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Vincent Muczynski, Caterina Casari, François Moreau, Gabriel Aymé, Charlotte Kawecki, et al.. A factor VIII–nanobody fusion protein forming an ultrastable complex with VWF: effect on clearance and antibody formation. *Blood*, 2018, 132 (11), pp.1193-1197. 10.1182/blood-2018-01-829523 . hal-04456589

HAL Id: hal-04456589

<https://hal.science/hal-04456589v1>

Submitted on 14 Feb 2024

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1 **A Factor VIII-nanobody fusion protein forming an ultra-stable complex with**
2 **VWF: effect on clearance & antibody formation**

3

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19

20 **Running title:** A FVIII-nanobody fusion protein

21

22 **Keywords:** Hemophilia, Factor VIII, single-domain antibody

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24

25 Abstract count: 196 words

26 Word count: 1198 (max 1200)

27 Figures: 2

28 References: 25

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1 **Abstract**

2 Von Willebrand factor (VWF) modulates factor VIII (FVIII) clearance and the anti-
3 FVIII immune response. Despite the high affinity that defines the FVIII/VWF
4 interaction, association/dissociation kinetics dictates 2-5% FVIII being present as free
5 protein. To avoid free FVIII when studying the FVIII-VWF complex in vivo, we
6 designed a FVIII-nanobody fusion protein, with the nanobody part being directed
7 against VWF. This fusion protein, designated FVIII-KB013bv, had a 25-fold higher
8 affinity compared to B-domainless FVIII (BDD-FVIII) for VWF. In vitro analysis
9 revealed full cofactor activity in one-stage clotting and chromogenic assays
10 (activity/antigen ratio 1.0 ± 0.3 and 1.1 ± 0.3 , respectively). In vivo, FVIII-013bv
11 displayed a two-fold increased mean residence time compared to BDD-FVIII (3.0h
12 versus 1.6h). In a tail clip-bleeding assay performed 24h after FVIII infusion, blood
13 loss was significantly reduced in mice receiving FVIII-KB013bv versus BDD-FVIII
14 (15 ± 7 microliter versus 194 ± 146 microliter; $p=0.0043$). Unexpectedly, when
15 examining anti-FVIII antibody formation in FVIII-deficient mice, the immune-response
16 towards FVIII-KB013bv was significantly reduced compared to BDD-FVIII (1/8 versus
17 14/16 mice produced anti-FVIII antibodies after treatment with FVIII-KB013bv and
18 BDD-FVIII, respectively). Our data show that a stabilized interaction between FVIII
19 and VWF is associated with a prolonged survival of FVIII and a reduced immune
20 response against FVIII.

21

22 **Key points:**

- 23 - The fusion between factor VIII and anti-VWF nanobodies increases affinity for VWF
24 25-fold without compromising FVIII activity
25 - Stabilized VWF binding results in a 2-fold enhanced circulatory survival of FVIII and
26 reduced anti-FVIII antibody formation

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29

1 **Introduction**

2 Factor VIII (FVIII) is a protein pre-cofactor critical to coagulation. Following its
3 secretion, FVIII circulates bound to von Willebrand factor (VWF), which is important
4 to stabilize FVIII and to prevent its premature degradation and clearance.¹⁻⁵ VWF
5 also modulates anti-FVIII antibody development in hemophilia A-patients, in part by
6 regulating FVIII uptake by antigen-presenting cells.⁶⁻⁸ Noteworthy, FVIII is
7 endocytosed following binding of the VWF-FVIII complex to the surface of antigen-
8 presenting cells, whereas VWF is protected from internalization.⁹

9

10 The molecular basis of VWF-FVIII complex formation is elucidated in great detail,
11 and involves interactions between the VWF D'-D3 region and two regions within
12 FVIII: the acidic region a3 including sulfated-Tyr1680 (Tyr1699 in HGVS-numbering),
13 and the C-terminal C1-C2 domains.¹⁰⁻¹² About 2-5% of FVIII circulates as free protein
14 despite its high affinity for VWF (0.2-1.5 nM), and is cleared faster than VWF-bound
15 FVIII.¹²⁻¹⁸ The pool of free FVIII is maintained via continuous release of FVIII from the
16 FVIII-VWF complex.¹⁹ Investigating the VWF-FVIII complex *in vivo* is therefore
17 complicated by the constitutive presence of free FVIII, which displays differential
18 clearance and uptake by antigen-presenting cells compared to VWF-bound FVIII. To
19 minimize the contribution of free FVIII, we designed a FVIII-variant containing a
20 nanobody against the VWF D'-D3 domain. Promoted by this nanobody, an ultra-
21 stable FVIII-VWF complex is formed, resulting in a prolonged FVIII survival and
22 reduced anti-FVIII immune response.

23

24

1 **Study design**

2 An extensive description of the experimental procedures is given in the
3 Supplementary Materials.

4

5 *Clearance*

6 F8-deficient mice were given FVIII (250U/kg) and residual FVIII activity was
7 determined using a chromogenic assay in plasma taken between 3 min and 48h.

8

9 *Tail clip assay*

10 F8-deficient mice were given FVIII (500U/kg) and after 24h the terminal 3mm of the
11 tail was amputated. Blood loss was monitored over 30 min.

12

13 *Formation of anti-FVIII antibodies*

14 F8-deficient mice were given 4 FVIII injections (50U/kg) at a weekly interval, and a 5th
15 injection at day 40. The presence of anti-FVIII antibodies was analyzed in plasma
16 taken at day 43.

17

18

1 Results and discussion

2 To improve FVIII-VWF complex formation, we used the notion that antibodies often
3 have low dissociation rates, and that small-sized nanobodies display high-affinity
4 antigen binding. From our library of anti-VWF nanobodies²⁰, we isolated three
5 nanobodies that recognize the FVIII-binding D'D3-region (Fig. 1A), while leaving FVIII
6 binding to VWF unaffected. These nanobodies have slow dissociation rates
7 (apparent dissociation rate constants ($k_{\text{off,app}}$): $2.0 \pm 1.1 \times 10^{-5} \text{ s}^{-1}$, $0.6 \pm 0.5 \times 10^{-5} \text{ s}^{-1}$, and
8 $2.2 \pm 1.2 \times 10^{-5} \text{ s}^{-1}$ for KB-VWF-008, -011 and -013, respectively; Fig. 1B). These values
9 are about 100-fold lower compared to the average k_{off} , reported in different studies for
10 the FVIII-VWF interaction (*i.e.* $k_{\text{off,app}} = 2.2 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$, range $2.3 \times 10^{-4} \text{ s}^{-1}$ to 6.6×10^{-3}
11 s^{-1}).^{16-18,21,22}

12
13 Although KB-VWF-011 had the lowest $k_{\text{off,app}}$, KB-VWF-013 was fused with FVIII,
14 because its cross-reactivity with murine VWF facilitates *in vivo* experiments. In this
15 fusion protein, named FVIII-KB013bv, two copies of KB-VWF-013 are replacing B-
16 domain residues Gln744-Arg1648, allowing FVIII to be secreted as a single chain
17 protein (Fig. 1C-D; Supplementary Fig. S1). Natural thrombin-activation sites at
18 Arg372, Arg740 and Arg1689 are maintained. Consequently, thrombin generates
19 similar proteolytic products upon activation of FVIII-KB013bv as for B-domainless
20 FVIII (BDD-FVIII), while the nanobody portion is released (Fig. 1D). Probably, this
21 nanobody portion will remain bound to VWF *in vivo*. FVIII-KB013bv exhibits full
22 cofactor activity in one-stage clotting and chromogenic activity assays
23 (activity/antigen ratios: 1.0 ± 0.3 (n=5) and 1.1 ± 0.3 (n=5), respectively; Supplementary
24 Table S1). When comparing VWF binding in an ELISA, FVIII-KB013bv was about 40-
25 fold more efficient than BDD-FVIII, with half-maximal binding being $4.0 \pm 2.1 \text{ ng/ml}$
26 ($2.1 \pm 1.1 \times 10^{-11} \text{ nM}$) and $170 \pm 22 \text{ ng/ml}$ ($1.1 \pm 0.1 \times 10^{-9} \text{ nM}$; Fig. 1E), respectively. Kinetic
27 measurements revealed an apparent affinity constant of $13 \pm 11 \text{ pM}$, 25-fold more
28 efficient compared to BDD-FVIII ($330 \pm 127 \text{ pM}$; Supplementary Fig. S2). Based on
29 this affinity, we calculated that 0.14% of FVIII-KB013bv is present as free protein in
30 plasma, compared to 3.3% of FVIII (Supplementary Materials). Binding of FVIII-
31 KB013bv but not BDD-FVIII to VWF was preserved after introduction of a
32 Tyr1680Phe mutation (Supplementary Fig. S3), which eliminates natural VWF
33 binding in FVIII. Thus, FVIII-KB013bv has full cofactor activity *in vitro*, and binds
34 more efficiently than BDD-FVIII to VWF.

35
36 We then tested survival of FVIII-KB013bv in F8-deficient mice, that have normal
37 VWF:Ag levels (Supplementary Fig. S4). Compared to BDD-FVIII, the mean

1 residence time for FVIII-KB013bv was increased about 2-fold (1.6h (95%-CI: 1.3-
2 2.1h) and 3.0h (95%-CI: 2.4-4.2h) for BDD-FVIII and FVIII-KB013bv, respectively;
3 $p=0.044$; Fig. 2A). Since FVIII-KB013bv remains associated to VWF, these data
4 support the view that FVIII is cleared as part of the FVIII-VWF complex rather than as
5 free protein.^{1,23} The prolonged survival was confirmed in a tail clip-assay performed
6 24h after injection. In our hands, this model requires 0.2U/ml FVIII to arrest bleeding,
7 and according to the pharmacokinetic parameters, BDD-FVIII levels are about
8 0.07U/ml compared to 0.27U/ml for FVIII-KB013bv 24h after infusion of 500U/kg.
9 Indeed, blood loss was significantly higher in BDD-FVIII *versus* FVIII-KB013bv
10 treated mice ($194\pm 146\mu\text{l}$ ($n=5$) *versus* $15\pm 7\mu\text{l}$ ($n=6$); $\text{mean}\pm\text{SD}$; $p=0.0043$; Fig. 2B).
11 Importantly, prolonged expression of FVIII-KB013bv at supra-physiological levels
12 (4U/ml) did not induce an increase in thrombotic makers (D-dimer, thrombin-
13 antithrombin complexes; Supplementary Fig. S5). Apparently, improved FVIII-VWF
14 binding is associated with prolonged circulatory survival of FVIII, and FVIII-KB013bv
15 is functionally active *in vivo*. Furthermore, the nanobody fragment that remains bound
16 to VWF does not seem to impair VWF function.

17
18 VWF may reduce the development of anti-FVIII antibodies.^{6,7} However, both free and
19 VWF-bound FVIII are present in *in vivo* studies, making it difficult to distinguish
20 whether free or VWF-bound FVIII favors antibody development. We tested if
21 improved complex formation affects inhibitor development. FVIII-deficient mice
22 received repeated doses of BDD-FVIII or FVIII-KB013bv (50 U/kg; Fig. 2C). Of note,
23 all dosing was based on Units activity/weight basis. Since BDD-FVIII and FVIII-
24 KB013bv have different molecular weights (166kDa vs 196kDa), this may have
25 resulted in different protein dosing/mouse. The presence of anti-FVIII antibodies was
26 detected in 14 of 16 mice (87.5%) treated with BDD-FVIII, with a median titer of 12.7
27 $\mu\text{g/ml}$ (Fig. 2C). In contrast, only one mouse out of 8 (12.5%) treated with FVIII-
28 KB013bv developed anti-FVIII antibodies ($p=0.0056$ vs BDD-FVIII). A Bethesda-
29 assay was used to measure inhibitory antibodies in a subset of the samples
30 (Supplementary Fig. S6). Mice having inhibitory antibodies were also positive in the
31 ELISA-assay. In none of the mice (treated with BDD-FVIII or FVIII-KB013bv) the
32 presence of anti-nanobody antibodies was detected. These data suggest that
33 enhanced VWF binding may result in reduced anti-FVIII antibody formation.
34 When the FVIII-VWF complex binds to antigen-presenting cells, VWF predominantly
35 remains at the cell-surface, while FVIII is internalized and presented by the MHCII-
36 complex (Fig. 2D).^{7,9} Our data suggest that improved VWF binding prevents

1 internalization and subsequent presentation of FVIII by the MHCII-complex (Fig. 2E).
2 These data are further compatible with the immunogenic pathway (which involves *eg.*
3 CD206, but not LRP1) being different from the catabolic pathway (which involves
4 LRP1 among others, but not CD206).^{5,24,25} Why receptors contribute to FVIII uptake
5 in some cell-types but not others is currently unknown, but deserves further studies.

6
7 Together, our data indicate that increased VWF-binding favors FVIII survival and
8 reduces anti-FVIII antibody formation. From a therapeutic perspective, it would be
9 interesting to combine this FVIII-nanobody fusion protein with long-acting VWF (*eg.*
10 PEGylated-VWF) to further prolong FVIII half-life, perhaps beyond the 1.5-1.8-fold
11 limitation that is observed with current FVIII-variants with an extended half-life (FVIII-
12 Fc and PEG-FVIII).¹

13

1 **Acknowledgements:**

2 We thank Amine Bazaa and Amelie Harel for their contribution in the early stage of
3 the study. This study was supported by a Proof-of-Concept grant from Inserm-
4 transfert (Paris, France).

5

6 **Author contributions:**

7 VM, FM, GA, CK, PL performed experiments and analyzed data. CC and VP
8 provided important intellectual input. OCD, CVD and PJL designed and supervised
9 the study and analyzed data. PJL wrote the manuscript, and all authors contributed
10 to its editing.

11

12 **Conflict of interest:**

13 GA, OCD, CVD and PJL are inventors on a patent application involving FVIII-
14 KB013bv. The other authors declare no conflict of interest.

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1 **Figure legends**

2 **Figure 1: Anti-VWF nanobodies in a FVIII-nanobody fusion protein**

3 *Panel A:* Immobilized nanobodies (5 $\mu\text{g/ml}$) were incubated with hVWF, mVWF,
4 proteolytic fragment SpII (*i.e.* VWF residues 2129-2813), fragment SpIII (residues
5 764-2128), D'-D3-HPC4 (residues 764-1247) and A1-A2-A3-HPC4 (residues 1260-
6 1874). Bound proteins were probed using polyclonal anti-VWF antibodies or with
7 monoclonal antibody HPC4 and detected via peroxidase-mediated hydrolysis of
8 TMB. Plotted are OD-values corrected for binding of the proteins to albumin-coated
9 wells (mean \pm SD; n=3). *Panel B:* Association and dissociation of hVWF (25 $\mu\text{g/ml}$,
10 grey lines or 250 $\mu\text{g/ml}$, black lines) to immobilized nanobodies (10 $\mu\text{g/ml}$) was
11 analyzed via BLI-analysis using Octet-QK-equipment. Association was allowed for
12 600s and dissociation was monitored during 900s. Representative sensorgrams for
13 each nanobody are presented. *Panel C:* Schematic representation of FVIII-KB013bv,
14 in which FVIII B-domain residues Gln744-Arg1648 are replaced by two copies of
15 nanobody KB-VWF-013. Original thrombin activation sites and VWF binding sites are
16 conserved in this protein, while Arg1648 is no longer present. *Panel D:* Purified FVIII-
17 KB013bv and BDD-FVIII (1 $\mu\text{g/ml}$) were incubated in the absence or presence of
18 thrombin (10 nM) for 30min at 37°C, and analyzed via Western blotting using
19 polyclonal anti-FVIII and anti-nanobody antibodies. Sc-FVIII: single chain FVIII; FVIII-
20 HC: FVIII heavy chain; FVIII-LC: FVIII light chain. *Panel E:* Wells coated with purified
21 VWF (10 $\mu\text{g/ml}$) were incubated with FVIII-KB013bv (0-25ng/ml; grey symbols) or
22 BDD-FVIII (0-1 $\mu\text{g/ml}$; white symbols). Bound FVIII was probed using peroxidase-
23 labeled monoclonal anti-FVIII antibody 833 and detected via peroxidase-mediated
24 hydrolysis of TMB. Plotted are typical binding curves of an experiment performed in
25 duplicate. Half-maximal binding values were calculated from three independent
26 experiments, and were 4.0 \pm 2.1 ng/ml and 170 \pm 22 ng/ml for FVIII-KB013bv and BDD-
27 FVIII, respectively.

29 **Figure 2: In vivo analysis of FVIII-KB013bv**

30 *Panel A:* FVIII-deficient mice were given BDD-FVIII (open circles) or FVIII-KB013bv
31 (closed circles) at a dose of 250 U/kg via tail vein injection, then blood samples were
32 taken at various timepoints, and plasma was analyzed for residual FVIII activity.
33 Plotted are FVIII activities relative to the activity at t=3 min, which was arbitrarily set
34 at 100%. Each data point represents mean \pm SD of 3-5 mice, and each mouse was
35 bled 1-2 times. *Panel B:* FVIII-deficient mice received BDD-FVIII or FVIII-KB013bv
36 (500 U/kg) via tail vein injection, and 24h after injection a tail clip-bleeding assay was

1 performed. Clipped tails were immersed into saline at 37°C, and blood was collected
2 for 30 min. Blood loss for each individual mouse is indicated. *Panel C:* Mice were
3 given BDD-FVIII or FVIII-KB013bv (50 U/kg) at days 0, 7, 14, 21 and 40 via tail vein-
4 injection. At day 43, blood samples were taken, and plasma was analyzed for the
5 presence of murine anti-FVIII antibodies. Briefly, wells coated with BDD-FVIII were
6 incubated with murine plasma, and bound murine antibodies were detected via
7 peroxidase-labeled polyclonal goat anti-mouse antibodies. As standard, a
8 monoclonal anti-FVIII antibody was used. The limit of detection (LOD) in this assay
9 was 0.1 µg/ml. The immune-response for each individual mouse is presented.
10 Statistical analyses were performed using a Mann-Whitney test. *Panel D:* According
11 to Sorvillo *et al.*, the VWF-FVIII complex separates at the cellular surface of antigen-
12 presenting cells, with FVIII being endocytosed and most of the VWF molecules
13 remaining outside the cell. *Panel E:* Based on the model described by Sorvillo *et al.*,
14 we anticipate that the complex between FVIII-KB013bv and VWF will not dissociate,
15 and consequently there will be reduced uptake of FVIII-KB013bv by antigen-
16 presenting cells. Fewer FVIII-derived peptides will then be presented to T-cells, and
17 in turn, there will be reduced development of anti-FVIII antibodies.

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19
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Figure 1
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