Laboratory Testing for von Willebrand Disease: The Past, Present, and Future State of Play for von Willebrand Factor Assays that Measure Platelet Binding Activity, with or without Ristocetin

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Abstract

von Willebrand disease (VWD) was first described nearly a century ago in 1924 by Erik Adolf von Willebrand. Diagnostic testing at the time was very limited and it was not until the mid to late 1900s that more tests became available to assist with the diagnosis and classification of VWD. Two of these tests are based on ristocetin, one being ristocetin-induced platelet aggregation (RIPA) and the other the von Willebrand factor (VWF) ristocetin cofactor assay (VWF:RCo). The VWF:RCo assay provides functional assessment of in vitro VWF binding to the platelet glycoprotein (Gp) complex, GPIb-IX-V. Despite some advancements and newer technologies utilizing the principles of the original VWF:RCo assay, the original assay is still referred to as the gold standard for measurement of VWF activity. This article will review the history of VWD diagnostic assays, including RIPA and VWF:RCo over the past 40 years, as well as the newer assays that measure platelet binding with or without ristocetin, and which have been developed with the aim to potentially replace platelet-based ristocetin-dependent assays.

Keywords

- ristocetin cofactor
- ristocetin induced platelet aggregation
- von Willebrand disease
- laboratory testing
- von Willebrand factor
- von Willebrand factor activity

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Introduction and Historical Background

von Willebrand factor (VWF) is a complex multimeric molecule ranging in size from small to large to ultra large (UL) molecular weight forms. VWF mediates platelet adhesion and carries coagulant factor VIII (FVIII), protecting FVIII from premature clearance. Platelet adhesion at time of vascular injury is largely mediated by initial binding of subendothelial collagen to the VWF A3 domain, which then triggers a conformational change, with exposure of a binding site in the VWF A1 domain for platelet glycoprotein complex: Gp Ib-IX-V. In terms of platelet adhesion, the larger the VWF molecule, the greater the number of VWF adhesion sites for collagen and platelets.

von Willebrand disease (VWD) is a heterogeneous disorder defined by a reduction or absence of high molecular weight (HMW) VWF or of all forms of VWF or a loss of a specific function of VWF.[1] Comprehensive laboratory testing is required to identify specific defects and correctly type VWD patients, with tests including ristocetin-induced platelet aggregation (RIPA) and VWF ristocetin cofactor (VWF:RCo), along with FVIII and VWF antigen (VWF:Ag) recommended by the International Society of Thrombosis and Haemostasis (ISTH).[2]

VWD was first described nearly a century ago in 1924 by Erik Adolf von Willebrand[3] when investigating a 5-year-old girl with a family history of significant bleeding that differed from classic hemophilia. The tests that were available to Erik von Willebrand at the time were a blood count (in this patient, normal with slight anemia and slight thrombocytopenia), clotting time and clot retraction (both normal), bleeding time

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(prolonged at more than 2 hours), and a tourniquet test (highly positive). He concluded that the disorder was due to platelet dysfunction along with a defect in the vessel walls.

Diagnostic hemostasis testing in the early to mid-1900s continued to be very limited, particularly tests related to 'platelet dysfunction'. There was the skin bleeding time[4,5] and several platelet adhesion tests,[6–8] but all of these tests were nonspecific, difficult to perform, time consuming, and most importantly not reliable predictors of bleeding.[9,10]

It was not until the mid to late 1900s that newer tests became available to assist with the diagnosis and classification of VWD. Two of these then 'newer' tests were based on ristocetin, one being RIPA and the other VWF:RCo. The VWF:RCo assay provides a functional assessment of in vitro VWF binding to GPIb-IX-V, and measures the ability of VWF to bind normal platelets in the presence of ristocetin, with a preferential detection of HMW forms of VWF. Despite some advancements and newer technologies utilizing the principles of the original VWF:RCo assay, the original assay is still today referred to as the gold standard for measuring VWF function. ►**Table 1** provides a timeline of developments in the use of ristocetin and VWF:RCo assays in VWD diagnosis over the past 40 years.

This article will review the history of VWD diagnostic assays, including RIPA and VWF:RCo over the past 40 years, as well as the newer assays that measure platelet binding, with or without ristocetin, and which have been developed to potentially replace these two platelet ristocetin-based assays.

Ristocetin-Induced Platelet Aggregation

The discovery of the use of ristocetin and the role it has in VWD testing was made in the late 1960s. Margaret Howard and Barry Firkin, two Australia-based pioneers in hemostasis,

Table 1 Timeline of developments in the use of ristocetin, VWF:RCo, and VWF:RCo-like assays in VWD diagnosis over the past 40 years

Event	Assay name/type	References (year)
RIPA	Ristocetin induced platelet aggregation (RIPA)	Howard and Firkin (1971)[11], Howard et al (1973)[12]
VWF:RCo	VWF:ristocetin cofactor assay	Weis et al (1973)[13], Jenkins et al (1974)[14], Olsen et al (1975)[15]
Patients with increased responsiveness of VWF to ristocetin (recognized as type 2B VWD)	Ristocetin-induced platelet aggregation studies	Ruggeri et al (1980)[16]
Patients with increased responsiveness of platelet GPIb to ristocetin identified (recognized as PT-VWD)	RIPA studies with mixing	Takahashi (1980)[17], Miller and Castella (1982)[18], Weiss et al (1982)[19]
Ristocetin used to help define the interaction of platelet GPIb and VWF	Various	Phillips et al (1980)[20], Coller et al (1983)[21]
Revised/simplified classification of VWD—based on phenotypic test results initially and then on more comprehensive testing panel	Various	Sadler et al (1994)[22], Sadler et al (2006)[2]
Automation of VWF:RCo platelet agglutination assay	BC von Willebrand reagent initially on BCS analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) and then adopted to other systems	Miller et al (2002)[23], Lattuada et al. (2004)[24], Redaelli et al (2005)[25], Strandberg et al (2006)[26], Bowyer et al (2011)[27], Lawrie et al (2011)[28]
Modifications to automated VWF:RCo platelet agglutination assay to improve lower limit of detection	BC von Willebrand reagent (Siemens Healthcare Diagnostics, Marburg, Germany)	Hillarp et al (2010)[29], Favaloro et al (2010,2014)[30,31]
VWF:RCo by ELISA	In-house assays	Vanhoorelbeke et al (2000)[32], Federici et al (2004)[33];
VWF 'RCo-like activity' by ELISA (ristocetin not used)	In-house assay	Murdock et al (1997)[34], Flood et al (2011)[35]
VWF:RCo by flow cytometry	In-house assays	Lindahl et al (2003)[36], Giannini et al (2007)[37], Chen et al (2008)[38]
Platelet-free VWF:RCo-like assay	HemosIL VWF activity (IL)	Pinol et al (2003, 2007)[39,40], Vleeschawer et al (2006)[41], Sucker et al (2006)[42], Salem et al (2007)[43], Trossaert et al (2011)[44], Chen et al (2011)[45], Lasne et al (2012)[46]
Platelet-free VWF:RCo assay	HemosIL VWF:RCoF (IL)	Pinol et al (2009)[47], Stufano et al. (2014)[48]
Platelet-free GpIb binding assay	Innovance VWF activity (Siemens Healthcare Diagnostics, Marburg, Germany)	Lawrie et al (2013)[49], Geisen et al (2014)[50], Reilly-Stitt et al (2014)[51], Graf et al (2014)[52], Patzke et al (2014)[53], Favaloro et al (2014)[31], Timm et al (2015)[54]
VWF:RCo assay using chemiluminescence technology	HemosIL AcuStar assay (Instrumentation Laboratory, Barcelona, Spain)	Verfaille et al (2013)[55], Cabrera et al (2013)[56], de Maistre et al (2014)[57], Costa-Pinto et al (2014)[58], Favaloro et al (2015)[59], Sagheer et al (2016)[60]
New nomenclature from ISTH SSC for platelet-dependent VWF activity assays	Various	Bodo et al (2015)[61]

Abbreviations: BCS, Behring coagulation system; ELISA, enzyme linked immunosorbent assay; GpIb, glyocprotein Ib; ISTH SSC, International Society on Thrombosis and Haemostasis Scientific Subcommittee; PT-VWD, platelet type von Willebrand disease; RIPA, ristocetin induced platelet aggregation; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:RCo, VWF Ristocetin Cofactor assay.

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began investigations into agents that had been reported to cause thrombocytopenia. Utilizing a relatively new laboratory technique for the time, platelet aggregation, as described a little earlier by Born,[62] they assessed a variety of different drugs, one of which was ristocetin.

Ristocetin had been used successfully as an antibiotic and is prepared by fermenting the broth of *Nocardia lurida*.[63] Ristocetin was initially used to combat gram-positive cocci including penicillinase-producing organisms. However, ristocetin was withdrawn from clinical use due to an associated incidence of dose-related thrombocytopenia in a significant number of patients.[63,64]

In 1971, Howard and Firkin showed that ristocetin was able to trigger platelet aggregation in normal platelet rich plasma (PRP) and in patients with thrombasthenia, but not in two of three patients they tested with VWD. They proposed that VWD may therefore be divided into two types, and that ristocetin could prove a valuable technique to further study and classify VWD patients.[11] A few years later they subsequently reported that platelets from VWD patients aggregated with ristocetin when normal plasma was added to the test system, and that normal platelets did not aggregate when VWD patient plasma and ristocetin was added to the test system. They therefore indicated that ristocetin could be a useful tool in studying the underlying plasma deficiency of VWD, which manifests as decreased platelet adhesiveness, and that it might be of diagnostic value in identifying patients with VWD.[12] These studies formed the basis for further development of the RIPA assay.

Several publications subsequently reported RIPA defects in several other acquired platelet disorders. These disorders included acute leukaemia,[65] idiopathic thrombocytopenic purpura,[66] infectious mononucleosis,[67] and the inherited platelet disorder of Bernard–Soulier syndrome.[68] However, it was only in VWD that this defect was corrected with the addition of normal plasma to the test system. This confirmed, as postulated originally by Howard and Firkin,[12] that the deficiency in VWD was due to a plasma component; this component is now known to be VWF.

With the advent of this laboratory test to assist in the diagnosis of VWD, several different variants of VWD were subsequently discovered utilizing the RIPA assay. Ruggeri et al[16] described a group of patients that showed an increased responsiveness to ristocetin; these patients are now known to represent type 2B VWD. Another series of patients were described in the early 1980s by several groups, similarly showing an enhanced interaction between VWF and platelets at low concentrations of ristocetin, but where the apparent defect rested in the platelets; this group of patients was subsequently designated platelet type (PT)- or pseudo VWD.[17–19]

The use of RIPA in these early studies provided several insights into the structure and function of VWF and the GPIb-IX platelet receptor complex.[20,21] These early breakthroughs in VWD testing, using ristocetin and platelet aggregation, still provide us with the critical tool to continue to diagnose type 2B and PT-VWD variants in the modern hemostasis laboratory.[69,70]

Ristocetin Cofactor Assays: The Early Years

After the early work of Howard and Firkin, essentially discovering the value of ristocetin as a diagnostic tool in VWD, subsequent studies showed that ristocetin was able to also aggregate washed platelets in the presence of normal plasma. This led to the development of a quantitative assay, also utilizing platelet aggregometry, to measure what was then known as the FVIII:VWF. The earlier versions of this assay utilized washed platelets.[13,14] These early assays showed that a log–log relationship existed between the amount of ristocetin-induced aggregation of normal platelets and the concentration of VWF activity present in the test system.

A similar assay was developed utilizing washed gel filtered platelets,[15] whereby the authors initiated several recommendations for performance of the assay; including that the standard curve have a correlation coefficient greater than 0.97 and a standard deviation about the regression line lower than 0.05. They mentioned that, if this was not achieved, the platelets should be rewashed. Three conditions were mentioned in the preparation of the washed gel filtered platelets that affected the sensitivity of the assay: the platelets needed to be refractile in the platelet concentrate, centrifugation of the gel filtered platelets for more than 10 minutes at 500 g decreased their sensitivity, and using concentrations of less than 200,000 platelets per mm^3 also gave decreased sensitivity to the assay. The authors also discussed the importance of diluting out the patient's plasma to achieve rates of aggregation in the linear portion of the standard curve. They also evaluated the effect of storage on platelet concentrates and found a decreased sensitivity in the assay after more than 3 days of storage. They also assessed the buffering system utilized in the assay, finding that the assay was more stable over the 3 to 4 hours required to perform testing by eliminating glucose from the buffer. They also discussed a variation in the potency of different lots of ristocetin and mentioned the need to standardize the assay with each new lot of ristocetin. This was important information for laboratories intending to set up their own assays. The authors also mentioned the importance of establishing the normal rate and extent of aggregation with ristocetin in each individual laboratory due to variation in instrumentation and techniques in performing aggregometry. Allain et al[71] described an adaptation to this method that allowed larger batches of platelets to be prepared and then fixed with paraformaldehyde, thus allowing increased stability of the platelet suspension of up to 1 month when stored at 4 degrees.

For laboratories that did not have an aggregometer, another method was also described in the literature, essentially utilizing the same principles but where aggregation was measured by macroscopic visualization.[72]

These earlier versions of what we now know as VWF:RCo were advances for the field of VWD diagnosis, providing a relatively reliable quantitative assay to further categorize patients with the disorder at that time.[73]

The VWF:RCo remained relatively unchanged for another 30 years, despite its increasingly well-known limitations, including increased coefficient of variation (CV) compared

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with other laboratory assays and lack of harmonization between laboratories. Indeed, standardization of the assay became increasingly poorer with increasing numbers of variations to the method developed over time. These variations contributed to the poor precision (increased CV) seen with the largely manual or semiautomated VWF:RCo assay performed by platelet aggregation.[74–76] Laboratories often prepared their own washed and/or fixed platelet suspensions, utilizing different buffers and centrifugation speeds for washing and preparation. There was also variability in the pooled platelet concentrates that were used to prepare these suspensions, with most laboratories utilizing unused/expired bags of platelets from their blood bank laboratory, which introduced biological variation from the donor platelets.

There was also variation in the standards used to calibrate the reference curve; most laboratories would use local pooled normal plasma preparations without reference to any VWF international reference plasmas, as these were not available until several decades later. There was also potential variation in the quality control products and lack of standardization with the ristocetin reagent itself.[77] Overall, the assay was comparatively very time consuming and complex to perform, requiring experienced laboratory staff to perform the testing and correctly measure the slope of the aggregation response.

The assay has been reported in several reviews of international external quality assessment (EQA) programs over the years to have issues with precision, high CVs, and problems in terms of the lower limit of sensitivity for VWF.[78–84] This is due to the many variables mentioned earlier in terms of poor laboratory standardization of the earlier VWF:RCo assay by aggregation and agglutination.

Automation of von Willebrand Factor: Ristocetin Cofactor

With the advent of automation to the hemostasis laboratory, VWF:RCo was developed further to enable its performance on routine coagulation analyzers, with the hope to improve on the limitations of the semiquantitative and manual assays involving aggregation and agglutination.[76,77,84] Automation also aimed to decrease the time taken to perform the assay, to simplify the testing process to enable it to be performed more frequently, and at the same time to improve the noted limitations of decreased sensitivity at low levels and to improve the reproducibility of results.

BC von Willebrand Reagent

In the late 1990s, Dade Behring (now Siemens Healthcare Diagnostics; Marburg, Germany) introduced a reagent called the BC von Willebrand reagent (a lyophilized platelet suspension including ristocetin and ethylenediaminetetraacetic acid) to be used on their Behring Coagulation System (BCS) analyzer. When sample and reagent were mixed, the instrument measured the change in absorbance at 570 nm, as caused by the platelet agglutination. A calibration curve was plotted of the time to achieve a reduction of 0.300 absorbance units against the concentration of VWF:RCo activity using pooled normal plasma with known VWF:RCo activity.

This automated assay was subsequently modified to be used on other automated analyzers and numerous studies were performed comparing the automated BC von Willebrand reagent and different analyzer combinations with the traditional VWF:RCo by platelet aggregation. At least eight separate groups have evaluated this reagent on automated analyzers (Diagnostica Stago [STAR][23], Werfen IL [ACL],[24,25] Siemens [BCS],[23,26,29,30] and Sysmex [CS series]),[27,28,31] with the results of these studies summarized in ►**Table 2**. In summary, results demonstrated comparable results to the then reference methods by platelet aggregometry, but with better precision and greater sensitivity, including improved low level VWF sensitivity with assay modifications. Thus, Hillarp et al[29] modified the automated protocol on a BCS XP coagulation analyzer to include two calibration curves as described previously by Strandberg et al.[26] Further modifications included diluting the BC von Willebrand reagent with sodium chloride solution, increasing the ristocetin content by addition of extra ristocetin, and later using VWF depleted plasma as the diluent in the assay instead of saline.[30] A comparison of this modified protocol with the standard automated analyzer on an expanded range of VWD patient samples by Favaloro et al confirmed the improvements in low level VWF sensitivity and also in discrimination of VWD types.[30,31]

All of these studies have thus shown this reagent to be suitable as a VWD screening and diagnostic reagent and has allowed the assay to be automated on several analyzers with subsequent improvements in ease of testing and improved sensitivity.

Enzyme Linked Immunosorbent Assay, Flow-Based Assays, and Other Newer Challengers to von Willebrand Factor:Ristocetin Cofactor —Ristocetin Is No Longer Always Utilized

Several groups have furthermore adopted the VWF:RCo assay principles to both enzyme linked immunosorbent assay (ELISA) and flow cytometric techniques, as both can be standardized and automated (refer to ►**Table 3**, which summarizes the studies using VWF:RCo by ELISA and flow cytometry). Other workers have also described assay methods that do not utilize ristocetin, but which provide results largely comparable to VWF:RCo (►**Table 3**). All these assays have in common a focus on exploring the VWF–GPIb interface, and thus the failure of this interaction in some forms of VWD. For the purpose of this review, then, these assays will be referred to as VWF:RCo-like assays.

Assays Performed by Enzyme Linked Immunosorbent Assay

Murdock et al[34] developed an ELISA method to measure VWF utilizing a monoclonal antibody that recognizes the epitope on VWF involved in its binding to GPIb α. This method does not employ ristocetin, and therefore cannot be referred to as a VWF:RCo assay. The authors compared this assay against VWF:RCo using aggregometry and fresh washed platelets and found largely comparable results, but with some noted improvements in sensitivity and reproducibility.

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Table 2 Summary of studies using the BC von Willebrand reagent

Key references	Samples assessed	Assays compared	Main findings and conclusions
Miller et al (2002)[23]	123 normal women, 79 women with menorrhagia, 20 VWD patients	BC VW reagent on BCS analyzer (Siemens, Marburg, Germany). BC VW reagent modified for STAR analyzer (Diagnostica Stago). Traditional VWF:RCo with platelet aggregometer.	Automated methods results equivalent/superior to traditional VWF:RCo.
Lattuada et al (2004)[24]	95 samples in total: 67 normal patients and 28 VWD patients (type 1 [*n* = 3], type 2A [*n* = 8], type 2B [*n* = 4], type 3 [*n* = 6], type 2M [*n* = 5], type 2M Vincenza [*n* = 2])	BC VW reagent on ACL 9000 coagulometer (IL). Traditional aggregometry using formalin fixed human platelets from blood bank donors.	Automated VWF:RCo improved turnaround times, sensitivity, detection down to < 1 IU/dL (vs 6.25 IU/dL). Improved precision: within-assay and between-assay CVs of < 5% (vs 10 and 15%). More accurate classification VWD subtypes.
Redaelli et al (2005)[25]	105 samples overall: 50 normal subjects, 24 type 1 VWD, 24 type 2 VWD, and 7 type 3 VWD patients.	BC VW reagent on ACL 7000 coagulometer (IL). Traditional VWF:RCo by aggregometry using lyophilized platelets.	Automated assay improved precision: CVs 10% (vs 19%). High correlation with traditional VWF:RCo. Easy to perform, with time and cost savings.
Strandberg et al (2006)[26]	478 patients overall: 251 normal patients, 103 VWD patients, 144 patients with other coagulation factor deficiency or platelet dysfunction	BC VW reagent on BCS analyzer (Siemens, Marburg, Germany), modified to include two calibration curves to improve lower limit of detection. VWF:RCo by agglutination method (VWF:RCo Agg).	Modified automated method superior to VWF: RCo Agg. Lower detection limit 3.0 IU/dL, Imprecision lower CVs 6–8% (vs 13–15%). Significantly more cost-effective and easier to perform.
Bowyer et al (2011)[27]	53 known VWD patients: 27 type 1, 15 type 2A or 2M, 4 type 2N, 3 type 3 VWD, 25 patients tested prior to and after receiving desmopressin or VWF-concentrates.	BC VW reagent on CS-2100i (Sysmex, Kobe, Japan). In-house manual visual agglutination assay using ristocetin and formaldehyde-fixed donor platelets.	Good correlation between methods. Improved lower limit of detection down to 5 IU/dL. Improved precision with intra and inter assay CV% 2.3 and 3.8% (vs 7% manual method).
Lawrie et al (2011)[28]	30 healthy normal subjects, 39 VWD patients.	BC VW reagent on Sysmex CS-2000i analyzer (Sysmex UK Ltd). Traditional VWF:RCo platelet aggregation assay.	Good correlation between methods. Automated method lower sensitivity limit 10 IU/dL (vs 20 IU/dL). No significant variation seen with three different BC VW reagent lots. Significant labor savings.
Hillarp et al (2010)[29]	Small number of patient samples: 7 type 2 VWD, 7 type 3 VWD patients. Commercial VWF concentrates with known potencies.	Modified protocol for BC VW reagent on a BCS XP coagulation analyzer (Siemens, Marburg, Germany) to include two calibration curves, diluting BC VW reagent with NaCl, addition of extra ristocetin, and using VWF depleted plasma as diluent instead of saline. Final platelet count difference of 900×10^{9} L in original BC reagent (vs 500×10^{9} L in modified), final ristocetin concentration of 0.53 mg/mL (vs 1.45 mg/mL in modified). Compared with manual agglutination based assay (VWF:RCo Agg).	Improved limit of detection from 10 IU/dL in original method (vs 3 IU/dL modified method). Potency estimates of the VWF concentrates close to assigned potency value. Modified assay had superior discrimination power in low VWF range and with type 3 patients, yet still accurately measure VWF functionality in type 2 patients.
Favaloro et al (2010)[30]	Type 1 VWD (*n* = 17), type 2A (*n* = 15), type 2B (*n* = 4), type 3 (*n* = 4), a commercial VWF deficient sample, borderline VWD/possible VWD patients (*n* = 11).	Modified protocol for BC VW reagent described by Hillarp et al (2010)[29]	Modified method: superior VWF lower limit of detection 3 IU/dL (vs 10–15 IU/dL). Better discrimination severe type 1 versus type 3 VWD.
Favaloro & Mohammed (2014)[31]	600 samples in total across the methods: normals (*n* = 56), low VWF samples (*n* = 60), type 1 VWD (*n* = 12), type 3 VWD (*n* = 6), type 2A (*n* = 28), type 2M (*n* = 24), type 2B (*n* = 11), platelet type VWD (PT-VWD; *n* = 2), non-VWD cases (*n* = 242), and others including post concentrate or DDAVP or miscellaneous samples (*n* = 156).	VWF:RCo using BC VW reagent on BCS analyzer (reference method; Siemens, Marburg, Germany) and the BC VW reagent on CS-5100 analyzer (Sysmex, Kobe, Japan) Innovance VWF Activity (VWF Ac) on CS-5100 (Sysmex, Kobe, Japan), and other VWD screening assays.	Correlation between methods good: VWF:RCo on BCS vs VWF:RCo on CS-5100 (r = 0.962), VWF:RCo on BCS vs VWF Ac on CS 5100 (r = 0.958). VWF:RCo assay on CS-5100 may need to be optimized to improve lower limit of detection to below 9 IU/dL. Overall, VWF Ac assay comparable to VWF:RCo, with no major discrepancies in VWD identification or provisional VWD type observed with VWF:RCo vs VWF Ac assays.

Abbreviations BCA, Behring coagulation system; IL, instrumentation laboratory; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:RCo, VWF Ristocetin Cofactor assay;.VWF Ac, Innovance VWF activity assay; VWF:RCo Agg, VWF:RCo by agglutination method.

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Table 3 Summary of studies using the VWF:RCo or VWF:RCo-like assays by ELISA and flow cytometry

Key references	Samples assessed	Assays compared	Main findings and conclusions
Murdock et al (1997)[34]	192 VWD patients: type 1 (*n* = 156), type 2A (*n* = 26), type 2B (*n* = 8)	ELISA method to measure VWF Mab recognizing epitope on VWF involved in binding to GPIb α (no ristocetin required). VWF:RCo assay using aggregometry and fresh washed platelets.	Mab-based ELISA better sensitivity and reproducibility than traditional VWF:RCo.
Vanhoorelbeke et al (2000)[32]	Normal individuals (*n* = 24) and VWD patients: type 1 (*n* = 17), type 2A (*n* = 18), type 2B (*n* = 4), type 2N (*n* = 2), type 3 (*n* = 3).	VWF:RCo by ELISA: plasma VWF binds to captured rGPIb α fragment in the presence of ristocetin. VWF:RCo by agglutination (VWF:RCo Agg).	Good correlation between assays. Improved intra- and inter-assay variability by ELISA CVs < 14%. Increased sensitivity with lower limit of detection 0.05 IU/dL (vs 10–12 IU/dL VWF: RCo Agg).
Federici et al (2004)[33]	Normal subjects (*n* = 60), 62 patients with VWD: type 1 (*n* = 8), type 2A (*n* = 16), type 2B (*n* = 13), type 2M (*n* = 17) and type 2M Vincenza (*n* = 8) with low VWF levels.	VWF:RCo by ELISA. In-house VWF:RCo, platelet agglutination using human formalin fixed donor platelets.	ELISA assay similar results to platelet agglutination assay with less variability and increased sensitivity at lower limit of detection (0.1 vs 6.25 IU/dL)
Flood et al (2011)[35]	Normal healthy controls (*n* = 113), known VWD patients: type 1 (*n* = 107), type 2a (*n* = 18), type 2B (*n* = 11), type 2M (*n* = 6), type 3 (*n* = 17)	ELISA-based assay of VWF GPIb interactions using gain of function GPIb construct, that preferentially binds HMW VWF, without the need for ristocetin, measuring VWF activity (termed the assay 'VWF:IbCo'). Does not employ ristocetin. Compared against other VWD screening assays including VWF:RCo (BC VW reagent on BCS analyzer, Siemens Healthcare Diagnostics, Marburg, Germany).	VWFIb:Co ELISA correlated with VWF:RCo for type 1, type 2A, type 2M, and type 2N VWD. VWF:IbCo yielded undetectable levels for type 3 VWD patients compared with VWF:RCo. Elevated VWFGpIb:Co values with type 2B subjects, suggesting assay may discriminate type 2A and 2B VWD. VWFGpIb:Co ELISA useful in testing VWF binding to GpIb, discrimination of type 2 variants with advantages over traditional VWF:RCo assays.
Lindahl et al (2003)[36]	Normal donors and known VWD patients: type 1 (*n* = 24), type 2B (*n* = 2), type 2M (*n* = 1)	VWF:RCo by flow cytometry using formalin-fixed platelets, fluorescein isothiocyanate-conjugated chicken anti-VWF antibodies (Fab-fragments) and phycoerythrin-conjugated anti-GPIIb/IIIa antibodies. VWF:RCo platelet aggregation method (BC VW reagent).	Flow method showed good within-assay and total precision (CVs 4.2%, and 7.5%) at a mean concentration of 40 IU/dL. Type 2B VWD showed discordance between VWF:Ag and flow VWF:RCo, diagnostic of type 2 VWD.
Giannini et al (2007)[37]	Normal donors (*n* = 34), VWD patients: type 1 (*n* = 24), type 2A (*n* = 1), type 2B (*n* = 3), type 2M (*n* = 6). Twelve VWD patients were also tested at baseline and 1 h after a desmopressin (DDAVP) infusion.	VWF:RCo by flow cytometry using formalin-fixed platelets incubated with ristocetin and patient plasma, labeled with anti-VWF monoclonal antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody, an alternative method using autologous platelets (platelet rich plasma [PRP])—similar to RIPA, compared both flow methods to VWF:RCo platelet aggregation method	Flow cytometry method good correlation with the platelet aggregation method (r = 0.90, fixed-formalin platelets and r = 0.69, autologous platelets). Flow cytometry method formalin fixed platelets: within-assay and total precision (CVs 5.6–7.9%), similar to Lindahl et al.[35] Sensitivity flow method using fixed platelets was 0.92 for patients with VWD type I patients and 0.88 for all types of VWD, similar sensitivities seen with autologous platelets. Both methods able to discriminate changes in plasma VWF concentration and function after DDAVP infusion.
Chen et al (2008)[38]	Normal donors (*n* = 51), VWD patients: type 1 (*n* = 16), type 2 (*n* = 17)	VWF:RCo by flow cytometry using colored flourochrome-labeled, fixed normal platelets incubated with ristocetin and patient plasma. The different molecular weight (MW) VWF multimers bind to platelets as either double events for higher MW or single events for lower MW. The extent of platelet microaggregates (double positive colors) correlates therefore with the plasma VWF:RCo activity. Compared with platelet aggregation VWF: RCo utilizing in-house washed donor platelet concentrates.	Flow-based assay showed good correlation with platelet aggregation method for normal donors and type 1 VWD patients. Better sensitivity than platelet aggregation method for detecting type 2 VWD, due to the assay's ability to distinguish between the different VWF multimers present.

Abbreviations: BCA, Behring coagulation system; ELISA, enzyme linked immunosorbent assay; GpIb, glycoprotein Ib; Mab, monoclonal antibody; MW, molecular weight; rGpIb, recombinant fragment of glycoprotein platelet receptor; RIPA, ristocetin induced platelet aggregation; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:RCo, von Willebrand factor ristocetin cofactor assay; VWF:RCo Agg, von Willebrand factor ristocetin cofactor assay by agglutination.

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Vanhoorelbeke et al[32] also developed an ELISA method, but this time employing ristocetin, where plasma VWF binds to a captured recombinant (r) GPIb α fragment in the presence of ristocetin; again, results were largely comparable to standard platelet-based VWF:RCo, but with noted improvements. Federici et al[33] described a similar ELISA technique to Vanhoorelbeke[32]; however, their method utilized a rGPIb α fragment bound to a different anti-GpIb α monoclonal antibody (LJ-P3) immobilized onto a microtiter plate, with this complex capturing plasma VWF in the presence of ristocetin. Both these assays therefore reflected ELISA-based VWF:RCo assays.

Later, Flood et al[35] described an ELISA-based assay of VWF–GPIb interactions using a gain of function rGPIb construct that preferentially binds HMW forms of VWF, to enable spontaneous binding without the need for ristocetin in the assay. They compared this new ELISA assay, measuring VWF activity (they used the term 'VWF:IbCo') against other VWF:RCo assays, again identifying largely comparable results but with noted improvements. This assay, not employing ristocetin, is therefore not a VWF: RCo assay, but could be described as VWF-GPIb-binding assay.

All the ELISA methods described earlier were noted by the respective authors to provide comparable results to standard VWF:RCo assays, but with some perceived improvements. However, none of these have been developed to commercialization, so that they remain in-house assays that are not widely utilized.

Platelet-Based Assays Performed by Flow Cytometry

Lindahl et al[36] developed a flow cytometric assay using formalin-fixed platelets, fluorescein isothiocyanate-conjugated chicken anti-VWF antibodies (Fab-fragments) and phycoerythrin-conjugated anti-GPIIb/IIIa antibodies, and compared this to a platelet aggregation method using BC von Willebrand reagent. Giannini et al[37] developed a flow cytometric assay using formalin-fixed platelets incubated with ristocetin and patient plasma, labeled with anti-VWF monoclonal antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody. They also developed an alternative method using autologous platelets (PRP)–similar to RIPA. Both flow methods were compared with a platelet aggregation method.

Chen et al[38] developed a flow cytometric assay using colored flourochrome-labeled, fixed normal platelets incubated with ristocetin and patient plasma. The different molecular weight (MW) VWF multimers bound to the platelets as either double events for higher molecular weight or single events for lower MW. The extent of platelet microaggregates (double positive colors) correlated therefore with the plasma VWF:RCo activity.

Again, all the platelet-based flow cytometry methods described earlier were noted by the respective authors to provide comparable results to standard VWF:RCo assays, but with some perceived improvements. However, none have been developed to commercialization, so that they also remain in-house assays that are not widely utilized.

Other Platelet Free von Willebrand Factor: Ristocetin Cofactor Assays and von Willebrand Factor:Ristocetin Cofactor-Like Assays Including Glycoprotein-Ib Binding

Some of the more recent improvements to the 'VWF:RCo class' of assays have been based around the development of platelet-free assays; some of these utilize the GPIb component of ristocetin binding and others assess GPIb binding or other activity assessment without the use of ristocetin. All assays comprise latex particle-based assays and rely on agglutination of the latex particles rather than platelet aggregation, but some utilize ristocetin to promote the aggregation, but others do not. The target on the latex particle varies according to the manufacturer with most using a recombinant form of GPIb and one a monoclonal antibody directed against the epitope on VWF involved in GpIb binding. The advantages of these assays over the traditional platelet-based VWF:RCo assays is their improved standardization, reduced variability, and improved lower limit of detection. ►**Table 4** provides a summary of studies using platelet-free VWF:RCo and also the VWF:RCo-like assays, including GPIb binding assays.

HemosIL von Willebrand Factor Activity Assay

The HemosIL von Willebrand Factor Activity assay (Instrumentation Laboratory, Bedford, MA) was the first of these platelet-free assays, being introduced in the early 2000s. This is a latex particle enhanced immunoturbidimetric assay that does not employ ristocetin. The latex particle is coated with a monoclonal antibody that recognizes the epitope on VWF involved in its binding to GPIb α, similar to that originally described by Murdock et al.[34] Due to the lack of ristocetin in the assay system, it is not a true VWF:RCo assay, and is alternatively termed a 'VWF activity' assay (VWF:Act) by the manufacturer.

At least eight groups have evaluated this assay, on a variety of instrumentation (►**Table 4**), including IL/Werfen (ACL Futura and TOP analyzers)[39,40,42,43,45,46] and Diagnostica Stago (STAC and STAR analyzers).[41,44] All investigators found the assay suitable as a VWD screening assay and for VWD diagnosis, but with some limitations/caveats. Most groups recommend that any low values obtained with this assay be screened with a traditional VWF:RCo assay.

HemosIL von Willebrand Factor:Ristocetin Cofactor Assay

The HemosIL VWF:RCo (Instrumentation Laboratory, Bedford, MA) is another platelet-free assay released in 2011. This immunoturbidimetric assay utilizes latex particles coated with a recombinant fragment of glycoprotein platelet receptor (rGpIb α) using a specific monoclonal antibody on the particle to orientate the rGpIb α in a way to enable it to interact with VWF in the patient sample in the presence of ristocetin. The degree of agglutination of the latex particles is directly proportional to the activity of VWF in the sample and is measured by the decrease in light transmission as the particles agglutinate. As the assay utilizes ristocetin, it can be referred to as a VWF:RCo assay.

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Table 4 Summary of studies using Platelet free VWF:RCo and also the VWF:RCo-like assays including GPIb binding assays

Key References	Samples Assessed	Assays Compared	Main findings and conclusions
Pinol et al (2003)[39]	Normal donors ($n = 18$); VWD patients ($n = 15$): type 1 ($n = 1$), type 2A ($n = 2$), type 2B ($n = 6$), type 2M ($n = 5$), type 3 ($n = 1$)	HemosIL VWF Activity on ACL Futura analyzer (IL).	Linearity up to 200 IU/dL, detection limit of 2.2 IU/dL. Precision studies CVs 5.8–7.3%, using normal and pathological controls. All normal and type 1 VWD had activity/Ag ratio of > 0.7. All type 2 VWD samples had activity/Ag ratio < 0.7, except for one type 2A (activity/Ag ratio 0.71). HemosIL able to discriminate qualitative differences seen in VWD.
Vleeschawer et al (2006)[41]	Normal donors ($n = 20$); VWD patients ($n = 51$): type 1 ($n = 42$), type 2 ($n = 6$), type 3 ($n = 3$); Plus other patients being screened for VWD ($n = 77$)	HemosIL VWF activity (VWF:Act) on STAC analyzer (Diagnostica Stago). Traditional VWF:RCo assay aggregation with normal fixed platelets.	Passing–Bablok regression: slope, 1.25 and intercept, 1.40; correlation coefficient 0.84 (95% CI, 0.78–0.89). Bland–Altman difference plot: mean difference, 5.7% (VWF:RCo vs VWF:Act). VWF:Act assay sensitivity of 1.00 and a specificity of 0.88 for detecting VWD when using cut-off value 60% VWF:Act. VWF:Act is reliable assay for VWD diagnosis, but recommend traditional VWF:RCo assay still be performed if low value detected by VWF:Act.
Sucker et al (2006)[42]	300 blood samples with all four assays and assessed their ability to predict loss of high molecular weight VWF multimers (HMWM).	HemosIL VWF activity assay on ACL Futura (IL). Traditional VWF:RCo, VWF:CB, and GPIb binding ELISA assay.	None of the assays showed sufficient sensitivity or specificity to detect or rule out absence of HMWM. All three assays compared with HemosIL, assess different functional properties of plasma VWF, differences seen between assay results. HemosIL assay, fully automated was easier to perform than GpIb-binding ELISA and could potentially replace ELISA-based GpIb binding assays and be part of a screening panel of VWF activity assays.
Pinol et al (2007)[40]	Normal controls ($n = 57$) and VWD patients ($n = 70$).	HemosIL VWF activity on ACL Futura analyzer (IL). VWF:RCo using aggregometer and normal fixed platelets.	HemosIL VWF:Act assay correlated with traditional VWF:RCo assay with VWD type 1 and 2 patients, unlike other indirect tests based on monoclonal epitopes of VWF A1 domain.[31] VWF:Act assay fully automated and faster than traditional VWF:RCo, sensitivity of 12.5 IU/dL, slightly less than traditional assay.
Salem and Van Cott (2007)[43]	61 samples from previously tested patients (type 1, type 2 VWD patients, nondiagnostic abnormalities, normal and elevated levels VWD)	HemosIL VWF activity (VWF:Act) on an ACL Top analyzer(IL). VWF:RCo with aggregometry using lyophilized platelets (Helena laboratories, Beaumont, TX). Ability to screen for VWD in conjunction with VWF:Ag, FVIII, and blood group was assessed.	Automated VWF:Act assay performed well as a screening assay, with 1.00 sensitivity and 0.86 specificity for detection VWF abnormalities. Automated VWF:Act reduced the need for traditional VWF:RCo testing by 67%, due to normal VWF activity. Recommended to detect all possible VWD that traditional VWF:RCo still be performed if VWF:Act < 60 IU/dL and VWF Act/VWF:Ag ratio was < 0.7.
Trossaert et al (2011)[44]	Prospectively screened patients ($n = 268$)	HemosIL VWF Activity (VWF:Act) on Stago STA-R analyser (Diagnostica Stago). VWF:RCo assay using BC VW reagent by platelet aggregometry. Assays ability for diagnosis of VWD.	VWF:Act and VWF:RCo correlated well ($r = 0.89$, $p < 0.0001$). Sensitivity for accurate VWD diagnosis was 75%, compared with expert using ISTH SSC guidelines when using either assay alone.[2] If assays were used in conjunction with PFA-100 results there was considerable improvement in sensitivity for both assays, up to 0.98 in screening for VWD.
Trossaert et al (2011)[44]	Retrospectively studied known VWD patients ($n = 111$)	As above.	VWF:Act and VWF:RCo assays correlated well ($p < 0.0001$), with no statistical difference between methods. Discrepant results between VWF:Act and VWF:RCo assay in certain VWD subtypes: type 2B VWD higher VWF:RCo vs VWF:Act, although not significant ($p = 0.16$). In three family members, type 2M (VWF gene missense mutation: PGly1324 A1a), significant discrepancies were seen between the two assays—VWF:RCo: < 3, 6, and 22 IU/dL compared with VWF:Act: 32, 23, and 59 IU/dL. One patient had normal VWF:Act compared with a reduced VWF:RCo.

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Table 4 (*Continued*)

Key References	Samples Assessed	Assays Compared	Main findings and conclusions
Chen et al (2011)[45]	492 patient samples: normals ($n = 368$), type 1 VWD ($n = 57$), type 2A/2B ($n = 31$), type 2M VWD ($n = 4$), severe type 1 vs type 2M VWD ($n = 8$), and acquired von Willebrand abnormalities (AVWA) ($n = 24$).	HemosIL VWF Activity (VWF:Act) on ACL TOP analyzer (IL). VWF:RCo by aggregation using washed platelets, VWF:RCo by flow cytometry.	VWF:Act showed excellent correlation with VWF:RCo assays without significant bias ($r = 0.93$, $p < 0.001$). Automated VWF:Act showed improved LLOD < 3 U/dL, excellent linearity between 3–350 U/dL and CV < 10%. Traditional VWF:RCo insensitive to AVWA—subtle loss of very high molecular weight multimers. VWF:Act showed superior sensitivity for detecting AVWS, close to 0.90 sensitivity and specificity (using VWF:Act/VWF:Ag ratio value <0.7). Changing this ratio to < 0.8 improved sensitivity to 1.00 for detecting congenital VWD, sensitivity 0.83 for detecting AVWS, and specificity 0.92 for detecting normal patients. Some rare discrepant results were observed with more work required before replacing VWF:RCo.
Lasne et al (2012)[46]	Patients referred for VWD screening ($n = 160$).	HemosIL VWF Activity on ACL TOP analyzer (IL). VWF:RCo by platelet aggregation using BC VW reagent and PFA-100 (Siemens, Marburg, Germany) results.	Acceptable linearity 0.10–1.00 IU/dL and precision: within-run and between-run CVs 2.3–14.1%. Bland–Altman difference plot showed good agreement between two methods, with differences seen in 10 samples. Seven had VWF:RCo values > 100 IU/dL and two had VWF:RCo between 42–74 IU/dL with corresponding higher VWF:Act values. One patient lower VWF:Act vs VWF:RCo (18 vs 65 IU/dL), potential misdiagnosis with VWF:Act assay. VWF:Ac assay suitable for VWD screening along with clinical assessment.
Pinol et al (2009)[47]	Normal individuals ($n = 74$) and known VWD patients ($n = 93$)	HemosIL VWF:RCo assay on ACL TOP system (IL). VWF:RCo by platelet aggregation using lyophilized platelets at two sites.	HemosIL VWF:RCo good linearity up to 130 IU/dL, lower limit of detection 3.5 IU/dL. Precision good with CVs 1.3–4.0% and 1.8–3.0% with normal and pathological control plasmas. Similar performance between automated and manual VWF:RCo methods. HemosIL VWF:RCo faster, fully automated, and demonstrated better reproducibility.
Stufano et al (2014)[48]	Healthy normal subjects ($n = 172$); VWD patients ($n = 80$): type 1 ($n = 16$), type 2 ($n = 58$), type 3 ($n = 6$), AVWS ($n = 7$)	HemosIL VWF:RCoF on ACL TOP500 analyzer (IL). VWD and AVWS patients previously tested for VWF:RCo using platelet-based assays performed with an aggregometer and an ACL 9000 analyzer (IL).[24,32]	HemosIL VWF:RCo inter-assay precision 3.8 and 5.2% with normal and pathological control plasmas. Lower limit of detection 4.4 IU/dL. Patients gave consistent results with the HemosIL VWF:RCo compared with traditional platelet-based assays. Three patients showed discrepancies between assays: type I VWD (29 vs 41 IU/dL), AVWS (33 vs 9 IUdL), and type 2A/IIE (18 vs < 6 IU/dL), higher values seen with HemosIL VWF:RCo.
Lawrie et al (2013)[49]	Healthy normal samples ($n = 50$); known VWD ($n = 80$): type 1 VWD ($n = 12$), type 2 VWD ($n = 60$), type 3 VWD ($n = 6$), acquired VWD ($n = 1$), unclassified ($n = 1$), samples with hemolysis, icterus, and lipaemia ($n = 50$).	Innovance VWF activity (VWF Ac). Automated platelet-based VWF:RCo assay using BC von Willebrand reagent. Both performed on CS: 2000i (Sysmex, Kobe, Japan).	Good correlation between clinical and normal samples between assays ($r = 0.99$ and $p < 0.001$), mean bias of 5.6 IU/dL. VWF Ac had superior lower limit of detection (LLOD) compared with VWF:RCo (3 vs 5 IU/dL). Allowing numerical detection of activity in 17 VWD samples previously below LLOD of VWF:RCo assay. Five samples (three type 1, one type 2A, and one type 2M) gave different VWF: RCo/Ag ratios (<0.7) to VWF: Ac/Ag ratios (>0.7) compared with their initial diagnosis. The authors were unsure as to why this occurred, possibly due to inherent variability of assays. Innovance VWF Ac assay had improved sensitivity and low levels of imprecision compared with VWF:RCo.

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Table 4 (*Continued*)

Key References	Samples Assessed	Assays Compared	Main findings and conclusions
Geisen et al (2014)[50]	Total patients ($n = 423$); normals ($n = 234$), known VWD ($n = 62$), acquired VWD (AVWD) ($n = 89$), and other conditions ($n = 47$).	Innovance VWF Activity (VWF Ac). Traditional BC VW reagent. Both performed on BCS XP analyzer (Siemens, Marburg, Germany).	VWF Activity and VWF:RCo and their ratios to VWF:Ag compared well. AVWS was more reliably detected by VWF Ac/VWF:Ag ratio. Data set largely non-VWD and AVWD cases, but results VWF Ac were comparable to VWF:RCo assay. VWF Ac could replace VWF:RCo for AVWS screening when prompt diagnosis is required.
Reilly-Stitt et al (2014)[51]	Healthy controls ($n = 27$) and patients with VWD ($n = 10$), type 1 ($n = 5$), type 2 ($n = 4$), and type 3 ($n = 1$).	Innovance VWF Activity (VWF Ac). BC VW reagent. Both performed on CS2000i analyser (Sysmex UK Ltd, Milton Keynes, UK).	Good correlation between assays for 36/37 samples, with small positive bias toward the VWF Ac assay. One patient, a significant outlier on correlation and agreement plots, due to high levels of HAMA which reacted with the antibody coated particles in Innovance assay. This resulted in increased turbidity and overestimation of VWF activity. A common source of error in immunoassays, with most manufacturers including heterophile antibody blocking agent in the reagent formulation.[85,86] In this patient, the blocking agent was not sufficient to eliminate cross-reactivity in the assay. This artifact needs to be considered with the Innovance VWF Ac assay and any immunoturbidimetric assay when interpreting results. The authors suggested performing VWF:RCo and VWF:Ag assay when there is high suspicion of such an event to rule out.
Graf et al (2014)[52]	100 normal plasma samples, 262 samples from 217 patients referred for VWD testing (197 adult and 64 children with one age unknown). Previously diagnosed VWD: type 1 ($n = 12$), type 2A ($n = 4$), type 2B ($n = 4$), type 2M 9 ($n = 7$), type 2N ($n = 1$), type 3 ($n = 3$), and acquired VWD ($n = 3$). Patients with VWF: D1427H polymorphism, characterized by reduced ristocetin-dependent binding VWF to GPIb, resulting in lower VWF activity relative to VWF:Ag.[87,88] ($n = 6$)	Innovance VWF activity (VWF Ac) on CS2000i (Sysmex, Kobe, Japan) and Stago STAR Evolution (Diagnostica Stago). VWF:RCo assay by aggregometry using Helena Aggram (Helena Laboratories, Beaumont, TX).	VWF Ac assay had better precision and sensitivity, and improved LLOD 0.04 (vs 0.10 IU/mL VWF:RCo). Subjects with gain-of-function defects (type 2B VWD), or loss-of-function defects (type 2A or 2M VWD), had reduced VWF Ac. Excellent agreement with VWF Ac run across both analyzer systems ($r = 0.96$). VWF Ac showed acceptable performance for VWD diagnosis and therapy monitoring, although VWF Ac results were on average, 0.07 IU/mL lower than VWF:RCo.
Patzke et al (2014)[53]	Normal controls ($n = 240$), patients with elevated VWF levels, and not classified ($n = 147$), VWD type 1 ($n = 82$) type 2A ($n = 25$), type 2B ($n = 21$), type 2M ($n = 12$), type 2N ($n = 6$), type 3 ($n = 7$), AVWS ($n = 8$), and patients treated with DDAVP ($n = 39$).	Innovance VWF activity (VWF Ac) on BCS/Sysmex coagulation systems (Siemens Healthcare Diagnostics, Marburg, Germany), VWF:RCo: BC VW reagent on BCS XP system (Siemens Healthcare Diagnostics, Marburg, Germany).	For all samples: correlation coefficient 0.99 and slope 0.96 using Passing–Bablok regression. VWF Ac values compared well to VWF:RCo for all VWD types, along with VWF:RCo/Ag ratios compared with VWF Ac/Ag ratio. Three patients were exceptions: with low VWF Ac/Ag ratios of 0.4 compared with VWF:RCo/Ag ratio (0.85, 1.20, 1.35). These patients had very low VWF:RCo values close to 0.1 IU/dL, which most likely caused the discrepancy. Linearity VWF Ac 0.4–6.0 IU/dL and within run imprecision 3.6%. Correlation studies between different reagent lots and different BCS/Sysmex coagulation systems showed excellent results. VWF Ac assay is reliable, precise, sensitive, and fully automated assay, superior to the VWF:RCo assay.
Favaloro & Mohammed (2014)[31]	600 samples in total across the methods: normals ($n = 56$), low VWF samples ($n = 60$), type 1 VWD ($n = 12$), type 3 VWD ($n = 6$), type 2A ($n = 28$), type 2M ($n = 24$), type 2B ($n = 11$), platelet type VWD (PT-VWD)($n = 2$), non-VWD cases ($n = 242$), and others including post concentrate or DDAVP or miscellaneous samples ($n = 156$).	Innovance VWF activity (VWF Ac) on CS-5100 (Sysmex, Kobe, Japan). VWF:RCo on BSC analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) using BC VW reagent (reference method) and the BC VW reagent on CS-5100 analyzer, and other VWD screening assays.	Correlation between methods good: VWF:RCo on BCS vs VWF:RCo on CS-5100 ($r = 0.962$), VWF:RCo on BCS vs VWF Ac on CS 5100 ($r = 0.958$). VWF:RCo assay on CS-5100 may need to be optimized to improve lower limit of detection to below 9 IU/dL. Overall, VWF Ac assay comparable to VWF:RCo, with no major discrepancies in VWD identification or provisional VWD type observed with VWF:RCo vs VWF Ac assays.

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Table 4 (*Continued*)

Key References	Samples Assessed	Assays Compared	Main findings and conclusions
Timm et al (2015)[54]	170 samples from a mixed population of patients referred for evaluation of bleeding tendency.	Four assays evaluated: Innovance VWF activity on BCS XP system (Siemens Healthcare Diagnostics, Marburg, Germany). BC VW reagent on Dade Behring automated coagulation timer (Siemens Healthcare Diagnostics, Marburg, Germany). Modified BC VW reagent protocol on BCS XP system (Siemens Healthcare Diagnostics, Marburg, Germany)[29] and HemosIL VWF:RCo on ACL TOP 500 analyzer (IL).	All methods correlated well, concordance correlation coefficients 0.90–0.95, Bland–Altman plots showed mean percentage differences 0.03–12%, with larger differences seen in lower measuring range. Overall, using cut-off 0.4 IU/mL for classification of VWD, a discrepancy was observed between samples across the four assays. Overall, 14 samples (8%) did not reach consensus between all assays. Looking at VWF:RCo/VWF:Ag ratio cut-off < 0.7 to discriminate between type 1 and type 2 VWD, differences were found for classification between the assays, with samples with ratio < 0.7 differing between 8–16%. Twenty-two samples having VWF activity < 0.4 IU/mL (13% overall) did not reach consensus between all assays using the 0.7 ratio to discriminate between type 1 and type 2 VWD. However, Favaloro et al[31] discussed that these differences may be due to the additive effects of assay variations, when data from two different assays are used to determine the ratios.

Abbreviations: AVWA, acquired von Willebrand abnormalities; AVWD, acquired von Willebrand disease; AVWS, acquired von Willebrand syndrome; BC VW, BC von Willebrand reagent; ELISA, enzyme linked immunosorbent assay; FVIII, Factor VIII; GpIb, Glycoprotein Ib; HAMA, human anti-mouse antibodies; HMWM, High molecular weight multimers; IL - Instrumentation Laboratory; ISTH SSC, International Society Thrombosis and Haemostasis Scientific Subcommittee; LLOD, lower limit of detection; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF Ac, Innovance von Willebrand factor activity assay; VWF:Act, von Willebrand activity assay; VWF:Ag, von Willebrand factor antigen; VWF:CB, von Willebrand collagen binding assay; VWF:RCo, von Willebrand ristocetin cofactor assay.

Two groups have published evaluations of the HemosIL VWF:RCo assay on ACL TOP analyzers (IL/Werfen) compared with traditional platelet aggregation-based assays[47,48]. Both showed the assay had improved lower limit of detection and improved precision, with benefits of being automated compared with traditional platelet aggregation VWF:RCo.

Innovance von Willebrand Factor Activity Assay

In 2011, a new VWF activity assay, Innovance VWF Ac (Siemens Healthcare Diagnostics, Marburg, Germany), was introduced to the market. This assay was promoted as being rapid and easily automated compared with the traditional VWF:RCo by aggregometry. The assay utilizes polystyrene particles coated with a murine anti-human GpIb antibody. Recombinant human GpIb (with two gain-of- function modifications) is added and binds to the polystyrene coated particles. When present in the patient sample, plasma VWF binds to the rGpIb in complex with the antibody coated particles and this causes particle agglutination, which is detected turbidimetrically on an automated coagulation analyzer. The use of the two gain-of-function modifications allows the assay to be performed without ristocetin.[89] As the assay does not use ristocetin, it is therefore not a VWF:RCo assay, and is thus best described as VWF-GPIb binding assay, but as noted has been designated as VWF Ac by the manufacturer.

At least seven groups have evaluated this assay (►**Table 4**). Assays compared with include VWF:RCo assay using BC von Willebrand reagent, both on Sysmex CS:2000i analyzer (Sysmex UK Ltd)[49]; the traditional VWF:RCo BC von Willebrand reagent on a Behring Coagulation system (BCS XP, Siemens, Marburg, Germany)[50]; VWF:RCo BC von Willebrand reagent on a CS2000i analyzer (Sysmex UK Ltd)[51]; VWF Ac on Sysmex CS2000i, and the Stago STAR Evolution (Diagnostica Stago) compared with VWF:RCo assays by aggregometry[52]; Patzke et al,[53] the inventors of the Innovance VWF Ac assay, described their evaluation of this assay on the BCS/Sysmex coagulation systems (Siemens, Marburg, Germany); Favaloro and Mohammed[31] evaluated the Innovance VWF Ac assay on a Sysmex CS-5100 analyzer (Sysmex, Kobe, Japan), and compared it to several other VWD screening assays including VWF:RCo by agglutination on BSC analyzer using Siemens BC VWF reagent (reference method) and the BC VWF reagent on the Sysmex CS-5100 analyzer; finally, Timm et al[54] evaluated four automated agglutination VWF:RCo assays: two platelet-based using BC von Willebrand reagent, Innovance VWF Ac on a BCS XP system (Siemens Healthcare Diagnostics, Marburg, Germany), and the HemosIL VWF:RCo on an ACL TOP 500 analyzer (IL). All of these assays as described previously, measure the change in optical density from VWF activation until agglutination. All seven groups showed the Innovance VWF Ac assay had improved precision, sensitivity, and lower limit of detection with the additional benefits of being automated compared with traditional platelet aggregation VWF:RCo. Some issues were found in a patient with high human anti-mouse antibodies.[51,85,86]

Overall, the three above mentioned platelet-free assays have provided improvements over the traditional platelet-based VWF:RCo assay with automation, reduced variability, and improved lower limit of detection. However, in several studies it was mentioned that there was still a need to

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confirm low levels of VWF activity with a VWF:RCo assay to aid in the correct diagnosis and subtype of VWD. In terms of the Innovance VWF Ac assay, the limitations are that some patients have lower levels of activity compared with VWF: RCo. Larger prospective studies and genotype–phenotype investigations would be required to determine if this VWF Ac assay would improve VWD detection, classification, and monitoring of treatment.

Chemiluminescence Technology: A New Chapter in von Willebrand disease Diagnostics

HemosIL Acustar–von Willebrand Factor:Ristocetin Cofactor

A relatively new development in the VWF:RCo assay market is the use of a rGPIb fragment immobilized onto a solid phase (hence platelet-free) instead of using stabilized human platelets. The previous examples highlighted in earlier sections have used latex particles or solid phase (ELISA) capture instead of platelets. In the chemiluminescence system developed for the HemosIL AcuStar (Instrumentation Laboratory, Barcelona, Spain) instrument, VWF in the patient plasma binds to the rGpIb fragment (similar to the one used in the HemosIL VWF: RCo assay discussed earlier) but this time coated on to magnetic particles through a highly specific monoclonal antibody, that orientates the fragment to permit binding of plasma VWF after incubation with a ristocetin containing buffer. Detection is by chemiluminescence, using isoluminol-labeled secondary anti-VWF antibodies and trigger solutions. The emitted light is directly proportional to the VWF:RCo activity. The AcuStar VWF:RCo reagent is supplied in ready-to-use cartridges that have an on-board analyzer stability of 8 weeks. At least seven groups have evaluated this new technology and compared it to various other VWF:RCo assays available on the market (findings summarized in ►**Table 5**).

Table 5 VWF:RCo assay using chemiluminescence technology: HemosIL AcuStar assay

Key references	Samples assessed	Assays compared	Main findings and conclusions
Verfaille et al (2013)[55]	Healthy volunteers (*n* = 40); patient samples (*n* = 61)—first time screening for VWD (*n* = 36), previously diagnosed VWD (*n* = 15) or desmopressin therapy trial (*n* = 10).	Three assays evaluated: HemosIL AcuStar VWF:RCo assay, traditional VWF:RCo assay on aggregometer, HemosIL VWF activity on STA analyzer (Diagnostica Stago).	Good agreement between the three methods, although slightly lower results were achieved with AcuStar VWF:RCo, due to improved lower limit of detection (LLOD) 0.2 IU/dL. HemosIL AcuStar assay improved precision 7%, including the lower range, a good sensitivity to low VWF activity and a large linearity range 0–200 IU/dL.
Cabrera et al (2013)[56]	Healthy volunteers (*n* = 18) and 73 patient samples with low VWF:RCo levels (< 50IU/dL). Including type 1 VWD (*n* = 29), type 2A (*n* = 13), type 2B (*n* = 5), type 2M (*n* = 5), type 2N (*n* = 3), type 3 (*n* = 5), type 3 under treatment (*n* = 4), type 3 carriers (*n* = 5), and other pathologies (*n* = 4).	HemosIL AcuStar VWF:RCo assay, agglutination assay with BC VW reagent on BCT analyzer (Siemens Healthcare Diagnostics, Marburg, Germany).	No statistically significant difference between AcuStar VWF:RCo vs BC VW reagent method, except in type 2B and type 2N group of patients. In type 2B and 2N VWD group, mean VWF:RCo values were lower with AcuStar assay. HemosIL AcuStar had improved LLOD, was fast and easier to perform than traditional assay. AcuStar assay was 0.96 sensitive (VWF:RCo < 50 IU/dL) and 1.00 specific for detecting VWF abnormalities (no false positive VWD diagnosis were made), using ISTH guidelines.[2]
Stufano et al (2014)[48]	Healthy normal subjects (*n* = 172); VWD patients (*n* = 88): type 1 (*n* = 16), type 2A (*n* = 19), Ttpe 2B (*n* = 20), type 2M (*n* = 20), type 3 (*n* = 6), AVWS (*n* = 7)	HemosIL VWF:RCo AcuStar assay, HemosIL VWF:RCo on TOP500 analyzer (IL). VWD and AVWS results compared with previous VWF:RCo results obtained with platelet-based assays using an aggregometer[33] and ACL 9000 analyzer (IL)[24].	Good correlation between AcuStar VWF:RCo vs HemosIL VWF:RCo (r = 0.82) on all samples tested. AcuStar VWF:RCo inter-assay precision CVs 3.3–6.9% vs 3.8–6.3% VWF:RCo ACL TOP with normal and pathological controls. AcuStar method LLOD 0.5 IU/dL vs HemosIL VWF: RCo TOP 500 LLOD 4.4 IU/dL. This allowed measurement of VWF:RCo in 16 samples below LLOD with platelet-based VWF:RCo. Three type 1 VWD patients with HemosIL assays gave lower values compared with platelet-based assays. AVWS patients with barely detectable VWF levels gave lower VWF:RCo results by AcuStar compared with traditional platelet-based and ACL TOP methods. One AVWS patient, AcuStar VWF:RCo was comparable to the traditional platelet-based assays (9 vs 8 IU/dL) vs HemosIL VWF:RCo (33 IU/dL). AcuStar method was very simplistic, with ready-to-use cartridges, long on-board stability, greater sensitivity at low concentrations VWF, making it suitable for diagnosis VWD and AVWS in nonspecialized and reference laboratories. Higher sensitivity AcuStar method (LLOD 0.5 IU/dL) would allow more accurate monitoring residual

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Table 5 (*Continued*)

Key references	Samples assessed	Assays compared	Main findings and conclusions
			VWF:RCo in type 3 VWD patients receiving prophylaxis.
de Maistre et al (2014)[57]	122 patient samples previously diagnosed with VWD or moderate quantitative VWF deficiency (30–50 IU/dL) and 11 healthy blood donors.	HemosIL AcuStar VWF:RCo assay, traditional VWF:RCo by aggregation, Innovance VWF activity (VWF Ac) assay on BCS analyzer (Siemens Healthcare diagnostics, Marburg, Germany).	Good correlation and concordance between all three assays. Type 2 VWD patients (confirmed by genotype) showed correlation between VWF:RCo AcuStar and VWF:RCo by aggregation was better than that between VWF Ac and VWF:RCo by aggregation. Authors explain, similarity in VWF:RCo AcuStar and VWF:RCo assay principles and lack of ristocetin in VWF Ac assay. The VWF Ac/antigen ratio with Innovance VWF Ac seemed better than the other two methods in predicting type 2B VWD (ratio < 0.7). Again the methodology of the assay could explain its high sensitivity to type 2B VWD, with the mutated VWF in these patients more easily recognizing gain of function r-GpIb in the assay. This needs to be confirmed in larger group of type 2B VWD, only 13 tested in this study. AcuStar VWF:RCo easy to perform, suitable for emergency use with high sensitivity to low levels VWF:RCo compared with traditional VWF:RCo by aggregation.
Costa-Pinto et al (2014)[58]	Normal subjects ($n = 30$) and known VWD patients ($n = 146$): type 1 ($n = 51$), type 2A ($n = 34$), type 2B ($n = 16$), type 2M ($n = 31$), type 2N ($n = 5$), and type 3 ($n = 9$).	HemosIL AcuStar VWF:RCo assay, VWF:RCo by aggregometry using lyophilized platelets (Helena Biosciences, Europe).	AcuStar VWF:RCo assay sensitive, specific, and reliable alternative to VWF:RCo by aggregation. There was a slightly lower correlation between AcuStar VWF:RCo and VWF:RCo by aggregation, due to lack of sensitivity of aggregation method to low VWF < 4 IU/dL and LLOD 0.5 IU/dL AcuStar VWF:RCo . This was the cause of some thirteen samples with discrepant results between original diagnosis and this study, with changes seen in VWF:RCo/VWF:Ag ratios, due to higher sensitivity and improved LLOD VWF:RCo AcuStar. Final VWD diagnosis based on the obtained ratios was generally consistent with previous diagnosis using traditional methods. AcuStar VWF:RCo method was sensitive, reliable, specific, and useful alternative to conventional VWF:RCo by aggregation.
Favaloro and Mohammed (2016)[59]	Total samples ($n = 535$): normals ($n = 13$), type 1 VWD ($n = 20$), type 2A ($n = 24$), type 2B ($n = 11$), type 2M ($n = 8$), type 3 ($n = 5$), probable VWD ($n = 67$), platelet type VWD ($n = 3$), non-VWD cases ($n = 107$), and other samples including post treatment with DDAVP or VWF concentrate, platelet lysate samples ($n = 177$).	HemosIL AcuStar VWF:RCo assay, VWF:RCo using BC VW reagent on CS-5100 analyzer (Sysmex, Kobe, Japan) and BCS analyser (Siemens Healthcare Diagnostics, Marburg, Germany), Innovance VWF Ac assay on CS-5100 analyzer (Sysmex, Kobe, Japan).	AcuStar VWF:RCo against the reference method VWF:RCo on CS5100 analyzer were highly correlated ($r = 0.928$) and comparable to the historical method VWF:RCo performed on the BCS analyzer and Innovance VWF Ac assay. A substantial bias was evident for all assays. Analysis of the data across all assays showed the current VWF:RCo assay on CS-5100 to be the outlier of this group of platelet GPIb binding assays. AcuStar method had excellent reproducibility, low inter-assay variability, and excellent low level VWF sensitivity, important in identification of type 3 VWD and discrimination of type 1 and 2 VWD patients.
Sagheer et al (2016)[60]	Total samples ($n = 60$) across all three assays: normals ($n = 25$), type 1 VWD ($n = 17$), type 2A VWD ($n = 3$), type 2B ($n = 4$), and type 2M ($n = 11$).	HemosIL AcuStar VWF:RCo assay, HemosIL VWF activity assay on a STAR Evolution analyzer (Diagnostica Stago), traditional VWF:RCo by aggregation using washed platelet concentrates.	AcuStar VWF:RCo between run precision CVs 4.1 and 4.2% with normal and abnormal control plasmas compared with HemosIL VWF activity precision 8.8–16.0%. LLOD with AcuStar was 0.17 IU/dL, verified with type 3 VWD patient. Good correlation VWF:RCo AcuStar and VWF:RCo by aggregation ($r = 0.91$) with AcuStar method showing a negative bias 5 IU/dL. Sensitivity for detection type 2 VWD was 100, 82, and 94% (AcuStar, HemosIL activity and VWF:RCo aggregation) and specificity 82, 100, and 88%. VWF:RCo AcuStar identified all type 2 VWD patients, was automated, faster turnaround time, and no need for platelets, with potential to replace traditional VWF:RCo.

Abbreviations: AVWS, acquired von Willebrand syndrome; BC VW, BC von Willebrand reagent; IL, Instrumentation Laboratory; ISTH, International Society Thrombosis and Haemostasis; LLOD, lower limit of detection; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF Ac, Innovance von Willebrand activity assay; VWF:RCo, von Willebrand factor ristocetin cofactor assay.

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Assays compared with AcuStar VWF:RCo include traditional VWF:RCo assay on an aggregometer and the HemosIL VWF Activity assay on STA analyzer (Diagnostica Stago)[55]; BC von Willebrand reagent on BCT analyzer (Siemens Health Care Diagnostics, Marburg, Germany)[56]; VWF:RCo assay on TOP500 analyzer (IL/Werfen)[48]; VWF:RCo by aggregation and the Innovance VWF activity (VWF Ac) assay on BCS analyzer (Siemens Healthcare Diagnostics, Marburg, Germany)[57]; traditional VWF: RCo by aggregometry[58,60]; VWF:RCo on CS-5100 and BCS analyzers using BC von Willebrand reagent and the Innovance VWF Ac assay on CS-5100 (Kobe, Japan),[59] and HemosIL VWF activity assay on STAR analyzer (Diagnostica Stago).[60]

All groups found that the assay was easy to perform with improved lower limit of detection compared with all compared assays, along with improvements in precision and sensitivity and significant improvements to turnaround times compared with traditional VWF:RCo by aggregation.

Conclusion: Where Are We Now?

Functional VWF assays including VWF:RCo are critical in diagnosing VWD and are essential for the correct classification of VWD patients. They are also used in the monitoring of treatment in the various subtypes of VWD.[1,2,74,90]

The VWF:RCo assay remained relatively unchanged for almost 30 years since its initial development in the 1970s. However, in the past 10 years there has been major redevelopment of the assay, from being a largely manual in-house assay to now being automated on many of the modern day coagulation analyzers. This has enabled significant improvements in the assays' sensitivity and precision in the diagnosis and typing of VWD patients and in monitoring of treatment in these patients. This has been evidenced, for example, by review of data from several international EQA programs.[80–84,91]

The name(s) applied to this assay and the functional ability of ristocetin in terms of VWD diagnosis has undergone many changes over time. The test was originally described as FVIIIR: Rco (factor VIII related:ristocetin cofactor) and later VWF:RCof by the Scientific and Standardisation committee (SSC) of the ISTH.[22] This was revised in 2001 to what is currently known as VWF:RCo.[92] Due to the emergence of the newer VWF:RCo and 'VWF:RCo-like' assays in the past 10 years, the VWF subcommittee of the SSC of the ISTH developed a new nomenclature for platelet-dependent VWF activity, as published in 2015.[61] According to this recommendation, VWF:RCo (ristocetin cofactor activity) now describes all assays that use platelets and ristocetin, VWF:GPIbR all assays that are based on ristocetin-induced binding of VWF to a recombinant wild type (WT) GPIb fragment, VWF:GPIbM all assays that are based on the spontaneous binding of VWF to gain-of-function mutant GPIb fragment and VWF:Ab all assays that are based on the binding of a monoclonal antibody (mAb) to a VWF A1 domain epitope.

In my own laboratory, we have recently switched from the VWF:RCo assay performed by platelet aggregometry to the HemosIL Acustar technology and this has provided significant improvement to our diagnosis and monitoring of treatment in VWD patients with improved assay sensitivity and precision. Assay results are now available 24 hours, 7 days a week with reduction in staff labor and cost savings to the laboratory. As laboratories become more automated, and with reduction of specialized qualified scientific staff,[93] the traditional platelet aggregation-based VWF:RCo assay will eventually become something mentioned only in textbooks from a historical teaching perspective. In the future, the newer automated assays will be a routine hemostasis test part of a diagnostic panel of VWD assays: FVIII, VWF:Ag, and functional VWF assays available for clinicians on-demand.

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