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REVIEW ARTICLE

von Willebrand's disease diagnosis and laboratory issues

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Summary. In this paper, the recent developments in the diagnosis and laboratory issues of von Willebrand's disease (VWD) are presented. Dr. Castaman reviews the functional tests available for the diagnosis of VWD and their pathophysiological significance, focusing on which tests are best used in the diagnosis and classification of VWD. Dr Montgomery reviews an emerging issue that is accelerated clearance of von Willebrand factor (VWF) occurring in some variants of VWD. This phenotype can be suspected by the presence of an increased ratio between the VWF propeptide and the VWF antigen. These patients have typically a robust, but short-lived increase of FVIII and VWF after desmopressin. Dr Meschengieser reviews the determinants of bleeding after surgery in patients with VWD, emphasizing the role of bleeding history in predicting this risk.

Keywords: von Willebrand factor, von Willebrand disease, inherited bleeding disorders, desmopressin

Functional tests for the diagnosis of von Willebrand's disease

Giancarlo Castaman

von Willebrand factor (VWF) is a multimeric glycoprotein synthesized by endothelial cells and megakaryocytes and plays two major functions in haemostasis [1]. First, it is essential for platelet adhesion to the subendothelium and platelet-to-platelet interactions as well as platelet aggregation in vessels in which rapid blood flow results in elevated shear stress. Adhesion is promoted by the interaction of the A1 domain of the VWF with glycoprotein Ib (GpIb) on platelet membrane and binding to collagen present in the subendothelial matrix mainly through the A3 domain of VWF. Furthermore, GpIb and VWF are also necessary for platelet-to-platelet cohesion [2]. Aggregation of platelets within the growing haemostatic plug is promoted by the interaction with a second receptor on platelets, the GpIIb/IIIa (or integrin αIIbβ3) which after activation binds to VWF and fibrinogen, recruiting more platelets into a stable plug. Both these binding activities of VWF are the highest in the largest VWF multimers. Second, VWF is a 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 monomer of VWF has one binding domain, located in the first 272 amino acids of the mature subunit (D'–D3 domain) able to bind one FVIII molecule.

These functions are explored by an array of laboratory assays, but no one reflects the whole spectrum of VWF activities. The deficiency or abnormal function of VWF causes von Willebrand's disease (VWD), the most frequent inherited bleeding disorder [3]. VWD is heterogeneous because molecular defects can occur in more than one of the functional domains of the multimeric glycoprotein. As a consequence VWD is classified in three different types: partial quantitative deficiency (type 1), qualitative deficiency (type 2) and complete quantitative deficiency (type 3). Tests for the correct diagnosis of VWD ideally have to

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explore the most important VWF properties: the antigenic level of VWF (VWF:Ag); the VWF–platelet GpIb interaction (VWF:RCo); the VWF–subendothelium–collagen interaction (VWF:CB); the VWF–FVIII interaction (VWF:FVIIIB) and the capacity of VWF to be organized into multimers. Factor VIII procoagulant activity (FVIII:C) is also included in the diagnostic work-up, because it reflects the ability of VWF to protect it from degradation and is a useful complement in suspecting type 2N variants (see below).

'*Functional tests*' *for laboratory diagnosis of VWD*

Table 1 summarizes the functional test for VWD diagnosis. With these tests, a useful classification in VWD types can be reached. The VWF:RCo explores the interaction of VWF with the platelet GpIb/IX/V complex and is still the standard method for measuring VWF activity. Abnormal VWF:RCo/VWF:Ag ratio (<0.6) usually indicates the presence of qualitative variants (type 2 VWD). Both aggregometric and turbidimetric methods appear useful. The aggregometric test however may be difficult to standardize and presents a low sensitivity at very low VWF concentrations (usually <10 U dL^{-1}). Furthermore, careful calibration and standardization are essential [4]. In the recent years, the sensitivity of VWF:RCo has been significantly improved by using ELISA assays with recombinant GpIb [5,6]. The improved sensitivity of the ELISA VWF:RCo assay should enhance the reliability of the ratio determination, which however is difficult to obtain at very low VWF:Ag concentration.

VWF:CB is particularly sensitive to VWD variants characterized by the absence of larger VWF multimers, and it has been suggested that a mixture of type I (95%) and type III (5%) collagen is best used for this purpose [7]. Thus, VWF:CBA is sometimes used as an alternative to multimeric analysis, and the ratio of VWF:CB to VWF:Ag levels appears to be useful for distinguishing between type 1 and 2 VWD [8]. However, this concept has been recently challenged by the identification of rare VWD mutations located in A3 domain (W1745C and S1783A) characterized by a normal multimeric pattern, but with a discrepant low VWF:CB/VWF:Ag ratio [9]. In some of these patients, the diagnosis of VWD could be missed as VWF:RCo level may be border-line. In general, this test seems not to provide substantial advantage compared with VWF:RCo, and furthermore, it is not well standardized yet. Previous studies have shown that a wide variety of animal sources and collagen types are used in this test and that this significantly affects the results [10].

The ristocetin-induced platelet aggregation (RIPA) using patient platelets explores the threshold ristocetin concentration, which induces aggregation of the patient platelet-rich plasma. Aggregation occurring

Table 1. 'Functional' laboratory assays for the diagnosis of von Willebrand's disease (VWD).

Test	Pathophysiological significance	Diagnostic significance
Ristocetin cofactor (VWF:RCo) using formalin-fixed platelets and fixed ristocetin concentration (1 mg mL^{-1})	VWF–Gp Ib interaction as mediated by ristocetin *in vitro* (ristocetin, normal platelets, patient's plasma)	'Functional test'; most sensitive screening test; turbidimetric method available with similar results
ELISA-based VWF:RCo	Measures' interaction between VWF and captured rGp Ibα fragment in the presence of ristocetin	Promising new test proposed as a substitute for VWF:RCo; higher sensitivity; validation on larger patient series required
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; not yet well standardized
Ristocetin-induced platelet aggregation (RIPA) using patient platelets	Threshold ristocetin concentration inducing patient's platelet-rich plasma aggregation	Allows the discrimination of type 2B, characterized by reduced threshold; absent in type 3 at every ristocetin concentration
Binding of VIII:C to VWF	Interaction of normal FVIII with patient plasma VWF	Allows the identification of type 2N, characterized by low binding values and suspected in case of reduced VIII:C/VWF:Ag
Closure time PFA-100	Simulates primary haemostasis after injury to a small vessel	More sensitive than BT in screening for VWD; not tested in bleeding subjects without specific diagnosis; specificity unknown; more data needed before recommendation for clinical laboratory
Propeptide assay	Measures the amount of VWFpp released in plasma	Increased VWFpp/VWF:Ag ratio identifies patients with shortened VWF survival after desmopressin; still for research purposes

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at low concentrations identifies type 2B VWD cases, in whom desmopressin may cause thrombocytopenia. This test is critical, especially when multimeric pattern evaluation is not feasible.

An additional test typically used in VWD diagnosis is the closure time (CT). The evaluation of CT with PFA-100 (platelet function analyzer) (Dade, Miami, FL, USA) allows rapid and simple determination of VWF-dependent platelet function at high-shear stress. This system was demonstrated to be sensitive and reproducible when screening for severe reduction in VWF, but it is normal in type 2N VWD and it has been questioned as an aid in screening for mild VWF deficiencies [11].

Type 2N VWD is suspected when the FVIII:C level is disproportionately decreased compared with levels of VWF:Ag and VWF:RCo [12]. Usually, plasma VWF:Ag levels are normal or subnormal depending on the ABO blood group [13] and genotype of the patient (i.e., presence of a silent allele) [14]. As a consequence, the FVIII:C to VWF:Ag ratio is reduced (<0.5) in all the patients with type 2N VWD. The diagnosis relies on the measurement of the affinity of VWF to FVIII (VWF:FVIIIB), which is markedly decreased. The original assay is a solid phase immunoassay, but several modifications have enabled simplification and even automation of the assay [15–18].

Recently, the assay for von Willebrand factor propeptide (VWFpp) has been developed. Even though the assay is based on an ELISA, it provides information on VWF 'function' of some VWD variants. The half-life of VWFpp is around 2–3 h, whereas normal VWF has a half-life of 8–12 h. An increased ratio of steady-state plasma VWFpp to VWF:Ag has been demonstrated to identify patients and VWF mutations with increased VWF clearance [reviewed in 20]. Typically, they show a severe VWF reduction at baseline and a marked but short-lived VWF increase after desmopressin. Thus, the measurement of VWFpp in plasma could help to identify the pathophysiological mechanism responsible for low VWF in a given patient, predicting his/her response to desmopressin. The assay is still used for research purposes, but it is likely that it could be soon widely available.

Conclusions

While VWF:RCo appears to still be a useful screening test for VWD in a patient investigated for a possible bleeding disorder, an array of different tests is required for the full characterization of a patient with VWD. This approach is still fundamental to individualize the most appropriate therapeutic approach. It should be borne in mind, however, that most FVIII/VWF concentrates are labelled according to their FVIII:C and VWF:RCo content, and these tests appear crucial in monitoring the safety and efficacy of replacement therapy in VWD.

von Willebrand's disease caused by accelerated clearance of VWF

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Type 1 VWD has a similar reduction of VWF protein (VWF:Ag) and VWF activity (VWF:RCo) that has usually been ascribed to the reduced synthesis of structurally normal VWF. Twenty-five years ago, a subgroup of type 1 VWD was first identified as having platelets with normal levels of stored VWF suggesting 'normal synthesis' of VWF, [21,22], but the cause of this was not clear until more recently. A variant of VWD – termed the Vicenza variant – was then identified and characterized by the *in vivo* response to desmopressin, in which the levels of VWF were dramatically increased, even more than normal, after desmopressin and the plasma VWF half-life was reduced. VWF levels were only transiently normalized [23,24]. When proVWF is synthesized, equal amounts of VWF monomer and the VWF propeptide, VWFpp, are synthesized, stored and released [25]. A ratio of the plasma concentration of VWFpp and VWF (VWFpp/VWF:Ag) at steady-state is therefore approximately 1.0 [26]. When VWF has a reduced half-life, the ratio is increased so that the steady-state VWFpp/VWF:Ag increases [19,27]. When these assays were carried out on a large population of type 1 VWD patients, 12% were found to have an abnormal VWFpp/VWF:Ag ratio suggesting accelerated clearance. Mutations have been demonstrated in the D3 domain (W1144G, C1130G/F/R, Vicenza variant R1205H) and the D4 domain (S2179F) [19,20,28].

Patients with type 2B VWD or platelet-type pseudo-VWD have accelerated clearance of their VWF and therefore have an elevated VWFpp/VWF:Ag ratio. In some patients with type 2A VWF, accelerated clearance is observed, but these have not been extensively studied except in recent abstracts [29].

The initial mouse model of mild VWD was the RIIIS/J mouse, in which the VWF is reduced secondary to accelerated clearance [30,31]. The cause of the reduced VWF is secondary to a glycosylation defect in which *N*-acetylgalactosaminyl transferase, B4GALNT2, is expressed ectopically in endothelial cells resulting in accelerated clearance in VWF. This is an example of a non-VWF linked cause of low VWF.

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Although in humans such a defect has not been described, the glycosylation of VWF differs based on blood type. Thus, VWF levels are reduced in individuals who have a blood group O, and this reduction is secondary to a reduced half-life with a corresponding increase in VWFpp/VWF:Ag ratio [32,33].

Accelerated clearance of VWF is also seen in individuals with an autoimmune antibody to VWF [6]. This results in a rapid clearance of VWF but not VWFpp. Thus, the VWFpp/VWF:Ag ratio is markedly increased.

Clinical syndromes with accelerated clearance of VWF are therefore suggested by (i) an elevated VWF/VWF:Ag ratio, (ii) normal platelet VWF:Ag with reduced plasma VWF, (iii) an excessive desmopressin response between steady-state plasma VWF and the 30 min or 1 h assay of plasma VWF or (iv) a reduced VWF survival after desmopressin. Interestingly, when VWF has increased clearance, FVIII also appears to have accelerated clearance. The converse is not usually seen. If there is an antibody to FVIII (acquired haemophilia), VWF is not usually reduced. With the exception of an acquired antibody to VWF, other causes of reduced VWF are the result of an abnormal host VWF. Treatment with exogenous VWF would be expected to be normal. In contrast, acquired autoimmune VWD will result in accelerated clearance of exogenous VWF. Although infused r-FVIII has a reduced survival in the absence of VWF, that survival is not affected by an antibody to VWF. Thus, continuous FVIII has been clinically effective in some cases of acquired autoimmune VWD.

Clinical and laboratory markers predicting VWD-associated bleeding in surgery

Susana S. Meschengieser, Adriana I. Woods, Maria A. Lazzari

Few studies have established the biological predictive markers of surgical bleeding in VWD [34,35]. Ziv reported [36] that postoperative bleeding could have been avoided in 83% of the cases if a preoperative family or bleeding history had been obtained. However, until recently, no quantitative description of bleeding symptoms in VWD has been available to fully appreciate their diagnostic relevance to discriminate between a significant bleeding history and trivial symptoms in VWD. A further question for the clinician is whether patients with a history of severe bleeding may be at higher risk of bleeding during invasive procedures (e.g., tooth extraction, surgery). This is clinically relevant, because the laboratory evaluation of mild bleeding disorders has no practical value as a guideline for the optimal antihaemorrhagic prophylaxis, as abnormal laboratory tests do not predict clinical bleeding. In the MCMDM-1VWD study, by using a standardized bleeding questionnaire to establish a bleeding score (BS), the association between spontaneous, mucocutaneous bleeding symptoms (epistaxis, cutaneous bleeding and menorrhagia) and bleeding after surgery or tooth extraction in patients with type 1 VWD was evaluated [37]. Interestingly, the BS showed a predictive value similar to VWF level for bleeding after tooth extraction, but was superior to VWF measurement for the prediction of bleeding after surgery. Although these findings are retrospective and therefore could not be immediately applied to the clinical practice, they suggest that antihaemorrhagic prophylaxis should be always considered in VWD patients with increased BS [37].

We have retrospectively evaluated the predictive markers of peri-operative major haemorrhages in a large single-centre population (n = 2455) of patients with VWF:RCo <50 IU dL^{-1} and type 1 VWD, possible type 1 and type 2 VWD. Diagnostic criteria for type 1 and possible type1 (VWF:RCo 15–30 IU dL^{-1} and 31–49 IU dL^{-1}, respectively), VWF:RCoVWF:Ag ratio >0.6 and type 2 with VWF:RCo/VWF:Ag <0.6 were used. For each patient, the severity of each symptom was summarized using the BS system ranging from 0 to 3 [38], according to ISTH recommendations [39], and taking into account the most severe episode for each symptom [40]. The BS was considered useful for the identification of a significant bleeding history (≥5 in females and ≥3 in males) for the diagnosis of type 1 VWD. This approach can also be useful in all VWD types [41,42].

Patient characteristics of group A (without surgical bleeding) and group B (with surgical bleeding) are shown in Table 2. Major surgical bleeding appeared in 26% of all type1 patients (32.6% type1 and 24.8% possible type1) and 54.9% of type 2. Considering surgeries, major haemorrhage was observed in 17.8% of all type1 and 50% of type 2 (Table 3). No significant differences were observed in family history, blood group, age, gender, BS, the number of bleeding sites (Table 1) and laboratory parameters (Table 4), between groups A and B. FVIII levels were not useful as predictors of postoperative bleeding.

In possible type 1, group B, a higher frequency of bleeding after tooth extraction (Table 5) and a higher BS in females were found. Postpartum bleeding was the most frequent symptom in type 2 VWD, although not significant. Caesarean section and

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Table 2. Patient characteristics grouped according to the absence or presence of surgical bleeding (surgical event was excluded from the analysis).

	VWD patients					
	Group A			Group B		
Clinical profile	Type 1 (n = 33)	Possible type 1 (n = 197)	Type 2 (n = 23)	Type 1 (n = 16)	Possible type 1 (n = 65)	Type 2 (n = 28)
Females (%)	61.3	74.6	65.2	56.2	70.9	67.8
O blood group (%)	86.2	74.0	80.0	70.0	78.0	70.0
Median age, years (range)	36 (5–62)	31 (6–64)	25 (7–68)	34 (6–63)	32 (7–66)	31 (7–82)
Positive family history (%)	85.2	57.2	63.2	81.8	57.8	84.2
Surgical procedures (n)	51	297	29	25	125	47
BS median (range)						
Females	3 (1–6)	2 (0–6)	3 (1–6)	3 (1–9)	3 (1–8)*	5 (4–12)
Males	1 (1–4)	1 (0–4)	1 (1–4)	2 (1–4)	1 (1–4)	5 (3–8)
BS ≥ 5 (%) females	25.0	9.3	33.3	33.3	21.3	36.8
BS ≥ 3 (%) males	16.6	12.5	37.5	42.8	5.5	70.0
Bleeding sites mean and SD	2.2 ± 1.4	2.1 ± 1.3	2.5 ± 1.6	2.4 ± 1.5	2.19 ± 1.4	3.0 ± 1.6
≥3 bleeding sites (%)	36.3	33.0	47.8	47.0	40.0	53.6

*P: possible type 1 group B vs. possible type 1 group A = 0.027.

Table 3. Major surgical haemorrhages in von Willebrand's disease (VWD) patients.

Surgeries	Type 1 (%)	Type 2 (%)	P value	RR (95% CI)
Major	12.6	41.6	0.000	3.66 (1.79–7.49)
Minor	15.3	38.0	0.027	2.74 (1.25–5.99)
Adenotonsillectomies	22.3	62.5	0.002	4.57 (1.78–11.7)
Caesarean section	24.6	66.6	0.005	4.15 (1.58–10.9)
Total	17.8	50.0	0.000	3.59 (2.4–5.38)

Table 4. Laboratory parameters of the patients grouped according to surgical bleeding tendency.

	VWD patients					
	Group A			Group B		
Laboratory tests	Type 1 (n = 33)	Possible type 1 (n = 197)	Type 2 (n = 23)	Type 1 (n = 16)	Possible type 1 (n = 65)	Type 2 (n = 28)
FVIII:C (IU dL^{-1})	35.2 ± 11.4	48.6 ± 11.0	60.2 ± 26.8	35.3 ± 9.8	51.0 ± 11.4	55.5 ± 30.3
VWF:Ag (IU dL^{-1})	28.7 ± 7.9	43.3 ± 9.5	81.3 ± 39.5	27.9 ± 8.6	41.2 ± 8.4	76.9 ± 34.4
VWF:RCo (IU dL^{-1})	26.0 ± 5.2	40.6 ± 5.1	21.3 ± 14.0	26.2 ± 8.4	40.5 ± 4.6	22.9 ± 19.4

VWD, von Willebrand's disease; VWF, von Willebrand factor.

Table 5. Bleeding symptoms, according to the absence or presence of major surgical haemorrhages.

	VWD patients					
	Group A			Group B		
Clinical profile	Type 1 (n = 33)	Possible type 1 (n = 197)	Type 2 (n = 23)	Type 1 (n = 16)	Possible type 1 (n = 65)	Type 2 (n = 28)
Bleeding after tooth extraction (%)	40.1	35.8	62.5	62.5	61.2*	70.5
Epistaxis (%)	48.3	39.4	60.8	50.0	44.6	53.5
Menorrhagia (%)	57.8	51.3	71.4	62.5	55.3	64.7
Postpartum haemorrhage (%)	25.0	24.3	28.5	37.5	45.0	61.5

VWD, von Willebrand's disease.
*P: possible type 1 group B vs. possible type 1 group A < 0.000.

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adeno-tonsillectomy showed the highest frequency of major haemorrhage.

Personal bleeding history, especially bleeding after tooth extraction in type 1 VWD [43], and post-partum haemorrhage in type 2 and the type of surgery appear to be predictive markers of major postoperative haemorrhage. The relative risk (RR) between type 1 and 2 was as expected. Possible type 1 VWD patients showed similar risk of peri-operative major bleeding compared with type 1, again emphasizing the superiority of symptoms over laboratory parameters. Neither the family history nor laboratory parameters could anticipate surgical bleeding.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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