Received: 27 November 2024 | Revised: 23 January 2025 | Accepted: 19 February 2025

https://doi.org/10.1016/j.rpth.2025.102730

ORIGINAL ARTICLE

Clinical utility of panel-based genetic sequencing for von Willebrand disease

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Handling Editor: Dr Suely Meireles Rezende

Abstract

Background: von Willebrand disease (VWD) is the most prevalent inherited bleeding disorder with a wide spectrum of causative variants. Next-generation sequencing analyzes the entire *VWF* gene and provides concomitant assessment of other genes, allowing differentiation between genocopies.

Objectives: We aimed to assess the clinical impact of panel-based sequencing in all VWD patients sequenced at UZ Leuven.

Methods: We conducted a single-center retrospective study of all patients with confirmed or suspected VWD who were screened with panel-based whole-exome sequencing. Presequencing diagnosis was performed using laboratory measures of VWF activity and quantity. Postsequencing diagnosis was informed by variant curation in combination with laboratory measures. We measured clinically meaningful changes in the pre- vs postgenetic sequencing diagnosis and subtyping.

Results: The study included 108 patients. The population was predominantly composed of pediatric patients <18 years old (77/108; 71%) and females (66/108; 61%). The largest presequencing subgroup was those with low VWF (61/108; 56%), followed by type 1 VWD (21/108; 19%) and type 2 not otherwise specified (18/108; 17%). A clinically meaningful change in management occurred in 19% (20/108) of the study population. The largest effect was seen in the presequencing type 2 group (16/24; 67%). In the type 2 group who could not be accurately subtyped into 2A/B/M/N prior to sequencing (type 2 not otherwise specified), 15/18 (83%) were able to be subtyped or given a different diagnosis postsequencing.

Conclusion: Panel-based sequencing for VWD in a well-selected cohort, particularly those with type 2 and type 3 VWD, was clinically relevant in differentiating genocopies, directing therapies, and family planning. Sequencing in those with low VWF and type 1 VWD rarely changed management.

KEYWORDS
genotype, high-throughput nucleotide sequencing, phenotype, von Willebrand diseases

Essentials

- VWD can be difficult to diagnose and subtype.
- Full laboratory testing for VWD is not always available or consistently accurate.
- Panel-based sequencing impacted management in 67% of type 2 VWD.
- Sequencing results differentiate genocopies, guide therapies, and support family planning.

1 | INTRODUCTION

von Willebrand disease (VWD) is the most prevalent inherited bleeding disorder. The prevalence is commonly described as ~1% based on population studies [1,2], but ~1/10,000 based on symptomatic presentation to specialists [3]. Inheritance can be autosomal dominant or recessive, and varies between subtypes. The disorder is caused by deficient or defective von Willebrand factor (VWF), a protein crucial for platelet adhesion and aggregation at sites of endothelial injury and chaperoning coagulation factor (F)VIII in plasma [4]. The degree and type of VWF defect informs the VWD subtype. The diagnosis and subtyping of VWD have traditionally relied on criteria based on VWF assay results [5]. However, recent advancements in genetic testing technology have accelerated its early use in the work-up of VWD in clinical practice. The spectrum of genetic variants in VWD occurs over the wide breadth of the large ~180 kb, 52 exon long *VWF* gene [6]. Next-generation sequencing is becoming a useful modality in the diagnosis of this condition as it allows the entire gene to be analyzed. It also allows concomitant assessment of other genes, which can help to distinguish between genocopies such as platelet-type VWD and hemophilia A (HA).

We aimed to evaluate the clinical impact of panel-based sequencing in all VWD patients who have undergone sequencing at Universitair Ziekenhuis (UZ) Leuven, Belgium. We identified cases in which a change in diagnosis or more precise prediction of subtype was made possible by genetic sequencing. We also evaluated how frequently a clinically meaningful impact resulted from a sequencing result. This included a change in diagnosis from VWD to a different disorder, or when the VWD subtype was changed or specified such that management would change.

2 | METHODS

2.1 | Patient selection

We conducted a single-center retrospective study of all patients with suspected VWD and low VWF who had panel-based whole-exome sequencing (WES) performed at UZ Leuven, Belgium, from January 2019 to April 2024. The study was approved by the ethics committee of Katholieke Universiteit (KU) Leuven (S69290). Supplementary Figure 1 illustrates the flow chart for patient selection. Patients were selected for study inclusion if they had the coagulation disorder WES panel performed [7] (Supplementary Table) and had laboratory measures fitting the criteria for low VWF or VWD [8]. Diagnosis and subtyping were undertaken with reference to the original guidelines by 1994 [5] and 2006 [9] guidelines by Sadler et al. and the more recent American Society of Hematology (ASH), the International Society on Thrombosis and Haemostasis (ISTH), the National Hemophilia Foundation (NHF), and the World Federation of Hemophilia (WFH) 2021 guidelines on the diagnosis of VWD [10] (Figure 1).

The set of results associated with the lowest platelet-binding activity of VWF (VWF:recombinant glycoprotein Ib [GPIbR]) result was used for each patient in the event of multiple testing. This was done to reduce collecting results impacted by confounding influences on VWF levels that can occur with inflammation, malignancy, elevated estrogen levels, and other physiological stressors [11–14] and also to avoid using elevated levels associated with desmopressin response testing.

2.2 | Laboratory testing and presequencing diagnosis

The laboratory testing included VWF antigen (VWF:Ag), platelet-binding VWF activity (VWF:GPIbR), and FVIII activity (FVIII:C) for all patients. If available, ristocetin-induced platelet agglutination (RIPA) and VWF multimer analysis were also used in the diagnostic and subtyping evaluation. VWF:Ag testing was performed by an immunoturbidimetric assay using latex microparticles covered in anti-VWF antibodies (HemosIL AcuStar VWF:Ag; Werfen). VWF:GPIbR testing was performed by a chemiluminescent immunoassay using magnetic beads coated in GPIbR in the presence of ristocetin (HemosIL AcuStar VWF:RCo; Werfen). FVIII:C was assessed using a 1-stage activated partial thromboplastin time on an ACL TOP (Werfen). Multimer analysis was performed using gel electrophoresis. A validated FVIII binding assay is not performed in this laboratory.

Low VWF was based on either VWF:Ag or VWF:GPIbR being <50% and both levels being above 30%. Bleeding data were not available, so it is unclear how many of these patients with VWF levels between 30% and 50% had a bleeding phenotype. Type 1C VWD (type 1 VWD with increased VWF clearance) was not able to be diagnosed as the propeptide level was not measured, and data on response to desmopressin were not collected. Subtyping for types 2A, 2B, and 2M was first based on a functional VWF level between 5% and 30% with an activity/antigen ratio of <0.7. Type 2A was then based on the absence of enhanced agglutination to low-dose ristocetin in RIPA and the loss of high-molecular-weight multimers in multimer analysis. Type 2M was based on the absence of enhanced agglutination to

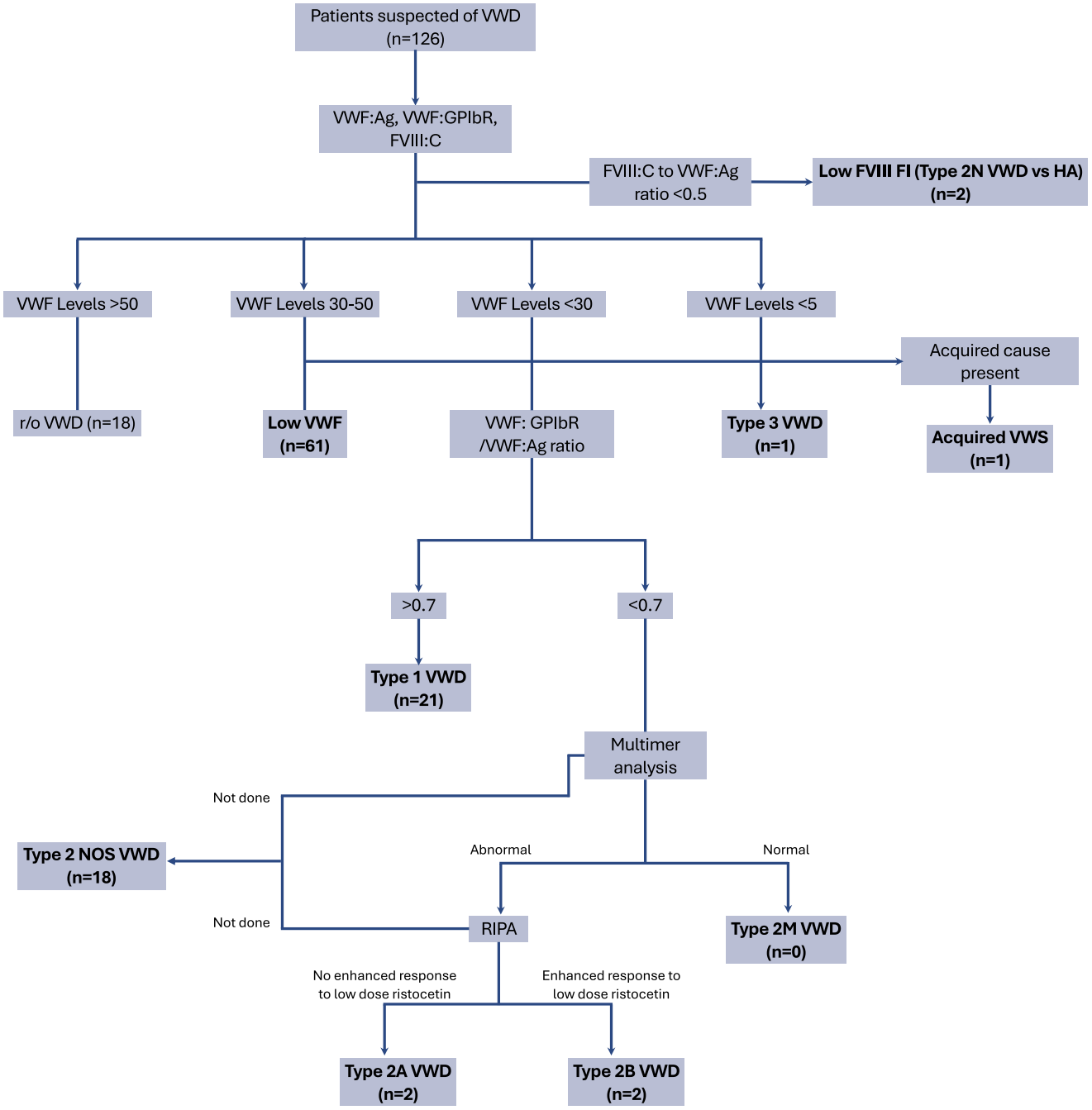

FIGURE 1 Patient subtyping flowchart. FI, for investigation; FVIII, factor VIII; FVIII:C, factor VIII activity assay; HA, hemophilia A; NOS, not otherwise specified; RIPA, ristocetin-induced platelet agglutination; r/o, rule out; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, VWF antigen assay; VWF:GPIbR, VWF platelet-binding activity of VWF using recombinant glycoprotein Ib on magnetic beads in the presence of ristocetin; VWS, von Willebrand syndrome.

low-dose ristocetin in RIPA and a normal multimer pattern. Type 2B was based presence of enhanced agglutination to low-dose ristocetin in RIPA. Type 2N was suspected based on a FVIII/VWF ratio < 0.5, irrespective of how low the antigenic level was; however, it could not be confirmed using laboratory testing. If a type 2 subtype could not be allocated based on the above, a type 2 not otherwise specified (NOS) diagnosis was allocated. Type 3 was diagnosed based on a VWF:Ag < 5%.

2.3 | Genetic sequencing and postsequencing diagnosis

Genetic testing was performed by the coagulation subpanel of the UZ Leuven Thrombosis-Haemostasis gene panel test using WES [7]. As described previously by Van Laer et al. [7], this 31-gene coagulation panel is targeted at genes associated with inherited bleeding disorders and is included in the Supplementary Table. If type 2B VWD was

suspected, *GP1BA* was additionally analyzed to exclude platelet-type VWD. WES was performed on the sequencing platform NovaSeq 6000 (Illumina), and Alissa Interpret software v5.2-5.4 (Agilent) was used for variant interpretation. Assessment of copy number variation was validated within the bioinformatics pipeline. The American College of Medical Genetics and Genomics and the Association of Molecular Pathology guidelines were used to classify variants as pathogenic (P), likely pathogenic (LP), a variant of uncertain significance (VUS), or (likely) benign (LB, B) variants [12,15]. Note that all variants were annotated according to the Human Genome Variation Society nomenclature in reference to the Matched Annotation from NCBI and EMBL-EBI (MANE) Select transcript for the *VWF* gene (NM_000552.5).

Postsequencing subtyping and diagnosis were informed by variant curation, including a literature review. A literature search for each sequencing result was performed with the use of the genomic search engine Mastermind [16] and a review in ClinVar, a publicly accessible genomic database maintained by the National Centre for Biotechnology Information [17]. The postsequencing diagnosis/subtype was then assigned based on the subtype associated with each variant published in the identified literature in conjunction with a rereview of the VWF laboratory measures. If there were conflicting results even after examination of the specific laboratory testing (if provided) used to assign a subtype in the publication, all possible subtypes were included. All variants were submitted to the publicly accessible GoldVariants database which streamlines variant entry into ClinVar [18].

The change in diagnosis/subtype from presequencing to postsequencing was evaluated in terms of clinical utility. A clinically meaningful change was defined as:

- Confirmation of type 2 VWD subtype as it informs the use of desmopressin.
- Differentiation of type 2B VWD from platelet-type VWD as it affects hemostatic product use.
- Differentiation of type 2N VWD from nonsevere HA as it affects hemostatic product use.
- Differentiation of congenital VWD from acquired von Willebrand syndrome (AVWS) as it drives the search for an underlying cause if acquired and impacts management (ie, of underlying cause vs purely hemostatic therapies).
- Characterization of type 3 VWD as it assists in prenatal diagnosis/family planning purposes and predicts inhibitor risk.

3 | RESULTS

3.1 | Presequencing analysis

This study analyzed 108 patients (107 unrelated patients) who underwent panel-based genetic sequencing during the investigation of VWD. Clinical characteristics of the study population divided into presequencing subgroups are provided in Table 1. The population was predominantly composed of pediatric patients <18 years old (77/108; 71%) and females (66/108; 61%).

The largest presequencing subgroup was those with low VWF (*n* = 61; 57%), followed by type 1 VWD (*n* = 21; 20%) and type 2 NOS (*n* = 18; 17%). There were small numbers of patients with type 2A (*n* = 2; 2%), type 2B (*n* = 2; 2%), low FVIII for investigation (FI; *n* = 2; 2%), type 3 (*n* = 1; 1%), and suspected acquired von Willebrand syndrome (AVWS; *n* = 1; 1%). The VWF:Ag, VWF:GPIbR, and FVIII:C levels were available for all patients, given that the absence of one of these results was an exclusion criterion. Collagen binding was not performed. In terms of second-line testing, RIPA testing was performed in 18/23 (78%) and multimer testing in 4/23 (17%) presequencing types 2A and 2B and type 2 NOS patients. Levels of VWF quantity and activity, FVIII:C, platelet count, and blood group O frequency are presented in Table 1. In the low VWF cohort, 84% (42/50) of patients who had blood grouping results available were blood group O, with a lower proportion in the type 1 cohort (56%; 9/16; Table 1, Figure 2A, B). Both are higher than the frequency of blood group O in the general Belgian population, which is reported as 45% by the Belgian Red Cross [19].

3.2 | Postsequencing analysis

Postsequencing, there were *n* = 61 patients in the low VWF cohort, *n* = 20 in the type 1 subgroup, *n* = 22 in the type 2 subgroup, *n* = 1 with type 3 VWD, and *n* = 1 with AVWS. The mutational landscape in the *VWF* gene is provided in Figure 2. Correlating with published cohorts of VWD patients [6,20], variants occurred over the entire breadth of the gene. Most variants occurred in the A1 domain. The p.Tyr1584Cys mutation in the A2 domain was the most frequent variant detected. This variant is associated with mild quantitative reductions in VWF, ie, a low VWF or type 1 phenotype, both in the literature [21–24] and in our study population. The pie charts in Supplementary Figure 2 illustrate the breakdown of variant type in the postsequencing low VWF, type 1, and type 2 subgroups. Patients with low VWF were most likely to have no variant identified (43/61; 70%), with no variant detected in 20% (4/20) and 14% (3/22) of type 1 and type 2 patients, respectively. The predominant variant type was missense, present in 60% (12/20) of type 1 and 82% (18/22) of type 2 patients. A further breakdown by blood group and variant classification for the presequencing low VWF and type 1 patients is illustrated in Figure 3A, B. Only 16% (10/61) of those with low VWF had a (L)P (ie, causative) variant identified compared with 52% (11/21) of type 1 VWD patients. In those with low VWF who had blood grouping results available (*n* = 50), 33 out of the 42 group O patients (79%) had no variant detected, and 5/8 (63%) of nongroup O patients had a variant detected.

Of the 24 presequencing type 2 patients (including the low FVIII FI), 83% (20/24) had a causative variant identified by sequencing. The single patient with type 3 VWD had 2 causative variants identified in

TABLE 1 Characteristics of the presequencing study population.

Patient characteristics	Low VWF (*n* = 61)	Type 1 VWD (*n* = 21)	Type 2A VWD (*n* = 2)	Type 2B VWD (*n* = 2)	Low FVIII FI (*n* = 2)	Type 2 NOS VWD (*n* = 18)	Type 3 VWD (*n* = 1)	AVWS (*n* = 1)	Total VWD population (*N* = 108)
Age (y) at testing, median (range)	6 (0-48)	13 (0-55)	6 (2-10)	20 (8-32)	16 (1-31)	12.5 (0-64)	1	47	6 (0-64)
Paediatric (<18 y), *n* (%)	50 (82)	13 (62)	2 (100)	1 (50)	1 (50)	9 (50)	1 (100)	0 (0)	77 (71)
Adult (≥ 18 y), *n* (%)	11 (18)	8 (38)	0 (0)	1 (50)	1 (50)	9 (50)	0 (0)	1 (100)	31 (29)
Sex at birth *n* (%)									
Female	36 (59)	14 (67)	1 (50)	1 (50)	1 (50)	11 (61)	1 (100)	1 (100)	66 (61)
Male	25 (41)	7 (33)	1 (50)	1 (50)	1 (50)	7 (39)	0 (0)	0 (0)	42 (39)
Laboratory assays for VWD									
VWF:Ag (%), median (range)	44.7 (30.8-67.5)	22.8 (6.5-38.8)	18.15 (16.1-20.2)	40.15 (29.3-51)	44.4 (39.5-49.3)	26.9 (8.2-74.6)	1.5	2.6	41.3 (1.5-74.6)
VWF:GPIbR (%), median (range)	41.4 (30.2-59.2)	19.8 (4.6-32.1)	5.95 (3.4-8.5)	21.8 (13.6-24.8)	39.85 (35.8-43.9)	11.5 (2.1-28.4)	0.6	2.2	35.4 (0.6-59.2)
FVIII:C (%), median (range)	63.4 (27.2-159.1)	38.7 (8-94.6)	22.5 (17-28)	58.05 (48-68.1)	4.5 (2.5-6.5)	34.45 (10-89.3)	12.2	7	56.45 (2.5-159.1)
Platelet count ($\times 10^9$/L), median (range)	330.5 (96-757)	278 (121-395)	389.5 (242-537)	220 (176-264)	231 (110-352)	330.5 (142-570)	369	325	323.5 (96-757)
RIPA performed, *n* (%)	49 (80)	15 (71)	2 (100)	2 (100)	1 (50)	14 (78)	0 (0)	1 (100)	84 (78)
Multimers performed, *n* (%)	1 (2)	0 (0)	2 (100)	1 (50)	0 (0)	1 (6)	0 (0)	0 (0)	5 (5)
Blood group O (of those who had blood group results available), *n* (%)	42/50 (84)	9/16 (56)	1/2 (50)	1/2 (50)	1/1 (100)	5/10 (50)	1/1 (100)	0/1 (0)	60/83 (72)

AVWS, acquired von Willebrand syndrome; FI, for investigation; FVIII, factor VIII; FVIII:C, factor VIII activity assay; NOS, not otherwise specified; RIPA, ristocetin-induced platelet agglutination; VWD, von Willebrand disease; VWF, von Willebrand factor; VWF:Ag, VWF antigen assay; VWF:GPIbR, VWF platelet-binding activity using recombinant glycoprotein Ib on magnetic beads in the presence of ristocetin.

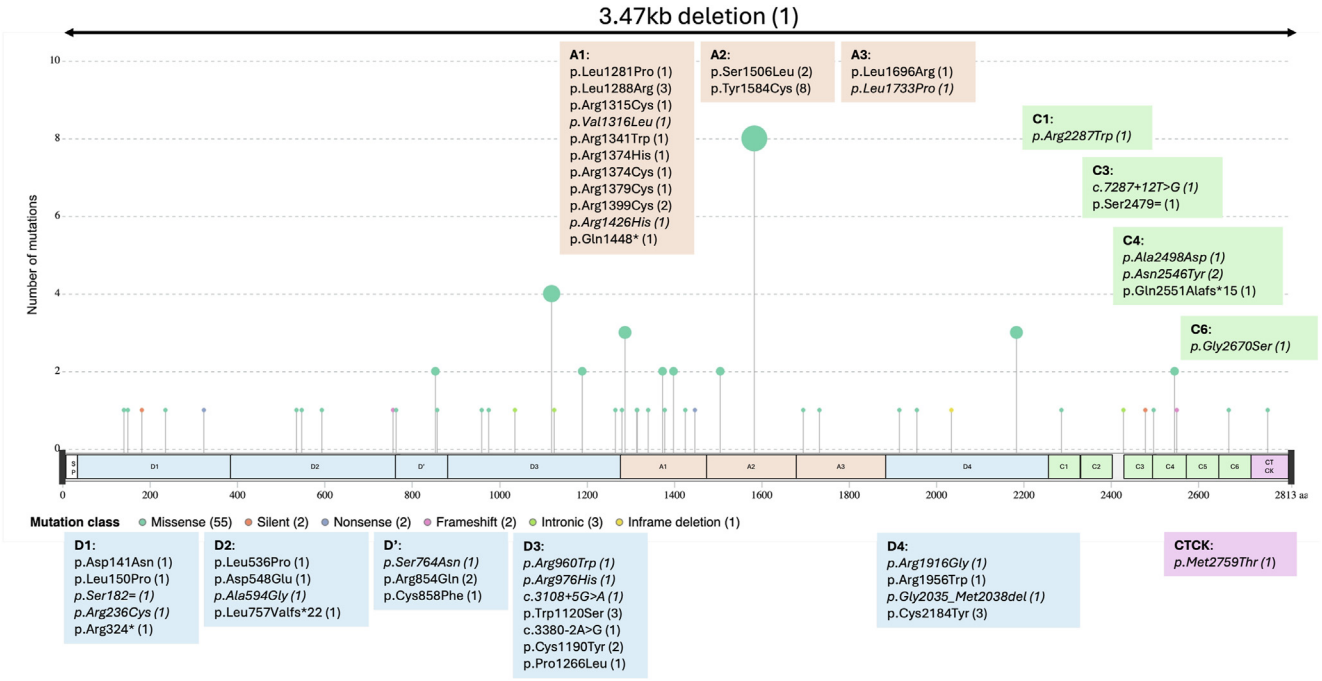

FIGURE 2 Lollipop plot outlining variants identified in *n* = 107 kindreds (2 related family members in the *N* = 108 population), separated by domains. The number in brackets is the number of kindreds with variants. Variants in italics are variants of uncertain significance; all nonitalicized variants are (likely) pathogenic. CTCK, C-terminal cystine knot.

addition to a VUS. Table 2 outlines the 7 novel variants detected in this cohort, deemed novel by virtue of not having been described previously in the published literature nor submitted to ClinVar apart from a submission by UZ Leuven. The *in silico* prediction scores used were the ensemble predictor REVEL (rare exome variant ensemble learner) [25] and spliceAI [26] for splicing prediction, with a REVEL score of >0.7 and a spliceAI score of >0.2, raising suspicion for a deleterious effect.

3.3 | Changes in pre- to postsequencing diagnosis/subtyping and clinical utility

The pre- to postsequencing change in diagnosis or subtype for VWD and AVWS is illustrated in Figure 3C. The low VWF cohort all remained as low VWF after genetic sequencing, given that this entity was purely based on minor reductions in VWF levels. In only 1 of these low VWF patients, a variant was identified that was associated with a functional rather than quantitative VWF reduction in the literature. This p.Leu536Pro variant was listed in only 1 publication, in which the variant was associated with type 2A VWD [26,27] (Figure 3A). There were 3 type 1 VWD patients with possible or likely type 2M variants (Figure 3C).

Of the 2 patients with a presequencing 2A subtype, 1 was deemed more likely to occur in type 2M. One of the 2 type 2B patients harbored a 2B Malmo/New York variant (p.Pro1266Gln), which is a subtype of type 2B with a distinct laboratory and clinical profile. There were 2 patients initially queried as having type 2N VWD due to low FVIII, who were subsequently both confirmed to have causative variants in *F8* and thus diagnosed as HA. One of these patients was an adult 31-year-old female with a mild FVIII reduction of 6.5%, while the other was a 1-year-old infant with a moderately reduced FVIII level of 2.5%. Both patients had mild reductions in VWF:Ag to 49.3% and 39.5%, respectively, with the latter, ie, the infant male, having blood group O. The 31-year-old female had a VUS variant in *VWF* (p.Ala594Gly), while the infant had no *VWF* variants. Another patient with a presequencing type 2 NOS phenotype had both a variant associated with type 2B (*VWF* p.Val1316Leu) and an *F8* variant (*F8* p.Trp1961*) identified, resulting in a combined diagnosis of both type 2B VWD and HA.

The largest change in diagnosis or subtype occurred in the type 2 NOS subgroup. In this type 2 NOS group, 15/18 (83%) patients had a more specific subtype or a different diagnosis made after genetic sequencing. Conflicting results occurred 4 times due to the inability to distinguish once between type 1 and type 2M (this case was included as a postsequencing type 1 case as they were classified type 1 presequencing) and 3 times between type 2A and type 2M.

A clinically meaningful impact from sequencing results, as defined in the Methods section, occurred in 19% (20/108) of the study population (Table 3 [28–57], Figure 3D). The largest effect was seen in the type 2 group, with 67% (16/24) of presequencing type 2 patients clinically impacted by the sequencing result. We included the 2 patients with low FVIII FI in this type 2 presequencing group, given the suspicion of type 2N VWD. The most frequent clinical impact was the

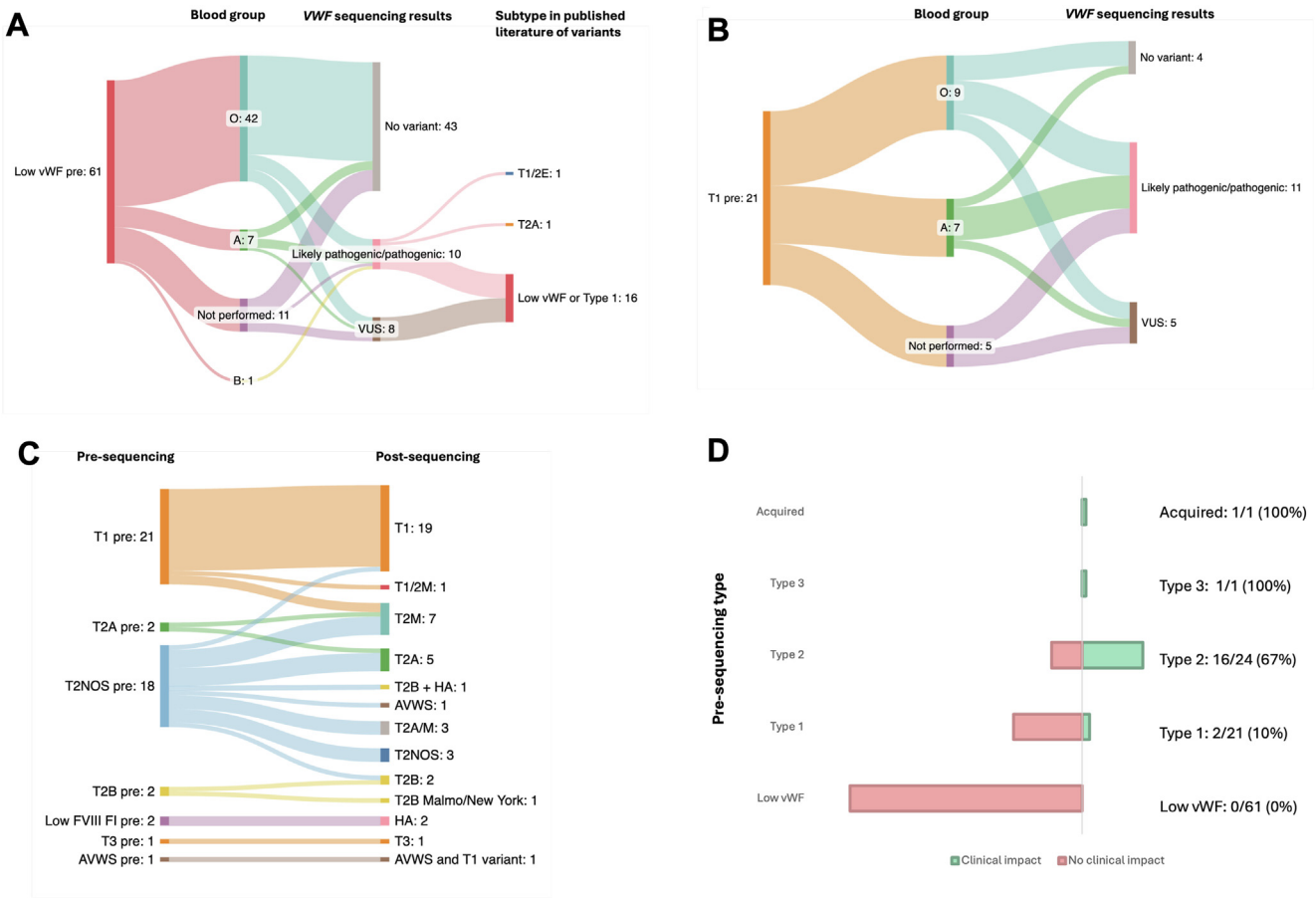

FIGURE 3 Sankey plot demonstrating a breakdown by blood group and sequencing results for the (A) low von Willebrand factor (VWF) cohort and (B) type 1 von Willebrand disease cohort. (C) Sankey plot demonstrating the change in subtype/diagnosis postsequencing in von Willebrand disease and acquired von Willebrand syndrome (AVWS) cases. Low VWF patients were excluded from this figure. (D) Clinical impact of sequencing result with a breakdown by postsequencing subtype. FI, for investigation; FVIII, factor VIII; HA, hemophilia A; NOS, not otherwise specified; VUS, variant of uncertain significance.

clarification of the type 2 subtype in those with presequencing type 2 NOS VWD (83%; 15/18), informing desmopressin use in those with type 2B vs those with type 2A, 2M or variant 2B subtypes. The 2 patients with low FVIII FI and another with type 2 NOS had *F8* gene variants, confirming a HA diagnosis that has significant implications for the choice of hemostatic therapies and familial genetic counseling. Of the 21 presequencing type 1 patients, 2/21 (10%) had a clinically significant sequencing result due to reclassification as type 2M VWD based on a literature review. For the patient with type 3 VWD, identifying the variants was required for carrier testing to inform familial risk and pregnancy planning and predict inhibitor risk, which tends to be highest in those with deletions [58,59].

In the patient with suspected AVWS secondary to a monoclonal gammopathy, the recurrent p.Tyr1584Cys variant was identified. However, the laboratory parameters in this patient were much lower than typically observed in cases with this variant [21–24], and thus, an acquired component was still deemed highly likely. It is unclear what contribution, if any, the p.Tyr1584Cys variant had in the development of the AVWS in this patient. A larger series of AVWS with sequencing results would be needed to identify if *VWF* variants are enriched in this cohort. The presence of the variant would explain why VWF parameters may not return to normal levels after addressing the AVWS trigger and is thus clinically informative.

4 | DISCUSSION

Our study demonstrates that the limited availability and precision of the full gamut of laboratory testing for VWD contributes to inaccurate diagnosis and subtyping in a proportion of patients with VWD. In these patients, panel-based genetic sequencing can clarify the diagnosis and impact management. The utility of sequencing was highest in type 2 and type 3 VWD, and low in low VWF and most type 1 patients. Concomitant variants were frequently present and may modulate phenotype, although clinical phenotyping was beyond the scope of this study.

The largest group within this study population were those labeled "low VWF." Some bodies advocate the inclusion of those with low VWF and a bleeding phenotype into type 1 VWD [10]; however, this suggestion is not universally followed for classification in routine

TABLE 2 Novel mutations with *in silico* predictions, frequency in the Genome Aggregation Database version 4, and American College of Medical Genetics and Genomics/Association of Molecular Pathology classification.

Age (y)	Sex	Presequencing	Postsequencing	*VWF* variant cDNA	*VWF* variant protein	REVEL	spliceAI	gnomAD v4 allele frequency (%)	ACMG/AMP classification
0	M	Low VWF	Low VWF	c.4342C>T	p.Gln1448*	NA	NA	0	LP
2	M	Low VWF	Low VWF	c.5746C>G	p.Arg1916Gly	0.21	0.00	0.00048	VUS
3	M	Low VWF	Low VWF	c.7287+12T>G	Splicing defect	NA	0.03	0.00027	VUS
4	F	Type 1 VWD	Type 1 VWD	c.6104_6115delGTGGGAACATGG	p.Gly2035_Met2038del	NA	0.02	0	VUS
17	M	Type 1 VWD	Type 1 VWD	g.5853982_6200592del	Null	NA	NA	0	LP
1	M	Type 2 NOS VWD	Type 1 VWD	c.4342C>T	p.Gln1448*	NA	NA	0	LP
5	F	Type 2 NOS VWD	Type 2A/M VWD	c.4277G>A	p.Arg1426His	0.416	0.00	0.0006	VUS

ACMG, American College of Medical Genetics and Genomics; AMP, Association of Molecular Pathology; cDNA, complementary DNA; gnomAD, Genome Aggregation Database; LP, likely pathogenic; NA, not available; NOS, not otherwise specified; VUS, variant of uncertain significance; VWD, von Willebrand disease; VWF, von Willebrand factor.

practice. We did not include clinical bleeding data, and thus, phenotype did not impact whether a patient was placed in the "low VWF" subgroup. Genetic screening in this population did not result in a change in diagnosis. The absence of a detected variant does not preclude a low VWF diagnosis, nor does a particular variant result in a change in the diagnosis to another subtype. It is clear that there are other modifiers of VWF levels apart from the *VWF* gene, which only contributes to between 25% and 75% of VWF level heritability [60–63]. Chief among these is the *ABO* gene locus [64]. Those with low VWF were highly likely to be blood group O (84%); if blood group O, they were unlikely to have a *VWF* variant identified (79%). We also note that given that this was a WES assay, we did not screen the *VWF* noncoding and regulatory regions. In a study of patients with low VWF, those without a variant were more likely to normalize their VWF parameters over time; however, the difference in bleeding risk in those with vs those without variants was not analyzed [65]. Repeat intraindividual assessments in this study were also not done, so it is unclear if bleeding risk in a patient changes once VWF levels normalize. Future research directed toward these questions may assist in decision-making regarding the utility of sequencing in this low VWF cohort. Additionally, knowing the genetic status of low VWF and type 1 VWD patients may be of benefit to clinicians in providing information about the genetic origins of the condition and predicted natural history of laboratory parameters to patients.

One patient with type 2B VWD had the Malmo/New York variant (p.Pro1266Leu). Type 2B VWD Malmo/New York is characterized by increased RIPA, normal multimer patterns, no thrombocytopenia even with a desmopressin challenge, and a milder bleeding phenotype than classical type 2B VWD [34,35,37,66,67]. Further subtyping of type 2B has been recommended by Casonato et al. [36] based on the presence (2B-II) or absence (2B-I) of high-molecular-weight multimers, which correlates to the absence or presence of thrombocytopenia in response to desmopressin, respectively. Discerning between 2B and the 2B variant Malmo/New York (Pro1266Leu/Gln) ie, type 2B-II, is important as desmopressin may be used safely in the latter [33] but has historically been avoided in the former, despite low-level evidence that it may be safe [68]. Testing the platelet count can also help in this distinction, although not all 2B-I subtypes have a low platelet count [36]. Thus, genetic sequencing is a valuable tool for such granular subtyping.

The knowledge that a proportion of type 2 VWD has been misidentified as type 1 using the 3-prong testing approach of VWF:Ag, VWF:GPIbR assays, and FVIII is not novel [69]. In our study, 3 patients with presequencing type 1 VWD harbored variants that were possibly or likely secondary to type 2M VWD [28–32]. The addition of improved functional assessments (eg, VWF collagen binding, VWF:CB) and consistent multimer analysis may improve subtyping. However, multimer analysis is time-consuming, requires expertise to perform, and can misidentify subtypes based on the multimer pattern identified [70]. Whether there is a clear clinical benefit in distinguishing type 1 from type 2M is also debatable, although there is an argument for potentially greater bleeding and poorer response to desmopressin in type 2M compared with type 1 [46]. A similar challenge occurs in

TABLE 3 Genetic sequencing results that impacted clinical management.

Age (y)	Sex	Presequencing	Postsequencing	*VWF* variant DNA	*VWF* variant protein	Classification	Comutations	Clinical relevance	References
0	F	Type 1 VWD	Type 2M VWD	c.5087T>G	p.Leu1696Arg	LP		Type change Confirmed not type 2B VWD, informs desmopressin use	[28,29]
33	F	Type 1 VWD	Type 2M VWD	c.3863T>G	p.Leu1288Arg	LP		Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[30–32]
32	F	Type 2B VWD	Type 2B Malmo/New York VWD	c.3797C>T	p.Pro1266Leu	P		Milder phenotype than other classical type 2B VWD, informs desmopressin use	[33–37]
1	M	Low FVIII FI	HA	NA	NA	NA	*F8* p.Trp1836Arg (LP)	Exclude type 2N VWD and confirm HA	NA
31	F	Low FVIII FI	HA	c.1781C>G	p.Ala594Gly	VUS	*F8* p.Met1? (LP)	Exclude type 2N VWD and confirm HA. Genetic counseling (HA carrier status–the risk of male offspring with HA)	[38]
1	M	Type 2 NOS VWD	Type 1 VWD	c.4342C>T	p.Gln1448*	LP		Type change	No publications of this variant identified
2	F	Type 2 NOS VWD	Type 2B VWD + HA	c.3946G>T	p.Val1316Leu	VUS	*F8* p.Trp1961* (LP)	Clarified subtype Confirmed type 2B VWD, informs desmopressin use (HA carrier status–the risk of male offspring with HA)	[33,36,39]
4	M	Type 2 NOS VWD	Type 2M VWD	c.3863T>G	p.Leu1288Arg	LP		Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[30–32]
5	F	Type 2 NOS VWD	Type 2A/M VWD	c.4277G>A	p.Arg1426His	VUS	*F11*:c.1481-9T>A (VUS) *F5* p.Gly774Ala (VUS)	Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	No publications of this variant identified
6	F	Type 2 NOS VWD	Type 2M VWD	c.4195C>T	p.Arg1399Cys	LP		Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[32,40]
19	F	Type 2 NOS VWD	Type 2A VWD	c.4517C>T	p.Ser1506Leu	LP		Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[41–45]
28	M	Type 2 NOS VWD	Type 2M VWD	c.3943C>T	p.Arg1315Cys	P		Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[41,46]
37	F	Type 2 NOS VWD	Type 2A VWD	c.3569G>A	p.Cys1190Tyr	LP	*F7* p.Arg326Gln (VUS)	Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[47–49]
39	F	Type 2 NOS VWD	Type 2A VWD	c.3569G>A	p.Cys1190Tyr	LP		Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[47–49]
40	M	Type 2 NOS VWD	Type 2M VWD	c.4195C>T	p.Arg1399Cys	LP		Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[32,40]
43	F	Type 2 NOS VWD	Type 2A/M VWD	c.4120C>T	p.Arg1374Cys	P		Clarified subtype Confirmed not type 2B VWD, informs desmopressin use	[41,50]

(Continues)

TABLE 3 (Continued)

Age (y)	Sex	Presequencing	Postsequencing	*VWF* variant DNA	*VWF* variant protein	Classification	Comutations	Clinical relevance	References
48	F	Type 2 NOS VWD	Type 2B VWD	c.3842T>C	p.Leu1281Pro	LP		Clarified subtype Confirmed type 2B VWD, informs desmopressin use	[51]
64	M	Type 2 NOS VWD	AVWS	NA	NA	NA		Diagnosis change	
1	F	Type 3 VWD	Type 3 VWD	c.421G>A c.7437G>A	p.Asp141Asn p.Ser2479=	P	*VWF* p.Thr2647Met (VUS)	Genetic counseling	[52–57]
47	F	AVWS	AVWS + type 1 VWD variant	c.4751A>G	p.Tyr1584Cys	LP	*FGG* p.Ala108Gly (LP) *F7* p.Pro70Ser (VUS)	Diagnosis change	[21–24]

AVWS, acquired von Willebrand syndrome; FI, for investigation; FVIII, factor VIII; HA, hemophilia A; LP, likely pathogenic; NA, not available; NOS, not otherwise specified; P, pathogenic; VWD, von Willebrand disease; VWF, von Willebrand factor; VUS, variant of uncertain significance.

discrimination between types 2A and 2M, which was further obscured in our study by the infrequent performance of multimer assessment. Here again, the question about the importance of this distinction is raised, noting, however, that a prospective study suggested increased bleeding risk in type 2A compared with 2M [71]. Seidizadeh et al. [41] have recently proposed a new classification for type 2 VWD with the mutations p.Arg1315Leu, p.Arg1374His, and p.Arg1374Cys as an overlap type 2M/A, given the shared laboratory phenotype present in cases harboring these variants. We identified 2 such patients with a variant at the p.Arg1374 position in our study population. A validated FVIII binding assay is not performed in this laboratory, and thus, confirmation of a type 2N VWD can only be performed using genetic testing. These issues with the availability and reliability of laboratory phenotyping are realistic challenges faced by many laboratories, where the full gamut of laboratory phenotyping required for accurate subtyping is not consistently available and, even if available, does not always result in a clearly defined subtype [72,73].

A strength of this study is the use of the VWF:GPIbR assay to measure VWF platelet-binding activity. Recommendations from the ASH ISTH NHF WFH 2021 guidelines suggest using platelet-binding activity assays such as the VWF:GPIbR or VWF:GPIbM (mutant GPIbR without ristocetin) for measurement of VWF function over the VWF:RCo [10]. This approach reduces the limitations of the traditional VWF:RCo assay, such as its wide coefficient of variation [73] and unreliability with a VWF:Ag less than 15% to 20% [9,74]. In addition, the WES assay and bioinformatics pipeline were validated for copy number change and structural variants in addition to missense variants, allowing, for instance, the large deletion in a type 1 VWD patient to be identified. The capability of panel-based sequencing to accurately identify copy number change will become increasingly important given that these structural variants are not infrequent in inherited bleeding and thrombotic disorders, occurring in 2% (40/2396) of the patients in the ThromboGenomics cohort [75], of which 14/40 occurred in the *VWF* gene.

A limitation of this study includes the lack of clinical data, including bleeding events, given that standardized bleeding scores were not routinely documented for all patients. This data would be particularly useful in the low VWF and type 1 groups to evaluate if the presence of a variant affects bleeding risk. The cohort mostly consisted of low VWF and type 1 VWD patients, with smaller numbers of type 2 VWD and only 1 type 3 patient. Unfortunately, data on the ethnicity of participants, which provide useful information about variant frequency in various populations, were not available.

In addition, RIPA testing, though performed in most, was not universally performed in type 2 patients, and multimer analysis was rarely performed. However, as articulated above, the full gamut of functional laboratory testing is not consistently available and is not always accurate. Our laboratory testing process, limited to an activity and antigen VWF assay, likely reflects the standard practice used in many laboratories that perform VWD testing. This retrospective study was designed to evaluate the real-world utility of combined laboratory phenotyping and sequencing under conditions similar to those in many laboratories. It does not aim to directly compare full laboratory

phenotyping with sequencing. Rather, the data highlight the potential for panel-based sequencing to replace labor-intensive sequential testing (eg, multimer analysis, collagen binding, and RIPA) with a single test that accurately subtypes the majority of patients. This is particularly relevant given that the discrimination between types 1, 2A, and 2M VWD may have minimal clinical impact, and thus using a single test that not only discriminates between these subtypes but also allows the more relevant identification of type 2B and type 2N VWD is practical and efficient. Current ASH ISTH NHF WFH 2021 guidelines suggest genetic sequencing for type 2B and type 2N VWD over RIPA or as an alternative to FVIII binding studies respectively [10]. Multimer analysis or collagen binding assays are conditionally recommended for differentiating types 2A, 2B, or 2M based on very low certainty evidence. Given these formal suggestions, using genetic sequencing as a streamlined method to differentiate all type 2 VWD subtypes is a reasonable and practical approach. A proposed diagnostic algorithm would be the use of panel-based sequencing following accurate and reproducible laboratory measures of VWF quantity and activity in presequencing severe types 1, 2, and 3 VWD patients and in those with a suspected acquired cause.

Lastly, the identification of a VUS can hinder the diagnostic clarification expected from sequencing. Familial segregation testing to identify affected carriers can assist in the reclassification of a VUS; however, extensive segregation analysis is typically required. Functional validation, for example, using cell-based models transfected with the variant of interest can more definitively clarify the nature of an uncertain variant.

5 | CONCLUSION

Panel-based genetic sequencing for VWD in a well-selected cohort, particularly those diagnosed with type 2 and type 3 VWD, was clinically relevant in differentiating genocopies, informing the use of hemostatic therapies, subtyping, and family planning. Those with low VWF and most type 1 VWD patients did not have an alteration in their clinical management implemented after sequencing. Future research is required to determine whether the bleeding risk in low VWF is affected by the presence of a *VWF* variant and if concomitant variants in other genes modify the clinical phenotype in VWD more generally.

DATA AVAILABILITY

The data that support the findings of this study are attached in the Supplementary Material.

FUNDING

R.R. is a recipient of an International Society on Thrombosis and Haemostasis Training Fellowship Award 2023 and a recipient of the Mike and Carole Ralston Travelling Fellowship Award 2024 from the Royal College of Pathologists of Australasia Foundation.

AUTHOR CONTRIBUTIONS

K.F. supervised the project. C.V.L. and R.R. created the study design. C.V.L. collected the data and performed the data extracts. K.F., S.B., C.K., and C.V.G. analyzed and reported the genetic testing results. R.R., C.V.L., K.F., and V.L. participated in project meetings to develop research methods and data interpretation. R.R. analyzed the data. R.R. created the tables and figures. R.R. wrote the manuscript. All authors critically reviewed the manuscript.

RELATIONSHIP DISCLOSURE

R.R. reports financial support by the Royal College of Pathologists of Australasia (RCPA) and International Society on Thrombosis and Haemostasis (ISTH). All other authors have no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.rpth.2025.102730.