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ORIGINAL ARTICLE

CLEC4M and *STXBP5* gene variations contribute to von Willebrand factor level variation in von Willebrand disease

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Summary. *Background:* von Willebrand factor (VWF) levels in healthy individuals are influenced by variations in genetic loci other than the *VWF* gene, whose contribution to VWF levels in patients with von Willebrand disease (VWD) is largely unknown. *Objectives:* To investigate the association between single-nucleotide polymorphisms (SNPs), VWF levels, and bleeding phenotype. *Patients/Methods:* In 364 type 1 VWD and 240 type 2 VWD patients from the nationwide cross-sectional 'Willebrand in The Netherlands' (WiN) study, we studied the association between eight SNPs in *STXBP5*, *SCARA5*, *ABO*, *VWF*, *STAB2*, *STX2*, *TC2N*, and *CLEC4M*, and VWF antigen (VWF:Ag), VWF activity (VWF:Act), and bleeding phenotype as assessed with the Tosetto bleeding score. *Results:* In type 1 patients, *STXBP5* was associated with a lower VWF:Ag level (adjusted difference of -3.0 IU dL^{-1} per allele; 95% confidence interval [CI] -6.0 to 0.1) and *CLEC4M* with both a lower VWF:Ag level (-4.3 IU dL^{-1} per allele; 95% CI -7.9 to -0.6) and lower VWF:Act (-5.7 IU dL^{-1} per allele; 95% CI -10.9 to -0.5). In type 2 patients, none of the SNPs was associated with VWF levels. None of the genetic variants was associated with bleeding score. *Conclusions:* Genetic variations in *STXBP5* and *CLEC4M* are associated with VWF level variation in type 1 VWD, but not in type 2 VWD. This study increases our understanding of the pathophysiology of VWD, and provides a further indication of the involvement of *STXBP5* and *CLEC4M* in determining VWF levels in VWD.

Keywords: CLEC4M protein, human; polymorphism, single nucleotide; STXBP5 protein, human; von Willebrand disease; von Willebrand factor.

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Introduction

von Willebrand disease (VWD), the commonest inherited bleeding disorder, is caused by a reduced concentration or aberrant activity of von Willebrand factor (VWF), and is characterized by recurrent mucocutaneous bleeding [1,2]. Type 1 VWD is characterized by a reduced level of VWF, and type 3 VWD by the complete absence of normal VWF, whereas type 2 VWD patients have functionally abnormal VWF [3].

Even in VWD patients with identical *VWF* gene mutations, VWF levels are highly variable, and the clinical expression is very heterogeneous [4–6]. Studies on the molecular pathology of type 1 VWD have shown that mutations in the *VWF* gene are common in more severe VWD cases [4,7]. However, in milder cases, the genetic model is more complex. Incomplete penetrance and variations in other genes probably play a greater role [6,7].

The Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium recently discovered novel genetic loci that regulate VWF antigen (VWF:Ag) levels (Table 1) in non-VWD patients [8,9]. Two of these genes (*STX2* and *STXBP5*) are likely to be involved in VWF secretion by interacting with soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which have been shown to be involved in Weibel–Palade body exocytosis [10–12]. *ABO*, *CLEC4M*, *SCARA5* and *STAB2* probably play a role in the clearance of VWF [8,13–16]. *TC2N* was also identified, and has since been shown to be associated with venous thrombosis [17].

Except for the single-nucleotide polymorphism (SNP) determining ABO blood group, which is the most important genetic determinant of VWF:Ag levels, and the SNP in *VWF*, these genes encode proteins that have not been linked to VWF levels [15]. We have recently shown that genetic variation in *STX2* affects VWF:Ag levels in type 1 VWD patients, and Rydz *et al*. showed that polymorphisms in *CLEC4M* contribute to variability in VWF levels [13,18]. However, it is still unknown whether these genetic modifiers also determine the variability of VWF levels and influence the bleeding phenotype in moderately and severely affected type 1 and type 2 VWD patients. Insights into these associations will increase our understanding of the pathophysiology of VWD, and possibly lead to new treatment options for patients with VWD. We therefore investigated the effects of genetic variations in *STXBP5*, *SCARA5*, *ABO*, *VWF*, *STAB2*, *STX2*, *TC2N* and *CLEC4M* on VWF levels and bleeding phenotype in a large cohort of moderately and severely affected type 1 and type 2 VWD patients from the nationwide cross-sectional 'Willebrand in The Netherlands' (WiN) study.

Patients and methods

Participants

This study is part of the WiN study, a nationwide cross-sectional multicenter study among VWD patients in the Netherlands that included 804 patients who had previously been diagnosed with type 1, type 2 or type 3 VWD [2,19–22]. The inclusion criteria for the WiN study were: (i) hemorrhagic diathesis or a family history of VWD; and (ii) historically lowest levels of VWF:Ag of ≤ 30 U dL^{-1} and/or VWF activity (VWF ristocetin cofactor activity) of ≤ 30 U dL^{-1} and/or factor VIII coagulation activity of ≤ 40 U dL^{-1} (for type 2N VWD). Patients were excluded if they were known to have other hemostatic disorders resulting in a hemorrhagic diathesis. Medical Ethical Committees at all participating centers (see Appendix) approved this study, and all participants gave informed consent.

For the current study, only patients with type 1 ($n = 364$) and type 2 ($n = 240$) VWD for whom centrally measured VWF levels were available were selected. Exclusion criteria were pregnancy and the recent use of desmopressin or replacement therapy at the time of blood sampling.

Assessment methods

All patients completed an extensive questionnaire on bleeding episodes and treatment of VWD [2,20–22]. To calculate a bleeding score (BS), as previously described by Tosetto [23], we used information on the severest lifetime event of each of 12 specific bleeding symptoms. To avoid prophylaxis bias, we did not score for a bleeding symptom if patients had received prophylactic desmopressin or prophylactic replacement therapy before a surgical intervention, dental extraction, or delivery [2, 24]. In addition, to gain insights into the heritability of the polymorphisms and the *VWF* mutations, we obtained pedigrees from the

Table 1 Genetic loci associated with von Willebrand factor identified in the Cohorts for Heart and Aging Research in Genomic Epidemiology consortium and analyzed in the Willebrand in The Netherlands study

Region	SNP	Gene	Gene name	(Possible) biological pathway
6q24	rs9390459	*STXBP5*	Syntaxin-binding protein 5	Vesicular trafficking and exocytosis
8p21	rs2726953	*SCARA5*	Scavenger receptor class A, member 5	Clearance
9q34	rs687621	*ABO*	ABO blood group	Clearance
12p13	rs1063857	*VWF*	von Willebrand factor	–
12q23	rs4981022	*STAB2*	Stabilin-2	Clearance
12q24.3	rs7978987	*STX2*	Syntaxin-2	Vesicular trafficking and exocytosis
14q32	rs2402074	*TC2N*	Tandem C2 domains, nuclear	Not yet known
19p13.2	rs868875	*CLEC4M*	C-type lectin domain family 4, member M	Clearance

SNP, single-nucleotide polymorphism.

392 families that we had identified with type 1 and type 2 VWD patients.

Laboratory measurements

At inclusion in the study, venous blood was collected in 0.105 M sodium citrate tubes (1 : 10) and centrifuged twice at 2200 × *g* for 10 min at room temperature; plasma was stored at − 80 °C. Plasma levels of VWF:Ag and VWF activity (VWF:Act) were measured centrally (Erasmus University Medical Center, Rotterdam). VWF:Ag levels were determined with an in-house ELISA using polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase-conjugated anti-human VWF antibodies (DakoCytomation, Glostrup, Denmark) for detection. VWF:Act was assessed with a LIA test, which uses mAbs directed against the glycoprotein (GP)Ibα-binding domain of VWF and thereby reflects the binding of VWF to GPIbα (HemosIL von Willebrand Factor Activity; Instrumentation Laboratory, Breda, the Netherlands). Phenotypic blood group was determined by mixing plasma from patients with red blood cells from donors with a known blood group [25]. If the phenotypic blood group was unknown, blood group was determined by genotyping the ABO blood group-specific SNPs rs687289 (marker for blood group O), rs507666 (marker for blood group A1), rs8176704 (marker for blood group A2), and rs8176749 (marker for blood group B) [26]. Details of the blood-sampling procedure and laboratory measurements at inclusion in the study have been described in more detail elsewhere [2].

Genotyping analysis

For DNA isolation, blood was collected in tubes containing EDTA (Beckon Dickinson, Plymouth, UK). From this, genomic DNA was extracted according to standard salting-out procedures [27], and stored at − 20 °C. From a subset of patients, saliva was collected in a DNA self-collection kit (Oragene-DNA OG-250; DNA Genotek, Ottawa, ON, Canada), and the DNA was purified with the Puregene DNA purification kit (DNA Genotek).

Using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA, USA), we genotyped eight SNPs that had been identified in the CHARGE consortium meta-analysis (Table 1) [8]. In *TC2N*, rs10133762 had the highest genome-wide significance level for VWF levels in the CHARGE consortium meta-analysis. However, because this SNP was not available in predesigned form (Applied Biosystems), we selected polymorphism rs2402074 in *TC2N*, which is in high linkage disequilibrium with rs10133762 ($D' = 1$, $R^2 = 1$) (International HapMap project, phases I + II + III, August 2010; http://www.hapmap.org and HAPLOVIEW software version 4.2 [28]). We excluded the CHARGE-identified SNP rs17057285 (*UFM1*) [9] from our analyses, because of the very low minor allele frequency (MAF) of rs17057285 (MAF = 0.005), which meant that our study would be underpowered to assess an association between *UFM1* and VWF levels.

Endpoint fluorescence was measured on the ABI 7900HT instrument, and clustered according to genotype with SDS 2.1 software (both Applied Biosystems). To ensure DNA quality, we included only patients in whom genotyping had been successful for > 75% of SNPs.

Statistical methods

Descriptive statistics for categorical data are presented as frequencies and percentages (*n*, %), and those for continuous variables as median and 25–75% interquartile range (IQR). As VWF:Ag and VWF:Act levels were skewed, these data were quadratically transformed (square root) for the regression analysis. As the regression results for non-transformed and transformed data were similar, we describe the untransformed data, as they are more easily interpreted. As an appropriate transformation for BS, which was skewed to the right, was not found, we used Kruskal–Wallis tests to test the statistical significance of differences in BS between genotypes. As type 1 and type 2 VWD have different pathophysiologies, all analyses were stratified for type of VWD.

Allele frequencies were calculated by genotype counting. For each SNP, the deviation from Hardy–Weinberg equilibrium was tested by means of a chi-squared test with one degree of freedom. To compare MAFs between CHARGE and WiN, the chi-squared test was used.

We performed linear regression analysis with additive genetic models to quantify differences in VWF:Ag or VWF:Act levels between patients with different genotypes, using the genotype of each SNP as a continuous variable. All models were adjusted for age, sex, and blood group, except for the SNP in *ABO* (rs687621), which was only adjusted for age and sex (model 1). In model 2, we also adjusted for pedigree structure with SOLAR, version 6.6.2 (Texas Biomedical Research Institute, San Antonio, TX, USA). Beta-coefficients (β) are interpreted as a reduction in VWF:Ag or VWF:Act levels per VWF-reducing allele with a 95% confidence interval (CI).

We also calculated each individual's total number of VWF-reducing alleles of the SNPs that were associated with VWF:Ag levels (rs9390459 in *STXBP5* and rs868875 in *CLEC4M*) (maximum of 4). The Kruskal–Wallis test was used to test differences in VWF:Ag and VWF:Act between numbers of *STXBP5* and *CLEC4M* VWF-reducing alleles in type 1 and type 2 VWD. The Mann–Whitney *U*-test was used to test the statistical significance of differences between numbers of *STXBP5* and *CLEC4M* VWF-reducing alleles. We performed linear regression analysis to quantify the differences in VWF:Ag or VWF:Act between patients with different numbers of *STXBP5*

and *CLEC4M* VWF-reducing alleles. Statistical analyses were performed with SPSS for Windows, version 21.0 (SPSS, Chicago, IL, USA). A *P*-value of < 0.05 was considered to be statistically significant. With the Bonferroni correction method for multiple testing, the significance level was set at 0.006 (0.05/8).

Results

Participants

A total of 804 VWD patients participated in the WiN study; DNA was obtained from 752 of them. The following patients were excluded from the present analyses: all type 3 VWD patients ($n = 43$); patients without centrally measured VWF levels ($n = 76$); pregnant patients ($n = 8$); patients who used desmopressin or clotting factor concentrate < 72 h before blood sampling ($n = 10$); and patients in whom the success rate of genotyping was < 75% ($n = 11$). Therefore, a total of 604 VWD patients were included, 364 of whom had type 1 VWD and 240 of whom had type 2 VWD (Fig. 1). BS was available for 350 type 1 patients and 224 type 2 patients. The baseline characteristics are shown in Table 2.

MAFs in VWD patients relative to the general population

Table 3 shows the MAFs of the SNPs in VWD patients. Among type 1 VWD patients, the MAF of rs687621 (*ABO*) was 19%, which is lower than that previously reported (34%) in white Caucasian subjects (CHARGE consortium) [8] and in the Dutch population [29] ($P < 0.001$). This reflects the higher prevalence of blood group O in our study population, as this SNP reflects the O allele. The MAF of rs1063857 (in *VWF*), the minor allele being the one associated with higher VWF:Ag levels, was decreased among type 1 VWD patients (24% versus 36% in CHARGE, $P < 0.001$) and type 2 VWD patients (24% versus 36% in CHARGE, $P < 0.001$). In addition, the MAF of rs4981022 (*STAB2*), the frequency of the allele associated with lower VWF:Ag levels, was slightly increased among type 1 VWD patients; 35%

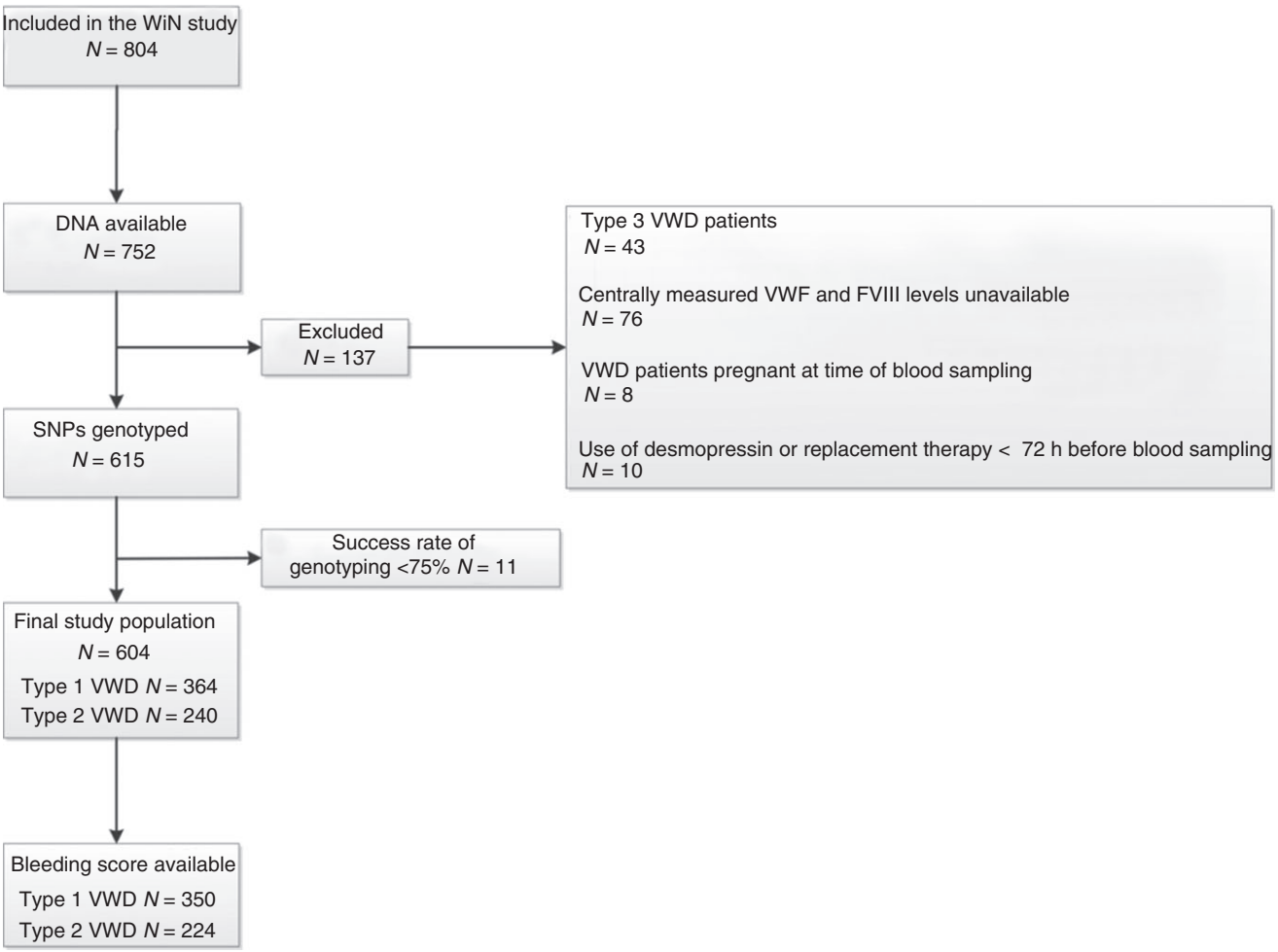

Fig. 1. Flowchart of included patients. SNP, single-nucleotide polymorphism; VWD, von Willebrand disease; VWF, von Willebrand factor; WiN, Willebrand in The Netherlands study.

Table 2 Baseline characteristics

Characteristics	Type 1 VWD (n = 364)	Type 2 VWD (n = 240)
Age (years), median (range)	45 (1–81)	43 (3–83)
Child (0–16 years), *n* (%)	33 (9)	22 (9)
Male sex, *n* (%)	121 (33)	107 (45)
VWD subtype 2, *n* (%)		
2A	–	155 (65)
2B	–	45 (19)
2M	–	26 (11)
2N	–	14 (6)
Blood group O, *n* (%)	245 (67)	120 (50)
VWF:Ag (IU dL^{-1}), median (IQR)	37 (23–53)	25 (16–34)
VWF:Act (IU dL^{-1}), median (IQR)	45 (24–70)	9 (4–16)
Bleeding score, median (IQR)	9 (5–14.3)	12 (8–17)

IQR, interquartile range; VWD, von Willebrand disease; VWF:Act, von Willebrand factor activity; VWF:Ag, von Willebrand factor antigen. VWF:Ag and VWF:Act levels were measured centrally at time of inclusion in the study.

versus 32% in CHARGE (P = 0.054). The MAF of rs9390459 (*STXBP5*) was lower in type 2 VWD patients than previously reported in CHARGE; 39% versus 44% (P = 0.026). The MAFs of the other SNPs did not differ between VWD patients and CHARGE subjects.

Association between genetic variations and VWF parameters

In type 1 VWD patients, rs868875 in *CLEC4M* was associated with lower VWF:Ag levels (adjusted difference of –4.3 IU dL^{-1} per allele; 95% CI −7.9 to −0.6), and rs9390459 in *STXBP5* was borderline associated with lower VWF:Ag levels (−3.0 IU dL^{-1} per allele; 95% CI −6.0 to 0.1) (Fig. 2; Table 4: model 1). After additional adjustment for pedigree (model 2), a similar but not significant trend was observed for these two SNPs in *CLEC4M* and *STXBP5* (for *CLEC4M*, adjusted difference of −3.3 IU dL^{-1} per allele [95% CI −6.8 to 0.2]; and for *STXBP5*, −2.6 IU dL^{-1} per allele [95% CI −5.5 to 0.4]). In addition, after adjustment for multiple testing with the Bonferroni method, the difference lost its statistical significance. The contributions of *CLEC4M* and *STXBP5* to VWF:Ag levels in a subgroup of type 1 VWD patients with VWF:Ag levels of > 20 IU dL^{-1} were larger, with −5.1 IU dL^{-1} per allele (95% CI −8.7 to −1.5) for *CLEC4M*, and −4.2 IU dL^{-1} per allele (95% CI −7.3 to −1.2) for *STXBP5*, and remained significant after Bonferroni correction. All associations were similar for VWF:Act (Fig. 2; Table 4). In type 1 VWD patients, the remaining SNPs were not associated with VWF:Ag or VWF:Act. In type 2 VWD, rs1063857 in *VWF* was associated with lower VWF:Act levels (adjusted difference of −5.1 IU dL^{-1} per allele; 95% CI −9.0 to −1.2), but not with VWF:Ag. None of the other SNPs was associated with VWF:Ag or VWF:Act levels (Table 4).

Numbers of STXBP5 *and* CLEC4M *VWF-reducing alleles and VWF levels*

Among patients with type 1 VWD, an increasing number of VWF-reducing alleles of *STXBP5* or *CLEC4M* was associated with increasingly lower VWF:Ag and VWF:Act levels. VWF:Ag levels were reduced by 3.5 IU dL^{-1} (95% CI 1.3 to 5.8) per allele of *STXBP5* or *CLEC4M*, and VWF:Act levels were reduced by 4.7 IU dL^{-1} (95% CI 1.5 to 8.0) per allele (adjusted for age, sex, and blood group). Type 1 patients with two VWF-reducing alleles of *STXBP5* or *CLEC4M* had the lowest VWF:Ag levels (median VWF:Ag level of 34 IU dL^{-1}; IQR 21–47) (Fig. 3).

Among type 2 VWD patients, numbers of *STXBP5* and *CLEC4M* VWF-reducing alleles were not associated with VWF:Ag or VWF:Act (for VWF:Ag, adjusted difference of 1.4 IU dL^{-1} [95% CI −0.9 to 3.6]; and for VWF:Act, −0.3 IU dL^{-1} [95% CI −2.9 to 2.2]) (Fig. 3).

Association between genetic variations and bleeding phenotype

Among type 1 VWD patients, homozygous carriers for the VWF-reducing alleles of *ABO* had a slightly higher BS: CC (n = 15), median BS of 6.0 (IQR 2.0–9.0); CT (n = 102), median BS of 8.5 (IQR 5.0–14.3); and TT (n = 232), median BS of 9.0 (IQR 5.0–15.0) (P for trend = 0.072). Median BS did not differ between the

Table 3 Minor allele frequencies (MAFs)

SNP	Gene	CHARGE	Type 1 VWD (95% CI)	Type 2 VWD (95% CI)
rs9390459	*STXBP5*	0.44	0.47 (0.44 to 0.51)	0.39† (0.35 to 0.44)
rs2726953	*SCARA5*	0.31	0.29 (0.26 to 0.33)	0.29 (0.24 to 0.33)
rs687621	*ABO*	0.34	0.19* (0.17 to 0.22)	0.31 (0.27 to 0.35)
rs1063857	*VWF*	0.36	0.24* (0.21 to 0.27)	0.24* (0.21 to 0.28)
rs4981022	*STAB2*	0.32	0.35 (0.31 to 0.39)	0.32 (0.28 to 0.36)
rs7978987	*STX2*	0.35	0.33 (0.30 to 0.37)	0.37 (0.32 to 0.41)
rs2402074	*TC2N*	0.44	0.43 (0.40 to 0.47)	0.43 (0.38 to 0.47)
rs868875	*CLEC4M*	0.26	0.29 (0.26 to 0.32)	0.29 (0.25 to 0.34)

CHARGE, the previously reported minor allele frequency in healthy individuals in the Cohorts for Heart and Aging Research in Genomic Epidemiology study [8]; CI, confidence interval; SNP, single-nucleotide polymorphism; VWD, von Willebrand disease. *P < 0.001. †P = 0.025.

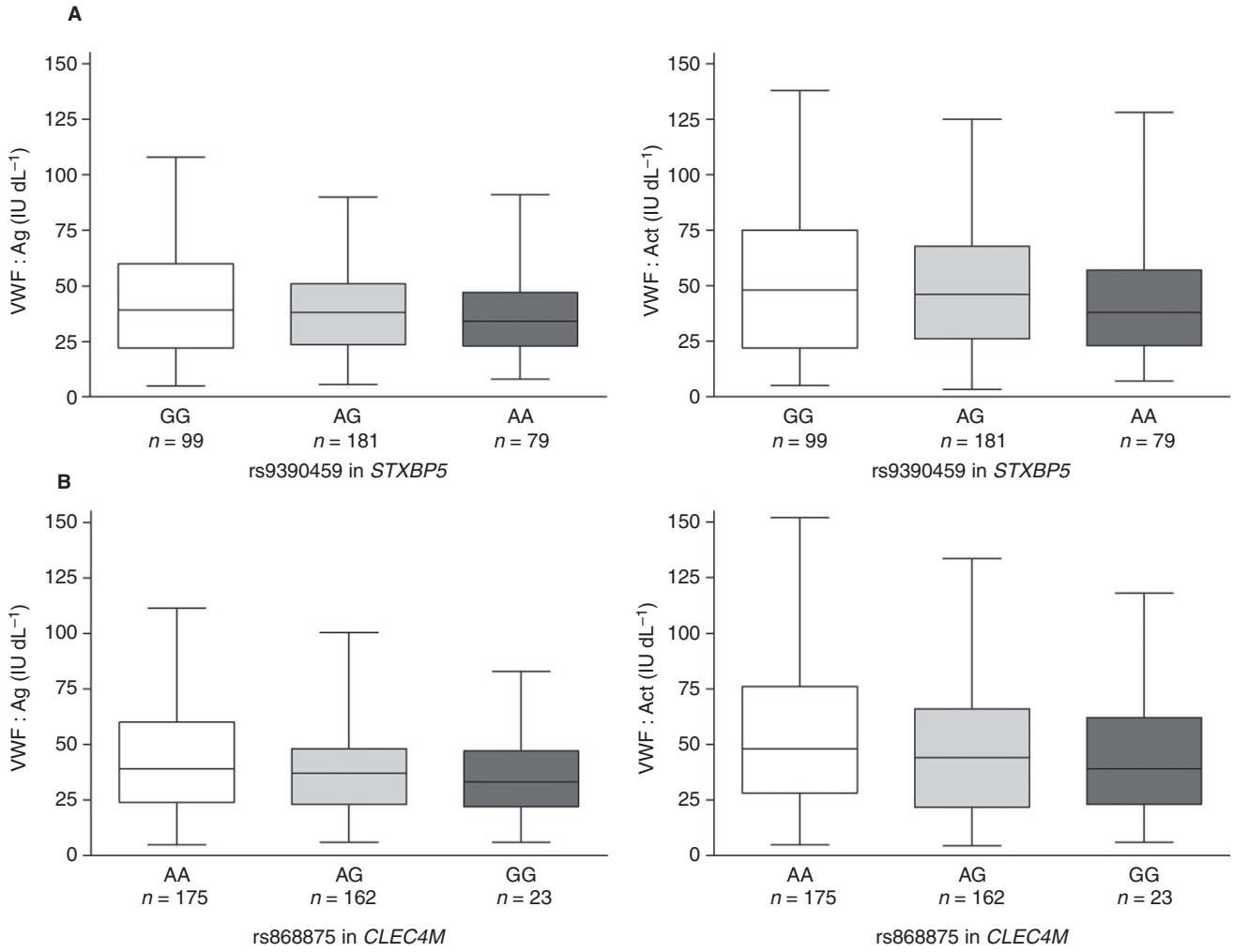

Fig. 2. von Willebrand factor antigen (VWF:Ag) and von Willebrand factor activity (VWF:Act) level per genotype of (A) rs9390459 in *STXBP5* and (B) rs868875 in *CLEC4M* in type 1 von Willebrand disease patients. The boxplots indicate the median, 25–75% interquartile range, and 1–99 percentile.

genotypes of *STAB2* ($P = 0.531$), *STXBP5* ($P = 0.484$), *SCARA5* ($P = 0.904$), *VWF* ($P = 0.628$), *CLEC4M* ($P = 0.273$), *TC2N* ($P = 0.362$), and *STX2* ($P = 0.743$). None of the SNPs was associated with BS in type 2 VWD patients.

Discussion

In this cohort of moderately and severely affected VWD patients from the WiN study, we observed that genetic variations in *CLEC4M* and *STXBP5* contribute to the variability in VWF:Ag and VWF:Act levels in type 1 VWD, but not in type 2 VWD. Bleeding phenotype appeared not to be associated with variations in these genetic loci outside the *VWF* gene that have been shown to contribute to variability in VWF levels.

It is well known that VWF levels are highly variable in VWD patients [4,5,7]. Besides mutations or polymorphisms in *VWF*, genetic variations in other genes may also affect VWF levels [6,7]. We have now found that this variability in VWF levels in VWD patients is partly explained by polymorphisms in *CLEC4M* and *STXBP5*. The effect sizes that we found for these SNPs were similar to those observed by the CHARGE consortium [8]. It should be noted that, after correction for multiple testing, this finding lost its significance, but this might also have introduced a type 2 error. It remained significant for VWD type 1 patients with VWF:Ag levels of > 20 IU dL^{-1}, who are known to have *VWF* mutations less frequently than type 1 patients with VWF:Ag levels of < 20 IU dL^{-1} or type 2 VWD patients. VWF has previously been shown to undergo receptor-mediated endocytosis after binding to the CLEC4M receptor [13]. It is probably involved in VWF clearance, which is a mechanism leading to lower VWF levels in VWD patients. STXBP5 interacts with SNARE proteins, which drive vesicle exocytosis through the fusion of granules and target membranes [10]. These SNARE proteins have been shown to be involved in Weibel–Palade Body exocytosis, a well-known mechanism for VWF secretion by endothelial cells [11], and a defect in which is

Table 4 Effects of various genetic loci on von Willebrand factor antigen (VWF:Ag) and von Willebrand factor activity (VWF:Act) in type 1 and type 2 von Willebrand disease

		Type 1 von Willebrand disease				Type 2 von Willebrand disease			
		VWF:Ag (IU dL^{-1})		VWF:Act (IU dL^{-1})		VWF:Ag (IU dL^{-1})		VWF:Act (IU dL^{-1})	
SNP	Gene	Model 1 β (95% CI)	Model 2 β (95% CI)	Model 1 β (95% CI)	Model 2 β (95% CI)	Model 1 β (95% CI)	Model 2 β (95% CI)	Model 1 β (95% CI)	Model 2 β (95% CI)
rs9390459	*STXBP5*	−3.0 (−6.0 to 0.1)	−2.6 (−5.5 to 0.4)	−3.9 (−8.3 to 0.5)	−3.4 (−7.7 to 0.8)	0.9 (−2.0 to 3.9)	−0.7 (−3.7 to 2.4)	−1.6 (−4.9 to 1.8)	−2.5 (−5.9 to 0.9)
rs2726953	*SCARA5*	0.4 (−3.1 to 3.9)	−0.3 (−3.0 to 3.6)	−0.1 (−5.1 to 4.9)	−0.4 (−5.3 to 4.4)	2.7 (−0.5 to 6.0)	2.2 (−1.0 to 5.4)	0.8 (−2.9 to 4.5)	0.9 (−2.7 to 4.5)
rs687621	*ABO*	0.4 (−3.5 to 4.3)	−1.0 (−4.8 to 2.7)	3.4 (−2.2 to 9.1)	1.2 (−4.2 to 6.7)	−1.4 (−4.5 to 1.7)	−1.5 (−4.6 to 1.6)	1.5 (−2.0 to 5.0)	0.7 (−2.8 to 4.1)
rs10638857	*VWF*	−1.8 (−5.5 to 1.8)	−1.6 (−18.9 to 15.7)	−1.6 (−6.9 to 3.8)	−0.6 (−18.0 to 16.8)	−2.5 (−5.9 to 1.0)	−1.3 (−23.2 to −20.6)	−5.1 (−9.0 to −1.2)*	−4.4 (−26.5 to 17.7)
rs4981022	*STAB2*	−0.7 (−4.1 to 2.7)	−0.9 (−4.2 to 2.4)	−1.1 (−6.1 to 3.8)	−0.9 (−5.6 to 3.9)	−1.3 (−4.5 to 1.9)	−0.7 (−4.0 to 2.5)	0.0 (−3.6 to 3.6)	0.4 (−3.1 to 4.0)
rs7978987	*STX2*	0.6 (−2.6 to 3.8)	0.4 (−2.6 to 3.5)	−1.2 (−5.8 to 3.4)	−1.2 (−5.6 to 3.3)	0.6 (−2.4 to 3.6)	0.7 (−2.3 to 3.8)	0.7 (−2.7 to 4.1)	1.2 (−2.2 to 4.5)
rs2402074	*TC2N*	−0.9 (−4.0 to 2.2)	−0.5 (−3.6 to 2.5)	−0.2 (−4.7 to 4.4)	0.3 (−4.1 to 4.6)	−1.5 (−4.3 to 1.4)	−1.8 (−4.7 to 1.1)	−1.6 (−4.8 to 1.6)	−1.5 (−4.7 to 1.8)
rs868875	*CLEC4M*	*−4.3 (−7.9 to −0.6)**	−3.3 (−6.8 to 0.2)	*−5.7 (−10.9 to −0.5)**	−4.3 (−9.3 to 0.7)	1.7 (−1.8 to 5.1)	1.8 (−1.6 to 5.2)	1.1 (−2.7 to 5.0)	1.2 (−2.5 to 5.0)

CI, confidence interval; SNP, single-nucleotide polymorphism. Model 1: linear regression analysis with additive genetic model adjusted for sex, age, and blood group (O versus non-O), except for rs687621 (*ABO*), which is adjusted for sex and age. Model 2: linear regression analysis with additive genetic model adjusted for sex, age, blood group (O versus non-O), and pedigree structure, except for rs687621 (*ABO*), which is adjusted for pedigree structure, sex, and age. The beta-coefficient (β) represents the increase in VWF:Ag or VWF:Act per VWF-decreasing allele with a 95% CI. *$P < 0.05$.

likely to lead to VWD. This is further supported by the recent observation that a *STXBP1* mutation lowers VWF:Ag levels and reduces VWF secretion from the endothelium. This mutation was identified in an early infantile epileptic encephalopathy type 4 patient, and the protein STXBP1 is also a member of the SNARE family [30]. By combining the numbers of VWF-reducing alleles of *STXBP5* and *CLEC4M*, we found a strong association between VWF levels and these numbers of VWF-reducing alleles. This indicates that these two genes have an additive effect on VWF levels in type 1 VWD patients, and that carriage of both results in even lower VWF levels.

It is common knowledge that ABO blood group influences VWF levels [31]. *ABO* was also shown within the CHARGE consortium to be the major genetic determinant of VWF:Ag levels, with an effect size of 24.1% per VWF-reducing allele. However, in our cohort of VWD patients, blood group O was not associated with lower VWF levels. This is explained by a phenomenon called index event bias [32]. The WiN cohort comprised patients with VWF levels lower than 30%, resulting in an overrepresentation of blood group O in our cohort of type 1 VWD patients. Patients with non-O blood groups have different causes for having low VWF levels. These other causes explain why patients with non-O blood groups have similar VWF levels as patients with blood group O in our study. The index event bias may also play a role in the *VWF* SNP. It is of importance that we observed lower frequencies of the *ABO* SNP and the *VWF* SNP that are associated with increased levels of VWF in our type 1 VWD group as compared with those observed in healthy individuals in CHARGE. This indicates that these genetic variations, by influencing VWF levels, may also determine whether an individual is diagnosed as a moderately or severely affected VWD patient (levels of < 30 IU dL^{-1}) and therefore be included in the WiN study.

In 2012, we were the first to report that genetic variation in *STX2*, which encodes a binding substrate for STXBP5 [10], was associated with VWF:Ag levels in type 1 VWD patients [18]. Our current study found no such association. This may have been because in our previous study the lowest VWF levels ever measured were used for analyses, and for our current analysis we used VWF parameters measured centrally at inclusion in the study. We now have a larger sample size, and we adjusted not only for blood group – which has been shown to determine 25% of the VWF levels – but also for pedigrees. Of the 364 type 1 VWD patients from the current study, 131 (36%) were included in both analyses.

In most of the type 2 VWD patients and in the majority of the type 1 VWD patients, mutations in *VWF* can be detected. The fact that molecular studies have been unable to find causative *VWF* gene mutations in 35% of type 1 VWD patients, especially in those with levels above 20 IU dL^{-1}, suggests that other genetic loci contribute to low VWF levels. The involvement of other

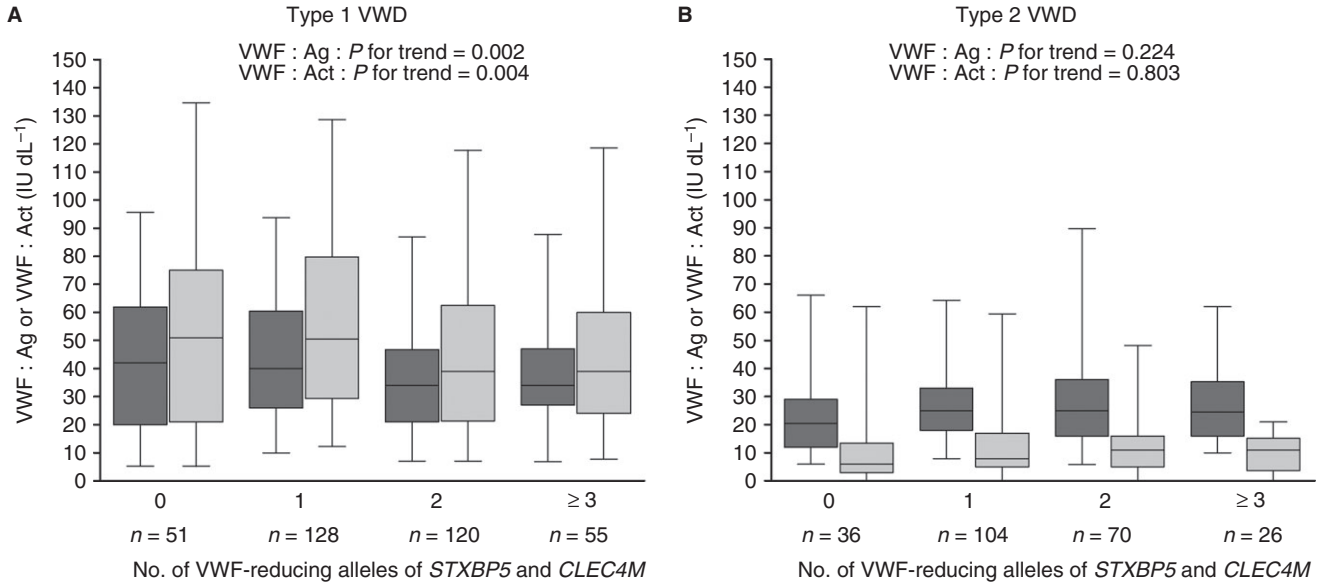

Fig. 3. Numbers of von Willebrand factor (VWF)-reducing alleles of *STXBP5* and *CLEC4M* in type 1 and type 2 von Willebrand disease (VWD), and VWF antigen (VWF:Ag) and VWF activity (VWF:Act) levels per number of VWF-reducing alleles of *STXBP5* and *CLEC4M* in (A) type 1 and (B) type 2 VWD. The boxplots indicate the median, 25–75% interquartile range (IQR), and extreme values. In type 1 VWD patients with no, one, two or three or more VWF-reducing alleles of *STXBP5* and *CLEC4M*, the median VWF:Ag levels were: 42 IU dL^{-1} (IQR 20–62), 40 IU dL^{-1} (IQR 26–60), 34 IU dL^{-1} (IQR 21–47), and 34 IU dL^{-1} (IQR 27–47), respectively, and the median VWF:Act levels were 51 IU dL^{-1} (IQR 21–75), 51 IU dL^{-1} (IQR 30–80), 39 IU dL^{-1} (IQR 21–63), and 39 IU dL^{-1} (IQR 24–60), respectively. In type 2 VWD patients with no VWF-reducing alleles of *STXBP5* and *CLEC4M*, the median VWF:Ag level was 20 IU dL^{-1} (IQR 12–29) and the median VWF:Act level was 6 IU dL^{-1} (IQR 3–14). In type 2 VWD patients with one VWF-reducing allele of *STXBP5* and *CLEC4M*, the median VWF:Ag and VWF:Act levels were 25 IU dL^{-1} (IQR 8–33) and 8 IU dL^{-1} (IQR 5–17), respectively. In type 2 VWD patients with two VWF-reducing alleles of *STXBP5* and *CLEC4M*, the median VWF:Ag and VWF:Act levels were 25 IU dL^{-1} (IQR 16–36) and 11 IU dL^{-1} (IQR 5–16), respectively. In type 2 VWD patients with more than three VWF-reducing alleles of *STXBP5* and *CLEC4M*, the median VWF:Ag and VWF:Act levels were 25 IU dL^{-1} (IQR 16–35) and 11 IU dL^{-1} (IQR 4–15), respectively.

genes is more likely in the mildly affected patients, in which the genetic model is more complex [6,7]. This is also observed in our study, because, in mildly affected VWD patients, the contribution of polymorphisms in *STXBP5* and *CLEC4M* to VWF levels is even larger. Our study supports the concept of the involvement of STXBP5 and CLEC4M in determining the variability of VWF levels, not only in healthy individuals, but also in type 1 VWD patients.

In type 2 VWD patients, we found no association between these genetic loci and VWF levels, probably because of the different pathophysiology of this type of VWD. Because the reduced VWF levels in these type 2 VWD patients mainly result from a specific mutation in *VWF* that causes a functionally aberrant VWF protein to be produced, the additional effect of genetic variations on VWF levels may be extremely small. Unfortunately, the number of type 2 VWD types in our cohort was relatively low, so we may not have had enough power to establish an association between genetic variations and VWF levels in type 2 VWD and its different subtypes.

If genetic loci outside *VWF* contribute to the variability of VWF levels in VWD patients, it may be expected that they will affect the bleeding phenotype in these patients, as VWF levels and bleeding phenotype are associated [2,23,33]. In our cohort, however, we found no association between these genetic loci and bleeding phenotype, as determined with the Tosetto BS [23]. The known limitations of the Tosetto BS (cumulative score, ceiling effect, and prophylaxis bias), possible selection bias or the relatively small influence of the genetic variants on VWF levels may explain the lack of association between bleeding phenotype and genetic variations [2,23,24]. However, this BS is the best currently available method for evaluating bleeding phenotype in VWD.

The strength of our study is that it is the first to examine the relationship between genetic variations in *STXBP5*, *SCARA5*, *ABO*, *STAB2*, *STX2*, *TC2N* and *CLEC4M* in patients with moderate or severe type 1 and type 2 VWD. Another very important strength is our use of pedigrees from all patients in the association analysis. In addition, our population covered almost all of the patients with moderate or severe VWD in the Netherlands, and centrally measured VWF and FVIII levels were available from all included patients. A study limitation is that *VWF* mutation analysis has not yet been performed in all patients. To compensate for this, we used pedigrees to adjust for the effect size of large families on VWF levels, and to avoid bias resulting from polymorphisms that may be in high linkage with the

mutation in *VWF*. Also, even though this is the largest cohort study of type 1 and type 2 VWD patients in whom associations between genetic variations and VWF levels and bleeding phenotype have been analyzed, the size of the study population is still relatively small for a genetic association study, and the findings should be interpreted with care.

In conclusion, VWF level variation in type 1 VWD is influenced by genetic variation in *CLEC4M* and *STXBP5*. In type 2 VWD, no associations were found between genetic loci outside *VWF* and VWF level variation. Although genetic variants modestly affected VWF levels, they were not associated with bleeding phenotype. By increasing our understanding of the pathophysiologic mechanisms of VWD, this study may contribute to the search for novel causes and new therapeutic options for this bleeding disorder.

Addendum

F. W. G. Leebeek designed research, analyzed and interpreted data, and wrote the manuscript. Y. V. Sanders performed research, analyzed and interpreted data, and wrote the manuscript. J. Eikenboom, J. G. van der Bom, M. H. Cnossen, K. Fijnvandraat, A. Isaacs, B. A. P. Laros-van Gorkom, M. P. M. de Maat, K. Meijer, and E. P. Mauser-Bunschoten designed research, interpreted data, and critically reviewed the manuscript. C. M. van Duijn critically reviewed the manuscript. All authors gave their consent to the final version of the manuscript.

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Disclosure of Conflict of Interests

F. W. G. Leebeek has received research support from CSL Behring for performing the WiN study, and has served on advisory boards of CSL Behring and Baxter in the past. Y. V. Sanders has been a teacher on educational activities of Baxter. J. Eikenboom has received research support from CSL Behring, and has been a teacher on educational activities of Roche. E. P. Mauser-Bunschoten has received research/educational support from CSL Behring, Bayer, Baxter, Novo Nordisk, Pfizer, Biovitrum, and Sanquin. J. G. van der Bom has received unrestricted research/educational funding for various projects from the following companies: Bayer Schering Pharma, Baxter, CSL Behring, Novo Nordisk, and Pfizer. In addition, she has been a consultant to Baxter and Pfizer, and she has been a teacher on educational activities of Bayer Schering Pharma. M. H. Cnossen has received unrestricted research/educational funding for various projects from the following companies: Pfizer, Baxter, Bayer Schering Pharma, Novo Nordisk, and Novartis. K. Fijnvandraat is a member of the European Hemophilia Treatment and Standardization Board sponsored by Baxter, has received unrestricted research grants from CSL Behring and Bayer, and has given lectures at educational symposiums organized by Pfizer, Bayer, and Baxter. K. Meijer has received research support from Bayer and Baxter, served on an advisory board for CSL Behring, received travel fees from Pfizer, and received speaker fees from Sanquin and Boehringer Ingelheim. B. Laros-van Gorkom has received unrestricted educational grants from Baxter and CSL Behring, and speaker fees from Sanquin. The other authors state that they have no conflict of interest.

Appendix: The WiN Study Group

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