Laboratory Diagnosis of von Willebrand Disease

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Abstract
von Willebrand disease (VWD) is a bleeding disorder caused by either quantitative (type 1 and 3) or qualitative (type 2) defects of von Willebrand factor (VWF). No single available test provides comprehensive information about the various functions of VWF, and the laboratory diagnosis of VWD is based on a panel of tests, including the measurement of factor VIII coagulant activity (FVIII: C), VWF antigen levels (VWF: Ag), VWF activity as measured by the ristocetin co-factor activity (VWF:RCo), the collagen-binding activity of VWF (VWF:CB), VWF multimer analysis, ristocetin-induced platelet agglutination (RIPA), the factor-VIII-binding assay of plasma VWF and VWF propeptide levels. Due to the heterogeneity of VWF defects and the variables that interfere with VWF levels, a correct diagnosis of types and subtypes may sometimes be difficult, but is very important for therapy. Furthermore, the RCo assay and the RIPA test are based on platelet agglutination in reaction with the non-physiological antibiotic ristocetin. These tests also have low sensitivity and are difficult to standardise. Therefore, several analyses (tests) are required to diagnose VWD and it is important to be aware of the pitfalls to which these tests are subjected in terms of the diagnosis. In this article, the laboratory diagnosis of patients with type 1, 2A, 2B, 2M, 2N and 3 VWD will be explained by using a modified algorithm that was first proposed by the guidelines for diagnosis and treatment of VWD in Italy.

Keywords
von Willebrand factor, von Willebrand disease, classification, subtypes

von Willebrand disease (VWD) is a bleeding disorder caused by quantitative or qualitative defects of von Willebrand factor (VWF). VWF is a high-molecular-weight (HMW) glycoprotein that plays an essential part in the early phases of haemostasis by promoting platelet adhesion to the subendothelium and platelet aggregation under high shear stress conditions. VWF is also the carrier of factor VIII (FVIII) in plasma, and a deficiency or abnormality of VWF also results in an impairment of blood coagulation. By the non-covalent interaction between VWF and FVIII, FVIII is protected against binding to membrane surfaces and to proteolytic attack by a variety of serine proteases, including activated protein C. In the majority of cases, VWD is a congenital disease that is inherited in an autosomal-dominant fashion. Patients with VWD may have had a mild, moderate or severe bleeding tendency since childhood, usually proportional to the degree of the VWF defect. Inherited VWD has been classified into three types that reflect its pathophysiology. Type 1 VWD is characterised by partial quantitative deficiency of VWF. It is the most common type, with a prevalence of about 80%. The mechanisms involved include reduced synthesis and secretion of VWF or increased clearance of VWF from plasma. The treatment is simple, as 1-deamino-8-D-arginine vasopressin (DDAVP) causes the release of structurally normal VWF from endothelial stores. However, in patients with increased clearance of VWF, DDAVP treatment would not be effective as VWF in plasma is cleared very quickly from the circulation. Therefore, it is important to diagnose patients with an increased clearance rate of VWF.

Type 3 VWD is the least common subtype and reflects a virtually complete absence of VWF. Again, DDAVP treatment is not effective in type 3 VWD. The current treatment of choice for type 3 VWD, as well as most type 2 VWD, is transfusional therapy with plasma-derived FVIII/VWF concentrates.

Type 2 VWD is a quantitative defect that is subdivided into four subtypes (2A, 2B, 2M and 2N VWD). Type 2A refers to variants with decreased platelet-dependent function and is associated with the absence of HMW multimers; type 2B refers to variants with increased affinity for platelet glycoprotein Ib; type 2M refers to variants with decreased platelet-dependent function not caused by the absence of HMW multimers; and type 2N refers to variants with markedly decreased affinity for FVIII. DDAVP treatment is not recommended in patients with type 2 VWD, as it would only increase the dysfunctional VWF.

Platelet-type VWD (PT-VWD) is a rare autosomal-dominant bleeding disorder. The genetic defect is in platelets rather than VWF, and the disease is characterised by abnormally high binding affinity of the platelets to the VWF, similar to type 2B VWD. Therefore, most people with PT-VWD are misdiagnosed as type 2B VWD. However, these may require different therapeutic management, so discrimination is clinically important. The spectrum and severity of VWD is wide, ranging from a few doubtful haemorrhagic symptoms to severe life-threatening bleeding episodes. This is due not only to the heterogeneous VWF gene,
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Figure 1: Algorithm for a Laboratory Diagnosis of von Willebrand Disease

- **Type 1**
  - VWF:Ag
  - Plasma RIPA versus VWF:Ag
  - Type 2N
- **Type 2**
  - VWF:Ag
  - Plasma FVIII versus VWF:Ag
  - Type 2B
- **Type 3**
  - VWF:Ag
  - Plasma FVIII versus VWF:Ag
  - Type 3

which may impair its haemostatic function, but also to the influence exerted by other genes (e.g., those for ABO blood groups). In addition, many acquired conditions — either physiological (stress, pregnancy) or pathological (inflammation) — can induce fluctuations in VWF levels. This highly variable clinical picture and the presence of many different defects in the VWF molecule complicate the diagnosis of VWD. The guidelines for diagnosis and treatment of VWD in Italy propose the use of an algorithm (see Figure 1). We adopted and modified these guidelines in our VWD testing facility.

**Laboratory Diagnosis of von Willebrand Disease**

**Sample Collection**

Blood samples must be collected into tubes containing 0.105M sodium citrate in a ratio of 1:9 with blood. Platelet-poor plasma (PPP) is prepared by centrifugation of whole blood at 2,000g for 20 minutes at room temperature. Samples must be stored immediately after centrifugation in polypropylene tubes at -70°C until analysis. It is important to note that a cryoprecipitate may form if plasma samples are stored at temperatures over -70°C. Cryoprecipitate contains large quantities of VWF, especially HMW multimers. All tests must be performed on original aliquots that were not previously thawed, and plasma samples should be thawed to 37°C before performing the diagnostic tests. Special care should be taken to ensure that no cryoprecipitate is present in the samples; therefore, it must be dissolved before the tests are performed as it will influence the results.

**Screening Tests**

Screening tests for bleeding disorders include a platelet count, a bleeding time, prothrombin time (PT) and activated partial thromboplastin time (APTT), plasma FVIII levels and the blood group of a patient. These tests are usually carried out by a routine coagulation laboratory.

**Laboratory Tests**

The first line of tests includes the VWF concentration in plasma (VWF:Ag), the ristocetin co-factor assay (VWF:RCo) and the collagen binding assay (VWF:CB). VWF:Ag is measured with an enzyme-linked immunosorbent assay (ELISA). An ELISA plate is coated with a specific rabbit anti-human VWF antibody that captures the VWF to be measured. The plasma to be measured is added in 1:50 and 1:100 dilutions in blocking buffer. Afterwards, a rabbit anti-human VWF antibody conjugated to peroxidase is added. This antibody binds to the remaining free antigenic determinants of VWF, subsequently forming a ‘sandwich’. The bound enzyme peroxidase is revealed by its activity in a pre-determined time on the substrate orthophenylenediamine (OPD) in the presence of hydrogen peroxide. After stopping the reaction with a strong acid, the intensity of the colour produced bears a direct relationship to the VWF concentration initially present in the plasma sample. A standard curve of calibrated human plasma is used as the standard against which the patient’s plasma is measured.

The RCo assay is performed with formalin-fixed washed platelets in an aggregometer with software suited for the test. Formalin-fixed washed platelets can be obtained commercially or can be self-prepared from normal platelet-rich plasma (PRP). Washed platelets do not agglutinate in the presence of the antibiotic ristocetin unless normal plasma is added as a source of VWF. The agglutination follows a dose–response curve that is dependent on the amount of plasma VWF added. Usually, plasma concentrations of 1:2 and 1:4 in Tris-buffered saline (TBS) are used. A standard curve of calibrated human plasma is used as the standard against which the patient’s plasma is measured. Another functional assay that more laboratories have been starting to use over the last seven years is the collagen-binding assay of von Willebrand factor.
type 3 or a combination thereof. The amount of bound VWF is determined by using an anti-horseradish peroxidase (HRP)-conjugated VWF antibody. The values are expressed in U/dl or per cent. A standard curve of calibrated human plasma is used as the standard against which the patient’s plasma is measured. This assay has been shown to be sensitive in the discrimination of type 1, 2A and 2B VWD. Casonato et al. demonstrated that the CBA was consistently more sensitive to large- and intermediate-VWF multimer representation than the RCo assay. The decreased values of the CBA in type 2A and 2B patients are more consistent than that of the RCo assay. However, the CBA is insensitive to type 2M VWD patients.

The ristocetin-induced platelet agglutination (RIPA) and the VWF multimer patterns are performed to diagnose the type 2 subtypes. RIPA is measured by mixing different concentrations of ristocetin ranging from 0.2 to 2mg/ml with the patient’s PRP in an aggregometer. The results are expressed as the concentration of ristocetin (mg/ml) able to induce 30% agglutination. RIPA-mixing studies are performed to distinguish between type 2B VWD and PT-VWD. In short, the patient’s PRP and PRP from a control person are centrifuged and gently resuspended to 200x10⁹ platelets per millilitre in the following manner: patient platelets/patient plasma; normal platelets/normal plasma; control platelets/patient plasma; and patient platelets/normal plasma. Different ristocetin concentrations are added to each of the platelet suspensions and the agglutination is measured in an aggregometer, similar to the RIPA assay. PT-VWD is diagnosed when the RIPA-mixing studies confirmed a platelet origin. The multimeric structure of VWF in plasma is determined by a highly sensitive and rapid method originally described by Krizek and Rick in 2000. This method utilises submerged horizontal agarose gel electrophoresis followed by transfer of the VWF onto a polyvinylidine fluoride membrane and immunolocalisation and luminographic visualisation of the VWF multimer pattern. This method distinguishes type 1 from type 2A and 2B VWD. The density of the high-, intermediate- and low-molecular-weight multimers of each multimer pattern is determined using a gel-documentation system. VWF multimer patterns from normal plasma and type 1, 2A, 2B and 2M VWD is shown in Figure 2 and the density graphs in Figure 3.

The capacity of plasma VWF to bind exogenous FVIII is measured with an ELISA. A microplate (Maxisorp, Nunc, Denmark) is coated by incubation for two days at 4°C with a rabbit polyclonal antihuman VWF. After washing with a TBS buffer of 0.1% bovine serum albumin (BSA) and 0.05% Tween, the wells are saturated with TBS containing 3% BSA. Next, 100µl of serial dilutions of plasma from patients and normal pooled plasma are added and incubated overnight at 4°C. Each patient sample is tested in six serial dilutions, the first adjusted to 5% VWF-antigen level. After removal of endogenous FVIII using 350mmol/l CaCl₂ (twice for 10 minutes), 70mU of recombinant FVIII is added to each well. After incubation for two hours at 37°C and washing, bound FVIII is quantified using 1µg/ml of peroxidase-conjugated sheep polyclonal antihuman FVIII. After washing, immobilised VWF is measured using a peroxidase-conjugated rabbit polyclonal antihuman VWF. The colour is developed by addition of OPD and the optical density read at 490nm. Two reference curves are established in parallel. One is for the quantification of immobilised VWF and the other one for the quantification of bound FVIII. For each plasma dilution, the values of bound FVIII are plotted against the amount of immobilised VWF. The slopes of the obtained regression lines reflect the binding capacity of VWF to FVIII.

Diagnosis of von Willebrand Disease

The algorithm used for the laboratory diagnosis of VWD is outlined in Figure 1. A proportional reduction of both VWF:Ag and VWF:RCo with a RCo/Ag ratio ≥0.7 as well as a proportional reduction of both VWF:Ag and VWF:CB with a CB/Ag ratio ≥0.7 suggest type 1 VWD. If type 1 VWD is diagnosed, it is important to determine the clearance rate of VWF; consequently, the VWF:pp assay is used to set up a standard curve against which the patient’s plasma is measured. The VWF propeptide (VWF:pp) level in the patient’s plasma is also measured with an ELISA. Microwell plates are coated with CLB-Pro 35 antibody and incubated with the plasma sample. Subsequently, the wells are washed and the bound propeptide-containing protein is detected with CLB-Pro 14.3 coupled to peroxidase. Calibrators for the VWF:propeptide assay are used to set up a standard curve against which the patient’s plasma is measured.

The density of the different bands is plotted against the relative front (Rf value) of the different lanes.
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VWD) by performing the RIPA-mixing studies. Type 2A and 2M may have a low RIPA (>1.2mg/ml). Multimeric analysis in plasma is necessary to distinguish between type 2A VWD (lack of largest and intermediate multimers) and type 2M VWD (all the multimers are present). The VWF-CB is usually normal in type 2M VWD due to the presence of the HMW multimers, except where a collagen-binding defect is diagnosed in patients with type 2M VWD. In type 1 VWD, the ratio between FVIII and VWF:Ag is always concordant. When this ratio is discrepant with a FVIII level of <20%, type 2N VWD is suspected, and this type of VWD can be confirmed by performing a FVIII-binding assay.

The mechanisms involved in type 1 VWD include reduced synthesis of VWF or increased clearance of VWF from plasma. 20 In contrast, however, the RCo test has a poor sensitivity of 50%, which is difficult to standardise, 21 and lacks a physiological analogue; however, it remains the standard method for measuring VWF activity approved by the Standardisation Committee of the ISTH. Casonato et al. 22 demonstrated that the VWF-CB was consistently more sensitive to large and intermediate VWF-multimer representation than the VWF-RCo. None of the type 1 VWD patients studied showed a greater decrease in CBA than in Ag levels, which was not the case with the RCo assay. The decreased values of the CBA in type 2A and 2B patients were more consistent than those of the RCo assay. The VWF-CB is normal in type 2M VWD patients due to the presence of the HMW multimers. This assists in the diagnosis of type 2M VWD.

The mechanisms involved in type 1 VWD include reduced synthesis and secretion of VWF or increased clearance of VWF from plasma. 20 In patients with increased clearance of VWF, the DDAVP treatment would not be effective, as VWF in plasma is cleared from the circulation very quickly. The ratio between the propeptide and the antigen levels is used as an indication of VWF clearance in these patients.

The RIPA test is necessary to distinguish between type 2B and type 2A VWD. It is also important to carry out the RIPA test with ristocetin concentrations (0.5, 1.25mg/ml). It is sometimes difficult to determine whether the RIPA is enhanced or reduced on only three values. Laffan et al. 23 also found RIPA to be normal in patients with a VWF:RCo value of less than 30%. They found RIPA decreased only in severe forms of VWD. When RIPA is increased and type 2B is suspected, it is important to perform the RIPA-mixing studies to distinguish PT-VWD from type 2B VWF.

VWF multimeric analysis in plasma is necessary to distinguish between subtypes of type 2 VWD. Our method includes rapid processing, simplicity of gel preparation, high sensitivity to low concentrations of VWF and elimination of radioactivity. 24 Type 2N VWD can be suspected in case of discrepant values of FVIII. In typical type 2N cases, the FVIII level is usually less than 20%. 25 The diagnosis of type 2N VWD should be confirmed by the FVIII-binding assay. As VWD is such a complex disease to diagnose, this systematic approach makes the diagnosis of VWD more accurate, which is of vital importance for the treatment of the disease. However, there are pitfalls in this diagnostic process that are due to the limitations in sensitivity, reproducibility and inter-laboratory variability of the agglutination-based RCo and RIPA tests. 26

In conclusion, it is crucial to use a systematic method to diagnose VWD. Each laboratory test only forms one piece of the diagnostic puzzle and therefore it is necessary to put all of the puzzle pieces together for the whole diagnostic picture to emerge.