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Combination of FVIII and by-passing agent potentiates in vitro thrombin production in hemophilia A inhibitor plasma

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Introduction

The majority of patients with congenital haemophilia A are successfully treated with prophylactic replacement therapy with factor VIII-products. The ultimate goal is to prevent bleeding, mainly joint bleeds, and to maintain haemostasis (Pipe and Valentino 2007). However, as many as 30% of patients with severe haemophilia A (FVIII plasma level <1% of normal concentration) develop inhibitory antibodies against FVIII (Berntorp 2007). In order to control haemostasis there are different clinical strategies to handle these antibodies; for example giving saturating levels of FVIII (Astermark 2003), or to eradicate the inhibitors by immune tolerance induction (ITI) (Tellier, *et al* 2009). One can also use FVIII-bypassing agents which induce thrombin production independently of FVIII. These include the activated prothrombin complex concentrates (APCC) and recombinant factor VIIa (rFVIIa), products with different modes of action (Gomperts, *et al* 2008). APCC (FEIBA®) contains, among other zymogens and coagulation enzymes, FII and FXa which initiate thrombin formation downstream of the inhibited FVIII (Turecek, *et al* 2004a, Turecek, *et al* 2004b, Varadi, *et al* 2003). Recombinant FVIIa (NovoSeven®), in pharmacological doses, activates in a tissue factor dependent pathway FX leading to thrombin production (Mackman 2009, Monroe, *et al* 1997, Monroe, *et al* 1998). These products are normally used as monotherapy. However, occasionally patients develop haemorrhages refractory to monotherapy. Several case reports indicate, that it is efficacious to use a sequential combination therapy in such cases with FEIBA® and NovoSeven® (Key, *et al* 2002, Livnat, *et al* 2008, Schneiderman, *et al* 2007). Van Veen et al have shown in an *in vitro* setting, a synergistic effect on thrombin generation when combining APCC and rFVII (van Veen, *et al* 2009). There are also studies showing that adding FX to FVIIa increases the haemostatic potency both *in vitro* and *in vivo* compared with utilization of FVIIa alone (Tomokiyo, *et al* 2003). More over, case reports

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3 have been published presenting clinical protocols used for patients with a history of
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5 complicated bleedings who will undergo orthopaedic surgery where preoperative
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7 immunoadsorption for elimination of inhibitory antibodies are followed by sequential
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9 administration of either FEIBA® or NovoSeven® and factor VIII concentrates (Dargaud, *et*
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11 *al* 2005, Habermann, *et al* 2004). Recently, Martinowitz *et al*, reported on the use of
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13 thrombin generation assay to monitor patient with refractory bleedings to be able to find
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15 individual dose levels of by-passing agents to maintain haemostasis. When using
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17 combinations of APCC and rFVIIa the patients could receive a concomitant treatment
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19 regimen with doses far lower than recommended for the individual product (Martinowitz, *et*
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21 *al* 2009).

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28 It has been observed clinically for some years that in some patients undergoing immune
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30 tolerance induction therapy the bleeding frequency seems to decrease during the treatment
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32 period. The International Immune Tolerance Study was recently terminated because of less
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34 frequent haemorrhages in the high-dose arm (Clinical Trials Gov and Charles Hay by personal
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36 communication) which the Data Safety Monitoring Board considered to be a safety concern.
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38 We therefore wanted to investigate if there is an advantage *in vitro* in combining a by-passing
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40 agent with factor VIII clotting factor concentrates in order to increase the thrombin generating
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42 capacity in plasmas from haemophilia A patients with an inhibitor.
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50 51 **Materials and Methods**

52 53 54 55 *Patient's plasma samples*

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57 Plasma was obtained from patients with severe congenital FVIII deficiency (< 0.01 IU/mL),
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59 and with a measurable titer of inhibitory FVIII antibodies. The experiments with Alphanate®
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3 included five patients. In the experiments with APCC and Fanhdi® ten patients were
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5 included. Plasma samples from four patients were spiked with a combination of APCC and
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7 rFVIIa without the additive of FVIII. In all other experiments plasma samples from eleven
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9 patients were used.
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15 Blood samples were obtained by venipuncture and blood collected into tubes containing
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17 0.129M citrate and platelet-poor plasma was prepared by centrifuging the tubes for 20 min at
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19 2000 g twice. Samples were frozen at – 80 ° C and thawed at 37 ° C just before starting the
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21 assay.
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27 *Reagents*

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29 The activated prothrombin complex concentrate (APCC) FEIBA® and the recombinant
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31 FVIII-product Advate® were obtained from Baxter, Baxter Healthcare Corporation, IL, USA.
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33 Kogenate® was obtained from Bayer, Bayer Schering Pharma, Leverkusen, Germany.
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35 Recombinant activated FVII (rFVIIa), NovoSeven®, was obtained from Novo Nordisk,
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37 Bagsvaerd, Denmark. The plasma-derived von Willebrand factor containing FVIII
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39 concentrates Fanhdi® and Alphanate® were obtained from Grifols, Barcelona, Spain and
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41 Haemate® (Humate-P), from CSL Behring, CSL Behring L. L. C., PA, USA. The
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43 VWF:FVIII ratio is close to 1 in Fanhdi® and Alphanate® and 2.5 in Haemate® (Berntorp
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45 2009, Logan 2009, Ristol, *et al* 1996). Human thrombin was obtained from ERL Enzyme
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47 Research Lab, Dia-Service, Gothenburg, Sweden. Freeze dried phospholipid/recombinant
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49 high rTF in Tris-Hepes-NaCl buffer, containing a high concentration of phospholipid micelles
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51 with tissue factor (TECHNOTHROMBIN® TGA RC for the rFVIIa-assays and
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53 TECHNOTHROMBIN® TGA RB for the assays with APCC), was obtained from
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55 Technoclone GmH, Vienna, Austria. Fluorogenic substrate solution (1mM Z-Gly-Gly-Arg-
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AMC) was purchased from BACHEM Feinchemikalien AG, Bubendorf, Switzerland. FVIII-deficient plasma (immune depleted) was purchased from Biopool, Trinity Biotech, Ireland, and used for dilution of patient samples and factor concentrates. Plasma for normal pool was collected from 20 individuals (laboratory staff) and used as positive control in the thrombin generation assay. FVIII:C Chromogenic assay kits were purchased from Haemachrom Diagnostica, Mölndal, Sweden.

Inhibitor test

Patient plasma was diluted to give an inhibitor titer of 11 Malmö inhibitor units (MIU/mL) in the thrombin generation assay. One Malmö inhibitor unit corresponds to the number of IU of factor VIII:C that get neutralized by 1 mL of patient plasma. One MIU corresponds to about three Bethesda inhibitor units (BIU) (Nilsson and Hedner 1976).

Thrombin Generation Assay

The thrombin generation assay was performed using a relipidated high tissue factor (rhTF) (see Reagents above) as a trigger of the reaction (Varadi, *et al* 1999). 10 μ L rhTF was added to 50 μ L of a fluorogenic substrate solution and 15 mM CaCl_2 . To start the reaction 40 μ L of patient's plasma was supplemented with either 5 μ L rFVIIa or APCC and 5 μ L of FVIII-dilution. The final concentrations of rFVIIa were 0 μ g/mL, 1.0 μ g/mL, 2.5 μ g/mL and 5.0 μ g/mL, and for APCC 0 IU/mL, 0.25 IU/mL, 0.5 IU/mL and 1 IU/mL. FVIII:C was added to reach final concentrations of 0 IU/mL, 2.5 IU/mL and 5 IU/mL. The doses used in this study are based on pilot experiments in our laboratory, and are found to reach an optimal stability and sensitivity in the assays. Comparing with doses used in clinical context, the dose levels of FVIII:C in the study are higher than standard dosing. APCC is normally administered at a dose of 75 IU/kg which correspond to an *in vitro* dose of ~ 1.5-2 IU/mL. Finally, rFVIIa at a

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dose of 90 µg/kg, which correspond to the medium dose level (2.5 µg/mL) used in the experiment, is commonly used for treating patients. However, the work is performed *in vitro*, hence the conditions can never be exactly the same as *in vivo*.

All samples were analyzed in duplicates. In the experiment with only APCC and rFVIIa in combination without additive of FVIII, four patients were analyzed. The experiment was repeated two times for one patient and three times for the others. The samples were incubated at 37°C in a Microplate Fluorescence Reader (Tecan Genios, Tecan Austria GmbH, Grödig, Austria) where the fluorescence intensity was monitored every minute for 90 min. The fluorescence intensity (measured in relative fluorescence units (RFU)) is proportional to the amount thrombin generated in the assay (Varadi, *et al* 2004). Calculation of the thrombin concentration is made from comparison to a reference curve which is established from known concentrations of thrombin. The thrombin concentration is expressed as the rate of development of fluorescence intensity [fluorescence units (FU)] calculated for each reading (FU/min).

Statistics

To analyze the interactions between the products included the ANCOVA (analysis of covariance) program was used. The statistical calculations were performed at RHO Inc, Chapel Hill, NC,USA. *P* less than .05 was considered to indicate statistical significance.

Results

APCC-FVIII interaction

Thrombin generation assay was performed on the plasma samples with different dose levels of FVIII and APCC (see Materials and Methods). This experimental design gives 12 possible treatment combinations for each patient plasma sample. The samples without any APCC or

factor VIII concentrate added turned out to produce a small amount of thrombin in the thrombin generation assay ranging from 8.5 – 10.7 nM. Despite the presence of inhibitors in each plasma sample the thrombin production increased in a dose dependent way when adding factor VIII to the sample (see Table 1).

For each separate dose level of APCC the thrombin production (Tmax) was shown to increase significantly with increased factor VIII dose level, irrespective of what factor VIII product that was added. Similarly, for each dose level of the different factor VIII products the thrombin production increases significantly when the APCC dose is increased. When comparing the maximum thrombin production between the different factor VIII products it seems that all products reach approximately the same level (range 355.7 – 392.1 nM) apart from Advate® reaching a maximum thrombin production of 292.4 nM (see Table 1).

To validate whether the combination of the two products potentiate the thrombin generation *in vitro* or whether the increase of the dose level only gives an additive pharmacologic effect the ANCOVA (analysis of covariance) statistical program was used. Analysis of the interaction between APCC and the factor VIII products show that the combination of the preparations potentiates each other in a synergistic way, resulting in a significant increase in thrombin production for all products apart from for Alphanate® ($p=0.1470$), as exemplified with Advate® in Figure 1.

rFVIIa-FVIII interaction

As shown with APCC, thrombin was produced with significant increase in the absence of rFVIIa when adding additional factor VIII to the sample. The maximum thrombin production

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3 ranged from 290.9 - 370.3 nM, where Advate® resulted in the lowest amount of produced
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5 thrombin and Alphanate® reached the highest level (see Table 2).
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10 In the interaction test for rFVIIa and the different factor VIII concentrates we find an
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12 increased thrombin generation with increased dose level of either rFVIIa dose or factor VIII
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14 dose. However, in contrast to the findings in the APCC experiment, the increase in thrombin
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16 generating capacity is additive but not synergistic (see Table 2).
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22 *APCC-rFVIIa interaction*
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24 We further wanted to evaluate whether the combination of APCC and rFVIIa would give a
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26 synergistic effect on the maximum rates of thrombin generation (Tmax). Four patients were
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28 included in this experiment. The experimental design used was the same as in the experiments
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30 shown above, apart from that no FVIII was added. We found that increasing the dose of
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32 rFVIIa (p=0.01) and APCC (p<0.0001) gives a significant increase in the maximum thrombin
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34 concentration produced. However, we can not find any synergistic interaction between the
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36 two products (p= 0.9289) (see Table 3).
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43 **Discussion**
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46 We have investigated if there is an advantage in combining a by-passing agent (APCC
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48 (FEIBA®) or rFVIIa (NovoSeven®)) with a factor VIII clotting factor (Advate®, Haemate®,
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50 Fanhdi®, Kogenate® and Alphanate®) in order to increase the thrombin generating capacity
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52 *in vitro* in plasmas from haemophilia A patients with an inhibitor. Occasionally patients with
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54 haemophilia A and inhibitory antibodies develop haemorrhages that could not be controlled
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56 by monotherapy with either APCC or rFVIIa. There are case reports showing that it is
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58 efficacious to treat such bleedings with a sequential administration of a combination of APCC
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and rFVIIa (Key, *et al* 2002, Livnat, *et al* 2008, Schneiderman, *et al* 2007). There are also *in vitro* studies indicating that there is a synergistic pharmacological effect between APCC and rFVIIa resulting in an increased thrombin generation (van Veen, *et al* 2009).

We show that a combination of by-passing agent and FVIII has a positive effect on thrombin generation *in vitro* in plasma from patients with severe haemophilia A and excess of inhibitory antibodies. Since the patient plasma samples consist of other coagulation factors in normal concentrations a background activity of thrombin production is generated in the assays (see Table 1 and 2). All plasma samples were diluted to reach a level of inhibitory antibodies of 11 MIU/mL (~ 33 Bethesda U/mL). Despite the presence of inhibitory antibodies at a concentration enough to neutralize 11 IU/mL of FVIII:C, i.e. in excess to the FVIII added, the thrombin production increased in a dose dependent way when adding factor VIII alone to the plasma samples. This might be explained by the kinetics of the inhibitory antibody. The thrombin generation reaction starts immediately when the FVIII-concentrate is added to the plasma sample. Since the binding of the antibody to FVIII, and inhibition of its function are relatively slow processes it may explain why thrombin is produced. In experiments with incubation times up to 2 h we still see thrombin production in samples with excess of inhibitor (unpublished observations) which corroborates with the clinical observation that some patients experience less bleeds during ITI. The reason for this is not known. Hypothetically, the explanation could be a transient haemostatic effect of infused FVIII not measurable *ex vivo*.

We could not demonstrate any clear difference in FVIII interaction with regard to concentrate content of von Willebrand factor (VWF). This is somewhat surprising as it has been proposed by several authors that VWF may compete with inhibitor binding (Gensana, *et al* 2001, Goudemand, *et al* 2006, Kallas and Talpsep 2001) and also impact on thrombin generation.

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As an example, FVIII concentrates containing VWF has been shown to elicit more thrombin in inhibitor plasma (Salvagno, *et al* 2007). However, when present together with APCC other mechanisms affecting thrombin generation (see below), could tentatively be more important and overshadow a hypothetical VWF mechanism.

Analysis of the interactions between APCC and the factor VIII products show that the combination of the preparations potentiates each other in a synergistic way, resulting in a significant increase in thrombin production for all products apart from for Alphanate® (p=0.1470). Alphanate®, Haemate® and Fanhdi® are all plasma derived concentrates which contain von Willebrand factor (Berntorp 2009, Logan 2009, Ristol, *et al* 1996). It has earlier been shown that the immunoreactivity appears to be higher towards Advate® compared with Haemate® and Fanhdi® in plasmas with inhibitors directed mainly to light chain epitopes, resulting in decreased thrombin generation (Salvagno, *et al* 2007). Hypothetically, one would therefore expect a higher thrombin generation and a better synergistic or additive effect of the VWF containing concentrates in the present study, although this was not seen. The lesser effect elicited by Alphanate® may partly be explained by the lower number of inhibitor plasmas studied with that product. Since we have not performed epitope mapping in this study we can not eliminate the possibility that different epitopes affect the outcome of the thrombin generation (see Figure 1). Further on, APCC (FEIBA®) contains, among other zymogens and coagulation enzymes, FIX and FIXa to which FVIII act as a co-factor (Turecek, *et al* 2004a, Turecek, *et al* 2004b, Varadi, *et al* 2003), and this could explain why the outcome of a combination of the products potentiates thrombin generation.

When combining rFVIIa with a FVIII-concentrate, the effect, in our study, is additive and not synergistically potentiated (see Figure 2). The thrombin generation assay we use is performed

with platelet poor plasma. It is well known that rFVIIa needs the presence of phospholipids or platelets to reach its full pharmacologic capacity (Livnat, *et al* 2008, Monroe, *et al* 1997). In order to improve the conditions for the experiments containing rFVIIa we have used a reagent with a high concentration of phospholipid micelles with tissue factor as a trigger of the thrombin generation reaction (TECHNOTHROMBIN® TGA RC). This should be able to compensate, at least partly, for the absence of platelets in the plasma samples. Anyhow, it is known from earlier studies that the phospholipid content of APCC contributes to the effect on the thrombin generation assay (Livnat, *et al* 2008). This is another possible explanation for the difference between APCC and rFVIIa that we found in the outcome of the interaction tests. Further more, adding of FX to a plasma sample has been shown to increase the thrombin generation capacity of rFVIIa both *in vivo* and *in vitro* (Tomokiyo, *et al* 2003). As mentioned above, APCC consist of e.g. coagulation enzyme FXa and several case reports describing sequential therapy with APCC and rFVIIa propose an administration protocol where APCC is given prior to rFVIIa (Economou, *et al* 2008, Key, *et al* 2002). By using such protocol the FXa-content of APCC could, hypothetically, improve the effect of rFVIIa. However, since the experimental conditions for monitoring rFVIIa is not optimal, this study is not aiming at comparing the effect of APCC and rFVIIa. We believe that the improved effect of adding FVIII to a by-passing agent in the presence of a high-responding inhibitor, could, potentially, be a very useful treatment option in clinical situations where monotherapy with by-passing agent fail.

Livnat *et al* has shown *in vitro*, both in platelet rich plasma (PRP) and in platelet poor plasma (PPP), a synergistic effect between APCC and rFVIIa on thrombin generation (Livnat, *et al* 2008). We performed similar experiments in this study, with thrombin generation assay on plasma samples (PPP) with addition of APCC and rFVIIa in combination. In discrepancy to

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what is referred to above we did not observe any synergy between the two products, but a significant increase in thrombin generation where the interactive effect is additive (see Table 3).

The development of inhibitory antibodies is the major complication for patients with haemophilia. As mentioned above, occasionally the by-passing agents APCC or rFVIIa in monotherapy fails to control haemorrhages in patients with the severe form of haemophilia A or B, and case reports as well as *in vitro* data suggest a combination of the two products in such cases. However, this study shows for the first time that a combination of FVIII and a by-passing agent enhances thrombin generation. Our findings could be useful for the development of clinical strategies for patients with inhibitors and severe haemorrhages refractory to monotherapy with by-passing agent.

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Figure legends

Figure 1:

The interaction between APCC and FVIII clotting factor. The curves of the diagram are to be parallel in cases where the pharmacologic effect of increased dose level is additive. In the APCC-assay, here exemplified with Advate (a), the curves are not parallel, indicating synergistic interaction resulting in a significant increase in thrombin production for all FVIII products apart from for Alphanate (b).

Figure 2:

The interaction between rFVIIa and FVIII clotting factor. The increase in thrombin generating capacity is additive but not synergistic, here exemplified with Advate (a). b) P-values for all FVIII-clotting factors included in the assay.

Table 1:

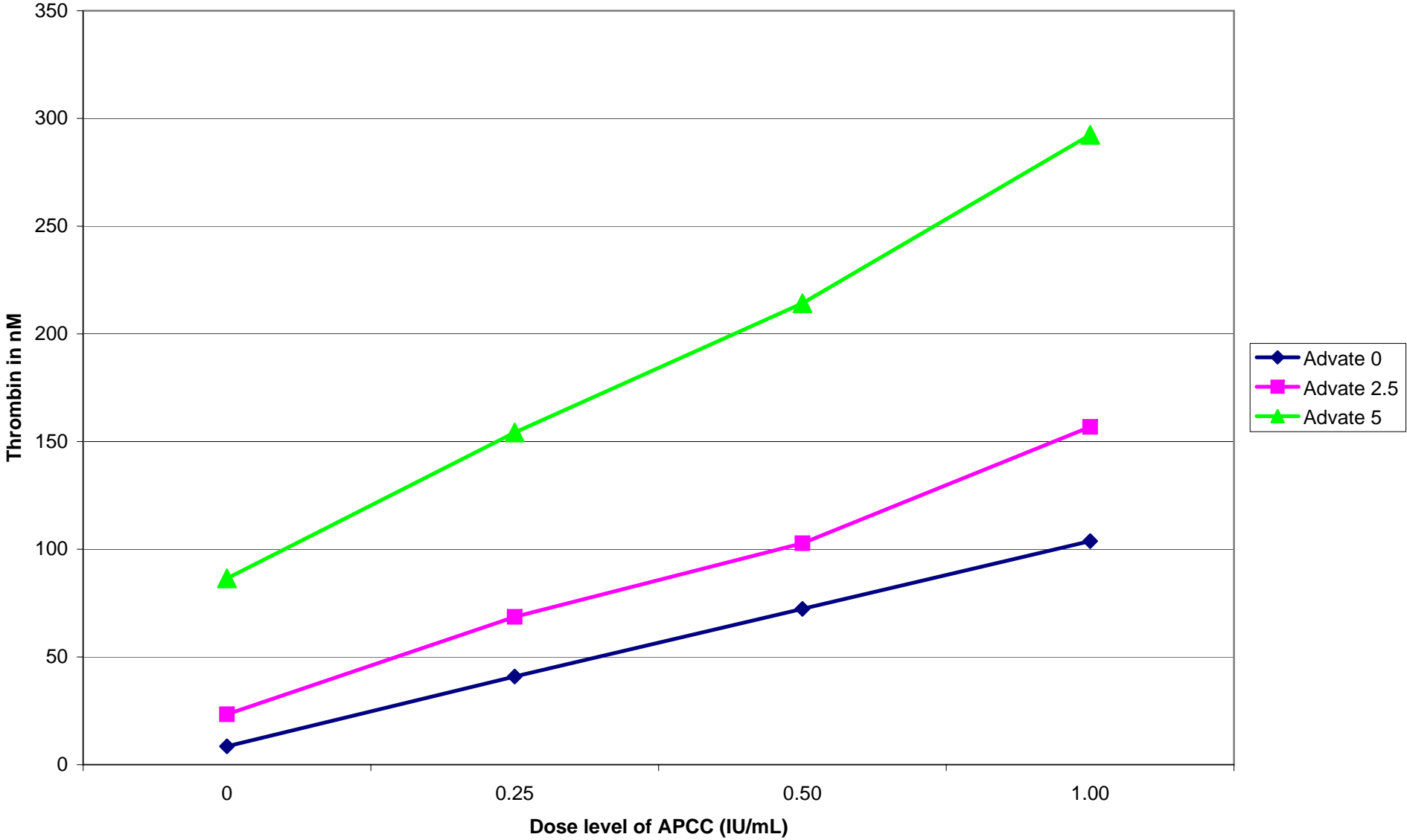
Thrombin production in nM for the assay combining APCC and FVIII. The maximum thrombin production shown within frame.

Table 2:

Thrombin production in nM for the assay combining rFVIIa and FVIII. The maximum thrombin production shown within frame.

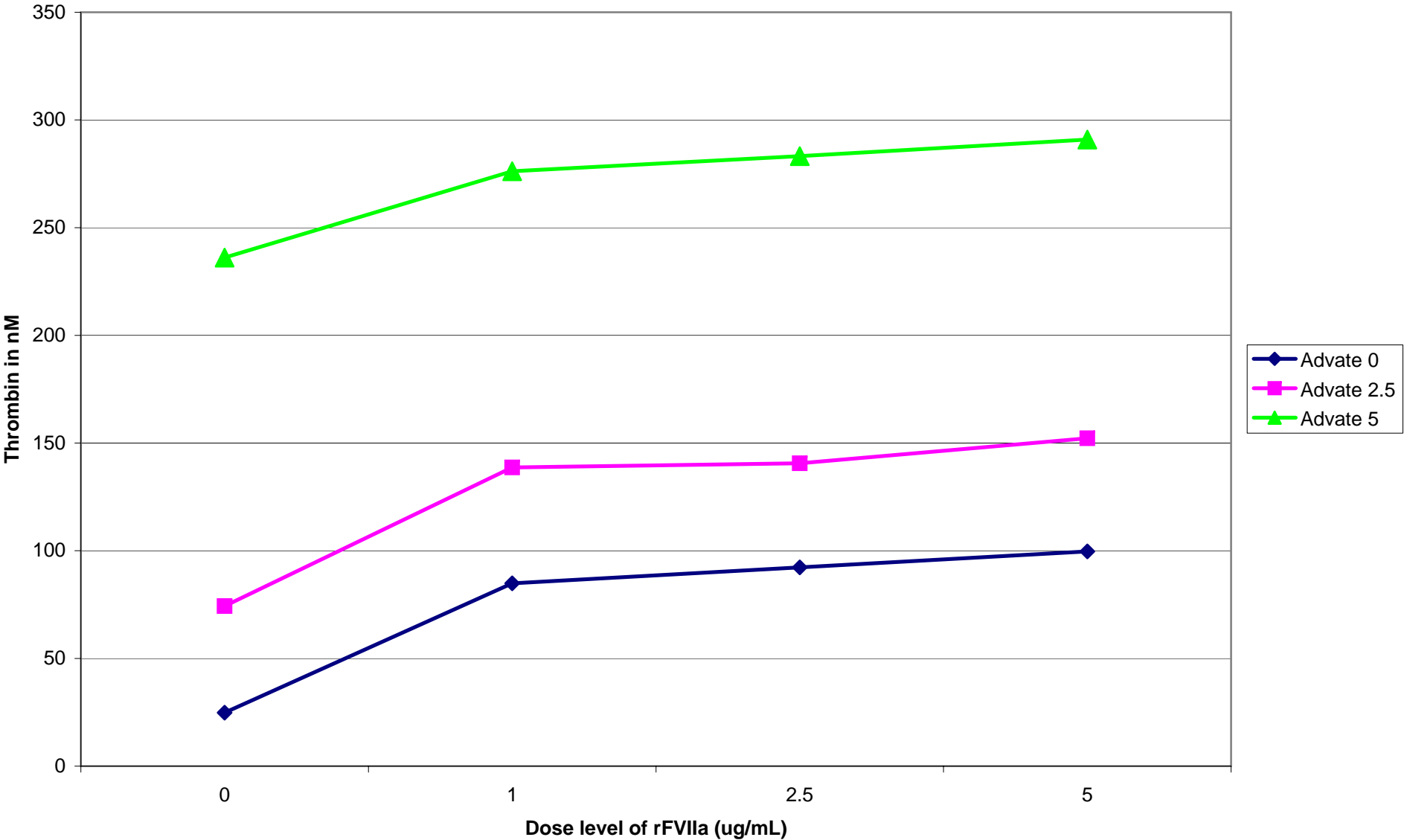
Table 3:

Thrombin production in nM for the assay combining rFVIIa and APCC.



Advate® (n=11)	Fanhdī® (n=10)	Haemate® (n=11)	Kogenate® (n=11)	Alphanate® (n=5)
p=0.02	p=0.01	p=0.04	p<0.0001	p=0.15

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Advate® (n=11)	Fanhdī® (n=11)	Haemate® (n=11)	Kogenate® (n=11)	Alphanate® (n=5)
p=0.98	p=0.99	p=0.97	p=0.90	p=0.99

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	APCC 0 IU/mL	APCC 0.25 IU/mL	APCC 0.50 IU/mL	APCC 1.00 IU/mL
FVIII dose level 0 IU/mL				
Advate	8.5	40.9	72.3	103.8
Fanhdi	10.6	46.2	71.3	115.7
Haemate	8.8	44.1	70.4	112.0
Kogenate	8.6	42.9	72.0	110.7
Alphanate	10.7	44.9	64.0	100.7
FVIII dose level 2.5 IU/mL				
Advate	23.4	68.6	102.8	156.8
Fanhdi	67.0	135.0	179.0	255.5
Haemate	68.9	134.1	173.2	248.8
Kogenate	23.3	75.6	109.5	174.3
Alphanate	49.4	115.7	150.7	215.1
FVIII dose level 5 IU/mL				
Advate	86.5	154.2	214.2	292.4
Fanhdi	139.5	221.4	282.1	376.6
Haemate	131.2	202.7	252.2	364.0
Kogenate	121.3	217.7	284.8	392.1
Alphanate	122.3	200.3	257.4	355.7

	rFVIIa 0 µg/mL	rFVIIa 1 µg/mL	rFVIIa 2.5 µg/mL	rFVIIa 5 µg/mL
FVIII dose level 0 IU/mL				
Advate	24.8	84.9	92.3	99.7
Fanhdi	22.7	80.4	88.2	94.9
Haemate	25.4	87.2	93.9	100.9
Kogenate	25.0	84.0	92.7	99.0
Alphanate	17.9	68.0	74.8	81.8
FVIII dose level 2.5 IU/mL				
Advate	74.3	138.7	140.6	152.2
Fanhdi	159.9	205.0	219.8	224.7
Haemate	204.2	239.8	246.3	246.3
Kogenate	71.2	140.0	176.1	159.7
Alphanate	147.5	201.2	203.9	205.4
FVIII dose level 5 IU/mL				
Advate	236.1	276.2	283.2	290.9
Fanhdi	284.2	322.1	325.7	343.0
Haemate	278.5	328.3	338.3	339.6
Kogenate	249.9	300.7	329.8	315.9
Alphanate	316.1	344.2	346.1	370.3

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rFVIIa (µg/mL)	APCC (IU/mL)			
	0	0.25	0.5	1
0	11.5	44.1	66.8	136.5
1	28.5	55.3	91.4	131.3
2.5	32.2	59.8	96.7	138.4
5	36.6	61.9	100.6	151.7

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Summary

By-passing agents, rFVIIa (recombinant factor VII) and APCC (activated prothrombin complex concentrates), are important tools in the treatment of patients with haemophilia A and high-responding inhibitory antibodies. It has been observed clinically that in some patients undergoing immune tolerance induction the bleeding frequency decreases, hypothetically caused by a transient haemostatic effect of infused FVIII not measurable *ex vivo*. We evaluated how by-passing agents and factor VIII affect thrombin generation (TG) *in vitro* using plasma from 11 patients with severe haemophilia A and high titer of inhibitors. Samples were spiked with combinations of APCC, rFVIIa and five different factor VIII products. Combination of APCC and factor VIII showed for four factor VIII products a synergistic effect in eliciting TG ($p < 0.005$). When rFVIIa and factor VIII were combined the interaction between the preparations was found to be additive. Further on, we combined APCC and rFVIIa without factor VIII, resulting in an additive effect on the thrombin production. Each product separately increased TG above baseline. In conclusion, the amount of thrombin formed *in vitro* by adding a by-passing agent, were higher in the presence of factor VIII. Our findings support the use of factor VIII in by-passing therapy to optimize the haemostatic effect.