Diagnosing von Willebrand Disease: A Short History of Laboratory Milestones and Innovations, Plus Current Status, Challenges, and Solutions*

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Abstract

von Willebrand disease (VWD) is a disorder characterized by deficiency of, or defects in, von Willebrand factor (VWF). VWD was originally identified by Erik Adolf von Willebrand, who in early 1924 investigated a large family suffering from a bleeding disorder that seemed to differ from hemophilia. Erik von Willebrand undertook some initial laboratory investigations to conclude the involvement of a plasma factor, the lack of which prolonged the bleeding time, but failed to impair coagulation times and clot retraction. By the end of the 1960s, VWD was accepted as a combined deficiency of factor VIII (FVIII) and another plasma factor responsible for normal platelet adhesion. Just how these two functions were related to each other was less clear and the diagnostic tests available at the time were poorly reproducible, cumbersome, and unreliable; thus, VWD was poorly delineated from other coagulation and platelet disorders. The early 1970s saw a revolution in diagnostics when ristocetin was identified to induce platelet aggregation, and this formed the basis of the first consistent and reliable VWF "activity" test, permitting quantification of the platelet adhesive function missing in VWD. Concurrently, immunoprecipitating techniques specific for VWF were defined, and the application of such technologies permitted a clearer understanding of both VWF and VWD heterogeneity. Continued exploration of the structure and function of VWF contributed greatly to the understanding of platelet physiology, ligand receptor interaction and pathways of cellular interaction and activation. Recently, additional assays evaluating other functions of VWF, including collagen binding, platelet glycoprotein Ib binding, and FVIII binding, have improved the diagnosis of VWD. The purpose of this narrative review is to explore the history of phenotypic VWD diagnostics, with a focus on laboratory milestones from the past as well highlighting recent and ongoing innovations, and ongoing challenges and possible solutions.

Keywords
- von Willebrand disease
- diagnosis
- von Willebrand factor
- laboratory testing
- ristocetin cofactor
- collagen binding
- VWF activity
- history

von Willebrand disease (VWD) is a bleeding disorder now recognized to be characterized by deficiency of, or defects in, an adhesive plasma protein called von Willebrand factor (VWF). This recognition, however, took many decades to evolve. This narrative historical review aims to highlight many of the important milestones in this journey, as well as recent and ongoing innovations. This article is dedicated to one of my mentors, Prof. Jerry Koutts (►**Fig. 1**), who recently passed away, as recently

* *Dedicated to the memory of Prof. Jerry Koutts (1944–2013).*

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Fig. 1 Jerry Koutts (in memoriam, 1944–2013).

reported in this journal.[1] In a fitting tribute to his memory, I have purposely and considerably borrowed from an earlier historical review that he penned for *Seminars in Thrombosis & Hemostasis* in 2006, and which in turn was dedicated to two of his own mentors.[2]

Early History of von Willebrand Disease

The disorder we now recognize as VWD was originally identified by Erik Adolf von Willebrand.[3,4] In 1924, he assessed a girl, aged 5 years, who presented at his Helsinki clinic with a history of recurrent severe mucosal bleeding, and who, according to the parents, was one of several female and some male family members who were bleeders. Physical examination of the young patient was normal apart from scattered small hematomas. Her blood count was also normal, apart from slight anemia and slight thrombocytopenia. Her clotting time and clot retraction were (perhaps unexpectedly) both normal, but her bleeding time (Duke) lasted more than 2 hours. Erik von Willebrand considered that the disorder was due to platelet dysfunction coupled with a defect of the vessel walls, and appeared to constitute a new bleeding entity with hereditary nature. He published his first article on the disease, which would much later bear his name, in 1926. His article includes a brief review of the hemorrhagic diathesis, which was notably distinct from "genuine" hemophilia, describes 58 individuals in a pedigree of two interrelated families spanning four generations, as well as an analysis of the heredity involvement (►**Table 1**).

That this "new" bleeding disorder was clinically serious was evidenced early on in this history. Several sisters of the index case had previously died from mucosal bleeds, and even the index case died at the age of 13 years with her fourth menstrual period. In his subsequent publications, Erik von Willebrand gave a detailed description of the disorder as being probably autosomal dominant in inheritance, with nose, wound, and menstrual bleeding the predominant features. He emphasized that hemarthrosis was rare, the skin bleeding time was prolonged, and these features differentiated it from classical hemophilia. The clinical picture of VWD was independently reported by Minot in the United States in 1928.[5]

Despite some early progress, it was appreciated even in the early 20th century that clinical manifestations of VWD were variable and inheritance was inconsistent. The diagnostic tests available in the early to mid-1900s, particularly of platelet function, included the skin bleeding time,[6,7] and various platelet adhesion tests[8–10] all of which were nonspecific, poorly reproducible, unreliable, cumbersome, time consuming,[11,12] and ultimately determined to be poor predictors of bleeding.[12,13]

Reliable methods of measuring FVIII coagulation function (FVIII:C) were first developed in the mid-1900s.[14,15] The finding that FVIII:C was deficient in both "classical hemophilia" plus the clinically heterogeneous condition later defined to be VWD caused further diagnostic confusion, and the disorder was called "pseudo hemophilia" or "vascular hemophilia," as most workers at the time believed that the prolonged skin bleeding time was because of some defect in the capillary vessels.[16–19] In the late 1950s, it was also demonstrated that one of the newly developed plasma fractions (called Cohn Fraction 1, which contained FVIII) corrected the prolonged skin bleeding time. Importantly, Cohn Fraction 1 prepared from hemophilic plasma was equally effective, confirming that the bleeding disorder later termed VWD was because of a deficiency in a plasma factor that was present in normal as well as in hemophilic plasma. Key workers at the time also observed that the prolonged increase in FVIII:C after plasma infusion characteristic of VWD was different to the linear response in patients with hemophilia A.[20,21] However, most of these observations were difficult to explain and essentially fuelled further confusion at that time. It was also not understood how a plasma factor could correct the skin bleeding time and improve primary hemostasis.

In 1961, Borchgrevink[8] demonstrated decreased platelet adhesion in VWD, and in 1963 Salzman[9] reported decreased platelet adhesion to glass beads, which was corrected by both normal and hemophilic plasma. By the end of the 1960s, it became accepted that VWD represented a combined deficiency in FVIII and another (as yet unidentified) plasma factor responsible for normal platelet adhesion. However, other than the measurement of FVIII:C, there was no reliable and consistent method of separating out these activities, or of measuring the "platelet component" of this combined functionality. It was also unclear just how these two functions were related to each other, and particularly whether they represented a combined function of one molecule, or separate functions of two molecules. Essentially, the lack of either sensitivity or specificity of the diagnostic tests available at the time resulted in the condition being poorly delineated from other coagulation and platelet disorders.[11]

Table 1 A short history of von Willebrand disease diagnostics: selected milestones

Milestone	References
Erik Adolf von Willebrand initiates the investigation of this "new" bleeding disorder	1924 (published in 1926)[3,4]
Independent recognition of VWD in the United States	Minot (1928)[5]
Skin bleeding times	Duke (1912)[6]; Ivy et al (1941)[7]
Platelet adhesion test	Borchgrevink (1961)[8]
Glass bead adhesion tests	Salzman (1963)[9]; Bowie et al (1969)[10]
Assays for factor VIII coagulant (FVIII:C) activity	Biggs et al (1955)[14]; Hardisty and Macpherson (1962)[15]
Platelet aggregation	Born (1962)[22]
The "birth" of VWF "antigen" testing	Zimmerman et al (1971, 1975)[65,66]; Peake and Bloom (1977)[67]
FVIII and VWF recognized as separate molecules, and VWF recognized to stabilize and protect FVIII:C function	Weiss and Kochwa (1970)[98]; Weiss and Hoyer (1973)[99]; Weiss et al (1977)[106]; Owen and Wagner (1972)[100]; Zimmerman and Edgington (1976)[101]; Koutts et al (1976, 1977, 1979)[102,103,105]
RIPA	Howard and Firkin (1971)[25] and Howard et al (1973)[26]
VWF:RCo	Weiss et al (1973)[31]; Jenkins et al (1974)[32]; Olson et al (1975)[33]
The "birth" of VWF multimer analysis	van Mourik and Mochtar (1970)[68]; Counts et al (1978)[69]; Hoyer and Shainoff (1980)[70]
Patients with increased responsiveness of VWF to ristocetin identified (later assigned to type 2B VWD)	Ruggeri et al (1980)[37]
Patients with increased responsiveness of platelet GPIb to ristocetin identified (later assigned to PT-VWD)	Takahashi (1980)[42]; Miller and Castella (1982)[43]; Weiss et al (1982)[44]
Ristocetin used to help define the interaction of VWF with platelet GPIb	Phillips et al (1980)[47]; Coller et al (1983)[48]
VWF-FVIII binding defect (type 2N VWD) identified	Nishino et al (1989)[107]
VWF:CB assay first described	Brown and Bosak (1986)[108]
VWF:CB assay refined by Westmead laboratory	Favaloro et al (1991–current)[109–121]
VWF:CB assay by flow cytometry	Kempfer et al (1999)[131]; Mina et al (2012)[132]
Simplified/revised classification of VWD partially based on phenotypic test results, then rerevised partially based on more comprehensive phenotypic test results	Sadler et al (1994)[34]; Sadler et al (2006)[41]
NHLBI Expert Panel VWD Guidelines	Nichols et al (2008)[57]
VWF:RCo platelet agglutination assay automated	Miller et al (2002)[134]; Lattuada et al (2004)[134]; Redaelli et al (2005)[135]; Strandberg et al (2006)[136]
VWF:RCo automated platelet agglutination assay refined to improve lower limit of VWF detection	Hillarp et al (2010)[139]; Favaloro et al (2010)[140]
VWF:RCo assay by ELISA	Vanhoorelbeke et al (2000, 2005)[141,143]; Federici et al (2004)[142]
VWF:RCo assay by flow cytometry	Giannini et al (2007)[144]; Chen et al (2008)[145]
VWF:RCo assay using latex-enhanced method, and development of alternative VWF activity assays and technical methods including chemiluminescence	Stufano et al (2014)[146]
Development of alternative VWF activity assays based on GPIb binding	Flood et al (2011)[148]; Lawrie et al (2013)[150]; Graf et al (2014)[151]

Abbreviations: CB, collagen binding; ELISA, enzyme-linked immunosorbent assay; FVIII, factor VIII; GPIb, glycoprotein Ib; NHLBI, National Heart, Lung, and Blood Institute; PT, platelet type; RCo, ristocetin cofactor; RIPA, ristocetin-induced platelet aggregation; VWD, von Willebrand disease; VWF, von Willebrand factor.

Ristocetin, Laboratory Tests Developed from Ristocetin, and Initial Important Advances in VWD Diagnostics

In the late 1960s, some early Australian-based pioneers in the hemostasis field, Margaret Howard and Barry Firkin, began to systematically study agents that had been reported to cause thrombocytopenia, using the relatively newly described technique of platelet aggregometry.[22] One of the agents tested was ristocetin, an antibiotic prepared from the fermentation broth of Nocardia lurida, and active against gram-positive cocci, including penicillinase-producing organisms.[23] Interestingly, ristocetin was withdrawn from clinical use as an antibiotic because of an associated incidence of thrombocytopenia of up to 20% that appeared to be dose related.[24,25] This would have likely caused the demise of ristocetin, had not Howard and Firkin pioneered an alternative utility for the antibiotic.

Notably, they reported in 1971 that ristocetin caused platelet aggregation in normal platelet-rich plasma, but not in two of three patients with VWD.[25] They also later demonstrated that VWD platelets aggregated to ristocetin with the addition of normal or hemophilic plasma.[26] Ristocetin-induced platelet aggregation (RIPA) defects were subsequently reported in a variety of acquired platelet disorders including idiopathic thrombocytopenic purpura, acute leukemia, infectious mononucleosis, and in the inherited disorder Bernard–Soulier syndrome.[27–30] Only in VWD, however, was this defect corrected by the addition of normal plasma, pointing to deficiency of a functional plasma component in VWD.

Later, ristocetin was also shown to aggregate washed platelets in the presence of normal plasma. This finding was rapidly developed into a quantitative assay using platelet aggregometry with either washed or fixed platelets.[31–33] This activity was originally designated as VIIIR:Rco and later VWF:RCof[34] but is currently known as VWF:RCo or the ristocetin cofactor assay.[35] The development of what was then considered to be a reliable quantitative assay provided the impetus to investigate the clinical heterogeneity of VWD and define, at least initially, two distinct groups—a severe form with no detectable VWF activity in plasma and which appeared to be usually, but not invariably, inherited as an autosomal recessive disorder (i.e., homozygote) and a less severe heterozygous form.[36] Many variant patterns were later described, the nature of which became clearer as an understanding of the relationship between the VWF protein (or antigen) and its various functions gradually evolved (see later sections).

In 1980, Ruggeri et al[37] described some patients that showed an increased responsiveness to ristocetin, and this was subsequently shown to be due to "gain of function mutations" in which VWF expresses an increased affinity for the platelet ligand receptor, later identified to be glycoprotein Ib (GPIb).[38–40] The higher affinity, larger molecular weight forms of VWF are disproportionately decreased in the plasma of this type of VWD, now designated "type 2B" VWD,[41] and (generally mild) thrombocytopenia may also develop as a result of the unregulated interaction and subsequent clearance of both VWF and platelets from the circulation.

Another condition associated with enhanced interaction between VWF and platelets at low concentrations of ristocetin was also subsequently identified, and this was designated "platelet-type" or "pseudo-" VWD.[42–44] In these cases, the VWF is normal, and instead mutations of the platelet GPIb receptor locus lead to an increased affinity between VWF and the platelet.[45,46]

Given that type 2B VWD and platelet-type (PT) VWD are both characterized by enhanced RIPA, but reduced levels of VWF:RCo and high molecular weight (HMW) forms of VWF as well as thrombocytopenia, they represent disorders that can be difficult to differentiate without additional testing strategies (this is explored in later sections).

Ristocetin subsequently became a tool to investigate the interaction between the VWF molecule and the platelet, which was shown to occur at the GPIb/IX platelet receptor complex.[47,48] Bernard Soulier platelets, which also characteristically failed to aggregate to ristocetin, were shown to have a deficiency of this receptor complex, notably of the GPIb molecule.[49,50] Conversely, tryptic digestion of the VWF molecule and the identification of VWF fragments that inhibited RIPA provided the initial characterization of the GPIb binding loci on the VWF molecule.[51]

The laboratory use of RIPA therefore provided many initial insights into the structure and function of VWF and its platelet receptor, GPIb, and helped to explain some of the functional abnormalities observed in some patients, particularly those with "variant" VWD. The interaction induced by ristocetin between VWF and the platelet represented one of several surrogates for the actual physiological events, since under normal circumstances, VWF does not interact directly with platelets.[52,53] The physiological equivalent of ristocetin appears to be high shear forces in the circulation, as may occur in the microcirculation or within pathologically narrowed arteries.[54,55] Under such shear forces, VWF undergoes conformational change that permits high affinity interaction with the platelet GPIb receptor, initiating – when "conditions are right" - intracellular signaling leading to activation of GPIIb/IIIa and consequently platelet aggregation due to GPIIb/IIIa binding with fibrinogen and VWF. At sites of vascular injury, VWF binding to exposed collagen appears to create the conformational changes leading to VWF-GPIb/IX interaction, and this both anchors the platelet to the site of injury and activates the platelets, inducing further aggregation and the formation of a hemostatic plug. This additional VWF activity (binding to collagen) provides the in vivo correlate to another test of VWF function now commonly used in laboratory test practice, the collagen binding assay, as explored later in this review.

As diagnostic tools, tests utilizing ristocetin have many limitations, as discussed in more detail later in this review. Nevertheless, RIPA studies using ristocetin at low concentrations with the patient's platelet rich plasma are mandatory for the diagnosis of type 2B and PT-VWD variants.[41,56,57] Measurement of VWF:RCo is also generally satisfactory for the diagnosis of severe and moderately severe VWD. However, the coefficient of variation of this test is very high, inter-laboratory reproducibility and standardization is

poor, and the lower limit of sensitivity for VWF is also poor.[58–64] Consequently, the discriminatory power of this test between normal and mild VWD is poor, discrimination between type 1 versus type 2 VWD is compromised, as is type 3 versus severe type 1 VWD discrimination. Many attempts to improve the performance of VWF:RCo tests have been promoted, including modifications of assay set ups on automated instrumentation, new tests using recombinant GPIb fragments or antibodies against functional epitopes of the VWF molecule, and these are discussed in greater detail later in this review. Alternate tests, independent of ristocetin, have also been developed, as also explored later.

Factor VIII and von Willebrand Factor "Antigen": The Confusion Continues, then Gives Rise to the Birth of Multimer Analysis

In 1971, Zimmerman et al[65] raised a rabbit antisera against human FVIII–enriched material, which both neutralized FVIII:C and precipitated what appeared to be a single protein from human plasma. They demonstrated material quantitatively and antigenically similar to this "FVIII", by immunoelectrophoresis, in the plasma of all 22 hemophiliac patients tested. In contrast, this antigen was present in reduced amounts in the plasma of each of 11 patients with VWD. These workers initially suggested that VWD was a disorder of a true deficiency of FVIII whereas in classic hemophilia, nonfunctioning antigen was produced. This conclusion would only be valid if the two functions resided on the same molecule. This hypothesis was later proved to be incorrect.

Irrespective of this speculation, the application of reliable immunoprecipitating techniques such as the Laurell quantitative immunoelectrophoresis, and of two-dimensional immunoelectophoresis[66,67] rapidly changed the diagnostic criteria for VWD and provided a major contribution to the understanding of the molecular structure, synthesis, and tissue localization of VWF, and its discrimination from FVIII.

By the mid-1970s, with improved purification techniques,[68] it was demonstrated that plasma VWF was actually composed of a series of multimers, ranging from approximately 500,000 Da (which subsequently proved to be dimers of the basic VWD subunit) to over 20,000,000 Da.[69,70] Functional assays with RIPA indicated that the HMW forms had disproportionately higher platelet aggregating activity.[71–74]

This evaluation of VWF multimers saw a burst of reevaluation into VWD, and the identification of many "variant forms" of VWD,[36,75–81] which was subsequently rationalized into an accepted, clinically relevant, classification and common nomenclature,[34] comprising a primary group of three VWD types (1, 2, and 3), and a secondary subgroup of four type 2 subtypes (2A, 2B, 2M, and 2N). At this time, the VWF antigen was measured by immunoprecipitating techniques, usually involving rabbit polyclonal antihuman VWF, and this test came to be called VWF:Ag (VWF "antigen"). Subsequently, combining measurement of VWF activity (initially using VWF:RCo and later including VWF:CB) with VWF:Ag showed that normally, and in the majority (~70–80%) of patients with VWD, there was assay concordance, reflecting lower VWF:Ag but essentially with normal activity. This group of patients with VWD were designated "type I" and later "type 1" VWD.[34]

A small proportion of severely affected patients had very little (< 2%) or no detectable levels of VWF:RCo or VWF:Ag. These patients were designated type III and later type 3 VWD.[34] Most of these patients could be shown to have autosomal dominant inheritance, although the clinical manifestations in related family members were extremely variable and up to half of obligatory carriers were asymptomatic.[82–85] Until recently, no consistent mutation was identified for the majority of patients with type 1 VWD. In the severe type 3 patients, nonsense and frame-shift mutations, large gene deletions, and mRNA defects have been described. Most of them are homozygous, some compound heterozygous. Such patients may develop alloantibody inhibitors to the VWF after replacement therapy.[86]

The remaining VWD patients demonstrate a VWF:RCo (and/or VWF:CB and/or other VWF activity assay) level which is disproportionately reduced compared with normal, near normal, or low levels of VWF:Ag. This group with discordant levels were originally labeled as "Variant", later as "type II", and now as "type 2" VWD.[34,41] Many cases of type 2 VWD were later shown to be particularly deficient in the more biologically active HMW multimers of VWF,[87] and these are now classified as type 2A VWD.[34,41] The reduction in HMW multimers in these patients was shown in most cases examined to be the result of mutations that cause either impaired intracellular transport, or enhanced proteolysis by specific proteases or defective posttranslational processing.[88–90]

On the contrary, type 2M VWD patients may produce adequate HMW multimers, but the VWF produced is otherwise functionally defective.[91–95] These patients still gave discrepant results between VWF activity and antigen assays, but the patterns observed can be distinguished from type 2A VWD.

Type 2 VWD also includes the group, now designated type 2B, that have reduced HMW multimers as a result of enhanced affinity of the VWF (particularly the HMW forms) for the GPIb receptor, as described in the previous section.[37–40] The specific mutations are located on restricted regions of the A1 domain of the genome. Details of the mutations of the VWF molecule are now available on an online database.[96,97]Type 2 VWD also includes a group of patients that suffer from defective VWF–FVIII binding, now called type 2N VWD, as covered in the next section.

The Factor VIII–VWF Relationship Further Explored and Type 2N von Willebrand Disease Identified

Another important piece in the VWD jigsaw puzzle was the developing understanding of the relationship between the coagulation activity (FVIII driven = FVIII:C) and the platelet function activity of VWF, as well as the later definition of a disorder that involved this relationship.

For the early investigators, the presence of both functional abnormalities in the single inherited condition of VWD, particularly as evident in the more severe forms of VWD

such as type 3 VWD, and the copurification and coimmunoprecipitation of the two activities with rabbit antisera raised against fractions enriched in FVIII:C activity, clearly suggested a single entity. This assumption, that a single molecule expressed both activities, became increasingly untenable. This model neither was consistent with several clinical syndromes described at that time nor was it consistent with the results of transfusion studies in patients with VWD, and with findings that megakaryocytes and endothelial cells synthesized just the platelet functional component of VWF, but without FVIII.

Original reports of experimental dissociation of FVIII from VWF were based on high ionic strength[98–102] or affinity chromatographic separation,[101–103] and these were followed by further purification and binding studies which have mapped the binding sites between FVIII and VWF[104] that also identified the separate genetic locus and site of synthesis of FVIII. Several authors at the time also provided evidence suggesting that the binding of FVIII to VWF provided stabilization or protection of the biological function of FVIII.[105,106] In a fitting tribute to a recently passed colleague to whom this current review is dedicated, readers should note that Jerry Koutts (►**Fig. 1**) was one of the early pioneers who provided evidence that VWF and FVIII are composed of separate molecules.[102,103,105]

A specific inherited defect at the VWF binding site between VIII and VWF has since been elucidated,[107] which probably explains some of the earlier reported cases of "autosomal" hemophilia. FVIII, when unbound to VWF, is subject to accelerated proteolysis, resulting in a syndrome, which is autosomal recessive in inheritance, with mild-to-moderate reduction in FVIIIC levels and low-to-normal VWF:Ag and VWF:RCo levels, but with low-relative FVIII:C/VWF:Ag. This condition phenotypically resembles hemophilia A, and is now termed as type 2N VWD.[34,41]

Investigation of 2N VWD now involves a sequential process that combines personal and family histories with extended phenotypic testing, as well as potentially genetic testing. In essence, the basic phenotypic test profile including FVIII:C, VWF:Ag, and VWF:RCo will be similar for hemophilia A and type 2N VWD. However, family histories are often different, given the sex-linked inheritance nature of hemophilia A versus the autosomal recessive inheritance nature of 2N VWD. Discrimination of hemophilia A and 2N VWD involves performance of a specific VWF-FVIII binding assay, or else genetic testing of the *VWF* and *FVIII* genes to identify which is affected. This is explored toward the end of this review.

Collagen Binding and the Collagen-Binding Assay

As explored at the beginning of this historical review, the VWF:RCo represented the first truly useful and reasonably reproducible VWF activity assay that helped identify different types of VWD. The use of ristocetin also provided experimental means to identify the interaction between VWF and its platelet receptor, GPIb. VWF:RCo in essence represented a surrogate for VWF-platelet–GPIb interaction, with ristocetin acting to alter the VWF conformation and bind to platelets, as partly analogous to shear stress-induced VWF conformation changes (with apologies here to the purist scientists for the oversimplification). However, it became increasingly recognized that platelet GPIb binding represented just one function of VWF, as does FVIII binding, collagen binding, etc. Indeed, as previously highlighted, collagen binding—representing in vivo binding of VWF to damaged subendothelial matrix—will lead to later platelet recruitment, activation, and aggregation, leading to formation of the platelet plug.

The collagen binding ability of VWF was initially explored in a laboratory assay by Brown and Bosak in 1986.[108] These workers used an enzyme-linked immunosorbent assay (ELISA) design and a single source of collagen, a single collagen-coating procedure, and a small number of VWD patient samples, to investigate the potential utility of this assay to assist in the diagnosis of VWD. Their results indicated broad comparability of VWF:CB and VWF:RCo, and hence limited additional utility for VWF:CB over performance of VWF:RCo. The VWF:CB story could therefore have ended there, except for the encouragement for me to pursue the assay further from my then "bosses", Jerry Koutts, the Clinical head of our unit, to whom this review is dedicated (►**Fig. 1**), and Thomas Exner, the then Principle Scientist of our unit. Some of this history is explored elsewhere.[1] Our first article on this assay appeared in 1991,[109] with many others following over the subsequent decades.[58,110–124] Several reviews on the VWF:CB, including comparative evaluations with other VWF activity assays, have also been published by this author.[121–128] Contrary to the original report from Brown and Bosak, we saw many advantages for the VWF:CB over the VWF:RCo, and we also recognized that the utility of the VWF:CB was highly dependent on the assay design, something that Brown and Bosak did not explore, and which has also recently become recognized for VWF:RCo (see the next section). In brief, the source of collagen and how it is coated onto 96-well plates, as well as the source and types of plates themselves, are each critical for the optimized utility of this assay. There are now several different commercial options for VWF:CB, but even today, these differ in terms of diagnostic utility.[120]

Others have also reported on the utility of the VWF:CB, as has been extensively reviewed elsewhere.[121–128] In brief, the VWF:CB has several strengths. Directly compared with the VWF:RCo, an optimally designed VWF:CB has reduced variability, better sensitivity to HMW VWF, and better sensitivity to low levels of VWF. This is also true when considering interlaboratory data. Thus, compared with use of the VWF:RCo, the error rate in misdiagnosis of VWD because of assay issues will be approximately halved by use of the VWF:CB.[61,62,64] However, the true value of the VWF:CB lies in its use as a companion assay to the VWF:RCo, or to another GPIb-binding assay surrogate. Combining the VWF:CB with the VWF:RCo (or similar assay) will lead to a reduction in VWD misdiagnosis error rates, as well as providing a firmer diagnostic strategy for VWD investigations, particularly when combined with a desmopressin (DDAVP) trial.[111,117,118] This is summarized toward the end of this review, and reflects a

combination of favorable technical strengths as well as reflecting a distinct activity of VWF. In summary, rather than being a replacement for VWF:RCo, the VWF:CB when combined with VWF:RCo will strengthen VWD diagnostics and will in fact be able to replace performance of multimer analysis for the vast majority of VWD investigations.[129,130] Although alternate procedures have been explored for performance of VWF:CB, for example, by flow cytometry,[131,132] most laboratories continue to perform this assay by ELISA.

Improving Ristocetin Cofactor Assays and Development of Alternative von Willebrand Factor: Glycoprotein Ib–Binding Assays

The major recent improvements in performance of VWF:RCo assays comprised of the following: (1) development of automated assays that utilized common coagulation equipment to replace the manual semiquantitative visual agglutination and semimanual platelet aggregometer-based methods to reduce performance time and complexity and improve reproducibility,[133–138] and (2) improving the assay design to improve low level VWF sensitivity.[139,140]

Alternate assay designs for VWF:RCo have involved either performance by ELISA[141–143] or flow cytometry.[144,145] However, none of these alternative assay designs have been taken up in routine practice, which still today largely comprise platelet aggregation, mainly using automated assays on routine coagulation equipment, or increasingly less commonly by platelet aggregometry. Nevertheless, the development and theoretical advantages of some of these assays can be noted.[141–145] Recently, however, newer assays based on latex agglutination (rather than platelets), and a recombinant form of GPIb, have been marketed and these have several theoretical advantages to the platelet-based assay, including better standardization, reduced variability, and improved lower limit of VWF detection.[146] One such methodology involves chemiluminescence technology.[146]

Recognized limitations to the VWF:RCo also saw the development of alternative GPIb-binding (VWF activity) assays.[147–150] In addition to the previously noted limitations, it also became recognized that VWF-ristocetin–binding variants were apparently common in the African American population, and this could lead to their potential false identification as type 2A or type 2M VWD.[147]

Most recently, a novel GPIb binding assay has been released that utilizes two gain of function GPIb mutations in a recombinant GPIb molecule bound to latex particles to permit spontaneous binding of native VWF in a ristocetin-independent manner.[150,151] The utility of this assay in VWD diagnostics has surprisingly been underexplored in the literature, with only two publications till date and despite several meeting presentations in previous years. Although this assay does not completely replicate the VWF:RCo assay, it does provide broadly similar test findings in the majority of cases cotested, and it does have several advantages over classical VWF:RCo. Actual and theoretical advantages include the following: (1) reduced assay variation; (2) improved low level VWF detection; (3) simpler assay design; and (4) no interference from VWF-ristocetin–binding variants apparently common in the African American population. On the contrary, even the limited comparative evaluations till date have identified some discrepancies between VWF:RCo and this new assay is a small subset of patients, suggesting that replacement of VWF:RCo with the new assay may as yet be premature, and which assay better reflects the VWF dysfunction in these patients is not as yet entirely clear.

Development of Other von Willebrand Factor Activity Assays

Recognized limitations to the VWF:RCo also saw the development of alternative VWF "activity" assays over the past decades. One of these was based on a monoclonal antibody (MAB) that bound to VWF at the epitope that otherwise bound to platelet GPIb. This MAB therefore recognized some of the VWF structural variants that would now be classified into type 2A or 2M VWD, due to altered VWF structure reducing the binding of the MAB. The assay was also shown to have some sensitivity to HMW VWF. The original assay was developed as a radioimmuno assay, and later as an ELISA assay in various formats that were commercialized and then used by laboratories for several years.[152–155] Later, the principle was utilized to develop a commercial latex-based assay.[156–162]

Our own experience with these MAB-based "VWF activity" assays, either in direct comparisons or else by evaluation of cross-laboratory test data suggests that the MAB-based assays have both advantages and disadvantages in VWD diagnosis.[64,115,116,163] In particular, the assays seem to be less sensitive to HMW VWF, and thus to the presence of type 2A VWD, than either VWF:RCo or VWF:CB.[64,163] Although assay variation is less than VWF:RCo, it is sometimes higher than VWF:CB[64]; similarly, while sensitivity to low levels of VWF is often better than VWF:RCo, it is generally not as good as VWF:CB.[63,64] Nevertheless, the current latex-based assay does show comparable findings to VWF:RCo for the majority of cases tested, and because of automation and broad availability, it is still used successfully as a screening assay instead of VWF:RCo by some laboratories.[64,161,162]

Current Status of Diagnostic Testing for von Willebrand Disease

A summary of the phenotypic characteristics of each subtype of VWD is given in ►**Table 2**, and an algorithm that describes my laboratory's current approach to laboratory-aided diagnosis and typing of VWD is shown in ►**Fig. 2**.

In brief, type 1 VWD is identified by lowered levels (i.e., less than ~ 50 U/dL) of "functionally normal" VWF, which can be identified by normal VWF "adhesive" activity to VWF antigen ratios. Essentially, all such ratios, be they VWF:RCo/Ag, VWF:CB/Ag, VWF:Ac/Ag, or other GpIb-binding assay mimic/Ag, will be normal. What may differ between assays and laboratories is the actual normal/qualitative defect ratio cut off value, which will usually range from 0.5 to 0.7. However, it is important to note that the actual cut off value may be different between different VWF activity assays, as

Table 2 A practical guide to identification of VWD and VWD type

VWD type	VWF:Ag	VWF:RCo[a]	VWF:CB	FVIII:C	Multimers	RCo/Ag[a]	CB/Ag	FVIII/VWF	Comments
1	↓to ↓ ↓	↓to ↓ ↓	↓to ↓ ↓	N to ↓ ↓	Normal pattern but reduced intensity	> (0.5–0.7)	> (0.5–0.7)	> (0.5–0.7)	VWF levels between ~30 and 50 U/dL will generally not be associated with *VWF* mutations and can be considered as representing "low" VWF as a risk factor for bleeding. VWF levels below ~30 U/dL will often be associated with *VWF* mutations and can be considered as representing "true" VWD.
2A	↓to ↓ ↓	↓ ↓ to ↓ ↓ ↓	↓ ↓ to ↓ ↓ ↓	↓to ↓ ↓	Loss of HMW VWF	< (0.5–0.7)	< (0.5–0.7)	> (0.5–0.7)	2A and 2B VWD can only be distinguished by means of RIPA. PT-VWD phenotypically resembles 2B VWD; these can be distinguished by means of RIPA mixing studies, or by genetic analysis of *VWF* and/or platelet *GPIb* genes.
2B	N to ↓ ↓	↓ to ↓ ↓ ↓	↓ to ↓ ↓ ↓	N to ↓ ↓	Loss of HMW VWF	< (0.5–0.7)	< (0.5–0.7)	> (0.5–0.7)	
2N	N to ↓ ↓	N to ↓ ↓	N to ↓ ↓	↓ ↓to ↓ ↓ ↓	Normal pattern	> (0.5–0.7)	> (0.5–0.7)	< (0.5–0.7)	Phenotypically similar to hemophilia A; distinguish using VWF:FVIII binding assay or genetic analysis of *FVIII* and/or *VWF* genes.
2M	↓to ↓ ↓	(↓ to ↓ ↓ ↓)	(↓ to ↓ ↓ ↓)	↓to ↓ ↓	No loss of HMW VWF; some multimer defects may however be observed	< (0.5–0.7) (platelet binding defect) or > (0.5–0.7) (collagen binding defect)	< (0.5–0.7) (collagen binding defect) or > (0.5–0.7) (platelet binding defect)	> (0.5–0.7)	2A and 2M VWD can only be distinguished by comprehensive or composite panel testing.
3	↓ ↓ ↓ (absent)	↓ ↓ ↓ (absent)	↓ ↓ ↓ (absent)	↓ ↓ ↓	No VWF present	NA	NA	NA	Type 3 VWD can only be identified when VWF tests are performed and these are sensitive to very low levels of VWF.

Abbreviations: Ag, antigen; CB, collagen binding; FVIII, factor VIIII; GPIb, glycoprotein Ib (the platelet VWF receptor); HMW, high molecular weight; N, normal; NA, not applicable; RCo, ristocetin cofactor; RIPA, ristocetin-induced platelet aggregation; VWD, von Willebrand disease; VWF, von Willebrand factor.

[a]Other VWF GPIb binding 'activity' assays (including the Siemens VWF:Ac assay) will provide results that will most closely resemble VWF:RCo.

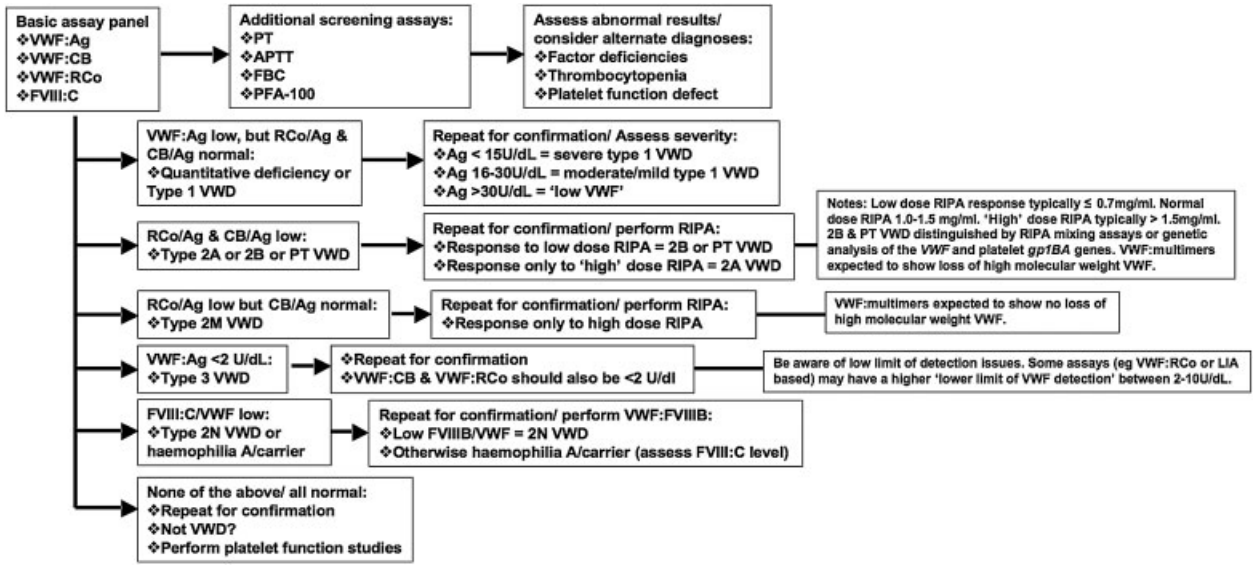

Fig. 2 An algorithm that describes our current laboratory approach to the laboratory aided identification and diagnosis of VWD and VWD type. Ag, antigen; APTT, activated partial thromboplastin time; CB, collagen binding; FBC, full blood count; FVIII:C, factor VIII coagulant (activity); FVIIIB, factor VIII bound (to VWF in a VWF:FVIII binding assay); LIA, latex-based immunoassay; PFA, platelet function analyzer; PT, prothrombin time (assay) or platelet-type (VWD); RCo, ristocetin cofactor; RIPA, ristocetin induced platelet aggregation; VWD, von Willebrand disease; VWF, von Willebrand factor.

well as between different methods of the same activity assay. For example, in our most recent exploration from the RCPA-QAP,[64] a cut off of 0.6 to 0.7 would be more generally applicable for VWF:CB/Ag and VWF:Act/Ag, but a more effective cut off for VWF:RCo/Ag would be 0.5 to 0.6. Similarly, for VWF:CB, our in-house method cut off would also be around 0.6 to 0.7, but for some commercial VWF:CB methods a cut off closer to 0.5 to 0.6 would be better.[120] RIPA will also usually be normal in type 1 VWD patients, unless VWF is at very low level ($<$ 15 U/dL). VWF multimers will show normal multimer patterns but reduced intensity consistent with the presence of all VWF multimer forms in lower than normal amounts. Platelet function analyser (PFA-100 or PFA-200; Siemens Healthcare, Marburg, Germany) closure times will be prolonged when VWF levels fall below approximately 30 to 40 U/dL.

In contrast, type 2A VWD is defined by low VWF activity/Ag ratios; again, all the earlier noted ratios will be low, because all will (albeit to potentially different extents) be sensitive to the loss of HMW VWF in these VWD samples. Again, the actual ratio cut off value may differ between assays and laboratories but will usually range from 0.5 to 0.7. VWF:Ag levels are also generally below 50 U/dL in type 2A VWD, and RIPA will generally only show activity with higher levels of ristocetin ($>$ 1.5 mg/mL). VWF multimers will show a loss of HMW VWF multimer forms. PFA closure times will generally be prolonged.

Type 2B VWD will typically also show low VWF activity/Ag ratios, again because of the loss of HMW VWF in these samples, although some patients may not show this pattern. VWF:Ag levels may or may not be low, and although VWF activity assays are usually below the normal cut off value, some patients may show normal values. RIPA will evidence heightened responsiveness, with aggregation with lower levels of ristocetin ($\leq$ 0.7 mg/mL). VWF multimers will typically show a loss of HMW VWF multimer forms. PFA closure times will generally be prolonged. Although the loss of HMW VWF multimers is usually greater in 2A than 2B VWD, assessment of VWF multimers will not permit their discrimination as there are overlaps of patterns between types 2A and 2B VWD samples. Types 2A and 2B VWD are distinguished phenotypically by RIPA analysis, although 2B VWD may also be associated with (mild) thrombocytopenia.

Type 2M VWD will typically also show low VWF activity/Ag ratios, but in these patients patterns will differ from types 2A and 2B VWD, and will depend on the type of VWF defect present. As HMW VWF is not missing in these patients, the low VWF activity/Ag ratios reflect specific functional VWF defects. Thus, patients with collagen-binding defects will show low VWF:CB/Ag ratios, but possibly normal VWF:RCo/Ag ratios. In contrast, patients with platelet GPIb-binding defects will show low VWF:RCo/Ag ratios, but possibly normal VWF:CB/Ag ratios (►**Table 2**). The newer latex-based GPIb-binding assays will more generally follow VWF:RCo patterns but not VWF:CB patterns. VWF:Ag levels are often, but not always, low in these patients. RIPA will usually evidence reduced responsiveness in patients with a GPIb-binding defect, with aggregation only to high levels of ristocetin ($>$ 1.5 mg/mL). Although VWF multimers will typically not show a loss of HMW VWF multimer forms, some abnormal VWF structure may be evident. PFA closure times will generally be prolonged.

Type 2N VWD reflects a dysfunction of VWF–FVIII binding, and so will typically represent phenotypically like mild hemophilia A, with normal or low VWF, but relatively lower FVIII:C, and thus low FVIII:C/VWF:Ag ratios. VWF

multimers will reflect the level of VWF present and will otherwise be (structurally) normal, as will RIPA and PFA closure times. Diagnosis of type 2N VWD requires performance of a VWF:FVIII binding assay, and/or identification of a causative *VWF* gene mutation (rather than *FVIII* mutation as in hemophilia A).

Type 3 VWD is identified by an absence of VWF in plasma. All VWF assays will therefore result below 5 U/dL; note, however, that many VWF assays have low VWF level detection issues and so may not be sensitive enough to show the absence of VWF in these patients (see the next section).[63,64] RIPA will be absent, without aggregation even to very high levels of ristocetin ($>$ 2.0 mg/mL). VWF multimers will typically show an absence of all multimer forms, and PFA closure times will be maximally prolonged.

Controversies and Ongoing Difficulties in the Diagnosis of von Willebrand Disease

Despite the considerable improvement in the array of tests over the years, some areas of the diagnosis of VWD remain difficult and/or controversial. These issues are summarized in ►**Table 3**, as are some possible solutions. Some of these issues have already been covered in previous sections, notably poor assay reproducibility (or high assay variability), poor low level of VWF sensitivity, and thus high resultant diagnostic error rates. My laboratory, in collaboration with the RCPAQAP Haematology, reported on diagnostic error rates in 2005 and 2006,[61,62] and again in 2007[164] and 2014,[64] and some of this data are summarized in ►**Figs. 3** to **8**. In brief, using a composite of data from the start of the RCPAQAP Haematology VWF/VWD surveys in 1998 to the year 2004, we could identify differential diagnostic error rates (►**Fig. 3**) for "misidentification" of type 1 VWD as type 2 VWD, "misidentification" of type 2 VWD as type 1 VWD, and "misidentification" of normal samples as being VWD; error rates were highest when the VWF:RCo was used as the only VWF activity assay in conjunction with VWF:Ag and FVIII:C, respectively reaching approximately 20, 30, and 5%, for these scenarios. These error rates could be more than halved by replacement of VWF:RCo with VWF:CB, or by inclusion of VWF:CB to the panel of VWF:RCo, VWF:Ag, and FVIII:C (►**Fig. 3**). Error rates were primarily due to a composite of assay variability (especially VWF:RCo), poor sensitivity to low VWF (especially VWF:RCo), and laboratory misinterpretation of test data that was consistent with the sample type, but interpreted incorrectly. We updated this finding in a separate study focusing on type 2B VWD in 2007,[164] where we could identify an error rate of "misidentification" of type 2B VWD samples of nearly 80% when laboratories performed testing using only FVIII:C and VWF:Ag or FVIII:C and VWF:RCo (►**Fig. 4**). This is because such a limited test panel will not be able to identify a qualitative VWF defect. However, the error rate was still as high as almost 30% when laboratories performed testing using FVIII:C, VWF:Ag, and VWF:RCo; this error rate again more than halved by addition of the VWF:CB. We again recently updated our findings in 2014 (►**Fig. 5**).[64] We identified overall error rates of close to 10% for "misidentification" of type 1 (quantitative) deficiencies as well as of type 2 (qualitative) defects (►**Fig. 5A**). However, the reasons for errors differed somewhat according to the sample type. For type 1 (quantitative deficiencies), the main reason for errors was false VWF:activity to antigen discordance (►**Fig. 5B**), and this was primarily driven by false VWF:RCo/VWF:Ag discordance (►**Fig. 5C**). In contrast, for type 2 (qualitative defects), the main reason for errors was laboratory misinterpretation of test results which were often reported as "type 1" but were actually consistent with the sample type being type 2 (qualitative defect) (►**Fig. 5B**). For the updated data,[64] a summary of comparative data for VWF activity/Ag ratios, and interlaboratory variation data for various assays is respectively shown in ►**Figs. 6** and **7**. An update on low level VWF sensitivity is given in ►**Fig. 8**.

Many other issues and controversies in VWD diagnostics exist. Some of the more important ones are outlined later and in ►**Table 3**.

Type 1 von Willebrand Disease

One topical issue is the diagnosis of mild Type 1 VWD and the suggestion that this diagnosis be discarded.[2,165] VWD is an inherited, systemic bleeding disorder, presumably as a result of a mutation in the *VWF* gene. This definition assumes three diagnostic components: (1) Inheritance; (2) Evidence of a systemic bleeding tendency, different from the normal population; and (3) Confirmatory, objective, testing which confidently delineates the diseased group from the normal population. Ideally, such testing should predict, or correlate with, the risk of bleeding. There are problems with each of these three components when it comes to making the diagnosis of type 1 VWD in the majority of patients. There is no consistent genetic defect associated with the majority of type 1 VWD. The expression of the VWF gene is significantly modified by a range of epigenetic (i.e., other genes) and environmental factors such as exercise, inflammation, pregnancy, and drugs. Except blood group, with O populations reported to have VWF levels 25 to 35% lower than other blood types, most epigenetic factors remain unexplained. Extraneous factors contribute to the wide normal range for VWF levels and the final degree of penetrance of any specific VWF gene defect, with resulting significant overlap between normal and affected individuals, both in terms of levels of VWF as well as clinical bleeding histories.

Normal ranges for tests in hematology are traditionally established to capture some 95% of the normal population, which causes problems in diagnosing low-frequency disorders because 2.5% of the normal healthy population falls below the normal range and this exceeds the incidence of VWD by at least one order of magnitude. Natural heterogeneity is further compounded by the imprecision of the existing tests. As a consequence of overlapping criteria for diagnosis, there have been widely varying estimates of VWD prevalence. Population studies give reported prevalence of over 1%, but more stringent diagnostic criteria give figures of 0.01 to 0.02%.[166]

It follows that many, if not most, patients diagnosed with "mild" type 1 VWD are false positives as the diagnosis does

Table 3 A summary of problems and solutions in von Willebrand disease diagnostics

Problem or issue	Comments	Solution
Mild type 1 VWD or low VWF level as a risk factor for bleeding?	Most patients with VWF levels above 30 U/dL will not have an identifiable *VWF* mutation. Such levels will overlap with normal individuals without evident bleeding diathesis, and other patients may have similar bleeding diathesis with normal levels of VWF.	International expert or consensus recommendations?
No standard definitions of severity for type 1 VWD based on laboratory phenotype.	Unlike the case for hemophilia A, where severe hemophilia is defined by FVIII:C < 1U/dL, moderate by FVIII:C of 1–5 U/dL, and mild by FVIII:C of 6–30 U/dL	International expert or consensus recommendations?
High diagnostic error rate with incomplete test panels.	Most laboratories only perform limited test panels, most commonly, FVIII:C, VWF:Ag and VWF:RCo.	Laboratories should utilize comprehensive test panels including (at a minimum) FVIII:C, VWF:Ag, VWF:RCo, and VWF:CB. Some GPIb binding assays may in time be found to be able to replace VWF:RCo. Additional assays should be employed to help type VWD (e.g., VWF multimers if available, VWF:FVIIIB for 2N/hemophilia A discrimination, RIPA for 2A/2M vs. 2B vs. PT-VWD discrimination).
High variability with some assays.	Especially VWF:RCo	Laboratories should select the best assays for use in VWD diagnostics, and optimize them. All assays should be repeated at least once for confirmation using a fresh plasma sample.
Poor sensitivity to low levels of VWF.	Especially VWF:RCo	Laboratories should select the best assays for use in VWD diagnostics, and also recognize the level of VWF sensitivity for all assays they perform, and also consider modifying the assay-design if required to improve this sensitivity. A VWF deficient plasma sample may be a useful control in this setting.
All VWF activity assays measure slightly different VWF activities, so not strictly interchangeable (different sensitivities to HMW VWF/type 2 structural changes/variants).	In particular, GPIb binding activity mimic assays (e.g., VWF:RCo, VWF:Ac) will show different test patterns to collagen binding (VWF:CB) assays.	Laboratories should understand the HMW VWF sensitivity for all assays they perform, as well as their behavior in the presence of functional VWF defects. HMW VWF deficient/type 2A-like/2M VWD-like plasma samples would be useful in this evaluation, and also useful controls for on-going testing.
VWF:CB assays, including commercial options, differ in terms of sensitivities to HMW VWF.	And thus their utility for discrimination of type 1 vs. 2 VWD.	Laboratories should recognize the HMW VWF sensitivity of the VWF:CB assay they utilize, and chose a more sensitive assay should their current assay be determined to be inadequate.
Cut off values for VWF activity/Ag ratios for discrimination of quantitative deficiencies of VWF vs. qualitative defects in VWF differ between assays.	Values typically range between 0.5 and 0.7. The cut off value chosen will affect their utility for discrimination of type 1 vs. 2 VWD.	Laboratories should optimize these cut off values to maximize their diagnostic utility.
Regulation of IVDs	Differs between geographies, forcing manufacturers to seek many different approvals; this duplication is costly and may preclude some IVDs being introduced into some localities. Some regulators	Regulators should better harmonize regulatory processes to avoid the need for manufacturers to seek different approvals in different geographies. Regulators should

(*Continued*)

Table 3 (*Continued*)

Problem or issue	Comments	Solution
	appear to be overzealous in regards to requirements, raising the issue of "risk" beyond that of clinical utility, forcing some manufacturers to undertake unnecessary assessments and delaying or precluding some IVDs being introduced into some localities.	focus more on clinical utility and less on risk for low risk IVDs.
Genetic testing is complex, costly, not always successful, and only rarely affects therapy or patient management.	For quantitative VWF deficiency patients (type 1 VWD), a search for a *VWF* mutation may require a full evaluation of the entire *VWF* gene, and thus be very costly, and will not be successful in most cases. A search for a *VWF* mutation in type 3 VWD may also require a full evaluation of the entire *VWF* gene, and will be clinically limited except in select cases. Although such a search will be more successful for qualitative VWF defect cases (type 2 VWD), and can usually be achieved for using a more restrictive search in the *VWF* gene, such a search can be restricted to cases when clinically useful.	Add genetic testing only in select cases (e.g., whenever type 2A/2M/2B/2N/HA is suspected, but be more selectively in types 1 and 3). Most cases investigated for VWD in general practice are found not to be VWD; thus, genetic testing is warranted in $<$ 1% of all VWF test requests and probably $<$ 10% of VWD cases.
Unclear VWD type?	Sometimes laboratory testing leads to suspicion of VWD, but the type is unclear, and not clarified by repeat testing.	Repeat all tests at least once on a fresh sample for confirmation/clarification. Also perform DDAVP trial as an aid to diagnosis/subtype discrimination whenever subtype not clear.

Abbreviations: Ag, antigen; CB, collagen binding; DDAVP, desmopressin ; GPIb, glycoprotein Ib; HMW, high molecular weight; IVD, in vitro diagnostic; PT, platelet type; RCo, ristocetin cofactor; RIPA, Ristocetin induced platelet aggregation; VWD, von Willebrand disease; VWF, von Willebrand factor.

not exhibit consistent linkage to the *VWF* gene. Such a "disease" label is not without medical, social, and personal consequences. Laboratories need therefore to be cautious in interpreting mild or moderately reduced levels of VWF activity and VWF antigen (which they often have to do in the absence of a detailed history) as the statistical probability is that this is not VWD. Sadler, the main proponent of this argument,[165] suggests that type 1 VWD is used only for the severe patients with markedly reduced levels and that the others be reported simply as "low VWF," just as one would

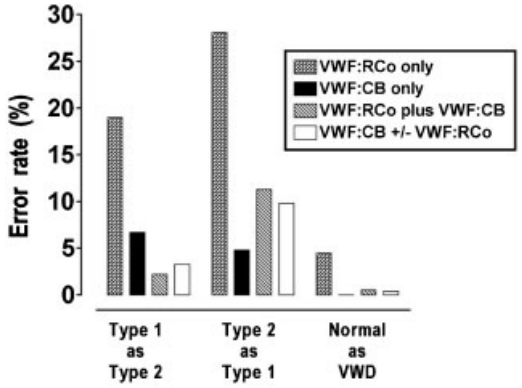

Fig. 3 Error rates in misidentification of VWD type 1 as type 2, VWD type 2 as type 1, or normal individuals as VWD. Data from the RCPAQAP Haematology program[61] covering the period from 1998 to 2004, and separated according to laboratory performance of FVIII:C plus VWF:Ag plus (A) VWF:RCo as the only VWF activity assay, (B) VWF:CB as the only VWF activity assay, (C) both VWF:RCo and VWF:CB activity assays, or (D) VWF:RCo and/or VWF:CB as VWF activity assays. Ag, antigen; CB, collagen binding; FVIII:C, factor VIII coagulant (activity); RCo, ristocetin cofactor; VWD, von Willebrand disease; VWF, von Willebrand factor.

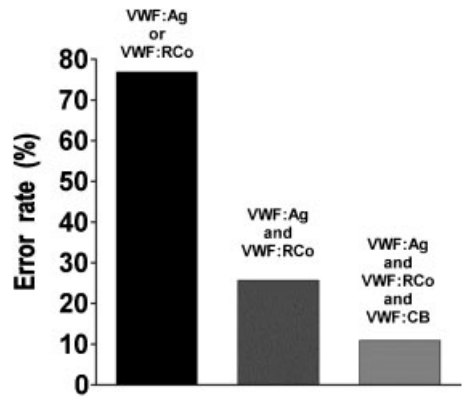

Fig. 4 Error rates in misidentification of VWD type 2B. Data from the RCPAQAP Haematology program,[164] and separated according to laboratory performance of FVIII:C plus (1) VWF:Ag or VWF:RCo, (2) VWF:Ag and VWF:RCo, (3) VWF:Ag and VWF:RCo and VWF:CB. Ag, antigen; CB, collagen binding; FVIII:C, factor VIII coagulant (activity); RCo, ristocetin cofactor; VWD, von Willebrand disease; VWF, von Willebrand factor.

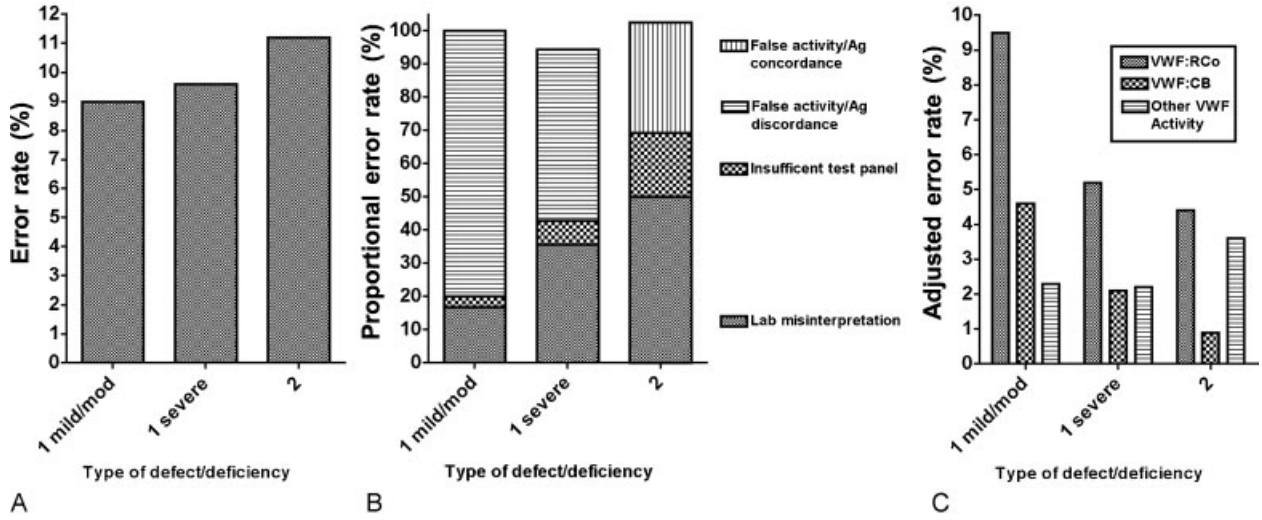

Fig. 5 Error rate data from the RCPAQAP Haematology program[64] and covering the period from 2006 to 2013. (A) Overall error rates in misidentification of mild/moderate or severe quantitative VWF deficiencies ("type 1" VWD), and of qualitative VWF defects ("type 2" VWD) each approximates 10%. (B) This data expressed as a proportional error rate and due to (1) false VWF activity/Ag concordance or discordance, (2) performance of too limited a VWF test panel, or (3) due to the laboratory's misinterpretation of its own test data. (C) The same data for false VWF activity/Ag concordance or discordance separated according to test performed (VWF:RCo, VWF:CB, or other VWF activity assay). Ag, antigen; CB, collagen binding; FVIII:C, factor VIII coagulant (activity); RCo, ristocetin cofactor; VWD, von Willebrand disease; VWF, von Willebrand factor.

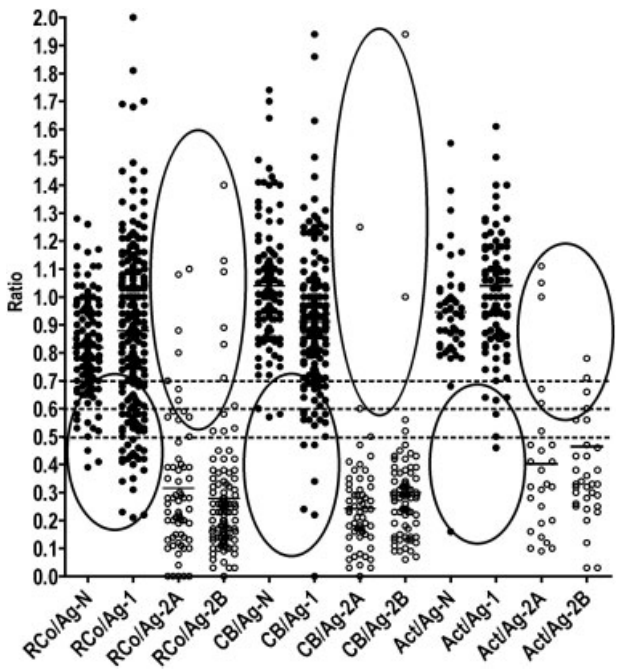

Fig. 6 Recent data from the RCPAQAP Haematology program[64] covering the period from 2006 to 2013 and showing VWF activity to antigen ratios for sets of (a) normal samples (N), (b) quantitative deficiency ("type 1" samples (1)), (c) qualitative defect (high molecular weight VWF deficiency samples = "type 2A" (2A)), and (d) type 2B VWD samples (2B) and separated according to the activity assay. False activity/Ag discordance for N and 1, and false activity/Ag concordance in 2A and 2B are respectively represented by false low and false high ratios (circled). Cut off values for quantitative versus qualitative VWD disorders (i.e., type 1 vs. type 2 VWD) would generally range from 0.5 to 0.7 (see horizontal dashed lines), but the actual cut off value may differ for different assays and laboratories. Act, other VWF activity assays (i.e., excludes RCo and CB); Ag, antigen; CB, collagen binding; RCo, ristocetin cofactor; VWD, von Willebrand disease; VWF, von Willebrand factor.

report an out of range cholesterol or white cell count or platelet count without necessarily making a specific diagnosis. This point has been taken up and championed most recently by the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel group.[57]

Another less well-defined issue relates to severity of type 1 VWD as related to phenotypic tests. In hemophilia A, severe hemophilia is defined by FVIII:C < 1 U/dL, moderate by FVIII:C of 1 to 5 U/dL, and mild by FVIII:C of 6 to 30 U/dL.[167] There is no such characterization definition for type 1 VWD, and the level of VWD only partially defines the clinical severity of the bleeding disorder. Our own "working definition" is shown in ►**Fig. 2**, until such time as this can be better defined by expert consensus.

Type 2M von Willebrand Disease

Type 2M VWD is conceptually a difficult VWD type for many clinicians and scientists to grasp. Type 2M VWD reflects a functional VWF disorder unrelated to loss of HMW VWF. The easiest way to consider type 2M VWD is by exclusion of the other forms of VWD; if a patient has VWD, but the type is not 1, 2A, 2B, 3, or PT, then it is type 2M.

Most patients with type 2M VWD are misdiagnosed either as types 2A or 1.[166,168,169] This reflects a combination of many factors, including: (1) limited recognition of type 2M VWD (so that a diagnosis of types 1 or 2A VWD is made preferentially); (2) performance of limited test panels by many laboratories (e.g., performance of only VWF:Ag and VWF:RCo will provide similar findings in types 2A and most 2M VWD; accordingly, type 2A VWD will preferentially be identified); this can be alleviated by additional performance of VWF:CB, which will aid the discrimination of 2A versus 2M VWD; (3) assay limitations such as high assay variability (particularly VWF:RCo) and poor sensitivity to low levels of VWF (again

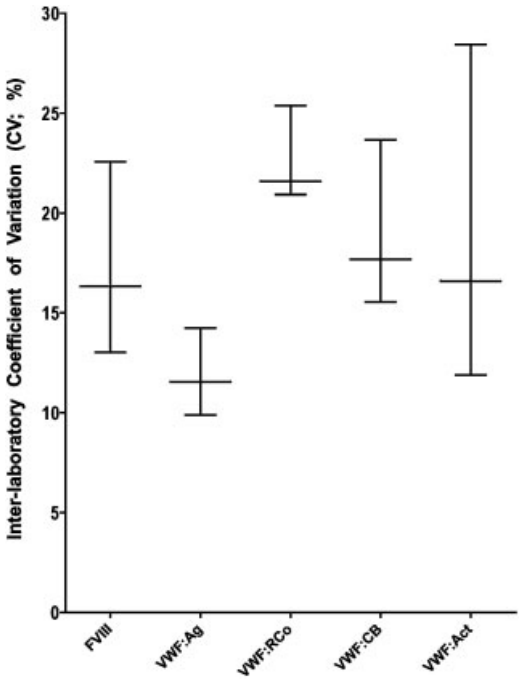

Fig. 7 Recent data from the RCPAQAP Haematology program,[64] covering the period from 2006 to 2013 and showing the inter laboratory variation (shown as coefficient of variation;%) for FVIII:C, VWF:Ag, VWF:CB, and other VWF activity assays (VWF:Act) combined (but primarily reflecting the monoclonal antibody–based latex assay from IL, Werfen). Data shown as 25th percentile, median and 75th percentile. In general, the lowest variation was observed with VWF:Ag and the highest with VWF:RCo; variation for the other assays was intermediate. The large range of variation observed with FVIII:C and the VWF:Act group reflects the varied samples represented in this data. Act, other VWF activity assays (i.e., excludes RCo and CB); Ag, antigen; CB, collagen binding; FVIII:C, factor VIII coagulant (activity); RCo, ristocetin cofactor; VWF, von Willebrand factor.

particularly for VWF:RCo); this leads to false concordance of VWF:RCo and VWF:Ag, and thus, false identification of type 2M as type 1 VWD.

Type 3 von Willebrand Disease

Conceptually, type 3 VWD should be fairly straightforward to diagnose, reflecting an absence of VWF. However, given poor sensitivity of many assays for low levels of VWF, false values for VWF of upward of 20 U/dL or more may be reported by laboratories when testing samples devoid of VWF (and thus reflecting a type 3 VWD pattern) (►**Fig. 8**).[63,64] Thus, type 3 VWD may be missed and alternatively be identified as types 1 or 2 VWD depending on the test results reported by the laboratory.

Type 3 VWD is sometimes also misdiagnosed as hemophilia A. This may occur because a clinician has only requested, or a laboratory has only performed, FVIII:C testing for a patient with type 3 VWD. The resultant FVIII:C level will be between 2 and 8 U/dL, and the patient may be diagnosed as a moderate hemophilia A, simply because VWF testing has not been performed, and because hemophilia A is a better known bleeding disorder.[166,170]

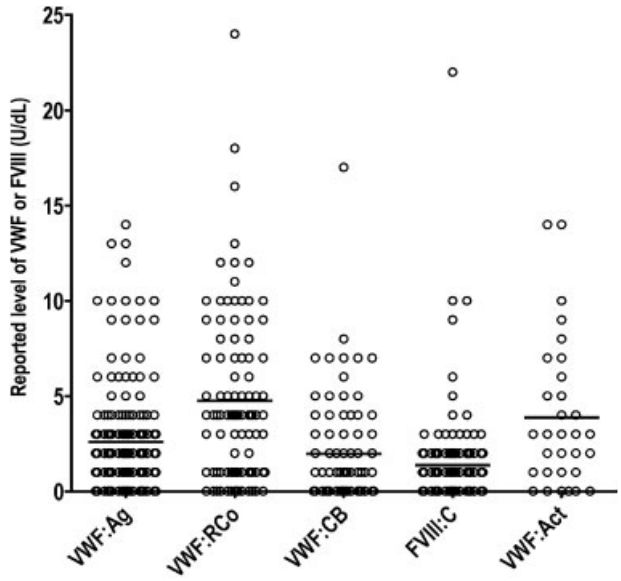

Fig. 8 Recent data from the RCPAQAP Haematology program,[64] covering the period from 2006 to 2013 and showing participant returned data for FVIII:C, VWF:Ag, VWF:CB and other VWF activity assays (VWF:Act) combined (but primarily reflecting the monoclonal antibody based latex assay from IL, Werfen) for distributed samples that were devoid of VWF (three samples in total). In general, the worst lower limit of VWF detection is represented by VWF:RCo; excluding occasional outliers, the best general performance is seen with VWF:CB and FVIII:C. Act, other VWF activity assays (i.e., excludes RCo and CB); Ag, antigen; CB, collagen binding; FVIII:C, factor VIII coagulant (activity); RCo, ristocetin cofactor; VWF, von Willebrand factor.

Type 1 versus Type 2 von Willebrand Disease Discrimination

As highlighted earlier, this can only be achieved when laboratories perform a full battery of reliable VWF tests, and repeat test results at least once on a new plasma sample for confirmation. Performance of too few tests, or of inferiorly performed tests, will not enable discrimination (►**Fig. 6**), and this has important clinical implications given possible differential therapeutic management.

Discrimination of Type 2B VWD versus PT-VWD

Type 2B VWD describes a VWF defect, and PT-VWD describes a platelet GPIb defect; both are managed differently (VWF replacement in type 2B VWD versus platelets in PT-VWD), thus discrimination is important. Phenotypically, 2B and PT-VWD yield similar laboratory findings—low to normal VWF:Ag, reduced VWF:RCo/Ag ratio, reduced VWF:CB/Ag ratio, loss of HMW VWF, mild thrombocytopenia, and enhanced RIPA response (low-dose ristocetin). Discrimination therefore requires either performance of RIPA mixing studies (to identify whether the defect is plasma or platelet based), and/or genetic investigation of the *VWF* and/or platelet *GPIb* genes.[171]

Discrimination of Type 2N von Willebrand Disease versus Hemophilia A

Type 2N VWD describes a VWF defect, whereas hemophilia A describes a FVIII:C defect; both are managed differently

(VWF replacement in type 2N VWD vs FVIII replacement in hemophilia A), thus discrimination is important. Phenotypically, 2N VWD and hemophilia A yield similar laboratory findings—low to normal VWF:Ag, reduced FVIII:C, and reduced FVIII/VWF ratios. Discrimination therefore requires either performance of a specific VWF:FVIII binding assay[172] (to identify whether there is a VWF based defect), and/or genetic investigation of the *VWF* and/or *FVIII* genes. Sometimes, familial inheritance provides the clue—hemophilia A is sex linked and affects mostly males, whereas type 2N VWD is not sex linked and will affect both males and females "equally" (note however, that the additional hemostatic challenges of menstruation and child birth in females may instead lead to identification of type 2N VWD more often in females than males).

Genetic Testing in von Willebrand Disease

Genetic testing in VWD investigations is typically complex, costly, not always successful, and only rarely affects therapy or patient management.[173,174] For quantitative VWF deficiency patients (type 1 VWD), a search for a *VWF* mutation may require a full evaluation of the entire *VWF* gene, and thus be very costly, and will not be successful in most cases. A search for a *VWF* mutation in type 3 VWD may also require a full evaluation of the entire *VWF* gene, and will be clinically limited except in select cases. Although such a search will be more successful for qualitative VWF defect cases (type 2 VWD), and can usually be achieved for using a more restrictive search in the *VWF* gene, such a search can be restricted to cases when clinically useful. In general, our recommendation would be to only request/perform genetic testing in select cases (e.g., whenever type 2A/2M/2B/2N/HA is suspected, but be more selectively in types 1 and 3 VWD). As most cases investigated for VWD in general practice are found not to be VWD, genetic testing is warranted in $< 1\%$ of all VWF test requests and probably in $< 10\%$ of VWD cases.

Regulation of In Vitro Diagnostics

Another less well-recognized issue in VWD diagnostics (as with all diagnostics) is the lack of some methodologies in some geographies simply because they have not been cleared by regulatory authorities.[175–177] This may be because of several factors. Importantly, different regulatory systems exist in different countries, and this necessitates multiple submissions from manufacturers wishing to market their product internationally. This causes additional expense, duplication, and sometimes prohibits release of some products into some countries as the process is determined to be not cost effective by that manufacturer. On other occasions, companies may obtain clearance of their product in one country, but fail to get clearance in another country. Despite being perceived as a liberal country, the United States, in particular, has one of the tightest and most difficult regulatory clearance procedures, so that diagnostic testing in that country is several years behind that of Europe and Australia.[177]

Conclusion and Final Comments

The diagnosis of the majority of type 2 patients can be confidently made on the basis of clinical history and markedly reduced levels of VWF:RCo (and/or VWF:CB and/or other VWF activity assay) compared with FVIII:C and VWF:Ag levels (i.e., evidence of "VWF functional discordance"). Type 3 VWD should be a fairly straightforward diagnosis, although it may be challenged by poor or limited test selection or performance. Only a small proportion of type 1 patients have clearly abnormal clinical symptoms, levels of VWF:Ag and VWF: RCo (and/or VWF:CB and/or other VWF activity assay) below 25%, and usually well-defined genetic abnormalities. Although VWF multimer analysis may provide further subdivision, this has limited clinical relevance for a very few patients. In my own laboratory practice, VWF multimer analysis is utilized for $< 1\%$ of investigated patients, and test patterns obtained with VWF:Ag, VWF:RCo, and VWF:CB will inform on the likely multimer pattern in more than 99% of patients investigated; thus, multimer analysis can be omitted for most patients when the laboratory performs this three VWF test composite panel. This has recently been confirmed by others.[129,130]

Ideally, for all type 2 VWD patients (as initially defined by phenotypic data with FVIII:C, VWF:Ag, VWF:RCo, and VWF:CB), performance of the RIPA test is more strongly recommended than multimer analysis. This will identify types 2A and 2M VWD patients (reduced responsiveness) as well as type 2B and PT-VWD patients (enhanced responsiveness) and will more likely influence treatment decisions. Types 2A and 2M VWD can be distinguished by VWF:RCo versus VWF:CB test patterns (or comparative VWF:RCo/Ag vs. VWF:CB/Ag ratios), but treatment will be similar and so their differentiation is largely academic. In contrast, discrimination of type 2B and PT-VWD is more important, and this can be accomplished by either RIPA mixing studies, or genetic testing of the *VWF* and *GPIb* genes to identify where the defect lies. Similarly, discrimination of type 2N VWD and hemophilia A is also important, and this can be accomplished by either VWF:FVIII binding studies, or genetic testing of the *VWF* and *FVIII* genes to identify where the defect lies.

Current laboratory limitations in VWD diagnostics are reflected by a composite of poor test performance, poor assay choice, limited test panels, and inability to properly interpret laboratory test patterns. New VWF assays in progress promise a new horizon in VWD diagnostics, but this needs to be coupled to improved understanding within laboratories of the assays they perform, as well as overall interpretation.

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