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

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Supplementary materials

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Supplementary methods

Animals and ethics statement

F8-deficient mice[1] 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 H_2O 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 H_2O 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 $CaCl_2$ (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, 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 $CaCl_2$ (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 immerged 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 immerged 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]

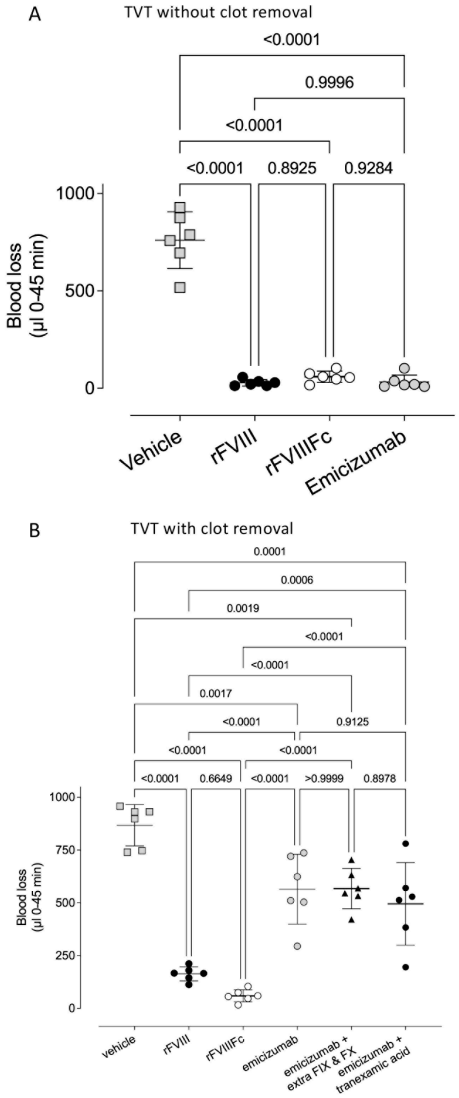
Image analysis

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.

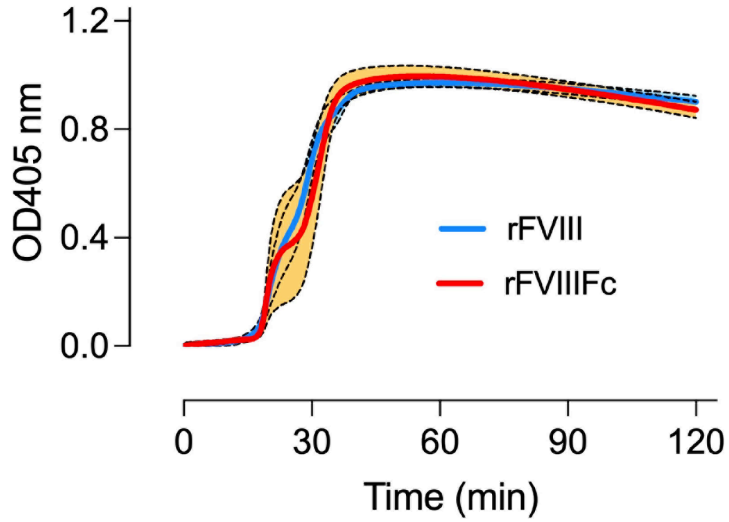
Supplementary figure S1



Supplementary figure S1: Blood loss in FVIII-deficient mice after treatment with rFVIII, rFVIIIFc or emicizumab.

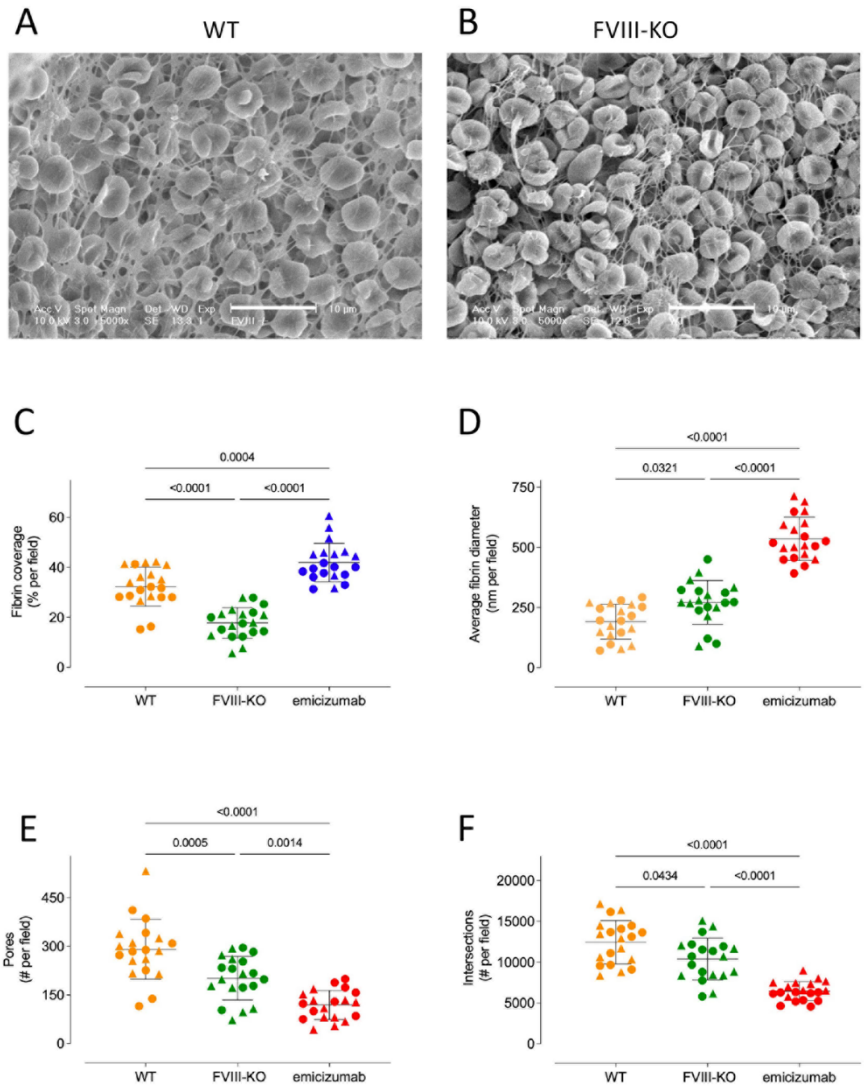
At a tail-diameter of 2.3 mm, an incision with a depth of 0.5 mm in the left lateral vein was made, and blood loss was monitored for 45 min. Mice were given emicizumab 24 h before injury, allowing plasma concentrations of 55 μg/ml at the start of the TVT-procedure. In addition, mice received human factor IX and factor X (both 100 IU/kg) 5 min before injury. Concentrations of rFVIII and rFVIIIFc 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



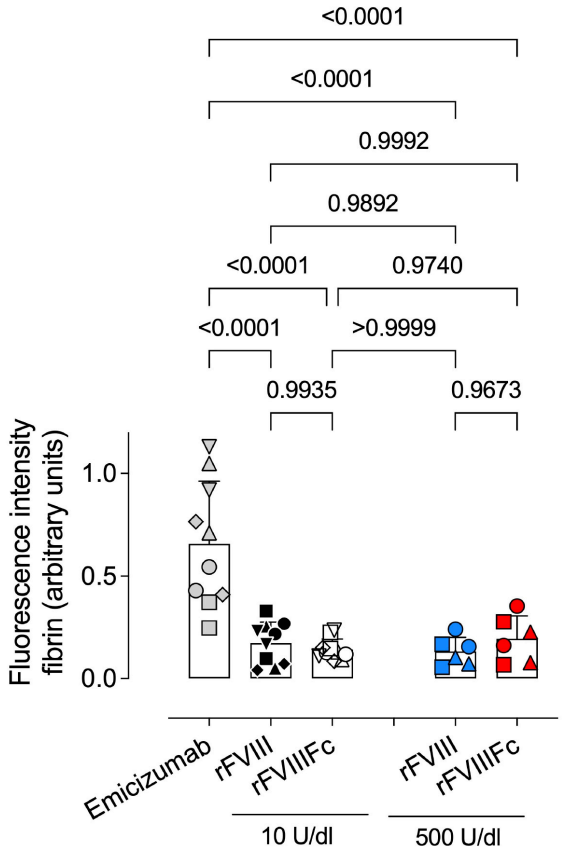
Legend Supplementary figure S2: In vitro fibrin formation comparing rFVIII to rFVIIIFc. FVIII-deficient plasma was supplemented with rFVIII or rFVIIIFc at 100 IU/dl. In addition, fibrinogen was added (1 mg/ml final concentration) and the reaction was initiated by the addition of $CaCl_2$. No other additives (tissue factor or factor XIa) 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.

Supplementary figure S3



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

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