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ORIGINAL ARTICLE

Characterization of copy-number variants in a large cohort of patients with von Willebrand disease reveals a relationship between disrupted regions and disease type

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Abstract

Background: Genetic analysis for von Willebrand disease (VWD) commonly utilizes DNA sequencing to identify variants in the von Willebrand factor (*VWF*) gene; however, this technique cannot always detect copy-number variants (CNVs). Additional mapping of CNVs in patients with VWD is needed.

Objectives: This study aimed to characterize CNVs in a large sample of VWF mutation-negative VWD patients.

Methods: To determine the role of CNVs in VWD, a VWF high-resolution comparative genomic hybridization array was custom-designed to avoid multiple sequence variations, repeated sequences, and the *VWF* pseudogene. This was performed on 204 mutation-negative subjects for whom clinical variables were also available.

Results: Among the 204 patients, 7 unique CNVs were found, with a total of 24 CNVs (12%). Of the 7 unique CNVs, 1 was novel, 1 was found in a VWF database, and 5 were previously reported. All patients with type 1C VWD and a CNV had the same exon 33 and 34 in-frame deletion. Certain clinical variables were also significantly different between those with and without CNVs.

Conclusion: The in-frame deletion in patients with type 1C VWD exactly matches the D4N module of the D4 domain, a region where mutations and deletions are known to affect clearance. We observed significantly higher VWF–to–ristocetin cofactor levels in patients with type 1C VWD and a CNV than in patients without a CNV, suggesting a relationship between CNVs and the increased clearance observed in patients with type 1C VWD. Glycoprotein IbM activity was significantly lower in patients with type 1 VWD and a CNV than in patients without a CNV, suggesting that platelet binding is more affected by CNVs than single base pair mutations. This work elucidates some of the underlying genetic mechanisms of CNVs in these patients.

KEYWORDS
bleeding score, comparative genomic hybridization, copy number variants, ristocetin cofactor, von Willebrand disease, von Willebrand factor

Essentials

- Copy-number variants (CNVs) are not usually evaluated in patients with von Willebrand disease (VWD).
- Significant phenotypic differences exist among patients with and without CNVs in the *VWF* gene.
- All Type 1C VWD patients had the same partial deletion of the D4 domain, affecting clearance.
- This work elucidates some of the underlying genetic mechanisms of CNVs in VWD.

1 | INTRODUCTION

von Willebrand disease (VWD) is a common inherited bleeding disorder caused by a quantitative or qualitative abnormality of von Willebrand factor (VWF). VWD is often caused by genetic mutations within the coding and regulatory regions of the *VWF* gene and can be divided into 3 major subtypes. VWD type 3 is a recessive disorder categorized by a complete lack of VWF and requiring 2 distinct genetic knockout events. VWD type 2 is characterized by a functional disturbance of VWF, including enhanced (2B) or reduced (2A, 2M) platelet interaction and impaired binding to factor VIII (2N) [1]. VWD type 1 stands as the prevailing form of VWD. It is marked by reduced levels of VWF in the plasma, coupled with intact VWF structure and operational capabilities. This form of VWD is predominantly inherited in an autosomal dominant manner [2] and its underlying cause is largely linked to insufficient VWF production due to haploinsufficiency at the VWF gene locus [3]. These individuals are additionally classified into either a low VWF (LVWF) phenotype or VWD type 1 depending on their VWF plasma levels falling below 30 IU/dL and between 30 and 50 IU/dL, respectively [4], although the validity of these thresholds has been a subject of prolonged discussion [5].

Type 1 VWD involves genetic mutations that lead to disruptions in VWF equilibrium, distinct from the breakdown of VWF multimers by ADAMTS-13 enzyme [6]. These mutations primarily impact the balance of VWF levels in the circulating plasma, encompassing processes such as VWF synthesis, secretion, storage, and clearance. Those with VWD type 1C experience a notable increase in the clearance of VWF. Approximately 35% of individuals with type 1 VWD lack a known pathogenic variant in the *VWF* gene [7]. Previous genetic studies revealed that merely 41% of families with type 1 VWD showed a connection to the *VWF* locus [8]. This percentage is even smaller among individuals with LVWF as their reduced VWF levels are typically attributed to problems with synthesis and secretion rather than overactive clearance [9]. In fact, the 2006 subcommittee dedicated to VWD guidelines expanded their recommendations to encompass potential genetic origins of VWD beyond the *VWF* gene itself [10]. Further complicating the genetics of VWD is the presence of an unprocessed pseudogene (*VWFP1*) located on chromosome 22. This pseudogene spans exons 23 to 34 of the full *VWF* gene found on chromosome 12 and has 97% sequence homology to the *VWF* gene for that region [11].

Due to the fact that VWD is a complex disorder with multiple genetic risk factors, it is necessary to move beyond looking for putatively causal single nucleotide polymorphisms in the *VWF* gene. Copy-number variants (CNVs) are a type of structural variation involving duplication or deletion of segments of chromosomes and have important roles in disease. CNVs have been found to cause all subtypes of VWD [1]. In the past, variant screening methods were unable to detect heterozygous CNVs due to amplification of the wild-type allele by polymerase chain reaction (PCR)–based methods [12]. Comparative genomic hybridization (aCGH) is a sensitive and powerful method to identify CNVs in the *VWF* gene, narrow the breakpoint locations for mapping, and characterize the different mechanisms for VWD types 1 and 3. Both in-frame and out-of-frame deletions may be a common mechanism of VWD type 1 and result in reduced levels of VWF in plasma. Furthermore, a number of studies have employed the technique of multiplex ligation–dependent probe amplification to examine VWF, enabling the detection of substantial homozygous and heterozygous copy-number variants (CNVs) within individuals afflicted by VWD. However, only a small subset of CNVs has been examined using this method [12]. Here, we report the results from 204 mutation-negative patients with VWD; using aCGH, we found that approximately 12% of these patients harbor a CNV, many of which were found multiple times. These results highlight the importance of CNVs in VWD as well as the need for further investigation into the pathological mechanisms underlying these CNVs.

2 | METHODS

2.1 | Cohort description

All subjects with VWD were enrolled in the Zimmerman Program of the Molecular and Clinical Biology of VWD study (Zimmerman Program) through 8 primary and 23 secondary clinical hematology centers across the United States. The institutional review board approved this study and all patients and/or parents provided informed consent. Patients found to have a mutation in their *VWF* gene with Sanger

sequencing were excluded, leaving 204 patients. Sixty-nine (34%) patients were male, and 135 (66%) patients were female. The demographic breakdown (self-reported) of the patient population is as follows: 162 (80%) were White, 27 (13%) were Latino, 5 (2.5%) were Asian, 5 (2.5%) were of unknown ethnicity, 2 (1%) were American Indian, 2 (1%) were Black, and 1 (0.5%) was Hawaiian/Pacific Islander. The age range of patients was between 0 and 65 years. Clinical phenotypes ascertained for the patients included VWF antigen (VWF:Ag), VWF ristocetin cofactor (VWF:RCo), VWF propeptide (VWFpp), VWF glycoprotein IbM (VWF:GPIbM), VWF binding to collagen III and IV, and multimer analysis [4]. International Society on Thrombosis and Haemostasis (ISTH) bleeding assessment tool (BAT) score was also obtained. Subjects were classified according to the criteria established by the 2007 National Heart, Lung, and Blood Institute guidelines: VWD type 1 was defined as having VWF levels (VWF:Ag or VWF:RCo) of <30 IU/dL and LVWF was defined as having VWF:Ag or VWF:RCo levels of 30 to 50 IU/dL. Subjects with a VWF:Ag level of <30 IU/dL and a VWFpp-to-VWF:Ag ratio of >3 were categorized as having VWD type 1C. Subjects with type 3 VWD had undetectable VWF.

2.2 | DNA sequencing

EDTA whole blood was collected from each subject, and DNA was isolated by the Qiagen Gentra Puregene method in the Molecular Diagnostic Laboratory at Versiti Diagnostic Laboratories. Full-length *VWF* Sanger sequencing was performed, including intron/exon boundaries at Harvard Partners Genome Center, Versiti, or Functional Biosciences using *VWF* reference sequence NM_000552, and the results were analyzed using SoftGenetics Mutation Surveyor DNA Variant Analysis Software. Rare *VWF* variants (<1% of Zimmerman Program healthy controls) were considered potentially causative [13].

2.3 | CNV validation using custom aCGH

CNVs are identified by mean log ratio of fluorescence hybridization of a test sample vs reference sample. In the aCGH workflow, the same concentration of genomic DNA of the sample and the reference are denatured, each labeled with fluorescent dyes (Cy3 and Cy5, respectively), combined, and competitively hybridized to probes on an oligonucleotide array. After hybridization, the array is washed and scanned for fluorescent signal intensity. The log ratio of the sample signal intensity vs the reference is analyzed for each probe. Copy-number variants are represented as mean log ratio changes of probes within a chromosomal region. A high-resolution aCGH array was custom-designed using long oligonucleotide probes approximately 60 bp in length to avoid multiple sequence variations, repeated sequences, and overlap with the pseudogene. A total of 645 probes spanned the *VWF* exon and exon/intron boundaries, and only 32 of these probes cross-hybridized with the *VWF* pseudogene region. For much of the pseudogene region, unique probes could not be identified. Instead of removing nonunique probes, they were retained, with the knowledge that if a CNV was observed in some regions, it would have to be shown whether they were in *VWF* or the pseudogene. Data were manually inspected and analyzed with CytoSure Interpret Software (Oxford Gene Technology), and QC was performed as in the study of Askree et al. [14].

2.4 | Confirming and mapping breakpoints

A multiplex PCR approach was used to map deletion breakpoints and confirm the presence and zygosity of the deletions in index cases and associated family members. Oligonucleotide PCR primers, 20-30 bases in length, were designed based on the maximum deletion call according to the CytoSure software and were mapped directly surrounding the area of the potential CNV. One set of primers was designed to hybridize outside of the deletion within 200 bp on both ends, and it amplified the deletion product and the normal allele product, if not too large. A second set of primers was designed to include at least 1 primer that was within the deletion, which would amplify a product only found in the normal allele and served as a control. Multiplex PCR conditions were optimized, and reactions were assembled using Promega's GoTaq Green Master Mix. Thermal cycler conditions were optimized based on the primer properties and expected length of products. A normal control DNA was included in each run.

Agarose gel electrophoresis was performed, and the ProteinSimple AlphaImager (ProteinSimple) was used to visualize and calculate the band sizes. Zygosity was determined according to the bands present. Bands indicating the size expected for the deletion product were excised and purified using Macherey-Nagel's NucleoSpin Extract II kit. Purified products were sequenced using the outer set of primers.

2.5 | Statistical analysis of clinical phenotypes

First, all patients with no *VWF* sequence variant for whom aCGH was performed were divided into 3 types: 1) type 1, 2) type 1C, and 3) type 3. We did not perform this analysis for patients with type 2 VWD as only 1 had a CNV. Next, we compared clinical phenotype data within each type listed above. To determine whether any statistically significant differences existed between those with and without a CNV, *t*-tests were performed in the R statistical software package by the R Project, using the *t*-test function [15]. This comparison was performed for all clinical phenotypes for which we had data.

3 | RESULTS

Here, we have systematically identified the frequency of clinically relevant CNVs in the *VWF* gene in a sample of mutation-negative patients with LVWF and VWD with no single base pair protein-altering *VWF* variant. No patients with LVWF were found to have a

TABLE 1 Copy-number variant counts found in the sample, stratified by von Willebrand disease type.

Exons	Total	LVWF	Type 1	Type 1C	Type 3	Type 2A
Ex 1-3 del[a]	1	0	0	0	1	0
Ex 4-5 del	13	0	10	0	3	0
Ex 18 del[a]	2	0	0	0	2	0
Ex 33-34 del	5	0	0	5	0	0
Ex 35-37 del[b]	1	0	1	0	0	0
Ex 35-38 dup[a]	1	0	0	0	1	0
Ex 9-10 dup	1	0	0	0	0	1
Total	24	0	11	5	7	1

del, deletion; dup, duplication; Ex, exon; LVWF, low von Willebrand factor.
[a]Not in-frame.
[b]Novel at the time of publishing.

protein-altering CNV in *VWF*. However, 11 patients with type 1 VWD, 1 patient with type 2A VWD, and 7 patients with type 3 VWD were found to have 7 recurrent CNVs disrupting the coding sequence of *VWF* (Table 1). Of these 7 CNVs, 3 were not in-frame, and 1 was novel. Although all but 1 patient with type 1 VWD had the same in-frame deletion of exons 4 and 5 (c.221-977_532+7059del) and all patients with type 1C VWD had the same in-frame deletion of exons 33 and 34 (c.5621-478_5842+2440delinsGCAGCATAAGCATAAAGC), patients with type 3 VWD had CNVs in 4 locations across the *VWF* gene, with the majority causing a frameshift and a truncated VWF. The lone patient with type 2A VWD harbored the only duplication found in the patient sample (Figure). The prevalence of CNVs in males vs females was not significantly different (P = .61).

3.1 | Type 1C

In the patient population, we found that 5 of 13 mutation-negative patients with type 1C VWD (38%) had an in-frame deletion of exons 33 and 34 (c.5621-478_5842+2440delinsGCAGCATAAGCATAAAGC). No other CNV was present in the *VWF* gene of these patients. This 3.5 kb deletion results in a loss of 9 cysteine residues, which may be associated with reduced plasma survival of the VWF protein in these patients. This exon 33 and 34 deletion was previously reported by Cartwright et al. [12]. However, in that study, the exon 33 and 34 deletion was found in a patient with type 2A VWD, along with 2 other nonsynonymous *VWF* mutations in trans. They found that this patient had a mild reduction in VWF:Ag and a reduction in high-molecular-weight VWF, but expressed pseudo–Weibel-Palade bodies similar to wild type in appearance and localization. This deletion has also previously been reported in a patient with VWD type 1 [16] and a patient with type 2A VWD, hemophilia, and a mutation in the *F8* gene [17,18]. As expected, the patients with type 1C VWD had a higher VWFpp-to-VWF:Ag ratio than the patients with type 1 VWD ($P = 1.43 \times 10^{-8}$).

When we compared the clinical phenotypes of those patients with VWD type 1C with (n = 5) and without a CNV (n = 8), we found that those with a CNV have significantly higher ristocetin cofactor levels on average (18.6) than those type 1C without a CNV (11.6; $P = 2.2 \times 10^{-3}$). They also had a significantly lower bleeding score (3.4) than those without a CNV (9.9; P = .02) (Table 2).

3.2 | Type 1

In the patient population, we found that 12 of 37 (32.4%) patients with VWD type 1 had a portion of the *VWF* gene deleted. Ten out of 11 of these are the well-known exon 4 and 5 in-frame deletion (c.221-977_532 _+7059del [p.Asp75_Gly178del]) and the last is a novel in-frame deletion of exons 35 to 37 (c.5843-2158_6598+32del). This exon 4 and 5 deletion has been previously found in both patients with VWD type 1 and patients with VWD type 3 of European ancestry and was absent in patients of Asian ancestry [19]. This deletion was inherited in an autosomal dominant manner and associated with a particular haplotype, possibly indicating a founder effect. Expression studies showed decreased secretion and defective multimerization. Another study [20] also reported this deletion in a patient with type 3 VWD of European ancestry, while it was absent in their Iranian VWD population. Among the patients with VWD type 1, those with a CNV had significantly lower VWF:GPIbM (28.3) than those without a CNV(35.9; P = .03) (Table 2).

3.3 | Type 3

We found 3 unique deletions among the patients with type 3 VWD. The frameshift deletions of exons 1 to 3 (c.-30029_220+3487del), 18 (c.2282-809_2442+2811delinsT), and 35 to 38 (c.5843-2754_6799-1517del) were previously reported by our group in patients with type 3 VWD [21]. The exon 1 to 3 frameshift deletion had been previously reported in a Hungarian patient group with type 3 VWD; however, this study described different breakpoints from those in the present study [22]. The authors suggested that as there were Alu Y and Alu SP repetitive sequences at the ends of the deletions, the deletion was caused by an Alu-mediated recombination event and rose to high frequency through a founder effect. An additional 3 of these patients had the exon 4 and 5 deletion seen in patients with VWD type 1, including 1 patient who had both the exon 1 to 3 and 4 and 5 deletions. Among the clinical phenotypes in the patients with type 3 VWD, we found no significant differences between those with or without a CNV.

3.4 | Type 2A

The only duplication we found in the entire sample was in a patient with type 2A VWD and was an in-frame duplication of exons 9 and 10 (c.998_1156dup; p.Glu333_385dup). This duplication has been

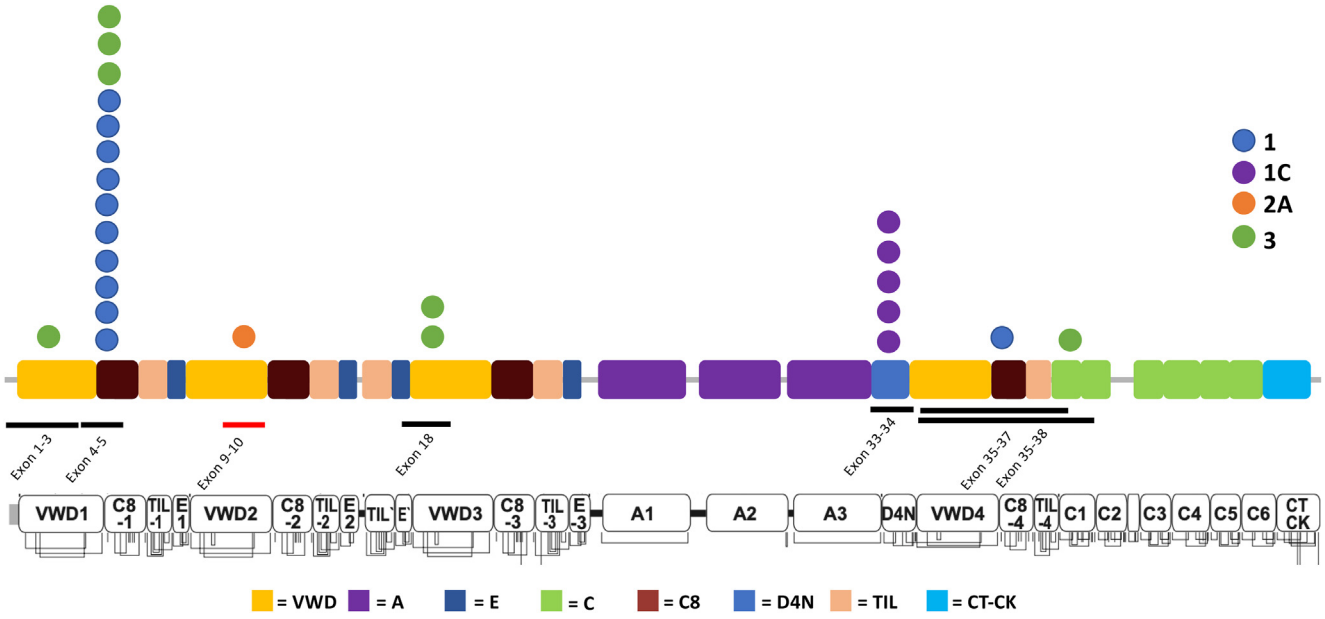

FIGURE Mapping of *VWF* comparative genomic hybridization copy-number variants across the *VWF* gene by von Willebrand disease type and domain (black lines indicate deletions; red lines indicate duplications).

previously described [23]. This individual in our study had a VWF:Ag level of 180 IU/dL, a VWFpp-to-VWF:Ag ratio of 0.4, a VWF:RCo level of 46 IU/dL, a VWF:GPIbM level of 26 IU/dL, and an ISTH BAT score of 4, with loss of high-molecular-weight multimers.

4 | DISCUSSION

All 5 of the patients with VWD type 1C and a CNV had the same in-frame deletion of exons 33 and 34. This directly corresponds to the D4N module of the D4 domain in the *VWF* gene [24] as amino acids 1873 to 1948 encompass this module, and the deletion present in the patients includes amino acids 1875 to 1947. The D4 domain is known to harbor mutations that affect clearance [25]. One study [26] examined the effect of various truncated forms of VWF on clearance in mice and found that deleting amino acids 1875 to 2813 (D4N module to CK domains) actually resulted in reduced clearance. A subsequent study measured the expression of VWF fragments in HEK 293 cells and found that omission of the D4N module from the D4-TIL4 domains abolished the expression of VWF [24].

Our group previously reported that reduced plasma survival of VWF can be a determinant of VWD type 1C, as can mutations upstream of the D4 that causes processing defects [25]. So, it is possible that the deletion of the D4N module could be 1 such mutation. *In vitro* expression studies of this deletion have shown decreased secretion and defective multimerization, which indicate that this mutation behaves in a dominant negative manner when in the heterozygous state [19]. The fact that the patients with VWD type 1C in the sample with a CNV had significantly higher VWF:RCo than that in patients with type 1C VWD without a CNV could indicate that deletion of the D4N module either impairs clearance more than in those with VWD type 1C and no deletion of this module or renders the molecule less active. Further evidence of the increased clinical severity of patients with VWD type 1C with no D4N deletion comes from the significantly lower ISTH BAT bleeding scores in those patients with VWD type 1C with the deletion than without it. More investigation is needed to determine the role of the D4N module in clearance and bleeding severity in patients with VWD type 1C.

All but 1 of the VWD type 1 patients had the same recurrent in-frame deletion of exons 4 and 5. VWF secretion is strongly reduced in VWD type 1, while the ratio of VWFpp to VWF:Ag remains close to 1 [25]. However, patients with VWD type 1 and a CNV had significantly lesser VWF:GPIbM binding than that in those without, suggesting a role for this deletion in platelet binding. We also found a novel in-frame deletion of exons 35 to 37 in a patient with VWD type 1 that spanned the D4-TIL4 domains. Further work is needed to characterize the underlying functional mechanisms of this deletion.

Limitations of the present study include a small sample size, making it difficult to observe significant differences. Additionally, we only performed aCGH on mutation-negative subjects, so we could have missed subjects who have a VWF variant and a CNV.

In the patient population, CNVs in VWD type 1C and type 1 were primarily localized to exons 33 and 34 and 4 and 5, respectively. However, in patients with VWD type 3, CNVs were spread across the *VWF* gene and all but the exon 4 and 5 deletion were frameshifting. This could be due to the fact that there are multiple mechanisms that lead to complete lack of VWF:Ag as seen in patients with VWD type 3. Here, we have shown that there is a relationship between the regions deleted in VWD type 1C and type 1 as well as significant differences in clinical laboratory values among those with and without CNVs in the *VWF* gene. This work both confirms previous mechanisms associated with these deletions and elucidates possible novel mechanisms

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TABLE 2 Mean values and ranges of clinical characteristics (except International Society on Thrombosis and Haemostasis bleeding assessment tool score, which is the median value) by type and stratified by the presence or absence of a copy-number variant, along with results of significance testing.

Type 1C	**VWF:Ag**	**VWFpp**	**VWFpp-to-VWF:Ag ratio**	**VWF:CB4**	**VWF:CB3**	**VWF:GPIbM**	**VWF:RCo**	**ISTH BAT score**
Mean + CNV (*n* = 5)	20	83	4	16	17	14	19	3.00
(range)	(10-32)	(46-119)	(4-5)	(3-43)	(6-32)	(5-23)	(12-28)	(0-7)
Mean − CNV (*n* = 8)	16	68	5	8	10	9	12	10.5
(range)	(6-26)	(33-187)	(3-12)	(1-14)	(1-15)	(1-14)	(5-15)	(2-17)
P value (*t*-test)	.37	1.00	.42	.17	.09	.09	$\mathbf{2.2 \times 10^{-3}}$	**.02**
Type 1	**VWF:Ag**	**VWFpp**	**VWFpp-to-VWF:Ag ratio**	**VWF:CB4**	**VWF:CB3**	**VWF:GPIbM**	**VWF:RCo**	**ISTH BAT score**
Mean + CNV (*n* = 11)	29	32	1	25	35	28	25	4
(range)	(16-42)	(20-41)	(0.94-1.61)	(7-38)	(16-45)	(16-37)	(14-35)	(0-10)
Mean − CNV (*n* = 26)	31	51	2	27	37	36	29	5
(range)	(22-42)	(31-77)	(1.50-2.99)	(10-40)	(11-58)	(15-54)	(18-50)	(0-16)
P value (*t*-test)	.31	.001[a]	.11	.46	.72	.03[a]	.16	.33
Type 3	**VWF:Ag**	**VWFpp**	**VWFpp-to-VWF:Ag ratio**	**VWF:CB4**	**VWF:CB3**	**VWF:GPIbM**	**VWF:RCo**	**ISTH BAT score**
Mean + CNV (*n* = 7)	4	2	2	4	3	2	5	17
(range)	(1-21)	(1-3)	(0-4)	(1-17)	(1-16)	(1-10)	(1-5)	(8-21)
Mean − CNV (*n* = 12)	10	2	2	8	6	7	9	16.5
(range)	(1-87)	(1-5)	(0-6)	(1-75)	(1-73)	(1-57)	(5-46)	(4-29)
P value (*t*-test)	.61	.68	.94	.66	.66	.55	.40	.74

All values except ISTH BAT scores are in IU/dL.

Ag, antigen; BAT, bleeding assessment tool; CB3, collagen III; CB4, collagen IV; CNV, copy-number variant; GPIbM, glycoprotein IbM; ISTH, International Society on Thrombosis and Haemostasis; RCo, ristocetin cofactor; VWF, von Willebrand factor; VWFpp, von Willebrand factor propeptide.

as well. This study highlights the importance of incorporating CNV analysis to genotype VWD type 1 and type 3 cases when no pathogenic *VWF* variant has been identified. CNV analysis is not routinely performed on patients with VWD; however, it can be an important diagnostic tool.

APPENDIX

Zimmerman Program Investigators

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ETHICS STATEMENT

The Children's Hospital of Wisconsin Institutional Review Board (IRB) approved this study, and all patients and/or parents provided informed consent.

AUTHOR CONTRIBUTIONS

B.S. wrote the manuscript. J.D.P., R.R.M., S.L.H., V.H.F., and P.A.C. conceived the manuscript. B.S. and G.H. performed all statistical analyses. D.B.B. and the Zimmerman Program Investigators collected samples. L.A. advised analyses.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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