The role of thrombin-activatable fibrinolysis inhibitor and sex on thrombus stability and pulmonary embolism in murine venous thromboembolism

The role of thrombin-activatable fibrinolysis inhibitor and sex on thrombus stability and pulmonary embolism in murine venous thromboembolism

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TITLE:	The role of thrombin-activatable fibrinolysis inhibitor and sex on thrombus stability and pulmonary embolism in murine venous thromboembolism
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Lay Abstract

Venous thromboembolism (VTE) encompasses clotting within a major vein (deep vein thrombosis; DVT) and clot fragmentation that accumulates within lungs (pulmonary embolism; PE). Primary VTE treatment is anticoagulation that prevents progression and recurrence, at the cost of increased risk of bleeding. Alternate approach is to accelerate clot breakdown, or fibrinolysis. Thrombin-activatable fibrinolysis inhibitor (TAFI), when activated (TAFIa), suppresses fibrinolysis. How sex affects VTE is also uncertain. Therefore, this study aims to investigate how (a) sex, and (b) the loss of TAFI affects DVT stability and consequent PE. Overall, female mice showed more unstable thrombi that also embolized more than males. However, PE burden did not mirror this trend. Loss of TAFI in both males and females exacerbated PE burden, whereby the emboli contained greater fibrin composition in mice. Thus, VTE treatment involving TAFIa activity reduction to enhance fibrinolysis may require further stabilization of the thrombi to reduce PE risk.

Abstract

Thrombin-activatable fibrinolysis inhibitor (TAFI) levels correlate with the risk of thrombosis. However, the role of sex or TAFI in venous thromboembolism (VTE) remains uncertain, this study determines the effect of sex and TAFI on thrombus stability using a mouse model of VTE. $FeCl_3$-induced thrombi were formed within femoral veins of male and female wild-type (WT) or TAFI-knockout (*Cpb2*$^{-/-}$) mice. Thrombi were imaged at 10-minute intervals over 2-hours using fluorescent intravital videomicroscopy to quantify embolization and thrombus size. Lungs were examined by histology to identify quantity and composition of pulmonary emboli (PE) within pulmonary arteries/tissues. Emboli are considered large when >4 standard deviations above the average. Compared with WT mice, thrombi in *Cpb2*$^{-/-}$ mice exhibited 3.2-fold and 1.3-fold greater embolization for males and females, respectively. When comparing by sex, female mice embolization events were 7.9-fold and 3.1-fold greater than male WT and *Cpb2*$^{-/-}$ mice, respectively. Male *Cpb2*$^{-/-}$ displayed a 10.3-fold greater thrombus size increase compared with male WT mice, while the opposite was true for female mice (5.8-fold greater in WT). Between WT, females had greater thrombus size increase (4.8-fold), while the opposite was true in *Cpb2*$^{-/-}$ males (12.4-fold). TAFI deficiency led to a 2.2-fold and 2.5-fold increase in PE burden in males and females, respectively, while sex had no influence. Quantitation demonstrated that lungs of female *Cpb2*$^{-/-}$ mice contained pulmonary emboli with higher fibrin composition compared with WT. Clot lysis times remained similar between male and female WT mice using nascent plasma. Similarly, no differences were observed in circulating TAT concentrations between mice using plasma isolated after imaging. Overall, Sex affects

venous thrombus stability, with female mice at increased risk of embolization. Removing TAFI/TAFIa significantly increases PE burden in males and females and increases PE fibrin composition. Inhibition of TAFI may lead to unstable thrombi and increased PE, particularly in females.

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VTE	Venous thromboembolism
DVT	Deep vein thrombosis
PE	Pulmonary embolism
TM	Thrombomodulin
EPCR	Endothelial protein C receptor
PSGL-1	P-selectin glycoprotein ligand-1
TF	Tissue factor
vWF	Von Willebrand Factor
RBCs	Red blood cells
AT	Antithrombin
TAT	Thrombin-antithrombin complex
CTEPH	Chronic thromboembolic pulmonary hypertension
HIT	Heparin induced thrombocytopenia
rt-PA	Recombinant tissue-type plasminogen activator
PTS	Post-thrombotic syndrome
u-PA	Urokinase plasminogen activator
Fn’	Plasmin-modified fibrin
FDPs	Fibrin degradation products
PAI-1	Plasminogen activator inhibitor – 1
α_2-AP	α_2–antiplasmin
TAFI	Thrombin activatable fibrinolysis inhibitor
TAFIa	Activated thrombin activatable fibrinolysis inhibitor
T-TM	Thrombin-thrombomodulin complex
PTCI	Potato tuber carboxypeptidase inhibitor
GEMSA	2-guanidinoethylmercaptosuccinate
MERGEPTA	2-mercaptomethyl-3-guanindinoethylthiopropannoic acid
ε-ACA	ε-aminocaproic acid
CPN	Carboxypeptidase N
WT	Wild type
IVC	Inferior vena cava
IVM	Intravital video microscopy
DAPI	4′,6-diamidino-2-phenylindole
TNK-tPA	Tenecteplase
PPP	Platelet-poor plasma
EE	Embolic events
RFU	Relative fluorescence units
LT	Lysis time
GH	Growth hormone

Declaration of Academic Achievements

James E. Chessum contributed to the development of the study, obtained scholarship funding to support the project, completed all experiments and associated data analysis, interpreted data and performed all statistical analyses, and presented the findings at symposia.

Paul Y. Kim contributed to the development of the study, obtained grant funding to support the project, critically assessed results and analyses.

Peter L. Gross contributed to the development of the study and critically assessed results and analyses.

1 Introduction

1.1 Venous Thromboembolism

Venous thromboembolism (VTE) is the third major cause of cardiovascular related mortality worldwide, affecting roughly 1 to 2 in 1,000 Canadians annually (Heit, 2015; Tagalakis, 2019). VTE is a collective term encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), whereby a thrombus is formed within a deep vein that subsequently embolizes, enters the bloodstream, and accumulates within pulmonary microvasculature (Figure 1) (Budnik & Brill, 2018). As a multifactorial condition, the risk factors of VTE vary with genetic and acquired causes including infection, surgery, immobilization, cancer, trauma, or sometimes idiopathic onset, and may exhibit up to a 30% rate of recurrence following anticoagulant treatment (Heit, 2015; Weitz et al., 2020). Incidence of VTE increases drastically with age, with patients 80 years or older exhibiting an incidence rate 8-fold greater than those under the age of 50, with a case fatality rate up to 20-fold greater in older populations (Tagalakis et al., 2013).

1.1.1 Deep Vein Thrombosis

As VTE is multifactorial, initiation of DVT may occur through a variety of mechanisms. There are three general contributors to the beginning of thrombus formation as outlined by Virchow's Triad: venous stasis, vascular injury, and hypercoagulability (Chan & Weitz, 2020). A contributor to asymptomatic lower extremity DVT is venous stasis, which typically occurs around venous valve sinuses within deep veins, leading to

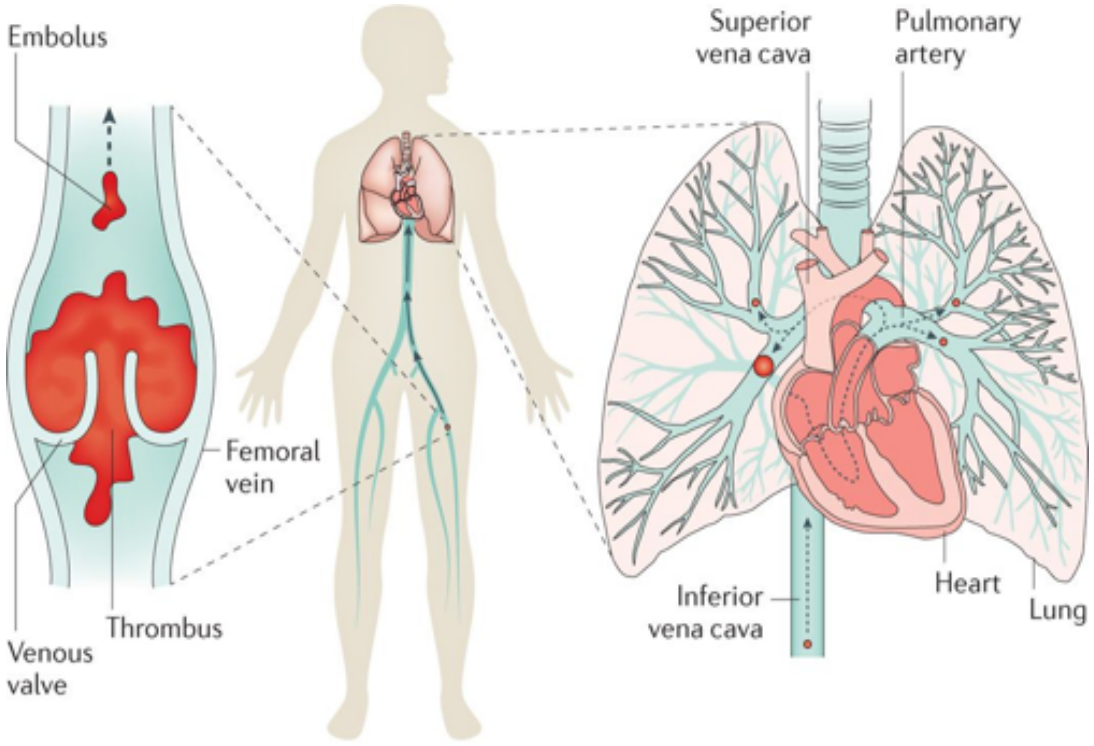

Figure 1. Properties of deep vein thrombosis and pulmonary embolism making up venous thromboembolism.
Deep vein thrombosis is characterized by the formation of a blood clot within a deep vein, typically around the venous valves within the leg. The thrombus then breaks into fragments (emboli) that flow up through the inferior vena cava to the heart and eventually enter the lungs through the pulmonary artery (dotted line). In the lungs, emboli lodge and accumulate in the narrowing vessels (pulmonary embolism), eventually leading to an occlusion that may pose serious life-threatening consequences. (Figure adapted from Huisman et al., 2018).

the development of hypoxic microenvironments, increased hematocrit, and reduced efflux of procoagulant factors and influx of inhibitors surrounding the sinus (Stone et al., 2017). The resulting hypoxia causes the shedding of heparin-like glycosaminoglycans from the endothelial cell surface, reduced surface expression of anticoagulant proteins (such as thrombomodulin (TM) and endothelial protein C receptor (EPCR), and promotes the procoagulant adhesion molecule P-selectin (Stone et al., 2017). This contributes to a hypercoagulable environment, another contributor from Virchow's triad. P-selectin and associated P-selectin glycoprotein ligand 1 (PSGL-1) are involved with the recruitment of immunologic cells expressing tissue factor (TF) to the site of endothelial activation (Stone et al., 2017). Platelets that have aggregated, in response to exposed collagen and the release of von Willebrand Factor (vWF) from activated endothelial cells, are also activated and release prothrombotic factors including coagulation factors (F) V, XI, VIII, IX and XIII, amplifying the growth of the thrombus (Golebiewska & Poole, 2015; Yau et al., 2015). This process also occurs following trauma or direct injury to the endothelium, whereby vascular injury activates surrounding endothelial cells and exposes TF and collagen present in the sub-endothelium, inducing the binding and activation of platelets via vWF (Yau et al., 2015).

When thrombi are formed within a major vessel, such as a femoral vein, there is an increased risk of clot fragmentation, possibly leading to larger embolic events that travel downstream, through the heart, and into the lungs. The stability profile of clots varies, depending on individual factors including the circulating concentrations of pro- and anti-coagulants (Kattula et al., 2017). Increased fibrin density and FXIII cross-linking of fibrin

have been shown to improve clot stability by reducing the efficiency of fibrinolysis, the process of clot degradation (Kattula et al., 2017; Shaya, Gani, et al., 2019). Additionally, the stability of thrombi in DVT also contributes to the development and progression of PE, with more unstable thrombi increasing the risk of PE (Shaya et al., 2016; Shaya, Gani, et al., 2019).

1.1.2 Pulmonary Embolism

PE is the primary cause of death in VTE patients, with between 5-10% of all in-hospital deaths attributable to PE through serious complications such as myocardial infarction, stroke, hypoxia, and organ failure (Lavorini et al., 2013; Morrone & Morrone, 2018; Turetz et al., 2018). When emboli break away and enter the bloodstream, they accumulate within the lung and restrict blood flow through major pulmonary arteries, increasing pressure in the right cardiac ventricle (Lavorini et al., 2013). If left untreated, up to 30% of cases result in death, with increased risk of post-PE complications including chronic thromboembolic pulmonary hypertension (CTEPH) and VTE recurrence occurring after treatment (Carson et al., 1992; Huisman et al., 2018). Thrombi present in the lower extremities are more frequently associated with PE, including DVT cases constituting VTE, and risk of developing PE depends on a variety of factors including age, smoking, its recurrence, and prolonged presence of DVT (Huisman et al., 2018). The incidence of PE has been shown to correlate with decreased venous thrombus stability in mice, with stability being defined as the shedding of deep vein thrombi over time that contribute to PE (Shaya et al., 2016; Shaya, Gani, et al., 2019; Zhang et al., 2020).

1.1.3 Sex and VTE

Sex-specific contributions to VTE risk are an ongoing discussion. Although incidence of VTE increases with age, several cohort studies have assessed the incidence of first VTE between men and women, which remains similar when adjusted for age (Tagalakis et al., 2013). When adjusted for reproductive risk factors, men exhibit increased risk of first VTE than women and increased risk of recurrent VTE overall (Baglin et al., 2004; Martinez et al., 2014; Roach et al., 2014). When experiencing PE, it has been reported that women exhibit significantly increased mortality rates during acute PE, along with increased risk of major bleeding events during anticoagulant treatment for acute VTE compared with men (Barrios et al., 2017; Blanco-Molina et al., 2014). Sex differences contributing to the progression of VTE and severity of PE are not fully understood, with some studies suggesting a higher risk in men than women at a ratio of 1.2:1 (Silverstein et al., 1998), whereas others report up to 1.3-fold higher risk of VTE in women than in men (Naess et al., 2007; Roach et al., 2014; Scheres et al., 2017). However, the risk of recurrent VTE has consistently been shown to be up to 2-fold higher in men than in women (Douketis et al., 2011; Linnemann et al., 2008; McRae et al., 2006), suggesting the role of sex as a potential risk factor for VTE.

1.2 Coagulation

TF is a 47 kDa membrane-bound glycoprotein that initiates the formation of a thrombus through the extrinsic coagulation pathway (Palta et al., 2014). Coagulation can be described in four main phases: initiation, amplification, propagation, and stabilization.

During the initiation phase, expression of TF leads to the formation of the extrinsic tenase complex with FVIIa, creating a potent activator of FIX and FX. FVII (50 kDa), FIX (57 kDa), and FX (59 kDa) are synthesized in the liver and undergo post-translational vitamin K-dependent γ-carboxylation of glutamic acid residues before circulating in plasma at 0.5µg/mL, 5µg/mL, and 5-10µg/mL, respectively (Furie & Furie, 1988; Hao et al., 2020; Periayah et al., 2017). Additional activation of FX may occur through the formation of the intrinsic tenase complex consisting of FIXa and cofactor FVIIIa, thus initiating the intrinsic coagulation pathway. These tenase complexes are mediated by calcium and occur on negatively charged lipid surfaces, such as the surface of activated platelets (Mann et al., 1990). FXa then colocalizes with cofactor FVa and calcium on a lipid surface to create the prothrombinase complex, activating the zymogen prothrombin to serine protease thrombin. Prothrombin (72 kDa) is synthesized in the liver and circulates at a concentration of 100 µg/mL before FXa-mediated cleavage at Arg^{320} and Arg^{271} to release thrombin (37-kDa) and fragment 1.2 (Bradford et al., 2010; Palta et al., 2014). The interaction of calcium with negatively charged γ-carboxylated glutamic acids in the GLA domain of prothrombin induces a conformational change that exposes a hydrophobic patch on the protein structure that interacts with the hydrophobic tails within the lipid bilayer, allowing for contact with the prothrombinase complex on the surface of activated platelets (Huang et al., 2003; Sunnerhagen et al., 1995). FXa proteolytic cleavage of prothrombin is relatively inefficient until sufficient levels of FXa assemble with FVa to form the prothrombinase complex, at which point the efficient catalytic conversion of prothrombin increases by over 300,000-fold (Nesheim et al., 1979). This rapid increase in thrombin concentration translates to

increased fibrinogen proteolytic cleavage and subsequent fibrin polymerization in addition to further activation of platelets (Smith et al., 2015).

Cofactors FV (330kDa) and FVIII (170-280kDa) are synthesized primarily in the liver and circulate in plasma at 10 μg/mL and 0.1-0.2 μg/mL, respectively (Furie & Furie, 1988). As little thrombin is produced during initiation, the amplification phase involves the activation of platelets, FV, FVIII, and FXI by thrombin. The rate of FX activation is also enhanced as FXIa then activates FIX. FXI can also be activated via FXIIa, the serine protease resulting from activation of the zymogen FXII by negatively charged molecules such as polyphosphates or autoactivation (Emsley et al., 2010). This thrombin-mediated positive feedback cycle produces robust quantities of thrombin throughout the propagation phase, allowing for subsequent fibrin generation.

Produced in the liver, fibrinogen (340 kDa) circulates in plasma at a concentration of 2-5 mg/mL and is the precursor of fibrin, an essential insoluble protein that assembles into a fibrous mesh supporting the thrombus (Kattula et al., 2017). Thrombin cleaves the N-terminal ends of the α and β chains of fibrinogen, which results in the release of fibrinopeptides A and B, respectively. This converts fibrinogen into fibrin, which is then able to polymerize with other adjacent fibrin monomers (Kattula et al., 2017). The growing thrombus, therefore, is primarily composed of platelets and red blood cells (RBCs) surrounded by a fibrin mesh (Chernysh et al., 2020). Finally, during stabilization, thrombin activates FXIII to FXIIIa which then covalently crosslinks fibrin, further strengthening and stabilizing the growing thrombus (Kattula et al., 2018; Shaya, Gani, et al., 2019). FXIII

circulates in plasma at 14-28 µg/mL as a heterotetrameric protein complex consisting of two dimeric subunits, the $FXIIIA_2$ (83 kDa) subunit containing a 37 amino acid activation peptide and the non-enzymatic $FXIIIB_2$ (80 kDa) subunit (Gupta et al., 2016). Additionally, a FXIIIA homodimer exists in the cytoplasm of platelets, at concentrations 150-fold greater than in plasma, and is externalized upon platelet activation (Kattula et al., 2018).

To maintain localized thrombus formation, endogenous anticoagulants such as antithrombin (AT) and protein C continuously circulate (Palta et al., 2014). Protein C is activated by the thrombin-thrombomodulin complex (T-TM) forming activated protein C (APC) which colocalizes with protein S and phospholipids. In the presence of calcium, APC inactivates FVa and FVIIIa, with protein S also acting as an important cofactor for tissue factor pathway inhibitor in an APC-independent manner (Griffin et al., 2012; Hackeng et al., 2006). Furthermore, binding of AT to heparin and heparin sulfates enhances the inhibitory function of AT by (a) inducing conformational changes that increase AT recognition of substrates and (b) providing a scaffold for substrate-AT colocalization (O'Donnell et al., 2019). AT is a potent regulator of coagulation as it irreversibly binds and inactivates thrombin, FIXa, FXa, and FXIa (Palta et al., 2014). As thrombin rapidly binds AT in the presence of heparins, the thrombin-antithrombin (TAT) complex can be measured in plasma as a biomarker of thrombin generation.

1.3 VTE, Anticoagulation, and Thrombolysis

Symptoms of VTE may vary, where prolonged and more severe cases of DVT may show limb swelling, warmness, and pain, PE may show signs of chest pain and shortness of breath (Heit, 2015; Lavorini et al., 2013; Morrone & Morrone, 2018). Due to difficulties in diagnosis and the multifactorial nature of VTE, anticoagulant therapy is the primary treatment for suspected VTE patients (Key & Kasthuri, 2010; Laryea & Champagne, 2013). Commonly used anticoagulants include 1) the vitamin-K antagonist warfarin that prohibits post-translational vitamin K-dependent γ-carboxylation of thrombin, FVII, FIX, FX, protein C and S, 2) antithrombin activating low molecular weight heparins (such as dalteparin, enoxaparin, and tinzaparin), and more recently, 3) direct oral anticoagulants that directly inhibit FXa (rivaroxaban, apixaban) or thrombin (dabigatran) (Budnik & Brill, 2018; Fredenburgh & Weitz, 2021; Harter et al., 2015; Key & Kasthuri, 2010). Adversities of such anticoagulants may include heparin-induced thrombocytopenia (HIT), difficult monitoring strategies, and dosage (Harter et al., 2015), however the major issue with anticoagulant therapy is an increase in major bleeding risk/events in patients.

The standard 3 to 6 months of oral anticoagulation is effective at limiting the continued growth of thrombi and may reduce the risk of recurrent DVT, however, the inability to degrade existing clots within the vasculature remains apparent, relying mostly on the body's endogenous thrombolytic capabilities (Fleck et al., 2017; Weitz et al., 2020). Increasing systemic perfusion through the use of thrombolytic agents such as streptokinase, urokinase, or recombinant tissue-type plasminogen activator (rt-PA), as well as catheter-directed intervention by delivering the thrombolytic agent directly into the thrombus, may provide rapid clot dissolution. This strategy, along with mechanical clot removal, is only

undertaken in severe instances and is not advantageous for the majority of cases because of increased bleeding risks over standard anticoagulation (DeRoo et al., 2021; Ucar, 2019). Therefore, incomplete thrombus resolution may occur following anticoagulation therapy, with persistent thrombi leading to debilitating conditions such as post-thrombotic syndrome in the leg and CTEPH in the lung. (Nicholson et al., 2020). A demand exists for the development of thrombolytic agents that enhance fibrinolysis and dissolve existing clots more effectively without increased bleeding risk.

1.4 Fibrinolysis

Fibrinolysis is the process of degrading existing thrombi. The presence of fibrin along an activated endothelium allows for the cleavage of plasminogen to an active state, plasmin, via the endothelial cell-derived serine protease tissue-type plasminogen activator (t-PA) and the monocyte/macrophage-derived urokinase-type plasminogen activator (u-PA) (Chapin & Hajjar, 2015; Mahmood et al., 2018). Plasminogen activation by t-PA is enhanced by the presence of fibrin 1000-fold, which acts as a cofactor for plasmin generation and consequently, aids in its own degradation (Henderson et al., 2018; Rijken & Sakharov, 2001) (Figure 2). This occurs as fibrin acts as a template onto which plasminogen and t-PA can localize, with plasminogen binding via its kringle domains in a lysine-dependent manner while t-PA binds fibrin in a lysine-dependent (kringle 2 domain) and lysine-independent (finger domain) manner (Urano et al., 2018). Upon initial modification of fibrin by plasmin to generate plasmin-modified fibrin (Fn’), newly exposed

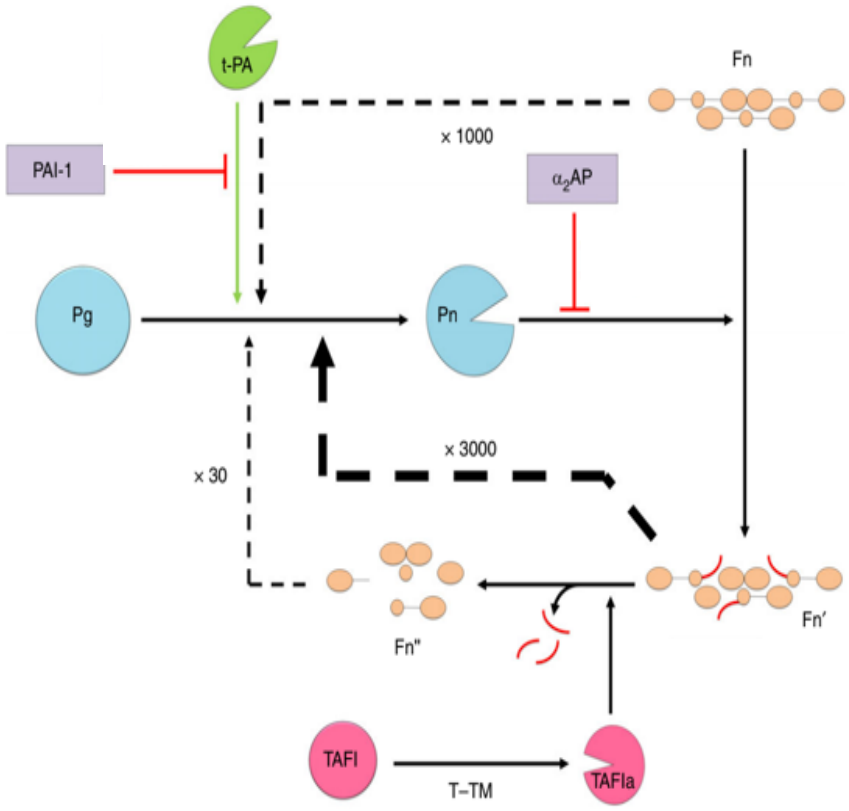

Figure 2. A schematic representation of the fibrinolytic system.
Plasminogen (Pg) is cleaved to its active form plasmin (Pn) by endothelial cell-derived tissue-type plasminogen activator (t-PA). This specific process is regulated by plasminogen activator inhibitor 1 (PAI-1). Fibrin clot (Fn) accelerates 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 α_2-antiplasmin (α_2-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 surface. 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"). (Figure adapted from: Henderson *et al*., 2018).

C-terminal lysine residues enhance the cofactor activity of plasminogen and t-PA by a factor of 3, creating a positive feedback system (Henderson et al., 2018). Plasmin binds to specific areas of fibrin, typically after basic arginine or lysine residues, allowing for the cleavage of fibrin α-, β-, and γ-chains, which results in the production of soluble fibrin degradation products (FDPs) (Urano et al., 2018; Walker & Nesheim, 1999). The smallest of the FDPs, D-dimers, are only produced as a result of crosslinked fibrin degradation, thus can be used as a marker of fibrinolysis (Chapin & Hajjar, 2015; Weisel & Litvinov, 2017). Plasmin is protected from inhibition when bound to fibrin and may also exhibit broad specificity, whereby it can also target fibrinogen, FV, FVIII, FIX, FXI, FXII, extracellular matrix proteins, and growth factors in addition to fibrin (Chapin & Hajjar, 2015; Henderson et al., 2018). Due to this potential impairment in coagulability, it is critical to regulate plasmin generation.

Fibrinolysis is regulated by various inhibitors such as plasminogen activator inhibitor-1 (PAI-1), α_2-antiplasmin (α_2AP), and thrombin-activatable fibrinolysis inhibitor (TAFI). The serine protease inhibitors, PAI-1 and α_2AP, play important roles by binding and directly inhibiting t-PA and plasmin, respectively (Cesari et al., 2010; Zakrzewski et al., 2016). While TAFI, when activated to TAFIa by thrombin, plasmin, or the T-TM complex, attenuates fibrinolysis by down-regulating plasmin generation while also promoting plasmin inhibition by reducing fibrin-mediated plasmin protection (Henderson et al., 2018).

1.4.1 Thrombin Activatable Fibrinolysis Inhibitor (TAFI)

TAFI, also known as plasma procarboxypeptidase (proCP) B, proCPB2, proCPR, and proCPU, is a circulating zymogen (56 kDa) in plasma that undergoes proteolytic cleavage at Arg^{92} to release the activation peptide (20 kDa) that liberates the pre-formed catalytically active domain (36 kDa) (Bazzi et al., 2016). Primarily synthesized in the liver and megakaryocytes, TAFI is present in blood and platelet α-granules, with platelet-derived TAFI accounting for <0.1% of all TAFI (Foley et al., 2013). Circulating concentrations of TAFI vary widely between 73-275 nM, which may be a result of TAFI variants identified containing either Thr or Ile at the 325 position that react differently between commercial ELISAs (Foley et al., 2013). Other genetic and/or acquired factors may also contribute to variation in TAFI concentrations, such as genetic polymorphisms (including SNPs), infection/injury/inflammation, and hormones (Foley et al., 2013). Activation of TAFI can be mediated by plasmin, thrombin, or the T-TM complex to form activated TAFI (TAFIa), which specifically targets and removes exposed C-terminal arginine and lysine residues of macromolecules including fibrin and cell-surface plasminogen receptors (Bazzi et al., 2016, 2017; Foley et al., 2013). Thrombin is a relatively weak activator compared with T-TM, which is 1250-fold more efficient, and plasmin has been proposed to activate TAFI in close proximity to fibrin (Foley et al., 2013). Activation of TAFI typically occurs in environments with high levels of thrombin present or vascular beds with increased thrombomodulin presence (Eichinger et al., 2004). Removal of the newly generated C-terminal lysines, and therefore removing plasminogen and t-PA binding sites on plasmin-modified fibrin, greatly decreases the rate of fibrinolysis

by up to 100-fold, solidifying the role of TAFIa as a fibrinolytic regulator by indirectly inhibiting plasmin generation (Henderson et al., 2018) (Figure 2).

TAFIa has no known endogenous inhibitor in circulation, most likely due to its intrinsic thermal instability with a functional half-life of ~8-15 minutes at 37ºC depending on the variant (Bazzi et al., 2016, 2017; M. Schneider et al., 2002). Nevertheless, small molecule TAFIa inhibitors have been developed such as potato tuber carboxypeptidase inhibitor (PTCI) and 2-guanidinoethylmercaptosuccinic acid (GEMSA) that are effective at improving t-PA mediated fibrinolysis *in vitro* and *in vivo* without increasing major bleeding complications (Henderson et al., 2018; M. Schneider & Nesheim, 2003; X. Wang et al., 2006). Both inhibitors exhibit biphasic activity, whereby low doses stabilize TAFIa (*i.e*. increase its half-life) and reduce fibrinolytic activity, enhancing clot lysis only in higher concentrations (Bazzi et al., 2017; Zhou et al., 2017). Studies investigating the efficacy of other fibrinolytic enhancers (such as those inhibiting PAI-1 and α_2-AP) may yield promising results. However, these studies were not translated successfully and the resulting effect on bleeding phenotypes and PE burden remains under discussion (Henderson et al., 2018). As thrombus stability is inversely related to PE burden in a ferric chloride induced DVT model (Shaya, Gani, et al., 2019; Shaya, Westrick, et al., 2019), it is advantageous to consider the effect TAFI/TAFIa has on thrombus stability and resulting PE *in vivo* between sexes, studying this effect may lead to more individualized medical treatment strategies for VTE.

1.4.2 Fibrinolysis and VTE

Targeting and enhancing the fibrinolytic system is an alternative treatment for thrombotic disorders including VTE. rt-PA and purified uPA are both approved as clinical thrombolytic agents, with preference given to rt-PA due to its fibrin colocalizing capabilities (Lin et al., 2020). rt-PAs such as Alteplase, Retaplase, and Tenecteplase are primarily used for severe, usually acute, thrombotic diseases as treatment with rt-PA produces a substantial increase in bleeding risk (Lin et al., 2020). Additionally, treatment with uPA may result in increased fibrinogenolysis due to the activation of both circulating and fibrin-bound plasminogen. Monoclonal antibodies against PAI-1 and α_2-AP have also been developed to enhance t-PA-mediated plasminogen activation and plasmin-mediated fibrin cleavage, respectively. Although unsuccessful in human trials, inhibition of PAI-1 exhibited decreased fibrin deposition during thrombosis in animal models, with α_2-AP inhibitors showing reduced microvascular thrombosis, ischemic brain injury or bleeding, and death (Lin et al., 2020; Reed et al., 2017). In addition to monoclonal antibodies against TAFIa, multiple inhibitors have been developed that target the active site of TAFIa by (a) competitive inhibition, (b) chelating the essential zinc ion, or (c) disrupting the disulfide bonds within the site, as well as inhibition through arginine analogs (MERGEPTA, GEMSA) or the lysine analog ε-ACA (Sillen & Declerck, 2021). The primary drawback of these inhibitors involves the non-specific binding of other plasma carboxypeptidases including carboxypeptidase N (CPN), pancreatic CPB, and stabilization of TAFIa (Sillen & Declerck, 2021). The effect of such inhibitors on existing thrombus stability *in vivo* has not been determined and may increase the risk of PE events. Considering the potential role

of TAFI/TAFIa in VTE has been suggested previously, whereby elevated levels of TAFI correlate with increased risk of unprovoked VTE and VTE recurrence by up to 3-fold (Eichinger et al., 2004; Foley et al., 2013; Rosendaal & LUMC, 2000). It may be advantageous to determine the potential role of TAFI/TAFIa on thrombus stability in VTE and consider how sex influences this role in a mouse model.

1.5 Mouse Models

Mice generally do not develop deep vein thrombosis naturally and therefore do not experience VTE. Thrombus formation must be induced to study the mechanisms of VTE progression, and over time, varying murine models of VTE have been developed to deliver robust quantifiable thrombus formation that mimic DVT cases seen in humans. Commonly used models may include the inferior vena cava (IVC) stasis and stenosis models, the electrolysis model, and the ferric chloride ($FeCl_3$) induced model. The stasis and stenosis models ligate the IVC either completely or partially, respectively. This restricts blood flow through the vein, creating a static procoagulant environment that produces robust thrombus growth. Issues with these models include inconsistent thrombus sizes and reduced perfusion of systemic drug treatments when fully occluded (Diaz et al., 2012; Zhou et al., 2009). Alternatively, the electrolysis model involves inserting a needle into the vein and running an electrical current to induce robust non-occlusive thrombus formation. Limitations may include prolonged time for thrombus growth and potential damage to the endothelium at the site of probe insertion (Cooley, 2011; Cooley et al., 2005). The $FeCl_3$

induced model applies $FeCl_3$-soaked filter paper to the surface of a vein, damaging the endothelium through oxidation, and producing non-occlusive thrombus growth within minutes (Mukhopadhyay et al., 2019; X. Wang et al., 2006). Although effective at producing venous thrombi that mimic acute DVT cases in humans, this represents only a small portion of total DVT cases and is therefore difficult to translate (Diaz et al., 2012). To determine the influence of TAFI on thrombus stability using this model, mice with its TAFI gene knockout on C57BL/6 background (*Cpb2*$^{-/-}$) were used (Velde et al., 2003). These *Cpb2*$^{-/-}$ mice have shown to be viable, carry offspring to term, show no abnormalities in circulating fibrinogen, and do not exhibit increased bleeding or thrombus occlusion times (Nagashima et al., 2002).

1.6 Hypothesis and Specific Aims

Hypotheses: (1) Absence of TAFI/TAFIa enhances fibrinolysis and results in faster resolution of deep vein thrombi, which may also lead to increased PE burden. (2) Sex affects thrombus stability and embolization.

Specific Aims:

Aim 1*:* To determine the frequency and size of embolic events in real-time between *Cpb2*$^{-/-}$ mice and wild-type (WT) mice via intravital video-microscopy (IVM).

Aim 2: To determine the quantity and fibrin content of pulmonary emboli present in the lungs using histology.

Aim 3: To identify femoral vein thrombus composition using histology and immunofluorescence.

Aim 4: To investigate differences in hemostatic potential between male and female WT mice using clot lysis analysis.

2 Materials and Methods

2.1 Materials

Sodium pentobarbital was purchased from CevaSante Animale (Cambridge, Ontario, Canada). Polyethylene tubing (PE10, PE100), Whatman filter paper, and 10% formalin solution were purchased from VWR (Mississauga, Ontario, Canada). Rat-anti-mouse CD41 antibodies (MwReg30) were purchased from Emfret Analytics (Eibelstadt, Germany). F_{abs} were purified using the Pierce F_{ab} Purification Kit and labeled using the Alexa Fluor 488 Labeling Kit purchased from Thermo Scientific (Mississauga, Ontario, Canada). 5-0 silk sutures were purchased from the Surgical Specialties Corporation (Wyomissing, PA). Ferric chloride solution was purchased from Sigma-Aldrich (St Louis, MO). Tissue samples were sectioned using a Leica RM 2125RT Microtome purchased from Leica Microsystems Inc. (Richmond Hill, Ontario, Canada). Carstair's staining kit was purchased from Electron Microscopy Sciences (Hatfield, PA). For immunofluorescent staining, Proteinase K Solution, DAPI (4',6-diamidino-2-phenylindole), and goat anti-rabbit IgG conjugated with Alexa Fluor 594 were purchased from Invitrogen (Burlington, ON). Normal goat serum was purchased from Vector Laboratories (Burlington, ON) and rabbit anti-human fibrin/fibrinogen antibody (Gromov et al., 2006) and antibody diluent were from Dako (Ref# A0080; Burlington, ON). Mounting medium was purchased from Thermo Fisher Scientific and tissue sections were imaged using an Olympus BX41 microscope with Olympus DP72 camera and illuminated using an X-Cite Series 120Q laser. Fluorescence intensity was quantified using the RGB Measure plugin on ImageJ (v1.53i)

image analysis software developed by the National Institutes of Health (Parker et al., 2020; Rasband, 2004; C. A. Schneider et al., 2012). For clot lysis assays, thrombin was purchased from Enzyme Research Laboratories (South Bend, IN) and Tenecteplase (TNK-tPA) was manufactured by Genentech and purchased from the Hamilton General Hospital pharmacy (Hamilton, ON). The ELISA kit to measure murine thrombin-antithrombin (TAT) complex levels was purchased from Abcam (ab137994, Cambridge, MA).

2.2 Mouse Model of VTE

Cpb2$^{-/-}$ mice on C57BL/6 background were a generous gift from Dr. J.C.M. Meijers (Amsterdam, Netherlands) (Plug & Meijers, 2016), and WT C57BL/6 mice, were purchased from Charles River Laboratories (Sherbrooke, QC), were maintained in temperature/humidity-controlled micro-isolator cages that allowed access to food and water *ad libitum*, with cycled 12-hour lighting. Protocols were undertaken with approval from the Animal Research Ethics Board at McMaster University, conforming to Canadian Council of Animal Care guidelines.

Male and female *Cpb2*$^{-/-}$ and WT mice (20-30g) were anesthetized with an intraperitoneal injection of a mixture of ketamine (0.125 mg g^{-1}), xylazine (0.0125 mg g^{-1}) and atropine sulfate (0.0025 mg g^{-1}), as described previously (Gross et al., 2005). Sodium pentobarbital (0.05 mg g^{-1}) was used to maintain anesthesia (Shaya et al., 2016). The trachea and jugular vein were cannulated with PE100 and PE10 polyethylene tubing,

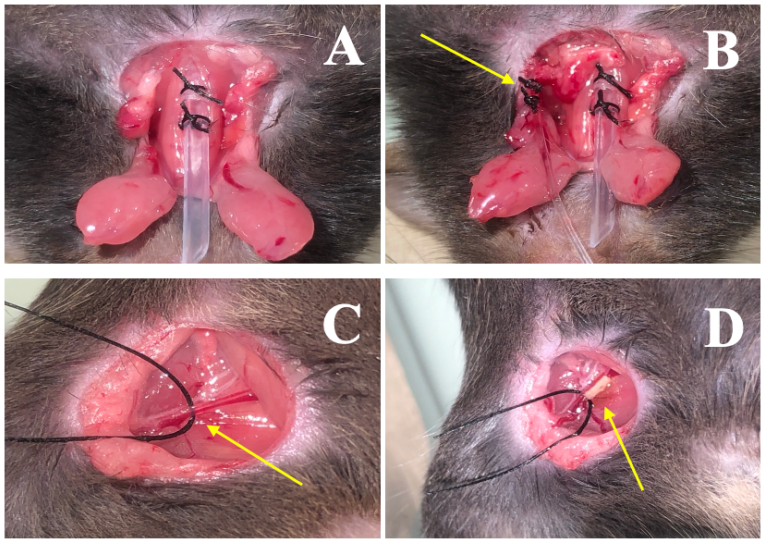

Figure 3. $FeCl_3$-induced mouse model of VTE.
Representative images of thrombus induction illustrating (A) trachea isolation and cannulation, (B) jugular vein isolation and cannulation, (C) femoral vein isolation, and (D) application of $FeCl_3$-soaked filter paper to the medial side of the femoral vein. Non-occlusive venous thrombus growth occurred following the application of 2% $FeCl_3$ to the vein for 5-minutes.

respectively (Figure 3A & B). Platelets were labeled with rat-anti-mouse CD41 F_{ab} fragments, generated with the purification kit listed above, conjugated to Alexa Fluor 488 and injected through the jugular vein (0.625 μg g^{-1}). A 5-0 silk suture was used to isolate the femoral vein (Figure 3C), where a 1 mm x 1.5 mm Whatman filter paper saturated with 2% ferric chloride ($FeCl_3$) was placed on the medial side of the vein for 5 minutes (Figure 3D). The paper was then removed, and the femoral vein was irrigated with saline prior to imaging. After imaging, blood was extracted, and mice were euthanized by cervical dislocation, followed by excision of the lungs and femoral vein.

2.3 Intravital Microscopy (IVM)

Thrombi were imaged in real-time via IVM utilizing an Olympus BX series microscope controlled by SlideBook (v6.0) from Intelligent Imaging Innovations (Denver, CO). Montage images illustrating thrombus size were recorded at 10-minute intervals (10x objective, 1x1 binning, 100 ms exposure) (Figure 4A-B). Embolic events were recorded for 1-minute (20x objective, 4x4 binning, 5000 frames, 10 ms exposure) at staggered 10-minute intervals for 2-hours (Figure 4C). The gain on the image intensifier was constant throughout imaging.

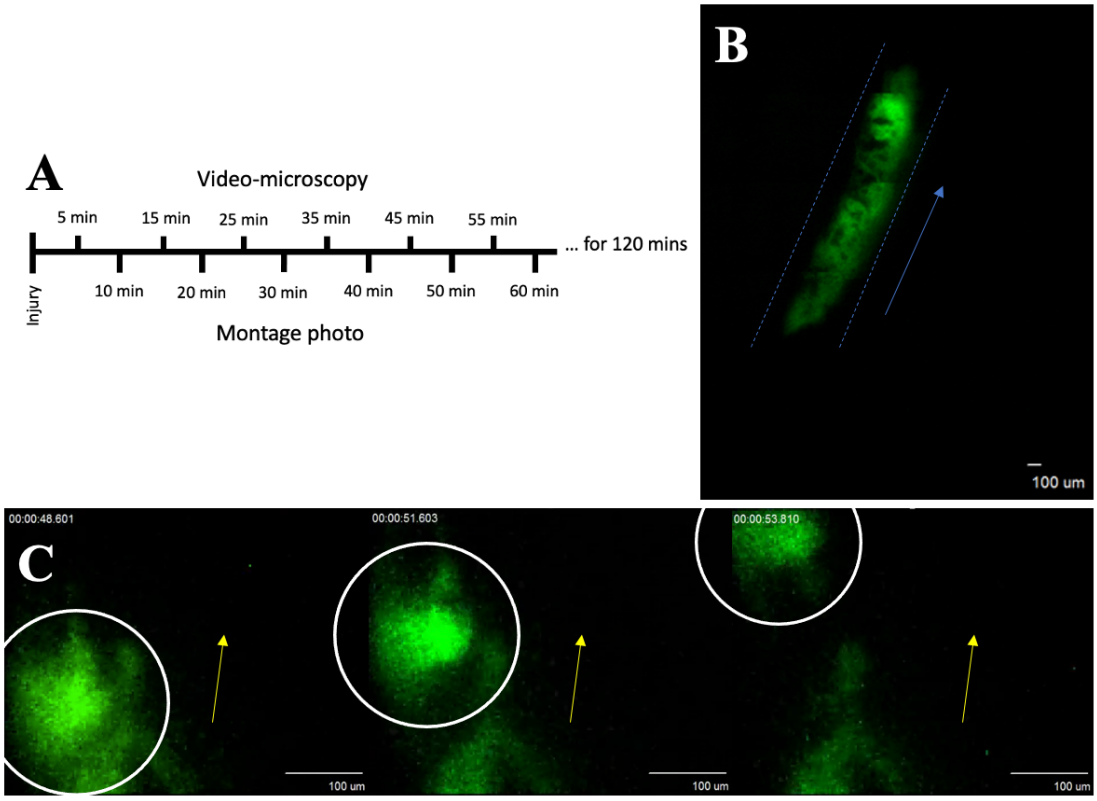

Figure 4. Imaging $FeCl_3$-induced thrombi in real-time.

(A) The imaging timeline between staggered time-lapse videos and montage images. Each video was captured at 10-minute intervals, staggered with montage images by 5-minutes. Representative images of (B) a montage image of a venous thrombus tagged with anti-CD41 antibody and (C) an embolic event breaking away (white circle) from the thrombus and entering circulation in the direction of flow (yellow arrow). Montage images were captured at 10X objective, 1 x 1 binning, and 100 ms exposure. Time-lapse images were captured at 20X objective, 4 x 4 binning, and 10 ms exposure.

2.4 Sample Collection and Handling

Blood was collected into 3.2% citrate buffer solution (10% of final volume) via a carotid artery cannulus. After centrifugation at 6000 x g for 5 minutes, platelet-poor plasma (PPP) was harvested and was stored at -80ºC after a second round of centrifugation.

After exsanguination, femoral veins and lungs were isolated, collected, and preserved in 10% formalin for at least 48 hours before dehydration (3 rounds of 30-minutes in 70%, 85%, 100% ethanol, respectively, followed by 45-minutes in xylene). Tissues were stored overnight in paraffin and paraffin blocks were sectioned into 4.5 μm samples using a Leica RM 2125RT Microtome. Sections were mounted on slides, dried overnight, and then deparaffinization and rehydrated, and subjected to Carstair's staining, which differentiates fibrin (red), platelets (light blue), and the vessel wall (dark blue). Sections were then dehydrated, suspended in xylene, placed on a xylene-based mounting medium, and dried before imaging.

2.5 Immunofluorescent Staining

To better identify the fibrin content of thrombi and emboli, immunofluorescent staining with a fibrin targeted antibody was undertaken. Briefly, after incubating the hydrated slides with proteinase K working solution for 10-minutes at 37ºC and washing, samples were incubated for 1-hour at room temperature (RT) with 5% normal goat serum, followed by a 1-hour incubation with rabbit anti-human fibrin/fibrinogen antibody diluted

with antibody diluent. Slides were then rinsed twice with 0.5% tween 80 and incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 594 for 45-minutes before a second wash with 0.5% tween 80. Slides were incubated with DAPI for 5-minutes, mounted with fluorescent mounting media, sealed with nail polish, and dried overnight. Slides were then imaged using an Olympus DP72 camera on a BX series microscope equipped with an X-Cite Series 120Q laser to identify cellular nuclei (blue), CD41 (green), and fibrin (red).

2.6 Data Analysis

2.6.1 Thrombus Size Quantification

Using montage images, fluorescence intensity of the entire thrombus was quantified at each time-point. The change in thrombus size from baseline was determined by subtracting the baseline intensity values from the average values obtained during the final hour of imaging. The changes in thrombus size were then divided by the baseline values to illustrate relative change in thrombus size.

2.6.2 Quantification of Emboli

The proximal end of the thrombus was imaged at 5000-frame intervals (about 1-minute in length) to capture emboli, which were defined as fluorescent fragments of the thrombus that break away and flow past the region of interest (Figure 4C). Emboli in each video sample were identified and counted manually to derive a sum of embolic events per mouse. An embolus was considered large if the fluorescence intensity was greater than 4 standard deviations above the average intensity of the region of interest. The number of

large emboli among the counted emboli was determined and expressed as the total number of large emboli.

2.6.3 Quantification of Pulmonary Emboli

Lungs were sectioned completely and immobilized on slides (~4 sections per slide) yielding roughly 100 slides per lung. One in five slides were stained with Carstair's stain and one section per slide was subjected to analysis by light microscopy and the total number of thrombi in the pulmonary arteries was determined by manual counting. The number of PE within the section was normalized with the number of sections and slides per mouse. Highest and lowest values from each group were excluded as outliers.

2.6.4 Composition of Pulmonary Emboli and Venous Thrombi

Images of sections stained with immunofluorescent antibodies for fibrin were analyzed using ImageJ image analysis software (Parker et al., 2020; C. A. Schneider et al., 2012). Fluorescent images were captured using Slidebook (v5.0) and pulmonary emboli were identified within pulmonary arteries using ImageJ. A mask was drawn manually overlaying each individual PE, using surrounding tissues/cells that were illuminated by DAPI staining as a guide. Using the mask selection containing the embolus, fibrin fluorescence intensity readings could be determined within individual PE while excluding surrounding tissues. Fibrin fluorescence intensities were quantified using the RGB Measure ImageJ plugin (Rasband, 2004), followed by subtraction of background fluorescence.

2.7 Clot Lysis Assay

Fresh plasma samples isolated from male and female WT mice (n=3) were used to determine the lysis times (LTs) of clots formed *in vitro*, utilizing a previously described turbidity-based clot lysis assay (Kim et al., 2007). To avoid non-specific absorption of proteins, 96-well clear bottom microtiter plates were pre-treated with Hepes Buffered Saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4) containing 1% Tween80. To initiate clot formation in mouse plasma, human thrombin (10 nM) and a mutant variant of t-PA (TNK-tPA) was used due to the inefficiency of human t-PA activating the murine fibrinolytic system (Matsuo et al., 2007). Placed independently within each reaction well, 2 µL of $CaCl_2$ (15 mM final), 5 µL of thrombin (10 nM final), and 5 µL of TNK-tPA (20 nM final) were added before initiating the reaction with an 88 µL mixture comprised of 49.5µL fresh mouse plasma (33µL of mouse plasma and 16.5 µL of PIPES buffer) and 38.5 µL of HBS with 0.01% Tween 80. Reactions were completed in triplicates at 37ºC and measured by absorbance at 400 nm for 8-hours at 1-minute intervals using a SpectraMax M2 microplate reader (Molecular Devices, CA). The time to reach the half-maximal OD change during clot degradation from the half-maximal change during clot formation is defined as the LT.

2.8 Measurement of TAT Complexes in Isolated Plasma

To determine the level of thrombin generation occurring between these mice, we measured TAT complex levels in plasma samples using a sandwich-type ELISA kit. Briefly, TAT complex standards and plasma samples (diluted 1:50) were added to a pre-

coated microtiter plate and incubated for 2 hours at 25ºC. Liquid was decanted from each well and irrigated 5 times with wash buffer before adding biotinylated TAT complex antibodies and let incubate for 2 hours. Following a second wash, streptavidin-peroxidase conjugate was added to each well and incubated for 30 minutes at 25ºC. Following a third wash, chromogen substrate was incubated in each well for 30 minutes. Stop solution was added and absorbance was measured at 450 nm immediately. The absorbance was plotted against the known concentration of TAT complexes of the standard to determine TAT complex levels in male and female WT and *Cpb2*$^{-/-}$ mice following induced VTE.

2.9 Statistical Analysis

Values are presented as the means ± standard errors of the mean (SEM). Unpaired Student's t-tests were used to compare differences in embolic events, large emboli, and pulmonary emboli in male and female mice. GraphPad Prism 8.0 was used to perform statistical analyses and significance was determined when $p < 0.05$. To determine the sample size, power calculation was performed. With the assumption that 40% to 45% change in embolic events is biologically significant with an estimated standard deviation of 25% and power estimation of 0.8, The appropriate sample size was deemed to be near 6 per group.

3 Results

3.1 Thrombus Size Quantification

Thrombus size was measured throughout each experiment to determine disparities in thrombus growth between mice. Due to the wide variation in thrombus size throughout the 2-hour timeframe between individual mice (Figure 5A-D), initial and maximum thrombus size did not differ between mouse groups (Figure 6A-B). The relative changes in thrombus size were significantly different between mice (Figure 6C). There was a 10.3-fold greater increase in thrombus size over baseline in male *Cpb2*$^{-/-}$ mice than in male WT mice (P=0.015). In contrast, there was a 5.6-fold greater increase in thrombus size over baseline in female WT mice compared with female *Cpb2*$^{-/-}$ mice (P=0.026). In WT mice, there was a 4.8-fold greater increase in thrombus size over baseline in females than in males (P=0.038). The opposite was observed in *Cpb2*$^{-/-}$ mice, where there was a 12.4-fold greater increase in thrombus size in males than in females (P=0.013).

3.2 Venous thrombi in female mice exhibit greater embolization

We quantified EEs as an indicator of thrombus instability, with increased embolization suggesting decreased stability. Compared with thrombi in male WT mice (Figure 7A), thrombi in male *Cpb2*$^{-/-}$ mice exhibited 3.2-fold greater embolization (Figure 7B). However, this difference was not statistically significant (P=0.102). Likewise, compared with thrombi in female WT mice (Figure 7C), thrombi in female *Cpb2*$^{-/-}$ mice exhibited 1.3-fold greater embolization (P=0.572) (Figure 7D). However, thrombi in

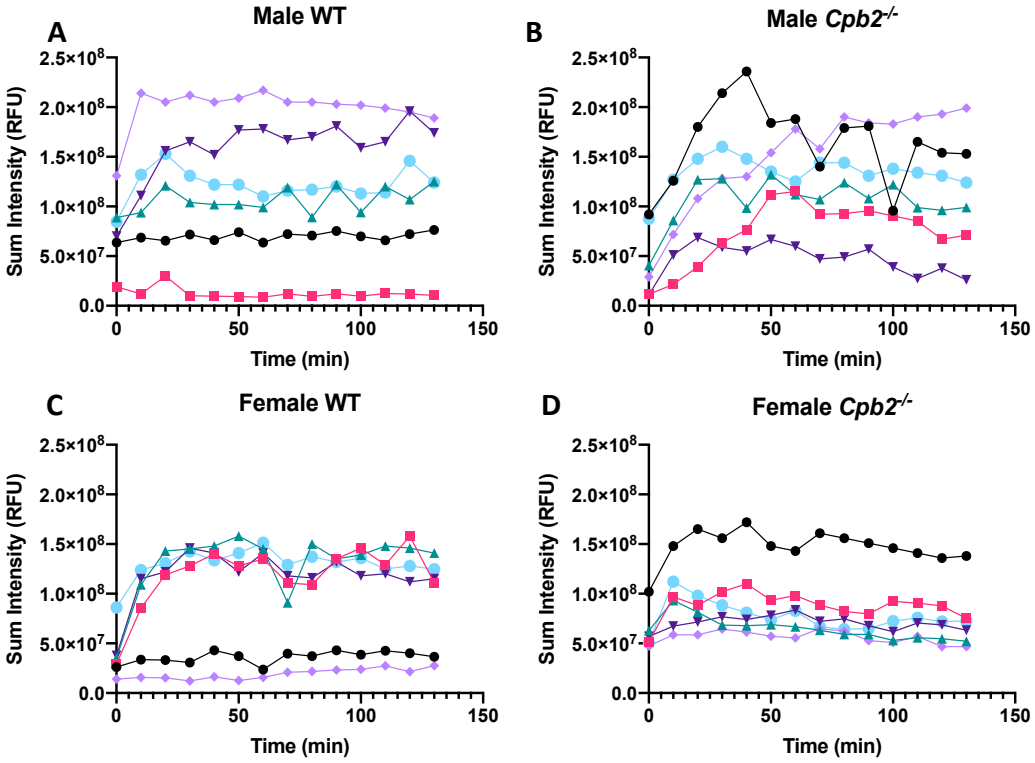

Figure 5. Individual time courses of thrombus size.
Each time point represents the sum intensity within the thrombus area when background is subtracted. Montage images of the thrombi were captured every 10-minutes (staggered from time-lapse images) over a 2-hour timeframe, with a total of 14 timepoints per mouse (n=6 per group). (A) male WT, (B) male *Cpb2*$^{-/-}$, (C) female WT, and (D) female *Cpb2*$^{-/-}$.

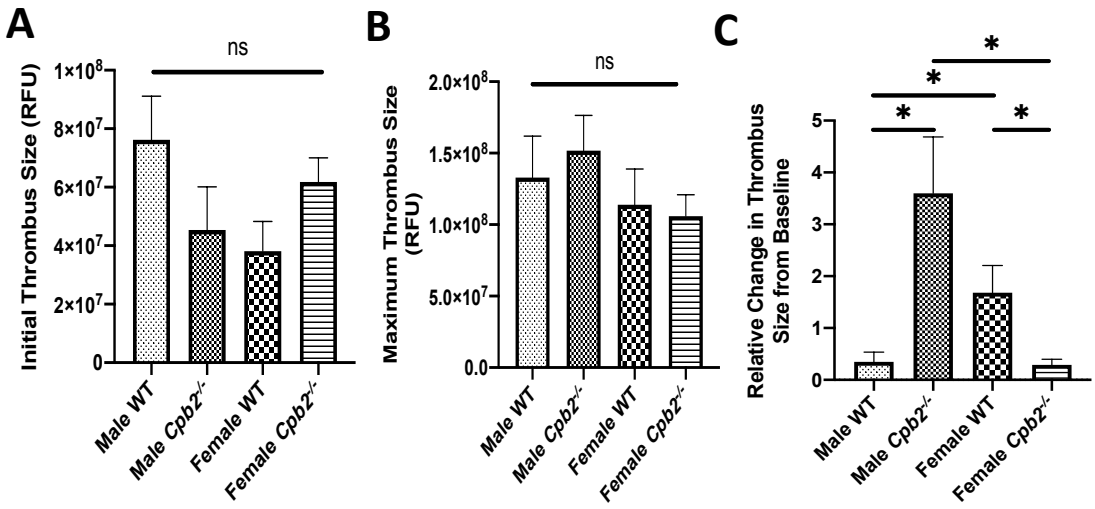

Figure 6. Thrombus size dynamics between mice.
(A) Initial thrombus size fluorescence intensity recordings at the first timepoint for each mouse. (B) Maximum thrombus size fluorescence intensities recorded throughout each experiment for each mouse. The maximum intensity of the surrounding area was considered background and was subtracted from each value. (C) Relative change in thrombus size towards the end of imaging from baseline fluorescence intensities. All values are expressed as the mean ± SEM and significance was determined when $P<0.05$ (n=6 per group).

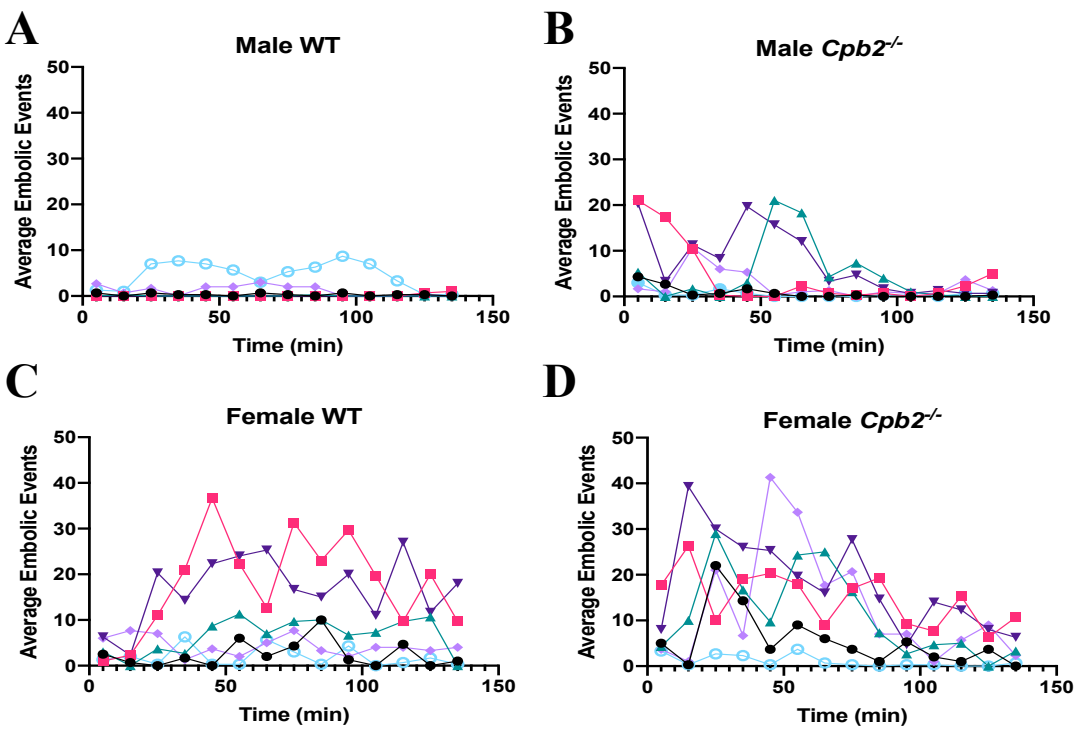

Figure 7. Individual time courses of embolic events.
Each time point represents an average of three one-minute time-lapse videos quantified for embolic events. Images were captured every 10-minutes over a 2-hour timeframe, with a total of 14 timepoints per mouse (n=6 per group). (A) male WT, (B) male *Cpb2*$^{-/-}$, (C) female WT, and (D) female *Cpb2*$^{-/-}$.

female mice embolize significantly more compared with thrombi in male mice. There were 7.9-fold (P=0.037) and 3.1-fold (P=0.026) greater embolization events in female WT and *Cpb2*$^{-/-}$ mice, respectively, than their male counterparts (Figure 8A).

3.3 Quantification of Large Emboli

The size of EEs observed was quantified and large emboli were classified as having a fluorescence intensity at least 4 standard deviations greater than the average within the area of interest. There were no statistically significant differences in the total quantity of large emboli between male mice (P=0.775) or female mice (P=0.773) (Figure 8B). However, thrombi in females illustrate a trend indicating an increased incidence of large embolic events compared with male thrombi. Compared with thrombi in male WT and *Cpb2*$^{-/-}$ mice, thrombi in female WT and *Cpb2*$^{-/-}$ mice produced a greater quantity of large emboli by up to 3.2-fold (P=0.211) and 2.8-fold (P=0.062), respectively (Figure 8B).

3.4 Quantification of Pulmonary Emboli

Using the lung histology sections treated with Carstair's staining, PE were counted manually by visually identifying the emboli (Figure 9A). There were no significant differences in the PE burden between male and female WT mice (P=0.884) or between male and female *Cpb2*$^{-/-}$ mice (P=0.562) (Figure 9A-B). However, when compared by sex, PE burden in the *Cpb2*$^{-/-}$ mice were 2.2-fold and 2.5-fold greater than the WT mice in males (P=0.040) and females (P=0.016), respectively (Figure 9B).

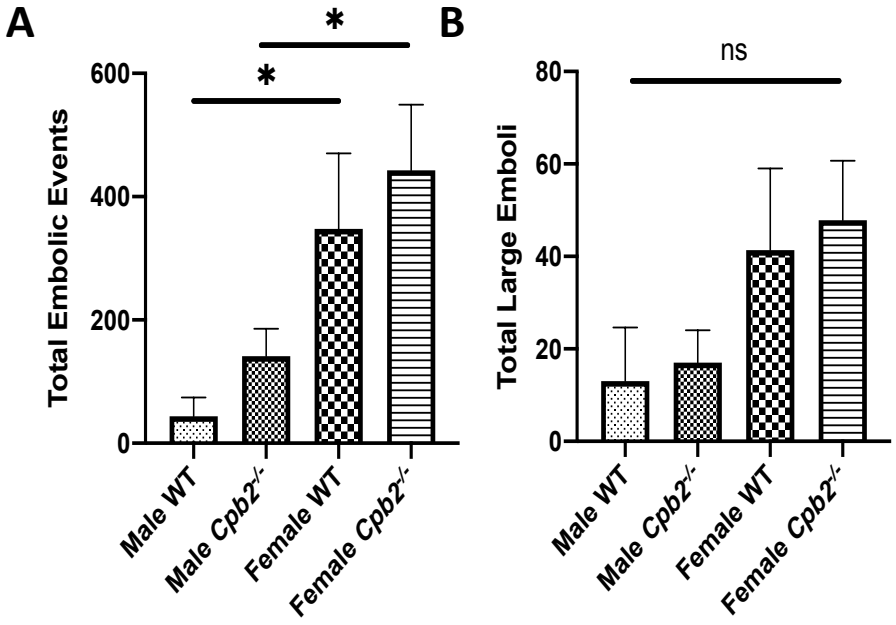

Figure 8. The role of TAFI and sex on venous thrombus stability.
(A) Total and (B) Large embolic events were quantified for the four groups of mice. Focusing on the proximal end of the thrombus, emboli moving away from the thrombus in the direction of blood flow and that passed through a region of interest were counted manually. Large emboli were defined as emboli with fluorescence intensity greater than 4 standard deviations above the mean intensity of embolic events. Significance was determined if $P<0.05$ (n=6 per group).

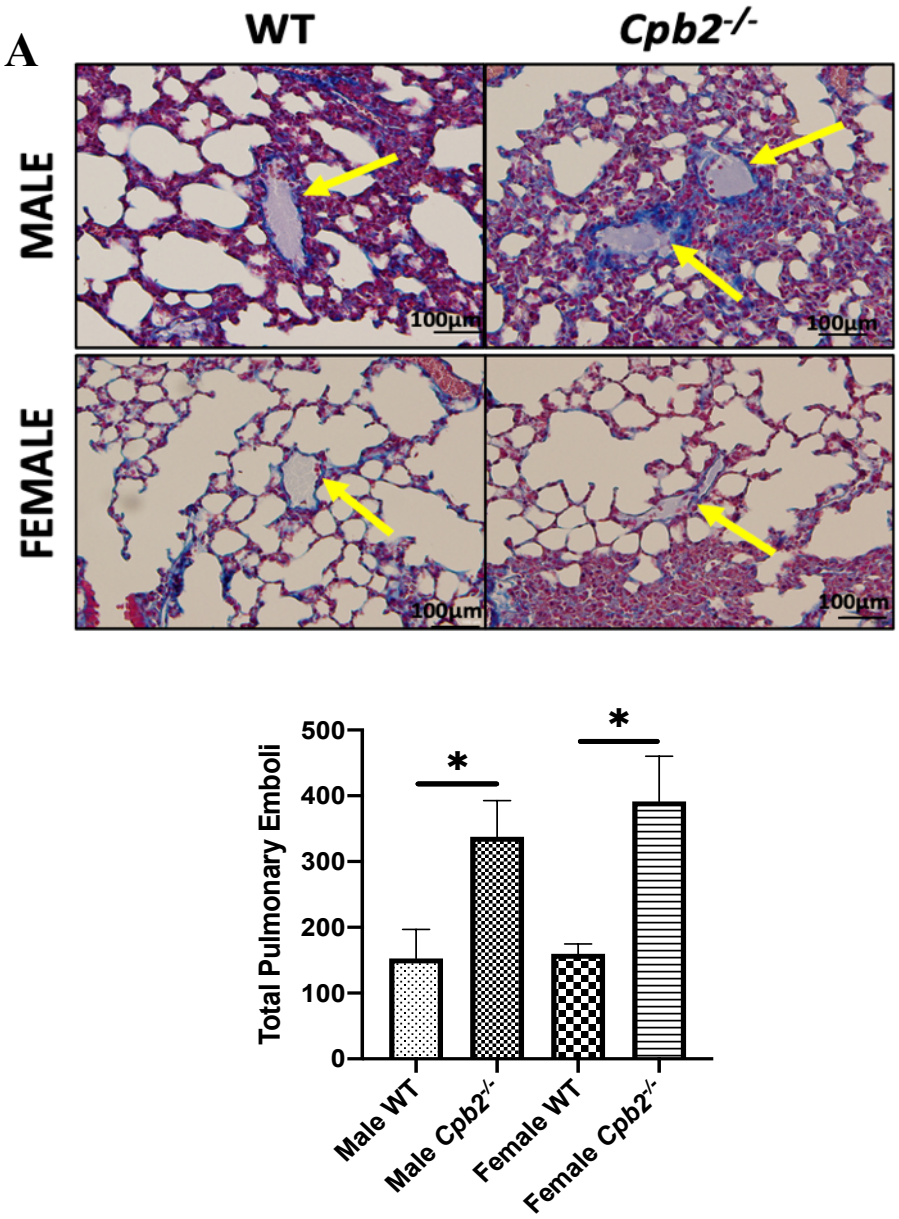

Figure 9. The role of TAFI and sex on PE burden.
After the experiment, lung sections were quantified for total pulmonary emboli present when visualized using the Carstair's stain. (A) Representative Carstair's images of lung tissue with pulmonary emboli (yellow arrows). (B) Total PE burden measured in mice by manually quantifying PE present. Highest and lowest values from each group were removed as outliers and the remaining values were averaged. All values are presented as the mean ± standard error of the mean (SEM) and significance was determined if $P<0.05$ (n=6 per group).

3.5 Composition of Pulmonary Emboli and Venous Thrombi

Immunofluorescent staining was used to quantify the fibrin content of PE (Figure 10A). The fibrin content of pulmonary emboli in female *Cpb2*$^{-/-}$ mice was significantly greater than the WT female mice (P=0.034) (Figure 10B). Although the fibrin content of pulmonary emboli in male *Cpb2*$^{-/-}$ mice was 1.4-fold greater than the male WT mice, this difference was not significant (P=0.452). Similarly, lung tissues from female *Cpb2*$^{-/-}$ mice contained up to 1.8-fold higher fibrin content compared with male *Cpb2*$^{-/-}$ mice, but the difference was not significantly different (P=0.196). No differences were observed between male and female WT mice (P=0.666).

Similarly, venous thrombi were also stained to quantify their composition using the same antibodies targeting fibrin or CD41 on platelets (Figure 11). Initial observation of the venous thrombi in all mice indicate a greater CD41 fluorescence intensity compared with fluorescence using the fibrin stain, with brighter CD41 fluorescence appearing towards the centre of the thrombus and fibrin fluorescence appearing around the edges of the samples.

3.6 Clot lysis using fresh mouse plasma

Ex vivo analysis of fresh mouse plasma isolated from male and female WT mice without injury was used to identify any potential mechanisms that could explain the observed disparities between male and female mice and their deep vein thrombi stability *in vivo*. Mice were 15-17 weeks of age and weighed 27 ± 4 grams. Lysis times did not differ significantly between sexes (P=0.334). Plasma isolated from male WT mice lysed in 41

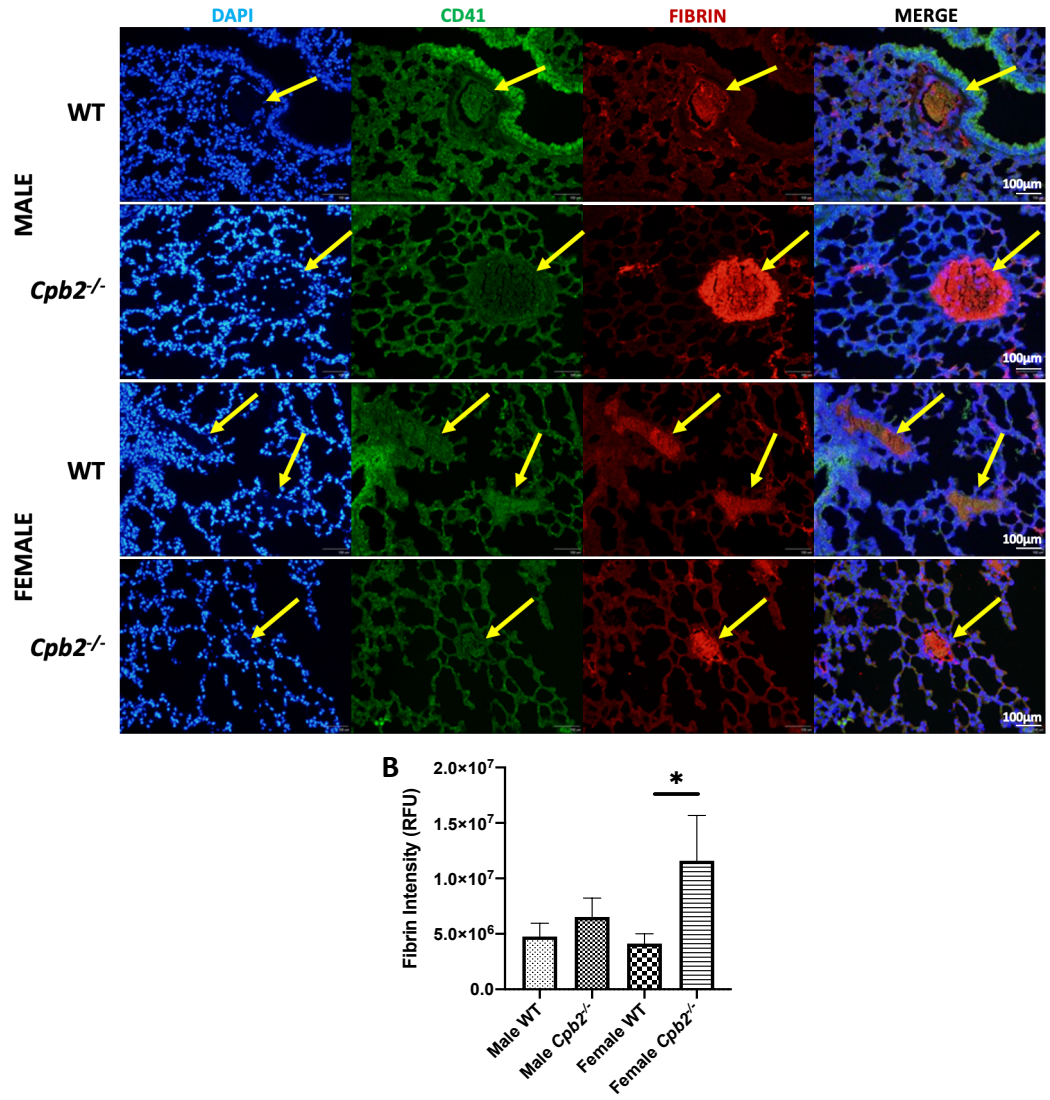

Figure 10. The role of TAFI and sex on PE composition.
Lung sections were stained using CD41-specific and fibrin-specific antibodies conjugated to Alexa-Fluor 488 and 594, respectively. (A) Emboli are illuminated red representing fibrin with blue (DAPI) representing surrounding pulmonary nucleated cells. (B) The relative fibrin fluorescence intensities of emboli were adjusted for background and plotted as relative fluorescence units (RFU). Values are presented as the mean ± SEM and significance was determined when $P<0.05$.

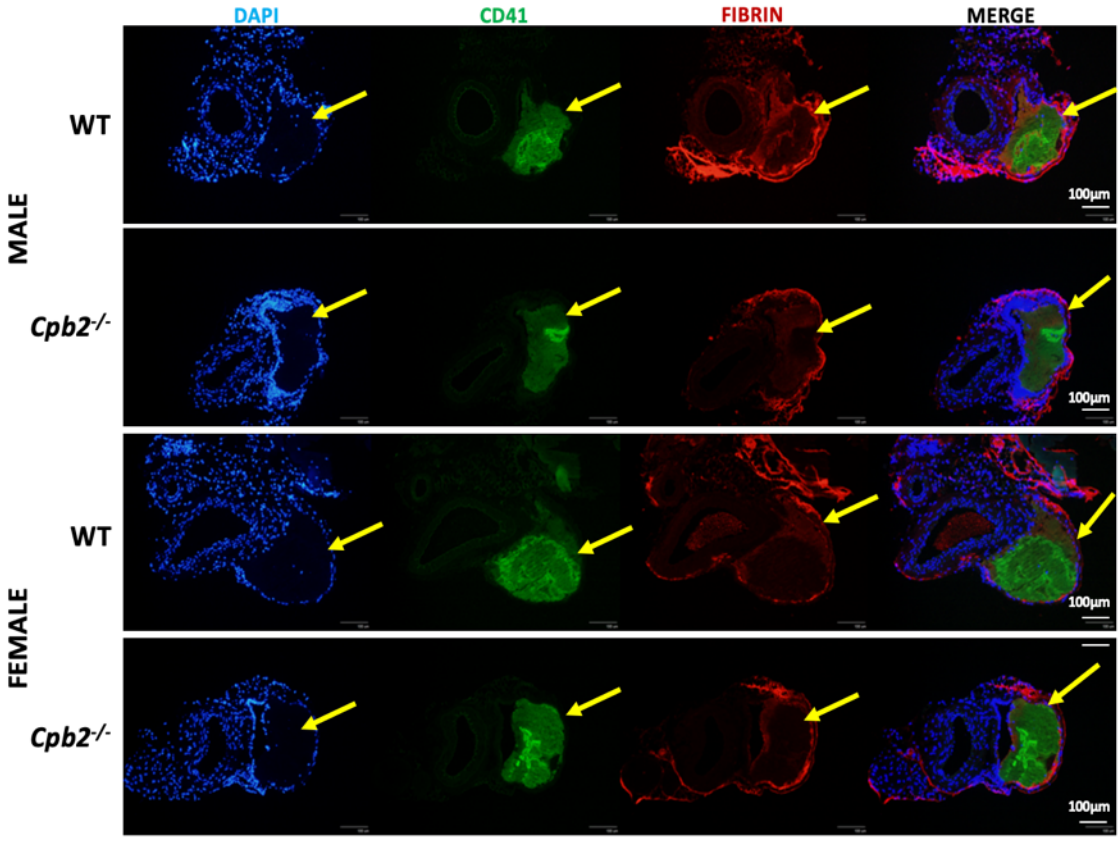

Figure 11. Representative cross-section images of venous thrombi composition. Femoral vein sections were stained using a CD41-specific antibody fragment conjugated to Alexa Fluor 488 and a fibrin-specific antibody conjugated to Alexa Fluor 594. The vein wall and surrounding nucleated cells are illuminated blue by DAPI (column 1), platelets within the thrombus are illuminated green (column 2), and fibrin is illuminated red (column 3), with a merged image in column 4.

minutes on average (40.7 ± 16.3), with plasma from female WT mice lysing in 51 minutes on average (51.4 ± 4.6) (Figure 12).

3.7 Thrombin-antithrombin complex concentrations in mouse plasma

To determine if thrombin generation was altered between mice used in this study, we measured TAT complex levels in isolated plasma from uninjured mice and mice post-surgery using a murine-specific TAT ELISA. Between mice that were subjected to thrombosis, TAT levels in males ranged between 93.3 pg/mL and 773.1 pg/mL for WT mice and 167.2 pg/mL and 1004.7 pg/mL for *Cpb2*$^{-/-}$ mice, without achieving statistical significance (P=0.555). For females, TAT levels ranged between 217.4 pg/mL and 701.3 pg/mL for WT mice and 251.6 pg/mL and 458.5 pg/mL for *Cpb2*$^{-/-}$ mice (P=0.742). No statistically significant differences were observed between male and female WT or *Cpb2*$^{-/-}$ mice (P=0.988 and P=0.402, respectively) (Table 1).

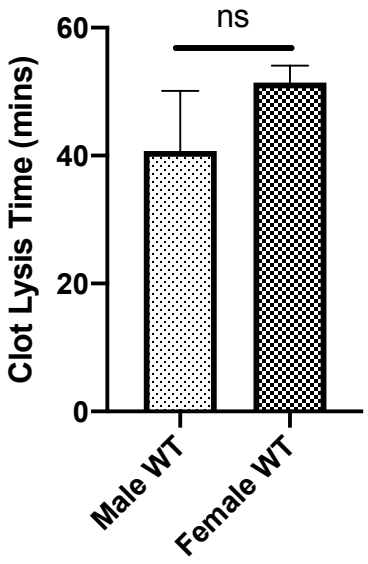

Figure 12. Clot lysis analysis of nascent WT mouse plasma.
Freshly collected mouse plasma was clotted using human thrombin and lysed using TNK-tPA at 37ºC. Clot lysis time was plotted as mean ± SEM between male and female mice. Clot lysis profiles were generated using turbidity at 400nm. The difference of half maximal OD change between clotting and lysis was considered as the clot lysis time.

Sample	Male	Female	P-value
WT TAT (pg/mL)	373.4 ± 259.1 n = 6	375.4 ± 175.2 n = 6	P = 0.9878
Cpb2$^{-/-}$ TAT (pg/mL)	489.3 ± 385.2 n = 6	349.2 ± 72.1 n = 6	P = 0.4019
P-value	P = 0.5547	P = 0.7416	

Table 1. TAT complex levels in mouse plasma after IVM.
Isolated mouse plasma was used to determine TAT concentrations using a mouse-specific TAT ELISA. (A) mean TAT levels between mice with values expressed ± SEM. (B) TAT levels between mice and associated P-values. Table values are expressed as mean ± standard deviation and significance determined if $P<0.05$.

4 Discussion

Quantification of emboli breaking away from deep vein thrombi effectively indicates the stability of the thrombi. We determined that knocking out TAFI in mice decreases thrombus stability (*i.e.* trends indicating increased embolic events) and consequently increases PE burden. Thrombus size increased for the duration of imaging predominantly in male *Cpb2*$^{-/-}$ and female WT mice. Female mice embolized to a greater extent than male mice, with or without the presence of TAFI, but did not exhibit significant increases in large emboli. Histological analysis of pulmonary tissue following the surgical procedures revealed increased PE within pulmonary arteries in male and female *Cpb2*$^{-/-}$ mice compared with respective WT counterparts, with emboli in females showing increased fibrin content. These findings suggest that TAFI may function to stabilize thrombi in DVT and reduce the risk of PE. Therefore, females may appear to be at a greater risk of developing PE when experiencing DVT.

The apparent sex-dependence of thrombus stability whereby female mice, both WT and *Cpb2*$^{-/-}$, had significantly greater embolization events than their respective male mice, were not associated with their (a) fibrinolytic potential as assessed by the in vitro clot lysis assay on nascent plasma (Figure 12), nor (b) TAT levels measured in plasma samples collected at the end of the 2-hour IVM experiment (Table 1). Additionally, TAT levels were measured in nascent plasma isolated from male (301.6 ± 46.2 pg/mL) and female (467.9 ± 193.8 pg/mL) WT mice, however, no significant differences were observed (P=0.2218). The disproportional incidence of VTE and/or PE between males and females is controversial, and the mechanisms behind these observations are incompletely understood.

One potential mechanism focuses on sex-specific growth hormone (GH) secretion patterns, which regulate hepatic gene expression (Wong et al., 2008). GH has been shown to modulate the expression of coagulation inhibitors in the liver including antithrombin, heparin cofactor II, and protein C inhibitor (Wong et al., 2008). As TAFI is synthesized in the liver, it is currently unknown how GH influences *Cpb2* gene expression and may contribute to the disparities between sex shown by our work.

Another proposed mechanism involves downregulation of the *Cpb2* gene by estrogen and progesterone, two major female sex hormones (Garand et al., 2013). An *in vitro* study determined a correlation between increased estrogen/progesterone levels and decreased *Cpb2* mRNA concentrations, suggesting a potentially novel indirect pathway of TAFI regulation (Garand et al., 2013). An increase in estrogen and progesterone seen in females and resulting decreased TAFI expression may also contribute to decreased thrombus stability seen in WT female mice. Interestingly, females have been noted to be at increased risk of first VTE during child-bearing age, pregnancy, and postpartum with oral contraceptives also contributing to risk depending on estrogen/progesterone levels (Gomes & Deitcher, 2004; Tormene et al., 2011). Sex-specific hormones including estrogen and progesterone may be a risk factor between women, as a surge of estrogen can be observed during ovulation and decreased levels post-menopause (Koebele & Bimonte-Nelson, 2016). Additionally, the murine estrous cycle, which begins at roughly 4 weeks of age and occurs every 4-5 days, follows similar fluctuations in hormone levels as seen in the human menstruation cycle (Koebele & Bimonte-Nelson, 2016). Due to this, it may be necessary to account for such disparities in circulating hormones in future studies. The effect of

testosterone on *Cpb2* expression remains elusive, therefore further studies must be completed to determine the mechanisms contributing to this difference in risk. However, studies have not demonstrated differences in circulating levels of TAFI between male and female mice. Furthermore, the similar differences in thrombus stability observed between male and female *Cpb2*$^{-/-}$ mice and WT mice suggest that the sex-dependent VTE outcome lies beyond TAFI. It is likely that TAFI accentuates this process since a trend exists suggesting that total embolic events were increased in both male and female *Cpb2*$^{-/-}$ mice when compared with their respective WT counterpart, although statistically insignificant in our study, this may suggest increased degradation of the fibrin matrix within the thrombus in the absence of TAFI.

We determined thrombus size by targeting CD41 on platelets. Although initial and maximum thrombus sizes remained similar between mice (Figure 6A-B), the differences in thrombus size towards the end of imaging, relative to baseline fluorescence intensities, increased for all mice. However, this increase was more profound in male *Cpb2*$^{-/-}$ and female WT mice. Studies investigating thrombus size in mice are limited due to different thrombus induction methods. However, one study investigating differences in thrombus size between sex in mice determined that male mice develop significantly larger thrombi than females when using an inferior vena cava electrolysis model (Alvarado et al., 2011). Interestingly, this is seen in our $FeCl_3$ femoral vein model with male *Cpb2*$^{-/-}$ mice, but not WT mice, which may suggest potential differences in vascular biology and inflammation between WT and *Cpb2*$^{-/-}$ mice (Myles et al., 2003). In female mice, the difference in thrombus size between WT and *Cpb2*$^{-/-}$ mice may be due to the increased embolization seen

in *Cpb2*$^{-/-}$ mice. However, this is not consistent with the male thrombus size data and it has been shown previously that thrombus size poorly correlates with PE burden (Shaya et al., 2016).

When compared with males, the significant increase in embolization seen in female mice did not result in a statistically significant increase in large emboli. A previous study by Shaya *et al*. observed a strong positive correlation between the quantity of large embolic events and PE burden in female mice, suggesting that large emboli entering circulation may be significantly contributing to PE burden (Shaya et al., 2016). However, no such correlation could be determined from our study. Based on our findings, although insignificant, the difference of large embolic events entering circulation appears to be influenced by sex to a greater extent than TAFI. Furthermore, these trends were not translated to PE burden, where only *Cpb2*$^{-/-}$ mice showed significantly elevated PE burden relative to respective WT mice. This is validated by comparing the emboli composition, whereby significantly greater levels of fibrin were detected in the emboli of female *Cpb2*$^{-/-}$ mice compared with WT mice. Emboli in male *Cpb2*$^{-/-}$ mice showed a slight increasing trend in fibrin levels compared with WT, however this difference was not significant. This seems counter-intuitive as absence of TAFI should lead to enhanced systemic fibrinolysis and fibrin degradation. However, it is likely that lack of TAFI leads to enhanced fibrinolysis at the site of the thrombus, particularly in females, which may lead to less stable thrombi with 1) greater embolic events and 2) potential generation of larger emboli that are also fibrin-rich in composition. One recent study looking at human thrombi reported that the composition of pulmonary emboli exhibited similar composition to its venous thrombi, of

which they are primarily composed of fibrin and red blood cells (RBCs) (Chernysh et al., 2020). Given the high fibrin composition of PE in our female *Cpb2*$^{-/-}$ mice, it is feasible that more stable thrombi generated in WT mice may have allowed for further fibrin degradation/thrombolysis prior to embolization. Additionally, coagulation factor XIII has been shown previously to improve the stability of thrombi and reduce PE burden in WT mice, even after treatment with dalteparin or dabigatran, which raises the question if administration of TAFI/TAFIa inhibitors along with supplemental FXIII would produce a stable thrombus while enhancing endogenous fibrinolysis.

The role of TAFI in thrombosis and hemostasis has been investigated using *Cpb2*$^{-/-}$ mice by various groups over the last two decades (Kraft et al., 2010; Leung & Morser, 2018; Morser et al., 2010; Nagashima et al., 2002; Qin et al., 2010). Most notably, studies have suggested that *Cpb2*$^{-/-}$ mice, compared with WT, do not exhibit changes to thrombosis or fibrinolysis *in vivo*, and therefore the loss of TAFI offers no consistent beneficial fibrinolytic phenotype (Foley et al., 2013; Velde et al., 2003). To better characterize the role of TAFI in VTE, we implemented our recently published novel murine model of VTE, which specifically quantifies thrombus size, stability, as well as downstream PE burden. The advantage of our model is that 1) it follows a specific non-occlusive injury to standardize thrombus generation *in vivo*, 2) the thrombi are monitored through real-time imaging and quantitation over hours thus allowing sufficient TAFI activation and TAFIa activity to affect fibrinolysis, and 3) PE burden can be observed and quantified using histology as a function of embolization events/thrombus stability (Shaya et al., 2016; Shaya, Gani, et al., 2019; Shaya, Westrick, et al., 2019). Using this approach, our study

reports a potential role of TAFI in VTE. Furthermore, it is challenging to directly compare many of the models used and their outcomes due to the various methods used to induce the thrombi (*e.g.* photochemical, stasis, or $FeCl_3$, concentration of the $FeCl_3$ or filter paper size, and length of contact) as well as the vasculature used (mesenteric vein, inferior vena cava, or femoral vein) (Vercauteren et al., 2012; Y.-X. Wang et al., 2007). In addition, a large majority of these studies used murine models of thrombosis that were biased against expressing TAFIa function towards thrombosis and fibrinolysis, as well as lacked the sensitivity and specificity to characterize the temporal changes to the thrombi. This is demonstrated by a recent study that reported the effect of TAFI on hemophilic arthropathy using the joint bleeding model on *Cpb2*$^{-/-}$ mice (Wyseure et al., 2016, 2018, 2019). Additionally, it is difficult to directly compare studies investigating the effects of TAFIa inhibition with total lack of TAFI zymogen on PE burden for two major reasons. First, TAFIa inhibition studies often require an initial stage of thrombus formation, usually by administering a procoagulant such as tissue factor, allowing thrombi generation to take place, and subsequently infusing TAFIa inhibitor of choice. While this is necessary to allow for the formation of either high thrombin accumulation or the thrombin-TM complex to efficiently activate TAFI, this enables the system to allow TAFIa formation/activity prior to its inhibition, albeit rapid, which may be sufficient to alter the overall outcome. Second, studies of PE fibrin deposition tend use a crude method of lung isolation and homogenization, followed by a fibrin-specific ELISA or other antigen-based assays. As we utilize a more sensitive and specific histological analysis to quantify PE burden, along with

immunohistochemistry to quantify fibrin deposition specific to the PE, direct comparisons of results from different methods should be avoided.

Overall, this study demonstrates that sex influences venous thrombus stability *in vivo*, with female mice at increased risk of VTE. We also demonstrate that TAFI may function to stabilize thrombi in DVT and subsequently reduce the risk of PE. Thus, the loss of TAFI/TAFIa significantly increases PE burden in both male and female mice and increases fibrin-rich PE in females. Further development of treatment strategies to minimize the role of TAFIa by reducing TAFI expression or applying direct TAFIa inhibitors to enhance fibrinolysis may require additional strategies to stabilize the thrombi during its breakdown.

5 Future Directions

As described by our data, the complete removal of TAFI from circulation contributes to thrombus instability. To further demonstrate the role of TAFI in VTE, administering WT mice with a TAFIa-specific inhibitor, such as potato tuber carboxypeptidase inhibitor (PTCI) as described previously (M. Schneider & Nesheim, 2003), may provide additional insight into the specific roles that TAFI and TAFIa each have on thrombi *in vivo*. Additionally, supplementation of endogenous TAFI or TAFIa in WT, knockout, and TAFIa inhibited mice may also provide further information on thrombus stability dynamics and potentially novel interactions or mechanisms of action.

Recently, small interfering RNA (siRNA) targeting TAFI have been gaining traction as a potential therapeutic strategy against breast cancer and liver injury (Okumura et al., 2009; Yu et al., 2017). This strategy could also be extrapolated to potentially be used as an anti-thrombotic therapy in hospitalized patients to prevent VTE. As animal studies have reported the loss of TAFI does not lead to hemostatic imbalance, this is an attractive strategy whereby reduced circulating TAFI levels would only impact rapid degradation of any thrombi formed without increased bleeding risks. Therefore, investigation of how anti-TAFI siRNA treatment affects acute thrombus growth in the femoral vein is currently being studied by our lab in collaboration with Dr. Christian Kastrup (University of British Columbia, BC). The effect on thrombus stability and resulting PE burden is yet to be

determined. Investigation of anti-TAFI siRNA may lead to alternative treatments for VTE in efforts to reduce PE burden and accelerate thrombus degradation.

Considering meaningful data collected from this project, it would be interesting to examine the role of TAFIa in arterial thrombosis by altering the described murine model of VTE to focus on arterial and brain tissue as opposed to venous and lung tissue. If TAFIa does have an important role in the regulation of venous thrombus stability, disparities in arterial thrombus stability may provide new insights into ischemic stroke treatment and management.

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