CHAPTER 6

Von Willebrand factor propeptide and the phenotypic classification of von Willebrand disease

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Abstract

The ratios between von Willebrand factor propeptide (VWFpp) or FVIII coagulant activity (FVIII:C) and VWF antigen (VWF:Ag) reflect synthesis, secretion and clearance of VWF. We aimed to define the pathophysiology of 658 types 1, 2 and 3 von Willebrand disease (VWD) patients with VWF levels ≤30 U/dL from the "Willebrand in the Netherlands" (WiN-)study using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios. We also evaluated the use of VWFpp in the classification and diagnosis of VWD. Based on the ratios, reduced VWF synthesis was observed in 18% (67/380) of type 1 patients and in only 2% (5/240) of type 2 patients. A significant proportion of type 3 patients had detectable VWFpp (14/37, 41%). These patients had a lower bleeding score than type 3 patients with complete absence of both VWF:Ag and VWFpp: 14.0 vs. 19.5, p=0.025. The majority of these patients had missense mutations associated with rapid VWF clearance, whereas "true" type 3 patients were homozygous for null alleles. In conclusion, VWFpp identified severe type 1 VWD with very low VWF levels in patients who had previously been classified as type 3 VWD. This study underlines the clinical significance of the VWFpp assay in the diagnosis and classification of VWD.

Introduction

Von Willebrand factor (VWF) is a large multimeric glycoprotein that mediates platelet adhesion and aggregation at sites of vascular injury, and serves as the carrier of factor VIII (FVIII) to prevent its premature clearance [1,2]. Synthesis of VWF is restricted to endothelial cells and megakaryocytes [3], where it is formed as a precursor protein, pre-proVWF, with a signal peptide, a propeptide and a mature subunit [4]. After translocation to the endoplasmic reticulum the signal peptide is cleaved, and the pro-VWF undergoes extensive post-translational modifications, including dimerization in the endoplasmic reticulum and multimerization in the Golgi system [5,6]. The VWF propeptide (VWFpp) is subsequently removed from the mature VWF, however it stays noncovalently bound [7]. Part of VWF is secreted constitutively into the plasma and the remaining part is stored in Weibel-Palade bodies in the endothelium or α-granules in megakaryocytes [8.9]. After release into the circulation, the VWFpp and the mature VWF completely dissociate [8].

Plasma VWF concentrations represent a balance between synthesis, secretion and clearance from the circulation. VWF and VWFpp are secreted equimolarly, but are cleared independently with estimated half-lives of 8-12 hours for VWF and 2 hours for VWFpp [10,11]. The ratio between VWFpp and VWF antigen (VWF:Ag) can therefore be used to assess the rates of synthesis, secretion and clearance of VWF [10,12,13]. In addition to VWFpp, the ratio between FVIII coagulant activity (FVIII:C) and VWF:Ag can also be used to assess VWF synthesis and clearance [14]. Since FVIII and VWF circulate in blood as a complex and are cleared together, their half-lives are strongly related [15]. VWF is deficient or defective in von Willebrand disease (VWD), the most com-

mon inherited bleeding disorder [16,17]. Low VWF levels are caused by reduced VWF synthesis or secretion, increased VWF clearance, or a combination thereof [18,19]. Recently, Eikenboom *et al.* have shown that the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios represent the pathophysiology of VWD and correspond to different *VWF* gene mutations in type 1 VWD [20]. However, it is still unknown whether these ratios can also provide insight into the pathophysiological mechanism in types 2 and 3 VWD.

VWD patients are classified as type 1, 2 or 3 VWD according to their remaining level of functional VWF [16,21]. Type 1 VWD is characterized by partially reduced VWF levels, while type 3 VWD show completely absent VWF plasma levels. In type 2 VWD, functionally abnormal variants of VWF are synthesized. Type 2 VWD is subclassified as 2A, 2B, 2M or 2N, based on the type of functional defect in VWF [16,21]. In some patients it is difficult to classify VWD correctly, as the available diagnostic laboratory tests do not provide adequate information to distinguish between VWD (sub)types. In addition, these laboratory assays are less sensitive in measuring extremely low VWF and FVIII levels and are therefore less reliable.

In this large nationwide cross-sectional Willebrand in the Netherlands (WiN)-study, we aimed to define the pathophysiology of type 1, 2 and 3 VWD using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios irrespective of the *VWF* gene mutation. In addition, we evaluated if the use of VWFpp can improve the diagnosis and classification of VWD.

Methods

Participants

In the nationwide cross-sectional "Willebrand in the Netherlands" (WiN-)study 804 VWD patients who had previously been diagnosed with types 1, 2 or 3 VWD, were included [23-25]. The inclusion criteria for the WiN-study were (1) hemorrhagic diathesis or a family history of VWD and (2) historically lowest VWF levels ≤30 U/dL (VWF:Ag and/or VWF:RCo) and/or FVIII:C levels ≤40 U/dL. Patients were excluded if they were known to have other hemostatic disorders resulting in hemorrhagic diathesis. The Medical Ethical Committees at all participating centers approved this study, and all participants gave informed consent.

For the current study, only patients from whom plasma was obtained were selected (n=681). Exclusion criteria were pregnancy (n=7) and the recent use of desmopressin or replacement therapy at the time of blood sampling (n=16). Therefore, a total of 658 VWD patients were included in the current study.

Assessment of bleeding symptoms

All patients completed a questionnaire regarding bleeding episodes, treatment of VWD, co-morbidity, and Quality of Life [24-27]. To calculate a bleeding score (BS), as

previously described by Tosetto [28], we used information on the severest life-time event of each of twelve specific bleeding symptoms. To avoid prophylaxis-bias, bleeding symptoms were not scored if patients had received prophylactic desmopressin or prophylactic replacement therapy before a surgical intervention, dental extraction or delivery [24,29]. However, if bleeding did occur despite prophylactic treatment, this bleeding was scored according to the Tosetto BS.

Laboratory measurements

Patients' plasma was obtained at inclusion in the study. Venous whole blood was collected in 0.105M sodium citrate tubes and centrifuged twice at 2200x *g* for 10 minutes at room temperature and stored at -80°C. VWF:Ag, VWF activity (VWF:Act), FVIII:C, VWF binding to FVIII (VWF:FVIIIB), and VWF multimers were determined centrally at the Erasmus University Medical Center, Rotterdam, as described [24]. VWFpp was measured centrally at the Leiden University Medical Center, Leiden. This assay was also used in the previous paper from Eikenboom *et al.* [20], and was measured at the same center. VWFpp antigen was determined with an ELISA using antibodies from Sanquin (Amsterdam, the Netherlands) as described [10,20]. First microtiter plates were coated with antibody CLB-Pro 35 overnight at 4°C, then blocked with 1% Bovine Serum Albumin (BSA) at room temperature for 2 hours. Next the diluted samples were incubated for 2 hours at 37°C. VWFpp was detected with peroxidase-conjugated antibody CLB-Pro 14.3. Pooled normal plasma was used to create a standard curve. At the time of the study, no international standard was available for VWFpp and therefore the plasma pool was arbitrarily set at 100 U/dL. Details on the blood-sampling procedure and laboratory measurements at inclusion in the study have been described in more detail by de Wee *et al* [24].

Definitions

Determination of VWD type was based on the current ISTH guidelines [16], using centrally measured plasma concentrations of VWF:Ag, VWF:Act and FVIII:C; VWF multimer patterns; VWF:FVIIIB assay; and locally performed ristocetin-induced platelet aggregation (RIPA) tests [24].

Type 1 VWD patients had a VWF:Act/VWF:Ag ratio ≥0.70, whereas type 2 patients had a ratio <0.70. If type 2 patients had normal multimers they were classified as 2M. Type 2A or 2B VWD patients showed abnormal multimers. If the locally performed RIPA test showed increased VWF affinity for platelets, patients were classified as type 2B. Type 2N patients had a FVIII:C/VWF:Ag ratio <0.70, and VWF:FVIIIB <60%. Type 3 VWD was defined as having both VWF:Ag and VWF:Act levels <5 IU/dL, irrespective of FVIII:C level.

The normal range (2.5th to 97.5th percentile) used for VWFpp was 82-173 U/dL, for the VWFpp/VWF:Ag ratio 0.8-2.2, and for the FVIII:C/VWF:Ag ratio 0.6-1.9, as reported before by Eikenboom *et al*. [20].

Statistical methods

Descriptive statistics for categorical data are presented as numbers with percentages (n, %). As data were non-normally distributed, continuous variables are presented as median with 25-75% interquartile ranges [IQR]. For comparison of proportions, chi-squared tests were used. The Kruskal-Wallis test was used to test differences in VWF:pp, VWFpp/VWF:Ag ratio and FVIII:C/VWF:Ag ratio between types 1, 2A, 2B, 2M, 2N and 3 VWD. Mann-Whitney U-test were applied to detect differences between types and to compare bleedings score between groups. Statistical analyses were performed with SPSS for Windows, version 21.0 (SPSS Inc, Chicago, USA). A p-value <0.05 was considered statistically significant.

Results

A total of 658 VWD patients were included in the current analyses, 381 of whom had type 1 VWD, 240 type 2 VWD, and 37 type 3 VWD, according to the current ISTH guidelines [16]. Baseline characteristics are presented in table 1.

VWF parameters and ratios per type of VWD

In type 1 patients, median VWFpp was 91 U/dL [IQR 68-116], median VWFpp/VWF:Ag ratio 2.2 [IQR 1.7-3.1] and median FVIII:C/VWF:Ag ratio 1.8 [IQR 1.4-2.3] (Figure 1A and table 2). This is in accordance with previously published data by Eikenboom *et al.*[20]. In type 2 patients, median VWFpp was 104 U/dL [81-136], which was higher than in type 1 patients (p<0.001). Median VWFpp/VWF:Ag ratio was 4.5 [3.2-6.0] and median

Table 1. Baseline characteristics

Characteristics			VWD Patients (n=658)
Age (years), median (range)			43.5 (1-83)
Male sex, n (%)			251 (38)
VWD type, n (%)			
	1		380 (58)
	2		241 (37)
		2A	*158*
		2B	*42*
		2M	*27*
		2N	*14*
	3		37(6)
Blood group O, n (%)*			398(61)
VWF:Ag (IU/dL), median [IQR]			29[18-45]
VWF:Act (IU/dL), median [IQR]			22 [8-53]
FVIII:C (IU/dL), median [IQR]			51 [32-73]
Bleeding score, median [IQR]†			11 [6-16]

*4 missing, †32 missing

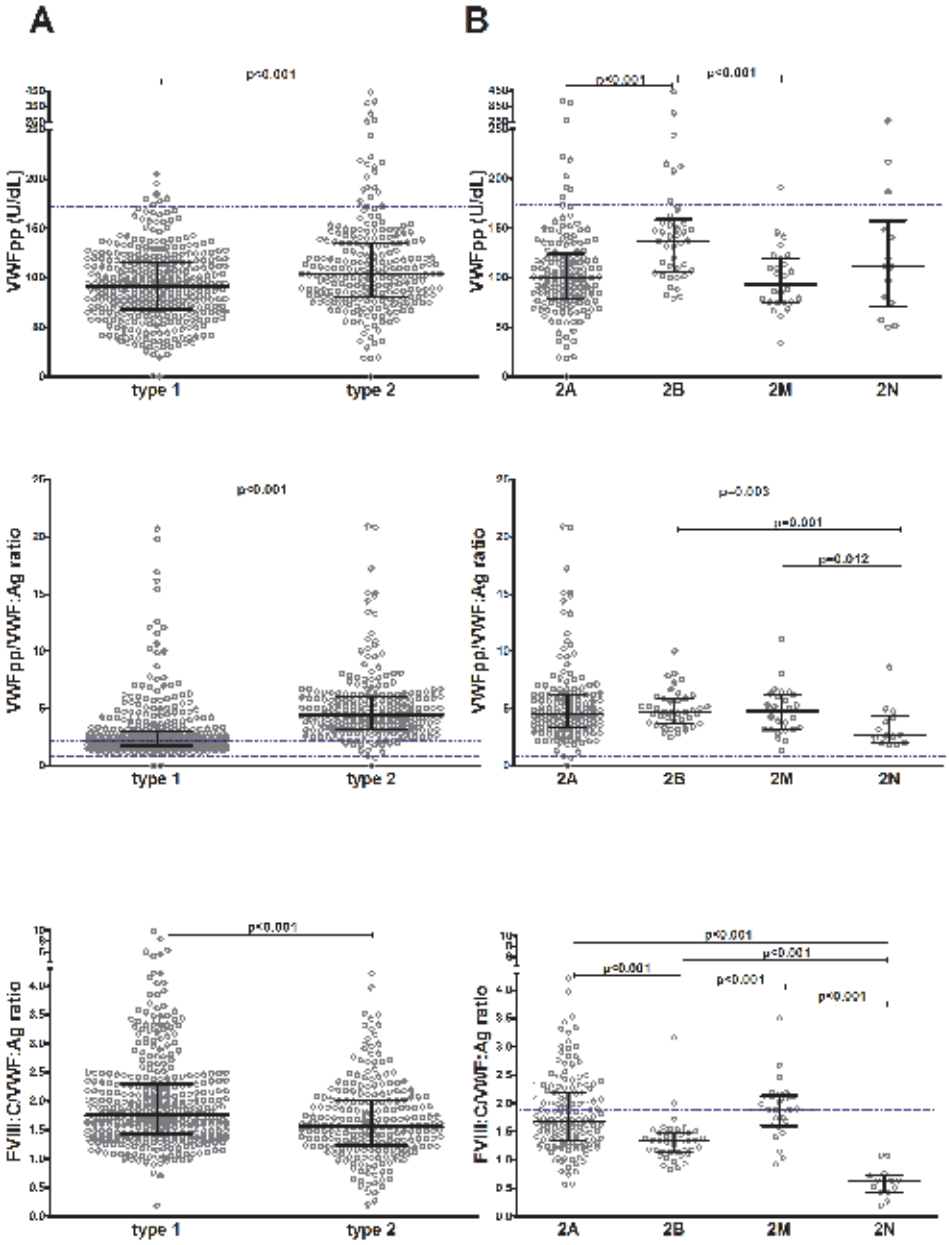

Figure 1: Ratio's per type of VWD A: VWFpp levels, VWFpp/VWF:Ag ratios and FVIII:C/VWF:Ag ratios are shown for type 1 VWD and type 2 VWD patients. For a better presentation of the graphic VWFpp/VWF:Ag ratio, one type 1 VWD patient with both a high VWFpp/Ag ratio (33.7) and a FVIII:C/VWF:Ag ratio of 3.4 was omitted. B: For all patients with type 2A, 2B, 2M and 2N VWD the VWFpp levels, VWFpp/VWF:Ag ratios and FVIII:C/VWF:Ag ratios are shown. The central horizontal line represents the median. The upper and lower horizontal lines show the 25-75% interquartile ranges. The dash lines illustrate the normal ranges (2.5th to 97.5th percentile) for VWFpp (82-173 U/dL), for VWFpp/VWF:Ag ratio (0.8-2.2), and for FVIII:C/VWF:Ag ratio (0.6-1.9).

Table 2. VWF parameters and ratios per type of VWD

Type of VWD	n	VWF:Ag (IU/dL) *median [IQR]*	VWF:Act (IU/dL) *median [IQR]*	FVIII:C (IU/dL) *median [IQR]*	VWFpp (U/dL) *median [IQR]*	VWFAct/VWF:Ag ratio *median [IQR]*	VWFpp/VWF:Ag ratio *median [IQR]*	FVIII:C/VWF:Ag ratio *median [IQR]*
1*	381	38 [23-53]	46 [24-72]	67 [49-88]	91 [68-116]	1.2 [1.0-1.4]	2.2 [1.7-3.1]	1.8 [1.4-2.3]
2	240	24 [16-34]	8 [4-16]	37 [27-48]	104 [81-136]	0.4 [0.2-0.5]	4.5 [3.2-6.0]	1.6 [1.2-2.0]
2A	157	22 [14-32]	8 [3-15]	37 [27-47]	99 [79-123]	0.4 [0.2-0.5]	4.5 [3.3-6.2]	1.7 [1.3-2.2]
2B	42	28 [23-40]	8 [6-14]	39 [28-48]	137 [106-157]	0.3 [0.2-0.4]	4.6 [3.7-5.8]	1.3 [1.1-1.5]
2M	27	24 [16-30]	5 [3-13]	42 [31-52]	93 [75-119]	0.3 [0.1-0.4]	4.8 [3.1-6.2]	1.9 [1.6-2.1]
2N	14	34 [28-44]	45 [29-54]	19 [13-28]	111 [71-157]	1.2 [1.0-1.5]	2.7 [2.0-4.4]	0.6 [0.4-0.7]
3	37	1 [0-4]	0 [0-1]	3 [1-9]	0 [0-60]**	NA	NA	NA

*Median and 25-75% interquartile range (IQR) of VWFpp level and ratios in type 1 VWD patients are in accordance with the results from Eikenboom et al. [20] **VWFpp levels below the assay detection limit (<4 U/dL) were considered 0 (n=25). Abbreviations: VWF = von Willebrand factor, VWD = von Willebrand disease, n = number, VWF:Ag = VWF antigen, VWF:Act = VWF activity, FVIII:C = factor VIII coagulant activity, VWFpp = von Willebrand factor propeptide, IQR = 25-75% interquartile range, NA = not applicable

FVIII:C/VWF:Ag ratio 1.6 [1.2-2.0] in the type 2 patients. The VWFpp/VWF:Ag ratio was higher in type 2 VWD than in type 1 VWD (p<0.001) and the FVIII:C/VWF:Ag ratio was lower in type 2 VWD compared with type 1 VWD (p<0.001) (table 2 and Figure 1A). This difference in VWFpp, VWFpp/VWF:Ag and FVIII:C/VWF:Ag between type 1 and 2 VWD was also observed after exclusion of type 2N patients (all p<0.001): median VWFpp in type 2A, 2B and 2M VWD was 103 U/dL [81-135], median VWF:pp/VWF:Ag was 4.6 [3.4-6.2], and median FVIII:C/VWF:Ag was 1.6 [1.3-2.1].

VWF parameters and ratios per subtype of type 2 VWD

In VWD 2B patients, median VWFpp was significantly higher than in 2A patients or 2M patients: 137 U/dL [IQR 106-157] vs. 99 U/dL [79-123] or 93 U/dL [IQR 75-119], both p<0.001 (Figure 1B and table 2). VWFpp/VWF:Ag ratio was increased in 2A, 2B and 2M patients based on the normal range (0.8-2.2)[20]; 4.5 [3.3-6.2] in 2A, 4.6 [3.7-5.8] in 2B, and 4.8 [3.1-6.2] in 2M VWD. Type 2B patients had significantly lower FVIII:C/VWF:Ag ratios than 2A or 2M patients (1.3 [1.1-1.5] vs. 1.7 [1.3-2.2]or 1.9 [1.6-2.1], both p<0.001). Median VWFpp and median VWFpp/VWF:Ag ratio were similar between type 2N and type 1 VWD. Type 2N patients had a significantly decreased FVIII:C/VWF:Ag ratio compared with type 1, 2A, 2B and 2M VWD (all p<0.001), as expected by the decreased FVIII binding affinity in type 2N.

Pathophysiological mechanisms in type 1 VWD

An increased VWFpp/VWF:Ag ratio (> 2.2) defines accelerated clearance of VWF and an increased FVIII:C/VWF:Ag ratio (>1.9) correlates with reduced VWF synthesis (Figure 2A). The pathophysiological mechanisms of type 1 VWD were variable with increased clearance of VWF in 23% of type 1 patients (89/380), reduced synthesis of VWF in 18% (67/380), and a combination of these in 23% (86/380). In 36% (138/380) of type 1 patients other pathophysiological mechanisms may play a role, as VWFpp/VWF:Ag and FVIII:C/VWF:Ag were within normal ranges (Figure 2).

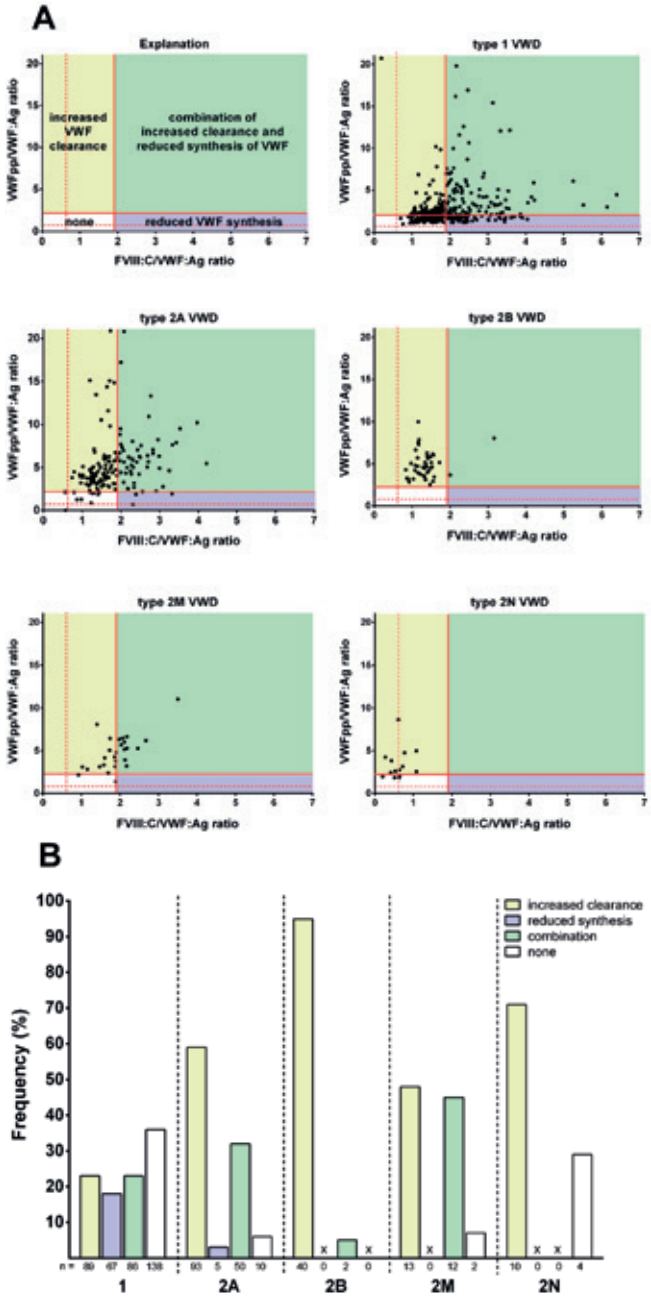

Figure 2: Pathophysiological mechanisms per type of VWD A: Scatter plots of FVIII:C/VWF:Ag (x-axis) versus VWFpp/VWF:Ag (y-axis) for type 1 VWD and type 2A, 2B, 2M and 2N VWD. The yellow area represents the patients with increased clearance, the purple area the patients with reduced synthesis, the green area the patients with a combination of both mechanisms, and the white area represents the absence of these pathophysiological mechanisms. For a better presentation of the graphics: one type 1 VWD patient with a very high VWFpp/Ag ratio (33.7), and two type 1 VWD patients with high FVIII:C/VWF:Ag ratios (8.4 and 9.7) were omitted. **B:** Proportion of VWD patients with increased clearance (yellow), reduced synthesis (purple), combination of increased clearance and reduced synthesis (green), and none of these mechanisms (white). X = not present.

Pathophysiological mechanisms in type 2 VWD variants

Based on increased VWFpp/VWF:Ag or FVIII:C/VWF:Ag ratios, the majority of type 2A VWD was characterized by an increased clearance of VWF (93/158, 59%), followed by a combination of increased clearance and reduced synthesis (50/158, 32%). In only five (of 158, 3%) patients with 2A VWD, reduced synthesis of VWF predominated. In ten (of 158, 6%) 2A patients, the VWD was characterized by neither increased clearance nor reduced synthesis. Ninety-five percent (40/42) of type 2B patients showed an increased VWFpp/VWF:Ag ratio with a normal FVIII:C/VWF:Ag ratio, suggesting that 2B VWD is completely characterized by an increased clearance of normally synthesized and secreted VWF. The pathophysiological mechanisms of type 2M VWD were an increased clearance in almost half of the patients (13/27, 48%) and a combination of increased clearance and reduced synthesis in the other half (12/27, 44%). None of the 2M patients had an increased FVIII:C/VWF:Ag ratio, indicating that 2M VWD is not characterized by reduced synthesis solely. Ten (of 14, 71%) 2N patients had an increased VWFpp/VWF:Ag ratio, indicating that apart from reduced FVIII binding the type 2N VWD is also characterized by increased clearance (Figure 2).

Type 3 VWD patients

Thirty-seven patients with VWF:Ag and VWF:Act <5 IU/dL were classified as type 3 VWD, according to the current ISTH guidelines [16]. VWFpp was undetectable in 59% of the (22/37) type 3 patients, which confirms the definition of type 3 VWD with complete absence of VWF. We therefore designated these patients: "true type 3 VWD patients". In all other type 3 patients (15/37, 41%), VWFpp was detectable with a median of 72 U/dL [IQR 37-94] (table 3).

Table 3. Characteristics of type 3 VWD patients with and without VWFpp

Characteristics		Type 3 VWD patients without VWFpp n=22	Type 3 VWD patients With VWFpp n=15	p-value
Age (years)		22 (2-60)	35 (4-65)	0.636
Male sex, n (%)		9 (41)	7 (47)	0.729
Blood group O, n (%)		8 (36)	12 (80)	0.009
VWFpp (U/dL)		0 [0-0]	72 [37-94]	<0.001
VWF:Ag (IU/dL)		0 [0-1]	4 [2-4]	<0.001
VWF:Act (IU/dL		0 [0-0]	1 [0-3]	0.036
FVIII:C (IU/dL)		2 [1-3]	9 [8-13]	<0.001
Multimers	absent	19 (86)	3 (20)	<0.001
	abnormal	3 (14)	12 (80)	
Bleeding score		19.5 [11.3-23.8]*	14.0 [7.0-17.0]	0.025
Mutations identified†		10 out of 12 homozygous or compound heterozygous for null alleles	14 patients genotyped, 10 of whom had a mutation that is associated with increased clearance	

*2 missing, †11 missing. Levels and bleeding score are presented as median and 25-75% interquartile range [IQR]. Age is presented as median and range. Abbreviations: VWD = von Willebrand disease; VWFpp = von Willebrand factor propeptide; VWF:Ag = von Willebrand factor antigen; VWF:Act = von Willebrand factor activity; FVIII:C = factor VIII coagulation activity. VWFpp, VWF:Ag, VWF:Act and FVIII:C levels were measured centrally at time of inclusion in the study.

Compared with the "true" type 3 VWD patients with complete absence of both VWFpp and VWF:Ag, type 3 patients with detectable VWFpp had higher VWF:Ag levels (0 vs. 4 IU/dL, p<0.001), higher VWF:Act levels (0 vs. 1 IU/dL, p=0.036), and higher FVIII:C levels (2 vs. 9 IU/dL, p<0.001). The frequency of blood group O was significantly higher in the type 3 patients with detectable VWFpp than in the "true" type 3 patients (12/15 vs. 8/22, p=0.009).

In 12/22 "true" type 3 patients, the *VWF* gene was sequenced. Ten of whom were homozygous or compound heterozygous for a null allele (p.R2535X, p.N2546Y, p.Y1570X, p.S330KfsX4, p.D1333EfsX43, and deletion of exons 17-25). Of the 15 patients with detectable VWFpp, the *VWF* gene mutation was known in 14. Ten of whom had missense mutations in regions associated with rapid clearance of VWF (p.R1205H and p.S1285P) or mutations that are considered to rapidly clear VWF from the circulation (p.Y1584C and p.Y1146C)[12,18,30,31]. Additionally, three were compound heterozygous for a null allele and an unknown defect on the other allele.

Bleeding phenotype, VWFpp and pathophysiological mechanism

Bleeding score was significantly higher in the "true" type 3 patients with undetectable VWFpp than in those with detectable VWFpp level: 19.5 [11.3-23.8] vs. 14.0 [7.0-17.0], p=0.025 (table 3). In patients with type 1 VWD, the BS was not associated with the pathophysiological mechanisms of VWD, i.e. increased clearance, reduced synthesis, combination of increased clearance and reduced synthesis, or none of these mechanisms: median BS 10.0 vs. 9.0 vs. 8.5 vs. 9.0 respectively (Figure 3A). However, all type 2 patients with increased clearance, reduced synthesis or combination, had significantly higher BS than type 2 patients with none of these pathophysiologic mechanisms (p=0.010). Median BS in type 2 patients with increased clearance (13.0 [9.0-17.5], p=0.001) or a combination of increased clearance and reduced synthesis (13.0 [8.0-16.3], p=0.007) was significantly higher than in those with normal ratios: (7.5 [5.5-10.8]). Median BS in type 2 VWD characterized by reduced synthesis was 17.0 [8.0-26.0] vs. 7.5 [5.5-10.8] in those with normal ratios (p=0.051) (Figure 3B).

Discussion

In the present study from the WiN-cohort, we present as first that VWFpp improved the discrimination of true type 3 VWD patients with a total absence of both VWF:Ag and VWFpp, and VWD patients with extremely rapid VWF clearance leading to very low VWF plasma levels. Moreover, the ratios of VWF:Ag with VWFpp or FVIII:C differ between type 1 and 2 VWD. Type 2 VWD was mainly characterized by an increased clearance of VWF and type 1 VWD had a more heterogeneous pathophysiology. Finally, low VWFpp was associated with higher BS in type 3 and type 2 VWD.

VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios have been shown to identify type 1 VWD patients by giving insight into the pathophysiological mechanisms behind the low

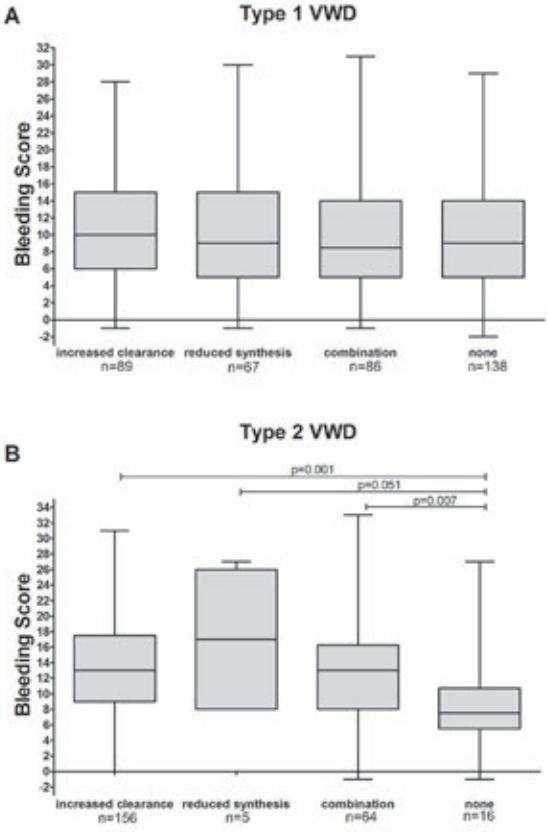

Figure 3: Bleeding score per pathophysiological mechanisms for type 1 and 2 VWD Bleeding score according to different pathophysiological mechanisms of VWD for (A) type 1 VWD and (B) type 2 VWD. Boxplots show median, 25-75% interquartile ranges and minimum and maximum score.

VWF levels, i.e. increased clearance and reduced synthesis of VWF [12,20,32]. We confirmed this in our study and showed that the pathophysiology of type 1 VWD is diverse, as our type 1 patients showed a mixture between VWF synthesis and clearance defects using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios. Almost two fifth of patients did not have increased ratios. Probably VWD in these patients results from other not yet identified mechanisms. This confirms the heterogeneity of type 1 VWD and the various causes of low VWF levels.

We hypothesized that as a result of our inclusion criteria (historically lowest VWF levels <30 U/dL), we selected more type 1 patients with accelerated clearance of VWF or reduced synthesis, and therefore patients with higher ratios. However, the VWFpp levels and ratios thereof in our type 1 patients are in agreement with a previous study in type 1 VWD patients [20]. This is remarkable as in the MCMDM-1VWD study also milder cases were included. Regression to the mean or the effect of aging on VWF and FVIII levels may have been observed [27].

We observed a higher VWFpp/VWF:Ag ratio in type 2 VWD patients compared with type 1 VWD patients. However, type 1 patients had a significantly higher FVIII:C/VWF:Ag ratio, revealing reduced synthesis as a pathophysiological mechanism of VWD in type 1, but rarely or not in type 2 VWD. This also suggests that these ratios may discriminate between type 2 en type 1 VWD, which has been stated before [33].

The main pathophysiological mechanism of type 2 VWD is increased VWF clearance, as the majority of type 2A, 2B and 2M patients showed an increased VWFpp/VWF:Ag ratio. In addition, a small proportion of type 2 patients showed a combination of reduced VWF survival and reduced synthesis, but this was only seen in 2A and 2M VWD. The effect of proteolysis of VWF on clearance has been argued [3,30,34,35]. We found no differences between 2A VWD with defects in multimerization and enhanced ADAMTS-13-mediated proteolysis, and 2M VWD with normal multimer patterns. Because of small sample size these findings should be interpreted with care. Our data indicate that 2B VWD is completely characterized by a normal synthesis and an increased clearance of VWF, which has been shown before in 18 type 2B VWD patients [36]. It has been suggested that the mutant VWF is cleared faster in these patients due to the spontaneous binding of VWF to platelets [17,19,37,38]. Our results also showed that the mutant type 2N VWF is cleared faster than normal VWF.

To the best of our knowledge, this is the first study to assess VWFpp in VWD patients with VWF:Ag and VWF:Act <5 IU/dL, the diagnostic criteria used in the current ISTH classification for type 3 VWD [16]. We showed that VWFpp is detectable in half of the type 3 VWD patients, indicating that VWF is actually produced but very rapidly cleared from the circulation in these patients. Our findings suggest that these type 3 VWD patients should actually reclassify as severe type 1 VWD patients. We therefore conclude that VWFpp can discriminate between "true" type 3 VWD with complete absence of both VWF:Ag and VWFpp, and severe type 1 VWD with extremely low VWF levels. A significant proportion of these severely affected type 1 VWD patients had blood group O, which may have contributed to the low VWF levels in these patients. Distinguishing between true type 3 VWD and severe type 1 VWD is of clinical importance as VWD patients with lack of VWF but detectable VWFpp have different clinical characteristics than those with a complete absence of both VWF and VWFpp.

Because VWF:Ag and FVIII:C levels are associated with bleeding phenotype in VWD [24,28,39], it may be expected that VWFpp also associates with bleeding phenotype in VWD patients: indirectly through its association with VWF:Ag or by binding to mature VWF in the circulation [40]. Interestingly, we found that the presence of VWFpp in patients with VWF:Ag levels <5 IU/dL correlates with a milder bleeding phenotype. We also observed that increased clearance, reduced synthesis and a combination thereof associates with higher bleeding scores in type 2 patients.

Mutation analysis of the *VWF* gene has been advised in type 2N VWD, type 2B VWD, and in type 3 VWD [41]. However, sequencing of the *VWF* gene is expensive and not

widely available. Looking at the molecular background of the type 3 VWD patients, we observed homozygosity and compound heterozygosity for null alleles in the true type 3 VWD patients with complete absence of VWF:Ag and VWFpp. In the type 3 VWD patients with detectable VWFpp, missense mutations in the D3 and D4 domain of the *VWF* gene were identified that are associated with accelerated clearance of VWF (p.R1205H; p.S1285P) [12,18,30]. Therefore assessing VWFpp may substitute for VWF gene analyses as it identifies carriers of two null alleles and thus "true" type 3 VWD. In addition, the MCMDM-1VWD study has recently shown that a high VWFpp/VWF:Ag ratio predicts the detection of *VWF* gene mutations in type 1 VWD patients [20]. Additionally, patients with mutations in regions of the *VWF* gene associated with reduced VWF survival, can be identified with the VWFpp/VWF:Ag ratio [12,18,30]. Based on our results and the current literature, we believe that measurement of VWFpp should be implemented as a standard diagnostic in VWD for patients with low VWF levels and may render mutation analysis of the *VWF* gene unnecessary.

VWFpp measurement is of clinical importance because its ratio with VWF:Ag predicts reduced VWF survival and may therefore predict desmopressin response. In patients with increased VWF clearance, VWF disappears rapidly after desmopressin administration. The desmopressin response in these patients is probably sufficient for hemostasis in case of minor bleeding, but inadequate in case of major bleeding or surgery because they lack a sustained response [13,18,42]. The FVIII:C/VWF:Ag ratio can also help in the classification of VWD patients, as those patients with an increased FVIII:C/VWF:Ag mainly classify as type 1 VWD. With the current novel insights into the pathophysiology and molecular biology of VWD the classification of VWD should be reviewed in the near future.

In conclusion, VWFpp discriminates between true type 3 VWD patients with complete lack of VWF and severe type 1 VWD patients with very low VWF levels. An increased FVIII:C/VWF:Ag ratio may help identify type 1 VWD patients. This study shows the clinical significance of the VWFpp assay; it is of added value for the classification of VWD, for the assessment of the bleeding phenotype, for genetic counseling, and it also has therapeutic consequences.

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Authorship Contributions

FWG Leebeek and J Eikenboom designed research, analyzed and interpreted data, and wrote the manuscript. YV Sanders performed research, analyzed and interpreted data, and wrote the manuscript. D Groeneveld performed research, analyzed and interpreted data, and critically reviewed the manuscript. K Meijer, K Fijnvandraat, MH Cnossen, JG van der Bom , BAP Laros-van Gorkom, and EP Mauser-Bunschoten: designed research, analyzed and interpreted data, and critically reviewed the manuscript. M Coppens and J de Meris: analyzed and interpreted data, and critically reviewed the manuscript. All authors gave their consent to the final version of the manuscript.

Disclosures of Conflicts of Interest

FWG Leebeek received research support from CSL Behring for performing the WiN-study and has served on advisory boards of CSL Behring and Baxter. J Eikenboom received research support from CSL Behring and he has been a teacher on educational activities of Roche. EP Mauser-Bunschoten received research support from CSL Behring, Bayer, Baxter, Novo Nordisk, Pfizer and Sanquin. JG van der Bom has received unrestricted research/educational funding for various projects from the following companies: Bayer Schering Pharma, Baxter, CSL Behring, Novo Nordisk, and Pfizer. In addition, she has been a consultant to Baxter and Pfizer, and she has been a teacher on educational activities of Bayer Schering Pharma. MH Cnossen has received unrestricted research/educational funding for various projects from the following companies: Bayer Schering Pharma, Baxter, Novo Nordisk and Pfizer. K Meijer received research support from Bayer and Baxter, served on an advisory board for

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