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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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**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  
4 complicated bleedings who will undergo orthopaedic surgery where preoperative  
5 immunoadsorption for elimination of inhibitory antibodies are followed by sequential  
6  
7 immunoadsorption for elimination of inhibitory antibodies are followed by sequential  
8 administration of either FEIBA® or NovoSeven® and factor VIII concentrates (Dargaud, *et*  
9  
10 *al* 2005, Habermann, *et al* 2004). Recently, Martinowitz et al, reported on the use of  
11  
12 thrombin generation assay to monitor patient with refractory bleedings to be able to find  
13  
14 individual dose levels of by-passing agents to maintain haemostasis. When using  
15  
16 combinations of APCC and rFVIIa the patients could receive a concomitant treatment  
17  
18 regimen with doses far lower than recommended for the individual product (Martinowitz, *et*  
19  
20 *al* 2009).

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28 It has been observed clinically for some years that in some patients undergoing immune  
29  
30 tolerance induction therapy the bleeding frequency seems to decrease during the treatment  
31  
32 period. The International Immune Tolerance Study was recently terminated because of less  
33  
34 frequent haemorrhages in the high-dose arm (Clinical Trials Gov and Charles Hay by personal  
35  
36 communication) which the Data Safety Monitoring Board considered to be a safety concern.  
37  
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 **Materials and Methods**

### 51 *Patient's plasma samples*

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57 Plasma was obtained from patients with severe congenital FVIII deficiency (< 0.01 IU/mL),  
58  
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  
4  
5 included. Plasma samples from four patients were spiked with a combination of APCC and  
6  
7 rFVIIa without the additive of FVIII. In all other experiments plasma samples from eleven  
8  
9 patients were used.  
10  
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12  
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14  
15 Blood samples were obtained by venipuncture and blood collected into tubes containing  
16  
17 0.129M citrate and platelet-poor plasma was prepared by centrifuging the tubes for 20 min at  
18  
19 2000 g twice. Samples were frozen at – 80 ° C and thawed at 37 ° C just before starting the  
20  
21 assay.  
22  
23

### 24 25 26 27 *Reagents*

28  
29 The activated prothrombin complex concentrate (APCC) FEIBA® and the recombinant  
30  
31 FVIII-product Advate® were obtained from Baxter, Baxter Healthcare Corporation, IL, USA.  
32  
33 Kogenate® was obtained from Bayer, Bayer Schering Pharma, Leverkusen, Germany.  
34  
35 Recombinant activated FVII (rFVIIa), NovoSeven®, was obtained from Novo Nordisk,  
36  
37 Bagsvaerd, Denmark. The plasma-derived von Willebrand factor containing FVIII  
38  
39 concentrates Fanhdi® and Alphanate® were obtained from Grifols, Barcelona, Spain and  
40  
41 Haemate® (Humate-P), from CSL Behring, CSL Behring L. L. C., PA, USA. The  
42  
43 VWF:FVIII ratio is close to 1 in Fanhdi® and Alphanate® and 2.5 in Haemate® (Berntorp  
44  
45 2009, Logan 2009, Ristol, *et al* 1996). Human thrombin was obtained from ERL Enzyme  
46  
47 Research Lab, Dia-Service, Gothenburg, Sweden. Freeze dried phospholipid/recombinant  
48  
49 high rTF in Tris-Hepes-NaCl buffer, containing a high concentration of phospholipid micelles  
50  
51 with tissue factor (TECHNOTHROMBIN® TGA RC for the rFVIIa-assays and  
52  
53 TECHNOTHROMBIN® TGA RB for the assays with APCC), was obtained from  
54  
55 Technoclone GmhB, Vienna, Austria. Fluorogenic substrate solution (1mM Z-Gly-Gly-Arg-  
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3 AMC) was purchased from BACHEM Feinchemikalien AG, Bubendorf, Switzerland. FVIII-  
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5 deficient plasma (immune depleted) was purchased from Biopool, Trinity Biotech, Ireland,  
6  
7 and used for dilution of patient samples and factor concentrates. Plasma for normal pool was  
8  
9 collected from 20 individuals (laboratory staff) and used as positive control in the thrombin  
10  
11 generation assay. FVIII:C Chromogenic assay kits were purchased from Haemachrom  
12  
13 Diagnostica, Mölndal, Sweden.  
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### 20 *Inhibitor test*

21  
22 Patient plasma was diluted to give an inhibitor titer of 11 Malmö inhibitor units (MIU/mL) in  
23  
24 the thrombin generation assay. One Malmö inhibitor unit corresponds to the number of IU of  
25  
26 factor VIII:C that get neutralized by 1 mL of patient plasma. One MIU corresponds to about  
27  
28 three Bethesda inhibitor units (BIU) (Nilsson and Hedner 1976).  
29  
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### 34 *Thrombin Generation Assay*

35  
36 The thrombin generation assay was performed using a relipidated high tissue factor (rhTF)  
37  
38 (see Reagents above) as a trigger of the reaction (Varadi, *et al* 1999). 10  $\mu$ L rhTF was added  
39  
40 to 50  $\mu$ L of a fluorogenic substrate solution and 15 mM  $\text{CaCl}_2$ . To start the reaction 40  $\mu$ L of  
41  
42 patient's plasma was supplemented with either 5  $\mu$ L rFVIIa or APCC and 5  $\mu$ L of FVIII-  
43  
44 dilution. The final concentrations of rFVIIa were 0  $\mu$ g/mL, 1.0  $\mu$ g/mL, 2.5  $\mu$ g/mL and 5.0  
45  
46  $\mu$ 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  
47  
48 reach final concentrations of 0 IU/mL, 2.5 IU/mL and 5 IU/mL. The doses used in this study  
49  
50 are based on pilot experiments in our laboratory, and are found to reach an optimal stability  
51  
52 and sensitivity in the assays. Comparing with doses used in clinical context, the dose levels of  
53  
54 FVIII:C in the study are higher than standard dosing. APCC is normally administered at a  
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56 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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3 dose of 90  $\mu\text{g}/\text{kg}$ , which correspond to the medium dose level (2.5  $\mu\text{g}/\text{mL}$ ) used in the  
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5 experiment, is commonly used for treating patients. However, the work is performed *in vitro*,  
6  
7 hence the conditions can never be exactly the same as *in vivo*.  
8  
9

10 All samples were analyzed in duplicates. In the experiment with only APCC and rFVIIa in  
11  
12 combination without additive of FVIII, four patients were analyzed. The experiment was  
13  
14 repeated two times for one patient and three times for the others. The samples were incubated  
15  
16 at 37°C in a Microplate Fluorescence Reader (Tecan Genios, Tecan Austria GmbH, Grödig,  
17  
18 Austria) where the fluorescence intensity was monitored every minute for 90 min. The  
19  
20 fluorescence intensity (measured in relative fluorescence units (RFU)) is proportional to the  
21  
22 amount thrombin generated in the assay (Varadi, *et al* 2004). Calculation of the thrombin  
23  
24 concentration is made from comparison to a reference curve which is established from known  
25  
26 concentrations of thrombin. The thrombin concentration is expressed as the rate of  
27  
28 development of fluorescence intensity [fluorescence units (FU)] calculated for each reading  
29  
30 (FU/min).  
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### 39 *Statistics*

40  
41 To analyze the interactions between the products included the ANCOVA (analysis of  
42  
43 covariance) program was used. The statistical calculations were performed at RHO Inc,  
44  
45 Chapel Hill, NC,USA. *P* less than .05 was considered to indicate statistical significance.  
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49

## 50 **Results**

### 51 *APCC-FVIII interaction*

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54 Thrombin generation assay was performed on the plasma samples with different dose levels  
55  
56 of FVIII and APCC (see Materials and Methods). This experimental design gives 12 possible  
57  
58 treatment combinations for each patient plasma sample. The samples without any APCC or  
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3 factor VIII concentrate added turned out to produce a small amount of thrombin in the  
4  
5 thrombin generation assay ranging from 8.5 – 10.7 nM. Despite the presence of inhibitors in  
6  
7 each plasma sample the thrombin production increased in a dose dependent way when adding  
8  
9 factor VIII to the sample (see Table 1).  
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15 For each separate dose level of APCC the thrombin production (T<sub>max</sub>) was shown to increase  
16  
17 significantly with increased factor VIII dose level, irrespective of what factor VIII product  
18  
19 that was added. Similarly, for each dose level of the different factor VIII products the  
20  
21 thrombin production increases significantly when the APCC dose is increased. When  
22  
23 comparing the maximum thrombin production between the different factor VIII products it  
24  
25 seems that all products reach approximately the same level (range 355.7 – 392.1 nM) apart  
26  
27 from Advate® reaching a maximum thrombin production of 292.4 nM (see Table 1).  
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34 To validate whether the combination of the two products potentiate the thrombin generation *in*  
35  
36 *vitro* or whether the increase of the dose level only gives an additive pharmacologic effect the  
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38 ANCOVA (analysis of covariance) statistical program was used. Analysis of the interaction  
39  
40 between APCC and the factor VIII products show that the combination of the preparations  
41  
42 potentiates each other in a synergistic way, resulting in a significant increase in thrombin  
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44 production for all products apart from for Alphanate® (p=0.1470), as exemplified with  
45  
46 Advate® in Figure 1.  
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#### 50 51 52 53 *rFVIIa-FVIII interaction*

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55 As shown with APCC, thrombin was produced with significant increase in the absence of  
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57 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 Alphante® 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  
11  
12 increased thrombin generation with increased dose level of either rFVIIa dose or factor VIII  
13  
14 dose. However, in contrast to the findings in the APCC experiment, the increase in thrombin  
15  
16 generating capacity is additive but not synergistic (see Table 2).  
17  
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### 20 21 22 *APCC-rFVIIa interaction*

23  
24 We further wanted to evaluate whether the combination of APCC and rFVIIa would give a  
25  
26 synergistic effect on the maximum rates of thrombin generation (Tmax). Four patients were  
27  
28 included in this experiment. The experimental design used was the same as in the experiments  
29  
30 shown above, apart from that no FVIII was added. We found that increasing the dose of  
31  
32 rFVIIa (p=0.01) and APCC (p<0.0001) gives a significant increase in the maximum thrombin  
33  
34 concentration produced. However, we can not find any synergistic interaction between the  
35  
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®,  
49  
50 Fanhdi®, Kogenate® and Alphanate®) in order to increase the thrombin generating capacity  
51  
52 *in vitro* in plasmas from haemophilia A patients with an inhibitor. Occasionally patients with  
53  
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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59  
60 efficacious to treat such bleedings with a sequential administration of a combination of APCC

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3 and rFVIIa (Key, *et al* 2002, Livnat, *et al* 2008, Schneiderman, *et al* 2007). There are also *in*  
4  
5 *vitro* studies indicating that there is a synergistic pharmacological effect between APCC and  
6  
7 rFVIIa resulting in an increased thrombin generation (van Veen, *et al* 2009).  
8  
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10  
11 We show that a combination of by-passing agent and FVIII has a positive effect on thrombin  
12  
13 generation *in vitro* in plasma from patients with severe haemophilia A and excess of  
14  
15 inhibitory antibodies. Since the patient plasma samples consist of other coagulation factors in  
16  
17 normal concentrations a background activity of thrombin production is generated in the assays  
18  
19 (see Table 1 and 2). All plasma samples were diluted to reach a level of inhibitory antibodies  
20  
21 of 11 MIU/mL (~ 33 Bethesda U/mL). Despite the presence of inhibitory antibodies at a  
22  
23 concentration enough to neutralize 11 IU/mL of FVIII:C, i.e. in excess to the FVIII added, the  
24  
25 thrombin production increased in a dose dependent way when adding factor VIII alone to the  
26  
27 plasma samples. This might be explained by the kinetics of the inhibitory antibody. The  
28  
29 thrombin generation reaction starts immediately when the FVIII-concentrate is added to the  
30  
31 plasma sample. Since the binding of the antibody to FVIII, and inhibition of its function are  
32  
33 relatively slow processes it may explain why thrombin is produced. In experiments with  
34  
35 incubation times up to 2 h we still see thrombin production in samples with excess of inhibitor  
36  
37 (unpublished observations) which corroborates with the clinical observation that some  
38  
39 patients experience less bleeds during ITI. The reason for this is not known. Hypothetically,  
40  
41 the explanation could be a transient haemostatic effect of infused FVIII not measurable *ex*  
42  
43 *vivo*.  
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54 We could not demonstrate any clear difference in FVIII interaction with regard to concentrate  
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56 content of von Willebrand factor (VWF). This is somewhat surprising as it has been proposed  
57  
58 by several authors that VWF may compete with inhibitor binding (Gensana, *et al* 2001,  
59  
60 Goudemand, *et al* 2006, Kallas and Talpsep 2001) and also impact on thrombin generation.

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3 As an example, FVIII concentrates containing VWF has been shown to elicit more thrombin  
4 in inhibitor plasma (Salvagno, *et al* 2007). However, when present together with APCC other  
5 mechanisms affecting thrombin generation (see below), could tentatively be more important  
6 and overshadow a hypothetical VWF mechanism.  
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15 Analysis of the interactions between APCC and the factor VIII products show that the  
16 combination of the preparations potentiates each other in a synergistic way, resulting in a  
17 significant increase in thrombin production for all products apart from for Alphanate®  
18 (p=0.1470). Alphanate®, Haemate® and Fanhdi® are all plasma derived concentrates which  
19 contain von Willebrand factor (Berntorp 2009, Logan 2009, Ristol, *et al* 1996). It has earlier  
20 been shown that the immunoreactivity appears to be higher towards Advate® compared with  
21 Haemate® and Fanhdi® in plasmas with inhibitors directed mainly to light chain epitopes,  
22 resulting in decreased thrombin generation (Salvagno, *et al* 2007). Hypothetically, one would  
23 therefore expect a higher thrombin generation and a better synergistic or additive effect of the  
24 VWF containing concentrates in the present study, although this was not seen. The lesser  
25 effect elicited by Alphanate® may partly be explained by the lower number of inhibitor  
26 plasmas studied with that product. Since we have not performed epitope mapping in this study  
27 we can not eliminate the possibility that different epitopes affect the outcome of the thrombin  
28 generation (see Figure 1). Further on, APCC (FEIBA®) contains, among other zymogens and  
29 coagulation enzymes, FIX and FIXa to which FVIII act as a co-factor (Turecek, *et al* 2004a,  
30 Turecek, *et al* 2004b, Varadi, *et al* 2003), and this could explain why the outcome of a  
31 combination of the products potentiates thrombin generation.  
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58 When combining rFVIIa with a FVIII-concentrate, the effect, in our study, is additive and not  
59 synergistically potentiated (see Figure 2). The thrombin generation assay we use is performed  
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3 with platelet poor plasma. It is well known that rFVIIa needs the presence of phospholipids or  
4 platelets to reach its full pharmacologic capacity (Livnat, *et al* 2008, Monroe, *et al* 1997). In  
5  
6 order to improve the conditions for the experiments containing rFVIIa we have used a reagent  
7  
8 with a high concentration of phospholipid micelles with tissue factor as a trigger of the  
9  
10 thrombin generation reaction (TECHNOTHROMBIN® TGA RC). This should be able to  
11  
12 compensate, at least partly, for the absence of platelets in the plasma samples. Anyhow, it is  
13  
14 known from earlier studies that the phospholipid content of APCC contributes to the effect on  
15  
16 the thrombin generation assay (Livnat, *et al* 2008). This is another possible explanation for  
17  
18 the difference between APCC and rFVIIa that we found in the outcome of the interaction  
19  
20 tests. Further more, adding of FX to a plasma sample has been shown to increase the thrombin  
21  
22 generation capacity of rFVIIa both *in vivo* and *in vitro* (Tomokiyo, *et al* 2003). As mentioned  
23  
24 above, APCC consist of e.g. coagulation enzyme FXa and several case reports describing  
25  
26 sequential therapy with APCC and rFVIIa propose an administration protocol where APCC is  
27  
28 given prior to rFVIIa (Economou, *et al* 2008, Key, *et al* 2002). By using such protocol the  
29  
30 FXa-content of APCC could, hypothetically, improve the effect of rFVIIa. However, since the  
31  
32 experimental conditions for monitoring rFVIIa is not optimal, this study is not aiming at  
33  
34 comparing the effect of APCC and rFVIIa. We believe that the improved effect of adding  
35  
36 FVIII to a by-passing agent in the presence of a high-responding inhibitor, could, potentially,  
37  
38 be a very useful treatment option in clinical situations where monotherapy with by-passing  
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40 agent fail.  
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53 Livnat et al has shown *in vitro*, both in platelet rich plasma (PRP) and in platelet poor plasma  
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55 (PPP), a synergistic effect between APCC and rFVIIa on thrombin generation (Livnat, *et al*  
56  
57 2008). We performed similar experiments in this study, with thrombin generation assay on  
58  
59 plasma samples (PPP) with addition of APCC and rFVIIa in combination. In discrepancy to  
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2  
3 what is referred to above we did not observe any synergy between the two products, but a  
4  
5 significant increase in thrombin generation where the interactive effect is additive (see Table  
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8 3).  
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12 The development of inhibitory antibodies is the major complication for patients with  
13  
14 haemophilia. As mentioned above, occasionally the by-passing agents APCC or rFVIIa in  
15  
16 monotherapy fails to control haemorrhages in patients with the severe form of haemophilia A  
17  
18 or B, and case reports as well as *in vitro* data suggest a combination of the two products in  
19  
20 such cases. However, this study shows for the first time that a combination of FVIII and a by-  
21  
22 passing agent enhances thrombin generation. Our findings could be useful for the  
23  
24 development of clinical strategies for patients with inhibitors and severe haemorrhages  
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26 refractory to monotherapy with by-passing agent.  
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35  
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39  
40 Chapel Hill, NC, USA for help with the ANCOVA analysis.  
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43  
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46 Skane University Hospital.  
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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:

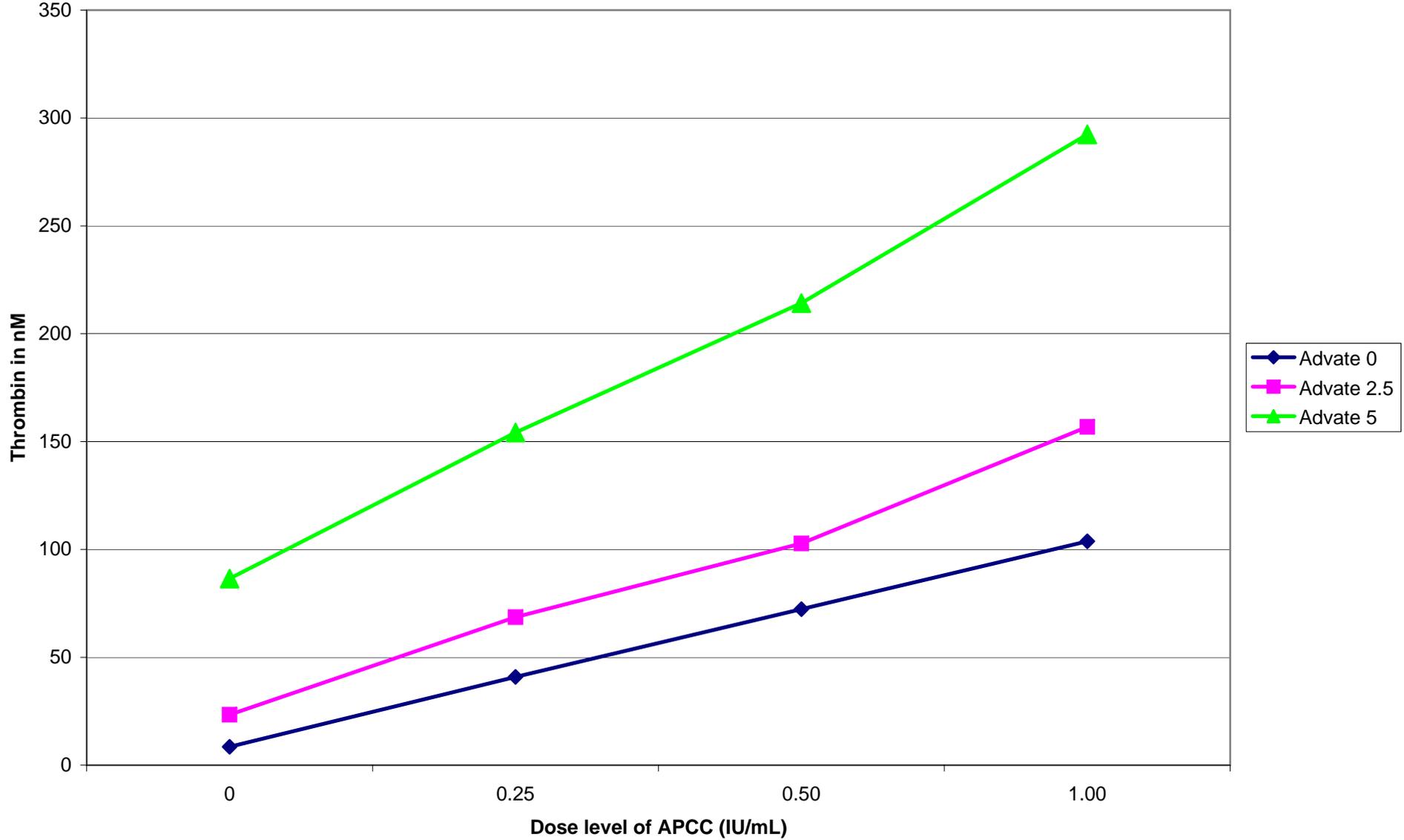
Thrombin production in nM for the assay combining APCC and FVIII. The maximum thrombin production shown within frame.

### 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.

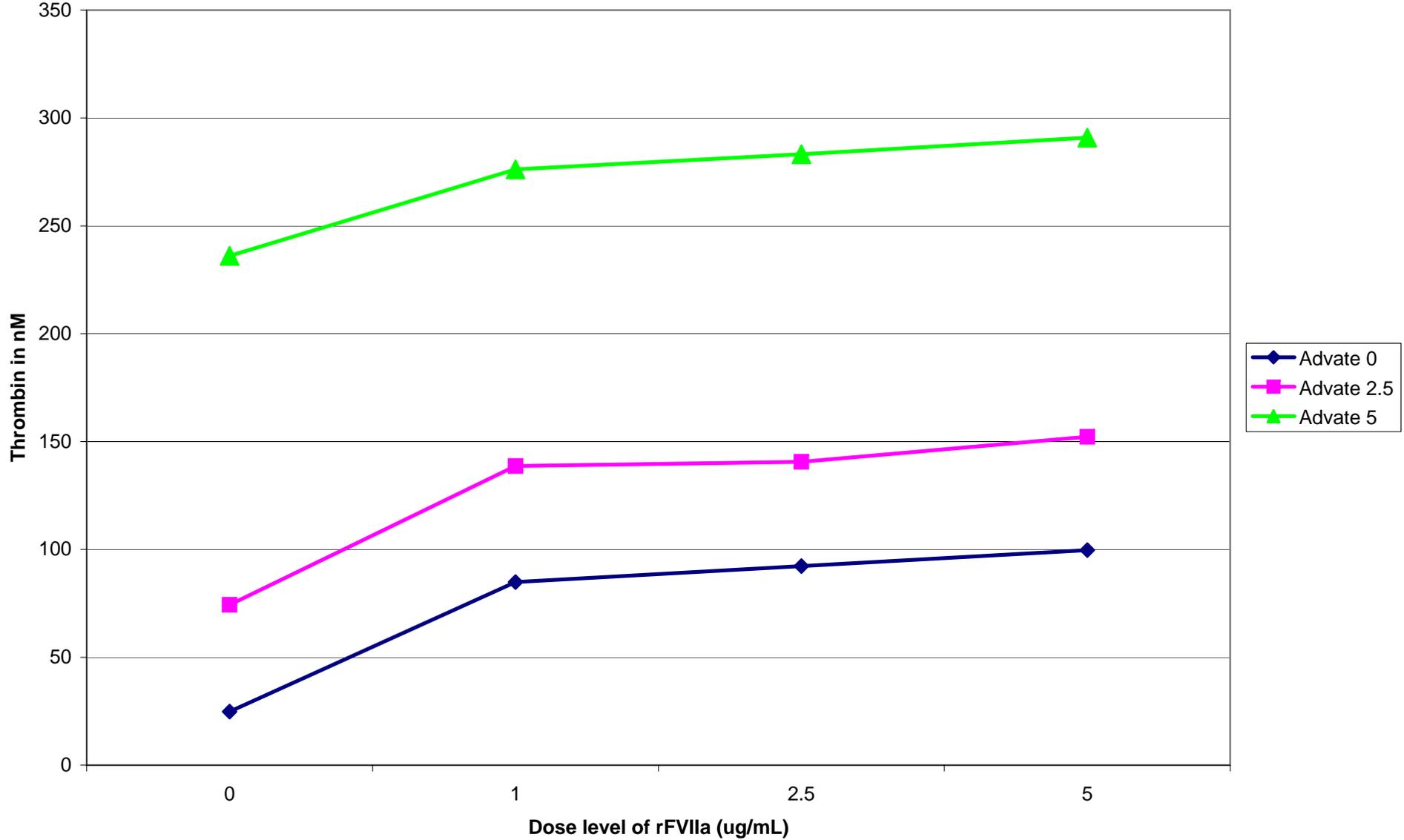


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Advate® (n=11)	Fanhdi® (n=10)	Haemate® (n=11)	Kogenate® (n=11)	Alphanate® (n=5)
p=0.02	p=0.01	p=0.04	p<0.0001	p=0.15

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Advate® (n=11)	Fanhdi® (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
<b>FVIII dose level 0 IU/mL</b>				
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
<b>FVIII dose level 2.5 IU/mL</b>				
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
<b>FVIII dose level 5 IU/mL</b>				
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

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	rFVIIa 0 µg/mL	rFVIIa 1 µg/mL	rFVIIa 2.5 µg/mL	rFVIIa 5 µg/mL
<b>FVIII dose level 0 IU/mL</b>				
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
<b>FVIII dose level 2.5 IU/mL</b>				
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
<b>FVIII dose level 5 IU/mL</b>				
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 ( $\mu\text{g/mL}$ )	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, rVIIa (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.