

DIAGNOSIS OF VON WILLEBRAND DISEASE

PHENOTYPIC CHARACTERIZATION

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DIAGNOSIS OF VON WILLEBRAND DISEASE

Phenotypic Characterization

Abstract

von Willebrand disease (VWD) is the most common bleeding disorder caused by inherited defects in the concentration, structure, and function of von Willebrand factor (VWF). VWD is transmitted as an autosomal dominant or recessive disorder affecting both males and females. VWF is a multimeric, high molecular weight glycoprotein that plays a pivotal role, under high shear conditions, in platelet adhesion and aggregation, and in stabilizing blood coagulation factor VIII (FVIII).

Several laboratory tests are necessary for an accurate diagnosis of VWD. These investigations are performed sequentially and subdivided into initial screening, first, and second level tests. Therefore, classification of VWD is relatively complex, but proper diagnosis is important for the therapeutic management of patients. Molecular characterization of the VWF gene (*VWF*) is not essential in the diagnosis of VWD if patients have undergone phenotypic tests. However, if some of these assays are unavailable, then molecular analysis is crucial in order to obtain an accurate diagnosis. Furthermore, molecular analysis may provide additional useful information for phenotypically well-characterized patients (i.e., when prenatal diagnosis is required). The companion monograph, #56 in the WFH Treatment of Hemophilia series *Molecular Diagnosis of von Willebrand Disease*, provides a detailed review of the genotypic approach to VWD diagnosis [1].

Together, these two monographs offer guidance to both clinicians and laboratory specialists regarding the accurate phenotypic and genotypic diagnosis of patients with bleeding symptoms and a positive family bleeding history, suspected of having VWD.

Introduction

von Willebrand factor (VWF) is a multimeric, high molecular weight glycoprotein involved in primary hemostasis,

supporting platelet adhesion and aggregation via binding to platelet glycoprotein (GP) receptors GPIb α and GPIIb-IIIa under high shear conditions at the site of vascular injury [2]. In addition, VWF acts as a carrier protein for coagulation factor VIII (FVIII), protecting it against proteolytic degradation and rapid clearance [3].

Definition of von Willebrand disease

von Willebrand disease (VWD) is the most common bleeding disorder caused by inherited defects in the concentration, structure, and function of VWF. The reported prevalence in the general population varies considerably among studies, ranging from 0.1% to 1%, and depends on the type of patient selection and the criteria used to diagnose the disease [2]. It is mainly transmitted as an autosomal dominant disorder and is caused by quantitative or qualitative defects in VWF [2,3]. The current classification of VWD is based on partial or complete quantitative (VWD type 1 and 3) or qualitative (VWD type 2) defects in VWF. VWD type 2 is further divided into four variants (2A, 2B, 2M, and 2N) on details of the phenotype [2]. VWD type 3 is transmitted as an autosomal recessive disorder with a prevalence of approximately one per million in the general population, but its frequency is significantly higher in areas with a high rate of consanguinity [4].

Overview of VWD diagnosis

Clinical diagnosis

The diagnostic approach starts with the clinical assessment of a person who reports mucocutaneous bleeding symptoms. The initial evaluation should take into account the personal and familial history of bleeding manifestations. Evaluation of the personal bleeding history aims to identify the characteristics of bleeding, such as spontaneity, severity, and localization.

Different tools have been developed to help in the diagnosis of bleeding disorders; some of them are general and

some are symptom-specific. The Bleeding Assessment Tools section of the World Federation of Hemophilia (WFH) Compendium of Assessment Tools¹ provides evaluations of the most useful tools in current practice (e.g., the Molecular and Clinical Markers for the Diagnosis and Management of Type 1 [MCMDM-1] VWD Bleeding Questionnaire², and the Pictorial Blood Loss Assessment Chart [PBAC]³).

The International Society on Thrombosis and Haemostasis (ISTH) has developed a bleeding assessment tool (BAT)⁴. This instrument is helpful in the clinical setting as it can be used to evaluate VWD or platelet dysfunction in suspected patients. In addition, it serves to rationalize laboratory bleeding investigation and evaluate the propensity of patients to bleed.

Laboratory diagnosis

VWD is an inherited bleeding disorder caused by a quantitative and/or qualitative deficiency of the VWF. A phenotypic approach to diagnosing VWD is described below. Molecular analysis can also contribute important information to further the laboratory diagnosis, and is described in detail in the accompanying monograph to this one, #56 in the WFH Treatment of Hemophilia series *Molecular Diagnosis of von Willebrand Disease* [1].

Phenotypic diagnosis

VWF is a multimeric and multifunctional glycoprotein with several domains containing different functional binding sites. Therefore, more than one test is needed to assess all VWF functions, and often, VWD diagnosis is complex. Different biochemical assays are available to analyse the functions of this complex glycoprotein, which needs to be performed step-by-step as follows.

The initial hemostasis laboratory evaluation, named ‘initial screening tests’, includes complete blood count (CBC), activated partial thromboplastin time (APTT), prothrombin time (PT), and fibrinogen (Figure 1). These tests cannot exclude or confirm VWD, but they can suggest whether a coagulation factor deficiency might be the potential cause of a patient’s bleeding symptoms [5]. As thrombocytopenia may occur in patients with VWD type 2B, platelet count should be evaluated. In VWD, FVIII deficiency is secondary to the deficiency or dysfunction of VWF, its carrier protein. Therefore, in VWD, APTT results are prolonged only if the FVIII level is sufficiently reduced (i.e., VWD type 3 or VWD type 2N). In some laboratories, bleeding time (BT) and platelet function analyser (PFA-100[®]) assays are also performed. However, BT results suffer from operator variability, and the test also lacks sensitivity [6]. Some studies have shown that PFA-100[®] assay results are abnormal in the majority of VWD patients; however, PFA-100[®] lacks sensitivity and specificity to be used alone as a screening test [7,8].

An algorithm for the diagnosis of VWD, including first and second level tests, is summarized in Figure 2.

First level tests for the diagnosis of VWD

First level tests measure the plasma level of FVIII coagulant activity (FVIII:C), the VWF antigen level (VWF:Ag), and platelet-dependent VWF function (VWF-platelet GPIIb/IIIa binding activity) usually measured as ristocetin cofactor activity (VWF:RCo). The results of these tests should be expressed in either international units per decilitre (IU/dl) or in international units per millilitre (IU/ml) based on the World Health Organization (WHO) standard [5].

FVIII:C

FVIII:C is performed by one-stage clotting assay⁵ based on the APTT assay or by two-stage clotting assay⁶. FVIII

1 WFH Compendium of Assessment Tools. <http://elearning.wfh.org/>

2 MCMDM-1 VWD Bleeding Questionnaire. <http://elearning.wfh.org/>

3 Pictorial Blood Loss Assessment Chart. <http://elearning.wfh.org/>

4 ISTH Bleeding Assessment Tool. <http://elearning.wfh.org/>

5 WFH Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual. <http://elearning.wfh.org/> Section 23: Factor Assays Based on APTT.

6 WFH Diagnosis of Hemophilia and Other Bleeding Disorders. A Laboratory Manual. <http://elearning.wfh.org/> Section 25: Two-Stage Clotting Assay for Factor VIII:C.

activity levels are also measured by a chromogenic assay⁷ [9]. Among these, the most common is the clotting assay, which measures the capacity of a patient's FVIII to reduce the clotting time of FVIII-deficient plasma.

VWF:Ag

VWF:Ag measures the concentration of the VWF glycoprotein in patient plasma. The VWF:Ag assay is performed by enzyme-linked immunosorbent assay (ELISA)⁸ or by automated latex immunoassay (LIA).

VWF-platelet GPIb α binding activity

The following explains the available tests to measure the main functional activity of VWF. VWF-platelet GPIb α binding activity is commonly assessed as VWF:RCo, which measures the ability of plasma VWF to agglutinate platelets in the presence of ristocetin [10]. In the past ten years, new assays have been introduced to evaluate VWF-platelet GPIb α binding activity in order to overcome limitations of the VWF:RCo assay [11]. Therefore, the ISTH Standardization and Scientific Committee (SSC) approved a new nomenclature that facilitates distinction of assays that differ in their functional details [12]. The consensus nomenclature classifies VWF-platelet GPIb α binding activity assays as follows:

1) *VWF:RCo assays*. These tests utilize intact platelets in different forms (native, formalin-fixed, or reconstituted lyophilized) and ristocetin. VWF:RCo assays are subdivided into first generation (manual), second generation (semi-automated), third generation (fully automated), fourth generation (modified fully automated), and flow cytometry methods.

However, these assays present some limitations, such as high coefficient of variation (CV; which can exceed 30% when values are <15 IU/dl), batch-to-batch variability of ristocetin or the platelet reagent (whether locally prepared or commercially lyophilized), and intrinsic instability of the assay system. First and second generation assays are also time consuming and poorly standardized. A further drawback is that VWF:RCo

measures two parameters: 1) the binding of ristocetin to VWF; 2) the binding of ristocetin-‘activated’ VWF to platelets [12]. Flood *et al.* [13,14] described two sequence variants in the ristocetin binding region of the A1 domain (p.P1467S and the H-allele of a common polymorphism p.D1472H) that cause spuriously decreased VWF:RCo levels. Nevertheless, VWF:RCo is widely used and still accepted as the reference method for assessing platelet-dependent VWF activity.

2) *Ristocetin-triggered GPIb α binding (VWF:GPIbR) assays*. These tests use ristocetin and a GPIb α fragment captured by a monoclonal antibody (mAb) coated onto an ELISA plate or on a latex or magnetic particle (for enhanced automated assays). As described by Vanhoorelbeke *et al.* [15] and Federici *et al.* [16], different antibodies are available for capturing the GPIb α fragment, which can be plasma-derived or a recombinant protein. There is good correlation between the VWF:RCo and VWF:GPIbR assays; although, VWF:GPIbR assays demonstrate improved precision, a better CV, and lower limit of detection [12].

3) *Gain-of-function mutant GPIb α binding (VWF:GPIbM) assays*. These tests use recombinant gain-of-function mutant GPIb α fragments (rGPIbM), which spontaneously bind to VWF without ristocetin. Different gain-of-function rGPIbM mutations can be used. However, no difference is detected in the binding between rGPIbM and VWF when any two of three gain-of-function mutations (p.G233V, p.D235Y, and p.M239V) are introduced [17]. VWF:GPIbM assays use rGPIbM captured by an ELISA plate or on a latex particle, coated with an mAb. VWF:GPIbM assays reportedly correlate with the standard VWF:RCo assay; however, the newer assays have better precision, CV, and sensitivity [11,18,19].

4) *mAb binding-based VWF activity (VWF:Ab)*. This is used for the commercial latex-enhanced automated immunoturbidimetric assay, where an mAb is directed against an epitope in the VWF A1 domain involved in

7 WFH Diagnosis of Hemophilia and Other Bleeding Disorders. A Laboratory Manual. <http://elearning.wfh.org/> Section 26: Chromogenic FVIII:C Assay.

8 WFH Diagnosis of Hemophilia and Other Bleeding Disorders. A Laboratory Manual. <http://elearning.wfh.org/> Section 30: Von Willebrand Factor Antigen by Elisa (VWF:Ag).

GPIIb α binding. The advantages of this assay include its applicability to different platforms, and therefore, its feasibility for routine laboratories. Since the VWF:Ab assay measures VWF binding to an mAb and not to GPIIb α , it is unclear if this antibody is able to accurately mimic GPIIb α binding to VWF [20]. Therefore, it cannot be considered a true activity assay nor recommended as a replacement for the VWF:RCo assay.

Second level tests for the diagnosis of VWD

Second level tests are necessary for defining and classifying VWD variants. They are applied when low levels of VWF are detected and/or when a discrepancy between the concentration of VWF protein and its platelet-dependent functions is found (i.e., VWF:RCo/VWF:Ag <0.6). Decreased FVIII levels associated with normal or nearly normal VWF values, leading to a FVIII:C/VWF:Ag ratio <1 [21], may be related to VWD type 2N or mild hemophilia A.

VWF:CB

The interaction of VWF with collagen is crucial under high shear conditions for the initiation of a platelet plug at sites of injury. The primary site of fibrillar collagen binding is the A3 domain of VWF [22], although the A1 domain has also been reported to interact with collagen types IV and VI [23,24]. The collagen binding activity (VWF:CB) assay measures the capacity of VWF to bind to collagen. As with VWF:RCo, the results of VWF:CB are dependent on the VWF multimers size, meaning that the largest multimers bind to collagen with higher affinity than the smaller forms. The sensitivity and capability of the VWF:CB assay to discriminate between VWD subtypes is dependent on the source and the type of collagen used [25]. The VWF:CB assay can be used as a surrogate of VWF multimer analysis, whenever the latter test is unavailable, in order to evaluate the absence of high molecular weight multimers (HMWM). Even if some studies suggest that VWF:CB testing can improve the differentiation of VWD type 2M from type 2A, in clinical and laboratory practice, the VWF:CB assay is not part of the routine workup for VWD [26,27]. Collagen-binding

defects are also described in VWD type 2M (VWF:CB/VWF:Ag <0.6) due to mutations in the A3 domain [28,29].

Nowadays, there are a number of commercially available ELISA kits to evaluate this VWF function. One example is described in the *Diagnosis of Hemophilia and Other Bleeding Disorder: A Laboratory Manual*⁹, but many others have been used with success.

RIPA

Only a few biochemical methods have been reported [30-33] to distinguish VWD type 2B from the other VWD types (e.g., VWD type 2A or type 1). Among these methods, ristocetin-induced platelet agglutination (RIPA) is the most widely used [34]. RIPA¹⁰, performed using patient platelet-rich plasma (PRP), evaluates the affinity of VWF for the platelet GPIIb α receptor at different concentrations of ristocetin. Therefore, for this assay, the availability of a fresh blood sample is mandatory. Low ristocetin concentrations (<0.7 mg/ml) do not cause platelets agglutination in the PRP of normal subjects, whereas the same amount of ristocetin might cause platelet agglutination in PRP from patients with VWD type 2B. On the contrary, at higher concentrations of ristocetin (between 0.7 and 1.2 mg/ml) capable of inducing platelet agglutination in normal subjects, RIPA is impaired in VWD type 3, 2A, and 2M patients. Gain-of-function mutations in the platelet receptor GPIIb α cause platelet-type VWD (PT-VWD), also known as pseudo-VWD. The RIPA assay is unable to distinguish VWD type 2B from PT-VWD; however, a differential diagnosis can be determined by platelet-mixing tests [35] or molecular analysis of VWF and the GPIIb α gene.

VWF multimer analysis

The multimeric pattern of VWF in plasma is important for its functional activity. The HMWM is the most hemostatically active form of VWF. These molecules bind to collagen and platelets with significantly higher affinity than the low molecular weight multimers. Due to their size, HMWMs are also more effective at mediating platelet adhesion and aggregation, particularly under elevated

9 WFH Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual. <http://elearning.wfh.org/> Section 31: Von Willebrand Factor Collagen Binding Assay (VWF:CB).

10 WFH Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual. <http://elearning.wfh.org/> Section 39: Platelet Function Testing.

shear conditions [36]. Analysis of the multimeric structure of VWF is complex and time consuming, but useful in the diagnosis of VWD.

Low resolution VWF multimer analysis¹¹ (low agarose concentration gels) can detect the loss of HMWM (Figure 3, panel A), allowing patients with VWD type 1 to be distinguished from type 2, and to distinguish type 2A (loss of HMWM and intermediate molecular weight multimers [MWM]) from type 2M (normal multimeric pattern). Intermediate resolution gels¹¹ (made with higher agarose concentrations) resolve the inner multimeric structure, showing the triplet structure of each single oligomer. In a normal individual, the triplet pattern consists of a central band and two outer sub-bands [37]. In some patients with VWD, alterations of the triplet structure can be observed (Figure 3, panel B). A semi-automated method for VWF multimer analysis at low resolution will soon be commercially available.

VWF:FVIII

The VWF-FVIII binding assay (VWF:FVIII) evaluates the capacity of VWF to bind FVIII. This assay is crucial to differentiate a diagnosis of VWD type 2N from mild hemophilia A, since both conditions are associated with a moderate reduction in FVIII plasma levels, and normal levels of VWF:Ag and VWF:RCO (FVIII:C/VWF:Ag <1) [21]. The VWF:FVIII test measures the capacity of plasma VWF to bind an exogenous FVIII using a solid-phase immunoassay¹² [38]. In the differential diagnosis, the results obtained from this assay can be: 1) a normal capacity of VWF to bind FVIII, resulting in a diagnosis of mild hemophilia A; 2) a markedly reduced capacity of VWF to bind FVIII, resulting in a diagnosis of VWD type 2N. A third outcome is possible using this assay; a moderately reduced capacity of VWF to bind FVIII. This finding is typical in asymptomatic carriers of VWD type 2N defects who usually present normal FVIII levels (FVIII:C/VWF:Ag >1), and it is also observed in some patients with VWD type 2A IIE variants.

Commercial ELISA kits to measure VWF:FVIII are available.

Intraplatelet VWF analysis

In addition to the Weibel–Palade bodies of endothelial cells, VWF is also stored within the α -granules of platelets. In contrast to plasma VWF, this pool of platelet VWF is enriched in hemostatically active ultra-large (or unusually large) VWF (UL-VWF) multimers [39]. Platelet VWF can be evaluated using the same assays adopted for plasma VWF (VWF:Ag, VWF:RCO, VWF:CB, and multimer analysis). However, in order to correctly interpret the results of each assay, it is necessary to first establish a normal range, obtained from a small population of healthy individuals. As with the RIPA assay, platelets must be isolated from a fresh blood sample. Few methods are available for the extraction of VWF from platelets, and none have been standardized. Mannucci *et al.* [40] described three different subgroups of patients based on platelet VWF levels: 1) ‘platelet-low’, those with reduced platelet VWF:Ag and VWF:RCO; 2) ‘platelet-normal’, those with platelet VWF:Ag and VWF:RCO within the normal range; and 3) ‘platelet-discordant’, those with platelets containing normal concentrations of VWF:Ag, but disproportionately reduced VWF:RCO. These subcategories have been shown to predict patient plasma VWF response after DDAVP (desmopressin: 1-desamino-8-D-arginine vasopressin) administration, and therefore could be clinically relevant.

DDAVP infusion test

DDAVP is a synthetic derivative of the antidiuretic hormone vasopressin that stimulates the release of VWF from the Weibel–Palade bodies of endothelial cells [41,42]. DDAVP has been used to treat the mild form of VWD, and its pharmacological mechanism of action and indications have been reviewed extensively [3,41]. The DDAVP test can be used to assess patient responsiveness prior to the administration of DDAVP for prevention or treatment of bleeding. To test patient response, a therapeutic dose of DDAVP (0.3 μ g/kg body weight) is administered either by subcutaneous or intravenous route, and VWF:Ag, VWF:RCO, and FVIII:C are measured at baseline and at 1,

11 WFH Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual. <http://elearning.wfh.org/> Section 33: VWF Multimer Analysis.

12 WFH Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual. <http://elearning.wfh.org/> Section 32: Factor VIII Binding Assay for Diagnosis of von Willebrand Disease Normandy.

2, and 4 hours after infusion. Assessment at 4 hours after infusion is necessary to identify patients with increased clearance of VWF who are possible candidates for alternative treatments [43].

VWFpp/VWF:Ag ratio

An increased ratio of VWF propeptide (VWFpp) to VWF (VWFpp/VWF:Ag), which is a marker for enhanced clearance of VWF, has been found in patients with VWD and in patients with disseminated intravascular coagulation [44-46]. Different ELISA kits are commercially available for the measurement of VWFpp plasma concentration. However, the assay is relatively new and not widely used in the diagnosis of VWD.

Evaluating the VWFpp/VWF:Ag ratio is a good alternative to the DDAVP test to investigate the rate of clearance of VWF [47].

Issues and considerations in laboratory testing

ABO blood types and aging

ABO blood types have a significant effect on plasma VWF and FVIII concentrations [48]. Subjects with blood group type O have VWF concentrations around 25% lower than individuals with non-O blood types, as a consequence of the higher clearance of VWF related to the presence of O antigens on VWF molecules in comparison to VWF molecules presenting non-O antigens [49].

Both VWF and FVIII levels increase with age. In adults, VWF increases approximately 1% to 2% per year [50].

Pre-analytical issues

The pre-analytical phase covers various procedures, from patient preparation to sample collection, handling, transportation, and storage. The pre-analytical phase is considered the most vulnerable part of the whole testing process, and pre-analytical issues account for about 70% of clinical laboratory errors.

Prior to laboratory testing it is important to consider the clinical history of the patient to detect any pathologic situations known to influence VWF levels (e.g., cardiovascular

diseases, autoimmune disorders, cancer, lymphoproliferative or myeloproliferative diseases, and hypothyroidism). Pregnancy and estrogen/oral contraceptives must be considered as these are associated with elevated VWF along with other coagulation factors. It is important to avoid stress (e.g., anxiety or crying, in the case of frightened children) before blood sampling [51] as it increases acute phase proteins (e.g., VWF and FVIII). Physical exercise is not recommended up to 2 hours before blood withdrawals and subjects should have a minimum of 5 minutes of rest prior to sampling. Other external factors, such as inflammation and surgery, increase VWF plasma levels [51]. Consequently, repeated testing in suspected VWD subjects is needed and low VWF levels must be confirmed on at least two blood withdrawals on separate occasions.

Collection, handling, and transportation of samples

The College of American Pathologists (CAP), as well as the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS), recommend blood collection in 3.2% buffered citrate solution. After collection, it is important to promptly mix the blood by inversion to ensure the distribution of anticoagulant, whereas shaking must be avoided as it induces hemolysis and/or platelet and factor activation. Hemolysed, icteric, and lipemic samples may compromise test results. Before laboratory processing, samples should be evaluated for the presence of precipitates, clot, hemolysis, icterus, and lipemia^{13,14}. However, most fully automated coagulation instruments have a multi-wavelength scanning detection system to detect unsuitable specimens.

Temperature and transportation are key variables in specimen management. Transportation of whole blood at refrigerated conditions is not recommended. Samples should be transported at room temperature (15°C–25°C) in the shortest possible time [52,53]. Indeed, mishandling of whole blood (i.e., transportation at 2°C–8°C) before centrifugation may cause spurious decreases in VWF and FVIII [51].

After withdrawal, whole blood must be processed quickly (ideally within 1 hour) to obtain plasma. Assays for VWF

13 NCCLS H51-A Ed.1 (2002): <https://infostore.saiglobal.com/>

14 NCCLS H21-A4 Ed.4 (2003): <https://infostore.saiglobal.com/>

should be performed on ‘platelet-poor’ or ‘platelet-free’ plasma¹³; to ensure platelet removal, sample tubes may require double centrifugation. Following centrifugation, if assays are to be completed within 2 hours, then sample tubes need to remain capped and at room temperature. If plasma testing is not possible within 2 hours, then samples should be frozen, preferably at or below -70°C , and analysed within 18 months [51]. The release of proteases or platelet membrane particles that can influence VWF tests, can occur during the freezing process if plasma has not been centrifuged properly.

Frozen samples may deteriorate during transportation; rapid delivery is recommended and aliquots should be delivered on dry ice. To avoid formation of cryoprecipitate, frozen plasma samples should be thawed for at least 5 minutes at 37°C in a waterbath prior to testing. After that, samples should be gently stirred.

Classification of VWD

As previously stated, the current classification of VWD is based on partial or complete quantitative (VWD type 1 and 3) or qualitative (VWD type 2) defects of VWF. The previous classification restricted VWD to mutations within *VWF* [54], but this criterion has been relaxed (Table 1). Mutations in other genes could conceivably produce a disorder indistinguishable from VWD that is caused by intragenic *VWF* mutations [34]. The companion monograph, TOH 56, *Molecular Diagnosis of von Willebrand Disease*, provides a detailed review of the genotypic approach to VWD diagnosis [1].

VWD type 1

VWD type 1 is characterized by a partial quantitative deficiency of VWF. The bleeding manifestations in VWD type 1 patients are attributed to a decrease in VWF concentration, not to a selective decrease in the hemostatically effective large multimers or to specific abnormalities in ligand binding sites. The typical laboratory finding in VWD type 1 is reduced VWF levels with normal VWF functional activities compared to VWF:Ag. The proportion of HMWM to the total of VWF multimers is normal or not particularly decreased [34]. VWD type 1 is usually inherited in an autosomal dominant pattern, although severe forms of VWD type 1 due to compound heterozygous mutations have been reported [55].

VWD type 2

VWD type 2 classification is based on specific functional defects of VWF that impair platelet aggregation and/or adhesion, or compromise plasma FVIII survival [34]. Table 2 provides a summary of the qualitative VWF variants (VWD type 2) that are described in detail below.

VWD type 2A

VWD type 2A is characterized by decreased VWF-dependent platelet adhesion due to a selective deficiency of HMWM. VWD type 2A is characterized by a variable relative deficiency of large multimers. The loss of large multimers is associated with decreases in VWF–platelet interactions (i.e., VWF:RCO) and VWF–connective tissue interactions (i.e., VWF:CB) [56] relative to VWF:Ag. VWD type 2A is a heterogeneous group of variants (previously classified as IIA, IIE, IID, and IIC) where each subgroup is due to a different molecular pathomechanism. With the exception of VWD type 2A IIC, the VWD type 2A variants are inherited in an autosomal dominant pattern. At present, the discrimination between different type 2A variants requires intermediate resolution multimer analysis or *VWF* sequencing and these techniques are not widely available [34]. The most common type 2A variants, reported in the previous version of the VWD classification as IIA [57], are characterized by the loss of high and intermediate MWM, that may result from defects in multimer assembly and secretion or from intrinsically increased susceptibility to cleavage by a disintegrin and metalloprotease with a thrombospondin type 1 motif member 13 (ADAMTS-13) [34]. As a consequence of the latter, these variants also show an enhancement of the triplet bands. The second most common variant of VWD type 2A, previously identified as IIE [58], is characterized by a modest loss of HMWM, associated with the absence of the satellite triplet bands due to a reduced susceptibility to ADAMTS-13 proteolysis. A rare variant of VWD type 2A, previously identified as IID [59], is characterized by the loss of HMWM, associated with the presence of ‘odd’ bands in the intermediate resolution multimer analysis. Another rare group of variants of VWD type 2A, previously identified as IIC [58], is characterized by the loss of high and intermediate MWM, associated with the absence of the satellite triplet bands due to reduced susceptibility to ADAMTS-13 proteolysis. As mentioned above, VWD type 2A IIC is inherited in an autosomal recessive pattern.

VWD type 2B

VWD type 2B is due to enhanced affinity of VWF for the platelet GPIIb/IIIa receptor and is characterized by an enhanced RIPA (i.e., platelet agglutination at low concentrations of ristocetin) [30]. The increased affinity of this VWF variant for platelet GPIIb/IIIa leads to spontaneous binding of VWF to platelets *in vivo*, resulting in the formation of aggregates with consequent loss of HMWVWF, and occasionally thrombocytopenia [60]. In addition, the spontaneous interaction between VWF and GPIIb/IIIa accelerates the cleavage of VWF by ADAMTS-13, resulting in further depletion of the HMWVWF [61]. VWD type 2B is transmitted as an autosomal dominant disease.

VWD type 2B shares most of the clinical and laboratory features of PT-VWD, which is a rare autosomal dominant bleeding disorder first described by Weiss *et al.* (1982) [62] and Miller & Castella (1982) [63]. PT-VWD is also characterized by an enhanced RIPA, but in this case the spontaneous interaction between VWF and the GPIIb/IIIa platelet receptor is due to the presence of gain-of-function mutations in the platelet receptor [64].

VWD type 2M

VWD type 2M is characterized by decreased VWF-dependent platelet adhesion not associated with the absence of HMWVWF. Mutations identified in patients with VWD type 2M compromise the interaction of VWF with platelet GPIIb/IIIa or with collagen, but do not impair multimer assembly (hence the M notation). Decreased platelet binding reduces the exposure of VWF subunits to cleavage by ADAMTS-13, resulting in a decrease in satellite bands. VWD type 2M is inherited in an autosomal dominant pattern. Most cases of VWD type 2M have been identified based upon a value for VWF:RCO that is disproportionately low compared to VWF:Ag. First level tests (i.e., FVIII:C, VWF:Ag, and VWF:RCO) for VWD type 2M and 2A are similar and a differential diagnosis is determined using multimer analysis [65]. Several cases of VWD type 2M patients with disproportionately low VWF:CB compared to VWF:Ag have been reported [28,29].

VWD type 2N

VWD type 2N is characterized by a decreased capacity of VWF to bind FVIII. The first description of this variant was in a patient from Normandy (hence the N notation). In particular, FVIII:C is decreased compared to the normal VWF:Ag level, and the VWF multimeric pattern is

normal. Therefore, VWD type 2N can be misdiagnosed as mild hemophilia A, although the former is a recessive autosomal disease and the latter is inherited as an X-linked disease. Differential diagnosis is determined using a solid-phase immunoassay to evaluate the capacity of a patient's VWF to bind FVIII (VWF:FVIIIIB) [38]. As an alternative, genetic analysis of both *F8* and *VWF* can be performed. In contrast to type 2B, type 2M, and most type 2A variants, VWD type 2N is inherited in an autosomal recessive pattern.

VWD type 3

VWD type 3 is characterized by virtually complete deficiency of VWF in both plasma and platelets [66]. VWD type 3 is inherited as a recessive trait, and heterozygous relatives usually have mild or no bleeding symptoms [67,68]. VWF:Ag, VWF:RCO, and VWF:CB values are <1 IU/dl and FVIII:C levels are also very low (<10 IU/dl) [4,69].

Acquired von Willebrand syndrome

Acquired von Willebrand syndrome (AVWS) is a rare bleeding disorder that refers to defects in VWF concentration, structure, or function. AVWS arises in individuals with no previous personal or family history of bleeding. The syndrome is associated with lymphoproliferative or myeloproliferative diseases, cardiovascular diseases, autoimmune disorders, and cancer, and usually presents in the elderly. Laboratory test results for AVWS are similar to VWD and may include decreased values of VWF:Ag, VWF:RCO, and FVIII:C. VWF multimer distribution may be normal, but may lack the HMWVWF, similar to VWD type 2A [70,71]. Increased VWF clearance from plasma is one of the pathogenic mechanisms that have been proposed to explain VWF deficiency in AVWS patients [72].

VWF alloantibodies and autoantibodies

Alloantibodies against VWF are a rare complication, with estimated prevalence ranging from 5.8% to 9.5% of VWD type 3 patients [73-75]. There are no reports of VWF alloantibody development in either VWD type 1 or type 2. Patients with anti-VWF alloantibodies usually lack or have a loss of a hemostatic response to infused VWF concentrates. Patients who have developed an inhibitor, especially those with high-titer anti-VWF alloantibodies, can experience severe or life-threatening anaphylactic reactions when reexposed to VWF. These cases are characterized by complement system activation and immune complex formation [75].

Whereas, anti-VWF alloantibodies are exclusive to type 3 VWD patients repeatedly exposed to VWF, autoantibodies play a role in the pathogenesis of some patients with AVWS, in particular those with lymphoproliferative disorders. In contrast to acquired hemophilia, which is attributable to neutralizing autoantibodies against FVIII, a variety of pathogenic mechanisms can cause structural or functional disturbances of VWF. These include autoantibodies, either interfering with VWF platelet [76,77] or collagen binding [78,79], or increasing VWF clearance from patient plasma [72]. Only 20% of patients with AVWS have autoantibodies against VWF, indicating that available methods may not be adequately sensitive to detect antibodies or that AVWS may not always have an autoimmune basis [5]. Nevertheless, the presence of anti-VWF autoantibodies, together with late onset bleeding diathesis, negative bleeding family history, laboratory findings mimicking VWD, and the presence of an underlying disease usually associated with AVWS, further support the diagnosis of AVWS. Furthermore, the follow-up of levels of VWF and anti-VWF autoantibodies could help physicians to monitor treatment efficacy.

Assay for anti-VWF alloantibody detection

There is no standard laboratory approach for the identification of anti-VWF alloantibodies. Available assays are based on the principle of a mixing study to demonstrate the inhibition of the platelet-dependent function of VWF, although recommendations to evaluate VWF function more broadly (including collagen and FVIII binding) exist [80]. The mixing tests mimic the Bethesda assays for hemophilia inhibitors. VWF activities are evaluated¹⁵ in a mixture of patient plasma and normal pool plasma after 2 hours incubation at 37°C. The titer of anti-VWF inhibitor is calculated by the current dilution of patient plasma inhibiting 50% of normal plasma pool diluted 1:2 compared to control mixture. Negative results by mixing tests cannot completely exclude inhibitors, since alloantibodies might affect non-functional regions of the VWF protein.

Assays for anti-VWF autoantibody detection

The presence of autoantibodies appears to be associated with a more severe bleeding tendency in patients with AVWS [81,82]. Unlike acquired hemophilia, which is always characterized by neutralizing anti-FVIII autoantibodies, in AVWS, autoantibodies against VWF are rarely identified by mixing tests that detect VWF functional activity. These tests might fail to detect low-titer, but clinically important, autoantibodies. Therefore, the mixing studies are rarely useful in the clinical diagnosis of AVWS. Siaka and Tiede described two ELISA assays, using purified VWF bound to the plates, to detect anti-VWF autoantibodies [81,83] independent of their neutralizing activity. Recently, Franchi *et al.* [84] described a new combined approach (an ELISA assay followed by a confirmation assay) to detect anti-VWF autoantibodies.

Diagnosis of VWD in low resource countries

Diagnosing VWD requires more than one assay, and some of them are not available worldwide. There are, however, just a few assays that are essential for VWD diagnosis. Essential first level tests are FVIII:C and VWF:Ag, with VWF:RCo in order to confirm or exclude a diagnosis of VWD type 3. In most cases, these tests are also able to diagnose VWD type 1 and type 2, though they have no power to distinguish between different VWD type 2 variants (i.e., 2A, 2B, 2M and 2N).

FVIII:C can be determined by a one-stage clotting assay based on the APTT assay¹⁶, a technique which can be carried out with very limited resources.¹⁷ VWF:Ag can be measured using the ELISA method with either pre-coated microtitre plates, or coating the microtitre plates in-house.¹⁸ In order to determine the VWF:RCo, ideally laboratories in developing countries would access an aggregometer and perform in-house preparation of platelets, in the

15 WFH Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual <http://elearning.wfh.org/> Section 34: Quantitative Measurement of FVIII Inhibitors.

16 WFH Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual. <http://elearning.wfh.org/> Section 23: Factor Assays Based on APTT (One-Stage Assay of FVIII:C, FIX, FXI, or FXII)

17 One-Stage Assay of Factor VIII (FVIII) – Lab Manual Video. <http://elearning.wfh.org/>

18 WFH Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual. <http://elearning.wfh.org/> Section 30. Von Willebrand Factor Antigen by Elisa (VWF:Ag)

presence of ristocetin. Manual methods also exist for the determination of VWF:RCO using plates that have been washed and fixed in-house, or those available in commercial kits [85]. If none of these can be accessed, the APTT and bleeding time determinations can yield evidence to support or exclude a VWD type 3 diagnosis.

It is very important to exclude the presence of an inhibitor to VWF (anti-VWF alloantibodies) in VWD type 3 patients, especially those who have only been exposed to VWF replacement therapy once or a few times. The presence of an inhibitor may result in a severe, possibly even life-threatening, anaphylactic reaction should these patients be re-exposed to VWF. Therefore, mixing tests that mimic the Bethesda assay constitute an essential test

for the second level of VWD diagnosis, even in the context of restricted resources.

Conclusion

Laboratory diagnosis of VWD can be complex due to external factors and laboratory variables that may influence VWF plasma levels. Good collaboration between clinicians and laboratory specialists is needed to perform an accurate diagnosis. A phenotypic diagnosis of VWD can be confirmed by genotypic characterization. The companion monograph, TOH 56, *Molecular Diagnosis of von Willebrand Disease*, provides a detailed review of the genotypic approach to VWD diagnosis [1].

TABLE 1. Changes to the classification of VWD

PREVIOUS	CURRENT
VWD is caused by mutations at the <i>VWF</i> locus	VWD is not restricted to <i>VWF</i> gene mutations
VWD type 1 includes partial quantitative deficiency of VWF. The multimers distribution and structure of plasma VWF is indistinguishable from normal	VWD type 1 includes partial quantitative deficiency of VWF. Plasma VWF may contain mutant subunits, but has normal functional activity relative to antigen level. The proportion of large multimers is not decreased significantly

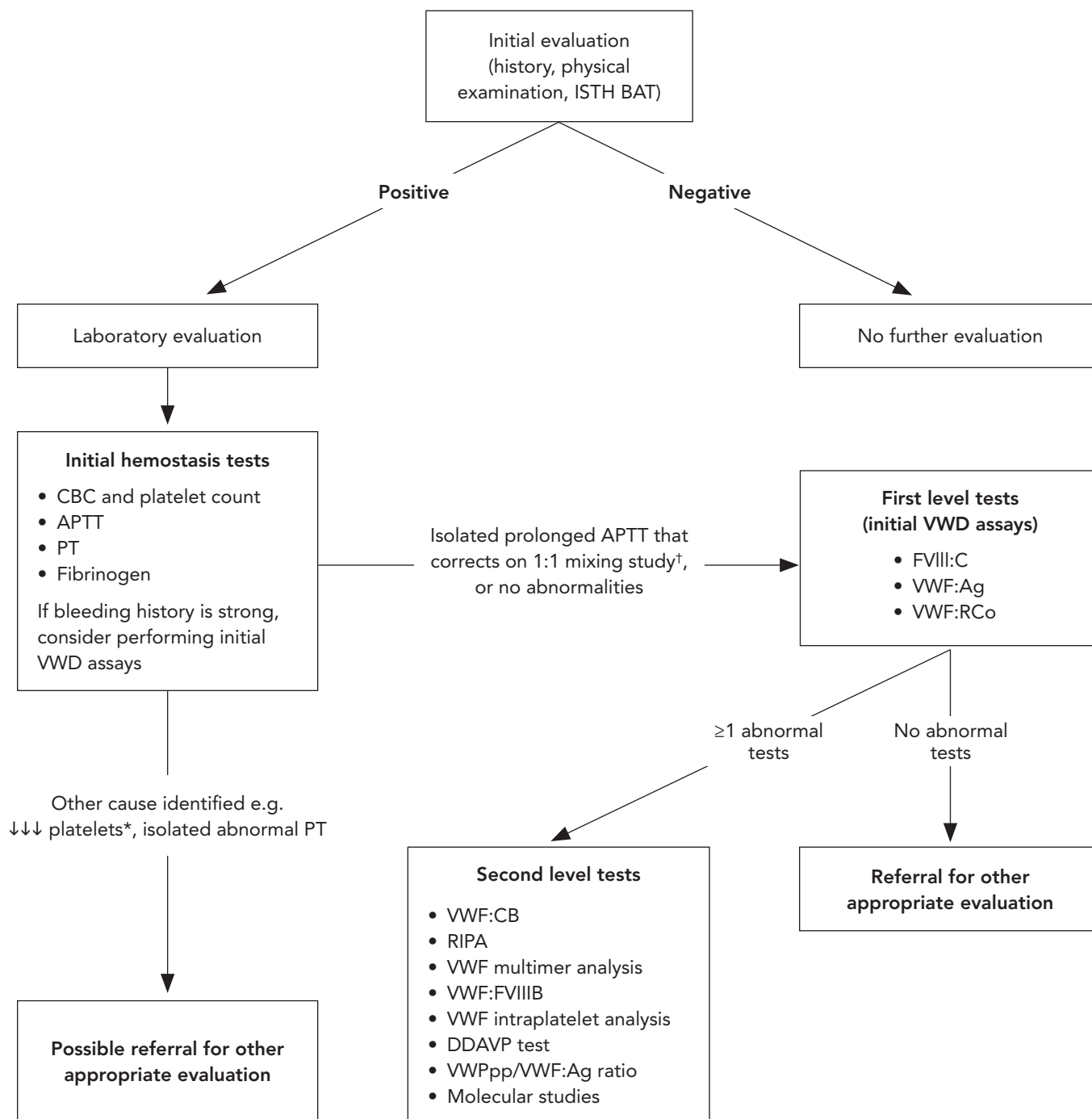
VWD, von Willebrand disease; VWF, von Willebrand factor. (Adapted with permission from Sadler et al., JTH 2006) [34]

TABLE 2. Main features of VWD type 2

VWD type	Multimeric pattern	Inheritance	Main diagnostic hallmark
2A	Variable deficiency of high and intermediate MWM, see specific section below	Variable, see specific section below	Decreases in VWF platelet binding (i.e., VWF:RCo) or VWF-connective tissue interactions (i.e., VWF:CB) relative to VWF:Ag due to loss of high and intermediate MWM
IIA	Loss of high and intermediate MWM, associated with an enhancement of the triplet bands	Dominant	Loss of high and intermediate MWM. Increased susceptibility to ADAMTS-13 proteolysis
IIE	Modest loss of HMWM, associated with the absence the satellite triplet bands	Dominant	Modest loss of HMWM. Reduced susceptibility to ADAMTS-13 proteolysis
IID	Loss of HMWM, associated with the presence of odd numbered multimers between regular oligomers	Dominant	Loss of HMWM associated with the presence of odd bands
IIC	Loss of high and intermediate MWM, associated with the absence of the satellite triplet bands	Recessive	Loss of high and intermediate MWM. Reduced susceptibility to ADAMTS-13 proteolysis
2B	Modest loss or loss of HMWM	Dominant	Enhanced affinity for platelet GPIIb/IIIa. Occasionally thrombocytopenia
2M	Normal multimeric pattern with a decrease in satellite bands	Dominant	Decreased VWF-dependent platelet adhesion not associated with the absence of HMWM
2N	Normal multimeric pattern	Recessive	Decreased binding capacity to FVIII

ADAMTS-13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13; FVIII, factor VIII; GPIIb/IIIa, glycoprotein IIb/IIIa; HMWM, high molecular weight multimers; MWM, molecular weight multimers; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:CB, VWF collagen binding activity; VWF:RCo, VWF ristocetin cofactor activity.

FIGURE 1. Initial hemostasis laboratory evaluation

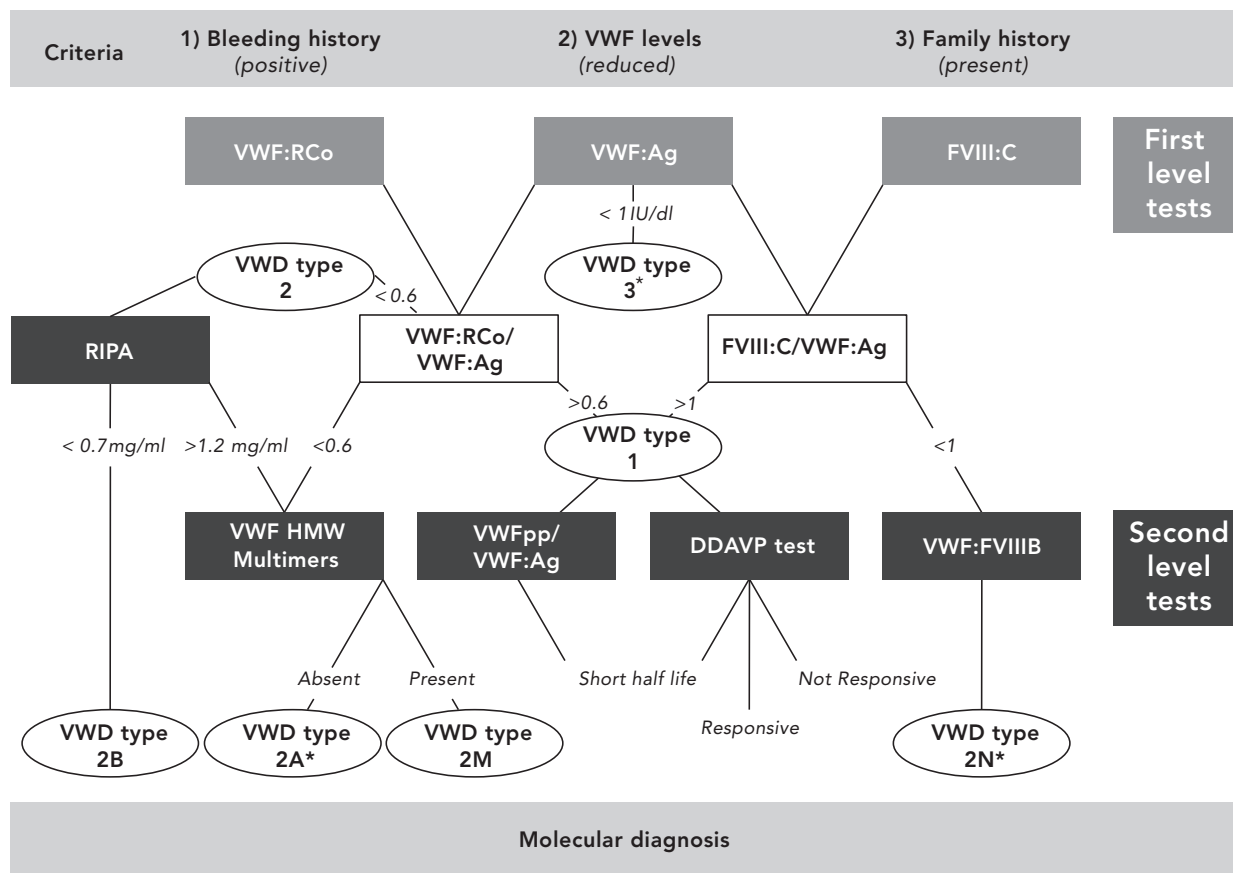


If the initial clinical evaluation suggests a bleeding disorder, the 'initial hemostasis test' should be performed, followed by or along with the next tests ('initial VWD assays') indicated in the algorithm (adapted from Nichols, 2008) [5].

* Isolated decreased platelet count may occasionally occur in VWD type 2B.

† The presence of a FVIII inhibitor is no longer suspected if correction is obtained both immediately and after 2-hour incubation in the APTT mixing study. Investigation of other intrinsic factors and lupus anticoagulant may be indicated.

APTT, activated partial thromboplastin time; CBC, complete blood count; DDAVP, desmopressin (1-desamino-8-D-arginine vasopressin); FVIII, factor VIII; FVIII:C, FVIII coagulant activity; PT, prothrombin time; RIPA, ristocetin-induced platelet agglutination; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:CB, VWF collagen binding activity; VWF:FVIII B, VWF-FVIII binding assay; VWF:RCO, VWF ristocetin cofactor activity; VWFpp, VWF propeptide.

FIGURE 2. Algorithm proposed for the diagnosis and classification of VWD


DDAVP, desmopressin:1-desamino-8-D-arginine vasopressin; FVIII, factor VIII; FVIII:C, FVIII coagulant activity, HMW, high molecular weight; RIPA, ristocetin-induced platelet agglutination; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:FVIIIIB, VWF-FVIII binding assay; VWFpp, VWF propeptide; VWF:RCo, VWF ristocetin cofactor activity.

Adapted with permission from Federici *et al.*, Haematologica 2009 [21].

The initial evaluation of a suspected VWD patient should take into account the personal and familial history of bleeding manifestations. Then, FVIII:C, VWF:Ag, and VWF:RCo levels should be evaluated.

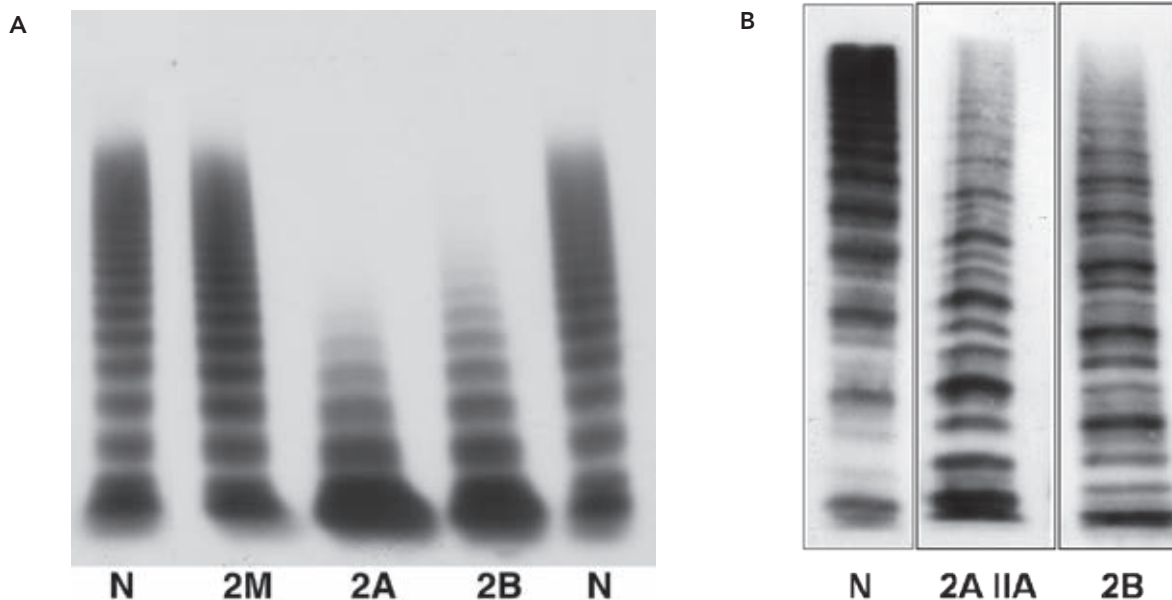
*However, in the case of VWD types with a recessive inheritance (i.e., VWD types 3, 2A1C, and 2N) a patient's family history may be negative.

First level tests: VWD type 3 can be diagnosed in case of undetectable VWF:Ag. FVIII:C is always reduced in VWD type 3 and 2N; it can be reduced or normal in all other VWD types. A proportionate reduction of both VWF:Ag and VWF:RCo with a VWF:RCo/VWF:Ag ratio >0.6 suggests VWD type 1. When VWF:RCo/VWF:Ag is <0.6 , VWD type 2A, 2B, and 2M should be suspected. If FVIII:C/VWF:Ag is <1 , then VWD type 2N or mild hemophilia A should be suspected, whereas FVIII:C/VWF:Ag >1 is usually associated with VWD type 1.

Second level tests: VWD type 2B is characterized by an enhanced RIPA (platelets agglutination at low concentrations of ristocetin, <0.7 mg/ml), whereas RIPA is impaired in VWD type 2A and 2M patients (>1.2 mg/ml). Low resolution VWF multimer analysis can detect the loss of HMWM, allowing the differentiation of VWD type 2A (loss of high and intermediate MWM) from type 2M (normal multimeric pattern). VWFpp/VWF:Ag is increased in VWD type 1 with short VWF half-life. DDAVP infusion tests can identify patients with no biological response, short biological response, or response to DDAVP. VWF:FVIIIIB is crucial to determine a differential diagnosis between mild hemophilia A and VWD type 2N, and should be performed whenever FVIII:C/VWF:Ag is <1 .

The phenotypic diagnosis can be followed by molecular analysis as a further contribution.

FIGURE 3. Comparison of the multimeric structure of plasma VWF from VWD patients and normal individuals. Low (A) and intermediate (B) resolution VWF multimer analysis



Panel A. Lanes 1 and 5 show a normal multimeric structure from a normal control (N). Lane 2 (VWD type 2M) shows a normal multimeric pattern. Lane 3 (VWD type 2A IIA) and Lane 4 (VWD type 2B) show lack of HMWM.

Panel B. Lane 1 (N) shows a normal multimeric structure from a normal individual (control). Lane 2 (VWD type 2A IIA) and Lane 3 (VWD type 2B) show an enhancement of the triplet bands.

HMWM, high molecular weight multimers; VWD, von Willebrand disease; VWF, von Willebrand factor.

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FP, LB, and FS have no interests that might be perceived as posing a conflict or bias with respect to this monograph.

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Authorship

FS wrote the manuscript. FP and LB critically revised the manuscript. All the authors approved the submitted and final version of the manuscript.

Abbreviations

ADAMTS-13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13
APTT	Activated partial thromboplastin time
AVWS	Acquired von Willebrand syndrome
BAT	Bleeding assessment tool
BT	Bleeding time
CAP	College of American Pathologists
CBC	Complete blood count
CLSI	Clinical Laboratory Standards Institute
CV	Coefficient of variation
DDAVP	Desmopressin (1-desamino-8-D-arginine vasopressin)
ELISA	Enzyme-linked immunosorbent assay
FVIII	Factor VIII
FVIII:C	Factor VIII coagulant activity
GP	Glycoprotein
HMWM	High molecular weight multimers
ISTH	International Society on Thrombosis and Haemostasis
IU	International unit
LIA	Latex immunoassay
mAB	Monoclonal antibody
MCMDM-1	Molecular and Clinical Markers for the Diagnosis and Management of Type 1
MWM	Molecular weight multimers
PBAC	Pictorial Blood Loss Assessment Chart
PFA	Platelet function analyser
PRP	Platelet-rich plasma
PT	Prothrombin time
PT-VWD	Platelet-type von Willebrand disease
rGPIbM	Recombinant gain-of-function mutant GPIb α fragments
RIPA	Ristocetin-induced platelet agglutination
SSC	Standardization and Scientific Committee
TOH	Treatment of Hemophilia
UL-VWF	Ultra-large (or unusually large) von Willebrand factor
VWD	von Willebrand disease
VWF	von Willebrand factor
VWFpp	von Willebrand factor propeptide
VWF:FVIII B	von Willebrand factor-factor VIII binding assay
VWF:Ab	Monoclonal antibody binding based von Willebrand factor activity
VWF:Ag	von Willebrand factor antigen level
VWF:CB	von Willebrand factor collagen binding activity
VWF:GPIbM	von Willebrand factor gain-of-function GPIb α binding
VWF:GPIbR	von Willebrand factor ristocetin-triggered GPIb α binding
VWF:RC α	von Willebrand factor ristocetin cofactor activity
WFH	World Federation of Hemophilia
WHO	World Health Organization

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