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Diagnosis and classification of von Willebrand disease

A B S T R A C T

von Willebrand disease (VWD) is the most frequent inherited disorder of hemostasis and is due to quantitative (VWD type 1 and 3) or qualitative (VWD type 2) defects of von Willebrand factor (VWF). Due to the large heterogeneity of VWF defects and to the external variables (blood groups and other physiologic modifiers) influencing VWF levels in the circulation, VWD diagnosis can be difficult especially in relatively mild forms. Three criteria should be always satisfied for a correct VWD diagnosis: 1) a positive bleeding history in the patients; 2) reduced levels of VWF activity in plasma; 3) a positive family history suggestive of VWD. According to the most recent clinical prospective studies, bleeding history in the patients and in their family members should be now derived from a detailed questionnaire on 11 bleeding symptoms and a bleeding severity score (BSS) can be calculated. The ristocetin cofactor activity of VWF (VWF:RCo) is the most useful test for VWD screening in the general population because it reproduces *in vitro* the first VWF interactions with its platelet receptor: however other assays are required to identify and classify the different VWD types The current classification in different VWD types (1, 2A, 2B, 2M, 2N, 3) is important to understand the basic mechanisms of VWF defects, to determine the risk of bleeding and to select the best therapeutic approach. Molecular screening can be important to confirm phenotypic diagnosis. Compared to hemophilia, most VWD patients show relatively mild bleeding symptoms. Therefore, prenatal diagnosis is required mainly in case of parents already known to be carrier of VWD type 3, with gene defects identified in their first affected child. No major bleeding problems usually occur at birth also in severe type 3 VWD. Neonatal diagnosis can be performed in case of children born from parents with VWF defects already characterized, but phenotypic diagnosis of VWD should be always confirmed and compared with the other affected members within the same family. Since young children with VWD type 3 might carry deletions of VWF gene that predispose to the allo-antibodies to VWF, every new child with VWD type 3 should be intensively investigated by searching deletions, before starting extensive therapy with exogenous VWF concentrates.

Introduction

When Erik von Willebrand in 1926 described a novel bleeding disorder in a large family from Foglo on the islands of Aland in the Gulf of Bothnia, he provided an impressive and exhaustive description of its clinical and genetic features. Unlike hemophilia, the epitome of inherited bleeding disorders, both sexes were affected, and mucosal bleeding was the dominant symptom. Prolonged bleeding time (BT) with normal platelet count was the most important laboratory abnormality and a functional disorder of the platelets associated with systemic lesion of the vessel wall was suggested as a possible cause of the disorder. However, he called the disease *hereditary pseudohaemophilia*. To further complicate the issue, some authors subsequently called the disorder *vascular hemophilia*. Only in the 1950s, was it demonstrated that the prolonged BT in these patients was associated with reduced FVIII, but we had to wait until the 1970s to clarify that the deficiency of a new factor, called von Willebrand factor and different from FVIII, was actually responsible for the disease. Surprisingly, the reduction of this factor caused low FVIII, pointing to the close relationships between the two proteins. In the 1980s, the cloning of the VWF gene set the basis for unraveling the molecular causes of the disorder. The history of von Willebrand disease (VWD) has been the subject of two reviews:[1,2] the list of major milestones on VWD diagnosis from 1926 to 2006 is summarized in Table 1. In this review article we discuss the progress and the problems of VWD diagnosis and classification today, 80 years after the original description by Erik von Willebrand.

Table 1. List of major milestones on VWD diagnosis from 1926 to 2006.

1. First description of VWD (index case Hjördis) by Erik von Willebrand (1926)
2. First case reported of VWD in USA (1928)
3. Bleeding time in inherited bleeding disorders (1941)
4. First assay for FVIII in hemophilia A (1950)
5. VWD defect corrected by infusion with fraction I-O (1956)
5. Platelet abnormal adhesiveness in VWD (1965)
6. First case of acquired von Willebrand syndrome (1968)
7. Immunologic differentiation of classic hemophilia A and VWD (1971)
8. RIPA and ristocetin cofactor activity (1971-1973)
9. Synthesis of VWF by cultured human endothelial cells (1973)
10. Detailed descriptions of VWD families in European countries (1973-77)
11. Crossed-immuno-electrophoresis of VWF in VWD (1974)
12. Decreased adhesion to sub-endothelium in VWD (1974-86)
13. FVIII related antigen in platelets of VWD patients (1975)
14. Localization of VWF antigen in vascular endothelial cells (1978-80)
15. Multimeric structure of VWF and VWD 2A versus 2B variants (1980)
16. Epidemiological studies on VWD in general population (1982)
17. Plasma VWF and its binding to platelet receptors (1983-89)
18. Discovery of VWF gene by four independent groups (1985)
19. Molecular diagnosis of VWD types and expression of mutants (1986-2006)
20. VWF binding assays to collagen (1986)
21. VWF binding assay to FVIII and VWD 2N variant (1989)
22. Classification of VWD different types (1994)
23. Identification of the protease /ADAMTS-13) cleaving VWF (1996)
23. National guidelines for diagnosis of VWD (1998-2002)
24. Registry on acquired von Willebrand syndrome (2000)
25. European and Canadian Studies on VWD type 1 (2001-2006)
26. Standardized bleeding score for VWD (2002-2006)

Structure-function of von Willebrand factor

Von Willebrand factor (VWF) is synthesized by endothelial cells and megakaryocytes.[3] The gene coding for VWF has been cloned and located at chromosome 12p13.2. It is a large gene composed of about 178 kilobases and containing 52 exons. A noncoding, partial, highly homologous pseudogene has been identified in chromosome 22. The pseudogene spans the gene sequence from exon 23 to 34.[4] The primary product of the VWF gene is a 2,813 amino acid protein made of a signal peptide of 22 amino acids (also called a pre-peptide), a large pro-peptide of 741 amino acids and a mature VWF molecule containing 2,050 amino acids. In keeping with a recently pro-

posed nomenclature,[5] numbering starts from the first amino acid of the signal peptide, so 764 is the first amino acid of the mature protein. Different protein regions, corresponding to four types of repeated domains (D1, D2, D', D3, A1, A2, A3, D4, B, C1, C2) of cDNA, are responsible for the different binding functions of the molecule (Figure 1).

VWF is the result of ordered intra-cellular processing, leading to the storage and/or secretion of a heterogeneous array of multimeric multi-domain glycoproteins, referred to as VWF.

VWF has two major functions in hemostasis. First, it is essential for platelet-subendothelium adhesion and platelet-to-platelet interactions as well as platelet aggregation in vessels in which rapid blood flow results in elevated shear stress, a function partially explored *in vivo* by measuring the BT. Adhesion is promoted by the interaction of a region of the A1 domain of VWF with GpIbα on platelet membrane. It is thought that high shear stress activates the A1 domain of the collagen-bound VWF by stretching VWF multimers into their filamentous form. Furthermore GPIbα and VWF are also necessary for platelet-to-platelet interactions.[3] The interaction between GPIb· and VWF can be mimicked in platelet–rich plasma by addition of the antibiotic ristocetin, which promotes the binding of VWF to GPIb· of fresh or formalin fixed platelets. Aggregation of platelets within the growing haemostatic plug is promoted by the interaction with a sec-

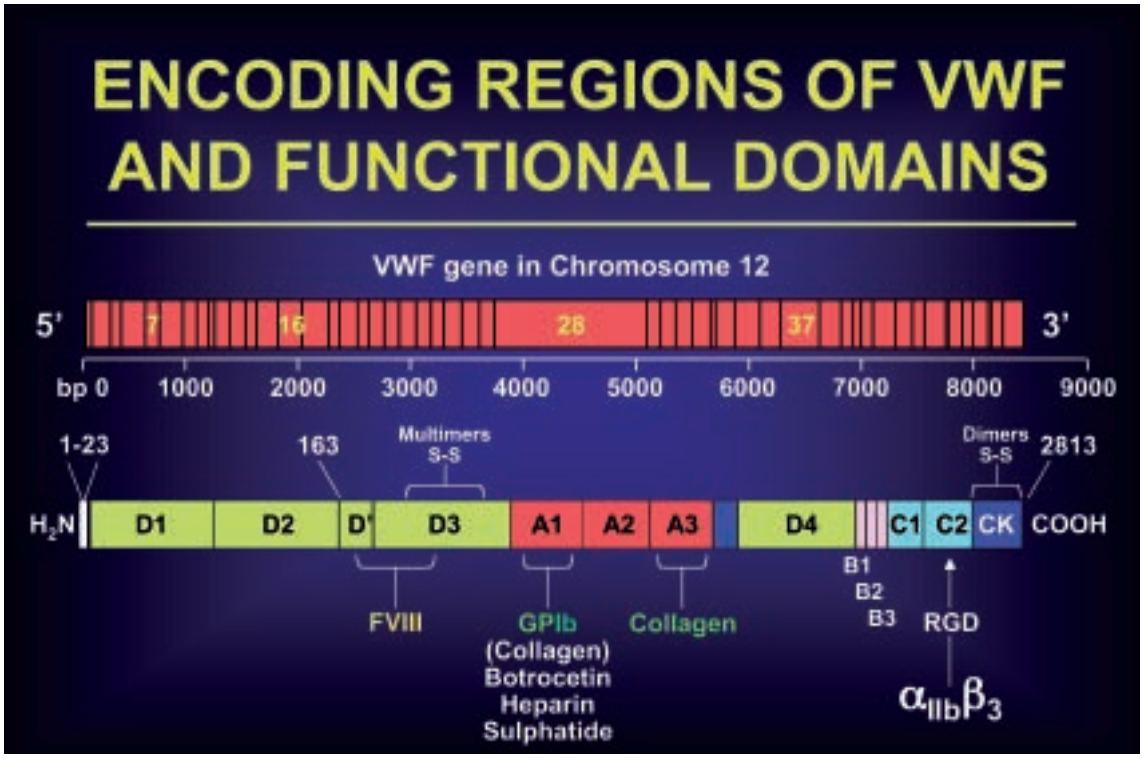

Figure 1. Schematic representation of the VWF gene located in chromosome 12: the main exons are indicated with the number of base pairs from 5' to 3' (upper panel). The structure of VWF functional domains: the pre-pro-VWF is indicated with amino acids numbered from the amino- (aa 1) to carboxy-terminal portions (aa 2813) of VWF. Note the important CK and D3 domains for formation of VWF dimers and multimers. The native mature subunit of VWF, after the cleaving of the pre-pro VWF, is described with its functional domains: the VWF binding sites for factor VIII (D' and D3), GpIb, botrocetin, heparin, sulfatide, collagen (A1), collagen (A3) and the RGD sequence for binding to αIIβ,3 (lower panel).

Table 2. Recommended nomenclature of factor VIII/von Willebrand factor complex.

Factor VIII	
Protein	VIII
Antigen	VIII:Ag
Function	VIII:C
Von Willebrand factor	
Mature protein	VWF
Antigen	VWF:Ag
Ristocetin cofactor activity	VWF:RCo
Collagen binding capacity	VWF:CB
Factor VIII binding capacity	VWF:FVIIIB

(see reference 7)

Table 3. Classification of von Willebrand disease.

Quantitative deficiency of VWF
Type 1 Partial quantitative deficiency of VWF Type 3 Virtually complete deficiency of VWF
Qualitative deficiency of VWF
Type 2 Qualitative deficiency of VWF
A) Type 2A Qualitative variants with decreased platelet-dependent fuction associated with tha absence of high-molecular-weight VWF multimers
B) Type 2B Qualitative variants with increased affinity for platelet GPIbα
C) Type 2M Qualitative variants withdecreased platelet-dependent fuction not caused by the abssence of high-molecular-weight VWF multimers
D) Type 2N Qualitative variants with markedly decreased affinity for factor VIII

(see references 11 and 12)

ond receptor on platelets, GPIIb-IIIa (or integrin αIIb,3) which, once activated, binds to VWF and fibrinogen, recruiting more platelets into a stable plug. Both these binding activities of VWF are highly expressed in the largest VWF multimers.

Second, VWF is the specific carrier of factor VIII (FVIII) in plasma. VWF protects FVIII from proteolytic degradation, prolonging its half-life in circulation and efficiently localizing it at the site of vascular injury. Each VWF monomer has one binding domain, located in the first 272 amino acids of the mature subunit (D' domain) which can bind one FVIII molecule, *in vivo*, however only 1-2% of available monomers are occupied by FVIII.[6] Therefore, any change in plasma VWF level is usually associated with a concordant change in FVIII plasma concentration. The correct nomenclature with abbreviations of the different FVIII/VWF activities, as approved by the Scientific Standardization Committees – Sub-Committee on VWF – of the International Society of Thrombosis and Haemostasis (ISTH-SSC on VWF), are summarized in Table 2.[7]

The mature native VWF circulates in plasma of normal individuals at a concentration of 5-15 ug/mL: subjects with blood group O show lower plasma levels of VWF than those with blood group non-O.[8] During fetal growth, VWF retains ultra-large molecular weight forms and plasma levels of VWF are higher in the newborn than in children: only after six months from birth, children show their actual levels of VWF and factor VIII.[9,10] These data can explain why neonates with severe forms of VWD do not usually bleed but should be also taken into consideration when VWD diagnosis is suspected in young children during their first six-eight months.

Classification of von Willebrand disease

The current classification of VWD, summarized in Table 3, was proposed originally by Sadler in 1994 and updated in 2006 on behalf of the ISTH-SSC on VWF.[11,12] The revised classification of VWD identifies two major categories, characterized by quantitative (types 1 and 3) or qualitative (type 2) VWF defects. A

partial quantitative deficiency of VWF in plasma and/or platelets identifies type 1, whereas type 3 is marked by the total absence or only traces amounts of VWF in plasma and platelets. Type 1 is easily distinguished from type 3 by the milder VWF deficiency (usually in the range of 10-40 U/dL), the autosomal dominant inheritance pattern and the presence of milder bleeding symptoms.[13] Four type 2 VWD subtypes have been identified, reflecting different pathophysiologic mechanisms. Type 2A and 2B VWD are marked by the absence of high molecular weight VWF multimers of in plasma; in type 2B, there is increased affinity for platelet glycoprotein Ib-IX-V complex (GpIbα). The identification of qualitatively abnormal variants with decreased platelet-dependent function and the presence of normal multimers on gel electrophoresis has led the addition of a new subtype, called 2M. If this definition is followed and more stringent criteria are applied to VWD diagnosis, many cases previously identified as type 1 should now be classified as type 2M because they are caused by single missense mutations affecting VWF function but not its multimeric structure and assembly. Furthermore, type 2N (Normandy) also shows a full array of multimers since the defect lies in the N-terminal region of the VWF where the binding domain for factor VIII resides. This type is phenotypically identified only by an abnormal FVIII/VWF binding test: in fact, this test is always normal in mild hemophiliacs or carrier of hemophilia A.[14]

Prevalence and frequency of different VWD types

VWD is the most frequent inherited bleed-

Figure 2. Distribution of VWD types in 1234 patients enrolled into the Italian VWD registry by 16 haemophilia centres. Note that only 73 (6%) of the entire cohort of 1234 VWD patients are children with age below 10 years.

ing disorder, with prevalence up to 1% in certain geographic areas according to population studies. On the other hand, prevalence based on the number of patients registered at specialized centers ranges from 4 to 10 cases/100,000 inhabitants: symptomatic VWD requiring specific treatment are 50-100 per million.[13] In the past type 1 was reported as the most frequent form of VWD. A recent retrospective study based on reappraisal of type 1 diagnoses after ten years (1994 versus 2004) in 1234 VWD patients followed by 16 Italian Hemophilia Centers, found that VWD type 1 were only 671/1234 (54%), because most of previously diagnosed VWD type 1 were re-diagnosed type 2 according to their discrepant VWF activities (VWF:RCo/Ag ratio <0.7). Age distribution of the 1,234 Italian VWD patients was 5-86 years, with 267/1234 (22%) cases with age below 20 years (Figure 2): however, the 16 Italian Hemophilia Centers followed only 73/1234 (6%) children at age below 10 years.[15,16] Since most patients enrolled in the study are not located in pediatric hospitals, probably the pediatric population of VWD in Italy is greatly underestimated.

Criteria for evaluation of the bleeding history: a bleeding severity score

Several attempts have been made recently by clinicians, experts in VWD, to evaluate the sensitivity and specificity of bleeding symptoms, which are important especially in the mild cases of type 1 VWD, with VWF:RCo levels >30 U/dL. In a multicentre study about the clinical presentation of type 1 VWD in obligatory carriers, it has been shown that menorrhagia and epistaxis are not good predictor of type 1 VWD while cutaneous bleeding and bleeding after dental extractions should be considered the most sensitive symptoms.[17] Therefore, a specific bleeding severity score (BSS) has been proposed (Table 4). This bleeding score has been tested in affected and non-affected members of 154 VWD families enrolled prospectively in a large European study, as well as 200 normal individuals.[18]

Patterns of inheritance

The inheritance pattern of VWD type 3 is autosomal recessive. In type 2 VWD patients, the pattern of inheritance is mainly autosomal dominant, even though rare cases with recessive pattern have been reported.[13] The inheritance of the mild type 1 VWD is usually autosomal dominant, with variable phenotype and penetrance. Despite its high prevalence, the precise genetic cause of type 1 VWD is still elusive in most cases, especially those with a mild phenotype. In type 1 VWD, in fact, a number of genetic and non-genetic factors are likely to contribute to the wide variability of the clinical and laboratory phenotype. About 60% of the variation in VWF plasma is due to genetic factors, with ABO group accounting for only about 30%. In type O subjects the VWF level is 25-35% lower than in non-O individuals.[8,19] Other factors outside the VWF gene, such as platelet polymorphysms, have been proposed to modify the bleeding tendency of type 1 VWD, as reported.[20]

Clinical features and bleeding symptoms in different VWD types

The clinical expression of VWD is usually mild in most type 1, increasing the severity in types 2 and 3. In general, the severity of bleeding correlates with the degree of the reduction of VWF:RCo and FVIII:C activities, but not with the magnitude of BT prolongation or with the patient ABO blood type. Mucocutaneous bleeding (epistaxis, menorrhagia) is a typical manifes-

tation of the disease and may even affect the quality of life. VWD may be highly prevalent in patients with isolated menorrhagia[13] To date, only a few detailed descriptions of symptoms in VWD patients have been provided[21,22] but only one study took into account the differentiation according to the VWD types.[15,16] Table 5 shows the relative frequency of bleeding symptoms in three large series of patients with VWD diagnosed at specialized centers.

Table 4. Bleeding sseverity score used to evalutate bleeding history in VWD.

Symptom	-1	0	1	2	3	4
Epistaxis	-	No or trivial (less than 5)	> 5 or more than 10'	Consultation only	Packing or Cauterization or Antifibrinolytic	Blood transfusion or Replacement therapy or Desmopressin
Cutaneous	-	No or trivial (<1 cm)	> 1 cm and no trauma	Consultation only		
Bleeding from minor wounds	-	No or trivial (less than 5)	> 5 or more than 5'	Consultation only	Surgical hemostasis	Blood transfusion or Replacement therapy or Desmopressin
Oral cavity	-	No	Referred at least one	Consultation only	Surgical hemostasis or Antifibrinolytic	Blood transfusion or Replacement therapy or Desmopressin
GI bleeding	-	No	Associated with ulcer, portal hypertension., hemorrhoids, angiodysplasia	Spontaneous	Surgical hemostasis, Blood transfusion, Replacement therapy, Desmopressin, Antifibrinolytic	
Tooth extraction	No bleeding in at least 2 extraction	None done or no bleeding in 1 extraction	Referred in <25% of all procedures	Referred in >25% of all procedures, no intervention	Resuturing or packing	Blood transfusion or Replacement therapy or Desmopressin
Surgery	No bleeding in at least two surgeries	None done or no bleeding in 1 surgery	Referred in <25% of all surgeries	Referred in >25% of all procedures, no intervention	Surgical hemostasis or Antifibrinolytic	Blood transfusion or Replacement therapy or Desmopressin
Menorrhagia	-	No	Consultation only	Antifibrinolytics, Pill use	Dilatation & Curettage, Iron therapy	Blood transfusion or Replacement therapy or Desmopressin or Hysterectomy
Post-partum hemorrhage	No bleeding in at least two deliveries	No deliveries or no bleeding in 1 delivery	Consultation only	Dilatation & Curettage, Iron therapy, Antifibrinolytics	Blood transfusion or Replacement therapy or Desmopressin	Hysterectomy
Muscle hematomas	-	Never	Post trauma no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring Desmopressin or Replacement therapy	Spontaneous or traumatic, requiring Surgical intervention or blood transfusion
Hemarthrosis	-	Never	Post trauma no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring Desmopressin or Replacement therapy	Spontaneous or traumatic, requiring Surgical intervention or blood transfusion
CNS bleeding	-	Never	-	-	Subdural, any intervention	Intracerebral, any intervention

(derived from reference 18)

Laboratory diagnosis of VWD types

VWD encompasses a wide spectrum of severity, ranging from few, doubtful bleeds to severe life-threatening bleeding episodes. This is due not only to the heterogeneous defects of the VWF gene which may impair its hemostatic 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 affect fluctuations in VWF levels. Thus, the diagnosis of mild forms of VWD, particularly type 1, may require several laboratory tests.

Screening tests. These tests are usually applied for patients with suspected bleeding tendency and Table 6 summarizes the different steps for VWD diagnosis. The *platelet count* is usually normal, but mild thrombocytopenia may occur in patients with type 2B. The *bleeding time (BT)* is usually prolonged, though it may be normal in patients with mild forms of VWD such as those with type 1 and normal platelet VWF content. The *prothrombin time (PT)* is normal whereas the *partial thromboplastin time (PTT)* may be prolonged to a variable degree, depending on the plasma FVIII levels.

Diagnosis of VWD and identification of the type. The list of tests used for VWD diagnosis and their pathophysiologic and diagnostic signif-

Table 6. Clinical and laboratory parameters used for VWD diagnosis.

i) Patients at risk for VWD
- 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).

ii) Diagnosis and definition of VWD
- VWF antigen *[a]*
- VWF: Ristocetin cofactor activity *[b]*
- Factor VIII *[c]*
- VWF multimeric structure on low resolution gels *[e]*

iii) Diagnosis of VWD types
- Ristocetin Induced Platelet Agglutination (RIPA) *[d]*
- VWF multimeric structure on high resolution gels *[e]*
- Platelet VWF content *[f]*
- Factor VIII binding assay *[g]*

For the use of these tests see the diagnostic flow-chart reported in Figure 3 and also reference 15.

Table 5. Incidence (%) of bleeding symptoms in patients with VWD and in normal subjects (adapted from Federici *et al.*,[15,16] Silwer[21]; Lak *et al.*[22]

	Iranian VWD	*Italian VWD (n = 1234) (*)*			*Scandinavia*	
Symptoms	*Type 3 (n = 348)*	*Type 1 (n = 671)*	*Type 2 (n = 497)*	*Type 3 (n = 66)*	*VWD (n= 264)*	*Normals (n = 500)*
Epistaxis	77	61	63	66	62	5
Menorrhagia	69	32	32	56	60	25
Post-extraction bleeding	70	31	39	53	51	5
Hematomas	n. r.	13	14	33	49	12
Bleeding from minor wounds	n. r.	36	40	50	36	0.2
Gum bleeding	n. r..	31	35	56	35	7
Post-surgical bleeding	41	20	23	41	28	1
Post-partum bleeding	15	17	18	26	23	19
Gastrointestinal bleeding	20	5	8	20	14	1
Joint bleeding	37	3	4	45	8	0

n.r.: not reported; (*)bleeding symptoms in Italian patients have been recently recalculated according to the updated results of the Italian Registry of VWD and therefore are different from previously reported[1]

Table 7a. Basic and discriminating laboratory assays for the diagnosis of VWD.

Test	*Pathophysiologic significance*	*Diagnostic significance*
Ristocetin Cofactor (VWF:RCo) using formalin-fixed platelets and fixed ristocetin concentration (1 mg/ml)	VWF-GpIb· interaction as mediated by ristocetin *in vitro* (ristocetin at fixed concentration, normal platelets, patient plasma)	*Functional test*; most sensitive screening test
Immunological assay with polyclonal antibody (VWF:Ag)	Antigen concentration	Correlates with VWF:RCo in VWD type 1; reduced ratio VWF:RCo/Ag suggests type 2
FVIII:C level (one-stage assay)	FVIII/VWF interaction	not specific, but useful for patient management
Bleeding time (Ivy method)	Platelet-vessel wall VWF-mediated interaction	not specific; correlates with platelet VWF content in type 1 VWD. Screens for qualitative platelet defects
Ristocetin-Induced palatelet aggregation (RIPA)	Threshold of Ristocetin concentration inducing patient platelet-rich plasma aggregation	Allows the discrimination with type 2B, characterized by reduced threshold
Multimeric analysis	Multimeric composition of VWF	Full range of multimers in types 1, 2M, 2N Loss of high and intermediate multimers in 2A 2B
Platelet VWF	Reflects endothelial stores	Useful to predict responsiveness to desmopressin in type 1
Binding of FVIII to VWF	Interaction of normal FVIII with patient plasma VWF	Allows the discrimination with type 2N, characterized by low binding values

Table 7b. Additional laboratory assays for the diagnosis of VWD.

Test	*Pathophysiologic significance*	*Diagnostic significance*
Binding of VWF to collagen	VWF-collagen interaction	Correlates with VWF :RCo in type 1 VWD; some collagen preparations more sensitive to high molecular weight multimers
Closure time PFA-100	Simulates primary hemostasis after injury to a small vessel	More sensitive than bleeding time in screening for VWD; not tested in bleeding subjects without specific diagnosis; specificity unknown; more data needed before recommendation for clinical laboratory
Monoclonal antibody-based ELISA	Moab against an epitope of VWF involved in the interaction with GpIb·	Correlation with VWF:RCo not confirmed; not suggested instead of VWF:RCo
ELISA-based *VWF:RCo*	Measure interaction between VWF and captured rGpIbα fragment in the presence of ristocetin	Promising new test for using instead of VWF:RCo; more data needed

icance is reported in Table 7a. *VWF antigen (VWF:Ag)* is undetectable in type 3 VWD, whereas it may be low in type 1 and low or normal in type 2. The assay for *ristocetin cofactor activity (VWF:RCo)* explores the interaction of VWF with the platelet GPIbα and is still the standard method for measuring VWF activity. It is based on the property of the antibiotic ristocetin to agglutinate formalin-fixed normal platelets in the presence of VWF. Besides the original method, new *ex vivo* methods have been proposed to measure the interactions between VWF and its platelet receptors in the presence of ristocetin, as reported in Table 7b. In patients with a normal VWF structure (type 1 VWD), VWF:RCo values are similar to VWF:Ag. Levels lower than VWF:Ag (VWF:RCo/Ag ratio < 0.7) are characteristic of type 2 VWD, as recently reported in the guidelines for diagnosis and treatment of VWD in Italy. *FVIII:C* plasma levels are very low (1-5 %) in patients with type 3 VWD. In patients with type 1 or type 2 VWD, FVIII may be decreased to a variable extent but is sometimes normal. 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.

Characterization of the type. For a correct diagnosis of patients with VWD and to establish their treatment, other assays are used to define specific subtypes. Ristocetin-induced platelet agglutination (RIPA) is measured by mixing in the aggregometer different concentrations of ristocetin and patient platelet rich-plasma (PRP). Results are expressed as the concentrations of ristocetin (mg/mL) able to induce 30% agglutination. Most VWD types and subtypes show a low response to ristocetin, but an important exception is type 2B VWD, in which there is hyper-responsiveness to ristocetin, due to a higher than normal affinity of VWF for platelet GPIbα. *VWF multimeric analysis* with high-resolution agarose gels identifies 1 and 2 VWD subtypes better. *Platelet VWF* plays an important role in primary hemostasis, since it can be released from α-granules directly to the site of vascular injury. On the basis of its measurement, type 1 VWD can be classified in three subtypes: *type 1 platelet normal*, with a normal content of functionally normal VWF; *type 1 platelet low*, with low concentrations of functionally normal VWF; *type 1 platelet discordant*, with normal concentrations of dysfunctional VWF.[23] The Factor VIII binding assay measures the affinity of VWF for FVIII. In this assay, anti-VWF antibody is coated on wells of a micro titer plate and test plasma is added to the wells. The factor VIII/WF complex from the plasma is bound by the antibody after which factor VIII is removed from the complex by a high ionic strength buffer.Excess recombinant FVIII (rFVIII) is then added and, after removal of unbound rFVIII, the VWF and the bound rFVIII are assayed. This assay allows type 2N VWD to be distinguished from mild to moderate hemophilia A.

Additional tests for VWD diagnosis include the *Closure Time (CT)* and assays of VWF activity based on binding to collagen *(VWF:CB)*. Evaluation of CT with the Platelet Function Analyzer (PFA-100) gives rapid and simple measure of VWF dependent platelet function at high shear stress. This system is sensitive and reproducible for VWD screening, even though the CT is normal in type 2N. Assays are also available for VWF:CB and the ratio of VWF:CB to VWF:Ag appears useful for distinguishing types 1 and 2 (Table 7b). Neither assay has been well standardized yet and thus are not officially approved by the Scientific Standardization Committee Sub-

Committee on VWF of the International Society of Thrombosis and Haemostasis. Differential diagnosis of VWD types can be done by using these laboratory tests, following the flow chart already proposed by the guidelines for diagnosis and treatment of VWD in Italy (Figure 3). Type 3 VWD can be diagnosed in case of undetectable VWF:Ag. A proportionate reduction of both VWF:Ag and VWF:RCo with a RCo/Ag ratio > 0.7 suggests type 1 VWD. If the VWF:RCo/Ag ratio is < 0.7 type 2 is diagnosed. Type 2B VWD can be identified in case of an enhanced RIPA (<0.8 mg/mL) while type 2A and 2M cause low RIPA (>1.2 mg/mL). Multimeric analysis in plasma is necessary to distinguish between type 2A VWD (lack of the largest and intermediate multimers) and type 2M VWD (all the multimers present). Type 2N VWD can be suspected in case of discrepant values for factor VIII and VWF:Ag (ratio <1) and diagnosis should be confirmed by the specific test of VWF:FVIIIB. In type 1 VWD the ratio between FVIII and VWF:Ag is always >1 and the severity of type 1 VWD phenotype can usually be evaluated from platelet VWF measurements.[15]

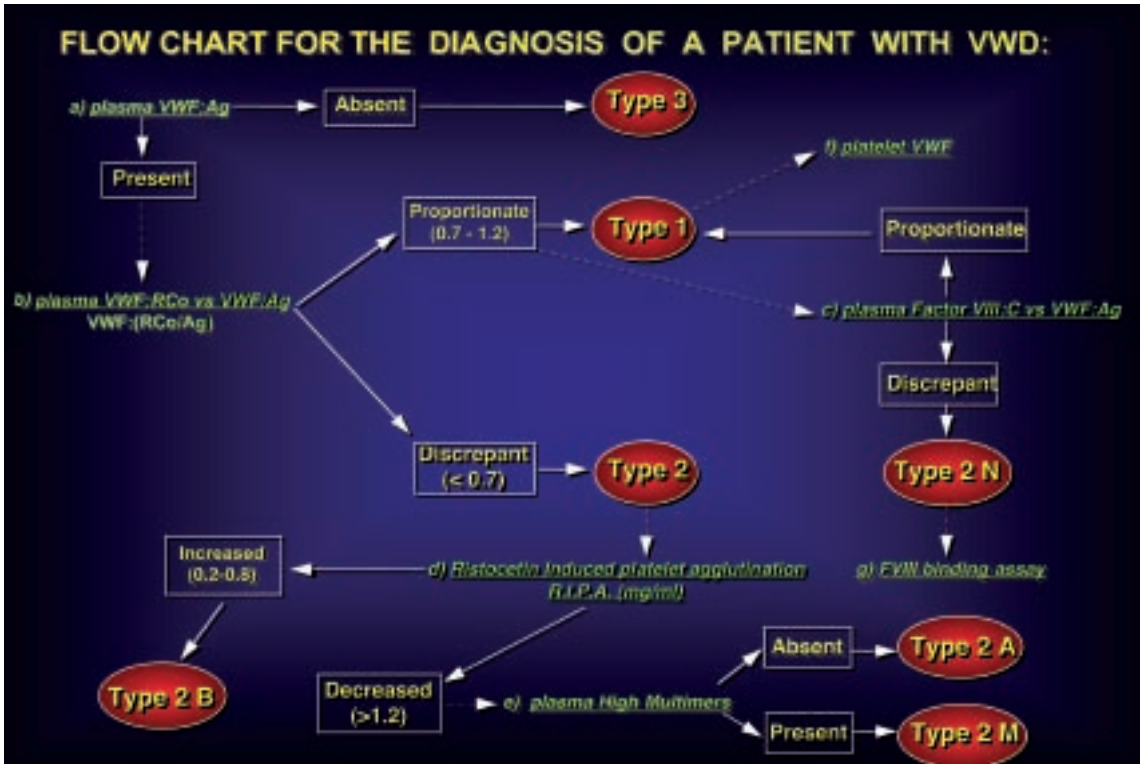

Figure 3. Flow chart proposed for the diagnosis of different VWD types. Type 3 VWD can be diagnosed in case of unmeasurable VWF:Ag (a). A proportionate reduction of both VWF:Ag and VWF:RCo with a RCo/Ag ratio > 0.7 suggests type 1 VWD (b). If the VWF:RCo/Ag ratio is < 0.7 type 2 is diagnosed. Type 2B VWD (d) can be identified in case heightend RIPA (< 0.8 mg/mL) whereas types 2A and 2M cause low RIPA (> 1.2 mg/mL). Multimeric analysis in plasma (e) is necessary to distinguish between type 2A VWD (lack of the largest and intermediate multimers) and type 2M VWD (all the multimers present). Type 2N VWD can be suspected in case of discrepant values for FVIII (c) and VWF:Ag (ratio <1) and diagnosis should be confirmed by the specific test (g) of VWF:factor VIII binding capacity (VWF:FVIIIB). In type 1 VWD the ratio between Factor VIII and VWF:Ag is always > 1 and the severity of type 1 VWD phenotype can usually be evaluated from platelet VWF (f) measurements.[15]

Molecular and prenatal diagnosis of VWD

Cloning the VWF gene has allowed the identification of several suitable restriction fragment length polymorphisms (RFLP) which demonstrate the co-segregation of VWD phenotype with haplotype-specific RFLP patterns in family members of different kindred with VWD.[13] Knowledge of the crucial segments of VWF involved in the interaction with GPIbα initially prompted the fruitful search for mutations in exon 28 of the VWF gene which encodes for the A1 and A2 domains of mature VWF as reported in Figure 1 (*for review see ref. 13*). The search for mutations has been extended to additional VWF exons encoding for the other functional domains of VWF. The most frequent mutations reported in types 2A, 2B, 2M, 2N are listed in Table 8 according to the specific VWF domains and are currently updated in the web site organized on behalf of the ISTH-SSC on VWF *(www.shef.ac.uk/vwf)*. Most type 2A cases are due to missense mutations in the A1 domain, with R1597W or Q or Y and S1506L accounting for about 60%.[24,25] The majority of type 2B cases are due to missense mutations in the A1 domain, about 90% being caused by R1306W, R1308C, V1316M and R1341Q mutations (Table 8). A few heterogeneous mutations are responsible for type 2M cases and are also located within the A1 domain. Therefore most mutations are expressed and the mutated recombinant VWF have been compared with others found within the same domain.[24] A recurrent mutation in type 2M Vicenza has been recently reported in families from Europe (R1205H), associated with a second nucleotide change (M740I) exclusively identified in some families from the Vicenza area.[26,27] Missense mutations in the FVIII-binding domain at the amino-terminal portion of VWF are responsible for type 2N (*for review see ref. 13*). The genetic causes of type 1 VWD is still elusive in many cases, especially in those with a mild phenotype. More information on the molecular basis of

Table 8. List of most frequent mutations in type 2A, 2B, 2M and 2N according to VWF domains.

Localization of VWF defects	*VWD Types*	*VWF Mutations associated with specific types*
-D2 domain	Type 2A (formerly IIC)	F404insNP -R436del6 -N528S -G550R C623W -A625insG
-D' – D3 domains	Type 2N	R782W -G785E -E787K -C788R C788Y -T791M -Y795C -M800V R816W -R816Q -H817Q -R854Q R854W -C858F -D879N -Q1053H C1060R -C1225G
-D3 domain	Type 2M R1205H -Y1146C (formerly 1 Vicenza) Type 2A C1143Y -C1173R (formerly IIE)	
-A1 domain	Type 2B (formerly IIB)	P1266L -H1268D -C1272G - C1272R M1304insM-R1306Q -R1306L - R1306W R1308C -R1308P -I1309V - S1310F W1313C -V1314F -V1314L - V1316M P1337L -R1341L -R1341Q - R1341W L1460V -A1461V
	Type 2M	G1324S -G1324A -E1359K -F1369I I1425F -Q1191del1-K1408delK
	Type2M/2A	L1276P -R1374C -R1374H -C1458Y R1374R
-A2 domain	Type 2A (formerly IIA)	G1505E -G1505R -S1506L - F1514C K1518E -L1540P -S1543F - Q1556R L1562P -R1597G -R1597Q - R1597W V1604F -V1607D -V1609R - P1627H I1628T -G1629R -V1630F -E1638K L1639P -P1648S -L1657I -V1665E G1672R
-CK domain	Type 2A (formerly IID)	C2773R

For an updated list of VWF mutations according to VWD types the web site www.shef.ac.uk/vwf can be checked

type 1 has been collected by two multicenter international studies. In the European study, recruitment was based on the historical diagnosis of type 1 VWD as made by 12 expert centers, which included 278 affected cases, 312 non-affected family members and 1166 controls.[28] Three broad groups of patients were identified: 53 had a normal multimeric structure, a VWF:RCo/Ag ratio equal or greater than 0.7 and mutations in the VWF gene; 55 had VWF gene mutations but abnormal multimers and a ratio lower than 0.7, and 43 had normal multimers, a ratio equal of greater than 0.7 but no detectable mutation. The Canadian investigators could recruit 123 families for which the idex case had bleeding symptoms and VWF levels between 5 and 50 U/dL.[23] In this study, subjects with abnormal multimeric patterns or other evidence of qualitative defects were excluded. The most important conclusions from both studies are the following: a) despite the selection of patients based on bleeding history, candidate VWF mutations were not found for 27% (Canadian) and 36% (European) of index cases diagnosed with VWD type 1; b) the spectrum of VWD type 1 mutations was different from that found in VWF type 3 (see later), since about 90% of patients in whom mutations were found had at least one missense mutation, often associated with the loss or creation of cysteine residues.Thefore, VWD type 1 is not at all like heterozygous VWD type 3 because VWF defects that occur in VWD type 1 usually is caused by dominant VWF abnormalities that affect VWF secretion or clearance without substantially altering multimeric patterns or platelet binding. In type 3 VWD, partial or total gene deletions have been initially reported (for review)[30]. Notably, homozygous states for gene deletion may be associated with the appearance of allo¬antibodies against VWF, which may render replacement therapy ineffective and stimulate anaphylactic reactions to treatment.[13] Gene defects of type 3 VWD patients from different populations have now been studied, but there was no founder effect and mutations were distributed throughout the entire VWF gene.[31] Compared to hemophilia, most VWD patients show relatively mild bleeding symptoms. Therefore, prenatal diagnosis is required mainly in case of parents already known to be carrier of VWD type 3, with gene defects identified in their first affected child. Neonatal diagnosis can be performed in case of children born from parents with VWF defects already characterized, but phenotypic diagnosis of VWD should be always confirmed later on in the child and compared with the other affected members within the same family. Since young children with VWD type 3 might carry deletions of VWF gene that predispose to the alloantibodies to VWF, every new child with VWD type 3 should be intensively investigated by searching fordeletions, as reported,[32] before starting extensive therapy with exogenous VWF concentrates.

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