Received: 12 July 2017 | Accepted: 19 December 2017

DOI: 10.1002/rth2.12077

ORIGINAL ARTICLE

rpth research & practice in thrombosis & haemostasis

Common *VWF* sequence variants associated with higher VWF and FVIII are less frequent in subjects diagnosed with type 1 VWD

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Funding information
National Institutes of Health, Grant/Award Number: HL081588, HL102260 and HL126810; BloodCenter Research Fund and MACC Fund

[Article updated on March 22, 2018, after first online publication on January 23, 2018: Figures 1 and 2 were updated for clarity.]

Abstract

Background: Genetic variation in the *VWF* gene is associated with von Willebrand factor (VWF) and factor VIII (FVIII) levels in healthy individuals.

Objectives: We hypothesized that *VWF* sequence variants associated with higher VWF or FVIII could impact the diagnosis of type 1 von Willebrand disease (VWD).

Methods: We examined VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), VWF propeptide (VWFpp), and FVIII levels along with *VWF* gene sequencing in 256 healthy control and 97 type 1 VWD subjects as part of a cross-sectional study.

Results: We found several VWF sequence variants (*VWF* c.2880G>A and *VWF* c.2365A>G(;)c.2385T>C, found in linkage disequilibrium) associated with higher VWF and FVIII levels in healthy controls ($P < .001$ for both variants). In addition, these variants were significantly more common in controls than in subjects diagnosed with type 1 VWD and VWF:Ag <30 ($P < .005$). The decreased variant frequencies in type 1 VWD was not seen in other VWD types. VWF:Ag, VWF:RCo, and FVIII were not statistically different in type 1 VWD subjects who had these *VWF* variants compared to type 1 VWD patients without them. There was no difference in ABO blood group, VWF propeptide levels (excluding subjects with known VWF clearance defects), or bleeding score using the ISTH bleeding assessment tool.

Conclusions: These data suggest that certain *VWF* sequence variants associated with elevated FVIII and VWF levels may protect against reduced VWF levels. These findings were independent of other pathogenic sequence variants in *VWF*, suggesting a possible independent effect of c.2880G>A and c.2365A>G(;)c.2385T>C on VWF levels.

KEYWORDS

factor VIII, genetics, hemostasis, von Willebrand disease, von Willebrand factor

Essentials

- Specific sequence variants in the VWF D′D3 region are associated with elevated VWF antigen levels.
- VWF levels and variant frequencies were examined in both European and Caucasian Americans.
- Subjects homozygous for D′D3 variants had the highest VWF and factor VIII levels.
- D′D3 variants are less frequent in type 1 VWD, suggesting a potential protective effect.

1 | INTRODUCTION

Von Willebrand disease occurs when there is a defect in the quantity or quality of von Willebrand factor. Many cases are due to genetic defects in the *VWF* gene, but other patients have VWD without an identified associated *VWF* genetic defect. Genetic changes in *VWF* are clearly linked to type 2 VWD where specific genetic defects in regions of the *VWF* gene coding for specific functional domains of the protein cause predictable defects due to the location.[1] For example, type 2B VWD genetic defects map to exon 28, which encodes for the VWF A1 domain responsible for binding GPIb. Type 2N VWD genetic defects map to the D′D3 region which binds factor VIII (FVIII). Many patients with type 3 VWD, where the VWF protein is absent, have been found to have homozygosity or compound heterozygosity for *VWF* genetic changes coding for stop codons or large deletions in the VWF gene.[2,3]

In type 1 VWD, the most common VWD variant, sequence variants are spread across the *VWF* gene, with no particular predilection for a specific location.[1] Occasionally heterozygosity for type 3 VWD variants such as large deletions are seen, but missense mutations make up the vast majority of type 1 VWD sequence variants.[2] When *VWF* gene sequencing is performed in type 1 VWD, only about 2/3 of patients are found to have genetic variants that might account for their low VWF levels.[4] This percentage has held relatively stable across several large studies of type 1 VWD.[5–8] Lower VWF levels are in general more likely to be associated with a genetic variant.[6,7] Other genes outside the *VWF* locus have been implicated in altering VWF levels, such as *ABO* and *CLEC4M*.[9,10]

While much work has been done on deleterious genetic variants that decrease VWF, there are potential variants that increase VWF levels as well. The CHARGE Consortium reported on a number of variants in both *FVIII* and *VWF*.[11] Recently, Johnsen and colleagues reported on *VWF* variants in African Americans associated with higher VWF and FVIII levels.[12] Because the D′D3 region contains the only binding site for FVIII found in VWF, we focused on variants found in this region. These include synonymous variants c.2880G>A (p.Arg960=)[12,13] and c.2385C>T (p.Tyr795=)[12,13] and nonsynonymous variant c.2365A>G (p.Thr789Ala).[12] We hypothesized that such variants might be under-represented in patients diagnosed with type 1 VWD, where a mild decrease in VWF level is typically present, by conferring an increase in VWF level.

2 | METHODS

Subjects were enrolled in the Zimmerman Program for the Molecular and Clinical Biology of VWD as previously described.[14] Informed consent was obtained from all subjects or their guardian if the subject was under age 18. Healthy control subjects 18 and older were recruited from participating centers via advertisement and were included if they were over the age of 18 and willing to complete study enrollment. VWD subjects were enrolled at clinic visit and through advertisement at some centers. Exclusion criteria included a pre-existing diagnosis of VWD. Specific attempts were made to enroll diverse ethnic groups. Healthy control subjects reported bleeding symptoms and donated a single blood sample for VWF testing and genetic analysis. VWD subjects reported bleeding symptoms and donated a blood sample at the time of enrollment, but were also eligible for follow-up visits (not addressed here). Participants self-identified race and ethnicity, but were given the option to decline answering. Sanger sequencing of the full length *VWF* gene, including intron and exon boundaries, was performed as previously described.[15] VWF antigen was performed by ELISA assay, VWF ristocetin cofactor activity was performed by automated agglutination, and FVIII levels were obtained via one stage clotting assay as previously described.[14] VWF propeptide was measured by ELISA as previously described.[16] ABO blood group was assigned by detection of isohemagglutinins.[9] Bleeding score was calculated using the ISTH bleeding assessment tool.[17] Only subjects with data available for all categories were analyzed. Sample size was determined based on the total number of subjects enrolled during the course of the study.

Subjects were divided into cohorts based on their underlying diagnosis. Healthy controls were adult subjects recruited as controls with no previous diagnosis of a bleeding disorder or VWD. Type 1 VWD subjects were those with a VWF:Ag and/or VWF:RCo <30 IU/dL at the time of study enrollment. Type 2 VWD subjects had laboratory findings consistent with the type 2 VWD variant in question. Type 3 VWD subjects had undetectable VWF (either at study entry or on a historical sample if the study entry sample was obtained while on treatment).

Expression of *VWF* sequence variants was performed using site-directed mutagenesis as previously described.[18] *VWF* c.2880G>A, *VWF* c.2365A>G, and *VWF* c.2385T>C were made as individual constructs containing a single variant in full length *VWF*. An additional construct was made with both *VWF* c.2365A>G and *VWF* c.2385T>C as they were in linkage disequilibrium in our subjects. As some individuals had both sets of variants, a construct was also made containing all three variants together. Each construct was expressed in HEK293T cells by transfection of equal amounts of vector DNA. Secretion of VWF was measured by VWF:Ag as described above. The VWF-FVIII interaction was studied in detail due to the location of the *VWF* variants. VWF binding to FVIII was measured first by capturing recombinant full length FVIII with an anti-FVIII monoclonal antibody (103.3,

Blood Research Institute, Milwaukee, WI), adding recombinant normal or variant VWF, and detecting VWF bound to FVIII with an anti-VWF antibody (AVW-4 and AVW-15, Blood Research Institute). Then FVIII activity bound to VWF was measured by capturing VWF with an anti-VWF monoclonal antibody (AVW-1, Blood Research Institute), adding recombinant full length FVIII, and detecting FVIII activity using the chromogenic Coatest assay (Diapharma, West Chester, OH).

Statistical analysis was performed using GraphPad Prism 7. The Mann–Whitney test was used for comparisons as the data did not have a normal distribution.

3 | RESULTS

VWF parameters, FVIII activity levels, and *VWF* gene sequencing were tested for a group of healthy control subjects and a group of type 1 VWD subjects enrolled in a larger study of VWD, the Zimmerman Program.

3.1 | VWF c.2880G>A

We first looked at 256 healthy control subjects and compared those with or without *VWF* c.2880G>A. Healthy control subjects with c.2880G>A had higher mean VWF:Ag (149 IU/dL) and FVIII (116 IU/dL) whereas healthy control subjects lacking this variant had lower mean VWF:Ag (107 IU/dL) and FVIII (103 IU/dL) as shown in Figure 1. This difference was statistically significant ($P < .001$) for both VWF:Ag and FVIII. Since our healthy controls had a higher number of African American subjects, we also compared VWF and FVIII levels by race. As expected, the c.2880G>A variant was more common in African Americans (61% of controls) as compared to European Americans (36% of controls). Of the African American controls, 52% were heterozygous and 9% were homozygous for c.2880G>A, while of the European Americans, 30% were heterozygous and 7% were homozygous for c.2880G>A. An association of high VWF levels with these variants was observed in both the European American only group and African American only group, with presence of c.2880G>A associated with higher mean VWF levels ($P < .001$). There was no significant difference in FVIII (P = NS). This allele was also observed at high frequencies in the ExAC database, including in European (25%) and African (31%) ancestries (Table 1).[19]

3.2 | VWF c.2365A>G and VWF c.2385T>C

Two other *VWF* variants, c.2365A>G and c.2385T>C, showed evidence of being in linkage disequilibrium. All of our subjects with these variants were heterozygous for both, homozygous for both, or found to have neither variant. Healthy control subjects with c.2365A>G(;)c.2385T>C had higher mean VWF:Ag (143 IU/dL) and FVIII (114 IU/dL) whereas healthy control subjects lacking this variant had lower mean VWF:Ag (100 IU/dL) and FVIII (101 IU/dL) as shown in Figure 2. This difference was statistically significant ($P < 0.005$) for both VWF:Ag and FVIII. We also compared VWF and FVIII levels by race. As expected, the c.2365A>G and c.2385T>C variants were more common in African Americans (88% of controls) as compared to European Americans (49% of controls). Of the African American controls, 46% were heterozygous and 42% were homozygous for these variants, while of the European Americans, 36% were heterozygous and 12% were homozygous for c.2365A>G(;)c.2385T>C. When only African Americans or European Americans were considered for the presence of the c.2365A>G(;)c.2385T>C variant, the difference in VWF:Ag was significant ($P < .05$ for African Americans, $P < .001$ for European Americans). No difference however was observed in mean FVIII levels (P = NS). Both *VWF* variants were observed at nearly identical high frequencies (consistent

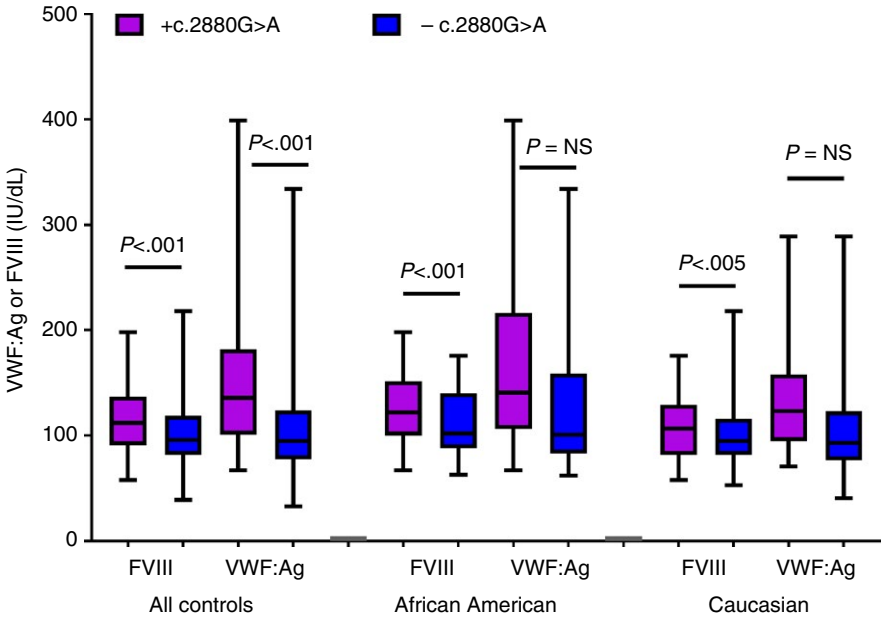

FIGURE 1 FVIII and VWF levels for healthy control subjects with or without variant c.2880G>A. Data are shown as a box and whisker plot with box representing 25th to 75th percentile. Purple bars represent subjects with the *VWF* c.2880G>A variant and blue bars represent subjects without the c.2880G>A variant. VWF and FVIII levels are first shown for all healthy controls combined, and then shown for only African Americans and only European Americans. Levels of both FVIII and VWF were higher when the c.2880G>A variant was present

TABLE 1 Characteristics of *VWF* variants associated with higher VWF levels

VWF cDNA variant	c.2365G>A	c.2385C>T	c.2880G>A
Genomic annotation			
Chr position (Hg19)	12:6153534	12:6153514	12:6138595
Genomic variant	T/C	A/G	C/T
rsID	rs1063856	rs1063857	rs1800380
Predicted VWF protein	p.Thr789Ala	p.Tyr795Tyr	p.Arg960Arg
ExAC allele frequencies[19]			
Total	0.3232	0.3231	0.2081
African	0.5805	0.5797	0.315
European (Finnish)	0.3648	0.3639	0.2539
European (Non-Finnish)	0.3561	0.3563	0.2524
Latino	0.1654	0.1655	0.1129
East Asian	0.07398	0.07391	0.0008096
South Asian	0.2522	0.2519	0.1179
Other	0.3293	0.3293	0.2192
Predictive Tools[30]			
PolyPhen[a]	0	unknown	unknown
Grantham Score[b]	58	NA	NA
PhastCons Score[c]	0.995	0.766	0.113
GERP Score[d]	2.17	−5.47	−5.07
CADD Score[e]	3.009	10.4	7.277

Chr, chromosome; Hg, human genome browser hg19 assembly; rsID, reference SNP cluster ID; ExAC, Exome Aggregation Consortium (http://exac.broadinstitute.org/).

[a]PolyPhen (http://genetics.bwh.harvard.edu/pph2/) uses a scoring system of 0 to 1 with 0 representing a benign variant and 1 representing a damaging variant.[31]

[b]Grantham Score uses a scoring system from 15 (least radical change) to 200 (most radical change).[32]

[c]PhastCons Score (http://genome.ucsc.edu provides a conservation score using values between 0 and 1.[33]

[d]GERP Score (http://mendel.stanford.edu/SidowLab/downloads/gerp/) assesses conservation with higher scores representing greater degree of conservation.[34]

[e]CADD Score (http://cadd.gs.washington.edu/) also has higher scores representing more likely deleterious variants.[35]

with linkage disequilibrium) in the ExAC database (Table 1, in 36% of European and 58% of African ancestries).[19]

3.3 | Type 1 VWD

We then looked at the prevalence of these *VWF* variants in 97 type 1 VWD subjects with VWF:Ag or VWF:RCo <30 IU/dL. As shown in table 2, *VWF* c.2880G>A was significantly more common in controls (36% European American, 61% African American) than in subjects diagnosed with type 1 VWD (20%, $P < .005$). *VWF* c.2365A>G(;)c.2385T>C was significantly more common in controls (49% European American, 88% African American) than in subjects diagnosed with type 1 VWD (35%, $P < .001$). VWF:Ag, VWF:RCo, and FVIII levels were not statistically different in type 1 VWD subjects who had these *VWF* D′D3 region variants compared to type 1 VWD patients without them (Figure 3). The bleeding score also did not differ significantly between those with and without the variants under study. For *VWF* c.2880G>A, the median bleeding score was 7 for those subjects with the variant as compared to 6 for those without (P = NS). For *VWF* c.2365A>G(;)c.2385T>C, the median bleeding score was 7 for those subjects with the variant as compared to 5 for those without (P = NS). *VWF* variant frequencies were similar to healthy controls for subjects with a historical diagnosis of type 1 VWD but current VWF:Ag >50 or with a diagnosis of type 2 VWD. However, the frequencies of each variant in those subjects with type 1 VWD and levels 30-50 IU/dL was similar to those with levels <30 IU/dL (20% for c.2880G>A and 38% for c.2365A>G(;)c.2385T>C).

There is an association of low VWF with blood group O.[9] In our type 1 subjects, however, there was no difference in ABO blood group frequency when subjects with the D′D3 region variants were compared to those without the variants.

To investigate the possible effect of these variants on production and clearance of VWF, we also examined VWF propeptide (excluding those subjects with a known clearance defect and VWF propeptide/VWF:Ag ratio >3. No difference in VWF propeptide was seen for either c.2880G>A or c.2365A>G(;)c.2385T>C when compared to type 1 VWD subjects without one of these variants (P = NS). Since the presence of a large deletion, stop codon, or other deleterious loss-of-function *VWF* variant could confound the analysis, we analyzed the type 1 subjects excluding those who had a potentially deleterious sequence variant found. Similar results were obtained. We also looked at bleeding score using the ISTH bleeding assessment tool. No difference in bleeding score was seen between type 1 VWD subjects with or without the D′D3 region variants under study.

3.4 | Allele effect and effect size

The number of subjects homozygous for either variant was small, but there was a trend towards an allele effect, with the highest VWF and FVIII levels in subjects homozygous for c.2880G>A or homozygous for c.2365A>G(;)c.2385T>C (Table 2). Heterozygotes for either variant combination had intermediate levels, while those homozygous for the *VWF* "wild-type" reference allele had the lowest VWF and FVIII levels. Table 3 shows the VWF levels observed in subjects homozygous and heterozygous for these variants. Both homozygous and heterozygous subjects had significantly higher levels compared to wild-type with the exception of c.2880G>A homozygotes (who were small in number) for FVIII and propeptide levels.

On average, VWF and FVIII levels were higher by about 50% in subjects homozygous for either set of *VWF* variants. There were fewer subjects with these variants in the type 1 VWD group, when compared with the control group. Based on the prevalence of these variants in

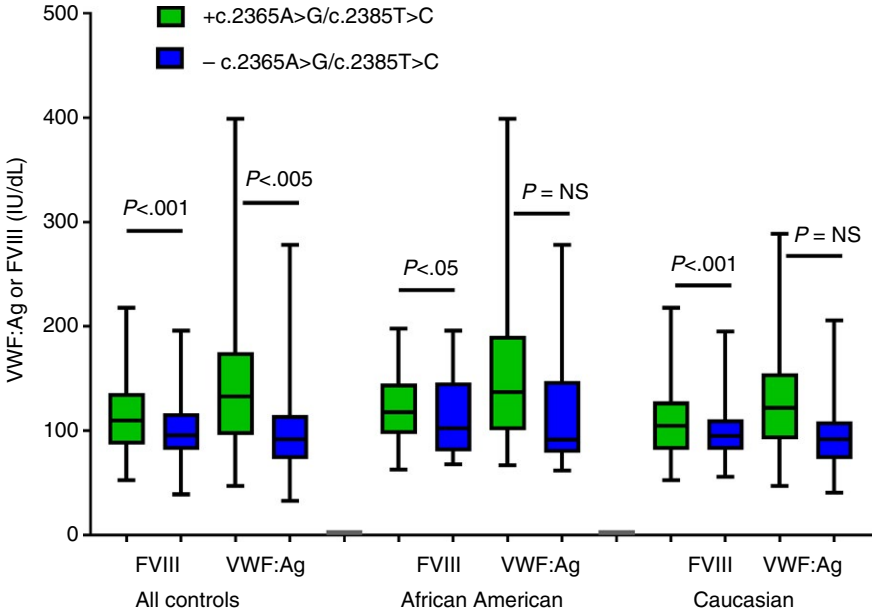

FIGURE 2 FVIII and VWF levels by *VWF* variant status for healthy control subjects with or without variants c.2365A>G and c.2385T>C. Data are shown as a box and whisker plot with box representing 25th to 75th percentile (the box and whisker plot graphs the median value). Green bars represent subjects with the c.2365A>G and c.2385T>C variants and blue bars represent subjects without the c.2365A>G and c.2385T>C variants. VWF and FVIII levels are first shown for all healthy controls combined, and then shown for only African Americans and only European Americans. Levels of both FVIII and VWF were higher when the c.2365A>G and c.2385T>C variants were present

the control population, about 15% more type 1 VWD subjects would have been expected to have these variants, suggesting that the presence of one or more of these variants protects against a diagnosis of VWD.

3.5 | Secretion and function of recombinant VWF variants

In an effort to determine the effect of each specific variant, recombinant VWF constructs were expressed containing each *VWF* variant singly as well as in combination. No difference in VWF secretion was seen when *VWF* c.2880G>A, c.2365A>G, and c.2385T>C were expressed in a mammalian cell line (Figure 4). No difference in VWF interactions with FVIII was seen in vitro for any of the variants either singly or in combination. VWF binding to FVIII for each variant was similar to that seen for control wild-type recombinant VWF. Similarly, no difference was seen in FVIII activity bound to captured VWF. As would be expected based on the clinical data, no difference in propeptide, GPIb binding, or collagen binding was seen for the variants as compared to wild-type recombinant VWF.

4 | DISCUSSION

These data confirm previous studies demonstrating increase in FVIII and VWF in association with VWF D'D3 domain sequence variations.[12] Our data also show that this association held true regardless of race, with both African American and European American subjects displaying the same trend toward higher VWF and FVIII values in the presence of these *VWF* sequence variations. The higher VWF levels associated with African American race may reflect higher frequency of D'D3 region sequence variants in that population. The c.2365A>G(;)

TABLE 2 Demographic data and *VWF* variant frequencies in healthy control subjects as compared to type 1 VWD subjects

	Healthy Control Subjects (n = 256)	Type 1 VWD Subjects (n = 97)
Race		
African American	26%	10%
European American	54%	81%
Asian	17%	1%
Other[a]	3%	7%
Ethnicity		
Hispanic	18%	7%
Sex (% female: % male)	52:48	57:43
Age (years, ±1 SD)	38 ± 11	19 ± 17
c.2880G>A	36%	20%
heterozygote	30%	16%
homozygote	6%	3%
c.2365A>G(;)c.2385T>C	50%	34%
heterozygote	33%	28%
homozygote	18%	6%
c.2880G>A (African American only)	61%	10%
c.2880G>A (European American only)	36%	23%
c.2365A>G(;)c.2385T>C (African American only)	88%	40%
c.2365A>G(;)c.2385T>C (European American only)	49%	33%

[a]Includes American Indian/Alaskan native, Hawaiian or other Pacific Islander, Middle Eastern, multiple races, and unknown/refused to answer (<5 subjects each).

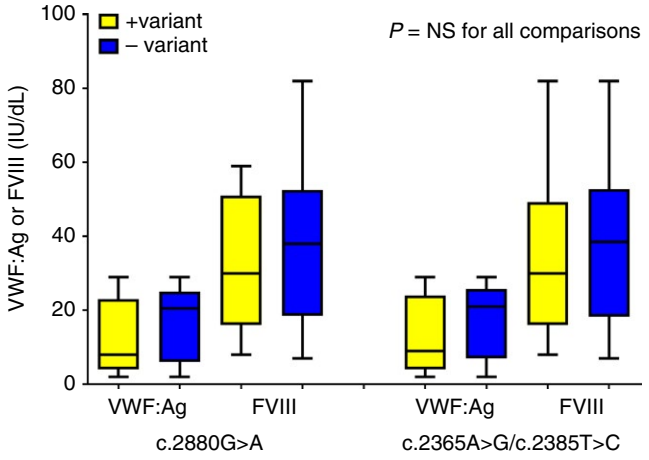

FIGURE 3 FVIII and VWF levels by *VWF* variant status for type 1 VWD subjects with or without variant c.2880G>A and variants c.2365A>G and c.2385T>C. Data are shown as a box and whisker plot with box representing 25th to 75th percentile. Yellow bars represent VWF and FVIII levels for subjects with the indicated variant(s) and blue bars represent VWF and FVIII levels for subjects without the indicated variant(s). No difference was seen in type 1 subjects when those with the D′D3 region variants were compared to those subjects without the variants

c.2385T>C variants were always found together, consistent with linkage disequilibrium. A priori, the c.2365A>G change was evaluated to be most likely to be a causative variant since it is nonsynonymous, but in vitro expression of this variant alone did not result in a significant increase in VWF levels. The c.2880G>A variant was independently associated with higher VWF levels despite being synonymous.

The frequencies of both *VWF* c.2880G>A and c.2365A>G(;)c.2385T>C were reduced in type 1 VWD, suggesting that subjects with these *VWF* alleles are less likely to be diagnosed with type 1 VWD. A similar pattern was seen in subjects who had VWF:Ag ranging from 30-50 IU/dL. In type 1 VWD, no difference in bleeding score was seen between subjects with or without these variants. This suggests that the variants may affect VWF (and FVIII) levels but do not directly affect bleeding symptoms. Subjects enrolled in the Zimmerman Program with type 2A or type 2B VWD had these higher VWF-associated *VWF* variants at frequencies comparable to the control group rather than the type 1 VWD group. This is consistent with the known mechanisms of the type 2 VWD subtypes, which are defined by functionally defective VWF proteins rather than quantitatively low (but functionally normal) VWF levels. Thus, common *VWF* polymorphisms associated with increased overall VWF levels would not be expected to rescue the qualitative functional defects that define type 2 VWD disease phenotypes.

There is evidence that sequence variants in *VWF* can affect VWF levels via clearance, and therefore lead to reduced VWF survival, with the clearest example p.R1205H, or the Vicenza variant.[20] There is evidence that sequence variations in other genes can affect VWF levels, with the strongest cases currently for ABO blood group[9] and *CLEC4M*, which binds and clears VWF.[10,21] It therefore seems reasonable that other variation within or outside *VWF* could confer increased VWF levels, either by directly increasing VWF expression, by prolonging survival, or by influencing interactions with other VWF modifiers.

FVIII levels in circulation are in large part dependent on the presence of VWF. Type 3 VWD patients have low FVIII simply because the VWF is not present to protect circulating FVIII. Acquired elevations in VWF are also accompanied by elevations in FVIII, such as after demopressin challenge.[22] Higher VWF levels due to *VWF* genetic variation can be expected to be associated with higher levels of FVIII. African Americans in general have higher VWF and FVIII levels.[12,23,24] Higher VWF and/or FVIII levels are also associated with thrombosis.

TABLE 3 Higher mean VWF and FVIII levels in healthy control subjects with variant *VWF* alleles

c.2880G>A	Homozygous (n = 15)	Heterozygous (n = 76)	Wild-type (n = 165)
FVIII	118 ± 36	116 ± 32*	103 ± 31
VWF:Ag	162 ± 86*	147 ± 58*	107 ± 47
VWFpp	103 ± 32	97 ± 22*	88 ± 25
FVIII/VWF:Ag ratio	0.79 ± 0.19*	0.84 ± 0.20*	1.04 ± 0.28
VWFpp/VWF:Ag ratio	0.72 ± 0.25*	0.71 ± 0.19*	0.90 ± 0.27
c.2365A>G(;)c.2385T>C	**Homozygous (n = 45)**	**Heterozygous (n = 84)**	**Wild-type (n = 127)**
FVIII	118 ± 31*	112 ± 34**	101 ± 30
VWF:Ag	155 ± 59*	137 ± 63*	100 ± 41
VWFpp	98 ± 24*	98 ± 28*	85 ± 22
FVIII/VWF:Ag ratio	0.80 ± 0.16*	0.88 ± 0.22*	1.09 ± 0.29
VWFpp/VWF:Ag ratio	0.68 ± 0.18*	0.77 ± 0.24*	0.93 ± 0.26

All data are given in IU/dL and represent the mean ± 1 SD.
*$P < 0.005$ as compared to wild-type.
**$P < 0.05$ as compared to wild-type.

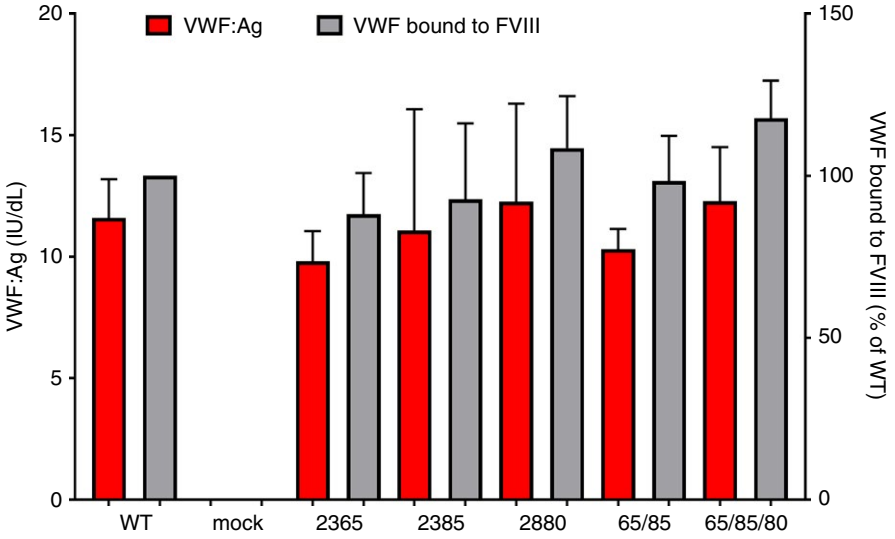

FIGURE 4 Recombinant VWF containing the candidate *VWF* variants under study did not show differences in VWF secretion or FVIII binding. Each variant was expressed individually and in combination and compared to wild-type recombinant VWF ('WT') and an empty vector negative control ('mock'). Results for VWF secretion as measured by VWF:Ag are shown with the red bars and FVIII binding with the gray bars. $N \geq 3$ for each assay on 3 separate transfections. Error bars represent one standard deviation. No difference was seen when for any of the variants as compared to WT VWF. 2365 = c.2365G, 2385 = c.2385C, 2880 = c.2880A, 65/85 = combined c.2365G and c.2385C, 65/85/80 = combined c.2365G and c.2385C and c.2880A

Consistent with this, the *VWF* c.2365G>A variant has been associated with deep vein thrombosis.[25]

We did not find evidence that these variants directly increase VWF secretion in vitro and genomic computational tools find these positions to be poorly conserved. It is possible that these variants do not impact protein or transcript function directly (Table 1). However the in vivo propeptide data suggest that either synthesis is increased, given the higher antigen levels, or survival is increased, given the lower VWFpp/VWF:Ag ratios (Table 3). It is also possible that these variants mark *VWF* alleles with functional genetic elements that influence VWF levels but lie outside of the *VWF* gene regions sequenced in this study. Alternately, these variants could directly influence *VWF* gene function and VWF levels by an as yet undetected mechanism such as directing tissue specific gene regulation, transcript processing, cell synthesis, storage, or release of VWF.

Due to their location in the VWF D'D3 region, we hypothesized that these variants could show a difference in the ability of recombinant variant VWF to bind FVIII, but our in vitro studies found no difference in VWF-FVIII binding. This suggests these variants do not directly influence VWF-FVIII binding capacity, with the caveat that there may be important functional differences between recombinantly expressed VWF with endogenous VWF circulating in vivo. There are numerous post translational modifications that can affect in vivo function.[26] Additionally, the in vitro secretion experiments used HEK293T cells without the endogenous VWF promotor, which may have affected recombinant VWF function results. The glycosylation pattern of VWF is critically important and recombinant VWF differs from the typical glycosylation pattern found when VWF is naturally expressed by endothelial cells.[27,28] It seems likely that higher circulating VWF levels in subjects with these variants allow for higher total amounts of FVIII bound to VWF and therefore higher overall FVIII levels. There may also be limitations in the methods of detecting gain-of-function variants, and this method relies on in vitro expression of recombinant VWF with additional of extracellular FVIII which may be different than the mechanisms of VWF and FVIII homeostasis in vivo. Recombinant VWF has been shown to bind similar amounts of FVIII as plasma-derived VWF, and so in the case of these variants increased VWF levels would presumably lead to an increase in total FVIII binding capacity.[29] There may also be other variants associated with the haplotype containing the D'D3 region variants.

The strengths of this study include the ability to compare variant frequencies and VWF levels in both healthy individuals and in a well-defined cohort of subjects with type 1 VWD and the ability to functionally test recombinant expression of naturally occurring *VWF* DNA variants in vitro. There are some limitations, however, in the retrospective study design and relatively small number of homozygous subjects available for study. In addition, the control population does not necessarily represent the demographics of the general population of type 1 VWD, although efforts were made to obtain good representation of all races.

In conclusion, specific sequence variants in *VWF* are associated with higher VWF and FVIII levels. These variants may protect against quantitative decreases in VWF levels and a diagnosis of type 1 VWD. The genetic diagnosis of type 1 VWD remains complicated, and may require consideration of the effect of multiple sequence variants within *VWF*.

ACKNOWLEDGMENTS

The authors would like to acknowledge funding from the National Institutes of Health (HL081588, HL102260, and HL126810).

Additional support was provided by the MACC Fund (Midwest Athletes Against Childhood Cancer) and the BloodCenter Research Fund. The authors would also like to acknowledge the patients, nurses, and research coordinators who make the Zimmerman Program a success.

RELATIONSHIP DISCLOSURES

VHF has served as a consultant for Baxalta/Shire and CSL Behring. JMJ has served as a consultant for CSL Behring and Octapharma. KDF has served as a consultant for Alexion, Baxalta/Shire, CSL Behring, Genentech, and NovoNordisk and has served on a speakers bureau for Alexion.

AUTHOR CONTRIBUTIONS

VHF designed and performed the research and wrote the manuscript. JMJ and RRM designed the research and wrote the manuscript. CK, TLS, PAC, SLH, RU, DBB, and KDF performed the research and edited the manuscript.

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How to cite this article: Flood VH, Johnsen JM, Kochelek C, et al. Common *VWF* sequence variants associated with higher VWF and FVIII are less frequent in subjects diagnosed with type 1 VWD. *Res Pract Thromb Haemost*. 2018;2:390–398. https://doi.org/10.1002/rth2.12077