Microfluidic hemophilia models using blood from healthy donors

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Abstract
Background: Microfluidic clotting assays permit drug action studies for hemophilia therapeutics under flow. However, limited availability of patient samples and Inter-donor variability limit the application of such assays, especially with many patients on prophylaxis.

Objective: To develop approaches to phenocopy hemophilia using modified healthy blood in microfluidic assays.

Methods: Corn trypsin inhibitor (4 µg/mL)-treated healthy blood was dosed with either anti–factor VIII (FVIII; hemophilia A model) or a recombinant factor IX (FIX) missense variant (FIX-V181T; hemophilia B model). Treated blood was perfused at 100 s⁻¹ wall shear rate over collagen/tissue factor (TF) or collagen/factor XIa (FXIa).

Results: Anti-FVIII partially blocked fibrin production on collagen/TF, but completely blocked fibrin production on collagen/FXIa, a phenotype reversed with 1 µmol/L bispecific antibody (emicizumab), which binds FIXa and factor X. As expected, emicizumab had no significant effect on healthy blood (no anti-FVIII present) perfused over collagen/FXIa. The efficacy of emicizumab in anti-FVIII-treated healthy blood phenocopied the action of emicizumab in the blood of a patient with hemophilia A perfused over collagen/FXIIa. Interestingly, a patient-derived FVIII-neutralizing antibody reduced fibrin production when added to healthy blood perfused over collagen/FXIIa. For low TF surfaces, reFIX-V181T (50 µg/mL) fully blocked platelet and fibrin deposition, a phenotype fully reversed with anti-TFPI.

Conclusion: Two new microfluidic hemophilia A and B models demonstrate the potency of anti-TF pathway inhibitor, emicizumab, and a patient-derived inhibitory antibody. Using collagen/FXIIa-coated surfaces resulted in reliable and highly sensitive hemophilia models.

Keywords
drug evaluation, fibrin, hemophilia, hemostasis, microfluidics
Essentials

- Limited availability of patient samples is a major challenge for hemophilia drug action studies.
- Microfluidic assays using blood from healthy donors were developed to phenocopy hemophilia.
- A hemophilia A assay demonstrated the potency of emicizumab on collagen/FXa-coated surfaces.
- A hemophilia B assay demonstrated potency of anti-TFPI on collagen/TF-coated surfaces.

1 | INTRODUCTION

Congenital hemophilia is a genetic disorder that increases bleeding risk in affected individuals. The 2 major types of the bleeding disorder are hemophilia A, with a deficiency in coagulation factor VIII (FVIII), and hemophilia B, with a deficiency in factor IX (FIX). In healthy subjects, FVIIla (activated FVII) acts as a cofactor for FIXa, serving to increase the affinity of FIXa for factor X (FX) by 10,000-fold. FIXa then converts FX to Fx. Both FVIII and FIX are parts of the intrinsic pathway of coagulation, which is impaired in patients with hemophilia. Based on the residual factor levels, the bleeding disorder can be categorized into severe (<1% residual factor activity), moderate (1%-5%), and mild (5%-40%). However, while residual FVIII/FIX activity is useful for the stratification of patients, the bleeding risk among these groups can vary considerably and is influenced by multiple factors such as genetic mutation types or von Willebrand factor levels. Individuals with hemophilia A or hemophilia B are more likely to have bleeding in the joints where tissue factor (TF) expression is considered low and weight/impact-induced biomechanical perturbation of the joint is high.

Conventional treatment for patients with hemophilia is the administration of intravenous factor replacements to restore their residual factor levels, and this can be done prophylactically or on demand. One third of patients with severe hemophilia A develop neutralizing antibodies against FVIII and 1.5% to 3% of patients with hemophilia B develop FIX-neutralizing antibodies. These “inhibitor” patients are treated with bypassing agents such as activated prothrombin complex concentrates or recombinant FVIIa (rFVIIa). rFVIIa enhances FX activation through TF-dependent, cellular surface-dependent, and endothelial protein C receptor-dependent pathways. A recent advance is the development of a bispecific antibody (emicizumab), which mimics FVIIla function by transiently binding FIXa and its substrate FX to mediate FXa generation. Emicizumab is advantageous, as it can be subcutaneously administered, has a long half-life (4 weeks), and no immunoglobulin G (IgG)-based immune responses have been reported so far. More importantly, the bispecific antibody can be used in patients with and without FVIII inhibitors. In addition to the traditional bypassing agents and FVIIla-mimicking bispecific antibodies, several other novel agents are being investigated. For example, 3 monoclonal antibodies against tissue factor pathway inhibitor (TFPI) are currently in different phases of development.

Various in vitro models/assays have been used to study the effect of coagulation factor modulation on fibrin formation under flow conditions. Sakurai et al demonstrated that perfusion of FIX-deficient blood (5% FIX) over collagen/TF microposts led to impaired fibrin formation. Thomassen et al showed that TFPI-α antagonism was able to increase fibrin formation in blood from both healthy donors and patients with hemophilia.

The determination of residual FVIII/FIX activity is assessed in the clinic using a static assay that uses plasma rather than whole blood. Microfluidic assays allow the phenotyping of whole blood from patients with hemophilia and provide a platform to assess the efficacy of various therapeutics under flow in a high-throughput fashion. In such assays, whole blood is perfused over prothrombotic surfaces such as collagen or collagen/TF, and clot growth is measured. Blood from patients with severe hemophilia displays a defect in both platelet deposition and fibrin formation under flow. In contrast, blood from patients with moderate and mild hemophilia displays relatively normal platelet deposition with deficits in fibrin formation. Patient recruitment, variability in their clinical presentation, and interference from prophylactic products all pose significant challenges to the development of whole blood microfluidic assays to study drug potency or mechanism of action on a background of hemophilia. As more patients switch to novel therapeutics like emicizumab with a longer half-life (4 weeks as opposed to 1 week for the traditional FVIII products), drug testing in patient blood without interference from prophylactics may become increasingly difficult. Therefore, it would be useful to recapitulate the hemophilic phenotypes ex vivo using treated blood from healthy donors. Such hemophilia models allow significantly greater throughput and standardization compared to using blood from patients with hemophilia. Donated blood from healthy donors is more readily available and excludes the potential interference of other drugs. We present 2 hemophilia microfluidic assays using healthy adult blood to study the effect of bypassing agents. The use of highly diluted TF or FXa in the triggering surface allowed the dose-response testing of anti-TFPI, emicizumab, and a patient-derived FVIII-neutralizing antibody.

2 | METHODS

2.1 | Blood collection

Whole blood (1 mL) was collected into a syringe containing corn trypsin inhibitor (CTI, 4 µg/mL) to lightly suppress contact pathway activation of FXIIIa during sample collection and handling. Whole blood was subjected to microfluidic assay within 10 minutes of collection.
2.2 | Reagents

Reagents were obtained as follows: Alexa Fluor 488 conjugated anti-human CD61 (Bio-Rad), AF647 conjugated human fibrinogen (ThermoFisher Scientific), type I fibrillar collagen (Chronolab), Dade Innovin lipitated tissue factor (TF, Siemens), CTI and FXIa (Haematologic Technologies), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Fisher Scientific), Sigmacote (Sigma), Anti-FVIII (4A4) and anti-FIX (BC2; Green Mountain Antibodies), Anti-TFPI (gA200 hIgG1, Bayer, Whippany), bispecific antibody (emicizumab), reFIX-V181T variant,25 and a recombinant rgG4 containing complementarity-determining regions originally derived from a patient (NB41, as described in van den Brink).26

2.3 | Microfluidic clotting assay

Whole blood was perfused over a patterned prothrombotic surface to trigger clot formation in microfluidic devices at 1 µL/min per channel. A single 250-µm-wide channel polydimethylsiloxane (PDMS) patterning device was vacuum-sealed to a Sigmacote-treated glass slide, as previously described.27,28 A total of 5 µL of fibrillar collagen was perfused through the channel to pattern a stripe of aligned collagen fibers. For TF-dependent assays, this step was followed by TF perfusion (20 nmol/L or 1 nmol/L, 5 µL) and incubated under static conditions for 30 minutes to allow binding to collagen. For intrinsic pathway-dependent assays, FXIa (4.9 µg/mL, 5 µL) was used instead of TF. The channel was then rinsed with 20 µL of 1% bovine serum albumin. The patterning device was then replaced by an 8-channel PDMS device positioned perpendicularly to the surface pattern stripe to form 8 uniformly distributed prothrombotic patches (250 µm × 250 µm) (Figure S1A). Anti-FVIII (4A4) and patient-derived NB41 were used to inhibit FVIII activity in healthy blood to recapitulate hemophilia A clotting defect. Anti-FIX (BC2) and reFIX-V181T variant were used to inhibit FIX activity in healthy blood to recapitulate hemophilia B clotting defect.

2.4 | Imaging

Platelet and fibrin were detected by an epifluorescence microscopy (IX81; Olympus America Inc.) and a charge-coupled device camera (Hamamatsu, Bridgewater, NJ, USA). A 10X objective/0.30NA was used in all of the experiments. ImageJ (NIH, Bethesda, MD, USA) was used to analyze acquired images. The mean fluorescent intensity was measured over the central 75% of the channel to eliminate side-wall effects. The fluorescence values were background-corrected by subtracting the signals at the first time point.

2.5 | Statistical analysis

Differences between control and treated groups were analyzed with 2-way analysis of variance (with Bonferroni posttest) or Student t-test. The difference was considered significant at \( P < 0.05 \). The number of donors (N) and number of clots (n) are shown in the figures or figure legends.

3 | RESULTS

3.1 | Hemophilia A model

A commercially available murine FVIII-neutralizing antibody (anti-FVIII) was added to healthy blood to recapitulate the hemophilia A phenotype in our device (Figure 1A). We have previously shown that whole blood perfused over collagen/TF (Figure 1B) could be used to study TF-dependent therapeutics.19 FVIII inhibition did not result in marked decreases in platelet accumulation (Figure 1D), but all 3 doses of anti-FVIII significantly reduced fibrin generation. In the presence of TF, the effect of FVIII inhibition only started to manifest at later time points. We have previously used collagen or collagen/kaolin to activate the contact pathway24; however, factors such as device surface treatment and incubation time could influence the onset and the extent of contact activation. Here, we present a modified assay using collagen/FXa as the surface trigger (Figure 1C) to activate the intrinsic pathway (Figure S5). On collagen/FXa surfaces, FVIII inhibition resulted in a slight reduction in platelet deposition (Figure 1F) after 7 minutes. More importantly, anti-FVIII at concentrations ≥ 25 µg/mL abolished fibrin generation (Figure 1G). Platelet deposition in FVIII-inhibited blood (Figure 1F) began to deviate from the control shortly after onset of fibrin polymerization (Figure 1G). We also treated healthy blood with a neutralizing FVIII-binding antibody (15 µg/mL) derived from patients with hemophilia A (Figure 1H,I), and similar results were observed.

3.2 | The effect of an FVIIIa-mimetic bispecific antibody in hemophilia A assay

A humanized bispecific antibody (emicizumab) mimics FVIIIa function and restores hemostasis by bringing FIXa and FX together to

![Figure 1](image-url) Hemophilia A assay. In a microfluidic device (A), whole blood was perfused over collagen/TF surfaces (B) and collagen/FXa surfaces (C) to form thrombi. Murine anti-FVIII (4A4) caused no change in platelet deposition (D) and a reduction in fibrin generation (E) on collagen/TF. The same concentrations of the FVIII inhibitory antibody slightly reduced platelet deposition (F) and abolished fibrin accumulation (G) on collagen/FXa, similar to results obtained with a patient-derived anti-FVIII (H, I). Differences between control and treated groups were analyzed with 2-way analysis of variance with Bonferroni posttest. * \( P < 0.05 \). Shaded areas represent standard deviation. CTI, corn trypsin inhibitor; FI, fluorescence intensity; FVIII, factor VIII; FXIa, factor Xla; TF, tissue factor; WB, whole blood
(A) [Image of a platelet-rich plasma sample]

(B) CTI WB → Collagen/TF (100 s⁻¹)

(C) CTI WB → Collagen/FXIIa (100 s⁻¹)

(D) Platelet FI
- control
- 25 µg/mL Anti-FVIII
- 50 µg/mL Anti-FVIII
- 100 µg/mL Anti-FVIII

N = 2 donors, n = 4 clots

(E) Fibrin FI
- control
- 25 µg/mL Anti-FVIII
- 50 µg/mL Anti-FVIII
- 100 µg/mL Anti-FVIII

N = 3 donors, n = 6 clots

(F) Platelet FI
- control
- 25 µg/mL Anti-FVIII
- 50 µg/mL Anti-FVIII
- 100 µg/mL Anti-FVIII

N = 2 donors, n = 4 clots

(G) Fibrin FI
- control
- 25 µg/mL Anti-FVIII
- 50 µg/mL Anti-FVIII
- 100 µg/mL Anti-FVIII

N = 3 donors, n = 6 clots

(H) Platelet FI
- control
- 15 µg/mL NB41

N = 3 donors, n = 6 clots

(I) Fibrin FI
- control
- 15 µg/mL NB41

N = 3 donors, n = 6 clots
support FX activation. Adding the bispecific antibody to healthy blood (no anti-FVIII) did not increase platelet accumulation (Figure 2A), but it slightly shortened the lag time preceding fibrin polymerization (Figure 2B) in a dose-dependent manner. The rate of fibrin polymerization following the onset appeared to be unchanged since the fibrin generation curves obtained at different doses can be superimposed by horizontal shifts along the time axis. Similar to the platelet response in healthy blood, emicizumab did not alter platelet accumulation (Figure 2C) in our hemophilia A model. In contrast, emicizumab dose-dependently reduced fibrin generation lag time and increased fibrin polymerization rate (Figure 2D).

The results at the time of control clot occlusion are shown in Figure S1 and plotted in bar graphs (Figure 3). Neither emicizumab nor the anti-FVIII antibody caused any significant changes in platelet fluorescein intensity (Figure 3A). All 3 doses of emicizumab (0.01, 0.1, 1 μmol/L) significantly increased fibrin polymerization (P < .05, Figure 3B) in whole blood treated with anti-FVIII, with a modest dose-dependent effect on untreated whole blood. The highest dose (1 μmol/L) of the bispecific antibody largely restored fibrin polymerization compared to the healthy adult control level. We also tested emicizumab in blood obtained from a patient with mild hemophilia. In Figure 3B (N = 3 donors, n ≥ 5 clots) at occlusion, the coefficient of variation was < 15% for high bispecific antibody
in unmodified healthy blood. Similarly, the acquired hemophilia A model generated essentially no fibrin and high levels of bispecific antibody absolutely rescued fibrin production \((P = 1.77 \times 10^{-5})\) over 3 donors. Similar to the results in our hemophilia A assay, platelet accumulation (Figure 4A) was insensitive to emicizumab, whereas the drug dose-dependently rescued fibrin polymerization (Figure 4B) in the blood of the patient with hemophilia A (7% of normal FVIII). This was a single hemophilia patient experiment (2 clots per condition) that demonstrated a striking 554.5% increase in fibrin with drug. In addition to emicizumab, Advate also restored fibrin generation in blood of the patient with hemophilia A (Figures S2 and S3).

### 3.3 Hemophilia B model

To recreate the phenotype of hemophilia B, we tested the effect of a murine FIX-neutralizing antibody (anti-FIX) on healthy blood clotting. Because anti-FIX only partially inhibited fibrin polymerization...
on collagen/TF (Figure S4), we used a recombinant FIX variant (reFIX-V181T) in our hemophilia B model to achieve FIX inhibition. It has been shown that certain mutations at position 181 (legacy numbering) lead to moderate to severe hemophilia B.\textsuperscript{29,30} FIX-V181T has significantly reduced activity causing prolonged clotting time in activated partial thromboplastin time assays.\textsuperscript{25} When added to healthy blood, low-activity variants like reFIX-V181T can compete with endogenous FIX to impair FXa generation. CTI-treated whole blood (±50 μg/mL FIX-V181T) was perfused over collagen/TF surfaces at an initial venous shear rate of 100 s\textsuperscript{-1}. Recombinant FIX-V181T reduced both platelet deposition (Figure 5A) and fibrin generation (Figure 5B). The FIX variant also reduced platelet accumulation (Figure 5C) and fibrin polymerization (Figure 5D) on collagen surfaces with diluted TF (20-fold TF dilution factor). Although less fibrin was made at low TF in comparison to the high TF condition, the 95% inhibition by reFIX-V181T was more pronounced on low TF surfaces providing a larger dynamic range for the assay.

### 3.4 | The effect of anti-TFPI in hemophilia B assay

The use of reFIX-V181T to recreate hemophilia B phenotype allowed us to test the effect of bypassing agents such as anti-TFPI. The control experiment in Figure 5 tested the effect of TF level on the clotting response and demonstrated low TF has better dynamic
range for subsequent figures: Low TF was then used in Figure 6. Adding anti-TFPI did not significantly affect platelet accumulation (Figure 6A) in healthy adult blood (no reFIX-V181T present) on low TF surfaces likely because collagen alone was a potent platelet agonist. However, all 3 doses of anti-TFPI significantly enhanced fibrin polymerization (Figure 6B) to the same level. Also, the control channels occluded by 8 minutes. When healthy blood was dosed with 50 µg/mL of reFIX-V181T, anti-TFPI (≤12 µg/mL) dose-dependently restored platelet deposition (Figure 6C) and increased fibrin polymerization (Figure 6D). This was in agreement with results of concizumab from a previous clinical trial.34

**DISCUSSION**

Here, we report ex vivo hemophilia models that allow the assessment of novel FVIII/FIX replacements and bypassing agents. In the past, we have used blood inhibited with low levels of CTI to investigate hemophilia A phenotypes on collagen/±TF surfaces. Consistent with the previous observation that high levels of TF can partially compensate for the FVIII deficiency,19 adding the anti-FVIII inhibitory antibody to the whole blood from healthy individuals reduced but did not abolish platelet and fibrin deposition. Platelet accumulation was unaffected possibly due to the strong

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**FIGURE 6** Testing anti-TFPI in hemophilia B assay. The effect of anti-TFPI on platelet and fibrin deposition were investigated in healthy blood (A, B) and hemophilia B mimetic blood (C, D). Anti-TFPI not only restored coagulation in hemophilia A model (D), but it also has a procoagulant effect in blood with normal FIX activity (B). This is in agreement with results of another anti-TFPI (concizumab) from a previous clinical trial.34 Differences between control and treated groups were analyzed with 2-way analysis of variance with Bonferroni posttest. *P < 0.05. Shaded areas represent standard deviation. CTI, corn trypsin inhibitor; FI, fluorescence intensity; n.s., nonsignificant; TF, tissue factor; TFPI, tissue factor pathway inhibitor; WB, whole blood
activation by the collagen trigger and ADP/thromboxane A₂ release. While the TF-dependent pathway alone was able to generate enough thrombin for fibrin polymerization, all 3 doses of anti-FVIII reduced fibrin generation after 5 minutes due to the defect in the FXI-thrombin feedback loop. This reduction in the late-stage fibrin generation was similar to the previously reported results for FXI-inhibited clot formation.

Traditionally, kaolin is used to study TF-independent clotting in healthy/hemophilic blood assays. Here, we developed a new method using collagen/FXIIIa surfaces to potently and locally activate the intrinsic pathway. The use of FXIIIa bypasses the need for FXII activation; therefore, blood can be collected in high levels of CTI (40 µg/mL), which alleviates the need to start the assay right after the blood collection. In Figure 4, all 3 concentrations of the murine FVIII-neutralizing antibody (4A4) abolished fibrin generation on collagen/FXIIIa within the experimental time frame. This was also true for the recombinant IgG4 NM41, which was derived from an inhibitor patient. Because collagen alone was potent enough to drive platelet aggregation to full occlusion, it was difficult to capture the effect of anti-FVIII on platelets. In contrast, the sensitivity and the large dynamic range of fibrin polymerization makes the system a good platform to study novel therapeutics such as FVIII-mimetics like bispicific antibodies in a low-TF environment. Using this collagen/FXIIIa-based assay, we demonstrated that Advate and emicizumab were able to restore coagulation in hemophilia A blood, therefore recapitulating the situation in vivo.

We also quantitatively validated our microfluidic hemophilia A model by measuring the restoration of fibrin polymerization in hemophilia A-mimetic blood by emicizumab. These experiments highlight the advantage of being able to run a paired control alongside the treatment sample in our hemophilia assay. Because the time of blood collection from patients can be unpredictable, it has previously been difficult to coordinate a side-by-side comparison like this between healthy and hemophilic patient blood without having an on-demand healthy donor available. Using healthy blood not only generates standard curves of normal clot growth but also provides insights into potential thrombotic risks. For example, emicizumab caused a small dose-dependent shift toward earlier time points for fibrin generation in blood with normal FVIII activities. This is likely due to the strong activation of FIX by the concentrated FXIIIa on the surface.

The use of a FIX missense variant allowed us to recreate a hemophilia B phenotype. The surface trigger concentration is tunable and can be adjusted to represent different levels of TF expression in different sites in the body. By reducing the surface TF concentration, we were able to increase the dynamic range for platelet and fibrin deposition. Adding anti-TFPI to blood in the presence of reFIX-V181T dose-dependently rescued platelet deposition and fibrin formation. In contrast, adding anti-TFPI to healthy adult blood did not alter the platelet response but it did enhance fibrin polymerization at all 3 doses. This indicates that anti-TFPI is potentially effective at restoring hemostatic activity in patients with severe hemophilia.

For anti-FVIII > 25 µg/mL, fibrin polymerization was abolished on collagen + FXIIIa. Similarly, FIX-V181T > 50 µg/mL caused a very substantial reduction of fibrin generation on collagen + low TF. This extent of coagulation defect was equivalent to observations of fibrin formation previously reported for severe FVIII- and FIX-deficient patients, where residual activities of FVIII or FIX were <1% of normal.

In conclusion, we have developed 2 hemophilia assays that mimic hemophilia A and hemophilia B phenotypes through dosing readily accessible healthy blood with a FVIII-neutralizing antibody and a recombinant FIX variant, respectively. The surface trigger concentration is adjustable and either collagen/TF or collagen/FXIIIa can be used depending on the pathway of interest. While factor inhibition is what enables us to phenocopy hemophilia clotting profile, the 2 highly sensitive models allow for high-throughput evaluation of drug responses. A limitation of the models is that they cannot be used to test traditional factor replacement therapeutics because the FVIII-neutralizing antibody and the recombinant FIX variant will interfere with the activity of the exogenous factors. Nonetheless, the hemophilia assays could enable rapid screening/evaluation of novel hemophilic agents in whole blood under flow in a high-throughput fashion, which helps narrow the therapeutic candidates before testing them in hemophilic patient blood. The hemophilia assays also have the added advantage of allowing side-by-side comparisons to clotting at healthy factor levels. Future work is needed to recapitulate different hemophilia severities/residual factor levels using different anti-FVIII/reFIX doses. The platelet agonist on the prothrombotic surface can be optimized to increase the dynamic range for platelet deposition by reducing thrombin-independent platelet activation/aggregation. Alternatively, as fibrin generation is more sensitive to factor level changes, the TF trigger alone may be used to initiate coagulation. In addition, patient-derived neutralizing antibodies and factor variants can be explored to predict the effect of therapeutics in patients with hemophilia with the specific inhibitors/mutations.

### RELATIONSHIP DISCLOSURE

XY, KP, RMC, CHC, and SLD have nothing to disclose. Li reports grants from Bayer Hemophilia Award Program, during the conduct of the study; and a pending patent “Compositions and Methods for Modulating Factor IX Function,” 2019:US20190024071A1. AC reports personal fees from Synergy, Kedrion, and Genzyme; and grants from Alexion, Bayer, Bioverativ, Novo Nordisk, Shire, Spark, and Syntimmune outside the submitted work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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