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ORIGINAL ARTICLE

Concizumab improves clot formation in hemophilia A under flow

Megan P. Jewell[1] | Zaina Ashour[1] | Christine H. Baird[2,3] | Marilyn Manco Johnson[2,3] | Beth Boulden Warren[2,3] | Adam R. Wufsus[4] | Chiara Pallini[5] | Michael Dockal[5] | Marianne Kjalke[5] | Keith B. Neeves[1,2,3]

[1]Department of Bioengineering, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado, USA

[2]Department of Pediatrics, Section of Hematology, Oncology, and Bone Marrow Transplant, University of Colorado, Anschutz Medical Campus, Aurora, Colorado, USA

[3]Hemophilia and Thrombosis Center, University of Colorado, Anschutz Medical Campus, Aurora, Colorado, USA

[4]Rare Blood Disorders, Medical Affairs Rare Disease, Novo Nordisk Inc, Plainsboro, New Jersey, USA

[5]Rare Blood Disorders, Rare Disease Research, Novo Nordisk, Måløv, Denmark

Correspondence
Keith B. Neeves, University of Colorado Anschutz Medical Campus, 12800 East 19th St, Mail Stop 8302, Aurora, CO 80045, USA.
Email: keith.neeves@cuanschutz.edu

Abstract

Background: Inhibition of tissue factor pathway inhibitor (TFPI) is an emerging therapeutic strategy for treatment of hemophilia. Concizumab is a monoclonal antibody that binds TFPI and blocks its inhibition of factor (F)Xa thereby extending the initiation of coagulation and compensating for lack of FVIII or FIX.

Objectives: The objective of this *in vitro* study was to evaluate how concizumab affects clot formation in hemophilia A under flow.

Methods: Blood was collected from normal controls or people with hemophilia A. An anti-FVIII antibody was added to normal controls to simulate hemophilia A with inhibitory antibodies to FVIII. Whole blood and recombinant activated FVII (rFVIIa, 25 nM) or concizumab (200, 1000, and 4000 ng/mL) were perfused at 100 s^{-1} over a surface micropatterned with tissue factor (TF) and collagen-related peptide. Platelet and fibrin(ogen) accumulation were measured by confocal microscopy. Static thrombin generation in plasma was measured in response to rFVIIa and concizumab.

Results: Concizumab (1000 and 4000 ng/mL) and rFVIIa both rescued (93%-101%) total platelet accumulation, but only partially rescued (53%-63%) fibrin(ogen) incorporation to normal control levels in simulated hemophilia A. Results using congenital hemophilia A blood confirmed effects of rFVIIa and concizumab. While these 2 agents had similar effect on clot formation under flow, concizumab enhanced thrombin generation in plasma under static conditions to a greater extent than rFVIIa.

Conclusion: TFPI inhibition by concizumab enhanced activation and aggregation of platelets and fibrin clot formation in hemophilia A to levels comparable with that of rFVIIa.

KEYWORDS
blood coagulation, hemophilia A, hemorheology, lipoprotein-associated coagulation inhibitor, microfluidics

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1 | INTRODUCTION

Hemophilia A and B are bleeding disorders characterized by deficiencies in factor (F)VIII or FIX that causes diminished thrombin generation and consequently result in spontaneous bleeding in the joints, muscles, and soft tissues. Bleeding and arthropathy are most problematic in people with severe or moderate FVIII or FIX deficiencies (<5%) but can be present in mild deficiencies as well [1,2]. Currently, guidelines for hemophilia treatment recommend replacement therapy, which involves the intravenous injection of plasma-derived or recombinant FVIII or FIX products [3]. Replacement therapy has successfully reduced morbidity and improved quality of life for people with hemophilia [4]. However, replacement therapy comes with drawbacks including frequent intravenous injections when used prophylactically and suboptimal protection against bleeds, particularly at low trough levels toward the end of the dosing intervals [3]. Furthermore, the development of neutralizing inhibitors, which occurs in 25% to 30% of people with severe FVIII deficiencies, represents another complication of factor replacement therapy [5–7]. Both people with congenital hemophilia A that develop inhibitors against FVIII replacement therapies and people that spontaneously develop FVIII autoantibodies (acquired hemophilia A) are often treated with bypassing agents such as recombinant activated FVII (rFVIIa) or activated prothrombin complex concentrate. Emicizumab, a bispecific antibody that mimics FVIIIa cofactor function, is an alternative nonfactor product for hemophilia A administered subcutaneously that can also bypass inhibitors [8–11].

The concept of rebalancing therapies wherein endogenous anticoagulant pathways are inhibited has gained traction for subcutaneous prophylaxis in hemophilia [12]. One target of rebalancing therapy is tissue factor pathway inhibitor (TFPI). TFPI is a serine protease inhibitor that inhibits activated FX (FXa) and the tissue factor (TF)–activated FVII (FVIIa) complex in an FXa-dependent manner, as well as early forms of the activated FV (FVa)–FXa (prothrombinase) complex [13]. Concizumab is a high affinity, humanized monoclonal immunoglobin G4 antibody against the TFPI-K2 domain that prevents TFPI binding to and its inhibition of FXa [14]. In a rabbit model of hemophilia A, concizumab reduces cuticle bleeding when delivered either intravenously or subcutaneously [14]. A series of clinical trials called the Explorer studies have examined the safety and efficacy of concizumab in humans. These include a phase I single-dose escalation study [15], a phase I multiple dose study [16], and 2 phase 2 multiple dose studies in people with hemophilia with and without inhibitors [17,18]. Clinical data in phase 3 trials demonstrated safety and efficacy with a daily subcutaneous treatment [19].

Concizumab can rescue thrombin generation in hemophilia A plasma in closed systems without flow in a concentration-dependent manner [14,20,21]. However, clots formed under flow in an open system—specifically blood flow in a blood vessel—are subject to additional biophysical mechanisms that regulate thrombin generation. These include the following: (i) platelet adhesion to the subendothelium inhibits TF-initiated coagulation by physically blocking access to the TF–FVIIa complex [22–24]; (ii) blood flow dictates the rate of zymogen, cofactor, and enzyme transport to and from an evolving clot [25–32]; and (iii) platelets and other blood cells play an essential role in protecting the coagulation reaction from dilution by flow and serving as the nidus and anchor for fibrin generation [33–40]. These mechanisms may be relevant to the efficacy of hemostatic agents. This is illustrated by the enhanced effect of rFVIIa on thrombin generation under flow compared with that under static conditions [41].

The objective of this study was to measure the effect of concizumab on clot formation in whole blood under physiologic flow conditions. For this purpose, we developed a new method to copattern TF-laden liposomes and collagen-related peptides as a repeatable, homogeneous procoagulant substrate. In a microfluidic flow assay using this substrate, we measured the kinetics of clot formation in whole blood from people with hemophilia A and simulated hemophilia A using a FVIII function–blocking antibody, which parallels the development of inhibitors in congenital and acquired hemophilia A, when treated with rFVIIa and concizumab. The results show a near complete rescue of platelet accumulation and partial rescue of fibrin formation at a saturating dose of concizumab that is comparable with those of rFVIIa.

2 | MATERIALS AND METHODS

2.1 | Materials

Methanol, hydrochloric acid, (3-aminopropyl)triethoxysilane (APTES), bovine serum albumin (BSA), glutaraldehyde, acetic acid, 2-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and CF405S amine were purchased from Sigma Aldrich. 3,3-Dihexyloxacarbocyanine iodide ($DiOC_6$), sodium chloride, and Alexa Fluor 647 Protein Labeling Kit were from purchased from Thermo Fisher. (Tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane was purchased from Gelest. Dade Innovin lipidated TF was purchased from Siemens Health Diagnostics. The collagen peptides CRP-XL (GPVI ligand), VWF-BP (von Willebrand factor A3-binding peptide), and GFOGER ($\alpha 2\beta 1$ ligand) were purchased from CambCol. HEPES-buffered saline (pH = 7.4) consisting of 20 mM HEPES and 100 mM sodium chloride was made in house. A function-blocking anti-FVIII antibody (GMA-8015) was purchased from Green Mountain Antibodies. PE-labeled Annexin V and Annexin V binding buffer were purchased from BioLegend. FVIII-deficient plasma (<1% activity) was purchased from George King Bio-Medical. Recombinant FVIIa (rFVIIa, NovoSeven) and concizumab were provided by Novo Nordisk.

2.2 | Glass slide functionalization

Glass slides were cleaned with a 1:1 solution of methanol and hydrochloric acid (37%), thoroughly rinsed with deionized water (diH_2O), dried with a nitrogen air brush, and then treated with an oxygen plasma (Harrick Plasma Cleaner) for 1 minute. Cleaned slides were

incubated with 1% APTES (v/v diH_2O) for 2 minutes, rinsed in diH_2O, dried with nitrogen air brush, and dehydrated for 2 minutes at 110 °C on a hot plate. Next, slides were incubated in 8% glutaraldehyde (v/v diH_2O) at room temperature (RT) for 30 minutes, rinsed in diH_2O, dried with nitrogen air brush, and stored in a desiccating chamber for up to 1 week.

2.3 | Copatterning TF and collagen peptides

A sequential patterning was used wherein liposomal TF was first bound to the glutaraldehyde functionalized slide followed by collagen peptide binding. A stock solution of Dade Innovin TF or stock solutions diluted (1:10 or 1:100) with HEPES-buffered saline (pH = 7.4) were perfused through a rectangular microfluidic channel with dimensions 50 μm × 250 μm × 55 mm (height × width × length, respectively) at a flow rate of 3 μL/min for 1 minute. The TF solution was incubated for 10 minutes in the filled channel at RT. This flow-incubate process was repeated 4 times to achieve even distribution of liposomes across the patterning channel (Supplementary Figure S1). Next, the 3 collagen peptides CRP-XL, VWF-BP, and GFOGER were used at final concentrations of 350 μg/mL, 257 μg/mL, and 257 μg/mL, respectively, in 10 mM acetic acid. The peptide solution was drawn through the same channel used for TF patterning. The slide with peptide solution was kept in a humid environment incubated overnight at 4 °C. The next day, the patterning channel was washed with 10 mM acetic acid containing 600 μg/mL CF405S-amine to identify the location of the patterned procoagulant substrate. The microfluidic patterning device was removed, and the slide was allowed to dry briefly.

2.4 | Annexin V staining of immobilized liposomal TF

The staining solution was prepared by diluting 5 μL of PE-labeled Annexin V into 495 μL of Annexin V Binding Buffer. The glass slide was patterned with TF solution and collagen peptides, and was subsequently washed with HBS. Staining solution was then drawn through the patterning channel, and the slide was incubated in the dark for 15 minutes at RT. The channel was then washed with Annexin V Binding Buffer and imaged using confocal microscope (Olympus IX83 [40× objective, NA = 0.6]; Yokogawa CSU-W1 spinning disk confocal unit; Thorlabs laser lines—405 nm, 488 nm, and 640 nm; and Hamamatsu ORCA-Flash4.0 Digital CMOS camera)

2.5 | Blood collection and labeling

Human whole blood was collected by venipuncture with 19-gauge butterfly needles into vacutainer tubes. The study and consent process received approval from the Colorado Multiple Institutional Review Board in accordance with the Declaration of Helsinki (COMIRB #09-0816). Three vacutainer tubes were drawn in the following order and with the following uses: 4.5 mL of 3.2% sodium citrate tube that was discarded, 4.5 mL of 11 mM sodium citrate with 50-μg/mL corn trypsin inhibitor (CTI) tube (Prolytix/Haemtech) that was used for flow assays, and EDTA tube that was used for blood cell counts using a Micros 60 hematology analyzer (Horiba). Whole blood from the citrate/CTI tube was incubated for 10 minutes at 37 °C with 1 μM $DiOC_6$ and 75 μg/mL Alexa Fluor 647–labeled fibrinogen, and in conditions to simulate hemophilia A, 2 μg/mL of GMA-8015 was added. All experiments with whole blood were conducted within 90 minutes of phlebotomy, a time scale where no significant changes in platelet activation were observed as measured by P-selectin exposure (Supplementary Figure S2).

2.6 | Factor VIII activity

FVIII activity was measured using a modified activated partial thromboplastin time assay. The assay used calibration curves based on dilutions of normal pooled platelet-poor plasma (NPP, generated in-house from 38 individuals) and FVIII-deficient plasma using the STAR Max (Stago) coagulation analyzer following manufacturer's instructions. The FVIII activity assay has a standard deviation of 10%. To measure the inhibition of FVIII by GMA-8015, the antibody was added to whole blood (n = 5 donors) and incubated for 10 minutes at 37 °C to mimic the conditions used for microfluidic flow assays, followed by plasma isolation by centrifugation and measurement of FVIII activity.

2.7 | TF concentration

The concentration of TF in the Dade Innovin reagent was measured by enzyme-linked immunosorbent assay (Human Coagulation Factor III enzyme-linked immunosorbent assay, Thermo Fisher) according to manufacturer's instructions.

2.8 | Microfluidic device assembly

The polydimethylsiloxane (PDMS) microfluidic flow device consists of 4 channels with dimensions of 50 μm × 500 μm × 10 mm (height × width × length, respectively). The flow device is placed on the slide with the micropatterned strip of TF-collagen peptides such that the flow channels run perpendicular to the strip. The flow device and slide are then placed in a custom 3D-printed holder that seals them together. To block the channel walls, 2% BSA (w/v in HBS, pH = 7.4) was drawn through the 4 channels and incubated for at least 45 minutes at RT in a humid environment. While the assay device was being blocked, tubing (Saint-Gobain Tygon AAD04091-CP), syringes (Hamilton Gastight nos. 1702 and 1725), and blunt-tip Luer Lok needles (CML Supply Cat. no 901-30-050) were assembled and blocked with 2% BSA.

2.9 | Microfluidic flow assay

Whole blood and recalcification buffer (HBS with 75 mM $CaCl_2$ and 37.5 mM $MgCl_2$) were perfused from the syringe pump into the PDMS mixer at a relative flow rate of 9:1 using a syringe pump (PHD 2000, Harvard Apparatus) to yield a final concentration of 7.5 mM $CaCl_2$ and 3.75 mM $MgCl_2$. Figure 1 shows the experimental setup including the separate split-flow microfluidic mixer used to combine the whole blood and the recalcification buffer. The following conditions were tested: simulated hemophilia A (SimHA), SimHA with rFVIIa (25 nM), SimHA with concizumab (200, 1000, and 4000 ng/mL), and a vehicle control reflecting healthy normal conditions. rFVIIa and concizumab were added to the recalcification buffer. This was done to avoid any preactivation of coagulation prior to the assay with rFVIIa and used with the concizumab for consistency across treatments. The recalcified whole blood was perfused at a wall shear rate of 100 s^{-1} through the microfluidic channels. Platelet ($DiOC_6$) and Alexa Fluor 647–fibrin(ogen) accumulation were measured in each channel (40×, NA = 0.6). Fluorescence time series were collected with CellSens Dimension software. Images were collected every 20 seconds for 20 minutes, starting when the recalcified whole blood reached the TF-collagen peptide strip.

2.10 | Image analysis

All image processing was performed in FIJI/ImageJ and data analysis was conducted in GraphPad Prism 9. Six-plane Z-stacks were taken at each timepoint to account for drift and to fully capture the clot formation in the channel. Rolling ball background subtraction (radius = 50 pixels) was applied to remove illumination artifacts that would obscure the labeled fibrin(ogen) response. The t = 0 timepoint of each experiment was set as an internal baseline and subtracted from subsequent frames. The maximum intensity projections of these Z-stacks were used to track the mean intensity of the field of view over time. To provide an internal control for each donor, the mean intensity is normalized by the maximum intensity of the vehicle control for each fluorophore. Three metrics were calculated to quantify platelet and fibrin(ogen) kinetics: (1) maximum intensity defined as the maximum integrated fluorescence for each channel over the time course of the assay, (2) lag time defined as the time at which the fluorescence intensity reaches 10% of the maximum intensity of the vehicle control, and (3) velocity defined by the second derivative of each kinetic curve and expressed as change in fluorescence intensity per unit time (DI_f/min). A region was included in the velocity calculation if the second derivative deviated from 0 (inflection point) by less than 10% of the range. Linear regression was performed on the time points within the selected region.

2.11 | Thrombin generation

Thrombin generation under static conditions was measured by Calibrated Automated Thrombogram (CAT, Thrombinoscope BV) using 1 pM TF, phospholipids, and calcium ions. Samples included NPP with 2 μg/mL of GMA-8015 (SimHA), FVIII-deficient plasma, and plasma from an individual with moderate FVIII deficiency (6% FVIII). rFVIIa (25 nM) or concizumab (4000 ng/mL) or vehicle control was added to each sample and incubated for 10 minutes before addition of CAT reagents.

2.12 | Statistical analysis

All statistical analysis was performed in GraphPad Prism 9. For each metric, Kruskal–Wallis analysis of variance was performed across the conditions. Post-hoc multiple comparisons were performed using Dunn's test. The mean of each condition was compared with the mean of every other condition for a total of 12 comparisons per metric. Mean rank difference and multiplicity adjusted P values were reported for each comparison and can be found in the Supplementary Tables S1–S6.

3 | RESULTS

A 2-step patterning process was established wherein lipidated TF was first bound to the surface, followed by binding of the collagen-related peptides (Figure 2A). To prevent desorption during the patterning or

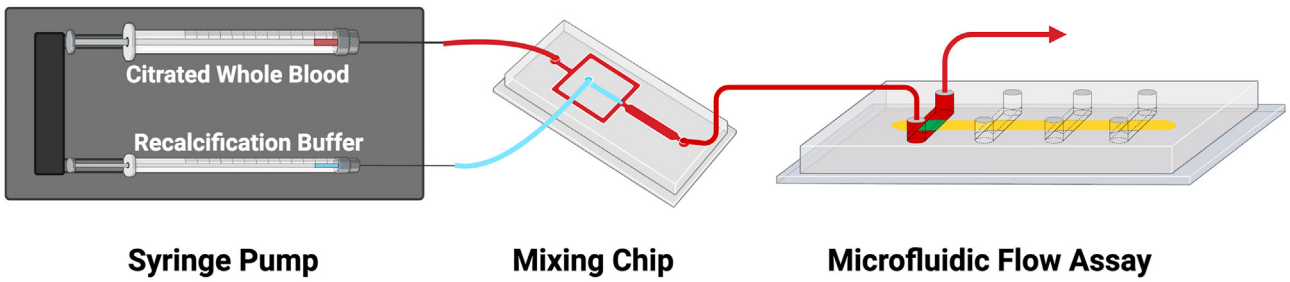

FIGURE 1 Schematic for microfluidic flow assay. Whole blood (red) and recalcification buffer (cyan) are injected separately via a syringe pump at volumetric flow rate ratio of 9:1 into microfluidic mixing chip that enhances mixing by reducing length scale for diffusion (~20 μm) of divalent cations into the blood. Recalcified whole blood is then perfused over the procoagulant substrate (yellow) in the microfluidic flow assay device forming a clot (green) that is captured by optical microscopy.

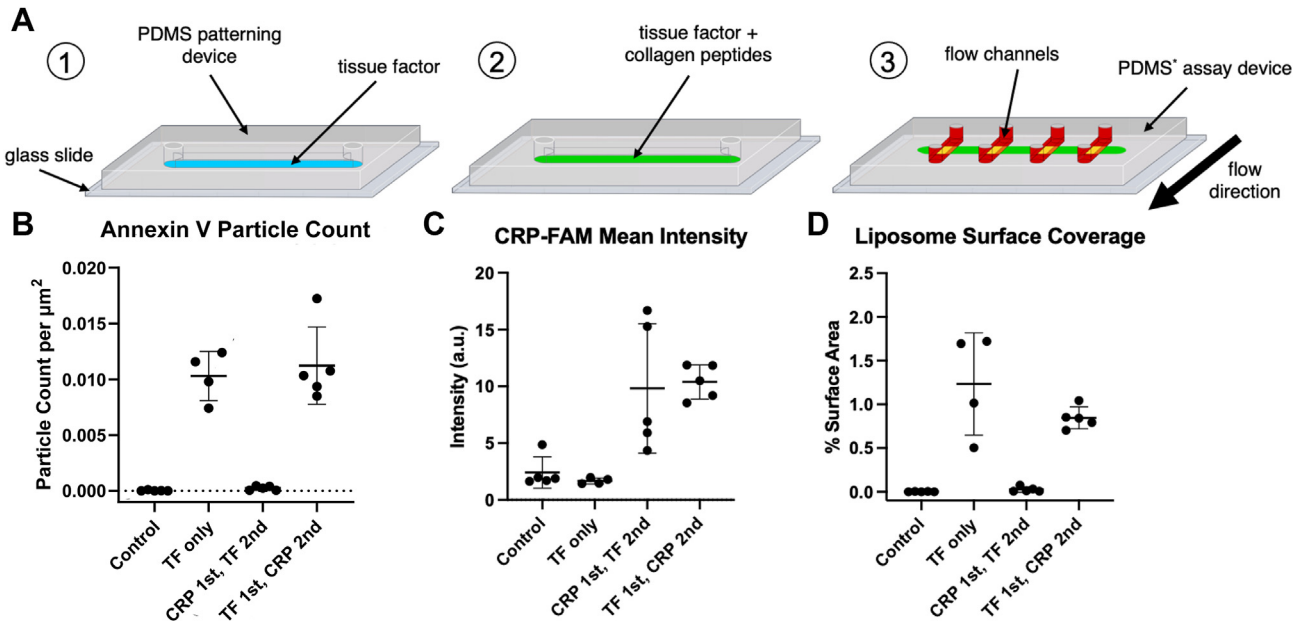

FIGURE 2 Micropatterning tissue factor and collagen peptides sequentially. (A) Step 1: Lipidated TF was first perfused through the patterning device and allowed to bind to the glutaraldehyde-activated surface. This step was repeated 4 times. Step 2: A mixture of CRPs was then perfused through the same patterning device and incubated overnight. Step 3: An assay device was then placed with channels perpendicular to the patterned area to create a precise procoagulant area within the channel of 250 µm × 500 µm. Patterning the TF liposomes first, followed by the collagen-related peptides yielded a substrate with both components: (B) number of TF liposomes in the procoagulant strip as detected by PE-labeled Annexin V staining and (C) collagen peptide surface coverage based on FAM-labeled CRP-XL fluorescence. (D) % surface area coverage of TF liposomes detected. CRP, collagen-related peptide; TF, tissue factor.

flow assay, both the lipidated TF and peptides were covalently bound to the glass surfaces: first, clean glass slides were treated with an aminosilane (APTES) whose primary amine is used to bind the cross-linker glutaraldehyde. The aldehyde in the glutaraldehyde group reacts with primary amines to form covalent bonds with phosphatidylserine head groups of the TF liposomes and the collagen-related peptides. As previously shown, PDMS absorbs collagen-related peptides. Vapor deposition of a different silane with a large alkane chain (OTS) blocks peptide adsorption onto the PDMS patterning device prior to use [42].

The TF concentration in the undiluted, reconstituted reagent was 193 pM. To achieve even TF liposome deposition across the patterning channel, a semibatch approach was used: the TF reagent was introduced into a channel, allowed to bind to the glass surface, and then replaced with a fresh volume of reagent. This process was repeated 4 times. To vary TF surface density, the TF reagent was diluted by 10- and 100-fold. The amount of TF liposomes was quantified by labeling the phosphatidylserine (PS) lipids with annexin V. The semibatch patterning yielded a consistent surface density of TF liposomes along the entire patterning channel (Supplementary Figure S1A) however there was considerable variance in the 100-fold diluted reagent (Supplementary Figure S1B). The liposomes occupied only a small fraction of the surface (<5%) leaving sufficient area for pattering the collagen-related peptides in the next step (Figure 2D).

To support platelet function, 3 collagen peptides were used that together recreate the function of collagen fibers—ligand of platelet GPVI and α2β1 receptors and binding partner for the A3 domain of VWF—but without the impurities and spatial heterogeneity of animal-derived collagen fibers [43]. Following TF–liposome binding, the 3 collagen peptides were introduced into the patterning channel and incubated overnight at 4 °C. The sequence of these 2 steps is important to enable equal surface area coverage of collagen peptides and TF liposomes; if the collagen peptides were incubated first, they occupied all binding sites and there was little stable binding of the liposomes (Figure 2B, D). However, when peptides were incubated after TF liposomes, there was a similar amount of collagen peptides on the surface (Figure 2C).

Simulated hemophilia A (SimHA) was achieved using a function-blocking anti-FVIII antibody. In a 1-stage clotting assay, the FVIII activity of the SimHA condition was 1.4% ± 0.3% (range, 1.1%-1.9%) of normal. As treatments, either rFVIIa or concizumab in the recalcification buffer were added to the SimHA whole blood in the mixing chip with upstream of the microfluidic flow assay device (Figure 1). A normal control condition included only vehicle.

To identify a large dynamic range in platelet and fibrin response to FVIII levels, 3 TF surface densities were considered (Supplementary Figure S1C–N). The highest density of 0.078 ± 0.018 TF liposomes/µm^2 showed slower, but similar maximum fibrin(ogen) accumulation for SimHA conditions compared with normal controls suggesting this concentration of TF is too high to detect impaired fibrin(ogen) deposition in hemophilia. The low and middle surface densities demonstrated similar platelet and fibrin(ogen) accumulation. The middle TF surface density (0.0098 ± 0.0020 TF liposomes/µm^2) was used for

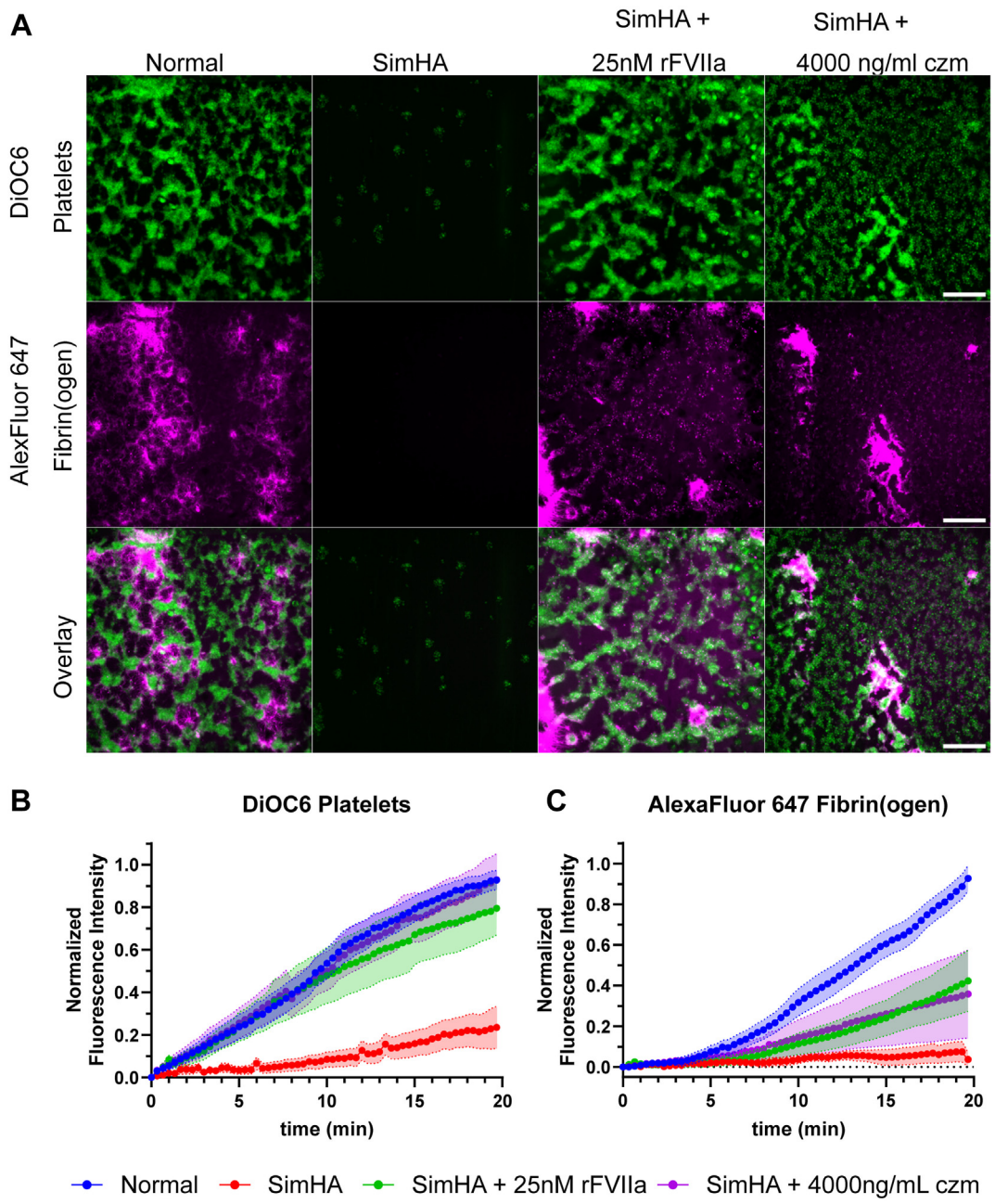

FIGURE 3 (A) Representative images of DIOC6-labeled platelets and Alexa Fluor 647–labeled fibrin(ogen) accumulation after 20 minute perfusion over collagen peptide–TF surface at 100 s^{-1}. Scale bar = 50 μm. Kinetics of platelet (B) and fibrin(ogen) (C) accumulation. Fluorescence intensity is normalized by the maximum fluorescence intensity of the vehicle control. SHA, simulated hemophilia A (anti-FVIII antibody); czm, concizumab. Data points represent averages and shaded regions represent SEM of *n* = 9 donors.

subsequent experiments because of the high variance of the low density (0.0057 ± 0.0029 TF liposomes/μm^2; Supplementary Figure S1A). Under the SimHA condition, both rFVIIa and concizumab treatments enhanced clot formation under flow at a wall shear rate of 100 s^{-1} (Figure 3A). Images taken at the end of the 20-minute assay show that platelet accumulation in the presence of concizumab or rFVIIa was similar to the normal control, while fibrin fibers are mostly present around large platelet aggregates. Indeed, fibrin maximum intensity appears to correlate with platelet maximum intensity in most cases (Supplementary Figure S3).

Qualitatively, the average of the kinetic curves showed that both treatments rescue platelet accumulation to normal control levels (Figure 3B) and partially rescue fibrin(ogen) accumulation (Figure 3C) over the 20-minute assay. This trend is confirmed when quantifying these curves with 3 metrics: lag time, velocity, and maximum intensity (Figure 4, Supplementary Tables S1–S6) as well the response to concizumab for low (200 ng/mL), medium (1000 ng/mL), and high (4000 ng/mL) concentrations. The lag time for platelet accumulation was significantly prolonged with SimHA conditions (13.8 ± 6.8 minutes) and rescued to lag times comparable with the normal control

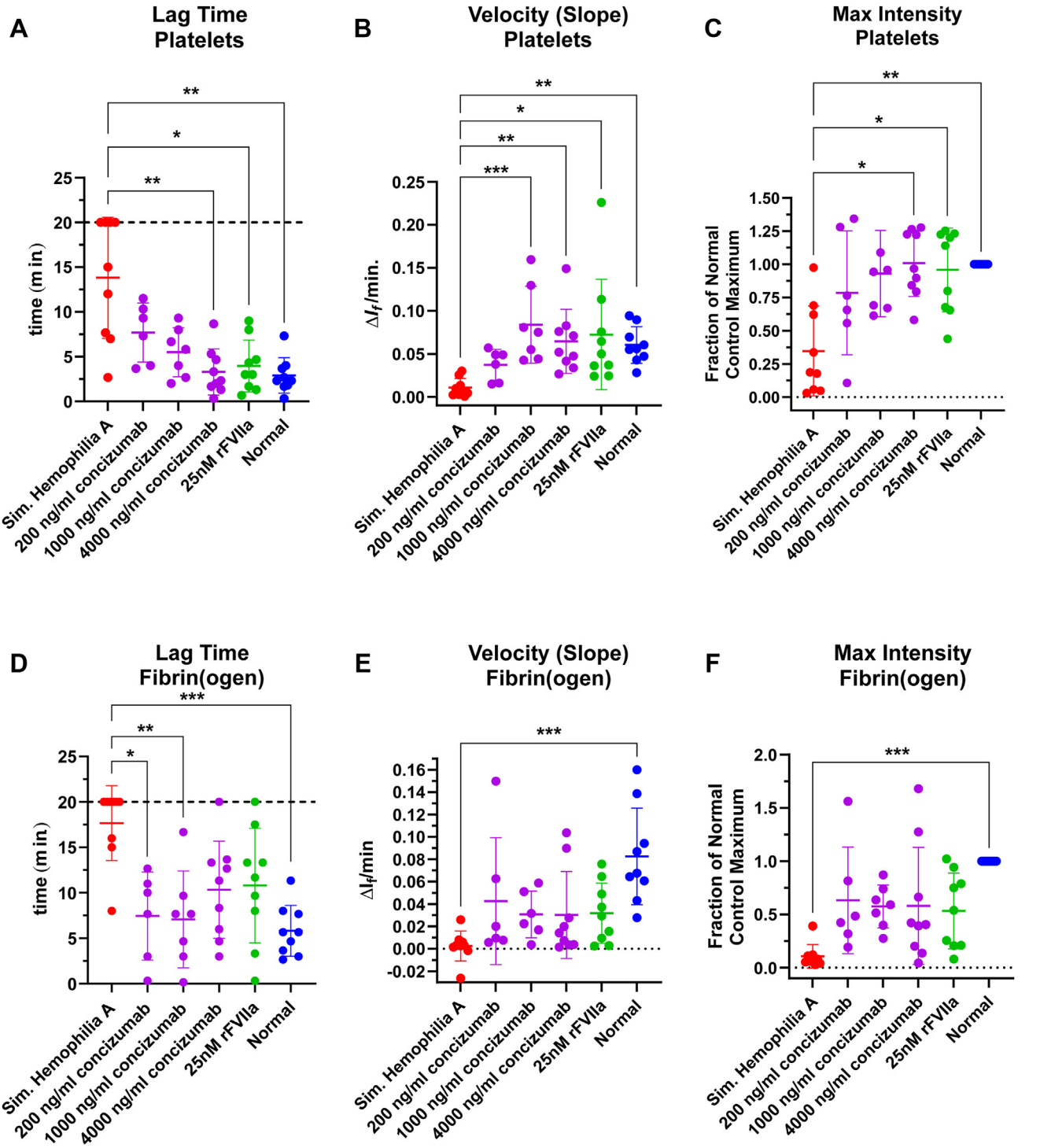

FIGURE 4 Metrics of platelet (A–C) and fibrin(ogen) (D–F) kinetics on collagen peptide–TF surfaces at a wall shear rate of 100 s^{-1}. Each data point is an individual donor with mean and SD of n = 6 to 9 donors. P values denoted as $^{*}P < .05$; $^{**}P < .01$; and $^{***}P < .001$.

(2.9 ± 2.0 minutes) for rFVIIa (4.0 ± 3.0 minutes) and the high concentration of concizumab (3.3 ± 2.6 minutes, Figure 4A, Supplementary Table S1). The rate of platelet accumulation as quantified by the velocity was similar to the normal control (0.060 ± 0.021 DI_f/min) for both rFVIIa (0.072 ± 0.064 DI_f/min) and medium (0.084 ± 0.045 DI_f/min) and high (0.065 ± 0.037 DI_f/min) concentration concizumab treatments of SimHA (Figure 4B, C; Supplementary Tables S2 and S3). Similarly, the maximum intensity normalized to the normal control shows full rescue of platelet accumulation for rFVIIa (0.96 ± 0.31) and medium (0.93 ± 0.33) and high (1.01 ± 0.25) concentrations of concizumab.

In contrast to platelet accumulation, fibrin(ogen) accumulation was only partially rescued by rFVIIa and concizumab treatments. The lag time in fibrin(ogen) accumulation was not measurable over the

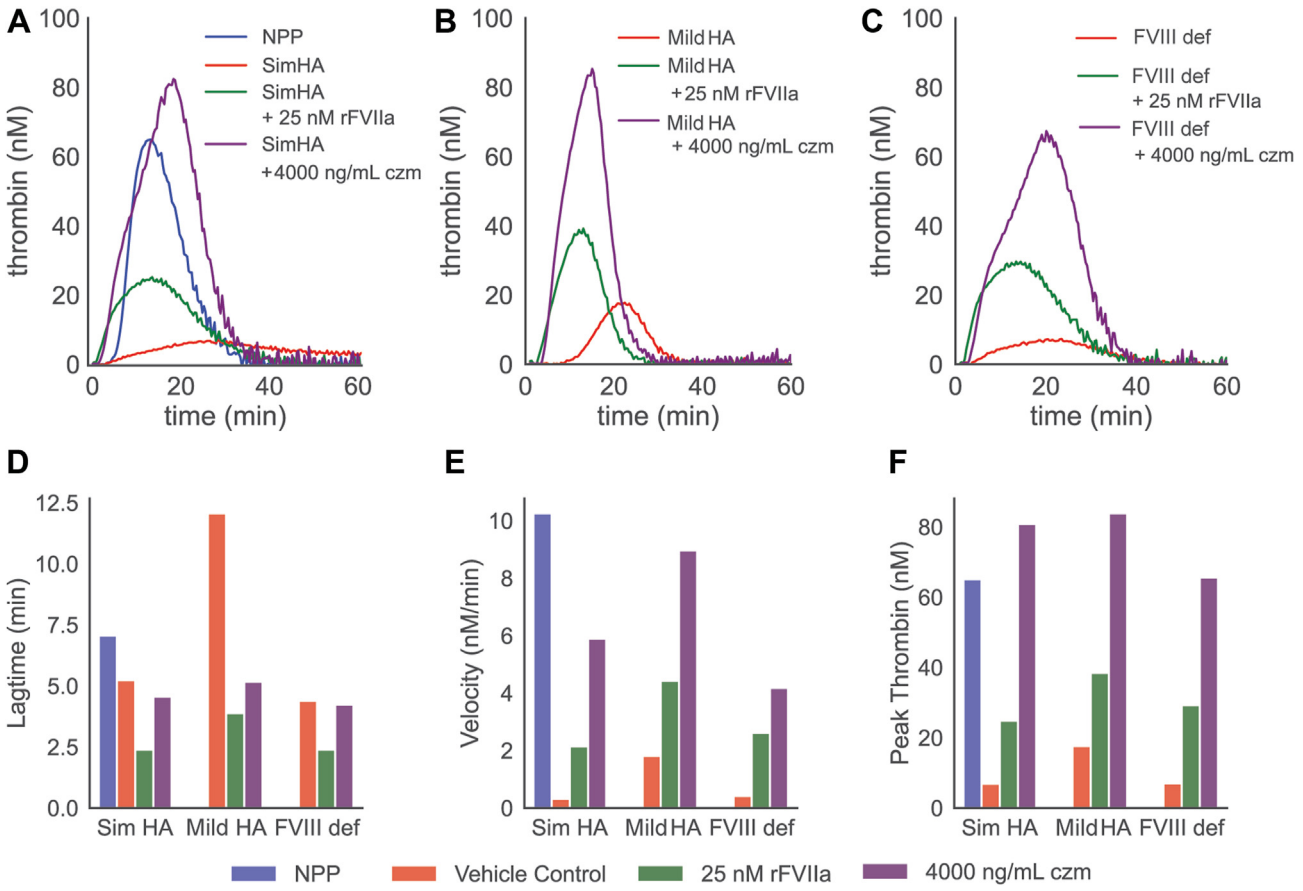

FIGURE 5 Static thrombin generation (1 pM TF). (A–C) Kinetics and (D–F) metrics of static thrombin generation in plasma for simulated hemophilia A (SimA, anti-FVIII), mild hemophilia A (FVIII = 6%), and FVIII deficient (FVIII < 1%) in response to 25 nM rFVIIa and 4000 ng/mL concizumab.

course of the 20 minutes in the majority of SimHA cases as there was little to no fibrin(ogen) deposited (Figure 4D). Lag times were measurable for rFVIIa (10.8 ± 6.3 minutes) and all concentrations of concizumab (7.4 ± 4.8, 7.1 ± 5.3, and 10.3 ± 5.3 minutes for low, medium, and high concentrations, respectively) but were prolonged compared with those of the normal control (5.8 ± 2.8 minutes, Figure 4D, Supplementary Table S4). The same trend was observed for velocity, which on average increased for rFVIIa (0.032 ± 0.027 DI_f/min) and concizumab (0.043 ± 0.021, 0.031 ± 0.057, and 0.030 ± 0.039 DI_f/min for low, medium, and high concentrations, respectively) compared with those of SimHA (0.002 ± 0.001 DI_f/min), but were not rescued to the normal control velocity (0.083 ± 0.043 DI_f/min, Figure 4E; Supplementary Table S5). The total fibrin(ogen) accumulation was only partially rescued with treatment by rFVIIa (0.53 ± 0.35) and concizumab (0.63 ± 0.50, 0.58 ± 0.20, and 0.58 ± 0.55 for low, medium, and high concentrations, respectively; Figure 4F, Supplementary Table S6). Inspection of images near the lag time suggests that this decreased lag time with treatments is primarily driven by accumulation of platelet-bound fibrinogen rather than fibrin fibers. Data using blood from a person with hemphilia A (HA,FVIII = 2.4%) confirmed a more pronounced overall effect on platelet accumulation than on fibrin(ogen) deposition (Supplementary Figure S4). There was a 2- to 3-fold increase in platelet accumulation with marked improvement over the untreated sample, whereas fibrin(ogen) deposition showed inconsistent results.

Thrombin generation was used to compare the open, flow-based system represented by the microfluidic flow assay to a closed, static system. We measured the response to 25 nM rFVIIa and 4000 ng/mL concizumab in SimHA plasma, plasma from an individual with FVIII deficiency (FVIII = 6%, mild HA), and congenital FVIII deficiency (FVIII < 1%, FVIII def) in TF-initiated thrombin generation assay (Figure 5). In contrast to the microfluidic assay, concizumab was able to rescue peak thrombin in SimHA, while rFVIIa only provided a partial rescue. In severe and moderate HA plasma, a similar trend was observed; 4000 ng/mL concizumab enhanced thrombin generation more than rFVIIa illustrating the difference between the flow-based model in whole blood and a static, plasma-based system.

4 | DISCUSSION

In this study, a microfluidic flow assay sensitive to FVIII function to evaluate the effect of concizumab in simulated hemophilia A was developed. Motivated by the rescue of thrombin generation in closed

systems by saturating concentrations of concizumab [14,21], the degree to which similar concentrations of concizumab could enhance clot formation under flow was investigated.

Establishing a well-defined procoagulant substrate was essential for repeatable results in the flow assay, a challenge that is compounded with the small areas defined by micropatterning methods often used in microfluidic assays. To avoid nonuniform patterning and impurities in animal-derived collagens [44], 3 collagen peptides [43] that in combination provide comparable levels of platelet accumulation to type I collagen and have previously been shown to have a uniform and repeatable surface via microfluidic patterning were used [42]. To integrate TF into the collagen peptide surface, a 2-step approach was necessary because the small, high concentration peptides outcompete the large, low concentration TF-laden liposomes for binding sites. Thus, TF liposomes were first covalently bound to the surface at a relatively low surface coverage (<5%), and then collagen peptides were covalently bound to fill in the remaining surface. Covalent bonding is necessary to prevent peptide desorption over the time course of the flow assay. The resultant procoagulant surface promoted both platelet and fibrin accumulation in a FVIII-dependent manner.

The microfluidic flow assay was then used to investigate the ability of concizumab to enhance clot formation under flow under simulated hemophilia A conditions prepared by adding a neutralizing antibody to FVIII. This condition also simulates hemophilia A with inhibitors and acquired hemophilia A. Concizumab supported platelet activation and aggregation to levels that were at or near normal controls. As opposed to static, TF-initiated thrombin generation assays in a closed system, concizumab improved but could only partially rescue fibrin formation owing to the additional biophysical constraints placed on coagulation and fibrin polymerization in an open, flow-based system [30].

The differences in these 2 systems are further underscored by the observation that concizumab enhances thrombin generation to a greater degree than rFVIIa in thrombin generation assay, but these 2 agents provide similar enhancements in clot formation under flow. This may reflect how these agents act on different phases of coagulation. The main action of rFVIIa is during amplification of thrombin generation on the platelet surface [45], while concizumab extends the initiation phase by inhibiting TFPI's binding to FXa and TF:FVIIa [14]. This is supported by observations that thrombin generation in platelet-poor plasma is weakly enhanced with rFVIIa, while an increased response of FVIIa is seen in platelet-rich plasma [46]. Furthermore, rFVIIa provides a more pronounced response in whole blood thromboelastography than in thrombin generation assay [47].

The ability of concizumab and rFVIIa to rescue platelet accumulation, but only to partially rescue fibrin accumulation under flow, may be due to several reasons. First, the thrombin concentration required to activate platelets is significantly lower than that to support fibrin fiber formation under flow. Platelets can be fully activated by 1 nM of thrombin through protease activated receptor 1 (PAR1) [48]; however, for fibrin fibers to form under flow, thrombin concentrations exceeding 100 nM are necessary even at low shear rates ($\sim$10 s^{-1}) [49]. This is further supported by data from flow assays on type I collagen-TF surfaces with whole blood from people with hemophilia A; platelet accumulation is more sensitive to FVIII levels than fibrin accumulation [39]. Similar to observations in this study, fibrin accumulation in flow assays with FVIII deficiencies was limited to areas on and peripheral to large platelet aggregates [41]. Second, both rFVIIa and concizumab have mechanisms of action that include the TF:FVIIa complex. This complex is physically blocked by adhered platelets, which may in turn inhibit their activity once a significant fraction of the surface is covered with platelets [23,24]. Third, both rFVIIa and inhibition of TFPIα have platelet-dependent activity, likely by enhancing coagulation reactions on the surface of activated platelets [50–52]. Higher levels of platelet accumulation, and specifically large platelet aggregates, were associated with higher levels of local fibrin accumulation. In the cell-based model of coagulation, high levels of rFVIIa can produce FXa on platelet surfaces, however it is less efficient than when bound to TF [52,53]. Indeed, in agreement with previous reports [54,55], we found that rFVIIa cannot completely rescue fibrin formation under flow for severe FVIII deficiencies. Our observation that concizumab partially rescued fibrin formation is also in agreement with previous work showing that antagonism of TFPIα reduced the time to fibrin formation in hemophilia A and B plasma perfused over preformed activated platelets but does not rescue it to the level of normal plasma [51].

The variance in the rFVIIa and concizumab data is notable as some donors demonstrate platelet and fibrin accumulation comparable with that of the normal control while others demonstrate only a modest effect compared with that of the untreated SimHA condition. As a whole blood assay, there are several sources of variability that affect the outputs of flow assays including blood cell counts and VWF levels [50,56–58]. Plasma and platelet levels of the various coagulation factors could also affect response to rFVIIa or concizumab. With prophylaxis being the current standard of care for people with hemophilia A in the United States, it proved challenging to recruit subjects with FVIII levels of <5% as their numbers are small and growing smaller. However, we were able to recruit 1 individual with moderate FVIII deficiency and confirmed a robust, enhanced platelet accumulation with both agents.

In summary, we established a micropatterning protocol for procoagulant substrate of TF liposomes and collagen-related peptides that is sensitive to FVIII levels and demonstrated its suitability to evaluate the impact of therapeutic agents in a simulated hemophilia A condition. Concizumab was able to enhance clot formation under flow under hemophilia A conditions, highlighting a yet underrecognized role of concizumab in the platelet-dependent part of the hemostatic process.

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AUTHOR CONTRIBUTIONS

M.P.J., A.R.W., C.P., M.D., M.K., M.M.J., and K.B.N. conceptualized the study. M.P.J. and K.B.N. devised the methodology. M.P.J., Z.A., and

C.H.B. carried out the investigation. M.P.J. undertook the data analysis. M.P.J. and K.B.N. prepared the original draft. B.B.W. and M.M.J. recruited patients. All authors reviewed and edited the manuscript. K.B.N. acquired the funding. 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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Keith B. Neeves 𝕏 @neeveslab

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SUPPLEMENTARY MATERIAL

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