CAT
Critically Appraised Topic

Utility of the functional collagen binding assay in the diagnostic approach of VWD.

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Date: 08/03/2016

CLINICAL BOTTOM LINE

Von Willebrand disease is the most common inherited bleeding disorder and is caused by a deficiency or dysfunction of the plasma protein von Willebrand factor. There are currently six types of defined VWD: type 1 VWD (partial quantitative deficiency of VWF), type 2 VWD (qualitative deficiency of VWF), which is divided in four distinct types (2A, 2B, 2M and 2N), and type 3 VWD (total quantitative deficiency of VWF). The VWD diagnosis is complex due to the heterogeneity of the disease. VWD testing is undertaken in a step-wise process. A typical VWD screen panel, consisting of a VWF antigen assay, minimum one functional VWF assay and a FVIII assay, is followed by one or more confirmation assays depending on the initial test results.

VWF:CB is a functional assay, which provides useful information for the VWD diagnosis. VWF:CB can be used to differentiate between HMWM deficient samples from type 2A or type 2B VWD patients and samples from "normal" or type I VWD patients. In addition and more importantly, VWF:CB is necessary to detect a subtype of 2M VWD. VWD type 2M includes qualitative variants with a decreased VWF-dependent platelet adhesion due to a GpIbα-IX-V binding defect or a collagen binding defect. Collagen binding defects may be uncommon, however their real incidence will remain unknown until more data are available about the use of VWF:CB in the diagnostic approach of VWD. All reported cases with an exclusive collagen binding defect (due to a mutation in the A3 domain of VWF) had only a mild decreased VWF antigen level, a normal VWF:RCo/VWF:Ag ratio and a normal multimer distribution. Patients with an exclusive collagen binding defect escape clinical diagnosis unless a VWF:CB is performed.

The inclusion of VWF:CB in our VWD diagnostic panel would add valuable information and would reduce the potential for underdiagnosis of VWD.

CLINICAL/DIAGNOSTIC SCENARIO

Von Willebrand disease (VWD) is considered as the most common inherited bleeding disorder and arises from deficiency or dysfunction of the plasma protein von Willebrand factor (VWF).[1-5] The prevalence of VWD ranges from approx. 0.01% to 1% depending on the method used to identify patients, either by actual symptomatic patients seen at specialized medical centers (0.01%) or by population screening (1%).[2,5-8,12,13] The most common symptoms of VWD reflect the characteristic defect in platelet adhesion and include epistaxis, gum bleeding, easy bruising, petechial rash, menorrhagia and postsurgical or -trauma bleeding. In type 3 and 2N VWD, factor VIII (FVIII) level can be sufficiently low and bleeding symptoms similar to those of severe hemophilia, f.e. soft tissue and joint bleeding, can occur.[2,3,6,9,14]

The VWF gene is located on the short arm of chromosome 12.[2,9,10,13-15] VWF is a large multimeric adhesive glycoprotein composed of identical subunits with a complex role in thrombosis and hemostasis.[2,9,14,15] The biosynthesis of VWF is restricted to endothelial cells and megakaryocytes.[2,3,5,8,9,15] Endothelial cells synthesize VWF as a pre-pro-VWF consisting of a signal peptide, the pro-peptide and the mature VWF subunit.[9,15] The VWF synthesized within the endothelial cells is either directly released in the plasma and in the subendothelial matrix or stored into the Weibel-Palade bodies (WPBs). The second storage site for VWF is within the α-granules of platelets derived from megakaryocytes. VWF multimers and VWF propeptide are secreted in 1:1 stoichiometric amounts and VWF propeptide is immediately dissociated from the multimers after secretion. VWF multimers have a half-life of 12 hours and the plasma concentration average is 10 µg/mL.[2,5,8,9] VWF activity is dependent on the extent and pattern of multimerization. Plasma VWF exists as multimers of various sizes. UL VWF multimers and high molecular weight multimers are the most hemostatically active forms (UL VWF > HMWM VWF). UL VWF multimers are stored in the WPBs and α-granules. They can only be detected transiently in normal plasma and their release is triggered by a variety of agonists f.e. adenosine diphosphate, collagen, histamine, fibrin and thrombin. The activity of UL VWF should be tightly regulated. Therefore, UL VWF are quickly cleaved by the metalloprotease ADAMTS-13.[2,3,5,7,9,15]

VWF exists as a series of oligomers containing a variable number of subunits from a minimum of two to a maximum of 40, with the largest multimers having molecular weights in excess of 20,000 kDA. Each subunit consists of three A-domains, three B-domains, two C-domains and four D-domains (*figure 1*.) These domains are arranged in the following sequence: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK.[3,5,8-10,15]

The main functional domains are:

(1) A1 domain: binding site for the platelet receptor GpIbα-IX-V, heparin, nonfibrillar collagen type VI and fibrillar collagen type I and III
(2) A2 domain: cleavage site for ADAMTS-13
(3) A3 domain: main collagen binding site for fibrillar collagen type I and III
(4) C1-C2 domains: binding site for the platelet integrin αIIbβ3
(5) D'-D3 domains: binding site for FVIII and heparin

VWF has two main functions:[5,8,9,13,15]

(1) VWF serves as an adhesive bridge between platelets and damaged vascular subendothelium and promotes platelet-platelet interactions at sites of injury or high shear stress. At sites of injury, VWF binds to collagen. This binding, and/or high shear stress exerted on immobilized VWF, induces exposure of the A1 domain, which allows adhesion of platelets from the circulation through interactions with platelet receptor GpIbα-IX-V. Because of the fast on- and off-rates of the GpIbα-VWF interaction, platelets are decelerated, allowing establishment of more firm interactions between platelets (through platelet glycoprotein GPIa-IIa) and collagen. Platelet adhesion is followed by platelet aggregation, which involves interaction of activated integrin αIIbβ3 with fibrinogen and the C1 domain of VWF.

(2) VWF is a carrier protein and stabilizer of FVIII in the circulation. The formation of a non-covalent VWF-FVIII complex protects FVIII from degradation by activated protein C. This complex localizes FVIII at sites of platelet plug formation. After thrombin cleavage, activated FVIII (FVIIIa) is released from VWF and becomes an active cofactor.

There are currently six types of defined VWD, which are classified into three major categories: partial quantitative deficiency of VWF (type 1 VWD), qualitative deficiency of VWF (type 2 VWD) and a total quantitative deficiency of VWF (type 3 VWD). **Type 1 VWD**, an autosomal dominant disease, is the most common VWD (approx. 60 to 80% of all symptomatic VWD patients) and has reduced levels of functionally normal VWF due to a decreased cellular secretion or mild increased VWF clearance. **Type 2 VWD** (approx. 7 to 30% of all symptomatic VWD patients) comprises four distinct types. In brief, **type 2A** is characterized by a selective HMWM deficiency leading to a decreased binding to platelet receptor GpIbα-IX-V. **Type 2B** is caused by gain-of-function mutation leading to an increased affinity of plasma VWF to platelet GpIbα-IX-V, resulting in an increased platelet binding and clearance of VWF-platelet complexes from the circulation. Most type 2B VWD patients lack HMWM, they may have thrombocytopenia, sometimes associated with giant platelets and spontaneous platelet aggregation. A phenotype similar to type 2B VWD is the platelet-type VWD, also known as pseudo-VWD. This disease is caused by a rare gain-of-function mutation in the platelet receptor GpIbα-IX-V that leads to an increased affinity for VWF. **Type 2M VWD** includes platelet and collagen binding defects with a relatively preserved multimer distribution. **Type 2N VWD** is characterized by a reduced binding affinity of VWF to FVIII. **Type 3 VWD** (approx. 5 to 20% of all symptomatic VWD patients), an autosomal recessive disease, is the most severe form and shows virtually a total absent of VWF.[1-4,6,8-10,13,14,16-20,26] These six categories correspond to distinct pathophysiologic mechanisms and correlate to distinct clinical features and therapeutic requirements.[8,10,21]

The correct diagnosis of VWD is complex due to the heterogeneity of this disease, the variety of the available assays, the limitations of the different test procedures and the different opinions regarding the optimum testing methodologies and algorithms.[2,3,7,16-18,22,23] Therefore, we want to revise our test strategy and investigate the utility of the functional collagen binding assay (VWF:CB) in the test panel for VWD diagnosis.

QUESTIONS

1) *Question 1: How to diagnose VWD?*

2) *Question 2: Which is the added value of VWF:CB in the diagnostic approach of VWD?*

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term: "Von Willebrand diseases " [Mesh], "von Willebrand Diseases/diagnosis"[Mesh], "von Willebrand Diseases/classification"[Mesh], "von Willebrand Factor/diagnostic use"[Mesh] "von Willebrand Diseases/diagnosis"[Mesh] OR "von Willebrand Diseases/epidemiology"[Mesh]*
2) *Pubmed (Medline; from 1966).*
3) *SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/), Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane, Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm)*
4) *National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/), International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp), Clinical Laboratory Improvement Amendments (CLIA; http://www.cms.hhs.gov/clia/)*
5) *UpToDate Online version 12.2 (2004:*
    a. *Classification and pathophysiology of von Willebrand disease. Author: Margaret E Rick MD. Last update: May 15, 2015.*
    b. *Biology and normal function of von Willebrand factor. Author: Margaret E Rick MD. Last update: Jun 11, 2015.*
6) *Gene Database: "von Willebrand Factor"*

RELEVANT EVIDENCE/REFERENCES

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3) Original Articles

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51. Manual Von Willebrand Factor Antigen (HemosIL®AcuStar, Insert revision 04/2014)
52. Manual Von Willebrand Factor Ristocetin Cofactor Activity (HemosIL®Acustar, Insert revision 12/2014)
53. Manual FVIII deficient plasma (HemosIL®, insert revision 07/2012)

APPRAISAL

1. ***How to diagnose VWD?***

1.1. *General diagnostic approach of VWD.*

An appropriate VWD diagnosis is crucial for optimal patient management since treatment varies by VWD type. The diagnosis of VWD is being made by a clinical and physical examination, with personal and familial evidence of primarily mucocutaneous bleeding, and confirmed by laboratory testing.[1,2,7,10,13,27] An initial hemostasis laboratory evaluation usually includes a complete blood count with platelet count, the activated partial thromboplastin time (aPTT) and the prothrombin time. aPTT may be prolonged in type 3 or type 2N VWD, however in other VWD types, aPTT is mostly within the normal range. This panel of initial tests neither "rules in" nor "rules out" VWD. In most laboratories, VWD testing is undertaken in a staged or step-wise process.[1,2,7,8,10]

A typical VWD screen panel includes a VWF antigen assay (VWF:Ag), minimum one functional VWF assay and a FVIII assay. VWF activity is classically assessed by the VWF ristocetin cofactor activity assay (VWF:RCo), although more recent attention has focused on the VWF:CB. Dependent on the initial test results, additional laboratory testing might be necessary to confirm the diagnosis or to assist in subtyping VWD type 2, including VWF multimer analysis, VWF FVIII binding assay (VWF:FVIIIB), platelet aggregation with ristocetin as an agonist, and/or von Willebrand factor platelet binding assay (VWF:PB).[1-4,6,11,16-18,21,22,24,25] Many patients can be correctly classified using these phenotypic assays. Therefore, genetic testing should be limited to very specific situations f.e. to identify patients with a higher risk of developing neutralizing antibodies, to provide genetic counseling or to differentiate between VWD 2B and PT-VWD or VWD 2N and hemophilia A.[1-3,10,14,19] A summary of most commonly used VWD assays is given in table 1.[1,2,4,6,8,11,12,14,22,25]

The relative proportion of VWF activity compared to the total VWF antigen level is used to discriminate between type 1 and 2 VWD. The VWF:RCo/VWF:Ag ratio is typically used for this purpose and is concordant in type 1 VWD (above or equal to 0.7), whereas VWF:RCo/VWF:Ag ratios < 0.7 (or preferable < 0.5) are characteristic for 2A, 2M and most patients with 2B VWD.[1,2,14,16] Therefore, platelet aggregation with ristocetin is crucial for the diagnosis of 2B VWD.[2,8,14,25] The use of the VWF:RCo/VWF:Ag ratio is not recommended when there is an absence of VWF antigen and the diagnosis of type 3 VWD is being made.[1,2,6,14,24]

Assay	Utility in the diagnostic approach of VWD	Method
VWF:Ag	Screen panel.	Assessment of the total plasma VWF level. Measured by immunological methods, usually by ELISA-based methods or by automated immunoturbidimetric procedures.
VWF:RCo	Screen panel. Used to differentiate between VWD type 1 and 2. Used to identify 2M VWD.	The VWF:RCo assay is dependent on the presence of HMWM and an intact GpIbα-IX-V binding site. It measures the ability of VWF to bind platelet receptor GpIbα-IX-V. Classic method: ristocetin induced agglutination of formalin-fixed normal platelets in the presence of VWF. Newer methods: automated immunological-based assays with recombinant or plasma derived GpIbα(-IX-V) in the presence of ristocetin (with a turbidimetric or chemiluminescent detection system).
FVIII assay	Screen panel.	Quantifies the FVIII level in plasma. This assay is mostly performed as an aPTT based one-stage clotting assay or as a chromogenic assay.
VWF multimer analysis	Used to differentiate between VWD type 2A/2B and 2M.	Visualizes the VWF multimer distribution. Multimer distribution is classically analyzed by gel electrophoresis followed by visualization of the multimers.
VWF:CB	(Sometimes) screen panel. Used to differentiate between VWD type 1 and 2. Used to identify 2M VWD.	The VWF:CB assay is dependent on the presence of HMWM and an intact collagen binding site. It measures the ability of VWF to bind collagen. VWF:CB assay is mostly performed as an ELISA-based method.
VWF activity assay (VWF:Act)	Used to differentiate between VWD type 1 and type 2. Role in VWD diagnosis is still unclear/in development.	Non-ristocetin/non-collagen based method. VWF:Act assays use a monoclonal antibody directed against a functional binding site of VWF.
Platelet aggregation in platelet rich plasma (PRP)	Used to identify 2B VWD.	Measures an increased affinity of VWF to platelets when ristocetin is used as an agonist in a low (0.5 mg/mL) and high dose (1.2 mg/mL).
VWF:FVIIIB	Used to identify 2N VWD.	Measures the binding affinity of VWF to FVIII. The assay is usually performed by an ELISA-based method.
VWF:PB	Used to distinguish between type 2B VWD and PLT-VWD	Measures the binding of VWF to normal formalin-fixed platelets or a recombinant fragment of GpIbα(-IX-V) in the presence of a low concentration ristocetin. The bound amount of VWF is measured with a labeled antibody. VWD type 2B patients have increased VWF:PB results, while PLT-VWD patients have normal VWF:PB results. Other ways to discriminate between both diseases are ristocetin-induced platelet aggregation mixing studies and gene mutation studies.

Table 1. Most commonly used assays in the VWD diagnostic approach.

1.2. Current diagnostic approach of VWD in UH Leuven.

Our screen panel includes VWF:Ag, VWF:RCo and a FVIII coagulant assay (FVIII:C). The VWF:RCo/VWF:Ag ratio (reference value 0.7-1.4) is only reported when VWF multimer analysis is requested at the same time by the clinician. As recommended, we do not report the VWF:RCo/VWF:Ag ratio when there is an absence of VWF antigen. Instead, we suggest the diagnosis of type 3 VWD. VWF multimer analysis, platelet aggregation and VWF:FVIIIB are used to determine the type of VWD (*figure 3., 4., 5.)*. Our currently used VWD assays are summarized in table 2 and our test algorithm is shown in figure 2. [51-53]

Assay	Method	Reference value
VWF:Ag[a]	This two-step immunoassay uses magnetic particles, coated with anti-VWF polyclonal antibodies, as solid phase and a chemiluminescent detection system.	50-150%
VWF:RCo[a]	This two-step immunoassay uses magnetic particles, coated with a recombinant fragment of GpIbα, and a chemiluminescent detection system in the presence of ristocetin.	50-150%
FVIII:C[b]	The FVIII coagulant assay (FVIII:C) is an one-stage aPTT-based factor assay.	50-150%
VWF multimer analysis[c]	VWF multimer distribution analysis is performed by an in-house method: a gel electrophoresis with a concentration and a separation gel followed by western blotting and visualization of the multimers by a chemiluminescent detection system.	/
Platelet aggregation assay	Platelet aggregation assay is performed with the following eight agonists: adenosine diphosphate (5µM), collagen low dose (1µg/mL) and high dose (2µg/mL), ristocetin low dose (0.5 mg/mL) and high dose (1.2 mg/mL), arachidonic acid (1mM), U46 (1.33µM) and TRAP (18µM). Only the platelet aggregation results from ristocetin (low and high dose) are important for the diagnosis of VWD.	/
VWF:FVIIIB[c]	This assay is performed by an in-house ELISA, which uses a microtiter plate coated with anti-VWF antibodies. After isolation of the plasma VWF, purified FVIII is added and the bound FVIII is measured with a labeled antibody.	/

Table 2. VWD assays used in UH Leuven.
[a]VWF:Ag and VWF:RCo are from HemosIL® Acustar and are performed on Acustar (Instrumentation Laboratory) [b]FVIII:C assay is from HemosIL® and is performed on ACL TOP 700 (Instrumentation Laboratory). [c]VWF multimer analysis and VWF:FVIIIB assay are in-house, qualitative methods and are not mentioned in the laboratory test directory or on the standard laboratory request form.

1.3. Limitations of the general diagnostic approach of VWD.

There is always an overlap in assay results between unaffected "normal" individuals and affected VWD individuals. In addition, VWF level is influenced by several factors such as blood type, race, pregnancy, age, acute stress and inflammation, etc. These fluctuations can be a challenge for VWD diagnosis, especially in patients with mild deficiencies of VWF. Therefore, laboratory results should always be interpreted in the context of the patient's medical history and repeated testing for VWD on a fresh sample is sometimes needed to correctly differentiate between mild type I VWD patients and "normal" individuals.[1,7,8,10,14,19,27]

Many articles state that the high coefficient of variation (CV), both intra- and inter-laboratory, is one of the disadvantages of the VWF:RCo.[2-4,6,14,21] However, this poor reproducibility is mainly a problem of the classic VWF:RCo. Automation and optimization of VWF:RCo have certainly improved the reproducibility.[11,14,21,22] Another problem for all VWD assays is the poor lower limit of assay sensitivity.[11,14,21,22] Automation does not entirely overcome this problem.[2,4,6,28] It is important that each laboratory defines the performance characteristics and knows the limitations of their VWD assays.

Measurement of VWF multimer distribution is critical for accurate diagnosis of type 2A and 2B VWD, which both lack HMWM. Multimer distribution is classically analyzed by gel electrophoresis followed by visualization of the VWF multimers.[2,12,29] This assay is quickly disappearing in general laboratories because it is very labor-intensive, time consuming, requires specialized equipment and technical expertise and cannot be viewed as foolproof.[12,13,19,22,25,29] It takes several days to obtain results.[25,29] In UH Leuven, it takes at least 2 days before results are available.

A real limitation of all ristocetin based methods is the inference of some VWF polymorphisms leading to a significantly lower or higher VWF:RCo activity without a conferring hemorrhagic risk. For example, p.D1472H and p.P1467S result in a lower VWF:RCo/VWF:Ag ratio, without a conferring hemorrhagic risk.[1,11,19,31,32] p.D1472H is most common and results in a modest value reduction of approx. 28% and p.P1467S, which is less common, results in a reduction of approx. 92.5%. These patients are at risk of being misdiagnosed and/or inappropriately treated for VWD. These polymorphisms occur more frequently in African Americans compared to Caucasians.[11,30-34]

Many laboratories use only one activity VWF assay, classically the VWF:RCo, which has the disadvantage of missing a subtype of 2M VWD cases. Type 2M VWD is a qualitative disorder with dysfunctional VWF without a loss of HMWM. As previously stated, it includes platelet and collagen binding defects. Most 2M defects so far described adversely affect the binding of VWF to platelet GpIbα-IX-V.[19,21,35-37] However, some mutations, which affect the collagen binding, have already been described and those patients escape clinical diagnosis unless a VWF:CB is performed.[13,22,25,38-40]

2. ***Which is the added value of VWF:CB in the diagnostic approach of VWD?***

Collagen is an important ligand of VWF and can be used as an activity assay in the diagnostic approach of VWD. VWF binds to collagen through two sites: the A3 domain, which contains the main binding site for collagen type I and III, and the A1 domain, which binds collagen type I, III and VI. VWF:CB is mostly performed using an ELISA-based assay, which is easy to perform and minimal labor is required. There is a growing interest in VWF:CB because it may provide useful information.[6,19,22,25,29] VWF:CB is dependent on the presence of HMWM and an intact collagen binding site. Various collagen types are used in these assays: purified type I, type III, type IV, type VI collagen or a 95%/5% type I/III collagen mixture.[19,41,42] The collagen source can range from human placenta, equine tendon, bovine skin to calf skin.[42] The original assay, reported by Brown and Bosak, used bovine type I collagen.[43] Favaloro (2000) showed that the effectiveness of VWF:CB is influenced by the source of collagen, the type and concentration of collagen and sometimes by the used batch lot of collagen.[42] All VWF:CB assays should therefore be tested for suitability of use before they are implemented in routine.

As mentioned before, VWF multimer analysis is a difficult, labor-intensive, and costly technique. Therefore, many laboratories search for surrogate laboratory markers to identify a loss of HMWM, f.e. the VWF activity/VWF:Ag ratio, which should be below 0.7 (or preferable < 0.5). Several studies reported the utility of the VWF:CB to screen for multimer defects.[1,3,6,13,14,21-25,29,36,44-47]

Several studies already showed that the VWF:RCo/VWF:Ag ratio was generally less able to discriminate HMWM deficient samples from normal or quantitative VWF deficient samples compared to the VWF:CB/VWF:Ag ratio (*figure 6.,7.,8.,9.,10.*).[21-23,25,36,44] Favaloro (2000) investigated a large number of different collagen preparations (n=21) for their ability to discriminate between different VWD subtypes. Tissue sources varied from human placenta, calf skin, bovine tendon to equine tendon and collagen preparation included type I, type III, a mixture of type I and type III and type IV collagen. The best discrimination between type I and 2 VWD was observed using a bovine or an equine tendon type I/III collagen mixture.[42] In addition, Baronciani *et al.* (2006) have confirmed that VWF:CB assays based on a 95%/5% type I/III collagen mixture are best at detecting the loss of HMWM.[48] Performing VWF:CB, especially in combination with VWF:RCo, could possibly minimize the use of the VWF multimer assay (*table 3.*).

VWF:RCo and VWF:CB are also used to diagnose type 2M VWD.[45] As previously stated, type 2M VWD includes platelet and collagen binding defects, with a relatively preserved multimer distribution. Most 2M defects adversely affect the binding of VWF to platelet GpIbα-IX-V.[19,21,35-37] This condition is characterized by a relative low VWF:RCo value compared with the VWF:Ag result. These mutations do not seem to influence the binding of VWF to matrix components f.e. collagen. VWF:CB results will tend to parallel VWF:Ag results. It would be an inappropriate oversimplification to assume that this test pattern can be used to invariably identify all types of 2M VWD.[19,21,36,37] Genetic mutations that lead to a loss of relative binding to collagen with no or little effect on VWF binding to GpIbα-IX-V would present with a low VWF:CB/VWF:Ag ratio, a normal VWF:RCo/VWF:Ag ratio and a normal multimer distribution.[19,35,36]

Five naturally occurring mutations with exclusive collagen binding defects have already been reported: S1731T, W1745C, S1783A, M1761K and H1786D. These mutations are located in the collagen binding A3 domain of VWF and result in a mild bleeding phenotype. All reported cases were heterozygote for these mutations.[37-40] Three additional mutations, Q1734H, I1741T and Q1762R, have been described with a decreased collagen binding, however the affected subjects did not display profound bleeding symptoms.[39] Subtype 2M VWD with an exclusive collagen binding defect may be relatively rare or perhaps less clinically severe and therefore less diagnosed. However, type 2M due to exclusively collagen binding defects may also be less diagnosed because the VWF:RCo is mainly used as activity assay. It is possible that time and further study may redress the relative imbalance of differential detected cases of 2M VWD.[19,35] All cases had only a mild decreased VWF antigen level, a normal VWF:RCo/VWF:Ag ratio and a normal multimer distribution so not performing VWF:CB could lead to failure in the diagnosis of patients with exclusive collagen binding defects.[37-40] In summary, the detection of type 2M VWD requires both VWF:RCo and VWF:CB because they do not detect the same adhesive VWF function: VWF:RCo detects GpIbα-IX-V binding defects and VWF:CB detects collagen binding defects, which both impair platelet adhesion in vivo. [1,6,21,22,24,35,36]

We already know that VWF:CB based on a 95%/5% type I/III collagen mixture is best at detecting the loss of HMWM but not yet which type of collagen is best at detecting type 2M VWD with an isolated collagen binding defect. Riddell *et al.* (2009) investigated 3 unrelated families with following A3 domain mutations: W1745C, S1731T, S1783A. VWF:CB was performed using human placental type I and type III collagen. In family 1, the mother and her son were heterozygote for W1745C and had markedly reduced ratios of VWF:CB/VWF:Ag with both type I and III collagen. In family 2, mother and son were heterozygote for S1783A and VWF:CB/VWF:Ag was significantly reduced for both type I and III collagen. In family 3, the father, his daughter and his two brothers were heterozygote for S1731T and the VWF:CB/VWF:Ag ratio with type I collagen was reduced in all family members, whereas only one showed a reduction in the VWF:CB/VWF:Ag ratio with type III collagen (*Figure 11.*). All affected patients had a normal VWF multimeric pattern. The requirement of collagen in the PFA-100 cassettes suggests that it could be a sensitive test to detect defects of collagen binding, however Riddell *et al.* obtained normal clotting times in two affected subjects.[37] In the original report of mutation S1731T, the mutation resulted in a reduced binding to type I human collagen but binding to type III collagen was not assessed.[38] Keeling *et al.* (2012) reported the M1761K mutation. This mutation was picked up by their routine VWF:CB using equine type III collagen (11 IU/L, reference range 50-200 IU/L) but was missed by another VWF:CB using humane type III collagen (52 IU/L, reference range 50-200 IU/L). This finding suggests that not only the type of collagen is important but also the collagen source.[40] Flood *et al.* (2010) showed a reduction in binding to both types collagen for rVWF construct S1731T. The three other rVWF

constructs, H1786D, W1745C, S1783A, showed barely detectable binding to type I and III human collagen (*Figure 12.*).[39] Based on these limited studies, we can conclude that type I collagen is possibly best at detecting VWD type 2M patients with collagen binding defects.

Recently, several novel VWF A1 domain sequence variations have been demonstrated with a decreased binding to type VI collagen.[49,50] Data from Flood *et al.* (2012) demonstrated a decreased binding to type VI collagen for S1387I, R1399H, Q1402P and the 11 amino acid deletion 1392-1402 (all A1 domain variations or deletions). Subjects, which were heterozygote for S1387I (VWF:CB/VWF:Ag <0.7 with type I and VI collagen, not with type III), 11 amino acid deletion 1392-1402 (VWF:CB/VWF:Ag <0.7 with type I and VI collagen, not with type III) and Q1402P (VWF:CB/VWF:Ag <0.7 with type VI collagen, not with type I and III), had a decreased VWF:RCo/VWF:Ag ratio, and were diagnosed as type 2M VWD. However, one subject, which was heterozygote for R1399H mutation (VWF:CB/VWF:Ag <0.7 with type VI collagen, not with type I and III), had only a mild decrease in VWF levels and a normal VWF:RCo/VWF:Ag ratio and would therefore be missed by current VWD diagnostic approach. On the other hand, also two healthy control subjects, which were heterozygote for R1399H, were noted to have VWF:CB/VWF:Ag ratios below normal with collagen type VI but not with type I and III. Meaning that further work is needed to better characterize the in vivo effect of R1399H on hemostasis. Larsen *et al.* (2013) reported four A1 domain mutations in individuals with 2M VWD: S1358N, S1387I, S1394F and Q1402P. All subjects had a history of bleeding, low VWF:RCo results, a VWF:RCo/VWF:Ag ratio < 0.7 and a normal multimer distribution. The subject, which was heterozygote for S1394F, displayed normal binding to all three collagen types and the subject, which was heterozygote for S1358N, displayed a slightly reduced binding to type I collagen. Subjects, whom were heterozygote for S1387I and Q1402P, showed respectively a decreased binding to type I and type VI collagen (VWF:CB/VWF:Ag <0.7 with type I and VI collagen, not with type III) and a selective decreased binding to type VI collagen (VWF:CB/VWF:Ag <0.7 with type VI collagen, not with type I and III).[50]
Given that VWF A1 domain is responsible for the binding to GpIbα-IX-V and to collagen type VI, it is necessary to determine whether some mutations will impair selectively one of these interactions. The importance of type VI collagen in routine clinical laboratory testing requires further investigation.[49,50]

3. Conclusion

Several studies showed that the VWF:CB/VWF:Ag ratio can be used to detect the loss of HMWM. In addition and more importantly, the use of VWF:CB will detect mutations with exclusive collagen binding defects, which would otherwise be missed if VWF:RCo is the only used VWF activity assay.
The inclusion of VWF:CB in our VWF diagnostic panel would add valuable information and reduces the potential for underdiagnosis of VWD. The availability of this assay is important in our laboratory because UH Leuven is recognized as a national reference center for rare bleeding disorders.

TO DO/ACTIONS

1) To validate VWF:CB assays with type I, type III and type VI human collagen
2) Prospectively collect samples from patients with bleeding disorders
3) To evaluate the HemosIL®AcuStar VWF:CB (available within one year)
4) To evaluate the incidence of type 2M VWD with a reduced binding to collagen
5) To determine the mutations associated with type 2M VWD with a reduced binding to collagen (Bridge study)

ATTACHMENTS

Figures:

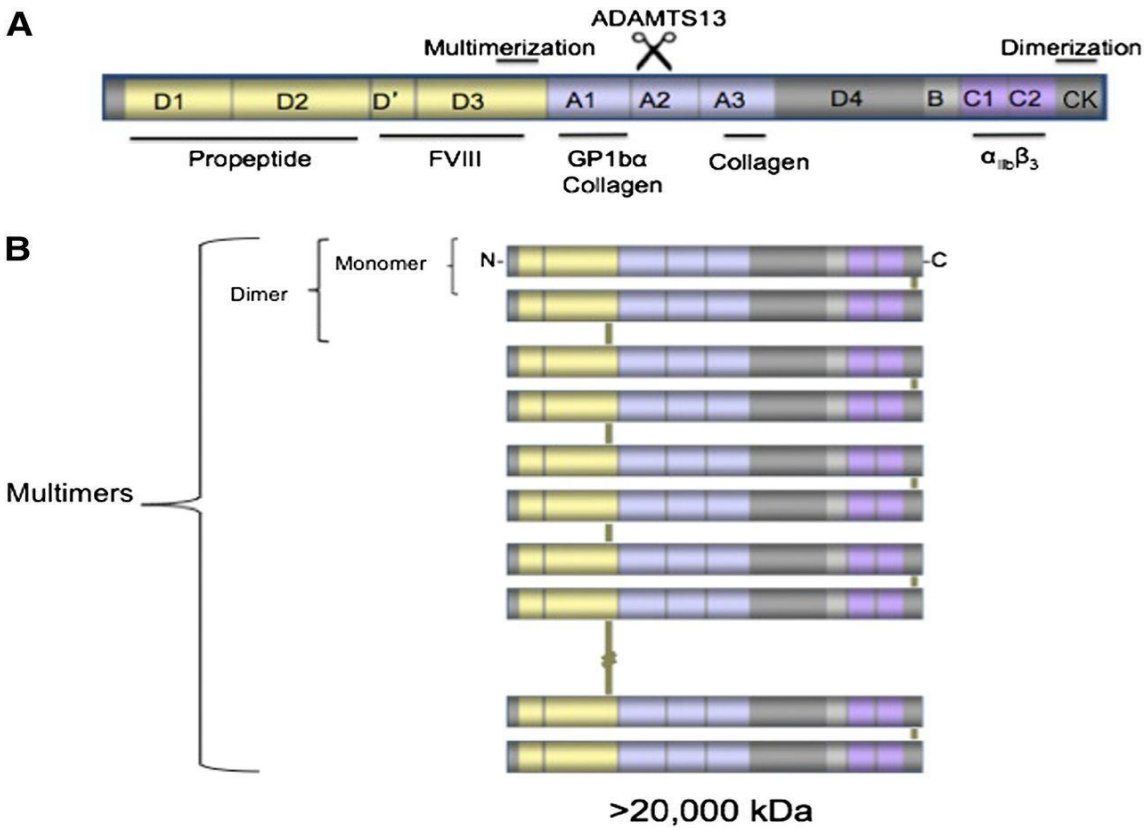
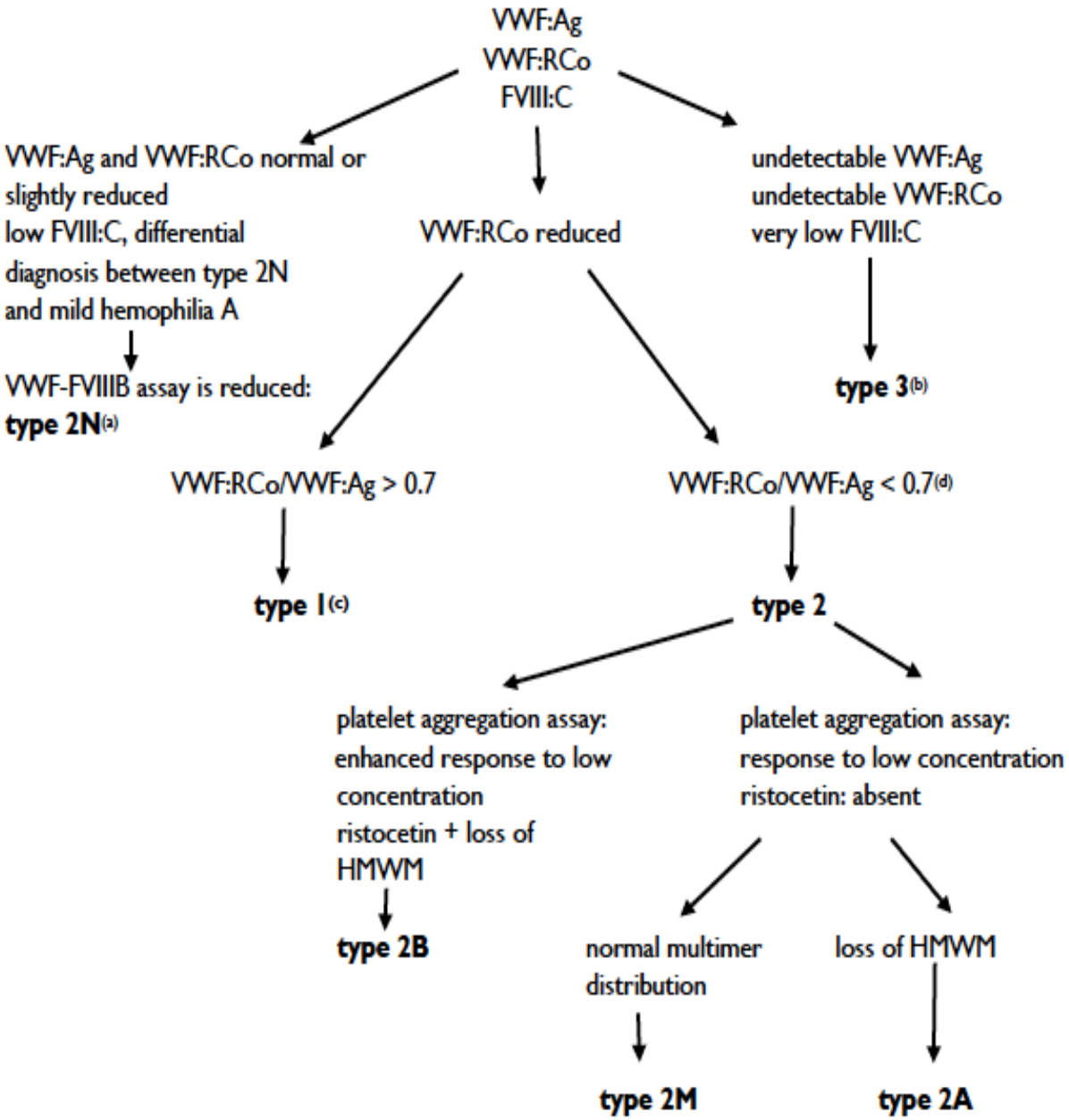


Figure 1. VWF structure. *(A) VWF monomer. (B) Multimerization process.*[8]



When additional tests are performed:

(a) normal multimer distribution, platelet aggregation assay: response to low concentration ristocetin: absent

(b) multimer pattern: absent, platelet aggregation assay: response to low concentration ristocetin: absent

(c) normal multimer distribution, platelet aggregation assay: response to low concentration ristocetin: absent

(d) VWF:RCo/VWF:Ag ratios < 0.7 are characteristic for VWD 2A, VWD 2M and most cases with VWD 2B

Figure 2. Current diagnostic approach of VWD in UH Leuven.

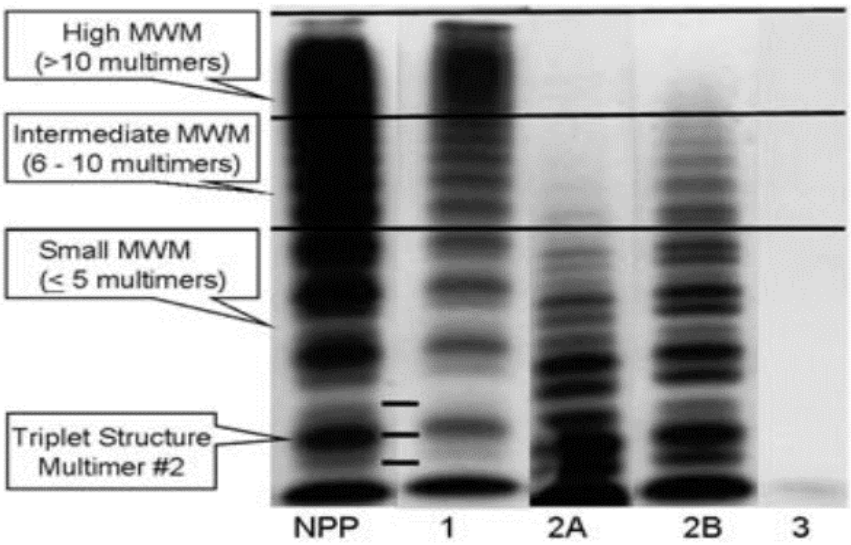


Figure 3. VWF multimer analysis.
Type 2A and 2B VWD are characterized by the loss of HMWM.

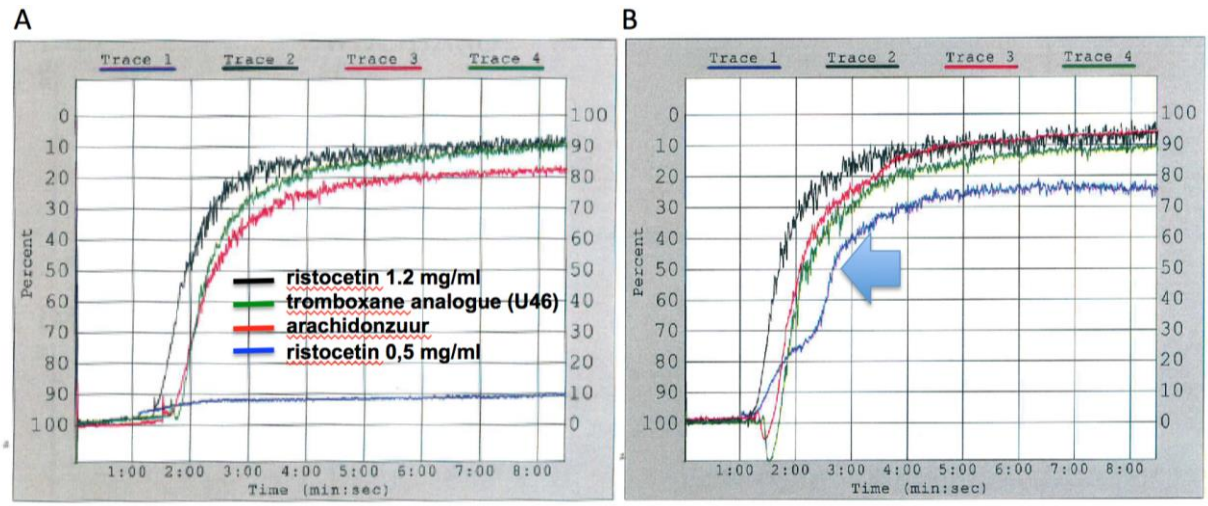


Figure 4. Platelet aggregation with ristocetin.
A. Platelet aggregation results from a normal patient. B. Platelet aggregation results from a patient with 2B VWD: Platelet agglutination with low dose ristocetin allows the diagnosis of type 2B VWD

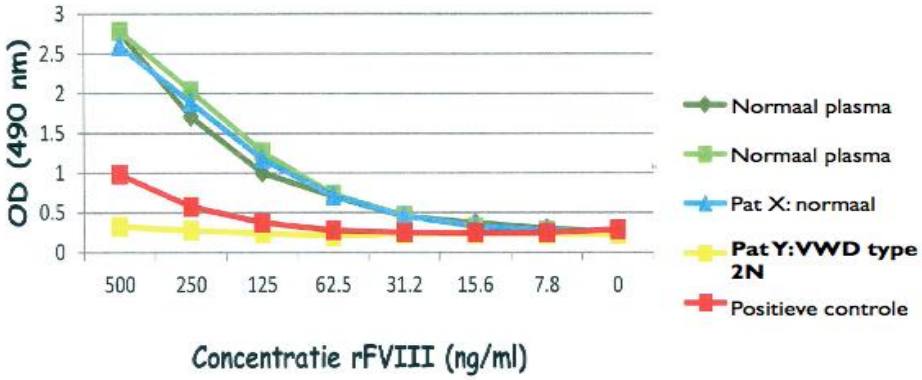


Figure 5. VWF:FVIIIB assay.

The yellow curve is from a patient with type 2N VWD. VWF shows a reduced FVIII binding capacity, which allows the diagnosis of type 2N VWD.

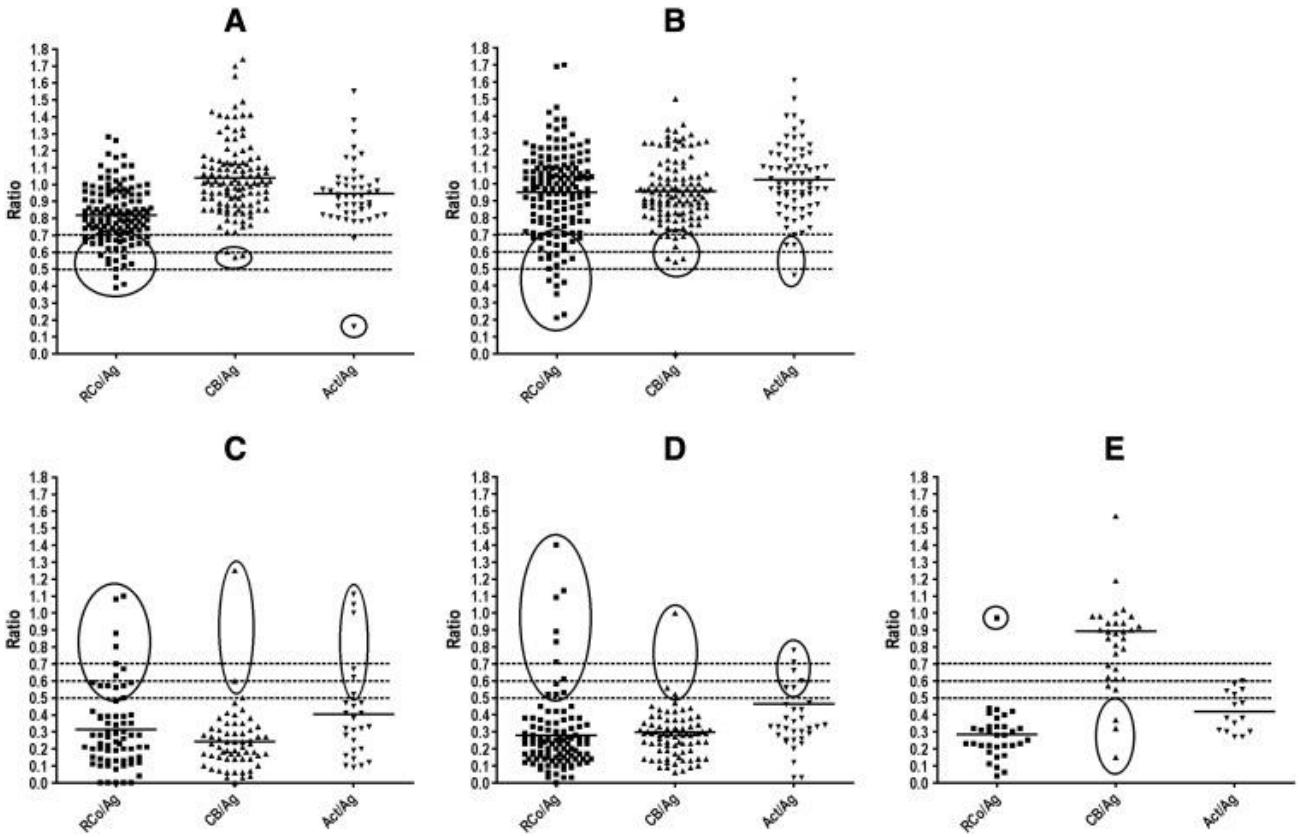


Figure 6. VWF activity/VWF:Ag ratios.

Favaloro et al. (2014) evaluated data from a large set of varied plasma samples comprising both "quantitative" and "qualitative" defects, which have been tested in a cross-laboratory (n=52-59) setting between 2006 and 2013.[21]

VWF:RCo: performed as platelet agglutination (using an aggregometer or a coagulaton based instrument) or as a LIA based method. VWF:CB: in-house or commercial ELISA. VWF:Act: non-ristocetin or non-collagen based methods using monoclonal antibodies against the VWF epitope containing the GpIb-binding site (ELISA or LIA).

A. Normal plasma with normal multimer pattern (n=4)

B. Mild/moderate VWF deficient samples with normal multimer pattern (n=4)

C. HMWM deficient samples: 2A VWD samples (n=2)

D. HMWM deficient samples: 2B VWD samples (n=3).

E. 2M VWD sample (n=1).

Circled values in each case reflect false (discordant) low ratios (< 0.7) for normal and VWF deficient samples (A and B) and false (concordant) high ratios (> 0.7) for HMWM deficient samples, or areas of overlap, and therefore causing difficulties and errors in quantitative vs qualitative VWD type assignment. Apart from noted (circled) outliers, the type 2M case (E) showed VWF:RCo/Ag ratios < 0.5 and VWF:CB/Ag ratios > 0.5, the VWF:Act/Ag ratios were intermediate between those for VWF:RCo/Ag and VWF:CB/Ag.

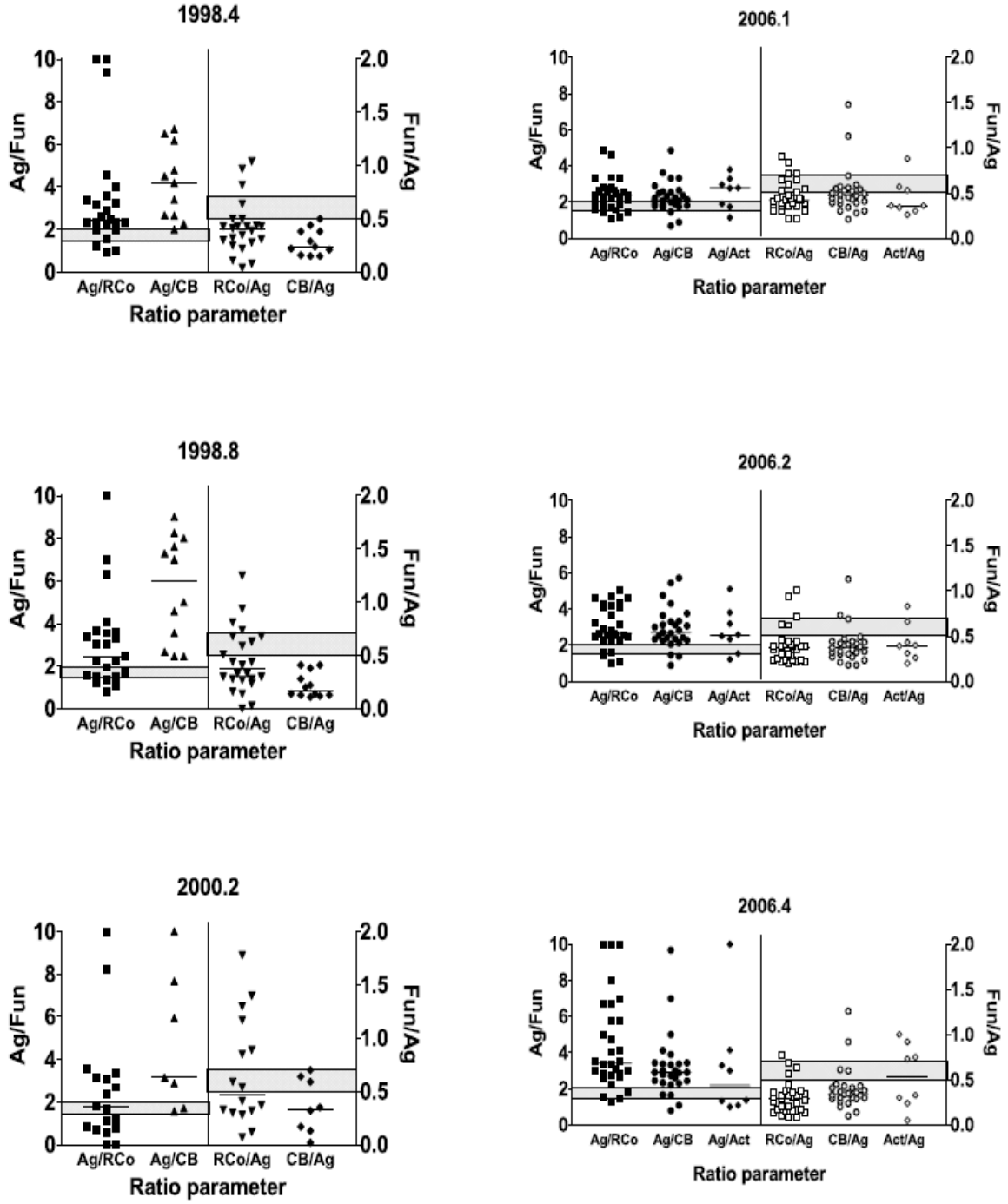


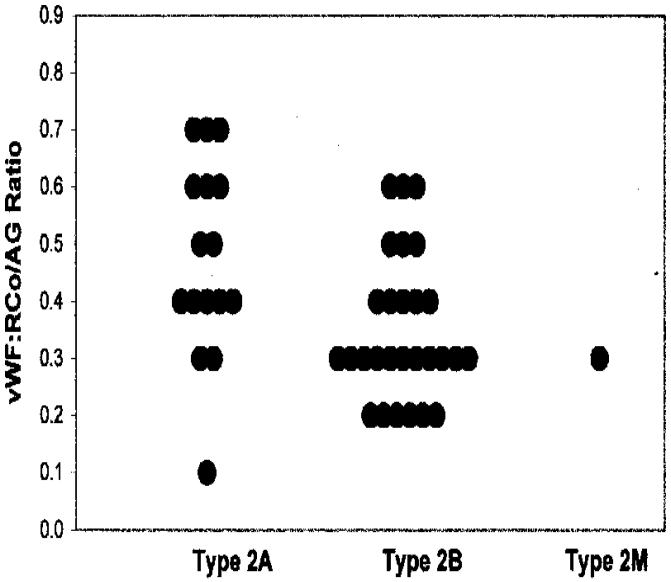
Figure 7. VWF activity/VWF antigen ratios for three type 2B samples (1998.4, 1998.8, 2000.2).
Figure 8. VWF activity/VWF antigen ratios for three type 2B samples (2006.1, 2006.2, 2006.3).

Favaloro et al. (2007) evaluated data from six well-characterized type 2B VWD samples, which have been tested by up to 52 laboratories.[44]

VWF:RCo: performed as platelet agglutination (using an aggregometer or a coagulaton based instrument) or as a LIA based method. WF:CB: in-house or commercial ELISA. VWF:Act: non-ristocetin or non-collagen based methods using monoclonal antibodies against the VWF epitope containing the GpIb-binding site (ELISA or LIA).

Results are expressed as a ratio of VWF activity to VWF antigen and VWF antigen to VWF activity (as preferred by some workers).

Results above the shaded bar for VWF antigen to VWF activity or below the shaded bar for VWF activity to VWF antigen would be acceptable evidence for a functional VWF discordance (type 2A, 2B or 2M). Most samples tended to show greater VWF discordance using the VWF:Ag to VWF:CB ratio or the VWF:CB to VWF:Ag ratio compared to the other ratios. This finding was more evident for the historical samples (1998.4, 1998.8, 2000.2) but notably less evident with the new samples.



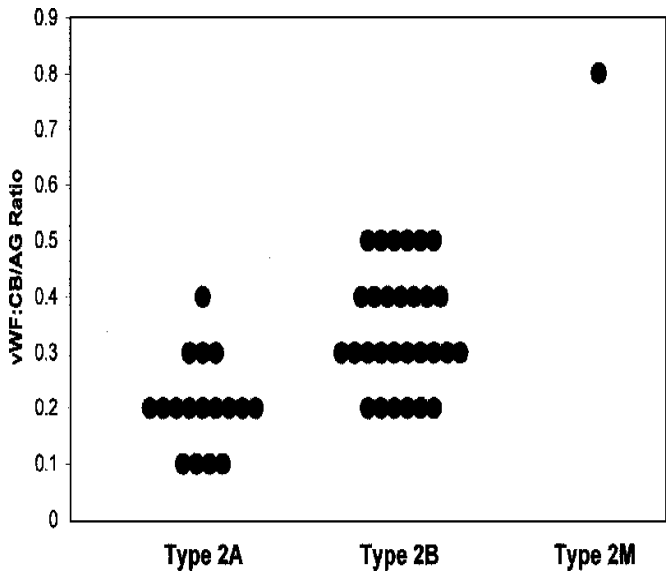


Figure 9. Distribution of VWF:RCo/VWF:Ag ratios and Figure 10. Distribution of VWF:CB/VWF:Ag ratios for all type 2A (n=18), 2B (n=29) and 2M (n=1) samples.

Adcock et al. (2006) reported data from a retrospective review of well-characterized VWD cases from 2003 to 2005.[22]
VWF:RCo was detemined using Dade-Behring vW reagent (lyophilized platelets) on a BTC analyzer (=automated coagulometer). VWF:CB assay was measured using the Gradipore ELISA assay by Corgenix (equine tendon-derived collagen).
For those samples classified as type 2A, the mean VWF:RCo/VWF:Ag ratio was 0.48 and the mean VWF:CB/VWF:Ag ratio was 0.2; for those samples classified as type 2B, the mean VWF:RCo/VWF:Ag ratio was 0.35 and the mean VWF:CB/VWF:Ag ratio was 0.36.

ID	Sex	Relationship to propositus	Bleeding Score	Main bleeding symptoms	Blood Group	Mutation	VWF:Ag	VWF:RCo	RCo : Ag Ratio	VWF:CB Activity			
										Human Type III		Human Type I	
							50-175 IU/dL	50-175 IU/dL	>0.7	50-175 IU/dL	CB (III) : Ag Ratio >0.7	50–175 IU/dL	CB (I) : Ag Ratio >0.7
1A	F	Propositus	8	Epistaxis, dental, menorrhagia, bruising	B	W1745C / R760H	52	39	0.8	7	0.1	10	0.2
1B	M	Son	4	Epistaxis	O	W1745C / WT	29	27	0.9	10	0.3	8	0.3
1C	F	Daughter	2	Epistaxis, menorrhagia	A	WT / R760H	39	41	1.1	34	0.9	52	1.3
2D	F	Propositus	10	Post-partum haemorrhage, menorrhagia	O	S1783A	88	57	0.7	16	0.2	22	0.3
2E	M	Son	5	Bruising, epistaxis	O	S1783A	68	66	1.0	19	0.3	27	0.4
3F	M	Father	1	Bruising	A	S1731T	46	48	1.0	25	0.5	30	0.7
3G	F	Propositus	2	Bruising, epistaxis	O	S1731T	43	40	0.9	40	0.9	18	0.4
3H	M	Brother	0	None	O	S1731T	72	63	0.9	63	0.9	39	0.6
3J	M	Brother	0	None	A	S1731T	75	64	0.9	63	0.9	45	0.6

Figure 11. Phenotypic data from the investigated families from Riddell *et al.* (2009).[37]

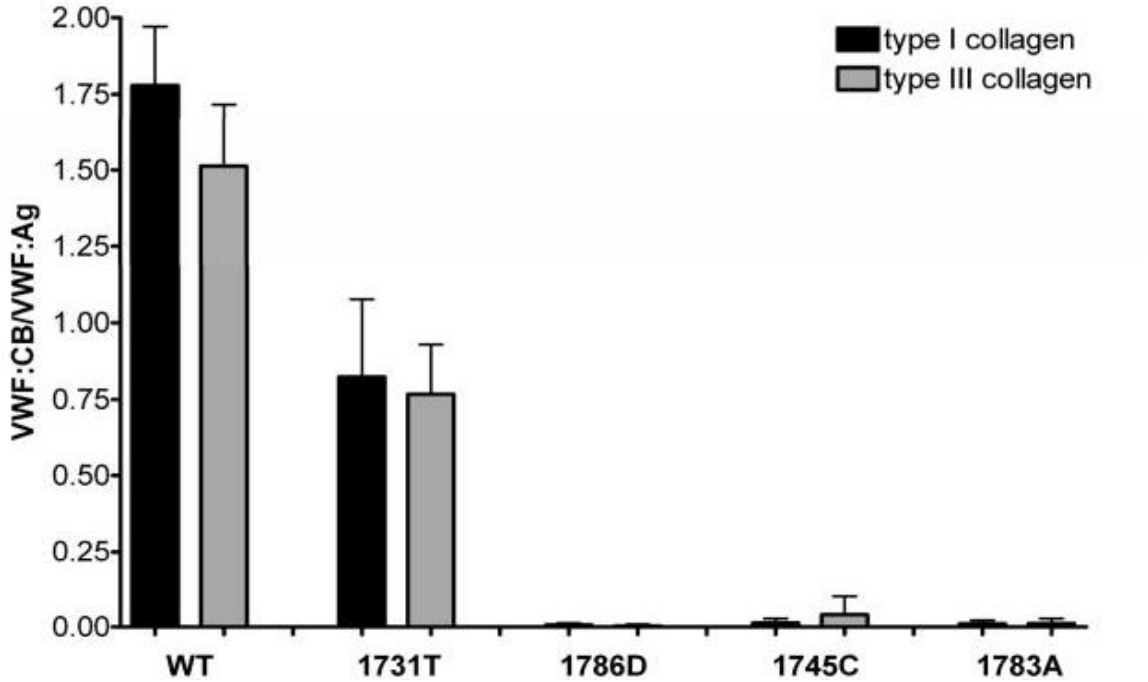


Figure 12. Collagen binding for recombinant VWF constructs from Flood *et al.* (2010).[39]

Tables:

Assay	Type 2A VWD	Type 2B VWD	Type 2M subtype GpIbα-IX-V defect	Type 2M subtype collagen defect
VWF:Ag	normal to mild decreased	normal to mild decreased	normal to mild decreased	normal to mild decreased
VWF:RCo	decreased	decreased	decreased	normal
VWF:RCo/ VWF:Ag	discordant (<0.7)	discordant (<0.7)	discordant (<0.7)	concordant (>0.7)
VWF:CB	decreased	decreased	normal	decreased
VWF:CB/ VWF:Ag	discordant (<0.7)	discordant (<0.7)	concordant (>0.7)	discordant (<0.7)
PLT aggregation with low dose ristocetin	absent	enhanced response	absent	absent

Table 3. The use of both VWF:RCo and VWF:CB allows a good discrimination between type 2A, 2B and 2M VWD.