In Focus Article

Identification of p.W246L As a Novel Mutation in the *GP1BA* Gene Responsible for Platelet-Type von Willebrand Disease

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Semin Thromb Hemost 2014;40:151–160.

Abstract

Platelet-type von Willebrand disease (PT-VWD) and type 2B von Willebrand disease (2B-VWD) are rare bleeding disorders characterized by increased ristocetin-induced platelet aggregation (RIPA) at low concentrations of ristocetin. Diagnosis of either condition is not easy and the differential diagnosis between the two entities is especially challenging as evidenced by high levels of misdiagnosis of both conditions, but particularly PT-VWD. Five mutations in the *GP1BA* gene related to PT-VWD and less than 50 patients are currently reported worldwide. We herein describe a patient with severe bleeding symptoms, macrothrombocytopenia, mild spontaneous platelet aggregation, positive RIPA at 0.3 and 0.4 mg/mL, von Willebrand factor ristocetin cofactor (VWF:RCo) to antigen (VWF:Ag) < 0.2, normal VWF propeptide/VWF:Ag ratio, and RIPA mixing tests and cryoprecipitate challenge positive for PT-VWD. *GP1BA* gene was studied in the patient, in his mother, and in 100 healthy control subjects. We identified a heterozygous substitution G $>$ T located at nucleotide 3805 in the g.DNA of the patient's *GP1BA* gene, resulting in a Trp to Leu amino acid change at residue 246 (p.W246L). This mutation was absent in his unaffected mother and also in the 100 controls, and was predicted as damaging by in silico analysis. The residue W246 is located within the VWF-binding region and exists in a strongly conserved position in the phylogenetic tree, which is expected to be unable to tolerate substitutions without changing its functional characteristics. These findings argue strongly in favor of the view that this substitution does not represent a polymorphism and is therefore responsible for the PT-VWD phenotype of the patient.

Keywords
- platelet-type von Willebrand disease
- p.W246L
- W230L
- RIPA mixing assay
- cryoprecipitate challenge assay

The von Willebrand factor (VWF) protein as present in plasma is variable in size, dependent on the level of multimerization, but the largest forms are around 20,000 kDa. VWF has two fundamental roles: in primary hemostasis, it initiates adhesion of platelets to sites of vascular damage and in blood clotting, it protects factor VIII (FVIII) from attack by proteases, prolonging its survival.[1] In recent years, an immune protective effect over FVIII to prevent the development of anti-FVIII inhibitors in hemophilia A has also been proposed and studied.[2] The primary structure of VWF comprises a subunit

published online January 28, 2014

Issue Theme Quality in Hemostasis and Thrombosis, Part III; Guest Editors, Emmanuel J. Favaloro, PhD, FFSc (RCPA), Giuseppe Lippi, MD, and Mario Plebani, MD.

DOI http://dx.doi.org/10.1055/s-0033-1364183.
ISSN 0094-6176.

of signal peptide, a propeptide, and the mature VWF. Each individual VWF subunit has binding sites for FVIII, heparin, glycoprotein Ibα (GPIbα), collagen, glycoprotein IIb-IIIa, some of which depend on the shear-rate inducing conformational changes.

According to several authors, von Willebrand disease (VWD) has a prevalence of 0.1[3] to 1%[4] in the general population. VWD is characterized by quantitative (types 1 and 3) or qualitative defects of VWF (types 2A, 2B, 2M, and 2N). The clinical manifestations are typical of a deficit in primary hemostasis, such as mucocutaneous bleeding symptoms. The hereditary nature of VWD is well established and, although clinical manifestations are highly variable, most patients develop their initial hemorrhagic symptoms during early childhood. Normal ranges of VWF have been described as overlapping with those of VWD.[5] A decreased level of VWF and bleeding symptoms unrelated to VWD may coexist with high likelihood.[5] We must also keep in mind that mild cases bleed only after challenges such as menstruation and childbirth in women or unscheduled surgery and trauma.[6]

There are several situations in which differential diagnosis in VWD is mandatory: between hereditary (autosomal dominant) congenital and acquired VWD,[7] between subtype 2N-VWD and mild hemophilia,[8] and between 2B-VWD and platelet-type VWD (PT-VWD).[9] An accurate diagnosis will likely require the patient to be referred to a specialized center or reference laboratory in order that the most appropriate tests are performed with the highest possible quality, together with validated controls.

Of particular relevance to the current report is that a differential diagnosis to distinguish 2B-VWD from PT-VWD is mandatory. Both disorders share similar autosomal dominant inheritance, and are characterized by abnormally high-binding affinity between the GPIbα and the VWF, leading to a platelet-VWF hyperresponsiveness, evidenced by the ristocetin-induced platelet aggregation (RIPA) test. Patients show a bleeding phenotype due to circulating platelet aggregates which are removed from circulation and result in thrombocytopenia together with the absence of high-molecular-weight multimers (HMWM) of VWF in plasma, but normal multimeric structure in platelets.[10] However, type 2B-VWD is characterized by gain-of-function mutations of the GPIbα-binding site in the VWF protein, leading to accelerated clearance of the platelet/VWF complex. In these cases, the mutant VWF binds spontaneously to platelets.[11] PT-VWD was first described in 1982 and was also referred as pseudo VWD.[10,12] Before its formal description, an earlier group reported an enhanced RIPA due to a platelet receptor abnormality, therefore, showing the first possibility of this new disorder.[13] PT-VWD has also been described as a "VWD-mimic" disorder.[14] However, the term "platelet-type" VWD is more descriptive of the pathophysiology, and has already achieved universal utility. PT-VWD is caused by the presence of mutations in the *GP1BA* gene, which result in a platelet function defect. Platelet macrocytosis possibly due to shortened platelet survival, increased turnover, and prolonged bleeding time (BT) can also be seen.[15]

Patients with PT-VWD have a mild-to-moderate mucocutaneous bleeding characterized by epistaxis, bleeding after tooth extraction, surgery, and pregnancy.[16,17] An international registry-based study[18] and the international online database supported by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (ISTH-SSC) (www.pt-vwd.org) have reported close to 50 patients and five distinct mutations worldwide with PT-VWD.

GPIbα and the *GP1BA* Gene

GPIbα is the largest component of the GPIb/IX/V platelet receptor complex and carries the VWF-binding site. Its structure reveals four functional domains: the 45-kDa extracytoplasmic N-terminal domain of approximately 300 amino acids, a heavily glycosylated mucin-like stalk known as macroglycopeptide region, a single transmembrane domain, and an intracytoplasmatic domain.[19] Regarding nomenclature for mutations and polymorphisms, sequence numbering may vary among publications. This is why, so as to avoid confusion, it is recommended[18,20–22] to use systematic names to describe gene sequence variations. A new nomenclature starts with the first methionine (Met); by this way, 16 amino acids were added to previous mutation/polymorphism numbers. We have adopted the new nomenclature for numbering mutations and other amino acids of GPIba (►**Table 1**).

The His17-Glu298 stretch within the 45-kDa extracytoplasmic N-terminal domain is known to contribute to direct VWF binding. In more detail, the positively charged VWF-A1 domain binds GPIbα via contacts with the R-loop and the negatively charged LRR domain.[22] Different secondary structures of the R-loop have been described: an extended β-hairpin conformation, a compact triangular conformation, and a partially disordered conformation. In addition, the Asp251-Lys253 salt bridge is a conserved feature of GPIbα, which is present in the R-loop compact triangular conformation but absent in the extended R-loop conformation.[22]

The *GP1BA* gene was cloned in 1987.[23] It is located in chromosome 17 (17p12), spans 2.4 kb of DNA, with two exons, and the mRNA comprises near 1,882 bp. The gene codes for a leader sequence of 16 amino acids, and a mature 610 amino acid protein. There are also 42 nucleotides of 5′ noncoding sequence and 497 nucleotides of 3′ noncoding sequence, including the poly "A" tail.

There has been functional characterization of five mutations found to be responsible for the PT-VWD phenotype: Gly249Val,[24] Gly249Ser,[21,25,26] Asp251Tyr,[15] Met255Val,[27,28] and a 27 bp deletion.[29] With the exception of the deletion mutant, which is located in the macroglycopeptide region of the GP1bα, the mutations are clustered in a limited zone that comprises the VWF-binding region.

Differential Laboratory Diagnosis of PT-VWD and 2B-VWD

Patients with PT-VWD and 2B-VWD share many similar clinical symptoms. The common features of PT-VWD and 2B-VWD include severe nosebleeds, excessive bleeding following tooth extraction, tonsillectomy, and other surgical operations. Bleeding becomes more pronounced after aspirin

Table 1 Amino acid numbering of *GP1BA* mutations, polymorphisms, and other important positions

GP1BA	Amino acid number		References
	Old nomenclature	New nomenclature	
Mutations	**Trp230Leu**	**Trp246Leu**	**Current report**
	Gly233Val	Gly249Val	Miller et al, 1991[24] Othman, 2008[20]
	Gly233Ser	Gly249Ser	Matsubara et al, 2003[25] Nurden et al, 2007[26] Favaloro et al, 2007[21]
	Asp235Tyr	Asp251Tyr	Enayat et al, 2012[15]
	Met239Val	Met255Val	Russell and Roth, 1993[27] Takahashi et al, 1995[28] Othman, 2008[20]
Polymorphisms	Leu70Phe	Leu86Phe	Othman, 2008[20]
	Thr145Met	Thr161Met	Othman, 2008[20]
VNTR D allele: 1 repeat	399–412	415–428	Othman, 2008[20]
VNTR C allele: 2 repeats	399–425	415–441	Othman, 2008[20]
VNTR B allele: 3 repeats	399–438	415–454	Othman, 2008[20]
VNTR A allele: 4 repeats	399–451	415–467	Othman, 2008[20]
Other important amino acid positions	His1-Glu282	His17-Glu298	
	Asp235-Lys237	Asp251-Lys253	

ingestion or drugs that have antiplatelet activity.[30] This is why distinguishing between these conditions is clinically a diagnostic challenge. PT-VWD incidence is unknown, given that most people with PT-VWD are misdiagnosed as 2B-VWD (approximately 15% of cases).[18] However, the therapeutic management of the two disorders is different, so that discrimination is clinically essential.

The laboratory phenotype in patients with 2B-VWD and PT-VWD is also similar; thus, the "characteristic" feature of RIPA positivity at 0.5 mg/mL ristocetin or less (or in some cases, RIPA positivity at 0.7 mg/mL ristocetin)[31] is present in both conditions. In addition, in both 2B-VWD and PT-VWD, VWF ristocetin cofactor (VWF:RCo) to antigen (VWF:Ag) and/or collagen binding to VWF:Ag ratios < 0.6 are frequently reported; FVIII levels are normal or reduced, similar to those of VWF:Ag levels[32]; mild or moderate intermittent thrombocytopenia may occur, which is aggravated by conditions that increase the endogenous release of VWF such as pregnancy, stress, and infection.[30,33] Therefore, in many cases investigated, both PT-VWD and 2B-VWD may be misdiagnosed as idiopathic thrombocytopenia (ITP), and/or PT-VWD may be misdiagnosed as 2B-VWD.[18] Thus, clinical and laboratory differentiation between PT-VWD and 2B-VWD can be very difficult.[9,34,35] Discriminating methods include RIPA mixing assay,[9,21] cryoprecipitate challenge assay,[36] flow cytometry-based RIPA,[37] and DNA analysis (the gold standard) of the *VWF* gene A1 domain (exon 28, where 100% of the candidate mutations for 2B-VWD are located), together with the platelet *GP1BA* gene for PT-VWD. However, the definitive diagnosis of PT-VWD remains the identification of mutations in the VWF-binding domain of the *GP1BA* gene.

We report here the identification of a new missense mutation, p.W246L, which expresses the PT-VWD phenotype in the affected patient, including positive (platelet basis) RIPA mixing assay and cryoprecipitate challenge assay. We have adopted the new nomenclature for numbering this new mutation.

Materials and Methods

Assessment of the Bleeding Phenotype

All patients involved in this study were evaluated in accordance with the Helsinki Declaration after obtaining their written informed consent, with collected information remaining confidential. The severity of each symptom was summarized using the bleeding score (BS) system ranging from 0 to 3, according to the ISTH recommendations.[38] BS is considered useful for the identification of a significant bleeding history (≥ 5 in females and ≥ 3 in males) in the diagnosis of all VWD types.[39] Menorrhagia was defined when the Pictorial Bleeding Assessment Chart score was ≥ 185.[40]

Sample Preparation

Peripheral venous blood was collected in polypropylene tubes with 109 mM sodium citrate (9:1) by clean antecubital venipuncture for clotting tests. For DNA analysis, blood was collected in polypropylene tubes with 2% ethylenediaminetetraacetic acid (EDTA). For aggregometric studies, venous blood was collected in polypropylene tubes with 129 mM sodium citrate (9:1).

Laboratory Analysis

The following coagulation tests were performed: platelet count, BT using Ivy's method,[41] activated partial thromboplastin time

(aPTT), FVIII using the one-stage clotting assay.[42] VWF:RCo was assayed by aggregometry, using fixed washed platelets (WP).[43] VWF:Ag[44] was assayed by enzyme-linked immunosorbent assay (ELISA) using polyclonal rabbit antihuman VWF (Code A 0082, Dako Denmark A/S), polyclonal rabbit antihuman VWF/HRP (Code P 0226, Dako Denmark A/S), and microwells CovaLink NH, F8 (Nunc, Roskilde, Denmark); the VWF propeptide (VWFpp)[45] were measured by ELISA using anti-VWFpp (Clone CLB-Pro 35, catalogue number M193902) and anti-VWFpp/HRP (Clone CLB-Pro 14.3, catalogue number M193904) (White label-Sanquin, The Netherlands), and microwells F8 Maxisorp Losse (catalogue number 469949 Nunc, Denmark). Mixing studies of the patient plasma with a normal control plasma to evaluate the presence of inhibitors were undertaken by aPTT and VWF:RCo. Calibration curves for FVIII, VWF:Ag, VWF:RCo, and VWFpp were made from a local normal plasma pool from 20 healthy donors used as a secondary standard calibrated against standard 07/316 of the National Institute of Biological Standards and Control.

VWF Multimeric Analysis

VWF multimeric analysis was performed by Sodium lauryl sulfate (SDS) 1 and 1.7% agarose gel electrophoresis and immunoenzymatic stain visualization of VWF multimers, as described,[46] with the following antibodies: anti-VWF (Code A0082, Dako Denmark A/S), antihuman immunoglobulin G (Code E0353, Dako Denmark A/S), and ABPComplex (Code K377 A y B, Dako Denmark A/S). Gels were subsequently scanned and image analysis was performed (ImageQuant TL software; Amersham Biosciences, Piscataway, NJ). The multimer pattern of the plasma samples studied was always compared with the normal plasma pattern on the same gel.

Platelet-Rich Plasma and Washed Platelets

To obtain platelet-rich plasma (PRP), blood was centrifuged at $300 \times g$ for 10 minutes, and the platelet-rich supernatant was collected and pooled into another polypropylene tube. The remaining plasma was recentrifuged at $1{,}300 \times g$ for 15 minutes to obtain platelet-poor plasma (PPP).

In case of samples with very large platelets, PRP should be prepared by blood sedimentation.[47] Aggregometric studies in PRP with low platelet count ($< 150 \times 10^9$/L) is possible, but the results should be treated with caution; a normal control should be analyzed, where the PRP count is adjusted to equal that of the test by dilution.[48] The platelet count of PRP was adjusted to 200×10^9/L using autologous PPP. A suspension of platelets was obtained from PRP by washing it twice in modified Tyrode buffer (138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.4mM NaH_2PO_4, 0.1% glucose, 0.35% bovine serum albumin, pH 6.5) with 50 ng/mL prostaglandin E_1. The final resuspension was performed in modified Tyrode buffer, pH 7.3 with 1 mM $CaCl_2$. All sample preparation was completed within 4 hours of collection.[49]

Platelet Aggregation

Studies of platelet aggregation were performed as usual[50] using an optical aggregometer (Chronolog, Havertown, PA). Spontaneous platelet aggregation was assayed in the absence of agonists during a 15-minute process of stirring at 37°C. RIPA analysis was performed by mixing 400 µL of PRP (200×10^9 platelets/L) with different ristocetin concentrations, looking for the minimal concentration that would promote aggregation of patient's PRP.

Methods to Differentiate between PT-VWD and 2B-VWD

RIPA mixing assay was performed as previously described.[9] Briefly, patient or control PPP was added to patient or control WP in the following combinations: patient WP/patient PPP, patient WP/control PPP, control WP/control PPP, and control WP/patient PPP. Platelet count of WP was 400×10^9/L, so the final concentration in the mix WP/PPP was 200×10^9/L. To perform RIPA mixing assay, 0.5 mg/mL ristocetin was used.

The cryoprecipitate challenge assay was performed as described.[9] In brief, cryoprecipitate diluted to 70 U/dL (fc) VWF:RCo activity was added to patient's PRP and normal PRP (200×10^9 platelets/L), and the percentage of aggregation was recorded.

Genotypic Analysis

Genomic DNA was extracted from peripheral blood collected in EDTA using a commercial kit (Wizard Genomic DNA Purification Kit, Promega Corp, Madison, WI). The complete exon 28 of the *VWF* gene and the *GP1BA* gene were amplified by polymerase chain reaction (PCR) and sequenced. We designed the primers for the PCR of exon 28 of *VWF* gene as previously described.[51] The primers of *GP1BA* gene were assayed as previously described.[29] Amplification was performed in a Perkin-Elmer Thermocycler 2400 (Norwalk, CT). PCR products were purified with the use of GFX (Piscataway, NJ) for DNA and Gel Band Purification kit (Amersham Pharmacia Biotech, Piscataway, NJ). Both the forward and reverse strands were directly sequenced by automated sequencing technology using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) with corresponding primers, and run in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). DNA samples of 100 normal individuals were also amplified for *GP1BA* gene, sequenced, and analyzed.

In Silico Analysis of the Functional Effect of Missense Changes and Sequence Alignment

Software for prediction of functional effect of protein changes is increasingly being identified as a useful tool.[52–54] The in silico analysis of the predicted missense changes was performed with the informatics applications: scale-invariant feature transform (SIFT) (http://sift.bii.a-star.edu.sg/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) to search for possible impact of the amino acid substitution on the structure and function of a human protein.

To determine whether the position W246 was conserved or not along the phylogenetic tree, the sequence alignment of the protein was performed using the informatics application UniProt KB. Fourteen mammalian species were compared.

Results

Clinical Profile of the Patient and His Relatives

The index case was a 23-year-old male at the moment of his first consultation in our institution due to severe bleeding history. He presented a history of severe epistaxis requiring hospitalization, gum bleeding, major bleeding after dental extraction requiring replacement therapy, despite receiving prophylaxis with antifibrinolytics and cryoprecipitates. At the age of 17 years, he suffered from a posttraumatic muscle hematoma requiring replacement therapy. His BS was 11 at the time of his most recent presentation (29 years). His 48-year-old mother presented mild mucocutaneous symptoms; she had two cesarean sections and one minor, and one major surgery without bleeding. Her BS was 2. His 50-year-old father did not have any bleeding symptoms, but living outside the country he was not available for laboratory testing. All the members of the family had a white origin.

Patient Phenotype and Laboratory Features

A summary of laboratory data of the patient and his mother is provided in ►**Table 2**. The patient always had VWF levels consistent with an initial diagnosis of 2B-VWD/PT-VWD, including VWF:RCo/VWF:Ag ratio < 0.2, together with loss of HMWM, low platelet count, 50% of macroplatelets, and 10% of spontaneous platelet aggregation. No inhibitory activity was seen in aPTT and VWF:RCo. The VWFpp/VWF:Ag ratio (VWFpp ratio) was normal in both the patient and his mother. HMWM are absent from the patient plasma (►**Fig. 1a**); ►**Fig. 1b** shows an exemplary multimer densitogram in type 2B-VWD or PT-VWD, the relative peak areas of the small multimers are increased, while those of the intermediate and large multimers are reduced.

The patient PRP showed RIPA positively at low concentrations of ristocetin (0.3 and 0.4 mg/mL in his two different studies) (►**Fig. 2**). The RIPA mixing assay was positive for PT-VWD and negative for 2B-VWD (►**Fig. 3**). In the cryoprecipitate challenge assay, the patient's PRP showed an enhanced aggregation in the presence of cryoprecipitate, which helped to confirm the diagnosis of PT-VWD (►**Fig. 4**). His mother's PRP aggregated at a threshold of 1.2 mg/mL ristocetin. Her additional studies did not reveal any abnormality in her phenotypic profile and the discriminating tests (RIPA mixing assay and cryoprecipitate challenge assay) were negative for both PT-VWD and 2B-VWD.

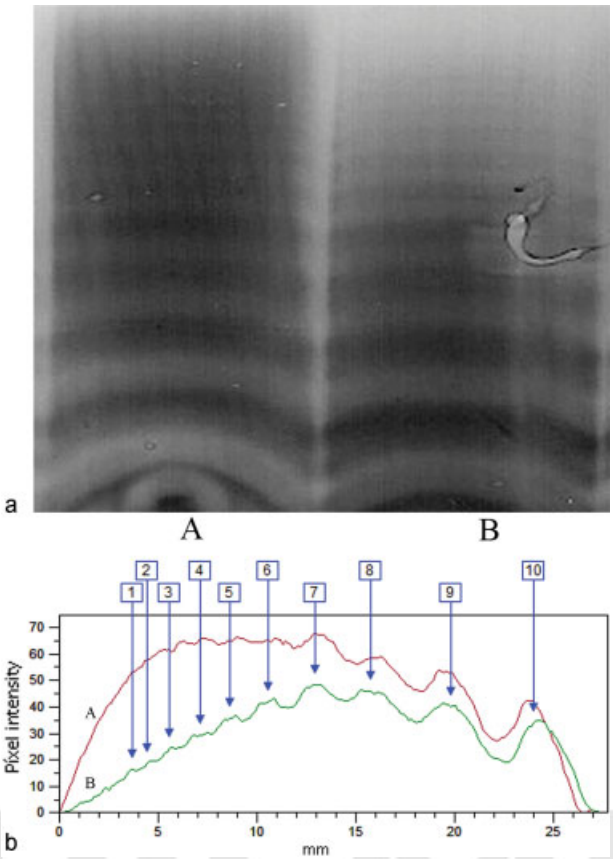

Fig. 1 (a) Plasma VWF multimer profiles. Plasma VWF multimers for normal plasma (lane A) and patient plasma (lane B). Plasma samples were electrophoresed through a 1.7% SDS-agarose gel and multimers were visualized by immunoenzymatic stain. (b) Densitometry of VWF multimers. Lane A: normal plasma; lane B: patient plasma. Densitometry was performed on the same 1.7% SDS-agarose gel as in (a). Peaks above peak 1 represent HMWM; peaks 1 through 5 represent the intermediate multimers (IMWM), and peaks 6 through 10 represent the low multimers (LMWM). For the normal plasma, the total relative peak areas are 374,339 (100%), with 179,222 (48%) LMWM, 135,260 (36%) IMWM, and 71,432 (19%) HMWM. For the patient plasma, the total is 213,803 (100%) with 146,537 (68%) LMWM, 57,039 (26%) IMWM, and 12,948 (6%) HMWM. Densitometry of VWF multimers for a patient previously diagnosed as 2B-VWD is: total 322,729 (100%) with 253,343 (78%) LMWM; 53,710 (17%) IMWM and 15,676 (5%) HMWM (data not shown). HMWM, high-molecular-weight multimers; IMWW, intermediate-molecular-weight multimers; LMWM, low-molecular-weight multimers.

Table 2 Laboratory data of the patient and his mother

	BT, min	PLT, $\times 10^9$/L	FVIII, IU/dL	VWF:Ag, IU/dL	VWF:RCo IU/dL	VWF:RCo/ VWF:Ag	VWFpp ratio	VWF HMWM	RIPA (+) lowest Rist (mg/mL)
Patient 1° study	> 9	75	26	47	< 10	< 0.2	Not done	Absent	0.4
2° study	–	41	46	61	< 10	< 0.2	1.65	Absent	0.3
Mother	2	165	150	140	101	0.72	1.17	Present	0.8

Abbreviations: BT, bleeding time; FVIII, factor VIII; HMWM, high-molecular-weight multimers; PLT, platelet count; Rist, ristocetin; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:RCo, ristocetin cofactor activity; VWFpp, propeptide.

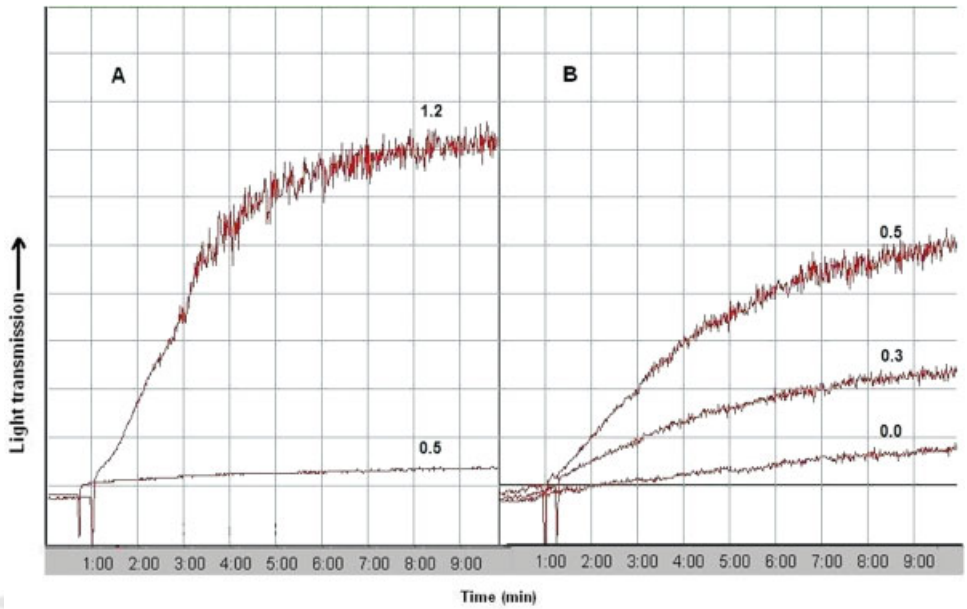

Fig. 2 Results of ristocetin-induced platelet aggregation (RIPA) for a sample of control and patient. Panels show RIPA performed on the following: (A) control platelet-rich plasma (PRP) sample; (B) patient PRP sample. Values within each panel represent final concentrations of ristocetin in mg/mL. The value "0.0" represents the spontaneous platelet aggregation in the patient (i.e., no added ristocetin).

Genetic Studies

DNA sequencing analysis for both the *GP1BA* and exon 28 of *VWF* genes were performed on genomic DNA from the proband and his mother. We identified a novel single substitution (G > T) located at nucleotide 3,805 in the leucine-rich repeat C-terminal domain of the *GP1BA* gene, in a heterozygous form in the proband, but neither in the unaffected mother nor in the 100 control subjects. This transition results in a Trp to Leu amino acid change at residue 246 (p.W246L) (GeneBank accession number AB086948; gene ID: 2811). Forward and reverse strands are shown in ►**Fig. 5**. No mutations were identified in the exon 28 of the *VWF* gene in the patient and his mother.

In Silico Analysis

PolyPhen-2 predicted this mutation as probably damaging with a score of 0.998 (sensitivity: 0.27; specificity: 0.99). SIFT predicted the substitution to affect the protein function with a score of 0.05, with median sequence conservation: 2.76.

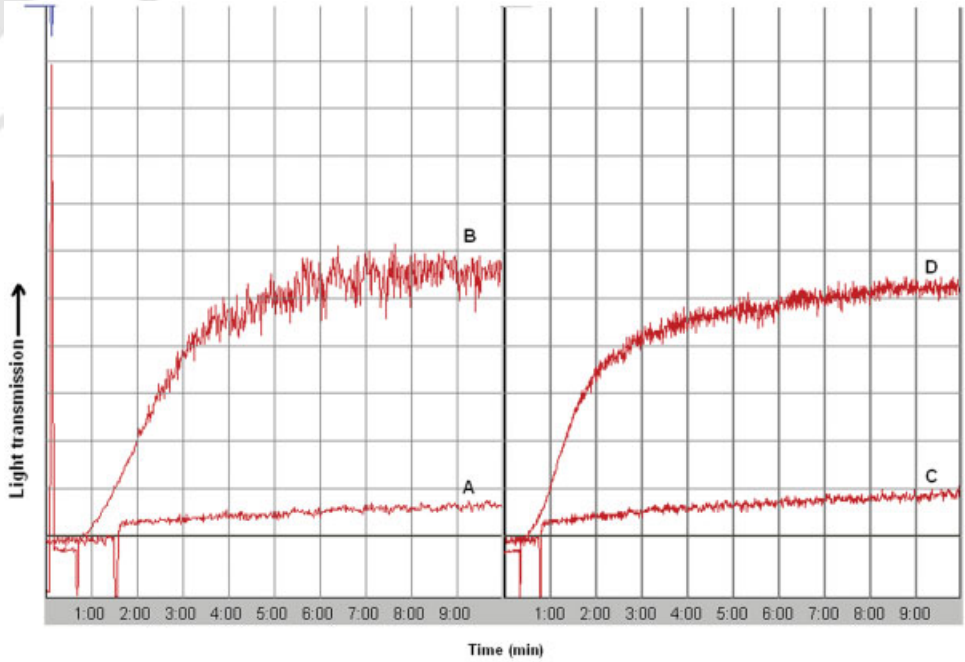

Fig. 3 Results of ristocetin-induced platelet aggregation (RIPA) mixing assay. The final concentration of ristocetin was 0.5 mg/mL. Panels show: (A) control washed platelet (WP)/control platelet-poor plasma (PPP) as negative control; (B) patient WP/control PPP; (C): control WP/patient PPP; (D) patient WP/patient PPP as positive control.

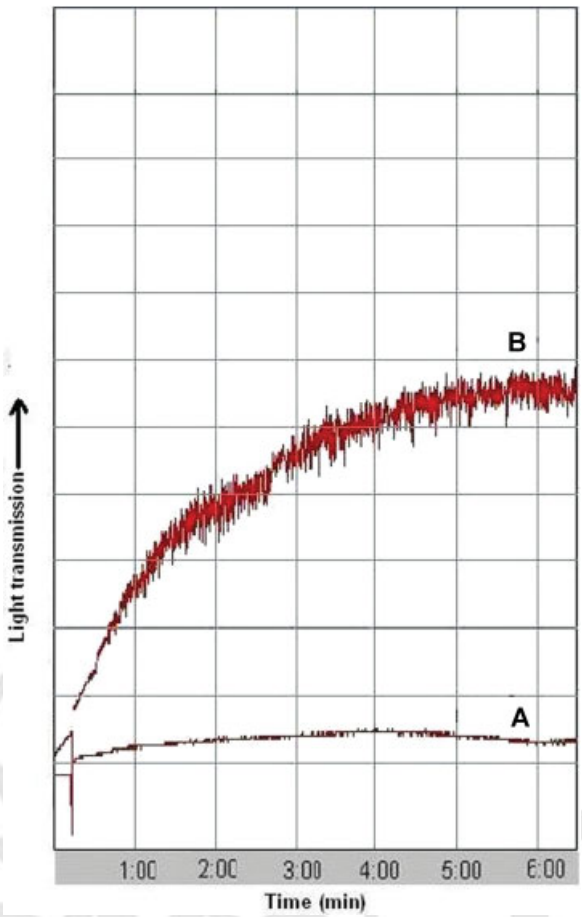

Fig. 4 Results of cryoprecipitate challenge assay. The cryoprecipitate was diluted to 70 IU/dL (fc) of VWF:RCo activity. Panels show: (A) control PRP; (B) patient PRP.

Sequence Alignment

According to sequence alignment, the residue W246 is conserved in the 14 mammalian species compared (Chimpanzee; Crab-eating macaque; Lowland gorilla; Northern white-cheeked gibbon; Sumatran orangutan; Rhesus macaque; White-tufted-ear marmoset; Rattus norvegicus; *Mus musculus*; *Sus scrofa*; 13-lined ground squirrel; David's *Myotis*; Giant panda; Naked mole rat).

Discussion

We report here the identification of a novel missense mutation in the heterozygosity state in *GP1BA* gene, the p.W246L, which expressed the PT-VWD phenotype in a patient with very low VWF:RCo/VWF:Ag, normal VWFpp ratio, macrothrombocytopenia, mild spontaneous platelet aggregation, absence of HMWM, positive RIPA at low concentrations of ristocetin, and RIPA mixing assay, and cryoprecipitate challenge assay confirming a PT-VWD diagnosis. This substitution was absent in the 100 normal individuals (200 alleles) from the same region and was predicted as damaging by in silico analysis.

The residue W246 is located in a strongly conserved position in the phylogenetic tree, which is expected to be unable to tolerate substitutions without changing its functional characteristics. These findings argue strongly in favor of the view that this substitution does not represent a polymorphism, and is responsible for the PT-VWD phenotype of the patient.

PT-VWD and 2B-VWD patients have similar phenotypes, that is, increased RIPA at low concentrations given the hypersensitivity of the VWF/GPIbα interaction, intermittent thrombocytopenia, and absence of HMWM forms of VWF in plasma. Clinical manifestations are also similar, characterized by mucocutaneous bleeding or excessive bleeding after surgeries or invasive procedures. The distinction between PT-VWD and 2B-VWD is important not only for the treatment of bleeding manifestations, which need VWF/FVIII concentrates in type 2B-VWD and administration of platelet concentrates in PT-VWD, but also in particular situations such as pregnancy and delivery, in which it becomes critical because the VWF:Ag levels increase and may lead to severe thrombocytopenia.[16] Others recommend considering PT-VWD whenever investigating pregnant woman with thrombocytopenia.[17]

The cutoff values of ristocetin concentration in RIPA can contribute to false-negative or false-positive results.[55] On the basis of our experience, it is necessary to perform the assay with decreasing ristocetin concentrations, until reaching the lowest concentration that causes aggregation. This may vary between patients, especially according to the VWF:RCo levels.[56]

The VWFpp ratio provides information about VWF survival. If VWF survival is not impaired, this ratio is close to 1. Alternatively, when the VWF survival is reduced, the ratio is higher. It has been demonstrated that the mean VWFpp ratio is higher than normal in 2B-VWD.[57] No information has been available on VWFpp in PT-VWD patients so far. The normal value of VWFpp ratio in our patient was consistent with normal survival of VWF, which seems therefore unaffected in this case of PT-VWD. It has been described that RIPA mixing assay has superiority over cryoprecipitate challenge assay and standard RIPA because it provides simultaneous answers regarding the PT-VWD and 2B-VWD.[21] In our experience, the RIPA mixing assay and the cryoprecipitate challenge assay were very useful as discriminating methods to prevent misdiagnosis between PT-VWD and 2B-VWD in our patient.

Regarding the in silico prediction tools, the goal of prediction programs is to identify how severe or not a nucleotide change is and how this change affects or not the phenotype. Analyzing this before mutagenesis studies could reduce the number of functional assays required and yield a higher proportion of affected phenotypes.[58] We believe that this would be a very helpful tool for characterizing new nucleotide missense substitutions, when expression studies are difficult to perform.

In summary, we describe here a novel missense mutation, p.W246L in the *GP1BA* gene responsible of PT-VWD, in a patient with moderate-to-severe bleeding manifestations, whereas the cases reported in the literature are generally of mild-to-moderate bleeding tendency.[28,29] The absence of this mutation in the normal population is tightly linked to the phenotypic expression of disease, and the absence of any additional abnormality of GPIbα provides strong support for the Trp to Leu mutation underlying the functional abnormality of the VWF receptor in PT-VWD in this patient. All reported PT-VWD missense mutations were shown to be located within the R-loop of GPIbα. It has also been described

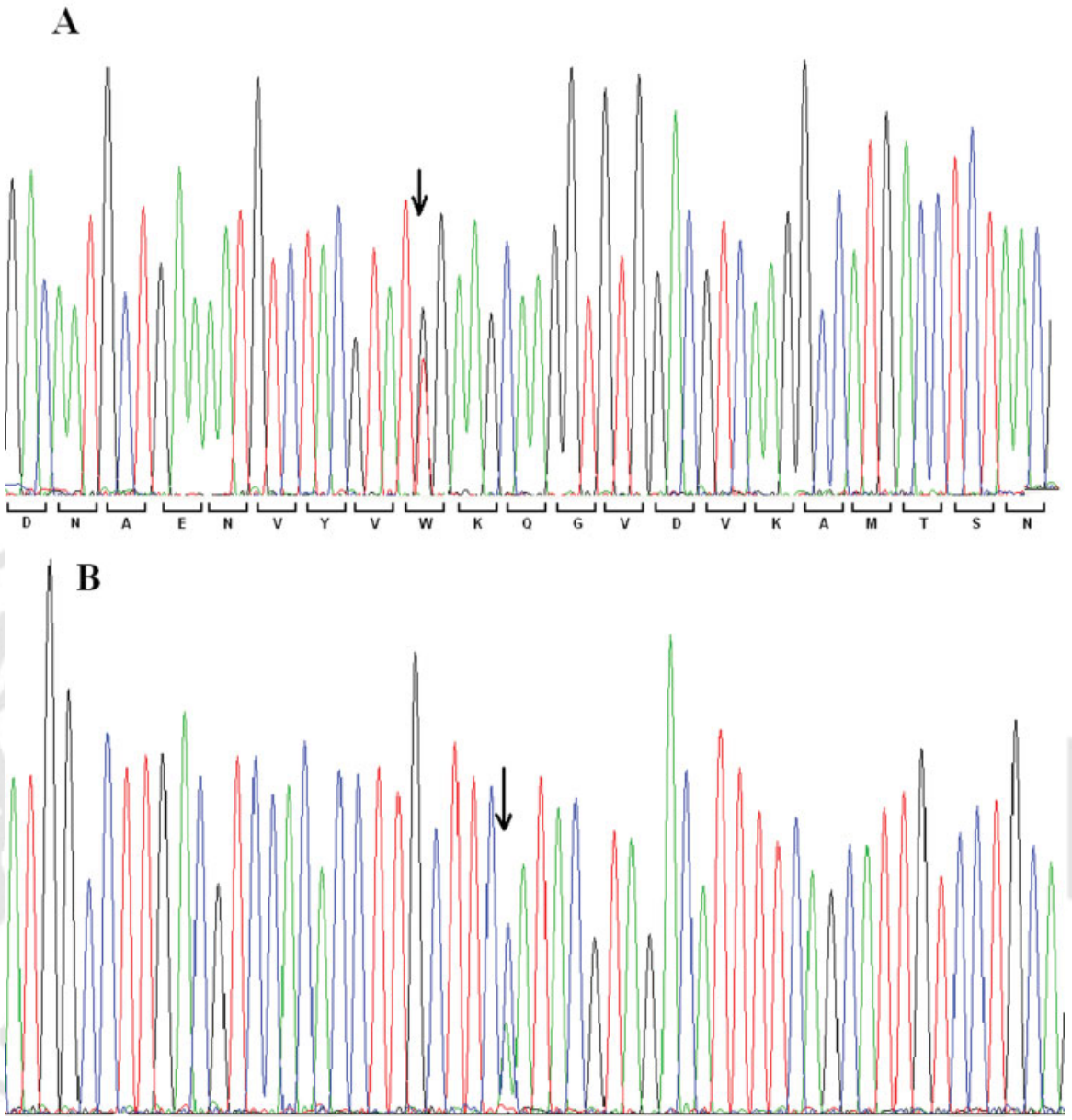

Fig. 5 Sequence chromatogram showing fragments of DNA strand of *GP1BA* with the new mutation identified. (A) forward strand; (B) reverse strand.

that the W246 forms hydrophobic contacts with M255 in the R-loop.[22] This new missense mutation, p.W246L, may also destabilize the compact triangular form of the R-loop by affecting the interaction with VWF-A1 domain. In addition, this is the first report of VWFpp ratio in PT-VWD.

In this study, the differential diagnosis of PT-VWD from 2B-VWD was achieved using a wide range of approaches. The use of a more limited assessment process will lead to incomplete characterization of PT-VWD, and thus false identification of PT-VWD as either 2B-VWD or ITP.[18] Expression studies are now planned to more fully investigate the identified mutation.

Acknowledgments
This work was supported by CONICET, SECYT, Fundación Rene Barón, and Academia Nacional de Medicina (Buenos Aires), Argentina.

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