Diagnosis of von Willebrand disease in Argentina: a single institution experience

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Background: von Willebrand disease (VWD) is the most common autosomal bleeding disorder, mostly inherited as dominant trait. VWD is due to deficiency/abnormality of von Willebrand factor (VWF). The true prevalence of VWD is unknown, but estimated as 0.1% to 1% of the general population.

Methods: The bleeding score (BS) was used to evaluate the bleeding status of patients presenting at our institution. Laboratory analyses include: VWF:Ag, VWF:RCo, VWF:CB, multimeric pattern, VWF propeptide, Desmopressin challenge test. Genotypic analysis comprises the study of specific exons of VWF, depending on the suspected variant.

Results: We describe the main characteristics of our VWD population, including phenotypic and genotypic methods used to achieve the diagnosis. From our 2,482 patients registered, quantitative variants account for 83.2% of cases, and qualitative, 16.8% of cases. Platelet-type VWD (PT-VWD) was diagnosed in two patients and acquired von Willebrand syndrome (AVWS) in six. From quantitative variants, relative frequencies are: possible type 1, 78.7%; type 1, 14.9%; type 1S, 1.5%; type 1C, 0.4%; type 3, 1.6%. From qualitative variants: 2M, 21.9%; 2B, 10.3%; 2A, 8.4%; 2N, 3.4%; patients currently without complete diagnosis: 56%. Family history of bleeding: 64.7% of cases. Type 3, 2A and 2B VWD showed the highest major bleeding (MB) frequency. Genotypic diagnosis was reached in eight type 1C, 72 type 2M, 38 type 2B, 30 type 2A, 14 type 2N, and two PT-VWD patients. Five *de novo* mutations were identified among 52 families (9.61%): two mutations were associated with type 2B VWD (p.V1316M and p.S1310F), one mutation associated with type 1C (Vicenza) (p.R1205H) described in combination with p.R924Q, one deletion associated to type 1 VWD according to the ISTH VWF database (p.Pro1648fs*45) in combination with p.R1426C resulting in a 2M phenotype and one mutation related to type 2A VWD (p.Y1542D). Six patients are under prophylactic treatment due to their severe bleeding: four are type 3 VWD, one type 2M VWD, and one, the patient with p.Pro1648fs*45 and p.R1426C.

Conclusions: The wide heterogeneity of clinical symptoms and laboratory data from possible type 1 VWD implies a risk for bleeding that becomes crucial in challenging situations. In our population, type 2M was found to be more frequent than the type 2A variant. DDAVP (1-deamino-8-D-arginine vasopressin) challenge test, VWF propeptide/antigen ratio and genotypic studies are useful tools for discriminating cases representing difficult diagnoses such as type 2M versus type 1C VWD. Elevated propeptide ratio, the detection of antiphospholipid antibodies, platelet-associated antibodies or anti-VWF immunoglobulins and negative genotypic screening is useful to discriminate inherited VWD from AVWS. Genotypic testing assisted discrimination between types 2B versus platelet-type-VWD and type 2N VWD versus mild hemophilia A.

Keywords: Desmopressin; hemorrhage; mutations; von Willebrand disease (VWD); von Willebrand factor (VWF)

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Introduction

von Willebrand disease (VWD) is the most common inherited bleeding disorder, affecting 0.1% to 1% of the total population (1,2), although the prevalence of symptomatic patients is closer to 0.01% (1). However, the true prevalence of VWD is unknown, and difficult to determine (3). Since the early eighties, our institution has been studying patients referred to us because of personal and/or family bleeding histories or abnormal pre-surgery hemostasis assays. Since then, we have established a reference center for VWD diagnosis, laboratory testing, and treatment in Argentina, with growing experience over the last four decades. During these years, we have improved our evaluation of patients through the incorporation of additional discriminating assays, including RIPA mixing tests and cryoprecipitate challenge for the differential diagnosis between platelet-type VWD (PT-VWD) and type 2B VWD, and the genotyping study of *GP1BA*.

In recent years, considerable advances have been made world-wide regarding the diagnosis of VWD, and including:

(I) the age-related increase of von Willebrand factor (VWF) levels in type 1 VWD patients may result in a normalization of hemostatic parameters and sometimes bleeding symptoms, thereby potentially also changing a patient's 'diagnosis' (4); this finding has only been confirmed in the 'mild' VWD subgroup, with less definitive changes seen in the definite type 1 VWD subgroup (5). Increasing bleeding score (BS) in type 1 VWD (6) but no change in VWF and FVIII levels with an increased bleeding risk in type 2 VWD with aging was described (7). However, it has less extensively been explored as to whether the hemorrhagic phenotype improves with aging (8).

(II) findings related to the involvement of different polymorphisms on VWF:Ag levels among different ethnic and racial groups (9,10). This makes necessary the better characterization of ethnic and racial variations in VWF, otherwise resulting in ambiguity or erroneous conclusions that might have an impact on the diagnosis of VWD;

(III) the development of new methods to measure VWF activity (11), including: (i) VWF:GPIbR: based on the ristocetin-induced binding of VWF to a recombinant wild-type GPIb fragment (12); (ii) VWF:GPIbM: based on the spontaneous binding of VWF to a gain-of-function variant GPIb fragment (13); (iii) VWF:Ab: based on the binding of a monoclonal antibody to a VWF A1 domain epitope (12); (iv) VWF:2b3aB: to measure VWF binding to αIIb β3 (14); (v) the use of type IV and VI collagen for VWF:CB assay (15); (vi) the development of automated methods for VWF:CB assay (16);

(IV) the development of new methods for molecular genetic testing approaches that include the use of a multi-gene panel, and next-generation sequencing (12);

(V) the last approach of ISTH-bleeding assessment tool (ISTH-BAT), the semi quantitative system to measure the amount of clinical bleeding, named BS, applied to VWD patients (17,18);

(VI) the description of pathogenic variants, which have been identified in 60–65% of individuals with type 1 VWD (19,20).

Our aim in this paper is to show the phenotypic and genotypic characteristics of the VWD population assessed by our institution. Genetic testing was unfortunately only performed in a minority of our patients, given the high cost of these studies. We have therefore focused genetic testing for type 2 VWD patients.

Criteria and strategy for diagnosis

The rational diagnostic strategy for VWD patients is based on phenotypic analysis and, in specific cases, additionally on genotypic analysis. All patients undergo a minimum of two separate laboratory studies. As usual in clinical practice, the most abnormal value for any given test is considered for the final diagnosis. Possible associations between VWD and other disorders, such as hemophilia A, and B, and different

thrombopathies should also be considered (21). Previously, the BS was calculated according to the "Vicenza" BS; subsequently, it has been changed to the ISTH- BAT (17), being defined as normal if 0–3 in adult males, 0–5 in adult females, and 0–2 in both male and female children (18).

Major bleeding (MB) is defined as per Schulman *et al*. (22), and includes transfusion requirements [recombinant or blood derivatives infusion, DDAVP (1-deamino-8-D-arginine vasopressin) or antifibrinolytic agents], need of hospitalization, surgical re-intervention, or any other urgent procedures or decisions.

Types 1 and 3 variants of VWD

It is relatively easy to distinguish type 1 from type 3 VWD by its milder bleeding tendency and VWF deficiency. Differentiation of type 1 and possible type 1 requires more effort, given the incomplete penetrance of the phenotype, the variable expression of bleeding symptoms, and difficulties in standardizing diagnostic tests (23). Subjects with low VWF (31–50 IU/dL) and mild bleeding symptoms may be considered as "low VWF" (24) or "possible" type 1 VWD (25). In these cases, family history may be negative (12). To be considered as having type 1 VWD, the subject should have VWF:RCo below the blood group specific normal range, bleeding symptoms (26) and a family history or a causative mutation. Notably, variations in the VWF sequence have been described in only 44% of type 1 patients with VWF:Ag levels ≥30 IU/dL (3).

Changes in VWF:Ag and VWF:RCo levels according to aging have been described, with an increase of 0.17 and 0.15 IU/mL per decade (4) in the normal population. It is also known that individual with blood O group have VWF levels around 25% lower than those of non-O group, probably resulting from differences in glycosylation that may alter VWF clearance rates (27). The difference is more pronounced in elderly individuals, where the VWF increases only 1.25-fold in O-group, against 1.71-fold increase in non-O group (27). This consideration may be particularly important in type 1 VWD.

Our criteria for diagnosing quantitative disorders of VWD are: possible type 1: VWF:RCo within 31–49 IU/dL, type 1 VWD with a VWF:RCo within 15–30 IU/dL, and severe type 1 (type 1S) with VWF:RCo within 5–15 IU/dL, and with a poor response to DDAVP. Type 1C comprises those cases in which VWF shows a shortened survival after DDAVP challenge test and high VWFpp ratio (28).In all these cases, VWF:RCo/VWF:Ag (RCo/Ag) are ≥0.6. Type 3 are those patients with VWF <5 IU/dL, FVIII:C usually <10 IU/dL. The use of DDAVP in type 3 VWD patients is known to be ineffective.

Type 2 variants of VWD

Type 2 VWD is characterized by an impaired VWF function, towards platelets or FVIII (29). Type 2A, 2B and 2M VWD is intially identified by RCo/Ag <0.6, with normal or slightly reduced levels of VWF:Ag and FVIII:C. In type 2N, the deficiency of VWF resides in the binding of FVIII:C to VWF; type 2N VWD is suspected when FVIII:C is very low, the FVIII:C/VWF:Ag is <0.7 and furthermore suggested when the FVIII-VWF binding assay (VWF:FVIIIB/VWF:Ag) is <0.8, with normal VWF. Until we find a candidate mutation, however, we refer to these cases as "probable VWD2N".

Type 2A and 2M show low affinity of VWF for GPIb, reduced or absent RIPA at 1.2 mg/mL ristocetin, with normal multimers in type 2M. Type 2B shows a gain of function of VWF for GPIb and subsequently enhanced RIPA at low doses (<0.8 mg/mL), with absence of large multimers of VWF. The platelet count may range from low to normal (30). In situations of stress, such as pregnancy or response to DDAVP, thrombocytopenia is accentuated. An atypical form of type 2B has also been described, characterized by normal platelet count, normal multimers, and normal RCo/Ag (31). A proposal has been made to distinguish these as type 2B-I, being those patients lacking in large multimers, versus type 2B-II, being those with normal multimeric profile, with or without a normal VWF synthesis, in order to better classify type 2B VWD patients (32). PT-VWD is a very rare bleeding disorder, caused by the presence of mutations in *GP1BA*, which result in a platelet function defect, evidenced by an increased affinity of platelets for VWF. Seven mutations in the *GP1BA* gene related to PT-VWD have so far been described, and about 50 patients are currently reported world-wide (http://pt-vwd.org/. Accessed: November 2017). One of these mutations has been newly described by us (33). Herein, VWF is not involved *per se*. However, because PT-VWD patients show similar bleeding symptoms and laboratory abnormalities as type 2B VWD, their study is typically included in VWD reviews. The differential diagnosis between the two entities is especially challenging, as evidenced by high levels of misdiagnosis of both conditions,

but particularly PT-VWD, which has been reported as type 2B VWD in ~15% of cases (34). Both PT-VWD and type 2BVWD may also be misdiagnosed as idiopathic thrombocytopenia (34). For this reason, the real PT-VWD incidence is unknown (34). The therapeutic management of the two disorders is different, so that discrimination is clinically essential.

Methods

Sample preparation

Peripheral venous blood is drawn in 3.13% sodium citrate for clotting tests, in 2% EDTA for DNA analysis, and with anti-proteases (2.8% sodium citrate; 60 mM N-ethylmaleimide, 50 mM EDTA (disodium ethylenediaminetetraacetate dihydrate), and 2,000 KIU/mL aprotinin) for multimeric profile. Laboratory investigation is offered to all available members of affected families.

Phenotypic analysis

The following tests are performed: complete blood count that includes platelet count (Plt); bleeding time (BT) [Ivy method, normal value (nv) ≤4.5 min]; factor VIII (FVIII:C) (35) [one-stage method, normal range (nr): 50-150 IU/dL]; VWF antigen (VWF:Ag) (36) (ELISA, nr: 50–150 IU/dL); VWF ristocetin cofactor (VWF:RCo) (37) (aggregometry, nr: 50–150 IU/dL); VWF collagen binding (VWF:CB) (ELISA, collagen type 1, nr: 60–130 IU/dL); VWF FVIII binding (VWF:FVIIIB), VWF propeptide (VWFpp) (38) (ELISA); VWF multimeric analysis (39), using SDS 1% and 1.7% agarose gel electrophoresis. FVIII/VWF:Ag (nv ≥0.7), VWF:RCo/VWF:Ag (RCo/Ag) (nv ≥0.6), VWF:CB/VWF:Ag (CB/Ag) (nv ≥0.6), and VWFpp/VWF:Ag (VWFpp ratio) (nr: 0.92–2.14) are calculated. The presence of anti-VWF antibodies in patients with either prolonged activated partial thromboplastin time (APTT) or VWF:RCo <10 IU/dL or with clinical suspicion of acquired von Willebrand syndrome (AVWS) is performed as previously described (40); however, antibodies to nonfunctional domain will be missed on such functional testing. We therefore developed an ELISA technique to detect anti-VWF antibodies as previously described (41), but with some modifications. Ristocetin induced platelet aggregation (RIPA) at 1.2 mg ristocetin/mL is the first RIPA test performed. Where RIPA 1.2 mg/mL is normal, testing is repeated using ristocetin 0.5–0.7 mg/mL, looking for the threshold ristocetin concentration; where RIPA 1.2 mg/mL is absent, testing is repeated using ristocetin at 1.5 and if required 2 mg/mL (http://www.isth.org/?page=ssc_minutes/; 56th SSC meeting of ISTH 2010. Accessed: November 2017).

When PT-VWD is suspected, discriminating methods (RIPA mixing assay and cryoprecipitate challenge assay) are performed as previously described (42,43).

Not all these tests are routinely performed on all patients. VWF:CB, multimeric analysis, and VWFpp are performed in selected patients, according to the results of previous phenotypic studies. A local plasma-pool obtained from 20 healthy donors is used as a secondary standard calibrated against the standard 07/316 of the National Institute of Biological Standards and Control (NIBSC).

As described before (3), given our lower limit of detection for VWF:RCo is 10 IU/dL, levels <10 IU/dL were taken as 5 IU/dL for calculation of means and ratios.

Genotypic analysis

There are several situations in which differential diagnosis in VWD is mandatory: between hereditary congenital and AVWS (44), between type 2N VWD and mild hemophilia A (45) and between type 2BVWD and PT-VWD (42). In all these situations, the definitive diagnosis remains the identification of mutations.

Genetic testing for VWD is not performed routinely. Since 1998, we have performed genotypic studies in patients with suspected type 2N, and subsequently in patients with suspected 2A, 2M, 2B, and PT-VWD. Genomic DNA is extracted from peripheral blood using standard methods. Exons of interest are amplified using polymerase chain reaction (PCR) technique. In probable type 2N VWD patients, exons 17 to 27 of *VWF* gene are analyzed. In type 2A, 2M and 2B patients, given the high frequency of mutations located in the exon 28, we start genotypic studies through the analysis of this exon (46). Subsequently, where no mutations are found, exons 26, 27, 29, 30, 31 and 52 are also analyzed. When PT-VWD is suspected, *GPIBA* is analyzed. Primers for PCR were designed in our laboratory. Both forward and reverse strands are directly sequenced by automated sequencing technology (ABI Prism 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). When mutations are found in the index cases, genotypic studies are offered to all available relatives to confirm familial transmission.

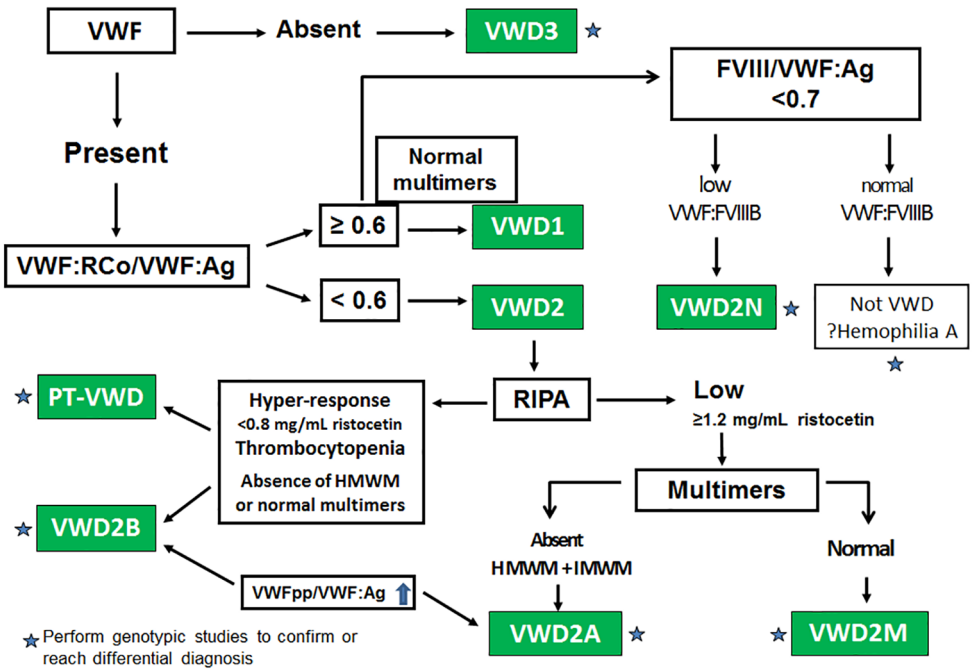

Figure 1 The algorithm of VWD diagnostic strategy. VWF, von Willebrand factor; VWD, von Willebrand disease; RIPA, ristocetin-induced platelet aggregation; HMWM, high molecular weight multimers; IMWM, intermediate molecular weight multimers.

Figure 1 shows an algorithm that describes the VWD diagnostic strategy.

Biologic response to desmopressin

DDAVP is a synthetic analog of the vasopressin, which increases two to five times the basal level of circulating FVIII/VWF within 30–60 min and last by 6–8 hours post infusion, returning to basal levels after 24 hours (47). This effect is achieved by the FVIII/VWF releases from storage. In cases of increased clearance, levels return to base-line after 1–4 hours. DDAVP does not normally show significant side effects and it is safe from the risk of transmitting blood viruses. It is generally contraindicated in type 2B VWD because of the transient appearance or aggravation of thrombocytopenia (30). However, DDAVP was described as appropriate in type 2B VWD patients with normal multimeric pattern and platelet count (32). Because of its antidiuretic properties, such as hyponatremia, and volume overload, it should be used with caution in elderly subjects with cardiovascular or atherosclerosis disease (25), and in children below the age of 2 years (25).

In our institute, DDAVP is infused intravenously (0.3 μg/kg body weight during 20 min in 50 mL saline solution). Blood samples are obtained before, 60 and 120 min after the end of the infusion. In case of children below 20kg of body weight, samples are obtained before and 90 min after (48). In accordance with the National Heart, Lung and Blood Institute guidelines on VWD (25) we do not use DDAVP in children <2 years of age. The time courses of Plt, BT, FVIII:C, VWF:Ag, VWF:RCo and euglobulin clot lysis time are analyzed. A good response is considered when all abnormal parameters reach normal levels. A poor response is when FVIII, VWF:Ag or VWF:RCo do not reach normal levels or when these levels fall rapidly (i.e., within 2 h) after the infusion. No response means that all the parameters remain abnormal, or in type 2N VWD patients, where FVIII:C does not reach plasma levels required for hemostasis (≥50 IU/dL) (49). In these cases of accelerated fibrinolytic response, the dose of DDAVP is reduced to 0.2 μg/kg body weight, for further treatments. The success of the test depends on the infusion time, which must be strictly observed and the time between the end of the infusion and the start of the bleeding event to

Table 1 Classification of our VWD patients, according to their phenotype

Type of VWD	Overall, n (%)	Females (%)	Blood O group (%)	≤13 years-old (%)	With family history (%)	With MB (%)
Quantitative variants, n=2,066 (83.2%)						
Possible type 1	1,626 (78.7)	70	76.1	24	61.6	12.8
Type 1	308 (14.9)	66.4	83.3	24	67.8	15.2
Type 1S	30 (1.5)	54.8	72,2	41.9	66.6	33.3
Type 1C	8 (0.4)	62.5	50	20	100	40
1+probable 2N	60 (2.9)	13.9	74.2	46.8	60.8	60.2
Type 3	34 (1.6)	55.8	73.6	41.2	82.1	62
Qualitative variants, n=416 (16.8%)						
Probable 2N	31 (7.5)	37.6	53.5	17	80.5	18
2N	14 (3.4)	2.4	50	28.5	90	30
2A	35 (8.4)	55.5	37.5	25	93.1	60
2B	43 (10.3)	53.4	70.5	25.6	84.6	60.4
2M	91 (21.9)	56.1	62.3	39.3	88.4	38.4
2 unclassified	202 (48.6)	54.5	64.4	30.5	54.8	20.6
AVWS	6	40	50	0	0	100
PT-VWD	2	0	50	0	50	100

Number and percentage of patients within their variant group. VWD, von Willebrand disease; MB, major bleeding; AVWS, acquired von Willebrand

be prevented.

Minor local bleeding (nasal or oral bleeding) may be managed with topical hemostatic agents or local pressure for long time. The most common topical agents are: fibrin sealants, topical thrombin, micronized collagen strips (25).

The most frequent pharmacologic agents are: fibrinolysis inhibitors such as aminocaproic acid, and tranexamic acid, which are very frequently used in dental procedures and menorrhagia (50), and hormonal treatments with estrogen-progesterone combined oral contraceptives that are useful in treatment of menorrhagia (25).

In silico evaluation

In silico evaluation is being used more and more frequently by several groups to predict effects of nucleotidic changes on the integrity of the resulting protein. In our laboratory, the *in silico* analysis was performed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/. Accessed: November 2017), Panther (http://pantherdb.org/ Accessed: November 2017), and Mutation Taster (http://www.mutationtaster.org/. Accessed: November 2017). The sequence alignment of the proteins is performed using the informatics application UniProt KB (www.uniprot.org. Accessed: November 2017).

Results

Characteristics of our VWD population

Our country has nearly 44 million inhabitants. From 1982 to December 2016, a total of 5,439 patients were studied at our institution and the final VWD diagnosis was reached in 2,482 patients. We do not have an estimation of prevalence of VWD in our country, because this is a report from a single institution. A total of 83.2% of patients were diagnosed as having a quantitative VWD, whereas 16.8% were type 2 VWD. Two patients were diagnosed as PT-VWD and six patients as AVWS. The true global prevalence of the different VWD variants is unknown, but type 1 VWD is believed to represent 65–80% of all the VWD population (51). The prevalence of type 1 VWD variants in our population is quite similar to those of other

Table 2 Laboratory data of quantitative variants

VWD	BS	VWF:Ag, IU/dL	FVIII:C, U/dL	FVIII/Ag	VWF:RCo, IU/dL	RCo/Ag	BT, min	VWFpp ratio
Possible type 1	3.5±2.4	43.5±11.7	51±19	1.18±0.3	41.6±5.3	1.2±0.6	4.8±1.8	ND
Type 1	4.2±2.8	35±11.6	50.6±30.3	1.4±0.5	25.8±4.2	0.9±0.3	5.2±2.6	ND
Type 1S	5.7±3.2	14.8±8.6	38±11.8	2.7±0.4	7.9±6.0	NA	6.2±2.7	1.5±0.2
Type 1C	4.7±2	14.1±5.8	35.6±13.1	2.5±0.9	10.9±5.3	0.9±0.2	5.8±2.4	5.3±3.5
Type 1 probable 2N	6.4±3.1	43±9.6	9.7±8.5	0.22±0.1	40.2±7	1±0.25	4.6±1.7	ND
Type 3	9±4.8	1.7±1.9	12.4±8.9	7.3±1.5	1±0	NA	11.8±2.9	ND

VWD, von Willebrand disease; BS, bleeding score; FVIII:C, factor VIII; VWF:Ag, von Willebrand antigen; VWF:RCo, ristocetin cofactor activity; RCo/Ag, ristocetin cofactor activity/antigen ratio; BT, bleeding time; VWFpp ratio, propeptide/antigen ratio. NA, not applicant; ND, not done.

reports (*Table 1*). Seventy-five percentage of patients were under 13 years of age at enrollment; the more severe VWD, the younger the patients. Blood O group was shown in 74% of all patients. The highest frequency of this blood group was observed in type 1 (83.1%), possible type 1 (76.1%), and type 2B (70.5%), whereas in our normal population is near 50% (52). Only 64.7% of the patients have family history of bleeding. The prevalence of females was 60.7%, especially in type 1 VWD. In patients with type probable 2N and combined type 1+probable 2N, the frequency of males is higher than females (P<0.0001).

Although 0.29% of parental consanguinity rate in normal births were reported in our country, we have one case of recognized parental consanguinity in one 2-year girl with type 3 VWD whose parents (brother and sister) were diagnosed as type 1 VWD.

Quantitative subtypes

Phenotypic analysis

Within our patients belonging to quantitative variants, possible type 1 is the most frequent. Type 3 accounts for only 1.6%, and type 1C that includes type Vicenza, for 0.4%. The laboratory data of the quantitative variants are summarized in *Table 2*.

The most frequent bleeding symptoms seen in quantitative variants were bleeding after tooth extraction, bleeding related to surgery, epistaxis, bleeding related to parturition/delivery, and in females of fertile age, menorrhagia. Joint bleeding was reported in 0.77% of patients (14.7% of type 3 VWD patients). Overall, lower levels of VWF:RCo, a higher percentage of patients with bleeding symptoms, especially epistaxis and bleeding, related to surgeries.

The median of age at the enrollment in possible type 1 was 22 years (range: 1–73 years), and in type 1 patients 23 years (range: 1–67 years), both variants with 24% of patients below 13 years old. In these cases, it is important to consider that at that age of diagnosis, patients have had less time to accumulate bleeding symptoms, as highlighted previously (3). This is the case for possible type 1 VWD patients, who show a wide heterogeneity not only in their BS, but also in their MB incidence. Unexpectedly for their VWF levels, BS ≥10 was observed in 2% of patients, quite similar to that observed in type 1 (5% of patients). MB incidence was 12.8% in possible type 1 VWD, versus 15.2%in type 1 VWD (P=0.01; RR, 1.19; 95% CI: 0.89–1.60). On the other hand, 10.2% of possible type 1 VWD patients do not have personal bleeding symptoms, quite similar to type 1 patients (9.5%) (P=ns), with no difference in the frequency of blood O group (P=ns). In addition, normal levels of FVIII are found in 44% of type 1 and possible type 1 variants. Then, considering all these findings and the variability in FVIII and VWF over time, the differences between the possible types 1 and type 1 are subtle. Asymptomatic patients with low values of VWF should be regarded with caution, as many of them might not have had a hemostatic challenge to manifest a bleeding tendency. This implies a risk for bleeding that acquires an important value in challenging situations, such as surgeries.

Recently a non-significant difference in BS between blood O group and non-O blood group was described in type 1 VWD patients, with no correlation with VWF:Ag (3). In agreement with the authors, in our cohort, when possible

Table 3 Comparison between different ranges of BS versus blood group and laboratory tests in possible type 1 and type 1 VWD patients

VWD	Laboratory tests	Blood group (O group/non-O group)		
		BS ≥10	BS from normal to 9	Normal BS
Type 1	% of patients	7.3/4*	40.2/24*	52.5/72*
	FVIII:C, IU/dL	60±28/20	48±17/62±36*	49±18/54±21*
	VWF:Ag, IU/dL	31±6.9/20	32±11/41±14*	34±11/35±17*
	VWF:RCo, IU/dL	25±4.5/25	26±4/28±3*	27±4/24±4*
	BT, min	6±2.5/–	5±2/3±1*	5±2/5±2*
Possible type 1	% of patients	2.6/2.3*	27.3/21*	70/76.7*
	FVIII:C, IU/dL	44.2±18/63±27*	51±18/52±25*	50±17/53±22*
	VWF:Ag, IU/dL	42.6±8/47±4.7*	42±9/43±10*	42±11/45±12*
	VWF:RCo, IU/dL	41.2±8/43±6.2*	41±5/43±6*	42±5/42±5*
	BT, min	4.9±2.2/6±1.7*	5±2/5±2*	5±2/5±2*

VWD, von Willebrand disease; BS, bleeding score; FVIII:C, factor VIII; VWF:Ag, von Willebrand antigen; VWF:RCo, ristocetin cofactor activity; BT, bleeding time; *, not significant.

type 1 and type 1 VWD patients were grouped according to their BS and blood group, no correlation was observed between FVIII:C, VWF:Ag, VWF:RCo and BT between blood O group and non-O group (*Table 3*). In addition, no difference was observed in the percentage of patients with similar BS between blood O group and non-O group.

Individuals with both low VWF and FVIII/VWF:Ag <0.7 resulted in a more severe bleeding phenotype: patients with type 1+ probable 2N showed higher frequency of MB (P<0.0001; RR, 3.37, 95% CI, 2.42–4.68) than probable 2N. The same finding was observed when we compared type 1+ probable 2N versus possible type 1 (P=0.0001; RR, 4.71; 95% CI, 3.78–5.87).

Regarding type 3 VWD, the median age of enrollment was 16 years (range: 1–61years), with 41.2% of patients below 13 years and 29.4% ≥40 years. Joint bleeding was reported in 14.7% of patients. Four patients (11.7%) within seven months to 6 years of age are currently under long-term prophylactic treatment because of the severity of their bleeding episodes.

Genotypic analysis

Eight cases of type 1C VWD have been identified: two patients with type Vicenza: one of them, with heterozygous p.R1205H, and the other patient, with combined heterozygous p.R1205H and p.R924Q (53). In the remaining six patients, p.R1315C was identified in heterozygous state.

Qualitative subtypes

Phenotypic analysis

It has been described that type 2M VWD is more often misidentified than correctly identified as 2M VWD (47), giving an under-reported incidence of this variant in the literature. In our patients belonging to qualitative variants, type 2M was the most frequent, followed by type 2B, and type 2A, and type 2N. In 56% of patients overall, the subtype could not be identified because the necessary additional phenotypic and genotypic studies could not be performed.

The most frequent symptoms were bleeding after tooth extraction, bleeding related to surgery, menorrhagia, hematomas, and epistaxis. Joint bleeding was reported in 8.6% of patients (5.7% of type 2A VWD patients). The qualitative group showed higher bleeding tendency than the quantitative group (P=0.000; RR, 1.57; 95% CI, 1.33–1.87). Type 2B VWD was the group with the highest MB frequency (60.4%), followed by type 2A (60%), 2M (38.4%), type 2N (30%), and probably 2N (18%) (*Table 1*). Type 2A patients showed a significant higher bleeding tendency than type 2M patients (P=0.025; RR, 1.67; 95% CI 1.13–2.46), but not significantly higher than those of type 2B patients. However, a 3-year-old girl with type 2M VWD is currently under long-term prophylactic treatment.

The laboratory data are summarized in *Table 4*.

Table 4 Laboratory data of qualitative variants

VWD	BS	VWF:Ag, IU/dL	FVIII:C, IU/dL	FVIII/Ag	VWF:RCo, IU/dL	RCo/Ag	VWF:CB, IU/dL	CB/Ag	BT, min	VWFpp ratio
Probable 2N	5.4±3.2	88.4±30.7	24.4±15.3	0.27±0.15	81.4±24.4	0.95±0.2	ND	ND	4.5±1.7	ND
2A	7.5±4.1	43.5±23.6	42.2±18.7	0.97±1.2	2.9±4.5	0.1±0.1	11.3±5.6	0.3±0.1	9±3.4	2.7±1.1
2B	5.8±2.5	53.3±30.8	52.2±24.9	0.96±0.1	28.7±22.4	0.6±0.4	22±18.2	0.41±0.6	8.3±2.1	2.3±0.5
2M	5.1±3.7	48.3±26.8	51.8±27.2	1.07±0.2	8.9±11.5	0.2±0.2	23.6±29	0.7±0.6	7±2.9	2.07±0.8
2 unclassified	4.1±3	78.1±41.9	51±28.8	0.70±0.1	26.6±15.6	0.3±0.2	71±48	1±0.4	6.6±3.3	ND
PT-VWD	10±1	95±68	73±38	0.76±0.1	5±4.5	0.08±0.02	50.5±27.5	0.58±0.03	>9	1.5±0.2

VWD, von Willebrand disease; BS, bleeding score; FVIII:C, factor VIII; VWF:Ag, von Willebrand antigen; VWF:RCo, ristocetin cofactor activity; RCo/Ag, ristocetin cofactor activity/antigen ratio; CB/Ag, collagen binding/antigen ratio; BT, bleeding time; VWFpp ratio, propeptide/antigen ratio; ND, not done.

Genotypic analysis

From the 416 patients with qualitative phenotype, responsible mutations were identified in 156 patients (37.5%).

Patients with type 2A VWD

From our total of 35 type 2A patients, the exon 28 was sequenced in 33 individuals. Six mutations were identified in heterozygosis in 30 patients: p.C1272F (n=2), p.G1505R (n=3), p.Y1542D (n=1), p.R1597W (n=16), p.I1628T (n=6) and p.F1654L (n=2). In three patients, no mutations were observed in this exon. The p.R1597W, located in the A2 domain, was the most frequent mutation in this variant.

Patients with type 2B VWD

From our total of 43 type 2B patients, seven mutations in heterozygosis were identified in 38 patients: p.Y1258C (n=1), p.P1266L (n=1), p.M1304V (n=3), p.R1306W (n=8), p.R1308C (n=9), p.S1310F (n=4), and p.V1316M (n=12). Two mutations, p.P1266L and p.M1304V were associated with atypical type 2B VWD, given the presence of normal HMWM and platelet count (54), according to Casonato's description (31). The p.V1316M was the most frequent mutation in this variant.

Patients with type 2M VWD

Eighty out of 89 type 2M VWD patients were available for sequencing of exon 28; 14 mutations were identified in 74 patients: in heterozygosis: p.F1293C (n=2), p.R1315C (n=2), p.G1324S (n=1), p.S1325F (n=1), p.R1334Q (n=1), p.R1374C (n=18), p.R1374L (n=1), p.A1437T (n=5), p.T1468I (n=1), p.L1503P (n=2), p.E1549K (n=32), p.R1564W (n=1), p.R1583W (n=1), and p.I1628T (n=4); and in homozygosis: p.R1374C (n=2; two sisters). In the remaining 6 patients, no mutations were observed in this exon. The p.E1549K was the most frequent mutation, observed in a big family (four generations) and in one unrelated individual.

Patients with type probable 2N and type 1+probable 2N VWD

We could genotypically study 215 patients with FVIII/VWF:Ag <0.7. In this group, we found three mutations in heterozygosis in 14 patients: p.R854Q (n=6); p.R924Q (n=6), p.R816W (n=1), and a novel mutation p.D1195Y (n=1). Although type 2N VWD has been described as a recessive trait, in our case, all the patients had mutations in heterozygous state. No mutations were found in the

exons 17–27 of *VWF* gene in the remaining 201 patients. Given that 20% of patients with responsible mutations showed normal VWF:FVIIIB/VWF:Ag, this test was not performed anymore. These patients were referred to look for mutations related to hemophilia A.

Patients with PT-VWD

Two patients with phenotypic tests suggesting type 2B VWD were studied. Discriminating methods suggested PT-VWD diagnosis. Two mutations were found in *GP1BA*: p.W246L (33) in one patient, and p. M255V in the other patient. In these patients, no mutations were found in exon 28 of *VWF* gene.

Patients with AVWS

Six patients with bleeding symptoms of late appearance showed phenotypic studies suggesting type 2 VWD. The absence of mutations to explain the phenotype helped the medical staff to redirect the studies over the search of AVWS. Elevated VWFpp ratio, the detection of antiphospholipid antibodies, platelet-associated antibodies or anti-VWD immunoglobulins (IgG, IgA or IgM) were common features among these patients. AVWS related to monoclonal gammopathy of undetermined significance was diagnosed in four patients, and related to antiphospholipid syndrome was diagnosed in the other two patients.

Discrepancies in genotype-phenotype relationships

Diagnosis for VWD can also be complicated by incomplete penetrance and variable expressivity of the conditions described above. For example, ethnicity can influence the diversity seen in *VWF*. Some genomic variants of *VWF* are seemingly pathogenic in Caucasian populations, but benign in African individuals, which may contribute to an increase in the rate of false-positive diagnoses (10,55).

Discrepancies between the VWD variants and the candidate mutations related to different phenotypes have been reported in the ISTH-SSC VWF online database (http://www.ragtimedesign.com/vwf/. Accessed: November 2017). This is the case of p.R1315C, which has been reported in association with type 2M (56), type unclassified, type 1 (57), type 2A (58), type 1C (59) and type 3. In our population, this mutation was found in nine patients with normal multimers, in whom the initial phenotype was not clear. These cases could be solved through VWFpp ratio and the DDAVP challenge test. In six patients, with high basal VWFpp ratio, very low VWF and RCo/Ag <0.6, the normalization of RCo/Ag but a rapid clearance of VWF after DDAVP infusion, suggested a type 1C phenotype; another two patients, with normal VWFpp ratio and discordant RCo/Ag before and after DDAVP infusion showed a type 2M phenotype, and other patient could not be differentiated as type 1C or 2M.

We also found discrepancy in ten patients with p.I1628T; six patients showed a 2AVWD phenotype, whereas another four patients showed a 2M VWD phenotype. This mutation was reported in the ISTH-VWF database as type 2AVWD and polymorphism (SNP). Those patients with a 2A VWD phenotype showed significant longer BS than those with type 2M phenotype (P=0.03). These data show some diagnostic problems to be taken into account during patient investigations (46).

Another real challenge was the identification of two mutations with heterozygous and *trans* presentation in one of our patients with a type 2M VWD phenotype. The first mutation, p.R1426C, not previously described, responsible for probable type 1 in his mother, and the second mutation, the p.Pro1648fs*45, previously described associated with type 1VWD in the VWF SSC VWF database, which is absent in parents, suggesting a *de novo* presentation in the patient. The combined effect of both mutations resulted in a type 2M VWD, despite being individually associated to type 1 VWD (60).

De novo mutations

Occurrence of *de novo* mutations is a rare event in VWD families, although there are several cases described (61-64). Its frequency within families was reported as 14.3% (62), and 26.7% (61). In our cohort, five *de novo* mutations were identified among 52 families, with a frequency of 9.61%: two mutations associated with type 2B VWD (p.V1316M and p.S1310F) (54), one mutation associated with type 1C (Vicenza) (p.R1205H), which has been described in combination with p.R924Q (53), one deletion associated to type 1 VWD according to the ISTH VWF database (p.Pro1648fs*45) and another mutation related to type 2A VWD (p.Y1542D).

DDAVP challenge test

Results of DDAVP challenge tests in our VWD population are summarized in *Table 5*. The median age of patients at

Table 5 DDAVP challenge test in VWD variants

VWD type	Response % of patients		
	Good	Partial	Absent
Quantitative subtypes (n=843)			
Possible 1 (n=677)	99	1	0
Type 1 (n=119)	91.6	7.6	0.8
Type 1S (n=14)	43	57	0
Type 1C (n=5)	40	60	0
Type 1+ probable 2N (n=18)	38.8	50	11.2
Type 3 (n=10)	0	0	100
Qualitative subtypes (n=89)			
Probable 2N (n=18)	46.5	32.5	20.9
Type 2A (n=6)	16.6	83.4	0
Type 2B (n=9)	33.3	55.5	11.1
Type 2M (n=27)	44.4	48.1	7.4
Type 2 unclassified (n=29)	62.1	31	6.9

VWD, von Willebrand disease; DDAVP, 1-deamino-8-D-arginine vasopressin.

the moment of testing was 18.4 (range: 3–55) years. From a total of 932 tests performed, 138 (14.8%) were <13 years.

Quantitative subtypes: over a total of 843 tests done, 93% of patients showed a good response whereas 4.2% showed a partial response and 1.5% did not respond. A 12.3% of patients were <13 years. Only one type 1 VWD patient was 3 years old.

Qualitative subtypes: DDAVP response test was assessed in 89 patients. A total of 47.4% of these patients showed a good response, whereas 40.4% had a partial response, and 12.2% did not respond. A 38.2% of patients were <13 years, especially in probable 2N VWD. Only five patients were between 2–4 years old.

DDAVP challenge test was very useful in identifying some patients with difficult interpretation of tests results, and the subsequent classification such as discriminating type 3 from severe type 1, and type 2M from type 1C when the VWF levels were inconclusive or too low. This was the case of two patients, who developed thrombocytopenia, and by this way, the diagnostic approach towards type 2B VWD was addressed. Another similar case was our group of patients with the mutation p.R1315C, which was commented on previously.

Conclusions

The wide heterogeneity of clinical symptoms and laboratory data from possible type 1 implies a risk for bleeding that becomes crucial in challenging situations.

In our population, type 2M VWD was found more frequent than type 2A variant.

Because of the absence of significant side effects in our patients, its effectiveness and low cost, DDAVP should be considered as the first-choice therapy in VWD, especially in developing countries.

DDAVP challenge test, VWF propeptide and genotypic studies were very useful tools for discriminating those cases with difficult diagnoses, such as type 2M versus type 1C VWD. Elevated VWFpp ratio, the detection of antiphospholipid antibodies, platelet-associated antibodies or anti-VWF immunoglobulins (IgG, IgA or IgM) and the negative genotypic screening were useful to discriminate inherited VWD from AVWS. Genotypic testing also helped us to discriminate between type 2B versus PT-VWD and type 2N VWD versus mild hemophilia A.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study is included within the framework of the project whose number of minutes is 92/November 10/2016. Written informed consent was obtained from the patient for publication of this manuscript and any accompanying images.

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