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Letter to the Editors-in-Chief

Thrombin generation assay for testing hemostatic effect of factor VIII concentrates in patients with hemophilia A and inhibitors: *In vitro* results from the PredicTGA study

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The development of inhibitors to factor VIII (FVIII) is the most challenging and severe complication of hemophilia A as FVIII inhibitors make patients resistant to replacement therapy [1]. Although FVIII bypassing agents are commonly used in these patients [2], FVIII concentrates are still employed in low titer and low responding inhibitor patients. Main advantages of FVIII concentrates over bypassing agents would be their lower (reported) thrombogenicity [2] and the availability of simple laboratory monitoring based on FVIII measurement.

Earlier reports had shown that certain inhibitors are less inhibitory to FVIII when this protein is complexed with von Willebrand factor (VWF) [3]. It was therefore hypothesized that VWF-containing FVIII concentrates are possibly more effective than concentrates devoid of VWF in producing sustained thrombin generation [4]. However, this contention has never been supported by large *ex-vivo* studies comparing treatment with FVIII concentrates rich or devoid of VWF.

We organized a multicenter trial (approved by ethics committees of participating sites) with the primary aim to assess whether thrombin generation assays (TGA) may predict effectiveness of FVIII concentrates (devoid or rich of VWF) in patients with hemophilia A and inhibitors. A second objective was to assess the FVIII protective effect exerted by VWF from alloantibodies and the degree of correlation achieved by this protection with the hemostatic effectiveness of FVIII concentrate *in-vivo*. The study (*PredicTGA*, *NCT01505946*) consists of two phases. In the first (*in-vitro*), centralized spiking experiments were performed by mixing aliquots of each patient plasma with each of three FVIII concentrates. In the second phase (*in vivo*) still ongoing, patients started on treatment with FVIII standard products will undergo testing to assess *in-vivo* FVIII recovery and TGA before and after the first infusion. Patients will be similarly tested during follow up at fixed time points. This manuscript, reports results of the *in-vitro* phase.

Fifteen Italian Centers enrolled 41 patients aged 1–72 years. At enrollment, patients (or tutors) provided informed consent and were instructed to respect 72 h washout period from any concentrate infusion. Blood was collected into vacuum tubes containing 11 mM citrate (final concentration) and 50 μg/mL corn-trypsin inhibitor (CTI) (Haematologic Technologies, Essex, VT) or 10.5 mM citrate without CTI (Becton-Dickinson, Plymouth, UK). Citrate-plain tubes were used for FVIII inhibitor measurements and citrate-CTI was used for TGA. Participating centers were instructed on how to prepare and store plasma. Briefly, blood (with or without CTI) was centrifuged for 20 min at 2880*g* (controlled room temperature). Plasma was quickly frozen and stored at −70 °C until shipment in dry ice to the central laboratory (Hemophilia and Thrombosis Center, Milano).

Three commercially available FVIII concentrates were used for spiking experiments. They included the highly purified plasma derived VWF/FVIII complex concentrate (Alphanate®, Grifols); recombinant full-length FVIII (Advate®, Shire) and recombinant B-domain deleted FVIII (REfacto®, Pfizer). The FVIII potencies used for spiking experiments were those reported on the label of each concentrate. Aliquots of plasma from individual patients were spiked with appropriate amounts of each concentrate to obtain a concentration correspondent to that achieved in patients treated with 100 U/kg.

TGA was performed according to manufacturer instructions (Thrombinoscope™, Thrombinoscope BV, Maastricht, Netherlands) and based on the activation of coagulation of platelet-poor plasma (80 μL) with 20 μL PPP-reagent Low (1 pM human recombinant tissue factor and 4 μM synthetic phospholipids). The reaction was started by 20 μL FluCa (fluorogenic substrate and calcium chloride). Among the TGA parameters, we report on the area under the curve (endogenous thrombin potential, ETP), peak-thrombin and velocity index, defined as VI = [Peak height / (time to peak − lag time)].

FVIII inhibitor titers were assessed against the panel of the three FVIII concentrates or pooled normal plasma (PNP) by the Nijmegen-Bethesda assay [5].

Continuous variables are reported as median and range (interquartile). Friedman or Wilcoxon Signed Ranks non-parametric tests were used to test for statistical significance between groups with $p < 0.05$ considered as statistically significant. Analyses were performed with the IBM SPSS software (Chicago, IL).

Fifteen centers provided plasmas from 41 patients. Two were excluded, as CTI-plasma was unavailable and four were not analyzed because the volume of CTI-plasma was not sufficient. All patients had

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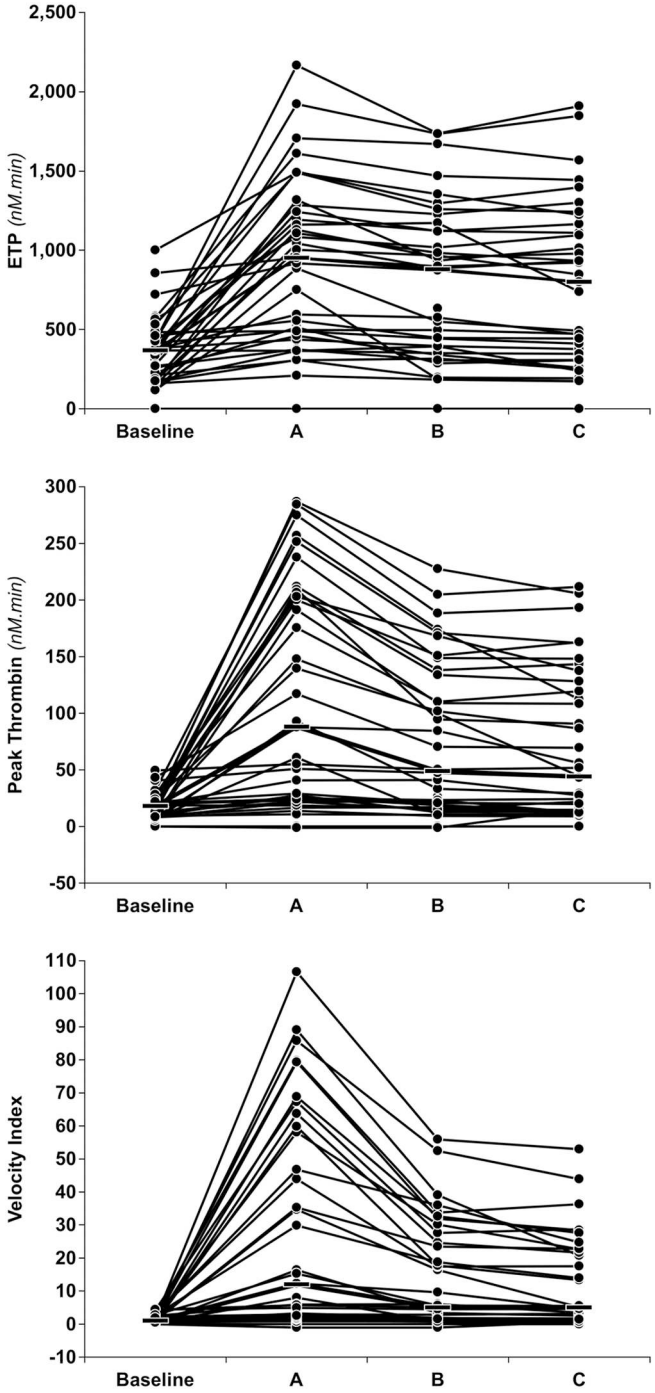

Fig. 1. Endogenous thrombin potential (ETP), peak thrombin and velocity index obtained for patients with hemophilia and inhibitors at baseline and after addition of the three factor VIII concentrates at a dose corresponding to 100 U/kg (see text for more details). A, Alphanate®. B, Advate®. C, REfacto®. Differences (spiked-*vs*-no spiked) for ETP, peak-thrombin and velocity index for all FVIII concentrates were statistically significant ($p < 0.001$). *p*-Values for between-concentrate differences were as follows: A-*vs*-B, A-*vs*-C and B-*vs*-C ($p < 0.001$) for all TGA parameters; ETP and peak-thrombin B-*vs*-C ($p > 0.05$).

pre-spiking FVIII levels < 1 U/dL as measured with the one-stage clotting assay based on APTT (Synthasil, Werfen) and FVIII-deficient plasma (Werfen). The median (interquartile) inhibitor titer measured centrally against PNP was 6 BU (1 − 23). The corresponding values when measured against the three investigated concentrates were the highest with REfacto® [9 BU (2 − 33)] and the lowest with Alphanate® [6 BU (1 − 22)] or with Advate® [6 BU (1–22)]. A minority of patients ($n = 6$) who has had measurable inhibitor titers at enrollment had inhibitors titers < 0.5BU at centralization. However, results and conclusions did not change after removal of these patients (not shown). TGA parameters are in Fig. 1. Although, there was a certain degree of between-patients variability, the median values of ETP and thrombin-peak that were relatively low at baseline increased significantly after spiking for all FVIII concentrates ($p < 0.001$). ETP and peak-thrombin after spiking were the highest for Alphanate®, intermediate for Advate® and the lowest for REfacto®. The velocity index was low at baseline and increased after spiking and the order of change for the three FVIII concentrates mirrored that observed for the ETP or peak-thrombin.

ETP and the inhibitor titers were inversely correlated for all FVIII concentrates with rho values ranging from −0.36 to −0.42 ($p < 0.05$).

VWF along with securing platelet adhesion to the sub-endothelial matrix and platelet aggregation one another is also the carrier for FVIII. Hence, the latter is protected from the digestion mediated by plasma proteases. It has been surmised that FVIII when bound to VWF is less immunogenic than its unbound counterpart [6,7]. This was shown by a recent study investigating recombinant *vs* human derived FVIII concentrates [8]. Furthermore, it has been surmised that FVIII when bound to VWF is less amenable to be inactivated by FVIII inhibitors [3,10]. The above properties generated the perception that FVIII concentrates that contain sufficient amounts of VWF might be more suitable to treat patients with hemophilia A and inhibitors than those derived from recombinant technology that do not contain VWF. Although *in-vivo* studies are not currently available to test this hypothesis, Salvagno et al. [9] have investigated in their *in vitro* study patients with hemophilia and FVIII inhibitors. They spiked a relatively small number ($n = 11$) of patient plasmas with four FVIII concentrates and measured the capacity of plasma to inhibit thrombin generation. They found that the inhibitor titers needed to inhibit 50% maximum thrombin generation were the highest for the concentrates that contained VWF and the lowest for those that did not, suggesting that VWF containing concentrates, added to hemophilic plasma with inhibitors, generate more thrombin than do concentrates devoid of VWF.

In this study we provide direct evidence that the above conclusions holds true by investigating a larger number ($n = 35$) of patients, whose plasma were added with three FVIII concentrates. The TGA parameters, including ETP, peak-thrombin and velocity index were the greatest when plasmas were added with the VWF containing concentrate (*i.e.*, Alphanate®) and the inhibitor titer measured against the three concentrates mirrored the results obtained with the TGA. Furthermore, the inhibitor titer and ETP were inversely correlated. These findings are consistent with the hypothesis that FVIII concentrates containing VWF might be more suited than those devoid of VWF to protect FVIII from neutralization by FVIII inhibitors and to support sustained thrombin generation.

Some limitations of the study should be recognized. Characteristics of the FVIII concentrates other than VWF could explain the observed differences in TGA. Purified VWF should have been added to the full length or B-domain deleted FVIII concentrates to prove or disprove conclusively that the increased thrombin generation is due to VWF. However, the aim of our study was to compare *in-vitro* three products and only one contained VWF. Therefore, the hypothesis that VWF is one of the determinants that explains our results is reasonable.

The inhibitor epitopes have not been determined, as the method was not available at the time of the study. Hence, we are unable to assess the relationship of inhibitor epitopes with FVIII-VWF. Although the above information would have been useful, it should be considered that the aim of the study was to compare different FVIII concentrates for thrombin generation under the same experimental conditions. The effect due to inhibitor epitopes (if any) should be similar for the three concentrates.

Finally, this is a preliminary *in-vitro* study and therefore no definitive conclusions can be drawn on the role played by VWF *in-vivo*. The above limitations notwithstanding, we believe that our observations are valuable to shed light on the effect that VWF may have on the capacity of FVIII concentrates to sustain thrombin generation.

In conclusion, TGA and inhibitor cross-reactivity showed between-concentrate differences, with the plasma derived FVIII/VWF concentrate resulting the least reactive against inhibitors and the most efficient in thrombin generation. Whether the *in-vitro* effect holds true after infusion of concentrates rich or devoid of VWF is being investigated by the *in-vivo* study that is still ongoing within the frame of the same project.

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Disclosure

AT, Advisory Board and/or Speaker bureau: Novo Nordisk, Baxalta/Shire, CSL Behring and Roche. FP, Consultation fees: Kedrion, LFB. Honoraria for participation as speaker at educational meetings: Ablynx, Grifols, Sobi, Shire, Roche, Alnylam. Member of the advisory board: Ablynx, Roche, Shire. ES, Advisory Board and/or Speaker bureau: Bayer; Grifols, Novo Nordisk, Octapharma, Baxalta/Shire, Pfizer, Kedrion, Sobi, Bioverativ, CSL Behring and Roche.

Authors contribution

AT, and ES conceived the study, reviewed results and revised the manuscript; AT, wrote the manuscript; VC, conceived the experimental design, collected data, made statistical analyses and reviewed results; MC, RB and MB, performed laboratory testing; FP, revised the manuscript.

Appendix A. List of PredicTGA collaborators

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