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ORIGINAL ARTICLE

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Plasma-derived FVIII/VWF complex shows higher protection against inhibitors than isolated FVIII after infusion in haemophilic patients: A translational study

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Abstract

Introduction: Presence of von Willebrand factor (VWF) in FVIII concentrates offers protection against neutralizing inhibitors in haemophilia A (HA). Whether this protection is more evident in plasma-derived (pd) FVIII/VWF or recombinant (r) FVIII concentrates remains controversial.

Aim: We investigated the protection exerted by VWF against FVIII inhibitors in an in vivo mouse model of HA exposed to pdFVIII/VWF or to various rFVIII concentrates.

Methods: Haemophilia A mice received the different FVIII concentrates after administration of vehicle or an inhibitory IgG purified from a commercial pool of HA plasma with inhibitors and FVIII:C recoveries were measured. Furthermore, using a novel clinically oriented *ex vivo* approach, Bethesda inhibitory activities (BU) of a commercial pool of HA plasma with inhibitors were assessed using normal plasma, or plasma from severe HA patients, without inhibitors, after treatment with the same concentrates.

Results: in vivo studies showed that pdFVIII/VWF offers markedly higher protection against inhibitors when compared with any of the FVIII products without VWF. More importantly, in the *ex vivo* studies, plasma from patients treated with pdFVIII/VWF showed higher protection against inhibitors (*P* values ranging .05-.001) in comparison with that observed in plasma from patients who received FVIII products without VWF, regardless of the type of product evaluated.

Conclusion: Data indicate that FVIII+VWF complexes assembled in the circulation after rFVIII infusion are not equivalent to the naturally formed complex in pdFVIII/VWF. Therefore, rFVIII infused into HA patients with inhibitors would be less protected by VWF than the FVIII in pdFVIII/VWF concentrates.

KEYWORDS
FVIII inhibitors, FVIII^{null} E16 Knockout mice, haemophilia A patients, haemophilia A treatment, plasma-derived FVIII, recombinant FVIII, von Willebrand factor

1 | INTRODUCTION

The development of neutralizing antibodies to factor VIII (FVIII) in patients with severe haemophilia A (HA) is the most important complication of FVIII replacement therapy. This complication can affect 25–35% of the patients and has been associated with significant increases in morbidity and socio-economic costs.[1–3] Development of inhibitory antibodies in HA is regulated by a complex balance of genetic factors, environmental variables, and inflammatory conditions developing in patients.[4] One hypothesized risk factor for the development of these inhibitory antibodies is the source of the FVIII concentrate used for treatment. FVIII concentrates can be plasma-derived (pd) or recombinant (r). Furthermore, biochemical modifications in the FVIII molecule are dependent on the molecular structure: full-length versus B-domain deleted (BDD), or source: hamster (Chinese hamster ovary [CHO]; baby hamster kidney [BHK]) versus human (Human embryonic kidney cells; HEK) cells.[5]

Differential immunogenicity of FVIII concentrates of various origins has been documented in various epidemiologic studies. Data from earlier clinical trials suggest that the presence of von Willebrand factor (VWF) in the therapeutic FVIII concentrates may confer protection from the development of neutralizing antibodies in severe HA patients.[6] In a randomized controlled trial in previously untreated patients, a significant increase in the risk of inhibitor development was observed in patients treated with rFVIII compared with those treated with pdFVIII/VWF.[7,8] The lower immunogenicity of pdFVIII/VWF is believed to derive from the natural presence of VWF in these concentrates.[8] However, it should be noted that the enhanced development of inhibitors with the use of rFVIII products had not been consistently observed in other studies.[9,10]

Concerning antigenicity, early experimental evidence indicated a differential reactivity to inhibitors when several commercial FVIII preparations were challenged in studies in vitro.[11,12] Protection against the inactivating actions of inhibitors seemed to be superior with concentrates containing native pdFVIII/VWF complexes than with preparations that contained complexes produced by combining the isolated proteins in a laboratory.[13–15] Mechanisms of the inactivation of FVIII in in vitro studies or in vivo animal models and their translational impact in the clinical practice have not been fully elucidated.

Our present studies were designed to investigate the possible differential protection against inhibitors of pdFVIII/VWF versus rFVIII products and their relevance to the clinical practice using a translational approach. With this goal in mind, we first performed a series of in vivo studies in a mouse experimental model of severe haemophilia A with inhibitors exposed to various commercial concentrates of different molecular structures and sources. In the second set of studies, we evaluated the protective role of VWF against FVIII inhibitors reproducing a situation closer to the clinical setting. For the latter purpose, a clinically oriented research study was conducted *ex vivo* using plasma samples from severe HA patients without inhibitors who received treatment with the same commercial concentrates evaluated in the mouse model.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The neutralizing activity of HA inhibitors on different FVIII/VWF products was investigated following a translational approach from an in vivo setting in a FVIII deficient mouse to a clinically oriented *ex vivo* study using plasma samples from HA patients who received FVIII concentrates from different sources.

In the in vivo approach, 8-week old FVIII^{null} E16 Knockout (KO) mice[16,17] were infused with either: (a) an IgG purified from a commercial pool of plasma from patients that had developed antibodies, or (b) a buffer vehicle as control. Immediately afterward, animals received different FVIII concentrates under investigation. FVIII:C recovery was calculated taking into consideration the information provided by the different manufacturers and the empirical assumption that 1 IUFVIII/kg body weight increases FVIII plasma levels by 2%. After 5 min,[18] plasma samples were obtained and FVIII:C recoveries were evaluated.

In the *ex vivo* studies, the reactivity of inhibitors (as determined using the Bethesda assay[13]) present in a commercial pool of plasma from HA patients was tested with normal pooled plasma or with plasma samples obtained from HA patients -with no history of inhibitory antibodies- after receiving FVIII concentrates of different sources and compositions. HA patients were recruited at Thrombosis and Haemostasis Unit from University Hospital La Paz (Spain). Blood samples were drawn 30 min after the administration of their usual therapeutic FVIII concentrate, without affecting the usual clinical practice. Samples from a minimum of three patients were processed for each of the FVIII concentrates.

The various FVIII concentrates investigated in studies in vivo were the same that patients received as substitutive treatment for their HA in the *ex vivo* studies. Refer to Table 1 in the text for details on commercial names and abbreviations. A diagram that summarizes both the in vivo and the *ex vivo* approaches is shown in Figure 1.

2.2 | Reagents and biologicals

The pdFVIII/VWF used in the study was Fanhdi® (Grifols, Barcelona, Spain). This product contains an approximate 1:1 ratio between FVIII:C and VWF:RCo activities. Inhibitor reactivity of products with ratio ≥ 1 is similar to that obtained with normal plasma.[14] The FVIII concentrates produced by recombinant technologies (third and fourth generation) were: Advate® rFVIII, CHO, full chain; Refacto® BDD-rFVIII, CHO, moroctocog alfa, B domain deleted; Nuwiq® BDD-hrFVIII, HEK, simoctocog alfa, B domain deleted, and Elocta® BDD-hrFVIII-Fc, HEK, Fc fusion protein efmoroctocog alfa, B domain deleted (refer to Table 1, for details on commercial names and abbreviations see Schiavoni et al[19]). The FVIII-deficient plasma (containing VWF) and normal pooled plasma were purchased from Diagnostic Grifols (Barcelona, Spain). Inhibitor human IgG was purified from a commercial pool

TABLE 1 Different FVIII products used in these investigations

Abbreviation	Source	Commercial name[19]
pdFVIII/VWF	Plasma-derived, von Willebrand Factor (VWF)-containing FVIII concentrate	Fanhdi®
rFVIII full-chain	Recombinant FVIII full-chain	Advate® (Third generation rFVIII)
BDD-rFVIII	B-domain deleted recombinant FVIII produced in Chinese hamster ovary (CHO) cells	Refacto® (Third generation rFVIII)
BDD-hrFVIII	B-domain deleted recombinant FVIII produced in human embryonic kidney cells (HEK) cells	Nuwiq® (Fourth generation rFVIII)
BDD-hrFVIII-Fc	B-domain deleted recombinant FVIII produced in HEK cell, Fc Fusion protein; extended half-life	Elocta® (Fourth generation rFVIII)

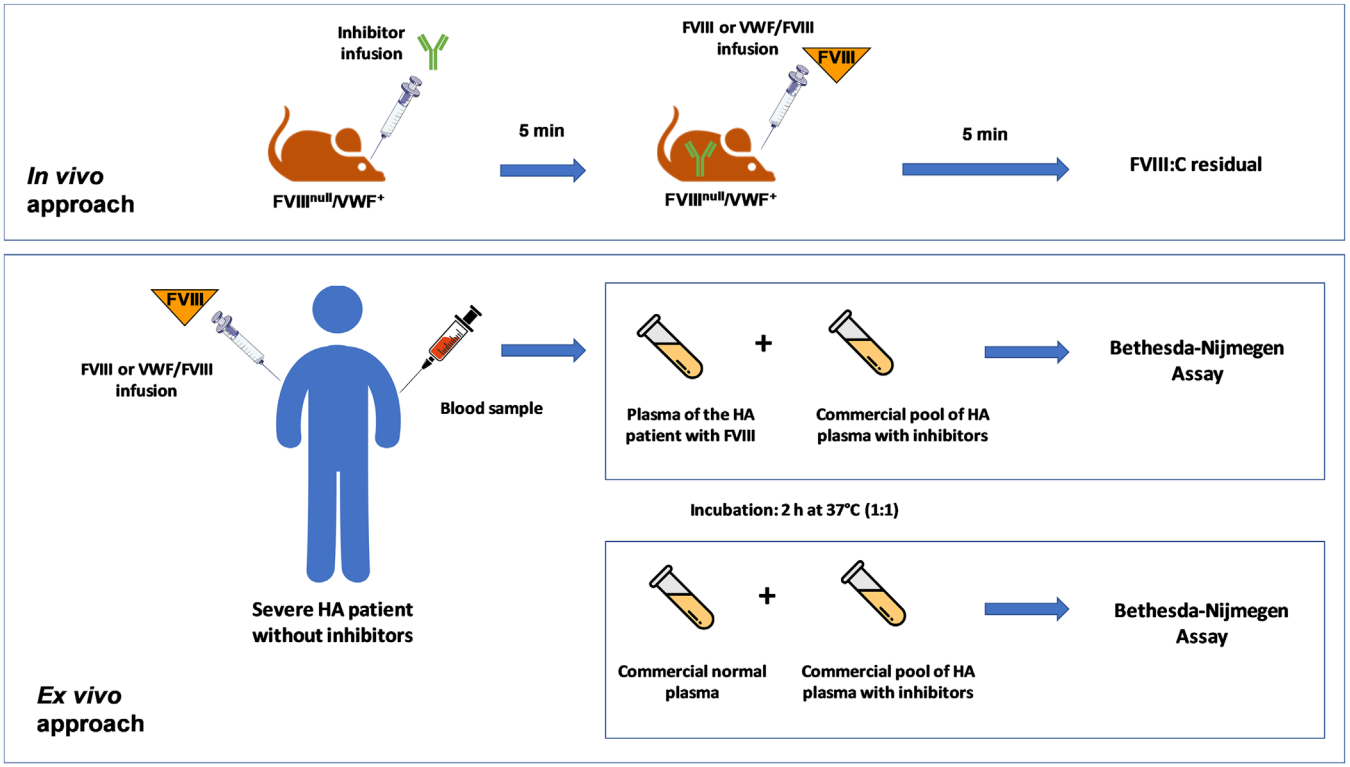

FIGURE 1 Diagram that summarizes both the in vivo and the *ex vivo* approaches performed in this study

of hemophilic plasmas with inhibitors (Technoclone, Vienna, Austria) using protein G Sepharose chromatography (GE Healthcare, Uppsala, Sweden). Characterization of the pool demonstrated the inhibitory activity and the presence of antibodies against both light and heavy (A1-A2) chains (data not shown).

2.3 | Ethical aspects

Studies in animals followed guidelines for ethical conduct in the care and use of animals in research and were approved by the Ethical Committee of Animal Experimentation of Parc Cientific de Barcelona (CEEA-PCB #10708). Studies described involving human subjects adhere to the principles of the Declaration of Helsinki. Blood samples from HA patients were obtained after informed consent. None of the patients were exposed to a FVIII source that was not customarily prescribed. The study was approved by the Ethical Committee of Hospital de la Paz (PI-2981).

2.4 | IgG purification and in vivo studies

Inhibitor IgG was purified from a pool of human plasma from HA patients (Technoclone GmbH, Vienna, Austria) by using Protein G Sepharose chromatography as described before.[20] Groups of FVIII^{null} E16 KO mice ($n \geq 4$, for each treatment group) received intravenously: (a) buffer vehicle (control), or (b) the purified IgG at concentrations tailored (250 Bethesda Units (BU)/kg) to achieve approximately 6 BU/ml

(the minimum above 5 BU/ml, the commonly accepted threshold to be considered high titre inhibitor). Preliminary experiments performed on three mice confirmed that the administered dose consistently yielded 6 BU/ml (data not shown). Five minutes later, animals were further infused with the different FVIII concentrates at 100 IU/kg from different sources, including native pdFVIII/VWF; full chain rFVIII, BDD-rFVIII, BDD-hrFVIII and BDD-hrFVIII-Fc. Five min after infusion of the different concentrates, plasma samples were obtained through abdominal vena cava sampling, and residual FVIII:C levels were measured using a chromogenic assay (Coamatic FVIII kit, Chromogenix, Bedford, USA). FVIII recovery was estimated according to the empirical finding that 1IUFVIII/kg body weight raises the plasma FVIII activity by 2% of normal activity.

2.5 | Determination of inhibitor activities in ex vivo studies

The titre (BU) of a commercial plasma pool of haemophiliac patients with inhibitors was determined using FVIII from a normal commercial plasma or plasma samples from the HA patients after infusion of the different FVIII concentrates. The inhibitor titre of the experimental samples was determined using the Bethesda assay as previously described.[13] The method is based on the quantification of residual FVIII from a series of mixtures containing different concentrations of the sample with inhibitor diluted with FVIII-deficient plasma (DG-FVIII #218063, Diagnostic Grifols) mixed 1:1 with normal plasma or with plasma of the HA patients containing FVIII (~1 UI/ml). FVIII-deficient plasma control mixtures were assayed in parallel. Samples were incubated for 2 h at 37°C. After incubation, residual FVIII:C was determined for each sample, using a chromogenic assay with the Coamatic FVIII kit (Chromogenix, Bedford, MA, USA) as described below. The inhibitor assay was repeated at least three times for each experimental condition.

2.6 | FVIII:C activity assays

FVIII:C levels were determined using a modified chromogenic method (Coamatic FVIII kit, Chromogenix, Bedford, USA). Briefly, 50 μl samples were added to 96-well microtitre plates in duplicate and warmed for 2 min at 37°C. Fifty microlitre of assay components, bovine Factor IXa, Factor X, thrombin colyophilized with $CaCl_2$ and phospholipids were added to each well, and the plates were incubated 10s with shaking and rested for 167s at 37°C. Subsequently, 50 μl of the chromogenic FXa substrate mixture S-2765/I-2581 was added to each plate and the plate was incubated 10s with shaking and 1 min without shaking at 37°C. Finally, 50 μl of acetic acid 20% was added to each well to stop the reaction. The plate was transferred to a microplate reader (Model ELX808, BioTek Instruments, Inc. Winooski VT, USA) preset at 37°C. The absorbance was read at 405 nm. A standard curve was plotted using a secondary standard calibrated against human coagulation FVIII concentrate (Pharmacopea Europea BRP).[14]

2.7 | Statistical analysis

All summary data are presented as mean ± standard deviation. Variations in FVIII recoveries in the in vivo mouse model and inhibitor BU titres in the *ex vivo* model were analysed. ANOVA and Kruskal Wallis were used for statistical comparisons among multiple groups for data following normality and non-normality, respectively. A minimum of $n = 4$ animals per group was used for the in vivo experiments. A minimum of $n = 3$ patients per group was included in the *ex vivo* studies. The lowest levels of statistical significance were established at $P < .05$. GraphPad Prism Statistics 9.0 (GraphPad Software Inc.; La Jolla, CA, USA) was used for analysis and graphic representations.

3 | RESULTS

3.1 | VWF-containing FVIII concentrate is more efficient in the restoration of FVIII circulating levels in the HA mouse model pre-infused with inhibitors

FVIII:C recovery was evaluated in a severe HA mouse model $FVIII^{null}$E16 KO infused with vehicle or with inhibitors (IgG) purified from a commercial plasma pool from HA patients with inhibitors. The final inhibitory activities achieved in the animals were approximately 6 BU/ml (ranging from 5.8 to 6.1 BU/ml). Results revealed a differential neutralizing effect against different FVIII concentrates in this mouse haemophilia model. In the absence of inhibitors (Figure 2A), in vivo FVIII recovery in $FVIII^{null}$E16 KO mice was similar for all FVIII concentrates, with values ranging from 106% to 135% (pdFVIII/VWF = 106.5±10.5%; rFVIII = 112.3±9.3%; BDD-rFVIII = 107.3±28.3%, BDD-hrFVIII = 134.9±46.2%; and BDD-hrFVIII-Fc = 131.6±30.8%). FVIII:C recoveries in animals exposed to the inhibitors (Figure 2B) showed marked differences among treatment groups. In the presence of inhibitory IgG at 6 BU, FVIII:C activity recovered was markedly higher for the native VWF-containing FVIII concentrate (pdFVIII/VWF = 23.0±6.0%) when compared to concentrates composed of isolated FVIII (rFVIII < 5.7±3.1%; BDD-rFVIII < .5%; BDD-hrFVIII < 1.1±1.1%; and BDD-hrFVIII-Fc < .5%).

3.2 | Protection against inhibitors to FVIII was superior in plasma samples from patients exposed to plasma-derived FVIII/VWF

Figure 3 summarizes the results of the clinically oriented research study using plasma samples from treated HA patients with no history of inhibitory antibodies. Plasma from patients who received pdFVIII/VWF consistently showed less inhibitory reactivity (higher residual FVIII activity) compared to plasma from patients who received rFVIII concentrates, regardless of the type of rFVIII product evaluated. Inhibitory values were 17.0±1.7 BU/ml for normal plasma. Results were slightly lower for pdFVIII/VWF = 15.1±2.2 BU/ml and significantly enhanced for the remaining recombinant products

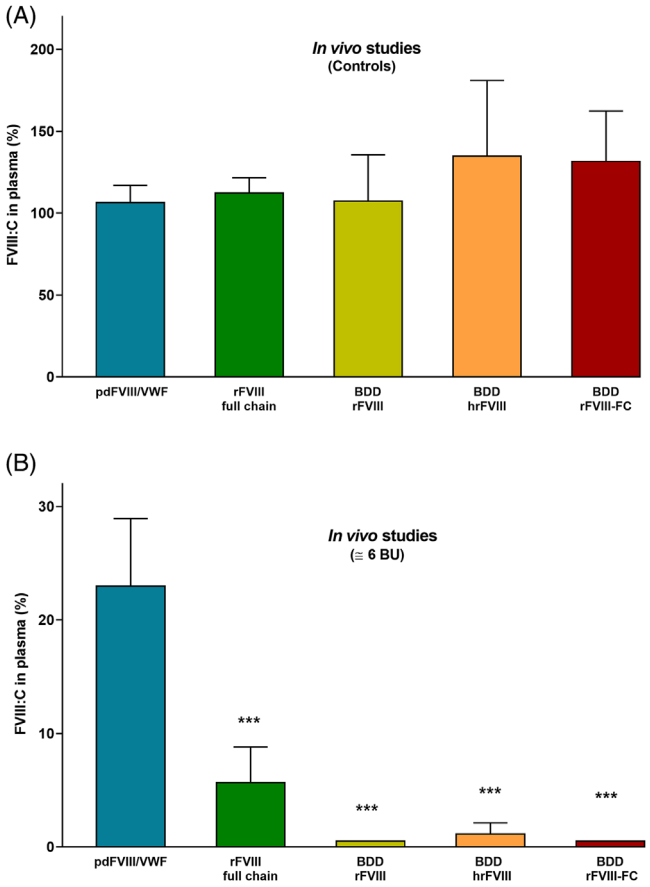

FIGURE 2 Recoveries of FVIII:C in the in vivo model of haemophilia in the FVIII^{null} E16 KO mice. (A) FVIII:C levels in control mice nonexposed to inhibitory antibodies and exposed to different FVIII concentrates. The FVIII:C levels achieved recoveries ≥100% after infusion with the different FVIII concentrates. No significant differences were detected among the different FVIII products (one way ANOVA analysis). (B) FVIII:C levels were markedly reduced in mice previously infused with the purified inhibitory IgG at 6 BU/ml. Recoveries were preserved up to approximately 25% in animals receiving pdFVIII/VWF and were markedly reduced in mice receiving rFVIII products in comparison with those exposed to pdFVIII/VWF. Bars represent average with SD derived from n ≥4 (****$P < .0001$ and ** $P < .01$ vs. pdFVIII/VWF; Kruskal Wallis analysis with Dunn's post-test)

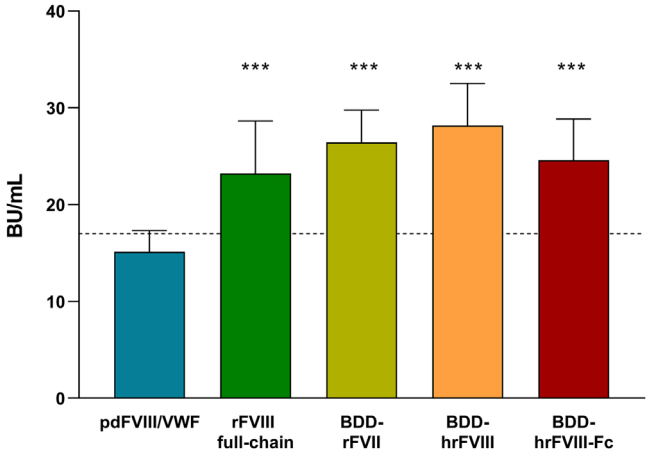

FIGURE 3 Inhibitor titres in Bethesda Units (BU/ml) measured with samples of plasma from HA patients without inhibitors, treated with FVIII concentrates of different sources and characteristics. Test samples were further exposed to a commercial pool of plasma from HA patients with inhibitors. The inhibitory activity against FVIII was significantly elevated in plasma from patients who received rFVIII, BDD-rFVIII, BDD-hrFVIII, or BDD-hrFVIII-Fc, in comparison with those plasma samples from patients who received pdFVIII/VWF. Each dot represents the BU/ml results obtained for each patient sample. Horizontal solid lines represent mean value with SD for each group. The dashed line represents BU/ml results obtained for normal plasma samples. (*$P < .05$; **$P < .01$; ***$P < .001$; vs. pdFVII/VWF; one-way ANOVA with Dunnett's post-test)

(rFVIII = 23.2±5.4 BU/ml ($P < .05$); BDD-rFVIII = 26.5±3.3 BU/ml ($P < .01$); BDD-hrFVIII = 28.2±4.4 BU/ml ($P < .001$); and BDD-hrFVIII-Fc = 24.6±4.3 BU/ml ($P < .01$)). Ratios between inhibitor titres calculated for FVIII in HA treated plasma versus those obtained with normal plasma were close to 1 for pdFVIII/VWF (.9±.1) and increased above 1 for the remaining rFVIII concentrates (1.3±.2 for rFVIII; 1.6±.2 for BDD-rFVIII; 1.7±.1 for BDD-hrFVIII, and 1.4±.2 for BDD-hrFVIII-Fc).

4 | DISCUSSION

Overall, the results of the present investigations indicate that FVIII+VWF complexes formed in circulating blood after administration of rFVIII products, in mice or humans, are not equivalent to the naturally occurring complexes present in pdFVIII/VWF. Moreover, our data support the concept that isolated FVIII infused into HA patients with inhibitors would be less protected by VWF than the FVIII in plasma concentrates natively containing FVIII/VWF complexes.

Mechanisms of immunogenicity induced by exogenous FVIII in HA may depend on the characteristics of the FVIII product,[21] and are regulated by a complex balance of genetic factors, environmental variables or additional conditions.[22,23] Neutralization or inactivation of the procoagulant activity of FVIII is the result of inhibitory antibodies developed in HA patients when they are re-exposed to a FVIII containing product. Immunogenicity of FVIII concentrates and neutralizing activity of inhibitors in HA patients are interrelated, but the mechanisms involved are not interchangeable. Our present investigations have concentrated on the neutralizing action of FVIII inhibitory antibodies during a single exposure to FVIII concentrates of different origins and the possible role of the preexisting or postinfusion formation of FVIII/VWF complexes. Our study is not intended to address the multifaceted innate and adaptive immune mechanisms involved in the development of inhibitory antibodies in patients after repeated exposure to FVIII.

Despite evidence for the presence of VWF in pdFVIII protecting from the development of inhibitors in HA patients,[7,8] there is no consensus in the literature that these concentrates are less immunogenic than FVIII without VWF.[9,24] In contrast, numerous experimental studies have demonstrated that VWF-containing pdFVIII concentrates provide better protection against inhibitor neutralization than rFVIII

products.[11,12,14,15,25] It has been proposed that VWF may act as a shield and chaperone, protecting FVIII from the neutralizing effects of inhibitors.[8] However, the precise in vivo molecular mechanisms of the FVIII-VWF interactions and the recognition and inactivation of FVIII by inhibitors are not well understood.

It is expected that, after infusion into a HA patient, isolated FVIII will spontaneously bind to circulating VWF at an apparent stoichiometric ratio of 1 IU FVIII:1 IUVWF.[26] Data from our studies in the in vivo model indicated that pdFVIII/VWF is more efficient in restoring circulating FVIII levels in the FVIII^{null}E16 KO mouse model preinfused with inhibitors. This information confirms the results of previous in vitro studies.[11–15,25] Moreover, our results in the in vivo model are compatible with those previously reported in similar mouse models of HA using different FVIII concentrates or mixes of the isolated proteins (FVIII+VWF) prepared in the lab.[15,27] Interestingly, our present data using high titre inhibitors (around 6 BU/ml) indicate that FVIII protection was still significantly greater for native pdFVIII/VWF than for the theoretical FVIII+VWF complexes formed in the circulation after infusion of rFVIII concentrates. These results imply that complexes generated in the circulation after the infusion of rFVIII concentrates differ from those naturally present in the pdFVIII/VWF concentrate. A question remained as to whether the protective action observed with the pdFVIII/VWF concentrate would be similar with FVIII+VWF complexes formed in the circulation of HA patients infused with rFVIII concentrates.

To answer this question, we carried out a series of translational studies in plasma from patients treated with different FVIII products. These plasma samples were challenged with a HA pool of plasma containing inhibitory antibodies. Our *ex vivo* studies demonstrated, for the first time in the clinical setting, that plasma from patients who received pdFVIII/VWF consistently showed less inhibitory reactivity regardless of the type of rFVIII product they received. Previous studies performed in vitro on different FVIII-containing products have shown that the rFVIII concentrates invariably contained a fraction of factor FVIII related antigen (FVIII:Ag) molecules (approximately 20%) which was unable to associate with VWF.[28,29] Results of the present clinically oriented *ex vivo* study confirmed a high level of correspondence with those of previous in vitro studies[14,30] (refer to Supporting information for indirect comparisons). Overall, these *ex vivo* and in vivo studies are in agreement with the concept that the natural FVIII/VWF complex conferred stronger protection to FVIII against inhibitors.

The different behaviour of pdFVIII containing VWF versus purified or rFVIII products exposed to inhibitors has been explained through qualitative differences causing a reduction of FVIII binding or through conformational changes in the binding of FVIII to VWF after concentrate has been infused into patients.[31–34] A free FVIII fraction -not protected by the shielding effects of VWF- would be more available to the neutralizing effects of inhibitors.

Molecular transformations occurring during the synthesis of FVIII and VWF and throughout the process of their noncovalent association may play a role in addition to the conformational changes so as FVIII would be protected in the interior of the globular VWF, less exposed to components of the immune system and partially shielded against inhibitors.[35–38] Considering the complexity of the biosynthesis and secretion processes of naturally forming FVIII/VWF complexes, it is not possible to assure that the delicate balance of molecular interactions occurring in vivo is replicated in mixtures combining the isolated proteins (FVIII+VWF), or in FVIII-VWF complexes formed in the circulation after the infusion of rFVIII products. Our present data indicate that FVIII is more protected in the constitutively formed FVIII-VWF complex, as it is found in plasma and in pdFVIII/VWF products. However, we should take into account that, although it has been demonstrated that human FVIII has comparable affinities for human and murine VWF,[18,39] we did not measure specifically the binding of each FVIII product to murine VW. Furthermore, the differential protection between pdFVIII/VWF and rFVIII that we have observed in the *ex vivo* studies using plasma from patients with HA, may suggest that naturally forming FVIII/VWF complexes would not be comparable to the FVIII-VWF complex formed in the circulation after administration of rFVIII products. This hypothesis deserves further investigation in dedicated mechanistic studies. However, as most patients were treated with rFVIII products and the risk of antibody formation is greater with these products,[7] it cannot be ruled out that the epitopes in the purified IgG are more specific for rFVIII than pdFVIII (i.e., antibody binding sites may be in the area covered by VWF).

Overall, our results suggest that treatment with plasma-derived products containing pdFVIII/VWF may offer higher protection in HA patients with inhibitors who required substitutive treatment in situations such as immunotolerance treatment.

5 | CONCLUSION

In summary, our results reveal intrinsic differences in the neutralizing effects of FVIII inhibitors against various FVIII concentrates in an animal model and in plasma from HA patients treated with various FVIII products. None of the single FVIII products evaluated showed better protection against inhibitors than the pdFVIII/VWF concentrate. The results presented here demonstrate that constitutively occurring FVIII/VWF complexes in plasma-derived products were not equivalent to those formed in the circulation after administration of rFVIII.

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CONFLICT OF INTEREST

Maria Isabel Bravo, Alba Pérez, Aida Raventós, Salvador Grancha, Juan Ignacio Jorquera, Montserrat Costa and Todd Willis are full-time employees of Grifols, a manufacturer of pd-FVIII/VWF (Alphanate®/Fanhdi®). Nora Viviana Butta has participated as speaker for Novo

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available within the manuscript and/or its supplemental material. Additional information is available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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