von Willebrand Disease

Clinical and Laboratory Aspects
Editorial

In 2011 we started with the first special issue of The Clotting Times and this year we are proud to present our second issue with the theme “von Willebrand Disease, clinical and laboratory aspects”.

The first contribution is an overview of the current classification, diagnosis and treatment of von Willebrand disease (F.W.G. Leebeek), followed by phenotype and genotype associations in von Willebrand disease (H.C.J. Eikenboom) and a contribution which describes the clinical, therapeutic and laboratory aspects of type 2N von Willebrand disease (C. Caron, J. Goudemand).

The second part of this special issue focuses on laboratory aspects and starts with a contribution which tells us the story of the past, the present and the future testing repertoire applied to von Willebrand disease written by D. Cheng and W.L. Nichols. An article about the multimer analysis is written by U. Budde, followed by an overview of the assay systems to measure platelet-dependent von Willebrand factor activity written by I. Bodó. In the last contribution P. Meijer gives an overview of observations from surveys related to von Willebrand disease diagnostics.

The editorial board is happy to provide you with this comprehensive special issue and we thank all the authors for their useful contributions.

Special thanks is due to Prof. dr. H.C.J. Eikenboom for his significant contribution as guest editor of this issue.

Petra ter Hark

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Current classification, diagnosis and treatment of von Willebrand disease

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Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder that affects both sexes and is characterised by mucocutaneous bleeding episodes, or bleeding after surgery or trauma. The disease was first described by Erik von Willebrand in 1926 [1]. He reported a family in which both men and women had severe bleeding symptoms. The family lived on the Åland islands in the Gulf of Bothnia between Sweden and Finland. Many family members had troublesome nose bleeds. Four children died from uncontrolled bleeding at an early age, and the proposita of the family died at the age of 13 during her fourth menstruation. The disease was first referred to as pseudo-haemophilia, but was named VWD after the discovery of von Willebrand factor (VWF). The disease is caused by a deficiency or abnormality of VWF, resulting in reduced VWF activity. VWF has two roles in blood coagulation: first it is involved in the adhesion of platelets to the subendothelium by interacting with the GPIb receptor on platelets; second it serves as a carrier protein for FVIII. Reduced levels of VWF will therefore not only lead to changes in primary haemostasis, i.e. reduced platelet adhesion and aggregation, but also in secondary haemostasis due to reduced FVIII levels. The prevalence of VWD is 0.5 -1%. However, in the general population approximately 1 in 10,000 individuals has VWD with clinically relevant severe bleeding, for which treatment is needed [2].

von Willebrand factor

VWF plays an important role in primary haemostasis. Primary haemostasis is the process of platelet plug formation after a vessel wall has been disrupted. VWF binds platelets to the exposed subendothelium, and mediates platelet-platelet interaction. In addition VWF is the carrier protein of FVIII and prevents degradation of FVIII, thereby determining the half-life of FVIII. The VWF gene is located at chromosome 12. It contains 52 exons and 180 kb. VWF is formed as a pre-propeptide; a signal peptide of 22 amino acids, a propeptide of 741 amino acids and a mature subunit of 2050 amino acids. The mature subunits have several structural domains. VWF is synthesized by endothelial cells and megakaryocytes. Various mutations in the VWF gene have been reported in patients with VWD (International Society on Thrombosis and Hemostasis [ISTH] SSC VWF database). Amongst these are large gene deletions, nonsense mutations, frameshift, splice site mutations, small insertions and missense mutations. These mutations are distributed throughout the VWF gene and have been found in most of the 52 exons [3].

<table>
<thead>
<tr>
<th>Type</th>
<th>Frequency (%)</th>
<th>Heritability</th>
<th>VWF:Ag</th>
<th>RCo/CB</th>
<th>FVIII:C</th>
<th>RIPA</th>
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</table>

AD = autosomal dominant, AR = autosomal recessive
von Willebrand disease

VWD is an inherited bleeding disorder, but may be acquired in rare cases. The hereditary VWD types are subdivided into type 1, 2, and 3. Type 1 VWD is characterized by a partial quantitative deficiency of VWF. This is the most common type of VWD (70-80%). Type 2 VWD is a qualitative defect, due to the synthesis of an abnormal VWF molecule (20-25%). On the basis of the current SSC-ISTH criteria four subtypes can be distinguished: 2A, 2B, 2M and 2N. The characteristics of these types are given in table 1 [4]. In type 2A there is an abnormal synthesis or increased proteolysis of VWF multimers resulting in the loss of high molecular weight multimers. It is characterized by a disproportionally low ristocetin co-factor activity compared to VWF antigen. Type 2B is characterized by a “gain of function” mutation of binding to GPIb, leading to spontaneous binding to platelets and a subsequent rapid clearance of the platelets and large VWF multimers from the circulation. Mild to severe thrombocytopenia may occur in type 2B patients. In type 2M a “loss of function” mutation of binding to GPIb is present, which is associated with reduced binding of VWF to platelets. The multimer pattern is normal in type 2M patients. Type 2N VWD is characterized by a reduced binding of VWF to FVIII. Patients suffering from this type of VWD have normal VWF levels but have low FVIII levels. Type 3 is the most severe form of VWD, defined as exhibiting no detectable VWF levels (< 5 %) in plasma, and is associated with strongly reduced FVIII levels.

VWD is a heterogeneous disorder with a large variability in bleeding frequency and severity between VWD patients. Patients with VWD have frequent bleeding episodes, mostly of mucocutaneous origin, varying from gum bleeds and epistaxis to intestinal bleeding. VWF and FVIII levels largely determine the bleeding tendency, though the variation in bleeding tendency between individuals with VWD is not completely related to VWF levels. Some patients bleed excessively, whereas others with similar VWF levels in plasma have no or only mild bleeding problems [5]. Also within families carrying the same mutation, there is a considerable difference in bleeding pattern. In patients with type 2B, the number of circulating platelets may also affect the bleeding phenotype [6]. Type 3 VWD patients have the most severe bleeding phenotype. Due to the absence of VWF they experience severe mucosa-associated bleeding, including nose bleeds, menorrhagia and sometimes life-threatening gastrointestinal bleeding. Because of the strongly reduced FVIII levels, they also have haemophilia-like bleeding symptoms, including bleeding in joints and muscles [7]. Theoretically, men and women are equally likely to be affected with VWD, but in women the disorder is more often clinically manifest because of the bleeding challenges that are associated with menstruation and childbirth. Several studies showed that women with VWD frequently have menorrhagia, with reported prevalences ranging from 74 to 92%. We recently reported that more than 20% of women with moderate to severe VWD underwent hysterectomy, which is significantly higher than the percentage for women without VWD [8].

Besides VWF and FVIII levels other coagulation parameters can also affect the bleeding phenotype. It has been demonstrated that thrombin generation capacity may influence the bleeding phenotype of VWD patients [9]. Another factor that may determine the variability in clinical expression of VWD is the rate of fibrinolysis, the process of degradation of a fibrin clot. We recently studied the effect of fibrinolysis on the bleeding tendency in VWD patients. The fibrinolytic potential was measured in over 600 adult patients with moderate to severe VWD. We could not establish an association between the fibrinolytic potential and bleeding phenotype [10].

The bleeding episodes and sequelae of frequent bleeding in joints or muscles, but also other bleeding episodes may affect health-related quality of life (HRQoL), especially in severe, mainly type 3 VWD patients. The bleeding symptoms not only affect physical functioning of patients with VWD, but also have an impact on emotional and psychosocial well-being. In patients with type 3 or severe type 2 VWD muscle and joint bleedings may result in arthropathy and disabilities. The effect of VWD on daily life has recently been evaluated by measuring health-related quality of life (QoL), a multidimensional construct that quantifies patient-perceived well-being and functioning in terms of physical, emotional, mental and social components. In two recent studies we showed that HR-QoL is strongly reduced in patients with severe VWD. This was seen both in children and adults [11-12]. This reduced HR-QoL was strongly dependent upon the severity of the bleeding phenotype.
Diagnosis

The diagnosis of VWD is based on both clinical and laboratory criteria. First the patient should suffer from a bleeding diathesis, mainly mucocutaneous bleeding. In addition the family history is in most cases positive for bleeding [4]. Type 1 VWD is sometimes hard to diagnose, due to the physiological variation of VWF levels and the influence of ABO blood group on VWF:Ag levels (blood group O individuals have 25-30% lower VWF:Ag levels than non-O individuals), and the clinical symptoms may overlap with normal bleedings occurring during life in healthy subjects [13]. In contrast type 2 and type 3 VWD are more easily diagnosed. If VWD is suspected, screening coagulation tests are performed (Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT), a screening test for primary haemostasis for instance bleeding time or Platelet Function Analyzer-100® (PFA), and platelet count. The next step is the measurement of VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), VWF:collagen-binding activity (VWF:CB) and FVIII coagulant activity (FVIII:C) levels. Testing should be repeated at least two times if results are inconclusive or there is a high clinical suspicion of VWD. In type 1 disease the VWF RCo:Ag ratio is >0.7, whereas in type 2 VWD the ratio is <0.7. If the patient’s results are compatible with the diagnosis VWD, multimer analysis and a ristocetin-induced platelet agglutination (RIPA) test is performed to classify the type of VWD, according to the current ISTH guidelines (see Table 1) [4]. Type 2N can be diagnosed by performing a FVIII binding assay of VWF. In type 3 disease the bleeding time is markedly prolonged, and the closure time measured by the PFA100® is usually >300 seconds. The definition of type 3 VWD disease varies in the literature. Strictly speaking no VWF should be present in plasma, though some definitions describe type 3 VWD as VWF antigen and activity below 5% [5]. VWF is also not detectable in platelets of patients with type 3 VWD. No multimers can be detected using SDS-protein electrophoresis. Usually FVIII:C levels are strongly reduced, and vary between 1 and 9%.

If the diagnosis of VWD is made, a DDAVP infusion test should be performed which defines all types of VWD, except for type 3 and 2B. DDAVP should also not be given to patients with contra-indications, such as coronary heart disease, previous stroke, etc. The DDAVP test determines the increase of the VWF:Ag and VWF:Ac (CB or RCo) levels after infusion of DDAVP, the duration of response and potential side effects. Finally, after the diagnostic strategy and the DDAVP testing have been completed, a personalized treatment protocol is drawn up, summarizing the exact diagnosis of VWD, treatment, and dosage according to bleeding severity or before surgery. Genetic testing for VWD is not routinely performed and would not influence treatment in most individuals with VWD. If type 2 VWD is suspected or diagnosed, genotyping is sometimes performed to distinguish between types 2A and 2B, and between type 2B and platelet-type VWD. It could be performed in those individuals who do not have all the characteristics typical of type 2B. Another reason for genetic testing is to differentiate between haemophilia A and VWD type 2N. In rare cases genetic testing is performed because of genetic counselling. Detailed guidelines have been published on the molecular analysis of von Willebrand disease by the UKHCDO [14].

Quantifying the bleeding phenotype for the diagnosis of VWD

Tosetto et al. have developed a bleeding score to quantify the number and severity of bleeding symptoms in order to discriminate between subjects with type 1 VWD and individuals without VWD [15]. This score was developed because a large proportion of healthy individuals have suffered bleeding symptoms in the past and it is difficult to distinguish normal from abnormal bleeding. Therefore bleeding symptoms were quantified using this score. The score ranges from -3 to 45. The patients with the most severe bleeding phenotype have the highest score. This score is very useful to distinguish between individuals with and without VWD and is now considered a valuable diagnostic tool for VWD type 1. The cut-off value of normal is 3 in men and 5 in women. This score has been validated in other patient groups with type 1 VWD. So far only limited data on the bleeding score in patients with type 2 and 3 VWD is available, and it is not known whether it can be used in these more severely affected patients to assess the severity of the bleeding.

Treatment of von Willebrand disease.

In recent years several guidelines have been published on the management of VWD, in the UK, USA, Italy and the Netherlands [16-17]. Although limited prospective management studies have been performed in VWD, these guidelines are evidence-based and provide the basis for current treatment strategies.
for patients with VWD to combat bleeding, in patients undergoing surgical procedures and in women during pregnancy and delivery. The treatment of VWD has as its aim to restore VWF antigen and activity levels and stop or prevent bleeding in VWD patients. This can be achieved by the use of desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) or FVIII/VWF concentrate. Other drugs used in VWD are antifibrinolytic agents. In women with menorrhagia oral contraceptives can be prescribed to reduce blood loss. The treatment depends on the type of VWD, the severity of the bleeding, or the extent of surgical or dental interventions. If it is possible to treat a VWD patient with DDAVP, this is the product of choice.

DDAVP is registered for intravenous (Minrin® (Ferring, Utrecht, The Netherlands) and intranasal (Octostim®, Ferring, Utrecht, The Netherlands) use. The intravenous dose is 0.3 microgram/kg body weight infused over 30 minutes in 50 ml saline. The intranasal dose is 150 microgram in both nostrils for bleeding or before interventions. The effect is seen within minutes and may last 3-24 hours depending upon the type of VWD. DDAVP is used for most patients with type 1, but is effective only in a minority of type 2 patients. It cannot be used in VWD type 3.

Patients not responding to DDAVP should be treated with coagulation factor concentrates. Several FVIII/VWF concentrates are available for the treatment of VWD. The most widely used is Haemate-P® (CSL Behring, Marburg, Germany). Other products include Wilate® (Octapharma, Wien, Austria) and Wilfactin® (LFB, Les Ulis, France). Recombinant VWF has recently been developed and is currently tested in phase II trials. The dosage of plasma coagulation factor concentrates is frequently based on IU FVIII activity per kilogram of body weight, because historically concentrates were labeled solely in terms of FVIII content. Furthermore, it is laborious to perform VWF:RCo, so levels are not readily available and the monitoring of treatment based on VWF:RCo is difficult. Guidelines for substitution with coagulation factor concentrates are shown in table 2 [18]. Fibrinolysis inhibitors can be used as adjunctive therapy, especially in mucosal bleeding or after surgical intervention involving mucosa (dental extraction, gastrointestinal bleeding). Tranexamic acid which inhibits fibrinolysis by interfering with the lysine binding site of plasminogen, thereby reducing the binding of plasminogen to fibrin, is most widely used. The recommended treatment with tranexamic acid in adults is 1 gram 3-4 times a day. It is regularly used in women with menorrhagia who do not respond to oral contraceptive drugs. In some patients with type 3 disease infusion of platelet concentrates can be beneficial because they contain VWF, in contrast to the patients’ endogenous platelets.

**Monitoring of treatment**

Besides careful clinical observation of the VWD patient, treatment should also be monitored using

<table>
<thead>
<tr>
<th>Indication for treatment</th>
<th>FVIII/VWF* dose</th>
<th>Duration of treatment</th>
<th>Therapeutic goal levels</th>
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<tr>
<td>Major surgery or bleeding</td>
<td>Initial 40-60 U/kg Maintenance 20-40 U/kg every 8-24 hours</td>
<td>7-14 days</td>
<td>At time of surgery VWF:RCo and FVIII:C 100 IU/dl; during maintenance &gt; 50 U/dl trough levels</td>
</tr>
<tr>
<td>Minor surgery or bleeding</td>
<td>Initial dose 30-60 U/Kg Maintenance 20-40 U/kg every 12-48 h</td>
<td>3-5 days</td>
<td>At time of surgery VWF:RCo and FVIII:C 50-100 IU/dl; during maintenance &gt;50 IU/dl trough levels</td>
</tr>
<tr>
<td>Delivery</td>
<td>Initial dose 30-40U/kg, maintenance 20-40 U/kg every 12-24 hours</td>
<td>3-5 days</td>
<td>At time of delivery** and during maintenance VWF:RCo and FVIII:C &gt; 50 IU/dl</td>
</tr>
</tbody>
</table>

*: based on VWF:RCo levels labelled on the concentrate. ** For Caesarean Section see major surgery.

VWF:RCo = VWF ristocetin cofactor activity.
laboratory techniques. Bleeding time cannot be used to determine the efficacy of treatment with FVIII/VWF concentrate, because this does not normalize despite adequate replacement therapy. Also other global primary haemostasis function tests, including PFA-100® are not useful, because these tests are not always correct after infusion of even high-dose concentrate [19]. This may be related to the lack of VWF in platelets in these individuals. FVIII:C levels are frequently used to monitor treatment, because they can be measured easily and fast, whereas functional tests for VWF are elaborate and time-consuming. It is evident however that FVIII:C levels do not always reflect VWF levels and may overestimate VWF:RCo activity. It is therefore recommended that both VWF:RCo and FVIII levels be measured to guide FVIII/VWF concentrate dosing. The dosages in table 2 are based on the VWF:RCo content of the concentrates.

Conclusion

VWD is the most common, inherited bleeding disorder. It is of utmost importance to diagnose VWD in patients complaining of bleeding, in order to treat bleeding episodes and to prevent bleeding during surgery or other interventions. Diagnosis should be based on both clinical and laboratory parameters. Treatment can be given with DDAVP or FVIII/VWF concentrates and will halt or prevent bleeding in most instances.

References

Phenotype and genotype associations in von Willebrand disease

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Introduction
By definition von Willebrand disease (VWD) is a bleeding disorder caused by inherited deficiencies in the concentration, structure or function of von Willebrand factor (VWF) [1]. The disease is classified into three major subtypes that distinguish between the quantitative deficiency of VWF (types 1 and 3, comprising partial and virtually complete deficiency, respectively) and the qualitative defects of VWF (type 2) [1]. In 1985 the cDNA of VWF was cloned [2,3] and in 1989 the structure of the VWF gene was reported [4]. Since then the search for genetic defects resulting in VWF defects and causing VWD has become possible. At first the research was focused on the severe type 3 VWD, later on type 2 VWD, and more recently the focus has shifted to the diagnostically more challenging type 1 VWD. In this article the associations between the VWD phenotypes and the VWF genotypes will be discussed.

Type 3 VWD
Type 3 VWD is a very severe form of VWD comprising less than 5% of all VWD patients, and is characterized by a virtually complete deficiency of VWF. Even before molecular genetic studies were available the autosomal recessive inheritance pattern had been described on the basis of the phenotypes of severely affected patients and their symptomless relatives. The patients are homozygous, often as a result of the consanguinity of the parents, or compound heterozygous. Because of the apparent complete deficiency of VWF the anticipated genetic defects were null-alleles; i.e. large or complete gene deletions, lack of mRNA production, premature stop codons, frame-shift mutations or splice-site mutations. Indeed, more than 85% of the genetic defects identified in type 3 VWD patients are null-alleles (http://www.vwf.group.shef.ac.uk) [5,6]. Only in a minor subset of type 3 VWD patients have missense mutations been identified. These missense mutations are heterogeneous, but a subgroup of mutated cysteine residues seems to cluster in the carboxy-terminal cystine knot (CK) domain of VWF leading to dimerization defects [5,6]. Heterozygous carriers of null-alleles in general have no or only very mild bleeding symptoms and may be recognized at the phenotypic level by an increased ratio of FVIII:C over VWF:Ag [7].

Type 2 VWD
Type 2 VWD includes all structural and functional variants of VWF. About 25% of VWD patients fall into this category. Many different subtypes have been described on the basis of specific phenotypic characteristics [1]. The inheritance pattern of most type 2 variants is autosomal dominant, with the exception of type 2N (characterized by decreased affinity of VWF for factor VIII) and the former subtype IIC (now included in type 2A) which are inherited in an autosomal recessive way. As expected, mutations identified among type 2 VWD patients are mainly missense mutations leading to a structural change of VWF or a defect in its function. Mutations in VWD type 2 have been identified relatively easily as the genetic analysis in VWD type 2 can be restricted to discrete parts of the VWF gene guided by the distinctive phenotypes of the different subtypes.

Type 2A is characterized by qualitative variants with decreased platelet adhesion and a selective deficiency of high-molecular-weight VWF multimers. This subtype 2A includes former subtypes IIA, IIC, IID and IIE that can be distinguished on the basis of characteristic differences in the VWF multimer pattern. The missense mutations responsible for these respective subtypes cluster within specific regions of VWF: IIA in the A2 domain, IIC in the propeptide, IID in the CK domain, and IIE in the D3 domain (Figure 1). Type 2B is characterized by qualitative variants with increased affinity for platelet glycoprotein Ib (GPIb) and the mutations are, as can be expected, clustered in the A1 domain of VWF which binds to GPIb. As type 2B is a kind of gain-of-function mutation the phenotype is caused by a small set of mutations. The majority of cases are caused by only four different mutations: p.Arg1306Trp, p.Arg1308Cys, p.Val1316Met, and p.Arg1341Gln. Type 2M mutations with decreased platelet adhesion also cluster mainly in the A1 domain. Type 2N VWD with decreased binding affinity
for factor VIII is caused by a limited number of mutations clustered in the D’D3 domains where the binding domain for factor VIII is located.

The location of most mutations in type 2 VWD correspond to the expected region of the VWF on the basis of the pathophysiology of the subtype.

**Type 1 VWD**

The majority of VWD patients (70%) have type 1 VWD with a partial quantitative deficiency of VWF. The phenotypic expression of type 1 VWD is variable and the penetrance is often low. Even though mutation analysis in VWD has been possible since the early 1990s, the genetic mechanism underlying type 1 VWD remained unknown for a long time. In 2001 there were only 9 entries of mutations in the VWF mutation database and by 2006 these were still only 14 entries (http://www.vwf.group.shef.ac.uk). Since then several multicentre studies have reported on linkage and the mutation spectrum of VWD type 1 [8-12]. Candidate mutations were identified in 63% of 32 index cases in the UK Haemophilia Centre Doctor’s Organisation (UKHCDO) study on type 1 VWD [9], in 70% of 150 index cases in the European multicentre study “Molecular and Clinical Markers for the Diagnosis and Management of Type 1 VWD (MCMDM-1VWD)” [10], in 63% of 123 index cases in the Canadian study on VWD type 1 [11], and in 56% of 54 index cases in the Swedish cohort [12]. Thus, overall, VWF gene mutations were identified in 65% of index cases. The large majority of these mutations are missense mutations and, unlike type 3 VWD where the majority of mutations predict null alleles, only 15% of mutations in type 1 VWD are null alleles. The mutations were not restricted to specific regions of VWF but distributed all over the VWF gene.

In the MCMDM-1VWD cohort overall linkage of the type1 VWD phenotype to the VWF gene was 70% [8]. When the analysis was performed in subgroups of families with index cases characterized by a VWF:Ag <30 IU/dL, or an abnormal VWF multimer pattern, or a VWF:RCo/VWF:Ag ratio <0.7 then linkage was 100%. However, linkage was only 50% in the subgroups with VWF:Ag >30 IU/dL, completely normal multimers, or VWF:RCo/VWF:Ag ratio >0.7 [8]. Blood group O clearly contributed to the type 1 VWD phenotype. In the group of index cases with complete co-segregation of the phenotype and the VWF gene the prevalence of blood group O was 56%, whereas in the group with incomplete co-segregation this prevalence was 76% [8].

Several of the identified missense mutations have been expressed in vitro and the predominant pathogenic mechanism of mutant VWF in type 1 VWD seems to be intracellular retention of mutant VWF [13]. Also, reduced VWF half-life has been identified as a disease mechanism in a subset of patients and
those patients can be recognized by an increased ratio of VWF propeptide over VWF:Ag [14]. Identification of fast clearance of mutant VWF is relevant as it may result in a short-lived response after DDAVP. The response to DDAVP was also associated with the location of the causative mutation. Most partial and non-responsive patients had mutations in the A1-A3 domains [15].

In contrast to type 2 and type 3 VWD there remain about 30% of type 1 VWD patients where no mutation can be identified. Probably the phenotype in those patients is determined by multiple other genetic loci, including the ABO blood group. The search for these loci is ongoing and only recently some potential modifier genes of VWF levels were identified [16].

Concluding remarks

The molecular genetic investigations of VWD patients have dramatically improved our pathophysiological knowledge and understanding of the disease. However, genetic testing in VWD is not routinely advised. It may be of use in type 3 VWD families to enable prenatal diagnosis, but it is not necessary for the diagnosis itself. Most type 2 VWD subtypes can be very well characterized at the phenotypic laboratory level, however genetic testing can be useful to confirm or distinguish type 2B VWD. Mutation analysis is mostly relevant to distinguish type 2N VWD from mild haemophilia A.

References

Type 2N von Willebrand disease: clinical, therapeutical and laboratory aspects

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Introduction

Plasma von Willebrand factor (VWF) is the carrier protein for factor VIII; the FVIII-binding site is located on the N terminal part of the mature VWF subunit. In type 2N von Willebrand disease (VWD), the presence of an altered FVIII-binding site results in a markedly decreased or no FVIII-VWF interaction.

Type 2N VWD was first identified in 1989 in a patient from Normandy in France; it is designated in the VWD classification [1] as “a qualitative variant with markedly decreased binding affinity of VWF for FVIII”. The classical type 2N phenotype shows a disproportionate decrease in coagulant FVIII activity relative to the VWF level; consequently it may be misclassified as mild or moderate haemophilia A or haemophilia A carrier. The differential diagnosis is crucial as it has strong implications for both therapy and genetic counselling [2]. For example, a pregnant woman who presents with an isolated FVIII deficiency without any familial history of bleeding diaphesis may be either a haemophilia A carrier with consequently a haemorrhagic risk for a male child, or a patient with a type 2N VWD which can exclude a bleeding risk for her child whatever the sex.

Laboratory diagnosis

The diagnosis of type 2N VWD is phenotypic rather than genotypic. The first step is based on the factor VIII level as FVIII:C deficiency is the hallmark of type 2N patients. Although some cases of very low FVIII:C deficiency (1-2 IU/dL) have been described, most patients with type 2N VWD have FVIII levels ranging from 5-40 IU/dL depending on the FVIII-binding capacity. Patients with no binding capacity have lower FVIII:C concentration (8.4 ± 5.2 IU/dL) than those with only a markedly reduced binding capacity (21.8 ± 5.4 IU/dL) [3]. The VWF:Ag is normal or subnormal depending on the ABO group and the presence or absence of a silent VWF allele. As a consequence the FVIII:C/VWF:Ag ratio is decreased lower than 0.6 [4], so it is advisable to evaluate the plasma VWF capacity to bind FVIII in all patients with a reduced FVIII to VWF ratio.

Table 1. Comparative description of three VWF:FVIIIB ELISA methods

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<td>Serial dilutions</td>
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</tr>
<tr>
<td>Removal of endogenous FVIII</td>
<td>CaCl2 0.35M</td>
<td>CaCl2 0.35M</td>
<td>Dilution buffer</td>
</tr>
<tr>
<td>Addition of immunopurified FVIII</td>
<td>Plasmatic immunopurified FVIII</td>
<td>Recombinant FVIII</td>
<td>Recombinant FVIII</td>
</tr>
<tr>
<td>Quantification of bound FVIII</td>
<td>Chromogenic assay</td>
<td>Immunoenzymatic assay</td>
<td>Immunoenzymatic assay</td>
</tr>
<tr>
<td></td>
<td>Polyclonal Ab antiFVIII-HRP (Kordia)</td>
<td>Monoclonal Ab antiFVIII-HRP</td>
<td></td>
</tr>
<tr>
<td>Quantification of immobilized VWF</td>
<td>Immunoradiometric assay</td>
<td>Immunoenzymatic assay</td>
<td>No quantification</td>
</tr>
<tr>
<td></td>
<td>Monoclonal Ab antiVWF-125</td>
<td>Polyclonal Ab antiVWF-HRP (Dako)</td>
<td></td>
</tr>
<tr>
<td>Limits of the method</td>
<td>Homemade reagents and assay</td>
<td>Homemade assay</td>
<td>VWF:Ag level &gt; 10 IU/dl</td>
</tr>
</tbody>
</table>

Table 1. Comparative description of three VWF:FVIIIB ELISA methods
when the FVIII deficiency is not clearly chromosome X-linked.

The second step of the phenotypic diagnosis consists of the in vitro measurement of the capacity of plasma VWF to bind exogenous factor VIII (VWF:FVIIIB). This assay is the only one enabling a distinction to be made between haemophilia A (normal VWF:FVIIIB) and type 2N VWD (markedly decreased VWF:FVIIIB). Assays measuring VWF binding to FVIII were initially described more than 20 years ago [5,2]; they have been progressively adapted in order to make them more suitable for medical laboratories, especially by using commercial reagents instead of home-made ones [6-8]. However the VWF:FVIIIB assay remained for several years a cumbersome technique poorly suited to non-specialized laboratories until the recent validation of the first commercial ELISA kit (Asserachrom VWF:FVIIIB®, Stago) [9]. Whatever the application, the main steps remain the same (table 1): capture of a sample of VWF by an anti-VWF antibody, followed by the removal of endogenous FVIII, the addition of a given amount of immunopurified or recombinant FVIII, and the subsequent quantification of bound FVIII and immobilized VWF (except in Asserachrom VWF:FVIIIB®).

The result of VWF-FVIII binding of patient plasma VWF is expressed as a percentage of normal plasma VWF. VWF:FVIIIB testing must be accurate enough to differentiate between low, intermediate or normal binding (figure 1). VWF:FVIIIB levels are considered as normal when higher than 80%, as severely decreased when lower than 15% and as moderately decreased between 30% and 65%, (figure 2). A zero or markedly reduced VWF:FVIIIB indicates a diagnosis of 2N VWD (homozygous or compound heterozygous). A moderately reduced VWF:FVIIIB value indicates the presence of a type 2N mutation at the heterozygous state which cannot by itself explain a plasma FVIII deficiency, as patients with heterozygous type 2N VWD have FVIII levels in the normal range. Therefore, patients who have an isolated FVIII deficiency associated with either a normal or moderately decreased VWF:FVIIIB are not patients with type 2N VWD and must be submitted to an analysis of the F8 gene to clarify the genetic cause of their FVIII deficiency (figure 2).

The French multicentre study comparing the reference method [7] to Asserachrom VWF:FVIIIB® Stago has shown the excellent sensitivity and specificity of both methods: 37 type 2N patients and 9 heterozygous carriers for a type 2N mutation, previously genetically characterized, were all detected ; none of the control subjects, consisting of 60 healthy volun-

---

**Figure 1. Representation of regression lines obtained for normal plasma, heterozygous type 2N and type 2N VWD (Caron C et al, 2002)**

![Figure 1](image-url)
teers, 37 haemophilia A and 17 haemophilia A carriers, 38 VWD patients other than type 2N, exhibited a marked VWF:FVIIIB decrease; a moderately decreased VWF:FVIIIB was found in one healthy subject and 3 haemophiliacs A, suggesting a heterozygous status for a type 2N mutation, without clinical consequence [9].

In type 2N VWD the VWF multimeric pattern is usually normal (mutations R854Q, R816W, T791M...) although some mutations especially those involving cysteine residues may affect the ability to form multimeric structures (C788R, C848F...).

The genotypic diagnosis enables the confirmation of the phenotypic data. The type 2N VWD variant is a recessive autosomal inherited disease caused by mutations located within the binding site for FVIII in D'-D3 VWF domains in exons 18-27. Today more than 20 mutations causing type 2N VWD have been identified (available at http://www.vwf.group.shef.ac.uk/vwd.htm). As a recessively inherited disease, type 2N VWD patients are either homozygous for one FVIII-binding missense mutation, or compound heterozygous for two FVIII-binding missense mutations or compound heterozygous for a FVIII-binding missense mutation and a silent allele (2N/3). The most frequent mutation in type 2N patients [3,10] is the R854Q substitution, present in either the homozygous or the heterozygous state. In the Type 2 French INSERM network experience, 60/73 (82%) unrelated patients showed at least one R854Q allele (45% homozygous, 37% heterozygous) while the other patients were either homozygous for R816W, C1060R, T791M or compound heterozygous for 2 type 2N mutations or type 2N/3 [10]. In the general population, the allele frequency of the R854Q mutation is reported to be as high as 1% [8]. The mutation correlates with the intensity of the FVIII-binding defect: R854Q, Q1053H type 2N patients exhibit a marked VWF:FVIIIB decrease while patients with the R816W or C1060R or T791M mutations present with a zero FVIII binding [11].

**Clinical manifestations**

The clinical expression of patients with type 2N VWD is strongly dependent on their FVIII:C level. The haemorrhagic symptoms will be very similar to those observed in patients with moderate or mild haemophilia A or haemophilia carriers: ecchymosis, easy bruising, epistaxis, menorrhagia, post-traumatic or post-operative bleeding. Most of the patients who are homozygous for R854Q have very few symptoms and are often diagnosed at the point of surgery and abnormal pre-operative coagulation tests. Patients with very low FVIII:C (<10 IU/dL) have more severe symptoms: severe muscle haematoma, joint bleeding, gastrointestinal or even intra-cranial bleedings, severe post-operative or post-partum bleedings. Those patients with abnormal multimeric pattern and consequently a low VWF:RCo activity may have more severe spontaneous mucocutaneous bleedings. Regular monitoring of the FVIII:C throughout pregnancy is necessary. As most women have moderate VWD, the FVIII:C in women carrying the R854Q mutation is expected to gradually increase and be normalized at the end of pregnancy but FVIII:C may remain low for those carrying other types of mutations. In that situation a specific treatment must be considered.

**Therapeutic management**

Desmopressin (DDAVP) is the preferred treatment for patients with type 2N VWD. After DDAVP infusion, FVIII:C measured at the peak increases by a factor to 3-20 regardless of the mutation. There is no correlation between the baseline level and the FVIII increment and no correction of the VWF:FVIIIB binding capacity. The FVIII half-life is shorter than in the usual forms of VWD (2.3 - 4.4 hours vs 5-8 hours) [12]. When meas-
ured 2 hours after infusion, FVIII:C is still normalized (>50 IU/dL) in most patients with the R854Q mutation who are considered as “good responders” while in the others the FVIII level drops rapidly. So DDAVP may be a useful prophylactic or curative treatment for type 2N patients provided that a test dose has been shown to be effective to reach and maintain a haemostatic FVIII:C level for an appropriate period of time.

When DDAVP is ineffective or contraindicated, the patients with type 2N VWD have to be treated with VWF concentrates in order to restore the binding to endogenous FVIII and thus to correct the FVIII plasma rate. Factor VIII concentrates (recombinant or immunopurified) are inappropriate because the half-life of infused FVIII is very short and clinically inefficient due to the absence of VWF able to bind FVIII.

Conclusion
Type 2N VWD is not a rare disease as it concerns about 10% of patients with both a decreased FVIII:C level and a FVIII:C/VWF:Ag ratio lower than 0.6, and represents in France about 25% of all type 2 VWD variants [4]. The differential diagnosis with haemophilia A is crucial for both clinical and therapeutic issues; it requires performing phenotypic assays as a first step to guide the genetic diagnosis strategy. Reference techniques have up till now been in-house methods only accessible to expert laboratories but now a commercial ELISA kit can be adapted to any laboratory for type 2N VWD phenotypical diagnosis. The study of large cohorts of type 2N patients clearly demonstrates that clinical features are well correlated with biology and genotype.

References
Von Willebrand disease testing repertoire: The past, present and future

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Abbreviations
AVWA, acquired von Willebrand factor abnormality (reflecting subtle loss of the highest molecular weight multimers)
AVWS, acquired von Willebrand syndrome
CV, coefficient of variation
FVIII, coagulation factor VIII
FVIII:C, coagulation factor VIII activity
GP, glycoprotein
HMWM, high molecular weight multimers
LLD, lower limit of detection
LLQ, lower limit of quantification
rGPIB, recombinant glycoprotein Ib.
RIPA: ristocetin-induced platelet aggregation assay
ROC, receiver operating characteristic
VHMWM, highest (or very high) molecular weight multimers
VWD, von Willebrand disease
VWF, von Willebrand factor
VWF:Ag, VWF antigen
VWF:FC, flow cytometry–based VWF ristocetin cofactor activity assay
VWF:FVIIIB: VWF and coagulation factor VIII-binding assay
VWF:Lx, latex particle–enhanced immunoturbidimetric VWF activity assay
VWF:RCo, VWF platelet-binding activity by ristocetin-induced platelet aggregation assay (ristocetin cofactor activity assay)

Von Willebrand disease (VWD), an autosomally inherited bleeding diathesis, affects about 1% of the general population, approximately one tenth of whom are clinically symptomatic [1-3]. Since its first clinical description in 1926 [4], over many years of extensive study, we have gradually come to understand its clinical features, biochemical characteristics and molecular mechanisms. Paralleling endeavours to understand VWD, the existence of acquired von Willebrand syndrome (AVWS) was subsequently recognized as secondary to various haematoproliferative, immunological or cardiovascular conditions [5]. Accurate diagnosis and classification of VWD or AVWS relies on a correlation between clinical observation and laboratory testing results.

Bleeding Symptom and History Assessment

Diagnosis of VWD starts with clinical assessment of the bleeding history. Thorough documentation of a patient’s personal and family bleeding histories is essential. The 2008 National Heart, Lung, and Blood Institute (NHLBI) VWD guidelines provide evidence-based questions [6] for guiding bleeding history information collection. To analyse the significance of bleeding symptoms and history, and ultimately to help predict the likelihood of VWD, several quantitative bleeding history scoring systems have recently been developed, validated [7, 8] and independently verified [9, 10]. A similar bleeding history quantification scheme has also been validated for the pediatric population [11]. To date, these scoring systems have not been routinely used in clinical practice.

VWD Classification

VWD is classified as a quantitative or qualitative deficiency of plasma von Willebrand factor (VWF), often accompanied by secondary deficiency of coagulation factor VIII activity (FVIII:C). There are three major types of VWD [12, 13]. Type 1 VWD, the most common type, reflects quantitative deficiency of normally functioning VWF. Type 3 VWD, a recessively inherited rare disorder, denotes virtual absence of VWF. Type 2 VWD represents qualitative VWF abnormalities that are categorized into four variant groups [6, 12, 13]. Type 2A VWD is characterized by the absence of the most haemostatically effective VWF high molecular weight multimers (HMWM), due either to defective multimer assembly or to accelerated proteolysis. Type 2B VWD is caused by secondary clearance of the HMWM due to their abnormally high binding affinity to the platelet surface glycoprotein (GP) Ib complex (GPIb). Type 2M (with M representing multimer) VWD exhibits decreased VWF platelet-binding activity without substantive deficiency of VWF HMWM. Finally, type 2N (Normandy) VWD has a phenotype of plasma FVIII
deficiency that is secondary to defective VWF binding of FVIII.

**Initial VWD Laboratory Testing**

Recommended initial laboratory tests include measurements of: 1) plasma VWF antigen (VWF:Ag); 2) VWF-platelet GPIb complex binding activity such as ristocetin cofactor activity (VWF:RCo); and 3) factor VIII coagulant activity (FVIII:C). Of the NHLBI/NIH and ISTH recommended supplemental tests, assays of VWF platelet-binding activity (RIPA: ristocetin-induced platelet aggregometry) and derived VWF activity/antigen ratios, in conjunction with plasma VWF multimer analysis, are particularly important for accurately classifying type 2 VWD (Table 1). Among various VWF activity assays [14-18], the VWF:RCo assay, measuring ristocetin-dependent VWF-mediated agglutination of washed or fixed platelets, is considered the gold standard method [6, 12, 13]. This method was initially developed from observations of ristocetin-induced VWF-dependent platelet agglutination, measured either by light transmission aggregometry (LTA) or by macroscopic agglutination of washed formalin-fixed platelets [19-26]. VWF:RCo by LTA (VWF:RCo-LTA) is considered the reference method. Except for type 2N VWD, the derived VWF:RCo/VWF:Ag ratio cut off at 0.5-0.7 has been suggested to help distinguish type 2 from type 1 VWD (Table 1).[12, 13].

Nevertheless, manual VWF:RCo assays have relatively poor precision [27-31] with inadequate analytic sensitivity and precision for samples with less than 20-30 IU/dL VWF:RCo activity[32]. Consequently, VWF:RCo assay results can sometimes cause misdiagnosis or misclassification of VWD [33, 34]. The imprecision (average coefficient of variation [CV] of 20-30%) is multifactorial. Possible causes include biological variation of the donor reagent platelets, suboptimal quality control and standardization of ristocetin chemical compound, and imprecise slope measurement. Consequently, the VWF:RCo-LTA continues to be problematic in numerous external proficiency exercises in Europe [27, 28], Australia [30, 31], and the United States [29]. The VWF:RCo assay has a relatively high lower limit of detection and lower limit of quantification (LLD and LLQ), usually 10-20 IU/dL, and becomes unreliable (CV ≥ 30%) when the VWF:Ag level

---

**Table 1. Common VWD laboratory tests**

<table>
<thead>
<tr>
<th>VWD Variants</th>
<th>Normal</th>
<th>1</th>
<th>2A</th>
<th>2B</th>
<th>2M</th>
<th>2N</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Screening Tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet Count</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N or ↓</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>FVIII:C</td>
<td>N</td>
<td>N or ↓</td>
<td>N or ↓</td>
<td>N or ↓</td>
<td>N</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>VWF:Ag</td>
<td>N</td>
<td>↓ or ↓↓</td>
<td>N or ↓↓/↓↓</td>
<td>N or ↓↓/↓↓</td>
<td>N or ↓↓/↓↓</td>
<td>N or ↓</td>
<td>Absent</td>
</tr>
<tr>
<td>VWF:RCo</td>
<td>N</td>
<td>↓ or ↓↓</td>
<td>↓/↓↓</td>
<td>N or ↓/↓↓</td>
<td>N or ↓/↓↓</td>
<td>N or ↓</td>
<td>Absent</td>
</tr>
<tr>
<td>VWF:RCo/VWF:Ag</td>
<td>&gt; 0.5–0.7</td>
<td>&gt; 0.5–0.7</td>
<td>&lt; 0.5–0.7</td>
<td>&lt; 0.5–0.7</td>
<td>&lt; 0.5–0.7</td>
<td>&gt; 0.5–0.7</td>
<td>NA</td>
</tr>
<tr>
<td>VWF:CB/VWF:Ag</td>
<td>&gt; 0.5–0.7</td>
<td>&gt; 0.5–0.7</td>
<td>&lt; 0.5–0.7</td>
<td>&lt; 0.5–0.7</td>
<td>&lt; 0.5–0.7*</td>
<td>&gt; 0.5–0.7</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Supplemental Tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWF Multimer Analysis Patterns</td>
<td>Normal multimer distribution</td>
<td>Normal multimer distribution</td>
<td>Decreased HMWM</td>
<td>Abnormal satellite bands</td>
<td>Decreased HMWM</td>
<td>Abnormal satellite bands</td>
<td>HMWM present</td>
</tr>
<tr>
<td>RIPA (low dose ristocetin)</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>↑</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>VWF:FVIIIIB</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>↓↓</td>
<td>Absent</td>
</tr>
</tbody>
</table>

N: normal, NA: Not applicable, *: May be unreliable.
is less than 20-30 IU/dL [32], with significant potential for misclassification of VWD.

Consequently, various alternative methods have been explored to supplement and ultimately replace the VWF:RCo-LTA assay (Table 2). These include:

1) VWF collagen-binding assays (VWF:CB) by ELISA methodologies [35, 36];
2) an automated ristocetin cofactor activity assay using lyophilized normal donor platelets and photo-optical coagulation analysers (VWF:RCo-auto) [37-39];
3) a latex particle–enhanced immunoturbidimetric VWF activity assay (VWF:Lx) using an optical coagulation analyser [40-45];
4) recombinant platelet wild-type or gain-of-function recombinant GPIb-based enzyme-linked immunosorbent assays (rGPIb-ELISA) [16, 17, 46, 47] and
5) flow cytomteric methods (VWF:FC) [18, 48, 49].

Nevertheless, satisfactorily to replace the classical VWF:RCo-LTA assay, any substitute method must demonstrate excellent laboratory characteristics which include diagnostic accuracy, high precision, detection limits less than 5 IU/dL (%), and high sensitivity for detecting the vast majority of VWD variants as well as AVWS cases.

Among these methods, three alternative VWF activity assays have emerged with promising laboratory characteristics (Table 2). VWF-CB has an excel-

---

**Table 2. Comparison of various VWF activity assays**

<table>
<thead>
<tr>
<th>Method</th>
<th>Testing Mechanism</th>
<th>Precision (CV %)</th>
<th>LLQ (IU/dL)</th>
<th>Automated vs. Manual</th>
<th>Commercial Kit Availability</th>
<th>Limitations</th>
<th>Independently Verified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF:RCo by microtiter plate</td>
<td>VWF ristocetin-induced platelet agglutination using washed/fixed platelets</td>
<td>15-30</td>
<td>7-25</td>
<td>Manual</td>
<td>No</td>
<td>Imprecise and laborious</td>
<td>Yes</td>
<td>[26, 75]</td>
</tr>
<tr>
<td>Automated VWF:RCo</td>
<td>VWF ristocetin-induced platelet agglutination using lyophilized platelets</td>
<td>&lt;10</td>
<td>3</td>
<td>Automated</td>
<td>Yes</td>
<td>Expense and occasional shortage of reagent platelets</td>
<td>Yes</td>
<td>[38, 39, 41, 52-56]</td>
</tr>
<tr>
<td>VWF:CB</td>
<td>VWF binding to collagen, using ELISA methodology</td>
<td>15-20</td>
<td>3</td>
<td>Manual</td>
<td>Yes</td>
<td>Collagen type -dependent; Occasionally misses type 2M VWD</td>
<td>Yes</td>
<td>[35, 76-78]</td>
</tr>
<tr>
<td>VWF:Lx</td>
<td>Specific latex immunoassay using monoclonal antibody against VWF A1 domain</td>
<td>&lt;10</td>
<td>3</td>
<td>Automated</td>
<td>Yes</td>
<td>Not a true function assay</td>
<td>Yes</td>
<td>[40-45]</td>
</tr>
<tr>
<td>VWF:FC</td>
<td>VWF ristocetin-induced platelet interaction measured by flow cytometry</td>
<td>10-15%</td>
<td>&lt;1</td>
<td>Manual</td>
<td>No</td>
<td>Highly complex; Requires flow cytometer</td>
<td>No</td>
<td>[18, 49, 79]</td>
</tr>
<tr>
<td>VWF:RCo by wild type rGPIb</td>
<td>VWF binding to wild-type rGPIb protein</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>No</td>
<td></td>
<td>No</td>
<td>[16, 17, 47]</td>
</tr>
<tr>
<td>VWF:RCo by gain-of-function rGPIb</td>
<td>VWF binding to mutated rGPIb protein</td>
<td>6-18%</td>
<td>1.6</td>
<td>Manual</td>
<td>No</td>
<td>NA</td>
<td>No</td>
<td>[46]</td>
</tr>
</tbody>
</table>
lent LLQ at 0-5 U/dL, good linearity and improved precision (15-20% CV) [32]. It could potentially be used as a supplementary test, especially when the VWF:Ag is less than 30 IU/dL. Limitations of VWF:CB testing include somewhat limited availability of commercial kits, differing collagen components, and occasional false negative results for rare type 2M VWD cases [50]. The second method employs lyophilized reagent platelets and a photo-optical coagulation analyser (VWF-RCo-auto). This method was first described in 2002 [51] and has been further optimized and validated in recent years by various groups [38, 39, 41, 52-56]. The third method is a latex particle-enhanced immunoturbidimetric assay (VWF:Lx) using an optical coagulation analyser and a previously characterized monoclonal antibody against the VWF-GPIb binding A1 domain [57, 58]. Both latter methods have promising laboratory characteristics (Table 2) including excellent precision, broad linear range, and substantially improved LLQ (about 3 IU/dL or U/dL). The excellent linearity and CV (<10%), even in the range of 3 to 30 IU/dL, will surely improve the accuracy of laboratory differentiation of type 1 vs. type 2M VWD. However, VWF:Lx has some limitations. First, it is not considered a true VWF function assay. A recent report suggests it could potentially miss rare type 2M VWD cases [44]. Second, whether the VWF:Lx assay can be satisfactorily used for laboratory monitoring of VWD treatment such as with VWF concentrates or DDAVP (desmopressin) remains to be determined. Therefore, VWF:Lx is considered a good screening tool; but whether it can completely replace VWF-RCo requires further investigation [45].

Supplementary Testing

There are three major supplementary tests for classifying type 2 VWD. VWF multimer analysis is usually required to confirm the diagnosis of type 2A, 2B and 2M VWD. There are various VWF multimer electrophoresis and imaging methods, [59, 60] and examples of common type 2 VWD variants are available on the ISTH-related web site (http://www.vwf.group.shef.ac.uk/). VWF-FVIII binding activity assays are used for diagnosing for type 2N VWD, in which an aberrantly low VWF-FVIII binding capacity is indicative of type 2N VWD [61-65]. Finally, an abnormal ristocetin-induced platelet aggregation (RIPA) with low-dose ristocetin (approximately 0.5 mg/ml) is diagnostic for type 2B VWD [66, 67].

VWF Activity Tests for Detecting AVWS

Another limitation of VWF-RCo assays is their low sensitivity for detecting AVWS due to subtle loss of the very high molecular weight multimers (VHMWM) of VWF, especially in patients who have aortic stenosis [68], marked thrombocytosis [69], left ventricular assist device implantation [70-72], or hypertrophic obstructive cardiomyopathy [73]. Among the VWF laboratory tests, VWF multimer analysis is the most sensitive and specific method for detecting such AVWS or AVWA (acquired von Willebrand factor abnormality without definite bleeding symptoms). However, due to its complexity and limited accessibility, VWF multimer analysis is often impractical. Of the VWF activity assays, VWF:CB and VWF:Lx appear to have good sensitivity (about 80%) and specificity (about 80%) for detecting AVWS and AVWA [45, 66, 67, 71-73]. A more sensitive, efficient and robust method is still needed to facilitate clinical diagnosis, grading and monitoring therapy in patients with high-shear related AVWS.

Conclusion

After more than 80 years of endeavour, and with the more recent advent of new method development and instrument improvement, VWD laboratory testing is poised for significant future advancements. We anticipate that VWD testing will become more automated and precise; and in conjunction with the evolving availability of molecular testing, laboratory testing will be significantly improved more efficiently and precisely to identify and classify various VWD or AVWS variants.

References:
Discrimination between type I and type II, von Willebrand’s disease.


The Clotting Times

U. Budde MD
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Introduction

Von Willebrand disease (VWD) is caused by a quantitative and/or qualitative defect of the von Willebrand factor (VWF), a multimeric high molecular weight glycoprotein. The heterogeneity of the disease manifests both in the clinical symptoms and in the variable laboratory parameters. The protein is encoded by a gene located on chromosome 12p and a pseudogene is located on chromosome 22. The primary translation product undergoes a complex series of processing steps leading to the assembly of large VWF multimers that are stored in specialized intracellular organelles (Weibel Palade bodies, a-granules), while smaller multimers leave the cell constitutively.

Multimer analysis showed that the circulating VWF has a complex multimeric structure. The smallest detectable subunit is a dimer with a molecular mass of approximately 540 kDa. The largest molecules exceed 10,000 kDa. Because the protomer or building block is a dimer, the polymers are formed by an even number of VWF subunits. After secretion into the blood the identical subunits undergo proteolytic modification by the specific metalloproteinase ADAMTS13. These processing steps decrease the molecular weight of plasma von Willebrand factor. The metalloproteinase cleaves the subunit between Tyr 1605 and Met 1606 of the native subunit, leading to fragments of 140 and 176 kDa, respectively. As a result of these post-translational modifications the initially regular spacing between adjacent oligomers is more complex than anticipated leading to a quintuplet structure of the individual oligomers.

von Willebrand factor multimers

The first methods using large pore agarose gels and SDS as an ionic detergent were published by Ruggeri and Zimmerman [1] and Hoyer and Shainoff [2]. Ruggeri and Zimmerman followed the method of Lämmli [3] with the exception that they used agarose gels, because the VWF is too large to enter polyacrylamide gels. After these pioneering methods were published, technical variations were described by several groups. These variations comprise flat bed vs. vertical chambers, brands of agarose, continuous vs. discontinuous gels, gel dimensions, native gels vs. blotting, radioactive vs. nonradioactive methods, colorimetric vs. luminescent, ultraviolet or fluorescence methods, visual evaluation vs. densitometry [4-12].

A worldwide prospective and blind study in 32 laboratories with lyophilized plasma from normal persons or patients with genetically defined VWD variants showed the following results: All 27 laboratories performing multimer analysis used different methods relying more or less closely on the published methods. Although ratios (VWF:RCo/VWF:Ag and VWF:CB/VWF:Ag) detected some of the abnormal samples, false positive or negative results were much more frequent when multimers were not performed. Thus VWF multimers are apparently required for the correct diagnosis of VWD subtypes. “However, performance of VWF multimers in some laboratories requires review, with problems in sensitivity and resolution” [13].

When we analyzed 4362 patients diagnosed with inherited VWD and acquired VWS between January 2004 and December 2010 [14], we could show that 22% of the 1673 patients with VWD type 1 had a peculiar structural defect with persistent supranormal multimers, a smeary pattern without a clear triplet structure and in many aberrant (faster) velocities of the oligomers. These patients with the former subtype IC [15] had mutations in the carboxy terminus and were for the first time genetically characterized in the MCMDM-1VWD study [16]. 20% of these “type 1” patients showed VWF-parameters well above 50% without any abnormality than the structural defect and would have been overseen as patients with VWF without performing multimer analysis.

Accordingly in the 1082 patients with acquired von Willebrand syndrome it turned out that the ratio VWF:CB (the routine functional test in our laboratory) over VWF:Ag performed much worse than in the inherited type 2 VWD patients. In 207 patients with left-heart assistant devices and a loss of the large VWF multimers (100% with acquired VWD type 2), only 60% showed a ratio below 0.8; 44% showed a ratio below 0.7 and just 29% had a ratio <0.6. Thus a good part of these patients with a high burden of bleeding complications would have been overlooked without multimer analysis.

Out of the methods published up to now only the
“Hamburg” method was able to show that a high phenotype-genotype correlation can be achieved when multimer analysis is included in the usual battery of tests. In the MCMDM-1VWD study [11,16,17] in 150 families historically classified as type 1 families multimeric analysis was performed centrally by our laboratory. Theoretically there should have been no or only a small chance to detect qualitative abnormalities by multimer analysis, because by definition type 1 should show only quantitative abnormalities [15] and only the most experienced laboratories in Europe participated in this study. It turned out that 38% of the index cases and their affected family members showed qualitative abnormalities that at the end were associated in all with mutations in the VWF gene that proved to be causative by expression studies [18]. Therefore this method will be presented with more details here.

**Equipment**

Electrophoresis chamber Multiphor II with glass cooling plates, Electric Power Supply EPS 3501 XL and cooling aggregate Multitemp III, and Electroblot chamber Transphor +4 are from GE Healthcare Europe (München, Germany). The Power supply BIO RAD Power Pac HC comes from Bio Rad Laboratorien (München, Germany) and the Video System from Alpha Innotech (San Leandro CA, USA).

*Electrophoresis and blotting:* SDS-agarose discontinuous gel electrophoresis is carried out essentially as described by Ruggeri and Zimmerman [1,11]. 1.5 mm thick medium (1.6%) and low resolution (1.2%) gels (LGT agarose type VII, Sigma, Munich, Germany) are prepared in 26 x 15 cm cassettes. A stacking gel consisting of 0.8% Seacma HGT (P) agarose (Lonza Rockland Inc., Rockland, ME USA) of 1.5 cm length is poured over the running gel. The samples are diluted according to their VWF content (1:20 for a sample with 100% VWF:Ag; end concentration 0.5 µg/ml = 10 ng/slot if 20 µl of the solution is applied), heated for 20 min at 56°C and pipetted into the rectangular slots. Electrophoresis is performed at 65 V until the dye has left the slots (~1h). Afterwards the empty slots are backfilled with agarose, the voltage is lowered to 55 volts and the electrophoresis is performed overnight until the tracking dyes have reached the end of the gel. VWF multimers are transferred to nitrocellulose filters by electroblotting at 1.95 Amp during 4 hours using transfer buffer (0.05M phosphate, pH7.4 with 0.04M SDS, without methanol). After blocking in ultrahigh heated low-fat commercial milk (with no additions) for 20 minutes the filters are incubated with a 1:2500 dilution of rabbit anti-human VWF antibody A82 (Dako A/S Glostrup, Denmark) and after 3 washings steps for 10 minutes in milk the filters are incubated for 1.5 hours in the second antibody (1:2500 dilution of anti-rabbit antibody, peroxydase labelled, Bio Rad Laboratorien München, Germany). After the last 3 washing steps

**Figure 1. Reproducibility of the method described**

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 / NP 1:20</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5 = NP 1:20</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9 = NP 1:20</td>
</tr>
</tbody>
</table>

Shown by running the same sample seven times at the same dilution together with 2 control samples (pool) in a medium resolution gel. The characteristic pattern of the structural abnormal sample (supranormal multimers, no triplet structure and faster mobility) is reproduced invariably in every lane.
in milk the filters are washed under running tap water free of milk and thereafter placed into the video-detection system consisting of a dark housing, a sensitive cooled (-30°C) CCD camera and software generating 12-bit computer graphics (Alpha Ease software from Alpha Innotech, San Leandro CA, USA). Filters are overlaid with 4 ml solution containing 0.4 mg/ml luminol (Sigma-Aldrich Chemie, Steinheim, Germany), 0.01 mg/ml 4-iodophenol (Sigma-Aldrich Chemie, Steinheim, Germany) and 2.5 µl/ml 30% H₂O₂ (Perhydrol, Merck, Darmstadt, Germany) in Tris buffer (20 mMTris-HCl, 500mM NaCl, pH7.5). Depending on the necessary sensitivity in the case of a low VWF content of the sample a commercial luminol (LumiLight plus Roche, Darmstadt, Germany) is added. Typical exposure times are between 30 sec and 5 min depending on the signal visible on the computer screen or according to the set from the computer when using the auto exposure tool.

**Qualitative and quantitative evaluation**

*Figure 2. Reproducibility of the method described*

**a) Gel**

Shown by running the same sample seven times at the same dilution together with 2 control samples (pool) in a low resolution gel. The characteristic pattern of the sample of patients with aVWS type 2 (loss of the large multimers and enhanced proteolysis) is reproduced invariably in every lane.

**b) densitometric evaluation**
Plasma samples are classified as either abnormal multimers (AbM) or normal multimers (NM) by comparison with the reference plasma (pool of 30 normal persons, Fig.1). AbM are defined as a deviation from a normal distribution; either loss of HMW multimers or presence of larger than normal (supranormal).

**Figure 3. The sensitivity of the method described**

![Image of gel electrophoresis showing sensitivity of the method described](image)

Shown by diluting the normal sample (Pool 100% ~10 µg/ml) from 1:20 (10 ng) to 1:5120 (4 pg). The 1:1520 solution can still be evaluated when using the contrast adjustment tools of the program.

**Figure 4. Different variants of VWD**

![Image of gel electrophoresis showing different variants of VWD](image)

#1 dimerisation defect (note the missing large multimers, very prominent central and faint intervening bands);
#2 multimerisation defect (prosequence, note the missing large multimers, and the complete lack of proteolysis. The protomer is enhanced); #3 classical type 2A (note the missing large and intermediate multimers and very prominent proteolytic bands); #4 type 2B (note the missing large multimers and prominent proteolytic bands); #5 multimerisation defect (D3-domain, note the relative decrease of the large multimers, and the decreased proteolytic bands);
#6 type 1 variant (carboxy terminus; note the faster mobility of the oligomers, complete severely compromised proteolysis and the smeary pattern); #7 type 1; #8 NP (pool).
multimers on low resolution gels or as abnormal migration of individual oligomers or abnormal separation into triplets/quintuplets on medium resolution gels (Fig.2). In some samples the inter-oligomer space is filled with anti-VWF antibody-positive material, leading to a smeary pattern; these samples are also designated abnormal. Further classification of samples into type 1 and type 2 VWD and subclassification are performed according to ISTH guidelines [15,19].

Quantitative, densitometric gel analysis is performed by hand using software provided with the video-detection system (AlphaEaseFC Stand Aalone software, Alpha Innotech Corp. San Leandro, CA). Whenever possible, samples with the same quantity of VWF:Ag are applied to the gels (Fig.3). However, the lowest plasma dilution is limited to 1:10 (1:5) to ensure proper separation. For densitometric evaluation, small, intermediate and large multimers are defined as oligomers 1-5, 6-10 and >10, respectively. In the low resolution gels the small multimers are very close together and may even leave the gel, so the first peak (protomer) is counted as two oligomers, while in higher resolutions gels, where the smallest oligomers are better separated, the protomer is counted as one oligomer.

Concluding remarks

The recently finished relatively large studies with different patient groups with VWD showed that VWF multimers are required for the correct diagnosis of VWD subtypes. With a method suitable for a good separation of the individual oligomers together with the proteolytic banding pattern, the fate of the VWF in the circulation of individual patients can be read with a certain level of experience (Fig. 4). To distinguish between inherited and acquired diseases with the involvement of the VWF, anamnestic data are compulsory.

References

17. Hampshire DJ, Burghel GJ, Goudemand J, Bouvet LCS, Eikenboom JCI et al. Polymorphic variation within the VWF gene contributes to the failure to detect mutations in patients historically diagnosed with type 1 VWD from the MCMDM-1VWD cohort. Haematologica 2010;95:2163-5
Assay systems to measure platelet-dependent von Willebrand factor activity (GPIbα binding)

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St. István and St. László Hospital, Budapest, Hungary

Von Willebrand disease (VWD) is the most common bleeding disorder in man [1,2]. Measuring von Willebrand factor (VWF) functional activity is critical for making the correct diagnosis and classification. Traditionally, glycoprotein Ib (GPIb) binding was assessed using the VWF ristocetin cofactor activity (VWF:RCo) assay. This assay was developed in the 1970s [3,4], and can still be considered the “gold standard” for evaluating platelet-dependent VWF function [5,6]. All versions of the ristocetin cofactor activity assays have in common the use of (i) intact (native, formaline-fixed, or reconstituted lyophilized) platelets, and (ii) ristocetin.

Imprecision and insensitivity of the ristocetin cofactor assay led to a remarkable evolution of new assays during the last decade. Innovation focused on replacing the use of intact platelets with: (i) monoclonal antibodies directed against the GPIbα-binding site in VWF (no ristocetin needed); (ii) recombinant fragments of wild-type GPIb (with ristocetin); or (iii) a recombinant fragment of a gain-of-function mutant of GPIb (without ristocetin). Since it is essential that users of these various assays recognize the differences between the functions being measured, I propose the nomenclature summarized in Table 1. It should be noted that this nomenclature is a proposal, not yet accepted by the VWF community, but serves to clearly distinguish the various assay principles, and will be used throughout this manuscript.

Ristocetin cofactor activity (VWF:RCo) assays.

First generation (manual) VWF:RCo assay.
The original description of the test used glass slides, on which the reaction was performed. Although still used in some laboratories, this method is laborious and subjective.

Second generation (semi-automated) VWF:RCo assay.
The VWF:RCo assay was adapted to an aggregometer, and commercial reagents became available. The reaction velocity (slope of the aggregation curve) is compared to the calibrator. This assay is used in many laboratories throughout the world. Unfortunately, the VWF:RCo test is insensitive (Limit of detection (LOD): > 10–20 IU/dl) and imprecise (CV: up to 20–30 %) [7].

Third generation (fully automated) VWF:RCo assay.
The VWF:RCo assay was successfully adapted to current automated coagulometers. Full automation has the advantage of improved precision and higher throughputs. Table 2 lists published protocols for various platforms. Currently, most coagulation laboratories use one of the protocols listed in Table 2.

Fourth generation (modified fully automated) VWF:RCo assay.
Recently, a set of modifications to the original automated assay were reported to markedly improve sensitivity and precision (LOD: 3 IU/dl) with maintained precision (CV: 6–9%) [8,9]. Modifications included higher ristocetin concentration, the use of two different dilutions and calibration curves for low (0–50 IU/dl) and high (25–200 IU/dl) VWF concentrations as well as the use of VWF-deficient plasma in

<table>
<thead>
<tr>
<th>Table 1. Proposed nomenclature for the various activities measured by current assay systems</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abbreviation for VWF activity</strong></td>
</tr>
<tr>
<td>VWF:RCo</td>
</tr>
<tr>
<td>VWF:mAbA1B</td>
</tr>
<tr>
<td>VWF:RGPIbB</td>
</tr>
<tr>
<td>VWF:MGPIbB</td>
</tr>
</tbody>
</table>
the dilutions [9].

**VWF:RCo flow cytometry methods**

The sensitive detection of ristocetin-induced platelet agglutination is also possible with flow cytometric analysis. Several methods have been described [10-13]. However, because of the requirement for equipment and expertise that is usually not available in haemostasis laboratories, these systems have not gained wide acceptance.

**Advantages and disadvantages of the VWF:RCo assay.**

For decades, the VWF:RCo assay has been the gold standard for judging VWF activity. Therefore, much experience is accumulated, and most data correlating VWF levels and treatment relate to VWF:RCo. For this reason, most experts feel that the VWF:RCo remains indispensable in the routine laboratory diagnosis of VWD for the moment [5,14,15].

On the other hand, the disadvantages are numerous:

*The poor sensitivity* [7] prevents the measuring of VWF activity <10 IU/dl, exactly in the range where most severe VWD patients fall. Since the VWF:RCo/VWF:Ag ratio is critical in the phenotypic classification, such classification is often based on uncertain data.

The *high coefficient of variation* [7] also creates uncertainty and may lead to false diagnoses in the moderately reduced VWF range. Potential sources of error include instability and batch-to-batch variability of ristocetin, batch-to-batch variability of the platelet reagent (whether locally prepared or lyophilized), and some intrinsic imprecision of the assay system. Manual and semi-automated assays are even less precise.

An additional disadvantage comes from the fact that the VWF:RCo actually measures activity triggered by an artifact: although believed to induce conformational changes resembling the physiological activation of VWF brought about by immobilization to the collagen-rich subendothelial surface [16,17], ristocetin

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**Table 2. Third and fourth generation fully automated VWF:RCo assays***

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Platform</th>
<th>Sample dilution</th>
<th>PLT count (x109/L)</th>
<th>Ristocetin cc (mg/ml)</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV (%)</th>
<th>LOD (IU/dl)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC von Willebrand reagent</td>
<td>Sysmex CS2100i</td>
<td>18x (10/180 µl)</td>
<td>900**</td>
<td>0,53**</td>
<td>2,3</td>
<td>3,8</td>
<td>NS</td>
<td>36</td>
</tr>
<tr>
<td>BC von Willebrand reagent</td>
<td>Sysmex CS2000i</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>1,02-11,09</td>
<td>7%*</td>
<td>10</td>
<td>37</td>
</tr>
<tr>
<td>BC von Willebrand reagent</td>
<td>Stago STAR</td>
<td>10x (30/290 µl)</td>
<td>740**</td>
<td>0,43**</td>
<td>8,9-10,5</td>
<td>5,9-9,0</td>
<td>NS</td>
<td>38</td>
</tr>
<tr>
<td>Liophylized PLT:</td>
<td>IL ACL 9000</td>
<td>40x (10-80 for Hi&amp;Lo)</td>
<td>340-360</td>
<td>1</td>
<td>1,3-4,2</td>
<td>7,9-9,3</td>
<td>NS</td>
<td>39</td>
</tr>
<tr>
<td>(Dade-Behring) Risto:</td>
<td>IL ACL 7000</td>
<td>variable</td>
<td>300</td>
<td>1</td>
<td>10,3-10,6</td>
<td>6,9-11,7</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>(Mascia Brumelli)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC von Willebrand reagent</td>
<td>Siemens BCS</td>
<td>6&amp;36x (30&amp;5/180 µl for Lo&amp;Hi)</td>
<td>500</td>
<td>1,45</td>
<td>6,8-8,6</td>
<td>N/S</td>
<td>3</td>
<td>8,9</td>
</tr>
</tbody>
</table>

NS – not specified.

*Between batches

** based on measurements from the identical reagent, by Hillarp et al. Some of the ristocetin may be absorbed to platelets, not accounted for in this figure

*** Several modifications of the original assay lead to improved CV and LOD (bottom line in this table; ref.), and is termed “fourth generation VWF:RCo” in this manuscript.
itself is not a physiological activator of VWF. Two polymorphisms, D1472H and P1467S, have been described in the ristocetin-binding region in the A1 domain (exon 28) [13,18]. The D1472H polymorphism is common in the African-American population, and results in a decreased VWF:RCo/VWF:Ag ratio [18]. These low VWF activities do not correlate with bleeding symptoms, but seem to reflect an assay artifact.

Monoclonal antibody binding-based VWF activity (VWF:mAbA1B)

The original ELISA assay used the monoclonal antibody REF-VIII:R/2, directed against a VWF epitope involved in VWF-GPIb binding [19-21]. The commercial LIA version is marketed as “VWF activity” assay, even abbreviated as VWF:Act. Since the term “VWF activity” contains no information about the function of VWF being measured, it seems preferable to call the test “monoclonal antibody binding activity” (VWF:mAbA1B).

The latex-enhanced automated immunoturbidimetric assay (LIA; HemosIL VWF activity) performed better than the ELISA [22,23] in discriminating type 2 VWF defects and showed good correlation with VWF:RCo [24-28]. Advantages of the HemosIL VWF activity include ease of use, applicability to several platforms, and thus, feasibility for routine laboratories. Popularity has even led some authors to take it for granted that the assay measures the ability of VWF to bind to GPIb [25,26]. However, it cannot be over-emphasized that the VWF:mAbA1B assay tests binding of VWF A1 domain to a mAb, not GPIb, and it is unclear to what extent the mAb actually mimics the GPIb-binding surface. Type 2 VWD is often caused by structural changes in the A1 domain which will likely be detected by the assay, but it is unclear at this time how many mutations miss detection, and whether some type 1 mutations are misclassified (eg. 36% type 1 samples were classified as type 2 in one report [27]). Along these lines, at least some 2M mutations (eg. p.Gly1324Ala) are not detected by the assay [28]. Moreover, it is not clear to what extent the assay is sensitive to the loss of HMW multimers.

Some authors reported excellent detection of type 2A patient samples (suggesting a sensitivity of the assay to the loss of HMW VWF) [24], while others noted missing the loss of HMW multimers [25]. Furthermore, it should also be noted that the VWF:mAbA1B assay did not resolve the problem with the lower limit of detection: linearity is reported [24] to be acceptable above 12.5 IU/dl. Taken together, the good overall correlation with the VWF:RCo assay probably does give this assay a role in the routine screening of VWF patients, probably combined with other tests. However, the VWF:mAbA1B test cannot currently be recommended as a replacement for the VWF:RCo assay.

Ristocetin-triggered GPIb binding (VWF:RGPIbB) assays

In an effort to replace the platelet with a more reliable reagent, Deckmyn and colleagues developed a revolutionary ELISA test using a recombinant GPIb fragment captured by a monoclonal antibody coated onto ELISA plates [29]. The assay seemed to have much improved LOD compared to the standard VWF:RCo. For the first time, samples with <1 IU/dl could be accurately measured [30-32]. In addition, precision (coefficient of variation, CV) was also improved (Table 3). Subsequently, the same assay principle was used to develop latex or magnetic particle-enhanced automated assays.

Correlation with the classic VWF:RCo test is reported to be excellent for all variants of the VWF:RGPIbB assays, for normal controls and patients with type 1, 2 and 3 VWD, proving the validity of the concept. Table 3 lists published variations to the assay principle. Although all based on the same basic principle, these assays use different reagents for capturing the GPIb fragment, different recombinant or plasma-derived (glycocalcin, the extracellular portion of GPIb cleaved from platelets) fragments (Table 3). The source and concentration of ristocetin is also variable. Epitope specificity of the monoclonal antibody capturing GPIb is critical: for example, of 42 different mAbs directed at the GPIb molecule, only one captured glycocalcin in the correct orientation to support VWF binding [30]. The optimal capturing antibody was different for a recombinant GPIb fragment. Thus, these assays need circumspect optimization, and reagents are not interchangeable.

Gain-of-function mutant GPIb binding (VWF: MGPIbB) assays

The VWF:RGPIbB activity assays still use ristocetin, with all the inherent problems listed above. Furthermore, none of the ristocetin-triggered assays are able to differentiate between 2A and 2B subtypes. The differentiation, which is critical in patient care, is only possible by performing a RIPA on fresh
patient samples. These limitations were addressed by the introduction of the newest platelet-dependent VWF activity assays, which use recombinant gain-of-function mutant GPIb fragments by-passing the need for ristocetin in the assay mixture.

Table 4 lists the published characteristics of ELISA-based and automated assays. The mutations engineered into the GPIb fragment were described in patients with platelet-type (pseudo-) von Willebrand disease, and allow spontaneous binding of VWF to the mutant GPIb without activation of VWF. The binding is optimized when any two of three such mutations are introduced[33]. Published preliminary data support the concept in that these assays correlate very well with the standard VWF:RCo assay, are precise, sensitive and not subject to the false positive result seen with the P1467S and D1472H polymorphisms [33,34]. Additionally, it may be possible to differentiate between 2A and 2B VWD subtypes [33].

Summary

In summary, the past few years have seen a remarkable (r)evolution of assays measuring platelet-dependent VWF activities. The original manual and semi-automated methods have mostly been replaced by automated techniques with higher precision. In newer applications, the platelets have been substituted by recombinant GPIb fragments immobilized on ELISA wells or latex particles providing improved coefficient of variation, and higher sensitivity, allowing accurate measurement of VWF activity in the very low (<1 to 10 IU/dl) range. Further innovation has led to the newest generation of ristocetin-free assays, which not only seem accurate and sensitive, but also avoid problems with ristocetin including false-positive results in certain populations. While this is most welcome in VWD diagnosis, the exact role for the particular assays is not clear at this time. Side-by-side comparison using normal as well as pathological (including type 1, type 2 and type 3

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Platform</th>
<th>Coat: mAb</th>
<th>GPIbα</th>
<th>Ristocetin</th>
<th>Secondary reagents</th>
<th>Detection</th>
<th>LOD IU/dl</th>
<th>CV (%)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>in-house</td>
<td>ELISA</td>
<td>2D4</td>
<td>1-289</td>
<td>ABP, 760 µg/ml</td>
<td>rabbit anti-VWF polyclonal-HRP</td>
<td>absorbance, 492 nm</td>
<td>0.05</td>
<td>4-13</td>
<td>29,31</td>
</tr>
<tr>
<td>in-house</td>
<td>ELISA</td>
<td>24B3</td>
<td>glycocalcin</td>
<td>ABP, 1 mg/ml</td>
<td>rabbit anti-VWF polyclonal-HRP</td>
<td>absorbance, 492 nm</td>
<td>0.05</td>
<td>5-10</td>
<td>30</td>
</tr>
<tr>
<td>in-house</td>
<td>ELISA</td>
<td>LJ-P3</td>
<td>1-290- C65A</td>
<td>Sigma, 0,8 mg/ml</td>
<td>rabbit anti-VWF polyclonal-HRP</td>
<td>absorbance, 492 nm</td>
<td>0.1</td>
<td>9-10</td>
<td>32</td>
</tr>
<tr>
<td>in-house</td>
<td>ELISA</td>
<td>SZ-151</td>
<td>1-289- IgG1Fc fusion protein</td>
<td>Sigma 760 µg/ml</td>
<td>rabbit anti-VWF polyclonal-HRP</td>
<td>absorbance, 450 nm</td>
<td>0.008</td>
<td>8-12</td>
<td>41</td>
</tr>
<tr>
<td>in-house</td>
<td>ELISA</td>
<td>MBC 142:16</td>
<td>His-1-290-C65A</td>
<td>ABP 1 mg/ml</td>
<td>2 mAbs: AVW-1 &amp; AVW-15-biotin</td>
<td>Streptavidin AlkPhos absorbance @405nm</td>
<td>1.6</td>
<td>7-21</td>
<td>13,33</td>
</tr>
<tr>
<td>IL, HemosIL VWF:R:Co</td>
<td>Latex beads ACL TOP</td>
<td>24B3</td>
<td>NS</td>
<td>YES</td>
<td>None</td>
<td>turbidimetry (LIA)</td>
<td>3.5</td>
<td>3-4</td>
<td>42</td>
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<tr>
<td>IL, under development</td>
<td>Magnetic particles AcuStar</td>
<td>2D4</td>
<td>1-289</td>
<td>YES</td>
<td>anti-VWF-Isoluminol</td>
<td>Chemilumincence</td>
<td>0.5</td>
<td>2-7</td>
<td>43</td>
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</tbody>
</table>

NS – not specified
patient) samples is urgently needed. An ongoing comparative study [35] is expected to provide useful information for clinicians and laboratory professionals alike, and will probably lead to the gradual replacement of the older assays. In addition to providing technical details for the currently available assays, this review offers a new nomenclature for distinguishing the various test principles clearly.

### References


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### Table 4. Gain-of-function mutant GPIb-Binding (VWF:MGPIbB) assays by ELISA and automated (coagulometer) systems

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>Platform</th>
<th>Coat: mAb</th>
<th>GPIbα</th>
<th>Ristocetin</th>
<th>Secondary reagents</th>
<th>Detection</th>
<th>LOD IU/dl</th>
<th>CV (%)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>in-house</td>
<td>ELISA</td>
<td>mAb (NOS)</td>
<td>1-289-G233V -M239V</td>
<td>NO</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>44</td>
</tr>
<tr>
<td>Siemens, INNOVANCE VWF Ac</td>
<td>Latex beads</td>
<td>mAb (NOS)</td>
<td>1-289-G233V -M239V</td>
<td>NO</td>
<td>NS</td>
<td>2 mAbs: AVW-1 &amp; AVW-15-biotin</td>
<td>Streptavidin alpHos absorbance @ 405 nm</td>
<td>1,6</td>
<td>6-23</td>
</tr>
<tr>
<td>GTI, under development</td>
<td>Latex beads</td>
<td>mAb (NOS)</td>
<td>His-1-290-C65A+D235Y -M239V</td>
<td>NO</td>
<td>NS</td>
<td>turbidimetry</td>
<td>2,2</td>
<td>2-5</td>
<td>34</td>
</tr>
</tbody>
</table>

NS – not specified
NOS – not otherwise specified


External quality assessment for von Willebrand factor laboratory analysis

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Introduction

Von Willebrand disease (VWD) is the most frequently occurring inherited bleeding disorder and needs proper laboratory diagnosis to differentiate between its subtypes. Therefore a panel of different tests is needed (see for details the contribution of Chen and Nichols).

Because of the importance of the laboratory diagnosis of VWD as well as its complexity a proper quality control of the performance of these laboratory tests is necessary. It was shown in several multi-laboratory survey studies that there is a wide variation in the performance of laboratory tests included in the VWD diagnosis [1-3].

Here an overview is given of inter-laboratory comparison for the measurement of VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (vWF:RCo) and factor VIII clotting activity (FVIII:C) for different types of VWD samples in the external quality assessment (EQA) programme of the ECAT Foundation.

VWF EQA surveys

The ECAT provides 4 surveys per year. In each survey, one lyophilised plasma sample is distributed to the participants who are asked to handle the sample as if it was a normal clinical laboratory sample using their routine method for the determination of VWF, including VWF:Ag, VWF:RCo, collagen-binding assay (VWF:CB), multimer analysis and FVIII:C for different types of VWD samples in the external quality assessment (EQA) programme of the ECAT Foundation.

Table 1. The classification of samples distributed in the ECAT VWF surveys.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>No classification #</th>
<th>Normal</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8 – 19%</td>
<td>73 – 92%</td>
<td>0 – 1%</td>
<td>0 – 8%</td>
<td>0%</td>
</tr>
<tr>
<td>Type 1</td>
<td>25 – 26%</td>
<td>0 – 11%</td>
<td>51 – 71%</td>
<td>2 – 13%</td>
<td>0%</td>
</tr>
<tr>
<td>Type 2</td>
<td>33 – 47%</td>
<td>0 – 1%</td>
<td>11 – 30%</td>
<td>26 – 41%</td>
<td>1 – 10%</td>
</tr>
</tbody>
</table>

# No classification: a participant could not give a final classification because of the lack of sufficient test results.

In this overview results of surveys in the period 2008 – 2012 are reviewed, including 4 normal control samples, 4 samples of a type 1 VWD patient and 4 samples of a type 2 VWD patient.

Classification

In each survey the participants were asked to give a conclusive assessment of the type of sample which was distributed. Between 20% and 35% of the participants did not give any conclusive assessment.

An overview is given in table 1 of those participants who gave a final classification. From this table it can be observed that the more complex a sample is to diagnose (normal ► type 2), the more participants indicated that it was not possible to give a conclusive assessment. The reason for this is the fact that not each participant had access to the complete repertoire of different VWF tests running in their laboratory. A normal sample is most times well classified, although some participants identify a normal sample sometimes as a type 1 or type 2 VWD. A type 1 VWD sample is sometimes misclassified as normal or a type 2 VWD sample, by more than 10% of the participants. A type 2 VWD sample could be misclassified by up to 30% as a type 1 VWD sample and 10% as a type 3 VWD sample. These figures show that specially for type 1 and type 2 VWD samples further improvement in the laboratory diagnosis is necessary.

Consensus values and between-laboratory variation

In table 2 a summary is given of the range of consensus values and between-laboratory variation observed for the different samples used in the surveys. It is clear that for both VWF antigen and activity, which includes both VWF:RCo and immunological...
The Clotting Times

assays, as well as for FVIII activity, there is no overlap in the consensus values between the different types of samples. However, when looking at the level of individual participant results (Fig. 1) it can be appreciated that there could be some overlap between the different types of samples for the three different parameters. This is especially the case for VWF activity and FVIII levels for type 1 and 2 VWD samples.

In general the between-laboratory variation in the measurement of VWF antigen and activity and FVIII is fairly comparable with that between normal plasma samples and samples from a type 1 VWD patient. Only the between-laboratory variation for VWF activity in a type 1 VWD sample could sometimes be higher than in a normal sample. Furthermore, samples from a type 2 VWD patient, with low values for all 3 parameters, show a significantly higher variation between individual laboratory results. Although this is a normal phenomenon in samples with a low concentration or activity, it may result in an overlap of results from a type 2 VWD patient with those from a type 1 VWD patient (see Fig. 1). This explains why a type 1 sample sometimes is classified as a type 2 sample and vice versa.

Interpretation of laboratory results

As mentioned most difficulties are observed in the proper classification of a type 1 or type 2 VWD sample (see table 1). One possibility to support a proper distinction between type 1 and 2 VWF samples is the use of the ratio between the VWF activity and antigen result [4, 5]. A ratio lower than 0.5 - 0.7 indicates a type 2 VWD sample, except for VWD type 2N. A ratio higher than 0.5 - 0.7 suggest a type 1 VWD sample, assuming that both the antigen and activity level are decreased. Otherwise it is a normal sample. Table 3 shows the evaluation of the percentage of participants with a ratio VWF activity/antigen below 0.5, 0.6 and 0.7, respectively, for normal and type 1 and type 2 VWD samples in the ECAT surveys.

For some typically normal type 1 and type 2 VWD

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Antigen</th>
<th>Activity</th>
<th>Factor VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level (U/dL)</td>
<td>CV (%)</td>
<td>Level (U/dL)</td>
</tr>
<tr>
<td>Normal</td>
<td>96 – 119</td>
<td>7.8 – 9.2</td>
<td>75 – 87</td>
</tr>
<tr>
<td>Type 1</td>
<td>32 – 44</td>
<td>7.8 – 11.1</td>
<td>23 - 37</td>
</tr>
<tr>
<td>Type 2</td>
<td>16 – 29</td>
<td>11.2 – 23.5</td>
<td>10 – 11</td>
</tr>
</tbody>
</table>

Table 2. The level and the between-laboratory variation for VWF antigen and activity and Factor VIII activity for normal samples and type 1 and 2 VWD samples used in the ECAT surveys.

<table>
<thead>
<tr>
<th>Normal</th>
<th>Type 1</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.5</td>
<td>&lt; 2%</td>
<td>37 – 38%</td>
</tr>
<tr>
<td>&lt; 0.6</td>
<td>6 – 9%</td>
<td>0 – 3%</td>
</tr>
<tr>
<td>&lt; 0.7</td>
<td>18 – 37%</td>
<td>4 – 14%</td>
</tr>
</tbody>
</table>

Table 3. The percentage of the ratio VWF activity / antigen below different cut-off levels for normal samples and type 1 and 2 VWD samples used in the ECAT surveys.
samples the ratio between the VWF activity and antigen has been evaluated. In Fig. 2 the results of this evaluation are shown. It is clear that the dispersion in the ratios for the normal and type 1 VWD samples are comparable. The range for the ratio is between 0.4 and 1.4. However, for the type 2 VWD samples a wide dispersion is observed (0.0 – 2.0) with a huge overlap with the normal and type 1 VWD samples. Because a significant number of participants classified a type 2 VWD sample as a type 1 or 3 VWD sample it is interesting to evaluate whether there is a relationship between the VWF activity/antigen ratio obtained and the final classification. A typical example of such a relationship is given in Fig. 3. Although a lower mean ratio is observed for the group that give a type 2 classification with respect to a type 1 or 3 classification, it can be appreciated from Fig. 3 that there is a huge overlap between the different classification groups.

Concluding remarks

The data presented in this summary of ECAT survey data for the period 2008 – 2012 shows that improvement in the measurement and interpretation of VWF parameters is needed. Assuming that the performance of the measurement of the VWD samples used in the ECAT surveys is comparable to those on fresh or frozen clinical samples, it is clear that laboratories may misclassify certain clinical samples. For a proper evaluation of VWD patient samples a dedicated test panel is needed. Not every individual laboratory is able to facilitate the full test panel. However, even when only VWF antigen and/or activity is measured the quality of the measurement should be sufficiently adequate to indicate what further laboratory investigations are needed.

References
