The Role of the von Willebrand Factor Collagen-Binding Assay (VWF:CB) in the Diagnosis and Treatment of von Willebrand Disease (VWD) and Way Beyond: A Comprehensive 36-Year History*

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Abstract

The von Willebrand factor (VWF) collagen binding (VWF:CB) assay was first reported for use in von Willebrand diagnostics in 1986, by Brown and Bosak. Since then, the VWF:CB has continued to be used to help diagnose von Willebrand disease (VWD) (*correctly*) and also to help assign the *correct* subtype, as well as to assist in the monitoring of VWD therapy, especially desmopressin (DDAVP). However, it is important to recognize that the specific value of any VWF:CB is predicated on the use of an optimized VWF:CB, and that not all VWF:CB assays are so optimized. There are some good commercial assays available, but there are also some "not-so-good" commercial assays available, and these may continue to give the VWF:CB "a bad reputation." In addition to VWD diagnosis and management, the VWF:CB found purpose in a variety of other applications, from assessing ADAMTS13 activity, to investigation into acquired von Willebrand syndrome (especially as associated with use of mechanical circulatory support or cardiac assist devices), to assessment of VWF activity in disease states in where an excess of high-molecular-weight VWF may accumulate, and lead to increased (micro) thrombosis risk (e.g., coronavirus disease 2019, thrombotic thrombocytopenic purpura). The VWF:CB turns 37 in 2023. This review is a celebration of the utility of the VWF:CB over this nearly 40-year history.

Keywords

- von Willebrand factor
- von Willebrand disease
- VWF:CB
- VWF:RCo
- VWF:GPIbR
- VWF:GPIbM
- VWF:Ag
- diagnosis
- management
- history

von Willebrand disease (VWD) is reportedly the most common inherited bleeding disorder, and can also arise as an acquired bleeding disorder called acquired von Willebrand syndrome (AVWS).[1,2] Both congenital and acquired VWD reflect a deficiency and/or dysfunction in a plasma protein called von Willebrand factor (VWF), which has multiple functions or activities. The main activities of VWF are (1) binding to platelets via the glycoprotein Ib (GPIb) receptor, (2) binding to collagen, and (3) binding to factor VIII (FVIII) and thereby protecting FVIII from degradation and thus preserving FVIII activity.[3,4] VWF exists in multimeric forms,

* *Dedicated to the memories of J. Evan Sadler (1951-2018), Elizabeth (Betsy) van Cott (1964-2021), and William L. Nichols (1940-2022).*

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with increasing molecular mass and increasing "activity" according to multimeric size. That is, the larger multimeric forms of VWF contain relatively larger numbers of binding sites and thus greater overall activity than do smaller multimers. VWD can be characterized or "typed" according to the loss of VWF, or of specific forms of VWF (especially the high-molecular-weight multimer [HMWM] versions), or of specific activities of VWF.[3–5]

There are six main types of VWD, with types 1 and 3, respectively, representing partial or complete loss of VWF (i.e., quantitative deficiencies or quantitative disorders).[5] Type 2 VWD is characterized by the loss of specific forms of VWF (e.g., HMWM), or loss of GPIb binding, and/or loss of collagen binding, or loss of FVIII binding. There are four different type 2 categories, all representing "qualitative" defects, being (1) type 2A (loss of HMWM due either to faulty assembly or increased VWF degradation); (2) type 2B (representing a hyper-adhesive form of VWF that binds too well, thereby leading to spontaneous VWF binding to platelets, and loss of HMWM and platelets [i.e., thrombocytopenia], due to their clearance); (3) type 2N (representing a dysfunction in VWF:FVIII binding, which then leads to increased degradation of FVIII and loss of plasma FVIII activity); and (4) type 2M (which essentially represents a loss of VWF activity not caused by loss of HMWM).

The characterization of patients with VWD into one of these "subtypes" has clinical relevance since different therapeutic/management options may apply according to both type and severity of VWD.[6] In brief, type 1 VWD, especially if only mild (i.e., only a slight loss of VWF), can be treated, at least for a short period, with a nontransfusional form of therapy using desmopressin (DDAVP), which facilitates release of stored reserves of VWF. However, most forms of type 2 VWD will require VWF replacement therapy, since DDAVP will only act to release "abnormal" VWF.

The diagnosis and then characterization of VWD patients is facilitated by laboratory testing, using tests that in some cases assess the activity of the VWF.[3,4] These in vitro "surrogates" try to mimic what occurs in vivo. While all tests are imperfect, some tests are more "imperfect" than others. In the current review, the author attempts to describe these assays in greater detail, but primarily focuses on the VWF collagen-binding assay (VWF:CB), and largely from a historical backdrop, given that this journal is celebrating 50 years of publication, and that the VWF:CB is celebrating nearly 40 years of utility.

Laboratory Tests for VWF Level and Activity

Before embarking on this rich history perspective, it is important to first overview the tests that are used to diagnose and type VWD. The greater the number of such tests that are performed and the better the tests that are performed, the more accurate the diagnosis and characterization of VWD. The most common tests are also summarized in ►**Table 1**.

Factor VIII

First, it is important to determine the FVIII level in any patient being investigated for VWD. The FVIII level is typically assessed using a FVIII activity assay, for which there are two main options—a chromogenic assay and a clot-based "coagulant" assay (FVIII:C).[7,8] The clot-based "coagulant" assay (FVIII:C) is easier to perform, and easier to automate, and thus more often performed than the chromogenic assay, and is also sufficient for investigation of VWD. Essentially, this assay can be performed on any automated hemostasis analyzer enabling clot detection, provided there are reagents available to perform the assay on said analyzer. FVIII:C levels are reported in percent of normal (%) or in units/mL (U/mL) or in units/dL (U/dL), with U/dL and % reflecting identical values. If the assay calibrant is linked to an international standard, the units may be reported in international units (IU). The normal reference range for FVIII:C should be determined for the specific method in use in any given laboratory, but would approximate 50 to 200 U/dL (or %), which is the actual range in use in our laboratory.

von Willebrand Factor "Level"

Second, the plasma level (or "quantity") of VWF should be assessed, with this most typically performed using an immunological assay employing antibodies against VWF to capture and quantify VWF.[3,4,9] Since VWF in these assays reflects the "antigen" against which the antibody was developed and is reactive against, these assays are called VWF antigen (VWF:Ag) assays. These are several methodological options, with Laurel gel rocket (or electroimmunodiffusion; EID) and radioimmunoassay (using radioisotopic detection) representing historical methods, but enzyme-linked immunosorbent assays (ELISA) and latex-immuno assay (LIA) representing the main contemporary options. For readership interest, and since this is a historical article, an example of a Laurel gel rocket VWF:Ag assay performed in our laboratory in the mid-1980s is shown in ►**Fig. 1**. The LIA method can essentially be performed on any automated hemostasis analyzer that is enabled with turbidimetric detection, provided there are reagents available to perform the assay on said instrument. A newer methodology based on chemiluminescence immunoassay ("CLIA") is also available in some locations with certain instrumentation, as performed on an AcuStar instrument (Werfen). Levels of VWF:Ag are reported in the same units as used for FVIII:C. The normal reference range for VWF:Ag should be determined for the specific method in use in any given laboratory, but would again approximate 50 to 200 U/dL (or %), which is the actual range in use in our laboratory.

von Willebrand Factor Glycoprotein Ib Binding Activity

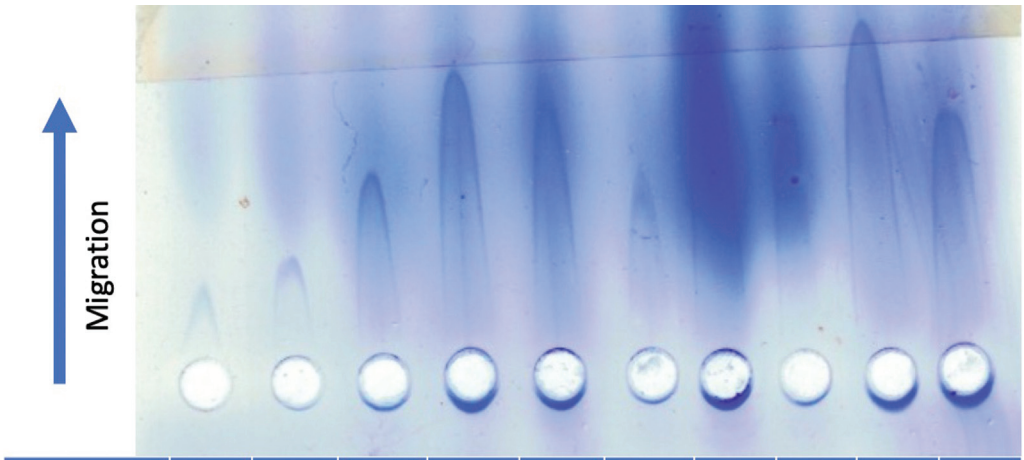
Third, the activity of VWF that measures platelet GPIb binding should be assessed. Historically, this was assessed using an assay called the VWF ristocetin cofactor (VWF:RCo) assay.[10] Ristocetin was originally developed as an antibiotic; although effective as an antibiotic, use in humans identified that ristocetin also caused thrombocytopenia. It was later

Table 1 A summary of tests used to diagnose VWD

Test	Abbreviation	Utility in VWD	Comments
Factor VIII coagulant activity	FVIII:C	VWD is due to loss of VWF or activity. Since VWF is a carrier of FVIII, and protects FVIII from proteolysis, a reduction in VWF or its activity is accompanied by a reduction in FVIII and its activity. FVIII:C is also proportionally low in type 2N VWD, where VWF:FVIII binding is impaired	Essential test for VWD diagnostics
VWF antigen	VWF:Ag	Measure of the level of VWF protein	Essential test for VWD diagnostics
VWF glycoprotein Ib binding activity	VWF:GPIbB	Generic term for an assay able to assess the VWF activity related to binding its platelet receptor, namely, glycoprotein Ib (GPIb). Can be performed in several ways (see below)	Essential test for VWD diagnostics
VWF ristocetin cofactor	VWF:RCo	One of the original VWF:GPIbB assays, measuring ristocetin-aided platelet agglutination	These assays are largely interchangeable; all laboratories should perform at least one of these assays for the diagnosis of VWD
VWF GPIb binding using recombinant GPIb	VWF:GPIbR	A second-generation VWF:GPIbB assay that uses recombinant GPIb coupled to an inert particle (e.g., latex or magnetic beads) to measure ristocetin-aided particle agglutination or binding	
VWF GPIb binding using recombinant mutated GPIb	VWF:GPIbM	A third-generation VWF:GPIbB assay that uses recombinant GPIb with gain-of-function mutation(s) coupled to an inert particle (e.g., latex or plastic well) to measure mutation-aided particle agglutination or binding	
VWF collagen binding activity	VWF:CB	An assay that measures the ability of VWF to bind to collagen (which is a subendothelial matrix protein)	Essential test for VWD diagnostics, especially to correctly identify types 2A, 2B, 2M VWD
VWF factor VIII binding activity	VWF:FVIIIB	An assay that measures the ability of VWF to bind to FVIII	An important assay in VWD diagnostics, especially for correctly identifying type 2N (including discriminating 2N VWD from hemophilia A)
Ristocetin-induced platelet aggregation/ agglutination	RIPA	An agglutination assay performed to identify/ exclude gain-of-function mutations in VWF (i.e., 2B VWD) or GPIb (i.e., platelet type [PT] VWD). Performed as part of platelet function studies using a platelet aggregometer	An important assay in VWD diagnostics, especially for correctly identifying or excluding type 2B or PT-VWD
VWF multimers	VWF:mult	VWF multimers can be separated according to size using agarose gels; with particular agarose gel, the individual bands of multimers of the same size can be visualized	An important assay in VWD diagnostics, especially when laboratories use only a three-test panel. Can often be omitted in laboratories using a four-test panel including an optimized VWF:CB

Abbreviations: GPIb, (platelet) glycoprotein Ib; VWD, von Willebrand disease; VWF, von Willebrand factor.

identified that this was since ristocetin bound to plasma VWF, and caused its "unfolding," which in turn uncovered a normally cryptic epitope on VWF that otherwise bound to platelet GPIb. In vivo, this would cause platelet agglutination and clearance, and thus thrombocytopenia. This adverse in vivo effect of ristocetin led to its eventual use to measure VWF GPIb binding in vitro, as facilitated by ristocetin.[10] The VWF:RCo assay can be performed on fresh platelets washed free of endogenous VWF; however, to enable assays to be used over a longer term, it became usual to either fix the platelets (using formalin) or to freeze-dry (or lyophilize) the platelets. The original VWF:RCo assays were either performed by qualitative visual assessment of platelet clumping or quantified using a platelet aggregometer. Later, the VWF:RCo assays were automated, and can now essentially be performed on several (but not all) automated hemostasis



Lane	1	2	3	4	5	6	7	8	9	10
Sample	PNP	PNP	PNP	PNP	Pat 1	Pat 1	Pat 2	Pat 2	Pat 3	Pat 4
Dilution	1/8	1/4	1/2	undil	undil	1/2	undil	1/2	undil	undil
Height (mm)	8	10.5	18	26	26	18	29	21	30	22
VWF:Ag (%)	12.5	25	50	100	96	58	111	73	116	76
Extrapolated					96	116	111	146	116	76
Average					106		129			

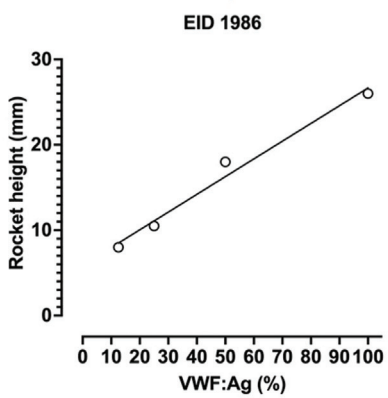


Fig. 1 A Laurel gel rocket (electro-immunodiffusion; EID) assay performed for von Willebrand factor antigen (VWF:Ag) in 1986. Shown at top is the gel, with rockets migrating upward. The level of plasma VWF:Ag is related to the height of the rocket. A calibration curve was hand drawn from the rocket heights of a series of dilutions for pool normal plasma (PNP), and patient values extrapolated from this curve. Since the patient values were unknown, it was usual to perform tests at two dilution points to ensure at least one point on the curve; the results could then be averaged, or in the case where values were very high or very low, then the value fitting within the curve would be taken.

analyzers, provided the right turbidimetric parameters and reagents are available for use on that instrument.[10,11] Several manufacturers can provide reagents to enable VWF:RCo testing, with at least two systems designed to be automated (Siemens and Diagnostica Stago).

Because classical VWF:RCo was associated with poor reproducibility, and/or poor low VWF level sensitivity, several commercial and research alternatives were developed. For example, it is possible to replace the platelets, as used in a classical VWF:RCo assay, with either latex or magnetic beads that have been coated with recombinant GPIb, and these assays are called VWF:GPIbR assays.[3,11,12] These assays still require ristocetin to unfold the plasma VWF and enable its capture by the recombinant GPIb. There is only a single manufacturer of commercial VWF:GPIbR assays by latex (LIA) or by CLIA method, namely, Werfen.

Alternately, latex beads can be coated with mutated recombinant forms of GPIb that reflect gain of VWF function mutations; these mutated forms bind to VWF spontaneously, and thus the assays do not require ristocetin. These assays are

abbreviated as VWF:GPIbM. There is only a single commercially available latex-based (i.e., LIA) VWF:GPIbM assay (Siemens), which is marketed as "VWF Ac."[12] An ELISA version of VWF:GPIbM can also be developed, provided there is access to suitable reagents.[13]

All the GPIb-binding assays measure VWF:GPIb binding (VWF:GPIbB), but they do so differently, with different reagents and detection methods. Thus, it is inevitable that there will be some differences in the VWF:GPIbB that they each detect.[14] Nevertheless, these three assay types (VWF:RCo, VWF:GPIbR, and VWF:GPIbM) are largely interchangeable and yield similar values for the same tested sample in the majority of tested samples, as, for example, can be evidenced in modern external quality assessment (EQA) exercises.[15–17] However, there remain differing opinions around which assays are best for diagnosis/classification of VWD. The latest VWD diagnosis guidelines,[18] for example, recommend the newer VWF:GPIbM and VWF:GPIbR assays over classical VWF:RCo, because they would be expected to show better reproducibility (or lower assay variation) as well as improved detection of low levels of VWF. Moreover, these guidelines also suggest VWF:GPIbM might be preferred over VWF:GPIbR, since use of ristocetin can identify false low VWF:RCo levels in some normal individuals, most notable in African Americans with particular VWF polymorphisms that prevent ristocetin from binding to their VWF.[19] However, these polymorphisms may not be a problem for VWF:GPIb testing in geographic areas in which the polymorphisms are absent or rare,[17] and there exists evidence that certain VWF:GPIbR assays (notably the CLIA method) have better reproducibility and low-level VWF sensitivity detection than VWF:GPIbM.[15–17] Also, commercial availability and regulatory clearance of assays is an important consideration, and not all assays are available in all locations. Finally, some type 2 VWD cases may show VWF dysfunction detectable by VWF:GPIbR and not by VWF:GPIbM (and potentially vice versa),[15–17] so laboratories need to weigh up all these factors to make the best assay choice of VWF:GPIbB assay, as available for their geographic locality, which may also be predicated by what instrumentation they use.

von Willebrand Factor Collagen-Binding Activity

The VWF:CB is an under-appreciated assay that is less widely available than VWF:GPIbB assays. Availability may also be constrained by lack of regulatory clearance. For example, no VWF:CB is regularly cleared or approved for use by the U.S. FDA (Food and Drug Administration), and so very few U.S. laboratories would perform this assay. As previously noted for VWF:GPIbB assays, where several versions are available, the situation for VWF:CB is similar and perhaps even more complex. For VWF:GPIbB, the choices are VWF:RCo (several manufacturers), VWF:GPIbR (one manufacturer, Werfen), and VWF:GPIbM (one manufacturer, Siemens). For VWF:CB, the main choices are either ELISA (several manufacturers or in-house assays) or CLIA (one manufacturer, Werfen).[3,20] For ELISA, the presence of several manufacturers or in-house assays (what U.S. laboratories call laboratory-developed tests [LDTs]), all potentially using different collagen sources (from different animals, from different anatomical locations [e.g., skin, tendon], different collagen extraction methods, different coated collagen concentrations, different plastic plates with different binding properties) makes for very varied assay utility. This is explored in far greater detail later; suffice to say for the moment that different VWF:CB assays can yield great variation between methods, and some users may find the assay problematic, whereas other users will indicate the VWF:CB to be an invaluable tool in VWD diagnostics, provided that you use an "optimized" assay.[21,22]

von Willebrand Factor:Factor VIII Binding Activity

This assay assesses the binding of FVIII to VWF, and is important for diagnosis/exclusion of type 2N VWD.[23,24] There is only one commercial option (Diagnostica Stago). It is possible to use an in-house (or LDT) version of VWF:FVIII binding (VWF:FVIIIB), but like the in-house VWF:CB, it needs to be properly "optimized."[23,24] Very few laboratories perform this assay. As an example for Australia, all laboratories that test for VWD will likely perform a FVIII:C, a VWF:Ag (most typically by LIA, and less so by CLIA), and a VWF:GPIbB, with method depending on preference and available instrumentation (i.e., VWF:RCo or VWF:GPIbR or VWF:GPIbM). Around 50% of Australian laboratories testing for VWD perform VWF:CB, mostly using a variety of ELISA methods, and increasingly by CLIA. But less than 5% of Australian laboratories testing for VWD would perform a VWF:FVIIIB assay.

Ristocetin-Induced Platelet Aggregation/Agglutination

The ristocetin-induced platelet aggregation/agglutination (RIPA) assay is typically performed as part of a platelet function assay using an aggregometer.[25] The assay utilizes ristocetin (like VWF:RCo), but markedly differs from the VWF:RCo assay. In VWF:RCo, a fixed concentration of platelets is mixed with a fixed concentration of ristocetin, and then mixed with a dilution of test plasma, which makes the VWF activity the assay-limiting step, and enables quantitation of VWF:RCo activity. In RIPA, patient platelet-rich plasma is mixed with varying concentrations of ristocetin to determine the sensitivity of the patient's VWF/platelets to ristocetin. Platelets aggregate according to the level of VWF and platelets, and their ristocetin sensitivity. The RIPA is primarily used to assess for the presence of type 2B VWD, reflecting hyper-adhesive VWF, or the presence of platelet type (PT-) VWD (also called pseudo-VWD), which reflects hyper-adhesive GPIb. Thus, RIPA is expected to show best sensitivity to ristocetin (i.e., lead to platelet aggregation at low concentrations of ristocetin) in 2B and PT-VWD. In contrast, in type 2A and severe type 1 VWD, platelet aggregation will occur only with high concentrations of ristocetin. Type 3 VWD, with an absence of VWF, will not show any aggregation to any concentration of ristocetin.[26]

von Willebrand Factor Multimers

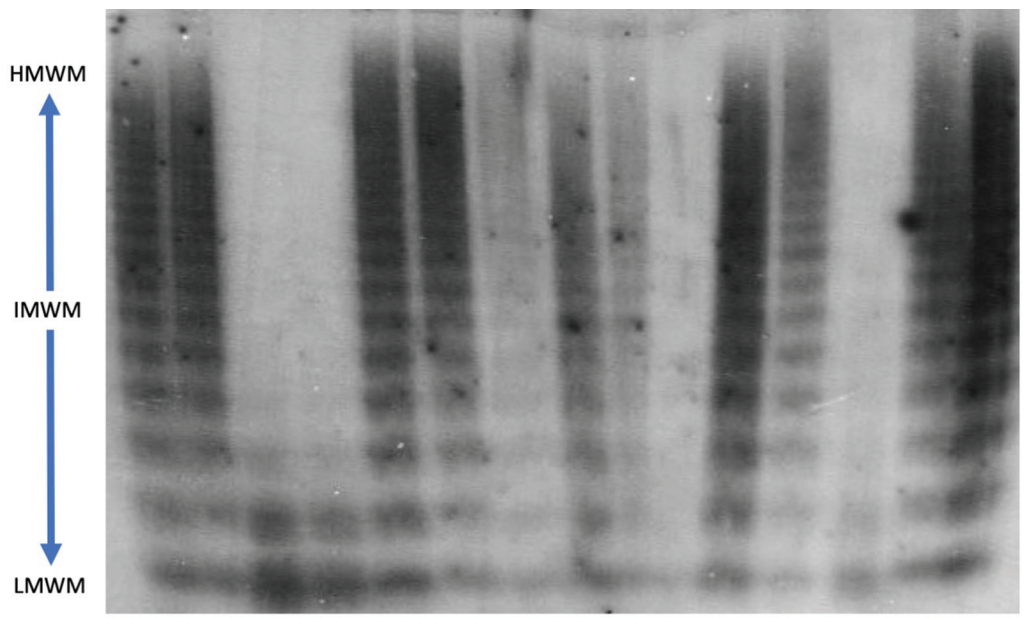
As mentioned, plasma VWF is constructed in vivo as a multimeric protein, which begins with inter-subunit

carboxyl termini disulphide bond formation of "pre-VWF" protein. This initial "tail-to-tail" dimerization is eventually further processed by additional multimerization of pro-VWF dimers that involves another round of disulphide bond formation near the amino-termini of the subunit.[5] In vivo biosynthesis of VWF is limited to endothelial cells and megakaryocytes. After "construction," mature VWF exits in the plasma as a series of oligomers containing a variable number of subunits, ranging from a minimum of 2 to a maximum of 40, with the largest multimers having molecular weights in excess of 20,000 kDa.

As also previously mentioned, the largest multimers have the greatest overall adhesive or functional ability since these contain the greatest number of overall binding sites for GPIb, collagen and FVIII. In my teachings, I often refer to VWF as being like "sticky string." The longer the VWF molecule (i.e., highest number of multimers), the greater the adhesion of this VWF to platelets (i.e., GPIb) and to damaged sub-endothelium (i.e., collagen), thereby the longest "sticky string" being able to arrest platelets at the site of injury and lead to more stable thrombus formation.

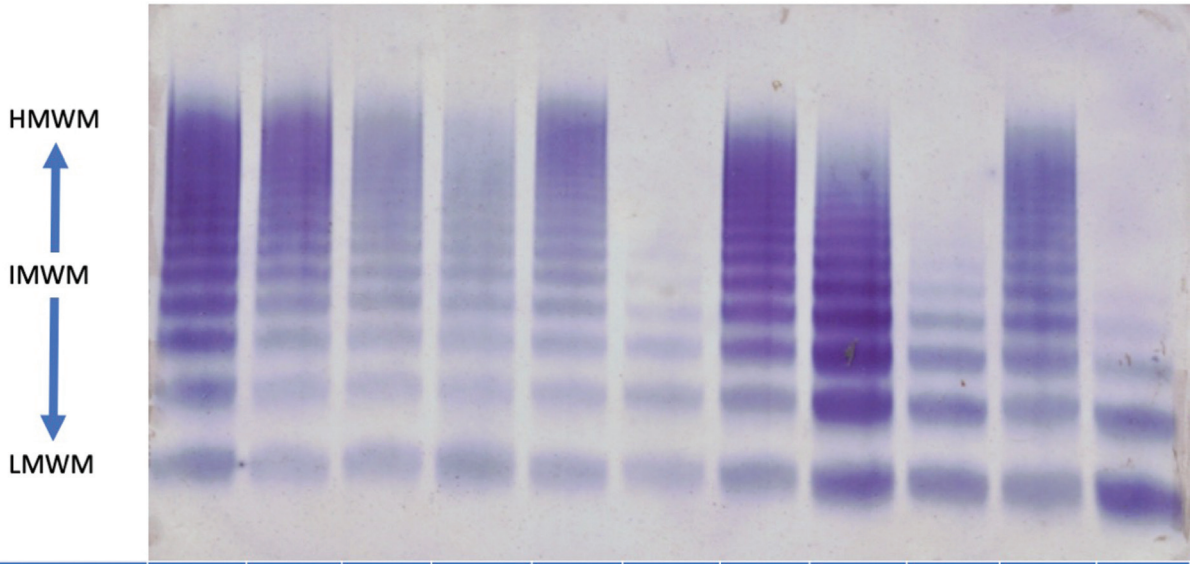
It is possible to visualize the multimeric pattern of plasma VWF by performing VWF multimer analysis. In the past, this used to be done using radio-labeled VWF and gel electrophoresis with X-ray visualization (►**Fig. 2**). These assays used to take upward of 2 to 4 days to perform for a total of 15 or so lanes, which meant 13 patient samples after inclusion of normal and type HMW-deficient 2A/2B VWD controls. The other problem with VWF multimers is that most laboratories are required to perform in-house assays (i.e., LDTs), and there is considerable variability in performance of these between laboratories. The outcome is that these assays suffer from poor accuracy, as has been highlighted in several reports. In one study, from the North American Specialized Coagulation Laboratory Association (NASCOLA), 5% (4/81) of participants incorrectly reported loss of HMWM in normal samples, 18% (14/80) incorrectly reported loss of HMWM in type 1 VWD samples, and 22% (8/36) incorrectly reported a normal multimer pattern in type 2 VWD samples; overall, 14.7% of VWF multimer survey responses were in error.[27] In another EQA report, from the European Concerted Action on Thrombosis and Disabilities Foundation (later to become ECAT, for external quality control for assays and tests), VWF multimer testing was associated with 10 to 23% error rates for normal samples, a 33% error rate for abnormal samples, and a 35 to 52% error rate for type 1 VWD.[28]

The situation has potentially improved in 2023, since a semiautomated VWF multimer assay method is now available.[29–32] Using the 11-lane system, nine patient samples and two controls can be run over a single day with approximately 4 hours of hands on time. The system can clearly show the loss or retention of HMWM and also intermediate MWM (IMWM), and thus is sufficiently powered to help correctly characterize the majority of VWD cases (►**Fig. 3**), for



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sample	PNP	Nor	2A	2A	Nor	Nor	T1	Nor	T1	T1 sev	Nor	Nor	2A	Nor	Nor

Fig. 2 An example of von Willebrand factor (VWF) multimer analysis using radio-isotopic visualization of multimers (from 1991). Shown is the multimers for a 15-lane gel, with interpretation shown in the table. A pool normal plasma (PNP) and a type 2A von Willebrand disease sample would be used as controls, and then the interpretation based on the multimer patterns. Normal samples would show retention of all multimers with intensity of bands close to the normal control. Type 1 VWD (T1) would be represented by retention of all multimers with intensity of bands significantly less than the normal control. Type 2 VWD samples would show loss of high-molecular-weight multimers (HMWM) and usually also intermediate-molecular-weight multimers (IMWM), with retention of only low-molecular-weight multimers (LMWM). Lanes 2, 5, 6, 8, 11, 12, 14, and 15 were interpreted as normal (Nor); lanes 7, 9, and 10 as type 1 (T1), with lane 10 sample also identified as severe (sev); finally, lanes 3, 4, and 13 show loss of IMWM and HMWM, and thus type 2A VWD.



Lane	1	2	3	4	5	6	7	8	9	10	11
Sample	PNP	T1	2M	T1	T1	T2A	Nor	PT-VWD	T2B	Nor	2A
VWF:Ag	~100	39	40	30	57	12	93	115	30	115	38
VWF:GPIbR	~100	35	18	31	50	5	97	59	6	94	12
VWF:CB	~100	49	40	25	41	3	76	39	5	99	11
GPIbR/Ag	~1.0	0.90	0.45	1.03	0.88	0.42	1.04	0.51	0.20	0.82	0.32
CB/Ag	~1.0	1.26	1.00	0.83	0.72	0.25	0.82	0.34	0.17	0.86	0.29

Fig. 3 An example of modern VWF multimer analysis using the Sebia system. Shown are results for an 11-lane gel, with controls run in lane 1 (Dade Standard Human Plasma [pool normal plasma]) with VWF results all close to 100 U/dL and assay ratios all close to 1.0) and in lane 11 (a type 2A VWD with loss of high- and intermediate-molecular-weight multimers (HMWM, IMWM). Lanes 2 and 4 show two type 1 VWD samples with retention of all multimers, and slight loss of VWF, but normal assay ratios. Lane 5 shows a borderline normal/low VWF sample, again with retention of all multimers and normal assay ratios. Lane 3 shows a type 2M VWD sample, with reduced VWF:GPIbR/Ag ratio, reflecting a loss of VWF:GPIb binding, but without loss of HMWM or IMWM (and normal VWF:CB/Ag ratio). Lanes 6 and 9, respectively, show a type 2A and 2B VWD sample, with loss of HMWM and IMWM. Lane 8 shows a platelet-type (PT-) VWD sample, with normal level of VWF:Ag, but low VWF:GPIbR /Ag and VWF:Ag/CB ratio reflective of the loss of HMWM. Red font numbers represent abnormal values for that test or parameter. LMWM, low-molecular-weight multimers.

example, distinguishing samples with loss of HMWM (and potentially IMWM) (being type 2A, 2B, or PT-VWD), from samples without loss of HMWM/IMWM (being type 1, 2M, 2N VWD, or normal samples), from samples without VWF (i.e., type 3 VWD). Because the system uses a fixed agarose gel concentration, it cannot distinguish the triplet structure of individual multimers, or the individual HMWMs; so, for fine detail VWF work, in-house methods would still be required.

►**Table 1** provides a summary of the assays used to diagnose/characterize VWD.

Use of Assay Ratios in Diagnosis/Characterization of von Willebrand Disease

The different assays previously discussed identify different aspects of VWF. The VWF:Ag assay is a primary assay that identifies the level of VWF, but detects both active and inactive (or nonfunctional) VWF. The VWF:RCo, and modern alternatives of VWF:GPIbR and VWF:GPIbM, identifies platelet GPIb binding, an important activity of VWF, and one which enables platelets to be clumped together and also immobilized at sites of vascular injury. These VWF:GPIbB assays are also constructed to be sensitive to the presence or absence of HMWM and IMWM forms of VWF, which accordingly express high or intermediate VWF:GPIbB activity. The assays are also sensitive, albeit somewhat variably, to VWF mutations affecting GPIb binding. Thus, one can calculate a ratio of VWF:GPIbB/Ag to determine the relative VWF:GPIbB activity, which will be normal when VWF:GPIbB is maintained, but which will be reduced when VWF:GPIbB activity (or HMWM) is reduced. In other words, VWF:GPIbB/Ag is normal in normal individuals and in patients with type 1 VWD, where VWF may be low but the VWF:GPIbB activity of the existing VWF is preserved and VWF:GPIbB/Ag will be low in patients with type 2A or 2B or PT-VWD, since HMWM VWF is reduced (2A, 2B, PT-VWD) and/or VWF:GPIbB activity is impaired (2A VWD). Most forms of 2M VWD also show reduced VWF:GPIbB activity, and thus VWF:GPIbB/Ag is also reduced in the majority (but not necessarily all) of type 2M VWD cases.

Similarly, VWF:CB assays also detect an activity of VWF, namely, collagen binding, and these assays can also be constructed to be sensitive to the presence or absence of

HMWM and IMWM forms of VWF, which accordingly express high or intermediate VWF:CB activity. The assays can also be made sensitive to VWF mutations affecting collagen binding. Thus, one can calculate a ratio of VWF:CB/Ag to determine the relative VWF:CB activity, which will be normal when VWF:CB is preserved, but which will be reduced when VWF:CB activity (or HMWM) is reduced. In other words, VWF:CB/Ag is normal in normal individuals and in patients with type 1 VWD, where VWF may be low but the VWF:CB activity of the existing VWF is preserved, and VWF:CB/Ag is low in patients with type 2A or 2B or PT-VWD, since HMWM VWF is reduced (2A, 2B, PT-VWD) and/or in patients in whom VWF:CB activity is impaired (2A VWD). Some forms of 2M VWD also show reduced VWF:CB activity, and thus VWF:CB/Ag is also reduced in some (but not most) type 2M VWD cases.

Finally, FVIII:C is low in most patients with VWD since VWF would normally act to stabilize and protect FVIII from degradation. The plasma level of FVIII:C therefore largely aligns to the plasma level of VWF, and as VWF reduces, so does FVIII:C. However, the level of FVIII:C is relatively lowered in type 2N VWD, since VWF no longer retains the ability to bind to FVIII and thus it is degraded. Remember, however, that a specific loss of FVIII will also occur in hemophilia A. In any case, one can calculate a ratio of FVIII:C/VWF:Ag to determine the relative FVIII:C activity, which will be normal when FVIII:C is maintained, but which will be reduced when FVIII:C activity is reduced. In other words, FVIII:C/VWF:Ag is normal in normal individuals and in patients with types 1, 2A, 2B, 2M, and PT-VWD, but FVIII:C/VWF:Ag will be low in patients with type 2N VWD or with hemophilia A.

►**Table 2** provides a summary of the utility of assay ratios used to help diagnose/characterize VWD. Indeed, the differential findings of these three activity/VWF:Ag ratios often provide clues to type of VWD present. If all three ratios are normal, and VWF levels are normal, then VWD is largely excluded. If both VWF:GPIbB/Ag (i.e., VWF:RCo/Ag, VWF:GPIb/Ag, or VWF:GPIbM/Ag) and VWF:CB/Ag are low, this usually means AVWS or type 2A or 2B VWD (however, further testing would be required to clarify which, or whether instead 2M or PT-VWD, are present; this would usually mean multimer analysis and/or RIPA testing). If only one of VWF:GPIbB/Ag and VWF:CB/Ag are low, this argues against a loss of HMWM, and thus against a diagnosis of 2A, 2B, or PT-VWD, and instead would suggest a 2M VWD. If FVIII:C/VWF:Ag is low, this would suggest either hemophilia A or 2N VWD; note, however, that FVIII:C is very heat and storage labile; so, false low FVIII:C can occur as a preanalytical event. If the low FVIII:C/VWF:Ag can be confirmed on a repeat test using a fresh sample, then hemophilia A and 2N VWD would need to be differentially diagnosed (using a VWF:FVIIIB, or genetic testing of the *F8* and *VWF* genes, respectively).

In terms of assay ratio cutoffs, used to discriminate type 1 (quantitative) from type 2A/2B (HMWM deficient), and namely VWF:GPIbB/Ag (i.e., VWF:RCo/Ag, VWF:GPIbR/Ag, VWF:GPIbM/Ag) and VWF:CB/Ag, these may range from 0.5 to 0.7 depending on the assays used.[14–17] The latest VWD diagnostic guidelines suggest using 0.7 in preference to 0.5 to not miss type 2 VWD; however, this will also falsely capture more type 1 VWD cases. In our laboratory, we tend to use 0.6, which works well with the CLIA methods we now use.[15–17]

Why Is the von Willebrand Factor Collagen-Binding Assay Important?

As noted earlier, both VWF:GPIbB and VWF:CB assays show some similar features. Both assay types, if properly constructed, will show sensitivity to the presence/absence of

Table 2 A summary of the utility of assay ratios used to diagnose von Willebrand disease (VWD)[a]

Assay ratio	Normal in	Low in	Comment
VWF:GPIbB/Ag (i.e., VWF:RCo/Ag, VWF:GPIbR/Ag, VWF:GPIbM/Ag)	Normal individuals (i.e., absence of VWD) Type 1 VWD	Type 2A and 2B VWD Most cases of 2M VWD	Can differentiate 2B and PT-VWD using RIPA and RIPA mixing. Can differentiate 2A/2B VWD (loss of HMWM) from 2M using VWF multimer analysis
VWF:CB/Ag	Normal individuals (i.e., absence of VWD) Type 1 VWD Most cases of 2M VWD (ELISA-based VWF:CB)	Type 2A and 2B VWD Some cases of 2M VWD (ELISA-based VWF:CB) Most cases of 2M VWD (CLIA-based VWF:CB)	
FVIII:C/VWF:Ag	Normal individuals (i.e., absence of VWD) Type 1 VWD Most cases of type 2 VWD (i.e., 2A, 2B, 2M)	Type 2N VWD Hemophilia A	Can differentiate hemophilia A and 2N VWD using a VWF:FVIIIB, or genetic testing of the *F8* and *VWF* genes, respectively

[a]The cutoff value to identify "normal" vs. "low" assay ratios depends on the assays and their methodologies. The latest VWD diagnosis guidelines[18] recommend using a cutoff of 0.7 for VWF:GPIbB/Ag (i.e., VWF:RCo/Ag, VWF:GPIbR/Ag, VWF:GPIbM/Ag) and VWF:CB/Ag ratios, ahead of a cutoff of 0.5, to not miss any type 2 VWD cases. Of course, the higher cutoff will mean that more patients with type 1 VWD will be falsely captured as "possible type 2." We tend to use a cutoff of 0.6 for VWF:GPIbB/Ag (i.e., VWF:RCo/Ag, VWF:GPIbR/Ag, VWF:GPIbM/Ag) and VWF:CB/Ag ratios, based on available data,[15–17,249] and noting this ratio was also recommended by the United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology.[324] For the FVIII:C/VWF:Ag, we tend to use 0.7 as the cutoff.

HMWM/IMWM. Also, both assay types, if properly constructed, will show sensitivity to the presence/absence of loss of VWF activity in 2A VWD. Thus, the question may arise as to why not just use one of these assay types; why do we need to use both? The best way to address this question is to consider the assay differences, and also the VWD diagnostic error rate when laboratories use a three-test panel (FVIII:C, VWF:Ag, VWF:GPIbB [i.e., VWF:RCo or VWF:GPIbR or VWF:GPIbM]) versus a four-test panel (FVIII:C, VWF:Ag, VWF:GPIbB, VWF:CB). Experience has shown that the VWD diagnostic error rate can be halved by using a four-test panel. In one early study,[33] the overall VWD diagnostic error rate among participants of the RCPAQAP (Royal College of Pathologists of Australasia Quality Assurance Program) was approximately 10%. That is, approximately 10% of all "VWD diagnostic" attempts were in error, with type 1 VWD misidentified as type 2 VWD (or vice versa), or VWD missed, etc. The VWF:GPIbB assays (then mostly VWF:RCo) were found to be the most problematic, and accounted for the majority of errors. So, adding the VWF:CB to the VWD test panel helps overcome some of the limitations associated with poor performance of VWF:GPIbB assays. In addition, VWF:GPIbB and VWF:GPIbB/Ag will be low in types 2A/2B/2M/PT-VWD, and the use of the VWF:CB can better characterize patients with 2M VWD, and help avoid their common misdiagnosis as type 2A VWD.[26,34] In one of the latest RCPAQAP reports, three test panels were associated with an overall error rate of 8.3%, whereas four test panels were associated with an overall error rate of 4.9%.[15] For type 2 HMWM VWF-deficient samples (viz, 2A, 2B VWD), the relative error rates were 16.3% (three-test panel) versus 7.3% (four-test panel).

In regard to 2M VWD, we have already well identified that use of insufficient panels comprising only one of VWF:GPIbB and VWF:CB will yield high diagnostic error rates. In one such report,[34] the RCPAQAP sent out two (duplicate) samples from two different type 2M VWD cases over a period of several years. For one 2M VWD case with ~50 U/dL of VWF:Ag, there were a total of 92 interpretative events received from participants, with near equal numbers of events for those performing both types of tests (i.e., VWF:GPIbB plus VWF:CB; $n = 45$) versus those performing only one type of test (i.e., VWF:GPIbB or VWF:CB; $n = 47$). Of these, 54.8% of those performing both types of tests correctly identified 2M VWD, compared with only 8.9% of those performing only one type of test. For the 2M VWD case with ~190 U/dL of VWF:Ag, there were a total of 88 interpretative events, again with near equal numbers of events for those performing both types of tests ($n = 45$) versus those performing only one type of test ($n = 43$). Of these, 66.8% of those performing both types of tests identified 2M VWD, compared with only 25.6% of those performing only one type of test. Since the majority of world-wide laboratories perform only a three-test panel for initial investigation of VWD, now unexpectedly supported by the new VWD guidelines,[18] the majority of world-wide laboratories will never be able to correctly diagnose 2M VWD, and indeed, this explains why 2M VWD is reportedly as common as 2A VWD in laboratories that perform four test panels (such as ours),[2] but may otherwise be considered a rare form of VWD when laboratories perform only a three-test panel (where most 2M VWD cases will be incorrectly identified as 2A VWD, or VWD will be incorrectly discounted).[34]

A History of the von Willebrand Factor Collagen-Binding Assay

In this section, I will provide a linear chronology of the use of the VWF:CB by various workers over the assay's near 40-year life time. The main portion of the content was identified using a PubMed search of "von Willebrand factor" "collagen binding" assay, with citations downloaded according to publication date (earliest to latest). The search was updated on January 9, 2023, to ensure the capture date was complete until the end of 2022. ►**Fig. 4** shows the timeline of captured citations per year. Although the first citation was for a 1984 study, this described collagen binding in a research study, and the first description of a collagen-binding assay for diagnosis of VWD was in 1986.[35] I apologize for the length of this section, but I think it is useful to document this chronology for the STH history series. Readers may opt to omit this section entirely and go directly to the "Discussion" section if they wish to just read a summary of the chronology and the main utility of the VWF:CB.

Early History—The 1990s

To my knowledge, the VWF:CB was originally reported by Brown and Bosak, in 1986, now some 36 years ago.[35] The VWF:CB was then coevaluated with the VWF:RCo assay, regarding its ability to both detect VWD and identify and discriminate potential type 2 VWD subtypes. Although this study was a relative landmark, being the first description of the potential utility of the VWF:CB in VWD, the data reported, and the conclusions raised, did little to endorse the continued use of the VWF:CB in VWD diagnostics. The study comprised a very small group of VWD patients and normal individuals, and used only a single source of collagen to coat ELISA plates. The comparative data between the VWF:CB and VWF:RCo were not strikingly dissimilar, and in hindsight, the VWF:CB utilized was not really optimized for discrimination of HMWM VWF. Indeed, the authors saw the VWF:CB as a potential replacement to the "problematic" and time-consuming VWF:RCo, rather than as a supplementary assay. The VWF:CB story might well have ended there, except that several other workers decided to more extensively test the utility of this novel assay. I was one of these workers, and was certainly tempted even by the possibility that an ELISA-based VWF:CB could potentially replace our laborious and highly variable platelet aggregometer-based VWF:RCo assay. We published our first study on the VWF:CB in 1991.[36] We tested several different collagen preparations, but finally settled on one particular preparation that contained a mixture of type I/III collagen. We also tested various concentrations of collagen for coating the ELISA plates. We assessed a panel of 42 normal individual samples, and an additional 39 samples from patients identified not to have VWD, and 53 samples from patients with VWD (37 type 1, and 16 type 2A/2B). Of interest, we

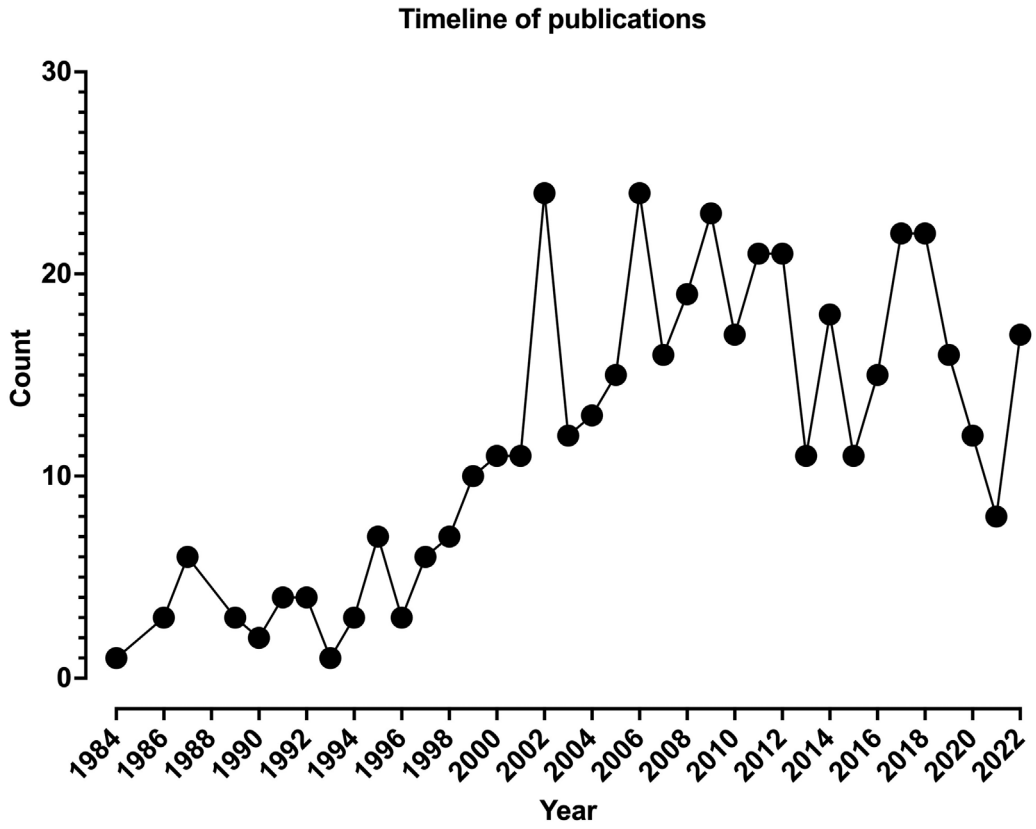


Fig. 4 Timeline of publications mentioning ("von Willebrand factor" "collagen binding" assay) in PubMed. This search term will not capture all publications reporting on the von Willebrand factor collagen-binding (VWF:CB) assay, as used for diagnosis of von Willebrand disease (VWD), being restricted to the term appearing in the abstract or title. Moreover, only around 50% of these publications actually refer to the VWF:CB, as used for diagnosis of VWD; some alternatively refer to collagen binding in research studies evaluating the function of VWF, or platelets. The first paper referring to a VWF:CB for use in VWD was in 1986 by Brown and Bosak. As can be seen, the VWF:CB was reported only in a small number of papers in the 1990s, with increasing use reported over subsequent decades.

identified that in our hands, the VWF:CB was much more sensitive to loss of HMWM VWF in type 2 VWD than was our VWF:RCo. This alone would have proved its worth for replacement of VWF:RCo (or inclusion in a wider test panel), since the VWF:RCo/Ag ratio was occasionally normal in type 2 VWD (i.e., yielded false functional concordance), which might lead to false misdiagnosis of type 2 as type 1 VWD, something we also consistently see in RCPAQAP surveys, even today.[15–17,33] Of course, the VWF:CB was to prove even more useful over time.

Naturally, there were many others who started to report on "collagen-binding activity assays" in those early years. For example, Perret and colleagues attempted to create a VWF:RCo-like assay using collagen.[37] They found that insoluble collagen from bovine aorta was able to agglutinate washed fixed platelets in the presence of VWF, similarly with the VWF:RCo assay. Their "VWF-collagen cofactor" activity assay showed good correlation ($r = 0.91$) with their VWF:RCo assay using 65 plasma samples containing various concentrations of VWF. It is not surprising that this assay never took off, since in essence it was similar to VWF:RCo, and just as complex and time consuming. In another early VWF "collagen-binding assay" exploration, collagen-mediated VWF absorption was investigated.[38] In this assay, plasma samples were incubated with aliquots of a collagen suspension, and following ultracentrifugation, values for (residual) VWF:Ag were assessed in the supernatant using a standard VWF:Ag ELISA, and compared with non–collagen-incubated samples. This was a time-consuming assay requiring ultracentrifugation, and thus similarly did not take off.

In terms of those who worked up versions of VWF:CB by ELISA, a smattering of individuals were developing variations of VWF:CB ELISA, or were using VWF:CB to characterize VWD or VWF or VWF concentrate, or exploring the VWF:CB in various disease states. For example, in 1989, Lawrie et al[39] used a VWF:CB as part of a panel of tests (also including VWF:Ag, VWF:RCo, and VWF multimers) to characterize "FVIII concentrates," then used for hemophilia A treatment, for potential use as VWD treatment (note that some of these "FVIII concentrates" were in fact also VWF concentrates). They found a significant correlation between VWF:RCo and VWF:CB for most concentrates, and based on VWF activity/Ag ratios, determined those concentrates most likely to be useful for symptomatic treatment of severe VWD patients. In 1994, Niesvizky et al[40] used a VWF:CB employing type III collagen to investigate 20 patients with end-stage renal disease. They also assessed VWF:Ag and VWF:RCo. They found increased levels of VWF:Ag in their patients, before and after dialysis. For VWF:RCo, values were elevated in patients who had not undergone dialysis, but values were

similar to control values in patients undergoing dialysis. Interestingly, VWF:CB values were similar to controls in patients who had not undergone dialysis. Of course, a relevant question in relation to this and all other studies using the VWF:CB is "was this an optimized VWF:CB"?

The next relevant study was from our laboratory,[41] and evaluated the potential utility of the VWF:CB in monitoring of desmopressin (DDAVP) therapy. We were then still using the same assay we described in 1991.[36] We evaluated the response of seven patients with VWD (four type 1, three type 2A) to DDAVP, administered using a standard protocol, and assessed levels of FVIII:C, FVIII antigen, VWF:Ag, VWF:RCo, VWF:CB, and VWF multimers, as well as performing skin bleeding times (SBT) prior to, and at sequential time points following, DDAVP administration. All patients showed an initial incremental increase in VWF and FVIII levels using all assays, and some showed some correction in SBT. Although the absolute levels of VWF and FVIII varied between patients, the VWF:CB was found to provide consistently the greatest proportional incremental increases (i.e., x-fold) compared with baseline (pre-DDAVP) levels. Accordingly, we consistently observed an increase in the VWF:CB/Ag ratio for all patients evaluated. This suggested that our specific VWF:CB bound preferentially to higher molecular weight (i.e., more functionally active) forms of VWF than did our VWF:RCo, and we therefore proposed that the use of the VWF:CB (and VWF:CB/Ag ratio) might provide a basis for more accurate estimation of a patient's functional responsiveness to DDAVP therapy in future studies.

Also in 1994,[42] van Genderen et al described an interesting case of an AVWS in a patient with non-Hodgkin lymphoma with a selective inhibitor directed against collagen, and as detected using their VWF:CB and mixing studies. There was no apparent inhibitor detected by their VWF:RCo assay. This pointed to the fact that the assays were identifying different VWF activities, and also pointed to the utility of the VWF:CB in supplement to the VWF:RCo.

In 1995, we used the VWF:CB to investigate patients with neurofibromatosis type 1.[43] In response to 1 and 2 µg/mL collagen, these patients expressed an attenuated rate of aggregation, aggregation lag phase and ATP release, as well as requiring higher collagen concentrations to attain threshold aggregation response. VWF:CB values were also reduced in these patients compared with controls. Thus, as a group, patients with neurofibromatosis type 1 displayed defective platelet function characterized by in vitro evidence of impaired responsiveness to collagen. Also in 1995,[44] Thomas et al used a VWF:CB to identify increased VWF activity in newborns and infants. They also reported elevated levels of VWF:Ag, but VWF:CB values were higher. They thus suggested caution be exercised when interpreting laboratory data and diagnosing VWD in newborns and young infants, which warranted the use of age-specific reference ranges. They also proposed that efficient hemostasis observed during early neonatal life may in part be due to the increased ability of VWF to interact with collagen.

In 1996,[45] van Genderen et al used the VWF:CB and VWF:RCo to investigate the relationship between platelet count and large VWF multimers in the plasma of 36 patients with essential thrombocythemia (ET) and 26 patients with reactive thrombocytosis (RT). In both ET and RT patients, an inverse relationship could be established between platelet count and large VWF multimers in plasma as well in relatively decreased VWF:RCo/Ag and VWF:CB/Ag ratios. A normalization of the platelet count was accompanied by restoration of a normal plasma VWF multimeric distribution. They concluded that these data suggested that increasing numbers of platelets circulating in blood result in increased removal of large VWF multimers from plasma. Also in 1996,[46] Fischer et al used the VWF:CB to investigate plasma-derived and recombinant VWF. Heparin affinity chromatography was used to isolate VWF polymers with different degrees of multimerization. Analysis of VWF:CB and platelet aggregation revealed that these activities increased with increasing degree of VWF multimerization. Thus, the relationship of the VWF:CB to preferentially detect HMWM was confirmed.

In 1997, Zieger et al[47] included a VWF:CB to characterize a new variant of type 2M VWD with supranormal VWF multimers in plasma similar to those seen in normal plasma after desmopressin infusion. Clinically, the patients presented with bleeding symptoms and expressed reduced laboratory values for VWF:Ag, FVII:C, VWF:RCo, and VWF:CB. Also in 1997, Chang and Aronson[48] investigated the VWF activity of various plasma-derived VWF preparations using VWF:CB and VWF:RCo, in addition to VWF:Ag. They found that some preparations had higher VWF:RCo/Ag and VWF:CB/Ag ratios than that found in normal plasma. VWF:RCo and VWF:CB activities were tightly correlated ($r = 0.95$). Ultracentrifugal analysis was used to compare the size distribution of VWF:Ag, VWF:RCo, and VWF:CB. The VWF:CB/Ag ratio decreased with decreasing VWF size. They concluded that assignment of potency to VWF-containing preparations utilizing the VWF:CB might be more precise and as accurate as with the traditional VWF:RCo assay. Also in 1997, van Genderen and colleagues[49] extended on their earlier 1996 study[45] to investigate whether the decrease in large VWF multimers in plasma with increasing platelet counts was the consequence of increased turnover of large VWF multimers in vivo. They measured the half-life times of endogenously released VWF:Ag and VWF:CB following DDAVP in nine ET patients and nine control subjects. Also, the half-life times of VWF:Ag and VWF:CB were measured in four ET patients after cytoreduction of the increased platelet count to normal or nearly normal values. Estimated half-life times of VWF:Ag did not differ between ET patients and normal, but estimated half-life times of VWF:CB were significantly lower in ET patients as compared with normal individuals. After cytoreduction of the increased platelet count to (nearly) normal values in all four ET patients, the half-life time of VWF:CB increased significantly. They concluded that their data suggested that platelets might play a role in the homeostasis of circulating VWF. Finally for 1997, the year recorded my first review on VWD and VWF assays in its diagnosis.[50] This review was written from the perspective of the relative contribution of different assays to the diagnosis of VWD.

The review also attempted to clarify some of the issues that led to confusion around the relative roles of different assays used to diagnose VWD. It also tried to put some perspective into the potential relative contribution of VWF:CB versus VWF:RCo.

In 1998, Mohri et al[51] described another interesting case of AVWS, this one in myeloma, with an autoantibody inhibiting VWF activity, this time against both VWF:GPIbB (using VWF:RCo) and VWF:CB, using type I collagen. Also in 1998, Siekmann et al[52] investigated the use of various collagens for VWF:CB testing, and found optimal results using a pepsin-digested type III collagen from human placenta covalently immobilized on a microtiter plate. Their VWF:CB data corresponded to the degree of VWF multimerization and proposed their assay would be useful for both clinical diagnosis and for the measurement of VWF functional activity in factor concentrates; moreover, in certain applications, the VWF:CB might also represent a suitable replacement for VWF:RCo. This study confirmed the observation we had made earlier that not all VWF:CB assays are the same.[36] In the same year, Fischer et al[53] also described an assay using type III collagen immobilized covalently on ELISA plates. Assay plates were simple to prepare and remained stable at 4 and −20 °C for at least 2 months. Sample testing confirmed proportionally low levels of VWF:CB/Ag in samples lacking HMWM, while higher VWF:CB/Ag values were obtained for samples containing these multimers. Furthermore, the VWF:CB/Ag ratio sensitively reflected the functional and structural intactness of the VWF molecules for all analyzed samples. Monoclonal antibody directed to the region within the A1 domain of VWF which interacts with the glycoprotein Ib completely inhibited VWF:RCo, while VWF:CB was not affected. Thus, VWF:CB and VWF:RCo clearly represented separate, noninterchangeable functional parameters of VWF. The authors concluded that their newly described method for the immobilization of collagen onto microtiter plates was suitable for the determination of VWF:CB, and that VWF:CB/Ag ratio would simplify the detection and classification of patients with VWD and assist in quality control during the purification of normal VWF. The exploration of the potential utility of the VWF:CB for measuring VWF activity in VWF concentrates continued in 1998, with the study from Ramasamy et al,[54] who flagged the recent recommendation by the European Pharmacopoeia for the characterization of factor VIII/VWF concentrates to utilize the VWF:CB. They optimized their VWF:CB to decrease reagent variability and to allow for interlaboratory comparison. A study of clinical samples of patients with VWD was performed to establish that a ratio of VWF:Ag/CB antigen ratio >3.7 was associated with loss of HMWM and a decrease in biological activity, whereas a ratio <1.4 was associated with normal multimeric distribution. The VWF:CB was also used to monitor changes in biological activity of VWF during the manufacture of concentrates. They used the opposite ratio to VWF:CB/Ag, as used by most other workers at that time. Also in 1998, Fischer et al[55] added to their earlier study[53] to compare findings with various VWF activity assays and using VWF purified from normal human plasma and then separated into three fractions containing high, medium, and low-molecular-weight VWF multimers. The VWF fractions were tested for VWF:Ag, VWF:RCo, VWF:CB, and a monoclonal antibody-binding ELISA (mAB-binding ELISA), based on VWF binding to an immobilized monoclonal antibody directed to the GPIb-binding region within the A1 domain of VWF. They reported that the three different fractions of VWF showed a correlation between multimer size and VWF:RCo/Ag and VWF:CB/Ag, but that results obtained with the mAB-binding ELISA showed identical levels of mAB-binding/Ag, without regard for the multimer size present in the tested fraction. Their results therefore suggested that in the case of structurally normal VWF, the mAB-binding ELISA reflected the concentration of VWF:Ag rather than VWF function. They also felt that it was feasible that while the mAB-binding ELISA might show reduced levels for abnormal VWF protein, structurally altered within the A1 domain of VWF, as might be found in some patients with type 2 VWD, this assay did not appear to be suitable for functional analysis of structurally intact VWF. This is actually a very important distinction, and was to prove true in subsequent studies (refer to later sections). In 2023, this mAB-binding ELISA would be called VWF:Ab, according to the latest nomenclature recommendation.[56]

The year 1999 also saw quite a few reports on the VWF:CB. Barington and Kaersgaard[57] used the VWF:CB to correlate with VWF multimers in a new process for large-scale production of VWF concentrate. Fischer continued to report on the potential utility of the VWF:CB in potential therapy with recombinant VWF.[58] Several studies were reported using the VWF:CB to investigate VWD in dogs,[59,60] including responsiveness to DDAVP. We reported our first series of RCPAQAP surveys on VWF/VWD, then with a small number of participants ($n = 25$).[61] This would prove to be the first of a long series of reports where we highlight the value of the VWF:CB via EQA findings. We explored the outcomes using a set of 10 plasmas, including a normal plasma pool (in duplicate), a 50% dilution of this pool (in duplicate), a normal individual (x1), a severe type 1 VWD individual (x1), two type 2B VWD individuals (unrelated donors), a type 3 VWD (x1), and a 2A VWD individual (x1). Laboratories were asked to perform all tests available to them to establish a laboratory diagnosis of VWD, and then to comment on the possibility or otherwise of VWD. Overall findings indicated a wide variation in test practice, in the effectiveness of various test procedures in detecting VWD, and in the ability of various composite test panels to identify type 2 VWD subtypes. First, while all laboratories ($n = 25$) performed tests for FVIII:C activity, VWF:Ag and at least one functional VWF assay (VWF:RCo; $n = 23$), and/or the VWF:CB ($n = 12$), only three laboratories performed VWF:multimer analysis. Second, for the three quantitative VWF assays, 10/25 (40%) laboratories performed all three, whereas 15/25 (60%) performed only two (VWF:Ag and VWF:RCo, $n = 13$; VWF:Ag and VWF:CBA, $n = 2$). Third, a variety of assay methodologies were evident for VWF:Ag (ELISA, EID, LIA, and VIDAS assay) and VWF:RCo (platelet agglutination/"aggregometry" and a "functional VWF:RCo alternative" ELISA assay—this being an early version of

VWF:Ab). Between method analysis for the quantitative VWF assays showed that the VWF:RCo yielded the greatest degree of interlaboratory assay variation, and had the poorest overall performance with respect to sensitivity to low levels of VWF. The VWF:CB also performed better than VWF:RCo in terms of ability to detect functional VWF "discordance" (i.e., type 2 VWD). Within VWF:Ag method, analysis showed that the EID assay procedure was associated with the greatest variation in assay results, while the EID and LIA test methods both showed poorer sensitivity at low VWF levels compared with the ELISA method. Within the VWF:RCo assay procedure, greatest variation in assay results and poorest sensitivity to low VWF levels was obtained using the agglutination method; however, the agglutination procedure showed better performance than the "functional VWF:RCo alternative" ELISA [VWF:Ab] assay in identifying type 2 VWD plasma samples. Finally, despite identified variations, most laboratories appeared to understand the complexities involved in the VWD-diagnostic process, and made appropriate diagnostic predictions regarding tested samples. From a total possible 246 interpretation events, laboratories in most cases correctly identified normal samples as normal (67/75 events = 89%), and VWD samples as derived from individuals with VWD (117/121 events = 97%). Moreover, when VWD was suggested by laboratory findings, laboratories usually correctly predicted the general subtype of VWD present (96/109 events = 88%). When "misinterpretations" occurred, these could often be linked to the test panels utilized by laboratories. That is, laboratories using the VWF:Ag and VWF:RCo combination were more likely to incorrectly identify samples derived from type 2 VWD patients as being type 1, type 1 VWD patients as being type 2, and normal plasma samples as potentially derived from patients with VWD, compared with those using the VWF:Ag and VWF:CB. It remains of interest to me that the essential findings from this inaugural RCPA-QAP report would continue to be found in subsequent reports, including those most recent.[15–17]

The 1999 VWF:CB reports continued, with Gerritsen et al[62] using the VWF:CB to assay levels of plasma VWF-cleavage protease (later to be identified as ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) as a tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). Prior methods by immunoblotting were time-intensive and cumbersome. They therefore developed a new functional assay based on the preferential binding of HMWM forms of VWF to collagen. In this assay, the diluted plasma sample to be tested is added to normal human plasma in which protease activity had been abolished (i.e., TTP sample). The VWF present in the protease-depleted plasma is digested by the VWF-cleaving protease (i.e., ADAMTS13) in the test plasma. The proteolytic degradation leads to low-molecular-weight forms of VWF, which show impaired binding in the VWF:CB (they used human collagen type III). Testing of plasma from patients with TTP and hemolytic-uremic syndrome (HUS) showed that the assay could be used to distinguish between the two syndromes. The presence of an inhibitor could be detected by carrying out the test after incubation of normal human plasma with the patient plasma sample, thus enabling differentiation of patients with familial TTP from those with nonfamilial TTP. This marked the start of the wider use of residual VWF:CB as a marker of ADAMTS13 activity.

Mauz-Körholz et al[63] used a VWF:CB, along with VWF:RCo, VWF:Ag, and FVIII:C to explore DDAVP treatment in a child with 2M VWD, with good increments in all 2 hours post-DDAVP, and concluding that in some type 2M VWD cases, DDAVP administration might be an effective treatment in cases of elective surgery, dispensing with VWF replacement by pooled blood products. Kertzscher et al[64] explored the potential utility of the VWF:CB to characterize platelet VWF content in 24 patients with various forms of VWD. No platelet VWF:Ag or VWF:CB was detectable in type 3 patients ($n = 4$). In contrast, six out of seven patients with type 2 VWD had normal or increased VWF levels. Two type 1 patients (out of $n = 13$) with low VWF levels in platelets had no increased bleeding tendency. In two other individuals with normal amounts of VWF in platelets and low plasmatic VWF and FVIII:C, more frequent bleeding episodes reflecting the low plasmatic levels were observed in a long-term follow-up. The authors concluded that in their patients, bleeding history corresponded to plasmatic levels of FVIII:C and VWF. Finally, the decade ended with my second review on VWF/VWD, again from the perspective of laboratory testing for VWD, and where I concluded that selection of an appropriate test panel is a critical component for the proper diagnosis and classification of VWD.[65]

The Next Century History—The 2000s

The 2000s started with another report from our laboratory, this one attempting to clarify that the utility of a VWF:CB depended on the source of collagen.[21] I evaluated 21 different collagen preparations for their ability to both detect VWD and discriminate different VWD subtypes (i.e., type 1 vs. 2A/2B). Collagen preparations were tested at a range of concentrations and included types I, III, and IV, collagen, and various mixtures of these, as aqueous supplied preparations and/or reconstituted from bulk lyophilized stock. Tissue sources for collagens ranged from human placenta to calf skin to equine tendon. Three of the collagen preparations tested did not support VWF binding in an ELISA process (and were therefore unable to facilitate VWD diagnosis). The ability of the remaining preparations to detect VWF was variable, as was their ability to discriminate VWD subtypes. Detection of VWF and discrimination of VWD subtypes were not mutually inclusive. Thus, some collagen preparations provided excellent detection systems for VWF, but comparatively poorer discrimination of type 2 VWD, while others provided good to acceptable detection and discrimination. Subtype discrimination was also dependent on the collagen concentration, and some batch-to-batch variation was evident with some preparations (particularly type I collagens). Overall, best discrimination was typically achieved with type I/III collagen mixtures, or type III collagen preparations (where effectiveness was highly dependent on concentration used). Good discrimination was also achieved with a commercial type III collagen-based VWF:CB method. Results of the various

"VWF:CB assays" are also compared with those VWF:RCo assay (by platelet agglutination) and that using a commercial "VWF:RCo alternative/activity" ELISA procedure (i.e., an early VWF:Ab assay). These VWF:Ab methodologies tended to be less sensitive to VWF discordance identified in type 2 VWD when compared with that detected by the majority of the VWF:CB procedures.

Another report from our laboratory soon followed, this one evaluating a novel sulfatide-binding assay for VWF, which was able to detect VWD, but seemed to offer no utility to discriminate VWD subtypes.[66] This was in contrast to both VWF:RCo and VWF:CB assessed at the same time in the same samples. Dean et al[67] then reported the use of both the VWF:CB and the platelet function analyzer (PFA)-100 in pediatric patients. They concluded that the PFA-100 was a better screening test for VWD than the SBT. They compared the VWF:CB as a functional test for VWF against the more routinely used VWF:RCo. They found that the VWF:CB detected 43/49 (88%) subjects with definite types 1, 2, or 3 VWD, performing as well as the VWF:RCo, which detected 42/48 (88%). They also showed that, used in conjunction with VWF:Ag levels, the VWF:CB might be useful in the classification of VWD subtypes.

Two more reports from our laboratory followed. The first directly compared several commercial VWF activity options for the identification and characterization of VWD,[68] and the second compared VWF:CB testing against various constructed or commercial VWF:Ab options.[69] At that time, two VWF:CB assay methods were commercially available, as was a monoclonal antibody (MAB)-based ELISA (now called VWF:Ab) by then reported to correlate with a standard VWF:RCo assay. This VWF:Ab assay was then marketed as a VWF:activity assay and was available in two assay version formats. These four VWF-activity options were directly compared with each other and in-house VWF:CB ELISAs for their ability to detect VWD, and discriminate qualitative VWD defects.[68,69] The two MAB-based systems detected VWD but could not specifically identify qualitative VWF defects, although the recently modified "Mark II" kit was more effective for the latter compared with the original "Mark I" kit. All VWF:CB methods, including in-house and commercial, also effectively detected VWD but differed in their ability to identify qualitative VWF defects. Effectiveness was highest using our in-house reference VWF:CB (based on a type I/III collagen mix product from equine tendon), the then available Gradipore VWF:CB (which also used equine tendon-derived collagen), or an in-house VWF:CB method using type III human collagen at a relatively low concentration (1 or 3 µg/mL, without covalent linkage). The then available IMMUNO VWF:CB seemed to be the least effective among all the compared VWF:CB methods for the detection of qualitative VWF defects. In the second study,[69] we adapted several anti-VWF MAB developed at Westmead for use in in-house ELISA assays to assess their utility for VWD diagnosis and subtype discrimination, and compared these assays with other assay systems. Thus, our in-house VWF:CB, VWF:RCo by agglutination, the commercial VWF:Ab assay, and in-house VWF:Ab assays were directly compared for their ability to discriminate type 1 from type 2A/2B VWD samples. All VWF:Ab assay systems effectively measured VWF and could confirm a diagnosis of VWD, as well as exhibiting some VWD-subtype discriminatory capabilities. However, better evidence of VWF-discordance in type 2 VWD was usually achieved using the VWF:RCo (agglutination) assay, and best performance in VWF-discordance was consistently observed using the VWF:CB assay. In conclusion, the VWF:CB assay proved to offer the best diagnostic predictive tool for a type 2 VWD defect, while VWF:Ab-based systems appeared to be least effective in this regard.

The second of our RCPAQAP VWF/VWD studies was also published in the year 2000.[70] This study reported an evaluation of current laboratory practice for the diagnosis of VWD by means of a multi-laboratory ($n = 19$) survey, and results compared with our earlier survey.[61] Samples comprised a new set of seven plasmas: a type 3 VWD, a type 2B VWD, a moderate type 1 VWD/hemophilia A combined defect, a normal individual, a mild type 1 VWD, a type 2A/2M VWD, and a type 2N VWD. Overall, many findings confirmed those reported earlier (including between-method analysis, within-method analysis, inter-laboratory assay variation, sensitivity to low levels of VWF, detection of functional VWF "discordance," and appropriateness of diagnostic predictions). Novel findings included: (1) although VWF:CB activity performed better than VWF:RCo in the identification of functional discordance in type 2B VWD, both assays performed equally in the identification of discordance in the type 2A/2M VWD; (2) most laboratories failed to identify the type 2N VWD as a potential type 2N VWD utilizing VWF:Ag and FVIII:C testing as a screening process. This latest survey was followed up by a dry workshop attended by over 45 scientists from Australia and New Zealand, and comprising representatives from most survey participants. Discussion covered many topics including the effect of blood group, the role (if any) of the SBT, the role of the PFA-100, confirmatory and additional tests, and the possibility of restricting testing to specialized centers. Consensus was reached on the following points: (1) diagnosis of VWD requires both clinical and laboratory assessment; (2) testing should comprise FVIII:C, VWF:Ag, and either/or both VWF:RCo and VWF:CB; (3) laboratory results should be reviewed in the light of clinical findings; and (4) confirmatory repeat testing should be performed on a sample taken 6 weeks later.

Federici et al[71] then published a short report on VWF:RCo/Ag and VWF:CB/Ag for a rapid diagnosis of type 2 VWD, including a comparison of four different assays. These comprised an in-house VWF:RCo, an in-house VWF:CB, a commercial VWF:CB (Immuno-Baxter), and a commercial "VWF activity" assay (i.e., a VWF:Ab). The latter two were the same as those we previously evaluated in the Australian studies.[68,69] The in-house VWF:CB and VWF:RCo assays were found to be better for VWD diagnosis than either of the commercial methods, and the VWF:Ab the least useful, confirming the findings of our earlier studies.

Casonato and colleagues fuelled the discussion of whether the VWF:CB could replace the VWF:RCo in their 2021 study of 10 type 2A and 12 type 2B VWD patients, together with 30

type 1 VWD patients with reduced platelet VWF content.[72] In both 2A and 2B VWD, VWF:CB and VWF:RCo were decreased, but reductions of VWF:CB were more consistent. The difference was more evident when values were expressed as a ratio, where the VWF:CB/Ag ratio was always below 0.2, while that for VWF:RCo was greater than 0.4, and in no patient was the VWF:CB/Ag ratio higher than VWF:RCo/Ag. In contrast, in type 1 VWD, the decrease in VWF:CB was similar to that seen in VWF:RCo with the ratios always within the normal range. The authors also assessed DDAVP response in type 2A and 2B VWD patients. The differences between the VWF:CB and VWF:RCo were even more evident after DDAVP, and in type 2A VWD, even though large multimers were persistently decreased, VWF:RCo was normalized, while VWF:CB remained defective. These findings clearly indicated to the authors that VWF:CB detected the absence of large and intermediate VWF multimers better than VWF:RCo. Hence, they suggested adding VWF:CB to the panel of tests employed in the diagnosis of VWD. Moreover, owing to the difficulty in performing VWF:RCo and its low reproducibility, they suggested that, when necessary, VWF:CB may be substituted for VWF:RCo.

Our own study of VWF:CB and PFA-100 testing in DDAVP-treated VWD patients soon followed.[73] From a panel of 125 patients undergoing evaluation for clinical hemostatic defects, 29/30 samples from patients with VWD (17/18 type 1, 1/1 type 3, 3/3 type 2A, 7/7 type 2B, and 1/1 PT-VWD) gave prolonged closure times (CTs) using the collagen/epinephrine (C/Epi) cartridge. For these patients, correction of an initially prolonged CT by DDAVP was accompanied by normalization of VWF:Ag, VWF:CB, and VWF:RCo in type 1 VWD ($n = 5$). In an individual with type 2A VWD, DDAVP normalized VWF:Ag and VWF:RCo, but had no apparent effect on the baseline maximally prolonged CT. In an individual with type 2B VWD, VWF concentrate also normalized VWF:Ag and VWF:RCo, but similarly had no apparent effect on the baseline maximally prolonged CT. VWF:CB did not normalize for either of these two individuals, potentially suggesting that normalization of VWF:CB might be required for normalization of CT. This concept was supported by correlation analysis undertaken between CT and various VWF parameters. Among these, VWF:CB held the strongest relationship in our dataset, which showed an inverse progressive rise in CT for falling VWF:CB. Based on our results, we concluded that the PFA-100 was highly sensitive to the presence of VWD, and might thus provide a valuable screening test for VWD. Furthermore, the combined utility of the PFA-100 and VWF:CB as markers of DDAVP responsiveness may prove to be simple, quick but powerful, predictors for its clinical efficacy.

A study on VWF test parameters, including VWF:Ag, VWF:CB, VWF:RCo, and VWF multimers, in patients with uremia followed,[74] and failed to identify a correlation with uremic bleeding. Calibration of the 4th International Standard (97/586) for FVIII and VWF followed, with the attempted inclusion of a new parameter, the VWF:CB, to complement the usual FVIII:C, FVIII:Ag, VWF:Ag, and VWF:RCo values.[75] Unfortunately, excessive inter-laboratory variability and a low number of estimates ($n = 6$) precluded the assignment of a potency for VWF:CB! The function of VWF in children with diarrhea-associated HUS was investigated by Sutor et al.[76] These children had a reduced VWF:CB/Ag ratio, despite high levels of VWF:Ag (mean: 253 U/dL). They proposed that the very high concentration of plasma VWF:Ag in HUS probably reflected endothelial cell damage or irritation. The presence of dysfunctional VWF might be caused either by a primary (due to enterohemorrhagic *Escherichia coli*) or secondary (due to consumption of functionally active VWF) process. This abnormality was not evident as structural anomaly by multimer analysis.

Kallas and Talpsep[77] evaluated a VWF:CB, based on type III collagen, for potential utility in VWD diagnosis and DDAVP response monitoring. The assay correlated with VWF:RCo, low VWF:CB/Ag ratios were observed in type 2 VWD, and VWF:CB/Ag ratios increased post DDAVP, consistent with release of HMWM. They concluded that their findings suggested the VWF:CB assay to be a useful test for measuring the functional activity of VWF in plasma samples, VWF concentrates, as well as for estimating the outcome of treatment.

Mannucci et al[78] used a VWF:CB to evaluate changes in the VWF-cleaving protease (i.e., ADAMTS13) in health and disease. They identified an inverse relation between low VWF-protease and high plasma levels of VWF:Ag and VWF:CB activity, and that low plasma levels of VWF-cleaving protease were not a specific beacon of TTP, because the protease was also low in several physiological and pathologic conditions. Váradi et al used a VWF:CB to explore thrombin-mediated in vitro processing of pro-VWF.[79]

In 2002, Riddell et al used a VWF:CB to explore type 2M VWD, and compared this with VWF:RCo.[80] They analyzed a group of 32 patients with type 2 VWD (25 patients with type 2M, six with type 2A, and one with type 2B) versus 22 normal control subjects. VWF:RCo/Ag and VWF:CB/Ag ratios were compared between the patient and control groups. In the six patients with type 2A VWD, both VWF:RCo/Ag ratios and VWF:CBA/Ag ratios were discordant (≤ 0.7). In the 25 type 2M VWD patients, the VWF:CBA/Ag ratios were concordant (> 0.7), but the VWF:RCo/Ag ratios were discordant (≤ 0.7) compared with control subjects. Thus, VWF:RCo/Ag ratios were discordant in both type 2M and 2A VWD patient groups indicating a functional abnormality. However, VWF:CB/Ag ratios were discordant in the type 2A VWD group but not in the type 2M VWD group. Their study showed that VWF:CB is sensitive to functional variants associated with the loss of HMWM (i.e., types 2A and 2B VWD), but was unable to discriminate defective platelet-binding VWD variants with normal multimeric patterns such as type 2M VWD. They concluded that the VWF:CB assay should be used in association with rather than as a replacement for the VWF:RCo assay. However, what this study also showed, but not expressed in the abstract, was that combination of VWF:RCo and VWF:CB could be used to discriminate between 2A and 2M VWD.

Turecek et al continued to explore the VWF:CB in recombinant pro-VWF processing.[81] Linder et al used a VWF:CB as part of a study to explore the attachment of whole blood

platelets on extracellular matrix under flow conditions in preterm infants.[82] Saenko et al described the development and application of a surface plasmon resonance-based VWF:CB assay.[83] Their assay correlated with an ELISA-based VWF:CB, but was more accurate and reproducible. They used their assay to explore VWF concentrates during production. They suggested their assay to be a useful tool in the development of industrial virus-inactivation procedures, allowing preservation of VWF activity and achieving the maximal therapeutic efficacy of FVIII/VWF concentrates.

Michiels et al explored the DDAVP response in patients with type 1 or 2 VWD from a diagnostic and therapeutic perspective.[84] They evaluated whether VWF:CB, VWF multimeric analysis, and the response to intravenous DDAVP could correctly diagnose and classify congenital VWD in 24 probands with mild to moderate type 1 VWD, 6 probands with severe VWD type 1, and 12 probands with type 2 VWD. Neugebauer et al compared two VWF:CB assays with different binding affinities for low, medium, and high VWF multimers.[85] They concluded that the assay with pepsin-digested collagen (human, type III) that was covalently linked to preactivated microtiter plates revealed a higher affinity for low and medium VWF multimers, whereas the assay with collagen fibrils (equine, type I) that were adsorbed to microtiter plates predominantly bound high VWF multimers. Turecek et al performed a comparative study on VWF:CB by ELISA versus VWF:RCo for the detection of functional VWF activity.[86] Measurement of functional VWF:CB activity could be performed with substantially higher interassay reproducibility than VWF:RCo. Both assay systems could be used for diagnosis and subtyping of VWD, but their VWF:CB was more sensitive than VWF:RCo. The analysis of VWF multimers in different fractions obtained by affinity chromatography on heparin Sepharose showed that VWF activity measured both with VWF:RCo and VWF:CB correlated with the degree of VWF multimerization. They suggested that measurement of VWF:RCo could be replaced by the more reliable VWF:CB, which appeared not only to be more sensitive and easier to carry out but was also found to have a higher reproducibility and allow better standardization.

Also in 2002, Budde et al used the VWF:CB as part of a test panel including VWF:Ag, VWF:RCo, and VWF multimers, to characterize 303 patients with VWD.[87] I wrote my first review focused on the VWF:CB.[88] Finkelstein et al used the VWF:CB as part of a study to explore platelet deposition on extracellular matrix under flow conditions in preterm neonatal sepsis.[89] Remuzzi et al used the VWF:CB as part of a study into TTP versus HUS to help address the question of whether ADAMTS13 levels can be used to distinguish TTP/HUS.[90] Hubbard et al again attempted to incorporate VWF:CB values into an international standard, this time the 1st International Standard for von Willebrand Factor concentrate (00/514).[91] As per the previous attempt for the plasma standard,[75] large interlaboratory variability of estimates precluded the assignment of a value for VWF:CB. Rick et al described the clinical use of a rapid VWF:CB to evaluate VWF-cleaving protease (i.e., ADAMTS13) in patients with TTP.[92] They reported 97.5% concordance between the VWF:CB and a VWF multimer gel assay. The VWF:CB identified low VWF-protease activity in 78% of patients who had a clinical syndrome consistent with TTP/HUS and in 2 of 10 sick controls, giving it a positive predictive value of 0.94. The VWF:CB also detected inhibitors of VWF-protease in 26 of 29 patients (90%) with TTP/HUS and low protease activity levels. The authors concluded the VWF:CB to be a useful clinical assay for examining VWF-protease activity and detecting associated inhibitors.

Paczuski assessed the effect of collagen source and coating conditions on the ability of a VWF:CB to diagnose VWD, and reported that modification of coating conditions and the use of an alkaline buffer permitted the use of a relatively low concentration of collagen, and that this procedure was useful in the diagnosis of VWD and to distinguish between types 1 and 2 VWD.[93] Sciahbasi et al used a VWF:CB to help explore the prothrombotic response to coronary angioplasty in patients with unstable angina and raised C-reactive protein (CRP).[94] After angioplasty, virtually all patients with unstable angina and raised preprocedural CRP showed increased VWF:Ag, VWF:CB, and CRP. Their data suggested that such changes may contribute to the worse prognosis of unstable patients with raised indices of inflammation. Haley et al explored the effect of ABO blood group on the VWF:CB, to show similar changes to VWF:RCo, with lower levels in O-blood group.[95] The potential role of the VWF:CB in assessing VWF activity in VWF concentrates was further explored,[96] as was the utility of the VWF:CB to measure VWF-cleaving protease (ADAMTS13) activity.[97–99]

In 2003, Vincentelli et al[100] used the VWF:CB to explore AVWS in aortic stenosis, to identify decreased VWF:CB with loss of HMWM, and reduced platelet function, in 67 to 92% of patients with severe aortic stenosis and results correlating significantly with the severity of valve stenosis. The use of the VWF:CB in measuring VWF-cleaving protease (ADAMTS13) activity continued with a comparison of methods by Studt et al.[101] Miller et al[102] reassessed changes in VWF level and activity (including the VWF:CB) according to ABO blood group and race.

By 2004, a Chinese group had also evaluated a VWF:CB by ELISA to conclude that the measurement of the functional activity of VWF by VWF:RCo or RIPA could be replaced by the more reliable VWF:CB.[103] In 2023, I no longer think of replacement of VWF:RCo (and never thought the VWF:CB could replace RIPA), but rather the VWF:CB has supplementary utility in VWD diagnostics. In Japan, Sakai et al used the VWF:CB to help characterize another AVWS due to autoantibodies[104]; in this case, the antibodies were not functionally inhibitory by either VWF:CB or VWF:RCo, and so VWF clearance was proposed as the likely mechanism for the AVWS. Another study utilizing the VWF:CB to help characterize VWF concentrates was performed by Lethagen et al.[105] VWF:RCo and VWF:CB correlated with each other, but not with VWF:Ag, and there were substantial differences noted between the six concentrates assessed. Yet more studies performed on VWF-cleaving protease (ADAMTS13) measured by residual VWF:CB activity by Gao et al.[106,107] The authors concluded that measurement of the VWF-cleaving

protease activity using residual VWF:CB activity was a simple and rapid method for diagnosing TTP, and that the VWF-cleaving protease activity in patients with TTP was markedly lower than those of patients with tumors. The VWF:CB continued to make inroads into transfusion practice when Burnouf et al used the VWF:CB to help assess the content and functional activity of VWF in apheresis plasma.[108] They concluded that the VWF:Ag, VWF:RCo activity, and the 11th to 15th group of VWF multimers were well preserved in all plasma units from each of the five apheresis procedures, but that the VWF:CB activity and the percentage of multimers greater than 15 in apheresis plasma were less than that in normal plasma pools and differed slightly among procedures. The subtle inference here is that the VWF:CB correlated better with HMWM than did the VWF:RCo. Hubbard and Heath had another attempt at assigning a VWF:CB value to a WHO (5th) International Standard, this time with success![109] Tripodi et al conducted an international collaborative study on ADAMTS13 testing involving 11 methods including the VWF:CB.[110] They concluded that overall, the best performance was observed for three methods measuring cleaved VWF by ristocetin cofactor, collagen binding, and immunoblotting of degraded multimers of VWF substrate, respectively. Casonato et al used the VWF:CB as part of a test panel to assess a new variant of VWD (L1446P; 4337T->C).[111]

2005 saw another study on the effect of the ABO blood group on VWF:CB (plus VWF:Ag and VWF:RCo), this one performed by Chng et al.[112] We published our third RCPAQAP VWF/VWD survey paper,[113] this one reporting on findings with 37 plasma samples which include 9 normal samples, 4 type 1 VWD samples, 8 type 2 VWD samples (2A × 3, 2B × 3, 2M × 1, and 2N × 1), and 4 type 3 VWD samples. Similar to earlier, laboratories performing the VWF:CB performed better than those that did not. On average, type 1 VWD plasma was misidentified as type 2 VWD plasma in 11% of cases, and laboratories that performed the VWF:RCo without performing the VWF:CB were six times more likely to make such an error than those that did perform the VWF:CB. Similarly, type 2 VWD plasma samples were misidentified as type 1 or type 3 VWD in an average of 20% of cases, and laboratories that performed the VWF:RCo without the VWF:CB were three times more likely to make such an error than those that performed the VWF:CB. Finally, normal plasma was misidentified as VWD plasma in an average of 5% of cases, and laboratories that performed the VWF:RCo without the VWF:CB were 10 times more likely to make such an error than those that performed the VWF:CB. We concluded that laboratories were generally proficient in their testing for VWD and that diagnostic error rates are substantially reduced when test panels are more comprehensive and include the VWF:CB.

Mühlhausen et al[114] used the VWF:CB (plus VWF:Ag and multimers) to help characterize 10 patients with glycogen storage disease type Ia, concluding that these showed a type 1 AVWS-type pattern. Hellström-Westas et al assessed ADAMTS13, VWF:Ag, and VWF:CB in premature infants and reported lowered ADAMTS13 and increased VWF:Ag and VWF:CB compared with term infants.[115] Reiter et al reported another study on ADAMTS13 activity measured using a VWF:CB.[116] Trasi et al used the VWF:CB as part of a test panel to investigate the prevalence and spectrum of VWD in western India.[117] Of 796 patients with bleeding manifestations, 58 were diagnosed with VWD. The majority of patients were type 3 (59.5%) with severe clinical manifestations, ~18% were type 1 VWD and the prevalence of qualitative variants (i.e., type 2 VWD) was 19% (2A, 9.52%; 2B, 4.76%; 2M, 1.2%; 2N, 3.6%). They concluded that the high prevalence of type 3 and the low prevalence of type 1 VWD were in contrast to western reports, suggesting low awareness of VWD and underdiagnosis of mild cases in India.

Callan et al[118] performed a DDAVP study utilizing the VWF:CB on dogs with type 1 VWD, while Heseltine et al[119] used the VWF:CB to help assess the effects of levothyroxine administration on dogs with VWD. Rojnuckarin et al[120] used the VWF:CB to help characterize VWF levels and activity in Thais Health, also looking at the ABO blood group and age, while O'Donnell et al[121] used the VWF:CB to help investigate a rare Bombay phenotype blood group associated with reduced plasma VWF levels and an increased susceptibility to ADAMTS13 proteolysis. Penas et al used the VWF:CB to help address the diagnostic question of 2A versus 2M VWD.[122] The authors investigated 21 patients who presented with low plasma levels of VWF:Ag and VWF:RCo, reduced VWF:RCo/Ag, and the presence of all sizes of multimers. The results for VWF:CB varied depending on the type of collagen used. Genetic analysis showed these type 2M patients had the mutation R1374C, and that a high frequency of the R1374C mutation was observed in northwestern Spain (Galicia). The VWF:CB (with type I collagen) assay was unable to discriminate defective platelet binding of the R1374C VWF, confirming that VWF:CB cannot substitute for VWF:RCo, and both should be tested when diagnosing VWD. Liu et al reported on a Chinese study assessing ADAMTS13 activity using residual VWF:CB activity.[123] We reported our own evaluation of VWF parameters according to blood group, age, and sex.[124]

In 2006, another study using the VWF:CB to assess VWD in dogs was reported, to assess the potential for type 2 VWD. Sabino et al showed that the inclusion of the VWF:CB resulted in reclassification of 5% of those previously identified as type 1 to type 2 VWD.[125] Couto et al evaluated dogs for possible VWD or platelet function defects using the PFA-100 combined with the VWF:CB (and VWF:Ag).[126] More Chinese studies assessing ADAMTS13 activity using residual VWF:CB activity were reported by Lu et al[127] and Liu et al.[128] Iwaki et al used the VWF:CB to help assess VWF activity in a mouse model of cholesterol-driven atherosclerosis.[129] Lisman et al used the VWF:CB (with the VWF:RCo) to show reduced VWF activity in liver cirrhosis, despite elevation of VWF:Ag.[130] Meijer and Haverkate[28] reported on their EQA findings for VWF/VWD on behalf of the European Concerted Action on Thrombosis and Disabilities Foundation, the forerunner to the ECAT program. Normal samples were interpreted correctly by the majority of the participants. However, type 1 VWD samples were wrongly interpreted by 20 to 40% of participants, which was mainly caused by a false discordance in the

VWF:RCo/Ag ratio. The between-laboratory variation was also higher for VWF:RCo than VWF:CB (respectively, 20–40% and 17–29%). Casonato et al[131] used the VWF:CB as an aide to diagnosis and follow-up of nine patients with TTP. VWF:CB was able to detect the absence or decrease of large VWF multimers better than VWF:RCo; moreover, VWF:CB was defective when large VWF multimers persisted to be decreased, in contrast with what was observed with VWF:RCo. They concluded the VWF:CB to be a simple test that appears to be useful, together with clinical symptoms and reduced platelet count, for the diagnosis and follow-up of TTP. Sucker et al[132] used the VWF:CB to help characterize a new commercial VWF activity test from Werfen (would now be termed VWF:Ab). Using 300 samples, including some with VWD, they reported good correlation with VWF activities determined as VWF:RCo ($r = 0.88$), VWF:CB ($r = 0.93$), and VWF:GPIbB ELISA ($r = 0.91$). The comparability of results obtained by the new "HemosIL" assay and VWF:GPIbB ELISA were excellent, whereas more variance was evident with VWF:RCo and VWF:CB. However, the HemosIL assay failed to indicate a loss of HMWM; so multimeric analysis was suggested as the procedure of choice for the differentiation of functional defects.

Shelat et al[133] used the VWF:CB assay in comparison with GST-VWF73 and FRETS-VWF73 assays to assess ADAMTS13 and inhibitory autoantibodies in TTP. Shenkman et al[134] evaluated the VWF:CB in comparison to a new flow-based method for differentiation between inherited and acquired TTP. Discussion on the utility of the VWF:CB in types 2A, 2B, and 2M VWD continued, with Baronciani et al[135] commenting on several previously published studies. I published a review highlighting the efficacy of the PFA-100 and VWF:CB activity as coupled strategies for laboratory monitoring of therapy in VWD.[136] Budde et al[137] undertook a comparative analysis and classification of VWF/FVIII concentrates, and the potential impact on treatment of patients with VWD, utilizing a comprehensive approach including the VWF:CB. They reported that both VWF:RCo and VWF:CB correlated well with the HMW VWF content of the assessed products, and that these products differed markedly based on relative VWF activity. Popov et al[138] reported on the performance and clinical utility of a commercial VWF:CB assay for laboratory diagnosis of VWD, to conclude that the assay provided reliable results and proved useful for laboratory diagnosis of VWD. We reported an interesting case of AVWS, and the potential utility of the VWF:CB to the identification of functionally inhibiting autoantibodies to VWF.[139]

In 2007, Lo et al[140] used the VWF:CB (with the VWF:RCo) to show relatively increased VWF activity after off-pump coronary artery bypass graft surgery. Meiring et al[141] reported on the performance and utility of their in-house VWF:CB for the cost-effective laboratory diagnosis of VWD, and recommending this assay together with VWF:RCo in the diagnostic workup of VWD. Gudmundsdottir et al[142] used a VWF:CB (with VWF:RCo) in a study to investigate risk of excessive bleeding in patients with marginally low VWF and mild platelet dysfunction. Starke et al[143] evaluated the clinical utility of various ADAMTS13 activity, antigen, and autoantibody assays, including the VWF:CB, in TTP, to confirm the utility of newer assays. Davies et al[144] used the VWF:CB to study the effect of the Y/C1584 change in VWF on in vivo protein level and function and interaction with ABO blood group. Veyradier et al[145] evaluated VWF:CB using a new commercial kit with type III collagen in type 2 VWD. Our laboratory used the VWF:CB as part of a large panel of VWF tests to compare the pharmacokinetics of two VWF concentrates in people with VWD for a randomized crossover, multicenter study.[146] In brief, VWF:CB and VWF:RCo values tended to follow VWF multimer patterns, and functional VWF appeared to be better in the newer product. Feys et al[147] used a VWF:CB assay method to assess ADAMTS13 activity to antigen ratio in various physiological and pathological conditions associated with an increased risk of thrombosis. Wadanoli et al[148] utilized a VWF:CB assay to help evaluate the efficacy of a novel VWF antagonist (GPG-290) to prevent coronary thrombosis without prolongation of bleeding time. Another study using the VWF:CB to assess ADAMTS13 activity in TTP, this time human immunodeficiency virus related, was reported by Gunther et al.[149] I published my second review on the VWF:CB,[150] and a separate article proposing the use of VWF:CB/Ag and VWF:RCo/Ag ratios to identify specific VWF activity in VWF concentrates.[151]

In 2008, Marcucci et al[152] reported on VWF:Ag, VWF:CB, and ADAMTS13 activity to help assess residual platelet reactivity in high-risk coronary patients on antiplatelet treatment. A lower ADAMTS13 activity was present in patients with VWF:Ag and VWF:CB in the upper tertile. Lu et al[153] published another Chinese study on ADAMTS13 activity measured by residual VWF:CB, this time in patients with chronic renal diseases. Tiede et al[154] used the VWF:CB as part of a large panel of tests to investigate patients with AVWS. A combination of VWF:Ag < 50 IU/dL, VWF:RCo/Ag ratio < 0.7, and VWF:CB/Ag ratio < 0.8 yielded a sensitivity of 86% for AVWS diagnosis. Gallinaro et al[155] used a VWF:CB as part of a panel to show that shorter VWF survival in O blood group subjects explains how ABO determinants influence plasma VWF. Geisen et al[156] used a VWF:CB as part of a panel to identify AVWS in patients with ventricular assist devices (VADs) and to explain nonsurgical bleeding in these patients. The loss of large multimers was paralleled by the reduction in VWF:CB and VWF:RCo. Chung et al[157] used a VWF:CB to help explore the degradation of circulating VWF and its regulator ADAMTS13 in anthrax consumptive coagulopathy. Hanebutt et al[158] utilized the VWF:CB (together with FVIII:C, VWF:Ag, PFA-100) to help evaluate DDAVP effects on hemostasis in children with congenital bleeding disorders. They concluded that DDAVP was effective in most, but not all patients, and so DDAVP testing was recommended to determine the individual hemostatic response. Lara-García et al[159] used the VWF:CB to help explain postoperative bleeding in retired racing greyhounds. Guerin et al[160] published a rebuttal to our earlier study on the utility of the VWF:CB to identify functionally inhibiting autoantibodies to VWF.[139] Burnouf et al[161] used a VWF:CB to help explore the properties of a concentrated minipool solvent-detergent–treated cryoprecipitate. They reported the feasibility of preparing virally inactivated cryoprecipitate minipools depleted of

isoagglutinins and enriched in functional FVIII, VWF, and clottable fibrinogen. Prohaska et al[162] used the VWF:CB with the PFA-100 to help investigate platelet dysfunction in patients with aortic valve disease. Song et al[163] explored the relationship between ADAMTS13 activity and VWF:CB in a study that aimed to explore platelet dynamics and relation to platelet count in patients with consumptive coagulopathy. The platelet count itself was not correlated with ADAMTS13 activity or VWF:CB; however, the rate of decline of log-scaled platelet count did correlate with both ADAMTS13 activity and VWF:CB. Burgess and Wood[164] reported the validation of a VWF:Ag ELISA and a newly developed VWF:CB in yet another study on dog VWD. Le Tourneau et al[165] used a VWF:CB to assess functional impairment of VWF in hypertrophic cardiomyopathy. Platelet adhesion time, VWF:CB/Ag ratio, and the percentage of HMWM all correlated closely and independently with the magnitude of outflow obstruction.

In 2009, there was continued interest in the differential patterns expressed in 2A versus 2M VWD in relation to VWF:CB/Ag versus VWF:RCo/Ag, and thus their potential to help characterize 2M.[166–168] The utility of the VWF:CB in measuring ADAMTS13 continued to be explored.[169] I continued to promote the VWF:CB and DDAVP response data for assisting diagnosis of VWD and characterizing of subtypes.[170] Larkin et al[171] reported on severe *Plasmodium falciparum* malaria, noting this was associated with circulating ultra-large VWF multimers, raised VWF:CB, and ADAMTS13 inhibition, with correlation of these markers. We published some findings around DDAVP therapy acting as a diagnostic aide for functional identification and characterization of VWD, and the combined use of VWF:CB and VWF:RCo to facilitate this.[172] In brief, (1) type 1 VWD displayed generally good absolute and relative rises in all test parameters, although relative rises were greatest for FVIII:C and VWF:CB, and CB/Ag ratio increases overshadowed those for RCo/Ag; (2) type 2A VWD patients showed good absolute and relative rises in both FVIII:C and VWF:Ag, but poor absolute rises in both VWF:CB and VWF:RCo; although small rises in both CB/Ag and RCo/Ag were also observed, both ratios tended to remain below 0.7; (3) finally, type 2 M VWD patients generally showed good absolute and relative rises in FVIII:C, VWF:Ag, and VWF:CB, but a poor absolute and relative rise in VWF:RCo; thus, there were good rises in CB/Ag ratios but little change in RCo/Ag, which tended to remain below 0.7. We proposed future multicenter prospective investigations to validate these findings and to investigate their therapeutic implications.

Another study exploring VWF level and activity (VWF:RCo and VWF:CB) in plasma products was reported.[173] Pérez-Rodríguez et al[174] included the VWF:CB in their study of autosomal dominant C1149R VWD, previously characterized as type 1 VWD, to be recharacterized as type 2A VWD. Varadi et al[175] included the VWF:CB in their study of species-dependent variability of ADAMTS13-mediated proteolysis of human recombinant VWF. Udvardy et al[176] included the VWF:CB (and VWF:RCo) in their evaluation of densitometric curves of VWF multimers to describe the degree of multimerization. We published a study exploring the potential supplementary utility of combined PFA-100 and functional VWF testing for the laboratory assessment of DDAVP and VWF concentrate therapy in VWD.[177] In brief, both therapies tended to normalize VWF test parameters and PFA-100 CTs in type 1 VWD, with the level of correction in CTs related to the level of normalization of VWF, particularly the VWF:CB. However, although occasional correction of CTs was observed in type 2A or type 2M VWD, these did not in general normalize PFA-100 CTs, either with DDAVP or VWF concentrate therapy. In these patients, improvement in CTs was more likely in those in whom VWF:CB values normalized or when VWF:CB/VWF:Ag ratios normalized. This study confirmed the strong relationship between the presenting levels of plasma VWF, especially the VWF:CB, and PFA-100 CTs, and that the supplementary combination of PFA-100 and VWF:CB testing might provide added clinical utility to current broadly applied testing strategies limited primarily to VWF:Ag, VWF:RCo, and FVIII:C. Burgess et al[178,179] published two studies utilizing the VWF:CB and PFA-100 to investigate dog VWD.

The decade ended with a landmark study in which Riddell et al[180] reported on the characterization of W1745C and S1783A as novel mutations causing defective collagen binding in the A3 domain of VWF. In other words, these cases could be classified as VWF:CB defective type 2M VWD cases. Their findings demonstrated that mutations causing an abnormality in the binding of VWF to collagen may contribute to clinically significant bleeding symptoms, and they proposed that isolated collagen-binding defects should be classified as a distinct subtype of VWD.

The Next Decade—The 2010s

Fuchs et al[181] reported on novel flow-based measurements of VWF function, namely, binding to collagen and platelet adhesion under physiological shear. We published a study that included VWF:CB to investigate short-activated partial thromboplastin times (APTTs).[182] Mori et al[183] evaluated VWF activity in FVIII/VWF concentrates with a newly described automated VWF activity (IL) test (i.e., VWF:Ab). The assay had good correlation with values determined by VWF:Ag, VWF:RCo, and VWF:CB. However, comparison of means using the Wilcoxon test proved no significant variation with VWF:Ag, but displayed a slight variation with VWF:RCo and VWF:CB. Bongers et al[184] included the VWF:CB in a study investigating reduced ADAMTS13 in children with severe meningococcal sepsis, and its association with severity and outcome. In the acute phase, both ADAMTS13 activity and antigen were decreased and VWF:CB and VWF:Ag levels were strongly increased. ADAMTS13 activity and VWF:CB were both correlated with the severity of the disease. Wang et al[185] evaluated a new method for detecting ADAMTS13 activity alongside their historical VWF:CB. Casonato et al[186] used the VWF:CB as a surrogate for VWF multimers to explore the reduced survival of type 2B VWF, which occurred irrespective of large multimer representation or thrombocytopenia. Wang et al[187] reported another study of ADAMTS13 activity utilizing a VWF:CB. Crow et al[188] and Meyer et al[189] utilized the VWF:CB to investigate AVWS in

VAD recipients. I published my evaluation of seven commercial VWF:CB assays for the discrimination of types 1 and 2A/2B VWD.[22] In brief, these VWF:CB assays varied in their ability to discriminate types 1 and 2A/2B VWD, and the optimal VWF:CB/Ag ratio at which optimal discrimination occurred which also differed between assays, with some improvements observed with some (but not all) assays following a harmonization process that aimed to correct for different calibrator effects. Assay variability also compromised assay utility in some test occasions. I suggested that future standardization and improvements in some commercial VWF:CB assays are needed before the VWF:CB assay could be more fully and globally utilized for discrimination of VWD types in diagnostic laboratories. Frontroth et al[190] included a VWF:CB in their prospective study of low-dose ristocetin-induced platelet aggregation (RIPA) to identify type 2B VWD and PT-VWD in children. van Loon et al[191] included the VWF:CB in a study investigating the effect of genetic variations in syntaxin-binding protein-5 and syntaxin-2 on VWF concentration and cardiovascular risk. Some alleles were associated with higher VWF plasma levels and activity, whereas others were associated with lower VWF plasma levels and activity. Shapovalov and Vitkovskiĭ[192] investigated VWF:CB and platelet aggregation in Russian patients with frostbite.

In 2011, another study relating VWF activity including VWF:CB to the risk for cardiovascular disease was reported by van Schie et al[193]; this one related to variations in the *VWF* gene. Chen et al[194] investigated the possibility that N-acetylcysteine (NAC), which reduces the size and activity of VWF in human plasma and mice, could represent a feasible therapy for TTP. In vitro, NAC reduced soluble plasma-type VWF multimers in a concentration-dependent manner and rapidly degraded ultra large VWF multimer strings extruded from activated endothelial cells. NAC also inhibited VWF-dependent platelet aggregation and collagen binding. Qin et al[195] investigated the phenotype and genotype analysis of three Chinese pedigrees with VWD utilizing the VWF:CB as part of a VWF test panel. Karger et al[196] included a VWF:CB to characterize a patient with type 2B-like AVWS. Yango et al[197] used the VWF:CB as part of a VWF test panel to investigate hypothyroidism, and showing reduction in VWF:Ag, VWF:CB, and FVIII:C in these patients. Solomon et al[198] utilized the VWF:CB to investigate type 2A-like AVWS caused by aortic valve disease, which was corrected following valve surgery. Steinlechner et al[199] used the VWF:CB and the PFA-100 to show that patients with severe aortic valve stenosis (AVS) and impaired platelet function benefit from preoperative desmopressin infusion. Tauer et al[200] utilized the PFA-100 and a VWF:CB to evaluate the DDAVP effect on primary hemostasis in pediatric patients with aspirin-like defect as hereditary thrombocytopathy. Topf et al[201] reported an evaluation of a modified thromboelastography assay for the screening of VWD, using patients previously diagnosed with VWD using a test panel including the VWF:CB. Sosothikul et al[202] included the VWF:CB in their study of reference values for thrombotic markers in children.

Chandler et al[27] reported on VWF assay proficiency testing from the perspective of the North American Specialized Coagulation Laboratory Association (NASCOLA). They reported increasing use of the VWF:CB in their participant laboratories. Overall interpretation error rates ranged from 3% for normal samples, 28% for type 1 VWD, to a staggering 60% for type 2 VWD. Type 2 VWD samples were correctly identified by all laboratories using VWF:CB/Ag ratios but by only one-third of laboratories using VWF:RCo/Ag or VWF:Ab/Ag ratios. In 2009, only 27% (12/45) of laboratories performed VWF multimer analysis, with error rates ranging from 7 to 22%. Lotta et al[203] reported on the platelet reactive conformation and multimeric pattern of VWF in acquired TTP during acute disease and remission. VWF:Ag, VWF:RCo, VWF:CB, and VWF multimeric pattern were investigated in patients with acquired TTP during acute disease, remission or both. VWF:Ag was higher in TTP patients than in controls. Larger VWF multimers were frequently lacking in acute TTP patients, who displayed ultra-large multimers at remission. The degree of loss of larger VWF multimers correlated with the degree of abnormality of hemoglobin, platelet counts, and serum lactate dehydrogenase (LDH) and was associated with low levels of both VWF:RCo/Ag and VWF:CB/Ag ratios. Pérez-Rodríguez et al[204] included VWF:CB testing in their study of AVWS and mitral valve prosthesis leakage. FVIII:C, VWF:RCo, and VWF:CB were all considerably elevated before surgery, with disproportionate VWF:RCo/Ag and VWF:CB/Ag ratios seen along with the loss of large VWF multimers. Following surgery, all parameters were markedly increased and the ratios and multimeric VWF profile became normal. Heilmann et al[205] also used the VWF:CB as part of a panel to assess AVWS as an early-onset problem in VAD patients. Diagnosis of AVWS was based on reduced VWF:RCo/Ag, VWF:CB/Ag, and multimeric analysis. No patient had an AVWS prior to VAD implantation. An AVWS was identified already in the very early postoperative period, that is, in almost all patients on the first day and in all patients on the third day. The AVWS was also detected in the majority of patients in the further course. Nine of all 17 patients suffered bleeding complications and required a total of 25 interventions due to hemorrhages. Dieckmann et al[206] used the VWF:CB to provide evidence for acute vascular toxicity of cisplatin-based chemotherapy in patients with germ cell tumor. Levels of both VWF:Ag and VWF:CB increased significantly upon initiation of therapy, suggesting early vascular toxicity and endothelial cell activation.

In 2012, Heilmann et al[207] utilized the VWF:CB to help investigate AVWS in patients with extracorporeal life support (ECLS). They analyzed 32 patients with ECLS and 19 of them without support. They used VWF:RCo/Ag and VWF:CB/Ag ratios to diagnose AVWS, alongside multimeric analysis. Reduced VWF:RCo/Ag ratios were identified in 28 ECLS patients, while reduced VWF:CB/Ag ratios were identified in 31 patients; HMWMs were missing in the same 31 patients. Thus, 31/32 ECLS patients presented with AVWS, and 22/32 patients suffered from bleeding complications. AVWS was not detectable in any analyzed patient without support. Choi et al[208] utilized a VWF:CB to help characterize a Korean

patient with type 2A VWD and a Gly1609Arg missense mutation in the *VWF* gene. Weiss et al[209] used high-resolution multimer analysis, the VWF:CB and the PFA-100, to detect type 2A VWD in patients previously showing a normal VWF:RCo/Ag. Czúcz et al[210] utilized a VWF:CB as part of a panel of tests to investigate endothelial cell function in patients with hereditary angioedema.

We published our evaluation into the differential sensitivity of VWF "activity" assays (VWF:RCo, VWF:CB, VWF:Ab) to large and small VWF molecular weight forms.[211] The data showed that the VWF:CB and VWF:RCo assays had higher sensitivity to the loss of HMW VWF than did the VWF:Ab assay. Moreover, within-method analysis identified better HMW VWF sensitivity of some VWF:CB assays than of others, with all VWF:CB assays still showing better sensitivity than the VWF:Ab assay. Differences were also identified between VWF:RCo methodologies on the basis of either platelet aggregometry or as performed on automated analyzers. We felt that our results had significant clinical implications for the diagnosis of VWD and monitoring of its therapy, as well as for the future diagnosis and therapy monitoring of TTP. Flood et al[212] published their findings using a type VI collagen VWF:CB assay. Healthy controls and index VWD cases were analyzed for VWF:Ag, VWF:RCo activity, and VWF:CB with types I, III, and VI collagen. *VWF* gene sequencing was performed for all subjects. Two healthy controls and one type 1 VWD subject were heterozygous for an A1 domain sequence variation, R1399H, and displayed a selective decreased binding to type VI collagen but not types I and III. Expression of recombinant 1399H VWF resulted in absent binding to type VI collagen. Two other VWF A1 domain mutations, S1387I and Q1402P, displayed diminished binding to type VI collagen. An 11 amino acid deletion in the A1 domain also abrogated binding to type VI collagen. The authors concluded that the VWF:CB may be useful in diagnosis of VWD, as a decreased VWF:CB/VWF:Ag ratio may reflect specific loss of collagen-binding ability. However, mutations that exclusively affect type VI collagen binding may be associated with bleeding, yet missed by current testing. Flood et al[213] also undertook a comparison of type I, type III, and type VI VWF:CB assays in the diagnosis of VWD. The mean VWF:CB in healthy controls was similar and highly correlated for types I, III, and VI collagen (means: 1.2–1.3). In type 1 VWD subjects, VWF:CB was also similar to VWF:Ag with similar mean VWF:CB/VWF:Ag ratios for types I, III, and VI collagen (means: 1.1–1.3). For type 2A and 2B subjects, VWF:CB was uniformly low, with mean ratios of 0.62 and 0.7 for type I collagen, 0.38 and 0.4 for type III collagen, and 0.5 and 0.47 for type VI collagen. The authors concluded that the low VWF:CB in type 2A and 2B subjects suggested that VWF:CB may also supplement analysis of multimer distribution.

Koyama et al[214] explored VWF test patterns (VWF:Ag, VWF:CB, VWF multimers) and ADAMTS13 in patients with obstructive sleep apnea (OSA). Hassan et al[215] explored potential VWD among 30 Malay patients with menorrhagia. Using the APTT, FVIII:C, VWF:Ag, VWF activity (VWF:Ab), and VWF:CB, VWD was diagnosed in four patients (13.3%), three with type 1 and one with type 2M VWD. Jiang et al[216,217] included the VWF:CB in their evaluation of VWD among their Chinese cohort of patients. Wiegand et al[218] included VWF:CB to help investigate the bleeding diathesis in 15 patients with Noonan syndrome. Nine patients displayed a relevant bleeding diathesis and complained of easy bruising; three reported spontaneous gum bleeding. Five patients had pulmonary valve stenosis, three of who had loss of HMWM and reduced VWF:CB, suggesting AVWS. Montilla et al[219] used a VWF:CB to help investigate the effect of polyphosphate on VWF activity. We published a novel VWF:CB method using flow cytometry.[220] Al-Awadhi et al included a VWF:CB in their assessment of smoking, VWF, and ADAMTS13 in healthy males.[221] Acute smokers had significantly higher levels of VWF:CB activity and ADAMTS13 antigen and activity levels compared with smokers at rest.

In 2013, Legendre et al[222] identified several mutations in the A3 domain of VWF that induced combined qualitative and quantitative defects in the protein, including decreased binding to types I and III collagen. I helped to establish and characterize a novel VWF:CB assay for the measurement of VWF activity.[223] Soares et al[224,225] employed a VWF:CB to help characterize ADAMTS13 and VWF level and activity in children with cyanotic congenital heart disease. Hugenholtz et al[226] included a VWF:CB to highlight an unbalance between VWF and ADAMTS13 in acute liver failure; in these patients, VWF:Ag was raised, but VWF:CB and VWF:RCo/Ag ratio was reduced, as was ADAMTS13 and the proportion of HMWM. Colombatti et al[227] included the VWF:CB in a study of coagulation activation in children with sickle cell disease, as associated with cerebral small vessel vasculopathy. Hu et al[228] used a VWF:CB to help investigate nonsurgical bleeding in heart failure patients supported by continuous-flow left VAD. Kraisin et al[229] used a VWF:CB to evaluate reduced ADAMTS13 activity in a malaria-like model. Mazur et al[230] included a VWF:CB to explore postoperative draining and blood product usage with coronary artery bypass grafting.

In 2014, van Loon et al[231] included the VWF:CB to study performance-related factors as the main determinants of the VWF response to exhaustive physical exercise. VWF:Ag, VWF:CB, and ADAMTS13 activity all increased after exhaustive exercise, with this increase strongly determined by physical fitness level and the intensity of the exercise. Rau et al[232] used a VWF:CB to investigate VWF activity in a model of in vitro hemodilution. Kajdácsi et al[233] used a VWF:CB to help study endothelial cell activation during edematous attacks of hereditary angioedema types I and II. Levels of several endothelial cell activation markers, including VWF:Ag and VWF:CB, significantly increased during such attacks. Goel et al[234] used the VWF:CB to help measure ADAMTS13 activity, alongside other methods, to investigate ADAMTS13 deficiency in patients with noncirrhotic portal hypertension. I wrote a historical review on VWD, including the VWF:CB and other VWF assays.[10] Mancini et al[235] utilized the VWF:CB in addition to a FRETS-VWF73 assay to show that the FRETS-VWF73 better reflects ADAMTS13 proteolytic activity in acquired TTP. Quiroga et al[236] utilized the VWF:CB as part

of a panel of tests to investigate type 1 VWD in Chile. We reported the next in our series of RCPQAP EQA for VWF/VWD.[33] In brief, a set of 29 plasma samples comprising both "quantitative" VWF deficiency ("type 1 and 3 VWD") versus "qualitative" defects ("type 2 VWD") were tested in a cross-laboratory setting. Different VWF assays and activity/antigen ratios show different utility in VWD and type identification. VWD identification errors were often linked to high inter-laboratory test variation and result misinterpretation (i.e., laboratories failed to correctly interpret their own test panel findings). Moderate quantitative VWF-deficient samples were misinterpreted as qualitative defects on 30/334 occasions (9% error rate); 17% of these errors were due to laboratories misinterpreting their own data, which was instead consistent with quantitative deficiencies. Conversely, while qualitative VWF defects were misinterpreted as quantitative deficiencies at a similar error rate (~9%), this was more often due to laboratories misinterpreting their data (~50% of errors). For test-associated errors, VWF:RCo was associated with the highest variability and error rate, which was at least twice that using VWF:CB.

Waldow et al[237] used a VWF:CB, the PFA-100, and multimeric analysis to help explore AVWS in adult patients with congenital heart disease, and Loeffelbein et al[238] utilized a VWF:CB to help explore shear-stress induced AVWS in children with congenital heart disease with and without stenosis; here, VWF:CB was lower in the stenosis group as was the VWF:CB/Ag; after intervention, VWF parameters normalized rapidly within the first 24 hours after the procedure and showed no group difference. Our laboratory published a study comparing several automated VWF antigen and activity assays.[14] We included a large sample test set ($n = 600$), and also evaluated DDAVP responsiveness plus differential sensitivity to high-molecular-weight VWF. We reported that VWF:Ag results from different methods were respectively largely comparable, although some notable differences were evident, including one high false normal VWF:Ag value (105 U/dL) on a type 3 VWD sample, possibly due to heterophile antibody interference in the latex-based CS-5100 methodology. VWF:RCo versus VWF:GPIbR was also largely comparable. VWF:GPIbM was largely comparable to VWF:RCo and GPIbR, but VWF:CB showed discrepant findings to VWF:RCo, VWF:GPIbR, and VWF:GPIbM with some patients, most notably patients with type 2M VWD. We concluded (1) VWF:Ag on different platforms were largely interchangeable, as were VWF:RCo versus VWF:GPIbR on different platforms, although occasional (some potentially important) differences may be present, and manufacturer-recommended methods may otherwise require some assay optimization; (2) VWF:RCo, VWF:GPIbR, and VWF:GPIbM were also largely interchangeable, except for occasional differences that may also relate to assay design (differing optimizations); (3) VWF:CB provides an additional activity to supplement VWF:RCo, VWF:GPIbR, and VWF:GPIbM activity assays, and is therefore not interchangeable with any of the VWF:GPIbB assays.

In 2015, Kalbhenn et al[239] included the VWF:CB in an evaluation of AVWS due to extracorporeal membrane oxygenation (ECMO) support. The diagnosis of AVWS was based on the VWF:CB/Ag ratio and VWF multimeric analysis. Bleeding episodes were also monitored. All 18 patients supported with ECMO developed AVWS, which was identified within 24 hours of ECMO implantation. In 17/18 patients, bleeding complications arose requiring transfusions of blood, fresh frozen plasma (FFP), and/or platelet concentrates. The AVWS was reversed after ECMO explantation. The authors concluded that making an early diagnosis of AVWS and providing appropriate treatment may reduce the incidence of life-threatening bleeding in ECMO. Bürgin-Maunder et al[240] utilized the VWF:CB (with VWF:Ag, and VWF multimers) to assess whether moderate dietary supplementation with omega-3 fatty acids impacted plasma VWF profile in mildly hypertensive subjects. These subjects often have raised VWF levels, but supplementation did not impact VWF level or activity. Caspar et al[241] included a VWF:CB to evaluate the effects of transcutaneous aortic valve implantation (TAVI) on aortic valve disease–related hemostatic disorders involving VWF. They prospectively enrolled 49 consecutive patients with severe AVS addressed for TAVI. At baseline, a significant link between VWF abnormalities and the severity of AVS was evident, and negatively associated with VWF:Ag, VWF:RCo, and VWF:CB, and consistent with AVWS (VWF:CB/Ag < 0.7). One week after TAVI, all VWF levels increased and VWF:CB/Ag normalized. Hugenholtz et al[242] investigated ADAMTS13 and VWF level and activity (VWF:RCo, VWF:CB, and VWF multimers) during lung transplantation, and reported an unbalance promoting the development of hyperactive primary hemostasis. Ferhat-Hamida et al[243] described the contribution of the VWF:CB in the range of tests for the diagnosis and classification of VWD. They reported that a comparison between VWF:CB and VWF:RCo showed good correlation for all types of VWD except for type 2, while comparison between VWF:CB and multimer pattern showed good concordance for all types of VWD. They concluded that the VWF:CB could be a good alternative to VWF:RCo for the diagnosis of quantitative deficiencies of VWF and could also replace VWF multimers. van Meegeren et al[244] included the VWF:CB in their panel of VWF tests to help phenotype genetically confirmed type 2N VWD patients. Bartoli et al[245] included the VWF:CB to help assess the effect of ADAMTS13 inhibition on VWF activity using doxycycline during supraphysiological shear stress.

In 2016, Periayah et al[246] included the VWF:CB to evaluate VWD in Malaysia. Qu et al[247] included the VWF:CB to assess the diagnostic value of plasma levels, activities, and their ratios of VWF and ADAMTS13 in patients with cerebral infarction. They found an association between reduced levels of VWF:CB/Ag, ADAMTS13/VWF:Ag, and ADAMTS13/VWF:RCo ratios and cerebral infarction. They suggested that increased levels of VWF and reduced levels of ADAMTS13 activity may contribute to the pathogenesis of cerebral infarction. Feys et al[248] included the VWF:CB in their study investigating the potential contribution of VWF:GPIbα interactions to persistent aggregate formation in apheresis platelet concentrates. Our laboratory reported our evaluation of the three VWF test panel using the chemiluminescent-based

assay AcuStar system for identification of, and therapy monitoring in, VWD.[249] This test system was compared with previously evaluated and validated test systems including VWF:RCo on CS-5100, the new Siemens VWF:GPIbM on CS-5100, and VWF:Ag and VWF:CB assays performed by automated ELISA. We employed a large total sample test set ($n = 535$) including plasma samples from individuals with and without VWD, some on treatment, normal plasmas, and normal and pathological controls. We also evaluated DDAVP responsiveness, plus differential sensitivity to reduction in HMWM. The chemiluminescent test panel (VWF:Ag, VWF:RCo, VWF:CB) showed good comparability to similar assays performed by alternate methods, and broadly similar data for identification of VWD, provisional VWD-type identification, DDAVP and VWD therapy, and HMWM sensitivity, although some notable differences were evident. The chemiluminescent system showed best low-level VWF sensitivity, and lowest interassay variability, compared with all other systems. Nagy et al[250] included the VWF:CB to help determine that circulating osteoprotegerin levels are associated with non–O blood groups. Muthiah et al[251] utilized the VWF:CB to help evaluate whether longitudinal changes in hemostatic parameters and reduced pulsatility contributed to nonsurgical bleeding in patients with centrifugal continuous-flow left VADs. Bleeding events occurred in 14/28 (50%) patients. VWF profile impairment (VWF:CB/Ag < 0.8) was demonstrated in 89% of patients at D30, with subsequent recovery but further deterioration after D180. Bleeding was associated with elevated preimplant sGPVI, and pulsatility was associated with higher VWF:CB/Ag and a trend to less bleeding. The residual VWF:CB level continued to be used in China as a measure of ADAMTS13 activity, as reported by Sun et al,[252,253] for various patient groups with prothrombotic status (atherosclerosis, diabetes, acute promyelocytic leukemia, cancer and sepsis, for a total of 260 cases) and in patients with hematologic malignancies before and after treatment. de Maat et al[254] included the VWF:CB in their investigation into the biological variation of hemostasis variables in thrombosis and bleeding.

In 2017, I published my third review focused on the VWF:CB,[255] as well as a methodological study.[20] Ezigbo et al[256] included the VWF:CB to help characterize VWD in the Nigerian population. Zhang et al[257] included the VWF:CB in their study of VWF level and activity in healthy Chinese. Heilmann et al[258] included the VWF:CB in their study of AVWS in 74 patients on long-term support with the VAD HeartMate II. Abnormally low values of VWF:RCo/Ag and VWF:CB/Ag were found in 69 and 97% of blood samples, respectively. Only 10/181 multimer analyses showed a normal pattern. The VWF:CB/Ag ratio correlated with the multimer patterns, whereas the VWF:RCo/VWF:Ag ratio seemed to be less sensitive for AVWS. Wang et al[259] included the VWF:CB in their evaluation into ABO blood group, age and gender on plasma FVIII:C, fibrinogen, VWF, and ADAMTS13 in a Chinese population. Frank et al[260] studied 21 consecutive patients with AVS before and 6 to 18 months after valve surgery. They assessed PFA-100 CTs, FVIII:C, VWF multimers, VWF:Ag, VWF:RCo, VWF:CB, and VWF:CB/Ag ratio. Large VWF multimers were strongly reduced in all patients with AVS, while all controls had normal multimers. Collagen/ADP (C/ADP) CTs were prolonged in patients with AVS compared with the controls and the VWF:CB/Ag ratio was pathological in 20/21 patients but normal in all controls. After surgery, VWF multimers normalized in all patients, C/ADP CTs shortened, and VWF:CB/Ag ratio strongly improved, normalizing in 14 of 17 patients. Thus, all consecutive patients with severe AVS had an AVWS, and the combination of C/ADP CT and VWF:CB/Ag ratio detected the AVWS in all patients. Kumar et al[261] included the VWF:CB in their evaluation of plasma ADAMTS13 activity (reduced) and VWF antigen and activity (both elevated) in patients with subarachnoid hemorrhage. Cluster analysis also demonstrated that patients with higher VWF:Ag and VWF:CB and/or lower ADAMTS13 activity might be at risk of increased mortality. Cibor et al[262] investigated levels and activities of VWF and ADAMTS13 in inflammatory bowel diseases (IBDs). They concluded that complex VWF-ADAMTS13-mediated mechanisms disturbed hemostasis in IBD; a reduced VWF:CB was a risk factor for bleeding, while a lower ADAMTS13 level combined with an elevated VWF:Ag could predispose to thrombosis. Doruelo et al[263] included the VWF:CB in their clinical and laboratory study into phenotype variability in type 2M VWD. Michiels et al[264] included the VWF:CB in their study into VWD and DDAVP. Chan et al[265] included the VWF:CB in their study on shear stress-induced total blood trauma in multiple species. Lavin et al[266] included the VWF:CB in their study into the clinical phenotype and pathophysiology underlying low VWF levels.

In 2018, Stufano et al[267] reported their comparative evaluation of the AcuStar chemiluminescence VWF:CB to the Stago ELISA VWF:CB. Geisen et al[268] included the VWF:CB in their investigation into platelet secretion defects and AVWS in patients with VAD. All 198 VAD patients developed AVWS. As soon as the VAD was explanted, the AVWS disappeared within hours. AVWS was less severe in the HeartMate III patients than in the HeartMate II patients. The HeartMate III patients also had fewer bleeding symptoms. Jousselme et al[269] also undertook a comparison of the automated AcuStar chemiluminescent assay versus the Stago ELISA assay using VWD plasma from patients previously diagnosed through *VWF* molecular analysis. Discrepancies of VWF:CB/Ag ratio were observed in type 2M–2A-like VWD, a finding that we reported in 2016.[34] The residual VWF:CB assay was still being used in China to determine ADAMTS13 activity.[270] Alharbi et al[271] included the VWF:CB in their assessment of ABO blood group effect on VWF tests in healthy Saudi blood donors. Russell et al[272] included the VWF:CB in their evaluation of coagulopathy, endothelial cell damage, and mortality after severe pediatric trauma, showing lowest plasma ADAMTS13 activity in patients who died from their injuries. Zayat et al[273] used the VWF:CB in their evaluation of survival in HeartMate II patients. Sherazi et al[274] included the VWF:CB in their prospective analysis of bleeding events in left VAD patients. We published our findings on the differential sensitivity of different VWF activity assays to reduced VWF molecular weight forms,[275] in essence an update of the study we reported in 2012.[211] In

brief, sensitivity for reduction of HMW was highest for VWF:CB and VWF:GPIbM, intermediate for VWF:RCo and VWF:GPIbR, and lowest for VWF:Ab, findings similar to those we previously reported.[211] We again felt that our results held significant clinical implications for diagnosis and therapy monitoring of VWD, as well as potential future diagnosis and therapy monitoring of TTP. Atiq et al[276] included the VWF:CB in their study on comorbidities associated with higher VWF levels, and the age-related increase of VWF in VWD. Torkildsen et al[277] included the VWF:CB in their comparison of multiple thawing techniques on thaw time and stability of hemostatic proteins in canine plasma products. Lasom et al[278] included a VWF:CB in their evaluation of coronary stenosis in type 2 diabetes mellitus patients. Palyu et al[279] included the VWF:CB in their study into cirrhotic patients with stable disease or acute decompensation (AD). VWF:Ag, VWF:RCo, and VWF:CB were elevated in both cirrhotic groups, with 24/54 AD patients showing presence of ultralarge VWF multimers, with this also associated with low ADAMTS13 activity and high CRP levels. Oliveira et al[280] compared three nonautomated VWF:CB assays, two by ELISA and the other by flow cytometry. The ELISA assays could differentiate 2A and 2M VWD. Pelland-Marcotte et al[281] included the VWF:CB to investigate AVWS in children with idiopathic pulmonary arterial hypertension. Overall, 8/14 children had mild to moderate bleeding symptoms and/or laboratory abnormalities in keeping with AVWS. Normalization of the hemostatic defects following lung transplantation and lack of family history of bleeding attested to the acquired nature of their defects. Icheva et al[282] included the VWF:CB in their study into AVWS in congenital heart disease surgery.

In 2019, Staley et al[283] included the VWF:CB in their study into clinical factors and biomarkers that predicted outcome in patients with immune-mediated TTP. Slobodianuk et al[284] reported defective VWF:CB and increased bleeding in a murine model of VWD affecting collagen IV binding. Moonla et al[285] reported the bleeding symptoms and VWF levels in their VWD cohort, representing a 30-year experience in a tertiary-care center. Low VWF:RCo, VWF:GPIbM, and VWF:CB were all able to predict increased bleeding risk. Colling et al[286] included the VWF:CB in their in vitro assessment of VWF in cryoprecipitate FVIII/VWF concentrate, and recombinant VWF. Pechmann et al[287] included the VWF:Ag and VWF:CB in their study reporting increased VWF parameters in children with febrile seizures, concluding that especially VWF:CB may serve as additional biomarker in the diagnosis of febrile seizures. Kumar et al[288] included VWF:Ag and VWF:CB (both increased) and ADAMTS13 activity (decreased) in their study of patients with traumatic brain injury. Yan et al[289] reported the establishment of a flow cytometric immunobead assay to detecting plasma VWF activity (VWF:GPIbR-like assay) and showed its clinical application in the prognosis of ischemic stroke, as compared with VWF:Ag, VWF:GPIbR, and VWF:CB. Kubicki et al[290] included the VWF:CB in their investigation into AVWS in 39 pediatric patients during mechanical circulatory support (MCS) (extracorporeal life support, $n = 13$; ECMO, $n = 5$; and VAD, $n = 12$). All children developed AVWS during MCS, usually during the early postoperative course, but no AVWS after device explantation. The authors detected a loss of HMWM, decreased VWF:CB/Ag ratios, and reduced VWF:CB levels; 20/30 patients experienced bleeding complications, and 53% of them required surgical revision. There were no deaths due to bleeding during support. Authors concluded that AVWS prevalence in pediatric patients on MCS is 100% regardless of the types of devices used, but that the bleeding propensity varies widely. Coghill et al[291] compared several commercially available VAD using a bench-top method and exampled the effect on various VWF parameters, with loss of VWF:Ag, VWF:CB, and HMWM evident with their use. Klaeske et al[292] investigated AVWS in HeartMate 3 patients compared with HeartWare VAD (HVAD), showing reduction of HMWM, VWF:Ag, VWF activity, and VWF:CB in patients with both devices, but that the defects were more severe with the HVAD device. McBride et al[293] included the VWF:CB in their investigation into primary hemostatic function in dogs with acute kidney injury (AKI), and reported a type 2 VWD-like picture (low VWF:CB/Ag) in these dogs.

The Current Decade—The 2020s

In 2020, Radley et al[294] included the VWF:CB in their in vitro benchmarking study of VAD in current clinical use, and showed lowest VWF:CB with the HVAD. Jakob et al[295] included the VWF:CB in their study of VWF parameters as potential biomarkers for disease activity and coronary artery lesion in patients with Kawasaki disease, and showed a significant reduction in VWF:CB/Ag ratio in these patients. Stufano et al[296] reported their evaluation of the AcuStar VWF assay panel (VWF:Ag, VWF:GPIbR, VWF:CB) for the diagnosis of VWD. They reported similarly with our prior 2016 study,[249] with AcuStar VWF:GPIbR/Ag and VWF:CB/Ag showing overall good concordance with corresponding data obtained at the time of the historical diagnosis with earlier VWF assays. When discrepancies occurred, these were generally due to the lower VWF:CB/VWF:Ag obtained with AcuStar as compared with those obtained with historical methods and this affected particularly the diagnosis of type 2M VWD. Together, the AcuStar VWF:GPIbR/Ag and VWF:CB/Ag were able to distinguish type 1 from types 2A, 2B, and 2M, whereas no distinction was possible between type 2A and 2B. The authors concluded that the AcuStar panel offered good performance for the differential diagnosis of VWD type 1 and 2A/2B patients, with a high rate of coincidence with historical diagnosis obtained for VWD types 3, 2A/2B, and 1. Although more tests (e.g., RIPA, VWF multimers) might be needed to complete an accurate VWD classification in some cases, the AcuStar panel was considered a sensitive, rapid, and reliable tool to diagnose VWD patients. Harsfalvi et al[297] included the VWF:CB (plus VWF:Ag, VWF multimers, ADAMTS13) in their study reporting long-lasting prothrombotic states implied by changes of plasma VWF parameters after radical prostatectomy for prostate malignancy, showing elevated VWF:Ag, VWF:CB, HMWM, and reduced ADAMTS13/VWF:Ag in these patients. We published another comparative assessment of VWF

multimers versus activity for VWD using modern contemporary methodologies.[32] In brief, all evaluated VWF activity/Ag ratios showed high correlation to the presence/absence of HMWM and very HMWM, although VWF:CB/Ag and VWF:GPIbR/Ag ratios using the automated chemiluminescence AcuStar method yielded highest correlation coefficients. Schlagenhauf et al[298] included the VWF:CB in their evaluation of AVWS and platelet function defects during ECMO. Sacco et al[299] included the VWF:CB in their investigation of a novel-type 2B VWD patient.

In 2021, we outlined our strategic algorithmic approach to distinguish 2M VWD from other VWD types, in which the VWF:CB held great importance.[26] Ladeira et al[300] included the VWF:CB to help investigate the ADAMTS13–VWF axis in sickle cell disease patients. Ranger et al[301] included the VWF:CB in their investigation of the heparin–VWF interaction in cardiopulmonary bypass. They reported raised VWF:CB and VWF:RCo but reduced VWF:CB/Ag and VWF:RCo/Ag, suggesting loss of HMWM. We published an extensive review of changes in VWF and ADAMTS13 in COVID-19 (coronavirus disease 2019), reporting large rises in VWF:Ag and activity (including VWF:CB), reduced ADAMTS13, and thus reduced ADAMTS13/VWF:Ag ratios, in severe COVID-19, and potentially explaining reported (micro-) thrombosis in these patients.[302] Philippe et al[303] reported that the VWF:CB was predictive of in-hospital mortality in COVID-19 patients, as further highlighted by the VWF/ADAMTS13 ratio imbalance in these patients. Abdulrehman et al[304] reported on diagnosis of VWD in North American laboratories using various VWF assays including the VWF:CB.

In 2022, Thangaraju et al[305] included the VWF:CB in their report on the impact of age and BMI on the VWF/ADAMTS13 axis in hospitalized COVID-19 patients. Similarly, Sinkovits et al[306] included the VWF:CB in their report on the associations between the VWF–ADAMTS13 axis, complement activation, and COVID-19 severity and mortality. Fels et al[307] used the VWF:CB to identify a novel type 2M VWD with a likely pathogenic variant in the A3 domain of VWF (c.5192C $>$ T; p.Ser1731Leu). Lapić et al[308] included the VWF:CB in their reevaluation of VWD diagnosis in a Croatian pediatric cohort combining bleeding scores, phenotypic laboratory assays, and next-generation sequencing. Nagy et al[309] included the VWF:CB in their investigation into severe peripheral arterial disease (PAD), to show positive correlation of VWF:CB with other markers of PAD. Lapić et al[310] included the VWF:CB in their study of VWD in Croatian patients. Bowyer et al[311] utilized VWF:CB (with FVIII:C, VWF:Ag, VWF multimers, and six other VWF activity assays) when they reported data in patients with acquired immune TTP treated with caplacizumab. Gowani et al[312] included the VWF:CB in their evaluation of a case of AVWS associated with recurrent gastrointestinal bleeding. Ziemba et al[313] updated previous findings[304] related to diagnostic testing for VWD, including the VWF:CB, in North American Laboratories. Laporte et al[314] included the VWF:CB and multimer analysis in their comparative evaluation of VWF activity assays to detect AVWS in myeloproliferative neoplasms. Gritsch et al[315] included the VWF:CB in their investigation into the structure and function of recombinant versus plasma-derived VWF and impact on multimer pharmacokinetics in VWD. Lapić et al[316] included the VWF:CB in their study of a case of type 1 VWD in a pediatric patient as caused by a novel heterozygous deletion of exons 1 to 6 of the *VWF* gene. Icheva et al[317] included the VWF:CB with multimer analysis in their study of AVWS in infants with systemic-to-pulmonary shunts.

At the time of writing, 2023 has just begun, but had already marked some citations incorporating the VWF:CB. Kalbhenn et al[318] included the VWF:CB in their study of COVID-19 patients. Icheva et al[319] included the VWF:CB in their study of AVWS in infants with congenital heart disease. Van Den Helm et al[320] included the VWF:CB in their study on pediatric ECMO, reporting changes in VWF multimers, VWF:Ag, and activity; in summary, VWF:Ag increased and HMWM, VWF:RCo/Ag, and VWF:CB/Ag ratios all decreased while patients were on ECMO, compared with baseline pre-ECMO samples and healthy children.

Discussion

Several different VWF:CB methodologies have been tried over the years. The initial reports used methods that were complex or time consuming, or like VWF:RCo.[37,38] Several workers, including us, have described flow-based VWF:CB assays,[220,289] or surface plasmon resonance-based VWF:CB assay.[83] However, none of these have ever taken off, and the most often used method remains the ELISA method, which have devolved from the original method of Brown and Bosak.[35] However, not all ELISA VWF:CB assays are equal in detection of HMWM, or in VWD diagnostics. The best performing ELISA VWF:CB assays are those that balance detection of VWF against selective detection of HMWM. All ELISA VWF:CB assays detect VWF, but some detect VWF like a VWF:Ag does, and so have poor discrimination for HMWM. For in-house methods, the use of a type I/III mixture (respectively ~95%/5%) will prove to be best, or else a type III collagen used at low concentration to maximize HMW VWF binding.[21,69] The most popular VWF:CB ELISA assay is also one of the worse performing.[15,16] Alternatively, the use of the CLIA AcuStar VWF:CB assay can also be recommended; however, the ELISA versus CLIA methods have differing sensitivity to type 2M VWD, and hence, different algorithmic approaches may be needed.[26] In brief, the CLIA VWF:CB assay will tend to be low, with low VWF:CB/Ag ratios, in types 2A, 2B, and 2M VWD, whereas ELISA VWF:CB assays tend low, with low VWF:CB/Ag ratios, in types 2A and 2B, but normal in most 2M VWD (unless a specific collagen-binding defect 2M).

Use of the VWF:CB to Assist in the Diagnosis and Management of Congenital VWD

Scott and colleagues[321] identified that VWF bound to collagens of differing type and form in 1981. Santoro reported in 1983 that collagen bound preferentially to HMWM forms of VWF.[322] Sixma and colleagues[323] identified that VWF contained separate binding sites for platelets and for collagen in 1984. This prior information was exploited by Brown and

Bosak in 1986[35] to enable the development of a VWF:CB ELISA assay for VWF that could be used to assist in the diagnosis of VWD. With the benefit of hindsight, we recognized that this assay was not fully optimized for VWD diagnostics. We first reported our own ELISA version of VWF:CB in 1991, and explored the assay more fully than Brown and Bosak, showing, for example, the ability of the assay to distinguish types 1 and 2 (2A, 2B) VWD.[36] We followed this with several additional reports over time, showing in 1994, for example, the utility of the VWF:CB to improve the laboratory monitoring of DDAVP therapy in VWD patients.[41] In the year 2000, I definitively showed that the detection of VWD, and discrimination of VWD subtypes, depended on the source of the collagen, and how it was used (collagen concentration, collagen dilution buffers used to coat 96-well plates, and even the type of plates).[21] Here we showed, for example, that the collagen and coating conditions originally described by Brown and Bosak could be markedly improved, and that VWF:CB assays were not all alike. Indeed, we could make VWF:CB assays VWF:Ag-like by using type III collagen at too high a coating concentration, thereby losing the ability of a VWF:CB to discriminate HMWM and thus type 1 versus 2A/2B VWD. Also in 2000, I was able to compare a range of commercial ELISA-based VWF activity options to show that our in-house VWF:CB was far superior to commercial VWF:CB and VWF:Ab options at that time.[68,69] Indeed, one commercial VWF:CB assay and the then available VWF:Ab ELISA assays completely failed to discriminate type 1 from 2A/2B VWD. Also in 2000, we showed we could develop our own VWF:Ab ELISA assays, using monoclonal antibodies against VWF that were developed by us, capable of discriminating type 1 from 2A/2B VWD almost as well as our in-house VWF:CB, but as these did not appear to offer any advantage over the VWF:CB we did not further pursue.[69] We published updates on the potential utility of the combined use of the VWF:CB and PFA-100 as a diagnostic strategy when assessing monitoring of DDAVP therapy in 2001,[73] 2006,[136] and 2009,[177] and followed up with our study of evaluating DDAVP therapy responses with VWF:CB and VWF:RCo to help characterize VWD when the diagnosis was less clear.[172] In 2010, we coevaluated several commercial options for VWF:CB, making some recommendations, and also warning against use of some commercial assays that were relatively incapable of distinguishing type 1 versus 2 VWD.[22] In 2012, we clearly showed the differences in the then available VWF activity assays to differentiate HMWM of VWF,[211] a study that we updated in 2018.[275] We continued to compare our VWF:CB with alternative assays over the subsequent years.[14,249] We introduced VWF multimers into our laboratory, and progressed several studies on the relationship between various VWF assays and VWF multimers over subsequent years.[29–32] We clarified our approached to diagnosis of type 2M VWD, a form of VWD that most laboratories do not correctly diagnose, and showed the value of the VWF:CB in the differentiation of types 2A/2B/2M VWD.[26]

The VWF:CB does not replace the VWF:RCo, or any other VWF:GPIbB assay. The VWF:CB and VWF:GPIbB assays reflect different activities, with both activities important in VWF function, and either or both activities potentially defective in different forms of VWD. Accordingly, both assay types are required to enable identification of the full spectrum of VWD. Moreover, in head-to-head comparisons, well-optimized VWF:CB assays tend to show better utility than VWF:GPIbB in the detection of HMWM and discrimination of type 1 (quantitative VWF deficiency) versus types 2A/2B (HMWM deficient) VWD.

Of course, we were not alone in using and promoting the VWF:CB as part of a diagnostic panel for VWD. Perhaps the key reports were those that saw value in the utility of the VWF:CB as part of this panel. Indeed, the United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology saw this utility and included the VWF:CB in its initial testing strategy as part of a four test panel (FVIII:C, VWF:Ag, VWF:CB, VWF:RCo).[324] I think the latest VWD guidelines[18] recommending only a three test panel (FVIII:C, VWF:Ag, VWF:RCo [or VWF:GPIbR or VWF:GPIbM]) as the initial test strategy, and thereby omitting the VWF:CB from early diagnostic use, will continue to see the misdiagnosis of VWD progress into the rest of this decade. Although the guidelines include the VWF:CB as part of the subsequent diagnostic pathway, the misdiagnosis may have already occurred before that step. We have provided evidence in the past, often through the EQA pathway,[15–17,61,70,113] where laboratories that perform a three-test panel are twice as likely to misdiagnose VWD as those performing a four-test panel. Moreover, the VWF:CB, especially when performed by CLIA, was virtually "error-free."[15–17] Finally, the diagnosis of 2M VWD will continue to be under-reported by laboratories not performing the VWF:CB.[2,26,34,80]

Also of some interest is the use of the VWF:CB to help correctly diagnose VWD in animal forms of VWD, especially in dogs.[59,60,1236,179,180]

Finally, the VWF:CB assay has been used in a multitude of studies exploring VWF factor concentrates or recombinant VWF, with the aim to improve therapy for VWD.

Use of the VWF:CB to Diagnose Acquired von Willebrand Syndrome

AVWS is an acquired form of VWD and can arise secondary to several disease processes, including malignancies and myeloproliferative disorders.[1,42,45,49,51,154] However, much more recent focus on AVWS has recently arisen as a result of the increasing use of MCS, such as VAD and ECMO.[156,188,189,207,239,260,273,281,290,292,294,298,320] AVWS can arise due to absorption of VWF, preferentially HMWM, onto artificial surfaces, or due to increased shear stress. The VWF:CB has been increasingly investigated in this arena, and shown to correlate with loss of HMWM, development of AVWS, and also increased bleeding risk.

Use of the VWF:CB to Measure VWF Activity in VWF Factor Concentrates

The VWF:CB was included in studies evaluating the level and VWF activity of VWF concentrates from as early as 1989.[39] Several investigations have been performed since then, including from our laboratory, and also including

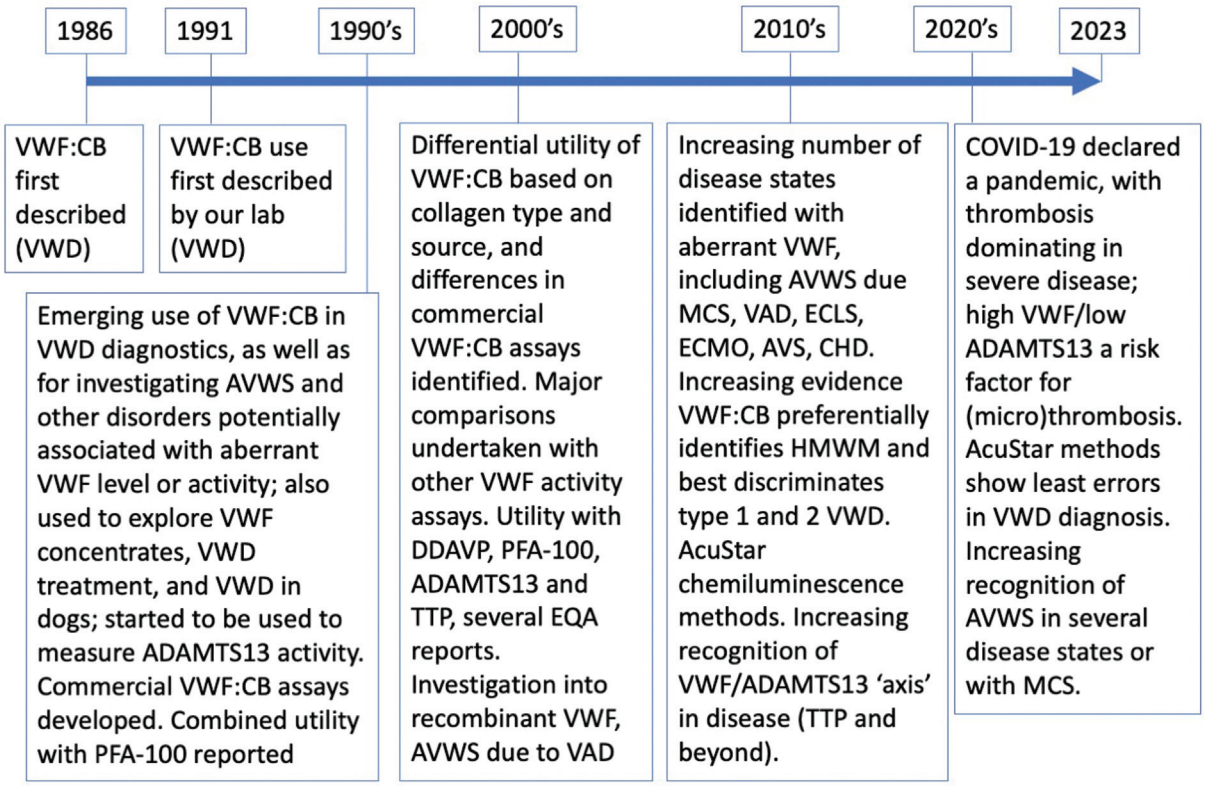


Fig. 5 Timeline of key events in the von Willebrand factor collagen-binding (VWF:CB) 36-year history. ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; AVWS, acquired von Willebrand syndrome; AVS, aortic valve stenosis; COVID-19, coronavirus disease 2019; CHD, congenital heart disease; DDAVP, desmopressin; ECLS, extracorporeal life support; ECMO, extracorporeal membrane oxygenation; EQA, external quality assessment; HMWM, high-molecular-weight multimers (of VWF); MCS, mechanical circulatory support; PFA, platelet function analyser; TTP, thrombotic thrombocytopenic purpura; VAD, ventricular assist devices; VWD, von Willebrand disease; VWF, von Willebrand factor.

recombinant VWF.[48,54,57,96,105,137,146,151,161,183,286,315] Perhaps one of the best such investigations comes from the laboratory of Budde et al,[137] who clearly showed that different VWF concentrates contained different levels of VWF activity, also correlating those activities to VWF multimers, and showing the particular value of the VWF:CB/Ag in these evaluations. More recently, the VWF activity of recombinant VWF, and then recombinant VWF concentrate, has been studied, again showing the particular value of the VWF:CB and the VWF:CB/Ag ratio as a marker of HMWM.[46,57,58,81,175,286,315] The level of HMWM in recombinant VWF concentrate is high since this material has never been exposed to ADAMTS13; this is expected to lead to much higher specific VWF activity in recombinant VWF concentrate compared with plasma-derived VWF concentrate.

Use of the VWF:CB to Measure ADAMTS13 Activity

ADAMTS13, also known earlier as VWF-cleaving protease, is the primary modifier of VWF activity, causing proteolysis of VWF multimers. Loss of ADAMTS13 leads to accumulation of VWF, especially ultra large VWF or HMWM, and is a risk factor for (micro)thrombosis, which manifests as TTP in the most severe ADAMTS13 deficiency state. In early days, the level of VWF activity could be estimated by the action on VWF multimers, or looking at residual multimers after incubation with patient plasma containing (or not) ADAMTS13. These assays were very time consuming and complex to perform, and did not really provide accurate data on ADAMTS13 activity quantification. Accordingly, researchers began to look for alternatives, and one such alternative was the residual VWF:CB as a marker of residual HMWM.[62] This assay could be performed in a fraction of the time that residual multimer testing could, and many more patient samples could be assessed at any given time; moreover, it permitted quantification of ADAMTS13 activity. Of course, more modern options are now available for measuring ADAMTS13 activity, including a chemiluminescence assay on the AcuStar.[134,235,325,326] Nevertheless, I was interested to see that the residual VWF:CB assay was still used for this purpose in some localities (namely China and Thailand) as late as 2018.[270,278]

Use of the VWF:CB to Measure High VWF Activity as a Measure of (Micro)Thrombosis

In part related to low ADAMTS13, leading to elevated HMWM, the VWF:CB has been investigated within studies assessing VWF-driven (micro)thrombosis, both in TTP and outside of TTP. In the latter, the situation with COVID-19 is perhaps most exemplary, since COVID-19 leads to the accumulation of VWF, both due to inflammation and activation of endothelial cells and platelets (representing the sources of plasma VWF), and also the resultant exhaustion of plasma ADAMTS13. Further

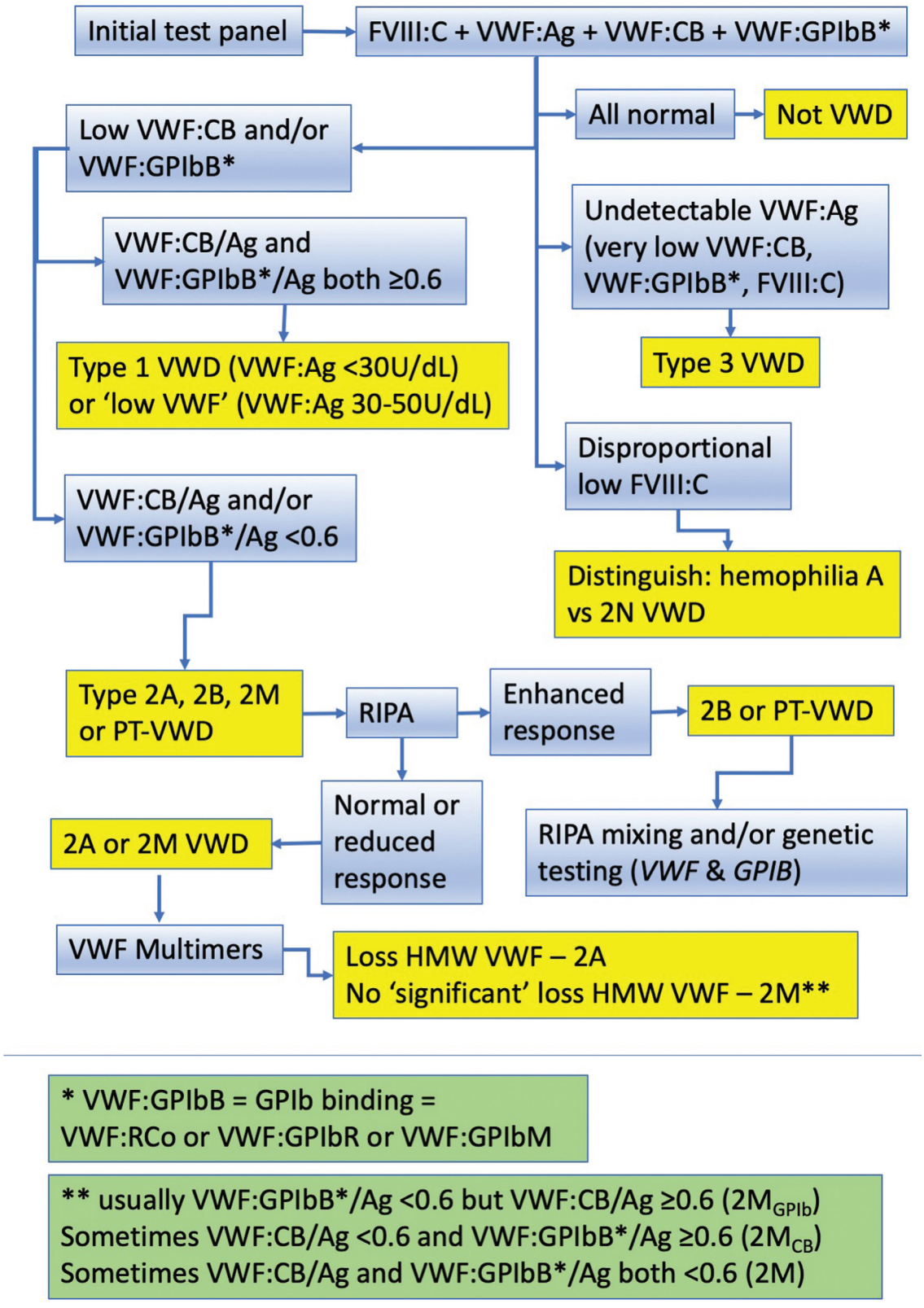


Fig. 6 An algorithmic approach to diagnosis of von Willebrand disease (VWD) and subtype assignment. Shown is our typical laboratory approach to diagnosis of VWD and assignment of subtype. We perform an initial four-test panel. If all test results are normal, then VWD is essentially excluded. If any test result is low, then the possibility of VWD (or hemophilia A) is evaluated according to the algorithm. Ag, antigen; CB, collagen binding; FVIII:C, factor VIII coagulant; GPIb, glycoprotein Ib; GPIbB, glycoprotein Ib binding; GPIbM, glycoprotein Ib "mutant"; GPIbR, glycoprotein Ib "recombinant"; PT, platelet type; RCo, ristocetin cofactor; VWF, von Willebrand factor.

details for COVID-19-related use are available in an earlier review.[302] Additional studies related to this, or the topic of the ADAMTS13/VWF "axis" or relative imbalance in various disease processes, are also evident in the reference list (e.g., references).[92,143,147,152,153,169,184,226,245,262,272,300 302,305,306]

Conclusion

Since the VWF:CB was first reported for use in VWD diagnostics by Brown and Bosak,[35] it has continued to be used to help *correctly* diagnose VWD and also to help assign a *correct* subtype, and also to assist in the monitoring of VWD therapy, especially DDAVP. Moreover, as highlighted in the review, laboratories that do not utilize the VWF:CB are at much greater risk of misdiagnosing patients with VWD, including misdiagnosis of type 1 VWD as type 2 VWD (and vice versa), and misdiagnosis of type 2M VWD as 2A VWD (or as not VWD). However, it is important to recognize that the specific value of any VWF:CB is predicated on the use of an optimized VWF:CB, and that not all VWF:CB assays are so optimized. One assay type that is excellent as a marker of loss of HMWM (e.g., in types 2A and 2B VWD), or as a marker of VWF dysfunction (i.e., 2A, 2B, 2M VWD) is the CLIA-based VWF:CB assay. Nevertheless, there are some good commercial ELISA-based assays also available; conversely, there are probably more "less-optimized" VWF:CB assays out there that may be giving the VWF:CB a bad name. Indeed, the most popular commercial VWF:CB assay is perhaps the least useful of the commercial VWF:CB assays, and certainly not one that I recommend laboratories to use.

In addition to VWD diagnosis and management, the VWF:CB found purpose in a variety of other applications, from assessing ADAMTS13 activity (e.g., in TTP diagnosis), for investigation into AVWS (especially as associated with cardiac assist devices (e.g., VAD, ECMO), to assessment of VWF activity in concentrates or in recombinant VWF, to assessment of VWF level and activity in situations of HMWM excess and (micro)thrombosis risk (e.g., COVID-19), and in partnership with ADAMTS13 in a variety of disorders assessing the VWF/ADAMTS13 axis.

►**Fig. 5** provides a summary of the timeline of utility of the VWF:CB over its nearly 40-year history, and ►**Fig. 6** provides an algorithm that permits identification of VWD, and discrimination of "subtype," using the VWF:CB as part of a four-test panel for laboratory investigation of VWD. The VWF:CB turns 37 in 2023; I wish it a happy birthday, and look forward to at least another 37 years of its ongoing use. I thank the readers who have managed to stick it out to the end! Those of you still wishing to have further insights into the VWF:CB and its history are directed to my earlier reviews.[26,88,136,148,150,255]

Conflict of Interest
None declared.

Acknowledgments
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