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Role of the factor VIII-binding capacity of endogenous von Willebrand factor on the development of factor VIII inhibitors in patients with severe hemophilia A

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Title: Role of the factor VIII-binding capacity of endogenous von Willebrand factor on the development of factor VIII inhibitors in patients with severe hemophilia A

Running title: Immune-protective role of endogenous VWF towards therapeutic FVIII

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The development of factor VIII (FVIII) inhibitors is the major complication of replacement therapy in patients with severe hemophilia A (HA). Experimental and clinical evidence suggest that the presence of exogenous von Willebrand factor (VWF) in FVIII products reduces the immunogenicity of therapeutic FVIII.[1-3] However, a direct immuno-protective effect of endogenous VWF remains unclear.[4, 5] The binding of VWF to FVIII implicates the first 272 amino acids of the mature VWF (D'-D3 region) encoded by the exons 18-23 of the *VWF* gene.[6] Mutations in the *VWF* gene that result in quantitative or qualitative defects in VWF lead to von Willebrand disease (VWD). Polymorphisms in the *VWF* gene were studied in the context of venous thrombosis and VWD,[7, 8] but, to our knowledge, not in that of HA. Here, we investigated whether the capacity of endogenous VWF in patients with severe HA to modulate inhibitor development depends on its capacity to bind to therapeutic FVIII. Our working hypothesis was that gene variations in the D'-D3 region result in qualitative changes in the capacity of circulating endogenous VWF to bind FVIII. While such polymorphisms do not translate into coagulation abnormalities, they might have an impact on the stabilization of the therapeutically administered exogenous FVIII in patient with HA. The consequence would be an increased ratio of free versus bound FVIII molecules and a potentially reduced immuno-protection of FVIII by VWF. Our result show that the relative binding of endogenous VWF to therapeutic FVIII is a poor predictor of inhibitor development, probably reflecting the multi-causal nature of the inhibitor risk.[9, 10]

We first evaluated the capacity of endogenous VWF in the plasma of 48 randomly selected patients with severe HA to bind rFVIII *in vitro*. VWF:Ag levels were 89.8% (standard error mean (SEM) 10.4) and 91.9% (SEM 13.0) for inhibitor-positive and inhibitor-negative patients, respectively (95% CI -30.9 to 35.0). The relative VWF binding to FVIII (referred to as VWF:FVIIIB) was determined in each sample using an immuno-assay initially validated for the diagnosis of type 2N VWD (See Methods in supplement). VWF:FVIIIB was normally distributed and ranged between 41.1% and 158.9%. Interestingly, the distribution of VWF:FVIIIB was different for inhibitor-positive and negative patients (Figure 1A) with means of 86.4% (SEM 5.1) for inhibitor-positive patients as opposed to 103.6% (SEM 5.8) for inhibitor-negative patients (95% CI 1.3-33.2). The ROC curve of VWF:FVIIIB as a predictor of inhibitor development in patients with severe HA yielded an area under the curve of 0.668 (95% CI 0.513-0.821, Figure 1B). Upon examination of the coordinates of the ROC curve, we chose a potential VWF:FVIIIB cut-off value of 95% that yielded the best relation between sensitivity and specificity. Using this cut-off value, a VWF:FVIIIB below 95% was more frequent among inhibitor-positive patients than inhibitor-negative patients (71% versus

37%), and a value below this cut-off was associated with an over fourfold increased risk of inhibitor development (odds ratio 4.3, 95% CI 1.3-14.5). The proposed cut-off value exhibited a sensitivity of 0.71 (95% CI 0.48-0.89) and specificity of 0.63 (95% CI 0.42-0.81). The calculated positive and negative predictive values (PPV and NPV) for the prediction of inhibitor development were 0.4 and 0.83, respectively, using an inhibitor prevalence of 30%. These data suggest the potential of the VWF:FVIIIB assay in the preventive identification of severe HA patients at a low risk of developing inhibitors during FVIII replacement therapy. It is noteworthy, however, that a substantial number (38%) of the inhibitor-negative patients included in the study had VWF:FVIIIB scores lower than the median of the whole population; conversely, 35% of the inhibitor-positive patients presented with VWF:FVIIIB scores greater than the median. These results highlight the multi-causal nature of inhibitor development.

Exons 18 to 23 were directly sequenced in order to characterize Single Nucleotide Polymorphisms (SNP) in the *VWF* gene from the 48 patients previously tested for VWF:FVIIIB (See Supplementary Methods). Four SNPs were identified with a prevalence equivalent to that previously described in different non-hemophilic populations[8]: c.2365 A>G, p.Thr789Ala (rs1063856); c.2385 T>C, p.Tyr795Tyr (rs1063857); c.2555 G>A, p.Arg852Gln (rs216321) and c.2880 G>A, p.Arg960Arg (rs1800380). The association between VWF:FVIIIB and SNP genotypes was assessed. The two silent SNPs (p.Tyr795Tyr and p.Arg960Arg) (data not shown) and the p.Thr789Ala had no impact on VWF:FVIIIB (Figure 1C). However, c.2555 G>A SNP, corresponding to the substitution of an arginine with a glutamine at position 852, was associated with a statistically significant reduction in VWF:FVIIIB in the case of plasma from the heterozygous G/A patients as compared to plasma from patients with the homozygous frequent G/G genotype ($P<0.001$, 95% CI 11.87-42.51) (Figure 1D). No patient with the rare A/A genotype was detected. Two patients carried either one of the p.Arg854Gln and p.Arg924Gln mutations associated with VWD. The transition p.Arg854Gln, described as a type 2N VWD causative mutation,[11] was found in one patients without inhibitor. Previously reported to be a polymorphism in a study of type 2N VWD mutations,[12] the p.Arg924Gln, which represents a non-conservative amino acid substitution in exon 21, was observed in one patient with inhibitor. These missense mutations were associated with normal VWF:Ag levels and reduced VWF:FVIIIB, 41% and 42% in one inhibitor-negative patient and one inhibitor-positive patient, respectively (lower 2 points in Figure 1D). A previous study by Nesbitt *et al.* had identified the c.2555 G>A SNP in 16 of 148 screened alleles.[13] In contrast to our findings, their results had suggested that the

VWF:FVIIIIB was not affected by the p.Arg852Gln polymorphism in VWF, possibly owing to the relatively low number of patients with the c.2555 G>A SNP included in their study.

In an attempt to determine whether the c.2555 G>A SNP in exon 18 of the *VWF* gene is associated with the occurrence of FVIII inhibitors in the patients, we searched for the SNP in 235 patients enrolled in the SIPPET study.[2] The cohort included 163 inhibitor-negative patients and 72 inhibitor-positive patients, encompassing 14 low-responder and 48 high-responder patients. Genotype frequencies of the polymorphism are summarized in Table 1. The distribution of the c.2555 G>A genotypes did not deviate from the Hardy-Weinberg equilibrium in both inhibitor-negative and inhibitor-positive patients. No clear association between the c.2555 G>A SNP genotypes and the development of inhibitors was observed (Table 1, OR 0.61, 95% CI 0.28-1.32). These data are in line with a similar analysis performed in parallel using biological samples from a multicentric retrospective cohort of 281 patients with severe HA[14] (supplementary Tables S1 and S2), suggesting that the different ethnic origin of patients in the SIPPET cohort[2] does not account for the results. Genotypes and alleles frequencies in both cohorts were identical to results from the 1000 Genomes Project (1111G).[8]

If our working hypothesis is correct, the nature of the VWF variant should play a role predominantly for patients receiving rFVIII products, but not for patients receiving exogenous VWF with the pdFVIII products. Among the 235 SIPPET patients included in the present study, 118 patients were treated with rFVIII concentrates and 117 patients received pdFVIII products following randomization (1:1). Associations between the genotype distribution and development of FVIII inhibitors were addressed in the two groups of patients (Table 1). There was again no clear association between the presence of A allele of the c.2555 G>A SNP and the presence of a FVIII inhibitor, both in the case of the rFVIII-treated group (OR 1.16, 95% CI 0.41-3.30) and of the pdFVIII-treated patients (OR 0.12, 95% CI 0.09-1.24). A genome-wide association study evaluated 13331 SNPs from 1,081 genes using the Illumina iSelect platform for associations with inhibitor development in patients with HA. The study group included 833 subjects from three independent cohorts. The authors identified 53 SNPs as significant predictors of the inhibitor status, thus highlighting the complexity of the anti-FVIII immune response.[15] However, the genome-wide association study did not find associations of SNPs in the *VWF* gene with the inhibitor status of the patients, which comforts the present findings.

A major limitation of the study is the discrepancy between our observations: i) an overall reduced relative endogenous VWF binding endogenous VWF in the plasma from inhibitor-

positive severe HA patients, ii) a reduced relative endogenous VWF binding with the c.2555 G>A SNP and iii) the lack of association of the 2555 G>A SNP with the inhibitory status of the patients. Recently, Muczynski et al. developed a recombinant FVIII (FVIII-KB013bv) that contains two VWF-specific nanobodies in place of the B domain.[16] FVIII-KB013bv has a 25-fold increased affinity for VWF as compared to B domain-deleted FVIII, and exhibits a prolonged blood residence time in FVIII-deficient mice. Interestingly, FVIII-KB013bv demonstrated an almost complete lack of immunogenicity *in vivo* in FVIII-deficient mice. In view of the latter information, the discrepancy between our observations may be explained by the fact that, owing to the multi-causal nature of the inhibitor risk, an affinity of the endogenous VWF for therapeutic FVIII in the high physiological range does not systematically play a major protective role. Instead, a stabilization of the complex beyond the physiological equilibrium affinity is required to exert blatant immune-protective functions.

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Authors contribution

Designed research: YR, SVK, SLD

Performed research: YR, CC, RP, EFM, AP, SLD

Participated to cohorts: CC, ABD, Rd'O, CR, AEB, ME, VR, PE, JO, PMM, FRR, FP

Analyzed data: YR, RP, FP, SLD

Wrote the paper: YR, RP, FP, SLD

The authors declare no conflict of interest

Appendix

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Table 1. c.2555 G>A genotypes distribution and association with the development of FVIII inhibitor in 235 patients with severe HA from the SIPPET study[2]

rFVIII + pdFVIII	Inh-negative (n=163)	Inh-positive (n=72)				
		LR (n=24)	HR (n=48)	LR+HR (n=72)	OR	95% CI
G/G	129 (79%)	22	40	62 (86%)	0.61	0.28-1.32
G/A + A/A	34 (21%)	2	8	10 (14%)		

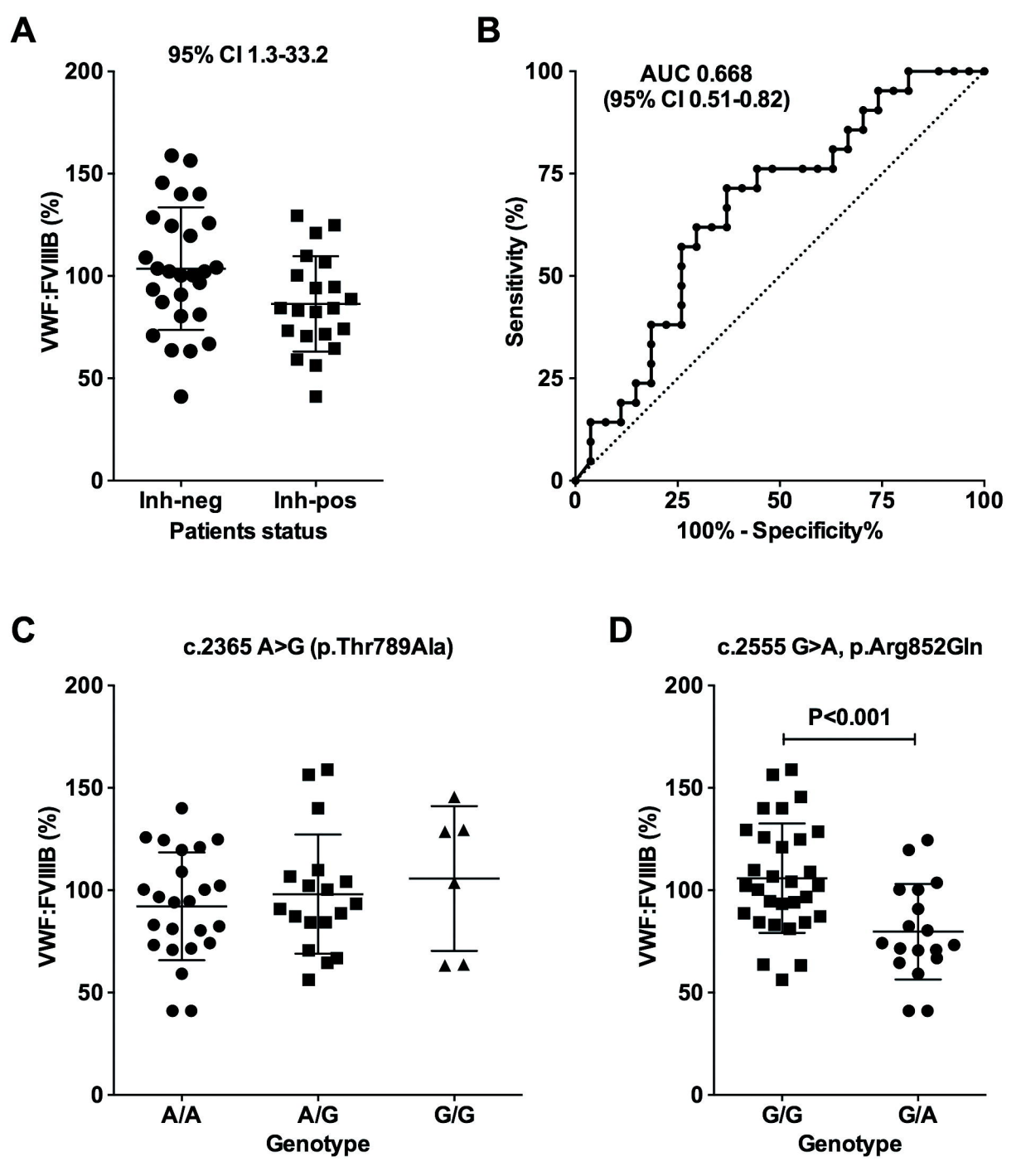
rFVIII-treated group	Inh-negative (n=73)	Inh-positive (n=45)				
		LR (n=16)	HR (n=29)	LR+HR (n=45)	OR	95% CI
G/G	63 (86%)	15	23	38 (84%)	1.16	0.41-3.30
G/A + A/A	10 (14%)	1	6	7 (16%)		

pdFVIII-treated group	Inh-negative (n=90)	Inh-positive (n=27)				
		LR (n=8)	HR (n=19)	LR+HR (n=27)	OR	95% CI
G/G	66 (73%)	7	17	24 (89%)	0.35	0.09-1.24
G/A + A/A	24 (27%)	1	2	3 (11%)		

LR: Low Responder; HR: High Responder; CI: confidence interval; OR: Odds Ratio.

Figure Legend

Figure 1. Relative endogenous VWF binding and inhibitory status in patients with severe HA. Panel A. Association between relative VWF binding (VWF:FVIIIB) and inhibitor status in severe HA patients (n=48). The X axis represents the inhibitor status: patients with HA without FVIII inhibitor (Inh-neg) and with FVIII inhibitor (Inh-pos). The Y axis represents the relative binding of recombinant FVIII to the endogenous VWF in the plasma of patients with severe HA measured by ELISA (expressed in %). The 95% confidence intervals (CI) was constructed with the standard errors derived from the Student's t distribution. Panel B. Receiver Operating Characteristic (ROC) curve for predicting inhibitor development in patients with severe HA by measurement of VWF:FVIIIB. The true positive rate (sensitivity) is plotted as a function of the false positive rate (100-specificity). AUC, Area Under Curve. Panels C and D. Association between VWF:FVIIIB and the p.Thr789Ala (c.2365 A>G) polymorphism (C) or the p.Arg852Gln (c.2555 G>A) polymorphism (D) in the exon 18 of the *VWF gene*. Statistical difference were determined using the Student's t test.


A
95% CI 1.3-33.2
VWF:FVIIIB (%)
200
150
100
50
0
Inh-neg
Inh-pos
Patients status
B
AUC 0.668
(95% CI 0.51-0.82)
Sensitivity (%)
100
75
50
25
0
0
25
50
75
100
100% - Specificity%
C
c.2365 A>G (p.Thr789Ala)
VWF:FVIIIB (%)
200
150
100
50
0
A/A
A/G
G/G
Genotype
D
c.2555 G>A, p.Arg852Gln
P<0.001
VWF:FVIIIB (%)
200
150
100
50
0
G/G
G/A
Genotype

Supplementary file

Supplementary Methods

Plasma samples

Plasma samples were obtained from frozen citrated plasma of 21 randomly selected inhibitor-positive and of 27 randomly selected inhibitor-negative patients (mean age 26 years; range 3-72) with severe hemophilia A (FVIII:C<0.01 UI/mL) treated with recombinant FVIII concentrates. Plasma had been collected during the usual clinical follow-up of patients with severe hemophilia A between 2008 and 2009 from hemophilia centers in France (Hôpital de Caen, Hôpital de Bicêtre) and Germany (University Clinic Bonn) and were analyzed in 2009. Approval for these studies was obtained from the Caen University institutional review board (Ethical committee agreement reference A16-D02-VOL.27). Written informed consent was provided by each patient according to the Declaration of Helsinki. Patients who had never developed an inhibitor after 150 cumulative exposure days (CED) or more were defined as inhibitor-negative patients. Inhibitor historical peak titers were documented for 20 of the 21 inhibitor-positive patients (mean 9.2; range 2-5000).

Randomized cohort of previously untreated patients with severe HA (SIPPET cohort)

All patients analyzed in this study were previously untreated or minimally-treated patients with severe HA enrolled in the multicenter, randomized, open-label clinical trial named SIPPET.[1] Patients included in SIPPET were randomized 1:1 to receive either plasma-derived FVIII (pdFVIII) or recombinant FVIII (rFVIII) and followed up for inhibitor development for 50 exposure days (EDs) or three years. Inclusion criteria included a severe deficiency of FVIII (FVIII:C <0.01 IU/mL) and the absence of FVIII inhibitor. Of the 251 patients enrolled in the SIPPET study, DNA samples were available from 235 patients (118 treated with rFVIII and 117 treated with pdFVIII) for analysis in *VWF* gene as per the original protocol

(ClinicalTrials.gov number, NCT01064284; EudraCT number, 2009-011186-88). Contributing centers to the SIPPET study group are listed in the Appendix.

Multicentric retrospective cohort

A multicentric retrospective cohort was constituted that included 281 patients with severe hemophilia A from different hemophilia centers in France (Hôpital de Caen, Hôpital de Bicêtre) and Germany (University Clinic Bonn). The cohort has been described previously.[2] The selection criterion was severe hemophilia A (FVIII:C<0.01 UI/mL). Inhibitor-negative patients (n=212) were defined as patients who had never developed an inhibitor after ≥150 cumulative exposure days (CED). Sixty-nine patients had been diagnosed with a FVIII inhibitor. Inhibitor historical peak titers were documented for 63 of the 69 inhibitor-positive patients: 20 patients had a historical peak titer <5 Bethesda units (BU)/mL (mean 2.6; range 1.0–4.8) and 43 patients had a historical peak titer ≥5 BU/mL (mean 1430; range 5 – 50000). The 212 inhibitor-negative patients matched with the 69 inhibitor-positive patients for the type of hemophilia A-causing mutation, with the exception of missense mutations that were more frequent among inhibitor-negative patients (Supplementary Table S1). Approval for this study was obtained from the Caen University institutional review board (ethical committee agreement reference A16-D02-VOL.27). Written informed consent was provided by each patient according to the Declaration of Helsinki.

Von Willebrand antigen (VWF:Ag) assay

VWF:Ag was determined in patients' plasma using the commercially available Asserachrom VWF:Ag assay (Stago®, Asnières, France), following the manufacturer's recommendations.

VWF-FVIII binding assay (VWF:FVIIIB)

The relative binding of plasma VWF to exogenous FVIII was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described,[3] with a slight modification. A microplate (Maxisorp, Nunc, Denmark) was coated by incubation for 24 hours at 2 to 8°C with 5 µg/ml of rabbit polyclonal anti-human VWF IgG (A0082, Dako®, Copenhagen, Denmark) in 0.05 mmol/L carbonate buffer, pH 9,6. After washing with Tris 50 mmol/L, NaCl 100 mmol/L (TBS) buffer containing 0.05% Tween 20, the wells were saturated with TBS containing 3% BSA for 1 hour at 37°C. Plasma samples were then incubated over night at 2 to 8°C in the saturation buffer. Normal plasma (NP) (Standart Human Plasma, Siemens, Marburg, Germany) and dilutions of a mixture (vol/vol) of NP with the plasma of a type 2N VWD patient homozygous for the p.Arg854Gln mutation (NP/2N mixture) were also included in each assay. Each patient's sample was tested in six serial dilutions, the first being adjusted to 0.05 IU/ml according to the VWF antigen (VWF:Ag) level. After removal of potential residual endogenous FVIII using 350 mmol/L $CaCl_2$ (10 min, twice at room temperature), 100 mIU of therapeutic rFVIII (Helixate® NexGen, CSL-Behring) diluted in TBS buffer with 10 mM of $CaCl_2$ were added to each well. After incubation (2 hours at 37°C) and washing with TBS buffer, bound FVIII was quantified using a mouse monoclonal anti-FVIII IgG (1 µg/mL) coupled to HRP directed to A2 domain (77IP52H7) of FVIII. After washing, captured VWF was quantified using 0.13 µg/mL of peroxidase-conjugated rabbit polyclonal anti-human VWF IgG (P0226, Dako®, Copenhagen, Denmark). The color was developed by addition of ortho- phenylene diamine dihydrochloride and the optical density (OD) was read at 490 nm. Two reference curves were established in parallel: for the quantification of immobilized VWF, 1:10 (10 mU) to 1:640 (0.156 mU) dilutions of NP were used; for the quantification of bound rFVIII, we added various amounts (2.23 to 143 mIU) of rFVIII to the wells in which VWF (1:5 dilution of NP) had been immobilized. For each plasma dilution, the values of bound rFVIII were plotted against the amount of immobilized VWF. The slopes of the obtained regression lines reflected

the relative VWF binding to FVIII (referred to as VWF:FVIIIB). The VWF:FVIIIB values are expressed in percentages compared with NP (100%).

Importantly, addition of monoclonal human anti-FVIII IgG to NP prior to incubation with immobilized anti-VWF antibodies did not alter the binding of recombinant FVIII to VWF (supplemental Figure S1), thus validating that the presence of anti-FVIII antibodies in plasma samples from inhibitor-positive patients does not interfere with the binding of endogenous VWF with the coated polyclonal anti-VWF IgG. Furthermore, addition of 350 mmol/L $CaCl_2$ was sufficient to remove residual FVIII and FVIII-anti-FVIII IgG complex potentially associated with endogenous VWF as depicted in supplemental Figure S2.

The VWF-FVIII binding assay is not designed for the measure of the binding constants (affinity) that govern the interaction between FVIII and VWF. It measures relative differences in VWF binding between different samples under identical conditions, thus eliminating the need for equilibrium conditions, and monitors global variations in FVIII-VWF interactions, independently from possible variations in association or dissociation constants.

Analysis of exons 18 to 23 of VWF gene

DNA was isolated using automated extraction kit. Exons 18 to 26 were amplified by PCR. Primer sequences are available on request. Reaction were carried out using 100 to 200 ng genomic DNA. Nucleotide sequencing was carried out using Sanger technique. All sequence changes were confirmed on both strands. The numbering of VWF mutations is based on the most recent recommendations of the ISTH Scientific and Standardization Subcommittee on von Willebrand factor.

Statistical analysis

The 95% confidence intervals (CI) for associations between the VWF:FVIIIB and patients groups as well as polymorphisms in the exon 18 of the *VWF gene*, were constructed with the standard errors derived from the Student's t distributions. A Receiving Operating Characteristic (ROC) curve was constructed to examine the predictive value of the VWF:FVIIIB on the development of FVIII inhibitors. Associations between groups and specific classes of allele, as well as genotypes were expressed as odds ratios (OR) and associated 95% CI. Statistical analyses were performed using GraphPad Prism software (La Jolla California, USA).

Supplementary Results

Exons 18 to 23 were directly sequenced in order to characterize Single Nucleotide Polymorphisms (SNP) in the D'-D3 region of the *VWF* gene from 281 severe patients with HA in the multicentric retrospective cohort. Supplementary table 1 describes the distribution of FVIII mutations in the cohort of patients. As previously reported, missense mutations were associated with a lower risk of inhibitor formation (supplementary Table S1).

In an attempt to determine whether the c.2555 G>A SNP in exon 18 of the *VWF* gene is associated with the occurrence of FVIII inhibitors in the patients, we determined c.2555 G>A genotype frequencies. The retrospective cohort included 212 inhibitor-negative patients and 69 inhibitor-positive patients. Genotype frequencies of the polymorphism are summarized in supplementary Table S2. The distribution of the c.2555 G>A genotypes did not deviate from the Hardy-Weinberg equilibrium for both inhibitor-negative and inhibitor-positive patients. No clear association between the c.2555 G>A SNP genotypes and the development of inhibitors was observed (OR 1.82, 95% CI 0.87-3.80).

References

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2. Repesse Y, Peyron I, Dimitrov JD, et al. Development of inhibitory antibodies to therapeutic factor VIII in severe hemophilia A is associated with microsatellite polymorphisms in the HMOX1 promoter. Haematologica. 2013;98(10):1650-1655.
3. Caron C, Mazurier C, Goudemand J. Large experience with a factor VIII binding assay of plasma von Willebrand factor. Br J Haematol. 2002;117(3):716-718.

Supplementary tables

Supplementary Table S1. Distribution of FVIII mutations for 281 severe HA patients.

	Inhibitor-negative patients (n=212)	**Inhibitor-positive patients (n=69)**				
		LR (n=20)	HR (n=49)	LR+HR (n=69)	OR	95% CI
Inversion of intron 22	106 (50%)	13	30	43 (62%)	0.6	0.35-1.06
Inversion of intron 1	3 (1%)	0	1	1 (1%)	0.98	0.1-9.55
Deletions/insertions	29 (14%)	3	6	9 (13%)	1.06	0.47-2.36
Non-sens mutations	25 (12%)	1	8	9 (14%)	0.89	0.39-2.02
Missense mutations	49 (23%)	5	4	7 (10%)	**2.66**	**1.14-6.20**

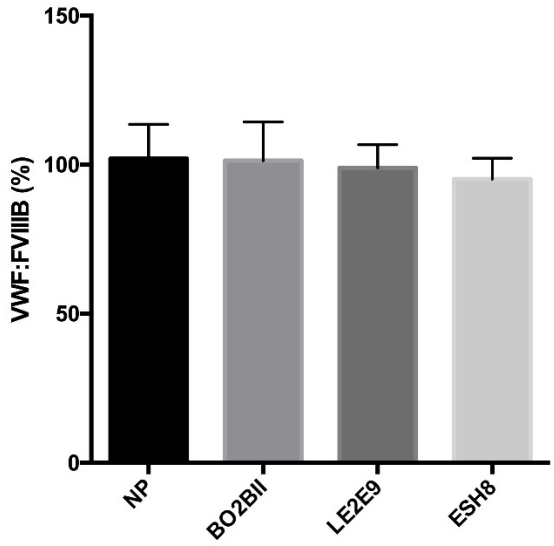
LR: Low Responder; HR: High Responder; CI: confidence interval; OR: Odds Ratio

Supplementary Table S2. c.2555 G>A genotypes distribution and association with the development of FVIII inhibitor for 281 severe HA patients.

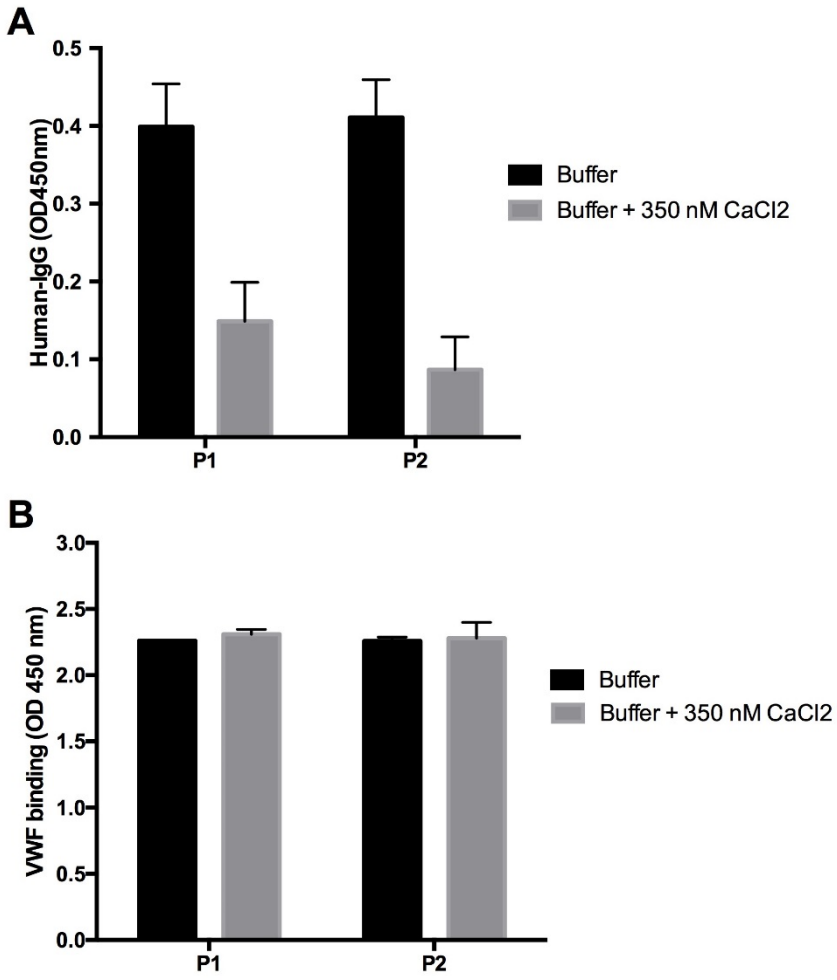
	Inhibitor-negative (n=212)	**Inhibitor-positive (n=69)**	OR	95% CI
G/G	188 (89%)	56 (81%)		
			1.82	0.87-3.80
G/A + A/A	24 (11%)	13 (19%)		

CI: confidence interval; OR: Odds Ratio.

Supplementary Figures



Supplementary Figure S1. Relative binding of FVIII to VWF in the presence of anti-FVIII antibodies. Normal plasma (NP) was incubated with 100-fold molar excess of anti-factor VIII antibodies directed against A2 domain (BO2BII), C1 domain (LE2E9) and C2 domain (ESH8) VWF:FVIIIB was then assessed for NP alone or NP incubated with anti-FVIII antibodies (mean±SD).



Supplementary Figure S2. Effect of CaCl2 incubation in inhibitor-positive samples for severe HA patients (P1:6.5 BU/mL; P2: 20 BU/mL). A microplate was coated with rabbit polyclonal anti-human VWF IgG (5µg/mL). Plasma samples were then incubated in absence or presence of 350 mmol/L of CaCl2 to remove endogenous FVIII and immune complexes (FVIII with anti-FVIII antibodies). (**A**) Following incubation, human IgG were detected using goat anti-human IgG antibody coupled with HRP. (**B**) Following incubation, bound VWF were revealed using polyclonal anti-VWF IgG coupled with HRP.