Journal of Thrombosis and Haemostasis, **16**: 2413–2424 DOI: 10.1111/jth.14319

ORIGINAL ARTICLE

Clinically relevant differences between assays for von Willebrand factor activity

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To cite this article: Boender J, Eikenboom J, van der Bom JG, Meijer K, de Meris J, Fijnvandraat K, Cnossen MH, Laros-van Gorkom BAP, van Heerde WL, Mauser-Bunschoten EP, de Maat MPM, Leebeek FWG, for the WiN Study Group. Clinically relevant differences between assays for von Willebrand factor activity. *J Thromb Haemost* 2018; **16**: 2413–24.

Essentials

- It is unclear whether there are differences between von Willebrand factor (VWF) activity assays.
- We compared the four most used VWF activity assays in 661 von Willebrand disease (VWD) patients.
- All assays correlated excellently, but a discrepant classification was seen in 20% of patients.
- Differences between VWF activity assays have a large impact on the classification of VWD.

Summary. *Background:* Measuring the ability of von Willebrand factor (VWF) to bind to platelets is crucial for the diagnosis and classification of von Willebrand disease (VWD). Several assays that measure this VWF activity using different principles are available, but the clinical relevance of different assay principles is unclear. *Objective:* To compare the four most widely used VWF activity assays in a large VWD patient population. *Methods:* We measured VWF:RCo (ristocetin to activate VWF + whole platelets), VWF:GPIbR (ristocetin + platelet glycoprotein Ib receptor [GPIb] fragments), VWF:GPIbM (gain-of-function GPIb fragments that bind VWF spontaneously without ristocetin) and VWF:Ab (monoclonal antibody directed against the GPIb binding epitope of VWF to mimic platelets) in 661 VWD patients from the nationwide 'Willebrand in the Netherlands' (WiN) Study. *Results:* All assays correlated excellently (Pearson $r > 0.9$), but discrepant results led to a different classification for up to one-fifth of VWD patients. VWF:RCo was not sensitive enough to classify 18% of patients and misclassified half of genotypic 2B VWD patients, especially those with p.Arg1306Trp. VWF:GPIbR was more sensitive, accurately classified the vast majority of patients, and was unaffected by the p.Asp1472His variant that causes artificially low VWF:RCo. VWF:GPIbM was the most precise assay but misclassified over a quarter of genotypic 2A, 2B and 3 patients. VWF:Ab, often not considered an actual VWF activity assay, performed at least equally to the other assays with regard to accurate VWD classification. *Conclusion:* Although the different VWF activity assays are often considered similar, differences between assays have a large impact on the classification of VWD.

Keywords: blood coagulation disorders; clinical laboratory techniques; subtype classification; von Willebrand disease; von Willebrand factor.

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Received: 7 June 2018
Manuscript handled by: R. Gosselin
Final decision: R. Gosselin, 9 October 2018

Introduction

Von Willebrand factor (VWF) plays an important role in primary hemostasis by mediating the adhesion of platelets

to exposed collagen in damaged vessels and subsequent platelet aggregation. In addition, VWF indirectly affects secondary hemostasis by protecting coagulation factor VIII from degradation and clearance. A deficiency or dysfunction of VWF causes the bleeding disorder von Willebrand disease (VWD) [1]. Von Willebrand disease is classified into three types: type 1 VWD is characterized by a partial deficiency of VWF; type 2 is caused by a dysfunctional VWF; type 3 is characterized by a virtually complete absence of VWF.

The diagnosis and classification of VWD are based on laboratory tests. The plasma concentration of VWF measured by VWF antigen (VWF:Ag) does not provide information on the functional activity of VWF. Because VWF:Ag may be normal in type 2 VWD, it is crucial to measure the ability of VWF to bind and activate platelets. Guidelines advise that both VWF:Ag and the ability of VWF to bind platelet glycoprotein Ib (GPIb) receptor (the 'platelet-dependent VWF activity', hereafter named 'VWF activity') be measured [2,3]. Moreover, the perioperative treatment of VWD patients is monitored using platelet-dependent VWF activity assays [1].

Since the 1970s, assays that use the antibiotic ristocetin and platelets (VWF:RCo) have been the standard for measuring VWF activity [4,5]. In this assay, ristocetin induces a conformational change in VWF, causing it to bind platelets, and the subsequent platelet agglutination is then measured. Despite improvements that have made the assay less elaborate and less time consuming, VWF:RCo still has a poor sensitivity and a high coefficient of variation [6,7]. In addition, several studies have shown that common genetic variants in the VWF gene that affect the sensitivity of VWF to ristocetin lead to artifactually low assay results [8,9].

Several other commercial assays that measure platelet-dependent VWF activity have been developed in recent decades. One of these assays measures the binding of VWF to a monoclonal antibody directed against the GPIb binding epitope of VWF as a surrogate for platelets (VWF:Ab), and is generally considered not to be a functional assay [10,11]. Another more recently developed assay still uses ristocetin, but uses recombinant GPIb fragments (rGPIb) adhered to microparticles instead of whole platelets (VWF:GPIbR) [12]. This assay is more sensitive and less variable than VWF:RCo, but may still be susceptible to the same genetic variants that affect VWF:RCo [6]. Another recently developed assay uses a rGPIb fragment with two gain-of-function mutations that enable binding to VWF without the need for ristocetin (VWF:GPIbM) [13]. Although VWF:GPIbM is reported to be precise and sensitive, data on its use in VWD patients are still limited [6]. While getting rid of ristocetin as a non-physiological VWF activator, the VWF:GPIbM assay introduces a non-physiological binding of VWF to a mutant receptor. In a recent worldwide survey by the External quality Control for Assays and Tests (ECAT) Foundation in 292 laboratories, 30% used a VWF:RCo assay, 27% the VWF:GPIbM assay, 19% the VWF:GPIbR assay and 16% the VWF:Ab assay [14].

Thus, there are currently several widely used assays that measure VWF activity using different principles. However, it is not known whether and to what extent these various assay principles translate into clinically relevant differences in test results. Comparisons have only been made in studies with relatively few VWD patients. To improve evidence-based diagnostics of VWD, a direct comparison of these assays in a large patient group is urgently needed [6]. We therefore performed the largest and most comprehensive such study to date, comparing the four most widely used VWF activity assays in 661 VWD patients.

Materials and methods

Patients

We included patients from the nationwide cross-sectional 'Willebrand in the Netherlands' (WiN) Study. Patients were included in the WiN Study if they had (i) a hemorrhagic diathesis or a positive family history for VWD and (ii) historically lowest VWF:Ag and/or VWF activity ≤ 30 U dL^{-1} or FVIII coagulant activity (FVIII:C) ≤ 40 U dL^{-1} (for type 2N patients). Details of the study design have been reported elsewhere [15]. Patients were excluded if they had also been diagnosed with another inherited bleeding disorder. This study has been approved by the medical ethics committees of all participating centers and all patients have given written informed consent.

Laboratory measurements

At inclusion in the WiN Study, venous blood was collected from patients in 0.105-M sodium citrate tubes, centrifuged twice at $2200 \times g$ for 10 min at room temperature and stored in aliquots at −80 °C. VWF:Ag was determined with an in-house ELISA using polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase (HRP)-conjugated anti-human VWF antibodies (DakoCytomation, Glostrup, Denmark) for detection. VWF:CB was measured with an in-house ELISA using collagen type 1 (Sigma-Aldrich, St Louis, MO, USA) for capture and HRP-conjugated anti-human VWF antibody (DakoCytomation) for detection. Other measurements such as FVIII:C, VWF:FVIIIB and VWF multimer analysis had been performed previously at Erasmus University Medical Center and VWF propeptide had been measured previously at Leiden University Medical Center [15,16]. For each of the four assays compared in the current study, we measured VWF activity from separate, previously unthawed aliquots. To limit aliquot to aliquot variability, plasma was derived from a single blood draw and pooled prior to making the aliquots. All assays were

performed on commercially available single automated platforms. All measurements were performed without prior knowledge of previous measurements or diagnosis.

VWF:RCo was measured with the commercial 'BC von Willebrand reagent' (Catalogue number OUBD37, Siemens Healthcare Diagnostics, Marburg, Germany) on a Sysmex CS-5100 analyzer (Sysmex, Kobe, Japan) using the manufacturer's protocol. To 18 μL patient plasma, 54 μL Owren's Veronal Buffer was added; after 10 s, 150 μL reagent (containing stabilized platelets and 1.25 mg mL^{-1} ristocetin) was added; platelet agglutination was then measured as change in turbidity.

VWF:GPIbR was measured with the 'HemosIL AcuStar VWF:RCo' reagent (catalogue number 0009802024, Werfen IL, Breda, the Netherlands) on an ACL AcuStar analyzer (Werfen IL) using the manufacturer's protocol. To 28 μL patient plasma, 112 μL diluent was added; 50 μL 'reagent 1B' (assay buffer containing ristocetin to an end concentration of 1 mg mL^{-1}) and 20 μL 'reagent 1A' (containing magnetic particles coated with a rGPIb fragment) were then added to 20 μL of the diluted plasma sample. After 114 s of incubation and a wash step, 85 μL 'reagent 1B' and 85 μL 'reagent 1C' (anti-VWF murine mAb labeled with isoluminol) were added. After 9.5 min of incubation, 190 μL sodium hydroxide and 200 μL hydrogen peroxide-urea were added and the subsequent chemiluminescense was measured.

VWF:GPIbM was measured with the 'INNOVANCE VWF Ac' reagent (Catalogue number OPHL03, Siemens Healthcare Diagnostics) on a Sysmex CS-5100 analyzer using the manufacturer's protocol. First, samples were measured using the 'High' protocol: 30 μL Owren's Veronal Buffer was added to 15 μL patient plasma; 20 s later, 70 μL 'Reagent II' (containing a heterophilic blocking reagent) was added; 20 s later 20 μL 'Reagent III' (containing rGPIb) was added; 30 s later 40 μL 'Reagent I' (containing polystyrene particles coated with anti-GPIb mAb) was added; particle agglutination was then measured as change in turbidity. If a test result using the 'High' protocol was below 15 IU dL^{-1}, the sample was measured again using the 'Low' protocol: 70 μL 'Reagent II' was added to 70 μL patient plasma; 20 s later, 20 μL 'Reagent III' was added; 30 s later, 40 μL 'Reagent I' was added; particle agglutination was measured as change in turbidity.

VWF:Ab was measured with the 'HemosIL VWF Activity' (catalogue number 0020004700, Werfen IL) on a Sysmex CA-1500 analyzer using the manufacturer's protocol. To 50 μL patient plasma, 50 μL 0.9% NaCl was added; 150 μL reagent (containing polystyrene latex particles coated with a murine mAb directed against the VWF GPIb binding epitope) was added to 30 μL of this mixture; particle agglutination was measured as change in absorbance.

Reference curves for VWF:RCo, VWF:GPIbM and VWF:Ab were constructed by diluting Standard Human Plasma (Siemens Healthcare Diagnostics) in Owren's Veronal Buffer. VWF:GPIbR could only be calibrated using the calibrator plasma provided in the reagent kit as described by the manufacturer and therefore we could not use the same standard curve for this assay as for the other assays. The lower limits of quantification were 12 IU dL^{-1} for VWF:RCo, 4 IU dL^{-1} for VWF:GPIbM, 1 IU dL^{-1} for VWF:Ab and 0.5 IU dL^{-1} for VWF:GPIbR.

The coefficients of variation (CV) were calculated for each assay using the quality control material (QC). For VWF:RCo, VWF:GPIbM and VWF:Ab, we used VisuCon™-F Frozen Normal Control Plasma with VWF activity ~100 IU dL^{-1} as high QC and VisuCon™-F Frozen Abnormal Control Plasma with VWF activity ~35 IU dL^{-1} as low QC. For VWF:GPIbR, we used the obligatory AcuStar von Willebrand Factor: Controls plasma (VWF activity for high QC ~90 IU dL^{-1}, VWF activity for low QC ~25 IU dL^{-1}). The CVs for VWF:GPIbM were 0.9% for the high QC and 1.2% for the low QC. The CVs for VWF:Ab were 1.0% for the high QC and 5.2% for the low QC. The ristocetin-containing assays were the most variable, with CVs of 5.2% (VWF:RCo) and 4.5% (VWF:GPIbR) for the high QC and 9.9% (VWF:RCo) and 8.3% (VWF:GPIbR) for the low QC.

Definitions

We classified every VWD patient for each of the assays according to current guidelines, independent from knowledge of the previous classification [1,3]. For VWF:GPIbR, VWF:GPIbM and VWF:Ab, type 1 VWD was defined as VWF activity/antigen ratio > 0.60, type 2 A/B/M as VWF activity/antigen ratio ≤ 0.60, and type 3 as VWF:Ag < 5 IU dL^{-1} and VWF activity < 5 IU dL^{-1}.

Because VWF:RCo is less sensitive than the other three assays, we had to use a different VWD classification for this assay. Patients with undetectably low VWF:RCo (< 12 IU dL^{-1}) and VWF:Ag ≥ 20 IU dL^{-1} were classified as type 2 A/B/M VWD because they had a VWF:RCo/VWF:Ag ratio < 0.6 (although the precise ratio could not be calculated). Because we were unable to determine if the VWF:RCo/VWF:Ag was above or below 0.6 for patients with VWF:RCo < 12 IU dL^{-1} and VWF:Ag 5–19 IU dL^{-1}, we classified these patients as having 'classification difficulties'. Patients with VWF:RCo < 12 IU dL^{-1} and VWF:Ag < 5 IU dL^{-1} were classified as type 3 VWD.

Patients were classified as type 2N if they had either abnormal VWF:FVIIIB or a type 2N *VWF* mutation. VWF gene mutations were defined as type 2A, 2B or 2M only if they were consistently reported as such in the EAHAD Coagulation Factor Variant Database for VWF (https://grenada.lumc.nl/LOVD2/VWF/home.php?select_db=VWF accessed October 2017). VWF gene mutations

that were previously reported for several subtypes of VWD, were not included in the VWD subtype analyses.

Statistical analysis

Correlation between assays was assessed with Deming regression and the Pearson correlation coefficient, and analytical agreement was assessed with Bland-Altman analysis. Test results were further compared using repeated measures one-way ANOVA with Greenhouse-Geisser correction for within-subjects effects and post-hoc analysis with Bonferroni correction for comparisons between assays. For these analyses, test results below the lower limits of quantification were set at half that value (i.e. 6 IU dL^{-1} for VWF:RCo, 2 IU dL^{-1} for VWF:GPIbM, 0.5 IU dL^{-1} for VWF:Ab and 0.25 IU dL^{-1} for VWF:GPIbR).

Clinical agreement was assessed by determining the agreement in VWD classification. Statistical significance was set at $P < 0.05$. Bland-Altman analysis was performed using GraphPad Prism, version 5 (GraphPad Software Inc., La Jolla, CA, USA); all other analyses were performed with SPSS for Windows, version 21 (IBM Corporation, Armonk, NY, USA).

Results

After exclusion of patients who were pregnant ($n = 5$) or who had used desmopressin or VWF concentrates within 72 h before blood sampling ($n = 15$), 661 patients were available for the current study (patient characteristics in Table 1). Of these, VWF:Ab was measured in 655 patients, VWF:RCo and VWF:GPIbM in 643 patients, and VWF:GPIbR in 623 patients. For 39% of patients ($n = 243$), VWF:RCo was below the lower limit of quantification. For the other assays, this was the case for 8% (VWF:Ab, $n = 54$) and 3% of patients (VWF:GPIbM, $n = 19$; VWF:GPIbR, $n = 18$).

In 618 patients all four assays were performed. Within these patients, the test results were significantly different (repeated measures one-way ANOVA with Greenhouse-Geisser correction for within-subjects effects $F(2.697, 1663.768) = 86.081$, $P < 0.001$). VWF:RCo and VWF:GPIbR gave similar results (mean difference, −0.1 IU dL^{-1}; 95% confidence interval (CI), −1.1 to 0.9). VWF:RCo gave lower results than VWF:GPIbM (mean difference, −4.7 IU dL^{-1}; 95% CI, −5.5 to −3.9) and VWF:Ab (mean difference, −3.1 IU dL^{-1}; 95% CI, −4.2 to −2.0). VWF:GPIbR also gave lower results than VWF:GPIbM (mean difference, −4.6 IU dL^{-1}; 95% CI, −5.3 to −3.8) and VWF:Ab (mean difference, −3.0 IU dL^{-1}; 95% CI, −4.0 to −2.1). Results for VWF:GPIbM were somewhat higher than for VWF:Ab (mean difference, 1.6 IU dL^{-1}; 95% CI, 0.6–2.5).

Table 1 Patient characteristics

	VWD patients ($n = 661$)	
Age, years	44	[29–57]
Female	411	62%
Blood group O*	400	61%
VWF:Ag, IU dL^{-1}	29	[18–44]
VWF:CB, IU dL^{-1}	22	[7–50]
VWF:RCo, IU dL^{-1}	20	[6–47]
VWF:GPIbR, IU dL^{-1}	22	[9–47]
VWF:GPIbM, IU dL^{-1}	24	[13–51]
VWF:Ab, IU dL^{-1}	22	[8–51]
FVIII:C, IU dL^{-1}	51	[32–73]

Data are shown as median, [interquartile range] or *n*, %. *Four missing. VWD, von Willebrand disease; VWF, von Willebrand factor.

There was an excellent correlation between assays (Fig. 1). Bland-Altman analysis revealed a small bias (< 5 IU dL^{-1}) between assays (Fig. 2). However, there were considerable differences in individual results: an absolute difference of 10 IU dL^{-1} or more was found in many patients, ranging from 15.7% of patients when comparing VWF:GPIbM and VWF:GPIbR to 24.5% of patients when comparing VWF:RCo and VWF:Ab (Fig. 3 and Table 2). Moreover, an absolute difference of 20 IU dL^{-1} or more was found in 3.9% (when comparing VWF:RCo and VWF:GPIbM) to 7.2% of patients (when comparing VWF:RCo and VWF:GPIbR). Compared with VWF:GPIbR, the positive bias for VWF:RCo, VWF:GPIbM and VWF:Ab increased at higher VWF activity (Fig. 2). All four assays also had a high correlation with VWF:CB with only a small bias, although in individual patients there were considerable differences between VWF activity and VWF:CB (Figure S1).

Because differences in test results have the biggest impact when results are low, absolute differences were also calculated when at least one of the compared assays was ≤ 30 IU dL^{-1}. In this group, an absolute difference of at least 10 IU dL^{-1} was still found in a significant proportion of patients, ranging from 4.5% when comparing VWF:GPIbR and VWF:Ab to 20.3% of patients when comparing VWF:RCo and VWF:GPIbM; an absolute difference of at least 20 IU dL^{-1} was found in 1.1% when comparing VWF:GPIbR and VWF:Ab, and 5.0% of patients when comparing VWF:RCo and VWF:Ab (Table 2).

To determine the effect of differences in test results on VWD classification, we calculated the classification agreement between assays after exclusion of type 2N patients. Of the 243 patients with VWF:RCo < 12 IU dL^{-1}, 113 (18% of total) had VWF:Ag < 20 IU dL^{-1}, leading to classification difficulties. None of the other assays had classification difficulties (Fig. 3A). After exclusion of patients with classification difficulties, classification agreement ranged from 82.5% when comparing VWF:GPIbM and VWF:Ab to 93.3% when comparing VWF:RCo and VWF:GPIbR (Table S1).

We next compared the assays in 57 patients with known type 2A mutations (list of mutations in Table S2). VWF:RCo had classification difficulties for 21% of

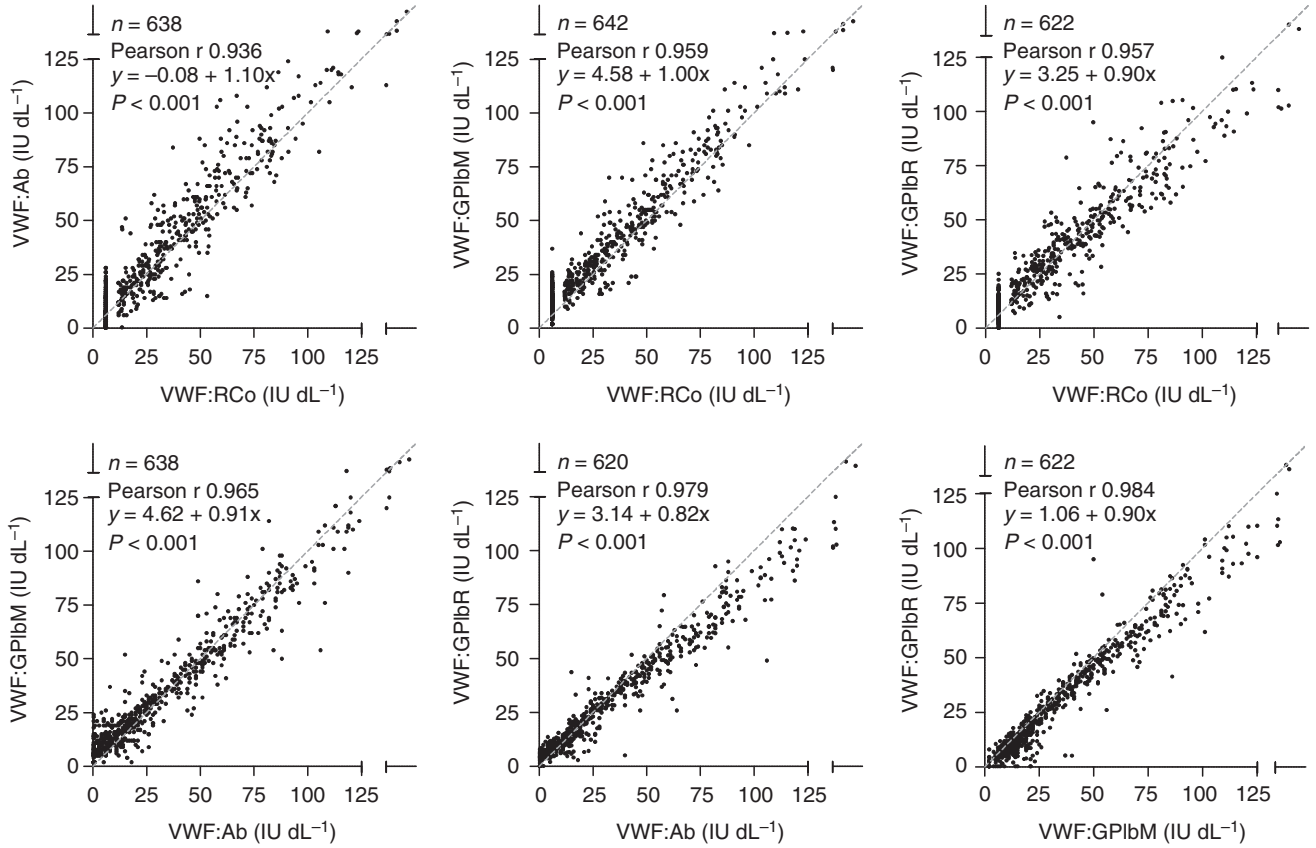

Fig. 1. Correlation between von Willebrand factor (VWF) activity assays. Correlation between assays was determined using the Pearson correlation coefficient. The slope was calculated using Deming regression. Dotted gray lines depict the line of identity.

genotype 2A patients, whereas it classified 72% of all genotype 2A patients as type 2 VWD (Fig. 3B). VWF:GPIbR and VWF:Ab classified 81% and 74% of genotype 2A patients as type 2. VWF:GPIbM classified 65% as type 2 VWD. Although VWF:GPIbM classified fewer patients as type 2, we did not observe differences in test results between VWF:GPIbM, VWF:GPIbR and VWF:Ab in these patients ($F(1.469, 82.278) = 1.952$, $P = 0.16$); VWF:RCo was excluded from this analysis because 51/57 patients had VWF:RCo below the lower limit of quantification (Fig. 4A). Of note, the vast majority of genotype 2A patients that were wrongly classified as type 1 VWD had an abnormal VWF multimeric pattern or VWF:CB to VWF:Ag ratio ≤ 0.60, which are both indicative of type 2A or 2B VWD (Table S3).

We then compared the assays in 53 patients with a known type 2B VWD mutation (list of mutations in Table S2). VWF:GPIbR and VWF:Ab classified 98% and 93% of genotype 2B patients as type 2 VWD, and VWF:GPIbM classified 81% of patients as type 2 (Fig. 3C). In contrast, VWF:RCo was unable to classify 19% of patients and classified 52% of all genotype 2B patients as type 2 VWD, while classifying the other 29% of patients as type 1 VWD. Test results differed in type 2B patients ($F(2.109, 109.691) = 8.784$, $P < 0.001$, Fig. 4B). These differences seemed dependent on the causal *VWF* mutation. In 28 patients with p.Arg1306Trp, VWF:RCo was 6.7 (95% CI, 2.1–11.2) IU dL^{-1} higher than VWF:GPIbR and 6.8 (95% CI, 2.0–11.5) IU dL^{-1} higher than VWF:Ab, whereas VWF:GPIbM was 4.5 IU dL^{-1} higher (95% CI, 0.9–8.0) than VWF:GPIbR and 4.6 IU dL^{-1} higher (95% CI, 0.5–8.6) than VWF:Ab (Fig. 4C). In 18 patients with p.Arg1308Cys, VWF:RCo was undetectable and the other assays gave similar results (Fig. 4D). It should be noted again that almost all genotype 2B patients that were classified as type 1 VWD had an abnormal VWF multimeric pattern or VWF:CB to VWF:Ag ratio ≤ 0.60 (Table S3).

Of all the assays, VWF:GPIbM least often classified patients as type 3 VWD (Fig. 3A and Table S1). We therefore also compared the assays in 21 patients with definite type 3 VWD, based on VWF:Ag and VWF propeptide < 5 U dL^{-1} [16]. Interestingly, eight of these type 3 patients had VWF:GPIbM $\geq$ 5 IU dL^{-1}, whereas results for the other assays were < 5 IU dL^{-1} (Fig. 4E). Two of these eight patients had detectable VWF multimers: one had VWF:GPIbM 5 IU dL^{-1}, VWF:GPIbR 2 IU dL^{-1} and VWF:Ag 4 IU dL^{-1} and the other had VWF:GPIbM 6 IU dL^{-1}, VWF:GPIbR 4.6 IU dL^{-1} and VWF:Ag 3 IU dL^{-1}; VWF:RCo and VWF:Ab were undetectable for both patients. VWF:Ag was < 1 IU dL^{-1} for all other type 3 VWD patients.

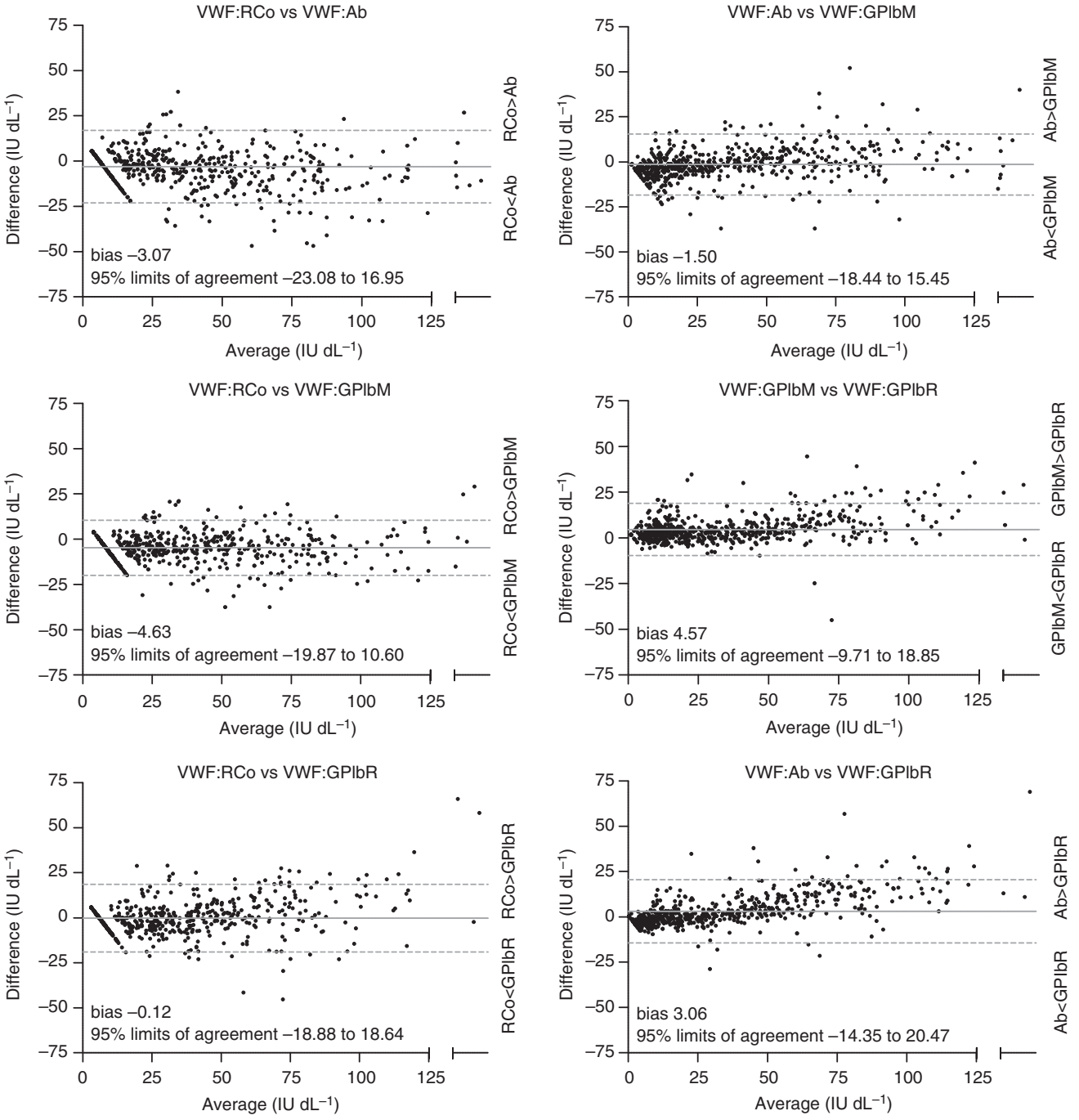

Fig. 2. Bland-Altman analysis. Bland-Altman plots show the difference in test results against the mean of the test results. The continuous gray lines depict bias between assays; dotted gray lines depict 95% limits of agreement (bias ± 1.96 SD) between test results. An absolute difference of over 10 IU dL^{-1} was found in more than 15% of patients (also when results were low).

We also compared the assays in the 47 patients with the *VWF* variant p.Asp1472His, which has been reported to cause 25% lower VWF:RCo results [9]. Indeed, test results differed in these patients ($F(1.962,\ 90.255) = 26.271$, $P < 0.0001$). VWF:RCo was 21% (mean difference, −7.7 IU dL^{-1}; 95% CI, −11.3 to −4.0) lower than VWF:GPIbR, 27% (mean difference, −10.7 IU dL^{-1}; 95% CI, −14.5 to −6.9) lower than VWF:GPIbM, and 25% (mean difference, −9.5 IU dL^{-1}; 95% CI, −14.6 to −4.3) lower than VWF:Ab (Fig. 4F). Interestingly, VWF:GPIbR was similar to VWF:Ab (mean difference, −1.8 IU dL^{-1}; 95% CI, −4.78 to 1.2), and only 8% (mean difference, −3.0 IU dL^{-1}; 95% CI, −5.2 to −0.8) lower than VWF:GPIbM.

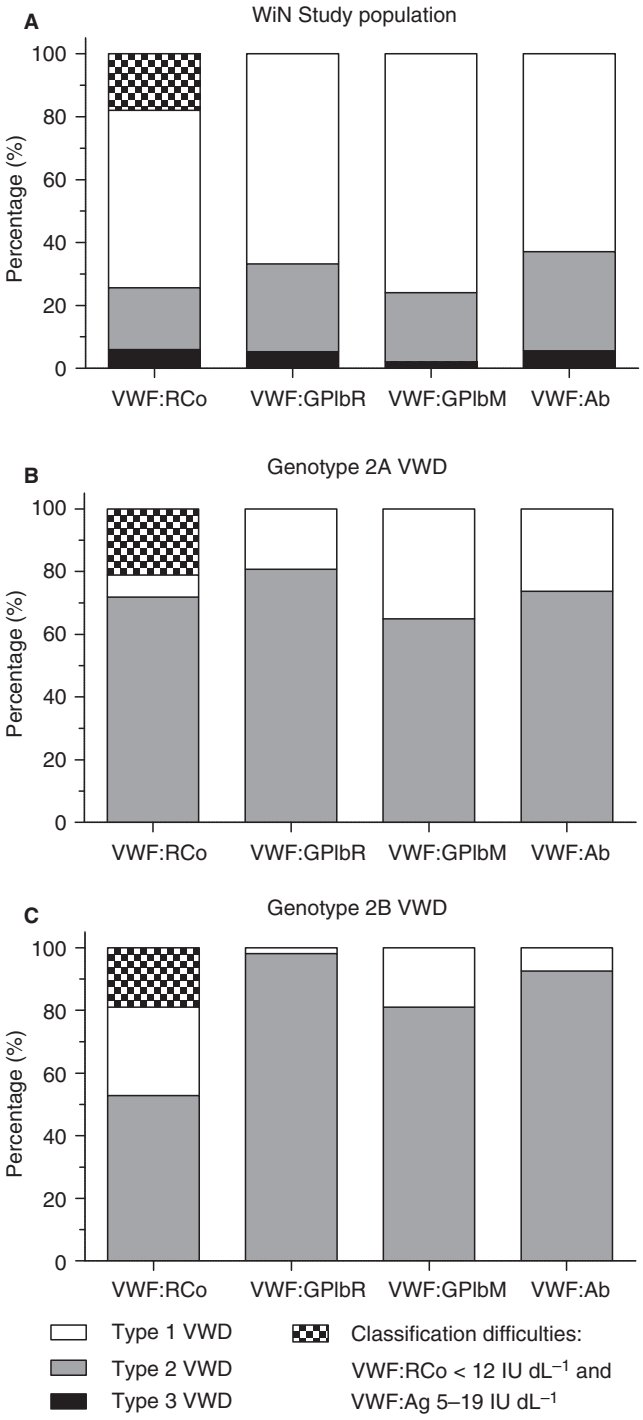

Fig. 3. von Willebrand disease classification per assay. For each assay, patients were classified according to current guidelines (type 1 VWD, VWF activity/antigen ratio > 0.60; type 2, VWF activity/antigen ratio ≤ 0.60; type 3, VWF activity and antigen both < 5 IU dL^{-1} or undetectable). Patients with type 2N VWD were excluded from analysis. (A) VWD classification for all patients. (B) VWD classification for patients with a known type 2A VWD mutation (n = 57). (C) VWD classification for patients with a known type 2B VWD mutation (n = 53). VWD, von Willebrand disease; VWF, von Willebrand factor.

Discussion

Currently there are several widely used assays that measure platelet-dependent VWF activity using different principles. Despite their widespread use, comparisons between these assays have mainly been performed in small groups of VWD patients and often only included two assays [7]. We compared the four by far most widely used VWF activity assays (VWF:RCo, VWF:GPIbR, VWF:GPIbM and VWF:Ab) in the most comprehensive study to date.

Table 2 Absolute difference between assays

Absolute difference	All results		Results ≤ 30 IU dL^{-1}*	
	n	%	*n*	%
VWF:RCo vs. VWF:GPIbR				
≥ 10 IU dL^{-1}	118/622	19.0	41/391	10.5
≥ 20 IU dL^{-1}	30/622	4.8	8/391	2.0
VWF:RCo vs. VWF:GPIbM				
≥ 10 IU dL^{-1}	147/642	22.9	81/400	20.3
≥ 20 IU dL^{-1}	25/642	3.9	7/400	1.8
VWF:RCo vs. VWF:Ab				
≥ 10 IU dL^{-1}	156/637	24.5	65/403	16.1
≥ 20 IU dL^{-1}	46/637	7.2	20/403	5.0
VWF:GPIbR vs. VWF:GPIbM				
≥ 10 IU dL^{-1}	98/622	15.8	29/377	7.7
≥ 20 IU dL^{-1}	30/622	4.8	6/377	1.6
VWF:GPIbR vs. VWF:Ab				
≥ 10 IU dL^{-1}	118/619	19.1	17/379	4.5
≥ 20 IU dL^{-1}	28/619	4.5	4/379	1.1
VWF:GPIbM vs. VWF:Ab				
≥ 10 IU dL^{-1}	110/629	17.5	56/384	14.6
≥ 20 IU dL^{-1}	27/629	4.3	11/384	2.9

*One or both assays ≤ 30 IU dL^{-1}. VWF, von Willebrand factor.

Although all four assays were highly correlated ($R^2 > 0.90$), we found clinically relevant differences in test results in one-fifth of our patient population. We also show that VWF genetics has a clear impact on the different assays and highlight limitations and advantages of each assay.

Although VWF:RCo is the historical standard for measuring VWF activity, it is well known for its high variability and lack of sensitivity [6]. In our study, VWF:RCo was below the detection limit of 12 IU dL^{-1} for almost 39% of patients. The ratio between VWF activity and antigen is calculated to distinguish type 1 (ratio > 0.6) and type 2 VWD (ratio ≤ 0.6) [1–3]. This cut-off could not be calculated accurately for 18% of patients with VWF:RCo, hampering VWD classification. VWF:GPIbR, VWF:Ab and VWF:GPIbM were much more sensitive, and we did not have classification difficulties in any patient using these assays. Hillarp *et al.* have developed an adaptation of VWF:RCo that improves its detection limit to 3 IU dL^{-1}, which may be of value for severe VWD patients, but this adaptation is not widely adopted and should be further validated [17].

Test results for VWF:RCo were high for many patients with genotype 2B VWD, especially patients with the p.Arg1306Trp mutation, and subsequently 29% of type 2B patients were misclassified as type 1. This impacts treatment for these patients because desmopressin is often given to type 1 VWD patients but is contraindicated in type 2B VWD patients. Trossaërt *et al.* also reported higher VWF:RCo in patients with type 2B VWD (compared with VWF:Ab), although the difference in their study was not statistically significant, probably as a result of small patient numbers. They, however, did not report the causal VWF mutation for their type 2B patients [18]. Importantly, ristocetin is also used in VWF:GPIbR, which correctly classified 98% of 2B patients.

Flood *et al.* have reported that the common *VWF* variant p.Asp1472His affects the ability of ristocetin to activate VWF, artifactually leading to 25% lower VWF:RCo results and possibly misdiagnosis of VWD in people with this variant [9]. We observed a similar difference in patients with this variant. The effect of p.Asp1472His on VWF:GPIbR has been hypothesized to be similar to that on VWF:RCo [19]. We are the first to investigate the effect of p.Asp1472His on VWF:GPIbR and show that this assay is not affected, meaning that people with p.Asp1472His are not at risk of VWD misdiagnosis when using VWF:GPIbR. The discrepancies between VWF:RCo and VWF:GPIbR in patients with type 2B mutations or p.Asp1472His may be explained by the difference in ristocetin concentration and use of a recombinant GPIb fragment rather than whole platelets.

VWF:GPIbM had the lowest coefficient of variation, making it the most precise assay in our study. However, VWF:GPIbM was 5 IU dL^{-1} or higher in eight of 21 patients with definite type 3 VWD (VWF:Ag and propeptide < 5 IU dL^{-1}). This finding is in contrast with previous studies in which VWF:GPIbM was undetectable in a combined total of 21 type 3 VWD patients, although a few case reports have reported a similar discrepancy in type 3 VWD patients [13,20–24]. Another VWF:GPIbM assay developed by the Zimmerman Program was undetectably low in all 13 type 3 VWD patients in their study. This assay, however, differs from the commercial assay we used (e.g. other gain-of-function mutations incorporated, ELISA versus particle-based assay) and comparability is limited.

It is well known that specific *VWF* mutations can cause distinct properties of the VWF protein that lead to artifactually high assay results, and possibly such mutations could explain the high VWF:GPIbM results in our study. However, six of the eight discrepant patients had absent VWF multimers, and all 21 type 3 VWD patients in our study had VWF:Ag and VWFpp < 5 IU dL^{-1}, indicating undetectably low circulating VWF. This rules out VWF properties that could cause such a discrepancy. [16,25] It is therefore likely that the discrepancy was caused by interference of other factors such as heterophilic antibodies, rheumatoid factors or human anti-mouse antibodies (HAMAs), which were thought to be the cause of previously reported discrepancies [23,24]. The prevalence of HAMAs may be as much as 11.7% of the population [26].

VWF:Ab has been reported to miss the type 2M VWD p.Gly1324Ala mutation [18]. Furthermore, VWF:Ab uses binding of an antibody directed against the VWF A1 domain as a surrogate for VWF-to-platelet binding. Because of this, VWF:Ab is generally considered an

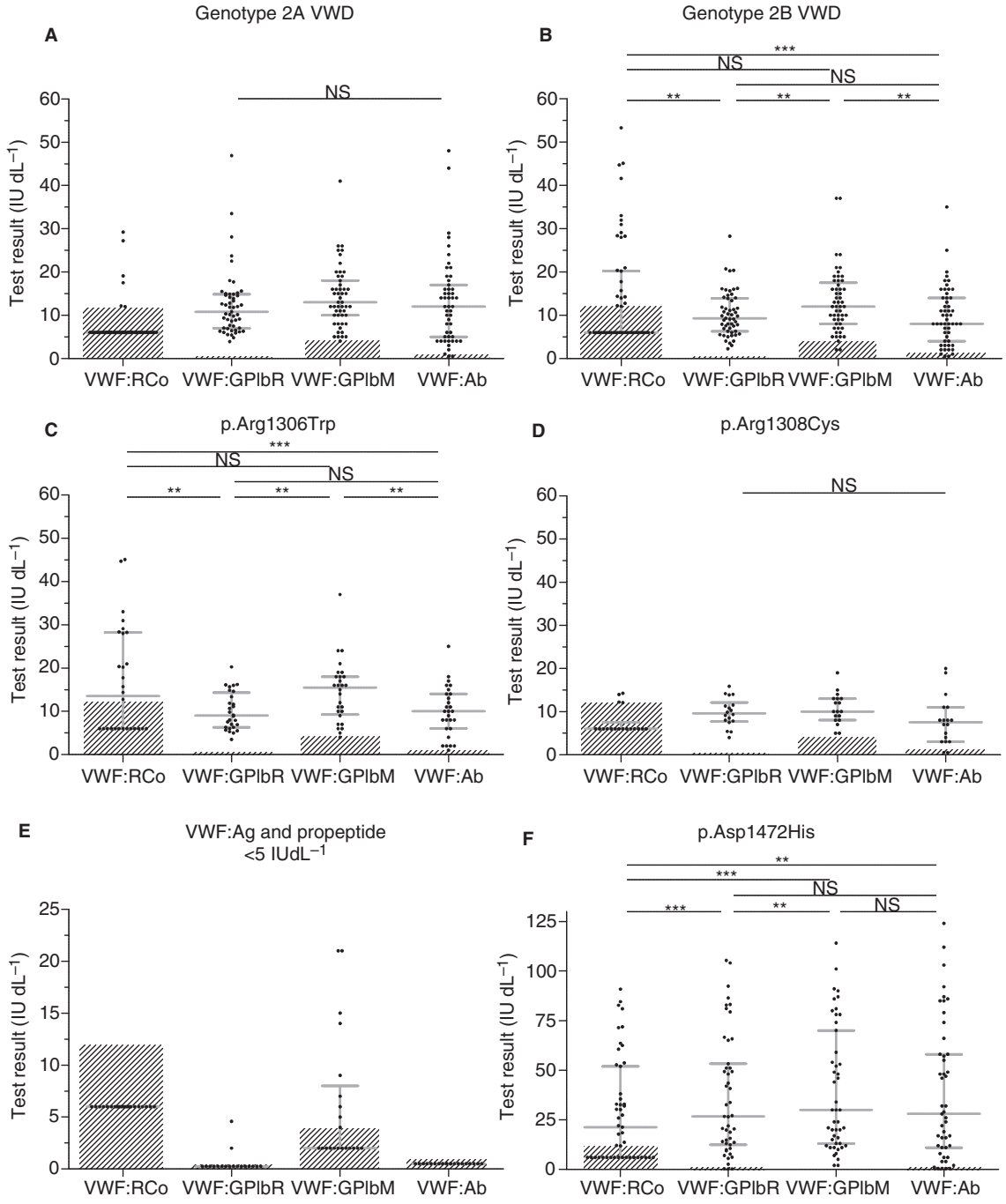

Fig. 4. Test results for subgroups of VWD patients. Test results are shown for (A) patients with a known type 2A VWD mutation (= 57), (B) patients with a known type 2B mutation (n = 53), (C) patients with the type 2B p.Arg1306Trp mutation (n = 28), (D) patients with the type 2B p.Arg1308Cys mutation (n = 18), (E) patients with type 3 VWD, defined as VWF:Ag and VWF propeptide < 5 IU dL^{-1} (n = 21), and (F) patients heterozygous (n = 46) or homozygous (n = 1) for the *VWF* variant p.Asp1472His. Striped areas depict the range below the lower limit of quantification for each assay; gray lines depict median and interquartile range. Results were compared using repeated measures one-way ANOVA with Greenhouse-Geisser correction and post-hoc analysis with Bonferroni correction. ***P < 0.001; **P < 0.01; NS, not significant; VWD, von Willebrand disease; VWF, von Willebrand factor.

adequate screening assay for VWD but not a replacement for VWF:RCo [6,19]. However, all currently available VWF activity assays and not just VWF:Ab use a surrogate (ristocetin or gain-of-function rGPIb) for the *in vivo* shear-dependent VWF to platelet binding. In our study, VWF:Ab was the assay that most consistently identified patients with type 2A, 2B and 3 VWD. No patient in our study had the p.Gly1324Ala mutation.

Although it measures the ability of VWF to bind collagen, making it essentially a different assay to the platelet-dependent VWF activity assays, VWF:CB is an important addition to the diagnostic panel for VWD. In our study, VWF:CB had excellent correlation with the VWF activity assays. Importantly, all type 2A and 2B patients undetected by single VWF activity assays were detected by VWF:CB. Additionally using a VWF:CB assay minimizes the risk of misclassifying type 2 VWD patients.

Our study has several limitations. Firstly, patients were included in the WiN Study if their historically lowest measured VWF antigen or activity in their local hospital laboratory was ≤ 30 U dL^{-1}. Because for decades VWF:RCo was used to measure VWF activity, patients may have been included solely based on a low VWF:RCo result, possibly causing a bias towards comparatively lower VWF:RCo results. Moreover, because of our inclusion criteria, this study does not provide information on the performance of VWF activity assays in the clinically relevant patient group with historically lowest VWF levels between 0.30 and 0.50 IU mL^{-1}. Secondly, there are several VWF:RCo assays, and subtle differences between these assays may limit the generalizability for the VWF:RCo assay that we used. Nevertheless, we used the VWF:RCo assay that is used by 61% of hemostasis laboratories that use a VWF:RCo assay [14]. Thirdly, for each assay we used commercially available assays from only one manufacturer, and therefore we may have missed subtle differences between manufacturers. Fourthly, our relatively short washout period for VWF concentrates of 72 h may have led to residual levels of exogenous VWF, which may have caused a small interference in the measurements. We also did not test multiple reagent lots, instead opting to use single lots to limit lot-to-lot variation. The results from our study might therefore partly reflect circumstances in the specific reagent lots used in our study rather than differences in assay principle.

Furthermore, although we were able to standardize the calibration method for three of the four assays, we were unable to do so for VWF:GPIbR. Differences between VWF:GPIbR and the other assays may therefore have been caused by the different calibration method rather than differences in assay principle. Unfortunately, we were not able to measure VWF:GPIbR in a number of patients. This assay was the latest measured and unfortunately there were no more fresh aliquots available from 20 patients (mainly children). However, we feel that this had a negligible impact on our results, because all four assays were used for the vast majority of patients. Lastly, there is currently no reference standard with which to compare all assays used in our study. We therefore used causal *VWF* mutations to identify patients with a clear VWD classification. Mutation analysis has now been performed in most patients in the WiN Study. Many *VWF* variants in our study have been linked to multiple VWD (sub)types, and were consequently excluded from subtype analysis [27]. An insufficient number of patients in our study had a mutation consistently linked to type 1 or 2M VWD. Nevertheless, our strict definition of type 2A or 2B mutation does increase the validity of our analysis.

In conclusion, VWF:RCo, long considered the reference assay for measuring VWF activity, has been surpassed by other assays in terms of sensitivity, variability and diagnostic accuracy. Laboratories that wish to use an assay that employs ristocetin should consider using VWF:GPIbR. VWF:GPIbM results should be interpreted with caution in patients with very low VWF:Ag. VWF:Ab, often not considered an actual VWF activity assay, performed at least equally to the other assays with regard to accuracy in VWD classification. The choice of VWF activity has a significant impact on the classification of VWD.

Addendum

F. W. G. Leebeek and M. P. M. de Maat designed the study, interpreted data and wrote the manuscript. J. Boender designed the study, performed laboratory measurements, analyzed and interpreted data, and wrote the manuscript. J. Eikenboom designed the study, interpreted data and critically revised the manuscript. J. G. van der Bom, K. Meijer, J. de Meris, K. Fijnvandraat, M. Cnossen, B. A. P. Laros-van Gorkom, W. van Heerde and E. P. Mauser-Bunschoten designed the study and critically revised the manuscript.

Acknowledgements

The authors thank all participating patients and E. de Wee and all the hemophilia nurses for their work on including patients. The authors also thank H. van Daele, R. Nieman, D. Priem and P. Beenhakker for their assistance in performing the laboratory measurements. A complete list of the members of the WiN Study appears in the Appendix. The WiN Study is supported by research funding from the Dutch Hemophilia Foundation (Stichting Haemophilia) and CSL Behring (unrestricted grant).

Disclosure of Conflict of Interests

B. A. P. Laros-van Gorkom has received unrestricted educational grants from Baxter and CSL Behring. K. Fijnvandraat has received unrestricted research grants from CSL Behring, Pfizer, Novo Nordisk and Bayer, and has given lectures at educational symposiums organized by Pfizer, Bayer, Baxter and Novo Nordisk. F. W. G. Leebeek has received research support from CSL Behring for performing the WiN Study (unrestricted grant), has received unrestricted research grants from Baxter for studies outside the submitted work, is a consultant for uniQure and Baxalta (Shire) and received a lecture fee from Roche. J. Boender reports grants from CSL Behring, outside the submitted work. K. Meijer has received research support from Bayer,

Baxter, Sanquin and Pfizer; speaker fees from Bayer, Sanquin, Boehringer Ingelheim and BMS; and consulting fees from Uniqure. E. P. Mauser-Bunschoten has received unrestricted research/educational support from CSL Behring, Bayer, Baxter, Griffols, Novo Nordisk, Pfizer, Biotest, Roche and Sanquin. M. Cnossen has received unrestricted research/educational funding for various projects from the following companies: Pfizer, Baxter, Bayer Schering Pharma, Novo Nordisk and Novartis. M. P. M. de Maat has collaborated with Roche, Siemens and Werfen for the evaluation of instruments and reagents. The other authors state that they have no conflict of interests.

Appendix

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1. Clinical agreement between VWF:RCo and VWF:GPIbM
Table S2. List of type 2A and 2B VWD mutations
Table S3. VWF:CB/VWF:Ag ratio and VWF multimer pattern in subgroups
Fig. S1. Correlations and Bland-Altman analyses for platelet-dependent VWF activity assays and VWF:CB. A, VWF:RCo; B, VWF:GPIbR; C, VWF:GPIbM; D, VWF:Ab. The slope was calculated using Deming regression. Dotted gray lines depict the line of identity.

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