MOLECULAR DIAGNOSIS OF VON WILLEBRAND DISEASE

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The role of molecular characterization in the diagnosis of von Willebrand disease (VWD) is not essential if the patients have been extensively investigated using phenotypic analysis. On the other hand, if some of these phenotype assays are not available, the identification of the mutation causing the disease could be crucial for an accurate diagnosis. Nevertheless, there are several reasons for performing molecular analysis in patients phenotypically well characterized, e.g. to identify the mutation causing VWD can be useful for patients and their family members when prenatal diagnosis is required (type 3 or severe type 2). In this manuscript, we report the techniques used for the molecular characterization of suspected VWD patients. We describe the use of online von Willebrand factor database and online single nucleotide variation databases, the former to verify whether a candidate mutation has been previously identified in other VWD patients and the latter to ascertain whether a putative mutation has been reported earlier in healthy individuals. We listed the available in silico analysis tools, to determine the predicted pathogenicity of a sequence variant and to establish its possible negative effect on the normal splicing process. We also report the strategy that can be used to identify VWD type 2 patients’ mutations in subjects who have been fully characterized using the phenotype assays.

Keywords: DNA sequence analysis, molecular characterization, Multiplex Ligation-dependent Probe Amplification, Next Generation Sequence analysis, von Willebrand disease, von Willebrand factor

Introduction

The role of molecular characterization in VWD diagnosis

The importance of molecular characterization in the diagnosis of von Willebrand disease (VWD) patients is inversely proportional to the number and quality of phenotypic assays run in the laboratory. If all von Willebrand factor (VWF) assays are used [1], the identification of the molecular defects will only confirm the patient’s phenotypic diagnosis. On the other hand, if some of these phenotypic assays are not available, e.g. the VWF:VIII binding assay (VWF: FVIIIB), ristocetin-induced platelet agglutination (RIPA) or multimer analysis, then the identification of the mutation causing VWD will be crucial for the patient’s diagnosis.

The VWF:FVIIIB assay can provide differential diagnosis between mild haemophilia and VWD type 2N (Normandy). However, the assay is not available in many laboratories, therefore DNA sequence analysis for the VWF gene (VWF) exons encoding the D/D3 domains can be a valuable alternative to the use of VWF:FVIIIB assay.

VWD types 2B and 2A (IIA) patients can present similar phenotypic parameters. The RIPA assay, performed using platelet-rich plasma, evaluates the affinity of VWF to bind to the glycoprotein (GP)Ib platelet receptor, allowing differential diagnosis between VWD types 2B and 2A (IIA). However, if the RIPA assay is not available or only patient’s frozen plasma is available, DNA sequence analysis of the VWF exon encoding the A1 domain (type 2B) or A2 domain (type 2A/IIA) can be used to establish the patient diagnosis. Even in the presence of an enhanced RIPA, outcome of the DNA sequence analysis should
be performed because the 2B-like phenotype can also be caused (although less frequently) by gain-of-function mutations in the GPIX gene (GP1BA). The identification of mutations in the VWF A1 domain (VWD type 2B) or in the GP1BA (Pseudo-VWD) is a valid alternative to platelet-mixing tests [1] required for the differential diagnosis.

Also in the case of VWD types 2A (IIA) and 2M, patients can present similar phenotypic parameters (i.e. markedly reduced VWF:RCo or other platelet-dependent VWF activity determination in comparison to VWF:Ag level and decreased RIPA) and the phenotypic diagnosis might be difficult if multimer analysis is not available. Once again the molecular analysis can be used to obtain, in the large majority of the cases, the patients diagnosis by investigating exon 28 encoding the A1 domain (VWD type 2M) or the A2 domain (VWD type 2A/IIA).

Nevertheless, there are several reasons for also performing molecular analysis in well phenotypically characterized diagnosed patients. VWD type 3 diagnosis can be performed using a reliable VWF:Ag assay. However, the identification of carrier status among family members might be uncertain using the phenotypic approach, whereas it can be easily ascertained once the proband’s mutation(s) have been identified.

The identification of homozygous large deletions in VWD type 3 is an important risk factor for the development of anti-VWF alloantibodies [2, 3] and these defects should always be investigated in previously untreated patients. Prenatal diagnosis (PND) or preimplantation diagnosis in VWD type 3, particularly in cases where the parents already have a severely affected child, may be required and can be based on analysis of DNA from chorionic villi/amniocytes or embryo cells. In addition, in families with severe VWD types 1 or 2, the molecular approach can be easily used to perform postnatal analysis [4–6].

The von Willebrand factor, gene, pseudogene, mRNA transcription, pro-VWF functional domains and its complex biosynthesis

The VWF has been localized to chromosome 12, containing 52 exons with a length of about 178 kilobases (kb) [7]. A partial unprocessed VWF pseudogene (VWFP1, exons 23–34) with 97% homology with the gene has been mapped to chromosome 22 [8]. Transcription of VWF results in a mRNA of approximately 8.7 kb in size. VWF is synthesized by endothelial cells and megakaryocytes as a precursor polypeptide of 2813 amino acids (pre-pro-VWF), including a 22-residue signal peptide, 741-residue propeptide and 2050-residue mature subunit [7]. The pre-VWF (260 kD) is organized into repeats of homologous domains (D1-D2-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK) [9, 10] for which specific functions have been identified (Fig. 1). The propeptide, D1-D2 domains have a role in the multimerization process and VWF localization to storage organelles, the D’-D3 domains exhibit a binding site for factor VIII (FVIII) and plays an important role in VWF multimerization. The A1 domain is the only known binding site for the platelet receptor GPIbα, in addition, the A1 domain also provides a binding site for type VI collagen. The A2 domain contains the cleavage site for ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif, member 13) [11]. The A3 domain is the binding site for fibrillar collagen types I and III. The C4 domain which includes the RGD sequence, is the binding site for the integrin αIIβ3. A complex post-translational biosynthesis is required in order to obtain multimeric VWF. After translocation into the endoplasmatic reticulum, the signal peptide is cleaved and the pro-VWF dimerizes through disulphide bonds between C-terminal cysteine residues of the CK domain [12]. Multimerization continues in the Golgi apparatus where dimers, through disulphide bonds at the N-terminal region of the subunits (D’ and D3 domain), yield multimers that may exceed 20 000 kD. The propeptide that is cleaved prior to protein secretion has a key role in the assembly of VWF multimers [13] and to traffic the VWF into the storage organelles (Weibel–Palade bodies of endothelial cells or platelet α-granules) [14, 15]. An update on the complex process of VWF biosynthesis, secretion and clearance has been reported recently by Lenting et al. [16].

Techniques used in the molecular characterization of VWD patients

The use of genomic DNA vs. mRNA

Usually, a mutation search is performed using genomic DNA, comprising either PCR amplification of VWF exons followed by Sanger sequencing or more recently, next generation DNA sequencing (NGS). Messenger RNA (mRNA) isolated from patient’s platelets can also be used. Nevertheless, the instability of the latter samples and the more laborious techniques required to obtain it often reduces the use of mRNA analysis. This strategy is usually applied to assess possible splicing mutations or intronic mutations identified in possible regulatory areas. The study of mRNA can be undertaken in VWD patients where no mutations have been previously identified after evaluation performed at the exonic DNA level. This approach is reasonable in VWD types 3 or 2 patients (with a proven family bleeding history), but not in VWD type 1 where mutations are not always identified. In this case, an alternative mRNA sequence due to altered © 2017 John Wiley & Sons Ltd Haemophilia (2017), 23, 188–197
splicing resulting from a possible deep intronic sequence change, may be found at the mRNA level. The identification of altered splicing is not always possible since mRNA decay in VWD patients presenting premature termination codons has been previously reported [17–19].

**Direct DNA sequence (Sanger) analysis**

The Sanger method allows establishing the nucleotide sequence of a DNA fragment (direct DNA sequence analysis). This technique is the most frequently adopted for the identification of VWF mutations. However, the new technology of NGS is reducing the use of the Sanger sequencing. Currently, the cost of NGS still makes the use of Sanger sequencing convenient in those cases where the mutation search is localized to specific VWF exons (i.e. most common VWD type 2 variants). In addition, this new technique is not yet widely available and therefore its use is limited to the more advanced genetic laboratories. For the investigation of the full VWF coding region along with the intronic flanking regions, the 5' and 3' untranslated regions and a portion of the promoter, using Sanger sequencing is extremely time-consuming, mainly due to the large size of the coding region of the gene itself (8442 bp). This has impaired the molecular characterization of patients with VWD types 1 and 3 in comparison with type 2 where mutation analysis is often performed for specific VWF regions. Direct sequencing is available in many laboratories and is particularly indicated to identify the gene mutation in VWD type 2 variants (see VWD type 2 variant analysis). However, Sanger sequencing is not able to identify large heterozygous deletions or duplications.

**Next Generation Sequence (NGS) analysis**

Newly developed genetic methods such as NGS are becoming faster and progressively cheaper, allowing wider study of VWF mutations at a very reasonable cost [20]. NGS enables investigation of the entire VWF coding region in a short time and therefore this approach is better suited to investigate VWD types 1 and 3 variants. When possible, the study of type 2 variants can also be undertaken using this technique. However, patients’ DNA samples should be sent to genetic laboratories that already have experience with this technique in order to contain the cost of the assay. Analysis can be performed using either PCR amplification or sequence capture through hybridization to prepare the template for NGS. All primers must be specifically designed to avoid amplification of VWFP1 and also to prevent the presence of common single nucleotide variants (SNV) within PCR primer sequences. This technique allows investigation of VWF in a relatively short time of many
patients. It is currently only available in larger genetic laboratories, but its adoption is spreading. Use of dosage analysis algorithms can enable detection of heterozygous large deletions and duplications and heterozygous gene conversions can also be detected. Candidate causative variants found with this technique require the use of Sanger sequencing to confirm the identified mutations. Recently, a large VWD cohort from Spain has been investigated using this approach [21]. The NGS analysis comprises three main phases: preparation of the DNA library, clonal amplification and cyclic array sequencing. Each phase can be done using more than one method. These authors used a modified version of the NGS method previously reported by Corrales et al. [20] and the reliability of this NGS approach has already been confirmed by Fidalgo and colleagues [22] on a cohort of Portuguese VWD patients. In Fig. 2, a schematic representation of this NGS procedure is summarized.

**Multiplex Ligation-dependent Probe Amplification (MLPA)**

Another technique, important in the molecular characterization of VWD variants, is MLPA analysis [23]. Large gene deletions and more rarely, large duplications may be present in VWF. Large gene deletions have been reported in all 3 disease types of VWD. If a deletion affects only one allele, PCR-based techniques are not able to identify the mutation. The semi-quantitative approach of the MLPA technique (MRC-Holland, The Netherlands) can identify large gene deletions or duplications even when these defects are heterozygous [24]. Most large deletions that have been identified have been seen in VWD type 3 patients, however, in-frame large deletions have also been identified in VWD types 1 and 2. This technique which can be adopted in all laboratories that are able to perform Sanger sequencing enables the identification of large gene deletions or duplications of an exon or more.

**Online VWF databases**

**Pathological mutations identified in VWF**

An online database on VWF is available through the Scientific and Standardization Committee on VWF of the International Society of Thrombosis and Haemostasis at the site of the University of Sheffield (http://www.vwf.group.shef.ac.uk/; accessed November 2016). The VWF mutation listing for the database is hosted through the European Association for Haemophilia and Allied Disorders (EAHAD) Coagulation Factor Variant Databases portal (https://grenada.lumc.nl/LOVD2/VWF/submit.php or http://www.eahad-db.org/index.php).

Sequence variants identified in VWF

There are now a number of databases that list SNV and their frequency within a number of different population groups. These include the single nucleotide polymorphism database, dbSNP (www.ncbi.nlm.nih.gov/SNP), the 1000 Genomes database (www.1000genomes.org), Exome Aggregation Consortium, ExAC (http://exac.broadinstitute.org) and Exome Variant Server, EVS (http://evs.gs.washington.edu/EVS), accessed November 2016. ExAC is particularly useful as it lists sequence variants in approximately 60 000 individuals from several different populations. The position of VWF SNV should be taken into consideration during the design of oligonucleotides that will be used in any of the techniques mentioned above, in order to avoid mis-amplification. The use of these databases is useful in order to verify if the same missense, synonymous or candidate splice site mutation has been already identified in several healthy individuals, helping to exclude a pathological role of the identified variant.

**In silico analysis**

Newly developed genetic methods are being used in many laboratories, allowing wider molecular studies in many different diseases. Most of these software tools can be freely accessed online and this has resulted in a rapid increase in the detection of rare or novel sequence variations. The use of *in silico* analysis represents a valuable tool to determine the predicted pathogenicity of exonic and intronic sequence variants within a limited timescale and budget [25, 26].

**Splice site prediction software**

Several different software tools are available to assess the likely impact of a mutation on mRNA splicing, examples are listed in Table 1. Use of at least three of the recommended prediction tools is reported to reach a consensus result. Where the tools do not agree, further predictions should be undertaken to try and reach a consensus.

Amino acid prediction software

The potential damaging effect on protein structure or function of a SNV can be evaluated using the *in silico*
tools listed in Table 2. Tools should be selected that rely on different types of algorithm to analyse potential pathogenicity. A minimum of about five tools should be used to seek a consensus on likelihood of pathogenicity or neutrality.

Molecular characterization of VWD patients

As previously mentioned, the role of molecular characterization in the diagnosis of VWD is not essential if the patients have been extensively investigated using phenotype analysis. However, identifying the mutations causing VWD can be useful for patients and their families in cases where prenatal diagnosis is required. In the following paragraphs, we report the strategy that can be used to identify patients’ mutations in subjects who have been fully characterized using the phenotype assays.

Table 1. Splice site prediction. Software tools available to assess the likely impact of a mutation on mRNA splicing.

<table>
<thead>
<tr>
<th>Program name</th>
<th>Type of tool</th>
<th>Web site address</th>
</tr>
</thead>
<tbody>
<tr>
<td>MaxEntScan (5')</td>
<td><a href="http://genes.mit.edu/burgelab/maxent/Xmaxe">http://genes.mit.edu/burgelab/maxent/Xmaxe</a> ntscan_scoreseq.acc.html</td>
<td></td>
</tr>
<tr>
<td>MaxEntScan (3')</td>
<td><a href="http://genes.mit.edu/burgelab/maxent/Xmaxe">http://genes.mit.edu/burgelab/maxent/Xmaxe</a> ntscan_scoreseq.html</td>
<td></td>
</tr>
<tr>
<td>NNSplice (FruitFly)</td>
<td><a href="http://www.fruitfly.org/seq_tools/splice.html">http://www.fruitfly.org/seq_tools/splice.html</a></td>
<td></td>
</tr>
<tr>
<td>NetGene 2</td>
<td><a href="http://www.cbs.dtu.dk/services/NetGene2/">http://www.cbs.dtu.dk/services/NetGene2/</a></td>
<td></td>
</tr>
<tr>
<td>MIT Splice Predictor</td>
<td><a href="http://genes.mit.edu/GENSCAN.html">http://genes.mit.edu/GENSCAN.html</a></td>
<td></td>
</tr>
<tr>
<td>Alternative Splice Site Predictor</td>
<td><a href="http://wangcomputing.com/assp/">http://wangcomputing.com/assp/</a></td>
<td></td>
</tr>
</tbody>
</table>

Use at least three of the reported predictions tools to reach a consensus result. Three of these prediction programs should agree, with a minimum reduction of 10% in the efficiency of a splice site being necessary to consider a deleterious effect on splicing.

Once the patients’ diagnosis as VWD types 1 or 3 has been undertaken using phenotypic methods, molecular analysis can be performed. The approach of investigating gene mutations causing types 1 and 3 is similar since in contrast to VWD type 2, where the large majority of mutations are located in exon 28 (see Fig. 1), there is no particular area where mutations are identified. The whole VWF coding region should be investigated along with the intronic flanking regions, the 5’ and 3’ untranslated regions and the proximal VWF promoter. For VWD type 3, that segregates as a recessive disease, both alleles may harbour different mutations (compound heterozygous) or two copies of the same mutation (homozygous). For VWD type 1, that mainly segregates as a dominant disease, only one allele is affected, although there are reports regarding VWD type 1 due to compound heterozygous mutations [22, 27]. In addition, the identification of both mutations in VWD type 3 patients is possible in almost all cases, whereas in VWD type 1 [27, 28], mutations are not identified in almost 30% of the investigated patients (mainly in those where the VWF:Ag level is above 30 IU dL⁻¹). Moreover, the mutations identified in VWD type 3 are often clearly disease-causing mutations (nonsense, frame-shift and large deletions), whereas in VWD type 1, missense mutations often identified can only be considered candidate mutations due to the lack of firm evidence of their causal effect. Figure 3 shows an algorithm for molecular characterization in patients affected with VWD types 1 and 3.

Prenatal diagnosis (PND) of VWD type 3

Compared with haemophilia, most patients with VWD show relatively mild bleeding symptoms. Therefore,
PND is not usually required, except for occasional type 2 cases and VWD type 3 families with a severe clinical history. PND is mainly required in cases where both parents are already known to be carriers of VWD type 3, with gene defects identified in their first affected child with severe bleeding symptoms. In the past, PND was performed using PCR of short tandem repeats (STR) in VWF [29]. To detect the gene mutations, the above mentioned sequencing and dosage analysis techniques can be used. For known mutations, sequence analysis is the first method of choice, whereas if the gene mutations are not known, NGS and if necessary, MLPA can be used [21, 24].

**Molecular characterization of VWD type 2 patients**

Differently to VWD types 1 and 3, the molecular diagnosis of type 2 variants can be performed in many laboratories. Characterization of VWD type 2 mutations does not require the study of the whole VWF gene, but only the evaluation of the exons encoding the specific functional domain(s) identified using the
Fig. 3. The algorithm of molecular characterization of VWD types 1 and 3 patients. VWD, von Willebrand disease; MLPA, Multiplex Ligation-dependent Probe Amplification; SNV, single nucleotide variant; rVWF, recombinant von Willebrand factor.
phenotypic approach. This strategy can be applied to all type 2 VWD (2A, 2B, 2M and 2N), although the evaluation of type 2A can be more complex due to the involvement of different functional domains implicated in the phenotype of these patients (see Fig. 1).

In VWD type 2B that segregates as dominant disease, only one mutation is expected. These mutations lie in the A1 domain, between p.Glu1260 and p.Gly1479 and are associated with spontaneous binding of VWF to the platelet GPIbα receptor (RIPA <0.7 mg mL⁻¹). To identify this defect, only the investigation of the 5′ portion of exon 28 is necessary. Therefore, one simple PCR, followed by sequence analysis can be used to identify the causative mutation. If no mutation is identified in the VWF A1 domain, in spite of a clearly enhanced RIPA result obtained from patient’s platelet-rich plasma, direct sequencing analysis of the GP1BA is necessary.

Pseudo-VWD, also known as platelet-type VWD, is due to missense mutations or an in-frame deletion in exon 2 of the GP1BA. Missense variants (between p.Trp246 and p.Asp265) and an in-frame deletion; a 27-bp in-frame deletion Pro449_Ser457del (1345_1371del27) [30], cause GPIbα to spontaneously bind to VWF resulting, as for VWD type 2B, in enhanced RIPA [31].

VWD type 2M also segregates as dominant disease. The missense mutations are more commonly located in the A1 domain. These defects are associated with reduced capacity of VWF to bind GPIbα receptor (VWF:RCo/VWF:Ag <0.6). Therefore, to identify these defects, only the investigation of the 5′ portion of exon 28 is necessary, similarly to VWD type 2B variants. However, collagen-binding defects are also seen in VWD type 2M (VWF:CB/VWF:Ag <0.6) [32, 33]. In this case, the majority of mutations are located in the A3 domain, and exons 29–32 should be investigated. A small proportion of A1 domain mutations result from collagen types IV or VI binding defects [34].

VWD type 2N segregates as a recessive disease; therefore, both alleles are expected to be mutated (either as compound heterozygous or homozygous mutations). Homozygous mutations may be found in patients from the consanguineous partnerships, but as also in European populations due to the high prevalence of the p.Arg854Gln mutation, often found in the homozygous form. Compound heterozygotes usually have a combination of a type 2N mutation with types 1 or 3 VWF defect. Mutations affecting binding of VWF to FVIII (VWF:FVIIIB) have been identified mainly in the first 272 amino acid residues of the mature subunit (domain D' and a portion of D3, encoded by exons 17–25), although mutations up to exon 26 have been reported to have mildly reduced VWF:FVIIIB. Therefore, in this VWD type, identification of the mutations responsible for decreased VWF: FVIIIB can be done by evaluating a relatively short region of VWF. However, for patients who are heterozygous for a type 2N missense mutation, identification of the second defect, requires the same approach used for VWD types 1 and 3 patients.

VWD type 2A patients have a heterogeneous group of mutations located in different VWF functional domains. All these mutations affect to different extents, the VWF multimer size; therefore, these patients present a variable loss of high molecular weight (HMW) multimers.

The most common form of VWD type 2A segregates as dominant disease and the variants are located in the A2 domain, which contains the ADAMTS13 cleavage site. These variants, identified in the previous VWD classification as IIA [35], are characterized by the loss of high and intermediate sized multimers, associated with enhancement of the triplet bands due to increased susceptibility to ADAMTS13 proteolysis. Therefore, to identify these defects, only the investigation of the 3′ portion of exon 28 is necessary.

The second most common form of type 2A VWD segregates as dominant disease and the variants are located in the D3 domain, which plays an important role in VWF multimerization. These variants, identified in the previous VWD classification as IIE [36], are characterized by a modest loss of HMW multimers, associated with the absence of satellite triplet bands due to reduced susceptibility to ADAMTS13 proteolysis. Therefore, to identify these defects, only the investigation of exons 22, 25–27 and the 5′ end of exon 28 is necessary [37, 38].

A rare form of VWD type 2A that also segregates as dominant disease is due to variants located in the CK domain, which plays an important role in VWF dimerization. These variants, identified in the previous VWD classification as IID [39], are characterized by the loss of HMW multimers, associated with the

Table 3. A strategy to identify the molecular defects in biochemically characterized type 2 VWD patients using PCR and Sanger sequencing.

<table>
<thead>
<tr>
<th>VWD type</th>
<th>2A(IIA)</th>
<th>2A(IIIE)</th>
<th>2A*(IIIC)</th>
<th>2A(IIID)</th>
<th>2A(IIID)</th>
<th>2B</th>
<th>2M</th>
<th>2M(CB)</th>
<th>2N*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domain</td>
<td>A2</td>
<td>D3</td>
<td>D1-D2</td>
<td>CK</td>
<td>A1</td>
<td>A1</td>
<td>A1</td>
<td>A3</td>
<td>D'–D3</td>
</tr>
<tr>
<td>Exon</td>
<td>3′ portion of 28</td>
<td>22, 25–27 and 5′ portion of 28</td>
<td>2–17</td>
<td>51–52</td>
<td>5′ portion of 28</td>
<td>29–32</td>
<td>17–25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each VWD type 2 variant results from sequence changes localized in a specific VWF functional domain. Although, some exceptions to this model have been reported, the large majority of the variants identified fit well with this pattern.

Recessive variants (*) are present in either the homozygous or compound heterozygous form. In the latter, it is likely that only the missense variant is responsible for patient type 2 phenotype, whereas the second defect may result in a null allele. Only the missense variant may be identified using this approach and the recognition of the second variant will require evaluation of the entire VWF coding sequence.
presence of 'odd' band sizes in the intermediate resolution multimer analysis. Therefore, to identify these defects, investigation of only exons 51–52 is necessary [39].

A further rare form of VWD type 2A that segregates as a recessive disease, is due to variants located in the D1-D2 domains (propeptide) which play an important role in VWF multimerization. The patients can be homozygous for a mutation in the D1 or D2 domains or compound heterozygous with a second defect elsewhere in VWF. These variants, identified in the previous VWD classification as IIC [36], are characterized by the loss of high and intermediate molecular size multimers associated with the absence of the satellite triplet bands due to reduced susceptibility to ADAMTS13 proteolysis. Therefore, to identify these defects, the investigation of exons 2–17 should be performed, although to date, mutations have been identified in the D1 domain, exons 6–7 and in exons 11–15 [40].

The above strategy to molecularly characterize the type 2 VWF variants is summarized in Table 3.

**Conclusion**

VWD diagnosis can be achieved through both genetic analysis and phenotypic characterization of VWF.

Sanger sequence analysis or NGS can identify mutations in most type 3 and 2 patients, but other aetiology than VWF defects may contribute to reduced VWF levels in one-third of type 1 VWD patients, since 30% of patients remain without mutations in the VWF protein coding sequence. Increasing use of NGS combined with dosage analysis derived from the NGS data will facilitate more complete analysis of VWF for mutations.

**Disclosures**

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**Author contributions**

LB wrote the manuscript. FP and AG critically revised the manuscript. All the authors approved the submitted and final version of the manuscript.

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