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**[Page 1] A comparative analysis of different automated von Willebrand factor Glycoprotein Ib-binding activity assays in well typed von Willebrand Disease patients.**

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### Essentials

- Von Willebrand Ristocetin Cofactor Activity (VWF:RCo) is not a completely reliable assay.
- Three automated VWF activity assays were compared within a von Willebrand Disease (VWD) cohort.
- Raw values for all three assays were virtually the same.

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- An overall problem within type 2A/IIE VWD using VWF:GPIb-binding activity/VWF:Ag was observed.

## [Page 2] Abstract

*Background:* von Willebrand Disease (VWD) is an inherited bleeding disorder caused by quantitative (type 1, 3) or qualitative (type 2) von Willebrand factor (VWF) defect. VWD diagnosis and classification require numerous laboratory tests. VWF: Glycoprotein Ib (GPIb)-binding activity assays are used to distinguish type 1 from type 2 VWD.

*Objectives:* Three different automated VWF:GPIb-binding activity assays were compared.

*Patients and methods:* BC-VWF:RCo (Siemens Healthcare Diagnostics), HemosIL® VWF:RCo (Instrumentation Laboratory) and INNOVANCE® VWF:Ac (Siemens Healthcare Diagnostics) were performed in a well typed VWD cohort (n=142).

*Results:* Based on three most used VWD parameters (FVIII:C, VWF:Ag, VWF:GPIb-binding activity) and using cutoff <0.70 for type 2 VWD revealed sensitivity and specificity of respectively 92% and 72.4% for VWF:RCo/VWF:Ag, 84% and 89.7% for VWF:GPIbR/VWF:Ag, 92% and 85.1% for VWF:GPIbM/VWF:Ag. Where a lowered cutoff <0.60 resulted in reduced sensitivity with increased specificity for all assays.

*Conclusion:* VWD classification based on FVIII:C, VWF:Ag and VWF:GPIb-binding activity revealed an overall problem with normal VWF:GPIb-binding activity/VWF:Ag within type 2, especially type 2A/IIE. Although all assays were practical identical, BC-VWF:RCo had higher %CV compared with both new assays but comparable LLOQ ~4IU/dl. No clear improved distinction between type 1 and 2 VWD with new assays was seen. BC-VWF:RCo and HemosIL® are ristocetin dependent where INNOVANCE® does not rely upon ristocetin, and is not influenced by VWF polymorphisms increasing VWF:GPIb-binding activity levels. INNOVANCE® seems to be the best choice as first-line VWF:GPIb-binding activity assay, providing best balance between sensitivity and specificity for type 2 VWD.

## Keywords

Von Willebrand Disease, von Willebrand Factor, Ristocetin Cofactor, Classification, subtypes

### [Page 3] Introduction

von Willebrand Disease (VWD) is the most common (autosomally) inherited bleeding disorder. It is caused by defects in concentration, structure or function of von Willebrand Factor (VWF), a pivotal component of primary haemostasis that promotes platelet binding to sub-endothelial structures exposed on vessel trauma. VWD is characterized by mucocutaneous bleeding, prolonged bleeding after trauma and surgery, epistaxis and menorrhagia [1]. Based upon the phenotypic findings, VWD can be classified according to the International Society on Thrombosis and Haemostasis and its Scientific and Standardization Committee (ISTH-SSC) classification [2] into three primary categories. Type 1 VWD which is caused by a partial quantitative deficiency of VWF and type 3 VWD by a complete deficiency. Type 2 VWD (subtype 2A, 2B, 2M, 2N) is characterized by a subtype specific qualitative VWF defect. Within type 2A four subtypes can be distinguished (IIA, IIC, IID, IIE), which are not included in the current ISTH-SSC classification [3], and this based on their typical VWF multimeric patterns due to mutations influencing the sensitivity to ADAMTS13 cleavage, multimerization assembly or dimerization.

For the complete diagnosis and (sub)typing of VWD numerous laboratory tests are required; Ristocetin induced platelet aggregation (RIPA), Factor VIII procoagulant activity (FVIII:C), VWF antigen assay (VWF:Ag), VWF Ristocetin Cofactor Activity (VWF:RCo), VWF Collagen binding (VWF:CB), VWF - FVIII Binding (VWF:FVIII:B) if indicated, VWF multimer analysis (VWF:MM) [4] and genetic analysis of the VWF gene. The VWF:RCo assay is the historically preferred assay to detect functional VWF defects present in type 2A, 2B and 2M VWD by measuring the capacity of VWF A1 domain to bind Glycoprotein Ib (GPIb) in the presence of ristocetin (VWF:GPIb-binding activity). The original VWF:RCo assay by manual light transmission platelet agglutination has a limit of quantification (LLOQ) of 10 – 20IU/dl which is insufficiently low to distinguish dominant type 1 versus 2M versus 2A/IIE in severe VWD, but this has been improved upon by an automated version of the VWF:RCo assay. Some VWF polymorphisms (e.g. P/S1467, D/H1472) have been reported to cause false lower VWF:GPIb-binding activity to VWF:Ag ratios probably due to decreased binding of ristocetin to VWF A1 domain [5]. Despite the limitations of the VWF:RCo assay it remains one of the most frequently performed VWF functional tests. The VWF:CB, commonly assayed by enzyme-linked immunosorbent assay (ELISA), measures the ability of VWF to bind to collagen type III or I, and is also a measure preferentially of the High molecular weight multimers (HMWM) of VWF (sensitive to the loss of HMWM). The development of the VWF:CB stems from concerns over sensitivity, reproducibility and inter laboratory variability of the platelet agglutination-based VWF:RCo. VWF:CB has less inter-assay and inter-laboratory variability than the VWF:RCo. The source and types of collagen are important variables [6]. Type III collagen from human placenta is sensitive to the loss of

VWF HMWM with high avidity for VWF, type I low avidity (~sensitivity of test). VWF:CB has been shown of some value in identification of VWD type 1, type 2A and type 2B. However, a very rare type 2M with normal VWF:GPIb-binding but reduced VWF:CB was described by Ribba AS et al [7]. Despite the limitations of VWF:RCo, VWF:CB is not a replacement for VWF:RCo in VWD diagnosis/classification as it does not measure the same function [8].

Clinical application of the current VWD classification is compromised by wide variations in sensitivity and reproducibility of these diagnostic assays, neither does it take into account the VWF gene mutations, nor the [Page 4] VWF domain structure of the protein. Also, there is no “golden standard” against which classification can be checked. The VWD ISTH/EAHAD database on VWD mutations reports different VWD subtypes for the same VWF gene variation. It has been shown in several studies that many “mistakes” are commonly made in the diagnosis and subtyping of VWD [9-12].

In our study two new automated VWF:GPIb-binding activity assays; the HemosIL® VWF:RCo, ISTH nomenclature VWF:GPIbR [13] (Instrumentation laboratory, Bedford, MA, USA) and the INNOVANCE® VWF Ac, ISTH nomenclature VWF:GPIbM [13] (Siemens Healthcare Diagnostics, Marburg, Germany) were evaluated in a previously extensively (sub)typed VWD population (manuscript submitted) [14], to see whether they were more able to clearly distinguish type 1 and type 2 VWD by using the VWF:GPIb-binding activity/VWF antigen ratio, and also whether they were able to improve the diagnosis and subtyping of VWD in this cohort.

## **Patients and methods**

### *Study design*

The aim of the cross-sectional study was to compare two new generation automated VWF:GPIb-binding activity assays; HemosIL® VWF:RCo (Instrumentation laboratory), a ristocetin-triggered GPIb binding (ISTH nomenclature VWF:GPIbR) and INNOVANCE® VWF Ac (Siemens Healthcare Diagnostics), a gain-of-function mutant GPIb-binding assay (ISTH nomenclature VWF:GPIbM) in a population previously characterized with an extensive panel of techniques beyond that normally employed in the routine diagnostic environment. In this population the VWF:GPIb-binding activity was determined by BC-VWF:RCo (Siemens Healthcare Diagnostics) and we wanted to evaluate whether there would be a diagnostic improvement using the new assays.

### *Patient samples*

The study made use of the data and plasma samples from a cross-sectional study into VWD in the Czech Republic (BRNO-VWD Study, manuscript submitted) [14]. This was a cooperative study between the University Hospital Brno (Czech Republic) and Antwerp University hospital (Belgium), in which VWD patients based on their historical values were included by the University Hospital Brno and fully analyzed in Antwerp with all available techniques. The blood samples were collected in line with the guidelines from the Brno University Hospital ethical committee, and the patients signed an informed consent form. The current comparative study was covered by the original informed consent of the Brno-VWD study. The VWD classification was done according to the ISTH-SSC classification [2], also recognizing type 1 Vicenza within type 1 and an additional subdivision of subtype 2A into type 2A/IIA, 2A/IIC, 2A/IID and 2A/IIE [3, 15-18]. 142 samples were confirmed of having VWD and underwent extensive (sub)typing and these were used for this study.

More than the half (61.3%) of the patient cohort could be classified as being a type 1 VWD. Type 2A (23.3%), further subdivided into 2A/IIA (12.7%) and 2A/IIE (10.6%), represented the second large group within these population. Type 2B and 2M VWD represented respectively 6.3% and 5.6% of this cohort. There were no homozygous type 2N VWD samples, but eleven heterozygous type 2N carriers were identified in type 1 VWD [Page 5] patients. All type 3 VWD patients (5/142, 3.5%) were found in type 1 VWD families. Final diagnosis and typing was made by an expert panel after reviewing all the test results (table 1).

### *VWD Classification*

To diagnose and classify the samples, the study made use of platelet function analysis (PFA-100) using Collagen/ADP and Collagen/Ephineprin cartridges (Siemens Healthcare Diagnostics) and RIPA with two concentrations (1.2 and 0.6 mg/ml) of ristocetin (Helena Biosciences, Gateshead, UK) using the APACK-4 aggregometer (Helena Biosciences). FVIII:C (STA<sup>®</sup>-PTT Automate 5 Diagnostica Stago, Asnières sur Siene, France; deficient plasma VIII, Grifols, Barcelona, Spain), VWF:Ag with STA-LIATEST<sup>®</sup> VWF:Ag (Diagnostica Stago), and VWF:RCo with BC von Willebrand Factor RCo reagent (Siemens Healthcare Diagnostics) were all performed using the STA-R Evolution analyzer (Diagnostica Stago). Definitive VWD classification was done using additional tests measured by commercial ELISA assays determined on a EZ Read 400 Microplate Reader (Biochrom Ltd, Cambourne, UK): VWF:CB with equine collagen type I/III using Zymutest VWF:CB (Hyphen Biomed, Neuville sur Oise, France), VWFpp using Tool Set 2 (Sanquin, Amsterdam, The Netherlands) and VWF:FVIIIIB with the Asserachrom<sup>®</sup>VWF-FVIIIIB Kit (Diagnostica Stago) where indicated. VWF:MM

was done by sodium dodecyl sulfate-agarose electrophoresis gels visualized by luminescence using an Optigo 750 CCD photo Imager (Isogen Life science, Utrecht, The Netherlands) according to the method developed by Budde et al [19]. Molecular analysis was done by direct DNA sequencing of the polymerase chain reaction products obtained after amplification of the complete VWF gene, including intron/exon boundaries, using the ABI Big Dye terminator Cycle Sequencing Ready Reaction Kit on a ABI prism 310 (Applied Biosystems, CA, USA). A multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P011 and P012 kit (MRC Holland, Amsterdam, The Netherlands) was performed to detect an abnormal copy numbers of genomic DNA.

#### *Analytical principles of VWF:GPIb-binding activity assays*

VWF:RCo was measured by using the BC-VWF:RCo (Siemens Healthcare Diagnostics), on STA-R Evolution (Diagnostica Stago). This reagent contains fixed, lyophilized platelets coated with GPIb on their surface which bind active VWF present in the plasma through its GPIb receptor in the presence of ristocetin [20]. VWF-platelet complexes are formed followed by platelet agglutination which is proportional to the capacity of plasma VWF to bind platelets and is determined by measuring the decrease of light transmission caused by this agglutination (figure 1). This assay is marked by a LLOQ of 2-3IU/dl with coefficient of variation (%CV) of 9.0.

The HemosIL® VWF:RCo; ISTH nomenclature VWF:GPIbR (Instrumentation Laboratory) on an ACL TOP 500 analyser (Werfen UK, Warrington, UK) involves binding of active plasma VWF, in the presence of ristocetin, to recombinant fragment of wild type GPIb coated onto latex particles through a monoclonal antibody. The microparticles agglutinate and cause a decreased light transmission which is directly proportional to the ristocetin-mediated plasma VWF:GPIb-binding activity [21] (figure 1).

The INNOVANCE® VWF:Ac; ISTH nomenclature VWF:GPIbM (Siemens Healthcare Diagnostics) on a Sysmex CS2000i analyser (Sysmex UK, Milton Keynes, UK) utilizes a recombinant GPIb $\alpha$  fragment containing two gain-of-function mutations (G233V, M239V) which bind plasma VWF via the GPIb receptor in the absence of ristocetin and shear stress. Added microparticles coated with an antibody against GPIb will bind the VWF-recombinant [Page 6] GPIb $\alpha$  complex inducing microparticle agglutination and decreased light transmission which is directly proportional to the VWF:GPIb-binding activity in the plasma [22,23]. The gain-of-function mutations introduced into the GPIb fragments are known from the platelet type- or pseudo VWD which are characterized by spontaneous binding of VWF to platelets with mutant GPIb [24], hence there being no requirement for ristocetin [22,23] (figure 1). These mutations play an important role in the binding of GPIb to VWF; A1 domain.

Both new assays were previously validated using a normal and Low control resulting in %CV of 4.2 for normal control and 3.2 for low control, and this for both assays. A LLOQ of 3.3 IU/dl for HemosIL® and 4.0IU/dl for INNOVANCE® was identified (manuscript submitted). A standard sample dilution was used and additional dilutions were only performed when the obtained results exceeded the standard curve. With every single batch of samples control plasma's were performed as a assay quality control. The whole procedure was performed according to manufacturer's instructions.

At the time of measuring the VWF:GPIIb-binding activity with the two assays under study, VWF:Ag was determined with HemosIL® VWF:Ag immunoassay (Instrumentation Laboratory) on an ACL TOP 500 (Werfen UK).

#### *Statistical analysis*

Assay differences were expressed in mean difference and 95% confidence interval (CI). IBM SPSS statistics software, version 21.0 (SPSS, Inc., IBM Corporation, U.S., Armonk, NY, USA) was used for the statistical analysis. For each activity assay a sensitivity was calculated to quantify the proportion of positives that were correctly identified as being a type 2 VWD, and specificity was determined to measures the proportion of negative samples that were correctly identified as not having type 2 VWD conditions.

## **RESULTS**

142 previously extensively (sub)typed VWD samples (table 1) were analyzed with both new generation VWF:GPIIb-binding activity assays; HemosIL® VWF:RCo (ISTH nomenclature VWF:GPIIbR, Instrumentation Laboratory), and the INNOVANCE® VWF:Ac (ISTH nomenclature VWF:GPIIbM, Siemens Healthcare Diagnostics ). The VWF:Ag levels were also measured with the HemosIL® VWF:Ag (Instrumentation Laboratory) at the same time and no significant difference was observed compared with the original LIATEST-VWF:Ag results  $\Delta=-0.8\%$  ,CI95% -2.4 – 0.8). Therefore the LIATEST-VWF:Ag (Diagnostica Stago) results were used for further analysis in this study, as they were also used for the initial classification.

Comparing the results of both new generation assays (expressed in percentages) with the test used in the initial classification (BC-VWF:RCo, Siemens Healthcare Diagnostics) no overall significant differences were found either for HemosIL® (  $\Delta=-0.6\%$  (CI95% -3.0 – 1.9)) or for INNOVANCE® (  $\Delta=0.2\%$  (CI95% -2.1 – 2.5)), nor between both new assays (  $\Delta= -0.6\%$  (CI95% -1.6 – 0.4)) (Figure 2a). Because the ratio between VWF:GPIIb-binding activity and VWF antigen is of prime importance to



distinguish between type 1 and type 2 VWD, the ratios for all three [Page 7] VWF:GPIIb-binding activity assays were compared. At first sight, a paired t-test evaluation (figure 2b) of the activity to antigen ratios for both new generation assays against the classification test (BC-VWF:RCo) showed a statistically significant difference for HemosIL® ( $\Delta=-0.08$  (CI95% -0.16 - -0.01)) but not for the INNOVANCE® ( $\Delta=-0.03$  (CI95% -0.08 – 0.02)), nor between the two new assays themselves ( $\Delta=-0.05$  (CI95% -0.11 – 0.01)). However, statistical significance could possibly have been reached with a larger sample size. More important is the way these differences may impact upon diagnosis/classification.

Most routine laboratories only provide 3 specialized parameters (FVIII:C, VWF:Ag and VWF:GPIIb-binding activity) to diagnose and classify VWD patients. Therefore, the specificity and sensitivity for type 2 VWD was evaluated based on the VWF:Ag and VWF:GPIIb-binding activity levels and their ratio using all three automated VWF:GPIIb-binding activity assays with the cut off  $\geq 0.70$  for type 1 as it was in the MCMDM-VWD1 study [25] and also for the more stringent cut off of 0.60 as it was in the Canadian type 1 VWD study [26], and advocated more recently by several publications [27,28] and recommended in the recent British guidelines [29].

Using the BC-VWF:RCo a sensitivity of 92% for type 2 VWD and a specificity of 72.4% was obtained when the 0.70 cut off was used. Using a 0.60 cut off resulted in a sensitivity and specificity of 86% and 87.3% respectively. The HemosIL® with a 0.70 and 0.60 cut off obtained a sensitivity of 84% and 80% for type 2 VWD with a specificity of 89.7% and 93.1% respectively. The VWF:GPIIb-binding activity/VWF antigen ratio (cut off 0.7 & 0.6) with the INNOVANCE® showed a sensitivity for type 2 VWD of 92% and 82% ,with a specificity of 85.1% and 90.8% .(table 2)

Because of the arguments in the literature that VWF:CB might be a replacement for VWF:GPIIb-binding activity [8] we judged it interesting to compare our results with VWF:CB especially for type 2A/IIIE and type 2M, and we evaluated the sensitivity and specificity for type 2 VWD based on the VWF:Ag and VWF:CB levels and their ratio with the same cut off of  $\geq 0.70$  and 0.60. A sensitivity of 76% and a specificity of 87% was obtained using a 0.70 cut off and a sensitivity of 62% and specificity of 97% for a 0.60cut off. As demonstrated in table 2, compared to VWF:GPIIb-binding activities, the same problems occurred within type 2A/IIIE and 2M VWD patients, and certain type 2 VWD would be misclassified when using only VWF:Ag and VWF:CB.

Using BC-VWF:RCo, 8% (4/50) of the samples had a ratio above 0.70 (1/9 type 2B and 3/15 type 2A/IIE (1/3 Cys1130Gly, 1/3 Trp1144Gly and 1/3 Tyr1146Cys)) and would be missed as being a type 2 (2A/IIE) VWD. This subtype is caused by mutations in the D3 multimerisation domain which can “easily” be diagnosed by VWF multimeric analysis [3,30].

Using HemosIL® 16% (8/50) type 2 samples (6/15 type 2A/IIE (2/6 Ser979Asn, 1/6 Cys1130Gly, 2/6 Trp1144Gly, 1/6 Tyr1146Cys) and 2/8 type 2M (Asp1691Glu+Gly1890Glu) VWD showed a normal ratio, and would be diagnosed as type 1.

The VWF:GPIb-binding activity/VWF antigen ratio obtained with the INNOVANCE® showed 8% (4/50) type 2 patients with a ratio above 0.70 which would have been classified as a type 1 VWD instead a type 2 VWD; 2/15 type 2A/IIE (1/2 Ser979Asn and 1/2 Tyr1146Cys,) and 2/8 type 2M VWD (Asp1691Glu+Gly1890Glu).

[Page 8] It has to be noted that within type 2 VWD all three assays showed problems within type 2A/IIE and type 2M VWD patients which would be missed as being a type 2 VWD based solely on the 3 most provided ‘routine’ VWD parameters (FVIII:C, VWF:Ag and VWF:GPIb-binding activity) present in general haemostasis laboratories. (table 2 and figure 3). Although their ratios were normal, all these “missed” type 2A/IIE patients were confirmed by the expert panel as being a type 2A/IIE based on their typical 2A/IIE VWF multimeric pattern (the lack of the outer bands and a loss of the high molecular weight multimers) and a corresponding mutation in the D3 domain of the VWF gene.

Despite their normal VWF:GPIb-binding activity/VWF Ag ratio using both new generation assays, the two “missed” type 2M patients were still classified as being a type 2M (A3 domain type) based on their normal VWF multimeric pattern and mutation predicted to be deleterious (DEOGEN Score >0.50) using the Mutaframe software (Interuniversity Institute of Bioinformatics ULB-VUB, Brussels, Belgium).

Within the type 1 VWD samples cohort, some samples (table 2) showed a reduced ratio (<0.7 or 0.6) with at least one VWF:GPIb-binding activity assay but these were after expert discussion classified as a type 1 VWD due to the presence of a normal VWF multimeric pattern and a type 1 VWD mutation according to the VWD ISTH/EAHAD database.

## DISCUSSION

In this study we compared two new automated assays for the measurement of the GPIb binding capacity of VWF in a previously extensively subtyped VWD population to determine whether the use of these new assays would have had benefits in reaching the right diagnosis and classification. The

Accepted Article

samples came from the Brno-VWD Study where patients were diagnosed and classified as far as possible with all currently available techniques, including VWF:MM, VWFpp and genetic analysis of the VWF gene, which are not routinely available for most laboratories. For VWD analysis, the majority rely on a few “core” assays like FVIII:C, VWF:Ag, VWF:GPIb-binding activity and RIPA, while others also add VWF:CB to this list [31]. With all guidelines using the VWF:RCo/VWF:Ag ratio to determine whether the patient has a type 1 (quantitative) or type 2 (qualitative) VWD the role of VWF:GPIb-binding activity in diagnosis is crucial.

The BC-VWF:RCo (BC von Willebrand Factor RCo reagent, Siemens Healthcare, Marburg, Germany) was used to measure the activity of the VWF to bind GPIb to diagnose/classify VWD in the Brno-VWD study population. The same VWD samples were analyzed with 2 new generation VWF activity assays; HemosIL® VWF:RCo, ISTH nomenclature VWF:GPIbR (Instrumentation Laboratory Bedford, MA, USA) and INNOVANCE® VWF Ac, ISTH nomenclature VWF:GPIbM (Siemens Healthcare Diagnostics, Marburg, Germany).

Although no significant difference between those three assays was seen (paired t-test with p-values >0.05), overall there was a slight difference in specificity and sensitivity for type 2 VWD between the three assays, if the evaluation was based solely on the “routine” VWD parameters (VWF:Ag, VWF activity) and their ratio. Initially a cut off ratio for VWF activity to antigen of 0.70 was used, as in the MCMDM-VWD1 study [25]. Lowering this ratio to 0.60 as advocated by several more recent publications and employed in the Canadian type 1 VWD study [Page 9] [26], resulted - as expected - in reduced sensitivity while increasing specificity for all assays. Even after taking the %CV of each assay into account; 5% for VWF:Ag, 9% for BC-VWF:RCo and 3.2% for HemosIL® and INNOVANCE® [32], which allowed some movement in the VWF activity/VWF:Ag ratio in both directions, this did not affect the differentiation of type 1 and type 2 VWD.

On the whole, there were no improvements in the distinction between type 1 from type 2 compared to the “established” BC-VWF:RCo. VWD diagnosis and classification based solely on the VWF:Ag and VWF:GPIb-binding activity levels revealed a problem within the type 2 VWD, especially in type 2A/IIIE and/or type 2M for all three VWF:GPIb-binding activity assays. Some of these type 2 VWD would, especially using HemosIL®, not have been classified as a type 2 VWD because of their normal ( $\geq 0.70$ ) VWF:GPIb-binding activity/VWF antigen ratio. All “missed” type 2A/IIIE samples had their classification “confirmed” by a typically abnormal VWF multimeric pattern with a lack of the triplet structure and a relative loss of the high molecular weight multimers, and by a corresponding mutation in the D3 domain, identified in the ISTH/EAHAD VWD database as causal for type 2A/IIIE.

The type 2M “mis-“classifications are more tenuous; these samples exhibited a normal VWF:CB and VWF:MM pattern which can suit either type 1 or type 2M. The current ISTH-SSC VWD classification [2] is based on RIPA, quantitative VWF:Ag and functional VWF:GPIb-binding activity and VWF:CB assays. VWD 2M due to loss of function mutation in the A1 domain impairing the VWF-platelet GPIb interaction is featured by decreased RIPA and a mostly normal VWF:CB (although VWF:CB is dependent on the type of collagen), and a decreased VWF:GPIb-binding activity compared to VWF:Ag. The VWF:MM may vary from normal to smeary patterns, or even show some loss of large multimers like in the Rotterdam and Hamburg studies [33,34,35]. Molecular analysis could not confirm the suspicion of a “typical” type 2M given the identified gene variation was unknown to the ISTH/EAHAD VWD database and was not located in the A1 but in the A3 domain. Mutation predicted programs qualified these “new” mutations as “deleterious” and these samples are now awaiting gene expression studies. Final classification as type 2M (A3 domain type) was made after expert discussion, but remains tenuous.

As shown in table 2, the same problems occurred when the VWD classification was based on only VWF:Ag and VWF:CB with misclassification of type 2 VWD as a type 1. This showed, as was previously suggested in the literature [8], that the VWF:GPIb-binding cannot be replaced by the VWF:CB but both assays should be performed in parallel in combination with VWF:Ag and FVIII:C.

One of the major limitations of this study is the unduplicated measurement of VWF:GPIb-binding activity for all three assays which inevitably decreases the precision of these results. However, all three activity tests underwent rigorous validation procedures including accuracy and reproducibility. The other major limitation is the diagnosis and classification of VWD itself which remains a challenge. Diagnosing/classifying of VWD is “incomplete” when it is based on only the VWF:Ag and VWF:GPIb-binding. Only by using an extensive laboratory panel including additional parameters like VWF:CB, VWFpp, VWF:MM and VWF gene analysis can a type 2 VWD be classified in the most “correct” way. The current ISTH-SSC VWD classification [2] tends to group together different previously existing variants in larger groups in order to provide a “simpler” classification system. Even the ISTH/EAHAD database on VWD mutations reports different types of VWD for the same gene variation.

[Page 10] All three assays measure binding of VWF to platelet GPIb but not under shear stress conditions, and therefore – inevitably – they are not “real” functional tests. As there was no clear improvement in the distinction between type 1 and 2 VWD in our cohort with the new assays compared to the “old” BC-VWF:RCo, also looked at other features like CV and LLOQ. Although all three assay results were practically identical, the BC-VWF:RCo had a higher %CV but the LLOQ

~4IU/dl was comparable. Two out of three assay are ristocetin dependent (BC-VWF:RCo and HemosIL®). The INNOVANCE® has the advantage of no longer having to rely upon ristocetin, and as such is not influenced by VWF polymorphisms (e.g. p.D/H1472 and p.P/S1467) [5] affecting the capacity of ristocetin to close the VWF A1 domain loop in vitro, but there could conceivably be circumstances in which the presence of the gain-of-function mutations could potentially misrepresent or even result in increased values for VWF:GPIIb-binding activity. Based on the current study the INNOVANCE® seems to be the best choice as first-line VWF:GPIIb-binding activity assay which also provides the best balance between sensitivity and specificity for a type 2 VWD, although differences were marginal.

#### **ADDENDUM**

J. J. Michiels, G. W. Moore and A. Gadisseur were responsible for the study initiation. O. Zapletal and P. Smejkal were responsible for the sample collection. I. Vangenechten and K. Mayger were involved in study design, data collection and performing laboratory analysis. I. Vangenechten and A. Gadisseur were responsible for analysis and interpretation of results and performed statistical analysis. I. Vangenechten was the lead author of the initial manuscript. K. Mayger P. Smejkal, J. J. Michiels, G. W. Moore and A. Gadisseur were responsible for revisions of the draft manuscripts. A. Gadisseur was responsible for review and approval of the final manuscript.

#### **Disclosure of Conflict of Interests**

G. W. Moore Moore is a member of the Coagulation Advisory Board for Roche Diagnostics International Ltd, outside the submitted work. The other authors state that they have no conflict of interest.

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