DEFINING THE ROLE OF FACTOR XIII IN VENOUS THROMBOEMBOLISM

Sravya Kattula

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology and Laboratory Medicine

Chapel Hill
2019

Approved by:

Alisa Wolberg

Cyrus Vaziri

Nigel Key

Christopher Mack

Wolfgang Bergmeier

"*Let us choose for ourselves our path in life, and let us try to strew that path with flowers*"
Émilie du Châtelet
French natural philosopher, mathematician, physicist, and author

ABSTRACT

Sravya Kattula: Defining the role of factor XIII in venous thromboembolism
(Under the direction of Alisa S. Wolberg)

Blood coagulation is a series of enzymatic reactions that form a clot and control bleeding in response to an injury. When this process goes awry and abnormal clots form in veins, it is called venous thromboembolism. This disease is a leading cause of death and disability in the Western world. Current anticoagulation treatments inhibit thrombus propagation but do not dissolve the existing thrombus or prevent embolization, and are also associated with bleeding risk. Further investigation is needed to identify therapeutic strategies that influence the structure and content of clots without increasing bleeding risk. We have discovered that the clotting protein, factor XIII (FXIII), plays an important role in mediating the pathophysiology of venous thrombosis. The studies presented in this dissertation give insight into FXIII's mechanistic contribution to venous thrombosis, hemostasis, and pulmonary embolism. We anticipate these findings will be important for the development of drugs targeting FXIII and may yield a new strategy for treating venous thromboembolism.

First, we determined the contribution of FXIII compartment (plasma vs. platelet FXIII) and level in clot contraction, composition, and size in in vitro and in vivo models of venous thrombosis and hemostasis. We found that plasma FXIII, but not platelet FXIII, produced high-molecular-weight fibrin crosslinks, promoted red blood cell retention, and mediated clot weight in vitro. We also demonstrated that 50% reduction in FXIII produced significantly smaller venous thrombi but did not increase bleeding in tail transection or saphenous vein puncture

models in vivo. These findings suggest that partial plasma FXIII reduction may be a therapeutic strategy for reducing venous thrombosis without increasing bleeding risk.

Second, we examined the effect of accelerated FXIII activation, through the FXIII Val34Leu polymorphism, on whole blood clot weight and composition. We found compared to the wild-type Val variant at high fibrinogen levels, presence of the Leu variant is associated with formation of smaller whole blood clots. These findings suggest the Val34Leu polymorphism may protect against venous thrombosis by decreasing red blood cell retention and, consequently, reducing clot size.

Third, we investigated the contribution of FXIII to thrombus stability and pulmonary embolism risk. Studying the mechanisms that may predispose a venous thrombus to embolize is limited by the lack of animal models that accurately recapitulate key characteristics of the human pathophysiology. Therefore, we developed a novel mouse model of venous thromboembolism that captures characteristics of human venous thrombosis (slow forming, red blood cell- and fibrin-rich thrombi) and pulmonary embolism (spontaneous embolism of existing venous thrombi). In this model, we determined that complete FXIII deficiency increases pulmonary embolization incidence, but partial deficiency does not. These findings suggest partial FXIII reduction may beneficially decrease venous thrombosis burden without increasing pulmonary embolization risk. Furthermore, we anticipate this novel mouse model can be used to evaluate the contribution of other pulmonary embolism risk factors and has to the potential to study disease progression and treatment intervention.

Collectively, these studies extend our understanding of the mechanisms that mediate venous thromboembolism and explore a previously-unrecognized, critical role for FXIII in

venous thromboembolism. This dissertation highlights basic research shaping translational efforts to discover novel approaches for prevention and treatment of diseases.

ACKNOWLEDGEMENTS

This dissertation is the accumulation of 5 years of hard work and commitment that could not have been possible without the guidance and support of many people who have helped shape the best version of me.

First and foremost, thank you to my PhD mentor, Dr. Alisa Wolberg. You have helped me to become a strong, critical thinking scientist and I will always be grateful for you creating the foundation of my scientific journey. I will carry the lessons you have taught me through every stage of my career and know I am better scientist because of it. I have truly loved being part of the thrombosis and hemostasis field and I would not have discovered where I fit in the world without you, so thank you again! Big, warm thank you to Dr. Lori Holle for literally everything you do for the lab and being my lab mom. From your scientific contributions to your kindness towards everyone, you have created a wonderful environment to work in. Dr. Adam Miszta, I am glad we have shaped up to be friends and even though you are back in the Netherlands, I know you will always be willing to talk science any time. Thank you Jean Marie Mwiza for your cheerful attitude every day and constant encouragement. I wish you the best of luck in finishing graduate school as well. Zhaoming Tang, thank you for your scientific discussions and good times in lab. Good luck with all your future endeavors. Although our time together has been short in lab, Dre Dobson and Emma Bouck, you both fit in well with the group dynamics and I wish the best for you two in graduate school and beyond. Michelle Luo, thank you for enthusiastically being an extra pair of hands to help our lab run smoothly. Thank you Dr.

Matt Flick for being a scientific mentor from afar when I put out my first publication and now locally with your lab set up right next to us. Welcome to UNC!

My current success could not have been possible with the support from former members of the Wolberg lab. Thank you Dr. James Byrnes for being such a vital part of my time in graduate school and beyond. From guiding me through experiments during my first days in lab to keeping up with me from the west coast at UCSF, I cannot image having done graduate school without you. Thank you Dr. Maria Aleman for your continued support on the FXIII projects I have extended since your days in the Wolberg lab and for helping set up the unbelievably, amazing opportunity to do an industry internship at Bioverativ. It has truly shaped my career path and goals. Dr. Joan Beckman, thank you following my progress in graduate school and always being available to talk. Dr. Bethany Walton, although we did not overlap much, thank you for always being reachable to talk science and life.

Beyond the lab, there are several people at UNC that have been key to my success. Thank you Anna Silver for being the first student I mentored in lab and my science sidekick, I am looking forward to seeing where the future takes you. Thank you Sara Martin and Seth Flynn for all of your hard work and help in pushing my projects forward during your summer research experiences through the SURE program. To my fellow thrombosis and hemostasis colleagues and friends on the 8th floor of MEJ 2.0 – Dorsaf Ghalloussi, Steve Grover, Silvio Antoniak, Bobby Lee, Denis Noubouossie, Mike Henderson, Yohei Michael, Anton Ilich, Dave Paul, Erica Sparkenbaugh, and Amandine Sotiaux – thank you for your support through the everyday ups and downs. Thank you Dr. Brian Cooley and the Animal Surgery Core Lab for helping me with all the in vivo studies that were essential to every part of my research. I will miss all the conversations we had while conducting mouse surgeries. Thank you to everyone at the Animal

Histopathology and Lab Medicine Core for helping me take on the mountain of a task to histologically process many mouse lungs and following the FIFA world cup games with me while I examine hundreds of slides under the microscopy. Thank you to the biological and biomedical science program team – Anna O'Connell, Beka Layton, Patrick Brandt, Dave McDonald – for helping me navigate the ups and downs of graduate school. Thank you Dr. Jon Homeister for creating an open environment for the students of the Pathobiology and Translational Science Program. Lastly, major thank you to my thesis committee members: Drs. Cyrus Vaziri, Nigel Key, Wolfgang Bergmeier, and Chris Mack. From helping shape my grant applications and thesis proposal in my early days of graduate school to the in-depth conversations that have shaped my science up until the final days, thank you for being my scientific mentors. Most of all, thank you for guiding me and ensuring my success for the next step in my career.

Beyond, the people at UNC, I would like to thank the different communities I have been grateful to be a part of and grow alongside with during my time at Chapel Hill. First, my friends who have become my family – Rachel Dee, Claire Trincot, Kate Geisen, Nicole Corbin, Carrie Wilzczewski, Katelyn Lavrich, Liz Muro, Sam Faber, Kristen Sieber Tucker, Andrea Bucker, and Brit Ciszek – thank you for all the memories we have made here in Chapel Hill, I cannot wait to make new ones and see where the future takes us all. Second, the family at The Coalition – Sheri Taylor, Jaimie Lee, DC Pacheco, Leith Rankine, Benay Hicks, and Jamin Rager – thank you for teaching me how to not just be strong physically but mentally as well to protect the peace in me. Third, my aerial and circus family – Liz Bliss, Kate Geisen, Amy Dreher, Kyla Garrett, Michael Dillion, and Mari Kearns – thank you for teaching me how to face challenges with courage and work through setbacks. Fourth, to all the wonderful friends I have made around the

world – Shana Shaya, Tammy Truong, Loyda Raquel, Aditi Patel, Annika de Jong, Jill Penafiel – thank you for making the world a smaller place and at the same time broadening my horizons. And last but not least, I would like to thank my family – Amma, Nana, Devika, and Radhika – thank you for being the foundations that have formed me and giving me the means to pursue my dreams and goals.

I would like to thank the American Heart Association (17PRE33660020) and National Institutes of Health, National Heart, Lung, and Blood Institute for funding the work in this dissertation and providing travel support for conferences through the Integrative Biology Program training grant (T32HL69768) and the Ruth L. Kirschstein Predoctoral Individual National Research Service Award (F31HL139100). I would also like to thank the UNC Pathobiology and Translational Sciences Program for awarding me the Katherine Pryzwansky Young Investigator Award which made it possible to attend and network at the International Society of Hemostasis and Thrombosis conference.

PREFACE

Chapter 1 contains text and figures from an article published prior to the writing of this dissertation with the following citation:

> Kattula S, Byrnes JR, and Wolberg AS. Fibrinogen and Fibrin in Thrombosis and Hemostasis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2017;e13-e21.

Permission to include portions of this article in a PhD dissertation was obtained from American Heart Association, the publisher of *Arteriosclerosis, Thrombosis, and Vascular Biology*.

Chapter 2 represents work that was published prior to writing this dissertation with the following citation:

> Kattula S, Byrnes JR, Martin SM, Holle LA, Cooley BC, Flick MJ, Wolberg AS. Factor XIII in plasma, but not in platelets, mediates red blood cell retention in clots and venous thrombus size in mice. *Blood Advances*. 2018;2(1):25-35.

Permission to include this article in its entirety in a PhD dissertation was obtained from The American Society of Hemotology, the publisher of *Blood Advances.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	Alpha
α_2-AP	α_2-antiplasmin
ANOVA	Analysis of variance
A.F.U	Arbitrary fluoresce unit
β	Beta
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
Fibrin(ogen)	Fibrinogen and fibrin, collectively
Fib$\gamma^{390\text{-}396A}$	Fibrinogen with alanine mutations in γ-chain residues 390-396
$FeCl_3$	Ferric Chloride
FXIII	Factor XIII
FXIIIa	Activated factor XIII
FXIII-A_2	Factor XIII-A_2 subunits
FXIII-A_2*	Activated FXIII-A
FXIII-A_2B_2	Plasma FXIII heterotetramer
FXIII-B_2	Factor XIII-B_2 subunits
$FXIII_{plasma}$	Plasma factor XIII
$FXIII_{plt}$	Platelet factor XIII
g	Gram
γ	Gamma
HBS	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMW	High molecular weight
II	Prothrombin
IIa	Thrombin
K_D	Dissociation constant
kDa	Kilodalton
kg	Kilogram
L	Liter
Leu	Leucine
M	Molar
Min	Minute
mg	Milligram
mL	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
M_w	Molecular weight
ng	Nanogram
nM	Nanomolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Pulmonary embolism
pM	Picomolar
PPP	Platelet-poor plasma
RBC	Red blood cell
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
T101	1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride
TAFI	Thrombin-activatable fibrinolysis inhibitor
TF	Tissue factor
U	Units
µL	Microliter
µM	Micromolar
µm	Micrometer
Val	Valine
VT	Venous thrombosis
VTE	Venous thromboembolism
WT	Wild-type

CHAPTER 1: INTRODUCTION[1]

1.1 Blood coagulation and venous thromboembolism.

Blood coagulation is the process that turns liquid blood into a gel-like clot in response to a vessel injury and thereby preventing blood loss. This process involves a series of enzymatic reactions through the intrinsic or extrinsic pathway, both of which ultimately lead to the common pathway to generate thrombin. Soluble fibrinogen is cleaved by thrombin to form fibrin monomers that become part of the insoluble structural framework of the clot. Thrombin also activates factor XIII (FXIII), which crosslinks the fibrin network and other plasma proteins to the fibrin network to stabilize clots against mechanical disruption and premature dissolution. Concurrently, activated platelets will pull on the fibrin network to contract the clot and restore blood flow.

Dysregulation of normal coagulation processes can lead to abnormal clot formation in the venous and arterial circulation, called thrombosis. Venous and arterial thrombosis are diseases with distinct pathophysiologies. Arterial thrombosis is typically associated with the rupture of an atherosclerotic plaque that exposes subendothelium and procoagulant material (e.g., collagen, tissue factor) to blood, which stimulates platelet activation and aggregation under high shear and produces platelet-rich "white thrombi".[1] In contrast, venous thrombosis (VT) is promoted by inappropriate expression of cell adhesion molecules and procoagulant activity on intact, dysfunctional endothelium in static blood, often in the presence of plasma hypercoagulability

[1] Parts of this chapter previously appeared in an article in *Arteriosclerosis, Thrombosis, and Vascular Biology*. The original citation is as follows: Kattula S, Byrnes JR, and Wolberg AS. Fibrinogen and Fibrin in Thrombosis and Hemostasis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2017;e13-e21.

(Virchow's triad), producing red blood cell-rich (RBC) "red thrombi".[1] VT and pulmonary embolism (PE), collectively venous thromboembolism (VTE), affect 1,000,000 individuals each year in the United States and in Europe.[2] VTE has ~30% mortality within the first 30 days of presentation that is usually associated with PE. Acute PE occurs when all or part of a thrombus detaches, travels through the vasculature, and occludes the pulmonary arteries. Surviving patients suffer further complications such as recurrent VT[3], post-thrombotic syndrome[4], and chronic thromboembolic pulmonary hypertension[5].

The initial goal of anticoagulation is to limit thrombus propagation, whereas the goal of long term treatment is to prevent recurrent VT. Although, traditional anticoagulants (e.g. fondaparinux, unfractionated heparin, low molecular weight heparin, and warfarin) accomplish these goals, they inhibit multiple coagulation proteins, require routine monitoring and dietary considerations, and increase the possibility of bleeding.[6] New direct oral anticoagulants (e.g. dabigatran, rivaroxaban, epixaban, and edoxaban) are highly effective at inhibiting a specific target and thus require less monitoring.[6] However, these anticoagulants still carry a bleeding risk because their targets are upstream of fibrin formation. While recent pursuits for novel anticoagulants with better safety profiles have lead researchers to target factors XI and XII[7-9], this dissertation explores the FXIII-fibrinogen axis as a therapeutic strategy for reducing VTE. FXIII's fibrin crosslinking function plays a role in the pathophysiology of VT by mediating RBC retention, and ultimately, thrombus size.[10] Targeting FXIII, which is downstream of thrombin generation and fibrin formation, could preserve normal fibrin clot formation and be associated with low bleeding risk while reducing VT. The dissertation examines the specific mechanisms by which the FXIII-fibrinogen axis mediate thrombosis and hemostasis.

1.2 Fibrinogen and factor XIII biology

The fibrinogen molecule is a 340-kDa homodimeric glycoprotein consisting of 2Aα, 2Bβ, and 2γ polypeptide chains linked by 29 disulfide bridges. Fibrinogen synthesis occurs primarily in hepatocytes (Figure 1). Assembly of the six chains takes place in a step-wise manner in which single chains assemble first into Aα-γ and Bβ-γ complexes, then into Aα/Bβ/γ half-molecules, and finally into hexameric complexes $(Aα/Bβ/γ)_2$ (reviewed in [11]). All six fibrinogen chains are assembled with their N-termini located in a central "E nodule," and extend outward in a coiled-coil arrangement. The Bβ and γ chains terminate in globular regions known as βC and γC modules, respectively. These regions collectively comprise the so-called "D nodule." The Aα chains are the longest; at the end of the coiled-coil region each chain extends into a highly-flexible series of repeats followed by a globular αC region. In healthy individuals, fibrinogen circulates in plasma at high concentrations (2-5 mg/mL). However, fibrinogen is an acute phase protein, and during acute inflammation, plasma fibrinogen levels can exceed 7 mg/mL.

During coagulation, fibrinogen is converted into insoluble fibrin (Figure 1.1). Fibrin formation involves thrombin-mediated proteolytic cleavage and removal of N-terminal fibrinopeptides from the Aα and Bβ chains. Insertion of these newly-exposed α- and β- "knobs" into a- and b- "holes" in the γC and βC regions of the D nodule, respectively, on another fibrin monomer permits the half-staggered association of fibrin monomers into protofibrils. Subsequent aggregation of protofibrils into fibers yields a fibrin meshwork that is essential for blood clot stability. Clot formation, structure, and stability are strongly influenced by the conditions present during fibrin generation. These include the concentrations of procoagulant, anticoagulant, fibrin(ogen)-binding proteins and molecules[12-20], and metal ions[21,22], as well as contributions of

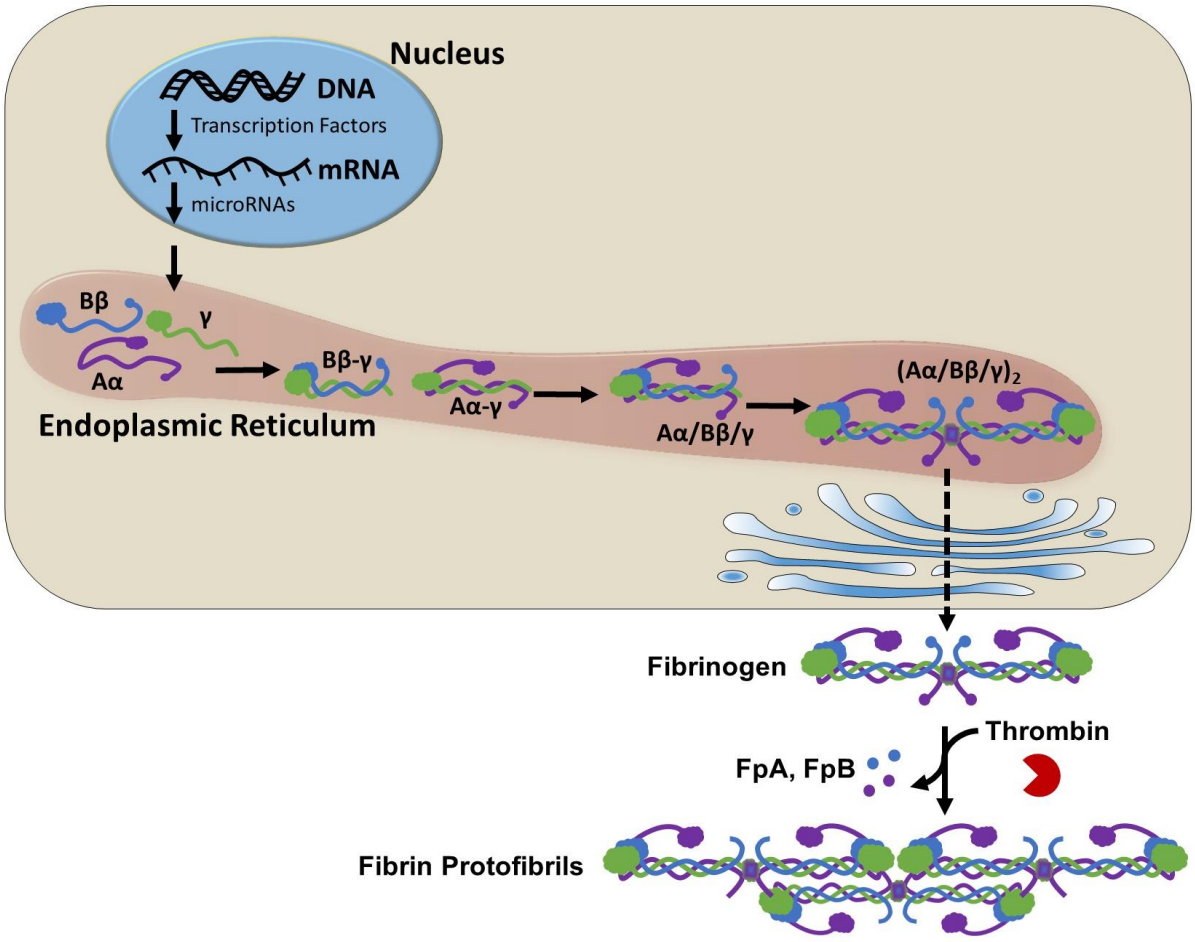


Figure 1.1. Fibrinogen synthesis. After individual fibrinogen chains are transcribed and translated, step-wise fibrinogen assembly begins with single chains assemble into Aα-γ and Bβ-γ precursors, then into Aα/Bβ/γ half-molecules, and finally into hexameric complexes $(Aα, Bβ, γ)_2$. This process occurs in the endoplasmic reticulum before fibrinogen is secreted from the cells and involved in fibrin network formation.

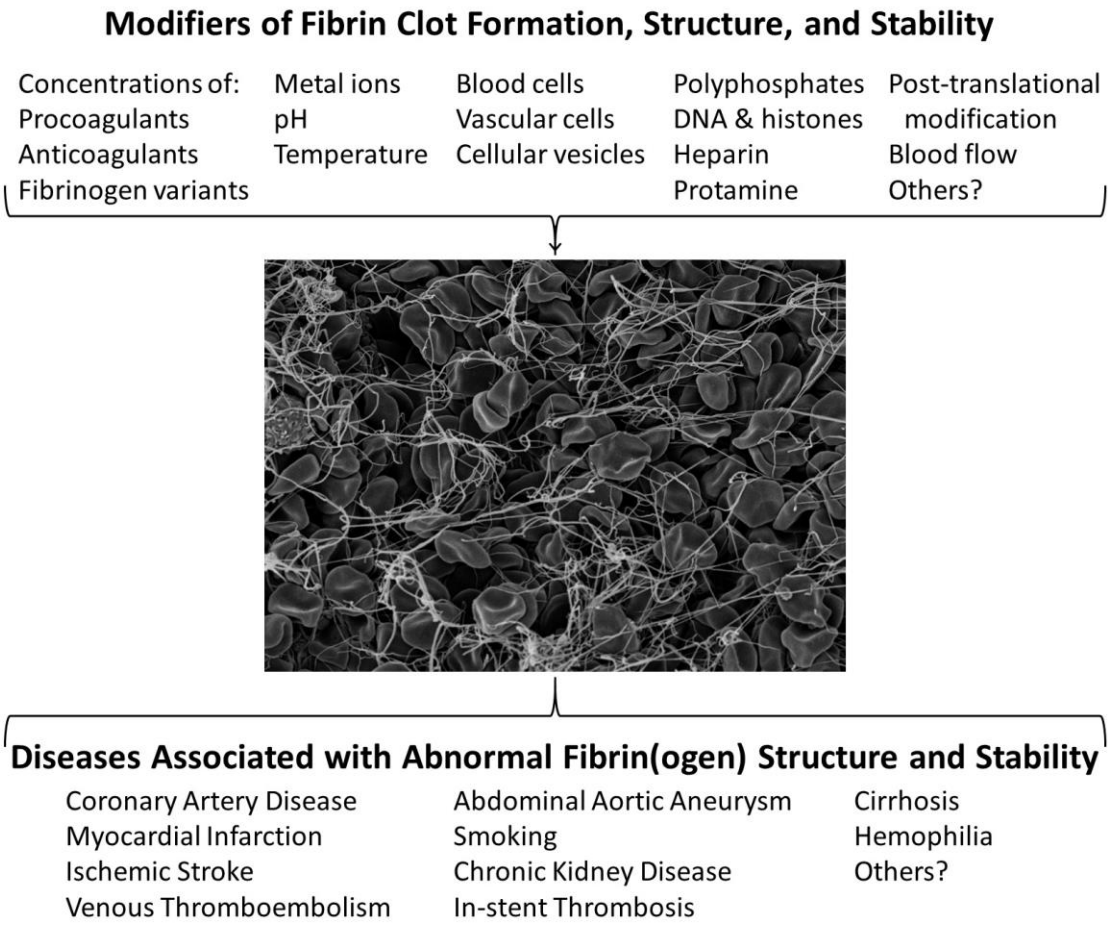


Figure 1.2. Modifiers of fibrin(ogen) and pathologic consequences. Clot formation, structure, and stability are influenced by the conditions present during fibrin generation. Fibrin clot abnormalities can be a biomarker for an operant pathophysiologic mechanism or causative in the disease etiology.

blood and vascular cells, and cell-derived microvesicles[23-30], and presence of blood flow[31,32] (Figure 1.2). Many of these mechanisms have been reviewed.[33,34]

FXIII is a protransglutaminase in plasma and cellular compartments, including platelets.[35] Plasma FXIII ($FXIII_{plasma}$) is a 320-kDa heterotetramer ($FXIII\text{-}A_2B_2$) consisting of two catalytic subunits ($FXIII\text{-}A_2$) tightly-associated ($K_d \sim 10^{-10}$ M)[36] with two non-catalytic subunits ($FXIII\text{-}B_2$). $FXIII_{plasma}$ circulates at ~70 nM (14-28 µg/mL)[37] in complex with fibrinogen. $FXIII_{plasma}$ is activated by thrombin-mediated cleavage of 37-amino acid (4-kDa) activation peptides from the N-termini of FXIII-A subunits, and calcium-mediated dissociation of the FXIII-B subunits.[38] Cellular FXIII is a dimer of the catalytic subunits ($FXIII\text{-}A_2$) and lacks B subunits. Cellular FXIII in platelets ($FXIII_{plt}$) is stored in the cytoplasm. During platelet activation, $FXIII_{plt}$ can be proteolytically cleaved and activated by thrombin, or non-proteolytically activated by high calcium concentrations. Following platelet activation, $FXIII_{plt}$ is externalized on the platelet surface; its exposure is maximized when platelets are activated by strong dual agonists.[39]

Catalytically-active FXIII (FXIIIa) induces ε-N-(γ-glutamyl)-lysyl crosslinks between glutamine and lysine residues on fibrin γ- and α-chains.[38] FXIII-mediated crosslinking of fibrin produces γ-γ dimers and high molecular weight species (γ-multimers, α-polymers, and αγ-hybrids). Assays in purified systems and platelet-poor plasma show $FXIII_{plasma}$ can crosslink fibrin, as well as other plasma proteins (e.g., α_2-antiplasmin [α_2-AP], thrombin-activatable fibrinolysis inhibitor [TAFI], fibronectin) to fibrin to stabilize the fibrin network against fibrinolysis and mechanical disruption.[40] Although the concentration of FXIII in platelets is 150-fold higher than in plasma, its role during hemostasis and thrombosis is unclear. $FXIII_{plt}$ can introduce crosslinks in fibrin and between fibrin and α_2-AP[41-44], and effects on clot stability can be detected when plasma levels of FXIII are low (≤10%).[39] Since fibrin crosslinking promotes

RBC retention in thrombi[40], these data suggest $FXIII_{plt}$ may promote RBC retention in thrombi and therefore, determine thrombus weight. It has also been proposed that $FXIII_{plt}$ is required for platelet-mediated clot contraction because impaired contraction was observed in FXIII-deficient platelet-rich plasma clots.[45,46] However, this finding is controversial, as Tutwiler et al. showed FXIII deficiency only delays clot contraction[46], and others report little-to-no influence of $FXIII_{plt}$ on platelet function or fibrin crosslinking.[42,47] The contribution of $FXIII_{plt}$ to RBC retention in thrombi and platelet-mediated clot contraction is unknown.

1.3 Factor XIII in venous thromboembolism

We recently showed FXIII activity mediates RBC retention in thrombi.[10] When *F13a*$^{+/+}$ and *F13a*$^{-/-}$ mice were subjected to experimental in vivo models of VT (inferior vein cava [IVC] ligation [stasis and stenosis]), thrombi harvested from *F13a*$^{-/-}$ mice had reduced RBC retention and consequently, were smaller.[10] Likewise, clots formed from *F13a*$^{-/-}$ mouse whole blood in vitro were smaller, further suggesting that FXIII deletion results in smaller clots due to decreased RBC retention.[10] We extended this finding to show that plasma-derived FXIII-A_2B_2 corrects RBC retention in $FXIII_{plasma}$-deficient clots in a dose-dependent manner.[10] These observations suggest $FXIII_{plasma}$ mediates clot composition and weight. However, the contribution of $FXIII_{plt}$ in these functions is unknown. In a study presented in chapter 2, we determined the role of the FXIII compartment and level in clot contraction, composition, and size in vitro and using in vivo models of hemostasis and venous thrombosis. Briefly, $FXIII_{plasma}$, but not $FXIII_{plt}$, produced high-molecular-weight fibrin crosslinks, promoted RBC retention, and increased clot weight. A 50% reduction in $FXIII_{plasma}$ produced significantly smaller venous thrombi but did not increase bleeding. These findings collectively suggest that partial $FXIII_{plasma}$ reduction may be a therapeutic strategy for reducing VT.

We also showed that the timing of FXIII activation influences thrombus RBC retention and size.[10] Specifically, *Fibγ*$^{390–396A}$ mice that have normal FXIII levels but delayed FXIII activation, show significantly decreased RBC retention and thrombus size.[10] This finding is interesting, given the existence of a FXIII polymorphism (FXIII in which Valine at codon 34 is a Leucine, Val34Leu) that exhibits accelerated FXIII activation and suggest the opposite result, increased clot weight, and consequently, increased VTE risk, but it paradoxically conveys moderate protection against VTE in certain human populations.[35,48,49] In plasma, the Val34Leu polymorphism affects fibrin clot structure in a fibrinogen concentration-dependent manner.[50] At high fibrinogen concentrations, the presence of the Val allele results in fibrin clots with thin, tightly-packed fibers.[50] However in the presence of the Leu allele, fibers are thicker and more loosely-packed, suggestive of a protective effect.[50] These interesting and apparent paradoxical findings raise important and clinically-relevant questions on the role of FXIII activation kinetics in thrombosis. The effect of the Val34Leu polymorphism has not been investigated in a physiologically-relevant whole blood system while modulating fibrinogen concentration. In an on-going study presented in Chapter 3, we determined the effect of the FXIII Val34Leu polymorphism on whole blood clot weight. Briefly, we demonstrated at high fibrinogen levels, the presence of the Leu variant is associated with formation of smaller whole blood clots. These findings suggest at prothrombotic fibrinogen concentration, the Val34Leu polymorphism may protect against VT by decreasing clot RBC retention and consequently, reducing clot size.

Our studies indicate reducing FXIII activity/levels decreases thrombus size, suggesting that FXIII inhibition is a potential strategy to reduce VT. FXIII is also known to play an essential role in clot stabilization to prevent premature lysis of a clot.[39,51] A meta-analysis by Girolami et al examining the prevalence of PE in congenital coagulation disorders in humans reported no

increase in embolism risk in FXIII deficient patients.[52] Conversely, Shaya et al detect increased pulmonary embolic events in *F13a*$^{-/-}$ mice; however, they used a $FeCl_3$ model of VT.[53] The effect of FXIII deficiency on thrombus stability and PE risk is unclear and requires further investigation. However, studies on the pathophysiological mechanisms that drive PE are currently limited by a lack of models that recapitulate human PE pathology. Previously reported mouse models of PE include exogenous thrombus injection[54-58] or administration of factors such as adenosine diphosphate[59,60], thromboplastin[61,62], tissue factor[59,63], thrombin[64-66] or collagen plus epinephrine[59,67-69]. These models result in microthrombi that get lodged in the lungs and do not result from spontaneous embolism of existing venous thrombi. These models do not fully recapitulate key aspects of PE in humans, which are thought to stem from spontaneous embolism of existing RBC- and fibrin-rich venous thrombi that travel through the circulation and get lodged in the lung. In an on-going study presented in Chapter 4, we aimed to develop a mouse PE model that is clinically-relevant to human PE pathophysiology to evaluate the contribution of FXIII to PE risk. In this novel PE model, complete FXIII deficiency increases PE incidence, but partial deficiency does not.

1.4 Focus of this dissertation

Traditional anticoagulants and newer direct oral anticoagulants are used to inhibit thrombus propagation and prevent VT recurrence. However, these anticoagulation strategies carry a bleeding risk because their targets are upstream of fibrin formation. There is a need to better understand the molecular mechanisms that promote VTE to develop targeted treatment strategies with better safety profiles. The focus of this dissertation is to expand our understanding of the role of FXIII activation and activity in VTE. This dissertation uses physiologically relevant in vitro and in vivo models to determine: 1) how plasma and platelet

FXIII mediate RBC retention and venous thrombus size, 2) how accelerated FXIII activation kinetics caused by the Val34Leu polymorphism modulates fibrinogen function and VT risk, and 3) how FXIII deficiency contributes to PE risk.

REFERENCES

1. Wolberg AS, Aleman MM, Leiderman K, Machlus KR. Procoagulant activity in hemostasis and thrombosis: Virchow's triad revisited. *Anesth Analg*. 2012;114(2):275-285.

2. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrom J. Incidence and mortality of venous thrombosis: a population-based study. *J Thromb Haemost*. 2007;5(4):692-699.

3. Heit JA. Venous thromboembolism epidemiology: implications for prevention and management. *Semin Thromb Hemost*. 2002;28 Suppl 2:3-13.

4. Ashrani AA, Heit JA. Incidence and cost burden of post-thrombotic syndrome. *J Thromb Thrombolysis*. 2009;28(4):465-476.

5. Goldhaber SZ, Visani L, De Rosa M. Acute pulmonary embolism: clinical outcomes in the International Cooperative Pulmonary Embolism Registry (ICOPER). *Lancet*. 1999;353(9162):1386-1389.

6. Wolberg AS, Rosendaal FR, Weitz JI, et al. Venous thrombosis. *Nat Rev Dis Primers*. 2015;1:15006.

7. Muller F, Gailani D, Renne T. Factor XI and XII as antithrombotic targets. *Curr Opin Hematol*. 2011;18(5):349-355.

8. Buller HR, Bethune C, Bhanot S, et al. Factor XI antisense oligonucleotide for prevention of venous thrombosis. *N Engl J Med*. 2015;372(3):232-240.

9. Larsson M, Rayzman V, Nolte MW, et al. A factor XIIa inhibitory antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk. *Sci Transl Med*. 2014;6(222):222ra217.

10. Aleman MM, Byrnes JR, Wang JG, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest*. 2014;124(8):3590-3600.

11. Redman CM, Xia H. Fibrinogen biosynthesis. Assembly, intracellular degradation, and association with lipid synthesis and secretion. *Ann N Y Acad Sci*. 2001;936:480-495.

12. Blomback B, Carlsson K, Fatah K, Hessel B, Procyk R. Fibrin in human plasma: gel architectures governed by rate and nature of fibrinogen activation. *Thromb Res*. 1994;75(5):521-538.

13. Francis CW, Marder VJ. Increased resistance to plasmic degradation of fibrin with highly crosslinked alpha-polymer chains formed at high factor XIII concentrations. *Blood*. 1988;71(5):1361-1365.

14. Glover CJ, McIntire LV, Brown CH, 3rd, Natelson EA. Rheological properties of fibrin clots. Effects of fibrinogen concentration, Factor XIII deficiency, and Factor XIII inhibition. *J Lab Clin Med*. 1975;86(4):644-656.

15. Machlus KR, Cardenas JC, Church FC, Wolberg AS. Causal relationship between hyperfibrinogenemia, thrombosis, and resistance to thrombolysis in mice. *Blood*. 2011;117(18):4953-4963.

16. Nair CH, Shah GA, Dhall DP. Effect of temperature, pH and ionic strength and composition on fibrin network structure and its development. *Thromb Res*. 1986;42(6):809-816.

17. Ryan EA, Mockros LF, Weisel JW, Lorand L. Structural origins of fibrin clot rheology. *Biophys J*. 1999;77(5):2813-2826.

18. Smith SA, Morrissey JH. Polyphosphate enhances fibrin clot structure. *Blood*. 2008;112(7):2810-2816.

19. Whyte CS, Chernysh IN, Domingues MM, et al. Polyphosphate delays fibrin polymerisation and alters the mechanical properties of the fibrin network. *Thromb Haemost*. 2016;116(5):897-903.

20. Smith SA, Morrissey JH. 2013 scientific sessions Sol Sherry distinguished lecture in thrombosis: polyphosphate: a novel modulator of hemostasis and thrombosis. *Arterioscler Thromb Vasc Biol*. 2015;35(6):1298-1305.

21. Henderson SJ, Xia J, Wu H, et al. Zinc promotes clot stability by accelerating clot formation and modifying fibrin structure. *Thromb Haemost*. 2016;115(3):533-542.

22. Wolberg AS, Gabriel DA, Hoffman M. Analyzing fibrin clot structure using a microplate reader. *Blood Coagul Fibrinolysis*. 2002;13(6):533-539.

23. Aleman MM, Gardiner C, Harrison P, Wolberg AS. Differential contributions of monocyte- and platelet-derived microparticles towards thrombin generation and fibrin formation and stability. *J Thromb Haemost*. 2011;9(11):2251-2261.

24. Campbell RA, Overmyer KA, Selzman CH, Sheridan BC, Wolberg AS. Contributions of extravascular and intravascular cells to fibrin network formation, structure, and stability. *Blood*. 2009;114(23):4886-4896.

25. Cines DB, Lebedeva T, Nagaswami C, et al. Clot contraction: compression of erythrocytes into tightly packed polyhedra and redistribution of platelets and fibrin. *Blood*. 2014;123(10):1596-1603.

26. Zubairova LD, Nabiullina RM, Nagaswami C, et al. Circulating Microparticles Alter Formation, Structure, and Properties of Fibrin Clots. *Sci Rep*. 2015;5:17611.

27. Wohner N, Sotonyi P, Machovich R, et al. Lytic resistance of fibrin containing red blood cells. *Arterioscler Thromb Vasc Biol*. 2011;31(10):2306-2313.

28. Gersh KC, Nagaswami C, Weisel JW. Fibrin network structure and clot mechanical properties are altered by incorporation of erythrocytes. *Thromb Haemost*. 2009;102(6):1169-1175.

29. Campbell RA, Overmyer KA, Bagnell CR, Wolberg AS. Cellular procoagulant activity dictates clot structure and stability as a function of distance from the cell surface. *Arterioscler Thromb Vasc Biol*. 2008;28(12):2247-2254.

30. Jerome WG, Handt S, Hantgan RR. Endothelial cells organize fibrin clots into structures that are more resistant to lysis. *Microsc Microanal*. 2005;11(3):268-277.

31. Campbell RA, Aleman M, Gray LD, Falvo MR, Wolberg AS. Flow profoundly influences fibrin network structure: implications for fibrin formation and clot stability in haemostasis. *Thromb Haemost*. 2010;104(6):1281-1284.

32. Gersh KC, Edmondson KE, Weisel JW. Flow rate and fibrin fiber alignment. *J Thromb Haemost*. 2010;8(12):2826-2828.

33. Weisel JW. Structure of fibrin: impact on clot stability. *J Thromb Haemost*. 2007;5 (Suppl 1):116-124.

34. Wolberg AS. Thrombin generation and fibrin clot structure. *Blood Rev*. 2007;21(3):131-142.

35. Bereczky Z, Muszbek L. Factor XIII and venous thromboembolism. *Semin Thromb Hemost*. 2011;37(3):305-314.

36. Katona E, Penzes K, Csapo A, et al. Interaction of factor XIII subunits. *Blood*. 2014;123(11):1757-1763.

37. Fickenscher K, Aab A, Stuber W. A photometric assay for blood coagulation factor XIII. *Thromb Haemost*. 1991;65(5):535-540.

38. Komaromi I, Bagoly Z, Muszbek L. Factor XIII: novel structural and functional aspects. *J Thromb Haemost*. 2011;9(1):9-20.

39. Mitchell JL, Lionikiene AS, Fraser SR, Whyte CS, Booth NA, Mutch NJ. Functional factor XIII-A is exposed on the stimulated platelet surface. *Blood*. 2014;124(26):3982-3990.

40. Byrnes JR, Duval C, Wang Y, et al. Factor XIIIa-dependent retention of red blood cells in clots is mediated by fibrin alpha-chain crosslinking. *Blood*. 2015;126(16):1940-1948.

41. Francis CW, Marder VJ. Rapid formation of large molecular weight alpha-polymers in cross-linked fibrin induced by high factor XIII concentrations. Role of platelet factor XIII. *J Clin Invest*. 1987;80(5):1459-1465.

42. Hevessy Z, Haramura G, Boda Z, Udvardy M, Muszbek L. Promotion of the crosslinking of fibrin and alpha 2-antiplasmin by platelets. *Thromb Haemost*. 1996;75(1):161-167.

43. Reed GL, Matsueda GR, Haber E. Fibrin-fibrin and alpha 2-antiplasmin-fibrin cross-linking by platelet factor XIII increases the resistance of platelet clots to fibrinolysis. *Trans Assoc Am Physicians*. 1991;104:21-28.

44. Reed GL, Matsueda GR, Haber E. Platelet factor XIII increases the fibrinolytic resistance of platelet-rich clots by accelerating the crosslinking of alpha 2-antiplasmin to fibrin. *Thromb Haemost*. 1992;68(3):315-320.

45. Kasahara K, Souri M, Kaneda M, Miki T, Yamamoto N, Ichinose A. Impaired clot retraction in factor XIII A subunit-deficient mice. *Blood*. 2010;115(6):1277-1279.

46. Tutwiler V, Litvinov RI, Lozhkin AP, et al. Kinetics and mechanics of clot contraction are governed by the molecular and cellular composition of the blood. *Blood*. 2016;127(1):149-159.

47. Rao KM, Newcomb TF. Clot retraction in a factor XIII free system. *Scand J Haematol*. 1980;24(2):142-148.

48. Balogh I, Szoke G, Karpati L, et al. Val34Leu polymorphism of plasma factor XIII: biochemistry and epidemiology in familial thrombophilia. *Blood*. 2000;96(7):2479-2486.

49. Catto AJ, Kohler HP, Coore J, Mansfield MW, Stickland MH, Grant PJ. Association of a common polymorphism in the factor XIII gene with venous thrombosis. *Blood*. 1999;93(3):906-908.

50. Lim BC, Ariens RA, Carter AM, Weisel JW, Grant PJ. Genetic regulation of fibrin structure and function: complex gene-environment interactions may modulate vascular risk. *Lancet*. 2003;361(9367):1424-1431.

51. Fraser SR, Booth NA, Mutch NJ. The antifibrinolytic function of factor XIII is exclusively expressed through alpha(2)-antiplasmin cross-linking. *Blood*. 2011;117(23):6371-6374.

52. Girolami A, Cosi E, Tasinato V, Peroni E, Girolami B, Lombardi AM. Pulmonary embolism in congenital bleeding disorders: intriguing discrepancies among different clotting factors deficiencies. *Blood Coagul Fibrinolysis*. 2016;27(5):517-525.

53. Shaya SA, Saldanha LJ, Vaezzadeh N, Zhou J, Ni R, Gross PL. Comparison of the effect of dabigatran and dalteparin on thrombus stability in a murine model of venous thromboembolism. *J Thromb Haemost*. 2016;14(1):143-152.

54. Bdeir K, Murciano JC, Tomaszewski J, et al. Urokinase mediates fibrinolysis in the pulmonary microvasculature. *Blood*. 2000;96(5):1820-1826.

55. Murciano JC, Harshaw D, Neschis DG, et al. Platelets inhibit the lysis of pulmonary microemboli. *Am J Physiol Lung Cell Mol Physiol*. 2002;282(3):L529-539.

56. Butte AN, Houng AK, Jang IK, Reed GL. Alpha 2-antiplasmin causes thrombi to resist fibrinolysis induced by tissue plasminogen activator in experimental pulmonary embolism. *Circulation*. 1997;95(7):1886-1891.

57. Carmeliet P, Stassen JM, Schoonjans L, et al. Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. *J Clin Invest*. 1993;92(6):2756-2760.

58. Kirchhof K, Welzel T, Zoubaa S, et al. New method of embolus preparation for standardized embolic stroke in rabbits. *Stroke*. 2002;33(9):2329-2333.

59. Smyth SS, Reis ED, Vaananen H, Zhang W, Coller BS. Variable protection of beta 3-integrin--deficient mice from thrombosis initiated by different mechanisms. *Blood*. 2001;98(4):1055-1062.

60. Nordoey A, Chandler AB. Platelet thrombosis induced by adenosine diphosphate in the rat. *Scand J Haematol*. 1964;1:16-25.

61. Leon C, Freund M, Ravanat C, Baurand A, Cazenave JP, Gachet C. Key role of the P2Y(1) receptor in tissue factor-induced thrombin-dependent acute thromboembolism: studies in P2Y(1)-knockout mice and mice treated with a P2Y(1) antagonist. *Circulation*. 2001;103(5):718-723.

62. Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. *Blood*. 2002;100(9):3240-3244.

63. Ellery PE, Maroney SA, Cooley BC, et al. A balance between TFPI and thrombin-mediated platelet activation is required for murine embryonic development. *Blood*. 2015;125(26):4078-4084.

64. Gresele P, Momi S, Berrettini M, et al. Activated human protein C prevents thrombin-induced thromboembolism in mice. Evidence that activated protein c reduces intravascular fibrin accumulation through the inhibition of additional thrombin generation. *J Clin Invest*. 1998;101(3):667-676.

65. Kumada T, Dittman WA, Majerus PW. A role for thrombomodulin in the pathogenesis of thrombin-induced thromboembolism in mice. *Blood*. 1988;71(3):728-733.

66. Paul W, Gresele P, Momi S, Bianchi G, Page CP. The effect of defibrotide on thromboembolism in the pulmonary vasculature of mice and rabbits and in the cerebral vasculature of rabbits. *Br J Pharmacol*. 1993;110(4):1565-1571.

67. Konstantinides S, Schafer K, Neels JG, Dellas C, Loskutoff DJ. Inhibition of endogenous leptin protects mice from arterial and venous thrombosis. *Arterioscler Thromb Vasc Biol*. 2004;24(11):2196-2201.

68. Beviglia L, Poggi A, Rossi C, et al. Mouse antithrombotic assay. Inhibition of platelet thromboembolism by disintegrins. *Thromb Res*. 1993;71(4):301-315.

69. DiMinno G, Silver MJ. Mouse antithrombotic assay: a simple method for the evaluation of antithrombotic agents in vivo. Potentiation of antithrombotic activity by ethyl alcohol. *J Pharmacol Exp Ther*. 1983;225(1):57-60.

CHAPTER 2: FACTOR XIII IN PLASMA, BUT NOT IN PLATELETS, MEDIATES RED BLOOD CELL RETENTION IN CLOTS AND VENOUS THROMBUS SIZE IN MICE[2]

2.1 Overview

The transglutaminase factor XIII (FXIII) stabilizes clots against mechanical and biochemical disruption, and is essential for hemostasis. In vitro and in vivo models of venous thrombosis demonstrate that FXIII mediates clot size by promoting red blood cell (RBC) retention. However, the key source of FXIII and whether FXIII activity can be reduced to suppress thrombosis without imposing deleterious hemostatic consequences are two critical unresolved questions. FXIII is present in multiple compartments including plasma ($FXIII_{plasma}$) as a heterotetramer of A_2 and B_2 subunits, and in platelets ($FXIII_{plt}$) as an A_2 homodimer. We determined the role of FXIII compartment and level in clot contraction, composition, and size both in vitro and using in vivo models of hemostasis and venous thrombosis. Reducing overall FXIII levels decreased whole blood clot weight, but did not alter thrombin generation or contraction of platelet-rich plasma clots. In reconstituted platelet-rich plasma and whole blood clot contraction assays, $FXIII_{plasma}$, but not $FXIII_{plt}$, produced high molecular weight fibrin crosslinks, promoted RBC retention, and increased clot weights. Genetically imposed reduction of FXIII delayed FXIII activation and fibrin crosslinking, suggesting FXIII levels mediate the kinetics of FXIII activation and activity, and that the timing of these processes is a critical

[2] This chapter previously appeared as an article in *Blood Advances*. The original citation is as follows: Kattula S, Byrnes JR, Martin SM, Holle LA, Cooley BC, Flick MJ, Wolberg AS. Factor XIII in plasma, but not in platelets, mediates red blood cell retention in clots and venous thrombus size in mice. Blood Advances. 2018;2(1):25-35.

determinant of RBC retention during clot formation and contraction. A 50% reduction in $FXIII_{plasma}$ produced significantly smaller venous thrombi, but did not increase bleeding in tail transection or saphenous vein puncture models in vivo. Collectively, these findings suggest that partial FXIII reduction may be a therapeutic strategy for reducing venous thrombosis.

2.2 Introduction

Coagulation factor XIII (FXIII) is a protransglutaminase that plays an important role in clot stability. Activated FXIII (FXIIIa) introduces ε-N-(γ-glutamyl)-lysyl crosslinks between glutamine and lysine residues. Crosslinks between fibrin γ- and α-chains, and between fibrin and other plasma proteins (e.g., α_2-antiplasmin, thrombin-activatable fibrinolysis inhibitor, fibronectin), stabilize clots against mechanical disruption and fibrinolysis.[1-4] FXIIIa also promotes red blood cell (RBC) retention in venous thrombi, and genetic deletion or inhibition of FXIII zymogen or FXIIIa (FXIII[a]), respectively, reduces thrombus weight both in vitro and in in vivo models of venous thrombosis.[5]

FXIII is present both in plasma and within cells, including platelets. Plasma FXIII ($FXIII_{plasma}$) is a 320-kDa non-covalent heterotetramer (FXIII-A_2B_2) consisting of two catalytic subunits (FXIII-A_2) tightly-associated (Kd ~10^{-10} M)[6] with two non-catalytic subunits (FXIII-B_2). $FXIII_{plasma}$ circulates at 14-28 μg/mL[7], nearly always in complex with fibrinogen. Assays in purified systems and platelet-poor plasma (PPP) show $FXIII_{plasma}$ can efficiently crosslink fibrin and other plasma proteins to fibrin to collectively stabilize the fibrin network.[2-4]

Platelet FXIII ($FXIII_{plt}$) exists as a homodimer (FXIII-A_2) in the platelet cytoplasm.[8] During platelet activation, $FXIII_{plt}$ is externalized on the platelet surface; exposure is maximized when platelets are activated by strong dual agonists (thrombin plus collagen).[9] Although the concentration of FXIII in platelets is 150-fold higher than in plasma, its role during hemostasis

and thrombosis is unclear. Three aspects of $FXIII_{plt}$ function have been particularly controversial. First, it has been proposed that $FXIII_{plt}$ is required for platelet contraction, as impaired contraction was observed in FXIII-deficient platelet-rich plasma (PRP) clots and in whole blood treated with transglutaminase inhibitors.[10,11] However, other studies have reported little-to-no influence of $FXIII_{plt}$ on platelet contractile events.[12-14] Thus, the contribution of $FXIII_{plt}$ to platelet-mediated clot contraction remains unclear. Second, a previous report suggested that FXIII-A deletion induces a compensatory increase in platelet-derived tissue transglutaminase activity.[15] Thus, this activity may have confounded earlier studies with *F13a*$^{-/-}$ mice. Third, although $FXIII_{plt}$ can introduce crosslinks in fibrin and between fibrin and α_2-antiplasmin[16-19], effects of $FXIII_{plt}$ on clot stability are only detected in vitro when $FXIII_{plasma}$ levels are low ($\leq$10%).[9] Thus, the contribution of $FXIII_{plt}$ to RBC retention in clots and during hemostasis and thrombosis in vivo are unclear. Determining the relative roles of $FXIII_{plasma}$ and $FXIII_{plt}$ in these processes is essential for understanding the biological importance of these compartments and the clinical significance of deficiencies in $FXIII_{plasma}$ or $FXIII_{plt}$.

Understanding FXIII function in vivo is also necessary for optimizing therapeutic approaches to modify FXIII level or activity to mitigate bleeding and thrombosis. Notably, in spite of the established role of FXIIIa in clot stabilization, the FXIII level necessary for hemostasis is unclear. Patients with <4% FXIIIa activity have a significantly increased risk of bleeding, including central nervous system and umbilical cord bleeding, hemarthroses, and hematomas, and experience recurrent miscarriage[20] However, patients with ~4-30% FXIIIa activity exhibit high variability in bleeding severity[20], and individuals with ≥30% FXIIIa activity, including those with acquired FXIII deficiency, are usually asymptomatic.[21,22] Interestingly, a recent analysis of hospitalized patients indicated 21% of adults and 52% of children had

$FXIII_{plasma}$ levels <50 U dL^{-1}, suggesting acquired FXIII deficiency is relatively common in patients after surgery and in the intensive care unit.[23] Since hospitalized patients have increased risk for both bleeding and thrombosis, the role of FXIII in these situations is difficult to predict. Thus, identifying a level of FXIII that reduces thrombus size without impairing hemostasis is critical for understanding the role of FXIII in clot function and for developing novel antithrombotic therapies targeting FXIII(a).

Here, we used murine models of FXIII deficiency to determine the roles of plasma and platelet FXIII in platelet-mediated clot contraction, thrombin generation, FXIII activation, fibrin crosslinking, and RBC retention in contracted clots. We also determined the effect of FXIII reduction using in vivo models of hemostasis and thrombosis. Collectively, our findings suggest moderate reduction of FXIII may reduce venous thrombosis without significantly increasing bleeding.

2.3 Methods

Murine blood draws and plasma preparation. Murine studies were approved by the University of North Carolina at Chapel Hill and Cincinnati Children's Hospital Medical Center Institution of Animal Care and Use Committees. *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice were backcrossed 6 generations on a C57BL/6J background.[24] A second line of *F13a*$^{-/-}$ mice (generous gift of CSL Behring) on a mixed 129Ola/CBACa background was maintained by homozygous breeding.[25]

Mice were anesthetized with 3-3.5% isoflurane in 2% oxygen and blood was drawn from the inferior vena cava (IVC) into 3.2% citrate (10% v/v, final) in a terminal procedure. PRP was prepared by first centrifuging whole blood (125*g*, 5 minutes) and then centrifuging the platelet-

enriched plasma fraction (100*g*, 5 minutes). PPP was prepared by centrifuging whole blood (5000*g*, 10 minutes).

Clot contraction assays. Whole blood was clotted with tissue factor (TF, Innovin diluted 1:12,000 [1 pM TF], final) and $CaCl_2$ (10 mM, final) in the absence or presence of the transglutaminase inhibitor T101 at the concentrations indicated. Clot formation and contraction proceeded in siliconized wells for 120 minutes at 37°C.

For PRP clot contraction, platelets in PRP were quantified (HV950FS Hemavet cell counter) and PRP was diluted with autologous PPP to obtain the final concentrations of platelets indicated. Clotting was triggered in siliconized aggregometer tubes at 37°C by adding TF and $CaCl_2$ (1 pM and 10 mM, final, respectively). Photos of contracting clots were recorded every 5 minutes for the first hour, every 10 minutes for the second hour, and at 24 hours.

For assays with reconstituted whole blood, PRP was treated with prostaglandin-I_2 (50 ng/mL, final) and platelets were pelleted by centrifugation (400*g*, 5 minutes). Platelet pellets were resuspended in Tyrode's buffer. PPP was obtained by centrifuging whole blood (5000*g*, 10 minutes). RBCs were isolated and washed with citrated glucose saline buffer (1.3 mM sodium citrate, 3.3 mM glucose, 1.2 mM NaCl, pH 7.2) and packed by centrifugation. PPP and washed platelets ($400x10^6$/mL, final) from C57BL/6J and *F13a*$^{-/-}$ mice were combined with RBCs ($3.5x10^9$/mL, final) from C57BL/6J mice to model reconstituted whole blood sufficient or deficient in $FXIII_{plasma}$ and $FXIII_{plt}$. Clotting was triggered at 37°C in siliconized wells with TF and $CaCl_2$ (1 pM and 10 mM, final, respectively) or collagen, thrombin, and calcium (20 mg/mL, 1 U/mL, and 10 mM final, respectively). Contracted clots were weighed at 2 hours.

Bleeding models. The tail vein bleeding model was performed on *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice on a C57BL/6J background, as described.[26] Briefly, mice were anesthetized with

ketamine/xylazine, 3-mm of the tail tip was excised using a scalpel blade (#11), and the tail was submerged in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.5) containing 2 mM $CaCl_2$ at 37°C. The time to blood flow cessation, defined as no flow for more than 15 seconds, was recorded as the bleeding time. The saphenous vein bleeding model was performed on *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice on a C57BL/6J background as described.[27] Briefly, the right saphenous vein was partially transected and opened further, longitudinally, with microscissors. Blood was gently wicked away under slow irrigation until hemostasis occurred. The clot was then disrupted using a 30-G needle, and blood was wicked away until hemostasis occurred again. Clot disruption was repeated after each incidence of hemostasis until 30 minutes after the initial injury. Two readouts were recorded: 1) the average time to hemostasis for each event in 30 minutes and 2) the total number of hemostatic events during the 30-minute period.

Venous thrombosis model. The IVC stasis model was performed as described[28] on 8- to 12-week-old *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice on a C57BL/6J background. Briefly, anesthetized mice were subjected to sterile laparotomy, the IVC was exposed, side branches were ligated, and lumbar branches were cauterized. The IVC was separated from the aorta by blunt dissection and completely ligated. Mice recovered with analgesia (subcutaneous buprenorphine, 0.05 mg/kg) and were maintained on acetaminophen (6 mg/mL) in their drinking water. After 24 hours, mice were anesthetized and blood was drawn from the suprarenal IVC into 3.2% sodium citrate (10% v/v, final concentration). Thrombi were separated from the vein wall and weighed. Blood samples were centrifuged to isolate PPP and platelets, and FXIII-A_2B_2 was quantified by western blotting using standard curves with the appropriate species (human or mouse FXIII-A_2B_2).

Statistical methods. Descriptive statistics (mean, standard error of the mean [SEM]) were calculated for each experiment, and Lilliefors test was used to assess normality. Experiments with 2 groups and normally-distributed data were compared by Student's *t* test with equal or unequal variance, as appropriate. Experiments with more than 2 conditions were analyzed by ANOVA with Bonferroni or Dunnett's post-hoc tests for between-group comparisons. $P<0.05$ was considered significant.

2.4 Results

FXIII deficiency decreases RBC retention and clot weight without altering thrombin generation or platelet contraction. We previously showed that genetic deletion of FXIII-A ($F13a^{-/-}$) reduces RBC retention in contracted clots and consequently, clot weight.[5] To now extend these findings to determine the dose-relationship between FXIII level and clot weight, we first analyzed in vitro clot contraction of whole blood from $F13a^{+/+}$, $F13a^{+/-}$, and $F13a^{-/-}$ mice. Complete blood counts indicate all three genotypes have normal levels of leukocytes, RBCs, and platelets (Supplemental Table 1). Compared to $F13a^{+/+}$ mice, both a 50% reduction in FXIII-A as well as complete FXIII-A deficiency increased RBC extrusion from contracted clots (~36% and ~50% for $F13a^{+/-}$ and $F13a^{-/-}$ mice, respectively, Figure 2.1A) and decreased clot weight (~32% and ~71% for $F13a^{+/-}$ and $F13a^{-/-}$ mice, respectively, Figure 2.1B). Inhibition of FXIIIa activity using the irreversible transglutaminase inhibitor T101 (20 μM, final, a concentration that maximally inhibits FXIIIa activity and reduces clot weight [Supplemental Figure 2.1A-B]) increased RBC extrusion from contracted clots (~69% and ~28% for $F13a^{+/+}$ and $F13a^{+/-}$ mice, respectively, Figure 2.1A) and reduced clot weight (~67% and ~70% for $F13a^{+/+}$ and $F13a^{+/-}$ mice, respectively, Figure 2.1B). T101 had no effect on RBC retention or whole blood clot weight from

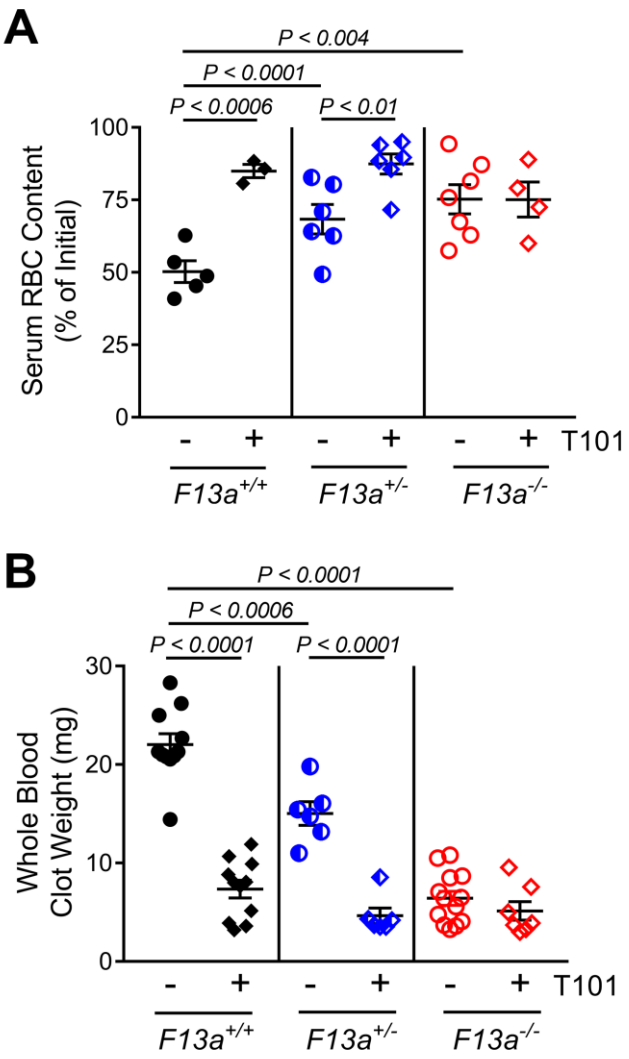


Figure 2.1. Inhibition or genetic deletion of FXIII(a) results in decreased clot weight. Whole blood from *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice was clotted with TF/$CaCl_2$ in the absence or presence of the FXIII inhibitor T101 (20 µM, final) for 2 hours. (A) Serum RBC content and (B) contracted whole blood clot weight were recorded. Each dot is a separate mouse; lines indicate means ± SEM.

F13a$^{-/-}$ mice, consistent with a specific role for FXIII(a)-dependent transglutaminase activity in determining clot composition and weight (Figure 2.1A-B).

Thrombin generation did not differ between genotypes in PRP or PPP (Table 2.1), indicating changes in RBC retention and clot weight were not due to altered plasma or platelet procoagulant activity. Since previous studies suggested FXIII is necessary for platelet-mediated contractile forces[10,11], we also analyzed the kinetics of clot contraction in *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ PRP. For all three genotypes, clot contraction occurred rapidly and was platelet concentration-dependent. In PRP with 400x10^6 platelets/mL, clots were 50% contracted within ~20 minutes of triggering clotting and were fully contracted within ~55 minutes. As the platelet concentration decreased, the onset time of clot contraction prolonged, and the rate and extent of maximum contraction decreased (Figure 2.2A-D, Table 2.2). However, there were no differences in contraction parameters between PRP clots from *F13a*$^{+/+}$, *F13a*$^{+/-}$, or *F13a*$^{-/-}$ mice (Figure 2.2A-D, Table 2.2), and inspection of contracted clots showed similar appearance in all three genotypes (Figure 2.2E). PRP clots from a separate line of *F13a*$^{-/-}$ mice on a 129Ola/CBACa background similarly showed 50% contraction with ~17 minutes, and complete clot contraction within ~42 minutes (data not shown). Finally, since a previous study suggested *F13a*$^{-/-}$ mice may express a FXIII-independent platelet transglutaminase activity[15] that could compensate for FXIII deficiency in these mice, we measured transglutaminase activity in platelets isolated from *F13a*$^{+/+}$ and *F13a*$^{-/-}$ mice. These experiments showed little-to-no transglutaminase activity in platelets from *F13a*$^{-/-}$ mice on a C57BL/6J background or in platelets from *F13a*$^{-/-}$ mice on a 129Ola/CBACa background (Figure 2.2F). Collectively, these data show FXIII deficiency decreases RBC retention and contracted whole blood clot weight in a dose-dependent manner, without altering plasma or platelet procoagulant activity, or platelet contraction.

	Platelet-Poor Plasma			Platelet-Rich Plasma		
	***F13a*$^{+/+}$**	***F13a*$^{+/-}$**	***F13a*$^{-/-}$**	***F13a*$^{+/+}$**	***F13a*$^{+/-}$**	***F13a*$^{-/-}$**
Lag time (min)	1.6 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	2.3 ± 0.2	2.3 ± 0.1	2.33 ± 0.2
Time to peak (min)	3.6 ± 0.5	3.7 ± 0.1	3.5 ± 0.1	5.6 ± 0.3	6.0 ± 0.4	4.9 ± 0.2
Velocity (nM/min)	35.8 ± 2.6	33.8 ± 1.2	34.1 ± 2.6	18.0 ± 2.7	13.8 ± 3.3	22.3 ± 5.3
Peak thrombin (nM)	70.1 ± 2.9	66.7 ± 1.5	65.3 ± 3.9	55.2 ± 6.0	46.4 ± 6.6	52.7 ± 3.7
Endogenous thrombin potential (nM*min)	387.7 ± 34.4	417.5 ± 68.7	284.6 ± 18.5	804.8 ± 164.6	621.0 ± 63.1	611.4 ± 50.0

Table 2.1. Thrombin generation is similar in *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice. Thrombin generation was measured by calibrated automated thrombography. Data show means ± SEM and were analyzed by ANOVA with Dunnett's post-hoc test (N=4-6).

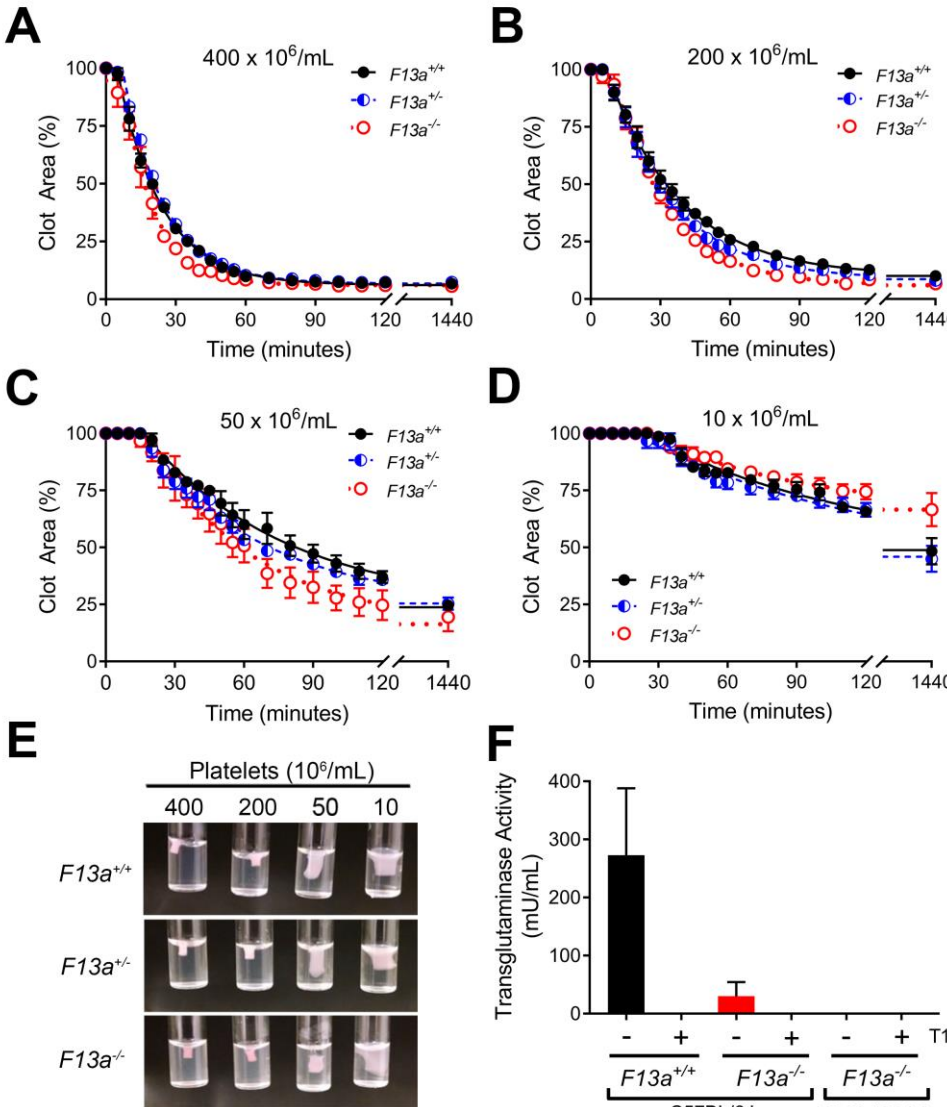


Figure 2.2. FXIII is not required for platelet-mediated clot contraction. PRP from *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice was clotted with TF/$CaCl_2$ at (A) 400, (B) 200, (C) 50, and (D) 10 x 10^6 platelets/mL. Clot area over time was assessed as a percentage of initial clot area. Dots show mean ± SEM (N=4); curves are fit to a non-linear regression with one-phase decay equation. (E) Photographs of contracted clots at 24 hours. (F) Transglutaminase activity was measured in platelets from *F13a*$^{+/+}$ and *F13a*$^{-/-}$ C57BL/6J mice, and *F13a*$^{-/-}$ 129Ola/CBACa mice. Data show mean ± SEM (N=3-5).

	Platelets (10^6/mL)	*F13a*$^{+/+}$	*F13a*$^{+/-}$	*F13a*$^{-/-}$
Onset time (minutes)	400	5.2 ± 0.9	7.1 ± 0.3	6.4 ± 1.5
	200	8.0 ± 1.6	7.8 ± 1.7	10.3 ± 1.6
	50	17.6 ± 1.6††	17.8 ± 4.4	19.8 ± 5.5
	10	22.2 ± 3.2†††	22.3 ± 4.5†	28.5 ± 4.6††
Rate (10^{-3}/minutes)	400	54.8 ± 1.4	55.7 ± 2.3	75.8 ± 5.1*
	200	33.6 ± 2.1†††	37.1 ± 1.9†	45.1 ± 1.1*,†
	50	17.4 ± 3.2†††	22.3 ± 0.2†††	26.4 ± 3.4††
	10	10.6 ± 1.1†††	16.4 ± 6.1†††	31.0 ± 13.0††
Maximum contraction (% of initial clot area)	400	6.4 ± 0.4	6.7 ± 0.1	6.1 ± 0.2
	200	10.5 ± 1.4	8.8 ± 1.0	6.7 ± 0.6*
	50	24.0 ± 0.9††	26.9 ± 2.7††	18.1 ± 6.5
	10	49.3 ± 5.6†††	47.5 ± 6.0†††	67.3 ± 7.3†††

Table 2.2. Kinetic parameters of PRP-mediated clot contraction in *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice. Clot contraction parameters were determined by a non-linear regression curve fit with plateau followed by one-phase decay. Data show means ± SEM and were analyzed by ANOVA with Dunnett's post-hoc test. *P < 0.005 between genotypes compared to F13a+/+. †P < 0.05, ††P < 0.005, and †††P < 0.0001 between platelet count, compared to 400x106/mL platelets (N=4).

$FXIII_{plasma}$, but not $FXIII_{plt}$, mediates clot weight. Genetic reduction of FXIII-A in mice reduces FXIII-A antigen in both plasma and platelets (Figure 2.3A-B). Therefore, to determine the relative contributions of $FXIII_{plasma}$ and $FXIII_{plt}$ to RBC retention and clot weight, we isolated plasma and platelets from FXIII-sufficient and -deficient (*F13a*$^{-/-}$) mice and recombined these with RBCs to yield reconstituted whole blood with specific deficiencies in $FXIII_{plasma}$ or $FXIII_{plt}$. We then triggered clotting with TF and $CaCl_2$, and measured fully contracted clot weights. As expected, absence of both $FXIII_{plasma}$ and $FXIII_{plt}$ significantly decreased clot weight (Figure 2.3C). Whereas reconstitution of $FXIII_{plt}$ had no effect on RBC retention or clot weight, reconstitution of $FXIII_{plasma}$ restored RBC retention and clot weight to levels seen in FXIII-replete reactions (Figure 2.3C). Since $FXIII_{plt}$ is maximally externalized on the platelet surface by strong, dual agonists[9], we also analyzed effects of $FXIII_{plasma}$ and $FXIII_{plt}$ on the weight of contracted clots triggered by addition of thrombin and collagen. Similar to experiments with TF, these reactions showed $FXIII_{plasma}$, but not $FXIII_{plt}$, promoted RBC retention in clots and increased clot weight (Figure 2.3C).

To determine effects of these FXIII compartments on fibrin crosslinking, we triggered clotting in reconstituted PRP that was sufficient or deficient in $FXIII_{plasma}$ or $FXIII_{plt}$, and assessed fibrin crosslinking by SDS-PAGE and western blotting. Only samples containing $FXIII_{plasma}$ showed crosslinked HMW fibrin species (Figure 2.3D), indicating $FXIII_{plasma}$, but not $FXIII_{plt}$, generates these species. This finding unites the observation that $FXIII_{plasma}$, but not $FXIII_{plt}$, increases RBC retention and weight of contracted clots (Figure 2.3C) with previous findings that FXIII(a)-dependent retention of RBCs in contracted clots is associated with the formation of HMW crosslinked fibrin species[29]. Together, these results indicate that $FXIII_{plasma}$, but not $FXIII_{plt}$, promotes RBC retention in contracted clots.

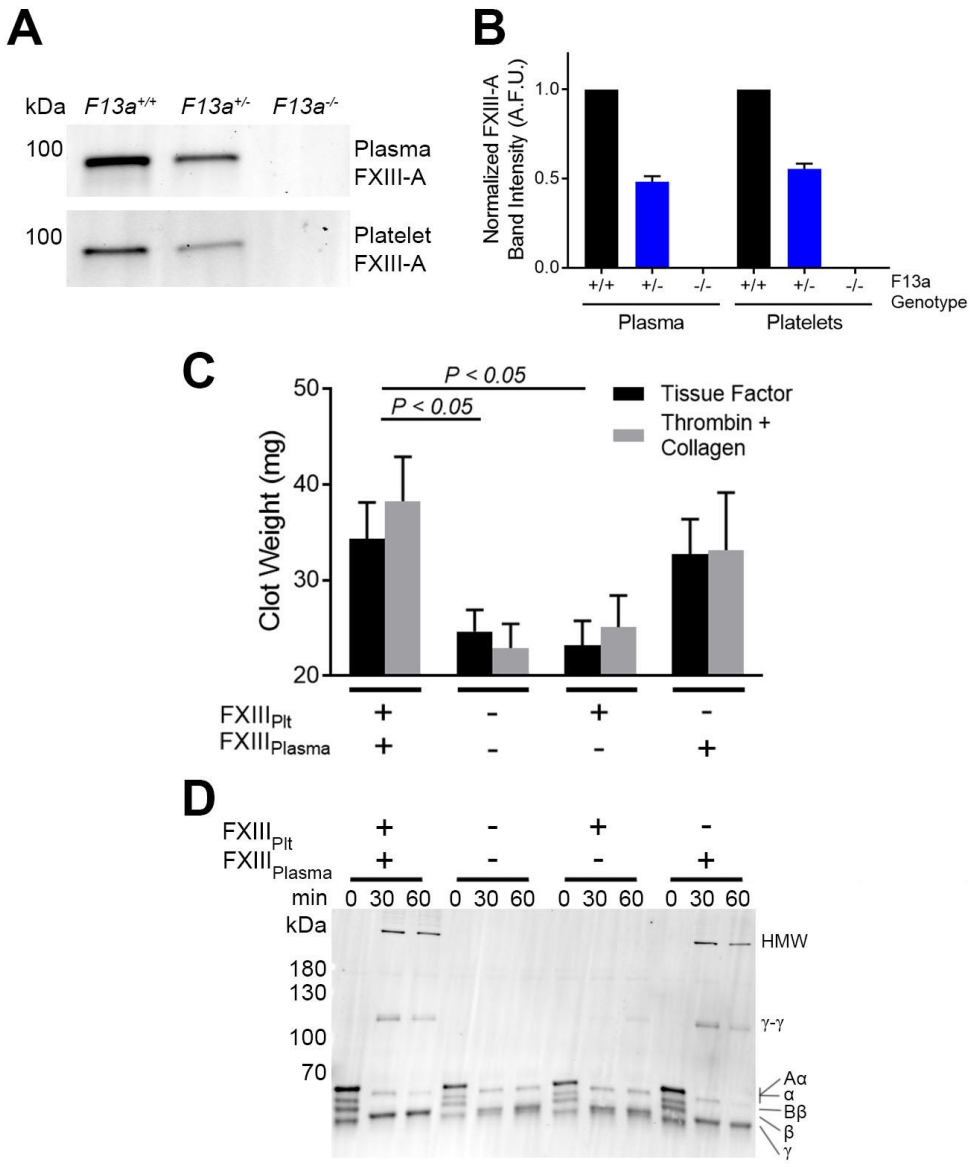


Figure 2.3. FXIII$_{plasma}$, but not FXIII$_{plt}$, mediates clot weight. (A) Representative western blot and (B) quantification for FXIII-A in plasma and platelets from *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice (N=3). (C) RBCs were reconstituted with C57BL6/J FXIII-sufficient (wild-type) and -deficient (*F13a*$^{-/-}$) plasma and platelets and clotting was triggered with TF/$CaCl_2$ (N=9) or thrombin/collagen/$CaCl_2$ (N=6). Data show means ± SEM. (D) Plasma and platelets from C57BL6/J FXIII-sufficient (wild-type) and -deficient (*F13a*$^{-/-}$) mice were recombined to make PRP sufficient or deficient in plasma or platelet FXIII. Clotting was triggered with TF/$CaCl_2$ and reactions were quenched at the indicated time points and analyzed by SDS-PAGE with western blotting (N=2). Fibrin crosslinking was detected using anti-fibrin(ogen) antibody, and identity of bands was confirmed by mass spectrometry.

FXIII level mediates FXIII activation kinetics and consequently, fibrin crosslinking. $FXIII_{plasma}$ is activated by thrombin-mediated cleavage of 37-amino acid (4-kDa) activation peptides on the N-termini of the FXIII-A subunits, and calcium-mediated dissociation of $FXIII\text{-}B_2$.[1] The observation that FXIII mediated clot weight in a gene dose-dependent manner (Figure 2.1) suggested that the concentration of FXIII zymogen present in plasma determines clot weight by modulating FXIIIa generation and consequently, fibrin crosslinking. To characterize this mechanism, we used SDS-PAGE and western blotting to compare FXIII activation and fibrin crosslinking kinetics during TF/$CaCl_2$-initiated clotting of $F13a^{+/+}$, $F13a^{+/-}$, and $F13a^{-/-}$ PRP. As expected, $F13a^{-/-}$ clots showed no FXIII-A antigen present and consequently, no fibrin crosslinking (data not shown). Compared to $F13a^{+/+}$, $F13a^{+/-}$ clots exhibited a ~3 minute delayed onset of FXIII activation, ~2.7-fold decreased rate of FXIII activation, and ~50% reduction in the total amount of FXIIIa generated at 15 minutes (Figure 2.4A-B). Moreover, compared to $F13a^{+/+}$, $F13a^{+/-}$ clots demonstrated ~3 minute delayed onset and ~2.5-fold decreased rate of γ-γ dimer formation (Figure 2.4C-E), and ~1.5 minute delayed onset and ~1.4-fold decreased rate of HMW crosslinked fibrin formation (Figure 2.4C-D, F). Both $F13a^{+/+}$ and $F13a^{+/-}$ clots showed complete crosslinking at ~15 minutes. These data suggest reducing plasma FXIII prolongs FXIII activation and delays, but does not prevent, maximal fibrin crosslinking.

Partial reduction of FXIII does not prolong the time to hemostasis, but significantly decreases thrombus size. FXIII is fundamentally different than other coagulation proteins because FXIII activation and FXIIIa activity occur downstream of thrombin generation. Given our data indicating that FXIII influenced clot size without altering thrombin generation (Table 2.1) or platelet contractile events (Figure 2.2), we hypothesized that partial FXIII reduction would not impair hemostatic clot formation. To test this hypothesis, we subjected $F13a^{+/+}$,

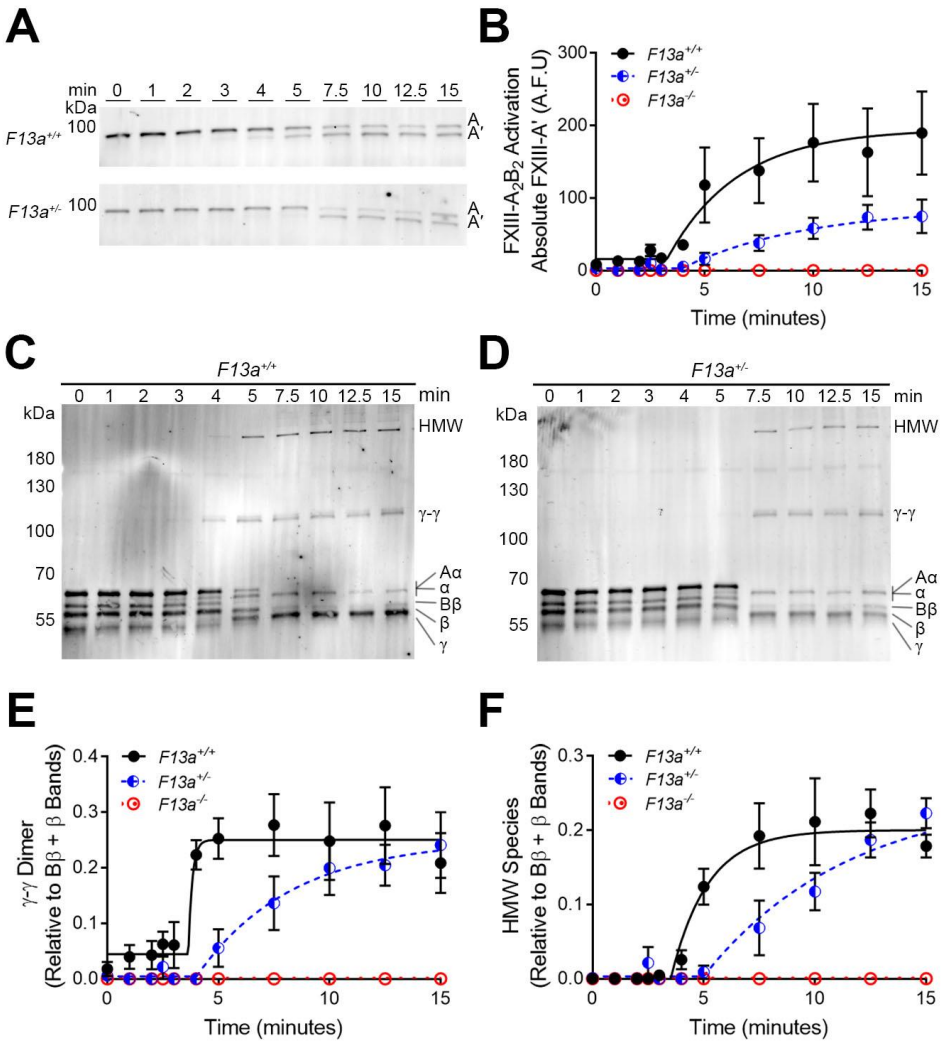


Figure 2.4. Compared to *F13a*$^{+/+}$ clots, *F13a*$^{+/-}$ clots have delayed FXIII activation and fibrin crosslinking. PRP was clotted with TF/$CaCl_2$ and quenched at the indicated time points. FXIII activation (generation of FXIII-A′) and fibrin crosslinking were analyzed by SDS-PAGE with western blotting. (A) Representative western blots and (B) quantitation of FXIII-A′ over time for clot formation in *F13a*$^{+/+}$ and *F13a*$^{+/-}$ PRP. Data show mean ± SEM (N=3-5). (C-D) Representative western blots for fibrin crosslinking in *F13a*$^{+/+}$and *F13a*$^{+/-}$ PRP clots and quantification of (E) γ-γ and (F) High molecular weight (HMW) crosslinked fibrin species formation. Data show mean ± SEM (N=3-5).

F13a$^{+/-}$, and anticoagulants, including unfractionated and low molecular weight heparin.[30-32] In the tail transection model, compared to *F13a*$^{+/+}$ mice, *F13a*$^{-/-}$ mice had prolonged tail-bleeding times, but *F13a*$^{+/-}$ mice did not (Figure 2.5A). In the saphenous vein model, *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice had a similar number of hemostatic events in 30 minutes (data not shown) and similar average hemostasis times (Figure 2.5B).

Finally, given our data demonstrating dose effects of $FXIII_{plasma}$ on clot size in vitro (Figure 2.3), we tested the hypothesis that $FXIII_{plasma}$ mediates venous thrombus weight in vivo. We first subjected *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice to the IVC ligation venous thrombosis model and harvested thrombi at 24 hours. We previously detected fully crosslinked fibrin in FXIII-sufficient mice, and significantly smaller thrombi in *F13a*$^{-/-}$ mice at this time point (MM Aleman, unpublished observation and [5]). We now demonstrated that this effect is gene dose-dependent during venous thrombosis in vivo (Figure 2.6A). To define the relationship between $FXIII_{plasma}$ and thrombus weight, we used human plasma-derived FXIII-A_2B_2 to restore $FXIII_{plasma}$ in *F13a*$^{-/-}$ mice to 10-100% of normal. Compared to mouse FXIII-A_2B_2, human FXIII-A_2B_2 has similar transglutaminase activity (data not shown), and similarly crosslinks mouse fibrin (Supplemental Figure 2.2). We induced thrombus formation by IVC ligation, harvested thrombi and plasmas at 24 hours, and determined plasma and platelet FXIII-A levels by western blot. Thrombus weights in *F13a*$^{+/+}$, *F13a*$^{+/-}$, *F13a*$^{-/-}$, and FXIII-A_2B_2-infused *F13a*$^{-/-}$ mice correlated positively and significantly with circulating FXIII-A antigen in plasma ($R=0.44$, $P<0.005$, Figure 2.6B). Importantly, since only a small amount of infused FXIII-A_2B_2 is

F13a$^{-/-}$ mice to tail transection and saphenous vein puncture bleeding models. Both of these models are sensitive to coagulation and platelet defects and effects of conventional

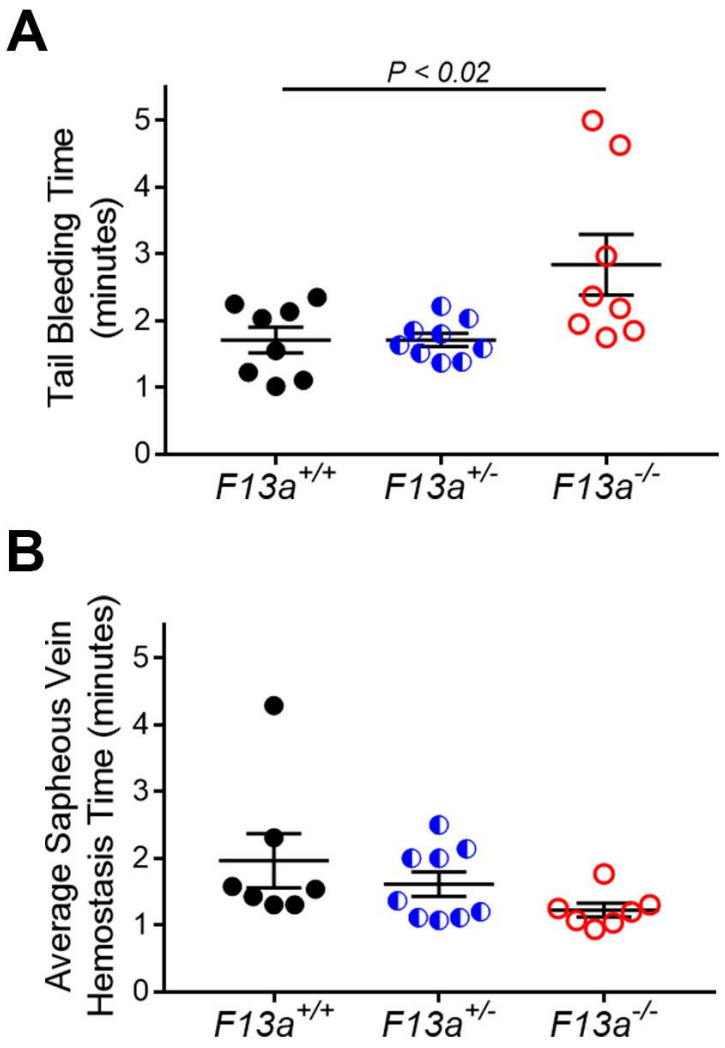


Figure 2.5. Partial FXIII deficiency does not increase the time to clot formation in murine models of hemostasis. *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice were subjected to (A) tail transection and (B) saphenous vein puncture. (A) Following 3-mm excision of the distal portion of the tail, bleeding was measured as described in Methods. Data show time to cessation of bleeding. (B) Following saphenous vein puncture, bleeding was measured after disruption of hemostatic clots as described in Methods. The data show average saphenous vein hemostasis times. Each dot represents a separate mouse; lines indicate mean ± SEM.

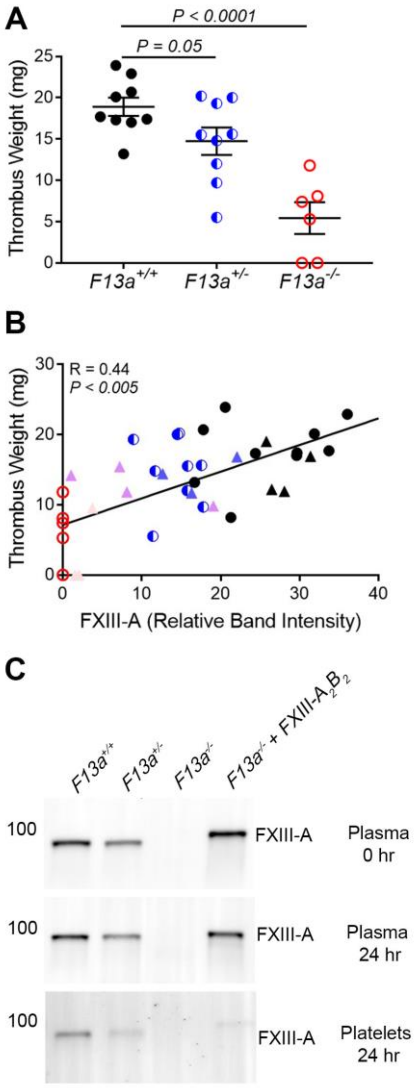


Figure 2.6. FXIII$_{plasma}$ infusion into *F13a*$^{-/-}$ mice rescues thrombus weight. *F13a*$^{+/+}$, *F13a*$^{+/-}$, *F13a*$^{-/-}$ mice infused with vehicle, and *F13a*$^{-/-}$ mice infused with FXIII-A_2B_2, were subjected to the IVC venous thrombosis model. (A) Thrombi were harvested after 24 hours and weighed. Each dot represents a separate mouse. (B) Correlation between FXIII-A level in plasma (measured by western blot and densitometry, shown as relative band intensity, arbitrary fluorescence units) and thrombus weights for *F13a*$^{+/+}$, *F13a*$^{+/-}$, *F13a*$^{-/-}$ (from panel A), and FXIII-A_2B_2-infused mice. Each dot represents a separate mouse; *F13a*$^{+/+}$ (black closed circles), *F13a*$^{+/-}$ (blue half circles), *F13a*$^{-/-}$ (red open circles) mice, and FXIII-A_2B_2-infused *F13a*$^{-/-}$ mice targeting 100% (black triangles), 50% (blue triangles), 25% (purple triangles), and 10% (pink triangles) FXIII-A_2B_2. (C) Representative western blots showing FXIII-A in plasma immediately after infusion of 50% FXIII-A_2B_2 (plasma final, top), and in plasma (middle) and platelets (bottom) 24 hours after infusion and ligation. Note, infused human FXIII-A_2B_2 is more strongly detected than mouse.

endocytosed into platelets ([33] and Figure 2.6C), these experiments show $FXIII_{plasma}$, but not $FXIII_{plt}$, mediates RBC retention and thrombus weight in vivo.

2.5 Discussion

FXIII deficiency decreases RBC retention in thrombi and consequently, decreases thrombus size.[5] This finding identifies FXIII(a) as an intriguing candidate therapeutic target for preventing venous thrombosis, but raises important questions. Herein, we sought to clarify the relative roles of $FXIII_{plasma}$ and $FXIII_{plt}$ and the effect of FXIII level on hemostasis and venous thrombosis. First, we showed that effects of FXIII reduction on clot weight are not due to reduced thrombin generation, decreased platelet-mediated clot contraction, or FXIII-independent transglutaminase activity. These findings address previously published controversial observations and inform understanding of FXIII biological function. Second, we demonstrated that $FXIII_{plasma}$, but not $FXIII_{plt}$, promotes formation of HMW crosslinked fibrin species and RBC retention in clots, and therefore, mediates clot weight. Third, we characterized kinetic mechanisms relating FXIII levels with thrombus composition. Finally, we showed that partial reduction in $FXIII_{plasma}$ reduces venous thrombus size, but does not increase bleeding in vivo. Collectively, these findings define a critical role for $FXIII_{plasma}$ during coagulation in vivo, and support the consideration of FXIII as a therapeutic target for anticoagulation.

In the course of this study, we investigated three observations that were previously, but controversially, associated with FXIII activity. First, although a subset of studies suggested FXIII contributes to platelet-mediated contractile events[10,11], our data indicate that PRP from FXIII-sufficient and -deficient mice show similar contraction kinetics. Possible discord between our results and the earlier findings may be explained by methodological differences, including the high centrifugal forces used for preparing PRP that may have resulted in lower platelet

concentrations in the prior experiments[10], or use of transglutaminase inhibitors (e.g., cystamine)[10,11] that can inhibit thrombin generation and activity[34]. Notably, our findings are consistent with several independent studies that showed no effect of FXIII on platelet contraction[12-14] and no effect on cytoskeletal dynamics in Chinese hamster ovary cells transfected with FXIII[35]. Second, although an earlier study detected transglutaminase-like activity in platelets from *F13a*$^{-/-}$ mice and suggested this activity compensates for FXIII deficiency in platelets from *F13a*$^{-/-}$ mice[15], we detected little-to-no transglutaminase activity in platelets from *F13a*$^{-/-}$ mice. Although tissue transglutaminase-2 (TG-2) antigen is present in mouse platelets, levels are 25-fold lower than FXIII.[36] Moreover, human platelets do not express TG-2[9,37] and TG-2 does not substantively contribute to platelet thrombus formation.[38] Thus, transglutaminase activity, be it from FXIII or other transglutaminase enzymes, appears dispensable for platelet contraction. Third, although lysates from unstimulated platelets can crosslink fibrin[9], we were unable to demonstrate a functional effect of $FXIII_{plt}$ on RBC retention in clots. This paradox may be explained by findings that FXIII exposed on the platelet surface is rapidly inactivated by an as yet unknown mechanism. Findings that $FXIII_{plasma}$ has functions not reproduced by $FXIII_{plt}$ are consistent with previous data[9], but extend these observations to provide a biochemical explanation for these differences. Notably, although $FXIII_{plasma}$ and $FXIII_{plt}$ both have catalytic FXIII-A_2 subunits, these compartments differ in subunit structure and activation kinetics. In $FXIII_{plasma}$, the FXIII-A_2 subunit dimer is tightly-bound to FXIII-B_2[6], which mediates its association with the fibrin(ogen) γ-chain in plasma[39]. Following activation of coagulation, $FXIII_{plasma}$ is activated rapidly by thrombin and is immediately available to crosslink fibrin. In contrast, $FXIII_{plt}$ lacks FXIII-B_2 and resides in the platelet cytoplasm, where calcium-mediated activation and externalization occur more slowly.[9] Indeed, presentation of platelet-

derived FXIII activity to the plasma/thrombus milieu likely happens *after* fibrin formation, crosslinking, and initiation of platelet-mediated clot contraction occur. Thus, since FXIIIa activity is essential for RBC retention in clots while the clot is undergoing contraction[29], only $FXIII_{plasma}$ can fulfill the immediate temporal requirement needed for this function. This premise is further supported by two previous observations. First, mice with normal $FXIII_{plt}$ but delayed FXIII activation secondary to reduced binding of $FXIII_{plasma}$ to fibrinogen (i.e., $Fib\gamma^{390\text{-}396A}$ mice) phenocopy $F13a^{-/-}$ mice with small thrombi and reduced RBC content (loss-of-function).[5] Second, addition of plasma-derived FXIII-A_2B_2 to FXIII-deficient human plasma fully restores RBC retention in reconstituted, contracted whole blood clots (gain-of-function).[5] Together, these experiments suggest $FXIII_{plasma}$, but not $FXIII_{plt}$, promotes RBC retention in thrombi and consequently, influences venous thrombus size.

Given the prominent effect of $FXIII_{plasma}$ on fibrin crosslinking, it is curious why FXIII is present in such high concentrations in platelets (3% of total protein).[9] $FXIII_{plt}$ has been reported to crosslink cytoskeletal proteins[40-42] and regulate bidirectional platelet signaling via reorganization of the cytoskeleton and αIIbβ3 integrin.[43] Although we did not detect platelet abnormalities in our assays, dysfunctions stemming from FXIII deficiency may be detectable in assays that assess platelet functions secondary to clot formation. In vitro studies and clinical observations suggest $FXIII_{plt}$ may contribute to hemostasis in certain situations. For example, FXIII-B-deficient patients have reduced $FXIII_{plasma}$, but normal $FXIII_{plt}$, and a generally milder phenotype than FXIII-A-deficient patients.[44] Although this difference has been attributed to only partial, versus total, loss of the FXIII-A_2 catalytic subunits in plasma[44], the milder phenotype may also reflect residual hemostatic activity provided by $FXIII_{plt}$. Alternately, or perhaps in addition, since FXIII has non-hemostatic functions during wound healing and the immune

response[45], $FXIII_{plt}$ may contribute to these processes. In this regard, delayed exposure of $FXIII_{plt}$ may be a critical aspect of its biological function.

The present study fills several critical knowledge gaps regarding FXIII biology and function, but has potential limitations. First, although *F13a*$^{+/-}$ mice did not demonstrate bleeding in either hemostasis assay, studies of human patients suggest moderate FXIII deficiency is associated with increased bleeding risk in certain situations, including delayed bleeding after injury or surgery.[20,23,46] Potential discord between mice and humans may reflect species differences, or may indicate murine hemostasis models are sensitive to initial clot formation, but not to clot stability – a parameter likely to be affected by FXIII-deficiency. Indeed, even *F13a*$^{-/-}$ mice show only moderate (~2-fold prolongation) in bleeding following tail transection assays (Fig 5A and [25]), and only slight, non-significant bleeding following digit amputation[24]. Development of in vivo assays that assess clot stability are needed to fully elucidate the relationship between FXIII level and bleeding risk. Second, the IVC ligation model involves complete restriction of blood flow, which may minimize effects of platelets, and consequently $FXIII_{plt}$, on venous thrombosis. However, mice with reduced or delayed FXIII activation have the same phenotype in both the IVC ligation (stasis) and stenosis (partial flow) models.[5] Thus, the effects of FXIII(a) on RBC retention and thrombus weight are independent of local blood flow and mediated by $FXIII_{plasma}$ in both models.

Identification of a role for $FXIII_{plasma}$ in promoting RBC retention in clots and determining clot weight has implications for developing FXIII(a) inhibitors to reduce venous thrombosis. First, although FXIII is found in two separate compartments, potential inhibitors only need to inhibit $FXIII_{plasma}$ to limit thrombosis severity, and would not need to traverse the platelet membrane to access $FXIII_{plt}$. This premise simplifies the molecular design of potential

drugs. Second, platelet proteins are somewhat "protected" from plasma inhibitors.[47,48] Therefore, although $FXIII_{plasma}$ and $FXIII_{plt}$ share structural homology, having two separate FXIII compartments may conserve sufficient FXIII activity to preserve hemostasis. Thus, FXIII may have advantages not present in other therapeutic targets for thrombosis.

In summary, we have shown that $FXIII_{plasma}$ mediates thrombus RBC retention and weight in a dose-dependent manner, and that partial FXIII reduction reduces venous thrombus size without increasing bleeding in murine models of thrombosis and hemostasis. Collectively, these data define specific physiologic roles of $FXIII_{plasma}$, and support the evaluation of FXIII(a) inhibition as a strategy to reduce venous thrombosis.

2.6 Supplemental Material

Materials. Sigmacote® (siliconizing agent) was from Sigma-Aldrich (St. Louis, MO). ProstaglandinI2 was from Cayman Chemical (Ann Arbor, MI). Lipidated tissue factor (TF, Innovin) was from Siemens (Munich, Germany). The transglutaminase inhibitor T101 was from Zedira (Darmstadt, Germany). Calibrated automated thrombography reagents (fluorogenic thrombin substrate, TF/Lipid Reagents [PPP-Low, PPP-High], and thrombin calibrator) were from Diagnostica Stago (Parsippany, NJ). Laemmli 6X sample buffer containing sodium dodecyl sulfate (SDS) was from Boston Bioproducts (Ashland, MA). Tris-glycine polyacrylamide gels (10%) were from Bio-Rad (Hercules, CA). Polyvinylidene difluoride membranes were from Invitrogen, (Carlsbad, CA) and scanned on a GE Typhoon FLA-9000 Imager (GE Healthcare, Pittsburgh, PA). Odyssey Blocking Buffer was from LICOR Biosciences (Lincoln, NE). Human α-thrombin and anti-human FXIII-A polyclonal antibody (SAF13A-AP) were from Enzyme Research Laboratories (South Bend, IN). Anti-human fibrinogen polyclonal antibody (A0080) was from Dako (Glostrup, Denmark). Alexa Fluor®-488 anti-rabbit and anti-sheep secondary

antibodies were from Jackson Immunoresearch (West Grove, PA). Type 1 collagen was from Bio/Data Corporation (Horsham, PA). FXIII-A2B2 (Corifact) was a generous gift from CSL Behring (King of Prussia, PA). The TG-Covtest transglutaminase activity was from Covalab (Lyon, France). Cell counts were determined on a HV950FS Hemavet cell counter from Drew Scientific (Miami Lakes, FL). Thrombin generation. Calibrated automated thrombography was performed as described.1 Briefly, thrombin generation was triggered in platelet-poor plasma with TF, phospholipids, and CaCl2 (1 pM, 4 µM, and 16.7 mM, final, respectively). Platelet-rich plasma (PRP) was analyzed under similar conditions, but without phospholipids.

Transglutaminase activity assay. PRP was centrifuged (400g, 5 minutes) to isolate platelets. Platelet pellets were washed, resuspended in Tyrode's buffer (15 mM HEPES, 3.3 mM NaH2PO4, pH 7.4, 138 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5.5 mM dextrose), and then incubated with thrombin (1 U/mL) for 15 minutes. Platelet lysates were prepared by adding cell lysis buffer (2% Triton X-100, 100 mM Tris, pH 7.3) to the resuspended, activated platelet pellets. Transglutaminase activity was measured using the TG-Covtest colorimetric activity microassay.

FXIII activation and fibrin crosslinking. PRP was clotted with TF and CaCl2 (1 pM and 10 mM, final, respectively). Samples were then dissolved in 50 mM dithiothreitol, 12.5 mM EDTA, and 8 M urea at 60°C for 1 hour, diluted 120-fold in 6X reducing SDS sample buffer, boiled, separated on 10% Tris-Glycine gels, and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour at room temperature with Odyssey Blocking Buffer, incubated overnight at 4°C with primary antibodies against FXIII-A or fibrin(ogen), and then

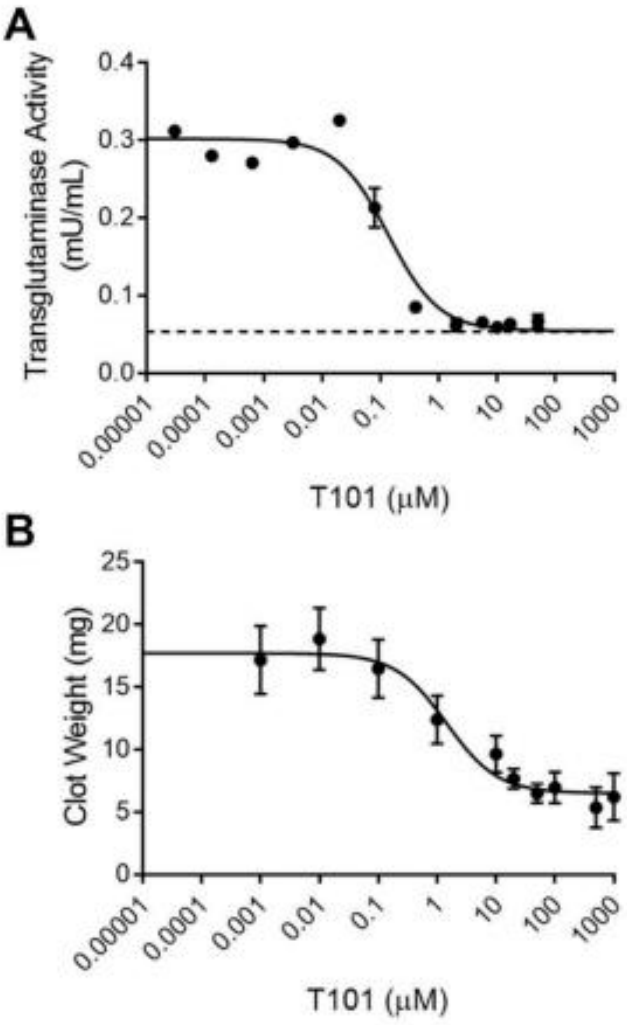
incubated with fluorescence-labeled anti-sheep and anti-rabbit secondary antibodies for 1 hour at room temperature. FXIII-A and fibrin(ogen) bands were quantified by densitometry (ImageJ 1.48v). FXIII-A′ band intensity was reported as total arbitrary fluorescence units (A.F.U.). Band intensities of fibrin γ-γ dimers and HMW crosslinked fibrin species were normalized to the fibrin(ogen) Bβ+β-chain before normalizing to time zero. Identity of murine fibrinogen bands was confirmed by mass spectrometry. Briefly, bands were visualized by colloidal blue 2 silver stain2, excised, and analyzed by the University of North Carolina Michael Hooker Proteomics Core using a MALDI TOF/TOF 4800 Mass Analyzer (ABSciex).

Supplemental references

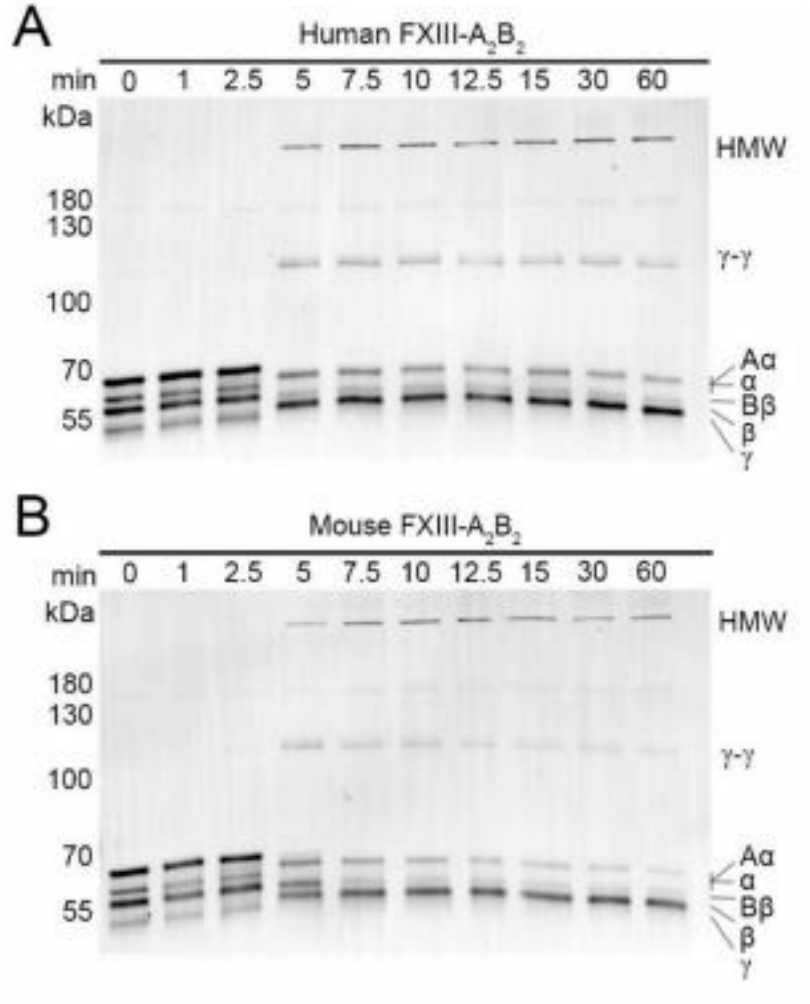
1. Machlus KR, Colby EA, Wu JR, Koch GG, Key NS, Wolberg AS. Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram. Thromb Haemost. 2009;102(5):936-944.
2. Candiano G, Bruschi M, Musante L, et al. Blue silver: a very sensitive colloidal Coomassie G250 staining for proteome analysis. Electrophoresis. 2004;25(9):1327-1333.

Supplemental Table 2.1. Complete blood count for *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice. Data show means ± SEM (N=3).

	***F13a*$^{+/+}$**	***F13a*$^{+/-}$**	***F13a*$^{-/-}$**
White blood cells (10^6/mL)	6.7 ± 0.5	5.5 ± 0.6	5.5 ± 0.8
Red blood cells (10^9/mL)	8.5 ± 0.1	9.0 ± 0.6	8.2 ± 0.4
Hemoglobin (g/dL)	11.8 ± 0.3	12.6 ± 1.0	12.2 ± 0.7
Hematocrit (%)	39.8 ± 1.2	41.6 ± 1.1	39.4 ± 0.9
Red cell distribution width (%)	15.6 ± 0.4	15.8 ± 0.4	16.5 ± 0.6
Platelets (10^6/mL)	748.0 ± 48.0	754.7 ± 90.4	701.0 ± 38.4



Supplemental Figure 2.1. Factor XIII inhibitor T101 reduces mouse whole blood clot weight. (A) Transglutaminase activity of human $FXIIIA_2B_2$ was measured in the presence of T101 at the concentrations indicated. Data show means ± standard error of the means (N=1-4). Dashed line indicates baseline activity in the assay (no $FXIIIA_2B_2$ present). (B) Whole blood from *F13a*$^{+/+}$ mice was clotted with tissue factor/$CaCl_2$ in the presence of T101 at the concentrations indicated. Contracted clots were weighed after 2 hours (N=3-4).



Supplemental Figure 2.2. Both human and mouse FXIII-A_2B_2 crosslink mouse fibrin. Clotting was triggered with tissue factor/$CaCl_2$ in *F13a*$^{-/-}$ plasma supplemented with human (A) or mouse (B) FXIIIA_2B_2 (62 µM, final), and reactions were quenched at the indicated time points. Fibrin crosslinking was analyzed by SDS-PAGE with western blotting (N=2).

REFERENCES

1. Komaromi I, Bagoly Z, Muszbek L. Factor XIII: novel structural and functional aspects. *J Thromb Haemost*. 2011;9(1):9-20.

2. Fraser SR, Booth NA, Mutch NJ. The antifibrinolytic function of factor XIII is exclusively expressed through alpha(2)-antiplasmin cross-linking. *Blood*. 2011;117(23):6371-6374.

3. Jensen PH, Lorand L, Ebbesen P, Gliemann J. Type-2 plasminogen-activator inhibitor is a substrate for trophoblast transglutaminase and factor XIIIa. Transglutaminase-catalyzed cross-linking to cellular and extracellular structures. *Eur J Biochem*. 1993;214(1):141-146.

4. Valnickova Z, Enghild JJ. Human procarboxypeptidase U, or thrombin-activable fibrinolysis inhibitor, is a substrate for transglutaminases. Evidence for transglutaminase-catalyzed cross-linking to fibrin. *J Biol Chem*. 1998;273(42):27220-27224.

5. Aleman MM, Byrnes JR, Wang JG, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest*. 2014;124(8):3590-3600.

6. Katona E, Penzes K, Csapo A, et al. Interaction of factor XIII subunits. *Blood*. 2014;123(11):1757-1763.

7. Fickenscher K, Aab A, Stuber W. A photometric assay for blood coagulation factor XIII. *Thromb Haemost*. 1991;65(5):535-540.

8. Bagoly Z, Katona E, Muszbek L. Factor XIII and inflammatory cells. *Thromb Res*. 2012;129 Suppl 2:S77-81.

9. Mitchell JL, Lionikiene AS, Fraser SR, Whyte CS, Booth NA, Mutch NJ. Functional factor XIII-A is exposed on the stimulated platelet surface. *Blood*. 2014;124(26):3982-3990.

10. Kasahara K, Souri M, Kaneda M, Miki T, Yamamoto N, Ichinose A. Impaired clot retraction in factor XIII A subunit-deficient mice. *Blood*. 2010;115(6):1277-1279.

11. Tutwiler V, Litvinov RI, Lozhkin AP, et al. Kinetics and mechanics of clot contraction are governed by the molecular and cellular composition of the blood. *Blood*. 2016;127(1):149-159.

12. Rao KM, Newcomb TF. Clot retraction in a factor XIII free system. *Scand J Haematol*. 1980;24(2):142-148.

13. Jelenska M, Kopec M, Breddin K. On the retraction of collagen and fibrin induced by normal, defective and modified platelets. *Haemostasis*. 1985;15(3):169-175.

14. Rijken DC, Uitte de Willige S. Inhibition of Fibrinolysis by Coagulation Factor XIII. *Biomed Res Int*. 2017;2017:1209676.

15. Jobe SM, Leo L, Eastvold JS, et al. Role of FcRgamma and factor XIIIA in coated platelet formation. *Blood*. 2005;106(13):4146-4151.

16. Francis CW, Marder VJ. Rapid formation of large molecular weight alpha-polymers in cross-linked fibrin induced by high factor XIII concentrations. Role of platelet factor XIII. *J Clin Invest*. 1987;80(5):1459-1465.

17. Hevessy Z, Haramura G, Boda Z, Udvardy M, Muszbek L. Promotion of the crosslinking of fibrin and alpha 2-antiplasmin by platelets. *Thromb Haemost*. 1996;75(1):161-167.

18. Reed GL, Matsueda GR, Haber E. Fibrin-fibrin and alpha 2-antiplasmin-fibrin cross-linking by platelet factor XIII increases the resistance of platelet clots to fibrinolysis. *Trans Assoc Am Physicians*. 1991;104:21-28.

19. Reed GL, Matsueda GR, Haber E. Platelet factor XIII increases the fibrinolytic resistance of platelet-rich clots by accelerating the crosslinking of alpha 2-antiplasmin to fibrin. *Thromb Haemost*. 1992;68(3):315-320.

20. Menegatti M, Palla R, Bucciarelli P, Peyvandi F. Minimal factor XIII activity level to prevent major spontaneous bleeds: reply. *J Thromb Haemost*. 2017;15(11):2280-2282.

21. de Moerloose P, Schved JF, Nugent D. Rare coagulation disorders: fibrinogen, factor VII and factor XIII. *Haemophilia*. 2016;22 Suppl 5:61-65.

22. Biswas A, Ivaskevicius V, Thomas A, Oldenburg J. Coagulation factor XIII deficiency. Diagnosis, prevalence and management of inherited and acquired forms. *Hamostaseologie*. 2014;34(2):160-166.

23. Lawrie AS, Green L, Mackie IJ, Liesner R, Machin SJ, Peyvandi F. Factor XIII--an under diagnosed deficiency--are we using the right assays? *J Thromb Haemost*. 2010;8(11):2478-2482.

24. Souri M, Koseki-Kuno S, Takeda N, Degen JL, Ichinose A. Administration of factor XIII B subunit increased plasma factor XIII A subunit levels in factor XIII B subunit knock-out mice. *Int J Hematol*. 2008;87(1):60-68.

25. Lauer P, Metzner HJ, Zettlmeissl G, et al. Targeted inactivation of the mouse locus encoding coagulation factor XIII-A: hemostatic abnormalities in mutant mice and characterization of the coagulation deficit. *Thromb Haemost*. 2002;88(6):967-974.

26. Prasad JM, Gorkun OV, Raghu H, et al. Mice expressing a mutant form of fibrinogen that cannot support fibrin formation exhibit compromised antimicrobial host defense. *Blood*. 2015;126(17):2047-2058.

27. Cooley B, Funkhouser W, Monroe D, et al. Prophylactic efficacy of BeneFIX vs Alprolix in hemophilia B mice. *Blood*. 2016;128(2):286-292.

28. Aleman MM, Walton BL, Byrnes JR, et al. Elevated prothrombin promotes venous, but not arterial, thrombosis in mice. *Arterioscler Thromb Vasc Biol*. 2013;33(8):1829-1836.

29. Byrnes JR, Duval C, Wang Y, et al. Factor XIIIa-dependent retention of red blood cells in clots is mediated by fibrin alpha-chain crosslinking. *Blood*. 2015;126(16):1940-1948.

30. Buyue Y, Whinna HC, Sheehan JP. The heparin-binding exosite of factor IXa is a critical regulator of plasma thrombin generation and venous thrombosis. *Blood*. 2008;112(8):3234-3241.

31. Pastoft AE, Lykkesfeldt J, Ezban M, Tranholm M, Whinna HC, Lauritzen B. A sensitive venous bleeding model in haemophilia A mice: effects of two recombinant FVIII products (N8 and Advate®). *Haemophilia*. 2012;18(5):782-788.

32. Vaezzadeh N, Ni R, Kim PY, Weitz JI, Gross PL. Comparison of the effect of coagulation and platelet function impairments on various mouse bleeding models. *Thromb Haemost*. 2014;112(2):412-418.

33. Marx G, Korner G, Mou X, Gorodetsky R. Packaging zinc, fibrinogen, and factor XIII in platelet alpha-granules. *J Cell Physiol*. 1993;156(3):437-442.

34. Aleman MM, Holle LA, Stember KG, Devette CI, Monroe DM, Wolberg AS. Cystamine preparations exhibit anticoagulant activity. *PLoS One*. 2015;10(4):e0124448.

35. Vijayan KV, Goldschmidt-Clermont PJ, Roos C, Bray PF. The Pl(A2) polymorphism of integrin beta(3) enhances outside-in signaling and adhesive functions. *J Clin Invest*. 2000;105(6):793-802.

36. Zeiler M, Moser M, Mann M. Copy number analysis of the murine platelet proteome spanning the complete abundance range. *Mol Cell Proteomics*. 2014;13(12):3435-3445.

37. Burkhart JM, Vaudel M, Gambaryan S, et al. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012;120(15):e73-82.

38. Mattheij NJ, Swieringa F, Mastenbroek TG, et al. Coated platelets function in platelet-dependent fibrin formation via integrin alphaIIbbeta3 and transglutaminase factor XIII. *Haematologica*. 2016;101(4):427-436.

39. Byrnes JR, Wilson C, Boutelle AM, et al. The interaction between fibrinogen and zymogen FXIII-A2B2 is mediated by fibrinogen residues gamma390-396 and the FXIII-B subunits. *Blood*. 2016;128(15):1969-1978.

40. Asijee GM, Muszbek L, Kappelmayer J, Polgar J, Horvath A, Sturk A. Platelet vinculin: a substrate of activated factor XIII. *Biochim Biophys Acta*. 1988;954(3):303-308.

41. Cohen I, Glaser T, Veis A, Bruner-Lorand J. Ca2+-dependent cross-linking processes in human platelets. *Biochim Biophys Acta*. 1981;676(2):137-147.

42. Serrano K, Devine DV. Intracellular factor XIII crosslinks platelet cytoskeletal elements upon platelet activation. *Thromb Haemost*. 2002;88(2):315-320.

43. Kasahara K, Kaneda M, Miki T, et al. Clot retraction is mediated by factor XIII-dependent fibrin-alphaIIbbeta3-myosin axis in platelet sphingomyelin-rich membrane rafts. *Blood*. 2013;122(19):3340-3348.

44. Saito M, Asakura H, Yoshida T, et al. A familial factor XIII subunit B deficiency. *Br J Haematol*. 1990;74(3):290-294.

45. Dickneite G, Herwald H, Korte W, Allanore Y, Denton CP, Matucci Cerinic M. Coagulation factor XIII: a multifunctional transglutaminase with clinical potential in a range of conditions. *Thromb Haemost*. 2015;113(4):686-697.

46. Duckert F, Jung E, Shmerling DH. A hitherto undescribed congenital haemorrhagic diathesis probably due to fibrin stabilizing factor deficiency. *Thromb Diath Haemorrh*. 1960;5:179-186.

47. Kuether EL, Schroeder JA, Fahs SA, et al. Lentivirus-mediated platelet gene therapy of murine hemophilia A with pre-existing anti-factor VIII immunity. *J Thromb Haemost*. 2012;10(8):1570-1580.

48. Gewirtz J, Thornton MA, Rauova L, Poncz M. Platelet-delivered factor VIII provides limited resistance to anti-factor VIII inhibitors. *J Thromb Haemost*. 2008;6(7):1160-1166.

CHAPTER 3: THE FACTOR XIII VAL34LEU POLYMORPHISM DECREASES WHOLE BLOOD CLOT WEIGHT AT HIGH FIBRINOGEN CONCENTRATIONS

3.1 Overview

Coagulation factor XIII (FXIII) is a protransglutaminase that plays an important role in fibrin crosslinking and clot red blood cell (RBC) retention. The FXIII polymorphism, Val34Leu, is associated with protection against venous thrombosis. This effect is hypothesized to result from fibrinogen concentration-dependent changes in clot structure, but has not been investigated in a whole blood system. We determined the effect of the FXIII Val34Leu polymorphism on whole blood clot weight, by reconstituted platelet-poor plasmas from human donors with platelets and RBCs. Clotting was triggered by recalcifcation and tissue factor. In reconstituted clots with donor plasmas, clot weight did not differ between Val variant ($FXIII^{Val/Val}$ and $FXIII^{Val/Leu}$) and homozygous presence of the Leu variant ($FXIII^{Leu/Leu}$). Through univariate analysis, we assessed the contribution of donor sex, age, clotting times, thrombin generation, FXIII activity, and fibrinogen concentration to contracted clot weight. In a multiple linear regression analysis adjusting for fibrinogen concentration, the Val variant positively correlated with clot weight, however, the Leu variant did not. To isolate the effect of fibrinogen, we reconstituted FXIII-depleted plasma with platelets, RBCs, and purified $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$, while varying fibrinogen concentration. At high fibrinogen concentrations, compared to $FXIII^{Val/Val}$, reconstituted clots with purified $FXIII^{Leu/Leu}$ were smaller. The Leu variant mitigates the effect of high fibrinogen concentration and results in smaller whole blood clots. The Val34Leu

polymorphism may protect against venous thrombosis by decreasing clot RBC retention and consequently, reducing clot size.

3.2 Introduction

Protransglutaminase factor XIII (FXIII) is a non-covalent heterotetramer (FXIII-A_2B_2) bound to circulating fibrinogen[1], consisting of two catalytic subunits (FXIII-A_2) and two non-catalytic carrier subunits (FXIII-B_2). FXIII is activated by thrombin-mediated cleavage of 37-amino acid (4-kDa) activation peptides from the N-termini of FXIII-A_2 subunits, followed by calcium-mediated dissociation of the FXIII-B_2 subunits.[2] Activated FXIII (FXIIIa) catalyzes the formation of ε-N-(γ-glutamyl)-lysyl crosslinks between γ- and α-chains of fibrin[3,4] and between fibrin and other plasma proteins[5-7], enhancing clot mechanical and biochemical stability, respectively. FXIII activity modulates RBC retention during clot formation and contraction, and thus, is centrally involved in venous thrombosis (VT).[8-10]

Interestingly, a common gene variant in FXIII coding for a valine (Val) to a leucine (Leu) at codon 34 (Val34Leu), present in ~25% of European Caucasians[11], is implicated with protection against VT.[12-15] Two early studies in separate populations (North White European and Southeastern Brazilian) report the FXIII Val34Leu polymorphism as a novel genetic factor involved in VT etiology.[12,13] A meta-analysis of 12 epidemiological studies support evidence that the Val34Leu polymorphism provides a modest, but significant protection against VT.[14] However, there are inconsistencies in the literature; some studies evaluating coagulation protein genetic variants and VT epidemiologic data in patients with thrombophilia, find no association of the Val34Leu polymorphism with VT.[16-18] Earlier studies had not yet understood the complex gene-plasma environment interactions that modulate the effect of Val34Leu polymorphism on thrombosis.[19] In the Leiden Thrombophilia Study, the Leu variant showed a weak association

with VT[20], but when the effect of fibrinogen concentration was factored into the analysis, the association was stronger[21]. Factoring in genetic modifiers, such fibrinogen, addresses some discrepancies between different population studies but more refined mechanistic studies are needed to interpret the contribution of the FXIII Val34Leu polymorphism to VT risk.

The Val34Leu polymorphism is located 3 amino acids from the thrombin cleavage site.[22] It results in ~2.5-fold accelerated FXIII activation[11], and consequently, earlier fibrin crosslinking activity[16,23,24]. In a plasma based system, the presence of wild-type Val variant at high fibrinogen concentrations results in fibrin clots with thin, tightly-packed fibers, however, the Leu variant results in fibrin clots with thicker, loosely-packed fibers.[19] A clot profile with increased susceptibility to fibrinolysis could suggest a protective effect but additionally, fibrin network density can also influence RBC retention in clots and, therein for, clot size[10]. The Val34Leu polymorphism has not been investigated in a physiologically-relevant whole blood system while modulating fibrinogen concentration.

The kinetics of FXIII activation and activity is a critical determinant of RBC retention during clot formation and contraction.[8,9] Three collective observations on the relationship between FXIII and RBC retention, warrant the need to explore accelerated FXIII activation by the Val34Leu polymorphism in a whole blood system. First, reduced FXIII levels in mice ($F13a^{+/-}$) demonstrate decreased RBC retention and clot weight. Second, hemophilic samples with rFVIIa/rFVIII and FXIII co-treatment, demonstrate elevated FXIII levels result in increased RBC retention and clot weight.[25] Third, mice with normal levels of FXIII but delayed FXIII activation due to reduced FXIII binding to fibrinogen ($Fib\gamma^{390–396A}$), in the inferior vena cava stasis model, show significantly decreased RBC retention and thrombus size.[9] Therefore, individuals with normal levels of FXIII[16] but accelerated FXIII activation would suggest

increased clot weight, and consequently, increased VT risk. However, it is interesting that the Val34Leu polymorphism is paradoxically associated with protection against VT[12-15]. Determining the impact of the Leu variant on RBC retention in clots is essential for understanding the clinical significance of the FXIII Val34Leu polymorphism in VT risk.

Herein we used reconstituted whole blood ex vivo systems to determine the relationship between accelerated FXIII activation and VT. Collectively, our data demonstrate compared to $FXIII^{Val/Val}$ clots at high fibrinogen concentrations, $FXIII^{Leu/Leu}$ is associated with the formation of smaller whole blood clots, suggesting the Leu variant mitigates the effect of fibrinogen on whole blood clot weight.

3.3 Methods

Materials. Sigmacote® was from Sigma-Aldrich (St. Louis, MO). Prostaglandin-I_2 was from Cayman Chemical (Ann Arbor, MI). Lipidated tissue factor (TF, Innovin) was from Siemens (Munich, Germany). Non-reducing 6X sample buffer containing sodium dodecyl sulfate (SDS) was from Boston Bioproducts (Ashland, MA). β-mercaptoethanol was from Fisher Scientific (Hampton, NH). Tris-glycine polyacrylamide gels (10%) were from Bio-Rad (Hercules, CA). Odyssey Blocking Buffer was from LI-COR Biosciences (Lincoln, NE). Human anti-human FXIII-A polyclonal antibody (SAF13A-AP) was from Enzyme Research Laboratories (South Bend, IN). FXIII-deficient plasma was from Affinity Biologicals (Ancaster, Ontario). Anti-human fibrinogen polyclonal antibody (A0080) was from Dako (Glostrup, Denmark). Alexa Fluor®-488 anti-rabbit and anti-sheep secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Polyvinylidene difluoride membranes were from Invitrogen (Carlsbad, CA) and scanned on a GE Typhoon FLA-9000 Imager (GE Healthcare, Pittsburgh, PA). Calibrated

automated thrombography reagents (fluorogenic thrombin substrate, TF/Lipid Reagents [PPP-Low], and thrombin calibrator) were from Diagnostica Stago (Parsippany, NJ).

Human blood draws and plasma preparation. Ethical approval was obtained from the University of Debrecen Ethics Committee (number: DE RKEB/IKEB: 3189-2010). Human whole blood was obtained by venipuncture and drawn into 3.2% citrate (10% v/v, final) from 86 healthy individuals [$FXIII^{Val/Val}$ (N=40), $FXIII^{Val/Leu}$ (N=28), and $FXIII^{Leu/Leu}$ (N=18)]. All participants provided signed informed consent in accordance with the Helsinki Declaration. DNA isolation was performed from buffy coat of blood samples by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). FXIII-A p.Val34Leu (c.103G>T; rs5985) polymorphism was identified by a real-time PCR method[26] using fluorescence resonance energy transfer detection and melting curve analysis on a LightCycler® 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany; primers are available from the authors upon request).

FXIII zymogen preparation. Highly purified human FXIII was isolated from pooled plasma, as described[27], from individuals possessing only 34Val or only 34Leu FXIII-A alleles (Supplemental Figure 1).

Western blotting. FXIII antigen and activity were analyzed in Peak 1 (FXIII-free) fibrinogen preparations by western blot detection of FXIII-A subunit and fibrin crosslinking. Human fibrinogen preparations were clotted with TF and $CaCl_2$ (1 pM and 10 mM, final, respectively). Samples were then dissolved in 50 mM dithiothreitol, 12.5 mM EDTA, and 8 M urea at 60°C for 1 hour, diluted 120-fold in 6X reducing SDS sample buffer, boiled, separated on 10% Tris-

Glycine gels, and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour at room temperature with blocking buffer, incubated overnight at 4°C with primary antibodies against FXIII-A or fibrin(ogen), and then incubated with fluorescence-labeled secondary antibodies for 1 hour at room temperature.

Reconstituted whole blood assays. Human whole blood was centrifuged (150*g*, 20 minutes). The platelet-rich plasma fraction was isolated and treated with prostaglandin-I_2 (50 ng/mL, final) was then was either centrifuged to obtain platelet-poor plasma (PPP) (700*g*, 10 minutes) or platelets (400*g*, 20 minutes). Platelet pellets were resuspended in Tyrode's buffer (15 mM HEPES, 3.3 mM NaH_2PO_4, pH 7.4, 138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM dextrose). The RBC fraction was isolated, washed with citrated glycine saline buffer, and packed by centrifugation (400*g*, 5 minutes 3x). In the first series of experiments, reconstituted whole blood was prepared by combining resuspended platelets (100 K/μL, final), washed RBCs (4.5 million/μL, final), and PPP from donors with $FXIII^{Val/Val}$, $FXIII^{Val/Leu}$, or $FXIII^{Leu/Leu}$. In the second series of experiments, reconstituted whole blood was prepared by combining resuspended platelets (100 K/μL, final), washed RBCs (4.5 million/μL, final), and FXIII-deficient plasma with purified $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$ zymogen and FXIII-free fibrinogen (to achieve the final concentrations indicated). For both series, clotting was triggered in siliconized wells with TF and $CaCl_2$ (1 pM and 10 mM, final, respectively) at 37°C. Contracted clots were weighed at two hours.

Clinical assays. FXIII activity was measured according to Karpati et al.[28] Reference ranges were determined according to guidelines of the Clinical and Laboratory Standards Institute.

Fibrinogen concentration was determined by the Clauss method[29], using reagents from Labexpert LTD (Debrecen, Hungary). Prothrombin time (PT) and activated partial thromboplastin time (APTT) analyses were performed on a BCS coagulometer (Siemens Healthcare Diagnostics, Marburg, Germany), using DADE Innovin PT and Actin FS reagents from Siemens (Munich, Germany). Normal control values represent the result of mixed samples of 25 healthy individuals with no hemostasis disorders, measured with the same reagent and coagulometer. Exclusion criteria for healthy individuals: known bleeding or thrombotic disorder, liver- or kidney disease, systemic disease or cancer, acute inflammation, surgery or thrombosis event (including acute myocardial infarction, stroke) within the past 1 month, pregnancy, and use of antiplatelet or anticoagulant therapy.

Thrombin generation. Thrombin generation was measured by calibrated automated thrombography, as described.[30] Briefly, thrombin generation was triggered in duplicate PPP samples with TF, phospholipids, and $CaCl_2$ (1 pM, 4 μM, and 16.7 mM, final, respectively). Fluorescence was detected by a Fluoroskan Ascent® fluorimeter (Thermo Fischer Scientific, Waltham, MA). Thrombograms were analysed by the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands).

Statistical methods. Descriptive statistics (mean, standard deviation [SD]) were calculated for each experiment. Experiments with normally-distributed data in 2 groups were compared by Student's *t* test and $P < 0.05$ was considered significant. Univariate analysis correlating parameters with clot weight and multiple regression analysis adjusting for fibrinogen level was

done using R. ANOVA with Bonferroni post hoc test was used for between-group comparisons and $P < 0.005$ was considered significant.

3.4 Results

In reconstituted whole blood with donor plasma, FXIII$^{Leu/Leu}$ mitigates the effect of fibrinogen on clot weight. To determine the effect of the FXIIII Val34Leu polymorphism on whole blood clot weight in human plasma, we reconstituted PPP from 86 healthy human donors with washed platelets and O-negative RBCs (Figure 3.1A). Following clot contraction, we analyzed clot weights as two groups: presence of the wild-type Val allele (FXIII$^{Val/Val}$ and FXIII$^{Val/Leu}$) or homozygous for the Leu allele (FXIII$^{Leu/Leu}$). These two groups were well-matched in clinical characteristics, clotting test times, and all thrombin generation parameters except endogenous thrombin potential (Table 3.1). Comparison of average raw clot weights (data not shown) or weights normalized per day of experiment showed no difference between groups (Figure 3.1B). Univariate analysis indicated significant positive correlations between clot weight and age, fibrinogen, and the lagtime and time to peak thrombin generation in plasmas carrying the 34Val allele (Table 3.2, Figure 3.1C-F). None of the measured characteristics correlated significantly with clot weight in plasmas with homozygous 34Leu (Table 3.2). Prior Val34Leu studies[19,21] demonstrate the necessity of examining the contribution of gene modifiers interpret the effect of the FXIII Val34Leu polymorphism.

To identify potential gene modifiers that effect clot composition in relation with the Val34Leu polymorphism, we constructed a univariate model to correlate clot weight with clinical characteristics, clotting test times, and thrombin generation parameters. In this analysis, donor sex, FXIII activity, PT and APTT clotting times, and thrombin generation parameters: peak thrombin and endogenous thrombin potential, did not correlate with clot weight for either

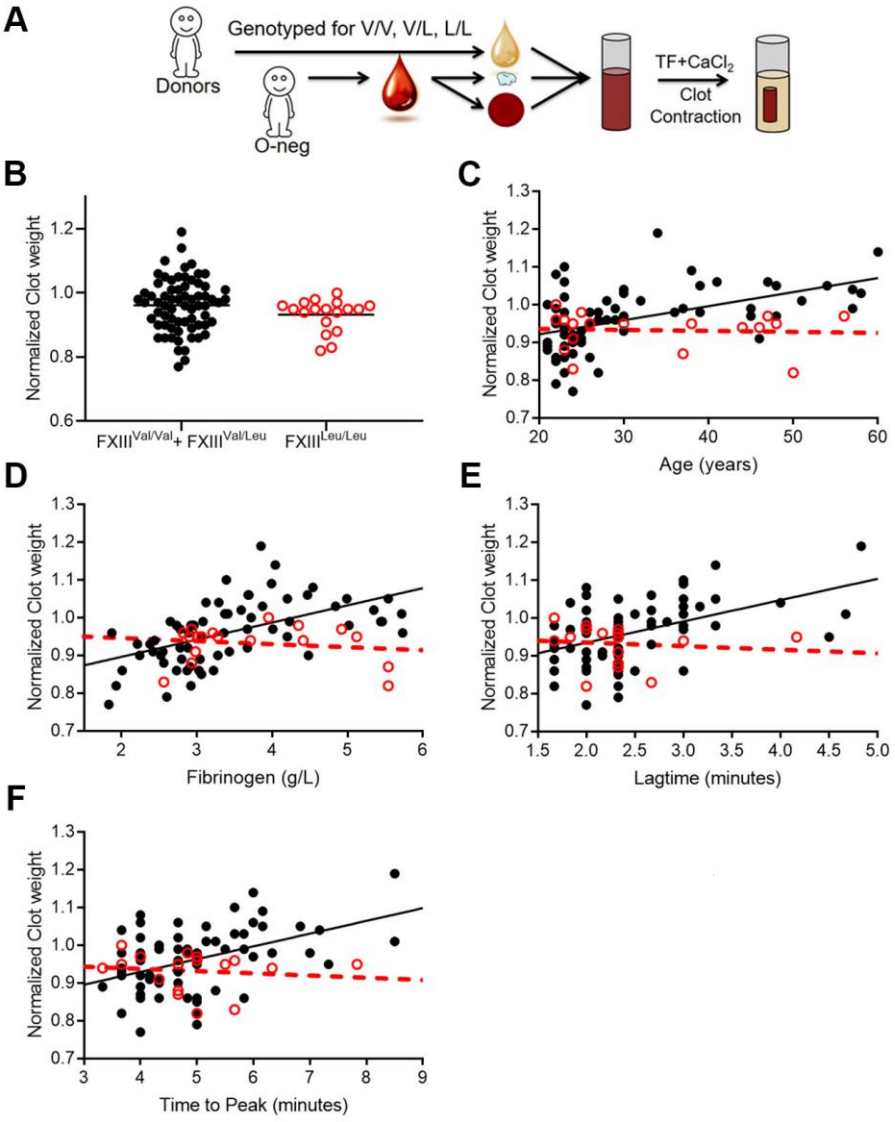


Figure 3.1. In reconstituted whole blood with human donor plasma, the 34Leu variant mitigates the effect of fibrinogen on contracted clot weight. (A) Platelet-poor plasma samples from 86 healthy human donors (40 FXIII$^{Val/Val}$, 28 FXIII$^{Val/Leu}$, and 18 FXIII$^{Leu/Leu}$) were reconstituted with washed platelets and O-negative red blood cells. Clotting was triggered with tissue factor and $CaCl_2$, and contracted clots were weighed after 2 hours. Clot weight was normalized between assays performed on different days. (B) Normalized clot weights. (C-F) Normalized clot weights for clots containing the 34Val allele or homozygous 34Leu were correlated with (C) age, (D) fibrinogen concentration, (E) prothrombin time, and (F) thrombin generation lagtime. FXIII$^{Val/Val}$ and FXIII$^{Val/Leu}$ (black closed circles) and FXIII$^{Leu/Leu}$ (red open circles).

FXIII, factor XIII; TF, tissue factor; V/V, Val/Val; V/L, Val/Leu; L/L, Leu/Leu.

Table 3.1. Clinical characteristics of human donor study population.

	Reference	$FXIII^{Val/Val}$ $FXIII^{Val/Leu}$ (N = 68)	$FXIII^{Leu/Leu}$ (N = 18)	P
Clinical Characteristics				
Sex (female/male)	-	57/11	14/4	1
Age (years)	-	31 ± 11	34 ± 12	0.3
FXIII Activity (%)	69-143	115 ± 22	110 ± 15	0.4
Fibrinogen (g/L)	1.5-4	3.4 ± 1.0	3.7 ± 1.0	0.3
Clotting Tests				
APTT (sec)	28.6-37.6	29.8 ± 3.0	29.8 ± 2.7	0.9
Prothrombin Time (sec)	8.2-12.2	8.3 ± 0.6	8.2 ± 0.5	0.8
Thrombin Generation Parameters				
Lag Time (min)	-	2.5 ± 0.6	2.3 ± 0.6	0.4
Time to Peak (min)	-	4.9 ± 1.0	4.9 ± 1.1	0.9
Peak Thrombin (nM)	-	327 ± 93	275 ± 79	0.03
ETP (nM*min)	-	1496 ± 318	1252 ± 218	0.003

Data show means ± SEM and were analyzed by t-test. $P < 0.005$ was considered significant.

Table 3.2. Factors associated with Val34Leu polymorphism and clot weight in a univariate analysis.

	$FXIII^{Val/Val}$ + $FXIII^{Val/Leu}$		$FXIII^{Leu/Leu}$	
	R	**P**	**R**	**P**
Clinical Characteristics				
Age (years)	0.2	<0.001	0.004	0.8
FXIII Activity (%)	0.002	0.7	0.02	0.6
Fibrinogen (g/L)	0.3	<0.001	0.03	0.5
Clotting Tests				
APTT (sec)	0.003	0.6	0.1	0.2
Prothrombin Time (sec)	0.1	0.01	0.001	0.9
Thrombin Generation Parameters				
Lag Time (min)	0.2	<0.001	0.01	0.7
Time to Peak (min)	0.2	<0.001	0.02	0.6
Peak Thrombin (nM)	0.003	0.5	0.02	0.6
ETP (nM*min)	0.06	0.05	0.07	0.3

$P < 0.005$ was considered significant.

population group. However, age, fibrinogen concentration, and thrombin generation parameters: lagtime and time to peak, demonstrated significant positive correlation with $FXIII^{Val/Val}$/$FXIII^{Val/Leu}$ clot weights (Table 3.2). No factor significantly correlated with $FXIII^{Leu/Leu}$ clot weights. To determine the effect of fibrinogen concentration as modifier of clot weight, we constructed a multiple regression model to adjust for fibrinogen as a confounding factor (Table 3.3). In this analysis, only age showed significant correlation with $FXIII^{Val/Val}$/$FXIII^{Val/Leu}$ clot weights. The association of thrombin generation parameters lagtime and time to peak with $FXIII^{Val/Val}$/$FXIII^{Val/Leu}$ clot weight was no longer observed. This suggests the correlations observed in the univariate analysis were strongly driven by fibrinogen concentration. As fibrinogen concentration increases, $FXIII^{Val/Val}$/$FXIII^{Val/Leu}$ clot weight increased (Figure 3.1C) and in contrast, $FXIII^{Leu/Leu}$ clot weights remained unchanged (Figure 3.1C). Furthermore, at high fibrinogen concentrations, compared to $FXIII^{Val/Val}$/$FXIII^{Val/Leu}$ clot weights, $FXIII^{Leu/Leu}$ clot weights were smaller. These human population data demonstrate the Leu variant mitigates the effect of fibrinogen concentration on contracted whole blood clot weight.

$FXIII^{Leu/Leu}$ alters whole blood clot weight in a fibrinogen concentration-dependent manner. To investigate the isolated effect of fibrinogen concentration on the Val34Leu polymorphism and whole blood clot weight, we reconstituted FXIII-depleted plasma with washed O-negative RBCs, washed platelets ($FXIII^{Val/Val}$), FXIII-free fibrinogen (Supplemental Figure 3.2), and purified $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$ zymogen (Figure 3.2A, Supplemental Figure 3.1). Following clot contraction, we correlated FXIII variant and fibrinogen concentration with whole blood clot weight. Importantly, we previously showed that plasma FXIII, not platelet FXIII, promotes RBC retention and mediates clot weight[8], therefore, the contribution of cellular

Table 3.3. Factors associated with Val34Leu polymorphism and clot weight in a multivariate analysis adjusting for fibrinogen.

	$FXIII^{Val/Val}$ + $FXIII^{Val/Leu}$		$FXIII^{Leu/Leu}$	
	R	**P**	**R**	**P**
Clinical Characteristics				
Age (years)	0.4	0.002	0.03	1
FXIII Activity (%)	0.3	1	0.04	0.6
Fibrinogen (g/L)	-	-	-	-
Clotting Tests				
APTT (sec)	0.3	1	0.1	0.2
Prothrombin Time (sec)	0.4	0.01	0.03	0.9
Thrombin Generation Parameters				
Lag Time (min)	0.4	0.02	0.05	0.6
Time to Peak (min)	0.3	0.03	0.04	0.6
Peak Thrombin (nM)	0.3	0.9	0.04	0.7
ETP (nM*min)	0.3	0.1	0.07	0.4

$P < 0.005$ was considered significant.

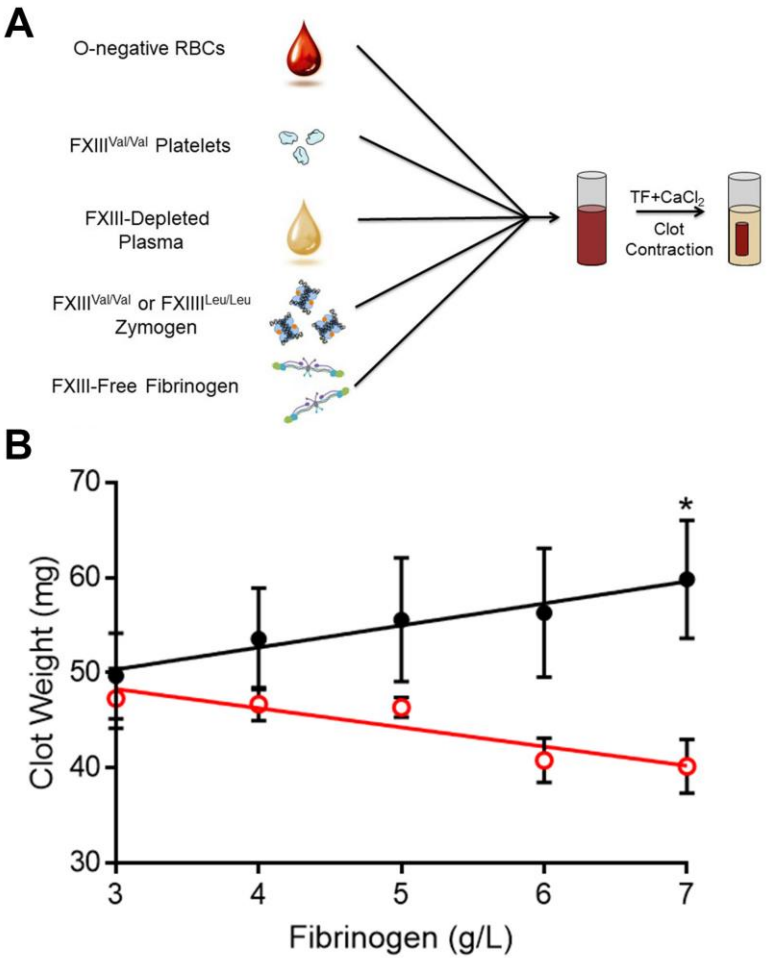


Figure 3.2. In reconstituted whole blood with purified FXIII, the 34Leu variant alters contracted clot weight in fibrinogen concentration-dependent manner. (A) O-negative RBCs, $FXIII^{Val/Val}$ platelets, and FXIII-depleted plasma reconstituted with either $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$ zymogen, were combined. FXIII-free fibrinogen was added at the indicated final concentrations. Clotting was triggered with TF/$CaCl_2$ and contracted clots were weighed after 2 hours. (B) Significance between $FXIII^{Val/Val}$ (black closed circles) and $FXIII^{Leu/Leu}$ (red open circles) clot weights of the same fibrinogen level was compared by t-test. Data show mean ± SEM (N=3). Asterisk indicates $P < 0.05$. FXIII, factor XIII; RBCs, red blood cells.

platelet FXIII to final clot weight was negligible. In reconstituted $FXIII^{Val/Val}$ clots, as fibrinogen concentration increased (from 3-7 g/L, final), contracted whole blood weight increased (Figure 3.2B). In reconstituted $FXIII^{Leu/Leu}$ clots, as fibrinogen concentration increased, contracted whole blood weight decreased (Figure 3.2B). These data show that fibrinogen concentration modulates the effect of the Val34Leu polymorphism on whole blood clot weight.

3.5 Discussion

The FXIII Val34Leu polymorphism is a naturally occurring polymorphism that results in accelerated FXIII activation. Molecular modeling stimulations of the enzyme-substrate complex formation between FXIII and thrombin, reveal the Leu variant results in a side-chain that forms a more energetically favorable hydrophobic interaction[16] and increased substrate specificity[31,32] with thrombin. Meta-analysis of epidemiological studies examining the Val34Leu polymorphism suggest the Leu variant offers a significant, but modest protection against VT.[14] This effect is hypothesized to result from fibrinogen concentration-dependent clot structure changes.[19] At high fibrinogen concentrations, the Val variant produces a dense, thin fibrin network, whereas the Leu variant reduces fibrin network density.[19] Concurrently, premature activation of FXIII potentially "depletes" local FXIII levels before the fibrin network is formed, resulting in decreased fibrin crosslinking.[32] Since both fibrin network density and fibrin crosslinking positively correlate with RBC retention[10], these mechanisms may reduce RBC retention in clots, and consequently, clot size. The effect of the Val34Leu polymorphism on whole blood clot weight has not been investigated.

Herein, we investigated the role of the Val34Leu polymorphism in a human whole blood system through two complementary approaches: experiments with individual donor plasmas that

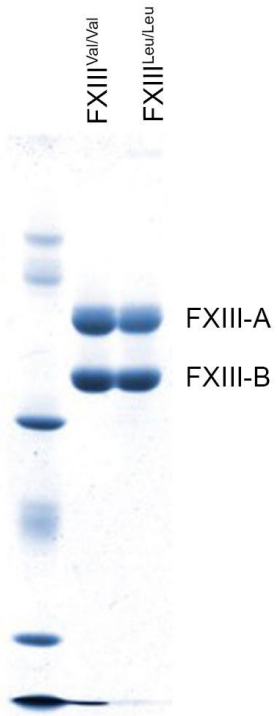
provided a more relevant context to the human population and enabled us to correlate laboratory characteristics (FXIII, fibrinogen, clotting times, thrombin generation) with genotype and clot weight, and experiments with purified FXIII that enabled us to control fibrinogen concentration. In reconstituted whole blood with individual plasmas, $FXIII^{Val/Val}$ and $FXIII^{Val/Leu}$ donors with high fibrinogen concentrations correlated with larger clot weights. In $FXIII^{Leu/Leu}$ donors, clot weight did not correlate with fibrinogen concentration and compared to $FXIII^{Val/Val}$ and $FXIII^{Val/Leu}$ donors at high fibrinogen concentrations, clot weights were smaller. We observed in reconstituted clots with purified FXIII, at the highest fibrinogen concentration tested (7 g/L), compared to $FXIII^{Val/Val}$, $FXIII^{Leu/Leu}$ resulted in smaller clot weights. Combined observations through the two complimentary approaches suggest a protective effect of the Leu variant under elevated fibrinogen levels considered prothrombotic.

This study has potential limitations. First, our study has a small population sample size, particularly in individuals homozygous for the Leu allele. In preliminary experiments with limited samples, we observed trends between theVal34Leu polymorphism and clot weight. We performed a power calculation (80% power, type 1 error $\alpha = 0.05$) to predetermine a sample size needed to observe statistical significance trends, which prompted additional donor sample collection. In the final sampling of 68 donors, the prevalence of homozygous Leu individuals (26%) was consistent with the prevalence in the human population (~25%) and the two population groups ($FXIII^{Val/Val}$/$FXIII^{Val/Leu}$ and $FXIII^{Leu/Leu}$) were well-matched and sufficiently powered to observe significant trends in the data. Second, our reporting of an association of a genetic polymorphism with VT gives insight into how genetic regulation of fibrin(ogen) function can modulate VT risk but is only one aspect of the complex gene-gene and gene-environment interactions that promote venous thrombogenesis. Our findings suggest that Val34Leu status in

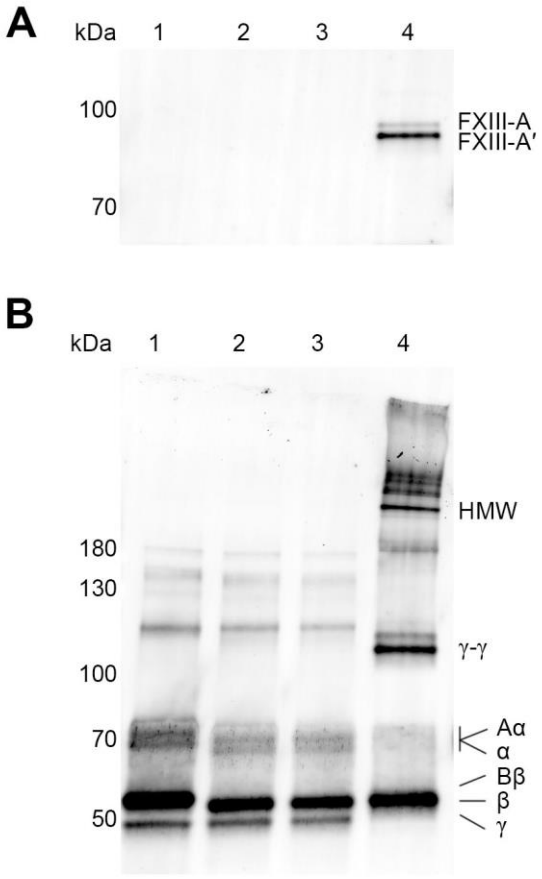
conjunction with fibrinogen level should be considered in clinical risk predictors to identify individuals at higher risk of VT.

In summary, we have shown in human plasma with prothrombotic high fibrinogen levels, the presence of the Leu variant is associated with formation of smaller whole blood clots. The Val34Leu polymorphism may protect against VT by decreasing clot RBC retention and consequently, reducing clot size.

3.6 Supplemental Material



Supplemental Figure 3.1. Preparation of purified FXIII$^{Val/Val}$ and FXIII$^{Leu/Leu}$ zymogen. FXIII$^{Val/Val}$ and FXIII$^{Leu/Leu}$ proteins were purified from human plasma; 10 µg were separated by non-reducing SDS-PAGE (7.5% gel) to allow for the separation of the FXIII-A and -B subunits. FXIII, factor XIII.



Supplemental Figure 3.2. FXIII is not present in peak 1 fibrinogen preparation. FXIII antigen and activity were analyzed in fibrinogen preparations by western blot detection of (A) FXIII-A subunit and (B) fibrin crosslinking, respectively. Lanes are: (1) peak 1 fibrinogen (unclotted negative control), (2) peak 1 fibrinogen + thrombin + EDTA (uncrosslinked negative control), (3) peak 1 fibrinogen + thrombin + calcium (10 mM, final), and (4) unfractionated fibrinogen + thrombin + calcium (crosslinked positive control). FXIII, factor XIII; HMW, high molecular weight fibrin species.

REFERENCES

1. Greenberg CS, Shuman MA. The zymogen forms of blood coagulation factor XIII bind specifically to fibrinogen. *J Biol Chem*. 1982;257(11):6096-6101.

2. Komaromi I, Bagoly Z, Muszbek L. Factor XIII: novel structural and functional aspects. *J Thromb Haemost*. 2011;9(1):9-20.

3. Glover CJ, McIntire LV, Brown CH, 3rd, Natelson EA. Rheological properties of fibrin clots. Effects of fibrinogen concentration, Factor XIII deficiency, and Factor XIII inhibition. *J Lab Clin Med*. 1975;86(4):644-656.

4. Liu W, Jawerth LM, Sparks EA, et al. Fibrin fibers have extraordinary extensibility and elasticity. *Science*. 2006;313(5787):634.

5. Fraser SR, Booth NA, Mutch NJ. The antifibrinolytic function of factor XIII is exclusively expressed through alpha(2)-antiplasmin cross-linking. *Blood*. 2011;117(23):6371-6374.

6. Valnickova Z, Enghild JJ. Human procarboxypeptidase U, or thrombin-activable fibrinolysis inhibitor, is a substrate for transglutaminases. Evidence for transglutaminase-catalyzed cross-linking to fibrin. *J Biol Chem*. 1998;273(42):27220-27224.

7. van Giezen JJ, Minkema J, Bouma BN, Jansen JW. Cross-linking of alpha 2-antiplasmin to fibrin is a key factor in regulating blood clot lysis: species differences. *Blood Coagul Fibrinolysis*. 1993;4(6):869-875.

8. Kattula S, Byrnes JR, Martin SM, et al. Factor XIII in plasma, but not in platelets, mediates red blood cell retention in clots and venous thrombus size in mice. *Blood Adv*. 2018;2(1):25-35.

9. Aleman MM, Byrnes JR, Wang JG, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest*. 2014;124(8):3590-3600.

10. Byrnes JR, Duval C, Wang Y, et al. Factor XIIIa-dependent retention of red blood cells in clots is mediated by fibrin alpha-chain crosslinking. *Blood*. 2015;126(16):1940-1948.

11. Muszbek L. Deficiency causing mutations and common polymorphisms in the factor XIII-A gene. *Thromb Haemost*. 2000;84(4):524-527.

12. Catto AJ, Kohler HP, Coore J, Mansfield MW, Stickland MH, Grant PJ. Association of a common polymorphism in the factor XIII gene with venous thrombosis. *Blood*. 1999;93(3):906-908.

13. Franco RF, Reitsma PH, Lourenco D, et al. Factor XIII Val34Leu is a genetic factor involved in the etiology of venous thrombosis. *Thromb Haemost*. 1999;81(5):676-679.

14. Wells PS, Anderson JL, Scarvelis DK, Doucette SP, Gagnon F. Factor XIII Val34Leu variant is protective against venous thromboembolism: a HuGE review and meta-analysis. *Am J Epidemiol*. 2006;164(2):101-109.

15. Renner W, Koppel H, Hoffmann C, et al. Prothrombin G20210A, factor V Leiden, and factor XIII Val34Leu: common mutations of blood coagulation factors and deep vein thrombosis in Austria. *Thromb Res*. 2000;99(1):35-39.

16. Balogh I, Szoke G, Karpati L, et al. Val34Leu polymorphism of plasma factor XIII: biochemistry and epidemiology in familial thrombophilia. *Blood*. 2000;96(7):2479-2486.

17. Margaglione M, Bossone A, Brancaccio V, Ciampa A, Di Minno G. Factor XIII Val34Leu polymorphism and risk of deep vein thrombosis. *Thromb Haemost*. 2000;84(6):1118-1119.

18. Corral J, Gonzalez-Conejero R, Iniesta JA, Rivera J, Martinez C, Vicente V. The FXIII Val34Leu polymorphism in venous and arterial thromboembolism. *Haematologica*. 2000;85(3):293-297.

19. Lim BC, Ariens RA, Carter AM, Weisel JW, Grant PJ. Genetic regulation of fibrin structure and function: complex gene-environment interactions may modulate vascular risk. *Lancet*. 2003;361(9367):1424-1431.

20. Van Hylckama Vlieg A, Komanasin N, Ariens RA, et al. Factor XIII Val34Leu polymorphism, factor XIII antigen levels and activity and the risk of deep venous thrombosis. *Br J Haematol*. 2002;119(1):169-175.

21. Vossen CY, Rosendaal FR. The protective effect of the factor XIII Val34Leu mutation on the risk of deep venous thrombosis is dependent on the fibrinogen level. *J Thromb Haemost*. 2005;3(5):1102-1103.

22. Mikkola H, Syrjala M, Rasi V, et al. Deficiency in the A-subunit of coagulation factor XIII: two novel point mutations demonstrate different effects on transcript levels. *Blood*. 1994;84(2):517-525.

23. Ariens RA, Philippou H, Nagaswami C, Weisel JW, Lane DA, Grant PJ. The factor XIII V34L polymorphism accelerates thrombin activation of factor XIII and affects cross-linked fibrin structure. *Blood*. 2000;96(3):988-995.

24. Wartiovaara U, Mikkola H, Szoke G, et al. Effect of Val34Leu polymorphism on the activation of the coagulation factor XIII-A. *Thromb Haemost*. 2000;84(4):595-600.

25. Beckman JD, Holle LA, Wolberg AS. Factor XIII cotreatment with hemostatic agents in hemophilia A increases fibrin alpha-chain crosslinking. *J Thromb Haemost*. 2018;16(1):131-141.

26. Shemirani AH, Muszbek L. Rapid detection of the factor XIII Val34Leu (163 G-->T) polymorphism by real-time PCR using fluorescence resonance energy transfer detection and melting curve analysis. *Clin Chem Lab Med*. 2004;42(8):877-879.

27. Lorand L, Credo RB, Janus TJ. Factor XIII (fibrin-stabilizing factor). *Methods Enzymol*. 1981;80 Pt C:333-341.

28. Karpati L, Penke B, Katona E, Balogh I, Vamosi G, Muszbek L. A modified, optimized kinetic photometric assay for the determination of blood coagulation factor XIII activity in plasma. *Clin Chem*. 2000;46(12):1946-1955.

29. Clauss A. [Rapid physiological coagulation method in determination of fibrinogen]. *Acta Haematol*. 1957;17(4):237-246.

30. Machlus KR, Colby EA, Wu JR, Koch GG, Key NS, Wolberg AS. Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram. *Thromb Haemost*. 2009;102(5):936-944.

31. Isetti G, Maurer MC. Probing thrombin's ability to accommodate a V34F substitution within the factor XIII activation peptide segment (28-41). *J Pept Res*. 2004;63(3):241-252.

32. Trumbo TA, Maurer MC. Examining thrombin hydrolysis of the factor XIII activation peptide segment leads to a proposal for explaining the cardioprotective effects observed with the factor XIII V34L mutation. *J Biol Chem*. 2000;275(27):20627-20631.

CHAPTER 4: NOVEL MURINE VENOUS THROMBOEMOBOLISM MODEL TO EVALUATE THE ROLE OF FACTOR XIII IN PULMONARY EMBOLISM RISK

4.1 Overview

Venous thrombosis and pulmonary embolism (PE), collectively venous thromboembolism, cause high mortality and morbidity. Factor XIII (FXIII) crosslinks fibrin to enhance thrombus stability and consequently, may influence PE risk. Elucidating mechanisms contributing to PE is limited by a lack of models that recapitulate human PE characteristics. We aimed to develop a mouse model that permits embolization of red blood cell (RBC)- and fibrin-rich venous thrombi formed during blood stasis and determine the contribution of FXIII to PE risk. We compared the composition of human PEs to wild-type mouse venous thrombi (electrolytic, ferric chloride, and inferior vena cava [IVC] stasis) and PEs generated by intravenous thrombin infusion. Femoral vein electrolytic and ferric chloride injury produced small thrombi with few RBCs (5% and 4%, respectively), whereas IVC stasis produced large thrombi with higher RBC content (68%), similar to human PEs (68%). Although $F13a^{+/+}$, $F13a^{+/-}$, and $F13a^{-/-}$ mice had similar PE incidence in the thrombin infusion model, PE were small, with low RBC content (≤ 7%), unlike human PEs. We used IVC stasis with subsequent ligature removal to permit thrombus embolization. Prior to ligature removal, $F13a^{-/-}$ thrombi showed no fibrin crosslinking, however, $F13a^{+/+}$ and $F13a^{+/-}$ thrombi demonstrated similar fibrin crosslinking. After ligature removal, compared to $F13a^{+/+}$ (13%), $F13a^{+/-}$ and $F13a^{-/-}$ mice had similar or increased PE incidence (17% and 64%, respectively). Complete FXIII deficiency increases PE incidence, but partial deficiency does not.

4.2 Introduction

Venous thrombosis (VT) and/or pulmonary embolism (PE), collectively venous thromboembolism (VTE), affect 1-2/1000 people annually.[1] VTE has ~30% mortality within the first 30 days of presentation; mortality is usually associated with PE.[2] Acute PE occurs when part or all of the thrombus detaches, travels through the vasculature, and occludes the pulmonary arteries. Anticoagulants inhibit thrombus propagation but do not dissolve existing thrombi. Thrombolytics dissolve thrombi, but are associated with bleeding risk.[3,4] There is a need to better understand molecular mechanisms that promote PE and develop effective therapies to prevent VTE without increasing bleeding risk.

Factor XIII (FXIII) is a zymogen of a transglutaminase that crosslinks fibrin and stabilizes clots from mechanical disruption and premature dissolution (fibrinolysis).[5,6] FXIII also promotes fibrin-mediated red blood cell (RBC) retention in contracted thrombi, and consequently, determines thrombus size.[7] Recent studies using ferric chloride ($FeCl_3$) to induce thrombosis in the femoral vein suggested complete FXIII deficiency increases thrombus embolization.[8,9] However, a meta-analysis examining the prevalence of PE in patients with congenital coagulation disorders reported no increase in PE risk in FXIII-deficient patients.[10] Although congenital FXIII deficiency is rare, acquired FXIII deficiency secondary to ongoing disease has been reported in patients with inflammatory bowel disease, sepsis, major surgery, trauma, and disseminated intravascular coagulation.[11,12] FXIII deficiency is prevalent in hospitalized patients (21% of adults, and 52% of children have a plasma FXIII concentration <50 U dL^{-1}).[13] Thus, determining the role of FXIII in VTE warrants further investigation.

Elucidating PE pathophysiology is limited by a lack of animal models that recapitulate key characteristics of human PE: embolization of existing RBC- and fibrin-rich thrombi.[14]

Established mouse models of PE include injection of clots generated ex vivo[15-20] or intravenous infusion of platelet agonists (adenosine diphosphate[21,22] or collagen plus epinephrine[21,23-25]) or coagulation activators (e.g., thrombin[26-29] or tissue factor/thromboplastin[21,30-32]). These models produce thrombi that form in flowing blood and subsequently lodge in the lung. This situation is unlike PE in humans, which typically arise from existing venous thrombi. Developing a mouse model of PE that better recapitulates VTE in humans is necessary to identify molecular mechanisms that contribute to PE pathophysiology.

Herein, we developed a novel VTE mouse model that produces RBC- and fibrin-rich thrombi histologically-comparable to human PE and permits subsequent embolization. Using this model, we evaluated the contribution of FXIII to PE risk.

4.3 Methods

Materials. Alexa Fluor® 647 protein labeling kits were from Thermofisher (Waltham, MA) and IRDye 800CW was from LICOR (Lincoln, NE). Bio-Spin 30 chromatography columns were from Bio-Rad (Hercules, CA). Fibrinogen (FXIII-free peak 1) was from Enzyme Research Laboratories (South Bend, IN). Anti-GPIX antibody (Clone Xia.B4) was from Emfret analytics (Eibelstadt, Germany). Hematoxylin and eosin (H&E, Cat: 26043-05) was from Electron Microscopy Services (Hatfield, PA). Target retrieval solution (Cat: 00-4955-58) was from Invitrogen (Carlsbad, CA) and mouse-on-mouse blocking reagent, protein concentrate, and biotinylated anti-mouse IgG kit reagents (Cat: BMK-2202) were from Vector Laboratories (Burlingame, CA). DAB (3,3′-Diaminobenzidine) chromogenic substrate and buffer (Cat: K3467) was from Dako (Glostrup, Denmark). Lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 mg/mL leupeptin) was from Cell Signaling (Danvers,

MA). Phenylmethylsulfonyl fluoride was from MP Biomedicals (Solon, OH). Non-reducing 6X SDS-sample buffer was from Boston BioProducts (Ashland, MA). Tris-glycine polyacrylamide gels (10%) were from Bio-Rad (Hercules, CA). Polyvinylidene difluoride membranes were from Invitrogen (Carlsbad, CA). Odyssey Blocking Buffer was from LICOR Biosciences (Lincoln, NE). Rabbit anti-human fibrinogen polyclonal antibody (A0080) was from Dako (Glostrup, Denmark), and goat anti-rabbit IRDye 800CW was from LICOR Biosciences (Lincoln, NE). Mouse anti-human fibrin primary antibody (59D8, was a generous gift from Dr. Charles Esmon, Oklahoma Medical Research Foundation.

Human PE. PEs were obtained from deceased patients from University of North Carolina (UNC) Hospitals with approval from the UNC Institutional Review Board. Clots were fixed with 10% buffered formalin for 24-72 hours and stored in 70% ethanol. PE sample 1 was from a 58-year-old male with a history of coronary artery disease, hypertension, obesity, and tobacco use. Autopsy results revealed pulmonary saddle embolus, multiple peripheral PEs, and pulmonary infarct. PE sample 2 was from a 17-year-old female with a history of pelvic germ cell tumor with metastasis to lung, liver, and bone, stage 2 kidney disease, and deep vein thrombosis of the femoral vein, detected by Doppler and D-dimer levels. PE sample 3 was from a 63-year-old male with a history of coronary artery disease, myocardial infarction, hypertension, and Crohn's disease. PE sample 4 was from a 46-year-old female with a history of lymphedema, hypertension, renal failure, obesity, and undifferentiated carcinoma of likely gynecologic origin with metastases. Autopsy results revealed a large thrombus occluding the left pulmonary artery and smaller thrombi occluding the initial branches of the right pulmonary artery.

Mice. Murine procedures were approved by the UNC Institutional Animal Care and Use Committee. *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice were backcrossed 6 generations on a C57BL/6J background.[33] Additional 8-12 week-old C57BL/6N mice were obtained from Charles River Laboratories and maintained by homozygous breeding. Mice were maintained on a 12-hour light cycle (7 am-7 pm) and fed 2920X irradiated regular feed or 2919X irradiated breeder feed (Envigo) (Huntingdon, UK). Male mice (8-12 week-old) were used for all experiments and were anesthetized with 1.5% isoflurane in oxygen (2 L/min).

Thrombin infusion PE model. Fibrinogen (FXIII-free peak 1) was labeled with an Alexa Fluor® 647 protein labeling kit, per manufacturer recommendations. Anti-GPIX antibodies were incubated with IRDye 800CW for 1 hour at room temperature and eluted on a Bio-Spin 30 chromatography column. Anesthetized *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice were injected retro-orbitally with Alexa Fluor® 647-labeled fibrinogen (0.15 mg/mL, , final plasma concentration) and 800-labeled anti-GPIX antibodies (1 µg/g body weight), followed by thrombin injection (1250 U/kg) via tail vein. Symptoms of thrombotic shock were assessed for 5 minutes and recorded as none, moderate (lethargy and/or shallow respiration), or severe (impaired mobility, irregular respiration, and/or loss of consciousness). Lungs were perfused with 10 mL of 1X phosphate buffered saline (3 mL/min), harvested, and imaged on a GE Typhoon FLA-9000 Imager (GE Healthcare, Pittsburgh, PA) or LI-COR Odyssey FC Scanner (LICOR Biosciences). Lungs were then fixed in 10% buffered formalin for 24 hours and stored in 70% ethanol.

VT models. VT models were compared using wild-type C57Bl/6N mice. Electrolytic injury was induced via 3-minute application of 3-V direct current by touching the femoral vein surface with

the blunt end of a 25-gauge needle connected to the anode and completing the circuit by contacting local subdermal tissue with the cathode, as described.[34] Ferric chloride ($FeCl_3$) injury was induced by exposing the dried femoral vein to 4% $FeCl_3$ (1.0 × 2.0-mm filter paper) for 5 minutes. In the inferior vena cava (IVC) stasis model, the IVC was separated from the aorta by blunt dissection. Lumbar branches were cauterized and the IVC and side branches were ligated, as described.[35] Thrombi from all 3 models were harvested at 24 hours. For electrolytic and $FeCl_3$ models, thrombi were collected with vein wall and weighed together. For the IVC stasis model, the thrombi were separated from the vein wall and weighed separately.

Ligature removal PE model. The ligature removal model was developed using *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ (C57Bl/6J) mice. Anesthetized mice were retro-orbitally injected with Alexa Fluor® 647-labeled fibrinogen and 800-labeled anti-GPIX antibodies and then subjected to IVC stasis. At 24 hours, a second surgery was performed to remove the ligature. Mice were allowed to recover and observed for symptoms of thrombotic shock. At 48 hours, lungs were perfused, harvested, imaged, and stored, as in thrombin infusion PE model. The thrombi were separated from the vein wall, weighed, fixed in 10% buffered formalin for 24 hours, and stored in 70% ethanol.

Histology. Formalin-fixed lungs were dehydrated and paraffin-embedded. Consecutive, 5-µm sections were cut and mounted, and every 4th slide was stained with H&E at the UNC Animal Histopathology and Lab Medicine Core. Immunohistochemistry was performed as described. Negative controls were stained in the absence of primary antibody.

Thrombus and PE analysis. PE burden was characterized by incidence, size, and composition. PEs were identified by histology and differentiated from clots formed post-mortem by defined criteria: 1) presence in pulmonary arteries and 2) layers of platelets, fibrin, and RBCs. PE incidence was confirmed by a blinded pathologist. RBC composition was quantified using Image J (version 1.48): pixels of demarcated RBC area(s) was reported as percentage of total thrombus/PE pixel area. Fibrin composition was quantified scored intensity of fibrin immunohistochemistry staining of thrombi/PEs by 5 independent, blinded observers on a scale of 0 to 3. Score of 1, 2, and 3, were considered weak, moderate, or strong, respectively. Whole lung fluorescent area was quantified using Image J (version 1.48): total pixels of fluorescent areas corresponding to platelets and fibrin(ogen) as percentage of total lung pixel area.

Thrombus analysis by Western blot. Frozen thrombi from *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice retrieved 24 hours after IVC stasis were analyzed by western blot, as previously described.[7] Membranes were incubated with primary polyclonal antibodies against fibrin(ogen), and incubated with fluorescence-labeled anti-rabbit secondary antibodies, each for 1 hour at room temperature. Fibrin(ogen) bands were quantified by densitometry (ImageJ) and band intensity was reported as total A.F.U.

Statistical methods. Descriptive statistics (mean, standard error of the mean [SEM]) were calculated for each experiment. Experiments with normally-distributed data in 2 groups were compared by Student's *t* test and more than 2 groups were analyzed by analysis of variance with Dunnett's post-hoc tests for between-group comparisons. Experiments with non-normally-distributed data in 2 groups were compared by Mann-Whitney test and more than 2 groups

Kruskal-Wallis with Dunn's post-hoc test for between-group comparisons. $P < 0.05$ was considered significant.

4.4 Results

FXIII does not impact PE incidence in the thrombin infusion model. We first characterized PE incidence, size, and composition in an established model of PE triggered by intravenous thrombin infusion[26-29]. For this model, we infused mice with fluorescently-labeled FXIII-free fibrinogen and anti-GPIX antibodies, followed by injection of thrombin (Figure 4.1A). In the 5-minute observation period post-injection, *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice showed no, or only moderate, signs of thrombotic shock and similar survival rates (Figure 4.1B-C). Inspection of H&E-stained lungs harvested from *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice showed similar incidence of numerous, scattered PEs (60%, 67%, and 70%, respectively, Figure 4.1D).

Thrombin infusion produces PEs histologically-dissimilar to human PEs. Previous studies have shown that venous thrombi and PE isolated from humans are characteristically rich in RBCs and have high fibrin content.[14,36-38] Accordingly, as expected, analysis of PE retrieved from 4 human subjects (Table 4.1) by H&E staining and immunohistochemistry demonstrated substantial RBC (68±5%) and strong fibrin staining (Figure 4.1E-F). This unique composition implies a role for RBCs and fibrin in venous thrombogenesis that is important to recapitulate in an animal model of VTE. We therefore examined the composition of PEs produced by thrombin infusion in mice and compared this to PEs isolated from humans. Imaging of perfused whole lungs from thrombin-infused mice showed similar fluorescence intensity for platelets and fibrin(ogen) between *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice (Figure 4.1G-H). Analysis by H&E staining showed PEs were small (Figure 4.1I) and scattered throughout the lung vasculature. Moreover, PEs from all *F13a*$^{+/+}$, *F13*$^{+/-}$, and *F13a*$^{-/-}$ mice had significantly lower RBC (7±3%,

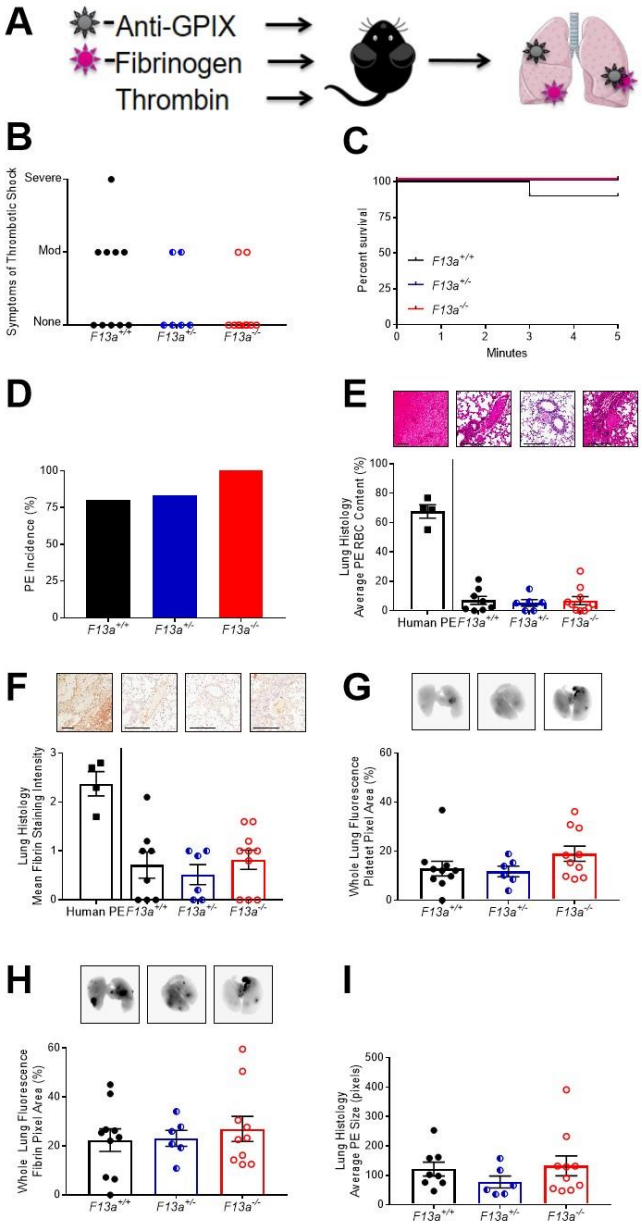


Figure 4.1. In the thrombin infusion model, *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice have similar incidence of fibrin- and platelet-rich PEs. (A) *F13a*$^{+/+}$ (N=10), *F13a*$^{+/-}$ (N=6), and *F13a*$^{-/-}$ (N=10) were injected retro-orbitally with Alexa Fluor-647 labeled fibrinogen and Alexa Fluor-800 labeled anti-GPIX antibodies and then injected intravenously with thrombin. Mice were perfused after observation period and lungs were harvested for histological analysis. (B) Thrombotic shock severity. (C) Survival. (D-F) PE (D) incidence, (E) RBC content, and (F) fibrin staining intensity by histology. (G-H) Fluorescent area from (G) AlexaFluor-800-anti-GPIX antibody and (H) AlexaFluor-647-fibrin(ogen) in perfused lungs. (I) PE size. Dots represent individual mice. Bars show 200 µm.

5±2%, and 7±3%, respectively) human PE (Figure 1E-F) and weak to moderate fibrin staining, this composition did not differ between genotype (Figure 4.1E-F). Collectively, these data show PEs generated by thrombin infusion have a substantially different composition than PEs isolated from humans.

IVC stasis produces RBC- and fibrin-rich thrombi histologically-similar to human PEs. Another limitation of the thrombin infusion model is that the PEs do not originate from an existing VT, but rather derive from microthrombi that form in circulation and become lodged in the lung microvasculature. To develop a model in which emboli arise from existing venous thrombi, we first generated thrombi in wild-type mice using 3 commonly-used mouse VT models: electrolytic/femoral vein, $FeCl_3$/femoral vein, and IVC stasis (Figure 4.2A)[34-36,39-42]. Thrombi from all 3 models were harvested at 24 hours for histological analysis by H&E and fibrin immunohistochemistry. We then compared the composition of these thrombi to that of human PEs. The femoral vein electrolytic and $FeCl_3$ models rapidly produced small thrombi (Figure 4.2B). Subsequent analysis by H&E staining and immunohistochemistry showed these thrombi were predominantely composed of proteinaceous material and had relatively low RBC (7±3% and 2±1%, respectively, (Figure 4.2A,C) compared to human PEs. Thrombi from the electrolytic model demonstrated weak to moderate fibrin staining intensity, whereas the thrombi from the $FeCl_3$ model showed weak fibrin staining intensity (Figure 2D). In contrast, IVC stasis produced large thrombi (Figure 4.2A,B) that had high RBC content (68±2%) (Figure 4.2A,C), similar to that seen in human PEs (Figure 4.1E) and moderate fibrin staining intensity (Figure 2D). These findings illustrate substantial differences in the composition of thrombi generated by these VT models, suggesting fundamental differences in the pathophysiologic processes leading to their generation.

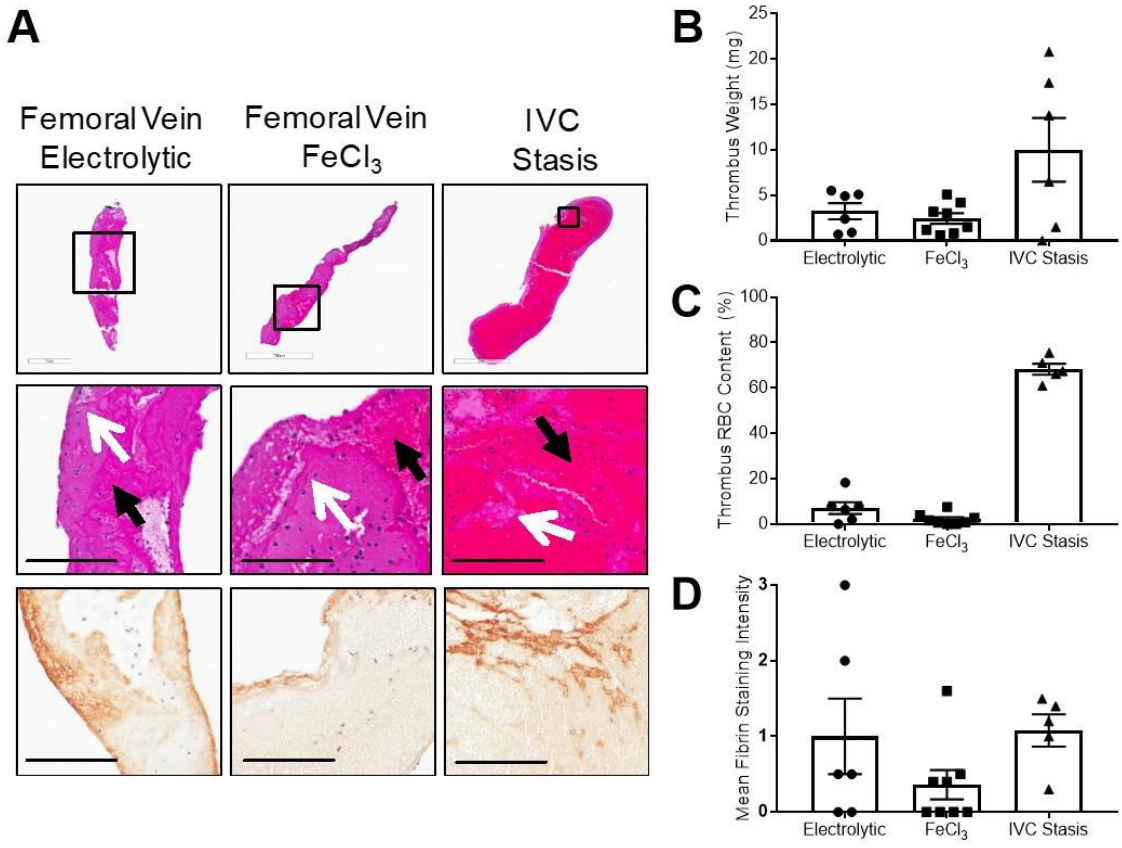


Figure 4.2. Femoral vein electrolytic and $FeCl_3$ models produce small, RBC- and fibrin-poor thrombi. IVC stasis produces large, RBC- and fibrin-rich thrombi. Thrombi from three mouse models of VT: electrolytic (N=6), $FeCl_3$ (N=8), and IVC stasis (N=5). (A) Representative whole (top row) thrombi; electrolytic and $FeCl_3$ thrombi were at 4x magnification and IVC stasis thrombus was 1x magnification. Representative magnified images showing H&E (middle row), and fibrin staining (bottom row). The black boxes outline the area that is magnified below; all magnified images are at 20x magnification. The black arrows indicate areas rich in RBCs and white arrows indicate proteinaeous material. (B) Weight (C) RBC content, and (D) fibrin staining intensity of the venous thrombi. Dots represent individual mice. Bars show 100 µm.

Ligature removal enables thrombus embolization, and complete FXIII deficiency increases incidence of PEs but partial FXIII deficiency does not. Given the similar composition of thrombi generated by IVC stasis to human VT[38] and PE ([14] and Figure 4.1), we further developed this method into a model of VTE. To enable thrombus embolization, we subjected mice to IVC stasis for 24 hours and then removed the ligature and allowed mice to recover under observation for signs of thrombotic shock. After another 24 hours (48 hours after IVC ligation), mice were sacrificed, residual thrombus in the IVC was isolated and weighed, and lungs were perfused, harvested, and processed for histology and fluorescence analysis (Figure 4.3A).

In the 60-minute observation period after ligature removal, 4/16 (25%) *F13a*$^{+/+}$ and 5/17 (30%) *F13a*$^{+/-}$ mice exhibited lethargy and irregular respiration consistent with clinical signs of thromboembolic events (Figure 4.3B). Compared to *F13a*$^{+/+}$ and *F13a*$^{+/-}$ mice, a higher number of *F13a*$^{-/-}$ mice (8/14, 57%) exhibited moderate or severe signs of thrombotic events. *F13a*$^{-/-}$ mice also had significantly decreased overall survival; deaths prior to ligature removal had no apparent signs of hemorrhage and deaths after ligature removal were likely due to thrombotic shock induced from PEs (Figure 4.3C). We previously showed that *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice subjected to IVC stasis have thrombi present in the IVC at 24 hours.[43] Interestingly, however, 24 hours after ligature removal, although thrombi were still adherent to the IVC in *F13a*$^{+/+}$ and *F13a*$^{+/-}$ mice, *F13a*$^{-/-}$ mice no longer had thrombi present in their IVC. Fluorescence imaging of whole perfused lungs indicated that compared to *F13a*$^{+/+}$ and *F13a*$^{+/-}$ mice, *F13a*$^{-/-}$ mice had increased fluorescence associated with fibrin(ogen) and platelets (Figure 4.3D-E). Histological examination of perfused lungs confirmed this finding and showed that compared to *F13a*$^{+/+}$ (4/16, 17%) and *F13a*$^{+/-}$ (2/17, 12%) mice, *F13a*$^{-/-}$ mice had increased PE incidence (9/14, 56%) (Figure 4.3F). Analysis using H&E staining and immunohistochemistry indicated

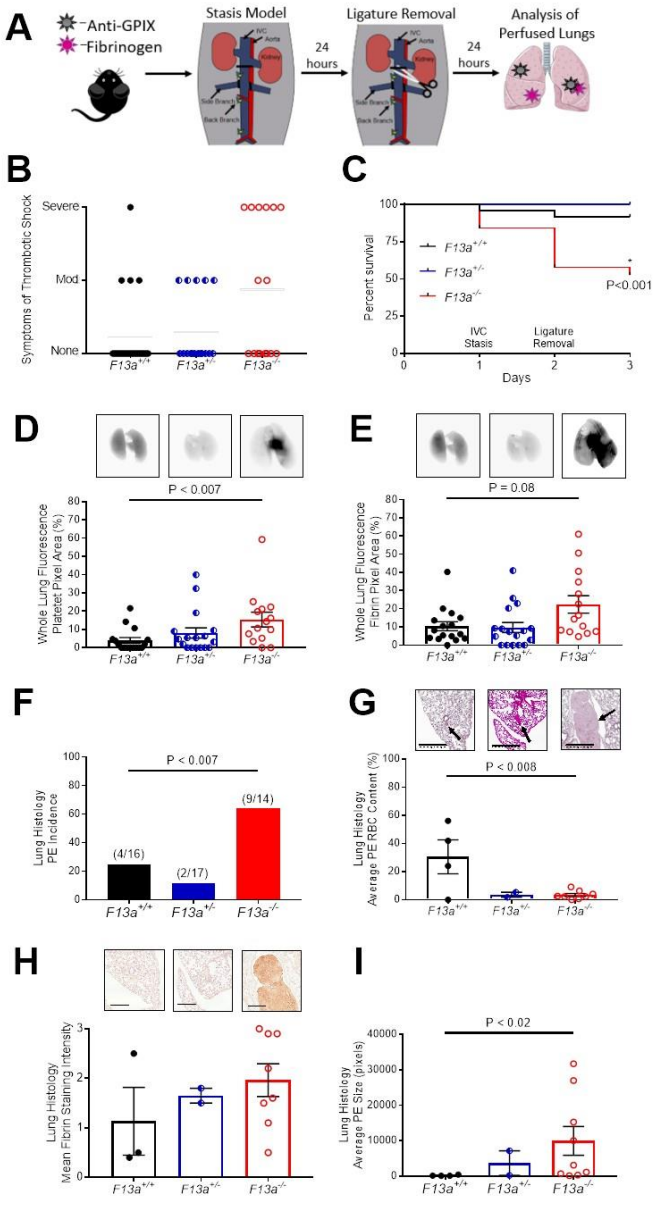


Figure 4.3. The ligature removal model allows for embolization of RBC- and fibrin-rich thrombi and complete FXIII deficiency increases PE incidence, but partial FXIII deficiency does not. (A) *F13a*$^{+/+}$ (N=16), *F13a*$^{+/-}$ (N=17), and *F13a*$^{-/-}$ (N=14) mice subjected to the ligature removal model. Mice were perfused after observation period and lungs were harvested for histological analysis. (B) Thrombotic shock severity. (C) Survival. (D-E) Fluorescent areal from (D) AlexaFluor-800-anti-GPIX antibodies and (E) AlexaFluor-647-fibrin(ogen) in perfused lungs. PE (F) size, (G) incidence, (H) RBC content, and (I) fibrin content. Dots represent individual mice. Bars show 500 µm.

that PEs present in *F13a*$^{+/+}$ mice had substantial RBC content (38±16% and 31±12%, respectively) and weak to moderate fibrin staining intensity (Figure 3G-H). In contrast, PEs present in *F13a*$^{-/-}$ mice had lower RBC content (3±1%) and moderate to strong fibrin staining intensity (61±11%) (Figure 3G-H). composition is consistent with previous findings that FXIII deficiency decreases RBC retention in contracted clot formed in vitro or in vivo.[7] Interestingly, PEs in *F13a*$^{-/-}$ mice were significantly larger than PEs in *F13a*$^{+/+}$ mice (Figure 3I), suggesting most or all thrombus present in the IVC at 24 hours had embolized fully to the lungs upon ligature removal. Together, these data show that IVC stasis followed by ligature removal permits embolization of existing thrombi. Moreover, although complete FXIII deficiency (*F13a*$^{-/-}$ mice) increases PE incidence, partial deficiency (*F13a*$^{+/-}$ mice) does not.

Partial FXIII deficiency results in maximal thrombus fibrin crosslinking. Previously we demonstrated that compared to *F13a*$^{+/+}$ clots in vitro, fibrin crosslinking is delayed in *F13a*$^{+/-}$ plasma clots, but both are completely crosslinked over time.[43] We examined lysates of thrombi formed in *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice subjected to IVC stasis. At 24 hours, high molecular weight (HMW) fibrin crosslinking was absent in *F13a*$^{-/-}$ thrombus lysates but HMW fibrin crosslinking in *F13a*$^{+/-}$ lysates was comparable to *F13a*$^{+/+}$ lysates (Figure 4). These findings suggest partial FXIII reduction does not prevent maximal fibrin crosslinking.

in *F13a*$^{+/+}$ mice had substantial RBC content (38±16% and 31±12%, respectively) and weak to moderate fibrin staining intensity (Figure 4.3G-H). In contrast, PEs present in *F13a*$^{-/-}$ mice had lower RBC content (3±1%) and moderate to strong fibrin staining intensity (61±11%) (Figure 4.3G-H). composition is consistent with previous findings that FXIII deficiency decreases RBC retention in contracted clot formed in vitro or in vivo.[7] Interestingly, PEs in *F13a*$^{-/-}$ mice were significantly larger than PEs in *F13a*$^{+/+}$ mice (Figure 4.3I), suggesting most or all thrombus

present in the IVC at 24 hours had embolized fully to the lungs upon ligature removal. Together, these data show that IVC stasis followed by ligature removal permits embolization of existing thrombi. Moreover, although complete FXIII deficiency (*F13a*$^{-/-}$ mice) increases PE incidence, partial deficiency (*F13a*$^{+/-}$ mice) does not.

Partial FXIII deficiency results in maximal thrombus fibrin crosslinking. Previously we demonstrated that compared to *F13a*$^{+/+}$ clots in vitro, fibrin crosslinking is delayed in *F13a*$^{+/-}$ plasma clots, but both are completely crosslinked over time.[43] We examined lysates of thrombi formed in *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice subjected to IVC stasis. At 24 hours, high molecular weight (HMW) fibrin crosslinking was absent in *F13a*$^{-/-}$ thrombus lysates but HMW fibrin crosslinking in *F13a*$^{+/-}$ lysates was comparable to *F13a*$^{+/+}$ lysates (Figure 4). These findings suggest partial FXIII reduction does not prevent maximal fibrin crosslinking.

4.5 Discussion

Elucidating PE pathophysiology and developing better therapeutic strategies to reduce VTE is paramount. Previous epidemiologic and biochemical studies have associated abnormal fibrin(ogen)[44-49] and/or FXIIIa activity[8,9] with abnormal thrombus stability and PE incidence. However, extension of these observations to the in vivo setting has been limited by a lack of animal models that recapitulate key characteristics of human VTE: embolization of existing RBC-rich venous thrombi to the lungs. Herein, we developed a new mouse model of VTE in which thrombogenesis is initiated by venous blood stasis in situ, and permits subsequent embolization of RBC- and fibrin-rich venous thrombi to the lungs. Our study advances the field in important ways. First, our analysis provides a head-to-head comparison of common mouse models of VT. Our findings reveal fundamental ways in which thrombi generated by electrolytic injury or $FeCl_3$ application differ from the composition of human PEs, but high similarity

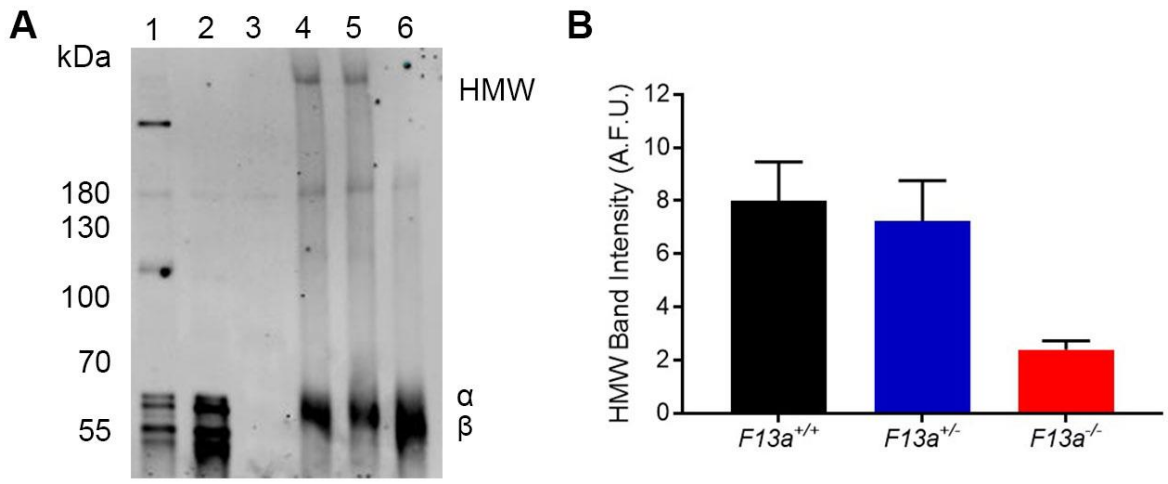


Figure 4.4. High molecular weight fibrin crosslinked species present in *F13a*$^{+/+}$ and *F13a*$^{+/-}$ thrombi but not *F13a*$^{-/-}$ thrombi. (A) Representative western blot for fibrin crosslinking. Lanes are: (1) *F13a*$^{+/+}$ (positive fibrin crosslinking control) (2) *F13a*$^{-/-}$ (negative fibrin crosslinking control), (3) Fgn$^{-/-}$ (negative control) PPP clots, and (4) *F13a*$^{+/+}$, (5) *F13a*$^{+/-}$, (6) *F13a*$^{-/-}$ thrombus lysates. (B) Quantification of high molecular weight fibrin species band intensity. FXIII, factor XIII; Fgn, fibrinogen; HMW, high molecular weight fibrin species.

between thrombi generated by IVC stasis and human PEs. Second, our demonstration that ligature removal after IVC stasis and thrombus formation permits embolization, and shows feasibility of this approach as a mouse model of PE. Third, using this model, we showed that whereas complete FXIII deficiency increases PE incidence, partial deficiency does not. These findings provide valuable confirmation of previous surprising findings on PE incidence in complete FXIII deficient mice[8,9], and also extends this knowledge to a broader and more common setting involving partial FXIII deficiency in which risk is mitigated.

Although PE is associated with high mortality and morbidity, in vivo models used in PE studies fail to recapitulate human pathology, making it problematic to identify molecular mechanisms that contribute to PE pathophysiology. A major advance of our work is the development of a mouse model that recapitulates key aspects of VTE in humans and offers several advantages of these other models. First, the ligature removal model forms RBC- and fibrin-rich venous thrombi via blood stasis. This setting may better mirror the cell-cell contacts that arise during the accrual of RBCs and plasma proteins during venous thrombus formation than models in which flow is preserved and produce platelet-rich thrombi. Although IVC stenosis also produces RBC- and fibrin-rich thrombi (data not shown), the higher reproducibility offered by IVC stasis compared to stenosis[50] provides a more robust method for standardizing thrombus formation. Second, unlike PE models that involve intravenous infusion of platelet or coagulation activators that form PEs in circulation, in ligature removal model, PEs originate from a pre-existing thrombus, similar to human pathophysiology. In humans, although a small subset of patients with PE fail to demonstrate co-existing residual thrombus in a distal vein[51-54], most cases of PE (85-90%) present with an accounted source. A third advantage of the ligature removal model is the control it permits over the relative timing of thrombus formation and

embolic events. Overall, the ligature removal model captures key aspects of VTE in humans and has several advantageous that makes it an amenable model to study PE risk factors.

The finding that PE risk is increased in FXIII-deficient mice ([8,9] and present study) but does not appear to be increased FXIII-deficient humans[10] is unexpected and raises questions about VT and the fate of venous thrombi in FXIII-deficient patients. Since FXIII deficiency is rare, affecting only 1 in ~3 million people worldwide[55], the lack of reported PE in FXIII-deficient patients may reflect the small population of affected individuals. Alternatively, the lack of fibrin crosslinking may facilitate rapid dissolution via endogenous fibrinolytic mechanisms in the lung[56], wherein PE in FXIII-deficient patients is asymptomatic. Long term PE resolution studies with FXIII deficient mice in the ligature removal model could address the discrepancy in reported PE incidence between FXIII deficient humans and mice.

We previously showed that partial FXIII deficiency decreases venous thrombus size without increasing bleeding.[43] These observations support consideration of FXIII as a therapeutic target for anticoagulation. In our prior study[43], we demonstrated partial FXIII deficiency results in delayed fibrin crosslinking, but does not prevent maximal fibrin crosslinking. Collectively, with findings from the present study, partial FXIII deficiency decreases RBC retention and thrombus size, and restores thrombus stability over time, thus not affecting PE risk. Partial FXIII inhibition may be a novel strategy to reduce VTE that has advantages not present in other therapeutic targets for treating VTE.

This study has potential limitations. First, although IVC ligation produces an occlusive thrombus with histological characteristics of human VT[38] and PE[14], it does not recapitulate all aspects of VT pathogenesis in humans. Notably, whereas venous thrombi in humans are thought to arise in valve pockets and extend in the direction of blood flow[57], thrombi produced by IVC

ligation initiate at the ligature site and extend distally, against the flow of blood. However, comparable to the situation in valve pockets, the IVC ligation creates an area of vortical flow that promotes hypoxia at the anatomical origin of the thrombus. Second, embolic events in humans are spontaneous, whereas the ligature removal model requires a second surgery to permit embolism. However, the observation that thrombi remain attached to the vessel wall post-ligature removal in *F13a*$^{+/+}$ and *F13a*$^{+/-}$ mice suggests embolization does not occur only as a result of ligature release but also reflects abnormal adherence of the thrombus to the vessel wall. Interestingly, fibrin and endothelial cell interaction has been linked to FXIII activity, specifically, that transglutaminase-mediated crosslinking and oligomerization of the fibrinogen αC domains plays a role in endothelial cell adhesion to their integrin receptors.[58,59] These studies suggest abnormal or absent FXIII crosslinking of fibrin can alter endothelial cell integrin interaction, potential mechanism related to PE risk in FXIII deficient mice and requires further investigation. Third, we only histologically examined portions of the lungs, and although this approach minimized costs and labor, we may have missed small PEs. However, by assessing thrombotic shock symptoms, residual thrombus in the IVC, and whole lung imaging of labeled fibrin and platelets, we could identify mice likely to have PEs, that underwent additional histological examination.

In humans and mice, FXIII is found in both plasma and cellular compartments.[60] Although findings from our study as well as that of Shaya et al show that complete FXIII deficiency increases PE incidence, the role of plasma vs platelet FXIII has not yet been defined. Interestingly, platelet FXIII mRNA is reduced in atrial fibrillation patients with PEs[61], suggesting platelet FXIII contributes to thrombus embolic propensity. The ligature removal model may enable studies to delineate the relative contributions of plasma and platelet FXIII in PE risk.

Development of this VTE model may also facilitate additional investigations into pathogenic mechanisms leading to PE, including settings of dysfibrinogenemia and other clinical situations associated with PE risk. Although the ligature removal model is sensitive to variables that increase PE risk, evaluation of conditions in which PE risk is decreased may be more challenging given the relatively low incidence of PE in wild-type mice.

In summary, we have developed a VTE model that permits embolization of existing venous thrombi that are histologically-similar to human PEs. Using this model, we show that complete FXIII deficiency increases PE incidence, but partial deficiency does not. We anticipate this model is an adaptable tool that can be used to evaluate the contribution of other factors to PE risk, as well as long-term PE progression and treatment.

REFERENCES

1. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrom J. Incidence and mortality of venous thrombosis: a population-based study. *J Thromb Haemost*. 2007;5(4):692-699.

2. Goldhaber SZ, Visani L, De Rosa M. Acute pulmonary embolism: clinical outcomes in the International Cooperative Pulmonary Embolism Registry (ICOPER). *Lancet*. 1999;353(9162):1386-1389.

3. Wan S, Quinlan DJ, Agnelli G, Eikelboom JW. Thrombolysis compared with heparin for the initial treatment of pulmonary embolism: a meta-analysis of the randomized controlled trials. *Circulation*. 2004;110(6):744-749.

4. Stein PD, Hull RD, Raskob G. Risks for major bleeding from thrombolytic therapy in patients with acute pulmonary embolism. Consideration of noninvasive management. *Ann Intern Med*. 1994;121(5):313-317.

5. Hevessy Z, Haramura G, Boda Z, Udvardy M, Muszbek L. Promotion of the crosslinking of fibrin and alpha 2-antiplasmin by platelets. *Thromb Haemost*. 1996;75(1):161-167.

6. Reed GL, Matsueda GR, Haber E. Fibrin-fibrin and alpha 2-antiplasmin-fibrin cross-linking by platelet factor XIII increases the resistance of platelet clots to fibrinolysis. *Trans Assoc Am Physicians*. 1991;104:21-28.

7. Aleman MM, Byrnes JR, Wang JG, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest*. 2014;124(8):3590-3600.

8. Shaya SA, Gani DM, Weitz JI, Kim PY, Gross PL. Factor XIII Prevents Pulmonary Emboli in Mice by Stabilizing Deep Vein Thrombi. *Thromb Haemost*. 2019.

9. Shaya SA, Saldanha LJ, Vaezzadeh N, Zhou J, Ni R, Gross PL. Comparison of the effect of dabigatran and dalteparin on thrombus stability in a murine model of venous thromboembolism. *J Thromb Haemost*. 2016;14(1):143-152.

10. Girolami A, Cosi E, Tasinato V, Peroni E, Girolami B, Lombardi AM. Pulmonary embolism in congenital bleeding disorders: intriguing discrepancies among different clotting factors deficiencies. *Blood Coagul Fibrinolysis*. 2016;27(5):517-525.

11. Godje O, Haushofer M, Lamm P, Reichart B. The effect of factor XIII on bleeding in coronary surgery. *Thorac Cardiovasc Surg*. 1998;46(5):263-267.

12. Biswas A, Ivaskevicius V, Thomas A, Oldenburg J. Coagulation factor XIII deficiency. Diagnosis, prevalence and management of inherited and acquired forms. *Hamostaseologie*. 2014;34(2):160-166.

13. Lawrie AS, Green L, Mackie IJ, Liesner R, Machin SJ, Peyvandi F. Factor XIII--an under diagnosed deficiency--are we using the right assays? *J Thromb Haemost*. 2010;8(11):2478-2482.

14. Undas A, Stepien E, Rudzinski P, Sadowski J. Architecture of a pulmonary thrombus removed during embolectomy in a patient with acute pulmonary embolism. *J Thorac Cardiovasc Surg*. 2010;140(3):e40-41.

15. Bdeir K, Murciano JC, Tomaszewski J, et al. Urokinase mediates fibrinolysis in the pulmonary microvasculature. *Blood*. 2000;96(5):1820-1826.

16. Murciano JC, Harshaw D, Neschis DG, et al. Platelets inhibit the lysis of pulmonary microemboli. *Am J Physiol Lung Cell Mol Physiol*. 2002;282(3):L529-539.

17. Butte AN, Houng AK, Jang IK, Reed GL. Alpha 2-antiplasmin causes thrombi to resist fibrinolysis induced by tissue plasminogen activator in experimental pulmonary embolism. *Circulation*. 1997;95(7):1886-1891.

18. Carmeliet P, Stassen JM, Schoonjans L, et al. Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. *J Clin Invest*. 1993;92(6):2756-2760.

19. Kirchhof K, Welzel T, Zoubaa S, et al. New method of embolus preparation for standardized embolic stroke in rabbits. *Stroke*. 2002;33(9):2329-2333.

20. Schultz J, Andersen A, Gade IL, Ringgaard S, Kjaergaard B, Nielsen-Kudsk JE. A porcine in-vivo model of acute pulmonary embolism. *Pulm Circ*. 2018;8(1):2045893217738217.

21. Smyth SS, Reis ED, Vaananen H, Zhang W, Coller BS. Variable protection of beta 3-integrin--deficient mice from thrombosis initiated by different mechanisms. *Blood*. 2001;98(4):1055-1062.

22. Nordoey A, Chandler AB. Platelet thrombosis induced by adenosine diphosphate in the rat. *Scand J Haematol*. 1964;1:16-25.

23. Konstantinides S, Schafer K, Neels JG, Dellas C, Loskutoff DJ. Inhibition of endogenous leptin protects mice from arterial and venous thrombosis. *Arterioscler Thromb Vasc Biol*. 2004;24(11):2196-2201.

24. Beviglia L, Poggi A, Rossi C, et al. Mouse antithrombotic assay. Inhibition of platelet thromboembolism by disintegrins. *Thromb Res*. 1993;71(4):301-315.

25. DiMinno G, Silver MJ. Mouse antithrombotic assay: a simple method for the evaluation of antithrombotic agents in vivo. Potentiation of antithrombotic activity by ethyl alcohol. *J Pharmacol Exp Ther*. 1983;225(1):57-60.

26. Gresele P, Momi S, Berrettini M, et al. Activated human protein C prevents thrombin-induced thromboembolism in mice. Evidence that activated protein c reduces intravascular

fibrin accumulation through the inhibition of additional thrombin generation. *J Clin Invest*. 1998;101(3):667-676.

27. Kumada T, Dittman WA, Majerus PW. A role for thrombomodulin in the pathogenesis of thrombin-induced thromboembolism in mice. *Blood*. 1988;71(3):728-733.

28. Paul W, Gresele P, Momi S, Bianchi G, Page CP. The effect of defibrotide on thromboembolism in the pulmonary vasculature of mice and rabbits and in the cerebral vasculature of rabbits. *Br J Pharmacol*. 1993;110(4):1565-1571.

29. Barbash IM, Schenke WH, Halabi M, et al. Experimental model of large pulmonary embolism employing controlled release of subacute caval thrombus in swine. *J Vasc Interv Radiol*. 2011;22(10):1471-1477.

30. Ellery PE, Maroney SA, Cooley BC, et al. A balance between TFPI and thrombin-mediated platelet activation is required for murine embryonic development. *Blood*. 2015;125(26):4078-4084.

31. Leon C, Freund M, Ravanat C, Baurand A, Cazenave JP, Gachet C. Key role of the P2Y(1) receptor in tissue factor-induced thrombin-dependent acute thromboembolism: studies in P2Y(1)-knockout mice and mice treated with a P2Y(1) antagonist. *Circulation*. 2001;103(5):718-723.

32. Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. *Blood*. 2002;100(9):3240-3244.

33. Souri M, Koseki-Kuno S, Takeda N, Degen JL, Ichinose A. Administration of factor XIII B subunit increased plasma factor XIII A subunit levels in factor XIII B subunit knock-out mice. *Int J Hematol*. 2008;87(1):60-68.

34. Cooley BC. In vivo fluorescence imaging of large-vessel thrombosis in mice. *Arterioscler Thromb Vasc Biol*. 2011;31(6):1351-1356.

35. Aleman MM, Walton BL, Byrnes JR, et al. Elevated prothrombin promotes venous, but not arterial, thrombosis in mice. *Arterioscler Thromb Vasc Biol*. 2013;33(8):1829-1836.

36. Aleman MM, Walton BL, Byrnes JR, Wolberg AS. Fibrinogen and red blood cells in venous thrombosis. *Thromb Res*. 2014;133 Suppl 1:S38-40.

37. Grover SP, Evans CE, Patel AS, Modarai B, Saha P, Smith A. Assessment of Venous Thrombosis in Animal Models. *Arterioscler Thromb Vasc Biol*. 2016;36(2):245-252.

38. Sevitt S. The structure and growth of valve-pocket thrombi in femoral veins. *J Clin Pathol*. 1974;27(7):517-528.

39. Buyue Y, Whinna HC, Sheehan JP. The heparin-binding exosite of factor IXa is a critical regulator of plasma thrombin generation and venous thrombosis. *Blood*. 2008;112(8):3234-3241.

40. Chauhan AK, Kisucka J, Lamb CB, Bergmeier W, Wagner DD. von Willebrand factor and factor VIII are independently required to form stable occlusive thrombi in injured veins. *Blood*. 2007;109(6):2424-2429.

41. Cooley BC, Szema L, Chen CY, Schwab JP, Schmeling G. A murine model of deep vein thrombosis: characterization and validation in transgenic mice. *Thromb Haemost*. 2005;94(3):498-503.

42. Zhou J, May L, Liao P, Gross PL, Weitz JI. Inferior vena cava ligation rapidly induces tissue factor expression and venous thrombosis in rats. *Arterioscler Thromb Vasc Biol*. 2009;29(6):863-869.

43. Kattula S, Byrnes JR, Martin SM, et al. Factor XIII in plasma, but not in platelets, mediates red blood cell retention in clots and venous thrombus size in mice. *Blood Adv*. 2018;2(1):25-35.

44. Undas A, Zawilska K, Ciesla-Dul M, et al. Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives. *Blood*. 2009;114(19):4272-4278.

45. Morris TA, Marsh JJ, Chiles PG, et al. High prevalence of dysfibrinogenemia among patients with chronic thromboembolic pulmonary hypertension. *Blood*. 2009;114(9):1929-1936.

46. Zabczyk M, Plens K, Wojtowicz W, Undas A. Prothrombotic Fibrin Clot Phenotype Is Associated With Recurrent Pulmonary Embolism After Discontinuation of Anticoagulant Therapy. *Arterioscler Thromb Vasc Biol*. 2017;37(2):365-373.

47. Marsh JJ, Chiles PG, Liang NC, Morris TA. Chronic thromboembolic pulmonary hypertension-associated dysfibrinogenemias exhibit disorganized fibrin structure. *Thromb Res*. 2013;132(6):729-734.

48. Morris TA, Marsh JJ, Chiles PG, Auger WR, Fedullo PF, Woods VL, Jr. Fibrin derived from patients with chronic thromboembolic pulmonary hypertension is resistant to lysis. *Am J Respir Crit Care Med*. 2006;173(11):1270-1275.

49. Morris TA, Marsh JJ, Chiles PG, et al. Abnormally sialylated fibrinogen gamma-chains in a patient with chronic thromboembolic pulmonary hypertension. *Thromb Res*. 2007;119(2):257-259.

50. Diaz JA, Saha P, Cooley B, et al. Choosing a Mouse Model of Venous Thrombosis. *Arterioscler Thromb Vasc Biol*. 2019;39(3):311-318.

51. Schwartz T, Hingorani A, Ascher E, et al. Pulmonary embolism without deep venous thrombosis. *Ann Vasc Surg*. 2012;26(7):973-976.

52. Lindblad B, Eriksson A, Bergqvist D. Autopsy-verified pulmonary embolism in a surgical department: analysis of the period from 1951 to 1988. *Br J Surg*. 1991;78(7):849-852.

53. Diebold J, Lohrs U. Venous thrombosis and pulmonary embolism. A study of 5039 autopsies. *Pathol Res Pract*. 1991;187(2-3):260-266.

54. Girard P, Musset D, Parent F, Maitre S, Phlippoteau C, Simonneau G. High prevalence of detectable deep venous thrombosis in patients with acute pulmonary embolism. *Chest*. 1999;116(4):903-908.

55. Karimi M, Peyvandi F, Naderi M, Shapiro A. Factor XIII deficiency diagnosis: Challenges and tools. *Int J Lab Hematol*. 2018;40(1):3-11.

56. Idell S, Zwieb C, Boggaram J, Holiday D, Johnson AR, Raghu G. Mechanisms of fibrin formation and lysis by human lung fibroblasts: influence of TGF-beta and TNF-alpha. *Am J Physiol*. 1992;263(4 Pt 1):L487-494.

57. Bovill EG, van der Vliet A. Venous valvular stasis-associated hypoxia and thrombosis: what is the link? *Annu Rev Physiol*. 2011;73:527-545.

58. Dallabrida SM, Falls LA, Farrell DH. Factor XIIIa supports microvascular endothelial cell adhesion and inhibits capillary tube formation in fibrin. *Blood*. 2000;95(8):2586-2592.

59. Belkin AM, Tsurupa G, Zemskov E, Veklich Y, Weisel JW, Medved L. Transglutaminase-mediated oligomerization of the fibrin(ogen) alphaC domains promotes integrin-dependent cell adhesion and signaling. *Blood*. 2005;105(9):3561-3568.

60. Bereczky Z, Muszbek L. Factor XIII and venous thromboembolism. *Semin Thromb Hemost*. 2011;37(3):305-314.

61. Gosk-Bierska I, McBane RD, Wu Y, et al. Platelet factor XIII gene expression and embolic propensity in atrial fibrillation. *Thromb Haemost*. 2011;106(1):75-82.

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

5.1 Summary and future directions

Traditional anticoagulants, such as warfarin, reduce the risk of thrombotic events but take several days to be effective, have multiple target interactions, high interpatient variability, and come at the cost of increased bleeding risk in patients.[1] Newer anticoagulants address some of these concerns with faster onset of action, increased target specificity and fewer interactions, and lower interpatient variability.[1] However, these new drugs are still associated with a bleeding risk, as they target coagulation proteins upstream of fibrin formation. The studies presented in this dissertation investigate the relationship between downstream components of the clotting cascade: FXIII, fibrinogen, and RBCs. These findings expand our understanding on the role of FXIII in clot formation, composition, and stability, and identify partial $FXIII_{plasma}$ inhibition as a novel therapeutic strategy to reduce VTE without increasing risk of bleeding and thrombus embolization.

In Chapter 2, we determined the role of $FXIII_{plasma}$ and $FXIII_{plt}$ in clot contraction, composition, and size.[2] This study extended our prior discovery that FXIII promotes RBC retention in clots.[3] Here, in reconstituted whole blood clot contraction assays, the absence of $FXIII_{plasma}$, but not $FXIII_{plt}$, decreased RBC retention, resulting in smaller clots. We observed $FXIII_{plasma}$, but not $FXIII_{plt}$, formed fibrin α-chain crosslinks to promote RBC retention and mediates clot weight. In this study we also explored the effect FXIII level in in vivo models of thrombosis and hemostasis. We determined FXIII deficiency results in a dose-dependent decrease in thrombus weight without altering thrombin generation or platelet contraction. In hemostatic challenges,

F13a$^{-/-}$, but not *F13a*$^{+/-}$, mice had prolonged tail bleeding times, whereas no genotype-dependent difference was observed following saphenous vein puncture. Collectively, these observations suggest imposition of partial $FXIII_{plasma}$ deficiency may reduce VT without simultaneously increasing bleeding risk.

These findings have important implications. Foremost, knowing that $FXIII_{plasma}$, but not $FXIII_{plt}$, mediated fibrin crosslinking, promoted RBC retention, and increased thrombus weight, suggests that potential FXIII inhibitors do not need to get intercellular to $FXIII_{plt}$, simplifying drug design. Furthermore, targeting only the plasma compartment, potentially preserves the hemostatic function of the $FXIII_{plt}$ compartment. These findings also further advance our understanding of the mechanism by which $FXIII_{plasma}$ mediates RBC retention. Only $FXIII_{plasma}$ can fulfill the immediate temporal requirement to impact RBC retention in clots undergoing contraction because it is bound to fibrinogen and activated concurrently as the fibrin network is forming. In contrast, $FXIII_{plt}$ activation and externalization occurs more slowly, likely after fibrin formation, and platelet-mediated clot contraction. Future direction of this research is to determine a clinically relevant titration range of $FXIII_{plasma}$ inhibition that reduces thrombus size without impairing hemostasis. To address this question, $FXIII_{plasma}$ level can be titrated in *F13a*$^{-/-}$ mice and subjected to in vivo models of hemostasis.

In Chapter 3, we determine the effect of accelerated FXIII activation on whole blood clot composition and size. This study is an extension of our previous findings that delayed/partial FXIII activation decreased RBC retention and clot weight.[2,3] A naturally-occurring FXIII polymorphism, Val34Leu, present in ~25% of European Caucasians, results in ~2.5-fold accelerated FXIII activation[4] and protection against VT[5-7]. This effect is hypothesized to result from fibrinogen concentration-dependent clot structure changes to fibrin network density.[8]

Notably, we have previously observed both FXIII activity and fibrin network density are positively associated with RBC retention in contracted clots and consequently, clot weight.[3,9] We conducted experiments with individual donor plasmas that provided a relevant context to the human population, and experiments with purified FXIII that enabled us to control fibrinogen concentration. We observed that at high fibrinogen concentration homozygous presence of the Leu allele resulted in decreased RBC retention and smaller whole blood clots. The Val34Leu polymorphism may be protective against VT by mitigating the effect of elevated fibrinogen levels considered prothrombotic.

This study adds an interesting new finding to a long standing paradoxical observation. Inconsistencies in evaluating the association of the Val34Leu polymorphism with VT were prevalent in the literature prior to understanding the complex gene-plasma environment interactions that modulate the effect of Val34Leu polymorphism on thrombosis. Factoring in fibrinogen concentration as gene modifier addresses some discrepancies between population studies but more refined mechanistic studies are needed to interpret the contribution of the FXIII Val34Leu polymorphism to VT risk. Our study evaluated the Val34Leu polymorphism in a physiologically-relevant whole blood system while modulating fibrinogen concentration. Our findings suggest that Val34Leu status in conjunction with fibrinogen level should be considered in clinical risk predictors to identify individuals at higher risk of VT. Future direction of this research is to translate these findings in vivo models of VT. Interestingly, in the ferric chloride model of femoral vein thrombosis, compared to human recombinant Val variant-infused *F13a*$^{-/-}$ mice, the Leu variant-infused *F13a*$^{-/-}$ mice showed no difference in thrombus size.[10] However, the mice were not studied under hyperfibrinogenmia conditions; thus it may not be surprising no difference in thrombus size was found. Furthermore, the ferric chloride model induces rapid

formation of platelet-rich thrombi and does not recapitulate RBC-rich venous thrombi. The need to study the Val34Leu polymorphism in a physiologically-relevant in vivo model is necessary.

In Chapter 4, we developed a VTE mouse model that permits embolization of stasis induced fibrin- and RBC-rich thrombi to determine the contribution of FXIII to PE risk. Our previous studies indicate reducing FXIII activity/levels decreases thrombus size, suggesting that FXIII inhibition is a potential strategy to reduce VT.[2,3] Since FXIII also plays an essential role in clot stabilization to prevent premature lysis of a clot[11,12], this study is an important follow-up looking at downstream effects of FXIII inhibition. However, elucidating FXIII's role in PE is limited by a lack of models that recapitulate human PE characteristics. We characterized and compared the composition of human PEs, WT mouse venous thrombi (electrolytic, ferric chloride, inferior and IVC stasis), and mouse PEs generated by intravenous thrombin infusion or IVC stasis with subsequent ligature removal to permit embolization. Although FXIII level did not affect PE incidence in the thrombin infusion model, it produced microthrombi histologically-dissimilar to human PEs. The ligature removal model in WT mice produced fibrin- and RBC-rich PEs similar to human PEs and complete FXIII deficiency increased PE incidence, but partial deficiency did not. These findings suggest partial FXIII reduction may decrease VT burden without increasing PE.

The implication of these findings are multifaceted. First, our findings reveal substantial differences in the composition of thrombi generated from different mouse VT models and therein for fundamental differences in the pathophysiologic processes of thrombogenesis between models. This warrants caution in choosing the appropriate in vivo model for every research question. Second, our ligature removal model better recapitulates human PE pathophysiology and offers advantages over other commonly used PE models that include 1) formation of RBC-

and fibrin-rich venous thrombi via blood stasis and 2) control over the timing of thrombus formation and embolic events. We anticipate that development of this model will facilitate continued investigations into the pathogenic mechanisms that lead to PE. Third, the findings suggest FXIII level can be titrated to decrease VT burden without increasing PE. This finding in conjunction with the findings from Chapter 2, supports the pursuit of partial FXIII inhibition as a novel therapeutic strategy to reduce VTE. Future directions of this study is to expand our understanding of the relationship between FXIII source (plasma vs platelet) and PE. A study found that mRNA for $FXIII_{plt}$ is reduced in atrial fibrillation patients with thrombus embolization[13], suggesting $FXIII_{plasma}$ and $FXIII_{plt}$ may contribute differently to thrombus stability and PE risk. Using methods developed in Chapter 2 to make $FXIII_{plasma}$ sufficient and $FXIII_{plt}$ deficient mice and the ligature removal model developed in Chapter 4, we can delineate the relative contributions of $FXIII_{plasma}$ and $FXIII_{plt}$ in PE risk.

In conclusion, this dissertation further expands our understanding of the relationship between FXIII, fibrinogen, and RBCs, and efforts to therapeutically target FXIII to reduce VTE.

REFERENCES

1. Wolberg AS, Rosendaal FR, Weitz JI, et al. Venous thrombosis. *Nat Rev Dis Primers*. 2015;1:15006.

2. Kattula S, Byrnes JR, Martin SM, et al. Factor XIII in plasma, but not in platelets, mediates red blood cell retention in clots and venous thrombus size in mice. *Blood Adv*. 2018;2(1):25-35.

3. Aleman MM, Byrnes JR, Wang JG, et al. Factor XIII activity mediates red blood cell retention in venous thrombi. *J Clin Invest*. 2014;124(8):3590-3600.

4. Muszbek L. Deficiency causing mutations and common polymorphisms in the factor XIII-A gene. *Thromb Haemost*. 2000;84(4):524-527.

5. Catto AJ, Kohler HP, Coore J, Mansfield MW, Stickland MH, Grant PJ. Association of a common polymorphism in the factor XIII gene with venous thrombosis. *Blood*. 1999;93(3):906-908.

6. Franco RF, Reitsma PH, Lourenco D, et al. Factor XIII Val34Leu is a genetic factor involved in the etiology of venous thrombosis. *Thromb Haemost*. 1999;81(5):676-679.e

7. Wells PS, Anderson JL, Scarvelis DK, Doucette SP, Gagnon F. Factor XIII Val34Leu variant is protective against venous thromboembolism: a HuGE review and meta-analysis. *Am J Epidemiol*. 2006;164(2):101-109.

8. Lim BC, Ariens RA, Carter AM, Weisel JW, Grant PJ. Genetic regulation of fibrin structure and function: complex gene-environment interactions may modulate vascular risk. *Lancet*. 2003;361(9367):1424-1431.

9. Byrnes JR, Duval C, Wang Y, et al. Factor XIIIa-dependent retention of red blood cells in clots is mediated by fibrin alpha-chain crosslinking. *Blood*. 2015;126(16):1940-1948.

10. Duval C, Ali M, Chaudhry WW, Ridger VC, Ariens RA, Philippou H. Factor XIII A-Subunit V34L Variant Affects Thrombus Cross-Linking in a Murine Model of Thrombosis. *Arterioscler Thromb Vasc Biol*. 2016;36(2):308-316.

11. Mitchell JL, Lionikiene AS, Fraser SR, Whyte CS, Booth NA, Mutch NJ. Functional factor XIII-A is exposed on the stimulated platelet surface. *Blood*. 2014;124(26):3982-3990.

12. Fraser SR, Booth NA, Mutch NJ. The antifibrinolytic function of factor XIII is exclusively expressed through alpha(2)-antiplasmin cross-linking. *Blood*. 2011;117(23):6371-6374.

13. Gosk-Bierska I, McBane RD, Wu Y, et al. Platelet factor XIII gene expression and embolic propensity in atrial fibrillation. *Thromb Haemost*. 2011;106(1):75-82.