Published in final edited form as:

Semin Thromb Hemost. 2014 February ; 40(1): 41–48. doi:10.1055/s-0033-1363166.

Perils, Problems, and Progress in Laboratory Diagnosis of Von Willebrand Disease

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Abstract

Diagnosis of von Willebrand disease (VWD) merits consideration of personal and family history of bleeding symptoms along with confirmatory laboratory testing. Because the latter yields quantifiable results, over-reliance on a laboratory diagnosis may occur. However, existing tests for VWD contain potential sources for error. Both intrinsic and extrinsic factors affecting these assays can contribute to either falsely normal or falsely abnormal results. This article will discuss presently available assays as well as new developments in diagnostic testing. A clear understanding of the limitations of VWD testing is helpful for ensuring the correct diagnosis of affected patients.

Keywords

Von Willebrand factor; von Willebrand disease; bleeding; collagen; platelets

Von Willebrand Disease

Von Willebrand factor (VWF) is a multimeric glycoprotein that plays several critical roles in hemostasis. VWF serves as a carrier protein for factor VIII (FVIII), protecting FVIII from degradation in plasma.¹ VWF also functions to tether clots to injured endothelium through binding sites for platelets and collagen. The binding site for platelet glycoprotein Ib (GPIb), located in the VWF A1 domain, changes conformation under shear stress *in vivo*, enabling platelets to bind VWF.² The binding sites for collagen, located in the VWF A3 and A1 domains, facilitate the localization of VWF to sites of injury.³

Disruptions in these functions, either through quantitative or qualitative defects in the VWF protein, lead to von Willebrand disease (VWD). Type 1 VWD includes mild to moderate quantitative deficiencies of VWF protein.⁴ Type 3 VWD, the most severe form, is characterized by absent protein secondary to genetic defects in VWF.⁵ Qualitative defects in VWF are classified as type 2 VWD, and divided into 4 categories. Types 2A and 2B both demonstrate loss of the high molecular weight multimer (HMWM) forms of VWF. In type

2A VWD, this is due to a defect in secretion or in clearance of VWF.⁶ Type 2B VWD is caused by gain-of-function mutations leading to increased platelet binding and clearance of VWF-platelet complexes.⁷ Type 2M VWD includes platelet binding and collagen binding defects, with relatively preserved multimer distribution.⁸ Type 2N VWD refers to those variants with a defect in binding FVIII.⁹

Diagnosis of quantitative defects in VWF is relatively straightforward, as levels of the protein are either reduced, in type 1 VWD, or absent, in type 3 VWD. Testing for qualitative defects is more problematic as no single test can diagnose type 2 VWD.¹⁰ Variability in common laboratory tests can lead to misdiagnosis and inadequate or inappropriate treatment for affected patients. Since treatment varies by type of VWD, accurate laboratory testing is critical for optimal patient care.

Laboratory Testing for Von Willebrand Disease

In order to support a suspected diagnosis of VWD, laboratory testing is required. A typical screening panel consists of VWF antigen (VWF:Ag) to quantify the amount of VWF present, and the VWF ristocetin cofactor assay (VWF:RCo) to assess the ability of VWF to bind platelets. FVIII activity is often assessed as well, since VWF serves as a carrier protein for FVIII. VWF multimer distribution is useful to distinguish several of the type 2 variants. Specialized assays, as detailed in the next section, are performed under certain circumstances to help elucidate the diagnosis. Table 1 details both diagnostic and additional specialized laboratory testing for VWD.

VWF antigen (VWF:Ag)

VWF:Ag measures the total amount of VWF present, and is a critical part of the workup for VWD. It is most typically performed by either enzyme-linked immunosorbent assays (ELISA) or by automated latex immunoassay (LIA).^{11,12} Assessment by other methods including flow cytometry have also been described, but have not been taken up in general practice.^{13,14} While VWF:Ag ELISA and LIA assays are in themselves highly reproducible, a number of external factors influence VWF levels. Patients with blood group O have decreased VWF:Ag compared to patients with other blood groups.¹⁵ Race, age, pregnancy, and stress all influence VWF:Ag, with higher VWF levels seen in African Americans and as people age.^{15,16} Levels also rise during pregnancy.¹⁷ Exercise and stress will elevate VWF:Ag, and possibly obscure a diagnosis of mild VWD.¹⁸ A single normal VWF:Ag does not exclude mild VWD, particularly if the patient may have been stressed at the time of sampling.

VWF:Ag results should also be interpreted in the context of patient history. Because the normal range is defined as $\pm$ a certain percent (usually 2 standard deviations of the average normal value), this means that a not insubstantial number of people (2.5% if using 2 standard deviations as the cut-off) will have VWF:Ag levels below the official lower limit of normal for a given laboratory. Since the prevalence of VWD is clearly less than 2.5%, many of these patients will simply have low VWF without associated bleeding, and care is needed to avoid over-diagnosis of VWD.¹⁹

VWF Ristocetin Cofactor Activity (VWF:RCo)

While the VWF:Ag can document the presence or absence of VWF protein, additional testing is required to determine whether the protein is functional. This is most typically evaluated using the VWF:RCo, where the antibiotic ristocetin is used to induce a conformational change that enables VWF to bind platelet GPIb.²⁰ VWF:RCo is a surrogate for the ability of VWF to interact with platelets, since *in vivo* this relationship is initiated by shear stress.²¹ A VWF:RCo/VWF:Ag ratio of less than 0.6–0.7 is thought to represent a functional VWF defect.^{4,22–24} Types 2A, 2B, and 2M VWD all have low VWF:RCo/VWF:Ag values.²⁴

Limitations of the VWF:RCo are its high coefficient of variation (i.e., poor reproducibility) and its use of ristocetin to initiate VWF-platelet interactions. VWF:RCo in its current form has both high intra- and inter-laboratory coefficient of variation, ranging from 10 to 40%.^{25,26} This has the unfortunate consequence that if the test is repeated several times on even a normal person with borderline VWF levels, at least one of the results is likely to be abnormal and the patient potentially diagnosed with VWD. Conversely, if the test is repeated several times on a patient with mild VWD and borderline low VWF levels, then one of the results may lie in the normal range and confuse the diagnosis.

In addition, the non-physiologic agonist ristocetin may introduce another source of variability, as sequence variations in the VWF protein may lead to altered ristocetin binding in the absence of any clinically important effect on VWF function.²⁷ The D1472H sequence variation, present in 63% of African Americans and 17% of Caucasians in one study, is associated with a decreased VWF:RCo/VWF:Ag ratio but is not associated with an elevated bleeding score.^{27,28} VWF:RCo/VWF:Ag ratios are inversely correlated with the number of 1472H alleles, as demonstrated in figure 1.

Newer versions of the VWF:RCo are under development, with the goal of improving on the limitations described above. Performing the VWF:RCo by ELISA has been explored using a monoclonal antibody capture²⁹ or nanobody capture.³⁰ Others have used recombinant GPIb with or without ristocetin to capture plasma VWF.^{31–35} Utilization of flow cytometry and latex particle-enhanced immunoturbidimetric assays have also been described.^{35,36}

Factor VIII activity (FVIII:C)

FVIII is most typically tested through evaluation of FVIII coagulant activity (FVIII:C). A one-stage clotting assay is most frequently employed³⁷ but two-stage and chromogenic assays for FVIII activity may also be used.³⁸ Type 2N VWD results in a significant decrease of FVIII:C, with either normal or low VWF:Ag.³⁹ These test results can alternatively be expressed as a low FVIII/VWF:Ag ratio (typically < 0.7). This pattern is similar to that observed in hemophilia A. Patients with type 1 VWD or type 2 variants 2A, 2B, or 2M will have FVIII:C levels at or higher than the measured VWF:Ag (i.e., usually FVIII/VWF:Ag ratio > 1.0). FVIII:C is low in type 3 VWD, typically at the level of a mild-moderate hemophilia patient (i.e., 4–8 U/dL).⁴⁰

VWF Multimer Distribution

Visualization of the VWF multimer distribution is critical in distinguishing types 2A and 2B VWD from type 2M (or type 1) VWD. Unfortunately, multimer distribution is technically complex, labor intensive and at present restricted to specialty laboratories with the resources and expertise available to perform the analysis. The process involves agarose gel electrophoresis to separate multimers based on size, followed by staining of the VWF multimers and subsequent visualization.⁴¹ Subtle differences in multimer distribution are associated with variant VWD but most laboratories are not equipped to distinguish these.⁴² Classification of VWD variants based on multimer distribution is problematic when considering some of these subtle multimer defects.⁴³

Other conditions besides VWD and acquired von Willebrand syndrome may lead to loss of the high molecular weight multimers, such as cardiac valve lesions.^{44,45} Multimer distribution is subject to artifact due to improper specimen handling, which may cause false concern for a diagnosis of type 2 VWD.⁴⁶ Newer techniques such as quantitative multimers may help alleviate some of the variability in results.⁴⁷

Specialized Laboratory Testing for Von Willebrand Disease

Diagnosis of variant VWD often requires additional testing. The assays listed below are helpful when initial testing is equivocal or when a variant other than type 1 VWD is suspected. This would therefore include test patterns suggestive of type 2A, 2B, 2M, 2N and type 3 VWD. Table 2 summarizes the expected laboratory results for each type of VWD.

VWF Collagen Binding (VWF:CB)

Collagen is also an important ligand for VWF.^{3,48} Types I and III collagen bind to the A3 domain of VWF, with an alternate binding site in the A1 domain.³ Type VI collagen binds solely to the A1 domain of VWF.⁴⁹ This interaction is not assessed by the typical VWD screen, but may provide useful information regarding the ability of the VWF protein to bind exposed subendothelial collagen and to form high molecular weight multimers. Importantly, whereas the VWF:RCO assay is considered to be an assay of VWF function related to the A1 domain of VWF, the VWF:CB may be considered an additional assay of VWF function related to the A3 domain of VWF. As VWF mutations leading to VWD may arise in either A1 or A3 or even elsewhere on VWF, it is important to perform multiple VWF functional assays to identify possible VWF functional abnormalities. Several cases of VWD due to mutations that affect collagen binding have also been described.^{50–52} As many of these cases had only mild decreases in VWF:Ag, omission of VWF:CB could result in failure to diagnose patients with exclusive collagen defects.

The standard VWF:CB assay is performed by ELISA using various collagen types.⁵³ Current VWF:CB ELISA assays use type I or III collagen, or their combination, with varying sensitivity,^{54,55} with their combination reportedly providing the greatest specificity for type 2 VWD diagnosis.⁵⁴ Assessment of VWF:CB by flow cytometry has also been described, but has not been incorporated into general practice.^{13,14} For further analysis of this important issue, the reader is directed to a 2007 paper by Favaloro in this journal.⁵⁶

The VWF:CB also has the potential to diagnose loss of high molecular weight multimers. A decreased VWF:CB/VWF:Ag ratio is typically seen in type 2A and type 2B VWD due to the loss of high molecular weight multimers.⁵⁷ A ratio of 0.6 to 0.7 or below supports the diagnosis of a defect in multimer distribution.^{22,58,59} The VWF:CB can therefore potentially substitute for multimer distribution in the initial workup of VWD, although caution should still be used in interpretation of results.^{47,56}

Recent reports have demonstrated that type VI collagen is a ligand for VWF, and that VWF A1 domain sequence variations may affect this interaction.^{60,61} Approximately 2% of Caucasian subjects are heterozygous for the R1399H sequence variation, with concomitant reduction in VWF:CB/VWF:Ag ratio with type VI collagen. This would result in 1:10,000 homozygotes with absent binding to type VI collagen, but the clinical significance of this requires further investigation.

VWF propeptide (VWFpp)

Although not routinely performed as part of the initial VWD workup, assessment of the VWFpp can provide valuable information, specifically in determining whether a defect in VWF clearance is present. Measurement is performed using ELISA to quantify the amount of VWFpp in a sample, which can then be compared to the VWF:Ag.⁶² Patients with increased VWF clearance (Vicenza or type 1C VWD) have an elevated VWFpp/VWF:Ag ratio.⁶³ Diagnosis of a clearance defect is critical because of the implications for treatment. These patients have a heightened response to DDAVP, but decreased survival of their VWF, such that they have a rapid return to baseline VWF levels. While they do experience a rise in VWF:Ag following desmopressin administration, the rapid clearance of endogenous VWF from circulation may present a problem for longer term management (for example of surgical bleeding).⁶⁴ Therefore, treatment with VWD-containing products is generally recommended for situations where significant bleeding is expected.

VWF gene sequencing

The advent of DNA sequencing has simplified the diagnosis of many genetic conditions. Unfortunately, the large size of the *VWF* gene and the frequency of non-pathologic sequence variations limit the usefulness of genetic diagnosis for VWD. The *VWF* gene has a total of 52 exons, and the coding sequence spans 8.4 kb.⁶⁵ Mutations in variant VWD map to specific regions of the VWF protein. Mutations in the A1 domain are associated with types 2B and 2M VWD, while mutations in the FVIII-binding region are associated with type 2N VWD.⁵ A database housing the reported mutations can be found online at https://grenada.lumc.nl/LOVD2/VWF/home.php?select_db=VWF.

One problem with genetic diagnosis is that not all patients, particularly those with type 1 VWD, have an identifiable mutation in the *VWF* coding sequence. Recent studies suggest that genetic mutations are common in those patients with VWF:Ag <20–30 IU/dL.^{66,67} Some patients have large gene deletions accounting for their low VWF levels; these may not be identified using conventional sequencing.⁶⁸ Factors other than VWF gene mutations can also lead to reduction in plasma VWF; for example, blood group has been demonstrated to affect VWF levels, with lower levels in blood type O thought to be related to increased

clearance of VWF.^{15,69} VWF levels may also be influenced by genes that affect processing of VWF, such as CLEC4M.⁷⁰

Another problem is the large number of sequence variations in the VWF gene that are likely benign. Certain ethnic groups, such as African Americans, have a higher prevalence of these sequence variations.⁷¹ Investigation of novel sequence variations is required to determine if they are causative of VWD, or simply a normal variant.

Type 2B VWD Testing

Confirmatory testing for type 2B VWD (and platelet-type or pseudo-VWD) can be performed using either low dose ristocetin-induced platelet aggregation or a direct binding assay looking for gain of VWF binding to platelets. Low dose ristocetin-induced platelet aggregation uses the patient's platelet-rich plasma with a smaller dose of ristocetin than used in the VWF:RCO, typically less than 0.6 mg/mL.²⁴ Platelet aggregation will occur in patients with a gain-of-function mutation as seen in type 2B VWD.⁷² VWF-platelet binding assays, using patient plasma and fixed platelets, can also detect spontaneous binding and confirm a diagnosis of type 2B VWD.⁷³ Platelet aggregation with low concentration ristocetin can also occur in platelet type VWD, which represents an abnormality or dysfunction of the platelet VWF receptor, glycoprotein Ib. As 2B VWD and platelet-type VWD require differential management but provide indistinguishable phenotypic results with standard VWF tests including ristocetin-induced platelet aggregation, further study is required for their differentiation. This can be achieved by a process of ristocetin-induced platelet aggregation mixing studies to determine if the enhanced response is plasma based (i.e., 2B VWD) or platelet based (i.e., platelet-type VWD), or by genetic analysis of the *VWF* and platelet glycoprotein Ib (*GPIB*) genes respectively.⁷⁴ Some centers evaluate patients with gain of function abnormalities in ristocetin induced platelet aggregation directly by genetic testing. If the testing is negative for type 2B von Willebrand disease, the possibility of platelet type von Willebrand disease is investigated.

Type 2N VWD Testing

Confirmatory testing for type 2N VWD can be performed using a direct binding assay looking for VWF bound to FVIII. This assay measures binding of recombinant FVIII to VWF from the patient's plasma, and this will be low in type 2N VWD.^{39,75} This assay is most useful in distinguishing type 2N VWD from hemophilia A. Sequencing of the VWF gene may be useful as patients with clinically significant type 2N VWD will have either two type 2N mutations or 1 type 2N mutation along with a null allele.⁵ Consideration of type 2N VWD is critical in patients with low FVIII and no family history of hemophilia A.⁷⁶

Discussion

Diagnosis of VWD, and assignment of the correct type of VWD, is critical to ensure patients receive appropriate treatment. Several promising new methods for measuring VWF activity are on the horizon. Assays for measuring VWF activity independent of ristocetin have been proposed, including a GPIb ELISA³⁴ and an immunoturbidimetric GPIb assay⁷⁷ using gain of function mutations in GPIb to eliminate the requirement for ristocetin. Alternately, a

chemiluminescence system for measuring both VWF:Ag and VWF:RCo has been developed.⁷⁸ Such methods may reduce variability in the current VWF:RCo and provide more accurate diagnosis of VWD. Newer activity assays, however, do not necessarily provide the ability to distinguish loss of high molecular weight multimers.⁷⁹ Such screening may best be performed utilizing the VWF:CB, although as previously described, variability in collagen source and assay conditions limit generalization of results.

In some circumstances, a trial of desmopressin may be helpful in diagnosing VWD. Measurement of VWF activity is generally performed prior to desmopressin administration to obtain a baseline level and 1 hour post desmopressin to evaluate peak response. Measurement of VWF activity at 4 hours following desmopressin is helpful in patients where a clearance defect is suspected, as the peak VWF activity may be normal but the later timepoint enables documentation of the increased clearance. VWF activity for desmopressin trials is typically measured by VWF:RCo, but in cases of suspected type 2A, 2B or 2M VWD, analysis of multimer distribution or VWF:CB following desmopressin can also be useful.⁸⁰

Some variation in VWF levels is secondary to age, with VWF:Ag higher in older patients as compared to younger ones.¹⁵ Patients with borderline VWF levels may 'outgrow' their VWD over time. Comparison of VWF laboratory testing to symptoms is essential to avoid over-diagnosis or over-treatment of VWD.

Summary and future directions

There is no single diagnostic test for VWD. Results must be interpreted in the context of an individual patient, taking into consideration personal and family history of bleeding. The currently available laboratory testing for VWD is imperfect, as outlined above, but with careful interpretation provides significant clinical utility by categorizing affected patients by type of VWD. As the diagnosis of variant VWD has implications for treatment, laboratory testing is therefore critical for facilitating patient care. It is to be hoped that the future will bring newer, more reliable assays but in the meantime, laboratory confirmation of VWD remains a perilous journey.

Acknowledgments

The author would like to acknowledge the invaluable contributions of Dr. Robert Montgomery and the Zimmerman Program for the Molecular and Clinical Biology of VWD. Current support is provided by the National Institutes of Health K08 grant HL102260 and the MACC Fund (Midwest Athletes Against Childhood Cancer).

Abbreviations

VWF	von Willebrand factor
VWD	von Willebrand disease
VWF:Ag	von Willebrand factor antigen
VWF:RCo	von Willebrand factor ristocetin cofactor activity
VWF:CB	von Willebrand factor collagen binding

VWFpp	von Willebrand factor propeptide
FVIII	factor VIII
FVIII:C	factor VIII coagulant activity
HMWM	high molecular weight multimers
GPIb	glycoprotein Ib

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Figure 1. Association of decreased VWF:RCo/VWF:Ag ratio and the VWF D1472H sequence variation

This scatter plot shows the VWF:RCo/VWF:Ag ratio, on the y axis, for healthy control subjects by allele status for p.D1472H. Those subjects homozygous for the p.D1472H allele (homo. 1472H) had the lowest ratios, followed by the heterozygous subjects (het. D1472H), while the subjects homozygous for the more frequent allele (homo. D1472) had the highest ratios. These differences were statistically significant, with p values as shown.

Table 1

VWF Laboratory Testing

Typical diagnostic testing panel	VWF:Ag	Measures total VWF protein. Will be low in type 1 and absent in type 3 VWD. Typically low but may be normal in type 2 VWD.
	VWF:RCo	Measures VWF activity in terms of ristocetin-mediated platelet binding. A decreased VWF:RCo/VWF:Ag ratio is found in types 2A, 2B, and 2M VWD. In contrast, levels of VWF:RCo and VWF:Ag are low but concordant (i.e., similar to each other) in type 1 VWD. Should be absent in type 3 VWD, but some assay methods show poor sensitivity to low levels of VWF and thus false low levels may be seen.
	FVIII:C	Measures circulating FVIII. Will be relatively low in type 2N VWD (ratio of FVIII/VWF:Ag <0.7) and very low (typically 4-8U/dL) in type 3 VWD.
	VWF multimer distribution	Important to assess for types 2A and 2B VWD which have loss of high molecular weight multimers. Other qualitative defects may also be seen when multimers are performed well.
	VWF:CB	Measures ability of VWF to bind collagen. Surrogate test for presence of high molecular weight multimers. Will also be low if collagen binding defects are present. Will provide similar findings to VWF:RCo in types 1, 2A, 2B, 2N and 3 VWD, and thus supportive of the diagnosis of these patients. However, VWF:CB assays in general show better reproducibility than VWF:RCo, and better sensitivity to low levels of VWF, so use of this assay is associated with reduced diagnostic error rates in all cases of VWD. VWF:CB will show different test patterns to VWF:RCo in type 2M VWD, and exclusion of this assay from test panels will lead to misdiagnosis of 2M VWD as type 2A and occasionally as type 1 VWD.
Additional tests	VWFpp	Tests the amount of circulating VWFpp. An elevated VWFpp/VWF:Ag ratio suggests a VWF clearance defect.
	VWF gene sequencing	Tests for presence of genetic variants. Supports a specific diagnosis of type 2 VWD when specific mutations are found.
	Low dose ristocetin-induced platelet aggregation	Tests for increased aggregation of platelets due to increased binding of VWF to platelets. Confirms diagnosis of type 2B VWD or platelet type VWD, which can then be differentiated by additional testing.
	VWF-platelet binding	Tests for increased binding of VWF to platelets. Confirms diagnosis of type 2B VWD.
	VWF-FVIII binding	Tests for ability of VWF to bind FVIII. Confirms diagnosis of type 2N VWD.

Table 2

Expected Laboratory Findings in VWD

VWD Type	VWF:Ag	VWF:RC ₀	VWF:RC ₀ /VWF:Ag ratio	FVIII:C	Multimer distribution	VWF:CB	VWF:CB/VWF:Ag ratio	Additional testing
Type 1	Low	Low	Normal	Normal/Low	Normal	Low	Normal	May have VWF gene mutation, but not normally assessed
Type 1C	Low	Low	Normal or decreased	Normal/Low	Normal or increased HMW ^a	Low	Normal	Elevated VWFpp/VWF:Ag ratio Usually have VWF gene defect
Type 3	Absent	Absent ^b	NA	Very low	Absent	Absent	NA	Should have two VWF gene mutations (includes large gene deletions)
Type 2A	Low	Very low	<0.6–0.7	Normal/Low	Loss of HMWM	Very low	<0.6–0.7	VWF gene mutations can usually be identified
Type 2B	Low	Very low	<0.6–0.7	Normal/Low	Loss of HMWM	Very low	<0.6–0.7	Increased VWF-platelet binding or increased low dose ristocetin-induced platelet aggregation VWF gene mutations can usually be identified
Type 2M	Normal/Low	Low	<0.6–0.7	Normal/Low	Normal	Normal or low	Normal or low	VWF gene mutations can usually be identified
Type 2N	Normal/Low	Normal/Low	Normal	Very low	Normal	Normal or low	Normal	Decreased VWF-FVIII binding VWF gene mutations can usually be identified

^a HMWM = high molecular weight multimers;^b some methods show poor sensitivity to low levels of VWF and may thus show false low levels up to 10U/dL;