Personalized treatment for von Willebrand disease by RNA-targeted therapies

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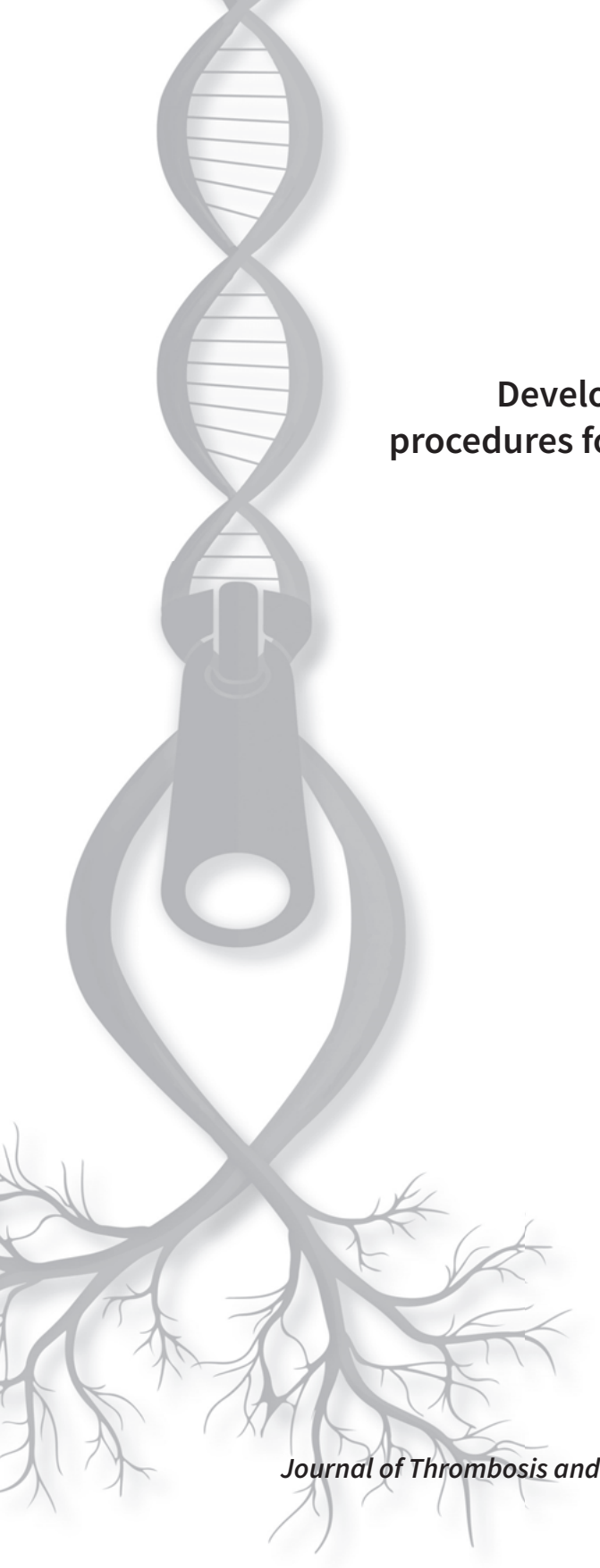

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Developments in the diagnostic procedures for von Willebrand disease

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Abstract

Von Willebrand disease (VWD) is the most common inherited bleeding disorder but its diagnosis can be challenging due to the heterogeneity of the disease. VWD is mainly associated with mild mucocutaneous bleeding, although there are more severe phenotypes with bleeding from the gastrointestinal tract or even the joints. Also, surgical interventions and trauma may lead to critical bleeding events. These bleeding episodes are all related to quantitative or qualitative defects of von Willebrand factor (VWF), a multimeric glycoprotein produced by endothelial cells and megakaryocytes, which mediates platelet adhesion and aggregation and binds factor VIII (FVIII) in the circulation. This review describes the diagnostic procedures required for correct diagnosis. Accurate diagnosis and classification is required for proper treatment and counseling. Assessment of bleeding starts with the medical history. After a positive bleeding or family history, subsequent laboratory investigations will start with a panel of standard screening tests for hemostatic defects. Patients suspected of having VWD will be tested for plasma VWF antigen levels, the ability of VWF to bind platelets and FVIII activity. When VWD is confirmed, a set of subtyping tests can classify the patients as VWD types 1, 2 (A, B, M or N) or 3. The performance of some additional assays and analyses, such as VWF propeptide measurement or genetic analysis, may help in identifying the pathological mechanism behind certain defects or can guide in the choice of treatment.

Introduction

von Willebrand disease (VWD) is the most common inherited bleeding disorder and is mainly associated with mucocutaneous and postoperative bleeding. The underlying cause of these symptoms is a qualitative or quantitative defect of the von Willebrand factor (VWF) protein. According to population studies, about 1% of the population shows defects of VWF fitting the diagnostic criteria; however, only about 0.01% of the population is reported to develop clinically significant bleeding.[1-5] The actual prevalence probably lies somewhere in the middle, with many people having undiagnosed VWD-related bleeding.

VWF is a large multimeric glycoprotein produced in endothelial cells and megakaryocytes. Endothelial cells are the main source of circulating VWF and store VWF multimers in cigar-shaped vesicles called Weibel-Palade bodies (WPBs).[6] Signals released after vascular injury stimulate the secretion of the contents of WPBs, including VWF, into the circulation. There, VWF unfolds under flow into ultra-long VWF (UL-VWF) strings that attract platelets by binding the platelet glycoprotein Ib (GPIb) receptor to the A1-domain of VWF. The multimeric size, and therefore the platelet binding activity of VWF, is regulated by cleavage of the UL-VWF strings by ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13).[7] In the circulation, VWF has a half-life of about 12 hours and functions, apart from its platelet binding activity, as a binding protein and stabilizer of coagulation factor VIII (FVIII).[8,9]

Defects in VWF synthesis, storage, secretion or clearance, or a combination thereof, can lead to a deficiency of plasma VWF.[10-15] This can be either a partial deficiency leading to VWD type 1 or a (virtually) complete deficiency causing VWD type 3. Qualitative defects of plasma VWF cause VWD type 2, which can be subcategorized into types 2A, 2B, 2M and 2N.[16] VWD type 2A is characterized by a decreased ability of VWF to bind platelet GPIb due to a decreased level of high-molecular-weight (HMW) VWF multimers. In VWD type 2B, a gain-of-function mutation in the GPIb binding site of VWF leads to the spontaneous binding of VWF to platelets without prior activation of VWF. The spontaneously formed VWF-platelet aggregates are quickly cleared, resulting in a variable degree of thrombocytopenia and consumption of HMW VWF multimers. In VWD type 2M, usually a loss-of-function mutation in the region of GPIb binding leads to a decreased binding affinity for platelets. However, other defects, such as an isolated defect in collagen binding, are also classified among VWD type 2M. Binding of FVIII to VWF is decreased in VWD type 2N, as a result of mutations in the FVIII binding site on VWF. Most VWD types 1 and 2 are inherited as an autosomal dominant trait with mainly dominant negative missense mutations in *VWF* as the causative factor.[17] VWD type 2N and type 3 are inherited as an autosomal recessive trait mainly caused by homozygous or compound heterozygous *VWF* mutations, although co-dominant *VWF* mutations are also observed (Table 1).[18]

Table 1. Classification and etiology of VWD

Type	Disease mechanism	Inheritance	Genetic defects*
1	Partial quantitative deficiency of VWF	Autosomal dominant	Missense mutations (85-90%), null alleles (10-15%), variable penetrance
2A	Decreased VWF-dependent platelet adhesion due to a selective deficiency of HMW VWF multimers	Autosomal dominant Autosomal recessive	Missense mutations, mainly in D3, A2, and CK domains Missense mutations in propeptide
2B	Increased affinity of VWF for platelet GPIb	Autosomal dominant	Missense mutations in A1 domain
2M	Decreased VWF-dependent platelet adhesion without a selective deficiency of HMW VWF multimers	Autosomal dominant	Missense mutations in A1 domain
2N	Decreased binding affinity of VWF for factor VIII	Autosomal recessive	Missense mutations in D' and D3 domains
3	Virtually complete deficiency of VWF	Autosomal recessive	Mainly null alleles, often consanguinity

* The majority of the genetic defects are found in the indicated domains, however there are exceptions. VWF, von Willebrand factor; HMW, high molecular weight; GPIb, glycoprotein Ib.

Accurate diagnosis and classification of VWD patients is of importance in the choice of treatment and in counseling regarding inheritance. However, a good diagnosis of VWD is difficult due to the heterogeneity of the disease and several different diagnostic tests are required. An overview of the diagnostic tests required can be found in Fig. 1. In this review we will describe and discuss the diagnostic approach from a first suspicion of a patient having a bleeding disorder to the specific sub-classification of VWD. Finally, additional tools will be described that can help to identify the pathophysiologic mechanisms behind VWF defects.

Assessment of bleeding history

The assessment of bleeding history is the first and possibly the most important test in the analysis of a suspected bleeding tendency, as laboratory evaluation will only be initiated after the suspicion has arisen. In general, hemostatic defects are more likely when someone has multiple bleeding symptoms or bleeding at different sites. The severity of the bleeding disorder can be judged from the frequency of bleeding and the age at which the bleeding problems started. The nature of the bleeding symptoms is also important and may already be suggestive of specific defects of hemostasis. In VWD, mucocutaneous bleeding is particularly present: bruises, bleeding from minor wounds, gum bleeding, epistaxis, menorrhagia, and bleeding from the gastrointestinal tract. Apart from spontaneous bleeding, patients with VWD also experience bleeding after procedures such as surgery or tooth extraction and at hemostatic challenges such as childbirth or trauma. The severity of VWD ranges from very mild with bleeding after major procedures only, up to spontaneous bleeding, including muscle and joint bleeding, in the most severe cases.

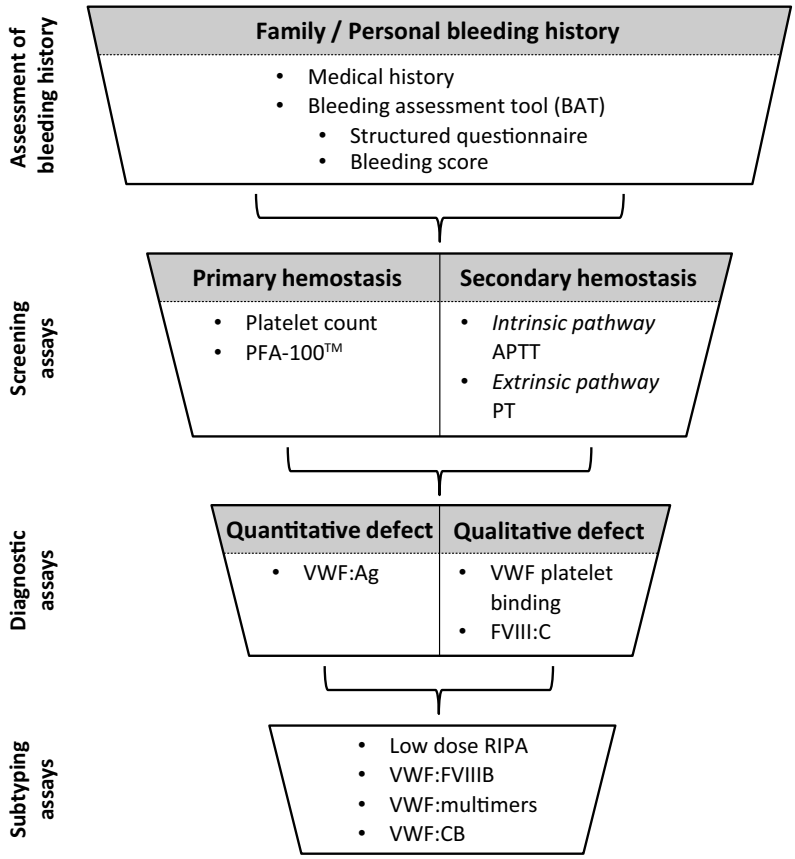

Figure 1. Overview of the phases in diagnosis of von Willebrand disease (VWD). Each phase describes the diagnostic tests required for diagnosing VWD. In the assessment of the bleeding history, hemostatic defects can be identified by the medical history, which can be guided by the bleeding assessment tool. Screening assays are performed to exclude other hemostatic disorders. Suggested VWD is confirmed by a set of standard diagnostic tests and the subtyping assays can categorize VWD into VWD types 1, 2 (A, B, M and N) and 3. PFA, platelet function analyzer; APTT, activated partial thromboplastin time; PT, prothrombin time; VWF:Ag, VWF antigen; FVIII:C, FVIII activity; RIPA, ristocetin-induced platelet aggregation; VWF:FVIIIB, VWF-FVIII binding assay; VWF:CB, VWF collagen binding.

To improve bleeding history as a diagnostic tool, structured questionnaires and quantitative scoring systems have been developed.[19-21] The bleeding score most often used, and frequently referred to as the 'Tosetto' bleeding score, was validated in a European population of 417 patients with a mild form of VWD, 295 unaffected family members of those patients and 195 healthy controls.[21] The scoring system evaluates 12 different bleeding symptoms and the symptoms are scored from -1 (e.g. no bleeding after multiple tooth extractions) to +4 (e.g. requirement of blood transfusion after tooth extraction) depending on the presence and severity of the symptom. This leads to a cumulative score reflecting the severity of the bleeding history. Recently, more condensed, pediatric and self-administered versions

of the bleeding score have been developed.[22-24] Also, a web-based version of the bleeding assessment tool, endorsed by the International Society on Thrombosis and Haemostasis, has been published (ISTH-BAT; https://bh.rockefeller.edu/ISTHBATR/). These bleeding scores are evidently valuable in research and allow quantitative comparison between different types of VWD patients and between cohorts of patients. However, the individual diagnostic value of the scores is limited: the scores are age dependent (increasing with age); the scores record the severest bleeding event only (lacking information on frequency); the symptoms are not diagnosis specific; and the questionnaires are time consuming. The main clinical value of the bleeding assessment tools may be in excluding a bleeding disorder. We found in a prospective study using a simplified version of the tool that a normal bleeding score (≤3) had a negative predictive value of 99.2%. The positive predictive value was limited when assuming a low prevalence of the bleeding disorder in the general population. However, the positive predictive value was reasonable (about 70%) among patients referred for evaluation of an abnormal clotting test or family investigation.[23]

Screening assays for bleeding disorders

When the bleeding or family history of a patient suggests the possible presence of a bleeding disorder, a series of standard hemostatic screening assays will usually be performed. In the context of this review on the diagnosis of VWD it should be remembered that those assays are not intended for the diagnosis of VWD per se, but are performed to exclude alternative diagnoses.

Screening tests for defects in primary hemostasis include platelet count and functional tests such as bleeding time (BT) or platelet function analyzer (PFA-100™), which has replaced BT measurements in most laboratories.[25-27] Decreased platelet count may be indicative of the thrombocytopenia that can be present in VWD type 2B. An increased PFA-100™ closure time indicates a defect in primary hemostasis. More severe platelet function disorders, such as Glanzmann thrombasthenia, Bernard–Soulier syndrome and severe forms of VWD, can be excluded using this test.[28] However, the test is not sensitive enough to detect all forms of VWD.[26,29,30] Therefore, more specific diagnostic tests for VWD are required.

Screening tests for defects in secondary hemostasis include the activated partial thromboplastin time (APTT) and the prothrombin time (PT). Prolonged APTT indicates a defect in the intrinsic pathway of coagulation, whereas prolonged PT identifies defects in the extrinsic pathway of coagulation. Prolongation of the APTT can be indicative of the diagnosis of VWD as it may reflect the concurrent decrease of FVIII in VWD, especially in VWD types 3 and 2N. The results of the above-mentioned screening tests can guide in the selection of further

specific VWD testing. A prolonged BT or PFA-100™ in combination with prolonged APTT may suggest VWD, but milder cases of VWD may show normal results for all these screening tests.

Diagnostic assays for VWD

To confirm or exclude VWD, a set of diagnostic tests needs to be performed. These tests include determination of the quantity of circulating VWF, VWF-platelet binding activity and FVIII activity. Based on these results, VWD can either be excluded or the patients can roughly be categorized as type 1, 2 or 3 VWD. Only when these tests indicate VWD, are additional subtyping assays required.

VWF antigen levels

The quantity of VWF antigen (VWF:Ag) in plasma can be determined by enzyme-linked immunosorbent assay (ELISA) or automated latex immunoassay (LIA).[31,32] LIA has the advantage that it is an automated assay and that it is very reproducible, but has limitations when measuring VWF plasma levels lower than 10 IU/dL or higher than 125 IU/dL.[33] Therefore it may not be possible to distinguish severe VWD type 1 and VWD type 3. The lower limit of detection of the VWF:Ag ELISA in the study by Castaman *et al*.[33] was 5 IU/dL, but with adaptation of the ELISA lower limits are possible. The VWF:Ag ELISA is, however, somewhat less reproducible than the LIA.

Plasma VWF:Ag levels of a healthy population are very variable and depend on many factors. Most studies have been performed on subjects from European descent and do not reflect all subjects worldwide. In a large European study among 1049 healthy individuals, the mean VWF:Ag level was determined to be 100.1 IU/dL, with a standard deviation (SD) of 31.9 IU/dL.[33] This results in a normal reference range for the Caucasian population, defined as mean ± 2 SD of 36.3-163.9 IU/dL. Higher levels of VWF:Ag are, however, found in subjects with African and Korean ancestry.[34-36] Blood group and age are other important determinants: blood group O is associated with 25% lower VWF:Ag levels[37,38] and VWF:Ag levels increase with age.[37,39,40] Other factors leading to fluctuating VWF:Ag levels are the menstrual cycle, stress, pregnancy and immune responses.[41-44]

Diagnosis of VWD by VWF:Ag measurements can only be conclusive in VWD type 3, where there is a virtually complete deficiency of VWF:Ag, or VWF:Ag is < 5 IU/dL.[16] In VWD type 1, VWF:Ag is by definition decreased. It is, however, very difficult to define a clear cut-off point. Usually, levels below 30 IU/dL are considered diagnostic; however, levels between 30 and 50 IU/dL should be interpreted in the context of the clinical bleeding phenotype. In VWD type 2, plasma

VWF:Ag may vary from normal to mildly reduced.[16] Due to the variable VWF:Ag levels, the interpretation of borderline levels may be ambiguous and repeated measurements are often required. It is important not to over-diagnose patients, which leads to unnecessary use of costly medication and unjustified labelling of patients. Depending on the clinical phenotype, borderline levels of VWF may be considered as a risk factor for bleeding rather than a disease.[45]

VWF-platelet binding activity

The major function of VWF is VWF-dependent platelet adhesion. VWF-platelet binding activity can be determined by several different assays. The VWF ristocetin cofactor activity (VWF:RCo) assay has been the gold standard for many years.[46] However, several new assays have been developed to overcome imprecision and insensitivity of the classical VWF:RCo assay. Recently, a new nomenclature for platelet-dependent VWF activity has been adopted and published by the VWF subcommittee of the Scientific and Standardization Committee (SSC) of the ISTH to recognize differences between the tests (Table 2).[47]

Table 2. Nomenclature of platelet-dependent VWF activity

Assay	Activator	Description of activity
VWF:RCo	Ristocetin	Ristocetin-induced VWF binding to GPIb on platelets
VWF:GPIbR	Ristocetin	Ristocetin-induced VWF binding to recombinant wild type GPIb fragment
VWF:GPIbM	-	Spontaneous VWF binding to recombinant gain-of-function mutant GPIb fragment
VWF:Ab	-	Binding of monoclonal antibody to the GPIb binding site in VWF (A1 domain epitope)

Adapted from Bodó *et al*.[47] VWF, von Willebrand factor; GPIb, glycoprotein Ib.

The VWF:RCo assay uses the antibiotic ristocetin with platelet-poor plasma to agglutinate an external source of formalin-fixed platelets. Low VWF:RCo indicates a defect in VWF-platelet binding. A drawback of the assay is the low sensitivity and precision; however, newer fully automated assays have been developed and show better sensitivity and precision.[48] The binding site of ristocetin in VWF is found to be inside the A1 domain and some mutations in this region affect ristocetin-mediated activation of VWF, leading to a false-positive VWD diagnosis. Mutations or variations known to interfere with VWF-ristocetin binding are p.Asp1472His and p.Pro1476Ser, with p.Asp1472His being a common variant found in the Afro-American population.[49,50]

An improved limit of detection and coefficient of variation (CV) compared with the standard VWF:RCo assays is obtained by ristocetin-triggered GPIb binding (VWF:GPIbR) assays.[47] In these assays, the external source of platelets has been replaced by a recombinant GPIb fragment bound to a monoclonal antibody[51,52] or latex or magnetic particles.[53] As the VWF:GPIbR assay

uses ristocetin to induce platelet agglutination, this assay has the same drawbacks as the VWF:RCo assay with respect to the variations in the ristocetin binding site. Another drawback is the use of different monoclonal antibodies and GPIb sources in all the assays, rendering substitution of reagents impossible.

The drawback of misclassification of certain subjects by the use of ristocetin can be bypassed by platelet-dependent VWF assays using recombinant mutant gain-of-function GPIb fragments (GPIbM assay). These GPIb fragments contain gain-of-function mutations that spontaneously bind VWF without the need for VWF activation by ristocetin. Several assays have been developed, including ELISA-based assays[54] and automated systems.[55]

In addition to the above-mentioned VWF-platelet binding activity assays, an alternative assay has been developed in which the platelets have been replaced by a monoclonal antibody detecting an epitope in the GPIb binding site (A1 domain) of VWF (indicated as VWF:Ab).[56,57] Although the results of this assay are closely correlated with the VWF:RCo assay, it is not an activity assay in the strict sense.

The VWF:RCo, VWF:GPIbR and GPIbM assays can be used to identify qualitative defects of VWF in the platelet binding. In VWD type 3 there is no platelet binding activity due to the absence of VWF:Ag and in VWD type 1 the platelet binding activity correspondingly decreases with the VWF:Ag level. Significantly decreased levels of platelet binding activity in comparison with VWF:Ag levels are found in VWD types 2A and 2M. This reduction, although less pronounced, is also observed in VWD type 2B. In VWD type 2N, there is no defect in platelet binding and hence no decrease in platelet binding activity in comparison with VWF:Ag levels (Table 3).

Table 3. Interpretation of laboratory assays in VWD

Assay	Type 1	Type 2A	Type 2B	Type 2M	Type 2N	Type 3	PT-VWD
Bleeding time or PFA-100™	n/↑	↑	↑	↑	n	↑↑	↑
VWF:Ag	↓	n/↓	n/↓	n/↓	n	↓↓↓	n/↓
VWF:RCo*	↓	↓↓	↓↓	↓↓	n	↓↓↓	↓
FVIII:C	n/↓	n/↓	n/↓	n/↓	↓↓	↓↓	n/↓
VWF:RCo*/VWF:Ag	> 0.6	< 0.6	< 0.6	< 0.6	> 0.6	NA	< 0.6
RIPA	n/↓	↓↓	↑	↓↓	n	absent	↑
Multimers	n	loss HMW	loss HMW	n	n	absent	loss HMW
VWF:FVIIIB	n	n	n	n	↓↓	NA	n
VWF:CB	↓	↓↓	↓↓	n/↓	n	↓↓↓	↓

PFA-100™, platelet function analyzer; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity; FVIII:C, FVIII coagulant activity; RIPA, ristocetin-induced platelet aggregation; VWF:FVIIIB, VWF-FVIII binding; HMW, high molecular weight; PT-VWD, platelet-type VWD; VWF:CB, VWF collagen binding; n, normal; ↑, increased (or prolonged for bleeding time or PFA-100™); ↓, decreased; NA, not applicable. *Or other assays measuring platelet binding activity (see Table 2).

VWF-platelet binding activity/VWF:Ag ratio

The ratio between VWF-platelet binding activity (VWF:RCo, VWF:GPIbR or VWF:GPIbM) and VWF:Ag level is used to roughly distinguish qualitative VWD from quantitative VWD.[16,58] Qualitative defects of VWF (except for VWD type 2N) are associated with decreased VWF-platelet binding and therefore a decreased VWF-platelet binding activity/VWF:Ag ratio. Most studies that analyzed ratios reported the VWF:RCo/VWF:Ag ratio; however, the results are likely to be equally true for the other platelet binding activity assays.[58] Generally, a VWF:RCo/VWF:Ag ratio below 0.6 is used for diagnosis of VWD type 2A, 2B and 2M. VWD type 2N patients do not have a defect in platelet binding and therefore no decreased VWF:RCo/VWF:Ag. When using VWF:RCo, repeated measurements are needed for correct diagnosis, due to a high CV of the VWF:RCo assay. In some cases with very low levels of VWF:Ag and/or VWF:RCo, the ratio may not be reliably calculated and the distinction between quantitative and qualitative defects may be difficult. However, after administration of desmopressin (DDAVP), VWF levels and potentially the VWF-platelet binding activity will rise above the detection limit. It will then be possible to calculate the VWF-platelet binding activity/VWF:Ag ratio. This may help in subtyping newly diagnosed VWD patients.

Factor VIII activity

VWF binds coagulation FVIII in the circulation, increasing the FVIII half-life.[9] Reduction of the FVIII activity (FVIII:C) can be due to either a decreased VWF:Ag level or a reduced binding affinity of VWF for FVIII. In VWD type 3, FVIII:C is severely decreased due to the absence of VWF:Ag. The remaining level of FVIII:C in VWD type 3 is an important determinant of the bleeding risk.[59] Due to very low FVIII:C levels, type 3 patients also experience hemophilia-like bleeding as joint and muscle bleeding.[59] In VWD types 1 and 2 (except for 2N), FVIII:C may be variable, sometimes reduced in line with the VWF:Ag level, sometimes within the normal range. Finally, decreased levels of FVIII:C may be the only detectable defect in VWD type 2N, which may thus be missed when relying on VWF:Ag and VWF:RCo levels as diagnostic tests only.

Subtyping assays

Diagnosis of VWD can be confirmed or excluded by the diagnostic assays described in the previous section. However, for conclusive diagnosis of the respective types of VWD, specific subtyping assays are required.

Ristocetin-induced platelet aggregation

VWF mutations found in VWD type 2B patients force a conformational change in the GPIb binding site leading to spontaneous binding of VWF to GPIb. Due to this increased affinity of VWF to GPIb, lower *in vitro* concentrations of ristocetin are already sufficient to induce binding of VWF to GPIb.[60] Using this feature, a distinction between VWD type 2B and VWD types 2A and 2M can be made by the ristocetin-induced platelet aggregation (RIPA) assay using low concentrations of ristocetin. However, an increased binding of VWF to GPIb can also be caused by a gain-of-function mutation in the GPIb receptor itself. Patients having such mutations are classified as platelet-type VWD (PT-VWD) and will also test positive in the low-dose RIPA assay.[61] To distinguish VWD type 2B from PT-VWD, one can vary the source of plasma and platelets in the low-dose RIPA assay.[62] It is, however, difficult to distinguish with certainty, so nowadays mutation analysis is usually performed to confirm either diagnosis.

VWF multimer analysis

A decreased VWF-dependent platelet adhesion may be due to a selective deficiency of HMW VWF multimers as seen in types 2A, 2B and PT-VWD or due to an intrinsic decreased GPIb binding affinity of VWF as seen in type 2M. To make this distinction, VWF multimers should be analyzed. Defects in the multimerization of VWF can be identified by VWF multimer analysis in which non-reduced plasma samples are run through medium (1.4–2%) and low (0.7–1.2%) resolution agarose gels. Subsequently, the VWF multimers can be visualized by Western blot.[63] The VWF multimer analysis is a complex and laborious assay, which is generally performed by specialized laboratories only. Even in those laboratories there is a high error rate, with inconsistent interpretation of multimer profiles and false classification.[64] The multimer pattern, when performed with high quality in specialized laboratories, may, however, be indicative of specific genetic defects in VWF.[65]

Normal plasma VWF shows an equal distribution of low, intermediate and HMW VWF multimers. In a high resolution gel, each multimeric band is reproduced as a triplet structure, with the outer bands reflecting the proteolytic cleavage of VWF by ADAMTS13. Decreased levels of HMW VWF multimers are found in VWD type 2A as a consequence of defects in dimerization and multimerization, or due to enhanced susceptibility to proteolysis by ADAMTS13. The decrease of HMW multimers in type 2B and PT-VWD is the consequence of enhanced *in vivo* binding of VWF to platelets, which leads to increased VWF proteolysis and clearance of the HMW multimers from the circulation. In VWD type 2M there is no decrease in HMW multimers as the decrease in VWF-dependent platelet adhesion is mainly due to loss-of-function mutations in the GPIb binding site of VWF (A1 domain). Although VWD type 1 is defined as a quantitative defect of VWF, a subtle decrease in HMW VWF multimers is occasionally observed. According to the recommendation of the SSC of the ISTH, this subtle decrease in HMW VWF multimers

(if VWF:RCo/VWF: Ag > 0.6) is classified as VWD type 1.[16] As these patients do show a minimal, qualitative defect in the multimerization of VWF, there is still an ongoing debate over whether they should be diagnosed as VWD type 1 or 2A. Nevertheless, this will not influence the management of treatment.

VWF-FVIII binding

Decreased FVIII:C can be found in all VWD types as a consequence of reduced VWF:Ag levels. However, in VWD type 2N there is a more obvious decrease in FVIII:C due to a mutation in the FVIII binding site of VWF disrupting the affinity for FVIII. Defects in the capacity of VWF to bind to FVIII, as found in VWD type 2N, can be identified by the VWF-FVIII binding assay (VWF:FVIIIB).[66] Decreased binding affinity of a patient's plasma VWF to recombinant FVIII in this ELISA-based assay indicates a binding defect. The assay is especially important for distinguishing VWD type 2N and mild hemophilia A, characterized, respectively, by reduced and normal VWF:FVIIIB. VWF:FVIIIB cannot be measured in type 3 due to absence of VWF:Ag. In all other VWD types, the VWF:FVIIIB is within the normal range.

VWF collagen binding

UL-VWF strings bind collagen types I and III in the VWF A3 domain and collagen types IV and VI in the A1 domain after vascular damage.[67-70] Reduced collagen binding may be a consequence of a decrease in VWF:Ag as in types 1 and 3, a specific lack of HMW VWF multimers as in types 2A and 2B, or specific collagen binding defects, categorized among type 2M.

The ability of VWF to bind collagen can be measured by the VWF collagen binding assay (VWF:CB). Many commercial VWF:CB assays are available, mainly using collagen I or III, or a combination of both. Drawbacks of these tests are the different sources of collagen and reference plasmas used, which makes the optimization and standardization of the VWF:CB assay difficult.[71] Furthermore, patients with defects in binding to collagen IV and VI will be missed using any of these assays.[68,69] However, defects in collagen IV and VI binding are found in affected patients as well as in healthy controls and are therefore considered to only increase the risk of bleeding rather than being a causative factor for VWD.[68,69]

Currently, the VWF:CB is not generally included among the standard diagnostic tests for VWD. However, there is reason to reconsider this. First of all, VWF:CB is able to identify patients with an isolated collagen binding defect that will go unnoticed with any of the other diagnostic assays.[68] This would, however, force to test all the collagen types separately. Secondly, the VWF:CB assay has been shown to be as effective in distinguishing VWD type 1 from type 2 as the VWF:RCo assay.[71,72] The best distinction between VWD types 1 and 2 has been observed

when a mixture of collagen I and III is used. As a subtyping test, the VWF:CB assay may even be a substitute for the complicated VWF multimer assay in the identification of patients with reduced HMW VWF multimers.[73] Although there are many reasons to include the VWF:CB assay in standard diagnosis, it is difficult to pinpoint which test is best. At this time, a mixture of collagen types I and III seems most appropriate. However, more optimization of the commercial tests is necessary.

The interpretation of the diagnostic and subtyping tests and the corresponding classification of VWD is summarized in Table 3 and a diagnostic algorithm is shown in Fig. 2.

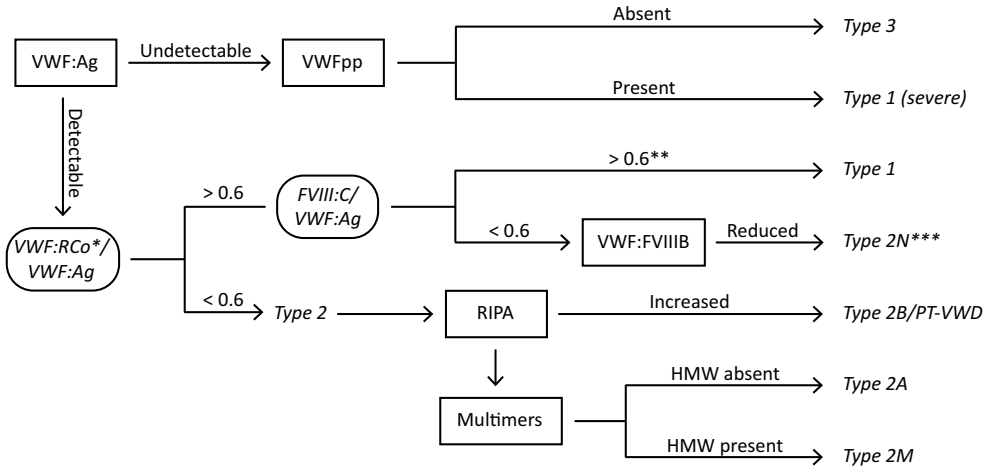

Figure 2. Diagnostic algorithm for von Willebrand disease (VWD) classification. Diagnosis of VWD is based on abnormal levels found in one or more of the standard diagnostic tests: VWF:Ag, VWF:RCo and FVIII:C. Specific VWD subtypes can be identified by performing the RIPA assay using low-dose ristocetin, VWF multimer analysis and the VWF:FVIIIB assay. VWFpp can be used to distinguish between true VWD type 3 and severe VWD type 1, and can help in the identification of the pathophysiological mechanism behind VWD. *VWF:RCo or other assays measuring VWF-platelet binding activity (see Table 2). **VWD is excluded when FVIII:C/VWF:Ag is > 0.6 and VWF:Ag and VWF:RCo are within the normal range. ***Normal VWF:FVIIIB but FVIII:C/VWF:Ag < 0.6 suggests hemophilia A. VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity; FVIII:C, FVIII activity; RIPA, ristocetin-induced platelet aggregation; VWF:FVIIIB, VWF-FVIII binding; VWFpp, VWF propeptide; PT-VWD, platelet-type VWD.

The value of VWF ratios and VWF mutation analysis

The tests outlined above are sufficient to diagnose and classify most VWD. Additional assays and analyses can be performed to confirm certain subtypes or identify the underlying pathophysiological mechanisms. The pathophysiological mechanisms are of scientific interest, but may also guide the choice of treatment.

VWF propeptide and its ratio to VWF:Ag

VWF is synthesized as pre-proVWF. The signal peptide is cleaved off right after synthesis. The propeptide is cleaved off in the endoplasmic reticulum (ER) and functions as a chaperone for the formation of VWF multimers.[74] VWF and its propeptide (VWFpp) are synthesized and secreted into the circulation in a 1:1 ratio.[75] However, in the circulation both molecules are cleared at different rates, with a circulating half-life of about 12 hours for VWF and about 2-3 hours for VWFpp.[8,76]

VWFpp levels in plasma can be determined by an ELISA-based assay and may be indicative of synthesis, secretion or clearance defects of VWF. Decreased VWFpp levels indicate reduced synthesis of VWF or impaired secretion of VWF from the WPBs, and may be found in VWD type 1 patients having a null allele or ER retention.[14,15] In VWD type 2, the qualitative defect of VWF is generally not caused by impaired synthesis or secretion and normal VWFpp plasma levels are expected.[14] Although VWD type 3 is associated with undetectable VWF:Ag levels, a subset of patients does show circulating VWFpp.[14] We found that patients historically diagnosed as VWD type 3 with undetectable VWFpp were homozygous or compound heterozygous for null alleles, whereas the majority of patients with detectable VWFpp were heterozygous for a missense mutation associated with increased VWF clearance.[14] The patients with detectable VWFpp also had a lower bleeding score. We therefore suggest that these patients should be reclassified as severe type 1 VWD instead of type 3. Altogether, VWFpp can discriminate between VWD type 3 with a complete absence of VWF:Ag and VWFpp and severe type 1 VWD with extremely low VWF:Ag levels.

The ratio of VWFpp to VWF:Ag gives a measure of the clearance rate of mature VWF, assuming a constant clearance rate for VWFpp.[14,15,77,78] Clearance defects can be found in all types of VWD and can be due to a mutation in VWF itself, such as the Vicenza mutation p.Arg1205His, or due to variations in proteins involved in the clearance of VWF.[13,79,80] In VWD type 2B, almost all patients show increased clearance rates. This is suggested to be caused by increased uptake of the VWF-platelet complex by macrophages, mediated by increased lipoprotein receptor 1 binding.[81]

Information about clearance defects may have therapeutic implications. DDAVP is for example the treatment of choice in VWD type 1 patients, but increasing the endogenous VWF levels may not be sufficient in patients with a major VWF clearance defect.

FVIII:C/VWF:Ag ratio

Binding of coagulation FVIII to VWF occurs at a 1:50 molar ratio. However, coagulation factors are determined in arbitrary units per milliliter and therefore the ratio of FVIII:C to VWF:Ag in

healthy controls is by definition approximately one.[82] Due to the excess of FVIII binding sites on VWF, a reduction in VWF synthesis will not lead to a concomitant decrease of FVIII levels. However, when VWF is reduced due to increased clearance of the protein, then the FVIII levels drop accordingly as VWF-FVIII is cleared as a complex. The ratio of FVIII:C to VWF:Ag could therefore identify defects in the synthesis or secretion of VWF.[14,83] Heterozygous VWF null mutations, mainly found in carriers of VWD type 3 and some carriers of VWD type 1, have been shown to be associated with a FVIII:C/VWF:Ag ratio of > 2.[15,83] As VWF and FVIII are cleared as a complex, FVIII:C/VWF:Ag is not altered in patients showing a pure clearance defect of VWF. However, a combined effect of decreased synthesis and increased clearance is possible and could be identified by plotting VWFpp/VWF:Ag to FVIII:C/VWF:Ag.[14] Finally, the FVIII:C/VWF:Ag ratio when reduced (< 0.6) suggests VWD type 2N or hemophilia A.

Mutation analysis

In most cases of VWD, a proper diagnosis and classification can be made based on the phenotypic assays and thus mutation analysis is not required. Also, because of the large size of *VWF*, it is suggested that mutation analysis should only be performed when phenotypic assays fall short and when additional mutation analysis is relevant for management or counseling of the patient. However, new developments, such as next generation sequencing, resulted in the implementation of routine gene analysis of *VWF* in some laboratories.[84] Deletions or duplication of exons can furthermore be identified by multiplex ligation-dependent probe amplification dosage analysis.[85] When it is decided to use sequencing for diagnosis, special care should be taken with regard to the *VWF* pseudogene located on chromosome 22.[86] The pseudogene corresponds to exons 23-34 of the *VWF* gene with a 97% similarity.

Quantitative defects of VWF found in VWD type 1 are mainly caused by missense mutations spread throughout the whole gene, and mutations have been identified only for ~ 70% of the sequenced patients.[17,87-89] Sequencing these patients would be labor intensive and costly, and would in most cases not benefit the patient. Especially in VWD type 1 with its variable penetrance, identification of a mutation is no proof of disease and not identifying a mutation does not reject the bleeding phenotype.

Qualitative defects, found in VWD type 2, are usually restricted to specific VWF domains and mutations have been identified in almost all sequenced patients.[17] When a qualitative defect of VWF is expected, sequencing the restricted domain would be sufficient and is usually performed to distinguish VWD from VWF-related diseases. An example is the distinction between VWD type 2N and mild hemophilia A, which has major implications for genetic counseling as well as the choice of treatment. Another example is the differentiation between

VWD type 2B and PT-VWD, which is also relevant for treatment.

The quantitative defect in VWD type 3 is largely explained by *VWF* null alleles.[17] Important reasons for sequencing these patients are genetic counseling and prenatal diagnosis. Furthermore, homozygous gene deletions that may predict the formation of VWF inhibitors after replacement therapy can be identified.[90]

Conclusion

The heterogeneity of VWD necessitates undertaking multiple diagnostic tests, which may have to be repeated several times before a proper diagnosis can be made. Adequate diagnosis and classification is important for the choice of treatment and for counseling with regard to inheritance. The broad diagnostic armamentarium for VWD is still insufficient as it does not take some very important aspects of VWF into account. All current tests are performed in static conditions, although flow plays a major role in the structural and functional properties of VWF. Also, the contribution of the vascular wall and the VWF string formation on the endothelial surface are not considered in the diagnosis. Recently, patient-derived blood outgrowth endothelial cells (BOECs) have been described as a model to study VWF in its native environment.[10,11] Although BOECs are not feasible for diagnostic purposes at this time, new innovative diagnostic tests could be focusing on combining patient-derived BOECs with aspects of flow for a more physiological evaluation of VWF function.

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Online supplemental data