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Laboratory aspects of von Willebrand disease: test repertoire and options for activity assays and genetic analysis

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Summary

The deficiency or abnormal function of von Willebrand factor (VWF) causes von Willebrand disease (VWD), the most frequent inherited bleeding disorder. The laboratory diagnosis of VWD can be difficult as the disease is heterogeneous and there is a need of an array of assays to describe the phenotype. Basic classification of quantitative (type 1 and 3) and qualitative (type 2) VWD variants requires determination of VWF antigenic levels (VWF:Ag) and VWF ristocetin cofactor activity assay (VWF:RCo), which assess the capacity of VWF to interact with platelet GPIb-receptor. VWF:RCo is essential for identifying, subtyping and monitoring VWD, but the assay is poorly standardised and many assay protocols do not fulfil the clinical need in all situations. This has led to the development of novel activity assays, independent of ristocetin, with enhanced assay characteristics. Results from the first independent clinical evaluations are promising showing that they are reliable and suitable for VWD diagnosis. The qualitative type 2 VWF deficiency can be further divided into four different sub-types (A, B, M and N) with specific assays that explore other activities or size distribution of VWF multimers and the methods are discussed here. However, in a number of patients it may be difficult to correctly classify the VWD phenotype and genetic analysis provides the best option to clarify the disorder through mutation identification.

Keywords

Laboratory diagnosis; von Willebrand disease; ristocetin cofactor; von Willebrand factor activity; genetic testing

Minimum or extensive VWD investigation in clinical routine (G. Castaman)

VWF is a multimeric glycoprotein, synthesized by endothelial cells and megakaryocytes, important for platelet adhesion to the subendothelium and for platelet- -platelet interactions,

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and the specific carrier of factor VIII (FVIII) in plasma. VWD is heterogeneous because molecular defects can occur in more than one of the functional domains of the multimeric glycoprotein [1, 2]. These functions are explored by an array of laboratory assays, but no one reflects the whole spectrum of VWF activities. VWD is classified into three different types: partial or complete VWF quantitative deficiency (Type 1 and 3), and qualitative deficiency (Type 2). Tests for the correct diagnosis of VWD have to explore the most important VWF properties: antigenic level of VWF (VWF:Ag); VWF-platelet GpIb interaction (VWF:RCo); VWF-subendothelium-collagen interaction (VWF:CB); VWF-factor VIII interaction (VWF:FVIIIB); and the capacity of VWF to be organised into multimers. FVIII activity (FVIII:C) is also included in the diagnostic work-up because it reflects the ability of VWF to protect FVIII from degradation and is a useful complement in suspected Type 2N variants.

Prior to laboratory tests, the diagnosis and appropriate classification of VWD requires evidence of a bleeding history, usually also present in other family members. The physician should take into consideration the practical advantage and the patient perspective of a specific diagnosis of VWD in any given patient, avoiding the risk of over-medicalization of patients with dubious or mild bleeding history [3]. Written bleeding questionnaires are increasingly used to improve the quality of data collection and to reduce both intra- and inter-observer variability. When the deficiency of VWF is mild, the risk of bleeding is usually small and thus diagnostic efforts are useful especially for patients with a significant bleeding history [4,5]. To better define what a significant bleeding history is, a bleeding score (BS), accounting for both the number and the severity of the bleeding symptoms, may be useful. It has been suggested that a bleeding score ≥ 3 or ≥ 5 in males and females respectively is a useful cut-off to identify adults in whom it is worthwhile measuring VWF-related activities [4]. The diagnosis of VWD is then based on the presence of reduced VWF:RCo (or VWF:CB) (<40 U/dL), with a further characterization of VWD type based on assessment of VWF:Ag, FVIII and multimer pattern. In general, VWF levels <30 U/dL are strongly associated with a significant clinical severity and the presence of mutations in the *VWF* gene in type 1 VWD [6,7]. However, levels <40 U/dL with other relatives with similar levels is a crucial clue for diagnosis of mild VWD [5], although bleeding history is milder and treatment usually rests on avoidance of anti-platelet drugs and use of antifibrinolytics.

Pediatric cases should be evaluated using less stringent criteria, although a recent study using the bleeding questionnaire adopted for adults showed that the threshold score for a significant bleeding history is ≥ 2 [8]. Table 1 summarizes a practical multistep approach to diagnosis.

VWF:RCo explores the interaction of VWF with platelet glycoprotein Ib/IX/V and is still the reference method for measuring VWF activity. Abnormal VWF:RCo/VWF:Ag ratio (<0.6) usually indicates the presence of qualitative variants (Type 2). VWF:CB is particularly sensitive to VWD variants characterized by the absence of larger VWF multimers [9]. VWF:CB is often used as an alternative to multimeric analysis and VWF:CB/VWF:Ag ratio appears useful for distinguishing between type 1 and 2 VWD [9]. However, rare VWD mutations in the A3 domain (p.W1745C and p.S1783A) with normal multimeric

pattern show a discrepantly low VWF:CB/VWF:Ag ratio [10]. In some of these patients, the diagnosis of VWD could be missed since VWF:RCo level may be borderline.

Ristocetin induced platelet aggregation (RIPA) using patient platelets explores the threshold ristocetin concentration which induces aggregation of patient platelet-rich plasma. Aggregation occurring at low concentrations identifies type 2B VWD cases, in whom desmopressin may cause thrombocytopenia [4]. This test is critical especially when multimeric pattern evaluation is not feasible.

The evaluation of closure time (CT) with PFA-100 (Platelet Function Analyzer) allows rapid and simple determination of VWF-dependent platelet function at high-shear stress, but this system is sensitive and reproducible for severe reduction of VWF, while it has a questionable role in screening for mild VWF deficiencies and type 2N VWD [11].

Type 2N VWD is suspected when the FVIII:C level is disproportionately decreased compared with normal or subnormal. VWF:Ag and VWF:RCo levels [1,2] As a consequence, the FVIII:C/VWF:Ag ratio is reduced (<0.5). The diagnosis relies on measurement of the affinity of VWF to FVIII (VWF:FVIIIB), which is markedly decreased.

Recently, an ELISA test for VWF propeptide (VWFpp) has been shown to provide information on VWF “function” of some VWD variants, since an increased ratio of steady-state plasma VWFpp to VWF:Ag identifies patients with increased VWF clearance [12]. Typically, they show a severe VWF reduction at baseline and a marked but short-lived VWF increase after desmopressin. Thus, measurement of VWFpp in plasma could help identify the pathophysiological mechanism responsible for low VWF, predicting response to desmopressin. To conclude, while VWF:RCo remains a useful screening test for VWD in patients investigated for a bleeding disorder, an array of different tests are required for full VWD characterization and should be used in the presence of a clear bleeding history to help select the best available treatment.

The VWF ristocetin cofactor assay (A. Hillarp)

The most important assay that probes the capacity of VWF to interact with the GPIb receptor on platelets is the VWF:RCo assay. The assay utilizes the antibiotic ristocetin sulphate that promotes the VWF-GPIb interaction under static conditions *in vitro*. Thus, VWF:RCo is a non-physiologic assay but it correlates well with the activity and multimeric distribution of VWF. However, it is well known that the VWF:RCo assay can be difficult to perform and suffers from poor precision and sensitivity, when assay protocols are based on manual visual agglutination or platelet aggregometry. The inter-laboratory CV is usually 30-40% when samples with low VWF content are analysed [13-16] and the limit of detection (LOD) is often as high as 10- 20 U/dl, which makes it difficult to use the test to identify and differentiate between VWD types with low activities.

In recent years, a number of modifications to the VWF:RCo assay have been published involving development of microplate-based assays (i.e. ELISA) or automation on various coagulation analysers. One of the driving forces for the diagnostic industry has been to produce reagents with improved characteristics that can be automated on common photo-

optical coagulation analysers. This allows turbidimetric measurements and faster availability combined with shorter result turnaround-times. The first commercially available automated VWF:RCo assay was performed by Siemens in the late 1990s (BC von Willebrand Reagent) and was restricted to Siemens BCS analysers. This assay gave better precision but the LOD was still unacceptable high. Nevertheless, this opened up local initiatives by users for improvements and applications on different photo-optical analysers. Instrumentation Laboratory is another diagnostic company that developed improved variants of the VWF:RCo assay. Recombinant wild-type GPIb has been coupled to uniform beads making the assay completely platelet-free. The assay comes in two versions, one based on turbidimetric detection and the other on chemiluminescence. Recent evaluations showed that the new assay protocols were precise and suitable for diagnosis of VWD [17-19].

GPIb-binding assays independent of ristocetin

With specific amino acid substitutions in the GPIb receptor it is possible to obtain constructs that bind VWF without need of ristocetin. With these gain-of-function GPIb peptides, novel assays based on the ELISA format or particle-based automated assays have successfully been developed [20, 21]. It appears also that activity is unaffected by a common VWF gene polymorphism known to result in false low VWF:RCo activity [20]. An automated version of this assay type was recently commercialised and has gained popularity in regions where it has been released. From the European external quality assessment (EQA) organisation ECAT, the number of laboratories that use a VWF:RCo assay protocol is steadily declining whereas laboratories using alternative activity assays are increasing. The method group for assays determining GPIb-binding capacity in the ECAT programme is now divided into two activity groups: classical VWF:RCo and novel “VWF activity” and the current number of participants is almost equal in the two groups. The majority use the latex VWF activity assay from Instrumentation Laboratory or the Innovance VWFAc from Siemens. The former is not a “true” activity assay as it relies on a monoclonal antibody that recognizes the functional GPIb-binding epitope on VWF. However, the Innovance VWFAc is an assay based on the gain-of-function GPIb construct that binds VWF without ristocetin. Despite the apparent success, based on the number of users in various EQAs, surprisingly few independent evaluations of the novel assays have been published [22]. Compared with the VWF:RCo assay, the novel assays have several practical advantages that probably explain the fast transition from VWF:RCo to novel activity assays. Moreover, the total number of users in the ECAT VWF module has increased recently and it is likely that the simplicity of the novel assays encourages less experienced laboratories to include an activity assay in their test repertoire.

With the increased diversity of VWF activity assays, other problems arise. First, the diagnostic industry provides assays developed for specific instruments that may not be available in all countries. Second, the assays themselves may not be approved for clinical use in large parts of the world, which is currently the case for the Siemens Innovance VWFAc assay, not yet approved by FDA in the US. As a consequence, it is difficult to suggest general recommendations on VWD testing that include the novel activity assays. Third, the lack of independent evaluation of these assays makes it difficult to ensure that they have acceptable sensitivity and specificity for all VWD types. The current ISTH-SSC

on VWF has appointed a working party that will compare all available activity tests with the VWF:RCo assay and will report during 2014.

In conclusion, the novel VWF activity assays appear to offer significant advantages over the VWF:RCo assay. However, lack of independent evaluations on all VWD types does not yet allow moving away from old assays to new ones. However, it can be argued that simplicity of the novel assays makes it feasible to improve diagnostic capability for VWD in laboratories with poor experience of the VWF:RCo assay.

When is genetic analysis an option in VWD? (A. Goodeve)

For many patients with an initial diagnosis of VWD, the testing described above provides sufficient information to type and subtype the patient's disorder. As treatment may differ with VWD type, it is important to ascertain disease classification, but for a small proportion of patients, specific laboratory tests for VWD do not adequately provide this information. Genetic analysis can help determine the molecular defect(s) responsible for the patient's bleeding and aid classification. Additionally, families with recessively inherited type 3 VWD may request prenatal diagnosis (PND) and ascertaining mutation(s) in an affected individual can facilitate this.

The von Willebrand factor gene (*VWF*) is relatively large spanning 178 kb of genomic DNA with 52 exons encoding the 8.8 kb mRNA and 2813 amino acid VWF monomer. Genetic analysis of *VWF* may include two main processes; 1) analysis of relevant regions of the gene for point mutations using Sanger DNA sequencing or a sequence variant-scanning process such as confirmation sensitive gel electrophoresis followed by Sanger sequencing to identify amplicons with altered behaviour in comparison with wild-type sequence. 2) Analysis of the gene for large deletions or duplications of an exon or more using multiplex ligation-dependent probe amplification (MLPA, MRC Holland) [23] or comparative genomic hybridisation [24].

Type 3 VWD

There is generally little doubt about diagnosis of this severe recessive form of VWD, apart from its discrimination from severe type 1 disease. Mutation analysis in the index case may be requested to determine the causative mutation(s) and to facilitate confirmation in each parent's DNA prior to prenatal diagnosis for a further pregnancy. Use of dosage analysis plus DNA sequence analysis can identify mutations in upwards of 90% of type 3 VWD alleles, but a small proportion of patients remain with only one or no mutations identified following these analyses [25]. mRNA analysis may help to identify missing mutations.

PND can be undertaken on chorionic villus samples obtained at 11-13 weeks gestation or on amniocentesis samples taken at 16-18 weeks, the latter requiring cell culture to obtain sufficient DNA. Analysis of maternal and fetal samples using microsatellite markers in different genomic locations can ensure that there is no significant maternal cell contamination of the fetal sample analysed (e.g. Promega Powerplex 16) [26]. Investigation can be conducted concurrently with fetal mutation analysis.

Type 2 VWD

Mutations in type 2 VWD are predominantly missense changes affecting specific functional domains and can be sought by targeted analysis of exons encoding these domains. This is simplest for type 2B and most complex for type 2A.

Patients may be referred for genetic testing when they have a discrepancy between VWF:RCo and VWF:Ag (≤0.6) indicating reduced platelet binding activity, but without disease type having been clarified. In these cases, exon 28 is the best starting point for analysis.

Type 2A—As further work is undertaken to understand the molecular basis of type 2A VWD, additional mutation locations are identified. The majority of mutations are found in the A2 and A1 domains encoded by exon 28. The D3 domain (exons 22 and 25-27) has recently been recognised as the site of >25% of 2A mutations [27]. Rarer mutations are found in the cysteine knot (CK, exon 52) and D2 domains (exons 11-17) with occasional mutations elsewhere in the gene [28, 29]. Targeted analysis of exon 28 is commonly available, but diagnostic laboratories may not analyse further exons.

Type 2B—This gain of function mutation type is characterised by enhanced RIPA. Conformational changes induced by the mutation result in spontaneous VWF-glycoprotein Ib (GPIb) binding. 2B VWD mutations have a restricted location within the A1 domain encoded by the 5' end of exon 28. Platelet-type pseudo VWD resulting from mutations in *GP1BA* is responsible for a similar phenotype and can be discriminated from 2B by analysis of exon 2 of that gene [30].

Type 2M—Mutations can affect either/both binding to GPIb/collagen. They are largely located in the A1 domain where they impair binding to GPIb and the A3 domain where they may affect binding only to collagen or to both collagen and GPIb Targeted analysis of exons 28-32 identifies most reported mutations [31].

Type 2N—Patients with this recessively inherited VWD type that mimics mild haemophilia A may have reduced FVIII coagulant activity with normal or reduced VWF levels. Mutations reduce the binding of FVIII by VWF thus reducing plasma half-life and level. Missense mutations responsible predominantly lie in the D' domain' encoded by exons 18-20. There are also reports of missense mutations that influence FVIII binding close to the propeptide cleavage site (exon 17) and in the D3 domain (exons 24-25) [29]. Putative 2N patients with no mutation in these *VWF* exons should be investigated for *F8* mutations.

Type 1 VWD

Parallel reductions of VWF:RCo and VWF:Ag indicate likely type 1 VWD. Genetic analysis may be requested to 1) help understand disease pathogenicity in patients that have particularly low VWF levels. Some individuals may be compound heterozygous for two different mutations that contribute to their severe reduction in VWF level, whilst others have a single dominantly inherited mutation; inheritance pattern has implications for family members. 2) Individuals who appear to have a rapid clearance phenotype indicated by

elevated VWFpp/VWF:Ag ratio where the majority of mutations reported lie between exons 25 and 31 [12].

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Table 1

A simplified practical approach to the diagnosis of von Willebrand disease (modified from ref.4)

1. VWD diagnosis should be considered within the context of an appropriate personal and/or familial bleeding history. The use of a standardized questionnaire for history collection is advisable to appreciate the severity of the bleeding tendency
2. Other common hemostatic defects should be excluded by performing a platelet count, APTT, PT and PFA-100 (or bleeding time).
3. If personal and/or familial bleeding history is significant, VWF:RCo assay should be carried out. If not possible, VWF:Ag assay or VWF:CB assay should be performed. VWF:Ag < 3 U/dL suggests type 3 VWD. VWF:Ag and VWF:RCo and FVIII:C should be measured on the same sample to assess the presence of a reduced VWF:RCo/VWF:Ag ratio (a ratio < 0.6 suggest type 2 VWD) or FVIII:C/VWF:Ag (a ratio < 0.6 suggests type 2N VWD, to be confirmed by binding study of FVIII to patient's VWF).
4. If any of these tests is below 40 U/dL, the diagnosis of VWD should be strongly considered.
5. Other family members with a possible bleeding history should be evaluated. Finding another member with bleeding and reduced VWF strongly supports the likelihood of diagnosis.
6. Aggregation of patient platelet rich plasma in the presence of increasing concentrations of ristocetin (0.25, 0.5, 1.0 mg/ml, final concentration) should be assessed. Aggregation at low concentration (≤0.5 mg) suggests type 2B (or platelet type) VWD.
7. Multimer pattern using an intermediate resolution gel should be evaluated. Lack of high molecular weight multimers suggests type 2A and/or 2B. Presence of full complement of multimers suggests type 1 (or 2N, 2M). Absence of multimers in type 3.
8. VWF genetic analysis could be advisable for differential diagnosis of mild haemophilia A vs 2N VWD in males, haemophilia A carriership vs 2N VWD in females and for type 2B VWD vs platelet type-VWD.
9. VWF genetic analysis may be required for prenatal diagnosis in type 3.