Received: 3 February 2017 | Accepted: 1 July 2017

DOI: 10.1002/rth2.12034

ORIGINAL ARTICLE

WILEY rpth research and practice in thrombosis and haemostasis | ISTH

Clot stability as a determinant of effective factor VIII replacement in hemophilia A

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Funding information
NIH; NHLBI, Grant/Award Number: UO1-HL116330; Engineering and Physical Sciences Research Council, Grant/Award Number: EP/C513037/1; Bayer

[Correction added on August 18, 2017 after first online publication on August 10, 2017: Dr. Chandrasekaran Nagaswami's education qualification was corrected from MS to MD.]

Abstract

Background: Factor VIII (FVIII) replacement is standard of care for patients with hemophilia A (HemA); however, patient response does not always correlate with FVIII levels. We hypothesize this may be in part due to the physical properties of clots and contributions of fibrin, platelets, and erythrocytes, which may be important for hemostasis.

Objective: To understand how FVIII contributes to effective hemostasis in terms of clot structure and mechanical properties

Patients/Methods: In vitro HemA clots in human plasma or whole blood were analyzed using turbidity waveform analysis, confocal microscopy, and rheometry with or without added FVIII. In vivo clots from saphenous vein puncture in wild-type and HemA mice with varying FVIII levels were examined using scanning electron microscopy.

Results: FVIII profoundly affected HemA clot structure and physical properties; added FVIII converted the open and porous fibrin meshwork and low stiffness of HemA clots to a highly branched and dense meshwork with higher stiffness. Platelets and erythrocytes incorporated into clots modulated clot properties. The clots formed in the mouse saphenous vein model contained variable amounts of compressed erythrocytes (polyhedrocytes), fibrin, and platelets depending on the levels of FVIII, correlating with bleeding times. FVIII effects on clot characteristics were dose-dependent and reached a maximum at ~25% FVIII, such that HemA clots formed with this level of FVIII resembled clots from unaffected controls.

Conclusions: Effective clot formation can be achieved in HemA by replacement therapy, which alters the architecture of the fibrin network and associated cells, thus increasing clot stiffness and decreasing clot permeability.

KEYWORDS

blood coagulation, factor VIII, fibrin, hemophilia A, thrombin

*Was an employee of Bayer when these studies were conducted.

Essentials

- Factor VIII (FVIII) replacement in hemophilia A corrects bleeding due to low thrombin generation.
- There is uncertainty about the minimal or trough level of FVIII needed for optimal treatment.
- FVIII improves physical properties of hemophilia clots, including stiffness and permeability.
- Fibrinogen and blood cells also stabilize hemophilia clots to decrease bleeding risk.

1 | INTRODUCTION

Hemophilia A (HemA) is an X-linked recessive disorder due to factor VIII (FVIII) deficiency. HemA disease severity is defined by plasma FVIII levels, such that levels <1%, 1% to <5%, and 5% to <40% are classified as severe, moderate, and mild, respectively.[1] Currently, the standard of care in HemA is FVIII replacement, although non-replacement new therapies that bypass the need for FVIII are in development.[2–4] With FVIII therapy, the goal for HemA treatment is to elevate plasma FVIII levels to minimize time spent at low trough levels, although the optimal trough level to prevent bleeding is uncertain.[5–8] Part of the uncertainty is related to variation in patient response to FVIII, likely due to the presence of disease modifiers.[9,10] However, with nonfactor therapies, it is unclear whether targeting a specific FVIII equivalence would yield the desired result of effective clot formation, especially because these new therapeutics usually mediate their effects through mechanisms of action other than FVIII replacement.[2,3] Therefore, it would be desirable to define what constitutes an effective clot, using FVIII replacement in HemA as a starting point, because the cofactor function of FVIII in coagulation is well understood. Additionally, because considerable variation exists in the response of HemA patients to therapy, it would be desirable to determine whether optimizing therapeutic dose (eg, restoring HemA plasma FVIII levels to 100%) would eliminate variation in individual responses and generate optimal clots with homogeneous characteristics and properties.

In a mouse model for hemostasis, our results show that polyhedrocytes, formed as a consequence of clot contraction, and fibrin were major components of saphenous wound clots in wild-type (unaffected) mice with replete FVIII. In contrast, fibrin and polyhedrocytes formed much smaller fractions in HemA mice. The lower fibrin content in HemA clots was not unexpected, although the difference in polyhedrocyte content between HemA and unaffected mouse clots was unexpected and would be consistent with the altered permeability and stability of the clots, functions attributed to polyhedrocytes.[11] Because factors in addition to FVIII regulate coagulation, a more precise determinant of adequate treatment in patients with HemA may be an individual's capacity to form clots with adequate structural and viscoelastic properties, rather than absolute plasma FVIII levels. Correlation of individual HemA patient capacity to form clots with specific structures and properties when treated with different therapies may provide a basis for more effective treatment (ie, minimizing bleeding frequency) while on therapy.

Titrating FVIII levels needed to restore HemA mouse clots to those from wild-type mice indicated that at plasma FVIII levels around 25%, HemA clots resemble those formed with plasma from unaffected individuals with respect to structure and biophysical properties. However, while FVIII improved clot structure and properties, it did not abrogate individual differences, which are still detectable in the fibrin meshwork and rheology changes.

Our findings not only addressed the fundamental question regarding how clots formed by individuals with HemA differed from those formed by unaffected individuals, but also provided insights into the clot characteristics that might need to be achieved at optimum FVIII trough levels (5-25% in our study).

2 | MATERIALS AND METHODS

2.1 | Clot time (CT), fibrinogen and clot structure in HemA plasma

Kinetics of turbidity development during activated partial thromboplastin time (aPTT) reactions allowed indirect assessment of clot structure. Plasma samples from individual HemA donors (HRF, Inc, Raleigh, NC, USA) were spiked with FVIII (0-100%), and the aPTT and fibrinogen assays were performed with the HemosIL APTT-SP and Fibrinogen-C kits, respectively (Instrumentation Laboratory [IL], Bedford, MA, USA), using on-board ACL TOP tests. HemA and control clots were prepared using similar conditions as the aPTT assay using HemA and pooled plasma from healthy donors (HRF, Inc), respectively. CT was determined from the second derivative of the turbidity change over time, and turbidity waveform data were extracted from the ACL TOP using proprietary software developed by Bayer Business Services (Leverkusen, Germany). Changes in turbidity parameters were correlated with changes in CTs and with fibrinogen levels.

Direct visualization of plasma clots was achieved by confocal microscopy of plasma. HemA plasma clots were prepared as described above for turbidity assessment, except that Alexa 488-labeled fibrinogen (Invitrogen, Carlsbad, CA, USA) was added to the plasma before clot formation. Three-dimensional image data sets of clots were collected with a Zeiss LSM510 confocal microscope (Zeiss, Oberkochen, Germany). Reconstructions of 60 z-sections were computed.

2.2 | Clot mechanical properties in HemA patient whole blood by rheometry

Whole blood (10 mL) was collected in acid-citrate-dextrose from adults with severe HemA (FVIII <1%). Patients in the study had plasma FVIII <1%, without detectable FVIII inhibitors (<0.6 Bethesda units, Table S1). The protocol was approved by the University of

Pennsylvania Institutional Review Board, and all patients signed informed consent.

Viscoelastic properties of whole blood clots were measured with a rheometer (AR-G2; TA Instruments, New Castle, DE, USA) during clot formation at 37°C. Whole blood clots were prepared between the 40-mm parallel plates of the rheometer using 20% of aPTT thromboplastin activator (IL) and different doses of FVIII (0-100%). To avoid drying, the outside of the forming clot was surrounded by mineral oil (Cannon Instruments, State College, PA, USA). A time-sweep test was performed for 1 hour under an oscillation procedure of 2% strain at an angular frequency of 5 radians/sec. The storage modulus (G′) and the loss modulus (G′′), measures of elastic and viscous properties, respectively, were recorded at 3-second intervals. Curves were averaged using Origin 6 (OriginLab, Northampton, MA) by averaging *y* values for each x-value time point.

2.3 | Composition of wound clots from HemA mice treated with rFVIII

This study utilized the saphenous vein model, which had been used previously to measure the bleeding times in HemA mice.[12–14] In these studies, untreated HemA mice did not form clots within 15 minutes. However, HemA mice with 5% FVIII formed small, fragile but visible clots and served as reference controls.

HemA mice (n=5/treatment) were given FVIII prior to puncture to achieve plasma levels of 5%, 25%, and 100% at 15 minutes after dosing when injury occurred. Saphenous veins were punctured with a 23-gauge needle, and the time required for cessation of bleeding was designated as primary CT. Some of these primary clots were examined as controls for determination of the structure and composition of normal clots. For the HemA mice, the nascent primary clot was disrupted again with a 23-gauge blunt needle, and the time required for cessation of re-bleeding was designated the secondary CT. The saphenous vein segment containing the wound was carefully separated from the rest of the tissues and excised, with the wound clot retained in the smaller, more distal segment of the vessel, so that the environment and orientation of each clot could be observed. Saphenous veins containing primary and secondary clots from excipient-treated, unaffected mice (n=5 each) were similarly generated and harvested.

The isolated saphenous vein segments were washed, fixed, and processed as described.[15] Samples were examined in an FEI Quanta 250 scanning electron microscope (FEI, Hillsboro, OR, USA). The predominant structures of clots, consisting of fibrin, platelets, microparticles, white blood cells, and various erythrocyte forms (normal biconcave, intermediate, polyhedral, balloon-like, and echinocyte) were quantified. Quantitative assessment of clot composition was carried out by previously used procedures that allow the determination of the relative volume of different components from the micrographs.[16] On a computer monitor, a fine grid (1.5 μm × 1.5 μm) was overlaid on each scanning electron microscope image using ImageJ (v1.48, National Institutes of Health, Bethesda, MD, USA), such that there was usually only 1 structure present in each grid square, and the structures in each image were quantified.[16]

2.4 | Statistical analysis

Differences between groups were tested using analysis of variance (ANOVA), with Dunnett correction for multiple comparisons and unpaired Student *t* test. Analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

3 | RESULTS

3.1 | Individual variation in HemA coagulation response to FVIII spike

CTs, fibrinogen levels, thrombin generation, and representative turbidity development of plasma clots derived from individual donors with severe HemA are shown (Figure 1A–C and Fig. S1[17]). The effect of FVIII on individual HemA plasma CT and maximal turbidity are shown in Figure 1D. FVIII decreased both CT and maximal turbidity, which reflects the thickness of fibrin fibers within a fibrin meshwork.[17] Interestingly, despite restoration of HemA CT to near normal with 25% FVIII, HemA plasma clots from different donors exhibited divergent fibrin fiber diameters, as assessed by maximum turbidity values. The variation in fibrin fiber diameters, however, is directly correlated with individual plasma fibrinogen levels (Figure 1B), indicating that clot structure (specifically average fibrin fiber cross-sectional area) is sensitive to both fibrinogen levels and thrombin generated during coagulation,[15] and hence, perhaps, a better clinical indicator than CT. Furthermore, these results raise the possibility that increasing HemA patient plasma FVIII levels to 25% with replacement therapy may uniformly achieve near-normal CT, without producing fibrin clots with the same network structure in all HemA patients. The results of Figure 1D suggest that variation in HemA patient plasma fibrinogen may partially account for the heterogeneity of clot structure as assessed indirectly by turbidity.

3.2 | Individual variation in turbidity also corresponds to individual changes in clot structure

Confocal microscopy of FVIII-spiked plasma clots from individual donors also demonstrated considerable individual variation in fibrin structure, despite their dose-dependent response to FVIII (Figure 2 and Fig. S2). FVIII increased fiber density, and the plasma clots consisted of thinner, more highly branched fibers, resulting in smaller pore sizes (Figure 2). Interestingly, although all plasma clots showed similar FVIII-dependent changes, individual differences in clot structures were still evident, particularly with respect to meshwork density and apparent fiber diameter. This may be related to the difference in fibrinogen content in plasma (Figure 1D and B), although the magnitude of the fibrinogen level difference had small effects in characteristics of in vitro clots formed with purified fibrinogen.[18]

3.3 | Individual variation in HemA whole blood clot properties in response to FVIII

To determine if the HemA plasma clot structure changes with FVIII correspond to changes in physical properties of clots, we assessed the

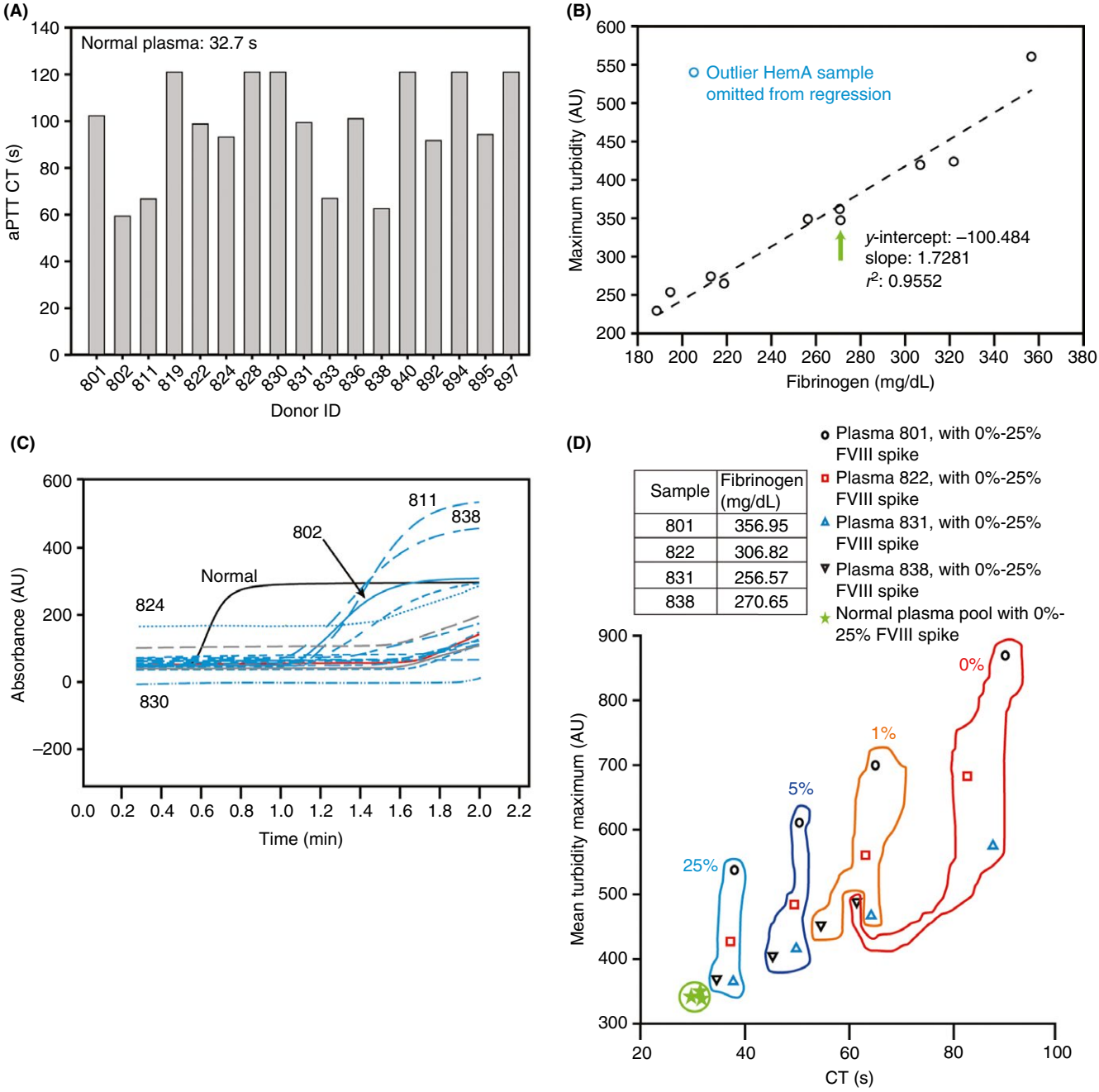

FIGURE 1 Turbidity profiles of HemA plasma clots with and without FVIII supplementation. The heterogeneity of individual HemA plasma is evident in the (A) CTs, (B) fibrinogen levels, and (C) maximum turbidity, which is directly proportional to the fibrin fiber cross-sectional area.[17] The correlation between fibrinogen and maximum turbidity is evident in the r^2 of ~1. Fibrinogen levels of normal pool in panel B are indicated with a green arrow. (D) Clot time and turbidity maxima of HemA donors with FVIII supplementation. Individual HemA plasma samples are denoted by different symbols: without FVIII (red outline) or with spikes of 1% FVIII (orange outline), 5% FVIII (blue outline), or 25% FVIII (cyan outline). aPTT, activated partial thromboplastin time; CT, clot time; FVIII, factor VIII; HemA, hemophilia A

changes in elastic (G′) and plastic (G′′) properties of whole blood clots derived from HemA patients (Figure 3A and B). Whole blood clots, rather than plasma clots, were examined to better capture the properties of HemA clots formed physiologically.

Unexpectedly, the baseline stiffness values (G′) of individual HemA clots formed were variable, ranging from ~100 to 200 Pa. Addition of FVIII up to 100% level caused a large total increase of stiffness in all HemA blood clots. However, despite the overall effect of FVIII on the G′ values of all the individual clots, the individual FVIII dose-response essentially maintained the rank order of baseline G′ values (ie, clots with low G′ values had lower G′ values with FVIII supplementation). These results suggest that the FVIII-mediated changes in clot characteristics are superimposed over individual baseline characteristics.

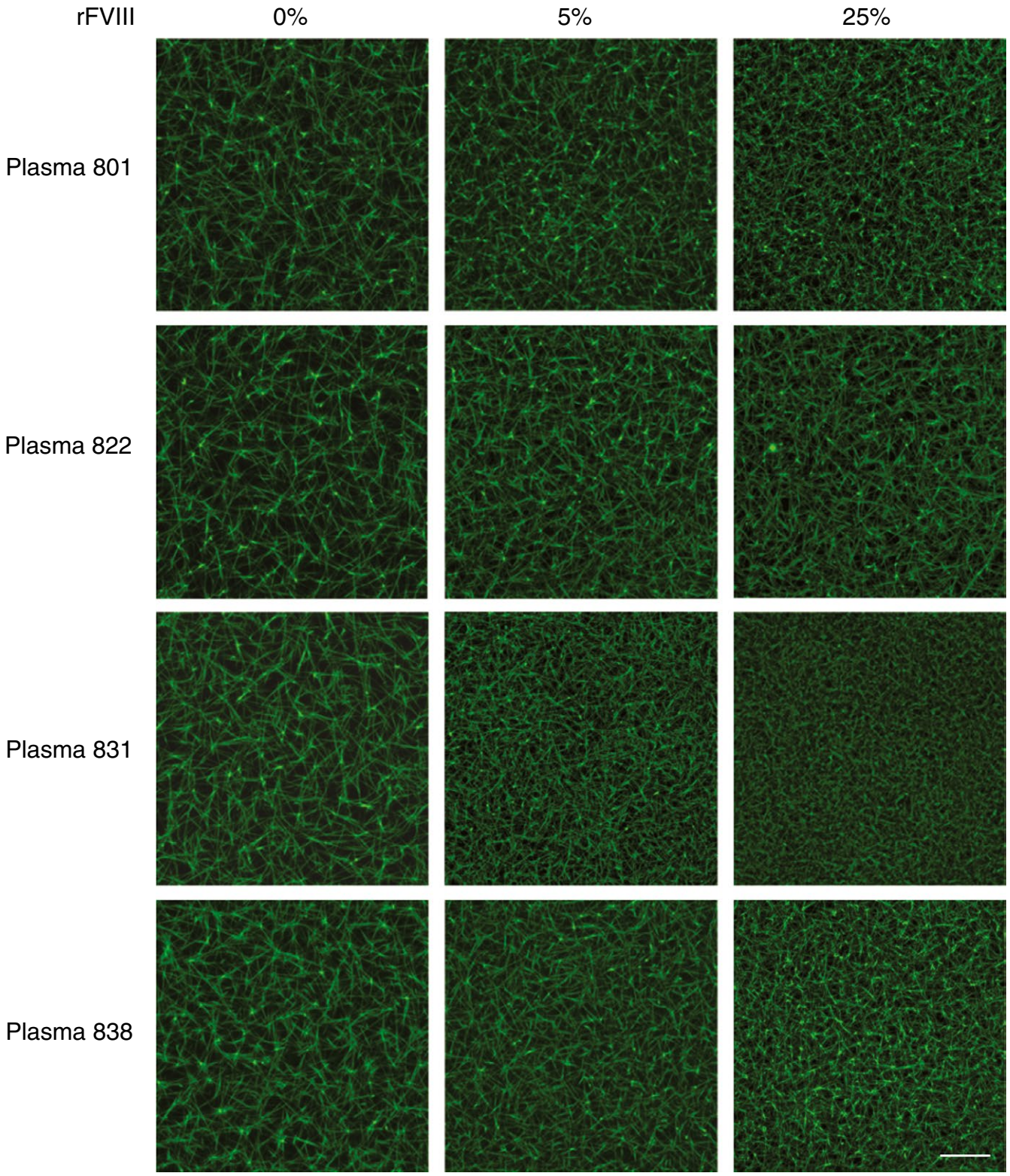

FIGURE 2 Effect of FVIII on HemA plasma clot structure. Changes in individual HemA plasma clot structure with the indicated FVIII supplementation were visualized by confocal microscopy as described in the Methods. Levels of FVIII depicted were chosen to capture potential clot structures formed in severe (0%) and mild (5% and 25%) HemA. Effects of other FVIII levels reflective of the continuum of HemA disease severity can be further visualized in Fig. S2. rFVIII, recombinant FVIII

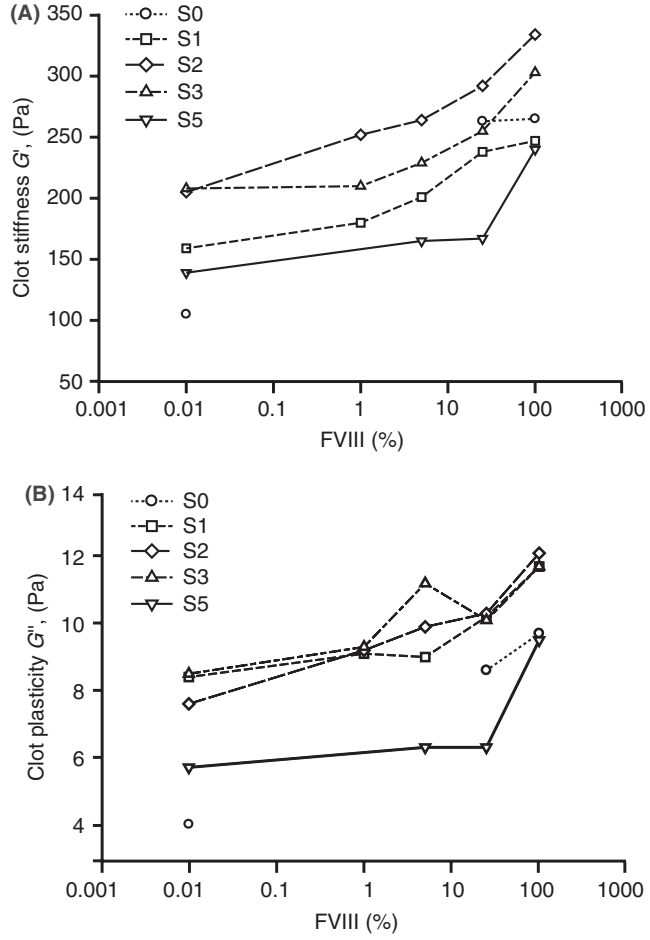

FIGURE 3 FVIII-mediated changes in whole blood rheological properties in HemA. (A and B) Rheology results of whole blood clots derived from individual HemA blood are indicated by different open symbols. Note that although FVIII increased both clot stiffness and clot plasticity, the individual HemA blood samples retained, in general, their baseline (no FVIII) rank orders, suggesting maintenance of individual characteristics. FVIII, factor VIII; HemA, hemophilia A

Because the HemA whole blood was derived from HemA patients with known clinical histories (Table S1), we attempted to correlate individual G′ results with bleeding phenotype. However, we were unable to assess the relationship between their bleeding history and rheology because all five HemA patients had histories of recurrent hemarthrosis, and the number of participants in the study was small and their treatments were variable.

In contrast to G′, clot plasticity G′′ changes were considerably smaller, increasing from a baseline of ~4 to 8 Pa to ~12 Pa with 100% FVIII supplementation (Figure 3B). Unlike G′, G′′ showed less FVIII-dependent increase, suggesting that G′ may be a more responsive parameter to FVIII replacement.

To better assess the relationship between bleeding, FVIII levels, and alterations in clot structure and properties in vivo, we assessed the effect of FVIII dosage on wound clots of HemA mice subjected to saphenous vein puncture. The saphenous vein is a commonly used hemostasis model owing to ease of access and histological preparation and because HemA mice do not spontaneously develop joint arthropathy.

3.4 | Effect of FVIII replacement on HemA wound clot structure and composition

HemA mice (n = 5/condition) were subjected to injury by puncture of the saphenous vein, as described previously.[12–14] In this model, the initial clot was disturbed for observation of the secondary (rFVIII-dependent) bleeding times and clot structure, (Figure 4A). Bleeding times in the HemA mice were greatly prolonged, but reduced by rFVIII (Figure 4B).

Despite the limitation of scanning electron microscopy, which allows visualization of exposed surfaces, details of the disrupted clot structure displayed areas with both fibrin and platelets (Figure 5A, D, E, and F; Figure 6B, D, and F). As in contracted clots in vitro, there were areas containing a dense meshwork of fibrin and/or platelets on the surface (Figure 5A, E, and F; Figure 6B), and, when visible, interior structures consisted mostly of polyhedrocytes (polyhedral erythrocytes) (Figure 5B–D; Figure 6C and H). There were also erythrocyte structures that were recognized from other studies[11,19] as forms

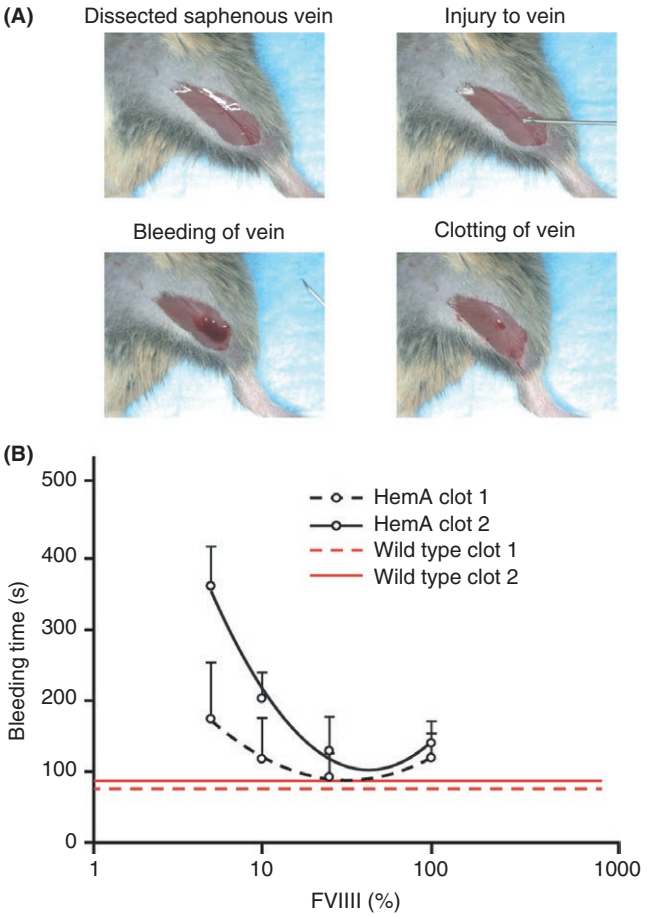

FIGURE 4 Saphenous vein injury model in HemA mice. HemA mice were given rFVIII such that at the time of vascular injury, the plasma FVIII level was predicted by pharmacokinetics to be at the desired level. (A) Vascular injury induction. (B) Bleeding times recorded for HemA mice dosed with the different FVIII (n=5/dose). Note that the bleeding time of HemA mice with 25% and 100% plasma FVIII were essentially comparable when the variance in bleeding time was taken into account. In these studies, C57BL mice treated with excipient (n=5) served as normal controls. FVIII, factor VIII; HemA, hemophilia A

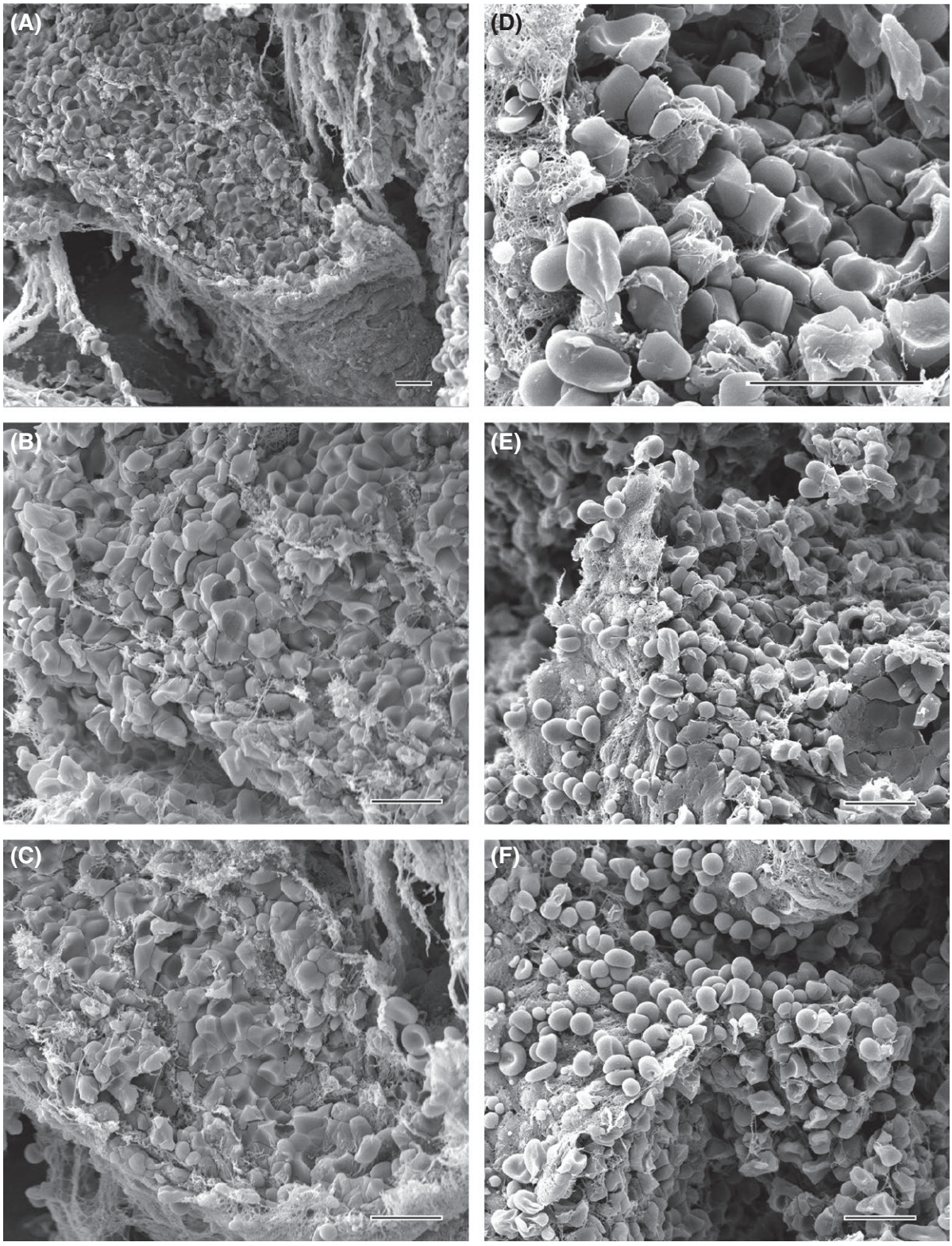

FIGURE 5 Structure of typical mouse saphenous vein clots. Scanning electron micrographs of the most typical areas of wild-type mouse saphenous vein clots. (A) Closely packed polyhedrocytes surrounded by a meshwork of fibrin and platelets. The top surface is mostly polyhedrocytes, and the sides are a dense mesh of fibrin and platelets. (B) Higher magnification view of the upper left part of the top surface, showing the tightly packed polyhedrocytes. (C) Higher magnification view of lower central part of the top surface, showing more polyhedrocytes. (D) Higher magnification view of another clot with closely packed polyhedrocytes and fibrin and platelets on the left. (E) Another clot with polyhedrocytes on the right and fibrin mesh with platelets on the left, including tethered balloon-like erythrocytes. (F) Another clot with fibrin mesh with platelets on the top, including tethered balloon-like erythrocytes, and polyhedrocytes below. Magnification bars for all panels=10 μm

intermediate between biconcave and polyhedral (Figures 5D and 6D). In addition, there were erythrocytes that were balloon-like, attached by a single point to pores in the fibrin mesh (Figure 5E and F; Figure 6G).

Comparison of the clots generated under different conditions demonstrated striking differences in the appearance of clots from HemA mice with low (5%) plasma FVIII compared with control clots from unaffected mice. The major components of control clots were erythrocytes and fibrin, as expected for venous clots, although unexpectedly most of the erythrocytes were polyhedral in shape (polyhedrocytes).[11] In contrast, in the clots from HemA mice with 5% FVIII, fibrin and erythrocytes formed a much smaller proportion, and platelets formed a disproportionately larger fraction of the total wound clot. It should also be noted that even in the HemA mice, nearly all erythrocytes present in these clots were polyhedrocytes or intermediate, between discoid and polyhedral in form, and only a very small proportion of typical biconcave discoid erythrocytes were present with no statistical difference between the treatment groups ($P > 0.05$).

Unlike clots formed in vitro,[11] these ex vivo clots were highly heterogeneous. Some areas were rich in platelets and microparticles (Figure 5A and E; Figure 6A, B, and E), and others were mostly fibrin (Figure 6B and I). Nevertheless, there were clear differences between clots from HemA mice with lower FVIII versus higher FVIII or controls. Relative to

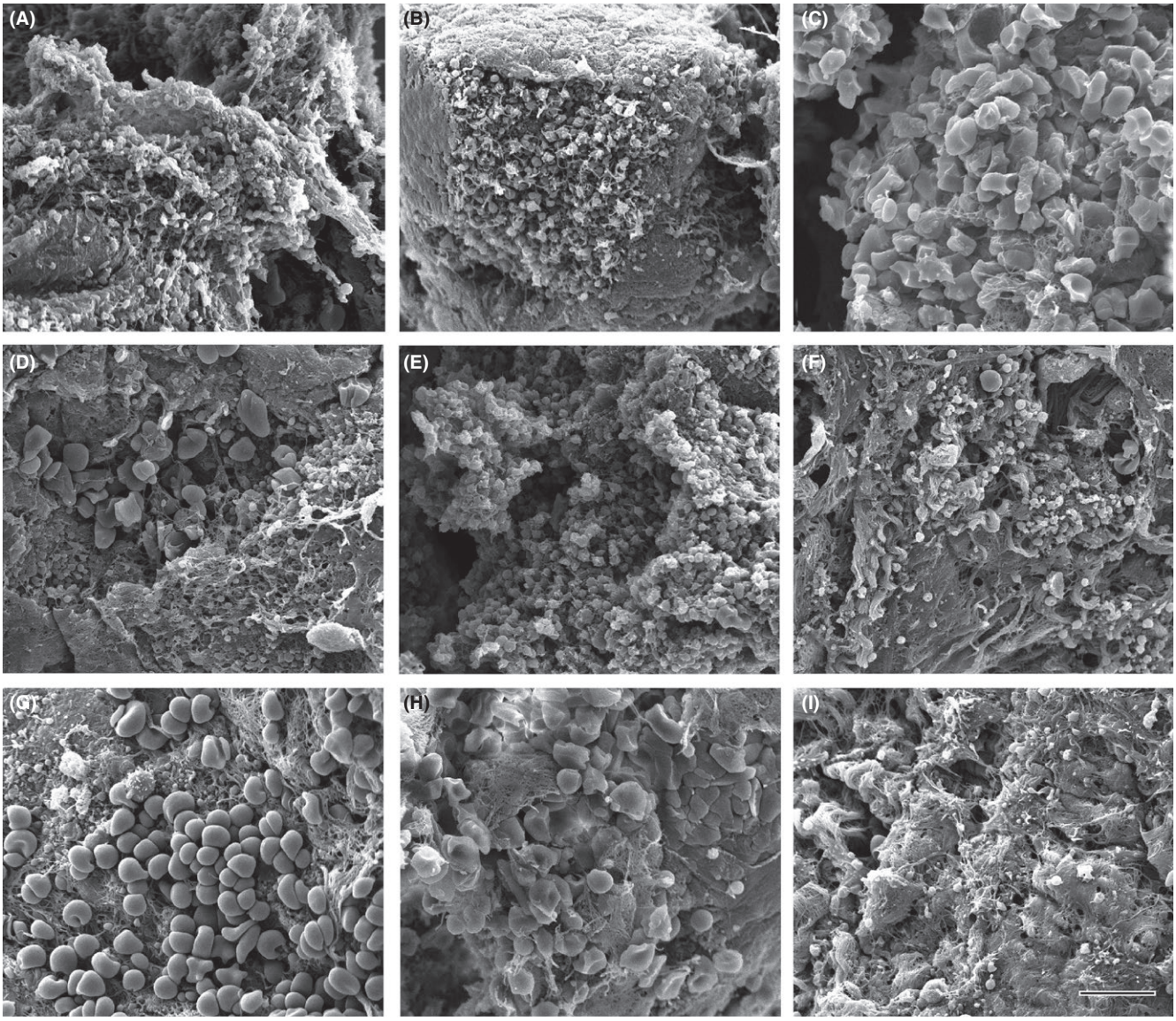

FIGURE 6 Structures identified in hemophilia A (HemA) saphenous vein clots. (A–C [16 images with 9796 structures]) Clots from HemA mice with low levels of plasma FVIII. (D–F) Clots from HemA mice with higher levels of FVIII (25%: D–E [11 images with 13,894 structures]; 100%: F [17 images with 14 059 structures]). (G–I) Clots derived from HemA mice with (G) 100% FVIII and from (H and I [16 images with 18 227 structures]) control, wild-type mice. (A) HemA mouse with 5% FVIII. Mostly platelets, some fibrin. (B) HemA mouse with 5% FVIII. Dense contracted clot with platelets and fibrin on the outside. (C) HemA mouse with 5% FVIII. Polyhedrocytes and fibrin. (D) HemA mouse with 25% FVIII. Fibrin and platelets with some intermediate forms of polyhedrocytes. (E) HemA mouse with 25% FVIII. Mostly aggregated platelets. (F) HemA mouse with 100% FVIII. Fibrin and platelets. (G) HemA mouse with 100% FVIII. Red blood cell balloons trapped in fibrin mesh. (H) Control mouse. Polyhedrocytes and fibrin mesh. (I) Control mouse. Mostly fibrin with a few platelets. Magnification bar for all panels=10 μm

unaffected control clots, platelet-rich areas were more common, and fibrin-rich areas were less common in HemA clots by general inspection.

Quantification of clot composition was carried out by accurate and robust methods as previously described, accounting for all structures observed.[16] In control clots, we found that in the 18 348 structures quantified, polyhedrocytes (29.6±2.5%) and intermediate forms (13.0±3.8%) (identified from other studies[11,19]) of compressed erythrocytes were a major component of these clots, altogether making up about 44.9% of the volume, with only 1.7±0.7% biconcave erythrocytes (Table S2). Other components were fibrin (40.1±7.7%), platelets (9.7±3.0%), microparticles (4.4±1.3%) and leukocytes (0.4±0.1%). Clots commonly contained closely packed polyhedrocytes surrounded by fibrin and platelets, as observed in contracted clots in vitro. An important conclusion of these studies is that polyhedrocytes comprise a large fraction of the mass of venous clots and are not merely an in vitro phenomenon or experimental artifact, and that there is segregation of much of the fibrin and platelets to the exterior of the contracted clot and polyhedrocytes on the interior.

In clots from HemA mice, >46 000 structures from four conditions were analyzed. Because clots were very small and sizes varied for each mouse, averages for each condition were normalized by the total number of structures in that particular clot to minimize the impact of outliers. These results confirmed and quantified the visual impressions mentioned above: fibrin is the major component (40%) of the unaffected control clots and HemA clots with a higher level (25% and 100%) of FVIII (Fig. S3 and Table S2). In contrast, HemA clots with low (5%) FVIII contained a high (37%) percentage of platelets in comparison with HemA clots with higher FVIII (or unaffected controls, 12%). Likewise, the microparticle content reflected the platelet composition. These differences were statistically significant.

In addition to having less fibrin and a higher proportion of platelets and microparticles, the distribution of erythrocyte forms varied in HemA clots with low versus higher FVIII. Polyhedrocytes formed a lower percentage (15%) of the clot in the presence of low FVIII (HemA with 5%) relative to the 30% found in clots from mice with higher FVIII. Moreover, biconcave normal erythrocytes constituted 3% of total in HemA mice with 5% FVIII versus the ~1.5% found in mice with higher FVIII levels. Surprisingly, the proportion of intermediate-form red blood cells for all groups was about the same (Table S2).

To compare clots with different levels of FVIII, normalization is needed to account for differences in clot size while clot composition is changing. For example, without normalization, it appears that as FVIII (and consequently thrombin) increased, the percentage of platelet aggregates decreased, which seems unlikely. With increasing FVIII (thrombin), fibrin is most likely to increase while platelet aggregates should remain either constant or perhaps increase. Because the simplest assumption is that the percentages of platelet aggregates are relatively constant, we chose to normalize other components with respect to platelet aggregate content. Comparison of the normalized clot composition of HemA mice with 5% plasma FVIII versus mice containing higher plasma levels of FVIII (HemA with 25% and 100% FVIII or unaffected control mice) affirmed significant differences in predominant components: fibrin, polyhedrocytes, and polyhedrocytes combined with intermediate-shaped red blood cells (Figure 7). However, in comparison with unaffected control clots, HemA clots containing higher FVIII levels (25% and 100%) showed nearly identical percentages of all components, indicating that infusion of even 25% FVIII effectively corrects the clot structure in the HemA mice. There were no significant differences between the unaffected group versus HemA mice with higher FVIII levels.

4 | DISCUSSION

Investigation of human HemA clotting by turbidity and confocal microscopy revealed effects on clotting rates and clot structures consistent with expectations from the reduced thrombin levels, but the effects on viscoelastic properties of the clots are more striking than effects measured by other methods. For example, by thromboelastography, it was difficult to discern differences between severe and moderate hemophilia.[20] On the other hand, by rheometry, the differences between HemA whole blood supplemented with 1% versus 5% were clearly discernible. While we were unable to study wound clots from HemA mice with plasma FVIII levels <5% because our focus on re-bleeding requires the formation of a discernible clot, our results suggest that methods more specific for clot structure and physical properties might provide more information. The differences between mouse HemA venous clots with different levels of FVIII and controls were striking and novel using scanning electron microscopy to assess whole blood clots. Our studies clearly demonstrated that plasma levels of ~25% and higher FVIII were sufficient to convert HemA clots toward unaffected clots, with respect to clot structure, rheology, and CTs. These results are consistent with recent findings that trough levels at ~15% FVIII may be adequate to prevent bleeding.[6] However, the observation that mild HemA patients with plasma levels between 5% and 40% have reduced range of motion,[21] suggestive of subclinical joint bleeding, indicates that plasma levels at 15% or higher do not necessarily equate to no bleeding risk.

Although further increases in FVIII could decrease HemA CT to the same level as unaffected controls (Figure 1D), the variations observed in fibrinogen levels (Figure 1B), fibrin fiber diameters, fiber branching, and the ability to form an interconnected meshwork and attain specific viscoelastic properties predict that the ability of HemA clots to stop bleeding may depend on more than circulating FVIII levels. For example, for plasma 831, the fibrin meshwork attained with 5% FVIII supplementation was comparable to that attained with higher FVIII supplementation (Figure 2). Not surprisingly, plasma 831 was one of the two plasmas tested that contained similar fibrinogen levels as normal pool (Figure 1B and D) and achieved closer maximal turbidity values approximating pooled normal plasma. In addition, different levels of FVIII are required to achieve a specific change in clot stiffness. For example, for some donors (S2 and S3), only 25% FVIII was required, while other donors (S1 and S5) required considerably more FVIII (Figure 3A).

Variations in HemA patient hemostatic response could reflect differences in plasma coagulation factors and cellular elements, including fibrinogen, that affect clot turbidity and fibrin polymerization characteristics,[15] and ultimately clot structure and viscoelastic properties. In our studies, supplementation of individual plasma to the same level of FVIII still revealed individual differences in fibrin meshwork and clot stiffness (Figures 1D and 3A).

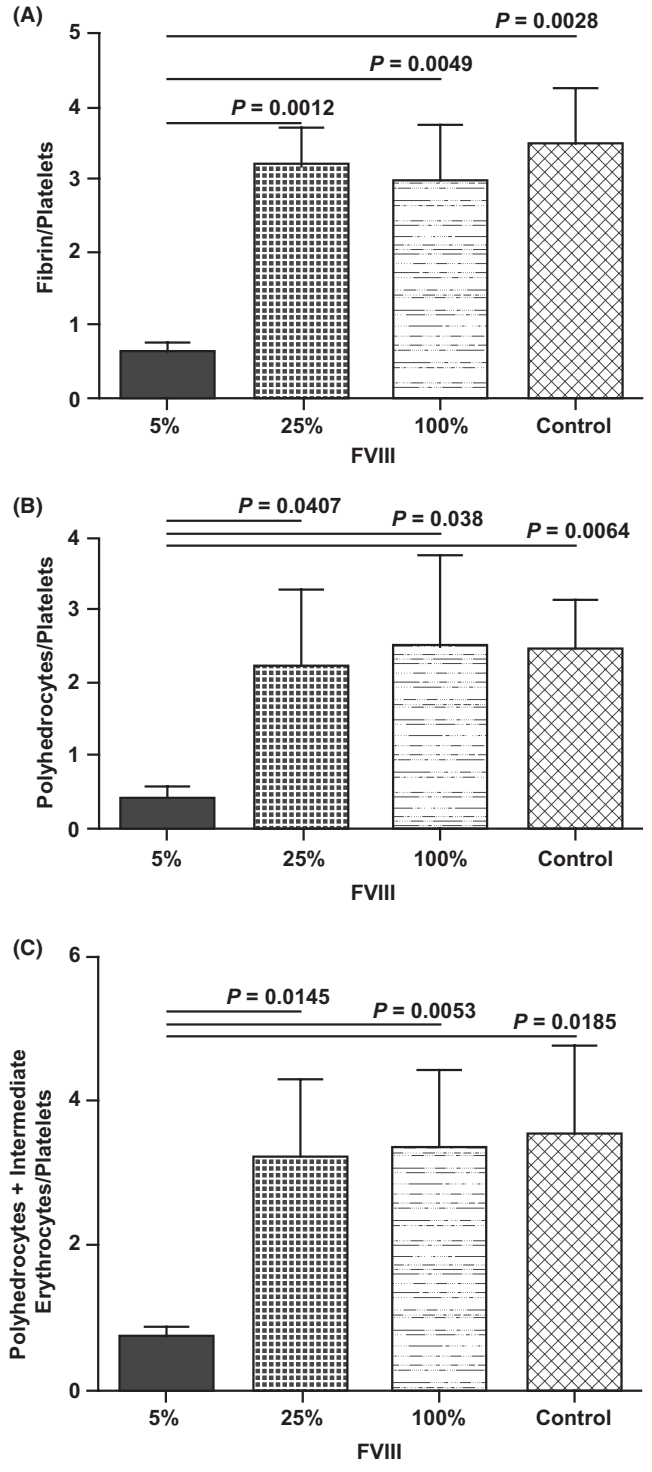

FIGURE 7 Quantitation of structures identified in saphenous vein clots. Saphenous vein clots visualized using scanning electron micrographs were quantified as described in the Section 2. The proportion of total visual field occupied by these structures were averaged. The fibrin and erythrocyte (polyhedrocyte as well as combined intermediate form and polyhedrocyte) structures identified in clots derived from HemA mice containing 5%, 25%, and 100% FVIII or wild-type C57BL mice are normalized against the platelet. Quantitative changes induced by FVIII were compared statistically by ANOVA test with Dunnett correction for multiple comparisons, and the levels of statistical significance for each comparison were done with a multiple Student *t* test. *P* values for differences between 5% FVIII, 25% FVIII, 100% FVII, and control are indicated. (A) Fibrin component. (B) Polyhedral-shaped erythrocytes. (C) Combination of polyhedrocytes and intermediate erythrocytes. ANOVA, analysis of variance: FVIII. factor VII

Other sources of individual variation in HemA response can include the formed elements of blood, specifically platelets and erythrocytes. Platelets have a positive effect on clot organization and resistance to lysis,[22] and erythrocytes modulate the organization and rheology of clots.[23]

The saphenous vein mouse model for hemostasis provides a wealth of new information about clotting in vivo. For wild-type mice, these results demonstrate that polyhedrocytes are generated in vivo during hemostasis of large vein injury. Since polyhedrocytes form a nearly impermeable barrier, their normal function could be to physically limit blood loss and stabilize clots by preventing access to fibrinolytic enzymes. The involvement of blood cells in modulating clot structure and viscoelastic property is also suggested by our data, which indicate differences in fibrin, platelet, and erythrocyte composition in HemA clots at low versus high levels of FVIII supplementation. At low FVIII (~5% or lower), when the fibrin content is lower and the fibrin meshwork is relatively open and porous (Figure 2; Figure 6A and B; Figure 7A), the presence of blood cells such as activated platelets and red blood cells likely contribute to the increase in HemA clot stiffness (Figure 3A, Table S2). With higher FVIII (25% or higher), the fibrin meshwork and polyhedrocytes assume greater roles in clot stability. With regard to the fibrin meshwork, higher FVIII induced the formation of thinner, highly branched fibers that interconnect to form a dense network with smaller pores. With regard to the whole blood clot, clots formed with low FVIII had a more friable appearance, in marked contrast to those formed with 25% and 100% FVIII, which contained platelets and red blood cells embedded within fibrin mats (Figure 6D, F, and I).

With whole blood clots formed at the higher level of FVIII, red blood cells, specifically polyhedrocytes, assumed an increasingly more prominent role (Figure 6C, D, and H; Figure 7B; Fig. S3A–D) when clots had higher stiffness (Figure 3B). This positive correlation between polyhedrocytes and FVIII levels would be consistent with their ascribed function, conferring clot stability (and resistance to lysis) to contracted clots.[11] This is the first evidence for polyhedrocytes in any model in response to vascular injury and suggests a function for these structures in normal hemostasis, because they form a highly impermeable barrier that mimics the function of the undamaged endothelium.[22] This function is entirely consistent with the correlation between increased polyhedrocyte content and decreased bleeding times with addition of FVIII to HemA mice.

The importance of platelet contraction in clot stabilization raises some interesting possibilities for approaches to improve bleeding in hemophilia. Increasing the level of FVIII replacement might be the simplest approach, as the consequent increased thrombin generation would both increase the fibrin polymer mass and density, and promote platelet activation and contraction. Fibrinogen administration may further improve HemA bleeding, particularly when patient fibrinogen levels may be lower. The use of platelet and red cell products may also be generally beneficial in controlling severe bleeding.

In conclusion, our findings indicate that the capacity to form an effective hemostatic clot depends on more than FVIII levels and raises the possibility that variation in HemA patient response to FVIII treatment may be reduced by differentially modulating the contribution of fibrin, platelets, and erythrocytes to hemostasis by increasing replacement FVIII levels, fibrinogen, or blood components like platelets and erythrocytes.

AUTHOR CONTRIBUTIONS

L. Leong, D. Sim, and J. W. Weisel formulated the study plan. I. N. Chernysh, C. Nagaswami, Y. Xu, L. Leong, Z. de Lange, and S. Kosolapova performed experiments and analyzed the data. A. Cuker contributed clinical expertise. L. Leong, J. W. Weisel, I. N. Chernysh, A. Cuker, D. Sim, and K. Kauser developed the manuscript. All authors reviewed each draft of the manuscript and approved the final draft.

ACKNOWLEDGMENTS

We acknowledge that editorial assistance was provided by Complete Healthcare Communications, LLC (Chadds Ford, PA, USA) and was fully funded by Bayer. The University of Pennsylvania acknowledges partial support of this research by a grant from Bayer and partial support by NIH NHLBI grant UO1-HL116330. We acknowledge grant EP/C513037/1 from the Engineering and Physical Sciences Research Council to P. Rhodri Williams (College of Engineering, Swansea University, Wales, UK) for the TA Instruments ARG2 rheometer. Additional funding support was provided by Bayer.

RELATIONSHIP DISCLOSURES

J. W. Weisel acknowledges partial support of this research by a grant from Bayer. L. Leong and Y. Xu are employees of Bayer. D. Sim and K. Kauser were employees of Bayer when these studies were conducted. I. N. Chernysh, C. Nagaswami, Z. de Lange, S. Kosolapova, and A. Cuker have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Leong L, Chernysh IN, Xu Y, et al. Clot stability as a determinant of effective factor VIII replacement in hemophilia A. *Res Pract Thromb Haemost*. 2017;1:231–241. https://doi.org/10.1002/rth2.12034