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Title: The factor XIII-A Val34Leu polymorphism decreases whole blood clot mass at high fibrinogen concentrations

Running Title: FXIII-A Val34Leu decreases clot mass at high fibrinogen concentrations

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ABSTRACT

Background: Factor XIII (FXIII) promotes fibrin crosslinking and red blood cell (RBC) retention in clots. The FXIII-A polymorphism, Val34Leu, is associated with protection against venous thrombosis. This effect is hypothesized to result from fibrinogen concentration-dependent changes in fibrin structure. Effects of the FXIII-A Val34Leu polymorphism in whole blood clots have not been investigated.

Aim: Characterize effects of FXIII-A Val34Leu polymorphism and fibrinogen on whole blood clots.

Methods: We isolated platelet-poor plasmas from human donors ($FXIII^{Val/Val}$, $FXIII^{Val/Leu}$, $FXIII^{Leu/Leu}$), reconstituted plasmas with platelets and RBCs, and triggered clotting. We assessed contributions of gender, age, clotting times, thrombin generation, FXIII activity, FXIII-A Val34Leu polymorphism, and fibrinogen to clot mass. We also reconstituted FXIII-depleted plasma with platelets, RBCs, and purified $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$, varied fibrinogen, and characterized effects on clot mass.

Results: Clot mass was associated with age, fibrinogen, prothrombin time, and thrombin generation. Clots reconstituted with plasmas from individuals with FXIII-$A^{Val/Val}$ and FXIII-$A^{Val/Leu}$ did not differ in mass from clots with FXIII-$A^{Leu/Leu}$. However, clots containing a 34Val allele demonstrated a fibrinogen concentration-dependent increase in mass, whereas clots with homozygous 34Leu did not. In plasmas with high fibrinogen, mass was higher for clots with 34Val alleles compared to clots with homozygous 34Leu. In clots reconstituted with purified FXIII, increasing fibrinogen enhanced clot mass in the presence of 34Val, but decreased mass in the presence of 34Leu.

Conclusions: FXIII 34Leu mitigates the effect of elevated fibrinogen on whole blood clot mass. The Val34Leu polymorphism may protect against venous thrombosis by reducing clot mass.

ESSENTIALS

- Factor XIII (FXIII) promotes fibrin crosslinking and red blood cell retention in clots
- FXIII-A Val34Leu polymorphism offers modest but significant protection against venous thrombosis
- Fibrinogen increases the mass of clots containing 34Val alleles, but not homozygous 34Leu
- The FXIII-A Val34Leu polymorphism may protect against venous thrombosis by reducing clot mass

KEY WORDS

Thrombosis, factor XIII, transglutaminase, fibrinogen, erythrocyte

INTRODUCTION

Factor XIII (FXIII) is a protransglutaminase present in cells and plasma. Plasma FXIII is a non-covalent heterotetramer (FXIII-A_2B_2) that circulates bound to fibrinogen [1]. During coagulation, FXIII is activated by thrombin-mediated cleavage and release of 37-amino acid (4-kDa) activation peptides from the N-termini of the catalytic FXIII-A_2 subunits, followed by calcium-mediated dissociation of the carrier 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] and between fibrin and other plasma proteins [4-6]. These crosslinks enhance clot mechanical and biochemical stability, respectively. FXIII deficiency is associated with poor wound healing, miscarriage, and intracranial hemorrhage [7].

The common gene variant in FXIII-A encoding a valine (Val) to leucine (Leu) substitution at codon 34 (Val34Leu) is present in ~25% of European Caucasians [8]. Findings on the role of this polymorphism in thrombosis risk have been inconsistent; however, both independent studies and meta-analyses suggest the 34Leu variant offers modest but significant protection against venous thrombosis [9-14]. Discovery that the effects of the Val34Leu polymorphism are mediated by gene-environment interactions between FXIII-A genotype and plasma fibrinogen concentration has resolved some of the discord [15]. For example, in the Leiden Thrombophilia Study, although Leu homozygosity showed weak protection against venous thrombosis in men [16], reanalysis adjusting for fibrinogen concentration strengthened the association for both men and women, especially in individuals older than 45 years [17].

The FXIII-A Val34Leu polymorphism is located 3 amino acids before the thrombin cleavage site and results in ~2.5-fold accelerated FXIII activation [18, 19] and accelerated fibrin crosslinking [20, 21]. Effects of the Val34Leu polymorphism on clot quality are hypothesized to manifest via fibrinogen-dependent changes in clot structure. Briefly, the normal range of fibrinogen in plasma is 1.5-4 g/L. Fibrinogen increases with age [22] and can exceed 7 g/L in individuals with congenital hyperfibrinogenemia or acquired hyperfibrinogenemia secondary to inflammatory disease or pregnancy. In plasmas with low/normal fibrinogen concentration, the 34Leu variant produces clots with thin fibrin fibers and low permeability. However, in plasmas with higher fibrinogen, it produces

thicker fibers in clots that have increased permeability and susceptibility to fibrinolysis [15]. Since increased fibrinogen is associated with increased risk of cardiovascular disease [23], these observations suggest a mechanism whereby the FXIII-A 34Leu polymorphism mitigates effects of hyperfibrinogenemia on clot quality and reduces thrombotic risk.

We recently showed that FXIIIa activity promotes red blood cell (RBC) retention during clot contraction and is a major determinant of venous thrombus composition and mass [24-26]. Mice with heterozygous deficiency in FXIII-A (*F13a1*$^{+/-}$) have delayed FXIII activation, decreased RBC retention in contracted whole blood clots, and reduced thrombus mass in vitro and in vivo [24, 25]. Mice with normal levels of FXIII but delayed FXIII activation secondary to decreased FXIII binding to fibrinogen (*Fib*$\gamma^{390–396A}$) also show delayed FXIII activation and reduced thrombus mass [25]. In whole blood from persons with hemophilia, co-treatment with hemostatic agents and supraphysiologic concentrations of FXIII accelerate FXIII activation and increase clot mass ex vivo [27]. By extension, one might speculate that individuals with the 34Leu variant and accelerated FXIII activation would produce larger clots carrying increased venous thrombosis risk. However, the paradoxical association of the 34Leu variant with decreased venous thrombosis risk [11-14] warrants an explicit analysis of the interaction between this polymorphism and fibrinogen concentration during whole blood clot formation. Determining the impact of the 34Leu variant on clot characteristics is essential for understanding the clinical significance of the FXIII-A Val34Leu polymorphism in thrombosis risk.

Herein we used independent but complementary methods to determine the relationships between the FXIII-A Val34Leu polymorphism, fibrinogen concentration, and whole blood clot formation. Collectively, our data suggest the 34Leu variant mitigates the effect of hyperfibrinogenemia on whole blood clot mass.

MATERIALS and 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). 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. Approval for the use of human subjects was obtained from the University of Debrecen Ethics Committee (number: DE RKEB/IKEB: 3189-2010) and University of North Carolina Institutional Review Board. All participants provided signed informed consent in accordance with the Declaration of Helsinki. Donors had no known bleeding disorder, liver or kidney disease, cancer, were not pregnant, had no history of surgery or thrombotic event (including acute myocardial infarction or stroke) within the past 3 months, and were not on antiplatelet or anticoagulant therapy. Whole blood was obtained by venipuncture and drawn into tubes containing 0.105 M citrate (Becton Dickinson, Franklin Lakes, NJ, 10% v/v, final). Blood samples were processed immediately by centrifugation twice at 1220x*g* (15 minutes, room temperature) to obtain platelet-poor plasma. Hemostasis screening tests (prothrombin time and activated partial thromboplastin time) were measured immediately from the plasma samples. Remaining plasma was stored at -70°C until further analysis. DNA isolation was performed from buffy coats of blood

samples by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The FXIII-A Val34Leu (c.103G>T; rs5985) polymorphism was identified by real-time PCR [28] 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). Normal, pooled plasma was prepared by sequential centrifugation as described [29] from a pool of 32 healthy donors with normal partial thromboplastin times; the fibrinogen concentration in the normal pool was 3.2 mg/mL (Clauss assay).

FXIII zymogen preparation. Purified FXIII-A_2B_2 was isolated as described [30] from pooled plasma from individuals possessing only 34Val or only 34Leu FXIII-A alleles.

Western blotting. FXIII antigen and activity were analyzed in Peak 1 (FXIII-free) fibrinogen preparations by immunoblot detection of FXIII-A subunit and fibrin crosslinking. Fibrinogen preparations were clotted with thrombin (1 U/mL) and EDTA (10 mM, final) or $CaCl_2$ (10 mM, final) for 2 hours. 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. Whole blood was centrifuged (150x*g*, 20 minutes). Platelet-rich plasma from a donor carrying the $FXIII^{Val/Val}$ genotype was isolated and treated with prostaglandin-I_2 (50 ng/mL, final) and then centrifuged to obtain platelet-poor plasma (700x*g*, 10 minutes) or platelets (400x*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). RBCs were isolated from an O-negative donor; the RBC fraction was isolated, washed with citrated glycine saline buffer, and packed three times by centrifugation (400x*g*, 5 minutes). Platelets and RBCs were prepared and used on the day of the experiment.

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 thawed platelet-poor plasma from donors with $FXIII^{Val/Val}$, $FXIII^{Val/Leu}$, or $FXIII^{Leu/Leu}$. Clotting was triggered in siliconized wells with TF and $CaCl_2$ (1 pM and 10 mM, final, respectively) at 37°C. After 2 hours, contracted clots were removed from the wells, dabbed on a Kimwipe to remove the serum, and weighed. These experiments were performed on three separate days, and clots from individual plasmas were normalized to the average mass of control clots made with normal pooled plasma reconstituted with platelets and RBCs on each day (36.8±2.5 mg [N=3 clots], 35.4±0.6 mg [N=2 clots], and 30.0±0.2 mg [N=2 clots], respectively).

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 thawed FXIII-deficient plasma with purified $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$ zymogen (21 μg/mL, final) and FXIII-free fibrinogen (to achieve the final concentrations indicated). Clotting was triggered in siliconized wells with TF and $CaCl_2$ (1 pM and 10 mM, final, respectively) at 37°C. After 2 hours, contracted clots were removed from the wells, dabbed on a Kimwipe to remove the serum, and weighed.

Hemostasis assays. FXIII activity was measured according to Karpati et al [31]. Reference ranges were determined according to guidelines of the Clinical and Laboratory Standards Institute. Fibrinogen concentration was determined by the Clauss method [32] 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 are the result of mixed samples of 25 healthy individuals with no hemostasis disorders, measured with the same reagent and coagulometer.

Thrombin generation. Thrombin generation was measured by calibrated automated thrombography, as described [29]. Briefly, thrombin generation was triggered in duplicate platelet-poor plasma samples with TF, phospholipids, and $CaCl_2$ (1 pM, 4 μM, and 16.7 mM, final, respectively). Fluorescence was detected by a Fluoroskan Ascent® fluorometer (Thermo Fischer Scientific,

Waltham, MA). Thrombograms were analysed with Thrombinoscope software v5.0 (Thrombinoscope BV, Maastricht, The Netherlands).

Statistical methods. Normality of data was evaluated by Shapiro-Wilk test. Descriptive statistics (mean, standard deviation or median, interquartile range) were calculated for continuous variables. Differences between categorical variables were assessed by Fisher's exact or χ^2 test. In all two-group analyses, unpaired Student's *t* test, or in case of non-parametric data Mann-Whitney U test, was used. ANOVA using Bonferroni post-hoc test or Kruskal-Wallis test using Dunn's post-hoc test were applied for multiple comparisons. Pearson's or Spearman's correlation coefficient was used to determine the strength of correlation between parameters of normalized clot mass and other continuous variables. To test for differences between adjusted means, univariate analysis incorporating covariate testing (one-way ANCOVA) was performed. $P<0.05$ was considered significant. Statistical analysis was performed using the Statistical Package for Social Sciences 22.0 (SPSS, Chicago, IL), GraphPad Prism 5.0 (GraphPad Prism Inc., La Jolla, CA), and Stata 12.0 software (Stata Corp LP, TX).

RESULTS

Effects of the FXIII-A Val34Leu polymorphism on clinical presentation and clot properties has been challenging to characterize because of individual variation in other potential modifiers, including overall procoagulant activity, fibrinogen concentration, and co-existing polymorphisms [10]. We therefore approached this question using independent but complementary methods that combined the translational strengths of human samples with the experimental control offered by in vitro biochemical analyses.

Determinants of whole blood clot mass in a reconstituted model using individual plasmas. In the first series of experiments, we isolated platelet-poor plasma from 86 healthy individuals with homozygous or heterozygous 34Val or 34Leu alleles: $FXIII^{Val/Val}$ (N=40), $FXIII^{Val/Leu}$ (N=28), and $FXIII^{Leu/Leu}$ (N=18). Demographic and clinical characteristics of all donors and donors separated by genotype are shown in Table 1. Subjects with the three genotypes did not differ in gender, age, FXIII activity, APTT, PT, or thrombin generation lag time or time to peak. Subjects with the $FXIII^{Val/Val}$ genotype had lower fibrinogen than subjects with $FXIII^{Val/Leu}$ or $FXIII^{Leu/Leu}$ genotypes. Subjects with the $FXIII^{Val/Leu}$ genotype had increased thrombin peaks compared to individuals with $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$ genotypes. Endogenous thrombin potential (ETP) differed significantly between groups, although differences were small.

To identify relationships between general demographic and clinical characteristics and clot mass, we reconstituted the individual plasma samples with O-negative RBCs and washed platelets isolated from a $FXIII^{Val/Val}$ donor, and triggered clotting by addition of TF and $CaCl_2$ (Figure 1A). Following clot formation and contraction, we measured contracted clot mass. In aggregate, average normalized clot mass correlated significantly with age, fibrinogen, PT, and thrombin generation lag time, time to peak, and ETP, but did not correlate with total FXIII activity (Table 2).

Comparison of individual FXIII genotypes showed average normalized clot mass did not differ between groups (Figure 1B). Clot mass correlated strongly and significantly with fibrinogen in plasmas containing a 34Val allele ($FXIII^{Val/Val}$ [R=0.5496] and $FXIII^{Val/Leu}$ [R=0.6461], P=0.0002), whereas mass of clots with homozygous 34Leu did not change (R=-0.0298, P=0.9066) Table 2, Figure 1C). Clot mass also correlated with age, FXIII activity, PT, and thrombin generation lag time

and time to peak in clots with $FXIII^{Val/Val}$, but not in clots with $FXIII^{Val/Leu}$ (Table 2, Figure 1D-G). Clot mass did not correlate significantly with any of the measured demographic or clinical characteristic for clots with $FXIII^{Leu/Leu}$.

Interaction between FXIII-A Val34Leu and fibrinogen in a reconstituted model using individual plasmas. Given the modifying effects of fibrinogen concentration on FXIII-A genotype-dependent fibrin structure [15], we further interrogated specific relationships between fibrinogen and clot mass for each FXIII-A genotype. Clots formed from individual genotypes did not differ at low-to-normal fibrinogen concentrations. However, in plasmas with the highest fibrinogen (>3.5 g/L) the mass of clots formed from individuals carrying a 34Val allele was higher than those from individuals with homozygous 34Leu and these differences persisted even after adjusting for age, gender, and thrombin generation (Table 3).

Interaction between FXIII-A Val34Leu and fibrinogen in a reconstituted model using purified FXIII zymogen. To experimentally circumvent potential effects of age, thrombin generation, or other modifiers on clot mass, we then utilized a fully reconstituted system comprised of commercially-sourced FXIII-depleted plasma, washed O-negative RBCs, washed platelets ($FXIII^{Val/Val}$ donor), and FXIII-free fibrinogen (Supplemental Figure 1), in which we added either purified $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$ zymogen (Figure 2A, Supplemental Figure 2). We then triggered clotting by addition of TF and $CaCl_2$ and measured clot mass. Like that seen in experiments with individual plasmas, mass was similar for clots formed in the presence of lower fibrinogen (<4 g/L), and addition of fibrinogen induced a concentration-dependent increase in $FXIII^{Val/Val}$ clot mass (R^2=0.9536, P<0.005, Figure 2B). Interestingly, the biochemical control offered by this experimental design unveiled an inverse effect of fibrinogen on the mass of clots formed with $FXIII^{Leu/Leu}$, in which clot mass decreased with increasing fibrinogen (R^2=0.8416, P<0.03, Figure 2B). These surprising findings suggest an even higher specific impact of the FXIII-A Val34Leu polymorphism on clot structure than can be appreciated from analysis of heterogeneous plasma samples. Collectively, combined observations from these complimentary methods suggest the FXIII-A 34Leu allele mitigates prothrombotic effects of elevated fibrinogen on contracted whole blood clot mass.

DISCUSSION

Complex gene-environment interactions make univariable studies on humans or blood samples difficult to interpret. Early conflicting reports on the contribution of the FXIII-A Val34Leu polymorphism have been partly resolved by incorporation of co-modifiers into the analysis, revealing intriguing interactions with fibrinogen concentration as a determinant of fibrin structure. Although individuals with the 34Leu allele make clots with thinner fibrin fibers, effects of 34Leu are reversed in the presence of high fibrinogen [15]. These observations establish a rational, but complex, explanation for the protection of 34Leu against thrombosis by associating protective effects specifically with the high risk setting of elevated fibrinogen. Nonetheless, understanding the effects of the 34Leu allele on clot formation has remained challenging. A major advance of our study is the extension of observations from a reductionist setting in cell-free plasma to a more holistic model that includes blood cells, platelet-mediated contraction, and clot consolidation. Together with previous findings, our data suggest the 34Leu polymorphism affects not only fibrin network structure, but also the way in which fibrin modifies the composition and mass of whole blood clots.

Using a thrombosis model triggered by $FeCl_3$ injury to the femoral vein, Duval et al previously observed that the FXIII Val34Leu polymorphism accelerates fibrin crosslinking, but does not alter clot size [20]. $FeCl_3$ injures the endothelium in flowing blood and induces rapid formation (< 5 minutes) of a platelet-rich thrombus that remains unchanged in size over the 60-minute observation period [20]. In contrast, our in vitro clot contraction model recapitulates aspects of venous thrombosis in which stasis induces formation of RBC-rich thrombi that undergo subsequent clot contraction and consolidation. Indeed, we have observed differences in the contribution of FXIII to thrombi triggered by $FeCl_3$ under flow [33] and thrombi triggered by venous stasis [24, 25], suggesting different effects of the FXIII V34L polymorphism on clot size in these two studies stem from differences in the role of FXIII in these experimental models.

Although mechanism(s) mediating the effects of the Val34Leu polymorphism on FXIII(a) activation are unclear, biochemical and structural analyses suggest the leucine side chain forms a more energetically favorable interaction with thrombin, enhancing substrate specificity and facilitating FXIII activation [34]. Fibrin crosslinking has only minimal effects on fibrin network

density and pore size [26]. However, in plasmas with high fibrinogen, presence of the 34Leu allele produces thicker fibers in clots that have increased permeability [15], and we now show that these whole blood clots are smaller. Together with earlier studies [24-26], we speculate that changes in the timing of crosslinking affects the acquisition of viscoelastic properties of individual fibrin fibers within the clot, and disruption of this coordination compromises the events that stabilize fibrin, permitting loss of RBCs from the clot and the formation of smaller clots. It is interesting to note different effects of accelerated FXIII activation due to supraphysiologic FXIII [27] versus accelerated activation of physiologic levels of FXIII (present study); whereas supraphysiologic FXIII levels enhance crosslinking and increase clot size [27], accelerated activation of physiologic FXIII levels result in the production of smaller clots. These differences may reflect effects of altering the molecular ratio of FXIII:fibrin(ogen) and therefore the number of fibers that are crosslinked quickly in each setting. Studies that incorporate detailed analysis of crosslinking patterns induced during clot formation in concert with structural analysis of whole blood clots are needed to test this hypothesis.

Given the reproducible finding that 34Leu decreases the impact of elevated fibrinogen on clot mass, it is interesting to consider why this effect has been so difficult to detect in population studies. Other modifiers both within and external to the fibrinogen and FXIII loci likely alter the relative contribution of the Val34Leu polymorphism to clot formation, structure, and stability. For example, differential expression of the alternatively-spliced fibrinogen γ′ chain that binds and restricts thrombin activity also modifies the effect of elevated fibrinogen on clot formation [35-37]. In lieu of information on the relative expression of γA and γ′ fibrinogen in a given plasma, it would be difficult to predict the effect of increased fibrinogen *a priori* on clot formation. Fibrin(ogen) function may also be altered by post-translational modifications, including glycation and homocysteinylation in diabetes and other pathologies. Other polymorphisms in FXIII have also been implicated as modifiers of FXIII function, including the FXIII-B intron K c.1952+144 C>G polymorphism associated with lower FXIII activity and antigen, which appears to be protective against coronary artery disease only in the presence of both the FXIII-A 34Leu allele and elevated fibrinogen [38]. Thus, although our analysis resolved one aspect of these gene-environment interactions, complete understanding of how these mechanisms operate during thrombus formation in vivo will require further studies.

We previously showed that changes in fibrin crosslinking that reduce clot mass in vitro (observed in both human and mouse blood) decrease venous thrombus mass in mice [24-26]. We have also observed that elevated prothrombin, an established risk factor for venous thrombosis in humans, increases fibrin density and thrombus mass in mice [39]. Collectively, these observations suggest that mechanisms that enhance thrombus size promote thrombosis, whereas mechanisms that decrease thrombus size reduce venous thrombosis risk. It is unclear whether smaller thrombi would have reduced propensity to occlude blood flow, enhanced susceptibility to endogenous fibrinolytic mechanisms, or both. Further work is necessary to understand the implications of altered thrombus mass in venous thromboembolic risk in humans, as well how reduced thrombus RBC content caused by altered FXIII function affect thrombus formation and resolution.

In summary, we have shown that the FXIII-A 34Leu allele mitigates effects of elevated fibrinogen on the mass of contracted whole blood clots. The FXIII-A Val34Leu polymorphism may protect against venous thrombosis by decreasing clot RBC retention and consequently, reducing thrombus mass.

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Authorship: Contribution: S.K. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript; Z.B, N.K.T., and L.M. performed experiments and analyzed and interpreted data; A.S.W. designed the research, analyzed and interpreted the data, and wrote the manuscript. All authors have read and approved the final manuscript.

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TABLES

Table 1. Demographic and clinical characteristics for all plasma donors and donors separated by genotype.

						Comparison of Genotypes (*P*):		
	Reference	All (N=86)	FXIII$^{Val/Val}$ (N=40)	FXIII$^{Val/Leu}$ (N=28)	FXIII$^{Leu/Leu}$ (N=18)	*P*&	FXIII$^{Val/Val}$ vs FXIII$^{Val/Leu}$	FXIII$^{Val/Leu}$ vs FXIII$^{Leu/Leu}$	FXIII$^{Val/Val}$ vs FXIII$^{Leu/Leu}$
Clinical Characteristics									
Gender (female/male)	-	71/15	33/7	24/4	14/4	0.7868	-	-	-
Age (years)	-	26 (23.0-38.8)	25 (21.0-36.8)	28.5 (23.8-38.3)	28.0 (23.8-46.3)	0.4053	-	-	-
FXIII Activity (%)	69 - 143	114 ± 20	114 ± 18	115 ± 26	110 ± 15	0.7127	-	-	-
Fibrinogen (g/L)	1.5 - 4	3.3 (2.9-4.2)	3.0 (2.7-3.7)	4.0 (2.9-4.5)	3.2 (2.9-4.5)	0.0432	0.0401	-	0.0443
Clotting Tests									
APTT (seconds)	28.6 - 37.6	29.7 ± 2.9	29.7 ± 3.2	29.6 ± 2.6	29.8 ± 2.7	0.9546	-	-	-
PT (seconds)	8.2 - 12.2	8.2 (7.8-8.6)	8.3 (7.9-8.6)	7.9 (7.6-8.8)	8.2 (8.1-8.4)	0.3436	-	-	-
Thrombin Generation Parameters									
Lag time (minutes)	-	2.3 (2.0-2.7)	2.3 (2.0-2.7)	2.3 (2.0-2.8)	2.3 (2.0-2.3)	0.5985	-	-	-
Time to Peak (minutes)	-	4.7 (4.0-5.5)	4.7 (4.0-5.5)	4.7 (4.1-5.4)	4.8 (4.2-5.5)	0.9720	-	-	-
Peak Thrombin (nM)	-	318 ± 93	317 ± 80	345 ± 107	283 ± 83	0.0470	-	0.0420	-
ETP (nM*minute)	-	1393	1382	1498	1206	0.0002	0.0179	0.0001	0.0085

	(1211-1581)	(1223-1581)	(1368-1797)	(1148-1363)

APTT, activated partial thromboplastin time; PT, prothrombin time; ETP, endogenous thrombin potential. FXIII, factor XIII. Continuous data show means ± standard deviation or medians (interquartile range), as appropriate. [&]Differences between genotypes were analyzed by one-way ANOVA with Bonferroni post-hoc testing or Kruskal-Wallis test with Dunn's post-hoc testing.

Table 2. Characteristics associated with normalized clot mass.

	All (N=86)		$FXIII^{Val/Val}$ (N=40)		$FXIII^{Val/Leu}$ (N=28)		$FXIII^{Leu/Leu}$ (N=18)	
	R	***P***	**R**	***P***	**R**	***P***	**R**	***P***
Clinical Characteristics								
Age (years)	0.3602	0.0007	0.5244	0.0005	0.4113	0.0297	-0.1488	0.5557
FXIII Activity (%)	0.1126	0.3019	0.3506	0.0266	-0.1700	0.3872	0.2193	0.3819
Fibrinogen (g/L)	0.5267	<0.0001	0.5496	0.0002	0.6461	0.0002	-0.0298	0.9066
Clotting Tests								
APTT (seconds)	0.0573	0.6004	0.0286	0.8610	0.1230	0.5330	-0.1755	0.4862
PT (seconds)	0.2248	0.0374	0.3127	0.0495	0.1017	0.6065	0.0614	0.8088
Thrombin Generation Parameters								
Lag time (minutes)	0.3371	0.0015	0.4581	0.0030	0.2615	0.1790	-0.3436	0.1627
Time to Peak (minutes)	0.3036	0.0045	0.3159	0.0471	0.3595	0.0603	-0.1216	0.6306
Peak Thrombin (nM)	-0.0531	0.6272	-0.0297	0.8556	-0.1061	0.5911	0.0606	0.8113
ETP (nM*minute)	0.2495	0.0205	0.2597	0.1056	0.0252	0.8986	0.2486	0.3199

R, Spearman coefficients; APTT, activated partial thromboplastin time; PT, prothrombin time; ETP, endogenous thrombin potential

Table 3. Effects of fibrinogen and FXIII-A 34Val or 34Leu alleles on clot mass.

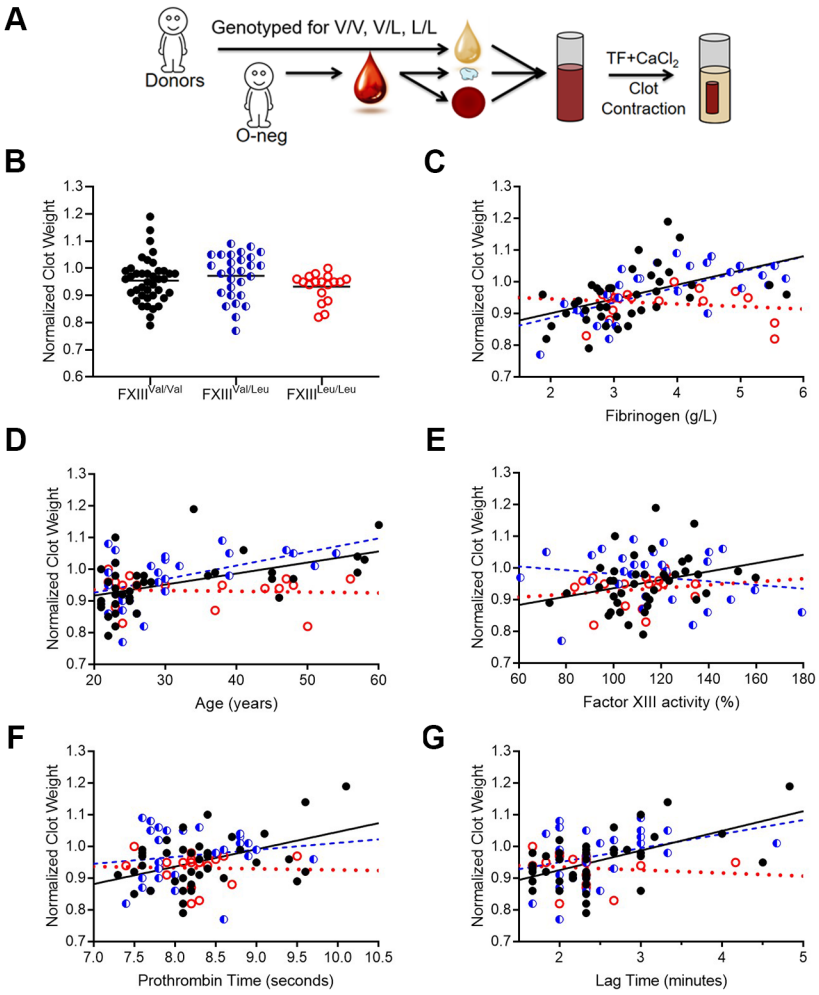
	$FXIII^{Val/Val}$			$FXIII^{Val/Leu}$			$FXIII^{Leu/Leu}$			
	fibrinogen < 3.5 g/L (N=28)	**fibrinogen > 3.5 g/L (N=12)**	***P***	**fibrinogen < 3.5 g/L (N=14)**	**fibrinogen > 3.5 g/L (N=14)**	***P***	**fibrinogen < 3.5 g/L (N=10)**	**fibrinogen > 3.5 g/L (N=8)**	***P***	***P*%**
Normalized clot weight	0.93±0.07	1.02±0.08	0.002*	0.92±0.08	1.02±0.05	0.001*	0.93±0.04	0.93±0.06	1.000*	0.0001
			0.002$			<0.0001$			0.622$	<0.0001
			0.010#			0.001#			0.851#	<0.0001

Data show mean ± standard deviation. Groups were compared using *ANOVA with Bonferroni post-hoc test, $ANCOVA with Bonferroni post-hoc test adjusted for age and gender, or #ANCOVA with Bonferroni post-hoc test adjusted for age, gender, and endogenous thrombin potential. Findings were similar when adjusted for age, gender, and lag time or time to peak. %ANOVA comparing all groups

FIGURE LEGENDS

Figure 1. In reconstituted whole blood with human donor plasma, the FXIII 34Leu variant mitigates the effect of high fibrinogen concentration on contracted clot mass. (A) Platelet-poor plasmas from 86 healthy 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 mass was normalized between assays performed on two different days. (B) Normalized clot mass. (C-F) Normalized clot mass were plotted as a function of (C) fibrinogen, (D) age, (E) FXIII activity, (F) prothrombin time, and (G) thrombin generation lag time. Lines indicate linear fits. Symbols are: $FXIII^{Val/Val}$ (black closed circles), $FXIII^{Val/Leu}$ (blue half-filled circles), and $FXIII^{Leu/Leu}$ (red open circles).

Figure 2. In reconstituted whole blood with purified FXIII, the FXIII 34Leu variant alters contracted clot mass in a fibrinogen concentration-dependent manner. (A) O-negative red blood cells, $FXIII^{Val/Val}$ platelets, and FXIII-depleted plasma reconstituted with either $FXIII^{Val/Val}$ or $FXIII^{Leu/Leu}$ zymogen, were combined. FXIII-free fibrinogen (peak 1) was added to reach the final concentrations indicated in panel B. Clotting was triggered with tissue factor and $CaCl_2$, and contracted clots were weighed at 2 hours. (B) Differences between $FXIII^{Val/Val}$ (black closed circles) and $FXIII^{Leu/Leu}$ (red open circles) clot mass for each fibrinogen concentration were compared by t-test. Data show mean ± standard error of the mean (N=3). *P<0.05



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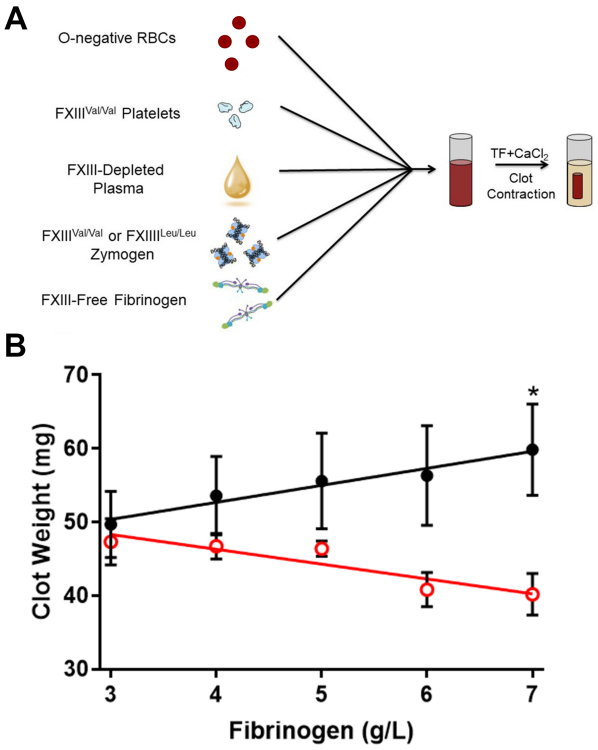

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