

Laboratory Diagnosis of von Willebrand's Disease

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Abstract

BACKGROUND: von Willebrand's disease (vWD) is an autosomally inherited bleeding disorder caused by a deficiency or abnormality of von Willebrand factor (vWF). vWF is a multimeric adhesive protein that plays an important role in primary hemostasis by promoting platelet adhesion to the subendothelium at the sites of vascular injury. It is also the carrier of factor VIII (FVIII), thus indirectly contributing to the coagulation process. Bleeding symptoms are usually mucocutaneous and postsurgical with varying severity. The diagnosis of vWD requires a personal and family history of bleeding and confirmation by laboratory analysis involving vWF antigen level, vWF ristocetin cofactor, FVIII activity, ristocetin-induced platelet aggregation, and vWF multimer analysis.

KEYWORDS: von Willebrand's disease, von Willebrand factor.

Introduction

von Willebrand's disease (vWD) is a hereditary abnormality of bleeding disorder that can be dominant or recessive autosomal, and is caused by a qualitative or quantitative disorder of von Willebrand factor (vWF). The revised classification of vWD identifies two major categories, characterized by quantitative (types 1 and 3) or qualitative

(type 2) vWF defects. (1-3) vWD has a prevalence of about 1% in the general population, but the figure for clinically relevant cases is lower. (4) Also, symptoms in individuals with mild-to-moderate vWD may improve with age. In contrast, the prevalence of type 3 vWD with the virtual absence of vWF has been estimated to be between 1 and 3 per million. (5)

vWD is characterized by mucocutaneous and postsurgical bleeding of varying severity. The disorder is very heterogenous, possibly as a result of multiple physiological functions of vWF. (5)

Two main function of vWF are: 1. Facilitating platelet adhesion when there is an endothelial damage by coupling the platelet membrane receptor (Glycoprotein Ib) to vascular subendothelial (6,7). 2. As a carrier protein for factor VIII (F VIII), an important blood coagulation protein (4,5).

Biosynthesis & Structure of vWF

The mRNA is translated as a pre-pro-polypeptide, comprises 2813 amino acids, composed of a signal peptide of 22 amino acids, a large propeptide of 741 amino acids, and a mature vWF subunit of 2050 amino acid residues with a molecular weight of 260 kDa (Figure 1) (4,5). The mature vWF peptide consists of 2050 amino acids organized into 4 domains (A-D). Each domain contains functional residues that mediate vWF biological functions of platelet adhesion and aggregation as well as protection of FVIII from premature inactivation (5,8).

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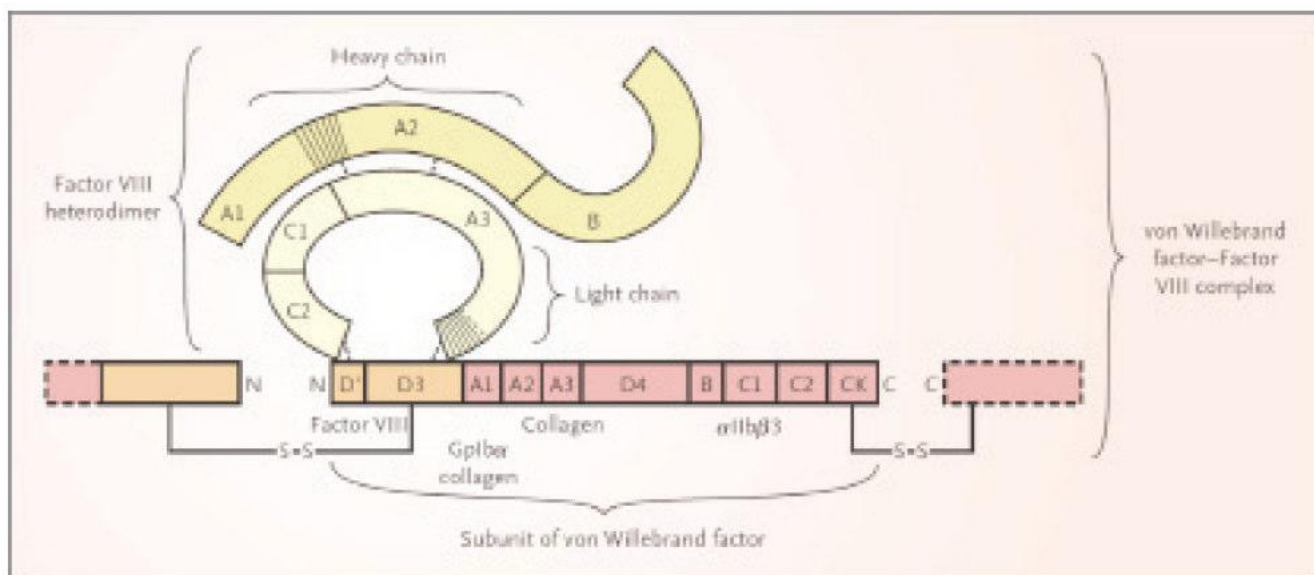


Figure 1. The structure of von Willebrand Factor (9).
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von Willebrand factor circulates as a complex with factor VIII, thereby protecting factor VIII from degradation by the naturally occurring anticoagulant-activated protein C and localizing factor VIII at the site of vascular injury (Figure 1) (9). Each mature von Willebrand factor subunit (domains D', D3, A1, A2, A3, D4, B, C1, C2, CK) dimerizes through disulfide bonds (shown as S-S) near the carboxy terminus (C). Each dimer further multimerizes through disulfide bonds near the amino terminus (N). Factor VIII (domains A1, A2, B, A3, C1, C2) is cleaved before secretion and binds to von Willebrand factor as a heterodimer (9). The acidic region (cross-hatching) of the A3 domain of factor VIII and the carboxy-terminal region on the C2 domain bind (noncovalent bonds are shown as dotted lines) in the amino-terminal region of the von Willebrand factor subunit (domain D'-D3). The A1 and A2 domains of the heavy chain of factor VIII are noncovalently bonded to the A3 domain of the light chain (dotted lines). At the bottom of the figure, areas of the von Willebrand factor subunit involved in the interaction with collagen and platelet glycoproteins Iba (GpIba) and aIIbβ3 are shown (9).

vWF is synthesized at endothelial cells and megakaryocytes and it is released as a series of multimers, including ultralarge forms that are rapidly cleaved to a slightly smaller size (6). Following synthesis, the vWF is secreted by one of two distinct pathways: a persistent,

constitutive release pathway or regulated release from sites of intracellular storage (6,10). The majority of vWF is secreted through the constitutive pathway. vWF is stored in the platelet α-granules, whereas in endothelial cells vWF is stored in the rod-shaped Weibel-Palade bodies (10). Only Weibel-Palade bodies or α-granules in platelets contain fully processed and functional vWF with unusually large multimers, which are usually not found in circulation (6,10).

Functions of vWF

In damaged vessels, vWF binds to the platelet Gp Ib and to subendothelial collagen, serving as a bridge between platelets and subendothelium (6,7). It also bridges between adjacent platelets in vessels with high shear stress leading to small platelet aggregates. In addition to functions in primary haemostasis, vWF also binds circulating factor VIII, independent of the size of the multimers, protecting it from proteolytic degradation in the circulation (10). In the absence of vWF, the half-life of the FVIII in the circulation decreases from 8 to 12 hours to about 2 hours. 4 vWF and factor VIII are released into the circulation, they form a tight, non-covalent complex. Each vWF monomer is able to bind one factor VIII molecule (4,10).

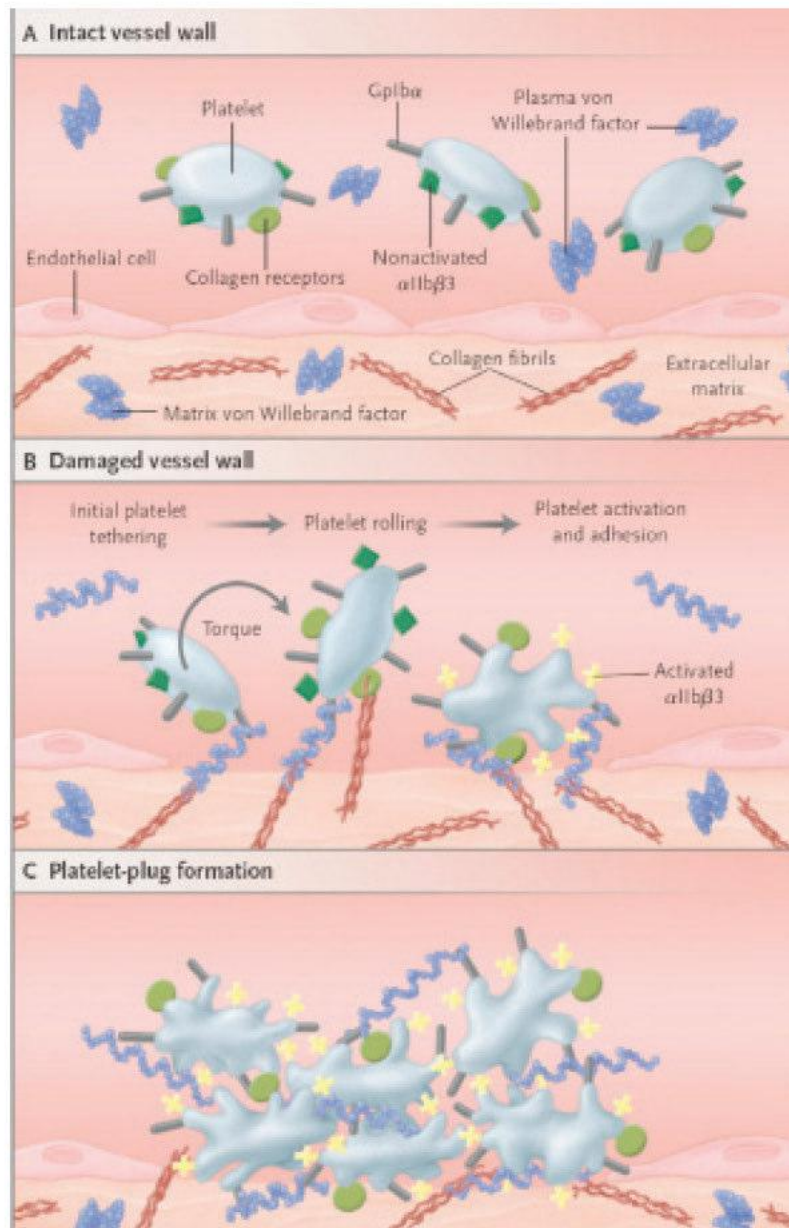


Figure 2. Simplified model of von Willebrand Factor functions in platelet-plug formation (9).
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Classification of vWD

Classification of vWD based on the quantitative and qualitative abnormality of vWF is as follows:

a. Quantitative Abnormality of vWF

Type 1 and 3 is characterized by the quantitative abnormality of vWF. Type 1 is a mild disorder and the most commonly found. In this type, 40% of the members of this group carry the allele of vWD, but has

a normal level of vWF. Type 3 is the most severe form and rarely occurs (10,11).

b. Qualitative Abnormality of vWF

Type 2, which consists of subtype 2A, 2B, 2M, and 2N, involves patients with qualitative abnormality of vWF. Type 2 includes mild to moderate abnormality, and characterized by moderate symptoms (10).

Type 2A is characterized by a decreased function of vWF and a deficiency in multimeric vWF. Type 2B is characterized by an increased affinity of vWF to platelet Gp Ib. Type 2M is characterized by a decreased function of vWF without a deficiency in multimeric vWF. Type 2N is characterized by an abnormality in vWF binding to F VIII (10,12).

Indication of vWF Tests

These tests are usually applied for patients with a suspected bleeding tendency, such as mucocutaneous bleeding (ecchymosis, epistaxis, gastrointestinal haemorrhage, and menorrhage) or post surgical bleeding (6,7), with normal platelet count, prolonged bleeding time (BT), normal prothrombin time (PT) whereas the activated partial thromboplastin time (aPTT) may be prolonged to a variable degree, depending on the plasma F VIII levels. (4)

Laboratory Diagnosis

A patient who presents with either spontaneous or postoperative hemorrhage or a family history of the same should undergo a detailed evaluation of personal and family history, physical examination, and laboratory testing for a bleeding disorder (5). vWD is suspected in a patient with clinical evidence of mucocutaneous bleeding (ecchymosis, epistaxis, gastrointestinal haemorrhage, and menorrhagia) or postsurgical bleeding (6,7). The large number of assays available reflects the wide spectrum of function of vWF, and no test by itself is sensitive or specific enough to diagnose all patients. The correct classification of a patient with vWD is important not only to predict the hemorrhagic risk, but also the treatment will differ substantially on this basis. Patients with type 2 vWD offer the greatest challenge, in terms of both diagnosis and therapeutic management. vWF and factor VIII are both acute phase reactants, increasing with stress, trauma, estrogen treatment, or pregnancy, however these factors are unlikely to obscure diagnosis in patients with more severe quantitative or qualitative defects (6). Classically screening tests, such as bleeding time (BT) and aPTT, are combined with specific tests such as vWF antigen (vWF:Ag) level, vWF activity and factor VIII activity (2).

1. SCREENING ASSAYS

These tests are usually applied for patients with a suspected bleeding tendency and Table 1 summarizes the different steps for diagnosing vWD (4).

A. Bleeding Time

This procedure is performed using a disposable apparatus to make a cut on the forearm, and the result is reported as the time taken for the cut to seal (6,7). There are some disadvantages of bleeding time such as, the relatively low sensitivity and specificity of bleeding time for vWD, its low efficacy as a predictor of surgical bleeding, operator dependence and the inconvenience to the patient. Also bleeding time may be normal in many patients with vWD (4,7). Bleeding time helps to the diagnosis of vWD if it is prolonged. After replacement therapy with vWF, the bleeding time may not be corrected, but surgical bleeding is generally prevented therefore monitoring of bleeding time is not recommended (7,13).

B. Activated Partial Thromboplastin Time (aPTT)

The aPTT is sensitive to deficiencies in factor VIII:C. Especially in patients with mild disease, the diagnosis of vWD may be missed by routine aPTT screening. It is important to know that a normal aPTT will not exclude vWD. If vWD is genuinely suspected, specific and sensitive assays should be performed (6,10).

C. Platelet Count

Usually platelet count is a component of a complete blood count when an automated blood counter is used. Both platelet count and platelet size should be evaluated. Patients with type 2B vWD and platelet type pseudo vWD will often present with a mild thrombocytopenia (7,13).

D. Automated Platelet Function Analyzer (PFA)

This test is used to evaluate the platelet function. In vitro techniques are developed to replace the bleeding time. The PFA-100 (Dade-Behring, Liederbach, Germany) is a new platelet function analyzer to evaluate platelet adhesion and aggregation to collagen in a whole blood assay under high shear conditions. This instrument uses citrate-anticoagulated whole blood flow through a capillary device to mimic the high shear stress conditions that occur in vivo (10,14). The PFA-100 gives a single end-point reading, when the blood flow through the capillary ceases, as a result of platelet adhesion and subsequent aggregation following exposure to platelet agonists coated onto a membrane in a disposable cartridge device. This end-point is

called the closure time. PFA closure times are sensitive to multiple factors, such as; vWF function, platelet count, platelet function, and haematocrit (14,15). The PFA is very sensitive to disturbances in vWF and to the presence of vWD. Reported data suggests that the sensitivity of the PFA-100 for vWD is around 90% (14,15,16,17).

2. CONFIRMATORY LABORATORY TESTS

Factor VIII Procoagulant Activity

The level of factor VIII:C should always be considered when evaluating patients for vWD. It is measured by the ability of patient plasma to correct the aPTT when diluted with factor VIII deficient plasma (10). An assessment of factor VIII:C will help to determine the severity of vWD, as this may suggest a low level of vWF. Factor VIII:C will also help to differentiate type 2N vWD and haemophilia A. However a normal factor VIII:C level is not sufficient to exclude vWD because it may be normal in some type 1 and 2 vWD (18).

3. vWF NON-FUNCTIONAL ASSAYS

Antigen Assay (vWF:Ag)

This is used to determine the total level of vWF protein and can be determined by immunoelectrophoresis, immunoradiometric, or more common method, such as ELISA (enzyme-linked immunosorbent assay) (1,8). Average levels of vWF antigen (vWF:Ag) obtained by each laboratory vary, but usually a level of 45-50 U/dl is the lower limit of normal with a normal range of 50-200 U/dl. The ratio of factor VIII:C to vWF:Ag also varies but generally ranges from 0.7 to 2.2. Plasma levels of vWF:Ag is determined by carbohydrate residues attached to the protein, lower levels are noted in patients with blood type O (18). In order to differentiate this situation from vWD type 1, some laboratories use blood group-specific reference ranges. A low vWF:Ag level alone will provide no information about vWD subtype. Patients with type 2 vWD may have normal vWF:Ag levels, consequently any clinical diagnosis solely on this assay is not recommended (18).

4. vWF FUNCTIONAL ASSAYS

A. Ristocetin Cofactor Activity (vWF:RCo)

The assay for ristocetin cofactor activity (vWF:RCo) quantitatively determines the ability of plasma vWF to bind to normal platelets in the presence of a compound called ristocetin and has some capacity preferentially to recognize HMW forms of vWF (18). Ristocetin, an antibiotic that induces an activation of vWF, then

it binds platelets by the platelet GpIb receptor, similar to the *in vivo* interaction between subendothelial tissue-bound vWF with platelets (1,3,8). This test is the most sensitive and specific assay for vWD. However the assay is a time-consuming procedure and more importantly is the problem of its reproducibility, with both high interassay and interlaboratory variability (10,18). A simpler ELISA alternative of ristocetin cofactor assay has also been described. This employs a monoclonal antibody to the GpIb binding site of vWF for estimating qualitative vWF defects (18).

Recently a sensitive vWF:RCo ELISA assay using a recombinant fragment of the α -subunit of GpIb for the diagnosis of patients with low vWF levels was described. vWF:RCo is sensitive to the loss of both intermediate and HMW multimers. Thus, patients with type 2A, type 2B and type 2M vWD will have lower vWF:RCo than vWF:Ag test results (<0.6) because of the relative absence of high and intermediate vWF molecular weight forms (type 2A), relative absence of HMW vWF forms (type 2B) or functionally defective vWF forms (type 2M) (10,12,18). Ristocetin cofactor activity is also generally used to follow a patient's response to therapy (18).

B. Ristocetin-induced Platelet Aggregation (RIPA)

This assay is a qualitative test that evaluates the rate or extent of agglutination of patient's platelet-rich plasma for sensitivity to ristocetin at various concentrations. At least two or three concentrations of ristocetin are used over a range between 0.5 to 1.5 mg/ml (10). RIPA is dependent on both the level and the function of vWF and platelets present in the test plasma, as well as on the amount of ristocetin. Normal individuals will show platelet aggregation at or above 1.0 mg/ml ristocetin but typically not below this concentration (18,20).

The level of platelet aggregation in type 1 vWD will depend on the level of vWF. Although RIPA test is somewhat insensitive to mild quantitative deficiencies, patients with severe type 1 vWD (<15% vWF) will tend to show no aggregation with 1.0 mg/ml or below ristocetin and no or mild aggregation at 1.5 mg/ml. Patients with severe type 2A vWD will show a similar pattern of no aggregation with up to 1.0 mg/ml ristocetin and no or little aggregation at 1.5 mg/ml (10,18,20). In contrast variants of vWD with 'gain of function' mutations (type 2B and platelet-type pseudo- vWD) show an enhanced aggregation response, typically aggregating with 0.6 mg/ml or below of ristocetin. RIPA is used primarily to assess if there is a gain of function mutation in the patient with vWD. Low dose ristocetin

aggregation cannot differentiate type 2B vWD from platelet-type pseudo vWD, and mixing studies using patient plasma and donor platelets have been devised (18,20). Patients with type 3 vWD will not show platelet aggregation at any ristocetin concentration as well as in patients with Bernard-Soulier syndrome in which platelets are missing the GpIb receptor (18).

C. von Willebrand Collagen-binding Activity (vWF: CB)

vWF contains collagen-binding sites, and collagen-binding activity (vWF:CB) is an important function of vWF to tether to platelets at sites of vascular injury (10). This quantitative and qualitative assay is performed by an ELISA procedure, in which type I or type III collagen is plated into microtiter wells, and the amount of vWF captured is assayed. The vWF:CB estimates the level of vWF, but its greatest strength is its ability to detect vWF of primarily HMW forms which are the most functional and adhesive forms (18,19). Thus, the vWF:CB contributes to the diagnosis of vWD by providing information on the quality of vWF present. Type 2A and type 2B vWD will yield very low vWF:CB values because of the absence of HMW vWF forms (19,20).

D. Factor VIII Binding Assay (vWF: FVIII)

FVIII binding by vWF is performed as an ELISA procedure and also involves a chromogenic assay step.

It is useful to diagnose type 2N vWD and assesses the ability of vWF to bind FVIII. In this ELISA format assay, ELISA platelet wells are coated with antibody to vWF to capture vWF from patient plasma. After washing patient-derived FVIII from the well, the ability of patient-derived vWF to capture recombinant FVIII (r-FVIII) is assessed using a chromogenic assay for FVIII, and the quantity of patient-derived vWF:Ag in the well is quantified by standard ELISA technique (10,18). The calculated ratio of r-FVIII to vWF:Ag is expected to be in the normal range in patients with haemophilia A, whereas it is abnormally low (ratio of less than 0.6) in type 2N vWD (18). Alternatively, a normal level of bound vWF and FVIII with similar proportional values, giving a ratio of around 1 is consistent with a normal sample or may reflect type 2B or 2M vWD; a low level of bound vWF and FVIII though with proportionally similar values, giving a ratio of around 1 is consistent with a type 1, 2A or 2B vWD, and a relative absence of both bound vWF and FVIII will suggest type 3 or very severe type 1 vWD (12,20).

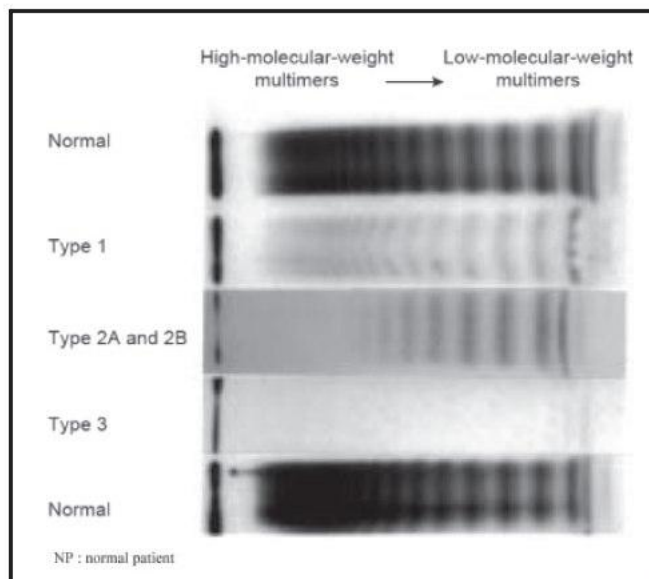


Figure 3. **von Willebrand factor multimers** (From Pruthi RK. A practical approach to genetic testing for von Willebrand's disease. *Mayo Clin Proc.* 2006;B1(5): 679-691. Used with permission.

E. Multimeric Analysis of vWF

This assay detects vWF of differing molecular weights, as well as identifying certain vWF structural abnormalities. It is performed by only a small number of expert coagulation reference laboratories, because of its complexity and cost (1). The procedure involves the agarose gel electrophoresis, and vWF multimers are imaged by immunologic methods such as autoradiography or immunoperoxidase technique (Figure 3) (6,10).

Normal vWF is composed of a complex series of multimers with molecular weight ranging from 800 to 20,000 kDa, which can be analyzed by agarose gel electrophoresis. Low-resolution agarose gels distinguish vWF multimers, which are conventionally indicated as high, intermediate and low molecular weight. In types 1, 2M and 2N vWD all multimers are present, whereas in types 2A and 2B the high and intermediate multimers are missing (4).

It is not appropriate to order vWF multimer assay during the initial vWD investigation process. The procedure is however, recommended when it is necessary to confirm or subtype type 2 vWD that has been diagnosed using clinical criteria and specific quantitative and qualitative diagnostic tests. This method confirms the loss of intermediate- or HMW multimers of vWF as is characteristic of the type 2A and type 2B defects (6,20). However, distinction between type 2A and type 2B vWD is not always possible using multimer analysis alone, necessitating further testing. Results of multimeric analysis may be helpful in predicting response to DDAVP treatment (9,20).

Table 1. Common laboratory findings associated with various types of vWD (8).

	Type 1	Type 3	Type 2A	Type 2B	Type 2M	Type 2N
vWF:Ag	↓ or ↓↓	absent (<0.05 U/mL)	↓	↓	↓	normal or ↓
vWF:RCo	↓ or ↓↓	absent (<0.05 U/mL)	↓↓ or ↓↓↓	↓↓	↓↓	normal or ↓
FVIII:C	normal or ↓	0.01-0.10 U/mL	normal or ↓	normal or ↓	normal or ↓	↓↓ or ↓↓↓
vWF:RCo / vWF:Ag ratio	>0.6	not useful	<0.6	<0.6	<0.6	>0.6
Multimers	normal	absent	loss of high (and possibly intermediate) molecular weight multimers	loss of high molecular weight	normal	normal

↓ slightly reduced ↓↓ moderately reduced ↓↓↓ severely reduced

Table 2. Clinical and laboratory parameters used for vWD diagnosis (4).

<i>Patients at risk of vWD</i>
<p>Clinical history: lifelong mucocutaneous and postoperative bleeding. Symptoms are sometimes present in other family members. Screening tests: prolonged bleeding time (maybe normal); normal platelet count; prolonged PTT (maybe normal).</p>
<i>Diagnosis and definition of vWD</i>
<p>vWF antigen vWF: Ristocetin cofactor activity Factor VIII vWF multimeric structure on low resolution gels</p>
<i>Diagnosis of vWD subtype</i>
<p>Ristocetin-induced platelet agglutination (RIPA) vWF multimeric structure on high resolution gels Platelet vWF content Factor VIII binding assay</p>

Summary

von Willebrand's disease (vWD) is the most common inherited bleeding disorder, caused by mutations in the von Willebrand factor (vWF) gene resulting in deficiency or abnormality of vWF. vWF is a multimeric adhesive protein which plays an important role in primary hemostasis by promoting platelet adhesion to the subendothelium at sites of vascular injury and it is also the carrier of factor VIII (FVIII), thus indirectly contributing to the coagulation process. The revised classification of vWD identifies two major categories, characterized by quantitative (types 1 and 3) or qualitative (type 2) vWF defects. Bleeding symptoms are usually mucocutaneous and postsurgical with varying severity. The diagnosis of vWD requires several laboratory tests involve vWF antigen level, vWF ristocetin cofactor, FVIII activity, ristocetin-induced platelet aggregation, and vWF multimer analysis.

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