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Differences in venous clot structures between hemophilic mice treated with emicizumab versus factor VIII or factor VIIIFc

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TS, HM, VM, XH, JD and PJL performed experiments and analyzed data; CEE provided patient data; ODC, CVD, CC and PJL designed and supervised the study. All authors contributed to the interpretation of the data. PJL wrote the first draft of the manuscript, and all authors contributed to the editing of the final manuscript.

Running head: Treatment-dependent venous clot structures

Data sharing statement: Data are available upon reasonable request to the corresponding author

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Abstract

Recombinant factor VIII (rFVIII), rFVIIIFc and emicizumab are established treatment options in the management of hemophilia A. Each has its unique mode of action, which can influence thrombin generation kinetics and therefore also the kinetics of thrombin substrates. Such differences may potentially result in clots with different structural and physical properties.

A starting observation of incomplete wound closure in a patient on emicizumab-prophylaxis led us employ a relevant mouse model in which we noticed that emicizumab-induced clots appeared less stable compared to FVIII-induced clots. We thus analyzed fibrin formation in vitro and in vivo. In vitro fibrin formation was faster and more abundant in the presence of emicizumab compared to rFVIII/rFVIIIFc. Furthermore, the time-interval between the initiation of fibrin formation and factor XIII activation was twice as long for emicizumab compared to rFVIII/rFVIIIFc. Scanning-electron microscopy and immunofluorescent spinning-disk confocal-microscopy of in vivo generated clots confirmed increased fibrin formation in the presence of emicizumab. Unexpectedly, we also detected a different morphology between rFVIII/rFVIIIFc- and emicizumab-induced clots. Contrary to the regular fibrin-mesh obtained with rFVIII/rFVIIIFc, fibrin-fibers appeared to be fused into large patches upon emicizumab-treatment. Moreover, fewer red blood cells were detected in regions where these fibrin patches were present. The presence of highly-dense fibrin-structures associated with a diffuse fiber-structure in emicizumab-induced clots was also observed when using super-resolution imaging.

We hypothesize that the modified kinetics of thrombin, fibrin and factor XIIIa generation contribute to differences in structural and physical properties between clots formed in the presence of FVIII or emicizumab.
Introduction

The blood coagulation cascade is a series of highly regulated enzymatic reactions designed to generate thrombin, which is needed to produce fibrin in a timely and spatially correct manner. To achieve this goal, the coagulation cascade consists (amongst others) of thrombin-dependent feedback loops that allow for the amplification of thrombin formation. In addition, thrombin is needed to generate activated factor XIII (FXIIIa), which converts the freshly produced fibrin protofibrils into a covalently-linked network, and activated thrombin-activatable fibrinolysis inhibitor (TAFIa), which delays fibrinolytic degradation of the fibrin network. Any disturbance of this complex process may potentially lead to an improperly formed fibrin network. Indeed, thrombin concentration has been shown to be a critical determinant of the fibrin-network structure and its physical properties.

One of the key cofactor proteins in the coagulation cascade is factor VIII (FVIII), the functional absence of which is associated with markedly reduced thrombin formation. The thrombin-activated derivative of FVIII, FVIIIa, functions as a cofactor in the tenase-complex, which participates in the amplifying portion of the coagulation cascade. Within the tenase complex, FVIIIa stimulates the generation of activated factor X (FXa) by the enzyme activated factor IX (FIXa). Its physiological relevance is illustrated by the severe bleeding complications associated with FVIII deficiency, a disorder known as hemophilia A.

To compensate for the deficiency of FVIII in hemophilia A patients, replacement therapy using FVIII concentrates has been the treatment of choice over the last four decades. Prophylactic treatment proved efficient in reducing spontaneous bleedings and subsequent joint damage. Despite its effectiveness, replacement therapy faced a number of disadvantages, including the need for frequent intravenous infusions and an immunologic response leading to the presence of neutralizing allo-antibodies against FVIII. These complications have led to the development of alternative treatment options, such as activated factor VII, extended half-life FVIII variants, gene therapy, and so-called non-factor therapies. One non-factor approach that has been approved for clinical use is emicizumab, a bispecific antibody that binds both the enzyme FIXa and its substrate FX, thereby mimicking part of the FVIIIa function. Emicizumab allows therefore the generation of a certain amount of thrombin without completely correcting coagulation. Clinical studies have shown marked therapeutic benefit of emicizumab, with average annual bleeding rates being below 2 among patients treated with emicizumab prophylactically. Because of its efficacy and its subcutaneous mode of application, increasing numbers of hemophilia A patients are using emicizumab as prophylactic therapy. Despite this success, it has been reported that about 5% of the patients receiving emicizumab may develop spontaneous or trauma-induced muscle bleeds, which sometimes are difficult to stop and require intensive factor replacement therapy.
It is important therefore to better understand the molecular basis by which emicizumab contributes to coagulation in comparison to FVIII. Indeed, their mode of action is quite different. Both components may differ in the timing by which they start acting and when their activity stops, as well as by the location where they may be acting. A similar point can also be made for extended half-life variants, such as recombinant FVIII Fc (rFVIII Fc), where the presence of the Fc portion may affect FVIII distribution and activation during coagulation. Knowing that thrombin formation requires a time- and spatial-dependent regulation, it is thus of relevance to investigate if and how emicizumab and rFVIII Fc may eventually differ from “classic” FVIII regarding the formation of a fibrin network.

It should be noted, that so far studies on fibrin formation using emicizumab have been restricted to in vitro settings. Moreover, activity of emicizumab is often examined in global assays, such as thrombin generation assays. Although this may provide insight into the potential of emicizumab to stimulate thrombin generation relative to FVIII, it provides little insight into the upstream processes that are required to generate a stable clot.

We have therefore explored in vivo clot formation by recombinant FVIII (rFVIII), rFVIII Fc and emicizumab using specific bleeding models and microscopic analysis, with focus on the structure of the fibrin network. We found differences in structural morphology between FVIII- and emicizumab-induced clots. Furthermore, kinetics of fibrin and FXIIIa generation are modified when using emicizumab, which could explain in part the differences in clot structure.
Methods
A description of the experimental procedures can be found in the Supplementary materials.

Ethics statement
Photos from patients’ wound were taken during consult, and informed consent was given by the parents for their anonymous use in this study. Animal housing and experiments were performed in accordance with French regulations and the experimental guidelines of the European Community. This project was approved by the local ethical committee of Université Paris-Saclay (Comité d'Ethique en Experimentation Animale n°26, protocol APAFIS#26510-202007061525281 v2).

Patient:
A 2-year-old boy with severe hemophilia A (FVIII <1%) on regular prophylaxis with emicizumab (6 mg/kg every 4 weeks, subcutaneously) experienced a deep laceration at his left foot and a consecutive insufficient wound healing. A more extensive description of the patient is available in the Supplementary materials.

In vitro assays
A description of fibrin formation and FXIIIa generation is available in the Supplementary materials.

In vivo models
A tail vein-transection model has been used in this study, which is described in the Supplementary materials.

Microscopy
The application of spinning disk confocal microscopy, scanning electron microscopy and Stimulated Emission Depletion (STED)-microscopy is described in the Supplementary materials.
Results

Patient
A 2-year-old patient with severe hemophilia A under regular emicizumab prophylaxis for 2 years (6 mg/kg every 4 weeks), experienced a deep laceration at his left foot (Figure 1). The wound was treated with local tranexamic acid application and covered with Steri-strips. Emicizumab prophylaxis continued, and bleeding stopped. However, when Steri-strips were removed after ten days, no wound closure or wound healing had occurred, and the covering clot appeared loose and fragile. Patient then received three consecutive FVIII administrations (66 IU/kg intravenously, followed by 33 IU/kg intravenously at 12-hourly intervals) at day 10 and 11 after injury. Complete wound closure was observed at day 11, and wound healing progressed naturally in the following days.

In vivo clot formation
To further explore clot formation in vivo, we compared mice receiving emicizumab, rFVIII or rFVIIIIFc using a previously validated mouse model. In this model, mice receive emicizumab in combination with human factors IX and X (100 IU/kg), or a single dose of rFVIII or rFVIIIIFc. Subsequently, bleeding is assessed in the tail vein-transection (TVT)-model. At the time of injury, plasma concentrations were 10 IU/dl for rFVIII and rFVIIIIFc, versus 55 μg/ml for emicizumab. These FVIII concentrations were chosen based on the apparent FVIII-equivalence for emicizumab that we observed in our previous in vivo studies. Whereas in vehicle-treated mice, all mice started to rebleed after the initial primary stop, treatment with rFVIII, rFVIIIIFc or emicizumab resulted in a permanent arrest of bleeding when the wound remained unchallenged (Figure 2A). Efficient bleeding arrest was accompanied by limited blood loss (Supplementary figure S1). In contrast, when the clot covering the injury was removed at 15 and 30 min after injury, differences in response between FVIII- and emicizumab-treated mice were observed. Mice receiving rFVIII or rFVIIIIFc readily stopped bleeding again, whereas emicizumab-treated mice displayed a pattern of frequent spontaneous re-bleeding (Figure 2B). Spontaneous re-bleeds were associated with increased blood loss (Supplementary figure S1). We considered the possibility that levels of factors IX and X had diminished below the necessary threshold to support emicizumab, since their respective half-lives are relatively short in mice (3-5 h) compared to human (>20h). However, re-injection of FIX and FX (100 IU/kg) 5 min before clot removal did not affect bleeding profile or blood loss (Figure 2B, Supplementary figure S1). We also examined if the use of tranexamic acid (10 mg/kg) could improve the phenotype of emicizumab-treated mice. Interestingly, tranexamic acid did not prevent spontaneous re-bleeds after clot removal (Figure 2B, Supplementary figure S1), suggesting that the clot instability is unrelated to
increased fibrinolysis of the fibrin network. In conclusion, these in vivo data point to potential differences in clot construction between FVIII- and emicizumab-treated mice.

In vitro fibrin formation
Given that a stable fibrin-network is key to the formation of a stable thrombus, we evaluated if differences in the mode of action between rFVIII and emicizumab could affect upstream fibrin formation. Therefore, in vitro experiments using human plasma were performed, in which rFVIII (both 10 IU/dl and 100 IU/dl) or emicizumab (55 μg/ml) were added to fibrinogen-enriched FVIII-deficient plasma (Figure 3A). Fibrin formation was monitored by measuring OD-values at 405nm. In the absence of rFVIII or emicizumab, the lag-time was 32 min, and half-maximal fibrin formation was reached at 42 min (Figure 3B). The addition of rFVIII (10 and 100 IU/dl) dose-dependently shortened the lag-time to 24 and 18 min, with half-maximal fibrin formation being at 33 and 26 min, respectively (Figure 3C-D). Similar data were obtained using rFVIIIFc, indicating that coagulation proceeds similarly for both FVIII variants (Supplementary figure S2). Interestingly, lag-time and time to half-maximal fibrin formation were strongly shortened in the presence of emicizumab, to 6 and 14 min, respectively (Figure 3E). In addition, the maximum OD reached with emicizumab (OD=1.17±0.03; n=6) was higher compared to both rFVIII and rFVIIIFc (OD=0.95±0.06 (n=6) and 0.97±0.08 (n=10), respectively; p<0.0001), pointing to rapid and increased fibrin formation. Together, these data suggest that modifying the kinetics of FXa generation affects upstream fibrin formation.

In vitro FXIIIa generation
Since fibrin formation involves two distinct steps following the conversion of fibrinogen into fibrin monomers (non-covalent polymerization of fibrin monomers into protofibrils, and subsequent covalent crosslinking via FXIIIa), we decided to monitor FXIII activation in parallel experiments. FXIIIa activity was detected in all conditions (Figure 3B-E). However, the synchronization between the initiation of fibrin formation and FXIIIa generation varied between the different conditions. In particular, a longer interval was observed between the initiation of fibrin and FXIIIa formation for emicizumab (12±2 min) compared to rFVIII at 100 IU/dl (7±1 min) or 10 IU/dl (4±1 min). Furthermore, more FXIIIa was generated in the presence of emicizumab compared to the presence of rFVIII at 100 IU/dl (0.14 OD/min, 95%CI: 0.13-0.15 OD/min versus 0.12 OD/min, 95%-CI: 0.11-0.13; p=0.026), rFVIII at 10 IU/dl (0.10 OD/min; p<0.0001) or FVIII-deficient plasma (0.11 OD/min; p<0.0001). Thus, relative to fibrin generation, activation of FXIII starts later in the presence of emicizumab compared to rFVIII, while once started, more FXIIIa is formed.
In vivo generation of fibrin: scanning-EM analysis

We next studied whether the in vitro differences in the kinetics of fibrin formation were also observed in vivo by applying the same TVT-model as described for Figure 2. Tails were collected 10 min after injury (in which bleeding had arrested for all treated mice; Figure 2), and tissue sections were prepared for whole-mount scanning-EM, which permits visualizing the outer portion of the clot (Figure 4A). rFVIII-derived clots contained a fibrin-network in which thin, tubular-shaped fibers with homogenous thickness were in a regular mesh over the clot, and cellular components were evenly distributed (Figure 4B). Similar structures were also observed in tail fragments obtained from wild-type mice, whereas FVIII-deficient mice displayed a disturbed network with less fibrin and fiber that had a 1.4-fold increased diameter size (p=0.032; Supplementary figure S3). The emicizumab-derived clots were characterized by a completely different fibrin morphology. They consisted of thick and uneven fiber structures that seemed to fuse together, thereby forming large layered structures. Strikingly, these patch-like structures were almost devoid of cellular components, and included small and large circular-shaped holes (Figure 4C). We analyzed these images using ImageJ-software for four different parameters: fibrin coverage, fiber diameter, number of pores and number of intersections (Figure 4D-G). In line with the in vitro fibrin generation experiments, more fibrin was detected in emicizumab-treated compared to rFVIII-treated mice (42±8% vs 24±8% per field; p<0.0001; Figure 4D). The average diameter of the fibrin fibers was also significantly increased 1.8-fold in emicizumab-treated mice (average of 534±90 nm versus 292±101 nm; p<0.0001; Figure 4E). Furthermore, there were 2-fold less pores in emicizumab-derived clots compared to rFVIII-derived clots (119±45 versus 248±58 per field; p<0.0001; Figure 4F). Finally, 1.7-fold more intersections per field of view were detected in rFVIII-induced clots compared to emicizumab-induced clots (11078±2935 versus 6510±1157 per field; p<0.0001; Figure 4G). These data indicate that clots derived from mice receiving emicizumab are having a different structure from clots generated in mice receiving rFVIII.

In vivo generation of fibrin: spinning-disk confocal immunofluorescence-analysis

To investigate whether differences in fibrin formation were also occurring within the interior of the injury, we first performed spinning-disk confocal imaging using anti-fibrin antibodies (Figure 5A). Five mice were analyzed for each group, with two tissue sections/mouse, and representative images are provided for each condition (wild-type mice, FVIII-deficient mice, and FVIII-deficient mice treated with rFVIII, rFVIIIFc or emicizumab; Figure 5B-F). Similar amounts of fibrin (in terms of mean fluorescence intensity) were found in clots from rFVIII- and rFVIIIFc-treated mice compared to wild-type mice (Figure 5G). This parameter did not further increase with higher doses of rFVIII or rFVIIIFc (500 IU/dl; Supplementary figure S4). Unexpectedly, the mean fluorescence intensity was significantly increased in clots from
emicizumab-treated mice (0.66±0.30 versus 0.14±0.05, 0.17±0.10 & 0.26±0.09 for emicizumab, rFVIII, rFVIIIFc & wild-type mice, respectively; p<0.0001; Figure 5B-F). These data are in line with our *in vitro* data, which showed that fibrin formation is more abundant in the presence of emicizumab compared to FVIII.

**In vivo generation of fibrin: Stimulated emission depletion (STED)-Microscopy**

Although spinning-disk confocal imaging allowed for the quantitative analysis of fibrin formation within the interior clot area, its resolution is insufficient to detect potential differences in structure. We therefore proceeded to perform STED-microscopy, a form of super-resolution microscopy, which is less suited for quantitative analysis of large regions, but enables imaging with sub-diffraction resolution of ≤50 nm. Detailed images of fibrin structures at the interior of the clot were obtained for wild-type, FVIII-deficient and rFVIIIFc- and emicizumab-treated mice (Figure 6A). As expected, typical fiber-like structures similar to those observed using scanning-EM, were readily distinguished in the clots of wild-type, FVIII-deficient and rFVIIIFc-treated mice. The fibrin-mesh was characterized by a homogeneous thickness of the fibers (Figure 6B-D). Interestingly, in each of the images (obtained from three different mice), intense-stained dot-like structures were observed, which for convenience we like to refer to as focal points. With regard to the structures observed in emicizumab-treated mice, fiber-like structures were also present, (Figure 6E). However, these appeared more diffuse and less homogenous in thickness. In addition, the number of focal points was considerably increased in these clots. When calculating the number of focal points using specific ImageJ-software, it was found that on average twice as many focal points were observed in emicizumab-derived clots compared to those under other conditions (0.36±0.12 for emicizumab versus 0.18±0.07, 0.21±0.08 & 0.18±0.08 for wild-type, FVIII-deficient and rFVIIIFc-treated mice, respectively (p<0.0001; Figure 6F). Together, these data are in support of the different fibrin structures that are being generated when using emicizumab in comparison to FVIII.
Discussion
There have been anecdotal communications on unusual bleeding episodes in patients using emicizumab. Here, we provide one example in which a laceration in the foot of a 2-year-old severe hemophilia A patient on emicizumab-prophylaxis was characterized by compromised wound closure and healing. This complication was readily corrected upon treatment with FVIII. Although this single example surely is not representative for the majority of hemophilia patients, it is noteworthy that Batsuli et al. reported that about 5% of patients on emicizumab prophylaxis develop spontaneous or trauma-induced muscle bleeds requiring intensive factor replacement-therapy. These observations may originate from emicizumab being a less efficient cofactor than FVIIIa for FIXa. Also, they may point to fundamental differences in how clots are generated in the presence of FVIII or emicizumab.

Based on the differences in functionality between FVIII and emicizumab, we have now explored fibrin formation and structure in the presence of rFVIII or rFVIIIFc versus emicizumab. Our data demonstrate that there is a difference not only in the amount of fibrin that is generated, but also in the structure of the fibrin-network that is formed.

To consistently assess clot structures in vivo, a bleeding model was used that involves the guided transection of the caudal vein, i.e., the tail vein-transection model (TVT). The advantage of this model lies in the reproducibility by which the injury is made using a specific template. FVIII-deficient mice are an established model to assess the hemostatic efficiency of FVIII and variants thereof. In contrast, less information exists with regard to the use of emicizumab in FVIII-deficient mice, because emicizumab is unable to bind to murine factors IXa and X. To overcome this limitation, we previously developed a protocol involving the co-infusion of human factors IX and X, allowing to test the functionality of emicizumab in FVIII-deficient mice. Importantly, we showed that addition of human FIX and FX did not alter hemostasis in FVIII-treated mice, and that human FIX was as functional as murine FIX in in vitro clotting assays using murine plasma. In the tail clip-model, emicizumab (55 μg/ml) significantly reduced blood loss, with an efficacy that was similar to FVIII plasma concentrations of about 10 IU/dl. These concentrations were therefore used in the present study.

rFVIII, rFVIIIFc and emicizumab were similar in that they all corrected bleeding in FVIII-deficient mice in the TVT-model (Figure 2). Bleeding stopped shortly after injury, and no spontaneous rebleeding was observed. In contrast, when clots were removed, emicizumab-treated mice displayed a pattern of frequent re-bleeds that was absent in rFVIII- or rFVIIIFc-treated mice. Control experiments verified that the re-bleeds were not due to an absence of human FIX or FX at the time of clot removal. Also increased fibrinolysis was excluded, since the use of tranexamic acid was unable to prevent these re-bleeds. Moreover, preliminary data presented by Locke et al., suggest that clot-lysis time of emicizumab-induced clots is
prolonged 1.6-fold when comparing FVIII-induced clots. Our in vivo data suggest the formation of instable clots when using emicizumab. With fibrin being key in stabilizing wound-covering clots, we focused the rest of this study on fibrin network formation. We realize that other factors, in particular platelet activation and aggregation, are also of relevance to maintain clot stability. We are currently investigating this specific aspect as part of a separate study. When examining unbiased fibrin formation using human FVIII-deficient plasma spiked with rFVIII or emicizumab, it was clear that fibrin generation was quickest in the presence of emicizumab, and also more fibrin was generated (Figure 3). Increased and more rapid fibrin formation is also compatible with the hyper-reactivity of emicizumab in standard aPTT-assays, which rely on fibrin clot formation. One possible explanation for increased and accelerated fibrin formation is related to the different kinetics by which FVIII and emicizumab stimulate coagulation. Emicizumab will promote FXa generation as soon as FIXa becomes available. In contrast, rFVIII (or rFVIIIFc) requires feedback activation by thrombin before it will exert its cofactor function. The modified kinetics in turn alter the kinetics by which thrombin is able to activate its various substrates, including fibrinogen and FXIII. The observation that emicizumab accelerates fibrin formation is not limited to the in vitro experiments, but is also detected in vivo. Two independent microscopic approaches (scanning-EM and immunofluorescent spinning-disk confocal imaging) revealed an excess of fibrin in emicizumab-treated mice compared to FVIII-treated mice (Figures 4 & 5). Moreover, a significant correlation between in vitro and in vivo fibrin formation was observed, irrespective whether scanning-EM or immunofluorescent spinning-disk confocal imaging was used (Figure 7).

To get insight in the structure of the in vivo generated fibrin network, we used two different microscopic techniques. By using scanning-EM, we were able to visualize the outer region of the clot, ie the portion that covers the injury. Unexpectedly, we observed quite an unusual fibrin-network in the clots of emicizumab-treated mice. In contrast to individual intertwined fibers that are present in the structures of clots from wild-type mice and FVIII-treated mice, fibrin-fibers appeared to be fused into large patches, with few individual fibers present. Consequently, average diameters were found to be excessively large (>500 nm; Figure 4). In addition, the presence of those patches seems to prevent the inclusion of red blood cells in these areas, resulting in a less dense clot structure.

Also, when using super-resolution (STED)-microscopy to study the interior of the clot, obvious differences were present between FVIII- and emicizumab-dependent clots (Figure 6). A normal fibrin mesh was found in the clots of FVIII-treated mice, whereas a less recognizable fibrin-staining was detected in the clots of emicizumab-treated mice. For the latter, very large, diffusely-stained patterns were observed, characterized by a large number of intense spots, which we referred to as focal points. As for now, we have no information
what these focal points represent in terms of fibrin structure, but they could correspond to the patches we have seen in the scanning-EM images. Another possibility is that these spots represent fibrin that is accumulated at the surface of platelets that are captured in the fibrin network. Additional studies will be performed to get more insight into this aspect.

An intriguing question is what causes the formation of such unusual fibrin-networks under the influence of emicizumab? We observed that the synchronization between fibrin formation and FXIII activation was different between emicizumab and FVIII. The interval between the initiation of fibrin and FXIIIa formation was longer when using emicizumab compared to FVIII (Figure 3). This suggests that the non-covalent polymerization of fibrin profibrils continues over a longer period before FXIIIa starts making the covalent connections. Furthermore, FXIIIa generation was more efficient in the presence of emicizumab, indicating that larger amounts of FXIIIa are available and more covalent connections can be made. These changes in kinetics could potentially contribute to the altered fibrin-network formation. The kinetics by which FXIII is activated has indeed been reported to have an important effect on the final structure of the fibrin network. For instance, the Val34Leu polymorphism in FXIII is associated with accelerated activation by thrombin, and results in a fibrin meshwork with thinner fibers and reduced porosity.31,32

It should be noted that discordant data have been described with regard to the formation of the fibrin network in the presence of emicizumab. Shimonishi et al. reported that the aPTT-reagent triggered fibrin-network generated in vitro in the presence of emicizumab was similar to that of FVIII-induced fibrin.16 In contrast, Janbain et al. found that fibrin-fibers produced in the presence of emicizumab were almost 2-fold thinner than those produced in the presence of FVIII at a concentration of 10 IU/dl.17,33 When comparing the protocols to obtain scanning-EM images to analyze the fibrin-structure, it appears that each of these studies used a different protocol for dehydration. Differences in dehydration-protocols may affect how fibers are represented within scanning-EM images. Second, artificial activating reagents were used in the in vitro studies by Shimonishi et al. and Janbain et al., whereas the fibrin structures in our study were obtained from in vivo generated clots, a more physiological environment that also contains other blood components (red blood cells, platelets). Each of these blood components contribute to the formation of the fibrin-network and its ultimate structure.34

Consequences of different clot architecture: It is tempting to speculate that the unstable clots originate from a modified fibrin-network. Although no direct evidence is provided in this study, the presented in vivo data are compatible with this scenario. In particular, a dense fibrin structure may modify the biomechanical properties of the clot, and the unusual thick fibers may reduce its elasticity.35 Such increased rigidity seems in keeping with the rebleeds that are observed upon clot removal in emicizumab-treated mice, but not rFVIII- or rFVIIIIFc-treated mice (Figure 2).
In conclusion, rFVIII and rFVIII Fc act similarly regarding clot formation and structure. In contrast, the different mode of action of emicizumab alters the kinetics of fibrin and FXIIIa formation, resulting in different clot morphologies. We are aware that these data represent only part of the differences that may occur in FVIII- versus emicizumab-dependent clot formation. Additional studies are in progress aiming to expand our notion of how non-factor molecules like emicizumab modify the clot architecture and therefore the hemostatic process.
References


Legends

Figure 1: Incomplete wound closure.
Hemophilic patient on emicizumab prophylaxis arrived in the hospital with a deep laceration in his foot (day 1). The wound was treated with local tranexamic acid application and covered with steri-strips. At day 10, steri-strips were removed, but the wound was still open, and no wound closure or healing was observed. Patient received FVIII treatment on days 10 and 11. At day 11, wound closure was observed and wound healing process progressed gradually during the following days (day 13 & day 16).

Figure 2: Hemostatic responses after tail vein-transection without and with clot removal.
At a tail-diameter of 2.3mm, an incision with a depth of 0.5mm in the left lateral vein was made. Mice receiving emicizumab also received human FIX and human FX (both 100 IU/kg) 5 min before injury. Both panels depict the bleeding profile of mice receiving vehicle, rFVIII, rFVIIIIFc or emicizumab. Plasma concentrations at the start of the procedure were 10 IU/dl for rFVIII and rFVIIIIFc and 55 μg/ml for emicizumab. Black represents period of bleeding, grey periods of minor bleeding, and white periods of non-bleeding. A: The wound remained unchallenged after the transection of the vein. B: Dotted lines at 900 seconds and 1800 seconds indicate timing of clot removal in mice that did not bleed at those instants. Emicizumab + FIX/FX 12’ refers to re-injection of FIX and FX at t=12 min in emicizumab-treated mice, ie. 3 min before the first clot removal. Emicizumab + TXA refers to emicizumab-treated mice that also received tranexamic acid (10 mg/kg) 5 min before injury. Amounts of blood loss measured in these mice are presented in supplementary figure S1.

Figure 3: In vitro fibrin formation and fibrinolysis. Panel A: Schematic representation of in vitro fibrin formation in human FVIII-deficient plasma supplemented with rFVIII or emicizumab. Panels B-E: FVIII-deficient plasma (panel B) was supplemented with rFVIII at 10 IU/dl (C) or 100 IU/dl (D) or emicizumab at 55 μg/ml (E). In addition, fibrinogen was added (1 mg/ml final concentration) and the reaction was initiated by the addition of CaCl₂. No other additives (tissue factor or factor XIa) were used. Fibrin formation was detected by monitoring OD-values at 405nm, while FXIII activity was measured via hydrolysis of the fluorescent A101 substrate (excitation 313 nm; emission 418 nm). Red line: fibrin formation (left Y-axis) and blue line: FXIII activity (right Y-axis). Presented are the mean (solid lines) and standard error (grey area around the solid line) of 6-8 measurements. AFU: arbitrary fluorescence units.
Figure 4: Scanning-EM imaging of in vivo generated fibrin networks. Panel A: experimental approach for the preparation of tissue sections. The black rectangle indicates the region examined using scanning-EM. Panels B-C: Tail fragments obtained 10 min after TVT were prepared for scanning-EM using standard pre-fixation with 4% glutaraldehyde and 1% OsO4 and dehydration. Presented are representative images of FVIII-deficient mice treated with rFVIII (panel B), or emicizumab (panel C). Plasma concentrations at the start of the procedure were 10 IU/dl for rFVIII and 55 μg/ml for emicizumab. Scale bars represent 10 microns in the large image, and 5 microns in the amplified images. White boxes indicate regions of numeric zooms. Panels D-G: ImageJ-plugin software was used to determine fibrin coverage (% per field; panel D), average fibrin diameter (in micron per field; panel E), number of pores per field (panel F) and the number of intersections per field (panel G). For each graph, 2 mice were included (round and triangle symbols) and 10 fields per mouse were examined. Each symbol represents the result of a single field. Statistical analysis was performed using unpaired Student’s t-test.

Figure 5: Fibrin content within the injured area. Panel A: experimental approach for the preparation of tissue sections. The black rectangle indicates the region examined using spinning disk confocal imaging. Panels B-F: Tail tissue sections obtained 10 min after TVT were prepared for immunofluorescence staining using an anti-mouse fibrin antibody. Panels B-F: representative images of wild-type (WT)-mice (panel B), untreated FVIII-deficient mice (FVIII-KO; panel C); FVIII-deficient mice treated with rFVIII (panel D1-D2), rFVIIIFc (panel E1-E2), or emicizumab (panel F1-F2). Plasma concentrations at the start of the procedure were 10 IU/dl for rFVIII and rFVIIIFc and 55 μg/ml for emicizumab. Panel G: For each condition, two non-subsequent tissue sections of five mice (represented by square, round, diamond, up-triangle and down-triangle symbols) were analyzed using ImageJ-software for the fluorescence intensity (panel E). Statistical analysis was performed using one-way ANOVA with Tukey’s corrections for multiple comparisons. Only statistically significant differences (p<0.05) are indicated. Each symbol represents a separate tissue section of an individual mice, and mean±SD are indicated for each condition.

Figure 6: Fibrin structures imaged using stimulated emission depletion (STED)-microscopy. Panel A: experimental approach for the preparation of tissue sections. The black rectangle indicates the region examined using STED-microscopy. Panels B-E: Representative images of wild-type mice (WT; panel B), FVIII-deficient mice treated with rFVIIIFc (panels C1-C3), untreated FVIII-deficient mice (FVIII-KO; panel D) or FVIII-deficient mice treated with emicizumab (panels E1-E3). Fibrin was detected using polyclonal goat anti-murine fibrin antibodies, and probed using STAR-RED-labeled donkey anti-goat antibodies.
Plasma concentrations at the start of the procedure were 10 IU/dl for rFVIIIFc and 55 μg/ml for emicizumab. Panel F: For each condition, 30 images from 3 different mice (10/mouse) were analyzed using ImageJ-software to calculate the presence of dot-like structures. White arrows in panels C₁-C₃ indicate examples of dot-like structures. Statistical analysis was performed using One-way ANOVA with Dunnet’s test for multiple comparisons.

Figure 7: Correlation between in vitro and in vivo fibrin generation. Panel A: The amount of fibrin generated (referred to as fibrin coverage) in vivo under different conditions (data presented in figure 4 and Supplementary figure S3) was plotted against the in vitro amount of fibrin generated (referred to as maximal (max) absorbance; data presented in figure 3). Panel B: The amount of fibrin generated (referred to as mean fluorescence intensity; MFI) in vivo under different conditions (data presented in figure 5) was plotted against the in vitro amount of fibrin generated (referred to as maximal (max) absorbance; data presented in figure 3). Red symbol: FVIII-KO mice vs FVIII-deficient plasma; orange symbol: FVIII-KO mice receiving 10 IU/dl rFVIII versus FVIII-deficient plasma spiked with 10 IU/dl rFVIII; Green symbol: wild-type (WT)-mice versus FVIII-deficient plasma spiked with 100 IU/dl rFVIII; Blue symbol: FVIII-KO mice receiving emicizumab (55 μg/ml) versus FVIII-deficient plasma spiked with emicizumab (55 μg/ml).
**Figure 3**

**A**

**Fibrin formation:**
- FVIII deficient plasma
- + rFVIII (10 & 100 IU/dl) or emicizumab (55 μg/ml)
- + Fibrinogen (1 mg/ml)
- + CaCl₂ (20 mM)

**B**

- FVIII-deficient plasma
- Time (min)

**C**

- FVIII-deficient plasma + 10 IU/dL rFVIII
- Time (min)

**D**

- FVIII-deficient plasma + 100 IU/dL rFVIII
- Time (min)

**E**

- FVIII-deficient plasma + emicizumab
- Time (min)
Figure 4

Skin

Caudal vein

Head Longitudinal axis Tail

rFVIII

emicizumab

Fibrin coverage (% per field)

Average fibrin diameter (nm per field)

Pores (# per field)

Intersections (# per field)

D

<0.0001

F

<0.0001

G

<0.0001
**Figure 5**

A

Skin

Caudal vein

Head → Longitudinal axis → Tail

B

WT

rFVIII

rFVIII-Fc

Emicizumab

100 μm

C

FVIII-KO

rFVIII

rFVIII-Fc

Emicizumab

100 μm

G

0.0230

<0.0001

<0.0001

<0.0001

<0.0001

Fluorescence intensity fibrin (arbitrary units)

WT

rFVIII

rFVIII-Fc

Emicizumab

FVIII-KO
Supplementary materials

Differences in venous clot structures between hemophilic mice treated with emicizumab versus factor VIII or factor VIIIFc

Thibaud Sefiane et al.

Supplementary methods

Animals and ethics statement

F8-deficient mice were backcrossed (>10 times) on a C57Bl/6 background. Males and females were used throughout the study (8-12 weeks old, 20-25 g). Housing and experiments were performed in accordance with French regulations and the experimental guidelines of the European Community. This project was approved by the local ethical committee of Université Paris-Saclay (Comité d’Ethique en Experimentation Animale n°26, protocol APAFIS#26510-202007061525281 v2).

Patient information

A 2-year-old boy with severe haemophilia A (FVIII <1%) on regular prophylaxis with emicizumab (6 mg/kg every 4 weeks, subcutaneously) since the age of 8 months (per policy of the local treatment center). The patient had no relevant bleeding history and has no history of FVIII inhibitors. He experienced a first small muscle bleed after intramuscular vaccination at the age of 2 months, which was treated with standard half-life rFVIII. A second bleed at the age of 5 months due to an injury of his finger, was also treated with standard half-life rFVIII. The patient has no comorbidities or other chronic treatment besides emicizumab. While on emicizumab, the patient had zero treated bleeds, but reported three non-treated bleeds (post-traumatic haematoma, small wound and dentition, for the latter of which the patient received additional tranexamic acid). The incidence described in this study concerns a glass-induced deep laceration at his left foot.

Proteins & materials

Emicizumab (marketed under Hemlibra) was from F. Hoffman-La Roche (Basel, Switzerland). Recombinant factor IX (FIX, Benefix) was from Pfizer (Paris, France). Plasma-derived factor X (FX) was from Cryopep (Montpellier, France). Recombinant factor VIII (rFVIII, Advate) was from Shire France SAS (Paris, France). Recombinant FVIIIFc (rFVIIIFc, Elocta) was from Sobi (Stockholm, Sweden). Tranexamic acid was from Mylan (Paris, France), and generously provided by Dr. Dominique Lasne. DAPI and Alexafluor 647-labeled chicken anti-goat antibodies were from Invitrogen (Saint Aubin, France). Polyclonal rabbit anti-human Fc was from Synobiological (Düsseldorf, Germany). Polyclonal goat anti-mouse fibrin antibodies (cat#
YNGMFBG7S) were from Accurate chemical (Carle Place, NY). Although categorized as anti-murine fibrinogen antibodies, these antibodies recognize fibrin and cross-linked fibrin but not murine fibrinogen in Elisa and tissue sections. STAR RED-labeled donkey anti goat antibodies used for Stimulated Emission Depletion (STED)-microscopy were from Abberior (Göttingen, Germany). FVIII-deficient plasma and purified fibrinogen (depleted for VWF and fibrin) were from Stago BNL (Leiden, the Netherlands). Fluorescent substrate A101 for FXIIIa was from Interchim (Montluçon, France).

**Emicizumab-compatible mouse model**
Mice were given emicizumab via retro-orbital intravenous injection 24 h before tail vein-transection (TVT) to obtain plasma concentrations of 55 µg/ml at the time of the transection.² A second retro-orbital injection was given 5 min before the TVT-procedure, using a solution containing human FIX and FX (both 100 U/kg).

**FVIII-mediated correction of bleeding**
Mice were given rFVIII or rFVIIIFc (100 µl volume) via retro-orbital injection 5 min before TVT to obtain plasma concentrations of 10 IU/dl at the time of the transection.

**Tail vein-transection**
For the tail vein-transection (TVT)-procedure, mice were anesthetized with isoflurane and a precise cut of the caudal tail vein was performed as described.³ After vein transection, the tail was immediately immersed in physiological saline, prewarmed at 37 °C. At the end of the assay (45 min observation time) animals were sacrificed by cervical dislocation. Mean values of the blood loss volume (µl) were reported. The mixture of collected blood and physiological saline was centrifuged at 1,500 g. The red blood cell pellet was then lysed in H₂O and the amount of hemoglobin was determined by reading the absorbance at 416 nm. The volume of blood loss in each sample was calculated from a standard curve, which was obtained by lysing defined volumes of mouse blood (20-40-60-80-100 µl) in H₂O to extract hemoglobin.

**Fibrin formation**
Fibrin formation experiments were performed in half-well microtiter-plates in a volume of 100 µl. Mixtures consisted of FVIII-deficient plasma (50 µl), purified fibrinogen (40 µl of 2.5 mg/ml), and rFVIII, rFVIIIFc or emicizumab (5 µl of 200 IU/dl to reach 10 IU/dl rFVIII or rFVIIIFc; 5 µl of 2000 IU/dl to reach 100 IU/dl rFVIII or rFVIIIFc; 5 µl of 1.1 mg/ml to reach 55 µg/ml emicizumab). The reaction was started by the addition of CaCl₂ (5 µl/well). OD at 405 nm was measured every 30 sec for a 2 h period.
**FXIIIa generation**

FXIIIa generation experiments were performed in half-well microtiter-plates in a volume of 100 µl. Mixtures consisted of FVIII-deficient plasma (50 µl), purified fibrinogen (40 µl of 2.5 mg/ml), and rFVIII, rFVIIIIFc or emicizumab (5 µl of 200 IU/dl to reach 10 IU/dl rFVIII or rFVIIIIFc; 5 µl of 2000 IU/dl to reach 100 IU/dl rFVIII or rFVIIIIFc; 5 µl of 1.1 mg/ml to reach 55 µg/ml emicizumab). The reaction was started by the addition of CaCl$_2$ (5 µl/well). FXIIIa generation was monitored using fluorescent substrate A101 (excitation 313 nm; emission 418 nm).

**Scanning electron microscopy**

**Dehydration-preparation:** 10 min after the TVT-procedure, the whole tail was dissected and immediately immersed in 4% PFA/1% glutaraldehyde and fixed overnight at 4 °C. Preparation of the sections for analysis was performed essentially as described.$^4$ A 1-cm tail section centered around the injury was progressively dehydrated via serial alcohol incubations: 2x 10 min in 50% ethanol, 2x 10 min in 70% ethanol, 2x 10 min in 95% ethanol, 3x 15 min in 100% ethanol. All incubation were performed under gentle agitation. Dehydrated sections were dried using hexamethyldisilazane (HMDS) as follows: 15 min in 2:1 100% ethanol/HMDS, 15 min in 1:1 100% ethanol/HMDS, 15 min in 1:2 100% ethanol/HMDS and 3x 15 min in HMDS. Samples were metallized using a sputtering device (Balzers Sputter Coater SCD 050; Leica) with the following setting: 10 nm of silver; 120 seconds at 5 cm; 30 mA. Samples were immediately analyzed using a Philips XL-Environmental Scanning Electron Microscope.

**Immunostaining of fibrin**

The whole tail was dissected 10 min after the TVT-procedure, immediately immersed in 4% paraformaldehyde (PFA), and fixed overnight at 4 °C. A 1-cm tail section centered around the injury was prepared and washed for 2h in PBS and embedded in Tissue-Tek optimum-cutting temperature (OCT)-compound. Tissues sections of 7 µm were collected using a cryostat (Leica) on glass slides. Staining of fibrin was performed using polyclonal goat antibodies against murine fibrin/fibrinogen (6 µg/ml) and Alexafluor 647-labeled chicken anti-goat antibodies (4 µg/ml). Images were obtained using high-resolution tilling reconstruction using spinning disk confocal microscopy (Olympus IX73 equipped with CrestOptics X-Light V1) as described previously.$^5$ Quantification of immunofluorescence was performed using ImageJ-software.

**Stimulated Emission Depletion (STED)-microscopy**
Tissues sections were generated and stained for fibrin as described under “Immunostaining for fibrin”, with the exception that STAR RED-labeled donkey anti goat antibodies (5 µg/ml) were used as secondary antibody. STED microscopy was performed on the Stedycon system (Abberior Instruments, Göttingen, Germany) composed of an AxioObserver 7 inverted microscope (Zeiss, Germany).6

*Image analysis*

Image analysis was performed using ImageJ-software. DiameterJ-plugin was used for the analysis of the Scanning-EM images of the fibrin-network.

*Statistical analysis*

All data are presented as mean±standard deviation (mean±SD) unless indicated otherwise. Number (n) refer to the number of independent experiments or animals. The statistical analysis was performed using GraphPad Prism 9 software for Mac (La Jolla, California, USA). One-way analysis of variance (1-way ANOVA) followed by Tukey’s or Dunnett’s multiple comparison test was performed when comparing multiple groups. Pairwise analysis was performed using the unpaired Student’s t-test or the Mann-Whitney test, where appropriate. P<0.05 was considered as statistically significant.
Supplementary figure S1: Blood loss in FVIII-deficient mice after treatment with rFVIII, rFVIII:Fc or emicizumab.

At a tail-diameter of 2.3 mm, an incision with a depth of 0.5 mm in the left lateral vein was made, and blood loss was monitored for 45 min. Mice were given emicizumab 24 h before injury, allowing plasma concentrations of 55 µg/ml at the start of the TVT-procedure. In addition, mice received human factor IX and factor X (both 100 IU/kg) 5 min before injury. Concentrations of rFVIII and rFVIII:Fc were 10 IU/dl at the time of injury. Panel A: Blood loss in mice without clot removal. Panel B: At 15 min and 30 min after injury, the clot was removed via gentle wiping, but only if the mouse was not bleeding at that particular moment. One series of mice (Emicizumab + extra FIX & FX) received additional FIX/FX at a dose of 100 IU/kg at 12 min after injury, ie. 3 min before the first clot removal. A second series of mice (Emicizumab + tranexamic acid) received tranexamic acid at a dose of 10 mg/kg at 5 min before injury. Statistical analysis was performed via One-way ANOVA with Tukey’s correction for multiple comparisons.
Supplementary figure S2

Legend Supplementary figure S2: In vitro fibrin formation comparing rFVIII to rFVIIIFc.

FVIII-deficient plasma was supplemented with rFVIII or rFVIIIFc at 100 IU/dl. In addition, fibrinogen was added (1 mg/ml final concentration) and the reaction was initiated by the addition of CaCl₂. No other additives (tissue factor or factor Xla) were used. Fibrin formation was detected by monitoring OD-values at 405 nm. Blue line: rFVIII and red line: rFVIIIFc. Presented are the mean (solid lines) and standard error (orange-colored areas around the solid line) of 6-8 measurements.
Legend Supplementary Figure S3: Scanning-EM imaging of *in vivo* generated fibrin networks. *Panel A-B*: Tail fragments obtained 10 min after TVT were prepared for scanning-EM using standard pre-fixation with 4% glutaraldehyde and 1% OsO4 and dehydration. Presented are representative images of wild-type (WT; *panel A*) and FVIII-deficient (FVIII-KO; *panel B*) mice. Scale bars represent 10 microns. *Panels C-F*: ImageJ-plugin software was used to determine fibrin coverage (% *per* field; *panel C*), average fibrin diameter (in micron *per* field; *panel D*), number of pores *per* field (panel *E*) and the number of intersections *per* field (panel *F*). For each graph, 2 mice were included (round and triangle symbols) and 10 fields *per* mouse were examined. Each symbol represents the result of a single field. Data for emicizumab (presented in Figure 4D-G) are included for comparison. Statistical analysis was performed using One-way ANOVA with Tukey’s test for multiple comparisons.
**Supplementary figure S4**

Legend **Supplementary figure S4**: Fibrin content within the injured area. Tail tissue sections obtained 10 min after TVT of FVIII-deficient mice that were given rFVIII, rFVIIIFc or emicizumab were prepared for immunofluorescence staining using an anti-mouse fibrin antibody. Plasma concentrations at the start of the procedure were 10 IU/dl or 500 IU/dl for rFVIII (black and blue symbols, respectively) and rFVIIIFc (white and red symbols, respectively) and 55 µg/ml for emicizumab (grey symbols). For each condition, two non-subsequent tissue sections of five mice (represented by square, round, diamond, up-triangle and down-triangle symbols) were analyzed using ImageJ software for the fluorescence intensity. Statistical analysis was performed using one-way ANOVA with Tukey's corrections for multiple comparisons. Each symbol represents a tissue section of an individual mice, and mean±SD are indicated for each condition. Data for emicizumab and 10 IU/dl for rFVIII and rFVIIIFc are identical to those presented in figure 5.
Supplementary references


