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Coagulation Factor VIII Regulates von Willebrand Factor Homeostasis In Vivo

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Summary

Background.—Coagulation factor VIII (FVIII) and von Willebrand factor (VWF) circulate as a noncovalent complex, but each has its distinct functions. Binding of FVIII to VWF results in a prolongation of FVIII's half-life in circulation and modulates FVIII's immunogenicity during hemophilia therapy. However, the biological effect of FVIII and VWF interaction on VWF homeostasis is not fully understood.

Methods.—Mouse models, recombinant FVIII infusion, and hemophilia A patients on a high dose FVIII for immune tolerance induction therapy or emicizumab for bleeding symptoms were included to address this question.

Results.—an intravenous infusion of a recombinant B-domain less FVIII (BDD-FVIII) (40 and 160 μg/kg) into wild-type mice significantly reduced plasma VWF multimer sizes and its antigen levels; an infusion of a high but not low dose of BDD-FVIII into *Adamts13*$^{+/-}$ and *Adamts13*$^{-/-}$ mice also significantly reduced the size of VWF multimers. However, plasma levels of VWF antigen remained unchanged following administration of any dose BDD-FVIII into *Adamts13*$^{-/-}$ mice, suggesting the partial ADAMTS13 dependency in FVIII-augmented VWF degradation. Moreover, persistent expression of BDD-FVIII at ~50–250 U/dL via AAV8 vector in hemophilia A mice also resulted in significant reduction of plasma VWF multimer sizes and

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Authors' contributions

WC and XLZ designed the study, analyzed the results, and wrote the manuscript. A.B., AB, NY, LZ, BSD, LAG, DES, and RMC performed experiments and data analysis and revised manuscript. All authors approved the final version of the manuscript for submission.

Disclosure

XLZ is a consultant and a member of the advisory boards for Alexion, Apollo, GC Biopharma, Sanofi, Stago, and Takeda. XLZ is also the co-founder of Clotsolution. WC holds equity in Ivygen. All other authors have declared no relevant conflict.

antigen levels. Finally, the sizes of plasma VWF multimers were significantly reduced in patients with hemophilia A who received a dose of recombinant or plasma derived FVIII for immune tolerance induction therapy.

Conclusion.—Our results demonstrate the pivotal role of FVIII as a cofactor regulating VWF proteolysis and homeostasis under various (patho) physiological conditions.

Keywords

Coagulation factor VIII; von Willebrand factor; regulation; proteolysis; ADAMTS13; thrombosis

Introduction

von Willebrand factor (VWF) is synthesized and released from vascular endothelium, which is primarily stored in Weibel-Palade bodies with small amount constitutively secreted into circulation[1, 2]. VWF polypeptide forms dimers via a tail-to-tail disulfide bond in the endoplasmic reticulum and multimerizes via a head-to-head disulfide bond, resulting in the formation of ultra large multimers of up to 50–100 polypeptides[2, 3]. VWF multimers also form bundles that remain anchored on endothelial surface through lateral-lateral association[4–6]. Endothelial and plasma VWF multimers play a critical role in regulating normal hemostasis and inflammatory responses by recruiting platelets and leukocytes from circulation[7–12]. Increased levels of plasma or endothelium-anchored ULVWF are shown to be associated with many inflammatory thrombotic disorders, including thrombotic thrombocytopenic purpura (TTP)[13, 14], myocardial infarction[15, 16], ischemic stroke[15, 17], preeclampsia[18, 19], and COVID-19 associated coagulopathy, etc.[20, 21].

The key enzyme regulating VWF homeostasis is plasma ADAMTS13[22, 23], which cleaves the Tyr^{1605}-Met^{1606} bond located in the central A2 domain of VWF[24, 25]. This proteolytic cleavage is essential for eliminating endothelium anchored ULVWF strings and for reducing the size of circulating VWF multimers[5]. Other serine proteases such as neutrophil proteinase 3, cathepsin G, and elastase, as well as plasmin have been shown to cleave VWF under certain experimental conditions[26, 27], but their physiological relevance in VWF homeostasis *in vivo* remains unknown. The proteolytic cleavage of soluble VWF by ADAMTS13 may be regulated at the substrate-level by fluidic shear[28–30]. For instance, binding of platelet glycoprotein (GP) 1b[31, 32] or FVIII[31, 33, 34] or ApoB100/LDL[35] to VWF appears to accelerate VWF proteolysis by ADAMTS13 under arterial shear. However, the pathophysiological relevance of FVIII dependent regulation of VWF proteolysis or homeostasis remains controversial. Chen et al. reported a normal plasma VWF multimer distribution in patients with severe hemophilia A. They conclude that VWF proteolysis may occur normally in the absence of FVIII[36]. However, a prolonged infusion of very high levels of recombinant FVIII into a patient with severe hemophilia A with inhibitors resulted in depletion of VWF, producing a laboratory picture which is consistent with the diagnosis of von Willebrand's disease[37].

Here, we employ multiple animal models and hemophilia A patient samples on immune tolerance treatment (ITI) to demonstrate that FVIII plays a critical role in regulating VWF homeostasis under (patho) physiological conditions. First, we show that an intravenous

infusion of recombinant FVIII in *wild-type* and *Adamts13*-deficient mice or over expression of recombinant FVIII in hemophilia A mice via AAV vector alters VWF homeostasis, resulting in dramatic reduction of plasma VWF multimer size and/or its antigen levels. Additionally, a daily infusion of high doses of recombinant or plasma derived FVIII for ITI treatment in hemophilia A patients significantly reduces plasma VWF multimer size. These results support the hypothesis that FVIII may be a physiological cofactor that regulate VWF homeostasis, primarily through enhanced proteolytic cleavage of VWF by ADAMTS13. Our results provide mechanistic insight into how FVIII may be used as an adjunctive therapy for hereditary thrombotic thrombocytopenic purpura (TTP) and perhaps other arterial thrombotic diseases resulting from an imbalance of VWF to ADAMTS13 axis.

Methods

Preparation of recombinant FVIII and ADAMTS13:

B-domain deleted FVIII variant (BDD-FVIII) was constructed as previously described[38]. Briefly, BDD-FVIII variant lacks residues 744–1637 and has a 14-aa linker between the heavy (1–740, A1–A2 domains) and light (1649–2332, a3–A3–C1–C2) chains[34]. BDD-FVIII ($M_{r,}$165,000) was stably expressed in a baby hamster kidney (BHK) cell line and purified using a combination of a SP-Sepharose FF and a Poros HQ/20 according to the procedure previously published[38, 39]. Recombinant full-length ADAMTS13 with a His-V5 tag at its C-terminus was expressed in stably transfected HEK293 cell line and purified with a combination of Q-fast flow and Ni-chelating affinity chromatography as previously described[30, 40].

Mice maintenance and administration of FVIII:

All animal experimental protocol were approved by the institutional animal care and use committees. *WT* and *Adamts13*$^{-/-}$ mice (CAST/Ei) and maintained at the University of Kansas Medical Center. Hemophilia A (HA) CD4 knockout (HA/CD4, C57Bl/6) mice were maintained at Children's Hospital of Philadelphia. Recombinant BDD-FVIII was intravenously administrated through tail vein in a total of 200 μL Hanks′ balanced salt solution (HBBS). Mouse blood was collected by retro-orbital bleeding and anticoagulated with 0.38% sodium citrate plus 10 mM ethylenediaminetetraacetic acid (EDTA).

Preparation and administration of AAV vector:

The Institutional Animal Care and Use Committees (IACUC) at the University of Kansas Medical Center and Children's Hospital of Philadelphia approved the animal protocols. A cDNA encoding the BDD-FVIII with a liver-specific transthyretin promoter was packaged into a recombinant AAV serotype 8 (AAV8) vector. The AAV8 vector was produced using a triple transfection protocol as previously described[41, 42]. The vector titer (vg/mL) was determined by SDS-polyacrylamide gel electrophoresis followed by silver staining and quantitative polymerase chain reaction (qPCR). Male HA/CD4 mice (C57Bl/6) were used for the AAV8 expression studies. Mouse blood was collected by a tail-clip following anesthesia and anticoagulated with 0.38% sodium citrate. Platelet-poor plasma was collected and aliquoted for storage at −80 °C until assay.

Collection of hemophilia A patient plasma:

The Institutional Review Board (IRB) at the University of Kansas Medical Center and Children's Hospital of Philadelphia approved the human study protocols. Citrated plasma was collected from patients with hemophilia A at different times and stored at −80 °C until assay. All assays were performed in a blind fashion and patient information was extracted from electronic medical records.

Plasma factor VIII activity and antigen:

FVIII activity in each sample was measured using a chromogenic Coatest assay (Diapharma, West Chester, OH). FVIII antigen was determined using paired commercial FVIII antibodies from Affinity Biologicals (Ancaster, Canada).

VWF multimer analysis:

Plasma VWF multimers were determined by mini-agarose gel electrophoresis followed by Western blotting with a rabbit anti-VWF IgG from Agilent Dako (Santa Clara, CA) followed by an IRDye 800CW-labeled goat anti-rabbit IgG from LI-COR Biosciences (Lincoln, NE). Odyssey imaging analysis, also from LI-COR Biosciences, was used to obtain the fluorescent signals. ImageJ software, available from National Institute of Healthy website, was used to determine the ratios of high molecular weight (H) to low molecular weight (L) portion of the VWF multimers as previously described[33, 43].

VWF antigen analysis:

Plasma VWF antigen was quantified by an in-house enzyme-linked immunosorbent assay (ELISA) using two different polyclonal antibodies from Dako as described previously[44].

Statistical analysis:

Paired student *t*-test (parametric) or Mann-Whitney test (non-parametric) was performed to determine the statistical significance in the ratios of H to L molecular weight of VWF multimers and plasma VWF antigen levels between pre and 24h-post infusion of BDD-FVIII. *P* values less than 0.05 and 0.01 are statistically significant and highly significant, respectively.

Results

Preparation and characterization of recombinant proteins and mice:

Human recombinant BDD-FVIII was expressed in baby hamster kidney (BHK) cells and purified to homogeneity as described in the Methods. The purified BDD-FVIII showed three distinct bands, corresponding to single chain BDD-FVIII, a heavy chain, and a light chain of BDD-FVIII following intracellular proteolytic cleavage on the SDS-polyacrylamide gel (PAGE) with Coomassie blue staining (Fig. 1A). *Adamts13*$^{-/-}$mice (CAST/Ei), originated from Dr. David Ginsburg laboratory at University of Michigan, Ann Arbor, MI)[45], were bred with *wild-type* (*WT*) mice (CAST/Ei) from Jackson Laboratory (Bar Harbor, ME) to generate *Adamts13*$^{+/-}$ and *Adamts13*$^{-/-}$ mice for the study. Plasma ADAMTS13 activity in *WT*, *Adamts13*$^{+/-}$, and *Adamts13*$^{-/-}$ mice was determined using a modified ELISA-based

assay (Technoclone, Vienna, Austria). The results showed no ADAMTS13 activity in plasma of *Adamts13*$^{-/-}$ mice, 25 to 100% of ADAMTS13 activity in *WT* mice, and ~50% of ADAMTS13 activity in *Adamts13*$^{+/-}$ mice (Fig. 1B). The variability of plasma ADAMTS13 activity in the *WT* mice is not fully understood. Of notes, the murine plasma ADAMTS13 activity was only about 50% of human plasma ADAMTS13 activity when a human specific VWF73 substrate was used in the assay. A chromogenic FVIII assay showed no FVIII activity in the plasma of *fVIII*$^{-/-}$ mice. Plasma FVIII activity ranged from 50 to 250 U/dL (corresponding to 0.5 to 2.5 nM of protein concentration) with an average of 190 U/dL (or 1.9 nM) in the *fVIII*$^{-/-}$ mice 13 weeks of post transduction with an AAV8 vector containing a human *BDD-FVIII cDNA* (Fig. 1C). These results demonstrate the availability of a crucial recombinant BDD-FVIII protein and two different animal models for the studies.

Infusion of recombinant human BDD-FVIII reduces plasma VWF multimer size and antigen levels in WT mice:

To determine if FVIII enhances the degradation of endogenous VWF under physiological conditions, a purified human recombinant BDD-FVIII was intravenously infused at 40 μg/kg (estimated plasma concentration, ~0.5 μg/mL or ~3.0 nM) or 160 μg/kg (estimated plasma concentration, ~2.0 μg/mL or ~12.1 nM) into *WT* mice that contain normal plasma levels of ADAMTS13 activity. Plasma VWF multimers and antigen levels were determined 24 hours following the infusion using agarose gel electrophoresis and ELISA-based assay, respectively. As shown, the ratios (mean ± SEM) of H molecular weight to L molecular weight of plasma VWF multimers were significantly reduced in *WT* mice receiving either a low dose (40 μg/kg or 3.0 nM) (2.6 ± 0.2 vs. 2.0 ± 0.2, $p<0.05$) (Fig. 2A, 2B, and 2C) ($p<0.005$) or a high dose (160 μg/kg or 12.1 nM) (2.7 ± 0.3 vs. 2.2 ± 0.2, $p<0.01$) (Fig. 2D, 2E, and 2F) of recombinant BDD-FVIII compared with those in the sample collected from same mice one week prior to the BDD-FVIII infusion. Additionally, there was a modest reduction of plasma VWF antigen levels in these mice following either a low (11.8 ± 0.9 vs. 10.4 ± 1.1 μg/mL, $p<0.05$) (Fig. 2G and 2H) or a high dose (13.1 ± 1.5 vs. 10.6 ± 1.4 μg/mL, $p<0.05$) (Fig. 2I and 2J) of recombinant BDD-FVIII infusion compared with those in the samples collected from the same mice one week prior to the BDD-FVIII infusion. These results demonstrate that recombinant BDD-FVIII may enhance the degradation of ultra large and high molecular weights of plasma VWF multimers *in vivo* and the degraded VWF fragments may be preferentially cleared via a mechanism that is not yet understood.

Infusion of recombinant BDD-FVIII also reduces the plasma VWF multimer size in *Adamts13*$^{+/-}$ mice:

To determine if FVIII influences VWF degradation when plasma ADAMTS13 activity is substantially reduced, we infused a recombinant human BDD-FVIII at both low (40 μg/kg or 3.0 nM) and high (160 μg/kg or 12.1 nM) doses into *Adamts13*$^{+/-}$ mice in which plasma ADAMTS13 activity is approximately one half of normal activity of the *WT* mice. Interestingly, the plasma ADAMTS13 activity was much less variable in *Adamts13*$^{+/-}$ than *WT* mice as shown in previous Fig. 1B. An infusion of a low dose of recombinant BDD-FVIII had little effect on plasma VWF multimer distribution with similar ratios of high to low molecular weight VWF multimers (3.1 ± 0.2 and 2.8 ± 0.3 before and after BDD-FVIII infusion, respectively. $p>0.05$) (Fig. 3A, 3B, and 3C). An infusion of a high

dose of recombinant BDD-FVIII resulted in significantly reduced ratios of high to low molecular weights of plasma VWF multimers (3.6 ± 0.2 vs. 2.5 ± 0.2, $p<0.0001$) (Fig. 3D, E, and F). However, no change in plasma VWF antigen levels regardless of dosage of recombinant BDD-FVIII administered (Fig. 3G, 3H, and 3I) ($p>0.05$). These results indicate that murine plasma VWF multimer degradation enhanced by recombinant FVIII at least in part depends on plasma ADAMTS13 activity.

Infusion of recombinant BDD-FVIII also reduced plasma VWF multimer sizes but has no effect on antigen levels in *Adamts13*$^{-/-}$ mice:

To determine if VWF degradation following recombinant FVIII infusion is dependent on plasma levels of ADAMTS13 activity, we infused recombinant BDD-FVIII at low and high doses into *Adamts13*$^{-/-}$ mice as described in the previous section. An infusion of low dose (40 μg/kg or 3.0 nM) of recombinant BDD-FVIII resulted in no change in plasma VWF multimer distribution with the ratios of high to low molecular weight VWF multimers of 3.4 ±0.3 and 3.2 ± 0.2, pre- and post-infusion, respectively ($p>0.05$) (Fig. 4A, 4B, and 4C). However, an infusion of a high dose (160 μg/kg or 12.8 nM) of recombinant BDD-FVIII did result in a significant reduction of the ratios of high to low molecular weight VWF multimers, with the ratios of 3.8 ± 0.3 and 2.8 ± 0.2, pre- and post-infusion, respectively, $p<0.005$) (Fig. 4D, 4E, and 4F). Interestingly, there was no difference in plasma VWF antigen levels following either a low or a high dose of recombinant BDD-FVIII infusion (Fig. 4G, 4I, and 4J) ($p>0.05$). These results suggest that while plasma ADAMTS13 may play a key role in mediating FVIII-augmented degradation of VWF multimers, other leukocyte proteases, such as elastase, proteinase 3, cathepsin G, and matrix metalloprotease 9 (MMP9)[26], may also participate in this process when plasma FVIII levels are at the super physiological levels.

Recombinant BDD-FVIII expressed via an AAV8 vector enhances the degradation of plasma VWF multimers and reduces its antigen levels in *fVIII*$^{-/-}$ mice:

To determine if recombinant FVIII reduces plasma VWF multimer sizes and antigen levels under physiological conditions, we intravenously administered an AAV8 vector containing a human recombinant BDD-FVIII expression cassette (1×10^{11}vg/kg) into the HA/CD4 *fVIII*$^{-/-}$ mice. Plasma VWF multimer distribution and antigen levels were assessed 3 months (or ~13 weeks) following the AAV8 administration as outlined (Fig. 5A). Multimer analysis demonstrated that in comparison with untreated *fVIII*$^{-/-}$ mice on the same genetic background (Fig. 5B and 5D), the ratios of high to low molecular weight VWF multimers (mean ± SEM) were significantly reduced in hemophilia A (or *fVIII*$^{-/-}$) mice received the dose of AAV8-BDD-FVIII (1.8 ± 0.1) compared with those that did not (0.9 ± 0.3) ($p<0.05$) (Fig. 5C and 5D). Additionally, plasma VWF antigen levels in hemophilia A mice received AAV8-BDD-FVIII were also significantly reduced compared with those in the untreated mice on the same genetic background (Fig. 5E) ($p<0.01$). These results demonstrate that FVIII may play a pivotal role in regulating VWF homeostasis under physiological conditions.

Immune tolerance induction therapy with FVIII reduces VWF multimer size in patients with hemophilia A:

Hemophilia A patients with inhibitors require treatment with large doses of recombinant or plasma-derived FVIII over a long period of time as immune tolerance induction (ITI) therapy[46, 47]. Alternatively, patients may be treated with emicizumab, a bivalent monoclonal antibody targeting both activated coagulation factor IX and factor X to activate factor X, as a prophylactic therapy against spontaneous bleeding[48, 49]. Plasma samples were obtained from 7 patients with severe hemophilia A ≥24 hours following FVIII exposure during daily ITI therapy, or during emicizumab prophylactic therapy, or at a trough period during a FVIII prophylactic therapy as detailed in Table 1. The median age of this cohort of patients was 3.5 years. All patients received a FVIII dosage between 65 units/kg (~93% normal level) and 200 units/kg (~285% of normal) daily during ITI. Emicizumab was given every 14–28 days at the doses from 3 to 6 mg/kg, respectively. 12/17 samples showed FVIII activity less than 1% at time of sample analysis. Multimer analysis demonstrated that the ratios of high to low molecular weight VWF multimers were significantly reduced in the plasma of hemophilia A patients following ITI therapy, compared with those who had no FVIII exposure or following treatment with emicizumab (Fig. 6A and 6B) ($p<0.01$). However, plasma VWF antigen levels were not significantly reduced in hemophilia A patients receiving ITI compared with those in patients who were not exposed to FVIII or treated with emicizumab (Fig. 6C) ($p>0.05$). As expected, patients who received ITI showed significantly higher plasma FVIII antigen levels than those without FVIII exposure or with emicizumab treatment (Fig. 6D) ($p<0.05$). The variability in FVIII antigen levels seen in the ITI cohort matches the time intervals between FVIII exposure and sample analysis (Table 1). These results corroborate the findings from two different animal models demonstrating the role of FVIII as a physiological cofactor augmenting the degradation of VWF by ADAMTS13 or perhaps other enzymes under various (patho)physiological conditions.

Discussion

The present study using several different animal models and human disease samples demonstrate the role of FVIII in regulation of VWF homeostasis under various physiological conditions. In mice, plasma VWF multimer sizes and antigen levels are significantly reduced following an intravenous infusion of recombinant human BDD-FVIII in a BDD-FVIII dose and ADAMTS13-dependent manner. Additionally, a restoration of plasma FVIII to the physiological levels in hemophilia A mice via an AAV8-mediated expression of recombinant BDD-FVIII also results in a significant reduction of plasma VWF multimer size and its antigen levels. Most importantly, patients with severe hemophilia A receiving an ITI therapy exhibit a significantly reduced VWF multimer sizes compared with those who do not have recent FVIII exposure or are treated with emicizumab for prophylaxis. Emicizumab, a bispecific humanized monoclonal antibody, binds factor IX and factor X to activate coagulation cascade without the need of FVIII[50, 51]. Together, our data support the hypothesis that FVIII is a cofactor that regulates VWF homeostasis by ADAMTS13 and perhaps other proteases under (patho)physiological conditions.

The current data are consistent with those previously reported using *in vitro* shear-based assay in which addition of recombinant FVIII to VWF accelerates the proteolytic cleavage of VWF by ADAMTS13 under arterial shear[31, 33]. Subsequently, Cao et al showed that an expression of recombinant human or canine FVIII expression via a hydrodynamic injection of a plasmid through a tail vein also resulted in a dramatic reduction in plasma VWF multimer sizes in *wild-type* mice[43]. However, these experimental conditions were criticized to be “too harsh” or “non-physiological”, resulting in massive release of endothelial VWF or causing endothelial damage. Our experimental conditions in this study, however, are all under physiological conditions which do not induce any significant stress as seen in the hydrodynamic challenge. The present results are also consistent with the previous study demonstrating that high dose recombinant FVIII treatment in hemophilia A rats[52] significantly reduces endogenous plasma VWF levels.

Additional evidence to support the role of FVIII as a physiological cofactor regulating VWF homeostasis comes from patients with hereditary TTP who received a treatment with plasma derived FVIII concentrate. Upon FVIII infusion, platelet counts rose quickly with concomitant reduction of serum lactate dehydrogenase activity[53, 54], indicative of TTP resolution. This therapeutic effect was interpreted as the addition of trace amount of contaminated ADAMTS13 in the FVIII concentrate preparation[55], although plasma ADAMTS13 activity in these patients following the infusion of FVIII concentrates was not significantly increased (data not shown). We believe that the therapeutic efficacy of plasma FVIII concentrates may primarily be mediated through an enhanced proteolysis of VWF by residual plasma ADAMTS13 enzyme.

However, the mechanism underlying FVIII-enhanced VWF proteolysis by ADAMTS13 or VWF homeostasis is not fully understood. Using the optical tweezer, Cao et al have demonstrated that binding of recombinant FVIII to VWF fragments containing D’D3-A1A2 or A2 domain alone induces a conformational change in the A2 domain, resulting in alteration of the pulling force profile[33], although direct kinetic of A2 cleavage by ADAMTS13 in the absence or presence of FVIII has not been determined due to the technical difficulty using the optical tweezer. In addition to ADAMTS13, other serine proteases such as cathepsin G, proteinase 3, and elastase[26], and plasmin[27] as well as MMP9[26] may cleave VWF when ADAMTS13 is absent. This hypothesis is further supported by the increase of VWF proteolysis in *Adamts13*$^{-/-}$ mice following an infusion of a high dose (160 μg/mL) of recombinant FVIII.

It is still not known whether FVIII or enhanced proteolysis of VWF accelerates VWF clearance. FVIII alone has a half-life of less than 2 hours as free FVIII molecules are rapidly cleared via low-density lipoprotein (LDL) receptor-related protein (LRP1)[56, 57]. Both LDL-receptor (LDLR)[58] and heparan-sulfate proteoglycans (HSPG)[59] facilitate the LRP1-mediated clearance of free FVIII. These receptors also have the potential to internalize FVIII by themselves. Other potential receptors for FVIII include megalin, asialoglycoprotein receptor (ASGPR) and other unidentified carbohydrate receptors[60]. In the presence of VWF, human FVIII’s half-life is significantly prolonged (to nearly 8 hours)[61, 62]. However, human FVIII only has a shorter half-life of 4.3 hours in mice[63]. This is reflected by less and transient effect of an infused human BDD-FVIII but more profound or persistent effect of

AAV8-mediated expression of human BDD-FVIII at physiological or supraphysiological levels on VWF homeostasis in mice. Plasma VWF, on the other hand, has a half-life of 12 hours[3]. It is cleared through macrophage galactose-type lectin (MGL), a C-type lectin that binds to glycoproteins like VWF which contains terminal N-acetyl galactosamine or galactose residues[64]. This is estimated in the setting of normal plasma FVIII concentration. In patients with hemophilia A or in mice with FVIII deficiency, the half-life of VWF may be prolonged. Conversely, in the presence of a high concentration of FVIII, the half-life of VWF may be shortened, resulting in a reduction of VWF multimer size and antigen levels. However, this hypothesis is yet to be tested in our future studies. It may also be possible that proteolytically cleaved fragments of VWF is cleared faster than the intact VWF multimer due to the lack of accumulation of smaller bands despite a significant reduction of ultra large VWF multimers in all *in vivo* studies.

We conclude that our results demonstrate that FVIII is a physiological cofactor that regulates VWF homeostasis via an enhanced proteolytic cleavage of VWF by ADAMTS13 and perhaps other proteases under arterial flow. Thus, FVIII may be useful as a potential adjunctive treatment for arterial thrombotic conditions associated with an imbalanced VWF/ADAMTS13 axis such as TTP, COVID-19, and trauma-associated coagulopathy. On the other hand, at clinical scenarios when a prolonged high dose FVIII therapy is needed, we may need to monitor the risk of bleeding due to enhanced VWF degradation.

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Key points

- The role of factor VIII in regulating VWF homeostasis *in vivo* remains to be established.
- Using various mouse models, we demonstrate the accelerated VWF degradation following an infusion of recombinant factor VIII.
- VWF multimers in hemophilia A patients on factor VIII immune tolerance induction are smaller than those on emicizumab.
- Our results indicate that factor VIII is a physiological regulator of VWF homeostasis.

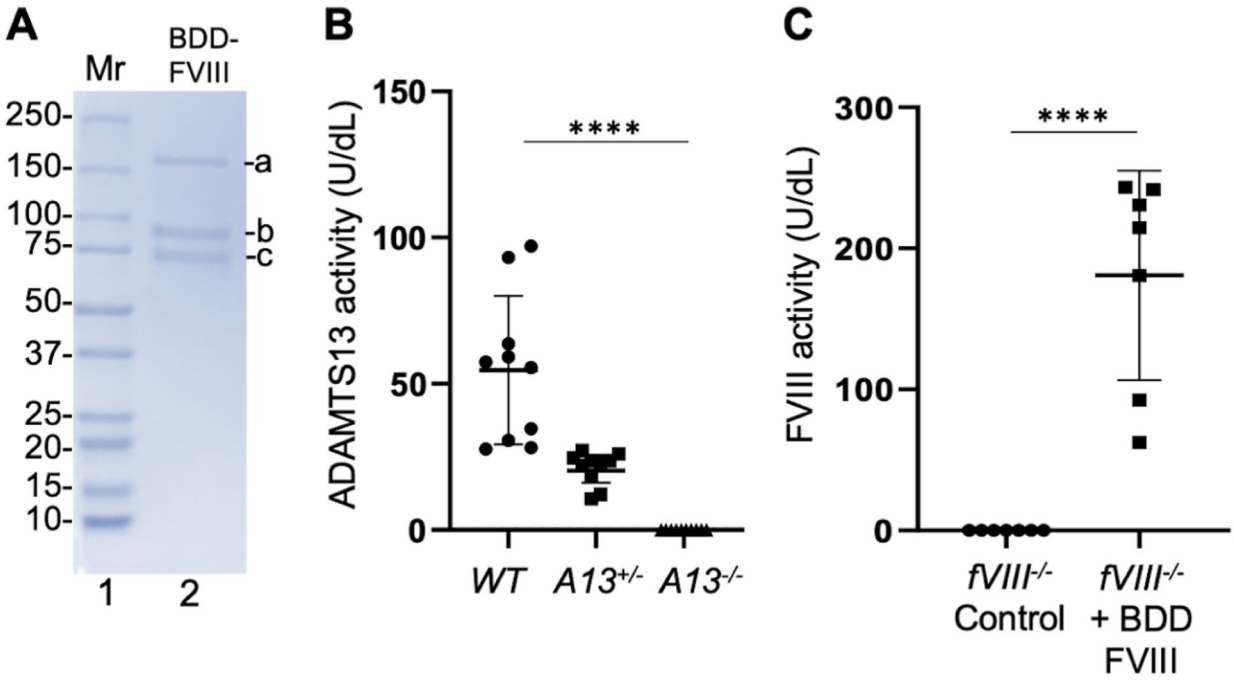

Fig. 1. Characterization of recombinant FVIII protein and mutant mice

. **A**. SDS-PAGE with Coomassie blue staining for a protein marker (Lane 1) and a purified recombinant B domain-deleted FVIII (DBBFVIII) (Lane 2); a, b, and c denote the intact, heavy chain, and light chain, respectively. **B**. Plasma ADAMTS13 activity in wild-type (*WT*), *Adamts13*$^{+/-}$ (*A13*$^{+/-}$), and *Adamts13*$^{-/-}$ (*A13*$^{-/-}$) mice determined by an ELISA-based assay using a pooled human normal plasma as a calibrator; **C**. Plasma FVIII activities in *fVIII*$^{-/-}$ mice 13 weeks following administration of nothing or AAV8-BDD-FVIII. Data in panels B and C were presented as the mean ± standard deviation (SD). Kruskal-Willis one way analysis and Mann-Whitney analysis were performed to determine the difference among three groups in panel B and between two groups in panel C. Here, four stars (****) indicates a p value <0.001.

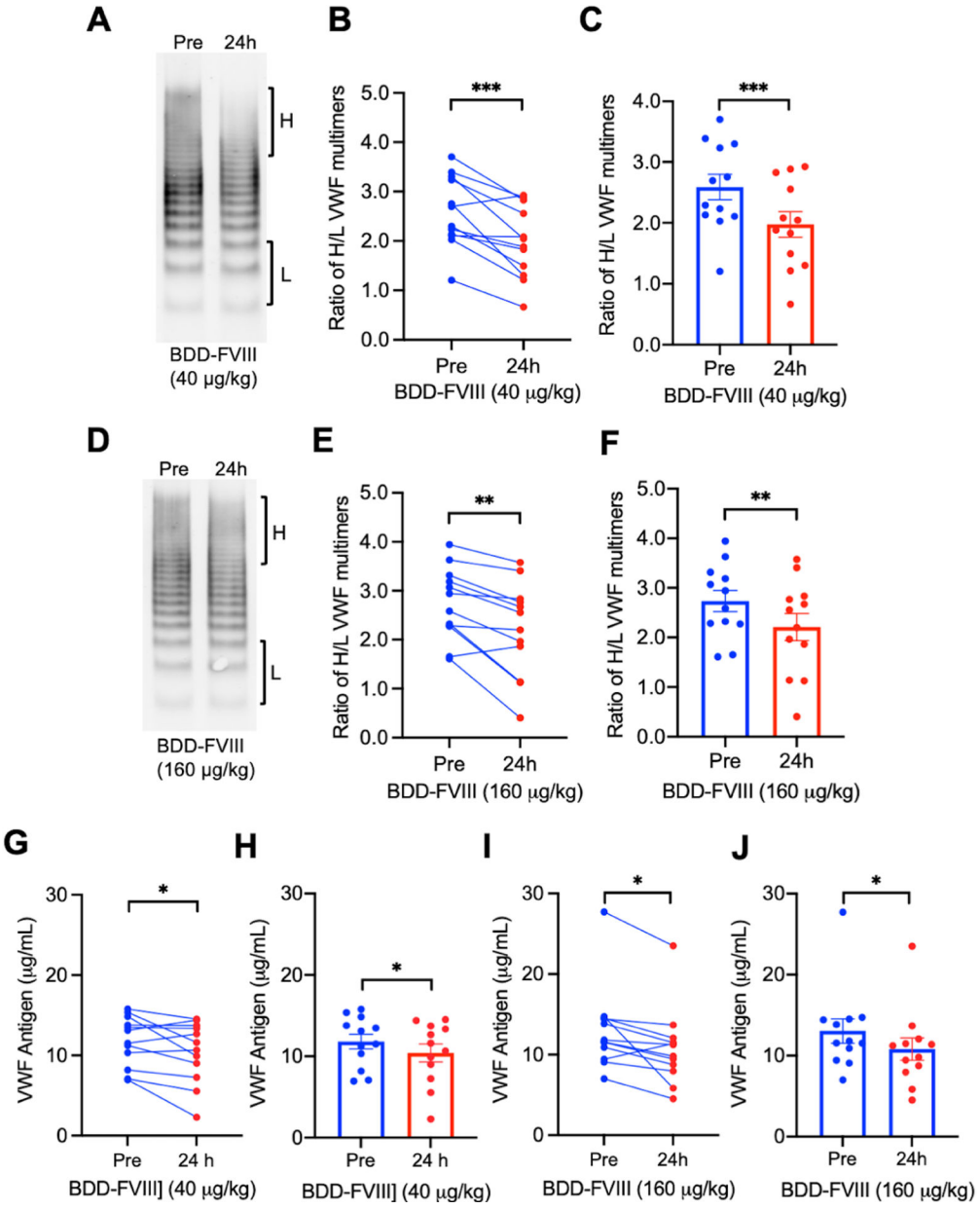

Fig. 2. Effect of recombinant BBD-FVIII on plasma VWF multimer distribution and antigen levels in *wild type* mice

. **A** and **B/C** show the multimer distribution and the ratios of high (H) to low (L) molecular weight of VWF multimers, respectively, in *wild-type (WT)* mice prior to (pre) and 24 hours following an infusion of recombinant BDD-FVIII (40 μg/kg or 3.0 nM). **D** and **E/F** are the multimer distribution and the H/L ratios of VWF multimers, respectively, in *WT* mice pre and 24 hours of post infusion of BDD-FVIII (160 μg/kg or 12.1 nM). **B/E**. Paired individual values of H/L ratios of VWF multimers pre and post BDD-FVIII infusion. **C/F**. Individual values and mean ± SEM of H/L ratios of VWF multimers pre and post BDD-FVIII infusion. **G/H and I/J** show the plasma VWF antigen levels pre and 24 hours of post BDD-FVIII infusion at 40 μg/kg (3.0 nM) and 160 μg/kg (12.1 nM), respectively. **G/I.** Paired individual

values of VWF antigen pre and post BDD-FVIII infusion. **H**/**J**. Individual values and mean ± SEM of VWF antigen pre and post BDD-FVIII infusion. A paired t-test was performed for determine the statistical significance of the difference of two different time points. Here *, **, and *** indicate a p value less than 0.05, 0.01, and 0.005, respectively.

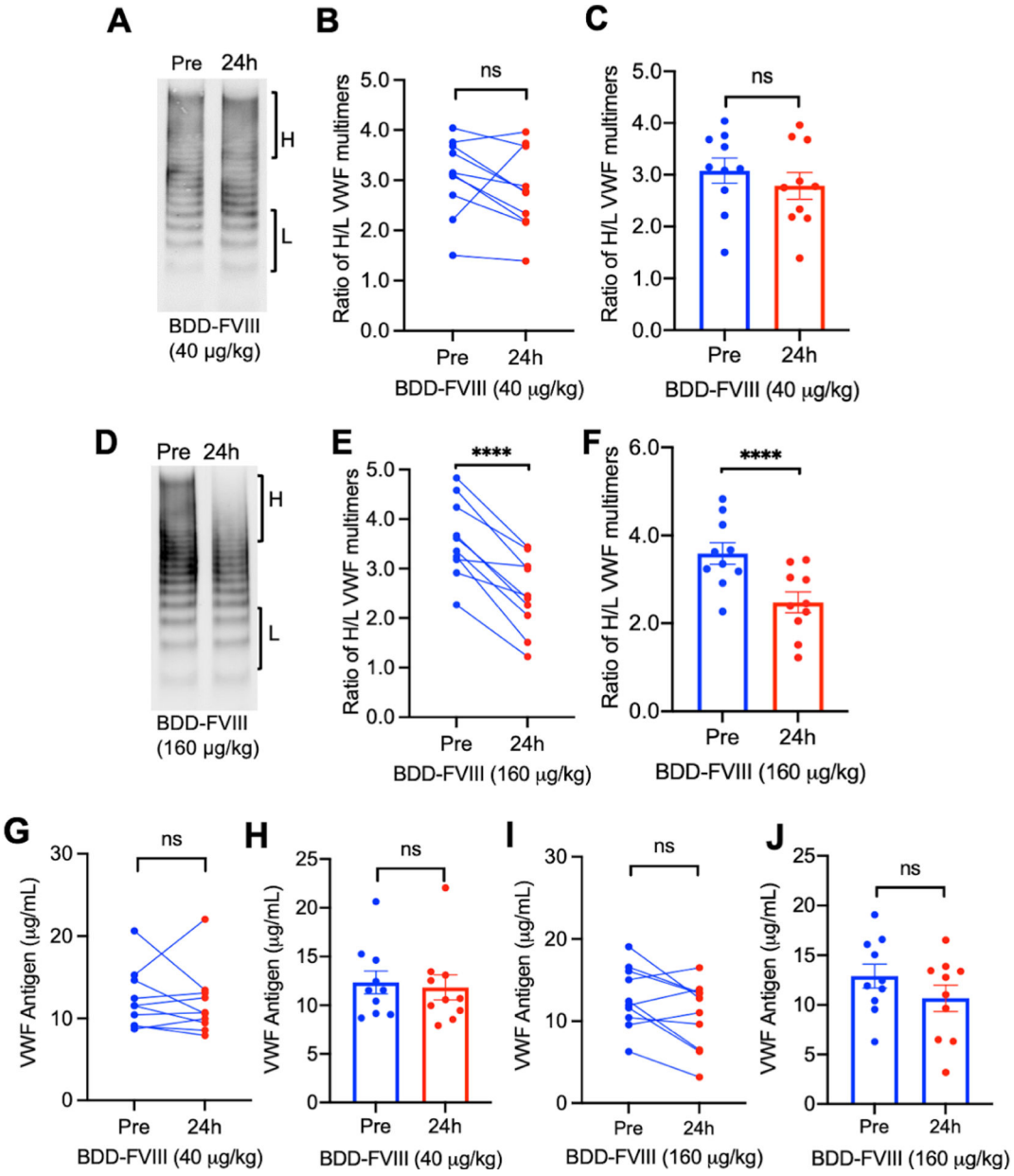

Fig. 3. Effect of recombinant BDD-FVIII on plasma VWF multimer distribution and its antigen levels in *Adamts13*$^{+/-}$ mice.

A and **B/C** show the multimer distribution and the ratios of high (H) to low (L) molecular weight of VWF multimers, respectively, in *Adamts13*$^{+/-}$ *(A13*$^{+/-}$*)* mice pre and 24 hours post Infusion of BDD-FVIII at 40 μg/kg (or 3.0 nM). **D and E/F** are the multimer distribution and the ratios of H/L molecular weight of VWF multimers, respectively, in *Adamts13*$^{+/-}$ *(A13*$^{+/-}$) mice pre and 24 hours post infusion of BDD-FVIII at 160 μg/kg (or 12.1 nM). **G/H** and **I/J** are the plasma VWF antigen levels pre and 24 hours of post infusion of BDD-FVIII at 40 μg/kg (or 3.0 nM) and 160 μg/kg (or 12.1 nM), respectively. A paired t-test was performed for determine the statistical significance of the difference of two different time points. Here, ns and **** indicate *p* value greater than 0.05 and less than 0.0001, respectively.

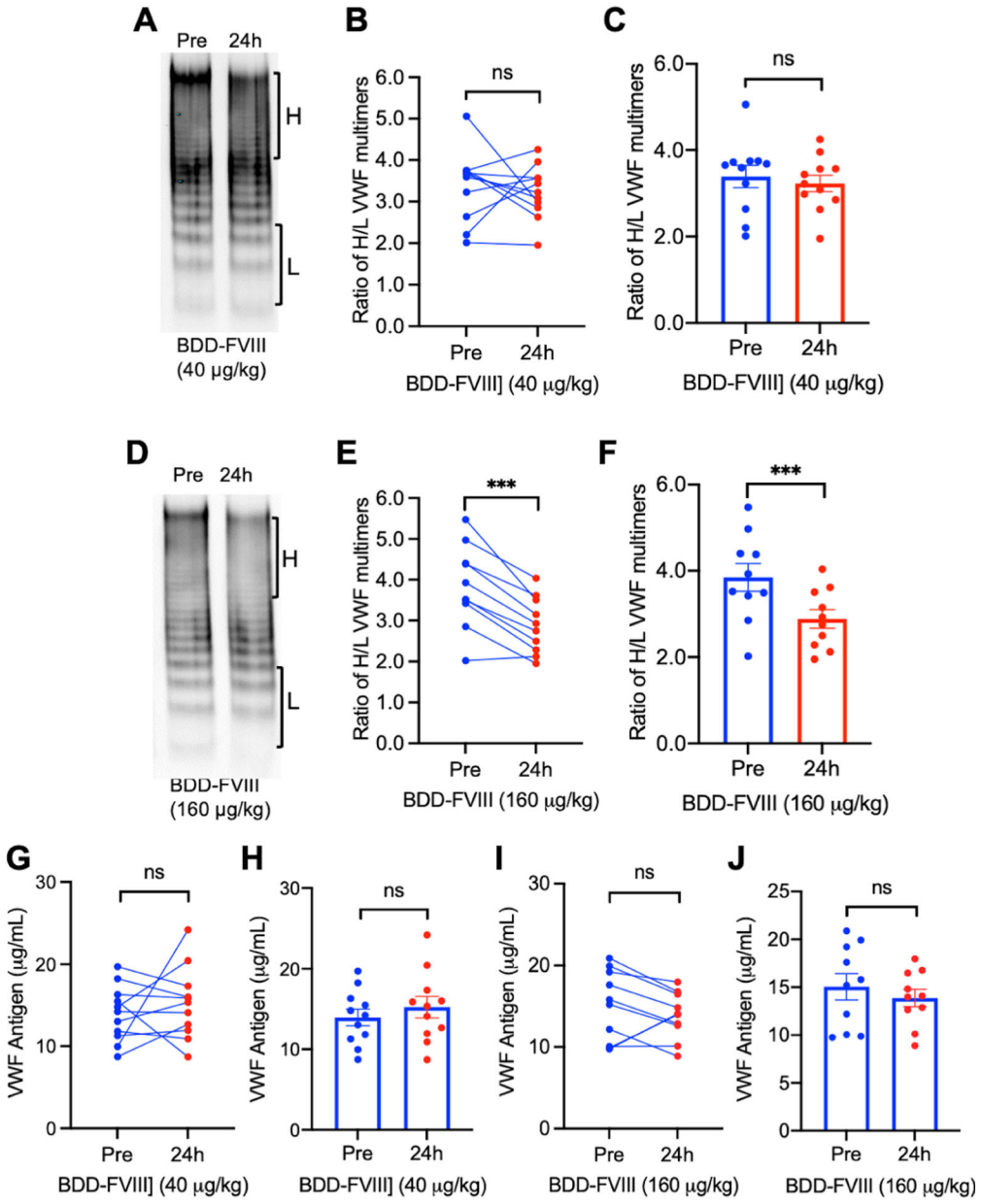

Fig. 4. Effect of recombinant BDD-FVIII on plasma VWF multimer distribution and antigen in *Adamts13*$^{-/-}$ mice.

A and **B/C** show the plasma multimer distribution and the ratios of high (H) to low (L) molecular weight of VWF multimers, respectively, in *Adamts13*$^{-/-}$ (*A13*$^{-/-}$) mice pre and 24 hours of post infusion of BDD-FVIII at 40 μg/kg (or 3.0 nM). **D** and **E/F** are the plasma multimer distribution and the ratios of H/L molecular weight of VWF multimers, respectively, in *Adamts13*$^{-/-}$ (*A13*$^{-/-}$) mice pre and 24 hours post infusion of BDD-FVIII at 160 μg/kg (or 12.1 nM). **G/H** and **I/J** show the plasma VWF antigen levels in *A13*$^{-/-}$ mice pre and 24 hours of post infusion of BDD-FVIII at 40 μg/kg (or 3.0 nM) and 160 μg/kg (or 12.1 nM), respectively. A paired t-test was performed for determine the statistical significance between two different time points. Here, ns and *** indicate *p* value greater than 0.05 and less than 0.005, respectively.

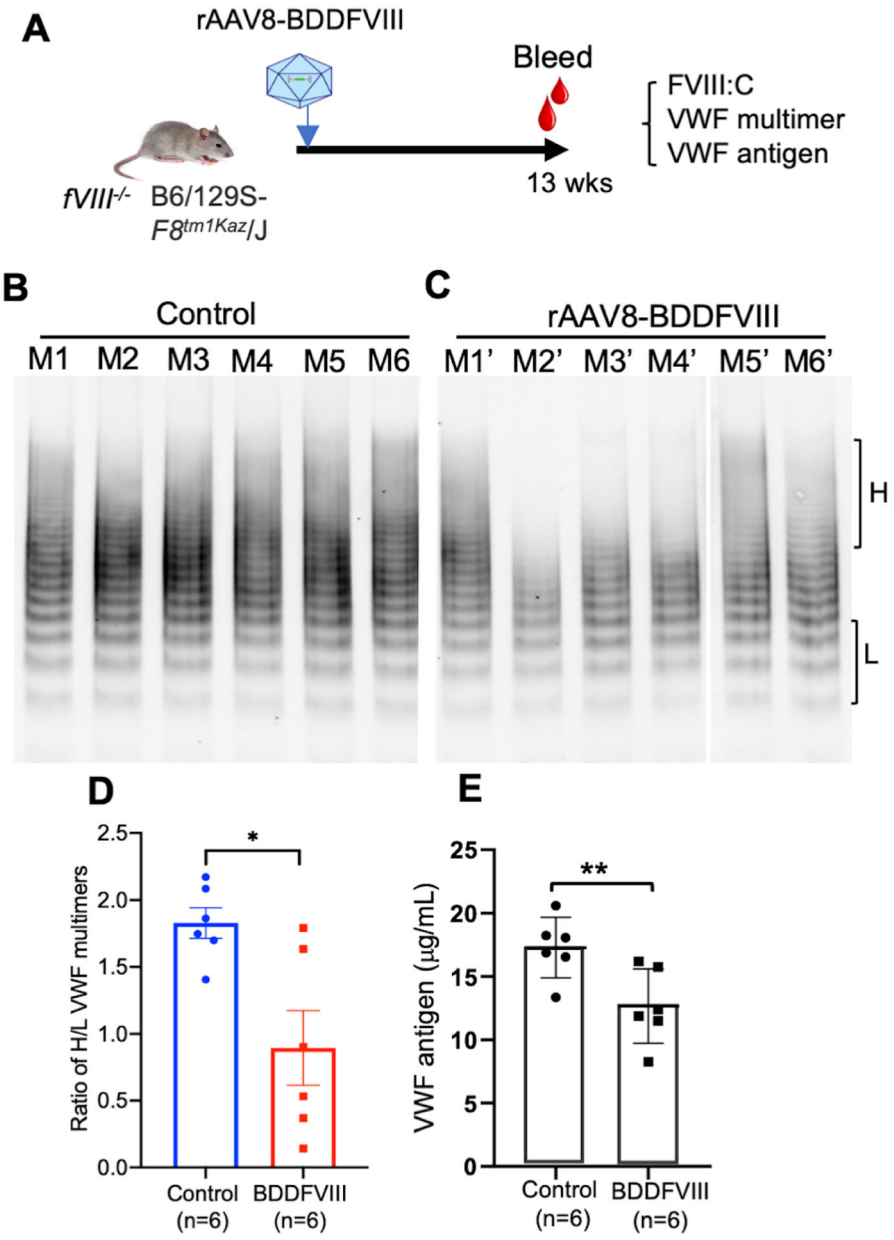

Fig. 5. Plasma VWF multimer distribution and antigen levels in *fVIII*$^{-/-}$ mice following AAV8-mediated expression of recombinant BDD-FVIII.

A. An outlined protocol for administration of AAV8-BDD-FVIII vector and blood collection. **B** and **C.** Plasma VWF multimer distribution in the untreated *fVIII*$^{-/-}$ mice (control) and mice received AAV8-BDD-FVIII (1×10^{11} vg/mouse), respectively. **D**. The individual values and mean ± SEM of the ratios of high (H) to low (L) molecular weight of VWF multimers in *fVIII*$^{-/-}$ mice without or with expression of BDD-FVIII. **E**. The individual and mean ± SEM of plasma VWF antigen levels. Mann-Whitney test was performed to determine the statistical significance. Here, * and ** indicate the *p* value less than 0.05 and 0.01, respectively.

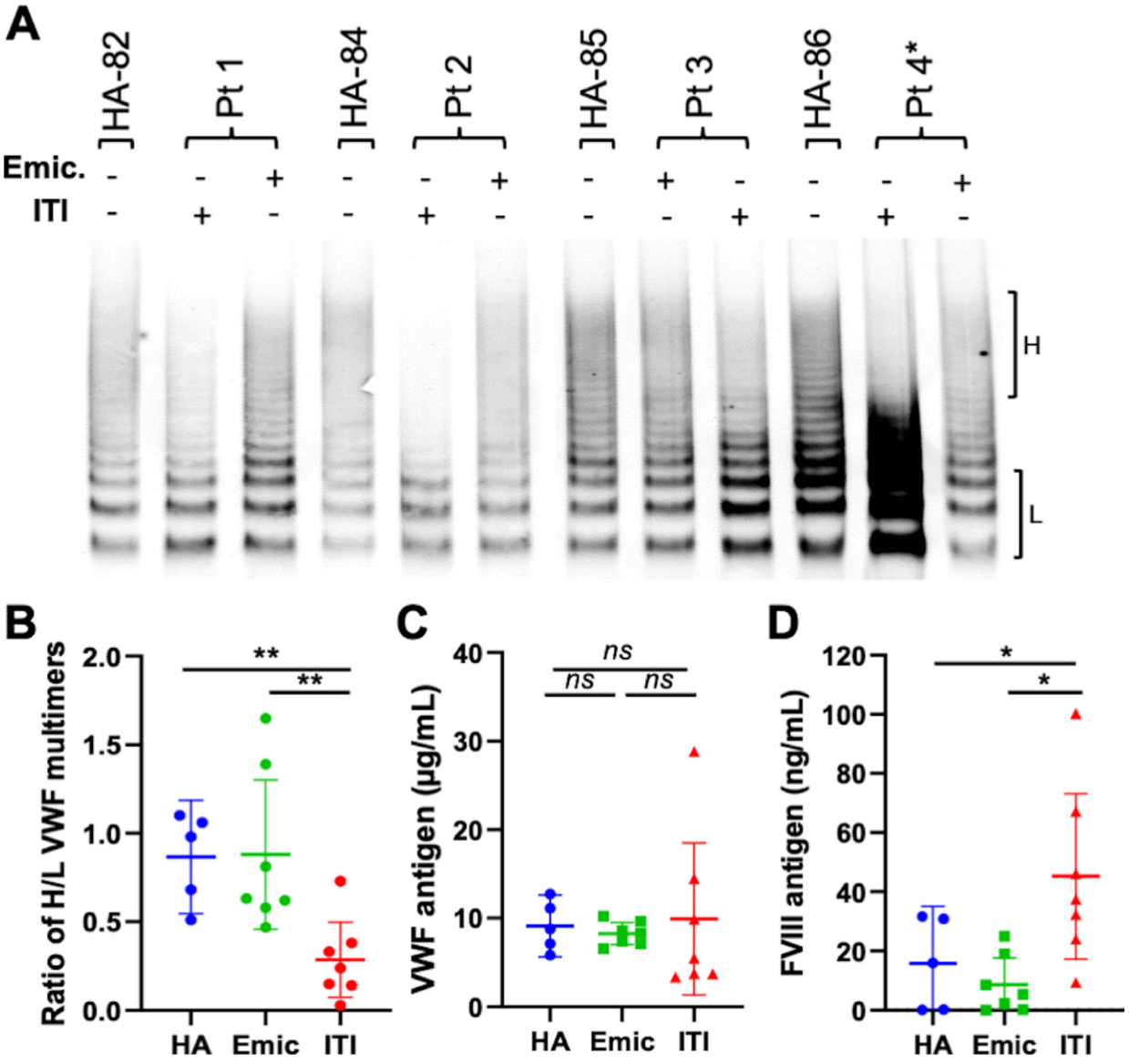

Fig. 6. Plasma VWF multimer distribution and its antigen levels in haemophilia A patients with immune tolerance induction or emicizumab therapy.

A. A representative image showing plasma VWF multimer distribution in 4 haemophilia A patients (Pt1-Pt4) who were treated with (+) immune tolerance induction (ITI) or emicizumab (Emic.) or without (−). Plasma from 4 other haemophilia A patients without recent FVIII exposure (HA-82, HA-84, HA-85, and HA-86) was used for additional controls. **B**, **C** and **D** show the ratios of high (H) to low (L) molecular weights of plasma VWF multimers, VWF antigen levels, and FVIII antigen, respectively, in haemophilia A patients who did not have recent FVIII exposure (HA), those who treated with ITI, and those with Emici. The data are presented as the median and interquartile range (IQR). Kruskal-Wallis test was performed to determine the statistical significance. Here, ns, *, and ** indicate the *p* value greater than 0.05, less than 0.05, and 0.01, respectively.

Table 1.

Demographic and clinical information of hemophilia A patients with inhibitors

Patients	Gender	Race	Age (yrs) at sampling	Prescribed Treatment	FVIII or Emicizumab dosage	Infusion Frequecy	Time since last dose (h)	Time since last FVIII exposure if on emicizumab	FVIII inhibitor titers (BU/mL)	FVIII: Act* (%)	FVIII:Ag (ng/mL)	VWF:Ag (μg/mL)
Pt 1	M	Other	4.6	rFVIII	100 units/kg	daily	24		0	< 1	9.4	5.5
			9.5	Emicizumab	3 mg/kg	every 14d	120	1 mo	0	267	2.3	10.2
Pt 2	M	Asian	14.5	rFVIII	100 units/kg	daily	UNK		53	< 1	0.3	3.8
			16.7	rFVIII	100 units/kg	daily	12		3	< 1	24.0	7.1
			18.1	Emicizumab	3 mg/kg	every 14d	UNK	> 1 yr	26	< 1	1.9	5.3
Pt 3	M	Caucasian	3.5	rFVIII (Xyntha)	200 units/kg	daily	24		74	< 1	67.2	9.9
			8.6	Emicizumab	1.5 mg/kg	weekly	120		4	< 1	24.9	8.6
Pt 4	M	Caucasian	4.4	pdFVIII	100 units/kg	daily	24		0	< 1	100.3	8.3
			9.2	Emicizumab	6 mg/kg	every 28d	24 days	> 1 yr	0	< 1	5.3	9.7
			11	Emicizumab	3 mg/kg	every 14d	UNK	6 mo	3	< 1	−0.3	28.8
Pt 5	M	Caucasian	2.1	rFVIII + FEIBA	75–200 units/kg	daily	24		3	< 1	37.4	3.7
			5.2	rFVIII-Fc (Eloctate)	65–115 units/kg	3x/wk	72**		0	2	20.3	7.9
Pt 6	M	African American	2.3	rFVIII	100 units/kg	daily	24		38	< 1	46.1	3.3
			3.1	rFVIII	100 units/kg	daily	26		0	36	63.7	8.7
			6.8	Emicizumab	3 mg/kg	every 14d	UNK	> 1 yr	0	UNK	8.6	7.4
Pt 7	M	Other	1.8	rFVIIa	2 mg	daily	UNK	several mo	14	< 1	19.0	6.6
			2.3	pdFVIII + FEIBA	75–100 units/kg	daily	22		2	UNK	32.4	14.5

Pt, patient; M, male; BU: Bethesda unit; rFVIII: recombinant FVIII (Advate); pdFVIII: plasma derived FVIII (Aphanate); FEIBA: factor VIII inhibitor bypass activity; rFVIIa, novoseven;

*FVIII activity was determined by one-stage APTT assay on emicizumab;

**the sample at 72 h trough after 115 units/kg dose; d, days; mo, months; yr, years; UNK, unknown.