Variant mapping using mass spectrometry-based proteotyping as a diagnostic tool in von Willebrand disease

Kreft, I.C.; Duijl, T.T. van; Kwawegen, C. van; Atiq, F.; Phan, W.; Schuller, M.B.P.; ... ; Willebrand Netherlands WiN Study Grp

Received: 17 January 2024 | Revised: 20 March 2024 | Accepted: 15 April 2024

https://doi.org/10.1016/j.jtha.2024.04.011

ORIGINAL ARTICLE

Variant mapping using mass spectrometry–based proteotyping as a diagnostic tool in von Willebrand disease

Iris C. Kreft[1] | Tirsa T. van Duijl[1] | Calvin van Kwawegen[2] | Ferdows Atiq[2] | Winny Phan[1] | Margo B. P. Schuller[1] | Mariëtte Boon-Spijker[1] | Carmen van der Zwaan[1] | Alexander B. Meijer[1,3] | Arie J. Hoogendijk[1] | Ruben Bierings[2] | Jeroen C. J. Eikenboom[4] | Frank W. G. Leebeek[2] | Maartje van den Biggelaar [1] | on behalf of the Willebrand in the Netherlands (WiN) study group

[1]Laboratory of Proteomics, Department of Molecular Hematology, Sanquin Research, Amsterdam, the Netherlands

[2]Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands

[3]Department of Biomolecular Mass Spectrometry and Proteomics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, the Netherlands

[4]Department of Internal Medicine, Division of Thrombosis and Hemostasis, Leiden University Medical Center, Leiden, the Netherlands

Correspondence
Maartje van den Biggelaar and Iris C. Kreft, Sanquin Research, Department of Molecular Hematology, Plesmanlaan 125, 1066 CX Amsterdam, the Netherlands.
Email: i.kreft@sanquin.nl and m.vandenbiggelaar@sanquin.nl

Funding information
The work presented here was supported by the product- en procesontwikkeling (PPOC) project PPOC18-11_L2334 to M.v.d.B.

Abstract

Background: von Willebrand disease (VWD) is the most common inherited bleeding disorder, characterized by either partial or complete von Willebrand factor (VWF) deficiency or by the occurrence of VWF proteoforms of altered functionality. The gene encoding VWF is highly polymorphic, giving rise to a variety of proteoforms with varying plasma concentrations and clinical significance.

Objectives: To address this complexity, we translated genomic variation in *VWF* to corresponding VWF proteoforms circulating in blood.

Methods: VWF was characterized in VWD patients (n = 64) participating in the Willebrand in the Netherlands study by conventional laboratory testing, DNA sequencing and complementary discovery, and targeted mass spectrometry–based plasma proteomic strategies.

Results: Unbiased plasma profiling combined with immune enrichment of VWF verified VWF and its binding partner factor VIII as key determinants of VWD and revealed a remarkable heterogeneity in VWF amino acid sequence coverage among patients. Subsequent VWF proteotyping enabled identification of both polymorphisms (eg, p.Thr789Ala, p.Gln852Arg, and p.Thr1381Ala), as well as pathogenic variants (n = 16) along with their corresponding canonical sequences. Targeted proteomics using stable isotope–labeled peptides confirmed unbiased proteotyping for 5 selected variants and suggested differential proteoform quantities in plasma. The variant-to-wild-type peptide ratio was determined in 6 type 2B patients heterozygous for p.Arg1306Trp, confirming the relatively low proteoform concentration of the pathogenic variant. The

Manuscript handled by: Karen Vanhoorelbeke

Final decision: Karen Vanhoorelbeke, 15 April 2024

Iris C. Kreft and Tirsa T. van Duijl contributed equally to this study.

elevated VWF propeptide/VWF ratio indicated increased clearance of specific VWF proteoforms.

Conclusion: This study highlights how VWF proteotyping from plasma could be the first step to bridge the gap between genotyping and functional testing in VWD.

KEYWORDS

mass spectrometry, plasma, proteomics, proteotyping, von Willebrand disease

1 | INTRODUCTION

von Willebrand disease (VWD) is the most common inherited bleeding disorder caused by deficiencies or abnormalities in von Willebrand factor (VWF) [1,2]. VWF is a large glycoprotein (GP) that is translated as a prepropolypeptide that is proteolytically processed to generate a signal peptide with a length of 22 amino acids (AAs), a propeptide (von Willebrand factor propeptide [VWFpp]) consisting of 741 AAs, and a large mature subunit containing 2050 AAs [3]. The mature VWF subunits contain a variety of functional domains (D′-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK) that are important for its hemostatic properties [4]. Specifically, the D′D3 domain interacts with coagulation factor (F) VIII, the A1 and A3 domains facilitate binding to collagen fibers, the A1 and C4 domains contain a platelet receptor glycoprotein Ib alpha (GPIbα) and a glycoprotein IIbIIIa (GPIIbIIIa) binding site that support platelet interactions, and the A2 domain interacts with "a disintegrin and metalloproteinase with thrombospondin type 1 motif member 13" (ADAMTS-13) to processes ultralarge VWF multimers in circulation [5].

Patients with VWD are subtyped for tailored monitoring and treatment [6] and classified by partial (type 1) or complete (type 3) VWF deficiency or by the presence of VWF proteoforms with altered function (type 2) [7]. Type 2 classification is primarily based on the discrepancy between VWF antigen (VWF:Ag) levels and platelet-dependent VWF activity (VWF:Act) or FVIII activity. Specifically, type 2A can be characterized by reduced or absent high-molecular-weight VWF multimers, type 2B by increased platelet binding, type 2M by altered platelet or collagen binding, and type 2N by reduced VWF binding to FVIII [8]. Recently, the International Society on Thrombosis and Haemostasis subclassification was expanded with the addition of type 1C, known for its accelerated VWF clearance from plasma [6].

Deficiencies in VWF are mostly caused by variants in the *VWF* gene, affecting messenger RNA transcription and translation, VWF biosynthesis, clearance, or protein functionality [9]. To facilitate the interpretation of variant pathogenicity, variances of unknown significance, benign variants, and pathogenic variants are registered in the Coagulation Factor Variant Databases portal, which is supported by the European Association for Haemophilia and Allied Disorders (EAHAD) [10], and the ClinVar archive. Despite these efforts, the pathogenicity of *VWF* variants remains elusive for the clinical phenotype in the individual patient, which may result in suboptimal VWD subclassification for clinical management [11]. In addition, in around 30% of VWD type 1 patients, no pathogenic variant in *VWF* can be identified [11,12]. Therefore, the added value of genetic counseling for disease phenotyping in the diagnostic pathway remains a topic of discussion [11] and laboratory tools are needed to facilitate the genotype-to-proteotype translation to interpret the clinical phenotype in a VWD patient [13].

The study of VWF proteoforms and their diverging concentrations in relation to the disease phenotype requires an analytical platform with sensitivity toward AA sequence variance to capture VWF heterogeneity. Here, we explored the potential of upcoming discovery-based and targeted mass spectrometry (MS)–based technologies in proteotyping in VWD [14–16]. To this end, we selected type 1, 2A, 2B, 2M, 2N, and 3 VWD patients with known genetic mutations and type 1 patients without a pathogenic variant in *VWF* [11] included in the Willebrand in the Netherlands (WiN) study [17]. First, we studied the whole plasma proteome by discovery proteomics. Hereafter, we analyzed the levels of VWF and VWFpp from plasma by quantitative proteomics and compared those with enzyme-linked immunosorbent assay (ELISA)–measured VWF plasma levels. Finally, we set out to detect VWF variants from plasma, independent of genetic variant information *a priori*. By combining different proteomics strategies, we demonstrate that MS-based proteomics is a valuable platform to proteotype VWD.

2 | METHODS

2.1 | Patient cohort and study design

Blood samples were collected from a subpopulation of 64 VWD patients from the WiN study (Supplementary Figure S1) [17]. Initially, for our discovery proteomics study, we selected 19 type 1 patients, 5 type 2A patients, 95 type 2B patients, 5 type 2M patients, 1 type 2N patient, and 4 type 3 patients. For our follow-up experiments, we expanded our cohort by including all accessible plasma samples from patients in the WiN study harboring p.Cys1190Arg, p.Phe1293Leu, p.Arg1306Trp, p.Arg1374His, or p.Tyr1584Cys to verify our initial observations with a targeted proteomics approach. VWD classification was according to standard practice of the medical centers, as described previously [18]. VWF:Ag, VWF Collagen Binding activity (VWF:CB), VWF:Act, FVIII activity levels (FVIII:C), and VWFpp were measured elsewhere [17,19]. The 52 exons of *VWF* gene including ±20 bp exon-intron boundaries were sequenced using ion semiconductor

sequencing (Ion-Torrent PGM, Thermo Fisher Scientific) and multiplex ligation-dependent probe amplification [11]. Blood samples were collected from anonymized healthy volunteers in accordance with Dutch regulations and Sanquin Ethical Advisory Board approval, following the Declaration of Helsinki. Blood samples collected at both institutes were centrifuged twice at 2200 × *g* for 10 minutes at room temperature, and citrated plasma aliquots were stored at −80 °C until analysis. VWF:Ag in healthy controls was determined by ELISA as detailed in the Supplementary Methods.

2.2 | Sample preparation

The different sample preparation methods for discovery and targeted proteomic analyses are detailed in the Supplementary Methods. In brief, plasma proteins were denatured, alkylated, and digested with trypsin and peptides were desalted with solid phase extraction prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS). Plasma samples from 4 type 3 patients and 10 healthy controls were depleted for the top 14 highly abundant proteins to study the proteome with increased proteomic depth. For plasma-derived protein variant mapping, VWF was immunocaptured using monoclonal antibodies CLB-Rag35 and CLB-Rag201 [20]. For targeted proteomics, plasma samples were supplemented with an internal standard (IS) mixture containing synthetic stable isotope–labeled (SIL) VWF peptides or a concatenated VWF polypeptide produced by stable isotope labeling by amino acids in cell culture (SILAC; Supplementary Figure S2). For absolute quantification, we prepared 5 calibrators by admixing VWF-deficient citrated plasma (Hyphen, catalog: DP150K) and an in-house value-assigned citrated plasma standard (Sanquin, PS18).

2.3 | LC-MS/MS analyses

Peptides were analyzed by LC-MS/MS in Data Dependent Acquisition mode for discovery proteomics and in Parallel Reaction Monitoring (PRM) mode for targeted proteomics using an orbitrap mass spectrometer (Fusion or Fusion Lumos Tribrid, Thermo Fisher Scientific). The LC-MS/MS setting is detailed in the Supplementary Methods, and the PRM mass list is provided in Supplementary Table S1.

2.4 | Variant database generation

A FASTA file was generated consisting of the Human UniProt database (UP000005640, 75 776 entries), supplemented with VWF protein variants reported in the EAHAD database (461 entries https://dbs.Eahad.org) and unique VWF variants identified through genetic sequencing in our cohort (8 entries).

2.5 | Data analysis

MS data acquired using Data-dependent acquisition were analyzed by MaxQuant software [21], and PRM data were processed in Skyline [22]. Data were further processed in Rstudio [23]. Prcomp function was used for principal component analysis (PCA) as an unsupervised method aimed to get a global overview of the data. PCA was performed on proteins quantified across all samples within each data set, with variation in protein expression visualized in a score plot. Protein variation was assessed by the coefficient of variation (CV) of non–$\log_2$-fold transformed intensities in proteins quantified in 50% of the samples. Missing values were imputed by a normal distribution (width = 0.3; shift = 1.8) and used for statistical analysis employing the Limma package [24], with *P* values corrected for multiple testing using the Benjamini–Hochberg method. Sequence coverage of immunocaptured VWF was determined using the MaxQuant peptide.txt output file. PRM data validity was checked by IS retention time agreement, IS intensity (area under the curve > 10 000), and consistent fragment ion ratios (median, ±0.15; Supplementary Figure S3). The relative responses (RRs) of endogenous and ISs for $_{220}$GLWEQCQLLK$_{229}$ and $_{2479}$SGFTYVLHEGECCGR$_{2493}$ were interpolated in the calibration curve to report concentrations in International Units per deciliter. For quantification of the variant-to-wild-type ratio, the RR to the peptide from SILAC concatenated VWF polypeptide of the variant peptide was divided by the RRs of the corresponding wild-type peptide sequence.

3 | RESULTS

3.1 | Studied VWD population characteristics

Sixty-four VWD patients were analyzed, including 30 type 1 patients, 30 type 2 patients, and 4 type 3 patients (Table 1). Type 1 VWD patients were divided into 10 patients without a *VWF* gene variant and 20 with a pathogenic *VWF* variant. Of the type 2 patients, 15 had type 2A, 9 had type 2B, 5 had type 2M, and 1 had type 2N VWD. The laboratory results of VWF:Ag, VWFpp:Ag, VWFpp:Ag/VWF:Ag, VWF:Act/VWF:Ag, and FVIII:C were grouped by VWD subclass (Supplementary Figure S4). All included subjects in this study were analyzed for pathogenic variants in the *VWF* gene, with an overview of sequencing results available in Supplementary Table S2.

3.2 | Unbiased plasma profiling indicated VWF variance, but a stable proteome in VWD

To explore significant proteome changes in plasma, unbiased proteomics was conducted in the first subset of VWD patients (*n* = 39) and healthy controls (*n* = 10). Based on those proteins identified in all samples, plasma proteomes from VWD patients did not separate from

TABLE 1 Studied von Willebrand disease and healthy population characteristics.

Patient characteristics	All VWD patients	VWD type 1	VWD type 2	VWD type 3	Healthy controls
n (%)	64 (100)	29 (45.3)	31 (48.4)	4 (6.3)	10 (100)
Age (IQR), y	46 (36.0-54.0)	47 (40.8-54.3)	45 (32.0-52.0)	26 (14.0-54.5)	52 (45.0-54.8)
Sex (% male)	22 (34.4)	10 (34.5)	11 (35.7)	1 (25.0)	5 (50)
Blood group O, (%)	34 (51.1)	19 (65.5)	13 (41.9)	2 (50.0)	6 (60)
Bleeding score (IQR)					NA
Female	10 (5.5-14.5)	10 (5.5-14)	17.5 (11.0-24.0)	20 (19.5-21.0)	
Male	9 (5.0-17.0)	10.5 (7.5-22.3)	7.5 (4.3-9.8)	23 (NA)	
Variant identified *VWF* gene, *n* (%)	54 (84.4)	19 (65.5)	31 (100)	4 (100)	NA
Blood biochemistry					
VWFpp (IU/dL)	87.0 (71.0-110)	79.0 (69.0-92.0)	110.0 (86.0-127.0)	<1	NA
VWF:Ag (IU/dL)	29.0 (19.0-37.0)	37.0 (0.28-0.40)	24.0 (19.0-37.0)	<1	96.7 (69.7-108.5)[a]
VWF:Act (%)	1.7 (0.3-0.4)	4.0 (3.0-5.5)	0.4 (0.2-1.3)	<0.01	NA
VWF:CB (%)	2.5 (0.8-4.3)	4.1 (3.0-5.6)	0.9 (0.6-2.0)	<0.01	NA
FVIII activity (%)	4.7 (3.2-6.4)	6.3 (4.8-7.0)	3.7 (3.1-4.7)	0.1 (0.1-0.1)	NA

Median (IQR) values are provided for numerical values.

VWD, von Willebrand disease; VWF, von Willebrand factor; VWFpp, von Willebrand factor propeptide; VWF:Ag, von Willebrand facto antigen; VWF:CB, von Willebrand factor collagen binding activity.

[a] Measured with other enzyme-linked immunosorbent assays.

healthy controls, and the predominant factors influencing the plasma proteome were immunoglobulins and hemoglobin (HB) subunits (Supplementary Figure S5A). The majority of quantified proteins (*n* = 239, Supplementary Table S3) were considered stable in abundance (CV ≤ 30%). As expected, VWF levels were most variable (CV = 269%) in this population, followed by acute phase protein serum amyloid A-1 (CV = 219%), pregnancy zone protein (CV = 214%), carbonic anhydrase 1 (CV = 121%), apolipoprotein(a) (CV = 149%), and HB subunits (HBA, CV = 140%; HBB, CV = 148%; HBD, CV = 149%; Figure 1A). The variations in HB subunits and carbonic anhydrase 1 levels were likely to be a result of erythrocyte contamination introduced during the preanalytical phase [25]. The variance in apolipoprotein(a) could be explained by the polymorphic kringle-domain structure between individuals but was also found at the lower end of the detection range [26]. Overall, the variation in VWF levels was subgroup-dependent, while plasma levels of serum amyloid A-1 were individual-specific (Figure 1B). The variation in pregnancy zone protein levels was sex-specific (Supplementary Figure S6), which is in line with previous studies [15,27].

Proteomic depth was gained by depletion of the top 14 highly abundant proteins, resulting in an increased number of protein identifications (from 422 to 1338) and quantifications (from 239 to 963; Supplementary Table S3). The interindividual proteomic variance was primarily driven by HB and immunoglobulins (Figure 1C, Supplementary Figure S7). VWF was the key determinant when comparing the depleted plasma proteome between the type 3 patients (*n* = 4) and healthy controls (*n* = 10), but also macrophage colony-stimulating factor 1 was found to be slightly increased in VWD type 3 patients (Figure 1D, E).

To examine potential alternative VWF binding partners in plasma or proteins with differential binding affinity in VWD specifically, we immunocaptured VWF from plasma and studied the proteome in an unbiased manner. In PCA, VWD subclasses represented the proteome variance, although type 2 subclasses and type 1 patients with and without a mutation in *VWF* could not be distinguished by their proteomic signature (Figure 1F). In addition, the proteomic variance was primarily driven by VWF and its binding partner FVIII (Figure 1G). Moreover, VWF levels strongly correlated with FVIII levels (R = 0.95), with the exception in the type 2N patients that is known for its reduced FVIII affinity (Figure 1H) [28]. Of note, in VWF-enriched samples, we did not detect other intracellular VWF interactors, such as low abundant cytokines osteoprotegerin, angiopoietin-2, apolipoprotein B-100, or the cell surface protein P-selectin. Taken together, we verified that among all quantified proteins, VWF and FVIII were the key discriminant proteins in VWD patients.

3.3 | Absolute quantification of VWF and VWFpp highlights proteoforms of aberrant clearance

Next, as a proof-of-concept for proteotyping in VWD, we developed an LC-MS/MS method to quantify VWF and VWFpp from plasma. Based on theoretical characteristics (including peptide uniqueness, minimal 7 AA tryptic peptide length, no posttranslational modifications [PTMs], and no common sequence variants) and experimental data obtained with unbiased proteomics, we selected one signature peptide $_{220}$GLWEQCQLLK$_{229}$ unique to the D1 domain of VWFpp and

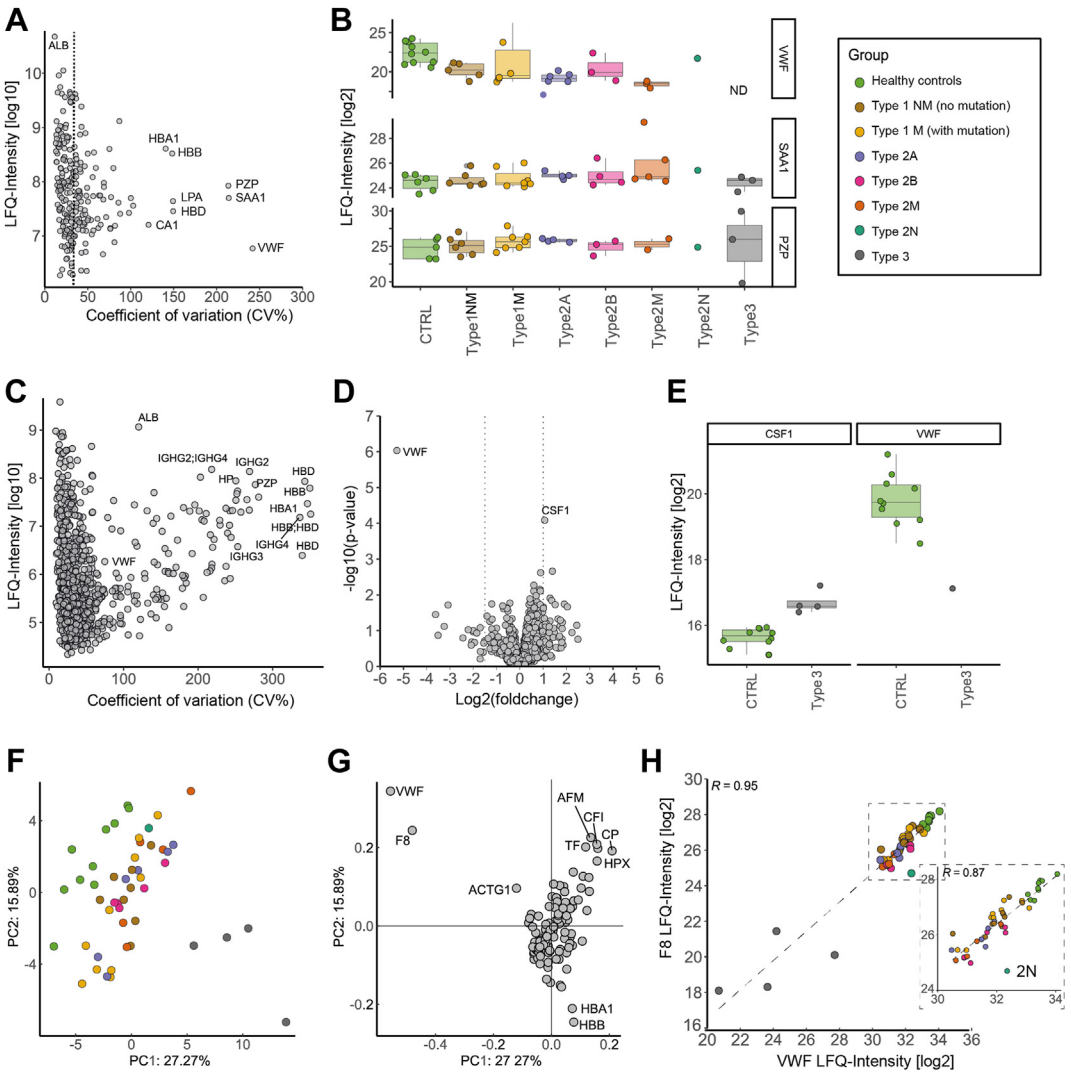

FIGURE 1 Unbiased profiling indicates a stable global plasma proteome in von Willebrand disease (VWD). (A) Coefficient of variation (CV, %) in global plasma protein quantification in relation to their Label-Free Quantification (LFQ) values from VWD patients (n = 39) and controls (n = 10). Proteins with the highest interindividual variation of a CV > 100% were annotated. (B) Distribution of LFQ intensity levels of proteins with highest variation, serum amyloid A1 (SAA1), von Willebrand factor (VWF), and pregnancy zone protein (PZP) categorized by VWD subtype. (C) Protein quantification variation (CV, %) in relation to protein abundance (LFQ) in depleted plasma from healthy (n = 10) and VWD type 3 patients (n = 4). (D) Comparison of the depleted plasma proteome in VWD type 3 patients and healthy controls. Volcano plot with the x-axis depicting the protein level fold change and the y-axis showing the $-\log_{10}$-fold t-test P values (adjusted Benjamini–Hochberg $P < .05$ and effect size $|\log FC| > 1$). (E) LFQ intensity levels of significantly altered VWF and colony-stimulating factor 1 (CSF1) in VWD type 3 compared with healthy controls after depletion of the top 14 highly abundant plasma proteins. (F) Immunoprecipitation of VWF from plasma combined with unbiased proteomic acquisition showed separation of healthy controls and VWD type 3 patients in principal component analysis (PCA) based on 131 proteins. (G) Loading protein plot from PCA with each dot representing a protein highlights VWF and FVIII as key determinants of proteomic variance. (H) Relation between the abundance (LFQ intensity) between VWF and FVIII after immunoprecipitation of VWF from plasma. A zoom-in of data from type 1 and type 2 VWD patients with annotation of type 2N is provided in the rectangle box. Color coding represents VWD subclasses type 1 (no mutation/mutation), type 2 (A, B, M, and N), and type 3.

peptide $_{2479}$SGFTYVLHEGECCGR$_{2493}$ unique to the C2 domain of mature VWF (Figure 2A). To report results in conventional units (IU/dL), the RR (Figure 2B) was interpolated in the plasma-based calibration curve (Figure 2C). A 7-leveled calibration curve for VWF and a single point-calibration strategy for VWFpp (0.12 RR represented 99 IU/dL) were obtained, as VWFpp remained present in VWF-deficient plasma.

The median VWF concentration was 2.3-fold lower in VWD patients compared with controls (Table 2). Eighteen VWD patients had VWF levels > 50 IU/dL. Interestingly, type 1 patients harboring the Y1584C variant had remarkably high levels of VWF (median, 58 IU/dL) and 6 out of 11 patients with this variant would not have met the 50 IU/dL threshold criteria for VWD diagnosis based on our LC-MS/MS analysis. The remaining 12 patients with mature VWF levels

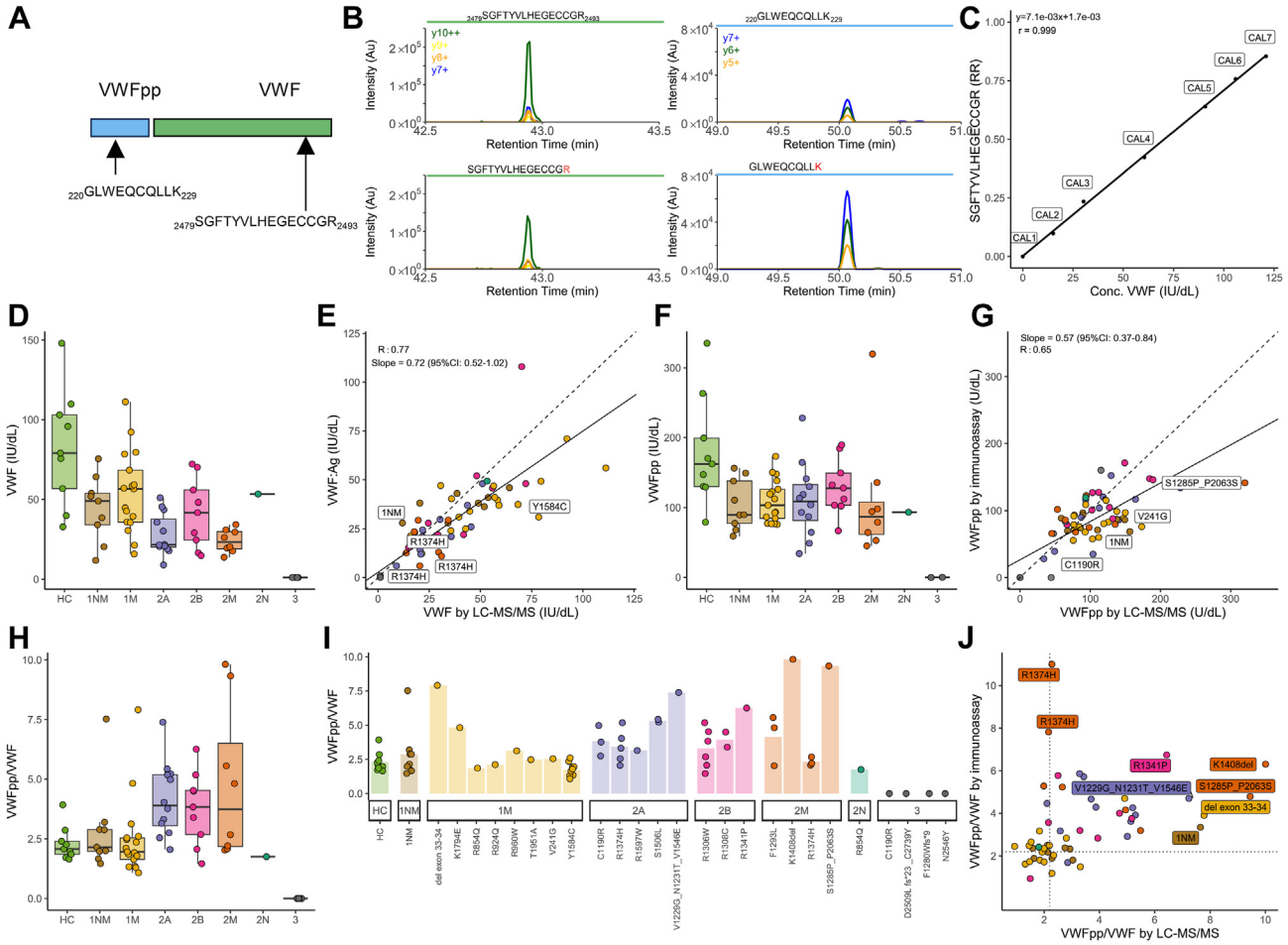

FIGURE 2 Quantification of von Willebrand factor (VWF) and VWF propeptide (VWFpp) by targeted mass spectrometry highlights proteoforms of increased clearance. (A) Mature VWF was quantified based on peptide $_{2479}$SGFTYVLHEGECCGR$_{2493}$ derived from the C2 domain, and VWFpp was quantified based on peptide $_{220}$GLWEQCQLLK$_{229}$ from the D1 domain. (B) The area under the curve (AUC) of the targeted tryptic peptide was divided by the AUC of the corresponding synthetic stable isotope–labeled peptide, functioning as an internal standard to obtain a relative response. (C) To convert results into conventional units, a calibration strategy was developed by admixing a value-assigned plasma standard and VWF-deficient plasma. The relative responses were interpolated in the calibration curve to report results in International Units per deciliter. (D) VWF concentrations among healthy controls and VWD patients. (E) Method comparison between liquid chromatography-tandem mass spectrometry (LC-MS/MS) and VWF antigen (VWF:Ag) in VWF quantification. (F) VWFpp concentrations among healthy controls and VWD patients. (G) Method comparison between LC-MS/MS and VWFpp:Ag in VWFpp quantification. (H) VWFpp/VWF ratios among controls and VWD patients. (I) VWFpp/VWF ratios grouped per VWD subtype and pathogenic variant. (J) Comparison of the VWFpp/VWF ratio determined by antigen-based testing and LC-MS/MS analysis with the previously proposed clinical cutoff value of 2.2 indicated by the dashed line. Data of healthy controls (n = 10) and VWD patients (n = 64) colored by subtype. 1NM = type 1 without a pathogenic mutant. 1M = type 1 with a pathogenic mutant. In method comparison, the solid slide represents Deming regression, the dashed line represents the line of identity, data are labeled when the difference between methods was ≥2-fold, and the Pearson correlation coefficient and quantification bias are provided.

> 50 IU/dL were as follows: (a) patients without a reported *VWF* mutation (n = 4), (b) patients with a p.Val241Gly variant in VWFpp (n = 1), (c) patients with a p.Arg854Gln (n = 2) or a p.Arg924Gln (n = 1) representing the VWD phenotype of reduced FVIII affinity rather than low VWF concentration, (d) patients with a p.Arg1374His (n = 1), and (e) patients with type 2B VWD (n = 3) with variants p.Arg1306Trp or p.Arg1308Cys. VWF levels varied remarkably among VWD subtypes, with the lowest levels found in type 2A and 2M patients, and VWF was below the analytical detection limit in type 3 patients (Figure 2D). Overall, VWF concentrations determined by LC-MS/MS were in agreement with conventional VWF:Ag levels with a positive absolute bias of 28% (Figure 2E). Compared with mature VWF, VWFpp levels were more stable across subtypes. The overall median VWFpp concentration in plasma was 96 IU/dL (IQR, 76-131; Figure 2F). Although VWFpp levels determined with LC-MS/MS were in the same range as quantified by immunoassay (50-200 IU/dL), data were scattered, and the correlation coefficient was r = 0.67 (Figure 2G). Noteworthy, based on nonnormalized and noncalibrated data, VWFpp concentrations in plasma were typically 8-fold lower than mature VWF (Table 2).

TABLE 2 von Willebrand factor and von Willebrand factor propeptide quantified by liquid chromatography-tandem mass spectrometry.

Measurand	Type 1: no variant (*n* = 9)	Type 1: variant (*n* = 17)	Type 2A (*n* = 12)	Type 2B (*n* = 9)	Type 2M (*n* = 8)	Type 2N (*n* = 1)	Type 3 (*n* = 4)	Healthy controls (*n* = 10)
VWFpp (IU/dL)	89 (78-138)	108 (85-135)	90 (61-121)	127 (103-149)	228 (97-320)	94	ND	162 (129-199)
VWF (IU/dL)	49 (34-54)	57 (37-66)	22 (20-32)	42 (25-56)	23 (18-32)	53	ND	79 (57-103)
VWFpp/VWF	2.2 (2.0-2.9)	2.0 (1.7-2.5)	3.3 (2.6-5.1)	3.8 (2.7-4.5)	7.4 (5.0-9.3)	1.75	ND	2.1 (1.9-2.4)

Median (IQR) values are provided. VWFpp was quantified based on peptide $_{220}$GLWEQCQLLK$_{229}$ and VWF based on $_{2479}$SGFTYVLHEGECCGR$_{2493}$. In type 3 patients, VWF was not detected directly from plasma without immune enrichment (*n* = 4). Quantitative VWF and VWFpp data were invalid for 4 patients (type 1/2).

ND, not detected; VWF, von Willebrand factor; VWFpp, von Willebrand factor propeptide.

Finally, we evaluated the VWFpp/VWF ratios as a surrogate measure of clearance of VWF from the circulation (Figure 2H). While we observed similar VWFpp/VWF ratios among controls (median, 2.1; IQR, 1.9-2.4), type 1 patients (median, 2.1; IQR, 1.7-2.8), and the type 2N patient (median, 1.8), this ratio was typically higher in type 2A, 2B, or 2M patients (median, 5.9; IQR, 2.6-12.7), indicating an accelerated clearance of VWF proteoforms in these patients. Since the VWFpp/VWF ratio was highly variable within VWD subtypes, we evaluated the VWFpp/VWF ratio per genetic variant (Figure 2I). VWFpp/VWF ratios tended to be higher in patients carrying multiple variants or a single pathogenic variant affecting the D3, A1, A2, or A3 domains. Subsequently, we compared VWFpp/VWF ratios examined by LC-MS/MS with those obtained from ELISA. With the LC-MS/MS approach, the highest ratios were found in patients heterozygous for mutants p.Lys1408del, p.Ser1285Pro, and p.Pro2063Ser; 33-34 exon deletion; or a combination of p.Val1229Gly, p.Asn1231Thr, and p.Val1546Glu (Figure 2J). Especially the p.Lys1408del and the exon 33-34 deletion have been described for their impaired plasma secretion or increased clearance [11]. Interestingly, 1 patient classified as type 1 without a pathogenic variant in the *VWF* gene showed a remarkably high VWFpp/VWF ratio of >7, which may indicate VWF deficiency due to accelerated clearance. Finally, we questioned whether the VWF levels or VWFpp/VWF ratios were determinants of the bleeding severity. The examined parameters, VWF:Ag, VWF:Act, VWF, and VWFpp/VWF ratios, were all poorly associated with bleeding severity expressed by the bleeding score (all R < (−)0.2).

3.4 | Protein sequence heterogeneity of plasma-derived VWF among healthy individuals and VWD patients

Since we did not identify alternative protein-based disease modifiers other than VWF in VWD, we studied circulating VWF in more detail by characterizing plasma-derived VWF variants. Upon VWF immunocapture from plasma, an AA sequence coverage of 74% for mature VWF (AAs 764-2813) was obtained. Peptides from VWFpp (AAs 23-763) were also detected with low signal intensity and covered 50% of its sequence (Supplementary Figure S5). The coverage of the functional domains of interest was 85% for D´D3, 79% for A1, 82% for A2, 74% for A3, and 73% for C4, which allowed for peptide mapping for variant characterization (Figure 3A). Interestingly, peptide intensity mapping per individual revealed a remarkable variation in sequence coverage within the population (Figure 3B). For VWD type 3 patients in specific, no peptides derived from the propeptide were identified, and limited coverage for mature VWF was acquired (<23%).

3.5 | Plasma-derived VWF proteotyping matches genotyping results of pathogenic variants and reveals benign variants *de novo*

To examine the observed heterogeneity of VWF proteoforms among individuals, we searched for benign and pathogenic variants by peptide mapping in a subset of VWD patients (*n* = 39). The acquired spectral matches were searched against an in-house generated sequence database including VWF variants. Based on peptide mapping using the extended sequence database, we identified 5 peptides unique to the polymorphisms p.Ala2178Ser, p.Val471Leu, p.Thr789Ala, p.Gln852Arg, and p.Thr1381Ala (Supplementary Table S4). For p.Thr789Ala, p.Gln852Arg, and p.Thr1381Ala, we detected both the wild-type (from UniProt) as well as the variant (noncanonical) sequence in the studied population (Figure 4A, B). In 27 of all 28 type 1 and 2 patients, the canonical variant p.Thr789 was detected either exclusively or in combination with the noncanonical variant p.Ala789. The individual with solely the noncanonical sequence p.Ala789 detected was a type 2N patient heterozygous for p.Arg854Gln. With respect to the polymorphism p.Gln852Arg, we detected the peptide noncanonical sequence, $_{844}$IGCNTCVCQDR$_{854}$ more frequently (*n* = 26) compared with the wild-type sequence $_{844}$IGCNTCVCR$_{852}$ (*n* = 8; Figure 4A). Comparison between the peptide frequency of p.Thr789Ala, p.Gln852Arg, and p.Thr1381Ala within our small study population and the genetic frequency of corresponding variant within the general population indicated that our MS-based proteotyping was in strong agreement with the expected minor allele frequencies reported in the Genome Aggregation Database (Figure 4C) [29].

In addition to proteotyping of polymorphisms, we aimed to detect the pathogenic variants in VWF by peptide mapping. In the studied VWD population, 27 different variants were identified by DNA

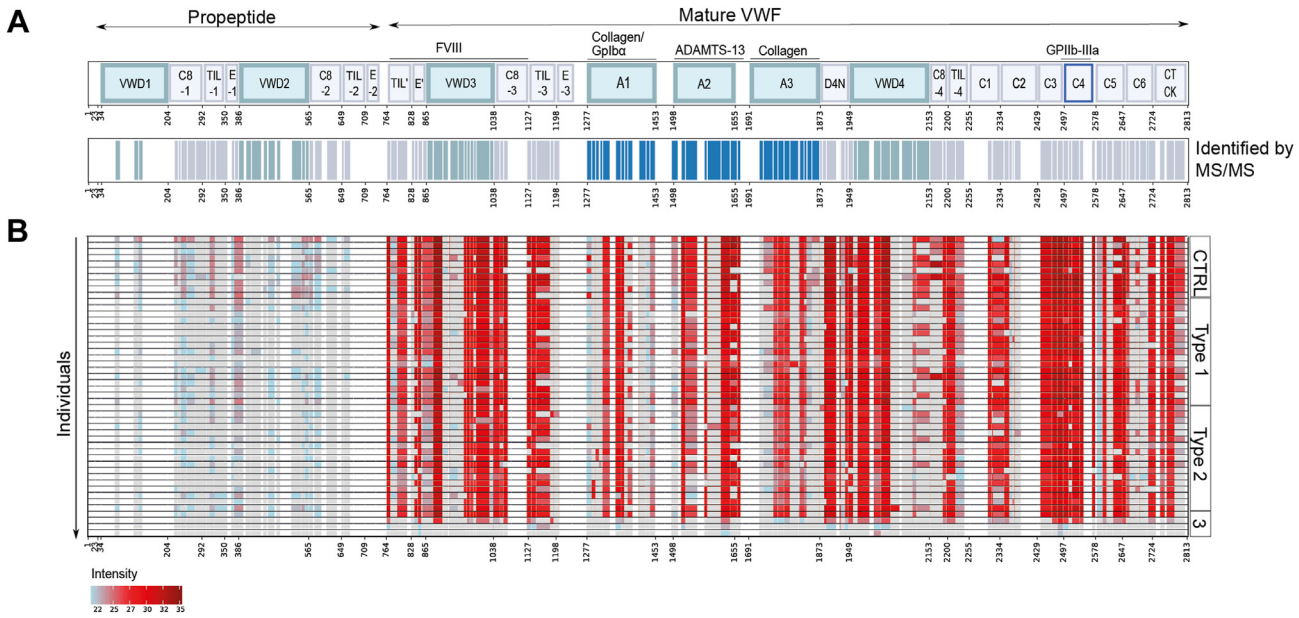

FIGURE 3 Canonical sequence coverage highlights interindividual variation in plasma-derived von Willebrand factor (VWF). (A) Schematic representation of the VWF domain organization adapted from [4] and complemented with the overall sequence coverage based on tandem mass spectrometry (MS/MS) peptide identification. The sequence was divided into propeptide and mature VWF, and the increased coverage indicates the higher abundance of mature VWF in plasma. (B) Overview of peptide coverage displayed per individual in the studied population with color coding based on peptide intensity. Gray indicates that the peptide is measured in one of the samples, blue denotes lower peptide intensity, and red signifies higher intensity. ADAMTS-13, a disintegrin and metalloproteinase with thrombospondin type 1 motif member 13; Ctrl, control; FVIII, factor VIII; GP, glycoprotein; VWD, von Willebrand disease.

sequencing, from which 24 were missense mutations leading to a single AA substitution [11]. We were able to identify 18 different pathogenic variants with peptide mapping, of which 16 were previously identified by DNA sequencing (Figure 5A). Noteworthy, the exon 33-34 deletion was recognized by the unique peptide sequence $_{1871}$LCSGVCTGSSTR$_{1955}$. Furthermore, 2 additional variants, p.Leu1383Pro (in type 2A and type 1) and p.Gly2518Ser (in type 1), were identified at the peptide level but initially not reported upon DNA sequencing, as the clinical relevance of these variants was considered uncertain. Two pathogenic variants identified by DNA sequencing could not be identified by peptide mapping using tryptic digestion and emphasize the need for multidigestion strategies to increase sequence coverage and thus sensitivity toward single nucleotide variance.

Next, we assessed whether we could differentiate in mutant allele zygosity by assessing the presence of both wild-type and variant peptide sequences. For the pathogenic variant p.Cys1190Arg, a patient heterozygous (type 2A) and a patient homozygous for this mutation (type 3) were included in the studied population, and, therefore, we selected this variant as a proof-of-principle. In a VWD patient without the p.Cys1190Arg variant, only the wild-type sequence $_{1182}$ILDELLQTCVDPEDCPVCEVAGR$_{1205}$ was detected; in the patient heterozygous for p.Cys1190Arg, both the wild-type and the variant peptide sequence $_{1182}$ILDELLQTR$_{1190}$ were detected, whereas in the patient homozygous for p.Cys1190Arg exclusively the variant peptide was identified (Figure 5B). Collectively, proteotyping enabled the identification of VWF variants at the peptide level and was in agreement with the allele zygosity for the p.Cys1190Arg variant.

3.6 | Verification of plasma-derived VWF proteotyping highlights distinct variant-to-wild-type ratios in plasma

To confirm our VWF proteotyping results, we analyzed the 5 most prevalent pathogenic variants in our cohort as proof-of-concept, namely p.Cys1190Arg, p.Phe1293Leu, p.Arg1306Trp, p.Arg1374His, and p.Tyr1584Cys, with targeted proteomics. First, peptides unique to wild-type or variant VWF were compared with MS/MS spectra of (light) synthetic reference peptides (Supplementary Figures S7–S18) and examined whether the variant peptides could be detected directly from plasma of VWD patients without prior immunocapture. As expected, the combination of wild-type and variant peptides was exclusively found in the VWD patients heterozygous for the specific mutation (Figure 6A–E).

Since both peptides unique to wild-type or variant VWF were detectable in plasma, we assessed whether these peptides were representative of the proteoform concentration. Wild-type and variant peptides were relatively quantified using SIL peptides as ISs for each target variant and their corresponding wild-type sequence. Overall, relative quantification (RR) highlighted the similarity of the wild-type and variant peptide levels with the concentration of VWF in

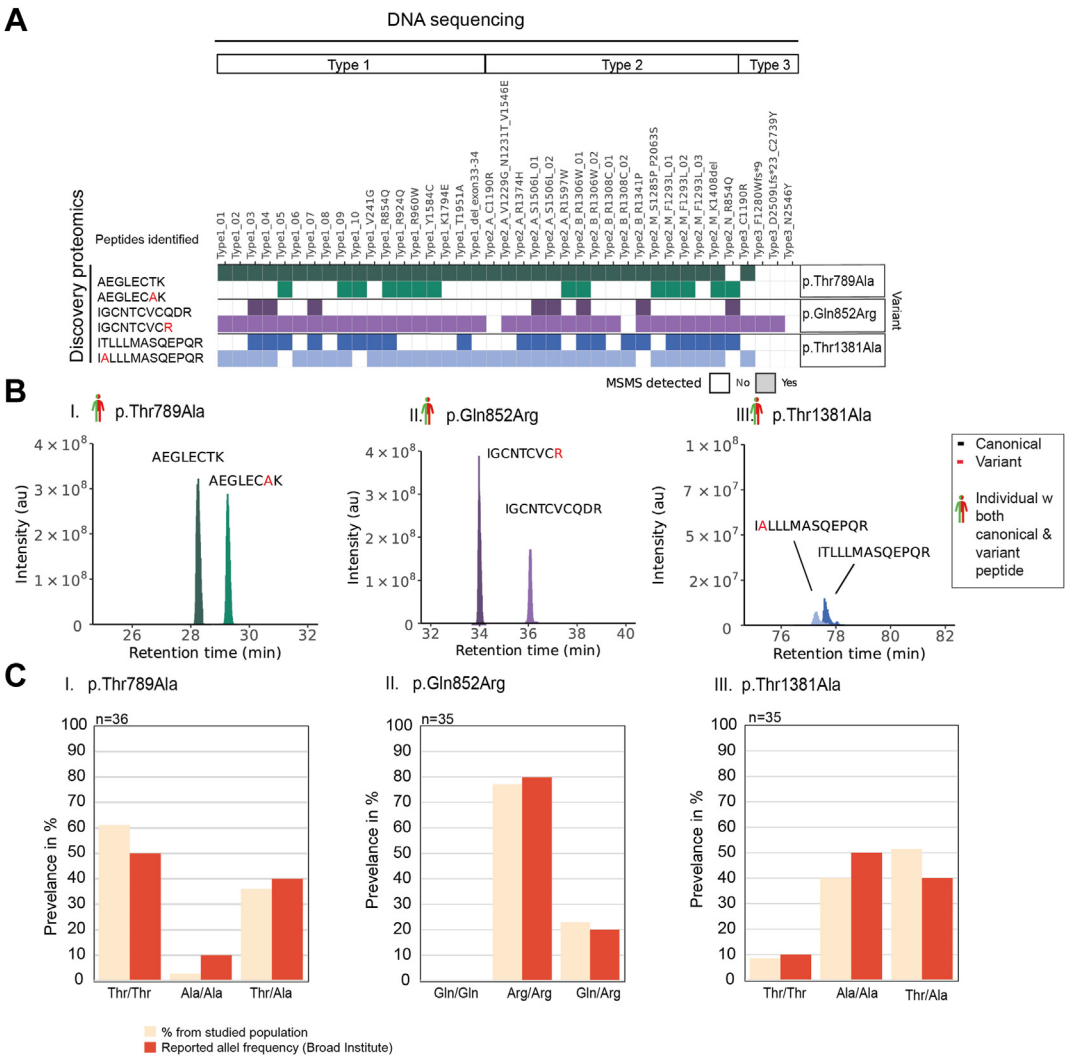

FIGURE 4 von Willebrand factor (VWF) polymorphisms identified by incorporation of the European Association for Haemophilia and Allied Disorders variant database. (A) Summary of the most frequent VWF polymorphisms identified within the study population by mass spectrometry–based proteotyping. Peptides correspond either to the canonical or variant of p.Thr789Ala, p.Gln852Arg, and p.Thr1381Ala. (B) Extracted ion chromatograms of peptides unique for polymorphisms (I) p.Thr789Ala, (II) p.Gln852Arg, and (III) p.Thr1381Ala in 3 representative individuals. The amino acid representing the canonical sequence reported in the Broad Institute's database is shown in black, whereas the substituted amino acid in the variant peptide is colored in red. (C) Comparison of the frequency of common VWF polymorphisms in the studied population (beige) by mass spectrometry–based proteotyping compared with the allele frequency reported in the Broad Institute's database (orange) for (I) Thr/Thr, Ala/Ala, and Thr/Ala at position 789, (II) Gln/Gln, Arg/Arg, and Gln/Arg at position 852, and (III) Thr/Thr, Ala/Ala, and Thr/Ala at position 1381. Ala, alanine; Arg, arginine; Gln, glutamine; MS/MS, tandem mass spectrometry; Thr, threonine.

plasma (Figure 6F). The variant-to-wild-type ratio could not be determined for all VWD patients due to low VWF levels, especially for individuals harboring p.Arg1374His and p.Cys1190Arg. However, for type 1 patients heterozygous for Tyr1584Cys and type 2M patients heterozygous for Phe1293Leu, the variant-to-wild-type ratio based on the RR was around 1. A relatively high variance in RR was observed for type 2B patients heterozygous for p.Arg1306Trp, although the RR of variant and wild-type peptide were in agreement, except for 1 patient. Surprisingly, for this type 2B patient, the variant-to-wild-type ratio was approximately 9-fold lower compared with that for the other patients heterozygous for p.Arg1306Trp, which was directed by relatively high levels of the wild-type peptide.

To overcome the limitation of a lacking calibration standard for noncanonical sequences, we designed a SILAC concatenated polypeptide including both the wild-type and variant sequence within the same molecule (Supplementary Figure S1) and used this for absolute quantification of the p.Arg1306Trp variant. For 5 out of the 6 type 2B patients heterozygous for the p.Arg1306Trp, the variant-to-wild-type ratio was between 0.5 and 0.6 (Figure 6G), suggesting that the VWF proteoform carrying a tryptophan residue at position 1306 was approximately 2-fold lower in abundance compared with the VWF proteoform carrying an arginine residue at this position. For the patient, we previously observed a relatively high wild-type VWF based on the RR using SIL peptides (Figure 6F); we again found a relatively

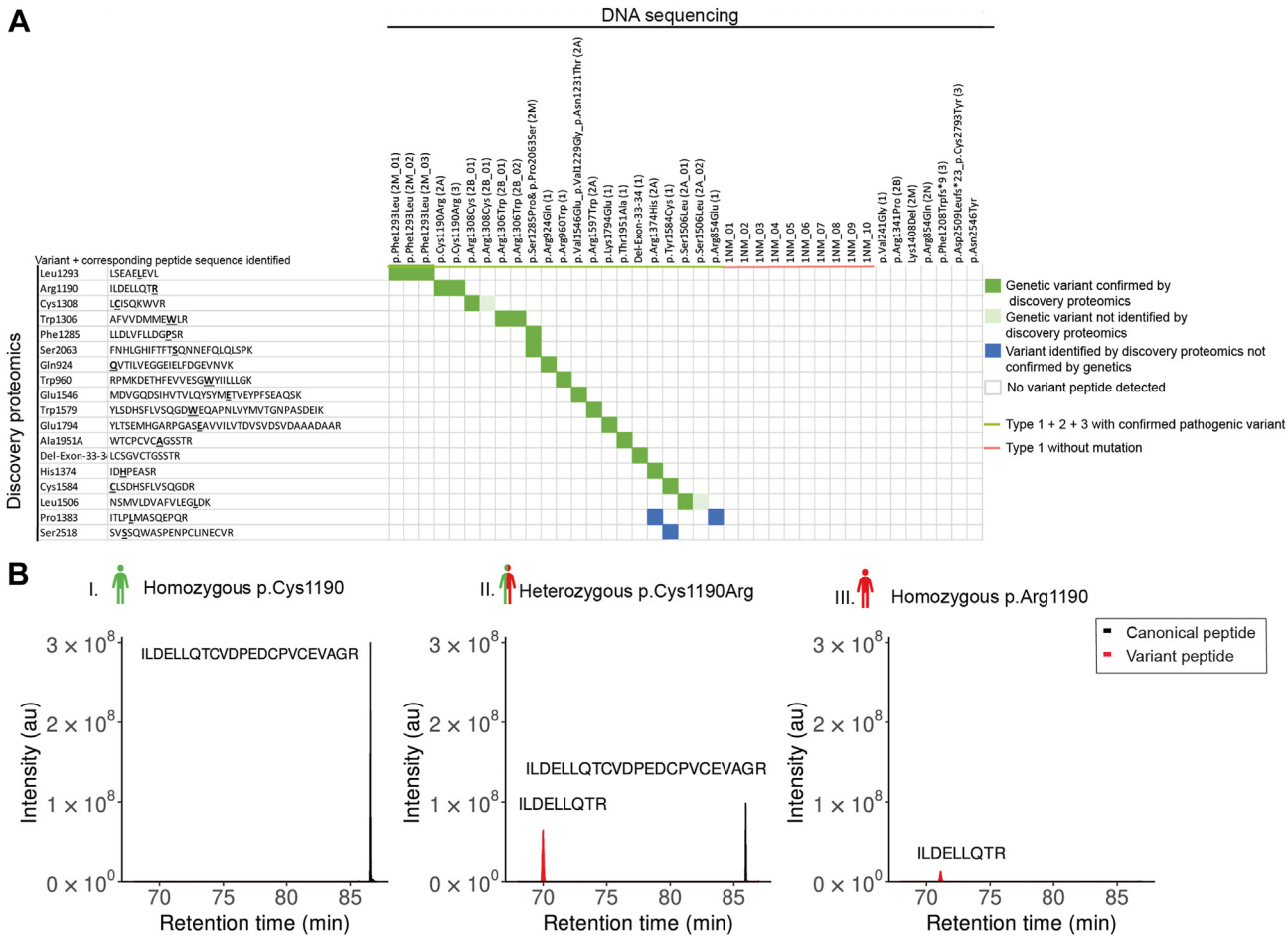

FIGURE 5 Verification of pathogenic variants in von Willebrand factor (VWF) as a result of abnormalities in the *VWF* gene causing von Willebrand disease. (A) Overview of all tryptic peptide sequences unique to pathogenic variants in the *VWF* gene identified by unbiased mass spectrometry–based proteotyping on VWF enriched from plasma. (B) Extracted ion chromatogram of VWF-derived peptides $_{1182}$ILDELLQTCVDPEDCPVCEVAGR$_{1204}$ (black) and variant peptide $_{1182}$ILDELLQTR$_{1190}$ (red) in (I) a patient control, (II) a type 2M patient heterozygous for p.Cys1190Arg variant, and (III) a type 3 patient homozygous for p.Cys1190Arg variant (type 3).

low variant-to-wild-type ratio of 0.1. This ratio implies a 10-fold higher level of the wild-type VWF proteoform compared with VWF with p.Arg1306Trp variant. Upon closer inspection of the clinical data, this 1 type 2B patient received recent VWF replacement therapy (3 times a week 25 E/kg antihemofilie factor/von Willebrand factor complex (Haemate(R) P, CSL Behring GmbH) recurrent gastrointestinal bleeding), suggesting that the relatively high wild-type VWF sequence may be exogenous and derived from VWF replacement therapy, despite a washout period of >72 hours [17].

4 | DISCUSSION

Genetic variation in *VWF* gives rise to a highly polymorphic protein with differential functionalities within hemostasis. For the majority of genetic variants, the clinical impact remains to be elucidated for the individual patient [6,11,30,31]. Analytical methodologies with sensitivity toward protein domains and sequence variants facilitate the translation from genetic variation to the (dys)functional proteoform involved in hemostasis. To bridge the gap between genotyping and functional testing in VWD, we quantified mature VWF, VWFpp, and sequence proteoforms from plasma using MS-based proteomics.

A VWF sequence database was developed, combining the canonical VWF sequence from UniProt, variant sequences registered in the EAHAD database, and unique variants present in this specific VWD cohort. Following this strategy, 16 unique pathogenic variants and 5 polymorphisms classified as likely benign were identified based on plasma-derived VWF [29]. Interestingly, 2 of these common polymorphisms are located within the D′D3 region of VWF, which constitutes the FVIII binding region. Although these variants are registered as "unlikely to be pathogenic" in the ClinVar database, they are associated with altered VWF and FVIII levels [13]. Specifically, the p.Gln852Arg variant has previously been shown to display reduced FVIII binding [32], whereas the p.Thr789Ala variant has been associated with slightly higher VWF levels and is described as protective modifier of the bleeding phenotype [33,34]. This raises the question of whether combinations of polymorphisms might act as (pathogenic) modifiers of hemostasis. Since variants in the D′D3 regions that affect

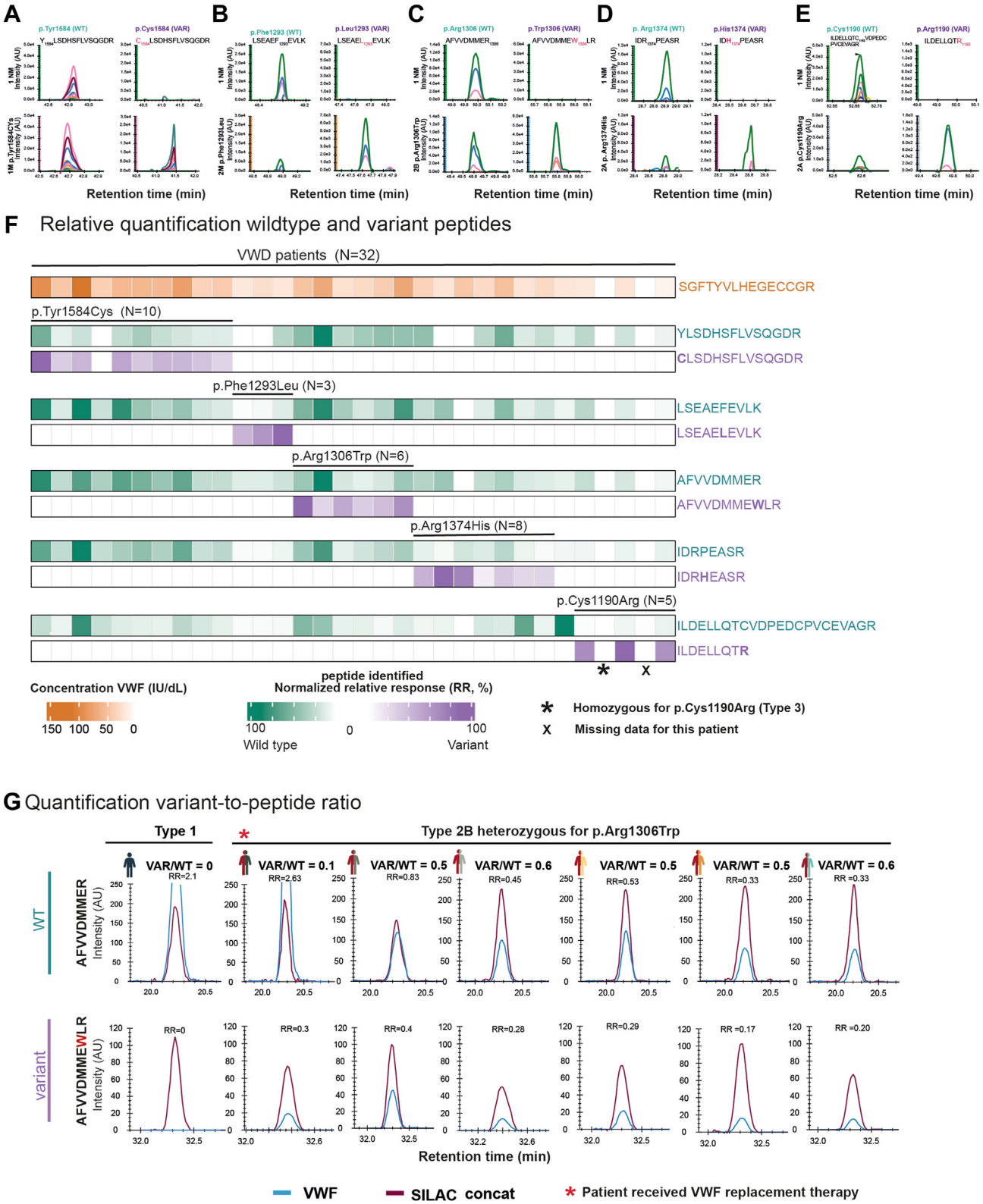

FIGURE 6 Quantification of pathogenic von Willebrand factor (VWF) variants from plasma reveals distinct variant-to-wild-type (VAR/WT) stoichiometry. Peptides characteristic to variants (A) p.Tyr1584Cys, (B) p.Phe1293Leu, (C) p.Arg1306Trp, (D) p.Arg1374His, and (E) p.Cys1190Arg, and their corresponding wild-type (WT) sequence were quantified by targeted mass spectrometry (MS). Representative MS2 extracted ion chromatograms are shown for each target variant found in a von Willebrand disease (VWD) patient heterozygous for this specific variant and a type 1 patient without this variant (control). (F) Relative quantification of 5 VWF variants from plasma using synthetic stable isotope–labeled peptides for each target variant and corresponding WT peptides. The acquired relative response (RR) was normalized per peptide sequence. (G) Quantification of the VAR/WT of VWF in plasma for 6 type 2B patients heterozygous for p.Arg1306Trp and 1 type 1 patient without a pathogenic variant (control). The MS1 extracted ion chromatogram is provided for both endogenous VWF and the stable isotope labeling by amino acids in cell culture concatenated (SILAC concat)–derived peptides. The SILAC VWF concat polypeptide comprised both the WT and variant sequence within the same molecule to enable comprehensive VAR/WT quantification.

FVIII interaction may contribute to a clinical phenotype resembling mild hemophilia A [35,36], it could be beneficial to monitor the plasma level of common VWF variants as a combined signature in relation to the clinical phenotype. Noteworthy in this respect, 2 VWD patients harbored the p.Arg854Gln variant, but only 1 patient was classified as type 2N (FVIII/VWF:Ag ratio = 0.4), while the other patient was classified as type 1 (FVIII/VWF:Ag ratio = 1.8). Based on VWF proteotyping, the type 2N patient was also suspected homozygous for p.Thr789Ala in the D′D3 domain, while the type 1 patient was suspected heterozygous for p.Thr789Ala.

Subsequently to polymorphic variant identification, the MS-based proteotyping strategy was verified for 6 distinct pathogenic variants in 29 of 32 affected VWD patients by coanalyzing synthetic peptides corresponding to canonical and noncanonical sequences. Additionally, we challenged the assumption that a minor allele heterozygosity results in 50% wild-type and 50% variant protein concentration in blood by determining the variant-to-wild-type ratio for p.Arg1306Trp in 6 VWD type 2B patients. The concentration of the pathogenic Trp1306 variant was 2-fold lower compared with that of the wild-type Arg1306 of VWF in plasma. VWF multimers with relatively more variant p.Trp1306 than wild-type p.Arg1306 incorporated have previously been shown to be more sensitive to shear flow-induced unfolding, resulting in enhanced interaction with the clearance receptor low-density lipoprotein receptor-related protein 1, increased platelet binding, and augmented proteolysis by ADAMTS-13, collectively leading to accelerated VWF clearance [37,38]. Interestingly, 1 type 2B patient heterozygous for p.Arg1306Trp had relatively higher concentration of wild-type than variant VWF, which could be explained by recent VWF replacement therapy.

There are some limitations and opportunities to this study that need to be addressed. First, the selection of 64 VWD patients does not represent the natural distribution of VWD patients in the general population. Evaluation of the diagnostic potential of plasma-derived proteotyping by MS requires follow-up studies with independent and larger cohorts of VWD patients. In addition, patients with variants known for impaired biosynthesis or increased clearance (eg, p.Arg1205His) [39] should be included to verify the proteomic strategy for VWFpp/VWF determination and accuracy of the variant-to-wild-type ratio. Second, we highlight the heterogeneity of circulating VWF among individuals. This not only exemplifies the inherent difficulties in assigning reference values for VWF standards across different laboratories for the variety of conventional VWF laboratory tests (VWF:Ag, VWF:CB, VWF:Act, and VWFpp) but also stresses the importance of careful consideration with respect to MS-based reference standards. For instance, we found a relatively high discrepancy in the VWFpp/VWF ratio determined by MS and antigen testing for individuals harboring the p.Arg1374His variant, which appeared to be mainly driven by the relatively low VWF:Ag levels in the ELISA-based assay using polyclonal antibodies (Figure 2E–J) [17,40]. Interestingly, VWD subclassification of patients harboring the p.Arg1374His has been described as being complex or even "unclassifiable" based on the laboratory phenotype [41]. Third, absolute quantification of noncanonical sequences by synthetic SIL peptides needs careful interpretation due to the lack of a calibration strategy when using a normal pooled plasma standard. The use of SILAC polypeptide comprising both canonical and noncanonical sequences facilitated the quantification but was not yet feasible for all target sequences. Fourth, in the type 1 patients without pathogenic *VWF* variants identified by DNA sequencing, no shared altered signatures were identified by our plasma profiling or proteogenomic screening. For 1 of these type 1 patients, VWF deficiency could be explained by increased VWF clearance (VWFpp/VWF ratio > 7). For the other type 1 patients, however, low VWF may be the result of alternative biological mechanisms, such as variants in genes encoding proteins involved in cellular VWF biosynthesis or trafficking [42,43]. Finally, MS-based proteotyping VWF was considered challenging in patients with low VWF plasma levels. Consequently, not all variants (eg, for 2 patients harboring p.Arg1308Cys or p.Ser1506Leu variant; Figure 5A) could be quantified in all patients because the (i) concentration was below the analytical limit of detection (peptide-dependent), (ii) detectability of variant sequences was dependent on single enzyme rather than multienzyme digestions, or (iii) sequence comprising PTMs were neglected in our analyses. In a follow-up study, both variant identification specificity and sensitivity could be improved via multienzyme digestion to advance the diagnostic accuracy of variant proteotyping in plasma [44].

This study is a first step toward bridging the gap between genotyping and functional assays. In this respect, proteoforms resulting from not only genetic variation but also PTMs on VWF are expected to be of great interest from both a biological as well as a clinical perspective [45,46]. A well-known PTM on VWF is glycosylation, which has been shown to be vastly different between plasma- and platelet-derived VWF, to be dependent on the ABO blood group system, and to be a key regulator of ADAMTS-13–mediated VWF cleavage [47]. Although we did not specifically focus on characterization of glycosylation sites in this study, we did note that certain areas in our peptide mapping studies are consistently not covered. It is highly likely that these areas contain PTMs, such as *N*-/*O*-glycosylation. Specifically, regions including $_{1212}TL\text{-}N_{1231}\text{-}CSR_{1274}$, $_{1509}IGEADFN_{1515}R_{1516}$, and $_{1570}YQGGN_{1574}R_{1575}$ were not covered by peptide mapping, which contain well-known *N*-glycosylation sites [48]. In addition, we observed interindividual variability in the sequence comprising the $N_{1147}SC$-glycosylation motif, which might be due to macro-heterogeneity in glycan occupancy previously observed for this specific site [48]. For further qualitative protein analysis, including PTM analysis, alternative analytical approaches could be applied, such as multienzyme digestion combined with *de novo* sequencing or limited proteolysis in combination with top/middle-down MS [49,50]. We expect that proteotypic characterization of plasma-, platelet-, and endothelial cell–derived VWF may shed light on the molecular composition of VWF proteoforms. Nevertheless, quantification of PTMs on plasma-derived VWF, beyond characterization, demands alternative analytical methodologies with appropriate robustness and quality control assurance that still need to be developed prior to its applicability in patient cohort studies.

To conclude, we show that identification of VWF variants from plasma of VWD patients is feasible without the need for genomic

information *a priori*. MS-based proteotyping facilitates (i) VWF and VWFpp quantification from plasma in conventional units and (ii) identification and quantification of VWF variant sequences. Antibody and manufacturer-independent quantification of VWFpp by MS may overcome the analytical hurdles of current immunoassays and can be used to study the additional value of VWFpp/VWF in relation to patient outcome in diagnostic accuracy studies [6]. In addition, the ability to distinguish between endogenous and exogenous VWF in this study exposes opportunities for MS-based protein quantification in pharmacokinetic studies, therapeutic drug monitoring, and assessment of gene therapy efficacy [51,52]. We envision a position for MS-based proteotyping to characterize and quantify pathogenic variants, contributing to the clinical (bleeding) phenotype, and support plasma-based VWD subclassification in the diagnostic care pathway.

ACKNOWLEDGMENTS

We thank all patients participating in the Willebrand in the Netherlands (WiN) study and healthy volunteers from Sanquin.

AUTHOR CONTRIBUTIONS

I.C.K. performed mass spectrometry analysis and data analysis, generated figures, and wrote the manuscript. T.T.v.D. performed mass spectrometry analysis and data analysis, generated figures, designed the concatenated polypeptide, and wrote the manuscript. F.A. and C.v.K. provided patient material and clinical data and revised the manuscript. W.P. and M.B.P.S. performed data analysis. C.v.d.Z. performed mass spectrometry analysis. M.B.-S. produced the concatenated von Willebrand Factor International Standard. J.C.J.E. and A.B.M. reviewed and revised the final manuscript. A.J.H. interpreted proteomics data and revised the manuscript. R.B. supervised the study and revised the manuscript. F.W.G.L. and M.v.d.B. designed and supervised the study and wrote the manuscript. All authors contributed to revisions of the manuscript and approved the final version.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

DATA AVAILABILITY

For original data, please contact m.vandenbiggelaar@sanquin.nl.

ORCID

Iris C. Kreft https://orcid.org/0000-0002-3595-992X

REFERENCES

[1] Castaman G, Linari S. Diagnosis and treatment of von Willebrand disease and rare bleeding disorders. *J Clin Med*. 2017;6:45.

[2] Du P, Bergamasco A, Moride Y, Tzivelekis S, Berthoz FT, Özen G. von Willebrand disease epidemiology, burden of illness and management: a systematic review. *J Blood Med*. 2023;14:189–208.

[3] Dong X, Leksa NC, Chhabra ES, Arndt JW, Lu Q, Knockenhauer KE, Peters RT, Springer TA. The von Willebrand factor D'D3 assembly and structural principles for factor VIII binding and concatemer biogenesis. *Blood*. 2019;133:1523–33.

[4] Zhou Y, Eng ET, Zhu J, Lu C, Walz T, Springer TA. Sequence and structure relationships within von Willebrand factor. *Blood*. 2012;120:449–58.

[5] Zanardelli S, Crawley JTB, Chan Kwo Chion CKN, Lam JK, Preston RJS, Lane DA. ADAMTS13 substrate recognition of von Willebrand factor A2 domain. *J Biol Chem*. 2006;281:1555–63.

[6] James PD, Connell NT, Ameer B, Di Paola J, Eikenboom J, Giraud N, Haberichter S, Jacobs-Pratt V, Konkle B, McLintock C, McRae S, Montgomery RR, O'Donnell JS, Scappe N, Sidonio R, Flood VH, Husainat N, Kalot MA, Mustafa RA. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. *Blood Adv*. 2021;5:280–300.

[7] Leebeek FWGEJ. von Willebrand's disease. *N Engl J Med*. 2016;375:2067–80.

[8] Jacobi PM, Gill JC, Flood VH, Jakab DA, Friedman KD, Haberichter SL. Intersection of mechanisms of type 2A VWD through defects in VWF multimerization, secretion, ADAMTS-13 susceptibility, and regulated storage. *Blood*. 2012;119:4543–53.

[9] de Jong A, Eikenboom J. von Willebrand disease mutation spectrum and associated mutation mechanisms. *Thromb Res*. 2017;159:65–75.

[10] Hampshire DJ, Goodeve AC. The International Society on Thrombosis and Haematosis von Willebrand disease database: an update. *Semin Thromb Hemost*. 2011;37:470–9.

[11] Atiq F, Boender J, van Heerde WL, Tellez Garcia JM, Schoormans SC, Krouwel S, Cnossen MH, Laros-Van Gorkom BAP, De Meris J, Fijnvandraat K, van Der Bom JG, Meijer K, van Galen KPM, Eikenboom J, Leebeek FWG. Importance of genotyping in von Willebrand disease to elucidate pathogenic mechanisms and variability in phenotype. *Hemasphere*. 2022;6:e718.

[12] James PD, Notley C, Hegadorn C, Leggo J, Tuttle A, Tinlin S, Brown C, Andrews C, Labelle A, Chirinian Y, O'Brien L, Othman M, Rivard G, Rapson D, Hough C, Lillicrap D. The mutational spectrum of type 1 von Willebrand disease: results from a Canadian cohort study. *Blood*. 2007;109:145–54.

[13] Keesler DA, Flood VH. Current issues in diagnosis and treatment of von Willebrand disease. *Res Pract Thromb Haemost*. 2018;2:34–41.

[14] Niu L, Stinson SE, Holm LA, Lund MAV, Fonvig CE, Cobuccio L, Meisner J, Juel HB, Thiele M, Krag A, Holm JC, Rasmussen S, Hansen T, Mann M. Plasma proteome variation and its genetic determinants in children and adolescents. *medRxiv*. 2023:2023.03.31.23287853. https://doi.org/10.1101/2023.03.31.23287853

[15] Kreft IC, Hoogendijk AJ, van der Zwaan C, van Alphen FPJ, Boon-Spijker M, Prinsze F, Meijer AB, de Korte D, van den Hurk K, van den Biggelaar M. Mass spectrometry-based analysis on the impact of whole blood donation on the global plasma proteome. *Transfusion*. 2023;63:564–73.

[16] Kreft IC, Huisman EJ, Cnossen MH, van Alphen FPJ, van der Zwaan C, van Leeuwen K, van Spaendonk R, Porcelijn L, Veen CSB, van den Biggelaar M, de Haas M, Meijer AB, Hoogendijk AJ. Proteomic landscapes of inherited platelet disorders with different etiologies. *J Thromb Haemost*. 2023;21:359–72.

[17] de Wee EM, Sanders YV, Mauser-Bunschoten EP, van der Bom JG, Degenaar-Dujardin MEL, Eikenboom J, de Goede-Bolder A, Laros-van Gorkom BAP, Meijer K, Hamulyák K, Nijziel MR, Fijnvandraat K, Leebeek FWG. WiN study group. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108:683–92.

[18] Sadler JE, Budde U, Eikenboom JCJ, Favaloro EJ, Hill FGH, Holmberg L, Ingerslev J, Lee CA, Lillicrap D, Mannucci PM, Mazurier C, Meyer D, Nichols WL, Nishino M, Peake IR, Rodeghiero F, Schneppenheim R, Ruggeri ZM, Srivastava A, Montgomery RR, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the

Subcommittee on von Willebrand factor. *J Thromb Haemost*. 2006;4:2103–14.

[19] Sanders YV, Groeneveld D, Meijer K, Fijnvandraat K, Cnossen MH, van der Bom JG, Coppens M, de Meris J, Laros-van Gorkom BAP, Mauser-Bunschoten EP, Leebeek FWG, Eikenboom J, WiN study group. von Willebrand factor propeptide and the phenotypic classification of von Willebrand disease. *Blood*. 2015;125:3006–13.

[20] Stel HV, Sakariassen KS, Scholte BJ, Veerman EC, van der Kwast TH, de Groot PG, Sixma JJ, van Mourik JA. Characterization of 25 monoclonal antibodies to factor VIII-von Willebrand factor: relationship between ristocetin-induced platelet aggregation and platelet adherence to subendothelium. *Blood*. 1984;63:1408–15.

[21] Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res*. 2011;10:1794–805.

[22] MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*. 2010;26:966–8.

[23] Allaire JJ. RStudio: integrated development environment for R, PBC, Boston, Massachusetts. http://www.rstudio.com/. [accessed January 20, 2020].

[24] Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43:e47.

[25] Geyer PE, Voytik E, Treit PV, Doll S, Kleinhempel A, Niu L, Müller JB, Bader J, Teupser D, Holdt LM, Mann M. Plasma proteome profiling to detect and avoid sample-related biases in biomarker studies. *EMBO Mol Med*. 2019;11:e10427.

[26] Geyer PE, Wewer Albrechtsen NJ, Tyanova S, Grassl N, Iepsen EW, Lundgren J, Madsbad S, Holst JJ, Torekov SS, Mann M. Proteomics reveals the effects of sustained weight loss on the human plasma proteome. *Mol Syst Biol*. 2016;12:901.

[27] Geyer PE, Kulak NA, Pichler G, Holdt LM, Teupser D, Mann M. Plasma proteome profiling to assess human health and disease. *Cell Syst*. 2016;2:185–95.

[28] Kalot MA, Husainat N, El Alayli A, Abughanimeh O, Diab O, Tayiem S, Madoukh B, Dimassi AB, Qureini A, Ameer B, Eikenboom JCJ, Giraud N, Mclintock C, Mcrae S, Montgomery RR, Donnell JSO, Scappe N, Sidonio RF, Brignardello-petersen JR, Flood VH, et al. von Willebrand factor levels in the diagnosis of von Willebrand disease: a systematic review and meta-analysis. *Blood Adv*. 2022;6:62–71.

[29] Broadinsitute. gnomAD browser. https://gnomad.broadinstitute.org/gene/ENSG00000110799?dataset=gnomad_r2_1. [accessed June 9, 2023].

[30] Favaloro EJ, Pasalic L, Lippi G. Getting smart with coagulation. *J Thromb Haemost*. 2022;20:1519–22.

[31] Woods AI, Paiva J, Primrose DM, Blanco AN, Sanchez-Luceros A. von Willebrand disease type 2M: correlation between genotype and phenotype: comment from Woods et al. *J Thromb Haemost*. 2022;20:1022–3.

[32] Paschal RD, Meeks SL, Neff AT. Development of factor VIII inhibitors in two patients with moderate haemophilia A. *Haemophilia*. 2013;19:369–72.

[33] Johnsen JM, Auer PL, Morrison AC, Jiao S, Wei P, Haessler J, Fox K, McGee SR, Smith JD, Carlson CS, Smith N, Boerwinkle E, Kooperberg C, Nickerson DA, Rich SS, Green D, Peters U, Cushman M, Reiner AP, NHLBI Exome Sequencing Project. Common and rare von Willebrand factor (VWF) coding variants, VWF levels, and factor VIII levels in African Americans: the NHLBI Exome Sequencing Project. *Blood*. 2013;122:590–7.

[34] Smith NL, Chen MH, Dehghan A, Strachan DP, Basu S, Soranzo N, Hayward C, Rudan I, Sabater-Lleal M, Bis JC, De Maat MPM, Rumley A, Kong X, Yang Q, Williams FMK, Vitart V, Campbell H, Mälarstig A, Wiggins KL, Van Duijn CM, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: the charge (cohorts for heart and aging research in genome epidemiology) consortium. *Circulation*. 2010;121:1382–92.

[35] Swystun LL, Ogiwara K, Rawley O, Brown C, Georgescu I, Hopman W, Labarque V, Male C, Thom K, Blanchette VS, Carcao MD, Lillicrap D. Genetic determinants of VWF clearance and FVIII binding modify FVIII pharmacokinetics in pediatric hemophilia A patients. *Blood*. 2019;134:880–91.

[36] Sadler B, Christopherson PA, Haller G, Montgomery RR, Di Paola J. von Willebrand factor antigen levels are associated with burden of rare nonsynonymous variants in the VWF gene. *Blood*. 2021;137:3277–83.

[37] De Cristofaro R, Scaglione GL, Lancellotti S, Pagliari MT, Papi M, De Spirito M, Maiorana A, Baronciani L, Arcovito A, Di Stasio E, Peyvandi F. The R1306W type 2B natural mutation of von Willebrand factor dramatically enhances the multimer sensitivity to shear stress. *Blood*. 2012;120:3306.

[38] Wohner N, Legendre P, Casari C, Christophe OD, Lenting PJ, Denis CV. Shear stress-independent binding of von Willebrand factor-type 2B mutants p.R1306Q & p.V1316M to LRP1 explains their increased clearance. *J Thromb Haemost*. 2015;13:815–20.

[39] Rawley O, O'Sullivan JM, Chion A, Keyes S, Lavin M, van Rooijen N, Brophy TM, Fallon P, Preston RJS, O'Donnell JS. von Willebrand factor arginine 1205 substitution results in accelerated macrophage-dependent clearance in vivo. *J Thromb Haemost*. 2015;13:821–6.

[40] Hoofnagle AN, Wener MH. The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. *J Immunol Methods*. 2009;347:3–11.

[41] Michiels JJ, Berneman Z, Gadisseur A, van Der Planken M, Schroyens W, van De Velde A, van Vliet H. Classification and characterization of hereditary types 2A, 2B, 2C, 2D, 2E, 2M, 2N, and 2U (unclassifiable) von Willebrand disease. *Clin Appl Thromb Hemost*. 2006;12:397–420.

[42] Sanders YV, van der Bom JG, Isaacs A, Cnossen MH, de Maat MPM, Laros-van Gorkom BAP, Fijnvandraat K, Meijer K, van Duijn CM, Mauser-Bunschoten EP, Eikenboom J, Leebeek FWG, Coppens M, Kors A, de Meris J, Nijziel MR, Tamminga RYJ, Ypma PF, Smiers FJW, Granzen B, et al. CLEC4M and STXBP5 gene variations contribute to von Willebrand factor level variation in von Willebrand disease. *J Thromb Haemost*. 2015;13:956–66.

[43] Kat M, Burgisser PE, Janssen H, De Cuyper IM, Conte IL, Hume AN, Carter T, Voorberg J, Margadant C, Bierings R. GDP/GTP exchange factor MADD drives activation and recruitment of secretory Rab GTPases to Weibel-Palade bodies. *Blood Adv*. 2021;5:5116–27.

[44] Sinitcyn P, Richards AL, Weatheritt RJ, Brademan DR, Marx H, Shishkova E, Meyer JG, Hebert AS, Westphall MS, Blencowe BJ, Cox J, Coon JJ. Global detection of human variants and isoforms by deep proteome sequencing. *Nat Biotechnol*. 2023;41:1776–86.

[45] Solecka BA, Weise C, Laffan MA, Kannicht C. Site-specific analysis of von Willebrand factor O-glycosylation. *J Thromb Haemost*. 2016;14:733–46.

[46] Canis K, McKinnon TAJ, Nowak A, Haslam SM, Panico M, Morris HR, Laffan MA, Dell A. Mapping the N-glycome of human von Willebrand factor. *Biochem J*. 2012;447:217–28.

[47] Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *J Thromb Haemost*. 2003;1:33–40.

[48] Ward S, O'Sullivan JM, O'Donnell JS. von Willebrand factor sialylation - a critical regulator of biological function. *J Thromb Haemost*. 2019;17:1018–29.

[49] Liu X, Dekker LJM, Wu S, Vanduijn MM, Luider TM, Tolić N, Kou Q, Dvorkin M, Alexandrova S, Vyatkina K, Paša-Tolić L, Pevzner PA. De novo protein sequencing by combining top-down and bottom-up tandem mass spectra. *J Proteome Res*. 2014;13:3241–8.

[50] Klaproth-Andrade D, Hingerl J, Bruns Y, Smith NH, Träuble J, Wilhelm M, Gagneur J. Deep learning-driven fragment ion series classification enables highly precise and sensitive de novo peptide sequencing. *Nat Commun*. 2024;15:151.
[51] Favaloro EJ, Lippi G. Translational aspects of developmental hemostasis: infants and children are not miniature adults and even adults may be different. *Ann Transl Med*. 2017;5:212.
[52] Pipe SW, Leebeek FWG, Recht M, Key NS, Castaman G, Miesbach W, Lattimore S, Peerlinck K, van der Valk P, Coppens M, Kampmann P, Meijer K, O'Connell N, Pasi KJ, Hart DP, Kazmi R, Astermark J, Hermans CRJR, Klamroth R, Lemons R, et al. Gene therapy with etranacogene dezaparvovec for hemophilia B. *N Engl J Med*. 2023;388:706–18.

SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2024.04.011