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ORIGINAL ARTICLE

Role of factor XIII in ischemic stroke: a key molecule promoting thrombus stabilization and resistance to lysis

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Abstract

Background: Active coagulation factor XIII (FXIII) catalyzing crosslinking of fibrin and other hemostatic factors plays a key role in clot stability and lysis.

Objectives: To evaluate the effect of FXIII inhibition in a mouse model of ischemic stroke (IS) and the role of activated FXIII (FXIIIa) in clot formation and lysis in patients with IS.

Methods: A ferric chloride IS murine model was performed before and after administration of a FXIIIa inhibitor (FXIIIinh). Thromboelastometry in human and mice blood was used to evaluate thrombus stiffness and lysis with FXIIIinh. FXIIIa-dependent fibrin crosslinking and lysis with fibrinolytic drugs (tissue plasminogen activator and tenecteplase) were studied on fibrin plates and on thrombi and clotted plasma of patients with IS. Finally, circulating and thrombus FXIIIa were measured in 85 patients with IS.

Results: FXIIIinh administration before stroke induction reduced infarct size, α2-antiplasmin (α2AP) crosslinking, and local microthrombosis, improving motor coordination and fibrinolysis without intracranial bleeds (24 hours). Interestingly, FXIII blockade after stroke also reduced brain damage and neurologic deficit. Thromboelastometry in human/mice blood with FXIIIinh showed delayed clot formation, reduced clot firmness, and shortened tissue plasminogen activator lysis time. FXIIIa fibrin crosslinking increased fibrin density and lysis resistance, which increased further after α2AP addition. FXIIIinh enhanced *ex vivo* lysis in stroke thrombi and fibrin plates. In patients with IS, thrombus FXIII and α2AP were associated with inflammatory and hemostatic components, and plasma FXIIIa correlated with thrombus α2AP and fibrin.

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Conclusion: Our results suggest a key role of FXIIIa in thrombus stabilization, α2AP crosslinking, and lysis resistance, with a protective effect of FXIIIinh in an IS experimental model.

KEYWORDS
factor XIII, fibrinolysis, stroke, thrombectomy, thrombosis

1 | INTRODUCTION

Ischemic stroke (IS) represents one of the leading causes of death worldwide and is the most frequent cause of permanent disability in adults in Europe [1]. Even after years of research, treatment options to attempt revascularization of the occluded vessel are limited and not always successful. Since IS is caused by occlusion of 1 or more cerebral arteries by a clot, coagulation pathways play a major role in its pathogenesis. Thus, factors implicated in hemostasia and endogenous fibrinolysis are expected to influence stroke thrombus structure and its susceptibility to lysis. A better understanding of the underlying mechanisms implicated in clot formation and resistance to thrombolytic agents in some patients is lacking.

Coagulation factor XIII (FXIII) is a zymogenic transglutaminase constituted by 2 potentially active A subunits (FXIII-A) and 2 carrier-inhibitory B subunits (FXIII-B). FXIII is present in plasma and cells, including platelets, endothelial cells, and monocytes, in an active homodimeric form (FXIII-A2) [2] and is a key player in the last step of coagulation. Its activation by thrombin into activated FXIII (FXIIIa) triggers the crosslinking of fibrin chains and stabilizes the fibrin meshwork, increasing the stiffness and resistance to the endogenous fibrinolysis system [3]. Moreover, the antifibrinolytic effect of FXIII has been proposed to depend in part on FXIIIa-mediated α2-antiplasmin (α2AP) crosslinking to the fibrin meshwork [4]. α2AP is an important member of the serine protease inhibitor family capable of covalently inhibiting plasmin in a very specific and fast manner. Higher circulating levels of α2AP have been associated with increased risk of IS, worse recanalization rates after tissue plasminogen activator (tPA) therapy [5], and a greater fibrinolytic effect in *ex vivo* α2AP-deficient thrombi [6].

Furthermore, FXIIIa has been reported to crosslink other hemostatic factors into the fibrin network, with potential effects on thrombus properties [2]. For example, von Willebrand factor (VWF) crosslinked by FXIIIa promotes thromboinflammation and leukocyte recruitment and activation/stabilization of platelets within the thrombus [7]. Thrombospondin-1, crosslinked by FXIIIa, increases fibrin clot density in a concentration-dependent manner [2]. Besides, other proteins with antifibrinolytic activity, such as thrombin-activable fibrinolysis inhibitor [8] or vitronectin [9] bound to the fibrin meshwork by FXIIIa, play a secondary role in modulating lysis [2]. Likewise, histone incorporation into fibrin by FXIIIa induces structural changes in the fibrin network, rendering a stronger and less lysable mesh [10].

Despite the importance of FXIII in thrombosis and hemostasis, relatively few studies describe its role in IS. Increased FXIII activity has been demonstrated in IS, especially in those of atherothrombotic origin, and small vessel infarcts [11] without predicting the success of tPA-mediated recanalization [5]. In addition, other studies have associated the drop in FXIII antigen levels with acute thromboembolic events and have postulated it as a possible marker of stroke [12] and pulmonary embolism [13]. Likewise, a decrease in FXIII levels in patients with IS after intravenous thrombolysis has been associated with a higher risk of hemorrhagic transformation [14,15] and mortality [11]. Therefore, FXIII may be a promising target for IS treatment.

In this study, we aimed to assess the effect of FXIIIa inhibition on lesion size and neurologic dysfunction in an experimental model of IS. In addition, we characterized its role in clot stabilization and resistance to current fibrinolytic drugs as well as the association of plasma and thrombus FXIII with other thrombus components and prognosis in patients with IS.

2 | METHODS

The data that support this study are available from the corresponding author upon reasonable request. The Stroke Therapy Academic Industry Roundtable 2009 guidelines [16] were taken into account in our experiments in order to promote reliability and reproducibility.

2.1 | Experimental models

The middle cerebral artery occlusion (MCAO) model with ferric chloride ($FeCl_3$; 30%, 4 minutes) [17] was applied to 9- to 13-week-old mice (C57Bl6J, Envigo) to evaluate the effect of intravenous administration (10% bolus plus 90% perfusion, for 40 minutes) of 1 mg (per mouse) of a specific irreversible FXIIIa inhibitor [18] (FXIIIinh; ZED1301, Zedira). First, a prestroke model was performed in 26 male mice (n = 13/group) by intravenous injection of ZED1301 15 minutes before stroke induction and during the next 40 minutes. Second, a prestroke model was performed in 32 male mice (n = 10-11/group) treated 15 minutes before stroke with a vehicle—

tranexamic acid (TXA, 300 mg/kg)—or TXA plus ZED 1301 during the next 40 minutes. Third, administration of ZED1301 10 minutes after IS was tested in 18 male mice (n = 9/group) and 15 female mice (n = 8 for the treatment group and n = 7 for the vehicle group) (poststroke model). In all models, 24 hours after stroke, animals were euthanized. Citrated plasma was obtained by intracardiac puncture, and animals were perfused with cold phosphate-buffered saline (PBS, Gibco). Brains were removed, frozen in isopentane, and stored at −80 °C until use. A more detailed experimental protocol is included in the Supplementary Methods with a graphic summary (Supplementary Figure S1).

In addition, the carotid thrombosis model with $FeCl_3$ (15%, 4 minutes) and IS stroke model were applied in 13 and 6 male mice, respectively, to obtain arterial thrombus samples 60 minutes after thrombus formation for histologic analysis (n = 3) and western blot (WB) studies (n = 16).

These experiments were approved by the University of Navarra Animal Research Review Committee (reference 52/18) and conducted according to the regulation of the European Council for the ethical care and use of laboratory animals (2019/1010).

2.2 | Pharmacokinetic studies

Pharmacokinetic studies were performed in 3 male mice treated with a single intravenous bolus of FXIIIinh (1 mg/mouse), and plasma FXIII activity was measured at different time points (5-30 minutes) on citrated plasma samples.

2.3 | Plasmatic D-dimer measurement

Mouse plasma D-dimer was determined by enzyme-linked immunosorbent assay (ELISA) (Mouse D-Dimer ELISA Kit, Abbexa) following the manufacturer's instructions.

2.4 | Infarct volume assessment

Frozen brain slides were stained with thionine (T7029-5G, Sigma-Aldrich) and scanned to analyze infarct volume with ImageJ (National Institutes of Health) [19] as specified in the Supplementary Methods. In addition, the presence of cerebral microbleeds was evaluated using Perls Prussian blue staining (HEMATOGNOST Fe, Sigma-Aldrich).

2.5 | Motor and behavioral assessment

Each mouse was functionally assessed by the Bederson scale and 5 seconds/60 seconds coat-hanger test 1 hour before and 24 hours after application of the experimental model. Twenty-four hours before MCAO surgery, mice were trained 3 times to perform the different tests (Supplementary Table S1).

2.6 | Immunohistochemical analysis

Immunofluorescence for CD31 (DIA-310, Dianova)/glycoprotein (GP) IX (LS-C334021, LSBio) in mice brains was performed as described in the Supplementary Methods (Supplementary Figure S2).

Mouse arterial thrombi were immunostained for FXIII (PA5-22110, Invitrogen), fibrin(ogen) (GAM/Fbg/7/S, Nordic Immunologic) and α2AP (PA5-47101, Invitrogen), as specified in the Supplementary Methods.

2.7 | α2AP WB analysis in carotid and brain thrombi

WB analysis for α2AP (0.1 μg/mL; PA5-47101, Invitrogen) was conducted in thrombi obtained after MCAO (n = 6), and carotid thrombosis (n = 10) in mice pretreated with FXIIIinh or vehicle.

2.8 | Thromboelastometry assay

Fresh citrated blood samples from mice (n = 7) and healthy human volunteers (n = 6) were assayed by rotational thromboelastometry (ROTEM, Tem Innovations GmbH) in the presence and absence of 20 μM of FXIIIinh (ZED1301) with and without 500 IU/mL of t-PA (Actilyse, Boehringer-Ingelheim) to evaluate recorded parameters. Additional experiments were performed in 5 human samples treated with 20 μM of FXIIIinh, 20 μM of TXA, or the combination of TXA + FXIIIinh in the presence of 500 IU/mL of t-PA. A more detailed protocol is included in the Supplementary Methods.

2.9 | Confocal microscopy studies of fibrin plates to analyze fibrin network and lysis

Fibrin polymerization assays were performed (n = 4) in the presence or absence of FXIIIa and α2AP (Supplementary Methods). After gold staining (1:100000, BBI), Z-stack images were obtained (LSM 880 NLO, Carl Zeiss) and analyzed by ImageJ software [19].

Similarly, fibrin clots were generated with plasma samples from 29 patients with IS (Supplementary Methods), gold-stained, and analyzed by confocal microscopy. Patient's main characteristics are presented in Supplementary Table S2.

Thrombolytic drug activities on FXIIIa and α2AP crosslinked fibrin plates (n = 6) were measured using 1.4 μM of plasminogen (HPG 2001, Enzyme Research Lab) with 1 IU/mL of tPA or 0.4IU/mL of tenecteplase (TNK, Metalyse, Boehringer-Ingelheim). Plates were scanned (Perfection 3200 scanner, Epson) at 3, 6, 8, and 24 hours to measure lysis area with ImageJ software [19]. The doses selected for the experiments are based on current TNK trials in IS [20].

Additional fibrin plates (n = 5) were generated with FXIIIa and FXIIIa + α2AP to assess tPA and TNK fibrinolytic activity in the presence of FXIIIinh (200 μM).

2.10 | *Ex vivo* lysis of IS thrombi

Next, FXIII activity was measured in 4 homogenized frozen IS thrombi to evaluate the effect of FXIII blockade (200 μM, ZED1301) in an already formed thrombus after 5 minutes of incubation. In addition, tissue transglutaminase (TG2) activity was measured in 4 other frozen IS thrombi with/without FXIIIinh using a reference curve of human TG2 (0.78-50 nM, T022, Zedira).

Then, we analyzed the effect of FXIII blockage on tPA fibrinolytic activity in IS thrombi. Briefly, 16 IS frozen thrombi, collected at Hospital Universitario Miguel Servet between February 2021-june 2021, were divided in half and exposed to tPA (580 IU/mL) with/without FXIIIinh (20 μM and 200 μM, ZED1301) and weighed every 15 minutes for 1 hour (Supplementary Methods). Patients' main characteristics are presented in Supplementary Table S3.

2.11 | Patients with IS

A total of 85 patients with IS with large vessel occlusion were recruited from 2 different Spanish tertiary hospitals: 57 patients from the Hospital Universitario de Navarra between November 2015 and July 2020 and 28 patients from the Hospital Universitario Vall d'Hebron between January 2017 and September 2019. These studies were approved by the ethics committee of the Navarra Government (84/2018) and the Catalonia Government (PR[AG]-234/2017). Written informed consent was obtained from all patients or their legally authorized representative. Samples and data from patients included in the study were provided by the Biobanks of the University of Navarra and the Aragon Health Sciences Institute in the framework of the Biobank of Aragon and were processed following standard operation procedures with the approval of the Ethical and Scientific Review Boards.

Patients' main characteristics are presented in Supplementary Table S4.

2.12 | Plasma levels of FXIII activity and α2AP

FXIII activity was measured on citrated plasma samples with a fluorescence assay kit (F001, Zedira) and calculated on a reference curve (0.625-10 U/mL) of human FXIIIa (T070, Zedira). α2AP levels were quantified by ELISA (LS-F10408, LSBio) following the manufacturer's instructions.

2.13 | α2AP WB analysis in plasma clots from patients with IS

Clots were formed in plasma samples of 29 patients by addition of 20 mM of $CaCl_2$ and 0.1 U/mL of thrombin. Subsequently, the levels of α2AP within these clots were quantified by WB using the primary antibody against α2AP (1 μg/mL, sc515771, Santa Cruz).

2.14 | Histological analysis

IS retrieved thrombi (n = 85) were embedded in paraffin, and serial sections from each thrombus were stained with Martius Scarlet Blue staining (Atom), hematoxylin–eosin (PanReac), and platelet GPIb (CD42b, 42C01, Invitrogen) to visualize their general internal organization. Moreover, specific immunostainings with antibodies against FXIII-A chain (HPA001804, Sigma-Aldrich), VWF (A0082, Dako), neutrophil elastase (NE, HPA066836, Sigma-Aldrich), α2AP (sc-515771, Santa Cruz), citrullinated histone H3 (ab5103, Abcam), T lymphocytes $CD3^+$ (A0452, Dako), leukocytes $CD45^+$ (NCL-LCA-RP, Leica Biosystems), and macrophages $CD68^+$ (M0814, Dako) were performed.

Double and triple immunofluorescence was performed in 2 thrombi to localize FXIII with specific cell types ($CD42b^+$ platelets and $CD68^+$ macrophages) and proteins in thrombi (fibrinogen and α2AP).

Immunostained slides were subsequently scanned (Aperio ImageScope, Leica Biosystems and Vectra Polaris, Perkin Elmer) and quantified with ImageJ software [19].

More detailed protocols are provided in the Supplementary Methods.

2.15 | Statistical analysis

Normality was assessed graphically and with the Shapiro–Wilk test, and skewed distributions were transformed by logarithmic or square-root transformation to achieve normal distribution. Continuous variables with normal distributions are presented as mean with SEM, whereas skewed variables are presented as median with IQR. Data were analyzed using a nonparametric Wilcoxon rank-sum test or parametric t-test for paired data or the Mann–Whitney U-test and the parametric t-test for comparison of unpaired data. To control the family-wise error rate when multiple comparisons were performed, the Holm–Sidak correction was applied. The Friedman test was used when analyzing 3 or more related groups that did not meet the assumptions of parametric tests, followed by Dunn's multiple comparisons test to compare each group with the control. Finally, Pearson correlation coefficient and linear regression models were used to evaluate associations between thrombus components and blood elements. Statistical significance was considered for all analyses if P value was <.05. STATA (version 16, StataCorp LLC) was used for statistical analyses in this study.

3 | RESULTS

3.1 | FXIII inhibition reduces infarct size and improves functional outcome in a mouse MCAO model

To assess the inhibitory activity of the selected FXIIIinh, we performed pharmacokinetic studies after a single bolus of the FXIIIinh (1 mg/mouse) in mice (n = 3). Twenty minutes after administration, a 64% decrease in plasma FXIII activity was observed (Supplementary Figure S3A).

Then, IS was induced in male mice (n = 13/group) treated with vehicle or FXIIIinh 15 minutes before surgery. Mice receiving FXIIIinh presented smaller infarct sizes (mean ± SEM, 16.4 ± 1.8 mm^3 vs 21.9 ± 1.8 mm^3; $P < .05$) with a better score in the 5 seconds coat-hanger test 24 hours after MCAO ($P < .05$; Figure 1A, B). To determine whether the beneficial effect of FXIIIinh in IS relies on plasmin activity, mice were treated with FXIIIinh and TXA. As shown in Figure 1E, FXIIIinh similarly tended to reduce the infarct size in the brains of mice subjected to MCAO regardless of the presence of TXA (residual significance, $P = .05$). A tendency toward a reduced infarct size in mice treated with TXA was also observed at a concentration able to reduce D-dimer levels (Supplementary Figure S3B).

No differences in plasma FXIII activity were observed between mice treated with FXIIIinh and the control group 24 hours after MCAO (Supplementary Figure S3C). However, D-dimer was significantly increased in treated mice at 24 hours, suggesting an enhancement in fibrin degradation in mice receiving FXIIIinh (Figure 1C).

To evaluate microvascular thrombosis after MCAO, double immunofluorescence for CD31 and activated platelets GPIX+ was performed in mice brains. As shown in Figure 1D, less microvascular thrombosis was observed in the ischemic hemisphere of treated mice compared to that in controls (median percentage of GPIX platelet-positive vessels, 12.1% [IQR, 8.5%-17.9%] vs 36.9% [IQR, 29.9%-77.3%]; $P < .05$). In addition, no macroscopic bleeding and few microhemorrhages (Perls staining) were observed in the infarct area of treated mice, similar to the control group (3/13 in pretreated mice and 4/13 in control mice).

Then, to better understand the therapeutic effect of FXIIIinh, it was administered to a group of 18 males and 15 females 10 minutes after MCAO (after stroke). Our results showed a reduction in the infarct volume (median, 9.0 mm^3 [IQR, 3.1-14.1 mm^3] vs 16.6 mm^3 [IQR, 11.7-18.4 mm^3]; $P < .01$) and a lower decline in motor coordination in the poststroke model ($P < .01$), as shown in Figure 1F, G. Interestingly, no significant interaction was observed between sex and treatment in terms of infarct size or functional status after stroke, which justifies the combined analysis.

3.2 | α2AP is poorly incorporated into thrombi after FXIII inhibition in 2 thrombosis models

To explore the molecular changes induced locally by FXIII inhibition, WB analysis for α2AP, a key fibrinolysis inhibitor, was performed on brain (n = 6) and carotid thrombi (n = 10) in mice pretreated with FXIIIinh or a vehicle. Thrombi of mice treated with FXIIIinh presented a reduction in α2AP compared with vehicle, indicating that lower FXIIIa reduces the incorporation of α2AP into the thrombus (Figure 1H).

3.3 | FXIII inhibition impairs clot formation and enhances clot lysis

Thromboelastometry assays were performed to analyze the effect of FXIIIinh in *ex vivo* clot formation and lysis.

In human blood samples, the addition of FXIIIinh delayed clot formation with increased clot formation time, a less pronounced α angle, and reduced clot burden with decreased maximum clot firmness. After addition of tPA, FXIIIinh enhanced fibrinolysis by reducing lysis time (Figure 2A, B). Similar results were obtained in mouse blood (Figure 2C).

To assess if FXIIIinh was still effective in the presence of an antifibrinolytic drug, thromboelastometry experiments were performed with TXA alone or in combination with FXIIIinh (Figure 2D). As expected, TXA increased clot firmness and delayed fibrinolysis, but in combination with FXIIIinh, these effects on clot firmness and lysis onset time were reduced. In addition, in blood samples treated with FXIIIinh and TXA, FXIIIinh consistently delayed clot enlargement (clot formation time and α angle), as observed with the FXIIIinh alone (Figure 2D).

3.4 | FXIII entangles fibrin fibers and crosslinks α2AP, influencing fibrinolysis resistance

To confirm the influence of FXIIIa and α2AP on fibrin crosslinking, fibrin plates were generated and evaluated by confocal microscopy in the presence or absence of FXIIIa and FXIIIa + α2AP at 24 hours (n = 4/condition). In the presence of FXIIIa and FXIIIa + α2AP, a higher fiber density (9.7 ± 1.2% and 10.8 ± 1.4% vs 8.4 ± 0.9% for control; $P < .01$), increased number of fibers (17963 ± 2326 and 20634 ± 2965 vs 13715 ± 948.8 for control; $P < .01$), and decreased pore diameter (2.9 ± 0.69 μm and 2.5 ± 0.66 μm vs 3.3 ± 0.67 μm for control; $P < .01$) were observed (Figure 3). However, fiber thickness and fiber length did not change.

In order to be closer to the clinical setting, clots were generated using plasma samples from patients with IS (n = 29, Supplementary Table S2). Unexpectedly, 45% of these samples failed to form consistent fibrin mesh structures. This clotting frequency was dependent on tPA treatment, being higher in those who did not receive tPA (71% vs 33%, $P = .02$). Interestingly, the formation of consistent fibrin mesh structures was associated with plasma FXIIIa, regardless of tPA treatment (Figure 3F). Moreover, WB analysis of these fibrin clots revealed a positive association of α2AP with both the number of fibers (r = 0.73; $P = .04$) and fiber density (r = 0.49; $P = .08$) and a negative association with pore diameter (r = −0.51; $P = .07$).

The lysis resistance was further analyzed in additional fibrin plates (n = 6) monitored for 24 hours (Supplementary Figure S4). We observed a reduction in tPA-induced lysis in the presence of FXIIIa that reached statistical significance at 24 hours (lysis activity vs control plates: median, 59.8%; IQR, 46.1%-75.4%; $P < .01$), and tPA-

induced lysis was completely inhibited when α2AP was crosslinked with FXIII. For TNK, lysis was also reduced on FXIIIa plates (lysis at 6 hours vs control plates: median, 76.8%; IQR, 63.9%-84.4%; $P < .01$) and further reduced on fibrin plates generated in the presence of FXIIIa + α2AP (median, 19%; IQR, 13.5%-21.6%; $P < .05$) (Figure 4). Moreover, the addition of FXIIIinh to the fibrinolytics enhanced the lysis of tPA and TNK in plates with FXIIIa but not in FXIIIa + α2AP fibrin plates (Figure 4B).

3.5 | FXIII inhibition enhances tPA lysis in *ex vivo* human stroke thrombi

Furthermore, we explored the effect of FXIIIinh on *ex vivo* fibrinolysis induced by tPA in 16 thrombi from patients with IS (Supplementary Table S3). The addition of 20 or 200 μM of FXIIIinh to tPA enhanced thrombus lysis throughout the experiment, reaching statistical significance with the higher dose (Figure 5).

FXIII activity was measured after homogenization of 4 IS retrieved thrombi, showing persistent FXIII activity (mean ± SEM, 2.6 ± 1.9 IU/mL) that was completely inhibited by FXIIIinh (200 μM) (Supplementary Figure S5). In contrast, scarce TG2 activity was found in IS thrombi (2.5 ± 0.5 nM) that were not modified for the FXIIIinh (3.4 ± 0.3 nM).

3.6 | FXIII and α2AP are present in thrombi and associated with other components

The local expression of FXIII and α2AP was studied by morphological analyses on mouse and human thrombi. In both thrombi, FXIII colocalized with fibrin mesh and α2AP (Figure 6A, B and Supplementary Figure S6). In human thrombi, FXIII also colocalized with platelets and macrophages. All these components were interspersed into platelet-rich regions, overall bordering red blood cell (RBC)–rich areas (Figure 6C–E).

Next, we quantified FXIII and α2AP in human stroke thrombi ($n = 85$) by immunostaining. The median age of the study population was 75.6

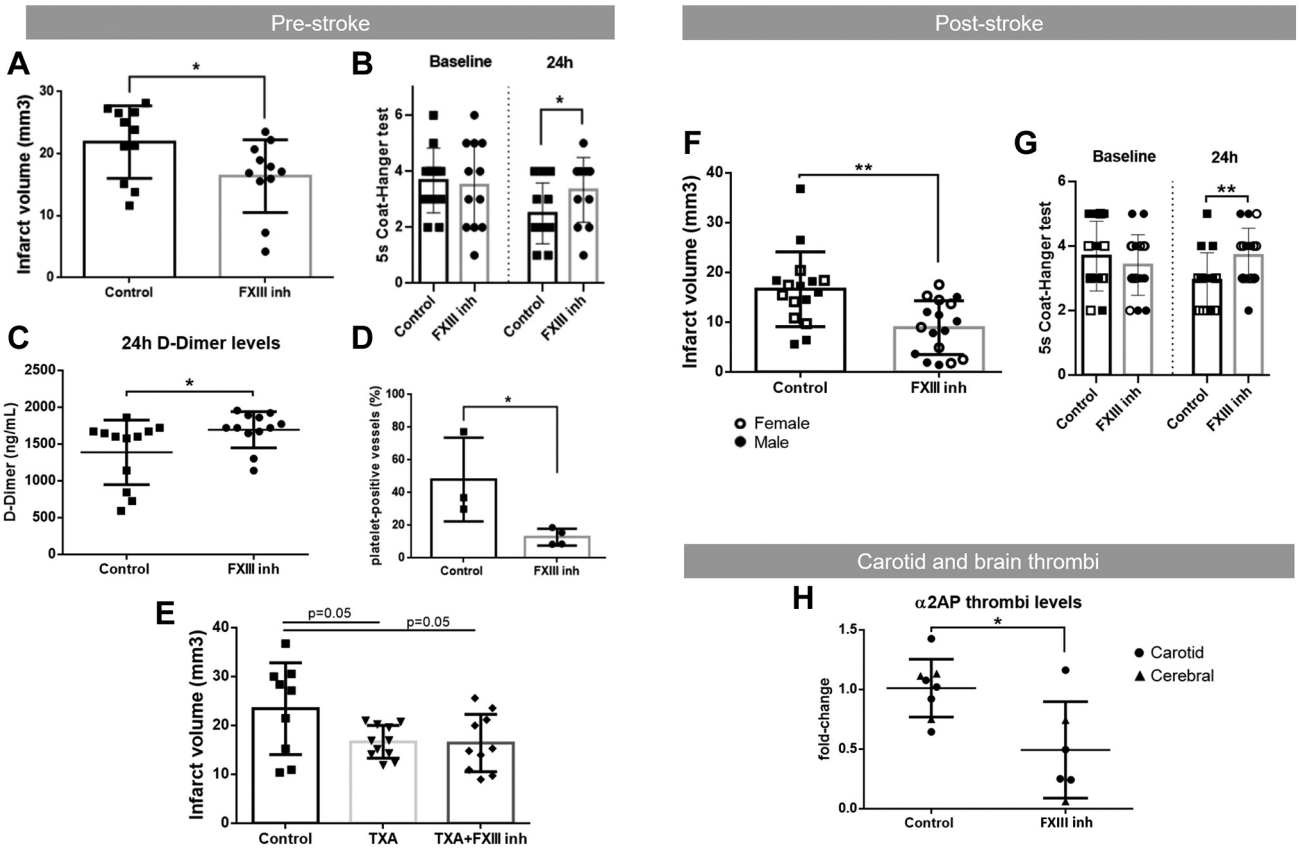

FIGURE 1 Effect of factor XIII (FXIII) inhibition in a middle cerebral artery occlusion (MCAO) mouse model. (A) Infarct size of mice treated with FXIII inhibitor (FXIIIinh) before stroke induction (prestroke administration) and (B) functional outcome 24 hours after MCAO (n = 13/group). *$P < .05$ by unpaired t-test. (C) Plasma D-dimer levels at 24 hours (n = 12/group). (D) Quantification of CD31/GPIX immunofluorescence (microthrombosis) in brain tissues 24 hours after stroke (n = 4/group). *$P < .05$ by Mann–Whitney U-test. (E) Infarct size of mice treated before stroke induction with tranexamic acid (TXA) alone or in combination with FXIIIinh. Analyzed by multiple unpaired t-test with Holm–Sidak correction. (F) Infarct size of mice treated with FXIIIinh after stroke (poststroke administration). (G) Functional outcome 24 hours after MCAO (n = 9 males/group and 8/7 females). (H) Western blot for α2-antiplasmin (α2AP) in carotid (circle) and brain (triangle) thrombi of mice treated with and without FXIIIinh. *$P < .05$; **$P < .01$ by Mann–Whitney U-test.

years (IQR, 62.9-80.3 years), and 47% were women. Patient characteristics are summarized in Supplementary Table S4. The most common vascular risk factor was arterial hypertension (60%), followed by dyslipidemia (52%). Approximately 20% were treated with antiplatelet or anticoagulants, and 61% received intravenous thrombolysis. The cardioembolic etiology represented 44% of the strokes, and 23% were atherothrombotic.

The thrombus FXIII positive area was 2.01% (IQR, 0.27%-5.27%), and its expression was associated with thrombus neutrophil extracellular traps (NETs) (exponential of B coefficient exp[B], 2.20; 95% CI, 1.16-4.20; $P < .02$), VWF (exp[B], 1.78; 95% CI, 1.28-2.46; $P < .01$), and NE expression (exp[B], 1.28; 95% CI, 1.06-1.54; $P = .01$) (Table).

Similarly, thrombus α2AP staining was quantified, giving a median value of 3.88% (IQR, 0.64%-8.16%) that was associated with VWF (exp[B], 1.30; 95% CI, 1.09-1.56; $P < .01$), and RBC (exp[B], 1.43; 95% CI, 1.08-1.91; $P < .02$) areas (Supplementary Table S5).

Neither FXIII nor α2AP stained areas in the thrombi were associated with recanalization rates, time from onset, etiology, and functional prognosis after stroke in our patients (data not shown).

3.7 | Plasma FXIII activity is associated with α2AP and fibrin crosslinking

Finally, we determined FXIII activity and α2AP levels in the blood of patients with IS to explore their possible association with thrombus components. Mean plasma FXIII activity was 0.90 IU/mL (SD, 0.31 IU/mL) and was positively associated with fibrin percentage (exp[B], 2.35; 95% CI, 1.30-4.26; $P < .01$) and α2AP expression in thrombi (exp[B], 2.74; 95% CI, 1.02-7.39; $P < .05$), supporting its role in fibrin and α2AP crosslinking within the thrombus (Supplementary Table S6).

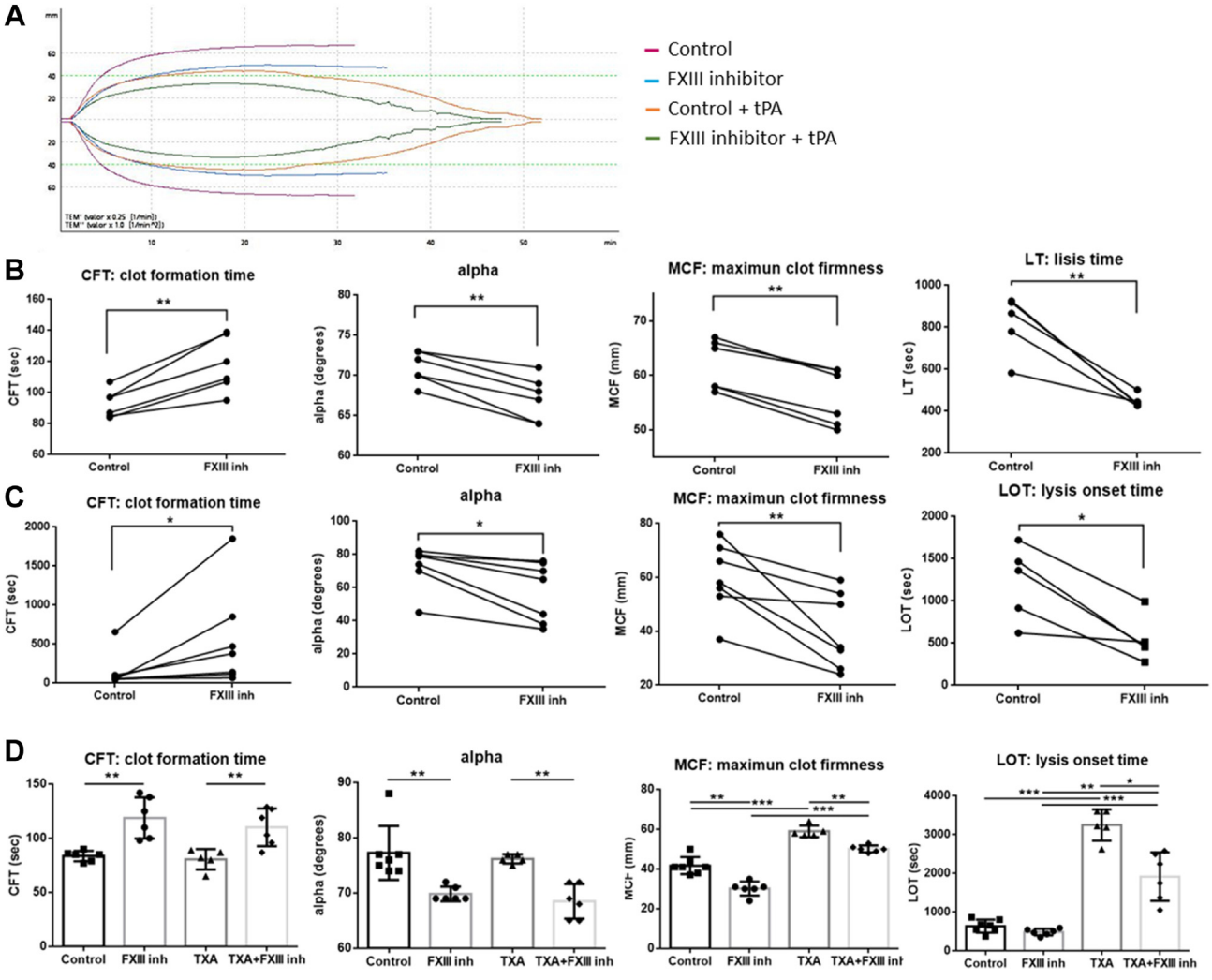

FIGURE 2 Effects of factor (F)XIII inhibition in rotational thromboelastometry (ROTEM) assay. (A) Temogram from human blood samples showing effects on clot formation and lysis with and without a FXIII inhibitor (FXIIIinh). (B) In human blood ($n = 6$), FXIIIinh increased clot formation time (CFT), reduced α angle and maximum clot firmness (MCF), and enhanced lysis. (C) Similar results were observed with mice blood ($n = 7$). (D) ROTEM of human blood samples treated with FXIIIinh, tranexamic acid (TXA), and TXA plus FXIIIinh. *$P < .05$; **$P < .01$; ***$P < .001$ by paired t-test and multiple unpaired t-tests with Holm–Sidak correction. LOT, lysis onset time; LT, lysis time; tPA, tissue plasminogen activator.

As expected, plasma α2AP was decreased in patients who received tPA (median, 40.0 μg/mL [IQR, 33.9-49.2 μg/mL] vs 50.8 μg/mL [IQR, 44.3-65.1 μg/mL]; $P < .01$), and this association remained significant after multivariate adjustment by cardioembolic stroke and previous anticoagulant treatment (odds ratio, 0.42; 95% CI, 0.21-0.82; $P < .02$).

FXIII and α2AP plasma levels were not associated with recanalization rates, time from onset, etiology, and functional prognosis after stroke in our patients.

4 | DISCUSSION

Our study showed, for the first time, that FXIII inhibition in an MCAO murine model reduced infarct size, improved functional outcome, and enhanced fibrin degradation product (D-dimer) 24 hours after IS. During thrombus formation, we demonstrated that FXIII is implicated in processes related to thrombus stiffness, α2AP crosslinking, and responsiveness to fibrinolytic treatments. With *in vitro* experiments, we confirmed that FXIII and α2AP increased fiber density and reduced pore size in the fibrin meshwork with direct consequences on lysis resistance to tPA and TNK that are reduced by FXIII inhibition. Furthermore, FXIII was present in IS human and mouse thrombi, colocalizing with fibrin and α2AP, and associated positively with thrombus VWF, NETs, NE, and inflammatory cells. In addition, circulating FXIII activity was associated with fibrin clot formation and the protein expression of fibrin and α2AP in the thrombus.

Even though FXIIIa plays a prominent role in fibrin crosslinking and resistance to fibrinolysis, only a few reports are available on the relationship between IS and FXIII, and no previous studies have been performed with the IS experimental model. Therefore, we explored the potential benefit of FXIIIinh on an MCAO mice model. We observed decreased infarct size and reduced functional sequelae with no increase in intracranial bleeding. Our results are in line with those of Pasternack et al. [21], showing an antithrombotic effect of FXIII inhibition by reducing clot burden without increasing bleeding risk in a venous thrombosis model in rabbits. In addition, we extended these results, showing an increase of the final end product of fibrinolysis (D-dimer), a reduction in local microthrombosis in the infarct area, and lower levels of α2AP trapped in thrombi 24 hours after FXIII inhibition, suggesting a higher level of fibrinolytic activity in the presence of FXIIIinh.

Interestingly, treatment with the FXIIIinh after stroke induction remained beneficial, reducing infarct size and functional damage. It has been reported that microvascular endothelium injury promotes platelet adhesion and proinflammatory pathways, leading to microthrombosis [22]. In this context, the cellular form of FXIII in platelets would become activated, stabilizing the thrombi and reducing reperfusion [23]. Together, these data suggest that FXIII inhibition could have a potential therapeutic effect not only in venous thrombosis but also in IS experimental models, leading to reduced brain damage.

In addition, we further studied whether the effects of FXIIIinh in the $FeCl_3$-induced IS model could be overruled by the antifibrinolytic drug TXA. We found a similar reduction in lesion size in FXIIIinh-treated and FXIIIinh + TXA–treated animals vs vehicles, suggesting that FXIIIinh activity might be independent of plasmin. Unexpectedly, TXA-treated mice also showed a tendency to exhibit a smaller infarct size than that in controls. TXA is a lysine analog

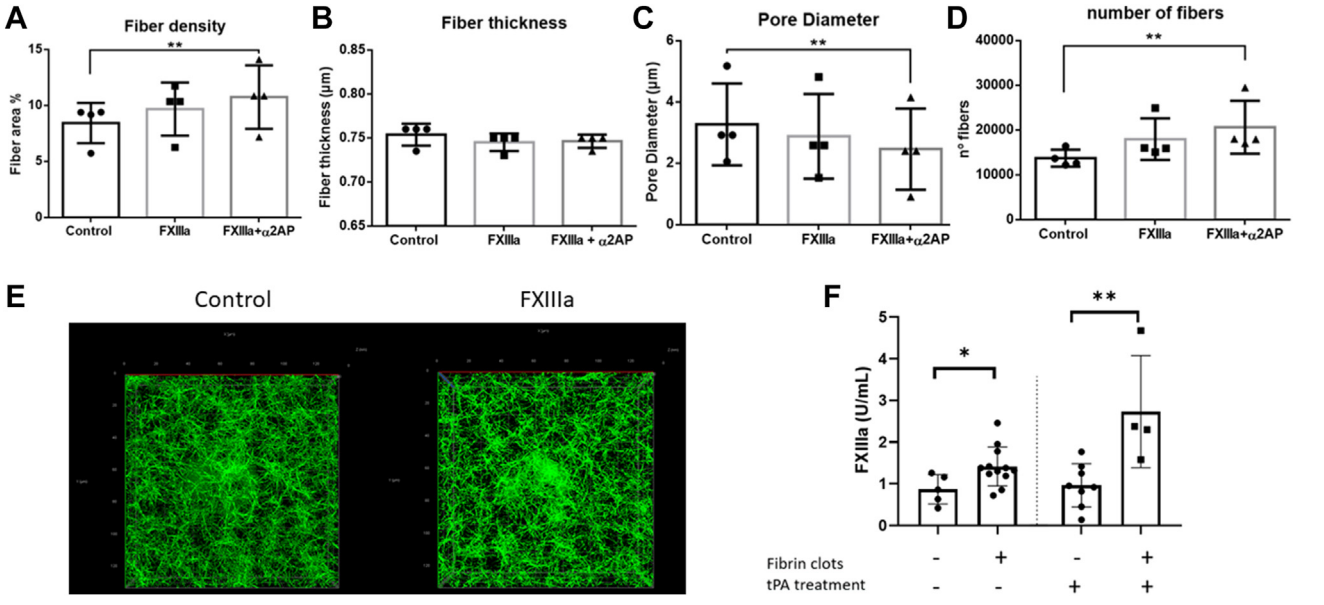

FIGURE 3 Addition of activated factor XIII (FXIIIa) and α2-antiplasmin (α2AP) increased fibrin mesh density. (A) Fiber density in fibrin plates generated in the presence of FXIIIa and FXIIIa + α2AP. (B) Fibrin thickness. (C) Pore diameter. (D) Number of fibers. (E) Extracted images of the fibrin network ($n = 3$). (F) Fibrin clot formation and plasma FXIIIa in patients with ischemic stroke treated or not treated with tissue plasminogen activator (tPA) ($n = 29$). $^*P < .05$ and $^{**}P < .01$ by the Mann-Whitney U-test, and by the Friedman test, followed by Dunn's multiple comparisons test.

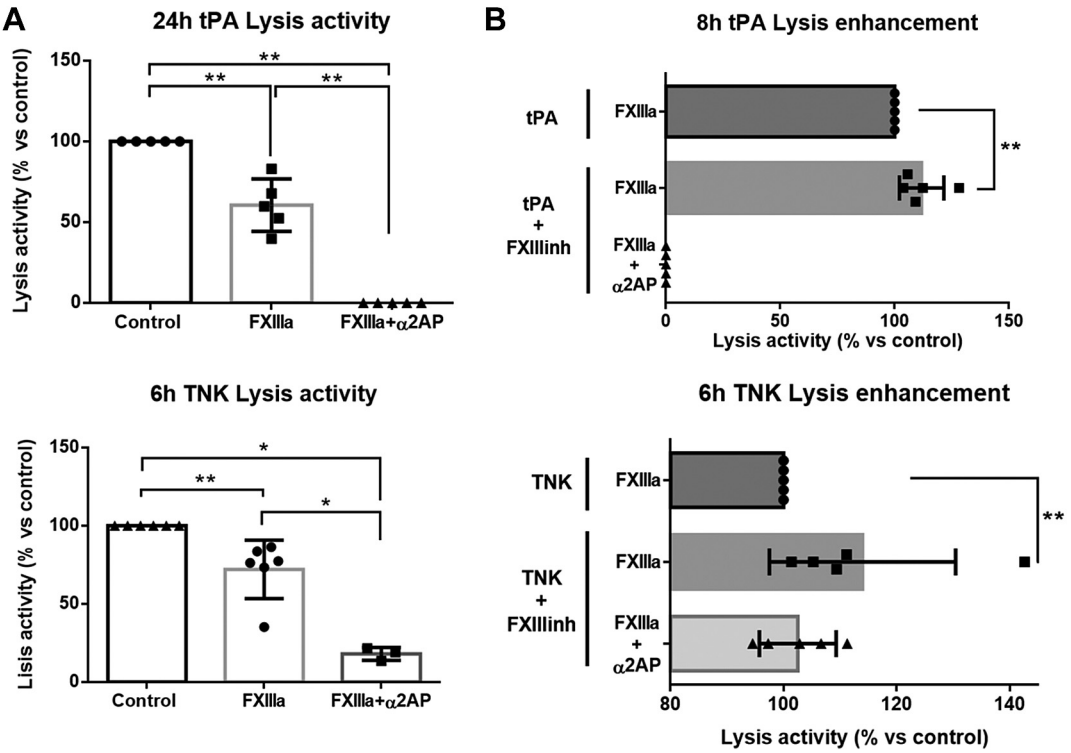

FIGURE 4 (A) Addition of activated factor XIII (FXIIIa) and α2-antiplasmin (α2AP) impairs fibrinolysis, reducing lysis activity at 24 hours for tissue plasminogen activator (tPA) (top) and at 6 hours for tenecteplase (TNK) (bottom). (B) Association of FXIII inhibitor (FXIIIinh) with fibrinolytics enhances lysis activity at 8 and 6 hours for tPA and TNK, respectively. $^{*}P < .05$; $^{**}P < .01$ by Mann–Whitney U-test.

that impairs plasmin generation. Plasmin, besides its activity on fibrin, participates in a myriad of biological processes with beneficial or detrimental effects [24], and IS models using knockout mice for fibrinolytic proteins have shown some contradictory results on infarct size [25]. Within the brain, plasmin generation by tPA has been linked to various aspects of neuronal function, including the promotion of synaptic plasticity, the increase in blood–brain barrier permeability [26–30], and the clearance of misfolded proteins, including amyloid-β [31,32]. In addition, the capacity of TXA to exert a direct effect (plasmin-independent) on inflammatory processes could be involved [24,33]. Based on these evidences, whether the observed reduction in infarct size after TXA administration could be related to the attenuation of the deleterious effects of plasmin on IS or through other mechanisms directly triggered by TXA should be further studied in future experiments.

FXIIIa-dependent crosslinking stabilizes the fibrin mesh by increasing its stiffness and making it able to withstand the activity of the endogenous fibrinolytic system better. To assess the impact of FXIIIinh on thrombus formation and lysis, we performed thromboelastometry assays in human and mouse samples. FXIIIinh significantly delayed clot formation, reduced clot burden, and enhanced tPA fibrinolysis even in the presence of an antifibrinolytic drug (TXA), although this inhibitor was not able to completely reverse the effects of TXA. These results confirmed previous data in patients, showing that the addition of FXIII improved clot formation, both alone and in combination with fibrinogen or platelet concentrate [34]. Likewise, Hethershaw et al. [3] reported a lysis reduction in fibrin clots generated in the presence of FXIII in static and flow systems, supporting an important role of FXIII in fibrin clot stiffness.

In a further step, using fibrin polymerization assays, we observed increased fiber density and decreased pore diameter in the presence of FXIII and α2AP. This is in agreement with previous data reporting a 12% increase in fiber density and 2.1-fold lower clot permeability in clots with the addition of FXIII [3]. In addition, confocal studies of clotted plasma from patients with IS also suggest an important role of FXIIIa in priming clot formation and an association between the levels of α2AP trapped in the clot and the fibrin mesh structure.

Moreover, we extended these results by investigating the effect of FXIII on lysis susceptibility to current fibrinolytic drugs. Our results revealed that FXIII supplementation decreased the fibrin lysis activity of TNK, and mainly tPA, and this effect was greater when FXIII and α2AP were present. Together, these results support FXIIIa interaction with fibrin, resulting in a less lysable network with a decreased pore size that could hinder fibrinolytic drug diffusion within the thrombus. In addition, we show an enhanced lysis with FXIIIinh even when fibrin mesh is already crosslinked by FXIII.

To assess whether this mechanism could also be operative in IS thrombi, we measured persistent FXIII activity in frozen IS thrombi and assayed *ex vivo* thrombus lysis in the presence of FXIIIinh. Here, we report that untreated IS thrombi presented persistent FXIIIa that could still be reduced by FXIIIinh. In this context, the inhibition of FXIIIa enhances tPA activity, thereby accelerating IS thrombus lysis,

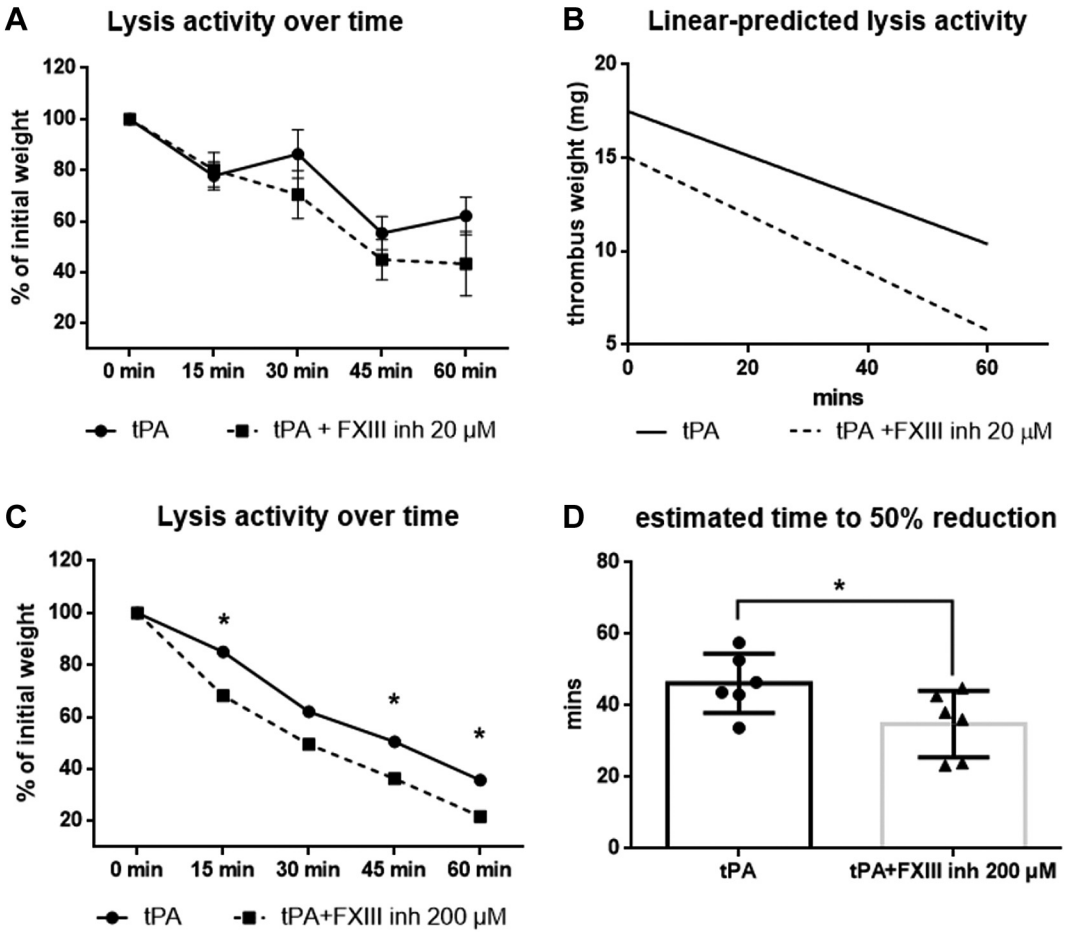

FIGURE 5 Addition of a factor XIII inhibitor (FXIIIinh) to tissue plasminogen activator (tPA) enhances *ex vivo* thrombus lysis. (A) Mean weight reduction during 60 minutes in thrombi exposed to tPA or tPA + FXIIIinh (20 µM). (B) Slope lysis activity of tPA or tPA + FXIIIinh (20 µM). (C) Mean weight reduction for 60 minutes in thrombi exposed to tPA and tPA + FXIIIinh (200 µM). $^*P < .05$ by multiple unpaired *t*-test with Holm–Sidak correction. (D) Estimated time to 50% weight reduction in thrombi exposed to tPA and tPA combined with FXIIIinh (200 µM). $^*P < .05$ by Wilcoxon test. (A and B) $n = 10$; (C and D) $n = 6$.

suggesting the possibility of using FXIII inhibitors to enhance the activity of thrombolytic drugs. The association of thrombus FXIII with inflammatory components and its potential role in binding to elements like NETs and other inflammatory proteins [7,10] suggest a potential beneficial effect of FXIII inhibition even hours after IS induction. Likewise, other thrombolytic cocktails have been previously assayed to enhance the fibrinolytic effect of current drugs [35,36]; so targeting specific mechanisms implicated in thrombolytic drug resistance remains promising. Moreover, platelet aggregation [37], clot retraction [38], and stretching [39] have also been described as FXIII-mediated cofactors implicated in thrombus lysis resistance. Similarly, FXIIIa-mediated α2AP retention in the clot seems to be critical to avoid α2AP expelling during clot retraction, and addition of α2AP prior to clot formation has been shown to reduce its lysis [40].

All these evidences indicate that FXIII exerts a wide range of effects on thrombus structure and composition that are not limited to fibrin crosslinking, opening an avenue to the use of FXIII inhibitors to achieve anticoagulation with limited bleeding risk [41] and supporting its use in IS as an adjuvant treatment in the acute phase.

We also investigated the expression of FXIII in human IS thrombi, which was mainly observed in the junction areas between platelet and RBC-rich regions, colocalizing with inflammatory cells. FXIII released by platelets and monocytes could promote fibrin fiber crosslinking along platelet-rich areas [42]. These findings support previous studies showing lesser dense packing of fibers and higher fiber diameters in RBC-rich areas [38], while more dense areas are present in platelet-rich regions [43], and suggest a different fibrin network between thrombus areas depending on cell interaction. It is still debated whether FXIII only mediates thrombosis or could also be involved in local inflammation, activation and recruitment of neutrophils at the thrombus level, and platelet–neutrophil interplay as major contributors to lysis resistance [43] and poststroke damage [44]. In our study, thrombus FXIII was associated with NETs, VWF, and NE within thrombi that could increase the fibrin clot density and impair fibrinolysis as previously described [7,43,45]. Similarly, Locke et al. [10] showed that fibrinolysis inhibition by NETs could depend on FXIIIa-mediated covalent crosslinking of histones to fibrin. Furthermore, FXIII-mediated VWF crosslinking into the fibrin network promotes

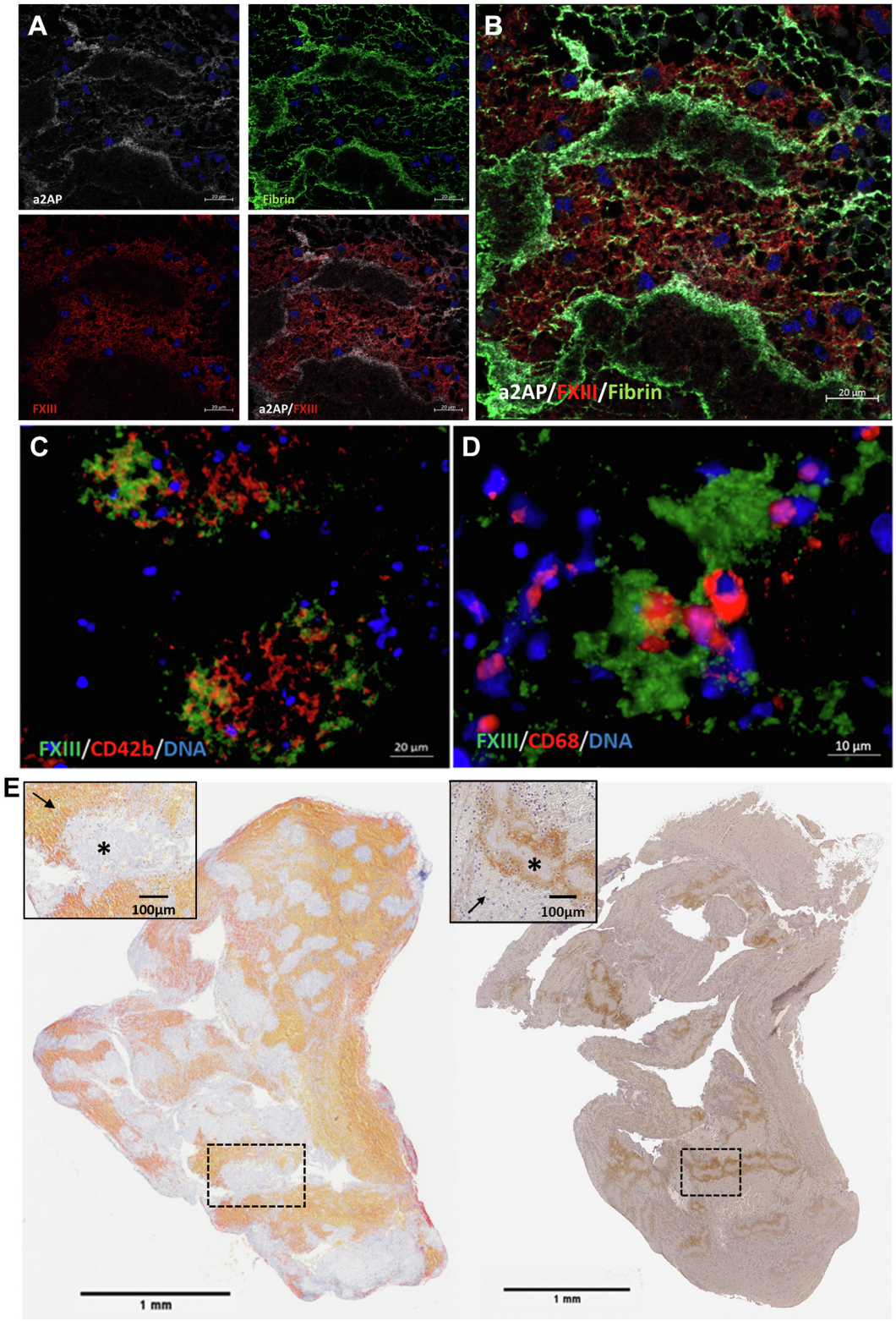

FIGURE 6 Factor XIII (FXIII) colocalizes with fibrin, α2-antiplasmin (α2AP), platelets, and macrophages in ischemic stroke thrombi. (A) Triple immunofluorescence for FXIII, α2AP, and fibrin, showing single channels and the colocalization of α2AP/FXIII. (B) Merging of α2AP, FXIII, and fibrin channels. (C) Double immunofluorescence for FXIII and platelets $CD42b^{+}$. (D) Double immunofluorescence for FXIII and macrophages/monocytes $CD68^{+}$. (E) Representative thrombus sections stained with Martius Scarlet Blue (red blood cells-yellow; fibrin-pink) on the left side and FXIII (stained in brown) on the right side where FXIII seems to be interspersed in platelet-rich areas (asterisk) surrounding red blood cells areas (arrow).

TABLE Associations of thrombus factor XIII–stained area quantification (n = 85) with other thrombus and blood components using a linear regression model.

Thrombus FXIII	exp(B)	*P* value	*n*
RBC (MSB)	1.11 (0.63-1.97)	.706	57
Platelets ($CD42b^+$)	1.51 (0.93-2.44)	.095	57
Leukocytes ($CD45^+$)[a]	1.44 (0.23-9.20)	.692	55
Macrophages ($CD68^+$)[a]	0.99 (0.71-1.37)	.938	55
T lymphocytes ($CD3^+$)[a]	0.89 (0.71-1.11)	.292	56
Fibrin (MSB)	1.02 (0.84-1.23)	.866	57
VWF	**1.78 (1.28-2.46)**	**.001**	**57**
α2AP	0.96 (0.71-1.31)	.789	54
NETs	**2.20 (1.16-4.20)**	**.017**	**54**
NE	**1.28 (1.06-1.54)**	**.010**	**85**
Blood levels			
FXIII activity	1.05 (0.91-1.21)	.499	54
α2AP	1.00 (0.78-1.28)	.987	51

α2AP, α2-antiplasmin; FXIII, factor XIII; MSB, Martius Scarlet Blue; NE, neutrophil elastase; NET, neutrophil extracellular trap; RBC, red blood cell; VWF, von Willebrand factor. Statistically significant associations are presented in bold type.
[a] Cellularity.

thromboinflammation by leukocyte recruitment and platelet activation [7]. In addition, after platelet activation, cellular FXIII is exposed in the cap region rich in procoagulant phosphatidylserine [23] with potential direct effects on thromboinflammation [46]. The potential involvement of FXIII in thromboinflammation suggests that the beneficial effect of FXIII inhibition observed in stroke experimental models may rely not only on its involvement in clot formation, characteristics, and resistance to fibrinolysis but also on the modulation of inflammation both within the thrombus and in the brain tissue. In this regard, further studies are needed to delve deeper into the potential beneficial role of FXIII inhibition and its potential relationship with thromboinflammation.

Finally, plasma FXIII activity was measured in our patients with IS and associated with concentrations of fibrin and α2AP in thrombi, supporting its role in the crosslinking of both elements. This direct association between plasma FXIII levels and α2AP and fibrin within thrombi was previously reported *in vitro* by Bagoly et al. [15] and was not associated with the worst functional outcome and recanalization rates.

There are some limitations in this report. First, any blockade of transglutaminases other than FXIII by ZED1301 with direct effects on observed results cannot be ruled out. Second, ROTEM functional tests were performed to evaluate how FXIII inhibition alters *ex vivo* clot formation and lysis, but this technique reflects static low-flow thrombus generation, which is more similar to venous conditions rather than arterial thrombosis. Furthermore, stroke thrombi included in the lysis assay had a wide range of sizes, and the potential lysis effect of the fibrinolytic drugs, although closer to clinical variability, could have been influenced by thrombus lysis due to the contact area. Moreover, tPA treatment in patients with IS can be an important confounding factor, as we have seen in the plasma clotting experiments. Finally, since all included patients underwent mechanical thrombectomy with thrombotic material retrieval, and thus the thrombi are inherently "fibrinolysis-resistant," this study is constrained in its ability to explore *in vivo* the potential link between FXIII and α2AP concentrations in thrombi and their resistance to current fibrinolytic treatments.

5 | CONCLUSION

Our results suggest a key role for FXIII in thrombus stabilization, resulting in a larger infarct size and the worst outcome in experimental IS. Furthermore, targeting FXIII could enhance the efficacy of fibrinolytic drugs and reduce resistance to lysis, opening new ways to improve therapies for patients with IS.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to all of the following: (1) the conception and design of the study, acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, and (3) final approval of the version to be submitted. J.M.-E., M.N.-O., F.J.D.M.M., J.-A.R., J.-A.P., C.R., R.M.-A., and J.O. designed and performed the experiments, analyzed the data, and drafted the manuscript. R.B., N.A., M.H., B.Z., J.P., J.J., and J.M.-M. contributed to the data acquisition and provided intellectual input. All authors read and approved the final version of the paper.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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