Laboratory diagnosis of von Willebrand disease

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Summary
Over the last decade, considerable progress has been made in the laboratory diagnosis of VWD. Precise, sensitive and automated VWF : Ag assays became widely available. The VWF : RCo performance was improved to a certain degree. However, the sensitivity, precision and general availability of automated applications is not yet optimal. Nevertheless, this type of assay is still recognized as superior to other activity assays, e.g. VWF : CBA assays and antibody-binding „activity“ assays, for the detection of defects in VWF function.

A decision limit of either 30 or 40 IU dl⁻¹ VWF (VWF : RCo or VWF : Ag) is recommended for a diagnosis of type 1 VWD. Type 2 VWD can be differentiated from type 1 by calculating the VWF : RCo/VWF : Ag ratio.

Improved and easier to perform multimer analysis and genetic testing are beginning to facilitate the diagnosis of the VWD type 1, 2A, 2B, 2N, 2M or 3. Within type 1 or 2, a decreased VWF survival can be detected by the VWFpp assay and its ratio to VWF : Ag.

A new type of VWF activity assay, based on the binding of VWF to a GPIb-box-fragment, has been developed. One assay variant does not need ristocetin as a cofactor anymore. The performance investigations presented so far are very promising. It is probable that these GPIb-box-binding assays will detect functional VWF defects as the VWF : RCo assay, but are much more sensitive and precise. Fully automated applications on routine analyzers are expected to be commercialized soon.

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Whenever screening assay results (PT, aPTT, complete blood count, fibrinogen, thrombin time, PFA-100® system) have led to the suspicion of von Willebrand disease (VWD), more specific assays as well as information on the patients bleeding history are required (1).

- In the first line of laboratory testing, either the activity or the antigen level of von Willebrand factor (VWF) should be significantly decreased for the diagnosis of VWD. Only in cases of the very rare type 2N (defective FVIII binding), VWF antigen and activity are sometimes normal while factor VIII clotting activity is clearly low. The complete absence of VWF activity or antigen allows the immediate diagnosis of VWD type 3.

- In second-line testing, more assays are needed to identify the different types 1, 2A, 2B, 2M, 2N and 3 of VWD which is essential for an appropriate therapeutic decision. The following assays are well established and accepted for subtyping purposes:
  - RIPA,
  - multimer analysis,
  - VWF : FVIIIB,
  - VWF : CBA,
  - ratio VWF : RCo/VWF : Ag and
  - VWF assays with sufficient sensitivity to detect type 3.

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Other assays are relatively new and/or their role and usefulness is not yet clearly defined: antibody-binding „activity” assay, GPIb/β-binding assays, VWFp p, VWFpp/ VWF:Ag ratio, genetic testing, flow-based assays, assays for detecting VWF antibodies, flow cytometry assays.

From our point of view, the following assay improvements and new assay developments represent the most relevant progress in the laboratory diagnosis of VWD over the preceding decade.

- Improved assays: automation, standardization and technical improvement of existing assay principles
  - VWF:RCo,
  - VWF:Ag,
  - antibody-binding „activity” assay,
  - multimer analysis,
- new assays
  - GPIb/β-binding assays,
  - VWFpp and
  - genetic testing.

Improved assays

VWF:RCo

The ristocetin cofactor assay uses stabilized platelets and ristocetin as a cofactor to activate VWF. The platelets agglutinate as a function of the VWF activity and functionality in the sample. This type of assay is the most widely accepted assay to screen for quantitative and functional VWF defects (1–3).

Originally, the reaction was observed visually on glass plates. Many laboratories prepared stabilized platelets. Later, commercial platelet reagents and assay applications on aggregometers were developed. In the late 1990s, a platelet-containing reagent, which already includes ristocetin, was adapted to fully automated haemostasis analyzers (4, 5). Such an automated analyzer has many advantages:

- fewer manual working steps,
- high throughput,
- measurement of VWF: RCo, VWF: Ag and FVIII:C on the same instrument, automated VWF: RCo/VWF: Ag ratio calculation,
- one calibrator for all these assays,
- improved CVs.

However, the lower limit of the measuring range as well as the precision at very low levels of VWF are still not satisfactory. Both assay properties could probably be improved by using an increased sample volume for the low measuring range (6).

Both, the VWF: RCo and the VWF: Ag assay can be used to diagnose type 1 VWD, the suggested decision limit is either 40 IU dl⁻¹ (7) or 30 IU dl⁻¹ (1). Nichols et al. (1) suggest applying 30 IU dl⁻¹ of VWF: RCo level also as a cutoff for type 2 A, B and M.

Although comprehensive studies on sensitivity and specificity are still missing, the ratio VWF: RCo/VWF: Ag is now recommended for discriminating between type 1 and type 2 VWD by using a limit of 0.5–0.7 (1).

VWF: Ag

This immunoassay measures the concentration of the VWF protein in the sample. Many ELISA assays and several particle-enhanced agglutination assays (latex assays, immunoturbidimetry) are commercially available. Most laboratories prefer automated methods on hemostasis instruments. Therefore, and because of an excellent overall performance, particle-enhanced assays on automated instruments have replaced the ELISAs in most routine laboratories. Single determinations, very stable and liquid reagents and very precise measurements are state of the art.

When using a particle-enhanced immunoassay, the interference of rheumatoid factors should be considered as a possible cause for implausible results (8).

For the diagnosis of type 3 VWD, a virtual absence of VWF should be measured. In this case, because of the required sensitivity of 1–3 IU dl⁻¹, an ELISA method might still be necessary.

Antibody-binding „activity” assay

One company has developed a particle-enhanced agglutination assay by using an antibody against VWF. The epitope of the antibody is located in the area of the binding site of VWF to GPIbα (domain A1).

Since VWD type 2 mutations often induce structural changes in domain A1, this antibody-based assay can detect many functional VWF defects. The overall correlation of the assay to VWF: RCo is good. Nevertheless, it is not clear if and how many functional defects might remain undetected or be falsely classified as type 1. One study (9) published a level of 21% (4 of 19), while another study (10) cited 5% (2 of 38), for the false classification of type 2 VWD samples as type 1 in comparison to the VWF: RCo result.

The application of the assay on haemostasis analyzers is fully automated and precise in the normal range, but the lower end of the test range remains unsatisfactory (19–21 %, package insert 01/2005) and a high level of inaccuracy up to 17 % at low levels of VWF was sometimes observed (9, 11).

The antibody-binding „activity” assay cannot replace the VWF: RCo assay. It is nevertheless a practical and precise alternative for laboratories not equipped with the instrumentation for automated VWF: RCo assays. Possibly, a screening strategy in combination with VWF: RCo might make sense (12). However, some false high results of VWD type 2 samples might occur.

Multimer analysis

Agarose gels are used to separate the different multimer sizes of the extremely large VWF protein. Originally, radioactive staining was used to visualize the different bands. The technique was complicated and only performed by specialized centers. Today, luminescence detection methods are available. They allow in combination with medium or high-resolution gels and digital imaging a practical, sensitive and precise analysis of the multimer pattern (13). Recently, a further improved method using HSFD (high-sensitivity fluorescence detection) was published (14). These technical improvements make the multimer analysis more practicable, feasible not only for highly specialized laboratories, and very helpful for subtyping decisions.
In the MCMDM-1VWD study, 15% of samples formerly classified as type 1 could be reclassified as type 2 on the basis of a detailed multimer analysis (15).

**New assays**

**GPIbα-binding assays**

The most important change over the next few years in the diagnosis of von Willebrand disease is expected to come from a new type of activity assay. It uses a recombinant GPIbα fragment instead of native, stabilized human platelets as for the VWF:RCo assay. The first assays were developed in an ELISA format. The ELISA well is coated with an anti-GPIbα antibody. After capture of the GPIbα, the sample and ristocetin is added. Bound VWF is detected by an anti-VWF antibody and a subsequent coloring reaction (16–18). The results for all types of VWD of GPIbα-binding assays and the classical VWF:RCo turned out to be very similar. The most important advantage of the ELISAs is the possibility to measure very low VWF activities (e.g. 0.1 IU dl-1) (18).

The assay principle was subsequently transferred to particle-enhanced agglutination assays and to a chemiluminescence assay which allows the development of fully automated instrument applications. Initial data from such applications were presented at the ISTH meeting in Boston (19, 20). Total CVs of 2–4% and a detection limit of 3.5 IU dl-1 were observed for the agglutination assay and total CVs of 6–7% and a lower limit of the linearity range of 0.5 IU dl-1 were measured by using the chemiluminescence assay. Good correlations to the platelet VWF:RCo assay, also by using VWD type 2 samples, were observed for both assays.

Ristocetin is still used by the assays described. It is known to be a problematic substance and very recent data implicate false low VWF:RCo results in the presence of ristocetin if the polymorphism P1467S or D1472H are present in the VWF molecule (21).

By using a recombinant rGPIbα with two gain-of-function mutations included, ristocetin-free GPIbα-binding assays were developed (21, 22). A particle agglutination assay format on a fully automated hemostasis system performed very well (calibrated range: 5–150% (IU dl-1), within-device CVs: 2–3%). Good correlations of VWF:RCo and GPIb-binding assay results for normal and pathological samples, including type 2 VWD, were observed (23, 24).

We expect that the automated GPIbα-binding agglutination assays, once available with precise and sensitive applications on routine haemostasis analyzers, will quickly replace a significant portion of the classical, platelet-using VWF:RCo assay.

**VWFpp**

Assaying the propeptide of VWF (VWFpp) and calculation of the VWFpp/VWF:Ag ratio can be used to detect VWD subtypes characterized by a decreased VWF survival, e.g. type 1, type Vicenza or a subgroup of 2A VWD (25, 26). Such a finding can influence the therapeutic approach and is therefore of special importance. Two ELISAs for VWFpp are commercially available and international standardization is in progress.

**Genetic testing**

Genetic testing in von Willebrand disease was recently controversially discussed by two authors (27, 28). Although immense progress has been made with respect to technology and costs, there is no general agreement if and under which conditions genetic testing should be performed. However, several specific indications for testing are suggested and the significance of genetic testing in von Willebrand disease is certainly increasing. This applies to the following issues:

**Genetic counseling**

The most severe form of VWD, type 3 VWD is recessively inherited. The diagnosis of patients with VWD type 3 is unequivocal in almost all cases merely by measuring VWF:Ag. However, neither quantitative nor qualitative VWF parameters can reliably identify heterozygous carriers of VWD type 3 mutations. This is further underlined by the finding that mutations causing a relative decrease of VWF in VWD type 1 are not just heterozygous VWD type 3 mutations but rather represent a molecular entity of its own (29).

Therefore, molecular genetic testing in VWD type 3, first of the index patient and subsequently of potential carriers seeking respective advice, is the most important application.

**VWD type 2**

In some cases the classification of patients with VWD type 2 may be difficult, since the intra-individual variation is sometimes quite remarkable. In such cases definition of the molecular background may help to find the correct diagnosis.

**Conclusion**

Considerable progress has been made in the laboratory diagnosis of von Willebrand disease in the recent years.

- The most widely accepted activity assay VWF:RCo was automated and improved but has not yet reached a really satisfying level of quality and availability.
- A very promising successor assay is based on recombinant GPIbα binding and will soon be commercially available.
- Genetic testing and multimer analysis as well as the VWF propeptide assay increasingly support the differentiation of von Willebrand types.

**Conflict of interest**

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