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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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

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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.