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ORIGINAL ARTICLE

Recombinant porcine factor VIII corrects thrombin generation in vitro in plasma from patients with congenital hemophilia A and inhibitors

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Abstract

Background: Neutralizing factor VIII (FVIII) antibodies are a major complication in hemophilia A. Antihemophilic factor VIII (recombinant), porcine sequence (rpFVIII; susoctocog alfa; Baxalta US Inc., a Takeda company) has low cross-reactivity to anti-human FVIII antibodies and can provide functional FVIII activity in the presence of FVIII inhibitors.

Objectives: Evaluate in vitro thrombin generation and clot formation responses to rpFVIII in blood from patients with congenital hemophilia A.

Methods: In this multicenter study, blood was obtained for in vitro analyses that included human and porcine FVIII inhibitors, low <5 Bethesda units (BU)/ml or high ≥5 BU/ml titer (Nijmegen-modified Bethesda assay); thrombin generation assay (TGA), clot viscoelasticity (thromboelastography), fibrin clot structure analysis (scanning electron microscopy), and epitope mapping.

Results: Blood samples were from 20 patients with congenital hemophilia A (FVIII activity <1%, mean [range] inhibitor titers: anti-human FVIII, 14 [1–427] BU/ml [n = 13 high, n = 6 low, n = 1 data unavailable]); anti-porcine FVIII, 12 (0–886) BU/ml (n = 11 high, n = 8 low, n = 1 data unavailable). Porcine inhibitor titer and TGA response measured by endogenous thrombin potential showed an inverse correlation (2.7–10.8 U/ml rpFVIII Spearman correlation coefficient: −0.594 to −0.773; $p < 0.01$). Clot structures in low anti-porcine inhibitor titer plasmas were similar to those in non-inhibitor plasma.

Conclusions: Recombinant porcine factor VIII demonstrated a dose-dependent correction of thrombin generation and clot formation in vitro, dependent on the anti-porcine FVIII inhibitor titer. Procoagulant responses to rpFVIII occurred in plasma containing FVIII inhibitors.

KEYWORDS
factor VIII inhibitor, hemophilia A, porcine factor VIII, recombinant, thrombin

Essentials

- Recombinant porcine factor VIII (rpFVIII) was evaluated in blood from patients with congenital hemophilia A and FVIII inhibitors.
- rpFVIII demonstrated a dose-dependent correction of thrombin generation and clot formation.
- Restoration of hemostatic markers depended on porcine- but not on human-FVIII inhibitor titers.
- In conclusion, responses to rpFVIII can occur in the presence of pre-existing FVIII inhibitors.

1 | INTRODUCTION

Congenital hemophilia A (CHA) is characterized by a deficiency of human factor VIII (FVIII), with most cases caused by inherited X-linked mutations in the *F8* clotting factor gene; approximately 30% of all hemophilia cases are caused by spontaneous mutations.[1,2] Development of inhibitory alloantibodies to FVIII is a major complication of replacement FVIII therapy for patients with hemophilia A because exogenous FVIII is no longer effective for control of bleeding in the presence of high titer inhibitors (>5 Bethesda units [BU]).[3-5] The lifetime risk (cumulative incidence) for development of anti-human FVIII inhibitory antibodies is approximately 30% in previously untreated patients with hemophilia A,[1] representing the most important challenge for the management of hemostasis in these patients.[6] Anti-human FVIII inhibitors are high-affinity polyclonal IgG neutralizing antibodies that primarily bind to the A2 and C2 domains and to the C-terminal region of the C1 domain of FVIII and inhibit FVIII hemostatic functions.[7-9] Bypassing agents such as recombinant activated factor VII or activated prothrombin complex concentrate are used for the management of acute bleeding in patients with hemophilia and human FVIII inhibitors.[10,11] There are reported variations in the clinical response to these agents[12] and, because routine assays are not able to accurately monitor treatment efficacy and inform treatment decisions, global coagulation assays may be required as an ancillary tool to support treatment decisions.[13,14] For patients with acquired hemophilia A (AHA), an additional disadvantage of therapy with bypassing agents is the risk of thrombosis that, although quite rare, is of importance when considering treatment of elderly patients with severe comorbidities.[13,15]

Porcine FVIII (pFVIII) has been used in patients with hemophilia A and human FVIII (hFVIII) inhibitors on account of its 84% and 76% homology with human FVIII A2 and C2 domains, respectively.[16,17] This is similar enough to achieve hemostatic functions of the human protein but sufficiently different to avoid recognition by neutralizing human FVIII antibodies.[18-20] pFVIII derived from pig plasma, Hyate:C, carried a risk of allergic reactions and thrombocytopenia when used in human patients, and concerns over potential viral contamination of the product ultimately led to production being suspended.[13]

Recombinant porcine factor VIII (rpFVIII, susoctocog alfa, Obizur, Baxalta US Inc., a Takeda company) is a B-domain deleted (BDD), 1448-amino acid heterodimer with a molecular mass of 170 kDa, composed of a 90-kDa heavy chain and an 80-kDa light chain.[21-23] In the clinical setting, rpFVIII has been evaluated in phase I and II studies in patients with CHA with inhibitors.[19,24,25] A phase II/III study reported the safety and efficacy of rpFVIII for the treatment of bleeding episodes in patients with AHA and pFVIII inhibitors (≤20 BU/ml).[26] These data supported regulatory approval of rpFVIII for the treatment of bleeding episodes in adults with AHA; rpFVIII is not approved for CHA.

Preclinical evidence suggests that the mode of action of rpFVIII is similar to that of human FVIII replacement products: the pairwise sequence homology is 86% compared with BDD human FVIII, thrombin activation of rpFVIII generates cleavage products comparable with human FVIII, and rpFVIII binds with high affinity to von Willebrand factor.[21,22,27] The functional characterization of rpFVIII demonstrated similar efficacy and half-life but higher recovery rate versus the plasma-derived pFVIII product Hyate:C.[19,22] RpFVIII displayed low cross-reactivity with human FVIII inhibitors and resulted in therapeutic levels of FVIII in patients with hemophilia A and in hemophilic plasmas in vitro, despite the presence of human FVIII inhibitors.[19,28]

Global assays that measure thrombin generation provide information on the functional status of blood coagulation as inferred from the overall balance between pro- and anticoagulant factors.[29] Fibrin clot ultrastructure analysis can provide complementary information on whether a solid fibrin clot is formed. The objectives of this study were, first, to evaluate the hemostatic efficacy of rpFVIII in vitro, including effects on thrombin generation, clot formation, and fibrin clot structure, using blood samples from patients with hemophilia A with inhibitors to human FVIII. The second objective was to further assess the differences in hemostatic efficacy based on the level of human FVIII and porcine FVIII inhibitor titers and to determine the epitope-specificity of human FVIII inhibitors in patient samples.

2 | METHODS

2.1 | Patients and sample preparation

In this study, blood was obtained for in vitro analyses that included human and porcine FVIII inhibitors. The study was conducted across three clinical sites in three countries. The study protocol was approved by the institutional review board at each institution and written and signed informed consent was obtained from all study participants before enrollment.

Individuals with congenital hemophilia A with inhibitors to hFVIII were recruited during routine outpatient visits to hemophilia centers in Bonn, Germany; Lyon, France; and Tel Aviv, Israel, between January and November 2011. The study aimed to recruit consecutive patients who attended for routine visits at their center during the study period and consented to contribute to the study by providing blood samples in addition to samples taken for their routine laboratory parameters.

Whole blood samples (20 ml) were obtained during routine outpatient visits with written, informed patient consent. A 10-ml aliquot was collected in sodium citrate tubes (final concentration, 10.9 mM); 1.2 ml was used for thromboelastography studies using whole blood, with the remainder undergoing centrifugation for preparation of plasma for FVIII activity assays. An additional 10-ml aliquot was collected in sodium citrate tubes (final concentration, 10.9 mM) containing corn trypsin inhibitor 1.45 μM for separation of platelet-poor plasma (PPP) as required for the thrombin generation assay (TGA) and scanning electron microscopy. Samples were centrifuged at 2000*g* at room temperature for 10 min, then plasma was removed and centrifuged again at 10,000*g* for 3 min. The PPP was removed, aliquoted, and stored at ≤−60°C until assayed.

2.2 | FVIII activity (FVIII:C) measurement

FVIII activity was measured at both local and central laboratories. A one-stage activated partial thromboplastin time-based assay was used. One-stage FVIII activity was measured at the central laboratory. In brief, citrated PPP samples were diluted in FVIII-deficient plasma and the activated partial thromboplastin time-reagent Actin FS (Siemens Healthcare Diagnostics, Marburg, Germany) was added. After incubation for 3 min at 37°C, 25 mM $CaCl_2$ was added, and clotting times were measured. Analyses were performed using a Sysmex CA 1500 or BCS XP instrument (Siemens Healthcare Diagnostics).

2.3 | FVIII inhibitor assays

Inhibitory titers against human or porcine FVIII were determined using the Nijmegen-modified Bethesda assay (NBA) at both the local (site of sample collection) and central laboratories.

For the anti-human FVIII NBA, heat-inactivated citrated plasma samples were mixed 1:2 with human reference plasma and incubated for 2 h (37°C). Residual FVIII:C was measured using the Sysmex CA 1500 or BCS XP instrument (Siemens Healthcare Diagnostics). The anti-porcine FVIII NBA was conducted as for the anti-human FVIII NBA except that rpFVIII (diluted in hFVIII-deficient plasma to approximately 1 U/ml) was used in place of normal human plasma.

2.4 | Thrombin generation assay

Lag time, peak height, and endogenous thrombin potential (ETP) were assessed using citrated-CTI PPP samples. The time course of thrombin generation was determined at a low tissue factor concentration (1 pM) using calibrated automated thrombography (CAT) (Thrombinoscope bv) and a Fluoroskan Ascent fluorometer (Thermo LabSystems, Inc.), as described previously.[30] The responses were determined at rpFVIII equivalent concentrations of 0, 100, 200, and 400 U/kg. Using standard assumptions for body weight (70 kg), plasma volume (40 ml/kg) and FVIII molecular weight (200 ng is equivalent to 1 U), the appropriate rpFVIII concentrations were calculated to be 2.7, 5.4, and 10.8 U/ml. Reference values for a thrombin generation response with the TGA were determined at the central laboratory using data generated from 100 healthy volunteer men. A full thrombin generation response referred to TGA results within the normal reference range (mean ± standard deviation) after in vitro addition of rpFVIII. A partial response referred to an increase or improvement of thrombin generation that could not reach the lower limit (mean ± standard deviation) of reference values.

2.5 | Thromboelasticity studies

Assessment of the viscoelastic properties of blood during clot formation and lysis were conducted on citrated whole blood at local study sites using either rotation thromboelastometry (ROTEM Pentapharm GmbH) or thromboelastography (TEG-5000, Haemonetics SARL) in the presence of recombinant human tissue factor (Innovin) diluted at 1:17,000 (final concentration). The maximum amplitude of fibrin clots obtained in the presence of tissue plasminogen activator was used as the main parameter. Measurements included clotting time and the observed maximum clot firmness.

2.6 | Fibrin clot structure assessment

Clots formed in the TGA assay were fixed with 1.5% glutaraldehyde (Sigma Aldrich) in phosphate buffered saline, pH 7.2, followed by a solution of OsO_4 in 1% sodium cacodylate 150 mM, pH 7.4. Fixed clots were dehydrated in serial ethanol solutions and hexamethyldisilazane and stored under vacuum. Samples were placed on carbon adhesive tabs, coated with gold-palladium ions, and viewed under a Hitachi S800 FEG scanning electron microscope (×10,000 magnification). Three to five representative images (mean of 250 fibrin fibers per sample) were analyzed using ImageJ software (National Institutes of Health). Clot quality was assessed according to the number, diameter, and density of fibrin fibers. For each patient sample, fibrin fiber diameters measured at baseline were compared with those measured after addition of rpFVIII 2.7, 5.4, and 10.8 U/ml. An increased number of thinner fibrin fibers with increased fiber density relative to baseline was considered an improvement in clot quality.

2.7 | Epitope mapping

Epitope mapping of inhibitors with respect to the A1, A2, A3, C1, and C2 domains of the human FVIII molecule was carried out at

the central laboratory in Atlanta, Georgia, USA, using citrated PPP samples loaded onto direct enzyme-linked immunosorbent assay (ELISA) on half-area 96-well plates with purified single human domain hybrid FVIII proteins as test antigens and BDD human FVIII and BDD porcine FVIII as positive control antigens.[31] Plasma samples were serially diluted from 1:20 in blocking buffer (0.15 M NaCl/20 mM HEPES/5 mM $CaCl_2$/0.05% sodium azide/0.05% Tween®80/0.25% BSA, pH 7.4) and dilutions added to the coated wells of the ELISA plate. Antibody binding was detected by goat anti-human IgG. Domain specificity was indicated by color reaction and ELISA titers.

2.8 | Statistical analyses

Samples were classified by human or porcine FVIII inhibitor titers as low titer (<5 BU/ml) or high titer (≥5 BU/ml)[32] and further stratified into the following subgroups: (1) low anti-human/low anti-porcine; (2) high anti-human/low anti-porcine; and (3) high anti-porcine inhibitor titers. Procoagulant response was classified as restored by a combination of increased peak height, increased ETP, and decreased time to peak and defined as positive, partial, or no response for each sample based on reference values.

Descriptive statistics were used for hemostatic assay results. In addition, Spearman rank correlation coefficients were used to analyze differences between rpFVIII inhibitor titers with respect to thrombogenic response. A Dunnett multiple comparison test was used to compare the differences between replicate clots used for analysis of clot formation within the same patient. A p value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Patient characteristics, FVIII activity, and FVIII inhibitor levels

Although the study aimed to recruit consecutive patients who attended for routine visits at their center during the study period, some patients were excluded because they were noncompliant, did not visit the hemophilia center regularly, or declined to participate in the study. FVIII inhibitor titers were determined in samples collected from 20 participants with congenital hemophilia A. Patients were aged 11–70 years and FVIII activity was <0.01 IU/ml, indicative of severe hemophilia A. Results from one patient with AHA were also available but are not reported with the main results. The patient with AHA was aged 74 years; no FVIII activity data were available for this patient, anti-human FVIII inhibitor titer was 7 BU/ml (classified as high), and anti-porcine FVIII inhibitors were not detected (0 BU/ml). Thrombin generation normalization was not achieved for this patient and fiber diameter changes (mean difference) from baseline in response to rpFVIII were: −19.8 (−27.6 to −12.0, $p < 0.01$) at rpFVIII 2.7 U/ml; −41.4 (−49.2 to −33.5, $p < 0.01$) at 5.4 U/ml; −41.8 (−49.6 to −33.9, $p < 0.01$) at 10.8 U/ml. Blood from this patient bound to all epitopes except C2.

In total, measurements from 22 patient blood samples were recorded (two of 20 patients provided a second sample at a later visit). Patients had varying FVIII inhibitor titers across the low and high titer range (Table 1). There was considerable variation in human FVIII and porcine FVIII inhibitor titers across the samples analyzed ($n = 20$ patients). The mean (range) FVIII inhibitor titer was 14 (1–427) BU/ml ($n = 13$ high, $n = 6$ low, $n = 1$ data unavailable) for human FVIII inhibitors and 12 (0–886) BU/ml ($n = 11$ high, $n = 8$ low, $n = 1$ data unavailable) for porcine FVIII inhibitors.

3.2 | Effects of rpFVIII on thrombin generation

A total of 20 samples from 19 patients were analyzed using CAT to assess thrombin generation (Table 2). Restoration of thrombin generation was determined by a combination of increased peak height, increased ETP, and decreased time to peak values in relation to a set of reference values obtained previously by the central laboratory from healthy volunteers. Thrombin generation was restored by rpFVIII in 13/20 (65%) patient plasma samples. In the majority of these samples (8/13), restoration was achieved with the lowest rpFVIII concentration (2.7 U/ml), compared with 1/13 and 4/13 for 5.4 U/mL and 10.8 U/mL concentrations of rpFVIII, respectively.

The CAT analysis data indicated correction of thrombin generation parameters was both rpFVIII dose dependent and porcine FVIII inhibitor titer dependent (Table 2) (Figure 1). rpFVIII correction of thrombin generation occurred in all patients with low porcine FVIII inhibitor titers (<5 BU/ml). All patient samples with low human FVIII inhibitors and low porcine FVIII inhibitor titers (subgroup 1) and all those with high human FVIII inhibitors and low porcine FVIII inhibitor titers (subgroup 2) displayed correction of thrombin generation with addition of rpFVIII. In patient samples with high porcine FVIII titers (subgroup 3), 6/10 (60%) showed no restoration of thrombin generation in response to the addition of rpFVIII in vitro, 1/10 (10%) showed partial restoration, and 3/10 (30%) showed complete restoration (Table 2).

Data from 18 patients with complete data for both ETP and peak height generated at the central laboratory were used for the Spearman rank correlation analysis to examine the effect of porcine inhibitor titers on restoration of thrombin generation after addition of rpFVIII in vitro. ETP and peak height data also varied substantially. A strong inverse relationship between porcine inhibitor titers and thrombin generation parameters was demonstrated using Spearman rank correlation coefficient (Table 3).

3.3 | Clot formation – thromboelasticity studies

Clot formation in response to increasing concentrations of rpFVIII (0, 2.7, 5.4, and 10.8 U/ml) was assessed by thromboelastography (Table 4). Overall, median quantifiable clotting times decreased with increasing concentrations of rpFVIII. Accordingly, observed values for maximum clot firmness increased with increasing concentrations of rpFVIII (Table 4).

TABLE 1 Patient characteristics, FVIII inhibitor titers, and classifications

		FVIII inhibitor titer (BU/ml) local laboratory		FVIII inhibitor titer group		FVIII inhibitor titer (BU/ml) central laboratory		
Patient	Age (years)	Anti-human	Anti-porcine	Anti-human	Anti-porcine	Anti-human	Anti-porcine	Subgroup
1	11	10.4	Not detected	High	Low	11.7	Not detected	2
2	17	27.3	2.6	High	Low	36.8	3	2
3	61	29.1	21.9	High	High	46.8	43.1	3
4	39	29.6	17.5	High	High	31.9	49	3
5	46	405.0	596.0	High	High	>500	>500	3
6	36	2.6	0.6	Low	Low	1.8	Not detected	1
7	38	2.6	5.0	Low	High	2.4	2.7	2
8	35	38.0	40.0	High	High	39.0	18.4	3
9	–	8.0	13.0	High	High	11.5	21	3
10	–	8.0	5.0	High	High	55.5	11.5	3
11	29	500.0	690.0	High	High	426.9	886.4	3
12	–	0.5	0.3	Low	Low	Not detected	Not detected	1
13	–	7.0	10.0	High	High	14.6	13.1	3
14	–	8.0	14.0	High	High	10.7	20.4	3
15	11	4.1	0.6	Low	Low	–	–	1
15b*		3.8	ND	Low	ND	2.5	<1	ND
16	15	59.3	5.1	High	High	54.0	13.1	3
16b*		102	ND	High	ND	46.2	11.4	ND
17	70	4.5	1.2	Low	Low	5.4	2.2	1
18	16	9.9	Not detected	High	Low	2.5	<1	2
19	48	4.0	1.8	Low	Low	2.3	4.3	1
20	52	–	–	–	–	2.3	4.3	–

Note: Subgroup 1: low/low = low anti-human FVIII inhibitors and low porcine FVIII inhibitors. Subgroup 2: high/low = high anti-human FVIII inhibitors and low porcine FVIII inhibitors. Subgroup 3: high porcine FVIII inhibitors (all patients in this group also had high anti-human FVIII inhibitors except for patient 7).

Abbreviations: –, data not available; BU, Bethesda units; FVIII, factor VIII; ND, not determined.

*Second sample taken from the same patient at a different study visit.

3.4 | Fibrin clot structure assessment – scanning electron microscopy

Electron micrographs of clots formed in response to different concentrations of rpFVIII added to PPP samples (Figure 2) were analyzed to assess clot quality (number, diameter, and density of fibrin fibers). Figure 2 shows examples of electron micrographs of clots from the same patient sample. An increase in the number of thinner fibrin fibers with increased fiber density was considered an improvement in clot quality.

The results of a Dunnett multiple comparison test on fibrin clot fiber diameters assessed by electron microscopy are shown in Table 5. Clot quality showed a dose-dependent improvement in response to rpFVIII added in vitro to samples with low porcine FVIII inhibitor titers. Clots formed in the presence of rpFVIII in samples with low porcine FVIII inhibitor titers were similar to those formed in control samples without inhibitors. In samples with high porcine FVIII inhibitor titers, little improvement in clot structure was observed, indicating that clots formed in response to rpFVIII were more vulnerable to fibrinolysis compared with noninhibitor control samples. Improvement in clot structure and thrombin generation were similarly dependent on rpFVIII dose and low anti-porcine inhibitor titer.

3.5 | Epitope mapping

Of 20 plasma samples available for this analysis, three had >75% porcine cross-reactivity, which prevented subdivision into domain specificity, and two samples had positive human FVIII inhibitor titers that were not high enough to subdivide into individual domain specificity. Anti-human FVIII inhibitory antibodies specifically bound to the A1, A2, C1, and C2 domains of the human FVIII protein (Figure 3).

Binding to the C2 domain was dominant in 11 samples, to the A1 domain in two samples, and to the A2 domain in five samples. In total, seven samples had no clear dominant epitopes; of these, three had no detectable epitopes and four had porcine cross-reactive antibodies. In plasma samples with no restoration of thrombin generation, either anti-human FVIII antibodies were detected to one or more FVIII domains or a dominant epitope could not be identified because of porcine cross-reactive antibodies.

TABLE 2 Thrombin generation parameters: calibrated automated thrombography (CAT) data

Patient	rpFVIII concentration (U/ml)	ETP (nmol/min)	Peak (nmol)	ttPeak (min)	Thrombin generation normalization achieved	Lowest normalizing rpFVIII dose (U/mL)	Inhibitor subgroup
		Central	Central	Central			
1*	0	3216	84	34	Yes	2.7	2
	2.7	4771	1040	11			
	5.4	4540	1212	9			
	10.8	4403	1217	8			
2	0	537	22	21	Yes	2.7	2
	2.7	1187	87	14			
	5.4	1165	147	10			
	10.8	1082	220	7			
3	0	558	27	15	No	NA	3
	2.7	561	26	15			
	5.4	510	23	15			
	10.8	538	20	15			
4	0	1267	44	17	No	NA	3
	2.7	1279	42	16			
	5.4	1263	40	16			
	10.8	1129	36	16			
5	0	108	4	29	No	NA	3
	2.7	109	4	31			
	5.4	111	4	32			
	10.8	0	5	46			
6	0	281	13	21	Yes	2.7	1
	2.7	1077	184	9			
	5.4	1013	210	8			
	10.8	853	208	7			
7	0	360	16	19	Yes	2.7	2
	2.7	2095	277	12			
	5.4	2228	390	9			
	10.8	2007	386	8			
8	0	177	7	21	No	NA	3
	2.7	170	6	22			
	5.4	150	6	23			
	10.8	597	43	16			
9	0	441	21	24	Yes	10.8	3
	2.7	370	16	26			
	5.4	335	14	28			
	10.8	1514	253	13			
10	0	386	13	21	Yes	5.4	3
	2.7	583	18	20			
	5.4	610	43	12			
	10.8	995	222	8			
11	0	201	7	25	No	NA	3
	2.7	173	6	25			
	5.4	174	6	26			
	10.8	160	5	30			

TABLE 2 (Continued)

Patient	rpFVIII concentration (U/ml)	ETP (nmol/min)	Peak (nmol)	ttPeak (min)	Thrombin generation normalization achieved	Lowest normalizing rpFVIII dose (U/mL)	Inhibitor subgroup
		Central	Central	Central			
12	0	91	4	25	Yes	2.7	1
	2.7	866	179	11			
	5.4	847	204	10			
	10.8	760	203	9			
13	0	337	14	21	Yes	10.8	3
	2.7	318	12	22			
	5.4	359	14	20			
	10.8	1100	122	12			
14	0	126	5	26	Yes (partial)	10.8	3
	2.7	115	5	26			
	5.4	199	7	27			
	10.8	389	29	16			
15	0	119	11	6	Yes	2.7	1
	2.7	2648	451	10			
	5.4	2626	574	9			
	10.8	2559	628	8			
16	0	–	–	–	No	NA	3
	2.7	–	–	–			
	5.4	–	–	–			
	10.8	–	–	–			
16b†	0	–	–	–	No	NA	ND
	2.7	–	–	–			
	5.4	–	–	–			
	10.8	–	–	–			
17	0	704	29	18	Yes	2.7	1
	2.7	1271	274	6			
	5.4	1154	291	5			
	10.8	1112	293	5			
19	0	744	26	18	Yes	2.7	1
	2.7	1189	179	9			
	5.4	1192	267	7			
	10.8	1114	259	6			
20	0	489	20	18	Yes	10.8	–
	2.7	448	20	13			
	5.4	474	18	13			
	10.8	1479	89	14			

Note: Subgroup 1: low/low =low anti-human FVIII inhibitors and low porcine FVIII inhibitors. Subgroup 2: high/low =high anti-human FVIII inhibitors and low porcine FVIII inhibitors. Subgroup 3: high porcine FVIII inhibitors (all patients in this group also had high anti-human FVIII inhibitors except for patient 7).

Abbreviations: –, data not available; ETP, endogenous thrombin potential; NA, not applicable; ND, not determined; rpFVIII, recombinant porcine Factor VIII; ttPeak, time to peak.

*The patient who provided this sample may have a thrombin generating capacity higher than expected for patients with hemophilia A and inhibitors.

†Second sample taken from the same patient at a different study visit.

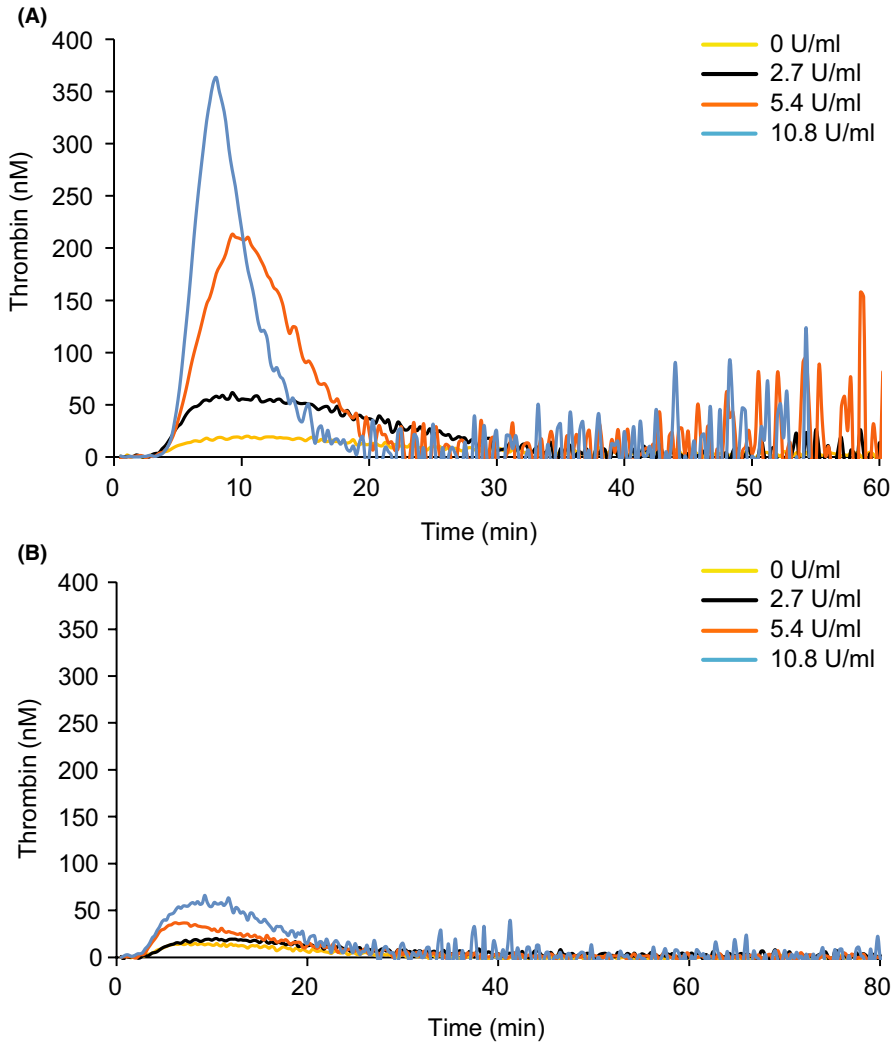

FIGURE 1 Correction of thrombin generation parameters in patient plasma. Thrombin generation profiles for two patient sample plasmas from local laboratories in Lyon after addition of varying recombinant porcine factor VIII (rpFVIII) concentrations (0–10.8 U/ml) in vitro. (A) Restoration of thrombin generation was dose dependent in a sample plasma with high human FVIII inhibitor titer and low porcine inhibitor titer. (B) In a sample plasma with high human FVIII inhibitor titer and high porcine inhibitor titer, there was a slight increase in thrombin generation observed with the highest rpFVIII concentration (10.8 U/ml) compared with vehicle control

TABLE 3 Results of Spearman rank correlation analysis of anti-porcine inhibitor titer and thrombin generation response

rpFVIII concentration (U/ml)	Number of patients	Spearman rank: anti-porcine inhibitor and ETP[a]	*p* value	Spearman rank: anti-porcine inhibitor titer and peak height[a]	*p* value
0	18	−0.171	0.5128	−0.266	0.3016
2.7	18	−0.718	0.0012	−0.848	<0.0001
5.4	18	−0.733	0.0008	−0.876	<0.0001
10.8	18	−0.594	0.0120	−0.789	0.0002

Abbreviations: ETP, endogenous thrombin potential; rpFVIII, recombinant porcine factor VIII.

[a]Anti-porcine inhibitor values as measured by local laboratories and ETP and peak height as measured by central laboratory.

4 | DISCUSSION

This study evaluated the hemostatic efficacy of rpFVIII in vitro in blood samples from 20 patients with congenital hemophilia A incorporating a wide range of anti-human FVIII and anti-porcine FVIII neutralizing antibody titers. Analysis of thrombin generation response and viscoelastic properties of clots provides evidence that rpFVIII can correct surrogate markers of hemostatic function in plasmas from patients with congenital hemophilia A who have inhibitors to FVIII. The analysis of in vitro rpFVIII activity included samples spanning a wide range of titers to human and porcine FVIII to gain insight into conditions in which rpFVIII could be useful for management of bleeding in patients with FVIII inhibitors.

Overall, rpFVIII increased thrombin generation responses in vitro in patient plasmas with porcine FVIII inhibitor titers. The effect was more pronounced when inhibitor titers were low. In such plasma samples, clot formation was comparable with clots formed in noninhibitor control plasmas as assessed by thromboelastography. Little or no restoration of the thrombin generation response was observed in plasma samples with high porcine FVIII inhibitor

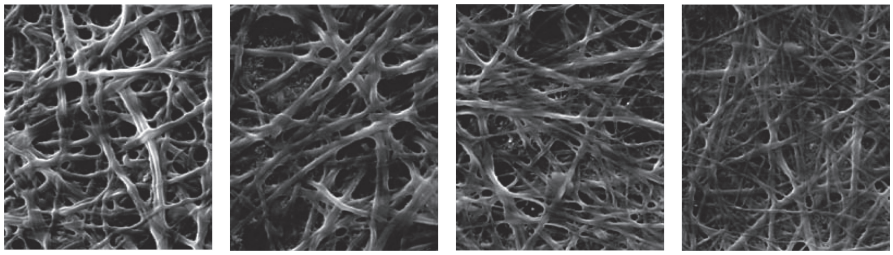

FIGURE 2 Scanning electron micrographs (×10,000; representative images) of fibrin clots formed in platelet-poor plasma from patient 2 with hemophilia A (high hFVIII inhibitor titer and low pFVIII inhibitor titer) in response to increasing concentrations of recombinant porcine factor VIII (rpFVIII), 0–10.8 U/ml. Fibrin clot structure assessment showed a dose-dependent improvement in clot quality with significant fiber and hole diameter reductions achieved with 5.4 and 10.8 U/ml of rpFVIII added in vitro. Thrombin generation normalization occurred at the lowest rpFVIII concentration (2.7 U/ml) for this patient sample (see Table 2)

TABLE 4 Overall rpFVIII-dependent clot formation as assessed by thromboelastometry (median [range])

rpFVIII concentration (U/ml)	Clotting time (s)	Maximum clot firmness (mm)
0	911 (265–3120)	41 (21–60)
2.7	667 (316–2257)	43 (28–58)
5.4	623 (221–1704)	44 (32–56)
10.8	520 (263–1208)	49 (36–61)

Note: Values apply across hFVIII and pFVIII inhibitor titer subgroups.
Abbreviations: hFVIII, human factor VIII; pFVIII, porcine factor VIII; rpFVIII, recombinant porcine FVIII.

titers. Ultrastructure analysis of fibrin fibers of the clots formed using scanning electron microscopy indicated that they were more vulnerable to fibrinolysis, as demonstrated in a previous report that showed a negative correlation between fibrin fiber diameters and clot resistance to fibrinolysis.[33]

In general, rpFVIII restoration of hemostatic markers function in vitro appeared to be dependent on rpFVIII dose and porcine FVIII inhibitory antibody titer but not on the titer of human FVIII inhibitors. These observations were supported by statistical testing (Spearman rank correlation analysis). An inverse correlation was found between the porcine FVIII inhibitor titer and thrombin generation response to rpFVIII as measured by ETP (2.7–5.4 U/ml rpFVIII) and peak height (2.7–10.8 U/ml rpFVIII) ($p < 0.03$).

Individual variability in responses to rpFVIII in vitro was noted. In some cases, hemostatic effects could be demonstrated in the presence of high human FVIII inhibitor titers ≥5 BU/ml (thrombin generation normalization was achieved in patients 1, 2, 9, 10, 13, and 14 with high human FVIII inhibitor titers; see Table 2), supportive of evidence that anti-human FVIII inhibitory antibodies might have a lower neutralizing capacity against porcine FVIII. [18-20] Furthermore, normalization of thrombin generation responses was also achieved in some patients from subgroup 3 with high titers for porcine FVIII inhibitors (patients 9, 10, 13, and 14; see Table 2).

In the present study, the observation that rpFVIII corrected thrombin generation responses in patient plasmas with low porcine FVIII inhibitor titers is consistent with findings from previous clinical studies. In a prospective phase II/III study of rpFVIII in patients with AHA, pretreatment anti-porcine FVIII inhibitory antibodies were detected in 35.7% of patients and appeared to affect the rise in FVIII activity levels, particularly if porcine FVIII inhibitor titers were >5 BU. However, therapeutic FVIII activity could still be achieved in the presence of high-titer porcine FVIII inhibitors.[26] A phase II study of rpFVIII treatment of nine patients with congenital hemophilia A with human FVIII inhibitory antibodies (<0.8–15.7 BU/ml) and porcine FVIII inhibitory antibodies (<0.8–6.2 BU/ml) also reported control of bleeding episodes in the presence of FVIII inhibitor levels.[25]

The low cross-reactivity of pFVIII (Hyate:C) or rpFVIII with human FVIII inhibitors in previous studies[19,20] may be related to amino acid sequence differences between porcine versus human FVIII at known human FVIII immunological epitopes on the A2 and C2 domains.[17] In a prospective cohort study of 70 patients with AHA, cross-reactivity with rpFVIII was demonstrated to be more likely in patients when anti-human FVIII antibodies were reactive against the C1 domain of FVIII, whereas cross-reactivity was rarely detected for inhibitors reactive only to the C2 domain.[34] Epitope mapping in the present study indicated that the dominant epitope was the C2 domain in 11 patients. The presence of antibodies to more than one domain or high cross-reactivity were properties common to samples in which thrombin generation could not be restored.

The in vitro setting and small patient sample numbers are obvious limitations of the present study. Although it was shown that rpFVIII corrected thrombin generation parameters when added to human hemophilic plasma containing FVIII inhibitors, these are surrogate markers that may not directly reflect clinical hemostatic efficacy. In addition, the participating centers aimed to include consecutive patients. However, the study may not have captured all eligible patients because some were noncompliant, missed appointments at their hemophilia center, or declined to participate in the study. Therefore, the study may have been biased toward motivated patients interested in the research.

In this in vitro study of 20 patients with congenital hemophilia A with human FVIII inhibitors, laboratory data support the restoration of hemostatic function with rpFVIII added to blood samples from

TABLE 5 Clot formation fibrin analysis: fiber diameter changes (mean difference) from baseline in response to rpFVIII in vitro

Patient[a]	Anti-porcine FVIII Ab titer	Anti-human FVIII Ab titer	Dunnett's multiple comparison test: Comparator (baseline vs. rpFVIII at dose shown)	Mean difference (95% CI)	*p* value
A					
3	High	High	2.7 U/ml	−7.3 (−15.1 to 0.61)	> 0.05
			5.4 U/ml	8.0 (0.1 to 15.8)	< 0.05
			10.8 U/ml	−18.8 (−26.7 to −11.0)	< 0.01
4	High	High	2.7 U/ml	43.3 (34.6 to 52.0)	< 0.01
			5.4 U/ml	34.0 (25.3 to 42.7)	< 0.01
			10.8 U/ml	12.9 (4.2 to 21.6)	< 0.01
5	High	High	2.7 U/ml	23.9 (16.2 to 31.6)	< 0.01
			5.4 U/ml	11.8 (4.0 to 19.5)	< 0.01
			10.8 U/ml	−33.5 (−41.3 to −25.8)	< 0.01
9	High	High	2.7 U/ml	18.9 (11.9 to 26.0)	< 0.01
			5.4 U/ml	12.6 (5.5 to 19.67)	< 0.01
			10.8 U/ml	27.2 (20.2 to 34.2)	< 0.01
10	High	High	2.7 U/ml	9.2 (1.5 to 16.9)	< 0.05
			5.4 U/ml	26.7 (19.0 to 34.4)	< 0.01
			10.8 U/ml	35.1 (27.4 to 42.8)	< 0.01
11	High	High	2.7 U/ml	25.4 (19.1 to 31.7)	< 0.01
			5.4 U/ml	58.9 (52.6 to 65.2)	< 0.01
			10.8 U/ml	45.5 (39.2 to 51.8)	< 0.01
13	High	High	2.7 U/ml	25.8 (17.3 to 34.4)	< 0.01
			5.4 U/ml	14.2 (5.7 to 22.8)	< 0.01
			10.8 U/ml	54.7 (46.1 to 63.2)	< 0.01
14	High	High	2.7 U/ml	−6.1 (−12.7 to 0.5)	> 0.05
			5.4 U/ml	49.6 (43.0 to 56.2)	< 0.01
			10.8 U/ml	3.8 (−2.8 to 10.4)	> 0.05
16	High	High	2.7 U/ml	−6.6 (−12.9 to −0.3)	< 0.05
			5.4 U/ml	10.0 (3.8 to 16.3)	< 0.01
			10.8 U/ml	−12.2 (−18.5 to −5.9)	< 0.01
B					
1	Low	High	2.7 U/ml	10.2 (2.5 to 17.9)	< 0.01
			5.4 U/ml	18.5 (10.8 to 26.2)	< 0.01
			10.8 U/ml	2.7 (−5.0 to 10.4)	> 0.05
2	Low	High	2.7 U/ml	−2.0 (−10.1 to 6.2)	> 0.05
			5.4 U/ml	29.1 (21.0 to 37.3)	< 0.01
			10.8 U/ml	31.6 (23.4 to 39.7)	< 0.01
12	Low	Low	2.7 U/ml	25.4 (18.2 to 32.5)	< 0.01
			5.4 U/ml	21.8 (14.7 to 29.0)	< 0.01
			10.8 U/ml	14.8 (7.7 to 22.0)	< 0.01
15	Low	Low	2.7 U/ml	17.0 (9.9 to 24.1)	< 0.01
			5.4 U/ml	8.6 (1.5 to 15.8)	< 0.05
			10.8 U/ml	−3.9 (−11.0 to 3.3)	> 0.05
17	Low	Low	2.7 U/ml	6.9 (1.0 to 12.7)	< 0.05
			5.4 U/ml	7.4 (1.6 to 13.3)	< 0.01
			10.8 U/ml	7.0 (1.1 to 12.8)	< 0.05
19	Low	Low	2.7 U/ml	15.4 (9.6 to 21.2)	< 0.01
			5.4 U/ml	−7.9 (−13.7 to −2.1)	< 0.01
			10.8 U/ml	34.3 (28.6 to 40.1)	< 0.01

Note: A: Patient samples with high titers for both anti-porcine FVIII inhibitory antibodies and anti-human FVIII inhibitory antibodies. B: Patient samples with low anti-porcine FVIII inhibitory antibodies.

Abbreviations: Ab, antibody; BL, baseline; CI, confidence interval; rpFVIII, recombinant porcine FVIII.

[a]Results are shown only for patients with available fibrin data.

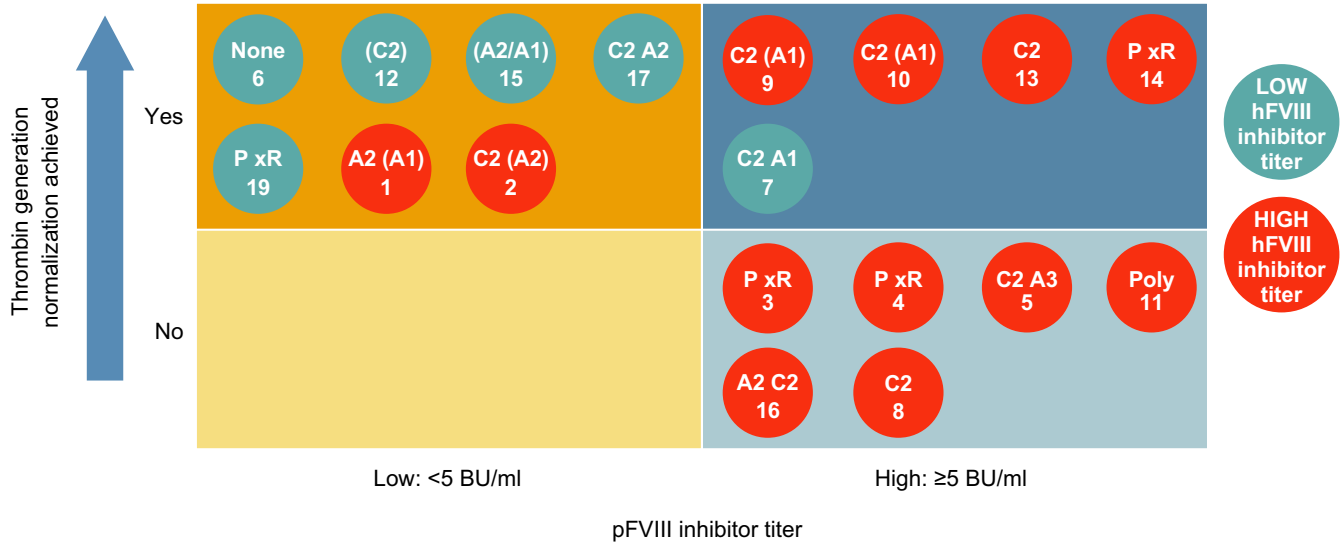

FIGURE 3 FVIII domain dominance and thrombin generation response map. Epitope mapping of inhibitor plasmas to domains of the human FVIII molecule analyzed by direct enzyme-linked immunosorbent assay. Dominant hFVIII domain(s) indicated in the top row of text in each circle (second text row is patient number). In some patients, >1 domain was dominant. Domains in parentheses indicate low signal. Thrombogenic response was defined as a positive response or no response for each sample. Results are shown only for samples with inhibitor titer information available for both hFVIII and pFVIII inhibitors (20 samples were tested from 18 patients). Excludes secondary sample results from (1) Patient 17 (high hFVIII inhibitor titer, pFVIII inhibitor titer not determined, thrombin generation response not restored): A2 + C2 dominant epitopes with high porcine antibody cross-reactivity, and (2) Patient 16 (low hFVIII inhibitor titer, porcine inhibitor titer not determined, no thrombin generation data): polyclonal. Abbreviations: BU, Bethesda units; FVIII, factor VIII; hFVIII, human factor VIII inhibitor; none, no predominant epitope; pFVIII, porcine factor VIII; poly, polyclonal antibodies; P xR, porcine crossreactive antibodies

patients with low porcine FVIII inhibitor titers independently of human FVIII inhibitor titers. This effect and the low thrombotic risk profile associated with rpFVIII could be important considerations when evaluating treatment options for the management of individual patients with inhibitors to FVIII, in particular those for whom the use of bypassing agents is unsuitable or ineffective. Although the procoagulant effect of rpFVIII in this study was assessed in vitro only, our results are in line with previous studies that have reported a correlation between TGA results and the clinical outcome of hemostatic treatment in patients with hemophilia A and inhibitors.[35-37] The measurement of trough FVIII levels as part of an rpFVIII dosing algorithm in patients with AHA has previously been shown to be useful for the treatment of bleeds.[38] Future studies that use a similar approach to incorporate the measurement of thrombin generation in the treatment of patients with hemophilia A are warranted.

AUTHOR CONTRIBUTIONS

Claude Négrier, Johannes Oldenburg, and Shannon Meeks: conception and study design; collection, analysis, and interpretation of the data. Gili Kenet, Jean-Claude Bordet, Jens Müller, Sandra Le Quellec, and Yesim Dargaud: collection, analysis, and interpretation of the data. Peter Turecek and Nikola Tripkovic: analysis and data interpretation. All authors revised the manuscript critically for intellectual content and gave their final approval for it to be published.

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DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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