A novel method to quantify fibrin-fibrin and fibrin-α2AP cross-links in thrombi formed from human trauma patient plasma.

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2024

jth

ORIGINAL ARTICLE

A novel method to quantify fibrin–fibrin and fibrin–α₂-antiplasmin cross-links in thrombi formed from human trauma patient plasma

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Abstract

Background: The widespread use of the antifibrinolytic agent, tranexamic acid (TXA), interferes with the quantification of fibrinolysis by dynamic laboratory assays such as clot lysis, making it difficult to measure fibrinolysis in many trauma patients. At the final stage of coagulation, factor (F)XIIIa catalyzes the formation of fibrin–fibrin and fibrin–α₂-antiplasmin (α₂AP) cross-links, which increases clot mechanical strength and resistance to fibrinolysis.

Objectives: Here, we developed a method to quantify fibrin–fibrin and fibrin–α₂AP cross-links that avoids the challenges posed by TXA in determining fibrinolytic resistance in conventional assays.

Methods: Fibrinogen alpha (FGA) chain (FGA–FGA), fibrinogen gamma (FGG) chain (FGG–FGG), and FGA–α_2AP cross-links were quantified using liquid chromatography–mass spectrometry (LC–MS) and parallel reaction monitoring in paired plasma samples from trauma patients prefibrinogen and postfibrinogen replacement. Differences in the abundance of cross-links in trauma patients receiving cryoprecipitate (cryo) or fibrinogen concentrate (Fg-C) were analyzed.

Results: The abundance of cross-links was significantly increased in trauma patients postcryo, but not Fg-C transfusion ($P < .0001$). The abundance of cross-links was positively correlated with the toughness of individual fibrin fibers, the peak thrombin concentration, and FXIII antigen ($P < .05$).

Conclusion: We have developed a novel method that allows us to quantify fibrin cross-links in trauma patients who have received TXA, providing an indirect measure of fibrinolytic resistance. Using this novel approach, we have avoided the effect of TXA and shown that cryo increases fibrin–fibrin and fibrin–α_2AP cross-linking when compared with Fg-C, highlighting the importance of FXIII in clot formation and stability in trauma patients.

KEYWORDS
cross-linking, fibrinogen, fibrinolysis, trauma coagulopathy, tranexamic acid

1 | INTRODUCTION

Traumatic injury accounts for 4.9 million deaths globally every year [1] and is the leading cause of death in persons under the age of 44 years [2]. Uncontrolled bleeding accounts for 1.2 million deaths each year, 25% of all injury-related deaths [3], and is exacerbated by the development of trauma-induced coagulopathy (TIC). TIC is associated with an increased need for massive transfusion and 3- to 4-fold increased risk of death [4]

Major studies have shown that there are 2 fundamental changes that underpin major hemorrhage during TIC: hypofibrinogenemia (fibrinogen depletion) and hyperfibrinolysis (excessive clot degradation) [5]. Fibrinogen is the key procoagulant factor for stable clot formation and is the first coagulation protein to reach critically low levels during traumatic hemorrhage [6–8]. Fibrinogen is cleaved by thrombin to insoluble fibrin, a viscoelastic polymer that is crucial in determining the physical and mechanical characteristics of the clot [9]. At the final stage of coagulation, the transglutaminase, activated factor (F)XIII, catalyzes the formation of cross-links between neighboring fibrin molecules to enhance clot stability against mechanical stress [10,11]. FXIIIa also exerts an antifibrinolytic effect by cross-linking the principal fibrinolysis inhibitor, alpha 2-antiplasmin (a_2AP), to fibrin to stabilize the clot against premature degradation by plasmin [12,13] and thus plays a key role in regulating fibrinolysis. Early during TIC, fibrinolysis is amplified, demonstrated by the increased levels of plasmin–a_2AP (PAP) complexes, D-dimer (degradation product of cross-linked fibrin), and tissue plasminogen activator (tPA) [14]. Depletion of fibrinolytic inhibitors, including a_2AP and plasminogen activator inhibitor 1 (PAI-1), is a likely contributor to hyperfibrinolysis in TIC.

The antifibrinolytic agent, tranexamic acid (TXA), is administered to trauma patients to stop bleeding. The CRASH-2, CRASH-3, and WOMAN trials found that early antifibrinolytic treatment with TXA effectively reduced mortality in trauma and obstetric hemorrhage [15–17]. TXA is widely used to treat patients with major hemorrhage due to its effectiveness and low cost. TXA inhibits fibrinolysis by binding to the lysine binding sites of plasminogen. This prevents plasminogen binding to fibrin and its activation to plasmin [18]. Consequently, TXA interferes with commonly used dynamic fibrinolysis tests such as the clot lysis assay, and alternative approaches are required to clearly understand the effects of factor replacement therapy on clot strength and susceptibility to fibrinolysis in trauma patients. Here we describe a novel method to indirectly assess clot susceptibility to fibrinolysis that avoids the effect of TXA by quantification of fibrin–fibrin and fibrin–α_2AP cross-links in trauma patients recruited to the Fibrinogen Early In Severe Trauma studY (FEISTY; NCT02745041) [19,20]. FEISTY trial participants were randomized to receive one of 2 fibrinogen replacement therapies: either cryoprecipitate (cryo) or fibrinogen concentrate (Fg-C).

Our previous data have shown that FXIII levels were significantly increased postcryo transfusion and significantly decreased post-Fg-C transfusion. Analysis of individual fibrin fibers revealed that clots formed from patients receiving cryo were composed of fibers that were more resistant to mechanical disruption [21]. Coagulation FXIIIa plays a critical role in the formation of fibrin–fibrin and fibrin–α_2AP cross-links [10–12]; therefore, we hypothesized that the abundance of cross-links may differ between patients who received cryo and Fg-C. Here, we utilize liquid chromatography–mass spectrometry (LC–MS) to quantify cross-linked peptides in clots formed from FEISTY trauma
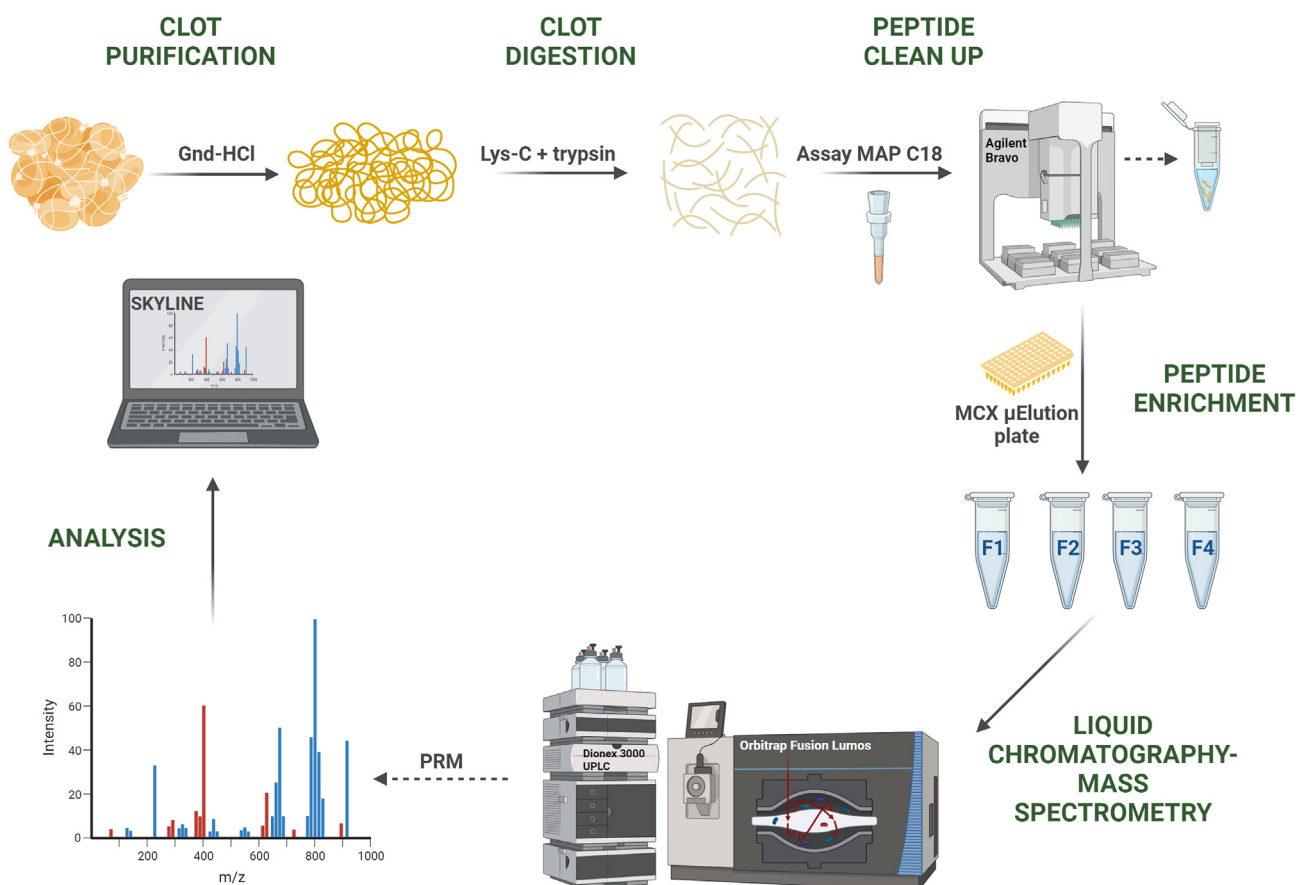
FIGURE 1 Mass spectrometry workflow. A schematic outline of the clot formation and its subsequent degradation for mass spectrometry analysis of fibrin cross-links. The fibrin clot is formed by addition of thrombin and calcium to trauma patient plasma. After clot formation, the clot undergoes a series of washing steps with Gnd-HCl to remove any constituents that are not covalently linked to the fibrin clot. The clot is then subjected to proteolytic digestion with trypsin and Lys-C to cleave carboxyterminal Arg and Lys residues. Digested peptides are subsequently desalted on AssayMAP C18 cartridges using the Agilent Bravo liquid handling platform prior to fractionation on an MCX µElution plate. The resulting cross-link–enriched peptide samples are separated by nano-HPLC and analyzed by an Orbitrap Fusion Lumos mass spectrometer operated in PRM mode. The resulting MS data were processed using Skyline to enable relative quantification of the selected cross-links in each trauma patient sample. HPLC, high-performance liquid chromatography; MCX, mixed-mode cationic exchange; MS, mass spectrometry; PRM, parallel reaction monitoring.

patient plasma (Figure 1) [22]. Parallel reaction monitoring (PRM) [23] was used to target cross-links between fibrinogen alpha (FGA) chains (FGA–FGA), fibrinogen gamma (FGG) chains (FGG–FGG), and fibrinogen alpha (FGA) and α_2AP (FGA–SERPINF2) chains. We report using LC–MS to quantify the relative abundance of cross-links as a novel method to indirectly quantify fibrinolytic resistance in patients who receive TXA and adjunctive transfusion therapy.

2 | METHODS

2.1 | Eligibility criteria and randomization

Full details of the FEISTY study are available [20,21]. Patients were randomly assigned to receive either Fg-C (Riastap, CSL Behring) or cryo (whole blood or apheresis cryo, Australian Red Cross Lifeblood) replacement during active trauma hemorrhage. All blood samples were

collected in 0.13 M trisodium citrate vacutainers and platelet-poor plasma was obtained by centrifugation of whole blood samples at 2500 g for 30 minutes at 4 °C. Plasma samples were stored at −80°C until analysis. Baseline characteristics of the patient cohorts were previously published and included as Supplementary Table S1 [21].

2.2 | Mass spectrometry quantification of fibrin cross-links

A workflow is shown in Figure 1.

2.2.1 | Clot formation

Plasma clots were formed at 37 °C for 30 minutes and clotting was initiated by addition of 1 nM thrombin (Sigma Aldrich), 2 mM MgCl₂,

and 18 mM CaCl₂. Clots were centrifuged at 17 000 *g* for 1 minute and the supernatant was discarded.

2.2.2 | Clot purification

Clots were treated with 6 M guanidine hydrochloride (Gnd-HCl) and 100 mM ammonium bicarbonate (ABC; pH 9) to remove any constituents that are not covalently linked to the fibrin clot. The tubes were placed on a vortex shaker for 5 minutes at room temperature. Clots were centrifuged at 17 000 *g* for 1 minute to remove supernatant. This purification step was repeated 3 times to ensure that the clots were translucent each time the vortex time was extended to 15 minutes, 30 minutes, and finally 16 hours, which was performed at 4 °C.

2.2.3 | Clot digestion

Our protocol was adapted from Schmitt et al. [24]. Clots were treated with hydroxylamine solution (1 M NH_2OH-HCl, 4.5 M Gnd-HCl, 0.2 M K_2CO_3; pH 9) at 45 °C on a vortex shaker for 16 hours. Following incubation, the samples were centrifuged at 17 000 *g* for 1 minute and the supernatant was removed. Clots were stored at −80 °C until ready for analysis. Prior to mass spectrometry analysis, clots were subjected to proteolytic digestion. Clots were washed with 100 mM triethylammonium bicarbonate buffer (TEAB; pH 8.5) prior to centrifugation at 17 000 *g* for 2 minutes at 4 °C and removal of the supernatant. Proteolytic digestion was performed using 25-μg PTMScan Lys-C Protease (Cell Signaling Technology) and 5 μg L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin (Sigma Aldrich) in 100 mM TEAB at 300 rpm for 16 hours at 37 °C. A further 5 μg L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin was added, and the digestion continued for another 2.5 hours.

2.2.4 | Peptide clean up

Following enzymatic digestion, 1% trifluoracetic acid was added to acidify the digests and the samples were centrifuged at 17 000 *g* for 30 minutes at 4 °C. The supernatant was desalted on C18 cartridges using the AssayMAP Peptide Cleanup protocol on the Agilent Bravo liquid handling platform (all from Agilent). Eluates were dried by vacuum centrifugation and stored at −80 °C.

2.2.5 | Enrichment of cross-linked peptides

Our protocol was adapted from Schmitt et al. [24]. Peptides were reconstituted in 50 μL 0.1% acetic acid prior to enrichment of hydrophobic peptides, including cross-linked peptides, on an Oasis mixed-mode cationic exchange (MCX) 96-well μElution Plate (Waters). Prior to sample addition, the MCX plate was primed with methanol

(MeOH) and then equilibrated twice with 200 μL wash buffer (500 mM ammonium acetate, 40% methanol, 0.5% acetic acid). The sample was applied and washed with another 200 μL wash buffer and then eluted in 4 50 μL fractions (F1-F4). Peptides were first eluted in 1 M ammonium acetate, 40% MeOH, and 0.5% acetic acid (F1), followed by 2 elutions with 2 M ammonium acetate, 40% MeOH, and 0.5% acetic acid (F2 and F3) and finally with 2 M ammonium acetate, 80% MeOH, and 0.5% acetic acid (F4). The eluted fractions were dried by vacuum centrifugation and later reconstituted in 30 μL 3% acetonitrile and 0.1% formic acid.

2.2.6 | LC–MS

Cross-link–enriched peptide samples were analyzed on a Dionex 3000 Ultimate high-performance liquid chromatography (HPLC) system coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). The peptides were loaded onto a trap column (PepMapC18; 300 μm × 5 mm, 5 μm particle size; Thermo Fisher Scientific) for 1 minute at a flowrate of 20 μL/min. The loaded peptides were separated on a 75 μm × 500 mm C18 EASY-Spray chromatographic column (Thermo Fisher Scientific) with a 2 to 35% acetonitrile gradient in 0.1% formic acid and 5% DMSO at a flow rate of 250 nL/min for 60 minutes. The Orbitrap Fusion Lumos was operated in PRM mode within a 10-minute retention window (Table 1). The MS1 spectra were acquired at a scan range of 350 to 1500 m/z at 120 000 resolution, an automated gain control target of 4×10^5, and a maximum injection time of 50 ms. MS2 spectra were acquired over a scan range of 400 to 1500 m/z at 7500 resolution, an isolation window of 1.2 m/z, and 25% higher-energy collision dissociation. Extracted ion chromatograms (XICs) of cross-linked peptides were generated from the raw data using Skyline v22.2 (MacCoss Lab, University of Washington) [25]. XICs were assessed for retention time alignment of transitions, mass error ppm (maximum ppm: 10), and the similarity of the measured fragment spectrum to the library spectrum (dotp value in Skyline). The sum of the fragment peak intensities (max height) and total fragment area were extracted from Skyline. The total fragment area parameter was used for quality control and the fragment peak intensity was used to quantify the cross-linked peptides (cross-link abundance).

2.3 | FXIII antigen

FXIII antigen levels were determined using a commercial enzyme-linked immunosorbent assays kit (Abcam).

2.4 | Thrombin generation

TG was triggered with 1 pM tissue factor in the presence of 4 μM phospholipids, $CaCl_2$, and a fluorogenic substrate for thrombin (Diagnostica Stago). Thrombin generation was measured using the

T A B L E 1 Parallel reaction monitoring method.

Protein name	Peptide cross-link	m/z	z	Retention time
FGA–FGA	ALTDMPQMR-GHAKSRPV-[Fibrin Î³-Î³ crosslink@7,4]	632.6575	3	34.87
FGA–FGA	ALTDMPQMR-GHAKSRPV-[Fibrin Î³-Î³ crosslink@7,4]	474.7449	4	34.87
FGA–FGA	ALTDMPQMR-GKSSSYSK-[Fibrin Î³-Î³ crosslink@7,2]	944.4506	2	33.05
FGA–FGA	ALTDMPQMR-GKSSSYSK-[Fibrin Î³-Î³ crosslink@7,2]	629.9695	3	33.05
FGA–FGA	ALTDMPQMR-GKSSSYSK-[Fibrin Î³-Î³ crosslink@7,2]	472.7289	4	33.05
FGA–FGA	ALTDMPQMR-LVTSKGDK-[Fibrin Î³-Î³ crosslink@7,5]	946.4844	2	32.39
FGA–FGA	ALTDMPQMR-LVTSKGDK-[Fibrin Î³-Î³ crosslink@7,5]	631.3254	3	32.39
FGA–FGA	ALTDMPQMR-LVTSKGDK-[Fibrin Î³-Î³ crosslink@7,5]	473.7459	4	32.39
FGA–FGA	ALTDMPQMR-TGKEK-[Fibrin Î³-Î³ crosslink@7,3]	536.2691	3	34.39
FGA–FGA	EKVTSGSTTTTR-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@2,7]	578.786	4	48.17
FGA–FGA	EYHTEKLVTSK-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@6,7]	793.7276	3	49.16
FGA–FGA	GDSTFESKSYK-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@8,7]	765.0207	3	44.39
FGA–FGA	GDSTFESKSYK-QFTSSTSYNR-[Fibrin Î³-Î³ crosslink@8,1]	807.6995	3	53.72
FGA–FGA	GSESGIFTNTKESSSHHPGIAEFPSR-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@11,7]	761.5609	5	48.33
FGA–FGA	GSESGIFTNTKESSSHHPGIAEFPSR-QFTSSTSYNR-[Fibrin Î³-Î³ crosslink@11,1]	983.7084	4	54.24
FGA–FGA	GSESGIFTNTKESSSHHPGIAEFPSR-QFTSSTSYNR-[Fibrin Î³-Î³ crosslink@11,1]	787.1682	5	54.24
FGA–FGA	GSESGIFTNTKESSSHHPGIAEFPSR-QFTSSTSYNRGDSTFESK-[Fibrin Î³-Î³ crosslink@11,1]	957.4414	5	59.36
FGA–FGA	MADEAGSEADHEGTHSTKR-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@18,7]	1025.118	3	38.5
FGA–FGA	MADEAGSEADHEGTHSTKR-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@18,7]	769.0907	4	38.5
FGA–FGA	MADEAGSEADHEGTHSTKR-QFTSSTSYNR-[Fibrin Î³-Î³ crosslink@18,1]	801.0998	4	52.16
FGA–FGA	MADEAGSEADHEGTHSTKR-QFTSSTSYNRGDSTFESK-[Fibrin Î³-Î³ crosslink@18,1]	811.3545	5	39.99
FGA–FGA	MADEAGSEADHEGTHSTKRGHAK-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@18,7]	1733.78	2	49.96
FGA–FGA	MADEAGSEADHEGTHSTKRGHAK-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@18,7]	1156.189	3	49.96
FGA–FGA	MADEAGSEADHEGTHSTKRGHAK-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@18,7]	867.3938	4	49.96
FGA–FGA	MKPVPDLVPGNFKSQLQK-ALTDMPQMR-[Fibrin Î³-Î³ crosslink@13,7]	614.9235	5	42.24
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPR-MADEAGSEADHEGTHSTKR-[Fibrin Î³-Î³ crosslink@25,18]	1208.269	4	53.31
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPR-MADEAGSEADHEGTHSTKR-[Fibrin Î³-Î³ crosslink@25,18]	966.8168	5	53.31
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-EKVTSGSTTTTR-[Fibrin Î³-Î³ crosslink@25,2]	1371.371	4	58.63
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-EKVTSGSTTTTR-[Fibrin Î³-Î³ crosslink@25,2]	1097.299	5	58.63
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-GDSTFESKSYK-[Fibrin Î³-Î³ crosslink@25,8]	1093.484	5	41.82
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-GKSSSYSK-[Fibrin Î³-Î³ crosslink@25,2]	1265.314	4	56.42

TABLE 1 (Continued)

Protein name	Peptide cross-link	*m/z*	*z*	Retention time
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-GKSSSYSK-[Fibrin γ-γ crosslink@25,2]	1012.453	5	56.42
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-LVTSKGDK-[Fibrin γ-γ crosslink@25,5]	1266.331	4	53.22
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-LVTSKGDK-[Fibrin γ-γ crosslink@25,5]	1013.266	5	53.22
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-SSSYSKQFTSSTSYNR-[Fibrin γ-γ crosslink@25,6]	1209.735	5	49.83
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-TVTKTVIGPDGHK-[Fibrin γ-γ crosslink@25,4]	1392.647	4	60.29
FGA–FGA	PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-TVTKTVIGPDGHK-[Fibrin γ-γ crosslink@25,4]	1114.319	5	60.29
FGA–FGA	QFTSSTSYNRGDSTFESK-GKSSSYSK-[Fibrin γ-γ crosslink@1,2]	717.5796	4	43.48
FGA–FGA	QFTSSTSYNRGDSTFESK-LVTSKGDK-[Fibrin γ-γ crosslink@1,5]	718.5965	4	44.31
FGA–FGA	QFTSSTSYNRGDSTFESK-MKPVPDLVPGNFK-[Fibrin γ-γ crosslink@1,2]	1155.893	3	49.52
FGA–FGA	QFTSSTSYNRGDSTFESKSYK-ALTDMPQMR-[Fibrin γ-γ crosslink@18,7]	866.8988	4	47.46
FGA–FGA	SSSYSKQFTSSTSYNR-ALTDMPQMR-[Fibrin γ-γ crosslink@6,7]	958.7726	3	41.24
FGA–FGA	SSSYSKQFTSSTSYNR-ALTDMPQMR-[Fibrin γ-γ crosslink@6,7]	719.3313	4	41.24
FGA–FGA	SSSYSKQFTSSTSYNRGDSTFESK-ALTDMPQMR-[Fibrin γ-γ crosslink@6,7]	932.1728	4	49.49
FGA–FGA	SSSYSKQFTSSTSYNRGDSTFESK-ALTDMPQMR-[Fibrin γ-γ crosslink@6,7]	745.9397	5	49.49
FGA–FGA	TVTKTVIGPDGHK-ALTDMPQMR-[Fibrin γ-γ crosslink@4,7]	600.0621	4	43.24
FGG–FGG	KCHAGHLNGVYYQGGTYSK-FEGNCAEQDGSGWWM[+16]N-[Fibrin γ-γ crosslink@1,8]	978.6622	4	50.18
FGG–FGG	KEGFGHLSPTGTTEFWLGNEK-NWIQY-[Fibrin γ-γ crosslink@1,4]	1014.154	3	44.56
FGG–FGG	KFEGNCAEQDGSGWWM[+16]NK-FEGNCAEQDGSGWWM[+16]N-[Fibrin γ-γ crosslink@1,8]	1311.177	3	56.78
FGG–FGG	KMLEEIMKYEASILTHDSSIR-AIQLTYNPDESSK-[Fibrin γ-γ crosslink@8,3]	986.2432	4	52.13
FGG–FGG	FFTSHNGMQFSTWDNDNDKFEGN-SHNGMQFSTWDNDNDK-[Fibrin γ-γ crosslink@19,6]	1539.288	3	44.58
FGG–FGG	FFTSHNGMQFSTWDNDNDKFEGN-SHNGMQFSTWDNDNDK-[Fibrin γ-γ crosslink@19,6]	1154.718	4	44.58
FGA-SERPINF2	LGNQEPGGQTALK-GKSSSYSK-[Fibrin γ-γ crosslink@9,2]	535.2736	4	23.76
FGA-SERPINF2	M[+16]ADEAGSEADHEGTHSTKR-LGNQEPGGQTALK-[Fibrin γ-γ crosslink@18,9]	835.634	4	31.71
FGA-SERPINF2	GSESGIFTNTKESSSHHPGIAEFPSR-LGNQEPGGQTALK-[Fibrin γ-γ crosslink@11,9]	1014.244	4	50.41
FGA-SERPINF2	LGNQEPGGQTALK-GDSTFESKSYK-[Fibrin γ-γ crosslink@9,8]	848.4135	3	51.9
FGA-SERPINF2	QLTSGPNQEQVSPLTLLK-SSSYSKQFTSSTSYNR-[Fibrin γ-γ crosslink@8,6]	941.9708	4	47.77
FGA-SERPINF2	TVTKTVIGPDGHK-LGNQEPGGQTALK-[Fibrin γ-γ crosslink@4,9]	662.6067	4	29.69
FGA-SERPINF2	GKSSSYSK-DFLQSLK-[Fibrin γ-γ crosslink@2,4]	838.4305	2	48.24
FGA-SERPINF2	SSSYSKQFTSSTSYNR-LGNQEPGGQTALK-[Fibrin γ-γ crosslink@6,9]	781.8759	4	50.29

(Continues)

TABLE 1 (Continued)

Protein name	Peptide cross-link	m/z	z	Retention time
FGA-SERPINF2	NQEQVSPLTLLK-GDSTFESKSYK-[Fibrin Î³-Î³ crosslink@2,8]	867.4412	3	59.31
FGA-SERPINF2	LGNQEPGGQTALK-EKVTSGSTTTTR-[Fibrin Î³-Î³ crosslink@9,2]	641.3306	4	45.97
FGA-SERPINF2	TVTKTVIGPDGHK-NQEQVSPLTLLK-[Fibrin Î³-Î³ crosslink@4,2]	676.8775	4	42.85
FGA-SERPINF2	NQEQVSPLTLLK-EYHTEKLVTSK-[Fibrin Î³-Î³ crosslink@2,6]	672.3629	4	48.1
FGA-SERPINF2	SSSYSKQFTSSTSYNR-NQEQVSPLTLLK-[Fibrin Î³-Î³ crosslink@6,2]	1061.193	3	51.77

PRM mass spectrometry was utilized for analysis of FGA–FGA, FGG–FGG, and FGA-SERPINF2 cross-linked peptides in clots formed from FEISTY trauma patient samples. The table lists the protein name, peptide cross-link sequence, mass to charge ratio (*m/z*), charge (*z*), and the elution window in which the peptides were detected (retention time). These data were used as the method on the MS for detection of the cross-linked peptides.

calibrated automated thrombogram (CAT) [26] and thrombinoscope v5 software. The peak height and endogenous thrombin potential parameters were extracted from the thrombogram and exported for statistical analysis.

2.5 | Lateral atomic force microscopy

This method is described in detail by Duval et al. [27]. Briefly, plasma was diluted to obtain a fibrinogen concentration of 0.5 mg/mL and clotting was initiated with 0.5-U/mL thrombin and 10.6 mM CaCl$_2$. Clots were allowed to form for 1.5 hours in a humid chamber prior to a washing step with Tris buffered saline. Fibers were then incubated with 20-nm yellow–green carboxylate FluoSpheres (Thermo Fisher Scientific) for 10 minutes and then washed again. A MFP3D atomic force microscope (Asylum Research, Oxford Instruments) combined with an Axiovert 200 optical fluorescence microscope (Zeiss) were used to measure the mechanical response of individual fibrin fibers upon lateral stretching. Individual fibers were pulled by CSC38 cantilevers (MikroMasch) until rupture, while the deformation was visualized with the fluorescence microscope. For each fiber, a stress (calculated from the lateral defection of the cantilever) vs strain (calculated from the position of the cantilever) curve was plotted and analyzed to obtain a range of parameters to quantify fiber mechanical properties. In this work, we report on toughness. Toughness is calculated as the area under the stress–strain curve and corresponds to the amount of energy required to rupture the fiber.

2.6 | Data analysis

Results are expressed as mean and SD. Chord diagrams were prepared in Flourish. Statistical analysis was performed using GraphPad Prism Software (v9.2.0). Normal distribution was assessed using a D'Agostino–Pearson omnibus test. Statistical significance prefibrinogen and postfibrinogen replacement was determined using a paired *t*-test (if the data were normally distributed) or nonparametric Wilcoxon matched-pairs signed rank test (if the data were not normally distributed). $P < .05$ was considered significant. Correlations were analyzed using Spearman correlation coefficients.

3 | RESULTS

Initial experiments utilized untargeted LC–MS analysis of fibrin clots formed from FEISTY trauma patient plasma to identify proteins of interest (fibrinogen). Analysis of the untargeted LC–MS spectral count revealed that the FGA, fibrinogen beta (FGB), and FGG chains were the most abundant proteins in the fibrin clot (Figure 2A). FGA, FGB, and FGG had a spectral count of 100, 63, and 70, respectively. An abundance of other plasma proteins was identified, including, but not limited to, serine proteases and their inhibitors (plasmin, α_1-antitrypsin, and α_2AP), lipoproteins (apolipoproteins A and B), extracellular matrix proteins (fibronectin and albumin), procoagulant factors (von Willebrand factor), complement factors (C3, C4A, and C4B), and contractile and structural proteins (actin, myosin, keratin, and talin) (Figure 2B).

We then developed a targeted LC–MS method to quantify the abundance of FGA–FGA, FGG–FGG, and FGA–SERPINF2 cross-links in fibrin clots formed from FEISTY trauma patient plasma (Table 1, Figure 1). Using the spectral count from the initial untargeted experiments, we utilized PRM to identify cross-links in fibrin clots formed from FEISTY trauma patient plasma (Table 1). The MS identified the cross-links based on their known mass and charge within a known time frame (the retention time, Table 1).

Analysis of the PRM LC–MS spectra identified 44 different cross-linked peptides in fibrin clots formed from FEISTY trauma patient plasma: 26 FGA–FGA, 5 FGG–FGG, and 13 FGA–SERPINF2 cross-links (Figure 3). Analysis of the cross-linked peptides in FEISTY trauma patient plasma was performed using Skyline [25] by extracting the max height (cross-link abundance) and total area parameters. We used fragment peak intensity to quantify fragment abundance, reasoning that it was less vulnerable to signal-to-noise differences between samples; however, the correlation with fragment total area in any case was extremely high ($r^2 = 0.98$, $P < .0001$; Figure 4A).

The FEISTY trauma patients were analyzed based on the fibrinogen replacement therapy they received (cryo or Fg-C) and were split into 4 groups; precryo transfusion, postcryo transfusion, pre–Fg-C transfusion, and post–Fg-C transfusion. Patients who received cryo had a 2.2-fold increase in FGA–FGA cross-links, 1.8-fold increase in FGG–FGG cross-links, and 1.4-fold increase in FGA–SERPINF2 cross-links ($P < .0001$; Figure 4B–D). In contrast, patients who received Fg-C
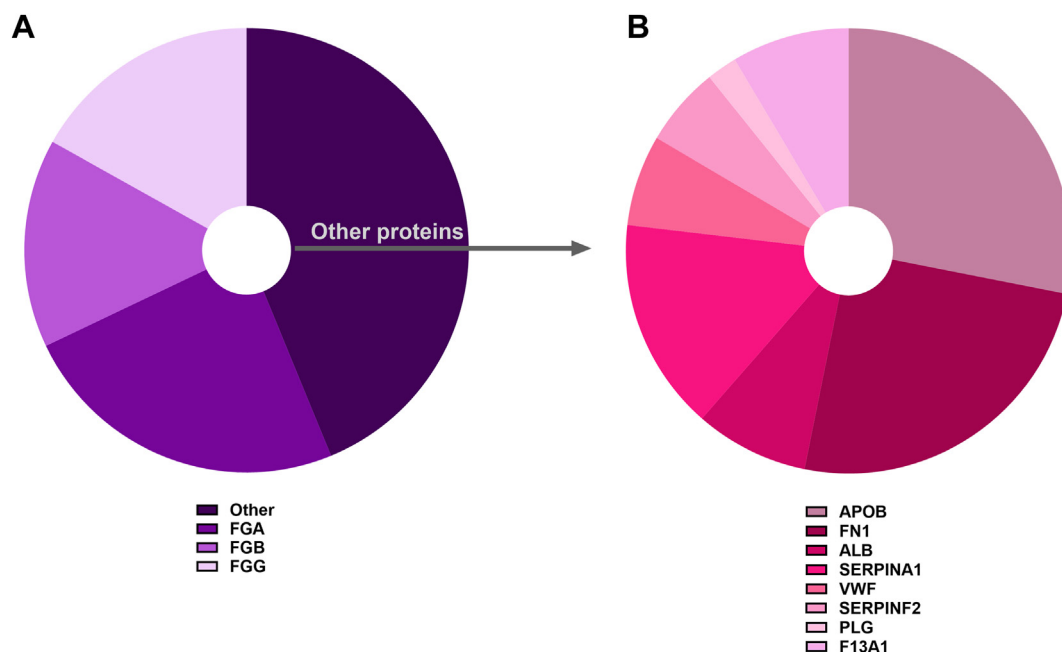
FIGURE 2 Protein abundance and identification of cross-links in the fibrin clot. Pie charts detailing the most abundant proteins detected in the fibrin clot. (A) FGA, FGB, and FGG chains were found in the highest abundance. (B) Other proteins identified were apolipoprotein B (APOB), fibronectin (FN1), albumin (ALB), α_1-antitrypsin (SERPINA1), von Willebrand factor (vWF), α_2-antiplasmin (SERPINF2), plasminogen (PLG), and factor XIII-A (F13A1). FGA, fibrinogen alpha; FGB, fibrinogen beta; FGG, fibrinogen gamma.

displayed a small decrease in FGA–FGA, FGG–FGG, and FGA–SERPINF2 cross-links (not significant; Figure 4B–D).

Analysis of each of the 44 individual cross-links in the FEISTY trauma patients revealed that patients who received cryo showed a
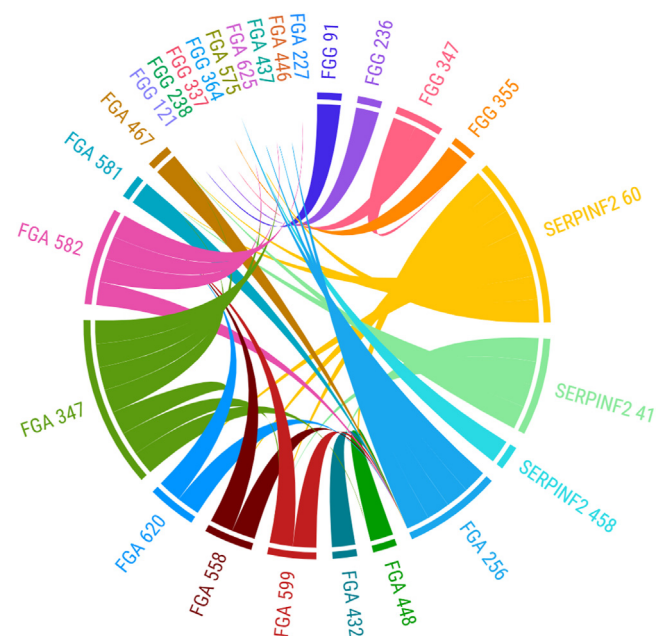

FIGURE 3 Identified FGA–FGA, FGG–FGG, and FGA-SERPINF2 cross-links. Chord diagram detailing the identified FGA–FGA, FGG–FGG, and FGA-SERPINF2 cross-links in fibrin clots formed from FEISTY trauma patient plasma. FGA, fibrinogen alpha; FGB, fibrinogen beta; FGG, fibrinogen gamma.

trend toward an increase in cross-link abundance, whereas those who received Fg-C were likely to show a trend toward a decrease (Table 2). Examples of a significant change in cross-link abundance pretransfusion and posttransfusion are the FGA–FGA cross-link (MADEAGSEADHEGTHSTKRGHAK-ALTDMPQMR) and FGA–SERPINF2 (TVTKTVIGPDGHK-NQEQVSPLTLLK; Figure 4E, F). Analysis of all 26 FGA–FGA cross-links revealed that 62% of these cross-links were significantly increased postcryo in FEISTY trauma patients, whereas no FGA–FGA cross-links were significantly increased in those who received Fg-C (Table 2). Out of the 5 FGG–FGG cross-links, 4 cross-links were significantly increased postcryo ($P < .05$, $P < .01$), with no statistically significant changes observed post–Fg-C transfusion (Table 2). Finally, 92% of the FGA–SERPINF2 cross-links were significantly increased postcryo transfusion ($P < .05$, $P < .01$), whereas only 15% of these cross-links were significantly increased post–Fg-C (Table 2).

The abundance of cross-links prefibrinogen and postfibrinogen replacement therapy in all FEISTY trauma patients was correlated with the toughness of individual fibrin fibers (Figure 5A, B). The toughness was measured by atomic force microscopy to determine the mechanical strength of individual fibrin fibers upon lateral stretching. There was no correlation between the two parameters pretransfusion (Figure 5A), but a positive correlation was observed postfibrinogen transfusion (Figure 5B). This was observed as an increase in individual fibrin fiber toughness that was associated with an increase in the abundance of cross-links ($r^2 = 0.5$, $P < .05$). The peak amount of thrombin generated in FEISTY patient plasma did not show any association with the abundance of cross-linked prefibrinogen
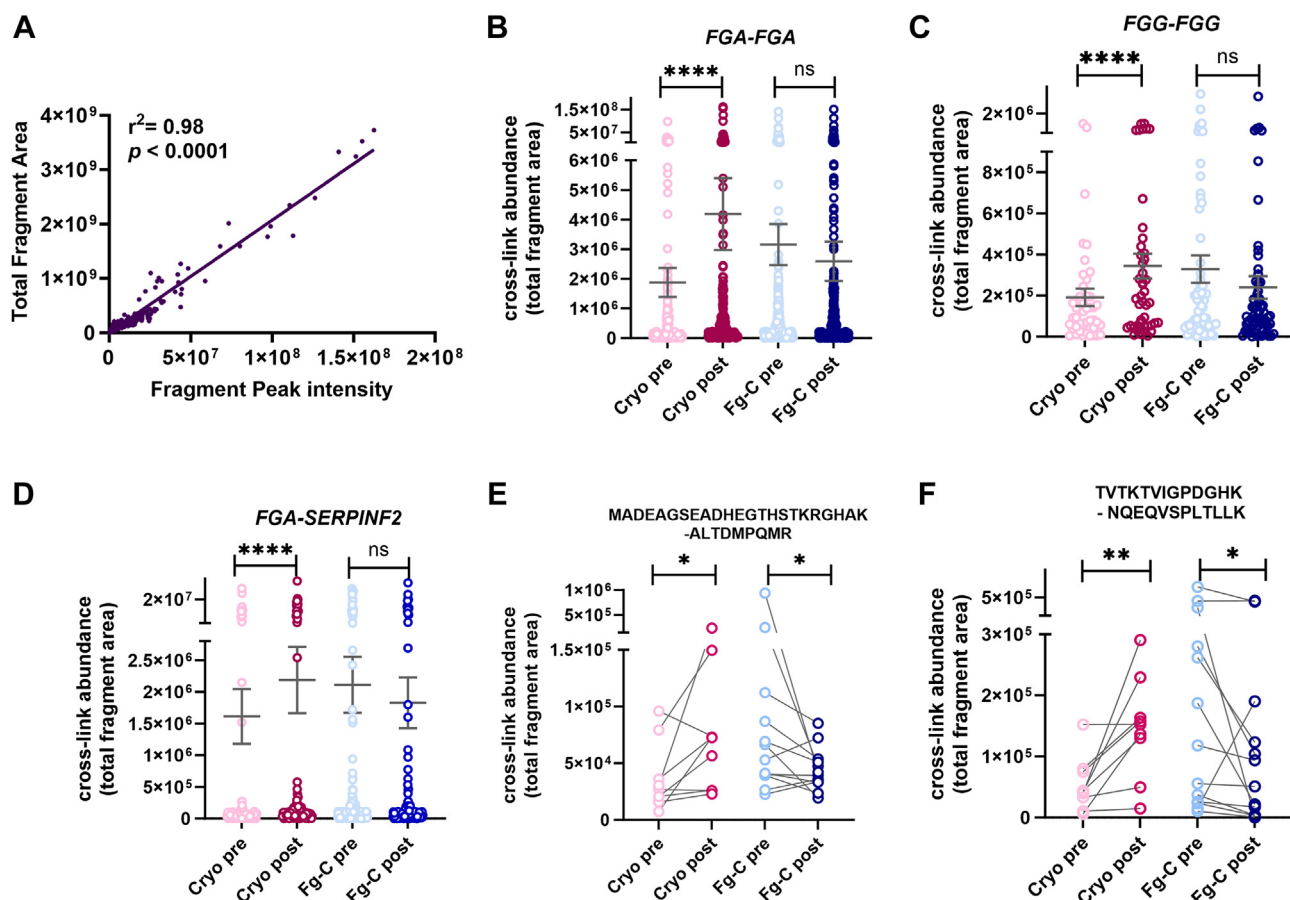
FIGURE 4 Cryoprecipitate, but not Fg-C, transfusion increases FGA–FGA, FGG–FGG, and FGA-SERPINF2 cross-links. (A) There was a strong positive correlation between the max height and total area parameters obtained from the extracted ion chromatograms (r^2 = 0.98). The abundances of cross-links were quantified in trauma patients pre- and postfibrinogen replacement therapy with either cryo or Fg-C. The number of (B) FGA–FGA, (C) FGG–FGG, and (D) FGA-SERPINF2 cross-links were analyzed. The percentage change in the cross-link abundance for each individual cross-link posttransfusion of fibrinogen replacement with either cryo or Fg-C is shown in Table 2. Visual examples of change in cross-link abundance in individual cross-links between (E) FGA–FGA and (F) FGA-SERPINF2 are shown. *P < .05, **P < .01, ****P < .0001. FGA, fibrinogen alpha; FGB, fibrinogen beta; FGG, fibrinogen gamma.

transfusion (Figure 5C). In postfibrinogen transfusion, there was a significant correlation between the abundance of cross-links and the peak thrombin generated; an increase in peak thrombin was associated with an increase in the abundance of cross-links (r^2 = 0.5, P < .05; Figure 5D). Finally, FXIII antigen levels correlated with the abundance of cross-linked prefibrinogen and postfibrinogen transfusion (Figure 5E, F). There was no association between FXIII antigen and the abundance of cross-links pretransfusion (Figure 5E); however, a weak but significant correlation was observed between FXIII antigen and the abundance of cross-links postfibrinogen replacement therapy (r^2 = 0.3, P < .05).

4 | DISCUSSION

The value of the PRM LC–MS method developed for this study is that it can be used to quantify the abundance of cross-links in patients with acute bleeding disorders who have received TXA and cannot be

analyzed using conventional fibrinolysis assays. We anticipate that this method may also have wider applicability in other hemorrhagic disorders and for analysis of pathological thrombi. Despite vast understanding of the molecular cascades that regulate coagulation, less is known about the end product, the insoluble fibrin matrix. Further development of this method could expand our understanding of venous and arterial thrombus structure at a molecular level and lead to identification of novel diagnostic targets of abnormal coagulation and fibrinolysis.

In the manuscript, we use PRM LC–MS to evaluate differences in two fibrinogen replacement therapies, cryo and Fg-C, in FEISTY trauma patients. Cryo is a pooled blood component derived from whole blood donations that has a variable but high fibrinogen concentration (8-16 g/L) [28]. Cryo is rich in a number of other coagulation factors that are not present in Fg-C [29]. These include antifibrinolytic factors such as PAI-1, α_2AP, and FXIII. Fg-C has a standard concentration of 20 g/L and has a favorable safety profile evident from its long term use in inherited dysfibrinogenemia,

TABLE 2 The percentage change in individual cross-links postfibrinogen replacement therapy with cryo or Fg-C.

Cross-link	Cryoprecipitate		Fibrinogen concentrate	
FGA–FGA	Percentage change posttransfusion (%)	Significance	Percentage change posttransfusion (%)	Significance
ALTDMPQMR-GHAKSRPV	210.1 ↑	ns	83.7 ↑	ns
ALTDMPQMR-GKSSSYSK	205.7 ↑	**	103.4 ↓	ns
ALTDMPQMR-LVTSKGDK	531.9 ↑	*	117.6 ↓	ns
ALTDMPQMR-TGKEK	165.4 ↑	*	267.1 ↑	ns
EKVTSGSTTTTR-ALTDMPQMR	130.4 ↑	ns	123.3 ↓	ns
EYHTEKLVTSK-ALTDMPQMR	178.9 ↑	*	123 ↓	ns
GDSTFESKSYK-ALTDMPQMR	98.3 ↓	ns	186.9 ↓	ns
GDSTFESKSYK-QFTSSTSYNR	93.6 ↓	*	130.3 ↑	ns
GSESGIFTNTKESSSHHPGIAEFPSR-ALTDMPQMR	294.9 ↑	ns	106.6 ↑	ns
GSESGIFTNTKESSSHHPGIAEFPSR-QFTSSTSYNR	82.6 ↓	ns	103.2 ↑	ns
GSESGIFTNTKESSSHHPGIAEFPSR-QFTSSTSYNRGDSTFESK	90.3 ↓	ns	116.9 ↓	ns
MADEAGSEADHEGTHSTKR-ALTDMPQMR	171.7 ↑	*	103.2 ↑	ns
MADEAGSEADHEGTHSTKR-QFTSSTSYNR	229.4 ↑	**	143 ↓	ns
MADEAGSEADHEGTHSTKRGHAK-ALTDMPQMR	233.9 ↑	*	1349.9 ↓	ns
MKPVPDLVPGNFKSQLQK-ALTDMPQMR	111.9 ↓	ns	108.6 ↓	ns
PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPR-MADEAGSEADHEGTHSTKR	120 ↑	ns	168.3 ↓	ns
PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-EKVTSGSTTTTR	373.6 ↑	ns	127.8 ↓	ns
PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-GKSSSYSK	237.3 ↑	ns	114.5 ↑	ns
PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-LVTSKGDK	219.9 ↑	*	252.1 ↓	ns
PGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSER-TVTKTVIGPDGHK	243 ↑	ns	100.3 ↓	ns
QFTSSTSYNRGDSTFESK-GKSSSYSK	122.7 ↑	ns	201.8 ↑	ns
QFTSSTSYNRGDSTFESK-LVTSKGDK	190.9 ↑	*	154.3 ↓	ns
QFTSSTSYNRGDSTFESK-MKPVPDLVPGNFK	178.5 ↑	*	178 ↓	ns
QFTSSTSYNRGDSTFESKSYK-ALTDMPQMR	128.4 ↑	*	103.5 ↓	ns
SSSYSKQFTSSTSYNRGDSTFESK-ALTDMPQMR	194.7 ↑	*	182.6 ↓	ns
TVTKTVIGPDGHK-ALTDMPQMR	106.4 ↑	ns	108.9 ↑	ns
FGG–FGG				
KCHAGHLNGVYYQGGTYSK-FEGNCAEQDGSGWWMN	310.5 ↑	**	114.5 ↓	ns
KEGFGHLSPTGTTEFWLGNEK-NWIQY	407.3 ↑	**	103.6 ↑	ns
KFEGNCAEQDGSGWWMNK-FEGNCAEQDGSGWWMN	143.4 ↑	ns	144.4 ↓	ns
KMLEEIMKYEASILTHDSSIR-AIQLTYNPDESSK	147.1 ↑	*	222.3 ↓	ns
FFTSHNGMQFSTWDNDNDKFEGN-SHNGMQFSTWDNDNDK	100.7 ↑	*	113.4 ↓	ns
FGA-SERPINF2				
QLTSGPNQEQVSPLTLLK-SSSYSKQFTSSTSYNR	171.1 ↑	ns	115.1 ↑	ns
LGNQEPGGQTALK-EKVTSGSTTTTR	348.7 ↑	*	128.3 ↓	ns

(Continues)

T A B L E   2   (Continued)

Cross-link	Cryoprecipitate		Fibrinogen concentrate	
FGA–FGA	Percentage change posttransfusion (%)	Significance	Percentage change posttransfusion (%)	Significance
NQEQVSPLTLLK-EYHTEKLVTSK	181.5 ↑	ns	138.5 ↓	ns
SSSYSKQFTSSTSYNR-NQEQVSPLTLLK	135.9 ↑	ns	229.4 ↓	ns
TVTKTVIGPDGHK-NQEQVSPLTLLK	275 ↑	**	162.5 ↓	*
NQEQVSPLTLLK-GDSTFESKSYK	258.6 ↑	**	122.4 ↓	ns
SSSYSKQFTSSTSYNR-LGNQEPGGQTALK	290.6 ↑	ns	167.9 ↓	ns
GKSSSYSK-DFLQSLK	194.9 ↑	ns	146.3 ↑	ns
LGNQEPGGQTALK-GDSTFESKSYK	108.5 ↑	ns	103.7 ↓	ns
TVTKTVIGPDGHK-LGNQEPGGQTALK	273 ↑	**	194.7 ↓	*
GSESGIFTNTKESSSHHPGIAEFPSR-LGNQEPGGQTALK	153.5 ↓	ns	123 ↑	ns
MADEAGSEADHEGTHSTKR-LGNQEPGGQTALK	391 ↑	**	191.6 ↓	ns
LGNQEPGGQTALK-GKSSSYSK	131.3 ↑	ns	119.8 ↑	ns

The percentage increase or decrease in the cross-link abundance for each individual cross-link posttransfusion of fibrinogen replacement with either cryo or Fg-C is shown. *$P < .05$, **$P < .01$. ns, not significant.

afibrinogenemia, and hypofibrinogenemia [30]. Our previous study showed that cryo supplementation reduced fibrinolytic activity, demonstrated by the attenuation of plasmin activation, an increase in PAI-1 and maintenance of thrombin activatable fibrinolysis inhibitor (TAFI) concentration [21]. Cryo clots displayed a more homogeneous fibrin network with an increased number of fibers than those formed post–Fg-C [21]. Analysis of FXIII and individual fibrin fibers indicated that these clots were composed of fibers that were more resistant to mechanical disruption [21]. These PRM LC–MS cross-linking data agree with our previous findings, in that patients who received cryo exhibit a significant increase in abundance of cross-links post-transfusion compared with those who received Fg-C.

Here we focus on cross-links formed between FGA–FGA, FGG–FGG, and FGA–SERPINF2. However, it is important to note that other fibrinolytic inhibitors and proteins can also be cross-linked to the fibrin clot by FXIIIa, including, but not limited to, plasminogen activator inhibitor 2 (PAI-2) [31], fibronectin [32], von Willebrand factor [33], TAFI [34], and complement C3 [35]. These cross-links were not searched for in our trauma patient samples but may warrant future study. Our findings highlight the importance of FXIIIa, the transglutaminase responsible for forming cross-links between neighboring fibrin molecules and the fibrinolytic inhibitor α_2AP(12). FXIII is present in cryo at a concentration of 57.7 μg/mL, whereas only trace amounts (0.4 μg/mL) were detected in Fg-C [21]. The Fg-C used in the FEISTY study is the CSL Behring product Riastap. Previous *in vitro* studies have evaluated different Fg-C preparations and have shown that Fg-C's that contain FXIII perform similarly to cryo in measures of clot strength and stability [36,37]. Our previous data suggest that there are differences in fibrin polymerization and degradation in patients who received cryo or Fg-C, most likely due to the differences in FXIII content [21]. Here we show that these differences in FXIII levels correlate with significant differences in the degree of cross-linking

between the cryo and Fg-C cohorts. Combined, these studies suggest that using a Fg-C that contains FXIII may have some benefits over one without to increase clot stability. Interestingly, the extent of fibrin cross-linking did not correlate with the fibrinogen concentration, however, this may be due to the small number of patients analyzed. Future studies might investigate the relationship with fibrinogen further and any crosstalk with inflammation.

A limitation of our study is the small number of patient samples; 13 in the Fg-C arm and 9 in the cryo arm. Despite the small number of patient samples, we have demonstrated statistically significant differences in fibrin clot polymerization between the 2 patient cohorts. The FEISTY trial was a pilot study to inform viability of a larger randomized controlled trial and these ongoing studies form part of the FEISTY II trial (NCT05449834), which will recruit 850 trauma patients across Australia and New Zealand. There are of course limitations to the PRM LC–MS method, which would impact its use in routine clinical practice, namely the requirement for highly specialized pieces of equipment and expert personnel in addition to a lack in real-time data analysis capacity and platform certification.

The results of CRYOSTAT-2, a randomized controlled trial evaluating whether early fibrinogen replacement with cryo improves survival in major trauma hemorrhage, were recently published [38]. There was no difference in mortality at 28 days if cryo was given early (<60 minutes), but interestingly, there was an improvement in patients who received cryo later (between 60 and 90 minutes) [38]. The findings highlight the importance of moving away from empiric transfusion and focus on the development of a personalized approach to diagnose coagulopathy and identify the best treatment. To do so, we need to improve our understanding of trauma coagulopathy at a molecular and cellular level, which requires additional studies of a similar nature to this one. Access to our targeted PRM LC–MS method and similar technologies will allow scientists and clinicians to improve
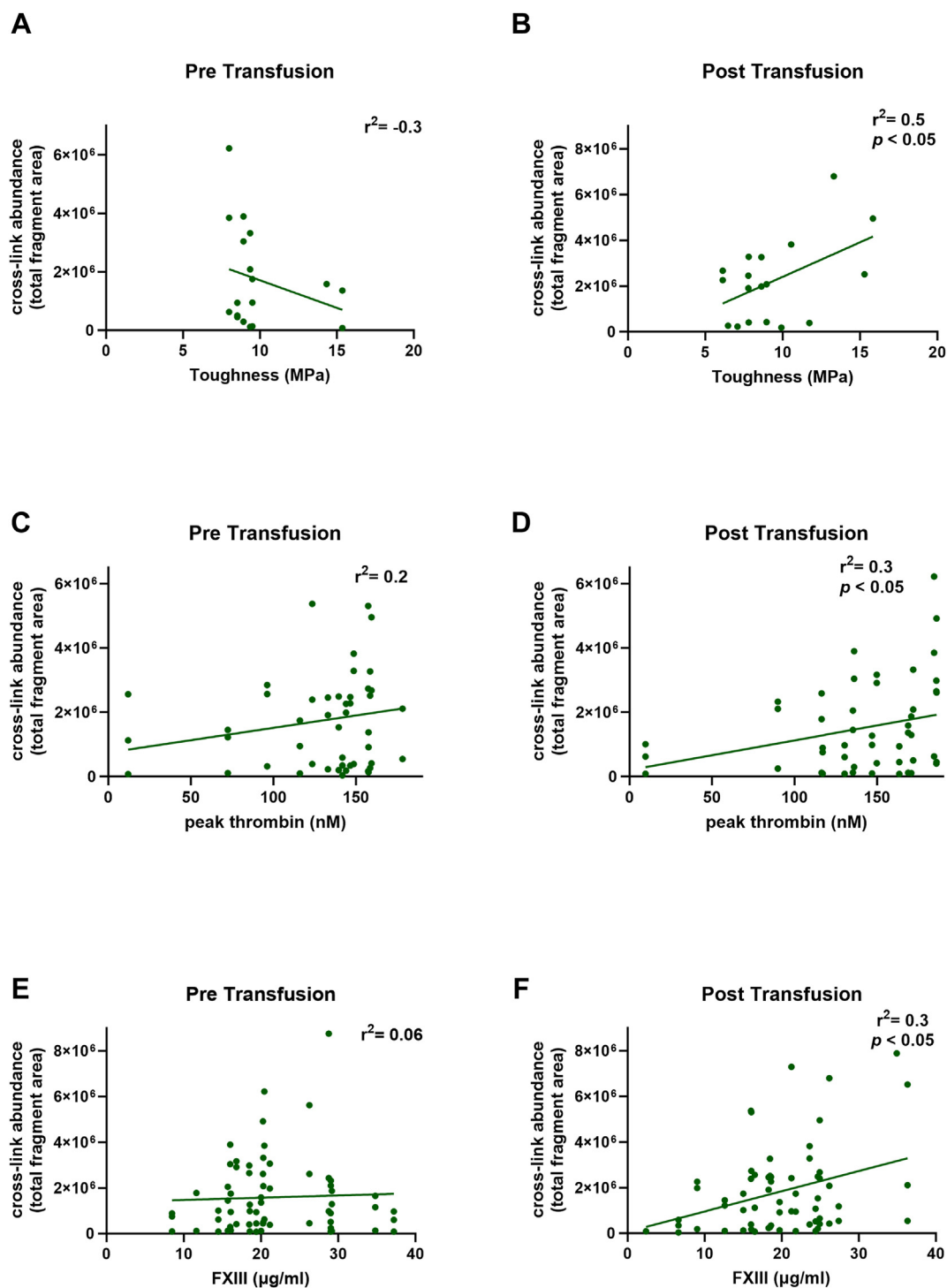
FIGURE 5 The abundance of cross-links correlates with individual fiber toughness, thrombin generation, and FXIII levels. The abundance of cross-links pre- and postfibrinogen replacement therapy was correlated with (A, B) individual fibrin fiber toughness, (C, D) peak thrombin concentration, and (E, F) FXIII antigen levels. *$P < .05$.

our understanding of trauma coagulopathy to develop precision transfusion therapies improving patient care and outcomes.

ETHICS STATEMENT

Investigations were conducted with the participants' informed consent in agreement with the Declaration of Helsinki. Ethical approval

was obtained from the Institutional Review Board of Oxford University Hospitals NHS Foundation Trust for analysis of the precollected trauma samples (18/EM/0056).

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2024.03.001

Table 1

		Cryo	Fg-C
Patients	**Number**	9	13
	Age (year)	41 (27-61)	48 (41-61)
	Male, *n* (%)	9 (100)	4 (31)
Admission	**Time to admission (min)**	43 (28-110)	30 (12-126)
	Heart rate (beats/min)	120 (91-144)	130 (110-143)
	Systolic blood pressure (mmHg)	141 (115-148)	130 (114-145)
	Clauss fibrinogen (mg/ml)	2 (1.7-2.7)	1.9 (1.6-2.3)
	Platelets (x 10^9 plt/L)	173 (155-251)	200.5 (125-238)
	Haemoglobin (g/L)	134 (101-146)	138 (126-158)
	PT (s)	16 (15-19)	16 (14-19)
	INR	1.3 (1.1-1.4)	1.2 (1.1-1.6)
	FIBTEM CA5 (mm)	9 (8.5-10)	7.5 (6.3-8.8)
	EXTEM CA5 (mm)	37 (30-40.5)	38 (32-39.5)
Injury	**ISS**	29 (20-35)	29 (18-33)
	GCS	15 (3-15)	3 (3-15)
	Blunt Injury, *n* (%)	8 (89)	10 (77)
	Multiple injuries, burns or other, *n* (%)	6 (67)	7 (54)
	Head and other associated injuries, *n* (%)	2 (22)	3 (23)
	Chest and/or abdominal injuries only, *n* (%)	1(11)	3 (23)
Pre-Hospital	**TXA, *n* patients (%)**	2 (22.2)	7 (54)
	FFP , *n* patients (%)	0 (0)	1 (8)
	RBC, *n* patients (%)	4 (44)	7 (54)
In Hospital	**ICU length of stay (days)**	8 (5-19)	5 (4-14)
	Hospital length of stay (days)	23 (17-45)	9 (4-45)
	Time to first dose (min)	112 (85-207)	48 (33-104)
	Time from first dose to sample (min)	74.5 (28-313)	25 (15-76)
	24 h blood products (units)	20 (19-38)	11 (7-20)
	Sepsis, *n* (%)	4 (44)	4 (31)
Arterial Thrombosis	**Myocardial Infarction, *n* (%)**	0 (0)	0 (0)
	Cerebrovascular Accident, *n* (%)	1 (11)	1 (8)
Venous Thrombosis	**Deep vein thrombosis, *n* (%)**	1 (11)	1 (8)
	Pulmonary Embolism, *n* (%)	3 (33)	0 (0)