Received: 20 November 2024 | Revised: 30 December 2024 | Accepted: 31 December 2024

https://doi.org/10.1016/j.rpth.2025.102686

rpth research & practice in thrombosis & haemostasis

REVIEW ARTICLE

Challenges and considerations of genetic testing in von Willebrand disease

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Handling Editor: Prof Michael Makris

Abstract

von Willebrand disease (VWD) is the most common inherited bleeding disorder characterized by defects in the quantity or function of the von Willebrand factor (VWF). The diagnosis of VWD is complex, requiring a battery of tests to evaluate the amount, functions, and multimeric structure of the VWF glycoprotein. The diagnosis can also be accomplished or confirmed by sequencing the VWF gene (*VWF*). Genetic testing of *VWF* has been around for 4 decades following the cloning of *VWF*, and nowadays, it has been integrated into the diagnostic panel of VWD. With the introduction of next-generation sequencing, genetic analysis of the *VWF* has become more practical than it was in the past, when Sanger sequencing was used. A number of laboratories have applied or started to use genetic testing with next-generation sequencing for VWD diagnosis. Considering the increasing application of genetic testing in VWD and the wide availability and decreasing cost of gene sequencing, we sought to discuss the challenges and considerations involved in applying genetic testing to VWD.

KEYWORDS
genetic, NGS, VWD, VWF, VWF gene

Essentials

- With the advent of next-generation sequencing, laboratories working on von Willebrand disease (VWD) are increasingly using von Willebrand factor genetic testing.
- Genetic testing is used to confirm/differentiate VWD diagnoses and to provide genetic counseling.
- The application of genetic testing in VWD is complex and requires careful considerations.
- Laboratories with less expertise in VWD diagnosis should be aware of these considerations.

1 | INTRODUCTION

von Willebrand disease (VWD) is the most common inherited bleeding disorder, caused by defects in the quantity or function of the von Willebrand factor (VWF) [1]. There are 3 principal types of VWD according to the Scientific and Standardization Committee Subcommittee on von Willebrand Factor of the International Society on Thrombosis and Haemostasis [2]. Type 1 is characterized by the partial deficiency of VWF, type 3 with complete absence of VWF, and type 2 with functionally abnormal VWF, including 2A, 2B, 2M, and 2N [2]. The diagnosis of VWD is complex, requiring a battery of tests to evaluate the amount, functions, and multimeric structure of the VWF glycoprotein [1]. The diagnosis can also be accomplished or confirmed by sequencing the VWF gene (*VWF*) [3].

Genetic testing of *VWF* has been around for 4 decades, following the *VWF* cloning, and nowadays, it has been integrated into the diagnostic panel of VWD [4]. With the introduction of next-generation sequencing (NGS), genetic analysis of *VWF* has become more practical than it was in the past, when Sanger sequencing was used [3]. Genetic testing of *VWF* should include the analysis of the coding regions (ie, all 52 exons), flanking regions including the splice site boundaries, and the 5′ and 3′ untranslated regions. Generally, it is recommended that the identified variants be confirmed by independent testing. A number of laboratories have applied or started to use genetic testing with NGS for VWD diagnosis. The wide spectrum applications of genetic testing in VWD have been comprehensively discussed elsewhere [3,5,6]. Considering the increasing use of genetic testing in VWD and the wide availability along with decreasing cost of gene sequencing, we sought to discuss the challenges and considerations involved in applying genetic testing to VWD.

2 | VWF GENE AND ITS PSEUDOGENE

VWF is located on the short arm of chromosome 12 (12p13.2) and contains 178 kb of DNA with 52 exons [7]. There is a partial, non-transcribed *VWF* pseudogene in chromosome 22q11.2 that spans 25 kb with 97% sequence homology with exons 23 to 34 of *VWF* [8] (Figure 1). With this strong sequence homology, one can expect that the pseudogene introduces challenges in the genetic testing of VWD at least through 2 ways: (i) difficulty in sequencing of the "real" *VWF* and (ii) difficulty in alignment of the obtained sequence against the reference *VWF* sequence. Specific primers should be designed to avoid amplifying the pseudogene along with *VWF*. In the case of NGS, attention should be exercised since the alignment of the sequence is problematic. NGS has shown limitations in covering exon 26 of *VWF*, primarily due to the great degree of homology between *VWF* and its pseudogene (Figure 2A). In fact, we found that the Genome Aggregation Database (gnomAD), which contains exome and genome sequencing of more than 800,000 subjects, also shows this significantly less coverage of *VWF* exon 26 (Figure 2B). Therefore, gene variants in this exon may be missed when applying NGS techniques for target panel, exome, or genome sequencing, and thus Sanger sequencing should be performed to search for potential variants. This is more emphasized in laboratories with less expertise in VWD diagnosis. The recently published UK guidelines on VWD [9] have also highlighted this aspect. It recommends that variants detected in *VWF* exons 23 to 34 using NGS should be confirmed, ideally using a different analysis method, to ensure their specificity. Additionally, since some NGS technologies are not capable of mapping sequence reads to specific regions of the gene, such as exon 26 in *VWF*, these regions should therefore be analyzed by Sanger sequencing [9]. Nanopore long-read sequencing seems to not be affected by this issue and shows good coverage of the entire *VWF*, including exon 26 (data not shown). In fact, several (relatively severe) type 2 variants are located on the *VWF* exon 26 such as p.Cys1101Arg, p.Cys1130Cys (p.Pro1127_Gly1180delinsArg), p.Cys1130Phe, p.Cys1130Tyr, p.Cys1142Phe p.Tyr1146Cys, p.Cys1149Arg, p.Cys1157Phe, p.Gly1172Cys, p.Cys1173Phe, and p.Cys1173Arg [10–12]. These variants, located in the VWF D3 domain, are associated with a specific type 2A phenotype, 2A(IIE), and were found to be relatively common among type 2A VWD in reports from Germany [11], France [13], and Italy [10].

Due to the strong homology between the *VWF* pseudogene and the *VWF*, recombination between them leads to the appearance of a group of pathogenic VWF variants known as gene conversions [14]. These gene conversions have 2 or more nucleotide substitutions that are all present in the same allele and can lead to various types of gene mutations: nonsense, missense, and synonymous variants [14]. Different phenotypical expressions can be caused by gene conversion depending upon its length, homozygous or heterozygous status, or compound heterozygosity with another genetic defect. These gene conversions are reported in type 1 [15], type 2B New York [10], 2M [10], and type 3 VWD [16]. Detecting (all nucleotide changes within) a gene conversion using NGS is also challenging and requires caution; hence, confirmatory testing using Sanger sequencing should be kept in mind.

3 | PENETRANCE OF VWF GENE VARIANTS

Penetrance is defined as "the probability that an individual with a pathogenic genetic variant develops a specific disease" [17]. Although almost all type 2 VWD variants are penetrant, several variants associated with type 1 showed incomplete penetrance [18]. A number of large cohorts of type 1 showed that variants that result in plasma VWF levels <25 IU/dL are often fully penetrant, while those resulting in higher VWF levels are often incompletely penetrant [19–21]. Therefore, genetic testing in these patients with a partial VWF deficiency should be interpreted with caution while considering the whole VWD test panel results for diagnosis. For example, variant p.Tyr1584Cys is the most common variant associated with type 1 VWD [22] with incomplete penetrance and a variable bleeding tendency [23]. Variants external to VWF may affect the severity or penetrance of type 1 VWD such as aging, blood group, or environmental exposures [24].

Numerous *VWF* variants have been previously thought to cause VWD and be fully penetrant. However, with the availability of access to population-scale genetic databases, we now learned that they are indeed benign or low-risk variants. We reported a list of variants, previously associated with VWD, that are common (allele frequency >1%) in the gnomAD population and thus are likely to be benign (p.Met576Ile, p.Met740Ile, p.His817Gln, p.Arg924Gln, p.Gly967Asp, p.Thr1034del, p.Pro2063Ser, p.Arg2185Gln, and p.Thr2647Met) [25]. Observation of *VWF* variants in healthy control populations should be interpreted with caution. For instance, p.Arg854Gln has been found in healthy individuals [26]; however, there is clear evidence (both family and functional data) that it contributes to VWD type 2N [27–29]. A genetic study of healthy individuals showed relatively high frequencies of genetic variants, particularly in the African American

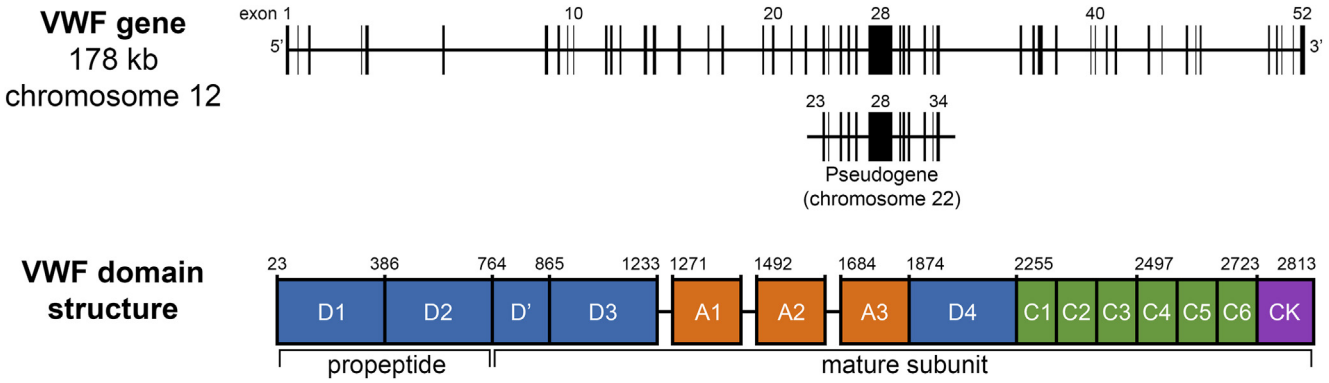

FIGURE 1 von Willebrand factor gene (*VWF*) and its pseudogene. *VWF* is located on the short arm of chromosome 12 (12p13.2) and contains 178 kb of DNA with 52 exons. A partial, nontranscribed *VWF* pseudogene in chromosome 22q11.2 that spans 25 kb with 97% sequence homology with exons 23 to 34 of *VWF* is present.

population, in whom several variants with high frequencies were found [26]. Of note, some of these variants had been previously considered as causative of VWD. Since *VWF* is highly polymorphic [30], the distinction between a disease-causing variant with a strong penetrance and nonpathogenic variants is challenging (see below—novel variants). With cooperation between ClinGen and an expert panel, efforts are being made to curate *VWF* genetic variants (https://clinicalgenome.org/affiliation/50051/). The curation of type 2 VWD variants has recently been finalized by the ClinGen expert panel [31].

4 | NOVEL VARIANTS

There have been numerous variants within *VWF* discovered as a result of high-throughput molecular technologies, but the clinical significance of these variants is yet to be determined. As more genetic studies on VWD patients are completed, we are observing an increasing number of novel variants in the *VWF* [13,16,32,33]. For example, in 4 large studies on VWD from Spain, France, Europe-Iran, and Germany, the rate of novel variants was 65% [32], 58% [13], 31%

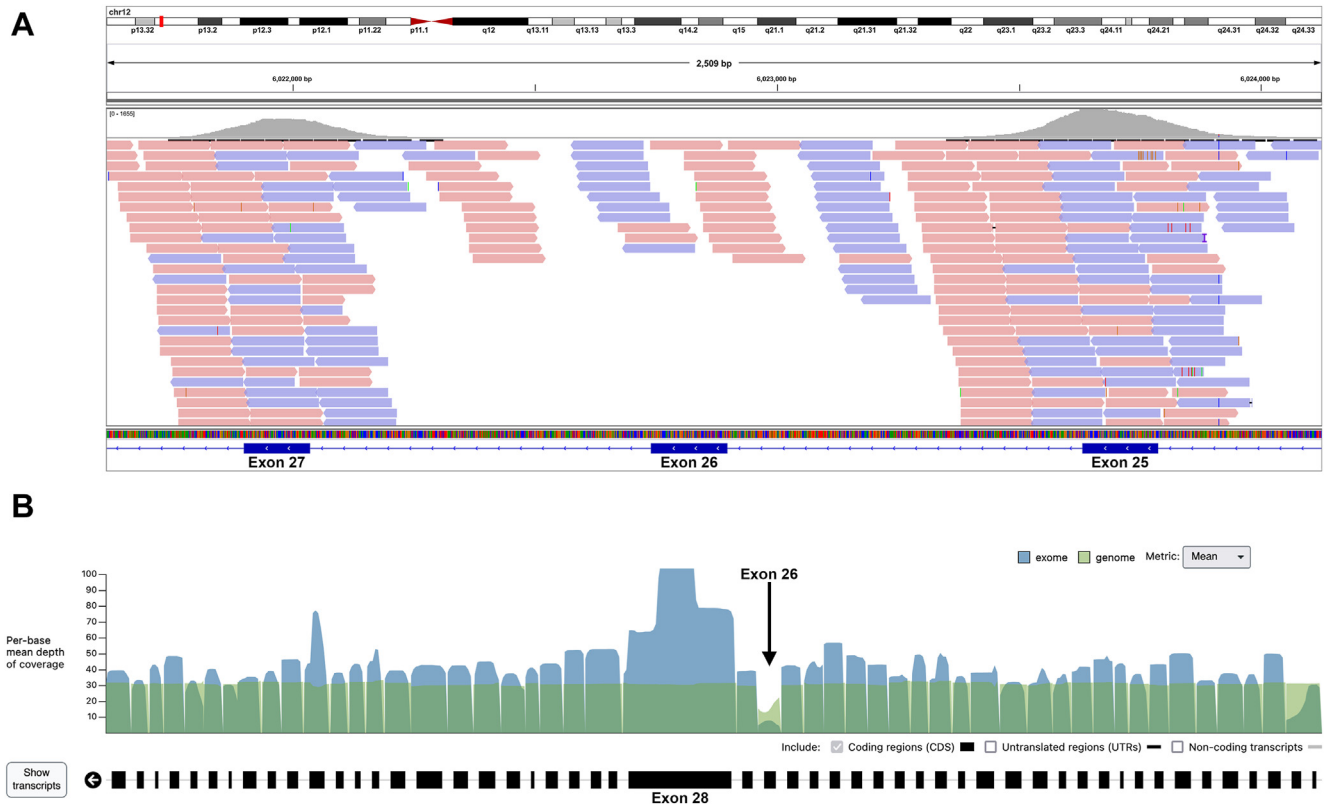

FIGURE 2 Coverage of the von Willebrand factor (VWF) gene (*VWF*) exon 26 using next-generation sequencing (NGS). (A) The coverage using our NGS technique and (B) the coverage of the gnomAD dataset in both exome and genome sequencing data. Since some NGS technologies are not capable of mapping sequence reads to specific regions of the gene, such as exon 26 in *VWF*, such regions should therefore be analyzed by Sanger sequencing.

[16], and 38% [34], respectively, totaling 447 different novel variants. This suggests that *VWF* is highly prone to nucleotide changes. However, the novel variants will introduce new challenges for understanding if these variants play a role in the disease mechanism of VWD. This is mainly due to the enormous number of *VWF* variants identified in VWD patients (>1500 distinct variants) and the fact that many of these variants have not been functionally tested based on cell or animal models. To investigate the novel variants, large population-based databases such as gnomAD should be utilized to filter out common variants, notwithstanding that some pathogenic VWF variants such as p.Arg854Gln, p.Tyr1584Cys, and p.Ser1731Thr can reach frequencies close to 1% in certain populations [25]. The effect of (missense and splicing) variants can be assessed using the combination of several *in silico* tools [3]. Investigating family members is important in understanding the pathogenicity of genetic variants and their inheritance patterns [3]. Another point is that many genetic variants responsible for VWD have not been identified and/or are not being reported yet, as was also shown in a population-scale genetic study in which the authors identified further 287 novel variants predicted to be pathogenic around the globe [25]. Laboratories are encouraged to report newly identified variants to international databases (such as the Human Gene Mutation Database, Leiden Open Variation Database, and ClinVar), along with associated clinical and laboratory characteristics.

A cautionary example of novel VWF variants is that of p.Asp1472His, a polymorphism associated with artificially decreased VWF ristocetin cofactor levels and VWF ristocetin cofactor/VWF antigen (VWF:Ag) ratios in several studies, resulting in VWF levels that may suggest type 2M VWD [35,36]. This is, indeed, a common variant with no clinical significance that is prevalent in the African American population (60%) [37], as well as moderately prevalent in Caucasians, with a minor allele frequency range of 7% to 11% in gnomAD. VWF p.Pro1467Ser, another variant located in the ristocetin-binding region of the A1 domain, has been reported in only one family [38]. Among more than 800,000 people from gnomAD, it was found only once, which, differently from p.Asp1472His, suggests the extreme rarity of the variant. These 2 variants (p.Asp1472His and p.Pro1467Ser) will provide normal activity levels when using an assay that does not require ristocetin [36].

5 | THE RATE OF MISSING VARIANTS IN VWD

VWD is known to be the result of genetic variants in *VWF*. However, over time, it has become evident that there are still patients with congenital VWD (all 3 VWD types) in whom the responsible VWF variant(s) is not identified. Disease-causing variants have been identified in more than 80% of type 1 cases with VWF levels <30 IU/dL [32], almost 60% of cases with VWF levels of 30 to 50 IU/dL [19], more than 95% of cases of type 2 VWD [32], and 85% to 95% of cases of type 3 VWD [16,32,39]. Thus, the lack of a pathogenic variant in the *VWF* does not exclude the diagnosis of VWD. In a recent study, *VWF* genetic variants were identified in only 88% of patients with VWF:Ag <30 IU/dL, 65% of patients with VWF:Ag of 30 to 50 IU/dL, and 92% of patients with type 3 VWD [34]. Since the authors also investigated patients for copy number variations, it is less likely that these patients with missing variants have variants within *VWF*, although variants in the deep intronic regions and distant regulatory sequences cannot be excluded. The importance of incorporating copy number variation analysis to genotype VWD types 1, 2, and 3 cases when no pathogenic *VWF* variant has been identified is highlighted in 2 independent studies [40,41]. In patients with VWD, copy number variation analysis is not routinely performed, although it is a valuable diagnostic tool. Of note, in the case of large deletions or duplications affecting only 1 allele, polymerase chain reaction–based techniques cannot identify the mutation.

The question regarding VWD cases with no pathogenic *VWF* variants that requires further investigation is (i) whether there are technological limitations to identifying the potential variants or (ii) whether genes other than *VWF* are involved in the VWD pathology. (i) As discussed earlier, NGS techniques have shown limitations in covering exon 26 of *VWF* [17]. Variants located in regulatory regions and deep intronic variants are not always recognized. Gross deletions and rearrangements may go undetected in exome or polymerase chain reaction–based sequencing. (ii) Several genes have been identified to influence VWF plasma levels by genome-wide association studies [42,43]. These genes are probably the next target of investigation in VWD patients with no obvious *VWF* variant, in particular, type 1 patients. Posttranslational modifications such as abnormal glycosylation have also been identified as VWF plasma level modifiers in VWD cases with partial deficiency [44]. Nonetheless, what is responsible for genetic defects in patients with more severe VWD forms (types 2 and 3) remains unknown.

6 | SHARED VARIANTS BETWEEN DIFFERENT VWD TYPES

For many VWD cases, the diagnosis and classification of VWD is straightforward, especially if both genotype and laboratory phenotype results are available. However, there is an overlapping phenotype between some cases of VWD that demands either performing specialized VWF assays or genetic testing to reach the final diagnosis and subtyping. This is the case for a large group of patients with type 2 phenotype, 2A(IIE), that overlaps with type 1 VWD. In addition, type 2B New York also has overlapping results with type 1 VWD. The classification of variants associated with quantitative VWD is further complicated by the following points. (i) There is evidence that severe type 1 VWD can rarely be autosomal recessive; this is distinct from type 3 VWD since VWF levels and multimers remain detectable [45]. (ii) Enhanced clearance of VWF is now classified as a distinct entity (type 1C VWD) [4], but whether this is a quantitative (ie, reduced levels due to enhanced clearance) or qualitative (ie, abnormal interaction of VWF with clearance receptors) trait remains to be determined. Among type 2 VWD also, there are other variants that

received different VWD classifications, eg, p.R1315L, p.R1374H, and p.R1374C. We proposed a new classification of VWD type 2M/2A for these variants because they share a common phenotype with 2M and 2A [46]. Due to the quantitative nature of the defects responsible for type 3 and type 1 VWD, some of the genetic variants identified in these 2 groups of patients are essentially the same [3]. However, type 1 is inherited as autosomal dominant and type 3 as a recessive or semidominant trait.

7 | DETERMINANTS OF DEVELOPING VWD

Recently Krahforst et al. [34] investigated 47 cases with VWF levels of 50 to 70 IU/dL and interestingly, almost 70% of them (33 of 47) had VWD-associated variants. Of the 36 identified variants, 22 were missense variants, 10 were null alleles, 2 were promoter variants, and 2 were gene conversions. Of note, the majority of the variants identified in this group were the same as those observed in type 1 with VWF <30 IU/dL or VWF of 30 to 50 IU/dL [34]. The question that arises here is why these individuals do not have a VWF level meeting diagnostic criteria (ie, <50 IU/dL) compared with those with type 1 VWD, notwithstanding having the same pathogenic variant(s). It probably can be explained by the fact that, in addition to the presence of a *VWF* pathogenic variant(s), several other players are important in determining the development of VWD (Figure 3). These include (i) the type of gene variants (dominant negative vs null variants), (ii) VWF level modifiers such as blood group, (iii) common *VWF* variants, and (iv) the presence of genetic variants other than those in *VWF*. (i) Because VWF has a quaternary structure (multimer), the circulating VWF is the product of both alleles; therefore, the dominant negative VWF variants in the mutated allele can compromise the structure, function, or amount of VWF produced from the normal *VWF* allele, leading to a more severe reduction of VWF. This is opposed to a null variant in 1 allele, which does not compromise the product of the other allele. (ii) The ABO blood group has a strong effect on plasma VWF levels; individuals with the O blood group have 20% to 30% lower VWF levels than those in individuals with the non-O blood group. It has been reported that the type of ABO blood group genotype can modify partial quantitative VWF deficiency, with type O individuals more likely to be diagnosed with type 1 VWD and more susceptible to severe bleeding diatheses than those type 1 VWD cases without O blood group [47,48]. Considering that the normal VWF range in the general population is 50 to 150 IU/dL, being heterozygous for a null allele variant, in combination with other VWF level modulators such as blood group, may create levels of about 25 to 75 IU/dL. Therefore, some people may have type 1 VWD, and many others will have VWF levels within the normal range [3]. (iii) While polymorphism alone does not have a strong effect on VWF levels, the accumulation of several of them can probably be impactful on the VWF levels [49]. A number of polymorphisms have been identified

FIGURE 3 Determinants of developing von Willebrand disease (VWD). In addition to the presence of a von Willebrand factor (*VWF*) pathogenic variant(s) and the penetrance of variants, there are several other players in VWD expression. These factors are arranged in clockwise order according to their approximately decreasing ability to affect VWF levels in VWD patient.

that reduce or even increase VWF levels [50,51] and hence are likely to influence VWD phenotype. (iv) As previously discussed, at least 18 genes are associated with VWF plasma levels through different mechanisms such as secretion (eg, *STXBP5* and *STX2*) and clearance (eg, *ABO*, *CLEC4M*, and *STAB2*) [42]. It is very likely that variants in these genes contribute to VWF levels and predispose some people to develop VWD or strengthen VWD severity in combination with *VWF* variants. Previous reports have shown that genetic variants in *STXBP5* and *CLEC4M* are associated with VWF level variation in type 1 VWD [52,53]. Other unknown modulators of VWF level cannot be excluded.

8 | PRESENCE OF MORE THAN ONE VARIANT IN THE VWF GENE

The presence of more than one variant in the *VWF* can modulate VWD phenotypic expression. It has been found that the number of rare nonsynonymous *VWF* genetic variants significantly affects VWF levels, regardless of VWD type, so the more rare nonsynonymous variants, the lower the VWF:Ag level [54]. The presence of an additional *VWF* variant has even been reported to change the VWD classification from a gain of function *VWF* variant to a loss of function variant (2B to 2M) [55]. The occurrence 2 two variants in the VWF D′D3 and D4 domains (p.Arg924Gln/p.Ala2178Ser) has also been reported to cause a type 2B-like phenotype [56]. Overall, these results emphasize that it is important to always consider the results of the full panel of VWD tests. Additionally, sequencing all *VWF* exons, flanking regions including the splice site boundaries, and the 5′ and 3′ untranslated regions is recommended, especially in severe VWD types or in cases where the phenotype cannot be explained by only one genetic variant.

9 | FREQUENT VWF GENE VARIANTS ASSOCIATED WITH VWD

Several variants have been reported to be frequent in specific VWD types, likely as a result of inheritance from a common ancestor (founder effects) or due to hotspots. In type 1 VWD patients, deletions of exons 4 and 5 [34,57], p.Tyr1584Cys [22,23], gene conversion harboring p.Val1229Gly and p.Asn1231Thr [34], and the Vicenza variant (p.Arg1205His [20]) are among the frequent ones. Within these, only p.Arg1205His segregates with a strong dominant pattern, whereas the remaining variants can also be found in patients with bleeding or individuals with no apparent bleeding symptoms. Among type 3 VWD, the most prevalent variants appear to be c.2435del, deletions of exons 4 and 5, and c.3931C>T (p.Gln1311*). The variant p.Gln1311* is often identified within a gene conversion including other nucleotide changes. The deletion c.2435del was identified in the original VWD family from the Åland Islands and has been frequently observed in European type 3 patients [58]. Since this deletion is in a stretch of 6 cytosine repeats, this may increase the possibility of a polymerase error during the replication [58]. In type 2 VWD, recurrent missense variants have been found in various studies for 2A (eg, p.Ile1628Thr, p.Arg1597Trp/Gln, and p.Ser1506Leu) [10,13], 2B (eg, p.Arg1306Trp/Gln/Leu, p.Arg1308Cys/Leu, p.Arg1341Trp/Gln, p.Val1316Met, and p.Pro1266Leu) [10,13], 2M (eg, p.Arg1315Cys and p.Arg1374Cys/His) [10,13], and 2N (eg, p.Arg854Gln and p.Arg816Trp) [13,28]. Some of these variants are either at CpG mutation hotspots and/or were found in the context of founder haplotypes [59,60].

10 | CONCLUSIONS

The utility of *VWF* genetic testing is increasing among laboratories working on VWD with the availability of NGS techniques. Genetic testing can be used to confirm or differentiate a VWD diagnosis and to provide genetic counseling [3]. Here, we attempt to highlight some of the common challenges and considerations when applying VWF molecular testing. Since NGS techniques are becoming more widely available, less specialized centers should be aware of these aspects, and caution needs to be exercised during genetic testing for VWD diagnosis.

ACKNOWLEDGMENTS

The Hemostasis & Thrombosis Unit of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico is member of the European Reference Network on Rare Haematological Diseases EuroBloodNet-Project ID No 101157011. ERN-EuroBloodNet is partly co-funded by the European Union within the framework of the Fourth EU Health Programme. The Department of Pathophysiology and Transplantation, University of Milan, is funded by the Italian Ministry of Education and Research (MUR): Dipartimenti di Eccellenza Program 2023 to 2027. We also acknowledge L.F. Ghilardini for the illustration work.

FUNDING

This work was partially supported by the Italian Ministry of Health (Bando Ricerca Corrente).

AUTHOR CONTRIBUTIONS

O.S. wrote the manuscript. L.B. and F.P. critically revised the manuscript. All authors have approved the final manuscript.

RELATIONSHIP DISCLOSURE

F.P. reports participation at educational meetings of Takeda and Spark and on the advisory board of CSL Behring, Biomarin, Roche, Sanofi, and Sobi. The other authors state that they have no conflict of interest.

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