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BRIEF REPORT

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Factor XIII deficiency does not prevent $FeCl_3$-induced carotid artery thrombus formation in mice

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Abstract

Background: The compositions of venous (red blood cell–rich) and arterial (platelet-rich) thrombi are mediated by distinct pathophysiologic processes; however, fibrin is a major structural component of both. The transglutaminase factor XIII (FXIII) stabilizes fibrin against mechanical and biochemical disruption and promotes red blood cell retention in contracted venous thrombi. Previous studies have shown factor XIII (FXIII) inhibition decreases whole blood clot mass and therefore, may be a therapeutic target for reducing venous thrombosis. The role of FXIII in arterial thrombogenesis is less studied, and the particular contribution of platelet FXIII remains unresolved.

Objective: To determine whether FXIII reduction prevents experimental arterial thrombogenesis.

Methods: Using wild-type mice and mice with genetically imposed deficiency in FXIII, we measured thrombus formation and stability following ferric chloride–induced arterial thrombosis. We also determined the impact of FXIII on the mass of contracted platelet-rich plasma clots.

Results: Following vessel injury, $F13a^{+/+}$, $F13a^{+/-}$, and $F13a^{-/-}$ mice developed occlusive arterial thrombi. FXIII deficiency did not significantly reduce the incidence or prolong the time to occlusion. FXIII deficiency also did not alter the timing of reflow events or decrease platelet-rich clot mass.

Conclusions: FXIII does not significantly alter the underlying pathophysiology of experimental arterial thrombus formation.

KEYWORDS
factor XIII, ferric chloride, fibrinogen, platelets, thrombosis, transglutaminase

Essentials

- Factor XIII (FXIII) stabilizes fibrin and promotes red cell retention in venous thrombi.
- The role of FXIII in arterial thrombosis is less understood.
- FXIII deficiency does not prevent ferric chloride–induced carotid artery thrombus formation in mice.
- FXIII has nonoverlapping roles in venous and arterial thrombosis.

1 | INTRODUCTION

Venous and arterial thrombosis are diseases with distinct pathophysiologic processes. Venous thrombosis/thromboembolism is promoted by inappropriate expression of cell adhesion molecules and procoagulant activity on intact, dysfunctional endothelium in static blood, often in concert with plasma hypercoagulability (Virchow's triad), producing red blood cell–rich "red thrombi."[1] In contrast, arterial thrombosis, commonly manifested in association with atherosclerosis, develops after rupture of an atherosclerotic plaque that exposes subendothelium and procoagulant material (eg, collagen, tissue factor) to blood, which stimulates platelet activation and aggregation under high shear flow and produces platelet-rich "white thrombi."[1,2] Although the compositions of red blood cell–rich venous thrombi and platelet-rich arterial thrombi are strikingly different, thrombolytic therapy to dissolve fibrin is effective in treating both arterial and venous thrombosis,[3,4] indicating that fibrin is a shared central component of both thrombus compositions.

During coagulation, thrombin cleaves fibrinogen to produce fibrin monomers that polymerize into an insoluble network.[5] The fibrin network is subsequently stabilized by factor XIII (FXIII), a protransglutaminase present in plasma and cells such as platelets.[6] Activated FXIII (FXIIIa) protects clots against mechanical disruption by introducing covalent ε-N-(γ-glutamyl)-lysyl crosslinks between fibrin γ- and α-chains[7,8] and increases resistance to fibrinolysis by crosslinking fibrinolysis inhibitors such as α_2-antiplasmin,[9–11] thrombin activatable fibrinolysis inhibitor,[12] and plasminogen activator inhibitor-2[13,14] to fibrin. Complete FXIII deficiency is rare but is associated with bleeding, delayed wound healing, and miscarriage.[15] Recent studies have shown that FXIIIa-mediated fibrin crosslinking also promotes red blood cell retention in contracted venous thrombi, and therefore determines thrombus size.[16–18] Consequently, FXIIIa inhibition is a potential therapeutic approach for reducing venous thrombosis.

The role of FXIII in arterial thrombosis is less established. In experimental models of thrombosis in rabbit femoral[19] and dog coronary[20] arteries, administration of the nonpeptidyl active site-directed FXIIIa inhibitor L-722-151[21] prior to thrombus induction increases thrombus susceptibility to tissue plasminogen activator–driven thrombolysis. Surprisingly, however, in spite of observations that FXIII facilitates platelet recruitment and adhesion under flow,[22] FXIIIa inhibition with L-722-151 failed to independently prolong the time to vessel occlusion or reduce thrombus mass.[19] These observations suggested that FXIII inhibition may be useful for accelerating pharmacologic thrombolysis but would not independently alter the course of arterial thrombus formation.

FXIII is highly concentrated within the platelet cytoplasm and is externalized during platelet activation.[23,24] Thus, given the ability of FXIII to promote platelet adhesion and stabilize fibrin,[7–14,22] the finding that FXIII inhibition did not significantly reduce arterial thrombus formation [19] was unexpected. Interestingly, studies testing ectopic expression of factors IX or VIII in platelets for treating hemophilic patients with inhibitors indicate that proteins residing within platelets are relatively protected from inhibitory antibodies.[25,26] Accordingly, platelet FXIII(a) may be similarly protected from small-molecule inhibitors and therefore able to contribute to platelet-rich arterial thrombus formation even in the presence of FXIIIa antagonists in plasma. It was not determined in the earlier studies[19,20] whether the FXIIIa inhibitor was able to fully block platelet FXIIIa during thrombus formation. Thus, the potential impact of FXIIIa reduction on arterial thrombogenesis remains unknown. Herein, we used a murine model of genetically imposed FXIII deficiency to fully eliminate platelet FXIII activity and investigate the contribution of this FXIII compartment to arterial thrombus formation and stability.

2 | METHODS

2.1 | Mice

Procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice were backcrossed 6 generations on a C57BL/6J background.[27]

2.2 | Murine arterial thrombosis model

Mice (8-19-week old [14.6 ± 2.6 weeks, mean ± standard deviation] male and female littermates) were anesthetized with 1.5% isoflurane in oxygen (2 L/min flow rate). The right common carotid artery was exposed after midline cervical incision. A Doppler flow probe connected to a flowmeter (Model TS420; Transonic Systems, Ithaca, NY, USA) was used to monitor flow in the carotid artery and data were acquired via a PowerLab 4/35 (ADInstruments, Dunedin, New Zealand). After the carotid artery was prepared, 10% or 7.5% ferric chloride ($FeCl_3$) on 1 × 1-mm filter paper was placed on the artery for 2 minutes, then removed, and the vessel was washed 3 times with warm saline. We previously showed that these conditions yield a mixture of stable and unstable vessel occlusions, providing sensitivity to mechanisms that increase or decrease thrombus formation.[28] After injury, blood flow was monitored continuously for 45 minutes. The time to occlusion was defined as the time between the onset of $FeCl_3$ administration and the onset of 60 consecutive seconds of loss of flow. Time to reflow was defined as time between the time to occlusion and time at which blood flow transiently or permanently increased to 10% of the baseline flow.

2.3 | Platelet-rich plasma clot contraction

Mice were anesthetized with 3% isoflurane in oxygen (2 L/min flow rate), and blood was drawn from the inferior vena cava into 3.2% citrate (10% vol/vol, final). Platelet-rich plasma (PRP) was prepared by sequential centrifugation, diluted with autologous platelet-poor plasma to obtain the concentrations of platelets indicated, and clotted in siliconized aggregometry tubes at 37°C by adding tissue factor and calcium chloride ($CaCl_2$; 1 PM and

10 mmol/L, final, respectively), as described.[18] Contracted clots were weighed at 2 hours.

2.4 | Statistical methods

Sample size calculations were based on simulations that used the observed occlusion rate as the true value to simulate 5000 data sets and the statistical power was calculated with a chi-squared test. For incidence of vessel occlusion, differences between genotype and type of occlusion (stable, unstable, or no occlusion) were analyzed with 2-way analysis of variance. For time to occlusion and time to reflow, differences between groups were analyzed by the Kruskal-Wallis test. For PRP clot weight, differences between groups were analyzed by 1-way analysis of variance with Dunnett's multiple comparisons post hoc test. Statistical analyses were performed using R 3.5.1[29] and Prism version 7 (GraphPad Software, La Jolla, CA, USA).

3 | RESULTS AND DISCUSSION

To investigate the contribution of FXIII to arterial thrombosis, we used a murine model of genetically imposed FXIII deficiency (*F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$) that enabled us to eliminate both plasma and platelet FXIII. Complete blood counts do not differ between these genotypes,[18] and P-selectin positivity and annexin V binding of FXIII-positive and -negative platelets in response to convulxin or thrombin, alone or in combination, are indistinguishable.[30] Compared to *F13a*$^{+/+}$ mice, *F13a*$^{+/-}$ and *F13a*$^{-/-}$ mice express reduced plasma and platelet FXIII in a gene dose-dependent manner.[18,30] Platelets from *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice undergo contraction.[16,18] Importantly, *F13a*$^{-/-}$ mice have no FXIII expression in plasma or platelets and no evidence of compensatory upregulation of transglutaminase activity in FXIII-deficient heart tissue or platelets.[18,31]

We applied $FeCl_3$ to the carotid artery of *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice; this model triggers robust formation of platelet-rich thrombi and is a commonly used model of arterial thrombosis.[32,33] Representative flow tracings for mice that did not experience vessel occlusion, mice with stable occlusions at the end of observation period, and mice with unstable occlusions are shown in Figure 1A-C. Following vessel injury, *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice developed occlusive arterial thrombi. FXIII deficiency did not significantly increase the frequency of nonoccluded vessels or alter the incidence of mice that had stable or unstable occlusions at the end of the observation period (Figure 1D). Although 3 more *F13a*$^{-/-}$ mice failed to develop occlusive thrombi compared to *F13a*$^{+/+}$ mice, a sample size calculation comparing the observed occlusion rate to 5000 simulations suggested more than 60 mice per genotype would be required to achieve statistically significant differences between these groups. A previous report detected sex-specific pathology in *F13a*$^{-/-}$ mice (males show increased cardiac fibrosis and reduced survival).[31] However, there was no difference in the incidence of vessel occlusion in males and females, and a subgroup analysis of male mice projected more than 20 mice per genotype would be required to reveal a significant difference in occlusion incidence. Of mice that exhibited an occlusive event, the time to occlusion was not different for *F13a*$^{+/+}$, *F13a*$^{+/-}$, or *F13a*$^{-/-}$ mice ($P = 0.9$, Figure 1E).

We also examined the impact of FXIII on thrombus stability by recording the first instance of spontaneous reflow (permanent or transient) in mice that initially exhibited vessel occlusion for at least 60 seconds. These included reflow in the 8 *F13a*$^{+/+}$, 7 *F13a*$^{+/-}$, and 6 *F13a*$^{-/-}$ mice with unstable occlusions, as well as transient events in 1 *F13a*$^{+/+}$, 2 *F13a*$^{+/-}$, and 2 *F13a*$^{-/-}$ mice that ultimately formed stable occlusions. Of these mice, the time to reflow was not different between genotypes ($P = .6$, Figure 1F). Extent of reflow (transient vs. permanent) were also similar between genotypes. An additional series of experiments using 7.5% $FeCl_3$ also did not show differences in the time to occlusion between *F13a*$^{+/+}$ and *F13a*$^{-/-}$ mice (data not shown). Thus, consistent with the prior studies using a pharmacologic FXIIIa inhibitor in rabbits,[19] our findings show that FXIII(a) reduction does not prevent arterial thrombus formation in mice.

Following activation and aggregation, platelets contract, which consolidates the thrombus over time; this process likely occurs after vessel occlusion.[34] During venous thrombosis, FXIII deficiency reduces red blood cell retention in thrombi during contraction, and therefore reduces venous thrombus mass.[16–18] Because arterial thrombi have low red blood cell content,[2] we also specifically assessed the impact of FXIII deficiency on contracted clot mass in the absence of red blood cells. Although increasing the platelet count in PRP reduced clot mass (by increasing serum extrusion), there was no effect of FXIII on final PRP clot mass (Figure 1G). Together with the observation that PRP from *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice shows similar clot contraction kinetics,[18] these data suggest that FXIII does not decrease the formation or mass of contracted platelet-rich arterial thrombi.

Previous studies in rabbits and dogs[19,20] showed that pharmacologic FXIII inhibition accelerates thrombolysis in response to administration of therapeutic lytic agents, suggesting that prophylactic FXIII inhibition may facilitate thrombus dissolution. Notably, however, in both the pharmacologic[19,20] and now genetically engineered animal models, FXIII reduction failed to alter the events leading to the formation of occlusive thrombi. Collectively, these data from multiple, independent experimental models of arterial thrombosis suggest FXIIIa does not contribute prominently to the molecular events that promote artery occlusion, and that inhibiting FXIIIa, alone, would not prevent the initial formation of arterial thrombi or associated tissue ischemia.

Although FXIII is abundant in platelets[23] and therefore present at high concentrations within the platelet-rich arterial thrombi, its potential functional role remains unclear. During platelet activation, cytoplasmic FXIII is released slowly and has a relatively short half-life on the platelet surface.[24] Thus, any functional effects of platelet FXIII activity on arterial thrombi may arise later in the pathologic

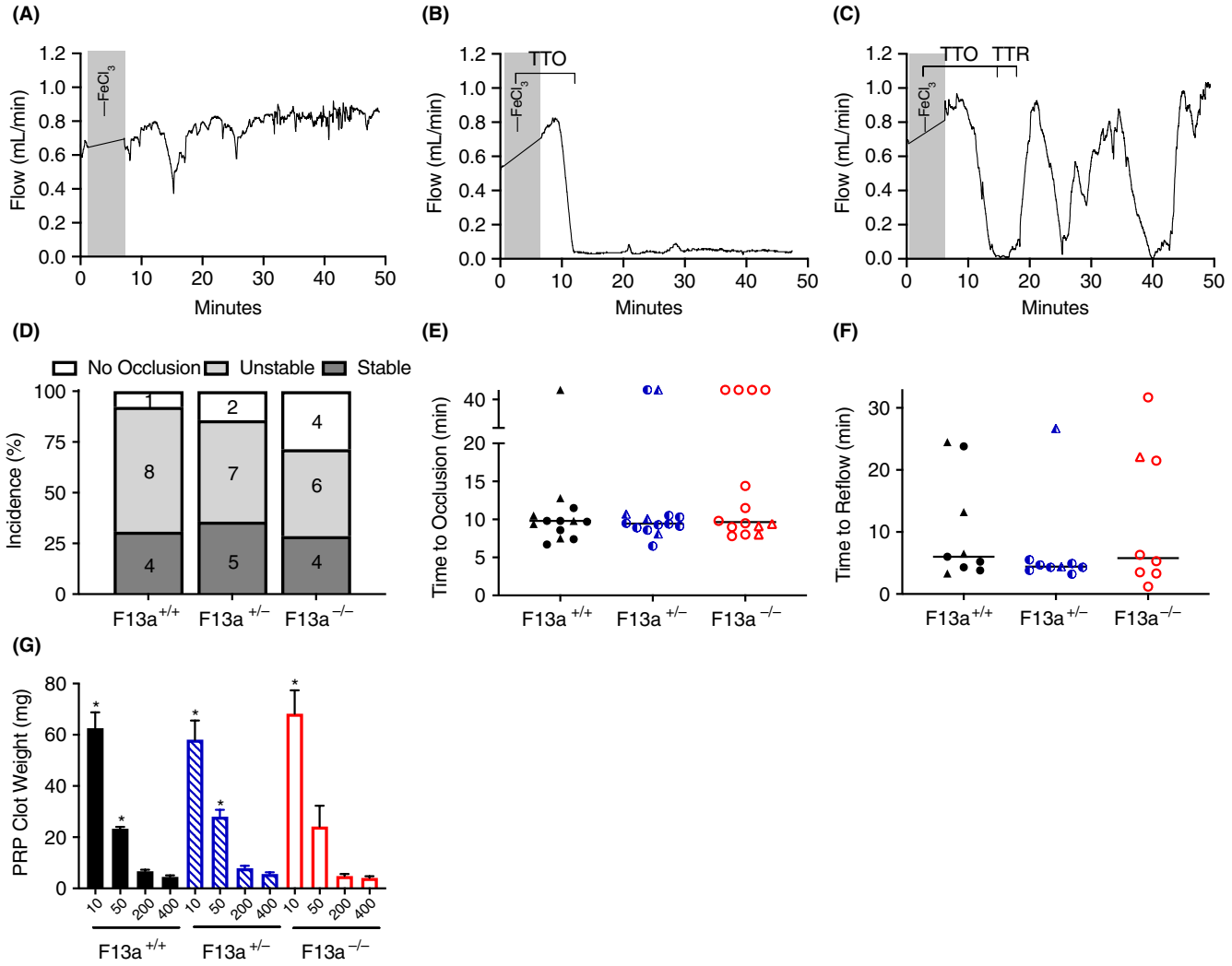

FIGURE 1 FXIII deficiency does not significantly reduce arterial thrombus incidence, formation, or mass. Thrombosis was induced in *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice by 10% $FeCl_3$ application to the carotid artery. Representative flow tracings that resulted in (A) no occlusion, (B) stable occlusion, or (C) unstable occlusion. Gray shaded areas represent time of vessel preparation, $FeCl_3$ administration, and vessel washing, during which flow could not be monitored (interpolated line added). The time to occlusion (TTO) and time to reflow (TTR) are indicated. (D) Incidence of mice with stable occlusions at the end of the observation period, unstable occlusions, and mice with no occlusion for each genotype. Numbers indicate the number of mice for each outcome. (E) Time to occlusion. Each point represents a separate mouse: *F13a*$^{+/+}$ (filled shapes), *F13a*$^{+/-}$ (half-filled shapes), *F13a*$^{-/-}$ (open shapes), males as circles, females as triangles; lines show medians. (F) Time to first reflow event (transient or permanent). Each point represents a separate mouse as in panel E; lines show medians. (G) Weight of contracted PRP clots from *F13a*$^{+/+}$, *F13a*$^{+/-}$, and *F13a*$^{-/-}$ mice. PRP contained 10, 50, 200, or 400 × 10^9 platelets/L, as indicated. Data show means ± standard error of the mean (N = 3-6 per condition); **P* < 0.005 compared to 400 × 10^9 platelets/L

process. Further studies are necessary to understand the function of the evolutionarily preserved abundance of FXIII in platelets.

The $FeCl_3$ model used here triggers thrombosis via oxidative stress and red blood cell adhesion to the vessel wall,[35] which could alter early events in thrombus initiation. In addition, the use of vessel occlusion as an end point may not detect subtle effects on thrombus formation. However, platelet accumulation and thrombus growth in the $FeCl_3$ model still recapitulate key aspects of arterial thrombosis,[35] and this model remains a gold standard in studies of arterial thrombosis.[32,33] Moreover, our findings are consistent with those of a prior model that used a copper coil to induce arterial thrombosis in rabbits.[19]

We previously showed FXIII promotes fibrin-mediated red blood cell retention in whole blood clots and, consequently, determines venous thrombus size in mice.[16–18] Therefore, FXIII is a potential therapeutic target for reducing venous thrombosis. Although fibrin is a major component of both arterial and venous thrombi, our data suggest that the potential of FXIII inhibition to reduce venous thrombus formation does not translate to the setting of arterial thrombosis. These negative findings are important for understanding the molecular mechanisms leading to thrombosis and honing the development of antithrombotic drugs to reduce thrombus formation. FXIII's nonoverlapping roles in these 2 presentations highlights the contrasting pathophysiologic mechanisms, with implications for prophylaxis and treatment strategies.

RELATIONSHIP DISCLOSURES

The authors report nothing to disclose.

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AUTHOR CONTRIBUTIONS

ZMT and SK performed experiments, analyzed data, and wrote the manuscript; LAH and BCC performed experiments; FCL performed statistical analysis; and ASW designed the research, analyzed and interpreted the data, and wrote the manuscript.

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