A factor IX variant that functions independently of factor VIII mitigates the hemophilia: a phenotype in patient plasma

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ORIGINAL ARTICLE

A factor IX variant that functions independently of factor VIII mitigates the hemophilia A phenotype in patient plasma

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Abstract

Background: Recombinant factor (F)IX-FIAV has previously been shown to function independently of activated FVIII (FVIIIa) and ameliorate the hemophilia A (HA) phenotype *in vitro* and *in vivo*.

Objectives: The aim of this study was to assess the efficacy of FIX-FIAV in plasma from HA patients using thrombin generation (TG) and intrinsic clotting activity (activated partial thromboplastin time [APTT]) analyses.

Methods: Plasma obtained from 21 patients with HA (>18 years; 7 mild, 7 moderate, and 7 severe patients) was spiked with FIX-FIAV. The FXIa-triggered TG lag time and APTT were quantified in terms of FVIII-equivalent activity using FVIII calibration for each patient plasma.

Results: The linear, dose-dependent improvement in the TG lag time and APTT reached its maximum with approximately 400% to 600% FIX-FIAV in severe HA plasma and with approximately 200% to 250% FIX-FIAV in nonsevere HA plasma. The cofactor-independent contribution of FIX-FIAV was therefore suggested and confirmed by the addition of inhibitory anti-FVIII antibodies to nonsevere HA plasma, resulting in a FIX-FIAV response similar to severe HA plasma. Addition of 100% (5 μg/mL) FIX-FIAV mitigated the HA phenotype from severe to moderate (from <0.01% to 2.9% [IQR 2.3%-3.9%] FVIII-equivalent activity), from moderate to mild (3.9% [IQR 3.3%-4.9%] to 16.1% [IQR 13.7%-18.1%] FVIII-equivalent activity), and from mild to normal (19.8% [IQR 9.2%-24.0%] to 48.0% [IQR 34.0%-67.5%] FVIII-equivalent activity). No substantial effects were observed when combining FIX-FIAV with current HA therapies.

Conclusion: FIX-FIAV is capable of increasing the FVIII-equivalent activity and coagulation activity in plasma from HA patients, thereby mitigating the HA phenotype. Hence, FIX-FIAV could serve as a potential treatment for HA patients with or without inhibitors.

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Viola J.F. Strijbis and Lorenzo G.R. Romano contributed equally to this study.

KEYWORDS
emicizumab, factor VIII, factor IX, hemophilia A, hemorrhage

1 | INTRODUCTION

Hemophilia A (HA) is an inherited bleeding disorder characterized by a deficiency of coagulation factor (F)VIII. Treatment consists of on-demand administration of FVIII concentrate in case of bleeding, or of regular FVIII infusions as prophylaxis to prevent bleeding. Emicizumab, a bispecific monoclonal antibody that mimics FVIII [1], can also be used for prophylaxis. FVIII replacement therapy may be associated with the development of anti–FVIII-inhibitory antibodies (FVIII inhibitors), which develop in 13% to 39% of patients with HA [2,3] . In inhibitor patients, treatment options are emicizumab and bypassing agents, such as recombinant FVIIa (rFVIIa) and activated prothrombin complex concentrate (aPCC) [4–7]. Although these treatments are effective in most patients, the use of rFVIIa may lead to thrombotic complications in rare cases and more often in combination with aPCC [8]. Furthermore, thrombotic microangiopathy has been observed in patients receiving emicizumab and aPCC simultaneously [9]. In addition, in up to 50% patients in prospective cohort studies, emicizumab treatment did not prevent breakthrough bleeds, although most of these were trauma-associated bleeds [10].

To further expand the current HA treatment strategies, we validated a novel bypassing agent. This agent is a modified FIX variant, FIX-FIAV, that functions independently of activated FVIII (FVIIIa) and was previously shown to ameliorate the HA phenotype *in vitro* and *in vivo* [11,12]. FIX-FIAV is named after its 4 amino acid substitutions: L6F, V181I, K265A, and I383V. These modifications have been implicated to be important for substrate recognition and binding and together lead to FVIII-independent FIX activity. The biochemical characteristics of FIX-FIAV were previously assessed [11]. In short, in a plasma-free system, FIXa-FIAV presented up to 5-fold increased factor X (FX) activation in the absence of FVIIIa compared with wild-type FIXa(-WT).

Here, we have assessed the prohemostatic effect of FIX-FIAV in a preclinical setting in the plasma of patients with severe, moderate, or mild HA with and without FVIII inhibitor. This allowed us to not only evaluate the FIX-FIAV response regardless of residual and baseline FVIII levels, but to also study the potential enhanced effects of FIX-FIAV in combination with the current HA therapies FVIII, emicizumab, FVIIa, or aPCC.

Essentials

- In previous studies, factor (F) IX-FIAV improved the hemophilia A phenotype *in vitro* and *in vivo*.
- FVIII-equivalent activity of FIX-FIAV was tested in (non-) severe hemophilia A plasma.
- FIX-FIAV improves thrombin formation and mitigates the hemophilia A phenotype in plasma.
- FIX-FIAV could serve as a potential hemophilia A treatment regardless of inhibitor status.

2 | METHODS

2.1 | Patient inclusion

Overall, 21 adults (>18 years) male patients with HA were included: 7 severe (historically lowest FVIII:C <0.01 IU/mL), 7 moderate (historically lowest FVIII:C 0.01-0.05 IU/mL), and 7 mild (historically lowest FVIII:C >0.05 to 0.40 IU/mL) patients. Patients with an additional bleeding disorder, chronic liver failure, or having received prophylactic treatment <48 hours within study participation or bypassing therapy in general, including prophylaxis with bypassing agents, (eg, emicizumab, FVIIa, and aPCC) were excluded. The median patient age was 38 years (IQR, 30-49 years). Included patients received a last dose of FVIII concentrate at least 3 days before inclusion, and none of the patients were treated with extended half-life FVIII products. Historically, lowest measured FVIII:C levels and known *F8* mutations were collected from patients' medical files. The study was approved by the local Medical Ethics Committee (MEC-2019-0617), and all patients gave written informed consent.

2.2 | Human plasma

Blood was collected in vacuum tubes containing 0.129 M sodium citrate with and without 25 μg/mL of the FXIIa inhibitor Thermostable Inhibitor of Contact Activation (TICA, provided by professor T.M. Hackeng, CARIM, Maastricht University) [13]. Platelet-poor plasma was prepared at room temperature by 2500 × *g* centrifugation for 15 minutes, followed by 10 minutes 14 000 × *g* centrifugation of the supernatant, and aliquots were stored at −80 °C. Normal pooled human plasma (NPP) comprising platelet-poor plasma from ≥20 male and female donors (18-66 years) was from Precision Biologic. FVIII- or FIX-immuno-depleted plasma was from Diagnostica Stago.

2.3 | Reagents and proteins

Benzamidine and Poly-D-lysine hydrobromide was from Sigma-Aldrich and all tissue culture reagents were from Thermo Fisher Scientific. Calibrator and fluorescent substrate (FluCa) were from a Thrombinoscope, TriniCLOT automated activated partial thromboplastin time (APTT) reagent, and Owren–Koller buffer (isotonic saline) from Diagnostica Stago. Phospholipid TGT containing phosphatidylserine,

phosphatidylcholine, and sphingomyelin was from Rossix, an anti-FVIII-inhibitory antibody (GMA-8015) from Green Mountain Antibodies. Human plasma-derived FIX, FIXa, and FXIa were from Prolytix, recombinant FVIII (NovoEight) and FVIIa (NovoSeven) from Novo Nordisk A/S, aPCC (FEIBA) from Takeda Pharmaceutical Company Limited, and emicizumab (HemLibra) from Roche. Molecular weights (Da) and extinction coefficients ($E_{0.1\%,}$ 280 nm) of the proteins used were taken as follows: FIX, 55 000 and 1.32; FIXa, 45 000 and 1.40; and FXIa, 160 000 and 1.34. For FIX-FIAV values for the human protein were used. All functional assays were performed in Hepes-buffered Saline (20 mM Hepes, 0.15 M NaCl, pH 7.5) (HBS buffer) supplemented with 0.1% (w/v) PEG8000 (dilution buffer) and 5 mM $CaCl_2$ (assay buffer).

2.4 | Plasma analysis

The APTT, FVIII:C using a one-stage FVIII-specific APTT assay (one-stage assay [OSA]) and chromogenic substrate assay (CSA), FIX:C (OSA and CSA), FVIII inhibitors according to the Nijmegen modification of the Bethesda assay, antithrombin, prothrombin, FX, and von Willebrand factor antigen (VWF:Ag) were measured in citrated plasma without the addition of TICA employing a Sysmex CS5100 (Siemens) using corresponding reagents according to the manufacturer. Bethesda units (BU) of >0.5 were considered clinically relevant. FVIII:Ag and FIX:Ag were assessed in TICA-comprising plasma employing enzyme-linked immunosorbent assays (ELISA) as described by the manufacturer (Cedarlane).

2.5 | Construction and expression of recombinant FIX

Constructs encoding for human wild-type FIX (FIX-WT) and FIX comprising L6F, V181I, K265A, and I383V substitutions (FIX-FIAV) were provided by uniQure Biopharma B.V. Human embryonic kidney 293 (HEK293, CRL-1573; American Type Culture Collection (ATCC)) cell lines stably expressing FIX were obtained following cotransfection of pcDNA3.1-FIX-WT or pcDNA3.1-FIX-FIAV with pcDNA3.1-Furin vectors employing Lipofectamine2000 per the manufacturer instructions and essentially as described previously for FX [14]. In brief, FIX expression of transfectants was assessed by conditioning individual clones for 24 hours in Dulbecco's modified Eagle's medium/F-12 without phenol red supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 μg/mL amphotericin B, 100 μg/mL geneticin, 10 μg/mL insulin-transferrin-sodium selenite, and 6 μg/mL vitamin K (Konakion, Roche) (FIX-specific expression media) and subsequently measuring the FIX-specific APTT clotting activity in a modified OSA by mixing conditioned media with FIX-depleted human plasma in a 1:1 ratio. A reference curve of NPP serially diluted in Owren–Koller buffer mixed in a 1:1 ratio with FIX-depleted human plasma was used to calculate the equivalent FIX Units per mL plasma, with 1 mL of NPP comprising 1 Unit of FIX activity. To monitor FIX-FIAV expression, up to 12 transfectants per variant with the highest FIX expression was assessed for FVIII-equivalent APTT clotting activity using FVIII-depleted human plasma to calculate the equivalent FVIII Units per mL plasma, with 1 mL of NPP comprising 1 Unit of FVIII activity. The transfectants with the highest FIX expression (and FVIII-equivalent expression for FIX-FIAV) were expanded into a 6320 cm^2 cell factory that was pretreated with Poly-D-lysine hydrobromide (5 mg for 1 hour at room temperature) and conditioned for 24 hours in FIX-specific expression media. Conditioned media was collected for 10 consecutive days, filtered over an 0.45 μm polyethersulfone membrane, and supplemented with 10 mM benzamidine before storage at −20 °C.

2.6 | Purification of FIX

Conditioned media (15 L) was thawed at 37 °C, applied to a size 6 A ultrafiltration hollow fiber cartridge using an Äkta flux 6 instrument (Cytiva), diafiltrated to approximately 500 mL in 20 mM Hepes, 0.15 M NaCl, 10 mM benzamidine, pH 7.4, and stored at −20 °C. After thawing at 37 °C, the concentrate was applied at room temperature to a 4.8 × 4 cm Q-Sepharose Fast Flow column (Cytiva) equilibrated in 20 mM Tris, 0.15 M NaCl, and 10 mM benzamidine, pH 7.4. After washing with the same buffer, bound protein was eluted with a linear 0.15 to 0.75 M NaCl gradient. Fractions containing FIX activity were stored at −80 °C. After thawing at 37 °C, the fractions were pooled and dialyzed at 4 °C, first for 3 hours to 1 mM EDTA, 20 mM Tris, 10 mM benzamidine, pH 7.0 (5 L), next for 3 hours to 40 mM Na2HPO4/NaH2PO4, 10 mM benzamidine, pH 6.8 (5 L), followed by overnight dialysis to the same buffer. The dialysate was centrifuged at 10 000 × *g* for 20 minutes at 4 °C, and the supernatant was applied at room temperature to a Bio-Scale CHT20-I hydroxyapatite column (Bio-Rad) equilibrated in 40 mM Na2HPO4/NaH2PO4, 10 mM benzamidine, pH 6.8. After washing with the same buffer, bound protein was eluted with a linear 40 to 400 mM Na2HPO4/NaH2PO4 gradient at a flow rate of 3 mL/min. Fractions containing FIX activity were analyzed employing sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, stored at −80 °C, pooled on thawing at 37 °C, precipitated with solid $(NH_4)_2SO_4$ (80% saturation) at 4 °C, collected by centrifugation (10 000 × *g* for 30 minutes at 4 °C), dissolved in HBS-buffer and 50% (v/v) glycerol, and stored at −20 °C. The typical yield of fully γ-carboxylated recombinant FIX was 0.15 to 0.20 mg/L conditioned medium. Purified products were visualized by SDS-PAGE analysis employing Quick Coomassie Stain (Protein Ark), indicating homogeneous protein preparations of >95% purity (Supplementary Figure S1).

2.7 | Anti–FVIII-inhibitory antibody

All plasma samples were measured with and without an anti–FVIII-inhibitory antibody. Plasma was pretreated with 0.5 μg/mL antibody (corresponding to approximately 8.0 BU) and incubated for 2 hours at 37 °C before addition of FIX-FIAV, rFVIII, rFVIIa, aPCC, or emicizumab, as described below. A Nijmegen Bethesda Assay was performed

(n = 2) to determine the BU of 0.5 μg/mL inhibitory antibody. In short, NPP was buffered with 0.1 M imidazole (pH 7.4) and supplemented with 0.5 μg/mL FVIII antibody. After 2 hours of incubation at 37 °C serial dilutions were prepared in FVIII-deficient plasma and the APTT was measured using the following ratios: 1 part diluted-treated NPP sample, 1 part untreated NPP, 1 part APTT reagent, and 1 part $CaCl_2$.

2.8 | Calibrated automated thrombography and APTT measurements

Thrombin generation (TG) was adapted from protocols using low plasma volumes as previously described [15,16]. All analyses involved supplementing FVIII-deficient plasma or HA patient plasma with 0% to 600% (0-30 μg/mL) FIX-FIAV, or 0% to 160% (0-160 IU/dL) rFVIII, or 1.5 μg/mL FVIIa, or 1 IU/mL aPCC, or 7 to 55 μg/mL emicizumab, or the same volume of dilution buffer. Different ranges of FIX-FIAV were tested per phenotype: 0% to 600% (0-30 μg/mL) for severe HA, 0% to 250% (0-12.5 μg/mL) for moderate, and 0% to 200% (0-10 μg/mL) for mild HA. Sufficient volumes of supplemented plasma samples were prepared to assess both TG and APTT. To convert FIX-FIAV protein concentrations to percentages, 5 μg/mL FIX-FIAV was assumed to be equivalent to 100% [17]. NPP was used as a control. Preheated (37 °C) FluCa was added to the plasma before the addition of the FXIa trigger. The concentration of the FXIa trigger was optimized in pilot experiments in which FIX-deficient plasma was reconstituted with FIX-WT or FIX-FIAV to obtain full TG correction (Supplementary Figure S2). The final reaction volume was 60 μL, with 40 μL being supplemented plasma, 10 μL phospholipid TGT (20 μM final)/FluCa mix, and 10 μL FXIa (5 nM). The thrombin formation was determined every 20 seconds for 90 to 120 minutes and corrected for the calibrator using Thrombinoscope software. The lag time, thrombin peak, endogenous thrombin potential (ETP), time to peak, and velocity index were calculated from duplicate measurements. The FVIII-equivalent activity (expressed in %) of FIX-FIAV was quantified using FVIII reference curves generated individually for each HA patient plasma in which the FVIII concentration was plotted vs the TG lag time. Plasma was supplemented with recombinant FVIII, and baseline FVIII:C (CSA) values determined the lowest value of the reference curve. Subsequently, employing a Start4 coagulation instrument (Diagnostica Stago) the APTT was measured in the same plasma sample as prepared for TG that was stored on ice. One part of the prepared plasma sample was incubated with an equal part of APTT reagent (micronized silica) and incubated for 180 seconds at 37 °C. The reaction was started by adding 1 part prewarmed $CaCl_2$.

2.9 | Data analysis

All continuous data are presented as median and IQR, unless otherwise stated. Categorical data are presented as frequency and proportions. A Wilcoxon signed-rank test (IBM SPSS statistics version 28) was performed to analyze continuous data before and after treatment with 100% FIX-FIAV. The level of significance was set at a p value of <.05.

2.10 | Data-sharing statement

For the original data, please email the corresponding author.

3 | RESULTS

3.1 | Patient plasma protein levels

FVIII:C analysis confirmed the patients with severe HA having <0.01 IU/mL plasma FVIII activity, the moderate patients with 0.03 to 0.05 IU/mL, and the mild patients with 0.07 to 0.21 IU/mL (Table). For all HA phenotypes, circulating FVIII protein was detected (FVIII:Ag, Table). FVIII inhibitor assessment indicated the presence of a clinically relevant inhibitor toward exogenous FVIII in 2 patients with moderate HA (0.8 and 5.2 BU) and in 2 patients with mild HA (1.4 and 2.8 BU). No FVIII inhibitor was detected for the severe patients. The plasma proteins von Willebrand factor, FIX, FX, prothrombin, and antithrombin were within the normal range (Table).

3.2 | FXIa-triggered thrombin formation detects FVIII activity <1%

To assess the FVIII-bypassing effect of FIX-FIAV in HA plasma, we made use of a FXIa-triggered TG assay for sensitive assessment of very low FVIII activity levels (<1%). Analysis of the TG lag time demonstrated a dose-dependent correlation with recombinant FVIII added to HA patient plasma (Figure 1) and allowed for the detection of FVIII levels as low as 0.05% in FVIII-deficient plasma (Supplementary Figure S3). Considering the well-described large interindividual variation in the TG response [18], the FVIII-equivalent activity following supplementation with FIX-FIAV was quantified in percentage using reference curves of known FVIII concentrations vs the TG lag time generated individually for each HA plasma (Figure 1D–F), where 100% FIX-FIAV equals 5 μg/mL.

3.3 | FIX-FIAV improves thrombin formation in HA plasma

Next, we demonstrated that the addition of purified recombinant FIX-FIAV to severe, moderate, or mild HA plasma improved TG, which was indicated by a shortening of the TG lag time corresponding with increasing concentrations of FIX-FIAV (Figure 2A–C, Supplementary Table S1). In contrast, supplementation of HA plasma with FIX-WT did not affect the TG parameters (Supplementary Figure S4), thereby confirming the FVIII-bypassing potency of FIX-FIAV. Interestingly, the conversion of the TG lag times obtained in the presence of FIX-FIAV to FVIII-equivalent activity demonstrated a linear correlation of FIX-FIAV levels with FVIII-equivalent activity (Figure 2D–F). This dose-dependent response was consistently observed for all severe, moderate, and mild HA plasmas (Figure 3). The maximum response of FIX-FIAV differed per HA phenotype and was attained

TABLE Coagulation parameters and plasma protein levels.

Patient baseline characteristics	Severe (*n* = 7)	Moderate (*n* = 7)	Mild (*n* = 7)
APTT (sec)	86.1 (65.7-88.2)	43.5 (38.8-46.1)	37.5 (33.8-39.3)
FVIII:C, OSA (IU/mL)	<0.01	0.04 (0.03-0.05)	0.14 (0.07-0.20)
FVIII:C, CSA (IU/mL)	<0.01	0.03 (0.03-0.05)	0.13 (0.07-0.22)
FVIII:Ag (%)	4.3 (1.7-5.0)	5.0 (3.8-8.2)	10.9 (8.6-22.8)
FVIII inhibitors (BU)	0.0 (0.0-0.1)	0.0 (0.0-0.5)	0.1 (0.0-0.8)
FIX:C, OSA (IU/mL)	1.13 (1.02-1.21)	0.96 (0.89-1.06)	0.99 (0.96-1.02)
FIX:C, CSA (IU/mL)	1.21 (1.05-1.35)	1.03 (0.99-1.13)	1.12 (1.00-1.20)
FIX:Ag (%)	85.0 (79.0-93.0)	79.0 (76.0-83.0)	88.0 (78.5-94.5)
AT (IU/mL)	1.02 (0.97-1.14)	1.08 (0.99-1.17)	1.06 (0.93-1.08)
FII (IU/mL)	1.13 (1.02-1.20)	1.09 (0.96-1.17)	1.11 (1.03-1.13)
FX (IU/mL)	1.10 (1.07-1.20)	0.98 (0.87-1.21)	1.02 (0.92-1.09)
VWF:Ag (IU/mL)	1.41 (0.91-2.23)	1.22 (0.98-1.34)	1.76 (1.36-2.26)
Days since the last FVIII concentrate dose	4 (3-5)	198 (118-262)	50 (39-614)
Gene mutation			
• p.Arg2169His	-	6 (86%)	2 (28.5%)
• Intron 22 inversion	4 (57%)	-	-
• p. LLE1213Phefs*5	2 (29%)	-	-
• p. Gln802X	1 (14%)	-	-
• p. Leu504Leu and p. Asp1241Glu	-	1 (14%)	
• p. Arg612Cys	-	-	1 (14.5%)
• p. Phe698Ser	-	-	1 (14.5%)
• p. Arg2178Cys	-	-	1 (14.5%)
• p. Pro149Arg	-	-	1 (14.5%)
• p. Ala563Gly	-	-	1 (14.5%)

Reported in median (IQR) or number (percentage).

Ag, antigen; APTT, activated partial thromboplastin time; AT, antithrombin; BU, Bethesda units; C, clotting activity; CSA, chromogenic substrate assay; FII, prothrombin; FX, factor X; OSA, one-stage assay; VWF, von Willebrand factor.

upon supplementation with approximately 600% FIX-FIAV for severe HA, resulting in a median FVIII-equivalent activity of 26% (IQR, 19%-31%). For moderate HA, the maximum response was observed at 250% FIX-FIAV leading to 33% (IQR, 27%-37%) FVIII-equivalent activity, and for mild HA at 200% FIX-FIAV producing 44% (IQR, 45%-97%) FVIII-equivalent activity (Figure 3, Supplementary Table S1). This is likely because of the plasma FVIII:C levels as this augments the FVIII-equivalent activity of FIX-FIAV in a dose-dependent manner (Supplementary Figure 5). The APTT of HA plasma was also shortened with increasing FIX-FIAV concentrations (Figure 4D–F) in a similar manner as observed for the TG lag time (Figure 4A–C). In contrast, the TG parameters ETP, thrombin peak, and velocity index did not show a dose-dependent response and plateaued at approximately 200% to 400% FIX-FIAV in severe HA, while in both moderate and mild HA, similar values were observed for all FIX-FIAV concentrations tested (Supplementary Table S1). Collectively, these data demonstrate that FIX-FIAV improves thrombin formation in HA plasma.

3.4 | FIX-FIAV ameliorates the HA phenotype in patient plasma

To gain more insight on how FIX-FIAV affects the HA phenotype in plasma *in vitro*, we specifically focused on the addition of 100% (5 μg/mL) FIX-FIAV to HA plasma because this results in an overall 1:1 ratio of endogenous FIX vs FIX-FIAV. For severe HA, a median FVIII-equivalent activity of 2.9% (IQR, 2.3%-3.9%) was observed following supplementation with 100% FIX-FIAV (Figure 5A–C), which

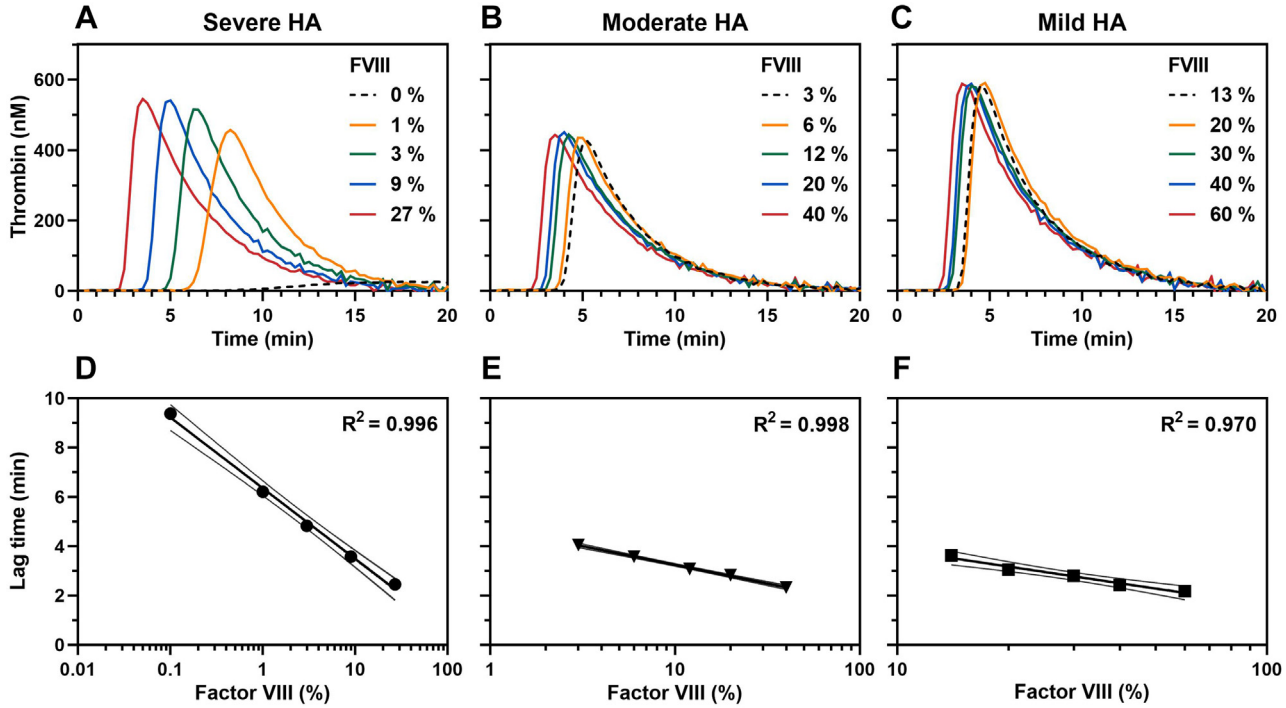

FIGURE 1 Correlation between factor VIII levels and the thrombin generation lag time. Thrombin generation was initiated with 5 nM FXIa in severe (A, D), moderate (B, E), or mild (C, F) HA plasma in the absence or presence of increasing concentrations of recombinant FVIII (%) (NovoEight) as indicated. (A-C) Representative curves are shown, with thrombin generation monitored in patient plasma with endogenous FVIII:C levels of <0.01 IU/mL (<1%) for severe HA, 0.03 IU/mL (3%) for moderate HA, or 0.13 IU/mL (13%) for mild HA. (D-F) The FVIII concentration was plotted vs the lag time of thrombin generation, and the data were fitted using a semilog fit and are displayed as mean ± 95% CI. HA, hemophilia A.

corresponds to a moderate laboratory HA phenotype. For moderate HA, the median FVIII-equivalent activity increased to 16.1% (IQR, 13.3%-18.1%) on adding 100% FIX-FIAV, leading to a mild laboratory HA phenotype. In mild HA, a median FVIII-equivalent activity of 48.0% (IQR, 34.0%-67.5%) was observed in the presence of 100% FIX-FIAV, corresponding to normal FVIII plasma levels. These findings indicate that FIX-FIAV levels equimolar to those of endogenous FIX are sufficient to improve the hemostatic potential and thereby mitigate the HA phenotype in patient plasma.

3.5 | The cofactor-independent activity of FIX-FIAV improves coagulation in HA plasma

The baseline FVIII activity in plasmas of both moderate and mild HA patients hampers the interpretation of the cofactor-independent effect of FIX-FIAV. We, therefore performed the same experiments as described above in the presence of an inhibitory anti-FVIII antibody to abolish all endogenous FVIII activity. In the absence of FIX-FIAV but with 5 μg/mL of the anti-FVIII antibody present, both the TG lag time and APTT clotting time were not affected in severe HA plasma given its low baseline FVIII activity (Figure 4, Supplementary Table S2). In contrast, analyses of moderate and mild HA plasma in the presence of the anti-FVIII antibody demonstrated prolonged TG lag times and APTT clotting times relative to conditions without the antibody, generating values similar to those observed for severe HA. Furthermore, the addition of increasing concentrations of FIX-FIAV to the antibody-spiked moderate and mild HA plasmas resulted in TG and APTT responses comparable to those observed for severe HA plasma. These data further substantiate that FIX-FIAV displays cofactor-independent FIX activity in a range of plasmas from HA patients.

We next assessed the efficacy of FIX-FIAV in the setting of circulating clinically relevant inhibitors toward exogenous FVIII, which were detected in 2 moderate (0.8 and 5.2 BU) and in 2 mild (1.4 and 2.8 BU) HA plasmas. As expected, a FVIII titration revealed a minimal shortening of the TG lag time in plasmas with an inhibitor titer of ≥1.4 BU, while an up to 1.7-fold shortened lag time was observed in plasma with an inhibitor titer of 0.8 BU, with a visible difference between high and low titer inhibitor patients (cut-off value 5 BU) (Figure 6A). In contrast, a dose-dependent shortening of the TG lag time was found for all inhibitor plasmas following FIX-FIAV addition (Figure 6B). Collectively, these findings indicate that the cofactor-independent FIX-FIAV activity is not affected by exogenous or endogenous inhibitory FVIII antibodies, thus allowing FIX-FIAV to function as a bypassing agent to improve hemostasis in HA plasma.

3.6 | FIX-FIAV combined with various HA treatment modalities shows a minor enhanced effect

To investigate the potential enhanced effect of bypassing agents on FIX-FIAV function, we have performed spiking experiments with the

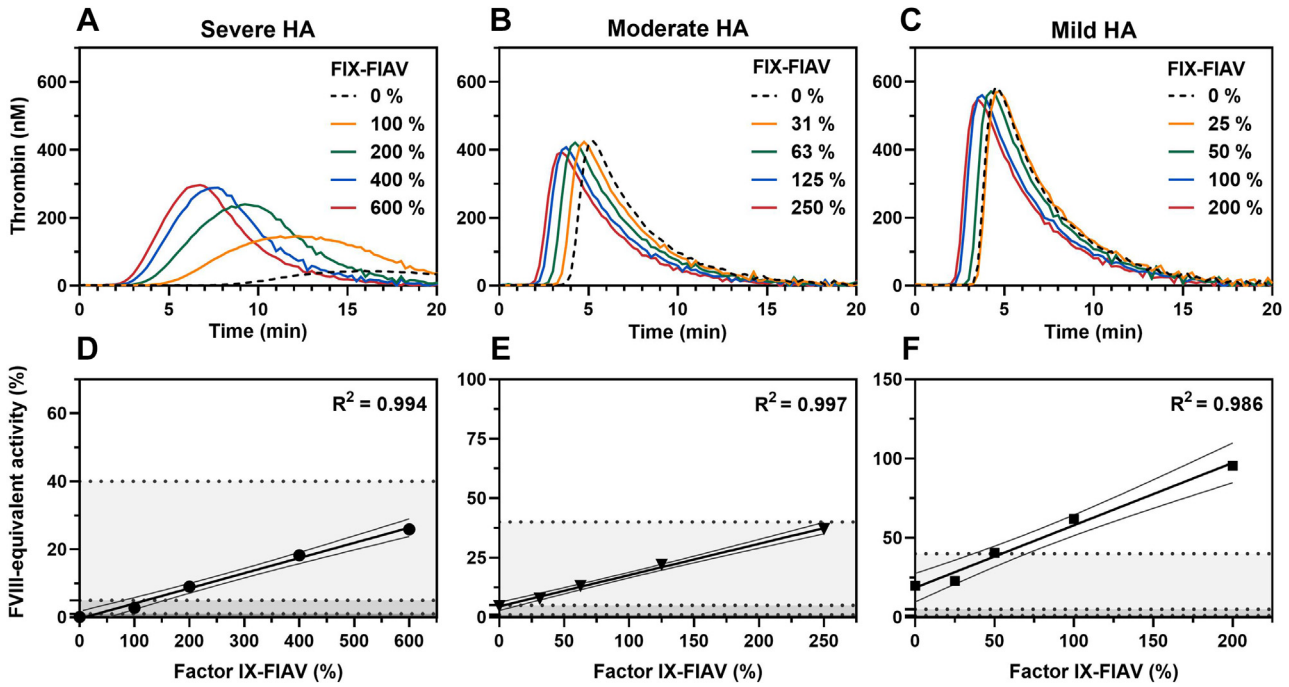

FIGURE 2 Factor IX-FIAV improves thrombin generation in hemophilia A plasma. Thrombin generation was initiated with 5 nM FXIa in severe (A, D), moderate (B, E), or mild (C, F) HA plasma supplemented with 0% to 600%, 0% to 250%, or 0% to 200% FIX-FIAV, respectively (100% equals 5 μg/mL FIX-FIAV). (A-C) Representative thrombin generation curves are shown. (D-F) The FIX-FIAV concentration was plotted vs the FVIII-equivalent activity, with the latter calculated employing the thrombin generation lag time as described in "Methods" section. The data were fitted using linear regression and are displayed as the mean ± 95% CI. The gray areas bordered by the dotted lines represent the FVIII activity cut-off values indicating severe (<1%), moderate (1%-5%), or mild (5%-40%) HA. HA, hemophilia A.

FVIII-bypassing agents aPCC or FVIIa in FIX-FIAV-treated HA plasmas (3 per phenotype). Although supplementation with 100% FIX-FIAV shortened the median TG lag time up to 1.5-fold compared with baseline, supplementation with 1 IU/mL aPCC or 1.5 μg/mL FVIIa, both peak plasma concentrations of respective drugs in typical clinical use, displayed a minimal effect on the TG lag time (maximum 1.2-fold reduced) (Figure 7A, Supplementary Table S3). Given that we made use of FXIa-triggered TG and as such primarily monitored the intrinsic coagulation system, none of the other TG parameters were affected on supplementation with FVIIa, corroborating previous observations [19]. When compared with supplementation with FIX-FIAV alone, the combination of aPCC or FVIIa with FIX-FIAV did not affect the TG lag time, whereas

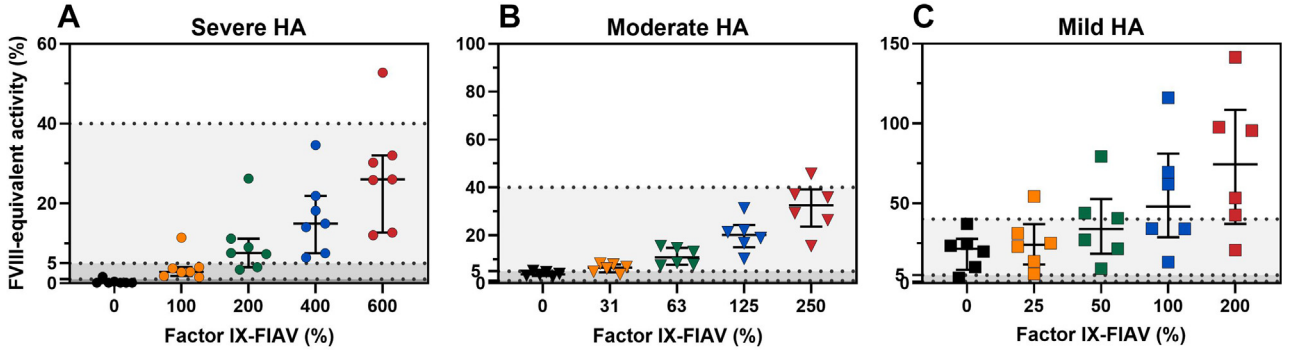

FIGURE 3 Factor IX-FIAV generates FVIII-equivalent activity in hemophilia A plasma. Thrombin generation was initiated with 5 nM FXIa in HA plasma supplemented with FIX-FIAV as indicated. The FIX-FIAV concentration (100% equals 5 μg/mL FIX-FIAV) of 0% to 600% for severe HA (A), 0% to 250% for moderate HA (B), or 0% to 200% for mild HA (C) was plotted vs the FVIII-equivalent activity, which was calculated individually for each HA plasma employing the thrombin generation lag time as described in "Methods" section. Analyses in plasmas with a FVIII inhibitor titer ≥2.8 BU were excluded, as the inhibitor did not allow for quantification of the FVIII-equivalent activity because of interference with the FVIII reference curves. All outliers in Figure 3A can be ascribed to 1 individual who presented with FVIII antigen levels of 2.2%, which can be explained by FVIII prophylaxis 3 days before inclusion. The gray areas bordered by the dotted lines represent the FVIII activity cut-off values indicating severe (<1%), moderate (1%-5%), or mild (5%-40%) HA. Median (IQR) values are provided in Supplementary Table S1. HA, hemophilia A.

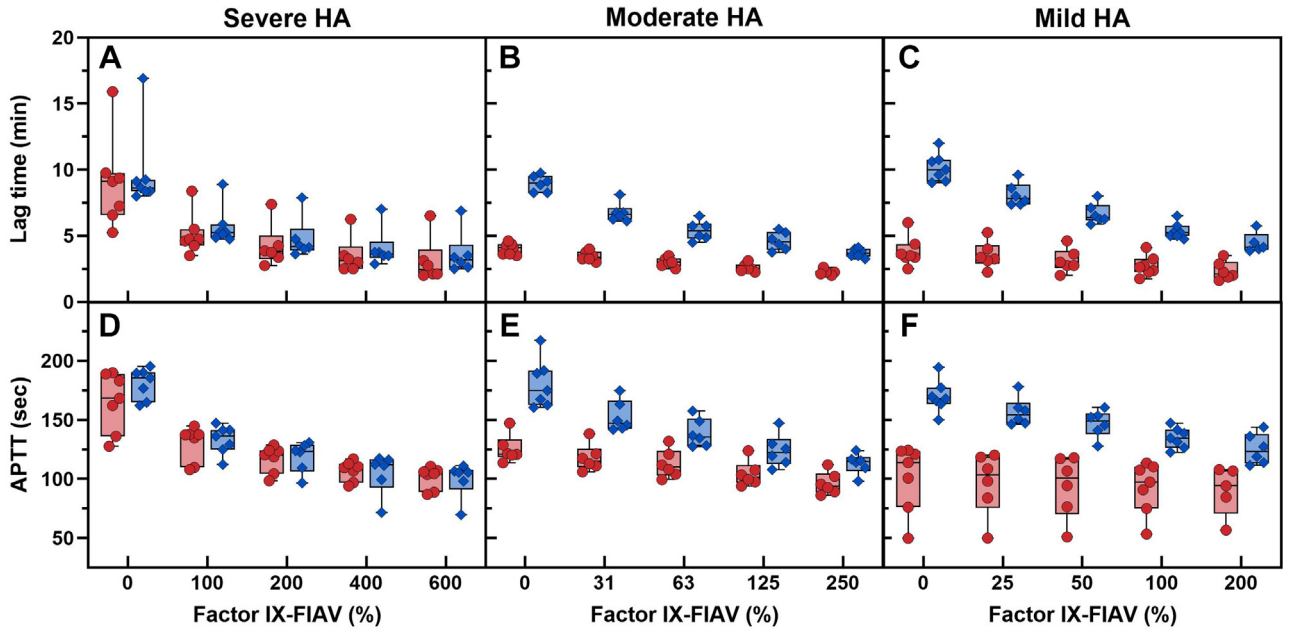

FIGURE 4 Cofactor-independent effect of factor IX-FIAV on coagulation parameters in hemophilia A plasma. The lag time of FXIa-triggered thrombin generation (A-C) or the APTT (D-F) was determined in severe (A, D), moderate (B, E), or mild (C, F) HA plasma supplemented with 0% to 600% for severe HA, 0% to 250% for moderate HA, and 0%-200% for mild HA (100% equals 5 μg/mL FIX-FIAV) FIX-FIAV in the absence (red circles) or presence of 5 μg/mL anti-FVIII antibody (blue diamonds) as described in "Methods" section. Analyses in plasmas with a FVIII inhibitor titer ≥2.8 BU were excluded, as the inhibitor did not allow for quantification of the FVIII-equivalent activity because of interference with the FVIII reference curves. Median (IQR) values are provided in Supplementary Tables S1 and S2. HA, hemophilia A.

the combination of aPCC and FIX-FIAV resulted in an up to 2.2-fold enhanced ETP. This was mostly caused by an aPCC-related increase in ETP of up to 2.3-fold relative to supplementation with FIX-FIAV. Similar results were obtained following APTT analyses (Supplementary Table S3). Hence, no substantial enhanced effect was observed when combining FIX-FIAV with the bypassing agents aPCC or FVIIa.

Similar experiments in which hemostatic levels of recombinant FVIII (1 U/mL) (Figure 7B) or emicizumab (55 μg/mL) (Figure 7C) were added to HA patient plasma presented a 2- to 6-fold shortened TG lag time compared with baseline, with the largest effect observed for severe HA plasma (Figure 7B, C, Supplementary Table S4). Addition of either FVIII or emicizumab to FIX-FIAV-spiked plasma shortened the TG lag time by 2- to 4-fold relative to FIX-FIAV alone. The latter was primarily because of the effect of supplementation with either FVIII or emicizumab on the TG lag time. No substantial effects were seen on other TG parameters for the combination of current HA treatments

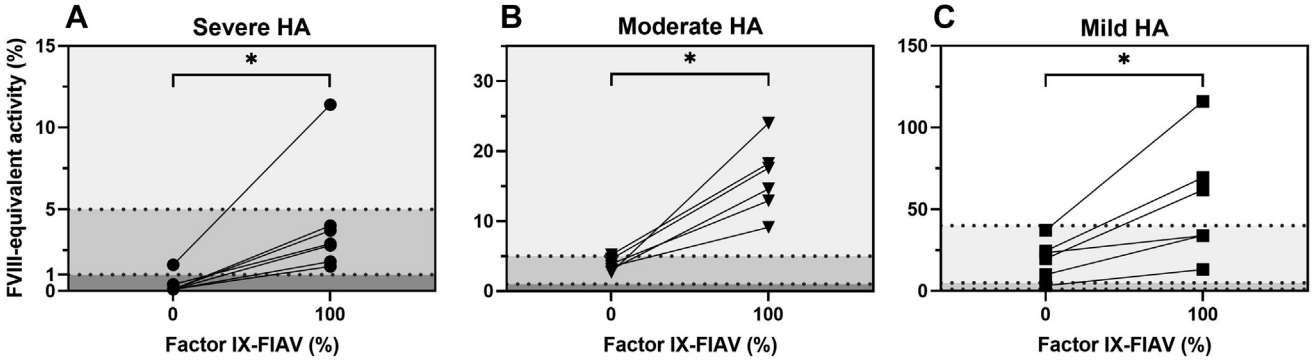

FIGURE 5 Factor IX-FIAV mitigates the hemophilia A phenotype in severe, moderate, and mild patient plasma. The lag time of FXIa-triggered thrombin generation in the absence or presence of 100% (5 μg/mL) FIX-FIAV determined in severe (A), moderate (B), or mild (C) HA plasma was converted to FVIII-equivalent activity as described in the "Methods." For moderate HA, the FVIII-equivalent activity at 100% FIX-FIAV was extrapolated from the individual FIX-FIAV response plots. Analyses in plasmas with a FVIII inhibitor titer ≥2.8 BU were excluded because the inhibitor did not allow for quantification of the FVIII-equivalent activity because of interference with the FVIII reference curves. The gray areas bordered by the dotted lines represent the FVIII activity cut-off values indicating severe (<1%), moderate (1%-5%), or mild (5%-40%) HA. Statistical significance: $^*p \leq 0.05$. Median (IQR) values are provided in Supplementary Table 1. HA, hemophilia A.

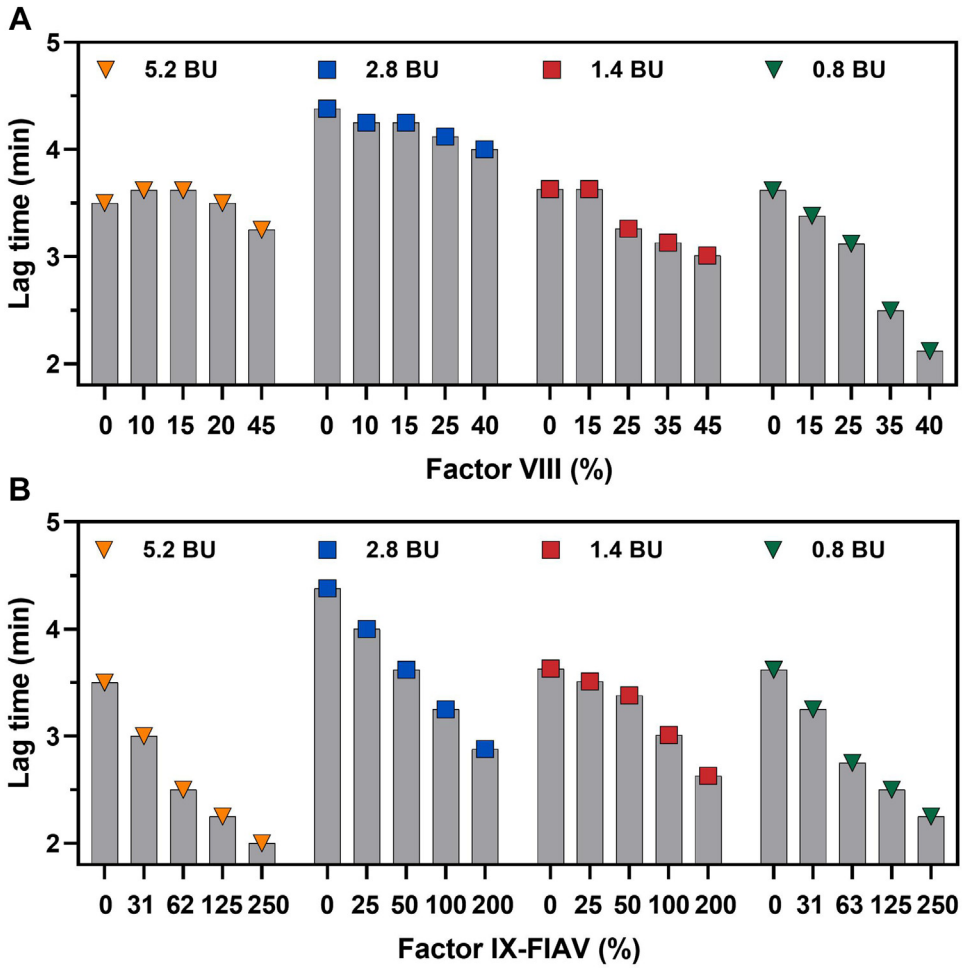

FIGURE 6 Factor IX-FIAV enhances thrombin generation in hemophilia A plasma of patients with clinically relevant circulating FVIII inhibitors. The lag time of FXIa-triggered thrombin generation was determined in HA plasmas with clinically relevant FVIII inhibitors, which were supplemented with either 0% to 45% FVIII (A) or 0% to 250% (0-12.5 μg/mL) FIX-FIAV (B) as described in the "Methods" section. The plasmas assessed were moderate HA with an inhibitor titer of 5.2 BU, FVIII:C (CSA) 0.07 IU/mL, FVIII:Ag 4% (yellow triangles), moderate HA with an inhibitor titer of 0.8 BU, FVIII:C 0.05 IU/mL, FVIII:Ag 27% (green triangles), mild HA plasma with an inhibitor titer of 2.8 BU, FVIII:C 0.10 IU/mL, FVIII:Ag 9% (blue squares), and mild HA plasma with an inhibitor titer of 1.4 BU, FVIII:C 0.07 IU/mL, FVIII:Ag 4% (red squares). HA, hemophilia A.

and FIX-FIAV (Supplementary Table S4). Collectively, using FXIa-triggered TG assessment, our findings indicate that no severe pro-coagulant effects were observed for the combination of FIX-FIAV with conventional HA therapeutics.

4 | DISCUSSION

To our knowledge, this is the first preclinical *in vitro* study on the effect of FIX-FIAV in plasma from HA patients. Previously, FIX-FIAV has been shown to improve hemostasis in *in vitro* and murine models of HA, with the specific substitutions introduced into FIX-FIAV giving rise to its FVIII-independent activity [11,12]. Here, we assessed the prohemostatic effects of FIX-FIAV alone and in combination with the currently available HA therapies employing FXIa-triggered TG assays. We demonstrated that FIX-FIAV improves hemostasis in HA regardless of residual FVIII, baseline FVIII, or FVIII inhibitor status. In addition, minimal enhanced effects were observed when FIX-FIAV and currently available HA therapies or FVIII-bypassing agents were combined.

To establish this, we made use of a modified FXIa-triggered calibrated automated thrombography assay to monitor thrombin formation in HA patient plasma. The International Society on Thrombosis and Haemostasiss (ISTH) Scientific and Standardization Committee (SSC) subcommittee has proposed to use low-tissue factor for hemostatic monitoring in HA [20]. Using a low amount of tissue factor as trigger, not only the extrinsic FX-mediated pathway in coagulation is initiated but also the intrinsic pathway through FIX activation, known as the Josso loop [21,22]. However, low-tissue factor-triggered TG has been reported to inadequately differentiate between moderate and severe HA [23,24]. To circumvent this, TG using a low FXIa trigger has previously been assessed, which allowed for enhanced sensitivity in quantifying FVIII/FIX levels in HA/B compared with the tissue factor-driven TG methods [23–26]. Here, we have built on this earlier work

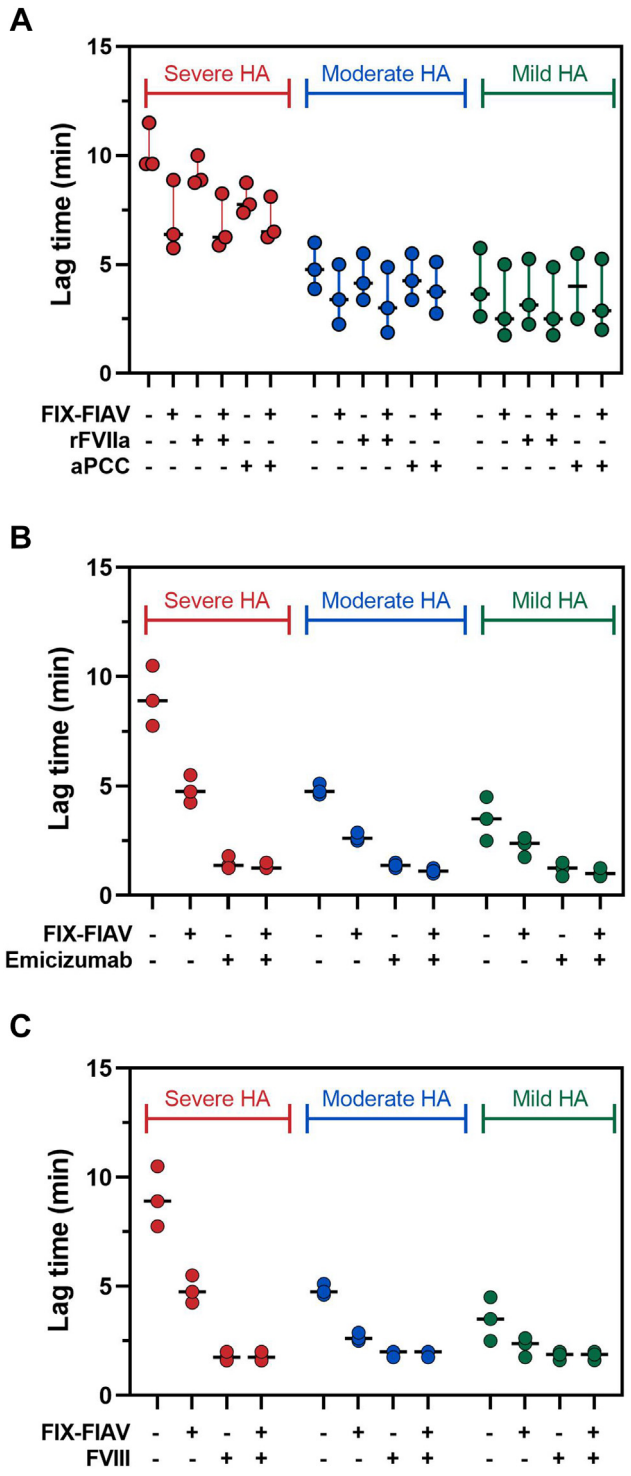

FIGURE 7 The effect of FIX-FIAV was combined with conventional HA therapies. Severe (red), moderate (blue), and mild (green) hemophilia A patient plasma with and without 100% (5 μg/mL) FIX-FIAV was tested with and without (A) 1.5 μg/mL of bypassing agents activated recombinant factor VII (FVIIa) or 1 U/mL activated prothrombin complex concentrates (aPCC), (B) 55 μg/mL emicizumab, or (C) 1 U/mL FVIII. Data are depicted as median and range of thrombin generation lag time and represent 2 to 3 independent experiments. HA, hemophilia A.

and made use of a relatively high FXIa concentration to initiate thrombin formation. Consequently, as higher amounts of FIX are activated and considered to be in excess, the limiting factor in this assay set-up is (activated) FVIII. We have shown that this approach allows for the assessment of FVIII(-equivalent) activity even at low FVIII plasma concentrations using the TG lag time as a parameter. The TG lag time consistently displayed a dose-dependent correlation with FVIII levels, specifically in plasma from HA patients, while this was less apparent for the ETP and thrombin peak, corroborating previous observations [27]. Accordingly, the high FXIa trigger allowed us to quantify the FVIII-independent activity of FIX-FIAV in terms of FVIII-equivalence.

In light of its FVIII-independent activity, we have demonstrated that FIX-FIAV is capable of improving thrombin formation in severe, moderate, and mild HA patient plasma. Moreover, we have revealed that 100% FIX-FIAV resulted in a phenotype shift in FVIII-equivalent activity: addition of 100% FIX-FIAV to severe, moderate, or mild HA plasma increased the median FVIII-equivalent activity to 3%, 16%, or 48%, respectively, compared with baseline. The higher efficacy observed for FIX-FIAV in moderate and mild HA suggests that this is related to FVIII-dependent FIX activity, while the lack of (detectable) FVIII in severe HA limits FIX-FIAV to its FVIII-independent function. Increasing the concentration of FIX-FIAV up to 600% in severe HA enhanced the FVIII-equivalent activity, although the maximum effect in nonsevere HA plasma was reached at 200% to 250% FIX-FIAV. The latter may be because of limited availability of the substrate FX as a result of consumption by FVIIIa-FIXa-FIAV. Interestingly, whereas shortened TG lag times correlated with increased FIX-FIAV, a trend toward a reduction in thrombin peak and ETP was observed for high FIX-FIAV in both mild and moderate HA. We hypothesize that this may result from the competition between the added FIX-FIAV and endogenous FIX for interaction with residual functional FVIII. This is confirmed by the addition of a neutralizing FVIII-inhibitory antibody to moderate/mild HA patient plasma, revealing results comparable with those observed for severe HA patient plasma.

The FVIII-independent effect of FIX-FIAV was repeatedly confirmed by adding a neutralizing FVIII-inhibitory antibody to all patient plasmas, thereby diminishing residual FVIII and improving the FVIII-equivalent activity of FIX-FIAV in all HA phenotypes in a similar manner. Interestingly, the plasma of 3 severe HA patients (OSA/CSA <1% FVIII baseline) who had received prophylaxis 48 to 72 hours before inclusion displayed increased TG parameters compared with other severe HA patient plasmas. After adding the FVIII-inhibitory antibody the TG parameters resembled those of the other severe HA plasmas, suggesting the former resulted from residual traces of FVIII. Furthermore, the improved FVIII-equivalent activity observed with a FVIII-inhibitory antibody present suggests that FIX-FIAV could serve as a potential treatment in HA with inhibitors. This is further underlined by the FIX-FIAV-induced shortened lag times observed for inhibitor patient plasma (BU > 0.5).

For the combination of recombinant FVIII or the FVIII-bypassing agents emicizumab, aPCC or FVIIa with FIX-FIAV minimal enhanced

effects were observed on the basis of the TG parameters. When comparing aPCC or FVIIa combined with FIX-FIAV with the effect of FIX-FIAV alone, similar TG lag times were obtained for all patient samples. Furthermore, for concentrations of emicizumab as low as 7 μg/mL, a minimal and nonsignificant enhanced effect was found when combined with FIX-FIAV. Concordantly, the combination of recombinant FVIII with FIX-FIAV resulted in a modest enhanced effect on lag time. More extensive analyses would be required to establish if the administration of FIX-FIAV affects current HA therapies.

Our study was adequately powered to ascertain the differences before and after the addition of FIX-FIAV and the primary end point of our study, namely FIX-FIAV's effect on TG lag time. Furthermore, employing a FXIa-driven TG allowed us to solely assess the intrinsic route of coagulation without the interference of the extrinsic route to evaluate the effect of FIX-FIAV, also with and without emicizumab, FVIIIa, FVIIa, or aPCC. However, 1 disadvantage of using the FXIa-driven TG is that it might underestimate the effect of aPCC and FVIIa. The small sample size of $n = 3$ per phenotype on the comparison and combination of FIX-FIAV with FVIII, emicizumab, and bypassing agents was chosen as a proof of concept. Of note, this could be considered a limiting factor; the results should therefore be interpreted with caution. Future clinical studies would be necessary to evaluate FIX-FIAV as a potential treatment for patients with HA.

5 | CONCLUSION

FIX-FIAV significantly improved the FVIII-equivalent activity in plasma from severe, moderate, and mild HA patients. The combination of current HA treatments with FIX-FIAV showed minor or modest effects on the TG lag time. As such, FIX-FIAV could serve as a potential treatment for patients with HA, regardless of the current treatment, inhibitor status, or FVIII levels. Clinical studies are needed to assess the hemostatic effects of FIX-FIAV in patients with HA with or without inhibitors to FVIII.

AUTHOR CONTRIBUTIONS

L.G.R.R., Y.P.L., A.C.M., F.W.G.L., and M.H.A.B. conceived and designed the study. V.J.F.S. and K.L.C. conceived of the experimental study design and performed the experiments. L.G.R.R. collected data. V.J.F.S., L.G.R.R., K.L.C., F.W.G.L., and M.H.A.B. analyzed and interpreted data. V.J.F.S. and L.G.R.R. drafted the manuscript. J.E., F.W.G.L., and M.H.A.B. critically revised the manuscript. All authors revised the manuscript, agreed with its content, and approved of submission.

DECLARATION OF COMPETING INTERESTS

L.G.R.R. received the Sobi Young Investigators Award 2020 and a travel grant from Sobi (2019). F.W.G.L. has received grants/research funding from CSL Behring, Sobi, Takeda for research unrelated to the current study, consultancy fees from BioMarin, CSL Behring, Takeda, and uniQure (all fees to the institution), and served as DSMB member for a study sponsored by Roche. J.E. received research support from CSL Behring unrelated to the current study and has been a teacher on educational activities of Roche (all fees to the institution). Y.P.L. is an employee and shareholder at uniQure. A.C.M. was an employee at uniQure when the study was conducted. M.H.A.B. has received research funding from Novo Nordisk Health Care A.G. and is inventor on intellectual property, both unrelated to this work. V.J.F.S. and K.L.C. have no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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