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Abstract:

The aim of the present study was to evaluate the *in vitro* function of the new recombinant factor VIII (FVIII) compound, N8. The specific activity of N8 as measured in a FVIII:C one stage clot assay was 9300±400 IU mg^{-1} based on analysis of seven individual batches. The ratio between the FVIII:C activity measured in clot and chromogenic assays was 1.00 (95% confidence interval 0.97–1.03). N8 bound to von Willebrand Factor with K_d values of 0.2 nM when measured by ELISA and by surface plasmon resonance. FVIIIa cofactor activity was determined from the kinetic parameters of factor IXa-catalyzed factor X (FX) activation. The rate of activation of N8 by thrombin as well as K_m and k_{cat} for FX activation were in the same range as those observed for Advate®. The rate of activated protein C (APC)-catalyzed inactivation was similar for activated N8 and Advate®. N8 improved thrombin generation in a dose-dependent manner and induced similar rates of thrombin generation as Advate® and the plasma-derived FVIII product Haemate®. Using thromboelastography (TEG®) N8 was shown to improve the clot formation and clot stability in whole blood from haemophilia A patients. Comparable potency and efficacy of N8 and Advate® was found based on TEG® parameters. Finally, similar binding profiles to immobilized lipoprotein receptor-related protein (LRP) of N8 and Advate® were observed. The study demonstrated that N8 is fully functional in a variety of assays measuring FVIII activity. No functional differences were found between N8 and comparator compounds.

Introduction:

Haemophilia A is an x-linked hereditary deficiency of functional coagulation factor VIII (FVIII). In the absence of treatment, patients with severe haemophilia A (plasma levels of FVIII below 1% of normal) will suffer repeated bleeds, often in muscles and joints. Joint bleeds especially are associated with long-term morbidity in the form of debilitating haemophilia arthropathy. To prevent this development, patients with severe haemophilia A are treated with recombinant or plasma-derived FVIII concentrates. It is well documented that such prophylactic replacement therapy reduces the number of acute bleeding episodes as well as the risk of joint destruction [1]. Hence, prophylactic treatment has become a cornerstone in the care of patients with haemophilia. However, due to inadequate access to coagulation factor concentrates many haemophilia A patients receive less than optimal treatment of their disease [2]. Therefore, a continuing development of safe and reliable treatment products is warranted. Recombinant products are safe treatment options as they eliminate the risk of transmission of human blood-borne infections [3]. Furthermore, recombinant coagulation factors can be produced in cell lines that can grow under conditions in which all human or animal derived components in the culture media have been substituted by chemically synthesized or recombinantly produced molecules. Such methods have been implemented in the production of a new recombinant FVIII (rFVIII) compound, N8, produced in chinese hamster ovary (CHO) cells and formulated without animal- or human-derived materials [4]. N8 consists of a heavy chain with the structure A1-*a1*-A2-*a2* followed by a 21 amino acid sequence of the natural B domain (amino acid 740-750 fused with 1638-1648) and a light chain containing the *a3*-A3-C1-C2 domains. Upon thrombin activation the B domain is removed rendering the activated N8 similar to activated FVIII (FVIIIa) originating from native human FVIII in blood. Analysis of the N8 protein confirmed the primary structure as well as the presence of the six known tyrosine sulfations relevant for interaction with other proteins including von Willebrand factor (VWF) [4].The aim of the present study was to analyze the function of N8 *in vitro* in order to ensure that a haemostatic effect similar to that of comparator FVIII compounds can be expected in haemophilia A patients. To accomplish this FVIII:C assays were employed as well as more detailed kinetic analyses of rate of thrombin

activation, the cofactor activity of activated FVIII (FVIIIa) and activated protein C (APC)-mediated inactivation of FVIIIa. Furthermore, the haemostatic effect was evaluated in thrombin generation assays at haemophilia A-like conditions as well as in whole blood from haemophilia A patients. Finally, binding to VWF and lipoprotein-related receptor protein (LRP) was evaluated. All analyses support that N8 is fully functional and has similar efficacy and potency as comparator FVIII compounds.

Materials and Methods:

Proteins

N8 was prepared as described [4]. As comparator molecules both a commercially available recombinant compound (Advate®, Baxter Bioscience, Vienna, Austria) [5] and a plasma-derived compound (Haemate®, CSL Behring, Marburg, Germany) were used. The protein concentration of N8 batches was determined by size exclusion HPLC using a N8 standard where the N8 protein content was determined by amino acid analysis [4]. In studies where binding to VWF or LRP was analyzed the molar concentration of Advate® was determined relative to that of N8 by a FVIII light chain ELISA (Asserachrom FVIII, American Diagnostica, Stamford, CT, USA) using the standard N8 as calibrator. For the thrombin generation and thromboelastography (TEG®) experiments, the labelled potency of Advate® and Haemate® was used. Before addition to the assays the FVIII:C was controlled by chromogenic assay.

The coagulation factors IXa (FIXa), X (FX) and Xa (FXa) were obtained from Enzyme Research Laboratories (Swansea, UK), coagulation factors V, IX, XI, antithrombin III and protein S were from Haematological Technologies Inc. (Essex Junction, VT, USA), APC was from Eli Lilly (Indianapolis, IN, USA) and hirudin and FVIII-free VWF were from American Diagnostica (Stamford, CT, USA). Human α-thrombin was from Roche (Basel, Switzerland). Pefabloc Xa was from Pentapharm (Basel, Switzerland). Phospholipid vesicles (Phospholipid-TGT) were from Rossix (Mölndal, Sweden) except for in the FVIIIa inactivation experiments in which vesicles from Haematological Technologies Inc. were used. Coagulation factors VII (FVII) and VIIa (FVIIa) and tissue factor pathway inhibitor (TFPI) were prepared in-house [6,7]. The murine

anti-A2 monoclonal antibody (mAb) FVIII-1F5 was produced in-house using standard techniques.

Human material

Blood samples were obtained from normal, healthy donors who were members of the Danish National Corps of Voluntary Blood Donors and met their criteria for blood donation. The Danish National Committee on Biomedical Research Ethics has approved the use of donor blood for research purposes in Novo Nordisk A/S. The donors received oral and written information regarding the use of their blood and signed informed consent prior to blood donation. The donors had not taken acetyl salicylic acid for 10 days or other non-steroidal anti-inflammatory drugs for at least 72 hours prior to blood sampling. Further, blood samples from four severe haemophilia A patients and four moderate haemophilia A patients were obtained. The study protocol was approved by the Danish National Committee on Biomedical Research Ethics. All patients received oral and written information about the study and signed informed consent prior to blood sampling.

Standard FVIII:C assays

FVIII:C activity was measured in a one-stage clot assay and a chromogenic assay. FVIII samples were diluted to approximately 11 IU mL^{-1} in a buffer containing 20 mM Hepes, 150 mM NaCl, pH 7.4 with 10 g L^{-1} bovine serum albumin (BSA) and subsequently diluted 10-fold in FVIII-deficient plasma containing a normal VWF level (Dade Behring, Marburg, Germany). Further dilutions were made in the Hepes buffer with BSA. The aPTT clotting time was measured on an ACL9000 instrument (Instrumentation Laboratory, Lexington, MA, USA). Synthasil (HemosIL®, Instrumentation Laboratory, Lexington, MA, USA) was used as aPTT reagent. The chromogenic activity was measured on an ACL9000 instrument using a Coamatic FVIII kit (Chromogenix, Milan, Italy). In both assays the 7th WHO FVIII standard (NIBSC code 99/678) was used as calibrator. The specific activity was determined by dividing the FVIII:C values with the protein concentration as described above.

Kinetic measurements of FVIIIa activity

FVIIIa was quantified by measuring its cofactor activity in FIXa-catalyzed FX activation in the presence of phospholipid vesicles, FIXa and FX in amounts ensuring that the rate of FX activation was linearly dependent on FVIIIa and constant during the assay (30-120 sec). All reactions were performed at 37°C. In general, FVIII was activated by 5 nM thrombin for 30 sec in a HBS/$CaCl_2$/BSA-buffer (20 mM Hepes pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, 5 g L^{-1} BSA). Activation was terminated by addition 14.3 ATU mL^{-1} hirudin. FVIIIa (0.1 nM final concentration) was transferred to six volumes of FX activation mixture containing 25 μM phospholipid vesicles, FIXa (5 nM) and FXa inhibitor Pefabloc Xa (2 μM) in HBS/$CaCl_2$/BSA-buffer and FX activation initiated by addition of FX to 100 nM. Activation of FX was allowed to proceed for 30-120 sec and was terminated by transferring aliquots to ice-cold EDTA-buffer (20 mM Hepes pH 7.4, 140 mM NaCl, 20 mM EDTA, 1 g L^{-1} BSA). The chromogenic FXa substrate S-2765 (Chromogenix, Milan, Italy) was added to 0.4 mM and color development was measured on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA). FXa was quantified by using FXa as calibrator. The FVIIIa cofactor activity was characterized by quantification of the apparent K_d for dissociation of the membrane-bound FIXa-FVIIIa complex ($K_{½}$FIXa) and the kinetic parameters K_m and k_{cat} for FX activation. Initially, reciprocal titrations of FVIIIa and FIXa were used to determine $K_{½}$FIXa and the functional concentration of FVIIIa [8]. FVIIIa titrations were performed by activating FVIII (0.35-105 nM) as described above and measuring the rate of FX activation using 0.1 nM FIXa. Titrations of FIXa were performed by activating FVIII (0.7 nM) as described above and measuring the rate of FX activation using 0.05–15 nM FIXa. The V_{max} of FX activation was obtained from plots of FX activation rate as a function of FVIIIa or FIXa concentration that were fitted to the Michaelis-Menten equation. $K_{½}$FIXa was obtained from the same plots fitted to the equation for a single site binding isotherm (hyperbola). The functional FVIIIa concentration was calculated as V_{max} obtained by FIXa titration divided by V_{max} obtained by FVIIIa titration as described [8].

Titrations of FX were used to determine the K_m and k_{cat} of FX activation by the FIXa-FVIIIa complex. For determination of k_{cat}, FVIII (0.7 nM) was activated as described above and the rate of FX activation was measured at 1–100 nM FX. For determination of K_m, FVIII (70 nM) was activated by 25 nM thrombin as described above and the rate of FX activation was measured at 0.02 nM FIXa and 1-100 nM FX. The K_m and k_{cat} of FX activation were obtained from plots of FX activation rate as a function of FX concentration that were fitted to the Michaelis-Menten equation. The fraction of FVIIIa incorporated in the FIXa-FVIIIa complex was calculated using the $K_{½}$FIXa determined above and used to correct the k_{cat} values [8].

The rate of thrombin-catalyzed FVIII activation was determined by measuring the initial formation of FVIIIa activity following incubation of FVIII with thrombin. FVIII (approximately 0.5 nM) was activated by 0.05 nM thrombin. FVIIIa activity was measured after 0, 1, 2 and 3 min by quantifying the rate of FX activation for 30 sec using 2 nM FIXa. The rate of FVIIIa activation was obtained from plots of FVIIIa activity as a function of time using linear regression and was expressed as nM of FVIIIa formed per min per nM of FVIII initially present ($v/[FVIII]_0$).

APC-catalyzed FVIIIa inactivation

The rate of APC-catalyzed FVIIIa inactivation was determined by measuring the initial loss of FVIIIa activity following incubation of FVIIIa with APC in the presence of 100 nM protein S and 25 µM phospholipid vesicles (25% PS, 75% PC). FVIII (approximately 2.4 nM) was activated as described above and activation was terminated by addition of hirudin (10 ATU mL^{-1}). FVIIIa activity was quantified immediately on an aliquot while APC (5 nM) was added to the remaining reaction volume. Subsequently, samples were removed and the residual FVIIIa activity was determined by measuring the rate of FX activation as described above using 1 nM FIXa. The rate of inactivation was obtained from plots of residual FVIIIa activity as a function of time by fitting to a monophasic decay model. The APC-independent loss of FVIIIa activity was quantified using parallel reactions without APC. The rate of inactivation was expressed as the rate (nM FVIIIa lost per min) observed in the absence of APC subtracted from the rate observed in the presence of APC divided by the APC concentration.

Thrombin generation assays

Monocytes were isolated from peripheral blood as described [9], plated in 96-well microtiter plates at a density of 5000 cells per well and stimulated overnight with LPS to express TF. Platelet-rich plasma (PRP) was prepared by centrifuging citrate-stabilized peripheral blood 10 min at 200 × g. The PRP was acidified by adding 1/10 volume acetate citrate dextrose (85 mM trisodiumcitrate, 71 mM citric acid and 111 mM glucose) before centrifuging 15 min at 330 × g. The pellet was gently re-suspended in 10 mL Hepes-Tyrodes buffer (15 mM Hepes, 138 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 5.5 mM dextrose and 1 mg mL^{-1} BSA, pH 6.5) with 5 µg mL^{-1} PGE_1 (Sigma, St. Louis, MO, USA) added. After a second 15 min centrifugation at 330 × g the platelets were re-suspended in 5 mL Hepes-Tyrodes buffer pH 7.4 and allowed to rest 30 min before use in the assay. The platelet density was determined as described [9] and adjusted to 111 × 10^9 L^{-1} (final density 100 × 10^9 L^{-1}) with buffer before addition to the proteins (final concentrations in parentheses) FVII (10 mM), FVIIa (0.1 nM), FX (8 µg mL^{-1}), FIX (5 µg mL^{-1}, FXI (5 µg mL^{-1}), prothrombin (86 µg mL^{-1}), TFPI (0.1 µg mL^{-1}), ATIII (120 µg mL^{-1}), FV (7 µg mL^{-1}), FVIII (1 U mL^{-1}; 0.1 U mL^{-1}; 0.01 U mL^{-1} or buffer without FVIII) and $CaCl_2$ (3 mM) before transfer to wells with the TF-expressing monocytes. Aliquots were analyzed for thrombin amidolytic activity as described [9]. The maximal rate of thrombin generation was calculated by linear regression of the part of the thrombin generation curve with a linear increase in thrombin activity.

The thrombin generation was also determined in a plasma-based model of thrombin generation as described by Hemker et al. [10]. Lyophilized FVIII-deficient plasma (Helena BioSciences, Gateshead, United Kingdom) was reconstituted with gel-filtered normal platelets [9] to a final density of 100 × 10^9 L^{-1}. FVIII dilutions (0.01 U mL^{-1}, 0.1 U mL^{-1} or 1 U mL^{-1}) were added together with lipidated TF (Innovin®, Dade Behring, Marburg, Germany) (final dilution 1:50.000) and a fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) (final concentration 417 nM) mixed with calcium (final concentration 16.7 mM). Substrate conversion was measured continuously for one hour on a Fluoroskan Ascent plate reader

(Thermo Fisher Scientific, Helsinki, Finland) with emission at 460 nm after excitation at 390 nM.The fluorescence signal was corrected for α2-macroglobulin-bound thrombin activity and converted to thrombin concentration by use of a calibrator and Thrombinoscope software (Synapse BV, Maastricht, The Netherlands) as described by Hemker [10].

Binding to VWF in ELISA

An ELISA assay was used to analyze binding of N8 and Advate® to immobilized VWF. Dilutions of N8 (0.05-6.4 nM) in 20 mM imidazole, 150 mM NaCl, 10 mM $CaCl_2$ and 10 g L^{-1} BSA at pH 7.3 were added to wells coated overnight with 2 µg mL^{-1} FVIII-free VWF. After 1 hour of incubation at room temperature and subsequent washing, bound FVIII was detected with a biotinylated anti-A2 mAb (FVIII-1F5) followed by peroxidase-conjugated steptavidin and finally the addition of 3, 3', 5, 5' – tetramethylbenzidine (TMB) substrate. Binding constants were calculated from the binding curves using a one-site binding model and GraFit software (Erithacus Software Ltd., Surrey, UK).

Binding to VWF and LRP by surface plasmon resonance (SPR)

FVIII-free VWF in Na-acetate, pH 4, was immobilized on a standard CM5 chip (Biacore, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to a level of approximately 500 RU. Dilutions of FVIII (0.05-12.5 nM) in 10 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 were injected over the VWF chip in a T100 Biacore instrument (Biacore, GE Healthcare Bio-Sciences AB). Regeneration of the chip was achieved with 20 mM Hepes, 0.35 M $CaCl_2$, 0.6 M NaCl, pH 7.2. To assess binding to LRP, LRP (affinity purified from human placenta, BioMac, Leipzig, Germany) in Na-acetate, pH 3, was immobilized on a standard CM5 chip to a level of 3500 RU. Dilutions of FVIII (0.8-50 nM) were injected over the LRP chip. Regeneration of the chip was achieved with 0.1 M phosphoric acid. SPR binding data were fitted to a 1:1 model in Biacore T100 Evaluation software.

Thromboelastography (TEG®) analysis in blood from haemophilia A patients

The effect of N8 was compared to that of Advate® in an open, single-centre, non-intervention, *in vitro* study using blood samples from 4 moderate and 4 severe haemophilia A patients. All were male, 18 years or older, in a non-bleeding state at the time of blood sampling and the wash out period since last administration of factor concentrate was at least 48 hours. Aliquots of N8 and Advate® were controlled by FVIII chromogenic activity assay to ensure correct additions to the blood samples. Blood was sampled in 3.2% citrate vacutainers (BD, Belliver Industrial Estate, Plymouth, UK) and following a 90 min resting period samples were spiked with dilutions of N8 or Advate® (0.00001-2 IU mL^{-1}) in 20 mM Hepes, 140 mM NaCl, 2% BSA, pH 7.4. Immediately hereafter TEG® analyses on TEG 5000® instruments (Haemoscope Corporation, Niles, Illinois, USA) [11,12] using commercial kaolin (TEG® Hemostasis System, Haemoscope) were started and allowed to proceed for a total of 180 min. Measurements of baseline and the spiked samples enabled assessment of dose-response effect of the FVIII compounds. For comparative purposes a normal range based on TEG® analyses of 11 healthy donors was generated. For each TEG® pattern the clot time (R-time), maximum thrombus generation (MTG) and maximum amplitude (MA) parameters were used for statistical analyses. To compare potency, individual EC_{50} ratios were estimated from the linear part of the response-log-concentration curves in a linear model for normal distributed data. The individual EC_{50} ratios were subsequently analyzed on log-scale in a normal linear mixed effects model that allowed for both random subject-to-subject variation and random residual variation. Efficacy (maximal effect) was estimated based on the highest concentration tested for each profile. The difference in efficacy between N8 and Advate® was estimated in a normal linear mixed effects model that allowed for random subject-to-subject, random day-to-day and random residual variation.

Results:

FVIII:C activity

The FVIII:C activity of N8 was determined in standard FVIII one-stage clot and two-stage chromogenic assays using the 7th WHO FVIII standard as calibrator and otherwise following the SSC recommendations for FVIII assays [13]. Analyses of seven batches of N8 in the one-stage

clot assay yielded a mean specific activity of 9300 ± 400 IU mg^{-1} (Table 1) thus demonstrating consistency of specific activity of the N8 batches. The ratio between the FVIII:C values obtained in the one-stage clot assay and the chromogenic assays was 1.00 (CI 95%: 0.97-1.03, n = 12-36) for N8 and 1.0 (CI 95%: 0.8-1.2, n=3) for comparator FVIII compounds Advate® and Haemate® (Table 1). The data demonstrate that N8 is fully functional in FVIII:C assays and that both clot and chromogenic assays can be used for measuring the FVIII:C activity of N8.

FVIII activation, co-factor activity and APC-mediated inactivation

The co-factor activity of N8 was further evaluated by kinetic analysis of the FIXa-FVIIIa complex assembled from purified human plasma FIXa and activated N8 and Advate® (Fig. 1 and Table 1). Initially, the maximal rate of FX activation was determined using a limiting concentration of FIXa and increasing concentrations of FVIIIa (Fig. 1A) or vice versa (reciprocal titrations). The functional concentration of FVIIIa was calculated by dividing the maximal rate of FX activation determined when FVIIIa was the limiting component (FIXa titrations, Fig. 1B) by the rate determined when FIXa was the limiting component [8]. Using this approach the FVIIIa concentration was expressed relative to a known amount of FIXa and was thus not dependent on FVIII activity or concentration determined in other assays. The apparent affinity for FIXa obtained from FIXa titrations ($K_{½}$FIXa, Table 1) was 0.78 ± 0.24 nM for activated N8 and 0.76 ± 0.25 nM for activated Advate® demonstrating that activated N8 and activated Advate® had similar affinity for FIXa.

Kinetic parameters of FX activation were determined from FX titrations (Fig. 1C) and showed that the Xase-complex comprising activated N8 had a Michaelis constant (K_m) of FX activation of 5.1 ± 0.5 nM which was not different from the K_m of the Xase-complex comprising activated Advate® (4.8 ± 0.4 nM). The turn-over number k_{cat} of FX activation obtained with FIXa in complex with activated N8 was 6.5 ± 2.2 sec^{-1} comparable to the k_{cat} value of 5.5 ± 1.0 sec^{-1} obtained with Advate®.

These kinetic parameters allowed determination of FVIIIa concentration based on measured rate of FX activation and thus quantification of the rate of FVIII activation and FVIIIa

inactivation (Table 1). Activation of N8 (0.35 nM) by thrombin (0.1 nM) had an initial rate ($v/[FVIII]_0$) of 13.5 ± 6.7 × 10^{-3} min^{-1} which was not different from the rate obtained for Advate® of 10.6 ± 3.5 × 10^{-3} min^{-1}. Inactivation of activated N8 and Advate® was monitored after addition of APC and protein S and residual FVIIIa activity was fitted to an exponential model. Results were corrected for the APC independent rate of spontaneous inactivation. The rate of APC-catalyzed inactivation of N8 was 0.14 ± 0.03 min^{-1} similar to that of Advate® which was 0.12 ± 0.02 min^{-1}.

VWF and LRP binding

The affinity of N8 to immobilized VWF was measured in an ELISA assay and by SPR (Fig..2 and Table 1), and compared to that of Advate®. To compare the two FVIII compounds containing different lengths of the B domain the antigen concentration of both was determined by a FVIII light chain ELISA after ensuring that the monoclonal antibodies used in the light chain ELISA bound N8 and Advate® with the same affinities. N8 bound VWF with K_d values of 0.24 ± 0.04 nM in the VWF-binding ELISA and 0.23 ± 0.13 nM by SPR. The K_d values obtained for Advate® were slightly higher, i.e. 0.48 ± 0.13 nM when measured by ELISA (p=0.023 when compared to that of N8) and 0.45 ± 0.17 nM when measured by SPR (p= 0.045 compared to N8). For both rFVIII compounds the VWF level in the blood (~50 nM) is sufficient for saturating the majority (≥ 99%) of circulating FVIII.

LRP is a potential clearance receptor for FVIII [14] and the binding of N8 to LRP was therefore analyzed by SPR (not shown). The binding curves did not fit a 1:1 stoichiometry model suggesting a complex interaction pattern of FVIII with LRP. Consequently, K_d values were not obtained. However, the sensorgrams showed similar binding profiles for N8 and Advate®. These findings suggest similar affinities and consequently similar clearance via LRP for N8 and Advate®.

Thrombin generation

The ability of N8 to generate thrombin in assay systems mimicking haemophilia A was evaluated using a reconstituted cell-based model [15] and a plasma-based model [10]. The reconstituted model system consisted of TF-expressing monocytes, non-stimulated normal platelets and purified coagulation factors V, VII, VIIa, IX, X, XI, prothrombin, TFPI and ATIII. When no FVIII was present a delayed and lowered thrombin generation was seen (Fig. 3). Addition of FVIII either N8 (Fig. 3A), Advate® (Fig. 3B) or Haemate® (Fig. 3C) improved the thrombin generation in a concentration-dependent manner. The maximal rate of thrombin generation increased from 0.74 ± 0.55 nM × min^{-1} without FVIII present to 1.9 ± 0.8, 2.6 ± 0.4 and 3.7 ± 0.8 nM × min^{-1} (n=3) when N8 was added to 0.01, 0.1 and 1 IU mL^{-1}, respectively. Similar values for maximal rate of thrombin generation were obtained for the three FVIII compounds (Table 1). Data from the plasma-based model system comprising FVIII-deficient plasma supplemented with normal platelets also showed similar concentration-dependent improvement of the thrombin generation by the three FVIII compounds analyzed (data not shown).

Thromboelastography (TEG®) analysis of N8 in blood from haemophilia A patients

The effect of N8 on clot formation and maximal mechanical strength in blood from haemophilia A patients was evaluated by thromboelastography (TEG®). N8 and Advate® (0.00001-2 IU mL^{-1}) were added to blood samples from four severe and four moderate haemophilia A patients followed by initiation of coagulation by kaolin. The individual TEG® patterns differed between patients most likely as a result of differences in trace FVIII in their blood either endogenous or remaining from their last administration of factor concentrate. However, in all baseline blood samples clot formation was delayed, only small clots were formed and all TEG® parameters were far outside the normal range (Table 2). Upon addition of N8 or Advate® to the blood samples the clot time (R-time) decreased and maximum thrombus generation (MTG) and maximal mechanical strength of the clot (maximum amplitude, MA) increased in a concentration-dependent manner. The mean dose-response curves for MTG are shown in Fig. 4. Addition of either N8 or Advate® at concentrations above 0.1 IU mL^{-1} resulted in MTG values

within the range of normal donors. The potency expressed as the ratio of EC_{50} values (the concentrations yielding half-maximal effect on the various TEG® parameters) of N8 relative to Advate® was calculated. The potency of N8 and Advate® was similar for R-time (p=0.99) and MA (p=0.76) and did not differ significantly for MTG (p=0.11) (Table 3). The efficacy of the FVIII compounds corresponded to the maximal effect obtained, i.e. the effect at the highest concentration analyzed (Table 2). No significant differences in efficacy were found for any of the TEG® parameters (R-time: p=0.94, MTG: p=0.48 and MA: p=0.49) indicating comparable efficacy of N8 and Advate®.

Discussion and Conclusion:

The aim of the present study was to evaluate the *in vitro* function of the new rFVIII compound N8 [4]. N8 is compared with the rFVIII product Advate® [5] in clinical trals, and therefore this rFVIII compound was chosen as comparator in the present study. In some experiments the plasma-derived FVIII product Haemate® was included. According to the package insert Haemate® contains a high amount of VWF and albumin which prevented the use of Haemate® in several of the analyses in the present study.

The specific FVIII:C activity of N8 was 9300 ± 400 IU mg^{-1} when seven individual N8 batches were analyzed. The FVIII:C activity of N8 did not differ when measured in clot and chromogenic FVIII assays. A clot/chromogenic activity ratio close to one was also found for the comparator FVIII products suggesting similar behaviour in standard FVIII:C assays of N8 and these products. The assessment of N8 kinetics showed that N8 and Advate® were activated by thrombin at similar rates. Likewise, the apparent affinity of activated N8 and activated Advate® towards FIXa was similar as were the Michaelis constants of FX activation (K_m) and turn-over numbers of FVIIIa-FIXa complexes assembled from activated N8 or activate Advate®. This is in line with similar effects of N8 and Advate® in assays reflecting the haemostatic effects of FVIII replacement. N8 restored the thrombin generation in mimicked haemophilia A and the whole blood clot formation in blood from haemophilia A patients as measured by TEG®. No functional differences of potency and efficacy were found between N8 and the comparator compounds

suggesting that a comparable haemostatic effect of N8 and the commercially available FVIII products can be expected.

FVIII interaction with endogenous VWF is important for protecting FVIII in circulation from degradation and clearance. As N8 contains the amino acid sequences and the tyrosine sulfations required for VWF interaction [4] normal VWF interaction was expected. This was confirmed in the present study where K_d values of 0.2 nM for VWF binding were obtained by ELISA and SPR. The high affinity of N8 for VWF is in good agreement with published data [5,16]. In plasma, the majority of FVIII is bound to VWF while a small pool (2–5%) at any given time exists in the non-bound state [14]. This small pool of free FVIII can be cleared by LRP or other receptors of the LRP family, and the interaction of N8 with LRP was therefore evaluated. As the data did not fit to a one-site binding model the binding is most likely complex with several binding sites involved in the interaction between N8 and LRP. However, the binding curves of N8 to LRP were similar to those obtained for Advate® indicating similar interaction of N8 with LRP as that of Advate®. This suggests similar potential for clearance via LRP of the two rFVIII proteins.

The role of the B domain in FVIII has been discussed [17]. The B domain influences the intracellular processing of FVIII but its role once FVIII is secreted from the cells into a VWF-containing environment in the blood is not evident and no clinical relevant function has been associated with the B domain. Preparations of rFVIII proteins based on translation from a full-length sequence contain variable lengths of the B domain attached to the heavy chain. The most predominant form is the one terminating at Arg1313 while other forms terminate at Ser817 and Lys1115 as well as at Arg740, the C-terminal amino acid in the A2-*a2* domain [18]. The latter form thus lacks the entire B domain. As the majority of the B domain is dispensable for activity [19] the acute pro-coagulant effect of all these forms of FVIII is unlikely to differ. This is indeed the case in the present study where the rate of thrombin activation as well as the pro-coagulant effect in thrombin generation assays and whole blood clot formation assays were not different for N8 and Advate®, the latter containing various lengths of the B domain. It should be noted that N8 contains the entire A2-*a2* (amino acid 373-740) as the purification of

N8 includes an affinity chromatography step using a recombinant antibody where the epitope is dependent on the presence of amino acids 720-740 [4,20]. Therefore, all N8 forms in the purified product contain the complete C-terminal part of A2-a2 rich in negatively charged amino acids important for interaction with e.g. thrombin [21].

The B domain has, however, been suggested to be relevant for APC-mediated inactivation of FVIII. In a study by Khrenov et al. [22] the APC-mediated inactivation of the commercially available B domain-deleted rFVIII product ReFacto® (Wyeth Pharmaceuticals Inc, Madison, NJ, USA) proceeded faster than the inactivation of a rFVIII product containing the entire repertoire of B domains, i.e. Kogenate® (Bayer Corporation, Elkhart, IN, USA). In our study, no differences in rate of APC-mediated inactivation were observed between activated N8 and Advate®. The experimental designs in the two studies differ as much higher concentrations of FVIIIa (300 nM) and APC (100 nM) were used by Khrenov et al. [22] in comparison to the present study (1 nM FVIIIa and 5 nM APC). The high concentrations of FVIIIa used by Khrenov et al. may shift the route of FVIIIa inactivation away from its physiological pathway by altering the relative importance of cleavage at Arg336 compared to Arg562. This notion is supported by the observation by Gale et al. that Arg336 cleavage was 5-fold faster than Arg562 cleavage at 2.3 nM FVIIIa (in the presence of protein S) and by Varfaj et al. who found Arg336 cleavage to be 25-fold faster than Arg562 cleavage at 100 nM FVIIIa (without protein S). On the contrary, Khrenov et al. found the rates of the two cleavages to be reversed such that cleavage at Arg336 was 2-fold *slower* than cleavage at Arg562 at 300 nM FVIIIa (in the presence of protein S) [8,22,23]. In contrast, the much lower concentrations of FVIIIa used in the present study mimic physiologic conditions which should allow the cleavage of the A1 and A2 domains to proceed as it would *in vivo*.

In summary, the present data demonstrate that N8 is fully functional in assays measuring FVIII:C. No functional differences were found between N8 and comparator FVIII compounds. The data obtained indicate that N8 has an effect similar to that of Advate® in mimicked haemophilia A as well as in the blood of haemophilia A patients. Therefore, it can be assumed

that the haemostatic effect of N8 in haemophilia A patients will be comparable to that of Advate®.

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References:

1 Manco-Johnson MJ, Abshire TC, Shapiro AD, Riske B, Hacker MR, Kilcoyne R *et al.* Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med* 2007; 357: 535-44.

2 Berntorp E. Progress in haemophilic care: ethical issues. *Haemophilia* 2002; 8: 435-38.

3 Key NS, Negrier C. Coagulation factor concentrates: past, present, and future. *Lancet* 2007; 370: 439-48.

4 Thim L, Vandahl B, Karlsson J, Klausen NK, Pedersen J, Krogh T *et al.* Purification and characterisation of a new recombinant Factor VIII (N8). *Haemophilia* 2010; 16(2): 349-59.

5 Ewenstein BM, Collins P, Tarantino MD, Negrier C, Blanchette V, Shapiro AD *et al.* Hemophilia therapy innovation: development of an advanced category recombinant factor VIII by a plasma/albumin-free method. Proceedings of a Special Symposium at the XIXth Congress of the International Society on Thrombosis and Haemostasis, July 12-18, 2003, Birmingham, UK. *Semin Hematol* 2004; 41: 1-16.

6 Pedersen AH, Nordfang O, Norris F, Wiberg FC, Christensen PM, Moeller KB *et al.* Recombinant human extrinsic pathway inhibitor. Production, isolation, and characterization of its inhibitory activity on tissue factor-initiated coagulation reactions. *J Biol Chem* 1990; 265: 16786-93.

7 Thim L, Bjoern S, Christensen M, Nicolaisen EM, Lund-Hansen T, Pedersen AH *et al.* Amino acid sequence and posttranslational modifications of human factor VIIa from plasma and transfected baby hamster kidney cells. *Biochemistry* 1988; 27: 7785-93.

8 Gale AJ, Cramer TJ, Rozenshteyn D, Cruz JR. Detailed mechanisms of the inactivation of factor VIIIa by activated protein C in the presence of its cofactors, protein S and factor V. *J Biol Chem* 2008; 283: 16355-62.

9 Kjalke M, Ezban M, Monroe DM, Hoffman M, Roberts HR, Hedner U. High-dose factor VIIa increases initial thrombin generation and mediates faster platelet activation in thrombocytopenia-like conditions in a cell-based model system. *Br J Haematol* 2001; 114: 114-20.

10 Hemker HC, Giesen P, Al DR, Regnault V, de SE, Wagenvoord R *et al.* Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb* 2003; 33: 4-15.

11 Carr ME, Jr., Martin EJ, Kuhn JG, Ambrose H, Fern S, Bryant PC. Monitoring of hemostatic status in four patients being treated with recombinant factor VIIa. *Clin Lab* 2004; 50: 529-38.

12 Sorensen B, Ingerslev J. Thromboelastography and recombinant factor VIIa-hemophilia and beyond. *Semin Hematol* 2004; 41: 140-44.

13 Barrowcliffe TW. Factor VIII and factor IX Sub-Committee. Recommendations for the assay of high-purity factor VIII concentrates. *Thromb Haemost* 1993; 70: 876-77.

14 Lenting PJ, VAN Schooten CJ, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. *J Thromb Haemost* 2007; 5: 1353-60.

15 Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. *Arterioscler Thromb Vasc Biol* 2002; 22: 1381-89.

16 Vlot AJ, Koppelman SJ, van den Berg MH, Bouma BN, Sixma JJ. The affinity and stoichiometry of binding of human factor VIII to von Willebrand factor. *Blood* 1995; 85: 3150-57.

17 Pipe SW. Functional roles of the factor VIII B domain. *Haemophilia* 2009; 15(6): 1187-96.

18 Jankowski MA, Patel H, Rouse JC, Marzilli LA, Weston SB, Sharpe PJ. Defining 'full-length' recombinant factor VIII: a comparative structural analysis. *Haemophilia* 2007; 13: 30-37.

19 Toole JJ, Pittman DD, Orr EC, Murtha P, Wasley LC, Kaufman RJ. A large region (approximately equal to 95 kDa) of human factor VIII is dispensable for in vitro procoagulant activity. *Proc Natl Acad Sci U S A* 1986; 83: 5939-42.

20 Hansen JJ, Bolt G, Kjaergaard K, Kristensen C, and Steenstrup TD. Generation of recombinant FVIII purification antibody for manufacturing of N8. J Thromb Haemost 7suppl 2, abstract, PP-WE-572. 2009.
Ref Type: Generic

21 Newell JL, Fay PJ. Acidic residues C-terminal to the A2 domain facilitate thrombin-catalyzed activation of factor VIII. *Biochemistry* 2008; 47: 8786-95.

22 Khrenov AV, Ananyeva NM, Saenko EL. Role of the B domain in proteolytic inactivation of activated coagulation factor VIII by activated protein C and activated factor X. *Blood Coagul Fibrinolysis* 2006; 17: 379-88.

23 Varfaj F, Neuberg J, Jenkins PV, Wakabayashi H, Fay PJ. Role of P1 residues Arg336 and Arg562 in the activated-Protein-C-catalysed inactivation of Factor VIIIa. *Biochem J* 2006; 396: 355-62.

Table 1 Summary of functional *in vitro* data of N8

	N8	Advate®	Haemate®
Specific FVIII:C activity (IU mg^{-1}) of seven individual batches†)	9300 ± 400	n/a	n/a
Clot/chromogenic FVIII:C activity ratio§	1.00 (0.97-1.03)	1.0 (0.8-1.2)	1.0 (0.8-1.2)
Apparent binding to FIXa, $K_{½FIXa}$ (nM) (n=13 for N8 and n=12 for Advate®)$	0.78 ± 0.24	0.76 ± 0.25	n/a
FX activation $			
K_m (nM) (n=10)	5.1 ± 0.5	4.8 ± 0.4	n/a
k_{cat} (sec^{-1}) (n=13 for N8 and n=12 for Advate®)	6.5 ± 2.2	5.5 ± 1.0	n/a
Rate of activation by thrombin (10^{-3} min^{-1}) (n=11 for N8 and n=12 for Advate®)$	13.5 ± 6.7	10.6 ± 3.5	n/a
Rate of APC-mediated inactivation (min^{-1}) (n=10 for N8 and n=12 for Advate®)$	0.14 ± 0.03	0.12 ± 0.02	n/a
VWF binding (ELISA) K_d (nM) (n=4)$	0.24 ± 0.04*	0.48 ± 0.13	n/a
VWF binding (SPR) (n=9 for N8 and n=2 for Advate®) $			
K_d (nM)	0.23 ± 0.13*	0.45 ± 0.07	n/a
k_{on} ($\times 10^6 M^{-1} sec^{-1}$)	4.5 ± 2.1	1.2 ± 0.6	n/a
k_{off} ($\times 10^{-4} sec^{-1}$)	9.3 ± 4.7	5.5 ± 1.9	n/a
Rate of thrombin generation after adding FVIII to 1 U/ml (nM min^{-1}) (n=3) $	3.7 ± 0.8	3.7 ± 0.7	4.2 ± 1.4

†) The FVIII:C activity as measured by one stage clot assay is mean and SD of values of seven individual batches of N8, each analyzed in three individual experiments each comprising four measurements.

§) The FVIII:C activity of N8 was analyzed in the clot assay (n=36) and in the chromogenic assay (n=12). For Advate® and Haemate® n=3 measurements of each were performed. The values are means with 95% confidence intervals.

$) Values are mean and SD of the numbers of experiments indicated.

*) Significantly different ($p<0.05$) from the value obtained for Advate® using one-tailed student's t-test.

Table 2 The maximal effect of N8 on whole blood thromboelastography (TEG®) using blood from haemophilia A patients (n=8).

	Pre-spiking†	N8	Advate®	Normal range
R-time (sec)	2228 (860-3596)	392 (369-414)	393 (370-415)	500 (459-541)
MTG (mm×100 sec^{-1})	6.3 (4.2-8.4)	13.1 (11.8-14.5)	12.9 (11.5-14.3)	12.8 (11.5-14.0)
MA (mm)	45.8 (37.0-54.6)	60.2 (58.0-62.5)	59.7 (57.4-61.9)	59.3 (56.2-62.5)

†) Values are mean with 95% confidence intervals

Table 3 Potency (ratio of EC_{50} values) of Advate® and N8 in blood from haemophilia A patients (n=8)

	EC_{50} Advate®/EC_{50} N8†	p-value
R-time	1.0 (0.7-1.4)	0.99
MTG	1.4 (0.9-2.3)	0.11
MA	1.1 (0.6-1.9)	0.76

†) Values are mean with 95% confidence intervals

Legends to Figures

Figure 1 Characterization of FVIII cofactor activity and APC-mediated inactivation of FVIIIa. Measured rate of FX activation upon (A) titration of activated N8 or Advate® against a fixed concentration of FIXa (0.1 nM), (B) titration of FIXa against a fixed concentration of activated N8 or Advate® (0.1 nM, nominal concentration) and (C) titration of FX against a fixed concentration of FIXa (5 nM) and activated N8 or Advate® (0.1 nM, nominal concentration). (D) Time course of initial loss of FVIIIa activity (1 nM) following addition of APC (5 nM) in the presence of phospholipids (25 μM) and protein S (100 nM). Plots are representative titrations and inactivations.

Figure 2 VWF binding measured in ELISA (A) or by surface plasmon resonance (B). (A) Binding isotherms of N8 and Advate® (mean and SD of n=4). The higher maximal signal observed with N8 presumably reflects its lower molecular mass which allows for a higher density on the VWF-coated surface. (B) Typical binding curve of N8 binding to immobilized VWF at a concentration range of 0.05-12.5 nM. Similar binding curves were achieved for binding of Advate® to VWF.

Figure 3 Thrombin generation at haemophilia A-like conditions in a reconstituted cell-based model. N8 (A), Advate® (B), or Haemate® (C) were added at 0.01 U mL^{-1} (squares, dotted lines), 0.1 U mL^{-1} (triangles, broken lines) or 1 U mL^{-1} (solid circles, solid lines) to purified coagulation proteins and mixed with washed platelets before addition to tissue factor-expressing cells. Timed samples were analyzed for thrombin activity. Haemophilia A-like conditions without FVIII added are shown with open circles and solid line. The data are representative of three individual experiments.

Figure 4 Thromboelastography TEG® analysis of blood from haemophilia A patients. Dose response relationship of the MTG (maximum thrombus generation) when adding N8 or Advate® to blood from haemophilia A patients (n=8). The data are mean and SD of data from the eight

patients. Normal range based on 11 healthy donors is shown as mean value (solid line) +/- one SD (dotted lines).

Figure 1

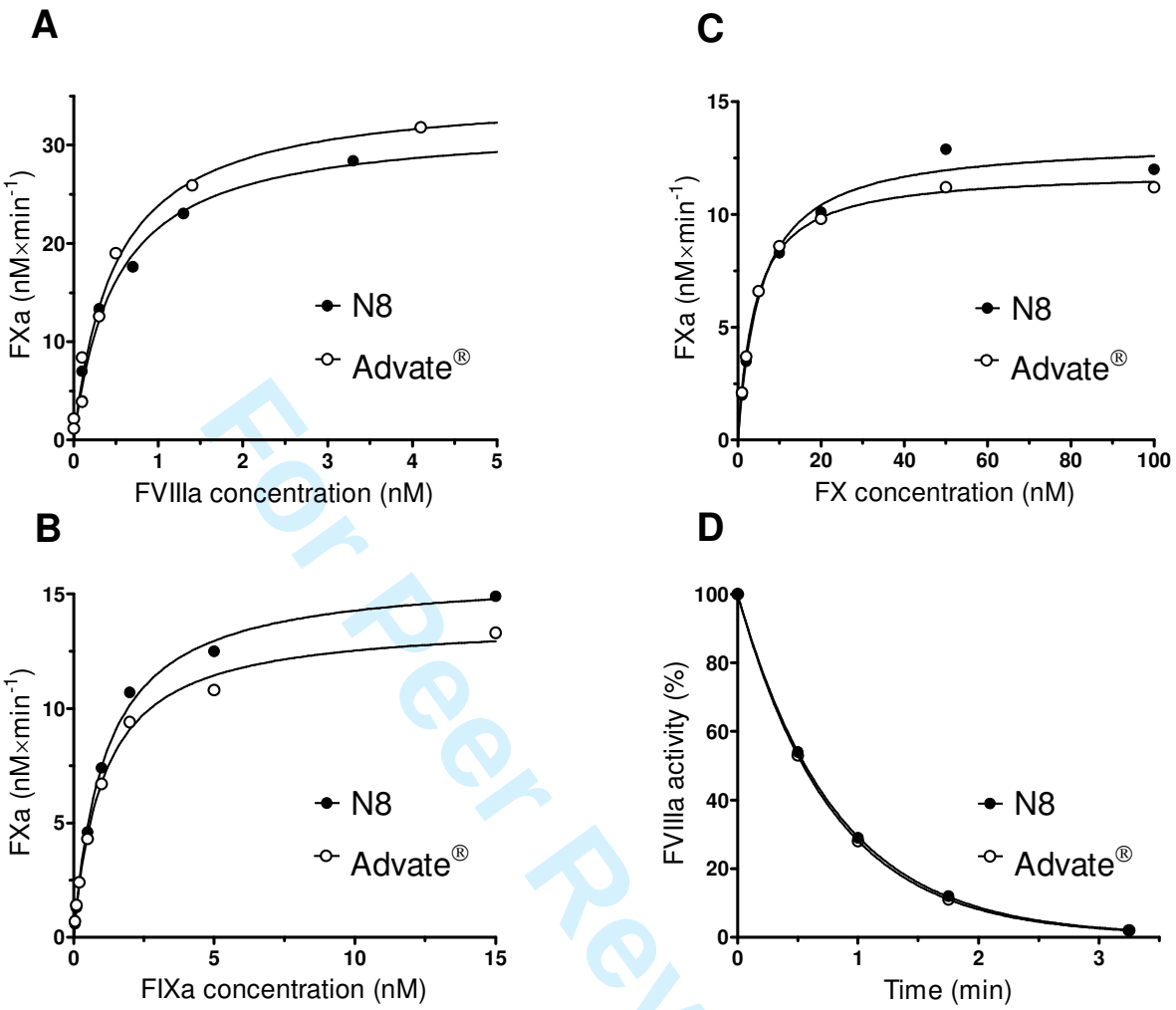

Figure 2

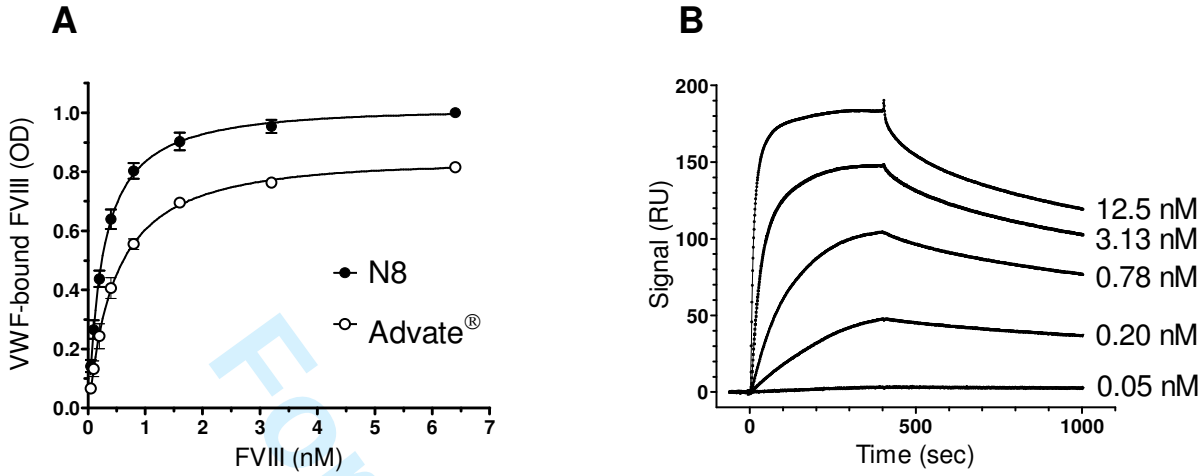

Figure 3

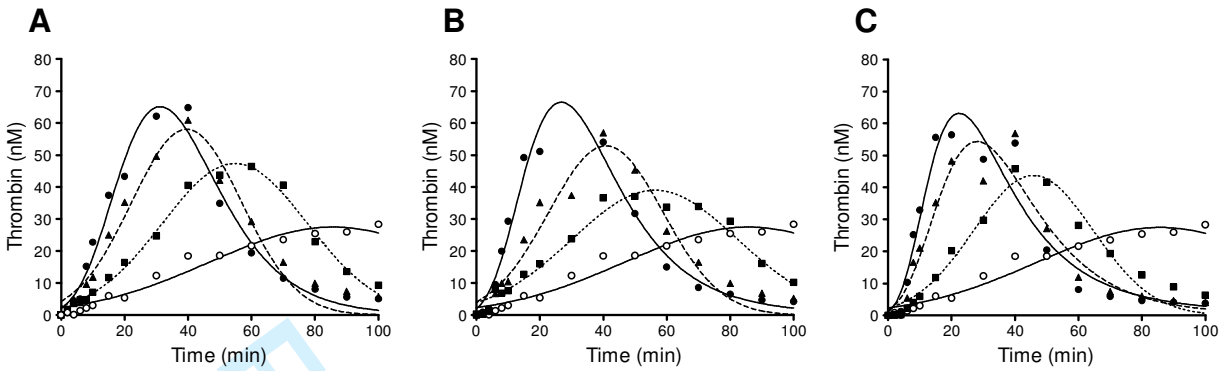

Figure 4