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ORIGINAL ARTICLE

Differential sensitivity of von Willebrand factor (VWF) 'activity' assays to large and small VWF molecular weight forms: a cross-laboratory study comparing ristocetin cofactor, collagen-binding and mAb-based assays

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Summary. *Background:* von Willebrand disease (VWD), the most common inherited bleeding disorder, is caused by deficiencies and/or defects in von Willebrand factor (VWF). An effective diagnostic and VWD typing strategy requires plasma testing for factor VIII, and VWF antigen plus one or more VWF 'activity' assays. VWF activity is classically assessed by using VWF ristocetin cofactor activity (VWF:RCo), although VWF collagen-binding (VWF:CB) and VWF mAb-based (VWF activity [VWF:Act]) assays are used by some laboratories. *Objective:* To perform a cross-laboratory study to specifically evaluate these three VWF activity assays for comparative sensitivity to loss of high molecular weight (HMW) VWF, representing the form of VWF that is most functionally active and that is absent in some types of VWD, namely 2A and 2B. *Methods:* A set of eight samples, including six selectively representing stepwise reduction in HMW VWF, were tested by 51 different laboratories using a variety of assays. *Results:* The combined data showed that the VWF:CB and VWF:RCo assays had higher sensitivity to the loss of HMW VWF than did the VWF:Act assay. Moreover, within-method analysis identified better HMW VWF sensitivity of some VWF:CB assays than of others, with all VWF:CB assays still showing better sensitivity than the VWF:Act assay. Differences were also identified between VWF:RCo methodologies on the basis of either platelet aggregometry or as performed on automated analyzers. *Conclusions:* We believe that these results have significant clinical implications for the diagnosis of VWD and monitoring of its therapy, as well as for the future diagnosis and therapy monitoring of thrombotic thrombocytopenic purpura.

Keywords: diagnosis, high molecular weight sensitivity, laboratory testing, von Willebrand disease, von Willebrand factor.

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Introduction

von Willebrand disease (VWD) is considered to be the most common inherited bleeding disorder. VWD is suspected following clinical and physical examination in individuals with personal and familial evidence of mucocutaneous bleeding, and the diagnosis is confirmed by laboratory testing [1,2]. VWD reflects quantitative or qualitative defects or deficiency in plasma von Willebrand factor (VWF), and is classified into six types. Types 1 and 3 define quantitative disorders with partial or complete loss of VWF, respectively, and types 2A, 2B, 2M and 2N define qualitative disorders [1,2]. Notably, type 2A VWD is characterized by decreased VWF-dependent platelet adhesion, owing to selective deficiency of high molecular weight (HMW) VWF multimers, type 2B VWD by increased affinity of VWF for platelet glycoprotein (GP)Ib, often also associated with loss of HMW VWF, and type 2M VWD by impaired VWF-dependent platelet adhesion without selective deficiency of HMW VWF [2].

Focused laboratory testing generally includes assessment of plasma factor VIII coagulant activity (FVIII:C), and VWF antigen (VWF:Ag) and VWF 'activity', supplemented with additional tests on a case-by-case basis [1–3]. VWF 'activity' is classically assessed with the VWF ristocetin cofactor (VWF:RCo) assay, originally described in the early 1970s [3–5]. Problems with this assay, including complexity, performance time, poor reproducibility, and poor sensitivity to low levels of

VWF (reviewed elsewhere [3]), has seen attention focused on newer assays, primarily the VWF collagen-binding (VWF:CB) assay and various so-called VWF 'activity' (VWF:Act) assays based on mAb technology. Used in conjunction with VWF:Ag and FVIII activity, VWF activity assays have the capacity to identify and discriminate the various types of VWD. For example, VWF:RCo/VWF:Ag (RCo/Ag) and VWF:CB/VWF:Ag (CB/Ag) ratios can be used to differentiate type 1 from types 2A, 2B and 2M VWD [1–3,6], given that these assays are sensitive to loss of HMW VWF and may also reflect VWF function (i.e. binding of VWF to platelet GPIb or collagen, respectively). Activity assays are also used as surrogate laboratory markers of HMW VWF, either for VWD diagnosis [1–3], VWF concentrate production, therapy, and pharmacokinetic studies [7], or assessment of VWF protease (e.g. ADAMTS-13 activity or inhibition for investigation of thrombotic thrombocytopenic purpura [TTP]) [8].

The VWF:CB assay is an ELISA-based assay first described by Brown and Bosak in 1986 [9]. mAb-based VWF:Act immunoassays, originally described in 1995, utilize a mAb directed against a functional epitope on VWF as the capture and/or detection antibody [3,10–12]. Later independent validation studies suggested inferiority of the commercial mAb ELISA-based assays to VWF:RCo and VWF:CB or even in-house mAb-based ELISA assays for discrimination of HMW VWF-deficient VWD types such as 2A and 2B [13–15], and as most recently highlighted for a case study of type 2A VWD [16]. The latex agglutination assay developed by Instrumentation Laboratory is currently the most popular mAb-based VWF:Act assay [17,18]. The true utility of this particular assay in VWD is only now emerging. Despite early reported high correlation with the VWF:RCo assay [17,18], these two assays are increasingly recognized as providing different values with many cases of VWD, notably qualitative variants [19,20].

Assessments of the respective performance of VWF activity assays within cross-laboratory testing exercises are limited. The Royal College of Pathologists of Australasia (RCPA) Haematology Quality Assurance Program (QAP) last reported findings in this area in 2007 [21]. At that time, the mAb latex-based VWF:Act assay had recently emerged, and was performed by approximately 20% of participant laboratories, often as a replacement for the VWF:RCo assay. Nevertheless, discrepant behavior of the three different activity assays (i.e. VWF:RCo, VWF:CB, and VWF:Act) for discrimination of several type 2B VWD cases was reported. More recently, the North American Specialized Coagulation Laboratory Association (NASCOLA) reported their experience [22], highlighting the emerging trend of the mAb VWF:Act assay replacing the VWF:RCo assay in many North American laboratories, and despite any evidence of equivalence. In that study, overall diagnostic interpretation error rates ranged from 3% for normal samples, to 28% for type 1 VWD, and to a staggering 60% for type 2 VWD. Notably, the type 2 VWD samples were identified correctly by all laboratories using CB/Ag ratios, but by only one-third using RCo/Ag or VWF:Act/VWF:Ag (Act/Ag) ratios.

A systematic, specific and comparative evaluation of VWF activity assays in terms of sensitivity to specific loss of HMW VWF has not, to our knowledge, ever been performed. Accordingly, we describe a study that aimed to compare results obtained with the three most commonly used activity assays (VWF:RCo, VWF:CB, and VWF:Act) by testing of a normal sample as well as samples selectively and sequentially depleted of HMW and intermediate molecular weight (IMW) VWF forms, using a cross-laboratory exercise involving 51 laboratories across a broad geographic region.

Materials and methods

Preparation and initial testing of main study samples

The main study sample set is summarized in Table 1, and extended information is provided in Table S1 and Data S1. The samples were derived primarily from a plasma pool of individual normal plasmas. A series of eight plasma samples was then initially produced from this pool by one of us (E.J.F.). These comprised the otherwise unmodified pool (identified as sample V1 in Table 1), and seven similar-volume aliquots of this normal pool, each of which was differentially treated to produce increasing stepwise loss of HMW and then IMW VWF forms (Data S1, Table S1), This was achieved through a propriety process of disulfide bond reduction with *N*-acetylcysteine (NAC), similar to that recently described by Chen *et al.* [23]. Although such treatment results in the loss of HMW VWF, and also IMW VWF with continued application, the native VWF is otherwise essentially normally active [23]. The generated study samples were later anonymized to permit blinded testing for the main study (Data S1, Table S1). The first few samples in the series (e.g. V2 and V3 in Table 1) represent a loss of HMW with a minor loss of IMW VWF, which might occur with a normal sample subsequent to a preanalytic event (e.g. filtration of a sample also intended for lupus anticoagulant testing, or refrigeration of a whole blood sample) [24–26]. Subsequent samples (e.g. V4 and V5 in Table 1) would then show increasing loss of HMW and IMW VWF, potentially reflective of type 2A or 2B VWD-like plasma, but with a relatively high level of VWF, which is a pattern that might occur in type 2A or 2B VWD during pregnancy. The final sample in the series (V6 in Table 1) was meant to be depleted of HMW and IMW VWF, and could reflect a type 2A VWD pattern.

The initial set comprised eight plasma samples (Table S1), which were tested for FVIII activity and various VWF test parameters at the host laboratory (Institute of Clinical Pathology and Medical Research [ICPMR], Westmead), prior to being frozen in both aliquot form and as large volume sets with stabilizers in preparation for lyophilization and later stability and homogeneity testing. Samples were subsequently lyophilized in vials in 0.5-mL volumes by a commercial lyophilization process, and thereafter stored refrigerated at 4 °C until required. All samples were retested by the host laboratory in a validation study, including homogeneity and

Table 1 Summary of test sample set and characteristics

Sample ID (current report)	Sample comprised:	Sample intended to, or that could feasibly, represent:
V1	Pool of normal plasma samples	Normal plasma
V2	Sample V1 treated to yield minor loss of HMW VWF	Normal plasma with minor loss of HMW VWF, as might be caused by a preanalytic event (see main text)
V3	Sample V1 treated to yield loss of HMW and minor loss of IMW VWF	Normal plasma with mild loss of HMW VWF, as might be caused by a preanalytic event (see main text)
V4	Sample V1 treated to yield loss of HMW VWF and moderate loss of IMW VWF	Type 2A or 2B VWD-like plasma as might be obtained in pregnancy
V5	Sample V1 treated to yield loss of HMW VWF and high loss of IMW VWF	Type 2A or 2B VWD-like plasma as might be obtained in pregnancy
V6	Sample V1 treated to yield complete loss of HMW and IMW VWF	Type 2A VWD-like plasma.
V7	Mixture of V1 and VWF-deficient plasma	Moderate type 1 VWD-like plasma with target VWF:Ag and FVIII:C = 20–30 U dL^{-1}
V8	Mixture of HMW VWF-deficient sample and VWF-deficient plasma	Type 2A or 2B VWD-like plasma with target VWF:Ag and FVIII:C = 20–30 U dL^{-1}

FVIII:C, factor VIII coagulant; HMW, high molecular weight; IMW, intermediate molecular weight; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, von Willebrand factor antigen. Sample V1 represents a normal pool plasma and the main plasma used to generate subsequent test samples. Samples V2–V6 were derived from sample V1, and reflect sequential increasing depletion of HMW VWF, followed by sequential loss of IMW VWF. Samples V7 and V8 were, respectively, derived from mixtures of VWF-deficient plasma and sample V1 or an HMW VWF-deficient sample to produce samples reflective of moderate type 1 VWD and 2A/2B VWD, but with a similar level of VWF:Ag.

stability, as extensively detailed in the Data S1. There were no obvious deleterious effects observed. Sample vials were also subsequently sent to four collaborative laboratories for local blind testing of FVIII:C and VWF test parameters including multimer analysis to further validate study samples (details in Data S1). This testing also confirmed the selective and sequential loss of HMW and, in some cases, IMW VWF (Fig. S1A,B), as well as the comparative reduction in functional VWF test parameters (Fig. S1C,D). A subset of six samples (Table 1, V1–V6) was finally selected as representing the best differential stepwise pattern for distribution to participants of the RCPA Haematology QAP for the main cross-laboratory study.

On the basis of the preliminary testing, another two samples were subsequently prepared to mimic a moderate type 1 VWD-like and a more classical type 2A VWD-like plasma (Table 1; V7 and V8). These were differentially and respectively prepared by dilution of either the normal pool sample or an HMW and IMW VWF-depleted sample into commercial VWF-deficient plasma (see Data S1). Notably, the respective plasmas were specifically designed to produce two test plasmas with similar levels of VWF:Ag (target of $\sim$ 25 U dL^{-1}) and FVIII:C (target of 20–25 U dL^{-1}), but with comparably differing VWF activities, reflective, respectively, of a type 1 or 2A VWD (see Table 1 and Results).

Further confirmation of test sample integrity

mAb-based assay ELISA testing The sample production process was intended to produce an incremental loss of HMW VWF, followed by a loss of IMW VWF, but was not intended or expected to substantially affect the core structure or activity of VWF [23]. In order to help confirm this, the samples were tested with several various well-characterized mAbs against VWF [14,27,28] (Data S1). These mAbs bind to various (different) sites on the VWF molecule, and include several that are reactive with the platelet GPIb-binding site on VWF, and which otherwise differentially block various VWF activities [27,28]. Notably, there was no evidence that production of the samples adversely affected the core VWF structure, as represented by a structurally intact GPIb-binding site, despite samples reflecting a sequential loss of HMW and IMW VWF (Data S1). However, it was also noted that: (i) mAb data were not identical to each other or to polyclonal antibody (RαVWF) data; (ii) the sample reflecting the greatest loss of HMW and IMW VWF (i.e. V6) yielded a lower VWF 'protein' level than the other samples, with all VWF detection test systems (i.e. RαVWF and all mAbs; Fig. S2) – thus, some alteration of core structure or internal disulfide reduction cannot be excluded for this sample; and (iii) the levels of VWF protein appeared to initially rise slightly in the first few samples, with a loss of HMW and IMW VWF (Fig. S1C). This would be consistent with the test sample generation process providing improved access for VWF antibody binding in these samples, rather than elevation of VWF as such (see Data S1 and [29]).

Another series of experiments were later performed with the same mAbs as VWF capture antibodies (i.e. rather than as detection antibodies as above) (see Data S1). We have shown in the past that, used in this way, some mAbs show some HMW selectivity [14], as is also shown by the commercial mAb-based 'activity' assays.

Main cross-laboratory study

All laboratories (n = 55) enrolled in the standard VWF/VWD test module (C) of the RCPA Haematology QAP (http://www.rcpaqap.com.au/haematology/) were invited to participate

in the main cross-laboratory study. This included three laboratories that also participated in the validation study described above (see also Data S1). Two additional international collaborating laboratories that were also involved in the validation study were similarly invited to participate in the main study. All invited laboratories (total $n = 57$) were sent three vials of each of eight anonymized samples (see Data S1; reidentified as V1 to V8 in Table 1), with sample reconstitution and test instructions, requesting laboratories to perform their normal test panels for testing of VWD. Laboratories were also asked for test methodology details and to interpret their test results. The choices offered included normal, equivocal, type 1 VWD (mild, moderate, or severe), type 2A or 2B VWD, type 2M VWD, type 2N VWD, type 3 VWD, or other (with a request to specify). Some of these choices (e.g. type 2N VWD and type 3 VWD) did not reflect any of the samples, but a complete range of possible interpretations was provided to avoid any potential biasing of returned data. Laboratory numerical data were used as reported by participants, except that values reported as '<' a given value were corrected downwards to permit numerical analysis (for example, an assay value of < 10 U dL^{-1} was corrected to 9 U dL^{-1}, and an assay ratio of < 0.2 was corrected to 0.1). This is consistent with our normal External Quality Assurance (EQA) practice. These events occurred only for samples with high loss of HMW VWF (i.e. V6 and V8), and only for VWF:RCo and VWF:CB testing (respectively: five events for V6 VWF:RCo, four events for V6 VWF:CB, four events for V8 VWF:RCo, and three events for V8 VWF:CB).

Statistical analysis

Numerical data were analyzed by means of comparative medians, means, ranges and interassay (interlaboratory) coefficients of variation, with GraphPad Prism (GraphPad Software, La Jolla, CA, USA; http://www.graphpad.com).

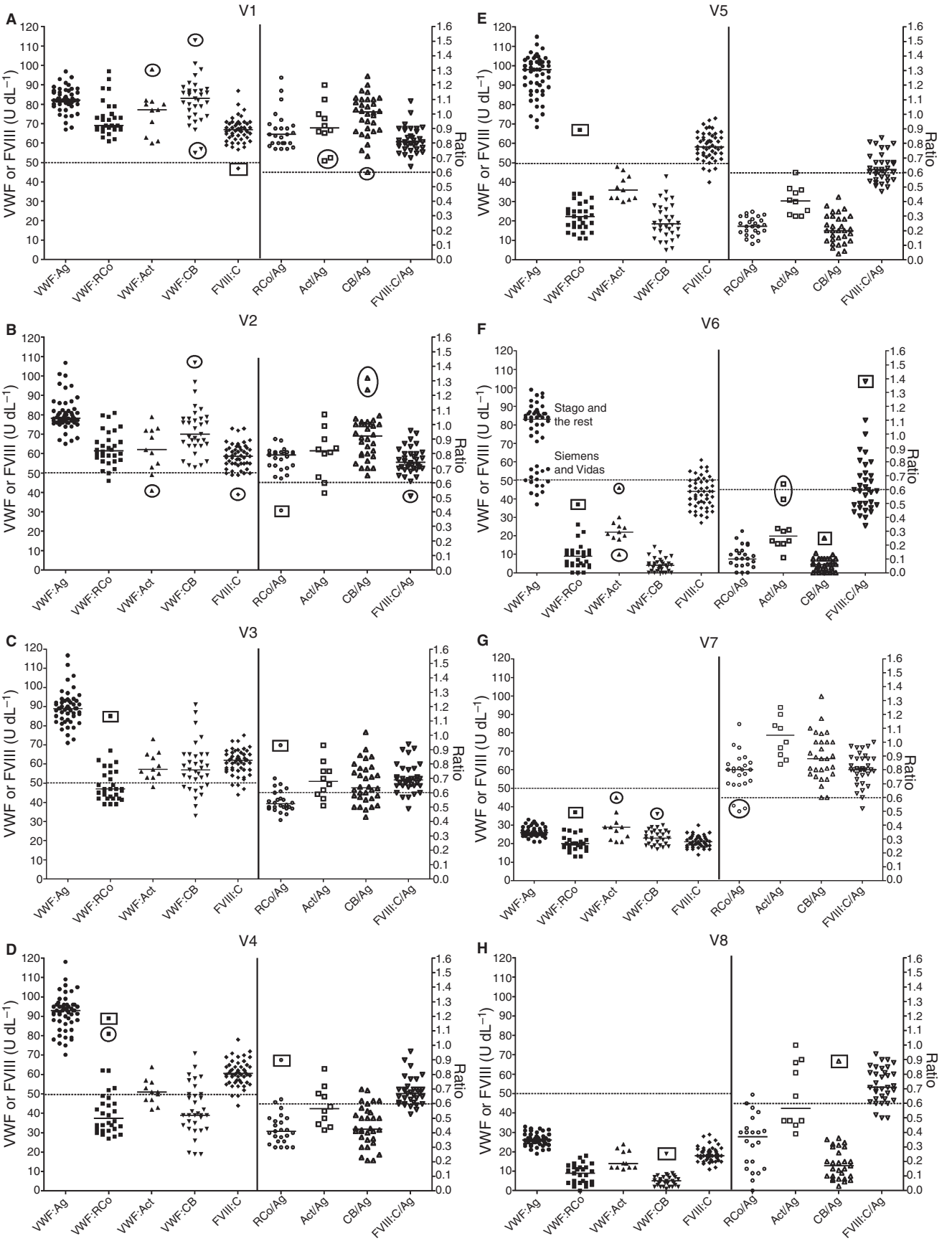
Results

Validation data for the samples prepared and dispatched in the main study are detailed in Data S1 and Figs 1–3. The main study data are derived from 51 participant laboratories (as not all invited laboratories [$n = 57$ in total] returned data), and are presented in Figs 1–3, with multimer patterns from one of the collaborating laboratories used as a reference site shown in Fig. 4. A breakdown of laboratory tests as used by laboratories, as well as summary statistical data, is given in Table 2.

All data for all samples and all laboratories for the main study test indices, namely VWF:Ag, VWF:RCo, VWF:CB, VWF:Act, and FVIII:C, as well as assay ratios, are shown in Fig. 1 as a scatter plot. This shows individual data and general trends, but also permits identification of outlier data, which may reflect aberrant testing from a few laboratories or potential transcription errors. In brief, outlier data were only identified in the case of activity assays, and values > 3 standard deviations from the mean were thereafter excluded from analysis. Outlier data were also checked for data source. It was noted that five laboratories were responsible for nearly half of all the data outliers identified in Fig. 1. These outlier data were also thereafter excluded from analysis. Summarized data (excluding outliers noted above), including corresponding assay ratios, are shown in Fig. 2, which attempts to focus on assay performance rather than participant performance. Note that VWF:Ag and FVIII:C testing identified similar levels across all samples tested except for sample V6, similar to the trend identified prior to the main study by collaborative laboratories (Fig. S1). In contrast, and as expected, VWF:RCo, VWF:CB and VWF:Act levels fell sequentially according to increasing loss (or decreasing levels) of HMW and IMW VWF. Notably, VWF:RCo and VWF:CB showed the greatest (similar) falls, particularly for samples showing the highest loss of HMW VWF, whereas VWF:Act showed intermediate results. Consistent with the trends observed for VWF:Ag and FVIII, the FVIII/VWF:Ag (FVIII:C/Ag) ratio showed only a moderate trend to reduction. Act/Ag ratios also showed a gradual trend to reduction, but this was overshadowed by the greater reductions observed in RCo/Ag and CB/Ag ratios. Comparative data for samples V7 and V8, intended to represent a moderate type 1 VWD and a type 2A VWD sample, respectively, are shown in Fig. 2C,D. The type 1 VWD mimic sample (V7) yielded similar numerical data for all test parameters (between 20 and 30 U mL^{-1}), with normal assay ratios (all > 0.7). In contrast, although the type 2A VWD mimic (V8) yielded similar numerical data for VWF:Ag and FVIII:C (around 20–30 U mL^{-1}) and similar data to sample V7, the VWF activity data (VWF:RCo, VWF:CB, and VWF:Act) showed decreased values, as did respective ratio data, and as expected for this sample. Consistent with the findings in Fig. 2B, VWF:RCo and VWF:CB (and thus RCo/Ag and CB/Ag ratios) provided the lowest comparative values.

A subanalysis of data according to type of VWF:CB and VWF:RCo assay is shown in Fig. 3. Although data should be interpreted cautiously, given low subsample test numbers, some VWF:CB assays appeared to be more sensitive to the loss of

Fig. 1. Main study data as derived from all samples tested where reported by 51 participating laboratories and shown as a scatter plot. The data for the main study test indices, namely von Willebrand factor (VWF) antigen (VWF:Ag), VWF ristocetin cofactor (VWF:RCo), VWF collagen binding (VWF:CB), VWF activity (VWF:Act), and factor VIII coagulant activity (FVIII:C), are shown on the left side of each figure. The horizontal dashed line represents a nominal 'normal' cut-off value of 50 U dL^{-1}. Data for assay ratios are shown on the right side of each figure. The horizontal dashed line represents a nominal 'normal' cut-off value of 0.6. (A)–(H), respectively, show data for samples V1–V8 (see Table 1). Some outlier data are evident; data in squares indicate values that are > 3 standard deviations from the mean. Data in circles show other visually appearing outlier data, in general between 2 and 3 standard deviations from the mean. All identified outlier data were checked for transcription error and for source of data.



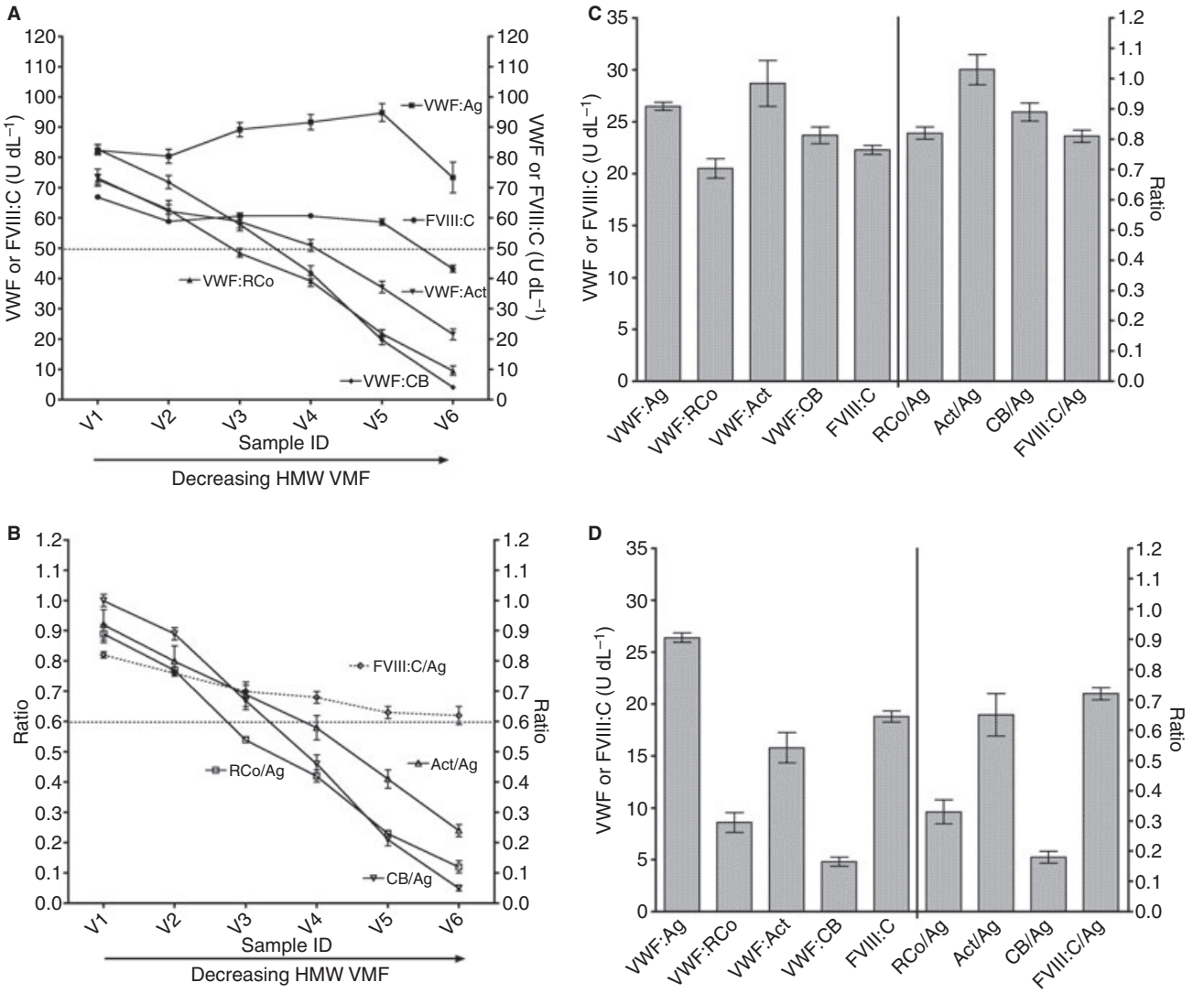


Fig. 2. Summarized main study data as derived from all samples tested where reported by 51 participating laboratories and shown as mean ± standard error of the mean. Some outlier data identified in Fig. 1 have been removed (refer to text) to permit a focus on method-based differences (i.e. to exclude potential participant-based problems). (A) Main study test indices, namely von Willebrand factor (VWF) antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), VWF collagen binding (VWF:CB), VWF activity (VWF:Act), and factor VIII coagulant activity (FVIII:C), as reported by all laboratories. The horizontal dashed line represents a nominal 'normal' cut-off value of 50 U dL^{-1}. (B) Data for corresponding assay ratios. The horizontal dashed line represents a nominal 'normal' cut-off value of 0.6. (C, D) Comparative data for samples V7 (C) and V8 (D), intended to, respectively, represent a moderate type 1 von Willebrand disease (VWD) and a type 2A VWD sample.

HMW VWF than others. Similarly, there also appeared to be a small difference in sensitivity between aggregometer-based and automated test processes for VWF:RCo. Figure 3C shows comparative data between the 'best' and 'least well' performing commercial VWF:CB assays, with VWF:RCo (aggregometry) and VWF:Act.

The reference laboratory multimer analysis shown in Fig. 4 is consistent with previous observations, and confirms the sequential loss of HMW and IMW VWF in the generated samples from V1 to V6, as well as the loss in sample V8 (type 2A VWD mimic), but not in sample V7 (moderate type 1 VWD mimic). The multimer analysis results submitted by two study participant laboratories yielded similar findings (data not shown).

In order to help explain some of the above observations, further studies (as outlined in Materials and methods) were performed by the host laboratory using several mAbs, including those known to react with VWF at the functional GPIb-binding site, which are presumed to be analogous to the mAb used in the commercial VWF:Act assay. The data are shown in Data S1 (Fig. S3). In brief, when used as the VWF capture system, some mAbs could be shown to yield HMW sensitivity data that were very similar to those of the VWF:Act assay. This sensitivity was increased by manipulation of the assay, most notably by reducing the concentration of coating mAb used (see Discussion).

Interpretative data from participants are shown in Fig. 5. Notably, all participants identified sample V1 as being normal,

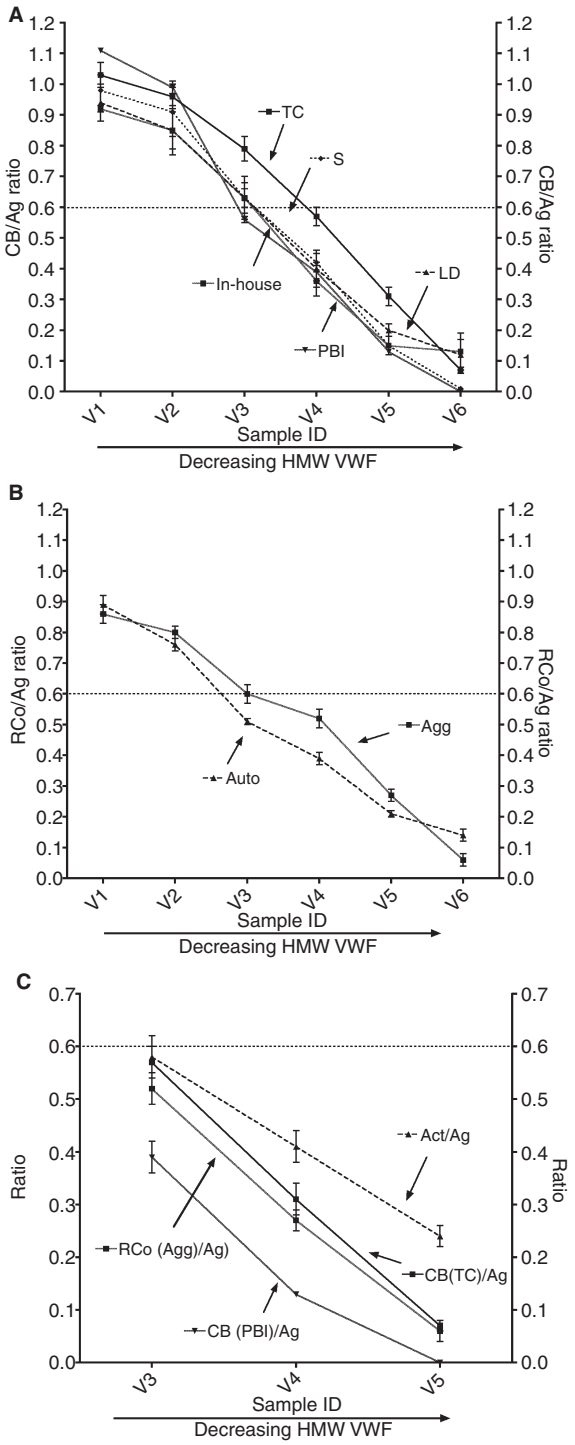


Fig. 3. Main study data from Fig. 2, but now showing submethod analysis for ratios of von Willebrand factor (VWF) collagen binding (VWF:CB)/VWF antigen (VWF:Ag) (CB/Ag) (A) and VWF ristocetin cofactor activity (VWF:RCo)/VWF antigen (VWF:Ag) (RCo/Ag) (B). (C) Comparative data for 'best' (Precision BioLogic Inc. [PBC]) and 'least well' (TechnoClone [TC]) performing method in the VWF:CB group vs. the RCo (Agg)/Ag and VWF activity (VWF:Act)/VWF antigen (VWF:Ag) (Act/Ag) groups for main three samples (V4, V5, and V6) showing the greatest loss of high molecular weight (HMW) VWF (see Fig. 4). Dashed horizontal line at 0.6 represents a typical nominal normal cut-off value. Agg, agglutination; Auto, automated; LD, Life Diagnostics; S, Stago.

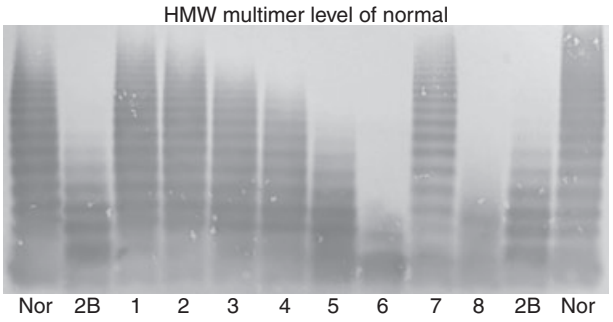


Fig. 4. Multimer patterns obtained on the main study sample set from one collaborating laboratory, and used as the reference multimers, and confirming sequential loss in high molecular weight (HMW) and intermediate molecular weight VWF in samples V1, V2, V3, V4, V5, and V6, as well as the loss in sample V8 (type 2A von Willebrand disease [VWD] mimic), but not sample V7 (moderate type 1 VWD mimic). Nor, normal plasma control; 2B, type 2B VWD plasma control.

which was reassuring. The vast majority of participants identified sample V6 (devoid of HMW VWF) as being type 2A, 2B or 2M VWD-like. There was also a trend for decreasing identification of the samples as normal, to that of them being increasingly identified as type 2A, 2B or 2M VWD-like, as the samples progressively lost HMW and then IMW VWF. Most participants identified sample V7 as type 1 VWD-like, and most also identified sample V8 as type 2A, 2B or 2M VWD-like. Somewhat concerning, perhaps, was that eight participants identified HMW VWF-deficient sample V4 as normal, with three of these same participants also identifying sample V5 as normal. Similarly concerning was that 10 participants identified the HMW VWF-deficient sample V8 as a type 1 VWD.

Discussion

To our knowledge, this study represents the first comparative assessment of the most widely used VWF 'activity'-based assays (i.e., VWF:Co, VWF:CB, and VWF:Act) for their specific sensitivity to loss of HMW VWF. VWF:RCo, the original functional VWF assay described in the early 1970s [4,5], reflects the ability of VWF to bind to its major platelet receptor (GPIb). The VWF:CB assay, first described in 1986 [9], represents another activity of VWF, namely its ability to bind to collagen, a subendothelial matrix component. The VWF:Act assay, first reported as an immunoradiometric assay in 1985, and later as an ELISA assay, is now most commonly performed with an immunolatex procedure [10–13,16,17].

The mAb-based VWF:Act assay is marketed as an 'activity' assay on the basis that the mAb used to capture VWF in the test sample recognizes the GPIb-binding site of VWF. Whether this then bestows functionality to the assay is debated. Nevertheless, the assay has been embraced by a high proportion of laboratories, and in many cases inappropriately used as a surrogate for the VWF:RCo assay. Although the RCPA Haematology QAP has noted such a trend in Australia [21], this was also recently noted in North America by the

Table 2 Summary test statistics and breakdown of main study methods used by study participants

Test/ methodology	No. of participants reporting results*	% of total†	% of method‡	Median (range); CV (of all data)§							
				V1	V2	V3	V4	V5	V6	V7	V8
FVIII:C	51	100	100	66.9 (47.0–87.0); 9.5	58.7 (39.0–73.0); 11.2	62.0 (44.0–75.0); 10.7	60.6 (44.0–78.0); 10.4	58.2 (40.0–73.0); 11.9	44.0 (27.0–61.0); 18.7	21.0 (14.0–30.0); 14.5	18.0 (11.0–29.0); 20.1
VWF:Ag	52	100	100	82.1 (67.0–96.9); 7.2	78.3 (65.0–107); 10.5	89.0 (71.0–117); 9.6	93.0 (70.2–118); 10.0	98.0 (68.4–115); 11.1	74.0 (37.0–99.0); 24.3	26.0 (21.0–33.0); 10.1	26.1 (19.0–33.0); 12.0
LIA	41	80.4	80.4	–	–	–	–	–	–	–	–
ELISA	6	11.8	11.8	–	–	–	–	–	–	–	–
ELFA	4	7.8	7.8	–	–	–	–	–	–	–	–
VWF:RCo	30	60.8	100	69.0 (61.0–97.0); 12.4	61.5 (46.0–81.0); 14.1	47.0 (39.0–85.0); 20.3	37.5 (27.0–89.0); 35.6	22.2 (11.0–67.0); 27.3	9.0 (0–37.0); 85.3	20.0 (13.0–37.0); 24.1	9.0 (0–18.0); 58.1
Aggregometer	9	17.6	29.0	–	–	–	–	–	–	–	–
Automated	22	43.1	71.0	–	–	–	–	–	–	–	–
VWF:CB	33	60.8	100	83.0 (55.0–113); 14.2	70.0 (53.0–107); 17.2	57.0 (33.0–91.0); 22.2	39.0 (19.0–71.0); 30.7	18.5 (5.0–43.0); 45.5	4.0 (0–14.0); 84.7	23.0 (17.0–36.0); 18.6	5.0 (1.0–19.0); 65.2
In-house	6	11.8	19.4	–	–	–	–	–	–	–	–
Stago	7	13.7	22.6	–	–	–	–	–	–	–	–
Technoclone	11	21.6	35.5	–	–	–	–	–	–	–	–
Life Therapeutics Group¶	5	9.8	16.1	–	–	–	–	–	–	–	–
Precision Biologic Inc.	2	3.9	6.5	–	–	–	–	–	–	–	–
VWF:Act**	11	21.6	100	77.2 (60.0–98); 14.9	62.0 (41.0–79.0); 19.0	57.2 (48.0–73.0); 12.2	51.0 (47.0–64.0); 12.6	36.0 (30.0–48.0); 17.4	22.0 (10.0–46.0); 37.9	28.9 (21.0–45.0); 25.4	14.0 (11.0–24.0); 30.6
Multimers	4	7.8	NA	NA	NA	NA	NA	NA	NA	NA	NA

CV, coefficient of variation; ELFA, enzyme-linked fluorescence assay; FVIII:C, factor VIII coagulant activity; LIA, latex immunoassay; NA, not applicable; VWF:Act, von Willebrand factor activity; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand factor collagen binding; VWF:RCo, von Willebrand factor ristocetin cofactor. *A total of 57 laboratories were invited to participate, but only 51 laboratories provided test data for main study samples. Some laboratories reported results for multiple test systems and methodologies, whereas other laboratories reported data only for selective tests and methodologies; thus, test numbers will differ in each case, and do not always add up to 51. †Percentage of total participants reporting results for each test group. ‡Percentage of participants reporting results for specific test or methodology groups (VWF:Ag, VWF:RCo, or VWF:CB, etc.). §Includes outlier data; data not reported for submethodology. ¶Comprises group of Life Therapeutics Ltd, Reaads Corgenix (Corgenix Medical Corporation) and Nusep Inc. **All participants reported using the Instrumentation Laboratory assay.

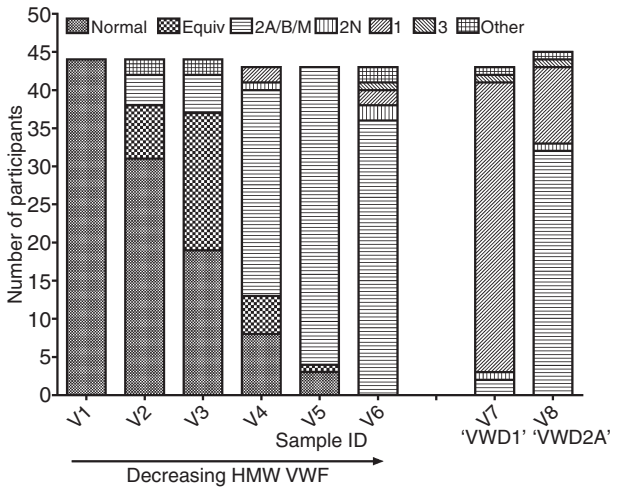


Fig. 5. Participant interpretations for tested samples. Note that: (A) not all participant laboratories provided an interpretation for test samples, so numbers are generally < 51; (B) all participants providing an interpretation identified sample V1 as being normal; (C) the vast majority identified the high molecular weight (HMW) von Willebrand factor (VWF)-devoid sample (V6) as being type 2A, 2B or 2M von Willebrand disease (VWD)-like; (D) there was a trend for decreasing identification of the samples as normal, to them increasingly being identified as type 2A, 2B or 2M VWD-like, as the samples progressively lost HMW and then intermediate molecular weight VWF (i.e. from sample V2 to sample V6); (E) most participants identified sample V7 as type 1 VWD-like; (F) most participants identified sample V8 as type 2A, 2B or 2M VWD-like. However, some participants identified HMW VWF-deficient samples V4 and V5 as normal, and some participants identified HMW-deficient sample V8 as a type 1 VWD.

NASCOLA [22]. This trend is presumably related to the assay's ease of use, and early (but incomplete) studies showing behavior similar to that of the VWF:RCo assay by regression analysis of selected samples. In some cases, preferential usage may also be driven by regulatory requirements. For example, although the VWF:Act assay has been cleared by the FDA for in vitro diagnostic use in North America, a VWF:CB assay has not. As clearly shown in the current study, the VWF:Act assay does show some selective discrimination of HMW VWF, but the VWF:RCo and VWF:CB assays are much more effective in this regard. This sensitivity of the VWF:Act assay for HMW VWF may be related to the manner in which the manufacturer has controlled the assay conditions, something that the host laboratory for the current study has shown is possible for ELISA mAb-based capture systems, simply by reducing the mAb concentration used (Fig. S3, Data S1, and [14]). Thus, the use of a lower concentration of mAb as a capture system makes the ELISA-based assay more selective for HMW VWF, presumably because low molecular weight VWF does not contain sufficient binding sites to permit stable binding of the VWF to the microplate. In a latex agglutination assay, it can be hypothesized that a similar process may occur; that is, cross-latex agglutination may also require VWF of a certain mass, according to the amount of latex-bound mAb. We have previously shown that, in discrimination of type 2 vs. type 1 VWD cases, the VWF:RCo and VWF:CB assays (and thus the RCo/Ag and CB/Ag ratios) generally perform better than the VWF:Act assay (and Act/Ag ratios) [21]. Interestingly, the NASCOLA study also identified better performance of the VWF:CB assay than either the VWF:Act or VWF:RCo assay in the context of such discrimination [22,30,31]. The current study expands on these findings, and in part may also explain possible reasons behind them, given that the VWF:Act assay appears to be less sensitive to the loss of HMW VWF than both the VWF:RCo and VWF:CB assays.

Although the VWF:CB and VWF:RCo assays (and thus the CB/Ag and RCo/Ag ratios) showed similar trends, the data were not identical. Methodology subanalysis was also performed, and although small subgroup numbers prevent any definitive conclusions, there did appear to be some differences in relation to methodology (Fig. 3). For example, the least sensitive VWF:CB assay for HMW VWF appeared to be that produced by Technoclone GmbH, an observation that is quite consistent with many previous evaluations of VWD cases by the host laboratory [15,32]. Even so, this 'least sensitive' VWF:CB assay for HMW VWF still appeared to be more sensitive than the Act/Ag ratio (Fig. 3C). For RCo/Ag ratios, the automated VWF:RCo method was as sensitive, if not more so, to the increasing loss of HMW and IMW VWF, except for sample V6, where it is suspected that low limit of VWF sensitivity issues compromise the assay's utility (that is, many laboratories cannot report assay values below 10 U dL^{-1} with this assay [33]). This lower limit of sensitivity can be improved by use of a low assay curve, as recently reported [34,35].

Participants in the main study provided interpretations of their data that were generally consistent with the sample type tested (Fig. 5). On occasion, however, interpretations appeared to be at odds with the sample type. Notably, several participants identified HMW VWF-decreased samples V4 (eight laboratories) and V5 (three laboratories) as 'normal', and sample V8 (10 laboratories) as 'type 1 VWD'. Interestingly, an analysis of these findings appears to identify the major problem as being that of limited test panels, rather than problems with 'activity' assays as such. Thus, these 21 occasions reflected testing by 15 laboratories, all of which performed the VWF:Ag assay. Three of the 15 laboratories performed no activity assay of any kind, eight laboratories performed the VWF:RCo assay, five performed the VWF:CB assay, and three performed the VWF:Act assay, with eight of 15 (53.3%) therefore performing only a single activity assay, and only four performing two activity assays. Thus, 11 of 15 (73.3%) laboratories performed only one or no VWF activity assays, compromising their ability to identify a loss of HMW VWF in this study (and a potential qualitative type 2 VWD otherwise). This finding is also consistent with our previous experience [36].

The current study utilized a propriety process that employed NAC to achieve a stepwise reduction in HMW and then IMW VWF. This in vitro process is thought to mimic a natural in vivo process, described but incompletely characterized, that permits reduction of VWF in the absence of ADAMTS-13 [37], and also possibly that assists in the formation of VWF complexes at a thrombus by a process of self-association [38].

The in vitro processing of VWF with NAC has most recently been described by Chen *et al.* [23] as a potential therapeutic aid in TTP, and, indeed, we believe that clinical trials of this agent have recently begun or being planned [37]. Should this agent become a treatment of choice for TTP, it can be envisaged that laboratories may be called upon to monitor treatment in TTP by using VWF assays, including the VWF:Ag assay and an HMW VWF 'activity surrogate'. The current study would then suggest caution in regard to the use of the VWF:Act assay for this purpose.

Finally, VWD therapy primarily involves the use of either desmopressin (DDAVP) or VWF factor concentrate [1,2]. The same assays that are used to diagnose VWD are also used to monitor therapy for VWD, and to assess the potential clinical utility of factor concentrates. Of additional interest, DDAVP therapy can also be used to assist in VWD diagnosis and typing [6], with various test patterns being observed in different VWD cases. Recently, the VWF:Act assay was proposed as a possible suitable 'alternative' to the VWF:RCo assay in terms of assessing VWF factor concentrates [39]. The current study, however, would caution against the expectation that the VWF:Act and VWF:RCo assays will provide the same information in such assessments. Indeed, although a given factor concentrate may provide the same value for VWF:RCo and for VWF:Act, this is not the same as identifying these assays as being equivalent for this purpose. Thus, on the basis of the current study, it can be predicted that the VWF:Act assay will provide higher VWF values than the VWF:RCo assay for VWF concentrates that are somewhat devoid of HMW VWF. Although potentially favorable to manufacturers of VWF concentrates, as this gives the impression of more favorable characteristics, this is not recommended practice.

Although the sample production used in this study reflected an in vitro process to reduce the levels of HMW and IMW VWF, we do not believe that this caused any substantial reduction in core VWF function as such. This was shown by testing with various mAbs against VWF, including functional sites on VWF, which showed comparable data to those obtained with polyclonal RαVWF material (Data S1). This would, in essence, suggest an intact VWF functional GPIb-binding site. This is also consistent with current knowledge that identifies very few cysteine molecules within the A domains of VWF [38]. Moreover, the FVIII/VWF:Ag ratio also remained fairly stable across the range of samples, again suggesting an intact VWF functional FVIII-binding site. Although there was a slight drop in the FVIII/VWF:Ag ratio across the samples, this was more likely related to the slight increase in VWF:Ag observed than to any fall in FVIII:C (Fig. 2A), with this presumably reflecting greater accessibility of the antibodies to the VWF in the samples [29] rather than an increase in VWF:Ag as such.

In conclusion, we report on a cross-laboratory evaluation of VWF testing with a range of assays and samples selectively depleted in HMW and, in some cases, IMW VWF. Differences in the ability of 'activity' assays to detect this loss were observed, with the VWF:RCo and VWF:CB assays being similar and showing the highest sensitivity, and the VWF:Act assay showing lower sensitivity. We believe that these findings have significant implications for clinical practice in a variety of settings, namely diagnostic and therapy management practice for both VWD and TTP. In particular, diagnostic and therapy management of both VWD and TTP rely, in part, on assays that are defined as being sensitive to the loss of HMW VWF, but not all 'activity' assays show similar sensitivities to the loss of HMW VWF. There now remains a stock of reserved samples prepared for this study that can be used for ongoing EQA, or for a more extensive, perhaps expert laboratory-based study, similar to that previously reported for patient samples [40]. A clinical validation study, showing comparative findings of plasma samples from NAC-treated TTP patients in a clinical trial setting and cotested by different VWF activity assays, would also seem to be warranted.

Addendum

E. J. Favaloro: conceived, designed and coordinated the study, prepared the study samples, undertook preliminary testing of study samples, participated in method validation, undertook data analysis, and wrote the manuscript; R. Bonar: arranged and oversaw the logistics of the main study, arranged for sample lyophilization, and contributed to data analysis; K. Chapman: assisted in study design, participated in preliminary testing of study samples and method validation, and participated in the main study; M. Meiring: participated in preliminary testing of study samples and method validation, and participated in the main study. D. Funk (Adcock): participated in preliminary testing of study samples and method validation, and participated in the main study. D. Funk (Adcock)'s laboratory acted as the study reference center for multimer analysis. All coauthors contributed to manuscript revision, and have approved the final manuscript for publication.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Extended description of materials and methods related to the validation study.
Figure S1. Main initial validation dataset.
Figure S2. VWF:Ag testing of test samples by the host laboratory with either rabbit antibody (RaVWF) or various mAbs (CR1, CR2, 5D2, 6G1) used as VWF detection antibodies.
Figure S3. VWF testing by ELISA with mAbs as VWF capture antibodies.
Table S1. Detailed summary of test sample set and characteristics.

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