MECHANISMS OF VENOUS THROMBUS STABILITY

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ABSTRACT

Whether a patient presents with deep vein thrombosis (DVT) or pulmonary embolism (PE) varies based on clinical factors. Patients with factor V Leiden (FVL) typically present with DVT while cancer patients present with PE. The biological mechanisms that determine DVT stability in the progression of DVT to PE are not known.

Thus, little is known about the mechanism of thrombus stability, the factors involved, or the effect of anticoagulants on embolization and PE burden. In order to answer these questions, we first need to (i) develop a mouse model to evaluate DVT stability and its relationship with PE burden when treated with anticoagulants, (ii) determine if anticoagulants, by inhibiting thrombin, require FXIII to decrease thrombus stability, (iii) determine the effects of attenuating fibrinolysis, using epsilon aminocaproic acid (ε-ACA or EACA), supplemental FXIII and α_2-AP, on clot stability and (iv) utilize our model to explain the FVL paradox.

For our thrombus stability model, the femoral vein of C57BL/6, FXIII deficient ($FXIII^{-/-}$), FVL heterozygous, or FVL homozygous female mice was subjected to ferric chloride ($FeCl_3$) injury to initiate a non-occlusive thrombus. Treatment with saline, dalteparin, dabigatran, EACA or FXIII was administered 12 minutes after thrombus formation. Intravital videomicroscopy recorded the thrombus sizes and embolic events leaving the thrombus for 2 hours. Lungs were harvested, sectioned and stained for the presence of PE.

Total and large embolic events were highest after dabigatran treatment compared to saline or dalteparin in wild-type (WT) mice. Variations in amounts of embolic events

were not attributed to variations in thrombus size since thrombus size was similar between the groups. The number of emboli per lung slice was higher in dabigatran-treated mice. Large embolic events correlated positively with the number of emboli per lung slice independent of treatment. Dabigatran treatment in $FXIII^{-/-}$ mice did not alter embolization patterns suggesting that FXIII is required for dabigatran to decrease thrombus stability.

EACA increases thrombus size significantly and therefore would not be a feasible alternative to IVC filters, as it will increase DVT size. FXIII marginally increased thrombus size. Treatment with FXIII decreases total and large embolic events in saline-, dalteparin- or dabigatran-treated mice, similar to EACA-treated mice. The number of emboli per lung slice was reduced after treatment with FXIII and EACA compared to non-treated mice. PE burden was not significantly different between FXIII anticoagulated mice or EACA-treated mice. The large embolic events correlate positively with PE burden.

FVL heterozygous and homozygous mice had significantly reduced embolization and thrombus size grew significantly over time, this contrasted with WT mice, where thrombus size remained similar to the initial injury. PE burden was significantly reduced in the FVL mice compared to WT.

Collectively, these data shows that we have successfully developed a mouse model of acute venous thrombus stability that can quantify emboli and PE burden. Consistent with clinical data, dabigatran, a DTI, was shown to acutely decrease thrombus stability and increase PE burden compared to LMWH or saline; an effect that was FXIII-dependent. Also, attenuating fibrinolysis with EACA, but not FXIII, increases thrombus

size; but both increase DVT stability and decrease PE burden. Supplementing α_2-AP did not alter thrombus stability. This suggests that administration of FXIII may be a better treatment option for DVT patients who are bleeding than EACA, since EACA may increase DVT size. Lastly, our model can explain the FVL paradox. Those with FVL have stable thrombus formation leading to an increased incidence of symptomatic DVT and a decreased risk of PE.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
APC	Activated Protein C
AP-FXIII	Factor XIII activation peptide
aPTT	Activatable partial thromboplastin time
AT	Antithrombin
α2-AP	Alpha 2- antiplasmin
CUS	Compression ultrasound
DTI	Direct thrombin inhibitor
DVT	Deep vein thrombosis
ELISA	enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
F1.2	Prothrombin fragment 1.2
FDP	Fibrin degradation products
$FeCl_3$	Ferric chloride
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
FV	Factor V
FVa	Activated factor V
$FV^{L/+}$	Factor V Leiden heterozygous
$FV^{L/L}$	Factor V Leiden homozygous
FVL	Factor V Leiden
FXIII	Factor XIII
FXIIIa	Activated factor XIII
$FXIII^{-/-}$	Factor XIII deficiency
FXIII-A	Factor XIII A-subunit
FXIII-B	Factor XIII B-subunit
GLA	γ-carboxy glutamic acid
HMWK	High molecular weight kininogen
HS	Heparan sulfate
INR	International normalized ratio
IVC	Inferior vena cava
IVM	Intravital videomicroscopy
LMWH	Low molecular weight heparin
Mbw	Mouse body weight
NOACS	Non-vitamin K oral anticoagulants
PAI-1	Plasminogen activator inhibitor type 1
PC	Protein C
PE	Pulmonary embolism
PK	Prekallikrein
PTS	Post thrombotic syndrome

Poly P	Polyphosphate
PTS	Post thrombotic syndrome
RBC	Red blood cells
TAFI	Thrombin activatable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
t-PA	Tissue plasminogen activator
TXA	Tranexamic acid
u-PA	Urokinase plasminogen activator
VKA	Vitamin K antagonist
VTE	Venous thromboembolism
VWF	von Willebrand factor
WT	Wild-type

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 OVERVIEW OF HEMOSTASIS

Hemostasis maintains the integrity of blood circulation after blood vessel rupture has occurred[1]. Hemostasis is attained when the procoagulant, anticoagulant and fibrinolytic processes are in balance. After damage to the endothelial lining of blood vessels occurs, a thrombus forms[2]. The end product of the hemostatic process is clot formation. Hemostatic clot formation involves vasoconstriction, platelet recruitment, activation and aggregation, and thrombin-mediated cleavage of fibrinogen to fibrin resulting from the coagulation cascade[3]. Hemostasis and thrombosis share three common phases: (i) formation of a loose and temporary platelet aggregate that binds to exposed collagen at the site of injury (ii) the formation of a fibrin mesh that binds to platelet aggregate and reinforces the platelet plug, and (iii) the dissolution of the thrombus by plasmin[2].

Upon vascular damage, sub-endothelial collagen is exposed to blood containing von Willebrand factor (vWF), which promotes platelet adhesion and activation. Activated platelets release the contents of storage granules, including adenosine diphosphate (ADP), and thromboxane A2, which serve to activate additional platelets, and result in the platelet plug.

Simultaneous to platelet activation, the coagulation cascade is initiated by two pathways: i) the extrinsic (tissue factor (TF)) pathway and ii) the intrinsic (contact) pathway. During coagulation, a series of proteolytic reactions occur which convert zymogens in the blood into active enzymes that result in the formation of thrombin.

Thrombin will further activate platelets and convert fibrinogen to fibrin, stabilizing the platelet plug[2].

Anticoagulant processes tightly control thrombin generation either through (i) feedback mechanisms at certain points in the coagulation cascade, (ii) by circulating inhibitors, and (iii) as well as the fibrinolytic system. The inhibitor antithrombin (AT) inhibits thrombin, activated Factor X (FXa), FIXa, FXIa, FXIIa, and FVIIa[4]. The presence of heparin sulfate and medical heparin catalyzes the inhibition rate of AT[5]. The protein C (PC) pathway is also important in regulating clotting[6]. Activated protein C (APC), with its cofactor protein S serves as an anticoagulant by inactivating FVa and FVIIIa, thereby suppressing thrombin generation[6,7]. Simultaneous fibrinolysis also contributes to the dynamic equilibrium of hemostasis by dissolving the formed fibrin clots. Fibrinolysis is initiated when plasminogen is converted to plasmin, which is responsible for degrading fibrin to soluble fibrin degradation products (FDP)[8].

Thus, hemostasis relies on the dynamic balance of forming clots and dissolving them, whereas persistent clotting can lead to blood obstruction, and unstable clots can degrade and lead to bleeding [9].

Venous stasis, a contributor to DVT formation, typically occurs around the venous valve sinuses within deep veins; this leads to the development of hypoxic microenvironments and reduced efflux of procoagulant factors. The resulting hypoxia results causes the shedding of heparin- like glycosaminoglycans from the endothelial cell surface, reduced surface expression of anticoagulant proteins (such as thrombomodulin and endothelial protein C receptor), and promotes the procoagulant adhesion molecule P-selectin. This contributes to a hypercoagulable environment. P-selectin and associated P-

selectin glycoprotein ligand 1 are involved in the recruitment of TF to the site of endothelial activation. Platelets that have aggregated, in response to exposed collagen and the release of von Willebrand Factor from activated endothelial cells, are also activated and release prothrombotic factors including coagulation factors V, XI, VIII, IX, and XIII, amplifying the growth of the thrombus.

1.2 OVERVIEW OF COAGULATION

Coagulation is normally described in three phases: i) initiation, which occurs on a TF-bearing cell, ii) amplification, in which platelets and cofactors are activated to set the stage for large scale thrombin generation and iii) propagation, in which large amounts of thrombin are generated often on the platelet surface [3]. Initiation of coagulation by tissue factor, when exposed at the damaged vessel wall, or the release of polyanions into the blood is carried out by the extrinsic or intrinsic pathway, respectively[2,6]. Both pathways trigger a stepwise series of enzymatic reactions that converge at the common pathway, which culminates in FXa generation, which leads to initial thrombin generation. This initial thrombin feeds back and activates important cofactors that are needed to produce a burst of thrombin and thus amplify the system. The burst of thrombin allows for platelet activation, which can in turn accelerate the formation of the intrinsic tenase and prothrombinase complex and localizes coagulation factors to a specific region, thus allowing for propagation[2].

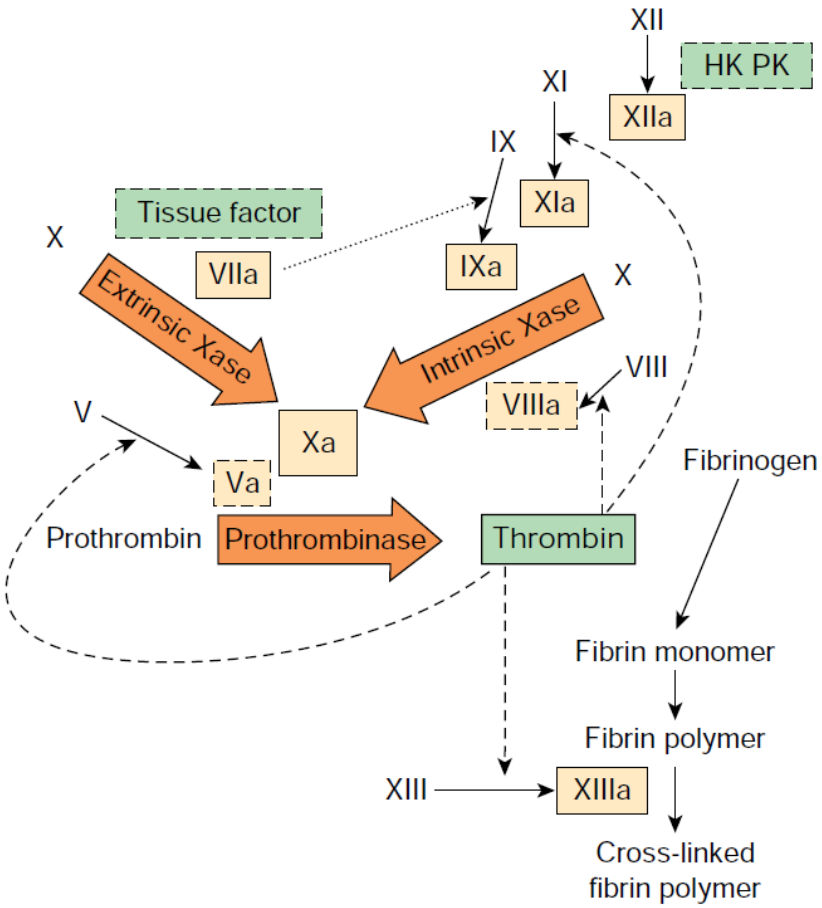

Figure 1: The Coagulation Cascade.

The coagulation cascade, comprised of two pathways, converging to a common pathway, with thrombin generation as the end point of the reactions. Both the extrinsic and intrinsic pathways result in activation of prothrombin to thrombin, which leads to the formation of fibrin by thrombin cleavage of fibrinogen. The coagulation cascade is complex and involves many specific interactions between cellular surfaces and plasma zymogen and cofactors. The proteins involved in these pathways can be classified into 5 types: (i) zymogens of serine-dependent proteases, (ii) cofactors, (iii) fibrinogen, (iv) a transglutaminase, and (v) regulatory proteins. Image taken from reference[2]

1.3 THE EXTRINSIC (TISSUE FACTOR) PATHWAY

Generally, the extrinsic pathway involves TF to sequentially activate zymogens (inactive serine proteases) factor VII (FVII), FX and prothrombin (FII), into the active serine proteases, FVIIa, FXa, and thrombin (FIIa) (Figure 1)[10].

Tissue factor, continuously expressed in the subendothelium on TF-bearing cells, becomes exposed to FVIIa in the blood. FVIIa is the only coagulation protein that routinely circulates in the blood in its active enzyme form, in very low concentrations, and rapidly binds to TF when TF becomes exposed to the blood[11]. The TF-FVIIa complex then activates additional FVII to FVIIa[11]. FVII undergoes proteolytic activation by cleavage of the peptide bond between Arg152 and Ilu153 by TF-FVIIa complex or other serine proteases (FXa, FIXa, FXIa, FXIIa, FIIa, or plasmin)[10]. The catalytic function of the binary complex TF/FVIIa (extrinsic tenase complex) is responsible for FX activation (also FIX activation, see below)[10]. FVIIa cleaves the Arg-Ile bond in FX to produce FXa[2]. The initial FXa produced, when bound to a membrane, activates tiny amounts of prothrombin to thrombin, and this initial thrombin is essential to the acceleration of the process by serving as the activator for platelets, FV and FVIII[12]. The interaction between tissue factor and FVIIa, is considered as a key role in initiation of the blood coagulation *in vivo* since it directly initiates the extrinsic pathway and indirectly initiates the intrinsic pathway (by activating FIX in the intrinsic pathway) [2].

1.4 THE INTRINSIC (CONTACT) PATHWAY

The intrinsic pathway involves factors XII, XI, IX, VIII, and X as well as prekallikrein (PK), high molecular weight kininogen (HMWK), Ca^{2+}, and phospholipids (Figure 1)[2].

The intrinsic pathway is initiated when FXII becomes activated on a charged surface by the "contact phase"[2]. However, in the last decade, naturally occurring polyphosphate containing molecules such as inorganic polyphosphates (polyP), DNA and RNA have been shown to activate FXII. Whereas polyP is stored in platelets and is released upon platelet activation, DNA and RNA are released from activated, damaged or dying cells[13–15]. This is when PK, HMWK, FXII and FXI assemble on an exposed negatively charged surface, and FXII will be activated to FXIIa[16]. Activation of FXII is followed sequentially by activation of factor XI and IX to the serine proteases FXIa and FIXa in the presence of calcium[16]. As stated previously, The TF-FVIIa complex catalyzes the activation of both FX and FIX. Once FVIIIa is formed, FIXa will combine with FVIIIa on activated platelet membranes to form the intrinsic tenase complex. That becomes the major activator of FX[12]. FIX also cleaves the Arg-Ile bond in FX to produce FXa in the intrinsic pathway[2].

1.5 THE COMMON PATHWAY

FXa acts as a molecular switch not only receiving upstream signals from the extrinsic and intrinsic pathways, but also dictating downstream coagulation[10]. FXa with FVa as a cofactor, assemble on the negatively charged membrane to form the prothrombinase complex, which converts prothrombin to thrombin. Thus, the clotting signals from both the extrinsic (FVII activation) and intrinsic (FIX activation) pathways converge in a common pathway to form thrombin. The assembly of the prothrombinase complex and tenase complexes takes place on the membrane of activated platelets[2]. Upon binding of FVa and FXa on platelet membranes, prothrombin is cleaved by FXa at two sites to generate thrombin, which is released from the membrane surface (this can occur on activated platelets)[11]. Thrombin will directly catalyze fibrinogen cleavage, releasing

fibrinopeptides. This allows for fibrin clot formation by association of the cleaved fibrinogen into protofibrils, later cross-linking of fibrin occurs[17].

1.6 REGULATION OF HEMOSTASIS

In normal patients, a balance between clot formation and clot breakdown is maintained to prevent excessive blood clotting. The level of thrombin in circulation is tightly controlled in two ways: (i) since thrombin is generated by activation of a catalytic cascade, there are feedback mechanisms at each point in the cascade that will up- and down-regulate its formation, and (ii) inactivate any thrombin formed with circulating inhibitors[2,3]. The fibrinolytic system as well as the antithrombotic properties of the endothelium also regulate thrombus formation[18].

The endothelial cells express heparan sulfate (HS), thrombomodulin (TM), and endothelial cell protein C receptor (EPCR)[4]. The protein C pathway is initiated when thrombin binds to thrombomodulin, a thrombin receptor on endothelial cells[6]. When thrombin is bound to thrombomodulin, forming a thrombin-TM complex, thrombin undergoes a conformational change that converts it from being a procoagulant enzyme into a potent activator of protein C on the EPCR[7]. Activated protein C, with its cofactor, protein S serves as an anticoagulant by inactivating FVa and FVIIIa and thereby suppressing thrombin generation[7].

Antithrombin is a member of the serine protease inhibitor (serpins) superfamily[4]. AT inhibits thrombin, FXa, FIXa, FXIa, FXIIa, and FVIIa[4]. The presence of HS catalyzes the inhibition rate of AT by binding to a specific cationic site of AT and inducing a conformation change that enhances it activity for inhibition of coagulation factors[5]

Tissue factor pathway inhibitor (TFPI) is also critical for the regulation of coagulation[19]. It inhibits TF-FVIIa complex in a FXa-dependent fashion[19]. TFPI recognizes FXa most effectively when it is in the prothrombinase complex[19]. First, TFPI binds to and inactivates FXa in a 1:1 stoichiometric complex[19]. The TFPI-FXa complex binds to and inhibits the TF-FVIIa complex, thus TFPI switches off the coagulation process from the extrinsic pathway to the intrinsic pathway[19].

Coagulation is maintained in a state of dynamic equilibrium, by forming fibrin clots, and dissolving them in a process known as fibrinolysis. Tissue plasminogen activator (t-PA) is a serine protease that is released into the circulation from endothelial cells during times of injury and physiological stress[2]. t-PA is catalytically inactive unless bound to fibrin, then t-PA will cleave plasminogen to plasmin[8]. Plasminogen is an inactive zymogen that binds to fibrin and becomes incorporated into the clot, and once the t-PA binds to fibrin, it is able to convert the inactive zymogen plasminogen to plasmin[8]. Plasmin is the serine protease that is mainly responsible for degrading fibrin to soluble fibrin degradation products[8].

There are three inhibitors for fibrinolysis: (i) plasminogen activator inhibitor-1 (PAI-1) (ii) thrombin activatable fibrinolysis inhibitor (TAFI) and (iii) α_2-anti-plasmin (AP). TAFI is able to inhibit fibrinolysis by removing the terminal lysines from fibrin[20]. The lysines help plasminogen, plasmin and t-PA bind to fibrin and are thus needed for the specificity of plasmin for fibrin[20]. TAFI is activated to TAFIa by thrombin, so stopping fibrinolysis occurs simultaneously with coagulation and helps regulate the hemostatic plug[20]. α_2-AP is the primary inhibitor of plasmin mediated fibrinolysis[2]. Plasmin that is found in the blood is inhibited by α_2-AP, but plasmin bound to fibrin is protected from

α_2-AP, and able to degrade fibrin[21]. However, α_2-AP is an efficient substrate of FXIIIa, and is cross-linked to fibrin(ogen), which will further increase the resistance of the clot to lysis[22].

1.7 FIBRINOLYSIS

Clot breakdown is known as fibrinolysis (Figure 2). Fibrinolysis is critical to prevent obstruction of flow. This begins when trauma to the blood vessel leads to t-PA release from endothelial cells, which can activate plasminogen to active plasmin. Urokinase plasminogen activator (uPA), produced by monocytes and macrophages, can also activate plasminogen to plasmin. Plasmin can degrade the fibrin clot to soluble fibrin degrading products and restore blow flow.

Damaged endothelial cells release t-PA, which cleaves the Arg561-Val562 bond in plasminogen to generate plasmin[23]. Plasmin can positively feedback on plasminogen activation by cleaving plasminogen at Lys77-Lys78, removing the NH_2-terminus from Glu-Plasminogen[23,24]. This induces a conformational change in Glu-Pg from a closed form to its open more readily activated form, Lys-Plasminogen[24]. In addition, Plasmin converts single-chain (sc)-uPA to its active form two-chain (tc)-uPA, which also augments plasminogen activation[24]. Fibrin generated from the coagulation cascade positively feeds back to enhance plasminogen activation by binding both t-PA and plasminogen[23,24]. The main function of plasmin is to hydrolyze fibrin into soluble fibrin degradation products (FDP). Fibrinolysis is regulated by serpins α_2-AP and PAI-1, which neutralize plasmin activity and plasminogen activation, respectively[23]. Formation of the thrombin/TM complex during coagulation results in the activation of TAFI to TAFIa[25].

This removes COOH-terminal Lys-residues on fibrin, hindering plasminogen, t-PA, and plasmin binding to fibrin[25].

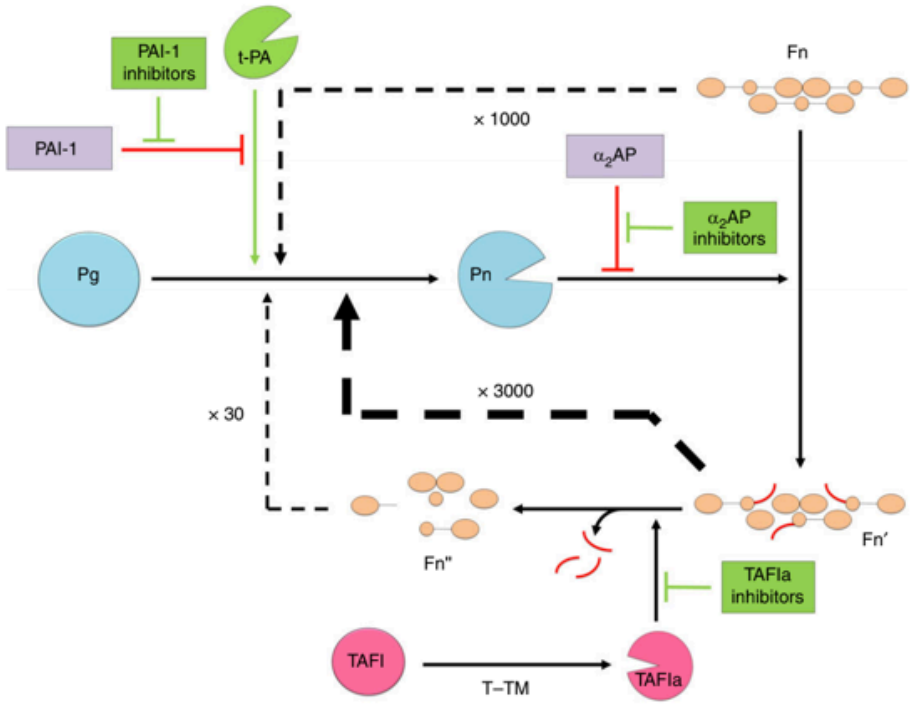

Figure 2: Overview of the fibrinolytic system.

Plasminogen (Pg) is cleaved to its active form plasmin (Pn) by endothelial cell-derived t-PA. This process is regulated by plasminogen activator inhibitor 1 (PAI-1). Fibrin clot (Fn) accelerates t-PA-mediated Pn formation by 1000-fold. Pn begins to cleave fibrin to produce plasmin-modified fibrin (Fn'), which is a better cofactor for Pn generation when compared with Fn by 3-fold due to the newly exposed C-terminal lysine residues. Plasmin is inhibited by α2-AP which prolongs clot lysis. Thrombin-activatable fibrinolysis inhibitor (TAFI) is activated to TAFIa by the thrombin-thrombomodulin complex (T-TM) likely present on endothelial cell surfaces. TAFIa reduces the cofactor activity of Fn' in Pn generation by removing the C-terminal lysine residues on Fn', thus generating TAFIa-modified fibrin (Fn"). Image taken from reference[26]

1.7.1 FIBRINOGEN AND FIBRIN

Fibrinogen is a plasma glycoprotein that is made up of three non-identical polypeptide chains covalently linked by disulfide bonds ($A\alpha$,$B\beta$, $\gamma)_2$ (Figure 3a)[2]. The molecular shape of fibrinogen is often described as a "highly asymmetric, elongated molecule" composed of a central E-domain and two lateral D-domains. The E-domain is formed by the convergence of the NH_2-termini of all six chains[27–29]. The D-domain is made up of the COOH-termini of the $B\beta$- and γ-chains, with an internal segment of the Aα-chain[27,28]. The COOH-termini of the Aα-chains exist the D-domains to form the αC-domain that resides near the E-domain (Figure 3a)[30]. Fibrinogen is soluble in plasma and that is due to the excess negative charge on the $A\alpha$ and Bβ portions of the A and B chains, designated as fibrinopeptide A (FPA) and fibrinopeptide B (FPB) [29,30]. The excess negative charge caused by the presence of aspartate and glutamate residues create electrostatic repulsion between fibrinogen molecules, preventing aggregation[30]. In circulation, the conversion of fibrinogen to fibrin is initiated by thrombin.

Thrombin will cleave the four Arg-Gly bonds, generating FPA and FPB, and fibrin monomers[2,29]. Thrombin initially releases FPA exposing a new amino terminal termed "knob A" which binds to pre-existing "hole a" located on the γ-nodules of D-domains of neighboring fibrin monomers[29]. The formation of "A:a" interaction lead to fibrin monomer polymerization and these half-staggered double-stranded polymers are referred to as protofibrils (Figure 3B) [29]. At slower rates, thrombin releases FPB from the Bβ chains exposing "knob b" which binds to complementary "hole b" on the β-nodule on the lateral D-domains[29]. The "B:b" interaction, while not essential for polymerization, enhances lateral association of protofibrils thus increasing fibrin diameter[29]. The release

of the fibrinopeptides allows the fibrin molecules to aggregate, forming a weak clot held together by non-covalent bonds (Figure 3B) [30].

Fibrin thickness influences the distribution and strength of fibers within the clot and is determined by the rate of fibrinopeptide release[21]. A reduction in thrombin concentration reduces the rate of fibrinopeptide cleavage and increases the number of fibrin monomers that associate laterally, and therefore, a slow rate of clot formation results in thicker fibers[21]. In contrast, increasing thrombin concentration leads to a faster rate of fibrinopeptide cleavage and promotes longitudinal growth rather than lateral aggregation. Thin fibrin fibers tend to be more densely compacted, whereas thick fibers tend to be loosely woven.

In addition, thrombin activates FXIII to FXIIIa, which is a transglutaminase that covalently cross-links the chains of fibrin, creating a more stable fibrin mesh and stabilizing the hemostatic plug or thrombus[31].

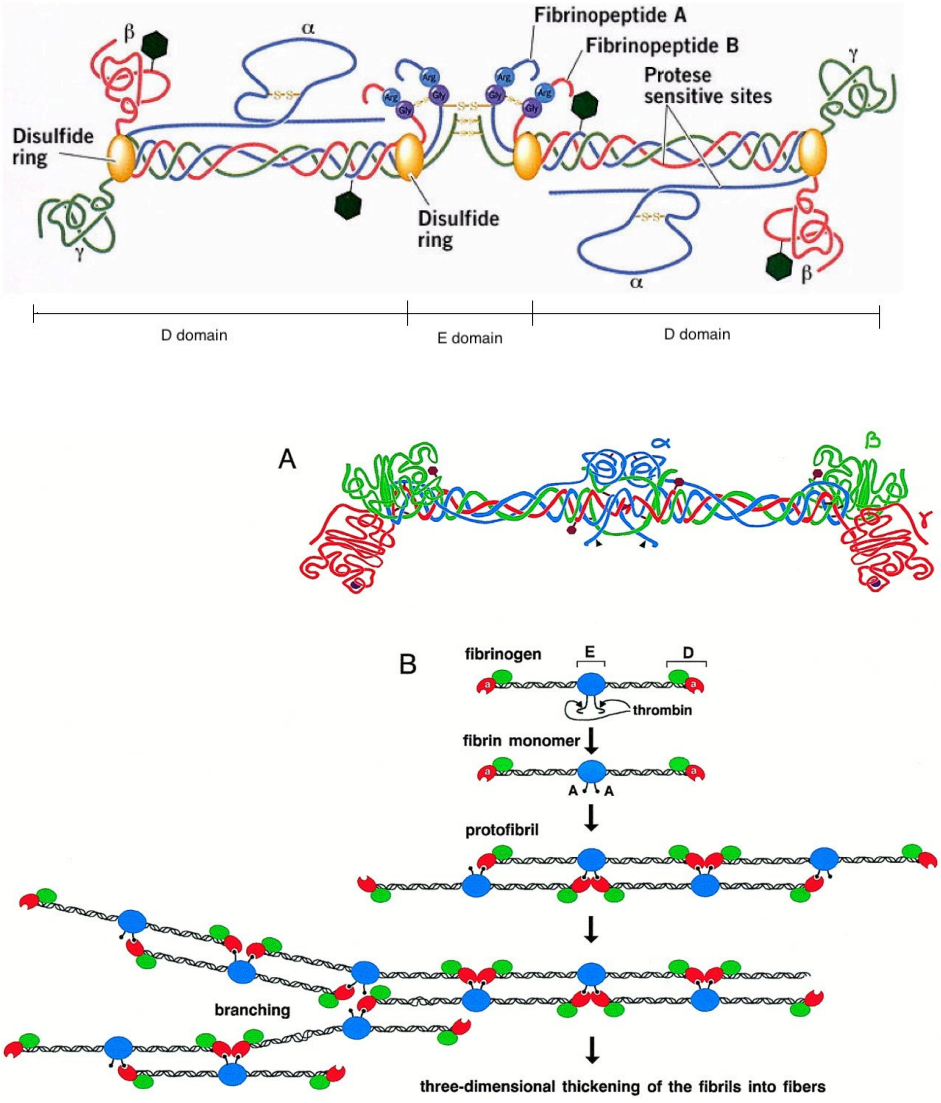

Figure 3: Structural Diagram of Fibrinogen.

(A) Fibrinogen, a blood-borne glycoprotein comprised of three pairs of non-identical polypeptide chains. Following vascular injury, thrombin cleaves the Arg-Gly bonds of FPA and FPB in the fibrinogen molecule to create fibrin monomers that will later be cross-linked by FXIIIa to form an insoluble stable fibrin mesh. (B) Polymerization of fibrin. Image taken from reference[32]

1.8 REGULATION OF FIBRINOLYSIS

Regulation of the fibrinolytic system, like that of coagulation cascade, is accomplished by a wide array of cofactors, receptors and inhibitors (Figure 2; red solid lines). Serpins, or serine protease inhibitors control circulating plasmin concentrations[23]. t-PA and uPA have short half-lives in circulation (4-8 minutes) due to the presence of high concentration of specific inhibitors, such as PAI-1 and α_2-AP[23]. Plasmin and α_2-AP bind with a 1:1 stoichiometry and both become inactive[23]. Plasmin bound to fibrin is protected from α_2-AP inhibition, allowing fibrinolysis to proceed[23]. Similarly, the plasminogen activators t-PA and uPA are rapidly inhibited by PAI-1 which is released into circulation by endothelial cells, platelets, and other cells[28].

Thrombin activated fibrinolysis inhibitor (TAFI) is a non-serpin fibrinolysis inhibitor that is activated by thrombin-TM[23]. TAFI removes c-terminal lysine and arginine residues on fibrin, thereby decreasing the binding sites for plasminogen and thus slowing plasmin generation and further stabilizing clots[23].

1.9 THE POTENTIAL of α2-AP INHIBITION OF PULMONARY EMBOLISM

Alpha 2-antiplasmin plays major roles in fibrinolysis since plasmin is inhibited by SERPIN (serine protease inhibitor) α_2-AP, forming a high-molecular-weight covalent complex[33,34]. This regulator has a long C-terminal sequence with a terminal lysine residue, which functions as a secondary binding site for free circulating plasmin and facilitates plasmin inactivation[33,34]. The binding is mediated by the lysine binding sites, located in the kringle domains, on plasmin[33,34]. However, when plasmin is bound to fibrin through the lysine binding site, α_2-AP can no longer bind plasmin, and plasmin remains active on

the fibrin surface[33,34]. Singh *et al.*, showed that effective thrombolysis was achieved by reducing α_2-AP activity using a neutralizing antibody targeting α_2-AP (α2AP-I)[35]. This antibody binds both circulating and fibrin-bound α_2-AP and thus neutralizes the activity of α_2-AP. In an in vivo model of pulmonary emboli, α_2-AP-I alone initiated spontaneous thrombolysis. Using a humanized α_2-AP mouse model, in which α_2-AP-deficient mice were pretreated using human α_2-AP, Singh *et al.,* also demonstrated that pulmonary emboli made of clotted human blood were efficiently dissolved by a combination of t-PA and α_2-AP-I[35].

1.10 FACTOR XIII STRUCTURE AND FUNCTION

Factor XIII is a zymogen that consists of two potentially active A-subunits (FXIII-A) and two inhibitory/carrier B-subunits (FXIII-B). Therefore, FXIII is a tetrameric coagulation factor (A2B2) that is activated by thrombin to activated Factor XIII (FXIIIa)[36]. Activation of plasma FXIII is initiated by proteolytic cleavage of the peptide bond Arg37-Gly38 by thrombin, followed by conformational changes, subunit dissociation and exposure of FXIIIA'S active site[36]. It has been previously shown that the activation peptide of FXIII (AP-FXIII) consisting of the 37 N-terminal amino acids of the FXIII-A subunit is released into plasma upon cleavage by thrombin[36,37]. The primary site of FXIII-A synthesis are cells of bone marrow origin, while FXIII-B is synthesized in the liver by hepatocytes, and the two subunits form a tetrameric complex in the plasma[37]. FXIII-A consists of 5 domains: an activation peptide, a central core domain, two B-barrel, and B-sandwich domain[37,38]. In circulation, FXIII-B carries and protects FXIII-A from spontaneous activation and clearance that may occur spontaneously in the presence of

plasma ionized calcium. FXIII-B is in excess in the plasma, ~50% of it exists in free non-complexed form[37,38].

Fibrin strength is increased by covalent crosslinking by FXIIIa. The activated enzyme FXIIIa catalyzes the formation of e-N-(γ -glutamyl)-lysyl protein crosslinks (Figure 4). Therefore, FXIIIa joins the c-terminus of the γ-chains by forming isopeptide bonds between the Gln on one γ- chain and a Lys on another[39]. The hemostatic function of FXIIIa is to crosslink fibrin γ -chains and α -chains into γ - γ dimers, high molecular weight α – α dimers, and γ - α polymers[39]. Thrombin activated FXIII also crosslinks α_2-AP to fibrin γ -chains preventing plasmin mediated fibrin degradation (see below). Fibrin crosslinking mechanically stabilizes the fibrin clot, protecting it from shear stress and enabling the retention of red blood cells (RBCs) during clot retraction, whereas α_2-AP crosslinking covalently incorporates this potent plasmin inhibitor into the forming clot, protecting the clot from premature degradation by the fibrinolytic system[40]. In addition to these hemostatic functions, FXIII also plays important roles in wound healing, angiogenesis, and maintenance of pregnancy[41,42].

In human plasma, FXIII is bound to a minor variant of the fibrinogen γ-chain, through its B subunit. In addition to plasma, FXIII is also present in platelets, monocytes/macrophages and in their bone marrow precursor cells[43]. Cellular FXIII lacks FXIII-B and exists as the dimer of FXIII-A (A2). The plasma concentration of FXIII A2B2 is 22 μg/mL (67.5 nmol/L). However, plasma FXIII circulates in complex with fibrinogen and becomes incorporated into the forming clot. It is estimated that the concentration of fibrin in a thrombus exceeds the fibrinogen concentration in plasma by 10-20 fold.

1.10.1 FXIII IN THE CLINIC

Individuals with congenital complete deficiency of FXIII (plasma activity <1% of normal) have a severe bleeding tendency characterized by formation of hematomas, soft-tissue hemorrhage and poor wound healing in the neonatal period[44–46]. Women with FXIII deficiency have an increased risk of spontaneous miscarriages[47]. FXIII-A subunit knockout mice can become pregnant and are fertile, but complications from uterine hemorrhage during gestation can occur[48].Patients with extensive pulmonary artery occlusion were found to have lower FXIII-A subunit, and the likelihood of PE was seven-fold higher in patients with A-subunit levels less than 60% of normal[48].

FXIII deficiency can be treated with cryoprecipitate or fresh frozen plasma, however these treatments carry a risk of transmitting blood-borne infections[49,50]. An alternative treatment is with plasma-derived FXIII concentrate. Fibrogammin is a purified FXIII concentrate derived from human plasma. Several studies have demonstrated that treatment with Fibrogammin is an effective prophylactic and on-demand treatment for congenital FXIII deficiency, reducing the incidence of both spontaneous and post-operative bleeding[51,52].

These findings are consistent with clinical data. In the context of invasive surgeries, including cardiopulmonary bypass in patients with high or moderate risk of blood loss postoperatively, supplemental recombinant FXIII did not increase the number of adverse events, including thromboembolic events[53–57]. In fact, the use of recombinant FXIII in deficient and healthy volunteers at high doses has not shown any evidence of increased risk of thromboembolism[58–60].

1.10.2 FXIII POLYMORPHISM

The most common polymorphism of FXIII is the Val34Leu polymorphism. This is caused by a G to T point mutation in codon 34 of exon 2 that results in the replacement of valine by leucine at position 34 (Val34Leu) in the activation peptide, three amino acids from the thrombin cleavage site[61–64]. There is a wide variation in the prevalence of the Val34Leu polymorphism in different ethnic groups; 2.5% of Asians, 28.9% of Blacks, 44.3% of Caucasians and 51.2% of Amerindians[65]. The 34Leu allele is virtually absent in the Japanese population and present in 11-27% of Australian Caucasians[65]. The Val34Leu polymorphism has been associated with decreased risk of myocardial infarction, in some but not all studies[66–68], and venous thrombosis, both DVT and PE[69–72]. The data on both hemorrhagic and ischemic stroke is too variable to draw any conclusions[73].

The presence of the Val34Leu polymorphism increases the rate of FXIII activation by thrombin. Thrombin cleaves the activation peptide from the Val34Leu more rapidly than from WT FXIII. With the Val34Leu mutation, fibrin γ -chain dimerization and the α -polymerization are enhanced[61], and there is more rapid cross-linking of fibrin γ- and α -chains[61]. Clots are composed of thinner fibrin fibers with less porosity[61].

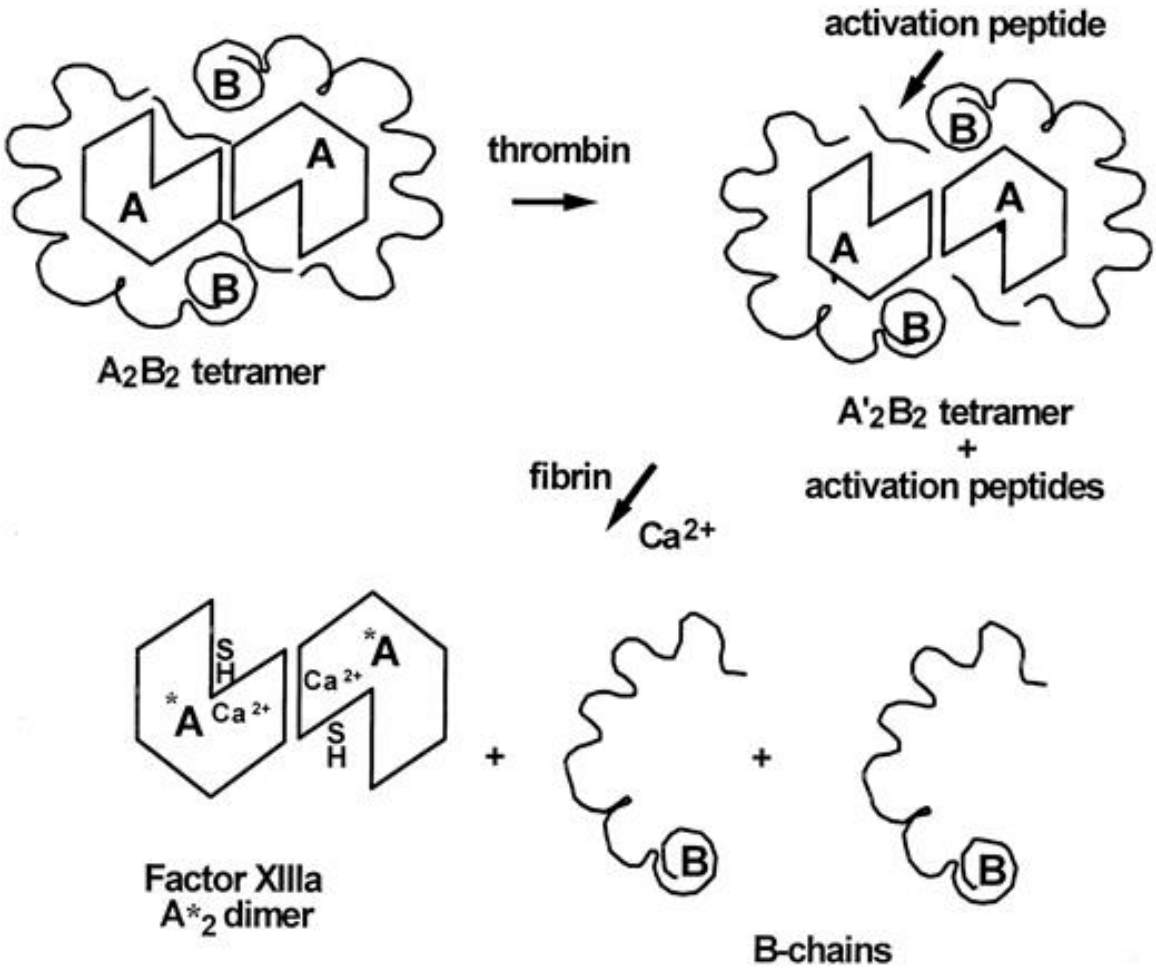

Figure 4: Structure and activation of FXIII.

Plasma FXIII is a tetramer consisting of 2A- and 2B-subunits. The A-subunits contain the activation site and the B-subunits serve a carrier function. Activation of FXIII involves cleavage of the activation peptide followed by the dissociation of the B-subunit in the presence of Ca^{2+} and fibrin. This exposes the active site of FXIIIa. Image taken from reference [31]

1.11 VENOUS THROMBOEMBOLISM (DEEP VEIN THROMBOSIS AND PULMONARY EMBOLISM)

Deep vein thrombosis (DVT) is a medical condition that occurs when a blood clot forms within the larger, deeper veins of the body, such as the thigh[9,74]. The most common site for initiation of thrombus appears to be at the venous valve pocket sinus, due to its tendency to become hypoxic[74]. This is a serious condition that can cause permanent damage to the leg, known as post-thrombotic syndrome (PTS), and life threatening if left untreated since it can cause pulmonary embolism[75]. Pulmonary embolism is a condition that occurs because some or part of the DVT can break off and embolize through the circulatory system, get trapped in the lungs, block a pulmonary artery, and cut off oxygen supply to the affected lung. This can lead to heart failure or death[9].

DVT can occur proximally (above the knee), or distally (in the calf) [74,76]. Blood clots formed in the popliteal vein or more proximal, such as the femoral vein are defined as proximal DVT, while blood clots formed beneath the popliteal vein are considered distal DVT[76]. Post-thrombotic syndrome is a constellation of signs and symptoms that occur as a long-term complication from DVT, and develops in nearly half of all the patients who experience a DVT[76]. Currently an algorithm strategy combining pretest probability, D-dimer testing and compression ultrasound imaging allows for safe and convenient investigation of suspected lower-extremity venous thrombosis. Patients with low pretest probability and a negative D-dimer test result can have proximal DVT excluded without the need for diagnostic imaging[77]. Compression ultrasonography is now the imaging test of choice to diagnose DVT. Lack of compressibility of a venous segment is the diagnostic criterion used, but the addition of Doppler (including colour flow) can

be useful to accurately identify vessels and to confirm the compressibility of a particular segment. When a DVT is treated with anticoagulants alone, the clot might remain in the leg since anticoagulants do not actively dissolve the clot but rather prevent the existing one from growing and prevent new ones from occurring[76]. Clot dissolution occurs slowly and incompletely via the fibrinolytic system. Due to this, a majority of patients develop irreversible damage in the affected leg vein (fibrosed valves from where the clot was)[74,75]. Symptoms of DVT include discolouration in the skin, calf or leg tenderness/pain, swelling of the lower limbs, warm skin with visible surface veins[78].

Symptoms of PE range from shortness of breath, sweating, coughing blood, rapid pulse, sharp chest pain, fainting and sudden death[78]. The clinical diagnosis of PE remains a problem due to the nonspecific presenting signs, symptoms, and chest X-ray changes. D-dimers are becoming a widely available clinical tool useful in the diagnostic management of suspected PE. The limitations of the imaging modalities for PE [ventilation –perfusion (V/Q) scanning, spiral computerized tomography, pulmonary angiography and leg venous imaging] necessitate the use of these tests in series and in combination with clinical pretest probability assessment and D-dimer in diagnostic management algorithms. These algorithms permit safe diagnostic management of patients with suspected PE[79].

Each year in the U.S., about 600,000 patients develop DVT, and over 100,000 patients die from PE[38]. In Canada, 10,000 people each year die from PE[80]. Annually, VTE causes more deaths in Canada than breast cancer or HIV[80]. The prevalence of PE in patients with proximal DVT has been estimated as 40%-50%[81]. Approximately 75% of patients with PE have DVT[81]. Venous thromboembolism (VTE), comprising of a DVT

and PE, is the third leading cause of cardiovascular-associated death, occurring in approximately 1 of every 1000 adults[82]. VTE is very uncommon before the age of 20 years, and at around 40-45 years of age the incidence rate increases sharply[83]. VTE is associated with an impaired life quality, especially when PTS develops or pulmonary hypertension[84]. About two-thirds of venous thromboembolism episodes manifest as DVT and one third as PE (with or without DVT)[84].

The major outcomes of VTE are death, PTS, pulmonary hypertension and major bleeding due to anticoagulants[85]. Death occurs within one month of an episode in about 6% of those with DVT and 10% of those with PE[85]. The mortality rate for PE has been estimated to be as high as 30% when including autopsy-based PE diagnosis, which points to the fact that many PE are not recognized clinically, but present as sudden death[85]. Furthermore, mortality rates are lower in patients with idiopathic VTE, and higher in patients whose thrombosis occurs in the setting of cancer[85]. VTE is a chronic disease that has episodic recurrences, and about 30% of patients develop recurrence within the next 10 years[85]. The hazard of recurrence varies with the time since the incident event, and is highest within the first 6 to 12 months[85].

1.12 RISK FACTORS FOR VTE

Risk factors for VTE are either acquired or inherited and about 80% of VTE patients present with at least one risk factor. Major acquired risk factors for thrombosis include exogenous factors such as surgery, hospitalization, immobility, trauma, and endogenous factors such as cancer, obesity and inherited or acquired disorders of hypercoagulation[86]. Most hospitalized patients have increased risk factors of venous

thrombosis, such as immobility, cancer, infection or surgery[86]. These events could lead to PE and about 10% of all deaths in the hospital are related to PE, with most cases going unnoticed before death[86]. Cancer patients also have increased risk of venous thrombosis because of a combination of risk factors (e.g., tumor cells activating coagulation, tumours compressing veins causing stasis, hospitalization, surgery, chemotherapy) [86]. In a cohort study that was conducted on 66, 329 patients with cancer, the incidence of venous thrombosis during the first 6 months after cancer diagnosis was 12.4 per 1000[86]. VTE is the second leading cause of death in cancer patients and the most common cause of death in the postoperative period, thus such patients often receive prophylaxis treatment against venous thromboembolism[86].

Inherited risk factors or inherited thrombophilia describes an inherited tendency for venous thrombosis (DVT with or without associated PE). Deficiency of AT was the first reported cause of familial thrombosis. Subsequently deficiencies of protein C and protein S were linked with thrombosis in individuals and families, whereas mutations in FV and prothrombin genes were discovered as risk factors for venous-thrombosis in case-control studies[87].

1.12.1 FACTOR V LEIDEN

Factor V is a high molecular weight glycoprotein, 330 kD, synthesized by hepatocytes and megakaryocytes as a single polypeptide chain[88]. In whole blood, FV is divided between two pools; approximately 80% circulates in plasma while the remaining 20% is found within the α -granules of platelets[89]. FV is synthesized as an inactive precursor in the liver, thus it has no cofactor activity until it is cleaved by thrombin to FVa[89]

FV/Va together with the serine protease FXa forms the prothombinase complex that converts prothrombin to active thrombin[90]. FVa is subsequently inactivated by the natural anticoagulant, activated protein C, which cleans FVa at amino acid arginine(R)-506, R306, and R678 in the heavy chain[91–93]. Cleavage at R506 is required for efficient cleavage at the two other sites[91–93].

The substitution of glutamine (Q) for R506 in FV, also known as FV Leiden (FVL), is identified in 20%-50% of patients with venous thromboembolic diseases[92]. The FVL mutation results in a mutant protein that has normal procoagulant activity but the R506Q mutation abolishes a cleavage site for APC, thereby reducing the rate of inactivation of FVa by APC and consequently inducing the formation of more thrombin[8].

1.13 ANTICOAGULANTS

Anticoagulants are used for the treatment of VTE. The role of anticoagulants is to prevent clot formation and the recurrence of thrombosis. Traditional anticoagulant therapy consisted of parenteral administration of fast-acting unfractionated heparin (UFH) followed by slow-acting vitamin K antagonist, warfarin[94,95]. However, the introduction of low-molecular weight heparin (LMWH) and fondaparinux simplified parenteral anticoagulants. The development of new oral anticoagulants called non-vitamin K oral anticoagulants (NOACs) or called, direct-acting oral anticoagulants (DOACs), have largely replaced heparins and vitamin k antagonists as the preferred treatment of VTE[94,95].

1.14 WARFARIN

Warfarin, a vitamin K antagonist (VKA), is an oral anticoagulant used for the long-term prevention and treatment of venous thrombosis (75)[96]. It inhibits the synthesis of clotting factors II, VII, IX, and X as well as the endogenous anticoagulant protein C and S[96]. Vitamin K is a co-factor for an enzyme that adds carboxylic acid to the glutamic acid at the N-terminal regions of these clotting factors. The formation of the γ-carboxy glutamic acid (GLA) results in a higher density of negative charge localized to the N-terminus[97]. As well, calcium, binding to GLA, can induce a conformation change in the coagulation protein to enhance or enable binding to the surface of activated platelets[97]. Warfarin inhibits the vitamin K conversion cycle and prevents γ-carboxylation of vitamin K dependent coagulation factors, and thereby reducing their procoagulant activity, by preventing the non-GLA clotting factors from assembling in the membrane bound coagulation complexes[97]. Extended treatment of venous thrombosis involves the use of warfarin, an oral anticoagulant available for long term use, however it has many limitations such as delayed onset of anticoagulation, many food and drug interactions, bleeding complications, narrow therapeutic range, variable dose requirements and the requirement for routine INR (international normalized ratio) monitoring[97]. Extensive research has been carried out on new drugs such as DOACs for use in extended treatment of venous thrombosis.

1.15 INHIBITING THROMBIN GENERATION

Thrombin is central in the clotting cascade[98]. Aside from the natural existing anticoagulants (TFPI, AT, protein C and protein S) which help restrict the formation of

the hemostatic plug, the discovery of direct thrombin inhibitors (DTIs) are a new class of anticoagulants that bind directly to thrombin and block its ability to cleave its substrates[98]. Rapid initial anticoagulation is given to reduce the risk of pulmonary embolism and thrombus extension[99]. This can be achieved by administering thrombin inhibitors such as heparin and LMWH, and indirectly, by FXa inhibitors, such as fondaparinux and rivaroxaban, which will result in less thrombin generation[100]. Thrombin inhibiting drugs block thrombin's action by binding to three domains: (i) the active/catalytic site (ii) exosite 1 and (iii) exosite 2[98]. Exosite 1 acts as a dock for substances like fibrin, and exosite 2 serves as a heparin-binding domain[98]. Heparin-bound thrombin can then bind fibrin or antithrombin. Once heparin-bound thrombin is joined to fibrin, both exosites are occupied and the complex is relatively protected from the effects of antithrombin. As a result, heparin has little effect on fibrin-bound thrombin. In contrast, DTIs differ from heparins because they can inhibit clot-bound, fibrin-bound and free thrombin[98]. Additionally, the DTIs reduce the platelet-aggregating effects of thrombin.

1.15.1 HEPARIN

Heparin is a naturally occurring negatively charged sulfated polysaccharide, and it is a glycosaminoglycan formed from alternating residues of D-glucosamine and L-iduronic acid[74]. Heparins contain a specific pentasaccharide fragment that binds and activates the plasma protease inhibitor antithrombin[97]. A heparin-thrombin-antithrombin complex is formed when heparin binds simultaneously to exosite 2 in thrombin and to antithrombin[98]. Heparin binding to AT facilitated by the core pentasaccharide present in heparin (40-50 saccharides in total), induces a conformational change in AT at the reactive site, which

results in an increase in its inhibitory activity against the key coagulation protease thrombin, FXa, FXIIa FXIa, FIXa, plasmin, and kallikrein[97]. The long tail ensures a certain orientation of thrombin with AT and fixes the AT portion in a ternary complex with heparin and thrombin[97]. The main role of heparin is to bring together the protease and its inhibitor, a process termed approximation, rather than just producing a conformational change[97]. This approximation and binding of the heparin chain are essential for the accelerated inhibition of thrombin by AT[97]. Heparin acts as a bridge between thrombin and fibrin by binding both to fibrin and exosite 2[97]. Because both of thrombin's exosites are occupied within this fibrin-heparin-thrombin complex, heparins have a reduced capacity for the inhibition of fibrin bound thrombin, which appears to be detrimental, because active thrombin further triggers thrombus growth[97]. Heparin alone has no direct effects on coagulation[82].

1.15.2 LOW MOLECULAR WEIGHT HEPARINS (LMWH)

Unfractionated heparin (UFH) is the standard preparation of heparin with a mean molecular weight of approximately 15000 DA consisting of 40-50 saccharides in length[97]. LMWH are produced from UFH by chemical or enzymatic depolymerization, with molecular weights of 4000-6500 Da[97]. LMWHs have increased bioavailability after subcutaneous injection, renal clearance that is dose-independent, and longer half-life (17-21 hours) when compared to UFH. Dalteparin, a commonly used LMWH, has been studied for the indication of treatment for VTE[101–107], prophylaxis of VTE after hip or major surgery[108–110], or prophylaxis of VTE in medically ill or surgical patients [111,112].

Multiple studies have evaluated the efficacy of dalteparin for the initial treatment of DVT and/or PE. Three of these studies were prospective, randomized, double blind clinical trials[105–107] and 3 were randomized open trials[102–104]. In all of these clinical trials, the diagnosis of DVT was confirmed with venography. Most of the patients received intravenous infusions of UFH for the first 24 hours until the diagnosis of DVT was established. Treatment regimens varied with respect to administration. Three trials compared subcutaneous dalteparin with intravenous infusion of UFH[102,103,107] and two trials compared subcutaneous administration of both UFH and dalteparin[102,105]. Dalteparin was administered once or twice daily either in fixed dosages or adjusted on the basis of anti-factor-Xa activity. These studies showed that dalteparin given at 100-120 IU/kg twice daily or 200 IU/kg once daily was comparable to activated partial thromboplastin time (aPTT)-adjusted UFH infusion in preventing recurrent thrombosis[102–105].

Three randomized trials have evaluated dalteparin for long-term therapy. The first was a small, open-label study that included patients who were unable to tolerate warfarin[113]. Patients were randomized to twice-daily of either dalteparin 5000 IU or UFH 10,000 IU. Only 3.3% of patients presented with recurrent VTE during 3 months of follow-up and no difference was observed between the two groups. The second trial primarily included patients without cancer and found no difference in efficacy or safety between dalteparin 5000 IU once-daily and warfarin adjusted to an INR of 2.0–3.0[114]. The third study is the largest trial investigating the use of LMWH for long-term therapy in cancer patients. The Comparison of Low-molecular-weight heparin versus Oral anti-coagulant Therapy for the prevention of recurrent venous thromboembolism (CLOT) trial

was a multicenter, randomized, open-label study in which 676 cancer patients with proximal DVT, PE or both[115]. Patients were randomized to usual treatment with dalteparin initially followed by Coumadin therapy or dalteparin therapy alone for 6 months[115]. In the dalteparin group, patients received therapeutic doses at 200 U/kg once-daily for the first month and then 75–80% of the full dose for the next 5 months. Over the 6-month treatment period, dalteparin significantly reduced the incidence of recurrent VTE from 17 to 9%. There were no differences in bleeding and overall mortality between the groups[115].

1.15.3 DIRECT THROMBIN INHIBITOR- XIMELAGATRAN

Ximelagatran is a direct thrombin inhibitor that had received approval for short-term prophylaxis in orthopedic surgery[116]. It was extensively studied for use in atrial fibrillation and for treatment of DVT[117,118]. These trials found an unexpected increased risk of hepatotoxicity with prolonged exposure to Ximelagatran. The manufacturer has now withdrawn the drug from the market worldwide[119].

The Thrombin Inhibitor in Venous Thromboembolism (THRIVE) trial looked at acute up-front treatment of ximelagatran compared to LMWH/VKA and showed an increased recurrence of VTE (on-treatment) in the ximelagatran arm[117]. This was driven by a 1.8-fold increase in PE; the increase in DVT was 1.2-fold. The early events suggest an early increase in all VTE, this was not statistically significant at the 30-day time point, but there was a trend for early increased recurrence in the ximelagatran arm.

1.15.4 DIRECTION THROMBIN INHIBITOR - DABIGATRAN

Dabigatran is a reversible, potent, competitive direct thrombin inhibitor[120]. Dabigatran binds to the active site of thrombin. Unlike heparin, which can only inhibit free thrombin, dabigatran is capable of binding and inhibiting both free and clot-bound thrombin[120]. Dabigatran is administered as the etexilate ester pro-drug, and following absorption, the etexilate moiety is hydrolyzed to the active compound dabigatran. Its bioavailability is only 3%-7% when the capsule is swallowed[121]. Peak plasma concentrations occur within 2 hours of ingestion[121]. Approximately 35% is bound to plasma proteins. In patients with normal renal function, approximately 80% of an intravenous dabigatran dose is excreted in the urine with an elimination half-life of 12-17 hours[121]. Renal impairment increases the elimination half-life to 15-34 hours. Dabigatran dosing therefore depends upon creatinine clearance[122]. Dabigatran is also contraindicated in patients with acute bleeding. The risk of bleeding increases when dabigatran is taken with other anticoagulant or antiplatelet agents such as warfarin or aspirin[120].

Two large randomized trials have compared the safety and effectiveness of dabigatran against standard warfarin therapy. The Randomized Evaluation of Long – Term Anticoagulant Therapy (RE-LY) compared dabigatran with warfarin for the prevention of embolic stroke in patients with atrial fibrillation[123]. Authors concluded that twice daily 110 mg dabigatran doses produced similar rates of stroke and systemic emboli as warfarin but with a lower rate of hemorrhage. 150 mg twice daily dabigatran produced reduced rate of stroke and systemic emboli as warfarin, but with a similar rate of hemorrhage. The Dabigatran vs Warfarin in the Treatment of Acute Venous Thrombosis (RE-COVER) trial compared dabigatran with warfarin for the treatment of

cute VTE[124]. 150 mg twice daily dabigatran produced no significant difference in the incidence of recurrent VTE or major bleeding compared with warfarin. Initial treatment with an approved parenteral anticoagulant (generally unfractionated heparin administered intravenously, or low-molecular-weight heparin administered subcutaneously) was usually started before random assignment.

1.16 FACTOR Xa INHIBITORS

FXa is also an attractive target for anticoagulants since it is responsible for dictating downstream coagulation by activating thrombin, thus FXa inhibition will attenuate further thrombin generation[98]. Direct FXa inhibitors can bind directly to free FXa and are able to inhibit prothrombinase – bound FXa, while indirect inhibitors will catalyze AT's anticoagulant ability and inhibit only free FXa[125].

Fondaparinux, an indirect FXa inhibitor, is a synthetic version of the heparin pentasaccharide. It binds to AT with high affinity and induces a conformational change in AT that increases its inhibition rate for FXa. It is administered subcutaneously and has a high bioavailability[126], and long half-life and is renally cleared[127].

Orally administered FXa inhibitors, rivaroxaban, apixaban and edoxaban, bind to the active site of FXa in a reversible fashion. They are characterized with having high bioavailability[127], rapid onset of action, short half-life, with limited monitoring and drug-drug interaction[127].

1.17 THROMBUS RESOLUTION

DVT resolution occurs through a complex process of inflammation and fibrinolysis (Figure 5). Inflammation begins with early neutrophil influx, followed by the eventual replacement with cells of the monocyte-macrophage lineage[126,128,129]. Studies show that reduction in monocyte recruitment severely restricts DVT resolution[130–132]. Monocyte/macrophages are important mediators of resolution, as they are capable of expressing a host of chemotactic agents that are known to affect tissue remodeling, recanalization, and ultimately complete resolution[132]. Fibrinolysis is thought to occur mostly through uPA, produced by the same monocytes that intensify the inflammatory process, leading to increased plasmin generation and clot degradation[133–135]. Complete venous occlusion may reduce leukocyte infiltration and fibrinolytic components to the clot, hence reducing the local inflammatory cytokines and fibrinolysis of DVT.

Multiple studies evaluated thrombus regression by CUS in patients with symptomatic or asymptomatic DVT of the lower limbs. Thrombus resolution rates at 6 months after a first episode of DVT ranged from 24%-44[136–140]. At 1 year or later, rates ranged from 36%-96%[137–150]. Thrombus burden[140,142,145,148], age[142], immobilization[150], previous DVT[142,143], absence of flow and duration of symptoms prior to treatment[143] were found to be unfavorable factors for thrombus resolution. This huge variation in the normalization rates being reported between studies has impaired our understanding of thrombus resolution.

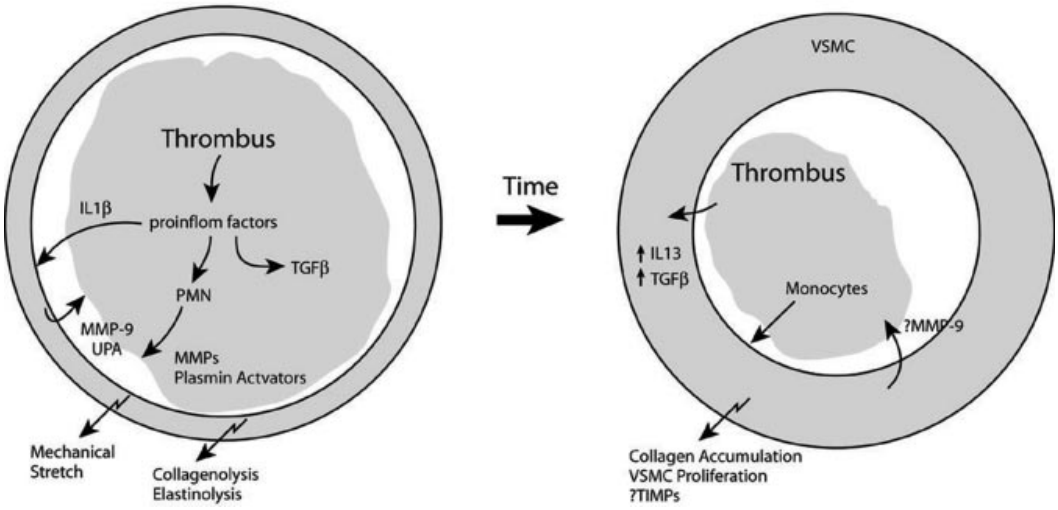

Figure 5: Proposed mechanism of DVT resolution.

Thrombus resolution is an active process of inflammation, with early neutrophil influx followed by the eventual replacement with cells of the monocyte-macrophage lineage. This contributes to the secretion of a number of pro-inflammatory cytokines that may direct extracellular matrix turnover. Image taken from reference [151]

1.18 THROMBUS COMPOSITION

Clots can display a diversity of structural, biological, physical, and chemical properties, depending on the conditions existing at the time of formation, which in turn, influence its stability and resistance to fibrinolysis. Arterial thrombi contain a large amount of fibrin in addition to platelets[152]. Venous thrombi and emboli contain red blood cells and fibrin, but contain a small amount of platelets too, as well, they contained few biconcave red blood cells but many polyhedrocytes or related forms of compressed red blood cells[152]. This was indicative of clot contraction that takes place. Pulmonary emboli mirror the most distal part of the venous thrombi from which they originated, and are significantly different from arterial thrombi[152]. Lastly, more fibrin bundles were observed in arterial than in venous thrombi and pulmonary emboli, but more individual fibrin fibers were observed in pulmonary emboli than in arterial or venous thrombi[152]. This is likely explained by the differences in the constriction of the vessel walls, blood flow, and shear present in their locations.

Different thrombin concentrations can also result in different fibrin clot thickness[153]. More specifically, greater thrombin concentration creates a stronger fibrin network formation less susceptible to lysis, and ultimately, greater thrombus stability[153]. Decreased thrombus stability or weaker fibrin clots become more susceptible to fibrinolysis and embolize more. Therefore, for the purpose of this thesis, the amount of embolization from a formed clot, was used as measure of DVT stability, ie., increased embolization meant decreased thrombus stability and vice versa.

When a DVT is treated with anticoagulants, the clot might remain in the leg since anticoagulants do not actively dissolve the clot but prevent existing ones from growing

and new ones from forming[154]. Clot dissolution occurs slowly and incompletely via the fibrinolytic system. The role of thrombus instability in the progression of DVT to PE remains undefined. Ultimately the role of anticoagulants should be to stabilize the deep vein thrombus with the goal of preventing PE-associated mortality.

Not much is known about how anticoagulants affect thrombus stability. In a study performed by Ammollo *et al.*, they show that dabigatran enhanced the clots susceptibility to lysis by at least two mechanisms; (i) by reducing activated thrombin-activatable fibrinolysis inhibition (TAFIa) generation; and (ii) by altering the viscoelastic properties of the clot[155]. Both effects are related to the ability of the drug to reduce thrombin generation and activity. As well, a study done by Nielsen *et al.*, showed that argatroban, a DTI, facilitates fibrinolysis mainly by inhibition of thrombin-mediated TAFI activation[156]. Both these studies and others that look at the effect of anticoagulants on fibrin structure, show that reduced thrombin generation does enhance fibrinolysis[155–157], however the precise mechanism involved and the relative concentration of various antifibrinolytic proteins (eg. TAFIa, FXIIIa)) in the process remains to be fully elucidated.

1.19 THROMBUS STABILITY

Thromboembolic diseases, such as pulmonary embolism, continue to be major causes of morbidity and mortality worldwide[158]. As of today, there is no way to predict the risk of PE development in patients diagnosed with, or at risk for a DVT. A method to measure or predict DVT stability might prove useful as a measure or prediction of PE risk. Ultimately, using thrombus stability as a measure of assessing the risk of PE development can help reduce PE associated mortality.

The correlation between DVT and PE, and progression of DVT to PE is not fully understood since it varies from patient to patient and with different clinical conditions. DVT usually recur as DVTs and PEs usually recur as PEs[76]. Questions that are left unanswered are: How does DVT correlate with PE? Is it a continuum of the same disease? If so, how does DVT stability affect this? And lastly, how do treatments of the disease alter this stability? Many researchers such as Caps *et al.*, Krupski *et al.*, Kakkar *et al.*, and Kashtan *et al.*, have all looked at the progression of DVT in patients from the first day of diagnosis using serial venous duplex scans[159–162]. Many find that most patients who had inadequate anticoagulation treatment immediately show progression of DVT as extension. During the acute phase of therapy, Caps *et al.*, saw that the risk of recurrent thrombus formation was significantly high[159]. This may suggest that acute treatment of DVT may lead to decreased thrombus stability, but the mechanism of how DVT instability accounts for this extension still remains unanswered. In almost all these studies they reported patients who developed PE and whether they were non-fatal or fatal, however none of them looked at thrombus embolization to see how it altered DVT stability during the acute phase of therapy and whether it correlated with prevalence of PE development. As of today, we don't know a lot about factors that alter thrombus stability, and no one has looked at whether therapy alters embolization during the acute phase.

1.20 MOUSE MODELS

Despite moderate success with some of the newer therapies, there is still much room for improvement in treatment of recurrent VTE and reduction of bleeding complications. A

major preclinical approach to understanding the biology of venous thrombosis requires the use of animal models. Animal models play a fundamental role in understanding the biology behind VTE[163].

Mouse models are suitable for the study because they're relatively low cost, and availability of different genetically manipulated strains[163]. Mice exhibit important differences compared to humans such as body size, life span, genetic differences, and vessel size[164]. Mice have a short life span and high metabolic rate compared to humans, which means that events occur faster in mice[164]. DVT in humans can be defined as acute, sub-acute, and chronic, and thus mice models become efficient in studying acute DVT[164].

Unlike humans, mice do not spontaneously develop venous thrombosis and thus it must be induced[165]. Several experimental approaches, such as photochemical injury, laser injury, and ferric chloride injury, or suture-based stenosis or ligation of a vein, such as the inferior vena cava (IVC), have been used.

1.20.1 MOUSE MODELS OF DEEP VEIN THROMBOSIS

A plethora of mouse models of venous thrombosis have been created to serve various purposes[166]. Of these animal models, the ferric chloride ($FeCl_3$) is the most commonly used agent to initiate vascular injury and thrombosis[167]. The $FeCl_3$ injury model was originally used to study arterial thrombosis but has been used in both microvascular and macrovascular models of mouse venous thrombosis[168,169]. Filter paper pre-soaked in $FeCl_3$ solution placed briefly on the side of the vein leads to the formation of fibrin rich thrombi within minutes post injury. The size and speed of thrombus formation is $FeCl_3$ concentration/time of exposure dependent[167].

The IVC stasis model is performed by placing a non-reactive suture ligature around the IVC and just below the renal vein to produce complete blood stasis anywhere from 24-48 hours, resulting in an occlusive thrombus[164]. The advantage of the IVC is that it yields quantifiable amounts of vein wall tissue and thrombus[163]. It has been proven useful for evaluating interaction between the vein wall and the occlusive thrombus[163]. The lack of blood flow however inhibits the maximal effect of administered systematic therapeutic agents on the thrombus and vein wall[164]. It also prevents evaluation of thrombus embolization or stability.

The IVC stenosis model, which is performed with a 7-0 prolene suture tied around the IVC with a 30 G needle, or other spacer, to allow some blood flow. This gives the model an advantage, in that it produced a laminar thrombus and cellular kinetics can be studied. However, the problem that arises with this model is that the degree of stenosis is inconsistent leading to variable thrombus sizes[164], in some cases none. There are also many variations in how this model is performed (side-branches ligated or not, vessel pinched or not, diameter and nature of spacer); in only one variation of this model pulmonary emboli have been found[170].

1.20.2 MOUSE MODELS OF PULMONARY EMBOLISM

Mouse models have also been used to study the pathophysiology of PE and the effect of antithrombotic and thrombolytic agents[171]. There are three main types of PE models, i) coagulation factor-induced, ii) photochemical reaction-induced and iii) exogenous clot injection-induced PE model. With the coagulation factor-induced model, strong coagulation factors such as thrombin[172,173], thromboplastin[174], collagen combined

with epinephrine[175], or adenosine diphosphate (ADP)[176,177], are injected intravenously through the jugular vein to induce widespread platelet activation, likely coagulation, and subsequent lethal pulmonary embolism.

In the photochemical reaction-induced model a laser light is used to directly irritate the vessel wall of the jugular vein, followed by intravenous infusion of Rose Bengal. Rose Bengal can react with the endothelial membrane protein and lipid to induce per-oxidative reactive and endothelial injury in the irradiated area. Histological evidence of thromboembolism in the lungs shows that the thrombi are rapidly established with platelet deposition starting approximately 30 s after irradiation, followed by complete vessel occlusion after 6-8 min. This method established a more physiological mice model of PE as a consequence of VTE.

In the exogenous clot injection-induced model, an exogenous thrombus is prepared in vitro, chopped-up, and injected into the venous system through the jugular vein or tail vein[178–180]; the chopped-up clots lodge in the lung and are evaluated by histology. Usually, there are two methods for preparing the clots; “red emboli,” which are made by the coagulation of the whole blood of the human or mouse, predominately consists of red blood cells, platelets and some fibrin. The components of this type of clots are similar to those formed in physiological conditions of the body. Another type of clot can be made by a mixture of human plasma or human fibrinogen with human thrombin. This type of clot is called “pale emboli” because of its constitution of almost no red blood cells. A clot made using this procedure consists of extensively cross-linked fibrin[181]. In vitro studies have demonstrated that fibrin-rich pale emboli retract more than red ones,

which reduced the permeability of the emboli and does not allow thrombolytic agents to pass through[182].

Recently, a spontaneous model of PE has been established. In this model, the femoral vein is surgically ligated with a 7-0 nylon suture to induce complete vessel occlusion, resulting in significant decrease of venous flow at the saphenous vein[183]. This model enables visualization the initiation of thromboembolism.

As well, recently a modified IVC stasis model that does develop PE has been established. Briefly, mice are subjected to IVC stasis for 24 hours. After 24 hours, thrombi are present, and the ligature is removed to allow mice to recover. After an additional 24 hours, mice are sacrificed, and the presence of PE is shown in lung histology[170,183,184].

While all these studies can produce large and quick PE for analysis and testing therapies, none of these PE models reflect the complex pathophysiology of venous thrombosis and PE in humans. To date, there is no animal model that recapitulates physiological venous thromboembolism, with PE that originates spontaneously from a DVT. A mouse model of DVT stability is necessary in order to understand the mechanism of DVT stability and the factors involved as well as correlation to PE burden.

CHAPTER TWO

2.0 THESIS OVERVIEW, RATIONAL, HYPOTHESIS, AND OBJECIVES

2.1 THESIS OVERVIEW

In the previous years researchers have wondered how to make "bad clots" into "good clots" or what makes a clot "bad" or "good" and have limited their scope to clot structure and stability in the sense of what makes a clot lyse[185]. Therefore, this has shaped research to simply make clots and study factors that will lead to clot lysis and conclude that these factors can have therapeutic potential. However, a clot that lyses or becomes smaller does not necessarily make the clot "good" which is what is perceived in literature. In order to make sure there are no downstream effects, studies that show decreased thrombus size need to make sure these clots are being dissolved and not embolizing to create PE.

In this dissertation we show that what has previously been perceived as a "good clot" is not the case when looking at the downstream effects. Pulmonary embolism, emboli that have broken off clots in the deep veins and travelled to a pulmonary artery causing sudden death, is primarily due to DVT, often asymptomatic, in patients. Therefore, we instead defined "good clots" as stable clots, thus making them less likely to break-off and embolize to the pulmonary artery and cause less PE. Increasing clot stability, using our novel mouse model of venous thromboembolism, leads to decreased PE burden in mice. When some anticoagulants, such as the direct thrombin inhibitor dabigatran, are used for upfront treatment of DVT, this leads to increased PE burden because of reduced clot stability. When treated with plasma purified human FXIII, mouse

thrombin can activate FXIII to FXIIIa and this stabilizes the clots without increasing the thrombus size. Treatment with FXIII also reduced PE burden. This dissertation suggests that in patients with a DVT, in addition to treatment using anticoagulants, which is the current standard of treatment, supplementing with FXIII would be beneficial since it would stabilize clots and minimize embolization to the lungs without increasing the existing clot.

2.2 RATIONALE FOR STUDIES

Naturally, many studies and researchers consider that reduced thrombus size in mice is a positive outcome because of reduced thrombus burden. However, none of these studies look at the downstream effects of these "reduced clots" and do not take into account the effect they have on PE. Therefore, venous thrombus stability should be studied and in the context of DVT and PE with a physiological mouse model of VTE, which currently there is none of. Thus, we need to redefine "good clots" in the literature.

Additionally, Caps *et al.,* published a preliminary study showing that in patients with a proximal DVT, diagnosed using compression ultrasonography, after a three-month treatment with warfarin, had extension of the thrombi with 40% of patients having recurrent events in the first three months of follow-up[159]. This suggests that despite treatment with anticoagulants, clot destabilization and extension can occur leading to PE in patients, thus an early recurrence of VTE. Furthermore, an all-oral DTI, ximelagtran caused increased VTE recurrence, within 90 days, compared to patients who were administered conventional anticoagulant therapy (LMWH bridging to warfarin)[117].

Therefore, we asked whether increasing the stability of an existing clot would lead to decreased pulmonary embolism without causing further clot extension.

The overall mechanism of thrombus stability has never been studied *in vivo* and therefore it is not well elucidated how attenuating fibrinolysis plays a role in DVT stability and its correlation to PE burden. While many mechanisms have been proposed to explain the FVL paradox, none have provided clear in vivo evidence because of the lack of a VTE mouse model that can study venous clots and PE.

2.3 CENTRAL HYPOTHESES

Using our venous thrombus stability model, we hypothesized that DVT stability is inversely related to embolization and PE burden. We hypothesized that thrombin-mediated FXIIIa promotes thrombus stability. Anticoagulants that inhibit thrombin will reduce FXIIIa generation and thus activity and decrease embolization. Reduced FXIII activity will decrease the mechanical stability of clots and therefore allow for increased embolization and PE burden in mice. When supplemented with FXIII, mechanical stability will be increased (either through crosslinking of α_2-AP onto fibrin(ogen) or crosslinking α-α, α-β, or γ-γ chains of fibrin) without increasing thrombus size, leading to reduced embolization and PE burden. Inhibiting fibrinolysis using ε-aminocaproic acid (EACA) would not have the same effect as FXIII since it will prevent clot breakdown completely and increasing thrombus size while. Last, we hypothesize that when using our mouse model of venous thrombosis, we will see increased thrombus stability and reduced PE burden in FVL mice compared to WT.

2.4 OVERALL OBJECTIVES

The overall objective of this dissertation is to i) develop a model of venous thrombus stability, in order to ii) better study thrombus stability and its correlation to PE burden, iii) understand the effect of anticoagulants on thrombus stability and PE burden, iii) study the effect of attenuating fibrinolysis on clot stability and iv) propose a mechanism for the FVL paradox so that we can uncover the mechanism of DVT stability and its correlation with PE burden. Therefore, the specific objectives of this dissertation are as follows:

2.4.1 Chapter 3 Objective

Objective: To develop a mouse model to assess thrombus stability and its link to PE burden, and identify whether DTIs, in contrast to LMWH, alter this correlation.

In order to understand the mechanism governing thrombus stability and its effects on PE burden, we need to develop a physiological mouse model of venous thromboembolism. Clinical trial data suggested an increase in thrombotic events when patients were treated with a direct thrombin inhibitor compared to low-molecular-weight-heparin. Previous to this study there are no animal models that produce PE directly from an induced DVT. We sought to develop a model to assess factor of thrombus stability and ultimately PE burden, and whether DTIs, as opposed to LMWH, alters the connection between DVT and PE. We hypothesized that an increase in embolic events would increase PE burden and acute treatment with dabigatran would increase embolic events. We tested this hypothesis in the femoral vein of C57BL/6 female mice that were subjected to ferric chloride injury to initiate thrombus formation. Platelets were fluorescently labelled and treatment with saline, dalteparin, or dabigatran, was

administered 12 minutes after thrombus formation. Intravital videomicroscopy recorded embolic events leaving the thrombus and thrombus sizes. Lungs were stained for the presence of PE.

2.4.2 Chapter 4 Objective

Investigate the consequence of attenuating fibrinolysis using FXIII, α_2-antiplasmin or EACA supplementation on clot lysis and venous thrombus stability.

Inferior vena cava filter use has increased over the decade despite the lack of evidence showing its benefit. This is likely due to the fact that clinicians do not have the confidence that an acutely anticoagulated deep vein thrombus will not embolize. Therefore, an ideal treatment for patients would be to stabilize clots in DVT patients, minimize embolization, and reduce PE. We have previously demonstrated that acute thrombin inhibition increases the number of embolic events and that this was FXIII-mediated. The role of fibrinolysis on thrombus stability and PE have never been studied. Antifibrinolytics, such as tranexamic acid (TXA) and ε-aminocaproic acid (EACA) are approved and clinically used to treat bleeding. Recombinant FXIII supplementation has been shown to reduce postoperative blood transfusions in the context of cardiopulmonary bypass and cancer surgery, and is also approved. Inhibition of α_2-AP has been shown to assist in dissolution of PE. However, the effects of these treatments on clot stability and its correlation to PE has never been studied. In this paper we discuss three methods of increasing thrombus stability, i) attenuating fibrinolysis, and ii) increasing mechanical stability with supplemented FXIII or iii) supplemented α_2-AP. We hypothesized that embolization and PE burden will decrease after treatment with EACA in WT mice

because of increased thrombus stability. However, this may increase over time. We tested this hypothesis by performing an *in vitro* clot lysis assay from plasma samples obtained from WT mice treated with the above mentioned agents as well as our previously described in vivo mouse model of venous thromboembolism. Treatment with saline, EACA (1 mg/g), α_2-AP (4.2 mg/kg), or FXIII (1mg/kg), with or without dalteparin or dabigatran, was administered 12 minutes after thrombus formation. Intravital videomicroscopy recorded embolic events leaving the thrombus, and thrombus sizes for 2 hours. Lungs were harvested, sectioned and stained for presence of PE.

2.4.3 Chapter 5 Objective

Use our mouse model of venous thromboembolism to explain why patients with FVL are more likely to present with DVT rather than PE.

People with FVL are often called hypercoagulable, and have a five-fold increase in the risk of venous thrombosis. There is a low prevalence of FVL among patients with fatal pulmonary embolism (PE) and a higher incidence of deep vein thrombosis (DVT) than PE in patients with FVL. This has been referred to as the FVL paradox in thrombosis. We wished to test whether FVL and normal patients are at the same risk of initial thrombus formation, expect FVL patients present with symptomatic DVT while normal patients do not present, because the small asymptomatic DVT in normal patients embolizes to become an asymptomatic PE. Therefore, to explain why patients with FVL are more likely to present with a DVT rather than a PE, we studied DVT stability in FVL mice using a modification of our previously published VTE mouse model. We tested this hypothesis using our previously established mouse model with a few changes. We chose

conditions such that only a small thrombus developed in wild type (WT) mice, and then compared them to thrombi in FVL heterozygous ($F5^{L/+}$) and FVL homozygous ($F5^{L/L}$) mice. Intravital videomicroscopy recorded embolic events leaving the thrombus and the thrombus sizes every 10 minutes for 2 hours. Lungs were harvested, sectioned and stained for the presence of PE.

CHAPTER THREE

3.0 COMPARISON OF THE EFFECT OF DABIGATRAN AND DALTEPARIN ON THROMBUS STABILITY IN A MURINE MODEL OF VENOUS THROMBOEMBOLISM

3.1 Forward

This manuscript describes the effect of dalteparin and dabigatran on clot stability using the newly developed mouse model of venous thromboembolism. This manuscript shows that i) thrombus stability is inversely related to PE burden, ii) dabigatran reduces thrombus stability significantly more than dalteparin and iii) in the absence of FXIII, thrombus stability is decreased.

Comparison of the Effect of Dabigatran and Dalteparin on Thrombus Stability in a Murine Model of Venous Thromboembolism

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Authorship Contributions: S.A Shaya performed all experiments, analyzed data and results, and wrote the manuscript. L.J. Saldanha designed the preliminary experiment model. R. Ni and N. Vaezzadeh helped teach surgical procedure and aPTT-like assay, as well as discussed results. J. Zhou taught histological techniques and helped in histological data interpretation. P.L. Gross supervised the research, conceived and designed the study, contributed to the interpretation of data and critically edited the manuscript.

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References: References for this Manuscript have been incorporated into the Bibliography (Chapter 7), at the end of this thesis.

3.2 ABSTRACT

Background: Venous thromboembolism (VTE) is a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE). Thrombus instability possibly contributes to progression of DVT to PE, and direct thrombin inhibitors (DTIs) may alter this. *Aim:* To develop a model to assess thrombus stability and its link to PE burden, and identify whether DTIs, in contrast to low-molecular-weight heparin (LMWH), alter this correlation. *Methods:* Twelve minutes after ferric chloride-induced thrombus formation in the femoral vein of female mice, saline, dalteparin (LMWH), or dabigatran (DTI) was administered. Thrombus size, and embolic events breaking off from the thrombus were quantified before treatment, and at 10-minute intervals after treatment for 2 hours using intravital videomicroscopy. Lungs were stained for the presence of PE. *Results:* Thrombus size was similar over time and between treatment groups. Total and large embolic events and pulmonary emboli were highest after treatment with dabigatran. Variations in amounts of pulmonary embolic events were not attributed to variations in thrombus size. Large embolic events correlated with the number of emboli per lung slice independent of treatment. Embolization in factor XIII deficient ($FXIII^{-/-}$) saline-treated mice was greater than wild-type (WT) saline-treated mice, but was similar to WT dabigatran-treated mice. *Conclusion:* We have developed a mouse model of VTE that can quantify emboli and correlate this to PE burden. Consistent with clinical data, dabigatran, a DTI, acutely decreases thrombus stability and increases PE burden compared to LMWH or saline, a FXIII-dependent effect.

3.3 KEYWORDS

(i) dabigatran (ii) dalteparin (iii) factor XIII (iv) pulmonary embolism (v) venous thrombosis

3.4 INTRODUCTION

Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is the third leading cause of cardiovascular-associated deaths [84]. Anticoagulant therapy, used for treatment of VTE, attempts to: (i) prevent the existing thrombus from growing, (ii) prevent new thrombi from forming, (iii) reduce PE-associated mortality, and (iv) reduce risk of recurrent VTE[186,187]. The risk of recurrent VTE in patients with symptomatic DVT is highest within the first month of diagnosis, so adequate initial anticoagulation therapy is critical[188].

Conventional anticoagulant therapy, used for treatment of VTE, involves use of low-molecular-weight heparin (LMWH), such as dalteparin, overlapped with warfarin[186]. New oral anticoagulants produced to replace this therapy include direct thrombin inhibitors (DTIs), such as dabigatran, and activated factor X (FXa) inhibitors, such as rivaroxaban and apixaban. While all-oral regimens for the FXa inhibitors have been evaluated for treatment of acute symptomatic VTE, and have proven to be as effective as traditional therapy[189–192], all-oral regimens for dabigatran have not. The evaluation of dabigatran for the treatment of VTE was only after acute initial treatment with LMWH [124,193].

In the THRIVE study, initial treatment of DVT with ximelagatran, a DTI, was compared to LMWH bridging to warfarin[117]. Thus, this study offers a direct comparison

between conventional LMWH therapy and a DTI for acute VTE treatment. Patients receiving ximelagatran had increased early VTE recurrence compared to those receiving conventional anticoagulant therapy[117]. Was the initial dose of ximelagatran too low? Or does acute treatment of DVT with a DTI, without initial use of LMWH, lead to decreased thrombus stability, leading to more PE? If so, what is the mechanism behind this?

Little is known about the mechanism of thrombus stability, the factors involved, and the effect of anticoagulants on embolization and PE burden. Factor XIII (FXIII), a transglutaminase activated to FXIIIa by thrombin, stabilizes fibrin by crosslinking fibrin monomers and crosslinking alpha$_2$-antiplasmin (α_2-AP) to fibrinogen and fibrin[31,194,195]. The crosslinking of α_2-AP, the primary inhibitor of plasmin, the fibrinolytic effector, may be more crucial for stability[22].

Our goal is to uncover factors that alter thrombus stability, and compare the effects of initial treatment with dabigatran and dalteparin on thrombus stability and PE. We developed a novel murine model of VTE to evaluate DVT stability and its relationship with PE burden. The model follows the progression of DVT to PE by imaging the thrombus in the femoral vein, and quantifying emboli from the DVT in the pulmonary arteries. Thrombus embolization correlates with PE burden, and both are increased after acute initial treatment with dabigatran. FXIII$^{-/-}$ mice were indistinguishable from wild-type (WT) dabigatran-treated mice, implying that DTI-dependant decrease in thrombus stability is mediated by FXIII.

3.5 MATERIALS AND METHODS

3.5.1 Animal handling

WT C57BL/6 female mice, purchased from Charles River Laboratories (Sherbrooke, Quebec, Canada), and FXIII$^{-/-}$ mice, a gift from CSL-Behring (Rossdorf, Germany)[196], were housed in micro-isolator cages on a constant 12-h light/12-h dark cycle with controlled temperature and humidity, and given access to food and water *ad libitum.* All animal use protocols were done according to Canadian Council of Animal Care Guidelines, and approved by Animal Research Ethics Board at McMaster University.

3.5.2 Surgical procedure

Mice weighing about 20 grams, were anaesthetized with an intraperitoneal mixture of ketamine (0.125 mg/g), xylazine (0.0125 mg/g), and atropine sulfate (0.0025 mg/g). The trachea and jugular vein were exposed and cannulated using a PE100 and PE10 polyethylene tubing respectively (VWR, Ontario, Canada). Sodium pentobarbital (0.05 mg/g, CevaSante Animale, New Jersey, USA) was given periodically to maintain sedation.

To label platelets *in vivo,* Alexa Fluor (AF)-488-tagged F_{ab} fragments of a rat-anti-mouse CD41 antibody (Emfret, Germany) were injected through the jugular vein at a concentration of 1 μg/g[197]. In some experiments, mouse anti-fibrin II β chain (Bβ 15-42) T2G1 antibody conjugated to AF-647 (Accurate, NY, USA) was injected. The femoral vein was exposed and isolated using a 5-0 silk surgical suture (Surgical Specialties Corporation, Pennsylvania, USA), bathed with saline, and dried. Freshly prepared 4%

ferric chloride ($FeCl_3$) solution (Sigma-Aldrich, Missouri, USA) was used to soak a strip of 1 mm x 2 mm Whatman filter paper (VWR), placed lengthwise on the medial side of the femoral vein for 5 minutes and removed. The vein was washed twice with normal saline.

3.5.3 Intravital videomicroscopy: imaging timeline

All *in vivo* thrombus images were visualized using IVM on an Olympus BX series microscope controlled by Slidebook 5 (Intelligent Imaging Innovations, Colorado, USA). Embolic events (EEs) were recorded for one minute (20X objective; 4x4 binning; 5000 frames; 10 ms exposure). Thrombus sizes were imaged (10X objective; 1x1 binning; 100 ms exposure) by creating a montage, a composite of multiple still images, encompassing the thrombus, grouped together. The gain setting on the Image Intensifier (Video Scope International, Ltd., Virginia, USA) was the same for all images.

Five and 10 minutes after removal of the $FeCl_3$-soaked filter paper, EEs and thrombus size were recorded before any treatment administration. Twelve minutes following injury, treatment with intravenous saline (5 μl/g), dalteparin (0.2 IU/g, Pfizer, Quebec, Canada), or dabigatran (0.033 mg/kg, dissolved in 1% DMSO and 1% HCl, Howsine, China)[198] was administered. Following treatment administration, EEs were recorded at 3 minutes and every 10 minutes until 123 minutes, and thrombus sizes were imaged at 8 minutes and every 10 minutes until 128 minutes.

3.5.4 Post surgery blood and tissue collection

After imaging, the carotid artery was cannulated to obtain blood (in 10% sodium citrate, BD, New Jersey, USA) and mice were euthanized by cervical dislocation. The lungs, and femoral vein segment containing the thrombus, were removed and stored in 10% formalin (Sigma-Aldrich) for subsequent histological analysis.

3.5.5 Blood and tissue handling

Blood was centrifuged at 6,000 × g for 5 minutes to obtain platelet poor plasma (PPP). PPP was centrifuged again, aliquoted and stored at -80°C. For the activated partial thromboplastin time (aPTT)-like assay, 30 µl of aPTT reagent (Instrumentation Laboratory, Massachusetts, USA) was added to PPP (40 µl) in a 96-well plate and incubated at 37°C for 5 minutes. PPP was recalcified with $CaCl_2$ (20 mM final [Ca^{2+}]) and absorbance was monitored at 350 nm (Spectramax 340PC^{384}, Molecular Devices)[199]. Clotting times were reported as time-to-reach-half-maximal-increase in absorbance. FXa generation was assessed by addition of chromogenic substrate S2765 (Aniara, West Chester, Ohio, 0.2 mM final) to PPP (50 µl). PPP was recalcified with $CaCl_2$ and absorbance was monitored at 405 nm[200]. Concentration of dabigatran and dalteparin in plasma was determined using a standard curve, made by adding known concentrations of anticoagulant to mouse plasma. Prothrombin fragment 1+2 (F1+2) levels were measured by ELISA (Cloud-Clone Corp. Houston, USA) following manufacturer's instructions.

Tissue handling: Lungs and femoral veins were processed as described: dehydrated for 30 minutes in 70%, 85%, and 100% ethanol, and 45 minutes in xylene (each step repeated 3 times) then stored overnight in paraffin. Samples were embedded in

paraffin blocks and sectioned entirely into 4 μm slices using a Leica RM 2125RT Microtome (Leica Microsystems Inc., Ontario, Canada). All sections were collected, placed on slides, left overnight to dry, and deparaffinized and rehydrated before being stained using Carstairs's staining kit (Electron Microscopy Sciences, Pennsylvania, USA). Sections were dehydrated, cleared in xylene, mounted with a xylene-based mounting medium, and allowed to dry before visualization.

In some experiments, lungs were snap frozen using liquid nitrogen, stored at -80°C for 24 hours, and later homogenized using liquid nitrogen and centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was collected, mixed with 1% Triton X-100 on a shaker for 1 hour, and fluorescence was measured.

3.5.6 Data analysis

Embolization Quantification: Embolization was observed in a limited region downstream from the proximal tip of the thrombus[201,202]. The region was imaged for 5000 frames, approximately one minute (Vid. S1). An EE was defined as visible fluorescence breaking off from the thrombus, moving downstream, and passing through the region of interest. The total amount of emboli were counted manually and expressed as a total number of emboli per minute. Large EEs were identified among counted EEs; an EE was defined as large if its fluorescence intensity was greater than 4 standard deviations above the average fluorescence intensity of EE for that time point. Large EEs were also expressed as events per minute.

Thrombus Size Quantification: Thrombus size at each time point was quantified by fluorescence intensity. The maximum fluorescence intensity from non-vessel regions

of the montage was used to calculate background. Changes in thrombus size (Thrombus $Size_{time(n)}$-Thrombus $Size_{before\ treatment}$) were plotted against time.

Percentage of thrombus that embolizes or "percentage of DVT turnover": To compare the sum of all observed emboli to the thrombus size, calculations to correct for the different imaging parameters were made. The sum of embolization over two hours was expressed as a percent of the baseline thrombus size and represents a percentage of DVT turnover.

Quantification of pulmonary emboli: All stained slides were examined for presence of emboli in the pulmonary arteries. The number of emboli in each lung section was counted and expressed as number of emboli per lung slice.

3.5.7 Statistical analysis

All values are expressed as mean ± standard error of mean (SEM). One-way ANOVA was used to analyze differences between treatments for F1+2 and the number of emboli per lung slices. Two-way ANOVA was used to analyze differences in thrombus size, total and large EEs, percent of DVT turnover between WT and $FXIII^{-/-}$ mice and treatments. A Student's t-test was used to analyze differences in relative fluorescence units (RFUs) between treatments. Pearson's r coefficient was used to analyze the association between thrombus size and large EEs with PE, and a regression analysis was used to compare the lines-of-best-fit between treatments. Statistical analyses were performed using GraphPad Prism version 6.0. Differences were considered significant when $p < 0.05$.

3.6 RESULTS

3.6.1 Evaluation of histological content from WT-treated mice

Following thrombus formation, the overall thrombus shape and size within the femoral vein of WT-treated mice was not noticeably different within and between treatment groups (Fig. 1A). EEs were observed as irregularly shaped aggregates of fluorescence detaching from the thrombus and releasing into the bloodstream (Fig. 1B). As seen from the video, emboli are of different shapes and sizes, and detach irregularly (Vid. S1: https://onlinelibrary.wiley.com/doi/full/10.1111/jth.13182).

Thrombi in the femoral veins of WT-treated mice subjected to $FeCl_3$ injury stained positive for fibrin and platelets; mice not subjected to injury did not have thrombi (Fig. 1C). Thrombi did not appear different between treatments. Pulmonary arteries of mice without DVT were free of any obstructions, while mice with DVT showed multiple emboli (Fig. 1C).

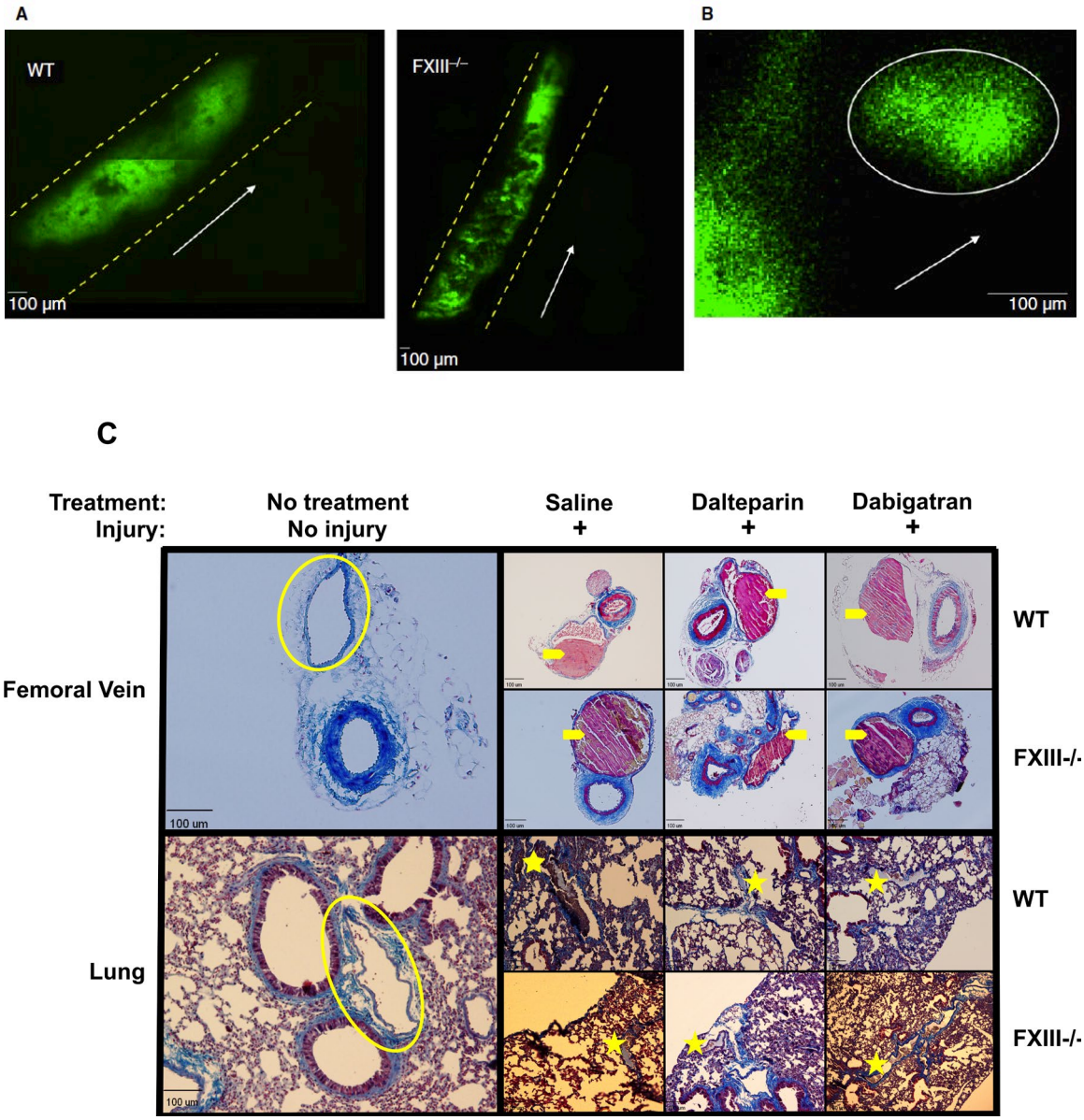

Figure 1: Images of femoral vein thrombi and pulmonary emboli.

Intravital microscopy images from a saline-treated thrombus showing (A) a representative montage of the whole thrombus from WT and FXIII$^{-/-}$ mice and (B) a representative counted embolic event. The field is proximal to the thrombus. The yellow dashed lines trace the outer walls of the femoral vein and the white arrows points in the direction of blood flow. The circle shows an embolus that has dislodged from the

thrombus and freely travelling in the same direction as blood flow (arrow). (C) Carstair's stained images of sectioned femoral vein and lung from WT and FXIII$^{-/-}$ mice treated with saline, dalteparin, or dabigatran. The circles outline femoral vein and pulmonary artery from mice without a DVT. The arrow points to fibrin-rich thrombus in the femoral vein of saline-, dabigatran-, and dalteparin-treated mice. The stars mark the presence of an embolus in a pulmonary artery. A blinded comparison test was performed using 10 femoral vein images from WT and FXIII$^{-/-}$ saline-treated clots, and had two independent blinded observers classify the images as either "fractured" or "non-fractured" clot appearance, to see whether they would identify WT clots as non-fractured clots, and FXIII$^{-/-}$ clots as fractured. The observers correctly assigned the clots (Fisher's exact test, $p<0.0001$).

3.6.2 Thrombus sizes do not change over two hours regardless of treatment in WT-treated mice

Thrombus size was measured over time to determine whether it was altered by treatment. The thrombus before and after treatment did not change significantly within ($p=0.44$) or among treatment groups ($p=0.08$, Fig. 2).

Changes in thrombus size were plotted against time for each treatment in the WT mice, to evaluate fluctuations in size. Fluctuation in thrombus size was used as a measure of thrombus stability; thrombi appeared least stable soon after injury, however, there was no difference among treatments ($p=0.29$) or over time ($p=0.22$, Fig. S1). Thus, independent of treatment, even though embolization was observed, the thrombi did not significantly change in size over two hours.

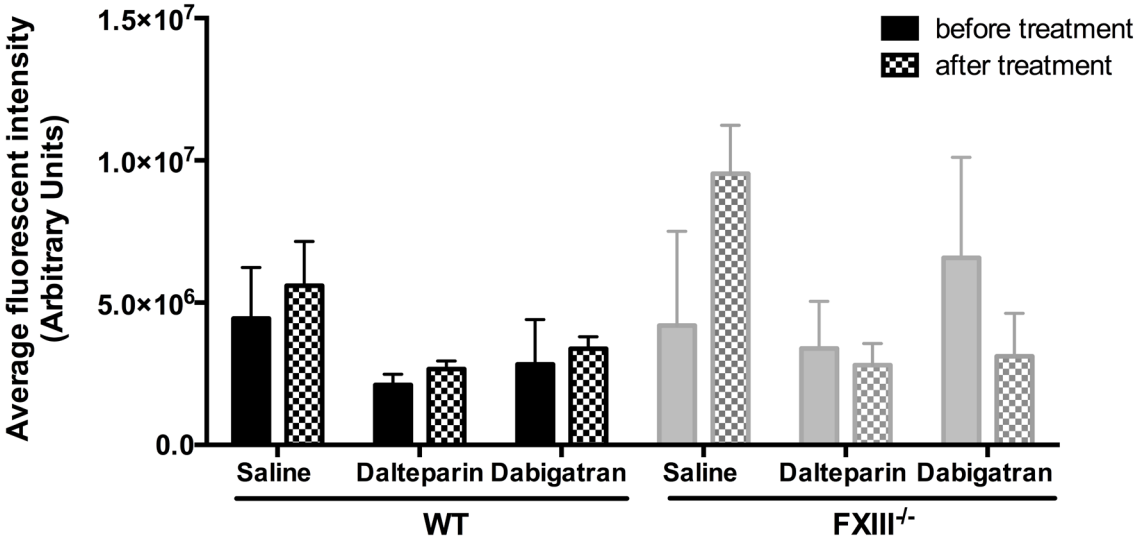

Figure 2: Thrombus size before and after treatment with saline, dalteparin, or dabigatran in WT and FXIII$^{-/-}$ mice.

The after treatment value is the average thrombus size throughout the entire 2-hour procedure. All values are expressed as mean ± SEM, n=10 and n=3 for each treatment in WT mice and FXIII$^{-/-}$ mice respectively, and analyzed using a 2-way ANOVA. For detailed depiction of thrombus size every 10 minutes see Supplemental Figure S1 (section 3.9).

3.6.3 Embolic events increase with dabigatran treatment in WT-treated mice

We quantified EEs, whereby increased embolization would be indicative of decreased thrombus stability. Compared with WT saline-treated mice, total and large EEs in WT dalteparin-treated mice did not differ (Fig. 3A, B). However, total and large EEs in WT mice significantly increased after dabigatran treatment compared with saline ($p<0.0001$ and $p<0.01$ respectively) or dalteparin treatment ($p<0.0001$ and $p<0.05$ respectively).

To assess whether embolization changed over time, the number of EEs within the 1st and 2nd hour after treatment were quantified (Fig. S2A, B in section 3.9). In WT saline-treated mice, total and large EEs decreased over time. When anticoagulants were administered in WT mice, total and large EEs increased over time, with the greatest increase observed in dabigatran-treated mice ($p<0.0001$).

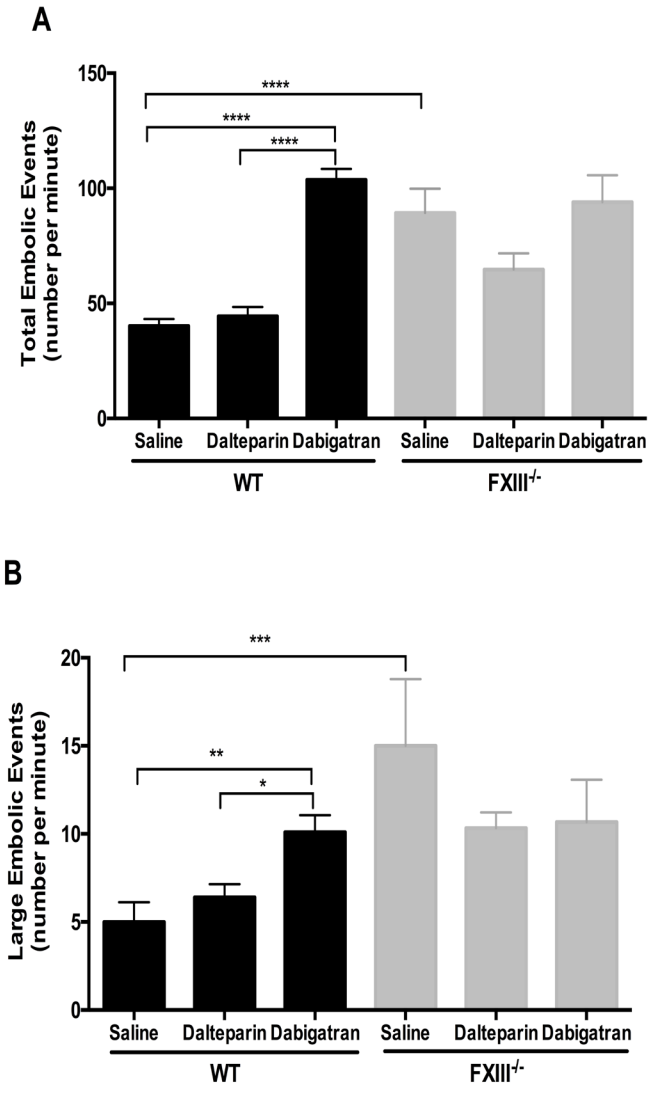

Figure 3: Embolic events after saline, dalteparin, or dabigatran-treatment in WT and FXIII$^{-/-}$ mice.

(A) Total and (B) large embolic events per minute over two hours are shown. All values expressed as mean ± SEM, n=10 and n=3 for each treatment in WT mice and FXIII$^{-/-}$ mice respectively. ****p-value<0.0001. ***p-value<0.001. **p-value<0.01. *p-value<0.05, using a 2-way ANOVA. For a comparison of these results between the first and second hour see Supplemental Figure S2 in section 3.9.

3.6.4 The percentage of thrombus that embolizes is greater in dabigatran-treated mice

Embolization increased over time in anticoagulated mice, yet thrombus size remained fairly similar to what was originally induced. Thus, variations in EEs are not due to variations in thrombus size. Therefore, the percentage of thrombus that embolizes, a measure of thrombus turnover, was compared. In WT dabigatran-treated mice, the percentage of DVT turnover in two hours was approximately eight-fold greater than WT saline-treated mice ($p<0.0001$), and three-fold greater than WT dalteparin-treated mice ($p<0.0001$, Fig. 4A).

No change was observed in the percentage of thrombus that embolized between the 1st to the 2nd hour of WT saline- or dalteparin-treatment mice ($p=0.97$ and $p=0.92$ respectively), however, this doubled from the 1st to the 2nd hour after dabigatran treatment ($p<0.0001$, Fig. S3 in section 3.9).

WT dabigatran-treated mice exhibited highest DVT turnover suggesting that more thrombin might have been generated. F1+2 was highest in WT dabigatran-treated mice compared to WT saline- or dalteparin-treated mice ($p<0.0001$ and $p<0.05$ respectively, Fig. 4B).

To determine levels of anticoagulants, aPTT and FXa activity were analyzed. The average concentration of dabigatran or dalteparin in dabigatran- or dalteparin-treated mice was 303 ± 32 ng/mL (n=9) or 1.5 ± 0.1 IU (anti-FXa)/mL of plasma (n=8), respectively. Thus, significant levels of anticoagulants remained in the bloodstream two hours after administration.

3.6.5 More emboli are detected in the pulmonary arteries of dabigatran-treated mice

The number of emboli per lung slice in WT-treated mice was highest after dabigatran treatment, but not significantly ($p=0.06$ for one-way ANOVA, but was significant using Dunnett's post-test comparison, $p=0.03$, Fig. 4C). To test if emboli were fibrin-rich, lungs were homogenized to compare anti-fibrin antibody fluorescence from lung extracts. WT dabigatran-treated mice had more fluorescent anti-fibrin antibody in lung extracts, than WT saline-treated mice, confirming the presence of fibrin-rich emboli and more PE ($p<0.05$, Fig. S4 in section 3.9).

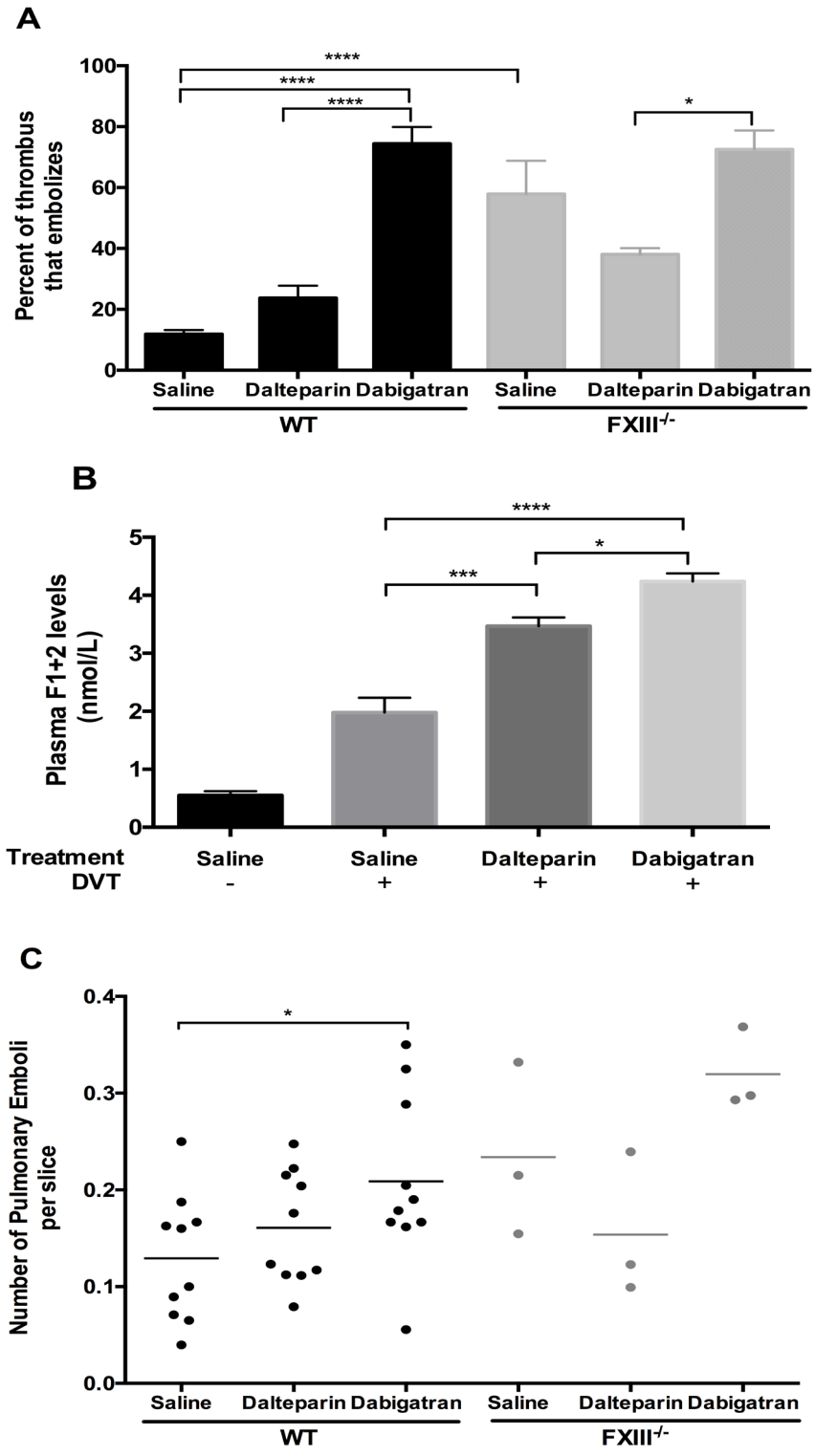

Figure 4: DVT turnover, F1+2, and PE risk after saline, dalteparin, or dabigatran treatment in WT and FXIII$^{-/-}$ mice.

(A) The percent of thrombus that embolized in two hours after saline, dalteparin, or dabigatran treatment in WT and $FXIII^{-/-}$ mice. For a comparison of the percent of thrombus that embolized between the first and second hour see Supplemental Figure S3. (B) Prothrombin fragment 1+2 plasma levels from WT mice treated with saline, dalteparin, or dabigatran and compared to WT mice without DVT. (C) The number of pulmonary emboli counted per lung slice for the treatment groups in both strains of mice. The average number of lung slices sectioned from each mouse was over 100. All values are expressed as mean ± SEM, n=10 and n=3 for each treatment in WT mice and $FXIII^{-/-}$ mice respectively. ****p-value<0.0001. ***p-value<0.001. **p-value<0.01. *p-value<0.05, using a 2-way ANOVA for (A) and a one-way ANOVA for (B) and (C).

3.6.6 Embolic events, but not thrombus size, correlate with PE burden in WT-treated mice

Quantified embolization, thrombus size, and percentage of DVT turnover are indicators of thrombus stability. To assess whether these indicators influence PE burden, we correlated thrombus size and EEs with the number of emboli per lung slice. Poor correlation exists between thrombus size and PE for all treatments (all $R^2 < 0.39$, Fig. 5A). Thus, thrombus size does not determine PE burden.

In contrast, a strong correlation exists between total and large EEs and PE for all treatments (all $R^2 > 0.90$ for total EEs, Fig. S5, and all $R^2 > 0.83$ for large EEs, Fig. 5B; two mice in the saline group did not exhibit large EEs and were excluded from analysis). Thus, in all treatment groups, PE burden increased linearly as a function of total and large EEs.

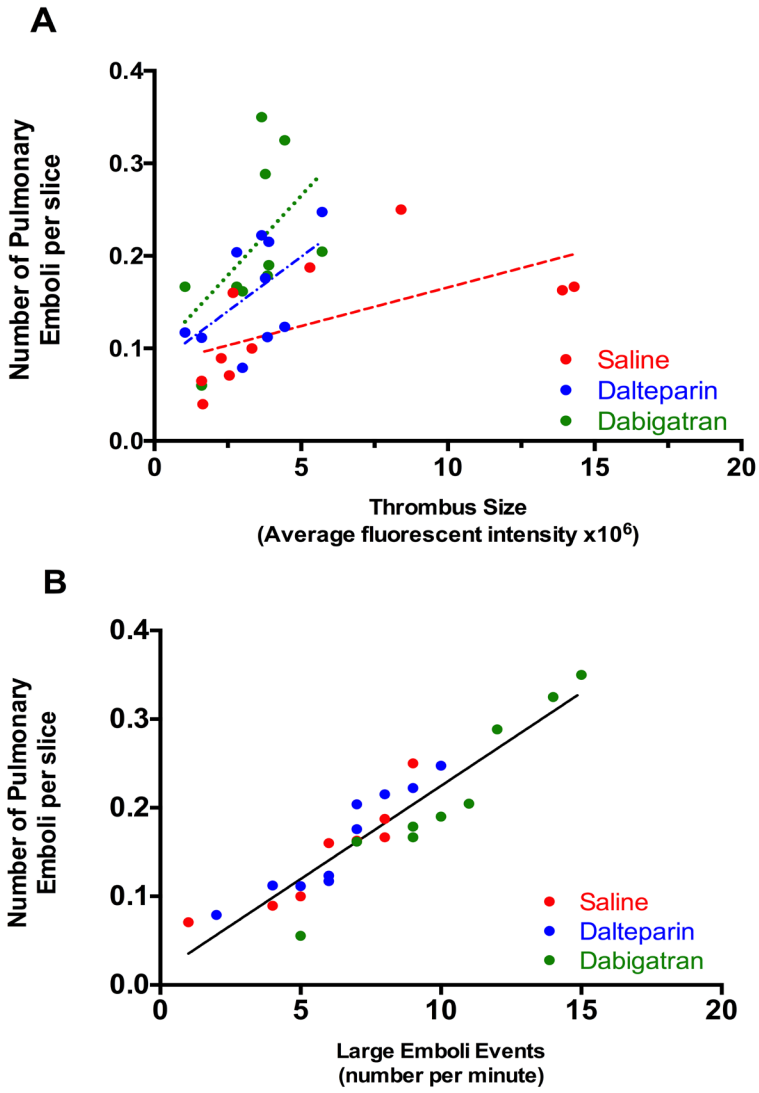

Figure 5: Correlation between the PE risk with thrombus size and large embolic events.

Correlation between the number of pulmonary emboli per lung slice and (A) thrombus size, and (B) large embolic events, for WT saline-, dalteparin-, and dabigatran-treated mice. All values are expressed as mean ± SEM, and n=10 for each treatment group. Regression values with p-value<0.05 were considered significant and represented with a solid regression line.

3.6.7 In WT-treated mice, large, but not total EEs, predict PE burden independently of treatment

To determine whether EEs could be used as predictors of PE burden independent of treatment, linear regression models correlating PE burden with total or large EEs were compared. The slopes were not significantly different between treatments ($p=0.13$), but the y-intercepts were ($p<0.0001$) for total EEs and PE burden (Fig. S5 in section 3.9). Thus, although total EEs strongly correlated with PE burden in all treatment groups, this relationship differed according to treatment type.

In contrast, for large EEs and PE, slopes and y-intercepts from the lines-of-best-fit were not significantly different between treatments. Therefore a pooled line-of-best-fit predicts the risk of PE burden independent of treatment (Fig. 5B). Thus, the number of large EEs quantified at a window near the proximal tip of the thrombus in the deep veins, appears to predict PE burden independent of treatment.

3.6.8 FXIII impacts thrombus stability

Since thrombin-mediates FXIII activation, and FXIIIa-mediated crosslinking of fibrin may be responsible for venous thrombus stability, we hypothesized that anticoagulants, by inhibiting thrombin, would require FXIII to decrease thrombus stability[194]. $FXIII^{-/-}$ mice were compared to WT mice to determine whether FXIII impacts thrombus stability. Thrombi in $FXIII^{-/-}$ mice appeared less compact with greater "voids" than WT mice (Fig. 1A). Fibrin in thrombi in the femoral veins of $FXIII^{-/-}$ mice appeared to have more fractures than WT mice, but emboli visually appeared to be similar to WT mice (Fig. 1C).

Thrombus size before and after treatment did not change significantly within or among treatments ($p=0.82$, $p=0.30$, Fig. 2). In contrast to WT mice, there was a significant change in thrombus size over time in FXIII$^{-/-}$ mice ($p=0.04$, Fig. S1), suggesting that FXIII$^{-/-}$ mice exhibit decreased thrombus stability compared to WT mice, and that FXIII impacts stability.

Comparing total EEs, large EEs, and percentage of DVT turnover between WT and FXIII$^{-/-}$ mice, FXIII$^{-/-}$ saline-treated mice mimicked the increased embolization and DVT turnover of WT dabigatran-treated mice (Fig. 3, 4A, S2 and S3). There was a significant difference in total and large EEs between WT and FXIII$^{-/-}$ saline-treated mice ($p<0.0001$ and $p<0.001$ respectively; Fig. 3A, B), suggesting FXIII prevents embolization and promotes thrombus stability. Percentage of DVT turnover over time in FXIII$^{-/-}$ mice were similar to WT, except in the saline treatment group, which doubled from the 1st to the 2nd hour ($p<0.005$, Fig. S3). Dabigatran treatment of FXIII$^{-/-}$ mice did not alter embolization patterns suggesting that it requires FXIII to decrease thrombus stability.

The number of emboli per lung slice in the FXIII$^{-/-}$ mice were not significantly different from WT mice, but were slightly higher in FXIII$^{-/-}$ saline-treated mice compared to WT saline-treated mice (Fig. 4C). Like WT mice, in FXIII$^{-/-}$ mice there was no correlation between thrombus size and PE; and total and large EEs did correlate with PE, however, this was dependent on treatment (data not shown).

3.7 DISCUSSION

We have established a mouse model of acute VTE that is unique in that it can assess thrombus size, quantify emboli that break off from the thrombus, and quantify the

resulting pulmonary emboli. This evaluation of thrombus stability allows us to show that states with altered thrombus stability can alter PE burden.

Embolization in WT mice occurred most frequently after dabigatran treatment. Over time, embolization decreased in saline-treated mice, but increased in dalteparin- and dabigatran-treated mice, suggesting that, at least in this acute phase, dabigatran, and to a lesser extent, dalteparin decrease thrombus stability. Without FXIII, embolization increased significantly compared to WT saline-treated mice, but this was not the case for dalteparin- or dabigatran-treated mice. Altered thrombus stability in $FXIII^{-/-}$ mice suggests that FXIII is responsible for thrombus stability. Thrombus size remained similar over two hours despite treatment with anticoagulants; thus thrombus lost by embolization was continually being replaced by thrombus growth. Dabigatran-treated mice had greater thrombus turnover and F1+2 compared to other treatments suggesting that more thrombin is activated in thrombus turnover. Thrombus size fluctuated more in $FXIII^{-/-}$ mice compared to WT, suggesting that FXIII stabilizes thrombi. The percentage of thrombus that embolized, as well as the number of emboli in the pulmonary arteries, was highest in dabigatran-treated mice. Thus, acute initial dabigatran treatment was associated with less femoral vein thrombus stability and more pulmonary emboli; this is also seen in $FXIII^{-/-}$ saline-treated mice. Lastly, large EEs correlate with PE burden independent of treatment. This suggests that quantifying larger EEs breaking off a DVT can be used to estimate PE burden.

We have shown that FXIII deficiency decreased thrombus stability, but specifically how FXIII impacts clot stability is not known. FXIII can stabilize clots by crosslinking fibrin γ-γ, γ-α, and α-α chains, and by crosslinking α_2-AP to fibrin, and to

fibrinogen[31,194,195]. However, the relative contribution of these crosslinking actions is debated[22,48,203].

DTIs such as dabigatran, are reported to be profibrinolytic by virtue of their ability to inhibit thrombin-induced resistance to clot lysis. Dabigatran enhances clot susceptibility to lysis by (at least) three possible mechanisms: (i) reducing activated thrombin activatable fibrinolysis inhibitor (TAFIa) generation; (ii) reducing FXIIIa generation; and (iii) altering the viscoelastic properties of clots (less thrombin leads to fewer and thicker fibrin strands that are prone to lysis)[155,156,185,204–207]. All effects are related to the ability of the drug to reduce thrombin generation and activity. These properties of DTIs, more specifically dabigatran, explains three things: 1) why we don't see this increased embolization and PE burden in mice treated with antithrombin-dependent FXa inhibitors, more specifically dalteparin, 2) why no difference was seen between FXIII$^{-/-}$ and WT dabigatran- or dalteparin-treated mice, and 3) why total EEs did not correlate with PE independent of treatment.

Firstly, antithrombin-dependent FXa inhibitors enhance FXa- more than thrombin-inhibition, and only inhibit free thrombin[208]. DTIs inhibit free and clot-bound thrombin. Therefore, the amount of residual thrombin left to generate FXIIIa, or any other thrombin-dependent factors required for thrombus stability, will be much higher with FXa inhibitors than with DTIs. This may explain why dalteparin-treated clots are more stable than dabigatran-treated clots. It has also been shown that dabigatran-treated clots are more porous, contain fewer and thicker fibrin strands than dalteparin-treated clots[209], and are lysed more quickly than those with dense, thin fibers[185]. It is important to note that thrombus stability can be dose-dependently altered by anticoagulants. Because

the anticoagulants are compared to no treatment, there is likely a lower level of anticoagulation that would not destabilize thrombi.

Secondly, when FXIII is deficient, there was no difference when dabigatran or dalteparin were administered, but saline-treated FXIII$^{-/-}$ mice differed from WT. Dabigatran decreases thrombin generation, thus there is less FXIII activation, producing an unstable clot. When the unstable fibrin clot begins to embolize, there is more thrombin generated to heal the injury (impaired wound healing)[210]. However, with dabigatran, there is not enough thrombin to generate FXIIIa, and therefore the clot becomes unstable leading to more embolization and ongoing thrombin production; this is exhibited by increased levels of F1+2 in dabigatran-treated mice[211,212]. This feedback loop may be responsible for increased PE burden seen in dabigatran-treated mice. Clinically, patients with PE have lower FXIIIA antigen levels than patients without PE[213].

Thirdly, the susceptibility of dabigatran-treated clots to lysis might explain why only large EEs predict PE burden independent of treatment. If dabigatran-treated clots and their emboli are more prone to lysis, perhaps smaller emboli in the pulmonary arteries have lysed, making them undetectable. Thus, total EEs cannot predict PE burden independent of treatment. However, larger EEs may be harder to lyse regardless of treatment, thus the size of an embolus may be proportional to its occlusive burden in the pulmonary circulation, making large EEs a better indicator of PE burden independent of treatment. The total EEs correlate positively with larger EEs independent of treatment (Fig. S6), and since dalteparin had fewer total and large EEs than dabigatran, this suggests that acute treatment of DVT with dalteparin is more effective because it results in lower PE burden. This may also explain why patients in the THRIVE study treated,

with ximelagatran, without initial treatment with LMWH, had higher early rates of VTE recurrence than those treated with conventional anticoagulants[117], and why all-oral therapy with FXa inhibitors do not have higher rates of VTE recurrence[189–192]. These findings also caution against the unapproved use of dabigatran for acute initial treatment of VTE.

There are limitations of this VTE stability model. The clinical significance of the altered PE burden cannot be determined. The finite time that we can evaluate thrombus stability limits our conclusions to acute treatment only. The mechanisms initiating thrombus formation in the $FeCl_3$ model and whether this models thrombus formation that occurs clinically remains incompletely understood[164,214–217]. Despite these limitations, the $FeCl_3$ model was chosen because the vein is not occluded and blood flow is required for embolization. Lastly, strain-dependent differences between WT and $FXIII^{-/-}$ mice may contribute to observations.

Development of a reproducible and standardized combined DVT and PE model is crucial to broaden understanding of PE and its relationship to DVT, and to allow better evaluation of antithrombotic strategies. Ultimately, anticoagulants should stabilize thrombi while preventing PE-associated mortality. This novel mouse model evaluates thrombus stability, and how states with altered thrombus stability influence PE burden, showing that FXIII is critical. We have shown that acute treatment of DVT with dabigatran, but not LMWH, decreases thrombus stability, and ultimately increases PE burden, which is consistent with clinical data[117].

3.8 ACKNOWLEDGMENTS

We thank Dr. Patricia Liaw for gifting us the chromogenic substrate, and Zahar Lysov for his help and advice with the FXa activation assay.

3.9 SUPPLEMENTARY INFORMATION

Video S1. IVM recording from the proximal tip of the thrombus used to count embolic events. https://onlinelibrary.wiley.com/doi/full/10.1111/jth.13182

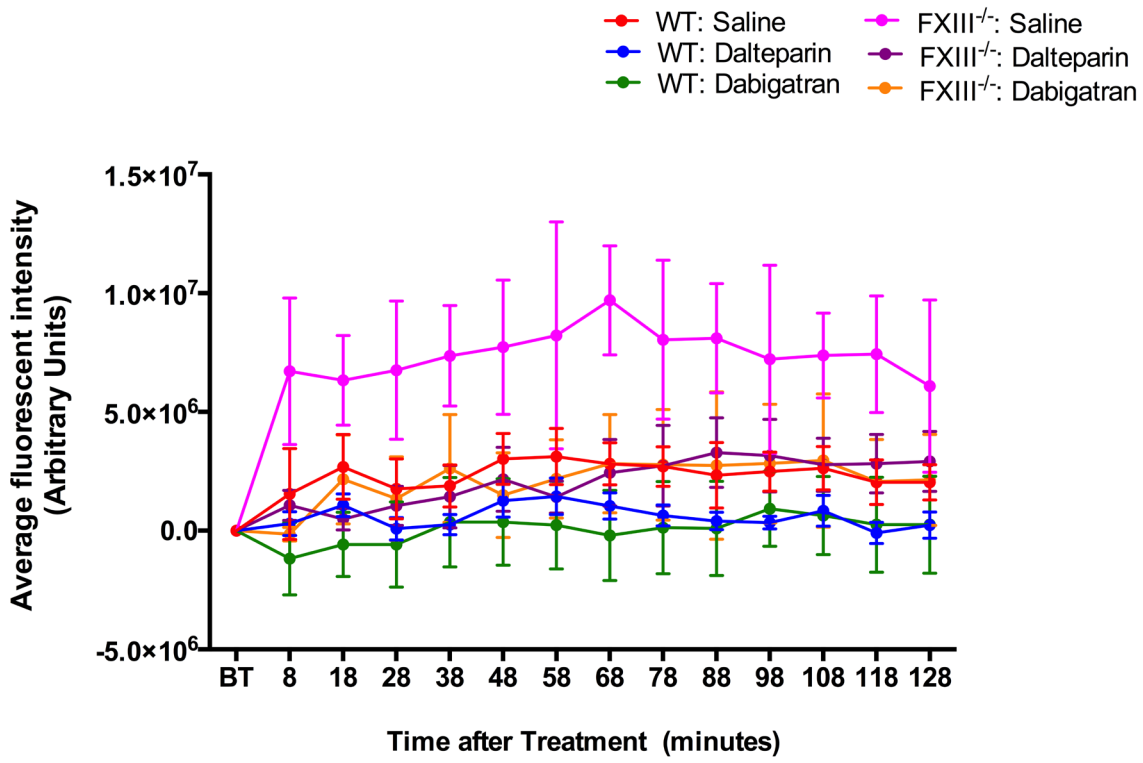

Figure S1: Change in thrombus size over two hours for WT and FXIII$^{-/-}$ mice treated with either saline, dalteparin, or dabigatran.

The change in thrombus size was calculated by subtracting the thrombus size at time *n* from the thrombus size before any treatment was administered (*i.e.* thrombus size at

baseline). All values are expressed as mean ± SEM, n=10 and n=3 for each treatment in WT mice and FXIII$^{-/-}$ mice respectively, using a 2-way ANOVA.

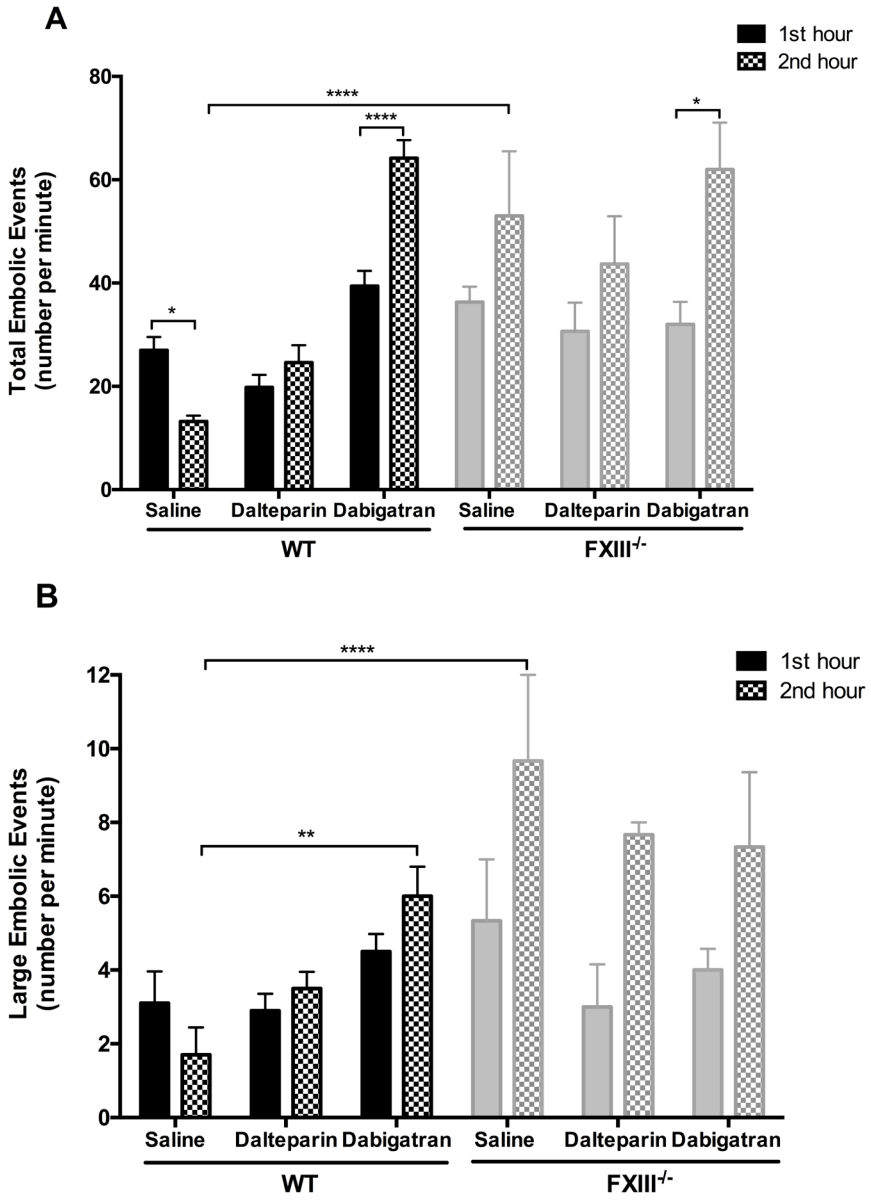

Figure S2: Changes in embolic events after treatment with saline, dalteparin, or dabigatran in WT and FXIII$^{-/-}$ mice.

Changes in (A) Total and (B) large embolic events in the 1st hour and 2nd hour after treatment administration. All values are expressed as mean ± SEM, n=10 and n=3 for each treatment in WT mice and FXIII$^{-/-}$ mice respectively. ****p-value<0.0001. ***p-value<0.001. **p-value<0.01. *p-value<0.05, using a 2-way ANOVA.

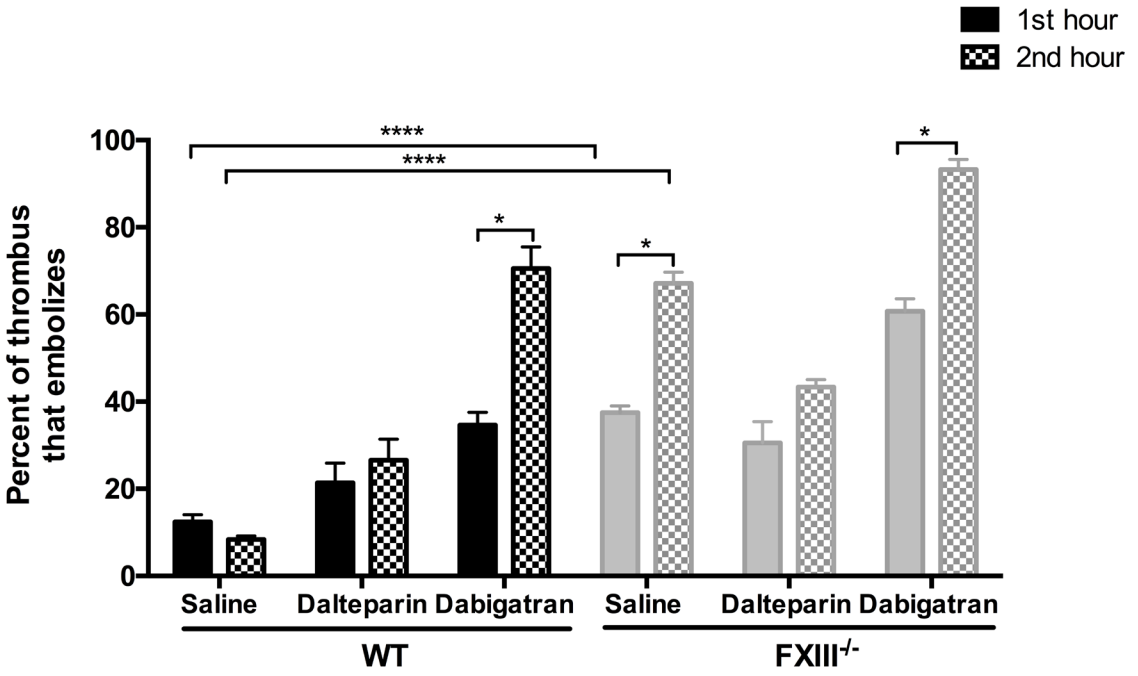

Figure S3: Percent of thrombus that embolizes in the first and second hour after saline, dalteparin, or dabigatran treatment in WT and FXIII$^{-/-}$ mice.

All values are expressed as mean ± SEM; n=10 and n=3 for each treatment in WT mice and FXIII$^{-/-}$ mice respectively. ****p-value<0.0001. *p-value<0.05, using a 2-way ANOVA.

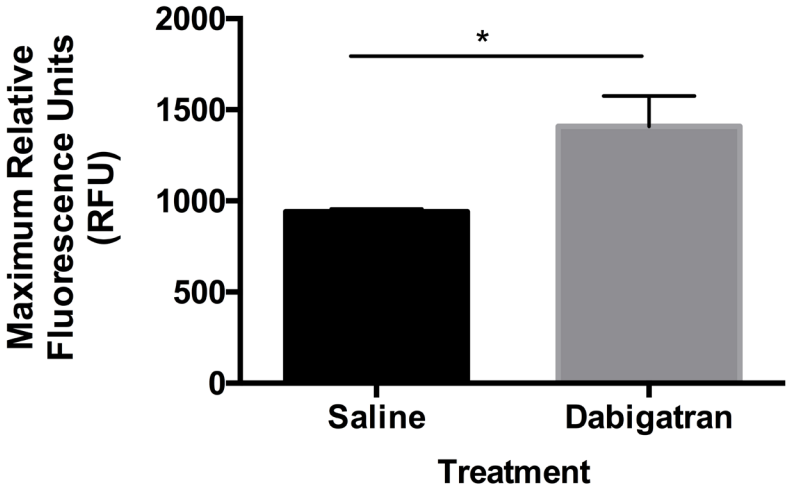

Figure S4: Maximum fluorescence units measured from homogenized lungs from WT saline- and dabigatran-treated mice after DVT and fluorescently tagged anti-fibrin antibody injection.

All values are expressed as mean ± SEM; n=3 for each treatment. *p-value<0.05, using an unpaired student's t-test.

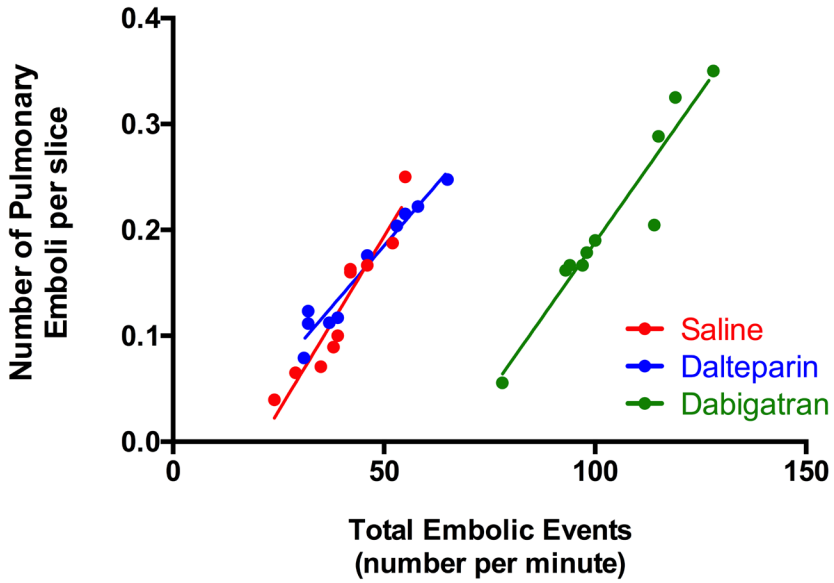

Figure S5: Association between the total embolic events with the number of pulmonary emboli per lung slice for WT saline-, dalteparin-, and dabigatran-treated mice.

All values expressed as mean ± SEM, n=10 for each. Regression values with p-value<0.05 were considered significant and represented with solid regression line.

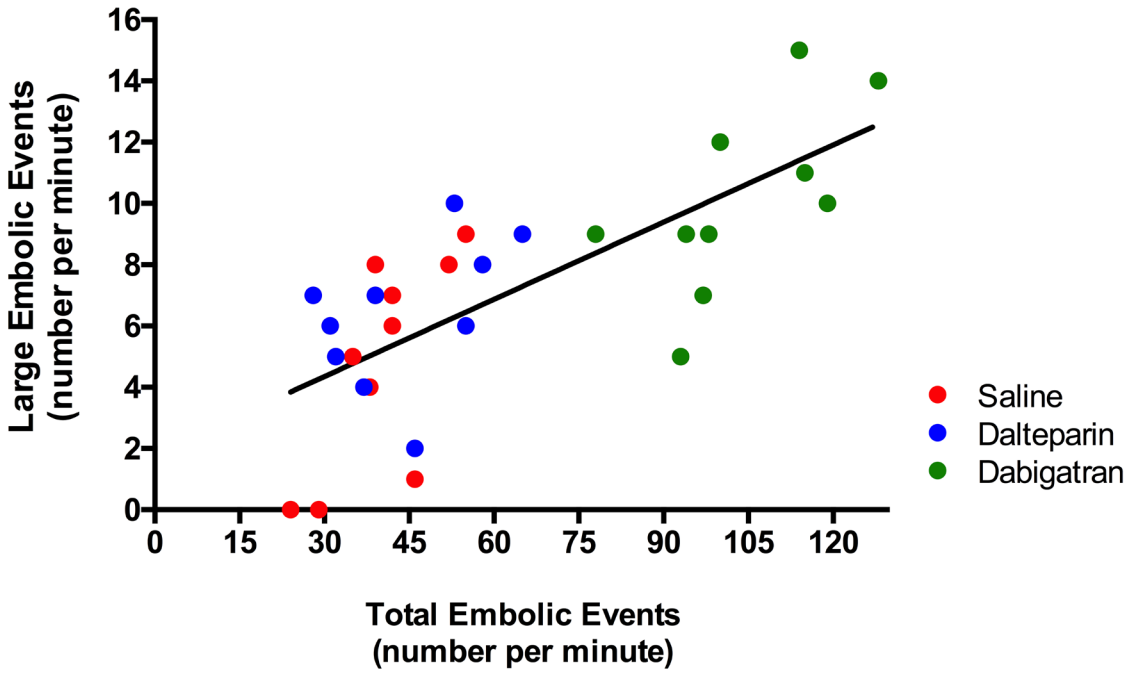

Figure S6: Correlation between the total and large embolic events in WT mice treated with saline, dalteparin, or dabigatran.

All values are expressed as mean ± SEM, n=10 for each. Regression values with p-value<0.05 were considered significant and represented with solid regression line.

CHAPTER FOUR

4.0 FACTOR XIII PREVENTS PULMONARY EMBOLI IN MICE BY STABILIZING DEEP VEIN THROMBOSIS

4.1 FORWARD

This manuscript describes the effect of attenuating fibrinolysis on thrombus stability using the *in vivo* mouse model of venous thromboembolism as well as clot lysis. This manuscript shows that i) with FXIII supplementation, thrombus stability is increased and pulmonary embolism risk is decreased without altering the size of the initial injury, ii) this is not seen with epsilon-aminocaproic acid and iii) *in vitro* clot lysis assays do not predict how agents will stabilize DVT *in vivo*.

Factor XIII Prevents Pulmonary Emboli in Mice by Stabilizing Deep Vein Thrombi

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Lay title: FXIII stabilizes venous thrombi

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Authorship Contributions: S.A. Shaya performed all experiments, analyzed data and results, and wrote the manuscript. D. Muzafar Gani performed the clot lysis assay. P.Y. Kim designed, and analyzed the clot lysis data, and edited the manuscript. P.L. Gross supervised the research, conceived and designed the study. P.L. Gross and J.I. Weitz contributed to the interpretation of data and critically edited the manuscript. All authors have read and approved the article.

*** This manuscript is published in the *Thrombosis and Hemostasis***. *Thromb Haemost.* 2019 Jun;119(6):992-999. [Epub 2019 Apr 20] Accepted February 22 2019, assigned to the April 2019 Issue.

References: References for this Manuscript have been incorporated into the Bibliography (Chapter 7), at the end of this thesis.

4.2 ABSTRACT

Background: Deep-vein thrombosis (DVT) can lead to pulmonary embolism (PE), but the mechanisms responsible for this progression are unknown. Previously, we showed that inhibition of thrombin-mediated activation of factor (F) XIII promotes venous thrombus stability in a murine model. *Aim:* In this study we investigate the consequence of attenuating fibrinolysis, using FXIII, alpha$_2$-antiplasmin (α_2-AP) or ε-aminocaproic acid (EACA) supplementation, on clot lysis and venous thrombus stability using the same mouse model. *Methods: In vitro* plasma clot lysis assay shows that EACA and α_2-AP but not FXIII, inhibit fibrinolysis. Ferric chloride induced thrombi in the femoral vein of mice. After thrombus formation, mice received saline, EACA, α_2-AP, or FXIII, with or without dalteparin or dabigatran. Thrombus sizes and embolization over two hours were visualized using intravital videomicroscopy. Lungs were sectioned to quantify emboli presence via histology. *Results:* The change in thrombus size over time was significantly greater after EACA treatment, but not FXIII or α_2-AP supplementation, compared to saline. α_2-AP-supplementation did not alter thrombus stability. Thrombi were more stable following EACA treatment and FXIII supplementation as evidenced by less embolic events and PE burden, even when they were anticoagulated with either dalteparin or dabigatran. *Conclusion:* FXIII supplementation stabilized venous thrombi, even in the presence of anticoagulants, and did not alter thrombus size. Supplemental FXIII may be useful to stabilize DVT and be an alternative adjunctive treatment to minimize PE, even when anticoagulants are used.

4.3 KEYWORDS

(i) factor XIII (ii) ε-aminocaproic acid (iii) pulmonary embolism (iv) fibrinolysis (v) alpha$_2$-antiplasmin.

4.4 WHAT IS KNOWN?

- The factors and mechanism involved in thrombus stability still remain largely undefined, therefore we asked if we can stabilize venous thrombi to reduce pulmonary embolism
- We have previously shown that acute upfront treatment with anticoagulants leads to decreased thrombus stability, as evidenced by increased embolization and increased pulmonary embolism risk, and this was factor XIII-dependent

4.5 WHAT DOES THIS PAPER ADD?

- ε-aminocaproic acid (EACA) and alpha$_2$-antiplasmin (α_2-AP), but not factor XIII (FXIII), reduced clot lysis *in vitro*
- In a mouse model, EACA stabilized thrombi and decreased PE risk, however DVT increased in size, while treatment with α_2-AP had no effect on embolization and DVT size compared to saline-treated mice
- Supplemental FXIII stabilized thrombi without increasing DVT size even in the presence of anticoagulants, therefore supplemental FXIII may stabilize deep vein thrombi and prevent PE

4.6 INTRODUCTION

Venous thromboembolism (VTE), comprising of deep-vein thrombosis (DVT) and pulmonary embolism (PE), occurs in at least 1 in 1,000 individuals and is the third most common cause of cardiovascular death[84]. Both the i) clinical manifestation of VTE (DVT or PE), and ii) progression of DVT to PE, which can be fatal, are unpredictable. An ideal treatment for DVT patients would stabilize clots, minimize embolization and reduce PE. Is there a way to minimize embolization from a DVT? The factors that determine venous thrombus stability and its link to PE risk remain undefined.

To address this, we have previously described a novel mouse model of VTE that can evaluate thrombus stability by quantifying the thrombus size, the number of embolic events breaking off the thrombus and the resulting PE burden[218]. We demonstrated that acute thrombin inhibition increased the number of embolic events and this was factor XIII- (FXIII) mediated. FXIII is activated by thrombin to activated FXIII (FXIIIa), a transglutaminase that stabilizes fibrin by crosslinking fibrin monomers and crosslinking alpha$_2$-antiplasmin (α_2-AP), the primary inhibitor of plasmin, to fibrinogen and fibrin[185,195,207,211]. Thus, FXIII activation by thrombi contributes to venous thrombus stability[218].

The purpose of this study was to compare the effects of ε-aminocaproic acid (EACA), FXIII or α_2-AP on thrombus stability. EACA is a synthetic lysine analogue that competes with tissue-type plasminogen activator (t-PA), plasminogen, and plasmin for binding fibrin, thus down-regulating fibrinolysis and increasing clot stability. It is used clinically to treat or prevent excessive blood loss during surgery[219]. Recombinant FXIII supplementation reduces postoperative blood transfusions in the context of

cardiopulmonary bypass and cancer surgery[53–56]. α_2-AP-inhibition assists in the dissolution of preformed pulmonary emboli[35]. Its effect on thrombus stability, however, has never been studied.

In this study, we compared the effects of EACA treatment, and FXIII or α_2-AP supplementation on clot lysis *in vitro*, and on venous thrombus stability using a characterized mouse model[218]. Only FXIII supplementation increased thrombus stability without increasing thrombus size.

4.7 MATERIALS AND METHODS

4.7.1 Animal handling

Wild type (WT) C57BL/6 female mice, purchased from Charles River Laboratories (Sherbrooke, Quebec, Canada), and FXIII deficient ($FXIII^{-/-}$) mice, a gift from CSL-Behring (Rossdorf, Germany)[196] were housed in micro-isolator cages on a constant 12h light/12h dark cycle with controlled temperature and humidity, and given access to food and water *ad libitum.* All animal use protocols were performed according to Canadian Council of Animal Care Guidelines, and were approved by the Animal Research Ethics Board at McMaster University.

4.7.2 Clot Lysis Assay

WT mice were administered saline (5 µL/g), FXIII (1 mg/kg, plasma purified human FXIIII A2B2 tetramer, Enzyme Research Laboratories)[210], or EACA (0.5 mg/g dose was used in the clot lysis experiment because pilot studies showed no clot lysis with 1 mg/g dose, Sigma Aldrich) and one hour later blood was collected as previously described[218].

Plasma from WT mice was mixed with either 0, 60, or 120 µg/mL of mouse α_2-AP (Abcam, Toronto, Ontario; to double or triple the physiological concentration which is approximately 60 µg/mL)[220]. Plasma from FXIII$^{-/-}$ mice was supplemented with 0, 5, 10, or 15 µg/mL of FXIII. The clot lysis assay was performed as previously described[221]. Briefly, a 1:3 dilution of mouse plasma was performed in HEPES buffered saline (HBS) containing 0.01% Tween-80 and incubated for 5 minutes at 25°C. Clotting was initiated with thrombin (10 nM) and lysis was initiated with 20 nM tenecteplase (Roche, Mississauga, Canada), in the presence of 15 mM $CaCl_2$ to a final volume of 100 µL. Absorbance was monitored at 400 nm at 25°C and the clot lysis time was defined as the time required to reach half-maximal decrease in absorbance during lysis.

4.7.3 Venous thrombus stability model

The model has been described previously[218]. Briefly, platelets were labeled with Alexa Fluor (AF)-488-tagged F_{ab} fragments of a rat-anti-mouse CD41 antibody (Emfret, Germany) that was injected through the jugular vein at a concentration of 1 µg/g[197]. In some experiments, mouse anti-fibrin II β chain (Bβ 15-42) T2G1 antibody conjugated to AF-647 (Accurate, NY, USA) was injected. Thrombus formation was initiated using a 4% ferric chloride ($FeCl_3$)-soaked filter paper applied to the medial side of the femoral vein. Twelve minutes after injury, treatment with intravenous EACA (1 mg/g)[222] alone, α_2-AP (4.2 mg/kg, Innovative Research, Burlington, Ontario)[220], alone, or saline (5 µl/g), dalteparin (0.2 IU/g, Pfizer, Quebec, Canada), dabigatran (0.033 mg/kg, dissolved in 1% DMSO and 1% HCl, Howsine, China)[198], then with or without FXIII (1 mg/kg) was administered.

Thrombi were imaged and embolic events recorded for two hours using intravital videomicroscopy. After imaging, blood was collected from the carotid artery and centrifuged to obtain platelet poor plasma (PPP). The lungs and the femoral vein were excised and sectioned; Carstairs staining was used to visualize the PE and DVT.

The change in thrombus size from baseline was quantified by subtracting the baseline thrombus size value from the average of the thrombus sizes over the last hour of observation. Total and large embolic events, the percentage of thrombus that embolized or DVT turnover, and the number of pulmonary emboli per lung slice were quantified as previously described[218].

4.7.4 FXIIIa Activity Assay

Normal human plasma (NHP) was supplemented with dabigatran (250 ng/mL) or dalteparin (1.5 U/mL) with or without FXIII (to achieve a level of 150% of physiological, Enzyme Research Laboratory, South Bend, Indiana). FXIIIa levels were measured using the Technochrom FXIII assay (Technoclone, Austria) following the manufacturer's instructions. Briefly, the assay measures ammonia released in the first step of the transglutaminase reaction with a sensitivity of 0.6%.

4.7.5 Statistical analysis

All values are expressed as mean ± standard error of mean (SEM), n=7 for α_2-AP supplementation and n=10 for the remaining treatments discussed. Student's t-test was used to analyze differences in clot lysis times, FXIIIa activity. The change in thrombus size from baseline was analyzed using a non-parametric ANOVA Kruskal-Wallis test

with Dunn's multiple comparison test. One-way ANOVA with Tukey's or Dunnett's multiple comparison test was used to analyze the differences in total and large embolic events, percentage of thrombus that embolized and the number of pulmonary emboli per lung slice among treatments. A two-way ANOVA with Tukey's multiple comparison test was used to analyze the differences in total and large embolic events, percentage of thrombus that embolized and number of PE per lung slide in dalteparin- or dabigatran-treated mice with and without FXIII supplementation. Analyses were performed using GraphPad Prism version 6.0 and P-values <0.05 were considered statistically significant.

4.8 RESULTS

4.8.1 Clot lysis is inhibited with EACA and α_2-AP but not with FXIII

In vitro clot lysis experiments showed no apparent lysis of clots generated from plasma collected from mice treated with EACA (0.5 mg/g; Figure 1A). Lysis times of clots generated from WT plasma treated with α_2-AP increased in a dose dependent manner (Figure 1B), with no apparent lysis observed at three-times physiological concentration of α_2-AP. However, there was no significant difference in clot lysis times in the plasma obtained from WT mice treated with either saline or supplemental FXIII (adding 50% to achieve 150% of normal; Figure 1C). Therefore, these data suggest that EACA and α_2-AP supplementation would inhibit fibrinolysis while FXIII supplementation would not alter fibrinolysis. When FXIII was added to plasma isolated from the $FXIII^{-/-}$ mice to reconstitute the plasma to 100% of FXIII, clot lysis times increased in a concentration-dependent manner. However, there was no further increase in lysis times with FXIII addition beyond 100% (Figure 1D).

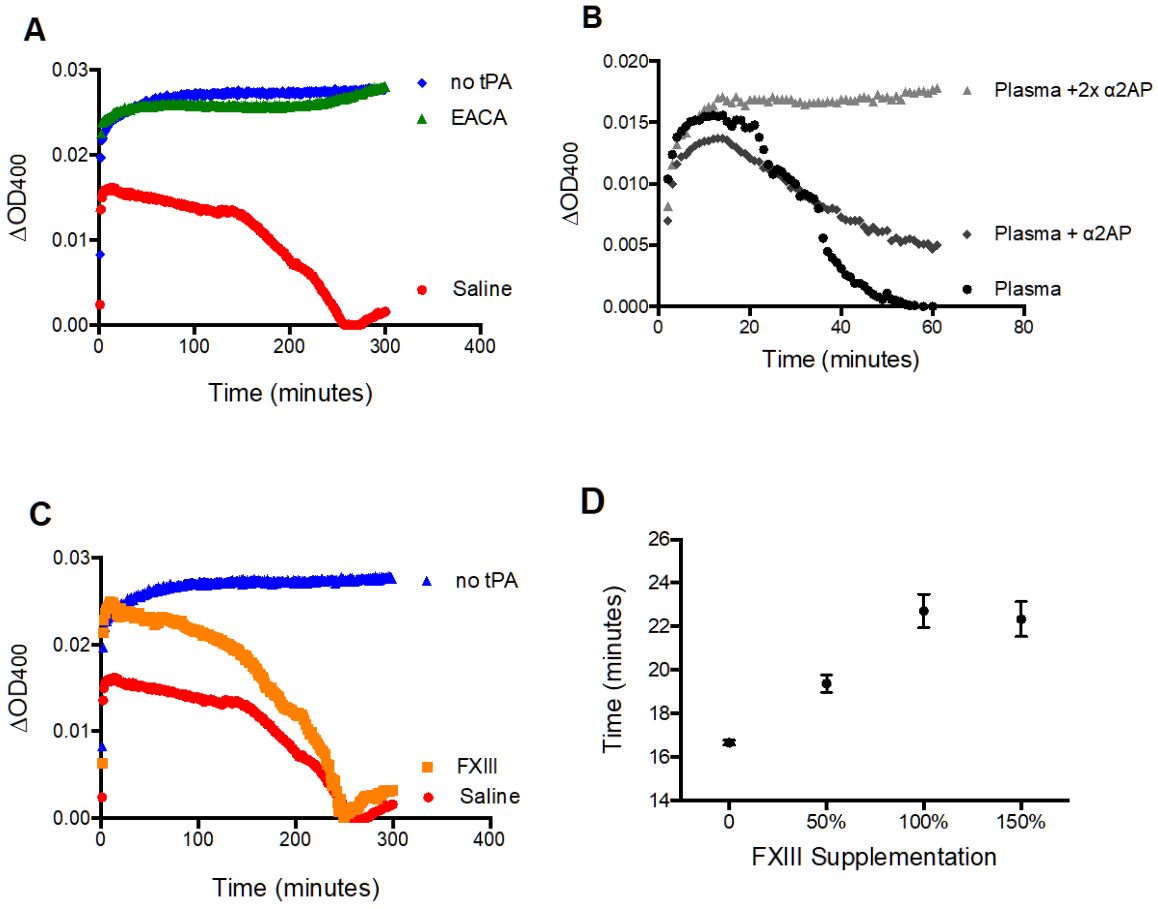

Figure 1: Clot lysis assays.

(A) WT mice treated with either saline or EACA (0.5 mg/g). (B) Plasma from WT mice was mixed with either no, or 60 µg/mL of mouse α_2-AP to double the physiological concentration which is approximately 60 µg/mL, or 120 µg/mL (to triple the concentration). (C) WT mice treated with either saline or FXIII supplementation (1 mg/kg). (D) Clot lysis times at different concentrations of FXIII. Clot lysis assay was performed using FXIII$^{-/-}$ plasma supplemented to achieve FXIII concentrations of 0 µg/mL, 5 µg/mL, 10 µg/mL (the physiological concentration of FXIII), or 15 µg/mL of FXIII.

4.8.2 Thrombus size increased with EACA treatment but not with FXIII or α_2-AP

Thrombus size was analyzed to see if the treatments affected DVT size. Compared to saline, EACA significantly ($p< 0.01$) increased thrombus size; a similar increase was observed with EACA at one-tenth the dose (data not shown). With FXIII or α_2-AP supplementation, however, the thrombus size did not change over the duration of the experiment (Figure 2A).

4.8.3 Thrombus stability increased with EACA, FXIII, but not α_2-AP

The number of total events and large embolic events were 40.2 ± 3.0 min^{-1} and 4.8 ± 1.1 min^{-1}, respectively, in mice given saline (Figure 2B, C). Similar numbers of total and large embolic events were observed with α_2-AP administration (35.3 ± 7.0 min^{-1} and 3.9 ± 1.7 min^{-1}, respectively; Figure 2B, C). In contrast, both FXIII and EACA significantly ($p<0.01$) reduced the number of total embolic events by approximately 40% to 23.4 ± 2.6 min^{-1} and 23.3 ± 2.2 min^{-1}, respectively. The large embolic events were reduced to 2.1 ± 0.8 min^{-1} with FXIII, albeit not statistically significantly ($p=0.06$), and to 0.8 ± 0.2 min^{-1}, ($p<0.05$) with EACA, respectively (Figure 2C).

The percentage of the thrombus that embolized was calculated in the 2-hour observation period as a measure of DVT turnover (Figure 2D)[218]. DVT turnover was higher in saline- and α_2-AP-treated mice than in those given EACA or FXIII (11.8% ± 1.4%, 10.9% ± 2.6%, 7.4% ± 0.7% and 8.9% ± 1.3%, respectively).

Compared to saline (0.13 ± 0.02 per lung slice), PE burden was reduced by 1.5-fold with FXIII supplementation (0.08 ± 0.01 per lung slice, $p=0.06$) and 2.5-fold with EACA treatment (0.05 ± 0.01 per lung slice, $p<0.01$) (Figure 2E).

Although EACA increased thrombus stability, it also significantly increased the thrombus size from baseline. α_2-AP supplementation did not alter the thrombus size or stability compared to control. In contrast, FXIII supplementation increased thrombus stability without increasing thrombus size significantly. Therefore, we evaluated whether FXIII would stabilize DVT when used as an adjunct to anticoagulation.

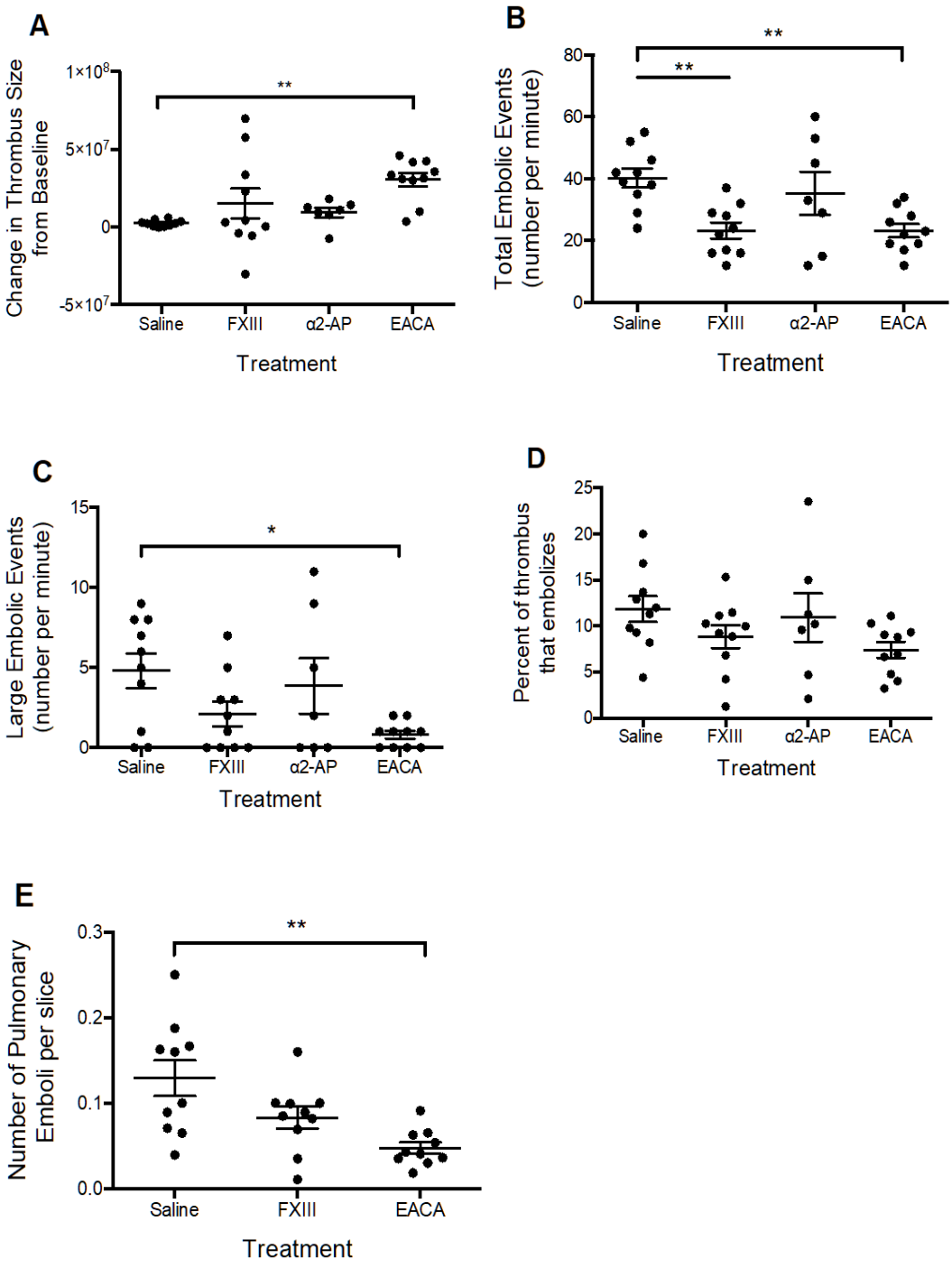

Figure 2: DVT stability after treatment with EACA (1 mg/g), FXIII (1 mg/kg) or α_2-AP (4.2 mg/kg) supplementation was compared to saline treatment in WT mice.

(A) The change in thrombus size from baseline. The (B) total and (C) large embolic events per minute, (D) percent of thrombus that embolized over two hours, and (E) number of pulmonary emboli counted per lung slice between the treatment groups are shown. All values are expressed as mean ± SEM, n=7 for α_2-AP experiments and n=10 for saline, EACA and FXIII experiments. **p-value<0.01. *p-value<0.05, using a Kruskal-Wallis test for (A) and one-way ANOVA for (B-E).

4.8.4 Treatment with FXIII increases thrombus stability in the presence of anticoagulants

We have previously shown that treatment with anticoagulants does not significantly alter the thrombus size over the two hours of observation[217]. When anticoagulated mice were supplemented with FXIII, thrombus sizes before and after treatment were not significantly different (data not shown). Therefore, the effect of FXIII supplementation on the number of embolic events in saline-, dalteparin- or dabigatran-treated mice was evaluated. Supplementation with FXIII significantly reduced total embolic events by approximately 50% in saline- (from 40.2 ± 3.0 to 23.3 ± 2.6 min^{-1}, $p<0.05$) and dalteparin-treated mice (from 44.4 ± 4.1 to 21.4 ± 3.1 min^{-1}, $p<0.01$), and by 70% in dabigatran-treated mice (from 103.76 ± 4.7 to 38 ± 4.5 min^{-1}, $p<0.0001$; Figure 3A). With FXIII supplementation, the frequency of embolization observed was similar with or without anticoagulation. A similar pattern was observed for large embolic events (Figure 3B). The number of large embolic events significantly decreased by more than 60% with FXIII supplementation in the dalteparin- (from 6.4 ± 0.7 to 2.3 ± 1.0 min^{-1}, $p<0.05$) and dabigatran-treated mice (from 10.1 ± 1.0 to 2.7 ± 0.7 min^{-1}, $p<0.0001$). Therefore, FXIII supplementation increases thrombus stability despite anticoagulation.

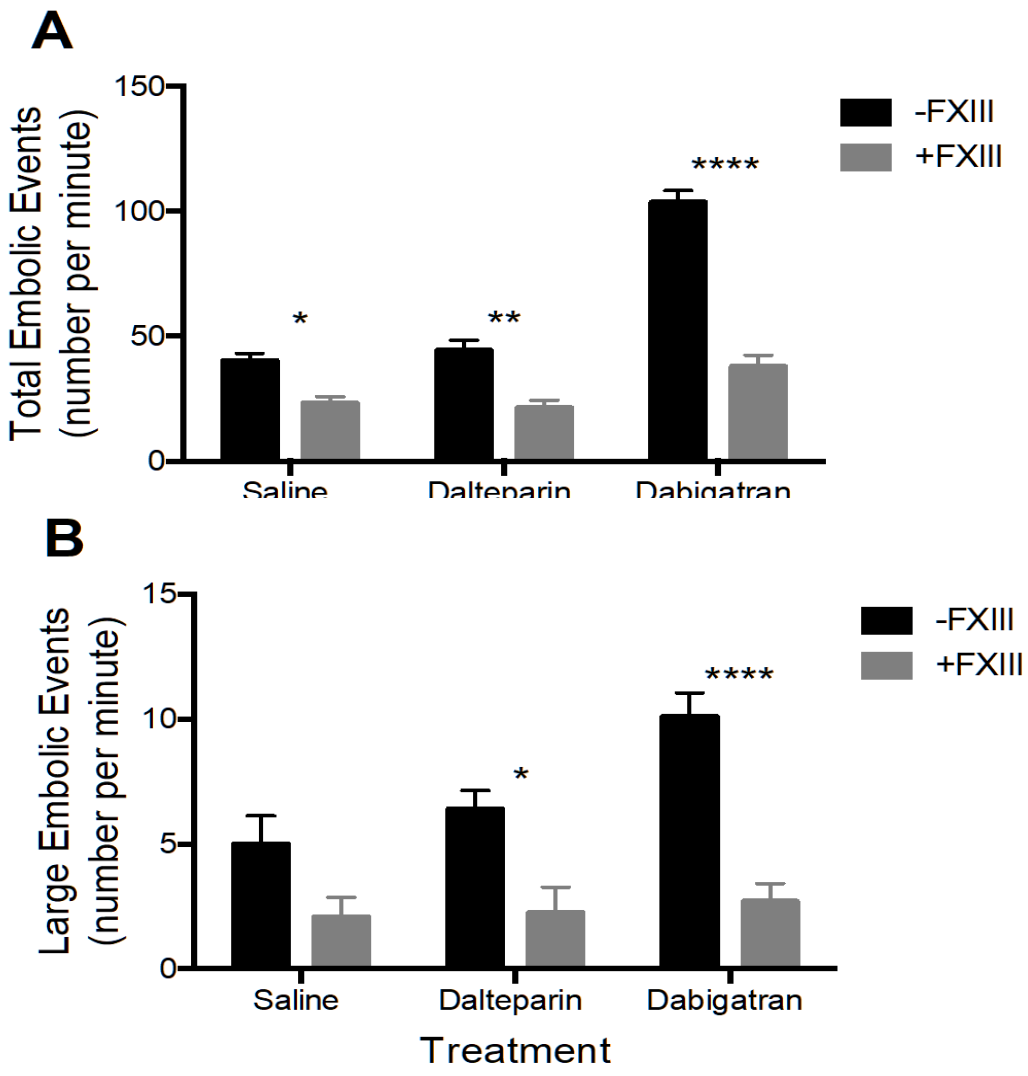

Figure 3: Embolic events after saline, dalteparin, or dabigatran treatment with or without FXIII supplementation.

The (A) total and (B) large embolic events per minute over two hours are shown. All values expressed as mean ± SEM, n=10 for each treatment in non-supplemented mice and FXIII-supplemented mice. ****p-value<0.0001. **p-value<0.01. *p-value<0.05, using a 2-way ANOVA.

4.8.5 FXIII supplementation reduced the percentage of thrombus that embolized and the PE burden in dabigatran-treated mice

With FXIII supplementation, DVT turnover decreased by approximately 50% in dalteparin-treated mice and by 75% in dabigatran-treated mice ($p<0.0001$; Figure 4A). This decrease correlated with the number of emboli per lung slice. FXIII supplementation significantly reduced PE burden by half in the dabigatran-treated mice (from 0.21 ± 0.03 to 0.11 ± 0.02 PE per lung slice, $p<0.01$; Figure 4B) and marginally reduced the number of emboli in the saline- or dalteparin-treated mice (Figure 4B). Therefore, FXIII supplementation exhibited the greatest increase in thrombus stability in dabigatran-treated mice, suggesting that the effect was dependent on the anticoagulant.

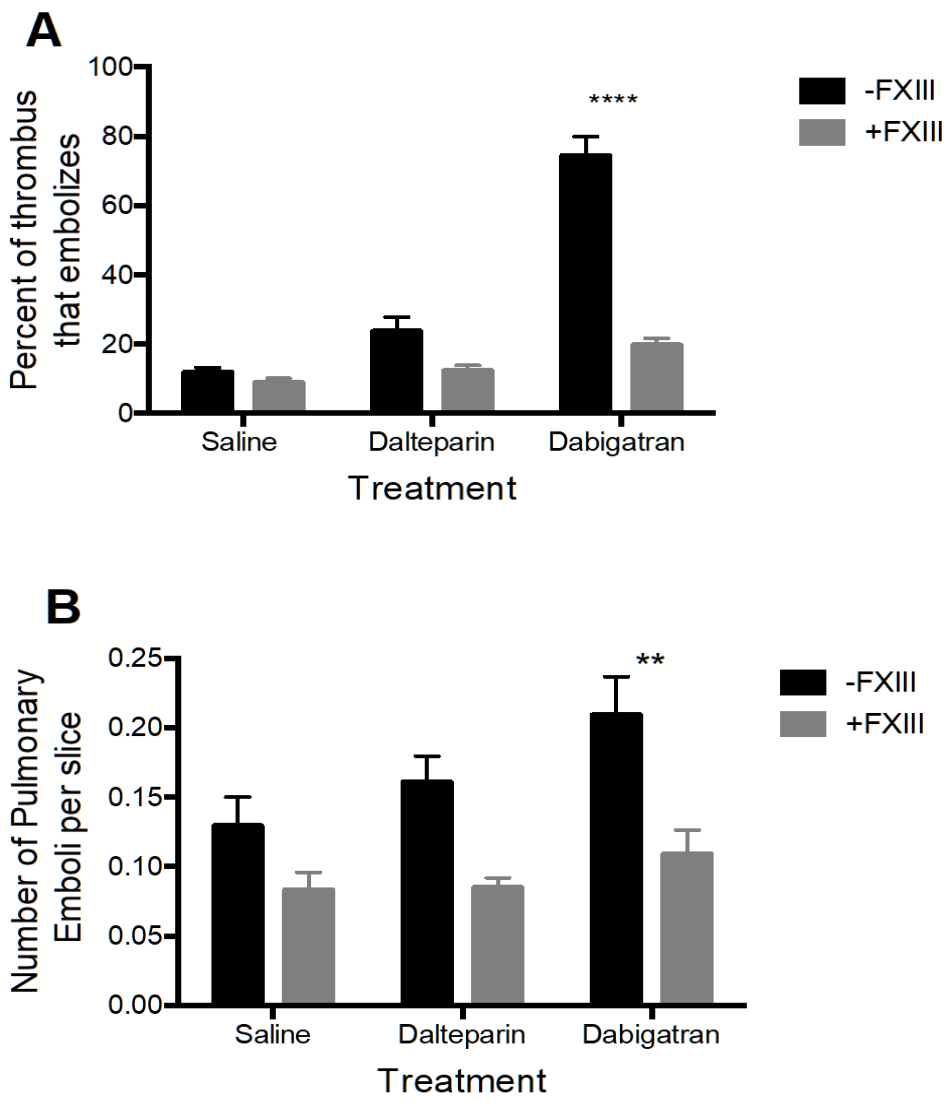

Figure 4: DVT turnover and PE risk in treated mine with, or without, FXIII supplementation.

(A) The percentage of thrombus that embolized in two hours and (B) the number of pulmonary emboli counted per lung slice after saline, dalteparin, or dabigatran treatment with or without FXIII supplementation. All values expressed as mean ± SEM, n=10 for each treatment in non-supplemented mice and FXIII-supplemented mice. ****p-value<0.0001. **p-value<0.01, using a 2-way ANOVA.

4.8.6 FXIII supplementation overcomes anticoagulant inhibition of FXIIIa generation

Dalteparin and dabigatran inhibit thrombin in an antithrombin-dependent and independent manner, respectively. We tested whether supplementing FXIII would overcome thrombin inhibition by dalteparin or dabigatran, to generate more FXIIIa. When therapeutic doses of dalteparin or dabigatran were added to NHP, FXIIIa activity was significantly reduced from 1 U to 0.82 ± 0.01 U and 0.62 ± 0.03 U, respectively (data not shown). FXIIIa activity was significantly higher in plasma containing dalteparin than in that containing dabigatran ($p<0.01$). When FXIII was added to 150% of the physiological level to plasma containing therapeutic doses of the anticoagulants, FXIIIa activity levels increased. With this additional FXIII, FXIIIa levels were significantly higher in plasma containing dalteparin than in plasma containing dabigatran (1.36 ± 0.06 U and 1.17 ± 0.03 U, respectively; $p<0.01$). Therefore, FXIIIa activity can be rescued by FXIII supplementation even in the context of anticoagulants that inhibit thrombin.

4.9 DISCUSSION

Thrombus stability determines whether or not a clot in the deep veins will embolize. We show that the thrombus stability increased with EACA treatment or supplemental FXIII but not with supplemental α_2-AP. However, thrombus size increased with EACA but not with FXIII or α_2-AP. Furthermore, supplemental FXIII reduced embolization and PE burden in mice given dalteparin or dabigatran without altering thrombus size. These data suggest that FXIII supplementation may stabilize DVT and reduce the risk of PE when used alone or in conjunction with anticoagulants.

The divergent effects of EACA and FXIII on DVT growth and embolization can be explained by their different effects on clot lysis. EACA attenuates fibrinolysis by competing with plasminogen and plasmin for fibrin binding, thereby further down-regulating fibrinolysis by reducing plasminogen activation and decreasing plasmin generation. In contrast, FXIIIa can render clots more resistant to lysis by i) crosslinking the fibrin, and ii) promoting crosslinking of α_2-AP onto fibrin[185,195,207,210]. Contrary to what would be expected, increasing FXIII beyond 100% of normal plasma levels does not significantly alter fibrinolysis (Figure 1D). Corroborating our finding, others have demonstrated that even in the presence of 10-fold higher than normal levels of FXIII (10 U/mL), clot lysis rates were only 1.5-fold slower compared with normal[223]. Crosslinking of α_2-AP to fibrin by FXIIIa also only partially inhibits fibrinolysis *in vitro*, because excess FXIII has been shown to crosslink only up to 20%-40% of available α_2-AP to fibrin clots[224–227]. Thus, enhancing the concentration of FXIII increases the rate of α_2-AP crosslinking but does not change the maximal incorporation[228]. Therefore, FXIII supplemented mice have more stable clots as a consequence of increased mechanical strength with little change in clot lysis. In contrast, EACA-treated mice not only have inhibited fibrinolysis but also have FXIIIa in the circulation that enhances the mechanical strength of the clot. Consequently, the equilibrium between thrombus growth and embolization is more disrupted with EACA treatment.

Our findings are consistent with clinical data with FXIII supplementation. In the context of invasive surgeries, including cardiopulmonary bypass in patients with high or moderate risk of blood loss postoperatively, supplemental recombinant FXIII reduced blood loss, but did not increase the number of adverse events, including thromboembolic

events[53–57]. In fact, the use of high doses of recombinant FXIII in deficient and healthy volunteers has not been shown to increase the risk of thromboembolism[58–60].

The observation that FXIII supplementation has a greater effect on restoring DVT stability in dabigatran-treated mice than in those given dalteparin or saline can be explained in two ways. First, the relative amount of residual thrombin left to generate FXIIIa is likely to be greater with indirect FXa inhibitors, such as dalteparin, than with a direct thrombin inhibitor such as dabigatran. This occurs because antithrombin-dependent FXa inhibitors only inhibit free thrombin[208]. In contrast, dabigatran inhibits both free and clot-bound thrombin[229]. Therefore, clots in dalteparin-treated mice retain greater residual thrombin activity, which can then activate FXIII. As such, FXIII supplementation has less of an effect on restoring thrombus stability. Alternatively, with dabigatran, the amount of FXIIIa generated is reduced and thrombus stability becomes compromised[218]. Therefore, FXIII supplementation has a greater effect on restoring the amount of FXIIIa activity.

Second, inadequate amounts of FXIIIa leads to decreased clot firmness. This phenomenon is observed in patients undergoing cardiopulmonary bypass who have bleeding despite an increased fibrin monomer concentration[55]. The reduction in clot firmness is restored if patients receive recombinant FXIII during surgery[230]. The imbalance or disequilibrium between thrombin generation and available FXIIIa may lead to decreased or impaired cross-linking capacity and thus reduced clot firmness, which leads to increased fibrin monomer formation[230]. In a previous study, dabigatran-treated mice had the least stable clots and yet the highest amount of thrombin generated compared with dalteparin-treated mice[218]. This imbalance may be greater after treatment

with dabigatran than with dalteparin. Therefore, the effect of supplemental FXIII on thrombus stability and thrombin generation will be greater in dabigatran-treated mice.

We previously showed that when mice were treated with dalteparin or dabigatran, thrombus size did not decrease despite embolization. Instead, there was constant thrombus turnover[218]. This phenomenon also was observed when dalteparin or dabigatran treated mice were given supplemental FXIII. These findings are consistent with clinical data where there is no evidence that the size of a DVT decreases in the first hours after an anticoagulant is given[159].

Our model has some limitations. These include its short duration, the use of ferric chloride to induce thrombus formation, and the use of a platelet label to monitor embolization. We are unable to do survival surgery once the femoral vein is isolated. More commonly used models of venous thrombosis, such as suture stenosis of the inferior vena cava, do not allow for spontaneous embolization, which is essential for evaluating thrombus stability. Lastly, we image emboli with a platelet label, because it is abundant and bright. Imaging with a fibrin-specific antibody T2G1 conjugated to AlexaFlour 647 (see Supplement Figure 1 in section 4.10) confirmed fibrin in the thrombus and the pulmonary emboli, but was not bright enough for adequate quantitation of dynamic embolization using intravital videomicroscopy. Also, Carstairs stain allows for fibrin visualization, thus the presence of fibrin in pulmonary emboli is confirmed in all groups. Increasing thrombus stability may have limitations. Stabilization of thrombi may increase the risk of the post-thrombotic syndrome (PTS). A slow rate of thrombus resolution has been correlated with valve reflux and PTS[145,150]. In contrast, rapid DVT resolution is associated with reduced valvular damage, less venous hypertension, and a

lower risk of PTS[231–233]. Therefore, it is unknown whether FXIII-mediated stabilization of DVT will increase the risk of PTS. However, if that were the case, patients with the FXIII Val34Leu polymorphism, who show increased rates of FXIII activation, and have more stable clots would be at an increased risk of PTS, but this has never been reported. In fact, patients with this polymorphism have a decreased risk of myocardial infarction [67,234], DVT and PE [235,236].

There is a clinical need for approaches that will reduce PE mortality. Inferior vena cava filters are still being used to prevent PE. It is these patients who would benefit from a treatment that could minimize embolization by stabilizing the DVT. We have shown that treatment with EACA or α_2-AP are not ideal. However, FXIII supplementation, even with dalteparin or dabigatran, stabilizes thrombi without increasing clot size. Hence, supplemental FXIII should be studied as an adjuvant treatment to reduce PE-associated morbidity.

4.10 SUPPLEMENTARY INFORMATION

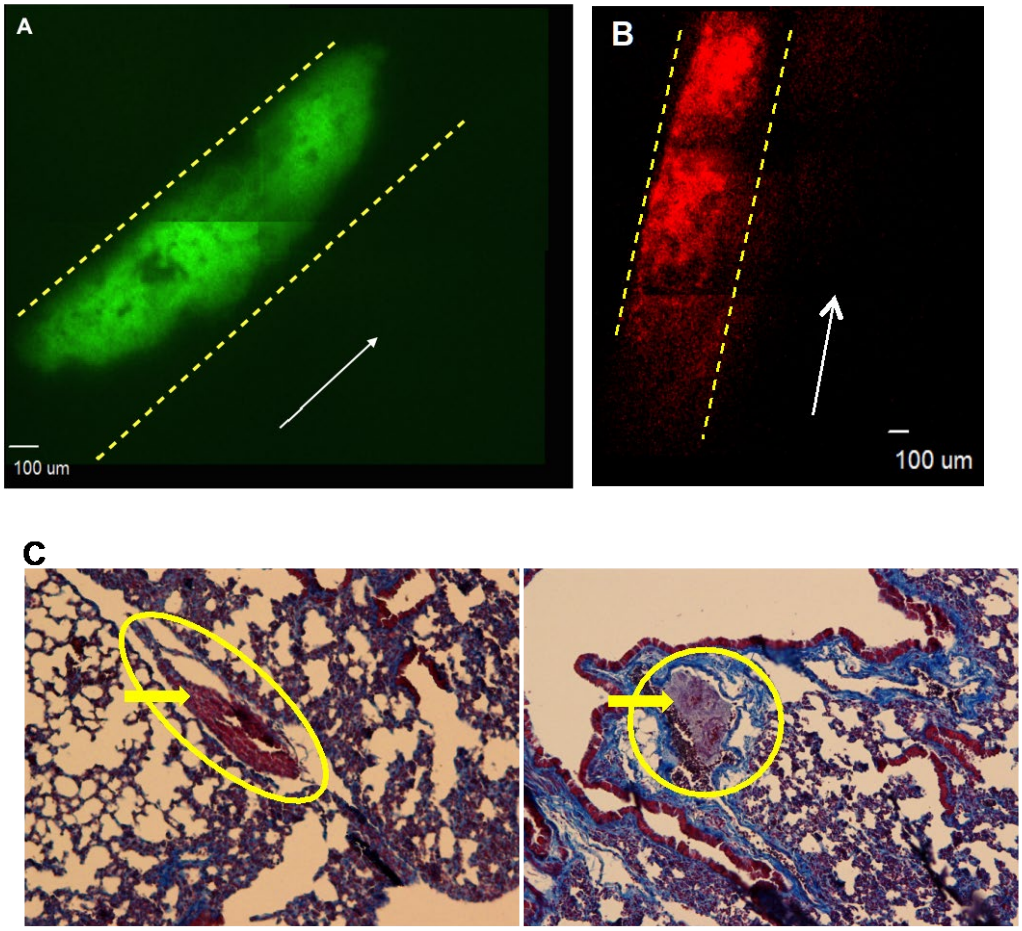

Figure S1: Representative montage of the whole thrombus and carstairs stained lung sections.

Representative montage of the whole thrombus labeled using either (A) AF488 labeled CD41 or (B) AF 647 labeled anti-fibrin β chain (Bβ 15-42) T2G1. The granular image is from look-up table compression because of the poor signal from the anti-fibrin antibody making thrombus size and emboli quantification difficult. (C) Carstair's stained images of sectioned lungs from dalteparin-treated mice. The circles outline the pulmonary arteries and he arrows point to fibrin-rick emboli.

CHAPTER FIVE

5.0 THROMBUS STABILITY EXPLAINS THE FACTOR V LEIDEN PARADOX: A MOUSE MODEL

5.1 FORWARD

This manuscript ascribes a mechanism for the factor V Leiden paradox seen in patients. FVL patients have more often venous thrombosis than pulmonary embolism due to enhanced clot stability. Mice with the FV Leiden mutation develop larger thrombi in a DVT model, which embolize at a lower rate than thrombi in WT mice.

Thrombus Stability Explains The Factor V Leiden Paradox: A Mouse Model

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Lay title: FVL stabilizes venous thrombi

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Authorship Contributions: S.A. Shaya performed all experiments, analyzed data and results, and wrote the manuscript. R.J. Westrick and P.L. Gross supervised the research, conceived and designed the study. P.L. Gross and R. Westrick contributed to the interpretation of data and critically edited the manuscript. All authors have read and approved the article.

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References: References for this Manuscript have been incorporated into the Bibliography (Chapter 7), at the end of this thesis.

5.2 KEY POINTS

- Factor V Leiden (FVL) homozygous mice have increased thrombus size and reduced pulmonary embolism burden compared with wild-type mice
- FVL carriers develop more stable thrombi which become clinical deep vein thrombosis more often than noncarriers, explaining the FVL paradox

5.3 ABSTRACT

Humans carrying the factor V Leiden (FVL) variant have a five-fold increased risk of venous thrombosis. However, incidence of deep vein thrombosis (DVT) is proportionally greater than pulmonary embolism (PE) in these individuals. This is known as the FVL paradox. We hypothesized that the rate of initial DVT development is similar in FVL and noncarriers, but thrombi in FVL carriers are more stable and develop into a clinically significant DVT more often than in noncarriers. To test this, we induced thrombi in the femoral vein of wild-type (WT), heterozygous ($F5^{L/+}$) and FVL homozygous ($F5^{L/L}$) mice. Using intravital microscopy, thrombus size and embolization were visualized, and emboli in the lungs were quantified. Compared to WT, femoral vein thrombi in $F5^{L/+}$ and $F5^{L/L}$ mice were larger and embolized less. Total and large embolic events, the percent of thrombus that embolized, and PE burden were significantly decreased in $F5^{L/L}$ mice. This suggests that noncarriers (reflected by WT), a minor injury initially resulting in a small DVT tends to remain small and asymptomatic due to the embolization of the otherwise growing thrombus. Alternatively, the same insult in people with FVL (reflected by $F5^{L/L}$) leads to thrombus growth due to less embolization and thus symptomatic DVT development.

5.4 INTRODUCTION

Activated factor V (FVa), together with the serine protease factor Xa (FXa) form the prothrombinase complex that converts prothrombin to active thrombin. FVa is subsequently inactivated by the natural anticoagulant, activated protein C (APC), which cleaves FVa at arginine(R)-506, R306, and R679 in the heavy chain[90]. Cleavage at R506 is required for efficient cleavage at the two other sites[90]. The substitution of glutamine (Q) for R506 in FV is a common mutation in humans known as factor V Leiden (FVL). The R506Q mutation abolishes a cleavage site for APC, thereby reducing the rate of FVa inactivation and consequently allowing for prolonged prothrombinase activity[90].

The clinical manifestation of the FVL variant varies. Whereas most individuals with FVL may never develop thrombosis[237], those with FVL heterozygosity are at increased risk of venous thrombosis, typically experiencing their first episode during adulthood[238], while those homozygous for FVL tend to present with their first episode at a younger age[239]. The relative risk for venous thrombosis is increased by 3- to 8-fold in those heterozygous for FVL[240,241]. While most studies report an increased VTE risk in those homozygous for FVL, there is more discrepancy within the relative risk estimates; 9 to 80-fold[240]and 9 to 12-fold[241,242].

FVL heterozygosity is an inherited autosomal dominant condition that is identified in 20-50% of patients with venous thromboembolic diseases[91,92], and 5-8% of the population in Canada[243]. There is a higher incidence of DVT than PE in those with FVL, and a low prevalence of FVL among people who suffer a fatal PE[91,92]. This has been referred to as the FVL paradox. While several mechanisms and hypotheses have been studied, none have explained the biological basis for the FVL paradox[244–246].

A study performed by van Langevelde *et al*., evaluated whether common venous thrombosis risk factors have differential effect on DVT and PE[247]. This study found that along with FVL, reproduction-related risk factors, such as pregnancy and use of oral contraceptives, as well as obesity also showed a higher risk for DVT than PE. Meanwhile, pulmonary conditions, such as chronic obstruction pulmonary disease, pneumonia, and sickle cell disease are a higher risk factor for PE but have little or no effect on DVT. Thus, this suggests a biology that underlies whether the presentation of VTE will be DVT or PE, thus understanding the FVL paradox may have broader implications.

Based on this previous work, we wondered if the incidence of subclinical thrombus formation are similar among FVL carriers and noncarriers, but FVL carriers develop a symptomatic DVT due to enhanced thrombus stability. In contrast, the thrombus remains subclinical in noncarriers due to micro-embolization. Therefore, to explain the FVL paradox, we assessed DVT stability after a small injury, in $F5^{L/+}$ and $F5^{L/L}$ compared to wild-type (WT) mice.

5.5 MATERIALS AND METHODS

FVL mice, gifted from Dr. Ginsburg's lab, were generated carrying the homologous mutation (R504Q) inserted into the endogenous murine *F5* gene. Adult heterozygous ($F5^{L/+}$) and homozygous ($F5^{L/L}$) mice are viable and fertile and exhibit normal survival[93]. Experiments were carried out using our *in vivo* mouse model of thrombus stability as previously described with slight modifications[218]. Previously, a 1 mm x 2 mm 4% ferric chloride ($FeCl_3$)- soaked filter paper placed on the medial side of the femoral vein for 5 minutes is used to initiate non-occlusive thrombi in WT mice for two hours. This

concentration and time of application of $FeCl_3$ occluded the femoral vein of $F5^{L/L}$ mice before the two-hour experiment window. Thus, we shortened the $FeCl_3$-soaked filter paper application time to 3 minutes to initiate smaller, reproducible and non-occlusive thrombi in both $F5^{L/L}$ and WT mice. Intravital videomicroscopy recorded embolic events leaving the thrombus and the thrombus sizes for two hours. Lungs were harvested, sectioned and stained for presence of PE. All other labeling, quantification and analysis has been previously described[218]. Prothrombin fragment 1 + 2 (F1 + 2) levels were measured by ELISA (Cloud Corp., Houston, TX, USA) following the manufacturer's instructions.

5.6 RESULTS AND DISCUSSION

Thrombi initially induced in the femoral vein of WT, $F5^{L/+}$ or $F5^{L/L}$ mice were not significantly different from each other in size (data not shown). After 2 hours, the $F5^{L/+}$ and $F5^{L/L}$ mice exhibited a significantly greater change in thrombus size from baseline compared to WT mice (Figure 1A). Emboli breaking off the proximal tip of the thrombus were recorded and manually counted. Both the total number and number of large embolic events were significantly more in WT mice compared with $F5^{L/+}$ and $F5^{L/L}$ mice (Figure 1B, C). The percent of thrombus that embolized in the two-hour observation period, a measure of DVT turnover, was significantly reduced in $F5^{L/+}$ and $F5^{L/L}$ mice compared with WT mice (Figure 1D). Taken together, these data suggest that the small thrombus induced in FVL heterozygous or homozygous mice stabilized over time, with reduced embolization. This enabled greater thrombus growth, while the small thrombus in WT mice continuously embolized without increasing the net thrombus size.

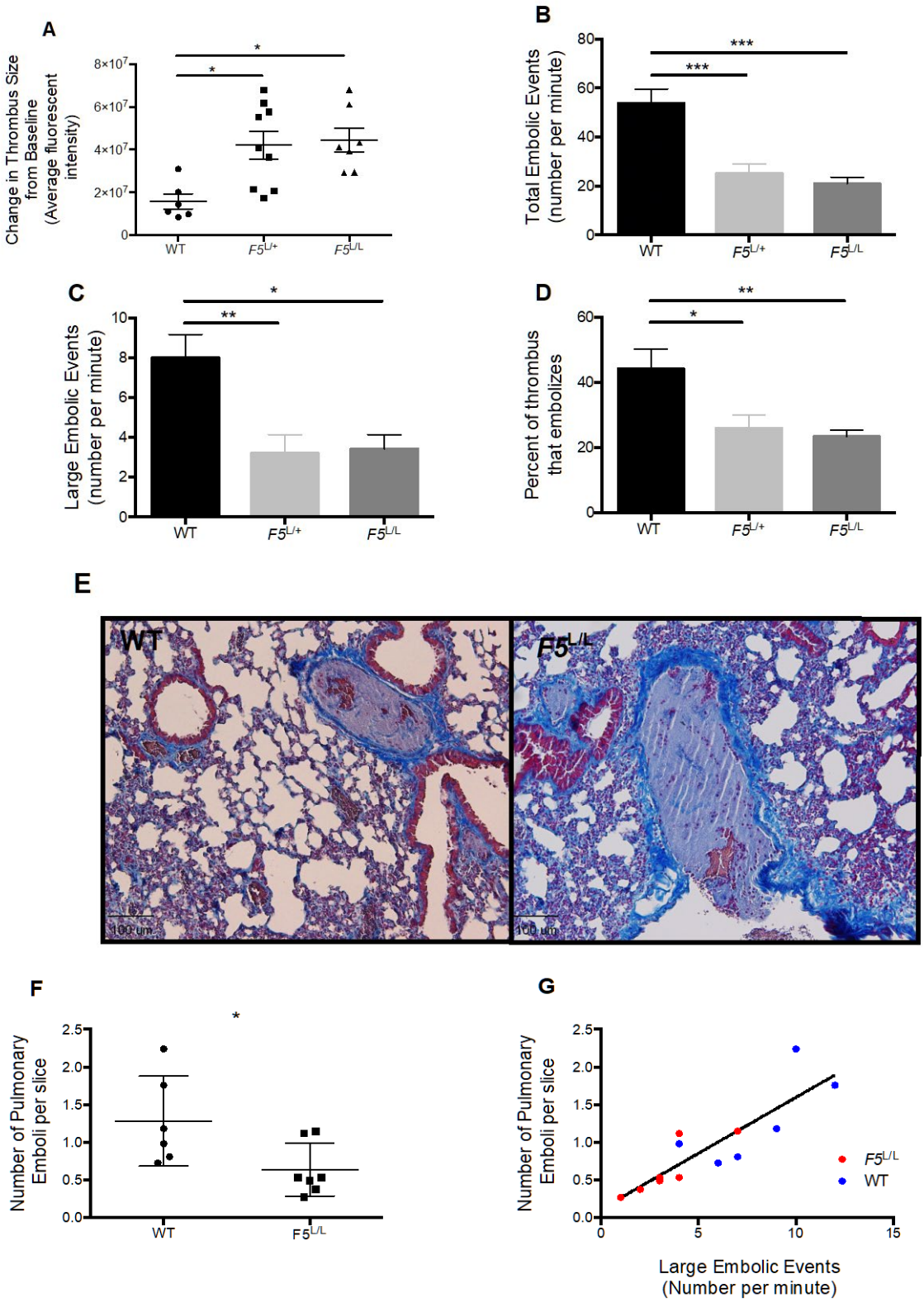
A
Change in Thrombus Size from Baseline (Average fluorescent intensity)
8×10^7
6×10^7
4×10^7
2×10^7
0
*
*
WT
F5L/+
F5L/L
B
Total Embolic Events (number per minute)
80
60
40
20
0
***
***
WT
F5L/+
F5L/L
C
Large Embolic Events (number per minute)
10
8
6
4
2
0
*
**
WT
F5L/+
F5L/L
D
Percent of thrombus that embolizes
60
40
20
0
**
*
WT
F5L/+
F5L/L
E
WT
F5L/L
100 um
100 um
F
Number of Pulmonary Emboli per slice
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
F5L/L
G
Number of Pulmonary Emboli per slice
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
F5L/L
WT
Large Embolic Events (Number per minute)

Figure 1: In vivo mouse model of thrombus stability in WT, $F5^{L/+}$ and $F5^{L/L}$ mice.

The (A) change in thrombus size from baseline, number of (B) total and (C) large embolic events and, (D) percentage of thrombus that embolized. (E) Representative emboli images found in the pulmonary arteries of WT and $F5^{L/L}$ mice. (F) Number of pulmonary emboli per lung slice and (G) the correlation between large embolic events and PE burden. All values are expressed as mean +/- SEM, n=6, 7, and 9 for WT, $F5^{L/+}$ and $F5^{L/L}$ mice respectively. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ using a One-way ANOVA for (A-D) and Student's T-test for (F) and linear regression for (G).

In order to determine whether the higher DVT turnover in WT mice led to increased PE burden, we quantified the number of pulmonary emboli per lung section in WT and *F5*$^{L/L}$ mice by histology (Figure 1E). PE burden was reduced in *F5*$^{L/L}$ mice compared to WT mice (Figure 1F). We have previously shown that the number of large embolic events correlate with PE burden[218]. Here again, large embolic events correlated positively with PE burden in WT and *F5*$^{L/L}$ mice (Figure 1G). The increased thrombus size and reduced PE burden in *F5*$^{L/L}$ mice compared with WT (likely due to enhanced thrombus stability) provides a physiological/biochemical rationale for the FVL paradox. FVL carriers are more likely to present with symptomatic DVT from a small vessel injury. In contrast, noncarrriers have a higher incidence of embolization, which restricts the DVT to a subclinical size while at the same time leading to increased PE burden.

Several hypotheses have been proposed to account for the FVL paradox. One of the original hypotheses was that the presence of the FVL mutation would lead to fatal PE resulting in a lower number of FVL subjects among those surviving with PE. However, autopsy studies have shown no difference in the proportion of FVL among those with fatal PE compared to PE survivors or the general population[248,249]. Other mechanisms such as thrombus location, the number of affected veins, thrombus growth and density have all been investigated to explain the FVL paradox, but none were sufficient to explain it[246]. A more recent hypothesis proposes that FVL may enhance local thrombin generation, thus intensifying the local inflammatory processes against the thrombus, as well as strengthening the clot structure by activation of thrombin-induced FXIII activity[246]. However other thrombophilic risk factors, such as antithrombin, and protein C and S deficiencies, increase the risk of DVT as much as they increase the risk of PE,

refuting this hypothesis. A second commonly proposed hypothesis involves an antifibrinolytic effect. Bajzar *et al*., showed that in FVL heterozygotes there is an impaired TAFI-dependent profibrinolytic response to activated protein C (APC)[245]. Parker *et al*., later confirmed these results and also showed that lysis of radiolabeled clots infused through the jugular vein and lodged in the lungs had less lysis in FVL homozygous mice compared to WT, supporting the hypothesis that FVL inhibits fibrinolysis[244]. However, FVL carriers do not show an increased risk of post-thrombotic syndrome[250,251], which would be expected if there was resistance to fibrinolysis. *F5*^{L} mice had significantly increased F1.2 compared to WT (54.1 ± 7.3 ng/ml and 33.8 ± 2.1 ng/ml, $p<0.01$). Therefore, we believe that after a small injury a stable thrombus forms in FVL mice, with reduced embolization compared to WT. However, since there is significantly more thrombin generation in *F5*^{L} mice (supported by the F1.2 data), this leads to thrombus growth and further stabilization, reducing embolization.

An increased rate of pulmonary microemboli in noncarriers compared to those with FVL has not been directly described. Indirectly, pulmonary microemboli are prevalent in the acute respiratory distress syndrome (ARDS), and those with FVL have a lower risk of death from ARDS than normal[252,253].

In mice with the *F5*$^{L/L}$ mutation, the thrombus grows and stabilizes over time. Increased thrombus stability is inversely related to embolization and PE burden. This suggests that those with FVL have stable thrombus formation leading to an increased incidence of symptomatic DVT and a decreased risk of PE.

5.7 ACKNOWEDGMENTS

We would like to thank Dr. David Ginsburg's lab for the FVL mice. The study was partly funded by Canadian Venous Thromboembolism Clinical Trials and Outcomes Research (CanVECTOR).

CHAPTER SIX

6.0 GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1. GENERAL DISCUSSION

Thrombosis is a primary contributor to coagulation-associated morbidity and mortality. Every year, there are approximately 10 million cases of VTE worldwide, resulting in more than half a million deaths in Europe and 100,000-300,000 deaths in the USA[254]. Thrombosis can occur in the arterial or venous circulation, leading to myocardial infarction, stroke, deep vein thrombosis and pulmonary embolism. Arterial thrombosis is usually associated with atherosclerotic plaque rupture that exposes sub-endothelial procoagulant material (eg. tissue factor, collagen) to blood, leading to platelet activation and aggregation in high-shear stress. Ultimately, platelet accumulation and fibrin deposition produce an occlusive platelet-rich "white thrombus"[255]. In contrast, venous thrombosis/thromboembolism, which has an incidence of about 1.5 in 1000 per year and a lifetime prevalence of more than 5%[256], is thought to be triggered by inappropriate expression of cell adhesion molecules on intact, but dysfunctional endothelium in the presence of plasma hypercoagulability and reduced blood flow (stasis)[257]. This is known as Virchow's triad. Because venous thrombi have a high RBC content, they are known as "red thrombi"[258]. Fibrin formation is a central component of both arterial and venous thrombosis, and is ultimately the target of thrombolytic enzymes used to treat both of those clinical presentations[258].

The term clot stability has been used differently throughout the literature. Studies evaluate clot stability in terms of i) platelet-platelet interactions, i) clot-vessel wall interactions, ii) fibrin formation and growth, or iii) different factors that alter clot

composition. As well, throughout these multiple studies, the formed clots are treated with various therapies, all with the same goal in mind, and that is to reduce their size. This is usually done by measuring the clot weight, looking at clot retraction, or looking for the reduction in thrombus size in vivo, before or after treatment. Thus, most studies in the literature will conclude that if the clot size or burden is reduced, the treatment used was successful. However, while some treatments may be promising in reducing the burden of a clot, this approach does not assess the downstream effects of embolization. This makes it difficult to understand the mechanism and factors that affect clot stability, not just looking at the clot, but also at the effect on embolization. Clots that have been defined as "stable" in literature, have been perceived as unfavorable since they are viewed as "harder to lyse and be cleared", and vice versa, whereby reducing thrombus stability is usually preferred, and therefore this has defined the ideals of thrombus stability[185].

Therapy for venous thromboembolism traditionally involved parenteral anticoagulation and subsequent warfarin[256]. However, this approach has changed with the introduction of direct oral anticoagulants (DOACs). Anticoagulation is usually needed to reduce the risk of fatal pulmonary embolism and morbidity from recurrent venous thrombosis, post-thrombotic syndrome, and pulmonary hypertension[256]. DOACs have major pharmacological advantages over vitamin K antagonists (eg, warfarin), including rapid onset/offset of action, few drug and food interactions, and predictable pharmacokinetics, eliminating the requirement of regular coagulation monitoring[259]. Some disadvantages of DOACs may be concerns regarding medication adherence without laboratory monitoring, uncertainty about dosing in some patient populations (eg,

renal dysfunction), higher drug costs compared to warfarin and most importantly the increased risk of gastrointestinal (GI) bleeding[259].

Therapies for pulmonary embolism, depending on the assessed severity of the PE burden, range from anticoagulant therapy, thrombectomy, thrombolytic therapy, or surgical removal of the emboli[260]. Thrombolytic agents are beneficial, but the ideal population is not yet defined. Thrombolytic treatment demonstrated superior efficacy in clot resolution and improvement in hemodynamics compared with anticoagulation alone, leading to approval of streptokinase, urokinase and alteplase in the treatment of massive PE[261]. However, the risk of major bleeding makes thrombolysis less desirable. Insertion of inferior vena cava (IVC) filters is also used in patients who have contraindications to anticoagulants, such as bleeding, to reduce the risk of the clot embolizing and leading to PE[262].

The purpose of this thesis was to study clot stability looking at both deep vein thrombosis and its correlation to PE burden. In this thesis we attempt to uncover the mechanism of thrombus stability and the factors that alter thrombus stability and how this affects PE burden in return. This work suggests that, despite what has long been reported in the literature, stabilizing a thrombus without increasing DVT size and reducing pulmonary embolism may be a better alternative than trying to break down a thrombus to prevent recurrent PE. This thesis demonstrates, using our novel *in vivo* mouse model of venous thrombus stability, that different anticoagulants, given acutely, have different effects on thrombus stability (Chapter 3). Also, supplementing FXIII can increase thrombus stability without increasing the thrombus size, which does not occur if fibrinolysis is attenuated by EACA (Chapter 4). Lastly, in mice with the FVL mutation, a

small vein injury-induced thrombus, stabilizes and grows significantly over time and embolizes less, thus explaining the FVL paradox (Chapter 5).

The purpose of this Chapter is to: A) discuss the overall significance of the data from Chapters 3 to 5 to highlight our novel findings about thrombus stability, B) review the possible mechanisms and factors involved in thrombus stability, and the link between DVT and PE, and finally, C) discuss the future directions for the model and clinical implications.

In the first paper we established that vein thrombus embolization is related to pulmonary embolism burden. The increase in embolization results in an increase in pulmonary embolism burden. We also found that when treating with some anticoagulants, embolization as well as pulmonary embolism burden increases. This suggests that some, but not all, anticoagulants reduce thrombus stability, which then increases embolization and PE. Thus, thrombus stability is inversely related to embolization and PE burden.

6.2 FERRIC CHLORIDE THROMBOSIS MODELS

Many rodent models have been established to study thrombosis. Ferric Chloride ($FeCl_3$) is the most used agent to initiate vascular injury and thrombosis[166]. The mechanism behind $FeCl_3$ thrombus formation is not well understood and heavily debated. The historical consensus is that application of the $FeCl_3$ to the surface of the vessel leads to denudation of the endothelium, which results in exposure of the sub-endothelial components such as collagen and TF[263,264]. More recent studies using electron microscopy to look at the vessel after $FeCl_3$ application demonstrate that, contrary to the generally accepted belief, the endothelium is intact and furthermore, the first cells to

adhere to the $FeCl_3$-treated endothelial surfaces are red blood cells rather than platelets[265]. An overlooked aspect of the $FeCl_3$ model is the influence of the severity of injury, which is dependent on $FeCl_3$ concentration, the filter paper size and the duration of its application on the surface of the vessel. Thus, whether the endothelium is denuded or not may be dependent on the extent of vascular injury. Wang *et al*., showed that reactive oxygen species induce vessel injury and lead to thrombus formation[266,267].

Nonetheless, $FeCl_3$ was chosen because it allows for the creation of a reproducible, size-controlled thrombus, including one that does not occlude for at least 2 hours. Regulating thrombus size was crucial since the thrombus size before treatment administration must be similar to draw conclusions about how the treatments alter thrombus size, and thus DVT stability and PE burden. Maintaining the presence of a non-occlusive thrombus was also important since blood flow is required to image embolization; other ligation models would prevent this. Besides our model, the only model where non-occlusive thrombi forms is the electrical injury model[268]. This model results in PE that is FXIII-dependent[184]. Lastly, the findings in Chapter 5 would not have been possible if another inducer of venous thrombosis was used. Therein we used a lower exposure to $FeCl_3$, one where the size of the thrombus in WT mice was barely large enough to be detected to demonstrate larger more stable thrombi in FVL mice.

Since the original experiments (chapter 3 and 4) were performed in WT mice undergoing a 5-minute $FeCl_3$ injury and experiments in chapter 5 were done in WT using a 3-minute $FeCl_3$ injury, we were able to compare the data from the different injury models. These data may give us a preliminary insight into the effect of blood flow on thrombus stability using our $FeCl_3$ venous thrombosis model. As seen in Figure 1. there is

a greater change in thrombus size from baseline in the 3-minute injury model. This suggests that a smaller initial injury, that has more blood flow present, will grow more and change from what was initially induced than if you had a bigger injury to start with little blood flow. We also observed an increase in embolization and the overall DVT turnover. This confirms that blood flow plays a role in embolization.

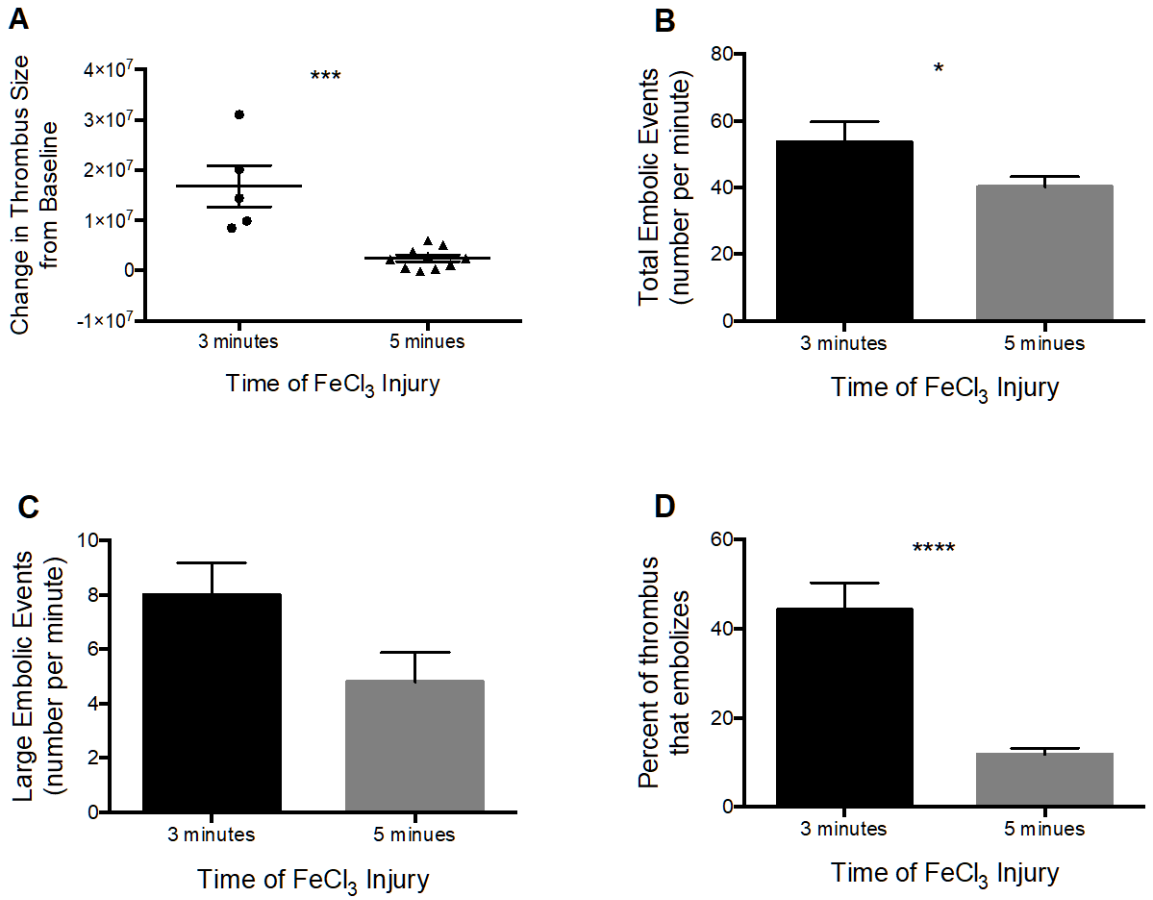

Figure 1: Comparing the $FeCl_3$ injury model using either 3-minute injury or a 5-minute injury.

Thrombi in WT mice were initiated with a 3-minute $FeCl_3$ injury or a 5-minute $FeCl_3$ injury and imaged for 2 hours. (A) The change in thrombus size from baseline, (B) total embolic events, (C) large embolic events, and (D) percentage of thrombus turnover were compared. All values are expressed as mean +/- SEM, n=5 and 10 for 3-minute and 5-minute injury in WT mice, respectively. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$ using a Student's T-test for A-D.

Then we correlated the thrombus size with the DVT turnover and found that there was a strong positive correlation in both models (Figure 2). This suggests that in the presence of a small injury, with presence of blood flow, this could lead to a less stable thrombus that embolizes more, and therefore DVT turnover increases. Whereas a bigger initial thrombus, with less blood flow, will embolize less and the DVT turnover is significantly less.

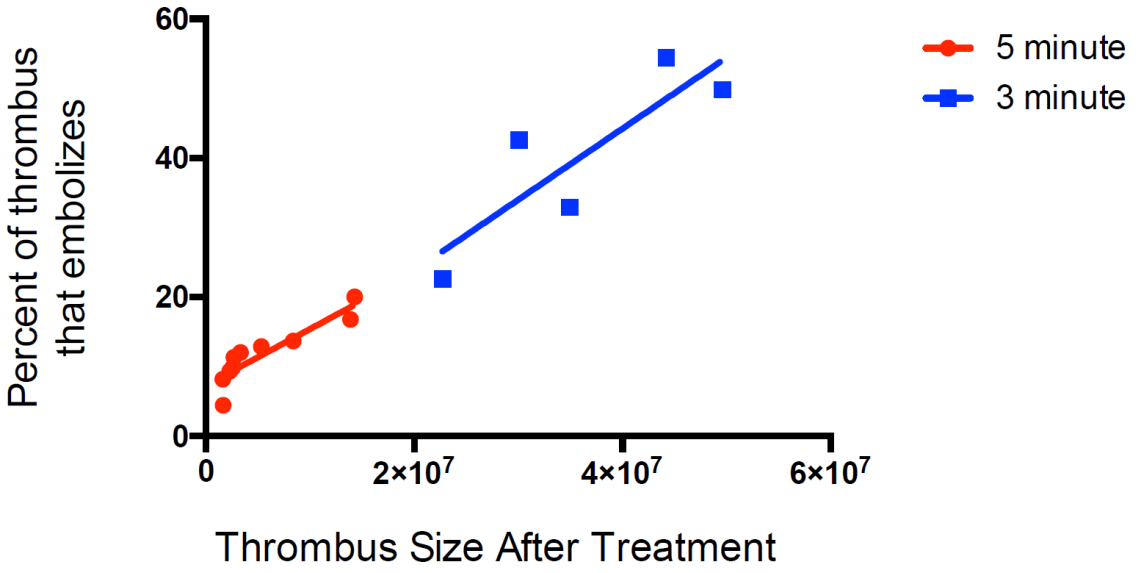

Figure 2: The percent of thrombus that embolized compared with the thrombus size in the 3-minute and 5-minute $FeCl_3$ injury model

All values are expressed as mean +/- SEM, n=5 and 10 for 3-minute and 5-minute injury in WT mice, respectively.

6.3 LIMITATION OF THIS MODEL

Like any other animal model, there are limitations: i) the model is acute; it is only imaged for 2 hours, ii) treatment was administered 12 minutes after thrombus formation; which is less clinically relevant, iii) the mechanism of thrombus formation is not the same as the formation of human DVT, iv) the thrombi formed are non-occlusive, v) we used only female mice in our studies, vi) the PE we found in our model differs by size and location from human PE, vii) we do not measure the clinical relevance of the PEs that occurred.

The model can be made to be "less acute" by extending the observation period to 5 hours, however the mouse cannot be sedated for longer. Thus, while we would accumulate more data and information about thrombus formation in the "less acute models" it would still overall be considered "acute". For the model to be chronic, a thrombus can be initiated in the femoral vein of a mouse, with the operative site sutured, to perform a survival surgery. However, in this case, we would not be able to image the thrombus size growth, and more importantly, quantification of embolic events would be impossible. After 24 hours to 48 hours, the thrombus in the femoral vein and the lungs can be removed to perform histology and quantify emboli, however it would be impossible to study the correlation between embolic events and PE burden. We could, in theory attempt to correlate thrombus size with PE burden. With the data collected from all studies, we assessed the correlation between the thrombus size with the number of PE per lung section as a measure of PE burden in the saline-treated group, as an example, and found there was no correlation (R^2= 0.3; Figure 3). This pattern was true in also the

dabigatran- and dalteparin-treated mice (R^2=0.2 and R^2= 0.02 respectively). Thus, thrombus size does not determine PE burden.

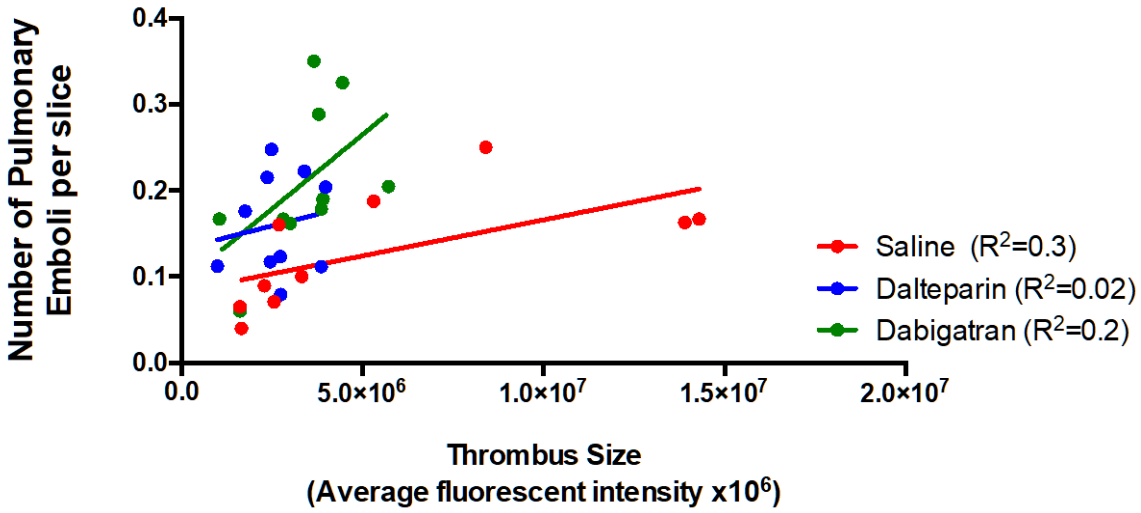

Figure 3: Thrombus size correlated with the number of pulmonary emboli per lung slice.

All values are expressed as mean ± SEM, and n=10 for each treatment group. Regression values all had p-value >0.05 and were considered insignificant and represented with solid regression lines.

Another limitation to the model is that treatment is administered 12 minutes after thrombus formation, which does not mimic clinical DVT treatment, where treatment occurs later. This limitation can be overcome by delaying treatment to study how delayed treatment would affect thrombus stability; however it would still be relatively "acute" even with delayed treatment. We have attempted to break-free of the dominant methodology in the literature, by treating an established clot with anticoagulants. Our work contrasts with forming clots in the presence of therapeutic anticoagulant levels, a practice that is ubiquitous in the literature, which has less clinical relevance. We chose not to administer antithrombotic drugs before injury, to better mimic clinical practice. We wanted to study the effects of drug treatment in the acute setting, and thus it makes sense to administer them acutely after thrombus injury rather than before. Furthermore, if we had chosen to administer the drugs before injury, we would have faced the difficulty that administration of anticoagulants before an injury would make it harder to form a thrombus. One could overcome this by titrating the injury, but this adds further complexity to the method and confuses the findings.

Another perceived limitation is that the technique used relies on the evaluation of labeled platelets, and that dabigatran may be interfering with platelet dependent clotting and the observed embolization is rather reflecting clot instability and platelet aggregate dissociation. However, we do not think that is the case. We chose to label platelets instead of fibrin because glycoprotein IIb/IIIa (CD41/CD61) receptor is one the most abundant cell surface receptors (~80,000 per platelet) and therefore provides a very good fluorescent signal. We have also measured fibrin content in venous thrombi by labeling fibrin using mouse anti-fibrin II β chain (Bβ 15-42) T2G1 antibody conjugated to AF 647

(Accurate, New York, USA), at a concentration of 1 μg/g. The thrombus can be observed very dimly under the microscope and tends to look similar to thrombi that were labeled using the AF488 labeled CD41 (Figure 4), however since the label is not as bright, it is difficult to quantify thrombus size and embolic events labeling fibrin. In addition, we detected fibrin-rich emboli in the lung sections of treated mice using Carstairs stain and we have quantified fibrin in lungs of saline and dabigatran treated mice (Figure 5 and 6) This was done with snap frozen lungs that were stored at -80°C for 24 hours, and later homogenized using liquid nitrogen and centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was collected, mixed with 1% Triton X-100 on a shaker for 1 hour, and fluorescence was measured.

Lastly another limitation to this model is the use of only female mice. We chose to use female mice so that in the future we could also study the effect of hysterectomy and how this would alter the correlation between DVT and PE. In a study done by Chessum *et al.*, they found that female WT mice exhibited greater embolization activity compared to WT male mice[269]. They also found that between WT males and females, the females showed greater thrombus size increase[269]. The mechanism behind these observations is still not understood.

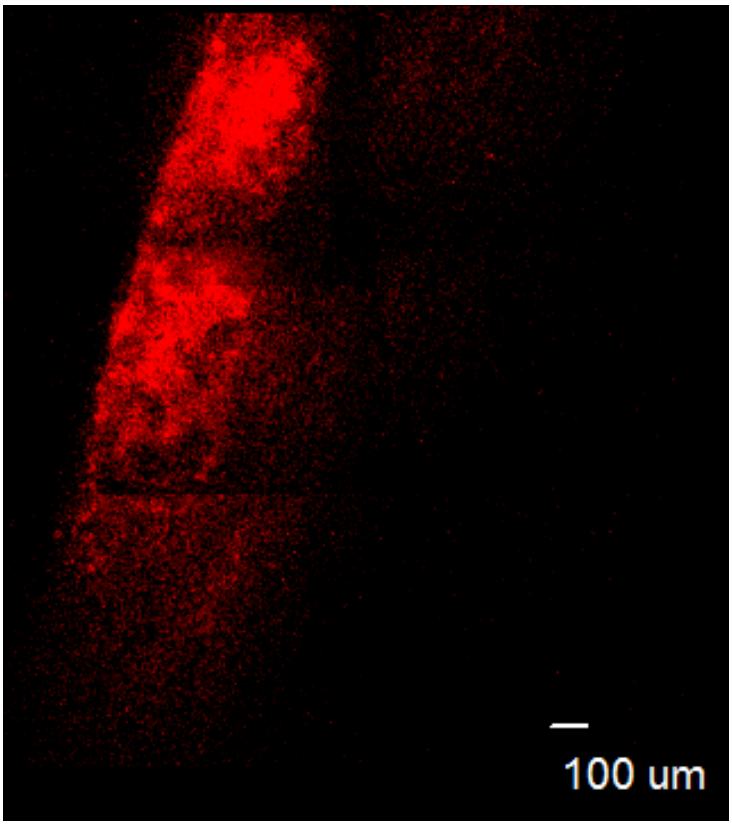

Figure 4: Intravital videomicroscopy of T2G1 labeled thrombus.

Representative montage of the whole thrombus labeled using T2G1 conjugated to AF647 to assess the fibrin content in venous thrombi. The granular image is a result of the poor signal from the anti-fibrin antibody. The ferric chloride femoral vein injury was induced as previously described in chapter 3.

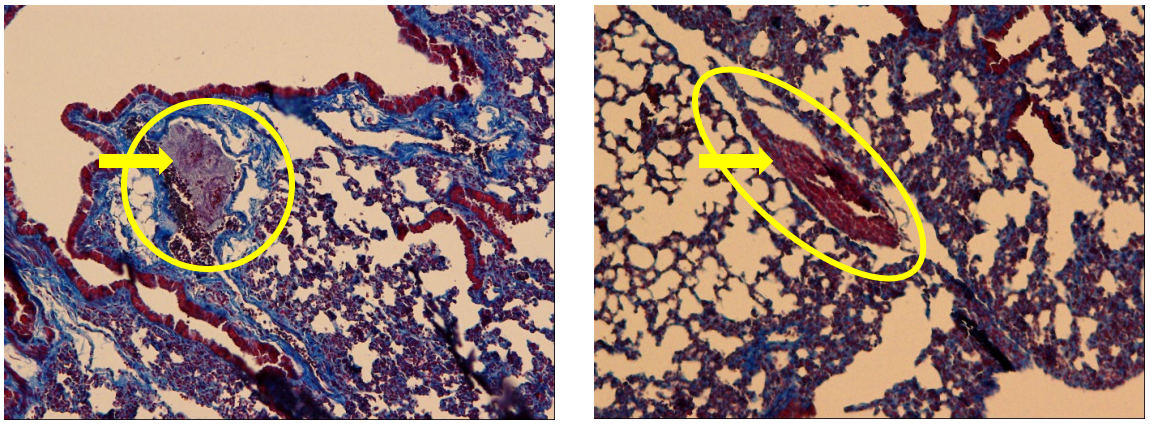

Figure 5: Carstair's stained images of sectioned lungs from dalteparin-treated mice.

Carstairs stain stains fibrin as red; the circles outline the pulmonary arteries with fibrin rich emboli shown by the arrows. Fibrin stains red and platelets stain blue.

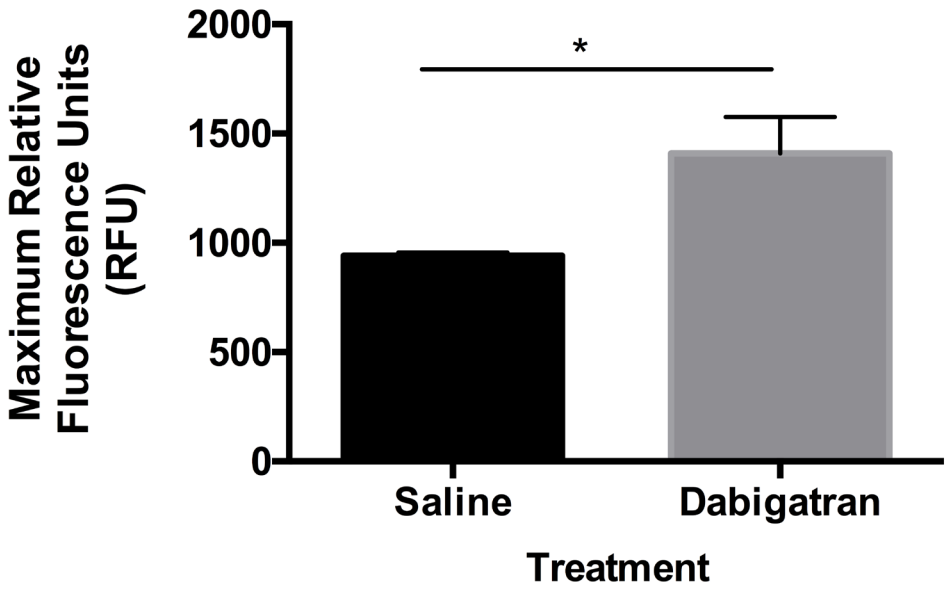

Figure 6: The graph shows maximum fluorescence units measured from homogenized lungs from WT saline- and dabigatran-treated mice after DVT and fluorescently tagged anti-fibrin antibody injection.

All values are expressed as mean ± SEM; n=3 for each treatment. *p-value<0.05, using an unpaired student's t-test.

6.4 CONTINUOUS THROMBIN GENERATION IN VIVO

In dabigatran-treated mice, the thrombus size remains the same over time, but the embolization and percent of DVT turnover is the highest. Thus there is continuous thrombus accretion and dissolution, and thus continuous thrombin generation (Chapter 3). These data suggest that thrombus formation is a dynamic process. This is demonstrated with dabigatran, once concentrations of active thrombin decrease, likely with less platelet activation and less fibrin formation, the thrombus starts to embolize. Then more thrombin is formed, because the injury, and whatever is initiating coagulation, has not been contained by fibrin and platelets, but that thrombus growth is unstable and it embolizes more (low concentrations of thrombin leads to thicker fibrin fibers that are weaker), and therefore more thrombin will be produced to repair the injury and this leads to the observed continuous thrombin generation. The analogy would be the same as that of "picking a scab". When there is an injury, you get the initial bleeding and then the scab or "clot" develops to stop the bleeding and repair the wound. Anticoagulants have been shown to impair wound healing[270,271]. When the scab is picked (embolization), bleeding will occur and the scab or clot will form again to repair the injury site. This was proven in Chapter 3 when dabigatran-treated mice exhibited highest DVT turnover suggesting that more thrombin might have been generated. F1+2 was also higher in WT dabigatran-treated mice compared to WT saline- or dalteparin-treated mice (Chapter 3 Figure 4B).

However, the question that remains is, where does thrombin come from if it is being inhibited from dabigatran? This can be explained by the residual thrombin hypothesis. Different antithrombin anticoagulants will inhibit different thrombin concentrations. Some anticoagulants are more potent than others, and therefore alter

thrombin concentrations differently. A potent thrombin inhibitor will inhibit thrombin, even at very low concentrations, and allow almost no thrombin activity (this would be done by a DTI, such as dabigatran). Whereas less potent inhibitors (such as FXa inhibitors) would inhibit thrombin formation, but still leave residual thrombin circulating. Less potent thrombin inhibitors (LMWH such as dalteparin) might also decrease thrombin formation but would leave very low concentrations of thrombin uninhibited. Thus, in the case of dabigatran, it may be that dabigatran being a potent inhibitor of thrombin, and since high levels of thrombin are needed to activate FXIII, FXIIIa generation is reduced significantly. In comparison to dalteparin, which is less potent, will inhibit thrombin, but there will still be enough thrombin to activate FXIII. We demonstrated this in vitro in chapter 4. This explains the reduced embolization and DVT turnover seen in the dalteparin-treated mice compared to the dabigatran-treated mice.

6.5 ACTIVATION OF FXIII AFTER REDUCED THROMBIN GENERATION

Thrombin has a number of substrates, and thrombin's Km for FXIII is one of the lowest[12]. Thus, even after administration of dabigatran and its inhibition of thrombin, there was higher prothrombin fragment F1+2 which was indicative of greater thrombin turnover. Thus, it may be that there is always enough residual thrombin to activate FXIII. As well, FXIIIa circulates in high concentrations, and α_2-AP bound to fibrinogen is found in the blood of healthy people[221]. This is likely from FXIIIa that is generated by "idling" concentrations of thrombin. Also, the activation peptide that thrombin cleaves off FXIII to make FXIIIa, inhibits FXIII activation, resulting in a relative deficiency[40]. In the contexts of anticoagulation, FXIII would be activated less, therefore increasing FXIII in

the context of thrombin inhibition would result in more FXIIIa activity. This also explains why administration of extra FXIII has an effect, as seen in Chapter 4.

6.6 HOW FXIII POLYMORPHISM VAL34LEU FITS OUR MOUSE MODEL

The common gene variant in FXIII-A encoding a valine (Val) to leucine (leu) substitution at codon 34 (Val34Leu) is present in ~25% of European Caucasians[272]. Findings on the role of this polymorphism in thrombosis have been inconsistent; however, both independent studies and meta-analyses suggest the 34Leu variant offers protection against venous thrombosis[39,71,273–276]. The FXIII-A Val34Leu polymorphism is located 3 amino acids before the thrombin cleavage site and therefore results in ~2.5 fold accelerated FXIII activation[63,277] and accelerated fibrin crosslinking[278,279]. Briefly, the normal range of fibrinogen in plasma is 1.5 to 4 g/L. In plasmas with low/normal fibrinogen concentrations, the 34Leu variant produced clots with thin fibrin fibers and low permeability. However, in plasmas with higher fibrinogen, it produces thicker fibers in clots that have increased permeability and susceptibility to fibrinolysis[280].

The presence of the polymorphism increases the rate of FXIII activation by thrombin. Thrombin cleaves the activation peptide from the Val34Leu FXII more rapidly than the WT FXIII[63]. It is still debated whether the cleaved activation peptide is released from FXIII upon activation or not. The polymorphism has been shown to reduce the risk of MI in multiple studies[64,234,281,282], the results of stroke studies are variable and not conclusive yet[283,284], and several studies demonstrated reduced risk of venous thrombosis[69,236,274,276]. Lastly the Val34Leu polymorphism has also been shown to alter fibrin clot structure[61]. There is enhanced γ-chain dimerization and α-chain polymerization

and these crosslinking mechanisms occur very early on compared to WT FXIII[279]. With turbimetric measurements showing shorter lag phase, thinner more porous fibers, why would these patients show reduced risk of venous thromboembolism? This could be explained by early crosslinking, and its effects on fibrin clot structure. This would result in a stable DVT without increasing the size, and thus reducing PE burden.

In another study, FXIII-A subunit levels have been shown to be lower in patients with extensive PE; the likelihood of PE is 7 times higher in patients with A subunit levels less than 60% of normal[213]. In patients with massive PE that had low FXIII-A subunit levels also had decreased clot firmness when measured by thromboelastography compared to normal of high patients[213].

So how does the polymorphism help explain our model of thrombus stability? The enhanced and early occurrence of γ-chain dimerization and α-chain polymerization seen in patients with Val34Leu polymorphism, could be resulting in an acutely formed thrombus that is "stable" (as seen in our model as well). This "stable" thrombus formation leads to reduced PE risk.

This can explain why we do not see a detrimental effect from the added FXIII in the FXIII supplemented mice (Chapter 4). It could be that extra FXIII is having the same effects as the polymorphism, where there is increased early crosslinking, and thus altering the effects on fibrin clot structure acutely. This makes the DVT stable without increasing the size and reduces the PE burden early on.

6.7 CONTRIBUTION OF FXIII TO THROMBUS STABILITY

FXIIIa activity influences the risk of embolization of venous thrombi as well as arterial thrombi. Platelet FXIIIa mRNA is significantly lower in patients with non-valvular arterial fibrillation and thrombus embolization than in similar patients with left arterial appendage thrombus and no history of embolization[213]. Accordingly, in mice, FXIIIa-mediated crosslinking of plasma fibronectin into thrombi formed in mesenteric arterioles enhances platelet aggregation and increased the stability of platelet-rich thrombi[285]. In our ferric chloride model of venous thrombosis, FXIII-deficient mice showed increased embolization compared to WT mice. Together these observations demonstrate critical FXIIIa contributions at several steps during thrombosis.

FXIII is unique among coagulation proteins, not only in the nature of its enzymatic activity that crosslinks, but also in its ability to directly impact both biochemical and biophysical properties of thrombi[38]. Growing data from many studies suggest FXIIIa is an important determinant of thrombus composition and stability.

6.8 MECHANISMS OF FXIIIa's ACTIONS

FXIII, a pro-transglutaminase found in plasma and platelets, plays multiple roles during thrombosis. During thrombosis, activated FXIII crosslinks fibrin, as well as α_2-AP to fibrin(ogen)[39]. Thus, FXIIIa is responsible for the mechanical stability of clots and also has an anti-fibrinolytic function[39]. As well, more recent studies have provided new information about FXIII activity during coagulation and its effect on clot composition and function[39].

6.8.1 FXIIIa MECHANISM OF CROSSLINKING

During coagulation, thrombin cleaves the N-terminal fibrinopeptides from the Aα - and Bβ – chains to produce fibrin monomers that polymerize into fibrin fibers[29]. FXIIIa catalyzes the formation of isopeptide bonds between glutamine residues 398/399 and lysine 406 in the fibrin γ-chain and subsequently between glutamine and lysine residues in the α-chain[39]. Fibrin crosslinking has a minor effect on the global network structure, but substantial effects on the structure and function of individual fibers, and consequently, mechanical stability and possibly clot composition[286].

6.8.2 THE EFFECTS OF CROSSLINKING ON FIBRIN STRUCTURE

Crosslinking has little effect on gross fibrin network morphology, producing only a minor (~12%) increase in fibrin network density. In contrast, crosslinking significantly alters the structure of individual fibrin fibers by promoting protofibril coupling within the fiber[207]. This effect, which is associated with the formation of α-chain-rich, high molecular weight (HMW) crosslinked species, is what causes clot compaction. Ryan *et al.*, showed that γ-chain crosslinking alone, is insufficient to stiffen fibrin clots, and that the increased stiffness is correlated with the formation of α-chain-rich HMW crosslinks[287]. Fiber compaction can also promote fiber stiffening, making fibers more resistant to deformation under low strain[207]. Since clot compaction decreases the size of pores within individual fibers, this may potentially have important effects on the diffusion of molecules like t-PA through the fibers, and fibrinolysis. Thus, the effect of FXIII on thrombus formation likely stems from its effects on the mechanical stability of fibrin fibers.

6.8.3 FXIII MECHANISM CROSSLINKING OF α_2-AP

FXIII has critical anti-fibrinolytic functions during coagulation, mediated by its ability to crosslink antifibrinolytic proteins, such as α_2-AP[22,288], TAFI[289], and type-2 plasminogen activator inhibitor (PAI-2)[290], to fibrin. The crosslinking of α_2-AP is essential for the inhibition of fibrinolysis, particularly by t-PA-induced plasminogen activation on the fibrin surface[291]. The crosslinking of α_2-AP to fibrin occurs rapidly. Maximal α_2-AP crosslinking is almost reached when α-chain polymerization has just started[228]. An unexplained phenomenon is that the crosslinking of α_2-AP stops at about 30% incorporation onto fibrin. Enhancing the concentrations of FXIIIa increases the rate of α_2-AP crosslinking but does not change the maximal incorporation[228]. It has also been suggested that FXIIIa not only accelerates the cross-linking of α_2-AP to fibrin but also accelerates the release of cross-linked α_2-AP from fibrin and that the latter activity explains the partial incorporation[225]. The mechanism by which this occurs is yet to be elucidated. Thus, the anti-fibrinolytic effect of FXIII via α_2-AP crosslinking is present, but likely does not play a similar role to its effects seen on increasing the mechanical stability of clots, however this is debated.

We also saw that in Chapter 4 (Figure 1D) a possible role for α_2-AP-linked to fibrinogen. In plasma from FXIII$^{-/-}$ mice supplemented with FXIII to normalize FXIII antigen levels, clot lysis times were shorter (23 mins) than in plasma from WT mice (35 mins). This could imply that α_2-AP linked to fibrinogen plays a role in resistance to fibrinolysis, likely formed by “idling” thrombin activating FXIII.

6.9 RELATIVE CONTRIBUTIONS OF FIBRIN CROSSLINKING AND α_2-AP CROSSLINKING

During blood clotting, FXIIIa introduces crosslinks within fibrin as well as between fibrin and α_2-AP. The relative contributions of these two types of crosslinks in the inhibition of fibrinolysis by FXIII has been thoroughly investigated. Jansen *et al*., studied the lysis rate of fresh whole blood clots containing t-PA that was added before clotting in vitro[292]. They reported that fibrin α_2-AP crosslinking explains the FXIIIa-induced resistance of blood clots to fibrinolysis, whereas fibrin-fibrin crosslinking has only a small, if any, influence. This was later confirmed by Fraser *et al*., who showed that the anti-fibrinolytic function of FXIII in plasma clots prepared in a Chandler loop and incubated in a buffer containing t-PA is independent of fibrin-fibrin crosslinking and is expressed exclusively though α_2-AP[22]. Reed and Houng studied t-PA-induced fibrinolysis in anaesthetized ferrets with pulmonary emboli and found, in contrast to the previous investigators, that both fibrin-fibrin and fibrin- α_2-AP crosslinking caused resistance to lysis[293].

From our *in vivo* mouse data in Chapter 4, α_2-AP administration after injury in WT mice did not alter thrombus size or embolic activity significantly compared with WT saline-treated mice. Thus, we suggest that α_2-AP crosslinked to fibrin does not have a large impact on clot stability and likely the anti-fibrinolytic effect of FXIII is primarily through crosslinking fibrin. In contrast, when looking at the clot lysis for α_2-AP data in Chapter 4, we saw that by increasing the amount of α_2-AP, the clot lysis times were shortened, suggesting that α_2-AP plays a role. The likely reason behind why our in vitro data does not mimic our *in vivo* data is because of inhibition of fibrinolysis by fibrin- α_2-

AP crosslinking occurs immediately after clotting, as these links are formed rapidly, which can be detected with an in vitro clot lysis but not in our model of thrombus stability that is two hours. This would also explain why α_2-AP had an effect on attenuating fibrinolysis in our clot lysis assays in Chapter 4 (Figure 1B) but that effect was not seen *in vivo* in our two-hour model of venous thromboembolism (Chapter 4 Figure 2 and 3). Alternatively, α_2-AP linked to fibrinogen might have a larger role than acute crosslinking of α_2-AP to fibrin during thrombus formation.

Significant inhibition of fibrinolysis by fibrin-fibrin crosslinks requires strong crosslinking conditions, resulting in very high molecular weight α -chain polymers and/or γ-chain trimers and tetramers, whereas significant inhibition of fibrinolysis by fibrin- α_2-AP crosslinks occurs immediately after clotting, as these links are formed rapidly. Therefore, it could be that the relative contribution of fibrin-fibrin crosslinks to the total inhibition of fibrinolysis by FXIII depends on the extent of crosslinking and increases with the age of the thrombus.

6.10 COMPARING EFFECTS OF α_2-AP AND EACA ON THROMBUS STABILITY

Secondly, popular belief suggesting that with increasing levels of FXIII, would lead to increased α_2-AP crosslinking to clots, and thus ultimately develop a clot resistant to any lysis by plasmin. In a paper published by Tamaki *et al*., they added increasing α_2-AP to fibrinogen and assessed crosslinking by SDS-PAGE and found that only found 40% of the potential α_2-AP had been crosslinked[228]. More interestingly, even at the highest

amount of FXIII, at 10 U/ml, there was still 50% lysis. Thus, they found that even with increasing FXIII levels, lysis by plasmin still occurs[228].

α_2-AP does circulate in plasma at higher concentrations than FXIII, but with physiological level of FXIII, only 30-50% of α_2-AP will be cross-linked to fibrin. Secondly, plasmin/ogen that has already been bound to fibrinogen or fibrin is protected from α_2-AP. Thus, when t-PA converts the plasminogen to plasmin, it will cleave C-terminal lysine residues on fibrin, creating fibrin. Since plasmin is not inhibited in this situation, plasmin has a higher affinity to fibrin' and more lysis occurs, irrespective of the presence of α_2-AP on the fibrin.

With EACA treatment (Chapter 4), physiological levels of FXIII and α_2-AP will perform their regular functions; in addition EACA inhibits fibrinolysis by inhibiting any plasmin-mediated cleavage. Thus, we have not only increased the mechanical strength of the clot through physiological levels of FXIII and α_2-AP, but also inhibited fibrinolysis via EACA administration. Therefore, with EACA administration, there will be less cleavage of the C-terminal lysine residues than seen in the FXIII-supplemented mice. Therefore, the balance between thrombin formation and lysis is disrupted more in EACA-treated mice and that explains why we see the significant thrombus growth over time than compared with FXIII-supplemented mice.

6.11 THROMBUS STABILITY AND ITS RELATIONSHIP TO INCREASING POST-THROMBOTIC SYNDROME

In Chapter 4, we have seen in our studies that increased FXIII through supplementation does not increase DVT size or increase PE burden in mice. The

relationship between thrombus stability and PTS has never been studied. Evidence suggests that a slow rate of thrombus resolution has been linked to increasing risk of PTS. However, the link between thrombus stability and thrombus resolution has never been studied. Does a stable thrombus mean it will take longer to resolve? We do not know. If that were true, PC, PS, or AT deficient patients who are prothrombotic and thus likely form stable clots, would be at increased risk of PTS and that has not been reported. These thrombophilias, per se, do not increase the incidence of PTS.

6.12 FACTOR V LEIDEN PARADOX

Clinical studies have shown that approximately 90% of the pulmonary emboli arise from thrombi in the deep veins of lower limbs[246,294]. Moreover, asymptomatic PE can be found in about half the patients presenting with DVT[295]. Therefore, in general DVT and PE are considered as two entities of a single disease and referenced to as venous thromboembolism.

However, several studies have repeatedly shown that carriers of the factor V Leiden (FVL) mutation have a significantly increased risk of DVT, whereas the risk of pulmonary embolism is only mildly increased compared with non-carriers[246]. After the discovery of the FVL mutation, it was hypothesized that the presence of FVL would often lead to fatal PE, resulting in lower number of FVL-positive subjects among those surviving PE[248,249]. In turn, this would explain the weak effect of FVL on the risk of PE found in studies of survivors on venous thrombosis (case-control studies). However, this hypothesis was rejected as autopsy studies have shown that among patients with fatal PE,

the proportion of individuals with FVL was no different from that in PE survivors or from that in the general population[248,249]. Thus, FVL may not be a risk for PE.

The differential effect of FVL on DVT and PE is known as the FVL paradox. So far, few studies have investigated mechanisms that could lead to FVL paradox[244–246]. Studies have sought to investigate several potential explanations for the mechanism such as (i) thrombus location, (ii) number of affected veins (iii) time until diagnosis (iv) growth speed, and (v) clot density[246]. None of these mechanisms presented a solid explanation for the mechanism of the FVL paradox.

A newer hypothesis proposes that FVL may enhance local thrombin generation and intensify the local inflammatory process against the thrombus, and strengthen the clot structure by activation of thrombin-induced FXIII activity[246]. However, the similar risk of DVT and PE observed for all other thrombophilias such as antithrombin, protein C or S deficiency, except FVL, makes this hypothesis unlikely[296]. A second commonly proposed hypothesis involves an antifibrinolytic effect. Bajzar *et al*., showed that in FVL heterozygotes there is an impaired TAFI-dependent profibrinolytic response to activated protein C (APC)[245]. Parker *et al*., later confirmed these results and showed that pulmonary clot lysis of radiolabeled, clots infused through the jugular vein had less lysis in FVL homozygous mice compared to WT, supporting the hypothesis that FVL inhibits fibrinolysis[244]. However, FVL carriers do not show an increased risk of post-thrombotic syndrome[250,251], which would be present if they had an increased resistance to fibrinolysis.

With our novel mouse model of venous thromboembolism, we were able to show that thrombus stability explains the FVL paradox. With a lower $FeCl_3$ injury that results in an initial small thrombus in WT mice, larger thrombi develop in FVL mice

(heterozygous and homozygous). Also, in the FVL mice, the thrombus grows and stabilizes over time, whereas in the WT mice the thrombus embolizes and does not grow. Thrombus stability is inversely related to embolization and PE burden. Therefore, this suggests that those with FVL form a larger stable thrombus compared to normals, thus leading to a symptomatic DVT but not an increased risk of PE.

6.13 PROPOSED MECHANISMS OF THROMBUS STABILITY

The proposed mechanism is that thrombin inhibitors reduce thrombin concentration which in turn reduced the amount of thrombin-induced FXIIIa generation, and clots that are not crosslinked become more susceptible to fibrinolysis. Further studies need to look at other factors that alter thrombus stability beyond what is shown in this thesis such as TAFI, thrombin concentration, and effect of blood flow.

6.13.1 POSSIBLE WORKING MECHANISMS: PART 1

The correlation between DVT and PE, and progression of DVT to PE is not fully understood since it varies from patient to patient and with different clinical conditions. Questions that are left unanswered are: How does DVT correlate with PE? Is it a continuum of the same disease? If so, how does DVT stability affect this? And lastly, how do treatments of the disease alter this stability? Alternatively, what alters embolization and how can we design therapies in order to reduce PE-associated mortality.

From Chapter 3, we found that with WT dalteparin- and dabigatran- treatment decreased the stability of the deep vein thrombus, increased embolization, and PE risk/burden compared to WT saline-treated mice. This is also consistent with clinically

data from Caps *et al*, Krupski *et al*, Kakkar *et al*, and Kashtan *et al*, who have all looked at the progression of DVT in patients from the first day of diagnosis using serial venous duplex scans[159–162]. They found that most patients who had inadequate anticoagulation treatment immediately show progression of DVT as extension. During the acute phase of therapy, Caps *et al*, saw that the risk of recurrent thrombus formation was significantly increased[159]. This may suggest that acute treatment of DVT may lead to decreased thrombus stability, but the mechanism of how DVT instability accounts for this extension remains unanswered.

Figure 7, shows the breakdown of results from chapter 3. In the 'pre-treatment stage', we formed a thrombus before any treatment is administered, that was relatively similar between treatment groups. During the 'treatment stage,' when treated with either saline, or dalteparin or dabigatran, there is no change in thrombus size (indicated by the dashed arrows) that was observed after treatment. However, there was an effect on embolization and PE risk. Thus, this suggests that treatment with dalteparin and dabigatran have an effect on embolization, and this is independent of thrombus size. It is not the size of the clot that plays a role in the amount of embolic activity but rather something else, thus we can alter embolization without altering thrombus size. Thus, the effects of the anticoagulants is on thrombus stability. Thrombus stability is determining the embolic activity independent of thrombus size.

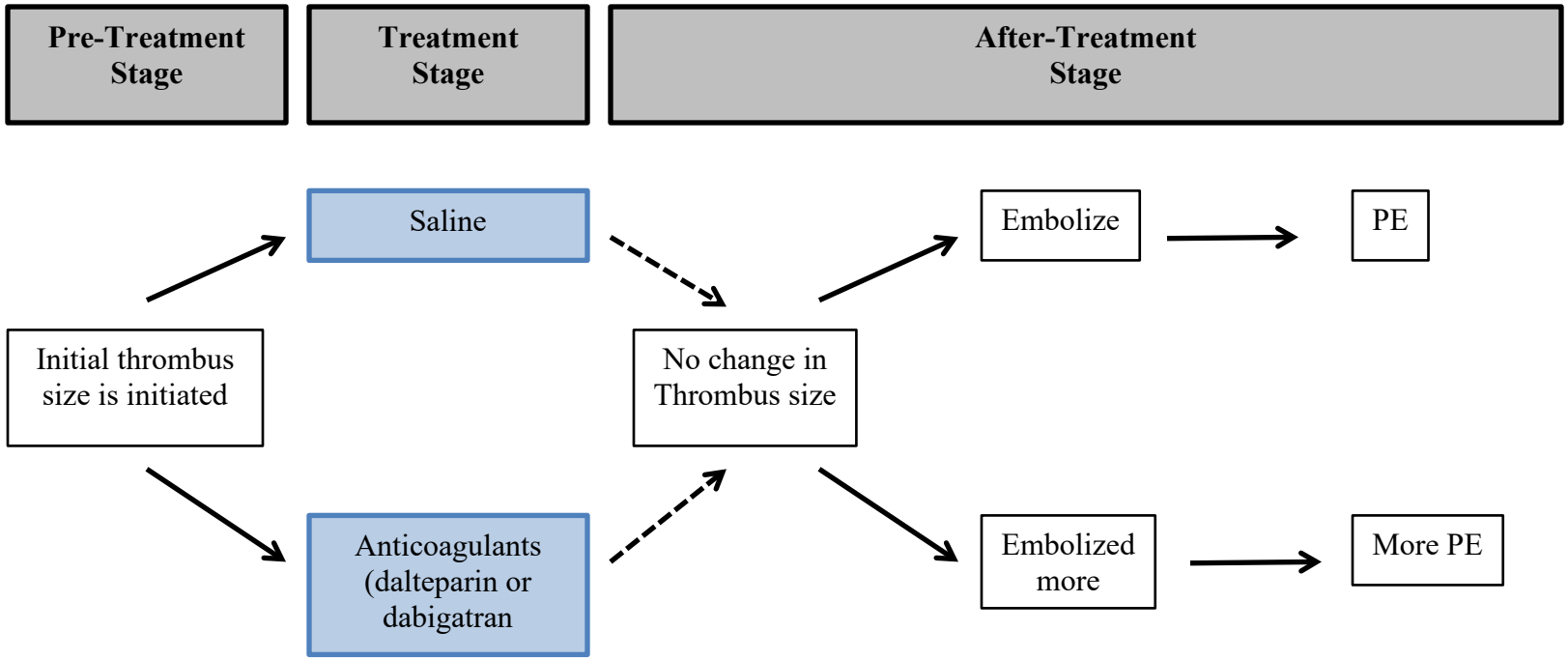

Figure 7: Outline of experimental results from Chapter 3.

A thrombus was induced before treatment was administered. Next, either saline, dalteparin or dabigtran was administered. After treatment, there was no change in thrombus size in any of the treatments (dashed line). However, treatment with dalteparin or dabigatran increased embolization and PE compared to saline (solid line)

This leads to the beginning of our working mechanism where embolization is inversely related to thrombus stability and thrombus stability is inversely related to pulmonary embolism. This means that an increase in embolization means decreased thrombus stability, which leads to increased pulmonary embolism (Figure 8). This can also be phrased that thrombus stability is inversely related to embolization and PE. Thus, a decrease in thrombus stability will lead to increased embolization and PE.

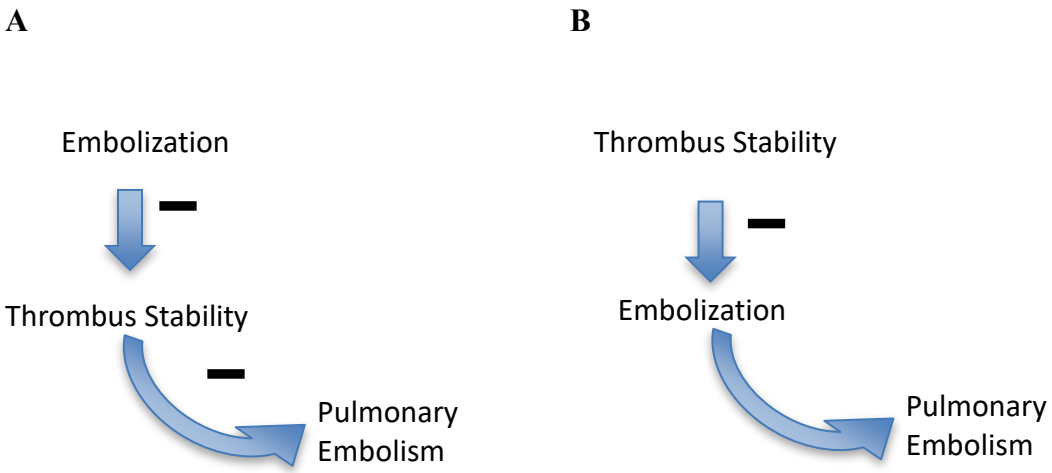

Figure 8: Thrombus stability is inversely related to embolization and PE.

The figure shows the start of our thrombus stability mechanism, which can be shown in two ways. (A) Embolization is inversely related to thrombus stability, which is inversely related to PE or (B) thrombus stability is inversely related to embolization and PE.

Next, in chapter 4, when supplementing with FXIII we saw a decrease in embolization and PE burden, but no change in thrombus size. In chapter 3, with $FXIII^{-/-}$ mice, there was an increase in embolization and PE, but no change in thrombus size. Thus, our mechanism still holds true when FXIII is lacking (Chapter 3) or supplemented (Chapter 4) (Figure 9). Thus, our initial mechanism of thrombus stability being inversely related to embolization and PE still holds true.

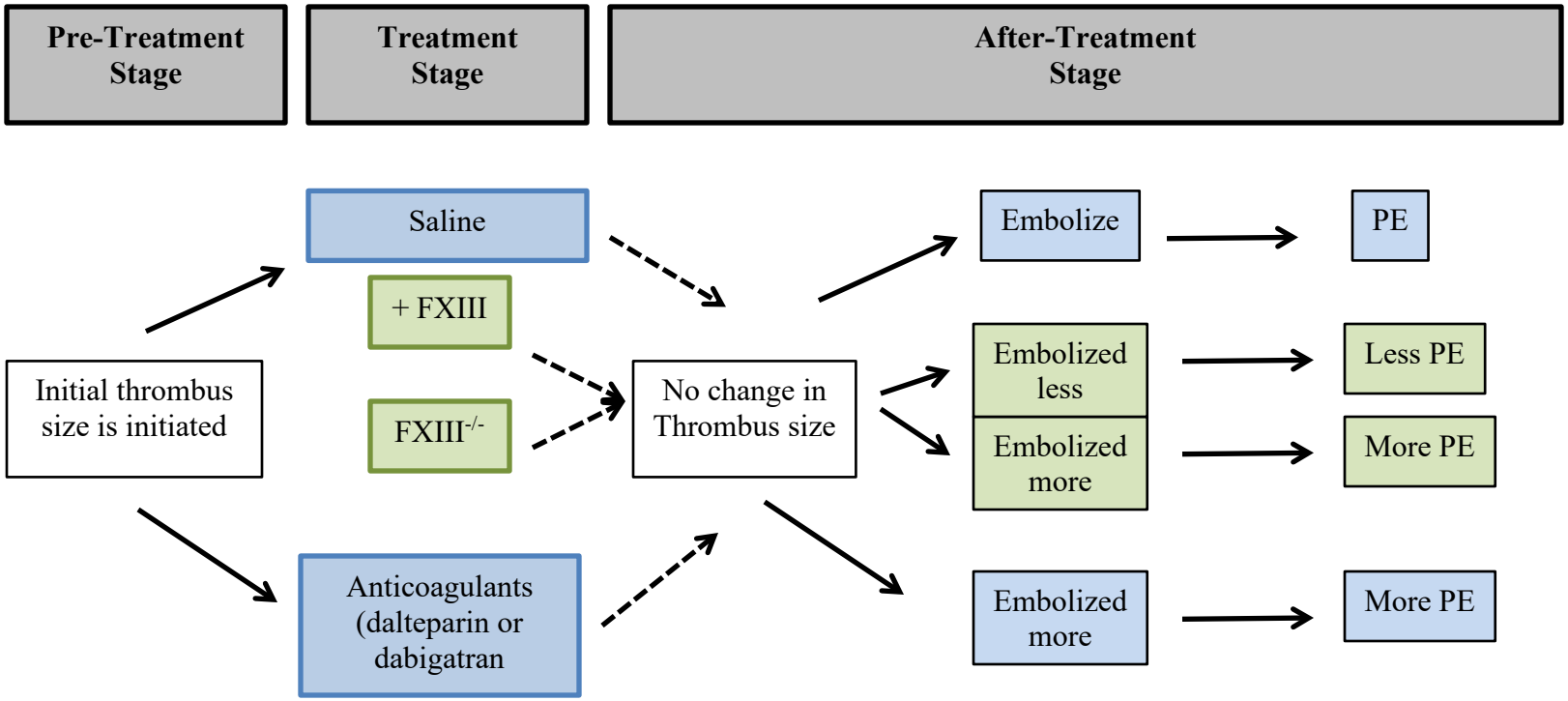

Figure 9: Experimental results from FXIII supplemented and FXIII$^{-/-}$ mice.

When mice were treated with FXIII supplementation (green box), no effect on thrombus size was seen (dashed line), but embolization and PE decreased (solid line). The opposite was observed for the untreated FXIII$^{-/-}$ mice.

6.13.2 POSSIBLE WORKING MECHANISMS: PART 2

What is the mechanism of how anticoagulants are affecting embolization and not size? We investigated this mechanism by administering dalteparin or dabigatran to both $FXIII^{-/-}$ and FXIII supplemented mice and found that, thrombin had a direct effect on embolic events through a FXIII mechanism. Thus, thrombin's effect on FXIII activation plays a role in altering thrombus stability directly (Figure 10). This information expanded our mechanism to suggest that thrombin's effect on FXIIIa is inversely related to embolization, which is inversely related to the stability of a thrombus, and pulmonary embolism. Thus decreased FXIIIa will reduce embolization and increase thrombus stability, leading to less PE burden.

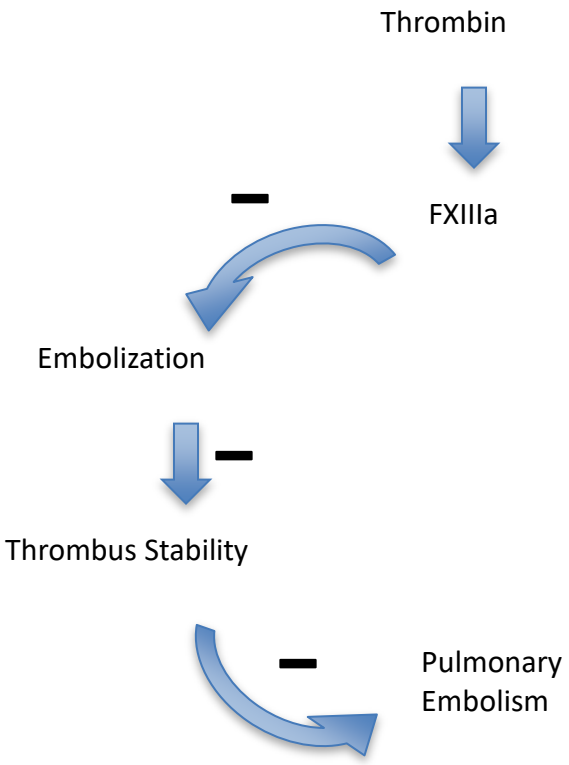

Figure 10: FXIII alters embolization, which alters thrombus stability and PE.

6.13.3 POSSIBLE WORKING MECHANISMS: PART 3

Next, we studied the effect of fibrinolysis, more specifically attenuating fibrinolysis using EACA, and compared its effect on thrombus stability to the previous results. In Chapter 4 we saw that when EACA was administered, it increased the thrombus size and significantly reduced total and large embolic events (Figure 5).

So now, the question is does the treatment (EACA inhibiting fibrinolysis) have a direct effect on the embolization, and with reduced embolization, the thrombus size grows? Or does the treatment (EACA inhibiting fibrinolysis) have a direct effect on thrombus size, and with increased thrombus size, the thrombi embolize less?

If we believe the first to be true, then this does not explain why there was no change in thrombus size in the FXIII-supplemented mice who also had reduced embolization and PE burden (Figure 11). If we believe the second to be true, then treatments that alter the size of the thrombus, alters embolization, as well as thrombus size would be correlated to embolization. However, this is not the case as seen from Chapter 3 in the dalteparin and dabigatran treated mice. The thrombus size in these mice does not correlate with embolization (Chapter 6 Figure 6).

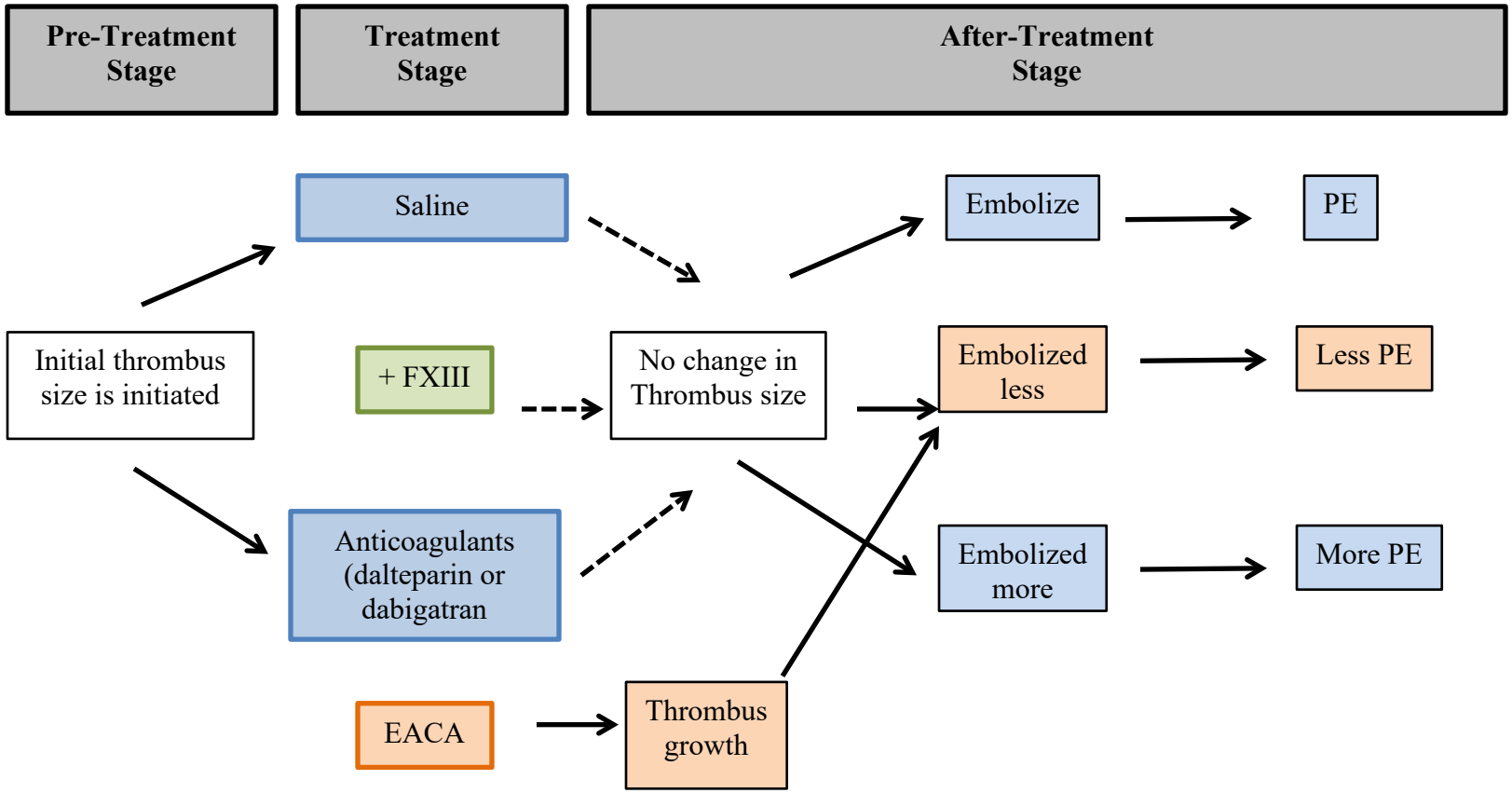

Figure 11: Experimental results from chapter 4 with EACA treatment.

Treatment with EACA (orange) had a direct effect on thrombus size (solid arrow). The thrombus was larger, and there was reduced embolization and PE burden.

Thus, this suggests that thrombus stability is likely to be determined by both thrombus size and embolization, or collectively, the composition of the thrombus. If the thrombus is large, it does not mean it will embolize less and be more stable, and if the thrombus is small it does not mean it will embolize more because it is less stable. As well, if a thrombus is embolizing frequently, it does not mean the thrombus is unstable and will shrink, and if the thrombus is not embolizing frequently, it does not mean it is stable and will grow. What is determining the thrombus stability is how the treatments are altering the thrombus composition and thus its effect on embolization and thrombus size will determine the stability of a thrombus (Figure 12).

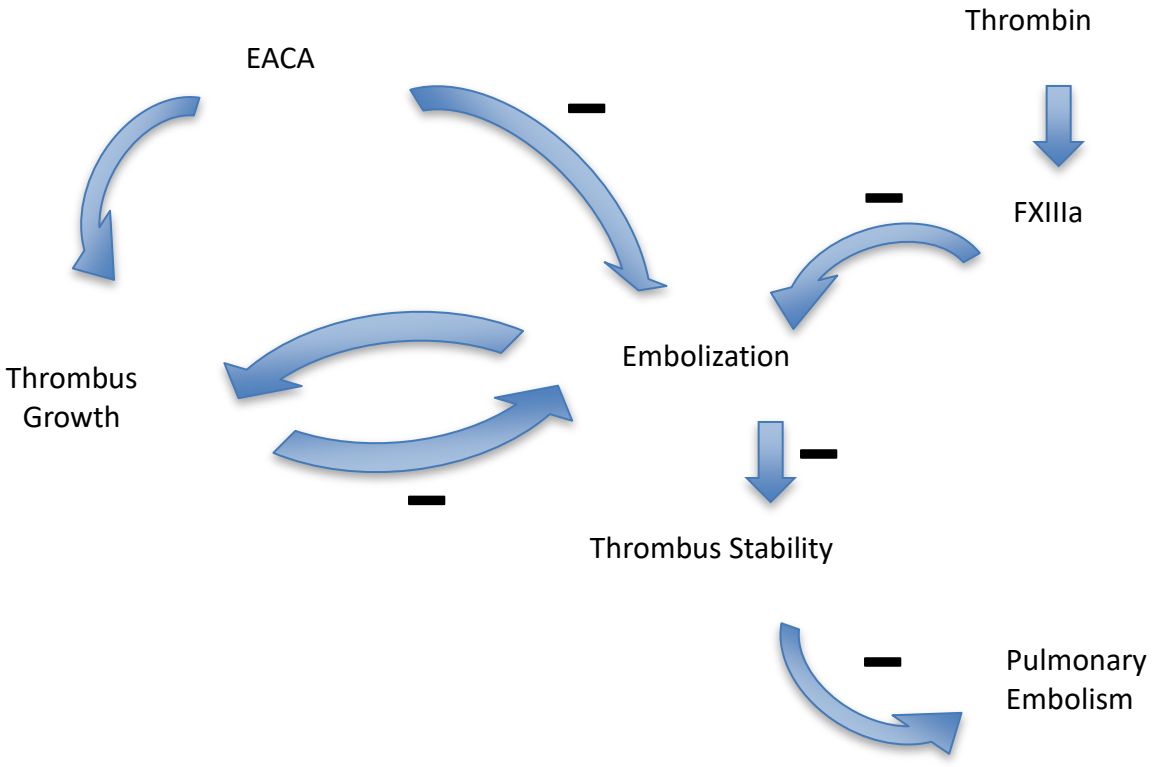

Figure 12: Factors altering thrombus stability.

6.14 OVERALL PROPOSED MECHANISM OF THROMBUS STABILITY

Our final proposed working mechanism model of thrombus stability is shown in Figure 13. We found that treatment with FXIII and dalteparin and dabigatran have more of an effect on the mechanical stability of the clot (blue arrows). These treatments altered mechanical stability of the clot via the FXIII-pathway. Treatment with EACA which increased thrombus size and reduced embolization and PE burden (not seen with FXIII supplementation), means it altered thrombus stability differently, likely through an antifibrinolytic mechanism (green arrows). Embolization and PE burden, are affected and altered by both i) the mechanical effects of FXIII (blue loop) as well as ii) the anti-fibrinolytic pathway (green loop). While these two pathways converge and have similar effects in reducing embolization and PE burden, the anti-fibrinolytic pathway (via EACA), also increased thrombus size significantly. Thus, the mechanism on which they alter embolization and PE burden differ. Altering mechanical strength of the clot has a different effect on thrombus stability than altering fibrinolysis because we do not see an effect on thrombus size in the mechanical pathway. Thus, these pathways are shown to be separate.

Regardless of the two pathways' differing effects on clot size and composition, they are likely still linked in many ways. For example, thrombin not only influences the mechanical loop of clot stability via FXIII activation, but thrombin concentrations have also been shown to alter fibrinolysis (as discussed before) and thus likely have a direct effect on altering the anti-fibrinolytic loop. Secondly, thrombin concentrations change the fibrin composition and the mechanical stability of clots by altering the rigidity, stiffness,

and elastic modulus measurements (also discussed previously in the chapter 1). Thus, thrombin likely also alters thrombus stability via the mechanical loop but not necessarily through FXIIIa alone. Thirdly, thrombin, when bound to thrombomodulin, will activate thrombin activatable fibrinolysis inhibitor (TAFI) which will alter embolization and PE burden through the anti-fibrinolytic pathway as well[297]. Lastly, FXIII not only alters the mechanical properties of clots, but does also have an effect on making clots resistant to fibrinolysis. Whether this is the predominant role of FXIII is still debated and discussed in the above sections of this chapter. Therefore, although our results are consistent with the mechanism depicted in Figure 13, there are more factors that play a role in determining thrombus stability and many inter-linking mechanisms that yet need to be studied.

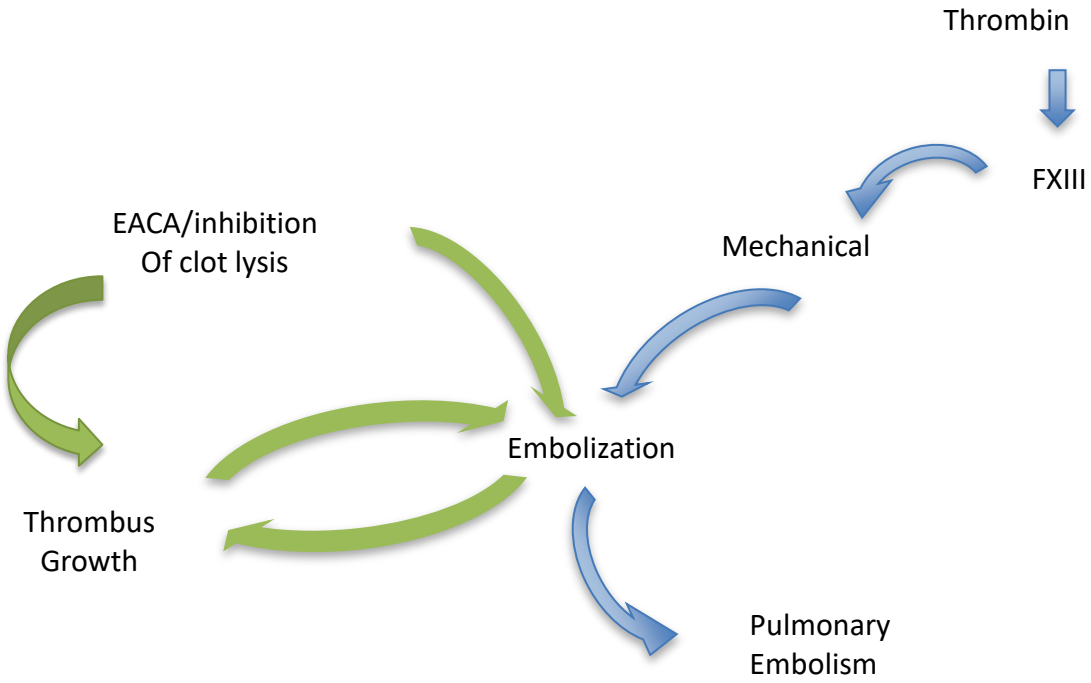

Figure 13: Mechanisms of thrombus stability.

Thrombus stability can be affected by altering (i) the mechanical strength of clots (via FXIIIa role in crosslinking; blue pathway) and (ii) by the anti-fibrinolytic pathway (via EACA; green pathway). Thrombin has an effect on FXIII activation, and this will alter the mechanical strength of clots and thus determine the embolization and PE burden. EACA inhibits fibrinolysis, and thus alters embolization and thrombus growth.

6.15 POTENTIAL IMPLICATIONS OF THE FINDINGS IN THIS THESIS

6.15.1 ALTERNATIVES TO IVC FILTERS

In patients with DVT and severe bleeding, anticoagulation is contraindicated and an inferior vena cava (IVC) filter might be placed to minimize PE[298,299]. IVC filters are associated with high morbidity[300–303] and in 2008 the American College of Chest Physician (ACCP) limited their use to lower extremity DVT in whom anticoagulation therapy is not possible because of the risk of bleeding[298]. Despite this guideline, IVC filter placement has increased dramatically over the decade[304], especially retrievable filters[305]. Approximately 80% of retrievable IVC filters are not removed from patients with DVT when bleeding has ceased[306,307]. Filters that remain *in situ* are associated with clinically important adverse events[305,308–310]. Filters are also being used for prophylactic use in the absence of bleeding and in the presence of anticoagulants without any evidence to support reduction in VTE-related mortality[304,311–313].

The use of IVC filters in addition to anticoagulation has been studied[314,315]. In the 1998 PREPIC study, patients with DVT were anticoagulated with enoxaparin or unfractionated heparin and either received IVC filters or did not[316]. Placement of an IVC filter reduced PE only within the first 12 days, but there was no difference in the rate of PE by the second year of follow-up[316]. In fact, in the filter arm of the study, filters that remained in place led to increased VTE recurrence[316]. In 2012 Barginear *et al*., investigated the benefit of IVC filters with fondaparinux, a FXa inhibitor, in cancer and VTE patients[317]. They found no added benefit from the use of IVC filters in terms of

safety, recurrent thrombosis or PE[317]. Finally, in the 2015 PREPIC2 study, the effect of retrievable filters plus anticoagulation in patients with acute pulmonary embolism was studied; filters did not reduce the risk of symptomatic recurrent pulmonary embolism at three months[318]. Yet, despite the lack of evidence for their benefit, IVC filters are still used in addition with anticoagulants by physicians because there is no alternative treatment to help minimize PE and its sequelae, especially when anticoagulation is contraindicated. This does vary by country. An ideal treatment for these patients would stabilize clots, minimize embolization, and reduce PE burden without causing further bleeding.

In chapter 4, we have shown that supplementing FXIII or treating with EACA will increase DVT stability, decrease embolization and PE burden. However, EACA increases thrombus size by 1.8-fold, whereas supplementing FXIII does not. Thus, EACA would not be a viable option for DVT patients in which anticoagulation is contraindicated. Nonetheless, when saline-, dalteparin-, or dabigatran-treated mice were supplemented with FXIII, embolization and PE burden was also reduced. However, with FXIII supplementation, thrombus size, which showed marginal growth, was not significantly different. These data suggest that FXIII supplementation is better at stabilizing DVT and may be a better option for DVT patients who have contraindications to anticoagulants, or to reduce the risk of PE in patients on anticoagulants; this needs clinical confirmation.

IVC filters, despite their extensive list of complications, are the only treatment option for DVT patients who have contraindications to anticoagulants. However, IVC filters are also used with anticoagulants, even though this is not a recommended

indication, because physicians most likely feel that the risks may be discounted in patients who present with less strictly defined indications such as i) a large clot burden, ii) medically unstable patients, and iii) those deemed at increased risk of recurrent PE or anticoagulant-related bleeding. Ultimately, the ideal treatment would reduce acute PE, as IVC filters do, without the associated complications. These patients would benefit from a treatment that could minimize embolization by stabilizing the DVT and prevent further bleeding, even with anticoagulation. We have shown that treatment with EACA, while decreasing PE burden, increases clot size, which is not ideal. FXIII supplementation, even with dalteparin or dabigatran, stabilizes the DVT without increasing clot size and reduces the PE burden. Hence, supplemental FXIII should be studied as an alternative treatment to reduce PE-associated mortality than IVC filters for DVT patients, including those who have contraindications to anticoagulants.

It is important to note that supplemental FXIIII has been used to decrease bleeding in cancer and trauma patients[54,55]. When supplemental FXIII was given during cardio-bypass surgery or in cancer surgery there were no adverse effects or new thrombosis in these patients[55]. However, in the case of EACA, there is literature suggesting that thrombosis is a side effect of anti-fibrinolytic use during trauma[319]. In contrast, the CRASH-2 clinical trial showed that administration of TXA reduced mortality in bleeding trauma patients with no apparent increase in side effects[320].

6.15.2 FVL AS A MODEL OF OTHER DETERMINANTS OF THROMBUS STABILITY

Much like the FVL paradox, there should be other genetic variants, with lower prevalence, that determine whether a patient might get a PE rather than a DVT. This is important, as it would help in understanding the biology of thrombus stability and allow us to reasonably predict which patients might be at risk for PE, rather than DVT. An approach that could help uncover these genetic variants would be with the use of the UK Biobank.

UK Biobank, a powerful resource to improve public health, is a large-scale biomedical database that is enabling new scientific discoveries to be made that improve public health. The UK Biobank provides access to medical and genetic data from half a million volunteer participants to improve our understanding o the prevention, diagnosis, and treatment of a wide range of life-threatening illnesses. This is the largest, most detailed and openly accessible research resource of its kind. The UK Biobank recruited >500,000 participants from the United Kingdom between 2006 and 2010. Age at baseline examination ranged between 40 and 69. Individual completed extensive questionnaires about sociodemographic, lifestyle, and health-related factors and completed a range of physical measure. From the 500,000 participants; whole genome sequencing has been completed on 50,000. The UK Biobank Showcase currently reports 15,000 VTE cases, and the ability to search for diagnosis of PE (code 20002) and DVT (code 6152).

6.16 FUTURE DIRECTIONS

This thesis has outlined the development of a clinically relevant mouse model for assessing venous thrombus stability, characterized by embolization and PE burden. We have just begun to piece together the mechanism of thrombus stability, which factors alter embolization and PE burden, and how it all connects. There are still many questions and lots of experiments that can be done to properly decipher the overall mechanism.

Acute treatment with the DTI dabigatran was found to decrease thrombus stability, and this was FXIII-dependent. Additionally, we found that FXIII supplementation can stabilize thrombi without increasing thrombus size because increasing the mechanical stability of the clot will allow for small amounts of embolization to occur and therefore the balance between thrombus growth and embolization is maintained. However, FXIII can crosslink α_2-AP and create covalent links between fibrin α-α, α-γ., γ-γ chains. It is unclear which of these mechanisms that FXIII stabilizes thrombi. Comparison of thrombus stability in the FXIII- and α_2-AP -deficient mouse would be of interest to tease apart the contribution of fibrin cross-linking and α_2-AP cross-linking to thrombus stability. Alternatively, this could be tested using an inhibitory antibody against α_2-AP or with α_2-AP knockdown using siRNA or an ASO. If thrombus stability in α_2-AP$^{-/-}$ mice was the same in WT mice, then this will imply that α_2-AP has no role in FXIII's ability to stabilize thrombi. Comparing thrombus stability between FXIII$^{-/-}$ and α_2-AP$^{-/-}$ would help decipher which of FXIII role is more important. For instance, if thrombus stability was worse in FXIII$^{-/-}$ mice than compared to α_2-AP$^{-/-}$ this would imply that the crosslinking is more important than FXIIIs role on α_2-AP crosslinking.

We have recently also shown that mice with Leiden mutation ($F5^{L/+}$ and $F5^{L/L}$) have greater thrombus stability and reduced PE compared with WT mice. With this model, we can investigate how much FVL is required to stabilize clots. FVL heterozygous mice, with 50% FVL, have stable thrombi; would lower FVL destabilize thrombi, and if so, how low? One way to explore this aim would be to infuse various amounts of mouse FV or mouse FVL into WT mice and use our established VTE model to induce a DVT and quantify PE and thrombus size.

Furthermore, TAFI levels have been correlated with the risk of thrombosis. However, the role of TAFI in VTE remains uncertain. A potential future direction would be to utilize WT and $TAFI^{-/-}$ using the same study design as described in the thesis to understand how it alters thrombus stability. It would be interesting to see if $TAFI^{-/-}$ mice would alter thrombus size. This would be important since it would help validate our working mechanism of thrombus stability. The effect of TAFI have been looked at by Chessum *et al.*, and they found that $TAFI^{-/-}$ mice had increased thrombus size, but embolization was also increased compared to WT[269].

While our ferric chloride model allowed us to study embolization in non-occlusive thrombi for 2 hours, this study can be repeated in an electrolytic model to validate our venous thrombosis model and our results. This is important since findings in animal models tend to be recognized as model specific. A requirement for non-occlusive thrombi to be formed, so that embolization can be imagined and thus quantified, limits our choices to the electrolytic model, which results in PE that is FXIII-dependant. Briefly, a direct current is sent through stainless-steel microsurgical needle which is placed on the

exposed femoral vein generates a thrombi. Thrombi can be formed in WT and FXIII$^{-/-}$ mice with or without treatments used throughout the thesis to see if we can duplicate our finding in this model.

Another interesting factor that likely plays a role, which we have not discussed yet, is blood flow. Flow, which delivers platelets and other factors to the site enabling thrombus growth, and subjects the growing thrombus to shearing forces, the removal of individual to small groups of platelets causes large scale embolization- have an impact on clot stability. The very high shear rates that exist in stenosed coronary arteries have great impact not only on the growth rate but also on the structure and the stability of the growing platelet thrombus. In contrast to erythrocyte-rich thrombi formed at low shear (125-250 s-1), thrombi formed at high shear rates have a thicker shell, a more densely packed platelet core, and an increased susceptibility to embolization[322]. How all this plays in our model of thrombus stability have yet to be tested.

CHAPTER SEVEN: BIBLIOGRPAHY

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