Collagen Binding Provides a Sensitive Screen for Variant von Willebrand Disease

Veronica H. Flood,1,2 Joan Cox Gill,1,2,3,4 Kenneth D. Friedman,3 Pamela A. Christopherson,3,4 Paula M. Jacobi,3,4 Raymond G. Hoffmann,5 Robert R. Montgomery,1,2,3,4 Sandra L. Haberichter,1,2,3,4* and the Zimmerman Program Investigators

BACKGROUND: von Willebrand factor (VWF) is a multimeric protein that binds platelets and collagen, facilitating hemostasis at sites of vessel injury. Measurement of VWF multimer distribution is critical for diagnosis of variant von Willebrand disease (VWD), particularly types 2A and 2B, but the typical measurement by gel electrophoresis is technically difficult and time-consuming. A comparison of VWF collagen binding (VWF:CB) and VWF multimer distribution was performed to evaluate the utility of VWF:CB as a diagnostic test.

METHODS: Participants were enrolled in the Zimmerman Program for the Molecular and Clinical Biology of VWD. VWF:CB was analyzed with type III collagen and multimer distribution by agarose gel electrophoresis. The study population included 146 healthy controls, 351 individuals with type 1 VWD, and 77 with type 2 VWD. Differences between individuals with multimer group results within (controls) and outside the reference intervals were assessed with Mann–Whitney tests.

RESULTS: The mean VWF:CB/VWF antigen ratio was 1.10 for individuals with multimer distribution within the reference intervals and 0.51 for those with multimer distribution outside the reference intervals (P < 0.001). Sensitivity of VWF:CB for multimer abnormalities was 100% for healthy controls, 99% for patients with type 1, and 100% for patients with type 2A and type 2B VWD using a VWF:CB/VWF antigen cutoff ratio of 0.6, and decreased to 99% for all patients with a ratio of 0.7. With the exception of individuals with novel or unclassified mutations, the VWF:CB was able to correctly categorize participants with variant VWD.

CONCLUSIONS: These findings suggest that VWF:CB may substitute for multimer distribution in initial VWD testing, although further studies are needed to validate the clinical utility of VWF:CB.

The function of von Willebrand factor (VWF) is dependent on the presence of high molecular weight multimers (HMWM). The monomeric protein is cotranslationally synthesized with C-terminal dimerization in the endoplasmic reticulum, then sent to the Golgi apparatus where N-terminal multimerization occurs (1). The 225-kD dimeric unit thus circulates in a multimeric structure of >20 000 kD that is capable of structural modification under shear stress, for the purpose of recruiting platelets to sites of vascular injury (2, 3). Binding sites for platelet glycoprotein Ib in the VWF A1 domain and for collagen in the VWF A1 and A3 domains facilitate this function.

Types 2A and 2B von Willebrand disease (VWD), both lacking HMWM, are characterized by excessive mucosal bleeding. Measurement of VWF multimer distribution is critical for accurate diagnosis of these subtypes of VWD and to guide effective treatment (4). Type 1 VWD, characterized by low levels of VWF but a multimer distribution within reference intervals, typically responds to desmopressin, whereas replacement of VWF may be required for treatment of types 2A and 2B VWD (5).

Multimer distribution is classically analyzed by gel electrophoresis (6). Studies have demonstrated that VWF collagen binding (VWF:CB) can serve as a surrogate measure for the presence of HMWM (7–9).
VWF:CB to VWF antigen (VWF:Ag) ratios of \(0.6\) or \(0.7\) have been considered to indicate results outside the reference interval, with ratios above that presumed to represent a multimer distribution within the reference interval \(8, 10\). In clinical practice, however, collagen binding assays are not typically performed as part of the routine workup for VWD.

To clarify the diagnostic role of VWF:CB, we compared the VWF multimer distribution with VWF:CB for a population of healthy controls and individuals with VWD who were enrolled in the Zimmerman Program for the Molecular and Clinical Biology of VWD (Zimmerman Program). The results show that the type III collagen binding assay can substitute for electrophoretic analysis of VWF multimer distribution as a part of the initial workup for VWD.

**Methods**

**STUDY POPULATION**

Informed consent was obtained for all participants following approval of the human research protocol by the institutional review boards of the participating institutions. Healthy controls with no preexisting diagnosis of a bleeding disorder were enrolled as a part of the Zimmerman Program from the local population of each of 7 primary centers (Atlanta, Detroit, Iowa City, Indianapolis, Milwaukee, New Orleans, and Pittsburgh). Study participants from 8 primary centers and from numerous secondary centers (listed in the Supplemental Appendix that accompanies the online version of this report at http://www.clinchem.org/content/vol59/issue4.) were enrolled if they had a preexisting diagnosis of VWD, of any type, as determined by the treating physician at each center. The study population included 146 healthy controls, 351 individuals with type 1 VWD, and 77 with type 2 VWD (Table 1). Several participants had study laboratory findings that were not consistent with the original diagnosis, requiring reclassification as detailed below.

**VWF TESTING**

Blood was collected in 3.2% sodium citrate and frozen plasma shipped to a central reference laboratory (Hemostasis Reference Laboratory at the BloodCenter of Wisconsin) for VWF testing. VWF:Ag, ristocetin cofactor activity (VWF:RCo), collagen binding with type III VWF:CB, and multimer distribution were performed on all samples as previously described \(11\). VWF:CB was performed on all index cases and healthy

![Table 1. Zimmerman Program multimer distribution and VWF:CB/VWF:Ag ratios by VWD diagnosis.](image-url)
controls by an ELISA assay using type III human placental collagen (Southern Biotech) coated at 1 µg/mL in carbonate coating buffer (15 mmol/L sodium carbonate, 35 mmol/L sodium bicarbonate, 3 mmol/L sodium azide, pH 9.5) on Immulon Ib plates (Thermo Scientific). A polyclonal anti-VWF antibody (Dako) was used for detection (12).

Multimer analysis was performed on all index cases and healthy controls by electrophoresis through a 0.65% high-gelling-temperature gel [HGT(P) agarose gel (Lonza)] containing 0.1% lithium dodecyl sulfate (LiDS) at 120 V for 4 h in a 0.1 mol/L Tris, 0.15 mol/L glycine, 0.1% LiDS (w/v) running buffer (13). Samples were transferred to an Immobilon-P membrane (Millipore), and we subsequently detected VWF by Western blot using a monoclonal anti-VWF antibody (Dako).

We performed quantitative multimer analysis by performing gel electrophoresis as described above and transferring proteins to nitrocellulose (Bio-Rad) by electroblotting at 5 V for 2 h in 0.05 mol/L sodium phosphate buffer, pH 7.8. Membranes were blocked with 0.05 mol/L Tris-buffered saline containing 1% BSA (w/v), pH 8.0, incubated with anti-VWF monoclonal antibodies AvW-1, AvW-15, and 105.4 (Hybridoma Core Laboratory, BloodCenter of Wisconsin) in 0.05 mol/L Tris-buffered saline containing 0.05% Tween-20, pH 8.0, followed by peroxidase-conjugated goat anti-mouse IgG (H+L) (Thermo Fisher Scientific). Protein was detected with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and visualized with the Fujifilm Luminescent Image Analyzer LAS-3000 (Fujifilm). Densitometry was performed using Multi Gauge ver. 3.0 analysis software (Fujifilm).

An additional sample was collected in EDTA for DNA extraction. Full-length von Willebrand factor (VWF) gene sequencing was performed for all index cases and healthy controls, including all intron–exon boundaries (14). A questionnaire about bleeding was administered to each participant, including the healthy controls, utilizing questions sufficient to determine a bleeding score as published by the European Union group (15).

**Statistical Analysis**
Statistical analysis was performed using Stata 11.1 (StataCorp LP). Mann–Whitney tests were used to compare multimer groups inside and outside the reference intervals. ROC curves were generated separately for the healthy control group, the type 1 VWD group, the type 2A VWD group, and the type 2B VWD group.

**Results**
The study population included both healthy controls and individuals with VWD as detailed in Table 1. Individuals with VWD were enrolled on the basis of a pre-existing diagnosis of VWD as made by their treating physician before study enrollment. Of these, 511 had a multimer distribution within the reference interval and 63 had a multimer distribution outside the reference interval. There was a statistically significant difference in VWF:CB between those with a multimer distribution within the reference interval and those with a multimer distribution outside the reference interval, with \( P < 0.001 \) (Fig. 1). Of those with multimers outside the reference interval, 51 had a diagnosis of type 2A or 2B VWD whereas the remaining 12 would have been expected to have a multimer distribution within the reference interval given the lack of a documented type 2A or 2B VWD mutation. Some of these represent subtle abnormalities in multimer distribution with loss of only the highest molecular weight multimers, whereas others are either unclassified or novel mutations, as discussed below. Loss of only the highest multimers has been reported with sample processing or transport-related artifacts and may not represent true VWD (7). Examples of the different multimer distributions ob-
and yielded high correlation, with an area under the ROC curve of 0.90 for the healthy controls, 0.82 for individuals with type 1 VWD, 0.98 for those with type 2A VWD, and 0.99 for those with type 2B VWD.

With regard to correct classification as type 2A or 2B VWD, the sensitivity of the VWF:CB/VWF:Ag ratio was 100% when all cases with an unclear phenotype were excluded, as discussed in detail below. All healthy controls had VWF:CB/VWF:Ag ratios within the reference intervals, and all individuals with type 2A and 2B VWD had low VWF:CB/VWF:Ag ratios. Specificity was only 99% for the individuals with type 1 VWD, owing to 4 individuals with a low VWF:CB/VWF:Ag ratio (all ≤5 IU/dL) but multimer distributions within the reference intervals and no documented type 2 VWD mutation on DNA sequencing. Omission of individuals with low VWF:Ag would have raised the specificity to 100%. No difference was seen in VWF:CB/VWF:Ag ratio for individuals with type 2A VWD as compared to those with type 2B VWD ($P = \text{not significant}$).

**VWF:CB and Multimer Distribution in Healthy Controls**

We then compared VWF:CB to multimer distribution for each group of participants on the basis of their clinical diagnosis. Data for 146 healthy controls were available for analysis. Only 2 (1.4%) had a multimer distribution outside the reference interval, both with loss of only the highest molecular weight multimers, a pattern associated in our laboratory with sample processing artifacts (7). All controls had VWF:CB/VWF:Ag ratios within the reference intervals, as did both individuals who had multimer distribution outside of the reference intervals. The lowest VWF:CB/VWF:Ag ratio seen in this group was 0.69, consistent with the use of a cutoff ratio of 0.6–0.7 for diagnosis of variant VWD. Both individuals with multimer distribution outside the reference interval had low VWF:RCo/VWF:Ag ratios (0.42 and 0.57) but bleeding scores (of 0 and 1, respectively) within reference intervals as evaluated by the European Union bleeding score questionnaire (15). Neither had a mutation in the VWF coding region.

**VWF:CB and Multimer Distribution in Type 1 VWD**

Next, VWF:CB was compared to multimer distribution for individuals with type 1 VWD. Of the 342 participants with type 1 VWD with multimers within the reference intervals, 4 had a VWF:CB/VWF:Ag ratio of <0.7, but 3 of these had very low VWF:Ag (≤5 IU/dL), for which the sensitivity of the ratio would be expected to be less optimal. Nine individuals with type 1 VWD had a multimer distribution outside the reference interval, with loss of the highest molecular weight multimers (6 participants), loss of all HMWM (1 participant), or a shift from high to low molecular weight...
multimers with relatively increased staining of the low molecular weight bands (2 participants). Of the 7 individuals with loss of HMWM, 5 had known type 1 VWD mutations and VWF:CB/VWF:Ag ratios within the reference interval, possibly representing sample processing or transport related artifact (7), as no multimer issues have been previously reported for these mutations (Table 3). One individual had a low VWF:RCo/VWF:Ag ratio and an unclassified mutation, p.R1374H, which has been alternately classified as type 1, type 2A, and type 2M VWD (16–18). One participant had no coding sequence mutation found. Two individuals had a full spectrum of multimers with a shift from high to low molecular weight multimers, both with novel A1 domain mutations (p.L1365P and p.V1934G) that are currently being investigated. The significance of this multimer pattern is unclear. None of the individuals with type 1 VWD with multimer distributions outside the reference intervals had mutations exclusively associated with type 2 VWD.

VWF:CB AND MULTIMER DISTRIBUTION IN TYPE 2 VWD
The comparison of multimer distribution to VWF:CB for individuals with type 2A VWD showed that the vast majority had loss of high and/or intermediate molecular weight multimers or a shift from high to low molecular weight bands, as would be expected for this diagnosis. Six participants with loss of HMWM had VWF:CB/VWF:Ag ratios >0.7, but none of those individuals had VWF mutations exclusively associated with type 2A VWD (Table 3). Two had the p.R1374C mutation, which was seen in individuals with type 1, type 2A, and type 2M VWD enrolled in our study. One had a p.M1304R mutation, which has also been seen in both type 2A and type 2M VWD patients (Jorge Di Paola, University of Colorado, personal communication). One had a novel mutation, p.C524Y, which has not yet been characterized by our group. One had the p.R2287W mutation, a known type 1 VWD mutation normally associated with a multimer pattern within the reference interval. The last individual with collagen binding within the reference interval had no mutation found and the VWD in this individual also may not represent true type 2A VWD, particularly given that this individual also had a VWF:RCo/VWF:Ag ratio within the reference interval. Two individuals with type 2A VWD had multimer distribution within the reference interval, both with unclassified mutations (1 with p.M1304R and 1 with p.R1374C). Characterization of these novel mutations is in progress because they may not represent typical type 2A VWD and therefore might be better classified in a different category (or termed unclassifiable). All participants with known type 2A VWD mutations had multimer distribution outside the reference interval and VWF:CB/VWF:Ag ratios <0.7.

Table 2. Sensitivity of the VWF:CB/VWF:Ag ratio for detection of multimer abnormalities.

<table>
<thead>
<tr>
<th>VWF:CB/VWF:Ag cutoff</th>
<th>Healthy Controls</th>
<th>Type 1</th>
<th>Type 2A</th>
<th>Type 2B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>100%</td>
<td>99%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>0.7</td>
<td>99%</td>
<td>99%</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>0.8</td>
<td>97%</td>
<td>97%</td>
<td>97%</td>
<td>97%</td>
</tr>
</tbody>
</table>

For healthy controls and for type 1 VWD, a VWF:CB/VWF:Ag ratio and a multimer distribution within reference intervals are expected. For types 2A and 2B VWD, a decreased VWF:CB/VWF:Ag ratio and a multimer distribution outside the reference intervals are expected.
All of the 17 participants with type 2B VWD had low VWF:CB/VWF:Ag ratios and corresponding multimer distribution outside the reference intervals, but 4 of the individuals with type 2B had VWF:RCo/VWF:Ag ratios < 0.7. This result suggests that either VWF:CB or multimer analysis is required to differentiate this group, because the VWF:RCo/VWF:Ag ratio alone would have been insufficient for diagnosis. All participants in this category had known type 2B VWD mutations on DNA sequencing.

All of the 17 individuals with type 2M VWD enrolled in the Zimmerman Program had VWF:CB/VWF:Ag ratios within the reference intervals. Seven individuals with type 2N VWD were enrolled, none of whom had multimer distribution outside the reference interval or decreased VWF:CB/VWF:Ag ratio.

### Discussion

This study shows that, in a population of previously diagnosed individuals with type 2A and 2B VWD, the VWF:CB assay provides a sensitive screen for detection of multimer distribution outside the reference interval. This finding is important because of both the technical challenges in testing multimer distribution and the subjective nature of this nonquantitative assay. No participants in this study would have been erroneously diagnosed as healthy or as having type 1 VWD on the basis of the VWF:CB assay if they had a type 2A or type 2B VWD mutation. Several individuals with type 1 VWD had low VWF:CB/VWF:Ag ratios, primarily due to very low VWF:Ag values (< 5 IU/dL); these individuals would then require further evaluation. However, because screening tests should be more inclusive, this likely would mean repeat testing for a few patients rather than misdiagnosis of VWD in an unaffected person. An alternate approach would be to omit reporting VWF:CB/VWF:Ag ratios for patients whose VWF:Ag is significantly reduced.

We are not the first to report on the utility of the VWF:CB assay in VWD diagnosis. In a recent review, the VWF:CB assay was championed by Favaloro for its ability to screen for multimer defects and reduce misdiagnosis of type 2 VWD (19). Federici and colleagues used the combination of VWF:RCo/VWF:Ag and VWF:CB/VWF:Ag ratios to categorize patients as type 1, type 2A, type 2B, or type 2M VWD (8). Adcock and colleagues also reported efficacy using the VWF:CB/VWF:Ag ratio to distinguish multimer distributions outside the reference intervals in types 2A and 2B VWD (9). It should be noted, however, that variability in collagen preparations and collagen coating techniques may hamper interpretation of the VWF:CB (10, 20, 21). We chose a preparation of type III collagen that has been well validated in our laboratory. Other groups have advocated for a combination of types I and III collagen (20).

One of the limitations of our study is that it used a single collagen source, and a single multimer technique, which may limit generalizability of these results. Another limitation is the retrospective nature of the analysis, in that participants had a preexisting diagnosis of VWD before study entry. A number of multimer

### Table 3. Sequence variations found in Zimmerman Program type 1 and type 2A VWD patients with inconsistent multimer results and VWF:CB within reference intervals.

<table>
<thead>
<tr>
<th>Sequence variation</th>
<th>No. of individuals</th>
<th>VWD type(s)*</th>
<th>Multimer distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.R924Qb</td>
<td>2</td>
<td>Type 1</td>
<td>Loss of HMWM</td>
</tr>
<tr>
<td>p.R1374H</td>
<td>1</td>
<td>Type 1</td>
<td>Loss of HMWM</td>
</tr>
<tr>
<td>p.Y1584Cb</td>
<td>2</td>
<td>Type 1</td>
<td>Loss of HMWM</td>
</tr>
<tr>
<td>p.C2693Yc</td>
<td>1</td>
<td>Type 1</td>
<td>Loss of HMWM</td>
</tr>
<tr>
<td>p.L1365Pc</td>
<td>1</td>
<td>Type 1</td>
<td>Shift high to low</td>
</tr>
<tr>
<td>p.V1934Gh,c</td>
<td>1</td>
<td>Type 1</td>
<td>Shift high to low</td>
</tr>
<tr>
<td>p.M1304Rc</td>
<td>1</td>
<td>Type 2A</td>
<td>Within reference intervals, shift high to low</td>
</tr>
<tr>
<td>p.R1374Cb</td>
<td>1</td>
<td>Type 2A</td>
<td>Within reference intervals, shift high to low, loss of HMWM</td>
</tr>
<tr>
<td>p.C524Yh,c</td>
<td>1</td>
<td>Type 2A</td>
<td>Shift high to low</td>
</tr>
<tr>
<td>p.Y1349Cc</td>
<td>1</td>
<td>Type 2A</td>
<td>Shift high to low</td>
</tr>
<tr>
<td>p.R2287Wb</td>
<td>1</td>
<td>Type 2A</td>
<td>Loss of HMWM</td>
</tr>
</tbody>
</table>

* Sequence variations are listed as the VWD type determined at the time of enrollment in the Zimmerman Program for affected individuals. VWD types in parentheses represent other reported classifications for that sequence variation.

† Also seen in the Zimmerman Program, patient(s) with type 1 VWD and multimer within reference intervals.

‡ Novel mutation not previously described; HMWM, loss of high molecular weight multimers; shift high to low, shift from HMWM to low molecular weight multimers.
distributions outside the reference intervals were seen in participants with VWF:Ag and VWF:CB within the reference intervals and no corresponding type 2A or type 2B VWD mutations. Quantitative multimer results for similar samples with loss of only the highest molecular weight multimers were within reference intervals, suggesting transport-related artifacts or sample-processing artifacts as a possible explanations (7). The current study design prevented confirmation of this hypothesis, but in future work we will incorporate repeat sampling of participants and additional collagen-coating conditions.

Multimer distribution is assessed by running plasma samples on an agarose gel, transferring to a nitrocellulose membrane, detecting the VWF present, and subjectively assessing the results (12). Results take several days to obtain and are nonquantitative. This is a difficult, labor-intensive, and costly technique. In contrast, the VWF collagen binding assay is ELISA based, using purified collagen coated on an ELISA plate (22). Results are available within hours and are quantitative. The technique is easy, and minimal labor is required, with the potential for automation of the assay. Even with the recent introduction of quantitative multimer techniques (23), VWF:CB remains more efficient and less costly. In our laboratory, the VWF:CB can be performed for less than half the cost of multimer distribution. Use of international, cross-referenced plasma samples such as those offered by the WHO allows collagen binding results to be standardized across laboratories (24).

This suggests that the real-world application of the VWF:CB may actually improve the detection of variant VWD, avoiding misclassification of patients as variant VWD when multimer distribution is within reference intervals. Preanalytical factors such as sample processing may result in loss of the highest molecular weight multimers, leading to a report of an abnormal finding, potential misdiagnosis, or costly additional testing. Acquired multimer abnormalities, as seen in patients with ventricular septal defect, aortic stenosis, or other cardiac abnormalities, may also lead to loss of the highest molecular weight multimers in the absence of a genetic defect in VWF (25–27). In addition, the VWF:CB will detect mutations in VWF–collagen interactions, a functional defect that may not be detected by either analysis of multimer distribution or VWF:RCo. Several such mutations that exclusively or disproportionately affect VWF:CB have been reported (12, 28–30).

The VWF:RCo/VWF:Ag ratio within the reference interval in several of the patients with type 2B VWD suggests that VWF:CB may actually outperform the VWF:RCo in this group. In addition, the VWF:RCo assay is known to have a high CV, unlike the VWF:CB (31–33). CVs in our laboratory for the VWF:CB are ≤5%. One recent study demonstrated greater sensitivity to loss of HMWM with collagen binding assays compared to monoclonal antibody–based VWF activity assays, suggesting that such VWF activity assays cannot substitute for either VWF:RCo or VWF:CB in assessment of multimer distribution (34).

A possible improvement in the diagnostic evaluation for VWD would combine the VWF:Ag, VWF:RCo, and VWF:CB as an initial screen (likely with the addition of factor VIII activity). Discrepancy between either VWF:Ag and VWF:RCo or VWF:Ag and VWF:CB might then prompt analysis of multimer distribution, but such analysis could be reserved for those patients with a result outside the reference interval on the initial testing. Our results corroborate the hypothesis that no patients, at least in our study, would have been missed by this mechanism. However, prospective analysis of this approach and standardization of collagen binding assays are required to validate elimination of the VWF multimer distribution from the initial diagnostic panel.
References


