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Clinical and Laboratory Variability in a Cohort of Patients Diagnosed with Type 1 VWD in the United States

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Key Points:

1. Type 1 VWD in the US is highly variable, including patients with very low VWF levels as well as those with mild or minimal VWF deficiency.

2. The frequency of sequence variants in the VWF gene increases with decreasing VWF level, but bleeding scores do not vary by VWF level.

Abstract

Von Willebrand disease (VWD) is the most common inherited bleeding disorder, and type 1 VWD is the most common VWD variant. Despite its frequency, diagnosis of type 1 VWD remains the subject of much debate. In order to study the spectrum of type 1 VWD in the United States, the Zimmerman Program enrolled 482 subjects with a previous diagnosis of type 1 VWD without stringent laboratory diagnostic criteria. VWF laboratory testing and full length *VWF* gene sequencing were performed for all index cases and healthy control subjects in a central laboratory. Bleeding phenotype was characterized using the ISTH Bleeding Assessment Tool. At study entry, 64% of subjects had VWF:Ag or VWF:RCo below the lower limit of normal, while 36% had normal VWF levels. VWF sequence variations were most frequent in subjects with VWF:Ag < 30 IU/dL (82%) while subjects with type 1 VWD and VWF:Ag ≥ 30 IU/dL had an intermediate frequency of variants (44%). Subjects whose VWF testing was normal at study entry had a similar rate of sequence variations as the healthy controls at 14% of subjects. All subjects with severe type 1 VWD and VWF:Ag ≤ 5 IU/dL had an abnormal bleeding score, but otherwise bleeding score did not correlate with VWF:Ag level. Subjects with a historical diagnosis of type 1 VWD had similar rates of abnormal bleeding scores compared to subjects with low VWF levels at study entry. Type 1 VWD in the United States is highly variable, and bleeding symptoms are frequent in this population.

Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder, affecting approximately 1:1000 individuals.[1] The most common variant of VWD in clinical practice is type 1 VWD, which presents with mild to moderate mucosal bleeding symptoms, typically associated with a family history of bleeding, and a quantitative reduction in VWF protein. The true incidence of VWD is difficult to determine. Low levels of VWF are seen in up to 1% of the population, but not all have clinically significant bleeding.[2,3] On the other hand, mild bleeding symptoms are not uncommon, such that the coincidental association of low VWF levels and bleeding may lead to an erroneous diagnosis.[4,5] Bleeding symptoms are difficult to quantify, but much recent work has been performed adapting bleeding assessment tools and assessing their reliability in VWD diagnosis.[6]

Lack of reliable screening tests for VWD also complicates diagnosis, in that no single screening test can confirm the presence or absence of VWD, and an array of tests is required to diagnose and classify the type of VWD present. VWF antigen (VWF:Ag) is used to measure total VWF protein present in a sample, while VWF ristocetin cofactor activity (VWF:RCo) is used as a surrogate measure of VWF-platelet binding.[7,8] Shear stress initiates VWF-platelet interactions *in vivo*, but no shear-based clinical assays are presently available to allow efficient diagnosis of VWD in the clinical laboratory setting.

The National Heart, Lung, and Blood Institute published guidelines in 2008 suggesting that laboratory values of VWF:Ag or VWF:RCo < 30 IU/dL serve as a cut-off for the diagnosis of

type 1 VWD.[9] Other groups have suggested 40 IU/dL as a cut-off, although this results in an increased number of VWD patients.[10,11] The hematology community has been cautioned about the risk of over-diagnosis, including an eloquent plea invoking VWD as a "diagnosis in search of a disease" by Dr. Sadler.[4] However, for clinicians faced with patients who bleed and have low VWF levels but an otherwise negative laboratory evaluation, assigning a diagnosis of VWD allows a route to treatment if needed.

Several groups have recently examined cohorts of type 1 VWD patients, including the UK HDCO VWD study,[12] the Canadian type 1 VWD study,[13] and the EU MCMDM-1VWD study.[14] We sought to evaluate the spectrum of type 1 VWD in the United States through a large multicenter NIH-supported study (The Zimmerman Program for the Molecular and Clinical Biology of VWD, or Zimmerman Program) that enrolled patients from 31 United States hematology centers and also evaluated healthy control subjects for comparison. In order to evaluate the true fidelity of VWD in clinical practice in the United States, inclusion was based on a past diagnosis of VWD and treatment as such by the patients' physicians without employing additional strict diagnostic criteria. Phenotypic evaluation of bleeding was measured by a Bleeding Assessment Tool (BAT); laboratory evaluation of VWF was examined through a series of VWF assays; and genetic evaluation of *VWF* was performed by Sanger sequencing and comparative genomic hybridization. The results presented here demonstrate the high degree of variability in bleeding symptoms and VWF laboratory testing observed in subjects with a diagnosis of type 1 VWD in the United States.

Methods

Subjects were enrolled in the Zimmerman Program through 8 primary centers and 23 secondary centers across the US (see appendix). The Institutional Review Board of each study center approved the study, and informed consent was obtained from each subject. A pre-existing diagnosis of VWD of any type was required for study entry. Although family members were enrolled, only probands were included in this current analysis. For this analysis, subjects were assigned to the "type 1 VWD" cohort if they had a current laboratory phenotypic diagnosis of type 1 VWD based on either VWF:Ag or VWF:RCo < 30 IU/dL as measured by the central laboratory at the time of study entry as per the NHLBI guidelines or low VWF with VWF:Ag 30-49 IU/dL and/or VWF:RCo 30-53 IU/dL at the time of study entry to include subjects with levels below the lower limit of normal for each assay. Subjects were assigned to the "historical type 1 VWD" cohort if they were enrolled with a diagnosis of type 1 VWD, but at the time of enrollment, their central laboratory findings did not support the laboratory criteria for diagnosis of VWD.

Phenotypic evaluation:

A bleeding questionnaire was administered to each subject, comprised of questions that enabled calculation of a formal bleeding score as well as Zimmerman Program specific questions. For the purpose of this report, bleeding scores were calculated using the ISTH Bleeding Assessment Tool (BAT).[15] The bleeding questionnaire and other questions were asked by a trained study coordinator, nurse, or physician. Race and ethnicity were as self-reported by subject. Subjects were encouraged but not required to answer all questions.

Laboratory evaluation:

Blood samples were collected from each subject at the time of study enrollment. Samples collected in 3.2% sodium citrate were centrifuged and plasma frozen at -80° C and sent to the central laboratory (the Hemostasis Reference Laboratory at the BloodCenter of Wisconsin) for further testing. All samples were maintained at -80° C for long term storage. VWF antigen (VWF:Ag) was performed by ELISA.[16] VWF ristocetin cofactor activity (VWF:RCo) was performed by automated platelet agglutination.[16] Factor VIII activity (FVIII:C) was performed by a one-stage clotting assay.[17] Multimer distribution was assayed by quantitative gel electrophoresis.[18] Ligand binding assays were performed as previously described, including VWF binding to type III collagen (VWF:CB3),[16] VWF binding to type IV collagen (VWF:CB4),[19] and VWF binding to mutant platelet GPIb (VWF:GPIbM).[20,21] VWF propeptide (VWFpp) was measured to evaluate for VWF clearance defects.[22] Blood type was ascertained by reverse typing.[16] When possible, results were compared to historical laboratory results available for the subject. The historical results were performed in a variety of different laboratories, and at a variable number of years prior to study enrollment.

Genetic evaluation:

One additional blood sample per subject was collected in EDTA, and the whole blood shipped at room temperature to the Hemostasis Reference Laboratory at the BloodCenter of Wisconsin. DNA was extracted and subjected to Sanger sequencing of all exons, including intron-exon boundaries and approximately 50-100 base pairs of intronic sequence at either the Harvard Partners Genome Center or the BloodCenter of Wisconsin using the VWF reference sequence NM_000552.[23] For the purpose of this report, sequence variations were stated as such if they

were seen in <1% of the Zimmerman Program healthy controls. Any variant present at higher frequencies in healthy control subjects was excluded from this analysis. Comparative genomic hybridization was used to evaluate for large deletions or insertions by analysis of copy number variation.[24,25]

Statistical analysis:

Chi-square tests were used to compare the categorical outcomes and Kruskal-Wallis/Mann-Whitney tests were used to compare the continuous outcomes across the groups. In addition, log transformed outcomes (since the outcomes were fairly skewed) were used for the multivariable model. A generalized linear model was used and a stepwise selection procedure (which included any variable with $\alpha <= 0.10$) was applied to select the best set of predictors for the respective outcomes.

Results

When considering all subjects originally enrolled as type 1 von Willebrand disease, the median VWF:Ag was 47 IU/dL and the median VWF:RCo was 45 IU/dL. The median bleeding score was 5. We substantiated low VWF levels in 64% of the subjects. These subjects were assigned to the "type 1 VWD" cohort for further analysis. In 36%, normal VWF levels were found at study entry. These subjects were assigned to the "historical type 1 VWD" cohort. The demographic characteristics of each cohort are given in table 1. The type 1 VWD cohort was further divided into clearance defects (type 1C), type 1 severe (type 1S, VWF:Ag 1-5 IU/dL), or type 1 (supplemental table 1). A propeptide to antigen ratio of greater than 3 was used to define

type 1C subjects.[22,26] The type 1C cohort had median VWF:Ag of 8 IU/dL and median VWF:RCo of 5 IU/dL. The median VWFpp/VWF:Ag ratio was elevated in type 1C subjects with a median ratio of 5.33. When the entire type 1 cohort with the exception of type 1C subjects was analyzed, the median VWF:Ag was 39 and the median VWF:RCo was 38 IU/dL. The historical type 1 VWD cohort had a median VWF:Ag of 76 IU/dL and a median VWF:RCo of 72 IU/dL. Differences in collagen binding were observed as previously reported.[19] At the time of initiation of this study, type 2M did not routinely include collagen binding variants so these subjects remained in the type 1 cohort for this report.

Similar racial and ethnic distributions were observed for each group of type 1 subjects, but increased numbers of African Americans were enrolled as healthy controls, potentially increasing the median VWF:Ag and decreasing the median VWF:RCo (table 1). Although the numbers of African American subjects with type 1 VWD were small, we did investigate the potential for laboratory differences based on race. No significant difference was seen for VWF:Ag, VWF:RCo, or VWF:RCo/VWF:Ag for African American type 1 subjects as compared to Caucasian type 1 VWD subjects (p=NS) although African American subjects did have a lower mean ratio of 0.87 as compared to a ratio of 1.00 for the Caucasian subjects. When subjects were analyzed based on ethnicity, no difference was seen for Hispanic compared to non-Hispanic subjects for VWF:Ag, VWF:RCo, or VWF:RCo/VWF:Ag (p=NS) although again VWF:RCo/VWF:Ag ratios trended lower in Hispanic subjects (mean ratio of 0.89).

We next examined the genotype of subjects with VWD. Sequence variations, defined as a variant present in <1% of the healthy control population, were found in 45% of subjects enrolled

as type 1 VWD (including both type 1 and historical type 1 subjects). In the group with type 1 VWD at study entry, 62% had a sequence variation in the *VWF* gene (84% of subjects with either VWF:Ag or VWF:RCo <30 IU/dL and 44% of subjects with levels >30 IU/dL). The historical type 1 VWD cohort had only 14% of subjects with sequence variations, similar to the 14% frequency seen in healthy controls. Figure 1 shows the percentage of subjects with sequence variations by VWF:Ag level. Many of the sequence variations found in the healthy control subjects were present in more than one subject, and African American healthy controls accounted for many of the sequence variations seen, as previously reported.[23] Of the subjects with severe type 1 VWD (VWF:Ag 1-5 IU/dL) 100% had a sequence variation, while 88% of type 1C subjects had a sequence variation (supplemental figure 1).

Figure 2 shows bleeding score by VWF:Ag for all type 1 subjects. When bleeding scores as assessed by the ISTH Bleeding Assessment Tool[15] were compared, there was no difference in bleeding score between the type 1 cohort and the historical VWD cohort, both with median BS of 5 (p=NS). Both type 1 and historical type 1 subjects had significantly higher bleeding scores than the healthy controls ($p<0.01$). When subdivided (supplemental figure 2), the type 1C subjects had a similar median bleeding score of 6 as compared to the remainder of the type 1 subjects (p=NS) but severe type 1 subjects had a much higher median bleeding score of 15 ($p<0.001$). Zimmerman Program type 2 VWD subjects as a combined group had a median bleeding score of 8 and type 3 VWD subjects had a median bleeding score of 15.[27]

Furthermore, bleeding scores within each of the VWD groups varied; 24% of type 1 VWD subjects had bleeding scores in the normal range, which is a score of 0-3 for adult males, 0-5 for

adult females, and 0-2 for all children <18 years of age.[28] This pattern was present in both pediatric and adult subjects ≥ 18 years of age, suggesting that the variable bleeding phenotype seen in type 1 VWD is not solely a function of age and exposure to hemostatic challenges. There was no difference in bleeding scores between males and females in the type 1 cohort, but there was a significant difference between females and males in the historical VWD cohort ($p<0.001$), with higher bleeding scores observed in adult female subjects. There was no difference in bleeding phenotype between boys and girls < 10 years of age. Comparison of bleeding scores for subjects with and without a sequence variation revealed no difference in adult subjects with historical type 1 VWD but a borderline significant difference in those subjects with type 1 VWD (figure 3).

Because blood group O is linked to lower VWF levels, we also investigated each group of subjects by blood type. While blood group O is present in approximately 45% of the general population, subjects with blood group O represented 73% of the type 1 subjects. Group A and B were underrepresented, each present at about half the frequency expected based on population data (21% and 4% respectively enrolled in Zimmerman program vs 40% and 11% in the general population). Only a few blood group AB subjects were enrolled, similar to frequencies seen in the normal population. Similar blood group distributions were observed in the historical type 1 VWD cohort. Sequence variations were more frequent in non-group O subjects. While only 54% of group O subjects had a sequence variation found, 75% of group A, 93% of group B, and 80% of group AB subjects in the type 1 cohort had a sequence variation in the *VWF* coding sequence. Figure 4 shows bleeding scores by blood group.

Alternate assays of VWF function were examined (table 2), including non-ristocetin mediated platelet binding (VWF:GPIbM) and collagen binding (VWF:CB3 and VWF:CB4). VWF platelet binding as measured by VWF:GPIbM was similar to the traditional VWF:RCo for type 1 VWD subjects. VWF:CB3/VWF:Ag ratios were normal for subjects with VWF:Ag > 10 IU/dL, with the exception of one subject with a previously reported p.H1786D sequence variant.[29] VWF:CB4/VWF:Ag ratios were normal for most subjects, but 12 subjects (4%) had low VWF:CB4/VWF:Ag ratios, as previously reported.[19] The median bleeding score for the subjects with low VWF:CB4 group was 10.5, as compared to a median bleeding score of 5 for the remainder of the type 1 subjects with VWF:CB4/VWF:Ag >0.5 or median bleeding score of 6 when comparison type 1 subjects were matched for comparable VWF:Ag, age, gender, race, and ethnicity. This difference was not statistically significant, but when subjects <18 were excluded (due to having less time to generate significant bleeding symptoms), the median bleeding score was 13.5 for those with low VWF:CB4/VWF:Ag ratio compared to 7 for those with normal ratios ($p<0.01$).

Historical VWF levels were available on 88% of the subjects enrolled initially as type 1 VWD (figure 5A). VWF:Ag values varied from 0 to 154 IU/dL, while VWF:RCo values varied from 0 to 223 IU/dL. These levels were obtained anywhere from 1 to 30 years prior to study enrollment. Recorded levels occasionally might have been obtained following treatment and were not all necessarily the original diagnostic laboratory findings, nor were all VWF:Ag and VWF:RCo necessarily drawn at the same time. Correlation between VWF:RCo and VWF:Ag was improved at study entry (figure 5B), as both levels were obtained from the same sample and all testing run in the same laboratory. Of the subjects with historical type 1 VWD and at least

one additional family member enrolled in the Zimmerman Program, 42% had family members with type 1 VWD (37 subjects) or a diagnosis of historical type 1 VWD (8 subjects) while 58% did not have affected family members. However, this data should be interpreted with caution given that not all family members were available for enrollment.

Discussion

While the initial subject enrollment was performed based on the pre-existing diagnoses from the referring center, this current assignment of diagnoses in this study was by phenotypic diagnosis based on careful review of central laboratory results. Discordance between study laboratory findings and the enrollment diagnosis was observed for a significant number of subjects (172 subjects, 36%) with some subjects having historically low VWF levels but normal levels at time of entry. Approximately one third (36%) of subjects were enrolled with a pre-existing diagnosis of type 1 VWD but did not have laboratory evidence of type 1 VWD at the time of study entry. There are a number of possible reasons for this lack of diagnostic fidelity. VWF levels increase with age, such that patients diagnosed many years prior to study entry may have "outgrown" their diagnosis;[30-32] furthermore, the appropriate reference interval for an older adult is not well defined. Assays for VWF function may not be ideal, resulting in potential false positive or false negative laboratory results. This is particularly true for the VWF:RCo with its high coefficient of variation.[33,34] Stress or underlying inflammatory conditions at the time of study entry may have also contributed to increased VWF levels.[35] Hormones and pregnancy can elevate VWF levels. Pre-analytical variability may come from specimen handling prior to reaching the laboratory, and laboratory techniques in measuring VWF activity are also subject to variation. It

is also plausible that in some cases presence of bleeding symptoms and a single low VWF level resulted in with a diagnosis of VWD. Almost half of subjects in the historical type 1 VWD category did have affected family members with current or historical low VWF. Since the subjects enrolled in this study represent only those subjects followed by an adult or pediatric hematologist, typically through a hemophilia treatment center, we suspect that variability in type 1 VWD seen in the community may be even greater than that demonstrated here.

Laboratory findings consistent with a diagnosis of type 1 VWD are generally considered to include decreased but proportional VWF antigen and activity. Typical VWF activity testing in the US includes ristocetin cofactor activity and often multimer distribution, and in some cases collagen binding with types 1 and/or 3 collagen. Our data showed that subjects with laboratory findings otherwise consistent with type 1 VWD but with low VWF:CB4 had increased bleeding symptoms as measured by bleeding score.[19] Although only 12 subjects were affected, this represented 4% of type 1 subjects. This raises the possibility that these subjects may be better classified as type 2M VWD on the basis of a functional defect in the VWF protein, even though the VWF:RCo/VWF:Ag ratio was normal. We elected to include them as type 1 for this analysis, since the collagen testing was performed following study entry as a research test, but they may best fit as type 2M variants.

Genetic analysis of *VWF* is currently not part of the typical laboratory workup for VWD. We have included as sequence variations any novel or previously reported variant found in <1% of our healthy control population, and excluded variants in 1% or more of the healthy controls for the purpose of this analysis. For example, the p.D1472H sequence variation is found at high

frequency in African Americans, and is associated with low VWF:RCo/VWF:Ag ratios but not with an increased risk of bleeding.[16] However, there are two potential limitations with this approach. First, not all sequence variations cause disease, and even unique variants may be benign. Therefore caution should be used in attributing VWD to any specific genetic variant until more careful analysis is performed. Second, even sequence variants occurring at relatively high frequency may result in changes in VWF, which might not be apparent in a healthy control but when inherited or expressed in conjunction with another hemostatic defect, might result in VWD. Further research is needed to clarify both these possibilities and their implications for diagnosis of VWD.

There are now several reported modifier genes not examined in our study that can affect VWF levels, apart from the *VWF* gene, including *CLEC4M* and *STXBP5*.[36,37] A chromosome 2 locus affecting VWF levels has also been identified from sibling studies.[38] However, our reported frequency of *VWF* sequence variations of 62% in all subjects with type 1 VWD is similar to that reported in several other studies, including the UK, Canadian, EU, and German studies.[12-14,39] Four subjects with *VWF* sequence variations had large deletions that would not have been picked up on conventional sequencing but were picked up via comparative genomic hybridization.

Phenotypic assessment of bleeding symptoms is challenging, but the advent of new bleeding assessment tools (BAT) allows for calculation of a numerical bleeding score for patients. In our study, there was little difference in median bleeding score for subjects with low VWF:Ag as compared to subjects with higher VWF:Ag. The relatively low scores could be due to inclusion of a large number of children, who have had less time to accumulate bleeding symptoms. It is

possible that the BAT may be less sensitive in children with fewer hemostatic challenges, although different normal ranges are used in children.[28] In addition, our BAT was performed following diagnosis, such that some patients may have acquired higher scores due to history of previous treatment of known VWD. Evaluation of BAT at time of diagnosis and following changes in BAT and VWF levels over time may be more predictive. Previous studies have showed that the BAT has excellent negative predictive value but lower positive predictive value when used as a screening tool.[40] It may also be very sensitive to mild decreases in VWF level, but in this study did not predict presence of a sequence variation or low VWF levels. Other investigators have examined use of bleeding score as a predictor of VWD and found that higher bleeding scores, particularly in families where multiple members have low VWF levels, were highly predictive of the presence of VWD.[41] However, the spectrum of type 1 von Willebrand disease includes mild bleeding that may not be easily distinguished from normal by current bleeding assessment tools, and an individualized approach that accounts for the observed bleeding rate in a given person may be more useful in terms of treatment.[42]

The Zimmerman Program type 1 cohort has several limitations. Historical VWF levels and the timing of those levels were not available to the study investigators for all subjects. Patients were not systematically investigated for non-VWF causes of a possible bleeding disorder, which may have confounded the results, particularly in the historical type 1 cohort. It is possible that mild factor deficiencies or platelet function defects could have been missed, and raises the question of the need for more thorough evaluation, including specific factor activities (factors IX, XI, XIII) and more extensive platelet testing (aggregation, release). The subjects were recruited from academic medical centers, meaning that these results may not fully represent the community

practice in the United States, and the relatively young median age of the subjects in this study may have influenced bleeding scores and reduce applicability to the general population. Individuals with lower VWF levels, however, are in theory more likely to present at an earlier age due to increased bleeding symptoms. The high numbers of subjects with low VWF levels in the range of 30-50 IU/dL and elevated bleeding scores suggest that this population merits further study, and consideration of the concept that low VWF may be a contributory risk factor for bleeding, even if it is insufficient to classify a patient as having VWD. Treatment for surgical procedures or bleeding episodes may in fact be indicated in this group based on symptoms.

Our study measured VWF activity using VWF:RCo, although we did also analyze a research laboratory VWF:GPIbM assay looking at direct binding of VWF to mutated GPIb in the absence of ristocetin.[20] We did not have available the current commercial VWF:GPIbM assay used in many clinical laboratories, particularly in Europe and Canada.[43] In our study, results with VWF:RCo and VWF:GPIbM were similar, however we did have a number of subjects with normal VWF:Ag included as type 1 VWD because of a low VWF:RCo, and a number of historical type 1 VWD subjects included due to a single low VWF:RCo as well. Unlike the VWF:GPIbM[43], the VWF:RCo is affected by *VWF* sequence variations that alter ristocetin binding but not VWF function.[16]

Despite these limitations, this study does demonstrate several key points. First, genetic analysis of *VWF* in type 1 VWD is not currently sufficient to confirm the diagnosis, although sequence variants are clearly more common in subjects with lower VWF levels. Genetic analysis of the VWF gene in type 1 VWD is not supported by current evidence. Second, VWF levels, and

sequence variations, do not always correlate with bleeding score. Bleeding scores may be more valuable at initial presentation, supported by data showing that bleeding scores were more predictive in family members than in the index case.[44] Obtaining a BAT at time of diagnosis, and following changes with time and age, may ultimately be more useful than retrospective assessment. Third, there appears to be a subgroup of patients who are potentially misclassified as type 1 VWD because standard assessment does not include evaluation of the interaction of VWF with collagen. Fourth, approximately one third of subjects who carried a diagnosis of type 1 VWD actually had VWF levels in the normal reference range upon study entry. The fact that these individuals had bleeding scores similar to those of subjects with type 1 VWD suggests that this group merits additional study. Assigning a diagnosis based on low VWF at one visit may mean limiting the exploration for alternate bleeding disorders, while merely stating they do not meet criteria for VWD may be denying these patients needed treatment for the ultimate cause of their bleeding. In addition, some information would suggest that individuals with higher bleeding scores are more likely to bleed in the future.[45,46]

This study highlights several critical areas in VWD diagnosis that require additional investigation. First, improved evaluation of phenotype, either through bleeding assessment tools or careful clinical evaluation, including repeat testing, should help define which patients require additional workup and treatment, the subject of further Zimmerman Program investigations. The ultimate goal is to accurately assess which patients require treatment, while limiting diagnosis of patients with low VWF levels who lack bleeding symptoms. Second, improved laboratory tests are needed to provide more accurate and efficient diagnosis of VWD. The advent of commercially available VWF:GPIbM assays may help reduce the variability seen with the

VWF:RCo, but repeat testing of borderline patients may still be necessary due to the numerous external influences on VWF levels. Third, improved understanding of both *VWF* genetics and potential modifier genes is required to interpret genotypic variation in type 1 VWD. These efforts will thus guide appropriate diagnosis and ultimately improve care of patients with type 1 VWD.

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Authorship Contributions

VHF and PAC designed the research, analyzed data and wrote the manuscript. JCG contributed to enrollment, analyzed data and edited the manuscript. KDF and SLH analyzed data and edited the manuscript. DBB, RU, and KTM performed DNA sequencing analysis and edited the manscript. MD and RGH performed the statistical analysis. MVR, ADS, JML, SRL, TCA, CL, WKH, MJMJ, RAG, LNG contributed to enrollment and edited the manuscript. ACG, PDJ, DL, IRP assisted with study design and edited the manuscript. RRM conceived the original study, designed the research, analyzed data and helped write the manuscript.

Conflict of Interest Disclosures

VHF has served as a consultant for Baxter and CSL Behring. JCG has served on advisory boards for Baxalta, Bayer, and CSL Behring. KDF has served as a consultant for CSL Behring, Novo-

Nordisk, and Werfen, and on the speakers bureau for Alexion. MVR serves on advisory board for Baxalta, Biogen, Biomarin, Dimensions, Shire, Tacere Benitec; and research funding from Alnylam, Baxalta, Biogen, Biomarin, CSL Behring, Dimension, Ferring, Genetech/Roche, Mescape, Pfizer, SPARK, Shire. SRL has served as a consultant for Novo Nordisk. TCA serves on the advisory board for CSL Behring. CL has served on advisory boards for Baxalta, Bayer, CSL Behring, and Kedrion. RRM is a consultant or advisor for AstraZeneca, Baxter, Bayer, Biogen Idec, CSL Behring, Grifols, Immucor, and Octapharma. None of the other authors have conflicts of interest to disclose.

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Table 1. **Characterization of VWD Cohorts.**

	Type 1 VWD cohort	Historical Type 1 VWD cohort	Healthy Control cohort
# of subjects	310	172	257
# (%) Caucasian	263 (85%)	152 (88%)	139 (54%)
# (%) African American	19 (6%)	7 (4%)	67 (26%)
# (%) Hispanic	35 11%)	16 (9%)	46 (18)
# (%) female	204 (66%)	115 (67%)	193 (75%)
Mean (SD) age at enrollment	19 (15)	21 (16)	38 (11)
# (%) under age 18	197 (64%)	100 (58%)	0

Subjects self categorized race (Caucasian, African American, Asian, American Indian, Native Hawaiian, multiple race) and ethnicity (Hispanic, Non-Hispanic) and had the option of not answering either question (>95% of each cohort had recorded answers for race and ethnicity). Race and ethnicity were separate questions and therefore the percentages do not always add up to 100%.

Table 2. **VWF Laboratory Testing in VWD Cohorts.**

	Type 1 VWD cohort (n=140)	Historical Type 1 VWD cohort (n=172)	Healthy Control cohort (n=257)
VWF:Ag (IU/dL)	36 (17-46)	76 (64-96)	104 (85-143)
VWF:RCo (IU/dL)	33 (19-44)	72 (61-97)	100 (76-141)
VWF:RCo/VWF:Ag ratio	0.98 (0.84-1.13)	1.00 (0.87-1.11)	0.96 (0.84-1.08)
# (%) with normal multimer distribution	285 (92%)	163 (95%)	255 (99%)
VWF:GPIbM	40 (19-54)	94 (75-121)	108 (80-142)
VWFpp	53 (39-66)	74 (66-88)	88 (75-102)
VWFpp/VWF:Ag ratio	1.50 (1.23-2.28)	0.98 (0.79-1.15)	0.80 (0.63-0.99)
FVIII:C	53 (38-71)	85 (73-101)	102 (84-125)
VWF:CB3	39 (20-52)	83 (72-104)	121 (3-167)
VWF:CB4	31 (19-42)	71 (56-99)	108 (74-163)
# (%) with sequence variants in *VWF*	193 (62%)	24 (14%)	36 (14%)
Bleeding score	5 (3-8)	5 (3-9)	1 (0-2)

Results are given as median (interquartile range). For VWF:RCo, the lower limit of detection is 10 IU/dL. Therefore levels < 10 IU/dL were given an average value of 5 IU/dL for calculation of means, with the underlying assumption that levels below 10 would be normally distributed.

Figure Legends

Figure 1. **Sequence variations in VWD are most common in subjects with VWF:Ag <30 IU/dL**. This graph shows the number of subjects with sequence variations (either point mutations or insertions or deletions) in the VWF coding sequence (dark grey) as compared to those without sequence variations in the VWF coding sequence (light grey) for the entire type 1 VWD cohort by VWF:Ag as compared to the healthy controls. The percent of each group with sequence variations is shown at the top of each column. Sequence variations were most common in those with VWF:Ag <30.

Figure 2. **No significant difference in bleeding score for type 1 VWD subjects regardless of VWF:Ag level**. This graph shows the number of subjects with abnormal bleeding scores (defined as >2 in children <18 years of age, >3 in adult males, and >5 in adult females) in dark grey as compared to those with normal bleeding scores (light grey) for the entire type 1 VWD cohort by VWF:Ag. The percent of each group with abnormal bleeding scores is shown at the top of each column. Bleeding scores were similar for type 1 subjects regardless of VWF:Ag.

Figure 3. **Correlation of sequence variations with bleeding scores**. This box and whisker plot compares bleeding scores using the ISTH Bleeding Assessment Tool (BAT) for adult subjects (≥18 years of age) with type 1 VWD (VWF:Ag and/or VWF:RCo below the lower limit of normal at study entry) in the first pair of columns ("VWD 1"), those with a historical diagnosis of type 1 VWD but normal laboratory findings at study entry in the second pair of columns ("VWD 1 Hist"), and a comparison group of healthy control subjects in the third pair of column

("Controls"). Those subjects with a sequence variation are shown in dark grey while those subjects without a sequence variation are shown in light grey. There was no significant difference in bleeding score between those with and those without a sequence variation for the historical type 1 cohort, and a borderline significant difference for the type 1 VWD cohort.

Figure 4. **Bleeding scores vary across blood groups in type 1 VWD subjects**. This graph shows bleeding scores for subjects with blood group A, AB, B, and O. Median bleeding scores are shown at the top of the graph. No significant difference was seen between blood group O and blood group B or AB. A borderline significant difference was seen comparing blood group O and blood group A ($p<0.015$).

Figure 5. **Variation in historical VWF testing for Zimmerman Program subjects**. Panels A and B compare the historical (panel A) and study entry (panel B) VWF:Ag (circles) and VWF:RCo (triangles) for all subjects enrolled with a diagnosis of type 1 VWD. The insets show the comparison of VWF:Ag on the x axis and VWF:RCo on the y axis for historical laboratory values (panel A) and study entry laboratory values (panel B). The correlation is much lower for historical values and improved for study entry values, as expected given that all study testing was performed in the same laboratory, and all testing was performed on the same sample for each subject. However, there still remain issues with the lower limit of the ristocetin cofactor assay, as seen by the number of VWF:RCo values at or below the lower limit of detection.

Figure 1

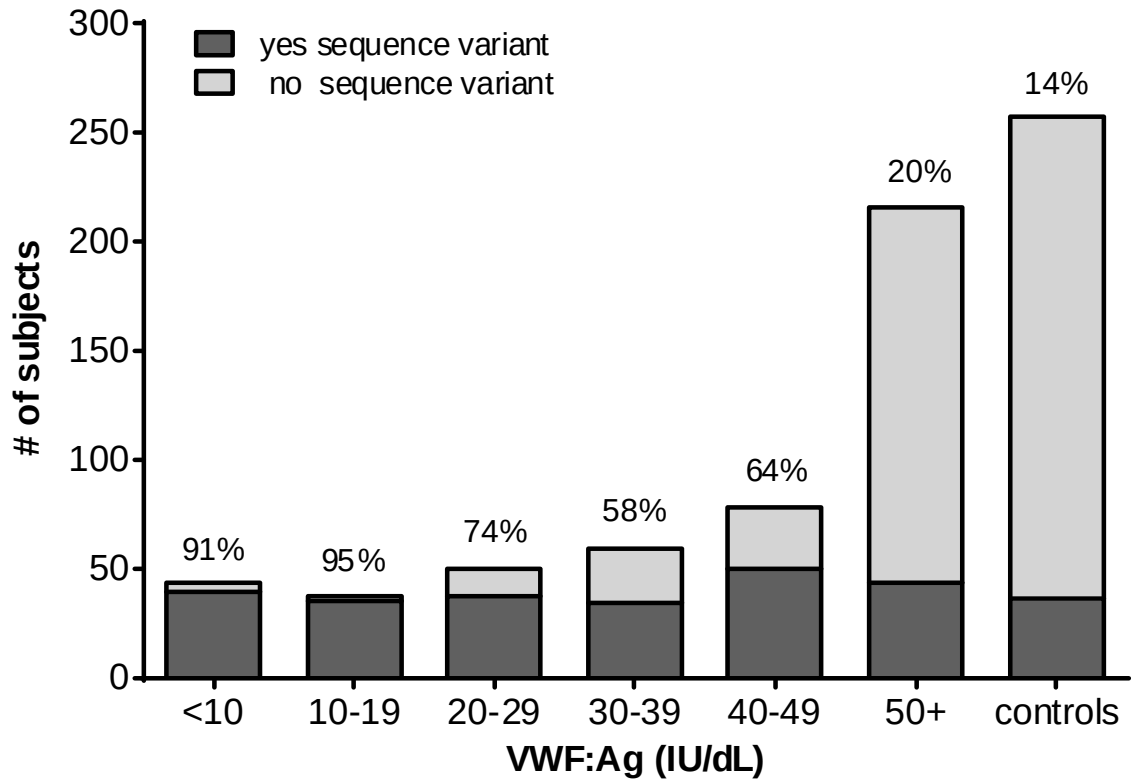


Figure 2

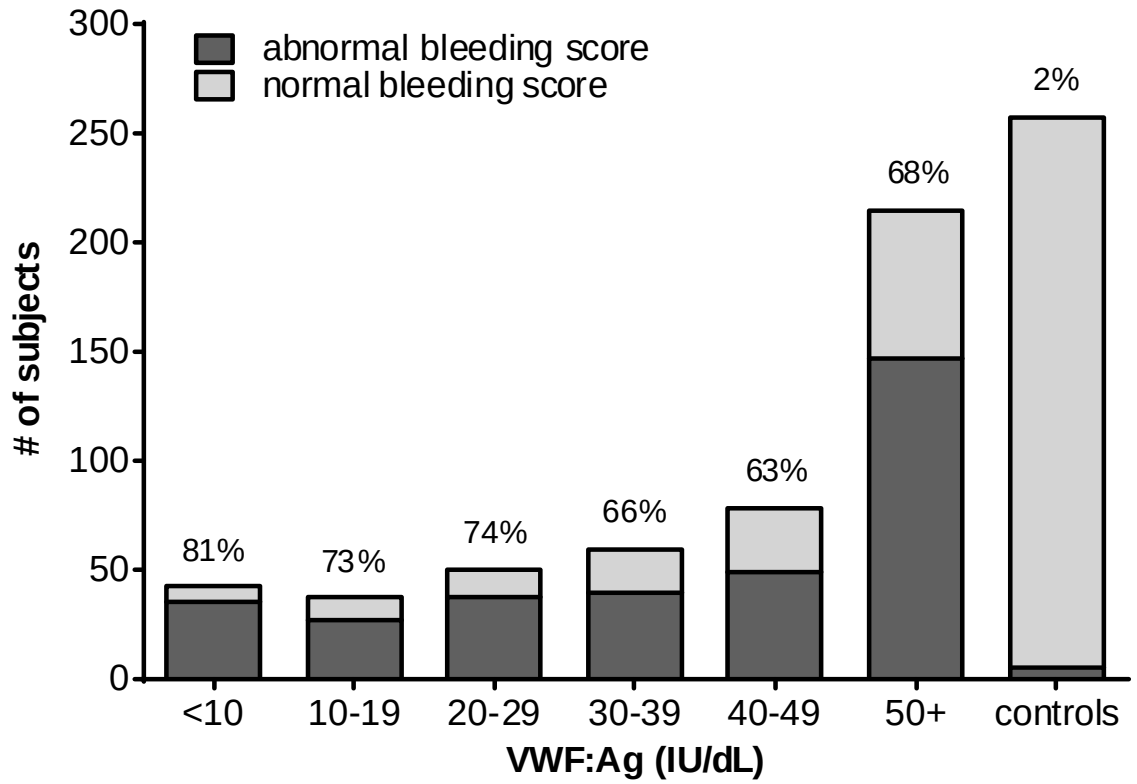


Figure 3

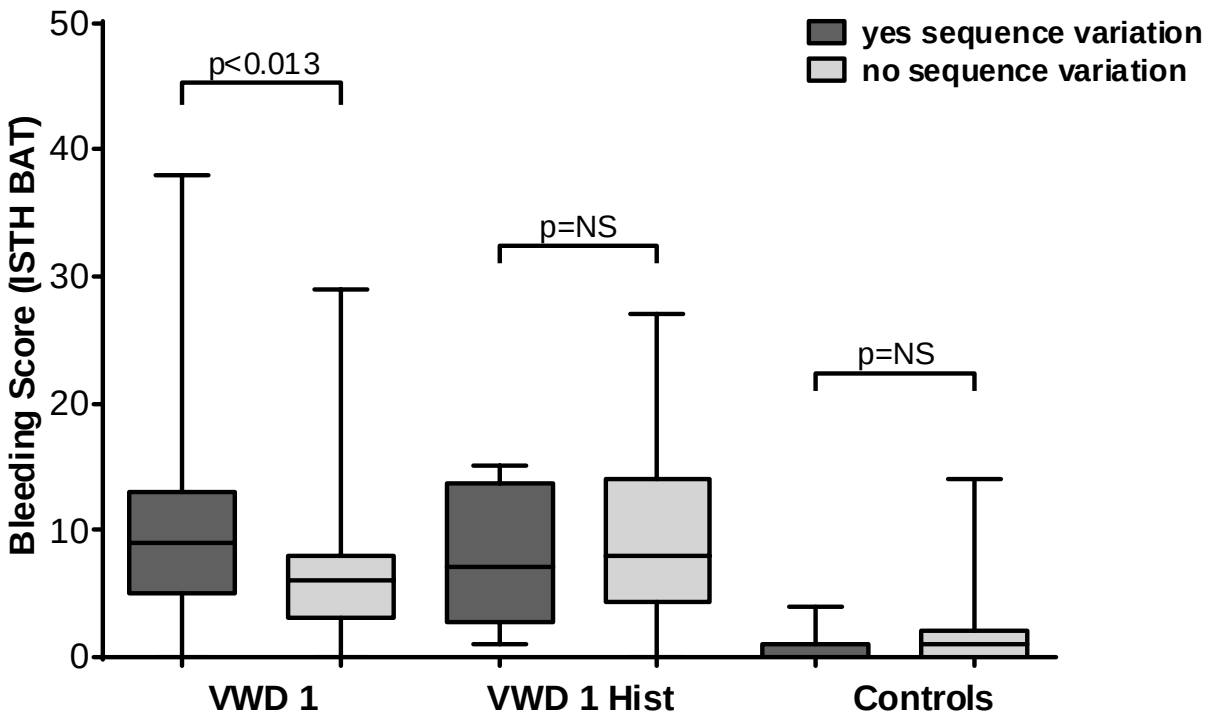


Figure 4

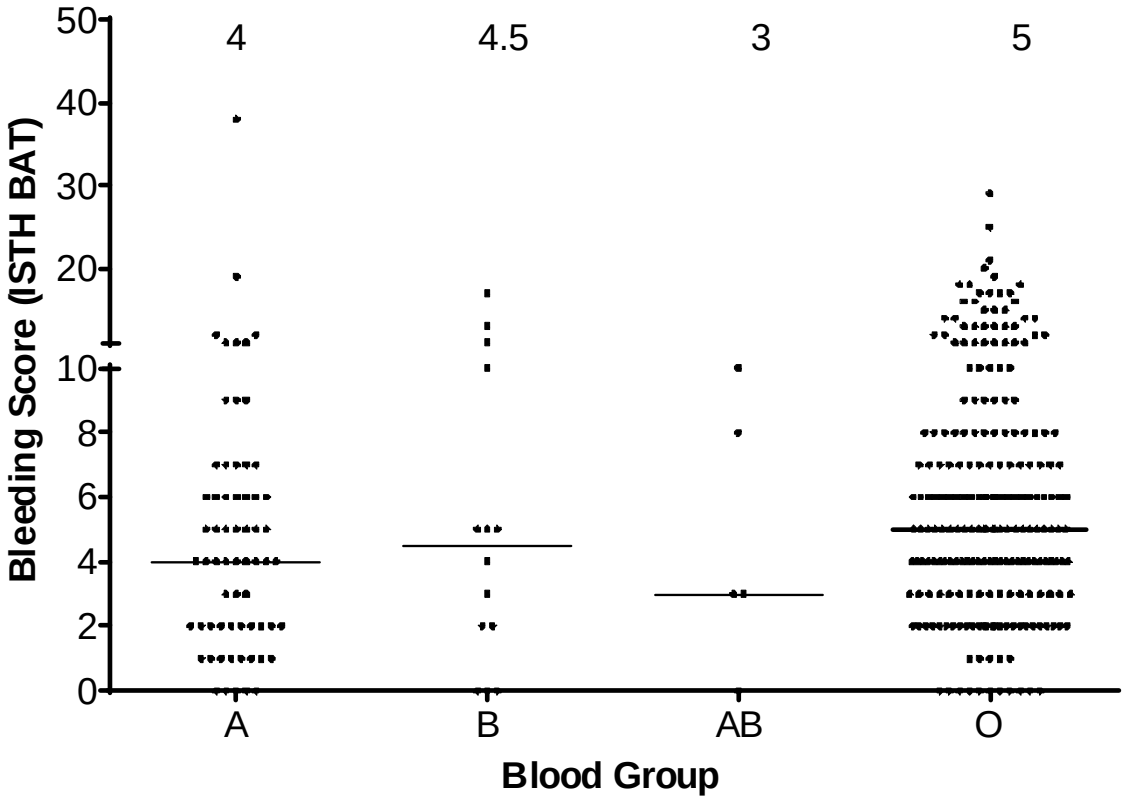


Figure 5A

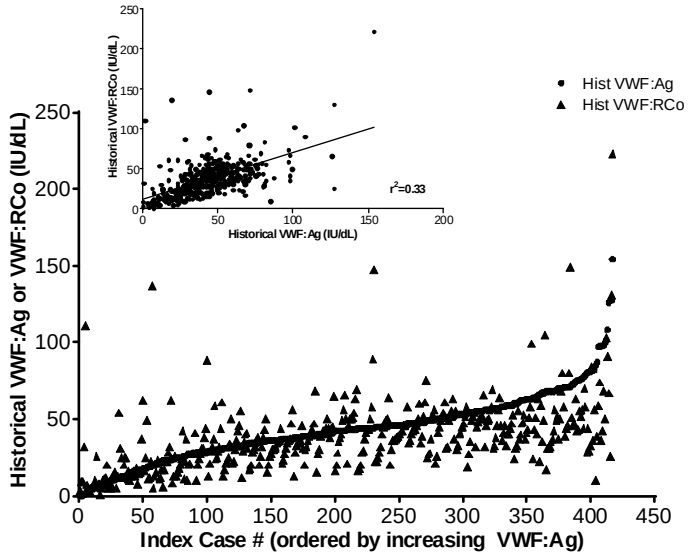
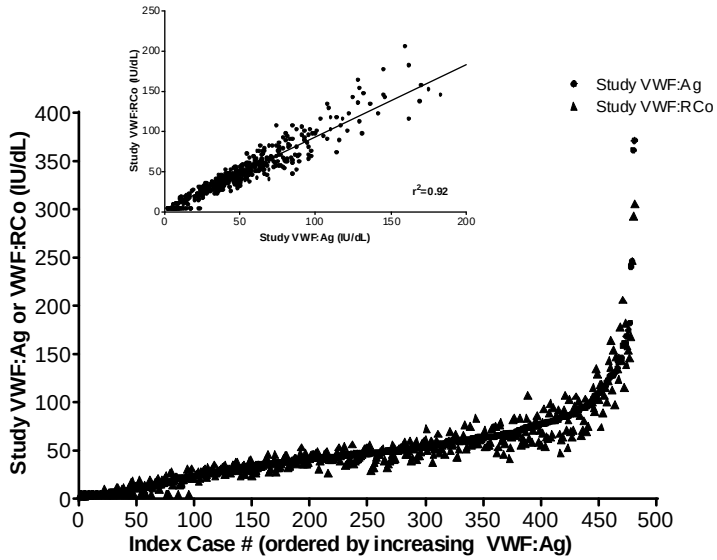


Figure 5B



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Clinical and laboratory variability in a cohort of patients diagnosed with type 1 VWD in the United States

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