Supplementary material to Rauch et al. “A novel ELISA-based diagnosis of acquired VWD with increased VWF proteolysis”
(Thromb Haemost 2016; 115.5)

Suppl. Methods

1) Standard
- VWF proteolysis standard
A normal pooled plasma (NPP Biopep®) was treated with Pefabloc 50mM and diluted (1/6.7) in Tris 5mM Urea 5mM buffer. ADAMTS13 was next activated by co-incubation of the former solution with BaCl2 at 37°C for 30 min. ADAMTS13 proceeding from the Pefabloc-BaCl2 treated NPP diluted in Tris-urea buffer was then incubated with plasma-derived purified VWF (Wilfactin® 100UI/mL, LFB® France) diluted 1/100 in Tris 5mM Urea 5mM buffer (ADAMTS13 and VWF final concentration respectively at 0.05nM and 25nM) for 16 hours at 37°C. Reaction was stopped by Na2SO4 1M. The resulting solution was centrifugated for 3 min at 3500 rpm and the supernatant was aliquoted and frozen at -80°C until ELISA analysis.

- Evaluation of standard for VWF proteolysis
Standard was evaluated for VWF proteolysis using SDS-agarose 1.4% electrophoresis as described [1] and immunoprecipitation / western blot. VWF was immuno-precipitated using rabbit polyclonal anti-VWF antibodies (50 μg/ml beads; Dako, Glostrup, Danmark) adsorbed onto Protein-G coated magnetic beads (Dynabeads Protein G, Invitrogen, Saint Aubin, France) for 2 hours at RT. After washing, immunoprecipitated VWF was released from the beads via a 5 min incubation at 100°C in 30 μL PBS/10 μL NuPAGE-LDS 4×sample buffer (Life Technologies, Saint Aubin, France) in the presence of 2 mM dithiothreitol. Electrophoresis was performed using discontinuous 4-12% SDS-page (Invitrogen) then transfert was run on an Immobilon P membrane (Millipore, Molsheim, France). The latter was incubated with a pool of 10 distinct monoclonal antibodies recognizing distinct epitopes of VWF (10μg/ml). Bound antibodies were probed using peroxidase-labelled goat anti-mouse antibodies (dilution 1/500; Santa Cruz, Heidelberg, Germany) and visualized with
SuperSignal West-Pico Enhanced Chemiluminescence Substrate (Thermo-Fischer Scientific, Villebon-sur-Yvette, France). Blots were analyzed via ImageJ-1.44 software (http://rsbweb.nih.gov/ij/index.html) in order to quantify uncleaved VWF (225 kDa bands) and cleaved VWF (140 kDa and 176 kDa bands).

- **Evaluation of inter-batch standard reproducibility**

Three independent standard preparations were diluted 1/8 to 1/512 in PBS Tween 0.05% BSA1 %, and then run in the same plate. OD₄₅₀ values were interpolated against the dilutions generating three calibration curves.

2) **ELISA: VWF proteolysis assay**

A 96-well microplate Immulon 4HBX was coated for 48 hours at 4°C with a commercially available anti-VWF monoclonal IgG (MAB27642, R&D Systems, Minneapolis, MN) previously diluted 1/125 (4 µg.ml⁻¹ final concentration) in bicarbonate buffer 0,5M. After washing step, the wells were blocked using 3% bovine serum albumine (BSA fraction V, Sigma) in phosphate buffered saline (PBS pH 7.4) for one hour at 37°C. After washing step, patients’ plasma samples and controls, diluted 1/8 and 1/16, were added to the plate and incubated for 2 hours at RT. After washing step, a polyclonal rabbit anti -VWF IgG (Dako®), horse-radish peroxidase (HRP) labelled, diluted 1/250 (5.2 µg.mL⁻¹ final concentration), was added to the plate and incubated for 2 hours at RT. Tetramethylbenzidine (TMB, Invitrogen, Frederick, MD) substrate was used for coloration. After being stopped with H2SO4 (1.5 M), optical density reading was assessed with an EL-808 Biotek® plate reader (Biotek Instruments, Winooski, Vermont, USA) at a 450 nm wavelength. Background correction was performed by substracting the average of the blank replicates from the raw measurement values. All washes step were performed with PBS Tween 0.1%. PBS Tween 0.05% BSA 3% was used as dilution buffer. The same reference controls, including one patient 2A (IIA) with mutation p.Arg1597Trp (high rate of VWF proteolysis) and one healthy subject (normal rate of VWF proteolysis), were run in each plate.

3) **Inner ELISA performances**

- **Lower detection limit (LDL) and lower quantification limits (LQL)**
Serial 2-fold dilutions of the standard (1/8 to 1/512) were performed in PBS Tween 0.05% BSA 3% to assess the LDL and LQL of the assay. Five replicates of each sample dilution and of the blank were then run in the same plate. Coefficients of variation (CVs) were calculated using results expressed as relative percentage.

- **Precision studies**

CVs were calculated for reference high rate of VWF proteolysis and normal rate of VWF proteolysis plasmas, respectively from a VWD-type 2A (IIA) and a healthy subject. For intra-assay precision, both samples were diluted 1/8 and 1/16 in PBS Tween 0.05% BSA 3%, and assayed 10 times into one plate. CVs were calculated using results expressed as relative percentage. The inter-assay CV was calculated using the same reference plasmas run on 17 consecutive ELISA.

- **Linearity experiment**

Linearity experiment was performed to assess the validity of using PBS Tween 0.05% BSA 3% buffer as diluent for the standard curve and samples. We elected the 2A (IIA) sample with the highest OD<sub>450</sub> value (p.Arg1597Trp) to assess linearity. 2-fold serial dilutions were performed on 2A (IIA) sample (1/8 to 1/512) in order to evaluate 7 different concentrations spanning the analytical range. Concentrations of 2A (IIA) sample dilutions were then interpolated from standard reference curve. Interpolated concentrations were next plotted versus the dilutions tested and statistical linear regression analysis was performed on the fitted data using Spearman correlation test.
**Figure 1: Assessment of VWF proteolysis on ELISA standard.** VWF proteolysis was assessed with SDS-agarose electrophoresis and western blot-immunoprecipitation analysis. A normal pooled plasma (Biopep®), a VWD-2A (IIA) plasma (p.Arg1597Trp) and purified plasma derived VWF (Wilfactin®) were used as controls. Loading of gels was normalized for VWF antigen. Representative SDS-agarose gel electrophoresis and western-blot with ELISA standard showing respectively HMW-multimers loss (A) and an increase in immunoprecipitated VWF proteolytic bands (140 and 176 kDa bands) vs uncleaved VWF (250 kDa band) (B).
**Suppl. Figure 2: Control of standard inter-batches variations.** Three independent standard preparations were diluted 1/8 to 1/512 in PBS Tween 0.05% BSA1 %, and then run in the same plate. OD_{450} values were interpolated against the dilutions generating three calibration curves.
Contributors to the French Reference Center for von Willebrand disease who included patients in the French cohort

Anne-Lise VOYER (CHU Amiens) ; Marie Anne BERTRAND (CHU Besançon)
Christine BOITEUX-VERGNES, Sabine-Marie CASTET, Mathieu FIORE, Yoann HUGUENIN, Chloe JAMES (CHU Bordeaux)
Brigitte PAN-PETESCH (CHU Brest)
Philippe GAUTIER, Yohann REPESSE (CHU Caen)
Valérie GAY (CH Chambery); Alain MARQUES-VERDIER (CHU Clermont-Ferrand)
Julien BOVET, Fabienne VOLOT (CHU Dijon)
Serge PIERRE LOUIS (CHU Fort de France)
Claire BARRO, Benoit POLACK(CHU Grenoble)
Tewfik BOUTEKEDJIRET, Stéphane GIRAULT, Caroline OUDOT (CHU Limoges)
Yésim DARGAUD, Anne LIENHART, Sandrine MEUNIER, Claude NEGRIER, Lucia RUGERI (HCL Lyon)
Katia POUYMAYOU, Hervé CHAMBOST, Céline FALAISE (APHM Marseille)
Jean François SCHVED, Marie-Christine BIRON-ANDREANI (CHU Montpellier)
Marie Elisabeth BRIQUEL, Birgit FROTSCHER (CHU Nancy)
Marianne SIGAUD, Marc TROSSAERT, Marc FOUASSIER, Mathilde GIRAUD (CHU Nantes)
Jean-Christophe GRIS (Nimes CHU)
Erwan CHOBLET (CH Nouméa)
Valérie PROULLE, Sophie COMBE, Roseline d'Oiron
Thierry LAMBERT (APHP, Kremlin-Bicêtre) ; Luc DARNIGE (APHP, HEGP)
Vasiliki GKALEA (APHP Tenon)
Annie HARROCHE, Chantal ROTHCHILD (APHP, Necker)
Valérie ROUSSEL-ROBERT, Natalie STIELTJES, Marie-Hélène HORELLOU, (APHP, Cochin)
Marie-Geneviève HUISSE (APHP, Bichat)
Marie-Françoise HURTAUD (APHP, Robert Debré)
Nathalie ITZHAR (APHP, Lariboisière)
Lotfi LAHJOMRI (APHP,GARCHES)
Eric BELTAN (CHU Pointe a Pitre)
Suppl. References
