Diagnostic Testing for von Willebrand Disease: Trends and Insights from North American Laboratories over the Last Decade

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Abstract

Accurate diagnosis of von Willebrand disease (VWD) depends on the quality, precision, and variability of the laboratory assays. The North American Specialized Coagulation Laboratory Association (NASCOLA) is a provider of external quality assessment (EQA) for approximately 60 specialized coagulation laboratories in North America. In this report, NASCOLA EQA data from 2010 to 2021 are reviewed for trends in methodology and precision among various assays. In particular, recent ASH ISTH NHF WFH (American Society of Hematology, International Society on Thrombosis and Haemostasis, National Hemophilia Foundation, and World Hemophilia Federation) guidelines for diagnosis of VWD are reviewed in light of EQA data. In contrast to other geographic regions, laboratories in North America predominantly use three-assay screening panels (antigen, platelet-binding activity, and factor VIII [FVIII] activity) rather than four-assay panels (antigen, platelet-binding activity, FVIII activity, and collagen-binding activity). They also use latex immunoassays rather than chemiluminescence immunoassays, and the classic ristocetin cofactor (VWF:RCo) assay and monoclonal antibody (VWF:Ab) assay to assess VWF platelet-binding activity over newer recommended assays (VWF:GPIbM and VWF:GPIbR). Factors that may be influencing these North American practice patterns include lack of Food and Drug Administration approval of the VWF:GPIbM, VWF:GPIbR, collagen binding assays, and chemiluminescence methodologies, and the influence of the 2008 National Heart, Lung, and Blood Institute guidelines on laboratory practice. Lastly, systems-based solutions are urgently needed to improve the overall accuracy of laboratory testing for VWD by minimizing preanalytical variables and adopting assay standardization.

Keywords
- coagulation tests
- external quality assessment
- proficiency testing
- ristocetin cofactor
- GPIbM
- GPIbR
- von Willebrand disease
- von Willebrand factor

von Willebrand Disease (VWD) is a bleeding diathesis caused by a quantitative or qualitative deficiency in von Willebrand factor (VWF). VWF functions as an essential primary hemostatic factor for platelet binding (largely through the platelet glycoprotein Ib [GpIb] receptor) to subendothelial collagen, and as a carrier protein in the plasma for factor VIII (FVIII), protecting it from degradation and delivering it to the site of injury.[1] VWF circulates in multimers of various molecular

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Table 1 Assays used in evaluation of VWD

Assay	Assay principle
VWF:Ag	Measures quantity of VWF antigen (does not imply functional activity)
VWF:RCo	Measures binding activity of VWF to platelets, GPIb receptor using reagents platelets and ristocetin
VWF:GPIbR	Measures binding activity of VWF to platelets, GPIb receptor using recombinant GPIb and ristocetin
VWF:GPIbM	Measures binding activity of VWF to platelets, GPIb receptor using recombinant GPIb with gain-of-function mutation
VWF:Ab	Measures binding activity of VWF to platelets, GPIb receptor using monoclonal antibodies to intact VWF GPIb binding site as a surrogate
VWF:CB	Measures binding activity of VWF to collagen
VWF:Mul	Measures size distribution of VWF multimers using protein electrophoresis

Abbreviations: VWF, von Willebrand factor; VWF:Ab, von Willebrand factor antibody assay; VWF:Ag, von Willebrand factor antigen assay; VWF:CB, von Willebrand factor collagen binding assay; VWF:GPIbM, GpIb binding activated by gain-of-function mutations; VWF:GPIbR, GpIb binding activated by ristocetin and using recombinant GPIb; VWF:Mul, von Willebrand factor multimer assay; VWF:RCo, von Willebrand factor ristocetin cofactor assay.
Note: Summarized from Mazurier and Rodeghiero[5] and Bodó et al[6].

weights. Increased multimer size displays increased affinity in platelet binding.[2] VWD is one of the commonest inherited bleeding disorders, being caused by numerous mutations that affect VWF and classified into three major types. Type 1 VWD is usually represented as a mild quantitative deficiency of VWF, type 2 VWD represents qualitative defects in VWF, and type 3 VWD represents a total or near total lack of VWF. Type 2 VWD is further subtyped into type 2A (results from a defect in VWF multimerization or increased clearance of large VWF multimers), 2B (results from spontaneous binding of VWF to platelets and increased clearance of higher molecular weight VWF multimers), 2M (results from a decreased affinity of VWF to platelets with normal VWF multimers), and 2N (results from decreased affinity in VWF binding to FVIII). The diagnosis of VWD is clinicopathological, based on either a moderate to high bleeding score on a standardized bleeding assessment or positive family history of VWD in conjunction with laboratory testing.[3]

Laboratory diagnosis of VWD consists of an initial screening panel that includes quantitative assessment of the level of VWF (antigen; VWF:Ag), VWF functional activity assays, and an assessment of FVIII activity (FVIII:C). VWF activity assays can assess platelet-binding activity, collagen-binding activity, or FVIII-binding activity; however, the majority of VWF activity assays in initial screening panels include an assessment of platelet-binding activity.[1,4] The different platelet-dependent VWF activity assays have been sub-classified based on assay principle given by the International Society on Thrombosis and Haemostasis (ISTH) as shown in ►**Table 1**.[5,6] The classic VWF activity assay—the ristocetin cofactor assay (VWF:RCo)—initially described by Margaret Howard and Barry Firkin in 1971,[7] measures the platelet-binding activity of VWF using the reagent platelets (as substrate) and ristocetin (as an activator of VWF). While this has been the classic standard for many decades, its high imprecision and suboptimal lower limit of VWF detection has prompted development of newer assays that are not affected by the instability of platelets or ristocetin as reagents. The first platelet-free VWF activity assay was developed by HemosIL in the early 2000s, and is marketed as the HemosIL von Willebrand Factor Activity assay (Instrumentation Laboratory, Bedford, MA).[8] This assay uses monoclonal antibodies directed against intact VWF GPIb-binding site as a surrogate measure for binding activity and is abbreviated as VWF:Ab (►**Table 1**). While it has been criticized as not being a true GPIb binding assay, it remains the only non-platelet (or GPIb)-based activity assay that is Food and Drug Administration (FDA)-approved and widely available in the United States and Canada.[9] The VWF:GPIbR assay measures the binding of VWF to recombinant GPIb fragments; thus, it is not dependent on platelets, but still depends on ristocetin for platelet activation. Instead of platelets, the VWF:GPIbR assay uses latex or magnetic particles depending on the analytical platform. The VWF:GPIbM uses a recombinant GPIb fragment (s) with a gain-of-function mutation that enables spontaneous VWF binding and does not use platelets or ristocetin. The VWF collagen binding assay (VWF:CB) measures the binding activity of VWF to collagen and is part of the initial VWD screening panel in some jurisdictions. Subtyping of VWD includes VWF multimer analysis where the size and distribution of VWF multimers is qualitatively or quantitatively assessed using protein electrophoresis.

External quality assessment (EQA), also known as proficiency testing, is an important quality practice that is required of laboratories in which participants compare their performance with their peer laboratories on the same sample with their usual analytical methods. While the primary purpose of EQA data is to assess the accuracy of measurement of the individual member laboratories, these data are also valuable in determining the availability of each assay in different regions, prevalence of the different methods and vendors, and most importantly in comparing the precision of each assay method with the same assay peer group as well as against other methods available for the same analyte. As VWD testing panels and assays vary by center, analysis of EQA data can provide important insights into the variability and accuracy of laboratory diagnosis of VWD. The North American Specialized Coagulation Laboratory Association (NASCOLA), in close collaboration with the ECAT (External Quality Control for Assays and Tests) Foundation, is one such nonprofit organization providing an EQA program for laboratories working in the

Table 2 Number of NASCOLA laboratories performing VWF antigen and VWF activity assays, by year and method

Assay	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
Antigen assays (*n*)	47	54	52	57	56	61	59	55	59	59	57	51
LIA (%)	91	94	94	96	93	95	97	96	97	97	96	96
ELISA (%)	9	7	6	4	7	5	3	4	3	3	4	4
IEP (%)	2	2	2	2	0	0	0	0	0	0	0	0
Platelet-based activity assays (*n*)	47	53	51	61	57	61	60	54	60	59	55	48
RCo (%)	77	74	74	66	45	53	43	45	42	42	41	39
Ab (%)	19	26	26	32	40	33	40	35	34	34	36	35
GPIbM (%)	0	0	0	1	9	4	10	6	8	15	15	16
GPIbR (%)	0	0	0	0	2	5	6	6	5	4	3	6
Homemade (%)	4	0	0	0	3	3	1	6	5	3	3	4
Other (%)	0	0	0	1	0	1	0	3	5	1	2	0
Collagen binding (*n*)	6	9	10	11	15	16	8	6	10	10	11	9
Multimers (*n*)	14	18	18	22	16	23	15	21	15	13	15	12

Abbreviations: Ab, VWF monoclonal antibody assay; ELISA, enzyme-linked immunoassay; IEP, Immuno-electrophoresis; GPIbM, GpIb binding activated by gain-of-function mutations; GPIbR, GpIb binding activated by ristocetin; LIA, latex immunoassay; RCo, ristocetin cofactor assay.
Note: All collagen binding assays were ELISA.

field of hemostasis and thrombosis in North America. In this report, we present an updated analysis of VWD EQA data from NASCOLA laboratories over the past decade and review the literature on the performance of VWF assays. We specifically discuss our data in the context of recent combined guidelines on the diagnosis of VWD by the American Society of Hematology (ASH), ISTH, the National Hemophilia Foundation (NHF), and the World Hemophilia Federation (WFH).[3]

Assessment of EQA Data from North American Laboratories

Our group has recently published an analysis of NASCOLA data from 2010 to 2019; the details of the analysis are available elsewhere.[10] For the purpose of this review, we updated our previously published data to include the last eight samples from the 2020 and 2021 surveys for a total of 42 proficiency samples. All samples analyzed in this study were true patient samples, lyophilized, and prepared by the ECAT (External Quality Control for Assays and Tests) Foundation. These consisted of 16 samples from type 1 VWD patients, 13 samples from type 2 VWD patients, and 13 samples of normal citrated pooled plasma. The number of laboratories that participated in NASCOLA proficiency testing from 2010 to 2021 remained relatively stable over that time ranging from 48 to 61. ►**Table 2** shows the number of unique laboratories that submitted responses for each assay during each year from 2010 to 2021, and also the breakdown of laboratories that used each method. Further details are provided in ►**Fig. 1**.

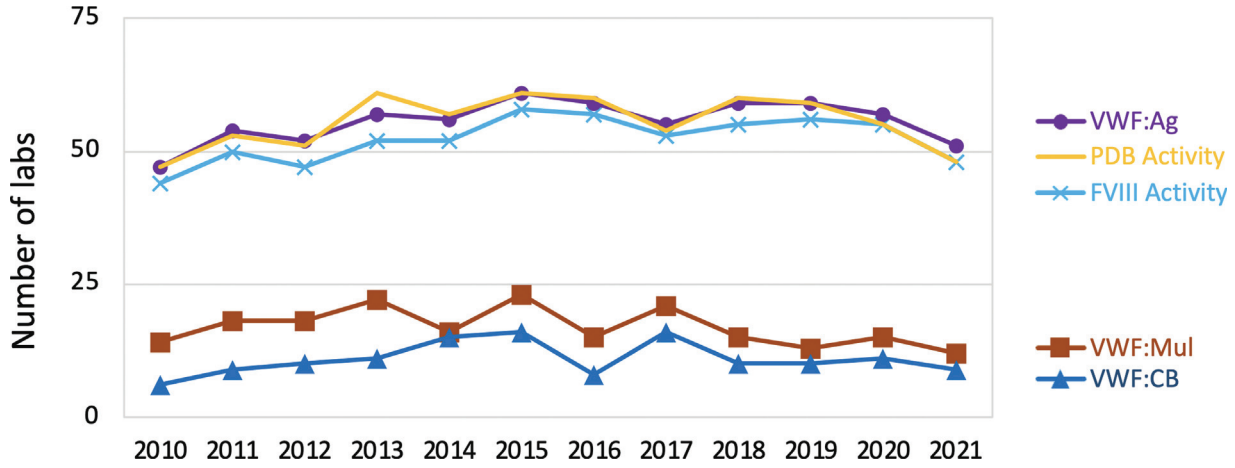

Fig. 1 Number of laboratories performing each assay (2010–2021).PDB activity, platelet-dependent binding VWF activity assay; FVIII activity, factor VIII activity assay; VWF:Ag, von Willebrand factor antigen assay; VWF:CB, von Willebrand factor collagen binding assay; VWF:Mul, von Willebrand factor multimer assay.

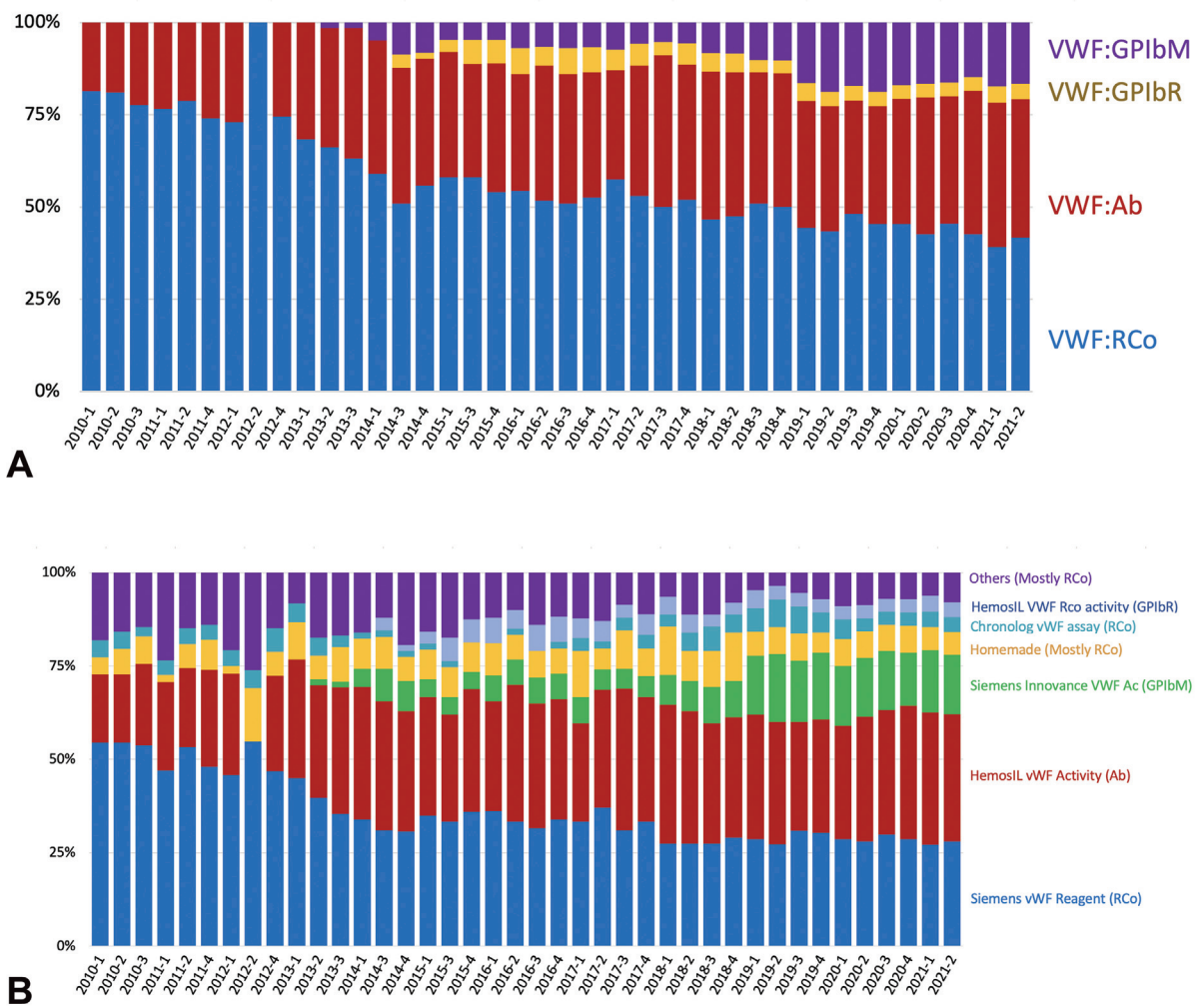

Fig. 2 (**A**) Distribution of VWF activity assays per quarterly survey using NASCOLA EQA data (2010–2021). There were no submissions for VWF:Ab in 2022–2. (**B**) Distribution of VWF GPIb-related activity assays by manufacturer using NASCOLA EQA data (2010–2021). Ab, VWF monoclonal antibody assay; GPIbM, GpIb binding activated by gain-of-function mutations; GPIbR, GpIb binding activated by ristocetin; RCo, von Willebrand factor ristocetin cofactor assay; VWF:Ab, von Willebrand factor antibody assay; VWF:GPIbM, GpIb binding activated by gain-of-function mutations; VWF:GPIbR, GpIb binding activated by ristocetin and using recombinant GPIb; VWF:RCo, von Willebrand factor ristocetin cofactor assay.

Several trends that occurred from 2010 to 2021 are apparent. For the VWF:Ag assay, the latex immunoassay (LIA) principle was consistently the most common and increased from 91 to 96%. In contrast, enzyme-linked immunoassay (ELISA) decreased from 9 to 4% during those years, reflecting the preference of laboratories toward automated assays over manual ELISAs as automated platforms became more widely available. For the VWF activity assays, use of platelet-dependent VWF:RCo assays decreased from 77 to 39%, while VWF:Ab assays increased from 19 to 35%, VWF:GPIbM assays increased from 0 to 16%, and VWF:GPIbR assays increased from 0 to 6%. These trends reflect the preference of many laboratories toward non-platelet-dependent assays, which are more precise, have a lower limit of detection, and are automated.[8] VWF:Ab is far more common than the other new assays among NASCOLA laboratories, likely because it is currently the only FDA-approved non-platelet-dependent activity assay. To visualize the trends within the activity assays, ►**Fig. 2(A, B)** shows year-by-year stacked bar graphs. ►**Fig. 2(A)** shows the distribution of VWF activity assays by ISTH type, and it is apparent that the VWF:RCo assay was the most common assay performed by the majority of laboratories in 2010, but is gradually being replaced by newer assays. ►**Fig. 2(B)** shows the same data by assay or reagent manufacturer.

To evaluate the precision of each assay, we calculated the coefficient of variation (CV) separately for each proficiency sample and assay type. These were calculated only from results that were submitted as a numerical value; results that were less than the lower limit of detection (e.g., a value reported as <10 U/dL) were excluded from our calculations for CV and for activity-to-antigen ratio. This is because the CV is used to quantify precision in measurements, and the lower limit of detection is never intended to be a measurement of the sample. Since CV is the standard deviation divided by the mean, a lower CV implies less variation in results and greater precision in the method. This is apparent in ►**Fig. 3**, where each box plot represents the range of CVs among the 42

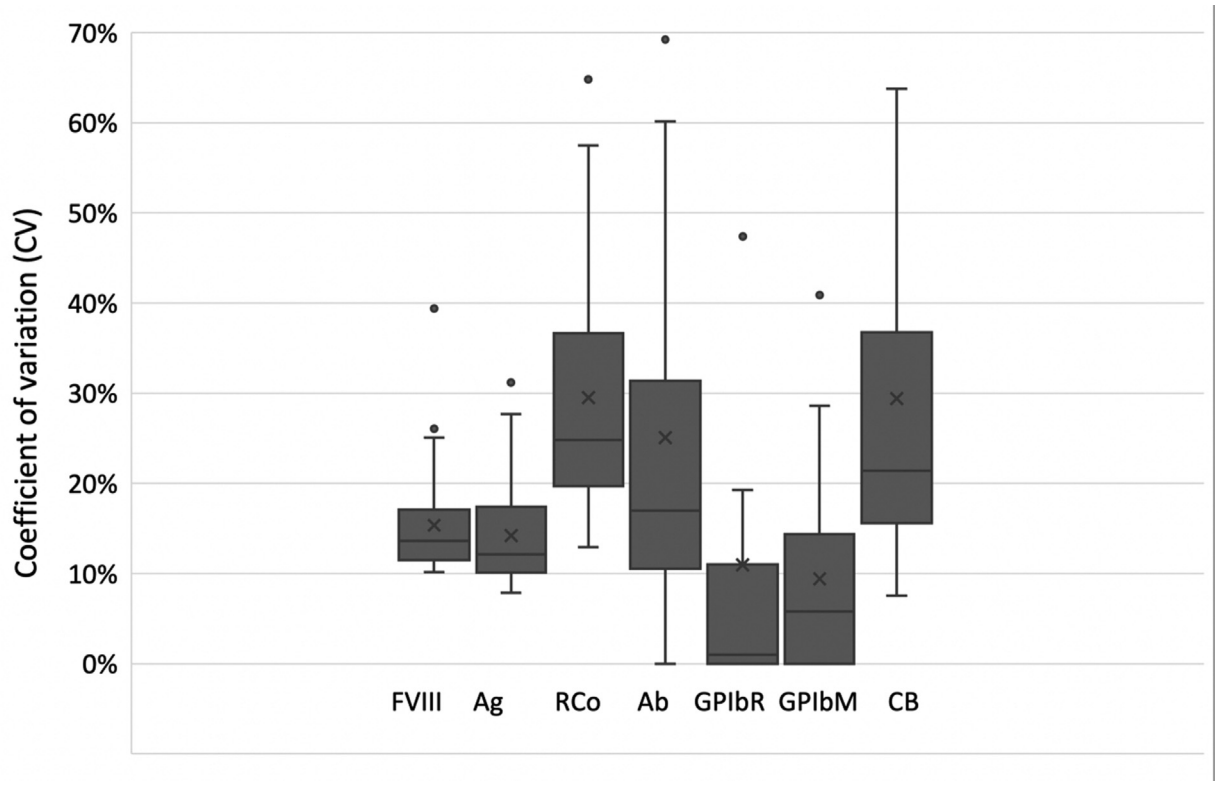

Fig. 3 Range in CV values for each assay type across the different assays using NASCOLA EQA data (2010–2021). Ab, von Willebrand factor monoclonal antibody assay; Ag, von Willebrand factor antigen assay; CB, von Willebrand factor collagen binding assay; CV, coefficient of variation; FVIII, factor VIII assay; GPIbM, GpIb binding activated by gain-of-function mutations; GPIbR, GpIb binding activated by ristocetin; RCo, von Willebrand factor ristocetin cofactor assay.

samples, and the vertical lines extending from the box show that some assays had nearly a 10-fold increase in CV among different samples. This variation is consistent with decreased precision in samples that have decreased VWF levels. ►**Fig. 4** shows the correlation between CV and VWF concentration as a scatterplot, and the dependence of the CV on concentration is apparent in all assay types.

In interpreting these CV values, it is important to consider three caveats. First, all the VWF:CB assays performed by NASCOLA laboratories are by ELISA, and our data cannot be used to evaluate VWF:CB assays performed by other methods. Second, since CV values are defined as the standard deviation divided by the mean, the CV will be lower when the mean result is higher. This effect is particularly relevant when comparing older assays that have higher limits of detection against newer assays that have lower limits of detection. When an older assay has a lower CV that is caused by this effect, it does not imply that the older assay is more precise, but rather the older assay is limited to high concentrations where all assays are more precise. Third, CV values are most reliable when calculated from 10 or more responses, and therefore our precision data for FVIII:C (clot-based), VWF:Ag, VWF:RCo, and VWF:Ab are very robust since the majority of laboratories performed these assays as part of their VWD screens, while the data for VWF:GPIbM, VWF:GPIbR, and VWF:CB are more limited among North American laboratories. However, the superior precision of the VWF:GpIbM and VWF:GpIbR assays is still evident despite their lower limit of detection and their limited use in our dataset. This supports their future use when these assays become more available in North America.

Influence of Clinical Guidelines on Practice Patterns

Evaluating laboratory testing trends can provide a glimpse into how geographic practice patterns and adherence to guidelines have evolved in the setting of VWD diagnostic guidelines published over the past 15 years. The guidelines published by several major societies (National Heart, Lung, and Blood Institute [NHLBI], British Committee for Standards in Haematology [BCSH], and ASH/ISTH/NHF/WFH)[3,4,10] and the important differences between them are summarized in ►**Table 3**. The NHLBI guidelines, published in 2008, recommended initial testing for VWD to consist of only three tests – FVIII, VWF:Ag, and a platelet-based activity assay—without need for VWF:CB.[4] This recommendation is consistent with the NHLBI's definition of type 2 VWF as disorders of "VWF-dependent platelet adhesion," implying that a decrease in VWF-dependent collagen binding was not part of the primary definition. As shown in ►**Fig. 1**, the majority of NASCOLA laboratories submitted results for the three assays recommended by NHLBI and not for VWF:CB, consistent with the adoption of these guidelines among North American laboratories. Furthermore, the diagnostic threshold for type 2 VWD was set by the NHLBI as an activity-to-antigen

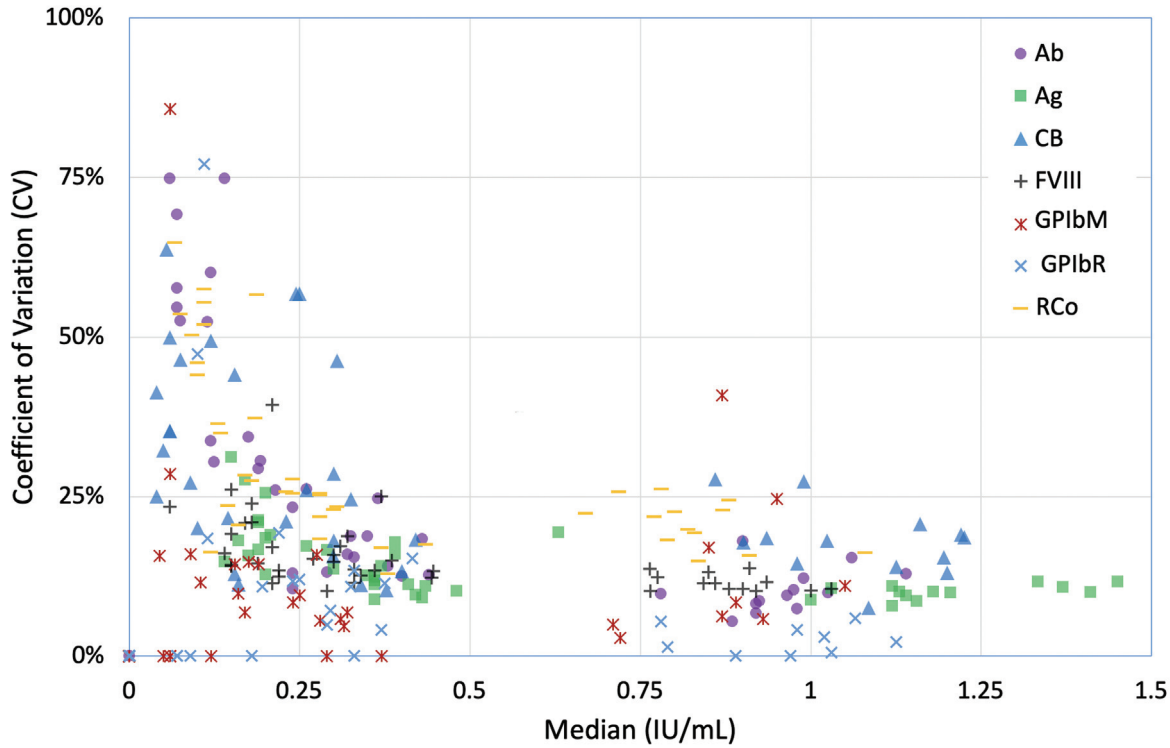

Fig. 4 Change in precision (CV) by VWF concentration (median IU/dL) across the different assays using NASCOLA EQA data (2010–2021). Ab, von Willebrand factor monoclonal antibody assay; Ag, von Willebrand factor antigen assay; CB, von Willebrand factor collagen binding assay; CV, coefficient of variation; FVIII, factor VIII assay; GPIbM, GpIb binding activated by gain-of-function mutations; GPIbR, GpIb binding activated by ristocetin; RCo, von Willebrand factor ristocetin cofactor assay.

ratio below 0.5 to 0.7, a deliberately loose specification that was put in place "until more research becomes available."

The BCSH guidelines[11] were published 6 years later in 2014 and differed in several important areas. The criteria to define VWD type 2 could be either decrease in VWF-dependent platelet binding OR decrease in VWF-dependent collagen binding. Consistent with this, the initial testing for VWD was to include four tests; the three mentioned above plus the VWF:CB assay. Furthermore, the diagnostic threshold for type 2 VWD was set as activity-to-antigen ratio below 0.6, which was exactly halfway between the range set by NHLBI.

The latest guidelines for VWD diagnosis were published in 2021 by ASH in conjunction with ISTH, NHF, and WFH.[3] This reverted back to the NHLBI standard in several areas. The VWF:CB assay is not included in the initial testing panel, and a decrease in collagen-binding activity alone does not qualify for VWD type 2 according to the published algorithm. Instead, the VWF:CB assay is recommended only in patients in need of additional testing, and primarily to discriminate VWD type 2M from other type 2 subtypes, as an alternative to multimers. Furthermore, the diagnostic threshold for type 2 VWD is set by these guidelines at 0.7, which classifies borderline patients as type 2 rather than type 1. While previous guidelines diagnosed VWD type 2B with ristocetin-induced platelet aggregation, the ASH/ISTH/NHF/WFH guidelines suggest targeted genetic testing instead. In addition, genetic analysis is listed as an additional option for diagnosis of VWD type 2N.

The conflicting guidelines regarding the VWF:CB assay is reflective of variations in regional practice patterns. A report in 2011 found that VWF:CB was performed by more than 50% of Australian laboratories participating in the RCPA (Royal College of Pathologists of Australasia) Haematology QAP (Quality Assurance Program) proficiency testing assessments, but by only 12% of North American laboratories participating in NASCOLA.[12] In addition, the studies that have been published about the utility of this assay are conflicting. In 2014, Favaloro et al,[13] studying a large set of proficiency testing data ($n = 29$ samples) to understand the source of real-world diagnostic errors in the laboratory identification of VWD, found superior performance in the VWF:CB assay, with an error rate that was substantially lower than that of VWF:RCo. This was reassessed by the authors in 2021 in another large set of more recent proficiency surveys ($n = 27$ samples), and again found that laboratories which did not include the VWF:CB assay in their initial screening panel were associated with greater error rates in classifying VWD.[14] It must be noted, however, that the erroneous classification of these patients may be a subjective interpretation in patients who were diagnosed with VWD based solely on VWF:CB assay. In addition, the findings of these studies have not been replicated in any population outside of Australasia. Ferhat-Hamida et al[15] evaluated and compared a panel of VWF assays on 19 patients suffering from recurrent bleeding to assess the contribution of the VWF:CB assay. They concluded that it

Table 3 Major international VWD guidelines—2008 to 2021

	NHLBI	BCSH	ASH, ISTH, NHF, WHF
Key reference	Nichols et al (2008) *Haemophilia [4]*	Laffan et al (2014) *British Journal of Haematology [10]*	James et al (2021) *Blood Advances [3]*
Initial screening panel	3 tests: antigen, FVIII, and platelet binding activity	4 tests: antigen, FVIII, platelet binding activity, and collagen binding activity	3 tests: antigen, FVIII, and platelet binding activity
Primary definition of VWD type 2	Low platelet binding activity relative to antigen level	Low platelet binding activity OR low collagen binding activity relative to antigen level	Low platelet binding activity relative to antigen level
Threshold for VWD type 1 (antigen assay)	<30 IU/dL for definitive diagnosis(30–50 IU/dL can be diagnosed as VWD if there is supporting clinical or family evidence)	<30 IU/dL(30–50 IU/dL should be regarded as "low VWF")	<50 IU/dL for patients with abnormal bleeding, or <30 IU/dL regardless of bleeding
Threshold for VWD type 2 (activity: antigen ratio)	0.5–0.7	0.6	0.7
GPIbR and GPIbM	GPIbR is acceptable, GPIbM is not mentioned	Acceptable	Preferred
VWF:Ab	Acceptable	Recommend against	No clear recommendation (panel reviewed data on this assay but "focused our deliberations" on the other assays)
Role of genetic analysis	Not included in diagnostic criteria	Recommended when "beneficial to clarify diagnosis and aid management"	Often first-line in type 2
Identify VWD type 2B	RIPA	RIPA	Genetic analysis (not RIPA)
Identify VWD type 2M	Multimer analysis	Multimer analysis	Multimer analysis or VWF:CB
Identify VWD type 2N	VWF:FVIIIB binding	VWF:FVIIIB binding or genetic analysis	VWF:FVIIIB binding or genetic analysis

Abbreviations: Act:Ag, ratio of functional activity to quantitative antigen level; ASH, American Society of Hematology; BCSH, British Committee for Standards in Haematology; FVIII, factor VIII activity assay; ISTH, International Society on Thrombosis and Haemostasis; GPIbM, GpIb binding activated by gain-of-function mutations; GpIbR, GpIb binding activated by ristocetin; IU/dL, international units per decaliter; NHF, National Hemophilia Foundation; NHLBI, National Heart, Lung, and Blood Institute; RIPA, ristocetin-induced platelet aggregation (low dose); VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ab, VWF monoclonal antibody assay; VWF:CB, VWF collagen-binding assay; VWF:FVIIIB, VWF factor VIII-binding assay; WFH, World Hemophilia Federation.

could be used as an alternative to VWF:RCo only in specific circumstances, and they do not consider it to be an additional test that is necessary after a platelet-binding activity assay has been performed. Baronciani et al[16] studied multiple VWF assays in a cohort of patients with genetically proven VWD type 2M ($n = 20$), type 2B ($n = 28$), and type 2A ($n = 14$). They concluded that there was little evidence to support the VWF:CB assay, and they also discuss the challenges in optimizing and standardizing the assay due to the different types of collagen sources. In sum, while some studies find great promise in the VWF:CB assay, positive findings have not been reported in any population outside of Australasia, challenges remain due to variations in collagen, and the assay is not commonly used for initial diagnosis in North America. In addition, study design in assessing the utility of this assay is challenging since the reference standard is not clear-cut and is influenced by the practice patterns in each region. Accuracy studies of VWF:CB assays are likely to show stronger performance in regions where the initial "gold-standard" diagnosis was rendered by VWF:CB assays, and weaker performance in regions where the initial "gold-standard" diagnosis was rendered solely by platelet-dependent assays.

The ASH ISTH NHF WFH guidelines suggest using newer assays that measure the platelet-binding activity of VWF using GPIb fragments (e.g., VWF:GPIbM, VWF:GPIbR) over the classic VWF:RCo assay. This recommendation is based on several factors. They reviewed studies of diagnostic accuracy, and members of the expert panel have recently published a separate report[17] with additional details on the systematic review and meta-analysis of 21 diagnostic accuracy studies that were used in the guideline deliberations, where sensitivity and specificity were calculated for VWF:GPIbM, VWF:GPIbR, and VWF:RCo. Although the findings that are quoted in the report show that VWF:GPIbM had significantly lower sensitivity but higher specificity than the two ristocetin-dependent assays, the panel ultimately concluded that the data do not support any specific assay since the overall test accuracy was generally comparable. In addition, they found the evidence from diagnostic accuracy studies to be of low certainty due to risk of bias in the case control design, and because the studies did not directly analyze the ability of

each assay to make a new VWD diagnosis, but rather to classify known patients into subtypes. Rather the critical consideration of the ASH ISTH NHF WFH panel in recommending the newer assays was the poor performance of the VWF:RCo assay in patients with benign genetic variants that cause a change in the ristocetin-binding domain, causing falsely decreased VWF:RCo results. A common example is the D1472H variant. The frequency of this variant has been found in one study to be 63% in subjects of African ancestry and 17% in subjects of Caucasian ancestry,[18] but these subjects were recruited solely from North America and the frequency is likely be different in other regions. The variants in ristocetin-binding domain have no effect on bleeding phenotype since this binding domain is not used in vivo.[19] Several points must be considered with regard to this reasoning. First, while the lower sensitivity but higher specificity in the VWF:GPIbM assay may be equivalent in terms of overall accuracy, it can still have significant impact on patient care. These parameters imply that the VWF:GPIbM assay would have fewer false positives and more false negatives. It would seem that the harm inflicted on patients from a false negative is greater than that of a false positive, since a false-positive diagnosis is likely to be followed by greater medical attention and further confirmatory testing to rule out VWD, while a false negative may not be investigated further. Second, diagnostic accuracy studies are valuable for development of clinical guidelines when the studies compare the test to a standardized reference test that is known to be more accurate, such as genetic testing or pathological diagnosis. In contrast, the reference standard in many of the studies accepted by ASH ISTH NHF WFH was clinical follow-up for symptoms alone.[17] Third, since the panel's primary consideration in recommending the newer assays is because of genetic variants in the ristocetin-binding domain, it is important to consider whether these variants affect the VWF:GPIbR assay. It would seem logical that they should affect any ristocetin-dependent assay, including VWF:GPIbR.[20,21] Interestingly, a clinical study in 47 patients with known D1472H variant reported that VWF activity based on the VWF:RCo assay was reduced by 25%, while VWF:GPIbR was not affected.[22] However, additional studies will be needed for this to become established.

In addition, the ASH ISTH NHF WFH 2021 guidelines are not clear about the role of the VWF:Ab assay, which is the most common VWF activity assay used by North American laboratories. The surrogate nature of the monoclonal antibodies has attracted some controversy. The ISTH[6] and BCSH[11] recommend that positive screens with the VWF:Ab assay should not be relied upon for final diagnosis, while the NHLBI panel lists this assay among the acceptable options. The ASH ISTH NHF WFH panel, however, does not directly address whether this assay is acceptable or recommended, stating "data were reviewed for all published methods for VWF:RCo, VWF:GPIbM, VWF:GPIbR, and VWF:Ab, however, consistent with the recommendation of the ISTH and other groups, we focused our deliberations on the first 3." While it may seem desirable to avoid surrogate markers when direct-binding assays are available, several caveats must be recognized with this reasoning. First, all assays depend on surrogates to some extent, since the in vivo activation of VWF by shear stress cannot be replicated in vitro, and VWF binding due to activation by ristocetin or mutated GPIb receptors also reflect nonphysiological surrogates, albeit to a lesser extent. Second, a large body of empirical evidence shows that the VWF:Ab assay is as good or better than VWF:RCo. Chen et al evaluated 492 patient plasma samples received by Mayo Clinic Rochester Special Coagulation Laboratory to compare lower detection limit, linearity, precision, and sample stability, and they reported that VWF:Ab has excellent laboratory characteristics compared with VWF:RCo.[23] In addition, the "Willebrand in the Netherlands" study compared all four activity assays (VWF:GPIbM, VWF:GPIbR, VWF:RCo, and VWF:Ab) for diagnostic accuracy in 661 known VWD patients, and found the VWF:Ab assay performed at least equally to the other assays with regard to accuracy in VWD classification.[22] Third, since there is no VWF:GPIbR or VWF:GPIbM assay that is currently FDA-cleared, clinical laboratories in the United States typically have only two available options to choose from, either VWF:RCo or VWF:Ab. Our data show that the VWF:Ab assay is currently the most used VWF activity assay and its popularity in North America has been growing steadily. In fact, out of 231 responses for activity results that were submitted to NASCOLA during the two surveys that are available from 2021, 31% used the VWF:Ab assay. This was greater than Homemade (3%), VWF:GPIbR (4%), VWF:GPIbM (14%) and VWF:CB (15%), and just slightly less than VWF:RCo (33%). Lack of access to the VWF:GpIbM and VWF:GpIbR assays due to lack of regulatory approval is likely the most important reason for the use of the VWF:Ab assay. The shift from the VWF:RCo assay to the VWF:Ab assay over time is likely related to the improved between-laboratory variation compared with the VWF:RCo assay, and also to the popularity of this manufacturer's instrument line for all coagulation tests. The implementation of the ASH ISTH NHF WFH guidelines by laboratories therefore faces an important barrier: the lack of FDA approval for their recommended assays. Of note, Siemens who markets the VWF:GPIbM assay as INNOVANCE VWF Ac Assay has recently announced that the assay is under review by the FDA as a de novo classification request.[24] This is a positive development that may cause VWF:GPIbM to become more accessible in North America.

The ASH ISTH NHF WFH panel suggests that for VWF activity:antigen ratios, the cutoff to distinguish VWD type 1 versus type 2 should be set at 0.7 rather than the lower or variable thresholds recommended by previous guidelines. This was intended to capture the borderline patients as type 2 and avoid false negatives for a type 2 diagnosis. This is further clarified in the "Evidence to decision" tables published as a supplement to the report. They write that false-negative VWD type 2 results are important to avoid since they would be labeled as VWD type 1 or 2N, and may receive inappropriate desmopressin treatment, and may be incorrectly counseled about the risk in their children. In contrast, false positives are less concerning as there is a tendency to confirm type 2 cases via genetic testing. We analyzed the ability of our assays to discriminate between VWD type 1

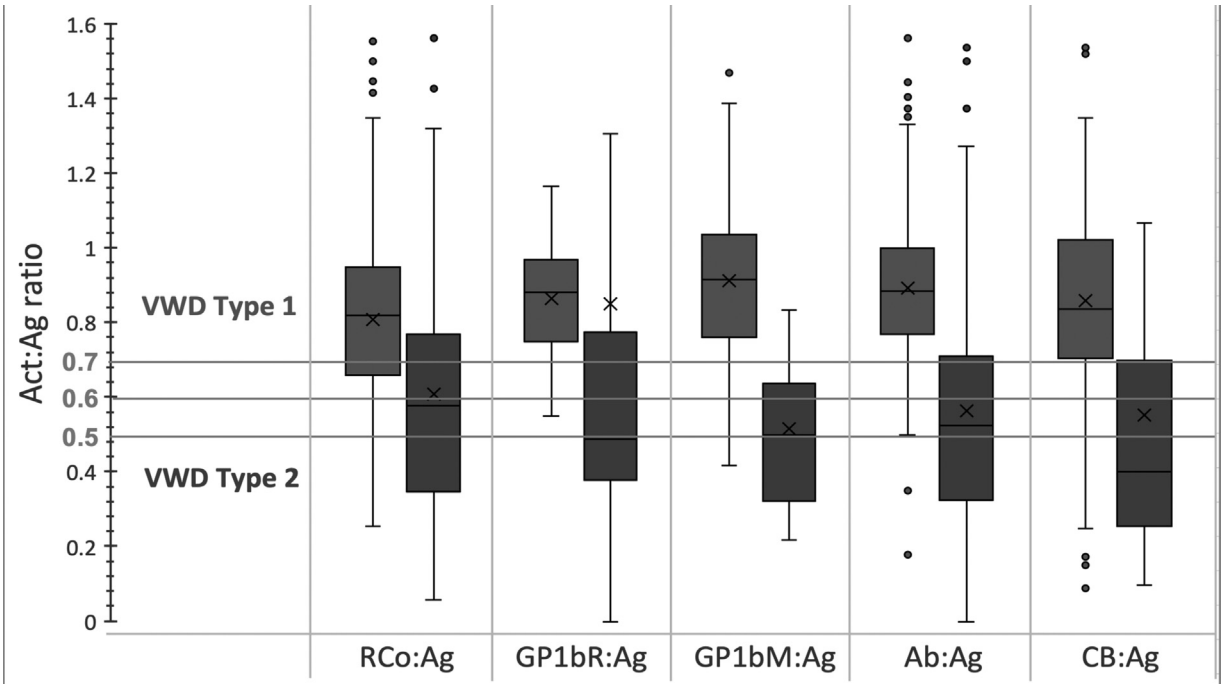

Fig. 5 Change in classification of VWD subtypes based on VWF activity:antigen cutoff ratios of 0.5, 0.6, and 0.7 using NASCOLA EQA data (2010–2021). Ratio results for VWD type 2 may be affected by the case mix of VWD subtypes. These data are compiled from 9 cases presumed to be VWD type 2A/B (based on abnormal multimer results) and 4 cases presumed to be VWD type 2M (based on normal multimer results). Ab:Ag, ratio of von Willebrand factor antibody assay to von Willebrand factor antigen assay; CB:Ag, ratio of von Willebrand factor collagen binding assay to von Willebrand factor antigen assay; GPIbM:Ag, ratio of von Willebrand factor GPIbM assay to von Willebrand factor antigen assay; GPIbR:Ag, ratio of von Willebrand factor GPIbR assay to von Willebrand factor antigen assay; RCo:Ag, ratio of von Willebrand factor ristocetin cofactor assay to von Willebrand factor antigen assay.

versus type 2 at the different cutoff levels in our dataset (►**Fig. 5**). Each box plot represents the range of VWF activity to antigen ratios across all laboratories and all samples for a given VWD type, with the potential cutoffs represented by horizontal lines. It is apparent that the 0.5 cutoff would misclassify approximately half of the type 2 cases for all assays, while the 0.7 cutoff would be far more accurate among the type 2 cases, and only slightly less accurate among the type 1 cases. The misclassifications by the 0.7 cutoff are most frequent with the VWF:RCo assay, which had the lowest result of all the activity assays. Therefore, our data additionally supports the 0.7 cutoff chosen by ASH ISTH NHF WFH. Interestingly, this novel method to visualize the effect of cutoff thresholds across assays was initially employed in a publication by Favaloro.[9] In this article, the author found that no single cutoff value was perfect for all assays, but 0.6 would be the best choice should a universal cutoff be required. It is not clear what causes the difference in our findings, although difference in VWD cases, assay methodology, and study methodology may explain some of the variance. For example, chemiluminescence is an emerging method in the data reported by Favaloro,[8] and tends to have lower detection limits, whereas this method is not available in North America. Also, VWF:GPIbR users were higher in number, and VWF:Ab much lower in number in the published study.[8] Lastly, the ratio calculations by Favaloro included results that were below the assay limit of detection (i.e., a value reported as $<$10 U/dL was taken as 9 U/dL),[25] whereas these values were excluded from ratio calculations in our analysis.

Additional Considerations to Improve Accuracy of Laboratory Testing for VWD

While EQA is of great importance in maintaining quality of practice, accurate laboratory testing for VWD requires consideration and optimization of preanalytical variables. It is well known that since VWF is an acute phase reactant, physiological stress before and during the blood draw can cause spurious "correction" of a mild deficiency and false-negative results.[26] This is a patient-specific factor that requires repeat laboratory testing to get a diagnostic result and is not a variable that is easy for the laboratory to control. However, more importantly, for VWF testing that requires specimens to be transported to a reference laboratory for testing, it is paramount that the temperature of the plasma specimen is always maintained at $-70°$ to $-20°$ along the transport route as a compromise in this cold chain and the resulting freeze–thaw cycles can decrease the measured level of VWF.[27,28] Moreover, this kind of inadequate sample cannot be easily detected and rejected by the receiving laboratory because a freeze–thaw artifact is usually undetectable. A degraded sample from a normal patient is usually indistinguishable from a fresh sample from a VWD patient, potentially leading to many unfortunate diagnostic errors resulting in a spurious diagnosis of type

1 VWD or type 1 getting interpreted as type 2 VWD. While concerns regarding this specific preanalytic error in VWD diagnosis have been discussed for decades, it is not clear whether significant progress has been made. Studies have shown that the preanalytical phase is the most common cause of errors and comprises between 46 and 75% of total errors in coagulation testing.[29,30] A large literature review published in 2002 tracked errors by analytical phase and laboratory department, and concluded by imploring the laboratory community to adopt more rigorous processes for error detection and reduction.[31] Unfortunately, as far as the preanalytical phase of testing for VWD is concerned, recent data suggest that this is still a significant problem. In 2020, Jaffray et al[26] published a large study to examine the effect on laboratory results when specimens were collected off-site and exposed to potential mishandling and degradation when transported long distances to the reference laboratory, as compared with results that were obtained when the same patient was collected on site. In this elegant study design that included 17 institutions and 263 patients, patients for whom a result had been rendered based on a transported specimen were brought in-person to the reference laboratory to repeat the testing. The study found that transported samples had significantly higher rates of abnormal results in all assays that were studied: VWF:Ag (38% abnormal in transported specimen vs. only 22% abnormal in fresh specimen), VWF:RCo (55 vs. 32%), and FVIII:C assay (29 vs. 13%), emphasizing the potential for erroneous diagnoses based on transported samples. The authors conclude that VWF assays should ideally be collected and processed at the same site under the guidance of a hematologist.

Another important issue that can impact diagnostic accuracy is assay harmonization. Assay harmonization refers to calibration between assays so that assays manufactured by different vendors will give the same result. Most assays are not harmonized, and normal reference ranges that are specific to each assay are needed to interpret the results. For harmonization of VWF testing, the World Health Organization 6th International Standard has provided reference standards for VWF:RCo since 2009, and more recently for VWF:GPIbR and VWF:GPIbM.[32] These are designed so that vendors calibrate their instruments to a uniform, traceable standard prior to marketing their test instruments. However, EQA data continue to show significant differences between assays, a fact that is not recognized by treating clinicians or published in expert guidelines. As discussed above, diagnosis of VWD is made based on measurement cutoffs (including ratio cutoffs) that have been recommended by clinical societies without specifying the appropriate laboratory measurement method or limitations of various methods. This may lead to guidelines that are impossible to apply uniformly. To address this, the American Association for Clinical Chemistry recently released a position statement[33] expressing concern that many health care providers are not aware that different measurement procedures may give different results for the same test. They make several recommendations to address this. First, laboratory organizations should provide education to providers and policymakers about the variability in non-harmonized test results and clinical guidelines should clearly identify the laboratory methods to be used. Second, EQA providers should use commutable materials such as true patient samples and accuracy-based acceptable limits whenever possible. Finally, government agencies should provide leadership and funding for harmonization efforts. Until system-based solutions address these issues of inaccurate VWD diagnoses due to preanalytical errors and lack of harmonization, significant advances cannot be made in improving the accuracy of VWD testing.

Conclusion

Although the recent ASH ISTH NHF WFH guidelines have recommended GPIb-binding assays based on their reliability in the face of genetic variants in the ristocetin-binding domain such as D1472H, these assays are not available in many regions in North America, and questions remain regarding the performance of VWF:GPIbR in these variants. In contrast to other geographic regions of the world, laboratories in North America predominantly use three-assay screening panels (antigen, platelet-binding activity, and FVIII activity) rather than four-assay panels (antigen, platelet-binding activity, FVIII activity, and collagen-binding activity). In assessing platelet-binding activity of VWF, VWF:RCo and VWF:Ab assays are the predominant assays in use in North America, rather than VWF:GPIbM and VWF:GPIbR assays recommended by the 2021 ASH ISTH NHF WFH guidelines, and widely available elsewhere. Moreover, LIAs (VWF:Ag and GPIb-based) or ELISA (VWF:CB) are used rather than chemiluminescence immunoassays. Factors that may be influencing these North American practice patterns include lack of FDA approval on VWF:GPIbM, VWF:GPIbR, collagen binding and chemiluminescence methodologies, and the widespread adoption of the 2008 NHLBI guidelines. Lastly, the lack of improvement in the preanalytical phase of VWD testing continues to be a significant problem in VWD diagnosis, as is the lack of assay standardization or harmonization; these can only be addressed by dedicated systems-based solutions.

Conflict of Interest
None declared.

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