

ORIGINAL ARTICLE

Genetic determinants of enhanced von Willebrand factor clearance from plasma

Omid Seidizadeh^{1,2} | Luciano Baronciani¹ | Maria Teresa Pagliari¹ |
 Giovanna Cozzi¹ | Paola Colpani¹ | Andrea Cairo¹ | Simona Maria Siboni¹ |
 Eugenia Biguzzi¹ | Flora Peyvandi^{1,2}

¹Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Milan, Italy

²Università degli Studi di Milano, Department of Pathophysiology and Transplantation, Milan, Italy

Correspondence

Flora Peyvandi, Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Via Pace 9, Milan 20122, Italy.
 Email: flora.peyvandi@unimi.it

Abstract

Background: Enhanced von Willebrand factor (VWF) clearance from plasma is associated with von Willebrand disease (VWD). However, the genetic background of this disease mechanism is not well defined.

Objective: To determine VWF variants that are associated with reduced VWF survival.

Methods: Two hundred fifty-four patients with VWD (type 1 = 50 and type 2 = 204) were investigated, and the results were compared with 120 healthy controls. The patients were comprehensively characterized for phenotypic and genetic features. The ratio of VWF propeptide (VWFpp)/VWF antigen (VWFpp ratio) was used to establish in each patient the VWF clearance state.

Results: Out of 92 variants associated with type 1 (7 were novel) and type 2 VWD, 19 had a VWFpp ratio ranging from 1.7 to 2.2, 24 had a VWFpp ratio between 2.3 and 2.9, and 24 variants had a ratio of ≥ 3 . The VWFpp median ratio in healthy controls was 0.98 (0.55–1.6) so that a cut-off value of >1.6 was considered an indicator of accelerated VWF clearance from plasma. An enhanced VWF clearance was observed in 34% of type 1 cases, 100% of type 1 Vicenza cases, 81% of 2A cases, 77% of 2B cases, 88% of 2M cases, and 36% of 2N cases.

Conclusions: An accelerated VWF clearance was found in most patients with type 2A, 2B, and 2M VWD, with a lower proportion of type 1 and 2N. Sixty-seven different variants alone or in combination with other variants were associated with an increased VWFpp ratio. The variants with the highest VWFpp ratio were mostly located in the D3-A1 VWF domains.

KEYWORDS

genetic, half-life, VWD, VWF, VWF clearance

Manuscript handled by: David Lillicrap

Final decision: David Lillicrap, 11 January 2023

© 2023 The Authors. Published by Elsevier Inc. on behalf of International Society on Thrombosis and Haemostasis. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1 | INTRODUCTION

Von Willebrand factor (VWF) is an adhesive multimeric glycoprotein that is exclusively synthesized in endothelial cells and megakaryocytes [1]. This multifunctional glycoprotein is a major player in thrombosis and hemostasis, vascular inflammation, angiogenesis, muscle cell proliferation, and tumor cell metastasis [2,3]. Among these functions, however, VWF is best known for supporting platelet adhesion to exposed collagen at the site of the injured vessel wall and for acting as a chaperon for coagulation factor VIII (FVIII) [4].

Being produced as a pre-pro-polypeptide of 2813 amino acids, VWF contains different domains arranged in the following order: D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK [5]. The D1-D2 domains represent the propeptide (VWFpp), whereas the remaining domains refer to the mature subunit of VWF [6]. Following the cleavage of a signal peptide in the endoplasmic reticulum, the pro-VWF undergoes extensive posttranslational modifications, including dimerization/N-glycosylation in the endoplasmic reticulum and multimerization/O-glycosylation in the Golgi [6]. VWFpp is cleaved from the mature subunit in the Golgi apparatus by furin, but it remains noncovalently bound to it. VWF and VWFpp are secreted concomitantly into the blood circulation but with different plasma half-lives (12–16 hours for VWF vs 2 hours for VWFpp) [7,8]. The glycans on VWF play a crucial role in the determination of its half-life by preventing an early removal through clearance receptors and maintaining its structure and function [9]. This is confirmed by the fact that individuals with O blood group show in average 25% lower VWF levels than those with non-O blood groups [10]. It seems that these lower levels are because of the enhanced clearance of VWF [11], but the exact mechanism is not yet understood.

The most common bleeding disorder, von Willebrand disease (VWD), is caused by quantitatively or qualitatively defective VWF that is inherited with autosomal dominant or recessive patterns of transmission [12]. The complex pathophysiology of VWD can be because of the reduced VWF synthesis/secretion, enhanced VWF plasma clearance, impaired multimerization, or altered binding affinity for such substrates as FVIII, platelet glycoprotein Ib, collagen, and A Disintegrin and Metalloproteinase with a Thrombospondin Type-1 Motif, Member 13 (ADAMTS-13) [13]. The clearance of VWF can be determined either by measuring the ratio of VWFpp to VWF antigen (hereinafter, VWFpp ratio) or by evaluating the half-life of VWF post desmopressin (DDAVP) administration [14].

VWD is classified into type 1 (partial quantitative deficiency of VWF), type 2 (qualitative abnormal VWF), and type 3 (virtual absence of VWF) [13]. A shorter plasma half-life of VWF is typically reported in patients with type 1 VWD [15–17] but also in other types of VWD. Indeed, Sanders et al. [18] showed that an enhanced clearance of VWF is a common finding in a number of patients with type 1 VWD and also in patients with type 2 and in severe type 1 with a previous diagnosis of type 3.

Because approximately 95% to 98% of the circulating FVIII is bound to VWF and FVIII-VWF circulates as a protein complex, the FVIII plasma half-life directly depends on VWF clearance [19].

Essentials

- The genetic background of increased von Willebrand factor (VWF) plasma clearance in von Willebrand disease (VWD) is not well defined.
- A higher proportion of patients with types 2A, 2B, and 2M VWD had accelerated VWF clearance compared with patients with types 1 and 2N VWD.
- Sixty-seven different variants were associated with increased VWF propeptide ratio, either alone or in combination with other variants.
- Variants with the highest VWF propeptide ratio were mostly located in the D3 and A1 domains.

Understanding the factors that contribute to VWF clearance is clinically relevant because DDAVP may not be a valid therapeutic option for patients with a shorter VWF half-life.

Because the genetic background of enhanced VWF clearance has not been extensively investigated in patients with VWD, especially in type 2, in this study, we aimed to determine variants in the VWF that are associated with reduced VWF plasma survival in a cohort of fully characterized patients with type 1 and type 2 VWD.

2 | PATIENTS AND METHODS

2.1 | Study population

We included in this study a total of 254 patients with type 1 (n = 50) and type 2 (n = 204) VWD. The included participants were patients referred to the A. Bianchi Bonomi Hemophilia and Thrombosis Center (Milan, Italy). Patients with type 2 VWD were selected from a cohort that is already characterized [20]. Patients with type 1 VWD were recruited from 2002 to 2022. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

2.2 | Definition

For this study, type 1 VWD was defined as having bleeding symptoms and decreased VWF levels (<50 IU/dL) with a normal ratio of platelet-dependent VWF activity ([VWF activity]/VWF:Ag >0.6). Given the peculiar clearance situation of type 1 Vicenza (p.Arg1205His), hereon, patients carrying this variant were isolated from other type 1 cases. Type 2A VWD was defined by a reduced VWF activity/VWF:Ag ratio (<0.6) and reduced VWF high-molecular-weight multimers (HMWMs). Because it is more practical, we subclassified type 2A as 2A(IIA), 2A(IIIE), and 2A(IIID) despite this being an outdated nomenclature: type 2A(IIA) with increased proteolysis of the A2 domain by ADAMTS-13, type 2A(IIIE) with an impaired VWF multimerization (D3 domain) and reduced proteolytic cleavage, and type 2A(IIIC) with multimerization

defects (D1-D2 domains) and reduced proteolytic cleavage. Type 2B was defined as hyperresponsiveness to ristocetin-induced platelet agglutination, with or without the reduced VWF activity/VWF:Ag ratio, and it was further divided into classical 2B (reduced HMWM) and 2B Malmo/New York (2B NY, normal HMWM). Type 2M was defined by a reduced VWF activity/VWF:Ag ratio or reduced VWF-collagen binding activity (VWF:CB)/VWF:Ag ratio (<0.6) with a normal multimer pattern. Type 2M was also subdivided into classical 2M with decreased VWF A1 domain affinity to glycoprotein Ib, 2MCB with a reduced VWF affinity to collagen, or type 2M/2A with a mixed phenotype of 2M and 2A [20]. Type 2N was defined by reduced binding affinity of VWF to FVIII as detected by the VWF: factor VIII binding (VWF:FVIIIb) test.

We used previous data [21] on 120 healthy volunteers for normal ranges of VWFpp, VWF:Ag, and VWFpp ratio. Accordingly, the reference range (the 95th percentile distribution) used was 51 to 165 IU/dL for VWF:Ag, 55 to 155 IU/dL for VWFpp, and 0.6 to 1.6 for the VWFpp ratio. A cutoff of >1.6 for the VWFpp ratio was defined as an index of enhanced clearance of plasma VWF [21].

2.3 | Laboratory measurements

Samples were collected in 3.2% buffered citrate solution (1:9 anticoagulant to whole blood) and centrifuged at 1500 g for 15 minutes at room temperature to obtain platelet-poor plasma. Several aliquots were stored at -80°C to the date of assessment. To characterize patients, the laboratory evaluation of VWD panel tests, including FVIII:C, VWF:Ag, VWF activity, VWF:CB, ristocetin-induced platelet agglutination, VWF multimer analysis, and VWF:FVIIIb were carried out as previously described [20,22].

We used a commercially available kit to measure the VWFpp antigen by the enzyme-linked immunoassay method (Sanquin). In brief, we first coated the microtiter plates with CLB-Pro 35 antibody overnight at 4°C . After washing the plate, it was blocked with 1% bovine serum albumin and incubated for 2 hours at room temperature. Next, the diluted samples were incubated for 2 hours at 37°C . The plate was then incubated for 2 additional hours with an HRP-conjugated CLB-Pro 14.3 antibody and then with a TMB-substrate solution. The absorbance was read at 450 nm immediately after stopping the reaction. A pool from normal subjects (calibrated against an international standard) was used to create the standard curve.

2.4 | Genetic analysis

Genomic DNA was extracted using standard methods [23]. Once the VWD diagnosis and its classification were established using the full set of laboratory tests as above, the polymerase chain reaction and Sanger sequencing were performed for patients as previously described [23,24]. For patients with type 2 VWD, the targeted sequencing approach was applied to amplify the exon encoding the specific VWF domains but also including intron-exon boundaries [20]. The sequencing of target exon (s) was based on the biochemical

results used to identify the defective VWF domains. In type 1 VWD, however, all exons were sequenced with Sanger sequencing or next-generation sequencing [25]. Of note, a few patients with type 1 VWD had been genetically characterized in the frame of the MCMDM-1VWD study [26]. For patients with more than one variant, we stated when it was unclear whether variants were in trans or cis position; otherwise, the position of the variants was reported.

2.5 | Statistical analysis

Continuous variables were described as medians (ranges), and categorical variables as counts (percentages). Statistical analyses were performed with SPSS for Windows, version 21.0 (SPSS). The Kruskal-Wallis test was used to test the differences in the VWFpp ratio between healthy controls and types 1, 2A, 2B, 2M, 2N, and 2 subclasses. The Mann-Whitney U-test was used to compare medians between 2 independent groups. A p value of $<.05$ was considered statistically significant.

3 | RESULTS

Patients included in this study were classified as type 1 VWD ($n = 38$), type 1 Vicenza ($n = 12$), type 2A ($n = 62$), type 2B ($n = 44$), type 2M ($n = 87$), and type 2N ($n = 11$). The characteristics of the study population and the phenotypic laboratory results are summarized in Table 1.

3.1 | Laboratory results and enhanced clearance of VWF in types 1 and 2 VWD

Type 1 Vicenza ($n = 12$) showed the highest median values for the VWFpp ratio (8.1, range: 5-16.4), with a median of 74 IU/dL (40-128 IU/dL) for VWFpp levels (Table 1). In patients with type 1 VWD ($n = 38$, Vicenza not included), the median VWFpp value was lower (46 IU/dL) than that in patients with type 1 Vicenza VWD, with a median VWFpp ratio of 1.3 (0.68-3.4, Table 1 and Figure 1). Both type 1 and Vicenza VWD had a significantly higher ratio than healthy controls ($p < .001$). We found an enhanced clearance in 34% of patients with type 1 (13/38) and 100% of patients with type 1 Vicenza.

In patients with type 2 VWD ($n = 204$), the overall median VWFpp was 73 IU/dL (9-376 IU/dL), with a VWFpp ratio of 2.2 (0.5-5.2), significantly higher than that in healthy controls ($p < .001$) and type 1 VWD ($p < .001$; Figure 1). We further subclassified type 2 based on phenotypic laboratory tests and a confirmed genetic diagnosis to determine the VWF clearance status among each group (Table 1).

The VWFpp ratio for different type 2 cases is shown in Figure 2. Patients with type 2A (IIA, $n = 36$; IIE, $n = 25$) had VWFpp median values of 82 IU/dL (18-174 IU/dL) and 63 IU/dL (33-88 IU/dL), respectively, with VWFpp ratios of 2 (0.78-3.5) and 2.8 (0.7-5.2). Type 2A (IIE) showed the highest ratio among all patients with type 2 VWD. Overall, 81% of patients with type 2A had a VWFpp ratio higher than

TABLE 1 The laboratory results of patients and controls.

| VWD type | N | FVIII:C (IU/dL) | VWF:Ag (IU/dL) | VWF activity (IU/dL) | VWFpp (IU/dL) | VWFpp ratio | % of patients with VWFpp ratio >1.6 |
|------------------|-----|-----------------|----------------|----------------------|---------------|----------------|-------------------------------------|
| Type 1 | 38 | 63 (20-110) | 37 (3-52) | 29 (3-57) | 46 (6-128) | 1.3 (0.68-3.4) | 34% |
| Vicenza | 12 | 16 (10-22) | 10 (3-18) | 8 (3-14) | 74 (40-128) | 8.1 (5-16.4) | 100% |
| All type 2 | 204 | 50 (6-133) | 33 (10-156) | 8 (3-112) | 73 (9-376) | 2.2 (0.5-5.2) | 80% |
| 2A(IIA) | 36 | 49 (21-91) | 43 (10-110) | 9 (3-24) | 82 (18-174) | 2 (0.78-3.5) | 77% |
| 2A(IIC) | 1 | 56 | 18 | 8 | 9 | 0.5 | |
| 2A(IIE) | 25 | 40 (25-77) | 21 (13-115) | 11 (6-45) | 63 (33-88) | 2.8 (0.7-5.2) | 84% |
| Classical 2B | 37 | 57 (6-132) | 48 (18-140) | 12 (4-90) | 102 (33-376) | 2.1 (0.85-3.8) | 81% |
| 2B NY | 7 | 62 (50-75) | 45 (21-80) | 26 (10-41) | 53 (35-126) | 1.8 (0.8-2.3) | 57% |
| Classical 2M | 34 | 45 (23-124) | 26 (12-130) | 11 (3-44) | 64 (32-137) | 2.5 (0.8-4.1) | 82% |
| 2M/2A | 28 | 48 (24-74) | 29 (12-61) | 10 (5-19) | 74 (37-136) | 2.5 (1.7-4) | 100% |
| 2MCB | 25 | 52 (35-133) | 28 (18-147) | 25 (14-101) | 57 (43-302) | 2.1 (0.8-3.2) | 84% |
| 2N | 11 | 35 (15-50) | 56 (15-156) | 42 (21-112) | 76 (25-140) | 1.1 (0.8-2.5) | 36% |
| Healthy controls | 120 | NA | 96 (51-165) | NA | 93 (55-155) | 0.98 (0.6-1.6) | |

All data are presented as median (range).

FVIII:C, factor VIII coagulant activity assay; VWD, von Willebrand disease; VWF activity, platelet-dependent VWF activity; VWF:Ag, von Willebrand factor antigen; VWFpp, von Willebrand factor propeptide; VWFpp ratio, VWFpp/VWF:Ag

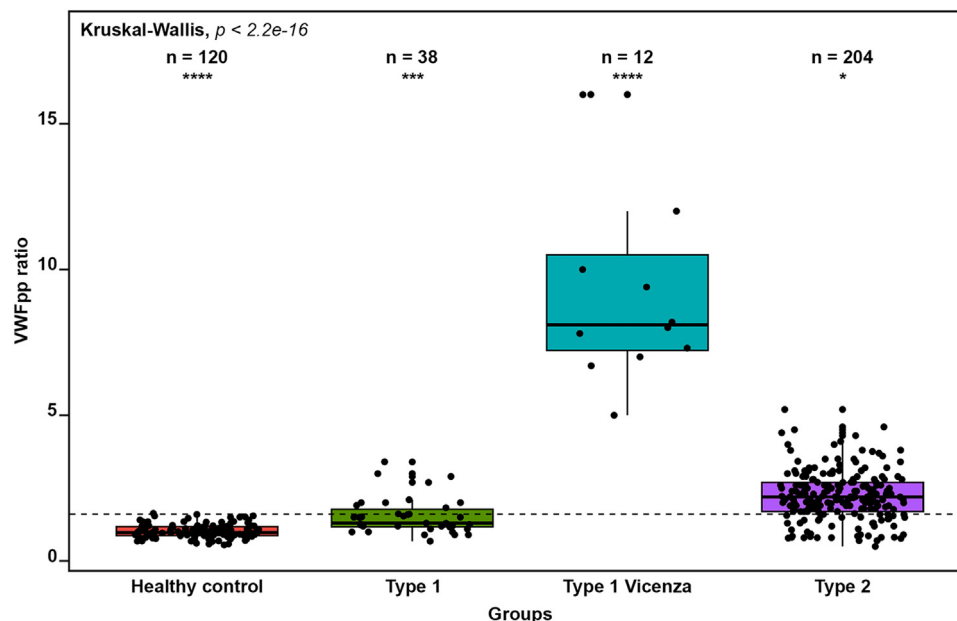


FIGURE 1 The box plot of VWFpp/VWF:Ag (VWFpp ratio) in healthy controls and patients with type 1, type 1 Vicenza, and type 2 VWD. The solid line indicates the median and the dotted line indicates the cutoff of 1.6 based on 120 healthy controls. The Kruskal-Wallis test was performed to assess the differences in the VWFpp ratio between healthy controls and type 1, type 1 Vicenza, and type 2 VWD groups. Using a VWFpp ratio of >1.6, the enhanced clearance of VWF was found in 34% of the patients with type 1 VWD, 100% of the patients with type 1 Vicenza VWD, and 80% of the patients with type 2 VWD. pp, propeptide; VWD, von Willebrand disease; VWF, von Willebrand factor. **p* value < .05, ***p* value < .01, ****p* value < .001, and **** *p* value < .0001.

1.6, including 77% (27/36) of type 2A(IIA) and 84% (21/25) of type 2A(IIE). The highest VWFpp value (102 IU/dL; range: 33-376 IU/dL) was found in patients with classical 2B (*n* = 37) as opposed to 2B NY

(*n* = 7) who had the lowest values (53 IU/dL; range, 35-126) among type 2. VWFpp ratios in both groups were higher than the determined normal ranges (2.1 and 1.8). Enhanced VWF clearance was found in

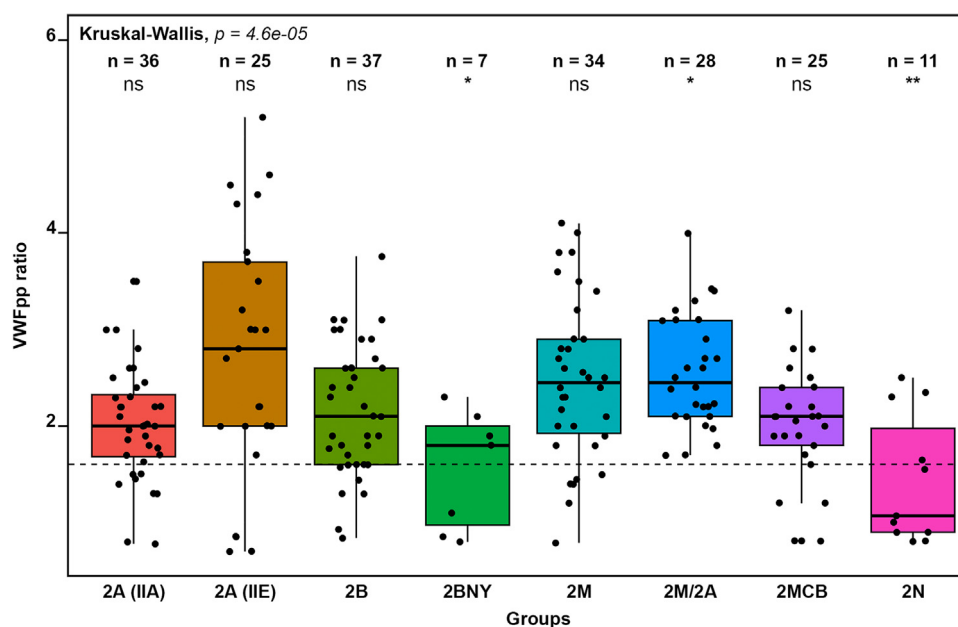


FIGURE 2 Box plot of the VWFpp/VWF:Ag (VWFpp ratio) in different groups of type 2 VWD. The boxplot solid line indicates the median and the dotted line indicates the cutoff of 1.6 based on 120 healthy controls. The Kruskal-Wallis test was performed to test the differences in the VWFpp ratio between different subclasses of type 2 VWD. The enhanced VWF clearance was identified in 77% of type 2A (IIA), 84% of 2A (IIE), 81% of classical 2B, 57% of 2BNY, 82% of classical 2M, 100% of 2M/2A, 84% of 2MCB, and 36% of 2N. Type 2A (IIC) is not shown in this figure. 2B, classical 2B; 2M, classical 2M; ns, non-significant; pp, propeptide; VWF, VWD, von Willebrand disease; von Willebrand factor. * p value $<.05$, ** p value $<.01$.

77% of all patients with type 2B VWD, including 81% of classical 2B (30/37) and 57% of 2B NY VWD (4/7). The VWFpp ratio was 2.5 (0.8-4.1, $n = 34$) in classical 2M, 2.5 (1.7-4, $n = 28$) in 2M/2A, and 2.1 (0.8-3.2, $n = 25$) in 2MCB. Type 2N ($n = 11$) had the lowest median VWFpp ratio among all type 2 cases (ratio, 1.1; range, 0.8-2.5), being the only type 2 group with no statistically significant difference in the VWFpp ratio compared with healthy controls (ratio of 1.1 vs that of 0.98, $p = .124$). The clearance of VWF was more enhanced in type 2M cases, with 88% of the cases showing a higher ratio than the defined cutoff. All the 28 cases of 2M/2A (100%), 28 of 34 cases with classical 2M (82%), and 21 of 25 cases with 2MCB (84%) had enhanced VWF clearance, whereas only 36% of patients with type 2N (4/11) presented with enhanced clearance.

3.2 | Gene variants associated with enhanced VWF clearance

Out of 92 unique variants investigated in this study, 22 caused type 1 (including 7 novel variants) and the remaining 70 were retrieved from our previous study [20]. Table 2 summarizes the novel variants and laboratory results of patients carrying these variants. Patients with p.Arg1205His showed a faster clearance than a type 1 patient carrying p.Arg1205Cys (ratio of 8.1 vs that of 3.4). All patients with type 1 were heterozygous for a single variant except for 4 cases; 2 patients with severe type 1 VWD (VWF levels between 2 and 10 IU/dL) who were compound heterozygous (c.5779T>C/c.8155+6T>C [p.Cys1927Arg/p.Cys2719Valfs*24] and c.1116C>A/c.1534-3C>A [p.Cys372*/p.Leu512Profs*11]) and 2 carrying a gene conversion

(c.3931C>T- c.3951C>T [p.Gln1311*-Ala1317Ala]). p.Tyr1584Cys was commonly found ($n = 9$) and patients carrying this variant had a median ratio of 1.6 (range, 1-2). Among all type 1 variants, the highest ratio (after p.Arg1205Cys) was found for c.5779T>C/c.8157+6T>C (p.Cys1927Arg/p.Cys2719Valfs*24) variants (ratio 3). However, a sibling of this patient carrying p.Cys1927Arg alone had a normal ratio (1.2). Two patients had p.Gln2520Pro with VWFpp ratios of 1.65 and 2.9. Enhanced VWF clearance was found for patients with p.Arg1379Cys (ratio 1.8 and 2.1) and for a patient with c.1116C>A/c.1534-3C>A variants (p.Cys372*/p.Leu512Profs*11; ratio, 2) as well. Other type 1 variants had a normal ratio (Figure 3, red columns).

We evaluated 26 distinct variants among patients with type 2A (IIA = 14, IIC = 2, and type IIE = 10). In type 2A (IIA), 2 patients with p.Arg1597Trp had the highest VWFpp ratio (3.5 and 3) and 1 patient who was heterozygous for 2 variants (p.Arg924Gln and p.Arg1597Trp) had a ratio of 2.6. Ten additional different type 2A (IIA) variants were also found to be associated with enhanced clearance (Figure 3, blue columns). The single case of type 2A (IIC) with 2 variants in the VWFpp (p.Asp366Leufs*16/p.Asn528Ser) showed the lowest ratio among all the studied cases (ratio 0.5). All the 10 different type 2A (IIE) variants had enhanced VWF clearance with the exception of p.Cys1142Phe. In type 2A (IIE), VWFpp ratios higher than 4 were observed in cases with p.Tyr1146Cys (ratio 4.4), p.Cys1126Tyr ($n = 4$; median, 4.1; range, 3.7-4.5) and c.3390C>T (p.Cys1130Cys, causing p.Pro1127_Gly1180delinsArg; $n = 8$; median, 3; range, 2-5.2). Several other type 2A (IIE) variants had ratios higher than 2 (Figure 3, light blue columns).

TABLE 2 Genotype-phenotype characterization and the in silico predication of novel variants in patients with VWD type 1.

| Family | Nucleotide change, amino acid change ^a | FVIII:C IU/dL | VWF:Ag IU/dL | VWF act IU/dL | VWF:CB IU/dL | VWF Act/VWF: Ag ratio | In silico analysis ^b | |
|--------|---|---------------|--------------|---------------|--------------|-----------------------|---|-------------------|
| | | | | | | | Predicted effects | VarSome |
| I | c.6798+9C>G | 61 | 35 | 29 | 36 | 0.82 | Likely benign, no splicing effect (CADD: 5.8, varSEAK: 1) | VUS |
| | c.6798+9C>G | 40 | 27 | 20 | 31 | 0.74 | Likely benign, no splicing effect (CADD: 5.8, varSEAK: 1) | VUS |
| II | c.324-10T>A | 56 | 28 | 23 | 24 | 0.82 | Damaging, splicing effect (CADD: 24.5, varSEAK: 5) | Likely pathogenic |
| III | c.7664_7665insAG, p.Cys2557Serfs*8 | 55 | 39 | 25 | 23 | 0.64 | Damaging (CADD: 32) | Pathogenic |
| IV | c.8249G>T, p.Cys2750Phe | 55 | 38 | 30 | - | 0.79 | Damaging (CADD: 26.8, PolyPhen-2: 0.99, PROVEAN: -5.53, SIFT: 0, M-CAP: 0.38) | VUS |
| | c.8249G>T, p.Cys2750Phe | 60 | 43 | 33 | - | 0.77 | Damaging (CADD: 26.8, PolyPhen-2: 0.99, PROVEAN: -5.53, SIFT: 0, M-CAP: 0.38) | VUS |
| V | c.105C>G, p.Cys35Trp | 66 | 29 | 24 | 27 | 0.83 | Damaging (CADD: 23.8, PolyPhen-2: 0.99, PROVEAN: -7.35, SIFT: 0, M-CAP: 0.88) | VUS |
| VI | c.7436C>A, p.Ser2479* | 79 | 37 | 28 | 29 | 0.74 | Damaging (CADD: 39) | pathogenic |
| VII | c.3072delC, p.Trp1025Glyfs*3 | 40 | 32 | 30 | 28 | 0.93 | Damaging (CADD: 26.5) | pathogenic |
| | c.3072delC, p.Trp1025Glyfs*3 | 45 | 41 | 34 | 30 | 0.83 | Damaging (CADD: 26.5) | pathogenic |

FVIII:C, factor VIII coagulant activity assay; VUS, variant of uncertain (or unknown) significance; VWD, von Willebrand disease; VWF activity, platelet-dependent VWF activity; VWF:Ag, von Willebrand factor antigen; VWF:CB, collagen binding activity.

^a None of these variants were found in the Genome Aggregation Database (gnomAD) indicating that they are extremely rare.

^b To predict the effect of novel variants, we used 6 different in silico tools as well as VarSome classification. These tools were including Polymorphism Phenotyping v2 (PolyPhen-2), Protein Variation Effect Analyzer (PROVEAN), Sorting Intolerant From Tolerant (SIFT), Combined Annotation Dependent Depletion (CADD), and Mendelian Clinically Applicable Pathogenicity (M-CAP). The varSEAK software and CADD score were used for splice variants. The nonsense variants were considered as damaging as also predicted by the CADD score.

Nineteen type 2B variants (14 2B classical and 5 2B NY) were investigated. Most cases with classical 2B variants were associated with increased ratios (Figure 3, green columns), such as p.Gly1172Val/p.Arg1308Cys (ratio, 3.1), p.Arg1306Gln (ratio, 2.9), p.Arg1306Trp (n = 9; median, 2.9; range, 1.7-3.8), p.Arg1306Leu (ratios, 2.4 and 2.3), and p.Arg1308Cys (n = 3; ratios, 3, 2.6, 1.8). In type 2B NY, 2 different gene conversions, including p.Ser1263Ser-Pro1266Leu (ratio, 2.1) and p.Val1229Gly-Asn1231Thr-Ser1263Ser-Pro1266Leu-Val1279Ile (ratio, 1.9), were associated with enhanced clearance. A patient with p.Ser1263Ser-Pro1266Leu also carried another variant (p.Cys2557-Tyr) and had a ratio of 2.3 (Figure 3, light green columns).

The majority of type 2M variants (16 of 19) were associated with enhanced clearance. In classical 2M, 2 cases with p.Ile1416Asn had a ratio of 4.1 and 3 and 1 patient with the p.Gly1415Asp had a ratio of

3.4. p.Ala1377Val-Arg1379Cys also was associated with enhanced clearance (n = 8; median, 2.6; range, 1.9-3.8). In addition to these missense variants, several gene conversions, such as p.Ile1343Val-Val1360Ala-Phe1369Ile-Ser1378Phe-Arg1379Cys (ratio, 3.5), p.Phe1369Ile-Ser1378Phe-Arg1379Cys (n = 3; ratios, 4, 2.7, 2), and p.Val1360Ala-Phe1369Ile-Ser1378Phe-Arg1379Cys (n = 4; median, 3.1; range, 2.5-3.8), were associated with enhanced VWF clearance. Other classical 2M variants with normal or high ratios can be seen in Figure 3 (orange columns). All the variants causing type 2M/2A were consistently associated with VWFpp ratios of >1.6: p.Arg1374His (n = 17; median, 2.6; range, 2-3.4), p.Arg1374Cys (n = 5; ratio, 2.6; range, 1.8-4), and p.Arg924Gln-p.Arg1315Leu (n = 6; ratio, 2.3; range, 1.7-3.1). Among type 2MCB variants (Figure 3, gray columns), 3 of 5 variants constantly led to enhanced clearance, including p.Ala1617Pro

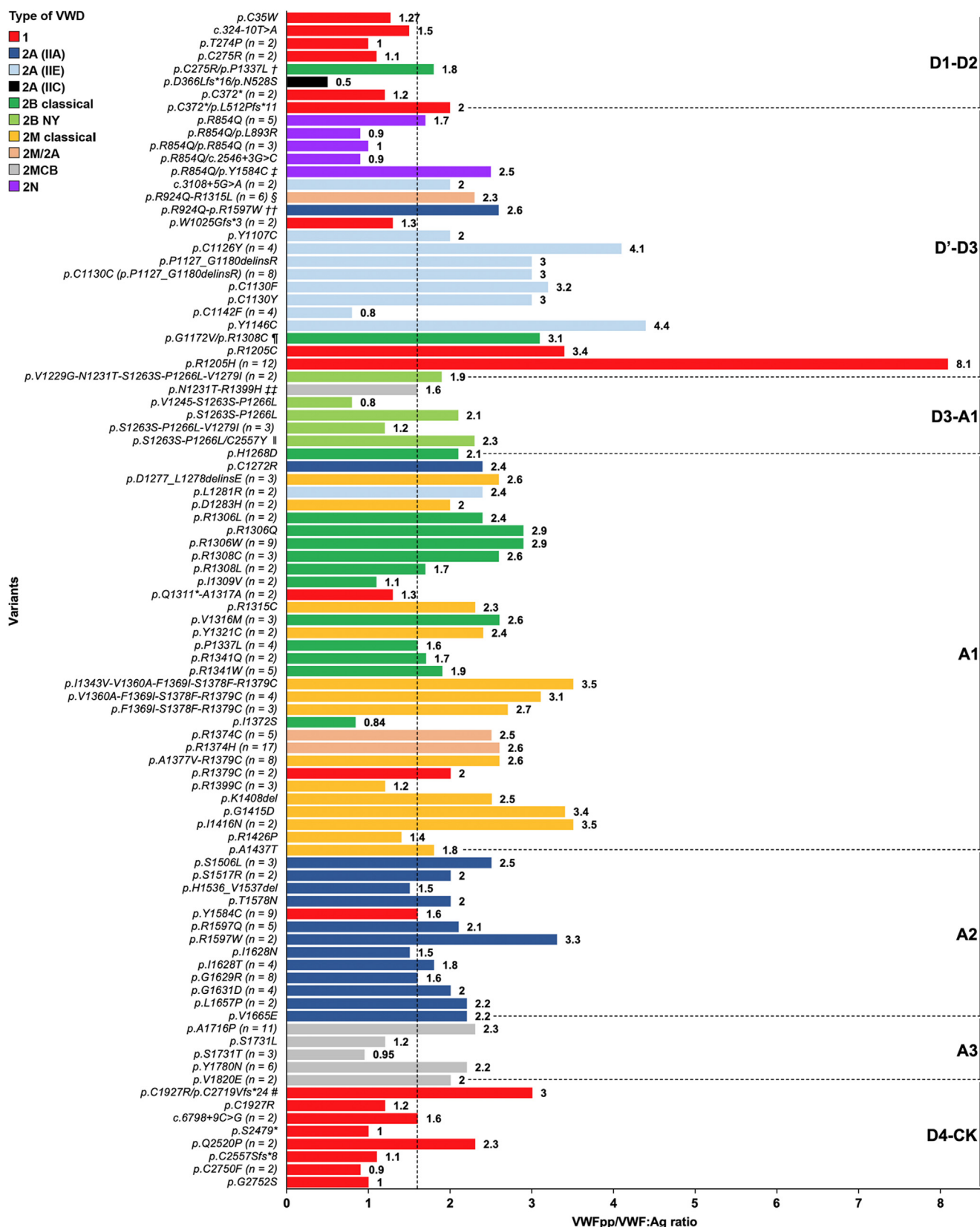


FIGURE 3 The VWFpp/VWF:Ag ratio (VWFpp ratio) of the individual genetic variants identified and the location of variants in the pro-VWF domains. Variants are ordered according to the protein position. If there were 2 variants in one patient, these variants were depicted based on the closer variant to the pro-VWF amino-terminal end. †p.Cys275Arg at D1 and p.Pro1337Leu at A1, ‡p.Arg854Gln at D' and p.Tyr1584Cys at A2, §p.Arg924Gln at D3 and p.Arg1315Leu at A1, ††p.Arg924Gln at D3 and p.Arg1597Trp at A2, ¶p.Gly1172Val at D3 and p.Arg1308Cys at A1, †††p.Asn1231Thr at D3 and p.Arg1399His at A1, ||p.Ser1263Ser-Pro1266Leu at D3 and p.Cys2557Tyr at C4, and #p.Cys1927Arg at D4 and

(n = 11, median, 2.3; range, 1.7-3.2), p.Tyr1780Asn (n = 6; median, 2.2; range, 1.8-2.8), and p.Val1820Glu (n = 2; ratios, 1.9, 2.1).

In type 2N (Figure 3, purple columns), a case with combined p.Arg854Q and p.Tyr1584Cys had a ratio of 2.5. Those who carried p.Arg854Gln in heterozygosity showed both normal and high ratios (n = 5; median, 1.7; range, 0.8-2.4). Although the phenotypic results of these 5 heterozygous patients suggest the potential existence of another variant, further investigations were impossible due to the lack of samples [20]. All 3 homozygous patients for p.Arg854Q had normal ratios (1.55, 1, and 0.8).

4 | DISCUSSION

This extensive phenotypic and genetic characterization of a large cohort of patients with type 1 and type 2 VWD newly demonstrates that an enhanced VWF clearance is more pronounced in type 2 than in type 1 VWD. Indeed, except for the type 1 Vicenza, only 34% of patients with type 1 had shorter VWF half-lives, whereas this phenomenon was observed in 80% of the entire VWD type 2 cohort. The overall VWFpp ratio taken as a criterion was higher in type 2 than in type 1 VWD ($p < .001$, Figure 1). A shortened survival of VWF was originally proposed as a mechanism of type 1 VWD, leading to the designation of these patients as type 1C [15]. This concept was confirmed in several cohorts of type 1 cases using the DDAVP trials [27,28] or the VWFpp ratio, even though each study used a different reference range for the VWFpp ratio [15,17,27].

The identification of variants associated with shorter plasma half-lives of VWF is crucial because DDAVP may not be a useful therapeutic approach for patients carrying these genetic variants. Indeed, the VWF half-life after DDAVP infusion was investigated for several VWF variants and results suggested that this medication may not be suitable for these patients because of the rapid removal of VWF and hence of FVIII [15,27,29].

We found a lower yet comparable enhanced VWF clearance in type 1 VWD (34%) in comparison with the Zimmerman study (41%) [30] and WiN study (46%) [18]. The present study found a higher rate of enhanced clearance in type 2A (81% vs 59%) and 2M (88% vs 48%) than in the WiN study [18], but a lower rate in type 2B (77% vs 95%) and 2N (36% vs 71%). The normal range of our VWFpp ratio was set to be 0.6 to 1.6, which is lower than that of the Zimmerman (68 healthy controls, 0.54-1.98) [15] and WiN studies (387 healthy controls, 0.8-2.2) [18]. Therefore, we used a lower cutoff of 1.6 as opposed to 2 [15] and 2.2 [18]. Some other studies also applied a ratio of >3 to diagnose patients with type 1C VWD [15,31] or those with low VWF levels and enhanced clearance [32]. Currently, there is no general agreement on a cutoff for the VWFpp ratio that warrants consideration, perhaps by the International Society on Thrombosis

and Haemostasis Scientific and Standardization Subcommittee on VWF. A desmopressin trial is a better way to comprehensively investigate VWF clearance because the ratio is influenced by the half-life of VWFpp itself. Indeed, the new guidelines suggest using desmopressin trials instead of relying on the VWFpp ratio to confirm elevated VWF clearance [33]. Nevertheless, the usefulness of the VWFpp ratio to predict increased VWF clearance has been confirmed in several studies in which both desmopressin trials and VWFpp ratio were used [11,15,16,34]. We observed a significant difference in the VWFpp ratio between healthy individuals with blood group O (n = 52, median = 1.14) and those with non-O blood groups (n = 68; median = 0.91, $p < .001$). This was also true for VWF:Ag levels (median = 80 IU/dL vs 105 IU/dL, $p < .005$). However, there was no difference between the 2 groups for VWFpp results (median = 93 IU/dL vs 99 IU/dL; $p = .365$), indicating a normal synthesis but also no difference for clearance in the 2 groups for the VWFpp [7,11]. These results confirm the hypothesis that VWF clearance is higher in people with the blood group O than that in people with non-O blood groups [11,15].

So far, no large study of type 2 VWD investigated the association between VWF variants and enhanced clearance. In this comprehensive phenotypic and genotypic analysis of 254 patients with type 1 and type 2 VWD, we attempted to address this issue. A total of 92 unique variants were identified, of which 7 were novel in type 1. The majority of patients (82%) had only 1 variant and the remainder had 2 (12%) or more (6%). In the literature, more than 20 variants have been associated with an enhanced VWF clearance [9,14,34]. Our study identified 67 VWF variants, either alone or in combination with other variants, to be associated with this phenomenon. The majority of these variants are reported for the first time to enhance VWF clearance. There were 19 variants with a VWFpp ratio from 1.7 to 2.2, 24 variants with a ratio between 2.3 and 2.9, and 24 variants with a ratio of ≥ 3 .

Clearance of VWF seems to be independent of its multimeric size and cleavage by ADAMTS-13 [35], but why patients with (2A and 2B) or without (2M) the lack of HMWM have a similar enhanced clearance remains to be determined. Recent studies demonstrated that D'-D3, A1, and D4 domains modulate VWF interaction with the low-density lipoprotein receptor-related protein 1 (LRP-1) and scavenger receptor class A member I [36,37]. The mutated VWF domains might have an altered binding affinity for macrophage-dependent clearance receptors. Among type 2 VWD, type 2B is expected to have the lowest VWF survival owing to the high affinity of the mutated A1 domain for platelet, thus triggering VWF and platelet removal from circulation. Casonato et al. [38] has found a similar increased VWFpp ratio in patients with type 2B VWD, regardless of their multimer composition or the degree of thrombocytopenia. In the present study, the classical type 2B had the highest VWFpp values among all studied patients (median, 102 IU/

p.Cys2719Valfs*25 at C6. Red: all type 1 variants, including the Vicenza variant (p.Arg1205His); blue: 2A(IIA); light blue: 2A(IIIE); black: 2A(IIIC); green: classical 2B; light green: 2B NY; orange: classical 2M; brown: 2M/2A; gray: 2MCB; and purple: 2N. Genetic variants are reported using the single letter amino acid code. The dotted line indicates the cutoff of 1.6 based on 120 healthy controls. pp, propeptide; VWD, von Willebrand disease; VWF, von Willebrand factor.

dL) and VWF:Ag (47 IU/dL). Because of the relatively high VWF:Ag levels, the VWFpp ratio did not increase, perhaps explaining the lower VWFpp ratio compared with what was expected. The normal value of VWFpp suggests a normal synthesis of VWF in classical 2B, as already established [18]. However, it is still unknown why the VWFpp ratio is not remarkably high in type 2B VWD.

Most variants with VWFpp ratios above our normal range were found in the D3 and A1 domains and, to a lesser extent, in the A2-A3 domains (Figure 3). The conformational structure of VWF (folded/unfolded) seems to be another player in VWF clearance through macrophages. In the presence of shear stress or ristocetin, macrophages and VWF interact more effectively [39,40], and thus, some genetic variants may cause VWF to be unfolded and lead to its early removal. Of note, the D3 domain appears to play an inhibitory role in VWF removal by LRP-1 [41,42]. Modifying the D3 domain by a variant may elucidate why several variants with a high VWFpp ratio are located in this region. In addition, studies demonstrated that LRP-1 requires shear stress to bind and clear VWF. Wohner et al. [36] showed that in type 2B variants (p.Arg1306Gln and p.Val1316Met), LRP-1 binds to VWF even in the absence of shear stress. A similar mechanism may exist for other types 2B variants with enhanced clearance. The cellular mechanisms of enhanced clearance for most VWF variants are unclear. The archetypal VWF variant associated with enhanced clearance (p.Arg1205His) was found to be mainly macrophage-mediated [42]. In fact, the macrophage scavenger receptor class A member I was recently reported to contribute to the accelerated removal of mutated VWF for p.Arg1205His and p.Ser2179Phe [37]. We had one case with p.Arg1205Cys that clearly showed a less severe reduction in both VWF:Ag (31 IU/dL) and activity (20 IU/dL) plasma levels compared with p.Arg1205His cases. More importantly, the VWFpp ratio was also lower in the case carrying p.Arg1205Cys (ratio, 3.4 vs 8.1). Therefore, it is evident that the type of amino acid at position 1205 plays a crucial role in the VWF half-life.

Variants that change cysteine are reported to cause enhanced VWF clearance [43]. Interestingly, in our study among variants associated with a high VWFpp ratio, approximately 30% of them were related to the disappearance of a cysteine residue. Most of these variants (such as p.Tyr1107Cys, p.Cys1126Tyr, p.Cys1130Phe, p.Cys1130Tyr, and p.Tyr1146Cys) were located in the D3 domain and associated with type 2A(IIE). Given the critical role of cysteine in the structural integrity of VWF [44], the mutated VWF may be better recognized by the clearance receptor and thus cleared from the blood flow.

A limitation of this study is that the 2 populations of patients evaluated (type 1 and type 2) are not equally represented. It has been widely reported that type 1 accounts for 75% of all VWD cases, but we have only a relatively small number of patients with type 1 diagnosis and VWF levels of <30 IU/dL at our center. This is probably because of the reclassification of previously diagnosed patients with type 1 as low VWF [33]. Another limitation is the lack of data regarding patients post-DDAVP response in comparison with the VWFpp ratio.

In conclusion, this study demonstrates that an accelerated VWF clearance is found in the vast majority of type 2A, 2B, and 2M but in a lower proportion of type 1 and 2N. Sixty-seven different genetic variants were associated with an increased VWFpp ratio, either alone or in combination with other variants. Variants with the highest VWFpp ratio were mostly located in D3 and A1 domains.

ACKNOWLEDGMENTS

This work was partially supported by the Italian Ministry of Health-Bando Ricerca Corrente. The authors thank Pier Mannuccio Mannucci for his critical advice and Luigi Ghilardini for the illustration work.

AUTHOR CONTRIBUTIONS

O.S., L.B., and F.P. conceptualized the study. O.S. collected and analyzed data and wrote the manuscript. M.T.P. and A.C. performed the genetic tests. P.C. and G.C. performed the phenotypic tests. S.M.S. and E.B. were involved in patient evaluation. L.B. and F.P. critically revised the manuscript. All authors read and approved the final paper.

DECLARATION OF COMPETING INTERESTS

F.P. has participated in educational meetings and the advisory board of Sanofi, Sobi, Takeda, Roche, and Biomarin. O.S. received congress support from Kedrion. All other authors report no competing interests to disclose.

REFERENCES

- [1] Wagner DD. Cell biology of von Willebrand factor. *Annu Rev Cell Biol.* 1990;6:217–46.
- [2] Luo GP, Ni B, Yang X, Wu YZ. Von Willebrand factor: more than a regulator of hemostasis and thrombosis. *Acta Haematol.* 2012;128:158–69.
- [3] Rauch A, Wohner N, Christophe OD, Denis CV, Susen S, Lenting PJ. On the versatility of von Willebrand factor. *Mediterr J Hematol Infect Dis.* 2013;5:e2013046.
- [4] Peyvandi F, Garagiola I, Baronciani L. Role of von Willebrand factor in the haemostasis. *Blood Transfus.* 2011;9:s3–8.
- [5] Springer TA. Von Willebrand factor, Jedi knight of the bloodstream. *Blood.* 2014;124:1412–25.
- [6] Lenting PJ, Christophe OD, Denis CV. Von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends. *Blood.* 2015;125:2019–28.
- [7] Habichter SL. Von Willebrand factor propeptide: biology and clinical utility. *Blood.* 2015;126:1753–61.
- [8] Rawley O, Lillicrap D. Functional roles of the von Willebrand factor propeptide. *Haemostaseologie.* 2021;41:63–8.
- [9] O'Sullivan JM, Ward S, Lavin M, O'Donnell JS. Von Willebrand factor clearance - biological mechanisms and clinical significance. *Br J Haematol.* 2018;183:185–95.
- [10] O'Donnell J, Laffan MA. The relationship between ABO histo-blood group, factor VIII and von Willebrand factor. *Transfus Med.* 2001;11:343–51.
- [11] Gallinaro L, Cattini MG, Sztukowska M, Padrini R, Sartorello F, Pontara E, Bertomoro A, Daidone V, Pagnan A, Casonato A. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood.* 2008;111:3540–5.

- [12] Sadler JE, Mannucci PM, Berntorp E, Bochkov N, Boulyjenkov V, Ginsburg D, Meyer D, Peake I, Rodeghiero F, Srivastava A. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost.* 2000;84:160–74.
- [13] Sadler JE, Budde U, Eikenboom JC, Favaloro EJ, Hill FG, Holmberg L, Ingerslev J, Lee CA, Lillicrap D, Mannucci PM, Mazurier C, Meyer D, Nichols WL, Nishino M, Peake IR, Rodeghiero F, Schneppenheim R, Ruggeri ZM, Srivastava A, Montgomery RR, 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;4:2103–14.
- [14] Casari C, Lenting PJ, Wohner N, Christophe OD, Denis CV. Clearance of von Willebrand factor. *J Thromb Haemost.* 2013;11:202–11.
- [15] Haberichter SL, Balistreri M, Christopherson P, Morateck P, Gavazova S, Bellissimo DB, Manco-Johnson MJ, Gill JC, Montgomery RR. Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood.* 2006;108:3344–51.
- [16] Haberichter SL, Castaman G, Budde U, Peake I, Goodeve A, Rodeghiero F, Federici AB, Batlle J, Meyer D, Mazurier C, Goudemand J, Eikenboom J, Schneppenheim R, Ingerslev J, Vorlova Z, Habart D, Holmberg L, Lethagen S, Pasi J, Hill FGH, et al. Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMDM-1VWD). *Blood.* 2008;111:4979–85.
- [17] Eikenboom J, Federici AB, Dirven RJ, Castaman G, Rodeghiero F, Budde U, MCMDM-1VWD Study Group. VWF propeptide and ratios between VWF, VWF propeptide, and FVIII in the characterization of type 1 von Willebrand disease. *Blood.* 2013;121:2336–9.
- [18] Sanders YV, Groeneveld D, Meijer K, Fijnvandraat K, Nossen MH, van der Bom JG, Coppens M, de Meris J, Laros-van Gorkom BA, Mauser-Bunschoten EP, Leebeek FW, Eikenboom J, WiN study group. Von Willebrand factor propeptide and the phenotypic classification of von Willebrand disease. *Blood.* 2015;125:3006–13.
- [19] Pipe SW, Montgomery RR, Pratt KP, Lenting PJ, Lillicrap D. Life in the shadow of a dominant partner: the FVIII-VWF association and its clinical implications for hemophilia A. *Blood.* 2016;128:2007–16.
- [20] Seidizadeh O, Baronciani L, Pagliari MT, Cozzi G, Colpani P, Cairo A, Siboni SM, Biguzzi E, Peyvandi F. Phenotypic and genetic characterizations of the Milan cohort of von Willebrand disease type 2. *Blood Adv.* 2022;6:4031–40.
- [21] Stufano F, Boscarino M, Bucciarelli P, Baronciani L, Maino A, Cozzi G, Peyvandi F. Evaluation of the utility of von Willebrand factor propeptide in the differential diagnosis of von Willebrand disease and acquired von Willebrand syndrome. *Semin Thromb Hemost.* 2019;45:36–42.
- [22] Biguzzi E, Siboni SM, le Cessie S, Baronciani L, Rosendaal FR, van Hylckama Vlieg A, Peyvandi F. Increasing levels of von Willebrand factor and factor VIII with age in patients affected by von Willebrand disease. *J Thromb Haemost.* 2021;19:96–106.
- [23] Baronciani L, Cozzi G, Canciani MT, Peyvandi F, Srivastava A, Federici AB, Mannucci PM. Molecular characterization of a multi-ethnic group of 21 patients with type 3 von Willebrand disease. *Thromb Haemost.* 2000;84:536–40.
- [24] Baronciani L, Cozzi G, Canciani MT, Peyvandi F, Srivastava A, Federici AB, Mannucci PM. Molecular defects in type 3 von Willebrand disease: updated results from 40 multiethnic patients. *Blood Cells Mol Dis.* 2003;30:264–70.
- [25] Baronciani L, Peake I, Schneppenheim R, Goodeve A, Ahmadinejad M, Badiee Z, Baghaipour MR, Benitez O, Bodó I, Budde U, Cairo A, Castaman G, Eshghi P, Goudemand J, Hassenpflug W, Hoorfar H, Karimi M, Keikhaei B, Lassila R, Leebeek FWG, et al. Genotypes of European and Iranian patients with type 3 von Willebrand disease enrolled in 3WINTERS-IPS. *Blood Adv.* 2021;5:2987–3001.
- [26] Goodeve A, Eikenboom J, Castaman G, Rodeghiero F, Federici AB, Batlle J, Meyer D, Mazurier C, Goudemand J, Schneppenheim R, Budde U, Ingerslev J, Habart D, Vorlova Z, Holmberg L, Lethagen S, Pasi J, Hill F, Hashemi Soteh M, Baronciani L, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood.* 2007;109:112–21.
- [27] Millar CM, Riddell AF, Brown SA, Starke R, Mackie I, Bowen DJ, Jenkins PV, van Mourik JA. Survival of von Willebrand factor released following DDAVP in a type 1 von Willebrand disease cohort: influence of glycosylation, proteolysis and gene mutations. *Thromb Haemost.* 2008;99:916–24.
- [28] Brown SA, Eldridge A, Collins PW, Bowen DJ. Increased clearance of von Willebrand factor antigen post-DDAVP in type 1 von Willebrand disease: is it a potential pathogenic process? *J Thromb Haemost.* 2003;1:1714–7.
- [29] Casonato A, Pontara E, Sartorello F, Cattini MG, Sartori MT, Padriani R, Girolami A. Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. *Blood.* 2002;99:180–4.
- [30] Flood VH, Gill JC, Friedman KD, Bellissimo DB, Haberichter SL, Montgomery RR. Von Willebrand disease in the United States: a perspective from Wisconsin. *Semin Thromb Hemost.* 2011;37:528–34.
- [31] Christopherson PA, Haberichter SL, Flood VH, Perry CL, Sadler BE, Bellissimo DB, Di Paola J, Montgomery RR, Zimmerman Program Investigators. Molecular pathogenesis and heterogeneity in type 3 VWD families in U.S. Zimmerman program. *J Thromb Haemost.* 2022;20:1576–88.
- [32] Aguila S, Lavin M, Dalton N, Patmore S, Chion A, Trahan GD, Jones KL, Keenan C, Brophy TM, O'Connell NM, Ryan K, Byrne M, Nolan M, Patel A, Preston RJS, James P, Di Paola J, O'Sullivan JM, O'Donnell JS. Increased galactose expression and enhanced clearance in patients with low von Willebrand factor. *Blood.* 2019;133:1585–96.
- [33] James PD, Connell NT, Ameer B, Di Paola J, Eikenboom J, Giraud N, Haberichter S, Jacobs-Pratt V, Konkle B, McLintock C, McRae S, Montgomery R, O'Donnell JS, Scappe N, Sidonio R, Flood VH, Husainat N, Kalot MA, Mustafa RA. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. *Blood Adv.* 2021;5:280–300.
- [34] Castaman G, Tosetto A, Rodeghiero F. Reduced von Willebrand factor survival in von Willebrand disease: pathophysiologic and clinical relevance. *J Thromb Haemost.* 2009;7:71–4.
- [35] Badirou I, Kurdi M, Rayes J, Legendre P, Christophe OD, Lenting PJ, Denis CV. Von Willebrand factor clearance does not involve proteolysis by ADAMTS-13. *J Thromb Haemost.* 2010;8:2338–40.
- [36] Wohner N, Legendre P, Casari C, Christophe OD, Lenting PJ, Denis CV. Shear stress-independent binding of von Willebrand factor-type 2B mutants p.R1306Q & p.V1316M to LRP1 explains their increased clearance. *J Thromb Haemost.* 2015;13:815–20.
- [37] Wohner N, Muczynski V, Mohamadi A, Legendre P, Proulle V, Aymé G, Christophe OD, Lenting PJ, Denis CV, Casari C. Macrophage scavenger receptor SR-AI contributes to the clearance of von Willebrand factor. *Haematologica.* 2018;103:728–37.
- [38] Casonato A, Gallinaro L, Cattini MG, Pontara E, Padriani R, Bertomoro A, Daidone V, Pagnan A. Reduced survival of type 2B von Willebrand factor, irrespective of large multimer representation or thrombocytopenia. *Haematologica.* 2010;95:1366–72.
- [39] Castro-Núñez L, Dienava-Verdoold I, Herczenik E, Mertens K, Meijer AB. Shear stress is required for the endocytic uptake of the factor VIII-von Willebrand factor complex by macrophages. *J Thromb Haemost.* 2012;10:1929–37.

- [40] van Schooten CJ, Shahbazi S, Groot E, Oortwijn BD, van den Berg HM, Denis CV, Lenting PJ. Macrophages contribute to the cellular uptake of von Willebrand factor and factor VIII in vivo. *Blood*. 2008;112:1704–12.
- [41] Lenting PJ, Westein E, Terraube V, Ribba AS, Huizinga EG, Meyer D, de Groot PG, Denis CV. An experimental model to study the in vivo survival of von Willebrand factor. Basic aspects and application to the R1205H mutation. *J Biol Chem*. 2004;279:12102–9.
- [42] Rawley O, O'Sullivan JM, Chion A, Keyes S, Lavin M, van Rooijen N, Brophy TM, Fallon P, Preston RJ, O'Donnell JS. Von Willebrand factor arginine 1205 substitution results in accelerated macrophage-dependent clearance in vivo. *J Thromb Haemost*. 2015;13:821–6.
- [43] Schooten CJ, Tjernberg P, Westein E, Terraube V, Castaman G, Mourik JA, Hollestelle MJ, Vos HL, Bertina RM, Berg HM, Eikenboom JC, Lenting PJ, Denis CV. Cysteine-mutations in von Willebrand factor associated with increased clearance. *J Thromb Haemost*. 2005;3:2228–37.
- [44] Purvis AR, Gross J, Dang LT, Huang RH, Kapadia M, Townsend RR, Sadler JE. Two Cys residues essential for von Willebrand factor multimer assembly in the Golgi. *Proc Natl Acad Sci U S A*. 2007;104:15647–52.