

Unraveling the effect of silent, intronic and missense mutations on VWF splicing: contribution of next generation sequencing in the study of mRNA



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ABSTRACT

Large studies in von Willebrand disease patients, including Spanish and Portuguese registries, led to the identification of >250 different mutations. It is a challenge to determine the pathogenic effect of potential splice site mutations on VWF mRNA. This study aimed to elucidate the true effects of 18 mutations on VWF mRNA processing, investigate the contribution of next-generation sequencing to *in vivo* mRNA study in von Willebrand disease, and compare the findings with *in silico* prediction. RNA extracted from patient platelets and leukocytes was amplified by RT-PCR and sequenced using Sanger and next generation sequencing techniques. Eight mutations affected VWF splicing: c.1533+1G>A, c.5664+2T>C and c.546G>A (p.=) prompted exon skipping; c.3223-7_3236dup and c.7082-2A>G resulted in activation of cryptic sites; c.3379+1G>A and c.7437G>A demonstrated both molecular pathogenic mechanisms simultaneously; and the p.Cys370Tyr missense mutation generated two aberrant transcripts. Of note, the complete

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effect of three mutations was provided by next generation sequencing alone because of low expression of the aberrant transcripts. In the remaining 10 mutations, no effect was elucidated in the experiments. However, the differential findings obtained in platelets and leukocytes provided substantial evidence that four of these would have an effect on VWF levels. In this first report using next generation sequencing technology to unravel the effects of *VWF* mutations on splicing, the technique yielded valuable information. Our data bring to light the importance of studying the effect of synonymous and missense mutations on *VWF* splicing to improve the current knowledge of the molecular mechanisms behind von Willebrand disease. *clinicaltrials.gov* identifier:02869074.

Introduction

Von Willebrand disease (VWD), the most common congenital bleeding disorder, is caused by a genetic defect in the von Willebrand factor gene (*VWF*).¹ *VWF* mutational analysis can be valuable for diagnosing and investigating the molecular etiology of VWD, as was seen in the Spanish (PCM-EVW-ES project) and Portuguese cohort of VWD patients.^{2,4} One interesting challenge in this condition is to elucidate the pathogenic mechanism of *VWF* mutations. *In silico* analysis is considered a suitable supporting tool to predict the pathogenicity of the variants identified.^{5,6} However, functional studies remain essential to unequivocally determine their deleterious effect.⁷

Functional studies can be performed by analyzing the potential effect of splice site mutations (PSSM) in RNA. In addition to splice site consensus sequence mutations, deep intronic, missense, and synonymous mutations can also disturb splicing. Along this line, 25% of synonymous mutations positioned at exon-intron boundaries result in altered splicing, which, in itself, can cause disease, modify the severity of the disease phenotype, or be linked with disease susceptibility.⁸ In *VWF*, heterozygotes for PSSM may be associated with mild forms of VWD type 1 or be phenotypically silent, but when two such mutations are found in different alleles, the phenotype is associated with VWD type 3.⁹

Almost all related studies of splicing effects have examined peripheral blood platelets, as *VWF* is exclusively expressed in these cells and endothelial cells.^{10,11} Platelets are anucleated, but they contain small amounts of translationally active megakaryocytic mRNA.^{12,13} In contrast, the amount of mRNA obtained from leukocytes is higher and contains mRNA transcripts for genes that are not normally expressed in these cells, known as “ectopic transcripts”. Their analysis has been used to investigate mutations in several inherited disorders,^{14,15} as they facilitate the study of mRNA of those genes expressed in hard to reach tissues.¹⁶ PSSM can affect mRNA reorganization and introduce premature termination codons (PTCs) into open reading frames, a common cause of genetic disorders. Most nonsense transcripts are recognized and degraded by nonsense-mediated mRNA decay (NMD),¹⁷ a degradation pathway to control synthesis of truncated proteins.¹⁸ The efficiency of NMD varies between cell types; hence, the use of RNA from platelets and leukocytes for *in vivo* study of *VWF* PSSM offers complementary results, particularly when NMD occurs in the allele carrying the mutation in platelets, as we reported.⁷

Since its development, next-generation sequencing (NGS) has been increasingly used in molecular genetics to identify mutations causing disease. However, few groups

have explored its potential for analyzing splicing variants following RT-PCR.^{19,20} In this new scenario, the procedure we previously described to analyze the effects of PSSM in *VWF*⁷ has been optimized and adapted to an NGS-based technique to investigate its value in this field. Our main objective was to elucidate the true effects of 18 selected mutations (intronic, synonymous, delins, and missense) on mRNA processing and their genotype/phenotype correspondence by analysis of leukocytes and platelets from clinical samples. Finally, the *in vivo* effects of the mutations were compared with the *in silico* predictions.

Methods

Patients

We studied 15 patients diagnosed with different types of VWD, 5 from Complejo Hospitalario Universitario A Coruña, 8 from Hospital Universitari Vall d'Hebron (HUVH), and 2 from Centro Hospitalar e Universitário de Coimbra. Samples from 4 healthy individuals were used as controls. The study was performed according to the guidelines of the Declaration of Helsinki and was approved by the local Research Ethics Committee. All participants provided written informed consent.

Splice site prediction software

The predicted impact of potential splice site mutations was analyzed with NetGene2²¹ and the splicing prediction module of Alamut Visual v.2.6.1 software (Interactive Biosoftware, Rouen, France), which integrates data from three methods: Splice Site Prediction by Neural Network (NNSplice), MaxEntScan, and Human Splicing Finder (HSF).

Platelet and leukocyte separation and RNA isolation

Leukocyte and platelet RNA from patients and controls was isolated from 10 mL of peripheral blood collected in EDTA tubes, as previously described.⁷

VWF mRNA amplification

After RNA isolation, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. The region including the mutation was amplified by Platinum Taq DNA Polymerase (Thermo Fisher Scientific) in leukocyte and platelet cDNA (*Online Supplementary Methods and Table S1*). PCR products were separated on 1% agarose gel and visualized by SYBR Safe DNA Gel Stain (Thermo Fisher Scientific).

Sanger sequencing and analysis

PCR products were sequenced as previously described.²² However, multiple-band PCR products were previously agarose-

Table 1. Laboratory and molecular data of VWD patients

| Patients code | VWD | VWF:Ag | VWF:RCo | FVIII:C | NT change | AA change | Exon | Intron | Domain |
|---------------------|-----------|--------|---------|---------|---|---|----------------|---------------|----------------------------|
| UMP01 | 3 carrier | 70 | 70.5 | 111 | c.1533+1G>A | – | – | 13 | intronic |
| UMP02 | 1H | 36 | 49 | 101 | c.3379+1G>A * c.-2627C>T * | – | – | 25 | intronic upstream |
| UMP03 | 1 | 26 | 21.2 | 64.8 | c.5664+2T>C * c.7220T>C * | – p.Leu2407Pro | – 42 | 33 | intronic C2 |
| UMP04 | 1 | 50 | 52 | 47 | c.7081+6G>T | – | – | 41 | intronic |
| UMP05 | 1 | 27 | 32 | 72 | c.7730-56C>T | – | – | 45 | intronic |
| UMP06 | 2A/2M | 12 | 4 | 24 | c.4120C>T * c.7730-4C>G * | p.Arg1374Cys – | 28 – | – 45 | A1 intronic |
| UMP07 | 1 | 37 | 28 | 60 | c.8254-5T>G | – | – | 51 | intronic |
| UMP08 | 1 | 42.4 | 41.7 | 73.4 | c.546G>A[†] c.4866C>T[†] | p.Ser182 p.Asp1622 | 6 28 | – | D1 A1-A3 |
| UMP09 [‡] | 1 | 51 | 49.8 | 71 | c.3291C>T | p.Cys1097 | 25 | – | D3 |
| UMP10 | 3 carrier | 46.4 | 45 | 105 | c.1109G>A | p.Cys370Tyr | 9 | – | D1 |
| UMP11 | 1 | 31 | 24 | 57 | c.3223-7_3236dup | p.Pro1079_Tyr1080ins LeuGlnValAspProGluPro | 25 | – | D3 |
| UMP12 | 3 | 3.7 | 5 | 6.7 | c.449T>C[†] c.7082-2A>G[†] | p.Leu150Pro – | 5 – | – 41 | D1 intronic |
| UMP13 | 2A | 117 | 6.5 | 90 | c.3426T>C[‡] c.3485_3486delinsTG[‡] c.8318G>C [†] | p.Cys1142 p.Prol162Leu p.Cys2773Ser | 26 26 52 | – | D3 D3 CK |
| UMP14 | 1 | 6 | 6 | 4.5 | c.6699_6702dup[†] c.7437G>A[†] | p.Cys2235ArgfsTer8 p.Ser2479 | 38 43 | – | D4 C3 |
| UMP15 | 3 | 2 | <1 | 5 | c.546G>A [§] c.7082-2A>G [§] c.8155+3G>C [†] | p.Ser182 – – | 6 – – | – 41 50 | D1 intronic intronic |

All mutations were identified in heterozygous state. In bold, mutations selected to study their effect on VWF mRNA. The subtype 1H (historical) refers to patients previously diagnosed as type 1 VWD that, at the time of enrollment at the PCM-EVW-ES project, show a slight decrease or even a normal VWF plasma levels. AA: amino acid; NT: nucleotide; VWD: von Willebrand disease. *The allelic phase (cis/trans) of mutations could not be determined since no informative relatives are available. †Mutations *in trans*. ‡Patient with an additional mutation in the F8 gene. ‡Mutations *in cis*. †Patient previously studied at mRNA level by Corrales *et al.*⁷

purified using the MiniElute Gel Extraction kit (Qiagen). The sequences obtained were assembled and aligned against the consensus wild-type (WT) VWF mRNA sequence (GenBank NM_000552) using SeqScape v2.7 software (Thermo Fisher Scientific).

Next-generation sequencing and analysis

PCR amplicons obtained per patient were equimolarly mixed in a single tube in a total amount of 250 ng. Subsequently, the libraries were fragmented and the adapter and barcodes were ligated using the NGSgo protocol (GenDX, Utrecht, Netherlands) following the manufacturer's recommendations. Resulting libraries were combined and sequenced on a MiSeq platform (Illumina, San Diego, CA).

After sequencing, barcoded sequences were demultiplexed and analyzed individually. The paired sequence files (fastq format) were used as input for analysis with the CLC Genomic Workbench v.11 software (Qiagen, Aarhus, Denmark) (*Online Supplementary Methods and Figure S1-S2*).

Results

An in-depth study was performed in PCM-EVW-ES,³ the Portuguese cohort,⁴ and HUVH patients to select previous-

ly undescribed VWF mutations and mutations with an unknown or controversial pathogenic mechanism. Eighteen mutations (15 patients) were selected: 8 intronic (4 in canonical, GT and AG, splice site sequences), 5 synonymous, 2 missense, and 3 delins. The patients' phenotypic and molecular data are summarized in Table 1 and *Online Supplementary Table S2*. Total mRNA was obtained from platelets and leukocytes of all patients, with the exception of patients UMP08 and UMP14, in whom platelet RNA isolation failed due to blood lysis. All mutations were analyzed by both Sanger sequencing and NGS, and results were compared to the predictions generated by *in silico* analysis (*Online Supplementary Table S3*).

Mutations in canonical splice site sequences

The c.1533+1G>A mutation (intron 13) was identified in a type 3 VWD carrier (UMP01). The exon 11-15 region was analyzed with a specific primer pair to avoid amplification of a prevalent alternative-splicing product (skipping of exons 14 and 15) in VWF from leukocytes.⁷ Whereas only the expected PCR product was observed in platelets, leukocyte amplification resulted in 4 PCR bands. Sanger analysis of leukocytes showed 2 mRNA aberrant transcripts: 1) lacking exon 13; and 2) lacking exon 13 and 14 (*Online Supplementary Figure S3*). By means of NGS, it was

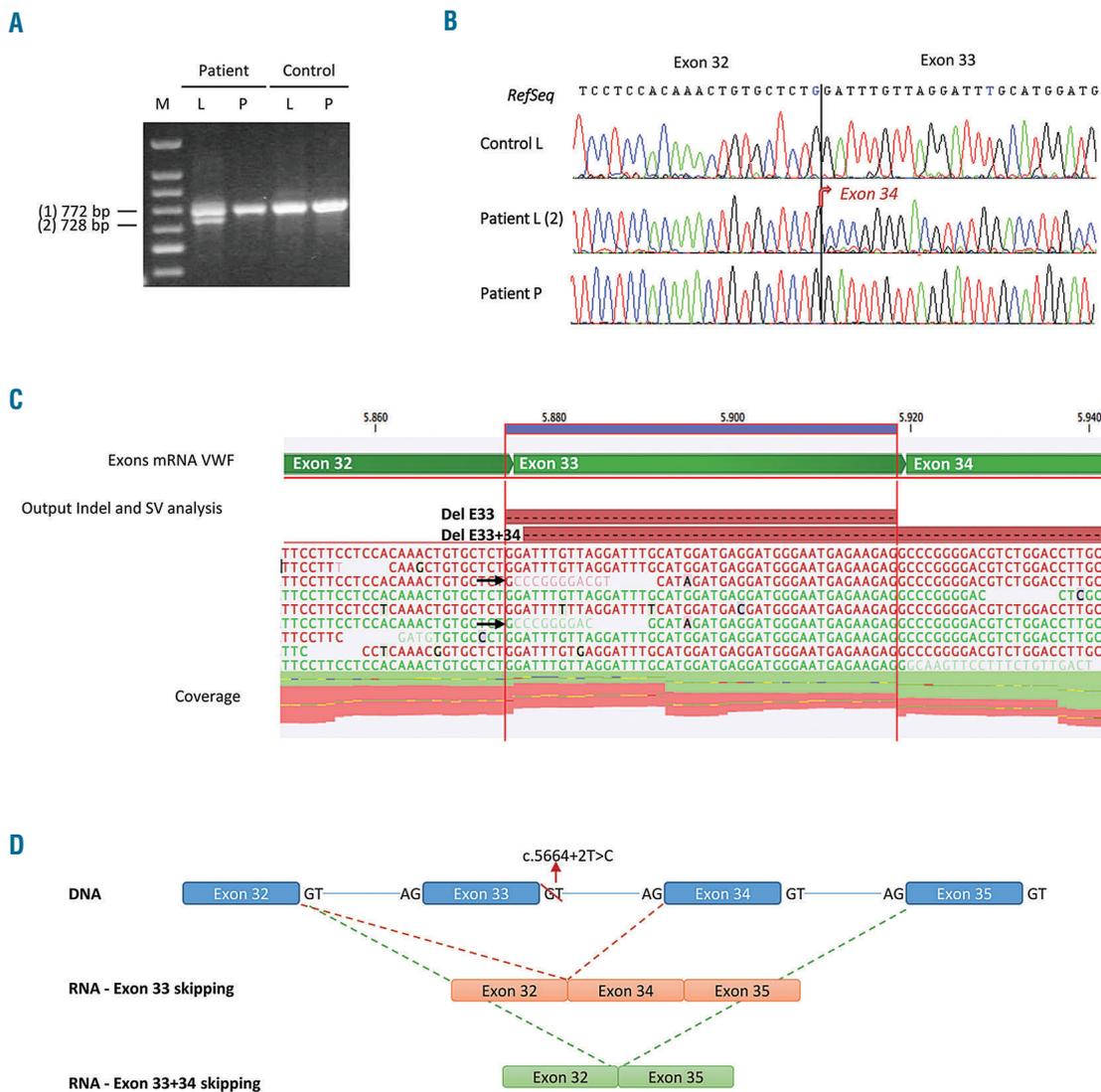


Figure 1. Analysis of the c.5664+2T>C mutation in patient UMP03. A) RT-PCR products amplified with primers located in exons 30 and 35 in leukocyte (L) and platelet (P) RNA, separated on 1% agarose gel. B) Traditional Sanger sequencing of PCR products from patient leukocytes (L2) and platelets (P), and control leukocytes (L). Analysis of the band 2 from patient leukocyte on agarose gel demonstrates exon 33 skipping. In platelets, only the allele without c.5664+2T>C mutation could be amplified. C) NGS of PCR products from leukocytes showed exon 33 skipping, indicated by arrows depict aberrant transcripts. However, exon 33-34 skipping was also detected, but in a really low of transcripts. D) Schematic representation of the mutation in genomic DNA and its effect on the VWF mRNA sequence. M indicates a 100-bp DNA ladder.

determined that 19% were transcripts without exon 13, 45% transcripts without exons 13 and 14, and an additional aberrant transcript without exon 14 was detected in 8% transcripts (*Online Supplementary Table S4*). By both techniques, no effect was visible in platelets due to NMD.

The c.3379+1G>A mutation (intron 25) was identified in a type 1H VWD patient (UMP02). We designed two new primers to analyze the exon 22-26 region. Amplification of leukocyte cDNA yielded two bands, one with the expected size and a smaller band, whereas in platelets only the expected PCR product was observed (*Online Supplementary Figure S4*). Sanger analysis of leukocytes showed that c.3379+1G>A caused exon 25 skipping, leading to a frameshift at position 1075 and adding 88 aberrant amino acids before a PTC was encountered (p.Pro1075ValfsTer88). On NGS, however, two aberrant

transcripts were detected: the major one, which lacked exon 25, was detected in 43% of all reads, and the minor one, which resulted from activation of a cryptic donor splice site (DSS) 31 nucleotides upstream from the WT-DSS, was found in 1.4% of reads (p.Pro1117ValfsTer88) (Table 2 and *Online Supplementary Table S4*). In platelets, however, the mutated allele had undergone NMD, which precluded observation of aberrant transcripts.

The c.5664+2T>C mutation was identified in a type 1 VWD patient (UMP03), *in trans* with the p.Leu2407Pro mutation. To analyze the PSSM in intron 33, the exon 30-35 region was amplified. The PCR product had the expected size in platelets, whereas an additional smaller one was observed in leukocytes (Figure 1). Sanger sequencing of the leukocyte PCR product confirmed exon 33 skipping (p.Gly1874AlafsTer32) (Table 2). NGS detected 2 aberrant

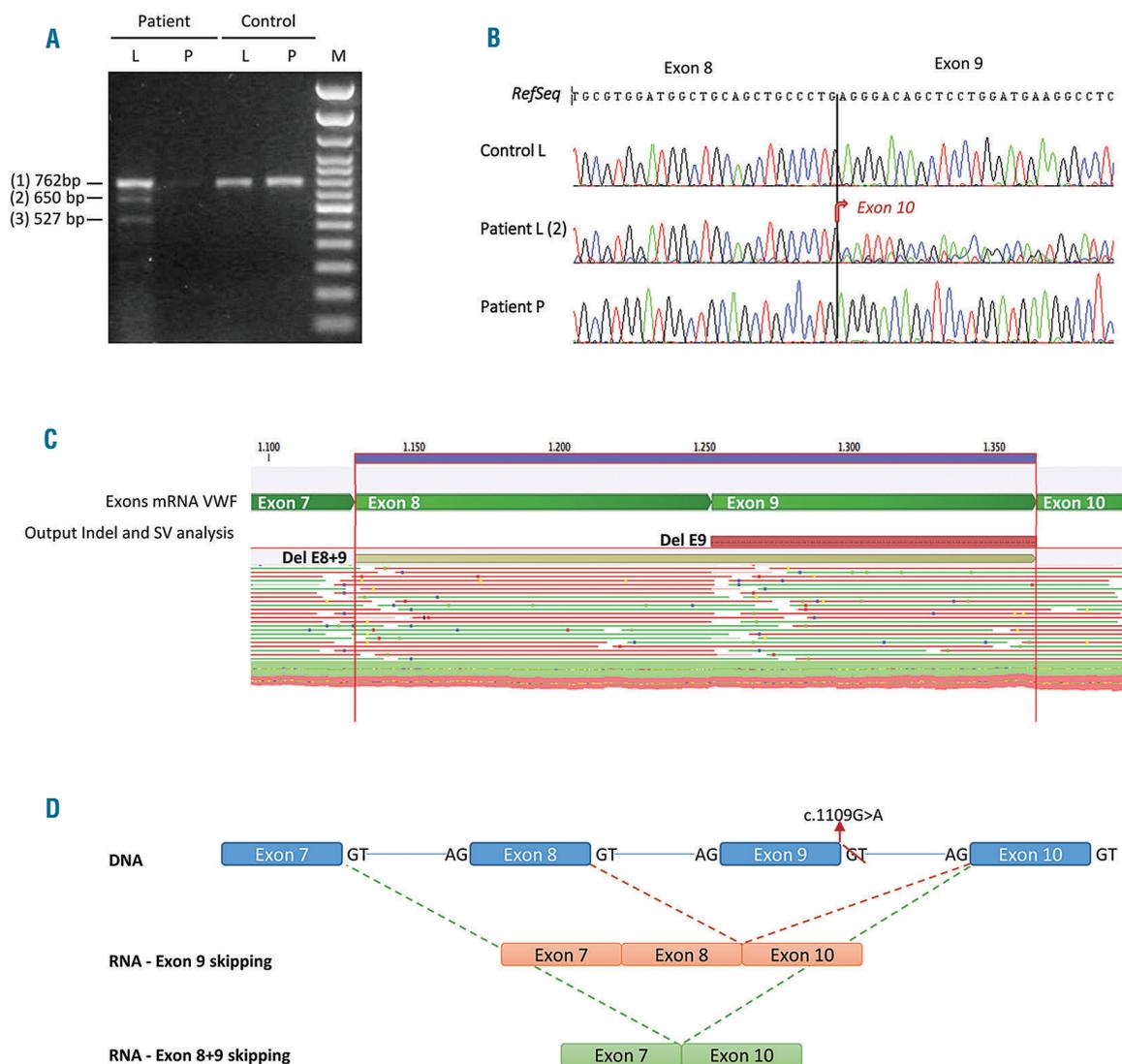


Figure 3. Analysis of the c.1109G>A (p.Cys370Tyr) mutation in patient UMP10, located in the last nucleotide of exon 9. A) RT-PCR products amplified with primers located in exons 6 and 12 in leukocyte (L) and platelet (P) RNA, separated on 1% agarose gel. B) Traditional Sanger sequencing of PCR product from patient L (2) showed exon 9 skipping. The L (3) band could not be purified in the agarose gel due to low concentration, thus it was not analyzed by Sanger. In platelets, only the non-mutated allele could be amplified. C) NGS of PCR products from leukocytes identified transcripts lacking exon 9, as well as transcripts lacking exons 8 and 9. D) Schematic representation of the mutation in genomic DNA and its effect on the VWF mRNA sequence. M indicates a 100-bp DNA ladder.

The c.8254-5T>G mutation (intron 51) was found in a type 1 VWD patient (UMP07). The exon 49-52 region was amplified, and the expected 546-bp size was observed in both cell types. Sequence analysis by the two techniques revealed no changes in VWF cDNA. Interestingly, this patient's two children, heterozygous for the c.8254-5T>G mutation, had bleeding symptoms. Hence, we performed complete sequencing of VWF cDNA from leukocytes. However, no changes were identified.

Synonymous mutations

The mutations c.546G>A (exon 6) and c.4866C>T (exon 28) were identified *in trans* in a type 1 VWD patient (UMP08). These mutations were studied only in leukocyte RNA. To investigate c.546G>A, the exon 4-7 region was amplified, which resulted in the expected band and a

slightly diffuse, smaller band. Sanger sequencing detected the c.546G>A change (Figure 2). However, NGS not only detected the nucleotide change, but also recognized a loss of 125 nucleotides corresponding to exon 6 (p.Thr179ProfsTer31) in small 2.3% of reads (Online Supplementary Table S4). The predicted impact of the c.546G>A mutation was discrepant and only two *in silico* algorithms predicted an effect on mRNA splicing (Online Supplementary Table S3) that was confirmed *in vivo* even though with a slight effect. To study c.4866C>T, the exon 25-31 region was amplified and sequenced, but no effect on mRNA processing was seen, as was predicted by *in silico* tools. Both nucleotides were identified in heterozygous state, indicating the presence of both alleles. Only offspring carrying the c.546G>A mutation (1 of 3 children) had lower levels of VWF:Ag and VWF:RCO compared to

Table 2. Effect of VWF mutations on mRNA from leukocytes and platelets.

| Mutation type | NT change | AA change | Exon | Intron | Leukocyte effect | Platelet effect | Protein prediction |
|---|---------------------|---|------|-------------------|---|---------------------------------------|---|
| Intronic | c.1533+1G>A | – | – | 13 | Exon 13 skipping (r.1433_1533del) | NMD | p.Gly478AlafsTer138 |
| | c.3379+1G>A* | – | – | 25 | Exon 25 skipping (r.3223_3379del) | NMD | p.Pro1075ValfsTer88 |
| | | | | | Activation of a cryptic site at +126 nt in exon 25 (r.3349_3379del) ‡ | NMD | p.Pro1117ValfsTer88 |
| | c.5664+2T>C | – | – | 33 | Exon 33 skipping (r.5621_5664del) | NMD | p.Gly1874AlafsTer32 |
| | | | | | Exon 33+34 skipping (r.5621_5842del) ‡ | NMD | p.Phe1875_Cys1948del |
| | c.7081+6G>T | – | – | 41 | No visible effect | No visible effect | – |
| | c.7082-2A>G † | – | – | 41 | Activation of a cryptic site +7 nt in exon 42 (r.7082_7088del) | NMD | p.Ala2361GlyfsTer40 |
| | | | | | No visible effect | No visible effect | – |
| c.7730-4C>G | – | – | 45 | No visible effect | No visible effect | – | |
| c.7730-56C>T | – | – | 45 | No visible effect | No visible effect | – | |
| c.8254-5T>G | – | – | 51 | No visible effect | No visible effect | – | |
| Synonymous | c.546G>A † | p.Ser182 | 6 | – | Exon 6 skipping (r.533_657del) ‡ | NA | p.Thr179ProfsTer31 |
| | c.3291C>T | p.Cys1097 | 25 | – | No visible effect | No visible effect | – |
| | c.3426T>C | p.Cys1142 | 26 | – | No visible effect | No visible effect | – |
| | c.4866C>T | p.Asp1622 | 28 | – | No visible effect | NA | – |
| | c.7437G>A | p.Ser2479 | 43 | – | Exon 43 skipping (r.7288_7437) | NA | p.Val2430GlyfsTer335 |
| Activation of a cryptic site at +146 nt in exon 43 (r.7434_7437del) | | | | | NA | p.Ser2479AlafsTer23 | |
| Missense | c.449T>C | p.Leu150Pro | 5 | – | No visible effect | No visible effect | – |
| | c.1109G>A | p.Cys370Tyr | 9 | – | Exon 9 skipping (r.998_1109del) | NMD | p.Glu333AlafsTer87 |
| Exon 8+9 skipping (r.875_1109del) | | | | | NMD | p.Ser292ThrfsTer87 | |
| Dellns | c.3485_3486delinsTG | p.Pro1162Leu | 26 | – | No visible effect | No visible effect | – |
| | c.3223-7_3236dup | p.Pro1079_Tyr1080insLeu GlnValAspProGluPro | 25 | – | (r.3477_3478instttgcaggtggaccccgagcc) | (r.3477_3478instttgcaggtggaccccgagcc) | p.Pro1079_Tyr1080insLeu GlnValAspProGluPro |
| | | | | | No visible effect | NMD | p.Cys2235ArgfsTer8 |

AA:amino acid; NA: not available; NMD: nonsense-mediated decay; NT:nucleotide. *Previous functional studies by Nesbitt *et al.*²³ †Previous functional studies by Corrales *et al.*⁷ ‡Leukocyte effect observed in a really low % of reads by NGS.

the others. This may be explained by the mutation and/or other factors not explored in the current study.

The c.3291C>T mutation (exon 25), was identified in patient UMP09 (female), as well as a novel mutation in *F8* (c.1346C>G, p.Ala430Gly). Of note, her brother, who was not included in this study, also had these mutations. As both siblings experienced bleeding, and the HSF prediction regarding c.3291C>T interpreted creation of an exonic splicing silencer site that could potentially alter splicing, we hypothesized that this synonymous mutation could have an effect on VWF mRNA processing. Hence, the exon 22-26 region was amplified, but no change in the VWF cDNA sequence was observed. In addition, we tested whether intron 25 was retained within mature VWF mRNA (*Online Supplementary Methods*), but no differences compared to control leukocyte cDNA were found (*data not shown*). Finally, as both siblings presented similar FVIII:C levels, we investigated X chromosome inactivation in patient UMP09. The results demonstrated skewed inactivation of the WT X chromosome in this patient (*Online Supplementary Figure S5*).

Missense mutations

The mutation c.1109G>A (p.Cys370Tyr, exon 9) was identified in a type 3 VWD carrier (UMP10). The exon 6-

12 region was investigated. Whereas the expected size was observed in platelets, amplification from leukocytes resulted in 2 additional bands of ~650 and ~500 bp (Figure 3). Sanger sequencing of leukocyte mRNA confirmed that the 650-bp band corresponded to a transcript lacking exon 9. NGS of leukocyte mRNA revealed 3 different transcripts: the WT, a transcript skipping exon 9 (p.Glu333AlafsTer87) (13% of reads), and a transcript skipping exons 8 and 9 (p.Ser292ThrfsTer87) (7% of reads)(Table 2). Sequencing of PCR products from platelets showed no changes, suggesting that the mutated allele had experienced NMD.

Duplication mutation

The c.3223-7_3236dup mutation, which generated an in-frame tandem duplication of 21 nucleotides, including 7 nucleotides from intron 24 and the first 14 from exon 25, was detected in a type 1 VWD patient (UMP11). Study of this mutation by the two techniques demonstrated a change in the native ASS of intron 24 in both cell types, which resulted in maintaining the 21-bp insert corresponding to 7 aberrant amino acids in mature VWF mRNA (p.Pro1079_Tyr1080insLeuGlnValAspProGluPro) (Table 2 and *Online Supplementary Figure S6*). Furthermore, NGS showed that the aberrant mRNA transcript was

present in ~14% of reads in both cell types (*Online Supplementary Table S4*).

Combined potential splice site mutations in individual patients

The c.7082-2A>G mutation (intron 41) was identified in a type 3 VWD patient (UMP12) combined *in trans* with the p.Leu150Pro (c.449C>T, exon 5) mutation. To study c.7082-2A>G, the exon 38-43 region was amplified in platelets and leukocytes, which yielded a band of the expected size. However, Sanger and NGS sequencing of the PCR products revealed activation of a cryptic ASS

within exon 42. This led to deletion of the 7 initial nucleotides of exon 42 (r.7082_7088del), as was predicted by the 4 algorithms, and resulted in a frameshift and a PTC (p.Ala2361GlyfsTer40) (Figure 4). Additionally, NGS showed that the aberrant transcript was present in 24% of reads. On sequencing of platelet amplicons, there were no changes, a finding highly suggestive of NMD. As type 3 VWD is characterized by absent or non-functional expression of both VWF alleles, we postulated that the p.Leu150Pro missense mutation could have an effect on VWF mRNA. Nonetheless, no sequence change was identified. These results were confirmed by analysis of 2

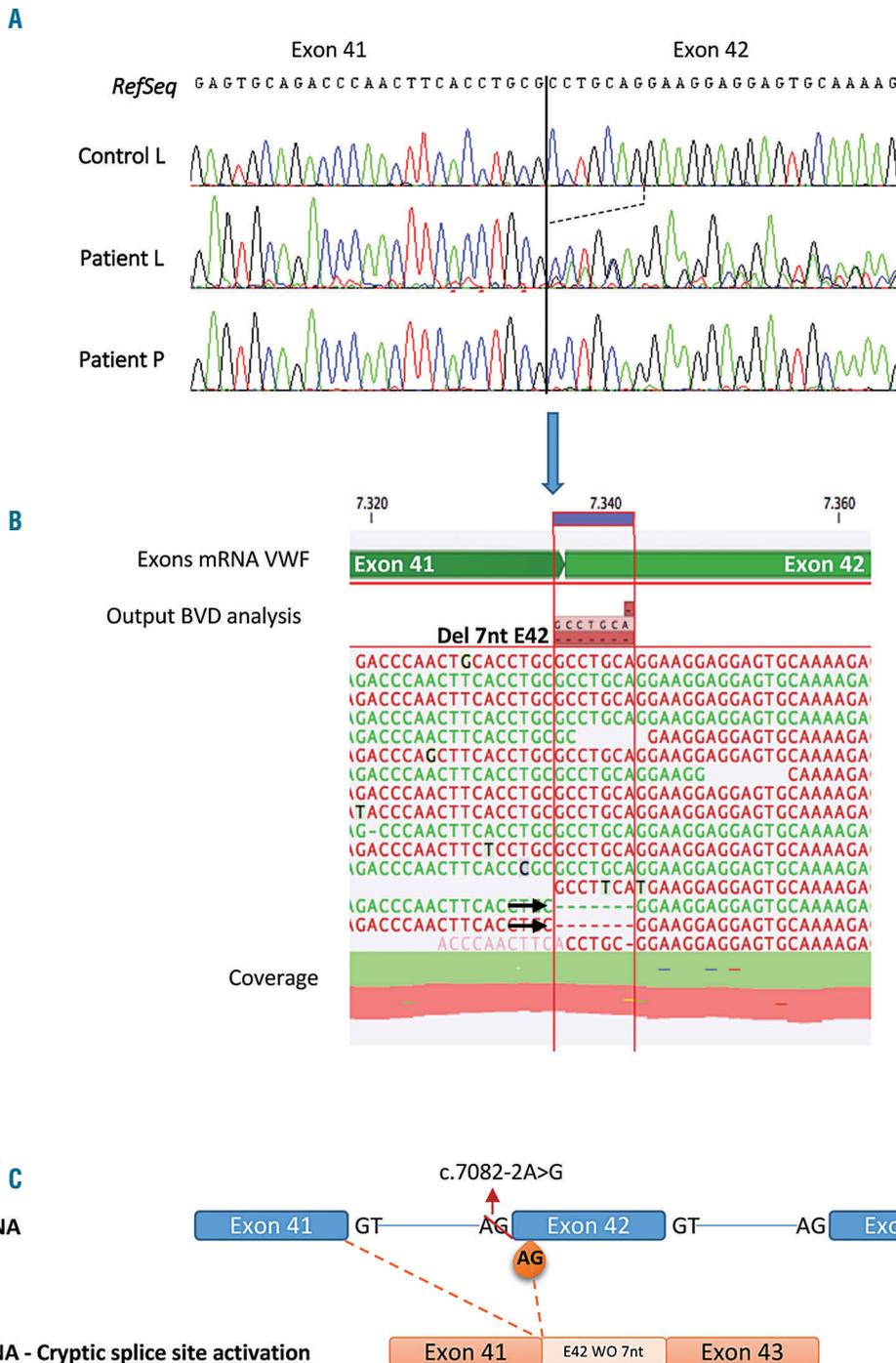


Figure 4. Analysis of the c.7082-2A>G mutation in patient UMP12. Agarose gel electrophoresis results of RT-PCR amplification of exon 38 to 43 using RNA from leukocytes and platelets were the same as those of healthy controls (*data not shown*). A) Traditional Sanger sequencing of PCR product from patient leukocyte (L) demonstrate activation of a cryptic splice site 7 nucleotides downstream of the native splice site within exon 42. In patient platelet (P), only the allele carrying the p.Leu150Pro mutation could be amplified. B) NGS of PCR products from leukocytes showed deletion of the 7 initial nucleotides of exon 42. Arrows show aberrant transcripts. C) Schematic representation of the mutation in genomic DNA and its effect on the VWF mRNA sequence. WO: without.

informative SNPs, rs1800375 and rs1800376, which were found in heterozygous state in leukocyte transcripts and homozygous state in platelets.

The c.[3426T>C; 3485_3486delinsTG] mutations (exon 26) were found in a type 2A VWD patient (UMP13), combined *in trans* with p.Cys2773Ser. *In silico* analysis predicted no impact on the splice site for c. 3485_3486delinsTG, but 2 of the 4 algorithms predicted that c.3426T>C would have an effect on splicing. The exon 25-28 region was investigated, but no effect was found on mRNA processing. Study of the p.Cys2773Ser mutation and 2 informative SNPs (rs2228317 and rs4021576) confirmed that the 2 alleles were present, suggesting that exon 26 mutations do not have an effect on VWF splicing.

The mutations (c.7437G>A exon 43), and c.6699_6702dup (p.Cys2235ArgfsTer8, exon 38) were identified *in trans* in a severe type 1 VWD patient (UMP14) and studied only in leukocyte RNA. For c.7437G>A, we amplified exons 41-45, and 2 bands emerged: one with the expected size, and a smaller band of ~350 bp (Figure 5). Sequencing analysis by both tech-

niques revealed 2 aberrant transcripts: one lacked exon 43 (p.Val2430GlyfsTer335) and the other showed activation of a cryptic DSS within exon 43, leading to deletion of its 4 final nucleotides (p.Ser2479AlafsTer23) (Table 2). Study of c.6699_6702dup in leukocyte mRNA showed no splicing changes, but the duplication introduced 4 nucleotides that changed the reading frame and generated a PTC (p.Cys2235ArgfsTer8).

The c.546G>A (p.=, exon 6) and c.7082-2A>G (intron 41) mutations *in trans* with c.8155+3G>C (intron 50) were detected in a type 3 VWD patient (UMP15). This patient had been described in our previous article, but at that time we were only able to identify the effect of c.8155+3G>C (p.Gly2706ValfsTer24) on splicing. On reanalysis of this case with NGS, we were able to characterize the effect of c.546G>A (p.Thr179ProfsTer31) and c.7082-2A>G (p.Ala2361GlyfsTer40) *in cis* in leukocyte mRNA. The results are consistent with those observed in patients UMP08 and UMP12, who harbored each mutation individually. In addition, in leukocytes, transcripts resulting from c.546G>A were detected in 2% of reads, transcripts

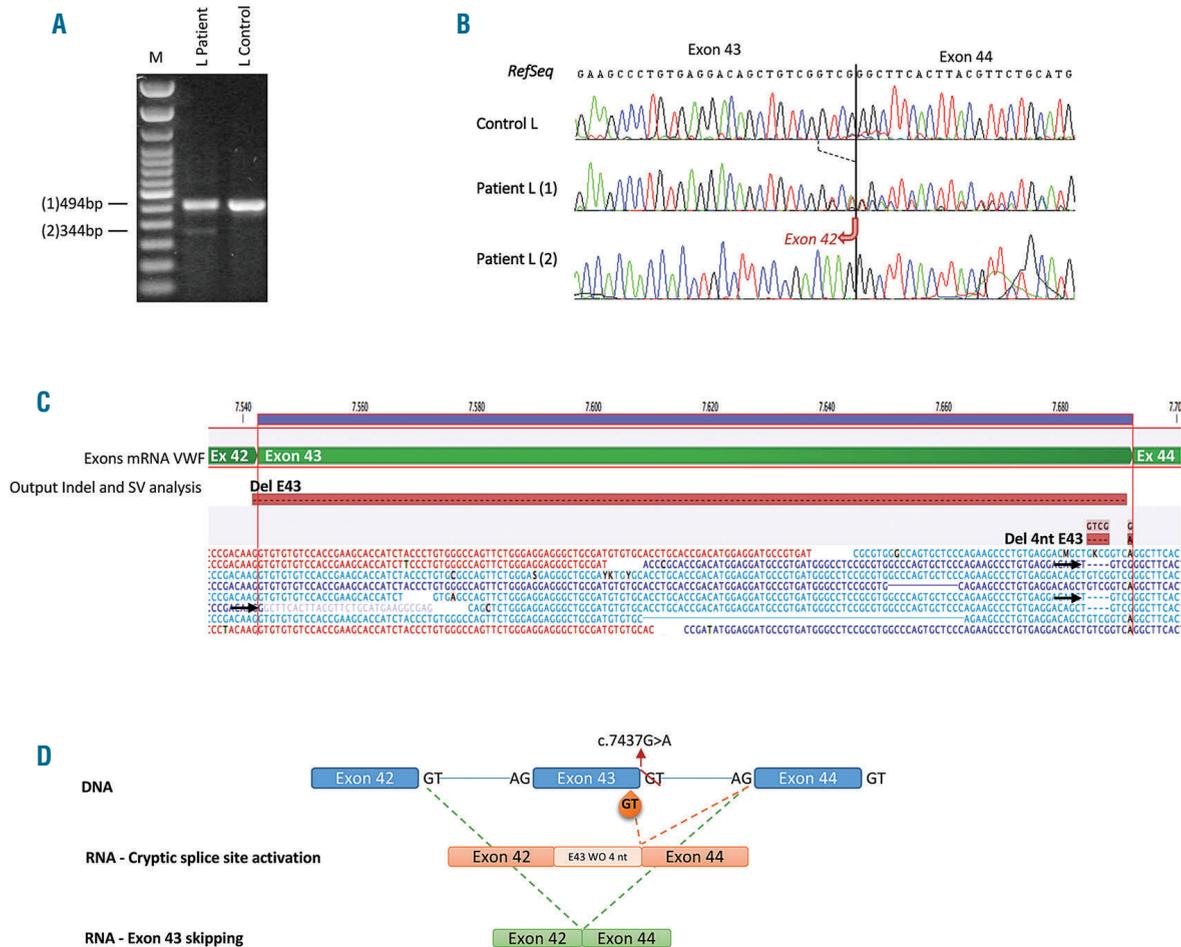


Figure 5. Analysis of the c.7437G>A (p.=) mutation in patient UMP14, located in the last nucleotide of exon 43. A) RT-PCR products amplified with primers located in exons 41 and 45 in leukocyte RNA (L), separated on 1% agarose gel. B) Traditional Sanger sequencing of PCR product from patient leukocyte (L) shows two aberrant transcripts: activation of a cryptic splice site - 4 nucleotides upstream to WT-DSS - in exon 43 (1), and exon 43 skipping (2). C) NGS of PCR products gave the same results than Sanger sequencing. D) Schematic representation of the mutation in genomic DNA and its effect on the VWF mRNA sequence. M indicates a 100-bp DNA ladder. WO: without.

from c.7082-2A>G in 6% and transcripts from c.8155+3G>C in 95% of reads. In platelet analyses, NGS detected transcripts without exon 6 resulting from the c.546G>A mutation in 11% of the total reads but no aberrant transcript derived from the mutation c.7082-2A>G indicating that NMD rate could differ between 5' and 3' regions of mRNA. These results indicate the c.[546G>A; 7082-2A>G] allele is under-represented compared to the allele carrying the c.8155+3G>C mutation, showing that this allele underwent NMD in both cell types, as was previously hypothesized.⁷

Discussion

This study reports the results of in-depth analysis of 18 PSSM in samples from VWD patients. The novel and robust procedure used, combining two-step RT-PCR and NGS sequencing, was faster and more sensitive than the method used in our previous article.⁷ This new approach provides several advantages, such as allele-specific individual sequences, higher sensitivity to detect transcript variants present at low copy numbers, and simplified sample preparation. Moreover, the NGS data on total reads obtained for each transcript is additional information that can provide an approximation of the expression levels of each VWF mRNA. Studies are currently ongoing to confirm that the relative expression of each transcript obtained by NGS is comparable to that provided by real-time RT-PCR.

The proven usefulness of leukocyte analysis to interpret mutations masked by NMD in platelets was seen in relation to the c.3379+1G>A mutation, which had been previously investigated by RT-PCR in platelets.²⁵ In that study, the true pathogenic effect of this mutation could not be determined due to NMD. In the present study, using leukocyte mRNA and NGS technology, we found that the mutation induces two aberrant transcripts.

Similarly, the pathogenic mechanism of 7 PSSM (c.1533+1G>A, c.5664+2T>C, c.7082-2A>G, c.546G>A, c.7437G>A, and p.Cys370Tyr) was determined in leukocytes, and only the c.3223-7_3236dup mutation effect was observed in both cell types. Because this mutation is located in the D3 domain implicated in multimerization, it is possible that the pathogenic mechanism leading to type 1 VWD may be related to impaired VWF secretion due to intracellular retention, as has been described for other D3 mutations such as p.Cys1130Phe.²⁴ To confirm this hypothesis, expression studies by means of heterologous cell lines or blood outgrowth endothelial cells (BOECs) will remain the gold standard.

As would be expected, mutations located in the consensus splicing sequence affected mRNA processing. These included c.1533+1G>A, c.5664+2T>C, leading to exon skipping, and c.7082-2A>G, activating a cryptic splice site. Both these molecular effects have been reported previously in splicing mutations causing VWD.^{7,25,26} The c.7082-2A>G *in trans* with p.Leu150Pro leads to the development of type 3 VWD. The p.Leu150Pro mutation does not affect splicing. However, other mutations located in the propeptide, such as p.Asp141Tyr, have been identified in type 3 patients,²⁷ and, as has been described in these cases, we suspect that p.Leu150Pro may compromise propeptide folding and affect intracellular survival and the capacity to mediate multimerization. The c.1533+1G>A mutation in

leukocytes generated three aberrant transcripts. One of them, which leads to the inframe deletion of exons 13-14, should have been detected in platelets since it would not generate a PTC and would not be degraded. However, because the mutation is located in a region with weak splicing signal sequences, as previously demonstrated,^{7,25,26} we suggest that the results obtained in leukocytes could be an artifact that would not occur in the natural cellular type of VWF expression. Based on these observations, we propose that the real effect of c.1533+1G>A is the skipping of exon 13, which predicts a frameshift at position 478 and addition of 138 aberrant amino acids before a PTC is encountered (p.Gly478AlafsTer138).

Two synonymous mutations included in our study, c.546G>A and c.7437G>A, affect splicing of VWF mRNA. To our knowledge, only three synonymous mutations affecting VWF mRNA processing have been reported: c.7056C>T,²⁸ c.7464C>T,²⁹ and c.3390C>T.²⁶ Of note, the results of the present study have almost doubled the number of reported mutations of this type causing VWD. The pathogenic effect of c.546G>A could only be documented by NGS; it eluded Sanger detection because of the low expression in leukocytes and platelets. The c.7437G>A mutation located at the last nucleotide of exon 43 was found in a patient with severe type 1 VWD (UMP14) *in trans* with c.6699_6702dup (p.Cys2235ArgfsTer8). This synonymous mutation produced 2 splicing variants. First, creation of a new DSS 4 nucleotides upstream of the WT-DSS in exon 43 that generated a PTC in exon 44 and would lead to NMD in platelets; and second, exon 43 skipping and generation of a PTC in exon 52. In this latter case, we would expect that NMD had been abolished in platelets, since this cellular mechanism is not effective when a PTC is encountered within 50 bp of the last exon-exon junction.³⁰

Study of the p.Cys370Tyr (c.1109G>A in exon 9) mutation, resulted in generation of 2 VWF transcripts, one of them identified only by NGS because of their low expression. Of note, only one other missense mutation, p.Gly1108Arg, has been reported to affect VWF splicing. Thus, these two mutations support the concept that missense mutations on the last exonic nucleotides can also have repercussions on the splicing process.³¹ Interestingly, the pathogenic effect of this mutation is the same as that described for c.1109+2T>C (intron 9).³² In addition to the patient reported here, the p.Cys370Tyr mutation was identified in 6 additional related patients included in the PCM-EVW-ES: in homozygous state in a type 3 VWD patient and in heterozygosis in 4 type 3 carriers and 1 patient with type 1 VWD, based on their phenotype levels. Certain genetic modifiers of VWF levels³³ and inter-individual variability in NMD efficiency between patients carrying identical mutations may lead to differences in the disease severity and clinical phenotype.³⁴

Lastly, study of c.7081+6G>T, c.7730-4C>G, c.7730-56C>T, c.8254-5T>G, c.3291C>T, c.4866C>T and c.[3426T>C; 3485_3486delinsTG] showed no visible effect on mRNA processing, although this does not necessarily mean that they have no effect on splicing. For instance, mRNA SNP analysis in patient UMP05 with c.7730-56C>T showed an absence of 1 allele in leukocytes and platelets, suggesting that the allele may have experienced NMD in both cell types or lack expression due to a mutation that was not detected by our sequencing protocol. Of particular note, deep intronic mutations such as

c.6599-20A>T have been found to cause VWD.³⁵ In patient UMP07 carrying the c.8254-5T>G mutation, no informative SNP was identified and we were unable to determine whether there was a lack of expression of 1 allele, which could explain cosegregation of the mutation in the family with bleeding symptoms.

Study of c.[3426T>C; 3485_3486delinsTG] and c.7730-4C>G showed no visible effect on splicing. However, these mutations have been identified in homozygous state in a type 3 patient (c.[3426T>C; 3485_3486delinsTG])³ and in a severe type 1 VWD patient (c.7730-4C>G)⁴ with VWF:Ag at 7% and VWF:RCo at 5%, combined with the heterozygous p.Ala631Val (previously reported in a healthy control).³⁶ Based on these findings, there is substantial evidence that these mutations would have an effect on VWF levels. Therefore, to unequivocally determine the potential deleterious effect of these variants, functional studies remain essential. These studies are traditionally carried out by *in vitro* analyses performed using heterologous cell lines (COS7, AtT-20 and HEK293). However, the advent of the possibility of obtaining BOECs from patients represents a valuable alternative, since this is the functional expression site of VWF.³⁷ Moreover, BOECs allows protein expression and mRNA studies simultaneously.

In silico algorithms used to assess the impact of mutations on splicing are more sensitive and accurate in determining the putative effect of intronic mutations than that of syn-

onymous or missense mutations, such as c.546G>A and p.Cys370Tyr. Therefore, the results here should not be considered definitive, and as with all analytical approaches, should form one aspect of a wider investigation.⁵

In conclusion, we present an extensive study reporting the effect of 18 candidate mutations on VWF mRNA processing. *In vivo* mRNA studies incorporating NGS technology together with traditional sequencing enabled us to determine the pathogenic effect of 8 PSSM (44%). Our study emphasizes the importance of examining selected mutations, including synonymous and missense mutations, to determine their pathogenic role in splicing. Taken together, our results add to the current knowledge about the molecular events leading to VWD.

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References

- Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*. 1987;69(2):454-459.
- Battle J, Perez-Rodriguez A, Corrales I, et al. Molecular and clinical profile of von Willebrand disease in Spain (PCM-EVW-ES): Proposal for a new diagnostic paradigm. *Thromb Haemost*. 2016;115(1):40-50.
- Borras N, Battle J, Perez-Rodriguez A, et al. Molecular and clinical profile of von Willebrand disease in Spain (PCM-EVW-ES): comprehensive genetic analysis by next-generation sequencing of 480 patients. *Haematologica*. 2017;102(12):2005-2014.
- Fidalgo T, Salvado R, Corrales I, et al. Genotype-phenotype correlation in a cohort of Portuguese patients comprising the entire spectrum of VWD types: impact of NGS. *Thromb Haemost*. 2016;116(1):17-31.
- Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17(5):405-424.
- Wang QY, Song J, Gibbs RA, Boerwinkle E, Dong JF, Yu FL. Characterizing polymorphisms and allelic diversity of von Willebrand factor gene in the 1000 Genomes. *J Thromb Haemost*. 2013;11(2):261-269.
- Corrales I, Ramirez L, Altisent C, Parra R, Vidal F. The study of the effect of splicing mutations in von Willebrand factor using RNA isolated from patients' platelets and leukocytes. *J Thromb Haemost*. 2011;9(4):679-688.
- Wang GS, Cooper TA. Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat Rev Genet*. 2007;8(10):749-761.
- Eikenboom JC, Reitsma PH, Peerlinck KM, Briët E. Recessive inheritance of von Willebrand's disease type I. *Lancet*. 1993;341(8851):982-986.
- Jaffe EA, Hoyer LW, Nachman RL. Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc Natl Acad Sci U S A*. 1974;71(5):1906-1909.
- Sporn LA, Chavin SI, Marder VJ, Wagner DD. Biosynthesis of von Willebrand protein by human megakaryocytes. *J Clin Invest*. 1985;76(3):1102-1106.
- Kieffer N, Guichard J, Farcet JP, Vainchenker W, Breton-Gorius J. Biosynthesis of major platelet proteins in human blood platelets. *Eur J Biochem*. 1987;164(1):189-195.
- Fink L, Holschermann H, Kwapiszewska G, et al. Characterization of platelet-specific mRNA by real-time PCR after laser-assisted microdissection. *Thromb Haemost*. 2003;90(4):749-756.
- McVey JH, Boswell EJ, Takamiya O, et al. Exclusion of the first EGF domain of factor VII by a splice site mutation causes lethal factor VII deficiency. *Blood*. 1998;92(3):920-926.
- David D, Santos IM, Johnson K, Tuddenham EG, McVey JH. Analysis of the consequences of premature termination codons within factor VIII coding sequences. *J Thromb Haemost*. 2003;1(1):139-146.
- Roberts RG, Bentley DR, Bobrow M. Infidelity in the structure of ectopic transcripts: a novel exon in lymphocyte dystrophin transcripts. *Hum Mutat*. 1993;2(4):293-299.
- Byers PH. Killing the messenger: new insights into nonsense-mediated mRNA decay. *J Clin Invest*. 2002;109(1):3-6.
- Lewis BF, Green RE, Brenner SE. Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proc Natl Acad Sci U S A*. 2003;100(1):189-192.
- Martorell L, Luce E, Vazquez JL, et al. Advanced cell-based modeling of the royal disease: characterization of the mutated F9 mRNA. *J Thromb Haemost*. 2017;15(11):2188-2197.
- Hojny J, Zemankova P, Lhota F, et al. Multiplex PCR and NGS-based identification of mRNA splicing variants: Analysis of BRCA1 splicing pattern as a model. *Gene*. 2017;637:41-49.
- Brunak S, Engelbrecht J, Knudsen S. Prediction of human mRNA donor and acceptor sites from the DNA sequence. *J Mol Biol*. 1991;220(1):49-65.
- Corrales I, Ramirez L, Altisent C, Parra R, Vidal F. Rapid molecular diagnosis of von Willebrand disease by direct sequencing. Detection of 12 novel putative mutations in VWF gene. *Thromb Haemost*. 2009;101(3):570-576.

23. Nesbitt IM, Hampton KK, Preston FE, Peake IR, Goodeve AC. A common splice site mutation is shared by two families with different type 2N von Willebrand disease mutations. *Thromb Haemost.* 1999;82(3):1061-1064.
24. Tjernberg P, Vos HL, Castaman G, Bertina RM, Eikenboom JC. Dimerization and multimerization defects of von Willebrand factor due to mutated cysteine residues. *J Thromb Haemost.* 2004;2(2):257-265.
25. Gallinaro L, Sartorello F, Pontara E, et al. Combined partial exon skipping and cryptic splice site activation as a new molecular mechanism for recessive type 1 von Willebrand disease. *Thromb Haemost.* 2006;96(6):711-716.
26. Pagliari MT, Baronciani L, Garcia Oya I, et al. A synonymous (c.3390C>T) or a splice-site (c.3380-2A>G) mutation causes exon 26 skipping in four patients with von Willebrand disease (2A/IIIE). *J Thromb Haemost.* 2013;11(7):1251-1259.
27. Baronciani L, Federici AB, Cozzi G, et al. Expression studies of missense mutations p.D141Y, p.C275S located in the propeptide of von Willebrand factor in patients with type 3 von Willebrand disease. *Haemophilia.* 2008;14(3):549-555.
28. Daidone V, Gallinaro L, Grazia Cattini M, et al. An apparently silent nucleotide substitution (c.7056C>T) in the von Willebrand factor gene is responsible for type 1 von Willebrand disease. *Haematologica.* 2011;96(6):881-887.
29. Yadegari H, Biswas A, Akhter MS, et al. Intron retention resulting from a silent mutation in the VWF gene that structurally influences the 5' splice site. *Blood.* 2016;128(17):2144-2152.
30. Maquat LE. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat Rev Mol Cell Biol.* 2004;5(2):89-99.
31. James PD, O'Brien LA, Hegadorn CA, et al. A novel type 2A von Willebrand factor mutation located at the last nucleotide of exon 26 (3538G>A) causes skipping of 2 nonadjacent exons. *Blood.* 2004;104(9):2739-2745.
32. Castaman G, Plate M, Giacomelli SH, Rodeghiero F, Duga S. Alterations of mRNA processing and stability as a pathogenic mechanism in von Willebrand factor quantitative deficiencies. *J Thromb Haemost.* 2010;8(12):2736-2742.
33. James PD, Lillicrap D. von Willebrand disease: clinical and laboratory lessons learned from the large von Willebrand disease studies. *Am J Hematol.* 2012;87 Suppl 1:S4-11.
34. Miller JN, Pearce DA. Nonsense-mediated decay in genetic disease: friend or foe? *Muta Res Rev Mutat Res.* 2014;762:52-64.
35. Hawke L, Bowman ML, Poon MC, Scully MF, Rivard GE, James PD. Characterization of aberrant splicing of von Willebrand factor in von Willebrand disease: an underrecognized mechanism. *Blood.* 2016;128(4):584-593.
36. Bellissimo DB, Christopherson PA, Flood VH, et al. VWF mutations and new sequence variations identified in healthy controls are more frequent in the African-American population. *Blood.* 2012;119(9):2135-2140.
37. Wang JW, Bouwens EA, Pintao MC, et al. Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease. *Blood.* 2013;121(14):2762-2772.