Pathophysiology of von Willebrand factor in bleeding and thrombosis

Pagliari, M.T.

PART II

TYPE 3 VON WILLEBRAND DISEASE: RESULTS FROM THE 3WINTERS-IPS

CHAPTER 5

VON WILLEBRAND FACTOR PROPEPTIDE AND PATHOPHYSIOLOGICAL MECHANISMS IN EUROPEAN AND IRANIAN PATIENTS WITH TYPE 3 VON WILLEBRAND DISEASE ENROLLED IN THE 3WINTERS-IPS STUDY

Pagliari MT, Rosendaal FR, Ahmadinejad M, Badiee Z, Baghaipour MR, Baronciani L, Benítez Hidalgo O, Bodó I, Budde U, Castaman G, Eshghi P, Goudemand J, Karimi M, Keikhaei B, Lassila R, Leebeek FWG, Lopez Fernandez MF, Mannucci PM, Marino R, Oldenburg J, Peake I, Santoro C, Schneppenheim R, Tiede A, Toogeh G, Tosetto A, Trossaert M, Yadegari H, Zetterberg EMK, Peyvandi F, Federici AB, Eikenboom J.

Journal of Thrombosis and Haemostasis. 2022;20(5):1106-1114.

ABSTRACT

Background

Type 3 von Willebrand disease (VWD) is a severe bleeding disorder caused by the virtually complete absence of von Willebrand factor (VWF). Pathophysiological mechanisms of VWD like defective synthesis, secretion and clearance of VWF have previously been evaluated using ratios of VWF propeptide (VWFpp) over VWF antigen (VWF:Ag) and factor (F)VIII coagulant activity (FVIII:C) over VWF:Ag.

Objective

To investigate whether the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios may also be applied to understand the pathophysiological mechanism underlying type 3 VWD and whether VWFpp is associated with bleeding severity.

Methods

European and Iranian type 3 patients were enrolled in the 3WINTERS-IPS study. Plasma samples and buffy coats were collected and a bleeding assessment tool was administered at enrolment. VWF:Ag, VWFpp, FVIII:C, and genetic analyses were performed centrally, to confirm patients' diagnoses. VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios were compared among different variant classes using the Mann-Whitney test. Median differences with 95% confidence intervals (CI) were estimated using the Hodges-Lehmann method. VWFpp association with bleeding symptoms was assessed using Spearman's rank correlation.

Results

Homozygosity/compound heterozygosity for missense variants showed higher VWFpp level and VWFpp/VWF:Ag ratio than homozygosity/compound heterozygosity for null variants ([VWFpp median difference, 1.4 IU/dL; 95%CI, 0.2–2.7; P= 0.016]; [VWFpp/VWF:Ag median difference, 1.4 IU/dL; 95%CI, 0–4.2; P= 0.054]). FVIII:C/VWF:Ag ratio was similarly increased in both. VWFpp level did not correlate with the bleeding symptoms (r= 0.024; P= 0.778).

Conclusions

An increased VWFpp/VWF:Ag ratio is indicative of missense variants, whereas FVIII:C/VWF:Ag ratio does not discriminate missense from null alleles. The VWFpp level was not associated with the severity of bleeding phenotype.

INTRODUCTION

Von Willebrand disease (VWD) is a common inherited bleeding disorder characterized by spontaneous mucocutaneous bleeding and caused by quantitative (types 1 and 3) or qualitative (types 2A, 2B, 2M and 2N) defects of von Willebrand factor (VWF) [1, 2]. VWF is a large multimeric glycoprotein involved in primary haemostasis, but it also acts as factor VIII (FVIII) carrier [3,4]. VWF is synthesized as pre-pro-VWF (2813 amino acids) in endothelial cells and megakaryocytes [5]. In the endoplasmic reticulum, the signal peptide of 22 amino acids is removed and pro-VWF undergoes C-terminal dimerization through cysteine disulfide bonds formation and post-translational modifications such as glycosylation [6]. Once in the Trans-Golgi apparatus, the VWF propeptide (VWFpp; 741 amino acids) is cleaved, although it remains non-covalently bound to the mature VWF [3]. In this phase, the VWFpp plays a role in the VWF multimerization which occurs through N-terminal interchain disulfide bonds [6, 7]. Mature VWF can be stored in platelet alpha granules and endothelial Weibel-Palade bodies or it can be constitutively secreted into the circulation [8]. After secretion, VWFpp dissociates from mature VWF and circulates and is cleared independently from VWF [3].

Although equimolarly secreted, VWF and VWFpp have a different half-life of 8–12 and 2 h, respectively [9]. Previous studies have demonstrated that the ratio of VWFpp over VWF antigen (VWF:Ag) along with the ratio of FVIII coagulant activity (FVIII:C) over VWF:Ag can be used to assess the properties of VWF synthesis, secretion and clearance [10, 11]. This allowed to discriminate whether reduced VWF levels in type 1 VWD patients are due to reduced synthesis, increased clearance, or a combination of both mechanisms [11]. The VWFpp/VWF:Ag ratio also discriminates between type 3 and severe type 1 VWD patients, which highlights the utility of VWFpp in the study of the pathophysiology of VWD and also in the diagnosis of VWD [12].

This study, which includes European and Iranian type 3 VWD patients enrolled in the Type *3* von *W*illebrand *Int*ernational *R*egistries *I*nhibitor *P*rospective *S*tudy (3WINTERS-IPS), aimed to assess the pathophysiological mechanisms leading to type 3 VWD using VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios in combination with the various underlying variants.

METHODS

Participants

The 3WINTERS-IPS study includes 265 patients previously diagnosed with type 3 VWD and recruited in Europe and Iran. The study was approved by the local ethical committees of all participating centers and patients gave their written informed consent. Briefly, inclusion criteria were: patients of both sexes and all ages, diagnosed by the recruiting centers as type 3 VWD; accessible information about previous bleeding episodes and treatment with concentrates containing VWF; and availability to follow-up.

For the analysis, we focus on patients who were genotyped for the underlying *VWF* variant, who were not using prophylactic concentrates containing VWF at the time of blood sampling as that precludes reliable VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios, and for whom the diagnosis of type 3 VWD was confirmed by a centrally measured VWF:Ag≤ 3 IU/dL (**Figure 1**).

Briefly, 265 patients were enrolled in the study; of these 52 patients were excluded because of the lack of DNA samples or multiple missing data. Of the 213 genotyped patients, a further 43 were excluded from analysis as they were on prophylaxis using concentrates containing VWF at the time of blood draw or because this information was missing. In the remaining group of 170 genotyped patients, 147 patients had a confirmed *VWF* genotype and this group forms the main study group, whereas in 8 patients no variant could be identified and 15 genotyped patients had a VWF:Ag level> 3 IU/dL not fulfilling the stringent diagnostic criterium for type 3 VWD.

5

Phenotypic and genotypic characterization

Plasma samples and buffy coats were collected to confirm patients' phenotype. Biochemical measurements were performed in centralized laboratories on samples taken at the time

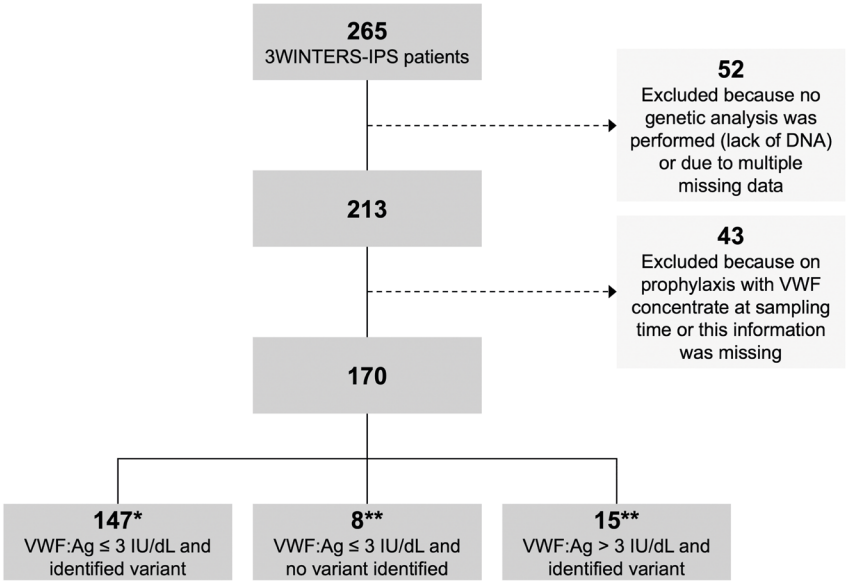

Figure 1. Flowchart of 3WINTERS-IPS patients analyzed in this study. Type 3 patients enrolled in the 3WINTERS-IPS study who met the inclusion criteria, were further divided depending on the availability of biochemical and molecular information. *Main study group included 147 fully characterized patients with VWF:Ag ≤ 3 IU/dL and a complete molecular characterization. ** The group of 8 patients with VWF:Ag≤ 3 IU/dL in whom no genetic defect could be identified and the remaining group of 15 patients with a genetic defect identified, but who did not meet inclusion criteria (VWF:Ag> 3 IU/dL) were considered separately for secondary analyses.

of recruitment. VWF:Ag was measured using a sensitive ELISA based assay. FVIII:C was measured by a one-stage clotting assay using FVIII deficient plasma (Siemens) and APTT reagent Triniclot (TCoag). VWFpp measurement was performed with an ELISA based assay using antibodies from Sanquin [9, 12]. Molecular analysis included next generation sequencing, polymerase chain reaction with Sanger sequencing and multiplex-ligation dependent probe amplification [13]. Bleeding score (BS) was calculated using a common bleeding assessment tool at time of enrolment [14].

Statistical analyses

Continuous variables were reported as median and interquartile range (IQR), whereas descriptive variables were reported as numbers with percentages.

The *VWF* gene defects identified in type 3 VWD patients were divided into missense and null defects. Missense defects included missense variants, gene conversions (not leading to null variants), small insertions and small deletions that do not alter the reading frame. Null defects included variants that introduce a stop codon, splice variants, large deletions, large insertions, small insertions, small deletions, indels, or small duplications which alter the reading frame and thereby cause a premature stop codon.

Based on these definitions, patients were grouped as homozygous/compound heterozygous for missense variants, homozygous/compound heterozygous for null variants and compound heterozygous null/missense variants. Patients with a partial molecular diagnosis because only one mutated allele was identified, were referred to as "other" and excluded from the statistical analysis due to their heterogeneity. Analyses were repeated by considering European and Iranian patients separately.

VWF:Ag, VWFpp, FVIII:C, VWFpp/VWF:Ag ratio, FVIII:C/VWF:Ag ratio, and bleeding score were analyzed in the different groups of patients classified as described above. As continuous variables were not normally distributed, comparisons (missense vs. null and missense vs. missense/null compounds) were performed using the non-parametric Mann-Whitney test. Median differences with 95% confidence intervals (CI) were estimated using the Hodges-Lehmann method. The correlation between VWFpp and bleeding score was evaluated using Spearman's rank distribution. All analyses were performed using SPSS statistics 27 (IBM Corporation).

RESULTS

The biochemical characteristics of 170 type 3 VWD patients are reported in **Table 1**. The main study group of 147 type 3 patients with a complete characterization had a median age of 27 years (IQR, 16.0-40.0) and included more women than men (59% and 41%, respectively). VWF:Ag was below the assay limit of detection (LOD, 0.8 IU/dL) in most of patients (n= 135; 92%); therefore it was arbitrarily set as 0.5 IU/dL. Median VWFpp was 1.5 IU/dL (IQR, 0.7-4.2), whereas median FVIII:C was 2.3 IU/dL (IQR, 1.8-2.9). Patients had a median bleeding score at inclusion of 14.0 (IQR, 8.0-19.0). Both VWFpp/

VWF:Ag and FVIII:C/VWF:Ag ratios were above the respective normal ranges (NR; VWFpp/VWF:Ag, NR: 0.8-2.2; FVIII:C/VWF:Ag NR: 0.6-1.9) [11].

The 8 patients in whom no *VWF* variant could be identified showed higher VWF:Ag (median, 1.8 vs. 0.5 IU/dL) and VWFpp values (median VWFpp, 2.8 vs. 1.5 IU/dL) than those of fully characterized type 3 patients, and the corresponding VWFpp/VWF:Ag ratio was lower (median, 1.6 vs. 2.8). They also had slightly lower FVIII:C (median, FVIII:C, 1.8 vs. 2.3 IU/dL), a lower FVIII:C/VWF:Ag ratio (median FVIII:C/VWF:Ag ratio 1.1 vs. 4.4) and a lower bleeding score (median 8.5 vs. 14.0) than the patients with a confirmed genotype. The 15 genotyped patients with an unconfirmed diagnosis of type 3 VWD (VWF:Ag> 3 IU/dL) were also analyzed (**Table 1**). They showed an increased median FVIII:C/VWF:Ag of 3.9, whereas the median VWFpp/VWF:Ag ratio (1.7) was within the normal range. Five patients were homozygous/compound heterozygous for null or missense variants (n= 4 and n= 1, respectively), five were compound heterozygous for null/missense variants,

Table 1. Baseline characteristics of the included patients

	Variants identified, VWF:Ag ≤ 3 IU/dL	No variants identified, VWF:Ag ≤ 3 IU/dL	Variants identified, VWF:Ag > 3 IU/dL
N	147	8	15
Age, years	27 (16-40)	18.5 (12.5-30.8)	41.0 (32-54)
Sex (female), n (%)	87 (59.0)	6 (75.0)	9 (60.0)
VWF:Ag, (IU/dL)	0.5 (0.5-0.5)	1.8 (1.2-2.4)	5.2 (4.1-6.3)
Missing, n	-	-	-
VWFpp, (IU/dL)	1.5 (0.7-4.2)	2.8 (2.3-4.4)	8.5 (3.4-15.9)
Missing, n	4	1	-
FVIII:C, (IU/dL)	2.3 (1.8-2.9)	1.8 (1.6-2.7)	19.1 (14.9-33.4)
Missing, n	1	1	
VWFpp/VWF:Ag [a]	2.8 (1.4-6.6)	1.6 (1.2-4.6)	1.7 (0.4-4.0)
Missing, n	4	1	-
FVIII:C/VWF:Ag [b]	4.4 (3.4-5.5)	1.1 (0.7-1.7)	3.9 (1.6-6.4)
Missing, n	1	1	-
Bleeding Score	14.0 (8.0-19.0)	8.5 (4.3-17.5)	11.0 (6.0-18.0)
Missing, n	6	-	-
Variant identified, n (%)	147 (100)	0 (0)	15 (100)

Note: Continuous variables were reported as median and interquartile range (IQR). Abbreviations: FVIII:C, Factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide.
[a] VWFpp/VWF:Ag, normal range 0.8–2.2, calculated as 2.5th to 97.5th percentile in 387 healthy controls [11].
[b] FVIII:C/VWF:Ag normal range 0.6–1.9, calculated as 2.5th to 97.5th percentile in 387 healthy controls [11].

Table 2. Main study group stratified by type of *VWF* variants

Patients, n (%)	VWF:Ag *(IU/dL)*	FVIII:C *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C/ VWF:Ag	VWFpp/ VWF:Ag	BS
Homozygous/compound heterozygous for missense variants, 20 (13.6)	0.5 (0.5 to 0.5)	2.6 (1.9 to 3.3)	3.6 (1.4 to 6.6)	4.4 (3.1 to 5.7)	4.9 (2.2 to 9.3)	11.5 (6.3 to 18.3)
Homozygous/compound heterozygous for null variants, 116 (78.9)	0.5 (0.5 to 0.5)	2.3 (1.8 to 2.8)	1.2 (0.6 to 3.4)	4.4 (3.4 to 5.2)	2.4 (1.2 to 5.8)	14.0 (8.0 to 19.0)
Compound heterozygous for missense-null variants 6 (4.1)	0.5 (0.5 to 1)	3.3 (2.1 to 6.1)	2.6 (1.2 to 7.8)	5.1 (4.2to 7.8)	2.8 (2.3 to 11.2)	18.5 (12.0 to 22.8)
Other, 5 (3.4)	0.5 (0.5 to 1.4)	1.9 (1.6 to 2.6)	3.1 (1.6 to 9.8)	3.8 (1.8 to 5.1)	6.2 (3.1 to 8.6)	18.5 (8.8 to 20.8)
Median Difference (95% CI), P missense vs. null	–	0.3 (–0.1 to 0.8) 0.154	1.4 (0.2 to 2.7) 0.016	0 (–0.8 to 0.8) 0.882	1.4 (0 to 4.2) 0.054	–1 (–5.0 to 3.0) 0.501
Median Difference (95% CI), P missense vs. compound missense-null	–	–0.7 (–2.3 to 0.5) 0.234	0.15 (-4.1 to 3.2) 0.838	–1.1 (3.0 to 0.8) 0.170	1.1 (–2.0 to 7.0) 0.683	–5.5 (–13.0 to 4.0) 0.234

Note: Continuous variables were reported as median and interquartile range (IQR). Comparisons between groups were performed using Mann-Whitney test. Median difference and 95% confidence intervals (CI) were estimated with Hodges-Lehmann method. Missing values: FVIII:C, n= 1; VWFpp, n= 4; FVIII:C/VWF:Ag, n= 1;VWFpp/VWF:Ag, n= 4; BS, n= 6. Abbreviations: BS, bleeding score; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide.

whereas the remaining five were referred as "other" because only one mutated allele was identified.

The main study group of 147 patients was grouped according to the molecular defects as described in the Methods section. Analyses of the VWF and FVIII parameters as well as the bleeding score are reported in **Table 2.** Type 3 patients homozygous/compound heterozygous for missense variants showed higher VWFpp levels than those homozygous/compound heterozygous for null variants (median, 3.6 vs. 1.2 IU/dL; P= 0.016, median difference of 1.4 IU/dL; 95% CI, 0.2-2.7). The group of patients compound heterozygous for a missense and a null variant showed a median VWFpp level of 2.6 IU/dL, which was intermediate between the patients homozygous/compound heterozygous for missense variants and those homozygous/compound heterozygous for null variants (**Table 2**).

VWFpp/VWF:Ag ratio was higher in those homozygous/compound heterozygous for missense variants than in those homozygous/compound heterozygous for null variants (median, 4.9 vs. 2.4, P= 0.054; median difference of 1.4 IU/dL; 95% CI, 0-4.2).

Compound heterozygosity for missense-null variants showed intermediate VWFpp/VWF:Ag ratio (2.8) between those homozygous/compound heterozygous for missense or null variants. All patients showed a similar FVIII:C/VWF:Ag ratio, regardless of the type of variant considered (**Table 2**).

Analyses were also performed by evaluating European and Iranian patients separately (**Table 3**). Patients homozygous/compound heterozygous for missense variants also showed higher VWFpp/VWF:Ag ratio than those with null variants, in both European (median, 4.7 vs. 2.4; P= 0.483; median difference of 1.0 IU/dL; 95% CI, –2.0 to 5.5) and Iranian patients (median, 5.4 vs. 2.4; P= 0.062, median difference of 1.6 IU/dL; 95% CI, –0.2 to 5.8). Compound heterozygosity for missense-null variants were mainly found among European patients (5 out of 6).

There was no association between VWFpp and the bleeding phenotype in type 3 VWD patients, assessed in the group of 147 completely characterized patients using Spearman's rank correlation (r= 0.024; P= 0.778).

DISCUSSION

Type 3 VWD is a severe recessively inherited bleeding disorder characterized by the virtual absence of VWF. It is mainly caused by homozygous or compound heterozygous *VWF* null variants [1, 2]. However, missense variants have also been identified as possible cause [15, 16]. While the pathomechanism of null variants consists of reduced VWF synthesis, the missense variants may lead to reduced VWF levels through a combination of factors like reduced synthesis, intracellular retention, impaired secretion or fast clearance from the circulation. The ratios of VWFpp/VWF:Ag and FVIII:C/VWF:Ag have previously been shown to be indicative of the pathogenic mechanism underlying the VWF deficiency for other types of VWD [10- 12].

Table 3. Main study group stratified by type of VWF variants in European and Iranian patients

European type 3 VWD patients, n (%) [a]	VWF:Ag *(IU/dL)*	FVIII:C *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C/ VWF:Ag	VWFpp/ VWF:Ag	BS
Homozygous/compound heterozygous for missense variants, 8 (13.3)	0.5 (0.5 to 1.8)	2.6 (2.2 to 4.0)	3.7 (3.0 to 7.3)	4.0 (2.8 to 4.9)	4.7 (1.7 to 12.7)	16.0 (15.0 to 24.3)
Homozygous/compound heterozygous for null variants, 43 (71.7)	0.5 (0.5 to 0.5)	2.4 (2.0 to 3.2)	1.2 (0.6 to 5.2)	4.4 (3.4 to 5.8)	2.4 (1.2 to 6.7)	18.0 (13.5 to 23.5)
Compound heterozygous for missense-null variants, 5 (8.3)	0.5 (0.5 to 1.5)	2.9 (2.0 to 7.8)	4.1 (1.1 to 8.2)	4.5 (4.0 to 7.3)	2.7 (2.2 to 13.6)	19.0 (10.5 to 24.5)
Other, 4 (6.7)	0.5 (0.5 to 1.8)	2.1 (1.8 to 2.7)	3.9 (1.2 to 11.9)	4.1 (1.5 to 5.4)	5.5 (2.4 to 9.7)	18.5 (8.8 to 20.8)
Median Difference (95% CI), P missense vs. null	-	0.3 (–0.4 to 1.1) 0.414	2.3 (–0.8 to 3.6) 0.190	–0.8 (–2 to 0.4) 0.228	1.0 (–2.0 to 5.5) 0.483	0 (–6.0 to 6.0) 0.989
Median Difference (95% CI), P missense vs. compound missense-null	-	–0.2 (–7.0 to 14.0) 0.712	0 (–0.6 to 1.4) 0.865	0.4 (–5.1 to 6.0) 0.122	–0.1 (–11.0 to 11.8) 1	–2.0 (–11.0 to 13.0) 0.607

Table 3 (continued). Main study group stratified by type of VWF variants in European and Iranian patients

Iranian type 3 VWD patients, n (%) [b]	VWF:Ag *(IU/dL)*	FVIII:C *(IU/dL)*	VWFpp *(IU/dL)*	FVIII:C/ VWF:Ag	VWFpp/ VWF:Ag	BS
Homozygous/compound heterozygous for missense variants, 12 (13.8)	0.5 (0.5 to 0.5)	2.6 (1.6 to 3.3)	2.7 (1.2 to 4.7)	5.2 (3.2 to 6.6)	5.4 (2.4 to 9.3)	7.5 (4.3 to 12.3)
Homozygous/compound heterozygous for null variants, 73 (83.9)	0.5 (0.5 to 0.5)	2.2 (1.7 to 2.6)	1.2 (0.6 to 2.7)	4.2 (3.5 to 5.2)	2.4 (1.2 to 5.4)	11.0 (4.0 to 17.0)
Compound heterozygous for missense-null variants, 1 (1.1)	0.5	3.7	2.6	7.4	5.2	15.0
Other, 1 (1.1)	0.5	1.4	3.1	2.8	6.2	-
Median Difference (95% CI), P missense vs. null	-	0.3 (–03 to 0.8) 0.271	0.8 (–0.1 to 2.9) 0.072	0.6 (–0.4 to 1.8) 0.229	1.6 (–0.2 to 5.8) 0.062	–3.5 (–7.0 to 2.0) 0.283
P missense vs. compound missense-null [c]	-	0.178	1	0.178	1	0.284

Note: Continuous variables were reported as median and interquartile range (IQR). Comparisons between groups were performed using the Mann-Whitney test. Median difference and 95% confidence intervals (CI) were estimated with the Hodges-Lehmann method. Abbreviations: BS, bleeding score; FVIII:C, factor VIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide. [a] Missing values in European type 3 VWD patients: VWFpp, n= 2; VWFpp/VWF:Ag, n= 2, BS, n= 1. [b] Missing values in Iranian type 3 VWD patients: FVIII:C, n= 1; VWFpp, n= 2; FVIII:C/VWF:Ag, n= 1; VWFpp/VWF:Ag, n= 2; BS, n= 5. [c] Median difference cannot be calculated due to the presence of only one patient in the compound heterozygous for missense-null variants group.

In this study, we investigated whether VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios can be applied to evaluate the pathophysiological mechanism underlying type 3 VWD and to predict the type of variant. The analysis of this large group of type 3 VWD patients showed that the VWFpp level as well as the VWFpp/VWF:Ag ratio were clearly higher in patients with missense variants than those with null variants, indicating that increased clearance of secreted mature VWF plays a role in the pathogenesis of the disease in the former. Fully in line with this observation the patients compound heterozygous for a null and a missense variant had intermediate levels of VWFpp and VWFpp/VWF:Ag.

In all type 3 VWD patients, we identified strongly reduced levels of VWF measurements (VWF:Ag and VWFpp). This is expected in patients characterized by a genotype of homozygous or compound heterozygous null variants, as these variants predict a defect in VWF synthesis leading to very low or undetectable levels of both VWFpp and VWF:Ag. However, in patients characterized by a genotype of homozygous or compound heterozygous missense variants it may be expected that the mutant VWF protein is still synthesized. Therefore, the low levels of circulating VWF:Ag measured in those patients could be explained by a combination of intracellular retention, secretion defect or fast clearance of mutant protein from the circulation. In the group of type 3 patients with homozygous or compound heterozygous missense variants the VWFpp level was indeed measurable at a low level (median 3.6 IU/dL), indicating the synthesis of the protein at low level and/or intracellular retention. The VWFpp/VWF:Ag ratio in this group was clearly increased and also higher than in the group characterized by null alleles, indicating that the secreted VWF is cleared faster from the circulation. Interestingly, most of missense variants were not localized in the propeptide, therefore a direct effect on propeptide clearance and as consequence on VWFpp/VWF:Ag ratio was excluded. Moreover, these findings showed that also in type 3 VWD an increased VWFpp/VWF:Ag ratio is indicative of the presence of missense variants. Sanders *et al.* previously reported the description of a group of 15 patients with VWF:Ag levels <5 IU/dL and high levels of VWFpp (median 72 IU/dL). Of them, 14 were genotyped and 10 were heterozygous for missense variants known to be associated with (very) rapid clearance of the protein from the circulation [12]. Indeed, those heterozygous patients should actually be reclassified as severe type 1. The VWFpp/VWF:Ag ratio can thus easily discriminate between type 3 and severe type 1 VWD. In contrast to Sanders *et al.* we have now studied patients that fulfil all criteria for type 3 VWD, including being homozygous or compound heterozygous for a *VWF* defect. Even then, the increased VWFpp/VWF:Ag ratio indicates the presence of missense variants versus null variants, highlighting the utility of VWFpp in studying the pathophysiology of type 3 VWD.

Previously, we have reported that increased FVIII:C/VWF:Ag ratio can discriminate in type 1 VWD between heterozygous carriers of null variants and missense variants, where a high FVIII:C/VWF:Ag indicates a synthetic defect as the pathophysiological mechanism in the case of null variants [11, 12]. In the current cohort of type 3 VWD patients we did not find a difference in FVIII:C/VWF:Ag ratio between patients homozygous/

compound heterozygous for null or missense variants, although the ratio was higher than the normal reference range. At very low levels of VWF:Ag the capacity to bind and stabilize FVIII is severely compromised, and the circulating FVIII may partly be unbound FVIII, and thus the FVIII:C/VWF:Ag ratio in type 3 VWD may not be reliable. Based on the higher circulating levels of VWFpp and the increased VWFpp/VWF:Ag ratio in type 3 patients homozygous/compound heterozygous for missense variants, it could be possible that more mature VWF:Ag is secreted to the circulation than in patients homozygous/compound heterozygous for null variants, although VWF is cleared rapidly. Therefore, we expected a milder bleeding phenotype for patients homozygous/compound heterozygous for missense variants. This hypothesis was partially supported by the fact that these patients showed a slightly lower BS than patients homozygous/compound heterozygous for null variants (11.5 vs. 14.0; P= 0.501). However, there was no association between VWFpp level and the bleeding symptoms represented by the BS (r= 0.024; P= 0.778). Our result may be affected by the use of the bleeding score, as it is easily saturated in type 3 patients, thus preventing to discriminate small differences.

The potential correlation between VWFpp level and bleeding phenotype has also been evaluated depending on the location of the genetic defects. To this purpose patients were grouped as: (i) homozygous/compound heterozygous carriers for variants in the VWFpp; (ii) homozygous/compound heterozygous carriers for variants in mature VWF; (iii) compound heterozygous carriers for a variant in the VWFpp and a variant in mature VWF and (iv) "excluded patients" as they do not meet the requirements to be coded in the previous groups (i.e., carriers for large deletions/duplications involving both VWFpp and mature VWF; **Table S1** in supporting information). However, none of these groups showed the association between VWFpp level and the bleeding symptoms. The 3WINTERS-IPS study represents the largest population of type 3 VWD patients so far collected. However, the principal limitation of this study consists in the considerable number of excluded patients because they did not fulfil the inclusion criteria, they had multiple missing data or were on prophylaxis at sampling time. Second, we did not have information about the distance between last treatment and sampling time. Therefore, we cannot exclude that the VWF:Ag≥ 3 IU/dL measured in some patients (e.g., the 15 subjects excluded from the main analysis group despite having a complete molecular characterization) were due to residual traces of concentrate containing VWF, thus contributing to the sample size reduction. Then, the choice to focus the genetic analysis on the coding region and intron/exon boundaries may have contributed to reducing the number of completely characterized patients such as for the eight subjects with VWF:Ag≤ 3 IU/dL excluded because of the inconclusive genotyping.

Last, we had considered whether our results could be affected by the genotype heterogeneity of European and Iranian patients. Indeed, the European population showed the highest number of different variants which were distributed along the VWF, whereas the Iranian population has the highest number of homozygous carriers of variants which were mainly localized at the VWF amino-terminal end [13]. Nevertheless, the results

obtained considering the two populations separately were in line with those of the main analysis thus suggesting good generalizability of our results.

In conclusion, this study showed that an increased VWFpp/VWF:Ag ratio can be indicative of the presence of missense variants as the cause of VWD even in type 3, whereas the FVIII:C/VWF:Ag ratio failed to discriminate the presence of null alleles. Discriminating homozygosity/compound heterozygosity of missense variants could be useful clinical information as it might be indicative of a milder bleeding phenotype; however the BS as used in this study failed to show an association with VWFpp levels.

ACKNOWLEDGMENTS

We would like to thank the Members and current Chair of the Sub-Committee on VWF of the Scientific Standardization Committee (SSC) of the ISTH who promoted the endorsement of 3WINTERS-IPS project among the scientific activities of this Sub-Committee. We also would like to thank Javier Battle, Erik Berntorp, Cosimo Ettore, Charles R.M. Hay, Hamid Hoorfar, Maria Gabriella Mazzucconi, Massimo Morfini, Rafael Parra Lòpez, and Omidreza Zekavat for patient enrolment and collections of clinical data and Anne Goodeve for her work on genotyping. The 3WINTERS-IPS project received unconditional research grants from several pharmaceutical companies and therefore we acknowledge the representatives of Baxter-Shire-Takeda, Grifols, CSL Behring, LFB, and Octapharma. This work was partially supported by the Hungarian National Research Development and Innovation Office (NFKI) grant OTKA-K19_131945 (I.B.). This work was partially supported by the Italian Ministry of Health - Bando Ricerca Corrente (F.P.). We wish to thank Paolo De Simoni, Luca Maravigna, and Elisabetta Musazzi of the CRO Sintesi Research for the study coordination. We acknowledge L. F. Ghilardini for the illustration work.

REFERENCES

1. Sadler JE, Budde U, Eikenboom JC, Favaloro EJ, Hill FG, Holmberg L, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. J Thromb Haemost. 2006; 4:2103-2114.

2. James PD, Connell NT, Ameer B, Di Paola J, Eikenboom J, Giraud N, et al. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. Blood Adv. 2021; 5:280-300.

3. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998; 67:395-424. 10.1146/annurev.biochem.67.1.395.

4. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. J Thromb Haemost. 2003; 1: 1335-1342.

5. Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. J Clin Invest. 1973; 52:2757-2764.

6. Wagner DD, Mayadas T, Marder VJ. Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. J Cell Biol. 1986; 102:1320-4.

7. Vischer UM, Wagner DD. von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. Blood. 1994; 83:3536-3544.

8. Wagner DD, Olmsted JB, Marder VJ. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. J Cell Biol. 1982; 95:355-360.

9. Borchiellini A, Fijnvandraat K, ten Cate JW, Pajkrt D, van Deventer SJ, Pasterkamp G, et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. Blood. 1996; 88:2951-2958.

10. Eikenboom JC, Castaman G, Kamphuisen PW, Rosendaal FR, Bertina RM. The factor VIII/von Willebrand factor ratio discriminates between reduced synthesis and increased clearance of von Willebrand factor. Thromb Haemost. 2002; 87:252-257.

11. Eikenboom J, Federici AB, Dirven RJ, Castaman G, Rodeghiero F, Budde U, et al. VWF propeptide and ratios between VWF, VWF propeptide, and FVIII in the characterization of type 1 von Willebrand disease. Blood. 2013; 121:2336-2339.

12. Sanders YV, Groeneveld D, Meijer K, Fijnvandraat K, Cnossen MH, van der Bom JG, et al. von Willebrand factor propeptide and the phenotypic classification of von Willebrand disease. Blood. 2015; 125:3006-3013.

13. Baronciani L, Peake I, Schneppenheim R, Goodeve A, Ahmadinejad M, Badiee Z, et al. Genotypes of European and Iranian patients with type 3 von Willebrand disease enrolled in 3WINTERS-IPS. Blood Adv. 2021; 5:2987-3001.

14. Tosetto A, Rodeghiero F, Castaman G, Goodeve A, Federici AB, Batlle J, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). J Thromb Haemost. 2006; 4:766-773.

15. Bowman M, Tuttle A, Notley C, Brown C, Tinlin S, Deforest M, et al. The genetics of Canadian type 3 von Willebrand disease: further evidence for co-dominant inheritance of mutant alleles. J Thromb Haemost. 2013; 11:512-520.

16. Goodeve AC. The genetic basis of von Willebrand disease. Blood Rev. 2010; 24:123-134.

SUPPLEMENTARY DATA

Table S1. Distribution of the 147 type 3 VWD patients based on variants' localization in the VWFpp or in mature VWF

	Homozygous/compound heterozygous for variants in VWFpp**	Compound heterozygous for a variant in VWFpp and a variant in mature VWF	Homozygous/compound heterozygous for variants in mature VWF††	"Excluded patients" who cannot be classified in the other groups‡‡
N	30	11	99	7
Age, years	26.0 (11.0-34.0)	22.0 (16.0-41.0)	26.0 (15.0-39.0)	42.0 (41.0-45.0)
Sex (female), n (%)	18 (60)	8 (73)	57 (58)	4 (57.0)
VWF:Ag, (IU/dL)	0.5 (0.5-0.5)	0.5 (0.5-0.5)	0.5 (0.5-0.5)	0.5 (0.5-0.5)
Missing, n	-	-	-	-
VWFpp, (IU/dL)	1.3 (0.6-3.4)	1.2 (0.7-8.0)	1.9 (0.8-4.9)	0.8 (0.3-1.3)
Missing, n	1	-	3	-
FVIII:C, (IU/dL)	2.3 (1.4-2.8)	2.3 (1.7-2.8)	2.3 (1.8-3.0)	2.4 (2.0-3.4)
Missing, n	-	-	1	-
VWFpp/VWF:Ag *	2.6 (1.2-5.4)	2.4 (1.0-16.0)	3.2 (1.6-7.2)	1.6 (0.6-2.6)
Missing, n	1	-	3	-
FVIII:C/VWF:Ag †	4.6 (2.8-5.7)	4.4 (3.4- 4.8)	4.3 (3.6-5.5)	4.8 (4.0-6.8)
Missing, n	-	-	1	-
Bleeding Score (BS)	14.0 (7.5-18.0)	14.0 (9.0-26.0)	14.0 (6.0-19.0)	20.0 (8.0-28.0)
Missing, n	5	-	1	-

Table S1 (continued). Distribution of the 147 type 3 VWD patients based on variants' localization in the VWFpp or in mature VWF

	Homozygous/compound heterozygous for variants in VWFpp**	Compound heterozygous for a variant in VWFpp and a variant in mature VWF	Homozygous/compound heterozygous for variants in mature VWF††	"Excluded patients" who cannot be classified in the other groups‡‡
Variant identified, n (%)	30 (100)	11 (100)	99 (100)	7 (100)
VWFpp/BS correlation‡, r (P-value)	-0.484 (0.016)	0.0336 (0.312)	0.099 (0.342)	0.036 (0.939)

Continuous variables were reported as median and interquartile range (IQR). VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide; FVIII:C, Factor VIII coagulant activity. *VWFpp/VWF:Ag, normal range 0.8-2.2, calculated as 2.5th to 97.5th percentile in 387 healthy controls; †FVIII:C/VWF:Ag normal range 0.6-1.9, calculated as 2.5th to 97.5th percentile in 387 healthy controls (Eikenboom J *et al*; Blood. 2013). ‡ The correlation between VWFpp and bleeding score was evaluated using the Spearman's rank distribution. ** Variant in VWFpp refers to all variants located in D1-D2 domains. †† Variants in mature VWF refers to all variants located in D'-CK domains. ‡‡ This group includes 7 patients who do not meet the requirements to be classified in the other groups. Of them, 6 patients are homozygous for large deletions involving both VWFpp and VWF and 1 patient is compound heterozygous for a large duplication and small deletion in exon 7.