Von Willebrand Disease in the Netherlands

from genetic variation to phenotypic variability

Yvonne Veroni Sanders

Von Willebrand Disease in the Netherlands – from genetic variation to phenotypic variability

Cover: Antelope Canyon, Page, Arizona, United States of America
Photographer: Bouke Michiel van der Werf
Cover design: Maaike van den Akker (www.maaikevandenakker.nl)
Layout and printing: Optima Grafische Communicatie, Rotterdam, the Netherlands

ISBN: 978-94-6169-723-3

The studies in this thesis were financially supported by Stichting Hemophilia and CSL Behring (unrestricted grant).

Printing and layouting of this thesis was financially supported by Van den Tol stichting, Octapharma, CSL Behring, Sanquin Blood Supply, Baxalta, Bayer HealthCare, Pfizer bv, Novo Nordisk, and Erasmus MC.

Von Willebrand Disease in the Netherlands

from genetic variation to phenotypic variability

De ziekte van von Willebrand in Nederland

van genetische variatie tot fenotypische variabiliteit

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 11 november 2015 om 15.30 uur

door

Yvonne Veroni Sanders
geboren te Winschoten

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotor:	Prof.dr. F.W.G. Leebeek
Overige leden:	Prof.dr. S. Middeldorp
	Prof.dr. J.L.C.M. van Saase
	Prof.dr. P. Sonneveld

De natuur haast zich niet,
en toch is alles af
Lao Tzu

CONTENTS

Chapter 1	General introduction and outline of the thesis	9
Chapter 2	Genotyping of von Willebrand Disease patients in the Netherlands: phenotype-genotype discrepancies and 27 novel *VWF* gene mutations	27
Chapter 3	Effect of genetic variation in *STXBP5* and *STX2* on von Willebrand Factor and bleeding phenotype in type 1 von Willebrand Disease patients	57
Chapter 4	*CLEC4M* and *STXBP5* gene variations contribute to von Willebrand Factor level variation in von Willebrand Disease	71
Chapter 5	Von Willebrand Factor propeptide and the phenotypic classification of von Willebrand Disease	89
Chapter 6	Dysregulated secretion of angiogenic mediators in patients with moderate and severe von Willebrand Disease	109
Chapter 7	Bleeding spectrum in children with moderate or severe von Willebrand Disease: relevance of pediatric-specific bleeding	127
Chapter 8	Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand Disease	145
Chapter 9	Joint bleeds in von Willebrand Disease patients have significant impact on quality of life and joint integrity: a cross-sectional study	167
Chapter 10	Reduced prevalence of arterial thrombosis in von Willebrand Disease	183
Chapter 11	Von Willebrand Disease and aging: an evolving phenotype	201
Chapter 12	General discussion	221
Chapter 13	Summary	241
	Samenvatting	249
Appendices	List of publications	259
	Authors and affiliations	261
	List of WiN study group members and participating hospitals	263
	List of abbreviations	265
	Dankwoord	269
	Curriculum vitae	275
	PhD portfolio summary	277

1

General introduction and outline of the thesis

partly published as
Von Willebrand Disease in the Netherlands: the WiN study
De ziekte van von Willebrand in Nederland: de WiN studie

YV Sanders, EM de Wee, K Meijer, J Eikenboom, JG van der Bom, K Fijnvandraat, BAP Laros-van Gorkom, MH Cnossen, EP Mauser-Bunschoten, FWG Leebeek, and the WiN study group

Nederlands Tijdschrift voor Geneeskunde. 2014;158:A6518

GENERAL INTRODUCTION

Von Willebrand Factor

Von Willebrand Factor (VWF) is a large multimeric glycoprotein that is essential for platelet-plug formation in hemostasis by mediating adhesion and aggregation of platelets at sites of vascular injury. In plasma, VWF also serves as a carrier protein of coagulation Factor VIII (FVIII) to prevent its premature clearance (figure 1).[1]

The *VWF* gene is located on chromosome 12, spans 178 kb and consists of 52 exons.[2] VWF synthesis is restricted to endothelial cells and megakaryocytes,[3] where it is formed as a precursor protein, pre-proVWF, with a signal peptide of 22 amino acids, a propeptide of 741 amino acids and a mature subunit of 2050 amino acids.[4] After translocation to the endoplasmic reticulum the signal peptide is removed, and the newly formed pro-VWF undergoes extensive post-translational modifications. First, VWF dimerizes through the formation of C-terminal disulfide bonds at the CK-domains of the VWF monomers.[5] Next, in the trans Golgi network dimers form multimers via N-terminal disulphide bonds at the D'D3 domain.[6] The VWF propeptide (VWFpp) is subsequently removed from the mature VWF, however it remains noncovalently bound.[7] Then, part of the synthesized VWF multimers is secreted constitutively into the plasma and the remaining part is stored in cell-specific organelles; the Weibel-Palade bodies in endothelial cells or α-granules in megakaryocytes.[8,9] After release into the circulation, the VWFpp and the mature VWF completely dissociate.[8] The mature subunit of VWF comprises several structural domains each with its own functions (figure 2).[10]

Normally, VWF levels in plasma range between 60-140 IU/dL. High VWF levels are shown to be a risk factor for arterial thrombosis, including coronary heart disease and ischemic stroke, and venous thrombosis.[11-16] Von Willebrand Disease (VWD), characterized by low VWF levels, results in an increased bleeding risk.[17,18]

Determinants of Von Willebrand Factor levels

Plasma VWF levels show high inter-individual and intra-individual variability. They are known to be influenced by several environmental factors, like physical exercise, stress, inflammation, hypertension, diabetes, hormones, and pregnancy.[19-25]

A major determinant of VWF antigen (VWF:Ag) levels is ABO blood group.[26] VWF plasma levels are approximately 25% lower in individuals with blood group O than in non-O individuals.[27] This is possibly regulated by the ABO blood group antigens on N-linked oligosaccharide chains of VWF and explained by increased clearance rate of VWF in individuals with blood group O.[28,29]

VWF and FVIII levels are known to increase with age in healthy individuals.[30,31] A recent study among over 5,000 normal, healthy blood donors showed a minor VWF:Ag increase with age up to the age of 40 years and a considerable increase of >8 IU/dL VWF:Ag per

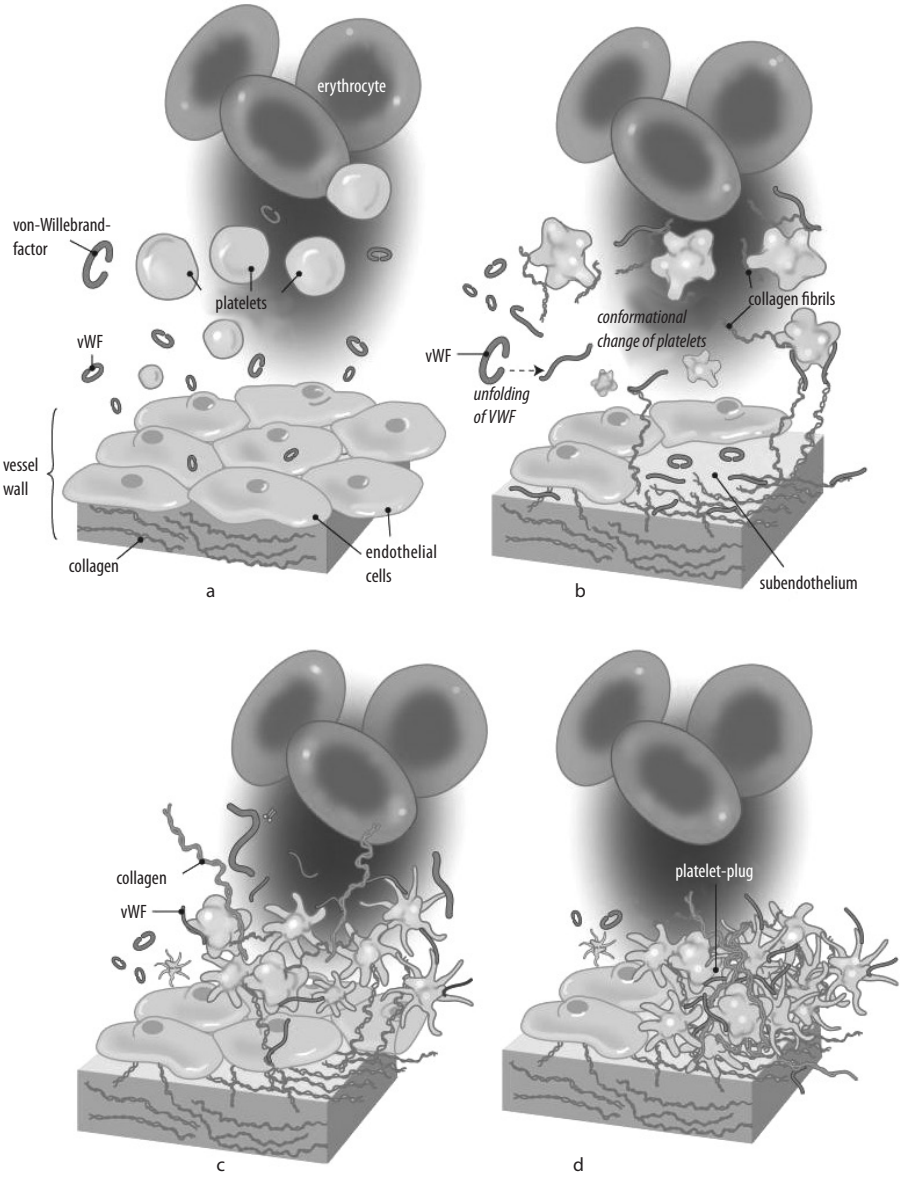


Figure 1. The function of von Willebrand Factor in primary hemostasis.
In this figure, the function of von Willebrand Factor (VWF) in primary hemostasis is schematically shown. (a) With an intact vessel wall the endothelium hampers interaction between collagen, VWF and platelets. In this situation VWF is stored in the subendothelium and circulates in plasma in a coiled structure. (b) If the vessel wall is injured, collagen becomes exposed and interacts with VWF. (c) Subsequently, the uncoiled and active VWF binds to the exposed collagen and to platelets, which become activated too. In this way VWF supports the adhesion of platelets to the damaged vessel wall. (d) VWF is also responsible for platelet aggregation that leads to platelet-plug formation. Finally fibrin strands are formed in the secondary hemostasis to strengthen the platelet-plug. (Adapted from Sanders et al [90])

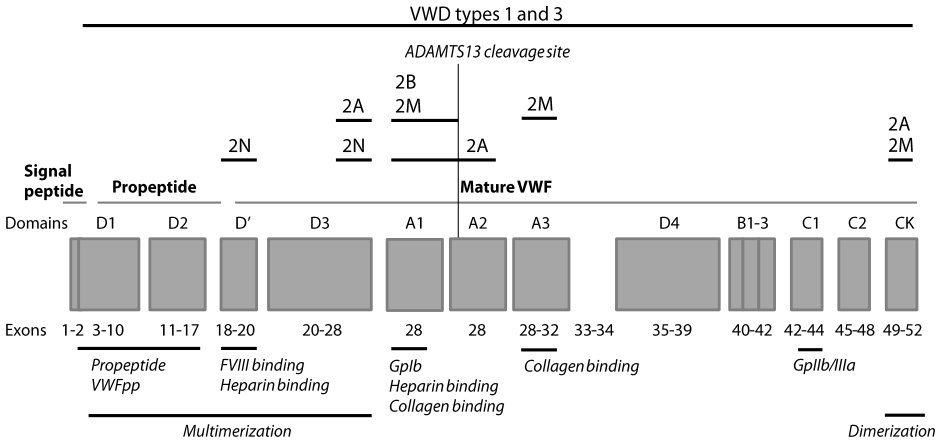


Figure 2. Schematic representation of the structure and function of the *VWF* gene and VWF protein. Exons encoding each functional domain are shown. Above the VWF protein, the location of *VWF* gene mutations for the various VWD types are illustrated. Types 1 and 3 VWD are caused by different types of mutations throughout the *VWF* gene, whereas mutations resulting in type 2 VWD are localized to distinct functional domains of VWF. Below the VWF protein, the different binding sites and functions are depicted. (In part adapted from Goodeve, AC and James, PD [80,91])

decade above 40 years of age.[32] The mechanisms of increase of VWF levels during aging are still unknown, but an association between rising VWF levels and increasing arterial rigidity has been suggested.[33] This is also indicated by a recent study from Sonneveld et al, as they found that VWF:Ag levels in ischemic stroke patients were strongly associated with the extent of atherosclerosis in the aortic arch and carotid arteries, measured by calcification volumes.[34]

Genetic variations inside and outside the VWF gene

Twin studies have shown that over half of the variability in VWF levels is caused by genetic factors.[35,36] Single nucleotide polymorphisms in coding regions of the *VWF* gene and in the VWF promotor have been demonstrated to determine VWF:Ag levels.[13,16,37-39] Interestingly, associations between polymorphisms in the VWF promotor and VWF levels were only found in blood group O individuals and were stronger above 40 years of age.[37,38]

Recently, genome-wide association studies have identified novel genetic loci that contribute to variability in VWF levels in healthy individuals.[40] Besides the *ABO* gene and loci within the *VWF* gene, also loci outside the VWF locus have been identified. These genes encode for proteins that had previously not been linked to VWF and therefore these proteins may be involved in secretion of VWF (*STX2* and *STXBP5*) and clearance of VWF (*CLEC4M*, *SCARA5*, and *STAB2*).[28] The *TC2N* gene was also identified, which has since been shown to be associated with venous thrombosis.[41] In this genetic associa-

tion study, ABO blood group has been shown to be the major genetic determinant of VWF:Ag levels in healthy individuals.

Von Willebrand Factor regulating mechanisms

It has been shown that accelerated clearance of VWF from plasma is an important mechanism for reduced VWF levels.[42,43] VWF clearance can be predicted using the VWFpp and mature VWF levels in plasma. VWF and VWFpp are secreted in equimolar amounts, but are cleared independently. Because the half-lives of VWFpp (2 hours) and VWF:Ag (8-12 hours) differ, the ratio between VWFpp and VWF:Ag can be used to assess synthesis, secretion and clearance rates of VWF.[44-47] Together with the VWFpp/VWF:Ag ratio, the ratio between FVIII coagulant activity (FVIII:C) and VWF:Ag can be used to assess VWF synthesis and clearance.[48] Since FVIII and VWF circulate in blood as a complex and are cleared together, their half-lives are related.[49] These ratios of VWFpp/VWF:Ag and FVIII:C/VWF:Ag have recently been shown to represent the pathophysiology of type 1 VWD.[50]

Von Willebrand Factor and angiogenesis

Recently, VWF has been shown to control angiogenesis as a negative regulator.[51,52] The angiogenic process is mediated by several proteins, like angiopoietin-2 (Ang-2), osteoprotegerin, galactin-3 and vascular endothelial growth factor (VEGF).[53,54] These proteins are stored in the Weibel-Palade bodies in endothelial cells, together with VWF.[55,56] Absent or reduced VWF, which lead to disturbed formation of Weibel-Palade bodies,[57] may result in an increased secretion of angiogenic mediators. The potential role of VWF in regulating angiogenesis is via increased VEGF receptor-2 dependent proliferation and migration, decreased integrin αvβ3 and increased Ang-2.[51] Disturbed angiogenesis leads to angiodysplasia most common in the gastrointestinal tract. Angiodysplasia related gastrointestinal bleeding is often seen in VWD patients and is associated with loss of VWF high molecular weight multimers and therefore more common in type 2A VWD patients.[58,59]

Von Willebrand Disease

VWD is the most common inherited bleeding disorder and is caused by reduced concentration or aberrant activity of VWF.[60] The clinical expression of VWD is very heterogeneous with a large variability in bleeding frequency and severity.

Bleeding symptoms and age-specific characteristics of Von Willebrand Disease

VWD patients regularly suffer from mucocutaneous bleeding episodes, varying from mucosal bleeds, epistaxis, gastrointestinal bleeding, and menorrhagia to post-surgical bleeding.[61-63] Joint bleeds also occur in patients with VWD, which can lead to structural joint damage and arthropathy (reviewed in [64]).

At every age-group different clinical characteristics of VWD are experienced. Children present with bleeding after change of teeth or bleeding after minor surgery, including tonsillectomy. Many young children often experience mouth and nose bleeds, which is less common in adults.[65,66] In addition, child-specific bleeding symptoms, like umbilical stump bleeding and cephalohematoma, may occur at very young age.[65] At higher age more gastrointestinal bleeding has been reported, which is also seen in the general population and may be associated with angiodysplasia.[67-69] In addition, with increasing age the bleeding risk may change due to comorbidities or changes in lifestyle.[70]

As mentioned before VWF and FVIII levels are known to increase with age in healthy individuals.[31,32] If the levels also increase with age in patients with VWD, this may in turn alter the bleeding phenotype and may change the necessity of treatment for VWD. Increasing VWF and FVIII levels with aging can also imply that elderly patients with mild type 1 VWD no longer meet the current diagnostic criteria for VWD.[71] However, the increase of VWF levels may still lead to levels that are too low for adequate hemostasis for this age group and may still result in a hemorrhagic diathesis.

Bleeding assessment tools

Recently, Tosetto et al developed a bleeding score with the aim to discriminate more easily between individuals with and without VWD.[72] This bleeding score assesses the severest life-time episodes of twelve specific types of bleeding and is able to quantify the number and severity of bleeding symptoms. Every bleeding symptom scores on a scale ranging from -1 to 4, with a minimum score of -3, and a maximum score of 37 for males and 45 for females. A bleeding questionnaire for pediatric patients has also been developed.[65] This score is similar to the Tosetto bleeding score, but is extended with child-specific bleeding symptoms, like umbilical stump bleeding, cephalohematoma, post-circumcision bleeding, post-venipuncture bleeding, and macroscopic hematuria. The currently advised version of the bleeding questionnaire is the ISTH-SSC Bleeding Assessment Tool.[73]

Diagnosis of Von Willebrand Disease

Figure 3 illustrates the diagnostic strategy for VWD testing. Patients with VWD have a bleeding history and usually also a positive family history. Additionally, the diagnosis of VWD is unlikely in individuals with a Tosetto bleeding score ≤3.[72] Laboratory assays are essential for a correct diagnosis of VWD.[18] If a patient presents with a bleeding diathesis, first the function of the primary and secondary are tested with the PFA (platelet function analyzer), Prothrombin Time (PT) and activated partial thromboplastin time (aPTT), platelet counts and fibrinogen. Next, in case of a prolonged PFA or a typical hemorrhagic diathesis, VWD-specific laboratory assays are performed to test the concentration and function of VWF and FVIII. This includes VWF:Ag, VWF collagen binding (VWF:CB), VWF

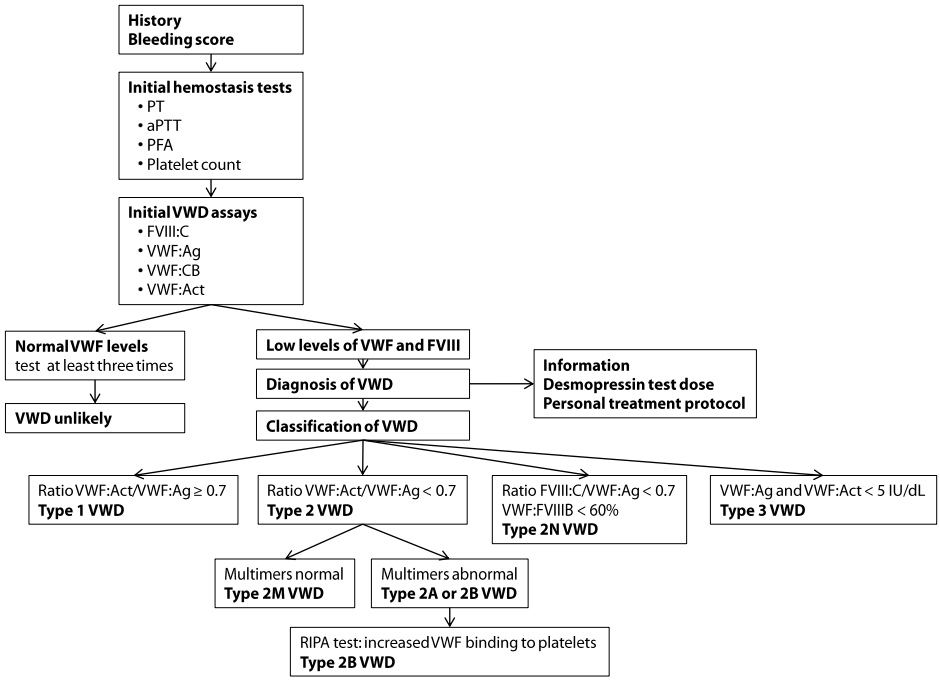


Figure 3. Diagnostic algorithm for von Willebrand Disease testing.
PT = Prothrombin Time, aPTT = activated partial thromboplasting time, PFA = platelet function analyzer, VWD = von Willebrand Disease, VWF = von Willebrand Factor, FVIII:C = FVIII coagulant activity, VWF:Ag = VWF antigen, VWF:CB = VWF collagen binding, VWF:Act = VWF activity, VWF:FVIIIB = VWF finding to FVIII , RIPA = ristocetin-induced platelet aggregation.

activity (VWF:Act), FVIII:C, and VWF multimer pattern. In addition, ristocetin-induced platelet aggregation (RIPA) tests or a VWF finding to FVIII (VWF:FVIIIB) assay will be performed in some cases. More details on epidemiology, pathophysiology, diagnosis and treatment of VWD are shown in table 1.

Classification of Von Willebrand Disease

According to the current guidelines of the International Society on Thrombosis and Haemostasis (ISTH),[71] VWD is classified into three types based on the remaining levels of functional VWF.[18,71] Type 1 VWD is characterized by partially reduced VWF levels and has a mostly mild clinical expression. Type 3 VWD is the severest and rarest form of VWD, and VWF is virtually completely absent (VWF:Ag and VWF:Act <5 IU/dL). These patients also have very low FVIII levels which may result in muscle hematoma and joint bleeds in addition to severe mucocutaneous bleeding. Type 2 VWD is characterized by functionally abnormal variants of VWF, and is further divided in four categories: 2A, 2B, 2M and 2N.[18,71] In type 2A, there are defects in multimerization, intracellular transport, secretion,

Table 1. Clinical characteristics of von Willebrand Disease.

Type of VWD		Type 1	Type 2				Type 3
			2A	2B	2M	2N	
Epidemiology and Pathophysiology	**Prevalence**	75%	20%				5%
	Quantitative or qualitative defect	quantitative	qualitative				quantitative
	Phenotype	mild	mild to severe, more severe phenotype than type 1 VWD				severe
	Cause	reduced levels of normal VWF	increased proteolysis or reduced synthesis of multimers	increased affinity of VWF to platelets	reduced VWF-dependent platelet adhesion, with normal multimers	reduced binding of FVIII to VWF	complete absence of VWF in plasma
	Inheritance pattern	AD	AD	AD	AD	AR	AR
Diagnosis	**PFA**	=/↑	↑	↑	↑	=/↑	↑↑
	VWF:Ag	↓	↓↓	↓↓	↓	=	absent
	VWF:Act	↓	↓↓↓	↓↓↓	↓↓↓	=	absent
	FVIII:C	=/↓	↓	↓	↓	↓↓	↓↓↓/absent
	Ratio VWF:Act/VWF:Ag	≥ 0.70	< 0.70	< 0.70	< 0.70	< 0.70	1
	RIPA	=	↓	↑	↓	=	absent
	HMW multimers	=	↓↓	↓	=	=	absent
	VWF:FVIII:B	=	=	=	=	↓ (to distinguish from hemophilia A)	=
	Platelet count	=	=	↓	=	=	=
Treatment		DDAVP antifibrinolytics	VWF/FVIII concentrate* or DDAVP antifibrinolytics	VWF/FVIII concentrate* antifibrinolytics	VWF/FVIII concentrate* or DDAVP antifibrinolytics	VWF/FVIII concentrate* antifibrinolytics	VWF/FVIII concentrate* antifibrinolytics

PFA = platelet function analyzer, VWF:Ag = VWF antigen, VWF:Act = VWF activity, FVIII:C = FVIII coagulant activity, RIPA = ristocetin-induced platelet aggregation, HMW multimers = high molecular weight multimers, VWF:FVIII:B = VWF binding to FVIII assay, AD = autosomal dominant, AR = autosomal recessive, DDAVP = desmopressin. = normal, ↓ slightly reduced, ↓↓ moderately reduced, ↓↓↓ strongly reduced, ↑ increased, ↑↑ moderately increased. *registered in the Netherlands: Haemate-P®, Wilfactin® en Wilate®. (In part adapted from Sanders Y.V. et al, Leebeek FWG et al, and Castaman et al. [18,90,92])

or increased ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin motif, member 13)-mediated proteolysis of VWF.[74,75] In type 2B VWD, the functionally aberrant VWF has an increased affinity for the GPIb receptor on platelets. Type 2M VWD is caused by a decreased VWF-platelet interaction or a reduced VWF-collagen binding.[76,77] In type 2N VWD the binding of FVIII to VWF is reduced which results in low FVIII levels.[18,71,78] More details on the classification of different types of VWD are shown in table 1.

VWF gene mutations in Von Willebrand Disease

The genetic background of VWD is very heterogeneous; as currently over 500 mutations in the *VWF* gene are known to cause VWD (EAHAD Coagulation Factor Variant database: https://grenada.lumc.nl/LOVD2/VWF/home.php; formerly ISTH-SSC VWF Online Database). These mutations are located throughout the *VWF* gene in type 1 and 3 VWD disease (figure 2). Some causative *VWF* gene mutations in type 1 VWD are associated with increased clearance of VWF, such as the well-known Vicenza mutation p.R1205H and p.C1130F, p.W1144G and p.C1149R.[42,45,79]

Most *VWF* gene mutations that result in type 2 VWD are located in the domain with its affected function. Classic type 2A VWD patients have mutations in the A2 and A1 domains, resulting in increased intracellular retention and therefore loss of high molecular weight multimers. Mutations in type 2A patients with enhanced ADAMTS13 cleavage surround the ADAMTS13 cleavage site. Type 2A VWD mutations that result in defects in dimerization and multimerization are located in the D2, D3 and CK domains.[80,81] The mutations involved in type 2B and type 2M VWD are predominantly located in the platelet-binding region of VWF (A1 domain) or in the A3 domain if it the VWD is caused by reduced VWF binding to collagen.[76-78] Mutations that cause type 2N VWD are located in the FVIII binding region of VWF (D' and D3 domains).[71] However, causative *VWF* gene mutations are not found in all VWD patients. In around 30% of type 1 VWD patients no mutations in the *VWF* gene are identified which implies that other genetic loci may be involved in the regulation of VWF levels.[82,83]

The Willebrand Disease in the Netherlands (WiN) study

In 2007 the "Willebrand in the Netherlands" (WiN) study was started, which aimed to obtain more insight into the clinical presentation, severity and impact of VWD in the Netherlands; and its influence on the quality of life.[84-86] All thirteen hemophilia treatment centers in the Netherlands participated in the WiN study. VWD patients with a hemorrhagic diathesis or a family history of VWD and historically lowest VWF levels ≤30 U/dL, were invited to participate in this study. In total, 804 patients were included in the WiN study, of whom 140 children and 664 adult patients. From these patients, a blood sample for the measurement of VWF and FVIII levels and for the isolation of DNA was

obtained. In addition, included patients completed an extensive questionnaire about bleeding symptoms, treatment of VWD, concomitant diseases and quality of life.[84]

Previously, the gynecological and obstetrical symptoms of 423 women with VWD were studied in this WiN study by de Wee et al.[87] Over 80% of females with VWD reported menorrhagia and twenty percent of females with VWD underwent a hysterectomy because of severe menstrual bleeding. In 37% of these patients, VWD was only diagnosed after the hysterectomy. Over 50% of females with VWD reported postpartum hemorrhage. A blood transfusion postpartum was needed in a significantly higher percentage of deliveries (11%) than in the general population (1%).[88]

The impact of VWD on health-related quality of life has been thoroughly studied in large cohorts of adults and children from the WiN study by de Wee et al.[85,86] Compared with the general population, the quality of life was reduced in children with VWD, in particular on the scales physical functioning, emotional-behavioral functioning, general health perceptions, and physical summary.[86] Interestingly, the parents of children aged 10-16 years experienced a remarkably lower quality of life than these children did. Also in adult patients, VWD was associated with lower quality of life than the general population, especially on the vitality domain.[85] Quality of life was strongly associated with bleeding severity and VWF plasma level, i.e. a more severe bleeding phenotype and lower VWF levels were associated with lower quality of life.

AIM AND OUTLINE OF THIS THESIS

The overall aim of this thesis is to investigate the genotypic and phenotypic determinants of VWD. In **chapter 2**, we will evaluate the genotype of a subgroup of VWD patients from the WiN study (n=199) and we will investigate the phenotype and genotype associations in these patients with moderate or severe VWD. We will compare the genotype-phenotype associations with the current diagnostic criteria from the ISTH to assess the necessity of molecular analysis of the *VWF* gene for the classification of VWD.[71] In this chapter, we also report newly discovered *VWF* gene mutations in the WiN-cohort.

No causative *VWF* gene mutation can be detected in around 30% of type 1 VWD.[82,83] This may imply that other genetic loci are involved in the occurrence of low VWF levels. Recently genetic loci have been identified that determine VWF:Ag levels in healthy individuals (*STXBP5*, *SCARA5*, *ABO*, *STAB2*, *STX2*, *TC2N*, and *CLEC4M*).[40] These genes may play a role in the secretion and clearance of VWF. In **chapters 3 and 4** we will investigate the influence of these genetic variations on VWF levels and bleeding phenotype in patients with type 1 VWD and type 2 VWD.

VWD is caused by reduced VWF synthesis or secretion, increased VWF clearance, or a combination thereof.[42,43] In **chapter 5**, we will study the pathophysiology of types 1, 2

and 3 VWD using the ratio between VWFpp and VWF:Ag and the ratio between FVIII:C and VWF:Ag. We aimed to evaluate if the use of VWFpp can improve the diagnosis and classification of VWD.

VWF has recently been shown to act as a negative regulator of angiogenesis.[51] Angiodysplasia is frequently seen in VWD patients with absent or reduced VWF levels. These patients may have an increased secretion of angiogenic mediators. We will therefore evaluate in **chapter 6** the association between angiogenic mediators, VWF levels and bleeding phenotype in patients with VWD.

It is well known that the phenotypic presentation of VWD is highly variable. We will therefore assess the determinants of bleeding phenotype and pattern of bleeding symptoms in VWD. First, the bleeding phenotype will be evaluated in 113 children with type 1, 2 and 3 VWD in **chapter 7**. We will investigate the occurrence and severity of various bleeding symptoms, including pediatric-specific bleeding symptoms, and their association with type of VWD and severity. Next, these determinants of the bleeding pattern and severity will be investigated in a large cohort of moderately and severely affected adult VWD patients. We aimed to assess the associations between bleeding phenotype and gender, age, type of VWD, VWF levels, FVIII levels, or blood group (**chapter 8**).

More severely affected VWD patients have a more severe bleeding phenotype including joint bleeds as a result of low FVIII levels.[64] Joint bleeds can lead to joint damage and arthropathy, however this has hardly been studied in the past. Therefore, in **chapter 9** the prevalence, onset and treatment of joint bleeds in VWD will be studied in patients from the WiN study.

High VWF levels are a known risk factor for arterial thrombosis, such as acute myocardial infarction and ischemic stroke. It is currently unknown whether individuals with very low levels of VWF, like VWD patients, are protected against arterial thrombosis. We therefore study the prevalence of arterial thrombosis in a large cohort of patients with moderate or severe VWD and compare it to the general population in **chapter 10** to obtain more insight in the pathophysiologic role of VWF in arterial thrombosis.

VWD patients may undergo changes with regard to VWF levels and FVIII levels with increasing age. Aging may also affect bleeding phenotype in patients with VWD.[89] These age-related changes in VWF levels and bleeding phenotype may have consequences for the diagnosis VWD and the intensity and dose of VWD concentrate treatment. We will therefore study if age-related changes in VWF and FVIII levels and in bleeding phenotype occur in elderly VWD patients (**chapter 11**). In these patients, bleeding frequency and severity may also be influenced by co-morbidities. We will therefore also study specific age-related comorbidity in elderly VWD patients in this chapter.

In the final chapter (**chapter 12**) the results of this thesis, the clinical implications and consequences for future research will be discussed.

REFERENCES

1. Ruggeri ZM. Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation. *Best Pract Res Clin Haematol*. 2001;14(2):257-279.
2. Mancuso DJ, Tuley EA, Westfield LA, et al. Structure of the gene for human von Willebrand factor. *J Biol Chem*. 1989;264(33):19514-19527.
3. Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J Clin Invest*. 1973;52(11):2757-2764.
4. Verweij CL, Diergaarde PJ, Hart M, Pannekoek H. Full-length von Willebrand factor (vWF) cDNA encodes a highly repetitive protein considerably larger than the mature vWF subunit. *EMBO J*. 1986; 5(8):1839-1847.
5. Marti T, Rosselet SJ, Titani K, Walsh KA. Identification of disulfide-bridged substructures within human von Willebrand factor. *Biochemistry*. 1987;26(25):8099-8109.
6. Wagner DD, Mayadas T, Marder VJ. Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. *J Cell Biol*. 1986;102(4):1320-1324.
7. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem*. 1998;67:395-424.
8. Vischer UM, Wagner DD. von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. *Blood*. 1994;83(12):3536-3544.
9. Wagner DD, Olmsted JB, Marder VJ. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J Cell Biol*. 1982;95(1):355-360.
10. Zhou YF, Eng ET, Zhu J, Lu C, Walz T, Springer TA. Sequence and structure relationships within von Willebrand factor. *Blood*. 2012;120(2):449-458.
11. Whincup PH, Danesh J, Walker M, et al. von Willebrand factor and coronary heart disease: prospective study and meta-analysis. *Eur Heart J*. 2002;23(22):1764-1770.
12. Wieberdink RG, van Schie MC, Koudstaal PJ, et al. High von Willebrand factor levels increase the risk of stroke: the Rotterdam study. *Stroke*. 2010;41(10):2151-2156.
13. van Schie MC, van Loon JE, de Maat MP, Leebeek FW. Genetic determinants of von Willebrand factor levels and activity in relation to the risk of cardiovascular disease: a review. *J Thromb Haemost*. 2011; 9(5):899-908.
14. Nossent AY, van Marion V, van Tilburg NH, et al. von Willebrand factor and its propeptide: the influence of secretion and clearance on protein levels and the risk of venous thrombosis. *J Thromb Haemost*. 2006;4(12):2556-2562.
15. van Loon JE, Kavousi M, Leebeek FW, et al. Von willebrand factor plasma levels, genetic variations, and coronary heart disease in an older population. *J Thromb Haemost*. 2012;10(7):1262-1269.
16. van Schie MC, de Maat MP, Isaacs A, et al. Variation in the von Willebrand factor gene is associated with von Willebrand factor levels and with the risk for cardiovascular disease. *Blood*. 2011;117(4): 1393-1399.
17. Sadler JE, Mannucci PM, Berntorp E, et al. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost*. 2000;84(2):160-174.
18. Castaman G, Goodeve A, Eikenboom J, European Group on von Willebrand Disease. Principles of care for the diagnosis and treatment of von Willebrand disease. *Haematologica*. 2013;98(5):667-674.
19. El-Sayed MS, Sale C, Jones PG, Chester M. Blood hemostasis in exercise and training. *Med Sci Sports Exerc*. 2000;32(5):918-925.
20. van Loon JE, Sonneveld MA, Praet SF, de Maat MP, Leebeek FW. Performance related factors are the main determinants of the von Willebrand factor response to exhaustive physical exercise. *PLoS One*. 2014;9(3):e91687.

21. Danesh J, Wheeler JG, Hirschfield GM, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med*. 2004;350(14):1387-1397.
22. Blann AD, Naqvi T, Waite M, McCollum CN. von Willebrand factor and endothelial damage in essential hypertension. *J Hum Hypertens*. 1993;7(2):107-111.
23. Frankel DS, Meigs JB, Massaro JM, et al. Von Willebrand factor, type 2 diabetes mellitus, and risk of cardiovascular disease: the framingham offspring study. *Circulation*. 2008;118(24):2533-2539.
24. Knol HM, Kemperman RF, Kluin-Nelemans HC, Mulder AB, Meijer K. Haemostatic variables during normal menstrual cycle. A systematic review. *Thromb Haemost*. 2012;107(1):22-29.
25. Huq FY, Kulkarni A, Agbim EC, Riddell A, Tuddenham E, Kadir RA. Changes in the levels of factor VIII and von Willebrand factor in the puerperium. *Haemophilia*. 2012;18(2):241-245.
26. McCallum CJ, Peake IR, Newcombe RG, Bloom AL. Factor VIII levels and blood group antigens. *Thromb Haemost*. 1983;50(3):757.
27. Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ, Jr., Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood*. 1987;69(6):1691-1695.
28. Gallinaro L, Cattini MG, Sztukowska M, et al. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*. 2008;111(7):3540-3545.
29. Jenkins PV, O'Donnell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? *Transfusion*. 2006;46(10):1836-1844.
30. Conlan MG, Folsom AR, Finch A, et al. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. *Thromb Haemost*. 1993;70(3):380-385.
31. Mari D, Coppola R, Provenzano R. Hemostasis factors and aging. *Exp Gerontol*. 2008;43(2):66-73.
32. Davies JA, Hathaway LS, Collins PW, Bowen DJ. von Willebrand factor: demographics of plasma protein level in a large blood donor cohort from South Wales in the United Kingdom. *Haemophilia*. 2012;18(3):e79-81.
33. Vischer UM, Herrmann FR, Peyrard T, Nzietchueng R, Benetos A. Plasma von Willebrand factor and arterial aging. *J Thromb Haemost*. 2005;3(4):794-795.
34. Sonneveld MA, van Dijk AC, van den Herik EG, et al. Relationship of Von Willebrand Factor with carotid artery and aortic arch calcification in ischemic stroke patients. *Atherosclerosis*. 2013;230(2):210-215.
35. de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. *Lancet*. 2001;357(9250):101-105.
36. Bladbjerg EM, de Maat MP, Christensen K, Bathum L, Jespersen J, Hjelmborg J. Genetic influence on thrombotic risk markers in the elderly--a Danish twin study. *J Thromb Haemost*. 2006;4(3):599-607.
37. Keightley AM, Lam YM, Brady JN, Cameron CL, Lillicrap D. Variation at the von Willebrand factor (vWF) gene locus is associated with plasma vWF:Ag levels: identification of three novel single nucleotide polymorphisms in the vWF gene promoter. *Blood*. 1999;93(12):4277-4283.
38. Harvey PJ, Keightley AM, Lam YM, Cameron C, Lillicrap D. A single nucleotide polymorphism at nucleotide -1793 in the von Willebrand factor (VWF) regulatory region is associated with plasma VWF:Ag levels. *Br J Haematol*. 2000;109(2):349-353.
39. Bongers TN, de Maat MP, Deckers JW, Dippel DW, Leebeek FW. Frequency of the von Willebrand factor Tyr1584Cys polymorphism in arterial thrombosis. *Br J Haematol*. 2008;140(5):578-579.
40. Smith NL, Chen MH, Dehghan A, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*. 2010;121(12):1382-1392.

41. Morange PE, Saut N, Antoni G, Emmerich J, Tregouet DA. Impact on venous thrombosis risk of newly discovered gene variants associated with FVIII and VWF plasma levels. *J Thromb Haemost*. 2011;9(1): 229-231.
42. Casonato A, Pontara E, Sartorello F, et al. Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. *Blood*. 2002;99(1):180-184.
43. Lenting PJ, Westein E, Terraube V, et al. An experimental model to study the in vivo survival of von Willebrand factor. Basic aspects and application to the R1205H mutation. *J Biol Chem*. 2004;279(13): 12102-12109.
44. Borchiellini A, Fijnvandraat K, ten Cate JW, et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood*. 1996;88(8):2951-2958.
45. Haberichter SL, Balistreri M, Christopherson P, et al. Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood*. 2006;108(10):3344-3351.
46. Sztukowska M, Gallinaro L, Cattini MG, et al. Von Willebrand factor propeptide makes it easy to identify the shorter Von Willebrand factor survival in patients with type 1 and type Vicenza von Willebrand disease. *Br J Haematol*. 2008;143(1):107-114.
47. Wagner DD, Fay PJ, Sporn LA, Sinha S, Lawrence SO, Marder VJ. Divergent fates of von Willebrand factor and its propolypeptide (von Willebrand antigen II) after secretion from endothelial cells. *Proc Natl Acad Sci U S A*. 1987;84(7):1955-1959.
48. Eikenboom JC, Castaman G, Kamphuisen PW, Rosendaal FR, Bertina RM. The factor VIII/von Willebrand factor ratio discriminates between reduced synthesis and increased clearance of von Willebrand factor. *Thromb Haemost*. 2002;87(2):252-257.
49. Lenting PJ, van Schooten CJ, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. *J Thromb Haemost*. 2007;5(7):1353-1360.
50. Eikenboom J, Federici AB, Dirven RJ, et al. VWF propeptide and ratios between VWF, VWF propeptide and FVIII in the characterization of type 1 von Willebrand disease. *Blood*. 2013;121(12):2336-2339.
51. Starke RD, Ferraro F, Paschalaki KE, et al. Endothelial von Willebrand factor regulates angiogenesis. *Blood*. 2011;117(3):1071-1080.
52. Lenting PJ, Casari C, Christophe OD, Denis CV. von Willebrand factor: the old, the new and the unknown. *J Thromb Haemost*. 2012;10(12):2428-2437.
53. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003;9(6):653-660.
54. Daly C, Eichten A, Castanaro C, et al. Angiopoietin-2 functions as a Tie2 agonist in tumor models, where it limits the effects of VEGF inhibition. *Cancer Res*. 2013;73(1):108-118.
55. Saint-Lu N, Oortwijn BD, Pegon JN, et al. Identification of galectin-1 and galectin-3 as novel partners for von Willebrand factor. *Arterioscler Thromb Vasc Biol*. 2012;32(4):894-901.
56. Shahbazi S, Lenting PJ, Fribourg C, Terraube V, Denis CV, Christophe OD. Characterization of the interaction between von Willebrand factor and osteoprotegerin. *J Thromb Haemost*. 2007;5(9):1956-1962.
57. Valentijn KM, Eikenboom J. Weibel-Palade bodies: a window to von Willebrand disease. *J Thromb Haemost*. 2013;11(4):581-592.
58. Fressinaud E, Meyer D. International survey of patients with von Willebrand disease and angiodysplasia. *Thromb Haemost*. 1993;70(3):546.
59. Castaman G, Federici AB, Tosetto A, et al. Different bleeding risk in type 2A and 2M von Willebrand disease: a 2-year prospective study in 107 patients. *J Thromb Haemost*. 2012;10(4):632-638.

60. James PD, Lillicrap D. von Willebrand disease: clinical and laboratory lessons learned from the large von Willebrand disease studies. *Am J Hematol*. 2012;87 Suppl 1:S4-11.
61. James AH. More than menorrhagia: a review of the obstetric and gynaecological manifestations of von Willebrand disease. *Thromb Res*. 2007;120 Suppl 1:S17-20.
62. Kadir RA, Chi C. Women and von Willebrand disease: controversies in diagnosis and management. *Semin Thromb Hemost*. 2006;32(6):605-615.
63. Kouides PA. Current understanding of von Willebrand's disease in women - some answers, more questions. *Haemophilia*. 2006;12 Suppl 3:143-151.
64. van Galen KP, Mauser-Bunschoten EP, Leebeek FW. Hemophilic arthropathy in patients with von Willebrand disease. *Blood Rev*. 2012;26(6):261-266.
65. Biss TT, Blanchette VS, Clark DS, et al. Quantitation of bleeding symptoms in children with von Willebrand disease: use of a standardized pediatric bleeding questionnaire. *J Thromb Haemost*. 2010; 8(5):950-956.
66. Bowman M, Hopman WM, Rapson D, Lillicrap D, James P. The prevalence of symptomatic von Willebrand disease in primary care practice. *J Thromb Haemost*. 2010;8(1):213-216.
67. Sharma R, Gorbien MJ. Angiodysplasia and lower gastrointestinal tract bleeding in elderly patients. *Arch Intern Med*. 1995;155(8):807-812.
68. Franchini M, Mannucci PM. Gastrointestinal angiodysplasia and bleeding in von Willebrand disease. *Thromb Haemost*. 2014;112(3):427-431.
69. Makris M. Gastrointestinal bleeding in von Willebrand disease. *Thromb Res*. 2006;118 Suppl 1:S13-17.
70. Mauser-Bunschoten EP, Fransen Van De Putte DE, Schutgens RE. Co-morbidity in the ageing haemophilia patient: the down side of increased life expectancy. *Haemophilia*. 2009;15(4):853-863.
71. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
72. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost*. 2006;4(4):766-773.
73. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063-2065.
74. Schneppenheim R, Michiels JJ, Obser T, et al. A cluster of mutations in the D3 domain of von Willebrand factor correlates with a distinct subgroup of von Willebrand disease: type 2A/IIE. *Blood*. 2010;115(23):4894-4901.
75. Jacobi PM, Gill JC, Flood VH, Jakab DA, Friedman KD, Haberichter SL. Intersection of mechanisms of type 2A VWD through defects in VWF multimerization, secretion, ADAMTS-13 susceptibility, and regulated storage. *Blood*. 2012;119(19):4543-4553.
76. Larsen DM, Haberichter SL, Gill JC, Shapiro AD, Flood VH. Variability in platelet- and collagen-binding defects in type 2M von Willebrand disease. *Haemophilia*. 2013;19(4):590-594.
77. James PD, Notley C, Hegadorn C, et al. Challenges in defining type 2M von Willebrand disease: results from a Canadian cohort study. *J Thromb Haemost*. 2007;5(9):1914-1922.
78. Schneppenheim R, Budde U, Ruggeri ZM. A molecular approach to the classification of von Willebrand disease. *Best Pract Res Clin Haematol*. 2001;14(2):281-298.
79. Schooten CJ, Tjernberg P, Westein E, et al. Cysteine-mutations in von Willebrand factor associated with increased clearance. *J Thromb Haemost*. 2005;3(10):2228-2237.
80. Goodeve AC. The genetic basis of von Willebrand disease. *Blood Rev*. 2010;24(3):123-134.

81. Lillicrap D. von Willebrand disease: advances in pathogenetic understanding, diagnosis, and therapy. *Blood*. 2013;122(23):3735-3740.
82. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*. 2007;109(1):112-121.
83. James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. *Blood*. 2007;109(1):145-154.
84. de Wee EM, Leebeek FW, Eikenboom JC. Diagnosis and management of von Willebrand disease in The Netherlands. *Semin Thromb Hemost*. 2011;37(5):480-487.
85. de Wee EM, Mauser-Bunschoten EP, van der Bom JG, et al. Health-related quality of life among adult patients with moderate and severe von Willebrand disease. *J Thromb Haemost*. 2010;8(7):1492-1499.
86. de Wee EM, Fijnvandraat K, de Goede-Bolder A, et al. Impact of von Willebrand disease on health-related quality of life in a pediatric population. *J Thromb Haemost*. 2011;9(3):502-509.
87. De Wee EM, Knol HM, Mauser-Bunschoten EP, et al. Gynaecological and obstetric bleeding in moderate and severe von Willebrand disease. *Thromb Haemost*. 2011;106(5):885-892.
88. Jansen AJ, van Rhenen DJ, Steegers EA, Duvekot JJ. Postpartum hemorrhage and transfusion of blood and blood components. *Obstet Gynecol Surv*. 2005;60(10):663-671.
89. Miesbach W. The effect of aging on persons with von Willebrand disease. *The International Monitor: reviews of current key literature on von Willebrand Disease*. 2011(6):3-6.
90. Sanders YV, de Wee EM, Meijer K, et al. [Von Willebrand disease in the Netherlands: the WiN study] De ziekte van von Willebrand in Nederland: de WiN-studie. *Ned Tijdschr Geneeskd*. 2014;158:A6518.
91. James PD, Lillicrap D. The molecular characterization of von Willebrand disease: good in parts. *Br J Haematol*. 2013;161(2):166-176.
92. Leebeek FWG, Mauser-Bunschoten EP, Editors. Nederlandse Vereniging van Hemofiliebehandelaars. Richtlijn diagnostiek en behandeling van hemofilie en aanverwante hemostasestoornissen. *Alphen aan de Rijn: Van Zuiden Communications BV*. 2009:13-23.

2

Genotyping of von Willebrand Disease patients in the Netherlands: phenotype-genotype discrepancies and 27 novel *VWF* gene mutations

YV Sanders, WL van Heerde, MH Cnossen, BAP Laros-van Gorkom, K Fijnvandraat, S Schoormans, N Dors, JG van der Bom, K Meijer, EP Mauser-Bunschoten, J Eikenboom, FWG Leebeek, and the WiN study group

Manuscript submitted for publication

ABSTRACT

The "Willebrand in the Netherlands" (WiN) study is a nationwide cross-sectional multicentre study among 804 moderate and severe von Willebrand Disease (VWD) patients in the Netherlands. *VWF* gene mutations and phenotypic characteristics of 199 patients from 122 families were evaluated in more detail. Mutations in the *VWF* gene were identified in 173 of 199 VWD patients (87%). This percentage differed between subtypes: 63% (38/60) in type 1, 99% (110/111) in type 2 and 89% (25/28) in type 3 VWD. In total, 75 different *VWF* gene mutations were found. Twenty-seven of these mutations have not been reported earlier: four novel mutations in type 1, twelve in type 2, and eleven in type 3 VWD. Mutation types include: sixteen missense mutations, four deletions, one duplication, two insertions, three nonsense mutations and a large deletion of exons 17-52. Applying the current phenotypic diagnostic classification for VWD, we observed phenotype-genotype discrepancies in 10% of VWD patients. In 6 phenotypic type 1 patients with a VWF:Act/VWF:Ag ratio >0.70, a type 2 genotype was found. Four genotypic 2B patients were previously classified as 2A VWD, because of normal ristocetin-induced platelet aggregation (RIPA) tests. Additionally, 8 patients with p.R1205H, the genotype associated with VWD "Vicenza", were classified as type 3 (n=5) or 2A (n=3) VWD. The heterogeneous molecular background of VWD is underlined by our study. Additionally, this study illustrates the difficulties of classifying VWD patients solely on phenotypic characteristics. Implementation of *VWF* gene mutation analysis might improve the VWD classification.

INTRODUCTION

Von Willebrand Disease (VWD) is the most common inherited bleeding disorder and is caused by reduced concentration or aberrant activity of von Willebrand Factor (VWF).[1] VWF is a large multimeric glycoprotein that mediates platelet adhesion and aggregation at sites of vascular injury, and serves as a carrier protein of Factor VIII (FVIII).[2] Patients with VWD mainly suffer from mucocutaneous bleeding.[3,4] VWD is classified in three types: type 1 VWD is characterized by reduced levels of normal VWF and type 3 VWD by virtually complete absence of VWF, whereas type 2 VWD patients have abnormal variants of VWF. Type 2 VWD is divided in four subcategories based on phenotypic characteristics: 2A, 2B, 2M and 2N.[1]

Mutations in the *VWF* gene are found in many patients with VWD. The *VWF* gene is located on chromosome 12, spans 178 kb, consists of 52 exons and has a highly homologous partial pseudogene on chromosome 22.[5] VWF is synthesized in endothelial cells and megakaryocytes as a pre-propeptide with a signal peptide of 22 amino acids, a propeptide of 741 amino acids and a mature subunit of 2050 amino acids.[6] The mature subunit comprises several structural domains each with its own function.[7] Previous studies on the genotype of VWD have identified over 500 different *VWF* gene mutations and have given important insights in the molecular biology of VWD.[8-11]

In the Netherlands, characterization of mutations in the *VWF* gene is not routinely performed, as it is costly and in most cases it has no direct impact on the treatment of individual VWD patients.[12] Exceptions are however made when molecular analysis facilitates a phenotypically difficult diagnose, for example when differentiation is necessary between type 2N VWD and mild haemophilia A or between type 2B VWD and platelet type VWD. Also in patients with type 3 VWD, which is inherited in an autosomal recessive pattern, *VWF* mutation analysis is of added value for genetic counselling.[12,13] In the present study, we describe newly discovered *VWF* mutations in the nationwide cross-sectional "Willebrand in the Netherlands" (WiN) study and additionally evaluate the genotype-phenotype associations in nearly 200 VWD patients.

MATERIALS AND METHODS

Patients

This study is part of a nationwide cross-sectional multicentre study among VWD patients in the Netherlands: "WiN study" (total of 837 patients included from October 1st, 2007 till October 1st 2009).[3,12,14] The WiN study consists of type 1, type 2 and type 3 VWD patients with a haemorrhagic diathesis or a family history of VWD, and historically lowest VWF levels ≤30 U/dL and/or FVIII coagulant activity levels (FVIII:C) ≤40 U/dL. Patients were

excluded if they were known to have other bleeding disorders. The Medical Ethical Committees at all participating centres approved this study, and all participants gave informed consent.

In the current study, we present findings of 199 patients in whom *VWF* gene mutation analysis had previously been performed. In three haemophilia treatment centres (Nijmegen, Utrecht, Amsterdam), the *VWF* gene was sequenced routinely in all moderate and severe VWD patients (n=143); in the other centres this was only carried out in case of diagnostic difficulties (n=42) or for research purposes (n=14).

Von Willebrand Disease classification

In the WiN study, determination of VWD type was based on the current ISTH guidelines.[1] Patients were classified using centrally measured plasma concentrations of VWF antigen (VWF:Ag), VWF Collagen Binding (VWF:CB), VWF activity (VWF:Act), and FVIII:C; VWF multimer patterns (centrally performed); VWF binding to FVIII (VWF:FVIIIB) assay (centrally performed); and locally performed ristocetin-induced platelet aggregation (RIPA) tests.[3]

Patients with a VWF:Act/VWF:Ag ratio ≥0.70 were classified as type 1 VWD, and those with a ratio <0.70 as type 2 VWD. Type 2 patients with normal multimers were classified as 2M VWD and type 2 patients with abnormal multimers as 2A or 2B VWD. RIPA tests were used to classify type 2B VWD. Type 2B patients show hyperactive ristocetin-induced platelet agglutination in contrast to other types of VWD. Type 2N patients had a FVIII:C/VWF:Ag ratio <0.70, and a VWF:FVIIIB <0.60. Type 3 VWD was defined as both VWF:Ag and VWF:Act levels <5 IU/dL, irrespective of FVIII:C level.

Assessment methods in the WiN study

Most patients (n=804) completed an extensive questionnaire on bleeding episodes and treatment of VWD.[3,14] We also collected information on the severest life-time episode of twelve specific bleeding symptoms by administration of the Tosetto Bleeding Questionnaire, generating a bleeding score (BS).[4] To avoid prophylaxis-bias, we did not score for a bleeding symptom if patients had received prophylactic desmopressin or prophylactic replacement therapy before undergoing surgery or dental extraction.[3,15]

Laboratory measurements in von Willebrand Disease patients

Plasma levels of VWF:Ag, VWF:CB, VWF:Act and FVIII:C were measured centrally at inclusion in the study (Erasmus University Medical Center, Rotterdam), as described in more detail by de Wee et al.[3] Venous whole blood was collected in 0.105M sodium citrate tubes and centrifuged twice at 2,200 x *g* for 10 minutes at room temperature and stored at -80°C. VWF:Ag was determined with an in-house ELISA using polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase (HRP-)conjugated anti-human VWF antibodies (DakoCytomation, Glostrup, Denmark) for detection. VWF:CB was measured

with an in-house ELISA using collagen type 1 (Sigma-Aldrich, St Louis, USA) for capture and a HRP-conjugated anti-human VWF antibody (DakoCytomation, Glostrup, Denmark) for detection. To measure VWF activity, we used a VWF:Act assay which uses monoclonal antibodies directed against the GpIbα binding domain of VWF and thereby reflects the binding activity of VWF to GpIbα (HemosIL™ VWF Activity, Instrumentation Laboratory B.V, Breda, Netherlands). We have previously validated the VWF:Act test (n=122) and obtained a Spearman correlation coefficient of 0.942 with our previously used VWF:RCo activity test ($p<0.0001$).[3] FVIII:C was measured in a one-stage clotting assay (TriniCLOT, Biomerieux, Marcy l'Etoile, France) with FVIII-deficient plasma (Biopool, Umea, Sweden) and reference plasma (Precision BioLogic, Kordia, Leiden, Netherlands). Multimeric pattern was analysed by low resolution 0.9% agarose (Bio-Rad Laboratories, Hercules, CA, USA) gel electrophoresis followed by capillary Western blotting, independently reviewed by JE and FWGL, and classified as abnormal or normal by comparison with the commercial reference plasma (Normal reference plasma, Precision biologic, Kordia, Leiden, Netherlands) according to the MCMDM-1VWD-study.[16] When type 2N VWD was suspected in patients, the VWF:FVIIIB was determined by ELISA. After coating with anti-VWF (DakoCytomation, Glostrup, Denmark) and incubation with patient plasma, the FVIII was removed from VWF with calcium chloride [350 mM]. Then, recombinant FVIII (Kogenate® FS, Bayer HealthCare, Leverkusen, Germany) was added and the amount of FVIII bound to VWF was determined using anti-FVIII (Affinity Biologicals Inc., Ancaster, ON, Canada).[3] Phenotypic blood group was determined by mixing plasma of patients with red blood cells of donors with known blood group, as has previously been described.[17] If 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 A1), rs8176704 (marker for A2), and rs8176749 (marker for B).[18]

DNA sequencing

VWF gene mutation analysis had been performed in 199 VWD patients. These analyses were performed in: Sanquin-AMC Landsteiner Laboratory Amsterdam (n=127); Radboud university medical center Nijmegen (n=54); Leiden University Medical Center (n=16); and Erasmus University Medical Center Rotterdam (n=2).

After collection of blood samples in EDTA, DNA was purified from peripheral blood leukocytes. At Radboud university medical center in Nijmegen, all 52 exons of the *VWF* gene, including exon-intron boundaries and promoter regions, were amplified by polymerase chain reaction (PCR) and Sanger sequenced using an automated system (Hamilton, Bonaduz, Switzerland) with M13-labeled *VWF* gene-specific primers (Biolegio, Nijmegen, Netherlands) directed at all exons of the *VWF* gene. At Leiden University Medical Center, PCR-fragments including intron-exon boundaries were sequenced by the dideoxynucleotide chain-termination method using PCR primers or internal prim-

ers. At Sanquin-AMC Landsteiner Laboratory Amsterdam, Sanger sequencing was performed for 28/52 exons (4, 7, 9, 11, 12, 14-28, 32, 37-38, 42-43, 45, 51-52) and at Erasmus University Medical Center Rotterdam only the A1 domain specifically for type 2B patients was sequenced. Primers were manually checked for known SNPs and mutations using the ensemble genome browser (www.ensembl.org) and they were *VWF* gene specific, so they won't detect the pseudogene.

Desmopressin test dose

Fifteen patients in whom a novel *VWF* mutation was found, received a desmopressin test dose intravenously (0.3 µg/kg body weight) at the local haemophilia treatment centre. Immediately before and after desmopressin administration, VWF:Act and FVIII:C levels were measured in all patients and VWF:Ag in nine of them. In eight patients, VWF:Act and FVIII:C were also determined 3-4 hours after infusion. Desmopressin response was defined as complete if VWF increased to ≥50 IU/dl after administration, partial if VWF ≥30 IU/dl but <50 IU/dl, and no response if VWF <30 IU/dl, based on previous studies.[19,20] The response one hour after administration was defined as initial response, and 3-4 hours after administration as sustained response.

Statistical analysis

Descriptive statistics for continuous variables are presented as median and 25-75% interquartile range (IQR), because data were not normally distributed. Mann-Whitney

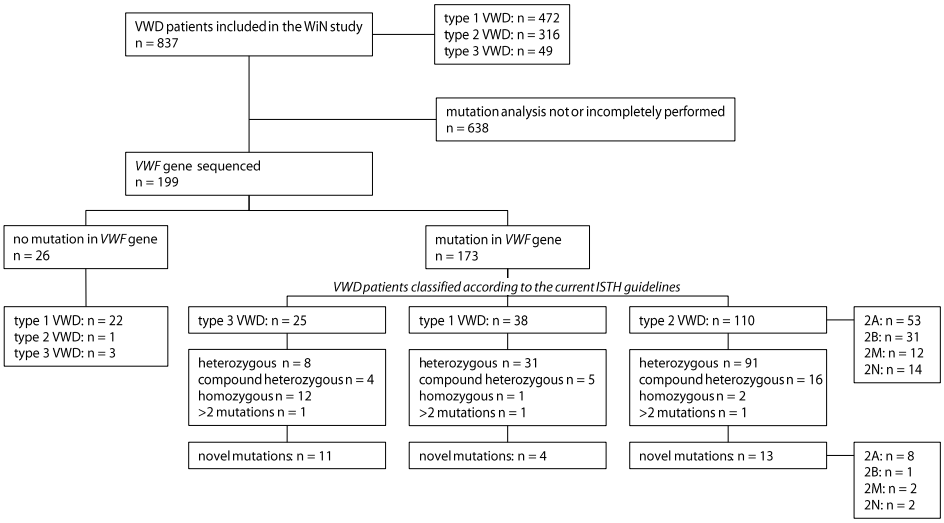


Figure 1. Flowchart of inclusion of patients with type 1, type 2 and type 3 von Willebrand Disease in this substudy of the Willebrand in the Netherlands (WiN) study.
VWF gene mutation analysis was performed in 199 patients and those were selected for the current study.

U-test was used to test the differences in patient characteristics between type 1 patients with and without a *VWF* gene mutation. Statistical analyses were performed with SPSS for Windows, version 21.0 (SPSS Inc, Chicago, USA). A p-value <0.05 was considered statistically significant.

RESULTS

Enrolled subjects and laboratory data

A total of 837 patients with moderate or severe VWD participated in the WiN study. Mutation analysis had been performed in 199 out of 837 (24%) of the patients: 60/472 (13%) type 1 patients, 111/316 (35%) type 2 patients and 28/49 (57%) type 3 patients (Figure

Table 1. Baseline characteristics of patients with moderate to severe von Willebrand Disease included in the WiN study in whom *VWF* gene sequencing had been performed.

Characteristics			VWD patients (n = 199)
Age (years), median (range)			35 (1-81)
Male sex, n (%)			106 (53)
VWD type, n (%)	1		60 (30)
	2		111 (56)
		2A	*53*
		2B	*31*
		2M	*12*
		2N	*14*
	3		28 (14)
No. of patients analysed per family		*1 patient*	82 families
		2 patients	21 families
		3 patients	10 families
		≥4 patients	9 families
Blood group O, n (%) *			105 (58)
VWF:Ag (IU/dL), median [IQR] †			23 [12-33]
VWF:CB (IU/dL), median [IQR] †			9 [5-24]
VWF:Act (IU/dL), median [IQR] †			14 [4-27]
FVIII:C (IU/dL), median [IQR] †			34 [20-50]
BS, median [IQR]‡			11 [6-17]

VWD = von Willebrand Disease, IQR = 25-75% interquartile range, VWF:Ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulation activity, BS = bleeding score.* Data available for n=181; † centrally measured VWF and FVIII levels available for n=161 (blood samples obtained within 72 hours after use of desmopressin or factor concentrate or during pregnancy are excluded). ‡ Data available for n=186.

1 and Table 1). The 199 VWD patients in whom mutation analysis had been performed belong to 122 different families.

Von Willebrand Disease patients without a *VWF* gene mutation

In 26/199 (13%) patients, belonging to sixteen families, no *VWF* gene mutations were detected. Based on their phenotype, most of these patients were classified as type 1 (n=22). In only one type 2A, and three type 3 patients no *VWF* mutation was detected (Table 2). In the 22 type 1 VWD patients without a *VWF* mutation, median VWF parameters were: VWF:Ag 23 IU/dL [25-75% IQR: 16-35], VWF:CB 19 IU/dL [IQR 11-43], VWF:Act 22 IU/dL [IQR 15-46] and FVIII:C 49 IU/dL [IQR 35-64]. Median BS was 8.0 [IQR 5.5-18.5]. Only half of these patients had a normal multimer pattern (12/22, 55%). No differences between the phenotypic characteristics of type 1 VWD patients with and without a causative *VWF* mutation were observed (VWF:Ag p=0.544; VWF:CB p=0.435; VWF:Act p=0.716; FVIII:C p=0.323; BS p=0.801).

Twenty-seven novel *VWF* gene mutations

In type 1 patients, five novel missense mutations in exons 22, 28, 37 and 38 were identified: p.S979N, p.P1240L, p.F1654I, p.C2184Y, p.C2248Y (Table 3). All type 1 patients had a VWF:Act/VWF:Ag ratio ≥0.70. However, four type 1 patients had an abnormal multimer pattern (p.F1654I, p.C2248Y, p.S979N).

We identified thirteen novel mutations in seventeen type 2 patients (Table 3). Eleven of the thirteen novel mutations (85%) were missense mutations. Two novel mutations were deletions (15%) that were identified in type 2N patients (p.S1090_C1099del and p.H1416Mfs*22) in compound heterozygosity with the well-known type 2N mutation p.R854Q. The p.H1416Mfs*22 mutation was also identified in a type 3 patient (compound heterozygous). One novel mutation (p.S979N) was identified in four individuals from one family. Two of the patients with this p.S979N mutation classified as type 1 VWD and two as type 2A VWD according to the current diagnostic criteria.[1]

Eleven novel mutations were identified in eleven type 3 patients. One of these novel mutations was identified in two brothers who were both homozygous for a duplication in the D1 domain (p.S330Kfs*4). Mutation p.D1333Efs*44 was identified in two siblings that were both homozygous for this insertion. In one type 3 patient three different mutations were found of which one novel: p.Y2160C. In two patients two different novel mutations were found: (I) p.H1416Mfs*22 and p.V1820fs*, and (II) p.Y1570* and p.G1609Efs*84. In one phenotypic type 3 patient only one mutation was found (p.E2553*).

Table 2. Characteristics of von Willebrand Disease patients without a *VWF* gene mutation (n=26).

no. of families	no. of patients	type of VWD*	BS	VWF:Ag (IU/dL)	VWF:CB (IU/dL)	VWF:Act (IU/dL)	FVIII:C (IU/dL)	VWF:Act/ VWF:Ag ratio	multimers	exons sequenced
1	1	1	6	13	10	15	21	≥0.7	abnormal	11-12, 14-16, 18-24, 26-28, 32, 37-38, 42-43, 45, 52
1	1	1	6	56	66	67	60	≥0.7	normal	11-12, 14-16, 18-24, 26-28, 32, 37-38, 42-43, 45, 52
1	3	1	8 - 14	20 - 23	12 - 14	17-22	29-39	≥0.7	abnormal	4, 7, 9, 11-12, 14-28, 32, 37-38, 42-43, 45, 51-52
1	3	1	7 - 27	7-23	7-28	8-29	40-61	≥0.7	normal/abnormal	4, 7, 9, 11-12, 14-28, 32, 37-38, 42-43, 45, 51-52
1	1	1	21	8	8	7	33	≥0.7	abnormal	4, 7, 9, 11-12, 14-28, 32, 37-38, 42-43, 45, 51-52
1	1	1	20	53	69	62	52	≥0.7	normal	11-12, 14-16, 18-24, 26-28, 32, 37-38, 42-43, 45, 52
1	2	1/2A	7-19	12-23	7-18	8-22	25-44	≥0.7 / <0.7	abnormal	11-12, 14-16, 18-24, 26-28, 32, 37-38, 42-43, 45, 52
1	1	3	12	0	0	0	2	NA	absent	4, 7, 9, 17, 25, 51
1	1	1	5	75	109	87	127	≥0.7	normal	4, 7, 9, 11-12, 14-28, 32, 37-38, 42-43, 45, 51-52
1	1	1†	2	45†	n/a	29†	46†	<0.7	normal†	4, 7, 9, 11-12, 14-24, 26-28, 32, 37-38, 42-43, 45, 52
1	2	1	18-24	12-18	10-14	9-14	30-37	≥0.7	abnormal	4,7,9,17,25,51
1	2	1	-1	30-34	35-39	29-40	52-72	≥0.7	normal	4,7,9,11-12,14-28,32,37,38,42-43,45,51-52
	1	3	11	0	0	0	1	NA	absent	
1	3	1	4-12	28-35	31-47	29-52	55-86	≥0.7	normal	11-12,14-16,18-24,26-28,32,37,38,42,43,45,52
1	1	1	7	62	84	84	98	≥0.7	normal	19,26,27,28,37,52
1	1	1	15	11	8	14	21	≥0.7	abnormal	11-12, 14-16, 18-24, 26-28, 32, 37-38, 42-43, 45, 52
1	1	3†	13	5†	n/a	4†	4†	≥0.7	absent†	11-12, 14-16, 18-24, 26-28, 32, 37-38, 42-43, 45, 52

no. = number, aa = amino acid, VWD = von Willebrand Disease, BS = bleeding score, VWF:Ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulation activity, n/a = not available, NA = not applicable. * VWD type according to current ISTH guidelines. † measured at patients own haemophilia treatment center.

Table 3. Twenty-seven novel *VWF* gene mutations in 34 patients with moderate and severe types 1, 2 or 3 von Willebrand Disease.

Type 1 VWD (n=6)

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	aa exchange	domain	exon	type of mutation	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF: FVIIIB	VWF:Act/ VWF:Ag ratio	multimers	BS
1	2	1 (also 2A, see below)	heterozygous	c.2936G>A	p.S979N	D3	22	missense	28-33	20-21	21-29	68-83		≥0.70	abnormal	7-13
1	1	1	>2	c.3614G>A ‡	p.R1205H	D3	27	missense	19†	n/a	24†	52†		≥0.70	normal†	4
				c.3719C>T	p.P1240L	D3	28	missense								
				c.3797C>A ‡	p.P1266Q	A1	28	missense								
1	1	1	heterozygous	c.4960T>A	p.F1654I	A2	28	missense	10	9	16	34		≥0.70	abnormal	9
1	1	1	heterozygous	c.6551G>A	p.C2184Y	D4	37	missense	10†	n/a	7†	49†		≥0.70	normal†	9
1	1	1	heterozygous	c.6753G>A	p.C2248Y	D4	38	missense	27	32	34	71		≥0.70	abnormal	14

Type 2 VWD (n=17)

no. of families	no. of patients	type of VWD*	genotype	nucleotide exchange	aa exchange	domain	exon	type of mutation	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF: FVIIIB	VWF:Act/ VWF:Ag ratio	multimers	BS
1	1	2A	compound heterozygous	c.2591A>G	p.D864G	D′	20	missense	15†	n/a	7†	11†		<0.70	n/a	3
				c.3614G>A ‡	p.R1205H	D3	27									
1	1	2A	compound heterozygous	c.2597C>T	p.T866M	D3	20	missense	31	9	17	65		<0.70	abnormal	16
				c.4789C>T ‡	p.R1597W	A2	28									
1	2	2A (also 1, see above)	heterozygous	c.2936G>A	p.S979N	D3	22	missense	16-33	12	11-15	31-32		<0.70	abnormal	2-21
1	1	2N	compound heterozygous	c.3269_98del30	p.S1090_C1099del	D3	25	deletion	27†	n/a	23†	12†	10†	≥0.70	abnormal†	11
				c.2561G>A ‡	p.R854Q	D′	20	missense								
				c.2561G>A ‡	p.R854Q	D′	20	missense								
1	3	2A	heterozygous	c.3863T>G	p.L1288R	A1	28	missense	25	29	16	68		<0.70	abnormal	10
		2M							12 - 14	n/a	4	26-42			normal	3-10
1	2	2M	heterozygous	c.3877T>C	p.F1293L	A1	28	missense	29-35	42	4-10	43-62		<0.70	normal	2-13

Table 3 (continued)

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	aa exchange	domain	exon	type of mutation	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF: FVIIIB	VWF:Act/ VWF:Ag ratio	multimers	BS
1	1	2N (also heterozygous in 3, see below)	compound heterozygous	c.4251delG	p.H1416Mfs*22	A1	28	deletion	19	26	25	12	41	≥0.70	normal	5
				c.2561G>A ‡	p.R854Q	D′	20	missense								
1	1	2M	heterozygous	c.4263C>A	p.N1421K	A1	28	missense	30	27	8	61		<0.70	normal	14
1	1	2A	compound heterozygous	c.4549A>C	p.S1517R	A2	28	missense	22†	n/a	4†	32†		<0.70	abnormal†	8
				5170+10C>T ‡		A3	29	splice								
1	1	2A	heterozygous	c.4717G>A	p.G1573S	A2	28	missense	51†	27†	19†	67†		<0.70	abnormal†	9
1	1	2A	heterozygous	c.4732A>C	p.T1578P	A2	28	missense	22	2	5	16		<0.70	abormal	15
1	1	2A	heterozygous	c.4892G>A	p.G1631D	A2	28	missense	36	6	23	43		<0.70	abnormal	15
1	1	2A	compound heterozygous	c.5015G>C	p.G1672A	A2	28	missense	17†	n/a	4†	44†		<0.70	abnormal†	13
				c.4994T>A ‡	p.V1665E	A2	28	missense								

Type 3 VWD (n=11)

no. of families	no. of patients	type of VWD*	genotype	nucleotide exchange	aa exchange	domain	exon	type of mutation	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF: FVIIIB	VWF:Act/ VWF:Ag ratio	multimers	BS
1	2	3	homozygous	c.988dupA	p.S330Kfs*4	D1	8	duplication	0-3	0	0-2	1-3		NA	absent	9-12
1	1	3	homozygous	c.1543_44ins8	p.V515fs*	D2	14	insertion	0	0	0	1		NA	absent	16
1	1	3	homozygous	exons 17-52 del		D2-CK	17-52	large deletion	0	0	0	1		NA	absent	29
1	2	3	homozygous	c.3998_9insA	p.D1333Efs*44	A1	28	insertion	0-5	0	0-7	2-4		NA	absent	9-21
1	1	3	compound heterozygous	c.4057C>T	p.Q1353*	A1	28	nonsense	5†	n/a	4†	3†		NA	absent†	10
				c.4751A>G ‡	p.Y1584C	A2	28	missense								
1	1	3 (also heterozygous in 2N, see above)	compound heterozygous	c.4251delG	p.H1416Mfs*22	A1	28	deletion	1†	n/a	7†	1†		NA	abnormal†	20
				c.5458delG	p.V1820fs*	A3	32	deletion								
1	1	3	compound heterozygous	c.4710C>A	p.Y1570*	A2	28	nonsense	0	0	0	1		NA	absent	7
				c.4826delG	p.G1609Efs*84	A2	28	deletion								

Table 3 (continued)

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	aa exchange	domain	exon	type of mutation	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF: FVIIIB	VWF:Act/ VWF:Ag ratio	multimers	BS
1	1	3	3 mutations	c.6479A>G	p.Y2160C	D4	37	missense	3	1	0	15		NA	abnormal	10
				c.4751A>G ‡	p.Y1584C	A2	28	missense								
				c.7636A>T ‡	p.N2546Y	C4	45	missense								
1	1	3	heterozygous	c.7657G>T	p.E2553*	C4	45	nonsense	0	0	2	1		NA	absent	19
				no other mutations found in *VWF* gene												

aa = amino acid, VWD = von Willebrand Disease, VWF:ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulation activity, VWF:FVIIIB = von Willebrand Factor binding to Factor VIII assay, BS = bleeding score, n/a = not available, NA = not applicable. * VWD type according to current ISTH guidelines. † measured at patients own haemophilia treatment center. ‡ published before and mentioned in ISTH-SSC VWF Online Database.

Response to desmopressin in types 1 and 2 von Willebrand Disease patients with novel mutations

Desmopressin response was tested in four (4/6, 67%) type 1 patients and eleven (11/17, 65%) type 2 patients with a novel *VWF* gene mutation. Initial desmopressin response was complete in the patients with p.C2248Y, p.S979N, p.F1293L, p.G1573S, and in the 2N patient with compound heterozygosity for p.S1090_C1099del and p.R854Q. One 2M patient with p.N1421K was a partial responder. No initial response to desmopressin was observed in six patients (Table 4).

Phenotypic and genotypic characteristics of types 1, 2 and 3 von Willebrand Disease patients

In 87% (173/199) of patients a *VWF* mutation had been identified. In total 75 different mutations were found of which 27 were novel. A *VWF* gene mutation was found in 38/60 (63%) type 1 VWD patients, 110/111 (99%) type 2 patients and in 25/28 (89%) type 3 patients. Phenotypic and genotypic details of all VWD patients with a *VWF* mutation are shown in figure 2, table 5 and supplementary tables 1, 2 and 3.

Mutations identified in type 1 patients were located throughout the *VWF* gene: from exon 4 to exon 49 (Figure 2 and Suppl. Table 1). In one patient a pseudogene conversion was identified: p.V1360A, p.F1369I and p.S1378F.

The majority of type 2 patients had one mutation, mostly located in exon 28 (n=81, 75%). Half of the mutations in type 2 patients were located in the A1 domain (n=52, 48%), and a quarter in the A2 domain (n=29, 27%). The most frequently identified mutations in type 2 patients were 2B mutations: p.R1306W (n=16, 15%, in seven families) and p.R1308C (n=11, 10%, in three families); and the 2A mutation p.R1597W (n=16, 15%, in six families). Mutation analysis was performed in fourteen type 2N patients. The majority (n=8) of these patients had the heterozygous missense mutations p.R854Q combined with a missense or null mutation on the other allele. Two type 2N patients were homozygous for a 2N mutation (p.R854Q and p.C1060Y). In two patients, only one mutation (p.R854Q or p.C1060Y) was identified and no other mutations on the second allele (Figure 2 and Suppl. Table 2).

Eleven (11/24, 46%) type 3 patients were homozygous and four (4/24, 17%) compound heterozygous. In one patient (1/24, 4%) three missense mutations were identified. In eight (8/24, 33%) type 3 patients only one mutation was observed. A total of seventeen different mutations were identified in type 3 patients, consisting of eight nonsense mutations, two insertions, six missense mutations and one large deletion (Figure 2 and Suppl. Table 3).

Table 4. Response to desmopressin in patients with novel *VWF* gene mutations.

	Genotype			Baseline			Peak			After 3-4 hours				
type of VWD	nucleotide exchange	aa exchange	domain	VWF:Ag (IU/dL)	VWF:Act (IU/dL)	FVIII:C (IU/dL)	VWF:Ag (IU/dL)	VWF:Act (IU/dL)	FVIII:C (IU/dL)	VWF:Ag (IU/dL)	VWF:Act (IU/dL)	FVIII:C (IU/dL)	initial response	sustained response
2A	c.2597C>T	p.T866M	D3	50	15	62	139	29	267	145	17	271	no response	no response
	c.4789C>T	p.R1597W	A2											
2A	c.2936G>A	p.S979N	D3	36	15	70	72	59	176	n/a	n/a	n/a	complete	NA
1	c.2936G>A	p.S979N	D3	n/a	26	90	n/a	>100	300	n/a	n/a	n/a	complete	NA
2A	c.2936G>A	p.S979N	D3	n/a	18	44	n/a	>100	180	n/a	82	130	complete	complete
1	c.2936G>A	p.S979N	D3	n/a	12	30	n/a	>100	200	n/a	53	120	complete	complete
2N	c.3269_98del30	p.S1090_ C1099del	D3	n/a	23	16	n/a	>100	250	n/a	97	88	complete	complete
	c.2561G>A	p.R854Q	D′											
2M	c.3863T>G	p.L1288R	A1	n/a	<10	27	n/a	20	97	n/a	16	80	no response	no response
2M	c.3877T>C	p.F1293L	A1	34	5	43	>100	59	170	n/a	n/a	n/a	complete	NA
2M	c.4263C>A	p.N1421K	A1	57	7	63	n/a	46	170	n/a	36	140	partial	partial
2A	c.4549A>C	p.S1517R	A2	22	10	32	28	10	35	n/a	n/a	n/a	no response	NA
	5170+10C>T		A3											
2A	c.4717G>A	p.G1573S	A2	n/a	19	63	n/a	70	160	n/a	68	130	complete	complete
2A	c.4892G>A	p.G1631D	A2	42	<15	26	160	17	143	69	15	102	no response	no response
2A	c.5015G>C	p.G1672A	A2	17	<13	46	45	<13	89	n/a	n/a	n/a	no response	NA
	c.4994T>A	p.V1665E	A2											
1	c.6551G>A	p.C2184Y	D4	10	<15	49	14	19	103	n/a	n/a	n/a	no response	NA
1	c.6753G>A	p.C2248Y	D4	29	29	100	100	153	354	n/a	n/a	n/a	complete	NA

aa = amino acid, VWD = von Willebrand Disease, VWF:ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulant activity, VWF:FVIIIB = von Willebrand Factor binding to Factor VIII assay, BS = bleeding score, n/a = not available, NA = not applicable. * VWD type according to current ISTH guidelines. † levels measured at patients own haemophilia treatment center.

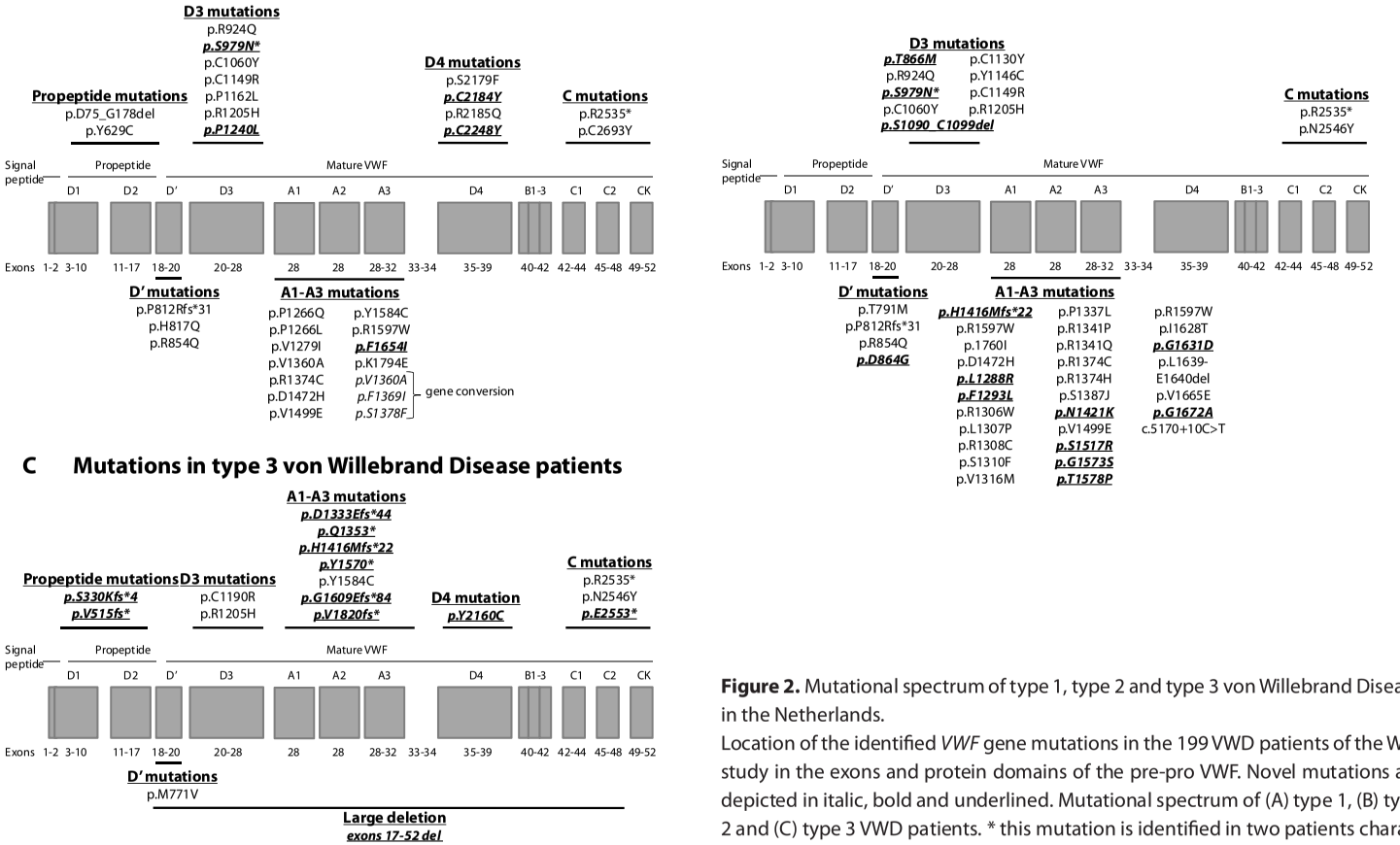


Figure 2. Mutational spectrum of type 1, type 2 and type 3 von Willebrand Disease in the Netherlands.
Location of the identified *VWF* gene mutations in the 199 VWD patients of the WiN study in the exons and protein domains of the pre-pro VWF. Novel mutations are depicted in italic, bold and underlined. Mutational spectrum of (A) type 1, (B) type 2 and (C) type 3 VWD patients. * this mutation is identified in two patients characterized as type 1 VWD and two patients as type 2A VWD

Table 5. Characteristics of von Willebrand Disease patients with a *VWF* gene mutation.

Characteristics	Type 1 VWD patients (n = 38)	Type 2 VWD patients (n = 110)	Type 3 VWD patients (n = 25)
Age (years), median (range)	38 (1-81)	35 (1-81)	17 (1-65)
Male sex, n (%)	20 (51)	59 (54)	14 (56)
Blood group O, n (%) *	25 (71)	53 (55)	13 (54)
VWF:Ag (IU/dL), median (IQR) †	26 [12-35]	28 [18-35]	0 [0-4]
VWF:CB (IU/dL), median (IQR) †	20 [9-40]	8 [5-19]	0 [0-2]
VWF:Act (IU/dL), median (IQR) †	26 [14-40]	11 [5-19]	0 [0-1]
FVIII:C (IU/dL), median (IQR) †	47 [26-60]	35 [23-47]	3 [1-9]
Bleeding score ‡	9.0 [5.5-13.5]	11.0 [6.0-16.0]	17.0 [9.3-20.8]

VWD = von Willebrand Disease, VWF:Ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulation activity. * Data available for n=156 (n=35 for type 1 VWD, n=97 for type 2 VWD, n=24 for type 3 VWD). † Centrally measured VWF and FVIII levels available for n=137 (n=34 for type 1 VWD, n=84 for type 2 VWD, n=19 for type 3 VWD) (blood samples obtained within 72 hours after use of desmopressin or factor concentrate or during pregnancy are excluded). ‡ Data available for n=160.

Difference between phenotypic and genotypic type of von Willebrand Disease

Nearly half of the patients who were originally classified as type 1 VWD based on VWF:Act/VWF:Ag ratio ≥0.7, had an abnormal multimer pattern (15/38; 39%) and had mutations that were previously reported in type 2 VWD, according to the EAHAD Coagulation Factor Variant database (https://grenada.lumc.nl/LOVD2/VWF/home.php; formerly ISTH-SSC VWF Online Database). The phenotypic type 1 patient homozygous for the previously described 2N mutation p.H817Q, had a FVIII:C/VWF:Ag ratio >0.70 and a VWF:FVIII:B of 0.69. Mutation p.V1360A has previously been described in a 2M patient, but was now found in a patient with a type 1 phenotype. One patient with p.V1499E had a VWF:Act/VWF:Ag ratio >0.70, and therefore classified as type 1 VWD. However, the VWF:CB was remarkably lower than VWF:Ag and VWF:Act, suggesting a type 2 phenotype. In contrast to 16 type 2A patients with p.R1597W, one patient with p.R1597W classified as type 1 VWD, because of a VWF:Act/Ag ratio >0.70. However, this patient had lower VWF:Ag levels than the others which may limit the reliability of the VWF:Act/VWF:Ag ratio. This patient also had a remarkably lower VWF:CB level than VWF:Ag and VWF:Act. Mutation p.R2185Q has previously been described in a phenotypic 2A patient, but was in our study cohort identified in three phenotypic type 1 patients. In addition, this mutation is found in exon 37, which is a region of the *VWF* gene in which so far no type 2 mutations have been identified. Mutation p.R924Q has been previously described in patients with a type 1 or 2N phenotype; we now found this mutation heterozygous in a type 2M patient. Three type 2 patients (2A, 2M and 2M) had mutation p.L1288R; in one of these patients the multimer pattern was abnormal and in two normal. According to the EAHAD Coagulation Factor Variant database these patients with normal multimers should be classified as type 2M patients. Three patients with p.R1308C and one with

p.P1337L did not classify as type 2B VWD, according to the current diagnostic criteria, as their RIPA test was normal.

Twenty-five patients were classified as type 3 VWD as their VWF:Ag and VWF:Act levels were below 5 IU/dL, irrespective of FVIII:C level. However, five of these patients were heterozygous for the "Vicenza" mutation (p.R1205H), which is a type 1 mutation characterized by accelerated clearance of VWF.[21] Three phenotypic type 2A patients were also heterozygous for this p.R1205H mutation, and should classify as type 1 VWD based on their genotype. Finally, in three type 3 patients only one mutation could be identified. One of these mutations have been linked to type 3 VWD before (p.R2535*).[22]

DISCUSSION

We evaluated genotype and phenotype in a cohort of 199 patients with moderate and severe VWD, with historically lowest VWF levels below 30 U/dL. A *VWF* gene mutation had been found in 63% of type 1 VWD patients, 99% of type 2 patients, and 89% of type 3 patients. We discovered 27 novel *VWF* mutations, mostly in type 2 and 3 VWD patients. Of these 27 novel mutations, sixteen were missense mutations, four deletions, one duplication, two insertions, three nonsense mutations and one large deletion of exons 17-52. This adds to the over 500 previously identified *VWF* gene mutations in VWD.[8-10] Characteristics vary per mutation, illustrating the genetic and clinical heterogeneity of all types of VWD.

In recent literature several *VWF* gene mutations have been associated with different VWD phenotypes.[13,23] These phenotype-genotype discrepancies are also observed in our study. We classified VWD according to the current ISTH guidelines.[1] According to this diagnostic algorithm, 472 patients from the WiN study classified as type 1 VWD. Only a minority (13%) of the type 1 patients in the WiN study were sequenced. A *VWF* mutation was identified in 63% of the patients, which is in accordance with international molecular epidemiological studies in type 1 VWD.[8,9,11] We found no clinical differences between these type 1 patients, but previous studies have shown higher VWF parameters in type 1 patients without a *VWF* mutation.[8,9]

In some phenotypic type 1 patients, we identified mutations that had previously been reported in type 2 VWD patients. We classified them as type 1 patients based on the VWF:Act/VWF:Ag ratio above 0.70.[1] However, the majority of these patients had abnormal multimers, which was also found in a considerable proportion of type 1 patients (38%) from the MCMDM-1VWD study.[8] Difficulties in the distinction between type 1, 2A and 2M VWD have been reported before.[8,9,11] Some phenotypic type 1 patients with mutations that were previously described in phenotypic type 2 patients had disproportionally low VWF:CB compared with VWF:Ag or VWF:Act. Currently, only the VWF:RCo/

VWF:Ag ratio is used to classify type 2 VWD, but from our current study it appears that the VWF:CB/VWF:Ag ratio may be used as an additional diagnostic criteria to classify type 2 VWD, as also suggested before.[24,25] We also show that VWF:Act/VWF:Ag ratio is less sensitive in patients with very low VWF:Ag levels, which support the importance of *VWF* gene mutation analysis in the classification of these patients.

Over 300 patients from the WiN study had a VWF:Act/VWF:Ag ratio <0.7 and therefore classified as type 2 VWD. In a third of these patients, the *VWF* gene was sequenced. Also subclassification of type 2 VWD can be difficult to perform correctly. Five patients with a typical 2B genotype (p.R1308C and p.P1337L) were classified as type 2A VWD based on the absence of hyperactive agglutination in the RIPA rest. The first paper that described genetic linkage with the *VWF* gene in a large type 2A VWD family, actually reported in a type 2B family as later on the p.R1308C mutation was identified in this family.[26] The extremely high consumption of VWF in these patients, which results in absence of high-molecular weight multimers and VWF activity, probably reduces the sensitivity of the RIPA test for the detection of hyperagglutination of the mutant VWF. Mutation analysis of the *VWF* gene is advisable in these patients for a clear diagnosis of 2B VWD.

Patients in the WiN study were defined as type 3 VWD if their VWF:Ag and VWF:Act levels were below 5 IU/dL, irrespective of FVIII:C level. According to these phenotypic diagnostic criteria,[1] five patients with p.R1205H were classified as type 3 VWD. However, this mutation is the well-known type 1 Vicenza mutation, in which VWF is normally synthesized, but rapidly cleared.[21] In the patients in our study the VWF levels were extremely low and even below 5 IU/dL and hence they were classified as type 3. This underlines the necessity to restrict the diagnosis of type 3 VWD to a recessive inheritance pattern, although this is not always clear and cannot always be distinguished from a *de novo* mutation. We also found this p.R1205H mutation in type 2 patients. Altogether, this mutation shows a poor correlation between phenotype and genotype.[16]

It is still debated if routine characterization of *VWF* gene mutations is required in clinical practice.[27,28] At the moment, the diagnosis VWD is still based on phenotypic testing, i.e. history and laboratory assays, and molecular analysis of the *VWF* gene is rarely performed in clinical practice.[1,12] Our study demonstrates the difficulty of diagnosing and classifying VWD on the basis of laboratory values alone. The above-mentioned discrepancy between laboratory based classification and genetic background of VWD, indicates that mutation analysis is important for the VWD classification. Expectedly, when the molecular biology of VWD is further unravelled and genetic analysis becomes easier available, the VWD classification will probably be revised. The development of newer sequencing techniques may add to this in the future.[29]

In 36% of our type 1 patients no causative mutation in the *VWF* gene could be detected. This can only partly be explained by the incomplete *VWF* gene sequencing. It may also imply that other genetic loci are involved in the regulation of VWF levels.[8,9] Re-

cently, a genome-wide association study has indeed identified new genetic loci that are associated with VWF levels in healthy individuals.[30] We and others have recently shown that genetic variations in the *STX2*, *CLEC4M* and *STXBP5* genes contribute to lower VWF levels in type 1 VWD patients.[31-33] Next generation sequencing, including whole exome sequencing, in those VWD patients without a causative *VWF* mutation may lead to the discovery of mutations in genes involved in the biosynthesis and clearance of VWF resulting in low levels of VWF.

The strengths of this study are that mutation analysis was performed in a large cohort of 199 types 1, 2 and 3 VWD patients, which is a large group of patients compared to previous studies,[10,34-36] and that 27 novel *VWF* gene mutations were identified in our study. Limitations are that mutation analysis was performed on selected patients and not in all patients from the WiN study. Furthermore, genetic analysis was performed in four different laboratories with slightly different protocols and in some patients only those exons in which the majority of *VWF* gene mutations are found, were sequenced for diagnostic purposes.

In conclusion, we observed 27 novel *VWF* gene mutations in our cohort of 199 patients with moderate and severe types 1, 2 and 3 VWD. Difficulties in classifying VWD patients remain, because of discrepancies between phenotype and genotype. Molecular analysis of the *VWF* gene provides important additional insight into the cause of VWD and should therefore be implemented to improve the classification of VWD.

REFERENCES

1. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
2. Ruggeri ZM. Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation. *Best Pract Res Clin Haematol*. 2001;14(2):257-279.
3. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108(4):683-692.
4. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost*. 2006;4(4):766-773.
5. Mancuso DJ, Tuley EA, Westfield LA, et al. Structure of the gene for human von Willebrand factor. *J Biol Chem*. 1989;264(33):19514-19527.
6. Verweij CL, Diergaarde PJ, Hart M, Pannekoek H. Full-length von Willebrand factor (vWF) cDNA encodes a highly repetitive protein considerably larger than the mature vWF subunit. *EMBO J*. 1986; 5(8):1839-1847.
7. Zhou YF, Eng ET, Zhu J, Lu C, Walz T, Springer TA. Sequence and structure relationships within von Willebrand factor. *Blood*. 2012;120(2):449-458.
8. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*. 2007;109(1):112-121.
9. James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. *Blood*. 2007;109(1):145-154.
10. Yadegari H, Driesen J, Pavlova A, Biswas A, Hertfelder HJ, Oldenburg J. Mutation distribution in the von Willebrand factor gene related to the different von Willebrand disease (VWD) types in a cohort of VWD patients. *Thromb Haemost*. 2012;108(4):662-671.
11. Cumming A, Grundy P, Keeney S, et al. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb Haemost*. 2006;96(5):630-641.
12. de Wee EM, Leebeek FW, Eikenboom JC. Diagnosis and management of von Willebrand disease in The Netherlands. *Semin Thromb Hemost*. 2011;37(5):480-487.
13. Castaman G, Goodeve A, Eikenboom J, European Group on von Willebrand Disease. Principles of care for the diagnosis and treatment of von Willebrand disease. *Haematologica*. 2013;98(5):667-674.
14. Sanders YV, Eikenboom J, de Wee EM, et al. Reduced prevalence of arterial thrombosis in von Willebrand disease. *J Thromb Haemost*. 2013;11(5):845-854.
15. Tosetto A, Castaman G, Rodeghiero F. Bleeding scores in inherited bleeding disorders: clinical or research tools? *Haemophilia*. 2008;14(3):415-422.
16. Budde U, Schneppenheim R, Eikenboom J, et al. Detailed von Willebrand factor multimer analysis in patients with von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 von Willebrand disease (MCMDM-1VWD). *J Thromb Haemost*. 2008;6(5):762-771.
17. Landsteiner K. On agglutination of normal human blood. *Transfusion*. 1961;1:5-8.
18. Pare G, Chasman DI, Kellogg M, et al. Novel association of ABO histo-blood group antigen with soluble ICAM-1: results of a genome-wide association study of 6,578 women. *PLoS Genet*. 2008;4(7): e1000118.

19. Stoof SC, Sanders YV, Petrij F, et al. Response to desmopressin is strongly dependent on F8 gene mutation type in mild and moderate haemophilia A. *Thromb Haemost*. 2013;109(3):440-449.
20. Castaman G, Lethagen S, Federici AB, et al. Response to desmopressin is influenced by the genotype and phenotype in type 1 von Willebrand disease (VWD): results from the European Study MCMDM-1VWD. *Blood*. 2008;111(7):3531-3539.
21. Schneppenheim R, Federici AB, Budde U, et al. Von Willebrand Disease type 2M "Vicenza" in Italian and German patients: identification of the first candidate mutation (G3864A; R1205H) in 8 families. *Thromb Haemost*. 2000;83(1):136-140.
22. Eikenboom JC, Ploos van Amstel HK, Reitsma PH, Briet E. Mutations in severe, type III von Willebrand's disease in the Dutch population: candidate missense and nonsense mutations associated with reduced levels of von Willebrand factor messenger RNA. *Thromb Haemost*. 1992;68(4):448-454.
23. Costa-Pinto J, Perez-Rodriguez A, del CG-d-CM, et al. Diagnosis of inherited von Willebrand disease: comparison of two methodologies and analysis of the discrepancies. *Haemophilia*. 2014;20(4): 559-567.
24. Favaloro EJ. Toward a new paradigm for the identification and functional characterization of von Willebrand disease. *Semin Thromb Hemost*. 2009;35(1):60-75.
25. Laffan MA, Lester W, O'Donnell JS, et al. The diagnosis and management of von Willebrand disease: a United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology. *Br J Haematol*. 2014;167(4):453-465.
26. Verweij CL, Quadt R, Briet E, Dubbeldam K, van Ommen GB, Pannekoek H. Genetic linkage of two intragenic restriction fragment length polymorphisms with von Willebrand's disease type IIA. Evidence for a defect in the von Willebrand factor gene. *J Clin Invest*. 1988;81(4):1116-1121.
27. Peake IR, Goodeve AC. Genetic testing for von Willebrand disease: the case for. *J Thromb Haemost*. 2010;8(1):13-16.
28. Favaloro EJ. Genetic testing for von Willebrand disease: the case against. *J Thromb Haemost*. 2010; 8(1):6-12.
29. Corrales I, Catarino S, Ayats J, et al. High-throughput molecular diagnosis of von Willebrand disease by next generation sequencing methods. *Haematologica*. 2012;97(7):1003-1007.
30. Smith NL, Chen MH, Dehghan A, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*. 2010;121(12):1382-1392.
31. van Loon JE, Sanders YV, de Wee EM, Kruip MJ, de Maat MP, Leebeek FW. Effect of Genetic Variation in STXBP5 and STX2 on von Willebrand Factor and Bleeding Phenotype in Type 1 von Willebrand Disease Patients. *PLoS One*. 2012;7(7):e40624.
32. Rydz N, Swystun LL, Notley C, et al. The C-type lectin receptor CLEC4M binds, internalizes, and clears von Willebrand factor and contributes to the variation in plasma von Willebrand factor levels. *Blood*. 2013;121(26):5228-5237.
33. Sanders YV, van der Bom JG, Isaacs A, et al. CLEC4M and STXBP5 gene variation contribute to von Willebrand factor level variation in von Willebrand disease. *J Thromb Haemost*. 2015;13(6):965-966.
34. Ahmad F, Jan R, Kannan M, et al. Characterisation of mutations and molecular studies of type 2 von Willebrand disease. *Thromb Haemost*. 2013;109(1):39-46.
35. Ahmad F, Budde U, Jan R, et al. Phenotypic and molecular characterisation of type 3 von Willebrand disease in a cohort of Indian patients. *Thromb Haemost*. 2013;109(4):652-660.
36. Hampshire DJ, Abuzenadah AM, Cartwright A, et al. Identification and characterisation of mutations associated with von Willebrand disease in a Turkish patient cohort. *Thromb Haemost*. 2013;110(2): 264-274.

Supplemental table 1. Genotype and phenotype of type 1 von Willebrand Disease patients (n=38).

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	aa exchange	domain	exon	type of mutation	novel	type based on genotype	BS	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF:Act/ VWF:Ag	multimers
1	1	1	compound heterozygous	c.221-977_532+7059del	p.D75_G178del	D1	4-5	deletion	no	1, 2N	18	24†	n/a	42†	26†	≥0.7	norm†
				c.2561G>A	p.R854Q	D′	20	missense	no								
1	1	1	compound heterozygous	c.1886A>G	p.Y629C	D2	15	missense	no	1, 2N	16	14	19	13	14	≥0.7	norm
				c.2561G>A	p.R854Q	D′	20	missense	no								
1	2	1	heterozygous	c.2435delC	p.P812Rfs*31	D′	18	nonsense	no	1	2-6	18-31	23-48	17-39	53-57	≥0.7	norm
1	1	1	compound heterozygous	c.2451T>A	p.H817Q	D′	19	missense	no	1, 2N	22	54	63	60	54	≥0.7	norm
				c.3485_6delinsTG	p.P1162L	D3	26	missense	no								
1	1	1	homozygous	c.2451T>A	p.H817Q	D′	19	missense	no	2N	4	40	45	49	37	≥0.7	norm
3	4	1	heterozygous	c.2561G>A	p.R854Q	D′	20	missense	no	1, 2N	6-12	26-68	14-84	22-82	35-86	≥0.7	abnorm/ norm
2	2	1	heterozygous	c.2771G>A	p.R924Q	D3	21	missense	no	1, 2N	3	13-27	9-11	17-31	26-52	≥0.7	norm/ abnorm
1	2	1	heterozygous	c.2936G>A	p.S979N	D3	22	missense	no	1, 2A	7-13	28-33	20-21	21-29	68-83	≥0.7	abnorm
1	1	1	heterozygous	c.3179G>A	p.C1060Y	D3	24	missense	no	1, 2N	6	23	28	29	36	≥0.7	abnorm
1	1	1	heterozygous	c.3445T>C	p.C1149R	D3	26	missense	no	1, 2A	n/a	10	7	7	20	≥0.7	abnorm
1	1	1	heterozygous	c.3614G>A	p.R1205H	D3	27	missense	no	1 vicenza	15	4	4	13	14	≥0.7	abnorm
1	1	1	>2	c.3614G>A	p.R1205H	D3	27	missense	no	1, 2M	4	19†	n/a	24†	52†	≥0.7	norm†
				c.3719C>T	p.P1240L	D3	28	missense	no								
				c.3797C>A	p.P1266Q	A1	28	missense	no								
1	1	1	heterozygous	c.3797C>T	p.P1266L	A1	28	missense	no	1, 2B, 2M	3	34	31	35	46	≥0.7	norm
1	1	1	compound heterozygous	c.3797C>T	p.P1266L	A1	28	missense	no	1, 2B, 2M	12	21	17	20	70	≥0.7	abnorm
				c.3853G>A	p.V1279I	A1	28	missense	no								
1	1	1	heterozygous	c.4079T>C	p.V1360A	A1	28	missense	no	2M	3	51	78	84	87	≥0.7	norm

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	aa exchange	domain	exon	type of mutation	novel	type based on genotype	BS	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF:Act/ VWF:Ag	multimers
1	1	1	compound heterozygous	c.4120C>T	p.R1374C	A1	28	missense	no	1	12	10	8	15	29	≥0.7	abnorm
				4079T>C (V1360A); 4105T>A (F1369I); 4133C>T (S1378F)				pseudogene conversion	no								
1	1	1	heterozygous	c.4414G>C	p.D1472H	A1	28	missense	no		7	28	38	32	56	≥0.7	norm
1	1	1	heterozygous	c.4496T>A	p.V1499E	A2	28	missense	no	2A	11	49	24	44	68	≥0.7	abnorm
2	2	1	heterozygous	c.4751A>G	p.Y1584C	A2	28	missense	no	1	8	10	5	16	29	≥0.7	norm
1	1	1	heterozygous	c.4789C>T	p.R1597W	A2	28	missense	no	2A	15	17	4	13	19	≥0.7	abnorm
1	1	1	heterozygous	c.4960T>A	p.F1654I	A2	28	missense	yes	1	9	10	9	16	34	≥0.7	abnorm
1	1	1	heterozygous	c.5380A>G	p.K1794E	A3	31	missense	no	1	21	12	11	9	26	≥0.7	norm
1	1	1	heterozygous	exons 33-34 del			33-34	deletion	no	2	7	14	9	11	26	≥0.7	abnorm
1	1	1	heterozygous	c.6536C>T	p.S2179F	D4	37	missense	no	1	8	6	7	14	20	≥0.7	abnorm
1	1	1	heterozygous	c.6551G>A	p.C2184Y	D4	37	missense	yes	1	9	10†	n/a	7†	49†	≥0.7	norm†
1	3	1	heterozygous	c.6554G>A	p.R2185Q	D4	37	missense	no	2A	5-13	19-45	18-85	31-42	48-55	≥0.7	norm
1	1	1	heterozygous	c.6753G>A	p.C2248Y	D4	38	missense	yes	1	14	27	32	34	71	≥0.7	norm
1	1	1	heterozygous	c.7603C>T	p.R2535*	C4	45	nonsense	no	1	23	6	3	6	52	≥0.7	abnorm
1	1	1	heterozygous	c.8078G>A	p.C2693Y	C6	49	missense	no	1	4	37	39	41	71	≥0.7	norm

no. = number, aa = amino acid, VWD = von Willebrand Disease, BS = bleeding score, VWF:Ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulation activity, norm = normal, abnorm = abnormal, n/a = not available. * VWD type according to current ISTH guidelines. † measured at patients own haemophilia treatment center.

Supplemental table 2. Genotype and phenotype of type 2 von Willebrand Disease patients (n=110).

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	mutation protein	domain	exon	type of mutation	novel	type based on genotype	BS	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF:Act/ VWF:Ag	VWF: FVIIIB	multimers
1	1	2N	compound heterozy	c.2372C>T	p.T791M	D′	18	missense	no	2N	11	29	40	62	6	≥0.7	0.06	norm
				c.2435delC	p.P812Rfs*31	D′	18	nonsense	no									
1	1	2N	heterozy	c.2561G>A	p.R854Q	D′	20	missense	no	2N	9	25	34	24	16	≥0.7	0.33	norm
				no other mutations found in *VWF* gene														
1	1	2N	>2	c.2561G>A	p.R854Q	D′	20	missense	no	2N	11	27†	n/a	23†	12†	≥0.7	0.10†	abnorm†
				c.2561G>A	p.R854Q	D′	20	missense	no									
				c.3269_ 98del30	p.S1090_ C1099del	D3	25	deletion	yes									
1	1	2N	compound heterozy	c.2561G>A	p.R854Q	D′	20	missense	no	2N	8	44	57	51	12	≥0.7	0.31	norm
				c.3179G>A	p.C1060Y	D3	24	missense	No									
1	1	2N	compound heterozy	c.2561G>A	p.R854Q	D′	20	missense	no	2N	5	19	26	25	12	≥0.7	0.41	norm
				c.4251delG	p.H1416 Mfs*22	A1	28	nonsense	yes									
3	4	2N	compound heterozy	c.2561G>A	p.R854Q	D′	20	missense	no	2N	7-30	31-56	45-72	45-65	22-27	≥0.7	0.28-0.39	norm
				c.7603C>T	p.R2535*	C4	45	nonsense	no									
1	1	2N	compound heterozy	c.2561G>A	p.R854Q	D′	20	missense	no	2N	11	17	24	27	13	≥0.7	0.39	norm
				c.7636A>T	p.N2546Y	C4	45	missense	no									
1	1	2N	homozygous	c.2561G>A	p.R854Q	D′	20	missense	no	2N	8	37	50	45	16	≥0.7	0.38	norm
1	1	2A	compound heterozy	c.2597C>T	p.T866M	D3	20	missense	yes	2A	16	31	9	17	65	<0.7		abnorm
				c.4789C>T	p.R1597W	A2	28	missense	no									
1	1	2M	heterozy	c.2771G>A	p.R924Q	D3	21	missense	no	1, 2N	2	20†	n/a	10†	59†	<0.7		norm†
1	2	2A	heterozy	c.2936G>A	p.S979N	D3	22	missense	yes	1, 2A	2-21	16-33	12	11-15	31-32	<0.7		abnorm

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	mutation protein	domain	exon	type of mutation	novel	type based on genotype	BS	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF:Act/ VWF:Ag	VWF: FVIIIB	multimers
1	1	2N	heterozy	c.3179G>A	p.C1060Y	D3	24	missense	no	2N	0	30	29	29	32	≥0.7	0.48	norm
				no other mutations found in *VWF* gene														
1	1	2N	compound heterozy	c.3179G>A	p.C1060Y	D3	24	missense	no	2N	11	44	44	41	47	≥0.7	0.47	norm
				c.5278G>A	p.V1760I	A3	30	missense	no									
1	1	2N	homozygous	c.3179G>A	p.C1060Y	D3	24	missense	no	2N	11	30	43	29	18	≥0.7	0.35	norm
1	1	2A	compound heterozy	c.3389G>A	p.C1130Y	D3	26	missense	no	1, 2A	1	21	8	12	32	<0.7		abnorm
				c.4414G>C	p.D1472H	A1	28	missense	no									
1	1	2A	heterozy	c.3569G>A	p.C1190Y	D3	27	missense	no	2A	10	72	23	24	63	<0.7		abnorm
1	2	2A	heterozy	c.3437A>G	p.Y1146C	D3	26	missense	no	1, 2A	9-11	18-41	0-19	7-12	23-36	<0.7		abnorm
3	4	2A	heterozy	c.3445T>C	p.C1149R	D3	26	missense	no	2A	5-14	8-17	6-10	4-5	16-28	<0.7		abnorm
1	1	2A	heterozy	c.3614G>A	p.R1205H	D3	27	missense	no	1	16	6	4	0	11	<0.7		abnorm
1	1	2A	compound heterozy	c.3614G>A	p.R1205H	D3	27	missense	no	1	3	15†	n/a	7†	11†	<0.7		abnorm†
				c.2591A>G	p.D864G	D′	20	missense	yes									
1	1	2A	compound heterozy	c.3614G>A	p.R1205H	D3	27	missense	no	1	24	9	5	0	11	<0.7		abnorm
				c.2771G>A	p.R924Q	D3	21	missense	no									
1	3	2A, 2M	heterozy	c.3863T>G	p.L1288R	A1	28	missense	yes	2M	3-10	12-25	29	4-16	26-68	<0.7		abnorm/ norm
1	2	2M	heterozy	c.3877T>C	p.F1293L	A1	28	missense	yes	2M	2-13	29-35	42	4-10	43-62	<0.7		norm
7	16	2B	heterozy	c.3916C>T	p.R1306W	A1	28	missense	no	2B	6-26	13-52	3-22	4-18	15-62	<0.7		abnorm
2	4	2A	heterozy	c.3920T>C	p.L1307P	A1	28	missense	no	2A	9-23	7-16	2-12	0-4	19-52	<0.7		abnorm
3	11	2A, 2B	heterozy	c.3922C>T	p.R1308C	A1	28	missense	no	2B	1-31	19-48	2-9	0-19	28-64	<0.7		abnorm
1	1	2B	heterozy	c.3929T>C	p.S1310F	A1	28	missense	no	2B	10	55	25	35	50	<0.7		abnorm
2	2	2B	heterozy	c.3946G>A	p.V1316M	A1	28	missense	no	2B	26	44-62	8-18	12-16	58-98	<0.7		abnorm
1	1	2A	heterozy	c.4010C>T	p.P1337L	A1	28	missense	no	2B	8	33	18	17	63	<0.7		abnorm

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	mutation protein	domain	exon	type of mutation	novel	type based on genotype	BS	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	VWF:Act/ VWF:Ag	VWF: FVIIIB	multimers
1	1	2B	heterozy	c.4022G>C	p.R1341P	A1	28	missense	no	2B	11	17	4	0	24	<0.7		abnorm
1	1	2B	heterozy	c.4022G>A	p.R1341Q	A1	28	missense	no	2B	6	33	20	16	48	<0.7		abnorm
2	2	2A, 2M	heterozy	c.4120C>T	p.R1374C	A1	28	missense	no	1, 2A, 2M	2,22	13	8	2	17	<0.7		abnorm
3	5	2A, 2M	heterozy	c.4121G>A	p.R1374H	A1	28	missense	no	2A	9-17	6-21	3-12	0-4	15-39	<0.7		abnorm/ norm
1	1	2M	compound heterozy	c.4121G>A	p.R1374H	A1	28	missense	no	1, 2A	8	30	23	16	44	<0.7		norm
				c.2771G>A	p.R924Q	D3	21	missense	no									
1	1	2M	heterozy	c.4160G>T	p.S1387J	A1	28	missense	no	2M	6	37†	n/a	9†	61†	<0.7		norm†
1	1	2M	heterozy	c.4263C>A	p.N1421K	A1	28	missense	yes	2M	14	30	27	8	61	<0.7		norm
1	6	2A	heterozy	c.4496T>A	p.V1499E	A2	28	missense	no	2A	0-12	22-32	5-14	9-21	38-59	<0.7		abnorm
1	1	2A	compound heterozy	c.4549A>C	p.S1517R	A2	28	missense	yes	2A	8	22†	n/a	4†	32†	<0.7		abnorm†
				c.5170+10C>T			29	splice	no									
1	1	2A	heterozy	c.4717G>A	p.G1573S	A2	28	missense	yes	2A	9	51†	27†	19†	67†	<0.7		abnorm†
1	1	2A	heterozy	c.4732A>C	p.T1578P	A2	28	missense	yes	2A	15	22	2	5	16	<0.7		abnorm
6	16	2A	heterozy	c.4789C>T	p.R1597W	A2	28	missense	no	2A	2-27	18-59	3-15	8-28	22-49	<0.7		abnorm
1	1	2A	heterozy	c.4883T>C	p.I1628T	A2	28	missense	no	2A	10	28	4	14	37	<0.7		abnorm
1	1	2A	heterozy	c.4892G>A	p.G1631D	A2	28	missense	yes	2A	15	36	6	23	43	<0.7		abnorm
1	1	2A	heterozy	c.4912delN	p.L1639-E1640del	A2	28	deletion	no	2A	28	21	5	9	33	<0.7		abnorm
1	1	2A	compound heterozy	c.4994T>A	p.V1665E	A2	28	missense	no	2A	13	17†	n/a	4†	44†	<0.7		abnorm†
				c.5015G>C	p.G1672A	A2	28	missense	yes									

no. = number; aa = amino acid; VWD = von Willebrand Disease; BS = bleeding score; VWF:Ag = von Willebrand Factor antigen; VWF:CB = von Willebrand Factor collagen binding; VWF:Act = von Willebrand Factor activity; FVIII:C = factor VIII coagulation activity; VWF:FVIIIB = von Willebrand Factor binding to Factor VIII assay; heterozy = heterozygous; norm = normal; abnorm = abnormal; n/a = not available; * VWD type according to current ISTH guidelines; † measured at patients own haemophilia treatment center

Supplemental table 3. Genotype and phenotype of type 3 von Willebrand Disease patients (n=25).

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	mutation protein	domain	exon	type of mutation	novel mutation	type based on genotype	BS	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	multimers
1	2	3†	homozygous	c.988dupA	p.S330Kfs*4	D1	8	nonsense	yes	3	9 - 12	0-3†	0	0-2†	1-3†	absent†
1	1	3	homozygous	c.1543_44ins8	p.V515fs*	D2	14	nonsense	yes	3	16	0	0	0	1	absent
1	1	3	homozygous	exons 17-52 del		D2-CK	17-52	large deletion	yes	3	29	0	0	0	1	absent
2	2	3	homozygous	c.2311A>G	p.M771V	D'	18	missense	no	3	n/a	4	2	0	2-3	absent
1	1	3	heterozygous	c.3568T>C	p.C1190R	D3	27	missense	no		17	6‡	6‡	1‡	29‡	abnorm
				no other mutations found in the *VWF* gene												
3	5	3	heterozygous	c.3614G>A	p.R1205H	D3	27	missense	no	1	1-17	4	2-4	0-7	8-16	abnorm
1	2	3	homozygous	c.3998_9insA	p.D1333Efs*44	A1	28	nonsense	yes	3	9-21	0	0	0	2	absent
1	1	3	compound heterozygous	c.4251delG	p.H1416Mfs*22	A1	28	nonsense	yes	3	20	17‡	8‡	8‡	52‡	abnorm
				c.5458delG	p.V1820fs*	A3	32	nonsense	yes							
1	1	3	compound heterozygous	c.4710C>A	p.Y1570*	A2	28	nonsense	yes	3	7	0	0	0	1	absent
				c.4826delG	p.G1609Efs*84	A2	28	nonsense	yes							
1	1	3†	compound heterozygous	c.4057C>T	p.Q1353*	A1	28	missense	no	3	10	5†	n/a	4†	3†	absent†
				c.4751A>G	p.Y1584C	A2	28	nonsense	yes							
1	1	3	>2	c.4751A>G	p.Y1584C	A2	28	missense	no	3	10	3	1	0	15	abnorm
				c.6479A>G	p.Y2160C	D4	37	missense	yes							
				c.7636A>T	p.N2546Y	C4	45	missense	no							
1	1	3	homozygous	c.7603C>T	p.R2535*	C4	45	nonsense	no	3	24	0	0	0	1	absent
1	1	3	heterozygous	c.7603C>T	p.R2535*	C4	45	nonsense	no		29	2	0	0	9	abnorm
				no other mutations found in the *VWF* gene												

no. of families	no. of patients	type of VWD*	no. of mutations	nucleotide exchange	mutation protein	domain	exon	type of mutation	novel mutation	type based on genotype	BS	VWF:Ag IU/dL	VWF:CB IU/dL	VWF:Act IU/dL	FVIII:C IU/dL	multimers
1	1	3	compound heterozygous	c.7603C>T	p.R2535*	C4	45	nonsense	no	3	17	0	0	0	1	absent
				c.7636A>T	p.N2546Y	C4	45	missense	no							
2	3	3	homozygous	c.7636A>T	p.N2546Y	C4	45	missense	no	3	20-24	0-3	0-3	0-2	0-3	absent
1	1	3	heterozygous	c.7657G>T	p.E2553*	C4	45	nonsense	yes		19	0	0	2	1	absent
				no other mutations found in the *VWF* gene												

no. = number, aa = amino acid, VWD = von Willebrand Disease, BS = bleeding score, VWF:Ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulation activity, norm = normal, abnorm = abnormal, n/a = not available. * VWD type according to current ISTH guidelines. † measured at patients own haemophilia treatment center. ‡ blood sampled within 72 hours after use of replacement therapy.

3

Effect of genetic variation in *STXBP5* and *STX2* on von Willebrand Factor and bleeding phenotype in type 1 von Willebrand Disease patients

JE van Loon, YV Sanders, EM de Wee, MJHA Kruip,
MPM de Maat, FWG Leebeek

PloS ONE. 2012;7:e40624

ABSTRACT

Background: In type 1 von Willebrand Disease (VWD) patients, von Willebrand Factor (VWF) levels and bleeding symptoms are highly variable. Recently, the association between genetic variations in *STXBP5* and *STX2* with VWF levels has been discovered in the general population. We assessed the relationship between genetic variations in *STXBP5* and *STX2*, VWF levels, and bleeding phenotype in type 1 VWD patients.

Methods: In 158 patients diagnosed with type 1 VWD according to the current ISTH guidelines, we genotyped three tagging-SNPs in *STXBP5* and *STX2* and analyzed their relationship with VWF:Ag levels and the severity of the bleeding phenotype, as assessed by the Tosetto bleeding score.

Results: In *STX2*, rs7978987 was significantly associated with VWF:Ag levels (bèta-coefficient (β) -0.04 IU/mL per allele; 95% confidence interval (CI) -0.07 to -0.001, p=0.04) and VWF:CB activity (β -0.12 IU/mL per allele; CI -0.17 to -0.06, p<0.0001). For rs1039084 in *STXBP5* a similar trend with VWF:Ag levels was observed: (β -0.03 IU/mL per allele; CI -0.06 to 0.003, p=0.07). In women, homozygous carriers of the minor alleles of both SNPs in *STXBP5* had a significantly higher bleeding score than homozygous carriers of the major alleles. (rs1039084 p=0.01 and rs9399599 p=0.02).

Conclusions: Genetic variation in *STX2* is associated with VWF:Ag levels in patients diagnosed with type 1 VWD. In addition, genetic variation in *STXBP5* is associated with bleeding phenotype in female VWD patients. Our findings may partly explain the variable VWF levels and bleeding phenotype in type 1 VWD patients.

INTRODUCTION

Von Willebrand Factor (VWF) is a multifunctional glycoprotein, which is involved in platelet adhesion and subsequent platelet aggregation during primary hemostasis.[1,2] Low VWF levels are a diagnostic criterion for von Willebrand Disease (VWD), the most common inherited bleeding disorder. VWD is classified as either a quantitative deficiency of VWF (type 1 and 3 VWD) or as a qualitative defect of VWF molecules (type 2 VWD). Furthermore, according to the current ISTH guidelines diagnosis of VWD is based on clinical criteria, including a mucocutaneous bleeding history and a family history of excessive bleeding.[3]

In type 1 VWD patients, who have reduced but functionally normal VWF molecules, VWF levels are highly variable. Also, the VWD phenotype penetrates incompletely leading to a variable clinical presentation. To date, we can only explain part of the variation in VWF levels and bleeding symptoms in these patients.

In recent years, genome-wide association studies have been performed and have enabled us to investigate the genetic component of common diseases and quantitative traits without a prior biological hypothesis. In this way, novel genetic determinants of VWF:Ag levels have been discovered: *STXBP5*, *SCARA5*, *STAB2*, *STX2*, *TC2N*, and *CLEC4M*.[4] Our interest was especially focussed on STXBP5 and STX2, because their encoding proteins are suggested to be involved in WPB exocytosis.[5]

Syntaxin 2 (*STX2*) is a binding substrate for the Syntaxin Binding Protein 5 (*STXBP5*) and is a member of the Soluble N-ethylmaleimide-sensitive factor (NSF) Attachment protein Receptor (SNARE) protein family. These proteins drive vesicle exocytosis by fusion of granules and target membranes, a process involved in the regulation of numerous secretory events.[6] STXBP5 and STX2 interact specifically with SNARE complex proteins, such as SNAP23 and syntaxin-4. These proteins have been shown to be involved in Weibel-Palade Body (WPB) exocytosis, the well-known mechanism for the secretion of VWF molecules by endothelial cells.[5]

Dysfunction of the WPB machinery is a likely contributor to the variation in VWF:Ag levels in type 1 VWD patients. Also, since we have recently confirmed the association between genetic variation in *STXBP5* and *STX2* and VWF:Ag levels in young patients with a first event of arterial thrombosis,[7] we hypothesized that genetic variation in *STXBP5* and *STX2* may also affect VWF:Ag levels in patients diagnosed with type 1 VWD according to the current ISTH guidelines. In addition, genetic variation in *STXBP5* and *STX2* may, by regulating VWF:Ag levels, influence the incomplete penetrance and the variable clinical presentation of type 1 VWD. Therefore, we aimed to assess the relationship between genetic variation in *STXBP5* and *STX2*, VWF:Ag levels, and the bleeding phenotype in patients previously diagnosed with type 1 VWD.

METHODS

Study population

In this study we included 158 patients, who were previously diagnosed with type 1 VWD in the Erasmus University Medical Center Rotterdam. The diagnosis of type 1 VWD was based on clinical bleeding symptoms, a family history of bleeding, and VWF:Ag levels or VWF:RCo activity ≤0.30 IU/mL, according to the current ISTH guidelines.[3]

Ethics statement

The study was approved by the medical research board at Erasmus University Medical Center and written informed consent was obtained from all participants at inclusion.

Laboratory measurements

Blood was drawn by venipuncture in the antecubital vein using Vacutainer system (Becton-Dickinson, Plymouth, UK). Blood for coagulation measurements was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within 1 hour at 2,000 x g for 10 minutes at 4°C. Plasma was additionally centrifuged at 14,000 x g for 10 minutes at 4°C and stored in aliquots at −80°C. For DNA isolation blood was collected in tubes containing ethylene diaminetetraacetic acid (EDTA; Beckon Dickinson) and genomic DNA was isolated according to standard salting-out procedures and stored at 4°C for genetic analysis.

VWF:Ag was determined with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase conjugated anti-human VWF antibodies (DakoCytomation, Glostrup, Denmark) for catching and tagging, respectively. For our analyses we used the lowest VWF:Ag level that was ever measured in a patient (historical VWF:Ag measurement). VWF collagen binding (VWF:CB) was measured with an in-house ELISA using type I collagen (Sigma, St. Louis, USA) for catching and horseradish peroxidase conjugated anti-human VWF antibodies for tagging. VWF:RCo was assayed with formalin-fixed platelets using a PAP4 Model Aggregometer (Bio-Data Corp. Hatboro, Pennsylvania) according to Macfarlane et al.[8]

Bleeding score

The Bleeding Score was used as previously described for bleeding severity in type 1 VWD by Tosetto et al.[9] It systematically evaluates bleeding symptoms, and accounts for both the number and severity of the bleeding symptoms. Each patient completed a questionnaire, which included the Bleeding Score assessment tool. The severity and frequency of twelve items are scored on a scale ranging from minus one to four points. Higher scores reflect a more severe bleeding phenotype characterized by more severe or more frequent bleeding. The total for all twelve items result in a Bleeding Score (range –3 to 45).

Genotyping analysis

The *STXBP5* gene spans 182 kbps and is located in the q24 region of chromosome 6. The *STX2* gene spans 50 kbp and is located in the q24.3 region of chromosome 12. Initially, we obtained data from the International HapMap project (phase II November 2008 http://www.hapmap.org) on the linkage disequilibrium (LD) pattern and selected haplotype-tagging single-nucleotide polymorphisms (ht-SNPs) using Haploview software (version 3.11; www.broad.mit.edu/mpg/haploview/index/php). For both genes blocks of haplotypes with a frequency of ≥3% were defined in order to select ht-SNPs. We took potential functionality into consideration by preferentially selecting non-synonymous ht-SNPs or SNPs that are located in known regulatory elements. We considered only SNPs that were present in a Caucasian population. Of these ht-SNPs, three were significantly associated with VWF:Ag levels in our previous study among young patients with arterial thrombosis and healthy controls.[7] Therefore, we selected and genotyped only these three SNPs in *STXBP5* (rs1039084 and rs9399599) and in *STX2* (rs7978987) for our current study using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The polymorphisms in *STXBP5*, rs1039084 and rs9399599, are in high linkage disequilibrium with rs9390459, which had the highest genome wide significance level for VWF plasma levels in the meta-analysis of the CHARGE consortium (D' = 1.00, R^2 = 0.87 for rs9399599 and D'= 0.96, R^2 = 0.86 for rs1039084) (phase II November 2008 http://www.hapmap.org). Also, rs7978987 in *STX2* had a highly significant *P* value of 3.82×10^{-11} in this meta-analysis. Endpoint fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, CA, USA) and clustered according to genotype using SDS 2.1 software (Applied Biosystems, Foster City, CA, USA). Genotyping was successful for each SNP in on average 96% of all subjects.

Statistical analysis

Allele frequencies were calculated by genotype counting. For each SNP the deviation from the Hardy-Weinberg equilibrium was tested by means of a Chi-squared test with one degree of freedom. VWF:Ag, VWF:RCo, and VWF:CB levels per genotype of each SNP were assessed by analysis of covariance (ANCOVA) adjusted for age and sex. Homozygous carriers of the VWF:Ag increasing allele were used as reference category. In addition, to analyze the trend across genotypes we performed linear regression analysis on VWF measures using the genotypes of each SNP as a continuous variable under the assumption of an additive genetic model (i.e. alleles have no dominant or recessive effect). To investigate the effect of ABO blood group on this association, the latter analysis was additionally adjusted for blood group (O and non-O).

The effect of genetic variation on the bleeding score has been assessed by ANCOVA adjusted for the age at the time of completing the questionnaire. Since the bleeding

score differs significantly between men and women, we stratified this analysis by sex additionally.

Statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS Inc, Chicago, USA). A two-sided value of $p<0.05$ was considered statistically significant.

RESULTS

Baseline characteristics

Baseline characteristics of the study population are displayed in table 1. We included 158 subjects diagnosed with type 1 VWD, who had a mean historical VWF:Ag level of 0.35 ± 0.13 IU/mL (mean ± standard deviation), VWF:CB of 0.29 ± 0.13 IU/mL, and VWF:RCo of 0.27 ± 0.14 IU/mL. The mean age at inclusion was 46.0 ± 16.8 years and 100 patients (63.3%) were female. Blood group O was the most frequent among these patients (78.5%). The allele frequency distributions of the genetic polymorphisms did not deviate from the Hardy-Weinberg equilibrium.

Table 1. Baseline characteristics of the study population.

	Type 1 VWD patients (n = 158)	Males (n = 58)	Females (n = 100)	p-value
Age (years)	46.0 ± 16.8	42.9 ± 19.4	47.8 ± 14.9	0.08
Female sex, n (%)	100 (63.3)	-	-	
Blood group O, n (%)	124 (78.5)	46 (79.3)	78 (78.0)	0.85
Bleeding score (points)	11 ± 7	8 ± 6	12 ± 7	0.001
VWF:Ag (IU/mL)	0.35 ± 0.13	0.34 ± 0.11	0.36 ± 0.14	0.38
VWF:CB (IU/mL)	0.29 ± 0.21	0.28 ± 0.16	0.30 ± 0.24	0.64
VWF:RCo (IU/mL)	0.27 ± 0.14	0.26 ± 0.11	0.28 ± 0.16	0.48

Summary statistics for continuous variables are presented as mean ± standard deviation. VWF = von Willebrand Factor, VWD = von Willebrand Disease. The p-value represents the difference between sexes for each variable.

Association between genetic polymorphisms in *STXBP5* and *STX2* and VWF:Ag levels

In *STX2*, rs7978987 (intron 9) was significantly associated with VWF:Ag levels (beta-coefficient (β) -0.04 IU/mL per allele; 95% confidence interval (CI) -0.07 to -0.001, p=0.04) and VWF:CB activity (β -0.12 IU/mL per allele; CI -0.17 to -0.06, p<0.0001), also after adjustment for blood group (p=0.04 for VWF:Ag and p<0.0001 for VWF:CB) (table 2). Interestingly, the frequency of the minor allele (MAF) of rs7978987, which corresponded with higher VWF levels, was lower among our patients with type 1 VWD (MAF = 0.29) than reported by dbSNP (http://www.ncbi.nlm.nih.gov/ projects/SNP/) (MAF = 0.38) and in the meta-analysis of the CHARGE consortium (MAF = 0.35).[4]

Table 2. VWF:Ag, VWF:RCo, and VWF:CB per genotype.

SNP#	Gene	N	VWF:Ag (IU/mL)	VWF:RCo (IU/mL)	VWF:CB (IU/mL)
rs1039084	*STXBP5*				
GG		37	0.38 ± 0.02	0.30 ± 0.02	0.33 ± 0.04
AG		79	0.35 ± 0.02	0.27 ± 0.02	0.31 ± 0.03
AA		34	0.33 ± 0.02	0.25 ± 0.03	0.25 ± 0.04
p for trend			0.07	0.09	0.12
rs9399599	*STXBP5*				
AA		36	0.36 ± 0.02	0.28 ± 0.02	0.33 ± 0.04
AT		76	0.35 ± 0.02	0.27 ± 0.02	0.28 ± 0.03
TT		39	0.34 ± 0.02	0.25 ± 0.02	0.30 ± 0.04
p for trend			0.40	0.31	0.62
rs7978987	*STX2*				
AA		10	0.40 ± 0.04	0.28 ± 0.05	0.53 ± 0.07
AG		67	0.37 ± 0.02	0.28 ± 0.02	0.32 ± 0.03
GG		73	0.33 ± 0.02	0.26 ± 0.02	0.24 ± 0.03
p for trend			0.04	0.43	< 0.0001

VWF:Ag, VWF:RCo, and VWF:CB levels (mean ± SE) per genotype of each SNP (ANCOVA adjusted for age and sex)). P for trend was calculated using linear regression on VWF:Ag measures with additive genetic models. SNP = single nucleotide polymorphism, MAF = minor allele frequency, VWF = von Willebrand Factor.

For rs1039084 in *STXBP5*, which is a missense mutation that encodes an amino acid substitution of asparagine into serine at position 436 a similar trend with VWF:Ag levels was observed: (β -0.03 IU/mL per allele; CI -0.06 to 0.003, p=0.07) (table 2). Rs9399599 in *STXBP5*, which is located in intron 25, was not associated with VWF:Ag levels nor with

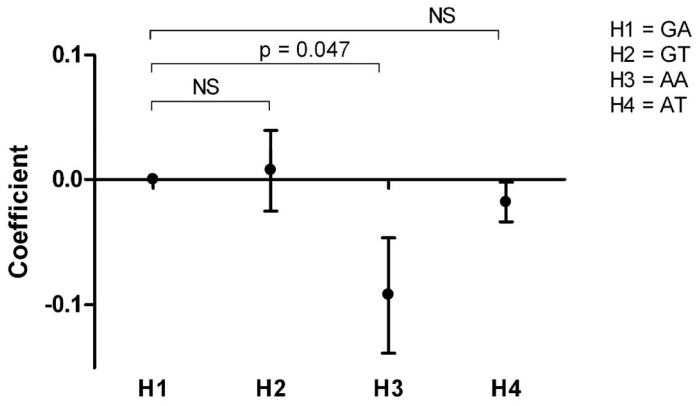


Figure 1. Haplotype analysis for rs1039084 and rs9399599.
Graph presents the coefficients with standard error per haplotype. Haplotype 1 was used as reference haplotype. NS = not significant.

VWF:CB, though carriers of the minor allele had lower VWF:Ag levels and VWF:CB activity as was also observed for rs1039084. Rs1039084 and rs9399599 have a strong linkage disequilibrium of D' = 0.88 and R^2 = 0.71. Haplotype analysis showed that both SNPs contribute to the variation in VWF:Ag levels (figure 1).

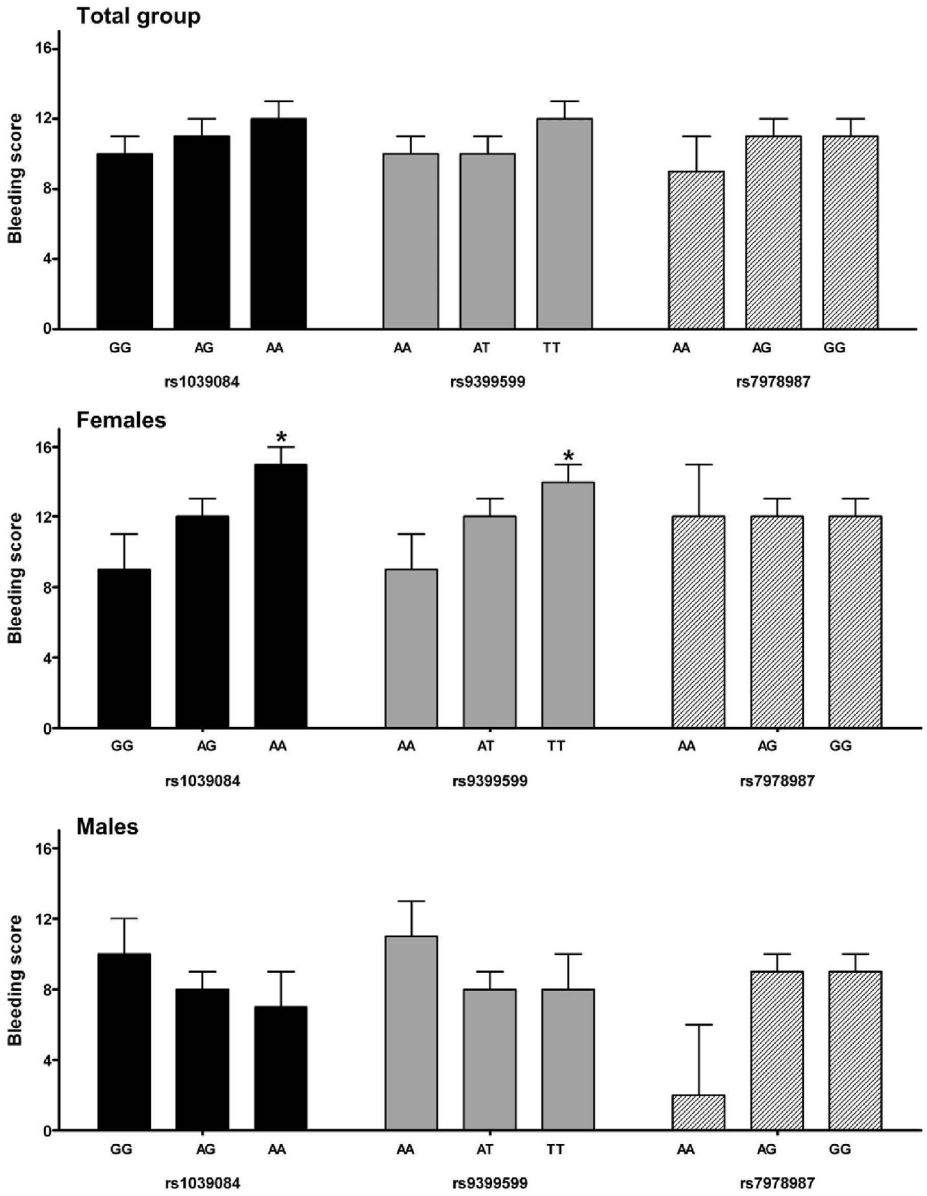


Figure 2. Bleeding score.
Bleeding score per genotype for each SNP for the total group and stratified by sex. * p<0.05

The VWF:RCo activity and VWF:CB activity showed similar associations for both SNPs in *STXBP5*, although not statistically significant (table 2).

Association between genetic polymorphisms in *STXBP5* and *STX2* and the bleeding phenotype

The mean bleeding score was 12 ± 8 in women (n=100) and 8 ± 6 in men (n=58) (p=0.001). In women, homozygous carriers of the minor alleles of both SNPs in *STXBP5* had a significantly higher bleeding score than homozygous carriers of the major allele (rs1039084: GG 9 ± 2 versus AA 15 ± 2, p=0.01 and rs9399599: AA 9 ± 2 versus TT 14 ± 1, p=0.02) (figure 2). Rs7978987 in *STX2* was not associated with the bleeding score.

DISCUSSION

In the current study we have shown that genetic variation in *STX2* and to a lesser extent in *STXBP5* contributes to VWF:Ag levels in patients diagnosed with type 1 VWD. In addition, we demonstrate that in women with type 1 VWD genetic variation in *STXBP5* is associated with the bleeding phenotype.

We are the first to describe the association between genetic variation in SNARE protein genes and VWF:Ag levels and VWF:CB in patients diagnosed with type 1 VWD. In our analysis we used historical levels, since we expect that the lowest levels ever measured are the least influenced by external factors, such as inflammation, hormones, and stress, and therefore reflect a steady state situation.

SNPs in *STXBP5* and *STX2* have been previously identified in the meta-analysis of the CHARGE consortium as determinants of VWF levels in the general population. The effect sizes we obtained were comparable with previous studies.[4,7] In our study, the selected SNPs are the same or are in high linkage disequilibrium with those identified in the CHARGE meta-analysis. Though the selected SNPs in *STXBP5* are in high LD ($D' = 0.88$ and $R^2 = 0.71$) the effect estimate of rs1039084 was slightly higher than for rs9399599. Haplotype analysis showed that both SNPs have an independent effect on VWF:Ag levels. It can be anticipated that since rs1039084 is a non-synonymous SNP and rs9399599 is intronic, rs1039084 may be the actual causal variant with a greater effect.

Interestingly, the frequency of the minor allele of rs7978987 that was significantly associated with higher VWF:Ag levels, was much lower in our type 1 VWD patients (MAF = 0.29) than reported by dbSNP (MAF = 0.38) and the CHARGE consortium (MAF = 0.35). Since the patients were selected on their VWF:Ag levels, it was expected that the frequency of genetic variants associated with higher VWF:Ag levels was much lower in our population.

Alongside the association between rs7978987 and VWF:Ag levels, this SNP was also significantly and more strongly associated with VWF:CB. Since VWF:CB is related to the multimer size of VWF and WPB's contain ultra-large VWF molecules only, this finding may point to actual involvement of *STX2* in WPB exocytosis. However, this hypothesis cannot be further substantiated and should await future research.

One striking finding is the association between genetic variation in *STXBP5* and the bleeding phenotype, as measured by the bleeding score, in women with type 1 VWD. In the total population and in men we did not observe this association. This can be explained by the fact that women experience generally more challenges to the hemostatic system during life, whereby the bleeding score in women may be a better reflector of clinically relevant bleeding tendency, than in men. Indeed, we observed that the menorraghia item of the bleeding score mainly drives the association between genetic variation in *STXBP5* and the bleeding score. Nevertheless, the association with bleeding score, which was assessed by a self-administrated questionnaire, should be interpreted with caution and replicated in larger cohorts, since the number of women per genotype was low.

The association between genetic variation in *STX2* and VWF:Ag levels was independent of blood group. Blood group is the most important determinant of VWF:Ag levels. The presence of blood group A and B antigens on VWF molecules leads to a decreased clearance of VWF molecules. Consequently, individuals with blood group O have approximately 25% lower VWF plasma concentrations than individuals with blood group non-O.[10] Furthermore, as blood group O corresponds with lower VWF:Ag levels, patients diagnosed with type 1 VWD more frequently have blood group O than non-O. We adjusted our statistical analysis for blood group, but this did not influence the effect size of the association. This finding meets our expectations, since we included only moderate and severe type 1 VWD. Also, genetic variation in *STXBP5* and *STX2* may affect the release of VWF molecules, which is expected to be similar in subjects with blood group O and in subjects with blood group non-O, rather than the clearance.

In today's clinical practice diagnosis of type 1 VWD is difficult, because of the high variability in VWF plasma levels and the incomplete penetrance of the phenotype. In addition, there is only a weak association between low VWF levels and bleeding symptoms, which are both highly frequent in the general population. For these reasons it is sometimes hard to distinguish between physiologically low VWF levels and low VWF levels because of type 1 VWD.[11] This problem is further underlined by the fact that a substantial number of individuals with low VWF levels have no clear family history of bleeding symptoms and no detectable mutations in the *VWF* gene (*VWF*), although it has been anticipated for a long time that type 1 VWD is caused by *VWF* mutations. Three population-based studies in Europe, the United Kingdom and Canada showed that only 65% of the type 1 VWD patients have candidate mutations, meaning that 35% have no apparent *VWF* mutations.[12-14] Therefore it is likely that genetic variations in genes other

than *VWF* may contribute to the variation in VWF levels and bleeding symptoms, as we present in the current study.

Our study had a few limitations. First, we have not investigated the combined effect of both SNARE protein gene variations and *VWF* mutations on VWF:Ag levels, since we have not performed *VWF* gene mutation analysis in our cohort. However, we expect that mutations in the *VWF* gene do not interact with genetic polymorphisms in *STXBP5* and *STX2*, since *STXBP5* is located on a different chromosome and *STX2* is located more than 120.000 kb from the *VWF* gene. Therefore, *VWF* mutations will be equally distributed across the genotypes. Finally, we are aware that our sample size is small and that our findings require replication in larger cohorts of patients with type 1 VWD. However, our findings are innovative and form the basis for further research on the role of SNARE protein genes in the clinical presentation of type 1 VWD.

In conclusion, we have shown that genetic variation in *STX2* is associated with VWF:Ag levels in patients previously diagnosed with type 1 VWD. In addition, genetic variation in *STXBP5* is associated with the bleeding phenotype in female type 1 VWD patients. Our findings may explain part of the variation in VWF levels and bleedings symptoms in patients with type 1 VWD. Also, alongside known VWF mutations, genetic variations in SNARE protein genes may help to diagnose individuals with low VWF levels in the future.

REFERENCES

1. Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. *J Clin Invest*. 2000;105:699-701.
2. Ruggeri ZM, Ware J. von Willebrand factor. *FASEB J*. 1993;7:308-316.
3. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4:2103-2114.
4. Smith NL, Chen MH, Dehghan A, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*. 2010;121:1382-1392.
5. Widberg CH, Bryant NJ, Girotti M, Rea S, James DE. Tomosyn interacts with the t-SNAREs syntaxin4 and SNAP23 and plays a role in insulin-stimulated GLUT4 translocation. *J Biol Chem*. 2003;278: 35093-35101.
6. Lowenstein CJ, Morrell CN, Yamakuchi M. Regulation of Weibel-Palade body exocytosis. *Trends Cardiovasc Med*. 2005;15:302-308.
7. van Loon JE, Leebeek FW, Deckers JW, et al. Effect of genetic variations in syntaxin-binding protein-5 and syntaxin-2 on von Willebrand factor concentration and cardiovascular risk. *Circ Cardiovasc Genet*. 2010;3:507-512.
8. Macfarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant RA, McPherson J. Letter: A method for assaying von Willebrand factor (ristocetin cofactor). *Thromb Diath Haemorrh*. 1975;34:306-308.
9. Tosetto A, Rodeghiero F, Castaman G, et al. Impact of plasma von Willebrand factor levels in the diagnosis of type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1VWD). *J Thromb Haemost*. 2007;5:715-721.
10. Gallinaro L, Cattini MG, Sztukowska M, et al. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*. 2008;111:3540-3545.
11. Sadler JE. Low von Willebrand factor: sometimes a risk factor and sometimes a disease. *Hematology Am Soc Hematol Educ Program*. 2009:106-112.
12. Cumming A, Grundy P, Keeney S, et al. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb Haemost*. 2006;96:630-641.
13. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*. 2007;109:112-121.
14. James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. *Blood*. 2007;109:145-154.

4

CLEC4M and *STXBP5* gene variations contribute to von Willebrand Factor level variation in von Willebrand Disease

YV Sanders, JG van der Bom, A Isaacs, MH Cnossen, MPM de Maat, BAP Laros-van Gorkom, K Fijnvandraat, K Meijer, CM van Duijn, EP Mauser-Bunschoten, J Eikenboom, FWG Leebeek, and the WiN study group

Journal of Thrombosis and Haemostasis. 2015;13(6):956-966

ABSTRACT

Background: Von Willebrand Factor (VWF) levels in healthy individuals are influenced by variation 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 lower VWF:Ag (adjusted difference of -3.0 IU/dL per allele; 95% confidence interval (CI) -6.0 to 0.1) and *CLEC4M* with both a lower VWF:Ag level (-4.3 IU/dL per allele; CI -7.9 to -0.6) and lower VWF:Act (-5.7 IU/dL per allele; CI -10.9 to -0.5). In type 2 patients, none of the SNPs were 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.

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 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 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 the *VWF* gene, these genes encode for proteins that had 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

Table 1. Genetic loci associated with von Willebrand Factor identified in the CHARGE consortium and analyzed in the WiN 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

also determine the variability of VWF levels and influence the bleeding phenotype in moderately and severely affected type 1 and type 2 VWD patients. Insight 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 effect 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 types 1, type 2 or type 3 VWD.[2,19-22] The inclusion criteria for the WiN study were: (1) hemorrhagic diathesis or a family history of VWD; and (2) historically lowest levels of VWF:Ag of ≤30 U/dL and/or VWF activity (VWF ristocetin cofactor activity (VWF:RCo)) of ≤30 U/dL and/or Factor VIII coagulation activity (FVIII:C) of ≤40 U/dL (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 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 life-time 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* gene mutations, we obtained pedigrees from the 392 families that we had identified with types 1 and 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 2,200 x *g* for 10 minutes 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 was 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, that uses monoclonal antibodies directed against the glycoprotein (GP)Ibα binding domain of VWF and thereby reflects the binding activity of VWF to GpIbα (HemosIL™ von Willebrand Factor Activity, Instrumentation Laboratory B.V, 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 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 A1), rs8176704 (marker for A2), and rs8176749 (marker for B).[26] Details on 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 using 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 the *TC2N* gene, 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 using SDS 2.1 software (both Applied Biosystems). To ensure DNA quality, we included only patients in whom genotyping had been successful for more than 75% of SNPs.

Statistical methods

Descriptive statistics for categorical data are presented as frequencies and percentages (n, %) and 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 described 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. Since 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 of VWF:Ag or VWF:Act levels between patients with different genotypes, using the genotypes 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 using 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 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 Inc, 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 hours 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 type 2 VWD (figure 1). BS was available for 350 type 1 patients and 224 type 2 patients. Baseline characteristics are shown in table 2.

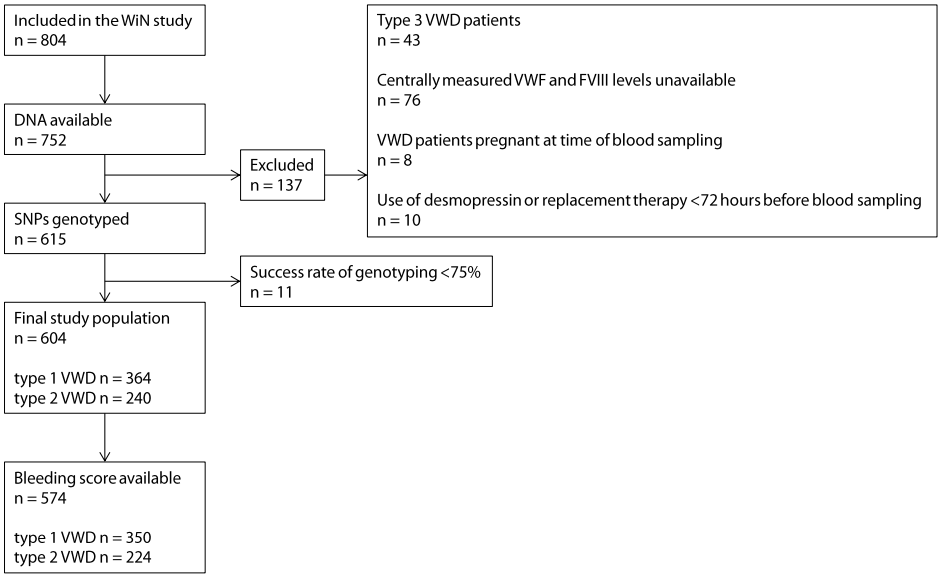


Figure 1. Flowchart of included patients.

Minor allele frequencies in von Willebrand Disease 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

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), median [IQR]		37 [23-53]	25 [16-34]
VWF:Act (IU/dL), 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:Ag = von Willebrand Factor antigen, VWF:Act = von Willebrand Factor activity. VWF:Ag and VWF:Act levels were measured centrally at time of inclusion in the study.

Table 3. Minor allele frequencies (MAFs).

SNP#	Gene	MAF CHARGE	MAF type 1 VWD (95% CI)	MAF 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)

MAF = minor allele frequency, VWD = Von Willebrand Disease, CI = confidence interval, CHARGE = the previously reported MAF in healthy individuals in the CHARGE study.[8] *p<0.001, †p=0.02.

associated with lower VWF:Ag levels, was slightly increased among type 1 VWD patients; 35% 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 -4.3 IU/dL per allele; CI -7.9 to -0.6); and rs9390459 in *STXBP5* was borderline associated with lower VWF:Ag levels (-3.0 IU/dL per allele; CI -6.0 to 0.1) (figure 2 and 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

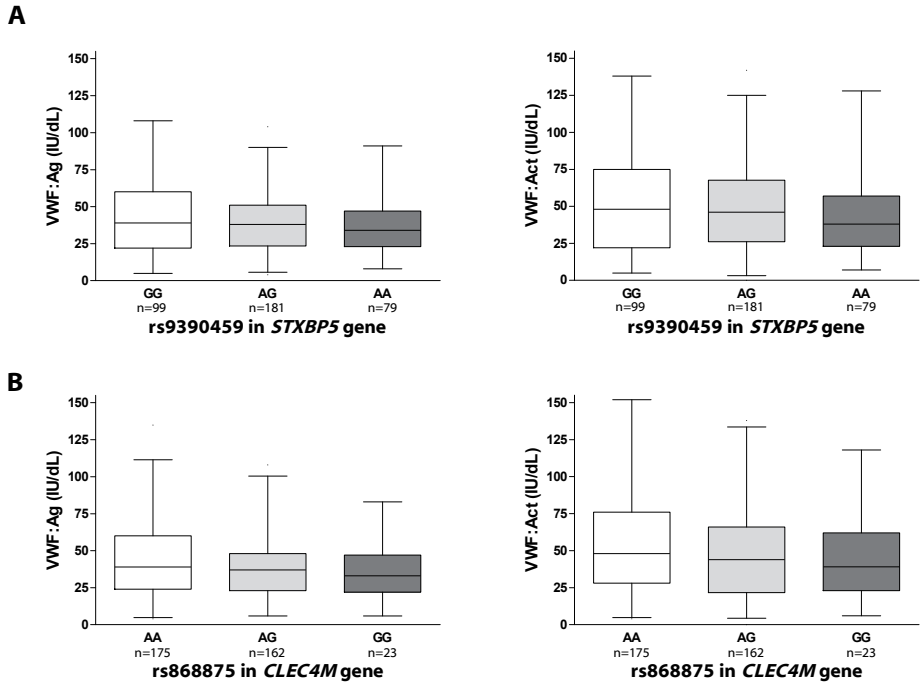


Figure 2. VWF:Ag and VWF:Act level per genotype of (A) rs9390459 in *STXBP5* and (B) rs868875 in *CLEC4M* in type 1 von Willebrand Disease patients.
The boxplots indicate the median, 25-75% interquartile range, and 1-99 percentile. VWF:Ag = von Willebrand Factor antigen, VWF:Act = von Willebrand Factor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

CLEC4M adjusted difference of -3.3 IU/dL per allele; CI -6.8 to 0.2; and for *STXBP5* -2.6 IU/dL per allele; CI -5.5 to 0.4). In addition, after adjustment for multiple testing, using 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 >20 IU/dL were larger, with -5.1 IU/dL per allele (CI -8.7 to -1.5) for *CLEC4M*, and -4.2 IU/dL per allele (CI -7.3 to -1.2) for *STXBP5*, and remained significant after Bonferroni correction. All associations were similar for VWF:Act (figure 2 and 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 -5.1 IU/dL per allele; 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 (CI 1.3 to 5.8) per allele of *STXBP5* or *CLEC4M*,

Table 4. Effect of various genetic loci on VWF:Ag and VWF:Act in type 1 and 2 von Willebrand Disease.

SNP	gene	type 1 von Willebrand disease								type 2 von Willebrand disease							
		VWF:Ag (IU/dL)				VWF:Act (IU/dL)				VWF:Ag (IU/dL)				VWF:Act (IU/dL)			
		Model 1		*Model 2*		*Model 1*		*Model 2*		*Model 1*		*Model 2*		*Model 1*		*Model 2*	
		β	(95% CI)	β	(95% CI)	β	(95% CI)	β	(95% CI)	β	(95% CI)	β	(95% CI)	β	(95% CI)	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)
rs1063857	***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)

*p<0.05. 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. Beta-coefficient (β) represents the increase in VWF:Ag or VWF:Act per VWF-decreasing allele with a 95% CI.

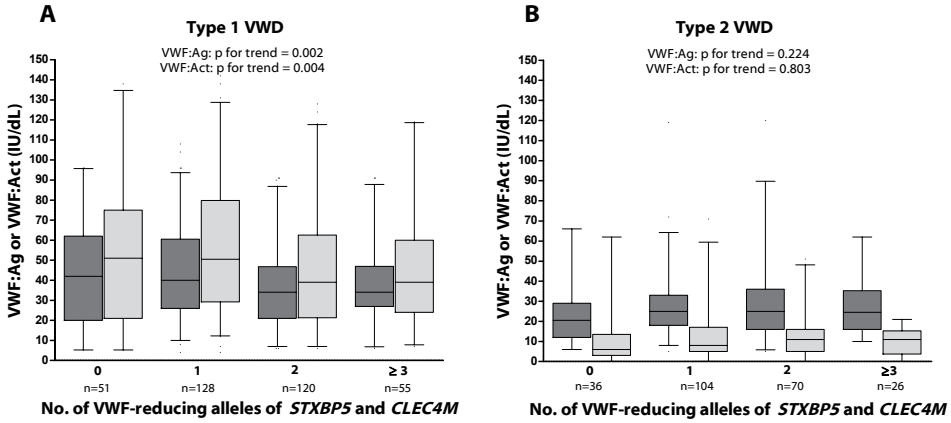


Figure 3. Numbers of VWF-reducing alleles of *STXBP5* and *CLEC4M* in type 1 and 2 von Willebrand Disease. VWF:Ag and VWF:Act levels per numbers of VWF-reducing alleles of *STXBP5* and *CLEC4M* in (A) type 1 and (B) type 2 von Willebrand Disease. The boxplots indicate the median, 25-75% interquartile range, and extreme values.

In type 1 VWD patients with 0, 1, 2 or ≥3 VWF-reducing alleles of *STXBP5* and *CLEC4M*, median VWF:Ag levels were: 42 IU/dL [IQR 20-62], 40 IU/dL [IQR 26-60], 34 IU/dL [IQR 21-47], and 34 IU/dL [IQR 27-47] respectively and median VWF:Act levels: 51 IU/dL [IQR 21-75], 51 IU/dL [IQR 30-80], 39 IU/dL [IQR 21-63], and 39 IU/dL [IQR 24-60].

In type 2 patients with 0 VWF-reducing alleles of *STXBP5* and *CLEC4M*, median VWF:Ag level was 20 IU/dL [IQR 12-29] and median VWF:Act level was 6 IU/dL [IQR 3-14]. Median VWF:Ag and VWF:Act levels were 25 IU/dL [IQR 18-33] and 8 IU/dL [IQR 5-17] in type 2 patients with 1 VWF-reducing alleles of *STXBP5* and *CLEC4M*. VWF:Ag and VWF:Act levels were 25 IU/dL [IQR 16-36] and 11 IU/dL [IQR 5-16] in type 2 patients with 2 VWF-reducing alleles of *STXBP5* and *CLEC4M*. In type 2 VWD patients with ≥3 VWF-reducing alleles of *STXBP5* and *CLEC4M* median VWF:Ag and VWF:Act levels were 25 IU/dL [IQR 16-35] and 11 IU/dL [IQR 4-15].

and VWF:Act levels were reduced by 4.7 IU/dL (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 34 IU/dL [IQR 21-47]) (figure 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; CI -0.9 to 3.6; and for VWF:Act, -0.3 IU/dL; CI -2.9 to 2.2) (figure 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 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* contributes to the variability in levels of 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, who are known to have *VWF* mutations less frequently than type 1 patients with VWF:Ag levels of <20 IU/dL 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 that 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 likely to lead to VWD. This is further supported by the recent observation that an *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 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 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 for 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 the VWF levels, may also determine whether an individual is diagnosed as moderately or severely affected VWD patient (levels of <30 IU/dL) 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 the *VWF* gene 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, suggests that other genetic loci contribute to low VWF levels. The involvement of other 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 as 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 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 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 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 the *VWF* gene. Also, even though this is the largest cohort study of type 1 and 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 the *CLEC4M* and *STXBP5* genes. 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.

REFERENCES

1. James PD, Lillicrap D. von Willebrand disease: clinical and laboratory lessons learned from the large von Willebrand disease studies. *Am J Hematol*. 2012;87 Suppl 1:S4-11.
2. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108(4):683-692.
3. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
4. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*. 2007;109(1):112-121.
5. Robertson JD, Yenson PR, Rand ML, et al. Expanded phenotype-genotype correlations in a pediatric population with type 1 von Willebrand disease. *J Thromb Haemost*. 2011;9(9):1752-1760.
6. Eikenboom J, Van Marion V, Putter H, et al. Linkage analysis in families diagnosed with type 1 von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 VWD. *J Thromb Haemost*. 2006;4(4):774-782.
7. James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. *Blood*. 2007;109(1):145-154.
8. Smith NL, Chen MH, Dehghan A, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*. 2010;121(12):1382-1392.
9. Van Loon JE. Genome-wide assocation studies identify genetic loci for low von Willebrand Factor levels. Genetic determinants of von Willebrand Factor and the risk of cardiovascular disease. Schiedam, the Netherlands; 2012:105-121.
10. Widberg CH, Bryant NJ, Girotti M, Rea S, James DE. Tomosyn interacts with the t-SNAREs syntaxin4 and SNAP23 and plays a role in insulin-stimulated GLUT4 translocation. *J Biol Chem*. 2003;278(37): 35093-35101.
11. Lowenstein CJ, Morrell CN, Yamakuchi M. Regulation of Weibel-Palade body exocytosis. *Trends Cardiovasc Med*. 2005;15(8):302-308.
12. van Loon JE, Leebeek FW, Deckers JW, et al. Effect of genetic variations in syntaxin-binding protein-5 and syntaxin-2 on von Willebrand factor concentration and cardiovascular risk. *Circ Cardiovasc Genet*. 2010;3(6):507-512.
13. Rydz N, Swystun LL, Notley C, et al. The C-type lectin receptor CLEC4M binds, internalizes, and clears von Willebrand factor and contributes to the variation in plasma von Willebrand factor levels. *Blood*. 2013;121(26):5228-5237.
14. Harris EN, Weigel PH. The ligand-binding profile of HARE: hyaluronan and chondroitin sulfates A, C, and D bind to overlapping sites distinct from the sites for heparin, acetylated low-density lipoprotein, dermatan sulfate, and CS-E. *Glycobiology*. 2008;18(8):638-648.
15. Gallinaro L, Cattini MG, Sztukowska M, et al. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*. 2008;111(7):3540-3545.
16. Jiang Y, Oliver P, Davies KE, Platt N. Identification and characterization of murine SCARA5, a novel class A scavenger receptor that is expressed by populations of epithelial cells. *J Biol Chem*. 2006; 281(17):11834-11845.

17. Morange PE, Saut N, Antoni G, Emmerich J, Tregouet DA. Impact on venous thrombosis risk of newly discovered gene variants associated with FVIII and VWF plasma levels. *J Thromb Haemost*. 2011;9(1): 229-231.
18. van Loon JE, Sanders YV, de Wee EM, Kruip MJ, de Maat MP, Leebeek FW. Effect of Genetic Variation in STXBP5 and STX2 on von Willebrand Factor and Bleeding Phenotype in Type 1 von Willebrand Disease Patients. *PLoS One*. 2012;7(7):e40624.
19. Sanders YV, Eikenboom J, de Wee EM, et al. Reduced prevalence of arterial thrombosis in von Willebrand disease. *J Thromb Haemost*. 2013;11(5):845-854.
20. De Wee EM, Knol HM, Mauser-Bunschoten EP, et al. Gynaecological and obstetric bleeding in moderate and severe von Willebrand disease. *Thromb Haemost*. 2011;106(5):885-892.
21. de Wee EM, Mauser-Bunschoten EP, van der Bom JG, et al. Health-related quality of life among adult patients with moderate and severe von Willebrand disease. *J Thromb Haemost*. 2010;8(7):1492-1499.
22. Sanders YV, Giezenaar MA, Laros-van Gorkom BA, et al. Von Willebrand disease and aging: an evolving phenotype. *J Thromb Haemost*. 2014;12(7):1066-1075.
23. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost*. 2006;4(4):766-773.
24. Tosetto A, Castaman G, Rodeghiero F. Bleeding scores in inherited bleeding disorders: clinical or research tools? *Haemophilia*. 2008;14(3):415-422.
25. Landsteiner K. On agglutination of normal human blood. *Transfusion*. 1961;1:5-8.
26. Pare G, Chasman DI, Kellogg M, et al. Novel association of ABO histo-blood group antigen with soluble ICAM-1: results of a genome-wide association study of 6,578 women. *PLoS Genet*. 2008;4(7): e1000118.
27. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988;16(3):1215.
28. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 2005;21(2):263-265.
29. Van Schie MC, Wieberdink RG, Koudstaal PJ, et al. Genetic determinants of von Willebrand factor plasma levels and the risk of stroke: the Rotterdam Study. *J Thromb Haemost*. 2012;10(4):550-556.
30. van Breevoort D, Snijders AP, Hellen N, et al. STXBP1 promotes Weibel-Palade body exocytosis through its interaction with the Rab27A effector Slp4-a. *Blood*. 2014;123(20):3185-3194.
31. Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ, Jr., Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood*. 1987;69(6):1691-1695.
32. Dahabreh IJ, Kent DM. Index event bias as an explanation for the paradoxes of recurrence risk research. *JAMA*. 2011;305(8):822-823.
33. Bowman M, Mundell G, Grabell J, et al. Generation and validation of the Condensed MCMDM-1VWD Bleeding Questionnaire for von Willebrand disease. *J Thromb Haemost*. 2008;6(12):2062-2066.

5

Von Willebrand Factor propeptide and the phenotypic classification of von Willebrand Disease

YV Sanders, D Groeneveld, K Meijer, K Fijnvandraat, MH Cnossen, JG van der Bom, M Coppens, J de Meris, BAP Laros-van Gorkom, EP Mauser-Bunschoten, FWG Leebeek, J Eikenboom, and the WiN study group

Blood. 2015;125(19):3006-3013

ABSTRACT

The ratios between von Willebrand Factor propeptide (VWFpp) or FVIII coagulant activity (FVIII:C) and VWF antigen (VWF:Ag) reflect synthesis, secretion and clearance of VWF. We aimed to define the pathophysiology of 658 patients with type 1, 2 or 3 von Willebrand Disease (VWD) with VWF levels ≤30 U/dL from the "Willebrand in the Netherlands" (WiN) study using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios. We evaluated the use of VWFpp in the classification and diagnosis of VWD. On the basis of the ratios, reduced VWF synthesis was observed in 18% (67/380) of type 1 patients, and in only 2% (5/240) of type 2 patients. A significant proportion of type 3 patients had detectable VWFpp (41%, 14/37). These patients had a lower bleeding score than type 3 patients who had a complete absence of both VWF:Ag and VWFpp: 14.0 vs. 19.5, p=0.025. The majority of these patients had missense mutations with rapid VWF clearance, whereas type 3 patients with no VWFpp were homozygous for null alleles. In conclusion, VWFpp identified severe type 1 VWD with very low VWF levels in patients who had previously been classified as type 3 VWD. This study underlines the clinical significance of the VWFpp assay in the diagnosis and classification of VWD.

INTRODUCTION

Von Willebrand Factor (VWF) is a large multimeric glycoprotein that mediates platelet adhesion and aggregation at sites of vascular injury and serves as the carrier of Factor VIII (FVIII) to prevent its premature clearance.[1] Synthesis of VWF is restricted to endothelial cells and megakaryocytes,[2] where it is formed as a precursor protein, pre-proVWF, with a signal peptide, a propeptide, and a mature subunit.[3] After translocation to the endoplasmic reticulum, the signal peptide is cleaved, and the pro-VWF undergoes extensive posttranslational modifications, including dimerization in the endoplasmic reticulum and multimerization in the Golgi system.[4] The VWF propeptide (VWFpp) is subsequently removed from the mature VWF; however, it stays noncovalently bound.[5] Part of VWF is thought to be secreted constitutively into the plasma, and the remaining part is stored in Weibel-Palade bodies in the endothelium or α-granules in megakaryocytes.[6] After release into the circulation, the VWFpp and the mature VWF completely dissociate.[6]

Plasma VWF concentrations represent a balance between synthesis, secretion, and clearance from the circulation. VWF and VWFpp are secreted equimolarly but are cleared independently with estimated half-lives of 8 to 12 hours for VWF and 2 hours for VWFpp.[7] The ratio between VWFpp and VWF antigen (VWF:Ag) can therefore be used to assess the rates of synthesis, secretion, and clearance of VWF.[7,8] In addition to VWFpp, the ratio between FVIII coagulant activity (FVIII:C) and VWF:Ag can also be used to assess VWF synthesis and clearance.[9] Because FVIII and VWF circulate in blood as a complex and are cleared together, their half-lives are strongly related.[10]

VWF is deficient or defective in von Willebrand Disease (VWD), the most common inherited bleeding disorder.[11] Low VWF levels are caused by reduced VWF synthesis or secretion, increased VWF clearance, or a combination thereof. Recently, Eikenboom et al have shown that the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios represent the pathophysiology of VWD and correspond to different *VWF* gene mutations in type 1 VWD.[12] However, it is still unknown whether these ratios can also provide insight into the pathophysiological mechanism in types 2 and 3 VWD.

VWD patients are classified as type 1, 2 or 3 VWD according to their remaining level of functional VWF.[11] Type 1 VWD is characterized by partially reduced VWF levels, whereas in type 3 VWD, plasma is completely devoid of VWF. In type 2 VWD, functionally abnormal variants of VWF are synthesized. Type 2 VWD is subclassified as 2A, 2B, 2M or 2N on the basis of the type of functional defect in VWF.[11] In some patients, it is difficult to classify VWD correctly because the available diagnostic laboratory tests do not provide adequate information to distinguish between VWD (sub)types. For instance, type 2M may be caused by a binding defect to collagen, which will be missed by the regular VWF assays. In addition, these laboratory assays are less sensitive in measuring extremely low VWF and FVIII levels and are therefore less reliable.

In this large nationwide cross-sectional Willebrand in the Netherlands (WiN) study, we aimed to define the pathophysiology of type 1, 2 and 3 VWD by using the VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios irrespective of the *VWF* gene mutation. In addition, we evaluated whether the use of VWFpp can improve the diagnosis and classification of VWD.

METHODS

Participants

The WiN study included 804 VWD patients who had previously been diagnosed with types 1, 2 or 3 VWD.[13,14] The inclusion criteria for the WiN study were (1) hemorrhagic diathesis or a family history of VWD and (2) historically lowest VWF levels ≤30 U/dL (VWF:Ag and/or VWF:RCo) and/or FVIII:C levels ≤40 U/dL. Patients were excluded if they were known to have other hemostatic disorders resulting in hemorrhagic diathesis. The Medical Ethical Committees at all participating centers approved this study, and all participants gave informed consent.

For this study, only patients from whom plasma was obtained were selected (n=681). Exclusion criteria were pregnancy (n=7) and the recent use of desmopressin or replacement therapy at the time of blood sampling (n=16). Therefore, a total of 658 VWD patients were included in this study.

Assessment of bleeding symptoms

All patients completed a questionnaire regarding bleeding episodes, treatment of VWD, co-morbidity, and Quality of Life.[14,15] To calculate a bleeding score (BS), as previously described by Tosetto,[16] we used information on the number and severity of clinically relevant bleeding of 12 specific bleeding symptoms. The Tosetto BS assigns a high score when desmopressin or replacement therapy is needed to control a bleeding; however, when this replacement therapy was used prophylactically before a surgical intervention, dental extraction or delivery to prevent bleeding, this bleeding symptom was not scored as a bleeding that required replacement therapy because that would overestimate the score.[14,17] However, if bleeding did occur despite prophylactic treatment, this bleeding was scored according to the Tosetto BS.

Laboratory measurements

Patients' plasma was obtained at inclusion in the study. Venous whole blood was collected in 0.105 M sodium citrate tubes and centrifuged twice at 2,200x *g* for 10 minutes at room temperature and stored at -80°C. VWF:Ag, VWF activity (VWF:Act), FVIII:C, VWF binding to FVIII (VWF:FVIIIB), and VWF multimers were determined centrally at the Erasmus University Medical Center (Rotterdam, the Netherlands) as described.[14]

VWF:Act was assessed with a latex immune assay (LIA) on an automated coagulometer. The LIA test uses monoclonal antibodies directed against the GpIbα binding site of VWF and thereby reflects the binding activity of VWF to GpIbα (HemosIL™ von Willebrand Factor Activity, Instrumentation Laboratory B.V, Breda, Netherlands).[18] We were not able to use the laborious VWF:RCo test in this study for logistical reasons, although it is the most widely used and preferable test. Because of the known variability of VWF testing in 13 different laboratories in the Netherlands, we preferred to perform all VWF tests centrally in a laboratory with expertise on VWF. We have previously found a very strong correlation (Spearman correlation coefficient 0.942, p<0.0001) between the VWF:RCo and the VWF:Act tests in a random set of patients (n=122). The diagnostic accuracy of this test was also confirmed by others.[19]

VWFpp was measured centrally at the Leiden University Medical Center (Leiden, the Netherlands). This assay was also used in the previous paper from Eikenboom et al[12] and was measured at the same center. VWFpp antigen was determined with an ELISA using antibodies from Sanquin (Amsterdam, the Netherlands) as described.[7,12] First, microtiter plates were coated with antibody CLB-Pro 35 overnight at 4°C, then blocked with 1% Bovine Serum Albumin (BSA) at room temperature for 2 hours. Next, the diluted samples were incubated for 2 hours at 37°C. VWFpp was detected with peroxidase-conjugated antibody CLB-Pro 14.3. Pooled normal plasma was used to create a standard curve. At the time of the study, no international standard was yet available for VWFpp, and therefore the plasma pool was arbitrarily set at 100 U/dL. Details on the blood-sampling procedure and laboratory measurements at inclusion in the WiN study have been described in more detail by de Wee et al.[14]

Definitions

Determination of VWD type was based on the current International Society on Thrombosis and Haemostasis (ISTH) guidelines[11] by using centrally measured plasma concentrations of VWF:Ag, VWF:Act and FVIII:C; VWF multimer patterns; VWF:FVIIIB assay; and locally performed ristocetin-induced platelet aggregation (RIPA) tests.[14]

Type 1 VWD patients had a VWF:Act/VWF:Ag ratio ≥0.70, whereas type 2 patients had a ratio <0.70. Considering the strong correlation between VWF:RCo and VWF:Act, we used the VWF:Act/VWF:Ag ratio instead of VWF:RCo/VWF:Ag. If type 2 patients had normal multimers, they were classified as 2M. Type 2A or 2B VWD patients showed abnormal multimers. If the locally performed RIPA test showed increased VWF affinity for platelets, patients were classified as type 2B. Type 2N patients had a FVIII:C/VWF:Ag ratio of <0.70 and a VWF:FVIIIB of <60%.[20] Type 3 VWD was defined as having both VWF:Ag and VWF:Act levels <5 U/dL, irrespective of FVIII:C level.

The normal range (2.5th to 97.5th percentile) used for VWFpp was 82 to 173 U/dL, for the VWFpp/VWF:Ag ratio 0.8 to 2.2, and for the FVIII:C/VWF:Ag ratio 0.6 to 1.9, as reported before by Eikenboom et al.[12]

Statistical methods

Descriptive statistics for categorical data are presented as numbers with percentages (n, %). Because data were non-normally distributed, continuous variables are presented as median with 25 to 75% interquartile ranges [IQR]. For comparison of proportions, chi-squared tests were used. The Kruskal-Wallis test was used to test differences in VWFpp, VWFpp/VWF:Ag ratio and FVIII:C/VWF:Ag ratio between types 1, 2A, 2B, 2M, 2N and 3 VWD. Mann-Whitney U-tests were applied to detect differences between types and to compare BS between groups. Statistical analyses were performed with SPSS for Windows, version 21.0 (SPSS Inc, Chicago, USA). A p-value <0.05 was considered statistically significant.

RESULTS

A total of 658 VWD patients were included in the current analyses, 381 of whom had type 1 VWD, 240 type 2 VWD, and 37 type 3 VWD, according to the current ISTH guidelines.[111] Baseline characteristics are presented in table 1.

Table 1. Baseline characteristics.

Characteristics	VWD patients (n = 658)
Age (years), median (range)	43.5 (1-83)
Male sex, n (%)	251 (38)
VWD type, n (%)	
1	380 (58)
2	241 (37)
2A	*158*
2B	*42*
2M	*27*
2N	*14*
3	37 (6)
Blood group O, n (%)*	398 (61)
VWF:Ag (IU/dL), median [IQR]	29 [18-45]
VWF:Act (U/dL), median [IQR]	22 [8-53]
FVIII:C (IU/dL), median [IQR]	51 [32-73]
Bleeding score, median [IQR]†	11 [6-16]

VWD = von Willebrand Disease, VWF:Ag = von Willebrand Factor antigen, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulant activity. VWF:Ag, VWF:Act and FVIII:C levels were measured centrally at time of inclusion in the study. *4 missing, †32 missing.

VWF parameters and ratios per type of von Willebrand Disease

In type 1 patients, median VWFpp was 91 U/dL [IQR 68-116], median VWFpp/VWF:Ag ratio 2.2 [IQR 1.7-3.1] and median FVIII:C/VWF:Ag ratio 1.8 [IQR 1.4-2.3] (table 2 and figure 1A). This is in accordance with previously published data by Eikenboom et al.[12] In type 2 patients, median VWFpp was 104 U/dL [IQR 81-136], which was higher than in type 1 patients (p<0.001). Median VWFpp/VWF:Ag ratio was 4.5 [IQR 3.2-6.0] and median FVIII:C/VWF:Ag ratio 1.6 [IQR 1.2-2.0] in the type 2 patients. The VWFpp/VWF:Ag ratio was higher in type 2 VWD than in type 1 VWD (p<0.001) and the FVIII:C/VWF:Ag ratio was lower in type 2 VWD compared with type 1 VWD (p<0.001) (table 2 and figure 1A). This difference in VWFpp, VWFpp/VWF:Ag, and FVIII:C/VWF:Ag between type 1 and type 2 VWD was also observed after exclusion of type 2N patients (all p<0.001). Median VWFpp in type 2A, 2B and 2M VWD was 103 U/dL [IQR 81-135], median VWFpp/VWF:Ag was 4.6 [IQR 3.4-6.2], and median FVIII:C/VWF:Ag was 1.6 [IQR 1.3-2.1].

Table 2. VWF parameters and ratios per type of von Willebrand Disease.

Type of VWD	n	VWF:Ag (IU/dL)	VWF:Act (U/dL)	FVIII:C (IU/dL)	VWFpp (U/dL)	VWF:Act/ VWF:Ag ratio	VWFpp/ VWF:Ag ratio	FVIII:C/ VWF:Ag ratio
1*	381	38 [23-53]	46 [24-72]	67 [49-88]	91 [68-116]	1.2 [1.0-1.4]	2.2 [1.7-3.1]	1.8 [1.4-2.3]
2	240	24 [16-34]	8 [4-16]	37 [27-48]	104 [81-136]	0.4 [0.2-0.5]	4.5 [3.2-6.0]	1.6 [1.2-2.0]
2A	157	22 [14-32]	8 [3-15]	37 [27-47]	99 [79-123]	0.4 [0.2-0.5]	4.5 [3.3-6.2]	1.7 [1.3-2.2]
2B	42	28 [23-40]	8 [6-14]	39 [28-48]	137 [106-157]	0.3 [0.2-0.4]	4.6 [3.7-5.8]	1.3 [1.1-1.5]
2M	27	24 [16-30]	5 [3-13]	42 [31-52]	93 [75-119]	0.3 [0.1-0.4]	4.8 [3.1-6.2]	1.9 [1.6-2.1]
2N	14	34 [28-44]	45 [29-54]	19 [13-28]	111 [71-157]	1.2 [1.0-1.5]	2.7 [2.0-4.4]	0.6 [0.4-0.7]
3	37	1 [0-4]	0 [0-1]	3 [1-9]	0 [0-60]†	NA	NA	NA
normal range‡		47-169	45-213	57-185	82-173	0.6-1.8	0.8-2.2	0.6-1.9

All levels are presented as median with 25-75% interquartile range (IQR) between square brackets. VWF = von Willebrand Factor, VWD = von Willebrand Disease, n = number, VWF:Ag = VWF antigen, VWF:Act = VWF activity, FVIII:C = factor VIII coagulant activity, VWFpp = von Willebrand Factor propeptide, IQR = 25-75% interquartile range, NA = not applicable. * median and 25-75% interquartile range (IQR) of VWFpp level and ratios in type 1 VWD patients are in accordance with the results from Eikenboom et al.[12] † VWFpp levels below the assay detection limit (<4 U/dL) were considered 0 (n=25). ‡ The normal reference ranges (2.5th to 97.5th percentile) are based on 387 healthy controls and were taken from our previous publication Eikenboom et al,[12] however VWF activity in that study was based on VWF:RCo. VWFpp in the WiN study was measured in this same laboratory.

VWF parameters and ratios for different type 2 von Willebrand Disease subtypes

Median VWFpp was significantly higher in VWD 2B patients than in 2A patients or 2M patients: 137 U/dL [IQR 106-157] vs. 99 U/dL [IQR 79-123] or 93 U/dL [IQR 75-119] with p<0.001 for both (figure 1B and table 2). VWFpp/VWF:Ag ratio was increased in 2A, 2B and 2M patients based on the normal range (range 0.8-2.2)[12]; 4.5 [IQR 3.3-6.2] in 2A, 4.6 [IQR 3.7-5.8] in 2B, and 4.8 [IQR 3.1-6.2] in 2M VWD. Type 2B patients had significantly lower FVIII:C/VWF:Ag ratios than 2A or 2M patients (1.3 [IQR 1.1-1.5] vs. 1.7 [IQR 1.3-2.2]

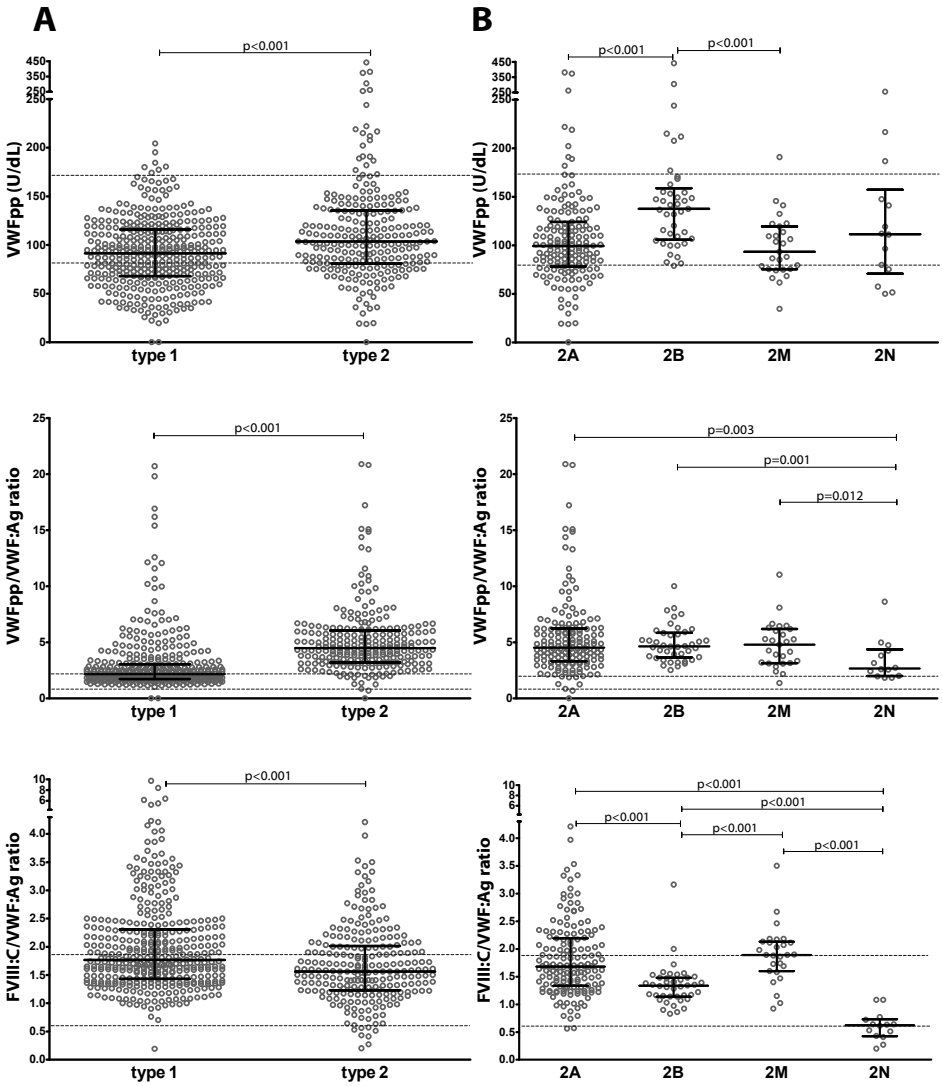


Figure 1. VWFpp levels and VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios per type of von Willebrand Disease.
A) VWFpp levels, VWFpp/VWF:Ag ratios and FVIII:C/VWF:Ag ratios are shown for type 1 VWD and type 2 VWD patients. For a better presentation of the graphic VWFpp/VWF:Ag ratio, one type 1 VWD patient with both a high VWFpp/Ag ratio (33.7) and a FVIII:C/VWF:Ag ratio of 3.4 was omitted.
B) For all patients with type 2A, 2B, 2M and 2N VWD the VWFpp levels, VWFpp/VWF:Ag ratios and FVIII:C/VWF:Ag ratios are shown.
The central horizontal line represents the median. The upper and lower horizontal lines show the 25-75% interquartile ranges. The dash lines illustrate the normal ranges (2.5th to 97.5th percentile) for VWFpp (82-173 U/dL), for VWFpp/VWF:Ag ratio (0.8-2.2), and for FVIII:C/VWF:Ag ratio (0.6-1.9).

or 1.9 [IQR 1.6-2.1], p<0.001 for both). Median VWFpp and median VWFpp/VWF:Ag ratio were similar between type 2N and type 1 VWD. Type 2N patients had a significantly decreased FVIII:C/VWF:Ag ratio compared with type 1, 2A, 2B and 2M VWD (all p<0.001), as expected by the decreased FVIII binding affinity in type 2N.

Pathophysiological mechanisms in type 1 von Willebrand Disease

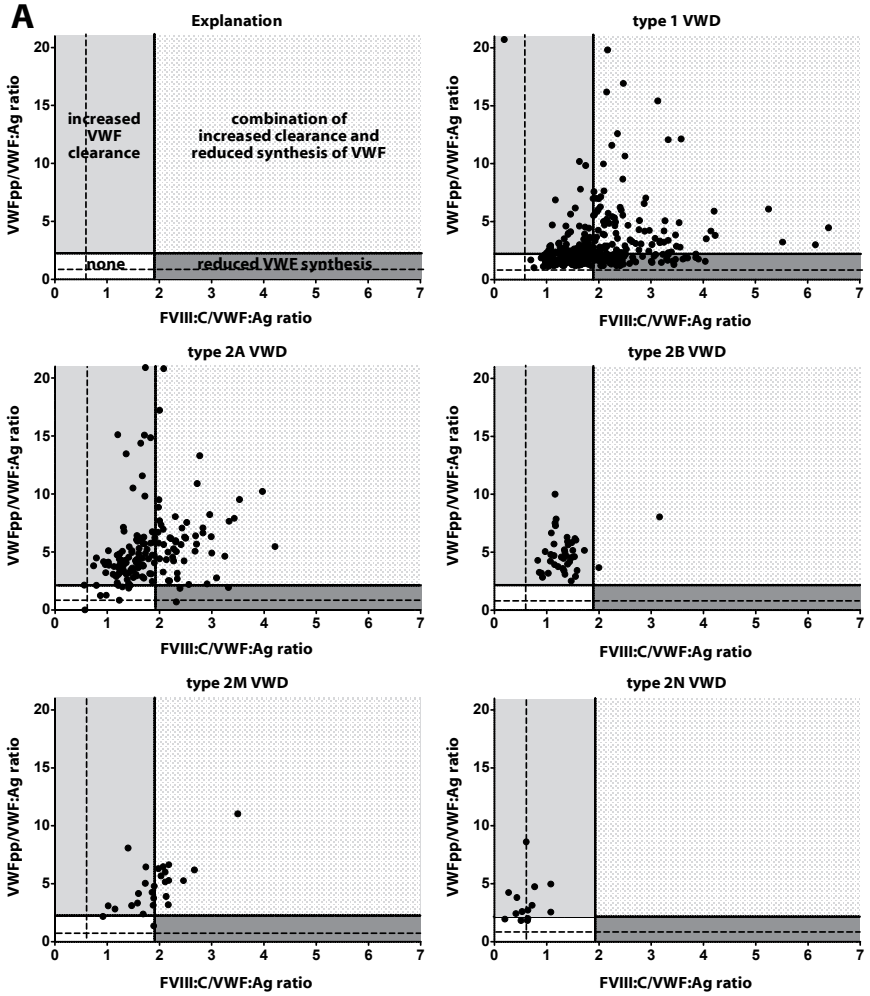
An increased VWFpp/VWF:Ag ratio (> 2.2) defines accelerated clearance of VWF, and an increased FVIII:C/VWF:Ag ratio (>1.9) correlates with reduced VWF synthesis (figure 2A). The pathophysiological mechanisms of type 1 VWD were variable, with increased clearance of VWF in 23% of type 1 patients (89/380), reduced synthesis of VWF in 18% (67/380), and a combination of these in 23% (86/380). In 36% (138/380) of type 1 patients other pathophysiological mechanisms may play a role, because VWFpp/VWF:Ag and FVIII:C/VWF:Ag were within normal ranges (figure 2).

Pathophysiological mechanisms in type 2 von Willebrand Disease variants

On the basis of increased VWFpp/VWF:Ag or FVIII:C/VWF:Ag ratios, the majority of type 2A VWD was characterized by an increased clearance of VWF (93/158, 59%), followed by a combination of increased clearance and reduced synthesis (50/158, 32%). In only five (of 158, 3%) patients with 2A VWD, reduced synthesis of VWF predominated. In ten (of 158, 6%) 2A patients, the VWD was characterized by neither increased clearance nor reduced synthesis. Ninety-five percent (40/42) of type 2B patients showed an increased VWFpp/VWF:Ag ratio with a normal FVIII:C/VWF:Ag ratio, suggesting that 2B VWD is completely characterized by an increased clearance of normally synthesized and secreted VWF. The pathophysiological mechanisms of type 2M VWD were an increased clearance in almost half of the patients (13/27, 48%) and a combination of increased clearance and reduced synthesis in the other half (12/27, 44%). None of the 2M patients had only an increased FVIII:C/VWF:Ag ratio, indicating that 2M VWD is not solely characterized by reduced synthesis. Ten (of 14, 71%) 2N patients had an increased VWFpp/VWF:Ag ratio, indicating that apart from reduced FVIII binding, the type 2N VWD is also characterized by increased clearance (figure 2).

Type 3 von Willebrand Disease patients

Thirty-seven patients with VWF:Ag and VWF:Act <5 U/dL were classified as type 3 VWD, according to the current ISTH guidelines.[11] VWFpp was undetectable in 59% (22/37) of those patients, which fits with type 3 VWD having complete absence of VWF production. In all other patients classified as type 3 (15/37, 41%), high levels of VWFpp were measured with a median of 72 U/dL (table 3). Compared with the type 3 VWD patients with virtually complete absence of VWFpp, the patients classified as type 3, but with ample VWFpp, had higher VWF:Ag levels (0 vs. 4 IU/dL, p<0.001), higher VWF:Act levels


A
Explanation
increased VWF clearance
combination of increased clearance and reduced synthesis of VWF
none
reduced VWF synthesis
type 1 VWD
type 2A VWD
type 2B VWD
type 2M VWD
type 2N VWD
VWFpp/VWF:Ag ratio
FVIII:C/VWF:Ag ratio

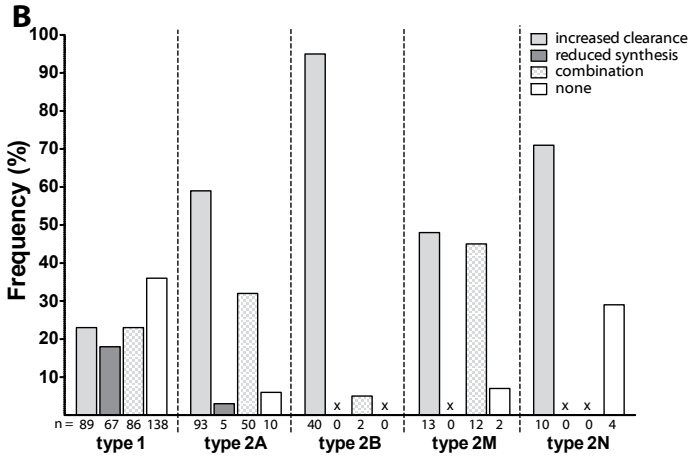

B
Frequency (%)
increased clearance
reduced synthesis
combination
none
n = 89 67 86 138
93 5 50 10
40 0 2 0
13 0 12 2
10 0 0 4
type 1
type 2A
type 2B
type 2M
type 2N

Figure 2. Pathophysiological mechanisms per type of von Willebrand Disease.
A) Scatter plots of FVIII:C/VWF:Ag (x-axis) versus VWFpp/VWF:Ag (y-axis) for type 1 VWD and type 2A, 2B, 2M and 2N VWD. The yellow area represents the patients with increased clearance, the purple area the patients with reduced synthesis, the green area the patients with a combination of both mechanisms, and the white area represents the absence of these pathophysiological mechanisms. Due to the FVIII binding defect in type 2N VWD the FVIII:C/VWF:Ag ratio is low by definition and cannot be assessed reliably with respect to aspects of synthesis in type 2N. For a better presentation of the graphics: one type 1 VWD patient with a very high VWFpp/Ag ratio (33.7), and two type 1 VWD patients with high FVIII:C/VWF:Ag ratios (8.4 and 9.7) were omitted. B) Proportion of VWD patients with increased clearance (yellow), reduced synthesis (purple), combination of increased clearance and reduced synthesis (green), and none of these mechanisms (white). X = not present.

(0 vs. 1 U/dL, p=0.036), and higher FVIII:C levels (2 vs. 9 IU/dL, p<0.001). The frequency of blood group O was significantly higher in patients with detectable VWFpp than in the type 3 patients without detectable VWFpp (12/15 vs. 8/22, p=0.009). In 12 of 22 type 3 patients without detectable VWFpp, historical data on *VWF* mutations were available; ten of those patients were homozygous or compound heterozygous for a null allele (p.R2535X, p.N2546Y, p.Y1570X, p.S330KfsX4, p.D1333EfsX43, and deletion of exons 17 to 25). Of the 15 patients with detectable VWFpp, the *VWF* gene mutation was known in 14, ten of whom had missense mutations in regions associated with rapid clearance of VWF (p.R1205H and p.S1285P) or mutations that are considered to rapidly clear VWF from the circulation (p.Y1584C and p.Y1146C).[8,21-23] Additionally, three patients were compound heterozygous for a null allele and an unknown defect on the other allele.

Table 3. Characteristics of type 3 von Willebrand Disease patients with and without VWFpp.

Characteristics		without VWFpp n=22	with VWFpp n=15	p-value
Age (years), median (range)		22 (2-60)	35 (4-65)	0.636
Male sex, n (%)		9 (41)	7 (47)	0.729
Blood group O, n (%)		8 (36)	12 (80)	0.009
VWFpp (U/dL), median [IQR]		0 [0-0]	72 [37-94]	<0.001
VWF:Ag (IU/dL), median [IQR]		0 [0-1]	4 [2-4]	<0.001
VWF:Act (U/dL), median [IQR]		0 [0-0]	1 [0-3]	0.036
FVIII:C (IU/dL), median [IQR]		2 [1-3]	9 [8-13]	<0.001
Multimers	Absent, n (%)	19 (86)	3 (20)	<0.001
	Abnormal, n (%)	3 (14)	12 (80)	
Bleeding score, median [IQR]		19.5 [11.3-23.8]*	14.0 [7.0-17.0]	0.025
Historical data on mutations†		10 out of 12 homozygous or compound heterozygous for null alleles	14 patients genotyped, 10 of whom had a mutation that is associated with increased clearance	

VWD = von Willebrand Disease, VWFpp = von Willebrand Factor propeptide, VWF:Ag = von Willebrand Factor antigen, VWF:Act = von Willebrand Factor activity, FVIII:C = factor VIII coagulation activity. VWFpp, VWF:Ag, VWF:Act and FVIII:C levels were measured centrally at time of inclusion in the study. *2 missing, †11 missing.

Bleeding phenotype, VWFpp and pathophysiological mechanism

Bleeding score was significantly higher in the type 3 patients with undetectable VWFpp than in those with detectable VWFpp levels: 19.5 [IQR 11.3-23.8] vs. 14.0 [IQR 7.0-17.0], p=0.025 (table 3). In patients with type 1 VWD, the BS was not associated with the pathophysiological mechanisms of VWD (i.e. increased clearance, reduced synthesis, combination of increased clearance and reduced synthesis, or none of these mechanisms: median BS 10.0 vs. 9.0 vs. 8.5 vs. 9.0 respectively) (figure 3A). However, all type 2 patients with increased clearance, reduced synthesis or combination, had significantly higher BS than type 2 patients with none of these pathophysiologic mechanisms (p=0.010). Median BS in type 2 patients with increased clearance or a combination of increased clearance and reduced synthesis was significantly higher than in those with normal ratios: 13.0 [IQR 9.0-17.5] or 13.0 [IQR 8.0-16.3] vs. 7.5 [IQR 5.5-10.8], p=0.001 or p=0.007. Median BS in type 2 VWD characterized by reduced synthesis was 17.0 [IQR 8.0-26.0] vs. 7.5 [IQR 5.5-10.8] in those with normal ratios, p=0.051 (figure 3B, see also Supplemental figure 1).

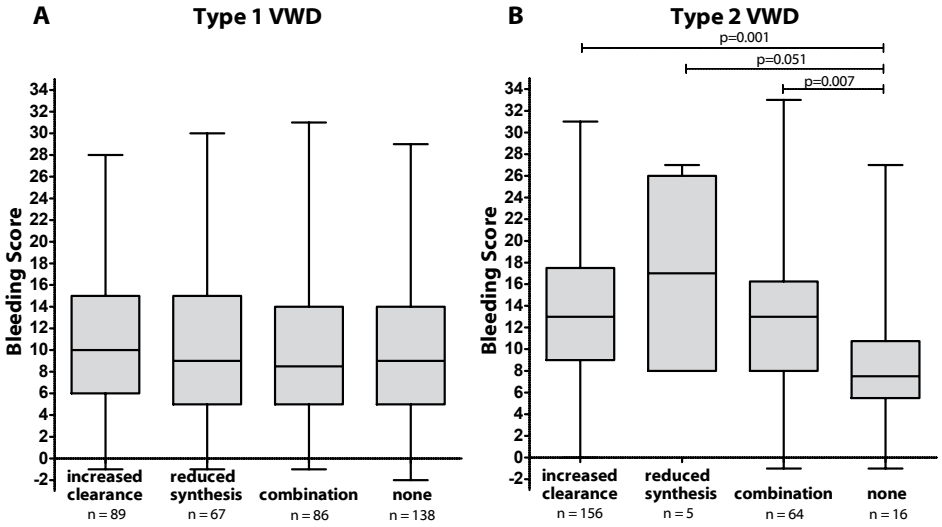


Figure 3. Bleeding score per pathophysiological mechanisms for type 1 and 2 von Willebrand Disease. Bleeding score according to different pathophysiological mechanisms of VWD for (A) type 1 VWD and (B) type 2 VWD. Boxplots show median, 25-75% interquartile ranges and minimum and maximum score.

DISCUSSION

Using data from our WiN study cohort, we are the first to report that VWFpp may help to improve the discrimination between type 3 VWD patients with a virtually complete absence of VWF:Ag and VWFpp and severe type 1 VWD patients with extremely rapid

VWF clearance leading to VWF:Ag plasma levels below 5 IU/dL and with relatively high VWFpp levels. Moreover, the ratios of VWF:Ag with VWFpp or FVIII:C differ between type 1 and 2 VWD. Type 2 VWD was mainly characterized by an increased clearance of VWF, and type 1 VWD had a more heterogeneous pathophysiology. Finally, low VWFpp was associated with higher BS in type 3 and type 2 VWD.

VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios have been shown to identify type 1 VWD patients by giving insight into the pathophysiological mechanisms behind the low VWF levels (i.e. increased clearance and reduced synthesis of VWF).[8,12,24] We confirmed this in our study and showed that the pathophysiology of type 1 VWD is diverse because our type 1 patients showed a mixture between VWF synthesis and clearance defects using the FVIII:C/VWF:Ag and VWFpp/VWF:Ag ratios. Almost two fifths of patients did not have increased ratios. VWD in these patients probably results from other yet unidentified mechanisms. Although VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios are correlated or consistent with increased clearance or reduced secretion of VWF, definitively demonstrating one mechanism or the other would require expression or clearance studies of the various VWD mutants.

We hypothesized that as a result of our inclusion criteria (historically lowest VWF levels <30 U/dL), we selected more type 1 patients with accelerated clearance of VWF or reduced synthesis, and therefore patients with higher ratios. However, the VWFpp levels and ratios thereof in our type 1 patients are in agreement with a previous study in type 1 VWD patients.[122] This is remarkable as in the MCMDM-1VWD study (Molecular and Clinical Markers for the Diagnosis and Management of Type 1 VWD), milder cases were also included. Regression to the mean or the effect of aging on VWF and FVIII levels may have been observed.

We observed a higher VWFpp/VWF:Ag ratio in type 2 VWD patients compared with type 1 VWD patients, suggesting a bigger contribution of increased clearance in type 2, whereas type 1 patients had a significantly higher FVIII:C/VWF:Ag ratio than type 2, indicating that reduced synthesis is more important as a pathophysiological mechanism in type 1 than type 2 VWD. Previous studies of type 2A mutations demonstrated reduced synthesis as a mechanism causing type 2A VWD; however, those *in vitro* expression studies could not analyze the component of clearance. In a recent expression study, it was shown that type 2A mutations are often characterized by a combination of intracellular retention, defective multimerization, loss of regulated secretion, and increased proteolysis by ADAMTS13.[25] Our data add to this increased clearance as an important component. This also suggests that these ratios may discriminate between type 1 and type 2 VWD, which has been stated before.[26]

The main pathophysiological mechanism of type 2 VWD is increased VWF clearance: as the majority of type 2A, 2B and 2M patients showed an increased VWFpp/VWF:Ag ratio. In addition, a small proportion of type 2 patients showed a combination of reduced VWF

survival and reduced synthesis, but this was only seen in 2A and 2M VWD. The effect of proteolysis of VWF on clearance has been argued.[22,27] Proteolysis itself probably does not play a major role in the VWF clearance. If increased proteolysis leads to loss of high molecular weight multimers only, with no shortening of the VWF half-life, then the ratio of VWFpp/VWF:Ag is not influenced by the proteolysis and this will not be picked up by the VWFpp/VWF:Ag ratio. The VWF type 2A mutations will often lead to a combination of pathophysiological mechanisms as reported by Jacobi et al.[25] In addition to increased susceptibility for ADAMTS13 proteolysis, the mutant VWF may also have a shortened half-life resulting in an increased VWFpp/VWF:Ag ratio. We found no differences between 2A VWD with defects in multimerization (and possibly enhanced ADAMTS13-mediated proteolysis) and 2M VWD with normal multimer patterns. Type 2M patients characterized by defects in collagen binding may have normal ratios and would require VWF collagen binding assay to be identified. Because of small sample size, these findings should be interpreted with care. Our data indicate that 2B VWD is completely characterized by a normal synthesis and an increased clearance of VWF, which has been shown before in 18 type 2B VWD patients.[28] It has been suggested that the mutant VWF is cleared faster in these patients because of the spontaneous binding of VWF to platelets.[29] Our results also showed that the mutant type 2N VWF is cleared faster than normal VWF.

To the best of our knowledge, this is the first study to assess VWFpp in VWD patients with VWF:Ag and VWF:Act <5 U/dL, the diagnostic criteria used in the current ISTH classification for type 3 VWD.[11] We showed that VWFpp is detectable in almost half of our type 3 VWD patients, indicating that VWF is actually produced but very rapidly cleared from the circulation in these patients. Our findings suggest that these type 3 VWD patients should actually reclassify as severe type 1 VWD patients. We therefore conclude that VWFpp can discriminate between type 3 VWD with complete absence of both VWF:Ag and VWFpp, and severe type 1 VWD with extremely low VWF levels. A significant proportion of these severely affected type 1 VWD patients had blood group O, which may have contributed to the low VWF levels in these patients. Distinguishing between type 3 VWD and severe type 1 VWD is of clinical importance because VWD patients with lack of VWF but with detectable VWFpp have clinical characteristics different from those of patients with a complete absence of both VWF and VWFpp.

Because VWF:Ag and FVIII:C levels are associated with bleeding phenotype in VWD,[14,16] it may be expected that VWFpp also associates with bleeding phenotype in VWD patients: either indirectly through its association with VWF:Ag or by binding to mature VWF in the circulation.[30] Interestingly, we found that the presence of VWFpp in patients with VWF:Ag levels <5 IU/dL correlates with a milder bleeding phenotype. We also observed that increased clearance, reduced synthesis and a combination thereof associates with higher BSs in type 2 patients.

To assess the functionality of VWF we used the HemosIL™ VWF Activity assay. We were not able to use the VWF:RCo test in this study for logistical reasons, although this is still the gold standard for assessing VWF functionality. To avoid the inevitable variability of VWF testing in the 13 different laboratories in the Netherlands, we preferred to perform all VWF tests centrally. This VWF:Act assay should be used with caution because it is not standard and does not completely replace the VWF:RCo test.

Mutation analysis of the *VWF* gene has been advised in type 2N VWD, type 2B VWD, and type 3 VWD.[31] However, sequencing of the *VWF* gene is expensive and not widely available. Looking at historical data on the molecular background of the type 3 VWD patients, we observed homozygosity and compound heterozygosity for null alleles in the type 3 VWD patients with complete absence of VWF:Ag and VWFpp. In the type 3 VWD patients with detectable VWFpp, missense mutations in the D3 and D4 domain of the *VWF* gene (p.R1205H, p.S1285P) were identified that are associated with accelerated clearance of VWF.[8,21,22] Therefore assessing VWFpp may substitute for *VWF* gene analyses because it identifies carriers of two null alleles and thus type 3 VWD without detectable VWFpp. In addition, the MCMDM-1VWD study has recently shown that a high VWFpp/VWF:Ag ratio predicts the detection of *VWF* gene mutations in type 1 VWD patients.[12] Furthermore, patients with mutations in regions of the *VWF* gene associated with reduced VWF survival can be identified with the VWFpp/VWF:Ag ratio.[8,21,22] On the basis of our results and the current literature, we believe that measurement of VWFpp should be implemented as a standard diagnostic in VWD for patients with low VWF levels and may render mutation analysis of the *VWF* gene unnecessary.

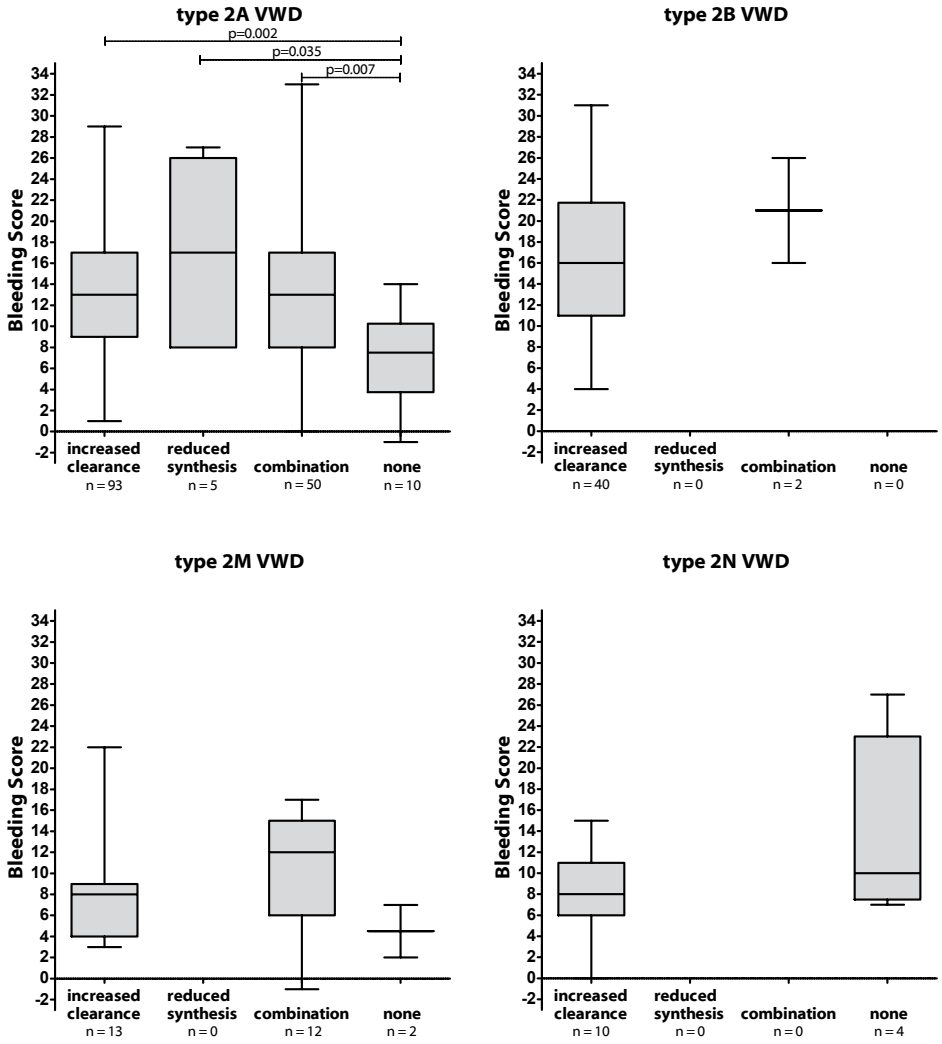
VWFpp measurement is of clinical importance because its ratio with VWF:Ag predicts reduced VWF survival and may therefore predict the VWF clearance after desmopressin treatment. In patients with increased VWF clearance, VWF disappears rapidly after desmopressin administration. The desmopressin response in these patients is probably sufficient for hemostasis in case of minor bleeding, but it is inadequate in case of major bleeding or surgery because these patients lack a sustained response.[21,32] The FVIII:C/VWF:Ag ratio can also help in the classification of VWD patients, as those patients with an increased FVIII:C/VWF:Ag mainly classify as type 1 VWD. With the current novel insights into the pathophysiology and molecular biology of VWD, the classification of VWD should be reviewed in the near future.

In conclusion, VWFpp discriminates between type 3 VWD patients with complete lack of VWF and severe type 1 VWD patients with very low VWF levels. An increased FVIII:C/VWF:Ag ratio may help identify type 1 VWD patients. This study shows the clinical significance of the VWFpp assay; it is of added value for the classification of VWD, for the assessment of the bleeding phenotype, and for genetic counseling. It also has therapeutic consequences.

REFERENCES

1. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost.* 2003;1(7):1335-1342.
2. Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J Clin Invest.* 1973;52(11):2757-2764.
3. Verweij CL, Diergaarde PJ, Hart M, Pannekoek H. Full-length von Willebrand factor (vWF) cDNA encodes a highly repetitive protein considerably larger than the mature vWF subunit. *EMBO J.* 1986; 5(8):1839-1847.
4. Wagner DD, Mayadas T, Marder VJ. Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. *J Cell Biol.* 1986;102(4):1320-1324.
5. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem.* 1998;67:395-424.
6. Vischer UM, Wagner DD. von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. *Blood.* 1994;83(12):3536-3544.
7. Borchiellini A, Fijnvandraat K, ten Cate JW, et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood.* 1996;88(8):2951-2958.
8. Haberichter SL, Balistreri M, Christopherson P, et al. Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood.* 2006;108(10):3344-3351.
9. Eikenboom JC, Castaman G, Kamphuisen PW, Rosendaal FR, Bertina RM. The factor VIII/von Willebrand factor ratio discriminates between reduced synthesis and increased clearance of von Willebrand factor. *Thromb Haemost.* 2002;87(2):252-257.
10. Lenting PJ, van Schooten CJ, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. *J Thromb Haemost.* 2007;5(7):1353-1360.
11. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost.* 2006;4(10):2103-2114.
12. Eikenboom J, Federici AB, Dirven RJ, et al. VWF propeptide and ratios between VWF, VWF propeptide and FVIII in the characterization of type 1 von Willebrand disease. *Blood.* 2013;121(12):2336-2339.
13. de Wee EM, Leebeek FW, Eikenboom JC. Diagnosis and management of von Willebrand disease in The Netherlands. *Semin Thromb Hemost.* 2011;37(5):480-487.
14. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost.* 2012;108(4):683-692.
15. de Wee EM, Mauser-Bunschoten EP, van der Bom JG, et al. Health-related quality of life among adult patients with moderate and severe von Willebrand disease. *J Thromb Haemost.* 2010;8(7):1492-1499.
16. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost.* 2006;4(4):766-773.
17. Tosetto A, Castaman G, Rodeghiero F. Bleeding scores in inherited bleeding disorders: clinical or research tools? *Haemophilia.* 2008;14(3):415-422.
18. Salem RO, Van Cott EM. A new automated screening assay for the diagnosis of von Willebrand disease. *Am J Clin Pathol.* 2007;127(5):730-735.
19. Chen D, Tange JI, Meyers BJ, Pruthi RK, Nichols WL, Heit JA. Validation of an automated latex particle-enhanced immunoturbidimetric von Willebrand factor activity assay. *J Thromb Haemost.* 2011;9(10): 1993-2002.

20. Caron C, Mazurier C, Goudemand J. Large experience with a factor VIII binding assay of plasma von Willebrand factor using commercial reagents. *Br J Haematol*. 2002;117(3):716-718.
21. Casonato A, Pontara E, Sartorello F, et al. Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. *Blood.* 2002;99(1):180-184.
22. Schooten CJ, Tjernberg P, Westein E, et al. Cysteine-mutations in von Willebrand factor associated with increased clearance. *J Thromb Haemost.* 2005;3(10):2228-2237.
23. Castaman G, Lethagen S, Federici AB, et al. Response to desmopressin is influenced by the genotype and phenotype in type 1 von Willebrand disease (VWD): results from the European Study MCMDM-1VWD. *Blood.* 2008;111(7):3531-3539.
24. Haberichter SL, Castaman G, Budde U, et al. Identification of type 1 von Willebrand disease patients with reduced von Willebrand factor survival by assay of the VWF propeptide in the European study: molecular and clinical markers for the diagnosis and management of type 1 VWD (MCMDM-1VWD). *Blood.* 2008;111(10):4979-4985.
25. Jacobi PM, Gill JC, Flood VH, Jakab DA, Friedman KD, Haberichter SL. Intersection of mechanisms of type 2A VWD through defects in VWF multimerization, secretion, ADAMTS-13 susceptibility, and regulated storage. *Blood*. 2012;119(19):4543-4553.
26. Casonato A, Daidone V, Padrini R. Assessment of von Willebrand factor propeptide improves the diagnosis of von Willebrand disease. *Semin Thromb Hemost.* 2011;37(5):456-463.
27. Stoddart JH, Jr., Andersen J, Lynch DC. Clearance of normal and type 2A von Willebrand factor in the rat. *Blood.* 1996;88(5):1692-1699.
28. Casonato A, Gallinaro L, Cattini MG, et al. Reduced survival of type 2B von Willebrand factor, irrespective of large multimer representation or thrombocytopenia. *Haematologica.* 2010;95(8):1366-1372.
29. Casari C, Du V, Wu YP, et al. Accelerated uptake of VWF/platelet complexes in macrophages contributes to VWD type 2B-associated thrombocytopenia. *Blood.* 2013;122(16):2893-2902.
30. Madabhushi SR, Shang C, Dayananda KM, et al. von Willebrand factor (VWF) propeptide binding to VWF D'D3 domain attenuates platelet activation and adhesion. *Blood.* 2012;119(20):4769-4778.
31. Peake IR, Goodeve AC. Genetic testing for von Willebrand disease: the case for. *J Thromb Haemost.* 2010;8(1):13-16.
32. Robertson JD, Yenson PR, Rand ML, et al. Expanded phenotype-genotype correlations in a pediatric population with type 1 von Willebrand disease. *J Thromb Haemost.* 2011;9(9):1752-1760.



Supplemental figure 1. Bleeding score per pathophysiological mechanisms for type 2 von Willebrand Disease variants.
Boxplots show median, 25-75% interquartile ranges and minimum and maximum score.

6

Dysregulated secretion of angiogenic mediators in patients with moderate and severe von Willebrand Disease

D Groeneveld, YV Sanders, J Adelmeijer, EP Mauser-Bunschoten, JG van der Bom, MH Cnossen, K Fijnvandraat, BAP Laros-van Gorkom, K Meijer, J de Meris, T Lisman, J Eikenboom, FWG Leebeek, and the WiN study group

Manuscript submitted for publication

ABSTRACT

Objective: it has recently been shown that von Willebrand Factor (VWF) can act as a negative regulator of angiogenesis. VWF is stored in endothelial cells in Weibel-Palade bodies (WPB) together with angiopoietin-2, osteoprotegerin and galectin-3. These proteins are known to mediate angiogenesis. We hypothesized that defects in VWF, potentially leading to impaired or loss of WPB formation, results in increased dysregulated release of angiogenic mediators in the circulation.

Approach and Results: We measured plasma levels of VEGF, angiopoietin-1 and 2, osteoprotegerin and galectin-3 in 380 type 1, 239 type 2 and 36 type 3 von Willebrand Disease (VWD) patients and 100 healthy controls. VWD patients had higher angiopoietin-1 and lower angiopoietin-2 levels compared with controls. VEGF levels were also higher in VWD patients and dependent upon VWF levels in plasma. These levels were higher in type 3 compared to type 1 patients. Type 1 and 2 VWD patients had lower angiopoietin-2 levels compared with controls, while type 3 VWD patients had similar angiopoietin-2 levels as controls. Type 3 VWD patients had higher osteoprotegerin levels compared with controls.

Conclusions: Patients with VWD have various disturbances in angiogenic markers in plasma. However, defects in VWF, leading to impaired or absent formation of WPB in VWD patients, do not consistently lead to dysregulated release of other WPB components. The variations in angiogenic mediators may contribute to the development of vascular malformations, such as angiodysplasia that are frequently observed in VWD patients.

INTRODUCTION

Von Willebrand Factor (VWF) is mainly produced in endothelial cells, although a small fraction of the total VWF pool is synthesized in megakaryocytes.[1-3] Upon formation of the multimeric VWF structure, the protein is either constitutively secreted into the blood, or stored in cell-specific organelles. In endothelial cells VWF is stored in the Weibel-Palade bodies (WPB).[4] VWF is essential for the formation of these WPBs, because absence of VWF leads to the loss of WPB formation.[5] Cells that normally do not express VWF form pseudo-WPB when transfected with VWF.[6,7]

Although VWF is best known for its role in hemostasis,[1,8] other functions have been identified in recent years, indicating that this protein is involved in multiple vascular processes (reviewed in [9]). Vascular malformations in von Willebrand Disease (VWD) patients, most frequently angiodysplasia in the gastrointestinal tract, have repeatedly been reported.[10,11] These malformations can be responsible for severe, intractable bleeding which is often not responsive to VWF replacement therapy and thus represent a significant unmet clinical challenge.[12] The pathological mechanism underlying vascular malformations in VWD is still largely unexplained. Recently it has been shown that VWF can act as a negative regulator of angiogenesis in vitro,[13] by inhibition of endothelial tube formation, migration and proliferation, in a Vascular Endothelial Growth Factor Receptor (VEGFR)-2 dependent process. Increased angiogenesis induced by VWF knockout in endothelial cells was associated with increased angiopoietin-2 (Ang-2) release into the medium. Blood Outgrowth Endothelial Cells (BOECs) of type 1 and 2 VWD patients confirmed low VWF levels lead to increased angiogenesis in vitro.[13]

The angiogenic mediators Ang-2, osteoprotegerin (OPG), galectin-3 (Gal-3) are synthesized in endothelial cells and stored in the WPB together with VWF.[14-16] Angiopoietin-1 (Ang-1) and Ang-2 work together with Vascular Endothelial Growth Factor-A (VEGF) to mediate angiogenesis. Ang-2 is an antagonist of Ang-1 and both angiopoietins bind to the TIE-2 receptor on endothelial cells.[17] Increased levels of Ang-2 results in destabilized vessels, while increased VEGF levels can lead to vascular malformations in humans.[18] OPG, a soluble decoy receptor for both receptor activator of nuclear factor-kappaB ligand (RANKL) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL), is a positive regulator of microvessel formation, promoting both endothelial cell sprouting and proliferation.[19] Gal-3 modulates VEGF-mediated angiogenesis by binding to integrin αVβ3 on endothelial cells, subsequently activating the signalling pathways that promote the growth of new vessels.[20] Since VWF is essential for the formation of WPB, other WPB components such as Ang-2, OPG and Gal-3 are dependent on VWF for their storage and secretion. We hypothesize that reduced levels or defects in VWF, which can lead to impaired WPB formation or complete loss of WPB, result in dysregulated release of other components of the WPB. The aim of this study was therefore to investigate

plasma levels of angiogenic mediators in patients with various types of VWD in order to obtain more insight in the development of vascular malformations frequently observed in these patients.

MATERIALS AND METHODS

Patients and controls

Stored plasma samples from the nationwide cross-sectional study among VWD patients in the Netherlands, the 'Willebrand in the Netherlands' (WiN) study were used for this study.[21-23] Patients diagnosed with type 1, 2 and 3 VWD who fulfilled the following inclusion criteria were initially included: (i) hemorrhagic symptoms or a family history of VWD; and (ii) historically lowest levels of VWF ≤30 U/dL (VWF:Ag and/or VWF:RCo) and/or FVIII:C ≤40 U/dL. Exclusion criteria for the current study were: patients who (i) were pregnant at time of inclusion, (ii) had blood products, (iii) had DDAVP infusion 72 hours prior to inclusion. Healthy individuals, who participated in the ATTAC study,[24] for which VWF:Ag and blood group were known, were included and matched for age, sex and blood group. A total of 100 controls were included in this study. The Medical Ethical Committees at all participating centers approved this study and written informed consent was obtained from all study participants.

Laboratory Measurements

Details regarding the blood sampling procedure, questionnaires and laboratory measurements of VWF:Ag and VWF:Act have been described in more detail by De Wee *et al*, Sanders *et al*, and De Bruijne *et al.*[22-24] VWFpp was measured as previously described.[25] At inclusion, peripheral venous blood was collected in tubes containing 3.2% (0.105 M) trisodium citrate. Subsequently, patient samples were centrifuged twice at 2,200 x g for 10 minutes at room temperature, control samples were centrifuged at 2000 x g for 10 minutes and plasma was additionally centrifuged at 20.000 x g for 10 minutes. Citrated platelet-poor plasma was aliquoted and stored at −80°C. Angiopoietin-1 levels were measured using the Human Angiopoietin-1 DuoSet ELISA kit (DY923, R&D Systems Europe, Abingdon, Oxon, UK). Angiopoietin-2 levels were measured using the Human Angiopoietin-2 DuoSet ELISA kit (DY623), galectin-3 levels with the Human Galectin-3 Duoset ELISA kit (DY1154), osteoprotegerin with the Human Osteoprotegerin/TNFRSF11B DuoSet ELISA kit (DY805), and VEGF levels with the Human VEGF DuoSet ELISA kit (DY293B, all R&D systems).

Statistical Analysis

Because data were not normally distributed, plasma levels are presented as median and 25-75% interquartile range (IQR). The Kruskal-Wallis test was used to test differences between types 1, 2A, 2B, 2M, 2N, 3 VWD and the control group. The Mann-Whitney U test was then used to detect differences between the groups. Correlations between different parameters were determined by Spearman correlation coefficient. A spearman r_s of <0.20 was regarded as very weak, 0.20-0.39 as weak, 0.40-0.59 as moderate and ≥ 0.6 as a strong correlation. Statistical analyses were performed with SPSS for Windows, version 22.0 (SPSS Inc, Chicago, USA). A p-value <0.05 was considered statistically significant.

RESULTS

A total of 804 moderate and severe VWD patients participated in the WiN-study. From 681 patients plasma samples were obtained, 26 patients were excluded because of pregnancy (n=7), recent use of desmopressin or replacement therapy (n=17) or insufficient plasma sample (n=2). Therefore a total of 655 VWD patients were included in this study; 380 patients were classified as type 1 VWD, 239 as type 2 VWD and 36 as type 3 VWD according to the 2006 classification.[26] Fifteen patients who were initially diagnosed with type 3 VWD, had VWFpp levels >5 U/dL, and as a consequence these patients were included in the type 1 VWD group for the current analysis. Patient characteristics are given in Table 1.

Angiogenic mediators according to age, VWF:Ag levels and von Willebrand Disease

Both in VWD patients and controls OPG and VWF:Ag levels were correlated with age. In VWD patients VEGF, OPG and Gal-3 levels were significantly correlated with VWF:Ag levels (r = -0.129, r = 0.089, r= 0.083) (Table 2). Considering all VWD patients together, patients had higher Ang-1 (median, 0.91 [IQR 0.51-1.56] ng/ml) and VEGF levels (median 5 [IQR 0-17] pg/ml) than healthy controls (0.14 [IQR 0.03-0.34] ng/ml, p=0.0002 and 0 [IQR 0-10] pg/ml, p<0.0001). Ang-2 levels were lower in VWD patients (median 0.12 [IQR 0-0.26] ng/ml) than in controls (0.20 [IQR 0.12-0.33] ng/ml, p<0.0001), while OPG and Gal-3 levels did not differ between patients and controls (Table 3).

Median Ang-1 levels were higher in type 1 (0.89 [IQR 0.51-1.54] ng/ml, p<0.0001), type 2 (0.95 [IQR 0.5-1.66] ng/ml, p<0.0001) and type 3 (1.32 [IQR 0.79-1.97] ng/ml, p<0.0001) VWD patients than in healthy controls (0.14 [IQR 0.03-0.34] ng/ml) (Table 3). In contrast, median levels of Ang-2 were lower in type 1 (0.1 [IQR 0-0.24] ng/ml, p<0.0001) and type 2 VWD patients (0.14 [IQR 0-0.29] ng/ml, p=0.003) than in healthy controls (0.20 [IQR 0.12-0.33] ng/ml). Type 3 VWD patients had comparable Ang-2 levels (median 0.16 [IQR 0.04-0.29] ng/ml, p=0.17) as controls (Table 3). Median VEGF levels were higher in type

Table 1. Baseline characteristics.

Characteristics	VWD patients (n = 655)	Controls (n = 100)
Age (years), median (range)	44 (1-83)	43 (18-56)
Male sex, n (%)	250 (38)	38 (38)
VWD type, n (%)		
1	395 (60)	
2	239 (36)	
2A	*156*	
2B	*43*	
2M	*26*	
2N	*14*	
3	21 (3)	
Blood group O, n (%)*	396 (61)	56 (56)
VWF:Ag (IU/dL), median [IQR]	29 [18-45]	91 [77-125]
VWF:CB (IU/dL), median [IQR]	22 [7-51]	108 [88-139]
VWF:Act (IU/dL), median [IQR]	23 [8-52]	
FVIII:C (IU/dL), median [IQR]	51 [32-73]	
Bleeding score, median [IQR] †	11 [6-16]	
Bleeding in last year, n (%) ‡		
Yes	180 (31)	
GI bleeding	*9 (5)*	
No	420 (69)	

VWF:Ag = von Willebrand Factor Antigen, VWF:CB = von Willebrand Factor Collagen Binding activity, VWF:Act = von Willebrand Factor Activity, FVIII:C = factor VIII coagulation activity, GI= gastrointestinal. * 6 missing, † 32 missing, ‡ 46 missing.

Table 2. Correlations between parameters.

Relation	Group	Spearman r	p-value
VEGF and VWF:Ag	Patients	-0.129	0.001
OPG and VWF:Ag	Patients	0.089	0.023
Gal-3 and VWF:Ag	Patients	0.083	0.033
VEGF and Age	Patients	-0.280	< 0.001
OPG and Age	Patients	0.382	< 0.001
Gal-3 and Age	Patients	0.260	<0.001
OPG and Age	Controls	0.294	0.003
VWF:Ag and Age	Patients	0.276	<0.001
VWF:Ag and Age	Controls	0.203	0.043

1, 2 and 3 VWD patients compared with healthy individuals (4 [IQR 0-15] pg/ml, p<0.001 for type 1 patients, 4 [IQR 0-21] pg/ml, p<0.001 for type 2 patients and 16 [IQR 7-52] pg/ml, p<0.0001 for type 3 patients vs 0 [IQR 0-10] pg/ml for controls) (Table 3).

Table 3. Angiogenic parameters in von Willebrand Disease patients and controls.

Angiogenic mediator	Controls (n = 100)	All Patients (n = 655)	Type 1 (n = 395)	Type 2 (n = 239)	Type 3 (n = 21)
Ang-1	0.14 [0.03-0.34]	0.91 [0.51-1.56] *	0.89 [0.51-1.54] *	0.95 [0.50-1.66] *	1.32 [0.79-1.97] *
Ang-2	0.20 [0.12-0.33]	0.12 [0-0.26] *	0.10 [0-0.24] * §	0.14 [0-0.29] ‡	0.16 [0.04-0.29]
VEGF	0 [0-10]	5 [0-17] †	4 [0-15] * ¶	4 [0-21] † ¶	16 [7-52] *
OPG	1.04 [0.77-1.29]	1.1 [0.85-1.48]	1.10 [0.85-1.44]	1.10 [0.84-1.56]	1.28 [0.89-1.86]
Gal-3	2.12 [1.6-2.76]	2.0 [1.28-2.77]	2.06 [1.36-2.78]	1.95 [1.2-2.78]	1.68 [0.93-2.70]

Variables are expressed as medians with 25% to 75% interquartile range between square brackets. * p<0.0001, † p<0.001, ‡ p<0.01 compared with controls. § p<0.05 compared with type 2. ¶ p<0.01 compared with type 3.

Type 3 VWD patients tended to have higher Ang-1 levels than type 1 VWD patients (median 1.32 [IQR 0.79-1.97] ng/ml vs 0.89 [IQR 0.51-1.54] ng/ml, p=0.07)(Table 3). Ang-2 levels were lower in type 1 VWD patients compared with type 2 VWD patients (median 0.1 [IQR 0-0.24] ng/ml vs 0.14 [IQR 0-0.29] ng/ml, p=0.04) (Table 3). In addition, type 3 VWD patients had the highest VEGF levels compared with both type 1 (16 pg/ml vs 4 pg/ml, p=0.0014) and type 2 VWD patients (16 pg/ml vs 4 pg/ml, p=0.006) (Table 3). OPG levels did not differ between type 1, 2 or 3 VWD patients and controls, although there was a trend towards higher levels in the type 3 VWD (median 1.28 [IQR 0.98-1.86] ng/ml) compared with type 1 (median 1.1 [IQR 0.85-1.44] ng/ml, p=0.08), type 2 VWD (median 1.1 [IQR 0.84-1.56] ng/ml, p=0.15) patients and controls (median 1.0 [IQR 0.77-1.29] ng/ml, p=0.02) (Table 3). Also Gal-3 levels did not differ between type 1, 2 or 3 VWD patients and healthy controls, although the type 3 VWD patients had slightly lower plasma levels (median 1.68 [IQR 0.93-2.7] ng/ml) compared with controls (median 2.12 [IQR 1.6-2.76] ng/ml, p=0.07) and type 1 and 2 VWD patients (median 2.06 [IQR 1.36-2.78] ng/ml, p=0.12 and 1.95 [IQR 1.2-2.78] ng/ml, p=0.26 respectively) (Table 3).

Angiogenic markers according to subtypes of type 2 von Willebrand Disease

Since type 2 VWD patients have different qualitative defects in their VWF, there might be diverse consequences on the various WPB components in the different VWD type 2 subtypes. Type 2 VWD patients were subdivided based on the ISTH subtype classification.[26] Our study had 156 type 2A, 43 type 2B, 26 type 2M and 14 type 2N patients. As shown in Table 4, compared with controls type 2A, 2B, 2M and 2N VWD patients had higher Ang-1 levels (p<0.0001). The median Ang-1 levels are higher in the type 2A (1.04 [IQR 0.61-1.78] ng/ml, p=0.0012) and 2M VWD (1.11 [IQR 0.65-1.69] ng/ml, p=0.02) compared with type 2B VWD (0.70 [IQR 0.27-1.1] ng/ml). Ang-1 levels were slightly lower in the type 2N VWD group (median 0.74 [IQR 0.35-1.41] ng/ml), but this difference did not reach statistical significance (Table 4). Ang-2 levels were only significantly lower in type 2A (median 0.14 [IQR 0.02-0.30] ng/ml, p=0.005) patients compared with controls (median 0.2 [IQR 0.12-0.33] ng/ml)(Table 4). Although there was no difference in Ang-2 levels within the type

Table 4. Angiogenic parameters in type 2 von Willebrand Disease patients and controls.

Angiogenic mediator	Controls (n = 100)	Type 2 (n = 239)	Type 2A (n = 156)	Type 2B (n = 43)	Type 2M (n = 26)	Type 2N (n = 14)
Ang-1	0.14 [0.03-0.34]	0.95 [0.50-1.66] *	1.04 [0.61-1.78] * ¶	0.70 [0.27-1.10] *	1.11 [0.65-1.69] * ††	0.75 [0.35-1.41] *
Ang-2	0.20 [0.12-0.33]	0.14 [0-0.29] ‡	0.14 [0.02-0.30]*	0.21 [0.03-0.34]	0.02 [0-0.32] §	0.11 [0.02-0.40]
VEGF	0 [0-10]	4 [0-21] †	4 [0-18] ‡	3 [0-26]	9 [0-28] ‡	7 [2-58] ‡
OPG	1.04 [0.77-1.29]	1.10 [0.84-1.56]	1.12 [0.84-1.56]	1.09 [0.84-1.59]	0.95 [0.61-1.19]	1.43 [0.94-1.75]
Gal-3	2.12 [1.60-2.76]	1.95 [1.20-2.78]	1.98 [1.27-2.78]	1.67 [0.99-2.39]	2.4 [1.68-3.23]	1.48 [0.99-3.43]

Variables are expressed as medians with 25% to 75% interquartile range between square brackets. [25-75% IQR]. * p<0.0001, † p<0.001, ‡ p<0.01, § p<0.05 compared with controls. ¶ p<0.01, †† p<0.05 compared with type 2B VWD.

2 VWD group, the type 2B VWD patients (median 0.21 [IQR 0.03-0.34] ng/ml) had slightly higher levels compared with the other type 2 VWD patients (Table 4). VEGF levels were higher in type 2A (median 4 [IQR 0-18] pg/ml, p=0.0048), 2M (median 9 [IQR 0-28] pg/ml, p=0.0038) and 2N (median 7 [IQR 1.5-58] pg/ml, p=0.016) patients compared with controls (median 0 [IQR 0-10] pg/ml), but not in type 2B patients (median 3 [IQR 0-26] pg/ml, p=0.054). VEGF, OPG and Gal-3 levels did not differ between the different type 2 VWD patients (Table 4).

VEGF levels are strongly dependent upon VWF levels in plasma

In order to study the association between VWF:Ag levels and the angiogenic markers, we divided the VWD patients into 4 groups based on their VWF:Ag level; <10 (n=79), 10-20 (n=115), 21-30 (n=154), and >30 IU/dL (n=306). As shown in Figure 1A, patients with VWF:Ag levels below 10 IU/dL had higher VEGF levels (median 14 pg/ml, IQR 4-42) compared with patients with VWF:Ag levels between 10-20 IU/dL (median 5 [IQR 0-18] pg/ml, p=0.0006), patients with VWF:Ag levels between 21-30 IU/dL (median 3 [IQR 0-16] pg/ml, p<0.0001) and patients with VWF:Ag levels >30 IU/dL (median 4 [IQR 0-14] pg/ml, p<0.0001). If only patients with type 1 and 2 with VWF:Ag levels <10 IU/dl were considered, also these patients had the highest VEGF levels (Figure 1B,C). For the type 2 patients this was still observed after exclusion of type 2B patients (data not shown). Ang-1, Ang-2, OPG and Gal-3 levels did not differ between the subgroups based on VWF:Ag levels (data not shown).

Increased VWF clearance and impaired VWF synthesis

The ratio between VWF propeptide (VWFpp) and VWF:Ag (VWFpp/VWF:Ag) can be used to assess VWF clearance, while the ratio between FVIII coagulant activity (FVIII:C) and VWF:Ag (FVIII:C/VWF:Ag) can be used to assess VWF synthesis.[27] A high VWFpp/VWF:Ag ratio indicates increased clearance of VWF and a high FVIII:C/VWF:Ag ratio decreased synthesis of VWF. Since Ang-2, OPG and Gal-3 are stored together with VWF inside the WPB, we investigated if increased VWF clearance and/or impaired VWF synthesis influ-

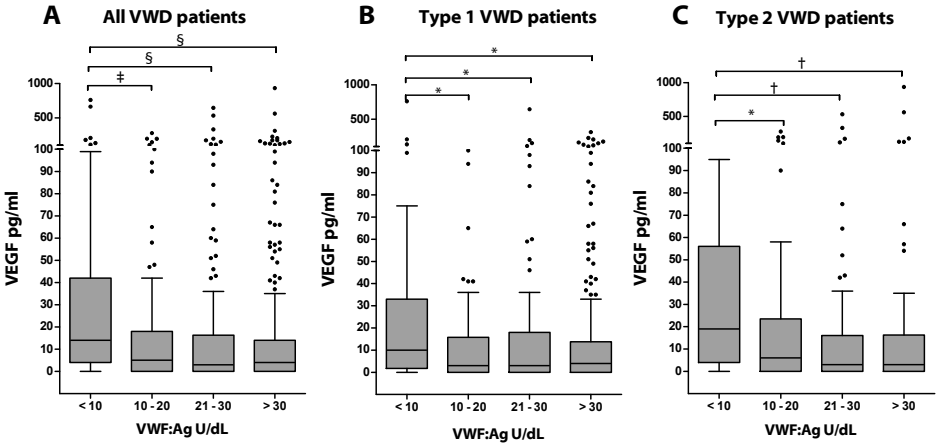


Figure 1. VEGF plasma levels in relation to VWF:Ag levels.
All patients were divided into four groups based on their VWF:Ag level: < 10U/dL, 10 - 20U/dL, 21 - 30U/dL and > 30U/dL. Panel A shows VEGF plasma levels of all VWD patients (< 10U/dL n=79, 10 - 20U/dL n=115, 21 - 30U/dL n=154, > 30U/dL n=306). In panel B only patients with type 1 VWD are plotted (n=34, n=50, n=79, n=232) and in panel C only patients with type 2 VWD are plotted (n=24, n=65, n=75, n=74). Box plots show median values and interquartile ranges. Whiskers show Tukey hinges; black circles are outliers. Asterisks denote significant deviations: * p≤0.05, † p≤0.01, ‡ p≤0.001, § p≤0.0001.

ences the plasma levels of the other WPB components. Within the type 1 VWD group we divided the patients into normal synthesis (n=219) or clearance (n=200) (based on the upper limit of normal range of VWFpp/VWF:Ag and FVIII:C/VWF:Ag ratios as described by Eikenboom et al[27]) or moderately and severely impaired synthesis (n=121 and 55) or increased clearance (n=137 and 58)(Figure 2). Median Ang-2 levels were higher in patients with very fast VWF clearance (arbitrary defined as a VWFpp/VWF:Ag ratio above 5) (median 0.16 [IQR 0.06-0.42] ng/ml), compared with patients with normal (median 0.09 [IQR 0-0.22] ng/ml, p=0.0021) or moderately increased clearance (median 0.09 [IQR 0-0.24] ng/ml, p=0.018) (Figure 2A). OPG and Gal-3 levels were similar in patients with normal and increased clearance of VWF (Figure 2B-C). Although not significant, Ang-2 levels were lower in patients with normal VWF synthesis (ratio of FVIII:C/VWF:Ag ≤1.9) compared with impaired VWF synthesis (ratio above 1.9)(Figure 2D). OPG levels were similar between patients with normal and impaired VWF synthesis (Figure 2E). Patients with normal VWF synthesis had significantly higher Gal-3 levels (median 2.15 [IQR 1.56-2.86] ng/ml) compared with patients with moderately impaired synthesis (median 1.91 [IQR 1.28-2.67] ng/ml, p=0.045) and severely impaired synthesis (median 1.74 [IQR 1.09-2.77] ng/ml, p=0.028)(Figure 2F).

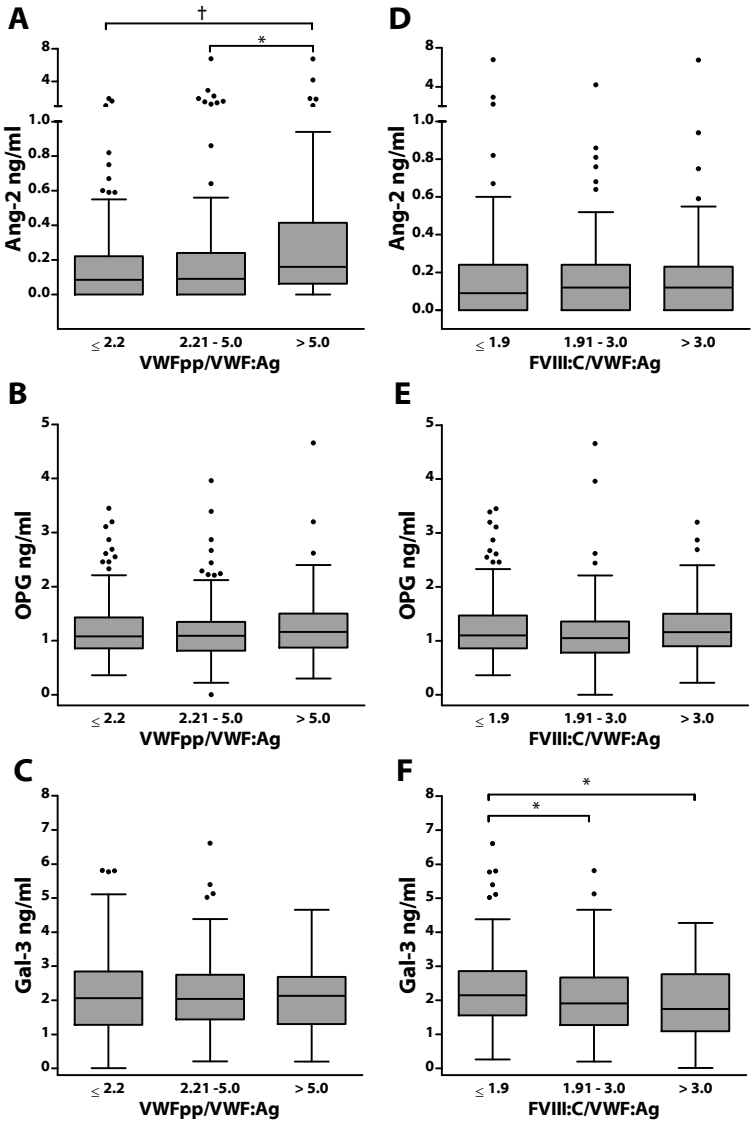


Figure 2. Plasma levels of WPB components Ang-2, OPG and Gal3 in relation to VWF synthesis and clearance.

VWFpp/VWF:Ag was used as a parameter for VWF clearance rate (A-C). Within the type 1 VWD group (n=395), patients were categorized in three groups: VWFpp/VWF:Ag ratio of ≤ 2.2 (n=200), 2.21 – 5 (n=137) and > 5 (n=58). Panel A shows levels of Ang-2, panel B shows OPG levels and panel C shows Gal-2 levels. The FVIII:C/VWF:Ag ratio was used as a parameter for VWF synthesis (D-F). Type 1 VWD patients were categorized into three groups based on their FVIII:C/VWF:Ag ratio: <1.9 (n=219), 1.9 – 3 (n=121) and >3 (n=55). Panel D shows levels of Ang-2, panel E shows OPG levels and panel F shows Gal-2 levels. The cutoffs are based on the upper limit of normal range as described by Eikenboom et al.[27] Box plots show median values and interquartile ranges. Whiskers show Tukey hinges; black circles are outliers. Asterisks denote significant deviations: * p≤0.05, † p≤0.01.

Angiogenic mediators and lack of Height Molecular Weight (HMW) Multimers

Angiodysplasia is mostly reported in type 2A, 2B[28-30] and type 3 VWD[10] patients, i.e. in patients lacking HMW multimers. Patients were divided into normal VWF multimer pattern (total n=339, type 1=300, type 2M =25, type 2N=14) or lacking the HMW multimers (total n=111, type 2A=76, type 2B=35) based on multimer analysis. We observed higher Ang-2 levels in the subgroups of VWD patients lacking the HMW multimers (median 0.14 [IQR 0.02-0.29] ng/ml) compared with those with normal multimeric pattern (median 0.09 [IQR 0-0.24] ng/ml, p=0.02) (Figure 3B). Although not significant, also Ang-1 levels are increased in patient lacking the HMW multimers (median 0.89 [IQR 0.51-1.47] ng/ml

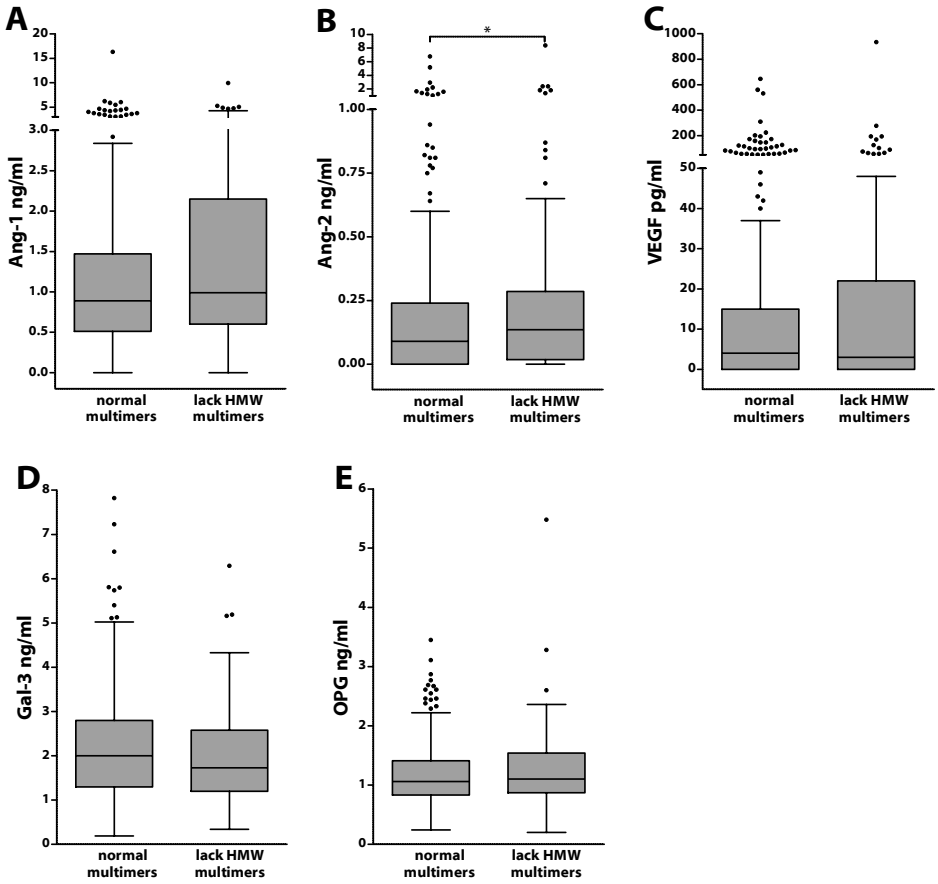


Figure 3. Plasma levels of angiogenic mediators in relation to VWF multimer pattern. Patients with normal VWF multimer pattern (Total n=339; type 1 = 300, type 2M = 25, type 2N = 14) and patients who lack the high molecular weight (HMW) multimers (Total n=111; type 2A = 76, type 2B = 35) are plotted for their plasma levels of (A) Ang-1, (B) Ang-2, (C) VEGF, (D) OPG, and (E) Gal-3. Box plots show median values and interquartile ranges. Whiskers show Tukey hinges; black circles are outliers. Asterisks denote significant deviations: * p≤0.05.

vs 0.99 [IQR 0.60-2.15] ng/ml, p=0.09), while Gal-3 levels were lower in these patients (median 2.0 [IQR 1.3-2.8] ng/ml vs 1.73 [IQR 1.2-2.58], p=0.0733) (Figure 3A, E). VEGF and OPG levels were comparable between the two patient groups (Figure 3C-D).

Angiogenic mediators and gastrointestinal bleeding

To investigate whether dysregulation of angiogenic mediators could contribute to vascular malformations leading to gastrointestinal bleedings, we analysed patients who experienced gastrointestinal bleedings in the last year (n=9) versus patients with bleedings elsewhere (n=180) and patients without bleedings in the year before inclusion (n=420). We observed higher Ang-2 levels in patients who bled (median 0.15 [IQR 0.01-0.29] ng/ml) compared with patients who did not experience bleedings (median 0.09 [IQR 0-0.25] ng/ml, p=0.03). Due to a lack of power, considering the small group of VWD patients with gastrointestinal bleedings we could not detect other differences between those with and without GI bleeding.

DISCUSSION

VWF is a negative regulator of (in vitro) angiogenesis and is stored in WPBs in endothelial cells.[13] Besides VWF also other angiogenic mediators are stored within the WPBs. In this study we investigated plasma levels of angiogenic mediators in patients with VWD in order to obtain more insight in the development of vascular malformations frequently observed in patients with VWD. We observed that several of the measured angiogenic mediators are significantly different in VWD patients compared to healthy controls. Also within the VWD patient groups variations in these levels were observed.

We found higher Ang-1 and VEGF plasma in patients with VWD, with the highest levels in type 3 patients, which is in line with a previously published study.[31] VEGF is the best characterized pro-angiogenic endothelial growth factor.[32,33] At high concentrations however, VEGF induces an irregular vasculature with fragile and frequently hemorrhagic vessels, reminiscent of angiodysplasia,[34] and increased VEGF levels have been reported in patients presenting with angiodysplasia and in patients with hereditary hemorrhagic telangiectasia.[35,36] Higher VEGF levels in VWD patients could therefore contribute to vascular malformations frequently seen in these patients.[12] However, care should be taken interpreting the increased VEGF and Ang-1 plasma levels. Since platelets store a large amount of Ang-1 and VEGF, circulating levels of these proteins may be biased by platelet count, *in vivo* platelet activation and unintentional platelet activation during blood collection.[37] Centrifugation steps were slightly different between patients and controls and we cannot exclude that this might have led to differences in *ex vivo* platelet activation. Since the first step of centrifugation is similar between patients and

controls, and most platelets are discarded after this step, potential *ex vivo* activation is presumably comparable between patients and controls. Nonetheless, plasma collection was identical for all VWD patients, and thus within the VWD group this is not an issue. Platelet counts were not documented in this study, but most VWD patients have normal platelet numbers.[36] It has been shown that megakaryocytopoiesis is modified by the enhanced VWF-GPIbα interactions in patients with type 2B VWD,[38] and VEGF and Ang-1 levels could be influenced by the abnormal megakaryocytopoiesis. However, when type 2B patients were excluded from the type 2 groups for analysis, differences were still observed. VEGF is also produced by endothelial cells and the higher levels of VEGF in VWD patients could be a consequence of perturbed intracellular and extracellular pathways leading to increased VEGF expression in the absence of VWF.[12,39] This may also explain why patients with the lowest VWF:Ag levels (type 3 VWD patients and patients with a VWF:Ag <10 IU/dL) have the highest VEGF levels.

We speculated that defects in VWF, potentially leading to impaired or absent WPB formation, enhanced the release of other components of the WPB. In accordance with this hypothesis we found higher Ang-2 and OPG levels in type 3 VWD patients compared with type 1 and 2 VWD patients. However, the opposite was observed for Gal-3 levels; Gal-3 levels were the lowest in type 3 VWD patients. In VWF deficient mice Gal-3 levels were also reduced compared with wild-type mice[14], confirming the results observed in our type 3 VWD patients. In VWF deficient mice the reduced Gal-3 levels were corrected following hydrodynamic gene transfer of VWF, indicating that VWF contributes to the regulation of Gal-3 plasma levels. In the circulation Gal-3 is bound to VWF,[14,40] and in the absence of VWF, Gal-3 co-precipitated with FVIII.[40] In type 3 VWD patients FVIII levels are very low (FVIII:C 1 IU/dL, IQR 1-3 in our group), because the half-life of free FVIII is very short compared with VWF-bound FVIII. In the absence of VWF, levels of circulating Gal-3 could be lower because complex formation with VWF, which may promote the circulatory lifespan of Gal-3, cannot occur or complex formation with free FVIII in the absence of VWF, promotes faster clearance.

The decreased Ang-2 levels observed in VWD patients compared with controls is contradictory to previous published results obtained from cultured endothelial cells from VWD patients.[12,41] The difference could be explained by lack of a clearance mechanism in the culture experiments. Since Ang-2 levels were measured in supernatants of cultured cells, clearance of Ang-2, which could play a role *in vivo*, is not affected in those experiments. Furthermore, Ang-2 levels were only measured in VWF siRNA-treated human umbilical vein endothelial cells, and levels in BOECs from VWD patients were unfortunately not reported. Both Ang-1 and Ang-2 bind to the TIE-2 (Tyrosine kinase with Immunoglobulin-like and EGF-like domains-2) receptor on endothelial cells[17,42] and activation of TIE-2 by Ang-1 binding induces intracellular signalling pathways leading to down-regulation of Ang-2 production in endothelial cells.[42] This interaction however,

does not occur in cultured endothelial cells because Ang-1 is absent in culture experiments.

The pathological mechanism underlying angiodysplasia in VWD is still largely unexplained. We observed higher Ang-2 levels in patients who had bleedings compared with patients who did not experience bleedings, but due to the small group of patients (n=9) with gastrointestinal bleedings we could not detect further significant differences. Higher Ang-2 levels, combined with lower Ang-1 levels could contribute to the formation of fragile vessels seen in angiodysplasia, but more patients with a recent history of gastrointestinal bleeding should be analyzed to validate this hypothesis.

With regard to the WPB components Gal-3, Ang-2 and OPG, the functional consequences of their binding to VWF have been investigated to a limited extent, if at all. Being in complex with VWF may prevent the interaction with their natural ligand via sterical hindrance, as is the case for FVIII. However, information is scarce on this issue, and it would be interesting to examine the shared functional effects between VWF and its binding partners. Since endothelial cells contain several other VWF-binding proteins with pro- and anti-angiogenic properties that we did not study, such as galectin-1, connective tissue growth factor and insulin-like growth factor binding protein-7, it is very likely that VWF plays an exceedingly complex role in regulating angiogenesis at different levels. Characterisation of the molecular pathways through which VWF regulates angiogenesis can provide novel therapeutic targets for the treatment of angiodysplastic gastrointestinal bleeding, since prophylaxis is less successful at reducing gastrointestinal blood loss than it is for reducing blood loss at different sites in the body.[43]

In conclusion, VWD patients have several abnormalities in the levels of angiogenic mediators in plasma compared to controls. Impaired formation or loss of WPB in VWD patients leads to both increased and decreased plasma levels of angiogenic mediators compared with healthy individuals. The dysregulation between these angiogenic mediators could contribute to the vascular malformation frequently observed in these patients.

REFERENCES

1. Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem*. 1998;67:395-424.
2. Wagner DD. Cell biology of von Willebrand factor. *Annu Rev Cell Biol*. 1990;6:217-246.
3. Sporn LA, Chavin SI, Marder VJ, Wagner DD. Biosynthesis of von Willebrand protein by human megakaryocytes. *J Clin Invest*. 1985;76(3):1102-1106.
4. Valentijn KM, Sadler JE, Valentijn JA, Voorberg J, Eikenboom J. Functional architecture of Weibel-Palade bodies. *Blood*. 2011;117(19):5033-5043.
5. Wagner DD, Saffaripour S, Bonfanti R, et al. Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell*. 1991;64(2):403-413.
6. Michaux G, Hewlett LJ, Messenger SL, et al. Analysis of intracellular storage and regulated secretion of 3 von Willebrand disease-causing variants of von Willebrand factor. *Blood*. 2003;102(7):2452-2458.
7. Metcalf DJ, Nightingale TD, Zenner HL, Lui-Roberts WW, Cutler DF. Formation and function of Weibel-Palade bodies. *J Cell Sci*. 2008;121(Pt 1):19-27.
8. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*. 1996;84(2):289-297.
9. Lenting PJ, Casari C, Christophe OD, Denis CV. von Willebrand factor: the old, the new and the unknown. *J Thromb Haemost*. 2012;10(12):2428-2437.
10. Fressinaud E, Meyer D. International survey of patients with von Willebrand disease and angiodysplasia. *Thromb Haemost*. 1993;70(3):546.
11. Koscielny JK, Latza R, Mursdorf S, et al. Capillary microscopic and rheological dimensions for the diagnosis of von Willebrand disease in comparison to other haemorrhagic diatheses. *Thromb Haemost*. 2000;84(6):981-988.
12. Randi AM, Laffan MA, Starke RD. Von Willebrand factor, angiodysplasia and angiogenesis. *Mediterr J Hematol Infect Dis*. 2013;5(1):e2013060.
13. Starke RD, Ferraro F, Paschalaki KE, et al. Endothelial von Willebrand factor regulates angiogenesis. *Blood*. 2011;117(3):1071-1080.
14. Saint-Lu N, Oortwijn BD, Pegon JN, et al. Identification of galectin-1 and galectin-3 as novel partners for von Willebrand factor. *Arterioscler Thromb Vasc Biol*. 2012;32(4):894-901.
15. Shahbazi S, Lenting PJ, Fribourg C, Terraube V, Denis CV, Christophe OD. Characterization of the interaction between von Willebrand factor and osteoprotegerin. *J Thromb Haemost*. 2007;5(9):1956-1962.
16. Fiedler U, Scharpfenecker M, Koidl S, et al. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood*. 2004;103(11):4150-4156.
17. Felcht M, Luck R, Schering A, et al. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. *J Clin Invest*. 2012;122(6):1991-2005.
18. Cao Y, Sonveaux P, Liu S, et al. Systemic overexpression of angiopoietin-2 promotes tumor microvessel regression and inhibits angiogenesis and tumor growth. *Cancer Res*. 2007;67(8):3835-3844.
19. McGonigle JS, Giachelli CM, Scatena M. Osteoprotegerin and RANKL differentially regulate angiogenesis and endothelial cell function. *Angiogenesis*. 2009;12(1):35-46.
20. Markowska AI, Liu FT, Panjwani N. Galectin-3 is an important mediator of VEGF- and bFGF-mediated angiogenic response. *J Exp Med*. 2010;207(9):1981-1993.
21. de Wee EM, Leebeek FW, Eikenboom JC. Diagnosis and management of von Willebrand disease in The Netherlands. *Semin Thromb Hemost*. 2011;37(5):480-487.

22. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108(4):683-692.
23. Sanders YV, Eikenboom J, de Wee EM, et al. Reduced prevalence of arterial thrombosis in von Willebrand disease. *J Thromb Haemost*. 2013;11(5):845-854.
24. de Bruijne EL, Gils A, Guimaraes AH, et al. The role of thrombin activatable fibrinolysis inhibitor in arterial thrombosis at a young age: the ATTAC study. *J Thromb Haemost*. 2009;7(6):919-927.
25. Borchiellini A, Fijnvandraat K, ten Cate JW, et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood*. 1996;88(8):2951-2958.
26. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
27. Eikenboom J, Federici AB, Dirven RJ, et al. VWF propeptide and ratios between VWF, VWF propeptide and FVIII in the characterization of type 1 von Willebrand disease. *Blood*. 2013;121(12):2336-2339.
28. Castaman G, Federici AB, Tosetto A, et al. Different bleeding risk in type 2A and 2M von Willebrand disease: a 2-year prospective study in 107 patients. *J Thromb Haemost*. 2012;10(4):632-638.
29. Morris ES, Hampton KK, Nesbitt IM, Preston FE, Thomas EG, Makris M. The management of von Willebrand's disease-associated gastrointestinal angiodysplasia. *Blood Coagul Fibrinolysis*. 2001;12(2):143-148.
30. Iannuzzi MC, Hidaka N, Boehnke M, et al. Analysis of the relationship of von Willebrand disease (vWD) and hereditary hemorrhagic telangiectasia and identification of a potential type IIA vWD mutation (Ile865 to Thr). *Am J Hum Genet*. 1991;48(4):757-763.
31. Gritti G, Cortelezzi A, Bucciarelli P, et al. Circulating and progenitor endothelial cells are abnormal in patients with different types of von Willebrand disease and correlate with markers of angiogenesis. *Am J Hematol*. 2011;86(8):650-656.
32. Ferrara N. VEGF-A: a critical regulator of blood vessel growth. *Eur Cytokine Netw*. 2009;20(4):158-163.
33. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9(6):669-676.
34. Ozawa CR, Banfi A, Glazer NL, et al. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. *J Clin Invest*. 2004;113(4):516-527.
35. Sadick H, Riedel F, Naim R, et al. Patients with hereditary hemorrhagic telangiectasia have increased plasma levels of vascular endothelial growth factor and transforming growth factor-beta1 as well as high ALK1 tissue expression. *Haematologica*. 2005;90(6):818-828.
36. Junquera F, Saperas E, de Torres I, Vidal MT, Malagelada JR. Increased expression of angiogenic factors in human colonic angiodysplasia. *Am J Gastroenterol*. 1999;94(4):1070-1076.
37. Brouwers J, Noviyanti R, Fijnheer R, et al. Platelet activation determines angiopoietin-1 and VEGF levels in malaria: implications for their use as biomarkers. *PLoS One*. 2014;8(6):e64850.
38. Nurden P, Gobbi G, Nurden A, et al. Abnormal VWF modifies megakaryocytopoiesis: studies of platelets and megakaryocyte cultures from patients with von Willebrand disease type 2B. *Blood*. 2010;115(13):2649-2656.
39. Shao ES, Lin L, Yao Y, Bostrom KI. Expression of vascular endothelial growth factor is coordinately regulated by the activin-like kinase receptors 1 and 5 in endothelial cells. *Blood*. 2009;114(10):2197-2206.
40. O'Sullivan JM, Rawley O, Jenkins V, Chion AC, Brophy TM, O'Donnell JS. Identification of Galectin-1 and Galectin-3 as novel binding partners for factor VIII. [Abstract]. *Blood*. 2013;122(21):28.

41. Starke R, Paschalaki KE, Dyer C, et al. Defective von Willebrand factor and angiopoietin-2 release from von Willebrand disease patients' blood outgrowth endothelial cells. [Abstract]. *Heart*. 2013; 99(suppl 2):A106.
42. Daly C, Wong V, Burova E, et al. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). *Genes Dev*. 2004;18(9):1060-1071.
43. Abshire TC, Federici AB, Alvarez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand Disease Prophylaxis Network (VWD PN). *Haemophilia*. 2013;19(1): 76-81.

7

Bleeding spectrum in children with moderate or severe von Willebrand Disease: relevance of pediatric-specific bleeding

YV Sanders, K Fijnvandraat, J Boender, EP Mauser-Bunschoten, JG van der Bom, J de Meris, FJ Smiers, P Brons, R Tamminga, MH Cnossen, FWG Leebeek, and the WiN study group

Manuscript submitted for publication

ABSTRACT

Background: The bleeding phenotype of children with Von Willebrand Disease (VWD) needs to be characterized in detail to facilitate diagnosis during childhood and aid in the planning of treatment strategies.

Objectives: to evaluate the occurrence, type and severity of bleeding in a large cohort of children with moderate and severe VWD.

Patients/methods: We included 113 children (aged 0-16 years) with type 1 (n=60), 2 (n=44) and 3 (n=9) VWD with VWF:Ag and/or VWF:RCo levels ≤30 U/dL in a nation-wide cross-sectional study (Willebrand in the Netherlands "WiN" study). Bleeding severity was determined using the ISTH-BAT with supplementary pediatric-specific bleeding symptoms (umbilical stump bleeding, cephalohematoma, cheek hematoma, conjunctival bleeding, post-circumcision bleeding and post-venipuncture bleeding).

Results: All 26 post-menarche girls experienced menorrhagia. Other frequently occurring bleedings were cutaneous (81%), oropharyngeal (64%), prolonged bleeding from minor wounds (58%), and epistaxis (56%). Pediatric-specific bleeding symptoms were present in 44% of patients. ISTH-BAT was higher in index cases than in affected family members (median 12.0 vs. 6.5, $p<0.001$), higher in type 3 VWD than in type 2 or 1 (17.0 vs. 10.5 or 6.5, $p<0.001$) and higher in children with severe (<10 U/dL) than moderate VWD (10-30 U/dL) (11.0 vs 7.0, $p<0.001$).

Conclusions: Pediatric-specific bleeding symptoms occurred in a large proportion (44%) of children with moderate or severe VWD. Impressively, all post-menarche girls suffered from menorrhagia. Determinants of bleeding severity were index case, VWD type, VWD severity, and age. When evaluating children suspected of VWD, pediatric-specific bleeding symptoms should be included in the bleeding score.

INTRODUCTION

Von Willebrand disease (VWD) is the most common inherited bleeding disorder and is caused by quantitative or qualitative defects in Von Willebrand Factor (VWF).[1,2] Although patients frequently suffer from mucocutaneous bleeding, clinical expression is heterogeneous with a large inter and intra-individual variability of symptoms.[3,4] In general, the frequency and severity of bleeding is associated with the type of VWD and the level of functional VWF.

VWD is classified into three types according to the current guidelines of the International Society on Thrombosis and Haemostasis (ISTH).[5] The most common type (75%), type 1 VWD is a quantitative partial deficiency of VWF which is usually characterized by mild bleeding symptoms. Type 2 VWD accounts for 20% of patients and is described as a qualitative defect of VWF. It is subdivided into four categories: 2A, 2B, 2M and 2N, based on functional VWF differences. Although clinical symptoms vary between patients, in general the phenotype is more severe than in type 1 VWD. Type 3 VWD is rarely diagnosed, but clinically the most severe type and characterized by a virtually complete absence of VWF.

Due to their young age, children generally lack exposure to hemostatic challenges and may therefore not yet manifest bleeding symptoms, even when affected with VWD. As healthy children may also suffer from mucocutaneous bleeding, such as epistaxis, bruising and oropharyngeal bleeding,[6-8] bleeding during childhood does not necessarily reflect the presence of a bleeding disorder. Therefore, diagnosing VWD can be especially difficult in younger age groups.[2]

In recent years, bleeding scores (BS) have been developed that aim to discriminate between individuals with and without VWD by quantifying the number and severity of various bleeding symptoms.[9-11] For children, a pediatric bleeding questionnaire including pediatric-specific bleeding symptoms was developed for this purpose, of which the ISTH-BAT is currently the most recommended.[11,12] Studies have demonstrated the value of a pediatric BS in diagnosis of mild VWD and mild bleeding disorder, but the tool has never been used to analyze frequency and severity of bleeding symptoms in children with more severe VWD.[12-14]

Therefore, we assessed the overall bleeding phenotype in a large cohort of children with moderate and severe VWD from the "Willebrand in the Netherlands" (WiN) study and evaluated the value of the ISTH-BAT with regard to this specific group.

PATIENTS AND METHODS

Patients

Children from the nation-wide cross-sectional "Willebrand in the Netherlands" (WiN) study who had been diagnosed earlier with type 1, 2 or 3 VWD were included if the following inclusion criteria were met: (1) a hemorrhagic diathesis or a family history of VWD and (2) historically lowest VWF plasma levels ≤30 U/dL (VWF:Ag and/or VWF:RCo) and/or FVIII:C plasma levels ≤40 U/dL.[4,15,16] Patients were excluded if they were known to have other hemostatic disorders. In total 140 children (aged <16 years) were included between 2007 and 2009 in the study. This cohort was approached by a trained physician between 2013 and 2014 to analyze bleeding pattern using the new ISTH-BAT score. The Medical Ethical Committees in all participating centers approved the study; all participants gave informed consent. This study was also approved by the ISTH-BAT SSC (chair F. Rodeghiero).

Bleeding questionnaires and assessment methods

The ISTH-BAT systematically evaluates the severity and frequency of thirteen specific bleeding symptoms and pediatric-specific bleeding symptoms (umbilical stump bleeding, cephalohematoma, cheek hematoma caused by sucking during breast or bottle feeding, conjunctival hemorrhage, excessive post-circumcision bleeding and excessive post-venipuncture bleeding). Based on treatment for the specific bleeding, the bleeding episodes are scored on a scale that ranges from 0 to 4 points. The cut-off value of the ISTH-BAT for an abnormal BS was recently determined as ≥3 for children.[17] For this study, we also collected information on four additional pediatric-specific bleeding symptoms: bleeding from fetal scalp electrode, excessive bruising after birth, excessive bleeding after heel prick test and post-vaccination bleeding to explore the occurrence and severity of these bleedings and to be sure no pediatric-specific bleeding symptoms were missed. We did not score post-partum hemorrhage in this pediatric cohort.

During the telephone interview by a trained physician (YVS) in addition to the ISTH-BAT bleeding questionnaire and additional pediatric-specific bleeding symptoms, we also collected information on the use of desmopressin or replacement therapy with clotting factor concentrate. The time investment of the interview was 15 to 45 minutes per child. An overall BS was independently calculated by two physicians (YVS, JB). The additional pediatric-specific bleedings were not scored in the BS. If a patient had multiple pediatric-specific bleeding symptoms the most severe episode of pediatric-specific bleeding was scored.

According to the ISTH-BAT, the absence of bleeding symptoms after surgery or dental extraction were not scored if patients had received prophylactic treatment with desmopressin or replacement therapy before the surgical intervention or dental extraction.[4,18]

However, if bleeding did occur during intervention despite this prophylactic treatment, we did score this bleeding according to the ISTH-BAT.

Definitions

Moderate VWD was defined as VWF levels (VWF:Ag and/or VWF:CB and/or VWF:Act) 10-30 U/dL, and/or FVIII:C 20-40 U/dL and severe VWD as VWF levels (VWF:Ag and/or VWF:CB and/or VWF:Act) ≤10 U/dL, and/or FVIII:C ≤20 U/dL.[19] Patients were classified as moderate or severe VWD based on centrally measured VWF and FVIII levels. If blood was not obtained centrally, historically measured VWF parameters were used.[4]

The first patient in a family referred to a medical specialist because of hemorrhagic diathesis and diagnosed with VWD was classified as the index case. A patient diagnosed with VWD because of a previously diagnosed family member with VWD was classified as an affected family member.

The time span between first bleeding and time of first consultation for bleeding was defined as "patient's delay". The time span between time of first consultation for bleeding and diagnosis was defined as "doctor's delay". Iatrogenic bleeding was defined as bleeding caused by medical examination or treatment (i.e. post-surgical bleeding, bleeding after tooth extraction, cephalohematoma after forceps or vacuum delivery, post-venipuncture bleeding, post-vaccination bleeding, post-circumcision bleeding, excessive bleeding after heel prick test).

Laboratory measurements of von Willebrand Disease

Patients' historically measured VWF parameters were determined earlier in local Hemophilia Treatment Centers. When blood was collected for the centrally performed assay, it was extracted by venipuncture into 3.2% (0.105 M) sodium citrate tubes. Subsequently, the tubes were centrifuged twice at 2200 x *g* for 10 minutes at room temperature and finally the citrated platelet-poor plasma (PPP) was aliquoted and stored at -80°C. All VWD assays were performed centrally (Erasmus University Medical Center, Rotterdam) and standard assays including VWF:Ag, VWF:CB, VWF:Act, FVIII:C, and multimer analysis were measured as previously described.[4,16,20] As the Ethical Committee only allowed extra blood sampling when clinically indicated, plasma was available for central measurements in only 40% of children.

Classification of von Willebrand Disease

The centrally measured VWF and FVIII levels were used to classify VWD according to the current ISTH guidelines.[5] If blood could not be obtained upon inclusion, VWD was classified based on the locally performed VWD assays. First, types 1 and 2 VWD were differentiated by the VWF:Act/VWF:Ag ratio (type 1 VWD ≥0.7 and type 2 VWD <0.7). Subsequently, type 2 VWD was subclassified further. Patients with normal multimers

were classified as 2M VWD and those with abnormal multimers as 2A or 2B VWD. Next, RIPA tests were performed to detect 2B VWD and VWF binding to FVIII (VWF:FVIIIB) tests to identify 2N VWD. In addition, type 2N patients had a disproportionately low FVIII:C compared with VWF:Ag with a FVIII:C/VWF:Ag ratio <0.7. Type 3 VWD was defined as both a VWF:Ag and VWF:Act level <5 IU/dL, irrespective of FVIII:C level.

Statistical methods

Descriptive statistics for categorical data are presented as numbers and percentages (n, %) and for continuous variables as median and 25 to 75% interquartile ranges [IQR] or ranges in case of age.

The chi-squared test or the Fisher's exact test was used to compare the prevalence of bleeding symptoms between groups. Since BS was skewed, we used Mann-Whitney U or Kruskal-Wallis tests to analyze differences in BS between groups. We performed linear regression analysis to model the association between BS and age. Statistical analyses were performed with SPSS for Windows, version 21.0 (SPSS Inc, Chicago, IL, USA). A p-value <0.05 was considered statistically significant.

RESULTS

Patients

Between 2007 and 2009, 140 children (aged 0-16 years) with VWD were included in the WiN study. All parents or caretakers of these children were approached separately by telephone for an additional interview between March 2013 and May 2014. Twenty-seven of these children could not be included in the current study due to different reasons (unreachable and no contact after several attempts in n=20, declined in n=5, and language barrier in n=2). In table 1 the characteristics of the 113 included children are reported.

Bleeding symptoms and required treatment

All twenty-six (100%) post-menarche girls had menorrhagia and a large proportion (21/26, 81%) required treatment (figure 1), most frequently by a combination of antifibrinolytics and hormonal therapy (9/26, 35%). Notably, two girls (with type 2A and type 3 VWD) had required emergency hospitalization and blood transfusions due to acute excessive menstrual bleeding.

A large proportion of patients (91/113, 81%) experienced clinically significant cutaneous bleeding, although in the majority of cases, no specific bleeding treatment was required (68/91, 75%) (figure 1). Frequently observed symptoms were: oropharyngeal bleeding (72/113, 64%), prolonged bleeding from minor wounds (65/113, 58%) and epistaxis (63/113, 56%). Sixty percent (9/15) of bleedings after surgery and 38% (6/16)

Table 1. Baseline patient and laboratory characteristics (n=113).

Characteristics			
Age (years), median (range)		13	(4-22)
Male sex, n (%)		68	(60)
VWD type, n (%)			
1		60	(53)
2		44	(39)
	2A	*33*	
	2B	*10*	
	2M	*1*	
	2N	*0*	
3		9	(8)
Blood group O, n (%) *		46	(58)
Index cases, n (%)		35	(31)
Number of pedigrees		79	
Interview completed by	Parents	92	(82)
	Parents together with child	15	(13)
	Child	6	(5)
Centrally measured VWF:Ag (IU/dL) †		21	[9-27]
Centrally measured VWF:CB (IU/dL) †		9	[4-27]
Centrally measured VWF:Act (IU/dL) †		13	[2-27]
Centrally measured FVIII:C (IU/dL) †		39	[23-53]

VWD = von Willebrand Disease, IQR, interquartile range; VWF:Ag, Von Willebrand Factor antigen; VWF:CB, Von Willebrand Factor collagen binding; VWF:Act, Von Willebrand Factor activity; FVIII:C: factor VIII coagulant activity. Levels are presented as median [25-75% interquartile range (IQR)]. * n=79 based on availability, 34 missing. † n=48, based on patients of whom plasma was available and patients who used desmopressin or replacement therapy ≤72 hours before blood sampling were excluded.

of bleedings after dental extraction were reported despite prophylactic treatment with replacement therapy. Applied therapy included antifibrinolytics, surgical hemostasis, desmopressin and replacement therapy (figure 1).

Bleeding symptoms and required treatment according to type of von Willebrand Disease

Figure 2A demonstrates the prevalence of various bleeding symptoms according to type of VWD. Type 3 VWD patients suffered significantly more frequently from joint bleeds and oropharyngeal bleeding than patients with type 1 and 2 VWD ($p<0.001$). Epistaxis was more frequent in type 2 and type 3 VWD patients than in type 1 VWD patients (73% and 67% vs. 42% respectively, $p=0.006$).

Figure 2B depicts the requirement of treatment with blood transfusion, desmopressin or factor replacement therapy for different bleeding types. The proportion of epistaxis,

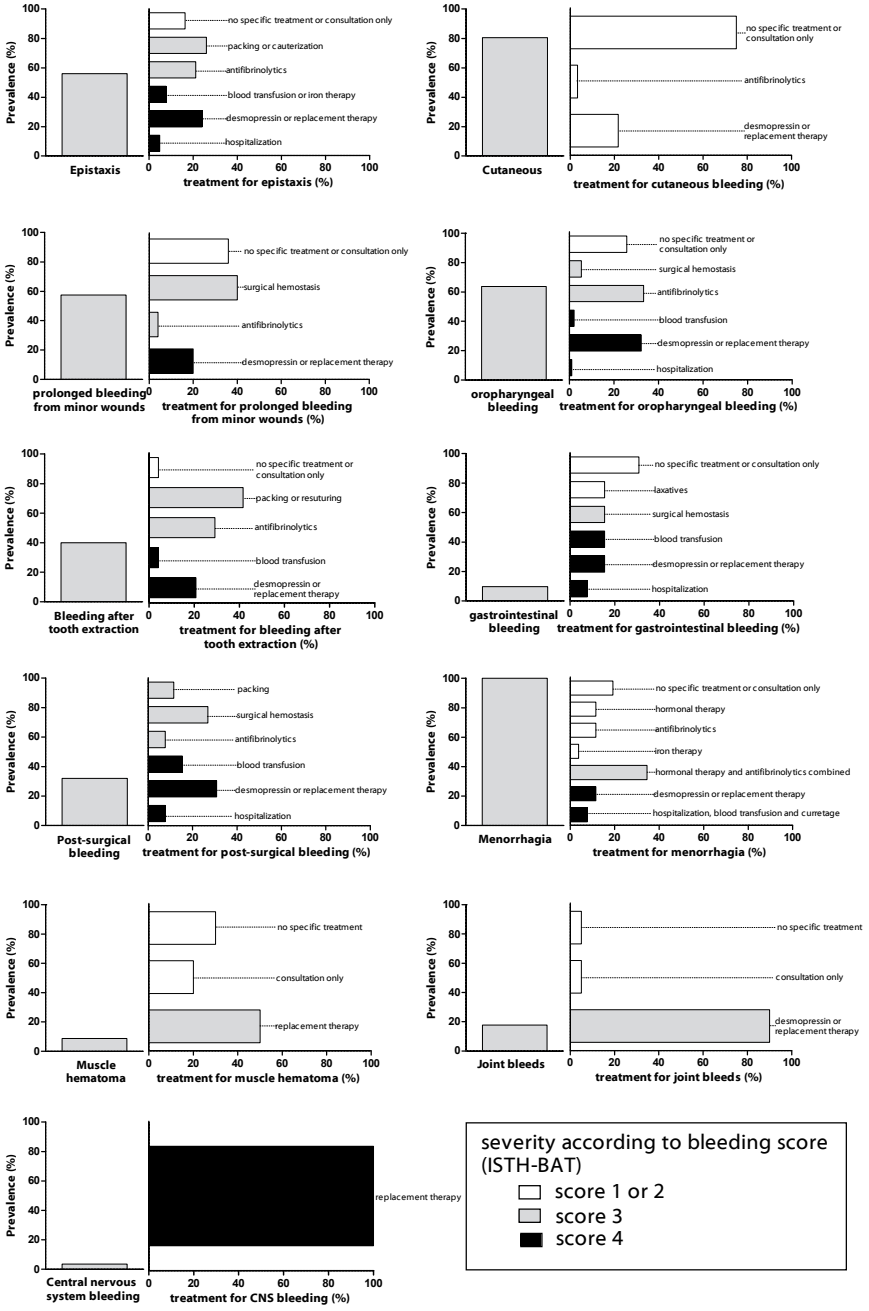


Figure 1. Prevalence and treatment of bleeding symptoms.

The prevalence (left) and percentage of received treatments (right) for the bleeding symptoms are shown. Hematuria did not occur in our cohort of children with VWD. The colors of the bars represent the different bleedings scores according to the ISTH-BAT: in white a bleeding score of 1 or 2, in grey a bleeding score of 3, and in black a bleeding score of 4.

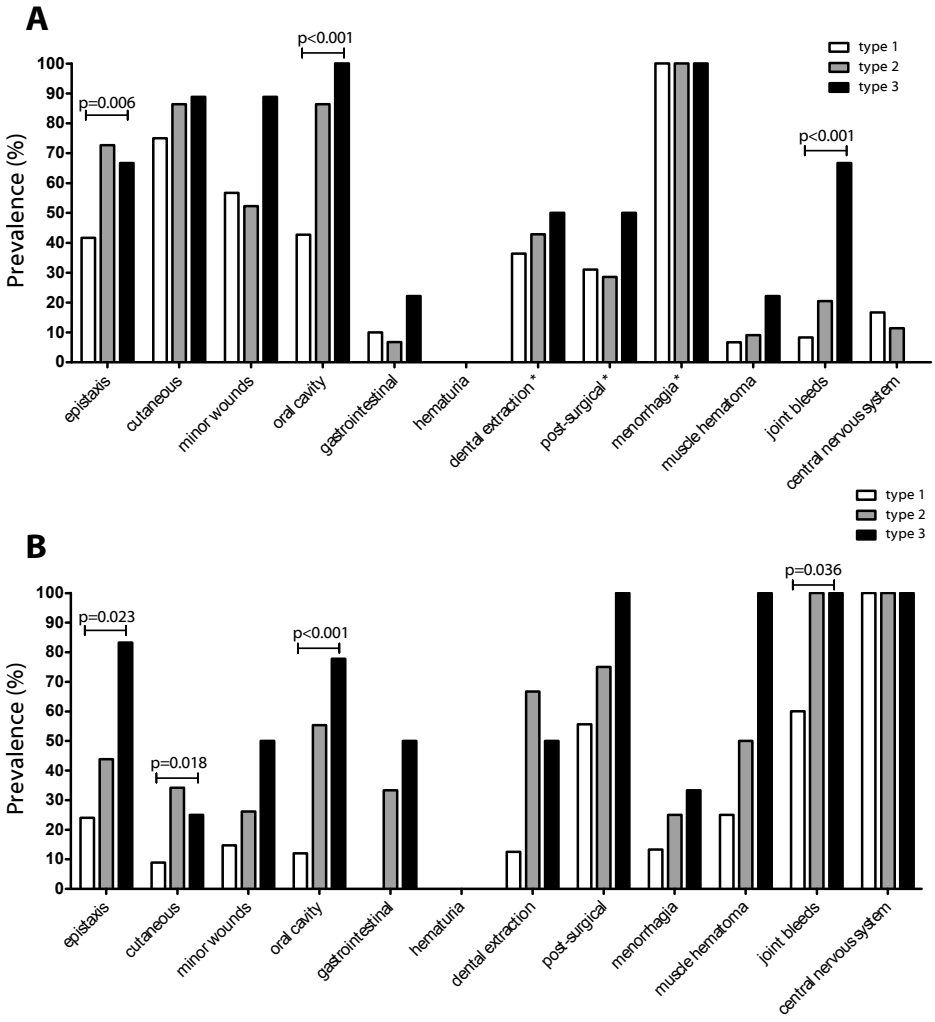


Figure 2. Prevalence of bleeding symptoms and need of treatment according to type of von Willebrand Disease.

A) Proportion of VWD patients who had experienced the bleeding symptom in the past according to types 1, 2 or 3 VWD. B) Proportion of patients, who had experienced the bleeding symptom and received a blood transfusion, desmopressin or FVIII/VWF factor concentrate because of bleeding according to type of VWD. *percentage of children that underwent surgery (n=15) or dental extraction (n=16) or percentage of girls who menstruate (n=26).

oropharyngeal bleeding, joint bleeds and cutaneous bleeding requiring treatment differed significantly between type 1, 2 and 3 VWD (figure 2B).

Compared with affected family members, index cases suffered more frequently from epistaxis (26/35, 74% vs. 37/78, 47%, p=0.008), prolonged bleeding from minor wounds (25/35, 71% vs. 40/78, 51%, p=0.045), post-surgical bleeding (9/18, 50% vs. 6/29, 21%,

p=0.036), gastrointestinal bleeding (7/35, 20% vs. 4/78, 5%, p=0.033), and joint bleeds (10/35, 29% vs. 10/78, 13%, p=0.043). The need for treatment for bleeding symptoms did not differ between index cases and affected family members (data not shown).

Prevalence of pediatric-specific bleedings and required treatment

Pediatric-specific bleeding symptoms included in the ISTH-BAT occurred in 44% (50/113) of patients, occurring more frequently in type 3 VWD patients than type 1 or 2 patients (78% vs. 48% or 32% (p=0.028) (table 2). This did not differ between index cases and affected family members: 19/35, 54% vs. 31/78, 40%, p=0.150. The proportion of patients with at least one pediatric-specific bleeding symptom increased to 52% (59/113) when the additional pediatric-specific bleedings were included.

The most frequently reported pediatric-specific bleeding was post-venipuncture bleeding (38/113, 34%), followed by post-vaccination bleeding (33/113, 29%) and post-circumcision bleeding (1/4, 25% of patients who underwent circumcision). A cephalohematoma was reported in 10/113 (9%) of children. Five of them (50%) were born by vacuum delivery one by emergency caesarean section and four spontaneously. After being born by vacuum delivery 71% of children (5/7) had a cephalohematoma (p<0.001).

Table 2. Prevalence of pediatric-specific bleeding symptoms.

Characteristics		Total cohort (n = 113)	type 1 VWD (n = 60)	Type 2 VWD (n = 44)	Type 3 VWD (n = 9)	p-value (p for trend between different types of VWD)
Umbilical stump bleeding	no.	7	6	1	0	0.197
	%	6.2	10.0	2.3	0	
Cephalohematoma	no.	10	5	4	1	0.961
	%	8.8	8.3	9.1	11.1	
Cheek hematoma caused by sucking	no.	5	3	0	2	0.012
	%	4.4	5.0	0	22.2	
Conjunctival hemorrhage	no.	2	1	0	1	0.070
	%	1.8	1.7	0	11.1	
Post-circumcision bleeding	no.	1	0 out 1	0 out 2	1 out 1	-
	%	0.9	0	0	100.0	
Post-venipuncture bleeding	no.	38	23	11	4	0.282
	%	33.6	38.3	25.0	44.4	
Total no. of children with pediatric-specific bleeding symptoms	no.	50	29	14	7	0.026
	%	44.2	48.3	31.8	77.8	
Bleeding score for pediatric-specific bleeding symptoms	median	0	0	0	1	0.010
	range	0-6	0-6	0-3	0-4	

Table 2. (continued)

Other pediatric-specific bleedings		Total cohort (n = 113)	type 1 VWD (n = 60)	Type 2 VWD (n = 44)	Type 3 VWD (n = 9)	p-value (p for trend between different types of VWD)
Post-vaccination bleeding	no.	33	16	14	3	0.816
	%	29.2	26.7	31.8	33.3	
Bleeding from fetal scalp electrode	no.	2	1	1	0	0.891
	%	1.8	1.7	2.3	0	
Excessive bruising after birth	no.	10	6	3	1	0.827
	%	8.8	10.0	6.8	11.1	
Excessive bleeding after Heel Prick	no.	15	10	5	0	0.347
Test	%	13.3	16.7	11.4	0	
Total no. of children with all	no.	59	30	22	7	0.278
pediatric-specific bleeding symptoms (ISTH-BAT plus additional)	%	52.2	50.0	50.0	77.8	

Age at presentation in children with von Willebrand Disease

The first bleeding occurred at a median age of 1.5 years [IQR 0.8-2.0]. Index cases and patients with type 3 VWD experienced their first bleeding episode at the youngest age and were the first to consult a physician and diagnosed with VWD (table 3). Median age at first consultation for bleeding was 3.0 years [1.0-7.0].

Median patient's delay was zero years [0-1.0] in index cases in comparison to 1.0 year [0-5.5] in affected family members, p=0.054. We could not demonstrate a statistically significant difference in patient delay between type 1, 2 and 3 VWD: 1.0 [0-6.0] year vs. 0.3 [0-2.0] year vs. 0.1 year [0-1] respectively, p=0.285. The majority of affected family members had already been diagnosed with VWD before they first consulted a doctor in relation to a bleeding episode (78%, 47 of 60 patients who have ever consulted a physician because of bleeding).

Table 3. Age at various presentations in children with von Willebrand Disease.

		Age at first bleeding		Age at first consultation for bleeding		Age at VWD diagnosis	
Characteristics		median	[IQR]	median	[IQR]	median	[IQR]
All VWD patients		1.5	[0.8-2.0]	3.0	[1.0-7.0]	2.0	[0.5-5.0]
Type of VWD	1	2.0	[1.0-3.0]	4.0	[2.2-8.8]	3.0	[1.0-6.0]
	2	1.0	[0.5-1.9]	1.5	[0.8-3.0]	0.8	[0.3-2.0]
	3	0.8	[0.3-3.3] †	1.0	[0.6-3.5] ‡	0.8	[0.3-2.0] ‡
Index cases (IC) or	IC	1.0	[0.5-2.0]	2.0	[0.7-4.0]	2.0	[0.8-5.0]
affected family member (AFM)	AFM	1.5	[1.0-3.0] *	3.5	[1.5-8.0] ‡	1.3	[0.3-4.3]

* p<0.05, † p<0.01, ‡ p<0.001

Presenting and diagnosing symptoms

The first bleeding was cutaneous bleeding in the majority of patients (32/101, 32%; 12 missing), followed by epistaxis (31/101, 31%) (figure 3). This pattern differed between type 1, 2 and 3 VWD patients (p=0.031). In type 2 and 3 patients the first bleeding was mainly from mucocutaneous origin (68% and 50%) and type 1 VWD patients mainly presented with hematomas (45%). A quarter of patients that consulted a doctor for the first time, suffered from epistaxis (26/95, 27%; 18 missing), 20% (19/95) for oropharyngeal bleeding, and 18% (17/95) for cutaneous bleeding.

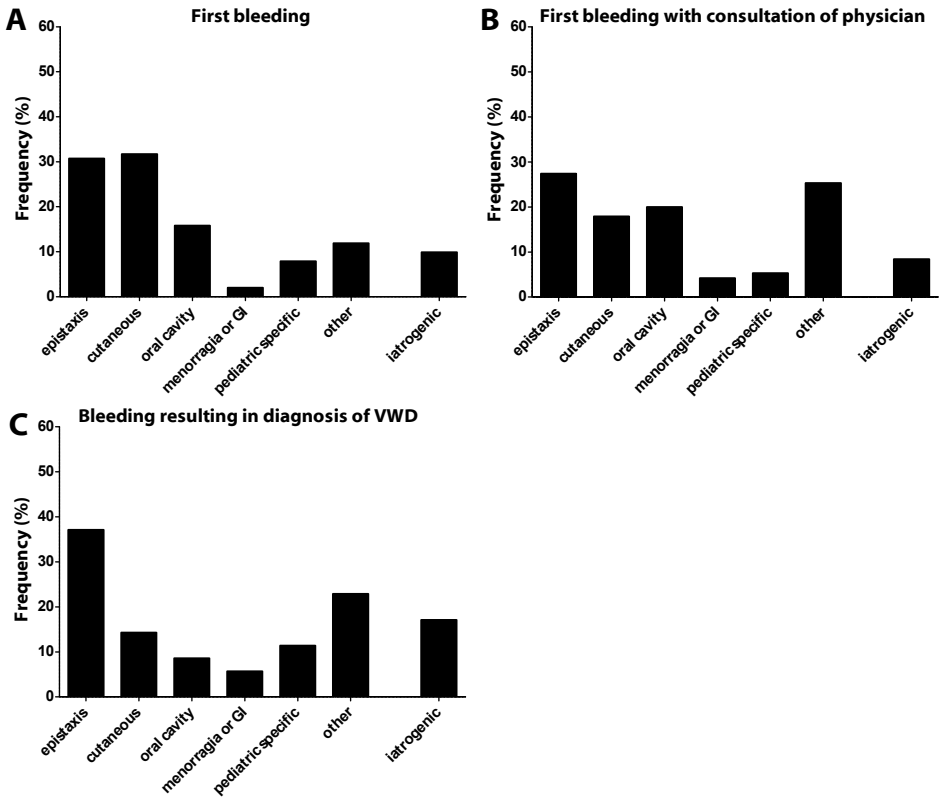


Figure 3. Prevalence of first bleeding symptoms and diagnosis.
A) Proportion of VWD patients who had experienced the bleeding symptom as first bleeding ever. B) Prevalence of bleeding symptoms for which a physician was consulted for the first time. C) Prevalence of bleeding symptom for which VWD was diagnosed.

Bleeding scores

In the total group of children with VWD, median BS (ISTH-BAT) was 9.0 [IQR 5.0-12.5]. BS did not differ between boys and girls: 9.0 [6.0-11.8] vs. 8.0 [4.0-14.5], p=0.963. Type 3 patients had the highest BS (17.0 [11.5-25.5]) compared with type 2 patients (10.5 [6.3-12.8]) or type 1 patients (6.5 [4.0-10.0]), p<0.001 (figure 4A). Median BS in type 2A

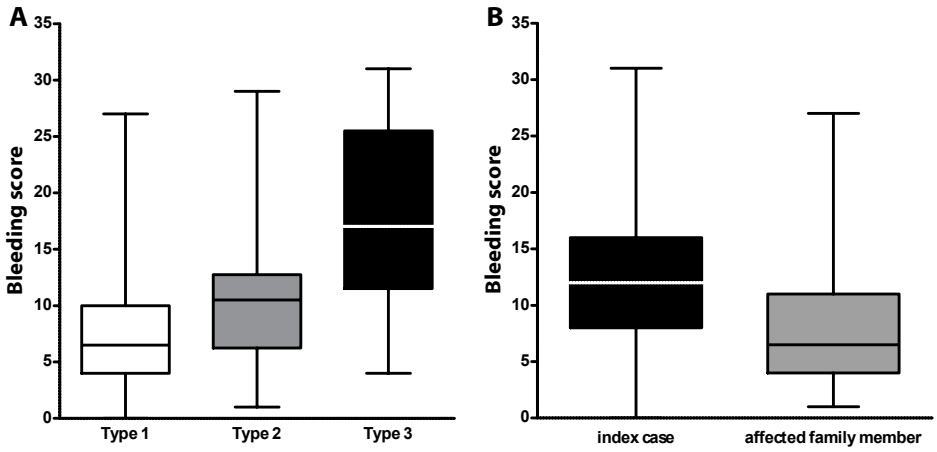


Figure 4. Bleeding scores per type of von Willebrand Disease and index cases or affected family members. A) Bleeding score according to type of VWD. B) Bleeding score for index cases and affected family members. The boxplots indicate the median, 25-75% interquartile range, and 0-100% range.

patients was 11.0 [7.0-13.5] and in type 2B patients 9.5 [5.8-12.3], p=0.470. Index cases had a higher BS than affected family members: 12.0 [8.0-15.0] vs. 6.5 [4.0-11.0], p<0.001 (figure 4B). Severe VWD patients had a higher BS than moderate VWD patients: 11.0 [6.0-15.0] vs. 7.0 [4.0-10.0], p<0.001. Twelve children had a BS below the recently determined cut-off (<3).[17] These children had a median age of 11.5 and were mainly affected family members with type 1 VWD (10/12).

BS increased with age, a one year age increase was associated with 0.4 point increase in BS (95% confidence interval (CI) 0.2 to 0.7). A one year increase in age was associated with a 0.5 point increase in BS (CI 0.2 to 0.8) in type 1 patients, a 0.3 point (CI -0.1 to 0.6) in type 2 patients, and 0.7 points (CI -1.0 to 2.4) in type 3 patients.

As described above, 50 children had bleeding symptoms in the additional bleeding symptom category "others" of the ISTH-BAT. Scores for bleedings in this category including umbilical stump bleeding, cephalohematoma, cheek hematoma caused by sucking during breast or bottle feeding, conjunctival hemorrhage, excessive post-circumcision bleeding and post-venipuncture bleeding, ranged from 1 to 4.

DISCUSSION

In this large pediatric cohort of 113 moderate or severe VWD patients from the WiN-study, the majority (52%) experienced pediatric-specific bleeding symptoms. In addition, all 26 post-menarche girls had menorrhagia. As expected, type 3 VWD children had a more severe bleeding phenotype than type 2 or type 1 VWD children, with earlier presenta-

tion in childhood, more frequent joint bleeds as well as oropharyngeal bleeding and more treatment moments with desmopressin, coagulation factor replacement therapy or blood transfusion. A significantly more severe bleeding phenotype was observed in index cases compared to affected family members and in older children. Mild bleeding symptoms like epistaxis and bruising are common in in healthy children with prevalences of 40% and 25%.[6,21] Recently, Mauer et al collected bleeding histories from 500 healthy adults and reported lower prevalences of 25% for epistaxis, 18% for easy bruising, 18% for prolonged bleeding after a tooth extraction, and 47% for menorrhagia.[22] In our pediatric cohort of moderate and severe VWD, significantly higher proportions of children of 50 up to 100% suffered from epistaxis, cutaneous bleeding, oropharyngeal bleeding and menorrhagia as is supported by other studies.[21] Interestingly, the odds of reporting epistaxis were also shown to decrease with age in healthy individuals.[22]

Hemostatic challenges such as surgery, dental extractions, or menstruation occur less frequently in children and the incidence increases with age.[14,23,24] However, if children with VWD are challenged, a large proportion suffers from bleeding as a result, a finding that was seen in our cohort and also in other pediatric VWD cohorts.[14] Even after prophylactic treatment with replacement therapy or desmopressin a large proportion of moderately or severely affected children still suffered from bleedings. Recently, it has been demonstrated that clinical bleeding outcome of VWD and patients that need prophylactic treatment can be predicted by the Tosetto BS.[9,25] It would be of interest to study prospectively if a BS can also predict bleeding in children and can be used to prevent bleedings after surgery and dental extraction in VWD patients.

In several cohorts, BS have been shown to discriminate between individuals with and without VWD or other bleeding disorders and to indicate the severity of the bleeding phenotype.[4,9,13,26-28] A few years ago, a Pediatric Bleeding Questionnaire was developed by Bowman et al and evaluated in healthy children and in a large cohort of children that were referred for evaluation of bleeding diathesis or preoperative screening.[12] The Pediatric Bleeding Questionnaire was similar to the adult version of the BS but supplemented with pediatric-specific bleeding symptoms, like umbilical stump bleeding, cephalohematoma, post-circumcision bleeding, post-venipuncture bleeding, and macroscopic hematuria. In their cohort of 151 children, 6 were diagnosed with VWD. None of these children presented with pediatric-specific bleeding symptoms. However, cheek hematoma and conjunctival hemorrhage were not evaluated in this study. Also in similar cohorts from the United States (n=104, 8 diagnosed with type 1 VWD) and from Germany (n=100, 12 type 1 VWD and 11 type 2 VWD) no pediatric-specific bleedings were reported.[13,29] In 2010, the Pediatric Bleeding Questionnaire was administered in 100 Canadian children with VWD, mostly type 1 VWD patients. Pediatric-specific bleedings were reported in 17% of these children.[14] In our more severely affected VWD cohort, the pediatric-specific bleedings, occurred in 44% of children, mainly in type 3 VWD patients

(78%), but also in type 2 (32%) and moderate to severe type 1 (48%) patients. These bleedings also occurred in children with a low overall BS. Adding the supplementary pediatric-specific bleeding symptoms increased the percentage of children reporting bleeding tot fifty-two. Importantly, 29% of patients experienced bleeding after vaccination, indicating that VWD patients should receive special care when being vaccinated. This demonstrates that pediatric-specific bleedings are definitely important to determine the bleeding phenotype and may help to discriminate children with and without VWD.

The strength of our study is that it is covers a large group of moderate and severe pediatric VWD patients with various types of VWD. In addition The ISTH-BAT has been thus far only been evaluated in healthy children and in children with mild VWD,[13,17] so we are the first to study the ISTH-BAT in a large group of moderate and severe pediatric VWD patients with various types of VWD.

In conclusion, pediatric-specific bleedings included in the ISTH-BAT but also other pediatric-specific bleedings occur in a large proportion of children with moderate to severe VWD. A bleeding score with supplementary pediatric-specific bleeding symptoms is therefore of important value in the diagnosis of VWD in children as it may predict bleeding phenotype and necessity of therapeutic interventions.

REFERENCES

1. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*. 1987;69(2):454-459.
2. Bowman M, Hopman WM, Rapson D, Lillicrap D, James P. The prevalence of symptomatic von Willebrand disease in primary care practice. *J Thromb Haemost*. 2010;8(1):213-216.
3. James AH. More than menorrhagia: a review of the obstetric and gynaecological manifestations of von Willebrand disease. *Thromb Res*. 2007;120 Suppl 1:S17-20.
4. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108(4):683-692.
5. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
6. Katsanis E, Luke KH, Hsu E, Li M, Lillicrap D. Prevalence and significance of mild bleeding disorders in children with recurrent epistaxis. *J Pediatr*. 1988;113(1 Pt 1):73-76.
7. Huq FY, Kulkarni A, Agbim EC, Riddell A, Tuddenham E, Kadir RA. Changes in the levels of factor VIII and von Willebrand factor in the puerperium. *Haemophilia*. 2012;18(2):241-245.
8. Knol HM, Kemperman RF, Kluin-Nelemans HC, Mulder AB, Meijer K. Haemostatic variables during normal menstrual cycle. A systematic review. *Thromb Haemost*. 2012;107(1):22-29.
9. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost*. 2006;4(4):766-773.
10. Rodeghiero F, Castaman G, Tosetto A, et al. The discriminant power of bleeding history for the diagnosis of type 1 von Willebrand disease: an international, multicenter study. *J Thromb Haemost*. 2005;3(12):2619-2626.
11. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063-2065.
12. Bowman M, Riddel J, Rand ML, Tosetto A, Silva M, James PD. Evaluation of the diagnostic utility for von Willebrand disease of a pediatric bleeding questionnaire. *J Thromb Haemost*. 2009;7(8):1418-1421.
13. Bidlingmaier C, Grote V, Budde U, Olivieri M, Kurnik K. Prospective evaluation of a pediatric bleeding questionnaire and the ISTH bleeding assessment tool in children and parents in routine clinical practice. *J Thromb Haemost*. 2012;10(7):1335-1341.
14. Biss TT, Blanchette VS, Clark DS, et al. Quantitation of bleeding symptoms in children with von Willebrand disease: use of a standardized pediatric bleeding questionnaire. *J Thromb Haemost*. 2010; 8(5):950-956.
15. de Wee EM, Leebeek FW, Eikenboom JC. Diagnosis and management of von Willebrand disease in The Netherlands. *Semin Thromb Hemost*. 2011;37(5):480-487.
16. Sanders YV, Eikenboom J, de Wee EM, et al. Reduced prevalence of arterial thrombosis in von Willebrand disease. *J Thromb Haemost*. 2013;11(5):845-854.
17. Elbatarny M, Mollah S, Grabell J, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. *Haemophilia*. 2014;20(6):831-835.
18. Tosetto A, Castaman G, Rodeghiero F. Bleeding scores in inherited bleeding disorders: clinical or research tools? *Haemophilia*. 2008;14(3):415-422.
19. Federici AB. Clinical diagnosis of von Willebrand disease. *Haemophilia*. 2004;10 Suppl 4:169-176.

20. Sanders YV, Giezenaar MA, Laros-van Gorkom BA, et al. Von Willebrand disease and aging: an evolving phenotype. *J Thromb Haemost*. 2014;12(7):1066-1075.
21. Nosek-Cenkowska B, Cheang MS, Pizzi NJ, Israels ED, Gerrard JM. Bleeding/bruising symptomatology in children with and without bleeding disorders. *Thromb Haemost*. 1991;65(3):237-241.
22. Mauer AC, Khazanov NA, Levenkova N, et al. Impact of sex, age, race, ethnicity and aspirin use on bleeding symptoms in healthy adults. *J Thromb Haemost*. 2011;9(1):100-108.
23. Montgomery RR. von Willebrand disease--the relevance of history. *J Thromb Haemost*. 2005;3(12): 2617-2618.
24. Bowman M, Hopman WM, Rapson D, Lillicrap D, Silva M, James P. A prospective evaluation of the prevalence of symptomatic von Willebrand disease (VWD) in a pediatric primary care population. *Pediatr Blood Cancer*. 2010;55(1):171-173.
25. Federici AB, Bucciarelli P, Castaman G, et al. The bleeding score predicts clinical outcomes and replacement therapy in adults with von Willebrand disease. *Blood*. 2014;123(26):4037-4044.
26. Bowman M, Mundell G, Grabell J, et al. Generation and validation of the Condensed MCMDM-1VWD Bleeding Questionnaire for von Willebrand disease. *J Thromb Haemost*. 2008;6(12):2062-2066.
27. Castaman G, Federici AB, Tosetto A, et al. Different bleeding risk in type 2A and 2M von Willebrand disease: a 2-year prospective study in 107 patients. *J Thromb Haemost*. 2012;10(4):632-638.
28. Boelaars MF, Peters M, Fijnvandraat K. Evaluation of a self-administrated pediatric bleeding questionnaire measuring bleeding severity in children. *Thromb Haemost*. 2012;108(5):1006-1007.
29. Marcus PD, Nire KG, Grooms L, Klima J, O'Brien SH. The power of a standardized bleeding score in diagnosing paediatric type 1 von Willebrand's disease and platelet function defects. *Haemophilia*. 2011;17(2):223-227.

8

Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand Disease

EM de Wee, YV Sanders, EP Mauser-Bunschoten, JG van der Bom, MEL Degenaar-Dujardin, J Eikenboom, A de Goede-Bolder, BAP Laros-van Gorkom, K Meijer, K Hamulyák, MR Nijziel, K Fijnvandraat, FWG Leebeek, and the WiN study group

Thrombosis and Haemostasis. 2012;108(4):683-692

ABSTRACT

We performed a nationwide cross-sectional study to evaluate determinants of bleeding symptoms in a large unselected cohort of adults with von Willebrand Disease (VWD). VWD patients were included (n=664), based on lowest historically measured VWF:Ag and VWF:Act levels ≤30 U/dL. Menorrhagia (85%), cutaneous bleeding (77%), bleeding from minor wounds (77%) and oral-cavity bleeding (62%) occurred most frequently. Higher age was associated with a higher bleeding score (BS), determined according to Tosetto, in females. A 10 year increase in age was associated with 0.8 point (95% confidence interval (CI) 0.4 to 1.1) higher BS. Females had higher BS than males (median 12 versus 10, p=0.012). BS differed significantly between VWD type 1, 2 and 3: median 9 [IQR -2-31], 13 [-1-33] and 19.5 [1-35] respectively (p<0.001). BS was strongly associated with VWF and FVIII levels: individuals with VWF:Ag levels ≤10 IU/dL, VWF:Act ≤10 IU/dL and FVIII:C ≤10 IU/dL had respectively 5.3 point (CI 3.2 to 7.3), 4.3 point (CI 2.9 to 5.8) and 9.6 point (CI 6.5 to 12.7) higher BS, than those with levels >30 IU/dL. In type 3 patients 1 IU/dL FVIII:C decrease was associated with 0.6 point (CI 0.1 to 1.1) BS increase (p=0.021). In conclusion, in VWD patients the bleeding phenotype is strongly associated with type of VWD and VWF and FVIII levels.

INTRODUCTION

Von Willebrand Disease (VWD) is the most common inherited bleeding disorder caused by a quantitative or qualitative defect in Von Willebrand Factor (VWF).[1] VWF plays a major role in haemostasis by promoting platelet adhesion and aggregation. In addition VWF is the carrier protein of Factor VIII (FVIII).[2] Patients with VWD regularly suffer from bleeding episodes, varying from gum bleeds, epistaxis, gastrointestinal bleeding, menorrhagia to bleeding after surgical intervention.[3-5] Type 1 VWD is characterised by a partial quantitative deficiency of VWF, whereas qualitative abnormal variants of VWF are classified as type 2 VWD. Type 3 VWD is characterised by a total deficiency of VWF.[6]

Clinical expression of VWD is very heterogeneous with a large variability in bleeding frequency and severity between patients and within one patient over time. Tosetto et al have developed a bleeding score (BS) to quantify the number and severity of bleeding symptoms,[7] in order to discriminate between subjects with type 1 VWD and individuals without VWD. This bleeding score was validated in a European study that included mainly type 1 patients who had mildly decreased VWF levels (<50 IU/dL). It is yet unknown whether the Tosetto BS can be used to assess the bleeding pattern and severity in more severely affected patients. Bowman et al determined a condensed BS in 42 subjects in whom VWD was previously diagnosed, including 16 type 1, 14 type 2 and 12 type 3 patients and found strong differences in scores.[8] Similarly within the Zimmerman Project, 35 patients with type 2 VWD and 28 with type 3 VWD have been investigated using the BS.[9] Federici et al reported on bleeding in type 2B patients and included the BS in their analysis.[10] Although previous studies have investigated the association between laboratory parameters of VWF levels, FVIII levels and the BS in type 1 VWD, this has not yet been assessed in type 2 and 3 VWD. Therefore it is of utmost interest to study the determinants of bleeding in a large group of adult patients with various types of VWD.[11]

The aim of our study is to evaluate the bleeding phenotype and pattern of bleeding symptoms in a large unselected cohort of adult patients with moderate or severe VWD defined as VWF levels ≤30 U/dL and to assess which factors influence bleeding pattern and severity.

MATERIALS AND METHODS

Subjects

We performed a nationwide cross-sectional study among patients with VWD in the Netherlands, the "Willebrand in the Netherlands" (WiN) study. Patients were recruited from all 13 Haemophilia Treatment Centres in the Netherlands between October 2007 and October 2009. We included patients diagnosed with type 1, type 2 and type 3 VWD

who fulfilled both of the following inclusion criteria: 1) haemorrhagic symptoms or a family history of VWD; 2) historically lowest levels of VWF antigen (VWF:Ag) ≤30 U/dL and/or VWF activity (VWF ristocetin cofactor activity (VWF:RCo)) ≤30 U/dL and/or Factor (F)VIII:C ≤40 U/dL, determined at the local Haemophilia Treatment Centre. Patients were excluded if haemophilia A or other disorders of haemostasis resulting in a haemorrhagic diathesis, were known.

For the current analysis only adult patients (≥16 years) were selected. Pregnant patients and patients who used desmopressin or clotting factor concentrate 72 hours prior to blood sampling, were excluded from all analyses using VWF and FVIII:C levels (n=18). The Medical Ethical Committees at all participating centres approved this study and written informed consent was obtained from all study participants.

Definitions

Determination of type of VWD was based on the current ISTH guidelines,[6,11] using centrally measured plasma concentrations of VWF:Ag, VWF Collagen Binding (VWF:CB), VWF activity (VWF:Act) and FVIII:C, performed between January and March 2010. An index case was defined as a patient who was referred to a Haemophilia Treatment Centre because of bleeding problems. An affected family member was defined as a patient who was screened for VWD because VWD was diagnosed previously in a family member.

Assessment methods

Participants were asked to complete an extensive self-administered questionnaire, containing questions on bleeding episodes, treatment of VWD, side effects of treatment, concomitant disease, Quality of Life and social aspects.[12,13] The questionnaire was sent by postal mail to all participants, followed by two reminders if necessary.

For the present investigation we used a condensed version of the Tosetto BS, also used in the European study,[7] retaining in the questionnaire only those questions relevant to compute the BS, as was previously described by Bowman.[8] The BS was computed using the BS study criteria.[7] The BS systematically evaluates the number and severity of twelve different bleeding symptoms and scores these on a scale ranging from -1 to 4 points. Higher scores reflect more severe or frequent bleeding. The total for all twelve items results in a BS. Minimum score for males and females was -3, maximum score for males was 37, whereas for females it was 45.

In our study a self-administered version of the condensed Tosetto BS was used, whereas the original Tosetto BS was developed as an expert-administered BS. To validate the use of our method we randomly selected 25 VWD patients from our cohort, who had similar baseline characteristics as the other patients from the cohort, and obtained both the self-completed BS and an expert-administered BS by detailed questioning by a well-trained physician. This revealed that the self-completed BS was comparable with

the expert-administered BS (median 13 (range 3 to 28) versus median 14 range (-2 to 25), p=0.253). The results of this comparison are also depicted in the supplemental figure.

A possible bias in our BS analysis is the use of short term (prophylactic) treatment with factor concentrates or desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) in patients undergoing surgery. In the Tosetto BS these individuals would score 4 points (use of desmopressin or factor concentrates), whereas this does not necessarily reflect a more severe bleeding phenotype. Therefore we did not score 4 points if patients received prophylactic factor concentrates or desmopressin before they underwent surgery, dental extraction or gave birth to eliminate a possible "prophylaxis-bias", as has been suggested before by Tosetto et al.[14]

Laboratory measurements in patients with von Willebrand Disease

Historically measured VWF and FVIII levels in the patient's own Haemophilia Treatment Centres were used as inclusion criteria for the WiN study. Patients with at least one measurement of VWF ≤30 U/dl or FVIII:C ≤40 U/dL (for type 2N) were included. Because we also wanted to include type 2N VWD, VWD patients with a level of FVIII:C ≤40 U/dL but VWF levels above 30 U/dl were also included. The historically lowest VWF parameters and FVIII levels are in U/dL or in %, according to the local laboratory. Most adult participants agreed to draw blood for measuring von Willebrand parameters in a central laboratory at inclusion in the study.

At inclusion in the study peripheral venous blood was collected in tubes containing 3.2% (0.105 M) sodium citrate. Subsequently, the tubes were centrifuged twice at 2,200 x *g* for 10 minutes at room temperature and finally the citrated platelet-poor plasma was aliquoted and stored at -80°C.

Plasma levels of VWF:Ag, VWF:CB, VWF:Act and FVIII:C were measured centrally (Erasmus University Medical Center, Rotterdam, The Netherlands). VWF:Ag level was measured with an in-house ELISA using a polyclonal rabbit anti-human VWF antibody (DakoCytomation, Glostrup, Denmark) for capturing and a HRP-conjugated anti-human VWF antibody (DakoCytomation, Glostrup, Denmark) for detecting. VWF:CB level was measured with an in-house ELISA using collagen type 1 (Sigma-Aldrich, St Louis, USA) for capturing and a HRP-conjugated anti-human VWF antibody (DakoCytomation, Glostrup, Denmark) for detecting. To assess VWF activity we used a VWF:Act assay that measures the ability of VWF to bind glycoprotein (Gp) Ibα. The VWF:Act assay uses latex particles coated with a monoclonal murine antibody directed against the GpIbα binding domain of VWF (HemosIL™ von Willebrand Factor Activity, Instrumentation Laboratory B.V, Breda, the Netherlands). These latex particles were incubated with the patient plasma and agglutination of the particles, proportionally to the GpIbα binding activity of VWF, was measured.[15] In the Erasmus University Medical Center Rotterdam, we have previously validated the VWF:Act test in plasma samples that were sent to our labora-

tory for diagnostic purposes (n=122) and studied the association with our previously used VWF:RCo activity test. We obtained a Spearman correlation coefficient of 0.942 (p<0.0001). FVIII:C was measured in an one-stage clotting assay (TriniCLOT, Biomerieux, Marcy l'Etoile, France) with FVIII-deficient plasma (Biopool, Umea, Sweden). All assays used commercial reference plasma (Normal reference plasma, Precision biologic, Kordia, Leiden, the Netherlands) which was standardised against the World Health Organization standard by the manufacturer. The new centrally measured VWF parameters and FVIII levels are expressed in IU/dL. Multimeric pattern was evaluated by low resolution 0.9% agarose (Bio-Rad Laboratories, Hercules, CA, USA) gel electrophoresis followed by capillary Western blotting.[16] VWF multimer patterns were evaluated by two independent reviewers (JE and FWGL). VWF multimers were classified as either abnormal, normal or absent by comparison with the commercial reference plasma (Normal reference plasma, Precision biologic, Kordia, Leiden, the Netherlands). Abnormal multimers were defined as a deviation from a normal distribution according to the MCMDM-1VWD study.[17] To ensure that all VWD type 2N patients indeed had VWD and no haemophilia A, VWF:FVIII binding (VWF:FVIIIB) assays were performed using an ELISA. First, a microtiter well was coated with anti-VWF (DakoCytomation, Glostrup, Denmark). After incubation with patient plasma, the FVIII was removed from VWF, its carrier, with calcium chloride [350 mM]. We then added recombinant FVIII (Kogenate® FS, Bayer HealthCare, Leverkusen, Germany) and determined the amount of FVIII that was bound to VWF using anti-FVIII (Affinity Biologicals Inc., Ancaster, Canada). In short, type 3 was defined as both a VWF:Ag and VWF:Act level of <5 IU/dL, irrespective of FVIII:C level. Type 2N patients had a FVIII/VWF:Ag ratio <0.70, and VWF:FVIIIB <60%. Type 1 patients were defined as a VWF:Act/VWF:Ag ratio ≥0.70, whereas type 2 patients had a ratio <0.70. If type 2 patients had normal multimers they were classified as 2M. If patients had abnormal multimers they were classified as 2A or 2B patients. For logistic reasons we used locally performed ristocetin-induced platelet aggregation (RIPA) tests and if available mutation analysis of the patient or a family member performed in the patient's own Haemophilia Treatment Centres in order to classify type 2B patients. Phenotypic blood group was determined by mixing plasma of patients with red blood cells of donors with known blood group, as has been described previously.[18] We have no information on prothrombotic factors in our cohort, such as Factor V Leiden or prothrombin gene variant.

Statistical methods

The continuous variables, such as age and VWF and FVIII levels were expressed as medians (ranges or 25-75% interquartile ranges [IQR]). BS presented a skewed distribution to the right. Due to a non-normal distribution, Mann-Whitney *U* or Kruskal Wallis tests were used to test statistical significance of differences in BS between groups. VWF and FVIII levels were categorised into four groups (0-10, 11-20, 21-30 and >30 IU/dL). We used

linear regression to model the association of BS with age, VWF levels and FVIII levels in a univariate model. Next, in multivariate models we adjusted for age and sex. A p-value of <0.05 was considered statistically significant.

RESULTS

Enrolled subjects and laboratory data

In the 13 Haemophilia Treatment Centres in the Netherlands 1,067 VWD patients were identified who fulfilled the inclusion criteria of the WiN study, based on the historically lowest measured plasma levels of VWF and FVIII. All these individuals received an invitation to participate in the study of whom 806 patients (76% response) were included. The questionnaire was completed by all individuals, including 666 adults and 140 children (<16 years). Patient characteristics between VWD patients who were included and VWD patients who were not, were similar regarding gender, VWD type, VWF and FVIII:C levels. Patients who participated in the WiN study were slightly older (median age 44 years; IQR 28-50) than patients who did not participate (median age 39 years; IQR 32-57) (p=0.001). The adult patients were included for the present analysis. Plasma was obtained from 589 (88%) adult patients. After performing VWF:FVIIIB assays in all patients previously diagnosed with type 2N VWD, two patients were diagnosed with haemophilia A based on normal FVIII binding and genotyping of the *F8* and *VWF* gene, and therefore excluded from the study.

Patient characteristics are summarised in table 1. The majority (64%) was female. The median age of males was 44 years and of females 46 years. The majority of patients had VWD type 1 (n=347, 59%), whereas 37% (n=214) had type 2 VWD and 4% (n=26) had type 3 VWD. Median [IQR] VWF levels and FVIII:C levels measured centrally were: VWF:Ag 30 IU/dL [19-46], VWF:CB 25 IU/dL [8-53], VWF:Act 24 IU/dL [9-55] and FVIII:C 53 IU/dL [35-75].

Bleeding score according to age and gender

Gender was strongly associated with bleeding severity. Females reported more bleeding symptoms than males (BS median 12 (range -2 to 35) versus median 10 (range -2 to 31), p=0.012). The BS calculated without the domains menorrhagia and postpartum haemorrhage for females was 9 (median; range -2-28) and for males 10 (median; range -2 to 31) (p=0.012). Index cases reported higher BS than affected family members: median 12 (range -2 to 35) versus median 11 (range -2 to 31), p=0.002. However, median VWF and FVIII levels in affected family members were significantly higher compared to index cases (all p<0.001). VWF and FVIII levels in index cases were: median VWF:Ag 38 IU/dL [24-55], median VWF:CB 40 IU/dL [14-66], median VWF:Act 44 IU/dL [18-71] and median FVIII:C 61 IU/dL [41-86]. VWF and FVIII levels in affected family members were: median

Table 1. Baseline characteristics of the WiN study population.

Total n=664			
Male sex, n (%)		241	(36)
Age (years), median (range)	Males	44	(16-85)
	Females	46	(16-83)
Type, n (%) *	1	347	(59)
	2	214	(37)
	2A	140	
	2B	37	
	2M	23	
	2N	14	
	3	26	(4)
Blood group O, n (%) *		359	(61)
Index case or affected family member, n (%)	Index case	324	(49)
	AFM	329	(49)
	Unknown	11	(2)
Historically lowest levels, median [IQR]	VWF:Ag (U/dL)	30	[21-41]
	VWF:CB (U/dL)	21	[13-31]
	VWF:RCo (U/dL)	16	[9-26]
	FVIII:C (U/dL)	44	[32-59]
New centrally measured levels, median [IQR] †	VWF:Ag (IU/dL)	30	[19-46]
	VWF:CB (IU/dL)	25	[8-53]
	VWF:Act (IU/dL)	24	[9-55]
	FVIII:C (IU/dL)	53	[35-75]

AFM = affected family member, Historically lowest levels = the historically lowest values measured previously in the patient's own hemophilia treatment center, New centrally measured values = centrally measured values in blood drawn at inclusion in the study, IQR = 25-75% interquartile range. * n=587: based on patients of whom plasma was available. † n=569: based on patients of whom plasma was available, pregnant patients and patients who used desmopressin or clotting factor concentrate 72 hours prior to blood sampling, were excluded.

VWF:Ag 48 IU/dL [24-71], median VWF:CB 61 IU/dL [28-97], median VWF:Act 63 IU/dL [30-103] and median FVIII:C 68 IU/dL [45-111].

Using linear regression, BS steadily prolonged with age, a 10 year increase of age was associated with 0.8 point increase in BS (95% confidence interval (CI) 0.4 to 1.1). In females every 10 year increase of age was associated with 1.1 point increase in BS (CI 0.6 to 1.5). In males this age effect was not observed (data not shown).

Bleeding symptoms and need of treatment in patients with von Willebrand Disease

Figure 1A shows the proportion of VWD patients who have experienced the bleeding symptoms in the past. Most frequently occurring bleeding symptoms were menorrhagia

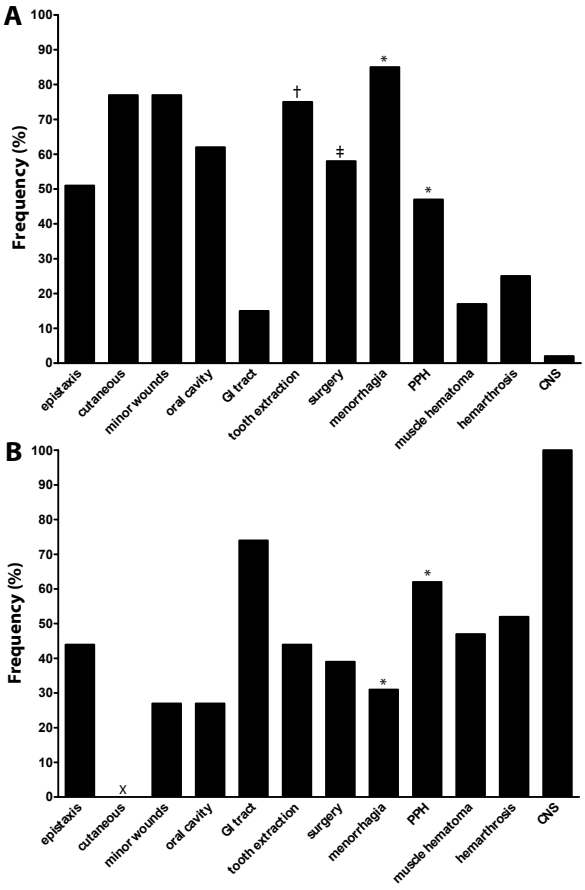


Figure 1. Prevalence of bleeding symptoms and need of treatment.
A) Proportion of VWD patients who had experienced the bleeding symptom in the past. B) Proportion of patients, who had experienced the bleeding symptom and received a blood transfusion, desmopressin or FVIII/VWF factor concentrate because of bleeding. X=not applicable. * frequencies are only of women who have been menstruating or who gave birth. GI = gastrointestinal, PPH = postpartum haemorrhage, CNS = central nervous system. † n=554 based on patients who underwent tooth extraction. ‡ n=559 based on patients who underwent surgery.

(85% of women who have been menstruating), cutaneous bleeding (77%), prolonged bleeding from minor wounds (77%), and oral cavity bleeding (63%). Central nervous system bleeding occurred in 8 out of 347 type 1 VWD patients (2%) and in 4 out of 214 type 2 VWD patients (2%). Central nervous system bleeding did not occur in type 3 VWD patients (p=0.707). Twenty-five percent of all VWD patients suffered from haemarthrosis, which occurred in 85 out of 347 type 1 VWD patients (24%), 44 out of 214 type 2 VWD patients (21%) and 15 out of 26 type 3 VWD patients (58%) (p<0.001). Gastrointestinal bleeding occurred in 37 out of 347 type 1 VWD patients (11%) and 44 out of 214 type 2

VWD patients (21%). Seven out of 26 type 3 VWD patients (27%) suffered from gastrointestinal bleeding (p for trend = 0.001).

In figure 1B the percentage of patients in need of treatment, including blood transfusion, desmopressin or coagulation-factor concentrate, is shown. Treatment was most frequently initiated for central nervous system bleeding (100%), gastrointestinal bleeding (74%), postpartum haemorrhage (62%), haemarthrosis (52%) and muscle haematoma (47%). Gastrointestinal bleeding for which treatment was necessary occurred in 7% of type 1 patients, 17% of type 2 patients and 27% of type 3 VWD patients. Haemarthrosis for which treatment was given, occurred mostly in type 3 patients (58%) and less often in type 2 (12%) and type 1 (10%).

Bleeding score according to type of von Willebrand Disease

The BS varied according to the type of VWD diagnosed with a median BS of 9 (range -2 to 31) in VWD type 1, 13 (range -1 to 33) in type 2 VWD and 19.5 (range 1 to 35) in type 3 (figure 2A and table 2). The BS differed significantly between the types of VWD ($p<0.001$). BS of the four type 2 sub-variants also differed significantly, as depicted in figure 2B. Of the patients with type 2, patients with type 2B had the highest median BS of 16 (range 4 to 31), whereas patients with type 2M had the lowest median BS of 9 (range -1 to 22) ($p<0.001$). The two most occurring mutations in the 30 genotyped type 2B patients in the Dutch WiN population were p.R1306W and p.R1308C. These individuals had a median BS of 20 (range 8-31, n=15) and median 16 (range 12-31, n=10), respectively.

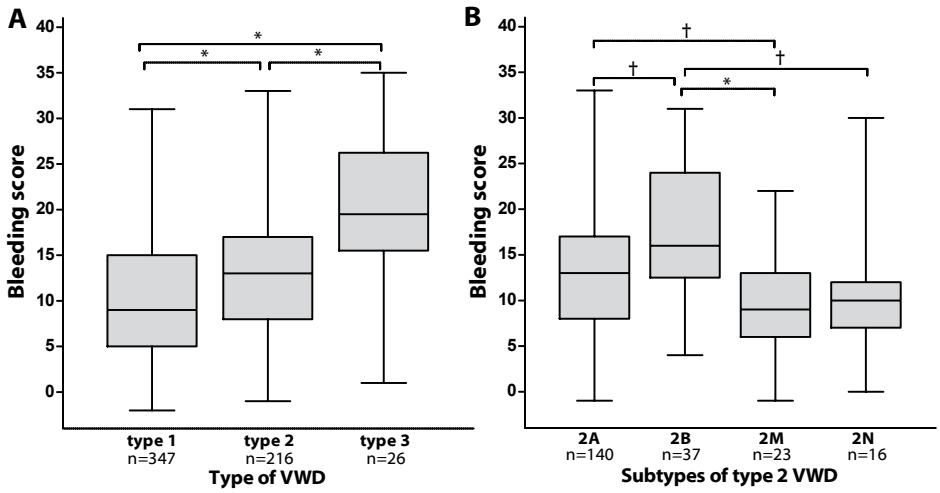


Figure 2. Bleeding score according to type of von Willebrand Disease.
A) Bleeding score according to type of VWD. B) Bleeding score according to type 2 variants in patients with VWD. * $p<0.001$, † $p<0.01$.

Table 2. Bleeding score and VWF/FVIII level per type of von Willebrand Disease.

Type*	n (%)	median BS (range)	VWF:Ag† (IU/dL)	VWF:CB† (IU/dL)	VWF:Act† (IU/dL)	FVIII:C† (IU/dL)
Type 1	346	9 (-2-31)	39 [25-54]	45 [26-68]	48 [26-72]	68 [52-90]
Type 2	215	13 (-1-33)	25 [16-35]	8 [6-16]	8 [4-17]	37 [27-49]
Type 3	26	19.5 (1-35)	0 [0-3]	0 [0-3]	0 [0-1]	2 [1-12]

* p for trend in VWF and FVIII:C levels between type 1, type 2 and type 3 VWD were all <0.001. † median levels in IU/dL [25-75% interquartile range], pregnant patients and patients who used desmopressin or clotting factor concentrates 72 hours prior to blood sampling, were excluded (type 1 n=337, type 2 n=209, type 3 n=23). New centrally measured levels of VWF and FVIII:C were used.

Association between bleeding score and VWF and FVIII levels

Figure 3 shows the BS according to centrally measured VWF:Ag, VWF:CB, VWF:Act and FVIII:C. The BS was inversely associated with centrally measured levels of VWF and FVIII, i.e. patients with the lowest VWF and FVIII levels had the highest BS. The regression coefficients are depicted in table 3, the BS is 5.3 points higher in VWD patients with VWF:Ag levels 0-10 IU/dL compared to patients with VWF:Ag levels >30 IU/dL (adjusted for age

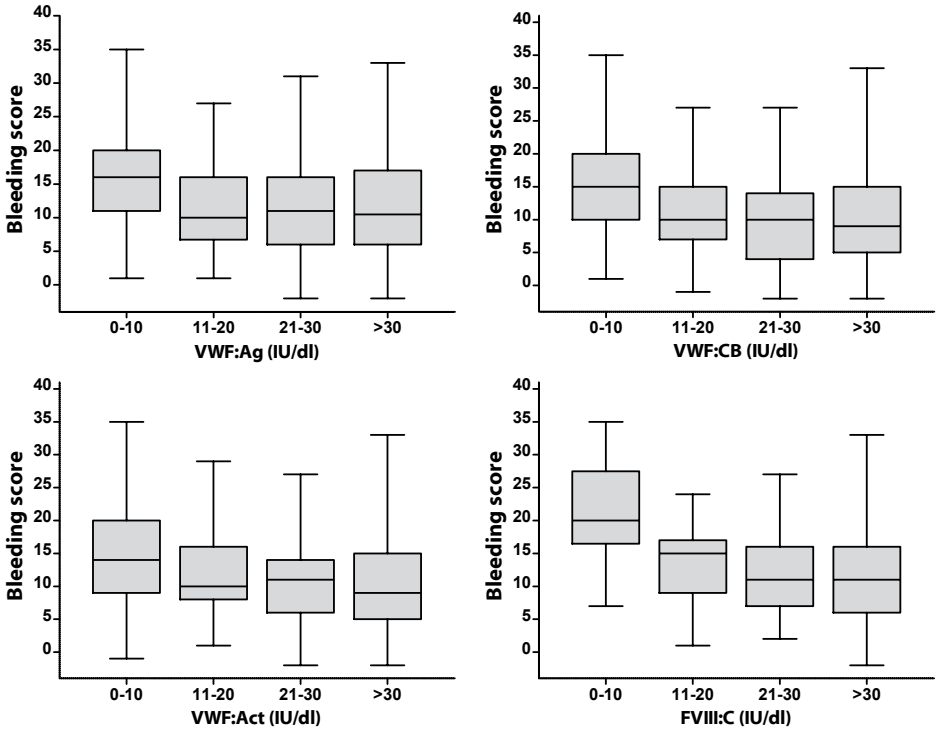


Figure 3. Bleeding score according to VWF and FVIII levels.
Association between bleeding score and VWF:Ag, VWF:CB, VWF:Act and FVIII:C levels.

Table 3. Crude and adjusted differences in bleeding score according to VWF and FVIII levels.

		n	crude difference*	adjusted† difference*
VWF:Ag	0-10 IU/dL	55	4.6 (2.5 to 6.7)	5.3 (3.2 to 7.3)
	11-20 IU/dL	102	-0.5 (-2.2 to 1.1)	0.3 (-1.3 to 1.9)
	21-30 IU/dL	130	-0.2 (-1.7 to 1.3)	0.1 (-1.4 to 1.6)
	>30 IU/dL	282	1 (ref)	1 (ref)
VWF:CB	0-10 IU/dL	171	4.4 (3.0 to 5.8)	5.0 (3.6 to 6.4)
	11-20 IU/dL	90	0.2 (-1.5 to 1.9)	0.4 (-1.2 to 2.1)
	21-30 IU/dL	57	-0.9 (-2.9 to 1.2)	-0.7 (-2.6 to 1.3)
	>30 IU/dL	251	1 (ref)	1 (ref)
VWF:Act	0-10 IU/dL	147	3.6 (2.1 to 5.1)	4.3 (2.9 to 5.8)
	11-20 IU/dL	103	1.3 (-0.4 to 3.0)	1.5 (-0.2 to 3.1)
	21-30 IU/dL	71	-0.2 (-2.1 to 1.8)	-0.1 (-2.0 to 1.7)
	>30 IU/dL	244	1 (ref)	1 (ref)
FVIII:C	0-10 IU/dL	21	9.2 (6.1 to 12.3)	9.6 (6.5 to 12.7)
	11-20 IU/dL	31	1.7 (-0.9 to 4.3)	2.4 (-0.2 to 4.9)
	21-30 IU/dL	57	0.9 (-1.1 to 2.9)	1.3 (-0.6 to 3.3)
	>30 IU/dL	460	1 (ref)	1 (ref)

† corrected for age and gender. * difference reflects the increase in BS (95% confidence interval). Compared to the group with levels >30 IU/dl. New centrally measured levels of VWF and FVIII:C were used.

and gender, p<0.001). Individuals with levels of VWF:CB, VWF:Act and FVIII:C ≤10 IU/dL have BS that are 5.0, 4.3 and 9.6 points higher than individuals with VWF:CB, VWF:Act and FVIII:C levels >30 IU/dL (adjusted for age and gender, p<0.001 for all).

In type 3 patients we found a strong inverse association between FVIII levels and BS: a 1 IU/dL decrease in FVIII was associated with 0.6 point (CI 0.1 to 1.1) increase in BS (p=0.021). This was not found in type 1 and type 2 patients (p=0.613 and p=0.459 respectively). Of VWD patients with FVIII level between 0-5 IU/dL, 6-10 IU/dL and 11-15 IU/dL, respectively 67% (10/15), 33% (2/6) and 50% (7/14) suffered haemarthrosis in the past.

Bleeding score and VWF/FVIII levels according to blood group

The frequency of blood group O in type 1 patients was 68% and in type 2 and type 3 VWD 51% and 58%, respectively. BS did not differ in the total group of VWD patients with blood group O (median 11, range -2 to 30) and non-O (median 11.5, range -2 to 35), p=0.641. Also in type 1, type 2 and type 3 patients separately no statistically significant differences in BS were found between blood group O and non-O (type 1 p=0.411, type 2 p=0.465, type 3 p=0.134). VWF:Ag levels between blood group O and non-O differs significantly in type 2 VWD (blood group O median VWF:Ag 22 IU/dL [15-31 IU/dL] and non-O median 28 IU/dL [18-38 IU/dL], p=0.009), whereas in type 1 and 3 no signicant differences were found in VWF:Ag between O and non-O (type 1 p=0.109, type 3 p=0.605).

DISCUSSION

We investigated the bleeding phenotype and determinants of bleeding in a large cohort of 664 patients with moderate or severe VWD, with lowest historically measured VWF levels of ≤30 U/dL. We found a strong association between bleeding phenotype, and age, sex, circulating plasma VWF and FVIII levels and type of VWD. Type 3 patients had a more severe bleeding phenotype than type 2 and type 1 patients. Within the group of type 2 patients bleeding phenotype was significantly more severe in type 2B compared to type 2A, 2M and 2N. VWF and FVIII:C levels below 10 IU/dL are associated with a more severe bleeding phenotype compared to those VWD patients with levels between 11-20 IU/dL, 21-30 IU/dL and above 30 IU/dL. In type 3 VWD bleeding phenotype is strongly dependent upon FVIII:C levels.

The BS was originally developed by Tosetto et al as a diagnostic tool for type 1 VWD.[7] We have used the BS to determine the severity of bleeding phenotype. Our study revealed higher BS for type 1 patients (median 10) than the cohort of Tosetto et al in which a median BS of 9 was found in index cases and median BS of 4 in affected family members. However, in the WiN study we have only included type 1 patients with low VWF levels (≤30 IU/dL) and therefore have a different cohort than studied in the European Type 1 study.[7] Bleeding phenotype was strongly dependent on type of VWD, which is found both in our large cohort as well as in other studies.[8,9] As others have successfully used the Tosetto BS to differentiate subgroups in other types of VWD,[9,10] its use seems to be justified in our study.

We observed a significantly more severe bleeding phenotype in type 2B and 2A VWD patients compared to 2M and 2N. In a recent study from Castaman et al bleeding tendency was prospectively analysed in type 2A and 2M VWD. In this study similar results were found, as bleeding phenotype in type 2A VWD patients was more severe than in type 2M,[19] which is explained by the fact that gastrointestinal bleeding occurred more frequent in type 2A patients. Anecdotal reports suggested that compared to other type 2 VWD patients bleeding may be more severe in type 2B VWD, in which also thrombocytopenia may exist.[20-22] In our study we observed a significantly higher BS in type 2B patients compared to all other types 2 VWD. Because platelet count was not available, we could not investigate the association between bleeding and platelet number. However, this association has already previously been demonstrated by Federici et al. In their study, lower BS was observed in type 2B patients than in our cohort.[10] This may be caused by the kind of mutation in the *VWF* gene causing type 2B. The two most occurring mutations in the 30 genotyped type 2B patients in the Dutch WiN population were p.R1306W and p.R1308C. Because mutation analysis was not performed in a large part of the WiN study, we cannot compare these data with other type 2B phenotypes. In the study of Federici et al these two mutations, p.R1306W and p.R1308C, were associated with a higher BS compared to other type 2B mutations.[10]

Severe bleeding complications like haemarthrosis, gastrointestinal bleeding and central nervous system bleeding occurred rarely in type 1 and 2 VWD. However, if these bleeding symptoms occurred they were frequently treated with a blood transfusion or factor concentrates. Gastrointestinal bleeding is a bleeding symptom causing severe morbidity, frequently associated with angiodysplasia, but can occur also without a visible lesion in the gastrointestinal tract. Several patients with gastrointestinal tract bleeding need frequent treatment with factor concentrates every day or every other day.[23,24] In our study especially patients with type 2A, type 2B and type 3 suffered from these burdensome bleeding symptoms. Indeed a previous study stated that gastrointestinal bleeding is occurring almost exclusively in types of VWD which are associated with a reduction in high-molecular-weight multimers of VWF.[19,24]

In our cohort the severe VWD patients might have experienced a ceiling effect, meaning that they could have fairly easy reached a maximum plateau of the BS. Patients who had a single gastrointestinal bleeding which was treated with factor concentrate, have the same score as patients with several gastrointestinal bleeding episodes requiring multiple transfusions. This is a limitation of using the Tosetto BS for assessing severity of the disease.

It has been previously reported that in type 1 VWD patients the bleeding phenotype was dependent on VWF and FVIII levels.[7] Also in our study the clinical severity of the disease was associated with VWF:Ag, VWF:CB, VWF:Act and FVIII:C levels (table 2). In type 3 patients, who all have VWF:Ag and VWF:Act levels <5 IU/dL, FVIII:C was a strong determinant of bleeding, especially for haemarthrosis which was mainly seen in individuals with FVIII:C levels <5 IU/dL. Metjian et al also found that lower FVIII levels predicted a higher risk of joint bleeding in VWD patients.[25] In our study haemarthrosis occurred more frequently than in the type 3 VWD study of Lak et al.[26] An explanation might be that the age in our study cohort is higher.

We found a higher BS in index cases than affected family members (p=0.002). However, median VWF and FVIII levels in index cases were significantly lower compared to affected family members (all p<0.001), so this might in part explain the differences in BS between index cases and affected family members.

We found that female gender was strongly associated with a higher BS. However, when we calculated in females the BS without the domains menorrhagia and postpartum haemorrage, this gender effect disappeared. Surprisingly, males even had a significant higher BS than females (p=0.012), using a BS without the domains menorrhagia and postpartum haemorrhage (median BS females 9 (range -2-28) and median BS males 10 (range -2-31)). However, this gender effect is known, has previously been described and is inherent to the BS developed by Tosetto.[7,8,27] Therefore the normal values of the BS are also different for males ≤ 3 and females ≤ 5.

In our cohort in women BS increased with age, as was also observed in the original study.[7] Older patients are more often exposed to risk of bleeding because of surgery and dental procedures. However, males in our cohort reached a BS plateau with increasing age, whereas women tend to increase their BS. An explanation might be that women may also suffer from menorrhagia, postpartum bleeding or dysfunctional uterine bleeding during the perimenopausal period.[28-30] The fact that the difference in age was limited to females could also be explained by historical reasons, as in the past less effective haemostatic interventions were used to prevent menorrhagia and postpartum haemorrhage.[30]

Blood group is a strong determinant of VWF levels.[31,32] As in other cohorts,[33-35] in our study blood group O was overrepresented in type 1 VWD patients compared to the general Dutch population (68% versus 47%). It has been debated in the past whether ABO-specific normal ranges for VWF should be used.[36-38] Because our study indicates that bleeding phenotype is clearly associated with VWF levels but not with blood group, we suggest that ABO-specific normal ranges should not be used in the diagnostic strategy of VWD.

The data in our study were gathered using a self-completed questionnaire, whereas the Tosetto BS was originally designed as an expert-administered questionnaire. In a pilot study in 25 patients we compared the self-reported Tosetto BS with the BS obtained by a physician using the condensed Tosetto BS.[7] We found some individual differences but the median score of these 25 patients was comparable. In previous analyses we showed that the BS was also strongly associated with Quality of Life.[12,13] This clearly shows that a self-administered BS represents the burden of the bleeding phenotype experienced by the patients. The observed differences in BS between patients with type 3 VWD and those with type 1 VWD patients in our study suggests that the self-administration of the questionnaire provides reliable results.[7,27] Furthermore the BS differences between types of VWD are comparable to other studies.[8,9]

This study has some limitations. A possible disadvantage of a self-completed questionnaire is the fact that it is unknown whether the respondent understood the questions properly. To overcome this we conducted a pilot study in which respondents filled in the questionnaire in the presence of the investigator using the "think aloud" method.[39] This resulted in rephrasing of some questions. Another limitation was that we included patients based on historical VWF levels measured in their own Haemophilia Treatment Centre, which use different VWF assays. To overcome inter-laboratory differences we obtained new plasma of nearly 90% of all patients and measured all VWF related parameters in a central laboratory. The VWF levels tended to be higher in the central measurement which may be due to the naturally occurring variation in levels of VWF and FVIII and due to the difference in age at the time of sampling.

The strength of our study is the large number of unselected patients with VWD included, with a response rate of 76%. The Dutch guidelines for treatment of haemophilia and allied disorders indicate that all patients with a bleeding disorder who are treated with factor concentrate replacement therapy, should be treated in a Haemophilia Treatment Centre or treated under responsibility of a Haemophilia Treatment Centre,[40] therefore our study covers almost all patients with moderate and severe VWD in the Netherlands.

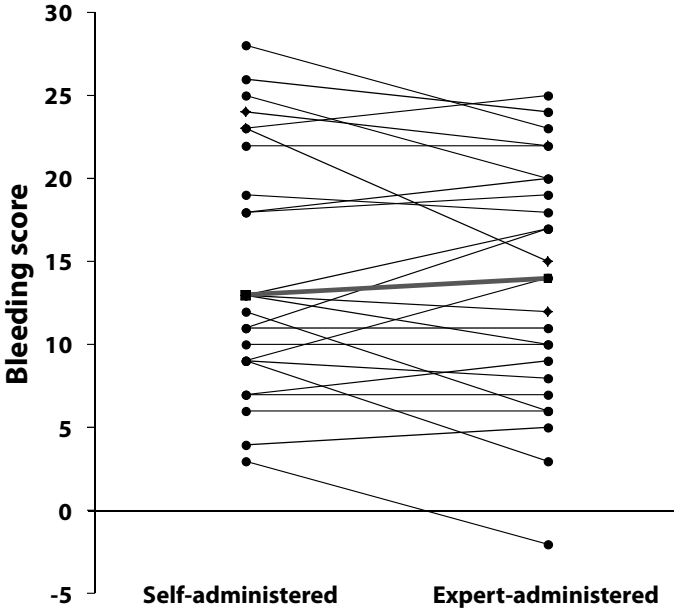
In conclusion, in patients with VWD increasing age, female sex, (sub)type of VWD and low VWF and FVIII:C levels (≤10 IU/dL) are associated with a more severe bleeding phenotype. In type 3 VWD bleeding phenotype is strongly dependent upon FVIII:C levels.

REFERENCES

1. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*. 1987;69(2):454-459.
2. Ruggeri ZM. Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation. *Best Pract Res Clin Haematol*. 2001;14(2):257-279.
3. James AH. More than menorrhagia: a review of the obstetric and gynaecological manifestations of von Willebrand disease. *Thromb Res*. 2007;120 Suppl 1:S17-20.
4. Kadir RA, Chi C. Women and von Willebrand disease: controversies in diagnosis and management. *Semin Thromb Hemost*. 2006;32(6):605-615.
5. Kouides PA. Current understanding of von Willebrand's disease in women - some answers, more questions. *Haemophilia*. 2006;12 Suppl 3:143-151.
6. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
7. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost*. 2006;4(4):766-773.
8. Bowman M, Mundell G, Grabell J, et al. Generation and validation of the Condensed MCMDM-1VWD Bleeding Questionnaire for von Willebrand disease. *J Thromb Haemost*. 2008;6(12):2062-2066.
9. Gill JC, Christoperson PA, Flood VH, Friedman KD, Montgomery RR. The Zimmerman Program Investigators. Bleeding Scores in Von Willebrand Disease (VWD) Re-Visited: Analysis of the TS Zimmerman Program for the Molecular and Clinical Biology of VWD. *Blood ASH Annual Meeting Abstracts*. 2008; 112(425):abstract.
10. Federici AB, Mannucci PM, Castaman G, et al. Clinical and molecular predictors of thrombocytopenia and risk of bleeding in patients with von Willebrand disease type 2B: a cohort study of 67 patients. *Blood*. 2009;113(3):526-534.
11. Nichols WL, Hultin MB, James AH, et al. von Willebrand disease (VWD): evidence-based diagnosis and management guidelines, the National Heart, Lung, and Blood Institute (NHLBI) Expert Panel report (USA). *Haemophilia*. 2008;14(2):171-232.
12. de Wee EM, Mauser-Bunschoten EP, Van Der Bom JG, et al. Health-related quality of life among adult patients with moderate and severe von Willebrand disease. *J Thromb Haemost*. 2010;8(7):1492-1499.
13. de Wee EM, Fijnvandraat K, de Goede-Bolder A, et al. Impact of von Willebrand disease on health-related quality of life in a pediatric population. *J Thromb Haemost*. 2011;9(3):502-509.
14. Tosetto A, Castaman G, Rodeghiero F. Bleeding scores in inherited bleeding disorders: clinical or research tools? *Haemophilia*. 2008;14(3):415-422.
15. Salem RO, Van Cott EM. A new automated screening assay for the diagnosis of von Willebrand disease. *Am J Clin Pathol*. 2007;127(5):730-735.
16. Smith DR, Murphy D. Capillary blotting of agarose gels. *Methods Mol Biol*. 1996;58:23-25.
17. Budde U, Schneppenheim R, Eikenboom J, et al. Detailed von Willebrand factor multimer analysis in patients with von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 von Willebrand disease (MCMDM-1VWD). *J Thromb Haemost*. 2008;6(5):762-771.
18. Landsteiner K. On agglutination of normal human blood. *Transfusion*. 1961;1:5-8.
19. Castaman G, Federici AB, Tosetto A, et al. Different bleeding risk in type 2A and 2M von Willebrand disease: a 2-year prospective study in 107 patients. *J Thromb Haemost*. 2012;10(4):632-638.

20. Mathew P, Greist A, Maahs JA, Lichtenberg EC, Shapiro AD. Type 2B vWD: the varied clinical manifestations in two kindreds. *Haemophilia*. 2003;9(1):137-144.
21. Casonato A, Sartorello F, Pontara E, et al. A novel von Willebrand factor mutation (I1372S) associated with type 2B-like von Willebrand disease: an elusive phenotype and a difficult diagnosis. *Thromb Haemost*. 2007;98(6):1182-1187.
22. Roland K, Rapson D, Lillicrap D, James P. The value of genetic testing for type 2B Von Willebrand disease. *Clin Lab Haematol*. 2006;28(1):17-21.
23. Federici AB. Prophylaxis of bleeding episodes in patients with von Willebrand's disease. *Blood Transfus*. 2008;6 Suppl 2:s26-32.
24. Makris M. Gastrointestinal bleeding in von Willebrand disease. *Thromb Res*. 2006;118 Suppl 1:S13-17.
25. Metjian AD, Wang C, Sood SL, et al. Bleeding symptoms and laboratory correlation in patients with severe von Willebrand disease. *Haemophilia*. 2009;15(4):918-925.
26. Lak M, Peyvandi F, Mannucci PM. Clinical manifestations and complications of childbirth and replacement therapy in 385 Iranian patients with type 3 von Willebrand disease. *Br J Haematol*. 2000; 111(4):1236-1239.
27. Rodeghiero F, Castaman G, Tosetto A, et al. The discriminant power of bleeding history for the diagnosis of type 1 von Willebrand disease: an international, multicenter study. *J Thromb Haemost*. 2005;3(12):2619-2626.
28. Astrup K, Olivarius Nde F, Moller S, Gottschau A, Karlslund W. Menstrual bleeding patterns in pre- and perimenopausal women: a population-based prospective diary study. *Acta Obstet Gynecol Scand*. 2004;83(2):197-202.
29. Duckitt K. Managing perimenopausal menorrhagia. *Maturitas*. 2010;66(3):251-256.
30. De Wee EM, Knol HM, Mauser-Bunschoten EP, et al. Gynaecological and obstetric bleeding in moderate and severe von Willebrand disease. *Thromb Haemost*. 2011;106(5):885-892.
31. Jenkins PV, O'Donnell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? *Transfusion*. 2006;46(10):1836-1844.
32. Gallinaro L, Cattini MG, Sztukowska M, et al. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*. 2008;111(7):3540-3545.
33. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*. 2007;109(1):112-121.
34. James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. *Blood*. 2007;109(1):145-154.
35. Cumming A, Grundy P, Keeney S, et al. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb Haemost*. 2006;96(5):630-641.
36. Collins PW, Cumming AM, Goodeve AC, Lillicrap D. Type 1 von Willebrand disease: application of emerging data to clinical practice. *Haemophilia*. 2008;14(4):685-696.
37. Klarmann D, Eggert C, Geisen C, et al. Association of ABO(H) and I blood group system development with von Willebrand factor and Factor VIII plasma levels in children and adolescents. *Transfusion*. 2010;50(7):1571-1580.
38. Favaloro EJ, Soltani S, McDonald J, Grezchnik E, Easton L, Favaloro JW. Reassessment of ABO blood group, sex, and age on laboratory parameters used to diagnose von Willebrand disorder: potential influence on the diagnosis vs the potential association with risk of thrombosis. *Am J Clin Pathol*. 2005;124(6):910-917.

39. Boren MT, Ramey J. Thinking aloud : Reconciling theory and practice. *IEEE transactions on professional communication* 2000;43(3):261-278.
40. de Wee EM, Leebeek FWG, Eikenboom HCJ. Diagnosis and management of Von Willebrand Disease in the Netherlands. *Semin Thromb Hemost*. 2011;37:480-487.



Supplementary figure 1. Differences in the self-administered and the expert-administered bleeding score. For the 25 randomly selected patients the self-administered and the expert-administered bleeding score are depicted. The thick line shows the median score.

9

Joint bleeds in von Willebrand Disease patients have significant impact on quality of life and joint integrity: a cross-sectional study

KPM van Galen, YV Sanders, U Vojinovic, J Eikenboom, MH Cnossen, REG Schutgens, JG van der Bom, K Fijnvandraat, BAP Laros-van Gorkom, K Meijer, FWG Leebeek, EP Mauser-Bunschoten, and the WiN study group

Haemophilia. 2015;21(3):e185-192

ABSTRACT

Background: Joint bleeds (JB) are reported in a minority of patients with Von Willebrand Disease (VWD) but may lead to structural joint damage. Prevalence, severity and impact of JB in VWD are largely unknown.

Objectives: The aim of this study was to assess JB prevalence, onset, treatment and impact on health-related quality of life (HR-QoL) and joint integrity in moderate and severe VWD.

Methods: In the Willebrand in the Netherlands study 804 moderate and severe VWD patients (von Willebrand Factor (VWF) activity ≤30 U/dL) completed a questionnaire on occurrence, sites and consequences of JB. To analyse JB number, onset, treatment and impact on joint integrity we additionally performed a patient-control study on medical file data comparing patients with JB to age, gender, Factor VIII (FVIII) and VWF activity matched VWD patients without JB.

Results: Of all VWD patients 23% (184/804) self-reported JB. These 184 patients also reported joint damage more often (54% versus 18%, $p<0.001$) and had lower HR-QoL (SF-36, $p<0.05$) compared to VWD patients not reporting JB. Of 55 patients with available JB data, 65% had the first JB before age 16. These 55 patients used more clotting factor concentrate (CFC; median dose 43 versus 0 IE FVIII/kg/year, $p<0.001$), more often had X-ray joint damage (44% versus 11%, $p=0.001$) and chronic joint pain (44% versus 18%, $p=0.008$) compared to 55 control VWD patients without JB.

Conclusions: In conclusion, JB are reported by 23% of moderate and severe VWD patients, mostly start in childhood, are associated with more CFC use, joint pain, lower HR-QoL and significantly more radiological and self-reported joint damage.

INTRODUCTION

Von Willebrand Disease (VWD) is a heterogeneous inherited bleeding disorder that affects up to 1% of the population.[1] Type 1 VWD is characterized by a quantitative deficiency of Von Willebrand Factor (VWF). In type 2 VWD four subtypes are recognized representing different functional VWF defects. In the most severe type 3 VWD, there is a complete lack of VWF. VWF is an essential clotting factor in primary hemostasis by interacting with platelets, but it also carries Factor VIII (FVIII) in the circulation. Therefore, besides VWF, FVIII can also be deficient in more severe VWD, resulting in hemophilia A like phenotype.[2,3] Accordingly, severe VWD patients have more severe bleedings including joint bleeds (JB).[3-6]

In hemophilia patients, JB can lead to structural joint damage due to synovial inflammation caused by hemosiderin deposition.[7] Synovial iron deposition and inflammatory cell proliferation provokes degeneration of joint surfaces and eventual loss of normal joint function.[7,8] Arthropathy caused by JB has occasionally been described in patients with VWD. Blood-induced arthropathy may develop in more than 10% of type 3 VWD patients.[6]

Although the occurrence of JB and subsequent joint damage has been recognized, the prevalence, severity, onset, impact and complications of JB in patients with VWD are largely unknown. This gap in knowledge hampers optimal prevention, treatment and education of VWD patients with JB. We have previously reported that moderate and severe VWD patients with the highest quartile bleeding score have lower health-related quality of life (HR-QoL) on the SF-36 domains of physical functioning, role limitations due to physical functioning, bodily pain, general health, social functioning and physical component summary, compared to VWD patients with the lowest quartile bleeding score.[9] Whether JB influence HR-QoL has not been assessed. The current cross-sectional study aims to improve the knowledge on JB in moderate and severe VWD by assessing the prevalence, onset and treatment of JB and impact on HR-QoL and joint integrity.

PATIENTS AND METHODS

Willebrand in the Netherlands study

The Willebrand in the Netherlands study (WiN) is a nationwide multicenter cross-sectional study in moderate and severe VWD patients. Patients with bleeding symptoms or a family history of VWD and historically lowest levels of VWF (VWF antigen level (VWF:Ag) or VWF ristocetin cofactor activity (VWF:RCo)) ≤30 U/dL or FVIII concentration (FVIII:C) ≤40 U/dL were included.[10] VWF:Ag, VWF collagen binding activity (VWF:CB), VWF:Act, FVIII:C and multimer analysis were measured centrally, as has been described

before.[3,11] Severe VWD was defined as a VWF activity or FVIII:C ≤10 U/dL and moderate VWD as VWF activity 11-30 U/dL or FVIII:C of 11-40 U/dL.[3] All participants completed a questionnaire between containing questions on bleeding symptoms including JB, treatment of VWD, co-morbidity, no, mild, moderate or severe permanent impairment of joint function after bleeding (joint damage) and surgery. Participants of 16 years or older (n=680) also completed the HR-QoL SF-36 questionnaire.[9]

Patient-control study

For all patients who reported in the WiN study questionnaire that they had received treatment with desmopressin or clotting factor concentrate (CFC) for JB, we studied the medical files. Patients of whom documentation was available, were included in the patient-control study. We obtained data on the site, number, cause and treatment of JB, age at first JB, use of therapeutic and prophylactic CFC, joint damage on X-rays, joint pain, pain medication and joint surgery. We calculated the mean CFC usage over a 5 year period (2005-2009) and defined CFC prophylaxis as at least one regular CFC infusion per week for at least 45 consecutive weeks.[12] X-ray confirmed joint damage comprised joint damage, indicated by bone or cartilage damage as described in the radiologists report (e.g. subchondral cysts, surface erosion or joint-space narrowing). Joint pain and the chronic use of pain medication were only recorded if not directly related to an active joint bleed.

To verify whether the recorded joint problems were related to JB and to study treatment consequences of JB in VWD, we additionally obtained medical file data from comparable VWD patients included in the WiN study who did not report JB in the questionnaire and had no JB treatment documented in their medical file. We matched these control VWD patients without JB 1:1 to the VWD patients with JB for gender, age (± 2 years) and FVIII:C (maximum ± 10%). The latter because of our previous finding that FVIII:C is the most important determinant for JB in VWD.[3] In cases without an available match on FVIII:C in the cohort, we matched on VWD severity instead (VWF:Act of ≤10 IU/dL, 11-30 IU/dL or >30 IU/dL, respectively). We matched on centrally measured clotting factor levels, if available (n=39), or else on historically lowest levels (n= 16) (figure 1).[3]

Statistical analysis

We performed all statistical analysis with IBM SPSS Statistics version 20 (IBM Corp.: Armonk, NY, USA). We checked if outcome measures were normally distributed with Q-Q plots and used chi-squared or Fisher's Exact test to compare proportions, depending on the expected cell counts. We used the Mann-Whitney *U t*est for non-parametric testing of the not normally distributed coagulation factor levels. We used linear regression to assess the influence of JB and self-reported joint damage on HR-QoL and logistic regression for dichotomous outcome variables to determine odds ratio (OR) and 95% confidence intervals (CI). We considered a p-value <0.05 as statistically significant.

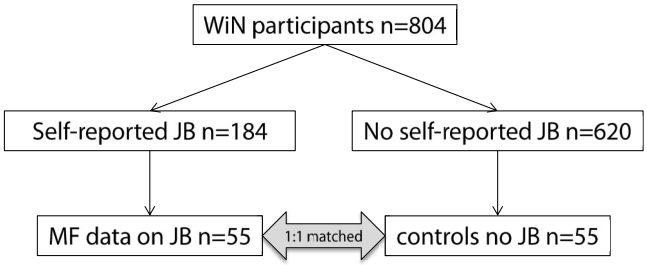


Figure 1. Flowchart of the patient-control study.
WiN = Willebrand in the Netherlands, MF = medical file, JB = joint bleeds. Matched control VWD patients did not report joint bleeds in the questionnaire and had no joint bleed treatment documented in their medical file. Matching on gender, age (+/- 2 years) and FVIII:C (+/- 10%) or VWD severity (VWF:Act level of ≤10 IU/dL, 11-30 IU/dL or >30 IU/dL, respectively) if no match on FVIII:C was available (n=13). We matched on centrally measured clotting factor levels, if available, or else on historically lowest levels (n=16: historically lowest FVIII:C, n=12, historically lowest VWF activity, n=4).

RESULTS

WiN study von Willebrand Disease questionnaire

In total 804 Dutch moderate and severe VWD patients who completed the questionnaire were included in the WiN study.[10,11,13] The median age at inclusion was 41 years (range 0 to 85, adults n=664) and the proportion of females 60% (n=481). Most patients had type 1 VWD (57%, n=454), 37% type 2 (n=301) and 6% type 3 VWD (n=46) (table 1).

Self-reported occurrence and consequences of joint bleeds

Twenty-three percent of the patients self-reported JB (184/804), most often in the knee (56%, n=103), followed by the ankle (50%, n=92) and elbow (24%, n=44) (table 2). Type 3 VWD patients reported JB most often (47%, 22/47). JB were reported by 21% (62/301) and 22% (100/454) of the type 2 and type 1 VWD patients, respectively. Compared to patients not reporting JB (n=620), patients reporting JB had more often severe VWD, based on centrally measured VWF activity or FVIII:C of ≤10 U/dL (OR 1.48, CI 1.01 to 2.12). Patients self-reporting JB also reported joint damage in large joints more frequently (54% versus 18%, p<0.001) as well as surgery of large joints (25% versus 11%, p<0.001) (table 2). The HR-QoL SF-36 questionnaire was completed by 617 participants, aged 16-83 years. Patients reporting JB had significantly lower HR-QoL compared to those not reporting JB (SF-36 physical component summary (PCS): β -4.03, CI -6.92 to -1.14, p=0.005 and mental component summary (MCS): β -2.78, CI -4.74 to -0.83, p=0.006) (table 2). The difference in HR-QoL was significantly lower (p<0.05) for all SF-36 domains except for physical functioning, which was rated high by all participants (supplemental table 2). When we added self-reported joint damage to the regression model, the effect of JB on

Table 1. Baseline characteristics of the WiN study population.

Total n=804			
Male sex, n (%)		323	(40)
Age (year), median (range)	All	41	(0-85)
	Males	36	(0-85)
	Females	44	(0-83)
	Aged ≥16 y, n (%)	680	(85)
Blood group O, n (%) *		434	(61)
Type, n (%) †	1	454	(57)
	2	301	(37)
	2A	202	
	2B	55	
	2M	30	
	2N	15	
	3	46	(6)
New centrally measured levels, median [IQR] ‡	VWF:Ag (IU/dL)	29	[18-45]
	VWF:CB (IU/dL)	23	[7-51]
	VWF:Act (IU/dL)	23	[8-53]
	FVIII:C (IU/dL)	51	[33-73]
Historically lowest levels, median [IQR]	VWF:Ag (U/dL)	29	[20-40]
	VWF:RCo (U/dL)	15	[7-25]
	FVIII:C (U/dL)	43	[30-59]

IQR = 25-75% inter quartile range, y = years, VWF:Ag = Von Willebrand Factor antigen level, VWF:CB = VWF collagen binding activity, VWF:Act = VWF activity level, VWF:RCo = VWF ristocetin cofactor binding activity, FVIII:C = Factor VIII level. * n=715 based on availability. † based on centrally determin 154, type known in the hemophilia treatment center, 1 missing). ‡ n=630, based on patients of whom plasma was available and after exclusion of pregnant patients and those who had received CFC or desmopressin <72hr before the laboratory assessment.

HR-QoL largely disappeared (PCS: β -2.73, CI -5.81 to 0.35, p=0.08 and MCS: β -0.27, CI -2.25 to 1.71, p=0.79), suggesting that joint damage due to JB is largely responsible for the association between JB and lower HR-QoL.

Patient-control study on joint bleeds in von Willebrand Disease

We included 55 patients who had self-reported treatment for JB and available medical file documentation on JB treatment in the patient-control study (figure 1). The characteristics of these patients and of 55 matched patients without JB are summarized in table 3. Remarkably, 66% of these patients with JB are male, a high proportion given the female predominance of 60% in the whole WiN cohort. By matching, FVIII:C and VWF activity levels between the two groups were not significantly different but in the JB patient group more patients had type 3 VWD compared to the control group (38% versus 20%, p=0.036). In the WiN questionnaire most JB occurred in the knee (41/55), followed by

Table 2. Characteristics of von Willebrand Disease patients self-reporting joint bleeds compared to those not reporting joint bleeds.

Data from WiN questionnaire n=804		**self-reported JB (n=184)**	**no JB reported (n= 620)**	**p-value**
Sex, n (%)	Males	81 (44)	242 (39)	0.230
	Females	103 (56)	378 (61)	
Age at inclusion (year),	Males	40 [26-59]	32 [13-52]	0.012
median [IQR]	Females	47 [29-58]	43 [28-55]	0.120
Type, n (%)*	1	100 (54)	354 (57)	0.510
	2	62 (34)	241 (39)	0.220
	3	22 (12)	24 (4)	<0.001
JB sites, n (%)	Knee	103 (56)	-	-
	Ankle	92 (50)		
	Elbow	44 (24)		
	Wrist	33 (18)		
	Shoulder	26 (14)		
	Hip	15 (8)		
Treatment reported for JB, n (%) †	Desmopressin	26 (14)	-	-
	CFC	62 (34)		
	Desmopressin and CFC	9 (5)		
Severe VWD, n (%) ‡	VWF:Act ≤10 IU/dL	52 (35)	125 (26)	0.048
	FVIII:C ≤10 IU/dL	17 (11)	16 (3)	<0.001
	VWF:Act or FVIII:C ≤10 IU/dL	56 (35)	133 (27)	0.044
Large joint damage, n (%) §	knee, ankle, elbow, shoulder, hip	99 (54)	110 (18)	<0.001
Comorbidity, n (%) ¶	Influencing muscle-joint function	19 (10)	39 (6)	0.06
Large joint surgery, n (%)	knee, ankle, elbow, shoulder, hip	46 (25)	69 (11)	<0.001
Quality of life, mean **	SF36 MCS	47	50	0.006
	SF36 PCS	73	77	0.005

JB = joint bleeds, y = years, CFC = clotting factor concentrate, WiN = Willebrand in the Netherlands study, SF36 MCS = mental component summary score of the SF36 health related quality of life questionnaire, SF36 PCS = physical component summary score of the SF36 health related quality of life questionnaire. * based on patients of whom plasma was available (n=649) or historical type vWD (n=154, 1 missing). † 18% of the patients reporting JB did not answer this question, 39% reported no JB treatment. ‡ based on n=151 JB patients and n=479 no-JB patients of whom plasma was available and after exclusion of pregnant patients and those who had received CFC or desmopressin <72hr before the laboratory assessment. § permanent mild, moderate or severe impairment of joint function after bleeding. ¶ complete list of relevant comorbidity in Supplemental Table 1. ** based on n=151 adult JB patients and n= 466 adult no-JB patients. No significant effect modification for sex (PCS p=0.69, MCS p=0.64). Results of the SF-36 subdomains are depicted in Supplemental Table 2.

the ankle (31/55) and elbow (14/55). Most patient had less than five JB (74%, 36/49, one joint bleed in 39%, 19/49). More than five JB occurred most often in type 3 VWD patients (9/19, 47%, p=0.009 compared to non-type 3 VWD), in 17% of type 2 patients (4/23) and none of the type 1 VWD patients. JB without an apparent traumatic cause occurred in 43% of the patients (20/46), mostly in type 3 VWD (14/20), but also in type 1 and 2 VWD patients (3/20 and 3/20, respectively). In most cases the first JB occurred before the age of 16 years (30/46, 65%, median age 12 years, range 3 to 69) (table 3).

Treatment of joint bleeds

Compared to the 55 matched control VWD patients without JB, the median CFC consumption per year was higher in the VWD patients with JB (43 versus 0 IE FVIII/kg/year, p<0.001). CFC prophylaxis was started because of JB in 24% of these patients (13/55) (table 3). The median duration of treatment for JB was 2 days (range 1 to 7.5 days) and

Table 3. Patient control study: characteristics and outcome of matched von Willebrand Disease patients with and without joint bleeds.

		n= 55 with JB	**n= 55 matched* controls no JB**	**p-value**
Sex, n (%)	Males Females	36 (66) 19 (34)	36 (66) 19 (34)	-
Age (year), median (range)	Males	32 (3-73)	31 (4-74)	-
	Females	42 (7-70)	40 (6-70)	
Type, n (%)	1	11 (20)	21 (38)	0.036
	2	23 (42)	23 (42)	1.000
	2A	17	17	
	2B	3	5	
	2N	2	0	
	2M	1	1	
	3	21 (38)	11 (20)	0.036
New centrally measured levels, median [IQR]†	VWF:Act (IU/dL)	2.5 [0-14]	6 [1-15]	0.170
	FVIII:C (IU/dL)	17 [2-46]	27 [15-47]	0.100
Severe VWD, n (%)	VWF:Act or FVIII:C ≤10 IU/dL	30 (68)	33 (72)	0.710
CFC use, n (%)	CFC use, all indications	52 (95)	44 (80)	0.022
	Median CFC dose (IE FVIII/kg/year) [IQR] ‡	43 [13-113]	0 [0-13]	<0.001
CFC prophylaxis, n (%)	At inclusion or in the past Because of JB	16 (29) 13 (24)	1 (2)	<0.001
Orthopedic joint surgery, n (%) §	knee, ankle, elbow, shoulder, hip	14 (26)	6 (11)	0.048
Age first JB ¶	Age 1th JB, median (range) Age 1th JB<16 years, n (%)	12 (3-69) 30 (65)		
Joint damage, n (%)	on X-ray	24 (44)	6 (11)	0.001
Joint pain, n (%)	Documented	24 (44)	10 (18)	0.008
Pain medication use (present or past), n (%)	Documented	6 (11)	3 (6)	0.490

Patient selection based on available medical file data. JB = joint bleeds, CFC = clotting factor concentrate, IQR = 25-75% interquartile range. * Matching on gender, age (+/- 2 years) and FVIII:C (+/- 10%) or VWD severity. † based on 44/55 patients and 46/55 controls of whom plasma was available and after exclusion of pregnant patients and those who had received CFC or desmopressin <72hr before the laboratory assessment. ‡ based on 80/110 cases wherein this information was available in the medical files. § Orthopedic joint surgery was performed in 14 joint bleed patients at median age 35 (range 13-66) and in 6 control VWD patients at median age 47 (range 20-60). ¶ based on n=46 cases wherein this information was available in the medical files.

median CFC dose used 3,666 IE FVIII per JB [25-75% IQR 2,000-6,000]. A clear decrease in JB frequency was noted in 13/14 VWD patients following the initiation of CFC prophylaxis. The duration of prophylaxis ranged from 10 months to 13 years (median duration 1.25 years) and most patients received CFC prophylaxis twice weekly to prevent JB (62%, 8/13).

Consequences of joint bleeds

Documented joint damage on X-rays occurred in 44% of the 55 patients with joint bleeds compared to 11% of the controls (p=0.001) (table 3). X-ray joint damage occurred more often in patients with more than five documented JB (69%, 9/13, of the patients with a total of >5 JB versus 36%, 15/42, of the patients with ≤5 JB, p=0.033). No association was observed between the first JB at younger age and joint damage on X-ray (15/30 with first JB<16 versus 4/16 with first JB≥16, p=0.100). Orthopedic joint surgery was performed in 14 JB patients (7 males and 7 females) and in 6 control VWD patients (26% versus 11%, p=0.048). The median age at surgery was 35 (range 13 to 66) in the JB patients and 47 (range 20 to 60) in the control VWD patients (p=0.42). Arthropathy due to JB was the main reason for joint surgery in 9 of the 14 joint bleed patients undergoing joint surgery. Of these 9 patients, 6 had type 3, 2 type 2A and 1 type 1 VWD. Joint pain was documented in 44% of the medical files of the VWD patients with JB, significantly more often compared to the control VWD patients (18%, p=0.008) and strongly associated with joint damage on X-rays in both patients (p<0.001) and controls (p=0.001) (table 3).

DISCUSSION

In this large Dutch cross-sectional study we found that JB in moderate and severe VWD patients (VWF activity ≤30 IU/dL) mostly start in childhood, are associated with more CFC use, joint pain, lower HR-QoL and significantly more radiologic and self-reported joint damage. In patients with hemophilia, arthropathy due to blood induced cartilage damage after JB has been shown to affect HR-QoL.[14] In VWD patients HR-QoL is lower compared to the general population and bleeding phenotype is a major determinant.[9] In this study, we found that JB are associated with significantly lower HR-QoL in VWD. This appeared to be related to joint damage, because the association between self-reported JB and HR-QoL largely disappeared after adding self-reported joint damage to the regression model. These findings suggest that arthropathy affects HR-QoL in VWD, as in hemophilia.

Almost a quarter (23%) of the moderate and severe VWD patients in our cohort self-reported JB, associated with lower FVIII:C and a more severe bleeding phenotype.[3,15] The 47% self-reported prevalence of JB in type 3 VWD patients is comparable with other

reports.[16-19] The 20-22% self-reported prevalence of JB in non-type 3 VWD is higher than previously reported, possibly because these previous studies also included mild type VWD patients.[5,20] Over-reporting of JB has been demonstrated in hemophilia.[21] Then again the occurrence of traumatic JB is not rare, even in the normal population, and likely to occur more often in patients with a coagulation disorder.[22] Better education of patients as well as physicians on the specific clinical characteristics and treatment of JB seems warranted and specific criteria are needed to determine whether joint symptoms are caused by JB.

The patient-control study revealed that patients receiving treatment for JB were predominantly male and had all three types VWD. The occurrence of JB in non-type 3 VWD patients mirrors the self-reported prevalence but also reflects the larger proportion of type 1 and 2 compared to type 3 VWD patients in the WiN cohort. Not surprisingly, in type 3 VWD the largest number of overall and non-traumatic JB occurred. Male over-representation in the occurrence of JB has not been reported before in VWD and could reflect differences in lifestyle. Joint damage on X-rays and chronic joint pain occurred significantly more often in VWD patients with JB, both male and female, compared to matched VWD patients without JB. Still, these problems occurred quite frequently in the controls. Subclinical or unattended JB, osteoarthritis or age-related joint problems could possibly cause joint symptoms and affect joint integrity in VWD patients without apparent JB. Prospective studies are needed to resolve these issues.

Based on literature, arthropathy occurs in 7% of VWD patients (all types) and over 10% of type 3 VWD patients.[6] In this study, almost half of the patients treated for JB had evidence of joint damage on X-rays. Such a high proportion of radiologic joint damage has previously been reported in VWD patients with JB in two Swedish series.[5,16] One Chinese study reported 10% joint deformity on X-rays but also included patients with mild VWD.[19] In a retrospective Italian study on 23 VWD patients with orthopedic surgery, arthropathy formed the indication for surgery in 16% of the procedures.[23] Arthropathy due to JB was the main indication for orthopedic intervention in more than half of our patients treated for bleeds, suggesting a significant burden of arthropathy in VWD. We are currently conducting a cross-sectional study to assess this burden (trial number NTR4548).

Prophylactic CFC infusion is known to prevent joint damage in severe hemophilia A.[24] CFC prophylaxis to prevent JB has been prescribed to 24% of VWD patients with JB (12/49) according to the patient-control study. From the patients with more than five recorded JB (n=13) most patients (n=8, 62%) have ever used CFC prophylaxis. The decreased frequency of JB after starting prophylaxis in 93% (13/14) of the patients in this study and 88% joint bleeding reduction within patients during prophylaxis in the VWD International Prophylaxis (VIP) study, strongly suggests that secondary CFC prophylaxis is indicated to prevent JB and thus joint damage.[25] The optimal timing to start

prophylaxis and the number of JB that can be accepted before its instigation remains to be determined in VWD. In hemophilia, JB before 2 years of age are associated with more arthropathy and progressive arthropathy has been reported to occur when patients reported more than five JB before the onset of prophylaxis.[26,27] Due to the use of existing radiology reports without uniform scoring of joint damage, we could not investigate the relationship between JB at younger age and more severe arthropathy, but we did find that joint damage was associated with more than five JB. Compared to hemophilia, the age at first JB seems higher in VWD. In a retrospective Swedish case series of 35 VWD patients on prophylaxis, clinical and radiological joint damage did occur in subjects starting prophylaxis over 15 years of age, but not in those starting prophylaxis before 5 years of age.[28] Prospective studies that are currently assessing the safety and efficacy of prophylactic CFC treatment in VWD have to be awaited to address the issues of optimal timing, cost-effectiveness and HR-QoL.[25,29,30]

The strength of this study is that we are the first to report on JB in a large cohort of moderate to severe VWD patients providing better insight into the occurrence, treatment and impact of JB. Combining self-reported and medical file data revealed novel information regarding JB in VWD including a possible role for male sex, the occurrence of JB in non-type 3 VWD and onset in childhood, as well as a probable impact on HR-QoL and a relatively large impact on joint integrity. Limitations are the use of a self-administered questionnaire, which may be associated with recall and reporting bias, and the inherent flaw of missing data in medical files. As joint aspiration and ultrasound are rarely performed in VWD we had to rely on the patients and physicians interpretation of joint symptoms to assess JB. These issues were partly overcome by comparing the findings in patients with physician-verified JB to a control group without JB. The matching of patients with documented treatment for JB to patients without JB, instead of studying all medical files, could, however, have led to loss of information due to a smaller sample size and the matching on FVIII:C led to an overrepresentation of type 3 VWD in de patient group, possibly influencing the results.

This study adds important information on the occurrence, treatment and impact of JB in VWD that could contribute to better prevention, treatment and patient education. JB are reported by almost a quarter of patients with moderate and severe VWD, start mostly before the age of 16 years and occur in all three types of moderate to severe VWD. JB in VWD are associated with more severe VWD, male sex, more CFC consumption, more self-reported and X-ray recorded joint damage and lower HR-QoL. CFC prophylaxis reduced joint bleed frequency and is likely to prevent joint damage when more than five JB occur. Joint pain occurs frequently in VWD patients with a history of JB. Taken together, our findings suggest that there is a significant burden of arthropathy in VWD. Further study is needed to assess this burden and to determine optimal treatment.

REFERENCES

1. Rodeghiero F, Castaman G, Dini E. Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*. 1987;69(2):454-459.
2. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
3. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108(4):683-692.
4. Federici AB. Clinical diagnosis of von Willebrand disease. *Haemophilia*. 2004;10 Suppl 4:169-176.
5. Silwer J. von Willebrand's disease in Sweden. *Acta Paediatr Scand Suppl*. 1973;238:1-159.
6. van Galen KP, Mauser-Bunschoten EP, Leebeek FW. Hemophilic arthropathy in patients with von Willebrand disease. *Blood Rev*. 2012;26(6):261-266.
7. Hooiveld M, Roosendaal G, Vianen M, van den Berg M, Bijlsma J, Lafeber F. Blood-induced joint damage: longterm effects in vitro and in vivo. *J Rheumatol*. 2003;30(2):339-344.
8. Valentino LA, Hakobyan N, Enockson C, et al. Exploring the biological basis of haemophilic joint disease: experimental studies. *Haemophilia*. 2012;18(3):310-318.
9. de Wee EM, Mauser-Bunschoten EP, van der Bom JG, et al. Health-related quality of life among adult patients with moderate and severe von Willebrand disease. *J Thromb Haemost*. 2010;8(7):1492-1499.
10. de Wee EM, Leebeek FW, Eikenboom JC. Diagnosis and management of von Willebrand disease in The Netherlands. *Semin Thromb Hemost*. 2011;37(5):480-487.
11. Sanders YV, Eikenboom J, de Wee EM, et al. Reduced prevalence of arterial thrombosis in von Willebrand disease. *J Thromb Haemost*. 2013;11(5):845-854.
12. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. *Haemophilia*. 2013;19(1):e1-47.
13. Sanders YV, de Wee EM, Meijer K, et al. [Von Willebrand disease in the Netherlands: the WiN study] De ziekte van von Willebrand in Nederland: de WiN-studie. *Ned Tijdschr Geneeskd*. 2014;158:A6518.
14. Fischer K, Bom JG, Mauser-Bunschoten EP, Roosendaal G, Berg HM. Effects of haemophilic arthropathy on health-related quality of life and socio-economic parameters. *Haemophilia*. 2005;11(1):43-48.
15. Sood SL, Cuker A, Wang C, et al. Similarity in joint function limitation in Type 3 von Willebrand's disease and moderate haemophilia A. *Haemophilia*. 2013;19(4):595-601.
16. Ahlberg A, Silwer J. Arthropathy in von Willebrand's disease. *Acta Orthop Scand*. 1970;41(5):539-544.
17. Metjian AD, Wang C, Sood SL, et al. Bleeding symptoms and laboratory correlation in patients with severe von Willebrand disease. *Haemophilia*. 2009;15(4):918-925.
18. Sumner M, Williams J. Type 3 von Willebrand disease: assessment of complications and approaches to treatment -- results of a patient and Hemophilia Treatment Center Survey in the United States. *Haemophilia*. 2004;10(4):360-366.
19. Zhang L, Li H, Zhao H, Zhang X, Ji L, Yang R. Retrospective analysis of 1312 patients with haemophilia and related disorders in a single Chinese institute. *Haemophilia*. 2003;9(6):696-702.
20. Ziv O, Ragni MV. Bleeding manifestations in males with von Willebrand disease. *Haemophilia*. 2004; 10(2):162-168.
21. Ceponis A, Wong-Sefidan I, Glass CS, von Drygalski A. Rapid musculoskeletal ultrasound for painful episodes in adult haemophilia patients. *Haemophilia*. 2013;19(5):790-798.
22. Sarimo J, Rantanen J, Heikkila J, Helttula I, Hiltunen A, Orava S. Acute traumatic hemarthrosis of the knee. Is routine arthroscopic examination necessary? A study of 320 consecutive patients. *Scand J Surg*. 2002;91(4):361-364.

23. Siboni SM, Biguzzi E, Solimeno LP, et al. Orthopaedic surgery in patients with von Willebrand disease. *Haemophilia*. 2014;20(1):133-140.
24. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*. 2007;357(6):535-544.
25. Abshire TC, Federici AB, Alvarez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand Disease Prophylaxis Network (VWD PN). *Haemophilia*. 2013;19(1): 76-81.
26. van Dijk K, Fischer K, van der Bom JG, Grobbee DE, van den Berg HM. Variability in clinical phenotype of severe haemophilia: the role of the first joint bleed. *Haemophilia*. 2005;11(5):438-443.
27. Kreuz W, Escuriola-Ettingshausen C, Funk M, Schmidt H, Kornhuber B. When should prophylactic treatment in patients with haemophilia A and B start?--The German experience. *Haemophilia*. 1998; 4(4):413-417.
28. Berntorp E, Petrini P. Long-term prophylaxis in von Willebrand disease. *Blood Coagul Fibrinolysis*. 2005;16 Suppl 1:S23-26.
29. Berntorp E. Prophylaxis in von Willebrand disease. *Haemophilia*. 2008;14 Suppl 5:47-53.
30. Federici AB. Highly purified VWF/FVIII concentrates in the treatment and prophylaxis of von Willebrand disease: the PRO. WILL Study. *Haemophilia*. 2007;13 Suppl 5:15-24.

Supplemental table 1. List of relevant comorbidity affecting joint or muscle function**.**

Osteoarthritis
Psoriatic arthritis
Ehlers Danlos syndrome
Fibromyalgia
Hip dysplasia
King syndrome
Club feet
Marfan syndrome
Mitochondrial myopathy
Rheumatoid arthritis
Sarcoidosis
Ankylosing spondylitis
Steinert myotonic dystrophy

Supplemental table 2. Results of the SF-36 health-related quality of life subdomains**.**

No self-reported joint bleed n=640					
	n	**mean**	**median**	**minimum**	**maximum**
SF36: physical functioning	620	0.57	1.0	0	1
SF36: role physical	483	85.95	95.0	0	100
SF36: bodily pain	483	80.73	100.0	0	100
SF36: general health	493	79.77	100.0	0	100
SF36: vitality	490	69.69	72.0	0	100
SF36: social functioning	491	64.96	70.0	5	100
SF36: role emotional	496	84.50	100.0	0	100
SF36: mental health	481	86.94	100.0	0	100
Self-reported joint bleed n=184					
	n	**mean**	**median**	**minimum**	**maximum**
SF36: physical functioning	184	0.55	1.0	0	1
SF36: role physical	162	80.94	90.0	5	100
SF36: bodily pain	156	73.08	100.0	0	100
SF36: general health	167	69.70	72.0	0	100
SF36: vitality	164	63.85	67.0	5	100
SF36: social functioning	165	58.59	60.0	0	100
SF36: role emotional	167	77.17	87.5	0	100
SF36: mental health	161	78.78	100.0	0	100

Results of the differences in SF-36 health-related quality of life (HR-QoL) subdomains between the patients reporting joint bleeds and those not reporting joint bleeds. The difference in HR-QoL was significantly lower ($p<0.05$) for all SF36 domains except for physical functioning which was rated high by all participants.

10

Reduced prevalence of arterial thrombosis in von Willebrand Disease

YV Sanders, J Eikenboom, EM de Wee, JG van der Bom, MH Cnossen, MEL Degenaar-Dujardin, K Fijnvandraat, PW Kamphuisen, BAP Laros-van Gorkom, K Meijer, EP Mauser-Bunschoten, FWG Leebeek, and the WiN study group

Journal of Thrombosis and Haemostasis. 2013;11(5):845-854

ABSTRACT

Background: High von Willebrand Factor (VWF) levels are an established risk factor for arterial thrombosis, including coronary heart disease and ischemic stroke. It has been hypothesized that von Willebrand Disease (VWD) patients are protected against arterial thrombosis; however, this has never been confirmed in clinical studies.

Objectives: to investigate the prevalence of arterial thrombosis in VWD patients relative to the general population.

Patients/Methods: We included 635 adult patients with VWF levels ≤30 U/dL, aged 16-85 years, from the nationwide cross-sectional "Willebrand in the Netherlands" (WiN) study and compared the prevalence of arterial thrombosis with two reference populations from the general Dutch population adjusted for age and sex as standardized morbidity ratios (SMRs).

Results: Twenty-nine arterial thrombotic events occurred in 21 patients (3.3%). Five patients suffered an acute myocardial infarction and three an ischemic stroke. Unstable angina pectoris was recorded twelve times, transient ischemic attack nine. The prevalence of all arterial thrombotic events combined (acute myocardial infarction, ischemic stroke and coronary heart disease) was 39% and 63% lower than in the two reference populations. The prevalence of cardiovascular disease in VWD was lower than in the general population, SMR 0.60 (95% confidence interval (CI) 0.32 to 0.98) for coronary heart disease and SMR 0.40 (CI 0.13 to 0.83) for acute myocardial infarction. For ischemic stroke the prevalence was 35-67% lower compared with two reference populations, SMR 0.65 (CI 0.12 to 1.59) and 0.33 (CI 0.06 to 0.80), respectively.

Conclusions: This is the first study showing that VWD patients have a reduced prevalence of arterial thrombosis and provides important insights into the role of VWF in the pathogenesis of arterial thrombosis.

INTRODUCTION

Von Willebrand Factor (VWF) plays an important role in primary hemostasis by facilitating adhesion of platelets to the endothelium, thereby initiating aggregation of platelets to form a platelet plug.[1] Von Willebrand Disease (VWD), the most common inherited bleeding disorder, is caused by reduced levels or activity of VWF and is mainly characterized by recurrent mucocutaneous bleeding.[2]

Several studies have shown that individuals with high VWF levels have an increased risk of coronary heart disease and ischemic stroke.[3,4] In addition, genetic variants of *VWF* resulting in higher VWF levels, are associated with an increased risk of coronary heart disease and ischemic stroke.[5] However, there is still debate whether VWF levels are causally associated with a reduced risk of arterial thrombosis. Nonetheless, VWF may be considered a risk modifier for arterial thrombosis, such as acute myocardial infarction and ischemic stroke. However, recent studies indicate that genetically determined high levels of VWF are not associated with arterial thrombosis, suggesting that VWF is merely a marker rather than a cause of the disease.[6,7] To further explore this important research question it is of utmost interest to study arterial thrombosis in individuals with very low levels of VWF, as is seen in VWD.

Only a few case series on arterial thrombosis in VWD patients have been reported. Large cohort studies on this subject have not yet been performed.[8] Therefore, it is currently unknown whether VWD patients are protected against arterial thrombosis. The objective of the present study was to study, in a large cohort of patients with moderate or severe VWD, the prevalence of arterial thrombosis relative to the general population.

PATIENTS AND METHODS

Participants

We performed a nationwide cross-sectional study among VWD patients in the Netherlands, "Willebrand in the Netherlands" (WiN) study. We included patients diagnosed with type 1, type 2 and type 3 VWD who fulfilled the following inclusion criteria: (I) hemorrhagic symptoms or a family history of VWD; and (II) historically lowest levels of VWF antigen (VWF:Ag) ≤30 U/dL and/or VWF activity (VWF ristocetin cofactor activity (VWF:RCo)) ≤30 U/dL and/or Factor VIII coagulation activity (FVIII:C) ≤40 U/dL (for type 2N VWD). Patients were excluded if hemophilia A (n=2) or other disorders of hemostasis resulting in a hemorrhagic diathesis, were known. Between October 2007 and October 2009, 804 individuals were included after completing a questionnaire, 664 individuals of whom were above the age of 16. The Medical Ethical Committees at all participating centers approved this study and written informed consent was obtained from all study participants.

Assessment methods in the study

All patients completed a questionnaire on bleeding symptoms, quality of life and co-morbidity.[9-11] Part of the questionnaire concerned the clinical history of acute myocardial infarction, ischemic stroke, transient ischemic attack (TIA), unstable angina pectoris or peripheral arterial disease. In addition, we included questions about co-morbidity and current medication use.

For each patient with arterial thrombosis the diagnosis was confirmed using medical records from the hospital of treatment or the general practitioner. To make sure that no arterial thrombotic events were missed, a random sample of medical records of VWD patients who reported no arterial thrombotic events themselves, was checked (n=234, 37% of the total WiN study population), and no additional arterial thrombotic events were found. Patients with an incomplete questionnaire on arterial thrombosis and cardiovascular risk factors were excluded from the current analysis (n=29).

Laboratory measurements in von Willebrand Disease patients

At inclusion in the study venous whole blood was collected in 0.105M sodium citrate tubes and centrifuged twice at 2,200 x *g* for 10 minutes at room temperature and stored at -80°C. Plasma levels of VWF:Ag, VWF:CB, VWF activity (VWF:Act) and FVIII:C were measured centrally (Erasmus University Medical Center, Rotterdam). VWF:Ag level was measured with an in-house ELISA using a polyclonal rabbit anti-human VWF antibody (DakoCytomation, Glostrup, Denmark) for capturing and a HRP-conjugated anti-human VWF antibody (DakoCytomation) for detecting. VWF:CB level was measured with an in-house ELISA using collagen type 1 (Sigma-Aldrich, St Louis, MI, USA) for capturing and a HRP-conjugated anti-human VWF antibody (DakoCytomation) for detecting. To assess VWF activity we used a VWF:Act assay that measures the ability of VWF to bind GpIbα (HemosIL™ von Willebrand Factor Activity, Instrumentation Laboratory B.V, Breda, the Netherlands). FVIII:C was measured in a one-stage clotting assay (TriniCLOT, Biomerieux, Marcy l'Etoile, France) with FVIII-deficient plasma (Biopool, Umea, Sweden) and reference plasma (Precision biologic, Kordia, Leiden, the Netherlands). Details on the blood sampling procedure and laboratory measurements have been described in more detail by de Wee et al.[11] We have no information on prothrombotic factors in our cohort, such as Factor V Leiden mutation or prothrombin gene variant.

Definitions

Acute myocardial infarction was defined as typical chest pain with matching characteristic electrocardiographic findings and a revascularization procedure, including percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) and drug treatment. Unstable angina pectoris was defined as typical chest pain while at rest and without electrocardiographic findings, in which additional information from

a general practitioner or cardiologist showed evidence of angina pectoris, drug treatment and a revascularization procedure, including PCI or CABG. Coronary heart disease was defined as acute myocardial infarction and/or unstable angina pectoris. Ischemic stroke was defined as the acute onset of focal cerebral dysfunction, which could not be explained other than by local cerebral ischemia and when irreversible damage was reported. Confirmation of the diagnosis was procured by neuroimaging with computed tomography or magnetic resonance. If the focal cerebral deficit resolved completely within 24 hours without signs of persistent cerebral lesions, it was defined as TIA. When a relevant hemorrhage was shown on computed tomography or magnetic resonance, hemorrhagic stroke was diagnosed. The diagnosis peripheral arterial disease was defined as peripheral arterial stenosis resulting in ischemia.

Risk factors for arterial thrombosis, such as history of smoking, diabetes mellitus, obesity, hypertension, hypercholesterolemia and family history of arterial thrombosis were recorded at the time of data collection. The use of antithrombotic medication was recorded at the time of and after the arterial thrombotic event. History of smoking was defined as former and/or current smoking. Obesity was defined as a body mass index (BMI) ≥30 kg/m^2. Patients with a medical history of hypertension, hypercholesterolemia or diabetes mellitus or patients using medication for these conditions were considered to have, respectively, hypertension, hypercholesterolemia or diabetes. A positive family history was defined as a first-degree relative with a history of coronary heart disease or ischemic stroke before the age of 60.

Reference group

The proportion of the Dutch general population who had suffered from arterial thrombosis, at a specific point in time (the prevalence of arterial thrombosis) was obtained from two independent Dutch sources who both publish statistics on health and disease in the Netherlands: National Public Health Compass (NPHC)[12] and Statistics Netherlands (CBS, Central Bureau of Statistics)[13]. CBS only provides prevalence data on acute myocardial infarction and ischemic stroke, and not on coronary heart disease, whereas NPHC provides prevalence data on coronary heart disease. Data from the NPHC were obtained from the General Practitioner Registration on 1 January 2007, and are based on the total number of inhabitants in the Netherlands on that date (age ≥20 years, n=12,400,889; males ≥20 years, n=6,064,361; females ≥20 years, n=6,336,528). The data on the prevalence of arterial thrombosis and cardiovascular risk factors from CBS were obtained by the Permanent Research Living Situation survey (POLS) questionnaire.[13] These data were acquired by questionnaires in a random sample of the Dutch general population in the year 2009 (n=9,118). These data are not validated by medical professionals.

Statistical methods

Descriptive statistics for continuous variables are presented as median and 25-75% interquartile range (IQR), because data were not normally distributed. For comparison of proportions, the chi-squared test or the Fisher's exact test were used. Mann-Whitney *U*-tests were performed for comparison of age and VWF and FVIII levels between groups. Standardized morbidity ratios (SMR) were calculated to estimate the rate of overall arterial thrombosis and of coronary heart disease, acute myocardial infarction and ischemic stroke separately in VWD patients relative to that of the reference population adjusted for age and sex. The SMR is the number of observed cases divided by the number that was expected if the prevalence in the cohort, with its specific age- and sex-distribution, was the same as that in the general population. SMR >1 indicates an excess and SMR <1 indicates a reduction of observed cases over the expected number. Ninety-five per cent confidence intervals (CI) were based on a Poisson's distribution for the observed number of events.

In the general population approximately 75% of the strokes are ischemic, 20% hemorrhagic and 5% are strokes caused by rare abnormalities.[12,14] To calculate the SMR for ischemic and hemorrhagic stroke separately, we used these proportions for the prevalence in the general population.

The levels of VWF:Ag, VWF:CB, VWF activity (VWF:Act) and FVIII:C in the case and total VWD cohort, were divided in tertiles based on the distribution in the control group. The association between levels of these variables and the occurrence of a cardiovascular event was determined using logistic regression. The lowest tertile was used as reference. We adjusted for age, sex, blood group, smoking, diabetes mellitus, obesity, hypertension, hypercholesterolemia and positive family history. Statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS Inc, Chicago, IL, USA). A p-value of <0.05 was considered statistically significant.

RESULTS

A total of 804 VWD patients participated in the WiN study, 664 of whom were above 16 years of age. In the present sub-study 29 patients were excluded, because of an incomplete questionnaire on arterial thrombosis and cardiovascular risk factors. Therefore, a total of 635 adult VWD patients were included, 36% of whom were male (Table 1). The median age was 45 years (range 16 to 85 years). The majority of patients had VWD type 1 (n=325, 58%), 37% (n=209) had type 2 VWD and 5% (n=25) had type 3 VWD. Median VWF and FVIII levels were: VWF:Ag 30 IU/dL [25-75% IQR 19-45], VWF:CB 24 IU/dL [8-51], VWF:Act 24 IU/dL [9-53] and FVIII:C 52 IU/dL [35-73].

Table 1. Baseline characteristics of von Willebrand Disease patients.

Characteristics	VWD patients (n = 635)	VWD patients with arterial thrombosis (n=21)	VWD patients without arterial thrombosis (n=614)	p-value*
Age, total group (years), median (range)	45 (16-85)	64 (42-81)	45 (16-85)	<0.001
Age, males (years), median (range)	44 (16-85)	68 (59-81)	43 (16-85)	<0.001
Age, females (years), median (range)	46 (16-83)	55 (42-78)	45 (16-83)	0.01
Male sex, n (%)	231 (36)	10 (48)	221 (36)	0.28
VWD type, n (%) †				
1	325 (58)	13 (65)	312 (58)	0.57 §
2	209 (37)	7 (35)	202 (38)	
2A	*136*	*5*	*131*	
2B	*36*	*2*	*34*	
2M	*23*	*0*	*23*	
2N	*14*	*0*	*14*	
3	25 (5)	0	25 (5)	
Blood group O, n (%) ‡	337 (61)	12 (60)	325 (61)	0.92
VWF:Ag (IU/dL), median [IQR] †	30 [19-45]	36 [22-59]	30 [19-45]	0.28
VWF:CB (IU/dL), median [IQR] †	24 [8-51]	26 [15-62]	24 [8-51]	0.26
VWF:Act (IU/dL), median [IQR] †	24 [9-53]	22 [15-78]	24 [8-53]	0.22
FVIII:C (IU/dL), median [IQR] †	52 [35-73]	68 [42-93]	52 [34-73]	0.06
History of smoking, n (%)	327 (52)	16 (76)	311 (51)	0.02
Diabetes Mellitus, n (%)	29 (5)	3 (14)	26 (4)	0.07
Obesity, n (%)	94 (15)	5 (24)	89 (15)	0.22
Hypertension, n (%)	137 (22)	15 (71)	122 (20)	<0.001
Hypercholesterolemia, n (%)	63 (10)	13 (62)	50 (8)	<0.001
Positive family history, n (%)	162 (26)	10 (48)	152 (25)	0.02
No cardiovascular risk factors, n (%)	170 (27)	1 (5)	169 (28)	0.02
At least two cardiovascular risk factors, n (%)	215 (34)	18 (86)	197 (32)	<0.001

VWD = von Willebrand Disease, IQR = interquartile range, VWF:Ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulation activity. * arterial thrombosis versus no arterial thrombosis. † n=559, based on patients from whom plasma was available (arterial thrombosis n=20, no arterial thrombosis n=539). ‡ n=552, based on availability (arterial thrombosis n=20, no arterial thrombosis n=532). § p for trend between type 1, type 2 and type 3.

Prevalence of arterial thrombosis

Twenty-nine arterial thrombotic events occurred in 21 of the 635 VWD patients (3.3%; CI 1.9 to 4.7) (Table 2). Five patients (0.8%; CI 0.1 to 1.5) suffered acute myocardial infarction and three patients suffered ischemic stroke (0.5%; CI 0 to 1.0). An unstable angina pectoris was recorded twelve times and nine events were a TIA. None of the VWD patients suffered peripheral arterial disease. Eight patients had more than one arterial thrombotic

event. Two patients had a TIA during pregnancy, one of whom had a second TIA during the same pregnancy. Two patients had an arterial thrombotic event within 12 hours after they received treatment for VWD: one TIA while using tranexamic acid and one TIA shortly after desmopressin administration. The patient with a TIA after desmopressin had a pulmonary embolism at the same time. This case has been reported before.[15]

Cardiovascular risk factors of von Willebrand Disease patients with and without arterial thrombosis

Forty-eight per cent of the VWD patients with arterial thrombosis were males. The median age was 64 years (range 42 to 81). We compared the cardiovascular risk factor profile in VWD patients with and without arterial thrombosis. As expected, cardiovascular risk factors were more prevalent in VWD patients with arterial thrombosis than in the VWD patients that did not experience an arterial thrombotic event (Table 1). There were no significant differences in VWF plasma levels in VWD patients with and without arterial thrombosis (table 1). FVIII:C levels showed a trend towards higher levels in VWD with arterial thrombosis (p=0.06).

We observed no reduced risk for arterial thrombosis in individuals in the lowest tertile of VWF:Ag, VWF:CB, VWF:Act and FVIII:C levels compared with the other VWD patients in the study.

Type of von Willebrand Disease and prevalence of arterial thrombosis

Thirteen type 1 VWD patients had a history of arterial thrombosis (13/325, 4.0%; CI 1.9 to 6.1). Seven type 2 VWD patients (7/209, 3.3%; CI 0.9 to 5.8) suffered from arterial thrombosis, five of whom had type 2A VWD (5/136, 3.7%; CI 0.5 to 6.8) and two type 2B VWD (2/36, 5.6%; CI 0 to 13.0). We observed no arterial thrombosis in type 3 VWD patients; however, the number of type 3 patients in our study cohort is limited (n=25) (Table 1). In type 1 and 2 VWD separately, no differences were found in VWF plasma levels between VWD patients with and without arterial thrombosis.

Cardiovascular drug treatment

Nineteen of the 21 (90.5%) patients who suffered an arterial thrombotic event, were treated with antiplatelet therapy or anticoagulant treatment. In the two other patients antiplatelet therapy was judged to be contraindicated by their treating physician because of VWD. Antiplatelet therapy consisting of aspirin (in a dose of 80-100 milligrams) was given to 15 patients (71%). Two of these patients also received clopidogrel and four patients additionally received short-term unfractionated heparin and long-term vitamin K antagonist. A minority of four patients received long-term medication other than aspirin, such as clopidogrel, dipyridamol or vitamin K antagonist. The patient with a TIA and a pulmonary embolism received low-molecular-weight heparin for 1 week and

vitamin K antagonist for 6 months. Of the 19 patients who received antiplatelet or anticoagulant treatment, 14 patients (74%) still used antiplatelet therapy at time of inclusion in the study. Two patients suffered gastrointestinal bleeding during aspirin use and had stopped treatment. One patient stopped treatment during follow-up for unknown reasons and one patient was only treated during pregnancies. The patient who suffered a pulmonary embolism and TIA received vitamin K antagonist for 6 months according to the Dutch guidelines for the treatment of venous thromboembolism (Table 2).

Prevalence of arterial thrombosis compared with the general population

Thirteen patients suffered coronary heart disease, whereas 21.5 patients would be expected based on prevalence data from the general population (NPHC)[12] taking the age and sex distribution into consideration. The SMR was 0.60 (CI 0.32 to 0.98), indicating that VWD patients had a 40% reduced prevalence for coronary heart disease when compared with the general population.

The observed number of ischemic strokes in the VWD cohort was 3 and the expected number 4.6 based on prevalence data from the general population (NPHC).[12] The SMR was 0.65 (CI 0.12 to 1.59). Overall the reduction in prevalence of arterial thrombosis (ischemic stroke and coronary heart disease) was 39% (SMR 0.61; CI 0.35 to 0.95), when compared with the age- and sex-matched general population (source NPHC).

Comparing the observed prevalence of acute myocardial infarction and ischemic stroke in the VWD cohort with a second age- and sex-matched general population (source CBS)[13], VWD patients had a significantly lower than expected prevalence of acute myocardial infarction and ischemic stroke. The SMRs were 0.40 (CI 0.13 to 0.83) for acute myocardial infarction and 0.33 (CI 0.06 to 0.80) for ischemic stroke, indicating a reduced prevalence of 60% for acute myocardial infarction and 67% for ischemic stroke in VWD patients. The reduction in overall arterial thrombotic morbidity was 63% (SMR 0.37; CI 0.16 to 0.67) in comparison with the age-and sex-matched general population (source CBS).[13]

Because arterial thrombosis occurs rarely below the age of 45 years, we also calculated the SMRs for only those patients aged 45 years and above (n=329) and observed similar SMRs. The prevalence of coronary heart disease in VWD was 37% lower than in the general population (SMR 0.63; CI 0.33 to 1.01). For acute myocardial infarction a 59% lower prevalence was observed (SMR 0.41; CI 0.13 to 0.85) and for ischemic stroke a 30-64% lower prevalence was observed (SMRs 0.70; CI 0.13 to 1.71 and 0.36; CI 0.07 to 0.89; using the two reference populations). Overall the reduction in prevalence of arterial thrombosis was 36% (SMR 0.64; CI 0.36 to 0.99) (source NPHC) and 61% (SMR 0.39; CI 0.17 to 0.71) (source CBS), when compared with the age- and sex-matched general population.

Interestingly, 12 VWD patients (67% male) suffered from hemorrhagic stroke. Based on prevalence data from the general population, the expected numbers were 1.2 (source

Table 2. Clinical characteristics of von Willebrand Disease patients with arterial thrombosis.

Pt	Age at inclusion (year)	Sex	Type VWD	Type AT	Age at event (year)	Interventional treatment	Antiplatelet or anticoagulant treatment	Risk factors	BMI	Current antiplatelet therapy	Details
1	59	m	1	AMI	52	CAG, CABG	ASA	S, D, HT, HC, FAM	27	ASA	
2	60	m	2B	AMI	48	PCI	none	S, O, HT	31	none	no anticoagulant treatment was given because of VWD
3	76	m	2A	AMI	75	CAG, CABG	ASA	S, HT, HC, FAM	26	ASA	
4	78	f	1	UAP, AMI	50, 60	1PCI 2 CABG	1 VKA 2 ASA	S, HT, HC, FAM	27	ASA	
5	66	m	2A	UAP, AMI	56, 64	1 PCI 2 CAG, PCI	dipyridamol	S, O, HT, HC, FAM	†	ASA	
6	77	f	1	UAP	66	PCI	UFH, ASA	S, D, HT, HC	23	ASA	
7	81	m	1	UAP	77	PCI	none	HT, HC	27	none	no antiplatelet treatment was given because of VWD
8	69	m	2B	UAP, UAP	48, 59	1 CAG, PCI 2 CABG	clopidogrel	S, HT, HC, FAM	29	none	stopping use of antiplatelet therapy was patient's choice
9	60	m	2A	UAP	50	CAG, CABG	ASA	S, HC	28	ASA	
10	69	m	2A	UAP, UAP	57, 67	1 PCI 2 PCI	1 ASA 2 clopidogrel	S, HT, HC, FAM	27	none	stopped use of antiplatelet therapy due to gastrointestinal bleeding
11	48	f	2A	UAP	46	CAG, PCI	ASA	HT, FAM	26	ASA	also protein S deficiency
12	64	m	1	UAP	59	CABG	ASA	S, HC, FAM	26	ASA	
13	75	m	1	TIA, UAP	60, 62	1 CEA 2 CAG, CABG	1,2 ASA	S, D, O, HT, HC	32	ASA	
14	59	f	1	IS	55	none	ASA	S, HT, HC, FAM	29	ASA	
15	78	f	1*	IS	54	none	ASA, clopidogrel	HT, HC	28	ASA, clopidogrel	
16	70	f	1	IS, TIA	59, 59	CEA	VKA	S, O, HT	27	VKA	

Table 2. (continued)

Pt	Age at inclusion (year)	Sex	Type VWD	Type AT	Age at event (year)	Interventional treatment	Antiplatelet or anticoagulant treatment	Risk factors	BMI	Current antiplatelet therapy	Details
17	42	f	1	TIA, TIA	25, 28	none	VKA, ASA	S	23	ASA	1 during pregnancy (4.5 month); 2 after tranexamic acid
18	42	f	1	TIA, TIA	26, 26	none	UFH, VKA, ASA	none	26	none	1, 2 during pregnancy (3.5 and 4 months), anticoagulant therapy only during pregnancies
19	55	f	1	TIA	50	none	ASA	O, HT	†	none	stopped ASA use due to gastrointestinal bleeding
20	55	f	1	TIA	52	none	LMWH, VKA	S, O, FAM	37	none	after desmopressin, also pulmonary embolism
21	45	f	1	TIA	41	none	ASA	S	20	ASA	

Pt = patient, VWD = von Willebrand disease, AT = arterial thrombosis, BMI = body mass index, f = female, m = male, UAP = unstabel angina pectoris, TIA = transient ischemic attack, IS = ischemic stroke, AMI = acute myocardial infarction, PCI = percutaneous coronary intervention, CAG = coronary angiography, CEA = carotid endarterectomy, CABG = coronary artery bypass graft surgery, UFH = unfractionated heparin, ASA = aspirin, VKA = vitamin K antagonist, LMWH = low-molecular-weight heparin, S = smoking, D = diabetes mellitus, O = obesity, HT = hypertension, HC = hypercholesterolaemia, FAM = positive family history. * type based on historic levels of von Willebrand Factor, because no plasma was available; † no length available.

NPHC) and 2.5 (source CBS). The SMRs were 9.71 (CI 5.00 to 15.99) and 4.88 (CI 2.51 to 8.03) respectively, so VWD patients had a 5-10 times higher risk of hemorrhagic stroke than the general population.

Prevalence of cardiovascular risk factors compared with the general population

The prevalence of hypertension in VWD patients was 21.6% (CI 18.4 to 24.8) compared with 13.5% (CI 12.7 to 14.3) in the general population (CBS).[13] Diabetes mellitus was present or diagnosed in 4.6% (CI 2.9 to 6.2) of the VWD patients and in 4.1% (CI 3.7 to 4.5) of the general population and obesity in, respectively, 14.8% (CI 12.0 to 17.6) and 11.8% (CI 11.0 to 12.6).

DISCUSSION

This study shows for the first time that VWD patients have a lower prevalence of coronary heart disease, acute myocardial infarction and ischemic stroke compared with the general population. From previous studies it is well known that individuals with high VWF levels have a higher risk of arterial thrombosis.[3,4] Our study suggests that VWD patients may be protected against arterial thrombotic events.

This study provides important insights in the pathogenesis of arterial thrombosis. Individuals with a defective primary hemostasis as in VWD, resulting in a life-long status of hypocoagulability, seem to be protected against the development of acute myocardial infarction and ischemic stroke. This stresses the importance of VWF in the pathogenesis on ischemic stroke and acute myocardial infarction and suggests that VWF may be a therapeutic target, as recently been suggested by de Meyer et al.[16] This is further substantiated by the recent studies with VWF inhibiting agents that have been developed.[17,18]

This study is the first large cohort study on VWD and the risk of arterial thrombosis. Our study in VWD patients reveals similar results as studies performed in hemophilia patients, hemophilia carriers and Factor XI-deficient patients.[19-22] For these coagulation disorders a reduced cardiovascular mortality has been demonstrated and was suggested to be caused by a life-long hypocoagulable state. Our prevalence data suggest that VWD patients may also be protected against coronary heart disease, acute myocardial infarction and ischemic stroke in comparison with the general population, as we observe a 35-67% risk reduction. The reduction of arterial thrombotic events in VWD may be caused by reduced atherosclerosis and/or by reduced thrombosis formation on pre-existing atherosclerotic plaques.

Previous animal studies have shown reduced atherosclerosis in VWF-deficient pigs and mice.[23,24] However, in more recent studies atherosclerosis was clearly present in type 3 and type 2B VWD patients.[25,26] In addition, the reduced atherosclerosis observed in pig

studies could have been caused by polymorphisms at the apolipoprotein B100 locus.[27] Therefore our finding that VWD patients are protected against arterial thrombosis seems not to be due to a reduction of atherosclerosis, but rather due to a life-long state of hypocoagulability and reduced thrombus formation. Performing new studies on the association between arterial thrombosis, VWF levels and atherosclerosis is recommended, to further unravel this intriguing aspect.

It can be expected that VWD patients with the lowest VWF levels have the lowest risk of arterial thrombosis. Furthermore, a difference in risk between blood group O and non-O VWD patients, due to lower VWF levels, is expected. However, despite the relatively large cohort of VWD patients included in this study, the sample size is not large enough to perform subgroup analysis and the study has not enough power to address these research questions. In VWD patients not only VWF levels, but also FVIII levels, are reduced. It is possible that in VWD patients low FVIII, next to VWF, contributes to the protection against arterial thrombosis. The median FVIII:C level in our study cohort was 52 IU/dL. Several studies in patients with low FVIII levels, as observed in hemophilia A patients or carriers of hemophilia, showed that they have a decreased mortality from coronary heart disease, which suggests that FVIII plays an important role in the development of arterial thrombosis.[19,20,22] However, more recently, Fransen van de Putte et al showed that severe hemophilia patients (FVIII levels <1%) have a significantly lower incidence of non-fatal myocardial infarction and ischemic stroke than in the general age-matched male population, whereas mild and moderate hemophilia patients had no reduced risk of arterial thrombosis.[28,29] This suggests that the reduction of arterial thrombosis in VWD patients is possibly mediated by the low VWF levels. Further research is needed to unravel the role of low FVIII levels and other pathogenic factors in the development of arterial thrombosis.

The strength of our study is that two independent control datasets from the general population were used.[12,13] The risk reduction observed with the CBS prevalence data may be overestimated, as CBS data are obtained by questionnaire from individuals and are not verified by medical professionals. Nevertheless, the risk reductions for ischemic stroke from both reference groups (NPHC, which is obtained from the General Practitioner Registration, and CBS) showed a reduction in prevalence and morbidity rate for VWD patients, which further supports the validity of our findings.

In the general population approximately 75% of the strokes are ischemic, 20% hemorrhagic and 5% are strokes caused by rare abnormalities.[12,14] As expected, we found that VWD patients have more hemorrhagic than ischemic strokes and a 5-10 times higher risk of suffering a hemorrhagic stroke than the age- and sex-matched general population. It would be of interest to study this prospectively to obtain information on prevalence and incidence of intracranial bleeding in VWD patients and to identify patients at highest risk of bleeding.

As we performed a retrospective cross-sectional study, a few fatal arterial thrombotic cases may have been missed. Survival bias or reverse causation could thus be a limitation in our study. However, we assume that the risk of dying of an acute myocardial infarction or ischemic stroke is similar for VWD patients and the general population. Another selection bias could be that in several VWD patients with coronary heart disease, interventions such as PCI or CABG are not performed due to higher risk of bleeding. However, when we reviewed all subjects and checked a random sample of medical records of VWD patients who reported no arterial thrombotic events themselves, no patients were identified with acute myocardial infarction or unstable angina pectoris who had not had a PCI or CABG performed.

In VWD patients with arterial thrombosis more classical cardiovascular risk factors were observed than in VWD patients without arterial thrombosis. However, information on risk factors was obtained at time of inclusion in the study and not at time of the arterial thrombotic event. The fact that the arterial thrombosis occurs more frequently in VWD patients with increasing age and the presence of more cardiovascular risk factors, is similar to the findings in the general population.

It has been suggested that a difference in cardiovascular risk profile could cause the reduced incidence and prevalence of arterial thrombosis in patients with coagulation deficiencies.[30,31] When we compared the cardiovascular risk factors in our VWD patients with the general population we observed that hypertension was more prevalent in VWD patients than the general population. The cause of a higher prevalence of hypertension might be that VWD patients are under regular medical control and therefore hypertension is diagnosed. This finding makes it even more likely that VWD protects against the development of arterial thrombosis and that this reduced prevalence of arterial thrombosis is due to a hypocoagulable state and a decreased thrombus formation.

In VWD patients a few cases of acute myocardial infarction and ischemic stroke after administration of clotting factor concentrates or desmopressin have been reported.[32] In a study on myocardial infarction in hemophilia patients more than half of the events occurred during or shortly after administration of clotting factor concentrates or desmopressin.[33] In our study, only one patient suffered from a TIA after desmopressin administration and no events after infusion of clotting factor concentrates were reported.

Due to the introduction of clotting factor concentrates in the last decades and the use of prophylactic treatment, the life expectancy of patients with hemophilia and VWD has increased and reaches the life expectancy of the general population. With increasing age, more individuals will suffer cardiovascular disease and the number of VWD patients requiring treatment will increase. It is still debated whether VWD patients with arterial thrombosis should receive long-term antiplatelet or anticoagulant therapy. However, VWD patients with a history of arterial thrombosis have demonstrated the ability to form an occlusive thrombus and therefore antiplatelet treatment with aspirin for arterial

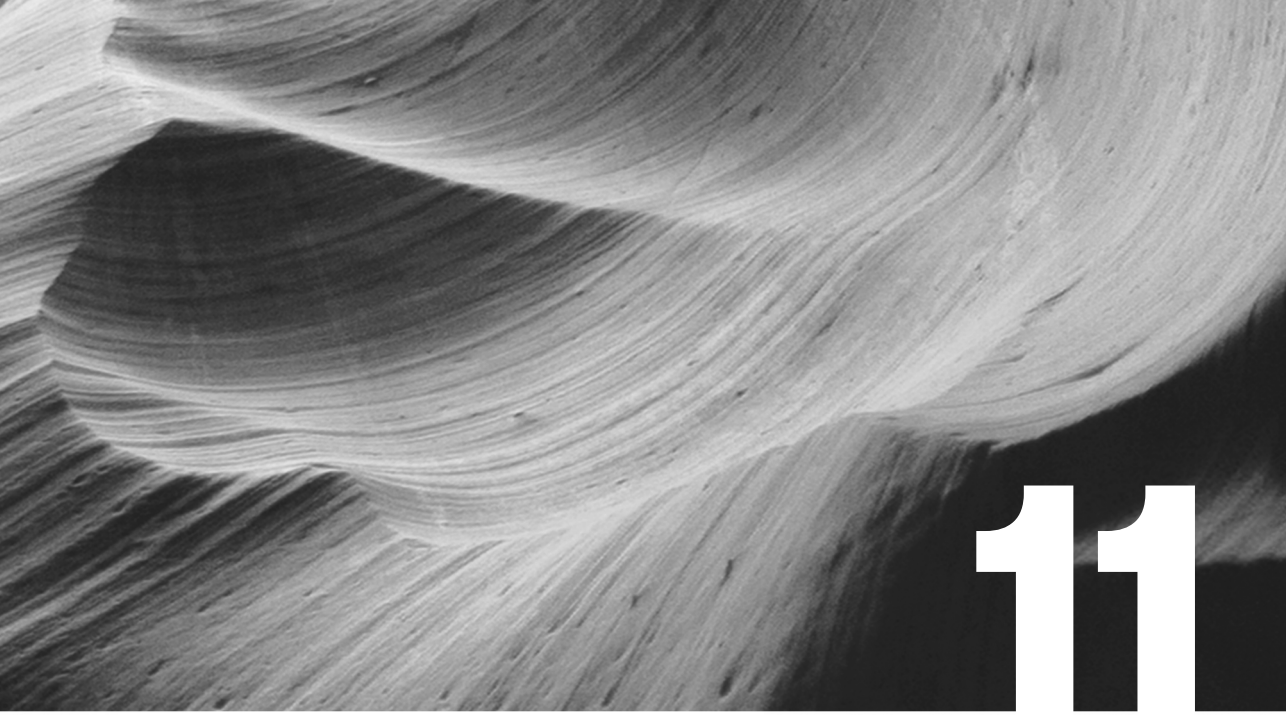
thrombosis is advised. On the other hand, the use of platelet inhibitory agents in VWD patients may result in more bleeding. In our study, two patients reported gastrointestinal bleeding and terminated antiplatelet therapy after they suffered arterial thrombosis. A prospective study is needed to obtain more detailed information on mortality, co-morbidity and life expectancy of VWD patients. We also recommend developing guidelines for the management of cardiovascular disease in VWD, as have recently been developed for hemophilia patients.[34,35] Recently Franchini and Coppola reported general and specific recommendations for optimizing management and minimizing bleeding risk for VWD patients with coronary heart disease.[8]

In conclusion, we are the first to report that VWD patients have a reduced prevalence of arterial thrombosis and therefore may be protected against arterial thrombosis, including coronary heart disease, acute myocardial infarction and ischemic stroke. This study provides important insights into the pathogenesis of arterial thrombosis and the role of VWF in this process.

REFERENCES

1. Ruggeri ZM. Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation. *Best Pract Res Clin Haematol*. 2001;14(2):257-279.
2. Sadler JE, Mannucci PM, Berntorp E, et al. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost*. 2000;84(2):160-174.
3. Whincup PH, Danesh J, Walker M, et al. von Willebrand factor and coronary heart disease: prospective study and meta-analysis. *Eur Heart J*. 2002;23(22):1764-1770.
4. Wieberdink RG, van Schie MC, Koudstaal PJ, et al. High von Willebrand factor levels increase the risk of stroke: the Rotterdam study. *Stroke*. 2010;41(10):2151-2156.
5. van Schie MC, de Maat MP, Isaacs A, et al. Variation in the von Willebrand factor gene is associated with von Willebrand factor levels and with the risk for cardiovascular disease. *Blood*. 2011;117(4): 1393-1399.
6. Van Schie MC, Wieberdink RG, Koudstaal PJ, et al. Genetic determinants of von Willebrand factor plasma levels and the risk of stroke: the Rotterdam Study. *J Thromb Haemost*. 2012;10(4):550-556.
7. van Loon JE, Kavousi M, Leebeek FW, et al. Von willebrand factor plasma levels, genetic variations, and coronary heart disease in an older population. *J Thromb Haemost*. 2012;10(7):1262-1269.
8. Franchini M, Coppola A. Atherothrombosis in von Willebrand disease: an analysis of the literature and implications for clinical management. *Semin Thromb Hemost*. 2012;38(2):185-199.
9. de Wee EM, Mauser-Bunschoten EP, van der Bom JG, et al. Health-related quality of life among adult patients with moderate and severe von Willebrand disease. *J Thromb Haemost*. 2010;8(7):1492-1499.
10. De Wee EM, Knol HM, Mauser-Bunschoten EP, et al. Gynaecological and obstetric bleeding in moderate and severe von Willebrand disease. *Thromb Haemost*. 2011;106(5):885-892.
11. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108(4):683-692.
12. Gommer AM, Poos MJJC. Prevalence, incidence and mortality according to age and gender. In: Volksgezondheid VTVNK ed. Vol. version 4.5. Bilthoven: RIVM: http://www.nationaalkompas.nl (Accessed 22 September 2011); 2010.
13. Central Bureau of Statistics. Permanent Research Living Situation (POLS) survey. Voorburg/Heerlen: CBS: http://statline.cbs.nl (Accessed 22 September 2011); 2009.
14. Feigin VL, Lawes CM, Bennett DA, Anderson CS. Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurol*. 2003;2(1): 43-53.
15. de Wee EM, Ikram MK, Dippel DW, Leebeek FW. Transient focal cerebral ischaemia and bilateral pulmonary embolism after desmopressin treatment for von Willebrand's disease. *Haemophilia*. 2008; 14(5):1133-1134.
16. De Meyer SF, Stoll G, Wagner DD, Kleinschnitz C. von Willebrand factor: an emerging target in stroke therapy. *Stroke*. 2012;43(2):599-606.
17. van Loon JE, de Jaegere PP, Ulrichts H, et al. The in vitro effect of the new antithrombotic drug candidate ALX-0081 on blood samples of patients undergoing percutaneous coronary intervention. *Thromb Haemost*. 2011;106(1):165-171.
18. Ulrichts H, Silence K, Schoolmeester A, et al. Antithrombotic drug candidate ALX-0081 shows superior preclinical efficacy and safety compared with currently marketed antiplatelet drugs. *Blood*. 2011;118(3):757-765.
19. Rosendaal FR, Varekamp I, Smit C, et al. Mortality and causes of death in Dutch haemophiliacs, 1973-86. *Br J Haematol*. 1989;71(1):71-76.

20. Darby SC, Kan SW, Spooner RJ, et al. Mortality rates, life expectancy, and causes of death in people with hemophilia A or B in the United Kingdom who were not infected with HIV. *Blood*. 2007;110(3): 815-825.
21. Salomon O, Steinberg DM, Koren-Morag N, Tanne D, Seligsohn U. Reduced incidence of ischemic stroke in patients with severe factor XI deficiency. *Blood*. 2008;111(8):4113-4117.
22. Sramek A, Kriek M, Rosendaal FR. Decreased mortality of ischaemic heart disease among carriers of haemophilia. *Lancet*. 2003;362(9381):351-354.
23. Fuster V, Lie JT, Badimon L, Rosemark JA, Badimon JJ, Bowie EJ. Spontaneous and diet-induced coronary atherosclerosis in normal swine and swine with von Willebrand disease. *Arteriosclerosis*. 1985;5(1):67-73.
24. Methia N, Andre P, Denis CV, Economopoulos M, Wagner DD. Localized reduction of atherosclerosis in von Willebrand factor-deficient mice. *Blood*. 2001;98(5):1424-1428.
25. Sramek A, Bucciarelli P, Federici AB, et al. Patients with type 3 severe von Willebrand disease are not protected against atherosclerosis: results from a multicenter study in 47 patients. *Circulation*. 2004; 109(6):740-744.
26. Federici AB, Mannucci PM, Fogato E, Ghidoni P, Matturri L. Autopsy findings in three patients with von Willebrand disease type IIB and type III: presence of atherosclerotic lesions without occlusive arterial thrombi. *Thromb Haemost*. 1993;70(5):758-761.
27. Denis CV, Wagner DD. Insights from von Willebrand disease animal models. *Cell Mol Life Sci*. 1999; 56(11-12):977-990.
28. Fransen van de Putte DE, Fischer K, Pulles AE, et al. Non-fatal cardiovascular disease, malignancies, and other co-morbidity in adult haemophilia patients. *Thromb Res*. 2012;130(2):157-162.
29. van de Putte DE, Fischer K, Makris M, et al. History of non-fatal cardiovascular disease in a cohort of Dutch and British patients with haemophilia. *Eur J Haematol*. 2012;89(4):336-339.
30. Rosendaal FR, Briet E, Stibbe J, et al. Haemophilia protects against ischaemic heart disease: a study of risk factors. *Br J Haematol*. 1990;75(4):525-530.
31. van de Putte DE, Fischer K, Makris M, et al. Increased prevalence of hypertension in haemophilia patients. *Thromb Haemost*. 2012;108(4):750-755.
32. Girolami A, Tezza F, Scapin M, Vettore S, Casonato A. Arterial and venous thrombosis in patients with von Willebrand's disease: a critical review of the literature. *J Thromb Thrombolysis*. 2006;21(2): 175-178.
33. Girolami A, Ruzzon E, Fabris F, Varvarikis C, Sartori R, Girolami B. Myocardial infarction and other arterial occlusions in hemophilia a patients. A cardiological evaluation of all 42 cases reported in the literature. *Acta Haematol*. 2006;116(2):120-125.
34. Mannucci PM, Mauser-Bunschoten EP. Cardiovascular disease in haemophilia patients: a contemporary issue. *Haemophilia*. 2010;16 Suppl 3:58-66.
35. Schutgens RE, Tuinenburg A, Roosendaal G, Guyomi SH, Mauser-Bunschoten EP. Treatment of ischaemic heart disease in haemophilia patients: an institutional guideline. *Haemophilia*. 2009;15(4): 952-958.



11

Von Willebrand Disease and aging: an evolving phenotype

YV Sanders, MA Giezenaar, BAP Laros-van Gorkom, K Meijer, JG van der Bom, MH Cnossen, MR Nijziel, PF Ypma, K Fijnvandraat, J Eikenboom, EP Mauser-Bunschoten, FWG Leebeek, and the WiN study group

Journal of Thrombosis and Haemostasis. 2014;12(7):1066-1075

ABSTRACT

Background: Because the number of elderly von Willebrand Disease (VWD) patients is increasing, the pathophysiology of aging in VWD has become increasingly relevant.
Objectives: To assess age-related changes in von Willebrand Factor (VWF) and Factor VIII (FVIII) levels and to compare age-related differences in bleeding phenotype between elderly VWD patients and those <65 years. We also studied co-morbidity in elderly patients.
Patients/Methods: We included VWD patients with VWF levels ≤30 U/dL in the nationwide cross-sectional "Willebrand in the Netherlands" (WiN) study. Patients reported bleeding episodes and treatment of VWD in the year preceding inclusion and during life. This was compared between VWD patients older (n=71) and younger (16-64 years, n=593) than 65 years. In elderly patients, age-related changes in VWF and FVIII levels were studied longitudinally by including all historically measured levels. All medical records were examined for co-morbidity.
Results: In elderly type 1 patients, a decade age increase was associated with 3.5 U/dL (95% confidence interval (CI) -0.6 to 7.6) VWF:Ag increase and 7.1 U/dL (CI 0.7 to13.4) FVIII:C increase. This increase was not observed in elderly type 2 patients. Elderly type 2 patients reported significantly more bleeding symptoms in the year preceding inclusion than younger patients (16/27, 59% versus 87/221, 39%; p=0.048), which was not observed in type 1 VWD.
Conclusions: VWF parameters and bleeding phenotype evolve with increasing age in VWD. VWF and FVIII levels increase with age in type 1 patients with no mitigation in bleeding phenotype. In type 2 patients VWF parameters do not increase with age and in these patients aging is accompanied by increased bleeding.

INTRODUCTION

Von Willebrand Disease (VWD) is the commonest inherited bleeding disorder, and is caused by reduced levels or reduced function of von Willebrand Factor (VWF).[1] VWF mediates the adhesion and aggregation of platelets at sites of vascular injury, and serves as a carrier of Factor VIII (FVIII).[2] Patients with VWD suffer from bleeding episodes of which the frequency and severity is associated with the type of VWD and the remaining level of functioning VWF.[3-5] Whereas type 1 VWD is characterized by partially reduced VWF levels and type 3 by complete absence of VWF in plasma, type 2 VWD patients have abnormal variants of VWF.[6]

With increasing age, VWD patients may undergo changes with regard to VWF levels, FVIII levels, bleeding symptoms, and health status. As the number of elderly VWD patients is increasing, the pathophysiology of aging in VWD has become increasingly relevant.[7]

Because plasma concentrations of VWF and FVIII have been shown to increase with age in healthy individuals[8-10], they are also likely to increase in aging VWD patients. Hypothetically, one would expect these higher levels to mitigate the bleeding phenotype, which may be associated with reductions in VWD concentrate treatment dose and intensity. When VWF and FVIII levels increase, they might also cross the threshold of current diagnostic criteria for VWD in some elderly patients with mild type 1 VWD.[6] However, these increased levels may still be too low for adequate hemostasis.

In elderly VWD patients, bleeding frequency and severity is influenced by various factors. For example, the risk of bleeding may be reduced by a more sedentary lifestyle with fewer physical challenges. Similarly, common co-morbidities in elderly patients, such as malignancies, may increase the risk of bleeding, as a result of disease or surgical procedures.[11] It has also been suggested that, due to degenerative intestinal changes and angiodysplasia, elderly VWD patients suffer from more frequent gastrointestinal bleeding than younger patients.[7]

In this nationwide cross-sectional study, we studied age-related changes in VWF and FVIII levels and compared the differences in bleeding phenotype between elderly VWD patients and patients younger than 65 years. We also studied specific age-related co-morbidity in elderly VWD patients.

PATIENTS AND METHODS

Participants

We performed a nationwide cross-sectional study among VWD patients in the Netherlands: "Willebrand in the Netherlands" (WiN) study.[3,12,13] We included patients diagnosed

with type 1, type 2 and type 3 VWD who had a hemorrhagic diathesis or a family history of VWD and had historically lowest VWF levels ≤30 U/dL (VWF:Ag and/or VWF:RCo) and/or FVIII levels (FVIII:C) ≤40 U/dL (for type 2N VWD). Patients were excluded if they were known to have hemophilia A or other hemostatic disorders resulting in hemorrhagic diathesis. Between October 2007 and October 2009, 804 individuals were included. In total, 664 patients aged ≥16 years participated, with 71 (11%) ≥65 years. The medical ethical committees at all participating centers approved this study, and all participants gave informed consent. This has been described in detail previously.[3]

Assessment methods in the study

All patients completed an extensive questionnaire on co-morbidity, hospitalization, bleeding episodes and treatment of VWD in the year preceding inclusion.[3,14,15] First a pilot study using the "think-aloud-method" was conducted in which respondents completed the questionnaire in the presence of the investigator, and then adjustments were made accordingly.[16] All patients were asked to report the bleeding episodes and treatment of VWD in the year preceding inclusion. We also collected information about the severest lifetime episode of twelve specific types of bleedings by administration of the Tosetto Bleeding Questionnaire.[17] We did not score for a bleeding symptom if patients received prophylactic desmopressin or prophylactic replacement therapy before they underwent surgery or dental extraction, to avoid prophylaxis-bias.[3,18] However, if bleeding after surgery or dental extraction had occurred despite prophylactic treatment, we scored for this bleeding according to the Tosetto bleeding score. Because of the limitations of the Tosetto bleeding score (cumulative score, ceiling effect and prophylaxis bias),[3,4,18] we mainly focused on bleeding episodes in the year preceding inclusion in the study. In addition, we reviewed all elderly patients' medical records and studied their co-morbidities and medication use.

In most elderly patients (n=66), VWF parameters had been determined at several time-points (at least twice) in the past 30 years in their own Hemophilia Treatment Centers. To study the age-related changes in VWF and FVIII levels within elderly patients over time, all such historical VWF and FVIII levels were included. Blood samples obtained within 72 hours after the use of desmopressin or replacement therapy and obtained during pregnancy have been excluded.

Laboratory measurements in von Willebrand Disease patients

Patients' historically measured VWF parameters had been determined previously in their Hemophilia Treatment Centers. Plasma levels of VWF antigen (VWF:Ag), VWF Collagen Binding (VWF:CB), VWF activity (VWF:Act) and FVIII coagulation activity (FVIII:C) were also measured centrally at inclusion in the study (Erasmus University Medical Center, Rotterdam).[3] Venous whole blood was collected in 0.105M sodium citrate tubes and

centrifuged twice at 2,200 x *g* for 10 minutes at room temperature and stored at -80°C. VWF:Ag was determined with an in-house ELISA using polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase (HRP) conjugated anti-human VWF antibodies (DakoCytomation, Glostrup, Denmark) for detection. VWF:CB was measured with an in-house ELISA using collagen type 1 (Sigma-Aldrich, St Louis, MO, USA) for capture and HRP-conjugated anti-human VWF antibody (DakoCytomation) for detection. A VWF:Act assay was used to assess VWF activity. This assay uses monoclonal antibodies directed against the glycoprotein (Gp) Ibα binding domain of VWF and thereby reflects the binding activity of VWF to GpIbα (HemosIL™ von Willebrand Factor Activity, Instrumentation Laboratory BV, Breda, the Netherlands). We have previously validated the VWF:Act test (n=122) and obtained a Spearman correlation coefficient of 0.942 with our previously used VWF:RCo activity test (p<0.0001).[3] FVIII:C was measured in a one-stage clotting assay (TriniCLOT, Biomerieux, Marcy l'Etoile, France) with FVIII-deficient plasma (Biopool, Umea, Sweden) and reference plasma (Precision biologic, Kordia, Leiden, the Netherlands). Details on the blood sampling procedure and laboratory measurements at inclusion in the study have been described in more detail by de Wee et al.[3]

Definitions

Elderly patients were defined as VWD patients ≥65 years of age at time of inclusion based on World Health Organization criteria[19] and younger patients as VWD patients 16-64 years of age at time of inclusion. The lifetime prevalence of bleeding symptoms was defined as clinically relevant bleeding symptoms that have occurred at least once during life, identified through the Tosetto Bleeding Questionnaire.[4,20] Co-morbidity was defined as the presence of any disease or condition other than the patient's VWD that required medical attention from a general practitioner or specialist. Part of the questionnaire concerned the clinical history of all kinds of co-morbidity, which was defined as self-reported co-morbidity. Co-morbidities were categorized using the following categories: HIV, hepatitis C, hepatitis B, overweight (BMI of 25-30), obesity (BMI>30), hypertension, hypercholesterolemia, chronic arthropathy, diabetes, renal disease, depression, respiratory disease, gastrointestinal disease, thyroid disease, neurological disease, cancer, cardiovascular disease, ocular disease, dermatological disease, urinary tract disease and other. The classification of co-morbidities is further specified in supplementary file 1.

Statistical methods

Because data were not normally distributed, continuous variables are presented as median and 25-75% interquartile range [IQR]. Descriptive statistics for categorical data are presented as frequencies and percentages (n, %). The chi-squared test or the Fisher's exact test is used to compare the prevalence of bleedings in the past and in the year preceding inclusion, and the prevalence of self-reported co-morbidity between patients

younger and older than 65 years. Mann-Whitney *U*-test is used to test the differences in VWF:Ag, VWF:Act and FVIII:C between patients younger and older than 65 years. First, in elderly patients (n=66), we used linear mixed models to model the association between age and all historically measured VWF:Ag, VWF:Act and FVIII:C levels. Secondly, linear regression analyses were used to determine the association between age, and centrally measured VWF:Ag, VWF:Act and FVIII:C levels in all type 1 and type 2 patients ≥16 years from the WiN study (n=664). Statistical analyses were performed with SPSS for Windows, version 20.0 (SPSS Inc, Chicago, IL, USA). A p-value <0.05 was considered statistically significant.

RESULTS

In total, 71 VWD patients aged 65-85 years were included in the WiN study and 593 VWD patients aged 16-64 years (Table 1). Over half of the elderly patients were female (n=47, 66%), had type 1 VWD (n=43, 61%) and blood group O (n=37, 52%).

Table 1. Baseline characteristics**.**

Characteristics	Elderly VWD patients (aged ≥65 years) (n = 71)	VWD patients (aged 16-64 years) (n = 593)	p-value
Age (years), median (range)	71 (65-85)	43 (16-64)	
Male sex, n (%)	24 (34)	217 (37)	0.644
VWD type, n (%)			0.458 ‡
1	43 (61)	344 (58)	
2	27 (38)	222 (37)	
2A	22	140	
2B	2	42	
2M	2	25	
2N	1	15	
3	1 (1)	27 (5)	
Blood group O, n (%) *	37 (52)	322 (54)	0.649
VWF:Ag (IU/dL), median [IQR] †	38 [24-53]	30 [19-46]	0.033
VWF:CB (IU/dL), median [IQR] †	27 [11-53]	24 [8-54]	0.296
VWF:Act (IU/dL), median [IQR] †	24 [12-70]	25 [9-55]	0.240
FVIII:C (IU/dL), median [IQR] †	62 [47-85]	52 [34-75]	0.011
Bleeding Score, median [IQR]	12 [8-18]	11 [6-16]	0.154

VWD = von Willebrand Disease, IQR = 25-75% interquartile range, VWF:Ag = von Willebrand Factor antigen, VWF:CB = von Willebrand Factor collagen binding, VWF:Act = von Willebrand Factor activity, FVIII:C = Factor VIII coagulation activity. * elderly n=63 and adults n=522, based on availability. † elderly n=62 and adults n=507, patients without centrally measured VWF parameters and patients who used desmopressin or clotting factor concentrate 72 hours before blood sampling were excluded. ‡ p for trend between type 1, type 2 and type 3.

Age-related changes in VWF and FVIII levels

Centrally measured VWF:Ag levels at inclusion were significantly higher in elderly patients than in those younger than 65 years: the VWF:Ag level in elderly patients was 38 IU/dL [median; IQR 24-53] and in patients <65 years 30 IU/dL [19-46] (p=0.033). Elderly patients also had significantly higher levels of FVIII:C (median 62 IU/dL [IQR 47-85]) than patients <65 years (52 IU/dL [34-75])(p=0.011) (Table 1).

In a subset of 66 elderly patients for whom historically measured VWF and FVIII levels were available over time, we studied the association between age and VWF parameters using linear mixed models. VWF:Ag, VWF:Act and FVIII:C levels increase with age in type 1 patients (n=40): a decade age increase was associated with a 3.5 U/dL (95% confidence interval (CI) -0.6 to 7.6) VWF:Ag increase, 9.5 U/dL (CI 3.7 to 15.3) VWF:Act increase and 7.1 U/dL (CI 0.7 to 13.4) FVIII:C increase. In type 2 patients (n=26), this age-related increase in VWF and FVIII levels was not observed: VWF:Ag: -1.6 U/dL (CI -10.3 to 7.2); VWF:Act: 0.5 U/dL (CI -2.8 to 3.7); and FVIII:C: -0.1 U/dL (CI -15.0 to 14.9).

In figure 1 centrally measured VWF:Ag, VWF:Act and FVIII:C levels for all individual type 1 and type 2 patients ≥16 years are depicted. In type 1 VWD, VWF:Ag increases by 2.7 IU/dL (CI 1.1 to 4.2) per decade, VWF:Act by 4.1 IU/dL (CI 2.0 to 6.3) and FVIII:C by 3.7 IU/dL (CI 1.6 to 5.9). In type 2 VWD, VWF:Ag increases by 2.1 IU/dL (CI 0.7 to 3.4) per decade and FVIII:C by 2.1 IU/dL (CI 0.2 to 4.0). VWF:Act did not increase in type 2 VWD: 1.0 IU/dL (CI -0.6 to 2.6).

Bleeding symptoms that required desmopressin or replacement therapy in the year preceding inclusion

At inclusion, patients were asked to report the bleeding symptoms encountered in the preceding year. Compared with the patients aged <65 years, more elderly patients reported a bleeding episode that required treatment in the year preceding inclusion (30/71, 42% versus 182/593, 31%; p=0.048), which was also observed after exclusion of the type 3 patients (29/70, 41% versus 165/566, 29%; p=0.035) (Figure 2A). This bleeding difference was explained by a difference among type 2 patients. Elderly type 2 patients reported significantly more bleeding that required treatment in the year preceding inclusion than those <65 years (16/27, 59% versus 87/222, 39%; p=0.046), whereas there was no difference in bleeding rate between younger and older type 1 patients (13/43, 30% versus 78/344, 23%; p=0.271). In elderly patients, gastrointestinal bleeding requiring treatment had occurred in a larger proportion in the year preceding inclusion than in younger patients (4/71, 6% versus 11/593, 2%; p=0.043) (Figure 2B).

Life-time prevalence of bleeding symptoms

The commonest bleeding symptoms reported by the elderly VWD population were cutaneous bleeding (52/71, 73%), bleeding after tooth extraction (47/61, 77% of the

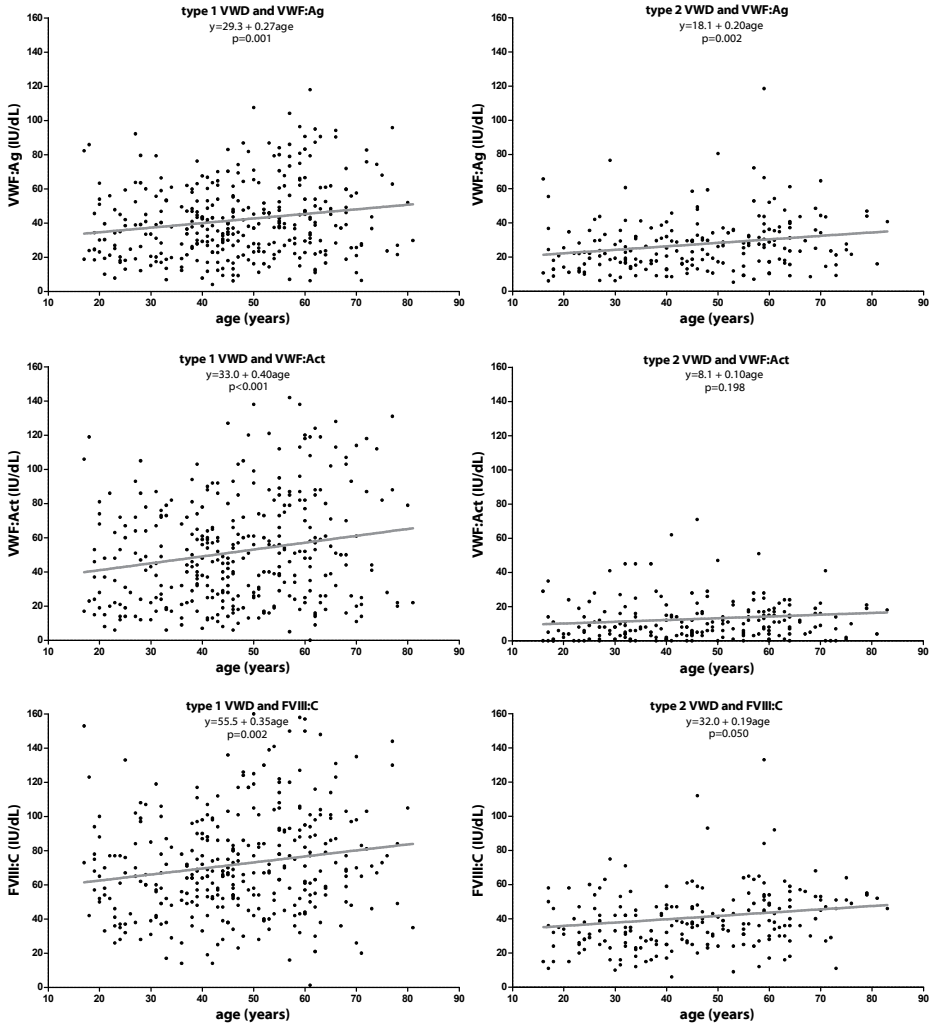


Figure 1. Centrally measured VWF and FVIII levels according to age.
Centrally measured VWF:Ag, VWF:Act and FVIII:C levels according to age in all type 1 and type 2 VWD patients aged 16-85 years at inclusion in the WiN study. Type 1 VWD n=338 and type 2 VWD n=208.

patients who underwent tooth extraction), and post-surgical bleeding (46/64, 72% of the patients who underwent surgery). Other reported bleeding symptoms were gastrointestinal bleeding (17/71, 24%), muscle hematoma (16/71, 23%); hemarthrosis (17/71, 24%) and central nervous system bleeding (1/71, 1%) (Figure 3).

Compared with patients <65 years, a higher number of elderly patients had had gastrointestinal bleeding (17/71, 24% versus 83/593, 14%; p=0.027). This was also observed after exclusion of the type 3 patients (16/70, 23% versus 76/566, 13%; p=0.034). In the

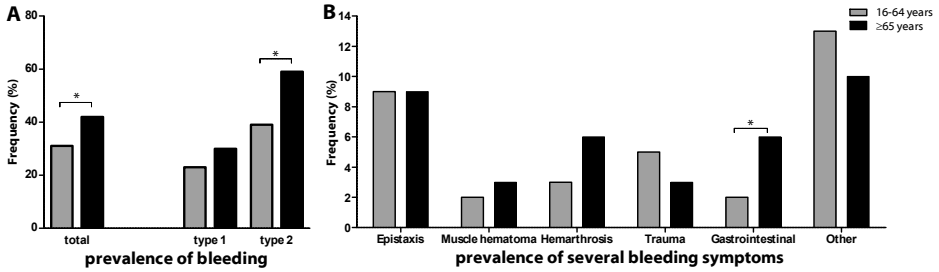


Figure 2. A) Prevalence of bleeding that required desmopressin or replacement therapy in the year preceding inclusion in the study. B) Prevalence of different bleeding symptoms that required desmopressin or replacement therapy in the year preceding inclusion in the study. Other bleeding symptoms include oral cavity bleeding, post-surgical bleeding, hematuria and ocular hemorrhage. * p<0.05

proportion of VWD patients who underwent a surgical procedure, more elderly patients reported post-surgical bleeding than patients <65 years (46/64, 72% versus 256/495, 52%; p=0.002), which was also observed after exclusion of the type 3 patients (41/57, 72% versus 220/424, 52%; p=0.004). Bleeding from minor wounds was reported significantly less by elderly patients (44/71, 62% versus 468/593, 79%; p=0.001). Also, if type 3 patients were included, elderly patients reported significantly less bleeding from minor wounds (44/70, 54% versus 446/566, 75%; p=0.003) (Figure 3).

Elderly type 2 patients reported significantly more post-surgical bleeding (15/20, 75% versus 75/152, 49%; p=0.031) and a trend was observed towards significantly more gastrointestinal bleeding (9/27, 33% versus 41/222, 18%; p=0.069). In type 1 VWD, the lifetime prevalence of all bleeding symptoms were similar between elderly patients and those <65 years of age.

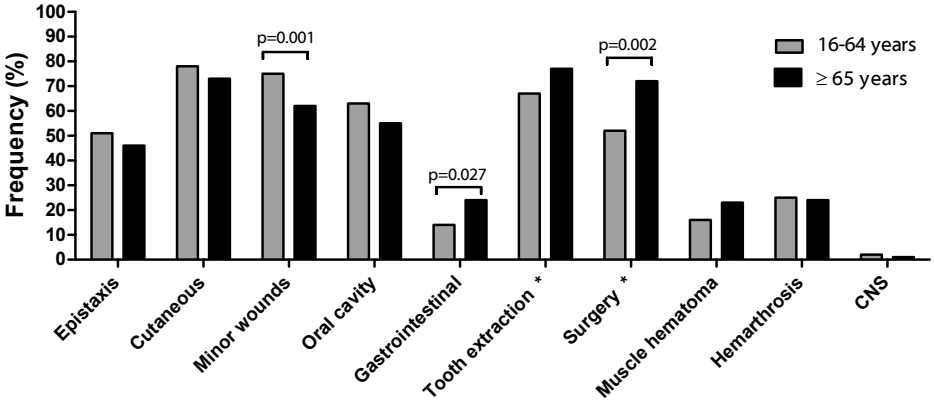


Figure 3. Lifetime prevalence of bleeding symptoms in elderly von Willebrand Disease patients compared with von Willebrand Disease patients below 65 years of age.
* Frequencies are from patients who ever underwent a tooth extraction or surgery.

Bleeding score did not differ between elderly and younger patients (12 [IQR 8-18] versus 11 [6-16]; p=0.154) (Table 1). Also for type 1 and type 2 VWD separately no difference was observed in bleeding score (p=0.293 and p=0.238, respectively).

Self-reported co-morbidity in elderly patients compared with younger patients

From 67 (67/71, 94%) elderly patients and from 580 (580/593, 98%) younger patients, self-reported co-morbidity data was available. Of those patients, more elderly patients reported one or more co-morbidities than younger ones (49/67, 73% versus 241/580, 42%; p<0.001). In both groups, hypertension was the most frequently self-reported co-morbidity, but in elderly the prevalence was significantly higher (35/67, 52% versus 81/580, 14%; p<0.001). Diabetes, cancer and cardiovascular disease were also more frequently self-reported by elderly than those <65 years (diabetes: 6/67, 9% versus 16/580, 3%; p=0.019; cancer: 8/67, 12% versus 23/580, 4%; p=0.010; and cardiovascular disease: 17/67, 25% versus 31/580, 5%; p<0.001). In addition, more elderly patients self-reported depression (6/67, 9% versus 8/580, 1%; p=0.002). The prevalence of all other classified co-morbidities (supplementary file 1) did not differ between those younger and older than 65 years of age. The above mentioned numbers of self-reported co-morbidities were not verified in medical files.

Co-morbidities in elderly von Willebrand Disease patients

Co-morbidity was assessed in elderly VWD patients by checking the medical charts of all individual patients. Nearly all elderly patients (66/71, 93%) suffered from co-morbidities and most of them had two or more co-morbidities (61/71, 86%). The prevalence of co-morbidities in elderly patients is shown in figure 4. Hypertension was the commonest co-morbidity among elderly patients (46/71, 65%). Viral infections were not common; there were no patients infected with HIV; one (1.4%) with chronic hepatitis B and four (5.6%) with chronic hepatitis C. Cardiovascular disease was present in 22 elderly patients (22/71, 31%), who reported a total of 40 different cardiovascular disorders. Cardiac arrhythmias were commonest (10/40, 25%), followed by unstable angina pectoris (6/40, 15%) and heart valve disease (6/40, 15%). Twenty patients (20/71, 28%) suffered from chronic arthropathy, occurring in 26% (11/43) of type 1 and 33% (9/27) of type 2 patients (p=0.485). Chronic arthropathy was not reported by the type 3 patient. The joints most commonly affected were the knee (n=5), hip (n=4) and spine (n=4).

Eleven out of 71 patients (15%) had gastrointestinal disease. The prevalence of gastrointestinal bleeding did not differ between patients with or without gastrointestinal disease (p=0.441). Seventeen elderly patients (24%) had a history of 20 different malignancies. Skin cancer, including basal cell carcinoma, squamous cell carcinoma and melanoma, was the commonest (7/20, 35%), followed by colon cancer (n=3), breast

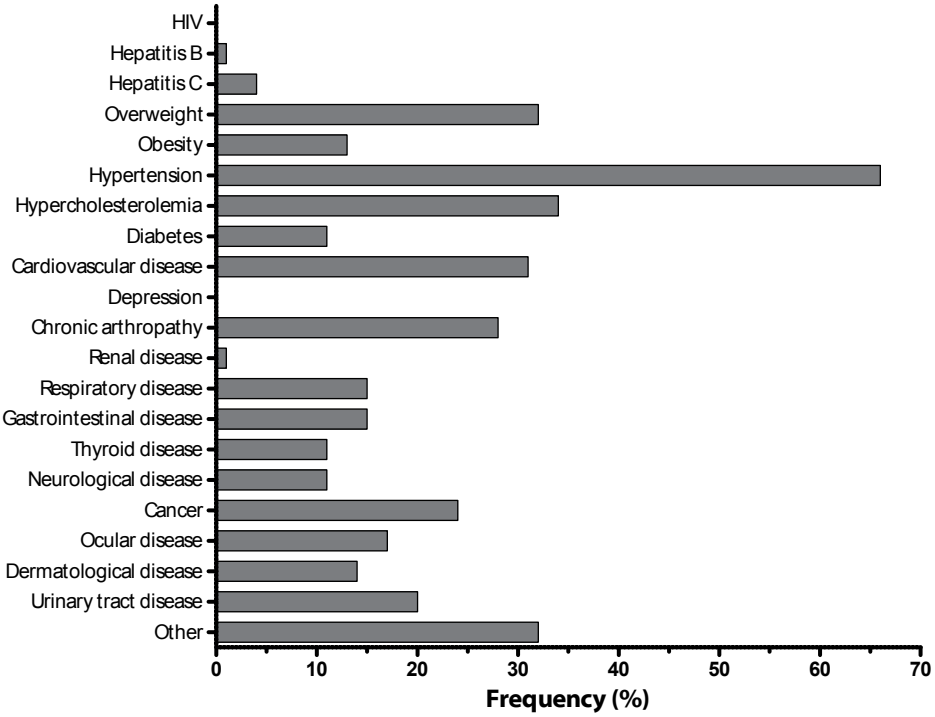


Figure 4. Prevalence of comorbidities in the elderly von Willebrand Disease patients.

cancer (n=3) and prostate cancer (n=3). Other malignancies reported were renal cell cancer, bladder tumor and a pheochromocytoma.

Hospitalizations and surgeries in the year preceding inclusion

Thirteen elderly patients (13/71, 19%) had been hospitalized in the year preceding inclusion, which was similar to patients <65 years (123/593, 21%; p=0.631). Median number of days of hospitalization was 3 [IQR 1.5-4.0]. The main reasons for hospitalization were surgery (9/13, 64%) and bleeding (2/13, 14%) (data not shown).

DISCUSSION

This first nationwide cross-sectional study of elderly VWD patients shows that VWF:Ag, VWF:Act and FVIII:C increase with age in elderly type 1 patients, but not in elderly type 2 patients. In addition, in the year preceding inclusion, bleeding that required treatment occurred more frequently in elderly type 2 patients than in younger type 2 patients, while such a difference was not found between type 1 patients younger and older than 65 years of age.

In healthy individuals, it is known that VWF and FVIII levels increase with age.[9,10,21] Our analyses of VWF and FVIII levels measured over time in a subset of elderly individuals showed that an increase with age also occurs in type 1 VWD patients, which is in accordance with a smaller recent study.[22] This longitudinally measured increase in VWF parameters with age within elderly patients was not found in type 2 VWD. In addition, centrally measured VWF:Act does not increase with age in type 2 VWD. Interestingly, compared with their respective <65 years age group, elderly type 2 patients suffered from more bleeding that required desmopressin or replacement therapy in the year preceding inclusion. Conversely, in type 1 patients, this bleeding rate was similar in elderly and younger patients. Unfortunately, we were not able to analyze difference within type 2 VWD, because of the low number of the various subtypes. However, we have previously shown that 2B and 2A patients had a more severe bleeding phenotype, which is in accordance with Castaman et al.[3,23] These data suggest that the increase in VWF and FVIII levels observed in elderly type 1 patients may mitigate age-related increase of bleeding. The increase in plasma levels is possibly physiological, as it also occurs in healthy individuals. Also, with increasing age, higher levels of VWF and FVIII may be necessary for adequate hemostasis in both healthy and VWD individuals. Tosetto et al have shown that bleeding score did not increase with age in healthy controls.[4] At the same time, as a consequence of the increase in VWF parameters elderly patients with mild type 1 VWD may no longer meet the current diagnostic criteria for VWD.[6]

The bleeding pattern in VWD observed in this study also clearly evolves with increasing age. In the year preceding inclusion in the study, more elderly patients suffered from gastrointestinal bleedings than younger patients. Also, the lifetime prevalence of gastrointestinal bleeding was higher in elderly patients than in younger patients. This has also been observed in the general elderly population,[24] and could be explained by the occurrence of degenerative intestinal changes and angiodysplasia in the elderly.[25] It is known from the general population that approximately 35-45% of all patients presenting with upper gastrointestinal bleeding are over the age of 60 and that the incidence rate of lower gastrointestinal bleeding increases more than 200-fold from the third to the ninth decade of life.[26,27] Gastrointestinal bleedings are often severe and difficult to treat in VWD patients, indicating a possible benefit from prophylactic treatment in elderly patients.[28,29] The increase in bleeding in the year before inclusion may also be driven by hemarthrosis, which could be related to degenerative osteoarticular changes in the elderly patients. However, In our study, chronic arthropathy occurred in 28% of elderly VWD patients, which is much lower than the prevalence reported in elderly hemophilia patients (69% and 95% for elderly with severe disease),[30,31] probably because hemarthrosis is less common in VWD than in hemophilia.

In VWD patients who underwent a surgical intervention, we also found that elderly patients reported more post-surgical bleeding than younger ones, possibly because

they had undergone more major surgical procedures, such as orthopedic surgery, which is characterized by a high bleeding risk.[32] Indeed, in our cohort more orthopedic interventions were performed in elderly patients than in younger ones (22/64, 34% versus 104/495, 21%; p=0.019). In order to reduce post-surgical bleeding in elderly VWD patients, it may be necessary to adjust preoperative and/or postoperative treatment on the basis of FVIII and VWF levels. However, before conclusions can be drawn, this should be studied prospectively.

To our knowledge, this is the first report on co-morbidities in elderly VWD patients. Many elderly individuals with VWD suffer from co-morbidities, which might interact with VWF levels or VWD treatment. If co-morbidity induces a less active lifestyle, patients may suffer fewer bleedings. On the other hand, the frequency and severity of bleeding may increase if more surgical procedures or treatment with antiplatelet and anticoagulant drugs or chemotherapy are needed because of co-morbidities. In our cohort of 71 elderly patients, HIV did not occur and chronic hepatitis B and C infections were rare, but is higher than in the general population with prevalences of 0.4% and 0.002%, respectively.[33,34] This is in contrast to elderly hemophilia patients[11,35], in whom the prevalence of HIV infections ranges from 3.5% to 13%, and that of hepatitis C infections ranges from 69% to 92%.[30,36] Although some patients may have died before inclusion, most VWD patients probably received less factor-replacement therapy than hemophilia patients, and therefore suffered fewer transfusion-transmitted viral infections.[37]

In our study, a quarter of elderly VWD patients had a history of malignancies and 5% of all patients ≥16 years self-reported a prevalent cancer, which is higher than the prevalence of 3.5% in the general Dutch population.[38] In VWD patients, the management of cancer remains a challenge for physicians. Diagnostic and elective procedures may require proper replacement therapy, and the fact that thrombocytopenia is a well-known side-effect of chemotherapy means that more treatment of VWD might be needed to prevent or treat a bleed.[39]

Several elderly patients suffered from various cardiovascular disorders, mainly cardiac arrhythmias, angina pectoris and heart valve disease. We have recently shown that VWD patients have a lower prevalence of coronary heart disease, acute myocardial infarction and ischemic stroke, a higher prevalence of hypertension, and a similar prevalence of diabetes and obesity than the general population.[12] In the current study, 66% of our elderly patients had hypertension, which is similar to the prevalence of hypertension in the general elderly population (between 46% and 70%).[17] VWD patients with cardiovascular disease are often treated with anticoagulation or antiplatelet drugs, which could result in a more severe bleeding phenotype. The balance between the risk of bleeding and the risk of re-thrombosis should be carefully evaluated in VWD patients with cardiovascular events. Guidelines on the management of cardiovascular disease in VWD

patients are lacking and further research on this subject is needed.[40] We also compared the co-morbidities between the patients <65 years and the elderly based on their self-reported co-morbidities. As expected, co-morbidities were reported more frequently by elderly patients, especially for hypertension, diabetes, cancer, cardiovascular disease and depression.

The strength of our study is that we included a large number of elderly VWD patients and that our population covered almost all the patients with moderate or severe VWD in the Netherlands. Secondly, this is the first study on bleeding phenotype and VWF parameters in elderly VWD patients. However, the study also has some limitations. First, laboratory assays for VWF:Ag, VWF:RCo and FVIII:C have improved over time and there may be variability in the results of these assays. Next, the more measurements of VWF and FVIII were available for a single patient in the past 30 years, regression to the mean may have been observed. In addition, there may be some recall bias with regard to bleeding episodes and severity, since it is a cross-sectional study based on a self-administered questionnaire. As we believe that recall bias was more likely to occur in older patients than in younger ones, and to be higher for less severe bleeding symptoms than for more severe bleedings, it might influence not only the rate of bleeding symptoms, but also the reported treatment and co-morbidity. To minimize recall bias, we verified the medical records of all elderly patients and analyzed separately data on bleeding symptoms in the year preceding inclusion in the study.

Another limitation is that the Tosetto Bleeding Questionnaire was used to calculate the lifetime prevalence of bleeding symptoms. This is a cumulative score, which indicates that the prevalence of bleeding symptoms potentially increases with each successive year of life. Patients might also have experienced a ceiling effect as they had already reached a maximum of the bleeding score early in life. We therefore probably observed a similar bleeding score between elderly and younger patients. In addition, patients born before 1964 may not have received high-quality clotting-factor concentrates in their first years of life, and may therefore have had severe bleedings that are included in the Tosetto Bleeding Score. We therefore also analyzed the bleeding symptoms occurring in the year preceding inclusion in the study, which is considered a better measurement for a patient's current bleeding phenotype.

In conclusion, VWF parameters and bleeding phenotype evolve with increasing age in VWD patients. VWF and FVIII levels increase with age in elderly type 1 patients with no amelioration in bleeding phenotype. In type 2 patients VWF and FVIII levels do not increase with age within the elderly population and in these patients aging is accompanied by increased bleeding.

REFERENCES

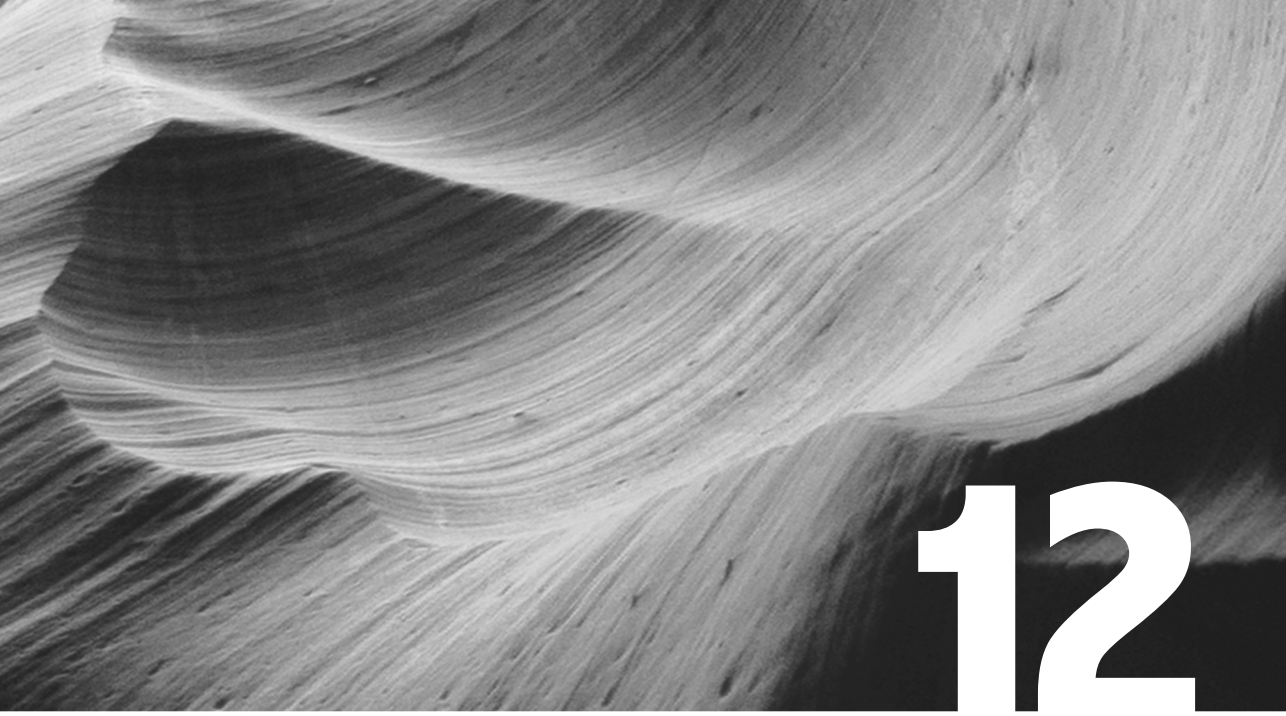
1. James PD, Lillicrap D. von Willebrand disease: clinical and laboratory lessons learned from the large von Willebrand disease studies. *Am J Hematol*. 2012;87 Suppl 1:S4-11.
2. Ruggeri ZM. Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation. *Best Pract Res Clin Haematol*. 2001;14(2):257-279.
3. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108(4):683-692.
4. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost*. 2006;4(4):766-773.
5. Sadler JE, Mannucci PM, Berntorp E, et al. Impact, diagnosis and treatment of von Willebrand disease. *Thromb Haemost*. 2000;84(2):160-174.
6. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
7. Miesbach W, Berntorp E. When von Willebrand disease comes into age - a matter of change? *Eur J Haematol*. 2011;86(6):496-501.
8. Vischer UM, Herrmann FR, Peyrard T, Nzietchueng R, Benetos A. Plasma von Willebrand factor and arterial aging. *J Thromb Haemost*. 2005;3(4):794-795.
9. Mari D, Coppola R, Provenzano R. Hemostasis factors and aging. *Exp Gerontol*. 2008;43(2):66-73.
10. van Loon JE, Kavousi M, Leebeek FW, et al. Von willebrand factor plasma levels, genetic variations, and coronary heart disease in an older population. *J Thromb Haemost*. 2012;10(7):1262-1269.
11. Mauser-Bunschoten EP, Fransen Van De Putte DE, Schutgens RE. Co-morbidity in the ageing haemophilia patient: the down side of increased life expectancy. *Haemophilia*. 2009;15(4):853-863.
12. Sanders YV, Eikenboom J, de Wee EM, et al. Reduced prevalence of arterial thrombosis in von Willebrand disease. *J Thromb Haemost*. 2013;11(5):845-854.
13. de Wee EM, Leebeek FW, Eikenboom JC. Diagnosis and management of von Willebrand disease in The Netherlands. *Semin Thromb Hemost*. 2011;37(5):480-487.
14. de Wee EM, Mauser-Bunschoten EP, van der Bom JG, et al. Health-related quality of life among adult patients with moderate and severe von Willebrand disease. *J Thromb Haemost*. 2010;8(7):1492-1499.
15. De Wee EM, Knol HM, Mauser-Bunschoten EP, et al. Gynaecological and obstetric bleeding in moderate and severe von Willebrand disease. *Thromb Haemost*. 2011;106(5):885-892.
16. Boren MT, Ramey J. Thinking aloud : Reconciling theory and practice. *IEEE transactions on professional communication* 2000;43(3):261-278.
17. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet*. 2005;365(9455):217-223.
18. Tosetto A, Castaman G, Rodeghiero F. Bleeding scores in inherited bleeding disorders: clinical or research tools? *Haemophilia*. 2008;14(3):415-422.
19. World Health Organization. Definition of an older or elderly person. Vol. 2011: WHO; 2001.
20. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063-2065.
21. Conlan MG, Folsom AR, Finch A, et al. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. *Thromb Haemost*. 1993;70(3):380-385.

22. Rydz N, Grabell J, Lillicrap D, P J. Changes in von Willebrand factor level and von Willebrand activity with age in type 1 von Willebrand disease. *Haemophilia*. 2012;18(Suppl. 3):PO-MO-249.
23. Castaman G, Federici AB, Tosetto A, et al. Different bleeding risk in type 2A and 2M von Willebrand disease: a 2-year prospective study in 107 patients. *J Thromb Haemost*. 2012;10(4):632-638.
24. Sharma R, Gorbien MJ. Angiodysplasia and lower gastrointestinal tract bleeding in elderly patients. *Arch Intern Med*. 1995;155(8):807-812.
25. Makris M. Gastrointestinal bleeding in von Willebrand disease. *Thromb Res*. 2006;118 Suppl 1:S13-17.
26. Cooper BT, Weston CF, Neumann CS. Acute upper gastrointestinal haemorrhage in patients aged 80 years or more. *Q J Med*. 1988;68(258):765-774.
27. Longstreth GF. Epidemiology of hospitalization for acute upper gastrointestinal hemorrhage: a population-based study. *Am J Gastroenterol*. 1995;90(2):206-210.
28. Federici AB. Prophylaxis of bleeding episodes in patients with von Willebrand's disease. *Blood Transfus*. 2008;6 Suppl 2:s26-32.
29. Abshire TC, Federici AB, Alvarez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand Disease Prophylaxis Network (VWD PN). *Haemophilia*. 2013;19(1): 76-81.
30. Siboni SM, Mannucci PM, Gringeri A, et al. Health status and quality of life of elderly persons with severe hemophilia born before the advent of modern replacement therapy. *J Thromb Haemost*. 2009;7(5):780-786.
31. Franchini M, Mannucci PM. Co-morbidities and quality of life in elderly persons with haemophilia. *Br J Haematol*. 2010;148(4):522-533.
32. Kistler U, Kramers-de Quervain I, Munzinger U, Kucher N. Bleeding complications after systematic switch of routine thromboprophylaxis for major orthopaedic surgery. *Thromb Haemost*. 2008;99(6): 1049-1052.
33. Gommer AM, Poos MJJC. Prevalence, incidence and mortality according to age and gender. In: Volksgezondheid VTVNK ed. Vol. version 4.5. Bilthoven: RIVM: http://www.nationaalkompas.nl (Accessed 22 September 2011); 2010.
34. Fransen van de Putte DE, Fischer K, Pulles AE, et al. Non-fatal cardiovascular disease, malignancies, and other co-morbidity in adult haemophilia patients. *Thromb Res*. 2012;130(2):157-162.
35. Mannucci PM, Schutgens RE, Santagostino E, Mauser-Bunschoten EP. How I treat age-related morbidities in elderly persons with hemophilia. *Blood*. 2009;114(26):5256-5263.
36. Putte DE, Makris M, Fischer K, et al. Long-term follow-up of hepatitis C infection in a large cohort of patients with inherited bleeding disorders. *J Hepatol*. 2013.
37. Federici AB, Santagostino E, Rumi MG, et al. The natural history of hepatitis C virus infection in Italian patients with von Willebrand's disease: a cohort study. *Haematologica*. 2006;91(4):503-508.
38. Dutch Cancer Registry. the Netherlands: IKNL http://www.cijfersoverkanker.nl (Accessed 21 January 2014); 2014.
39. Franchini M, Lippi G, Montagnana M, et al. Hemophilia and cancer: a new challenge for hemophilia centers. *Cancer Treat Rev*. 2009;35(4):374-377.
40. Franchini M, Coppola A. Atherothrombosis in von Willebrand disease: an analysis of the literature and implications for clinical management. *Semin Thromb Hemost*. 2012;38(2):185-199.

Supplemental table 1. Classification of comorbidities.

Category	Comorbidities
HIV	-
Hepatitis C	-
Hepatitis B	-
Overweight	BMI of 25-30
Obesity	BMI of >30
Hypertension	-
Hypercholesterolemia	-
Diabetes	-
Depression	-
Chronic arthropathy	Degenerative joint disease
	Arthrosis
Renal disease	Chronic renal failure
Respiratory disease	Asthma
	Chronic obstructive pulmonary disease
	Chronic restrictive pulmonary disease
Gastrointestinal disease	Diverticulosis
	Intestinal polyps
Thyroid disease	Hyperthyroidism
	Hypothyroidism
Neurological disease	Spinal canal stenosis
	Neuropathy
	Morbus Parkinson
	Dementia
	Epilepsy
Cancer	Breast cancer
	Prostate cancer
	MEN2A syndrome
	Skin cancer
	Colon cancer
	Renal cell carcinoma
	Bladder cancer
	Rectal cancer
Cardiovascular disease	Angina pectoris
	Cardiac arrhythmias
	Heart failure
	Heart valve disease
	Myocardial infarction
	Venous insufficiency
	Ventricular hypertrophy

Category	Comorbidities
	Coronary artery disease
	Cerebrovascular accident
Ocular disease	Cataract
	Glaucoma
	Graves orbitopathy
	Chronic inflammation
	Keratoconus
	Macular degeneration
Dermatological disease	Varices
	Psoriasis
	Eczema
	Atheroma cysts
	Actinic keratosis
Urinary tract disease	Urethral prolapse
	Urine retention
	Incontinence
	Benign prostatic hyperplasia
	Vaginal prolapse
	Cystocele
Other	Arthritis
	Venous ocular trombosis
	Chronic auto-immune thrombopenia
	Benign paraproteinemia
	Essential thrombocytemia
	Benign mammary tumor
	Pulmonary embolism



12

General discussion

GENERAL DISCUSSION

The overall aim of this thesis was to investigate genetic determinants of von Willebrand Factor (VWF) and genotypic and phenotypic determinants of von Willebrand Disease (VWD). The studies described in this thesis show the genetic and clinical heterogeneity of this bleeding disorder. The findings of the studies described in this thesis will be discussed and interpreted in this final chapter. In addition, recommendations to improve the classification, diagnostics and treatment of VWD and suggestions for future research are discussed.

Willebrand in the Netherlands study

The Willebrand in the Netherlands (WiN) study is the first large nationwide cross-sectional multicenter study on patients with all types of VWD and VWF levels below 30 IU/dL. The objective of the WiN study was to obtain information on clinical presentation, bleeding phenotype, treatment of VWD, and the influence of VWD on quality of life. Since the start in 2007, this study has resulted in several new insights into the quality of life of adults and children with VWD, and the gynecological and obstetrical bleeding symptoms in VWD patients,[1-3] described in more detail in the introduction of this thesis and in a recent review on VWD.[4]

Previous multicenter studies on VWD in particular focused on the molecular and clinical biology of mildly affected type 1 VWD patients, such as the European MCMDM-1VWD study, the Canadian type 1 study and the USA-based Zimmerman project.[5-7] Strengths of the WiN study are the inclusion of a large cohort of over 800 moderate and severe VWD patients with all types of VWD, the high response rate and inclusion rate of 76%, thereby the covering of a large proportion of patients with moderate and severe VWD in the Netherlands.

Causes and pathophysiology of von Willebrand Disease

Mutations in the VWF gene

Since the discovering of the *VWF* gene in 1985, over 500 *VWF* gene mutations have been identified in patients with VWD.[5,6,8,9] These *VWF* mutations are described in detail in the EAHAD Coagulation Factor Variant database (https://grenada.lumc.nl/LOVD2/VWF/home.php; formerly ISTH-SSC VWF Online Database). The genetic background of VWD is not yet fully unraveled and novel *VWF* gene mutations are still discovered, as was also described in chapter 2 of this thesis. Mutation analysis of the *VWF* gene had been performed in 199 patients of the WiN study. This is around 25% of all families. We collected information on pedigree structures from all patients. Performing *VWF* gene mutation analysis in all VWD patients from the WiN cohort in the near future will reveal

important insights in the pathophysiology of VWD and may result in novel insights in the genetic determinants of VWD.

In the ISTH classification of 1994, the diagnosis of VWD was still restricted to mutations within the *VWF* gene.[10] Indeed in nearly all types 2 and 3 VWD patients *VWF* gene mutations are found.[11-13] The VWD classification was revised in 2006, as *VWF* gene mutations could not be identified in a third of type 1 VWD patients.[5,6,14] Previous studies on the molecular pathology of type 1 VWD have shown that *VWF* gene mutations are common in the more severe VWD patients with levels below 20 IU/dL. However, in the mildly affected VWD patients the genetic background has shown to be more complex with incomplete penetrance and other genetic modifiers are more likely to be involved.[6,15]

Genetic variations outside the VWF gene determining VWF levels

Recently, the CHARGE consortium performed a meta-analysis of genome-wide association studies and identified new genetic associations between single nucleotide polymorphisms (SNPs) in *STXBP5*, *SCARA5*, *ABO*, *STAB2*, *STX2*, *TC2N* and *CLEC4M* genes and VWF:Ag levels in over 25,000 healthy individuals.[16] Except for the ABO blood group, which is the most important determinant of VWF:Ag levels, these genes encode for proteins that have not previously been linked to VWF levels.[16] These novel loci are likely to be involved in the secretion and clearance of VWF. Recently, Rydz et al confirmed that CLEC4M has a potential role in VWF clearance by VWF binding and internalization.[17] They also showed that polymorphisms in the CLEC4M gene contribute to the variation in VWF plasma levels.[17] We found that genetic variation in *STXBP5*, *STX2* and *CLEC4M* was associated with VWF:Ag levels in patients with type 1 VWD. In our study *CLEC4M* explains 4%, *STXBP5* 3%, and *STX2* 4% of the variation in VWF:Ag, which is accordance with the percentage VWF:Ag change observed by the CHARGE consortium.[16] From previously performed molecular studies in VWD patients it is known that 30% of mildly affected patients lack *VWF* gene mutations, which suggest that the contribution of other genetic loci is more likely in these patients with mildly decreased VWF levels.[6,15] We found that the percentage of variation in VWF:Ag explained by *CLEC4M* and *STXBP5* were larger in patients with VWF levels above 20 IU/dL than in patients with moderate to severe VWD in which a *VWF* gene mutation is more likely to be found.

As VWF levels and bleeding phenotype are highly associated,[18,19] we hypothesized that genetic variations that influence the VWF levels may also affect the bleeding phenotype. However, only genetic variation in *STXBP5* was associated with bleeding phenotype, measured with the Tosetto bleeding score, in type 1 VWD females.[18] The lack of association between bleeding phenotype and genetic variations may be explained by the known limitations of the Tosetto bleeding score (including cumulative score, ceiling effect, prophylaxis bias), possible selection in our cohort or the relatively small influence

of the genetic variants on VWF levels.[18-20] However, this bleeding score is the currently best available method for evaluating bleeding phenotype in VWD.

An association between genetic variation and VWF levels was not demonstrated in type 2 VWD, which may be explained by the different pathophysiology of this type of VWD. Type 2 VWD is caused by abnormal variants of the VWF protein and it is expected that the *VWF* gene mutation is to a greater extent responsible for the reduced VWF levels in these patients. In addition, a *VWF* gene mutation was found in 99% of the genotyped type 2 VWD patients from the WiN study, and this confirms that type 2 VWD in only caused by a mutation in the *VWF* gene.

Our findings confirm that proteins encoded by genes other than the *VWF* gene contribute to the variability in VWF levels. These genes may play an important role in reducing VWF levels and causing type 1 VWD. Next generation sequencing, including whole exome sequencing, in VWD patients without a causative mutation in the *VWF* gene may lead to the discovery of mutations in genes involved in the biological pathways of VWF and may thereby identify novel causes of VWD and new therapeutic options for VWD.

Recently, van Breevoort et al studied blood outgrowth endothelial cells of an individual with STXBP1-deficiency, which is characterized by early infantile epileptic encephalopathy type 4, and found reduced levels of VWF in this individual, which was caused by defective Weibel Palade Body exocytosis.[21] These findings strengthen our hypothesis that also other variants outside the *VWF* gene may result in low VWF levels, leading to VWD.

Pathophysiological mechanisms of von Willebrand Disease

Low VWF plasma levels in VWD patients result from defects in the biological pathway of VWF, like decreased VWF synthesis, impaired VWF secretion, increased proteolysis, increased VWF clearance, or a combination of these mechanisms. Impaired secretion and increased intracellular retention of VWF has shown to be the major pathophysiological mechanism in type 1 VWD patients with missense mutations.[22] Several mutations have been identified that cause increased clearance of VWF, including p.R1205H, p.C1130F, p.W1144G, and p.C1149R.[23-25] A high VWFpp/VWF:Ag ratio has shown to predict increased clearance of VWF and a high ratio FVIII:C/VWF:Ag ratio reduced synthesis of VWF.[24,26,27] We demonstrated reduced VWF synthesis in type 1 VWD, but rarely in type 2 VWD. The pathophysiological mechanism causing type 2 VWD was mainly increased clearance of VWF. A significant proportion of type 1 VWD patients did not have increased ratios, suggesting pathophysiological mechanisms that have not yet been identified. It would be of interest to study the pathophysiological mechanisms of VWD using Blood Outgrowth Endothelial Cells (BOEC) or Human Embryonic Kidney (HEK293) cells, which has shown to be a reliable model to study the intracellular biosynthesis of VWF.[28,29]

Classification of von Willebrand Disease

In the WiN study, VWD was classified according to the current ISTH guidelines.[30] First, types 1 and 2 VWD were distinguished by the VWF:RCo/VWF:Ag ratio (VWF:Act/VWF:Ag ratio in the WiN study): ≥0.7 for type 1 VWD and <0.7 for type 2 VWD. Next, multimer patterns were evaluated to discriminate between 2M VWD (normal) and 2A or 2B VWD (abnormal). Then, specific test for classification of type 2B VWD (ristocetin-induced platelet aggregation (RIPA) tests) and type 2N VWD (VWF binding to FVIII (VWF:FVIIIB) test) are performed. Additionally, type 2N VWD patients have a disproportional low FVIII:C compared with VWF:Ag. Type 3 VWD is defined as both a VWF:Ag and VWF:Act level of <5 IU/dL, irrespective of FVIII:C level. However, this classification is sometimes difficult, as ratios are less sensitive in patients with very low VWF levels and multimer analysis is difficult in these patients.

Recommendations for a revised von Willebrand Disease classification

The VWF:RCo/VWF:Ag ratio is used to distinguish between type 1 and type 2 VWD. However, in literature the cut-off value for this ratio has been defined as 0.6 and 0.7.[15,31-35] We used 0.7 in the WiN study, but defining an abnormal VWF:RCo/VWF:Ag ratio as 0.6 should have resulted in a different proportion of type 1 and type 2 VWD patients in our studies. Further study is needed to determine the correct cut-of value of these ratios for the classification of type 2 VWD.

In the literature several *VWF* gene mutations have been associated with different VWD phenotypes with highly variable VWF levels and a heterogeneous bleeding phenotype.[5,6,33,36,37] These phenotype-genotype discrepancies were also observed in our study. In some VWD patients with a VWF:Act/VWF:Ag ratio >0.70 and therefore classified as type 1 VWD patients, mutations previously found in type 2 VWD (p.V1499E, p.R1597W, p.R2185Q) were identified. In addition, some patients were classified as 2A or 2M VWD based on their multimer pattern, but all had the similar mutation p.L1288R. Difficulties also exist in type 3 VWD patients with VWF:Ag and VWF:Act levels below 5 IU/dL, because in some of them type 1 mutations were found that are characterized by accelerated clearance of VWF (Vicenza mutation p.R1205H, p.S1285P, p.Y1584C or p.Y1146C).[38] Our findings support the current literature on the difficulty of classifying VWD patients correctly. We therefore recommend reviewing the current VWD classification in the near future.

First, we advise to add the VWF:CB/VWF:Ag ratio to the diagnostic criteria of type 2 VWD, which previously has been shown to improve the diagnosis of VWD.[39,40] We found that a few type 1 VWD patients with type 2 VWD mutations had remarkably lower VWF:CB levels than VWF:Ag and VWF:Act, which suggests a type 2 VWD phenotype. The current classification of type 2 VWD is only based on a decreased VWF:RCo/VWF:Ag ratio, but our findings show that the VWF:CB/VWF:Ag ratio may be of important additional value in classifying type 2 VWD.

Next, we recommend implementing the VWFpp assay as a standard diagnostic in VWD, particularly in patient with low VWF levels. We demonstrated that the presence or absence of VWFpp in plasma discriminates clearly between type 3 VWD patients with complete lack of both VWF:Ag and VWFpp and severe type 1 VWD patients with extremely low VWF levels but detectable VWFpp. An increased VWFpp/VWF:Ag ratio (>3) has previously shown to be a good predictor of the identification of *VWF* gene mutations in type 1 VWD and of accelerated clearance of VWF.[23-25,41] We found that the type 3 VWD patients with complete lack of VWF, so called true type 3 VWD, are carriers of two null alleles and the majority of severe type 1 VWD has *VWF* gene mutations that are known to be associated with accelerated clearance of VWF. We therefore suggest adding VWFpp measurement to the classification of VWF as it also predicts the presence of *VWF* gene mutations and may therefore obviate the need of expensive mutation analysis of the *VWF* gene. Our results also show the importance of the recessive inheritance pattern in the definition and classification of type 3 VWD to detect the true type 3 VWD patients. Discrimination between type 3 VWD and severe type 1 VWD with both VWF levels below 5 IU/dL is very important as they have different clinical characteristics and bleeding phenotype. This may also have important implications for genetic counseling.

In some cases it is essential to perform *VWF* gene mutation analysis to classify patients with VWD. It has already been shown that molecular analysis of the *VWF* gene is useful to distinguish between type 2N VWD and mild hemophilia A or between type 2B VWD and platelet type VWD.[33,42] Our findings suggest that *VWF* gene mutation analysis is also advisable for discriminations between type 2A and 2B VWD patients, because of low sensitivity of the RIPA test in patients with absence of high-molecular weight multimers and very low VWF activity.[43-45] In addition, we found that the VWF:Act/VWF:Ag ratio is less sensitive in patients with very low VWF:Ag levels, which supports the importance of *VWF* gene mutation analysis in these VWD patients with very low VWF levels.[34,46] In type 3 VWD, genetic testing is also of importance to protect type 3 VWD patients from inhibitor development and anaphylactic reactions, as the presence of *VWF* gene deletions is an important risk factor for the development of anti-VWF alloantibodies.[47,48]

In the future, if the molecular background of VWD is completely unraveled, the bleeding risk associated with the *VWF* gene mutation can be more precisely estimated and used as clinical guidance, i.e. as a predictor of desmopressin response and the need for replacement therapy. In addition the observed discrepancy between laboratory based classification and genetic background of VWD indicates that mutation analysis is important for the VWD classification as it provides additional insight into the cause of VWD. Therefore molecular analysis of the *VWF* gene should be implemented to improve the classification of VWD.

Based on the studies described in this thesis, we conclude that the VWF:CB assay, VWFpp measurement and *VWF* gene mutation analysis are important for the correct classification of VWD.

Clinical presentation of von Willebrand Disease patients

Bleeding phenotype of von Willebrand Disease

A significant proportion of VWD patients from the WiN study suffered from menorrhagia, bruising, prolonged bleeding from cutaneous wounds and oral cavity bleeding (chapter 8).[19] Central nervous system bleeding rarely occurred, but we demonstrated that the risk of suffering a hemorrhagic stroke is 5-10 times higher in VWD patients risk than in the age- and sex-matched general population (sources National Public Health Compass (NPHC) and Statistics Netherlands (CBS, Central Bureau of Statistics)).[49-51] It has been shown in hemophilia patients that prophylactic treatment with clotting factor concentrate reduces the number and mortality of central nervous system bleeding.[52] Prospective studies on the prevalence and incidence of this severe bleeding symptom in VWD patients and their burden of disease would be of interest and are needed for optimal treatment, monitoring of VWD patients and possibly also prophylaxis.

We observed a more severe bleeding phenotype in adults and children type 3 VWD than in patients with types 1 and 2 VWD, based on the Tosetto bleeding score.[18] In type 3 VWD, the bleeding phenotype was strongly dependent upon FVIII:C levels, which was associated with the prevalence of joint bleeds. In addition, a more severe bleeding phenotype was found in patients with type 2B and 2A VWD than in 2M or 2N VWD, which has been reported before and explained by an increased frequency of gastrointestinal bleeding in type 2A VWD patients and by low platelet count in 2B VWD patients.[53,54] A possible explanation for the difference in prevalence of gastrointestinal bleeding between 2A and 2M VWD is the lack or presence of high-molecular-weight multimers.[53] Half of the children with moderate or severe VWD from the WiN study suffered from pediatric specific bleeding symptoms, such as umbilical stump bleeding, cephalohematoma, and excessive post-venipuncture bleeding. In other cohorts of children with bleeding disorders, very small numbers of children with VWD had child specific bleeding symptoms, but these children had less severe VWD than our cohort of VWD patients had.[55-58] Pediatric specific bleedings are therefore of important value in diagnosing VWD and may help to discriminate children with and without VWD.

Joint bleeds in von Willebrand Disease patients

Joint bleeds occur in a significant proportion of VWD patients and are known to be associated with lower FVIII:C levels and a more severe bleeding phenotype.[19,59,60] The occurrence of joint bleeds is not restricted to type 3 VWD patients, as 20% of type 1 or 2 VWD patients also self-reported joint bleeds in our study. Joint bleeds have a major

impact on joint damage and health-related quality of life, as shown in our study and before.[1,61,62] The VWD International Prophylaxis (VIP) study recently demonstrated that VWD patient with joint bleeds benefit from prophylactic clotting factor concentrate treatment to prevent recurrent joint bleeds and therefore joint damage, which is accordance with our findings and previous studies in severe haemophilia A patients.[63,64] Recall bias, reporting bias and missing data in medical files are drawbacks in our "Willebrand Arthropathy Study", therefore prospective studies are needed to assess the burden and impact of joint bleeds in VWD.

Gastrointestinal bleeding in Willebrand Disease patients

We showed that increasing age was a strong determinant of bleeding phenotype in adult VWD patients, measured with the Tosetto bleeding score.[18] Since the Tosetto bleeding score is a cumulative score, an association with age is anticipated, but this is the best available tool currently available. It has been suggested that the site of bleeding may change with age. We found that elderly VWD patients more frequently suffered from gastrointestinal bleeding than younger VWD patients, which has been suggested before.[65] The increased prevalence of gastrointestinal bleeding may be associated with gastrointestinal angiodysplasia.[66] Gastrointestinal bleeding in VWD patient is often severe, difficult to treat and due to frequent recurrence, secondary prophylaxis may be indicated in these patients.[67] The VWD International Prophylaxis (VIP) study recently showed a significant decrease in number of gastrointestinal bleeding episodes in VWD patients on secondary prophylaxis, but the effect was smaller than observed for joint bleeds.[63] Atorvastatin, which is anti-angiogenic at high doses, has shown to be successful in the treatment of angiodyplasia-related gastrointestinal bleeding in VWD patients, but larger studies are warranted to study the safety and efficacy.[68,69]

The use of bleeding assessment tools to assess bleeding phenotype

Bleedings scores for adults and children have been developed to discriminate between individuals with and without VWD by quantifying the number and severity of various bleeding symptoms.[18,55,70,71] Recently, normal ranges of bleeding scores for the ISTH-BAT, which is the currently advised version of the bleeding score, have been established: positive bleeding scores are ≥4 for adult males, ≥6 for adult females and ≥3 for children.[71,72] We found that age, gender, severity and type of VWD are important determinants of bleeding scores in pediatric and adult VWD patients. More recently, it has been demonstrated that clinical outcomes of VWD and patients that need prophylactic treatment can be predicted by the Tosetto bleeding score.[73]

Bleeding scores are originally designed as an expert-administered questionnaire.[18,70] In our pediatric cohort one physician administered the bleeding score, but our study of bleeding phenotype in adult patients was hampered by the use of a self-completed ver-

sion of the Tosetto bleeding score to establish the bleeding phenotype. Our pilot study (chapter 8) and a recent study of Boelaars et al showed similar results of self-reported and physician-administered bleeding scores.[19,74] Preliminary analysis of validation of the Self-BAT (a self-administered bleeding score) from James et al shows that the Self-BAT is a highly effective tool in the identification of VWD patients.[75] We prefer a self-administered bleeding score as it is more practical and less labor intensive, and hope this tool will be incorporated in the management of patients with VWD. However, bleeding scores have limitations, as recall bias may occur by obtaining the questionnaire, and severe VWD patients might easily reach a maximum plateau of the bleeding score, also known as a ceiling effect. In addition, prophylaxis-bias may occur if prophylactic treatment with clotting factor concentrates or desmopressin is given before surgery, dental extraction and delivery.[20] Therefore, further Improvement of this tool is still necessary in order to reduce the current limitations and to improve the diagnostic accuracy.

Angiogenesis in von Willebrand Disease

As mentioned above gastrointestinal bleeding is an important morbidity in VWD patients and is frequently caused by angiodysplasia. Gastrointestinal angiodysplastic lesions have been shown to occur frequently in patients with VWD and the prevalence increases with age.[76-78] Recently, Starke et al demonstrated a possible new function of VWF in regulating angiogenesis as VWF seems to inhibit angiogenesis.[79] Endothelial cells of VWF-deficient mice and endothelial cells of blood-derived endothelial progenitor cells of VWD patients showed increased angiogenesis in this study.[79] Markers of angiogenesis, such as angiopoietin-2, osteoprotegerin and galactin-3, are stored in Weibel-Palade bodies together with VWF.[80-83] As VWF has shown to be essential for the formation of Weibel-Palade bodies,[80,84] reduced levels of VWF or defects in VWF may implicate an altered balance of angiogenic markers and may therefore lead to increased angiogenesis and the development of vascular malformation. We found that VWD patients had various disturbances in angiogenic markers in plasma but defects in VWF did not consistently lead to dysregulated release of angiogenic markers from Weibel-Palade bodies. If the variations in angiogenic markers in VWD contributes to the development of angiodysplasia needs to be further explored in the near future.

Aging and von Willebrand Disease

Plasma concentrations of VWF and FVIII increase with age in healthy individuals.[85] Levels of VWF levels do not increase up to the age of 40, but after the age of 40 the increase of VWF:Ag is considerable with 10-20 IU/dL per decade.[86,87] This reflects an increasing prothrombotic status during aging.[88,89] The mechanism of age-related increase of VWF levels is still unknown, but an association with arterial rigidity and the extent of atherosclerosis in the aortic arch and carotid arteries has been suggested.[90,91] Changes in arterial rigidity

may induce endothelial activation and VWF secretion from the Weibel-Palade bodies. We demonstrated that VWF and FVIII levels also increase in type 1 VWD patients upon aging. Hence, some elderly patients with mild type 1 VWD no longer meet the current diagnostic criteria of VWD.[30] However, these increased levels of VWF could still result in levels that are too low for adequate hemostasis in this age group and may result in bleeding. We retrospectively studied the relationship between increase in VWF levels and change in bleeding phenotype in VWD patients upon aging. The age-related increase in VWF parameters did not alter the bleeding risk in type 1 VWD patients. In haemophilia patients, it has been shown that demopressin responses increase with age.[92] These age-related changes in VWD phenotype might also have consequences for desmopressin treatment in type 1 VWD patients, but this is still unknown and further research is necessary. We did not observe an increase in VWF parameters with age in elderly type 2 VWD patients. We found more frequent bleeding that required desmopressin or replacement therapy in the year before inclusion in these patients, so the bleeding phenotype in the more severe form of VWD even increases with aging.[93] We are the first to demonstrate this increase in VWF and FVIII and the change in bleeding risk upon aging, and further large prospective studies are needed before conclusions regarding age-specific cut-off levels, specific diagnostic VWD criteria for elderly or optimized age-specific VWD treatment, can be drawn.

Von Willebrand Disease and its risk for arterial thrombosis

Elderly frequently suffer from comorbidities. With a high prevalence of cardiovascular disease in the general population and the increasing life expectancy of VWD patients, it is very likely that VWD patients will also suffer from cardiovascular disease. It has been hypothesized that patients with VWD are protected against arterial thrombosis, as high VWF levels are a well-known risk factor for arterial thrombosis.[88,94] The WiN study is the first study that found a lower prevalence of acute myocardial infarction, coronary heart disease and ischemic stroke in patients with VWD compared with the healthy population in the Netherlands. This additionally shows that VWF plays an important role in the pathogenesis of arterial thrombosis. However, arterial thrombosis does occur in VWD patients and we therefore recommend developing guidelines for the management of cardiovascular disease in VWD patients. It is recommended to treat VWD patients that suffered from arterial thrombosis with long-term antiplatelet therapy to prevent them from recurrent occlusive thrombi. The bleeding risk in these patients should be monitored carefully and anti-thrombotic treatment needs to be adjusted accordingly. Further studies are needed to unravel the role of low VWF and FVIII levels in the development of arterial thrombosis. Additionally, VWF may be an important therapeutic target for the prevention of arterial thrombosis, and therefore VWF-inhibiting agents have recently been developed.[95-97] The efficacy and safety of these VWF-inhibiting drugs will be tested in the near future.

Suggestions for further research

In only a minority of VWD patients included in the WiN study mutation analysis of the *VWF* gene has been performed, but DNA has been collected from 94% of VWD patients. It would be of great interest to perform *VWF* gene mutation analysis in all VWD patients from the WiN cohort. Probably new *VWF* gene mutations causative for VWD will be found, which will give important new insights in the pathophysiology of patients with moderate and severe VWD. In addition, next generation sequencing, including whole exome sequencing, in VWD patients without a causative *VWF* gene mutation may discover mutations in genes other than the *VWF* gene that contribute to the variation in VWF levels and cause VWD. If novel genetic causes of VWD are found, this may imply new therapeutic options for VWD. Next step for further research would be analyzing the pathophysiological mechanisms of VWD for each genetic variation with Blood Outgrowth Endothelial Cells (BOEC) or Human Embryonic Kidney (HEK293) cells.

Prospective studies to analyze bleeding symptoms and the burden of VWD are necessary to provide insights into the factors that determine an individual's bleeding tendency and may have important clinical implications. In addition, it would be of interest to study prospectively whether a bleeding score can be used to improve the management of VWD patients and protect them against bleedings after surgery and dental extraction. If the molecular biology VWD, pathophysiology of VWD and the determinants of bleeding phenotype are further unraveled, the association between bleeding phenotypes and genetic, molecular, and environmental data could improve the management of VWD patients.

VWF has been shown to regulate angiogenesis. However, the exact mechanisms and the relation between angiodysplasia and gastrointestinal bleedings in VWD patients are still unknown. Further research is needed to address this issue. It would also be of interest to further unravel the role of VWF in the development of arterial thrombosis, as VWF may be an important therapeutic target for the prevention of arterial thrombosis. In addition, research on the management of arterial thrombosis, such as ischemic stroke or acute myocardial infarction, in VWD patients is needed to provide clinical guidelines for antithrombotic treatment in this vulnerable patient group.

Arterial rigidity or the extent of atherosclerosis may be the mechanism behind age-related increase of VWF levels in VWD patients and healthy individuals. This should be investigated in future studies. In addition, the relation between the change in VWF levels upon aging and bleeding phenotype needs to be explored in more detail to know if elderly patients with mild type 1 VWD cure from VWD or if age-adjusted normal values of VWF, age-adjusted diagnostic VWD criteria, and age-specific VWD treatment needs to be developed.

REFERENCES

1. de Wee EM, Mauser-Bunschoten EP, van der Bom JG, et al. Health-related quality of life among adult patients with moderate and severe von Willebrand disease. *J Thromb Haemost*. 2010;8(7):1492-1499.
2. de Wee EM, Fijnvandraat K, de Goede-Bolder A, et al. Impact of von Willebrand disease on health-related quality of life in a pediatric population. *J Thromb Haemost*. 2011;9(3):502-509.
3. De Wee EM, Knol HM, Mauser-Bunschoten EP, et al. Gynaecological and obstetric bleeding in moderate and severe von Willebrand disease. *Thromb Haemost*. 2011;106(5):885-892.
4. Sanders YV, de Wee EM, Meijer K, et al. [Von Willebrand disease in the Netherlands: the WiN study] De ziekte van von Willebrand in Nederland: de WiN-studie. *Ned Tijdschr Geneeskd*. 2014;158:A6518.
5. Goodeve A, Eikenboom J, Castaman G, et al. Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study, Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*. 2007;109(1):112-121.
6. James PD, Notley C, Hegadorn C, et al. The mutational spectrum of type 1 von Willebrand disease: Results from a Canadian cohort study. *Blood*. 2007;109(1):145-154.
7. Flood VH, Gill JC, Morateck PA, et al. Gain-of-function GPIb ELISA assay for VWF activity in the Zimmerman Program for the Molecular and Clinical Biology of VWD. *Blood*. 2011;117(6):e67-74.
8. Yadegari H, Driesen J, Pavlova A, Biswas A, Hertfelder HJ, Oldenburg J. Mutation distribution in the von Willebrand factor gene related to the different von Willebrand disease (VWD) types in a cohort of VWD patients. *Thromb Haemost*. 2012;108(4):662-671.
9. Ginsburg D, Handin RI, Bonthron DT, et al. Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization. *Science*. 1985;228(4706):1401-1406.
10. Sadler JE. A revised classification of von Willebrand disease. For the Subcommittee on von Willebrand Factor of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost*. 1994;71(4):520-525.
11. Eikenboom JC. Congenital von Willebrand disease type 3: clinical manifestations, pathophysiology and molecular biology. *Best Pract Res Clin Haematol*. 2001;14(2):365-379.
12. Bowman M, Lillicrap D, James P. The genetics of Canadian type 3 von Willebrand disease: further evidence for codominant inheritance of mutant alleles: a reply to a rebuttal. *J Thromb Haemost*. 2013;11(9):1786-1787.
13. Goodeve AC. The genetic basis of von Willebrand disease. *Blood Rev*. 2010;24(3):123-134.
14. Cumming A, Grundy P, Keeney S, et al. An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb Haemost*. 2006;96(5):630-641.
15. Eikenboom J, Van Marion V, Putter H, et al. Linkage analysis in families diagnosed with type 1 von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 VWD. *J Thromb Haemost*. 2006;4(4):774-782.
16. Smith NL, Chen MH, Dehghan A, et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*. 2010;121(12):1382-1392.
17. Rydz N, Swystun LL, Notley C, et al. The C-type lectin receptor CLEC4M binds, internalizes, and clears von Willebrand factor and contributes to the variation in plasma von Willebrand factor levels. *Blood*. 2013;121(26):5228-5237.
18. Tosetto A, Rodeghiero F, Castaman G, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). *J Thromb Haemost*. 2006;4(4):766-773.

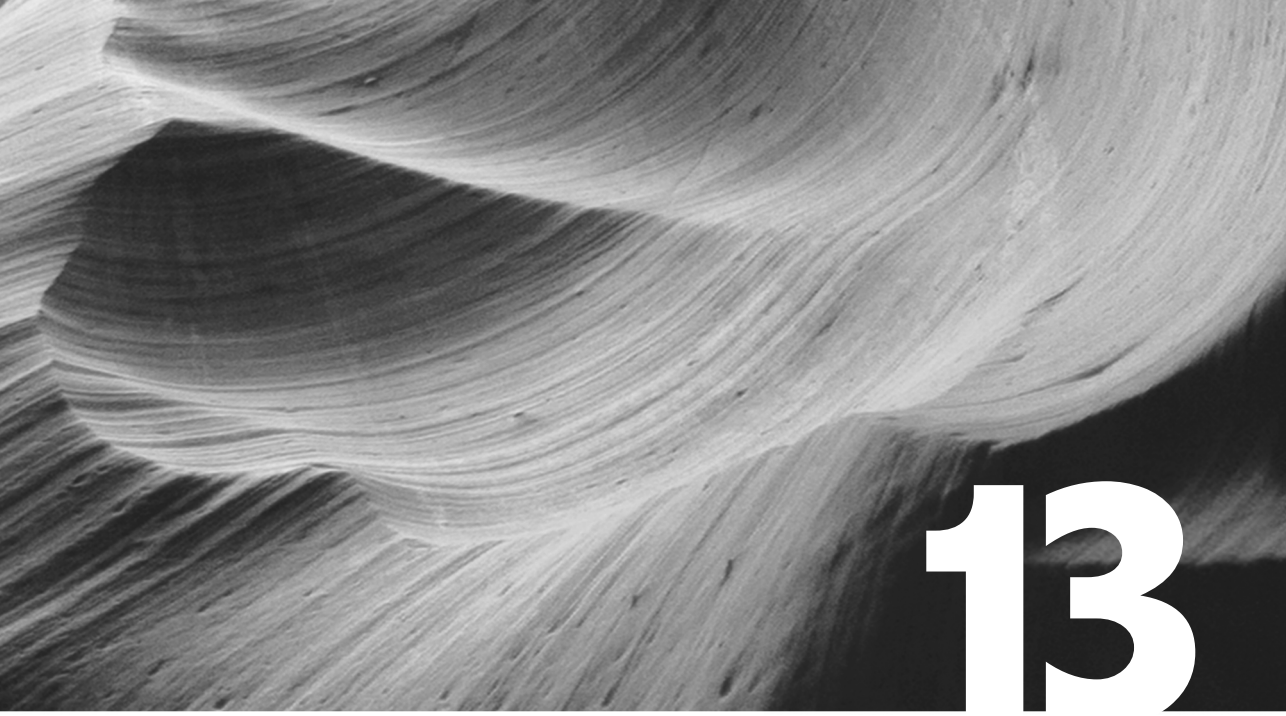
19. De Wee EM, Sanders YV, Mauser-Bunschoten EP, et al. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thromb Haemost*. 2012;108(4):683-692.
20. Tosetto A, Castaman G, Rodeghiero F. Bleeding scores in inherited bleeding disorders: clinical or research tools? *Haemophilia*. 2008;14(3):415-422.
21. van Breevoort D, Snijders AP, Hellen N, et al. STXBP1 promotes Weibel-Palade body exocytosis through its interaction with the Rab27A effector Slp4-a. *Blood*. 2014;123(20):3185-3194.
22. Eikenboom J, Hilbert L, Ribba AS, et al. Expression of 14 von Willebrand factor mutations identified in patients with type 1 von Willebrand disease from the MCMDM-1VWD study. *J Thromb Haemost*. 2009;7(8):1304-1312.
23. Schooten CJ, Tjernberg P, Westein E, et al. Cysteine-mutations in von Willebrand factor associated with increased clearance. *J Thromb Haemost*. 2005;3(10):2228-2237.
24. Haberichter SL, Balistreri M, Christopherson P, et al. Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood*. 2006;108(10):3344-3351.
25. Casonato A, Pontara E, Sartorello F, et al. Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. *Blood*. 2002;99(1):180-184.
26. Borchiellini A, Fijnvandraat K, ten Cate JW, et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood*. 1996;88(8):2951-2958.
27. Sztukowska M, Gallinaro L, Cattini MG, et al. Von Willebrand factor propeptide makes it easy to identify the shorter Von Willebrand factor survival in patients with type 1 and type Vicenza von Willebrand disease. *Br J Haematol*. 2008;143(1):107-114.
28. Wang JW, Bouwens EA, Pintao MC, et al. Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease. *Blood*. 2013;121(14):2762-2772.
29. Groeneveld DJ, Wang JW, Mourik MJ, et al. Storage and secretion of naturally occurring von Willebrand factor A domain variants. *Br J Haematol*. 2014;167(4):529-540.
30. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;4(10):2103-2114.
31. James PD, Paterson AD, Notley C, et al. Genetic linkage and association analysis in type 1 von Willebrand disease: results from the Canadian type 1 VWD study. *J Thromb Haemost*. 2006;4(4):783-792.
32. Laffan MA, Lester W, O'Donnell JS, et al. The diagnosis and management of von Willebrand disease: a United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology. *Br J Haematol*. 2014;167(4):453-465.
33. Castaman G, Goodeve A, Eikenboom J, European Group on von Willebrand Disease. Principles of care for the diagnosis and treatment of von Willebrand disease. *Haematologica*. 2013;98(5):667-674.
34. Federici AB, Canciani MT, Forza I, Cozzi G. Ristocetin cofactor and collagen binding activities normalized to antigen levels for a rapid diagnosis of type 2 von Willebrand disease--single center comparison of four different assays. *Thromb Haemost*. 2000;84(6):1127-1128.
35. de Wee EM, Leebeek FW, Eikenboom JC. Diagnosis and management of von Willebrand disease in The Netherlands. *Semin Thromb Hemost*. 2011;37(5):480-487.
36. Budde U, Schneppenheim R, Eikenboom J, et al. Detailed von Willebrand factor multimer analysis in patients with von Willebrand disease in the European study, molecular and clinical markers for the diagnosis and management of type 1 von Willebrand disease (MCMDM-1VWD). *J Thromb Haemost*. 2008;6(5):762-771.

37. Costa-Pinto J, Perez-Rodriguez A, del CG-d-CM, et al. Diagnosis of inherited von Willebrand disease: comparison of two methodologies and analysis of the discrepancies. *Haemophilia*. 2014;20(4): 559-567.
38. Schneppenheim R, Federici AB, Budde U, et al. Von Willebrand Disease type 2M "Vicenza" in Italian and German patients: identification of the first candidate mutation (G3864A; R1205H) in 8 families. *Thromb Haemost*. 2000;83(1):136-140.
39. Favaloro EJ, Mohammed S. Towards improved diagnosis of von Willebrand disease: comparative evaluations of several automated von Willebrand factor antigen and activity assays. *Thromb Res*. 2014;134(6):1292-1300.
40. Favaloro EJ, Bonar R, Kershaw G, et al. Reducing errors in identification of von Willebrand disease: the experience of the royal college of pathologists of australasia quality assurance program. *Semin Thromb Hemost*. 2006;32(5):505-513.
41. Eikenboom J, Federici AB, Dirven RJ, et al. VWF propeptide and ratios between VWF, VWF propeptide and FVIII in the characterization of type 1 von Willebrand disease. *Blood*. 2013;121(12):2336-2339.
42. Peake IR, Goodeve AC. Genetic testing for von Willebrand disease: the case for. *J Thromb Haemost*. 2010;8(1):13-16.
43. Gomez Garcia EB, Brouwers GJ, Leebeek FW. [From gene to disease; from mutations in the Von Willebrand factor gene to hemorrhagic diathesis and thrombocytopenia]. *Ned Tijdschr Geneeskd*. 2002; 146(25):1180-1182.
44. Weiss HJ, Sussman, II. A new von Willebrand variant (type I, New York): increased ristocetin-induced platelet aggregation and plasma von Willebrand factor containing the full range of multimers. *Blood*. 1986;68(1):149-156.
45. Frontroth JP, Hepner M, Sciuccati G, Feliu Torres A, Pieroni G, Bonduel M. Prospective study of low-dose ristocetin-induced platelet aggregation to identify type 2B von Willebrand disease (VWD) and platelet-type VWD in children. *Thromb Haemost*. 2010;104(6):1158-1165.
46. Favaloro EJ. Appropriate laboratory assessment as a critical facet in the proper diagnosis and classification of von Willebrand disorder. *Best Pract Res Clin Haematol*. 2001;14(2):299-319.
47. Mannucci PM. Genetic testing in von Willebrand disease: a rebuttal. *J Thromb Haemost*. 2010;8(4): 860; author reply 861.
48. James PD, Lillicrap D, Mannucci PM. Alloantibodies in von Willebrand disease. *Blood*. 2013;122(5): 636-640.
49. Gommer AM, Poos MJJC. Prevalence, incidence and mortality according to age and gender. In: Volksgezondheid VTVNK ed. Vol. version 4.5. Bilthoven: RIVM: http://www.nationaalkompas.nl (Accessed 22 September 2011); 2010.
50. Central Bureau of Statistics. Permanent Research Living Situation (POLS) survey. Voorburg/Heerlen: CBS: http://statline.cbs.nl (Accessed 22 September 2011); 2009.
51. Sanders YV, Eikenboom J, de Wee EM, et al. Reduced prevalence of arterial thrombosis in von Willebrand disease. *J Thromb Haemost*. 2013;11(5):845-854.
52. Witmer C, Presley R, Kulkarni R, Soucie JM, Manno CS, Raffini L. Associations between intracranial haemorrhage and prescribed prophylaxis in a large cohort of haemophilia patients in the United States. *Br J Haematol*. 2011;152(2):211-216.
53. Castaman G, Federici AB, Tosetto A, et al. Different bleeding risk in type 2A and 2M von Willebrand disease: a 2-year prospective study in 107 patients. *J Thromb Haemost*. 2012;10(4):632-638.
54. Federici AB, Mannucci PM, Castaman G, et al. Clinical and molecular predictors of thrombocytopenia and risk of bleeding in patients with von Willebrand disease type 2B: a cohort study of 67 patients. *Blood*. 2009;113(3):526-534.

55. Bowman M, Riddel J, Rand ML, Tosetto A, Silva M, James PD. Evaluation of the diagnostic utility for von Willebrand disease of a pediatric bleeding questionnaire. *J Thromb Haemost*. 2009;7(8):1418-1421.
56. Marcus PD, Nire KG, Grooms L, Klima J, O'Brien SH. The power of a standardized bleeding score in diagnosing paediatric type 1 von Willebrand's disease and platelet function defects. *Haemophilia*. 2011;17(2):223-227.
57. Bidlingmaier C, Grote V, Budde U, Olivieri M, Kurnik K. Prospective evaluation of a pediatric bleeding questionnaire and the ISTH bleeding assessment tool in children and parents in routine clinical practice. *J Thromb Haemost*. 2012;10(7):1335-1341.
58. Biss TT, Blanchette VS, Clark DS, et al. Quantitation of bleeding symptoms in children with von Willebrand disease: use of a standardized pediatric bleeding questionnaire. *J Thromb Haemost*. 2010; 8(5):950-956.
59. Sood SL, Cuker A, Wang C, et al. Similarity in joint function limitation in Type 3 von Willebrand's disease and moderate haemophilia A. *Haemophilia*. 2013;19(4):595-601.
60. Metjian AD, Wang C, Sood SL, et al. Bleeding symptoms and laboratory correlation in patients with severe von Willebrand disease. *Haemophilia*. 2009;15(4):918-925.
61. Jansen NW, Roosendaal G, Lafeber FP. Understanding haemophilic arthropathy: an exploration of current open issues. *Br J Haematol*. 2008;143(5):632-640.
62. Fischer K, Bom JG, Mauser-Bunschoten EP, Roosendaal G, Berg HM. Effects of haemophilic arthropathy on health-related quality of life and socio-economic parameters. *Haemophilia*. 2005;11(1):43-48.
63. Abshire TC, Federici AB, Alvarez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand Disease Prophylaxis Network (VWD PN). *Haemophilia*. 2013;19(1): 76-81.
64. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med*. 2007;357(6):535-544.
65. Miesbach W, Berntorp E. When von Willebrand disease comes into age - a matter of change? *Eur J Haematol*. 2011;86(6):496-501.
66. Franchini M, Mannucci PM. Gastrointestinal angiodysplasia and bleeding in von Willebrand disease. *Thromb Haemost*. 2014;112(3):427-431.
67. Franchini M, Targher G, Lippi G. Prophylaxis in von Willebrand disease. *Ann Hematol*. 2007;86(10): 699-704.
68. Sohal M, Laffan M. Von Willebrand disease and angiodysplasia responding to atorvastatin. *Br J Haematol*. 2008;142(2):308-309.
69. Alikhan R, Keeling D. Von Willebrand disease, angiodysplasia and atorvastatin. *Br J Haematol*. 2010; 149(1):159-160.
70. Rodeghiero F, Castaman G, Tosetto A, et al. The discriminant power of bleeding history for the diagnosis of type 1 von Willebrand disease: an international, multicenter study. *J Thromb Haemost*. 2005;3(12):2619-2626.
71. Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8(9):2063-2065.
72. Elbatarny M, Mollah S, Grabell J, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. *Haemophilia*. 2014;20(6):831-835.
73. Federici AB, Bucciarelli P, Castaman G, et al. The bleeding score predicts clinical outcomes and replacement therapy in adults with von Willebrand disease. *Blood*. 2014;123(26):4037-4044.

74. Boelaars MF, Peters M, Fijnvandraat K. Evaluation of a self-administrated pediatric bleeding questionnaire measuring bleeding severity in children. *Thromb Haemost*. 2012;108(5):1006-1007.
75. Young J, Grabell J, Tuttle A, et al. Validation of the Self-BAT (Self-administered Bleeding Assessment Tool) in hemophilia carriers: preliminary results. *Haemophilia*. 2014;20 Suppl. 3:Abstract FP-M-01.03.
76. Fressinaud E, Meyer D. International survey of patients with von Willebrand disease and angiodysplasia. *Thromb Haemost*. 1993;70(3):546.
77. Castaman G, Di Bona E, Rodeghiero F. Angiodysplasia and von Willebrand's disease. *Thromb Haemost*. 1994;71(4):527-528.
78. Siragusa S, Malato A, Lo Coco L, et al. Gastrointestinal bleeding due to angiodysplasia in patients with type 1 von Willebrand disease: report on association and management. *Haemophilia*. 2008; 14(1):150-152.
79. Starke RD, Ferraro F, Paschalaki KE, et al. Endothelial von Willebrand factor regulates angiogenesis. *Blood*. 2011;117(3):1071-1080.
80. Valentijn KM, Sadler JE, Valentijn JA, Voorberg J, Eikenboom J. Functional architecture of Weibel-Palade bodies. *Blood*. 2011;117(19):5033-5043.
81. Fiedler U, Scharpfenecker M, Koidl S, et al. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood*. 2004;103(11):4150-4156.
82. Saint-Lu N, Oortwijn BD, Pegon JN, et al. Identification of galectin-1 and galectin-3 as novel partners for von Willebrand factor. *Arterioscler Thromb Vasc Biol*. 2012;32(4):894-901.
83. Shahbazi S, Lenting PJ, Fribourg C, Terraube V, Denis CV, Christophe OD. Characterization of the interaction between von Willebrand factor and osteoprotegerin. *J Thromb Haemost*. 2007;5(9):1956-1962.
84. Wang JW, Groeneveld DJ, Cosemans G, et al. Biogenesis of Weibel-Palade bodies in von Willebrand's disease variants with impaired von Willebrand factor intrachain or interchain disulfide bond formation. *Haematologica*. 2012;97(6):859-866.
85. Mari D, Coppola R, Provenzano R. Hemostasis factors and aging. *Exp Gerontol*. 2008;43(2):66-73.
86. Davies JA, Hathaway LS, Collins PW, Bowen DJ. von Willebrand factor: demographics of plasma protein level in a large blood donor cohort from South Wales in the United Kingdom. *Haemophilia*. 2012;18(3):e79-81.
87. van Loon JE, Kavousi M, Leebeek FW, et al. Von willebrand factor plasma levels, genetic variations, and coronary heart disease in an older population. *J Thromb Haemost*. 2012;10(7):1262-1269.
88. Whincup PH, Danesh J, Walker M, et al. von Willebrand factor and coronary heart disease: prospective study and meta-analysis. *Eur Heart J*. 2002;23(22):1764-1770.
89. van Schie MC, de Maat MP, Isaacs A, et al. Variation in the von Willebrand factor gene is associated with von Willebrand factor levels and with the risk for cardiovascular disease. *Blood*. 2011;117(4): 1393-1399.
90. Sonneveld MA, van Dijk AC, van den Herik EG, et al. Relationship of Von Willebrand Factor with carotid artery and aortic arch calcification in ischemic stroke patients. *Atherosclerosis*. 2013;230(2): 210-215.
91. Vischer UM, Herrmann FR, Peyrard T, Nzietchueng R, Benetos A. Plasma von Willebrand factor and arterial aging. *J Thromb Haemost*. 2005;3(4):794-795.
92. Mauser-Bunschoten EP, Fransen van de Putte DE, Ploos van Amstel HK, Spoor M, Schutgens RE. Response to desmopressin in patients with mild hemophilia A caused by the F8 c.1910A>G, p.Asn637Ser mutation. *J Thromb Haemost*. 2013;11(12):2179-2181.

93. Miesbach W. The effect of aging on persons with von Willebrand disease. *The International Monitor: reviews of current key literature on von Willebrand Disease*. 2011(6):3-6.
94. Wieberdink RG, van Schie MC, Koudstaal PJ, et al. High von Willebrand factor levels increase the risk of stroke: the Rotterdam study. *Stroke*. 2010;41(10):2151-2156.
95. De Meyer SF, Stoll G, Wagner DD, Kleinschnitz C. von Willebrand factor: an emerging target in stroke therapy. *Stroke*. 2012;43(2):599-606.
96. van Loon JE, de Jaegere PP, Ulrichts H, et al. The in vitro effect of the new antithrombotic drug candidate ALX-0081 on blood samples of patients undergoing percutaneous coronary intervention. *Thromb Haemost*. 2011;106(1):165-171.
97. Ulrichts H, Silence K, Schoolmeester A, et al. Antithrombotic drug candidate ALX-0081 shows superior preclinical efficacy and safety compared with currently marketed antiplatelet drugs. *Blood*. 2011;118(3):757-765.



13

Summary

SUMMARY

Von Willebrand Disease (VWD) is the most common inherited bleeding disorder resulting in mucocutaneous bleeding, like epistaxis, oral cavity bleeding and menorrhagia. VWD is caused by reduced or dysfunctional von Willebrand Factor (VWF). VWF levels are highly variable between VWD patients and within a patient over time. Also the clinical expression of VWD is very heterogeneous with a large variability in bleeding frequency and severity. The overall aim of this thesis was to investigate the genotypic and phenotypic determinants of VWF levels and bleeding in patients with VWD.

Mutations in the *VWF* gene and the phenotype of von Willebrand Disease

In **chapter 2**, we studied mutations in the *VWF* gene of 199 patients with VWD and investigated the genotype-phenotype discrepancies in these patients. Seventy-five different mutations in the *VWF* gene were found. Mutations were identified in 63% (38/60) type 1 patients, 99% (110/111) type 2 patients and 89% (25/28) type 3 VWD patients. We observed discrepancies between phenotype and genotype in 10% of the VWD patients. We also reported 27 newly discovered *VWF* gene mutations. This study provides further insight into the molecular background of VWD and illustrates the difficulties of classifying the type of VWD correctly, as the association between phenotype and genotype is not always clear.

Variations in genes outside the *VWF* gene and the phenotype of von Willebrand Disease

Recently, new genetic loci have been identified that contribute to the variation in VWF levels in healthy individuals. These variations were located on different genes that have never been linked to VWF before, except for *ABO*: *STXBP5*, *SCARA5*, *ABO*, *STAB2*, *STX2*, *TC2N,* and *CLEC4M* genes. In **chapters 3 and 4** we investigated the relationship between these genetic variations, VWF levels, and bleeding phenotype in large cohorts of patients with types 1 and 2 VWD. Variation in the *STX2* gene was associated with VWF:Ag levels in 158 patients with type 1 VWD. Variation in the *STXBP5* gene was not associated with VWF levels, but with bleeding phenotype in females with type 1 VWD (**chapter 3**). In **chapter 4**, we studied the association between variations in abovementioned genes in 604 VWD patients included in the WiN study and VWF levels. We found an association between VWF levels and genetic variation in *STXBP5* and *CLEC4M* in type 1 VWD patients, but not in patients with type 2 VWD. No associations were found between bleeding symptoms and these genetic variations (**chapter 4**). Our findings may partly explain the variability in VWF levels and bleedings symptoms in VWD patients. These studies increase the understanding of the pathophysiology of VWD and provide further indication of the involvement of *STX2*, *STXBP5* and *CLEC4M* genes in determining VWF levels in VWD. In addition, these studies may contribute to the search for novel causes of VWD.

Pathophysiological mechanisms of von Willebrand Disease

Reduced plasma levels of VWF can be caused by several mechanisms, like impaired synthesis or secretion of VWF, and accelerated VWF clearance. In endothelial cells and megakaryocytes, VWF is formed as a pre-propeptide with a signal peptide, a propeptide and a mature subunit. After cleavage of the signal peptide and multimerization, the pro-VWF is stored in Weibel Palade bodies in the endothelium. The VWF propeptide (VWFpp) and the mature VWF (VWF antigen; VWF:Ag) completely dissociate after release from the endothelium into the circulation. VWF:Ag and VWFpp are secreted equimolarly, but are cleared independently and therefore have different half-lives: VWF:Ag 8-12 hours and VWFpp 2 hours. The ratio between VWFpp and VWF:Ag can therefore be used to assess synthesis, secretion and clearance of VWF. In addition, VWF is a carrier protein of Factor VIII (FVIII), so FVIII and VWF circulate as a complex and are cleared together. Therefore their half-lives are related and the ratio between FVIII and VWF:Ag can be used to assess VWF synthesis and clearance.

Using these ratios, we studied the pathophysiology of type 1, 2 and 3 VWD (**chapter 5**). Reduced VWF synthesis was often observed in type 1 VWD and type 2 VWD was mainly characterized by increased VWF clearance or a combination of increased clearance and reduced synthesis of VWF. Interestingly, in a significant proportion of VWD patients that classified as common international practice as type 3 based on VWF levels below 5 IU/dL, VWFpp was detectable. Compared with the patients with "true" type 3 VWD with complete absence of VWF:Ag and VWFpp, these type 3 patients with detectable VWFpp had a less severe bleeding phenotype and higher VWF and FVIII levels. The majority of these type 3 patients with detectable VWFpp had *VWF* gene mutations that were associated with rapid clearance of VWF. These findings indicate that these type 3 VWD patients with detectable VWFpp should actually reclassify as severe type 1 VWD patients. In this study we showed the clinical importance of the VWFpp assay in the diagnosis and classification of VWD.

Mediators of angiogenesis in von Willebrand Disease

VWF has recently been shown to negatively influence the angiogenesis, and that low VWF levels may result in more angiogenesis. Increased angiogenesis can contribute to the development of vascular malformations, like angiodysplasia, which may result in more gastro-intestinal bleeding, a common bleeding in elderly patients with VWD. The angiogenesis is mediated by several proteins, like angiopoietin-2, osteoprotegerin, galectin-3 and Vascular Endothelial Growth factor-A (VEGF). These proteins are stored in the Weibel-Palade bodies, together with VWF. Absent or reduced VWF leads to disturbed formation of Weibel Palade bodies and this may result in an increased secretion of angiogenic mediators. In **chapter 6**, we compared plasma levels of VEGF, angiopoietin-1 and 2, osteoprotegerin and galectin-3 between type 1, 2, 3 VWD patients and 100

healthy controls. VWD patients had higher angiopoietin-1 levels, higher VEGF levels and lower angiopoietin-2 levels than controls. VEGF levels were strongly dependent upon VWF levels in plasma. In VWD patients, the balance of angiogenic mediators in plasma is disturbed, which may result in vascular malformations, like angiodysplasia in these patients.

Bleeding symptoms in patients with von Willebrand Disease

VWD is a very heterogeneous bleeding disorder with a large inter-variability and intra-variability in bleeding phenotype. Clinical expression of VWD in children may be different than in adults, because younger aged patients generally lack exposure to hemostatic challenges and interventions. Therefore children may not yet have manifested bleeding symptoms. Because unaffected children may also suffer from mucocutaneous bleeding, like epistaxis and bruising, bleeding during childhood does not necessarily reflect the presence of a bleeding disorder. Pediatric bleeding scores including pediatric-specific bleeding symptoms (PBQ and ISTH-BAT) have recently been developed to discriminate between children with and without VWD. In **chapter 7**, we assessed the bleeding phenotype in a large cohort of children with moderate and severe VWD and evaluated the value of the ISTH-BAT. Half of the children suffered from pediatric specific bleeding symptoms. Children with type 3 VWD had a more severe bleeding phenotype, with earlier onset in childhood, more frequent joint bleeds and more often treatment with replacement therapy or blood transfusion than children with type 2 or 1 VWD. Important determinants of the ISTH-BAT were age, severity of VWD, type of VWD and index cases. This study shows that a bleeding score with supplementary pediatric-specific bleeding symptoms is of important value in diagnosing VWD.

In the next chapter, the clinical determinants of bleeding phenotype and pattern of bleeding symptoms, measured with the Tosetto bleeding score, were assessed in adult VWD patients (**chapter 8**). The most frequently reported bleeding symptoms were menorrhagia (85% of females who menstruate or have been menstruating), cutaneous bleeding (77%), prolonged bleeding from minor wounds (77%) and oral cavity bleeding (63%). Gastrointestinal bleeding mainly occurred in type 2 VWD patients (21%) and type 3 (27%) VWD patients. Central nervous system bleeding was reported by only 2% of patients. Joint bleeds were reported by 25% of all patients with moderate and severe VWD, and more often by patients with type 3 VWD (57%).

The bleeding score increased with age and females reported more bleeding symptoms than males. Type 3 VWD patients had a more severe bleeding phenotype than type 2 and type 1 patients. Within type 2 VWD, type 2B VWD patients had a more severe bleeding phenotype than type 2A, 2M and 2N VWD patients. Low VWF and FVIII:C levels (below 10 IU/dL) were also associated with a more severe bleeding phenotype. In type 3 VWD bleeding phenotype is strongly dependent upon FVIII:C levels.

Joint bleeds in patients with von Willebrand Disease

Previous studies have reported joint bleeds to be prevalent in 8-45% of patients with severe VWD. Joint bleeds can lead to joint damage and arthropathy. In **chapter 9** we analyzed the prevalence, onset and treatment of joint bleeds in VWD patients included in the WiN study. A quarter of VWD patients self-reported joint bleeds, which was mainly in patients with a more severe VWD phenotype. Joint bleeds in VWD resulted in more frequent joint damage and a lower quality of life. Two-thirds of VWD patients had their first joint bleed during childhood. Using medical files, we found that VWD patients with joint bleeds consumed more clotting factor concentrates, had more frequent joint damage on X-rays and more often had chronic joint pain than VWD patients with joint bleeds.

Von Willebrand Disease and its risk for arterial thrombosis

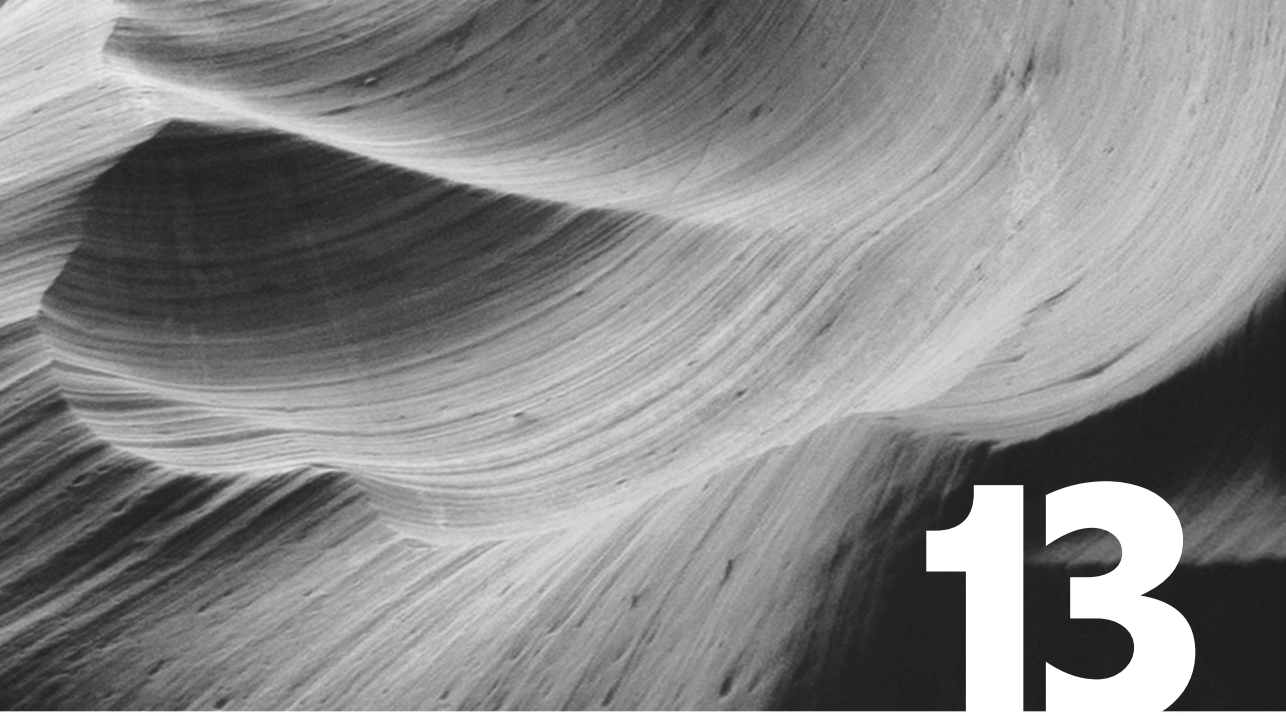
More and more studies report that VWF is a risk modifier for arterial thrombosis, such as acute myocardial infarction and ischemic stroke. These studies have shown that individuals with high VWF levels have an increased risk for arterial thrombosis. Conversely, it has been suggested that individuals with low VWF levels might have a reduced risk for arterial thrombosis; however this has never been analyzed in large studies. To further explore this, we studied for the first time the prevalence of arterial thrombosis in a large cohort of individuals with VWD from the WiN study (**chapter 10**). We found a lower prevalence of acute myocardial infarction, coronary heart disease and ischemic stroke in adult VWD patients compared with two age- and sex-matched reference population from the general Dutch population. Twenty-one out of 635 VWD patients suffered from arterial thrombosis. Compared with the general population, the prevalence of cardiovascular disease was 40% lower, the prevalence of acute myocardial infarction 60% lower and the prevalence of ischemic stroke 35-67% lower in patients with VWD. This is the first study that indicates that VWD patients may be protected against arterial thrombosis.

Aging and von Willebrand Disease

Aging may affect VWF parameters and bleeding phenotype in patients with VWD. From previous studies is known that VWF parameters increase with age in the general population. It is still unknown if this also occurs in patients with VWD. We therefore studied age-related changes in VWF and FVIII levels and differences in bleeding phenotype in elderly individuals with VWD (**chapter 11**). Per decade increase of age, VWF:Ag increase with 3.5 U/dL and FVIII:C with 7.1 U/dL in elderly type 1 VWD patients. The number of bleedings was similar in this group compared with younger patients with VWD. In contrast, VWF parameters did not change upon aging in type 2 VWD patients and elderly type 2 patients reported significantly more bleeding symptoms than younger VWD patients (16-65 years). In the general population, the elderly suffer more frequently from intesti-

nal bleeding. We found that also elderly patients with VWD more frequently suffer from gastro-intestinal bleedings and post-surgical bleeding than younger patients with VWD.

In conclusion, the studies described in this thesis illustrate the several genotypic and phenotypic determinants of VWF levels and bleeding in patients with VWD and thereby provide further insight into the pathophysiology of VWD.



13

Samenvatting

SAMENVATTING

De ziekte van von Willebrand (VWD) is de meest voorkomende erfelijke bloedingsziekte die zich uit in slijmvliesbloedingen, zoals epistaxis, orofaryngeale bloedingen en menorragie. VWD wordt veroorzaakt door een tekort aan of een afwijking in von Willebrand Factor (VWF). Het VWF gehalte varieert sterk tussen patiënten met VWD en ook binnen een patiënt varieert het VWF gehalte in de tijd. Het klinisch beeld van VWD is ook zeer heterogeen, waarbij de klinische presentatie sterk varieert in frequentie en ernst van bloedingen. Het algemene doel van dit proefschrift was het bestuderen van genotypische en fenotypische determinanten van het VWF gehalte en bloedingen in patiënten met VWD.

Mutaties in het *VWF* gen en het fenotype van de ziekte van von Willebrand

In **hoofdstuk 2**, hebben we de mutaties in het *VWF* gen bestudeerd in 199 patiënten met VWD en de discrepanties tussen genotype en fenotype in deze patiënten onderzocht. Er zijn 75 verschillende *VWF* gen mutaties gevonden. Mutaties werden gevonden in 63% (38/60) van de patiënten met type 1 VWD, 99% (110/111) van de patiënten met type 2 VWD en 89% (25/28) van de patiënten met type 3 VWD. Discrepanties tussen fenotype en genotype werden in 10% van de patiënten met VWD gezien. Daarnaast hebben we 27 nieuwe mutaties in het *VWF* gen gevonden die nog niet eerder gevonden waren. Dit onderzoek biedt nieuwe inzichten in de genetische achtergrond van VWD en laat zien dat het correcte classificeren van het type van VWD moeilijk kan zijn, omdat de relatie tussen fenotype en genotype niet altijd eenduidig is.

Variaties in genen buiten het *VWF* gen en het fenotype van de ziekte van von Willebrand

Een aantal jaren geleden zijn nieuwe genetische determinanten ontdekt die bijdragen aan de variatie in het VWF gehalte in gezonde individuen. Deze variaties liggen op verschillende genen die - met uitzondering van *ABO* - nog niet eerder met VWF in verband zijn gebracht: *STXBP5*, *SCARA5*, *STAB2*, *STX2*, *TC2N,* en *CLEC4M*. In de **hoofdstukken 3 en 4** analyseerden we de relatie tussen deze genetische variaties, de concentraties van VWF en de bloedingsneiging in grote groepen patiënten met type 1 en type 2 VWD. Variatie in het *STX2* gen was geassocieerd met de VWF concentratie in 158 patiënten met type 1 VWD. Variatie in het *STXBP5* gen was niet geassocieerd met VWF waarden, maar wel met de bloedingsneiging van vrouwen met type 1 VWD (**hoofdstuk 3**). In **hoofdstuk 4**, bestudeerden we de associatie tussen variaties in bovengenoemde genen in 604 VWD patiënten geïncludeerd in de WiN studie en VWF waarden. Wij vonden een associatie tussen de VWF concentratie en genetische variatie in *STXBP5* en *CLEC4M* in patiënten met type 1 VWD, maar niet in patiënten met type 2 VWD. In dit hoofdstuk konden we

geen verband tussen bloedingen en genetische variaties aantonen (**hoofdstuk 4**). Onze bevindingen zouden deels de variabiliteit in de VWF concentratie en de variatie in de bloedingsneiging van patiënten met VWD kunnen verklaren. Deze onderzoeken vergroten in ieder geval het inzicht in de pathofysiologie van VWD en geven verdere aanwijzingen over de betrokkenheid van de *STX2*, *STXBP5* en *CLEC4M* genen in het bepalen van de VWF concentratie bij VWD. Daarnaast zouden deze onderzoeken kunnen bijdragen aan de zoektocht naar nieuwe oorzaken van VWD.

Pathofysiologische mechanismen van de ziekte van von Willebrand

Er zijn verschillende mechanismen bekend die tot verlaagde VWF concentraties kunnen leiden, zoals vertraagde synthese of secretie van VWF en toegenomen klaring van VWF. VWF wordt gevormd als een pre-propeptide met een signaal peptide, een propeptide en een antigeen in het endotheel en megkaryocyten. Na verwijdering van het signaal peptide en multimerizatie, wordt het pro-VWF opgeslagen in Weibel Palade lichaampjes in het endotheel. Het VWF propeptide (VWFpp) en VWF komen los van elkaar nadat ze uit het endotheel in de circulatie zijn uitgescheiden. VWF:Ag en VWFpp worden equimolair uitgescheiden, maar worden onafhankelijk van elkaar geklaard en daarom hebben zij verschillende halfwaardetijden: VWF:Ag 8-12 uur en VWFpp 2 uur. De verhouding tussen VWFpp en VWF:Ag kan daarom gebruikt worden om de synthese, uitscheiding en klaring van VWF te beoordelen. Daarnaast is VWF een drager eiwit van Factor VIII (FVIII), dus FVIII en VWF circuleren als een complex en worden samen geklaard. Deze halfwaardetijden zijn dus aan elkaar gerelateerd en daarom kan de verhouding tussen FVIII en VWF:Ag gebruikt worden om de synthese en klaring van VWF te beoordelen.

Met behulp van deze ratio's, hebben we de pathofysiologie van type 1, 2 en 3 VWD bestudeerd (**hoofdstuk 5**). Type 1 VWD werd voornamelijk gekarakteriseerd door afgenomen synthese van VWF en type 2 VWD voornamelijk door toegenomen klaring van VWF of een combinatie van toegenomen klaring en afgenomen synthese van VWF. Een interessante observatie was dat VWFpp meetbaar was in een aanzienlijk deel van de patiënten die zoals internationaal gebruikelijk geclassificeerd waren als type 3 VWD op basis van een VWF concentratie beneden de 5 IU/dL. Vergeleken met patiënten met "echt" type 3 VWD bij wie de VWF en het VWFpp volledig afwezig zijn, hadden deze type 3 patiënten met meetbaar VWFpp een mildere bloedingsneiging en een hogere concentratie VWF en FVIII. Het overgrote deel van deze type 3 patiënten met meetbaar VWFpp hadden mutaties in het *VWF* gen die ervoor zorgen dat VWF versneld geklaard wordt. Uit deze bevindingen blijkt dat deze type 3 patiënten met meetbaar VWFpp eigenlijk opnieuw geclassificeerd zouden moeten worden tot patiënten met ernstige type 1 VWD. In dit onderzoek hebben we het klinisch belang van de VWFpp test in de diagnostiek en classificatie van VWD aangetoond.

Markers van angiogenese in de ziekte van von Willebrand

Recent is aangetoond dat VWF de angiogenese negatief beïnvloedt, en dat lage VWF waarden mogelijk leiden tot meer angiogenese. Toegenomen angiogenese kan bijdragen aan de ontwikkeling van vasculaire malformaties, zoals angiodysplasie, wat kan leiden tot meer gastro-intestinale bloedingen, een veelvoorkomende bloeding bij oudere patiënten met VWD. De angiogenese wordt gemedieerd door verschillende eiwitten, zoals angiopoietine-2, osteoprotegerine, galectine-3 en Vascular Endothelial Growth factor-A (VEGF). Deze eiwitten liggen opgeslagen in de Weibel Palade lichaampjes, samen met VWF. Verminderde of afwezig VWF leidt tot verstoorde vorming van de Weibel Palade lichaampjes en zou daarmee kunnen resulteren in een toegenomen uitstoot van angiogene markers. In **hoofdstuk 6**, hebben we het plasma gehalte van VEGF, angiopoietine-1 and 2, osteoprotegerine en galectine-3 van type 1, 2 and 3 VWD patiënten vergeleken met 100 gezonde controles. VWD patiënten hebben hogere angiopoietine-1 waarden, hogere VEGF waarden en lagere angiopoietine-2 waarden dan controles. Het VEGF gehalte was sterk afhankelijk van het VWF gehalte in plasma. Het evenwicht van angiogene mediatoren in plasma is verstoord in VWD patiënten en dat zou kunnen resulteren in vasculaire malformaties, zoals angiodysplasia in deze patiënten.

Bloedingssymptomen in patiënten met de ziekte van von Willebrand

VWD is een zeer heterogene bloedingsziekte met een grote inter- en intravariabiliteit in bloedingsfenotype. Klinische expressie van VWD kan verschillen tussen kinderen en volwassen, omdat jongere patiënten over algemeen minder bloot zijn gesteld aan bloedingsproblemen en interventies. Daarom hebben bloedingssymptomen zich in kinderen nog niet openbaart. Omdat niet-aangedane kinderen ook slijmvliesbloedingen, zoals neusbloedingen en blauwe plekken, kunnen krijgen, reflecteren bloedingen in de kindertijd niet altijd de aanwezigheid van een bloedingsziekte. Pediatrische bloedingsscores met daarin kind-specifieke bloedingssymptomen (PBQ en ISTH-BAT) zijn recent ontwikkeld om onderscheid te maken tussen kinderen met en zonder VWD. In **hoofdstuk 7**, onderzochten we het bloedingsfenotype in een grote cohort met kinderen met matig-ernstige en ernstige VWD en evalueerden we de waarden van de ISTH-BAT. De helft van de kinderen hebben kind-specifieke bloedingssymptomen gehad. Kinderen met type 3 VWD hadden een ernstiger bloedingsfenotype, met een vroeger ontstaan in de kindertijd, frequentere gewrichtsbloedingen en vakere behandelen met stollingsfactoren of bloedtransfusies dan kinderen met type 2 of 1 VWD. Belangrijke determinanten van de ISTH-BAT waren leeftijd, ernst van VWD, type of VWD en index cases. Deze studie toont dat een bloedingsscore met aanvullende kind-specifieke bloedingssymptomen van belangrijke waarde is bij de diagnostiek van VWD.

In het daaropvolgende hoofdstuk, evalueerden we in volwassen VWD patiënten de klinische determinanten van het bloedingsfenotype en het bloedingspatroon, gemeten

met de Tosetto bloedingsscore (**hoofdstuk 8**). De meest gerapporteerde bloedingssymptomen waren menorragie (85% van alle vrouwen die menstrueerden of gemenstrueerd hadden), hematomen (77%), langdurig bloeden na verwonding (77%) en orofaryngeale bloedingen (63%). Gastro-intestinale bloedingen kwamen voornamelijk voor bij patiënten met type 2 VWD (21%) en type 3 (27%) VWD. Bloedingen in het centrale zenuwstelsel kwamen bij slechts 2% van de patiënten voor. Gewrichtsbloedingen werden gerapporteerd door 25% van alle patiënten met matig-ernstige tot ernstige VWD, en vaker bij patiënten met type 3 VWD (57%).

De bloedingsscore nam toe met de leeftijd en vrouwen rapporteerden meer bloedingssymptomen dan mannen. Patiënten met type 3 VWD hadden een ernstiger bloedingsfenotype dan type 2 en 1 patiënten. Binnen type 2 VWD, hadden type 2B VWD patiënten een ernstiger bloedingsfenotype dan patiënten met type 2A, 2M en 2N VWD. Lage concentraties van VWF en FVIII (onder de 10 IU/dL) was geassocieerd met een ernstiger bloedingsfenotype. In type 3 VWD is het bloedingsfenotype sterk afhankelijk van het FVIII gehalte.

Gewrichtsbloedingen in patiënten met de ziekte van von Willebrand

Eerdere studies rapporteerden gewrichtsbloedingen in 8-45% van de patiënten met ernstige VWD. Gewrichtsbloedingen kunnen leiden tot gewrichtsschade en artropathie. In **hoofdstuk 9** analyseerden we het voorkomen, ontstaan en de behandeling van gewrichtsbloedingen in VWD patiënten geïncludeerd in de WiN studie. Een kwart van de VWD patiënten rapporteerde zelf gewrichtsbloedingen en dit was voornamelijk in patiënten met een ernstigere VWD fenotype. Gewrichtsbloedingen in VWD resulteerde vaker in gewrichtsschade en in een lagere kwaliteit van leven. Twee derde van de VWD patiënten had hun eerste gewrichtsbloeding in hun kindertijd. Met behulp van statusonderzoek, vonden we dat VWD patiënten met gewrichtsbloedingen meer stollingsfactoren gebruikten, vaker gewrichtsschade op röntgenfoto's hadden en vaker chronische gewrichtspijnen hadden dan VWD patiënten zonder doorgemaakte gewrichtsbloedingen.

Risico op arteriële trombose bij de ziekte van von Willebrand

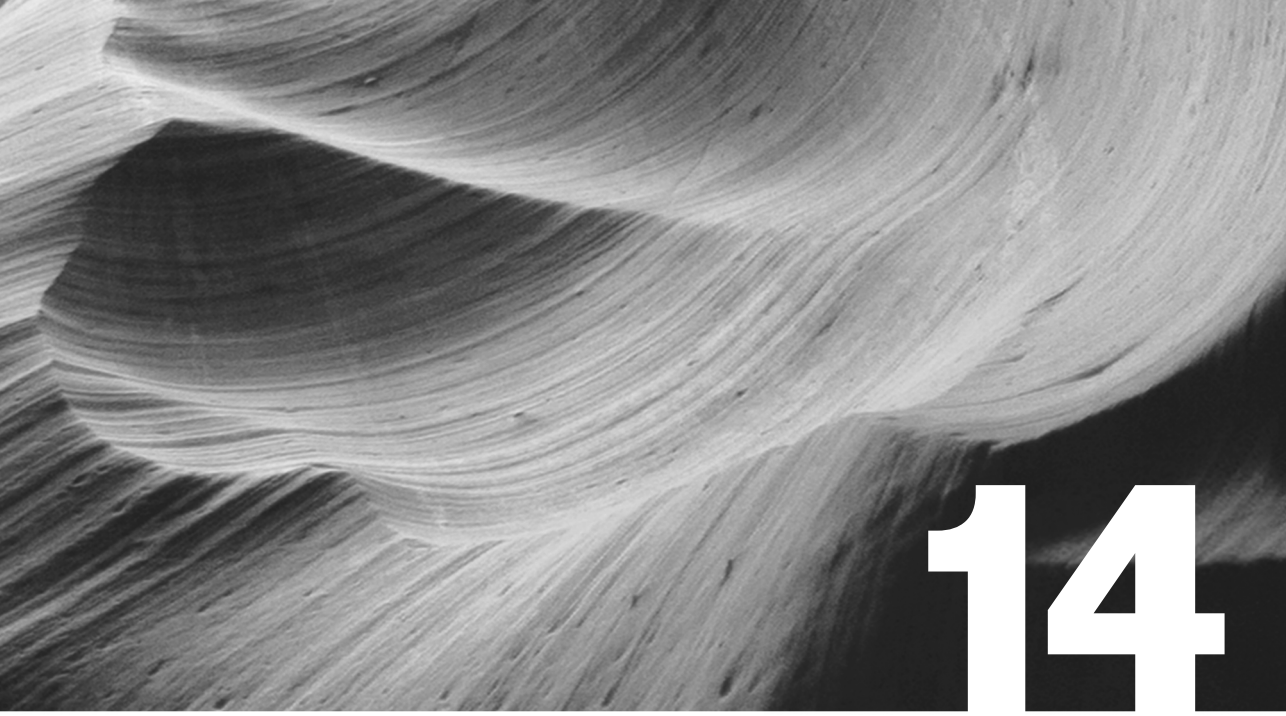
Steeds meer studies rapporteren dat VWF een risicofactor voor arteriële trombose is, zoals een hart- of herseninfarct. Deze onderzoeken hebben aangetoond dat individuen met een hoge concentratie VWF in het bloed een toegenomen kans hebben op arteriële trombose. Omgekeerd is gesuggereerd dat individuen met een lage concentratie VWF mogelijk beschermd zijn tegen arteriële trombose, echter dit is nooit eerder in grote studies onderzocht. Om dit nader te onderzoeken, hebben wij als eerste het vóórkomen van arteriële trombose in een groot cohort met individuen met de ziekte van von Willebrand uit de WiN studie bestudeerd (**hoofdstuk 10**). Wij vonden dat een acuut hartinfarct,

coronaire hartziekte en een herseninfarct minder vaak voorkwamen in volwassen VWD patiënten dan in de algemene Nederlands bevolking met dezelfde verdeling van leeftijd en geslacht. Eenentwintig van de 635 VWD patiënten hebben ooit arteriële trombose gehad. In vergelijking met de algemene bevolking, was de prevalentie van cardiovasculaire ziekte 40% lager, the prevalentie van een hartinfarct 60% lager en de prevalentie van een herseninfarct 35-67% lager in patiënten met VWD. Dit is de eerste studie waaruit blijkt dat VWD patiënten mogelijk beschermd zijn tegen arteriële trombose.

Ouder worden met de ziekte van von Willebrand

Ouder worden beïnvloedt mogelijk de VWF waarden en het bloedingsfenotype in patiënten met VWD. Uit voorgaande onderzoeken is bekend dat VWF waarden in het bloed met de leeftijd toenemen in de gezonde bevolking. Het is nog onbekend of dit ook in patiënten met VWD het geval is. Daarom bestudeerden we de leeftijdsgebonden veranderingen in VWF en FVIII waarden en de veranderingen in bloedingsfentoype in ouderen met VWD (**hoofdstuk 11**). Per 10 jaar leeftijdsstijging, nam het VWF:Ag met 3.5 U/dL toe en FVIII:C met 7.1 U/dL in oudere type 1 VWD patiënten. Het aantal bloedingen bleef gelijk in deze groep in vergelijking met de jongere patiënten met VWD. In patiënten met type 2 VWD daarentegen stegen de VWF waarden niet met het ouder worden en zagen we juist meer bloedingssymptomen bij oudere type 2 patiënten dan jongere VWD patiënten (16-65 jaar). In de algemene bevolking heeft de oudere generatie vaker last van bloedingen vanuit de darmen. Wij vonden dat ook oudere patiënten met VWD vaker last hebben van bloedingsproblemen vanuit het maag-darm kanaal. Tevens hebben zij vaker bloedingen na operaties dan de jongere patiënt met VWD.

De onderzoeken in dit proefschrift beschrijven de verschillende genotypische en fenotypische determinanten van het VWF gehalte en bloedingsneiging in patiënten met VWD. Deze onderzoeken hebben geleid tot vernieuwde inzichten in de pathofysiologie van VWD.



14

Appendices

List of publications
Authors and affiliations
List of WiN study group members and participating hospitals
List of abbreviations
Dankwoord
Curriculum vitae
PhD portfolio summary

LIST OF PUBLICATIONS

1. Van Loon JE, **Sanders YV**, de Wee EM, Kruip MJHA, de Maat MPM, Leebeek FWG. Effect of genetic variation in STXBP5 and STX2 on von Willebrand factor and bleeding phenotype in type 1 von Willebrand disease patients. *PloS One.* 2012;7(7):e40624.
2. De Wee EM, **Sanders YV**, Mauser-Bunschoten EP, van der Bom JG, Degenaar-Dujardin MEL, Eikenboom J, de Goede-Bolder A, Laros-van Gorkom BAP, Meijer K, Hamulyák K, Nijziel MR, Fijnvandraat K, Leebeek FWG, and the WiN study group. Determinants of bleeding phenotype in adult patients with moderate or severe von Willebrand disease. *Thrombosis and Haemostasis*. 2012;108(4):683-692.
3. Stoof SCM, **Sanders YV**, Petrij F, Cnossen MH, de Maat MPM, Leebeek FWG, Kruip MJHA. Response to desmopressin is strongly dependent on *F8* gene mutation type in mild and moderate Hemophilia A. *Thrombosis and Haemostasis*. 2013;109(3):440-449.
4. **Sanders YV**, Eikenboom J, de Wee EM, van der Bom JG, Cnossen MH, Degenaar-Dujardin MEL, Fijnvandraat K, Kamphuisen PW, Laros-van Gorkom BAP, Meijer K, Mauser-Bunschoten EP, Leebeek FWG, and the WiN study group. Reduced prevalence of arterial thrombosis in von Willebrand disease. *Journal of Thrombosis and Haemostasis.* 2013;11(5):845-854.
5. Stoof SCM, **Sanders YV**, Cnossen MH, de Maat MPM, Leebeek FWG, Kruip MJHA. Desmopressin response in Hemophilia A patients with FVIII:C <0.10 IU/ml. *Journal of Thrombosis and Haemostasis*. 2014;12(1):110-112.
6. **Sanders YV**, de Wee EM, Meijer K, Eikenboom J, van der Bom JG, Fijnvandraat K, Laros-van Gorkom BAP, Cnossen MH, Mauser-Bunschoten EP, Leebeek FWG. De ziekte van von Willebrand in Nederland: de WiN studie. *Nederlands Tijdschrift voor Geneeskunde*. 2014;158:A6518.
7. **Sanders YV**, Giezenaar MA, Laros-van Gorkom BAP, Meijer K, van der Bom JG, Cnossen MH, de Meris J, Nijziel MR, Ypma PF, Fijnvandraat K, Eikenboom J, Mauser-Bunschoten EP, Leebeek FWG, and the WiN study group. Von Willebrand disease and aging: an evolving phenotype. *Journal of Thrombosis and Haemostasis*. 2014;12(7):1066-1075.
8. **Sanders YV**, Groeneveld D, Meijer K, Fijnvandraat K, Cnossen MH, van der Bom JG, Middeldorp S, de Meris J, Laros-van Gorkom BAP, Mauser-Bunschoten EP, Leebeek FWG, Eikenboom J, and the WiN study group. Von Willebrand factor propeptide and the phenotypic classification of von Willebrand disease. *Blood.* 2015;125(19):3006-3013.
9. **Sanders YV**, van der Bom JG, Isaacs A, Cnossen MH, de Maat MPM, Laros-van Gorkom BAP, Fijnvandraat K, Meijer K, van Duijn CM, Mauser-Bunschoten EP, Eikenboom J, Leebeek FWG, and the WiN study group. *CLEC4M* and *STXBP5* gene variation

contribute to von Willebrand factor variation in von Willebrand disease. *Journal of Thrombosis and Haemostasis* 2015;13(6):956-966.

10. Van Galen K, **Sanders YV**, Vojinovic U, Eikenboom J, Cnossen MH, Zweegman S, Schutgens REG, van der Bom JG, Fijnvandraat K, Nijziel MR, Laros-van Gorkom B, Meijer K, Leebeek FWG, Mauser-Bunschoten EP, and the WiN study group. Joint bleeds in von Willebrand disease patients have significant impact on quality of life and joint integrity: a cross-sectional study. *Haemophilia*. 2015;21(3):e185-192.
11. Stoof SCM, van Steenbergen HW, Zwagemaker A, **Sanders YV**, Cannegieter SC, Duvekot JJ, Leebeek FWG, Peters M, Kruip MJHA, Eikenboom J. Primary postpartum haemorrhage in women with von Willebrand disease or carriership of hemophilia despite specialized care: a retrospective survey. *Haemophilia*. 2015;21(4):505-512.
12. Groeneveld D, **Sanders YV**, Adelmeijer J, Mauser-Bunschoten EP, van der Bom JG, Cnossen MH, Fijnvandraat K, Laros-van Gorkom BAP, Meijer K, de Meris J, Lisman T, Eikenboom J, Leebeek FWG, and the WiN study group. Dysregulated secretion of angiogenic mediators in patients with moderate and severe von Willebrand disease. *Manuscript submitted*.
13. **Sanders YV**, Fijnvandraat K, Boender J, Mauser-Bunschoten EP, van der Bom JG, de Meris J, Smiers FJ, Granzen B, Brons P, Tamminga R, Cnossen MH, Leebeek FWG, and the WiN study group. Bleeding spectrum in children with moderate or severe von Willebrand disease: relevance of pediatric-specific bleeding. *Manuscript submitted*.
14. **Sanders YV**, van der Heerde WL, Cnossen MH, Laros-van Gorkom BAP, Fijnvandraat K, Schoormans S, Dors N, van der Bom JG, Meijer K, Mauser-Bunschoten EP, Eikenboom J, Leebeek FWG, and the WiN study group. Genotyping of VWD patients in the Netherlands: phenotype-genotype discrepancies and 27 novel *VWF* gene mutations. *Manuscript submitted*.
15. Baaij M, Schutgens REG, Leung W, Laros-van Gorkom BAP, van Heerde WL, **Sanders YV**, Leebeek FWG, de Groot PhG, Urbanus RT, Roest M. Single-step whole blood micro assay for the rapid detection of von Willebrand disease. *Manuscript submitted*.
16. Eising HP, **Sanders YV**, Heijboer L, van der Bom JG, Cnossen MH, Eikenboom J, Fijnvandraat K, Laros-van Gorkom BAP, de Meris J, Mauser-Bunschoten EP, Leebeek FWG, Meijer K, and the WiN study group. Surgical intervention for heavy menstrual bleeding changes quality of life in women with Von Willebrand disease: a qualitative study. *Manuscript in preparation*.

AUTHORS AND AFFILIATIONS

Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
Emma Children's hospital, Department of Pediatric Hematology: Karin Fijnvandraat
Department of Vascular Medicine: Michiel Coppens

Catharina Hospital Eindhoven, Eindhoven, the Netherlands
Department of Pediatrics: Natasja Dors

Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands
Department of Hematology: Johan Boender, Marleen A. Giezenaar, Marieke J.H.A. Kruip, Frank W.G. Leebeek, Janine E. van Loon, Moniek P.M. de Maat, Yvonne V. Sanders, Una Vojinovic, Eva M. de Wee
Sophia Children's Hospital, Department of Pediatric Hematology and Oncology: Marjon H. Cnossen, Arja de Goede-Bolder
Genetic Epidemiology Unit, Department of Epidemiology: Cornelia M. van Duijn, Aaron J. Isaacs

HagaZiekenhuis, The Hague, the Netherlands
Department of Hematology: Paula F. Ypma

Leiden University Medical Center, Leiden, The Netherlands
Department of Clinical Epidemiology: Johanna G. van der Bom
Department of Pediatric Hematology: Frans J. Smiers
Department of Thrombosis and Haemostasis: Dafna Groeneveld, Jeroen Eikenboom

Maxima Medical Center, Eindhoven, The Netherlands
Department of Internal Medicine: Marten R. Nijziel

Netherlands Hemophilia Society, The Netherlands
Manon E.L. Degenaar-Dujardin, Joke de Meris

Radboud university medical center, Nijmegen, The Netherlands
Department of Hematology: Britta A.P. Laros-van Gorkom
Department of Laboratory Medicine, Laboratory of Hematology: Waander L. van Heerde, Selene Schoormans
Department of Pediatric Hematology: Paul Brons

University Hospital Maastricht, Maastricht, The Netherlands
Department of Hematology: Karly Hamulyák
Department of Pediatric Oncology and Hematology: Bernd Granzen

University Medical Center Utrecht, Utrecht, The Netherlands;
Van Creveldkliniek/Department of Hematology: Karin P.M. van Galen, Eveline P. Mauser-Bunschoten, Roger E.G. Schutgens

University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
Department of Hematology: Karina Meijer
Department of Pediatric Hematology: Rienk Y.J. Tamminga
Department of Surgery: Jelle Adelmeijer, Ton Lisman
Department of Vascular Medicine: Pieter W. Kamphuisen

LIST OF WIN STUDY GROUP MEMBERS AND PARTICIPATING HOSPITALS

Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
K Fijnvandraat, M Coppens

VU University Medical Center, Amsterdam, The Netherlands
A Kors, S Zweegman

Netherlands Hemophilia Society, The Netherlands
J de Meris

Amphia Hospital, Breda, The Netherlands

Catharina Hospital, Eindhoven, The Netherlands
N Dors

Maxima Medical Center, Eindhoven, The Netherlands
MR Nijziel

University Medical Center Groningen, Groningen, The Netherlands
K Meijer, RYJ Tamminga

Kennemer Gasthuis, Haarlem, The Netherlands

HagaZiekenhuis, The Hague, The Netherlands
PF Ypma

Leiden University Medical Center, Leiden, The Netherlands
JG van der Bom, HCJ Eikenboom, FJW Smiers

Maastricht University Medical Center, The Netherlands
B Granzen, K Hamulyák

Radboud university medical center, Nijmegen, The Netherlands
P Brons, BAP Laros-van Gorkom

Erasmus University Medical Center, Rotterdam, The Netherlands
MH Cnossen, FWG Leebeek (principal investigator), YV Sanders

Van Creveld, University Medical Center, Utrecht, The Netherlands
EP Mauser-Bunschoten (chairman steering committee)

LIST OF ABBREVIATIONS

ABO	ABO blood group
ADAMTS13	a disintegrin and metalloproteinase with a thrombospondin motif, member 13
AMI	acute myocardial infarction
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
aPTT	activated partial thromboplastin time
ASA	aspirin
β	Beta-coefficients
BMI	body mass index
BS	Bleeding Score
BOECs	Blood Outgrowth Endothelial Cells
CABG	coronary bypass grafting
CAG	coronary angiography
CBS	Statistics Netherlands/Central Bureau of Statistics
CEA	carotid endarterectomy
CFC	clotting factor concentrate
CHARGE	Cohorts for Heart and Aging Research in Genomic Epidemiology
CHD	coronary heart disease
CI	95% confidence interval
CLEC4M	C-type lectin domain family 4, member M
D	diabetes mellitus
EDTA	ethylene diaminetetraacetic acid
f	female
FAM	positive family history
FVIII	Factor VIII
FVIII:C	Factor VIII clotting activity
Gal-3	galectin-3
HC	hypercholesterolemia
HR-QoL	health-related quality of life
HT	hypertension
ht-SNPs	haplotype-tagging single-nucleotide polymorphisms
IQR	25-75% interquartile range
IS	ischemic stroke
ISTH	International Society on Thrombosis and Haemostasis
ISTH-BAT	ISTH bleeding assessment tool
JB	joint bleeds

LD	linkage disequilibrium
LMWH	low-molecular-weight heparin
m	male
MAF	minor allele frequency
MCS	SF-36 mental component summary
NPHC	National Public Health Compass
O	obesity
OPG	osteoprotegerin
PAD	peripheral arterial disease
PCI	percutaneous coronary intervention
PCS	SF-36 physical component summary
PFA	platelet function analyzer
PT	Prothrombin Time
RANKL	receptor activator of nuclear factor-kappaB ligand
RIPA	ristocetin-induced platelet aggregation
S	smoking
SCARA5	scavenger receptor class A, member 5
SMR	standardized morbidity ratio
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SNPs	single-nucleotide polymorphisms
STAB2	stabilin-2
STX2	syntaxin-2
STXBP5	syntaxin-binding protein 5
TC2N	tandem C2 domains, nuclear
TIA	transient ischemic attack
TIE-2	Tyrosine kinase with Immunoglobulin-like and EGF-like domains-
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
UAP	unstable angina pectoris
UFH	unfractionated heparin
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VKA	vitamin K antagonist
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:Act	von Willebrand factor activity
VWF:Ag	von Willebrand factor antigen
VWF:CB	von Willebrand factor collagen binding
VWF:FVIIIB	VWF finding to FVIII
VWFpp	von Willebrand factor propeptide

VWF:RCo	von Willebrand factor ristocetin cofactor activity
WiN	Willebrand in the Netherlands
WPB	Weibel-Palade bodies

DANKWOORD

Dankzij mijn onderzoek op de afdeling hematologie in het Erasmus MC en het nationale karakter van de WiN studie heb ik met veel bevlogen internisten, kinderartsen en biochemici uit heel Nederland mogen samenwerken en veel van hen mogen leren! Bedankt voor de goede momenten!

In dit dankwoord wil ik de volgende personen in het bijzonder bedanken.

Mijn promotor, beste prof.dr. Leebeek, beste **Frank**, vanaf het begin heb je mij weten te stimuleren. Mijn enthousiasme noemde je een groot pluspunt, maar daarin moest ik wel af en toe iets afgeremd worden. Ik heb veel geleerd van onze discussies en van je suggesties op alle versies van de artikelen uit dit proefschrift. In de 3 jaar dat ik onder jou werkzaam was, heb ik je leren kennen als een serieuze, gedreven, vrolijke, energieke baas. Ik ben blij met het vertrouwen dat je mij hebt gegeven en de kansen om mooie manuscripten te kunnen schrijven en op grote congressen te kunnen presenteren. De WiN studie is een prachtig project waar je trots op kan zijn!

Beste prof.dr. Eikenboom, beste **Jeroen**, wat was het fijn om zo'n belangrijke Willebrand-kenner dichtbij te hebben. De trein naar Leiden was zo gepakt en ook kon ik vragen gemakkelijk per e-mail stellen (met altijd een snelle respons). Ik ben dankbaar dat ik van jouw Willebrand-kennis gebruik heb mogen maken en dat het VWFpp-artikel een prachtige publicatie is geworden! Beste dr. Cnossen, lieve **Marjon**, jouw energieke instelling en creativiteit hadden altijd een positieve werking op mij. Je hebt met de Opticlot een mooie onderzoekslijn te pakken. Beste dr. Kruip, beste **Marieke**, ik was aangenomen om bij jou een project te leiden. Helaas, is de DuLac nooit van de grond gekomen. Ik ben erg blij met het vertrouwen dat je me toen gegeven hebt. Beste dr. De Maat, beste **Moniek**, je hebt mij veel bijgebracht over SNPs en genetische epidemiologie. Je kritische houding bij onze werkbesprekingen zijn ten goede gekomen aan de onderzoeken uit dit proefschrift. Verder de WiN stuurgroep – dr. **Karina** Meijer, dr. **Evelien** Mauser-Bunschoten, dr. **Karin** Fijnvandraat, dr. **Britta** Laros-van Gorkom, prof. dr. **Anske** van der Bom, **Joke** de Meris en **Lenka** Kerstens - hartelijk dank voor jullie kritische blik en jullie inzet om elk manuscript naar een hoger plan te tillen. Ik heb met groot plezier met jullie samengewerkt. Door jullie enthousiasme en kennis is de WiN studie een succes geworden!

De commissieleden: beste prof.dr. **P. Sonneveld**, prof.dr. **J.C.L.M. van Saase**, prof.dr. **S. Middeldorp**, prof.dr. **J.J. Voorberg**, prof.dr. **E.J.G. Sijbrands**, prof.dr. **H.C.J. Eikenboom** en dr. **M.P.M de Maat**, hartelijk dank voor plaatsnemen in de kleine of grote commissie.

De mensen die zijdelings bij verschillende projecten en artikelen hebben geholpen: **David Alexander**, through your enthusiastic support the English writing wasn't as difficult as it seemed. Thanks a lot, you've been a great help! **Aaron Isaacs**, thank you for your help with the genetic analyses for the SNP article. **Selene Schoormans**, de VWF-genmutaties zijn een passie voor je, dat merkte ik in ons vele e-mail contact over VWF-genmutaties van Willebrand-patiënten en de notaties hiervan. Ook **Waander van Heerde** dank voor jouw hulp hierbij en succes met jullie VWF-genetica lab.

Alle **hemofilieverpleegkundigen** en **hemofiliebehandelaren** in Nederland: de omvang van de WiN studie is vooral aan jullie inzet en enthousiasme te danken. Met plezier heb ik onderwijs gegeven aan de hemofilieverpleegkundigen via de NVHV. En natuurlijk was het leuk om een gezellig praatje te maken als ik jullie in de hemofiliebehandelcentra of op een congres tegen kwam. Lieve **Greta** en **Floor**, de Rotterdamse hemofilieverpleegkundigen, ik bewonder jullie tomeloze inzet voor en vrolijke benadering van alle hemofilie en von Willebrand-patiënten. Door jullie voelt het Rotterdamse HBC als een hechte familie!

De vrijwilligers van de patiëntenvereniging **NVHP**: ik vind het telkens weer bijzonder om te zien hoe jullie vol passie de kennis over bloedingsziekten bij alle patiënten in Nederland - en zelfs wereldwijd - willen vergroten, en dat met veel vrolijkheid en gezelligheid. Mijn complimenten!

Alle **Willebrand-patiënten** die ik heb mogen spreken (veelal telefonisch) en ontmoeten: Jullie verhalen inspireerden me en lieten me nog harder werken. Zonder jullie hulp had ik geen proefschrift. Ik hoop dat de zorg en kennis rondom de ziekte van von Willebrand toe zal nemen. Ik heb in ieder geval een bijdrage geleverd!

M'n mede-PhD-collega's van andere universitaire centra: Lieve **Karin**, je bent een fijne collega! Die Brusselse nep-bonbons van de Belgische Aziaat waren best lekker toch ;-)? Succes met de WAS en als hematoloog in Utrecht. En veel geluk met je gezin! Beste **Dafna**, onze verschillende kennis van VWF en VWD was een goede aanvulling bij onze artikelen VWFpp en angiogenese markers. Het was gezellig als je weer eens in het Rotterdamse was voor plasma of SPSS-hulp. Veel succes met je wetenschappelijke carrière. Beste **Heleen**, je bent een gedreven gynaecologe. Het was leuk om samen de focusgroepen te doen. Elke keer hoorden we weer indrukwekkende verhalen van deze vrouwen met Will(i)ebrand. Succes met je drukke baan. Beste **Marije**, jullie VWF-RCo assay wordt hopelijk een succes!

Dan mijn naaste collega's van het Erasmus MC. We hebben veel gekletst (heel veel…), veel gelachen, maar ook goede gesprekken, discussies en dilemma's gekend. Ik kijk

terug op een gezellige tijd! Lieve **Michelle**, ik heb jou het laatste jaar pas goed leren kennen. Wij hebben vaak hard gelachen samen (je lach werkt ook zo aanstekelijk ;-)) en veel gezellige koffiemomenten gekend. Jouw steun en vriendschap heb ik de afgelopen tijd erg gewaardeerd. Ik kijk uit naar jouw boekje (met een gouden randje?). Lieve **Carolien**, wij waren samen de gevoeligste mensen en hadden vaak maar een halve blik nodig. In Brussel hadden we een fantastische tijd en ook jouw eerste keer Amsterdam zal ik nooit vergeten. Ik heb bewondering voor je arbeidsethos. Stuur je me te zijner tijd de uitnodig van je oratie? Lieve **Carina**, met jou samen heb ik prachtige hoogtepunten beleefd (Trevi fontein, Champs-Elysées, Great Ocean Road, truffelrestaurant, survivallen en natuurlijk aangehouden worden voor speeding). Verder hebben we samen de eerste mijlpalen bereikt (eerste presentatie buiten het EMC, eerste poster, eerste gepubliceerde paper en eerste presentatie op groot internationaal congres). Het was fijn en vertrouwd om dit samen met jou te beleven. Heel veel succes nog even met de afronding van jouw proefschrift. Lieve **Janske**: Je was een hartelijke en spontane collega. Ik heb je vaak zien worstelen, maar gelukkig was er daarna weer die vrolijke lach. Ik hoop dat ik over een tijdje jouw boekje in handen krijg. Succes met die zware laatste loodjes en veel geluk met Rob, Jesse, Luuk en Nora. Lieve **Eva**, aan jou heb ik heel veel te danken. Ik kwam in een gespreid bedje terecht. Jouw energie en enthousiasme heb ik lang in de WiN studie gevoeld. Het is niet voor niets dat ik nog steeds de 2e Eva wordt genoemd. Een mooi compliment! Ook buiten werktijd zie ik je graag, je bent een fijn persoon! **Johan**, met je goedlachse en positieve instelling ben je een goede WiN-opvolger. Heel veel succes. **Janine**, bedankt voor het op weg helpen bij de Taqman- en SNP-analyses en je gezelligheid bij congressen, cursussen en etentjes. **Marc**, in Japan al de eerste dag samen bij de McDonald's belandden, betekende een goed begin van onze band. Het was fijn om elke maandag jouw nuchterheid in ons midden te hebben. Ook met alle andere stollingsvrienden en collega's (**Shiraaz**, **Jossi**, **Joyce**, **Shirley**, **Dick**, **Simone**, **Marianne**, **Jasper**, **Reinilde**, **Sjef**, **Hans**) heb ik gezellige en waardevolle tijden gekend. Ik wens jullie allemaal veel goeds, geluk en succes voor de toekomst. Mijn studenten **Marleen** en **Una** wil ik graag bedanken voor hun serieuze inzet en harde werken. Jullie hebben dat auteurschap meer dan verdiend! Ook **Robbie** en **Hassan** veel dank voor de Normandy-bepalingen en de verdeling van het DNA.

En dan alle collega's van de afdeling hematologie van het Erasmus MC (**secretariaat**, **researchverpleegkundigen**, **datamanagers**, **stollingslab**, **morfologielab** en **medische staf**). Er was vaak tijd voor een kletspraatje, lol en gezelligheid. Ik zal jullie missen!

Sinds september 2014 maak ik onderdeel uit van de art-assistenten Interne Geneeskunde van het Maasstad ziekenhuis. Speciale dank gaat uit naar dr. **Rene van den Dorpel**, opleider Interne Geneeskunde, en dr. **Joke van der Linden**, plaatsvervangend opleider,

voor hun steun, begrip en vertrouwen in mijn eerste Maasstad-tijd. Beste **Maasstad-collega's** dank voor de gezellig en leerzame momenten, ik hoop dat er nog vele zullen volgen!

Zonder muziek, geen leven. Beste **Philerianen** dank voor de vele mooie uurtjes getoeter en gezelligheid!

Lieve vriendinnetjes uit Winschoten en Rotterdam, lieve **Esther**, **Anneloes**, **Marlies**, **Betttina**, **Annet**, **Linda**, **Marjan**, **Adelina**, **Linda**: ik ben blij dat ik altijd mezelf bij jullie kan zijn. Jullie gezelligheid en vrolijkheid en doen mij veel goeds. Jullie vriendschap is fijn! Lieve **Esther**, je hebt zware tijden gekend, maar ik ben blij af en toe je lach weer te kunnen zien. Je betekent veel voor me en daarom mag jij deze grote dag niet aan mijn zijde ontbreken. Lieve **Linda**, fijn dat jij mijn paranimf wilt zijn. Dank voor alle goede gesprekken en hilarische momenten. Dank voor je fijne vriendschap.

Lieve **Wopke & Saskia**, ik waardeer jullie enorm en heb veel bewondering voor jullie. **Saskia**, jouw zelfstandigheid, kracht en eigenwaarde ervaar ik als een groot voorbeeld. Lieve **Opa (& Oma) Beverloo**, **Wiebe & Malena & Gustav & William**, **Sjoerd & Amilly**, **Wytse**, **Ulbe**, **Victor & Sandrine & Arthur & Lucas**, in het begin vond ik het moeilijk om me staande te houden tussen al die mannen met een sterke eigen mening. Nu zijn jullie voor mij één leuke grote familie: mijn tweede thuis.

Lieve **Henk & Tanja**, **Gert-Jan & Anouk**, ik kijk altijd uit naar ons weerzien. We hebben fijne gesprekken en jullie positieve woorden geven mij altijd veel kracht.

Lieve **Opa & Oma**, lieve surrogaat ouders, ik ben trots dat ik jullie kleinkind mag zijn. Jullie manier van lief hebben en liefde geven is een groot voorbeeld voor mij.

Lieve **Peter & Natascha & Ruben & Elisah**, onze levens zijn enorm verschillend, maar ik ben blij jullie (schoon)zus en tante te zijn. Ik ben er trots op dat jullie alles op eigen kracht zo goed voor elkaar hebben gekregen. Jullie liefde en gezelligheid doen mij veel goeds!

Lieve **Papa & Mama**: jullie hebben me altijd gestimuleerd en me de vrijheid gegeven om me te ontwikkelen tot wie ik ben. De afstand is soms groot, maar jullie onvoorwaardelijke liefde niet minder. Door jullie ben ik grotendeels wie ik ben en daar ben ik erg blij mee.

Als laatste mijn lieve **Bouke**, mijn rots in de branding. Bij jou voel ik me veilig. Je bent er altijd met veel liefde, geduld en vertrouwen, en soms de nodige kritische reflectie. Samen bereiken we grote hoogtes. Snel weer een topje beklimmen?

CURRICULUM VITAE

Yvonne Veroni Sanders werd op 17 maart 1986 in Winschoten geboren. Na het behalen van het gymnasium aan het Dollard College te Winschoten, studeerde zij vanaf september 2004 geneeskunde aan de Erasmus Universiteit te Rotterdam. In 2008 heeft zij vijf maanden wetenschappelijk onderzoek verricht aan de University of Iceland in Reykjavik, onder leiding van prof.dr. J.E. Eyfjörð en dr. P.M.J.J. Berns op het gebied van epigenetische silencing van het *BRCA1* gen in mammacarcinoom. Zij behaalde in 2009 haar doctoraal examen en in 2011 haar artsexamen (cum laude). In juni 2011 startte zij haar promotieonderzoek op de afdeling hematologie, onder supervisie van prof.dr. F.W.G. Leebeek. Het PhD project was deel van het Willebrand in Nederland (WiN) onderzoek en richtte zich op de variabiliteit in genotype en fenotype in patiënten met de ziekte van von Willebrand. Gedurende haar promotietraject kreeg zij in 2012 de NVTH Scientific Excellence Award van de Nederlandse Vereniging voor Trombose en Hemostase, in 2013 de ISTH Young Investigator Award van de International Society on Thrombosis and Haemostasis en in 2014 de WFH Young Researcher Award van de World Federation of Hemophilia. Sinds 1 september 2014 is zij werkzaam als artsassistent Interne Geneeskunde in het Maasstadziekenhuis.

PhD PORTFOLIO SUMMARY

Name of PhD student: Y.V. Sanders
Erasmus MC Department: Hematology
Research School: COEUR

PhD period: June 2011 – November 2015
Promotor: Prof. dr. F.W.G. Leebeek

1. PhD training	Year	Workload Hours/ECTS
General academic skills		
Biomedical English Writing course	2013	4.0
Research skills		
Introduction to clinical research (NIHES)	2012	0.9
Biostatistics for clinicians (NIHES)	2012	1.0
Regression analysis for clinicians (NIHES)	2012	1.9
SNP's and human diseases (MolMed)	2011	1.6
In-depth courses (e.g. Research school, Medical Training)		
1x COEUR course on cardiovascular medicine	2011	1.5
3x MolMed courses on SNPs and genetics	2011-2012	2.1
3x NVTH courses on thrombosis and hemostasis	2011-2013	3.0
Von Willebrand Disease – International case-oriented course	2011	1.5
Oral presentations		
5x oral presentations NVTH 2012-2014, ISTH 2013, WFH 2014	2012-2014	0.9
6x poster presentations EAHAD 2012, WFH 2012, ISTH 2013, EAHAD 2014	2012-2014	1.0
11x other oral presentations	2011-2014	2.4
National and international conferences or symposia		
2x ISTH, 2x WFH, 2x EAHAD	2011-2014	8.4
4x NVTH, 2x Dutch Hematology Congress	2011-2014	3.0
5x other symposia or conferences	2011-2014	1.8
Seminars and workshops		
4x COEUR research seminar	2011-2013	1.6
2x COEUR PhD day	2012, 2013	0.8
1x COEUR lecture	2012	0.1

2. Teaching activities	Year	Workload Hours/ECTS
Lecturing		
3x lecture "von Willebrand disease" to medical students (keuzeonderwijs 2e jaar)	2011-2012	0.2
Several times lecture "hemostasis and thrombosis" to nurses	2012-2014	1.1
Supervising Master thesis medical student (keuzeonderzoek 4e jaar)	2012	1.5
Supervising medical student continuously	2013-2014	1.0
Hematology PhD training		
AiO postdoc	2011-2014	1.5
Work discussions and literature discussions	2011-2014	7.0
Extra		
COEUR PhD committee and organizing COEUR PhD day	2011-2013	2.0
Total		**51.8**