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ORIGINAL ARTICLE

Defective lymphangiogenesis and iron removal after hemarthrosis in factor VIII-deficient mice are rectified with therapeutic factor VIII administration—implications for joint health

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Abstract

Background: Maladaptive lymphangiogenesis after hemarthrosis in factor(F)VIII-deficient (knockout [KO]) mice facilitates synovial iron accumulation.

Objectives: To investigate the effect of FVIII treatment on lymphangiogenesis, iron clearance, and joint health after hemarthrosis.

Methods: Two days after knee injury/bleed (subpatellar needle puncture) FVIII-KO mice were separated into 3 groups receiving (1) intravenous saline, (2) recombinant human FVIII for 2 days, or (3) murine (m)FcFVIII for 14 days. FVIII activity levels were measured repeatedly (peak/trough) for 14 days. Joint tissues were processed at 2 and 4 weeks postbleed for Prussian blue staining (iron), CD68 (macrophage), αSMA (vascular remodeling), and LYVE1 (lymphangiogenesis) immunohistochemistry, and Safranin-O-Green staining (cartilage health).

Results: Joint injury caused profound hemarthrosis. Mice treated with mFcFVIII maintained stable FVIII activity levels for 14 days (troughs 29%-38%). Pronounced synovial iron accumulation colocalizing with macrophages, along with severely impaired lymphangiogenesis and joint health parameters, were present in saline-treated mice at 2 and 4 weeks when compared with baseline. Short-term recombinant human FVIII administration resulted in partially impaired lymphangiogenesis and iron clearance with delayed recovery of joint health parameters. In contrast, mice treated with mFcFVIII experienced rapid iron clearance alongside normal lymphangiogenesis, associated with fast and effective normalization of joint health parameters, particularly with respect to cartilage health.

Conclusion: Prolonged FVIII availability in the “mild hemophilia range” (± Fc-mediated effects) after hemarthrosis seem critical for lymphangiogenesis, rapid iron removal, and joint repair, including glycosaminoglycan-dependent cartilage restoration.

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Intensified courses of FVIII treatment in patients may therefore be beneficial for postbleed management.

KEYWORDS
arthropathy, factor VIII, hemarthrosis, hemophilia, hemosiderin, inflammation, iron, lymphangiogenesis, macrophages

1 | INTRODUCTION

Hemophilia A is characterized by a lack of functional endogenous factor (F)VIII. This genetic condition occurs in roughly 1 in 10 000 live births, affecting individuals across various races and ethnicities [1]. Severe FVIII deficiency leads to unprovoked bleeding within the joints, resulting in persistent pain, joint deterioration, and disability that remain unpreventable even with the latest treatment options. Hemarthroses lead to progressive hemophilic arthropathy, which is marked by synovial inflammation, hypervascularity, and osteochondral damage [2–4].

Iron deposition due to blood breakdown is thought to be the incendiary of all processes leading to hemophilic arthropathy, including vascular remodeling and rebleeding [5–7], resulting in the production of catabolic enzymes and cytokines that damage cartilage and bone [8]. Synovial hemosiderin accumulation is frequently observed in individuals with hemophilia despite best bleed prophylaxis and probably plays a significant role in creating a toxic environment [9,10]. Hemosiderin can also accumulate in cartilage and subchondral cysts, which is associated with unfavorable joint health outcomes [4,10] as oxidative stress induced by iron contributes to chondrocyte apoptosis and disrupts osteoblast function [11].

Emerging evidence indicates that synovial iron accumulation after joint bleeding may be due to maladaptive lymphangiogenesis in FVIII deficiency. We previously demonstrated that accumulation and delayed clearance of iron-laden macrophages were associated with defective lymphangiogenesis after induced hemarthrosis in FVIII-deficient (FVIII-knockout [KO]) mice, which could be prevented with periprocedural FVIII prophylaxis. We also demonstrated that FVIII is necessary to restore lymphangiogenesis and remove iron-laden macrophages in mice made transiently hypocoagulable by FVIII inhibition and warfarin administration (^{Hypo}Bagg Albino Laboratory-Bred Mouse, substrain c [BALB/c] mice) after induced hemarthrosis with a large bleed volume [12].

Here, we sought to expand this line of investigation to determine the effect of FVIII treatment on lymphangiogenesis and iron clearance when administered after induced hemarthrosis during hemarthrosis resolution in FVIII-KO mice during the healing/recovery phase. Two FVIII preparations were employed, recombinant human (rh)FVIII and murine (m)FcFVIII, to elucidate the effects of more sustained FVIII plasma levels afforded by Fc half-life extension [13] vs peak and trough levels expected with rhFVIII [14]. We assumed that a prolonged phase of sustained FVIII availability (rather than short-term treatment) would be necessary based on previous observations delineating rescue effects with sustained FVIII availability during the healing phase [12]. Furthermore, we determined if prompt synovial iron clearance is meaningful for murine joint health.

2 | MATERIALS AND METHODS

2.1 | FVIII-KO mice breeding and colony maintenance

FVIII-deficient mice (FVIII-KO) with a BALB/c genetic background were provided by David Lillicrap (Queen's University, Kingston, Ontario, Canada) [15] and were subsequently bred at the University of California San Diego (UCSD). The breeding colonies were generated according to the guidelines and protocols of the Institutional Animal Care and Use Committee (IACUC) at UCSD, and the colonies were maintained with *ad libitum* food and water.

2.2 | Hemarthrosis in FVIII-KO mice

Skeletally mature (12-16 weeks old) FVIII deficient mice of both sexes (male and female) were used for all knee injury experiments. Hemarthrosis was induced by subpatellar puncture of the right knee with a 30-gauge needle, as previously described [5,7,16]. The extent of intra- and periarticular bleeding was inferred by the decrease in hematocrit 2 days after injury. Briefly, a small volume of blood was collected in heparinized capillary tubes, centrifuged, and the hematocrit was determined using a microhematocrit reader [5,7,16]. Subcutaneous injections of 400 μL saline were administered daily after injury for 2 days for fluid resuscitation. Before the joint injury, mice received intravenous saline (100 μL) and subcutaneous administration of Ethiqa (3.25 mg/kg) (Fidelis Animal Health) for pain control.

2.3 | FVIII treatment after hemarthrosis

On day 2 postinjury, the mice were separated into 3 groups, receiving either no FVIII treatment (group 1) or treatment with rhFVIII (group 2) or mFcFVIII (group 3), as depicted in Supplementary Figure S1. Group 1 (control) received 1 more dose of subcutaneous saline, whereas treatment with intravenous FVIII preparations was started

for group 2: rhFVIII (120 IU/kg) and group 3: mFcFVIII (100 IU/kg; to account for half-life prolongation and murine Fc species specificity) [17,18] on alternate days for an anticipated 7 doses (= 14-day treatment period).

2.4 | Blood collection and processing during the 14-day treatment period with FVIII preparations

To determine FVIII plasma activity levels and potential inhibitory antibody formation during the 14-day (2-week) treatment period after hemarthrosis, blood samples (75-100 μL) were collected retro-orbitally 10 minutes before and 10 minutes after FVIII infusion to evaluate trough and peak FVIII plasma activity levels. To minimize blood collection frequency for individual mice, one set of mice was used for trough and another set for peak collections only. Sampling was limited to 4 doses of FVIII after hemarthrosis because of IACUC animal welfare restrictions on frequency and volume of blood sampling. All samples were collected into 3.8% sodium citrate (1:9 volume dilution), centrifuged at 2000*g* for 10 minutes and 13,500*g* for 5 minutes to obtain platelet-poor plasma. All plasma samples were stored at −80 °C until analysis. FVIII chromogenic assays were performed using the Diapharma kit (Diapharma) according to the manufacturer's protocol.

2.5 | Inhibitor testing—clotting assay

To determine the presence of inhibitor development in those samples that had no FVIII activity, we performed serial plasma dilution studies (1:1 mix). The plasma samples were serially diluted with normal BALB/c baseline plasma (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32), and the activated partial thromboplastin time (APTT) was determined by mixing 25 μL diluted plasma with 25 μL APTT reagent (Diagnostica Stago) as previously described [19,20]. Additionally, mixing studies were performed in the peak plasma samples from mFcFVIII-treated mice 6 and 8 days after hemarthrosis.

2.6 | Knee joint tissue harvest, Perl's Prussian blue staining, and decalcification

Knee joint tissues harvested at 2 or 4 weeks after hemarthrosis were fixed in 10% zinc-buffered formalin (Z-Fix; Anatech), Prussian blue stained, decalcified, processed, and paraffin embedded as previously described [12]. The paraffin-embedded tissues were sectioned to a thickness of 4 μm, deparaffinized, and mounted using Refrax mounting medium (Anatech). The images were captured on a NanoZoomer 2.0-HT brightfield slide scanner at a magnification of 20× (Hamamatsu Photonics). The quantification of Prussian blue staining in synovial tissue was performed using ImageJ software (National Institutes of Health). Prussian blue staining was determined in synovial and stromal tissue between femoral and tibial growth plates [16].

2.7 | Immunohistochemical evaluation

The sectioned joints were stained for macrophage and macrophage-like synoviocyte marker CD68 (1:200 dilution) (rabbit polyclonal anti-CD68 [ab125212]; Abcam), vessel remodeling marker α-smooth muscle actin (αSMA) (1:400 dilution) (rabbit polyclonal anti-αSMA [ab5694]; Abcam), and the marker for lymphatic vessel endothelial receptor 1 (LYVE1) (1:100 dilution) (goat polyclonal anti-LYVE1 [AF2125]; R&D Systems). For CD68 and LYVE1 primary antibodies, the antigen retrieval step was carried out by incubating the tissues with pepsin solution (Abcam) at 37 °C for 10 minutes to unmask hidden or denatured target epitopes. Tissue sections were incubated with primary antibodies and were detected with ImmPress AP Horse AntiRabbit/AntiGoat IgG Polymer Detection Kits. The color was developed in solution from Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories). Slides were scanned using a NanoZoomer 2.0-HT brightfield slide scanner at 20× magnification. Quantification was determined using ImageJ [12]. The expression levels of Prussian blue, CD68, LYVE1, and αSMA were adjusted based on the soft tissue area and are represented as the fold difference relative to the median baseline value.

2.8 | Joint health—morphological analysis

Gross morphology, synovial hyperplasia, cartilage erosion, and hemosiderin deposition were visualized by Safranin-O-Green staining as previously described [5,7]. (Semi)quantitative scoring was performed using the modified Valentino scoring system (without vascularity assessment since assessed separately by αSMA) [21], assessing synovial hyperplasia, hemosiderin deposition, blood in the joint, villus formation, and cartilage erosion. Glycosaminoglycan (GAG) intensity (tibia and femur cartilage surfaces) was determined by Safranin-O-Green staining [5,22] and quantified using ImageJ software to determine GAG loss. Investigators (B.J.C., J.A.D.P.M., and A.v.D.) were blinded to the different groups. Differences in scores (a few scores, minor deviations) were reconciled in a group session (unblinded).

2.9 | Statistical analyses

Data are expressed as median plus IQR, and statistical comparisons were made using the nonparametric Mann–Whitney *U*-test. $P < .05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad).

3 | RESULTS

3.1 | Joint bleeding and plasma FVIII activity levels in FVIII-KO mice after induced hemarthrosis

Hemarthrosis was induced in the right knee joints of FVIII-KO mice by subpatellar injury. The median drop in hematocrit from 48% to

32% on day 2 postinjury indicated substantial intra and periarticular bleeding (n = 16-44; $P \leq .0001$) (Supplementary Figure S1). Mice were randomly assigned to 3 groups (n = 5-6 per group) 2 days after hemarthrosis induction. Group 1 received no treatment (saline), whereas groups 2 and 3 received rhFVIII or mFcFVIII, respectively, on alternate days for an anticipated period of 2 weeks (Supplementary Figure S1).

Mice treated with rhFVIII achieved a median FVIII plasma level of 46% shortly after the first dose, without noteworthy FVIII activity levels measurable before and after the second dose, indicating the development of inhibitory anti-FVIII antibodies ("inhibitors") (Figure 1A). Therefore, no additional doses of rhFVIII were administered. Serial plasma dilution studies (APTT, 1:1 mix) confirmed the presence of inhibitors with complete correction at a dilution titer of 1:16 (Supplementary Figure S2). In contrast, mice treated with mFcFVIII maintained stable trough (median 32%) and peak (median 41%) FVIII activity levels for the first 4 doses. Mixing studies with normalization of the APTT 6 and 8 days after hemarthrosis ruled out inhibitor formation (Supplementary Figure S3). Treatment was continued to complete 7 doses (14-day treatment period), although the continuous notable presence of FVIII could only be documented with certainty for an 8-day treatment period after hemarthrosis due to blood draw constraints (Figure 1B).

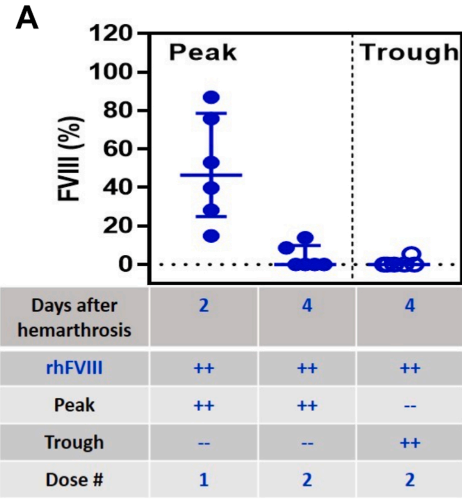
3.2 | Synovial hypertrophy and iron accumulation after hemarthrosis in FVIII-KO mice

We investigated the effect of FVIII availability during the healing process on synovial hypertrophy, inflammation, as well as accumulation and clearance of iron. Histological analysis of the harvested joint tissue revealed the presence of synovial hypertrophy 2 and 4 weeks after hemarthrosis. At 2 weeks, the synovial tissue area increased ~4-fold in mice treated with saline or rhFVIII compared with baseline, but only ~2-fold in mFcFVIII-treated mice ($P \leq .01$) (Supplementary Figure S4). Synovial hypertrophy remained unabated at 4 weeks in the saline-treated group, but (nearly) normalized to baseline in mice treated with rhFVIII or mFcFVIII ($P \leq .001$) (Supplementary Figure S4). These observations indicate a lesser extent and/or faster resolution of synovial hypertrophy with continuously detectable FVIII activity levels (mFcFVIII). However, it is noteworthy that even short-term availability of rhFVIII in the immediate postbleed period, prior to inhibitor development, resulted in late regression of synovial hypertrophy.

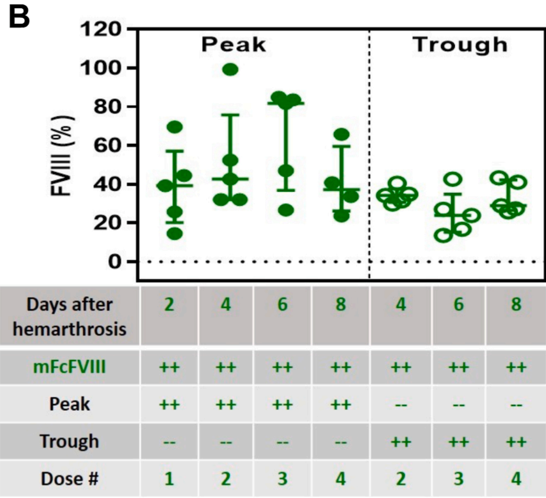
Prussian blue stain quantification demonstrated a significant increase in ferric iron accumulation in saline-treated mice at 2 weeks (94-fold; $P \leq .01$), with little improvement at 4 weeks (78-fold; $P \leq .0001$) compared with baseline (Figure 2A). In contrast, FVIII treatment facilitated iron clearance. Of note, prolonged treatment with mFcFVIII significantly reduced synovial iron accumulation at 2 weeks (16-fold; $P \leq .01$), with values returning to near baseline at 4 weeks (5-fold; $P \leq .01$) (Figure 2A). In contrast, brief treatment with rhFVIII reduced iron accumulation only partially (2 weeks, 54-fold; $P \leq .01$; 4 weeks, 48-fold; $P \leq .01$), with no further improvement at 4 weeks. These observations demonstrate that FVIII availability during hemarthrosis resolution is critical for synovial iron clearance. The distribution pattern of synovial iron changed over time, showing dispersion at 2 weeks and clustering at 4 weeks (Figure 2B).

3.3 | The relationship of synovial macrophage and iron accumulation after hemarthrosis in FVIII-KO mice

Synovial macrophage accumulation was assessed by CD68 expression in the 3 groups, 2 and 4 weeks after hemarthrosis, in association with Prussian blue staining.



Days after hemarthrosis	2	4	4
rhFVIII	++	++	++
Peak	++	++	--
Trough	--	--	++
Dose #	1	2	2



Days after hemarthrosis	2	4	6	8	4	6	8
mFcFVIII	++	++	++	++	++	++	++
Peak	++	++	++	++	--	--	--
Trough	--	--	--	--	++	++	++
Dose #	1	2	3	4	2	3	4

FIGURE 1 Chromogenic assay after FVIII treatment. FVIII-KO mice were randomly separated into 3 groups 2 days after induced knee hemarthrosis. Group 1 received saline, whereas groups 2 and 3 received rhFVIII (120 IU/kg) or mFcFVIII (100 IU/kg) on alternate days. Blood samples were collected 10 minutes before and 10 minutes after the FVIII infusion. Plasma FVIII activity levels were determined in the (A) rhFVIII and (B) mFcFVIII treatment groups (n = 4-6), and results are displayed in the graph. Error bars represent median values plus IQR. FVIII, factor VIII; KO, knockout; mFcFVIII, mouse-specific Fc-recombinant factor VIII; rhFVIII, recombinant human factor VIII.

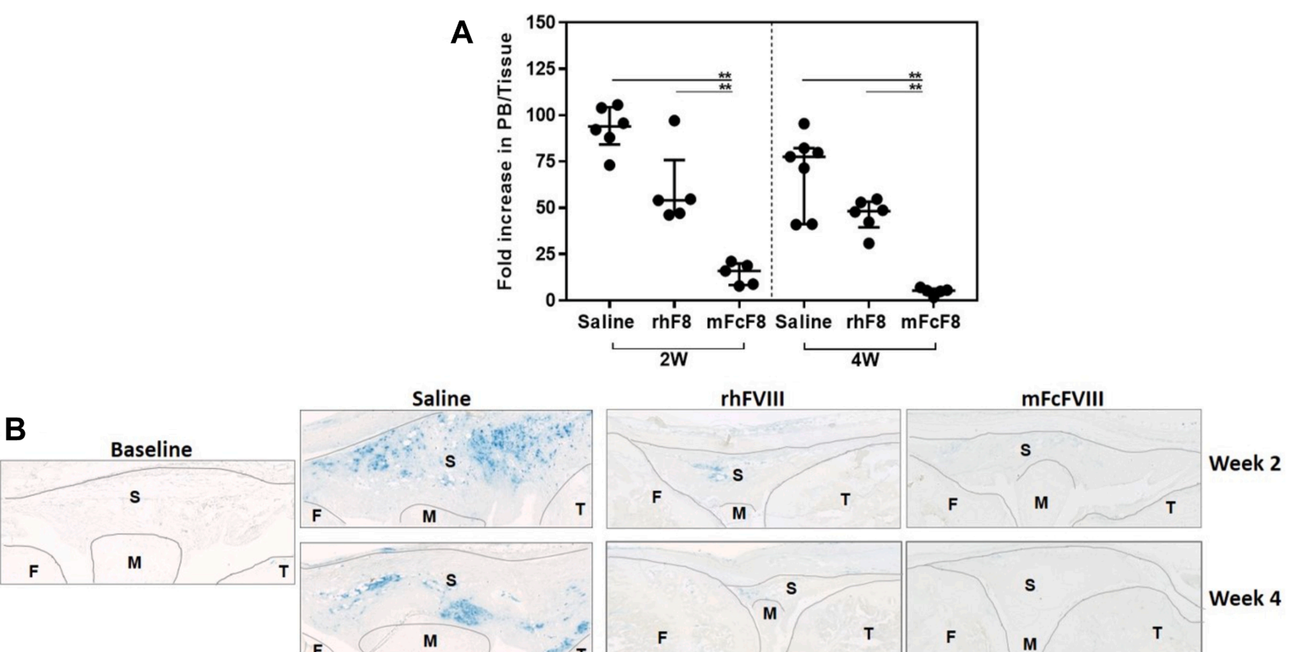


FIGURE 2 Ferric iron accumulation in synovial tissue after induced hemarthrosis and FVIII treatment. Joint tissue was harvested from mice at baseline, 2 weeks (2W), and 4 weeks (4W) after induced hemarthrosis (*n* = 5-7 per group). Mice were treated (treatment started 2 days after hemarthrosis induction) with saline, rhFVIII (120 IU/kg), or mFcFVIII (100 IU/kg) on alternate days (see Supplementary Figure S1). Paraffin-embedded tissue was sectioned (4 μm), deparaffinized, and imaged using a Hamamatsu NanoZoomer brightfield slide scanner at 20× magnification. Prussian blue staining was quantified in synovial tissue using ImageJ. (A) Synovial iron quantification is expressed as fold-increase compared with baseline (*n* = 5-7). Data are displayed as the median plus IQR (**$P \leq .01$). (B) Representative examples of changes in the distribution of synovial ferric iron over time. F, femur; M, meniscus; mFcFVIII/mFcF8, mouse-specific Fc-recombinant factor VIII; PB, Prussian blue; rhFVIII/rhF8, recombinant human factor VIII; S, synovium; T, tibia.

A significant increase in CD68 expression after hemarthrosis was observed in saline-treated mice at 2 weeks (268-fold; $P \leq .0001$), with improvement at 4 weeks (40-fold; $P \leq .001$) compared with baseline (Figure 3A). In contrast, FVIII treatment facilitated a reduction in CD68 expression at both time points, which was significantly more pronounced with mFcFVIII than with rhFVIII ($P \leq .01$) (Figure 3A). At 4 weeks, CD68 expression approached baseline levels with both FVIII preparations, but mFcFVIII reduced CD68 expression more profoundly than rhFVIII compared with baseline ($P < .05$). There was a strong positive correlation between synovial ferric iron and CD68 expression ($r = 0.8254$; $P \leq .0001$) (Figure 3B). Similar to synovial iron deposition, areas of CD68 expression were distributed throughout the synovium at 2 weeks, with clustering observed at 4 weeks, largely colocalizing with ferric iron deposits (Figure 3C). Together, these findings suggest a relationship between macrophage accumulation and ferric iron levels, with synovial clustering of iron-laden macrophages between 2 and 4 weeks postinjury and the need for FVIII availability for their clearance.

3.4 | Synovial lymphangiogenesis in relation to iron accumulation after hemarthrosis in FVIII-KO mice

We previously demonstrated that adequate lymphangiogenesis is required for removal of iron-laden macrophages [12]. Therefore, we examined and quantified the lymphatic endothelial cell marker, LYVE1, in FVIII-KO mice after hemarthrosis to determine the effects of FVIII treatments on lymphangiogenesis and synovial iron clearance.

In saline-treated mice, LYVE1 expression was reduced by 6-fold ($P \leq .0001$) compared with baseline at 2 weeks after hemarthrosis, without recovery at 4 weeks (8-fold; $P \leq .001$) (Figure 4A). LYVE1 expression in mice treated short-term with rhFVIII was comparable to the saline-treated group at 2 weeks (5-fold; $P \leq .001$), with some improvement at 4 weeks (3-fold; $P < .05$). In contrast, prolonged treatment with mFcFVIII maintained LYVE1 expression throughout the 4-week observation period (Figure 4A).

There were strong negative correlations between lymphatic vessel density and Prussian blue staining and between lymphatic vessel density and CD68 staining (r = approximately, −0.6; $P \leq .0001$) (Figure 4B, C). Representative examples of LYVE1 expression in relation to Prussian blue staining over the 4-week time course are displayed in Figure 4D. At baseline, LYVE1 expression revealed vessels appearing as small, incongruent structures. Later, lymphatic vessels evolved into longer, flat structures, which morphologically remained largely unchanged with short-term rhFVIII treatment. However, with prolonged mFcFVIII treatment, the structures returned to partial and full baseline appearance at 2 and 4 weeks, respectively (Figure 4D).

These findings suggest a profoundly maladaptive lymphatic response to injury-related hemarthrosis in FVIII-KO mice,

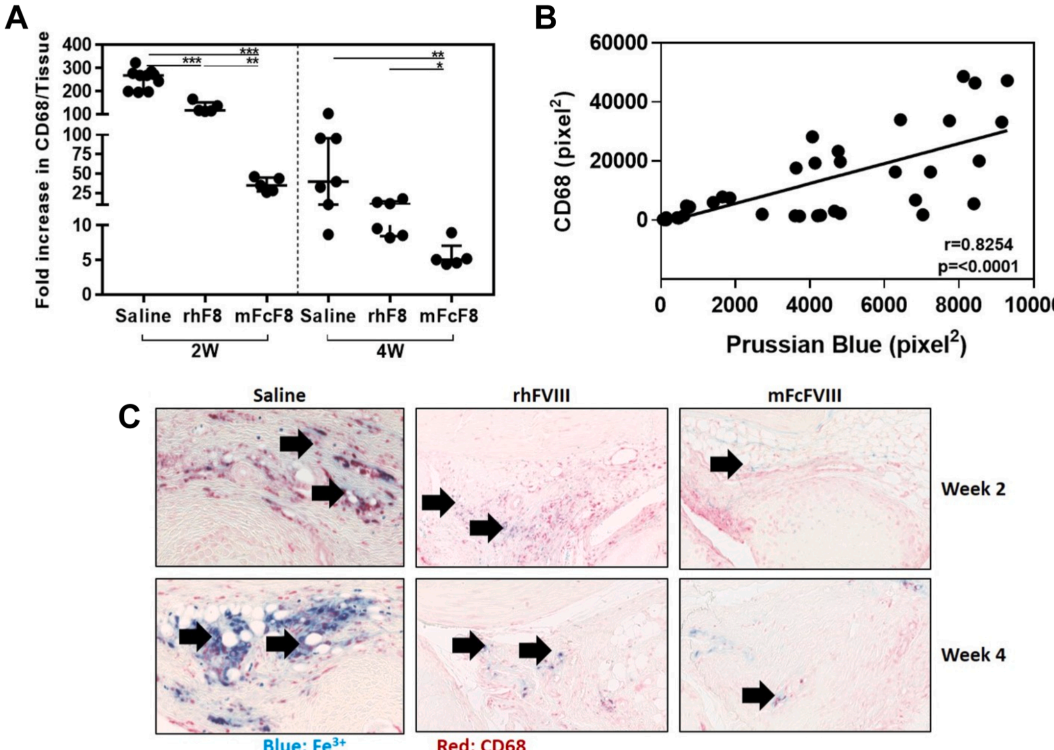


FIGURE 3 CD68 expression in synovial tissue after induced hemarthrosis and FVIII treatment. Joint tissue was harvested from mice at baseline, 2 weeks (2W), and 4 weeks (4W) after induced hemarthrosis (n = 5-7 per group). Mice were treated (treatment started 2 days after hemarthrosis induction) with saline, rhFVIII (120 IU/kg), or mFcFVIII (100 IU/kg) on alternate days (see Supplementary Figure S1). Paraffin-embedded tissue was sectioned (4 μm), deparaffinized, subjected to immunohistochemistry with an anti-CD68 antibody (red) and Prussian blue staining (blue) and imaged using a Hamamatsu NanoZoomer brightfield slide scanner at 20× magnification. (A) Synovial CD68 expression levels are expressed as fold-increase compared with baseline (n = 5-7). Data are displayed as the median plus IQR (***$P \leq .001$; **$P \leq .01$; *$P \leq .05$). (B) Spearman's rank correlation analysis of CD68 and Prussian blue staining (n = 41). (C) Representative images of synovium from FVIII-KO mice at 2 weeks (2W) or 4 weeks (4W) after hemarthrosis. Black arrows indicate areas of CD68 and Prussian blue colocalization. CD68, cluster of differentiation 68; KO, knockout; mFcFVIII/mFcF8, mouse-specific Fc-recombinant factor VIII; rhFVIII/rhF8, recombinant human factor VIII.

correctable with prolonged FVIII treatment during the hemarthrosis resolution and tissue restoration phase. Since synovial iron and macrophage accumulation were proportional to the impairment of lymphangiogenesis, the clearance of synovial iron appears contingent on FVIII-dependent lymphangiogenesis.

3.5 | Synovial and cartilage changes after hemarthrosis in FVIII-KO mice

To study vascular and cartilage changes associated with hemarthrosis in FVIII-KO mice, we performed morphological joint examinations using the Valentino score (omitting the vascularity parameter due to separate vascular staining with αSMA) [21]. Hemarthrosis resulted in pronounced synovial hyperplasia, increased hemosiderin deposition, cartilage erosion, and persistent presence of blood in the joint space at 2 weeks (median Valentino score 5 [maximum 7]), with partial resolution at 4 weeks (median Valentino score 2.5). Short-term treatment with rhFVIII after hemarthrosis resulted in similar scores (2 weeks, median Valentino score 4; 4 weeks, median Valentino score 2). In contrast, prolonged treatment with mFcFVIII resulted in significant improvement, already evident at 2 weeks (median Valentino score 1) and maintained at 4 weeks (Figure 5A).

Vascular remodeling was assessed by αSMA expression levels. Vascular remodeling increased significantly over baseline after hemarthrosis (14.6-fold; $P \leq .0001$) and persisted at 4 weeks (13.8-fold; $P \leq .001$). Short-term treatment with rhFVIII did not substantially affect this process at 2 weeks (15-fold; $P \leq .001$) but resulted in significant improvement at 4 weeks (2-fold; $P \leq .01$). In contrast, prolonged treatment with mFcFVIII resulted in early and significant improvement in vascular remodeling at 2 weeks (6.8-fold; $P \leq .01$), with further improvement at 4 weeks (1.6-fold; $P < .05$), similar to the effect of rhFVIII at 4 weeks (Figure 5B).

Cartilage GAG content decreased significantly after hemarthrosis ($P \leq .0001$) at 2 weeks but normalized at 4 weeks. Short-term treatment with rhFVIII did not improve GAG content at 2 weeks ($P \leq .01$) but augmented GAG content significantly above baseline at 4 weeks ($P \leq .01$). Prolonged treatment with mFcFVIII resulted in a similar "above baseline" augmentation already at 2 weeks ($P < .05$), with continuously high GAG content at 4 weeks ($P \leq .01$) (Figure 5C).

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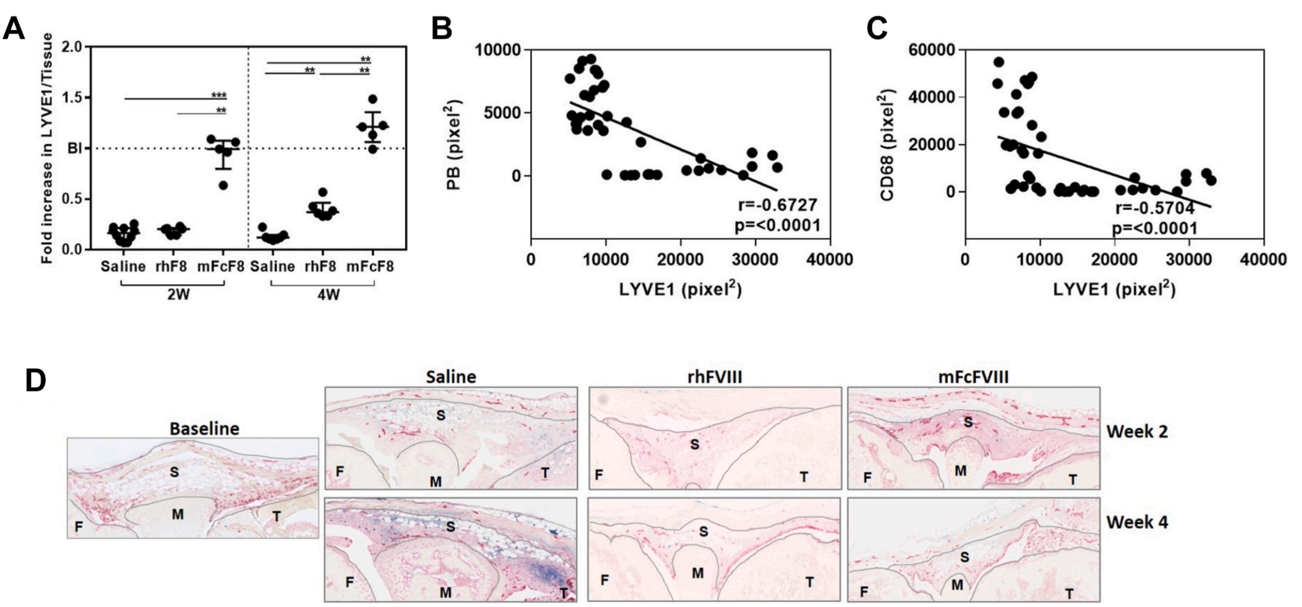

FIGURE 4 Lymphatic vessel density in synovial tissue after induced hemarthrosis and FVIII treatment. Joint tissue was harvested from mice at baseline, 2 weeks (2W), and 4 weeks (4W) after induced hemarthrosis (*n* = 5-7 per group). Mice were treated (treatment started 2 days after hemarthrosis induction) with saline, rhFVIII (120 IU/kg), or mFcFVIII (100 IU/kg) on alternate days (see Supplementary Figure S1). Paraffin-embedded tissue was sectioned (4 μm), deparaffinized, subjected to immunohistochemistry with an anti-LYVE1 antibody, and imaged using a Hamamatsu NanoZoomer brightfield slide scanner at 20× magnification. (A) Synovial LYVE1 expression levels are expressed as fold-increase compared with baseline (*n* = 5-7). Data are displayed as the median plus IQR (***$P \leq .001$; **$P \leq .01$). (B) Spearman's rank correlation analysis of LYVE1 and Prussian blue (PB) staining (*n* = 46). (C) Spearman's rank correlation analysis of LYVE1 and CD68 (*n* = 41). (D) Representative images of synovium from FVIII-KO mice at 2 weeks or 4 weeks after hemarthrosis stained with LYVE1. CD68, cluster of differentiation 68; F, femur; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1; M, meniscus; mFcFVIII/mFcF8, mouse-specific Fc-recombinant factor VIII; rhFVIII/rhF8, recombinant human factor VIII; S, synovium; T, tibia.

Cartilage erosions were determined with the Valentino scoring system [21]. At 2 weeks, erosions were only present in 1 in 5 (20%) of mice treated with mFcFVIII compared with mice treated with rhFVIII (5/6 [83.3%]) or saline (8/9 [89%]). At 4 weeks, the percentages of mice with erosions were similarly low between the FVIII treatment groups (mFcFVIII: 1/5 [20%] mice; rhFVIII: 2/6 [33.3%] mice), but remained higher in saline-treated mice (5/8 [63%]). Representative examples are depicted in Figure 5D.

Taken together, prolonged treatment with mFcFVIII afforded faster resolution of vascular remodeling and restoration of cartilage health, as well as early normalization of lymphangiogenesis and associated iron clearance, compared with short-term treatment with rhFVIII. However, at 4 weeks, morphological joint health parameters (synovial hyperplasia, vascular remodeling, and cartilage health) were similarly improved between the FVIII treatment groups despite persistent defective iron clearance with rhFVIII.

4 | DISCUSSION

Iron accumulation in the joint due to hemophilic joint bleeding is thought to ignite joint destruction [9,23]. Following hemarthrosis, iron deposited in the joint is absorbed by the synovial tissue, triggering an inflammatory response and the generation of reactive oxygen species. This promotes synovial proliferation and synovitis, ultimately leading to joint damage [24–26]. Recent studies in FVIII-KO mice revealed that iron accumulation in the joint may not only be a consequence of repeated joint bleeding, but also of impaired iron clearance [12]. We recently demonstrated that accumulation and delayed clearance of iron-laden macrophages were associated with defective lymphangiogenesis after hemarthrosis in FVIII-KO mice [12], preventable by periprocedural administration of FVIII [12]. In another mouse model (^{Hypo}BALB/c mice), the restoration of hemostatic capacity in wild-type BALB/c mice after induced hemarthrosis, made transiently "hypocoagulable" with anti-FVIII antibodies and warfarin [12], prevented lymphangiogenic dysregulation and the accumulation of iron-laden macrophages. These studies suggested that intact lymphatics are required for removal of iron-laden macrophages and that these processes depend on FVIII availability.

Here, we sought to expand our investigations to gain knowledge to what extent exogenous availability of FVIII during the postbleed healing phase in the FVIII-KO mouse may promote lymphangiogenesis and iron clearance. Furthermore, we explored effects of synovial iron accumulation on various joint health parameters including cartilage health.

As previously observed [12], FVIII-KO mice are unable to clear synovial iron-laden macrophages after induced hemarthrosis, which is associated with severely impaired lymphangiogenesis during the 4-week observation period. To determine if exogenous administration of FVIII after hemarthrosis could restore lymphangiogenesis and

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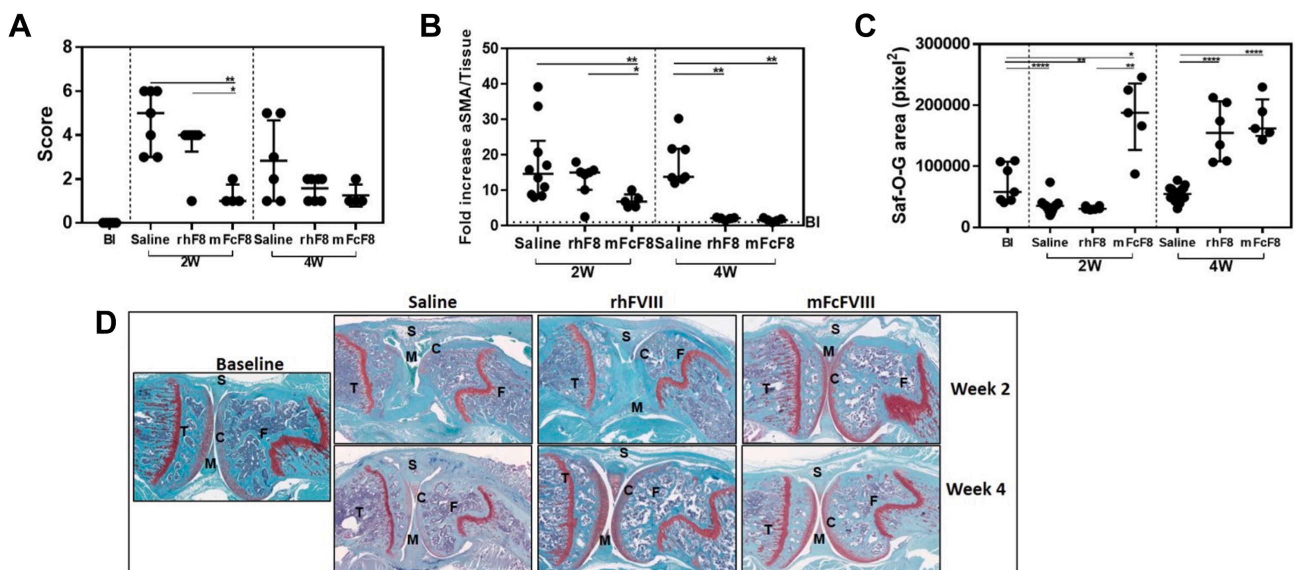

FIGURE 5 Soft tissue and cartilage changes after induced hemarthrosis in FVIII-KO mice. Joint tissue was harvested from mice at baseline, 2 weeks (2W), and 4 weeks (4W) after induced hemarthrosis (n = 5-19 per group). Mice were treated (treatment started 2 days after hemarthrosis induction) with saline, rhFVIII (120 IU/kg), or mFcFVIII (100 IU/kg) on alternate days (see Supplementary Figure S1). Paraffin-embedded tissue was sectioned (4 μm), deparaffinized, and stained with Safranin-O-Green. Another set of deparaffinized sections were stained with an anti-α-smooth muscle actin (αSMA) antibody and imaged using a Hamamatsu NanoZoomer brightfield slide scanner at 20× magnification. (A) Soft tissue damage was studied by histopathology using Valentino scoring (without vascularity assessment) after Safranin-O-Green staining (maximum score = 7). (B) Synovial αSMA expression levels are expressed as fold-increase compared with baseline. Data are displayed as the median plus interquartile range (****$P \leq .0001$; **$P \leq .01$; *$P \leq .05$). (C) Cartilage health was assessed by the intensity of Safranin-O-Green staining and quantified by ImageJ analysis of the cartilage area. (D) Representative images of synovium from FVIII-KO mice at 2 weeks or 4 weeks after hemarthrosis stained with Safranin-O-Green. Bl, baseline; C, cartilage; F, femur; KO, knockout; M, meniscus; mFcFVIII/mFcF8, mouse-specific Fc-fusion factor VIII; rhFVIII/rhF8, recombinant human factor VIII; S, synovium; Saf-O-G; Safranin-O-Green; T, tibia.

facilitate iron clearance, we randomized mice into 3 groups. Mice were treated either with saline (control) or with 1 of 2 different FVIII preparations (rhFVIII or half-life extended mFcFVIII), intended every other day for 2 weeks during hemarthrosis resolution, to study to what extent peak and trough levels (rhFVIII) vs continuous availability of FVIII (mFcVIII) may matter. However, mice injected with rhFVIII rapidly developed inhibitors after 1 to 2 doses with no further injections performed. Mice injected with mFcFVIII did not develop inhibitors and achieved continuously sustained FVIII levels at ∼30% to 40% for 2 weeks. A low tendency for inhibitor formation in mouse models with mFcFVIII was observed previously [27] and therefore proved beneficial for the design of this study. Because of inhibitor development, we switched course and delineated the effects of a short course of FVIII treatment (rhFVIII) on iron removal and joint health. Curiously, mFcFVIII showed little difference between peak and trough levels, which may be inherent to murine biology regarding interactions of Fc-fusion proteins with the neonatal Fc receptor [27,28]. Of note, a short course of FVIII treatment after hemarthrosis is the recommended approach by the World Federation of Hemophilia [29], but, to our best knowledge, without knowing the effects on joint health. We then compared short-term FVIII outcomes at 2 and 4 weeks with outcomes achieved with FVIII levels sustained over a 2-week time period (mFcFVIII).

Findings from this study clearly established that prolonged administration of mFcFVIII with continuous availability (∼30%-40% FVIII plasma levels for 2 weeks) resulted in rapid and complete clearance of iron-laden macrophages, associated with unabated lymphangiogenesis. These findings align with our previous observations in the ^{Hypo}BALB/c mouse demonstrating a lack of iron-laden macrophage accumulation and lymphangiogenesis impairment after hemarthrosis when warfarin was reversed and anti-FVIII antibody was cleared [12]. Strong negative correlations between lymphatic vessel density and Prussian blue staining and between lymphatic vessel density and macrophage accumulation further supported our contention that iron clearance is dependent on macrophage clearance via the lymphatics. Altogether, these findings suggest that restoration of hemostasis with FVIII during the healing phase is critical for synovial iron clearance in FVIII-KO mice and depends on lymphangiogenesis as a conduit for macrophage removal.

In terms of joint health, when compared with saline-treated mice, repeated injections of mFcFVIII also resulted in rapid and effective prevention/regression of profound synovial hypertrophy, vascular remodeling, cartilage defects, and achievement of low Valentino scores at 2 weeks, all maintained or further improved throughout the 4-week observation period. Of note, cartilage GAG formation became nearly undetectable 2 weeks after hemarthrosis in saline-

treated mice, with recovery to baseline 4 weeks later, revealing transiently severely impaired cartilage metabolism. GAGs are proteoglycans synthesized by chondrocytes that form the extracellular cartilage matrix, thereby contributing to structural integrity [30,31]. Similar findings from rabbit models demonstrated that intraarticular iron and blood components caused more severe cartilage and synovial pathology compared with plasma alone, implicating iron directly in tissue degeneration [24]. In contrast to saline-treated mice, however, repeated mFcFVIII injections not only corrected but significantly augmented GAG content above baseline at the 2- and 4-week time points, which was associated with substantial prevention/correction of cartilage erosions. Of note, cartilage erosions were present in saline-treated mice despite correction of GAG content. This observation indicates that normalization of GAG metabolism alone may not be sufficient to prevent lasting cartilage erosions and that FVIII, possibly because of rapid and complete synovial iron removal, is required to stimulate chondrocytes to boost GAG synthesis beyond baseline activity for cartilage repair after hemarthrosis.

Interestingly and unexpectedly, short-term treatment with rhFVIII (1-2 doses) also improved all joint health parameters (synovial hypertrophy, vascular remodeling, cartilage erosions, and Valentino scores). However, joint health repair was delayed compared with repeated injections of mFcFVIII, with improvements only becoming apparent at the 4-week timepoint. This included the GAG synthesis boost, which tracked late resolution of previous erosions, further establishing that stimulation of GAG metabolism is important for restoration of cartilage health during the postbleed period. However, in contrast to mFcFVIII, rhFVIII administration had no effects on iron-laden macrophage clearance and lymphangiogenesis correction at 2 weeks, with only partial improvements at 4 weeks. Therefore, a short course of rhFVIII was adequate to trigger hemarthrosis resolution and slow joint health recovery despite incomplete iron removal. These findings suggest a threshold of iron clearance is needed to achieve joint health restoration, which seems achievable rapidly and efficiently by prolonged and continuous exposure to FVIII in the “mild hemophilia range” (30%-40%).

Since mFcFVIII was used to achieve this prolonged and continuous availability of FVIII, potential immunomodulatory roles of the Fc domain must also be considered [32]. The neonatal Fc receptor (FcRn), which binds the Fc region of immunoglobulin (Ig) G in a pH-dependent manner [33], plays a critical role not only in extending IgG half-life [34] but also in modulating immune responses [35]. FcRn is widely expressed in adult epithelial, endothelial, and hematopoietic cells and functions in antigen presentation, phagocytosis regulation, and cytokine production [36–38]. Involvement of FcRn in class I and II human leukocyte antigen-processing pathways has been shown to enhance T cell activation, leading to B-cell response and IgG production [38], particularly in autoimmune and inflammatory conditions [39]. Thus, the Fc domain of mFcFVIII may have contributed to the anti-inflammatory and reparative environment observed in our study by engaging FcRn on immune and stromal cells, potentially improving chondrocyte recovery, lymphatic remodeling, and macrophage trafficking. Support for this hypothesis is also provided by previous synovial gene expression studies, where mFcFVIII influenced reparative processes after hemarthrosis differently from rhFVIII [40]. However, further investigation of such direct effects will require separate studies.

Observations from this study may be informative for clinical practice, where it is desirable to achieve fast resolution of symptoms after hemarthrosis (pain, swelling, and range of motion deficits). While we recognize that experimental observations in a murine model may not translate directly into human practice, extension of FVIII treatment beyond the usual 1 to 2 doses after hemarthrosis may have positive effects on iron removal and joint health restoration. Sometimes this strategy is pursued as “short-term prophylaxis” in regions without access to more permanent prophylaxis for persons with hemophilia [41,42] and is considered helpful to avoid rapid rebleeding and target joint formation [43,44]. Noteworthy in this context is the regression of synovial neoangiogenesis and vascular remodeling as a nidus for “vascular (re)bleeding” since vessels undergoing remodeling are fragile and leaky [4,5,7,16,45,46].

Although iron accumulation after a single FVIII-treated bleeding episode does not seem to cause immediate joint damage, persistent intraarticular iron loading of hemophilic joints is associated with worse clinical and imaging outcomes [4,10]. It is generally accepted that plasma FVIII activity levels $>15\%$ are protective against spontaneous joint bleeding [47,48], and it is therefore plausible that similar thresholds (mild hemophilic range) are applicable to iron removal and joint health restoration as well.

This study has several limitations. First, the number of mice per experimental group was relatively low ($n = \sim$4-7). While more mice in each group would have been preferable to solidify results, IACUC guidelines strongly recommend to limit the use of experimental animals to the minimum number required to show a biological effect. Second, the observation period was restricted to 4 weeks. Additional timepoints to determine time needed for full resolution of pathological findings may have provided insights regarding kinetics of restoration of lymphangiongenesis and synovial iron removal after short-term administration with rhFVIII. Third, we did not investigate effects of short-term administration of mFcFVIII on lymphangiogenesis and iron clearance and to what extent results would have differed from rhFVIII. This is an interesting question for future studies since it may reveal Fc-related effects on joint health. Fourth, it remains unclear to what extent inhibitor formation in mice treated with rhFVIII influenced joint health parameters.

5 | CONCLUSIONS

FVIII is critical for lymphangiogenesis and intraarticular iron removal in FVIII deficiency after hemarthrosis. However, it remains uncertain if these effects are due to improved hemostasis alone or if FVIII has specific effects in areas such as inflammation, immunogenicity, and tissue repair, which may be influenced further by the Fc-component [40]. To that end, it remains to be studied if these findings are

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relevant to hemophilia B (where FIX is the missing clotting protein) or to what extent restoration of hemostasis with new factor-less rebalancing agents [49–51] or gene therapy [52–54] would influence these processes.

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AUTHOR CONTRIBUTIONS

B.C.J. performed the experimental work and contributed to data analysis, interpretation, and manuscript drafting. J.A.D.P.M. contributed to ImageJ and statistical analysis, N.F. and M.L.C. performed the experimental work. A.v.D. provided concept and study oversight, and contributed to data analysis, interpretation and manuscript drafting.

DECLARATION OF COMPETING INTERESTS

A.v.D. has received honoraria for participating in scientific advisory board panels, consulting, and speaking engagements for Biomarin, Regeneron, Pfizer, Bioverativ/Sanofi, CSL-Behring, Novo Nordisk, Spark Therapeutics, and Genentech. A.v.D. is a cofounder and member of the Board of Directors of Hematherix LLC, a biotech company that is developing ^{super}FVa therapy for bleeding complications. B.C.J., J.A.D.P.M., N.F., and M.L.C. have no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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