(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 May 2018 (17.05.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/087271 A1

(51) International Patent Classification:
A61K 38/36 (2006.01) *A61P 7/04* (2006.01)
A61K 38/37 (2006.01) *A61K 36/36* (2006.01)
C07K 14/755 (2006.01) *A61K 36/37* (2006.01)

(21) International Application Number:
PCT/EP2017/078840

(22) International Filing Date:
10 November 2017 (10.11.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
16198497.6 11 November 2016 (11.11.2016) EP

(71) Applicant: **CSL BEHRING RECOMBINANT FACILITY AG** [CH/CH]; Wankdorfstrasse 10, 3014 Bern (CH).

(72) Inventors: **PESTEL, Sabine**; Stuempelstal 19, 35041 Marburg (DE). **RAQUET, Elmar**; Wilhelm-Tischbein-Strasse 12, 35066 Frankenberg (DE). **WEIMER, Thomas**; Richard-Wagner-Strasse 8, 35075 Gladenbach (DE).

(74) Agent: **LUDWIG, Holger** et al.; Emil-von-Behring-Strasse 76, 35041 Marburg (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2018/087271 A1

(54) Title: TRUNCATED VON WILLEBRAND FACTOR POLYPEPTIDES FOR EXTRAVASCULAR ADMINISTRATION IN THE TREATMENT OR PROPHYLAXIS OF A BLOOD COAGULATION DISORDER

(57) Abstract: The invention pertains to a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment or prophylaxis of a blood coagulation disorder, said treatment or prophylaxis comprising administering the recombinant polypeptide and a Factor VIII protein (FVIII) extravascular to a subject having a blood coagulation disorder, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

CSL Behring Recombinant Facility AG

Truncated Von Willebrand Factor Polypeptides for Extravascular Administration in the Treatment or Prophylaxis of a Blood Coagulation Disorder

FIELD OF THE INVENTION

The present invention relates to products and methods for improving treatment of blood coagulation disorders.

BACKGROUND OF THE INVENTION

There are various bleeding disorders caused by deficiencies of blood coagulation factors. The most common disorders are hemophilia A and B, resulting from deficiencies of blood coagulation Factor VIII (FVIII) and IX, respectively. Another known bleeding disorder is von Willebrand's disease (VWD).

In plasma FVIII exists mostly as a noncovalent complex with von Willebrand Factor (VWF), and its coagulant function is to accelerate Factor IXa dependent conversion of Factor X to Xa.

Classic hemophilia or hemophilia A is an inherited bleeding disorder. It results from a chromosome X-linked deficiency of blood coagulation FVIII, and affects almost exclusively males with an incidence of between one and two individuals per 10,000. The X-chromosome defect is transmitted by female carriers who are not themselves hemophiliacs. The clinical manifestation of hemophilia A is an increased bleeding tendency.

In severe hemophilia A patients undergoing prophylactic treatment FVIII has to be administered intravenously (i.v.) about 3 times per week due to the short plasma half-life of FVIII of about 12 to 14 hours. Each i.v. administration is cumbersome, associated with pain and entails the risk of an infection especially as this is mostly done at home by the patients themselves or by the parents of children having been diagnosed for hemophilia A.

It would thus be highly desirable to increase the half-life of FVIII so that pharmaceutical compositions containing such FVIII would have to be administered less frequently.

Several attempts have been made to prolong the half-life of non-activated FVIII either by reducing its interaction with cellular receptors (WO 2003/093313 A2, WO 2002/060951 A2), by covalently attaching polymers to FVIII (WO 1994/15625 A1, WO 1997/11957 A1 and US 4970300), by encapsulation of FVIII (WO 1999/55306 A1), by introduction of novel metal binding sites (WO 1997/03193 A1), by covalently attaching the A2 domain to the A3 domain either by peptidic (WO 1997/40145 A1 and WO 2003/087355 A1) or disulfide linkage (WO 2002/103024 A2) or by covalently attaching the A1 domain to the A2 domain (WO 2006/108590 A1).

Another approach to enhance the functional half-life of FVIII or VWF is by PEGylation of FVIII (WO 2007/126808 A1, WO 2006/053299 A2, WO 2004/075923 A2) or by PEGylation of VWF (WO 2006/071801 A2). The increased half-life of PEGylated VWF would indirectly also enhance the half-life of FVIII present in plasma. Also fusion proteins of FVIII have been described (WO 2004/101740 A2, WO2008/077616 A1 and WO 2009/156137 A1).

VWF, which is missing, functionally defect or only available in reduced quantity in different forms of von Willebrand disease (VWD), is a multimeric adhesive glycoprotein present in the plasma of mammals, which has multiple physiological functions. During primary hemostasis VWF acts as a mediator between specific receptors on the platelet surface and components of the extracellular matrix such as collagen. Moreover, VWF serves as a carrier and stabilizing protein for procoagulant FVIII. VWF is synthesized in endothelial cells and megakaryocytes as a 2813 amino acid precursor molecule. The amino acid sequence and the cDNA sequence of wild-type VWF are disclosed in Collins et al. 1987, Proc. Natl. Acad. Sci. USA 84:4393–4397. The precursor polypeptide, pre-pro-VWF, consists of an N-terminal 22-residue signal peptide, followed by a 741-residue pro-peptide and the 2050-residue polypeptide found in mature plasma VWF (Fischer et al., FEBS Lett. 351: 345-348, 1994). After cleavage of the signal peptide in the endoplasmatic reticulum a C-terminal disulfide bridge is formed between two monomers of VWF. During further transport through the secretory pathway 12 N-linked and 10 O-linked carbohydrate side chains are added. More important, VWF dimers are multimerized via N-terminal disulfide bridges and the propeptide of 741 amino acids length is cleaved off by the enzyme PACE/furin in the late Golgi apparatus.

Once secreted into plasma the protease ADAMTS13 can cleave high-molecular weight VWF multimers within the A1 domain of VWF. Plasma VWF therefore consists of a whole range of multimers ranging from single dimers of 500 kDa to multimers consisting of up to more than 20 dimers of a molecular weight of over 10,000 kDa. The VWF-HMWM hereby having the strongest hemostatic activity, which can be measured in ristocetin cofactor activity (VWF:RCo). The higher the ratio of VWF:RCo/VWF antigen, the higher the relative amount of high molecular weight multimers.

In plasma FVIII binds with high affinity to VWF, which protects it from premature elimination and thus, plays in addition to its role in primary hemostasis a crucial role to stabilize FVIII, regulate plasma levels of FVIII and as a consequence is also a central factor to control secondary hemostasis. The half-life of non-activated FVIII bound to VWF is about 12 to 14 hours in plasma. In von Willebrand disease type 3, where no or almost no VWF is present, the half-life of FVIII is only about 2 to 6 hours, leading to symptoms of mild to moderate hemophilia A in such patients due to decreased concentrations of FVIII. The stabilizing effect of VWF on FVIII has also been used to aid recombinant expression of FVIII in CHO cells (Kaufman et al. 1989, Mol Cell Biol 9:1233-1242). Von Willebrand disease type 2N is characterized by low FVIII levels due to mutations in VWF which affect the binding of FVIII to VWF. FVIII levels in VWD type 2N patients are in a range between about 3 IU/dL and 30 IU/dL, typically below 20 IU/dL, depending on the specific mutation in VWF (Sadler J.E. and Blinder M., Von Willebrand Disease: Diagnosis, Classification, and Treatment; in: Hemostasis and Thrombosis, eds. Colman, Marder, Clowes, George, Aird, and Goldhaber, Lippincott Williams & Wilkins 2006, pp 905-921).

VWF-derived polypeptides, in particular VWF fragments, have been described to stabilize FVIII *in vitro* and *in vivo*. WO 2013/106787 A1 is directed at chimeric proteins comprising a FVIII protein and certain VWF fragments. Those chimeric hetero-dimers of FVIII and VWF-fragment do have a fixed molar ratio of VWF to FVIII of 1:1.

WO 2014/198699 A2 and WO 2013/083858 A2 describe VWF fragments and their use in the treatment of hemophilia. It was found that bioavailability of FVIIIs may be significantly improved upon extravascular co-administration with similar molar amounts of VWF fragments. High molar excess of VWF over FVIII was said to be not desirable, and in experiments with VWF fragments co-administered s.c. with FVIII it was found that the VWF dose was not critical for FVIII bioavailability. Thus molar ratios of VWF fragments over FVIII as well as VWF dose were considered to be not critical for FVIII bioavailability.

WO 2011/060242 A2 discloses fusion polypeptides comprising certain VWF fragments and an antibody Fc region proposing specific molar ratios of VWF fragment over FVIII of up to 10:1. In addition, no *in vivo* data are presented with regard to said Fc-fusion constructs.

Yee et al. (2014) Blood 124(3):445-452 found that a VWF fragment containing the D'D3 domains fused to the Fc portion of immunoglobulin G1 is sufficient to stabilize endogenous Factor VIII in VWF-deficient mice. However, although a VWF D'D3-Fc fusion protein exhibited markedly prolonged survival when transfused into FVIII-deficient mice, the VWF D'D3-Fc fusion protein did not prolong the survival of co-transfused FVIII.

Until today the standard treatment of hemophilia A involves frequent intravenous infusions of FVIII, either as concentrates derived from the plasmas of human donors or as pharmaceutical preparations based on recombinant FVIII. While these replacement therapies are generally effective, e.g. in severe hemophilia A patients undergoing prophylactic treatment, as mentioned above Factor VIII has to be administered intravenously (i.v.) about 3 times per week due to the short plasma half-life of Factor VIII of about 12 hours. Already if levels of above 1% of the FVIII activity in healthy non-hemophiliacs is reached, e.g. by a raise of FVIII levels above 0.01 U/mL, severe hemophilia A is turned into moderate hemophilia A. In prophylactic therapy dosing regimens are designed such that the trough levels of FVIII activity do not fall below levels of 2-3% of the FVIII activity in healthy non-hemophiliacs. Each i.v. administration is cumbersome, associated with pain and entails the risk of an infection especially as this is mostly done in home treatment by the patients themselves or by the parents of children being diagnosed for hemophilia A. In addition the frequent i.v. injections inevitably result in scar formation, interfering with future infusions. As prophylactic treatment in severe hemophilia is started early in life, with children often being less than 2 years old, it is even more difficult to inject FVIII 3 times per week into the veins of such small patients. For a limited period, implantation of port systems may offer an alternative. Despite the fact that repeated infections may occur and ports can cause inconvenience during physical exercise, they are nevertheless typically considered to be favorable as compared to intravenous injections.

Thus there is still a great medical need to obviate the need to infuse FVIII intravenously.
As FVIII is a very large and labile molecule it exhibits a very low bioavailability due to insufficient absorption and severe degradation, if given subcutaneously, intramuscularly or intradermally, i.e. extravascularly.

EP 0710114 A1 discloses that FVIII formulations of a B-domain deleted FVIII in a concentration above 1000 IU/mL are suitable for subcutaneous administration, leading to a bioavailability of 5-10% after s.c. administration in monkeys measuring the area under the activity (FVIII:C)-time curve.

EP 0772452 discloses that FVIII formulations of a B-domain deleted FVIII in a concentration of at least 500 IU/mL together with an organic additive when administered subcutaneously can lead for more than 6 h to a FVIII plasma level of at least 1.5% of normal FVIII levels. Using hydrolyzed gelatin or soybean oil emulsion as the organic additive and a B-domain deleted FVIII in a concentration of 1100 IU/mL and a dose of 10000 IU/kg, more than 50% bioavailability as measured as the area under the activity (FVIII:C)-time curve was seen in cynomolgus monkeys.This is however not an appropriate clinical scenario for treatment of a patient having a blood coagulation disorder.

WO 1997/11957 A1 discloses a bioavailability of 5.3% when a B-domain deleted FVIII (specific activity 15000 IU/mg; dose 2500 IU/kg) was administered subcutaneously, whereas an mPEGylated conjugate of FVIII achieved bioavailabilities of 22% or 19% in cynomolgus monkeys.

According to WO 2015/185758 A2 a composition is presented comprising a non-covalent complex of Factor VIII and one or more von Willebrand Factor peptides, wherein the von Willebrand Factor peptides comprise at least the amino acids 764 to 1035 and 1691 to 1905. The molecular ratio of FVIII:VWF is between 1:1 to 1:20. In WO 2015/185758 A2 haemophilia A dogs were subjected to s.c. and subsequent i.v. injection of recombinant B-domain-deleted FVIII alone or in combination with five-fold molar excess of a VWF fragment yielded by digestion of pdVWF with *S. aureus* V-8 protease. Samples were analyzed for whole blood clotting time (WBCT) and activity in chromogenic FVIII activity assay. The subcutaneous administration of a VWF Fragment in complex with FVIII resulted in 1.4-fold increase in time required to exceed a clotting time for a normal dog comparing with s.c. administration of FVIII alone. The administration of VWF Fragment with FVIII resulted also in increased FVIII activity in dog plasma over time and in nearly doubled area under the curve (AUC) values for both, s.c. and i.v. application compared to administration of FVIII alone.

In WO 2008/151817 A1 it was shown that VWF can be taken up into the blood stream when administered extravascularly without any stabilizing covalent modifications, which can entail an increased risk of immune responses, and that VWF can be used to enhance the uptake of FVIII when co-administered with FVIII non-intravenously. The VWF was applied without any half-life extending modification. The ratio of VWF antigen over FVIII activity was larger than 2:1. Only multimer and monomer products comprising a full length VWF have been considered. By applying full length VWF, however, high ratios of VWF over FVIII may result in an elevated thrombogenic risk. In addition, when using full length VWF the protein amounts required for increasing the ratio would not be acceptable for administration. Further, multimeric and monomeric.

There is a medical need for alternatives to the intravenous administration of FVIII to patients. In addition, there is an ongoing need for methods providing Factor VIII absorption when administered extravascularly as well as for compounds or compositions suitable for such methods.

SUMMARY OF THE INVENTION

A first object of present invention was to provide an improved Factor VIII (FVIII) protein based treatment or prophylaxis of a blood coagulation disorder.

According to a second object, said treatment should allow for alternative routes of administration of FVIII to a subject in need thereof. In particular, subcutaneous administration of FVIII should be enabled.

According to a third object, said treatment should provide at least with regard to the administered FVIII pharmacokinetic parameters sufficient to treat a subject having a blood coagulation disorder.

According to a fourth object, said treatment should provide in particular for a half-life of FVIII which is sufficiently high to allow for a tolerable or improved administration frequency.

It has been surprisingly found by the inventors that a Factor VIII (FVIII) protein can be successfully administered via an extravascular route for treatment or prophylaxis of a blood coagulation disorder, provided that the FVIII is co-administered with a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF). Said recombinant polypeptide is capable of binding to said co-administered FVIII. The molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is preferably higher than 50. The recombinant polypeptide comprising a truncated VWF preferably comprises a half-life extending moiety (HLEM). Without wishing to be bound to any theory, it is believed that it is important to achieve a high excess of the administered recombinant polypeptide comprising a truncated VWF to minimize the binding of the co-administered FVIII to endogenous VWF which has a larger molecular structure probably leading to an increased catabolism as compared to the truncated VWF. By use of the herewith presented co-administration of FVIII and said recombinant polypeptide, it is demonstrated for the first time that extravascular route for application of FVIII is not only possible, but even achieved clinically relevant amounts of FVIII into circulation.

The invention further demonstrates that extravascular administration of the recombinant polypeptide provides for or increases bioavailability of a co-administered FVIII. In addition, subcutaneous administration of the recombinant polypeptide together with FVIII allows for extravascular administration of a FVIII associated with relevant absorption of FVIII into the bloodstream resulting in FVIII activity levels not only significantly above the detection limit, but furthermore suitable for therapeutic application. The recombinant polypeptide when co-administered with FVIII not only has a sufficiently long half-life, increases maintenance of FVIII in plasma once it reached this compartment, but also provides bioavailability of FVIII suitable for therapeutic application.

In addition, the invention further demonstrates that extravascular administration of the recombinant polypeptide may allow for a treatment option comprising an FVIII administration via a different route of administration than used for the recombinant polypeptide. In particular, benefits arising from a combination of an intravenously administered FVIII and a subcutaneously administered recombinant polypeptide are demonstrated.

The present invention therefore relates particularly to the following embodiments [1] to [73]:

[1] A recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment or prophylaxis of a blood coagulation disorder, said treatment or prophylaxis comprising administering the recombinant polypeptide and a Factor VIII protein (FVIII) extravascular to a subject having a blood coagulation disorder, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

[2] A recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment or prophylaxis of a blood coagulation disorder, said treatment or prophylaxis comprising administering the recombinant polypeptide extravascular and a Factor VIII protein (FVIII) to a subject having a blood coagulation disorder, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

[3] The recombinant polypeptide for use according to embodiment [1] or [2], wherein said polypeptide comprises a half-life extending moiety (HLEM).

[4] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the subject is a human subject.

[5] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the truncated VWF is a human truncated VWF.

[6] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein said polypeptide is administered either subcutaneously, intradermally or intramuscularly.

[7] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the FVIII is administered either subcutaneously, intradermally or intramuscularly. Preferably, both FVIII and said polypeptide are administered subcutaneously.

[8] The recombinant polypeptide for use according to embodiments [2] to [6], wherein the FVIII is administered via a different route of administration than the recombinant polypeptide, preferably FVIII is administered intravenously; more preferred the recombinant polypeptide is administered subcutaneously and the FVIII is administered intravenously.

[9] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the truncated VWF comprises an amino acid sequence having a sequence identity of at least 90% to amino acids 776 to 805 of SEQ ID NO:4, preferably comprises an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:4.

[10] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the truncated VWF lacks amino acids 1243 to 2813 of SEQ ID NO:4.

[11] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the truncated VWF consists either of (a) amino acids 764 to 1242 of SEQ ID NO:4, of (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:4, or of (c) a fragment of (a) or (b).

[12] The recombinant polypeptide for use according to any one of the embodiments [3] to [11], wherein the HLEM is a heterologous amino acid sequence fused to the truncated VWF.

[13] The recombinant polypeptide for use according to embodiment [12], wherein said heterologous amino acid sequence comprises or consists of a polypeptide selected from the group consisting of transferrin and fragments thereof, the C-terminal peptide of human chorionic gonadotropin, an XTEN sequence, homo-amino acid repeats (HAP), proline-alanine-serine repeats (PAS), albumin, afamin, alpha-fetoprotein, Vitamin D binding protein, polypeptides capable of binding under physiological conditions to albumin or immunoglobulin constant regions, polypeptides capable of binding to the neonatal Fc receptor (FcRn), particularly immunoglobulin constant regions and portions thereof, preferably the Fc portion of immunoglobulin, and combinations thereof. The immunoglobulin constant region or portions thereof is preferably an Fc fragment of immunoglobulin G1, an Fc fragment of immunoglobulin G2 or an Fc fragment of immunoglobulin A.

[14] The recombinant polypeptide for use according to any one of embodiments [3] to [11], wherein the HLEM is conjugated to the recombinant polypeptide.

[15] The recombinant polypeptide for use according to embodiment [14], wherein said HLEM is selected from the group consisting of hydroxyethyl starch (HES), polyethylene glycol (PEG), polysialic acids (PSAs), elastin-like polypeptides, heparosan polymers, hyaluronic acid and albumin binding ligands, e.g. fatty acid chains, and combinations thereof.

[16] The recombinant polypeptide for use according to any one of embodiments [3] to [13], wherein the recombinant polypeptide does not comprise any HLEM conjugated to the recombinant polypeptide.

[17] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein said polypeptide is a glycoprotein comprising N-glycans, and wherein preferably at least 75 %, preferably at least 85 % of said N-glycans comprise, on average, at least one sialic acid moiety.

[18] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein said recombinant polypeptide is present as a dimer or at least has a high proportion of dimers.

[19] The recombinant polypeptide for use according to embodiment [18], wherein said recombinant polypeptide is a homodimer preferably comprising two polypeptides as defined in one of the herein disclosed embodiments, and the two monomers forming the dimer are covalently linked to each other via at least one or more disulfide bridges formed by cysteine residues within the truncated VWF.

[20] The recombinant polypeptide for use according to embodiment [19], wherein the cysteine residues forming the one or more disulfide bridges is/are selected from the group consisting of Cys-1099, Cys-1142, Cys-1222, Cys-1225, Cys-1227and combinations thereof, preferably Cys-1099 and Cys-1142, wherein the amino acid numbering refers to SEQ ID NO:4.

[21] The recombinant polypeptide for use according to any one of embodiments [18] to [20], wherein the affinity of said dimer to FVIII is greater than the affinity of a monomeric polypeptide to FVIII, said monomeric polypeptide having the same amino acid sequence as a monomeric subunit of the dimeric polypeptide.

[22] The recombinant polypeptide for use according to any one of embodiments [18] to [21], wherein the ratio dimer : monomer of the polypeptide of the invention is at least 1.5, preferably at least 2, more preferably at least 2.5 or at least 3. Preferably, the recombinant

polypeptide of the invention does not comprise monomer and/or multimer forms of the polypeptide or at least is essentially free of monomer and/or multimer forms of the polypeptide. Most preferably all polypeptides of the invention are present as dimers.

[23] The recombinant polypeptide for use according to any one of embodiments [18] to [22], wherein the dimeric polypeptide has a FVIII binding affinity characterized by a dissociation constant K_D of less than 1 nM, preferably less than 500 pM, less than 200 pM, less than 100 pM, less than 90 pM or less than 80 pM.

[24] The recombinant polypeptide for use according to embodiment [23], wherein the K_D ranges from 0.1 pM to 500 pM, from 0.5 pM to 200 pM, from 0.75 pM to 100 pM or most preferred from 1 pM to 80 pM.

[25] The recombinant polypeptide for use according to any one of embodiments [18] to [24], wherein the polypeptide has a FVIII binding affinity characterized by a dissociation constant K_D and said dissociation constant K_D of the dimeric polypeptide is reduced compared to the dissociation constant K_D of a monomeric polypeptide, preferably by a factor of at least 10, by a factor of at least 100, by a factor of at least 500 or by a factor of at least 1000.

[26] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein said polypeptide comprises at least one amino acid substitution as compared to the amino acid sequence of the wild-type VWF, wherein the binding affinity of such a modified polypeptide to FVIII is preferably being further increased by introduction of said at least one substitution compared to the binding affinity of a reference polypeptide which has the same amino acid sequence except for said modifications.

[27] The recombinant polypeptide for use according to embodiment [26], wherein said substitutions within the truncated VWF have the capacity to further increase the half-life of co-administered FVIII following administration. Thereby, the treatment may also provide in particular an *in vivo* half-life of FVIII which is further increased to allow for a tolerable or improved administration frequency.

[28] The recombinant polypeptide for use according to embodiments [26] or [27], wherein the substitutions are selected from the group of combinations consisting of S764G/S766Y, S764P/S766I, S764P/S766M, S764V/S766Y, S764E/S766Y, S764Y/S766Y, S764L/S766Y, S764P/S766W, S766W/S806A, S766Y/P769K, S766Y/P769N, S766Y/P769R, S764P/S766L, and S764E/S766Y/V1083A, referring to the sequence of SEQ ID NO:4 with regard to the amino acid numbering.

[29] The recombinant polypeptide for use according to embodiment [28], wherein said substitution is the either the combination S764E/S766Y or S764E/S766Y/V1083A.

[30] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the mean residence time (MRT) of the administered FVIII is increased by the co-administration of the recombinant polypeptide, preferably by a factor of at least 1.5, at least 2, at least 3, at least 4 or at least 5, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention and/or except that no recombinant polypeptide has been administered.

[31] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the MRT of the administered FVIII is at least 10 h, preferably at least 15 h, at least 20 h or at least 25 h.

[32] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the MRT of the administered recombinant polypeptide is increased, preferably by a factor of at least 1.5, at least 2 or at least 3, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention, in particular below 50.

[33] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the terminal half-life of the administered FVIII is increased by the co-administration of the recombinant polypeptide, preferably by a factor of at least 1.2, at least 1.5, at least 2, at least 2.5, or at least 3, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention and/or except that no recombinant polypeptide has been administered. Thereby, the treatment may provide in particular an *in vivo* half-life of FVIII which is sufficiently high to allow for a tolerable or improved administration frequency.

[34] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the time period for reaching a 1% trough level of the FVIII co-administered with said polypeptide having a HLEM is prolonged compared to a reference

treatment, wherein said reference treatment is identical to said treatment, except the FVIII is administered with a recombinant polypeptide without having said HLEM and/or except that no recombinant polypeptide has been administered.

[35] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the time period either

(i) for reaching the 1% trough level of the FVIII co-administered with said polypeptide is at least about 30h, at least about 35h, at least about 38h, at least about 40h, or at least about 50h; or

(ii) for reaching the 5% trough level of the FVIII co-administered with said polypeptide is at least about 20h, at least about 22h, at least about 29h, at least about 34h, or at least about 43h; or

(iii) for reaching the 10% trough level of the FVIII co-administered with said polypeptide is at least about 5h, at least about 6h, at least about 10h, at least about 18h, or at least about 20h.

[36] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the plasma half-life of the polypeptide is increased compared to that of endogenous VWF and/or compared to that of VWF of normal human plasma (NHP), wherein the plasma half-life of the polypeptide is preferably at least 100%, at least 200% or preferably at least 400% higher than that of the endogenous VWF and/or compared to that of VWF of normal human plasma (NHP).

[37] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the blood coagulation disorder is hemophilia A or von-Willebrand disease.

[38] The recombinant polypeptide for use according to embodiment [37], wherein the blood coagulation disorder is hemophilia A and is either mild hemophilia A, typically associated with an endogenous FVIII activity level that is 5% to 40% of the endogenous FVIII activity level in normal human plasma (NHP), or moderate hemophilia A, typically associated with an endogenous FVIII activity level that is 1% to 5% of the endogenous FVIII activity level in NHP, or severe hemophilia A, typically associated with an endogenous FVIII activity level that is below 1% of the endogenous FVIII activity in NHP.

[39] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the polypeptide is used for (i) on-demand treatment and control of

bleeding episodes, (ii) routine prophylaxis, particularly to reduce the frequency of bleeding episodes, or (iii) perioperative management of bleeding.

[40] The recombinant polypeptide for use according to embodiment [39], wherein the polypeptide is used for routine prophylaxis to reduce the frequency of bleeding episodes of a patient with hemophilia A.

[41] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein co-administration of the recombinant polypeptide and the FVIII protein is achieved either

- (i) by administration together in a single composition comprising the recombinant polypeptide and the FVIII protein, or
- (ii) by administration of the recombinant polypeptide (first compound) and the FVIII protein (second compound) each provided in separate compositions, optionally as part of a combined therapy, wherein the first compound is administered before, after or concurrently with the second compound. Any suitable timing interval may be applied for administration of the first compound and the second compound when the first compound is administered before or after the second compound. In particular, for the purpose of routine prophylaxis, administration of the first compound and administration the second compound may be provided according to independent or coordinated dosing schedules.

[42] The recombinant polypeptide for use according to embodiment [41], wherein in case of (i) co-administration of the recombinant polypeptide and the FVIII protein is achieved either

by providing a combination product comprising the recombinant polypeptide and the FVIII blended in a single composition or

by providing a set or kit of at least two separate products arranged to be mixed before administration, whereby a first product comprises the recombinant polypeptide and a second product comprises the FVIII.

[43] The recombinant polypeptide for use according to embodiment [41], wherein in case of (ii) the recombinant polypeptide and the FVIII protein, in particular when administered concurrently and/or in particular when administered both extravascularly, are administered in close proximity, preferably, the injection sites are separated not more than 50 mm, not more than 40 mm, not more than 30 mm, in particular not more than 20 mm.

[44] The recombinant polypeptide for use according to embodiment [41] or [43], wherein in case of (ii) the recombinant polypeptide and the FVIII protein may be co-administered within 1

month, within three weeks, within two weeks, within one week, within one day, within about one hour, within 30 min, within 15 min or within 5 min.

[45] The recombinant polypeptide for use according to embodiment [41], [43] or [44], wherein in case of (ii) the recombinant polypeptide and the FVIII protein may be co-administered within a timing interval of no more than 1 month, no more than three weeks, no more than two weeks, no more than one week, no more than one day, no more than about one hour, preferably within 30 min, more preferably within 15 min and most preferably within 5 min.

[46] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the recombinant polypeptide does not comprise a FVIII protein and/or does not comprise a polypeptide having a FVIII activity.

[47] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the FVIII is a plasma derived FVIII protein or a recombinant FVIII protein, preferably a human FVIII protein.

[48] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the FVIII is a recombinant FVIII protein.

[49] The recombinant polypeptide for use according to any embodiment [48], wherein the recombinant FVIII has the natural B domain intact or has the B domain deleted, truncated or modified. Optionally, the recombinant FVIII protein may comprise at least one half-life extending moiety (HLEM). Suitable HLEMs are disclosed herein.

[50] The recombinant polypeptide for use according to embodiment [48], wherein the FVIII protein is a recombinant single-chain Factor VIII, preferably comprising or consisting of the amino acid sequence SEQ ID NO:5 or fragments thereof provided such fragments have FVIII activity.

[51] The recombinant polypeptide for use according to embodiment [48], wherein the recombinant FVIII has the B domain deleted or truncated provided that said deleted or truncated B domain comprises a heterologous insertion of at least one linker peptide and/or a half-life enhancing polypeptide.

[52] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein, when FVIII is administered extravascularly, the bioavailability of the administered FVIII following co-administration with the recombinant polypeptide is increased by the recombinant polypeptide when compared to a reference treatment wherein said reference treatment is identical to said treatment, except the FVIII is administered without said

recombinant polypeptide. Thereby, extravascular administration of the recombinant polypeptide provides for or increases bioavailability of the administered FVIII. Preferably, subcutaneous co-administration of the recombinant polypeptide together with FVIII allows for extravascular administration of a FVIII associated with relevant absorption of FVIII into the bloodstream resulting in FVIII activity levels not only significantly above the detection limit, but furthermore suitable for therapeutic application. Preferably, the recombinant polypeptide when co-administered with FVIII not only has a sufficiently long half-life, increases maintenance of FVIII in plasma once it reached this compartment, but also provides bioavailability of FVIII suitable for therapeutic application.

[53] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the bioavailability of extravascular administered FVIII following co-administration with the recombinant polypeptide is at least 2%, at least 3%, at least 5%, preferably at least 7%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35% or at least 40%.

[54] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the bioavailability of the recombinant polypeptide is at least 30%, preferably at least 35%, more preferably at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, or at least 80%.

[55] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the dosage of co-administered FVIII protein does not exceed 2500 IU/kg, preferably does not exceed 2000 IU/kg, does not exceed 1500 IU/kg, does not exceed 1000 IU/kg, does not exceed 600 IU/kg, does not exceed 500 IU/kg or does not exceed 400 IU/kg.

[56] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the maximal concentration (C_{max}) for FVIII is at 10 mIU/mL, at least 25 mIU/mL, at least 50 mIU/mL, at least 100 mIU/mL, at least 200 mIU/mL, at least 300 mIU/mL or at least 400 mIU/mL FVIII activity, preferably chromogenic FVIII activity.

[57] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the maximal concentration (C_{max}) for the recombinant polypeptide is at least 20 nmol/kg, at least 40 nmol/kg, at least 60 nmol/kg, at least 80 nmol/kg or at least 160 nmol/kg. Preferably, following co-administration of said recombinant polypeptide with FVIII the maximal

concentration (C_{max}) for the recombinant polypeptide is at least 5 µg HLEM/mL, at least 10 µg HLEM/mL, at least 15 µg HLEM/mL, or at least 20 µg HLEM /mL, whereby the values are based on a calculation for the HLEM, preferably, the values are based on a quantitation using a HLEM specific assay such as an immunoassay, preferably specific for human albumin. A further preferred embodiment pertains to the recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the maximal concentration (C_{max}) for the recombinant polypeptide is at least 3 fold higher as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

[58] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$) for the co-administered FVIII is at least 1,000 mIU*h/mL, at least 2,000 mIU*h/mL, at least 3,000 mIU*h/mL, at least 5,000 mIU*h/mL, at least 10,000 mIU*h/mL or at least 20,000 mIU*h/mL FVIII activity, preferably chromogenic FVIII activity.

[59] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$) for the co-administered recombinant polypeptide is at least 2 nmol * h/mL, at least 3 nmol * h/mL, at least 4 nmol * h/mL, at least 20 nmol * h/mL, at least 40 nmol * h/mL, or at least 80 nmol * h/mL. Preferably, following co-administration of said recombinant polypeptide with FVIII the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$) for the co-administered recombinant polypeptide is at least 500 µg HLEM *h/mL, at least 750 µg HLEM *h/mL, at least 1,000 µg HLEM *h/mL at least 5,000 µg HLEM*h/mL, or at least 10,000 µg HLEM *h/mL, whereby the values are based on a calculation for the HLEM, preferably, the values are based on a quantitation using a HLEM specific assay such as an immunoassay, preferably specific for human albumin. A further preferred embodiment pertains to the recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$) for the co-administered recombinant polypeptide is at least 5, is at least 10 or is at least 15 fold higher as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not

comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

[60] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the clearance (CL) value of the recombinant polypeptide amounts to a range between 1.0 to 2.5 mL/kg/h, or between 1.1 to 2.2 mL/kg/h or between 1.2 to 2.1 mL/kg/h.

[61] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the clearance (CL) value for the recombinant polypeptide is reduced by a factor of at least 2, at least 5, or at least 10, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

[62] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the clearance (CL) value of the administered FVIII is reduced compared to a reference treatment, preferably by a factor of at least 1.5, at least 2, at least 3, at least 5, at least 7.5 or at least 10, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

[63] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein following co-administration of said recombinant polypeptide with FVIII the clearance (CL) value of the administered FVIII is below 135 mL/kg/h, below 80 mL/kg/h, below 45 mL/kg/h, below 40 mL/kg/h, below 35 mL/kg/h, below 30 mL/kg/h or below 25 mL/kg/h. The clearance (CL) value of the administered FVIII is preferably lower than that of a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is lower below a molar ratio according to the invention.

[64] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the molar ratio of the recombinant polypeptide to the FVIII to be

administered is at least 75, at least 100, at least 200, at least 300, at least 400, at least 500 or at least 1000.

[65] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the recombinant polypeptide is administered at an amount of at least 0.01 mg/kg, at least 0.1 mg/kg, at least 0.2 mg/kg, at least 0.5 mg/kg, at least 1 mg/kg or at least 3 mg/kg recombinant polypeptide.

[66] The recombinant polypeptide for use according to any one of the preceding embodiments, wherein the recombinant polypeptide is administered with an amount not exceeding 20 mg/kg, not exceeding 15 mg/kg, not exceeding 10 mg/kg, or not exceeding 5 mg/kg of the recombinant polypeptide.

[67] A pharmaceutical composition for use in the treatment or prophylaxis of a blood coagulation disorder as defined in any one of embodiments [1] to [66], the composition comprising

(i) a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) according to any one of embodiments [1] to [7] or any one of embodiments [9] to [66] provided that the recombinant polypeptide and the FVIII are to be administered via the same route of administration, and

(ii) a Factor VIII protein (FVIII),

wherein the molar ratio of the recombinant polypeptide to the FVIII within the composition is greater than 50.

[68] A pharmaceutical composition for use according to embodiment [67], wherein said treatment comprising administering the composition extravascular to a subject with a blood coagulation disorder, and

said pharmaceutical composition is formulated suitable for extravascular co-administration. Preferably, at least portions of said recombinant polypeptide are bound to FVIII. According to a further preferred embodiment of the pharmaceutical composition, said recombinant polypeptide is non-covalently bound to FVIII. Preferably, the pharmaceutical composition comprises a high proportion of dimers of said polypeptide. Further preferred is that the pharmaceutical composition does not comprise monomer and/or multimer forms of the polypeptide or at least is essentially free of monomer and/or multimer forms of the polypeptide.

[69] A pharmaceutical kit comprising (i) a first composition comprising a Factor VIII protein (FVIII) and (ii) a second composition comprising a recombinant polypeptide comprising a

truncated von Willebrand Factor (VWF) for use according to any one of embodiments [1] to [66] for use in the treatment or prophylaxis of a blood coagulation disorder, said treatment comprising administering the recombinant polypeptide and the FVIII protein, preferably extravascular, to a subject having the blood coagulation disorder, wherein said FVIII and said recombinant polypeptide are provided within the kit. Preferably, said FVIII and said recombinant polypeptide are provided within the kit in order to allow prior to administration for at least a proportion of said recombinant polypeptide to bind to said FVIII, and provided that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50. Preferably, the second composition comprises a high proportion of dimers of said polypeptide. Further preferred is that the second composition does not comprise monomer and/or multimer forms of the polypeptide or at least is essentially free of monomer and/or multimer forms of the polypeptide.

[70] A method of treatment or prophylaxis of a blood coagulation disorder, the method comprising co-administering an effective amount of a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) and a Factor VIII protein (FVIII) to a subject having the blood coagulation disorder, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50. The recombinant polypeptide within this method may preferably be provided according to any one of embodiments [1] to [66]. Said polypeptide may be administered subcutaneously, intradermally or intramuscularly. The FVIII may be also administered subcutaneously, intradermally or intramuscularly. Preferably, both FVIII and said polypeptide are administered subcutaneously. According to another variation of the method, the FVIII is administered via a different route of administration than the recombinant polypeptide, preferably FVIII is then administered intravenously, more specifically the recombinant polypeptide is administered subcutaneously and the FVIII is administered intravenously.

[71] A method of treatment or prophylaxis of a blood coagulation disorder, the method comprising administering an effective amount of a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) and a Factor VIII protein (FVIII) via different routes of administration to a subject having the blood coagulation disorder, wherein said recombinant polypeptide is capable of binding to FVIII. The recombinant polypeptide within this method may be provided according to any one of embodiments [2] to [66]. Within this embodiment, the determination of the molar ratio of the recombinant polypeptide to the FVIII is not obligatory before administration of the recombinant polypeptide. Preferably, the FVIII

is administered intravenously, more preferred the recombinant polypeptide is administered subcutaneously and the FVIII is administered intravenously.

[72] The use of a recombinant polypeptide as defined in any one of embodiments [1] to [66] for the treatment or prophylaxis of a blood coagulation disorder, said recombinant polypeptide comprising a truncated von Willebrand Factor (VWF), said treatment or prophylaxis comprising administering the polypeptide and a Factor VIII (FVIII) protein, preferably extravascular, to a subject, wherein said recombinant polypeptide is capable of binding to said FVIII. The molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is preferably higher than 50.

[73] Use of a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) for the manufacture of a medicament for the treatment or prophylaxis of a blood coagulation disorder, said treatment or prophylaxis comprising administering the recombinant polypeptide and a Factor VIII (FVIII) protein, preferably extravascular, to a subject, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50 provided that said recombinant polypeptide is defined according to any one of embodiments [1] to [66].

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows levels of the recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) (hereinafter also: recombinant polypeptide) after subcutaneous or intravenous administration of rD'D3-FP or rD'D3-His with or without recombinant FVIII in FVIII ko mice. rD'D3-FP was quantified via its albumin component, and rD'D3-His data are calculated to equimolar concentrations. Data is given as mean ± SD for n=1-4 mice per timepoint. Solid lines represent s.c. and dotted lines i.v. treatment. Abbreviation: s.c.: subcutaneous; i.v.: intravenous;

Figure 2 shows maximal concentration and AUC of the recombinant polypeptide plasma levels after subcutaneous administration of rD'D3-FP or rD'D3-His with or without recombinant FVIII in FVIII ko mice. rD'D3-FP was quantified via its albumin component, and rD'D3-His data are calculated to equimolar concentrations. Data is given as mean ± SD for n=1-4 mice per timepoint. Estimation of C_{max} and AUC_{0-inf} was done by two-compartmental-resorption modelling;

Figure 3 shows bioavailability of rD'D3-FP or rD'D3-His after subcutaneous administration of rD'D3-FP or rD'D3-His with or without recombinant FVIII in FVIII ko mice. rD'D3-FP was quantified via its albumin component, and rD'D3-His data are calculated to equimolar concentrations. Data was calculated from the mean AUC_{0-inf} calculated from n=1-4 mice per timepoint. Estimation of AUC_{0-inf} was done by two-compartmental-resorption modelling. Bioavailability was calculated as the percentage of the AUC_{0-inf} after s.c. administration as compared to i.v. administration, in case of rD'D3-FP for the three different i.v. groups using rD'D3-FP at different doses with or without rFVIII;

Figure 4 shows FVIII activity plasma levels after subcutaneous or intravenous administration of rD'D3-FP or rD'D3-His with or without recombinant FVIII in FVIII ko mice. FVIII was quantified as chromogenic FVIII activity. Data is given as mean ± SD for n=2-3 mice per timepoint. Solid lines represent s.c. and dotted lines i.v. treatment; Abbreviation: s.c.: subcutaneous; i.v.: intravenous;

Figure 5 shows maximal concentration and AUC of FVIII activity plasma levels after subcutaneous administration of rD'D3-FP or rD'D3-His with or without recombinant FVIII in FVIII ko mice. FVIII was quantified as chromogenic FVIII activity. Data is given as mean ± SD for n=2-3 mice per timepoint. Estimation of C_{max} and AUC_{0-inf} was done by two-compartmental-resorption modelling;

Figure 6 shows bioavailability of chromogenic FVIII activity after subcutaneous administration of rD'D3-FP or rD'D3-His with recombinant FVIII in FVIII ko mice. Data was calculated from the mean AUC_{0-inf} calculated from n=2-3 mice per timepoint. Estimation of AUC_{0-inf} was done by two-compartmental-resorption modelling. Bioavailability was calculated as the percentage of the AUC_{0-inf} after s.c. administration as compared to i.v. administration, in case of rD'D3-FP for the two different i.v. groups using rD'D3-FP at different doses with or without rFVIII. FVIII doses and selected rD'D3-FP doses are given as numbers in the graph;

Figure 7 shows recombinant polypeptide plasma levels after subcutaneous or intravenous administration of rD'D3-FP with or without recombinant FVIII in pigs. rD'D3-FP was quantified via its albumin component. Data is given as mean ± SD for n=1-3 pigs per timepoint. Solid lines represent s.c. and dotted lines i.v. treatment. Abbreviation: s.c.: subcutaneous; i.v.: intravenous;

Figure 8 shows FVIII activity plasma levels after subcutaneous or intravenous administration of rD'D3-FP with or without recombinant FVIII in pigs. FVIII was quantified as chromogenic FVIII activity. Data is given as mean ± SD for n=1-3 pigs per timepoint. Solid lines represent s.c. and dotted lines i.v. treatment; Abbreviation: s.c.: subcutaneous; i.v.: intravenous;

Figure 9 shows recombinant polypeptide plasma levels after subcutaneous or intravenous administration of rD'D3-FP with or without different recombinant FVIII or a plasma derived FVIII in FVIII ko mice. rD'D3-FP was quantified via its albumin component. Data is given as mean ±

SD for n=2-3 mice per timepoint. Solid lines represent s.c. and dotted lines i.v. treatment. Abbreviation: s.c.: subcutaneous; i.v.: intravenous;

Figure 10 shows FVIII activity plasma levels after subcutaneous or intravenous administration of rD'D3-FP with or without different recombinant FVIII or a plasma derived FVIII in FVIII ko mice (Fig. 8A Beriate®, Fig. 8B Advate® and Fig. 8C ReFacto AF®). FVIII was quantified as chromogenic FVIII activity. Data is given as mean ± SD for n=2-3 mice per timepoint. Solid lines represent s.c. and dotted lines i.v. treatment; Abbreviation: s.c.: subcutaneous; i.v.: intravenous;

Figure 11 shows recombinant polypeptide plasma levels after subcutaneous or intravenous administration of rD'D3-FP EYA or rD'D3-CTP with recombinant FVIII in FVIII ko mice. rD'D3-FP EYA was quantified via its albumin component and rD'D3-CTP via its D'D3 component. Data is given as mean ± SD for n=3 mice per timepoint. Solid lines represent s.c. and dotted lines i.v. treatment. Abbreviation: s.c.: subcutaneous; i.v.: intravenous; and

Figure 12 shows FVIII activity plasma levels after subcutaneous or intravenous administration of rD'D3-FP EYA or rD'D3-CTP with recombinant FVIII in FVIII ko mice. FVIII was quantified as chromogenic FVIII activity. Data is given as mean ± SD for n=3 mice per timepoint. Solid lines represent s.c. and dotted lines i.v. treatment; Abbreviation: s.c.: subcutaneous; i.v.: intravenous.

DETAILED DESCRIPTION

In a first aspect, the present invention relates to a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment or prophylaxis of a blood coagulation disorder, said treatment comprising administering the recombinant polypeptide and a Factor VIII (FVIII) protein extravascular to a subject having a blood coagulation disorder, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

In a second aspect, the present invention pertains to a pharmaceutical composition for use in the treatment or prophylaxis of a blood coagulation disorder, the composition comprising

(i) the recombinant polypeptide of the invention comprising a truncated von Willebrand Factor (VWF), and

(ii) a Factor VIII protein (FVIII),

wherein the molar ratio of the recombinant polypeptide to the FVIII protein within the pharmaceutical composition is greater than 50 and wherein said recombinant polypeptide is capable of binding to said FVIII, said treatment comprising administering the pharmaceutical composition extravascular to a subject having a blood coagulation disorder, and said pharmaceutical composition is formulated for extravascular co-administration.

In a third aspect, the present invention pertains to a pharmaceutical kit comprising (i) a first composition comprising a Factor VIII (FVIII) protein and (ii) a second composition comprising the recombinant polypeptide of the invention comprising a truncated von Willebrand Factor (VWF) for use in the treatment or prophylaxis of a blood coagulation disorder as presented herein, said treatment comprising administering the recombinant polypeptide and the FVIII protein extravascular to a subject, wherein said FVIII and said recombinant polypeptide are provided within the kit in order to allow prior to administration for at least a proportion of said recombinant polypeptide to bind to said FVIII, and provided that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

According to a fourth aspect, the present invention pertains to a method of treatment or prophylaxis of a blood coagulation disorder, the method comprising co-administering an effective amount of the recombinant polypeptide of the invention comprising a truncated von Willebrand Factor (VWF) and a Factor VIII (FVIII) protein extravascular to a subject, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

In a fifth aspect, the present invention relates to the use of the recombinant polypeptide according to the invention for the treatment or prophylaxis of a blood coagulation disorder, said recombinant polypeptide comprising a truncated von Willebrand Factor (VWF), said treatment comprising administering the polypeptide and a Factor VIII (FVIII) protein extravascular to a subject, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

According to a further aspect, the present invention pertains to the use of the recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) for the manufacture of a medicament for the treatment or prophylaxis of a blood coagulation disorder, said treatment comprising administering the polypeptide and a Factor VIII (FVIII) protein extravascular to a subject, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

The polypeptide comprising a truncated von Willebrand Factor (VWF) will be referred to herein as “polypeptide of the invention” or “recombinant polypeptide”. The polypeptide of the invention preferably comprises a half-life extending moiety (HELM).

Ratios

As described in more detail below, the polypeptide of the invention may be a monomer, a dimer, or a mixture thereof. Any molar ratios according to the invention refer to a ratio of the molar concentration of the monomeric subunit of the polypeptide of the invention, whether actually present as monomer or dimer. Ratios are formed over the molar concentration of the co-administered FVIII. Any ratios of the polypeptide of the invention over FVIII in this application refer to the amount of monomers comprised in the polypeptide of the invention, which is preferably present as a dimer, to be administered (in mole) divided by the amount of FVIII to be administered (in mole), unless indicated otherwise. By way of non-limiting example the co-administration of 100 µM of a monomeric polypeptide of the invention with 1 µM of FVIII means a ratio of 100. The same ratio of 100 is obtained if 50 µM of a dimeric polypeptide of the invention are co-administered with 1 µM of FVIII.

The molar ratio of the polypeptide of the invention to be administered to FVIII to be administered is above 50, more preferably the ratio is greater than 60, or at least 75, at least 100, or greater than 100, or at least 200, most preferably at least 300, or at least 400, or at least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1,000, or at least 1,100, or at least 1,200, or at least 1,300, or at least 1,400, or at least 1,500, or at least 1,600, or at least 1,700, or at least 1,800, or at least 1,900, or at least 2,000, or at least 2,500, or at least 3,000 or at least 5,000, or at least 8,000 or up to 10,000. The molar ratio of the polypeptide of the invention to be administered to FVIII to be administered may according to certain embodiments not exceed a ratio of 10,000, a ratio of 5,000, a ratio of 2,500 or a ratio of 2,000.

The molar ratio of the polypeptide of the invention to be administered to FVIII to be administered may range from above 50 to 10,000, or from above 50 to 5,000, or from above 50 to 4,000, or from above 50 to 3,000, or from above 50 to 2,000, or from above 50 to 1,000. Preferably, the molar ratio of the polypeptide of the invention to be administered to FVIII to be administered ranges from 60 to 2,500, or from 110 to 2,000, or from 150 to 1,500, or from 200 to 1,000.

Normal human plasma (NHP) contains VWF in a concentration of 1 U/mL or 100% by definition. This corresponds to a protein concentration of approximately 10 µg/mL (Haberichter S.L. and Montgomery R.R., Structure and function of von Willebrand factor; in: Hemostasis and Thrombosis, eds. Marder, Aird, Bennett, Schulman and White, Lippincott Williams & Wilkins

2013, pp 197-207). Based on this VWF concentration in NHP and a molecular weight of the mature VWF monomer of approximately 267,500 Da including 18-19% of glycosylation a molar plasma concentration of the VWF monomer unit of approximately 37 x 10^{-9} Mol/L can be calculated for NHP. The half-life of endogenous VWF in human plasma is about 16h (Lenting PJ, Christophe OD, Denis CV. von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends. Blood. 2015.125(13):2019-28).

Further details of the treatment in accordance with the invention are described further below.

The truncated VWF

The term "von Willebrand Factor" (VWF) as used herein includes naturally occurring (native) VWF, but also variants thereof retaining at least the FVIII binding activity of naturally occurring VWF, e.g. sequence variants where one or more residues have been inserted, deleted or substituted. The FVIII binding activity is determined by a FVIII-VWF binding assay as described in Example 2.

A preferred VWF in accordance with this invention is human VWF represented by the amino acid sequence shown in SEQ ID NO:4. The cDNA encoding SEQ ID NO:4 is shown in SEQ ID NO:3.

The gene encoding human native VWF is transcribed into a 9 kb mRNA which is translated into a pre-propolypeptide of 2813 amino acids with an estimated molecular weight of 310,000 Da. The pre-propolypeptide contains an N-terminal 22 amino acids signal peptide, followed by a 741 amino acid pro-polypeptide (amino acids 23-763 of SEQ ID NO:4) and the mature subunit (amino acids 764-2813 of SEQ ID NO:4). Cleavage of the 741 amino acids propolypeptide from the N-terminus results in mature VWF consisting of 2050 amino acids. The amino acid sequence of the human native VWF pre-propolypeptide is shown in SEQ ID NO:4. Unless indicated otherwise, the amino acid numbering of VWF residues in this application refers to SEQ ID NO:4, even if the VWF molecule, in particular a truncated VWF, does not comprise all residues of SEQ ID NO:4.

The propolypeptide of native VWF comprises multiple domains. Different domain annotations can be found in the literature (see, e.g. Zhou et al. (2012) Blood 120(2): 449-458). The following domain annotation of native pre-propolypeptide of VWF is applied in this application:

D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK

With reference to SEQ ID NO:4, the D' domain consists of amino acids 764-865; and the D3 domain consists of amino acids 866-1242.

The feature "truncated" in terms of present invention means that the polypeptide does not comprise the entire amino acid sequence of mature VWF (amino acids 764-2813 of SEQ ID NO:4). Typically, the truncated VWF does not comprise all amino acids 764-2813 of SEQ ID NO:4 but only a fragment thereof. A truncated VWF may also be referred to as a VWF fragment, or in the plural as VWF fragments.

Typically, the truncated VWF is capable of binding to a Factor VIII. Preferably, the truncated VWF is capable of binding to the mature form of human native Factor VIII. In another embodiment, the truncated VWF is capable of binding to a recombinant FVIII, preferably to a FVIII as described herein, further preferred to a the single-chain Factor VIII consisting of the amino acid sequence SEQ ID NO:5. Binding of the truncated VWF to Factor VIII can be determined by a FVIII-VWF binding assay as described in Example 2.

The truncated VWF of the present invention preferably comprises or consists of an amino acid sequence having a sequence identity of at least 90% to amino acids 776 to 805 of SEQ ID NO:4 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 776 to 805 of SEQ ID NO:4 and is capable of binding to FVIII. Most preferably, the truncated VWF comprises or consists of amino acids 776 to 805 of SEQ ID NO:4. Unless indicated otherwise herein, sequence identities are determined over the entire length of the reference sequence (e.g. amino acids 776 to 805 of SEQ ID NO:4).

The truncated VWF of the present invention preferably comprises or consists of an amino acid sequence having a sequence identity of at least 90% to amino acids 766 to 864 of SEQ ID NO:4 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 766 to 864 of SEQ ID NO:4 and is capable of binding to FVIII. Most preferably, the truncated VWF comprises or consists of amino acids 766 to 864 of SEQ ID NO:4.

In another preferred embodiment, the truncated VWF consists of (a) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. More preferably, the truncated VWF consists of (a) an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 764 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Most preferably, the truncated VWF consists of (a) amino acids 764 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII.

As described in more detail below, the polypeptide of the invention may be prepared by a method which uses cells comprising a nucleic acid encoding the polypeptide comprising the truncated VWF. The nucleic acid is introduced into suitable host cells by techniques that are known *per se*.

In a preferred embodiment, the nucleic acid in the host cell encodes (a) an amino acid sequence having a sequence identity of at least 90% to amino acids 1 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated mature VWF is still capable of binding to FVIII. More preferably, the nucleic acid encodes (a) an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 1 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Most preferably, the nucleic acid encodes (a) amino acids 1 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Especially if the polypeptide in accordance with this invention is a dimer, the nucleic acid will comprise a sequence encoding amino acids 1 to 763 of VWF (e.g. SEQ ID NO:4), even if the truncated VWF in the polypeptide does not comprise amino acids 1 to 763 of VWF (e.g. SEQ ID NO:4).

The truncated VWF of the recombinant polypeptide of the invention according to a preferred embodiment may not comprise amino acid sequence 1 to 763 of VWF of SEQ ID NO:4.

According to further preferred embodiments, the truncated VWF comprises or consists of one of the following amino acid sequences, each referring to SEQ ID NO:4:
776-805; 766-805; 764-805; 776-810; 766-810; 764-810; 776-815; 766-815; 764-815;
776-820; 766-820; 764-820; 776-825; 766-825; 764-825; 776-830; 766-830; 764-830;
776-835; 766-835; 764-835; 776-840; 766-840; 764-840; 776-845; 766-845; 764-845;
776-850; 766-850; 764-850; 776-855; 766-855; 764-855; 776-860; 766-860; 764-860;
776-864; 766-864; 764-864; 776-865; 766-865; 764-865; 776-870; 766-870; 764-870;
776-875; 766-875; 764-875; 776-880; 766-880; 764-880; 776-885; 766-885; 764-885;
776-890; 766-890; 764-890; 776-895; 766-895; 764-895; 776-900; 766-900; 764-900;
776-905; 766-905; 764-905; 776-910; 766-910; 764-910; 776-915; 766-915; 764-915;
776-920; 766-920; 764-920; 776-925; 766-925; 764-925; 776-930; 766-930; 764-930;
776-935; 766-935; 764-935; 776-940; 766-940; 764-940; 776-945; 766-945; 764-945;

776-950; 766-950; 764-950; 776-955; 766-955; 764-955; 776-960; 766-960; 764-960; 776-965; 766-965; 764-965; 776-970; 766-970; 764-970; 776-975; 766-975; 764-975; 776-980; 766-980; 764-980; 776-985; 766-985; 764-985; 776-990; 766-990; 764-990; 776-995; 766-995; 764-995; 776-1000; 766-1000; 764-1000; 776-1005; 766-1005; 764-1005; 776-1010; 766-1010; 764-1010; 776-1015; 766-1015; 764-1015; 776-1020; 766-1020; 764-1020; 776-1025; 766-1025; 764-1025; 776-1030; 766-1030; 764-1030; 776-1035; 766-1035; 764-1035; 776-1040; 766-1040; 764-1040; 776-1045; 766-1045; 764-1045; 776-1050; 766-1050; 764-1050; 776-1055; 766-1055; 764-1055; 776-1060; 766-1060; 764-1060; 776-1065; 766-1065; 764-1065; 776-1070; 766-1070; 764-1070; 776-1075; 766-1075; 764-1075; 776-1080; 766-1080; 764-1080; 776-1085; 766-1085; 764-1085; 776-1090; 766-1090; 764-1090; 776-1095; 766-1095; 764-1095; 776-1100; 766-1100; 764-1100; 776-1105; 766-1105; 764-1105; 776-1110; 766-1110; 764-1110; 776-1115; 766-1115; 764-1115; 776-1120; 766-1120; 764-1120; 776-1125; 766-1125; 764-1125; 776-1130; 766-1130; 764-1130; 776-1135; 766-1135; 764-1135; 776-1140; 766-1140; 764-1140; 776-1145; 766-1145; 764-1145; 776-1150; 766-1150; 764-1150; 776-1155; 766-1155; 764-1155; 776-1160; 766-1160; 764-1160; 776-1165; 766-1165; 764-1165; 776-1170; 766-1170; 764-1170; 776-1175; 766-1175; 764-1175; 776-1180; 766-1180; 764-1180; 776-1185; 766-1185; 764-1185; 776-1190; 766-1190; 764-1190; 776-1195; 766-1195; 764-1195; 776-1200; 766-1200; 764-1200; 776-1205; 766-1205; 764-1205; 776-1210; 766-1210; 764-1210; 776-1215; 766-1215; 764-1215; 776-1220; 766-1220; 764-1220; 776-1225; 766-1225; 764-1225; 776-1230; 766-1230; 764-1230; 776-1235; 766-1235; 764-1235; 776-1240; 766-1240; 764-1240; 776-1242; 766-1242; 764-1242; 764-1464; 764-1250; 764-1041; 764-828; 764-865; 764-1045; 764-1035; 764-1128; 764-1198; 764-1268; 764-1261; 764-1264; 764-1459; 764-1463; 764-1464; 764-1683; 764-1873; 764-1482; 764-1479; 764-1672; and 764-1874.

In certain embodiments the truncated VWF has an internal deletion relative to mature wild type VWF. For example, the A1, A2, A3, D4, C1, C2, C3, C4, C5, C6, CK domains or combinations thereof may be deleted, and the D' domain and/or the D3 domain is retained. According to further embodiments, the truncated VWF lacks one or more of the domains A1, A2, A3, D4, C1, C2, C3, C4, C5, C6 or CK. According to further embodiments, the truncated VWF lacks amino acids 1243 to 2813 of SEQ ID NO:4, i.e. the domains A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK.

In further embodiments the truncated VWF does not comprise the binding sites for platelet glycoprotein Ibα (GPIbα), collagen and/or integrin αIIbβIII (RGDS sequence within the C1 domain). In other embodiments, the truncated VWF does not comprise the cleavage site (Tyr1605-Met1606) for ADAMTS13 which is located at the central A2 domain of VWF. In yet another embodiment, the truncated VWF does not comprise the binding sites for GPIbα, and/or does not comprise the binding site for collagen, and/or does not comprise the binding site for integrin αIIbβIII, and/or it does not comprise the cleavage site (Tyr1605-Met1606) for ADAMTS13 which is located at the central A2 domain of VWF.

In other embodiments the truncated VWF comprises or consists of an amino acid sequence that has a sequence identity of at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, to one of the amino acid sequences recited in the preceding paragraph, provided that the truncated VWF is capable of binding to FVIII.

A polypeptide of the invention is termed a "dimer" in the present invention if two monomers of the polypeptide of the invention are linked covalently. Preferably, the covalent bond is located within the truncated VWF portion of the polypeptide of the invention. Preferably, the two monomeric subunits are covalently linked via at least one disulfide bridge, e.g. by one, two, three or four disulfide bridges. The cysteine residues forming the at least one disulfide bridge are preferably located within the truncated VWF portion of the polypeptide of the invention. In one embodiment, these cysteine residues are Cys-1099, Cys-1142, Cys-1222, Cys-1225, or Cys-1227 or combinations thereof. Preferably, the dimeric polypeptide of the invention does not comprise any further covalent bond linking the monomers in addition to said covalent bond located within the truncated VWF portion of the polypeptide, in particular does not comprise any further covalent bond located within the HLEM or HLEP portion of the polypeptide. According to alternative embodiments, however, the dimeric polypeptide of the invention may comprise a covalent bond located in the HLEM or HLEP portion of the polypeptide linking the monomers.

The dimer is preferably a homo-dimer, whereby each monomer comprises preferably a HLEM as disclosed herein. If the polypeptide of the invention is a dimer, the truncated VWF preferably comprises or consists of two polypeptides each with an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:4 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:4 and is capable of binding to FVIII. Most preferably, the truncated VWF comprises or consists of amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, amino acids 764 to 1227 or amino acids 764 to 1242 of SEQ ID NO:4.

The truncated VWF may be any one of the VWF fragments disclosed in WO 2013/106787 A1, WO 2014/198699 A2, WO 2011/060242 A2 or WO 2013/093760 A2, the disclosure of which is incorporated herein by reference.

According to further preferred embodiments the truncated VWF as disclosed above may comprise at least one of the amino acid substitutions as disclosed in WO 2016/000039 A1. Those modified versions of the truncated VWF comprise at least one amino acid substitution within its D' domain, as compared to the amino acid sequence of the D' domain of wild-type VWF according to SEQ ID NO: 4. The amino acid sequence of the modified versions of the truncated VWF can have one or more amino acid substitutions relative to the respective wild type sequence. The amino acid sequence of the D' domain of the modified truncated VWF preferably has one or 2 amino acid substitutions relative to the D' domain of SEQ ID NO:4. It is preferred that S at position 764 of SEQ ID NO:4, corresponding to position 1 of SEQ ID NO:2, is substituted with an amino acid selected from the group consisting of G, P, V, E, Y, A and L. It is also preferred that S at position 766 of SEQ ID NO:4, corresponding to position 3 of SEQ ID NO:2 is substituted with an amino acid selected from the group consisting of Y, I, M, V, F, H, R and W. Preferred combinations of substitutions include S764G/S766Y, S764P/S766I, S764P/S766M, S764V/S766Y, S764E/S766Y, S764Y/S766Y, S764L/S766Y, S764P/S766W, S766W/S806A, S766Y/P769K, S766Y/P769N, S766Y/P769R and S764P/S766L, referring to the sequence of SEQ ID NO:4. The binding affinity of the polypeptide of the present invention to FVIII may be further increased by introduction of said substitutions compared to the binding affinity of a reference polypeptide which has the same amino acid sequence except for said modifications. Said substitutions within the truncated VWF may contribute to increase the half-life of co-administered FVIII.

The term “endogenous VWF” as used herein refers to monomeric subunits of VWF, independent of its degree of multimerization.

Half-life extending moiety (HLEM)

In addition to the truncated VWF, the polypeptide of the invention may in certain preferred embodiments further comprise a half-life extending moiety. The half-life-extending moiety may be a heterologous amino acid sequence fused to the truncated VWF. Alternatively, the half-life-extending moiety may be chemically conjugated to the polypeptide comprising the truncated VWF by a covalent bond different from a peptide bond.

In certain embodiments of the invention, the half-life of the polypeptide of the invention is extended by chemical modification, e.g. attachment of a half-life extending moiety such as polyethylene glycol (PEGylation), glycosylated PEG, hydroxyl ethyl starch (HESylation), polysialic acids, elastin-like polypeptides, heparosan polymers or hyaluronic acid. In another embodiment, the polypeptide of the invention is conjugated to a HLEM such as albumin via a chemical linker. The principle of this conjugation technology has been described in an exemplary manner by Conjuchem LLC (see, e.g., US patent No. 7,256,253).

In other embodiments, the half-life-extending moiety is a half-life enhancing protein (HLEP). Preferably, the HLEP is an albumin or a fragment thereof. The N-terminus of the albumin may be fused to the C-terminus of the truncated VWF. Alternatively, the C-terminus of the albumin may be fused to the N-terminus of the truncated VWF. One or more HLEPs may be fused to the N- or C-terminal part of VWF provided that they do not to interfere with or abolish the binding capability of the truncated VWF to FVIII.

The recombinant polypeptide further comprises preferably a chemical bond or a linker sequence positioned between the truncated VWF and the HLEM.

Said linker sequence may be a peptidic linker consisting of one or more amino acids, in particular of 1 to 50, 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5 or 1 to 3 (e.g. 1 , 2 or 3) amino acids and which may be equal or different from each other. Preferably, the linker sequence is not present at the corresponding position in the wild-type VWF. Preferred amino acids present in said linker sequence include Gly and Ser. The linker sequence should be non-immunogenic. Preferred linkers may be comprised of alternating glycine and serine residues. Suitable linkers are described for example in WO2007/090584.

In another embodiment of the invention the peptidic linker between the truncated VWF moiety and the HLEM consists of peptide sequences, which serve as natural interdomain linkers in human proteins. Preferably such peptide sequences in their natural environment are located close to the protein surface and are accessible to the immune system so that one can assume a natural tolerance against this sequence. Examples are given in WO 2007/090584. Cleavable linker sequences are described, e.g., in WO 2013/120939 A1.

In a preferred embodiment of the recombinant polypeptide the linker between the truncated VWF and the HLEM is a glycine/serine peptidic linker having or consisting of amino acid sequence 480 – 510 of SEQ ID NO:2.

In one embodiment the polypeptide has the following structure:
tVWF - L1 - H, [formula 1]

Wherein tVWF is the truncated VWF, L1 is a chemical bond or a linker sequence, and H is a HLEM, in particular a HLEP.

L1 may be a chemical bond or a linker sequence consisting of one or more amino acids, e.g. of 1 to 50, 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5 or 1 to 3 (e.g. 1 , 2 or 3) amino acids and which may be equal or different from each other. Usually, the linker sequences are not present at the corresponding position in the wild-type VWF. Examples of suitable amino acids present in L1 include Gly and Ser. The linker should be non-immunogenic and may be a non-cleavable or cleavable linker. Non-cleavable linkers may be comprised of alternating glycine and serine residues as exemplified in WO 2007/090584 A1. In another embodiment of the invention the peptidic linker between the truncated VWF moiety and the albumin moiety consists of peptide sequences, which serve as natural interdomain linkers in human proteins. Preferably such peptide sequences in their natural environment are located close to the protein surface and are accessible to the immune system so that one can assume a natural tolerance against this sequence. Examples are given in WO2007/090584. Cleavable linker sequences are described, e.g., in WO 2013/120939 A1.

Preferred HLEP sequences are described infra. Likewise encompassed by the invention are fusions to the exact "N-terminal amino acid" or to the exact "C-terminal amino acid" of the respective HLEP, or fusions to the "N-terminal part" or "C-terminal part" of the respective HLEP, which includes N-terminal deletions of one or more amino acids of the HLEP. The polypeptide may comprise more than one HLEP sequence, e.g. two or three HLEP sequences. These multiple HLEP sequences may be fused to the C-terminal part of VWF in tandem, e.g. as successive repeats.

Half-life enhancing polypeptides (HLEPs)

Preferably, the half-life extending moiety is a half-life extending polypeptide (HLEP). More preferably the HLEP is selected from the group consisting of albumin, a member of the albumin-family or fragments thereof, solvated random chains with large hydrodynamic volume (e.g. XTEN (Schellenberger et al. 2009; Nature Biotechnol. 27:1186-1190), homo-amino acid repeats (HAP) or proline-alanine-serine repeats (PAS), afamin, alpha-fetoprotein, Vitamin D binding protein, transferrin or variants or fragments thereof, carboxyl-terminal peptide (CTP) of human chorionic gonadotropin-ß subunit, a polypeptide capable of binding to the neonatal Fc receptor (FcRn), in particular an immunoglobulin constant region and portions thereof, e.g. the Fc fragment, polypeptides or lipids capable of binding under physiological conditions to albumin, to a member of the albumin-family or to fragments thereof or to an immunoglobulin constant region or portions thereof. The immunoglobulin constant region or portions thereof is preferably an Fc fragment of immunoglobulin G1, an Fc fragment of immunoglobulin G2 or an Fc fragment of immunoglobulin A.

A half-life enhancing polypeptide as used herein may be a full-length half-life-enhancing protein described herein or one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity or the biological activity of the coagulation factor, in particular of increasing the in vivo half-life of the polypeptide of the invention. Such fragments may be of 10 or more amino acids in length or may include at least about 15, at least about 20, at least about 25, at least about 30, at least about 50, at least about 100, or more contiguous amino acids from the HLEP sequence or may include part or all of specific domains of the respective HLEP, as long as the HLEP fragment provides a functional half-life extension of at least 25% compared to the respective polypeptide without the HLEP.

The HLEP portion of the polypeptide of the invention may be a variant of a wild type HLEP. The term "variants" includes insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the FVIII-binding activity of the truncated VWF.

In particular, the proposed truncated VWF-HLEP fusion constructs of the invention may include naturally occurring polymorphic variants of HLEPs and fragments of HLEPs. The HLEP may be derived from any vertebrate, especially any mammal, for example human, monkey, cow, sheep, or pig. Non-mammalian HLEPs include, but are not limited to, hen and salmon.

According to certain embodiments of present disclosure the HLEM, in particular a HLEP, portion of the recombinant polypeptide of the invention may be specified with the alternative term "FP". Preferably, the term "FP" represents a human albumin.

According to certain preferred embodiments, the recombinant polypeptide is a fusion protein. A fusion protein in terms of present invention is a protein created by in-frame joining of at least two DNA sequences encoding the truncated VWF as well as the HLEP. The skilled person understands that translation of the fusion protein DNA sequence will result in a single protein sequence. As a result of an in frame insertion of a DNA sequence encoding a peptidic linker according to a further preferred embodiment, a fusion protein comprising the truncated VWF, a suitable linker and the HELP may be obtained.

According to some embodiments, the co-administered FVIII does neither comprise any of the herein described HLEM or HLEP structures. According to certain other embodiments, the co-administered FVIII may comprise at least one of the herein described HLEM or HLEP structures.

Albumin as HLEP

The terms, "human serum albumin" (HSA) and "human albumin" (HA) and "albumin" (ALB) are used interchangeably in this application. The terms "albumin" and "serum albumin" are broader, and encompass human serum albumin (and fragments and variants thereof) as well as albumin from other species (and fragments and variants thereof).

As used herein, "albumin" refers collectively to albumin polypeptide or amino acid sequence, or an albumin fragment or variant, having one or more functional activities (e.g., biological activities) of albumin. In particular, "albumin" refers to human albumin or fragments thereof, especially the mature form of human albumin as shown in SEQ ID NO:6 herein or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

According to certain embodiments of present disclosure the alternative term "FP" is used to identify the HLEP, in particular to define albumin as HLEP.

In particular, the proposed polypeptides of the invention may include naturally occurring polymorphic variants of human albumin and fragments of human albumin. Generally speaking, an albumin fragment or variant will be at least 10, preferably at least 40, most preferably more than 70 amino acids long.

Preferred embodiments of the invention include albumin variants used as a HLEP of the polypeptide of the invention with enhanced binding to the FcRn receptor. Such albumin variants may lead to a longer plasma half-life of a truncated VWF albumin variant fusion protein as compared to a truncated VWF fusion with a wild-type albumin.

The albumin portion of the polypeptides of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.

Immunoglobulins as HLEPs

Immunoglobulin G (IgG) constant regions (Fc) are known in the art to increase the half-life of therapeutic proteins (Dumont J A et al. 2006. BioDrugs 20:151-160). The IgG constant region of the heavy chain consists of 3 domains (CH1-CH3) and a hinge region. The immunoglobulin sequence may be derived from any mammal, or from subclasses IgG1, IgG2, IgG3 or IgG4, respectively. IgG and IgG fragments without an antigen-binding domain may also be used as HLEPs. The therapeutic polypeptide portion is connected to the IgG or the IgG fragments preferably via the hinge region of the antibody or a peptidic linker, which may even be cleavable. Several patents and patent applications describe the fusion of therapeutic proteins to immunoglobulin constant regions to enhance the therapeutic proteins' in vivo half-lives. US 2004/0087778 and WO 2005/001025 describe fusion proteins of Fc domains or at least portions of immunoglobulin constant regions with biologically active peptides that increase the half-life of the peptide, which otherwise would be quickly eliminated in vivo. Fc-IFN-β fusion proteins were described that achieved enhanced biological activity, prolonged circulating half-life and greater solubility (WO 2006/000448 A2). Fc-EPO proteins with a prolonged serum half-life and increased in vivo potency were disclosed (WO 2005/063808 A1) as well as Fc fusions with G-CSF (WO 2003/076567 A2), glucagon-like peptide-1 (WO 2005/000892 A2), clotting factors (WO 2004/101740 A2) and interleukin-10 (U.S. Pat. No. 6,403,077), all with half-life enhancing properties.

Various HLEPs which can be used in accordance with this invention are described in detail in WO 2013/120939 A1.

N-Glycans and Sialylation of the polypeptide of the invention

The polypeptide of the invention preferably comprises N-glycans, and at least 75%, preferably at least 85%, more preferably at least 90% of said N-glycans comprise, on average, at least one sialic acid moiety. In preferred embodiments, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of said N-glycans comprise, on average, at least one sialic acid moiety. The inventors found that polypeptides comprising highly sialylated VWF fragments not only may have a further prolonged half-life themselves, but may also be capable to extend the half-life of co-administered FVIII further. In other words, administration of the polypeptide of the invention leads to an extended half-life and/or to a reduced clearance of co-administered FVIII.

The polypeptide of the invention preferably comprises N-glycans, and at least 50% of the sialyl groups of the N-glycans of the glycoproteins are α-2,6-linked sialyl groups. In general, terminal sialyl groups can be attached to the galactose groups via a α-2,3- or via a α-2,6-linkage. Typically, N-glycans of the polypeptide of the invention comprise more α-2,6-linked sialyl groups than α-2,3-linked sialyl groups. Preferably, at least 60%, or at least 70%, or at least 80%, or at least 90% of the sialyl groups of the N-glycans are α-2,6-linked sialyl groups. These embodiments can be obtained by, e.g., co-expressing human α-2,6-sialyltransferase in mammalian cells.

Suitable methods of producing such glycoproteins are described in pending PCT/EP2016/061440. Accordingly, a method of producing a glycoprotein comprising N-glycans with increased sialylation is described therein, which method comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF), and (ii) culturing said cells at a temperature of less than 36.0°C. In addition, a method of producing a dimer of a glycoprotein comprising a truncated von Willebrand Factor (VWF), or for increasing the dimerization of said glycoprotein is described, which method comprises (i) providing cells comprising a nucleic acid encoding the amino acid sequence of the glycoprotein, and (ii) culturing said cells at a temperature of less than 36.0°C. Further, a method of producing a glycoprotein comprising N-glycans with increased sialylation is described therein, which comprises (i) providing cells comprising a nucleic acid encoding a polypeptide comprising a truncated von Willebrand Factor (VWF) and a recombinant nucleic acid encoding an α-2,6-sialyltransferase, and (ii) culturing the cells under conditions that allow expression of the glycoprotein and of the α-2,6-sialyltransferase.

In one embodiment, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of the N-glycans of the polypeptide of the invention comprise at least one sialic acid group. In another embodiment, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of the N-glycans of the polypeptide of the invention comprise at least one sialic acid group.

In another embodiment, less than 15%, less than 12%, less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the polypeptide of the invention are asialo-N-glycans, i.e. they are N-glycans lacking a sialic acid group. In another embodiment, less than 15%, less than 12%, less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the polypeptide of the invention are asialo-N-glycans, i.e. they do not have a sialic acid group.

Other embodiments of the invention comprise a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein comprises N-glycans, wherein less than 35%, preferably less than 34%, preferably less than 33%, preferably less than 32%, preferably less than 31%, preferably less than 30%, preferably less than 29%, preferably less than 28%, preferably less than 27% preferably less than 26%, preferably less than 25%, preferably less than 24%, preferably less than 23%, preferably less than 22%, preferably less than 21%, preferably less than 20%, preferably less than 19%, preferably less than 18%, preferably less than 17%, preferably less than 16%, preferably less than 15%, preferably less than 14%, preferably less than 13%, preferably less than 12%, preferably less than 11%, preferably less than 10%, preferably less than 9%, preferably less than 8%, preferably less than 7%, preferably less than 6% and preferably less than 5% of said N-glycans comprise, on average, two or more terminal and non-sialylated galactose residues.

Still other embodiments of the invention comprise a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said truncated VWF comprises N-glycans, wherein less than 6%, preferably less than 5%, preferably less than 4%, preferably less than 3%, preferably less than 2%, and preferably less than 1% of said N-glycans comprise, on average, three or more terminal and non-sialylated galactose residues.

The above-described embodiments can be combined with each other. Any percentages of N-glycans mentioned above, or any indications of the degree of sialylation, are to be understood as average percentages or degrees, i.e. they refer to a population of molecules, not to a single molecule. It is clear that the glycosylation or sialylation of the individual glycoprotein molecules within a population of glycoproteins will show some heterogeneity.

Dimers

The polypeptides of this invention have a high proportion of dimers. The polypeptide of the invention is therefore preferably present as dimer. In one embodiment, at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% or about 100% of the polypeptides are present as dimers. In another embodiment, the ratio dimer : monomer of the polypeptide of the invention is at least 1.5, preferably at least 2, more preferably at least 2.5 or at least 3. Most preferably all polypeptides of the invention are present as dimers. Further preferred is that the polypeptide of the invention does not comprise multimeric forms. The use of dimers is favorable, as the dimer has an improved affinity to Factor VIII as compared to the monomer. The dimer content and the ratio of dimer to monomer of the polypeptide of the invention can be determined as described in Example 2.

In one embodiment, the affinity of the polypeptide of the invention to Factor VIII is greater than that of human native VWF to the same Factor VIII molecule. The Factor VIII affinity of the polypeptide may refer to human native, either plasma-derived or recombinant, Factor VIII, in particular to a recombinant Factor VIII molecule having a truncated o deleted B-domain, preferably a Factor VIII molecule as characterized by SEQ ID NO:5.

It has been found that preparations of the polypeptide of this invention with a high proportion of dimers do have an increased affinity to Factor VIII. Such increased affinity to Factor VIII does lead to an enhanced stabilization of Factor VIII by the polypeptides of the present invention. Alternatively to or in combination with an increased dimer proportion also polypeptides in accordance with the invention with mutations within the Factor VIII binding domain which do increase the affinity to Factor VIII are preferred embodiments of the invention. Suitable mutations are disclosed, e.g., in WO 2013/120939 A1.

Preparation of the polypeptide

The nucleic acid encoding the polypeptide of the invention can be prepared according to methods known in the art. Based on the cDNA sequence of pre-pro form of human native VWF (SEQ ID NO:3), recombinant DNA encoding the above-mentioned truncated VWF constructs or polypeptides of the invention can be designed and generated.

Even if the polypeptide which is secreted by the host cells does not comprise amino acids 1 to 763 of pre-pro form of human native VWF, it is preferred that the nucleic acid (e.g. the DNA) encoding the intracellular precursor of the polypeptide comprises a nucleotide sequence encoding an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 23 to 763 or preferably to amino acids 1 to 763 of SEQ ID NO:4. Most preferably, the nucleic acid (e.g. the DNA) encoding the intracellular precursor of the polypeptide comprises a nucleotide sequence encoding amino acids 23 to 763 of SEQ ID NO:4, or amino acids 1 to 763 of SEQ ID NO:4.

Constructs in which the DNA contains the entire open reading frame inserted in the correct orientation into an expression plasmid may be used for protein expression. Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the inserted nucleic acid in the plasmid-bearing cells. They may also include an origin of replication sequence allowing for their autonomous replication within the host organism, and sequences that increase the efficiency with which the synthesized mRNA is translated. Stable long-term vectors may be maintained as freely replicating entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines may also be produced that have integrated the vector into the genomic DNA, and in this manner the gene product is produced on a continuous basis.

Typically, the cells to be provided are obtained by introducing the nucleic acid encoding a polypeptide of the invention into mammalian host cells.

Any host cell susceptible to cell culture, and to expression of glycoproteins, may be utilized in accordance with the present invention. In certain embodiments, a host cell is mammalian. Non-limiting examples of mammalian cells that may be used in accordance with the present invention include BALB/c mouse myeloma line (NSO/ 1, ECACC No: 85110503); human retinoblasts (PER.C6 (CruCell, Leiden, The Netherlands)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59, 1977); baby hamster kidney cells (BHK, ATCC CCL10); Chinese hamster ovary cells +/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216, 1980); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243 251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1 587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (HepG2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals NY. Acad. Sci., 383:44-68, 1982); MRC 5 cells; PS4 cells; human amniocyte cells (CAP); and a human hepatoma line (Hep G2). Preferably, the cell line is a rodent cell line, especially a hamster cell line such as CHO or BHK.

Methods suitable for introducing nucleic acids sufficient to achieve expression of a glycoprotein of interest into mammalian host cells are known in the art. See, for example, Gething et al., Nature, 293:620-625, 1981; Mantei et al., Nature, 281:40-46, 1979; Levinson et al. EP 117,060; and EP 117,058. For mammalian cells, common methods of introducing genetic material into mammalian cells include the calcium phosphate precipitation method of Graham and van der Erb (Virology, 52:456-457, 1978) or the lipofectamine™ (Gibco BRL) Method of Hawley-Nelson (Focus 15:73, 1993). General aspects of mammalian cell host system transformations have been described by Axel in US. Pat. No. 4,399,216. For various techniques for introducing genetic material into mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537, 1990, and Mansour et al., Nature, 336:348-352, 1988.

The cells are cultured under conditions that allow expression of the polypeptide. The polypeptide can be recovered and purified using methods that are known to the skilled artisan.

Maximal concentration, area under the time-concentration curve, terminal half-life, MRT, clearance and bioavailability

Another aspect of the invention is the use of a polypeptide comprising a truncated VWF as defined hereinabove for providing or increasing bioavailability of FVIII after extravascular administration. Additionally, an aspect of the invention is its use for increasing the C_{max}, AUC, terminal half-life and/or mean residence time (MRT) and/or reducing the clearance of Factor VIII as compared to a reference treatment being identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention, in particular below a molar ratio of 50, below 60, below 75, below 100, below 200, below 300, below 400 or below 1000.

For evaluation of the pharmacokinetic data a two-compartment model (biphasic pharmacokinetic profile) was applied.

The maximal concentration (C_{max}) is the highest plasma concentration given by the model. Following co-administration of said recombinant polypeptide with FVIII the maximal concentration (C_{max}) for FVIII may be at least 10 IU/mL, at least 25 mIU/mL, at least 50 mIU/mL, at least 100 mIU/mL, at least 200 mIU/mL, at least 300 mIU/mL or at least 400 mIU/mL FVIII activity, preferably chromogenic FVIII activity.

Following co-administration of the recombinant polypeptide with FVIII the maximal concentration (C_{max}) for the recombinant polypeptide is according to certain embodiments at least 20 nmol/kg, at least 40 nmol/kg, at least 60 nmol/kg, at least 80 nmol/kg or at least 160 nmol/kg. Preferably, following co-administration of said recombinant polypeptide with FVIII the maximal concentration (C_{max}) for the recombinant polypeptide is at least 5 µg HLEM/mL, 10 µg HLEM/mL, at least 15 µg HLEM/mL, or at least 20 µg HLEM /mL, whereby the values are based on a calculation for the HLEM, preferably, the values are based on a quantitation using a HLEM specific assay such as an immunoassay, preferably specific for human albumin. The maximal concentration (C_{max}) for the recombinant polypeptide may be at least 3 fold higher as compared to a reference treatment, wherein said reference treatment is identical to the treatment according to the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

The $AUC_{0\text{-}inf}$ is the area under the plasma concentration-time curve from zero to infinity. Following co-administration of the recombinant polypeptide with FVIII the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$) for the co-administered FVIII may be at least 1000 mIU*h/mL, at least 2000 mIU*h/mL, at least 3000 mIU*h/mL, at least 5000 mIU*h/mL, at least 10000 mIU*h/mL or at least 20000 mIU*h/mL FVIII activity, preferably chromogenic FVIII activity.

Following co-administration of the recombinant polypeptide with FVIII the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$) for the co-administered recombinant polypeptide is at least 2 nmol * h/mL, at least 3 nmol * h/mL, at least 4 nmol * h/mL, at least 20 nmol * h/mL, at least 40 nmol * h/mL or at least 80 nmol * h/mL. Preferably, following co-administration of the recombinant polypeptide with FVIII the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$) for the co-administered recombinant polypeptide may be at least 500 µg HLEM *h/mL, at least 750 µg HLEM *h/mL, at least 1000 µg HLEM *h/mL at least 5000 µg HLEM*h/mL, or at least 10000 µg HLEM *h/m, whereby the values are based on a calculation for the HLEM, preferably, the values are based on a quantitation using a HLEM specific assay such as an immunoassay, preferably specific for human albumin.

Following co-administration of the recombinant polypeptide with FVIII the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$) for the co-administered recombinant polypeptide may be at least 5, is at least 10 or is at least 15 fold higher as compared to a reference treatment, wherein said reference treatment is identical to a treatment according to the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

The "half-life" T1/2(t) at a certain time t is the time it takes to halve the plasma concentration C(t) that is present at time t. The "terminal half-life" (in the latter text abbreviated as $t_{1/2}$) is the limit of T1/2(t) when t tends to infinity. It is calculated by dividing the natural logarithm of 2 by the terminal elimination constant.

The terminal half-life of the administered FVIII may be increased by the co-administration of the recombinant polypeptide, preferably by a factor of at least 1.2, at least 1.5, at least 2, at least 2.5, or at least 3, as compared to a reference treatment, wherein said reference treatment is identical to the treatment according to the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention. Preferably, the terminal half-life of the co-administered FVIII is increased as compared to a reference treatment, wherein said reference treatment is identical to the treatment according to the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

The terminal half-life of the administered FVIII followed by co-administration of the recombinant polypeptide, may amount to at least 5h, at least 6h, at least 7h, at least 9h, at least 10h or at least 15h.

The plasma half-life of the polypeptide of the invention may be increased compared to that of endogenous VWF, wherein the plasma half-life of the polypeptide is preferably at least 100%, at least 200% or preferably at least 400% higher than that of the endogenous VWF.

The terminal half-life of the recombinant polypeptide followed by co-administration with FVIII, may amount to at least 10 h, at least, 15 h, at least 20 h, at least 25 h, at least 30 h or at least 35 h. The terminal half-life of the recombinant polypeptide may be increased as compared to a reference treatment, wherein said reference treatment is identical to the treatment according to the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

The term “MRT”, as used herein, means the average time a drug molecule (e.g. the polypeptide of the invention or a FVIII) resides in the body. In a pharmacokinetic system with constant clearance MRT can be calculated as the area under the first moment curve ($AUMC_{0\text{-}inf}$) divided by the $AUC_{0\text{-}inf}$. The first moment curve is time multiplied by plasma concentration at that time. $AUMC_{0\text{-}inf}$ is calculated analog to $AUC_{0\text{-}inf}$.

The mean residence time (MRT) of the administered FVIII is increased by the co-administration of the recombinant polypeptide, preferably by a factor of at least 1.5, at least 2, at least 3, at least 4 or at least 5, as compared to a reference treatment, wherein said reference treatment is identical to the treatment according to the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

The MRT of the administered FVIII may amount to at least 10 h, preferably at least 15 h, at least 20 h or at least 25 h.

The MRT of the administered recombinant polypeptide may be increased, preferably by a factor of at least 1.5, at least 2 or at least 3, as compared to a reference treatment, wherein said reference treatment is identical to a treatment of the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

The term “clearance”, as used herein, refers to the rate at which plasma is cleared of drug. Specifically, it is the current elimination rate of a drug divided by its current plasma concentration. In a pharmacokinetic system after a single intravenous administration the clearance can be calculated as the ratio of dose over the $AUC_{0\text{-}inf}$, provided the clearance is constant. The lower the clearance the longer it takes until the plasma is cleared of the drug.

Following co-administration of the recombinant polypeptide with FVIII the clearance (CL) value of the administered FVIII is reduced compared to a reference treatment, preferably by a factor of at least 1.5, at least 2, at least 3, at least 5, at least 7.5 or at least 10, wherein said reference treatment is identical to a treatment of the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

Preferably, following co-administration of the recombinant polypeptide with FVIII the clearance (CL) value of the administered FVIII is below 135 mL/kg/h, below 80 mL/kg/h, below 45 mL/kg/h, below 40 mL/kg/h, below 35 mL/kg/h, below 30 mL/kg/h or below 25 mL/kg/h. The clearance (CL) value of the administered FVIII is preferably lower than that of a reference treatment, wherein said reference treatment is identical to the treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

Following co-administration of the recombinant polypeptide with FVIII the clearance (CL) value of the recombinant polypeptide may amount to a range between 1.0 to 2.5 mL/kg/h, or between 1.1 to 2.2 mL/kg/h or between 1.2 to 2.1 mL/kg/h.

Following co-administration of said recombinant polypeptide with FVIII the clearance (CL) value for the recombinant polypeptide is reduced by a factor of at least 2, at least 5, or at least 10, as compared to a reference treatment, wherein said reference treatment is identical to the treatment according to the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

The term bioavailability, as used herein, is defined as the percentage of the $AUC_{0\text{-}inf}$ of the polypeptide of the invention, for example rD'D3-FP, after s.c. administration, in relation to the $AUC_{0\text{-}inf}$ of the polypeptide of the invention, for example rD'D3-FP, after i.v. administration.

The invention further relates to the use of a polypeptide as defined hereinabove, e.g. but not limited to embodiments [1] to [66] above, for enabling subcutaneous FVIII administration. The invention in particular further relates to the use of a polypeptide as defined hereinabove for providing or increasing the bioavailability of FVIII.

The bioavailability of the administered FVIII may be increased following co-administration with the recombinant polypeptide by a factor of at least 2, at least 3, at least, 4, at least 5 or at least 10, as compared to a reference treatment, wherein said reference treatment is identical to the treatment according to the invention, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below a molar ratio according to the invention.

The bioavailability of the administered FVIII following co-administration with the recombinant polypeptide is preferably at least 2%, at least 3%, at least 5%, preferably at least 7%, at least 10%, at least 15%, at least 30%, at least 35% or at least 40%. Further preferred ranges for bioavailability of the administered FVIII following co-administration with the recombinant polypeptide are 5-80%, 5-70%, 5-60%, 5-50%, 5-40%, 5-30%, 5-25%, 10-25%, 10-15%, or 5-15%.

The bioavailability of the recombinant polypeptide following co-administration with the FVIII is at least 30%, preferably at least 35%, more preferably at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70% or at least 80%.

A further aspect of this invention is a method of treating a blood coagulation disorder, comprising administering to a patient in need thereof an effective amount of a polypeptide as defined hereinabove.

A further aspect is the use of a polypeptide as defined hereinabove, e.g. by any of but not limited to embodiments [1] to [66] above, for reducing the frequency of administration of FVIII in a treatment of hemophilia A. The frequency of subcutaneous administration of FVIII may be reduced to twice per week. Alternatively, the frequency of subcutaneous administration of FVIII may be reduced to once per week, or even lower, e.g. to once per 10 days or once per 14 days. The FVIII may be administered twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four days to every month, from every 10 days to every two weeks, etc.

The term “trough level” is used herein to define the plasma FVIII concentration at which in a prophylactic setting the next dose of FVIII would be applied. Currently, for patients with severe haemophilia A the recommended trough levels (i.e. the lowest level of coagulation factor present in the body) are set at 1%. Time to 1, 5 and 10 % trough levels is calculated by setting the model equation equal to 0.01, 0.05 or 0.1 IU/mL and solving for time.

Preferably, the time period for reaching a 1%, 5% or 10% trough level of the FVIII co-administered together with the polypeptide having a HLEM is prolonged compared to a reference treatment, wherein said reference treatment is identical to the treatment according to the invention, except the FVIII is administered with a recombinant polypeptide without having said HLEM.

The time period either for reaching the 1% trough level of the FVIII co-administered together with the polypeptide is at least about 30h, at least about 35h, at least about 38h, at least about 40h, or at least about 50h; or for reaching the 5% trough level of the FVIII co-administered together with said polypeptide is at least about 20h, at least about 22h, at least about 29h, at least about 34h, or at least about 43h; or for reaching the 10% trough level of the FVIII co-administered together with said polypeptide is at least about 5h, at least about 6h, at least about 10h, at least about 18h, or at least about 20h.

Treatment of coagulation disorder

The polypeptides of the invention are useful for treating coagulation disorders including hemophilia A and von-Willebrand disease. The term "hemophilia A" refers to a deficiency in functional coagulation FVIII, which is usually inherited. The von-Willebrand disease according to some preferred embodiments is selected from the group consisting of von-Willebrand disease type 2N, von-Willebrand disease type 3 and von-Willebrand disease type 1.

In one embodiment, the blood coagulation disorder is moderate hemophilia A. Moderate hemophilia A is preferably characterized by an endogenous FVIII activity level which is from about 1% to about 5% of the endogenous FVIII activity level in NHP. Typically, subjects having moderate hemophilia A have an endogenous FVIII activity level from 0.01 to 0.05 IU/mL in plasma.

In another embodiment, the blood coagulation disorder is mild hemophilia A. Mild hemophilia A is preferably characterized by an endogenous FVIII activity level which is from about 5% to about 40% of the endogenous FVIII activity level in NHP. Typically, subjects having mild hemophilia A have an endogenous FVIII activity level from 0.05 to 0.4 IU/mL in plasma.

In another embodiment, the blood coagulation disorder is severe hemophilia A, preferably associated with an endogenous FVIII activity level that is below 1% of the endogenous FVIII activity level in NHP.

In another embodiment, the blood coagulation disorder is von-Willebrand disease type 2N. von-Willebrand disease type 2N is preferably characterized by an endogenous FVIII activity level which is from about 3 IU/dL to about 30 IU/dL FVIII activity level corresponding to 3% to about 30% of the endogenous FVIII activity level in NHP. Most of the patients have an endogenous FVIII activity level below 20 IU/dL, thus a level below 20% of the endogenous FVIII activity level in NHP. Thus, subjects having von-Willebrand disease type 2N have an endogenous FVIII activity level from 0.03 IU/mL to 0.3 IU/mL in plasma, typically below 0.2 IU/mL.

In another embodiment, the blood coagulation disorder is von-Willebrand disease type 3, preferably characterized by an endogenous FVIII activity level before treatment which is usually in a range between about 1 IU/dL and about 20 IU/dL FVIII activity level, corresponding to about 1% to about 20% of the endogenous FVIII activity level in NHP. Most of the patients have an endogenous FVIII activity level below 10 IU/dL, thus a level below 10% of the endogenous FVIII activity level in NHP.

According to another embodiment, the blood coagulation disorder is von-Willebrand disease type 1, characterized by an endogenous FVIII activity level before treatment which is reduced compared to the endogenous FVIII activity level in NHP.

Treatment of a disease encompasses the treatment of patients already diagnosed as having any form of the disease at any clinical stage or manifestation; the delay of the onset or evolution or aggravation or deterioration of the symptoms or signs of the disease; and/or preventing and/or reducing the severity of the disease.

A "subject" or "patient" to whom a polypeptide of the invention is administered preferably is a human. In certain aspects, the human is a pediatric patient. In other aspects, the human is an adult patient.

Compositions comprising a polypeptide of the invention and, optionally FVIII, are described herein. The compositions typically are supplied as part of a sterile, pharmaceutical composition that includes a pharmaceutically acceptable carrier. This composition can be in any suitable form (depending upon the desired method of administering it to a patient).

The term "Factor VIII" and "FVIII" or "Factor VIII protein" are used interchangeably herein and encompass both plasma derived FVIII and recombinant FVIII. Recombinant FVIII encompasses without limitation full-length FVIII as well as two-chain B-domain deleted or truncated variants as well as single-chain B-domain deleted or truncated variants for example those described in WO 2004/067566 A1 and other FVIII variants with mutations outside the B-domain but having the biological activity of FVIII.

The polypeptide of the invention can be administered to a patient by a variety of extravascular routes such as subcutaneously, intradermally or intramuscularly. The most suitable route for administration in any given case will depend on the particular polypeptide, the subject, and the nature and severity of the disease and the physical condition of the subject. Preferably, a polypeptide of the invention will be administered subcutaneously.

The polypeptide and the FVIII are preferably co-administered subcutaneously.

Determination of the total number of doses and length of treatment with a polypeptide of the invention and FVIII is well within the capabilities of those skilled in the art. The dosage of the polypeptide of the invention as well as FVIII to be administered depends on the concentrations of the FVIII to be administered, the concentration of endogenous VWF in the patient to be treated, or both. An effective dosage based on the ratios defined by the inventors of this application can be determined by the skilled person, taking into account the molecular weight of the polypeptide of the invention as well as the molecular weight of the FVIII to be administered. The degree of severity of the blood coagulation disorder may also be considered to determine the appropriate dosage of the polypeptide of the invention as well as of FVIII to be administered. Typical dosages for FVIII may range from about 20 IU/kg body weight to about 1000 IU/kg body weight, preferably from about 20 IU/kg body weight to about 500 IU/kg body weight, further preferred from about 20 IU/kg body weight to about 400 IU/kg body weight, more preferred from about 20 IU/kg body weight to about 300 IU/kg body weight.

In accordance with this invention, the patient being treated with the polypeptide of the invention is also treated with blood coagulation Factor VIII. The polypeptide of the invention and the Factor VIII may preferably be administered simultaneously, i.e. together, although an administration in a sequential fashion could in principle also be performed, both modes of administration being encompassed by the term "combination therapy" and "co-administration". The polypeptide of the invention and the Factor VIII may be administered as a mixture, i.e.

within the same composition, or separately, i.e. as separate compositions. Co-administration of the recombinant polypeptide and the FVIII protein is preferably achieved by administration together in a single composition comprising the recombinant polypeptide and the FVIII protein. According to further preferred embodiments, co-administration of the recombinant polypeptide and the FVIII protein is achieved by providing a combination product comprising the recombinant polypeptide and the FVIII blended in a single composition or by providing a set or kit of at least two separate products arranged to be mixed before administration, whereby a first product comprises the recombinant polypeptide and a second product comprises the FVIII.

In particular, in case that the recombinant polypeptide and the FVIII protein are provided in separate compositions or products to be mixed prior to co-administration, the mixture may be treated before administration in such a manner to allow prior to administration for at least a proportion of said recombinant polypeptide to bind to said FVIII. For example, the mixture could be incubated for a certain time. Such incubation could be conducted in less than 1 min, or less than 5 min at either ambient temperature or, if appropriate, at elevated temperature, however, preferably at a temperature below 40°C. Such a quick incubation step may also be appropriate during reconstitution for a combination product comprising the recombinant polypeptide and the FVIII blended in a single composition.

The concentration of Factor VIII in the composition used is typically in the range of 10-10,000 IU/mL. In different embodiments, the concentration of FVIII in the compositions of the invention is in the range of 10-8,000 IU/mL, or 10-5,000 IU/mL, or 20-3,000 IU/mL, or 50-1,500 IU/mL, or 3,000 IU/mL, or 2,500 IU/mL, or 2,000 IU/mL, or 1,500 IU/mL, or 1,200 IU/mL, or 1,000 IU/mL, or 800 IU/mL, or 750 IU/mL, or 600 IU/mL, or 500 IU/mL, or 400 IU/mL, or 300 IU/mL, or 250 IU/mL, or 200 IU/mL, or 150 IU/mL, or 125 IU/mL, or 100 IU/mL, or 62.5 IU/mL, or 50 IU/mL, provided the requirements regarding the ratio with respect to the VWF polypeptide of the invention as defined herein are fulfilled.

"International Unit," or "IU," is a unit of measurement of the blood coagulation activity (potency) of FVIII as measured by a FVIII activity assay such as a one stage clotting assay or a chromogenic substrate FVIII activity assay using a standard calibrated in “IU” against an international standard preparation . One stage clotting assays are known to the art, such as that described in N Lee, Martin L, et al., An Effect of Predilution on Potency Assays of FVIII Concentrates, Thrombosis Research (Pergamon Press Ltd.) 30, 511 519 (1983). Principle of the one stage assay: The test is executed as a modified version of the activated Partial Thromboplastin Time (aPTT)-assay: Incubation of plasma with phospholipids and a surface activator leads to the activation of factors of the intrinsic coagulation system. Addition of calcium ions triggers the coagulation cascade. The time to formation of a measurable fibrin clot is determined. The assay is executed in the presence of Factor VIII deficient plasma. The coagulation capability of the deficient plasma is restored by Coagulation Factor VIII included in the sample to be tested. The shortening of coagulation time is proportional to the amount of Factor VIII present in the sample. The activity of Coagulation Factor VIII is quantified by direct comparison to a standard preparation with a known activity of Factor VIII in International Units.

Another standard assay is a chromogenic substrate assay. Chromogenic substrate assays may be purchased commercially, such as the Coamatic® FVIII test kit (Chromogenix-Instrumentation Laboratory SpA V. le Monza 338 - 20128 Milano, Italy). Principle of the chromogenic assay: In the presence of calcium and phospholipid, Factor X is activated by Factor IXa to Factor Xa. This reaction is stimulated by Factor VIIIa as cofactor. FVIIIa is formed by low amounts of thrombin in the reaction mixture from FVIII in the sample to be measured. When using the optimum concentrations of Ca2+, phospholipid and Factor IXa and an excess quantity of Factor X, activation of Factor X is proportional to the potency of Factor VIII. Activated Factor X releases the chromophore pNA from the chromogenic substrate S-2765. The release of pNA, measured at 405 nm, is therefore proportional to the amount of FXa formed, and, therefore, also to the Factor VIII activity of the sample.

Pharmaceutical compositions

Therapeutic formulations of the polypeptide of the invention suitable in the methods described herein can be prepared for storage as lyophilized formulations or aqueous solutions by mixing the polypeptide having the desired degree of purity with optional pharmaceutically-acceptable carriers, excipients or stabilizers typically employed in the art (all of which are referred to herein as "carriers"), i.e., buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic detergents, antioxidants, and other miscellaneous additives. See, Remington's Pharmaceutical Sciences, 16th edition (Osol, ed. 1980). Such additives must be nontoxic to the recipients at the dosages and concentrations employed.

Buffering agents help to maintain the pH in the range which approximates physiological conditions. They can present at concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additionally, phosphate buffers, histidine buffers and trimethylamine salts such as Tris can be used.

Preservatives can be added to retard microbial growth, and can be added in amounts ranging from 0.2%- 1% (w/v). Suitable preservatives include phenol, benzyl alcohol, meta- cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalconium halides (e.g., chloride, bromide, and iodide), hexamethonium chloride, and alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol. Isotonicifiers sometimes known as "stabilizers" can be added to ensure isotonicity of liquid compositions and include polhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thio sulfate; low molecular weight polypeptides (e.g., peptides of 10 residues or fewer); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophylic polymers, such as polyvinylpyrrolidone monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccacharides such as raffinose; and polysaccharides such as dextran. Stabilizers can be present in the range from 0.1 to 10,000 weights per part of weight active protein.

Non-ionic surfactants or detergents (also known as "wetting agents") can be added to help solubilize the therapeutic agent as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stressed without causing denaturation of the protein. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic polyols, polyoxyethylene sorbitan monoethers (TWEEN®-20, TWEEN®-80, etc.). Non-ionic surfactants can be present in a range of about 0.05 mg/ml to about 1.0 mg/ml, or in a range of about 0.07 mg/ml to about 0.2 mg/ml.

Additional miscellaneous excipients include bulking agents (e.g., starch), chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E), and cosolvents.

The formulation herein can also contain a second therapeutic agent in addition to a polypeptide of the invention. Examples of suitable second therapeutic agents are provided below.

The dosing schedule can vary from once a month to daily depending on a number of clinical factors, including the type of disease, severity of disease, and the patient's sensitivity to the polypeptide of the invention. In specific embodiments, a polypeptide of the invention is administered, twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four weeks to every month, from every 10 days to every two weeks, etc.

The dosage of a polypeptide of the invention to be administered will vary according to the particular polypeptide, the subject, and the nature and severity of the disease, the physical condition of the subject, the therapeutic regimen (e.g., whether a second therapeutic agent is used), and the selected route of administration; the appropriate dosage can be readily determined by a person skilled in the art.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a polypeptide of the invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the age and condition of the particular subject being treated, and that a physician will ultimately determine appropriate dosages to be used. This dosage can be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be altered or reduced, in accordance with normal clinical practice.

The pharmaceutical composition is preferably formulated to be administered extravascularly, preferably to be administered subcutaneously.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the spirit and scope. The invention also includes all of the features, compositions, steps, and compounds referred to or indicated in this specification, individually or collectively and any and all combinations of any two or more of said features, compositions, steps, and compounds.

The nucleotide and amino acid sequences shown in the sequence listing are summarized in the Table 1.

Table 1:

SEQ ID NO:	Remarks
1	DNA sequence encoding a polypeptide comprising acids 1 to 1242 of human VWF, a glycine/serine linker and human albumin; nucleotide positions (nt): nt 1-6: EcoRI restriction enzyme cleavage site nt 32 – 3757: coding sequence for VWF amino acids 1 to 1242 nt 3758 – 3850: coding sequence for glycine/serine linker nt 3851 – 5608: coding sequence for human albumin nt 5609 – 5616: NotI restriction enzyme cleavage site
2	Amino acid sequence encoded by SEQ ID NO:1 (mature form): amino acid positions (aa): aa 1 – 479: VWF D´D3 region (VWF amino acids 764 – 1242) aa 480 – 510: glycine/serine linker aa 511 – 1095: human albumin
3	DNA sequence encoding the pre-pro form of human native VWF
4	Amino acid sequence encoded by SEQ ID NO:3
5	Amino acid sequence of a single chain Factor VIII molecule
6	Amino acid sequence of mature human serum albumin
7	Amino acid sequence of D´D3-His aa 1 – 479: VWF D´D3 region (VWF amino acids 764 – 1242) aa 480 – 511: glycine/serine linker aa 512 – 519: polyhistidine tag
8	Amino acid sequence of D´D3-CTP aa 1 – 479: VWF D´D3 region (VWF amino acids 764 – 1242) aa 480 – 511: glycine/serine linker aa 512 – 576: C-terminal peptide of human chorionic gonadotropin-ß subunit aa 577 – 584: polyhistidine tag

Certain embodiments of the invention will now be described with reference to the following examples which are intended for the purpose of illustration only and are not intended to limit the scope of the generality hereinbefore described.

EXAMPLES

<u>Material and Methods</u>

Generation of D´D3 albumin fusion protein (D´D3-FP):

The expression cassette for D´D3-FP consisting of cDNA encoding VWF amino acids 1 to 1242, a glycine/serine linker and the cDNA of human albumin was prepared by custom gene synthesis (Eurofins Genomics, Ebersberg, Germany). Through flanking restriction sites (EcoRI, NotI) the expression cassette was excised from the cloning vector supplied and inserted into a pIRESneo3 vector (BD Biosciences, Franklin Lakes, NJ, USA) linearized with EcoRI and NotI. The resulting expression plasmid contained nucleotide sequences encoding the VWF propeptide, D´ and D3 (VWF amino acids 1 to 1242 of SEQ ID NO:4) fused to the albumin coding sequence through a short linker coding sequence under CMV promoter control. The nucleotide sequence of the coding sequence is displayed as SEQ ID NO:1, the amino acid sequence of the mature D´D3-FP is shown as SEQ ID NO:2. The presence of the D1D2 VWF propeptide (741 amino acids) during expression is crucial for dimerization of the synthesized polypeptide.

A similar approach was used to generate an expression plasmid for a His-tagged D´D3 protein (D´D3 and His8 linked by a glycine/serine linker) and a D´D3 fusion protein to the C-terminal peptide of human chorionic gonadotropin-ß subunit, also linked via a glycine/serine linker and tagged by 8 histidines at the C-terminus of the fusion protein. The amino acid sequence of the mature D´D3-His is shown as SEQ ID NO: 7 and the amino acid sequence of the mature D´D3-CTP is shown as SEQ ID NO: 8.

The expression plasmids as described above were grown up in XL10 Gold (Agilent Technologies) and purified using standard protocols (Qiagen, Hilden, Germany).

CHO K1 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) and grown up in serum-free medium (CD-CHO, Invitrogen) in the presence of 500-1000 µg/ml Geneticin. An expression plasmid encoding PACE/furin (pFu-797) as described in WO 2007/144173 A1 was cotransfected to maximize propeptide cleavage efficacy. Single cell derived clones were grown up and selected according to their D´D3-FP expression yield as quantified by an albumin specific enzyme immunoassay (see below). The cell line finally selected for D´D3-FP fermentation was called T2050-CL3.

Production of D´D3-FP was carried out in bioreactors applying a fermentation process in perfusion mode. The fermentation process for the production of D´D3-containing polypeptides started with the thaw of cell line T2050-CL3 followed by cell expansion in shake flasks and finally a fermentation process in perfusion mode using the Sartorius BioStat B-DCU 5 L bioreactor and the BioStat STR 50L single-use bioreactors. The BioSeps 10L or 200L (Applikon), respectively, were used as cell retention devices. Cell culture media were either PowerCHO3 (Lonza BESP1204) with 8 mM L-glutamine and 1 µM $CuSO_4$ or ProCHO5 (Lonza BESP1072) with 10 mM L-glutamine and 1 µM $CuSO_4$.

The seed trains in shake flasks were performed at 37°C, 7.5% CO_2 at a shaker speed of 160 rpm.

The 5L bioreactor was inoculated with a target VCD of 2.5×10^5 cells/mL. The cells were cultivated in PowerCHO3 with 8 mM L-glutamine and 1 µM $CuSO_4$ at a temperature of +37.0°C, a pH of 7.00, and at 30 % oxygen saturation. A temperature shift to +34.0°C (evaluated range +31°C to +35°C) was performed after initial harvests from the bioreactor run at +37°C had been taken. The pH was controlled using CO_2 sparged as acid and $NaHCO_3$ as base. The overlay air flow rate was set to 0.5 L/min. A ring sparger was used as a sparging unit. The agitation rate was 150 rpm with a 2fold pitch blade impeller in down pull mode.

The 50L bioreactor was inoculated with a target VCD of 3.0×10^5 cells/mL. The cells were cultivated in ProCHO5 medium with 10 mM L-glutamine and 1 µM $CuSO_4$ at a temperature of +37.0°C, a pH of 6.90, and at 30 % oxygen saturation. A temperature shift to +34.0°C was performed after the initial one or two harvests. PH control as above, the overlay air flow rate was set to 2 L/min. A micro sparger was used as a sparging unit. The agitation rate was 90 rpm with a 2fold pitch blade impeller in down pull mode.

The perfusion was initiated when the VCD in the bioreactor was $\geq 1.0 \times 10^6$ cells/mL. The perfusion rate was set to 1.0 volume/volume/day. The BioSep was operated in back flush mode with 5 (10) minutes runtime and 10 seconds back flush at a power input of 7 (30) W (numbers in brackets refer to the 50L bioreactor). The perfusate and the bleed were filtered inline and collected in bags over 48 hours at +2 to +8°C. The VCD was controlled by active bleeding using a turbidity probe using glucose consumption as parameter with a target of 2 g/L glucose. Harvest and bleed were filtered inline, the harvest system consisting of a disposable filter and disposable bag was changed every second day.

To prepare material for the PK analyses described below D´D3 albumin fusion protein harvests were purified by affinity and size exclusion chromatography. Briefly, the cell-free harvest from the bioreactor was concentrated 30-fold using a TFF system (e.g. Pall Centramate 500 S) with a 30 kD membrane (e.g Pall Centramate OS030T12). That concentrate was spiked with NaCl and

EDTA to a final concentration of 0.75 M NaCl and 5 mM EDTA and loaded overnight on a CaptureSelect Human Albumin column (Life Technologies) which was pre-equilibrated with 20 mM Tris buffer pH 7.4. After washing the column with equilibration buffer D'D3-FP was eluted with elution buffer (20 mM Tris, 2 M $MgCl_2$, pH 7.4). The eluate was then 10-fold concentrated and dialyzed against 50 mM Tris, 150 mM NaCl, pH 7.4 using Ultra Centrifugal Filters with a 30 kD cut-off (e.g. Amicon. UFC903024). To separate the D´D3-FP dimer from the monomer portion that material was loaded on a Superdex 200 pg column (GE Healthcare Code: 17-1069-01) pre-equilibrated with 50 mM Tris, 150 mM NaCl, pH 7.4 and the peak fractions containing the D'D3-FP dimer were pooled. The area under the curve for the dimer and monomer peak fractions was used to calculate dimer to monomer ratio. Dimer preparations of said D´D3 albumin fusion protein were used for the pharmacokinetic experiments. Such dimer preparations are referred to as D'D3-FP in the following, if not indicated otherwise.

The rD'D3-FP EYA variant has been generated by equivalent method steps.

His-tagged D´D3 proteins were purified by Ni-chelate affinity and size exclusion chromatography. Briefly, TFF concentrated cell-free bioreactor harvest (see above for details) was loaded on a preequilibrated (20mM sodium phosphate / 500 mM NaCl, pH 7.4) Ni-Sepharose column (HisTrap^{TM}, GE Healthcare) over night. After washing the column with 20mM sodium phosphate / 500 mM NaCl / 30 mM Imidazol, pH 7.4 the protein was eluted with 20mM sodium phosphate + 500 mM NaCl + 500 mM Imidazol, pH 7.4. The eluate was then concentrated and dialysed (TBS, pH7.4) using an Amicon Ultra Centrifugal Filter (see above). The final product was then loaded onto a SEC column (see above), the peak fractions containing the dimer were pooled and concentrated to about 7 mg/mL $OD_{280-320}$. Dimer preparations of His-tagged D´D3 proteins were used for the pharmacokinetic experiments. Such dimer preparations are referred to as rD'D3-His in the following, if not indicated otherwise.

Example 1: Subcutaneous bioavailability of a recombinant FVIII in the presence of rD'D3-FP or variants thereof

To assess, whether extravascular injections might be an option for an improved therapy with FVIII, a typical representative for an extravascular therapy, i.e. subcutaneous (s.c.) injection, was chosen. We aimed at characterizing the impact of a recombinant polypeptide comprising a truncated VWF on the subcutaneous bioavailability of FVIII in different approaches:

- Example 1.1: Investigation of rD'D3-FP and rVIII-SingleChain given both subcutaneously in a hemophilia A model, i.e. in FVIII ko mice.

- Example 1.2: Investigation of rD'D3-FP and rVIII-SingleChain given both subcutaneously in a model with physiological endogenous FVIII, i.e. in pigs.
- Example 1.3: Investigation of the effect of rD'D3-FP on different FVIII products, each given subcutaneously in a hemophilia A model, i.e. in FVIII ko mice.
- Example 1.4: Investigation of the effect of a rD'D3-FP affinity variant, a rD'D3 molecule with non-albumin HELP and rVIII-SingleChain given both subcutaneously in a mouse hemophilia A model, i.e. in FVIII ko mice.

Therefore, we investigated the impact of a recombinant polypeptide comprising the D' and D3 domains of VWF fused to albumin via a linker peptide when subcutaneously co-administered with a recombinant FVIII.

For the Examples, a polypeptide comprising a truncated VWF having an amino acid sequence as defined in SEQ ID NO:2 was used. This particular fusion protein consists of an N-terminal amino acid sequence from 1 – 479 representing the VWF D´D3 region (amino acids 764 – 1242 of human native VWF), followed by a 31 amino acid glycine/serine linker peptide and a C-terminal human albumin amino acid sequence from 511 – 1095. This fusion protein having a sequence as defined in SEQ ID NO:2 is referred to as rD'D3-FP or rD'D3-FP WT in the following.

For the purpose of the examples, a recombinant B-Domain-deleted single chain FVIII, i.e. rVIII-SingleChain, having an amino acid sequence as defined in SEQ ID NO:5 was used. In Example 1.3 different recombinant FVIII products have been tested.

Further, we investigated the impact of different ratios of rD'D3-FP to the rVIII-SingleChain.

The impact of the albumin fusion as a potential mediator for subcutaneous availability was investigated by comparing bioavailability of rD'D3-FP to that of a His-tagged rD'D3 (rD'D3-His). The amino acid sequence of the mature D´D3-His is shown as SEQ ID NO: 7 whereby D´D3 and His8 are being joined by a glycine/serine linker.

As an alternative for the albumin as half-life extending polypeptide (HLEP), in some Examples a rD'D3-FP variant is used having instead of albumin a CTP (C-terminal peptide of human chorionic gonadotropin-ß subunit) fused to rD'D3 via a glycine/serine linker which is referred to as rD'D3-CTP hereinafter. The fusion protein rD'D3-CTP has a sequence as defined in SEQ ID NO:8.

In certain Examples a high affinity variant of rD'D3-FP was used. This particular variant fusion protein consists of an N-terminal amino acid sequence from 1 – 479 representing the VWF D´D3 region (amino acids 764 – 1242 of human native VWF), followed by a 31 amino acid glycine/serine linker peptide and a C-terminal human albumin amino acid sequence from 511 – 1095, provided that within the D’D3 domain of said polypeptide three amino acid substitutions are present, i.e. S764E, S766Y and V1083A. This fusion protein consists of a sequence as defined in SEQ ID NO:2 having said three substitutions S764E, S766Y, and V1083A within the D´D3 region. Said variant is referred to as rD’D3-FP EYA hereinafter.

Material and Methods

Background information

For calculating ratios of the different rD‘D3-FP:rVIII-SingleChain combinations, the following assumptions were made:

- The drugs are diluted in 40 mL plasma per kg body weight after their administration
- Molecular weight of the polypeptide of the invention used: rD'D3-FP molecular weight of monomeric subunit (including glycosylation): 127,000 Da (HLEM = human albumin); the monomeric weight was used in the calculated ratios
- Molecular weight of rD’D3-His: rD'D3-His molecular weight of monomeric subunit (including glycosylation): 64,000 Da; the monomeric weight was used in the calculated ratios
- Molecular weight of rD’D3-FP EYA variant: rD'D3-FP molecular weight of monomeric subunit (including glycosylation): 127,000 Da; the monomeric weight was used in the calculated ratios
- Molecular weight of rD’D3-CTP: rD'D3-CTP molecular weight of monomeric subunit (including glycosylation): 69,800 Da; the monomeric weight was used in the calculated ratios
- Molecular weight of FVIII used: rVIII-SingleChain molecular weight (with glycosylation): 180,000 Da and specific activity: 11,000 IU/mg
- Molecular weight of other FVIII products used:
  - Beriate®: molecular weight: 285,000 Da and specific activity: 5,000 IU/mg
  - Advate®: molecular weight: 280,000 Da and specific activity: 7,000 IU/mg
  - ReFacto AF®: molecular weight: 170,000 Da and specific activity: 10,700 IU/mg

Beriate® is a plasma-derived human FVIII product from CSL Behring.
Advate® was purchased from Baxter AG, Vienna, Austria and is a recombinant full-length factor VIII preparation.
ReFacto AF® was purchased from Pfizer Limited, Kent, United Kingdom and is a recombinant factor VIII preparation having a deleted B-domain.

Analytics

rD'D3-FP (wildtype as well as the EYA variant) was applied at dose levels quantified by a human albumin ELISA, thereby measuring the albumin part of the protein. This rD'D3-FP ELISA was used for plasma samples as well.

The human albumin ELISA used a polyclonal goat anti-human albumin capture antibody from Bethyl Laboratories, Inc. (Montgomery, USA). The detection solution consists of a polyclonal peroxidase labelled anti-human albumin detection antibody preparation (Bethyl Laboratories Inc., Montgomery, USA). A chromogenic readout, i.e. TMB from Siemens Healthcare (Eschborn, Germany) was used for quantification in a microplate reader at 450/650 nm (ELx808, BioTek, USA) directly after stopping. As a standard, the drug formulation containing rD'D3-FP was used. rD'D3-FP amounts are given in mg albumin, i.e. no adjustment was done for the D'D3 part of the molecule.

The dose levels of the rD'D3-His and rD'D3-CTP construct were measured at OD_{280}, and the protein amount was adjusted to an equimolar concentration to the rD'D3-FP amount for rD'D3-His. Thereby, the unit for rD'D3-His is the same as for rD'D3-FP, i.e. it is plotted in the graphs as theoretical mg albumin. rD'D3-CTP was dosed in a similar molar ratio as rD'D3-FP (EYA variant) and the unit is not transferred to albumin but given as rD'D3-CTP. The plasma samples of the PK containing rD'D3-His and rD'D3-CTP were measured in an anti-D'D3 ELISA. This D'D3 ELISA used a monoclonal anti-human D'D3 capture antibody (in house research preparation). The detection solution consists of another monoclonal peroxidase labelled anti-human D'D3 detection antibody (in house research preparation). A chromogenic readout, i.e. TMB from Siemens Healthcare (Eschborn, Germany) was used for quantification in a microplate reader at 450/650 nm (ELx808, BioTek, Vermont, USA) directly after stopping. As a standard, the drug formulation containing rD'D3-His and rD'D3-CTP was used, and as before for rD'D3-His calculated to an equimolar concentration as compared to rD'D3-FP, i.e. again amounts are given as theoretical mg albumin. rD'D3-CTP amounts are given as rD'D3-CTP concentrations.

FVIII chromogenic activity plasma levels were detected by the COAMATIC® FVIII assay (FVIII:C chromogenic assay, Chromogenix, Instrumentation Laboratory SpA, Milan, Italy) according to the test instruction manual of the manufacturer. FVIII chromogenic activity is abbreviated as FVIII:C.

Human FVIII:Ag plasma levels were determined with the FVIII Asserachrom ELISA test kit from Stago, S.A.S., France according to the test instruction manual. The Asserachrom testkit contained all reagents with exception of the stop solution, which was obtained from Siemens

Healthcare (Eschborn, Germany). As a standard, the drug formulation containing rVIII-SingleChain was used.

Animals

FVIII ko mice

FVIII knock-out (ko) mice (representing a hemophilia A phenotype) were chosen, since they lack exons 16 and 17 of the FVIII gene, and thus have no plasma factor VIII activity (Bi L. et al, Nature genetics, 1995, Vol 10(1), 119-121; Bi L. et al, Blood, 1996, Vol 88(9), 3446-3450). This allows the analysis of FVIII activity levels following treatment with FVIII by quantification of FVIII activity in the plasma of these mice.

Male and female FVIII ko mice in a weight range of 17-35 g were breed at Charles River Laboratories (Sulzfeld, Germany). In house, the animals were kept at standard housing conditions, i.e. at 20-24°C under a 12 h/12 h light-darkness cycle. Animals were fed ad libitum with standard rat diet (Ssniff-Versuchsdiäten, Soest, Germany). Tap water was supplied ad libitum. Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations.

The group size was n=12, divided in 3 or 4 cohorts. Thus, n=3-4 animals per time-point were used.

Pigs

Pigs were chosen, since they represent a good model for subcutaneous bioavailability with respect to its predictivity for men.

Male pigs in a weight range of 23-27 kg were breed at Schlosser (Schwalmtal, Germany). In house, the animals were kept in a stable on straw at 18-21°C. Animals were fed with bruised grain. Tap water was supplied ad libitum. Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations.

The group size was 2 (intravenous) or 3 (subcutaneous).

Example 1.1: Investigation of rD'D3-FP and rVIII-SingleChain given both subcutaneously in a hemophilia A model, i.e. in FVIII ko mice.

Experimental details

The test articles were administered s.c. in the neck or i.v. into the lateral tail vein by a single injection, at a total volume of 5 mL/kg. Administered dose levels and routes are given in Table 2.

Table 2: Treatment groups

rD'D3-FP or rD'D3-His [mg albumin/kg]	rVIII-SingleChain [IU FVIII:C/kg]	Route and duration of observation	Ratio rD'D3-FP:rFVIII
-	400	sc (72h)	-
10	400	sc (72h)	745
3	400	sc (96h)	223
3	200	sc (96h)	447
3	100	sc (96h)	894
3	100	iv (96h)	894
3	50	sc (96h)	1787
3	-	sc (96h)	-
3	-	iv (96h)	-
1	400	sc (96h)	74
1	100	sc (96h)	298
1	100	iv (96h)	298
1	50	sc (96h)	596
0.3	200	sc (96h)	45
3 (rD'D3-His)	200	sc (96h)	447
3 (rD'D3-His)	200	iv (96h)	447

rD'D3-FP was applied in a dose range from 0.3 to 10 mg/kg based on human albumin values, rVIII-SingleChain doses ranged from 50 to 400 IU/kg chromogenic FVIII activity. rVIII-SingleChain was reconstituted with water for injection, and rD'D3-FP as well as rD'D3-His was thawed in a water bath. For co-administration, the compounds were incubated together for approximately 30 minutes at +37°C. In every case, a dose volume of 5 mL/kg was administered, with dilution buffer for FVIII being used for dissolution of the compounds if necessary.

Blood samples were taken retrobulbary under short term anaesthesia using an alternating sampling scheme. Timepoints in the s.c. groups were 3, 8, 16, 24, 48, 72, and 96 h p.a. (except for the 400 IU/kg rVIII-SingleChain and the 10 mg/kg rD'D3-FP + 400 IU/kg rVIII-SingleChain group), and in the i.v. groups 5 min, 3, 8, 24, 48, 72, and 96 h p.a. The PK profile was taken from 3 or 4 cohorts of mice per group, and n=3-4 animals per timepoint. Blood samples were anticoagulated using sodium citrate (1 parts sodium citrate 3.13% + 9 parts blood), processed to plasma and stored at –70°C for the determination of FVIII activity, FVIII antigen, albumin and/or rD'D3-His.

rD'D3-FP exposure was determined by measurement of the albumin part of the construct using a human albumin ELISA. Further, FVIII chromogenic activity and in selected groups FVIII antigen was measured.

Biostatistics

Estimation of the maximal concentration (C_{max}), the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$), mean residence time (MRT), clearance (CL) and terminal half-life ($t_{1/2}$) was done by two-compartmental modelling in the i.v. calculations, and by two-compartmental-resorption modelling in the s.c. calculations. For parameter estimation, a weighted least-squares cost function was applied. Bioavailability was calculated as the percentage of the $AUC_{0\text{-}inf}$ after s.c. administration as compared to i.v. administration. Time to 1, 5 and 10 % trough levels was calculated by setting the model equation equal to 0.01, 0.05 or 0.1 IU/mL and solving for time.

Results

Evaluation of D'D3 data

Both constructs of D'D3 (rD'D3-FP and rD'D3-His, with and without albumin fusion, respectively) were absorbed after s.c. administration. rD'D3-FP could be quantified over the whole period of observation of 96 h, even at the lowest dose of 0.3 mg/kg; i.e. it remained above the detection limit of 23.4 ng/mL (Fig. 1). However, rD'D3-FP could be detected at a significantly higher levels compared to rD'D3-His, in particular at the later time points.

It needs to be mentioned that some of the curves showed high similarity in the last two measurement points, which led to a "flattening out" of the plasma concentration curve in the terminal phase. Thereby estimation of clearance, MRT, $t_{1/2}$ and $AUC_{0\text{-}inf}$ was estimated extremely long when including the last point. A second calculation was done without the last timepoint in order to avoid an overestimation of bioavailability; a comparison of the data is given in Table 3 and shows good agreement of the data without the last timepoint with the other data.

Therefore, in the tables and graphs (except for Fig. 1), the second dataset without the 96 h datapoint was used, which may underestimate bioavailability of rD'D3-FP.

C_{max} and $AUC_{0\text{-}inf}$ showed dose-dependency in the tested range of 0.3-10 mg/kg rD'D3-FP and 0-400 IU/kg rVIII-SingleChain, independent of the added rFVIII (Table 3, Fig. 2). Related to the lower exposure, both, C_{max} as well as $AUC_{0\text{-}inf}$ of rD'D3-His, were relevantly lower than that for comparable rD'D3-FP doses. In detail, for s.c. administration, C_{max} was >3-fold and $AUC_{0\text{-}inf}$ was >16-fold lower.

Clearance, MRT and $t_{1/2}$ did not show a dose dependency for rD'D3-FP. The high variability in the s.c. estimates is prone to the difficulties with fitting the correct curves for the flat exposure over time curves. Clearance values were in the range of 1.2-2.1 mL/kg/h after s.c., and slightly lower (0.8-0.9 mL/kg/h) after i.v. administration. In line with this the MRT ($t_{1/2}$) range was 41-117 h (15-90 h) for s.c. and 55-83 h (39-69 h) for i.v. administration. In contrast, elimination of rD'D3-His was much quicker, i.e. clearance was 34.8 mL/kg/h after s.c. and 11.8 mL/kg/h after i.v. administration (>13 fold difference), MRT was 11 h after s.c. and 5 h after i.v. administration (>3 fold difference) and $t_{1/2}$ was 7 h after s.c. and 6 h after i.v. administration (>2 fold difference).

Importantly, bioavailability of rD'D3-FP after subcutaneous administration ranges from 40-79%, again with quite a high variability of the different groups within the experiment (Table 4, Fig. 3). Nevertheless, this bioavailability is independent of the rVIII-SingleChain or rD'D3-FP dose used in this experiment. rD'D3-His showed a lower bioavailability of 34%.

Table 3: Pharmacokinetic parameters of rD'D3-FP or rD'D3-His after s.c. or i.v. administration of rD'D3-FP or rD'D3-His and rVIII-SingleChain in FVIII ko mice

Treatment	C_{max}, extrap.	Clearance	MRT	Half-life, terminal	$AUC_{0\text{-}inf}$
		Albumin			
	[µg/mL]	[mL/kg/h]	[h]	[h]	[µg*h/mL]
3 mg/kg rD'D3-FP s.c.	21.5	1.9	65	42	1590
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	76.3	1.2	98	63	8234
3 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	17.1	2.0	78	46	1492
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	(24.6)* 25.2	(0.0)* 2.1	(99726)* 41	(69193)* 15	(734488)* 1398
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	20.6	1.7	71	44	1739
3 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	(33.3)* 33.0	(0.0)* 1.5	(99835)* 49	(69273)* 29	(919779)* 1989
1 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	16.1	1.2	117	90	844
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	8.8	1.4	73	48	719
1 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	(9.6)* 9.5	(0.0)* 1.6	(81484)* 55	(56588)* 34	(177600)* 613
0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	2.6	1.4	77	52	215
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain s.c.	7.2	34.8	11	7	86
3 mg/kg rD'D3-FP i.v.	90.0	0.9	48	34	3286
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.	71.0	0.8	83	69	3702
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.	27.1	0.9	55	39	1064
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain i.v.	81.6	11.7	5	6	257

* High similarity in the last two measurement points leads to an artificial "flattening out" of the plasma concentration curve in the terminal phase; thereby estimation of clearance, MRT, $t_{1/2}$ and $AUC_{0\text{-}inf}$ was estimated extremely long when including the last point. Therefore, an

additional calculation was done without the last timepoint in order to avoid an overestimation of bioavailability.

Table 4: Bioavailability of rD'D3-FP or rD'D3-His after s.c. administration in FVIII ko mice calculated against i.v. reference treatments

S.c. treatment	Bioavailability [%] to i.v. reference treatments§			
	3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.	1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.	3 mg/kg rD'D3-FP i.v.	3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain i.v.
3 mg/kg rD'D3-FP s.c.	43	50	**48**	n.a.
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	67	77	75	n.a.
3 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	40	47	45	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	38 *	44 *	43 *	n.a.
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	**47**	54	53	n.a.
3 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	54 *	62 *	61 *	n.a.
1 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	68	79	77	n.a.
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	58	**68**	66	n.a.
1 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	50 *	58 *	56 *	n.a.
0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	58	67	65	n.a.
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain s.c.	n.a.	n.a.	n.a.	**34**

n.a. = not applicable; * = lower confidence in AUC estimate of s.c. data (see above)

§ reference group with same treatment is given in bold

Evaluation of FVIII data

rVIII-SingleChain administered without any polypeptide of the invention, i.e. without any D'D3-FP construct, was not relevantly absorbed when administered s.c., at least no FVIII activity above the detection limit could be measured. Surprisingly however, FVIII was absorbed when co-administered s.c. with either of the two D'D3 constructs (rD'D3-FP and rD'D3-His, with and without albumin fusion, respectively); and FVIII activity endured the absorption process (Fig. 3).

It needs to be mentioned that also for FVIII activity one curve showed high similarity in the last two measurement points, which led to a "flattening out" of the plasma concentration curve in the terminal phase. Thereby estimation of clearance, MRT, $t_{1/2}$ and $AUC_{0\text{-}inf}$ was estimated extremely long when including the last point. A second calculation was done without the last timepoint in order to avoid an overestimation of bioavailability; a comparison of the data is given in Table 5. Therefore, in the tables and graphs (except for Fig. 4), the second dataset without the last datapoint was used, which may underestimate bioavailability of rD'D3-His.

Dependent on the FVIII dose, FVIII activity was quantified for at least 32 h (1 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain) and up to the last timepoint of 96 h (e.g. any dose with 400 IU/kg rVIII-SingleChain and 1-10 mg/kg rD'D3-FP); i.e. it remained over the detection limit of 3 or 10 mIU/mL (Fig. 4). As example, Fig. 4 represents FVIII plasma exposure after s.c. or i.v. administration of 1 or 3 mg/kg rD'D3-FP with 100 IU/kg rVIII-SingleChain compared with 3 mg/kg rD'D3-His with 200 IU/kg rVIII-SingleChain (the higher dose was administered to be able to monitor exposure). When no D'D3 construct was administered, rVIII-SingleChain remained below the detection limit, even at a s.c. dose of 400 IU/kg (data not shown).

C_{max} and $AUC_{0\text{-}inf}$ showed dose-dependency in the tested range of 0.3-10 mg/kg rD'D3-FP and 0-400 IU/kg rVIII-SingleChain, independent of the coadministered rD'D3-FP, while exposure was much lower when rD'D3-His was given (Table 5, Fig. 5).

When rD'D3-FP and rVIII-SingleChain were given at a molar ratio >50, CL for rVIII-SingleChain ranged from 7.5-23.7 mL/kg/h, and was thus lower than that for 0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c. (ratio 45) or rD'D3-His co-administration. In line with this, MRT and $t_{1/2}$ for rVIII-SingleChain were higher for rD'D3-FP and rVIII-SingleChain given at a ratio >50 as compared to rD'D3-His, except for the very low dose of rVIII-SingleChain of 50 IU/kg (range MRT: 24-37 h, range $t_{1/2}$: 8-20 h). For comparison, rVIII-SingleChain administered i.v. without rD'D3-FP was shown to have a CL of ~2-3 mL/kg/h, a MRT of 18h and a $t_{1/2}$ of 15h in FVIII ko mice, and a CL of ~2-3 mL/kg/h, a MRT of ~20h and a $t_{1/2}$ of ~14h in man (data not presented herein). Thus, pharmacokinetic parameters after s.c. administration were variable, but roughly comparable to those after i.v. administration.

Taken together, bioavailability of rVIII-SingleChain ranged from 11-25%, when given at a dose ≥3 mg/kg rD'D3-FP, between 6-14% when given at a dose of 1 mg/kg rD'D3-FP and ≤4% at a dose of 0.3 mg/kg rD'D3-FP (Table 6, Fig. 6). This bioavailability is dependent on the dose of rVIII-SingleChain in that sense that a potential saturation was observed at the highest tested dose of 400 IU/kg, which may be related to the available absorption area. Further, the rD'D3-FP dose limited availability of rVIII-SingleChain, i.e. the higher the rD'D3-FP dose, the better the rVIII-SingleChain availability. This can be transformed to relevant rD'D3-FP over rVIII-SingleChain tested ratios of at least 447 (≥3 mg/kg rD'D3-FP; excluding the 400 IU/kg rVIII-SingleChain dose with saturation), acceptable ratios in the tested range of 74-596 (1 mg/kg rD'D3-FP) and an unfavourable tested ratio of 45 (0.3 mg/kg rD'D3-FP). It was thus concluded that ratios <50 have shown an unfavourable bioavailability of FVIII, while those above 50 are favourable.

The bioavailability of rVIII-SingleChain was unproportionally lower when co-administered with rD'D3-His, i.e. 1% at a dose of 3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain, suggesting an advantage of the albumin fusion of rD'D3 for bioavailability of rVIIII-SingleChain.

Additionally time to trough was calculated for s.c. and i.v. administrations (Table 7). As for bioavailability, higher doses of rD'D3-FP and/or FVIII showed favourable trough levels, and within a constant FVIII or rD'D3-FP dose, an increase of the rD'D3-FP:rVIII-SingleChain ratio resulted in more favourable time to trough levels.

Table 5: Pharmacokinetic parameters of FVIII chromogenic activity after s.c. or i.v. administration of rD'D3-FP and rVIII-SingleChain in FVIII ko mice

Treatment	**C_{max}, extrap.**	**Clearance**	**MRT**	**Half-life, terminal**	**AUC_{0-inf}**
	FVIII:activity				
	mIU/mL	mL/kg/h	h	H	mIU*h/mL
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	692	12.2	37	17	32848
3 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	790	12.4	37	20	32387
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	621	7.5	32	11	26741
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	369	8.1	25	8	12409
3 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	186	8.8	24	6	5652
1 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	942	13.3	26	8	30028
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	138	23.7	27	16	4222
1 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	114	22.3	16	7	2243
0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	224	44.9	17	9	4454
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain s.c.	(82) * 81	(79.2) * 139.7	(30) * 7	(29) * 6	(1262) * 716
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.	2958	1.3	25	18	74850
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.	2323	1.8	23	16	54060
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain i.v.	5974	3.8	13	9	52824

* High similarity in the last two measurement points leads to an artificial "flattening out" of the plasma concentration curve in the terminal phase; thereby estimation of clearance, MRT, $t_{1/2}$ and AUC_{0-inf} was estimated extremely long when including the last point. Therefore, an additional calculation was done without the last timepoint in order to avoid an overestimation of bioavailability.

Table 6: Bioavailability of rVIII-SingleChain (FVIII chromogenic activity) after s.c. administration in FVIII ko mice calculated against i.v. reference treatments

S.c. treatment	**Bioavailability [%] to i..v. reference treatments[§]**		
	3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.	1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.	3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain i.v.
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	11	15	n.a.
3 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	11	15	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	18	25	n.a.
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	**17**	23	n.a.
3 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	15	21	n.a.
1 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	10	14	n.a.
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	6	**8**	n.a.
1 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	6	8	n.a.
0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	3	4	n.a.
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain s.c.	n.a.	n.a.	**1 ***

n.a. = not applicable; * = lower confidence in AUC estimate of s.c. data (see above)

[§] reference group with same treatment is given in bold

Table 7: Time to trough levels of rVIII-SingleChain (FVIII chromogenic activity) after s.c. administration in FVIII ko mice

Treatment	Time to 1% trough [h]	Time to 5% trough [h]	Time to 10% trough [h]
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	133	97	80
3 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	145	99	78
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	111	82	68
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	76	55	45
3 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	62	43	33
1 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	90	69	59
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	69	34	18
1 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.	38	22	6
0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	50	29	20
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain s.c.	15	7	3
3 mg/kg rDD3-FP & 100 IU/kg rVIII-SingleChain i.v.	127	87	69
1 mg/kg rDD3-FP & 100 IU/kg rVIII-SingleChain i.v.	109	73	57
3 mg/kg rDD3-His & 200 IU/kg rVIII-SingleChain i.v.	78	57	48

Example 1.2: Subcutaneous bioavailability of recombinant FVIII, rVIII-SingleChain, in the presence of rD'D3-FP in pigs

Experimental details

The test articles were administered s.c. in the flanks or i.v. into the ear vein by a single injection, at a total volume ranging from 0.211 to 0.751 mL/kg. Administered dose levels and routes are given in Table 8.

Table 8: Treatment groups

rD'D3-FP [mg albumin/kg]	rVIII-SingleChain [IU FVIII:C/kg]	Route and duration of observation	Ratio rD'D3-FP:rFVIII
10	400	sc (168h)	745
10	400	iv (168h)	745
3	200	sc (264h)	447
3	100	sc (264h)	894
3	-	sc (264h)	-

rD'D3-FP was applied in a dose range from 3 to 10 mg/kg based on human albumin values, rVIII-SingleChain doses ranged from 100 to 400 IU/kg chromogenic FVIII activity. rVIII-SingleChain was reconstituted with water for injection, and rD'D3-FP thawed in a water bath.

Blood samples were taken from the ear or saphenous vein. Timepoints in the 10 mg/kg rD'D3-FP s.c. groups were pre-dose, 3, 12, 24, 32, 48, 72, 96, 120, 144 and 168 h p.a. , and in the i.v. group pre-dose 5 min, 3, 12, 24, 32, 48, 72, 96, 120, 144 and 168 h p.a. Timepoints in the 3 mg/kg rD'D3-FP s.c. groups were pre-dose, 1, 3, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h p.a.

The PK profile was taken from individual animals. Blood samples were anticoagulated using sodium citrate (1 parts sodium citrate 3.13% + 9 parts blood), processed to plasma and stored at –70°C for the determination of FVIII antigen and albumin.

rD'D3-FP exposure was determined by measurement of the albumin part of the protein using a human albumin ELISA. Human FVIII:Ag plasma levels were determined with the FVIII Asserachrom ELISA.

Biostatistics

Estimation of the maximal concentration (C_{max}), the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$), mean residence time (MRT), clearance (CL) and terminal half-life ($t_{1/2}$) was done by two-compartmental modelling in the i.v. calculations, and by two-compartmental-resorption modelling in the s.c. calculations. For parameter estimation, a weighted least-squares cost function was applied. Bioavailability was calculated as the percentage of the $AUC_{0\text{-}inf}$ after s.c. administration as compared to i.v. administration. Time to 1, 5 and 10 % trough levels was calculated by setting the model equation equal to 0.01, 0.05 or 0.1 IU/mL and solving for time.

Results

Evaluation of D'D3 data

rD'D3-FP was absorbed after s.c. administration and quantified over the whole period of observation of up to 168 h at 3 and 10 mg/kg; i.e. it remained above the detection limit of 23.4 ng/mL (Fig. 7).

C_{max} and $AUC_{0\text{-}inf}$ showed dose-dependency in the tested range of 3-10 mg/kg rD'D3-FP (Table 9). C_{max} was independent of the added rFVIII in the range of 0-400 IU/kg rVIII-SingleChain, while $AUC_{0\text{-}inf}$ of rD'D3-FP increased with the dose of the added rVIII-SingleChain. Clearance, MRT and $t_{1/2}$ showed a longer PK profile for rD'D3-FP for animals treated with 200 or 400 IU/kg rVIII-SingleChain as compared to 100 IU/kg or rVIII-SingleChain given alone (Table 9), i.e. rD'D3-FP loaded with FVIII remained longer in the system than without relevant amounts of FVIII.

In line with this, bioavailability of rD'D3-FP after subcutaneous administration ranges from 59-187 % (Table 10), with higher values being reached with the highest co-administered FVIII doses. In conclusion, rVIII-SingleChain supported subcutaneous absorption of rD'D3-FP.

Table 9: Pharmacokinetic parameters of rD'D3-FP after s.c. or i.v. administration of rD'D3-FP and rVIII-SingleChain in pigs

Treatment	**C_{max}, extrap.**	**Clearance**	**MRT**	**Half-life, terminal**	**AUC_{0-inf}**
		Albumin			
	[µg/mL]	[mL/kg/h]	[h]	[h]	[µg*h/mL]
3 mg/kg rD'D3-FP s.c.	17.6	0.5	271	154	5968
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	61.7	0.16	979	671	62813
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	17.4	0.18	939	644	16861
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	18.2	0.43	318	191	7013

Table 10: Bioavailability of rD'D3-FP after s.c. administration in pigs calculated against i.v. reference treatments

S.c. treatment	**Bioavailability [%] to i.v. reference treatment: 10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain i.v. §**
3 mg/kg rD'D3-FP s.c.	59
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	**187**
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	167
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	70

§ reference group with same treatment is given in bold

Evaluation of FVIII data

FVIII was surprisingly absorbed when co-administered s.c. with rD'D3-FP and FVIII activity endured the absorption process (Fig. 8). Dependent on the FVIII dose, FVIII activity was quantified for at least 48 h (3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain) and up to 168 h (e.g. any dose with 400 IU/kg rVIII-SingleChain and 3 or 10 mg/kg rD'D3-FP); i.e. it remained over the detection limit of 117 mIU/mL.

C_{max} and AUC_{0-inf} showed dose-dependency in the tested range of 100-400 IU/kg rVIII-SingleChain. Values were expectedly higher after i.v. administration of the drugs (Table 10). Clearance of FVIII activity was higher (2.9-4.1 mL/kg/h) after s.c. than after i.v. (1.2 mL/kg/h) administration. Nevertheless, MRT and $t_{1/2}$ was comparable after s.c. and i.v. administration (82 & 85h vs. 77h and 52 & 59 h vs. 54 h, respectively) with higher rVIII-SingleChain doses of 200 or 400 IU/kg. At the lower dose of 100 IU/kg, MRT and $t_{1/2}$ were even longer for rVIII-SingleChain (130 and 83 h, respectively). Thus, pharmacokinetic parameters after s.c. administration were roughly comparable to those after i.v. administration with higher doses of rVIII-SingleChain, and superior at a dose of 100 IU/kg rVIII-SingleChain.

Bioavailability of rVIII-SingleChain ranged from 29-40 %, increasing with the dose of rVIII-SingleChain and/or rD'D3-FP (Table 12).

Additionally time to trough was calculated for s.c. and i.v. administrations (Table 13). Time to 1% trough levels were comparable for all s.c. doses, while time to 5% or 10% trough was comparable for 200 and 400 IU/kg rVIII-SingleChain + 3 or 10 mg/kg rD'D3-FP, and superior for 100 IU/kg rVIII-SingleChain + 3 mg/kg rD'D3-FP.

Table 11: Pharmacokinetic parameters of FVIII activity after s.c. or i.v. administration of rD'D3-FP and rVIII-SingleChain in pigs

Treatment	**C_{max}, extrap.**	**Clearance**	**MRT**	**Half-life, terminal**	**AUC_{0-inf}**
		FVIII:activity			
	[IU/mL]	[mL/kg/h]	[h]	[h]	[IU*h/mL]
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain i.v.	7.0	1.2	77	54	339
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	1.1	4.1	85	59	97
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	0.7	2.9	82	52	68
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	0.2	3.1	130	83	31

Table 12: Bioavailability of rVIII-SingleChain (FVIII activity) after s.c. administration in pigs calculated against i.v. reference treatments

S.c. treatment	**Bioavailability [%] to i.v. reference treatment: 10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain i.v.§**
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	29
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	**40**
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	37

§ reference group with same treatment is given in bold

Table 13: Time to trough levels of rVIII-SingleChain (FVIII antigen) after s.c. administration in pigs

Treatment		**Time to**	
	1% trough [h]	**5% trough** [h]	**10% trough** [h]
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain i.v.	319	195	141
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.	383	255	196
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.	349	249	198
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.	388	349	303

Example 1.3: Investigation of the effect of rD'D3-FP on different FVIII products, each given subcutaneously in a mouse hemophilia A model, i.e. in FVIII ko mice.

Experimental details

The test articles were administered s.c. in the neck or i.v. into the lateral tail vein by a single injection, at a total volume of 5 mL/kg. Administered dose levels and routes are given in Table 14.

Table 14: Treatment groups

rD'D3-FP [mg albumin/kg]	FVIII [IU FVIII:C/kg]	Route	Ratio rD'D3-FP:rFVIII
3	200 Beriate®	sc	322
3	200 Beriate®	iv	322
-	200 Beriate®	sc	-
3	200 Advate®	sc	442
3	200 Advate®	iv	442
-	200 Advate®	sc	-
3	200 ReFacto AF®	sc	410
3	200 ReFacto AF®	iv	410
-	200 ReFacto AF®	sc	-

rD'D3-FP was applied in a dose of 3 mg/kg based on human albumin values, and FVIII products at a dose of 200 IU/kg chromogenic FVIII activity (nominal: Advate® and ReFacto AF®, Certificate of Analysis: Beriate®). Advate® and ReFacto® AF were reconstituted according to the package insert. Beriate® was reconstituted with water for injection using a pipette. rD'D3-FP was thawed in a water bath and mixed with respective FVIII product. In every case, a dose volume of 5 mL/kg was administered, dilution buffer for FVIII was used for all products.

It shall be mentioned that the ratio of rD'D3-FP:rFVIII was in a comparably high range from 322 to 442 for the four different products, based on their different molecular weights and specific activities.

Blood samples were taken retrobulbary under short term anaesthesia using an alternating sampling scheme. Timepoints in the s.c. groups were 3, 8, 16, 24, 32, 48, 72, and 96 h p.a., and in the i.v. groups 5 min, 3, 8, 16, 24, 48, 72, and 96 h p.a. The PK profile was taken from four cohorts of mice per group, and n=3 per timepoint. Blood samples were anticoagulated using sodium citrate (1 parts sodium citrate 3.13% + 9 parts blood), processed to plasma and stored at –70°C for the determination of chromogenic FVIII activity and albumin.

rD'D3-FP exposure was determined by measurement of the albumin part of the construct using a human albumin ELISA. Further, FVIII chromogenic activity measured.

Biostatistics

Estimation of the maximal concentration (C_{max}), the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-}inf}$), mean residence time (MRT), clearance (CL) and terminal half-life ($t_{1/2}$) was done by two-compartmental modelling in the i.v. calculations, and by two-compartmental-resorption modelling in the s.c. calculations. For parameter estimation, a weighted least-squares cost function was applied. Bioavailability was calculated as the percentage of the $AUC_{0\text{-inf}}$ after s.c. administration as compared to i.v. administration. Time to 1, 5 and 10 % trough levels was calculated by setting the model equation equal to 0.01, 0.05 or 0.1 IU/mL and solving for time.

Results

Evaluation of D'D3 data

Independent of the co-administered FVIII product, rD'D3-FP was absorbed after s.c. administration. rD'D3-FP could be quantified over the whole period of observation of 96 h and remained above the detection limit of 23.4 ng/mL (Fig. 9).

There was no visible difference in the PK profiles of rD'D3-FP after i.v. or s.c. administration, respectively, in dependence of the co-administered FVIII. In line with this, the estimation of clearance, MRT, $t_{1/2}$ and $AUC_{0\text{-inf}}$ shows good agreement of the data for all s.c. or i.v. treatments, respectively (Table 15). In detail, clearance was in the range of 0.9 to 1.1 mL/kg/h for i.v. and was slightly higher after s.c. administration (1.0 to 1.5 mL/kg/h). In line with this, MRT and $t_{1/2}$ ranged between 40-56 h and 31-40 h for i.v and between 61-117 h and 35-89 h for s.c. treatment respectively; i.e. clearance was lower for i.v. but typically MRT and $t_{1/2}$ were nevertheless shorter for i.v. treatment.

Importantly, bioavailability of rD'D3-FP after subcutaneous administration ranges from 56-87% (Table 16), and does not differ relevantly between the different co-administered FVIII products. It is very comparable to that of rVIII-SingleChain (Table 4, range 40-79%).

Table 15: Pharmacokinetic parameters of rD'D3-FP after s.c. or i.v. administration of rD'D3-FP and different FVIIII products in FVIII ko mice

Treatment	$C_{max,}$ extrap.	Clearance	MRT	Half-life, terminal	AUC_{0-inf}
		Albumin			
	[µg/mL]	[mL/kg/h]	[h]	[h]	[µg*h/mL]
3 mg/kg rD'D3-FP & 200 IU/kg Beriate® s.c.	26.2	1.5	61	35	1940
3 mg/kg rD'D3-FP & 200 IU/kg Beriate® i.v.	74.3	1.1	40	31	2667
3 mg/kg rD'D3-FP & 200 IU/kg Advate® s.c.	35.4	1.1	85	71	2624
3 mg/kg rD'D3-FP & 200 IU/kg Advate® i.v.	101.3	0.9	46	33	3268
3 mg/kg rD'D3-FP & 200 IU/kg ReFacto® AF s.c.	30.3	1.0	117	89	2987
3 mg/kg rD'D3-FP & 200 IU/kg ReFacto® AF i.v.	99.4	0.9	56	40	3488

Table 16: Bioavailability of rD'D3-FP after s.c. administration in FVIII ko mice calculated against i.v. reference treatments

S.c. treatment	Bioavailability [%] to i.v. reference treatments: rD'D3-FP with respective FVIII product i.v.
3 mg/kg rD'D3-FP & 200 IU/kg Beriate® s.c.	56
3 mg/kg rD'D3-FP & 200 IU/kg Advate® s.c.	80
3 mg/kg rD'D3-FP & 200 IU/kg ReFacto® AF s.c.	87

Evaluation of FVIII data

All of the FVIII products were absorbed when co-administered s.c. with rD'D3-FP and FVIII activity endured the absorption process (Fig. 10). In contrast, none of the products showed relevant s.c. bioavailability when given alone.

Data from estimation of C_{max}, AUC_{0-inf}, clearance, MRT and $t_{1/2}$ is given in Table 17. While the different FVIII products showed different PK profiles when given alone, clearance was always increased after s.c. administration as compared to i.v. administration, i.e. from 3.1 to 51.5 mL/kg/h for Beriate®, from 4.4 to 78.5 mL/kg/h for Advate® and from 1.7 to 16.2 mL/kg/h for ReFacto AF®. MRT was about comparable between s.c. and i.v. administration (Beriate® and Advate®: range 17-19 h; ReFacto AF®: range 21-28 h), in line with the results from co-administration of rVIII-SingleChain (see Table 11). For these other FVIII products, $t_{1/2}$ tended to be longer after i.v. administration as compared to s.c. administration (Beriate: 8 h s.c. to 13 h i.v.; Advate®: 12 h s.c. to 14 h i.v.; ReFacto AF®: 11 h s.c. to 15 h i.v.).

Subcutanous bioavailability of the different FVIII products co-administered with rD'D3-FP ranged from 6-11%, suggesting no relevant difference between the FVIII products (Table 18). This is slightly less as compared with the observed 20% when co-administering rD'D3-FP with rVIII-SingleChain (see Table 12); nevertheless all bioavailabilities of FVIII products were within an acceptable range. This indicates that rD'D3-FP plays the key role for FVIII resorption after subcutaneous administration. However, a combination of a rD'D3-FP with rVIII-SingleChain may further improve bioavailability of FVIII.

Additionally time to trough was calculated for s.c. and i.v. administrations (Table 19). For 1% trough levels, data were about comparable after i.v. and s.c. administration (Beriate® 60 and 79 h, Advate® 66 and 68 h, ReFacto® AF 98 and 104 h). Time to trough for 5% or 10% levels was superior after s.c. as compared to i.v. administration: Beriate showed superiority of s.c. over i.v. by 4% (5% trough) and 17% (10% trough), Advate® by 29% (5% trough) and 50% (10% trough) and ReFacto® AF by 50% (5% trough) and 28% (10% trough), respectively.

Table 17: Pharmacokinetic parameters of FVIII antigen after s.c. or i.v. administration of rD'D3-FP and different FVIIII products in FVIII ko mice

Treatment	C_{max}, extrap.	Clearance	MRT	Half-life, terminal	$AUC_{0\text{-}inf}$
	FVIII antigen				
	[IU/mL]	[mL/kg/h]	[h]	[h]	[IU*h/mL]
200 IU/kg Beriate® s.c.	n.a.	n.a.	n.a.	n.a.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg Beriate® s.c.	0.17	51.5	18	8	4
3 mg/kg rD'D3-FP & 200 IU/kg Beriate® i.v.	5.31	3.1	19	13	66
200 IU/kg Advate® s.c.	n.a.	n.a.	n.a.	n.a.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg Advate® s.c.	0.13	78.5	19	12	3
3 mg/kg rD'D3-FP & 200 IU/kg Advate® i.v.	6.37	4.4	17	14	45
200 IU/kg ReFacto® AF s.c.	n.a.	n.a.	n.a.	n.a.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg ReFacto® AF s.c.	0.32	16.2	28	11	12
3 mg/kg rD'D3-FP & 200 IU/kg ReFacto® AF i.v.	5.46	1.7	21	15	116

n.a.: modelling not applicable (data below detection limit)

Table 18: Bioavailability of different FVIIII products (FVIII antigen) after s.c. administration in FVIII ko mice calculated against i.v. reference treatments

S.c. treatment	Bioavailability [%] to i.v. reference treatments: rD'D3-FP with respective FVIII product i.v.
200 IU/kg Beriate® s.c.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg Beriate® s.c.	6
200 IU/kg Advate® s.c.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg Advate® s.c.	6
200 IU/kg ReFacto® AF s.c.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg ReFacto® AF s.c.	11

n.a.: not applicable

Table 19: Time to trough levels of different FVIIII products (FVIII antigen) after s.c. or i.v. administration in FVIII ko mice

Treatment	**Time to**		
	1% trough [h]	**5% trough** [h]	**10% trough** [h]
200 IU/kg Beriate® s.c.	n.a.	n.a.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg Beriate® s.c.	60	50	42
3 mg/kg rD'D3-FP & 200 IU/kg Beriate® i.v.	79	48	35
200 IU/kg Advate® s.c.	n.a.	n.a.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg Advate® s.c.	66	51	44
3 mg/kg rD'D3-FP & 200 IU/kg Advate® i.v.	68	36	22
200 IU/kg ReFacto AF® s.c.	n.a.	n.a.	n.a.
3 mg/kg rD'D3-FP & 200 IU/kg ReFacto® AF s.c.	104	80	68
3 mg/kg rD'D3-FP & 200 IU/kg ReFacto® AF i.v.	98	64	49

n.a.: modelling not applicable (data below detection limit)

Example 1.4: Investigation of the effect of a rD'D3-FP affinity variant, a rD'D3 molecule with non-albumin HELP and rVIII-SingleChain given both subcutaneously in a mouse hemophilia A model, i.e. in FVIII ko mice.

Experimental details

The test articles were administered s.c. in the neck or i.v. into the lateral tail vein by a single injection, at a total volume of 5 mL/kg. Administered dose levels and routes are given in Table 20.

Table 20: Treatment groups

rD'D3 variant [mg/kg]	rVIII-SingleChain [IU FVIII:C/kg]	Route	Ratio rD'D3-FP:rFVIII
3 rD'D3-FP EYA	200	sc	447
3 rD'D3-FP EYA	200	iv	447
4.29 rD'D3-CTP	200	sc	608
4.29 rD'D3-CTP	200	iv	608

rD'D3-FP EYA was applied at a dose of 3 mg/kg based on human albumin values, rD'D3-CTP at a dose of 4.29 mg/kg based on protein content (leading both to high rD'D3 variant : rVIII-SingleChain doses, Table 20), and FVIII products at a dose of 200 IU/kg chromogenic FVIII activity. rVIII-SingleChain was reconstituted with water for injection, and rD'D3-FP EYA as well as rD'D3-CTP was thawed in a water bath. In every case, a dose volume of 5 mL/kg was administered, using dilution buffer for FVIII for dilution.

Blood samples were taken retrobulbary under short term anaesthesia using an alternating sampling scheme. Timepoints in the s.c. groups were 3, 8, 16, 24, 32, 48, 72, and 96 h p.a., and in the i.v. groups 5 min, 3, 8, 16, 24, 48, 72, and 96 h p.a. The PK profile was taken from four cohorts of mice per group, and n=3 per timepoint. Blood samples were anticoagulated using sodium citrate (1 parts sodium citrate 3.13% + 9 parts blood), processed to plasma and stored at –70°C for the determination of FVIII activity, albumin and/or rD'D3-CTP.

rD'D3-FP EYA exposure was determined by measurement of the albumin part of the construct using a human albumin ELISA. rD'D3-CTP was measured by an ELISA technique using antibodies against anti-human D'D3. Further, FVIII chromogenic activity was measured.

Biostatistics

Estimation of the maximal concentration (C_{max}), the area under the concentration over time curve from t=0 to t=∞ ($AUC_{0\text{-inf}}$), mean residence time (MRT), clearance (CL) and terminal half-life ($t_{1/2}$) was done by two-compartmental modelling in the i.v. calculations, and by two-compartmental-resorption modelling in the s.c. calculations. For parameter estimation, a weighted least-squares cost function was applied. Bioavailability was calculated as the percentage of the $AUC_{0\text{-inf}}$ after s.c. administration as compared to i.v. administration. Time to 1, 5 and 10 % trough levels was calculated by setting the model equation equal to 0.01, 0.05 or 0.1 IU/mL and solving for time.

Results

Evaluation of D'D3 data

Both, rD'D3-FP EYA and rD'D3-CTP, were absorbed after s.c. administration, and could both be quantified over the whole period of observation of 96 h, i.e. remaining above the detection limit of 23.4 ng/mL (Fig. 11).

Estimation of clearance, MRT, $t_{1/2}$ and $AUC_{0\text{-}inf}$ is given in Table 21, showing longer $t_{1/2}$ and MRT for rD'D3-FP EYA as compared to rD'D3-CTP after i.v. as well as after s.c. administration ($t_{1/2}$: 30 h i.v. and 32 h s.c. for EYA longer than 22 h for CTP; MRT: 42 h i.v. and 57 h s.c. for EYA longer than 27 h i.v. and 40 h s.c. for CTP). These data also show that s.c administration was equal or superior over i.v. administration for both rD'D3-FP variants. C_{max} was higher for rD'D3-CTP as compared to rD'D3-EYA, especially after i.v. administration. $AUC_{0\text{-}inf}$ was slightly higher for rD'D3-EYA as compared to rD'D3-CTP after s.c. administration (1094 and 825 µg*h/mL), but there was no major difference after i.v. administration (1669 and 1783 µg*h/mL). These data show that $AUC_{0\text{-}inf}$ is higher after i.v. administration, mostly due to the high initial values.

Bioavailability of rD'D3-FP EYA after subcutaneous administration was 66%, and of rD'D3-CTP was 47% (Table 22), and thus in the range of rD'D3-FP in FVIII ko mice (range 40-79%, Table 4).

Table 21: Pharmacokinetic parameters of rD'D3-FP EYA and rD'D3-CTP after s.c. or i.v. co-administration of rD'D3-FP EYA and rD'D3-CTP with rVIII-SingleChain in FVIII ko mice

Treatment	**C_{max}, extrap.** [µg/mL]	**Clearance** [mL/kg/h]	**MRT** [h]	**Half-life, terminal** [h]	**$AUC_{0\text{-}inf}$** [µg*h/mL]
3 mg/kg rD'D3-FP EYA s.c.	15.7	2.7	57	32	1094
3 mg/kg rD'D3-FP EYA i.v.	62.8	1.8	42	30	1669
4.29 mg/kg rD'D3-CTP s.c.	16.5	5.2	40	22	825
4.29 mg/kg rD'D3-CTP i.v.	144.1	2.4	27	22	1783

Table 22: Bioavailability of rD'D3 variants after s.c. administration in FVIII ko mice calculated against i.v. reference treatments calculated against i.v. reference treatments

S.c. treatment	Bioavailability [%] to respective i.v. reference treatment with rD'D3 variant
3 mg/kg rD'D3-FP EYA s.c.	66
4.29 rD'D3-CTP s.c.	47

Evaluation of FVIII data

FVIII was absorbed when co-administered s.c. with rD'D3-FP EYA or rD'D3-CTP and FVIII activity endured the absorption process (Fig. 12). FVIII activity was quantified over the whole observation period of 96h.

Estimation of clearance, MRT, $t_{1/2}$ and $AUC_{0\text{-}inf}$ is given in Table 23, showing comparable MRT and $t_{1/2}$ for rD'D3-FP EYA as compared to rD'D3-CTP after s.c. administration (MRT: 27 and 29 h, $t_{1/2}$ 13 and 12 h) and slightly higher MRT and $t_{1/2}$ for rD'D3-FP EYA after i.v. administration (MRT: 30 and 25 h, $t_{1/2}$ 21 and 18 h). No difference was observed for $AUC_{0\text{-}inf}$ for the two variants per route of administration (16 and 18 IU*h/mL for s.c. and 111 and 110 IU*h/mL for i.v.). C_{max} was lower for rD'D3-FP EYA compared to rD'D3-CTP after both, i.v. and s.c. administration (0.46 vs. 0.51 IU/mL after s.c. and 4.63 vs. 5.49 after i.v).

Bioavailability of rVIII-SingleChain was 14% for rD'D3-FP EYA and 16% for rD'D3-CTP (Table 24).

Additionally time to trough was calculated for s.c. administration, which showed comparable results for rD'D3-FP EYA and rD'D3-CTP at 1 % (105 and 104 h) and 5 % (76 and 78 h) trough, and a very slight advantage for rD'D3-FP EYA over rD'D3-CTP at 10 % (64 vs. 52 h) trough levels (Table 25). Together, these data demonstrate that the rD'D3 variant is responsible for the improved pharmacokinetics of FVIII, not primarily the type of the half-life extending principle attached to the rD'D3 variant. However, a rD'D3 polypeptide, which does not contain any HELP, is not capable of improving pharmacokinetics of FVIII or at least only with impaired efficacy (see Tables 5 and 7).

Table 23: Pharmacokinetic parameters of FVIII antigen after s.c. or i.v. administration of rD'D3-FP EYA or rD'D3-CTP and rVIII-SingleChain in FVIII ko mice

Treatment	$C_{max,}$ extrap.	Clearance	MRT	Half-life, terminal	$AUC_{0\text{-}inf}$
		FVIII antigen			
	[IU/mL]	[mL/kg/h]	[h]	[h]	[IU*h/mL]
3 mg/kg rD'D3-FP EYA s.c.	0.46	12.8	27	13	16
3 mg/kg rD'D3-FP EYA i.v.	4.63	1.8	30	21	111
4.29 mg/kg rD'D3-CTP s.c.	0.51	11.4	29	12	18
4.29 mg/kg rD'D3-CTP i.v.	5.49	1.8	25	18	110

Table 24: Bioavailability of rVIII-SingleChain (FVIII antigen) after s.c. administration in FVIII ko mice calculated against i.v. reference treatments

S.c. treatment	Bioavailability [%] to respective i.v. reference treatment with rD'D3 variant & 200 IU/kg rVIII-SingleChain
3 mg/kg rD'D3-FP EYA s.c.	14
4.29 mg/kg rD'D3-CTP s.c.	16

Table 25: Time to trough levels of rVIII-SingleChain (FVIII antigen) after s.c. administration in FVIII ko mice

Treatment		Time to	
	1% trough	5% trough	10% trough
	[h]	[h]	[h]
3 mg/kg rD'D3-FP EYA s.c.	105	76	64
4.29 mg/kg rD'D3-CTP s.c.	104	78	52

Conclusion from in vivo experiments

The invention demonstrates subcutaneous bioavailability of rD'D3-FP in different species (Table 26), and relevant bioavailability of a recombinant FVIII product, i.e. rVIII-SingleChain, Advate®, ReFacto AF® or Beriate®, when co-administered subcutaneously with rD'D3-FP (Table 27).

Table 26: Bioavailability of rD'D3-FP in different species

Treatment	Bioavailability	
	Mouse, FVIII ko	Pig
rD'D3-FP alone	43-50 %	59 %
rD'D3-FP with FVIII	40-87 %	70-187 %
rD'D3-His	34 %	n.d.
rD'D3-CTP	47 %	n.d.
rD'D3-FP EYA	66 %	n.d.

Table 27: Bioavailability of FVIII in different species

Treatment	Bioavailability	
	Mouse, FVIII ko	Pig
rD'D3-FP	3-25 %	29-40 %
rD'D3-His	1 %	n.d.
rD'D3-CTP	16 %	n.d.
rD'D3-FP EYA	14 %	n.d.

n.d., not determined

In fact, present results demonstrate that subcutaneous administration of rD'D3-FP together with FVIII allows for extravascular administration of a recombinant FVIII product, i.e. rVIII-SingleChain or other FVIII products, e.g. Beriate®, Advate® or ReFacto AF®, associated with unprecedented absorption of FVIII into the bloodstream (bioavailability range from 3-40 % with rVIII-SingleChain), resulting in FVIII activity levels significantly above the detection limit. rD'D3-CTP and rD'D3-FP EYA showed roughly comparable data to rD'D3-FP. Said resulting FVIII activity levels are suitable for therapeutic application.

rD'D3-FP or variants thereof are favourable over rD'D3-His, not only for the longer half-life of the rD'D3-FP, increasing maintenance of FVIII in plasma once it reached this compartment, but also for the unproportionally high increase in bioavailability of rVIII-SingleChain, when co-administered with rD'D3-FP or variants thereof as compared to rD'D3-His. This supports that half-life prolongation using albumin or other HLEP is a favourable approach.

Example 2: Determination of FVIII affinity to VWF fragment dimer and monomer

A VWF fragment (1-1242) albumin fusion (D´D3-FP) was expressed in a bioreactor; after purification as described above and isolation of monomer and dimer, the affinity of FVIII to these preparations was assessed through surface plasmon resonance via a Biacore instrument (T200, GE Healthcare).

An anti-albumin antibody (MA1-20124, Thermo Scientific) was covalently coupled via its N-terminus to an activated CM 3 chip by NHS (N-Hydroxysuccinimide) and EDC (Ethanolamine hydrochloride), both contained in the amine coupling kit (BR1000-50) from GE Healthcare. For immobilization 3 µg/mL of the antibody were diluted in sodium acetate buffer (10 mM, pH 5.0) and the antibody solution was flown over the chip for 7 min. at a flow rate of 10 µL/min. After the immobilization procedure non-coupled dextran filaments were saturated by flowing ethanolamine solution (1 M, pH 8.3) over the chip for 5 min (at a flow rate of 10 µL/min). The aim of saturating the flow cell was to minimize unspecific binding of the analytes to the chip. A reference flow cell was set up by saturating an empty flow cell with ethanolamine by using the same procedure as above.

Dimeric and monomeric D´D3-FP proteins, respectively, were immobilized to the covalently coupled anti-albumin antibody by a flow of the D´D3-FP proteins (5 µg/mL) over the chip for 3 min (flow rate of 10 µL/min).

To create binding curves for FVIII, each D´D3-FP protein preparation was diluted in running buffer (HBS-P+: 0.1 M HEPES, 1.5 M NaCl and 0.5% v/v Surfactant P20, pH 7.4; product code BR100671, GE Healthcare) to concentrations of 0.25 nM, 0.5 nM, 1 nM, 3nM and 4 nM. By performing a single cycle kinetic, samples with ascending concentrations of each dilution were flown over the chip for 2 min (flow rate 30µL/min.), followed by a dissociation time of 10 min. with running buffer HBS-P+. All measurements were performed twice. The temperature for the measuring procedure was adjusted to +25°C.

Binding parameters were calculated using BiaEvaluation Software. The curve fitting methods were based on Langmuir equations. The input data for calculations were the molar mass of the analyte FVIII (rVIII-SingleChain), other parameters like max. RU and slopes were automatically extracted out of the fitted association and dissociation curves. The outputs of BiaEvaluation Software are the association rate constants and the dissociation rate constants, from which the affinity constants were calculated. The results are shown in Table 28.

Table 28: rFVIII-SingleChain affinity data for D´D3-FP dimer and monomer

D'D3-FP preparation	**ka [1/Ms]**	**kd [1/s]**	**KD [M]**
D'D3-FP Dimer	4.5×10^{7}	1.5×10^{-3}	3.4×10^{-11}
D'D3-FP Monomer	9.9×10^{5}	3.0×10^{-2}	3.0×10^{-8}

The dimeric D'D3-FP shows a significantly (K_D = 34 pM) increased affinity to FVIII compared to the D'D3-FP monomer (K_D = 30 nM) which results both from a faster association and a slower dissociation of rVIII-SingleChain.

Claims

1. A recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment or prophylaxis of a blood coagulation disorder, said treatment or prophylaxis comprising administering the recombinant polypeptide and a Factor VIII protein (FVIII) extravascular to a subject having a blood coagulation disorder, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

2. A recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment or prophylaxis of a blood coagulation disorder, said treatment or prophylaxis comprising administering the recombinant polypeptide extravascular and a Factor VIII protein (FVIII) to a subject having a blood coagulation disorder, wherein said recombinant polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is higher than 50.

3. The recombinant polypeptide for use according to claim 1 or 2, wherein said polypeptide comprises a half-life extending moiety (HLEM).

4. The recombinant polypeptide for use according to any one of the preceding claims, wherein the subject is a human subject.

5. The recombinant polypeptide for use according to any one of the preceding claims, wherein the polypeptide is present as a dimer or at least has a high proportion of dimers.

6. The recombinant polypeptide for use according to claim 5, wherein the dimeric polypeptide has a FVIII binding affinity characterized by a dissociation constant K_D of less than 1 nM, preferably less than 500 pM, less than 200 pM, less than 100 pM, less than 90 pM or less than 80 pM.

7. The recombinant polypeptide for use according to any one of the preceding claims, wherein the polypeptide is administered either subcutaneously, intradermally or intramuscularly.

8. The recombinant polypeptide for use according to any one of the preceding claims, wherein the truncated VWF comprises an amino acid sequence having a sequence identity of at least 90% to amino acids 776 to 805 of SEQ ID NO:4, preferably comprises an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:4.

9. The recombinant polypeptide for use according to any one of claims 1 to 7, wherein the truncated VWF consists either of (a) amino acids 764 to 1242 of SEQ ID NO:4, of (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:4, or of (c) a fragment of (a) or (b).

10. The recombinant polypeptide for use according to any one of claims 3 to 9, wherein the HLEM is a heterologous amino acid sequence fused to the truncated VWF.

11. The recombinant polypeptide for use according to claim 10, wherein said heterologous amino acid sequence comprises or consists of a polypeptide selected from the group consisting of albumin or fragments thereof, transferrin or fragments thereof, the C-terminal peptide of human chorionic gonadotropin, an XTEN sequence, homo-amino acid repeats (HAP), proline-alanine-serine repeats (PAS), afamin, alpha-fetoprotein, Vitamin D binding protein, polypeptides capable of binding under physiological conditions to albumin or to immunoglobulin constant regions, polypeptides capable of binding to the neonatal Fc receptor (FcRn), particularly immunoglobulin constant regions and portions thereof, preferably the Fc portion of immunoglobulin, and combinations thereof.

12. The recombinant polypeptide for use according to any one of claims 3 to 9, wherein the HLEM is conjugated to the recombinant polypeptide.

13. The recombinant polypeptide for use according to claim 12, wherein said HLEM is selected from the group consisting of hydroxyethyl starch (HES), polyethylene glycol (PEG), polysialic acids (PSAs), elastin-like polypeptides, heparosan polymers,

hyaluronic acid and albumin binding ligands, e.g. fatty acid chains, and combinations thereof.

14. The recombinant polypeptide for use according to any one of claims 3 to 13, wherein the mean residence time (MRT) of the administered FVIII is increased by the co-administration of the recombinant polypeptide, preferably by a factor of at least 1.5, at least 2, at least 3, at least 4 or at least 5, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below 50.

15. The recombinant polypeptide for use according to any one of claims 3 to 14, wherein the mean residence time (MRT) of the administered recombinant polypeptide is increased, preferably by a factor of at least 1.5, at least 2 or at least 3, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below 50.

16. The recombinant polypeptide for use according to any one of claims 3 to 15, wherein the terminal half-life of the administered FVIII is increased by the co-administration of the recombinant polypeptide, preferably by a factor of at least 1.2, at least 1.5, at least 2, at least 2.5 or at least 3, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below 50.

17. The recombinant polypeptide for use according to any one of claims 3 to 16, wherein the time period for reaching a 1% trough level of the FVIII co-administered with said polypeptide having a HLEM is prolonged compared to a reference treatment, wherein said reference treatment is identical to said treatment, except the FVIII is administered with a recombinant polypeptide without having said HLEM.

18. The recombinant polypeptide for use according to any one of the preceding claims, wherein the plasma half-life of the polypeptide is increased compared to that of endogenous VWF and/or compared to that of VWF of normal human plasma (NHP), wherein the plasma half-life of the polypeptide is preferably at least 100%, at least 200% or preferably at least 400% higher than that of the endogenous VWF and/or compared to that of VWF of normal human plasma (NHP).

19. The recombinant polypeptide for use according to any one of the preceding claims, wherein the blood coagulation disorder is hemophilia A or von-Willebrand disease.

20. The recombinant polypeptide for use according to any one of the preceding claims, wherein co-administration of the recombinant polypeptide and the FVIII protein is achieved either (i) by administration together in a single composition comprising the recombinant polypeptide and the FVIII protein, or (ii) by administration of the recombinant polypeptide (first compound) and the FVIII protein (second compound) each provided in separate compositions, wherein the first compound is administered before, after or concurrently with the second compound.

21. The recombinant polypeptide for use according to any one of the preceding claims, wherein the FVIII is a plasma derived protein or a recombinant FVIII protein.

22. The recombinant polypeptide for use according to any one of the preceding claims, wherein FVIII is administered extravascularly and wherein the bioavailability of the administered FVIII following co-administration with the recombinant polypeptide is at least 2%, at least 3%, at least 5%, preferably at least 7%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35% or at least 40%.

23. The recombinant polypeptide for use according to any one of the preceding claims, wherein the bioavailability of the recombinant polypeptide is at least 30%, preferably at least 35%, more preferably at least 40%, at least 45%, at least 50%%, at least 55%, at least 60%, at least 65%, at least 70%, or at least 80%.

24. The recombinant polypeptide for use according to any one of the preceding claims, wherein the dosage of co-administered FVIII protein does not exceed 2500 IU/kg, 1500 IU/kg, 1000 IU/kg, 600 IU/kg, 500 IU/kg or 400 IU/kg.

25. The recombinant polypeptide for use according to any one of the preceding claims, wherein following co-administration of said recombinant polypeptide with FVIII the maximal concentration (C_{max}) for FVIII is at least 10 mIU/mL, at least 25 mIU/mL, at least 50 mIU/mL, at least 100 mIU/mL, at least 200 mIU/mL, at least 300 mIU/mL or at least 400 mIU/mL FVIII activity, preferably chromogenic FVIII activity.

26. The recombinant polypeptide for use according to any one of claims 3 to 25, wherein following co-administration of said recombinant polypeptide with FVIII the clearance (CL) value for the recombinant polypeptide is reduced by a factor of at least 2, at least 5 or at least 10, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below 50.

27. The recombinant polypeptide for use according to any one of claims 3 to 26, wherein following co-administration of said recombinant polypeptide with FVIII the clearance (CL) value of the administered FVIII is reduced compared to a reference treatment, preferably by a factor of at least 1.5, at least 2, at least 3, at least 5, at least 7.5 or at least 10, wherein said reference treatment is identical to said treatment, except that the recombinant polypeptide to be administered does not comprise a HLEM and/or except that the molar ratio of the recombinant polypeptide to be administered to the FVIII to be administered is below 50.

28. The recombinant polypeptide for use according to any one of the preceding claims, wherein the molar ratio of the recombinant polypeptide to the FVIII to be administered is at least 75, at least 100, at least 200, at least 300, at least 400, at least 500 or at least 1000.

29. A pharmaceutical composition for use in the treatment or prophylaxis of a blood coagulation disorder as defined in any one of claims 1 to 28, the composition comprising

(i) a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) according to any one of claims 1 to 28, and

(ii) a Factor VIII protein (FVIII);

wherein the molar ratio of the recombinant polypeptide to the FVIII protein within the pharmaceutical composition is greater than 50 and wherein said recombinant polypeptide is capable of binding to said FVIII,

said treatment comprising administering the pharmaceutical composition extravascular to a subject suffering from a blood coagulation disorder, and

said pharmaceutical composition is formulated for extravascular co-administration.

30. A pharmaceutical kit comprising (i) a first composition comprising a Factor VIII (FVIII) protein and (ii) a second composition comprising a recombinant polypeptide comprising a truncated von Willebrand Factor (VWF) provided for use according to any one of claims 1 to 28 for use in the treatment or prophylaxis of a blood coagulation disorder.

Figure 1

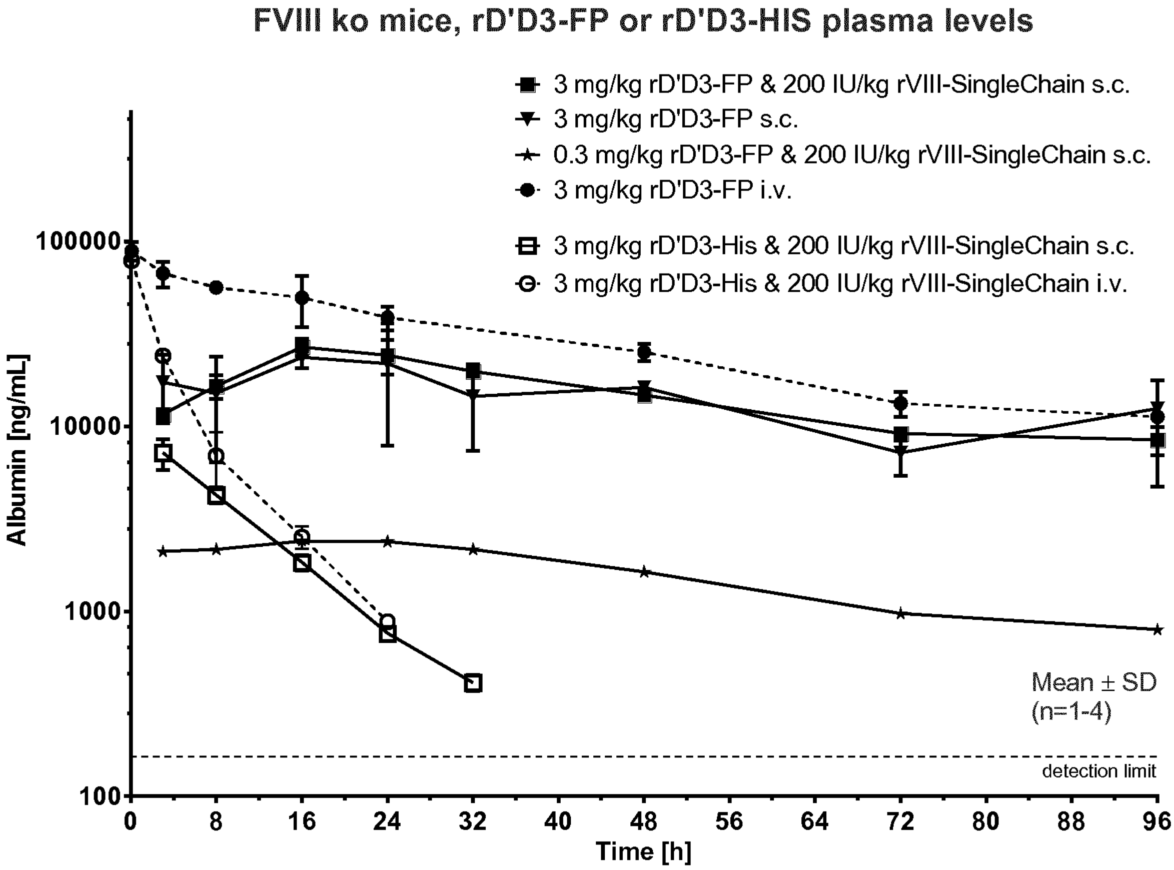
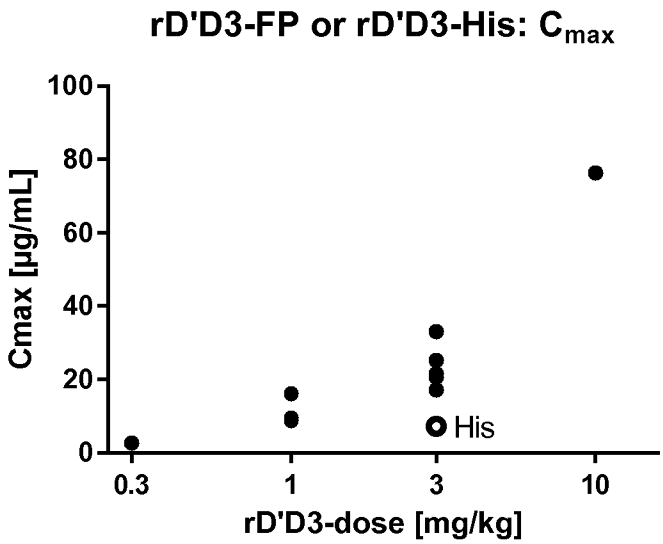

FVIII ko mice, rD'D3-FP or rD'D3-HIS plasma levels
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP s.c.
0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP i.v.
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain i.v.
Albumin [ng/mL]
100000
10000
1000
100
Mean ± SD
(n=1-4)
detection limit
0
8
16
24
32
40
48
56
64
72
80
88
96
Time [h]

Figure 2


rD'D3-FP or rD'D3-His: C_{max}
Cmax [µg/mL]
100
80
60
40
20
0
His
0.3
1
3
10
rD'D3-dose [mg/kg]

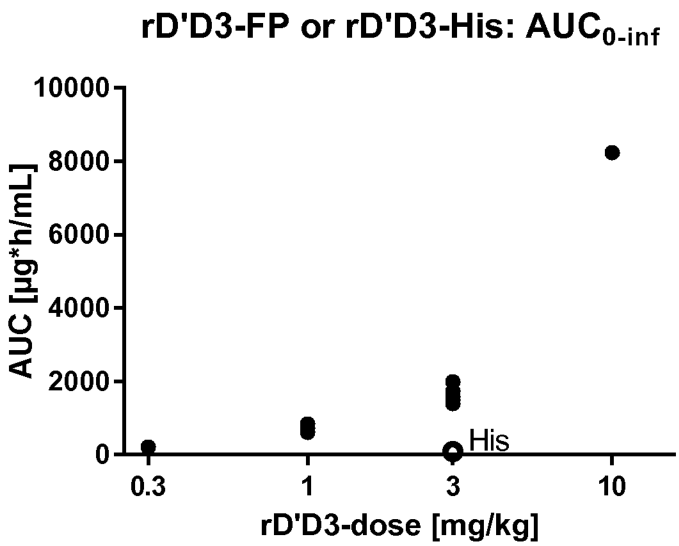

rD'D3-FP or rD'D3-His: AUC_{0-inf}
AUC [µg*h/mL]
10000
8000
6000
4000
2000
0
His
0.3
1
3
10
rD'D3-dose [mg/kg]

Figure 3

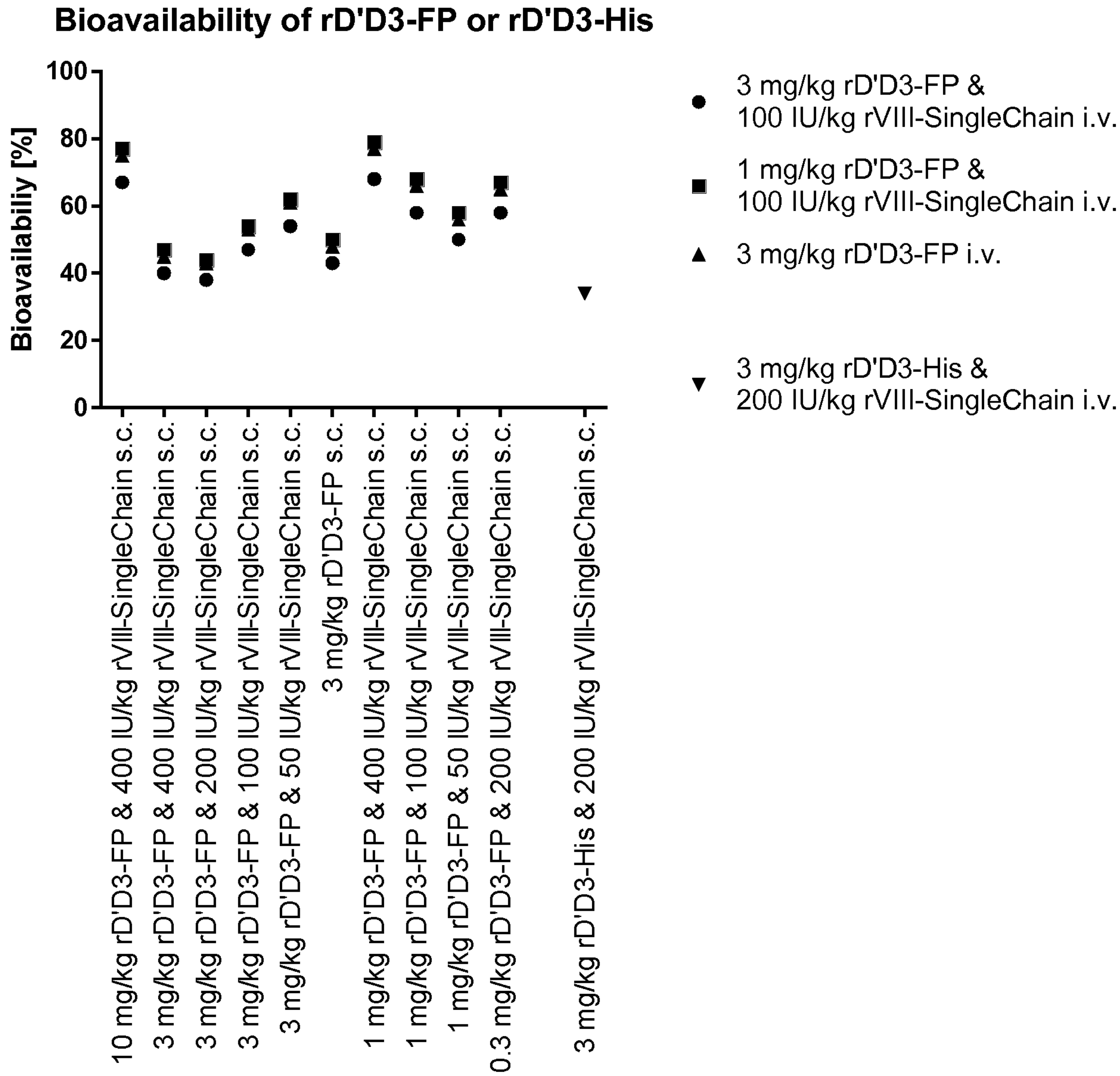

Bioavailability of rD'D3-FP or rD'D3-His
Bioavailabiliy [%]
100
80
60
40
20
0
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP s.c.
1 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.
1 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.
0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.
3 mg/kg rD'D3-FP i.v.
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain i.v.

Figure 4

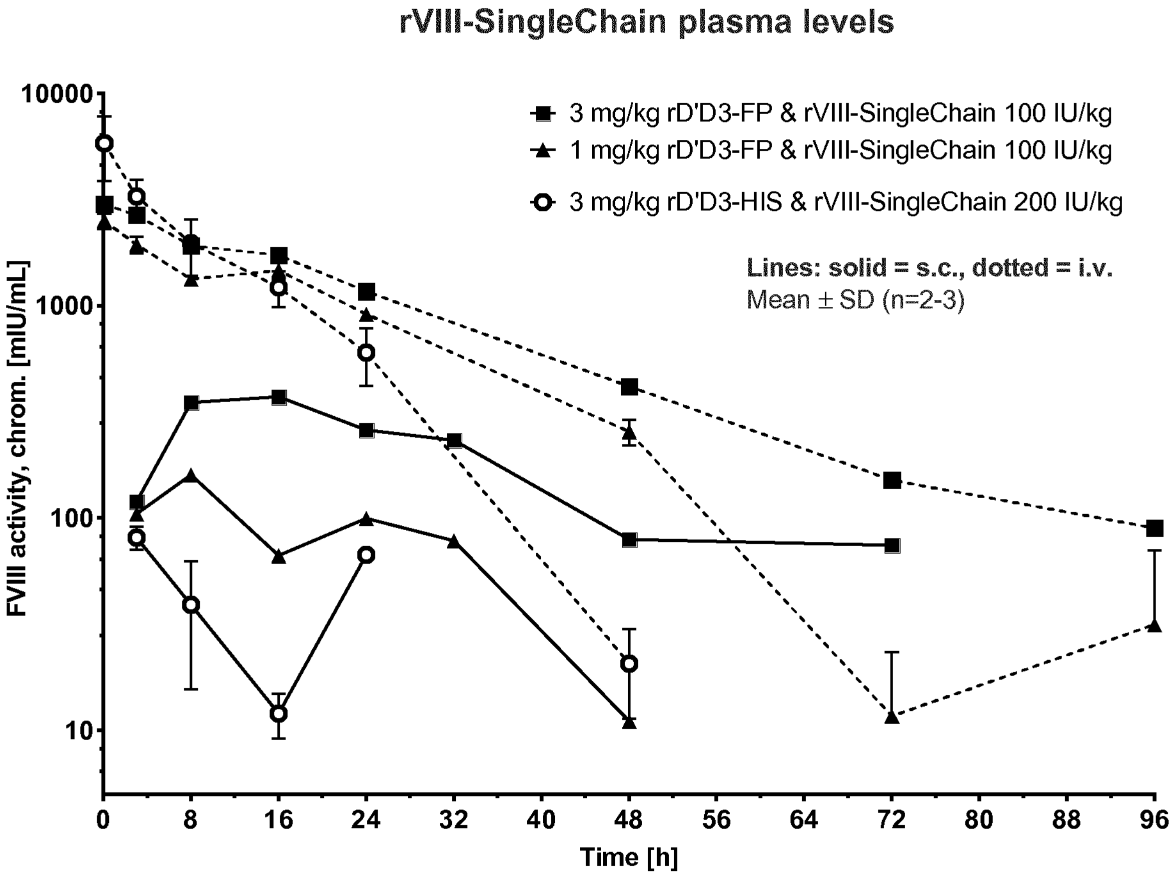
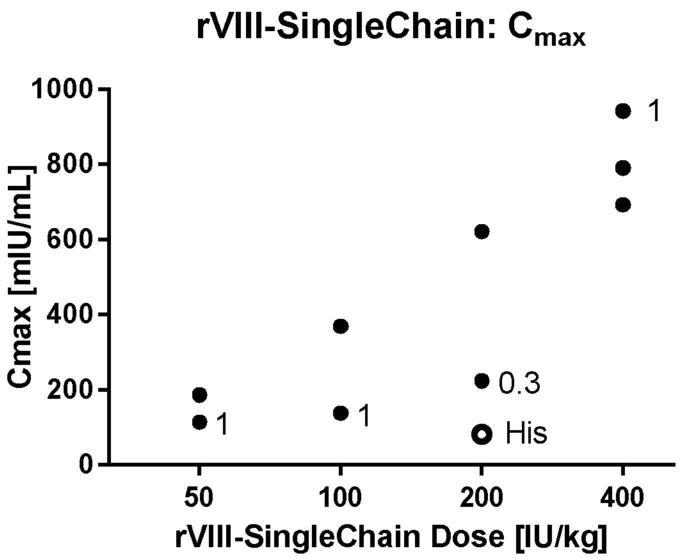

rVIII-SingleChain plasma levels
3 mg/kg rD'D3-FP & rVIII-SingleChain 100 IU/kg
1 mg/kg rD'D3-FP & rVIII-SingleChain 100 IU/kg
3 mg/kg rD'D3-HIS & rVIII-SingleChain 200 IU/kg
Lines: solid = s.c., dotted = i.v.
Mean ± SD (n=2-3)
FVIII activity, chrom. [mIU/mL]
10000
1000
100
10
0
8
16
24
32
40
48
56
64
72
80
88
96
Time [h]

Figure 5


rVIII-SingleChain: C_{max}
Cmax [mIU/mL]
1000
800
600
400
200
0
50
100
200
400
rVIII-SingleChain Dose [IU/kg]
1
1
1
0.3
His

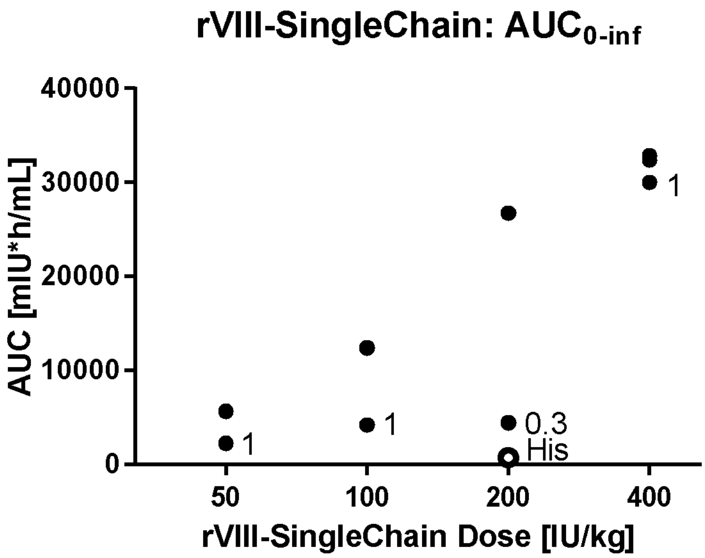

rVIII-SingleChain: AUC_{0-inf}
AUC [mIU*h/mL]
40000
30000
20000
10000
0
50
100
200
400
rVIII-SingleChain Dose [IU/kg]
1
1
1
0.3
His

Figure 6

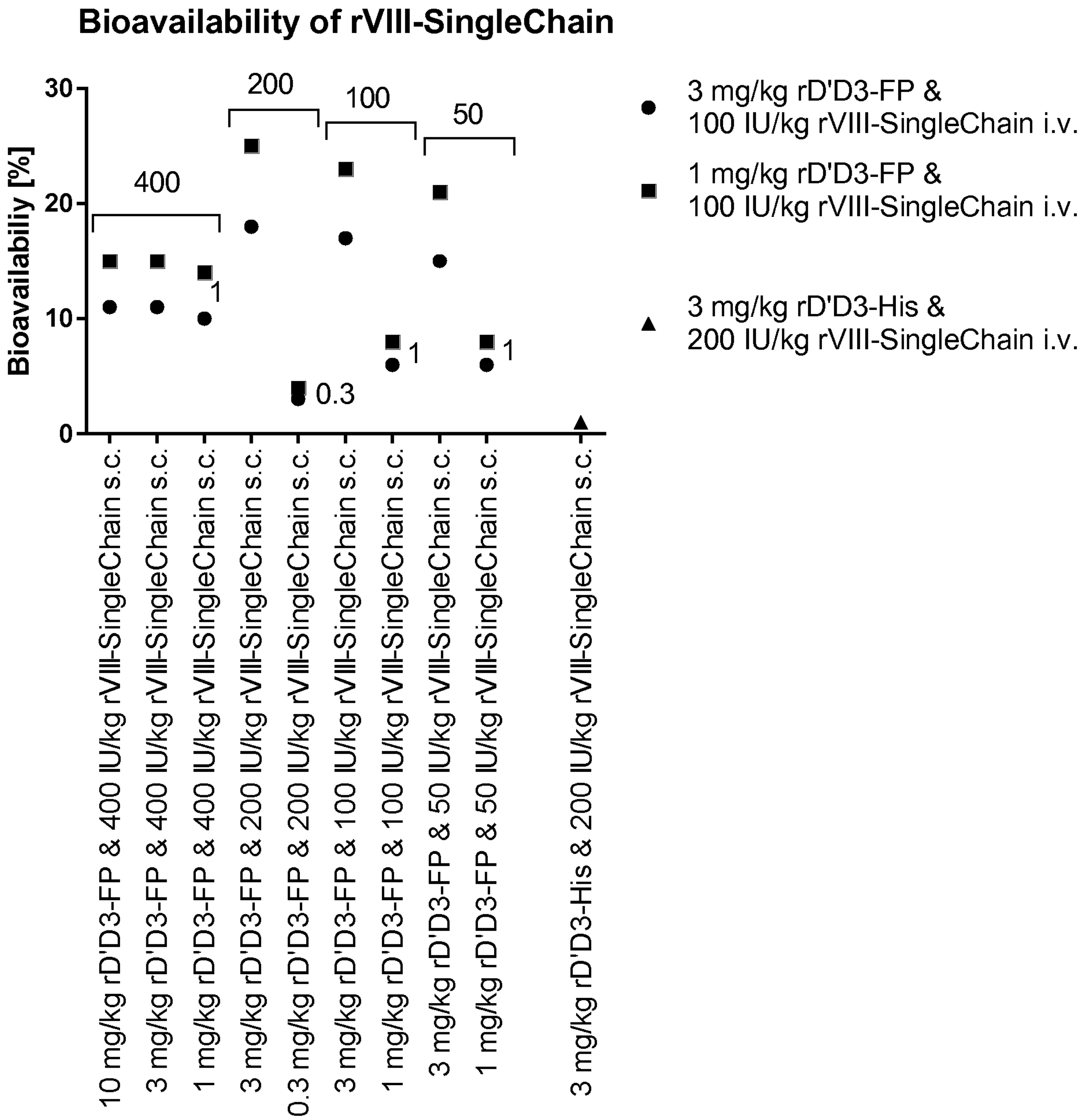

Bioavailability of rVIII-SingleChain
Bioavailabiliy [%]
30
20
10
0
400
200
100
50
1
0.3
1
1
10 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.
1 mg/kg rD'D3-FP & 400 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.
0.3 mg/kg rD'D3-FP & 200 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.
1 mg/kg rD'D3-FP & 50 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain s.c.
3 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.
1 mg/kg rD'D3-FP & 100 IU/kg rVIII-SingleChain i.v.
3 mg/kg rD'D3-His & 200 IU/kg rVIII-SingleChain i.v.

Figure 7

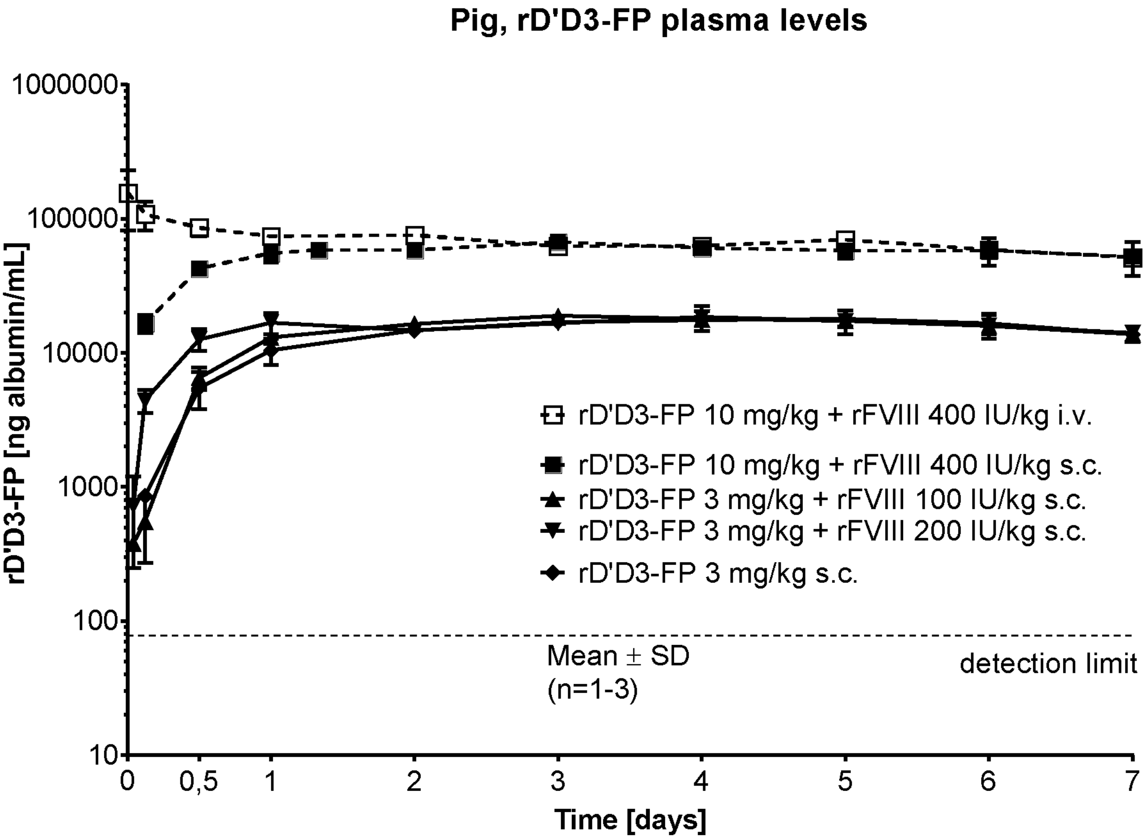

Pig, rD'D3-FP plasma levels
1000000
100000
10000
1000
100
10
rD'D3-FP [ng albumin/mL]
0
0,5
1
2
3
4
5
6
7
Time [days]
rD'D3-FP 10 mg/kg + rFVIII 400 IU/kg i.v.
rD'D3-FP 10 mg/kg + rFVIII 400 IU/kg s.c.
rD'D3-FP 3 mg/kg + rFVIII 100 IU/kg s.c.
rD'D3-FP 3 mg/kg + rFVIII 200 IU/kg s.c.
rD'D3-FP 3 mg/kg s.c.
Mean ± SD
(n=1-3)
detection limit

Figure 8

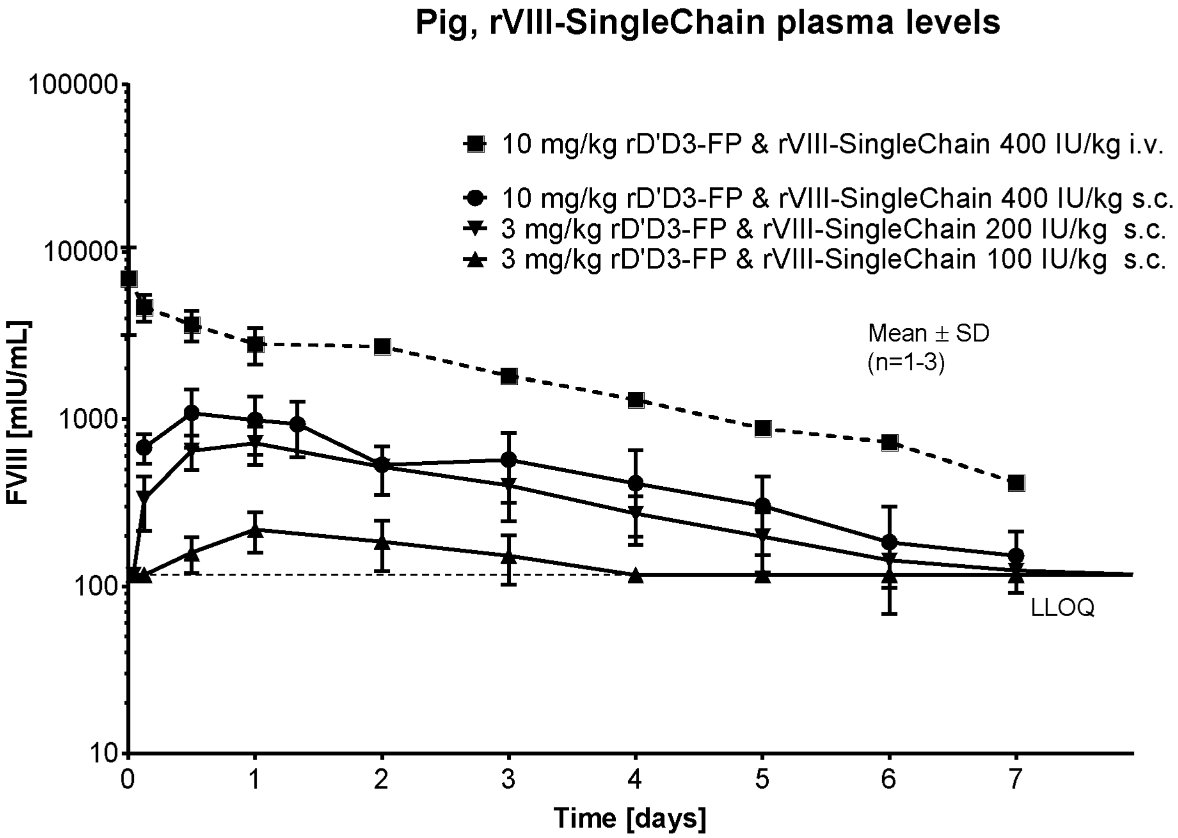

Pig, rVIII-SingleChain plasma levels
100000
10000
1000
100
10
FVIII [mIU/mL]
10 mg/kg rD'D3-FP & rVIII-SingleChain 400 IU/kg i.v.
10 mg/kg rD'D3-FP & rVIII-SingleChain 400 IU/kg s.c.
3 mg/kg rD'D3-FP & rVIII-SingleChain 200 IU/kg s.c.
3 mg/kg rD'D3-FP & rVIII-SingleChain 100 IU/kg s.c.
Mean ± SD
(n=1-3)
LLOQ
0
1
2
3
4
5
6
7
Time [days]

Figure 9

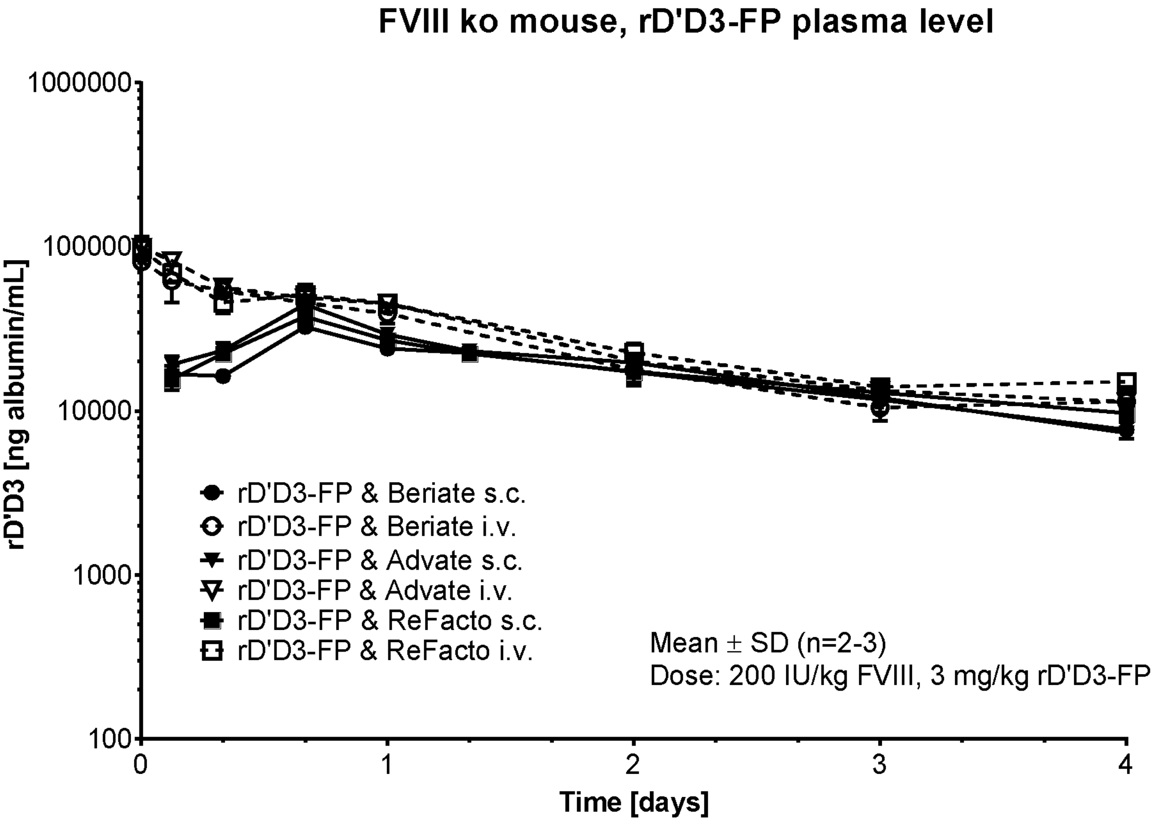
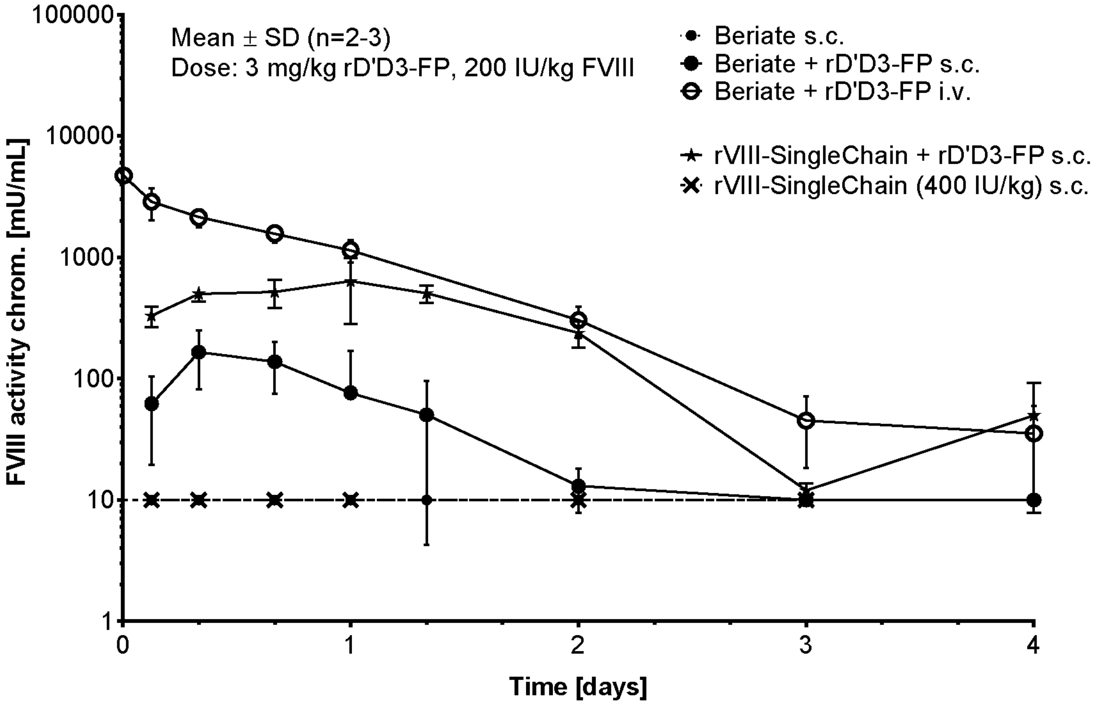

FVIII ko mouse, rD'D3-FP plasma level
1000000
100000
10000
1000
100
rD'D3 [ng albumin/mL]
0
1
2
3
4
Time [days]
rD'D3-FP & Beriate s.c.
rD'D3-FP & Beriate i.v.
rD'D3-FP & Advate s.c.
rD'D3-FP & Advate i.v.
rD'D3-FP & ReFacto s.c.
rD'D3-FP & ReFacto i.v.
Mean ± SD (n=2-3)
Dose: 200 IU/kg FVIII, 3 mg/kg rD'D3-FP

Figure 10

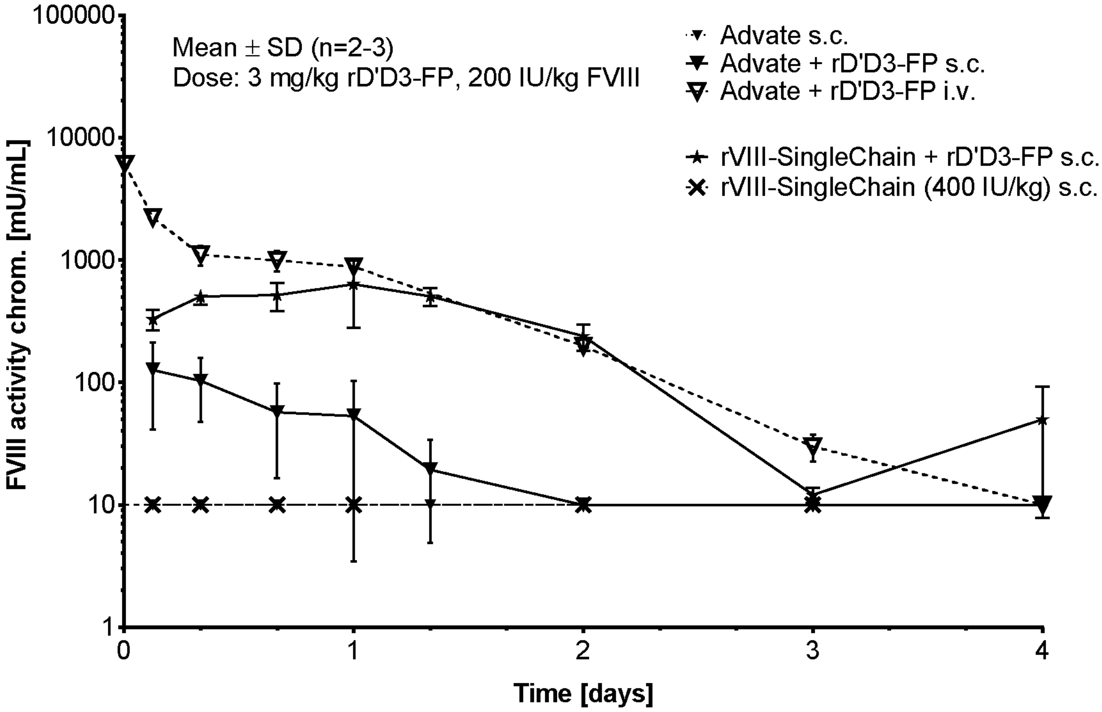
A

FVIII ko mouse, FVIII plasma level



B

FVIII ko mouse, FVIII plasma level



C

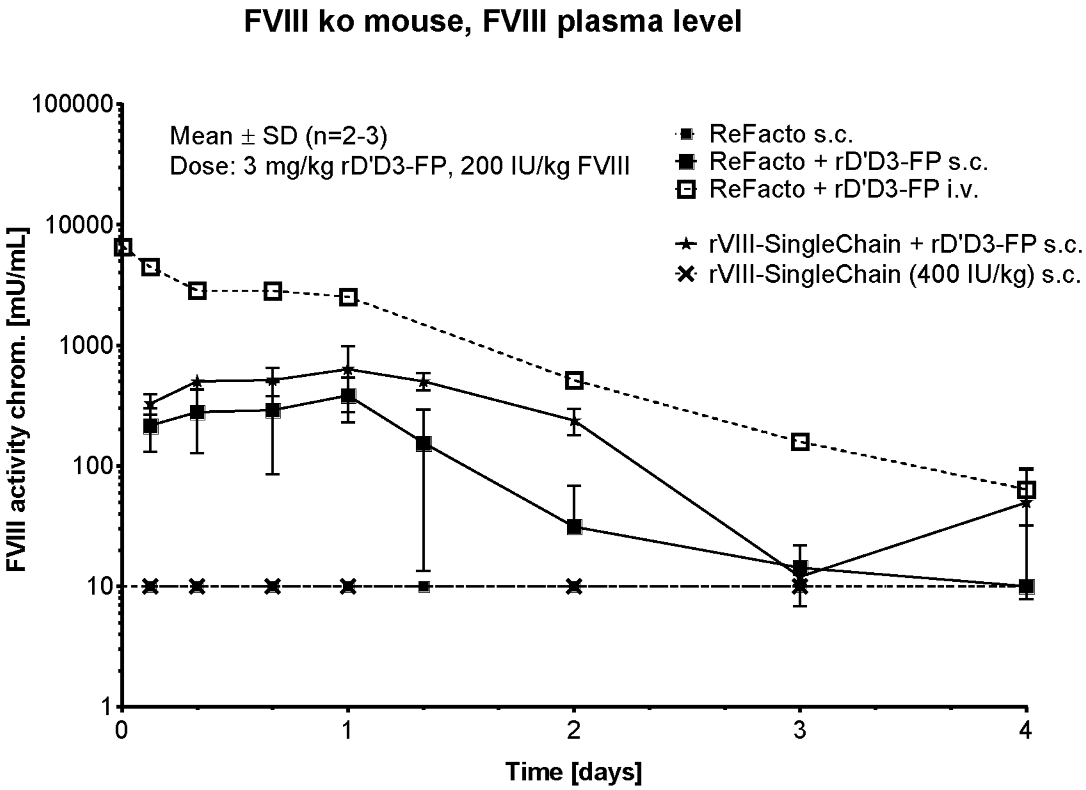

FVIII ko mouse, FVIII plasma level
Mean ± SD (n=2-3)
Dose: 3 mg/kg rD'D3-FP, 200 IU/kg FVIII
ReFacto s.c.
ReFacto + rD'D3-FP s.c.
ReFacto + rD'D3-FP i.v.
rVIII-SingleChain + rD'D3-FP s.c.
rVIII-SingleChain (400 IU/kg) s.c.
FVIII activity chrom. [mU/mL]
100000
10000
1000
100
10
1
0
1
2
3
4
Time [days]

Figure 11

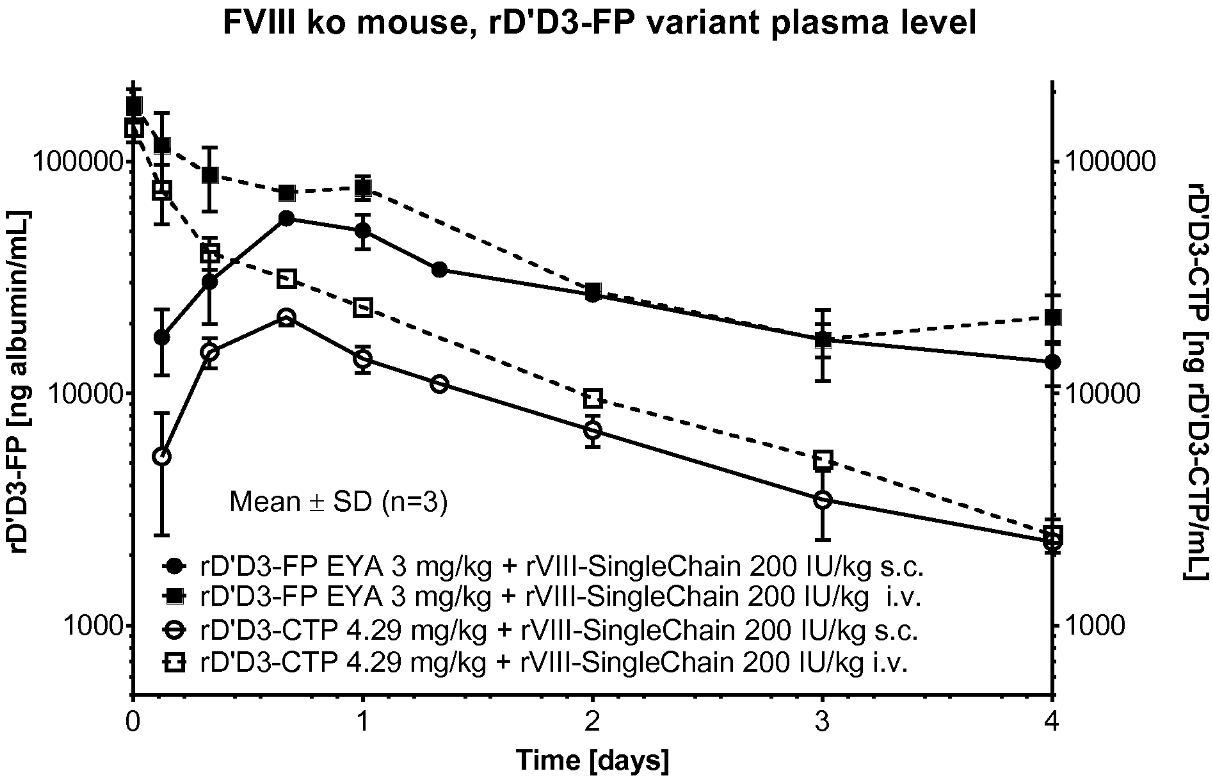
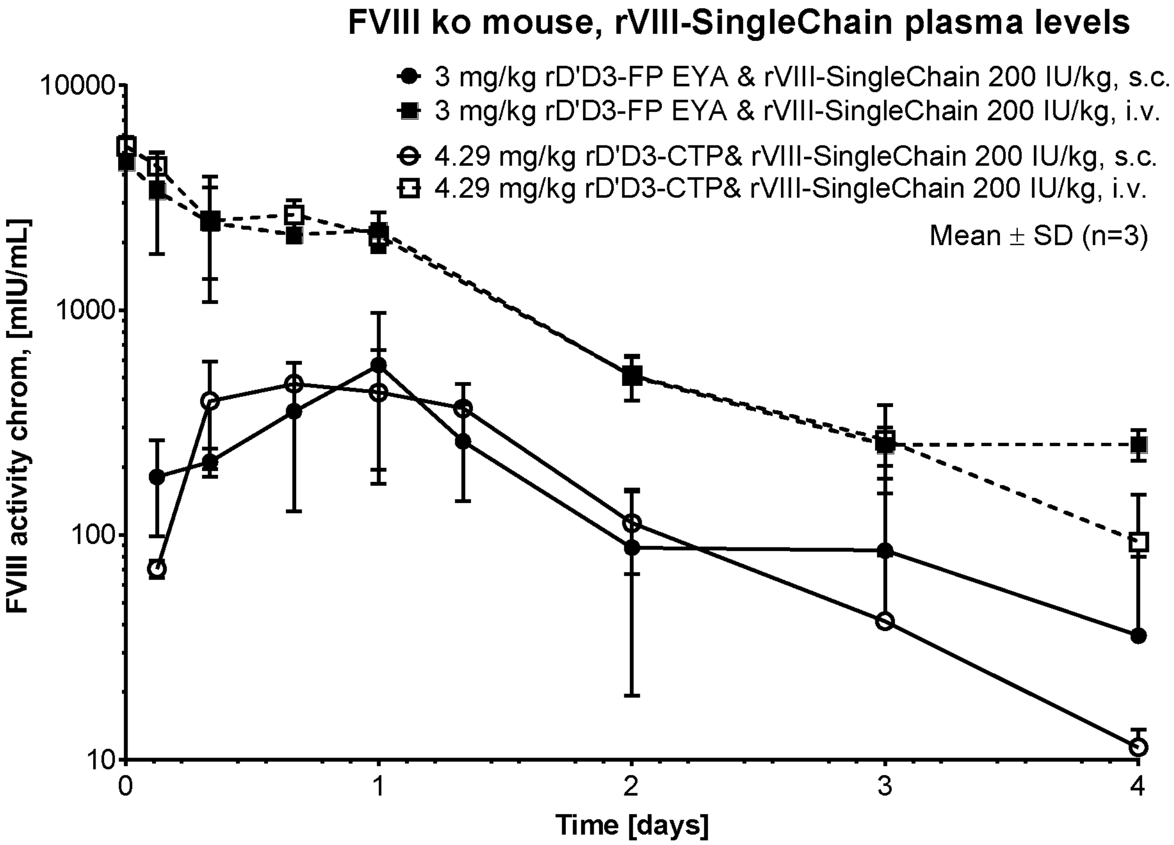

FVIII ko mouse, rD'D3-FP variant plasma level
rD'D3-FP [ng albumin/mL]
rD'D3-CTP [ng rD'D3-CTP/mL]
100000
10000
1000
Mean ± SD (n=3)
rD'D3-FP EYA 3 mg/kg + rVIII-SingleChain 200 IU/kg s.c.
rD'D3-FP EYA 3 mg/kg + rVIII-SingleChain 200 IU/kg i.v.
rD'D3-CTP 4.29 mg/kg + rVIII-SingleChain 200 IU/kg s.c.
rD'D3-CTP 4.29 mg/kg + rVIII-SingleChain 200 IU/kg i.v.
0
1
2
3
4
Time [days]

Figure 12


FVIII ko mouse, rVIII-SingleChain plasma levels
3 mg/kg rD'D3-FP EYA & rVIII-SingleChain 200 IU/kg, s.c.
3 mg/kg rD'D3-FP EYA & rVIII-SingleChain 200 IU/kg, i.v.
4.29 mg/kg rD'D3-CTP& rVIII-SingleChain 200 IU/kg, s.c.
4.29 mg/kg rD'D3-CTP& rVIII-SingleChain 200 IU/kg, i.v.
Mean ± SD (n=3)
FVIII activity chrom, [mIU/mL]
10000
1000
100
10
0
1
2
3
4
Time [days]

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/078840

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/36 A61K38/37 C07K14/755 A61P7/04 A61K36/36
A61K36/37
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, COMPENDEX, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/160005 A1 (NOVO NORDISK AS [DK]) 31 October 2013 (2013-10-31)	1-30
Y	page 1, line 3 page 2, line 14 - line 19 page 13, line 17 - line 20 page 22, line 13 - line 28 page 26, line 24 - line 35 -----	1-30
X	WO 2013/083858 A1 (NOVO NORDISK AS [DK]) 13 June 2013 (2013-06-13) cited in the application	1-30
Y	page 1, line 3 page 2, line 13 - line 26 page 22, line 1 - line 16 page 26, line 17 - line 28 page 31, line 5 - line 9 -----	1-30
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 January 2018	26/01/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pilling, Stephen

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/198699 A2 (NOVO NORDISK AS [DK]) 18 December 2014 (2014-12-18) cited in the application	1-30
Y	page 1, line 3 page 2, line 16 - line 26 page 22, line 28 - page 23, line 8 page 27, line 25 - page 28, line 2 page 29, line 25 - line 29 -----	1-30
X	L. L. SWYSTUN ET AL: "FVIII stabilization: VWF D'D3 will do", BLOOD, vol. 124, no. 3, 17 July 2014 (2014-07-17), pages 313-315, XP055330357, & 53RD ANNUAL MEETING AND EXPOSITION OF THE AMERICAN-SOCIETY-OF-HEMATOLOGY (ASH); SAN DIEGO, CA, USA; DECEMBER 10 -13, 2011 ISSN: 0006-4971, DOI: 10.1182/blood-2014-06-578955	1-30
Y	page 313, column 1, paragraph 1 page 314, column 1, paragraph 2 - column 2, paragraph 1 page 314, column 2, paragraph 2 -----	1-30
X	A. YEE ET AL: "A von Willebrand factor fragment containing the D'D3 domains is sufficient to stabilize coagulation factor VIII in mice", BLOOD, vol. 124, no. 3, 21 May 2014 (2014-05-21), pages 445-452, XP055225855, & 53RD ANNUAL MEETING AND EXPOSITION OF THE AMERICAN-SOCIETY-OF-HEMATOLOGY (ASH); SAN DIEGO, CA, USA; DECEMBER 10 -13, 2011 ISSN: 0006-4971, DOI: 10.1182/blood-2013-11-540534 cited in the application	1-30
Y	abstract -----	1-30
X	YEE ANDREW ET AL: "Partial in Vivo FVIII Stabilization by VWF Fragments", BLOOD, vol. 120, no. 21, November 2012 (2012-11), page 15, XP009194156, & 54TH ANNUAL MEETING AND EXPOSITION OF THE AMERICAN-SOCIETY-OF-HEMATOLOGY (ASH); ATLANTA, GA, USA; DECEMBER 08 -11, 2012 cited in the application	1-30
Y	abstract -----	1-30

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/078840

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013160005 A1	31-10-2013	CN 104411323 A	11-03-2015
		EP 2841091 A1	04-03-2015
		JP 2015519313 A	09-07-2015
		US 2015045303 A1	12-02-2015
		US 2016120954 A1	05-05-2016
		WO 2013160005 A1	31-10-2013
WO 2013083858 A1	13-06-2013	CN 104411716 A	11-03-2015
		EP 2841451 A1	04-03-2015
		JP 2015515482 A	28-05-2015
		US 2014018297 A1	16-01-2014
		US 2015080309 A1	19-03-2015
		US 2016318991 A1	03-11-2016
		WO 2013083858 A1	13-06-2013
WO 2014198699 A2	18-12-2014	CN 105452289 A	30-03-2016
		EP 3008084 A2	20-04-2016
		JP 2016522219 A	28-07-2016
		US 2016207977 A1	21-07-2016
		WO 2014198699 A2	18-12-2014