Phenotypic Parameters in Genotypically Selected Type 2B von Willebrand Disease Patients: A Large, Single-Center Experience Including a New Novel Mutation

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Abstract

von Willebrand disease type 2B (VWD2B) expresses gain-of-function mutations that enhance binding of an individual's von Willebrand factor (VWF) to its platelet ligand, glycoprotein Ib (GPIb), and which are usually identified by increased ristocetin-induced platelet aggregation (RIPA). We describe here the phenotypic profile of 38 genotypically selected VWD2B-affected family members (AFMs) belonging to 19 unrelated families. Major bleeding was observed in 68.4% of AFMs (previous to their diagnosis and registered by lifetime interviews), with a total of 46 episodes (1.21/patient), and was found to be highly related to the individual bleeding score and presence of thrombocytopenia, but otherwise unrelated to other laboratory parameters. Excessive mucocutaneous bleeding symptoms were often reported, the most frequent of which comprised menorrhagia, epistaxis, easy bruising, and bleeding after teeth extraction/in oral cavity. Eight unaffected family members were also studied. The prevalence of VWD2B within families was 0.826, and the penetrance of mutations was complete, making it mandatory to study entire family sets to complete diagnostic profiles. Seven heterozygous missense mutations were found, the most common being p.V1316M. In the p.R1308C group, 75% of the AFMs showed absence of RIPA at 0.5 mg/mL, 66.6% of whom had VWF:RCo $<$ 10 IU/dL, and 50% of whom had VWF:CB $<$ 10 IU/dL. In the p.S1310F group, none of the AFMs had VWF:RCo/VWF:Ag $<$ 0.6 (RCo/Ag), but 100% had VWF:CB/VWF:Ag $<$ 0.6/(CB/Ag). Patients with p.P1266L and p.R1304V were characterized as atypical VWD2B. Two de novo mutations were found in four AFMs belonging to two families. We also describe a novel mutation: p.Y1258C. Of our patients, 70.5% had O blood group. In conclusion, a normal RCo/Ag and a negative RIPA at 0.5 mg/mL do not necessarily rule out a diagnosis of VWD2B.

Keywords
- bleeding
- mutation
- RIPA
- von Willebrand disease type 2B
- von Willebrand factor

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von Willebrand disease type 2B (VWD2B) is inherited in an autosomal dominant manner. The patient's von Willebrand factor (VWF) has increased affinity for platelet glycoprotein Ibα (GPIbα), due to the presence of mutations clustering in the VWF A1 domain.[1] These mutations appear to inactivate a regulatory function of this domain, and allow binding to platelets in the absence of activation or vascular injury. The bleeding phenotype is highly variable and multifactorial, including being potentially due to spontaneous platelet agglutination, the clearance of VWF-platelet complexes, increased ADAMTS13-mediated VWF proteolysis,[2] the loss of high-molecular-weight multimers (HMWM), and thrombocytopenia. VWF:RCo/VWF:Ag ratio (RCo/Ag) ≥0.6 and giant platelets[3] are frequent findings.[4] Impaired megakaryocytopoiesis resulting from an abnormal interaction between platelet GPIbα and VWF/p.R1308P mutation has also been described.[5,6]

There are other VWD2B variants, including (1) "New York" and "Malmo," characterized by heightened ristocetin-induced platelet aggregation (RIPA) but normal multimers[7,8] and related to p.P1266L[9]; (2) the Montreal platelet syndrome, associated with p.V1316M[10,11]; and (3) type 2B Hiroshima, who express a chronic thrombocytopenia with normal multimers in plasma.[12] An atypical form of VWD2B was also described,[13] characterized by normal platelet count (PC), normal multimers, and normal RCo/Ag, but expressing heightened RIPA at low concentrations.

The aim of our study was to show the phenotypic behavior of a large cohort ($n = 38$) of VWD2B patients who were genotypically diagnosed. A novel mutation is also described.

Materials and Methods

Subjects

A total of 38 affected family members (AFMs) belonging to 19 unrelated families were enrolled according to the presence of a responsible mutation with a supportive VWF laboratory and clinical profile. Furthermore, 8 unaffected members of families were also studied, to calculate the penetrance of the causative mutations, and the prevalence of the disease in families, thus making the overall study population comprised 46 individuals. The AFMs represent 82.6% of all family members. Eight families were single generation with 10 members (9 AFMs), 9 families were two generation with 28 members (23 AFMs), and 2 families were three generation with 8 members (6 AFMs). No consanguinity was found, and all subjects were of Caucasian origin. Blood group was also analyzed.

One hundred random healthy controls were screened to estimate the potential findings of new variants. The participants were evaluated in accordance with the Helsinki declaration and signed a written informed consent at admission. The collected information remained confidential and anonymous.

Clinical Profile

Bleeding symptoms were obtained from clinical records. The ISTH/SSC bleeding assessment tool (ISTH/SSC BAT) is considered useful for the identification of a significant bleeding history in adults (≥5 in females; ≥3 in males).[14] The pediatric bleeding questionnaire (PBQ) was used in children younger than 16 years (≥2).[15] Menorrhagia was calculated in females older than 12 years, according to the Pictorial Bleeding Assessment Chart[16] (PBAC) score. Major bleeding (MB) was defined as per Schulman et al.[17]

Biologic Response to Desmopressin

Desmopressin (DDAVP) response was evaluated in 10 patients before the diagnosis of VWD2B was made. DDAVP was infused intravenously over a period of 20 minutes at a dose of 0.3 µg/kg body weight, in saline solution. Blood samples were obtained before as well as after 1 and 2 hours of infusion. The time courses of FVIII:C, VWF:Ag, VWF:RCo, and PC were analyzed.

Laboratory Methods

The following tests were performed: PC; bleeding time (BT) using Ivy's method[18]; FVIII:C as measured by one-stage method[19] (normal range: 50–150 IU/dL); and VWF:Ag[20] (normal range: 50–150 IU/dL). VWF propeptide (VWFpp)[21] and VWF:CB (Technozym # cat 5450311, Technoclone GmbH, Vienna Austria) were assayed by ELISA (normal range: 60–130 IU/dL). VWF:RCo was assayed by aggregometry, using fixed-washed platelets[22] (normal range: 50–150 IU/dL). The presence of anti-VWF-binding antibodies was performed as previously described.[23,24] RCo/Ag (normal values >0.6), VWF:CB/VWF:Ag (CB/Ag; normal values >0.6), and VWFpp/VWF:Ag (VWFpp ratio; normal range: 0.92–2.14) were calculated for each patient. VWF multimeric analysis was performed by SDS–1% and 1.7% agarose gel electrophoresis, and immunoenzymatic stain visualization of multimers.[25] Calibration curves for FVIII:C, VWF:Ag, VWF:RCo, VWF:CB, and VWFpp were made from a local plasma-pool obtained from 20 healthy donors used as a secondary standard calibrated against standard 07/316 of the National Institute of Biological Standards and Control (NIBSC). RIPA was performed using different ristocetin concentrations, observing the minimal aggregating concentration (MACR) which promoted at least 30% aggregation.

Genotypic Analysis

Genomic DNA was extracted from peripheral blood. The complete exon 28 of the *VWF* gene was amplified by polymerase chain reaction (PCR) and sequenced. We designed the primers for PCR as previously described.[26] Amplification was performed in a Perkin-Elmer Thermocycler 2400. Both the forward and reverse strands were directly sequenced by automated sequencing technology in an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

In Silico Bioinformatics Analysis and Sequence Alignment

The in silico analysis of the predicted missense changes was performed with the bioinformatics applications: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.bii.a-star.edu.sg/). The sequence alignment of the protein was performed using UniProt KB (www.uniprot.org).

Statistical Analysis

The statistical analysis was performed using the mean (x) and standard deviation (SD) for continuous variables. Comparisons among different data were performed using Student

t-test. Median was used to evaluate data with distribution bias. The comparative analysis was performed using chi-square test with Yates correction. *P*-value <0.05 was considered to be significant. Relative risk (RR) with 95% confidence interval (95% CI) and positive and negative likelihood ratios (LR+ and LR −) were used to measure effects within different phenotypic parameters. In families with ≥ 2 generations studied, the penetrance of the responsible mutation and the prevalence of the disease were calculated. Prevalence of patients with VWD2B within families was calculated. The 95% CI of the prevalence was calculated using the Agresti–Coull method.[27]

Results

Clinical Profile

All the AFMs reported excessive muco-cutaneous bleeding, the most frequently reported being menorrhagia (89.5% of >12-year-old females [PBAC: 288 ± 161]), epistaxis (74.3%), easy bruising (71.4%), and bleeding after teeth extraction/in oral cavity (66.6%) (►**Table 1**). The eight unaffected family members displayed both normal clinical and laboratory phenotype, and did not carry any causative mutations. In families with ≥ 2 studied members, the prevalence of VWD2B was calculated to be 0.826 (95% CI, 0.69–0.912). The penetrance of the mutations was complete, given that the mutations were present in all the generations. Overall, 70.5% of our VWD2B patients had O blood group (24/32 evaluated).

Abnormal bleeding score (BS) was observed in 78.3% of VWD2B patients, but was not associated with PC (RR: 1.23; 95% CI: 0.78–1.93), BT (RR: 0.65; 95% CI: 0.48–0.88), VWF:RCo (RR: 1.08; 95% CI: 0.36–3.24), VWF:CB (RR: 0.42; 95% CI: 0.08–2.11), or high VWFpp ratio (RR: 1.78; 95% CI: 0.90–3.51), or with any particular causative mutation. MB occurrence was observed in 26/38 (68.4%) of AFMs earlier to their diagnosis and registered by lifetime interviews with a total of 46 episodes (1.21/patient). The highest frequency of MB episodes was related to cesarean section (100%), delivery (50%), and surgery (44.4%). MB was highly related to BS (RR: 3.25; 95% CI: 1.44–3.45 and LR + : 3.25; 95% CI: 1.44–7.35 and LR − : 0.00; $p = 0.000$), and to low PC (RR: 3.10; 95% CI: 1.11–8.66 and LR + : 2.48; 95% CI: 1.06–5.79; $p = 0.017$ and LR − : 0.28; 95% CI: 0.09–0.84). No significant relationship was observed between the MB and the BT (RR: 1.03; 95% CI: 0.8–1.33), or between the MB and VWF:RCo $<$ 10 IU/dL (RR: 0.57; 95% CI: 0.25–1.28), VWF:CB $<$10 IU/dL (RR: 0.36; 95% CI: 0.06–2.05), high VWFpp ratio (RR: 1.78; 95% CI: 0.88–3.58), multimeric pattern (RR: 0.97; 95% CI: 0.35–2.74), and the responsible mutation.

Biological Response to Stress Conditions (DDAVP and Pregnancy)

The response to DDAVP in the 10 AFMs studied showed that FVIII:C, VWF:Ag, and VWF:RCo rose to 2.5 to 4 times that of the baseline value, with a drop of 40.8% in PC (►**Fig. 1**). Of the six patients with normal PC at baseline (patients CEJ, BP, BJI, CES, NM, CG), three patients developed thrombocytopenia after infusion, whereas the PC remained within normal values in the other three. The remaining four patients had thrombocytopenia before DDAVP infusion. Patients with p.M1304V and p.R1306W showed the smallest fall in PC, whereas those with p.R1308C and p.V1316M showed the most pronounced fall, with significant difference ($p = 0.032$).

Four women with VWD2B were managed during their pregnancy (►**Table 2**). Blood samples were taken at 26, 31, 37, or 38 weeks. All patients developed thrombocytopenia. Again, as in the DDAVP response test, the patient with p.V1316M (AMS) showed the most pronounced fall of PC at the delivery time.

In total, including both DDAVP and pregnancy, 6/14 (42.8%) of our AFMs had thrombocytopenia at baseline and 11/14 (78.6%) after these "stress" conditions.

Laboratory Parameters

In this study, 38 individuals with VWD2B were extensively characterized (►**Table 3**). A direct correlation was found between thrombocytopenia and absence of HMWM (RR, 1.40; 95% CI: 1.01–1.95; $p = 0.024$). A slightly high VWFpp ratio was found in 67.6% of our patients.

Comparison between RCo/Ag and CB/Ag

In a group of AFMs who had both VWF:RCo and VWF:CB performed, comparisons were made between VWF:RCo and VWF:CB, and between RCo/Ag and CB/Ag (►**Table 4**). In those with normal multimeric pattern ("atypical" VWD2B), no differences were observed between VWF:RCo and VWF:CB ($p = 0.83$), or between RCo/Ag and CB/Ag ($p = 0.974$). In those patients with absent HMWM, VWF:CB was lower than VWF:RCo, but this finding was not significantly different ($p = 0.086$). However, a significant difference was found when comparing RCo/Ag versus CB/Ag ($p = 0.006$) in this group. When RCo/Ag and CB/Ag were compared between atypical and low HMWM patients, significant statistical differences were also observed in RCo/Ag ($p = 0.013$) and, even more pronounced, in CB/Ag ($p = 0.000$).

The potential impact on VWF:RCo levels of two polymorphisms (SNP) (p.A1381T and p.D1472H) was calculated in the AFMs. Seventy-five percent of patients were homozygous for p.A1381 and p.D1472; 17.8% were heterozygous for p.A1381T; 7.2% were homozygous for p.1381T; and 25% were heterozygous for p.D1472H. The corresponding laboratory results were as follows: in homozygous p.A1381–p.D1472: VWF:RCo = 28.0 ± 16.1 IU/dL; in p.A1381–p.D1472H: VWF:RCo = 36.8 ± 22.9 IU/dL; in p.A1381T–p.D1472: VWF:RCo = 31.2 ± 44.3 IU/dL, with no statistical differences between groups.

Genotypic Analysis

Six different missense mutations in the heterozygous state and one novel mutation were found. The novel mutation was the result of the substitution A > G located in the position c.3773, causing the change of a tyrosine (Y) into a cysteine (C) at residue 1258 (p.Y1258C) (►**Fig. 2**). This change was found in a 33-year-old boy with a moderate to severe bleeding history (BS = 5), including epistaxis since childhood and an episode of severe hematoma requiring drainage. The patient

Table 1 Clinical profile in AFMs

Family	AFM code	Blood group	Sex	Age	BS	Bleeding symptoms	MB	Relationship
I	MJ	–	M	33	5	a; f	Yes	IC
II	CFA	–	F	13	1	b	No	IC
III	CEJ	O	F	15	5	a; f; g	Yes	IC
	CEN	O	F	36	5	a; b; e	Yes	Mother
	CML	O	F	25	3	a; b; f	No	Aunt
IV	AAB	A	F	6	5	a; f; r; anemia	Yes	IC
	AJM	O	M	5	3	a; f	No	Brother
	AL	O	M	6	2	a; f	No	Brother
V	BP	O	M	33	9	a; c; f; h; i	Yes	IC
	BJI	–	M	5	9	a; c; d; g	Yes	Son
VI	MMA	A	F	33	7	a; b; d; f	Yes	IC
VII	MCE	A	M	40	3	d; f	Yes	IC
	MMa	–	F	4	6	d; s	Yes	Daughter
VIII	NM	O	F	18	6	a; b; f; g; i; anemia	No	IC
	CES	B	F	44	11	a; b; c; d, r	Yes	Mother
	NA	O	M	18	1	No data	No	Sister
IX	FME	O	F	16	4	a; b; c; f	No	IC
	FDO	O	F	3	2	No data	No	Daughter
X	DBH	O	F	28	6	a; b; c; f	No	IC
XI	CG	O	F	3	3	a	Yes	IC
	CMDR	O	F	30	8	a; b; e	Yes	Mother
	COM	O	F	36	4	a; f; g, r	Yes	Grand-mother
XII	AGA	A	F	2	5	c; f; anemia	Yes	IC
	GD	O	F	21	7	a; b; d; anemia	Yes	Sister
	GS	A	F	14	9	a; b; d; f; anemia	Yes	Sister
XIII	LM	A	F	28	14	a; b; c; f; k; r	Yes	IC
XIV	AF	O	M	16	7	c; g; p at birth	Yes	IC
XV	AMS	O	F	37	9	a; f; g; n; r anemia	Yes	IC
	AA	A	M	62	1	No data	No	Father
	AEF	O	M	1	3	f	Yes	Son
XVI	ET	O	F	3	4	a; f	Yes	IC
	EMU	A	M	8	7	c; f; q	Yes	Brother
XVII	GJC	O	M	62	6	c; f; p	Yes	IC
XVIII	MEP	O	F	33	5	b; f; g; anemia	Yes	IC
	MG	O	M	41	6	a; c; f	Yes	Brother
	MM	O	F	14	4	a; b; f; g	No	MG's daughter
	MN	O	F	12	3	a; c	No	MG's daughter
XIX	MJD	A	F	14	9	a; b; d; f; g	Yes	IC

Abbreviations: a, epistaxis; AFMs, affected members; b, meno-metrorrhagia; BS, bleeding score; c, teeth extraction or oral cavity; d, surgery; e, delivery; f, easy bruising; g, gum; h, gastrointestinal; I, minor cuts; IC, index case; k, hemoperitoneum; MB, major bleeding; n, abdominal wall hematoma; p, pneumothorax; q, wound repair; r, cesarean section; s, cephalohematoma.

showed hyper-responsiveness to ristocetin (0.3 mg/mL); normal VWF:RCo, RCo/Ag, CB/Ag, and VWFpp ratio, but low VWF:CB, borderline PC; and a slight loss of HMWM (►**Table 3**). No members of his family were available for the study. This substitution was absent in 100 healthy individuals (200 alleles). Therefore, it was not considered to be a SNP. The in silico analysis showed that the novel missense changes are probably damaging. PolyPhen-2 predicted the p.Y1258C

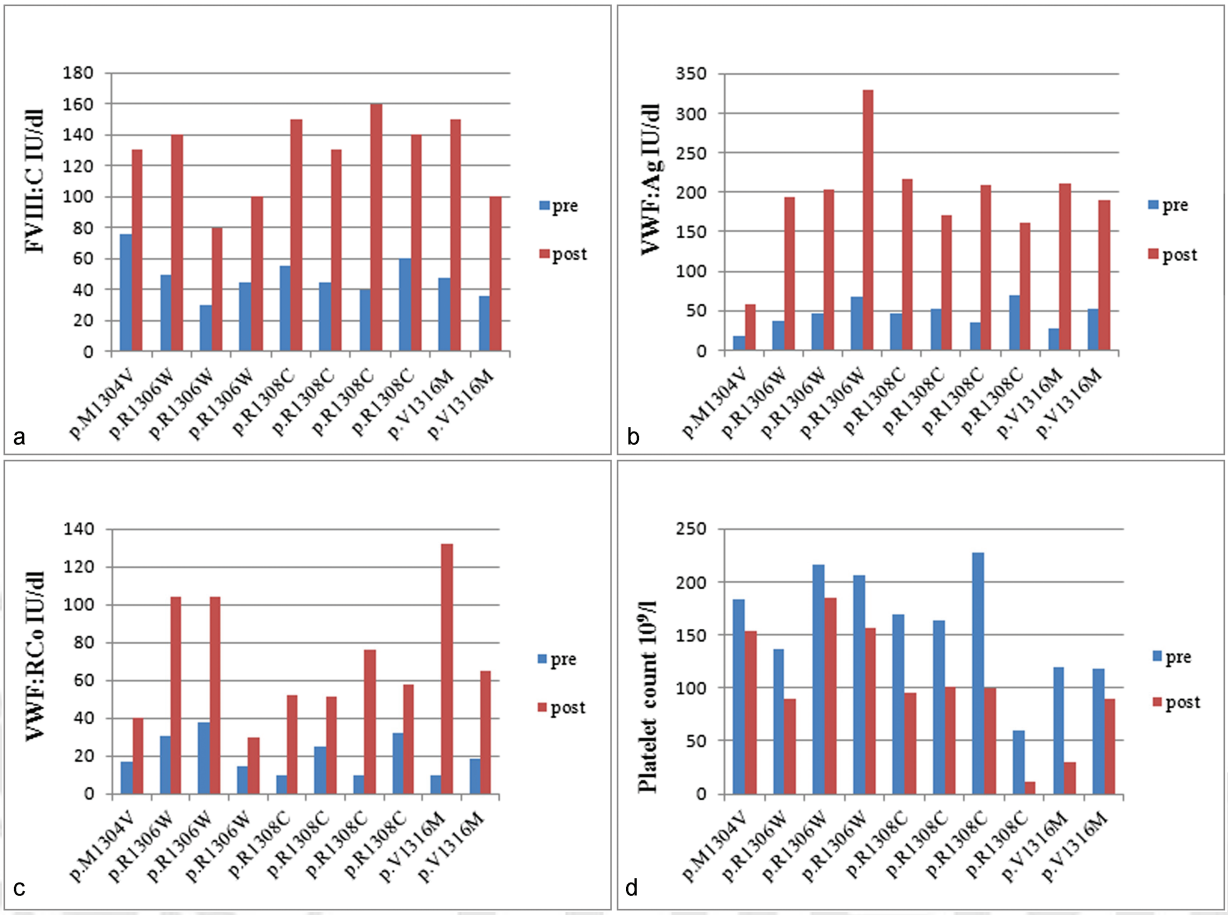

Fig. 1 Response to desmopressin (DDAVP) in affected members (AFMs). (A) FVIII:C, factor VIII coagulant activity; (B) VWF:Ag, von Willebrand antigen; (C) VWF:RCo, ristocetin cofactor activity; (D) platelet count.

Table 2 Laboratory profile before and after pregnancy in four AFMs

AFM code	Pregnancy period	FVIII:C IU/dL	VWF:Ag IU/dL	VWF:RCo IU/dL	PC 10^9/L	Mutation	Comments
AAB	Before 31 wk	35 44	59 93	54 78	153 35	p.R1306W	Cesarean section; with MB
FME	before 10 wk 26 wk	34 56 90	< 10 47 64	10 < 10 23	186 150 104	p.R1308C	Cesarean section with PDC; no bleeding
DBH	before 37 wk	44 45	33 87	< 10 15	92 75	p.R1308C	Delivery with PDC; no bleeding
AMS	before 38 wk	36 90	25 160	12 51	118 51	p.V1316M	Cesarean section with PDC and platelet transfusions; no bleeding

Abbreviations: AFMs, affected members; FVIII:C, factor VIII coagulant activity; MB, major bleeding; PC, platelet count; PDC, plasma-derived VWF concentrates; VWF:Ag, von Willebrand antigen; VWF:RCo, ristocetin cofactor activity.

mutation to be probably damaging with a score of 0.999 (sensitivity: 0.14; specificity: 0.99). According to SIFT, it was predicted to affect protein function with a score of 0.02. The sequence alignment showed that the residue p.Y1258 is located in a highly conserved *VWF* gene zone.

To compare the phenotypic profile of the AFMs, they were grouped according to the responsible mutation (►**Table 3**). Four AFMs (p.P1266L and p.M1304V) were associated with atypical VWD2B (frequency = 10.5%), given the presence of both normal PC and multimeric pattern. The p.V1316M was the most common mutation (31.6%), which similarly affects more families (31.6%). The p.R1308C group showed the highest percentage of AFMs with absent 0.5 mg/mL RIPA (75%), VWF:RCo < 10 IU/dL (66.6%), and VWF:CB < 10 IU/dL (50%). Patients with p.S1310F had the highest BS and the lowest PC; 0% of AFMs had RCo/Ag < 0.6; and 100% had CB/Ag < 0.6.

Table 3 Clinical and laboratory data of patients according to the responsible mutation

		p.Y1258C	p.P1266L[a]	p.M1304V[a]	p.R1306W	p.R1308C	p.S1310F	p.V1316M	Total
n families		1	1	1	4	4	2	6	19
n members		1	1	3	9	10	6	16	46
n AFMs		1	1	3	8	9	4	12	38
AFMs with MB		100%	0%	66.6%	75%	44.4%	100%	75%	68.4%
Age (md)		33	13	25	6	19.5	17.5	16	18
AFMs with O blood group		–	–	100%	50%	88.9%	33.3%	83.3%	70.5%
BS AFMs with abnormal value		5 100%	1 0%	4.3 ± 1.2 66.6%	5.5 ± 27 87.5%	4.7 ± 2.9 55.5%	8.7 ± 3.8 100%	5.7 ± 2.1 90.9%	5.4 ± 2.9 78.3%
BT		4	–	7.7 ± 2.3	9.2 ± 2.1	8.8 ± 1.8	>9	11.4 ± 1.5	9.4 ± 1.9
PC (/10^9/L) AFMs with low PC		149 100%	270 0%	191.7 ± 25.6 0%	153.3 ± 82.6 50%	169 ± 68.1 22.2%	54.8 ± 56.3 100%	110 ± 98.8 83.3%	130 ± 84.5 56.7%
FVIII:C (IU/dL)		50	46	53.3 ± 15.3	51.6 ± 7.8	40.6 ± 5.4	55.5 ± 3.3	57.5 ± 39	56.8 ± 27.9
VWF:Ag (IU/dL)		64	46	24.3 ± 6.7	48.6 ± 15.2	39.7 ± 18.4	64.8 ± 15.9	62.7 ± 48.3	50.8 ± 32.2
VWF:RCo (IU/dL) AFMs with <10 IU/dL with >50 IU/dL		56 0% 100%	56 0% 100%	26.7 ± 12.0 0% 0%	33.6 ± 16.5 12.5% 25%	13.5 ± 7.8 66.6% 0%	49.3 ± 14.9 0% 50%	31.8 ± 29.1 25% 16.6%	30.7 ± 22.7 24.3% 21.9%
RCo/Ag AFMs with ≤0.6		0.87 0%	0,57 0%	1.1 ± 0.2 0%	0.68 ± 0.2 50%	0.42 ± 0.31 77.7%	0.8 ± 0.1 0%	0.5 ± 0.2 83.3%	0.62 ± 0.3 56.7%
VWF:CB (IU/dL) AFMs with <10 IU/dL with >60 IU/dL		44 0% 0%	Nt	27.7 ± 17 0% 0%	22 ± 10.8 0% 0%	9.1 ± 5.8 50% 0%	19.2 ± 8.6 0% 0%	17.8 ± 6.1 0% 16.6%	23.1 ± 18.3 13% 4.3%
CB/Ag AFMs with <0.6		0.69 0%	Nt –	1.03 ± 0.45 33.4%	0.47 ± 0.21 66.6%	0.32 ± 0.24 83.3%	0.37 ± 0.19 100%	0.4 ± 0.1 100%	0.49 ± 0.32 78.3%
VWFpp ratio AFMs with abnormal value		1.5 0%	1.96 0%	2.5 ± 0.3 100%	2.5 ± 0.2 100%	2.3 ± 0.7 55.5%	2.2 ± 0.2 75%	2.17 ± 0.79 58.3%	2.23 ± 0.6 67.6%
HMWM AFMs with presence absence		Slightly low 0%	100% 0%	100% 0%	0% 100%	0% 100%	0% 100%	0% 100%	10.8% 89.2%
RIPA (mg/mL) AFMs with MACR	0.7	–	–	–	–	25.0%	33.3%	10%	12.9%
	0.6	–	–	–	20%	50.0%	–	10%	19.4%
	0.5	–	–	66.6%	20%	–	–	50%	25.8%
	0.4	–	100%	33.4%	20%	12.5%	66.6%	–	19.4%
	0.3	100%	–	–	40%	–	–	10%	13%
	0.2	–	–	–	–	12.5%	–	20%	9.6%
Penetrance		?	?	Complete	Complete	Complete	Complete	Complete	
De novo occurrence		?	?	No	No	No	1 family	1 family	

Abbreviations: AFMs, affected members; BS, bleeding score; BT, bleeding time; FVIII:C, factor VIII coagulant activity; HMWM, high-molecular-weight multimers of VWF; MACR, minimal aggregating concentration of ristocetin; MB, major bleeding; Md, median; Nt, not tested; PC, platelet count; RIPA, ristocetin-induced platelet aggregation; VWF:Ag: von Willebrand antigen; VWF:CB: collagen-binding assay; VWF:RCo: ristocetin cofactor activity; VWFpp ratio, propeptide/VWF:Ag.
[a]Atypical VWD2B.

We found two apparent de novo mutations. The former, p.V1316M, was found in a 16-year-old boy with moderate to severe bleeding symptoms (BS = 7), MB episodes in teeth change and pneumothorax, with easy bruising and gum bleeding. Both parents were asymptomatic, and neither was identified to have the mutation. The paternity was confirmed by performing the haplotypes of the father and the boy.

The other apparent de novo mutation, p.S1310F, was found in three sisters with severe bleeding symptoms, with a common mother but three different fathers. The mutation was not found in their mother. Thus, our frequency of apparent de novo mutations related to VWD2B was of 10.5% (2 of 19 families).

Discussion

The BS was abnormal in 78.3% of our patients, which is consistent with a value of 89% previously reported.[28] MB was a frequent finding in our patients, and was found to be highly related to abnormal BS and thrombocytopenia, but unrelated to prolonged BT, VWF:RCo and VWF:CB levels, high VWFpp ratio, the loss of HMWM, and the responsible mutation. This is in accordance with previous studies reporting that the clinical presentation of VWD2B was not always related to VWF:RCo and VWF:CB levels.[29]

Table 4 Comparison between RCo/Ag and CB/Ag in VWD2B patients

Multimeric pattern	HWMWM		*p*
	Normal	Absent	
No. of patients	4	19	
VWF:RCo IU/dL	34 ± 17.6	32.3 ± 28.1	0.909
VWF:CB IU/dL	31.2 ± 16.3	18.9 ± 17.5	0.211
p	0.83	0.086	
RCo/Ag	1.01 ± 0.2	0.58 ± 0.3	0.013
CB/Ag	0.95 ± 0.41	0.35 ± 0.17	0.000
p	0.974	0.006	

Abbreviations: VWF:RCo, ristocetin cofactor activity; VWF:CB, collagen-binding assay; RCo/Ag, VWF:RCo/VWF:Ag; CB/Ag, VWF:CB/VWF:Ag.

Considering stress conditions, we obtained similar results as previously described.[28] In the only atypical VWD2B patient evaluated, PC remained within normal values. The most severe fall in PC was observed in patients carrying p.R1308C and p.V1316M.

In our total of 38 patients with VWD2B, 7 different mutations were identified. All of them were missense, and in heterozygous state. We report one novel mutation, p.Y1258C. The p.M1304V mutation was first reported by Woods.[30,31]

Two mutations, p.P1266L and p.M1304V, were associated with atypical VWD2B, with a frequency of 10.5%, in agreement with a value of 16.6% (3/18) previously reported.[13] The p.P1266L was first reported as Malmo[9] or type I–New York.[8] Consistent with previous observations,[32] p.V1316M was the most common mutation in this report.

There are few studies describing de novo mutations in VWD.[33–35] Two apparent de novo mutations (p.V1316M and p.S1310F) have been reported here. In the p.S1310F group, given that the three sisters have a common mother but different fathers, we assume that the mutation has a maternal origin, possibly due to genetic mosaicism. However, it is difficult to discriminate if the mutation occurred at some point during the development of the germ line or postzygotically (germinal or somatic mosaicism).[33,36,37] A technique that may facilitate the quantitation of the level of the mutation in the mother (e.g., TaqMan analysis for the specific mutation) could be used to try and see if it is present in her somatic cells, analyzing DNA from other tissues, as buccal swap, hair roots, urine specimen, and skin biopsy. However, this may not be successful if it is a germline mosaic, in which case the mutation may only be present in her ova. Other reports identifying apparent de novo mutations in families with two and three affected siblings exist; for example: (1) Yang et al[38] described two affected siblings with an apparent de novo mutation, apparently from the father, and related to congenital cataracts; (2) Armaroli et al[39] described two half-siblings with a father in common and an apparent de novo mutation associated with collagen VI–related myopathies; (3) Nemirovsky et al[40] described three affected siblings, but parents and grandparents without apparent mutation in familial autism spectrum disorder; and (4) Aulitzky et al[41] described three affected siblings (two siblings and one half-sibling), with a common mother and two different fathers without apparent mutations. Ahmad et al[36] noticed that unaffected parents can have more than one child with autosomal dominant VWD. There may be other explanations for the apparent discrepancies in genetic presentations between parents and their offspring, including different sensitivities to the primers used for PCR.

Consistent with a previous report,[13] we found a direct correlation between the absence of HMWM and thrombocytopenia. The lowest documented PC was associated with the p.V1316M mutation.[11] In this report, the most severe thrombocytopenia was associated with the p.S1310F.

In this report, a high percentage of patients displayed a RCo/Ag > 0.6, in agreement with Federici et al,[28] who noted that RCo/Ag ratios were not always <0.7 as typically defined in type 2 (A, B, M) VWD. It is well known that VWF:RCo evaluates the VWF-A1 domain-platelet binding, whereas VWF:CB reflects a different functional property of VWF than VWF:RCo,[42] namely, VWF-collagen binding. Both assays are sensitive to the absence of HMWM.[43] VWF:CB and CB/Ag were found to be respectively lower than VWF:RCo and RCo/

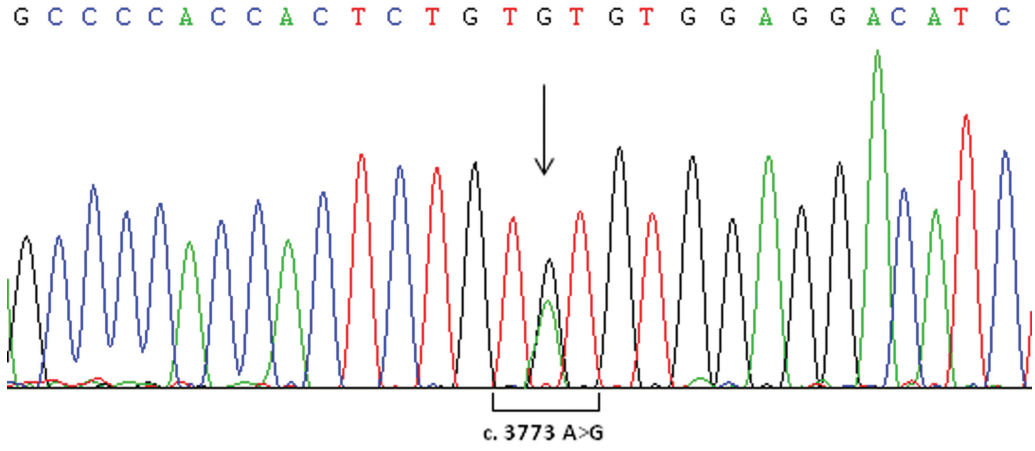

Fig. 2 Heterozygous substitution A > G located in the position c.3773 causing the change of tyrosine to cysteine at residue 1258 (p.Y1258C).

Ag in our VWD2B patients, a finding consistent with a previous report.[44] This was more evident in those patients with absent HMWM, mainly in those with p.S1310F. Trying to explain these differences, we analyzed the influence of p.A1381T and p.D1472H on VWF:RCo.[45,46] No changes in VWF:RCo were associated with the presence of these SNPs. Therefore, VWF:RCo would be overestimated in VWD2B, due to this known gain of function of VWF mutant, and by this way, many patients could be misdiagnosed. Taken together, this could explain the difference between RCo/Ag and CB/Ag seen in VWD2B patients. The difference in CB/Ag between atypical patients and those with low HMWM is closely related to the multimeric pattern. Therefore, both techniques, VWF:RCo and VWF:CB, must be performed in VWD2B patients, as previously recommended.[47]

It has been described that RIPA is the only specific assay for diagnosing VWD2B.[34] However, the ristocetin concentration to be used remains controversial.[48] Many authors suggest that 0.5 mg/mL should be used as the threshold.[49] In our case, the high number of patients with negative RIPA 0.5 mg/mL seen in p.R1308C mandates the evaluation of the threshold ristocetin concentration to avoid misdiagnosis. We should therefore keep in mind that a normal RCo/Ag and a negative 0.5 mg/mL RIPA do not exclude the diagnosis of VWD2B. In our study, the genotypic profile was the key to solve this problem. Finally, we have to point out the unexpected high presence of O blood group between our patients, like in type 1 VWD, without a clear explanation, considering that the frequency of this blood group in the normal population is near 50%.[50]

A shorter survival of VWF was observed in VWD2B patients with or without both HMWM and thrombocytopenia.[13] We found a slightly high VWFpp ratio in 67.6% of our patients.

We hope that these findings contribute to the overall knowledge of VWD2B as a bleeding disorder, and specifically knowledge of affected patients and their families, by providing further insight into this topic.

Conflict of Interest

The authors have no conflict of interests to report.

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