

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

Yohann Repessé,<sup>1,2,3</sup> Catherine Costa,<sup>4†</sup> Roberta Palla,<sup>5</sup> Erika Farrokhi Moshai,<sup>6</sup> Annie Borel-Derlon,<sup>1,2,3</sup> Roseline D'Oiron,<sup>7</sup> Chantal Rothschild,<sup>8</sup> Amal El-Beshlawy,<sup>9</sup> Mohsen Elalfy,<sup>10</sup> Vijay Ramanan,<sup>11</sup> Peyman Eshghi,<sup>12</sup> Johannes Oldenburg,<sup>13</sup> Anna Pavlova,<sup>13</sup> Frits R Rosendaal,<sup>14</sup> Flora Peyvandi,<sup>5</sup> Srinivas V. Kaveri<sup>6</sup> and Sébastien Lacroix-Desmazes<sup>6</sup>

*†This work is dedicated to the memory of our late colleague and friend Catherine Costa, an extraordinary geneticist and a wonderful human being.*

<sup>1</sup>CHU de Caen, Hematology Laboratory, Caen, France; <sup>2</sup>INSERM, U1237, GIP Cyceron, Caen, France; <sup>3</sup>Normandie Université, UNICAEN, UFR Santé, Caen, France; <sup>4</sup>Service de Génétique et Biologie Moléculaires, APHP, Groupe Hospitalier Cochin Broca, Hôtel-Dieu, Site Cochin, Paris, France; <sup>5</sup>Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Milano, Italy; <sup>6</sup>Centre de Recherche des Cordeliers, INSERM, Sorbonne Université, USPC, Université Paris Descartes, Université Paris Diderot, Paris, France; <sup>7</sup>Centres de Traitement de l'Hémophilie, APHP, Le Kremlin-Bicêtre, France; <sup>8</sup>Centres de Traitement de l'Hémophilie, APHP, Hôpital Necker, Paris, France; <sup>9</sup>Pediatric Hematology Department, Cairo University Pediatric Hospital, Cairo, Egypt; <sup>10</sup>Faculty of Medicine, Ain Shams Center, University - Department of Pediatrics, Cairo, Egypt; <sup>11</sup>Jehangir Clinical Development Center, Department of Hematology, Jehangir Hospital Premises, Pune, India; <sup>12</sup>Pediatric Congenital Hematologic Disorders Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran; <sup>13</sup>Institute of Experimental Hematology and Transfusion Medicine, University Clinic Bonn, Bonn, Germany and <sup>14</sup>Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, the Netherlands.

Correspondence: SEBASTIEN LACROIX-DESMAZES - [Sebastien.Lacroix-Desmazes@crc.jussieu.fr](mailto:Sebastien.Lacroix-Desmazes@crc.jussieu.fr)  
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## 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.<sup>1</sup> 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.<sup>2</sup> 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  $\geq 150$  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  $\geq 5$  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:FVIII B)*

The relative binding of plasma VWF to exogenous FVIII was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described,<sup>3</sup> 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<sub>2</sub> (10 min, twice at room temperature), 100 mIU of therapeutic rFVIII (Helixate® NexGen, CSL-Behring) diluted in TBS buffer with 10 mM of CaCl<sub>2</sub> 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:FVIIIIB). The VWF:FVIIIIB 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<sub>2</sub> 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:FVIIIIB 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:FVIIIIB 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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**Supplementary tables**

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

	Inhibitor-negative patients	Inhibitor-positive patients			OR	95% CI
	(n=212)	LR (n=20)	HR (n=49)	LR+HR (n=69)		
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%)	<b>2.66</b>	<b>1.14-6.20</b>

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

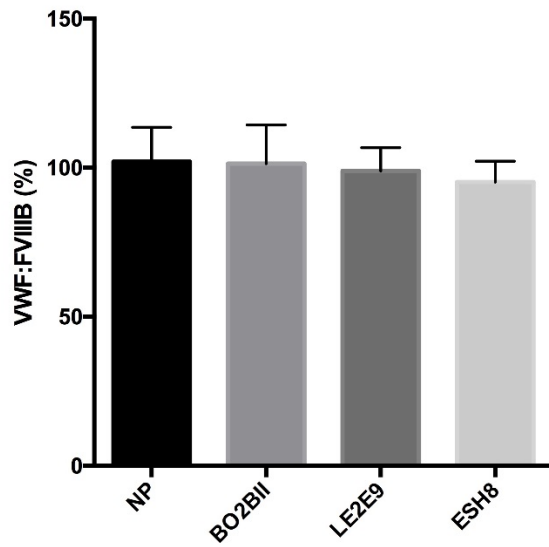


**Supplementary Table S2.** c.2555 G>A genotypes distribution and association with the development of FVIII inhibitor for 281 severe HA patients.

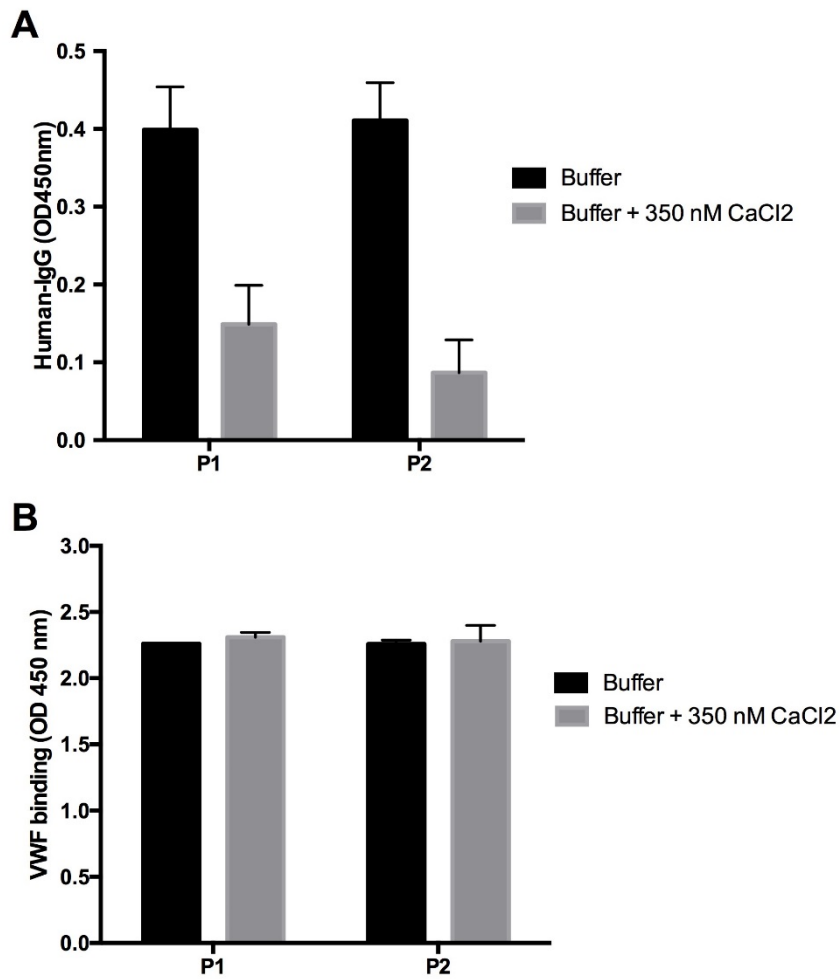
	<b>Inhibitor-negative (n=212)</b>	<b>Inhibitor-positive (n=69)</b>	OR	95% CI
G/G	188 (89%)	56 (81%)		
G/A + A/A	24 (11%)	13 (19%)	1.82	0.87-3.80

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:FVIIIIB was then assessed for NP alone or NP incubated with anti-FVIII antibodies (mean $\pm$ SD).



**Supplementary Figure S2.** Effect of CaCl<sub>2</sub> 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 (5 $\mu$ g/mL). Plasma samples were then incubated in absence or presence of 350 mmol/L of CaCl<sub>2</sub> 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.