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FVIII/VWF complex displays a greater pro-haemostatic activity than FVIII preparations devoid of VWF: Study in plasma and cell-based models

Concetta T. Ammollo[1] | Fabrizio Semeraro[1] | Antonia Vitulli[1] | Lavinia Dirienzo[1] | Anna M. Mezzasoma[2] | Nicola Semeraro[1] | Paolo Gresele[2] | Mario Colucci[1]

[1]Department of Biomedical Sciences and Human Oncology, University of Bari Aldo Moro, Bari, Italy

[2]Section of Internal and Cardiovascular Medicine, Department of Medicine, University of Perugia, Perugia, Italy

Correspondence
Mario Colucci, Department of Biomedical Sciences and Human Oncology, University of Bari Aldo Moro, Piazzale Giulio Cesare 11, 70124 Bari, Italy.
Email: mario.colucci@uniba.it

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Grifols (Barcelona, Spain); University of Bari

Abstract

Introduction: Plasma-derived FVIII/VWF complex was reported to be less sensitive to inhibitors than FVIII preparations devoid of VWF.

Aim: To compare the efficacy of FVIII/VWF complex (Fanhdi) and five different VWF-free FVIII preparations in restoring thrombin generation and activation of thrombin-activatable fibrinolysis inhibitor (TAFI) in haemophilic plasma, with and without inhibitor, and in cell-based models.

Methods: Experiments were performed in haemophilic plasma supplemented with inhibitory IgG or in plasma samples obtained from haemophilia A patients without (n = 11) and with inhibitor (n = 12). Thrombin generation was evaluated by calibrated automated thrombography (CAT) under standard conditions, in the presence of activated protein C (APC) or thrombomodulin (TM), and in cell-based models including endothelial cells, either alone or in combination with platelets or tissue factor-expressing blood mononuclear cells. The kinetics of TAFI activation was determined by a two-stage functional assay in the absence and in the presence of APC.

Results: In haemophilic plasma without inhibitor, Fanhdi enhanced thrombin generation and TAFI activation as well as recombinant (2nd-4th generation) and plasma-derived FVIII preparations devoid of VWF. On the contrary, in plasma with inhibitor, Fanhdi displayed a greater ability to restore thrombin generation and TAFI activation under all tested conditions. Notably, in cell-based models including endothelial cells, Fanhdi proved more efficient than all other preparations in improving thrombin generation even in the absence of inhibitor.

Conclusion: The greater pro-haemostatic activity of FVIII/VWF complex, either in haemophilic plasma with inhibitor or in the presence of endothelial cells, may offer therapeutic advantages.

KEYWORDS

coagulation, endothelial cells, fibrinolysis, inhibitor, thrombin, thrombin-activatable fibrinolysis inhibitor

1 | INTRODUCTION

The bleeding diathesis in haemophilia A is due to defective thrombin generation caused by factor VIII (FVIII) deficiency. Thrombin, besides converting fibrinogen to fibrin, plays a pivotal role in the regulation of clot resistance to fibrinolysis through several mechanisms, which include (a) cross-linking of fibrin monomers and binding of α_2-antiplasmin to fibrin strands through activation of FXIII,[1] (b) formation of more compact and lysis-resistant clots made up of thin and long fibres[2] and (c) inhibition of plasmin generation via activation of thrombin-activatable fibrinolysis inhibitor (TAFI), a procarboxypeptidase that, once activated (TAFIa), removes the binding sites for plasminogen from partially degraded fibrin.[3] Therefore, in conditions characterized by impaired thrombin generation, such as haemophilia, an important cause of bleeding is the premature lysis of the loose and unstable clot.[4,5] Among the thrombin-mediated mechanisms of fibrinolytic resistance, the most important in haemophilic patients seems to be the reduction in TAFI activation.[4-6]

The standard of care for patients with haemophilia A is replacement therapy with FVIII concentrates.[7] They can be broadly classified in plasma-derived products (pdFVIII) and recombinant FVIII (rFVIII), the most notable difference being the presence of von Willebrand factor (VWF) in the former. VWF is an important player of the haemostatic process and has, among others, the function to bind FVIII and protect it from proteolytic cleavage.[8] All FVIII concentrates efficiently correct both defective thrombin generation and hastened fibrinolysis. However, in the presence of FVIII inhibitors, their efficiency may differ remarkably. Development of FVIII-neutralizing antibodies occurs in about 30% of severe haemophilia A patients and seriously complicates the management of the disease.[9,10] Using a standard thrombin generation assay, some investigators showed that pdFVIII concentrates containing VWF generate more thrombin in plasma from haemophilic patients with inhibitors as compared to FVIII preparations lacking VWF,[11-13] suggesting that the former might represent better haemostatic agents. The superiority of FVIII/VWF over FVIII is most probably due to the capacity of VWF to protect FVIII from neutralization by FVIII inhibitors. Indeed, FVIII concentrates display remarkable differences in their reactivity with FVIII inhibitors, pdFVIII/VWF concentrates being the least reactive.[11-15]

To our knowledge, no study has evaluated the impact of FVIII preparations on thrombin generation under conditions that better mimic the 'in vivo' situation, that is presence of a functional protein C (PC) pathway and presence of endothelial and blood cells. Moreover, no data are available on the relative ability of different FVIII preparations in improving TAFI activation and fibrinolytic resistance in haemophilic plasma, particularly in the presence of inhibitors. Our study was undertaken to evaluate the efficiency of a pdFVIII/VWF concentrate, as compared to concentrates devoid of VWF (either recombinant or plasma-derived), in restoring thrombin generation, TAFI activation and fibrinolytic resistance in in vitro models of pathophysiologic relevance.

2 | MATERIALS AND METHODS

2.1 | FVIII preparations

The following FVIII preparations were compared: Fanhdi®, a pdFVIII/VWF complex (1:1.2 ratio based on units, Grifols); Beriate, a pdFVIII devoid of VWF (CSL Behring); Kogenate®, a 2nd-generation full length rFVIII and Kovaltry, a 3rd-generation full length rFVIII (Bayer Schering Pharma); Nuwiq, a 4th-generation B-domain-deleted rFVIII (Octapharma AG); and Elocta, a 4th-generation recombinant B-domain-deleted Fc-fusion protein (Sobi). FVIII activity of the various preparations was assessed by a one-stage clotting assay and ranged between 80% and 115% of that of Fanhdi. Unless otherwise specified, FVIII preparations were added to haemophilic plasma immediately before starting the experiment. In some experiments, we tested an in vitro formed FVIII/VWF complex that was freshly prepared by incubating purified VWF with pdFVIII (Beriate) at a 1:1 ratio for 15 min at 37°C. The complete list of reagents used in this study is provided in Appendix S1.

2.2 | Patients' samples

We collected samples from 23 patients with haemophilia A, 12 of whom had anti-FVIII inhibitors (from 1.5 to 11.5 BU/mL). The study was approved by the Institutional Review Board (CEAS Umbria), and written informed consent was obtained from each participant or a legal representative. The main characteristics of patients are reported in Tables S1 and S2. Citrated venous blood (0.129 mol/L) was collected by venipuncture in the morning under fasting conditions. Plasma was prepared by centrifugation at 2000 *g* for 20 min at room temperature and stored at −70°C until tested. Blood was generally collected ≥3 days after the last concentrate infusion, with few exceptions, and thus, detectable FVIII activity was observed in some patients (Table S1). Because of the limited volume of plasma samples available, we could not perform all assays in all patients.

2.3 | Cell preparations and IgG isolation

Freshly collected blood from healthy donors, anticoagulated with ACD (mixture of 39 mmol/L citric acid, 75 mmol/L sodium citrate and 135 mmol/L dextrose), was used for platelet and mononuclear cell (MNCs) isolation. Platelets were washed by differential centrifugation[16] and finally suspended in test plasma at 250×10^3 cells/μL. MNCs were isolated by density gradient centrifugation[17] and finally suspended in RPMI-1640 at 3×10^6 cells/mL. Tissue factor (TF) expression was induced by overnight incubation with 1 μg/mL LPS at 37°C. After incubation, cells were washed by centrifugation and suspended in RPMI-1640.

Endothelial cell line EA.hy926 was purchased from ATCC (LGC Standards Srl.) and cultured in high-glucose DMEM enriched with

10% foetal bovine serum, 2 mmol/L glutamine, 10 000 units/mL penicillin, 10 mg/mL streptomycin and 25 μg/mL amphotericin B in a humidified atmosphere containing 5% CO_2. Before use, subconfluent cells were detached by trypsin/EDTA, washed and suspended in serum-free DMEM at 1×10^6/mL.

IgG were isolated from the plasma of a patient with high inhibitor titre by affinity chromatography on protein G-Sepharose columns (GE Healthcare Europe GmbH), according to the manufacturer's instructions. Final IgG fraction was concentrated by centrifugation using Millipore Amicon Ultra concentration tubes and dissolved in Hepes. Inhibitor concentration was 2000 BU/mL.

2.4 | Assays

Thrombin generation was assessed by calibrated automated thrombography (CAT) as described by Hemker et al[18] with minor modifications.[19] The following parameters were considered: thrombin peak, endogenous thrombin potential (ETP, ie the total amount of thrombin generated) and thrombin formation velocity (velocity index). Besides standard conditions, the assay was performed in the presence of thrombomodulin (4 nmol/L), activated PC (APC, 0.1 μg/mL) or endothelial cells (50×10^3/well), either alone or in combination with platelets (250×10^3/μL) or TF-expressing MNC (5×10^3/well).

Clot lysis of tissue factor-induced plasma clots exposed to exogenous t-PA was studied by a turbidimetric assay.[20] Clot lysis times were calculated as the interval between the midpoint of the clear to maximum turbidity transition and the midpoint of the maximum turbidity to clear transition.

The kinetics of TAFI activation in clotting plasma was evaluated by a functional two-stage assay[21] with some modifications. In the first stage, plasma was clotted under the same conditions used for the CAT assay to induce TAFI activation. At intervals, aliquots of serum were transferred into refrigerated tubes containing hirudin (200 U/mL) to halt thrombin-mediated TAFI activation and preserve the activity of TAFIa. In the second stage, the antifibrinolytic activity of TAFIa was evaluated as the ability to prolong the lysis time of clots formed with purified fibrinogen. For that purpose, 80 μL of sample was added to microplate wells along with fibrinogen (1 mg/mL), t-PA (40 ng/mL) and reptilase (1:50, final dilution) as clotting reagent (final volume 120 μL). The plate was read at 405 nm at room temperature to reduce TAFIa decay, and the lysis times were calculated as described earlier. TAFIa activity was expressed as lysis time difference vs time zero, and TAFI activation was calculated as area under the TAFIa generation curve (AUC).

Both TAFI activation and clot lysis assays were performed in plasma alone and in plasma supplemented with APC (0.1 μg/mL). Neither assay could be done in the presence of thrombomodulin (either in purified form or in associated with endothelial cells) because under this condition, even a small concentration of thrombin leads to a normalization of TAFIa generation and fibrinolysis resistance.[4,5]

2.5 | Degradation of FVIII by APC

Fanhdi, Beriate or Beriate/VWF complex (2 IU/mL) was incubated at 37°C with APC (0.1 or 1 μg/mL), phospholipids (10 μg/mL) and $CaCl_2$ (5 mmol/L). At intervals, aliquots of the mixture were diluted 1:40 in Hepes buffer and tested for FVIII activity by a one-stage clotting assay. Residual FVIII activity was expressed as per cent of the initial activity by reference to calibration curves constructed with serial dilution of each FVIII preparation.

2.6 | Statistical analysis

Data are presented as mean ± SEM. Differences among FVIII preparations were assessed by Friedman test and pairwise comparison according to Conover. A two-sided *P*-value < .05 was considered significant. Statistical analyses were performed with MedCalc.

3 | RESULTS

The main objective of the study was to compare the pro-haemostatic activity of a pdFVIII/VWF concentrate (Fanhdi) with that of preparations devoid of VWF, either plasma-derived (Beriate) or recombinant (Kogenate), in haemophilic plasma supplemented with purified anti-FVIII IgG (model plasma) and in plasma samples from haemophilic patients without and with inhibitors. After completion of the main study, we undertook an ancillary study, using model plasma only, which aimed at comparing Fanhdi with 3rd- and 4th-generation recombinant products (Elocta, Kovaltry and Nuwiq).

3.1 | Effect of FVIII preparations on thrombin generation and TAFI activation in model plasma

Thrombin generation assay was performed in the absence and presence of inhibitor and under four different experimental settings: (a) standard conditions; (b) presence of soluble thrombomodulin; (c) presence of exogenous APC; and (d) cell-based models, in which we used endothelial cells as a source of cell-bound thrombomodulin, platelets as a natural source of phospholipids and LPS-stimulated monocytes as a source of TF. For the sake of brevity, we report only the thrombin peak as representative parameter of CAT. Qualitatively similar results were obtained with ETP and velocity (not shown).

Under standard conditions, in the absence of inhibitor, Fanhdi, Kogenate and Beriate displayed a similar efficacy in enhancing thrombin generation (Figure 1). On the contrary, upon addition of FVIII inhibitor (2.5 BU/mL), Fanhdi was significantly more effective than VWF-devoid FVIII preparations. Qualitatively similar results were obtained in plasma supplemented with APC or soluble thrombomodulin. When the assay was performed in the presence of intact endothelial cells, Fanhdi displayed a higher pro-haemostatic activity not only in the presence of inhibitor but also in its absence. Almost

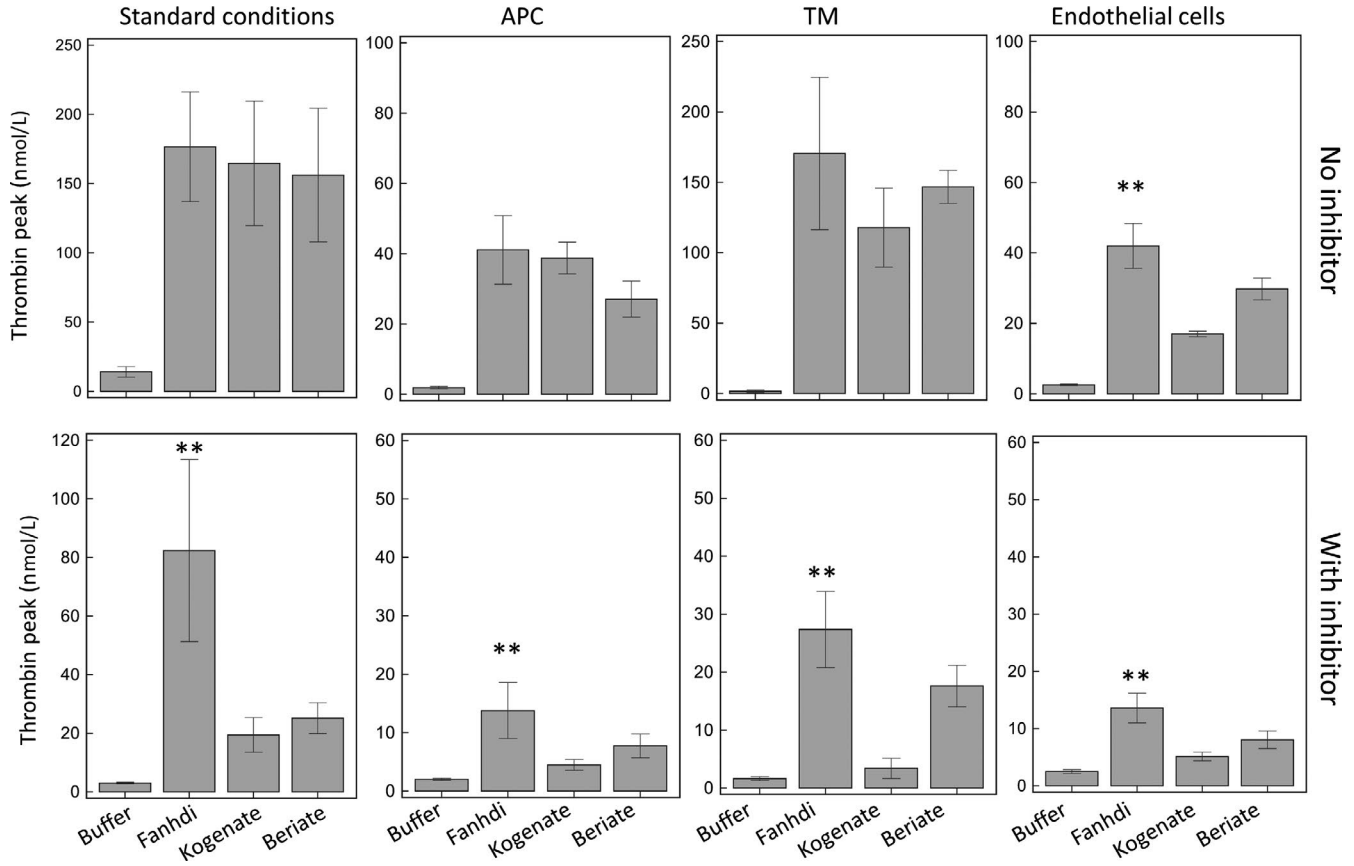

FIGURE 1 Effect of FVIII preparations (1 U/mL) on peak thrombin generation in the absence (top panels) and presence of inhibitor (2.5 BU/mL, bottom panels) in model plasma. Results are the mean ± SEM of 3-4 experiments. APC, activated protein C (0.1 μg/mL); TM, thrombomodulin (4 nmol/L). Endothelial cells were tested at a concentration of 50×10^3 per well. **Statistically different from Kogenate and Beriate by Friedman test and pairwise comparison according to Conover ($P < .05$). All drugs gave results significantly different from buffer (not shown in figure). Please note that the *Y*-axis scales are different

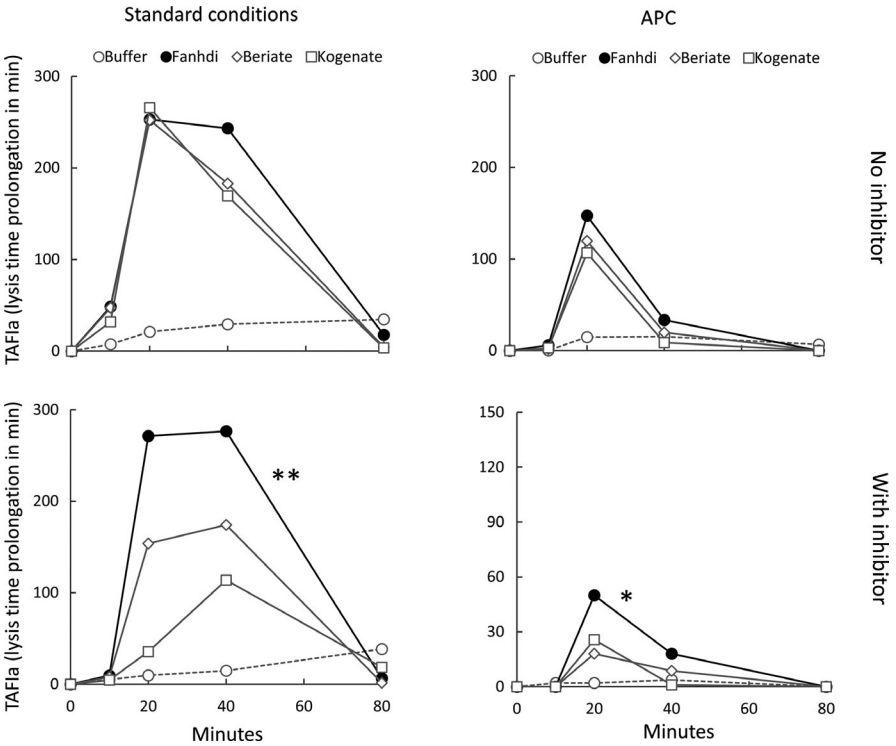

FIGURE 2 Effect of FVIII preparations (1 IU/mL) on TAFI activation kinetics in model plasma. TAFIa generation was evaluated under standard conditions (left panels) and upon addition of activated protein C (APC, 0.1 μg/mL, right panels), in the absence (top panels) and presence (bottom panels) of FVIII inhibitor (2.5 BU/mL). Results are expressed as lysis time prolongation over time zero and are the mean of 3-4 experiments. For clarity, SEM has been omitted. *Statistically different from Kogenate; **Statistically different from Kogenate and Beriate by comparing the AUC values (Friedman test and pairwise comparison according to Conover). All drugs gave results significantly different from buffer (not shown in figure). Please note that the *Y*-axis scales are different

identical results were obtained in models consisting of endothelial cells plus platelets or LPS-stimulated MNC (not shown).

Figure 2 shows the kinetic of TAFIa generated under standard conditions and after addition of APC. In both conditions, the three preparations had similar efficacy in promoting TAFI activation in the absence of inhibitor. In plasma with inhibitor, Fanhdi showed a greater ability in promoting the generation of TAFIa, even though the difference vs Beriate did not reach the statistical significance in the presence of APC.

3.2 | Effect of FVIII preparations on clot lysis in model plasma

The three FVIII preparations prolonged the clot lysis time to a similar extent both in the absence and in the presence of inhibitor (Figure 3, panels A and B). To explain the apparent discrepancy with thrombin and TAFIa generation data, we evaluated the changes in clot lysis time in response to decreasing concentrations of FVIII. As shown in Figure 3C, as little as 0.1 IU/mL was enough to reach the maximal prolongation of lysis time, meaning that, above this threshold, the lysis time will be the same even if the actual concentration of FVIII activity is strikingly different.

3.3 | Effect of FVIII preparations on thrombin generation in patients' plasma

The main characteristics of patients are reported in Tables S1 and S2. As shown in Figure 4, in samples from patients without inhibitor, the three preparations were equally effective in enhancing thrombin generation, both under standard conditions and in the presence of APC. In samples with inhibitor, Fanhdi was more effective in increasing thrombin generation as compared to the other two FVIII preparations, even though the difference vs Beriate did not reach statistical significance in the presence of APC. In agreement with model plasma data (see Figure 1), in the presence of endothelial cells, Fanhdi displayed a greater thrombin enhancing capacity than Kogenate and Beriate, both in samples without and with inhibitor.

3.4 | Comparison with 3rd- and 4th-generation FVIII preparations

Consistent with the above results, Fanhdi displayed a greater capacity to improve thrombin generation (Figure 5) and TAFI activation (Figure 6) in the presence of inhibitor, under all experimental conditions but one, that is in the presence of thrombomodulin in which case Fanhdi was not different from Elocta (Figure 5). Moreover, in the CAT assay in the presence of endothelial cells, Fanhdi proved superior to new generation FVIII preparations even in the absence of inhibitor (Figure 5).

3.5 | Role of VWF

When Beriate was preincubated with VWF, to form a FVIII/VWF complex, its ability to promote thrombin generation in model plasma with inhibitor was not increased (Figure 7 left panel). Similarly, no improvement was seen when plasma VWF concentration was raised by the addition of 1 IU/mL of purified VWF, or when Beriate was

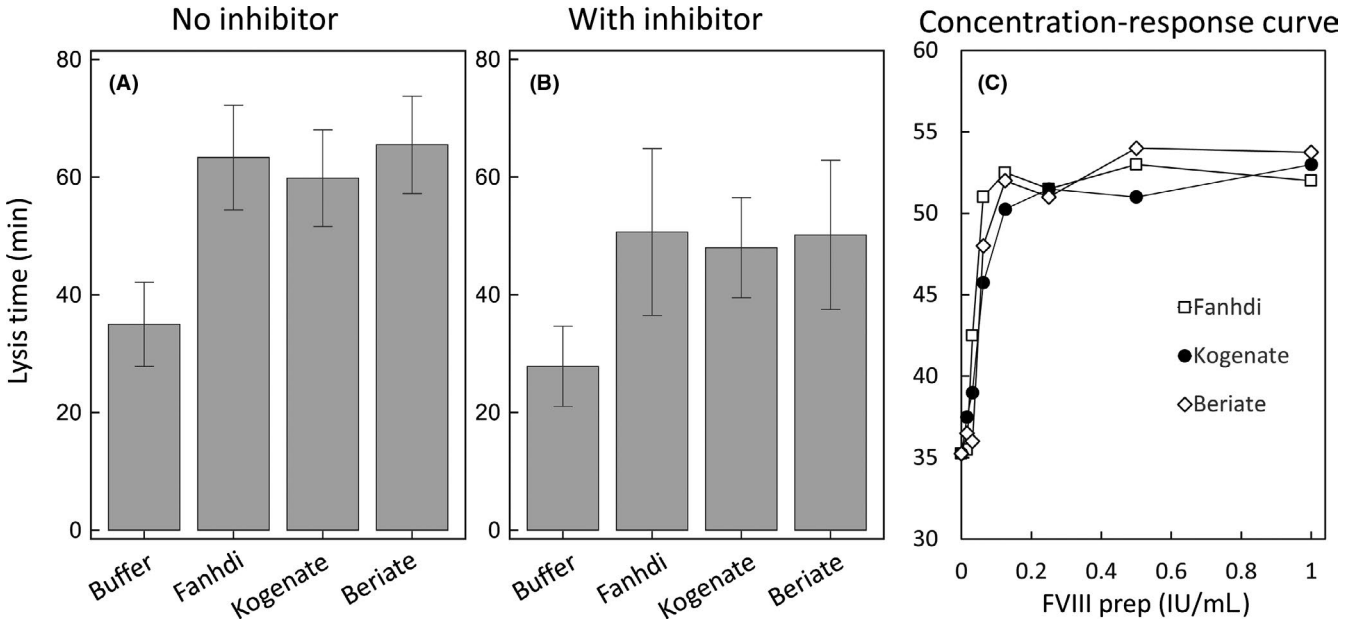

FIGURE 3 Effect of FVIII preparations on clot lysis time of model plasma. A and B, Clot lysis time of plasma without and with inhibitor (2.5 BU/mL) after addition of 1 IU/mL of FVIII preparations (mean ± SEM of three experiments). C, representative experiment of the prolongation of lysis time induced by decreasing concentrations of FVIII in model plasma (without inhibitor)

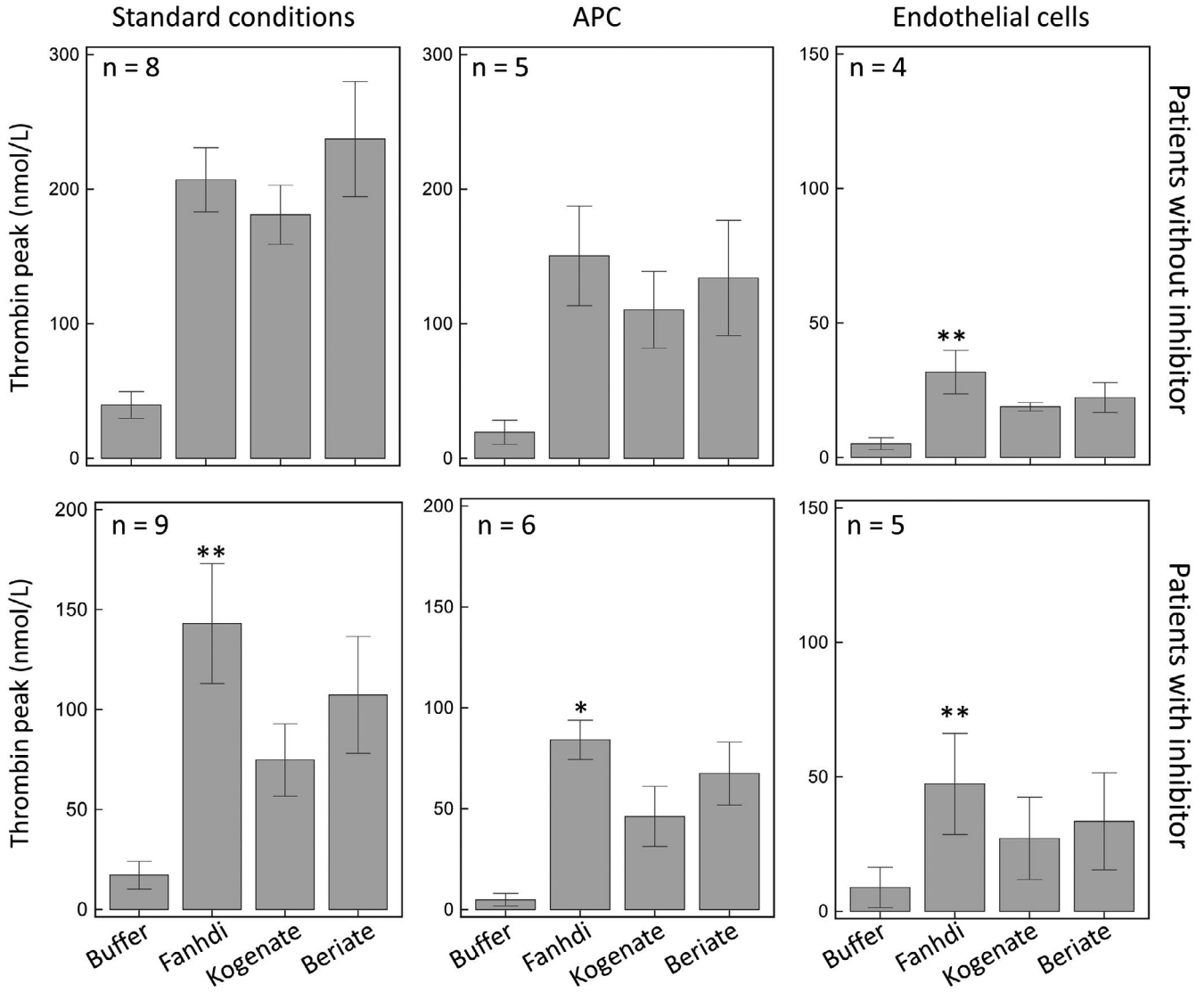

FIGURE 4 Effect of different FVIII preparations on peak thrombin activity in plasma from patients without inhibitor (top panels) and with inhibitor (bottom panels, 1.5-11.5 BU/mL). In the former, FVIII preparations were tested at the concentration of 1 IU/mL, whereas in samples with inhibitor FVIII concentration was equal to half the BU level. Results are mean ± SEM. The number of tested patients is reported in each panel. APC, activated protein C (0.1 μg/mL); endothelial cells were tested at a concentration of 50×10^3 per well. *Statistically different from Kogenate; **Statistically different from Kogenate and Beriate by Friedman test and pairwise comparison according to Conover ($P < .05$). All drugs gave results significantly different from buffer (not shown in figure). Please note that the *Y*-axis scales are different

preincubated in plasma (15 min at 37°C) to allow interaction with plasma VWF prior to the addition of inhibitory IgG.

Concerning FVIII sensitivity to APC, preincubation of Beriate with purified VWF resulted in marked protection against degradation by APC (1 μg/mL) (Figure 7 right panel).

4 | DISCUSSION

Our study adds in several ways to previous reports on the greater ability of FVIII/VWF complex in restoring thrombin generation in haemophilic plasma with inhibitor.[11,13,22-25] First, we performed the thrombin generation assay under different conditions in order to have a functioning PC pathway. Second, we evaluated thrombin generation in cell-based models. Third, we assessed to which extent the increase of thrombin generation translates in the enhancement of TAFI activation, which is one of the major mechanism of fibrinolytic resistance.

Under all tested conditions, FVIII/VWF complex (Fanhdi) proved superior to all other FVIII preparations in restoring thrombin generation and TAFI activation in haemophilic plasma with inhibitor. As to the role of VWF, we found that an in vitro formed FVIII/VWF complex, obtained either by incubating pdFVIII (Beriate) with purified VWF or by preincubating Beriate with plasma in order to allow the interaction with endogenous VWF, did not confer protection against inhibitory IgG (Figure 7A), suggesting that the native complex (Fanhdi) is not identical to the one formed in vitro, as hypothesized by others.[12]

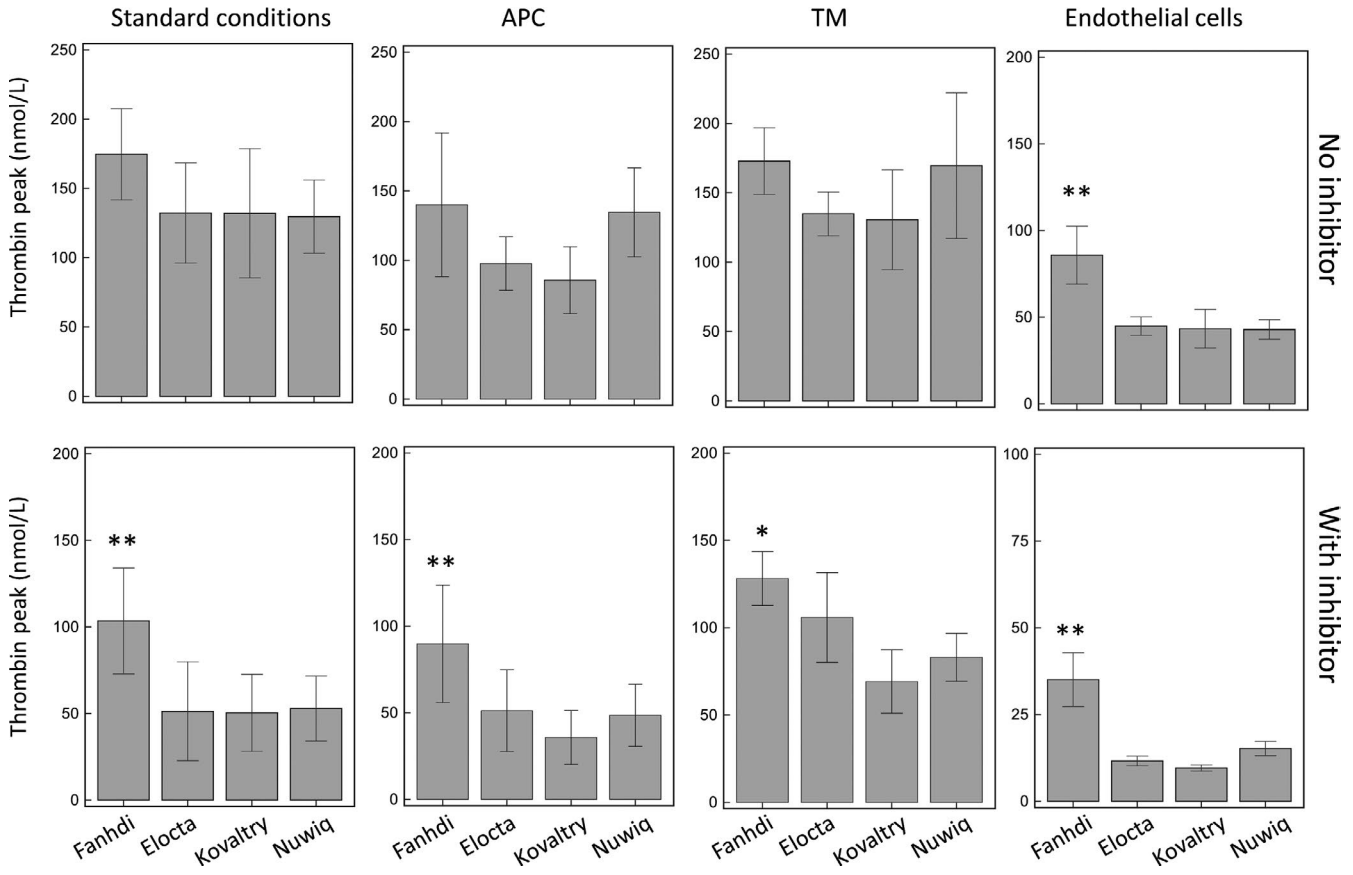

FIGURE 5 Effect of different FVIII preparations (1 IU/mL) on thrombin generation in model plasma without inhibitor (top panels) and with inhibitor (2.5 BU/mL, bottom panels) under different experimental conditions. Results are mean ± SEM of 4-6 experiments. APC, activated protein C (0.1 μg/mL); TM, thrombomodulin (4 nmol/L). Endothelial cells were tested at a concentration of 50×10^3 per well. *Statistically different from Kovaltry and Nuwiq; **Statistically different from all other three FVIII preparations by Friedman test and pairwise comparison according to Conover ($P < .05$). Please note that the *Y*-axis scales are different

A novel finding of our study is that Fanhdi displays a greater capacity in enhancing thrombin generation even in the absence of inhibitor when tested along with endothelial cells, either alone or in combination with TF-expressing MNCs or platelets. The mechanism behind this unexpected observation remains to be elucidated. Because VWF is known to protect FVIII from degradation by APC,[26] it can be hypothesized that the greater activity of Fanhdi results from its refractoriness to APC generated by endothelial cells through the expression of thrombomodulin and EPCR (endothelial protein C receptor).[27] This hypothesis, however, is not supported by our present data. First, the addition of APC or thrombomodulin to haemophilic plasma without inhibitor did not reproduce the results obtained with endothelial cells. Second, preincubation with purified VWF makes pdFVIII resistant to degradation by APC, suggesting that interaction of FVIII preparations with plasma VWF might elicit a similar effect. Therefore, considering the vast repertoire of endothelial cell properties,[28] alternative hypotheses should be tested through more targeted and in-depth studies.

Our finding that the greater thrombin generation induced by Fanhdi in the presence of FVIII inhibitor translates in a clear-cut increase in TAFI activation is relevant to the antihaemorrhagic treatment in haemophilia. In fact, the impairment of TAFI activation is considered a major mechanism of premature lysis of the haemostatic plug in haemophilia, as originally suggested by Broze and Higuchi[4] and more recently in an elegant study in mice, which provided compelling evidence that defective TAFI activation is a major contributor to joint bleeding, the hallmark of haemophilia.[6] Regrettably, TAFI activation data do not harmonize with fibrinolysis experiments, which did not reveal significant differences between Fanhdi and other preparations in their ability to prolong the lysis time of plasma clots. Most likely, the lack of difference is due to the fact that very little FVIII is required to restore fibrinolytic resistance in our model, as shown by ourselves (Figure 3) and by others,[4] implying that the lysis time will be the same even if FVIII activity and TAFIa levels are different, provided that they are above the low threshold at which the lysis time is normalized.[29,30] Notwithstanding these inconsistencies, which seem to be due to the in vitro clot lysis model used, the greater ability of Fanhdi to enhance TAFI activation is likely to translate in a greater capacity to improve fibrinolytic resistance.

Some limitations of our study should be acknowledged. First, the experiments aimed at evaluating the role of VWF must be taken with caution because they were performed using only pdFVIII and the inhibitor isolated from a single patient. Second, the inhibitor epitopes in patients' plasma were not identified, and thus, we could not establish

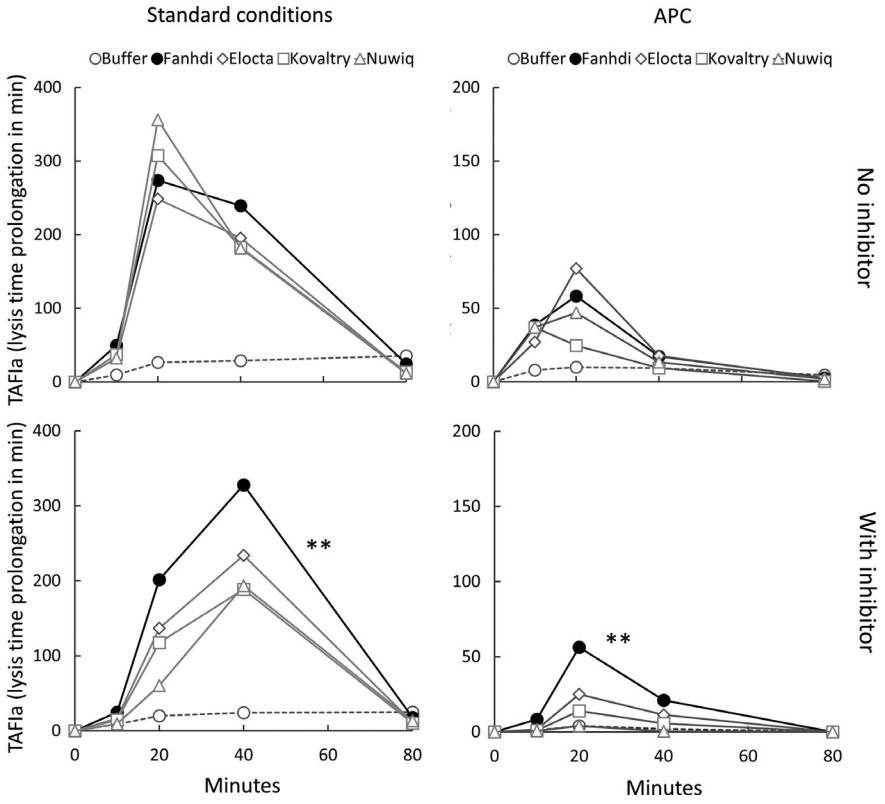

FIGURE 6 Effect of different FVIII preparations (1 IU/mL) on TAFI activation kinetics, evaluated under standard conditions (left panels) and upon addition of APC (0.1 µg/mL, right panels), in the absence (top panels) and presence (bottom panels) of FVIII inhibitor (2.5 BU/mL). Results are expressed as lysis time prolongation over time zero and are the mean of 3-4 experiments. For clarity, SEM has been omitted. **Statistically different from all other FVIII preparations by comparing AUC values (Friedman test and pairwise comparison according to Conover). Please note that the *Y*-axis scales are different

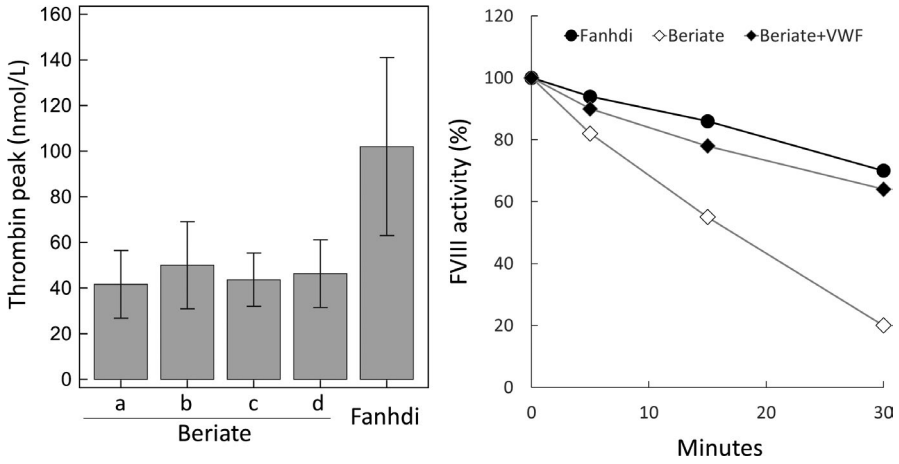

FIGURE 7 Role of VWF in the protection of FVIII against inhibitory IgG and APC. Left panel, Thrombin generation in model plasma with inhibitor (2.5 BU/mL) induced by Beriate (1 IU/mL) under different conditions: (a) Beriate was tested under standard conditions as in Figure 1; (b) Beriate was incubated for 15 min with purified VWF (1:1 ratio) before testing; (c) plasma was supplemented with 1 IU/mL of purified VWF before Beriate addition (to achieve a VWF concentration comparable to that obtained with Fanhdi); (d) Beriate was added to plasma 15 min before inhibitor addition to allow interaction with plasma VWF. Data are the mean ± SEM of three experiments. Fanhdi (1 IU/mL) was tested in parallel for reference. Right panel, Time course of APC (1 µg/mL)-induced degradation of Fanhdi, Beriate and Beriate complexed to purified VWF. Results represent residual FVIII activity and are the mean of two experiments (see methods for additional details). No appreciable degradation of any FVIII preparation was observed with 0.1 µg/mL APC (n = 2, not shown in the figure)

the possible relationship between epitope specificity and VWF role. Finally, some haemophilia samples had detectable levels of FVIII resulting from concentrate infusion. Such a heterogeneity, however, does not appear to have affected the results to a significant extent as the data in patients' samples were almost superimposable to those of model plasma.

5 | CONCLUSIONS

The development of inhibitors occurs in up to 30% of patients with severe haemophilia A and is the most significant complication of treatment.[9,10] Replacement therapy remains the preferred treatment in patients with low inhibitor titre, while bypassing agents

are used in those with high titre.[31] However, evidence has been provided that the combination of FVIII and bypassing agents improves thrombin generation in haemophilic patients with high inhibitor levels, both in vitro and in a pilot study in vivo.[32] In this context, the use of FVIII/VWF complex, which displays a greater capacity to improve thrombin generation and TAFI activation in haemophilic plasma with inhibitor, is likely to offer advantages both in terms of efficiency and costs. Furthermore, our finding that Fanhdi may, under certain conditions, exhibit a greater pro-haemostatic capacity even in the absence of inhibitor warrants further investigations as it may possibly widen the range of conditions under which the use of a FVIII/VWF complex may be preferable to concentrates devoid of VWF.

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DISCLOSURES

MC received an Investigator Sponsored Research (ISR) grant from Grifols and speaker fee from Bayer, Grifols and Werfen. PG has been in an advisory board for Sanofi Genzyme and received speaker fee from Werfen. All other authors state that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

MC designed and supervised the study and wrote the manuscript; CTA, FS, AV and LD performed the research and collected and analysed the data; AMM supervised the clinical study management; PG supervised the clinical study management and critically revised the manuscript; NS wrote part of the manuscript and critically revised the manuscript. All authors read the final version of the manuscript and approved it prior to submission.

ORCID

Mario Colucci https://orcid.org/0000-0001-9146-8095

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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