The Influence of Genetic Variation on Von Willebrand Factor Levels and the Risk of Stroke

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The Influence of Genetic Variation on Von Willebrand Factor Levels and the Risk of Stroke

De invloed van genetische variatie op von Willebrand factor levels en het risico op een herseninfarct

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Eenheid in het nodige,
vrijheid in het onzekere,
in alles de liefde

Contents

CHAPTER 1

GENERAL INTRODUCTION AND AIM OF THE THESIS

Cardiovascular disease (CVD), primarily due to arterial thrombus formation resulting in vascular occlusion, is one of the most important causes of morbidity and mortality in the world [1]. In the Netherlands, every day on average 51 males and 57 females die as a result of CVD. Every year, CVD is responsible for nearly one third of total deaths [2]. The pathogenesis of CVD is highly complex. Both genetic factors and environmental factors contribute, via complex interactions, to the pathogenesis of atherosclerosis, plaque rupture and subsequent thrombus formation and vascular occlusion [3,4].

In the past, several risk factors for cardiovascular disease, such as smoking, hypercholesterolemia and hypertension, have been identified. As coagulation factors, including von Willebrand factor (VWF), are of major importance in the regulation of thrombus formation, their role in CVD has also been investigated [5].

VWF has a key role in primary haemostasis, given that it facilitates platelet adhesion and aggregation at sites of vascular injury [6]. VWF is also a marker of endothelial cell activation, since it is synthesized by the endothelium and released upon endothelial cell activation [7,8]. Many studies have shown that high VWF levels are positively associated with the risk of coronary heart disease (CHD) [5,9,10]. The association between VWF levels and the risk of ischemic stroke has been investigated less frequently. However, also for stroke an association is suggested [11-13]. Whether VWF has a direct pathogenic role in the development of CVD or is mainly a marker of endothelial cell activation is still subject of investigation.

VWF plasma levels are influenced by environmental factors, such as stress and inflammation, but are also strongly heritable with estimates of heritability ranging from 53-75% [14,15]. The risk of CVD is strongly heritable as well. Genetic variants that determine VWF levels may partly explain the genetic susceptibility for CVD [16-18]. Identifying genetic variants associated with VWF levels, and with the risk of CVD will help to elucidate whether VWF is a direct or indirect risk factor for CVD.

Genetic and environmental factors also influence the biological variation of haemostatic factors such as VWF and factor VIII (FVIII). Variation over time in an individual is called the within-subject biological variation which is influenced by biological factors such as age, stress and inflammation [19]. Based on the obtained biological variation the number of repeated measurements needed to estimate an individual's homeostatic mean can be determined. This is important in epidemiological studies, since we are interested in the differences between homeostatic means of study participants and not in their short-term fluctuations. Biological variation has clinical implications as well. As low VWF and FVIII plasma levels and activity are important diagnostic criteria for von Willebrand disease (VWD), the estimation of an individual's true homeostatic mean is of vital importance for diagnostic precision. To date, knowledge on biological variation of VWF and FVIII is limited. The aim of this thesis is to unravel the association between genetic variation, VWF levels and CVD risk with emphasis on variation in the VWF gene, VWF:Ag levels and stroke. In addition,

the biological variation of VWF and FVIII is determined, based on which recommendations are provided that improve the assessment of VWF and FVIII as risk indicators for thrombotic disease and increase the diagnostic precision for VWD.

OUTLINE OF THE THESIS

In **Chapter 2** studies on the association between VWF levels and the risk of CHD and stroke are reviewed. The influence of non-genetic determinants on VWF plasma levels are being discussed. The main focus of the review however, is on the contribution of genetic variants to VWF levels, VWF activity and to the risk of CVD. An important question in the field is whether VWF is a direct causal factor in de development of CVD or mainly a marker of endothelial dysfunction. Since genetic association studies can be used as a tool to identify a causal role for VWF in the development of CVD, we will discuss whether the conducted genetic studies thus far help unravel the nature of the association between VWF and CVD. Finally, recommendations for future research will be provided.

Many studies have investigated the role of VWF levels in CHD, but only few investigated its role in stroke. In **Chapter 3** we study the association between VWF levels and the risk of stroke in The Rotterdam Study, a large population based cohort study amongst subjects aged 55 years and older [20].

A part of VWF in the circulation is it its active GPIbα-binding conformation. Only active VWF molecules are able to interact with GPIbα receptors on platelets [21]. Previous studies demonstrated that the level of circulating active VWF is increased during pathological conditions that increase the risk of thrombotic micro-angiopathies (TMA), such as thrombotic thrombocytopenic purpura (TTP) [22]. In **Chapter 4** we further explore the association between VWF and stroke by investigating the relationship between active VWF and the risk of ischemic stroke. The active GPIbα-binding conformation of VWF is measured in a case-control study in which patients with a first-ever ischemic stroke or transient attack (TIA) are included. The controls are a random set of the total population and are matched by age and sex .

VWF propeptide (VWFpp) has a critical role in VWF multimer assembly, processing and storage [23,24]. Since VWFpp and mature VWF are secreted equimolarly, VWFpp plasma levels reflect the rate of VWF secretion in steady state conditions [25]. Consequently, the association between VWF secretion and ischemic stroke can be investigated. In **Chapter 5**, we investigate the association between VWFpp and the occurrence of ischemic stroke in two independent case-control studies.

Information on the contribution of common VWF gene variation to VWF levels and CVD risk is limited. Therefore, in **Chapter 6**, the association between common variation spanning the

total VWF gene, VWF levels, VWF activity and the risk of CVD is investigated. Twenty-seven haplotype-tagging single nucleotide polymorphisms (ht-SNPs) representing common variation in the VWF gene are selected and subsequently genotyped in a case-control study including young patients with a first event of CHD or ischemic stroke and healthy controls. Specifically young subjects are included since previous research showed that the genetic contribution to the pathogenesis of arterial thrombotic disease is stronger at young age [17,26].

A meta-analysis of five large genome wide association studies indentified SNPs within eight genetic loci as determinants of VWF levels [27]. In **Chapter 7** we investigate the association between the top SNP within each of the eight loci and the risk of stroke in the Rotterdam Study. We additionally construct a genetic score that includes these eight top SNPs to investigate the total contribution to VWF plasma levels and their association with the risk of stroke.

In **Chapter 8** we determine the biological variation of VWF levels and activity and FVIII levels in a longitudinal study on 40 healthy individuals. From each participant blood was collected at 13 visits during a period of one year. Based on the obtained biological variations we will provide recommendations that improve the assessment of VWF and FVIII as risk indicators of thrombotic disease and increase the diagnostic precision for VWD.

In **Chapter 9**, the results of this thesis are summarized and discussed, and suggestions for future research are given.

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CHAPTER 2

GENETIC DETERMINANTS OF VON WILLEBRAND FACTOR LEVELS AND ACTIVITY IN RELATION TO THE RISK OF CARDIOVASCULAR DISEASE: A REVIEW

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SUMMARY

It has been well established that a high level of plasma von Willebrand factor (VWF) is associated with an increased risk of arterial thrombosis, including myocardial infarction and ischemic stroke. As plasma VWF levels are to a large extent genetically determined, numerous association studies have been performed to assess the effect of genetic variability in the VWF gene *(VWF)* on VWF antigen and activity levels, and on the risk of arterial thrombosis. Genetic variations in other regulators of VWF, including the ABO blood group, ADAMTS13, thrombospondin-1 and the recently identified SNARE protein genes, have also been investigated. In this article, we review the current literature as exploring the associations between genetic variations and the risk of arterial thrombosis may help elucidate the role of VWF in the pathogenesis of arterial thrombosis. However, as current studies frequently differ in design, population, and endpoint, and are often underpowered, it remains unclear whether VWF is causally related to the occurrence of arterial thrombosis or primarily mirrors endothelial dysfunction, which predisposes to atherosclerosis and subsequent arterial thrombosis. Nevertheless, current studies provide interesting results that do not exclude the possibility of VWF as causal mediator and justify further research into the relationship between VWF and arterial thrombosis. Large prospective studies are required to further establish the role of VWF in the occurrence of arterial thrombosis.

INTRODUCTION

von Willebrand factor (VWF) plays a key role in arterial thrombus formation, as this large multimeric glycoprotein facilitates platelet adhesion and aggregation at sites of vascular injury [1]. VWF is synthesized by endothelial cells as a precursor pro-VWF molecule and is a marker of endothelial cell activation [2]. Freshly synthesized VWF molecules are secreted via a basal secretion pathway and a constitutive pathway [2-4]. A small part of especially large VWF multimers is released from Weibel Palade Bodies (WPBs) of endothelial cells or alpha-granules of platelets via the regulated pathway, which requires stimulation by specific agonists, such as epinephrine, thrombin and ADP [5-7].

In healthy individuals, VWF plasma levels range from 0.60 to 1.40 IU/mL and are influenced by many lifestyle and environmental factors, such as inflammation, hormones, smoking and diabetes [6]. In addition, studies in human twins have demonstrated that plasma levels of VWF have a high heritability of approximately 60% [8-9]. An important genetic determinant of VWF levels is the ABO blood group, but also single nucleotide polymorphisms (SNPs) in the VWF gene (*VWF*) affect VWF levels [10-11]. As many studies have demonstrated that high VWF levels are associated with the risk of arterial thrombosis, such as coronary heart disease (CHD) and ischemic stroke, genetic polymorphisms that affect VWF levels may be directly associated with the risk of arterial thrombosis [12-14]. This is consistent with Mendel's second law, which suggests that the inheritance of a particular allele at a given locus is independent of the inheritance of other alleles at different loci [15]. Consequently, if VWF is causally associated with the risk of CVD, a genetic polymorphism that influences VWF plasma levels should increase the risk of CVD to same extent as predicted by its influence on VWF plasma levels [16].

Although high VWF plasma levels may be relevant in the development of arterial thrombosis, VWF activity, which depends strongly on its multimeric size, may also be an important mediator. ADAMTS-13 *("A Disintegrin and Metalloproteinase with a Thrombospondin type 1 motive")* and thrombospondin-1 (TSP-1) are regulators of VWF multimer size, which is important for controlling thrombus formation [17-18]. Consequently, genetic variants within the ADAMTS-13 gene (ADAMTS13) and/or TSP-1 (THBS1) gene may also influence the association between VWF levels and the risk of arterial thrombosis [19-21].

In the past two decades, numerous studies on genetic determinants of VWF plasma levels, VWF activity and the risk of arterial thrombosis have been conducted. In this article, we will review these studies comprehensively and discuss whether current evidence supports or questions a causal relationship between VWF and arterial thrombosis.

von Willebrand factor levels and cardiovascular disease

The association between high VWF levels and the occurrence of cardiovascular events has been investigated in initially healthy individuals as part of a number of case-control studies, but only in a limited number of prospective epidemiological studies. Most studies have focused on the relationship with CHD and showed that individuals with the highest VWF plasma levels had a higher risk of CHD than individuals with the lowest VWF plasma levels [14,22]. For example, in the prospective Reykjavik study the odds ratio (OR) for subjects with VWF levels in the highest tertile was 1.15 (95% CI 1.0-1.3) after adjustment for potential confounders [13]. A meta-analysis of 6 population-based prospective cohort studies yielded an adjusted OR of 1.5 (95% CI 1.1-2.0) [12]. Stronger associations were seen in studies on VWF in patients with pre-existent coronary artery disease or in other high risk populations [6,23].

The association between high VWF levels and the risk of incident ischemic stroke has been studied less frequently [24-28]. The prospective ARIC study, which included middle-aged individuals (45-64 years), found a 1.7-fold increased risk of ischemic stroke for individuals with VWF plasma levels in the highest quartile compared to the lowest quartile, after adjustment for potential confounders [14]. Recently, we have shown in the large prospective population-based Rotterdam study that the risk of ischemic stroke increases with increasing VWF levels (OR per standard deviation increase in VWF levels 1.12 95% CI [1.01-1.25]), independent of conventional cardiovascular risk factors and ABO blood group [29].

These results show that high VWF plasma levels are associated with the risk of CHD and ischemic stroke. However, to date the underlying mechanism of this association is not entirely clear.

Determinants of von Willebrand factor plasma levels

There are several non-genetic and genetic, non-modifiable factors that influence VWF levels. The most important determinant of VWF levels is ABO blood group. N-linked oligosaccharide side chains on VWF molecules contain A and B blood group antigens which are encoded by the blood group gene (*ABO*), which is located on the long arm of chromosome 9. These antigens decrease VWF clearance [30-31]. Consequently, individuals with blood group non-O (A, B, or AB) have at least 25% higher VWF levels than individuals with blood group O [32]. When studying a population without stratifying for blood group, the variability in VWF levels will increase and the statistical power will be reduced. In addition, as ABO blood group has been an established risk factor for arterial thrombosis [33-34], it should always be taken into account when investigating the relationship between VWF and the risk of arterial thrombosis.

Another important determinant of VWF plasma concentration is age [13,35]. It has been suggested that an increasing arterial rigidity explains the rising VWF levels during aging. However, the precise signal transduction events involved in this process are unknown [36-37]. The importance of the age of subjects in studies on genetic determinants of VWF levels has been demonstrated by Bladbjerg et al. In this study in healthy twins, it has been shown that the total genetic contribution to VWF plasma levels decreases with increasing age [9]. Consequently, associations between genetic polymorphisms and VWF plasma levels in older populations are expected to be weaker than in a younger population.

Elevated VWF levels have also been demonstrated in inflammatory disorders, such as rheumatoid arthritis and vasculitis. [38]. Also, C-reactive protein (CRP) has been associated with VWF levels [13].

Another disorder that may influence VWF levels is diabetes. Initially, it was anticipated that the elevated VWF levels in both type I and type II diabetics reflect only the diffuse endothelial dysfunction mediated by chronic hyperglycaemia. However, more recent studies showed that VWF is also related to insulin resistance, although the precise mechanism remains unclear [39]. The importance of diabetes in the association between VWF and arterial thrombosis was demonstrated by Frankel et al, who showed that VWF levels are associated with an increased risk of CHD in patients with type 2 diabetes, but not in non-diabetic subjects and insulin sensitive subjects [23].

Patients with hypertension have significantly increased VWF levels. VWF levels were especially correlated to the diastolic blood pressure. Interestingly, in patients who were treated with antihypertensive medication VWF levels normalized again [40-41]. Statins treatment may also reduce elevated VWF levels [42-43]. Consequently, as most patients with a previous event of arterial thrombosis are treated with antihypertensive agents and statins, it is important to consider their effect on VWF plasma levels in studies on the relationship between VWF and arterial thrombosis.

Genetic variability in the VWF gene

The VWF gene is large, consisting of 178 kilobasepairs and 52 exons. The gene is located on the short arm of chromosome 12, and encodes a protein of 2813 amino acids with several functional domains [44-45].

The VWF promoter region

As the VWF promoter is involved in the regulation of gene transcription, several studies focused on the VWF promoter region during their search for SNPs that affect VWF plasma concentration. These studies are summarized in table 1.

Table 1 | Association between polymorphisms in the promoter region of the VWF gene, VWF levels and activity, and arterial thrombosis

Characteristics study population				SNP ID	Nucleotide change	MAF	VWF:Ag levels (IU/mL)	Disease endpoints OR (95% CI)	Ref.
Participants	N	Ethnicity	Mean Age (years ± SD)						
Healthy blood group O donors	261	Caucasian	42.2 ± 10.9	rs7964777 rs7954855 rs7965413	-2709C>T -2661A>G -2527G>A	Haplotype 0.36	Haplotype 0.78 TT/GG/AA 0.87 TC/GA/AG 0.96 CC/AA/GG p=0.008	NA	[46]
Healthy blood group O donors	261	Caucasian	42.2 ± 10.9	rs7966230	-3268G>A	0.36	0.78 CC 0.86 CG 0.93 GG p=0.03	NA	
MI patients	525	Caucasian	62 ± 9.2	rs7954855	-2661A>G	Haplotype 0.37	NA	AG haplotypes 0.93 [0.77;1.22] MI	[49]
Controls	451		61.3 ± 9	rs7965413	-2527G>A				
Healthy subjects	210	Afro-Brazilian	32.4 ± 9.9	rs7954855	-2661A>G	0.62	1.13 *AA* 1.07 AG 1.05 GG NS	NA	[50]
Healthy subjects	210	Euro-Brazilian	33.5 ± 9.8	rs7954855	-2661A>G	0.41	1.14 AA 1.01 AG 0.98 GG NS		
CHD patients	352	Caucasian	66.5 ± 4.9	rs7966230	-3268G>A	0.38	NA	2.6 [1.0;6.8] CG+GG vs.CC CHD (advanced atherosclerosis)	[51]
Controls	736		65.2 ± 5.6						

Characteristics study population				SNP ID	Nucleotide change	MAF	VWF:Ag levels (IU/mL)	Disease endpoints OR (95% CI)	Ref.
Participants	N	Ethnicity	Mean Age (years ± SD)						
Twins	566	Caucasian	73-94	rs7954855	-2661A>G	0.36	2.05 GG 1.96 AG 1.99 AA p=0.31	NA	[9]
Stroke patients	124	Caucasian	56 ± 12	rs7966230	-3268G>A	0.30	NS	0.9 [0.5;1.5] CG vs. CC 0.7 [0.3;1.6] GG vs. CC	[24]
Controls	125		56 ± 12						
Healthy subjects	394	Unknown	39 ± 11	rs7966230	-3268G>A	Haplotype 0.37	0.95 1/1 0.97 1/2 0.99 2/2 p=0.70	NA	[48]
				rs7964777	-2709C>T				
				rs7954855	-2661A>G				
				rs7965413	-2527G>A				

VWF gene polymorphisms were numbered according to the latest recommendations of the ISTH Scientific Subcommittee on VWD, assigning +1 to the A of the initiator codon ATG. Abbreviations used in this table are *MAF* for minor allele frequency, NS for not significant, NA for not applicable, IHD for ischemic heart disease, MI for myocardial infarction, UAP for unstable angina pectoris, CHD for coronary heart disease, DMII for type 2 diabetes, IS for ischemic stroke.

To date, four SNPs in the VWF promoter region have been identified: rs7964777, rs7954855, rs7965413, rs7966230 [46-47]. In Caucasians, these polymorphisms are in strong linkage disequilibrium (LD) and segregate as two distinct haplotypes [46]. Consequently, information provided by these polymorphisms is interchangeable. Several studies showed that genetic variants within the VWF promoter region are significantly associated with higher VWF antigen (VWF:Ag) levels [46-47]. However, not all studies revealed similar findings [9,24,48-51]. These discrepancies may arise through heterogeneity of the study population, based on blood group, age, medical history, and ethnicity or lack of statistical power.

Interestingly, the association between the VWF promoter variant and VWF levels was only identified in healthy blood group O donors. Furthermore, the association was even stronger in subjects above the age of 40 years [46-47]. As the contribution of genetics is expected to be smaller at older age, this finding was surprising. However, it is possible that at older age non-genetic determinants of VWF may interact with genes, i.e. a gene-environment interaction, and in that way strengthen the association.

In patients with prevalent CHD or ischemic stroke, *VWF* promoter polymorphisms were not associated with plasma VWF levels [24,52-53]. In these patients cardiovascular risk factors are frequent and may – by interacting with genes – influence the association between VWF and arterial thrombosis via gene-environment interaction. Another important consideration in this is the use of antihypertensive medication or statins at the time of blood sampling, which may modify the actual association between genetic variants and VWF levels [42-43].

Allele frequencies of genetic variants, the LD pattern, and the genetic contribution to VWF levels may vary amongst different ethnic populations. Only one study has reported on the allele frequency of rs7954855 and its contribution to VWF levels in ethnic populations other than Caucasians. In this study, the distribution of the rs7954855 genotypes differed between Afro-Brazilians and Euro-Brazilians [50]. However, in neither group was an association with VWF plasma levels observed. When including different races in a study population, differences in allele frequencies may influence detection of associations. Also, SNPs that may be important in one ethnic population may not be relevant in other ethnic population.

A different type of genetic variation located in the *VWF* promoter region is a (GT)n repeat [54]. (GT)n repeats can be found in regulatory regions of genes, where they sometimes modulate transcriptional activity [55-56]. In vitro data showed that the *VWF* promoter activity can be enhanced by shear stress and that the magnitude of this induction depends on the length of the (GT)n repeat element [57]. However, in two independent studies that included healthy individuals, no association between the polymorphic GT(n) repeat and VWF plasma levels was observed [48,54].

Only a few studies have investigated the association between *VWF* promoter SNPs and the risk of CHD (table 1) [49,51]. In the Rotterdam Study, rs7966230 was associated with incident CHD, although only in a subgroup of subjects with advanced atherosclerosis. Carriers of the minor allele had a 2.6-fold increased risk of CHD (95% CI 1.0-6.8) [51]. These

results suggest that the association between the *VWF* promoter polymorphism and CHD may be modified by the severity of atherosclerosis.

We have studied for the first time the association between promoter SNPs and ischemic stroke. However, no association was found between genetic variations in the *VWF* promoter region and the risk of ischemic stroke [24].

The VWF coding regions

During screening of patients with von Willebrand disease for mutations, several polymorphisms in the coding regions of *VWF* were identified. These are summarized in table 2. Most studies have focused on exon 18 and 28, as these regions encode VWF protein domains with important haemostatic functions.

Exon 18 encodes part of the D' domain, which is involved in VWF multimerisation and binding of factor (F)VIII. The non-synonymous SNP rs1063856 (Thr789Ala) is located within this region. Carriers of Alanine have significantly higher VWF:Ag levels and VWF ristocetin co-factor (VWF:RCo) activity. This association was not only seen in healthy individuals, but also in patients with type 1 diabetes, type 2 diabetes, and CHD patients [10-11]. This indicates that the relationship between rs1063856, VWF:Ag levels, and VWF:Rco activity is independent of the disease state. In addition, rs1063856 was associated with an increased risk of CHD in patients with type I diabetes (OR 4.3, 95% CI [1.82;9.97]), but not in type 2 diabetes patients [11]. However, because of the small sample sizes these results should be interpreted with care.

The A1, A2 and A3 domains of the VWF protein are encoded by exon 28: the A1 domain contains binding sites for the GPIbα platelet receptor and collagen, the A2 domain contains the cleavage site for ADAMTS13, and the A3 domain is the primary binding site for collagen. Several SNPs have been identified in the genetic regions encoding these domains.

Rs216311 (Thr1381Ala) is located in the genetic region encoding the A1 domain. In patients with type 1 diabetes, rs216311 was not associated with VWF:Ag levels and the risk of CHD [11].

Rs1800386 (Tyr1584Cys) is located in the genetic region encoding the A2 domain of the VWF protein. In healthy Caucasians, the Tyr/Cys phenotype, which has a frequency of only 1%, was associated with significantly lower VWF:Ag levels, VWF collagen binding (VWF:CB) activity and VWF:RCo activity than the common Tyr/Tyr phenotype ($p<0.001$) [58]. In addition, VWF proteins containing cysteine at position 1584 were more sensitive to proteolytic degradation by ADAMTS13 [59-60]. In strata of blood group O and non-O, the effect size of rs1800386 was larger in individuals with blood group O than in individuals with blood group non-O [61]. This again shows the importance of considering ABO blood group as effect modifier in the association between SNPs and VWF plasma levels.

Table 2 | Association between polymorphisms in the coding regions of the VWF gene, VWF levels and activity, and arterial thrombosis

Characteristics study population				SNP ID	Alleles	Exon/ Intron no.	Amino acid change	MAF	VWF:Ag levels (IU/mL)	Disease endpoints OR (95% CI)	Ref.
Participants	N	Ethnicity	Mean Age (years ± SD)								
Patients type 1 diabetes	475	Caucasian	44	rs1063856	A/G	Exon 18	Thr789Ala	0.25	1.49 AA 1.64 AG 1.85 GG p=0.03	1.8 [0.88;3.71] A/G vs. A/A 4.3 [1.82;9.97] G/G vs. A/A For CHD	[11]
				rs216311	G/A	Exon 28	Ala1381Thr	0.40	NS (data not shown)	NS (data not shown)	
				rs216325	C/T	Intron 19	NA	0.37	NS (data not shown)	NS (data not shown)	
Patients	365	Unknown		rs1063856	A/G		Thr789Ala		DMII$^-$/CHD$^-$ vs. other groups	NS	[10]
DMII$^+$/CHD$^+$	204		68 ± 8					0.34	VWF:Ag p<0.001		
DMII$^+$/CHD$^-$	152		67 ± 10					0.40	VWF:Rco p=0.05		
DMII$^-$/CHD$^+$	22		64 ± 9					0.59			
DMII$^-$/CHD$^-$	100		38 ± 12					0.36			
Blood donors	200	Caucasian	Unknown	rs1800386	A/G		Tyr1584Cys	0.005	*VWF:Ag* 0.82 ± 0.35 AG 1.11 ± 0.37 GG p<0.001 *VWF:RCo* 0.73 ± 0.40 AG 1.10 ± 0.43 GG p<0.001 *VWF:CB* 0.83 ± 0.36 AG 1.24 ± 0.41 GG p<0.001 VWF proteolysis AG 49% greater than GG (p<0.001)	NA	[59]

Characteristics study population				SNP ID	Alleles	Exon/ Intron no.	Amino acid change	MAF	VWF:Ag levels (IU/mL)	Disease endpoints OR (95% CI)	Ref.
Participants	N	Ethnicity	Mean Age (years ± SD)								
IS patients Controls	124 125	Caucasian	56 ± 12	rs1800386	A/G	exon 28	Tyr1584Cys	0.016	NA	NS (data not shown)	[58]
AT patients controls	374 332	Caucasian	43 ± 7	rs1800386	A/G	exon 28	Tyr1584Cys	0.003	NA	NS (data not shown)	[58]
AT patients	421	Caucasian	43.2 ± 6.7	rs216293	C/A	Intron 17	NA	0.46	β=0.004 ± 0.02 per risk allele, p=0.86	1.44 [1.12;1.86] AT 1.50 [1.17;1.80] IS	[89]
Healthy controls				rs1063857	T/C	Exon 18	Tyr795Tyr	0.35	β=0.039 ± 0.02 per risk allele, p=0.08	1.32 [1.02;1.70] AT	
	409		39.6 ± 7.6	rs4764478	A/T	Intron 45	NA	0.25	*VWF:Ag* β=0.083 ± 0.03 per risk allele, p=0.002 *VWF:CB* β=0.087 ± 0.04 per risk allele, p=0.003	0.88 [0.67;1.17] AT	

Abbreviations used in this table are *MAF* for minor allele frequency, *NS* for not significant, *NA* for not applicable, *AT* arterial thrombosis, *IS* for ischemic stroke..

Only one study has investigated the association between rs1800386 and the risk of arterial thrombosis [58]. However, due to the very low minor allele frequency, the study did not have enough power to assess this association accurately.

Recently, we have investigated the total common variation in *VWF* (including the 3' and 5' flanking regions) by genotyping 27 haplotype tagging SNPs (ht-SNPs) in relation to VWF:Ag levels, VWF:CB activity, and the risk of arterial thrombosis in the ATTAC study, a case-control study of young patients with a first event of arterial thrombosis and healthy controls [62]. We identified one SNP, rs4764478 (intron 45), that was associated with VWF:Ag levels (beta-coefficient per risk allele 0.083 ± 0.026 U/ml, p=0.002) and VWF:CB activity (0.087 ± 0.044 U/ml, p=0.003). Two SNPs were associated with the risk of arterial thrombosis: rs216293 (intron 17) and rs1063857 (exon 18). The association between rs216293 and arterial thrombosis was predominantly seen in the subgroup of ischemic stroke patients (OR 1.50, 95% CI [1.17-1.80]). However, as rs216293 was not associated with VWF:Ag levels or activity, the underlying mechanism for the association between rs216293 and arterial thrombosis remains unclear.

The synonymous SNP rs1063857 (Tyr795Tyr) is in perfect LD with the non-synonymous SNP rs1063856, which has previously been associated with VWF:Ag levels [10-11]. Furthermore, the Cohorts for Heart and Aging Research in Genetic Epidemiology (CHARGE) consortium has replicated this association in a meta-analysis of genome-wide association studies in five large population-based cohort studies [63]. In the previously mentioned ATTAC study, rs1063857 was significantly associated with the risk of arterial thrombosis (OR per minor allele 1.32, 95% CI [1.02-1.70]). However, the association between rs1063857 and VWF:Ag levels was only borderline significant in this study of young individuals. Considering previous results on the association between rs1063856 and VWF:Ag levels, rs1063856 may be the actual functional variant that may be responsible for the association between rs1063857 and arterial thrombosis risk in the ATTAC study. Furthermore, these findings provide evidence for a causal relationship between VWF and arterial thrombosis.

Blood group, VWF levels and the risk of arterial thrombosis

Since blood group is the major determinant of VWF plasma levels, it is possible that the effect of ABO blood group on VWF plasma levels is the explanation for the relationship between VWF and arterial thrombosis [64]. Indeed, non-O blood groups confer an increased risk of arterial thrombosis, although this association has not yet been proven to be consistent [33-34].

A meta-analysis of studies on blood group and the risk of arterial thrombosis reported an overall significant association between blood group non-O and the risk of MI (pooled OR 1.25, 95% CI 1.14-1.36) and between blood group non-O and the risk of ischemic stroke (pooled OR 1.14, 95% CI 1.01-1.27) [34]. However, the association between blood group non-O and the risk of MI was seen in retrospective studies only. In addition, the relationship

between blood group and ischemic stroke has never been investigated in prospective studies. So, although the relationship between ABO blood group, VWF and arterial thrombosis seems plausible, there is a lack of well designed prospective population-based cohort studies in order to elucidate this relationship.

New genetic determinants of VWF plasma levels

Genome wide association studies enable us to investigate the genetic component of common diseases and quantitative traits without a prior biological hypothesis. With this approach new gene loci are identified that contain genes that contribute to CVD. However, to date no association between VWF gene loci or other coagulation-related genes and CVD endpoints have been established [65-71]. These findings raise the question whether VWF is relevant in the occurrence of arterial thrombosis at all. However, it is also possible that the effects of VWF-related genetic variations on the risk of arterial thrombosis are so small that very large populations are required in order to detect these effects.

Recently, the CHARGE consortium conducted a meta-analysis of GWAS in five large population-based cohort studies to identify new genetic determinants of VWF levels [63]. Besides confirmation of previously indentified candidate genes, such as *ABO* and *VWF*, the CHARGE consortium identified and replicated novel associations with six genetic loci: *STXBP5*, *SCARA5*, *STAB2*, *STX2*, *TC2N*, and *CLEC4M*. One of these new candidate genes, the syntaxin-2 (STX2) gene, encodes a binding substrate for syntaxin-binding-protein-5 (STXBP5), and is a member of the soluble *N*-ethylmaleimide sensitive fusion attachment protein receptor (SNARE) family. SNARE proteins drive vesicle exocytosis by fusion of granules and target membranes, a process involved in the regulation of numerous secretory events, such as Weibel Palade Body exocytosis [72]. Recently, we have confirmed the association between genetic variations in *STX2* and *STXBP5* and VWF:Ag levels in a large group of young patients with a first event of arterial thrombosis. In addition, we demonstrated that these genetic variations may also affect VWF:CB and even the risk of arterial thrombosis [73]. So, although large GWAS on CVD have not identified SNPs in coagulation- related genes, these new candidate genes provide additional possibilities for a causal role of VWF in the occurrence of arterial thrombosis.

The ADAMTS13 gene

ADAMTS13 is an important regulator of VWF multimer size. It specifically cleaves the Tyr1605-Met1606 bond within the A2 domain of activated VWF molecules, leading to smaller, less haemostatically active VWF molecules [18,74]. Congenital deficiency of ADAMTS13 is a risk factor for the development of thrombotic thrombocytopenic purpura (TTP), which can be complicated by micro-vascular thrombosis of organs including the heart and brain [75-76]. Therefore, it has been hypothesized that decreased ADAMTS13 levels may be a risk factor

for the development of arterial thrombosis in the general population. Indeed, several studies demonstrated that decreased ADAMTS-13 levels and activity are associated with an increased risk of MI and ischemic stroke [28,77-79]. We have additionally shown that the combination of decreased ADAMTS-13 levels and increased VWF levels confer an even higher risk [28].

A number of studies investigated the relationship between ADAMTS13 gene variants, ADAMTS13 levels and arterial thrombotic risk. The non-synonymous SNP rs11575933 (Pro475Ser), which is located in exon 12, was associated with a low ADAMTS13 activity in a Japanese population [80]. In other populations, this polymorphism is absent (Caucasians) or rare (Chinese) and will therefore not have a significant contribution to arterial thrombotic risk [81-82].

Rs685523 (Ala900Val) has been associated with a decreased ADAMTS13 activity [83]. In a prospective study that included Caucasians with chronic CHD, this polymorphism was associated with a significantly increased risk of all-cause death (OR 1.92, 95% CI 1.14-3.23), and death due to cardiac causes (OR 2.67, 95% CI 1.59-4.49) [21].

We have previously investigated the total common variation in the ADAMTS13 gene by genotyping four ht-SNPs [28]. Our study showed that one haplotype was associated with significant lower ADAMTS13 activity than the reference haplotype. However, none of the haplotypes was associated with the risk of arterial thrombosis.

Recently, Hanson et al. investigated the relationship between the total common variation in the ADAMTS13 gene and the risk of ischemic stroke. In this case-control study that included 600 ischemic stroke patients and 600 controls, rs4962153 was associated with an increased risk of ischemic stroke (OR per minor allele 1.24, 95% CI 1.01-1.54,), whereas rs2285489 and rs2301612 were associated with a decreased risk of ischemic stroke (OR 0.82, 95% CI [0.70;0.97], and OR 0.85, 95% CI [0.73;1.00], respectively) [84].

In conclusion, current studies suggest that ADAMTS13 polymorphisms influence ADAMTS13 activity and the risk of arterial thrombosis. Since processing VWF molecules is the most important function of ADAMTS13, the association between variation in the ADAMTS13 gene and the risk of arterial thrombosis can be explained by its effect on VWF. At this point it is not known whether SNPs in the ADAMTS13 gene influence VWF levels or activity as well.

The Thrombospondin-1 gene

TSP-1 is a multifunctional glycoprotein involved in numerous biological processes, including cell-cell interactions, inflammation, and thrombosis [85-88]. It is primarily synthesized by megakaryocytes as the major constituent of platelet α-granules, but can also be detected in the blood vessel wall [89]. Besides involvement in platelet adhesion and thrombus stabilization, TSP-1 can control VWF multimer size both directly via cleavage of the VWF linking disulfide bonds, and indirectly by competing with ADAMTS13 [17,89-90]. Considering

this ability to interact with VWF, TSP-1 evolved as an important candidate gene for arterial thrombosis. Indeed, a previously identified non-synonymous polymorphism on exon 13, rs2228262 (Asp700Ser) was associated with an increased risk of CHD [19,91]. This finding was replicated in at least two other studies [92-93]. However, a meta-analysis of studies on rs2228262 resulted in an odds ratio for myocardial infarction of 1.10 (95% CI 0.96-1.26) [20].

Only one small study investigated the effect of rs2228262 on VWF multimer size [92]. However, a different amount of high molecular weight multimers between homozygotes for serine (N=26) and aspartic acid (N=18) could not be identified.

Previous findings do not support a strong role of TSP-1 in the occurrence of arterial thrombosis. However, the effect of genetic variants on TSP-1 mediated regulation of VWF multimer size may be relevant for VWF activity.

DISCUSSION

For decades VWF is considered an important factor in the development of arterial thrombosis due to its crucial function in haemostasis and thrombus formation. Indeed, the relationship between elevated VWF plasma levels and the risk of arterial thrombosis has been well established, as described in the current review. However, it remains unclear whether VWF is causally related to the occurrence of CHD or primarily mirrors endothelial dysfunction. Studying a possible causal relationship is challenging, since current observational studies are limited by confounding and reverse causation. One way to overcome these problems is the "Mendelian Randomization" approach.

In this article we comprehensively reviewed the literature on common variations in the VWF gene and other VWF-related genes in relation to VWF plasma levels, VWF activity, and the risk of arterial thrombosis. When examining all the previous studies, one of our main observations was that only a limited number of studies simultaneously investigated the association between genetics, VWF levels and the risk of arterial thrombosis. In addition, studies differed in design, population (e.g. different age or ethnic background), and endpoint, (e.g. acute CHD versus chronic CHD, and ischemic stroke) and were often underpowered. All these limitations make it difficult to perform a Mendelian Randomization approach and would lead to the conclusion that current findings do not support causality between VWF and arterial thrombosis. However, individual studies have provided interesting results that do not exclude the possibility of VWF as causal mediator of arterial thrombosis, but rather make it even more plausible.

Genetic polymorphisms in the promoter region affect VWF levels in blood group O individuals. One of these was associated with an increased risk of CHD in subjects with advanced atherosclerosis. Also, rs1063856 is the only SNP in the coding region that is consistently associated with VWF levels and has been replicated in the large meta-analysis of the CHARGE consortium and our ATTAC study. In addition, rs1063856 was associated with

the risk of arterial thrombosis in two independent studies. These latter findings support the possibility that VWF has a causal role in the occurrence of arterial thrombosis.

Interest has also focused on other determinants of VWF, such as blood group, ADAMTS-13, TPS-1, and the recently identified SNARE protein genes, *STXBP5* and *STX2*. Blood group is the most important determinant of VWF levels. Moreover, blood group non-O is a risk factor for arterial thrombosis. Therefore, it could be anticipated that blood group increases the risk of arterial thrombosis by elevating VWF levels and supports causality between VWF and arterial thrombosis.

ADAMTS-13 and TSP-1 are important in processing VWF multimers. Genetic variations in *ADAMTS13* have been associated with ADAMTS13 activity and the risk of arterial thrombosis. However, to date no studies have investigated the effect of these variations on VWF levels, activity, or multimer pattern. So, although ADAMTS13 would be an interesting link between VWF and arterial thrombosis, current research has to be expanded.

STXBP5 and *STX2*, which have not been related to regulation of VWF levels before, are interesting new candidate genes that may explain the variability in VWF levels. It is been shown that genetic variations within these genes affect VWF levels. Recently, we have shown that variations in *STX2* are even related to the risk of arterial thrombosis [73]. Hence, these new candidates may contribute to our understanding of biological pathways involved in the regulation of VWF plasma levels and the occurrence of arterial thrombosis.

Besides genetic association studies, several animal studies, which fall outside the scope of this review, have provided evidence for a pathofysiological role of VWF in the occurrence of arterial thrombosis [94-95].

In conclusion, although current studies do not provide indisputable evidence for a causal relationship between VWF and arterial thrombosis, we believe that previous findings are convincing enough to suggest a role for VWF in the occurrence of arterial thrombosis. We should not neglect the complexity of the pathogenesis of arterial thrombosis and should not expect that arterial thrombosis can be clarified by one single factor, such as in monogenic disorders. As expected, findings of current large GWAS explain only a very small percentage of the incidence of arterial thrombosis. Therefore, other factors with modest effects remain to be discovered. We would suggest performing large prospective population-based cohort studies with well-defined homogeneous study endpoints, which discriminate between CHD and ischemic stroke, and have a long follow-up time. In these studies, not only VWF plasma levels should be taken into account as possible mediator, but also VWF activity and VWF multimer size. Finally, all genes that have been identified by the CHARGE consortium should be included. In this way we might be able to achieve consensus on the actual role of VWF in the development of arterial thrombosis in the future.

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CHAPTER 3

HIGH VON WILLEBRAND FACTOR LEVELS INCREASE THE RISK OF STROKE: THE ROTTERDAM STUDY

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ABSTRACT

Background and purpose: Many studies have investigated the role of plasma von Willebrand factor level in coronary heart disease, but few have investigated its role in stroke. The aim of this study was to determine if von Willebrand factor levels are associated with the risk of stroke.

Methods: The study was part of the Rotterdam Study, a large population-based cohort study among subjects aged 55 years and older. We included 6250 participants, who were free from stroke at baseline (1997-2001) and for whom blood samples were available. Follow-up for incident stroke was complete up to January 1, 2005. Data were analyzed with Cox' proportional hazards models adjusted for age and sex, and additionally with models adjusted for other potential confounders including ABO blood group. A subgroup analysis was performed in participants without atrial fibrillation. Effect modification by sex was tested on a multiplicative and on an additive scale.

Results: During an average follow-up time of 5.0 years, 290 first-ever strokes occurred, of which 197 were classified as ischemic. The risk of stroke increased with increasing von Willebrand factor levels (age and sex adjusted hazard ratios per standard deviation increase in von Willebrand factor level: 1.12 [95% CI: 1.01-1.25] for stroke, 1.13 [95% CI: 0.99-1.29] for ischemic stroke). Adjustments for additional confounders slightly attenuated the association. The association was also present in subjects without atrial fibrillation and did not differ between sexes.

Conclusion: High von Willebrand factor levels are associated with stroke risk in the general population.

INTRODUCTION

The plasma glycoprotein von Willebrand factor (VWF) has an essential role in hemostasis because it promotes platelet adhesion and aggregation at sites of vascular injury, and acts as a carrier protein for factor VIII [1]. VWF is almost exclusively synthesized, stored and secreted by endothelial cells [2]. The release of VWF is increased when endothelial cells are activated or damaged [3]. Therefore, plasma VWF level is considered a marker of endothelial dysfunction, a condition that predisposes to atherosclerosis and thrombosis [4]. Because of its direct role in hemostasis, and its indirect role as a marker of endothelial dysfunction, VWF is a potential risk indicator for cerebrovascular disease.

Although many investigators have studied the relationship between plasma VWF levels and coronary heart disease [5], little is known about the association between VWF levels and stroke. Several case-control studies did report an association with stroke, but VWF levels were measured after the stroke was diagnosed. Therefore it remains debatable whether high levels are a cause or a consequence of stroke [6-10]. Results from longitudinal studies are limited. Thus far, only one longitudinal study of a stroke-free cohort with a sufficiently large number of incident stroke cases has investigated the relationship between VWF levels and stroke in the general population [11]. Because the population of this study was relatively young (45-64 years old at baseline), it needs to be investigated if these findings also apply to elderly people, who are at the highest risk of stroke.

We investigated whether plasma VWF levels are associated with the risk of stroke and its subtype ischemic stroke in a large population-based cohort study among elderly subjects. We further examined if the association was different in men and women and whether it could be attributed to the effect of atrial fibrillation on VWF levels.

METHODS

Source population

This study is part of the Rotterdam Study, an ongoing prospective population-based cohort study, which started in 1990 [12]. Initially, 7983 persons (out of 10 215 invitees) who were aged 55 years or older and living in Ommoord, a district in the city of Rotterdam in the Netherlands, were enrolled in the cohort. In the year 2000, the cohort was expanded by 3011 persons (out of 4472 invitees) who had reached the age of 55 years or moved into the study district since the start of the study. Baseline examinations consisted of an interview at home and two visits to the research center for physical examination and blood sampling. These examinations were repeated every three to four years. All participants were continuously monitored for disease.

For the present study, we included participants of the third examination cycle of the original cohort (baseline 1997-1999), and participants of the first examination cycle of the extension of the cohort (baseline 2000-2001).

The study was approved by the Medical Ethics Committee of the Erasmus University Medical Center in Rotterdam. Written informed consent was obtained from all participants.

Assessment of stroke

History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records (n=419). After enrollment in the Rotterdam Study, participants were continuously monitored for incident strokes through automated linkage of the study database with files from general practitioners, the municipality, and nursing home physicians' files. Additional information was obtained from hospital records. Potential strokes were reviewed by research physicians, and verified by an experienced stroke neurologist (P.J.K.). Subarachnoid hemorrhages and retinal strokes were excluded.

Strokes were further classified as ischemic or hemorrhagic based on the following criteria: ischemic stroke was diagnosed if a CT or MRI scan carried out within four weeks after the event ruled out other diagnoses, or if indirect evidence (neurological deficit limited to one limb or completely recovered within 72 hours, or atrial fibrillation in the absence of anticoagulant therapy) indicated that the stroke was of an ischemic nature; a hemorrhagic stroke was diagnosed if a relevant hemorrhage was shown on CT or MRI scan, or if the person lost consciousness permanently or died within hours after the onset of focal signs. If a stroke did not match any of these criteria, it was classified as unspecified.

Participants were followed from baseline to stroke, death, last health status update where they were known to be free of stroke, or January 1, 2005, whichever came first. For the analysis of ischemic stroke, we censored participants who were diagnosed with hemorrhagic or unspecified stroke at the date of the event. Follow-up was complete up to January 1, 2005, for 98.6% of potential person years [13].

Blood sampling procedure and VWF plasma measurement

Fasting venous blood samples were taken at the research center and collected in citrated tubes. Samples were stored at -80°C. VWF antigen was determined with an in-house enzyme-linked immunosorbent assay (ELISA), using polyclonal rabbit antihuman VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging. The intra-assay coefficient of variation was 5.8% and the inter-assay coefficient of variation was 7.8%.

Baseline measurements

Smoking behavior and current medication use were assessed during the interview at home. Clinical measurements were obtained during two visits to the research center. Blood pressure was calculated as the mean of two measurements with the random-zero

sphygmomanometer at the right brachial artery while the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure of ≥ 90 mm Hg and/or a systolic blood pressure of ≥ 140 mm Hg and/or the use of antihypertensive medication indicated for the treatment of high blood pressure (≥ grade 1 hypertension according to World Health Organization criteria) [14]. Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured with an automated enzymatic procedure. Diabetes mellitus was defined as the use of serum glucose-lowering medication and/or a fasting serum glucose level ≥ 7.0 mmol/L. The waist-to-hip ratio was calculated by dividing the waist circumference (cm) by the hip circumference (cm). Body mass index was calculated as weight (in kg) divided by the square of height (in meters). History of myocardial infarction was determined during the baseline interview and verified in medical records. History of coronary heart disease was positive if the participant underwent a revascularization procedure, or fulfilled the criteria of myocardial infarction. Prevalent and incident atrial fibrillation were ascertained using the following methods: (1) An ECG was recorded during baseline visits and follow-up rounds; (2) information was obtained from general practitioners files, hospital records, and the national registration system of hospital discharge diagnoses [15]. The presence of peripheral arterial disease was evaluated by measuring the systolic blood pressure level of the posterior tibial artery at both legs using a Doppler probe and a random-zero sphygmomanometer, with the subject in supine position. The ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the brachial artery was calculated for each leg. Peripheral arterial disease was considered present if the ankle-brachial index was <0.9 in at least one leg [16]. Blood group antigen phenotypes were reconstructed by haplotype analysis of four single nucleotide polymorphisms (SNPs), rs687289, rs507666, rs8176704, and rs8176749, which collectively serve as tagging SNPs for the O, A1, A2 and B allele [17].

Population for analysis

A total of 8517 persons were free from stroke at baseline and eligible to participate. Of these, 2267 persons were not included in the analyses because they did not visit the research center (n=1869), or because blood draw or storage failed (n=398). In total, 6250 participants were included in the analyses.

Statistical analysis

VWF levels were truncated at the mean ± 4 standard deviations to remove outliers. Age and sex adjusted mean values (standard deviation) or percentages of stroke risk factors across VWF quartiles were computed by analysis of covariance (ANCOVA). We used Cox' proportional hazards regression to determine hazard ratios with 95% confidence intervals for the association between plasma VWF levels and stroke. Only first-ever strokes were included in the analyses. Hazard ratios were expressed per standard deviation increase in VWF level, and in strata of VWF quartile (relative to the lowest quartile). The linear trend

across quartiles was tested by including the quartile categories as a continuous variable in the model. All hazard ratios were adjusted for age and sex (model 1), and additionally for other putative confounders (systolic blood pressure, diabetes mellitus, total serum cholesterol level, HDL-cholesterol level, lipid lowering medication use, current cigarette smoking, waist-to-hip ratio, atrial fibrillation, coronary heart disease, peripheral arterial disease, and antithrombotic medication use; model 2), and ABO blood group (model 3). Missing values in covariates were imputed by using a linear regression model based on age and sex.

Subsequently, we analyzed if the association between VWF and stroke was different in men and women. Interaction, or effect modification, is usually determined by entering a product term in the regression model. Because the Cox regression model is a multiplicative model, adding a product term results in a measure of interaction as departure from multiplicity. However, the preferred approach to examine biologic interaction rather than statistical interaction is to estimate interaction as departure from additivity [18]. In this study we examined both types of interaction. We tested for interaction on a multiplicative scale by adding a product term to the model, and we tested for biologic interaction or effect measure modification on an additive scale by estimating the relative excess risk due to interaction (RERI) and its 95% confidence interval [19-21].

Finally, we performed a subgroup analysis among participants who were free from atrial fibrillation at baseline (n=5959). Subjects who developed atrial fibrillation during follow-up were censored at the date of onset of atrial fibrillation.

RESULTS

During 31 489 person years of follow-up (mean 5.0 years), 290 participants developed a stroke (153 women), of which 197 were ischemic, 28 hemorrhagic and 65 unspecified. CT or MRI imaging reports were available for 72.4% of strokes and for 92.4% of ischemic strokes. Baseline characteristics of the study population are shown in Table 1. At baseline, the mean age was 69.1 years, and 57.2 per cent of the participants were female.

Table 2 describes the baseline characteristics across quartiles of the VWF distribution. VWF levels rose with age. ABO blood group was strongly related to VWF level. Diabetes mellitus and cardiovascular disease were more prevalent in subjects with higher VWF levels.

Table 3 shows the association between plasma VWF level and risk of stroke and ischemic stroke. After adjustment for age and sex, higher VWF levels were associated with an increased risk of stroke. Additional adjustment for multiple putative confounders including ABO blood group had only a minor effect on the association. Effect estimates for the association between VWF level and ischemic stroke were of similar magnitude, although not statistically significant.

Table 1 | Baseline characteristics of the study population (n=6250)

Age, years		69.1 (8.2)
Female sex, %		57.2
Systolic blood pressure, mm Hg		143.2 (21.3)
Diastolic blood pressure, mm Hg		76.8 (11.2)
Hypertension, %		62.5
Antihypertensive medication, %		23.6
Glucose, mmol/L		6.0 (1.6)
Diabetes mellitus, %		12.8
Current cigarette smoking, %		17.4
Total cholesterol, mmol/L		5.81 (0.98)
HDL cholesterol, mmol/L		1.39 (0.40)
Lipid lowering medication, %		12.8
Waist-to-hip ratio		0.92 (0.10)
Body mass index, kg/m^2		27.0 (4.0)
History of cardiovascular disease, %		26.7
Atrial fibrillation, %		4.6
Myocardial infarction, %		8.0
Coronary heart disease, %		10.5
Peripheral arterial disease, %		15.3
Antithrombotic medication, %		19.1
ABO blood Group, %	O	45.6
	A	42.3
	B	8.8
	AB	3.3
Von Willebrand factor, IU/mL		1.31 (0.54)

Values are means (standard deviation) or percentages.

Sex was not a significant effect modifier of the association between VWF and stroke, either on a multiplicative scale (P=0.33), or on an additive scale (P=0.69), indicating that the association between VWF and stroke was not different in men and women.

The association between plasma VWF level and stroke in the subgroup of participants without atrial fibrillation was similar to the association we found in the total cohort. Age and sex adjusted hazard ratios per standard deviation increase in VWF level were 1.15 (95% CI: 1.03-1.18) for stroke and 1.15 (1.01-1.32) for ischemic stroke.

Table 2 | Baseline characteristics of the study population across von Willebrand factor quartiles

Von Willebrand factor quartile (range, IU/mL)		1 (0.24-0.92)	2 (0.92-1.20)	3 (1.20-1.59)	4 (1.60-3.64)	*P*
Number, n		1563	1556	1557	1554	
Age, years		66.7 (7.3)	68.0 (7.9)	69.7 (8.1)	71.9 (8.4)	<0.001
Female sex, %		59.2	56.9	55.8	56.9	0.26
Systolic blood pressure, mm Hg		142.9 (21.1)	143.6 (20.9)	144.0 (20.9)	142.2 (21.2)	0.10
Diastolic blood pressure, mm Hg		76.8 (11.1)	77.0 (11.0)	77.1 (11.0)	76.1 (11.1)	0.06
Hypertension, %		61.4	62.7	63.8	62.0	0.55
Antihypertensive medication, %		24.6	22.7	23.6	23.4	0.65
Glucose, mmol/L		5.8 (1.6)	5.9 (1.6)	6.0 (1.5)	6.2 (1.6)	<0.001
Diabetes mellitus, %		11.2	10.9	13.3	15.9	<0.001
Current cigarette smoking, %		17.2	16.8	17.9	18.0	0.79
Total cholesterol, mmol/L		5.85 (0.95)	5.81 (0.95)	5.81 (0.95)	5.77 (0.94)	0.15
HDL cholesterol, mmol/L		1.41 (0.39)	1.41 (0.35)	1.39 (0.36)	1.36 (0.39)	<0.001
Lipid lowering medication, %		11.0	12.2	13.2	14.8	0.02
Waist-to-hip ratio		0.91 (0.08)	0.92 (0.08)	0.92 (0.08)	0.92 (0.08)	0.04
Body mass index, kg/m^2		26.5 (4.0)	26.6 (3.9)	27.2 (3.9)	27.5 (4.0)	<0.001
History of cardiovascular disease, %		25.7	25.0	26.1	29.8	0.01
Atrial fibrillation, %		3.9	3.9	4.2	6.5	<0.001
Myocardial infarction, %		7.0	7.2	7.7	10.2	0.01
Coronary heart disease, %		9.6	9.5	9.7	13.1	<0.001
Peripheral arterial disease, %		15.3	15.5	15.7	14.7	0.87
Antithrombotic medication, %		18.5	17.7	18.8	21.4	0.05
ABO blood Group, %	O	70.7	52.3	37.0	22.2	<0.001
	A	25.3	38.0	47.5	58.6	<0.001
	B	3.3	6.8	12.0	13.2	<0.001
	AB	0.8	3.0	3.6	5.9	<0.001

Mean values (standard deviation) or percentages. Values are adjusted for age and sex when applicable.

Table 3 | Association between plasma von Willebrand factor level and stroke

	HR (95% CI)*		
Von Willebrand factor	Model 1	Model 2	Model 3
All strokes (n=290)			
Per SD increase	1.12 (1.01-1.25)	1.11 (1.00-1.24)	1.12 (1.00-1.25)
Quartile 1†	1 (ref)	1 (ref)	1 (ref)
Quartile 2	1.03 (0.70-1.51)	1.02 (0.69-1.49)	1.02 (0.70-1.50)
Quartile 3	1.29 (0.91-1.84)	1.26 (0.88-1.79)	1.27 (0.89-1.81)
Quartile 4	1.37 (0.97-1.93)	1.32 (0.93-1.87)	1.33 (0.93-1.91)
P trend	0.035	0.056	0.061
Ischemic strokes (n=197)			
Per SD increase	1.13 (0.99-1.29)	1.12 (0.98-1.27)	1.10 (0.95-1.26)
Quartile 1†	1 (ref)	1 (ref)	1 (ref)
Quartile 2	1.00 (0.64-1.58)	0.99 (0.63-1.55)	0.96 (0.61-1.52)
Quartile 3	1.26 (0.83-1.93)	1.23 (0.80-1.87)	1.19 (0.77-1.82)
Quartile 4	1.38 (0.91-2.09)	1.32 (0.87-2.00)	1.25 (0.81-1.92)
P trend	0.066	0.108	0.194

* Hazard ratios (95% confidence intervals).
† Von Willebrand factor quartiles (IU/mL): 0.24-0.92; 0.92-1.20; 1.20-1.59; 1.60-3.64.
Model 1: adjusted for age and sex.
Model 2: adjusted for age, sex, systolic blood pressure, diabetes mellitus, total cholesterol, HDL cholesterol, lipid lowering medication, current cigarette smoking, waist-to-hip ratio, atrial fibrillation, coronary heart disease, peripheral arterial disease and antithrombotic medication.
Model 3: as model 2, additionally adjusted for ABO blood group.

DISCUSSION

In the present study among subjects aged 55 years and over who were free from stroke at baseline, we found that plasma VWF levels were associated with the risk of stroke. The association was only slightly attenuated after adjustment for multiple potential confounders and was similar in subjects without atrial fibrillation. There was no evidence for effect modification by sex.

Before interpreting the results, some methodological issues need to be considered. The strengths of our study are its prospective and population-based design, the large number of participants, and the long and nearly complete follow-up (loss of potential person years only 1.4%). Thorough stroke monitoring procedures allowed us to include also stroke

patients who had not been referred to a neurologist. A disadvantage of this procedure was that neuroimaging had not been performed in 28% of stroke patients. However, 92% of ischemic strokes had been confirmed by CT or MRI. We used single imputation methods to replace missing values in covariates. Single imputation methods are considered to produce unbiased results, but too much precision, compared to multiple imputation methods [22]. However, as the overall number of missing values in our dataset was small we think the method we used did not have a strong influence on the results.

This is the first study that shows an association between VWF levels and stroke in an elderly population, independent from conventional cardiovascular risk factors and ABO blood group. Previous studies have investigated the relationship between VWF level and stroke. The majority of these studies used a case-control design in which VWF levels were determined after the stroke was diagnosed [6-10]. Results from these studies are likely to be biased by post-stroke changes in VWF levels. Four longitudinal studies have reported on the association between VWF and stroke in the general population [11,23-26]. In a matched case-control study among subjects with atrial fibrillation nested within the Rotterdam Study, no association was found between VWF level and stroke [23]. The Caerphilly Study among middle-aged men also did not find an association between VWF and stroke, after a median follow-up period of 13 year [24]. The Edinburgh Artery Study found a modest, non-significant association when participants were followed for a maximum of 5 years [25], but the association disappeared when the average follow-up period was extended to 17 years [26]. The lack of an association in the Caerphilly Study and the prolonged Edinburgh Artery Study might be explained by inter-individual variability in VWF levels in the long-term, resulting in dilution of the presumed effect. Furthermore, the results of these studies have to be interpreted carefully since they were small or had not excluded participants with prevalent stroke at baseline. Results of the Atherosclerosis Risk in Communities (ARIC) Study, the only prospective study of a cohort free of stroke at baseline with a large number of incident stroke cases, were very much in line with the results of our study [11]. Since ARIC Study participants were younger (45-64 years of age at baseline), our study is the first to provide information about VWF level and stroke risk in the elderly population (aged 55 years and older).

ABO blood group is a strong determinant of plasma VWF levels. Blood group A and B antigens, which are located on the surface of VWF molecules, decrease VWF clearance. As a result, VWF levels are about 25% higher in individuals with blood group non-O than in individuals with blood group O [27]. Because several studies have linked ABO blood group to stroke risk [28], blood group may be a confounder of the association between VWF level and stroke. However, adjustment for ABO blood group did not alter the association between VWF and stroke, suggesting that the association between VWF and stroke is independent from ABO blood group.

We performed a subgroup analysis among participants without atrial fibrillation and found that the association between VWF level and stroke was also present in this subgroup and of similar magnitude as the association we found in the total cohort. Unfortunately, we were not able to examine this association in subjects with atrial fibrillation, because the number of stroke cases in the subpopulation with atrial fibrillation was low. However, our findings in participants without atrial fibrillation suggest that the association between VWF and stroke is not principally driven by the presence of atrial fibrillation.

Recently, there has been increasing awareness of sex differences in stroke. Several studies have shown that the risk factor profile is different in male and female stroke patients. Men with stroke are more likely to have a history of heart disease, myocardial infarction, peripheral arterial disease, diabetes, and alcohol and tobacco use, whereas women with stroke are older at onset and more likely to have atrial fibrillation and hypertension [29-34]. Furthermore, it has been suggested that risk factors may have a different effect on stroke risk in men and women [34]. This motivated us to investigate if sex differences influenced the association between VWF and stroke risk. However, we did not detect any evidence for the presence of effect modification by sex in our study.

The association we found between VWF levels and the subtype ischemic stroke was of similar magnitude as the association we found with any stroke. However, probably due to the smaller number of events, the association was no longer statistically significant at the conventional α=0.05 level. Further studies and systematic reviews are required to establish the nature of the association with confidence.

In conclusion, plasma VWF levels are associated with the risk of stroke in the general population, independent from cardiovascular risk factors and ABO blood group. The association is also present in subjects without atrial fibrillation and does not differ between men and women.

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CHAPTER 4

VON WILLEBRAND FACTOR PROPEPTIDE AND THE OCCURRENCE OF A FIRST ISCHEMIC STROKE

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High von Willebrand factor (VWF) plasma levels are associated with the occurrence of arterial thrombotic disease, such as ischemic stroke (IS) [1]. Since VWF acts as a bridging molecule for platelet adhesion and aggregation at sides of vascular injury, a causal role for VWF in arterial thrombosis is anticipated [2]. However, as plasma VWF is mostly synthesized by endothelial cells and secreted upon endothelial cell activation, a high VWF level also marks endothelial cell activation and damage [3].

VWF propeptide (VWFpp), which has a critical role in VWF multimer assembly, processing and storage, is secreted together with mature VWF and is therefore a marker of endothelial cell activation as well [3,4]. Once secreted, VWF and VWFpp completely dissociate and have different life spans [5]. The half-life of VWF is twelve hours and of VWFpp two hours. Also, a part of secreted VWF, in contrast to secreted VWFpp, is trapped by the sub-endothelial connective tissue, and does not contribute to VWF levels in plasma [6].

Because VWFpp and mature VWF are secreted equimolarly, VWFpp plasma levels reflect the rate of VWF secretion at steady state conditions [7]. Consequently, the association between VWF secretion due to endothelial cell activation and arterial thrombotic disease, such as IS, can be studied separately from the association between total VWF level and IS. Previously, only the association between total VWF plasma levels and IS was studied [1].

We investigated the association between VWFpp, total VWF, and IS in two independent case-control studies (COCOS and ATTAC study) to obtain insight in the contribution of VWF secretion to the association between total VWF and IS.

The COCOS study is a case-control study in which first-ever ischemic stroke (IS) or transient ischemic attack (TIA) patients (aged 18-75) were included. Blood from the patients was collected 7 to 14 days after the IS.

The ATTAC study is a case-control study in which young patients (male aged 18-45, female aged 18-55), who suffered a first acute ischemic complication in the cardiac, or cerebral vascular system, were included. A total of 171 patients with a first IS or TIA were included. Blood from patients was collected three months after the IS. For both studies, controls were friends, neighbours or partners of the patients fulfilling the same age criteria but without a history of arterial thrombosis. The study design of both studies has been described in more detail [8,9]. Population characteristics are given in table 1.

VWF propeptide (VWFpp) and VWF antigen (VWF:Ag) levels were determined with an in-house ELISA as described previously [10,11]. Plasma was available of 101 patients and 103 controls for the COCOS study, and of 171 patients and 171 controls for the ATTAC study.

The VWFpp and VWF:Ag levels were divided into quartiles based on the distribution in the control group. The association between levels of these variables and the risk of IS was determined by means of logistic regression. The risk of IS for individuals in the highest quartile was compared with the risk of IS for individuals in the lower three quartiles, and expressed as odds ratio (OR) with 95 confidence interval (95% CI).

For both studies, the mean (± standard deviation (SD)) VWFpp, VWF:Ag levels, and the VWFpp/VWF:Ag ratio for patients and controls are presented in Table 1. In the COCOS study, the estimated relative risk for IS in the highest quartile of VWFpp levels was 1.9 (95% CI: 1.0-3.4) after adjustment for age and sex, and 1.9 (95% CI: 1.0-3.6) after additional adjustment for conventional cardiovascular risk factors (Table 1). For VWF:Ag levels, this was 2.1 (95% CI: 1.2-3.4), and 1.9 (95% CI: 1.0-3.3) respectively. In the ATTAC study, the estimated relative risk for IS in the highest quartile of VWFpp levels was 1.8 (95% CI: 1.1-2.8) after adjustment for age and sex, and 1.7 (95% CI: 1.1-2.8) after adjustment for conventional risk factors. For VWF:Ag levels this was 2.0 (95% CI: 1.2-3.2), and 1.9 (95% CI: 1.1-3.1) respectively. None of the relative risk estimates were influenced by additional adjustment for blood group.

The present study shows for the first time that in two independent case-control studies, increased VWF secretion, as measured by VWFpp levels, and increased total VWF:Ag levels both contribute substantially to the occurrence of IS. Plasma VWF levels are determined by the rate of VWF secretion and clearance. If VWF is a strong causal factor in the development of IS, it is expected that increased total VWF levels would be stronger associated with the risk of IS than increased VWF secretion alone. Our data suggest that increased VWF secretion, probably due to endothelial cell activation, is an important mechanism underlying the association between high total VWF plasma levels and the occurrence of IS. However, in both studies there is a trend towards a lower VWFpp/VWF:Ag ratio in patients compared to controls (Table 1), and therefore, a lower clearance of VWF may also contribute to the association between total VWF levels and IS.

Our study has some limitations. Firstly, in the two studies, blood was collected at different time points after the event which may raise the question whether the data are comparable and VWFpp and VWF:Ag levels reflect the same situation after the IS. Yet, in a subset of patients of the COCOS study (n=64), blood was also collected three months after the event. No significant differences were observed in VWFpp and VW:Ag levels between measurements one week and three months after the event (data not shown). Therefore, the different time points of blood sampling in the two studies probably have a small effect on the measured levels and calculated associations. Secondly, patients of both studies used statins at the time of inclusion. Statins decrease VWF plasma levels, probably by influencing VWF secretion. Consequently, differences in VWF:Ag levels between patients and controls might be underestimated. Whether statins influence VWFpp levels is unknown but this may be expected, as the two proteins are secreted in an equimolar ratio.

Table 1 | The characteristics of study participants

	Case-control study 1: COCOS study			Case-control study 2: ATTAC study		
	Patients (n=101)	Controls (n=106)	P value	Patients (n=171)	Controls (n=171)	P value
Demographics						
Age, Years	55 ± 12	56 ± 12	NS	42 ± 7.7	42.5 ± 5.8	NS
Female sex	49 (48%)	51 (47%)	NS	132 (77%)	113 (66%)	0.02
IS: TIA	96: 7			84: 87		
Blood Group:						
O	44 (45%)	54 (53%)	NS	80 (47%)	86 (50%)	NS
Non-O	53 (55%)	48 (47%)		91 (53%)	85 (50%)	
Stroke subtypes (TOAST):						
Large vessel disease	10			12		
Cardiac embolism	0			30		
Small vessel disease	36			18		
Dissection	0			8		
Other etiology	1			10		
Cryptogenic IS	54			93		
Cardiovascular risk factors						
Smoking	52 (53%)	30 (29%)	<0.001	111 (65%)	92 (54%)	0.05
Hypertension	42 (42%)	22 (21%)	<0.001	60 (35%)	17 (10%)	<0.001
Diabetes mellitus	13 (13%)	5 (5%)	0.05	11 (6%)	3 (2%)	0.04
Hyperlipidemia	61 (62%)	69 (66%)	NS	55 (32%)	11 (6%)	<0.001
VWFpp (U/ml)	1.29 (± 0.37)	1.20 (± 0.31)	0.05	0.96 (± 0.33)	0.89 (± 0.23)	0.08
VWF:Ag (U/ml)	1.47 (± 0.68)	1.25 (± 0.50)	0.03	1.25 (± 0.56)	1.10 (± 0.39)	<0.001
VWFpp/VWF:Ag ratio	0.99 (± 0.38)	1.07 (± 0.34)	0.05	0.83 (± 0.27)	0.89 (± 0.29)	0.09

Data for age, VWFpp levels, VWF:Ag levels and the VWFpp/VWF:Ag ratio are presented as mean ± SD, other data as counts and (percentages). IS indicates ischemic stroke; TIA, transient ischemic attack; NS, not significant and TOAST, Trial of Org 10172 in Acute Stroke Treatment.

The strength of our study is that we measured both VWFpp and VWF:Ag levels in two independent case-control studies in which well defined IS patient were included. The fact that the two studies show similar results, stresses the validity of the associations.

In conclusion, we showed that VWFpp and VWF were both associated with the occurrence of IS. This indicates that endothelial cell activation and subsequently secretion of VWF is an important mechanism underlying the association between total VWF levels and the occurrence of a first ischemic stroke.

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CHAPTER 5

ACTIVE VON WILLEBRAND FACTOR AND THE RISK OF STROKE

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Under conditions of high shear stress, von Willebrand factor (VWF) has an important function in recruiting platelets to injured vessel walls. In a resting state, VWF molecules circulate in an inactive conformation and are unable to interact spontaneously with platelets. Under the influence of high shear stress and by binding to collagen, these circulating VWF molecules are converted into an active GPIba-binding conformation [1]. By contrast, the ultra large VWF (UL-VWF) molecules secreted by activated endothelial cells are already in the active conformation [2]. Only active VWF molecules are able to interact with GPIba receptors on platelets.

A recombinant llama-derived antibody fragment (AU/VWF-11) enables direct and specific detection of active VWF in plasma of patients characterized by spontaneous VWF-platelet interaction [3]. Previous studies with this assay have demonstrated that the active proportion of circulating VWF is increased during pathological conditions which increase the risk of thrombotic micro-angiopathies (TMA), such as thrombotic thrombocytopenic purpura (TTP) [3].

Although high VWF plasma levels are associated with an increased risk of arterial thrombotic diseases, such as ischemic stroke (IS) [4], the association between active VWF and the risk of IS is unknown. Therefore, the aim of this study was to determine whether increased circulating active VWF levels are associated with an increased risk of IS.

We studied active VWF in a case-control study in patients with a first-ever ischemic stroke (IS) or transient ischemic attack (TIA), in which we previously observed an association between VWF and ADAMTS13 with IS [5]. Patients with a definite non-atherosclerotic cause of the IS were excluded from the study. The population-based controls were age- and sex-matched, and without a history of IS. Population characteristics are given in Table 1. Blood was collected 7-14 days after the event. Three months after the event blood was again collected from an unselected subgroup of 43 patients.

Circulating VWF in its active GPIba-binding conformation was measured using the AU/VWFa-11 llama-derived antibody, and expressed as VWF activation factor [3]. The ADAMTS13 activity (An Disintegrin and Metalloproteinase with a Thrombospondin type 1 motive), VWF ristocetin cofactor activity (VWF:RCo), and VWF antigen (VWF:Ag) levels were measured as described previously [6].

Since the measured levels were not normally distributed, the VWF activation factor in cases and controls was compared using a Mann-Whitney test. The VWF activation factors were divided into quartiles on the basis of their distribution in the control group and the relation with IS was determined by means of logistic regression, with the lowest quartile as the reference. We adjusted for well-known risk factors for cardiovascular diseases (Table 1). Correlations were estimated with the Spearman correlation coefficient (r_s).

The VWF activation factor (expressed as median, [25%-75% range]) was not different between IS patients and controls both one week (1.2 [0.9-1.8] versus 1.3 [0.9-1.7]; p=0.9), and three months after the event (1.2 [1.0-1.6] versus 1.3 [0.9-1.7]; p=0.4). The VWF activation

factor was also similar in the subgroup of 35 patients with IS caused by small vessel disease (1.2 [0.8-1.7]; p=0.8) and the control group. Also logistic regression showed no relationship between the VWF activation factor and the risk of IS.

Table 1 | The Characteristics of the Study participants

	Cases (n=94)	Controls (n=103)	P value
Demographics:			
Age, (years)	55 ± 12	56 ± 12	NS
Female sex	49 (48%)	51 (47%)	NS
Blood group:			
O	44 (45%)	54 (53%)	0.33
Non-O	53 (55%)	48 (47%)	
Index event:			
IS:TIA	88:6		
Stroke subtypes (TOAST criteria):			
Large vessel disease	9		
Cardiac embolism	3		
Small vessel disease	35		
Other etiology	1		
Undetermined etiology	46		
Risk factors:			
Smoking	52 (53%)	30 (29%)	0.001
Hypertension	42 (42%)	22 (21%)	0.001
Diabetes mellitus	13 (13%)	5 (5%)	0.05
Hypercholesterolemia	61 (62%)	69 (66%)	NS
Positive family history	81 (67%)	64 (53%)	0.03
VWF:Ag (μg/ml)	13.2 (9.8-18.5)	12.2 (8.4-15.6)	0.04
VWF activation factor	1.2 (0.9-1.8)	1.3 (0.9-1.7)	NS
VWF: Rco (U/ml)	1.2 (0.8-1.6)	1.07 (0.8-1.5)	0.09
ADAMTS13 (U/ml)	0.9 (0.7-1.2)	0.95 (0.7-1.3)	NS

Data for age are presented as mean ± SD. Data for VWF:Ag, VWF:Rco, VWF activation factor and ADAMTS13 activity are presented as median (25%-75% range). Other data are counts and (percentages). IS indicates ischemic stroke; TIA, transient ischemic attack; NS, not significant and TOAST, Trial of Org 10172 in Acute Stroke Treatment.

The difference between the results of our study and the studies on TMA, such as TTP, may be explained by differences in the underlying pathophysiologic mechanisms. In our study, the ischemic strokes are to a large extent caused by complications of large and small vessel

atherosclerosis. In addition, as we previously described, there are only subtle differences in ADAMTS13 activity between IS patients and controls, suggesting that the proteolyses of VWF by ADAMTS13 is normal [6]. In TTP however, a low ADAMTS13 activity results in high amounts of active UL-VWF in the circulation leading to microvascular thrombus formation [7]. Consumption of active VWF in patients could also explain why no increase in the VWF activation factor was seen. Furthermore, previous studies showed that VWF:Ag levels are decreased by drugs such as statins [8]. Whether drugs could also influences the VWF activation factor is unknown.

In the control group, we found a significant negative correlation between the VWF activation factor and ADAMTS13 (r_s=-0.30, p<0.001). No correlation was seen between the VWF activation factor and ADAMTS13 in IS patients (r_s=-0.14, p=0.2), suggesting a disturbed balance between these two proteins, which may be the result of an acute phase response. We found no correlation between VWF:RCo and ADAMTS13 in the subgroups of cases and controls (r_s=-0.15, p=0.14 versus r_s=-0.16, p=0.09). This is expected, since VWF:RCo levels show the total amount of VWF that could be potentially activated in plasma. However, the AU/VWF-11 assay measures the proportion of VWF in the plasma that is already in its activated state.

The VWF activation factor in the control group was significantly higher in blood group non-O than in blood group O (1.5, [1.1-1.8] versus 1.1, [0.8-1.5]; p<0.001). Previous studies showed that VWF levels are higher in subjects with blood group non-O than O [9]. Our study demonstrates for the first time that, in a steady state condition, healthy individuals with blood group non-O have more VWF in the active conformation per VWF molecule than healthy individuals with blood group O. Whether this may contribute to the increased risk of arterial thrombosis in individuals with blood group non-O, as reported in earlier studies, remains to be elucidated [10]. The VWF activation factor in the patient group was not different between individuals with blood group non-O and O, possibly due to the influence of the IS on the active VWF balance.

One of the limitations of our study is the small size of the study population. This results in limited power to detect moderate differences in VWF activation factor between patients and controls. Since the etiological subtype groups were small and patients with a definite non-atherosclerotic cause of the IS were excluded, it was not possible to do an extensive subtype analysis.

In conclusion, this study shows that the VWF activation factor was not different between patients with IS and controls. Interestingly, the active proportion of circulating VWF is higher in healthy individuals with blood group non-O than in healthy individuals with blood group O. Overall, our data suggest that circulating active VWF does not play an important role in the development of IS, and that two distinct processes cause arterial thrombosis (including small vessel disease) in IS patients, and microvascular thrombosis in TTP patients.

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CHAPTER 6

VARIATION IN THE VON WILLEBRAND FACTOR GENE IS ASSOCIATED WITH VON WILLEBRAND FACTOR LEVELS AND WITH THE RISK FOR CARDIOVASCULAR DISEASE

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ABSTRACT

High levels of von Willebrand factor (VWF) are associated with an increased risk for cardiovascular disease (CVD). Although VWF levels are strongly heritable and genetic susceptibility is an important risk factor for CVD, information on the contribution of common *VWF* gene variants to VWF levels and CVD risk is limited. In a case-control study of 421 young patients with a first event of acute coronary heart disease (CHD) or ischemic stroke (IS), and 409 healthy controls (men aged ≤45, women aged ≤55), 27 haplotype-tagging single nucleotide polymorphisms (ht-SNPs), covering the total common VWF gene variation, were selected and genotyped. The associations between these SNPs, VWF antigen (VWF:Ag) levels, VWF collagen binding (VWF:CB) activity, and CVD risk, was investigated. Two new associations were identified. For ht-SNP rs4764478 (intron 45), the increase in VWF:Ag levels and VWF:CB activity per minor allele was 0.082 (± 0.026) IU/ml (p=0.001) and 0.096 (± 0.030) IU/ml (p=0.002), respectively. Ht-SNP rs216293 (intron 17), was associated with CVD risk (odds ratio (OR) 1.44, 95% CI 1.12-1.86 per minor allele). We confirmed the association between rs1063857 and CVD risk. Our data show that common variants in the VWF gene are associated with VWF levels and with the risk of CVD.

INTRODUCTION

Von Willebrand factor (VWF) plays a key role in arterial thrombus formation because it facilitates platelet adhesion and aggregation at sites of vascular injury [1]. For this reason, an association between VWF plasma levels and cardiovascular disease (CVD) is anticipated, and has been investigated extensively. Many studies have shown that high VWF levels are positively associated with the risk of coronary heart disease (CHD) and ischemic stroke (IS) [2-4].

Plasma levels of VWF are not only influenced by non-genetic factors such as hormones and inflammation, but are also strongly heritable, with estimates of heritability ranging from 53-75% [5,6]. As genetic susceptibility is an important risk factor for CVD [7,8], several candidate studies have addressed the question whether single nucleotide polymorphisms (SNPs) in the VWF gene determine VWF levels, and subsequently contribute to the genetic susceptibility for CVD.

Because the VWF promoter region is involved in the regulation of gene transcription, and is therefore expected to be a determinant of VWF plasma levels, most studies have focused on SNPs in the promoter region of the VWF gene. In a population of healthy individuals, homozygotes for the minor allele of the VWF promoter variant rs7966230 had significantly higher VWF antigen (VWF:Ag) levels than homozygotes for the common allele [9]. In a case-control study, nested within a large prospective population-based cohort (Rotterdam study), the minor allele of this promoter variant was also associated with the risk of CHD, specifically in the sub-group of individuals with advanced atherosclerosis [10]. However, because several other studies could not demonstrate an association with VWF levels or CVD [5,11]. it remains unclear whether SNPs in the VWF promoter region contribute significantly to VWF:Ag levels and CVD risk.

SNPs in coding regions of the *VWF* gene are also of interest, as they may be related to protein changes that influence VWF structure and function. Several SNPs, located in exonic regions known to be involved in the regulation of VWF multimer size (exon 18 and exon 28), have been identified [12,13]. Although VWF multimeric composition is a strong determinant of its functional activity [14], the limited studies available on coding regions of the VWF gene mainly focussed on the contribution of common variants to VWF:Ag levels, and not on their contribution to VWF activity or CVD [12,13].

We investigated the association between common variation spanning the total VWF gene (including 12kb of the 5'and 3'flanking regions), VWF:Ag levels, VWF collagen binding activity (VWF:CB), and the risk of CVD. A total of 27 haplotype-tagging SNPs (ht-SNPs) representing common variation in the VWF gene were selected, and were subsequently genotyped in young patients with a first event of acute CHD or IS and in healthy control participants.

METHODS

Study population

In a single-center case-control study (Arterial Thrombosis at a young age: the role of TAFI and other Coagulation factors [ATTAC] study), we have included consecutive patients with arterial thrombotic disease at young age, and healthy control paricipants [15]. Male patients aged 18 to 45, and female patients aged 18 to 55, who experienced a first acute ischemic complication in either the heart or the brain, were eligible for inclusion. The patients had coronary heart disease (CHD), including acute myocardial infarction (AMI) or unstable angina pectoris (UAP), or ischemic stroke (IS) including patients with transient ischemic attacks (TIA). Patients were included one to three months after the event to avoid the effects of an acute phase response. Control participants were friends, neighbours or partners of the patients fulfilling the same age criteria but did not have a history of CVD, and were not related to the patients. For this study, we included only individuals with European ancestry. The study design has been described previously in more detail [15].

The study protocol is in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of Erasmus MC. Written informed consent was obtained from each participant.

Definitions

AMI was defined as typical chest pain, with elevated cardiac markers (CK, MB, troponin T) and/or characteristic electrocardiographic findings. UAP was defined as typical chest pain while at rest confirmed by characteristic electrocardiographic findings and normal levels of cardiac markers. IS was defined as suddenly occurring cerebral deficit, which cannot be explained otherwise than as local cerebral ischemia, and that lasted for longer than 24 hours after onset. TIA was defined similarly, but symptoms had to be temporary and lasting less than 24 hours after onset. Brain imaging by CT or MRI was required to confirm the initial diagnosis.

Clinical data were collected by means of a standard medical questionnaire and a physical examination was performed by a research physician. European ancestry was self reporting. To meet the definition of European ancestry grandparents of the included individuals had to be born in northern or western Europe. Smoking was defined as previous or current smoking. Hyperlipidemia was defined as either plasma total cholesterol >5.0 mmol/L or the use of lipid-lowering treatment on the day of the ischemic event. Patients with a medical history of diabetes, or patients using oral anti-diabetic medication or insulin on the day of the ischemic event, were considered to be diabetics. Hypertension was defined as a systolic blood pressure >140 mm Hg, a diastolic blood pressure >90 mm Hg, or the use of anti-hypertensive medication on the day of inclusion. Body mass index (BMI) was calculated for each participant by dividing weight (kg) by the square of height (m^2). A positive family

history was noted if the patient had a first-degree relative with a positive history of CVD before the age of 60.

Blood sampling procedure and plasma measurements

Under standardized conditions, blood was collected into citrate (0.105 mol/L) using the Vacutainer system (Beckton Dickinson, Plymouth, UK). Citrated blood was centrifuged at 2000 *g* for 10 min at 4°C. Plasma was additionally centrifuged at 20 000 *g* for 10 min at 4°C, and stored at -80°C until analysis. For DNA isolation, blood was collected in tubes containing EDTA (Beckton Dickinson). Genomic DNA was extracted and stored at 4°C for genetic analysis.

Von Willebrand factor antigen (VWF:Ag) levels were determined with an in-house enzyme-linked immunosorbent (ELISA) assay with use of polyclonal rabbit anti-human VWF antibodies (DakoCytomation) for capturing and detecting. The intra-assay coefficient of variation was 5.7% and the inter-assay coefficient of variation was 7.8%.

Von Willebrand factor collagen binding (VWF:CB) activity was measured by an in-house ELISA assay using bovine Achilles tendon collagen type I for capture (Sigma-Aldrich) and polyclonal rabbit antihuman VWF antibodies (DakoCytomation) for detection. The intra-assay coefficient of variation was 5.8% and the inter-assay coefficient of variation was 9.1%.

Both assays used commercial reference plasma (normal reference plasma, Precision biologic, Kordia) which were standardized against the WHO standard by the manufacturer.

Selection of SNPs in the *VWF* gene

The *VWF* gene, spanning 175.8 kbp, is located on the short arm of chromosome 12 (p13.3) and contains 52 exons. Thus far, more than 950 SNPs in this gene have been annotated in the National Center for Biotechnology Information's (NCBI's) SNP database (dbSNP, build 130). In our study, we considered only SNPs that were present in populations with northern and western European ancestry, with a minor allele frequency of at least 5%. From these SNPs, we selected haplotype tagging SNPs (ht-SNPs) in the *VWF* gene, including 12kb of the 5' and 3' flanking regions. The selection of ht-SNPs was performed on the basis of the linkage disequilibrium (LD) map of the *VWF* locus provided by the International HapMap Project (phase II, April 2007; www.HapMap.org). For the *VWF* gene, blocks of haplotypes with a frequency >5% were defined and their ht-SNPs were selected as implemented in the Haploview software (version 4.1; www.broadinstitute.org/mpg/haploview) [16]. This resulted in the selection of 27 ht-SNPs described in table 1 and presented in figure 1. The ht-SNPs cover all of the common variation in the *VWF* gene which is 70-95% of the total variation, depending on the LD in the haplotype blocks.

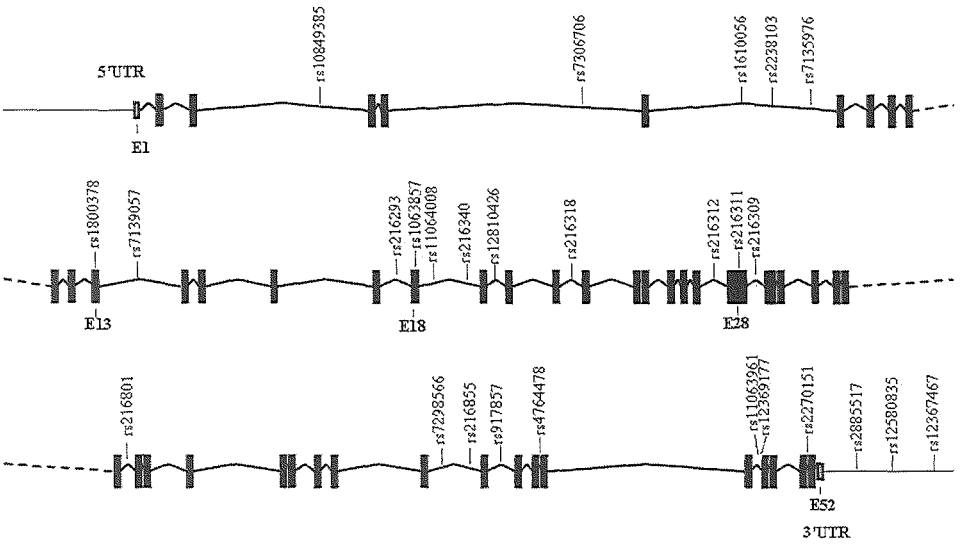

Figure 1 | Schematic representation of the *VWF* gene and the location of the ht-SNPs. Boxes are exons and lines connecting boxes are introns. Filled or darkened boxes are coding sequence, whereas empty, unfilled boxes are untranslated region (UTR). E indicates exon.

Genotyping

A total of 26 SNPs were genotyped with the use of pre-designed TaqMan Genotyping Assays (Applied Biosystems). One SNP was genotyped with a custom-designed TaqMan Genotyping Assay (Applied Biosystems).

The nucleotide sequences of the primers and probes used for each assay are available upon request. The context sequences for the SNPs are presented in supplemental Table 1. Endpoint fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems) and clustered according to genotype using SDS 2.2.2 software (Applied Biosystems). For 25 SNPs, genotyping was successful for >95% of the 830 subjects. SNPs rs7135976 and rs11064008 had call rates below 95% and were therefore excluded from the analyses.

A random selection of 10% of the samples was reanalyzed for every genotyping assay. This showed that the assays had a reproducibility of more than 99%. To ensure DNA quality, only participants who were successfully genotyped for more than 90% of the 25 SNPs (n=753) were included in the analysis. For the 25 SNPs, patients and control subjects were equally successfully genotyped and therefore missing genotype data was not patient related.

Table 1 | Haplotype-tagging SNPs in the *VWF* gene

Region VWF gene	position[a]	ht-SNP ID[b]	variant[c]	MAF[d]
Intron 3	6227919	rs10849385	A>G	0.36
Intron 5	6215634	rs7306706	A>G	0.43
Intron 6	6195397	rs1610056	C>G	0.09
Intron 6	6194183	rs2238103	A>T	0.43
Intron 6	6186116	rs7135976	T>C	0.46
Exon 13	6172202	rs1800378	c1451G>A	0.38
Intron 13	6169323	rs7139057	T>G	0.17
Intron 17	6153659	rs216293	T>G	0.46
Exon 18	6153514	rs1063857	c2385T>C	0.35
Intron 18	6153264	rs11064008	C>G	0.12
Intron 18	6149799	rs216340	G>A	0.19
Intron 19	6144138	rs12810426	G>A	0.14
Intron 21	6139261	rs216318	C>A	0.10
Intron 27	6128984	rs216312	C>T	0.48
Exon 28	6128443	rs216311	c4141G>A	0.38
Intron 28	6126927	rs216309	T>C	0.48
Intron 34	6108513	rs216801	G>A	0.37
Intron 42	6088103	rs7298566	A>C	0.13
Intron 42	6085513	rs216855	G>A	0.21
Intron 43	6081824	rs917857	G>A	0.44
Intron 45	6078125	rs4764478	T>A	0.25
Intron 47	6074016	rs11063961	A>G	0.22
Intron 47	6073520	rs12369177	G>T	0.33
Intron 50	6060960	rs2270151	G>A	0.17
3'FR	6050320	rs2885517	C>T	0.38
3'FR	6049784	rs12580835	T>C	0.45
3'FR	6049568	rs12367467	C>G	0.12

[a]Nucleotide position chromosome 12. [b]Haplotype tagging SNP ID according to NCBI dbSNP. [c]Nucleotide variant. For the SNPs located in exons the cDNA reference sequence has been provided. [d]MAF: minor allele frequency participants with European Ancestry. 3'FR: 3'flanking region.

Statistical analysis

Data on population demographics are presented as means and standard deviations (SD) for continuous variables and as counts and percentages for categorical variables. To test whether the genotype distributions deviated from that expected for a population in Hardy-Weinberg equilibrium, we used a chi-squared test with one degree of freedom. In the

original study population, individuals without European ancestry were also included. The allele frequencies in the different ethnic populations were compared using Fisher's exact test. The allele frequencies of SNPs and the linkage disequilibrium (LD) patterns between SNPs were significantly different between participants with, and without European ancestry. Therefore, for this study, the analyses were conducted in individuals with European ancestry only.

Because of non-normality, VWF:Ag levels and VWF:CB activity were rank normal transformed, as implemented in GenABEL [17]. The association between genotypes and VWF:Ag levels and VWF:CB activity were analyzed using linear regression models (under the assumption of an additive genetic model). The covariates included in the multivariable model were age, sex, case status, blood group, hormone use and time of blood sampling. The β represents the change per minor allele in standard deviations of the transformed outcomes. Due to this transformation, the change in VWF:Ag levels and VWF:CB activity in international units per milliliter (IU/ml) is an estimation of the real change.

The associations between genotypes for each SNP and the risk of CVD were analyzed using logistic regression models (under the assumption of an additive genetic effect). Two models were fitted. The first multivariable model included genotype, and the covariates age and sex. The second multivariable model additionally included the covariates smoking, hypertension, diabetes, hyperlipidemia, and blood group. Missing values in covariates were imputed at the series mean. Logistic regression analysis was additionally conducted for subgroups of CHD and IS patients.

We implemented multiple testing corrections by calculating the False Discovery Rate ("q-value") for each SNP. The q-value of a test measures the proportion of false positives incurred (the false positive rate), when the particular test is significant [18].

We inferred haplotypes using the R package haplo.stats [19,20]. The association between the haplotypes and the risk of arterial thrombosis was determined by weighted logistic regression analysis. This haplotype analysis calculates posterior probabilities for each possible haplotype for an individual and assigns an appropriate weight to account for this uncertainty in subsequent risk estimation [20]. The associations between the haplotypes and VWF:Ag levels and VWF:CB activity were similarly determined by linear regression analysis accounting for posterior uncertainty. The models used in the single SNP analyses were also utilized in the haplotype analysis. The most common haplotype was taken as the reference. Haplotype analysis was conducted in two ways. The first analysis was based on the haplotype block pattern in the VWF gene provided by the international hapmap project (phase II, April 2007; www.HapMap.org). In the second analysis, haplotypes in the *VWF* gene were analyzed with a sliding window approach.

We conducted the analyses using R, SPSS version 15 (SPSS Inc, Chicago, USA), Haploview, and the haplo.stats package. In statistical analyses, a p-value of <0.05 (two sided) was considered significant.

RESULTS

Characteristics of the study participants

A total of 421 patients and 409 controls with European ancestry were included in the study. The characteristics of the study participants are presented in Table 2. Of the patient group, 271 participants had CHD, and 150 participants had IS. Patients were somewhat older (43.2 ± 6.7 years) than control participants (39.6 ± 7.6 years, p=0.001). A total of 59% of the included individuals in the patient group and 65% of the included individuals in the control group were female (p=0.09). The distribution of blood group was similar in patients and control participants (p=0.78). As expected, the prevalence of classical CVD risk factors was significantly higher in patients than in control participants. In addition, at the time of inclusion, most patients used medications including anti-platelet drugs, antihypertensive drugs and statins. The VWF:Ag levels (mean±SD) were significantly higher in patients than in control participants (1.26 ± 0.53 IU/ml versus 1.09 ± 0.37 IU/ml, p<0.001). In addition, VWF:CB activity was significantly higher in patients than in control participants (1.38 ± 0.54 IU/ml versus 1.25 ± 0.42 IU/ml, p<0.001). There was no difference in VWFCB/VWF:Ag ratio between patients and control participants (1.15 ± 0.31 versus 1.17 ± 0.25, p=0.40).

VWF genotypes

The genotype distributions of 24 out of the 25 VWF SNPs were in Hardy-Weinberg equilibrium. In the control group, rs216311 was not in Hardy-Weinberg equilibrium (p<0.001), and was therefore excluded from the analyses. The allele frequencies for participants with European ancestry were similar to those reported by dbSNP.

Common genetic variation, VWF:Ag levels and VWF:CB activity

A total of 4 SNPs were associated with VWF:Ag levels and two with VWF:CB activity after adjustment for age, gender, case-status, blood group, hormone use and time of sampling (Table 3). SNP rs7306706 was nominally associated with a decrease in VWF:Ag level (-0.043 ± 0.021 IU/ml per minor allele, p=0.04) . SNP rs1063857 was marginally associated with an increase in VWF:Ag levels (0.039 ± 0.022 IU/ml per minor allele, p=0.08). SNP rs216318 was nominally associated with a decrease in VWF:Ag levels (-0.068 ± 0.036 IU/ml, p=0.05) and with a decrease in VWF:CB activity (-0.093 ± 0.042 IU/ml, p=0.03). There was no association between rs216318 and the VWFCB/VWF:Ag ratio (p=0.83). Finally, rs4764478 was nominally associated with an increase in VWF:Ag levels (0.082 ± 0.026 IU/ml, p=0.001), and an increase in VWF:CB activity (0.096 ± 0.030 IU/ml, p=0.002), but was not associated with the VWFCB/VWF:Ag ratio (p=0.88). After adjustment for multiple testing, only SNP rs4764478 remained associated with a significant increase in VWF:Ag levels (q=0.03) and with a significant increase in VWF:CB activity (q=0.03). For the respective common homzygotes, heterozygotes and rare homozygotes for SNP rs4764478, the mean VWF:Ag levels were

1.12 IU/ml, 1.20 IU/ml and 1.28 IU/ml, and the mean VWF:CB activity was 1.26 IU/ml, 1.34 IU/ml and 1.38 IU/ml.

Table 2 | Characteristics of the study population

	Patients (421)	Controls (409)	P value
Demographics:			
Age	43.2 (± 6.7)	39.6 (± 7.6)	0.001
Female gender	248 (59%)	265 (65%)	0.09
Classical CVD risk factors:			
Smoking[a]	332 (79%)	209 (51%)	<0.001
Hyperlipidemia	146 (35%)	15 (4%)	<0.001
Hypertension	119 (28%)	24 (6%)	<0.001
Diabetes Mellitus	30 (7%)	3 (1%)	<0.001
Positive family history	206 (49%)	91 (22%)	<0.001
BMI	26.7 (± 4.7)	25.5 (± 4.3)	<0.001
Blood group:			
O	187 (44%)	186 (45%)	0.78
Non-O	234 (56%)	223 (55%)	
Medication:			
Anti-hypertensive drugs	311 (74%)	18 (4.5%)	<0.001
Statins	344 (82%)	6 (1.5%)	<0.001
Anti-platelet drugs	404 (96%)	6 (1.5%)	<0.001
Hormone use[b]	84 (34%)	87 (33%)	0.80
Cardiovascular index event:			
Coronary heart disease	271 (64%)		
Ischemic stroke	150 (36%)		
VWF:Ag (IU/ml)	1.26 ± 0.53	1.09 ± 0.37	<0.001
VWF:CB (IU/ml)	1.38 ±0.54	1.25 ± 0.42	<0.001
VWF:CB/VWF:Ag ratio	1.15 ± 0.31	1.17 ± 0.25	0.40

Data for age, VWF:Ag levels, VWF:CB activity and VWF:CB/VWF:Ag ratio are presented as mean±SD, other data as counts and (percentages). BMI indicates body mass index [a]Previous and current smokers. index. [b]Hormone use includes oral contraceptives and hormone replacement therapy.

Table 3 | Association between SNPs, VWF:Ag levels and VWF:CB activity

SNP ID	VWF:Ag levels (±SE)	P-value	q-value	VWF:CB activity (±SE)	P-value	q-value
rs10849385	-0.003 (± 0.022)	0.88	0.90	0.008 (± 0.026)	0.76	0.70
rs7306706	***-0.043 (± 0.021)***	***0.04***	***0.42***	-0.037 (± 0.025)	0.14	0.58
rs1610056	-0.004 (± 0.032)	0.90	0.90	-0.001 (± 0.038)	0.98	0.74
rs2238103	-0.012 (± 0.022)	0.59	0.87	-0.027 (± 0.026)	0.32	0.58
rs1800378	0.019 (± 0.022)	0.39	0.87	0.033 (± 0.026)	0.21	0.58
rs7139057	-0.014 (± 0.027)	0.60	0.87	-0.035 (± 0.032)	0.27	0.58
rs216293	0.005 (± 0.022)	0.81	0.90	-0.011 (± 0.026)	0.66	0.70
rs1063857	***0.039 (± 0.022)***	***0.08***	***0.48***	0.030 (± 0.027)	0.26	0.58
rs216340	0.004 (± 0.026)	0.87	0.90	-0.007 (± 0.031)	0.82	0.70
rs12810426	0.004 (± 0.025)	0.88	0.90	-0.037 (± 0.030)	0.22	0.58
rs216318	***-0.068 (± 0.036)***	***0.05***	***0.42***	***-0.093 (± 0.042)***	***0.03***	***0.26***
rs216312	-0.013 (± 0.022)	0.56	0.87	-0.022 (± 0.026)	0.41	0.60
rs216309	-0.013 (± 0.022)	0.54	0.87	-0.016 (± 0.026)	0.53	0.60
rs216801	-0.013 (± 0.022)	0.54	0.87	0.006 (± 0.026)	0.83	0.69
rs7298566	-0.025 (± 0.030)	0.39	0.87	-0.023 (± 0.035)	0.52	0.59
rs216855	0.002 (± 0.023)	0.94	0.90	-0.004 (± 0.028)	0.88	0.70
rs917857	-0.009 (± 0.021)	0.66	0.89	-0.036 (± 0.025)	0.15	0.58
rs4764478	***0.082 (± 0.026)***	***0.001***	***0.03***	***0.096 (± 0.030)***	***0.002***	***0.03***
rs11063961	0.027 (± 0.024)	0.26	0.87	0.024 (± 0.029)	0.41	0.60
rs12369177	-0.007 (± 0.022)	0.76	0.90	0.004 (± 0.026)	0.88	0.70
rs2270151	0.021 (± 0.028)	0.46	0.87	0.035 (± 0.034)	0.29	0.58
rs2885517	-0.011 (± 0.022)	0.60	0.87	-0.010 (± 0.026)	0.70	0.69
rs12580835	-0.014 (± 0.022)	0.52	0.87	-0.019 (± 0.026)	0.47	0.59
rs12367467	0.037 (± 0.034)	0.28	0.87	0.030 (± 0.041)	0.46	0.60

VWF:Ag levels and VWF:CB activity in IU/ml (±SE): an estimated change in level per minor allele. The presented data in the table are adjusted for age, gender, case-status, blood group, hormone use and time of blood sampling.

Common genetic variation and the risk for CVD.

After adjustment for the full model, two SNPs were nominally associated with an increased risk of CVD (Table 4). For rs216293, the estimated increase in risk (OR, 95% CI) per minor allele was 1.44 (95% CI 1.12-1.86). For SNP rs1063857, the estimated increase in risk per minor allele was 1.32 (95% CI, 1.02-1.70). After adjustment for multiple testing, only rs216293 remained associated with a higher risk of CVD (q=0.03). It is interesting to note that the associations between the two SNPs and the risk for CVD was predominantly seen in the subgroup of IS patients. In this subgroup, the estimated increase in risk per minor allele

was 1.50 (95% CI, 1.17-1.8) for SNP rs216293, and 1.35 (95% CI, 1.03-1.68) for SNP rs1063857. In the subgroup of patients with CHD, the estimated increase in risk per minor allele was 1.26 (95% CI, 0.94-1.58) for SNP rs216293, and 1.15 (95% CI, 0.82-1.48) for SNP rs1063857.

VWF Haplotypes

Haplotype analysis confirmed the single SNP analyses; in 3-SNP sliding windows, haplotypes containing the associated SNPs were also related to VWF:Ag levels, and to CVD risk. Neither type of haplotype analysis, however, provided additional information on the contribution of common variation in the VWF gene to VWF:Ag levels, VWF:CB activity and the risk for CVD (data not shown).

Table 4 | Association between SNPs and the risk for CVD

SNP ID	OR [95% CI]	P-value	q-value
rs10849385	0.86 [0.67, 1.11]	0.25	0.19
rs7306706	0.85 [0.67, 1.08]	0.17	0.18
rs1610056	0.86 [0.60, 1.24]	0.42	0.19
rs2238103	1.07 [0.84, 1.37]	0.60	0.20
rs1800378	1.12 [0.88, 1.44]	0.37	0.19
rs7139057	0.93 [0.69, 1.26]	0.63	0.20
rs216293	***1.44 [1.12, 1.86]***	***0.004***	***0.03***
rs1063857	***1.32 [1.02, 1.70]***	***0.04***	***0.12***
rs216340	0.93 [0.69, 1.25]	0.64	0.20
rs12810426	1.04 [0.78, 1.39]	0.78	0.21
rs216318	1.25 [0.83, 1.89]	0.28	0.19
rs216312	1.07 [0.84, 1.38]	0.58	0.20
rs216309	1.10 [0.86, 1.40]	0.47	0.19
rs216801	1.10 [0.89, 1.45]	0.32	0.19
rs7298566	0.93 [0.66, 1.31]	0.68	0.20
rs216855	0.92 [0.71, 1.20]	0.55	0.20
rs917857	0.96 [0.76, 1.22]	0.74	0.21
rs4764478	0.88 [0.67, 1.17]	0.38	0.19
rs11063961	0.82 [0.62, 1.08]	0.15	0.19
rs12369177	1.00 [0.78, 1.28]	1.00	0.25
rs2270151	1.21 [0.88, 1.67]	0.23	0.19
rs2885517	1.23 [0.95, 1.58]	0.11	0.19
rs12580835	0.86 [0.67, 1.11]	0.24	0.19
rs12367467	0.85 [0.58, 1.25]	0.41	0.19

Data are presented as a change in the estimated relative risk per minor allele and are adjusted for age, gender, smoking, hypertension, diabetes, hyperlipidemia and blood group.

DISCUSSION

We genotyped common variants in the total *VWF* gene in a unique case-control study of young patients with a first AMI or IS. Our data show that the minor allele of SNP rs4764478 is associated with a significant increase in VWF levels and that the minor allele of SNP rs216293 is associated with a significantly increased risk for CVD. In addition, we confirmed the association between rs1063857 and the risk forCVD. The associations with CVD risk were predominantly seen in the subgroup of IS patients.

The present candidate gene study identified an association between ht-SNP rs4764478 and VWF:Ag levels (q=0.04). After adjustment for multiple testing, rs4764478, which is located in intron 45, was significantly associated with VWF:CB activity as well (q=0.03). The VWF:CB activity assay measures the binding of plasma VWF to collagen, which is particularly dependent upon the quantity of high molecular weight (HMW) multimers [14]. If the multimer composition is changed, (e.g. because of a reduction of HMW multimers, as is seen in von Willebrand disease type 2A), a lower VWF:CB activity to VWF:Ag ratio is found. For SNP rs4764478, the increase in VWF:CB activity per minor allele was similar to the increase in VWF:Ag levels. Therefore, our study suggests that ht-rs4764478 itself, or SNPs tagged by rs4764478, is not specifically involved in the regulation of VWF multimer size. SNP rs4764478 was not associated with the risk of CVD. However, considering the minor allele frequency of this SNP of 0.25 and an 8% increase of VWF a study with larger statistical power is needed to significantly detect this relationship.

Ht-SNP rs216293, which is located in intron 17, was associated with an increased risk for CVD, independent of other classical cardiovascular risk factors. Because SNP rs216293 was not located in exonic regions and did not tag any known non-synonymous SNP in the *VWF* gene. The associations with VWF:Ag levels and the risk for CVD are probably not the result of a change in amino-acid composition. However, previous studies on proteins involved in haemostasis such as fibrinogen show that other structural alterations exist, (e.g. alternations due to alternative splicing) [21]. Such a structural alteration may also be the underlying mechanism for the association between rs4764478 and VWF:Ag levels, and rs216293 and CVD risk. Functional studies are needed to elucidate the mechanism underlying these new identified associations.

The synonymous ht-SNP rs1063857, which is located in exon 18, was associated with an increased risk for CVD. Previously, it was shown that SNP rs1063856, which is also located in exon 18, and the only known SNP in this region resulting in a change in amino acid, was associated with the risk for CHD in a young Caucasian population of patients with diabetes mellitus type I [13]. This SNP is in perfect LD with SNP rs1063857 ($D'=1.0$; $R^2=1.0$) and is therefore likely to be the functional variant responsible for the association between SNP rs1063857 and the risk for CVD in our study population. Interestingly, in the candidate gene study of patients with diabetes mellitus type I, SNP rs1063856 was also associated with

VWF:Ag levels. The CHARGE *(Cohort for Heart and Aging Research in Genome Epidemiology)* Consortium recently published a meta-analysis of 5 studies in which the association between variation in the total human genome and VWF:Ag levels was investigated. This study confirmed the association between rs1063856, rs1063857 and VWF:Ag_level [22]. In our study, rs1063857 was borderline significantly associated with VWF:Ag levels, possibly due to the limited power of the study. Another explanations may be that at the time of inclusion all patients with CVD used statins, that are known to decrease VWF levels, probably by influencing VWF secretion [23,24]. Therefore, use of statins may have attenuated the association between genetic variation and VWF:Ag levels. Thus far, SNP rs1063857 was associated with an increase in VWF:Ag levels and with the risk of disease in two independent studies, which suggests that VWF may be a causal factor in the development of CVD.

It is also interesting to note that although the subgroup of patients with IS was smaller (N=150) than the subgroup of patients with CHD (N=371), the associations between SNP rs1063857, SNP rs216293, and CVD appeared to be driven largely by the subgroup of IS patients. Common genetic variants in other important proteins involved in arterial thrombus formation, such as the GpIbα platelet receptor, show different effect estimates for the risk for CHD and IS as well [25]. This finding indicates that genetic variants may have a different contribution to the development of CHD than to the development of IS.

Common variants in the *VWF* promoter region are tagged by the SNPs in intron 3 and 5. The previously identified association between promoter variants, VWF:Ag levels and the risk of CVD among other investigated in the Rotterdam Study [9,10], could not be replicated in our study. Differences in study design, and heterogeneity of the study population may have contributed to these contradictory findings.

A limitation of the case-control design of our study is that only survivors of CVD were included. This may have led to an underestimation of the effect of genetic variation on the risk for CVD. Another limitation of the study is that we investigated variation in the *VWF* gene only in a population of individuals with European ancestry. Risk factors for CVD, allele frequencies and LD patterns were dissimilar between different ethnic populations, and therefore the results of this study can not be generalized to other ethnic populations.

Several studies showed that the genetic contribution to VWF levels and to the pathogenesis of CVD is higher in younger patients than in older patients [5,26]. The strength of the present study is that we investigated variation in the *VWF* gene in a young population with early-onset cardiovascular disease that was well documented. In contrast to previous studies that focused on individual SNPs and VWF:Ag levels, we investigated ht-SNPs covering the entire *VWF* gene, and studied associations with VWF:Ag levels, VWF:CB activity and CVD risk. The results of such genetic association studies may initiate translational studies and may contribute to the understanding of VWF function and its role in the development of CVD.

In conclusion, our study showed that variants in the *VWF* gene are associated with VWF:Ag levels and with the risk for CVD. The association with the risk for CVD was largely driven by the subgroup of IS patients. SNP rs1063857, known to be associated with VWF:Ag levels, was also associated with CVD risk, which suggests a causal role of VWF in the development of CVD. However, polymorphisms that contributed to the risk for CVD were not always associated with VWF:Ag levels or VWF:CB activity. This suggests that other mechanisms than increased VWF levels might also mediate the association between VWF gene variation and the risk for CVD.

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CHAPTER 7

GENETIC DETERMINANTS OF VON WILLEBRAND FACTOR PLASMA LEVELS AND THE RISK OF STROKE: THE ROTTERDAM STUDY

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ABSTRACT

Background: High von Willebrand factor (VWF) plasma levels are associated with an increased risk of stroke. VWF levels are strongly heritable. A previous meta-analysis of five large genome-wide association studies identified single nucleotide polymorphisms (SNPs) within 8 genetic loci as determinants of VWF levels. Whether these SNPs are associated with stroke risk is not known. The aim of our study was to investigate the association between genetic determinants of VWF levels and stroke risk.

Methods: The study was part of the Rotterdam Study, a large population-based cohort study among subjects aged ≥55 years. A total of 5763 participants of whom DNA was available, and who were free of stroke at baseline, were eligible for analysis. VWF antigen (VWF:Ag) levels were measured in 3379 eligible participants. Within each of the 8 loci one top SNP was defined. The association between the 8 SNPs and the risk of stroke was analyzed. Then a genetic score, based on these 8 SNPs, was constructed, and its total contribution to VWF plasma levels and stroke risk was investigated.

Results: None of the 8 SNPs were individually associated with stroke risk. A higher genetic score was significantly associated with a higher VWF:Ag level, but was not associated with an increased risk of stroke.

Conclusion: Eight SNPs that strongly determine VWF levels, are not associated with stroke risk, neither individually, nor combined in a genetic score.

INTRODUCTION

The large multimeric glycoprotein von Willebrand factor (VWF) is involved in platelet adhesion and aggregation at sites of vascular injury and therefore has a central role in primary haemostasis [1,2]. Plasma levels of VWF are influenced by non-genetic factors such as hormones and inflammation, but are also strongly heritable with estimates of heritability ranging from 53-75% [3,4]. Although variants within the VWF gene have shown to be associated with VWF plasma levels [5,6], the blood group gene is considered the strongest genetic determinant. Variants within the ABO blood group gene encode blood group A and B antigens which increase VWF plasma levels by at least 25% [7]. Previously, a meta-analysis of five genome-wide association studies (GWAS) conducted in the Cohort for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium identified SNPs in 8 different loci that were genome-wide significantly associated with VWF levels [8]. Besides known SNPs located in the blood group gene and VWF gene, the meta-analysis revealed unknown SNPs within new candidate genes such as the syntaxin-2 (STX2) gene and the syntaxin-binding-protein-5 (STXBP5) gene.

Since several studies have shown that high VWF levels are associated with an increased risk of stroke [9-11], we hypothesized that genetic determinants of VWF levels may be associated with the risk of stroke as well.

Thus far the results of studies that investigated the relationship between determinants of VWF levels within the blood group gene and VWF gene and the risk of cardiovascular disease have been inconsistent, possibly because they were underpowered and heterogeneous with respect to study endpoint, study population and study design. In addition, these studies primarily focused on coronary heart disease rather than stroke. Also, the association between genetics and stroke has not been investigated in longitudinal studies [6,12-14]. Whether variants in the new candidate genes as identified by the CHARGE meta-analysis are associated with the risk of stroke has also not yet been investigated.

The aim of our study was to investigate whether genetic determinants of VWF levels are associated with the risk of stroke in the general elderly population, who are at the highest risk of stroke. The SNPs used for analyses were previously identified by the CHARGE meta-analysis as the variant strongest associated with VWF levels (top SNP) within each of the 8 currently identified genetic loci. We additionally constructed a genetic score that included these 8 top SNPs to investigate the total contribution to VWF plasma levels and their association with the risk of stroke.

METHODS

Source population

This study is part of the Rotterdam Study, an ongoing prospective population-based cohort study, which started in 1990 [15]. All inhabitants of Ommoord, a district of the city of Rotterdam in the Netherland, who were 55 years of age or older, were invited to participate, and 7983 persons agreed (response rate 78%). Invitation into the study occurred in random order. Baseline examinations consisted of an interview at home and two visits to the research center for physical examination and blood sampling. The examinations were repeated every three to four years. All participants are continuously followed for a variety of diseases that are frequent in the elderly, including stroke [15].

The study protocol was in accordance with the declaration of Helsinki and was approved by the Medical Ethics Committee of the Erasmus Medical Center in Rotterdam. All participants gave written informed consent.

Ascertainment of stroke

Stroke was defined as rapidly developing clinical signs of focal or global disturbance of cerebral function with no apparent cause other than a vascular origin [16]. History of stroke at baseline was assessed during the baseline interview and verified by reviewing medical records. After enrollment in the Rotterdam study, participants were continuously monitored for disease including stroke through automated linkage of the study database with files from general practitioners. Nursing home physicians' files and files from general practitioners of participants who moved out of the district were scrutinized as well. For reported strokes, additional information was obtained from hospital records. Potential strokes were reviewed by research physicians, and verified by an experienced stroke neurologist (P.J.K.).

Strokes were further classified as cerebral infarction or intracerebral hemorrhage based on neuro-imaging results, which was present for 72% of stroke cases. If neuro-imaging was not available, the stroke was classified as unspecified. Subarachnoid hemorrhages were excluded. Follow-up was complete up to January 1, 2005 for 98.6% of potential person years [17].

Blood sampling procedure, von Willebrand factor plasma measurement and genotyping

Fasting venous blood samples were taken at the research center. Samples were snap frozen in liquid nitrogen and stored at -80°C.

Von Willebrand factor antigen (VWF:Ag) levels were determined with an in-house enzyme-linked immunosorbent assay (ELISA), using polyclonal rabbit antihuman von Willebrand factor antibodies (DakoCytomation, Glostrop, Denmark) for capturing and detecting. The intra-assay coefficient of variation was 5.2% and the inter-assay coefficient of

variation was 6.3%. The assay used commercial reference plasma (Normal reference plasma, Precision Biologic, Kordia, Leiden, The Netherlands) which was standardized against the WHO standard by the manufacturer and expressed in IU/ml.

Genomic DNA was extracted from whole blood samples according to standard methods. In the Rotterdam Study, genotyping was attempted with the Infinium HumanHap 550 K chip (Illumina, version 3) in persons with high-quality extracted DNA (n=6449). From these 6449, samples with low call rate (<97.5%, n=209), with excess autosomal heterozygosity (>0.336, n=21), with sex-mismatch (n=36), or if there were outliers identified by the IBS clustering analysis (>3 standard deviations from population mean, n=102 or IBS probabilities >97%, n=129) were excluded from the study population with some persons meeting more than one exclusion criterion; in total, 5974 samples were available with good quality genotyping data. The genotype data were used to impute the ~2.6 million autosomal SNPs using HapMap phase II, release 22 CEU, build 36 as the reference population. The set of genotyped input SNPs used for imputation was selected based on their highest quality GWA data. We used a callrate >98%, a minor allele frequency >0.01; a Hardy-Weinberg p>1 x 10-6; and a test of differential missingness by the "mishap" test in PLINK p>1 x 10-9. We used the Markov Chain Haplotyping (MaCH) package for imputation. Imputation and quality-control measures have been described in more detail previously [8,18-20].

Baseline measurements

Smoking behavior was assessed during the interview at home. Clinical measurements were obtained during the visits to the research centre. Blood pressure was calculated as the mean of two measurements with the random-zero sphygmomanometer at the right brachial artery while the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure of ≥ 90 mm Hg and/or a systolic blood pressure of ≥140 mm Hg and/or the use of antihypertensive medication indicated for the treatment of high blood pressure (≥ grade 1 hypertension according to World Health Organization criteria) [21]. Total cholesterol was measured with an automated enzymatic procedure. Diabetes mellitus was defined as the use of serum glucose-lowering medication and/or a fasting serum glucose level ≥7.0 mmol/l. Body mass index was calculated as weight (in kg) divided by the square of height (in meters). History of myocardial infarction was determined during the baseline interview and verified in medical records. History of coronary heart disease was positive if the participant underwent a revascularization procedure, or fulfilled the criteria of myocardial infarction. Prevalent and incident atrial fibrillation were ascertained using the following methods: (1) An ECG was recorded during baseline visits and follow-up rounds; (2) information was obtained from general practitioners files, hospital records, and the national registration system of hospital discharge diagnoses [22]. The presence of peripheral arterial disease was evaluated by measuring the systolic blood pressure level of the posterior tibial artery at both legs using a Doppler probe and a random-zero sphygmomanometer, with

the subject in supine position. The ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the brachial artery was calculated for each leg. Peripheral arterial disease was considered present if the ankle-brachial index was <0.9 in at least one leg [23]. Blood group genotypes were reconstructed by haplotype analysis of four SNPs; rs687289, rs507666, rs8176704, and rs8176749, which collectively serve as tagging SNPs for the O, A1, A2 and B alleles [24]. Haplotypes were inferred using the R package haplo.stats [25,26]. The haplotype analysis calculates posterior probabilities for every possible haplotype, (and for each possible diplotype). In this way, the blood group genotype of all participants could be estimated with a probability of more than 99%.

Population for analysis

For the present study, all participants of whom DNA was available and who were free of stroke at baseline (n=5763) were eligible for analysis. VWF plasma levels were measured in blood collected during the third examination cycle (March 1997-December 1999) and could be determined for 3379 of the 5763 eligible participants.

Statistical analysis

Baseline characteristics of the study participants (n=5763) are presented as median and inter-quartile range or mean and standard deviation for continuous variables, and as counts and percentages for categorical variables.

The 8 SNPs used for analyses in the present study (rs9390459, rs2726953, rs687621, rs1063857, rs4981022, rs7978987, rs10133762 and rs868875) were previously identified by the CHARGE meta-analysis as the variant within each (VWF:Ag-level-influencing) genetic locus strongest associated with VWF levels (top SNPs) [8]. The Rotterdam Study was a significant contributor (20-25%) to the number of individuals in the CHARGE meta-analysis for which VWF level was measured. We analyzed the association between the VWF:Ag level increasing allele of the 8 top SNPs and VWF:Ag levels by means of linear regression.

Two genetic scores were constructed [27,28]. The first score included the 8 top SNPs as weighted sums of the number of VWF:Ag levels increasing alleles carried by an individual. Weighting was based on the effect estimates obtained from the CHARGE meta-analysis which were presented as percentage change in VWF:Ag level per allele. For instance, the genetic score for an individual with two VWF:Ag level increasing alleles for the blood group SNP rs687621, and one VWF:Ag level increasing allele for the VWF SNP rs1063857 was (2 x 24.1%) + (1 x 6.0%)=54.2. The age- and sex-adjusted mean VWF:Ag levels per quartile of the genetic score was calculated by analysis of covariance. The trend across quartiles was analyzed by linear regression. To analyze the effect of the score on VWF:Ag levels independent of blood group, we constructed a second genetic score without SNP rs687621. Age- and sex-adjusted mean VWF:Ag levels per quartile, and the linear trend across quartiles were calculated separately for participants with blood group O and blood group non-O.

We used the Cox' proportional hazards model, to determine hazard ratios (HR) and 95% confidence intervals (CI) for the association between individual SNPs and non-hemorrhagic stroke (unspecified and ischemic strokes) and for the association between the two genetic scores and non-hemorrhagic stroke. Unspecified strokes are likely of ischemic origin. Since a different pathofysiologic role for VWF is considered in hemorrhagic and ischemic stroke, hemorrhagic strokes were excluded from the analyses. The analyses were also conducted for the subgroup of ischemic stroke. HRs of individual SNPs were expressed per VWF level increasing allele, HRs of the genetic score were expressed in quartiles (relative to the lowest quartile). The linear trend across quartiles was tested by including the quartile categories as a continuous variable in the model. The association between the second genetic score (without blood group SNP rs687621) and the risk of stroke was investigated in strata of blood group O and non-O. All hazard ratios were adjusted for age and sex, and additionally for the potential confounders smoking, hypertension, diabetes, total cholesterol, body mass index and prevalent cardiovascular disease (CVD).

To investigate the contribution of blood group to VWF:Ag levels and stroke risk in more detail, we calculated the age- and sex-adjusted mean VWF:Ag level by analysis of covariance for each blood group genotype. Next, we calculated mean VWF:Ag levels per number of non-O blood group allele (0, 1, or 2), and per number of VWF:Ag level increasing blood group allele (0, 1, or 2). VWF:Ag level increasing alleles were all non-O alleles except the A_2 allele. In addition, Cox's proportional hazard model was used to calculate the association between the number of blood group non-O alleles and the risk of stroke, and between the number of VWF:Ag levels increasing blood group alleles (all relative to zero alleles), and the risk of stroke. For all analyses an additive and independent genetic effect model was assumed. Analyses were conducted using R, SPSS version 15 (SPSS Inc, Chicago, USA), Haploview, and the haplo.stats package. A p-value of <0.05 (two sided) was considered significant.

RESULTS

Baseline characteristics of the study population are presented in Table 1. During 58.371 person years of follow-up (mean, 10.1 years), 632 individuals suffered a stroke. Of the 392 strokes with neuro-imaging results, 348 (89%) were classified as ischemic and 44 (11%) as hemorrhagic. Two hundred and forty (240) stroke cases were classified as unspecified. At baseline, the median age was 68.0 (61.9-75.2) years. Of the study participants 59.4 % were female, and 28.0% had a history of CVD.

Associations between the 8 top SNPs and VWF:Ag levels in our study population are listed in Table 2. Six of the 8 studied SNPs were significantly associated with VWF:Ag levels.

Table 1 | Baseline characteristics of the study participants (n=5763)

Age, years	68.0 (61.9-75.2)
Female sex	3424 (59.4%)
European ancestry participants	5763 (100%)
Current cigarette smoking	1183 (20.5%)
Hypertension	2932 (50.9%)
Diabetes	561 (9.7%)
Total cholesterol, mmol/L	6.6 (1.2)
Body mass index, kg/m^2	25.9 (23.8-28.4)
Prevalent cardiovascular disease*	1615 (28.0%)
VWF:Ag level (IU/ml)	1.25 (0.95-1.66)

Values are presented as median (interquartile range), mean (SD) or counts and (percentage). *: Prevalent cardiovascular disease includes atrial fibrillation, coronary heart disease and peripheral arterial disease.

Table 2 | Association between the top SNP of every genetic locus and VWF:Ag levels (n=3379)

Region	Gene	SNP	Chromosome position	* The VWF:Ag level increasing allele with its allele frequency		β (± SE)	P-value
6q24	STXBP5	rs9390459	147722052	G/A	0.59	0.057 (± 0.02)	<0.001
8p21	SCARA5	rs2726953	27857224	T/C	0.31	0.034 (± 0.02)	0.04
9q34	ABO	rs687621	135126886	C/T	0.33	0.297 (± 0.02)	<0.001
12p13	VWF	rs1063857	6023775	C/T	0.35	0.055 (± 0.02)	<0.001
12q23	STAB2	rs4981022	102652341	T/C	0.68	0.044 (± 0.02)	<0.001
12q24.3	STX2	rs7978987	129806374	A/G	0.34	0.029 (± 0.02)	0.07
14q32	TC2N	rs10133762	91362522	T/G	0.45	0.008 (± 0.02)	0.58
19p13.2	CLEC4M	rs868875	7737166	A/G	0.75	0.047 (± 0.02)	0.04

SNP: Single Nucleotide Polymorphism. β: Change in VWF:Ag levels (± SE) presented in IU/ml per VWF:Ag level increasing allele. Data are adjusted for age and sex. * The underlined allele is the VWF:Ag level increasing allele.

The age- and sex-adjusted mean VWF:Ag levels per quartile of the genetic score are presented in Figure 1A. We found a strong linear association between an increasing genetic score and levels of plasma VWF:Ag (trend p<0.01).

The association between quartiles of the second genetic score (without the blood group SNP) and VWF:Ag levels is presented in figure 1B. Both in individuals with blood group O and non-O, an increasing genetic score was significantly associated with increasing VWF:Ag levels (trend p<0.01).

Table 3 shows the associations between individual SNPs and risk of non-hemorrhagic stroke and ischemic stroke. None of the 8 SNPs were associated with the risk of non-

hemorrhagic stroke or ischemic stroke. Additional adjustment for the potential confounders age, sex smoking, hypertension, diabetes, total cholesterol, body mass index and prevalent cardiovascular disease did not affect the associations (results not shown).

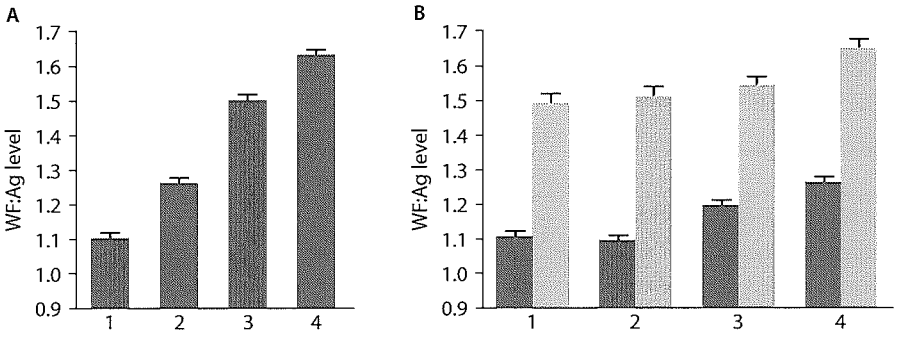

Figure 1 | A presents the mean VWF:Ag level in IU/ml (+SE) per quartile of the genetic score (trend $p<0.01$). Genetic score range per quartile: 4.98-29.77, 29.74-44.01, 44.03-56.32, 56.33-95.29. **B** presents the mean VWF:Ag level in IU/ml (+SE) per quartile of the genetic score for blood group O individuals (dark gray bars) and non-O individuals (light gray bars) separately (trend $p<0.01$ for both blood groups). Genetic score range per quartile for blood group O individuals: 4.98-23.51, 23.52-28.60, 28.61-33.79, 33.80-54.59, and for blood group non-O individuals: 3.51-23.63, 23.64-28.53, 28.54-33.56, 33.57-50.23. Mean VWF:Ag levels were adjusted for age and sex

Table 3 | Association between the top SNP of every gene and stroke risk (n=5763)

SNP	Gene	*Non-hemorrhagic stroke (n=588) HR (95% CI)	Ischemic stroke (n=348) HR (95% CI)
rs9390459	STXBP5	0.98 (0.87-1.10)	0.92 (0.79-1.07)
rs2726953	SCARA5	1.01 (0.89-1.15)	1.01 (0.86-1.19)
rs687621	ABO	0.98 (0.87-1.11)	1.02 (0.87-1.19)
rs1063857	VWF	1.03 (0.92-1.16)	1.10 (0.94-1.28)
rs4981022	STAB2	1.08 (0.96-1.22)	1.07 (0.91-1.25)
rs7978987	STX2	0.98 (0.87-1.10)	0.99 (0.85-1.16)
rs10133762	TC2N	0.96 (0.85-1.07)	1.04 (0.89-1.20)
rs868875	CLEC4M	1.03 (0.87-1.23)	1.02 (0.82-1.28)

Data are presented as hazard ratios (HR) with 95% confidence intervals (CI) per VWF:Ag level increasing allele. SNP: Single Nucleotide Polymorphism. *Non-hemorrhagic stroke = ischemic and unspecified stroke.

Table 4 shows that neither in the total cohort, nor in subgroups of blood group O and non-O, an increasing genetic score was associated with the risk of non-hemorrhagic stroke or ischemic stroke. Additional adjustment for the potential confounders age, sex, smoking, hypertension, diabetes, total cholesterol, body mass index and prevalent cardiovascular disease did not affect the associations (results not shown).

Table 4 | A ssociation between the genetic score and stroke risk (N=5763)

	HR (95% CI)	HR (95% CI)
Quartile (range genetic score)	***Non-hemorrhagic stroke (N=588)**	**Ischemic stroke (N=348)**
1 (00.50-29.68)	1 (ref)	1 (ref)
2 (29.69-44.60)	0.97 (0.77-1.22)	1.09 (0.80-1.48)
3 (44.61-56.38)	1.18 (0.94-1.47)	1.30 (0.97-1.74)
4 (56.39-98.39)	0.93 (0.74-1.18)	1.02 (0.75-1.39)
P trend	0.99	0.60
Blood group O individuals	(N=262)	(N=150)
1 (00.50-23.35)	1 (ref)	1 (ref)
2 (23.36-28.43)	1.15 (0.81-1.63)	1.45 (0.91-2.32)
3 (28.44-33.61)	1.16 (0.82-1.64)	1.33 (0.83-2.16)
4 (33.62-54.59)	1.07 (0.75-1.52)	1.33 (0.82-2.14)
P trend	0.73	0.36
Blood group non-O individuals	(N=326)	(N=197)
1 (3.51-23.62)	1 (ref)	1 (ref)
2 (23.63-28.52)	1.34 (0.99-1.83)	1.13 (0.76-1.66)
3 (28.54-33.55)	1.23 (0.90-1.69)	1.01 (0.68-1.51)
4 (33.56-53.27)	1.09 (0.79-1.51)	0.98 (0.65-1.47)
P trend	0.77	0.79

The association between the genetic score and non-hemorrhagic stroke, and ischemic stroke are presented as hazard ratios (HR, 95% CI). * Non-hemorrhagic stroke = ischemic and unspecified stroke.

Table 5 presents the mean VWF:Ag levels per blood group genotype. The A_2O genotype had the lowest mean VWF:Ag levels of all non-O genotypes. The age and sex adjusted mean VWF:Ag levels per number of VWF:Ag level increasing alleles was 1.09 (± 0.01) IU/ml for zero alleles, 1.47 (± 0.11) for one allele and 1.63 (± 0.02) for two alleles, whereas the age and sex adjusted mean VWF:Ag levels per number of blood group non-O alleles was 1.08 (± 0.01) IU/ml for zero, 1.39 (± 0.01) IU/ml for one, and 1.59 (± 0.02) IU/ml for two alleles. Neither the number of blood group increasing alleles nor the number of blood group non-O alleles were associated with the risk of any stroke or the risk of ischemic stroke.

Table 5 | VWF:Ag levels per blood group genotype (n=3376)

Blood group			
genotype	phenotype	N individuals (%)	VWF:Ag levels
OO	O	1536 (45.5%)	1.08 (± 0.01)
A_1O	A	872 (25.8%)	1.45 (± 0.01)
A_2O	A	300 (8.9%)	1.14 (± 0.02)
BO	B	284 (8.4%)	1.50 (± 0.02)
A_1A_1	A	146 (4.3%)	1.67 (± 0.03)
A_1A_2	A	104 (3.1%)	1.52 (± 0.04)
A_1B	AB	83 (2.5%)	1.58 (± 0.04)
A_2A_2	A	17 (0.5%)	1.34 (± 0.09)
A_2B	AB	26 (0.8%)	1.71 (± 0.08)
BB	B	8 (0.2%)	1.78 (± 0.14)

The age and sex adjusted VWF:Ag levels in IU/ml are presented as mean (± SE).

DISCUSSION

Our principal finding in this large prospective population-based cohort study is that genetic variants that strongly determine VWF plasma levels are not associated with the risk of stroke in the general elderly population.

By constructing a score with genetic determinants of VWF levels [8], we demonstrated that VWF plasma levels were strongly related to the genetic score in an elderly population. Since blood group is by far the strongest genetic determinant of VWF plasma levels, we investigated whether the association between the genetic score and VWF plasma levels was mainly driven by the blood group variant, or influenced by other genetic variants as well. We showed that in both blood group O and non-O, the genetic score without the blood group variant was still significantly associated with VWF plasma levels. Our data suggest that genetic constitution contributes substantially to VWF plasma levels, and strengthen the results of previous studies [3,4].

In the present study, SNP rs1063857, located in the VWF gene, was not associated with the risk of stroke (Table 3). This observation is not consistent with our previous findings on SNP rs1063857 and stroke risk in the ATTAC study. In the ATTAC study, a hospital-based case-control study among young individuals (males aged ≤ 45, and females aged ≤ 55) who had a first acute myocardial infarction or ischemic stroke, we found an association between rs1063857 and CVD, which was largely driven by ischemic stroke patients [6]. The conflicting results might be explained by the large difference in age range between the two studies.

Indeed, previous studies have shown that the genetic contribution to the development of CVD, such as stroke, is stronger in younger individuals [29,30].

This is the first prospective cohort study on the association between blood group and stroke risk. We found no association between blood group and stroke risk and therefore could not replicate the previously reported positive association between blood group non-O and the risk of stroke in a meta-analysis [14]. However, due to overlap in confidence intervals of the HR in the meta-analysis and our study, a positive association can not be excluded.

Most studies on the association between blood group and stroke risk compared the risk for blood group O individuals with the risk for blood group non-O individuals. It has been suggested that A_2 individuals have VWF levels comparable to blood group O individuals, and not to non-O individuals [31,32]. We reconstructed the blood group genotypes, and showed that the VWF levels were indeed lower for the A_2O genotype than for all other non-O genotypes (Table 5). Assuming that the association between blood group and stroke risk is mediated by VWF levels, we hypothesized that a different definition of the potential risk increasing allele could possibly affect the association between blood group and stroke risk. Therefore, we analyzed the effect of blood group non-O alleles, and VWF level increasing alleles on the risk of stroke. This different classification of the risk allele did influence VWF levels, but had no effect on the association between blood group and stroke risk.

Strengths of the present study are the large population based study cohort, the prospective setting and the long follow-up time of, on average, 10 years. In addition, since the Rotterdam Study included only elderly people, the results of the study are specifically applicable to the population at the highest risk of stroke. Our study has some limitations as well. In stroke subtypes different risk factors may be involved, but there were too few events to study the association between genetic determinants of VWF plasma levels and the risk of hemorrhagic stroke, or to study genotype-phenotype associations underlying the subtypes of ischemic stroke. To study the association between genetic determinants of VWF plasma levels and stroke subtypes in the future, larger study populations are needed. In this study, considering a powers of 80%, and a minor allele frequency of 30%, a minimum HR of 1.25 per risk allele could be identified. Expecting a hazard ratio of 1.25 is reasonable, since hazard ratios for the association between genetic variation and stroke were of the same magnitude [33] However, the power of the study may have been too low for detecting HR smaller than 1.25. Also, generalizability of the results to younger, and other ethnic populations than Caucasian may be a problem.

In conclusion, we showed that VWF levels are strongly influenced by genetic variants. However, genetic determinants of plasma VWF levels themselves are not associated with the risk of stroke in elderly people. Although previous studies showed that high VWF plasma levels are associated with an increased risk of stroke, our results suggest that VWF is not a strong causal factor in the development of stroke.

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CHAPTER 8

BIOLOGICAL VARIATION IN VON WILLEBRAND FACTOR AND FACTOR VIII

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ABSTRACT

Background/Objectives: Haemostatic factors such as von Willebrand factor (VWF) and factor VIII (FVIII) are subject to biological variation. The aim of our study was to determine the biological variation for VWF and FVIII in order to improve their assessment as risk indicator and diagnostic tool.

Methods: In 520 blood samples collected over a 1-year time period from 40 healthy individuals, the between-subject, within-subject and analytical variation for VWF antigen (VWF:Ag), VWF ristocetin cofactor activity (VWF:RCo), VWF collagen binding activity (VWF:CB) and FVIII were calculated and expressed as coefficients of variation (CV). *Results:* The between-subject, within-subject and analytical variation for VWF:Ag was 28.6%, 15.8%, and 4.2%, for VWF:CB activity 28%, 25.6%, and 12.9%, for VWF:RCo activity 31.2%, 21.3% and 9.6%, for FVIII 25.2%, 15.8% and 2.3%. The number of repeated measurements needed to estimate their homeostatic setting point was 2 for VWF:Ag and FVIII, 5 for VWF:RCo and 8 for VWF:CB.

Conclusion: This study provides insights into the biological variability of VWF and FVIII which may lead to a better assessment of VWF and FVIII as prognostic markers for the development of arterial and venous thrombosis and may improve the identification of VWD patients.

INTRODUCTION

High von Willebrand factor (VWF) and factor VIII (FVIII) levels are associated with an increased risk of arterial and venous thrombotic disease [1-5]. Low VWF and FVIII levels are associated with von Willebrand disease (VWD), the most common inherited bleeding disorder [6].

Plasma levels of VWF and FVIII vary largely when measured repeatedly in a certain time period. This variation over time in an individual is called the within-subject biological variation, and is influenced by age, and transient environmental factors including physical stress, common cold and seasonal variation.

Biological variation may have implications for the appraisal of VWF and FVIII as prognostic markers of arterial and venous thrombosis. For prognostic markers, the differences between individual's true homeostatic mean is of most interest and not differences in short time fluctuations due to transient factors. The estimation of an individual's homeostatic mean is improved by multiple measurements at different time points. However, in epidemiological studies, the value of a potential risk indicator such as VWF, is measured only once, and is then accepted as representative for an individuals homeostatic mean which may be incorrect.

Biological variation may have clinical implications as well. Low VWF and FVIII plasma levels and activity are important diagnostic criteria for VWD. Consequently, the estimation of an individual's true homeostatic mean is of vital importance for diagnostic precision.

The aim of this study was to investigate the biological variation of VWF antigen (VWF:Ag) levels, VWF ristocetin cofactor activity (VWF:RCo), VWF collagen binding activity (VWF:CB) and FVIII activity in 40 healthy individuals of whom blood was collected at 13 visits during a period of one year. Based on the obtained results, we will provide recommendations to improve the assessment of VWF and FVIII as risk indicators for thrombotic disease and the diagnostic precision for VWD.

METHODS

Study population

In the study, 40 healthy individuals were included. Individuals with symptoms of chronic or acute infectious diseases, and individuals who underwent surgical procedures within three months prior to the start of the study or during the study, were excluded. From each participant, blood was collected at 13 visits during a period of one year. Data on demographics, cardiovascular risk factors and use of medication were collected. Before each blood collection, study participants were asked whether they had recently used drugs, or suffered from infections, such as common cold. The study protocol is in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of Erasmus MC. Written informed consent was obtained from each participant.

Blood sampling procedure and plasma measurements

Blood was collected while the study participants were sitting and resting. They were allowed to have a light breakfast on the morning of blood collection. Blood was drawn by venapuncture using the Vacutainer system (Becton Dickinson, Plymouth, UK) containing sodium citrate (final concentration 0.105 mol L^{-1}). Platelet-poor plasma was obtained by centrifugation twice at 3500 x g for 15 minutes at 4°C and stored at -80°C until further analysis. To exclude inter-assay analytical variation, all samples from each participant were analyzed in a single run. In this run the samples were measured in duplicate.

Von Willebrand factor antigen (VWF:Ag) levels were determined with an in-house ELISA assay, using polyclonal rabbit antihuman VWF antibodies (DakoCytomation, Glostrop, Denmark) for capturing and detecting.

Von Willebrand factor collagen binding (VWF:CB) activity was measured by an in-house ELISA assay using bovine achilles tendon collagen type I for capturing (Sigma-Aldrich, St Louis, USA) and polyclonal rabbit antihuman VWF antibodies (DakoCytomation, Glostrop, Denmark) for detecting.

Von Willebrand factor ristocetin cofactor activity (VWF:RCo) was measured with a platelet aggregometer which measures the rate of aggregation of platelets in the presence of VWF and ristocetin.

Factor VIII (FVIII) activity was derived from the clotting time (APTT) measured with the Sysmex CA-1500 (Siemens, Breda, NL.).

All assays used commercial reference plasma (Normal reference plasma, Precision Biologic, Kordia, Leiden, The Netherlands) that was standardized against the WHO standard by the manufacturer.

Statistical analysis

Data on population demographics for continuous variables are presented as medians with inter-quartile range or mean with their standard deviation (SD), when applicable. Levels of investigated parameters are presented as means (SD). The components of variation are given as coefficients of variation (CV), calculated using ((SD/mean) × 100%).

In the present study, sources of variation were calculated using a nested random-effects analysis of variance model [7,8]:

$$Y_{ijr} = \mu + \alpha_i + \beta_{ij} + \varepsilon_{ijr}$$

Whereby Y_{ijr} is the value for subject *i* at time point *j* at the *r* replication, μ is the overall mean of the population, α_i is the deviation from the true mean of the *i*th person ($i = 1, 2, 340$), β_{ij} is the within subject variance, and ε_{ijr} is intra-serial analytical variance. α_i, β_{ij}, and ε_{ijr} are mutually uncorrelated random effects with zero means and respective variances σ_α^2, σ_β^2 and σ_ε^2. The variances were assumed to be normally distributed. The measurement number was not added as a factor because its deviations from the true mean were assumed to be independent of sampling order.

We have calculated the number of repeated measurements (*m*) that was needed to estimate with 95% probability a homeostatic setting point which does not deviate more than 20% from its true value. [9,10] As there are no strict criteria in the literature, 95% probability and 20% deviation were arbitrarily chosen:

$$m = [1.96^* (CV^2_{analytical} + CV^2_{within\text{-}subject})^{1/2} / 20]^2$$

Whereby $CV_{analytical}$ was the coefficient of analytical variation and $CV_{within\text{-}subject}$ was the coefficient of within-subject biological variation.

The indices of individuality were calculated, which represent how unique each test result was within the total study population [11]:

$$(CV^2_{analytical} + CV^2_{within\text{-}subject})^{1/2} / CV_{between\text{-}subject}$$

A low index of individuality indicates an analyte have marked individuality leading to large reference intervals. A marked change in a value for an individual may still lie within the conventional reference interval and may therefore not be noticed as unusual. In the case of marked individuality reference values for monitoring and case finding have low utility.

Reference change values (RCV) were calculated, which indicate how many percent a test result in an individual needs to deviate from the homeostatic point in order to be considered statistically different (that is to say in the present study outside the 95% of the distribution) [11]:

$$2^{1/2} * 1.96 (CV^2_{analytical} + CV^2_{within\text{-}subject})^{1/2}$$

Statistical analyses were performed with SPSS for Windows version 15 (SPSS Inc, Chicago IL, USA), and SAS/STAT 9.2. A two-sided p value <0.05 was considered statistically significant. Outliers were defined as values outside the 2.5%-97.5% range and according to the Cochran's test and Reed's criterion. The CVs from the total study population and the subgroups non-smokers only, and non-users of contraceptives were compared. In addition, the CVs between the subgroups men and women, blood group O and non-O, age <40 years and >40 years were compared.

RESULTS

Characteristics of the study population

The median age of the 40 included individuals in this study was 46 (interquartile range, 26-54). Twenty-six (65%) included individuals were female and 7 individuals were smokers. The mean BMI in the study population was 22.6 (±2.0) kg/m^2. Nine female participants (23%) used oral contraceptives throughout the follow-up period, 1 participant used lipid lowering medication and 2 participants used oral non-steroid anti-inflammatory drugs (NSAIDs).

Table 1 | Characteristics of the study population (n=40)

Variable	Values
Age, years	46 (26-54)
Females n (%)	26 (65)
Smokers n (%)	7 (18)
Body mass index kg/m^2	22.6 (± 2.0)
Oral contraceptives n (%)	9 (23)
Lipid lowering medication n (%)	1 (2.5)
#NSAIDs n (%)	2 (5)

Values are given as number (%) for categorical variable. The continues variable age is given as median (interquartile range) and body mass index as mean (± SD). #NSAIDs: non-steroid anti-inflammatory drugs.

Individual concentrations of VWF and FVIII

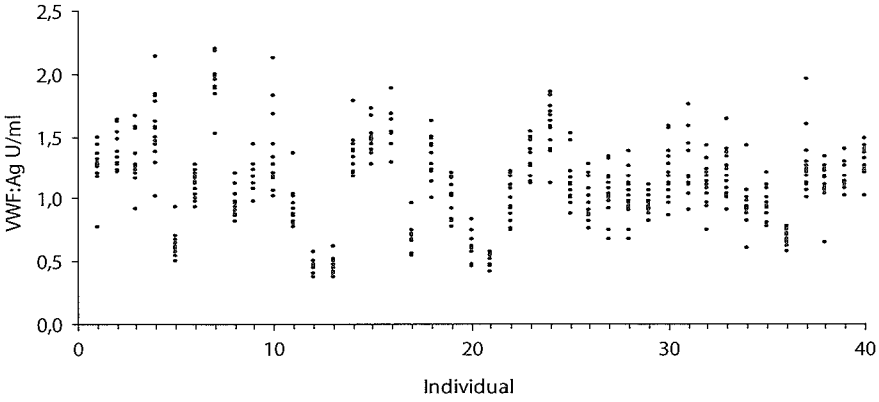
In total, 520 blood samples were collected on 197 different days that were spread throughout the study period of one year. The mean VWF:Ag plasma level measured in 516 samples was 1.11 (± 0.35) IU/ml (Figure 1A, Table 2). The CV for the total variation was 32.8%, for the between-subject variation 28.5%, for the within-subject variation 22.6% and for the analytical variation 4.1%. The mean VWF:CB activity measured in 515 samples was 1.12 (± 0.49) IU/ml (Figure 1B, Table 2) with CVs of 40.0%, 28.0%, 25.6% and 12.9% for the total, between-subject, within-subject and analytical variation, respectively. The mean VWF:RCo activity measured in 507 samples was 1.27 (± 0.39) IU/ml (Figure 1C, Table 2). The CVs were 39.0%, 31.2%, 21.3% and 9.6% respectively. The mean FVIII concentration measured in 520 samples was 1.03 (± 0.30) IU/ml (Figure 1D, Table 2) with CVs of 29.8%, 25.2%, 15.8% and 2.3% respectively.

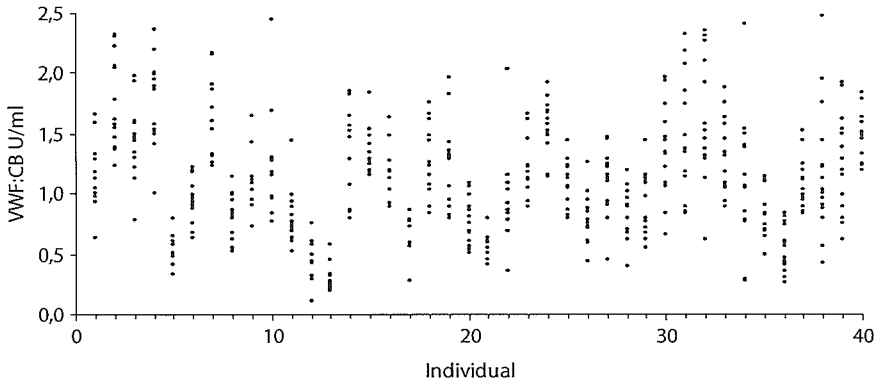
The CVs of the measured variables were similar when they were calculated for non-smokers only, for non-users of oral contraceptives, for males and females separately, for blood group O and non-O separately, and for participants <40 and >40 years of age (data not shown).

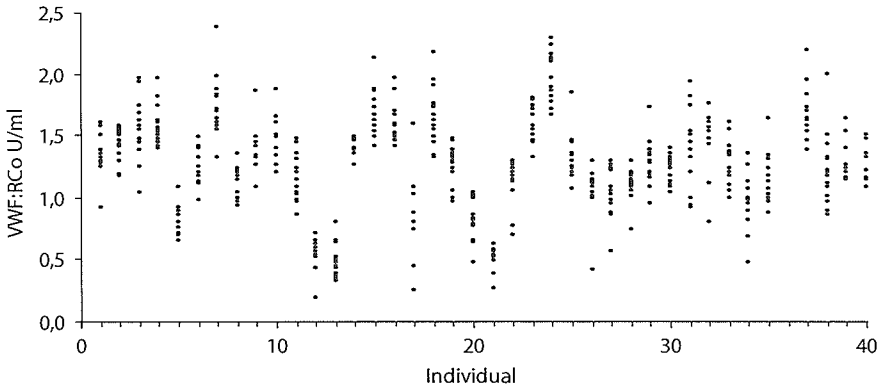
Table 2 | Components of variation in plasma concentrations of VWF and FVIII levels

Variable	N		Mean ± SD (IU/ml)	Total	Coefficients of variation		
	individuals	Observations			Between-subject	Within-subject	Analytical variation
VWF:Ag	40	516	1.11 (± 0.35)	32.8%	28.6%	15.8%	4.2%
VWF:CB	40	515	1.12 (± 0.49)	40.0%	28.0%	25.6%	12.9%
VWF:RCo	39	507	1.27 (± 0.39)	39.0%	31.2%	21.3%	9.6%
FVIII	40	520	1.03 (± 0.30)	29.8%	25.2%	15.8%	2.3%

Mean ± SD: the mean ± standard deviation expressed in international units per millilitre (IU/ml).


VWF:Ag U/ml
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
Individual


VWF:CB U/ml
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
Individual


VWF:RCo U/ml
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
Individual

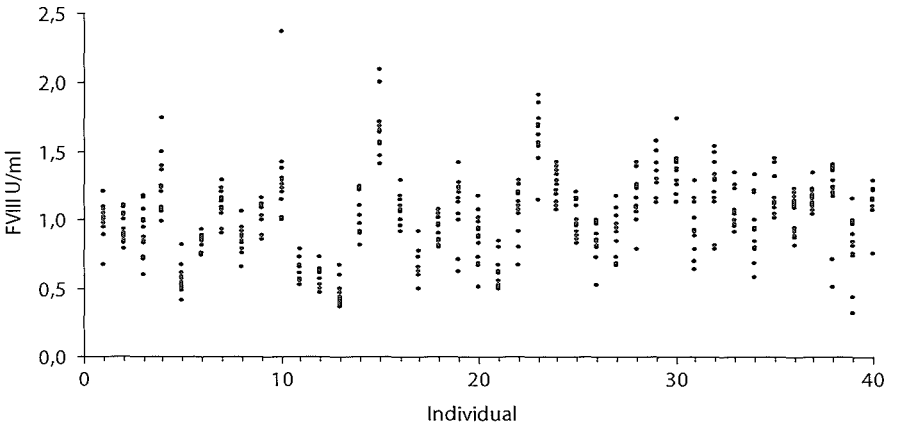


Figure 1 | Plasma concentrations of VWF:Ag (panel A), VWF:CB (panel B), VWF:RCo (panel C) and FVIII (panel D) (y-axis) for every individual (x-axis) measured at different time points throughout the year.

Repeated measurements

For VWF:Ag and FVIII activity the number of repeated measurements needed to estimate with 95% probability that a homeostatic setting point does not deviate more than 20% from its true value was 2. The number of repeated measurements was 5 for VWF:RCo and 8 for VWF:CB.

Reference change values and indices of individuality

Reference change values were 45.3% for VWF:Ag, 79.5 % for VWF:CB, 64.8% for VWF:RCo and 44.3% for FVIII. The indices of individuality were 0.57 for VWF:Ag, 1.02 for VWF:CB, 0.75 for VWF:RCo and 0.63 for FVIII.

DISCUSSION

The present study provides important new insights in the biological variation of VWF and FVIII. Based on these results we have provided several recommendations for measuring VWF and FVIII which improves their assessment as risk indicator and their precision as diagnostic tool.

Data of the few conducted studies on biological variation of VWF antigen levels (VWF:Ag) and FVIII activity vary largely and are not in line with our results [12-15]. One previous study showed CVs for the between- and within-subject biological variation of 71% and 28% for VWF:Ag levels, and 57% and 34% for FVIII [13], which is considerably larger than our data.

One other study displayed lower biological variations compared to our results. In this study, the CVs for the between- and within-subject biological variation of 18% and 5% for VWF:Ag levels and 19% and 5% for FVIII [14]. The differences in CVs might be explained by the small number of included individuals in previous studies. Also in these studies, individuals were sampled less frequently and in a shorter time period (for instance one week). To our knowledge, this is the first report on biological variation of von Willebrand factor ristocetin cofactor activity (VWF:RCo) and von Willebrand factor collagen binding activity (VWF:CB). The results show that for both analytes compared to VWF:Ag, the within-subject biological variation and analytical variation was larger (Table 2).

Based on the calculated CVs, for each analyte, the number of repeated measurements needed to estimate with 95% probability that a homeostatic setting point does not deviate more than 20% from its true value were calculated. Since the number of repeated measurements depends on the within-subject and analytical variation, the number of repeated measurements is considerably lower for VWF:Ag levels and FVIII activity (2 measurements) than for VWF:CB and VWF:RCo activity (5 and 8 measurement respectively).

Our results illustrate that one measurement per analyte is not representative for an individual's homeostatic setting points and we therefore give direction to the number of repeated measurements needed to achieve a representative value. An adequate number of measurements at different points in time will improve the assessment of VWF and FVIII as risk indicators and increase the power of epidemiological studies.

As laboratory evaluation of VWF:Ag, VWF:RCo and FVIII is critical for diagnosing VWD and the differentiation between VWD subtypes, the number of repeated measurements for the investigated analytes have clinical consequences too. In the clinical setting it is common practice to repeat a test in the face of normal initial results before drawing any conclusion. However, our results indicate that two measurements are not representative for an individual's homeostatic setting point for VWF:RCo and VWF:CB activity. As a consequence, VWD patients may be missed or individuals may incorrectly be diagnosed with VWD. Since biological variation can not be influenced, only by reducing the analytical variation the number of repeated measurements needed to estimate an individual's homeostatic setting points can be decreased. This will improve the clinical utility of analytes.

Also, based on the CVs from our study, we could calculate the reference change values (RCVs) which show how large the change in the concentration of an analyte in an individual should be in order to conclude the change is not attributable to the intrinsic sources of variation of the analyte. This may be important for monitoring, for instance in epidemiological studies, if one wants to investigate whether a significant change in FVIII levels during follow-up may influence the risk of thrombosis. RCVs are relevant in the clinical setting as well, since one can evaluate whether a specific treatment of patients with VWD significantly changes the level of an analyte. For instance in our study, the level of VWF:RCo activity has to be 64.8% higher or lower than the first measurement in order to be

significantly different. However, although a change in level is not statistically significant it may lead to a clinically relevant improvement of bleeding time.

Previous studies suggest that VWF levels are higher in females compared to males, VWF levels increase with age and are influenced by the use of medication [2,16,17]. If also the biological variation is influenced by these factors stratifying may improve monitoring of VWF and diagnosis of VWD (clinical decision making). However, we showed the CVs were similar for the total study population and the different subgroups [18].

The present study has several limitations. Since the inter quartile range for age in our study was 26-54, possibly the study group is not representative for the population with the highest risk for cardiovascular disease. In the present study the biological variation in healthy individuals was investigated, maybe this does not reflect biological variation in individuals with VWD. However, previous studies suggest that although the disease may influence the homeostatic setting point of an analyte, the variation around the setting point is not affected.

Strengths of the present study are that pre-analytical factors have been minimized and that individuals, in contrast to previous studies, were frequently sampled over a long time period.

In conclusion, the results of our study provide insights in the biological variability of VWF and FVIII. This will improve the design of new epidemiological studies investigating the association between haemostatic markers and the risk of thrombosis. In addition the results may improve monitoring and diagnostic precision for patients with VWD.

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CHAPTER 9

SUMMARY AND GENERAL DISCUSSION

SUMMARY

The main objective of this thesis was to unravel the association between genetic variation, von Willebrand factor (VWF) levels, and the risk of cardiovascular disease (CVD). We focussed on the relationship between variation in the VWF gene, VWF antigen (VWF:Ag) levels, and the risk of stroke. In addition, the biological variation of VWF and factor (F) VIII was determined, based on which recommendations are provided that improve the assessment of VWF and FVIII as risk indicators for thrombotic disease and increase the diagnostic precision for von Willebrand disease (VWD).

Since VWF has a key role in primary haemostasis and is a marker of endothelial cell activation, its association with risk of CVD is plausible and therefore, this association has frequently been the subject of investigation. In **Chapter 2** we reviewed studies that investigated the association between VWF levels and risk of CVD, including myocardial infarction and stroke. Also, studies that investigated the contribution of genetic variation to VWF:Ag, VWF activity levels and the risk of CVD were reviewed. It has been shown that besides the VWF gene, also genes coding for ABO blood group, ADAMTS13, thrombospondin-1 and SNARE proteins regulate VWF levels. Exploring the association between genetic variation, VWF levels and the risk of arterial thrombosis may help elucidate whether there is a causal role for VWF in the development of CVD. However, the published results on the contribution of these genetic variants to the risk of CVD were conflicting. This may be explained by the differences in study design and population, studied endpoint and also because the studies were often underpowered. Consequently, it remains unclear whether VWF is a causal factor in the development of CVD or primarily mirrors endothelial dysfunction which predisposes to atherosclerosis and thrombosis. Studies conducted so far provide interesting results that justify further exploration of the association between VWF and CVD in large prospective population-based cohorts with well-defined homogeneous study endpoints.

The association between VWF levels and stroke risk has not been investigated as extensively as the association between VWF levels and the risk of coronary heart disease (CHD). In **Chapter 3**, we studied the association between VWF:Ag levels and stroke risk in The Rotterdam Study. The Rotterdam Study is a large population-based cohort study amongst elderly (aged >55 years), who are at the highest risk of stroke. For the analyses, a total of 6250 participants, who were free of stroke at baseline and of whom blood samples were available, were included. During an average follow-up time of 5.0 years 290 first-ever strokes occurred, of which 197 strokes were classified as ischemic. We showed that the risk of stroke increased with increasing VWF levels. The age and sex adjusted hazard ratios (HR) per standard deviation increase in VWF level was 1.12 (95% CI: 1.01-1.25) for total stroke and 1.13 (95% CI: 0.99-1.29) for ischemic stroke. Adjustment for additional confounders slightly attenuated the association. Although previous research suggested the risk profile for stroke is different in male and female patients, we observed a similar association between VWF and

stroke in men and women. From this study, we conclude that high VWF levels are associated with stroke risk in elderly.

Since previous studies showed that the proportion of circulating active VWF, i.e. VWF in its active GPIbα-binding conformation, is increased during pathological conditions that increase the risk of micro-vascular thrombosis, we hypothesized that active VWF is associated with larger vessel thrombosis such as ischemic stroke. In **Chapter 4,** we investigated the association between the proportion of active VWF and stroke risk in a case-control study (COCOS Study) in which 124 patients with a first-ever ischemic stroke or transient attack (TIA) and 125 age- and sex matched controls, were included. In the cases, blood was collected 7-14 days after the event and from an unselected subgroup of these patients also three months after the event. The measured active GPIbα-binding conformation of VWF was expressed as VWF activation factor and could be measured in 94 cases and 103 controls. The VWF activation factor (expressed as median, [25%-75% range]) was not different between ischemic stroke cases and controls both one week (1.2 [0.9-1,8] versus 1.3 [0.9-1.7]; p=0.9), and three months after the event (1.2 [1.0-1.6] versus 1.3 [0.9-1.7]; p=0.4). Also in the subgroup of 35 cases with stroke due to small vessel disease the VWF activation factor was similar to the control group. In addition, no association between VWF activation factor and stroke risk was identified. Interestingly, we showed that the VWF activation factor in controls was higher in blood group non-O than in blood group O individuals, indicating that not only VWF levels but also the proportion of circulating active VWF may be influenced by blood group.

Since VWF propeptide (VWFpp) plasma levels reflect the rate of VWF secretion in steady state conditions, the association between VWF secretion and ischemic stroke can be investigated. In **Chapter 5** we investigated the association between VWFpp and the occurrence of ischemic stroke in two independent case-control studies. The first case-control study (COCOS Study) has previously been described in Chapter 4. In the second case-control study (ATTAC Study), young patients (males aged ≤45, females aged ≤55), who suffered from a first acute ischemic complication in the cardiac or cerebral vascular system were included. At the time this study was conducted, 171 patients with an ischemic stroke or TIA were included. Blood from patients was collected three months after the event. VWFpp and VWF:Ag levels were determined in 101 patients and 103 controls for the COCOS Study, and 171 patients and 171 controls for the ATTAC Study. In the COCOS Study the estimated relative risk of stroke in the highest quartile of VWFpp and VWF:Ag levels relative to the lower three quartiles was 1.9 (95% CI: 1.0-3.6) and 1.9 (95% CI: 1.0-3.3), respectively. In the ATTAC study the estimated relative risk of stroke was 1.7 (95% CI: 1.1-2.8) for VWFpp, and 1.9 (95% CI: 1.1-3.1) for VWF:Ag levels. We concluded that increased VWF secretion, measured by VWFpp levels, and a high total VWF level are both associated with the occurrence of ischemic stroke. This indicates that endothelial cell activation, reflected

by VWFpp secretion, is an important mechanism underlying the association between total VWF level and the occurrence of ischemic stroke.

Although VWF levels are strongly heritable and genetic susceptibility is an important risk factor for CVD, information on the contribution of common VWF gene variants to VWF levels and CVD risk is limited. In **Chapter 6,** 27 haplotype-tagging single nucleotide polymorphisms (ht-SNPs) covering the total VWF gene variation were selected, and genotyped in a case-control study of 421 young patients with a first event of acute coronary heart disease (CHD) or ischemic stroke, and 409 healthy population based controls (males aged ≤45, females aged ≤55). We investigated the association between common VWF gene variants, VWF antigen (VWF:Ag) levels, VWF collagen binding (VWF:CB) activity, and CVD risk. Two new associations were identified: For ht-SNP rs4764478 (intron 45), the increase in VWF:Ag levels and VWF:CB activity per minor allele was 0.082 (± 0.026) IU/ml (p=0.001) and 0.096 (± 0.030) IU/ml (p=0.002), respectively. Ht-SNP rs216293 (intron 17), was associated with CVD risk (odds ratio (OR) 1.44, 95% CI 1.12-1.86 per minor allele).We confirmed the association between rs1063857 and CVD risk indentified in previous studies. Our data show that common variants in the VWF gene are associated with VWF levels and with the risk of CVD.

A previous meta-analysis of five large genome-wide association studies identified SNPs within 8 genetic loci as determinants of VWF:Ag levels. Whether these SNPs are associated with stroke risk is not known. Therefore in **Chapter 7**, we investigated the association between genetic determinants of VWF:Ag levels and stroke risk. The study was part of The Rotterdam Study, a population-based cohort study among individuals of 55 years and older. A total of 5763 participants of whom DNA was available, and who were free of stroke at baseline, were eligible for analysis. VWF:Ag levels were measured in 3379 eligible participants. Within each of the eight loci one top SNP was defined. The association between the eight SNPs and the risk of stroke was analyzed. Then a genetic score, based on these eight SNPs, was constructed, and its total contribution to VWF plasma levels and stroke risk was investigated. None of the eight SNPs were individually associated with stroke risk. A higher genetic score was significantly associated with a higher VWF:Ag level, but was not associated with an increased risk of stroke. From this study we concluded that eight SNPs that strongly determine VWF levels, are not associated with stroke risk, neither individually, nor combined in a genetic score.

In **Chapter 8** we determined in 520 blood samples collected over a 1-year time period from 40 individuals, the between-subject biological variation, within-subject biological variation and analytical variation for VWF:Ag level, VWF ristocetin cofactor activity (VWF:RCo), VWF collagen binding activity (VWF:CB) and FVIII activity. Considerable differences in biological and analytical variation existed. Based on the obtained biological and analytical variation, the calculated number of repeated measurements needed to estimate a homeostatic setting point was 2 for VWF:Ag level and FVIII activity, 5 for VWF:RCo activity and 8 for

VWF:CB activity. Based on these results we concluded that one measurement per analyte is not representative for an individual's homeostatic setting point. In epidemiological studies, we are interested in the differences between homeostatic means of study participants and not in their short-term fluctuations. Therefore, depending on the analyte, 2, 5 or 8 measurements are needed to improve their usefulness as risk indicators. Our results have also clinical implications as laboratory evaluation of VWF parameters and FVIII is critical to diagnose VWD. Although it is common practice to repeat a test before drawing any conclusion, the presented results show that for VWF:RCo and VWF:CB activity more than 2 measurements are needed to increase diagnostic precision.

GENERAL DISCUSSION

Because of the pivotal role of von Willebrand factor (VWF) in platelet adhesion and aggregation, [1,2] a causal association between high VWF plasma levels and the development of cardiovascular disease (CVD) is anticipated. Numerous studies have investigated the association between VWF and coronary heart disease (CHD) and showed that high VWF levels are associated with an increased risk of CHD [3]. The association between VWF and stroke has been investigated less frequently. A few case-control studies identified a positive association between high VWF levels and risk of stroke [4-11]. In case-control studies VWF levels are measured after the stroke was diagnosed. Since VWF is largely synthesized by endothelial cells, and secreted when endothelial cell are activated or damaged [2,12], it remains debatable whether high VWF levels are a cause of stroke or a consequence of endothelial damage attributable to stroke [13-15].

By measuring VWF propeptide (VWFpp) we investigated VWF secretion due to endothelial cell activation in steady state conditions. Our data suggest that the association between total VWF and the occurrence of stroke has the same magnitude as the association between VWFpp and stroke. Therefore, we concluded that endothelial cell activation is a large contributor to the association between total VWF and stroke in case-control studies [16].

When investigating the association between VWF and stroke risk in a longitudinal setting, the increase in VWF during the acute phase post-stroke is avoided. Unfortunately, not many prospective studies on VWF and stroke risk have been conducted. We showed that VWF is an independent predictor of stroke in a cohort study of elderly individuals aged 55 and older [17]. Our results corroborate one previous conducted prospective study on this subject [18]. Also in prospective studies, an increased VWF may still merely reflect endothelial damage without being a causal mediator, because endothelial dysfunction predisposes to atherosclerosis, plaque rupture and subsequent arterial thrombosis. In future prospective studies, besides VWF, also VWFpp should be measured, and associations of both parameters

with stroke risk have to be calculated. Comparing the risk estimates for total VWF and VWFpp may reveal more insight in the pathogenetic role of VWF in stroke. In addition, since we have shown that a single measurement of VWF may not be representative for an individual's homeostatic setting point [19], in future epidemiological studies, an adequate number of measurements per individual is needed in order to increase the power of studies and to improve the assessment of VWF as risk indicator.

Previous studies have indicated that VWF is a strong independent risk factor for coronary events in patients with pre-existing vascular disease, particularly for myocardial infarction survivors [20-23]. The relationship between VWF and the risk of stroke in populations with pre-existing cerebral vascular disease has yet not been investigated. Future prospective studies should investigate the association of VWF with the risk of recurrent stroke in patients with a previous stroke (TIA or CVA). In addition, since several case-control studies indentified differences in risk for stroke-subtypes [4,7,10], also the association between VWF and stroke sub-types needs further exploration. If VWF is identified as a strong independent risk indicator for stroke or stroke sub-types, it may provide incremental information when added to standard risk markers for CVD. This may eventually improve the assessment of individuals at risk of developing stroke in the future [24].

In a resting state, VWF circulates in an inactive conformation which is not able to interact with platelets spontaneously [25]. High shear converts VWF into its active GP1bα-binding conformation. In pathological conditions such as atherosclerotic narrowing and plaque rupture shear forces increase. Only active VWF molecules are able to interact with platelets. Based on this knowledge is has been hypothesized that the association between active VWF and stroke may better reflect the association between VWF and CVD risk than VWF plasma concentration as measured with the VWF antigen (VWF:Ag) assay. Conducted studies thus far mainly focussed on VWF:Ag levels. Although we could not identify an association between active VWF and the risk of stroke in a case-control study [26], a more recent study on active VWF and the risk of myocardial infarction in young women identified a positive association [27]. In addition, active VWF has shown to be increased during pathological conditions that increase the risk of thrombotic micro-angiopathies, such as thrombotic thrombocytopenic purpura [28-30]. Overall, the results of these studies justify further exploration of the relationship between active VWF and the risk of CVD.

The previously mentioned suggestions for future studies may further explore the association of VWF with stroke and may improve the assessment and applicability of VWF as risk indicator for stroke (CVD). However, whether VWF has a causal role in the development of stroke and coronary heart disease can not be elucidated by observational epidemiologic studies.

The causal role of VWF in atherosclerosis and arterial thrombotic disease has been investigated in experimental and animal studies [31,32]. In a mouse model of transient middle cerebral artery occlusion, VWF-deficient mice were protected from brain injury.

Infarct sizes in VWF-deficient mice were 60% of the infarct volumes in wild-type controls [33]. Also, mice deficient of ADAMTS13, the principal regulator of VWF multimer size and subsequent activity, developed larger cerebral infarction compared to wild-type mice [34,35]. Overall, studies in animal models point towards a causal role of VWF in stroke development. However, due to large differences between animal models and humans, it remains debatable whether these results can be extrapolated to humans.

Evidence for a causal role of VWF in the development of CVD in humans may come from genetic association studies. In these *Mendalian Randomization* studies, genetic variants that proxy for potential, environmentally modifiable, risk factors such as VWF, are used as instruments for the specific exposure. Mendel's second law suggests that the inheritance of genetic variants is subject to random assortment of maternal and paternal alleles. Consequently, genotypes do not correlate with other risk factors for CVD. This minimizes confounding as potential confounders are distributed evenly among the different genotypes. In addition, bias due to reverse causation is avoided because genotypes are determined at conception and are not prone to modification by the onset of disease (i.e. atherosclerosis) [36,37]. If VWF is causally associated with the risk of CVD, a genetic variant that influence VWF plasma levels should increase the risk of CVD to the same extent as predicted by its influence on VWF plasma levels.

By investigating the association between total common variation in the VWF gene, VWF:Ag levels, VWF collagen binding activity (VWF:CB) and CVD risk in the ATTAC Study, we identified several variants associated with VWF levels and CVD in young individuals [38]. Our results were in accordance with results of other smaller studies on this subject [39-42]. The single nucleotide polymorphism (SNP) rs1063857, known to be associated with VWF:Ag levels was also associated with the risk of CVD, which indicates a causal role for VWF. However, in our study, variants associated with the risk of CVD were not always mediated by VWF:Ag levels or VWF:CB activity. This suggests that other mechanisms than increased VWF levels might also mediate the association between VWF gene variation and the risk of CVD. For instance by structural alterations of the VWF protein or by an increased portion of circulating active VWF.

In a subsequent study we showed that eight genetic variants located in eight different genes combined in a genetic score, which increases the power for detecting an association, strongly determines VWF:Ag levels in the Rotterdam Study. However, the genetic score was not associated with the risk of stroke in this population of elderly people, making a causal role of VWF in stroke less plausible [43]. The conflicting results with the ATTAC study may be attributable to the large difference in age range between the two studies. The ATTAC study included young individuals with a first stroke or myocardial infarction. Previous studies have shown that the genetic contribution to the development of CVD is stronger in younger individuals [44,45]. As a result, the statistical power of the study may have been too low to identify a significant association with CVD risk.

Overall, the results of the presented studies justify further exploration of the causal role of VWF by conducting genetic association studies via the method of *Mendelian Randomization*. The association between the genotype-intermediate phenotype and genotype-disease should be investigated in the same large population based prospective cohort as the association between the intermediate phenotype and disease. In the cohort a sufficient number of individuals with a well-defined homogenous study endpoint should be included. Not only VWF:Ag but also VWF:CB activity or VWF ristocetin cofactor activity and active VWF should be investigated as intermediate phenotypes. ADAMTS13 levels have been associated with the risk of CVD [5,11,46,47] In addition, variants in the ADAMTS13 gene have been associated with ADAMTS13 activity and with the risk of CVD [48-50]. Therefore, also genetic association studies with genes involved in the regulation of VWF could indentify a causal role for VWF.

Evidence for a causal role of VWF may also come from randomized controlled clinical trials with agents that exclusively inhibit VWF function. If treatment with a VWF inhibitor reduces VWF level or activity and the risk of CVD, convincing evidence for the causal nature of a high VWF in the development of CVD is delivered. The past several years, agents that inhibit the VWF-collagen or VWF-GP1bα interaction have been developed. Examples are monoclonal antibodies against VWF (82D6A3m AJvW2, AJW200, or GP1bα (6B4, h6B4), the nanobody ALX-0081 and the aptamer ARC1779 [51-53]. Most agents have been extensively tested in pre-clinical thrombosis models and showed strong antithrombotic activity without increasing the risk of bleeding [54-57]. Several agents were well tolerated in healthy volunteers in phase I trials. In a recent clinical trial, the aptamer ARC1779 reduced cerebral emboli in patients undergoing carotid endarterectomy [58]. Several others such as ALX-0081 have starting to enter Phase II clinical trials [59]. Future randomized controlled clinical trials have to show whether these VWF blockers may reduce ischemic injury in the acute phase of arterial-thrombosis and may be useful in CVD prevention. If so, strong evidence for a causal role of VWF has been provided.

Overall, conducted studies thus far have provided some evidence for a causal role of VWF in CVD. However, the matter is not fully resolved and additional experimental animal studies, genetic association studies, and randomized clinical trial are required to completely elucidate the role of VWF in CVD.

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NEDERLANDSE SAMENVATTING

In dit proefschrift is de associatie tussen genetische variatie, von Willebrand factor (VWF) levels, en het risico op het ontwikkelen van hart- en vaatziekten (HVZ) onderzocht. Er is voornamelijk gekeken naar de relatie tussen variatie in het VWF gen, VWF antigen (VWF:Ag) levels, variatie en het risico op het ontwikkelen van een herseninfarct. Ook is de biologische variatie in VWF en factor VIII (FVIII) levels bepaald. Op basis van deze biologische variatie worden aanbevelingen gedaan om de toepasbaarheid van VWF en FVIII als risico indicatoren voor thrombotische ziekten te verbeteren en de diagnostische precisie voor von Willebrand ziekte te (VWD) vergroten.

Omdat VWF een sleutelrol heeft in de primaire haemostase en tegelijkertijd een marker is van endotheel activatie kan men veronderstellen dat VWF geassocieerd is met het risico op HVZ. De relatie tussen VWF en HVZ is dan ook vaak onderzocht. In **Hoofdstuk 2** wordt een overzicht weergegeven van de studies waarbij de relatie tussen VWF levels en het risico op HVZ zoals een hartinfarct en een herseninfarct is onderzocht. In dit hoofdstuk worden echter voornamelijk studies besproken die de invloed van genetische variatie op VWF:Ag levels, VWF activiteit en HVZ beschrijven. De studies laten zien dat naast het VWF gen ook genen die coderen voor bloedgroep, ADAMTS13 en SNARE eiwitten VWF levels en activiteit beïnvloeden. Het onderzoeken van de associatie tussen genetische variatie, VWF levels en HVZ kan helpen de rol van VWF in de pathogenese van HVZ te verduidelijken. Echter, de onderzoeksresultaten over de bijdrage van genetische determinanten van VWF en het risico op HVZ zijn niet eenduidig. Dit kan veroorzaakt worden door een verschil in ontwerp van de uitgevoerde studies. Of omdat er onderzoek gedaan werd in heterogene studie populaties en er gekeken werd naar verschillende eindpunten. Ook waren veel studies underpowered. Hierdoor is het niet duidelijk of VWF een causale rol speelt in de ontwikkeling van HVZ of voornamelijk endotheelschade weerspiegelt op basis waarvan atherosclerose en thrombose ontstaan. Samenvattend, de gepubliceerde studies tot nu toe laten interessante resultaten zien en rechtvaardigen verder onderzoek naar de relatie tussen VWF en HVZ in grote prospectieve population-based studies met goed gedefinieerde homogene studie eindpunten.

De relatie tussen VWF levels en het risico op het ontwikkelen van een beroerte is niet zo frequent onderzocht als de relatie tussen VWF levels en het risico op het ontwikkelen van een hartinfarct. In **Hoofdstuk 3** is de relatie tussen VWF:Ag levels en het risico op het ontwikkelen van een beroerte weergegeven in De Rotterdam Studie. De Rotterdam studie is een grootschalig bevolkingsonderzoek onder ouderen (>55 jaar) die het hoogste risico lopen het krijgen van een beroerte. Voor de analyses werden in totaal 6250 personen geincludeerd, allen zonder beroerte in de voorgeschiedenis bij aanvang van de studie. Tijdens de follow-up periode van gemiddeld 5 jaar kregen 290 participanten een eerste

beroerte. Hiervan waren er 197 een herseninfarct. De resultaten van deze studie laten zien dat het risico op een beroerte toeneemt naarmate de VWF:Ag level hoger is. De leeftijd en geslacht gecorrigeerde hazard ratio (HR) per standaard deviatie toename van VWF level was 1.12 (95% CI 1.01-1.25) voor een beroerte en 1.13 (95% CI 0.99-1.29) voor een herseninfarct. Correctie voor additionele confounders verzwakte de associatie minimaal. Eerder onderzoek liet zien dat het risicoprofiel voor het ontwikkelen van een beroerte verschillend is voor mannen en voor vrouwen. In deze studie werd geen verschil tussen mannen en vrouwen gevonden. Uit deze studie concluderen wij dat hoge VWF levels geassocieerd zijn met een hoger risico op een beroerte bij ouderen.

Eerdere studies lieten zien dat circulerend actief VWF (VWF in de actieve GP1bα-bindings conformatie) verhoogd is tijdens pathologische omstandigheden waarbij er een verhoogd risico is op micro-vasculaire thrombose. Wij veronderstelden dat actief VWF ook verhoogd zou kunnen zijn bij thrombose van grote bloedvaten zoals bij een herseninfarct. In **Hoofdstuk 4** hebben we de relatie tussen actief VWF en het risico op het ontwikkelen van een herseninfarct onderzocht in de COCOS studie. In deze studie werden 124 patiënten met een eerste herseninfarct of TIA (transient ischemic attac) en 125 in leeftijd en geslacht overeenkomende controle personen die geen herseninfarct hadden doorgemaakt geincludeerd. Van de patiënten werd 7-14 dagen na het herseninfarct bloed afgenomen. Van een ongeselecteerde subgroep van deze patiënten werd drie maanden na het herseninfarct nogmaals bloed afgenomen. Actief VWF (uitgedrukt als mediaan, [25%-75% range]) was niet verschillend tussen patiënten en controle personen, zowel 1 week (1.2 [0.9-1.8] versus 1.3 [0.9-1.7]; p=0.9), als drie maanden na het herseninfarct (1.2 [1.0-1.6] versus 1.3 [0.9-1.7]; p=0.4). Ook in de subgroep van patiënten met een herseninfarct door ziekte van kleine vaten is actief VWF hetzelfde als in de controle personen. Echter, in deze studie is bij controle personen het actief VWF hoger in individuen met bloedgroep non-O dan bloedgroep O. Dit zou kunnen betekenen dat niet alleen VWF levels maar ook het circulerend actief VWF beïnvloed wordt door bloedgroep.

Omdat het VWF propeptide (VWFpp) level de mate van VWF secretie reflecteert in een steady-state situatie kan de associatie tussen VWF secretie en het voorkomen van een herseninfarct worden onderzocht. In **hoofdstuk 5** hebben we de associatie tussen VWFpp en het voorkomen van een herseninfarct onderzocht in twee onafhankelijke case-control studies. De eerste studie (COCOS studie) is eerder beschreven in hoofdstuk 4. In de tweede studie (ATTAC studie) worden jonge patiënten (mannen ≤ 45 en vrouwen ≤ 55 jaar) die zijn getroffen door een eerste hersen- of hartinfarct geincludeerd. Op het moment dat deze studie werd uitgevoerd waren 171 patiënten met een eerste herseninfarct of TIA opgenomen in de ATTAC studie. Bij deze patiënten werd drie maanden na het infarct bloed afgenomen. VWFpp en VWF:Ag levels werden gemeten in 101 patiënten en 103 controle personen van de COCOS studie en 171 patiënten en 171 controle personen van de ATTAC studie. In de COCOS studie was het geschatte relatieve risico op het ontwikkelen van een

herseninfarct in het hoogste quartiel vergeleken met de drie laagste quartielen 1.9 (95% CI: 1.0-3.6) voor VWFpp levels en 1.9 (95% CI: 1.0-3.3) voor VWF:Ag levels. In de ATTAC studie was het geschatte relatieve risico 1.7 (95% CI: 1.1-2.8) voor VWFpp en 1.9 (95% CI 1.1-3.1) voor VWF:Ag levels. Hieruit werd geconcludeerd dat zowel hoge VWF secretie gemeten door het bepalen van VWFpp levels, als een hoog totaal VWF level geassocieerd is met het voorkomen van een herseninfarct. Omdat VWF secretie is toegenomen bij endotheel activatie laat deze studie zien dat endotheel activatie een belangrijk onderliggend mechanisme is voor de associatie tussen totaal VWF level en het voorkomen van een herseninfarct.

Genetische predispositie is een belangrijke risicofactor voor het ontwikkelen van HVZ. Ook de hoogte van het VWF level wordt sterk genetisch bepaald. Informatie over de bijdrage van veel voorkomende genetische varianten in het VWF gen aan VWF levels en het risico op HVZ is echter beperkt. In **Hoofdstuk 6** worden 27 haplotype-tagging single nucleotide plymorphisms (ht-SNPs) geselecteerd die de totale (veel voorkomende) genetische variatie in het VWF gen vertegenwoordigen. Deze 27 SNPs worden gegenotypeerd in een studie van 421 jonge patiënten met een eerste hart- of herseninfarct en 409 gezonde controle personen (mannen ≤ 45 en vrouwen ≤ 55 jaar). De relatie tussen de veel voorkomende VWF-gen varianten, VWF:Ag levels, VWF collageen bindings (VWF:CB) activiteit en HVZ werd onderzocht. Twee nieuwe associaties werden gevonden. Voor ht-SNP rs4764478 (intron 45), was de toename in VWF:Ag level en VWF:CB activiteit per allel met de laagste frequentie respectievelijk 0.082 (± 0.026) IU/ml (p=0.001) en 0.096 (±0.030) IU/ml (p=0.002). Ht-SNP rs216293 (intron 17) was geassocieerd met een hoger risico op HVZ (odds ratio (OR) 1.44, 95% CI 1.12-1.86 per allel met de laagste frequentie). De studie bevestigt de in eerdere studies aangetoonde associatie tussen ht-SNP rs1063857 en HVZ. Op basis van de resultaten van deze studie kan worden geconcludeerd dat veel voorkomende genetische varianten in het VWF gen geassocieerd zijn met VWF levels en met het risico op HVZ.

Een eerdere meta-analyse van 5 grote GWA studies (genome-wide association studies) identificeerden associaties tussen SNPs en VWF levels in 8 verschillende genetische loci. Of deze SNPs ook geassocieerd zijn met het risico op HVZ was nog niet onderzocht. In **Hoofdstuk 7** onderzoeken we de associatie tussen genetische determinanten van VWF levels en het risico op een beroerte. Het onderzoek is uitgevoerd in de Rotterdam studie. Deze studie werd eerder beschreven in hoofdstuk 3. In totaal waren 5763 participanten die niet waren getroffen door een beroerte bij aanvang van de studie geschikt voor analyse. VWF:Ag levels werden bepaald in 3379 participanten. In ieder van de 8 genetische loci werd de SNP die het sterkst geassocieerd was met VWF:Ag levels geselecteerd. De associatie tussen deze 8 SNPs en het risico op een beroerte werd onderzocht. Hierna werd een genetische score gebaseerd op deze 8 SNPs geconstrueerd. De associatie tussen deze score, VWF:Ag levels en het risico op een beroerte werd onderzocht. Geen van de 8 individuele SNPs waren geassocieerd met het risico op een beroerte. Een hogere genetische score was sterk geassocieerd met VWF:Ag levels maar niet met het risico op een beroerte. Uit

deze studie kan worden geconcludeerd dat de 8 SNPs die in sterke mate het VWF level bepalen niet geassocieerd zijn met het risico op een beroerte, zowel niet individueel als gecombineerd in een genetische score.

In **Hoofdstuk 8** wordt een longitudinale studie beschreven waarin bij 40 gezonde vrijwilligers 520 bloedmonsters over een periode van 1 jaar worden afgenomen. Hierin wordt de tussenpersoons biologische variatie, binnenpersoons biologische variatie en analytische variatie bepaald voor VWF:Ag levels, VWF ristocetin cofactor activiteit (VWF:RCo), VWF collageen bindings activiteit (VWF:CB) en factor VIII (FVIII) activiteit. Er bestonden aanzienlijke verschillen in biologische en analytische variatie tussen de verschillende assays. Aan de hand van de verkregen variatie componenten hebben we het aantal herhaalde metingen berekend waarmee een homeostatisch setting point kon worden geschat. Dit was 2 voor VWF:Ag levels en FVIII activiteit, 5 voor VWF:RCo activiteit en 8 voor VWF:CB activiteit. Aan de hand van deze resultaten kan worden geconcludeerd dat een meting per assay niet representatief is voor het homeostatische setting point van een individu. In epidemiologische studies zijn de verschillen tussen homeostatische gemiddelden van participanten relevanter dan korte termijn fluctuaties. Afhankelijk van de assay zijn daarom 2, 5 of 8 metingen per individu nodig om hun toepasbaarheid als risico indicator te verbeteren. De resultaten hebben ook klinische implicaties omdat het meten van VWF en FVIII belangrijk is voor het diagnosticeren van von Willebrand ziekte. De resultaten van deze studie laten zien dat voor VWF:RCo en VWF:CB activiteit meer dan 2 metingen nodig zijn om hun diagnostische precisie te verbeteren.

DANKWOORD

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Van Schie MC*, van Loon JE*, de Maat MPM, Leebeek, FWG. Genetic determinants of von Willebrand factor levels and activity in relation to the risk of cardiovascular disease: a review. J Thromb. Haemost. 2011 May; 9(5): 899-908.

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J Thromb. Haemost. 2012 mar;10:550–6..

Van Schie MC, Rudez G, Meijer P, Kluft P, Leebeek FWG, de Maat MPM. Biological variation in von Willebrand factor and factor VIII. *Submitted.*

*Shared first authorship.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 1 mei 1981 te Kedichem. Na het behalen van haar VWO diplama aan de christelijke scholengemeenschap Oude Hoven te Gorinchem ging ze Biomedische Wetenschappen studeren aan de Vrije Universiteit te Amsterdam na uitgeloot te zijn voor de studie Geneeskunde. Ze behaalde haar Propedeuse en kon in 2000 beginnen met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. In 2004 werd het doctoraal examen afgelegd en in 2006 behaalde ze haar artsexamen met lof. Nadat ze een half jaar als ANIOS interne geneeskunde, cardiologie en longziekte in het Albert Schweitzer ziekenhuis te Dordrecht had gewerkt, begon ze als promovendus op de afdeling Hematologie van het Erasmus MC onder leiding van Prof. Dr. F.W.G. Leebeek en Dr M.P.M de Maat. Op 1 mei 2010 is ze gestart met de opleiding tot internist.

ERASMUS UNIVERSITEIT ROTTERDAM

PHD PORTFOLIO SUMMARY

SUMMARY OF PHD TRAINING AND TEACHING ACTIVITIES

Name PhD student: M C van Schie Erasmus MC Department: Hematology Research School: COEUR	PhD period: From April 2007 until May 2010 Promotor(s): Prof. Dr. F.W.G Leebeek Supervisor: Dr. M.P.M. de Maat

1. PhD training	Year	Workload (ECTS)
General academic skills – Biomedical English Writing and Communication	2008	3
Research skills – Statistics	2007	5.7
In-depth courses (e.g. Research school, Medical Training) – PhD Courses at COEUR (6x) and MolMed (2x) – PhD Courses of The Dutch Society of Thrombosis and Haemostasis (3x)	 2007-2010 2007-2010	 12 4.5
Presentations – Orals (5x) – Posters (3x)	 2007-2010 2007-2010	 0.5 0.3
International conferences – 4 International conferences	 2007-2010	 6.0
Seminars and workshops Coeur research seminars and lectures (4x)	 2007-2010	 1.6
Didactic skills		
Other		
2. Teaching activities		
Lecturing		
Supervising practicals and excursions		
Supervising Master's theses – Supervising 3 students with their master thesis (3x20 weeks)	 2007-2010	 4.5