Received: 14 December 2021 | Accepted: 21 April 2022

DOI: 10.1111/jth.15741

ORIGINAL ARTICLE

Efanesoctocog alfa elicits functional clot formation that is indistinguishable to that of recombinant factor VIII

Melanie Demers | Maria M. Aleman | Elena Kistanova | Robert Peters | Joe Salas | Ekta Seth Chhabra

Sanofi, Waltham, Massachusetts, USA

Correspondence
Ekta Seth Chhabra, 225 Second Avenue, Waltham, MA 02451, USA.
Email: Ekta.SethChhabra@sanofi.com

Funding information
Sanofi

Abstract

Background: Factor VIII (FVIII) binding to endogenous von Willebrand factor (VWF) has constrained half-life extension of recombinant FVIII (rFVIII) products for hemophilia A. Efanesoctocog alfa (rFVIIIFc-VWF-XTEN; BIVV001) is a novel fusion protein designed to decouple FVIII from VWF in circulation and maximize half-life prolongation by XTEN® polypeptides and Fc fusion. FVIII, VWF, and platelets interact to achieve normal hemostasis. Thus, bioengineered FVIII replacement products, such as efanesoctocog alfa, require comprehensive assessment of their hemostatic potential.

Objectives: We compared functional clot formation and injury-induced platelet accumulation between efanesoctocog alfa and rFVIII.

Patients/Methods: The hemostatic potential of efanesoctocog alfa and rFVIII were assessed by measuring their dose-dependent effects on *in vitro* fibrin generation in hemophilic plasma and *in vivo* injury-induced platelet accumulation using intravital microscopy and repeat saphenous vein laser-induced injuries in hemophilia A mice.

Results: Equal concentrations of efanesoctocog alfa or rFVIII (up to 1 IU/ml) added to plasma from patients with hemophilia A elicited similar kinetics for dose-dependent fibrin polymerization between factor products. In the presence of tissue plasminogen activator (tPA), clots formed had similar stability between products. Single intravenous doses (50, 100, or 150 IU/kg) of efanesoctocog alfa or rFVIII shortly before repeat saphenous vein laser-induced injuries increased platelet accumulation over time in a dose-dependent manner in hemophilia A mice. Platelet deposition kinetics were similar between products.

Conclusions: Equivalent doses of efanesoctocog alfa and rFVIII had similar efficacy in promoting fibrin clot formation and injury-induced platelet accumulation. The hemostatic potential of efanesoctocog alfa was indistinguishable from that of rFVIII.

KEYWORDS
blood platelets, factor VIII, fibrinogen, hemophilia A, von Willebrand factor

Melanie Demers and Maria M. Aleman contributed equally to the work. MD, MMA, RP, JS were employees of Sanofi at the time of the study.

1 | INTRODUCTION

Factor VIII (FVIII) has a key role in the amplification and propagation phases of coagulation by forming part of the tenase complex on platelets that catalyzes conversion of factor X to factor Xa, which in turn generates sufficient thrombin to form fibrin fibers.[1,2] In people with hemophilia A, the absence or reduced activity of FVIII results in fibrin clots of low density and poor stability.[3,4] People with hemophilia A therefore experience recurrent bleeding episodes, particularly in weight-bearing joints.[5] FVIII replacement therapy aims to increase circulating FVIII activity, thereby improving platelet activation and normalizing fibrin formation, to ultimately create a stable clot after injury.

Factor VIII interacts with von Willebrand factor (VWF) and platelets to generate a normal hemostatic response.[6,7] The interaction with VWF is complex. VWF acts as a chaperone for FVIII and, under normal conditions, more than 95% of FVIII circulating in plasma is bound to VWF in a high-affinity non-covalent association.[8,9] During clotting, thrombin cleavage releases activated FVIII from VWF. However, the FVIII-VWF interaction imposes a biological limit on the half-life of endogenous FVIII and replacement FVIII products, as the complex is subject to the VWF clearance pathway with a half-life of approximately 16 h.[7,10,11]

Efanesoctocog alfa (rFVIIIFc-VWF-XTEN; BIVV001) is a new class of FVIII replacement designed to circulate independently of endogenous VWF.[12,13] Efanesoctocog alfa is composed of a single B-domain–deleted recombinant FVIII (rFVIII) protein fused to dimeric Fc and covalently coupled to the FVIII binding D′D3 domain of VWF[14–16] and two XTEN® polypeptides (XTEN® is a registered trademark of Amunix Pharmaceuticals, Inc.). An XTEN® polypeptide is an unstructured polypeptide and comprises repeats of six hydrophilic amino acids (Gly, Ala, Pro, Thr, Ser, Glu).[17,18] Upon thrombin activation, both the B-domain XTEN® polypeptide (located between the FVIII A2 and A3 domains) and the D'D3 domain-XTEN® moiety are released, leaving behind an activated rFVIII-Fc fusion protein.[13] Appending the D′D3 domain of VWF to FVIII in efanesoctocog alfa prevents binding to endogenous VWF[12,19] and removes the limit on FVIII half-life imposed by VWF.[12,13] Fc fusion and XTEN®[17,18] polypeptide technologies provide further half-life extension.[20] In preclinical models, these modifications led to a four-fold longer half-life of FVIII and four-fold longer protection from bleeds compared with rFVIII.[13] In a Phase 1/2a clinical study of patients with severe hemophilia A, single-dose efanesoctocog alfa (65 IU/kg) resulted in a mean FVIII activity half-life of 42.5 h and provided high sustained mean FVIII activity levels in the normal to near-normal range (>40%) for 3 to 4 days post dose.[21] In the Phase 1 repeat-dose study, once-weekly efanesoctocog alfa at a dose of 50 IU/kg had a mean half-life of 41.3 h and provided high sustained FVIII activity levels in the normal to near-normal range for 3–4 days post dose and 10% at Day 7.[12]

Given the complex interaction between FVIII, endogenous VWF, and platelets for normal hemostasis, novel bioengineered FVIII replacement products, such as efanesoctocog alfa, require comprehensive preclinical assessment of their hemostatic potential. We aimed to directly compare and visualize the efficacy of efanesoctocog alfa versus rFVIII in clot formation. We assessed the ability of efanesoctocog alfa to form a fibrin network *in vitro* and platelet accumulation at the site of injury in mice using intravital microscopy and repeat saphenous vein laser-induced injury.

Essentials

- Efanesoctocog alfa is a novel recombinant FVIII decoupled from endogenous von Willebrand factor.
- The hemostatic effects of efanesoctocog alfa and rFVIII were compared in human plasma and mice.
- Efanesoctocog alfa induced similar fibrin polymerization and *in vitro* clot formation to rFVIII.
- Efanesoctocog alfa demonstrated comparable *in vivo* platelet adhesion to rFVIII.

2 | METHODS

Two experimental approaches were used to compare the hemostatic effects of efanesoctocog alfa and rFVIII. One approach was the *in vitro* evaluation of fibrin formation, network structure, and clot stability of FVIII-deficient human plasma (from donors with hemophilia A), spiked with rFVIII or efanesoctocog alfa. Congenital, FVIII-deficient, pooled plasma from either George King Bio-Medical Inc. (Catalog # 0800), Overland Park, Kansas, USA, or HRF Inc., Raleigh, North Carolina, USA, with confirmed negative inhibitor status were used during the development of assays or for FVIII molecule testing, respectively. A single lot of pooled FVIII-deficient plasma from HRF was used to test the two FVIII molecules. The plasma had a confirmed normal level of VWF activity and <1% FVIII activity by one-stage clotting assay.

The second approach was an *in vivo* evaluation of platelet accumulation during clot formation in mice with hemophilia A, followed by comparison of the effects of rFVIII and efanesoctocog alfa on restoring platelet deposition, using the saphenous vein injury model. For each experiment, 6–10-week-old hemophilia A male mice (n = 3–4/group) were randomly assigned to either the rFVIII or efanesoctocog alfa group. All animal studies were approved by the Institutional Animal Care and Use Committee of Biogen (Biogen separated its global hemophilia business to Bioverativ, which was later acquired by Sanofi). Full-length human rFVIII (Advate®, Takeda Pharmaceutical, Lexington, MA) was used as the comparator to efanesoctocog alfa. rFVIII was purchased and reconstituted according to manufacturers' guidelines.

2.1 | Integrated fibrin formation and network structure assay

We developed a novel integrated assay to evaluate fibrin formation and network structure *in vitro* by combining measurement of fibrin polymerization by absorbance with subsequent microscopy to capture fibrin network density (Figure 1).

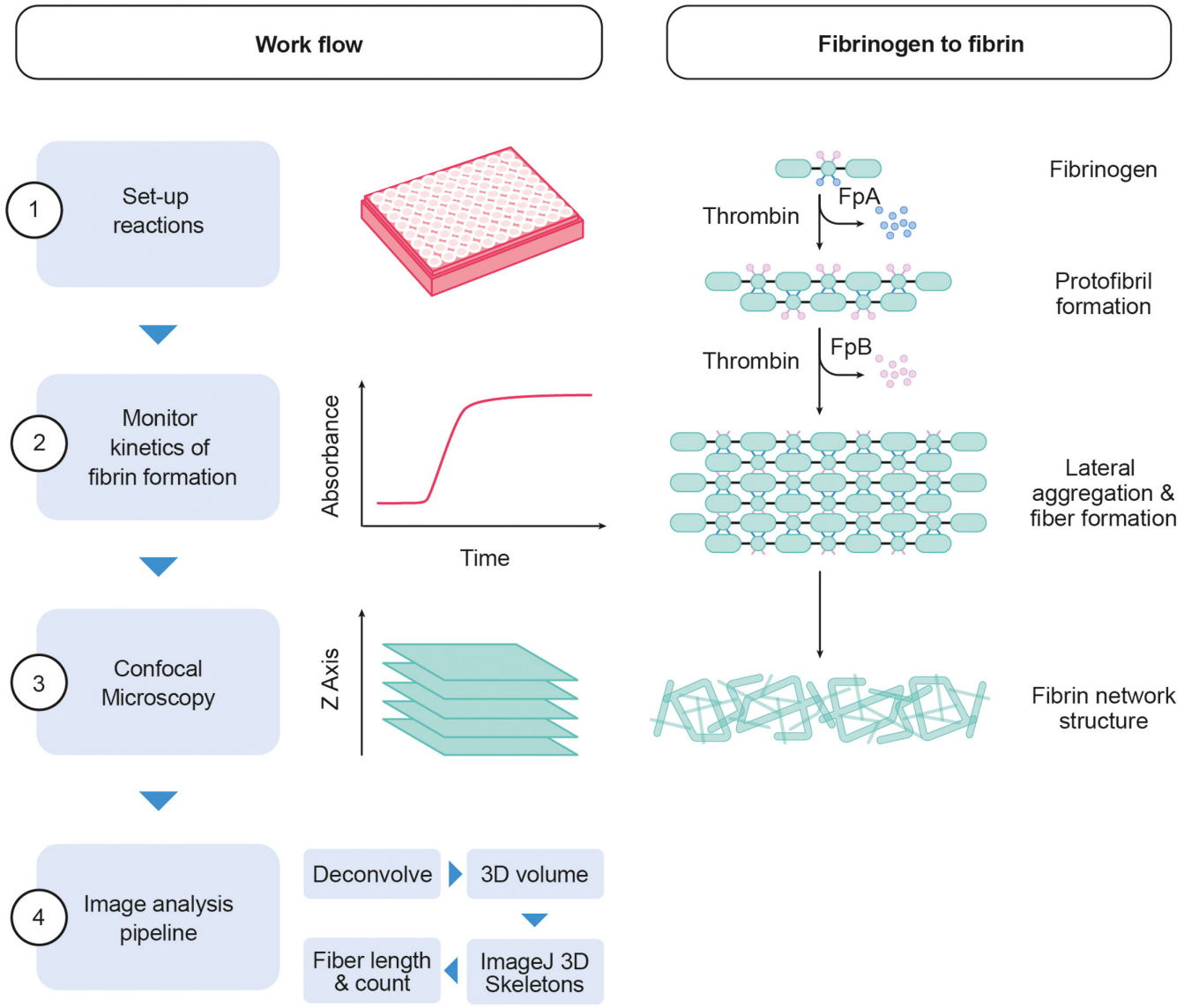

FIGURE 1 Integrated fibrin formation and network structure assay. 1. Reactions are set up in optically clear 96-well plates: 75 μg/ml Alexa Fluor 647–labeled human fibrinogen plus citrated human hemophilia A plasma spiked with increasing concentrations of efanesoctocog alfa or full-length recombinant factor VIII (Advate) up to 1 IU/ml (based on one-stage clotting assay activity [Actin FSL]). 2. Tissue factor/$CaCl_2$-triggered fibrin formation is monitored by absorbance at 405 nm over time, and kinetics of fibrin formation determined using SoftMax Pro v5.4.2 software (Molecular Devices). 3. Fibrin network structures of formed clots are captured by three-dimensional (3D) spinning disk confocal microscopy in multi-well capture mode (9 z-stacks per well). Z-stacks are deconvolved and fibrin network volumes measured using SlideBook v6 (Intelligent Imaging Innovations). 4. Fiber count and average fiber length per z-stack is quantified by 3D network skeletonization using ImageJ v1.51h (National Institutes of Health. Right image adapted from Blood Reviews, Volume 21, Issue 3, Alisa S. Wolberg, Thrombin generation and fibrin clot structure, Pages 131-142, Copyright 2006, with permission from Elsevier.

2.1.1 | Fibrin polymerization

To evaluate fibrin formation, increasing concentrations (up to 1 IU/ml) of efanesoctocog alfa or full-length human rFVIII were spiked into congenital FVIII-deficient platelet-poor plasma containing 75 μg/ml Alexa Fluor647-labeled human fibrinogen in optically clear 96-well plates.

Potency assignment for efanesoctocog alfa was based on the one-stage clotting assay (Actin® FSL reagent, Dade Behring).[13] Baseline was assessed using hemophilia A plasma alone. Briefly, the test FVIII molecule was diluted 100× in plasma to achieve the desired concentration of FVIII protein; 90 μl of FVIII or buffer containing plasma was mixed with 5 μl tissue factor (TF) solution (1:30,000 dilution of Innovin with 4 μM phospholipid vesicles [40% phosphatidylcholine; 40% phosphatidylethanolamine; 20% phosphatidylserine]) and incubated for 10 min at 37°C. Reactions were initiated by the addition of 5 μl calcium chloride ($CaCl_2$, 10 mM, final). Fibrin formation was monitored on a SpectraMax plate reader at 405 nm for 60 min using SoftMax Pro v5.4.2 software (Molecular Devices). The rate of fibrin polymerization was measured by the software from the maximum velocity of change in absorbance using a minimum of 10 data points. "Onset" represents time to fibrin protofibril lateral

aggregation. "Maximal polymerization rate" represents fibrin polymerization rate. "Delta absorbance" represents the change (increase) in fibrin network formation. Data from three separate experiments, run in duplicate, are presented (ie, a total of six readings per time point).

2.1.2 | Fibrin network density

Fibrin network structures of formed clots were captured by three-dimensional (3D) spinning disk confocal microscopy in multi-well capture mode (9 Z-stacks per well). Z-stacks were deconvolved and fibrin network volumes were measured using SlideBook v6 (Intelligent Imaging Innovations). To quantify fiber count and average fiber length per Z-stack, 3D network skeletonization was performed using ImageJ v1.51h (National Institutes of Health). Fibers that extended beyond the limits of the field of view were included in the analysis. Fibrin volume, fiber count, and fiber length are presented as mean ± standard error of the mean (SEM).

2.1.3 | Fibrin clot stability (tissue plasminogen activator challenge)

To assess clot stability, the fibrin formation assay was performed as described above but in the presence of tissue plasminogen activator (tPA) (0.5 μg/ml final). Resistance to lysis was determined by measuring parameters for fibrin polymerization, as described above, and included delta absorbance and area under the curve (AUC). Here, delta absorbance represents the increase in fibrin network formation before fibrinolysis takes over and AUC represents the overall resistance to lysis.

2.1.4 | Saphenous vein laser injury model

2.1.4.1 | *Dynamics of clot formation*

The laser-induced saphenous vein injury model was used to evaluate the dynamics of clot formation in wild-type (WT) and hemophilia A mice by measuring platelet accumulation over time in which (human) rFVIII binds normally to the mouse VWF. This model has been described previously but not in hemophilia A mice.[22] Briefly,

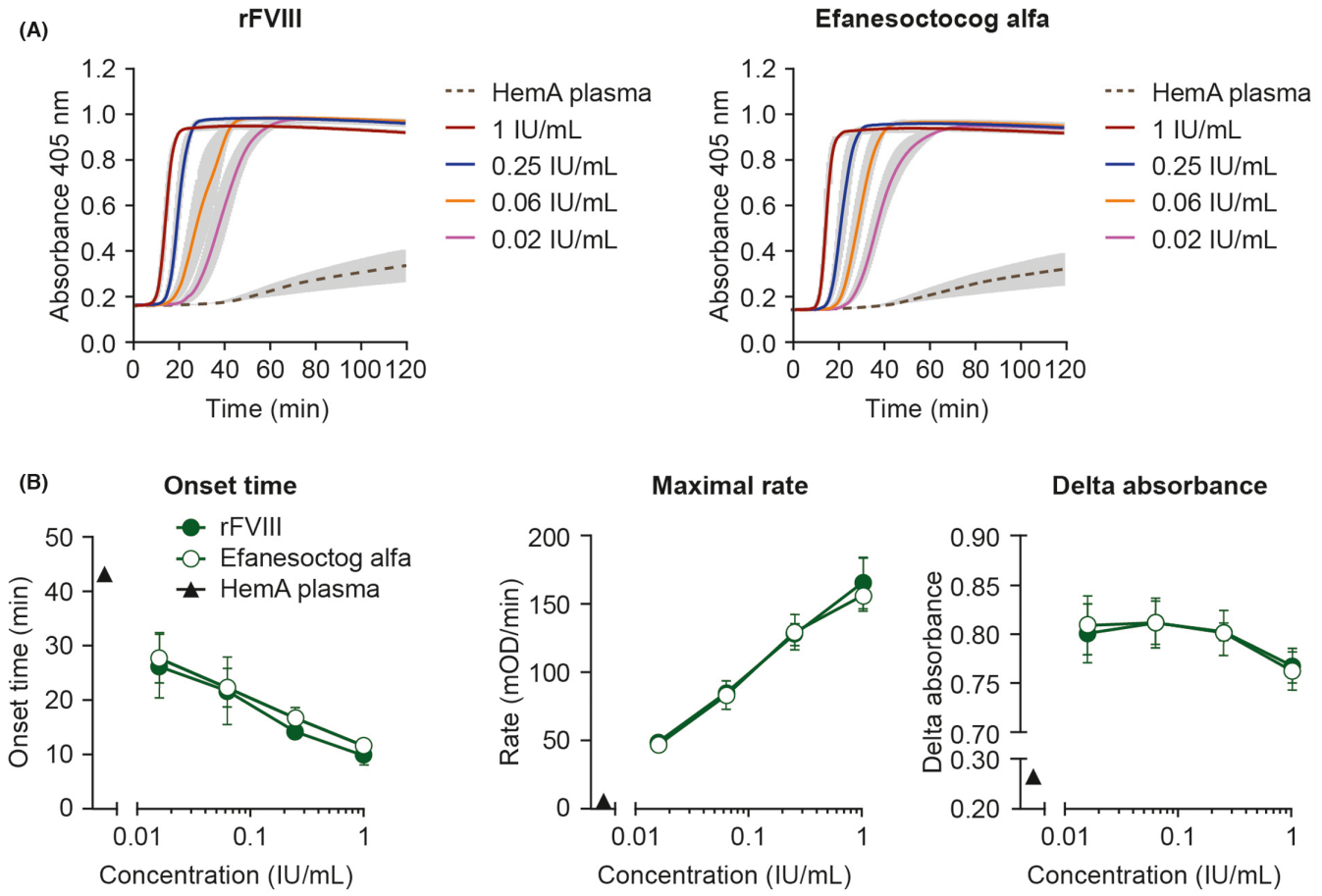

FIGURE 2 Fibrin polymerization in human FVIII-deficient plasma spiked with rFVIII or efanesoctocog alfa. OD, optical density; rFVIII, recombinant factor VIII; SEM, standard error of the mean. (A) Mean fibrin polymerization curves of rFVIII or efanesoctocog alfa. (B) Parameters of fibrin polymerization. Onset time represents the start of fibrin protofibril lateral aggregation, maximal rate represents the polymerization rate, and delta optical density (OD) represents the change in fibrin network density. HemA plasma refers to unspiked plasma from patients with severe hemophilia A (dotted line). Data are mean ± SEM (SEM shown as grey shading [A] or error bars [B]).

the three-hit laser-induced injury procedure was performed as follows (Figure S1): mice were anesthetized, platelet anti-glycoprotein Ib Alexa Fluor 647 conjugated antibody was injected intravenously before the saphenous vein was exposed, and laser-induced injury was initiated using 20 laser pulses (488 nm). The same site was reinjured after 3 min, and again after an additional 5 min.

Platelet deposition was recorded for a total of 13 min from the first laser hit and analyzed using the SlideBook software. Sum fluorescence intensity was recorded over time and used to analyze the kinetics of platelet accumulation after the third injury.

2.1.4.2 | *Effect of efanesoctocog alfa and rFVIII on platelet accumulation*

The laser-induced saphenous vein injury model was then used to assess the effects of efanesoctocog alfa versus rFVIII on platelet accumulation in hemophilia A mice. Mice were anesthetized and injected intravenously with one of three different doses (50, 100, or 150 IU/kg) of efanesoctocog alfa, rFVIII, or vehicle solution, 5–10 min before exposing the saphenous vein and performing the three-hit laser-induced injury procedure described above.

2.1.4.3 | *Statistical analysis*

Platelet accumulation over time and platelet kinetic parameters are shown as mean ± SEM. Analysis of platelet kinetics in WT and hemophilia A mice was performed by Mann-Whitney *t* test. Comparison of platelet kinetics in hemophilia A mice receiving vehicle, rFVIII, or efanesoctocog alfa was performed by one-way analysis of variance.

3 | RESULTS

3.1 | Integrated fibrin formation and network structure assay

3.1.1 | Fibrin polymerization

Minimal fibrin formation occurred in FVIII-deficient plasma from patients with hemophilia A (HemA plasma group in Figure 2A). The addition of rFVIII (0.02–1.00 IU/ml) or efanesoctocog alfa (0.02–1.00 IU/ml) elicited substantially more fibrin formation (Figure 2A). Dose-dependent increases in protofibril lateral aggregation (onset

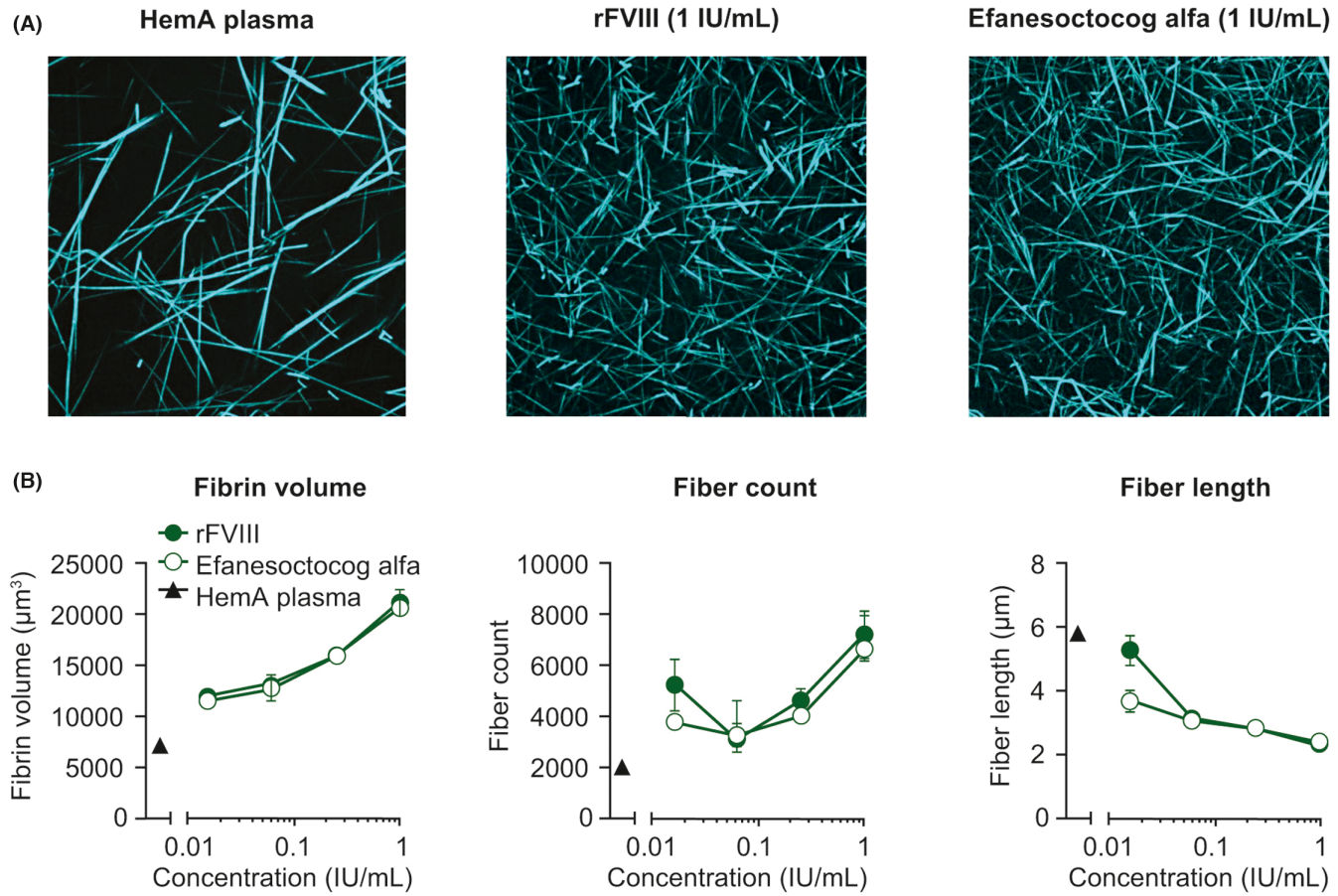

FIGURE 3 Fibrin network density in human hemophilia A plasma spiked with rFVIII or efanesoctocog alfa. rFVIII, recombinant factor VIII; SEM, standard error of the mean. (A) Representative of two-dimensional projections of z-stacks captured after measurement of fibrin polymerization by absorbance. (B) Mean fibrin network volume, mean fibrin fiber count, and mean fiber length as determined by ImageJ three-dimensional skeletonization. HemA plasma represents hemophilia A plasma alone. Data are mean ±SEM. Fiber count and fiber length are limited by inclusion in the analysis of fibers, which extend beyond limits of the field of view.

time), maximal polymerization rate, and change in fibrin network density (delta absorbance) were overlapping for rFVIII and efanesoctocog alfa, demonstrating that the kinetics of fibrin polymerization were similar for both products (Figure 2B).

3.1.2 | Fibrin network density

Fibrin networks formed using plasma from patients with severe hemophilia A were characterized by sparse and loosely arranged fibers (HemA plasma group in Figure 3A). When rFVIII and efanesoctocog alfa were added to the plasma, fibrin networks were denser compared with plasma alone (Figure 3A). Both factor products elicited similar dose-dependent increases in fibrin volume, fiber count, and a reduction in fiber length (Figure 3B).

3.1.3 | Clot stability (tPA challenge)

The clot formed using hemophilia A plasma was not stable in the presence of tPA resulting in only a small change in delta absorbance (HemA plasma group in Figure 4), whereas increasing concentrations of rFVIII and efanesoctocog alfa increased delta absorbance and AUC (Figure 4A,B). In contrast to the earlier experiment without tPA, neither factor achieved a maximal plateau absorbance (Figure 4). Instead, absorbance curves decayed and returned to baseline by 60–80 min. Clot stability in the presence of 0.5 μg/ml tPA was similar for both rFVIII and efanesoctocog alfa at the concentrations evaluated.

3.2 | Saphenous vein laser injury model

3.2.1 | Dynamics of clot formation

Repeat laser injury in WT and hemophilia A mice elicited platelet accumulation at the site of saphenous vein injury (Figure S2A). Peak platelet accumulation was similar between WT and hemophilia mice after the first injury (Figure S2A). In contrast, fewer platelets accumulated after the second and third injuries in hemophilia A versus WT mice. Platelet kinetics after the third injury as measured by time to maximal intensity, maximal intensity, slope,

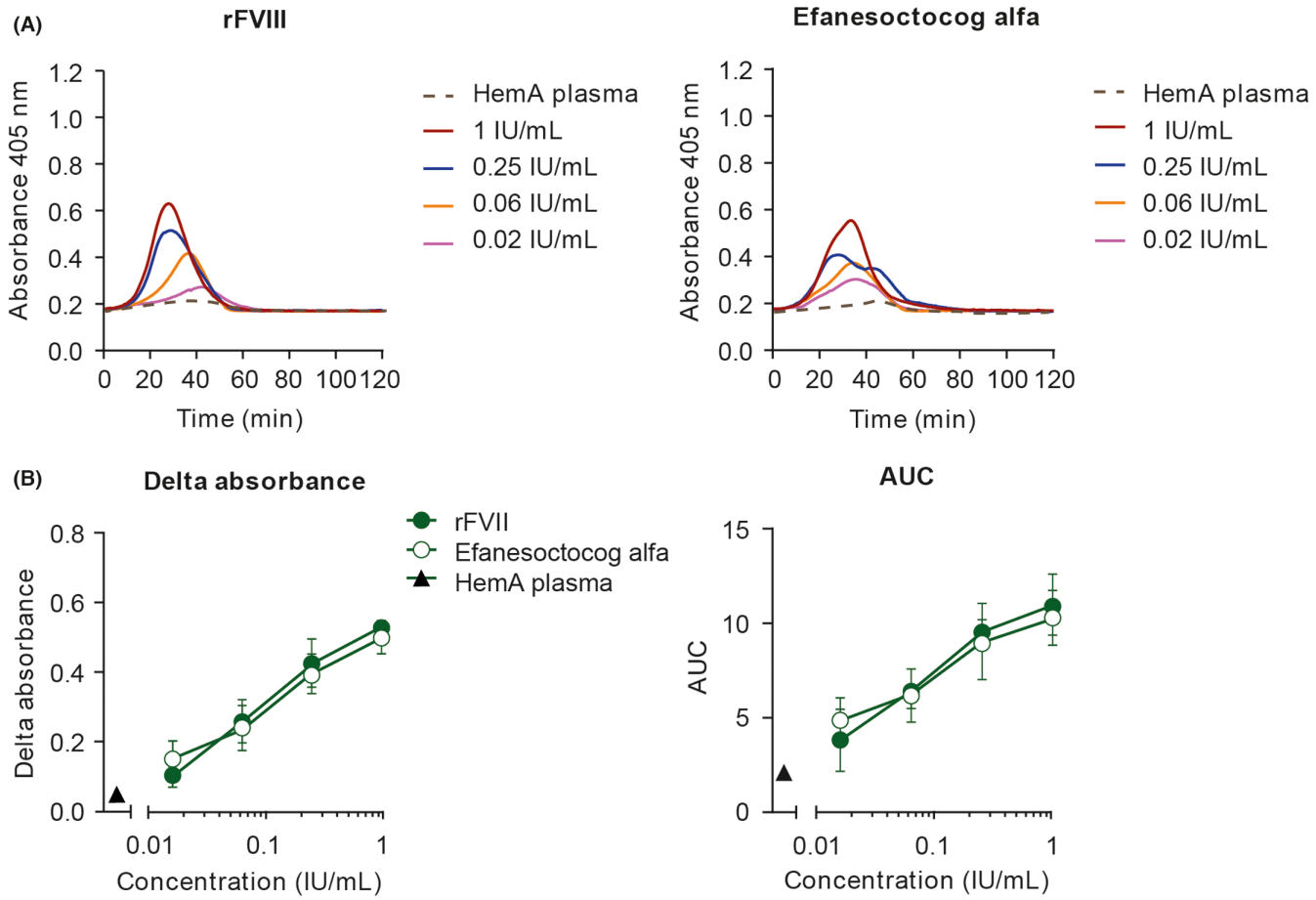

FIGURE 4 Fibrin clot stability in human hemophilia A plasma spiked with tPA and rFVIII or efanesoctocog alfa. rFVIII, recombinant factor VIII; SEM, standard error of the mean; tPA, tissue plasminogen activator. (A) Mean fibrin polymerization and lysis curves of rFVIII or efanesoctocog alfa ($n = 3$ per concentration, run in duplicate). (B) Parameters of clot stability, representing both polymerization (onset and rate, data not shown) and resistance to lysis (delta OD, area under the curve [AUC]). Data are mean ± SEM. Baseline (dotted line) represents hemophilia A plasma alone.

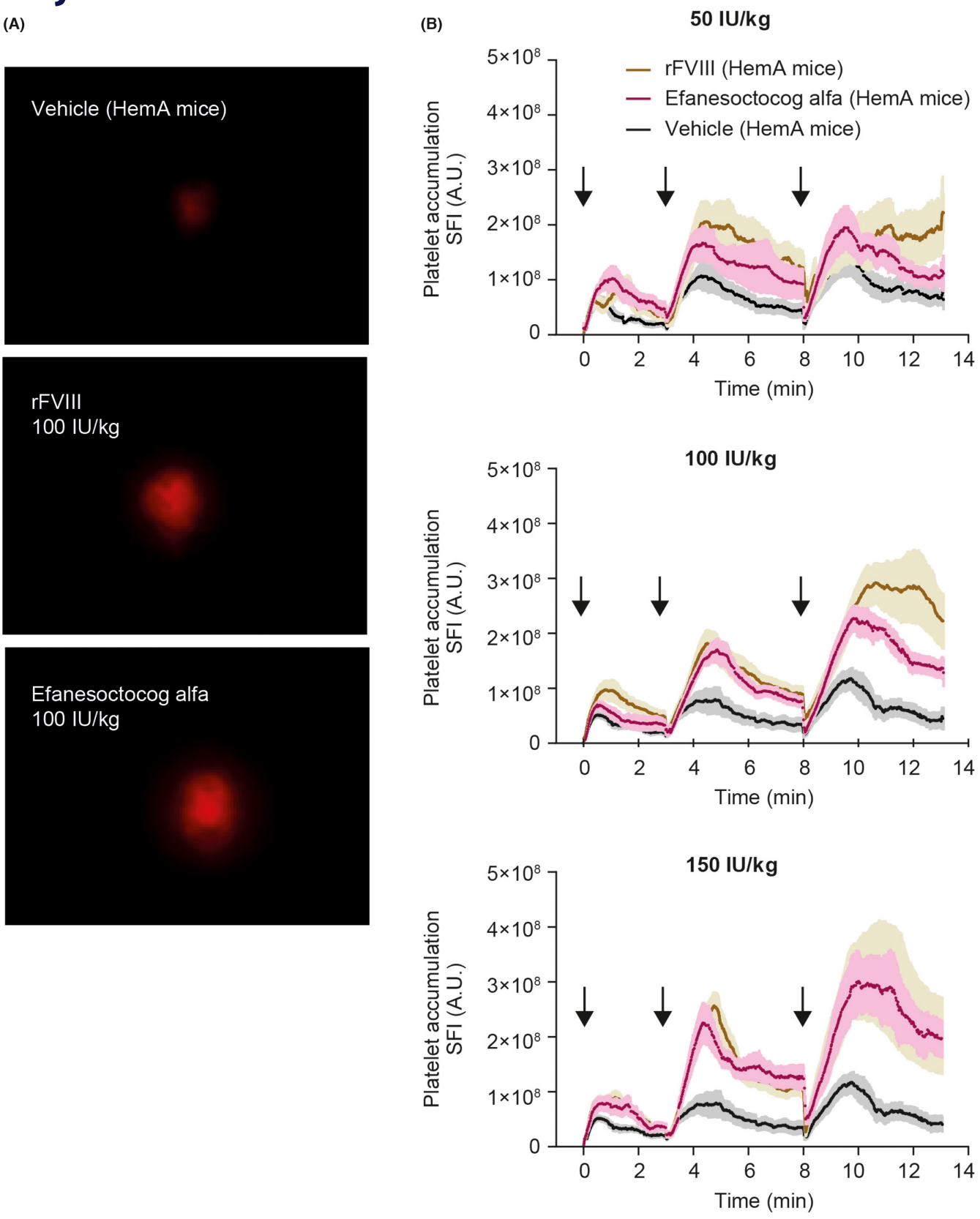

FIGURE 5 Platelet accumulation after three-hit saphenous vein laser injury in hemophilia A mice dosed acutely with rFVIII or efanesoctocog alfa. AU, arbitrary units; HemA, hemophilia A; rFVIII, recombinant factor VIII; SEM, standard error of the mean; SFI, sum fluorescence intensity. (A) Representative images of platelet deposition taken at the end of the third injury in hemophilia A mice given 100 IU/kg of rFVIII or efanesoctocog alfa, or vehicle (scale 50 μm). (B) Platelet accumulation over time in hemophilia A mice given one of three doses of rFVIII or efanesoctocog alfa, or vehicle. Results are shown as mean ± SEM (n = 3–5 mice; 8–14 injuries per group).

and AUC were significantly impaired in hemophilia mice compared with WT (Figure S2B).

3.2.2 | Effect of efanesoctocog alfa and rFVIII on platelet accumulation

Platelet accumulation was higher in hemophilia A mice dosed with rFVIII or efanesoctocog alfa compared with vehicle at all doses tested (Figure 5). Compared with vehicle, both rFVIII and efanesoctocog alfa showed a similar increase of the maximal intensity, slope, and AUC of platelet accumulation becoming significant at the 150 IU/kg doses suggesting that platelet kinetics were similar for both products (Figure 6).

4 | DISCUSSION

Efanesoctocog alfa (rFVIIIFc-VWF-XTEN; BIVV001) is a novel fusion protein designed to provide high sustained FVIII activity levels and extended hemostatic control in patients with hemophilia A, with a FVIII activity half-life of up to four times longer (ie, >40 h) than that for standard half-life FVIII products.[13,21] We have demonstrated that the hemostatic potential of efanesoctocog alfa is indistinguishable from equivalent doses of rFVIII based on similar fibrin formation kinetics and network structure, and injury-induced platelet deposition in hemophilia A mice. These findings corroborate previous findings[13] and confirm the potency assignment of efanesoctocog alfa.

High sustained FVIII activity levels are needed for more optimal bleed protection and to improve joint health outcomes. Products

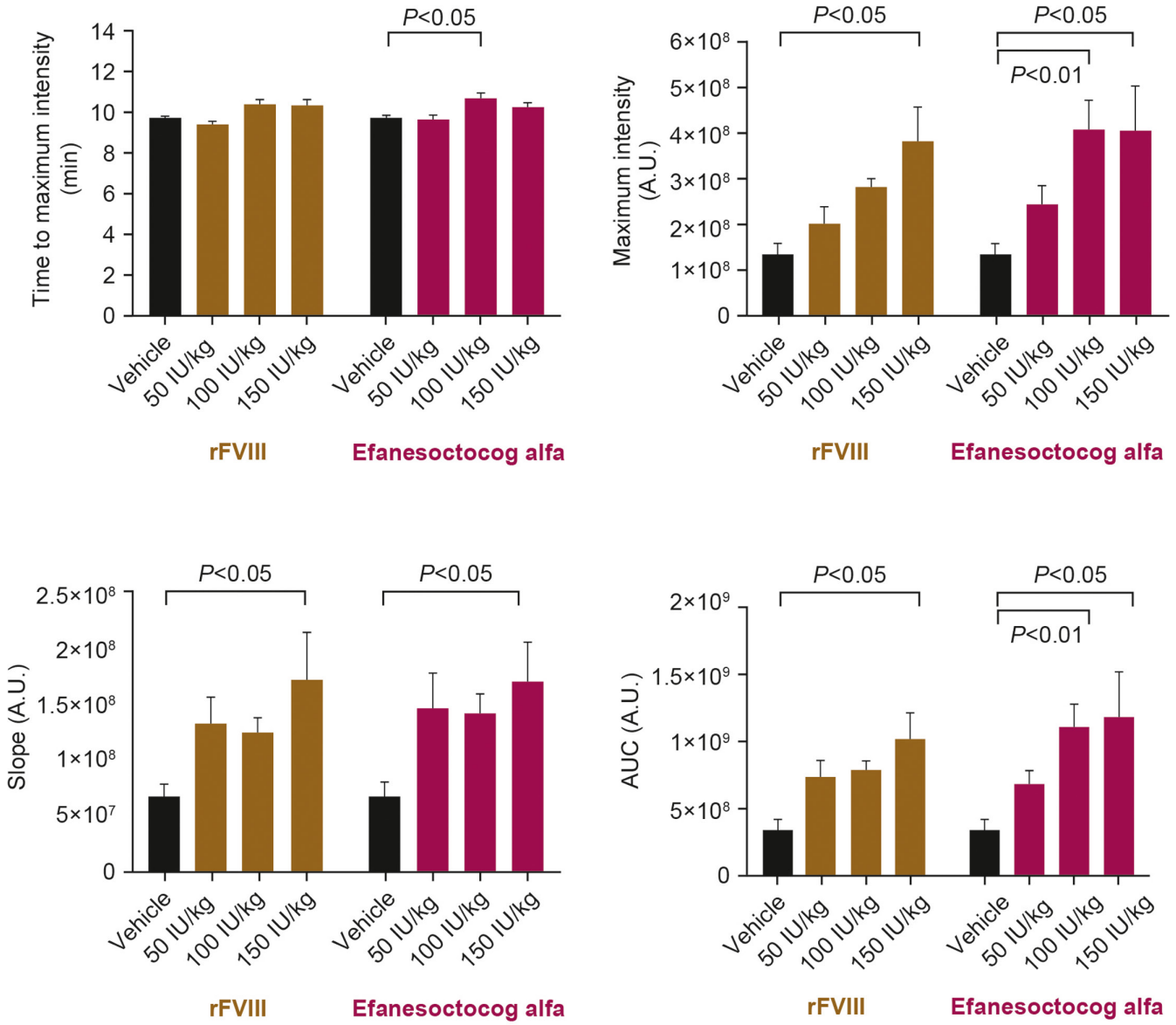

FIGURE 6 Kinetics of platelet accumulation after three-hit saphenous vein laser injury in hemophilia A mice dosed acutely with rFVIII or efanesoctocog alfa. AU, arbitrary units; AUC, area under the curve; rFVIII, recombinant factor VIII; SEM, standard error of the mean. Platelet kinetics of the third injury in hemophilia A mice given one of three doses of rFVIII or efanesoctocog alfa, or vehicle. Results are shown as mean ± SEM (n = 3–5 mice, 8–10 injuries per group). Statistical analysis by one-way analysis of variance.

that provide both high sustained FVIII activity levels and extended dosing intervals also can reduce patient treatment burden.[23] To date, strategies to extend the half-life of FVIII replacement products have achieved half-life increases of approximately 1.5-fold over standard half-life products. This is in part because recombinant FVIII half-life is constrained by the interaction of FVIII with endogenous VWF.[11] Efanesoctocog alfa is a new class of FVIII replacement designed to circulate independently of endogenous VWF, breaking the VWF-imposed half-life ceiling, and thereby offering high sustained FVIII activity levels with once-weekly dosing.[13,21]

Several innovations were used to extend the half-life of efanesoctocog alfa. These include the addition of XTEN® polypeptides, fusing rFVIII to dimeric Fc to utilize the neonatal Fc receptor (FcRn)-mediated recycling pathway, and covalently attaching the rFVIII-Fc fusion protein with the D′D3 domain of VWF to stabilize rFVIII in the circulation and prevent binding to endogenous VWF.[17–19] Efanesoctocog alfa potency is assigned using the activated partial thromboplastin time based one-stage clotting assay. Preclinical efficacy experiments have shown that efanesoctocog alfa potency assignment by the one-stage assay correlates well with its *in vivo* efficacy and is therefore therapeutically relevant.[13]

To evaluate the quality of clots formed in the presence of either efanesoctocog alfa or rFVIII, we assessed fibrin polymerization kinetics, fibrin network density, and clot stability. Efanesoctocog alfa increased fibrin polymerization, network density, and clot stability in human hemophilic plasma in a manner similar to rFVIII. A flattening of the lysis curve for efanesoctocog alfa relative to rFVIII was noted for the assessment of clot stability (Figure 4A). However, this is most likely the result of variability inherent to the tPA assay, rather than a reflection of differences in the clot stability profiles of the two molecules. These data are consistent with prior studies involving the thrombin generation assay and rotational thromboelastometry and confirm that the one-stage clotting assay is appropriate for efanesoctocog alfa potency assignment.[13] Additionally, our results suggest that the *in vitro* fibrin polymerization assay can be used as a tool to evaluate the procoagulant activity of traditional and novel clotting factors.

The quantification of platelet adhesion by intravital microscopy after repeat laser-induced injuries in the saphenous vein was first described by Getz et al.[22] The initial laser-induced injury on the surface of the endothelium results in tissue factor exposure, which then initiates the coagulation cascade resulting in thrombin generation, platelet adhesion and activation. The second and third injuries amplify the coagulation process, leading to accumulation of activated FVIII, FIX, and FX and the propagation of platelet activation, deposition, and clot formation.[24] Our results indicate that this model can be used to evaluate the role of FVIII in clot formation. Efanesoctocog alfa had similar efficacy to rFVIII in increasing platelet deposition in hemophilia A mice. Overlapping platelet kinetic parameters between the two factor products suggest that the association of rFVIII with endogenous VWF may not be essential for efficient clot formation. In addition, these data demonstrate that appending the D'D3 domains of VWF to rFVIII-Fc in efanesoctocog alfa does not interfere with recruitment to and activity of rFVIII at the site of injury. Additionally, once efanesoctocog alfa is activated by thrombin, the released D'D3-XTEN moiety does not interfere with any aspect of clot formation or platelet deposition.

In conclusion, these results show that efanesoctocog alfa has similar hemostatic potential to rFVIII in promoting both fibrin clot formation in plasma samples from patients with hemophilia A and platelet propagation at the site of laser injury in hemophilia A mice. When the potency of efanesoctocog alfa was assigned by the one-stage clotting assay, functional clot formation with efanesoctocog alfa was indistinguishable from that with rFVIII. The efficacy, safety, and tolerability of efanesoctocog alfa in patients with severe hemophilia is currently being evaluated in ongoing Phase 3 studies (XTEND-1 [NCT04161495], XTEND-Kids [NCT04759131], and XTEND-ed [NCT04644575]).

ACKNOWLEDGMENTS

This research was funded by Sanofi and Sobi. Medical writing and editorial assistance were provided by Francis John Golder, BVSc PhD and Timothy Davies, PhD, of Fishawack Health, and was funded by Sanofi and Sobi. Efanesoctocog alfa is codeveloped by Sanofi and Sobi. Both Sanofi and Sobi reviewed the manuscript during its development. The research reported in this manuscript was presented at the International Society on Thrombosis and Haemostasis (ISTH) Congress, July 8–13, 2017; Berlin, Germany.

CONFLICT OF INTEREST

All authors were employees of Sanofi at the time of the study and may hold shares and/or stock options in the company.

AUTHOR CONTRIBUTIONS

Contribution: M.D. and M.M.A. both contributed equally; M.D., M.M.A, E.K performed experiments; M.D., M.M.A, E.K, E.S.C, J.S analyzed results and M.D., M.M.A, E.K, made the figures; M.D., M.M.A, E.S.C, J.S designed the research; all authors reviewed the draft manuscript and approved the final version for submission.

ORCID

Melanie Demers https://orcid.org/0000-0002-3810-2008
Ekta Seth Chhabra https://orcid.org/0000-0002-0378-0909

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SUPPORTING INFORMATION

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How to cite this article: Demers M, Aleman MM, Kistanova E, Peters R, Salas J, Seth Chhabra E. Efanesoctocog alfa elicits functional clot formation that is indistinguishable to that of recombinant factor VIII. *J Thromb Haemost*. 2022;20:1674–1683. doi:10.1111/jth.15741