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Evaluation of an automated method for measuring von Willebrand factor activity in clinical samples without ristocetin

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SUMMARY

Introduction: The development of an automated, von Willebrand factor (VWF) activity assay, Innovance® VWF Ac (VWF:Ac), which measures VWF binding to the platelet receptor glycoprotein Ibα without ristocetin, led us to evaluate the assay for diagnosing von Willebrand disease (VWD) and monitoring therapy.
Methods: After validating that the assay could be performed on an instrument from a different manufacturer, we compared VWF:Ac to VWF ristocetin cofactor activity (VWF:RCo) findings, including ratios of activity/antigen, for 100 healthy controls and 262 consecutive clinical samples from 217 patients (197 adults, 64 children, $n = 1$ age unknown) referred for VWF testing.
Results: There was excellent correlation ($R^2 = 0.96$) between VWF:Ac results run at two different sites on two different instruments. VWF:Ac had greater precision and sensitivity to low levels of VWF than the VWF:RCo method. Although there was good correlation between VWF:Ac and VWF:RCo results among healthy controls and patient subjects, VWF:Ac results were undetectable and/or significantly lower than VWF:RCo among patients who had types 2A, 2B, or 2M VWD. Additionally, a higher proportion of patient samples were classified as showing qualitative defects using the VWF:Ac compared with VWF:RCo method. While most samples drawn on VWD therapy had similar VWF levels by VWF:Ac and VWF:RCo, a type 2B VWD subject on replacement had much lower activity estimated by VWF:Ac.
Conclusion: We conclude that Innovance® VWF Ac is suitable for the diagnosis, classification, and monitoring of VWD, and that it has a number of advantages over VWF:RCo method.

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INTRODUCTION

Von Willebrand factor (VWF) assays have an important role in the diagnostic evaluation, and treatment monitoring, of von Willebrand disease (VWD), which is one of the most common bleeding disorders [1–4]. The laboratory evaluation for VWD requires an assessment of plasma VWF activity, which is commonly done using an aggregometer to assess VWF ristocetin cofactor activity (VWF:RCo), a quantitative method that uses the antibiotic ristocetin to induce plasma VWF binding to the VWF receptor on target platelets and platelet agglutination [5]. Aggregometry-based VWF:RCo assays have important limitations, including a requirement for an antibiotic produced by a single manufacturer, a high coefficient of variation (CV) between laboratories [6], a higher within laboratory CV than automated methods [7–10], and poor precision and poor sensitivity to low levels of VWF [6, 11, 12]. To improve sensitivity and precision, ristocetin-dependent assays of VWF activity have been adapted for enzyme-linked immunoadsorbent assay (ELISA) platforms and for automated instruments [13]. Other adaptations have included using immunoturbidimetric or chemiluminescent end-points to quantitate VWF binding to immobilized glycoprotein (GP) Ibα instead of measuring the agglutination of target platelets [14, 15]. All ristocetin-dependent estimates of VWF activity have a limitation [16]: common *VWF* polymorphisms (e.g., D1472H), which are not associated with increased bleeding, reduce ristocetin-dependent binding of VWF to GPIbα, resulting in lower than expected levels of VWF activity relative to VWF antigen (VWF:Ag) [17, 18]. While VWF activity can also be assessed by collagen binding methods, such assays do not evaluate VWF binding to GPIbα, which is altered in many forms of VWD [19].

The results of VWF activity and antigen assays are important to distinguish between quantitative VWF deficiencies (type 1 and the more severe deficiencies of type 3 VWD) and qualitative defects (type 2 VWD) that often reduce the ratio of plasma VWF:RCo to VWF:Ag. Comparison studies and proficiency testing exercises have shown that VWF:RCo is less sensitive to the loss of high molecular weight VWF multimers (HMWM) than some collagen binding assays [19, 20]. Ristocetin-induced platelet aggregometry (RIPA) is required to distinguish between the gain-of-function defects associated with type 2B and platelet-type VWD and the loss-of-function defects associated with type 2A VWD, as these forms can similarly lead to a loss of HMWM and a reduced ratio of VWF:RCo/VWF:Ag [21, 22].

Recently, a commercial, automated, immunoturbidimetric VWF activity assay INNOVANCE® VWF Ac (abbreviated: VWF:Ac) (Siemens Healthcare Diagnostics, Marburg, Germany) became available that measures VWF binding to GPIbα without ristocetin. To quantitate VWF activity in plasma, VWF:Ac uses a recombinant form of the VWF receptor with two gain-of-function mutations, captured onto polystyrene particles that are coated with an antibody against GPIbα. The INNOVANCE® kit contains three different reagents (each in a ready-to-use liquid form) that include: a suspension of polystyrene particles coated with anti-GPIbα antibodies (reagent I), a heterophilic blocking reagent (reagent II), and recombinant GPIbα with activating mutations (reagent III).

While this assay has improved sensitivity and precision compared with VWF:RCo performed by aggregometry, and it detects abnormalities associated with VWD [8], there is only limited information on its performance in diagnosing and classifying VWD and no published information on its suitability for evaluating children or for monitoring VWD therapy.

To further address the performance of the VWF:Ac assay to diagnose, classify, and monitor VWD in adults and children, we conducted a prospective, comparison study of consecutive clinical samples after validating that an adapted version of VWF:Ac, run on an STA-R Evolution (Diagnostic Stago, Asnières, France), gave equivalent findings to the manufacturer's assay run on a Sysmex CS2000i (one of the instruments that Siemens Diagnostic Healthcare indicates can be used for the assay). To further evaluate the assay, we also assessed VWF:Ac findings for subjects with the *VWF* D1472H polymorphism.

METHODS

The study was conducted in accordance with the policies and procedures for Hamilton Regional Laboratory Medicine Program (HRLMP) assay validations and institutional Research Ethics Board (REB) requirements, which did not require informed consent for evaluating discard plasmas or to gather the medical

record information on VWD diagnosis, treatment, and VWF mutation analyses from Hamilton Health Sciences (if available). All subject identifiers were anonymized prior to data analysis.

Subjects and samples

All testing was carried out using plasma collected into buffered 0.105 M (approximately 3.2%) sodium citrate anticoagulant.

For reference interval (RI) validation, one hundred healthy volunteer plasma samples (donor ages: 18–64 years; blood groups not determined) were purchased from Affinity Biologicals (Ancaster, ON, Canada) and Precision Biologic (Halifax, NS, Canada). Assay performance was assessed using normal and abnormal quality control (QC) plasmas purchased from Siemens Healthcare Diagnostics.

The effect of pre-analytical errors on VWF:Ac compared with VWF:RCo results was compared by storing aliquots of a healthy control sample as whole blood or plasma at 4 °C or room temperature for up to 24 h before testing VWF activity.

To evaluate the findings for VWF:Ac run on different instruments, discard plasma samples, obtained for clinical testing by HRLMP (n = 48) and the Ottawa Hospital (n = 10), were exchanged. Additional, clinical samples tested (n = 262) included consecutive, discard plasma samples collected in Hamilton for VWD determinations (n = 261), along with a plasma obtained with informed consent from a type 2B VWD patient previously reported to have normal VWF:RCo and VWF:Ag levels [23].

The 262 samples from 217 patients evaluated included 188 samples (72%) from 153 females and 73 samples (28%) from 63 males (gender not provided for one subject). 197 samples were from adults (75%; ages 18–84) and 64 were from children (24%; ages 0–17 years) (age unknown for 1 sample). 67 samples (26%) were from patients with previously diagnosed VWD (n = 34, 91% adults, 9% children; type 1: n = 12; type 2A: n = 4; type 2B: n = 4; type 2M: n = 7; type 2N: n = 1; type 3: n = 3; acquired VWD: n = 3), including 29 samples that were used to assess VWD treatment.

Plasma samples (n = 6, anonymized) from persons with the *VWF* D1472H polymorphism were kindly provided by Dr. Robert Montgomery (Milwaukee Blood Center, Milwaukee, WI, USA).

Laboratory investigations

Plasma was tested by the VWF:Ac on an STA-R Evolution in Hamilton and on a Sysmex CS2000i instrument in Ottawa. On the STA-R Evolution, the VWF:Ac assay was modified as summarized in Table 1. During the validation stage that preceded the prospective, clinical sample evaluation, no prozone effect was observed. Curve graphics for each normal and abnormal sample were visually reviewed. The stability for all reagents was confirmed for up to 3 weeks.

VWF:RCo was performed by aggregometry on a Helena AggRAM instrument (Helena Laboratories, Beaumont, TX, USA) as recommended by the manufacturer. VWF:Ag was measured on an STA-R Evolution using the Diagnostica Stago LIATEST VWF:Ag assay.

Table 1. Summary of the protocol used for the Innovance® VWF Ac method run on an STA-R Evolution instrument

	Protocol for STA-R Evolution
Measurement	Delta absorbance at 540 nm
Curve fit	Polynomial, 3rd order
Scale	Lin Lin
Measurement range	0.04–0.75 IU/mL
Sample default dilution	1/7
Sample volume	60 μL
Diluent	Owren's Buffer
Reagent sequence	
Step 1	Reagent I (polystyrene particles coated with anti-GPIbα*)
Step 2	Reagent II (heterophilic blocking reagent)
Step 3	Reagent III (monoclonal anti-GPIbα)
Reagent volumes (μL)	
Step 1	60
Step 2	100
Step 3	25
Reagent incubation time (s)	
Sample	0
Step 1	0
Step 2	240
Step 3	0
Measurement analysis (s)	
Start time	30
End time	270

*Addition of 5 mL Owren's Buffer to reagent 1 (2 mL) (7 mL final volume).

Calibration curves for the VWF:Ac and VWF:Ag were prepared using reference plasmas calibrated against a WHO standard. For VWF:Ac, Standard Human Plasma (Siemens Healthcare Diagnostics) was used, and for VWF:RCo and VWF:Ag, the VWF:Ag Calibrator (Diagnostica Stago, Asnières, France) was used.

Statistical analyses

Reference intervals (RI) were determined by non-parametric estimates of the 95% confidence limits for 100 healthy controls, as described [24, 25]. Coefficients of variation (CV) and two-tailed t-test were determined using Microsoft Excel (Microsoft Canada, Mississauga, ON). Linear regressions, with determination of *P*-values, were performed using Stata 12 software (StataCorp LP, College Station, TX). Bland Altman plots were used to compare the results of VWF activity assays [26]. *P*-values <0.05 were considered statistically significant.

To determine whether using VWF:Ac instead of VWF:RCo influenced the interpretation of findings, two hematologists (L.G. and C.H.) reviewed data for each subject with one or more abnormal result and classified the findings in accordance with recent guidelines [5]. The subject's HRLMP results for multimer analysis and RIPA were considered, if available for current or previous samples. After establishing the consensus interpretation for each case, the results of *VWF* genetic investigations were reviewed, if available.

RESULTS

Validation analyses

The RI validated for VWF:Ac performed on the STA-R instrument [0.48–1.80 IU/mL] was similar to manufacturer's RI [0.48–1.73 IU/mL] for the assay. Comparison of healthy control samples from subjects of a specified gender (19 female and 30 male) indicated that results for both genders were similar ($P = 0.47$).

Results of samples exchanged between Hamilton and Ottawa ($n = 58$) indicated that there was excellent agreement ($R^2 = 0.96$, $P < 0.001$) between VWF:Ac estimated on STA-R Evolution and Sysmex CS2000i instruments (Figure 1). Additionally, VWF:Ac for an external quality assurance sample assessed on the STA-R (0.93 IU/mL) agreed with the results reported by peers for VWF:Ac (0.88 IU/mL, CV 11.6%) and for VWF:RCo (0.90 IU/mL, CV 16%; HRLMP result: 1.15 IU/mL). WHO 5th International Standard Factor VIII and von Willebrand Factor in plasma (NIBSC code: 02/150; assigned values, in IU/mL: VWF:Ag 0.91; VWF:RCo 0.78) had a VWF:Ac of 0.77 IU/mL on the STA-R Evolution, and a measured VWF:RCo value of 0.78 IU/mL.

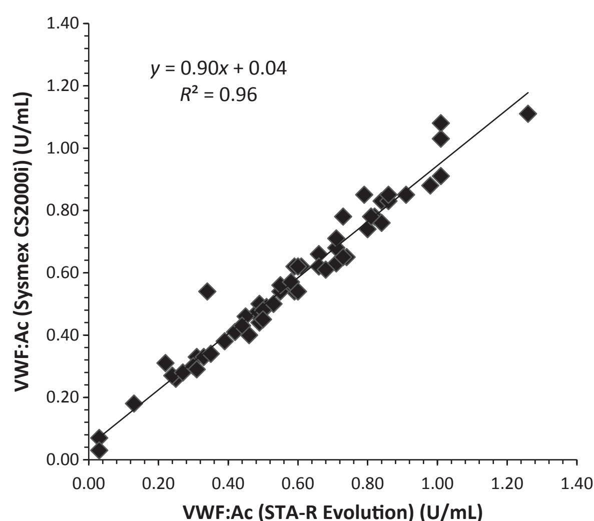

Figure 1. Comparison of Innovance® VWF Ac results for samples run on different instruments. The R^2 value indicates the correlation between results for the same samples, tested on an STA-R Evolution instrument in Hamilton and on a Sysmex CS2000i instrument in Ottawa.

Quality control sample analysis in Hamilton indicated that there was no trending of the VWF:Ac over a 3-h period. Within laboratory assessments (40 determinations, over 6 months with 4 lots of reagents) indicated that the VWF:Ac had better precision than the VWF:RCo assay (respective CV, normal sample: 5.6% *vs.* 13.0%, $P < 0.005$; abnormal sample: 9.4% *vs.* 14.5%, $P < 0.005$). The estimated lower limit for VWF:Ac performed on the STA-R Evolution (0.04 IU/mL) was lower than the VWF:RCo assay (0.10 IU/mL) and consistent with the manufacturer's specifications.

The estimated lower RI limit (2.5 percentile) for the VWF:activity/VWF:Ag ratio, based on healthy control samples, was lower for VWF:Ac/VWF:Ag (ratio: 0.63) than for VWF:RCo/VWF:Ag (ratio: 0.70) ($P < 0.001$).

Further analyses of healthy control samples indicated that there was good correlation between VWF:Ac and VWF:RCo ($R^2 = 0.68$, $P < 0.001$; Figure 2a) and between VWF:Ac and VWF:Ag ($R^2 = 0.73$, $P < 0.001$; Figure 2b).

As the VWF:RCo assay, the VWF:Ac assay was susceptible to pre-analytical errors as inappropriate storage of a healthy control whole-blood sample at 4 °C for 24 h led to striking reductions in VWF activity by both VWF:Ac and VWF:RCo (% loss of activity: VWF:Ac 51%; VWF:RCo 60%), whereas storage of the control's whole blood or plasma for 4 h at room temperature or 4 °C, or storage of the donor's plasma at 4 °C for 24 h, had minimal effects (≤15% loss of activity by both assays).

Analyses of consecutive patient samples referred for VWF testing

Bland Altman plot analysis of all clinical and healthy control samples ($n = 362$) indicated that there was some bias as VWF:Ac levels were, on average, 0.07 IU/mL (95% CI: 0.04–0.10 IU/mL) lower than VWF:RCo, with 95% agreement limits that ranged from −0.49 to 0.63 IU/mL (Figure 3). While patient samples ($n = 262$) showed a good overall correlation between VWF:Ac and VWF:RCo ($R^2 = 0.78$, $P < 0.001$; Figure 4a), and between VWF:Ac and VWF:Ag ($R^2 = 0.65$, $P < 0.001$; Figure 4b), outliers with low ratios of VWF:Ac/VWF:Ag were evident (Figure 4b) that included samples from patients with type 2 VWD, taken before and after treatment (presented later).

Further analyses of patient data by age (after excluding samples from subjects with known VWD on treatment) indicated that there was overlap between VWF:Ac results for adults and children (Figure 4c).

The majority of patient samples (173/262, 66%) had normal VWF:Ac/VWF:Ag and VWF:RCo/VWF:Ag ratios, whereas the others had either reduced ratios for both (51/262, 19%), only an abnormal VWF:Ac/VWF:Ag ratio (33/262, 13%), or only an abnormal VWF:RCo/VWF:Ag ratio (5/262, 2%). Among the 84/262 samples with an abnormal VWF:Ac/VWF:Ag ratio, many (50/84, 60%) were from patients with previously diagnosed VWD, including 23 samples taken on VWD treatment.

The data for 35 samples from 31 patients with previously diagnosed congenital VWD, who were not on therapy at the time of testing, are summarized in Figure 5. Type 1 VWD samples had similar VWF:Ac and VWF:RCo levels (means ± SD, in IU/mL: VWF:Ac 0.25 ± 0.13; VWF:RCo 0.30 ± 0.14; $P = 0.19$), as did the type 2N VWD (VWF:Ac 0.56 IU/mL *vs*. VWF:RCo 0.57 IU/mL) and type 3 VWD samples (3 from 3 patients; undetectable activity by both assays). Type 2M VWD samples (11 from 8 patients) had significantly lower activity by the VWF:Ac than by the VWF:RCo method (means ± SD in IU/mL: VWF:Ac 0.09 ± 0.06; VWF:RCo 0.13 ± 0.05; $P = 0.04$). While differences in VWF activity levels measured by the two assays were not significantly different for the small number of type 2A VWD ($n = 4$, 4 individuals; means ± SD: VWF:Ac 0.09 ± 0.08 IU/mL; VWF:RCo 0.24 ± 0.15 IU/mL; $P = 0.07$) and type 2B samples ($n = 4$, 4 individuals; means ± SD: VWF:Ac 0.19 ± 0.25 IU/mL; VWF:RCo 0.48 ± 0.33 IU/mL; $P = 0.10$), pooled analyses indicated that VWF:Ac was significantly lower among subjects with type 2A or 2B VWD

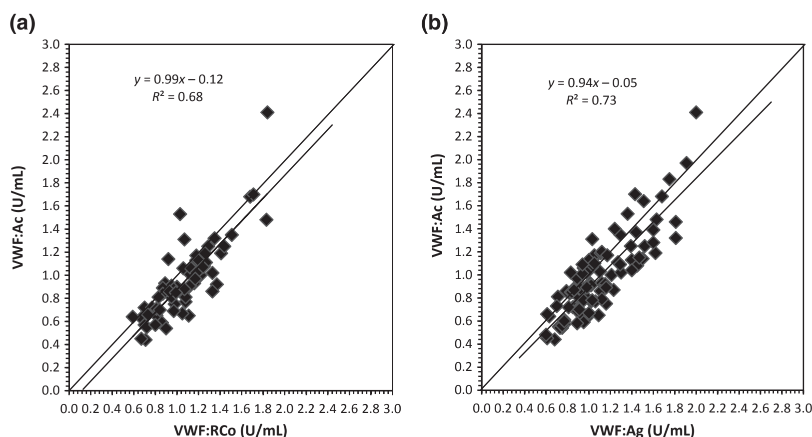

Figure 2. Comparison of Innovance® VWF Ac to VWF:RCo and VWF:Ag findings for healthy controls. Panels a and b illustrate the correlation between assay results (panel a, $n = 66$; panel b, $n = 100$; dotted lines indicate identity).

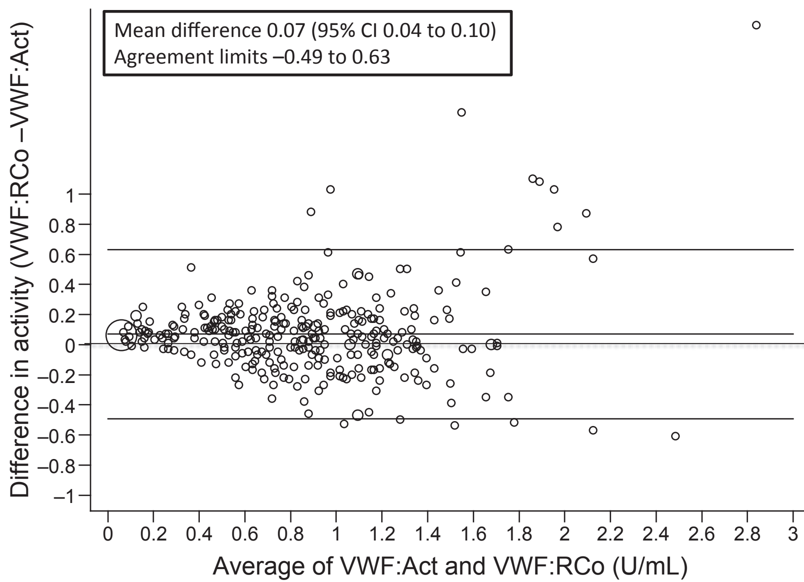

Figure 3. Bland Altman plot analysis of VWF activity measured by the Innovance® VWF Ac and VWF:RCo methods. The plot compares the difference between the measured values by both assays to the mean of both measures for individual samples (dotted line indicates identity; larger circles indicate overlapping results).

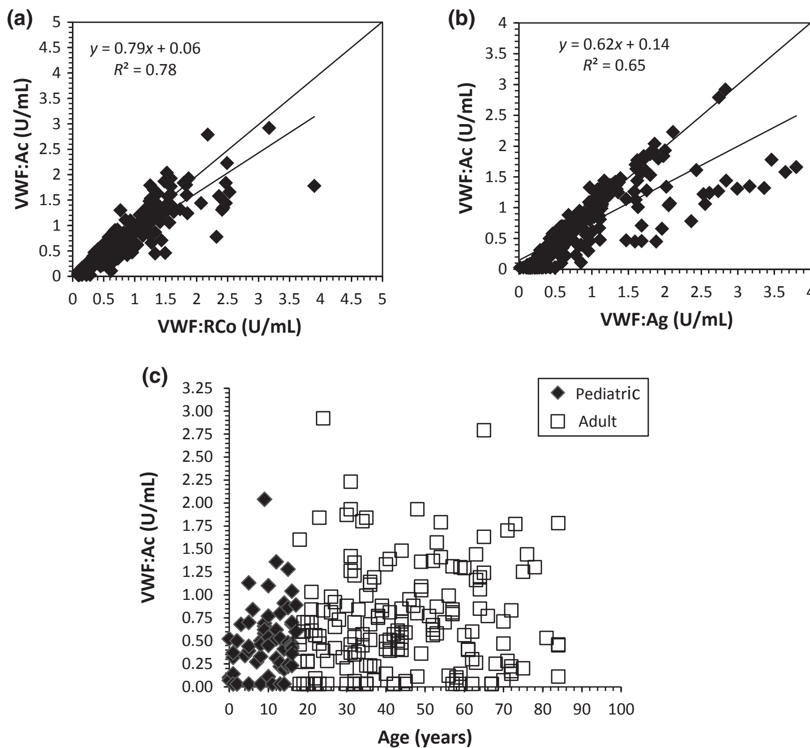

Figure 4. Comparison of Innovance® VWF Ac to VWF: RCo and VWF:Ag findings for clinical samples. Results for 262 consecutive samples from 217 patients are shown in Panels a and b (dotted line indicates identity; equation and R^2 indicate the relationship and correlation between assay results). Panel c shows Innovance® VWF Ac results for adults and children, by subject age, after exclusion of samples drawn on VWD treatment.

($P = 0.04$). Additionally, the only patient with type 2 VWD with a normal VWF:Ac result was the type 2B subject with a history of normal VWD screens (VWF: Ac 0.56 IU/mL; VWF:RCo 0.88 IU/mL), whose VWF: Ac/VWF:Ag was abnormal (0.61; lower limit: 0.63), unlike her VWF:RCo/VWF:Ag ratio (0.96; lower limit: 0.71). Her affected daughter had more striking abnormalities (VWF:Ac <0.04 IU/mL, VWF:RCo 0.28 IU/mL), like other type 2B VWD subjects.

The patients with acquired von Willebrand syndrome (AVWS) in association with an IgG paraprotein, who had samples drawn remote from therapy (three

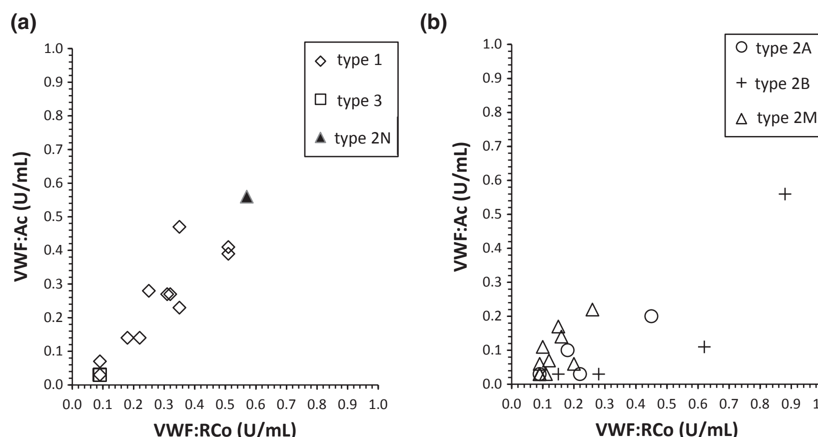

Figure 5. Comparison of Innovance® VWF Ac to VWF:RCo for patients with known VWD who had not received treatment. Panel a compares data for type 1, 3, and 2N VWD subjects, and Panel b compares data for type 2 VWD subjects (dotted lines indicate identity).

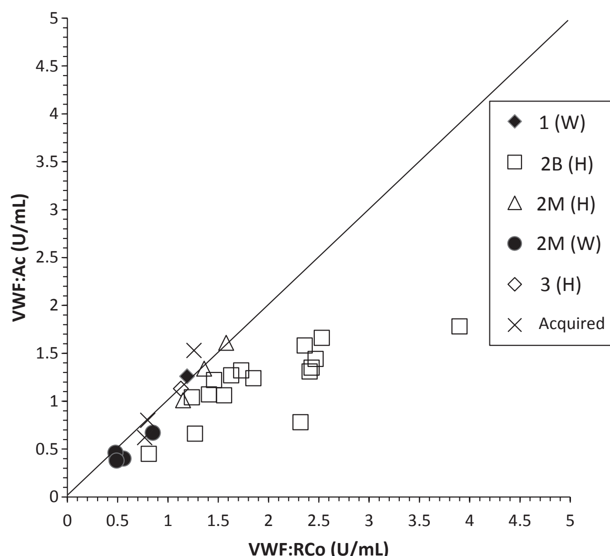

Figure 6. Comparison of Innovance® VWF Ac to VWF:RCo findings for patients with VWD on treatment. Results are shown for subjects with congenital VWD treated with Wilate (W), Humate P (H) and acquired VWD treated with intravenous gammaglobulin (X symbols) (dotted line indicates identity).

samples from three subjects), had VWF:Ac levels that were undetectable or lower (range: <0.04–0.07 IU/mL) than VWF:RCo levels (range: <0.10–0.18 IU/mL). The patient with essential thrombocythemia, who was evaluated twice for suspected AVWS (multimers not requested), had mildly reduced to normal VWF:Ac (0.47 and 0.71 IU/mL), normal VWF:RCo levels (0.96–1.43 IU/mL) and lower ratios for VWF:Ac/VWF:Ag (0.32–0.43 IU/mL) than for VWF:RCo/VWF:Ag (0.65–0.85 IU/mL).

Figure 6 shows VWF activity results for 29 samples from 9 VWD patients who had samples drawn to follow treatment of type 2M VWD with Wilate (n = 1; 5 samples) or Humate P (n = 2; 3 samples); type 1 VWD with Wilate (1 sample); type 3 VWD with Humate P (1 sample); type 2B VWD (n = 1, 16 samples) with Humate P; and AVWS (n = 2 with an IgG paraprotein, three samples) with IVIG. Treatment samples for all but the type 2B VWD patient showed acceptable correlation between the VWF:Ac and VWF:RCo (R^2 = 0.50). The type 2B subject (who required treatment for prolonged gastrointestinal bleeding) had consistently lower VWF:Ac than VWF:RCo on therapy (mean ± SD, in IU/mL: VWF:Ac 1.20 ± 0.36 IU/mL *vs*. VWF:RCo 1.96 ± 0.74 IU/mL; $P < 0.005$) (pretreatment levels: VWF:Ac 0.11 IU/mL *vs*. VWF:RCo 0.62 IU/mL).

Effect of the VWF activity assay on the detection and classification of abnormal findings

The effect of using VWF:Ac instead of VWF:RCo on the interpretation of findings was evaluated for 74 clinical samples from 66 patients with at least one of the following abnormalities: VWF:Ac <0.30 IU/mL, VWF:RCo <0.30 IU/mL, ratio VWF:Ac/VWF:Ag <0.63 or ratio VWF:RCo/VWF:Ag <0.70. There were no differences in the interpretations made by the two hematologists. Among first samples, 59% (39/66) were interpreted identically with both activity assays, whereas 41% (27/66) were interpreted differently, mainly because VWF:Ac data suggested more cases

had qualitative abnormalities (Figure 7). The repeat tests on 7 patients (one patient retested twice) confirmed original interpretations made with VWF:Ac, whereas interpretations made with VWF:RCo were discrepant for one patient (classified as having type 2 VWD than normal findings).

Among patients with one or more abnormal findings, 54 had data for multimer analyses, including 3 with type 3 VWD without detectable VWF. VWF:Ac was significantly lower than VWF:RCo among subjects with a loss of HMWM (n = 10, means ± SD, in IU/mL: VWF:Ac 0.09 ± 0.09; VWF:RCo 0.25 ± 0.16; P = 0.007). Smaller differences, which were close to the mean differences estimated by Bland Altman analysis, were evident among subjects (n = 40) without loss of HMWM (means ± SD, in IU/mL: VWF:Ac 0.15 ± 0.11; VWF:RCo 0.23 ± 0.16; P = 0.004).

VWF mutation analysis results were available for 10 patients with one or more abnormal findings. Among the six patients with identical interpretations by both assays, the *VWF* mutations included: heterozygosity for c.3614G>A [p.(R1205H)], associated with the Vicenza variant that accelerates VWF clearance [27] in a type 1 VWD subject; heterozygosity for c.4120C>T [p.(R1374C)], reported in type 2M and 2A VWD [28] in two type 2M subjects; heterozygosity for c.3943C>T [p.(R1315C)], reported in type 2M VWD [29] in another type 2M subject; homozygosity for c.817C>T [p.(R273W)], reported in type 1 and type 3 VWD [30] in a type 3 VWD subject; and no candidate mutation in the second type 3 subject. Among subjects who were classified differently using VWF:Ac or VWF:RCo findings (n = 4), the *VWF* mutations included heterozygosity for c.6536C>T [p.(S2179F)] associated with type 1 VWD with accelerated clearance [31] in the patient classified as type 1 VWD by VWF:RCo but as type 2M by VWF:Ac; heterozygosity for c.4378C>T [p.(L1460F)], a mutation reported for type 2B VWD [32] in the patient with type 2B VWD whose sole abnormality was a reduced VWF:Ac/VWF:Ag ratio; and no mutations in exon 28 in the patients classified as type 2M VWD based on VWF:Ac findings but as low VWF (n = 1) or type 1 VWD (n = 1) based on VWF:RCo results.

Interpretations based on VWF:Ac \ Interpretations based on VWF:RCo	Normal/ low VWF	Type 1	Type 1, 2M not excluded	Type 2M	Type 2A or 2B	Type 2 (A, B or M)	Type 3
Type 3							3
Type 2 (A, B or M)	4	1				5	
Type 2A or 2B		1			9		
Type 2M	6	6	4	16			
Type 1 , 2M not excluded							
Type 1	3	6		1			
Normal/ low VWF		1					

Figure 7. The effect of using Innovance® VWF Ac or VWF:RCo to interpret patient results with one or more abnormal finding. Shaded boxes indicate concordance, and numbers within cells indicate the number of patients with that classification.

VWF activity levels for samples from individuals with the VWF D1472H polymorphism

Among the 6 samples from subjects known to have the *VWF* polymorphism D1472H, all but one sample showed a higher VWF:Ac compared with VWF:RCo, but the differences were not statistically significant (means ± SD, ranges, in IU/mL: VWF:RCo 0.76 ± 0.20, 0.61–1.14; VWF:Ac 0.79 ± 0.04, 0.75–0.83; P = 0.35).

DISCUSSION

The goal of our study was to evaluate the performance of a new VWF activity assay, Innovance® VWF Ac (VWF:Ac), that assesses VWF binding to GP Ibα without added ristocetin, for the evaluation of VWD in adults and children, including the monitoring of replacement therapy. We validated that VWF:Ac can be run on another commonly used coagulation instrument, as there was excellent agreement between VWF:Ac run on Sysmex CS2000i and on Diagnostica Stago STA-R Evolution. Based on these data, we undertook an analysis of consecutive, clinical samples, from both adults and children, and found that the VWF:Ac assay has an acceptable performance for VWD diagnosis and therapy monitoring purposes, although VWF:Ac results were, on average, 0.07 IU/mL less

than VWF:RCo, based on 362 sample determinations. Interestingly, our estimate of bias is in good agreement with the −6% value reported by Lawrie *et al.* [8]. We confirmed that the VWF:Ac assay has better precision and sensitivity, and an improved lower limit of detection (0.04 *vs.* 0.10 IU/mL) compared with assessments of VWF:RCo on an aggregometer. Our study of a large number of consecutive clinical samples provides some unique observations. First, we found that VWF:Ac levels were undetectable and/or lower than VWF:RCo levels among patients, not on therapy, who had type 2A, 2B, 2M VWD, or AVWS due to an IgG paraprotein. Second, we observed that VWF:Ac was lower than VWF:RCo among patients with VWD and a loss of HMWM, whereas differences consistent with the overall assay bias were observed between patients with a normal multimer distribution. Third, we observed that more patients were classified as having qualitative rather than quantitative VWF defects based on the ratio of VWF:Ac/VWF:Ag, compared with VWF:RCo/VWF:Ag. Fourth, our analyses of patients with VWD receiving treatment indicated that the levels of VWF activity on replacement therapy agreed for most subjects but were much lower by VWF:Ac for a subject with type 2B VWD who had prolonged bleeding on replacement therapy and a notable discrepancy between VWF:Ac and VWF:RCo prior to therapy. The latter observation raises questions about whether VWF:Ac is more suitable than the VWF:RCo assay for dosing VWD replacement therapy in subjects with type 2B VWD.

From a laboratory perspective, the ability to run VWF:Ac on the same automated analyzer as other components of a VWD screen (e.g., factor VIII and VWF:Ag assays) is an important advantage for workflow, including the handling of STAT or urgent requests. We recommend that laboratories follow the sample collection and handling procedures recommended for other VWF assays as pre-examination errors (e.g., storage of whole blood at 4 °C for 24 h) had similar effects on VWF:Ac and VWF:RCo.

We found significant overlap in the VWF:Ac levels for children and adults in our study. We were unable to develop age-specific reference intervals for children because our study was designed to compare data for clinical samples, and healthy control samples from adults. While age-specific reference intervals are rarely used to evaluate VWF levels in diagnostic practice, Gill and colleagues reported that VWF:Ag levels increase by approximately 10% per decade of life, based on data for a large number of healthy blood donors [33].

A minority of the VWD subjects evaluated in our study had a known *VWF* mutation associated with VWD, and none had undergone complete gene sequencing. Given that a higher proportion of patient samples were classified as having qualitative abnormalities when VWF:Ac was used instead of VWF:RCo, the choice of activity assay could influence the rate of referrals for *VWF* mutation analysis. At present, the platelet based VWF:RCo is usually regarded as the gold standard for identifying functional defects. There is a need for improved functional assays as some mutations have been reported in association with more than one type of VWD [34]. While no exon 28 mutations had been found in the two subjects who were classified as having a qualitative defect (type 2M VWD) based on VWF:Ac but not VWF:RCo results, it is possible that these subjects could have mutations in other exons and/or subtle losses of HMWM that were not reported. It would be interesting to evaluate VWF:Ac in more subjects with mutations associated with type 1 VWD from accelerated clearance [31, 34] as a subject in our study with such a mutation was classified as type 2M VWD using VWF:Ac data, but as type 1 VWD using VWF:RCo data. Another with the Vicenza mutation that accelerates VWF clearance was classified as type 1 VWD based on both activity assays. Genotype–phenotype studies for a larger cohort would be helpful to better understand the effects of *VWF* mutations and polymorphisms on the findings for VWF:Ac and VWF:Ac/VWF:Ag ratios.

We observed that subjects with gain-of-function defects from type 2B VWD, or loss-of-function defects from type 2A or 2M VWD, had reduced VWF activity by the VWF:Ac method. This is important for diagnostic strategies as gain-of-function mutations increase the binding of VWF to GPIbα in ELISA [14]. Accordingly, RIPA still has a role in evaluating patients suspected to have type 2 VWD based on VWF:Ac and VWF:Ag results [35]. We had postulated that *VWF* polymorphisms that impair VWF:RCo activity without increased bleeding, which have no effect on ristocetin-independent binding of VWF to GPIbα (e.g., D1472H), would have higher VWF:Ac than VWF:RCo levels. While most subjects (5/6) that we tested with the D1472H polymorphism did have higher levels by

VWF:Ac, our study was underpowered to determine whether VWF:Ac levels were significantly higher than VWF:RCo levels in these subjects given that VWF:Ac levels were, on average, about 0.07 IU/mL lower than VWF:RCo for other samples.

Assays with high sensitivity and specificity are ideally suited for the laboratory evaluation of bleeding disorders. Historically, an assessment of VWF:RCo by aggregometry has been the most common method to assess VWF activity; however, ELISA, immunoturbidimetric, and chemiluminescent methods for assessing VWF activity have better precision and a lower limit of detection, and some have an improved sensitivity to HMWM loss, as some collagen binding assays [10, 36]. Our study, and the recent report [8], provide considerable evidence that replacing VWF:RCo with VWF:Ac in VWD screens is acceptable to evaluate patients for bleeding disorders with quantitative or qualitative defects in VWF binding to GPIbα. More patients were diagnosed as having qualitative VWF abnormalities using VWF:Ac than VWF:RCo, and we suspect that this reflects better sensitivity and precision along with an improved detection of some functional abnormalities. A large prospective study of subjects undergoing initial bleeding disorder investigations would be helpful to further assess the sensitivity and specificity of VWD screens that include VWF:Ac. Almost all subjects with known VWD that we tested in our study had reduced VWF:Ac, and the single patient with type 2B VWD with normal VWF:Ac and VWF:RCo had a reduced VWF:Ac/VWF:Ag ratio, but a normal VWF:RCo/VWF:Ag ratio. An additional patient, suspected to have AVWS, had an abnormal ratio of activity to antigen when VWF:Ac was used instead of VWF:RCo. These observations indirectly suggest that replacing VWF:RCo with VWF:Ac might reduce the number of false-negative VWD screens, for both congenital and acquired defects. However, a much larger study would be required to evaluate these possibilities.

In conclusion, the Innovance VWF:Ac method is an acceptable, automated alternative to the VWF:RCo method for assessment of VWF binding to the platelet receptor GPIbα that is sensitive to both quantitative and qualitative defects of VWF. Laboratories and clinicians need to be aware that some patients with VWD have much lower levels of VWF measured by the VWF:Ac assay than by VWF:RCo. A larger prospective study, and genotype–phenotype investigations, would be required to determine whether using VWF:Ac instead of VWF:RCo improves VWD detection, classification, and treatment monitoring.

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