexian will be administered via IV bolus and continuous infusion via the marginal ear at doses from low to high: 0.42 mg/kg (low; 0.25 mg/kg bolus + 0.17 mg/kg/h infusion), 1.67 mg/kg (medium; 1.0 mg/kg bolus + 0.67 mg/kg/h infusion), or 6.68 mg/kg (high; 4.0 mg/kg bolus + 2.68 mg/kg/h infusion) in a dosing volume of 5ml/3kg body weight. Heparin will be administered IV at a dose from low to high at 10, 20, and 40 anti-FXa U/kg/h as previously described (Agnelli et al., 1990).

Thrombus weight will be measured as the primary efficacy endpoint. After blood sample collection, ex vivo experiments, including aPTT, PT, and thrombin generation, will be conducted. Based on our preliminary data, we expect that conjunctive therapy of heparin and milvexian will demonstrate a greater antithrombotic effect than having either drug alone.

CHAPTER 9 – BIBLIOGRAPHY

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