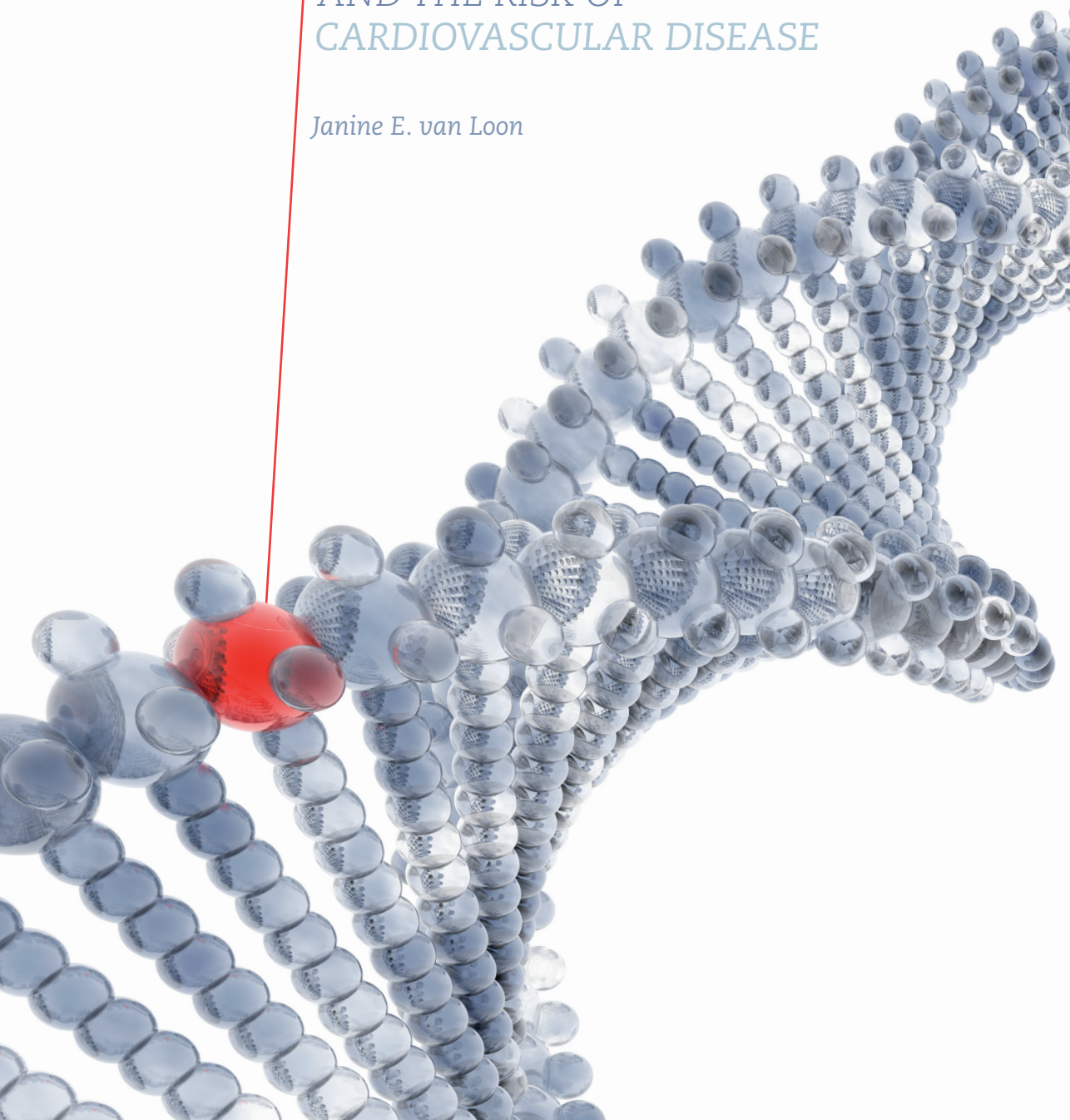
GENETIC DETERMINANTS OF VON WILLEBRAND FACTOR AND THE RISK OF CARDIOVASCULAR DISEASE

Janine E. van Loon



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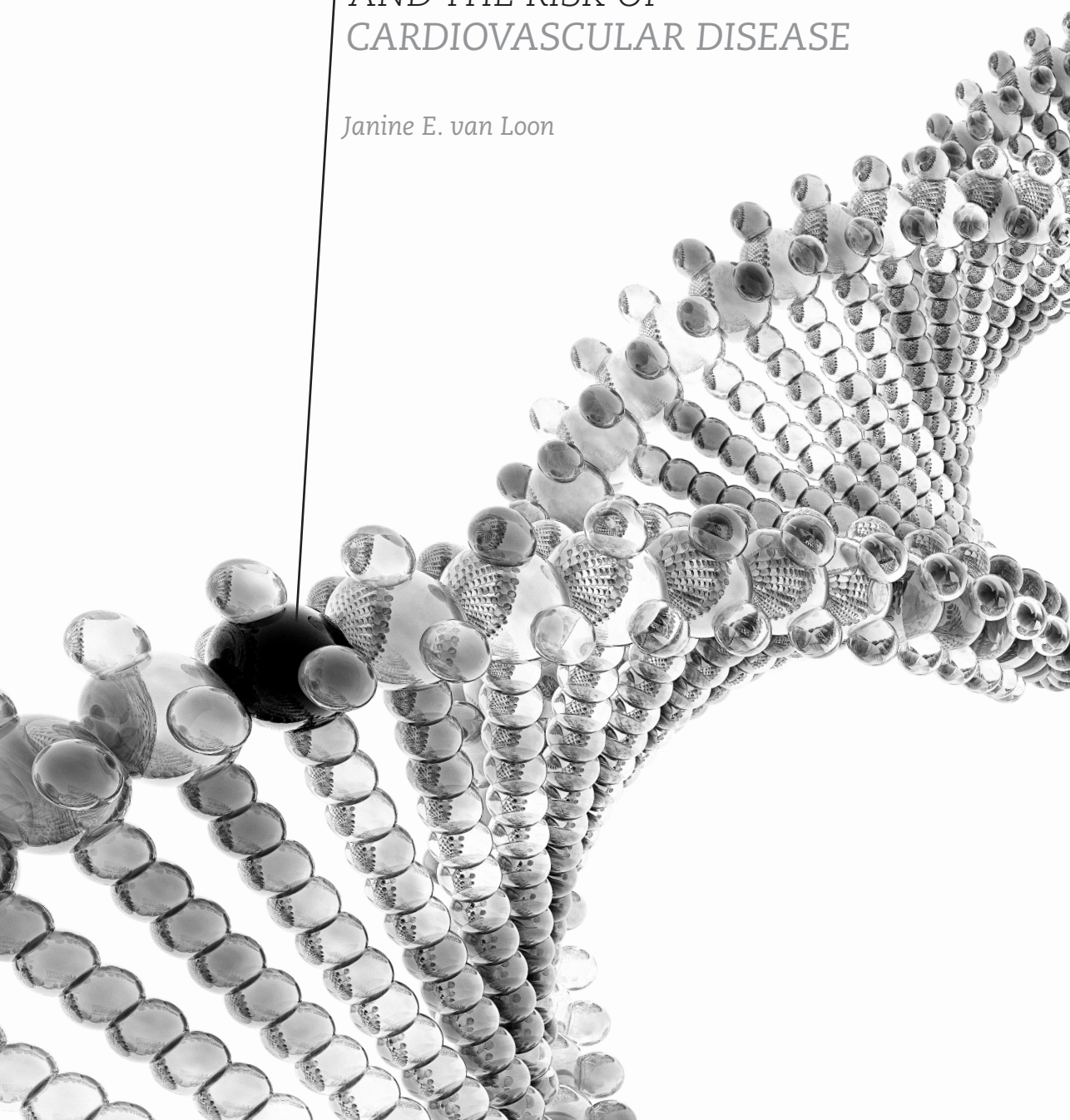

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Genetic determinants of von Willebrand Factor and the risk of cardiovascular disease

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GENETISCHE DETERMINANTEN VAN
von Willebrand Factor en het risico op hart- en vaatziekten

Proefschrift

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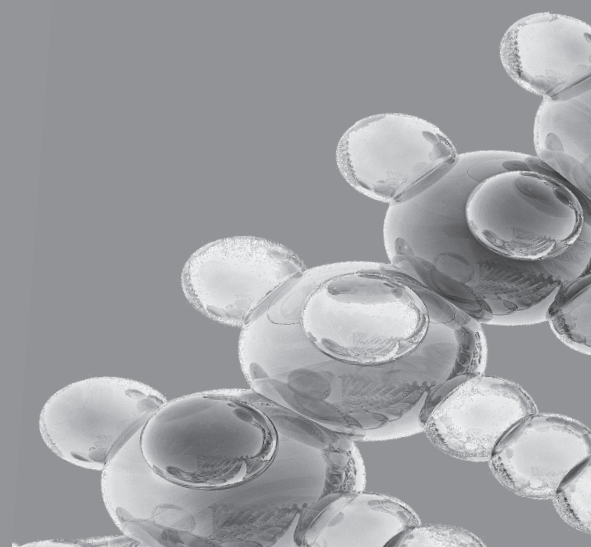
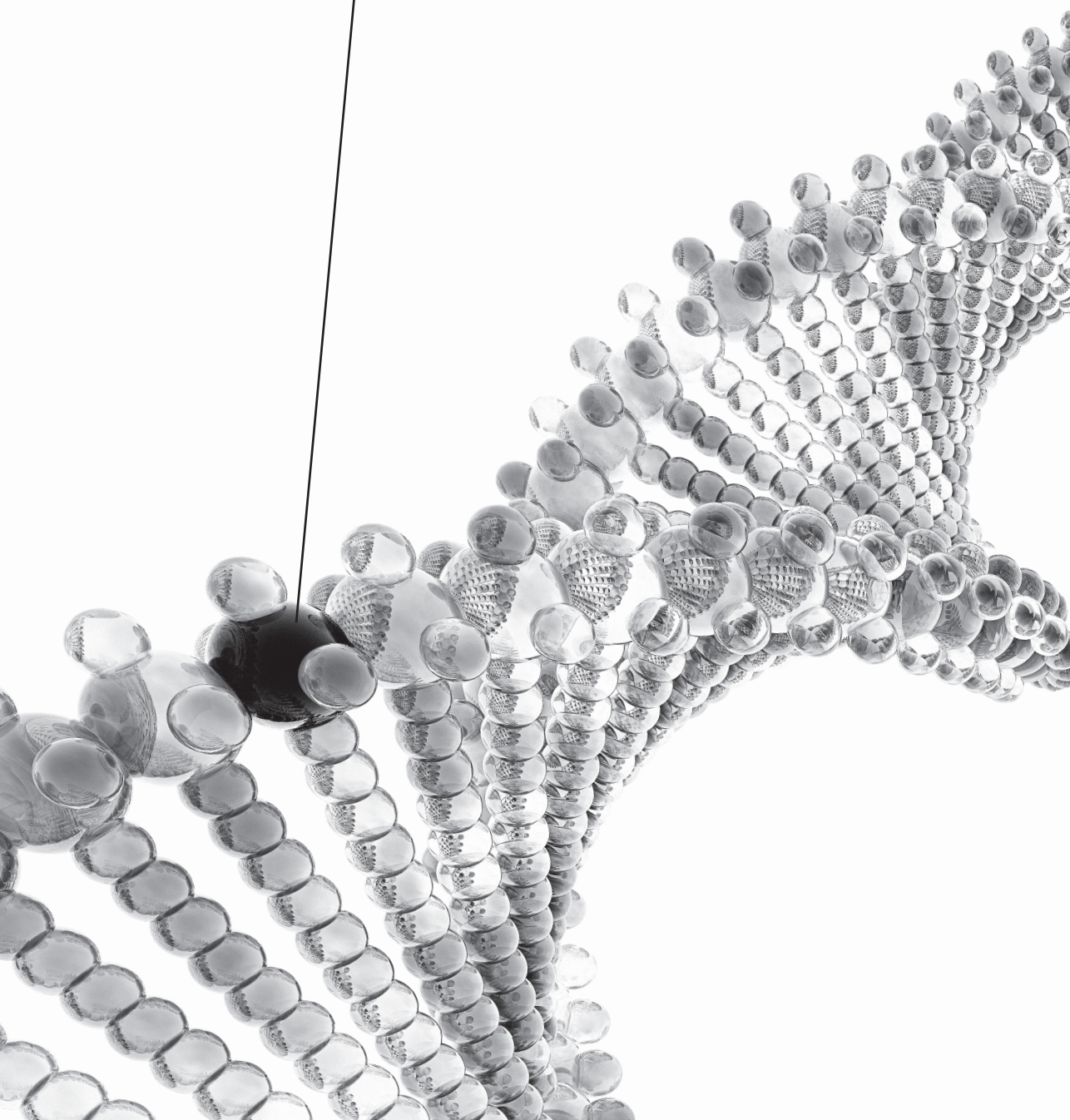


CHAPTER 1 *General introduction and outline of this thesis*



Normal haemostasis requires a delicate balance between procoagulant and anticoagulant factors. Disruption of this balance may lead to bleeding disorders, such as hemophilia, or thrombotic disorders, including deep vein thrombosis of the leg. As Virchow already reported in 1877, properties of the blood vessel wall, the blood flow and blood constituents contribute to the formation of thrombi in either veins or arteries[1]. However, through contemporary research the complexity of this process leading to thrombus formation has become more apparent.

An important player in thrombus formation is von Willebrand Factor (VWF), a large multifunctional glycoprotein. VWF initiates adherence of platelets to the injured vessel wall and subsequent platelet aggregation leading inevitably to the formation of thrombi[2-3]. In addition, VWF is a carrier protein of coagulation factor VIII (FVIII), thereby protecting it from clearance[4].

In healthy subjects normal plasma VWF levels range from 0.60 – 1.40 IU/mL and are characterized by a large variation. This can be partly attributed to a number of lifestyle and environmental factors, but most importantly to genetic factors[5-6].

The necessity of maintaining normal VWF levels in the circulation is illustrated by two clinical manifestations that may occur when VWF exceeds its normal range. Low levels of VWF may lead to bleeding, which is known as von Willebrand Disease (VWD), the most common inherited bleeding disorder in humans. To the contrary, high VWF antigen (VWF:Ag) levels are associated with an increased risk of venous thrombosis[7-8] and arterial thrombosis, including myocardial infarction (MI)[5, 9] and ischemic stroke[10].

Synthesis, secretion, and storage of von Willebrand Factor molecules

The VWF gene (*VWF*) is located on the short arm of chromosome 12 and encompasses 178 kilobases and 52 exons. A pseudogene of VWF is present on chromosome 22, which includes exons that correspond to regions of the original gene[11].

VWF molecules are mainly produced by endothelial cells and to a small extent by megakaryocytes[12-13]. VWF is synthesized as a precursor propeptide consisting of 2813 amino acids, which undergoes two proteolytic processing steps before it is released into the circulation (figure 1). In the endoplasmatic reticulum the proVWF subunits form dimers through disulfide bonds between the C-terminal ends of the VWF molecules. Also, N-linked carbohydrate residues are added, which are essential for dimerization and subsequent exit from this compartment[13]. The pro-VWF dimers are then transported to the Golgi apparatus where the propeptide facilitates formation of disulfide-linked VWF multimers. The VWF multimers differ in size and some multimers consists of more than 500 monomers. Finally, in the trans-Golgi network the propeptide is cleaved from the subunits. The remaining mature VWF proteins can either be secreted into the circulation constitutively or tubulized and packaged into Weibel Palade bodies (WPBs) within endothelial cells, from which they are released by basal and regulated secretion[14-17]. A

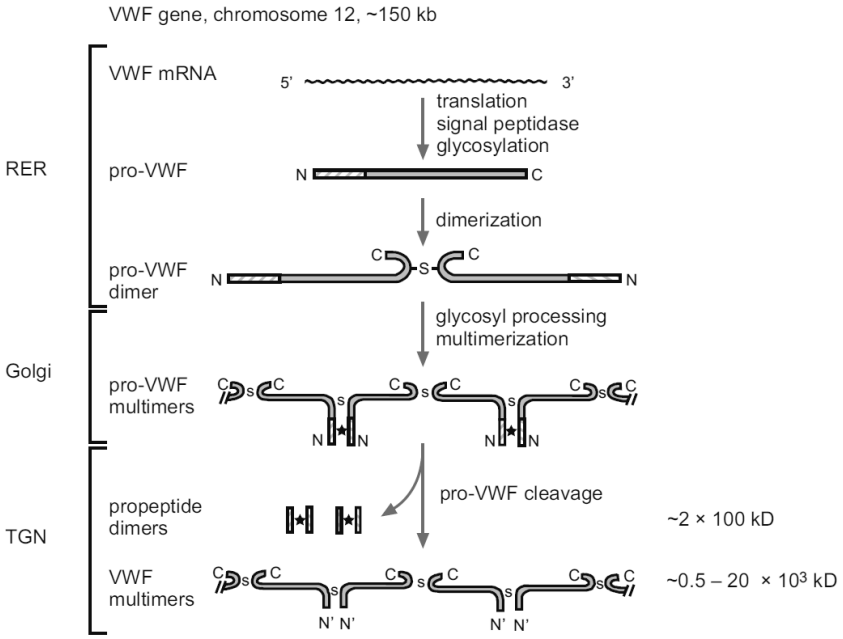


Figure 1 • Schematic representation of the processing steps involved in the biosynthesis of von Willebrand Factor. The hatched area represents the propeptide moiety of VWF and the dark area mature VWF. The compartments in which the processing steps take place are indicated on the left. * = non-covalent interaction; S = disulphide bonds. The molecular mass of the different VWF species is given on the right.
(Adapted from Verweij. Haemostasis. 1988;18:224-245 with permission)

small fraction (~5%) is stored in the alpha-granules of platelets. Especially the largest and most prothrombotic VWF multimers are stored in WPBs of endothelial cells. The release from these storage granules is induced by several physiological agonists, such as hypoxia[18], epinephrine, histamine, thrombin, fibrin and vasopressin[5].

Domain structure of VWF

The VWF protein consists of four types of repeated homologous domains (from the N- to the C-terminal region: D1, D2, D', D3, A1, A2, A3, D4, B, C1, and C2) (Figure 2). Each domain has a specific function and characteristic binding properties. The A1 domain contains binding sites for the platelet receptor GPIb, heparin, collagen, and ristocetin. The A2 domain holds a cleavage site for a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), which controls the multimer size of VWF molecules. The primary binding site for collagen is located in the A3 domain. The C1 domain has a binding site for another platelet receptor, GPIIb/IIIa. Lastly, the binding site for FVIII is located in de D' domain and partly in the adjacent D3 domain.

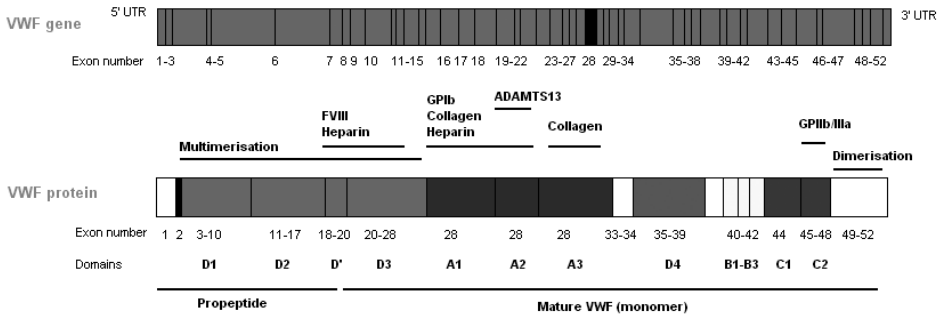


Figure 2 • Schematic representation of the von Willebrand Factor gene and protein. The upper part illustrates the structure of the VWF gene, including the positions of the exons and the identified SNPs. The lower part illustrates the protein structure of the pre-pro-VWF molecule with its various domains responsible for the different binding functions, as well as dimerisation and multimerisation.

Functions of VWF

At sites of vascular injury VWF facilitates primary haemostasis by forming adhesive bridges between platelets, exposed subendothelial structures, and adjacent platelets. VWF can only bind platelets, either activated or inactivated, when the A1 domain, which binds the GPIb-IX-V complex on platelets, is exposed. This can be induced by a conformational change following activation of the VWF molecule either through binding to subendothelial structures, such as collagen, or at high shear rates (>1000/second) as present in small arterioles and atherosclerotic arteries[15, 19]. Ultralarge VWF multimers are very haemostatically effective and are able to bind spontaneously to platelets in the circulation without previous activation[20]. GPIIb/IIIa platelet receptor can only bind VWF when platelets are activated[21-22]. This final step contributes to the irreversible binding of platelets to the subendothelium. Under very high shear conditions (>10.000/s) platelet aggregation is independent of the GPIIb/IIIa platelet receptor.

VWF functions also as a carrier protein for FVIII, which is an important protein cofactor in the thrombin generation pathway. VWF can only bind FVIII when FVIII is not cleaved by thrombin[23]. When FVIII is not attached to VWF, FVIII is proteolytically inactivated by activated protein C and its cofactor protein S, leading to a very short half-life of about two hours[4, 24-26].

Controlling multimer size

During the synthesis of VWF molecules, multimers of various sizes are formed. The freshly secreted and stored VWF molecules consist of ultralarge multimers, which are highly haemostatically effective in binding the GP Ib-IX-V complex. In order to avoid the occurrence of systemic thrombotic micro-angiopathies in the circulation, it is essential that the VWF multimers are proteolysed into smaller, less active VWF multimers. ADAMTS13 is a metalloprotease that reduces the size of large and ultralarge VWF multimers by

cleaving the Tyr842/Met843 peptide bond within the A2 domain of the VWF molecule. This scissile bond becomes exposed when VWF undergoes a conformational change, due to high shear forces or binding to subendothelial structures.

Thrombospondin-1 (TSP-1) is a multifunctional glycoprotein involved in numerous biological processes, including cell-cell interactions, inflammation, and thrombosis[27-30]. It is primarily synthesized by megakaryocytes as the major constituent of platelet α-granules, but can also be detected in the blood vessel wall[31]. Besides involvement in platelet adhesion and thrombus stabilization, TSP-1 can also control VWF multimer size. TSP-1 facilitates this directly via cleavage of the VWF linking disulfide bonds, and indirectly by competing with ADAMTS13[32].

Genetic association studies

In the last decade, knowledge of the structure of the human genome and genomic variation has extended enormously[33-34]. The most frequently occurring variations in the human genome are single nucleotide polymorphisms (SNPs). These variations can be mainly found in the noncoding regions of the genome. However, when variations occur in the coding region, they may alter the protein sequence or the level of gene expression leading to phenotypic effects. In contrast to rare mutations that may cause Mendelian diseases, such a cystic fibrosis and haemophilia, SNPs are generally not directly related to a disease but may only modulate the risk of disease. In addition, SNPs have by definition a frequency of >1% in the general population. According to the "common-disease-common-variant hypothesis" SNPs are specifically involved in the so-called complex diseases, such as cardiovascular disease, which is a common disease in the general population and has a multifactorial origin. So, although SNPs may have small effects, SNPs will affect larger groups and will have more impact than rare mutations.

SNPs mostly have an ancient origin, whereby their inheritance pattern is much more complex than the Mendelian inheritance pattern of rare mutations. However, due to natural genetic recombination throughout evolution the inheritance pattern of SNPs is not random. Some neighbouring SNPs in the genome are linked to each other and therefore always inherit together. This linkage structure, known as linkage disequilibrium (LD), facilitates studies to investigate the association between SNPs and disease risk, the so-called genetic association studies. By using the LD structure between SNPs it is not necessary to genotype all SNPs in a gene or even in the whole genome, but one can use a specific SNP as proxy for other SNPs (i.e. tagging SNP). The marker SNP and its corresponding SNPs form a so-called haplotype and always inherit together. By using this indirect approach of genetic association studies one can cover most of the human sequence variation and prior knowledge of the putative functional variants is not required.

The international HapMap project was created as a public resource to allow the indirect association approach to be applied for various purposes in the field of genetic association studies[35]. The international HapMap project has characterized common patterns of DNA sequence variation in the human genome, by characterizing sequence variants, their frequencies, and correlation between them, in DNA samples from populations with ancestry from parts of Africa, Asia, and Europe.

Initially, genetic association studies were only focused on single genes, i.e. candidate genes, of which the encoding proteins were thought to be involved in specific diseases based on their biological function. However, due to the start of the International HapMap project and new technical innovations, it is now possible to investigate common genetic variations in the whole genome in relation to complex diseases and phenotypes without a prior hypothesis (i.e. genome-wide association studies).

Although these recent developments have accelerated genetic association studies and the ability to study the whole genome, this manner of research also gave rise to new concerns. One of the main drawbacks of genome-wide association studies is the issue of multiple testing. By investigating associations between 1 million SNPs and a specific trait, a lot of associations would reach statistical significance at a p-value of 0.05 only by chance. Therefore, a new statistical significance threshold has been set for genome-wide association studies at a p–value of 5×10^{-8}. This measure led instantly to another problem. In order to reach such a highly significant p–value, the selected SNPs should comprise very large effects sizes or the study populations should be extremely large. Since SNPs have only small effects by definition, population-based studies started to collaborate and to pool their data in order to increase statistical power. One example of these collaborations is the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium[36].

Genetic determinants of von Willebrand Factor levels

Physiologically normal VWF levels have a very wide range. A number of lifestyle and environmental factors, such as age, smoking, hypertension, cholesterol and diabetes, are known to influence VWF levels[5]. However, studies in human twins have demonstrated that plasma levels of VWF have a high heritability of approximately 60%[6, 37].

The most important genetic 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. The presence of A and B antigens leads to a decreased VWF clearance[38-39]. Consequently, individuals with blood group non-O (A, B, or AB) have approximately 25% higher VWF:Ag levels than individuals with blood group O[40]. Also genetic polymorphisms in the VWF gene contribute to the variation in VWF:Ag levels[41-42].

Recently, the CHARGE consortium conducted a meta-analysis of genome-wide association studies of five large population-based cohort studies to identify new genetic determinants of VWF:Ag levels[43]. 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*. These novel loci point to other biological pathways, than were believed to be involved in the regulation of VWF:Ag levels. Two of the newly identified genetic loci, *STXBP5* and *STX2*, are of specific interest. Syntaxin 2 (*STX2*) is a binding substrate for the Syntaxin Binding Protein 5 (*STXBP5*) and is a member of the Soluble N-ethylmaleimide-sensitive factor (NSF) Attachment protein Receptor (SNARE) protein family. These proteins drive vesicle exocytosis by fusion of granules and target membranes, a process involved in the regulation of numerous secretory events[44]. STXBP5 and STX2 interact specifically with SNARE complex proteins, such as SNAP23 and syntaxin-4, which have been shown to be involved in Weibel Palade Body (WPB) exocytosis, the well known mechanisms for the secretion of VWF molecules by endothelial cells[45]. Dysfunction of the WPB machinery is a likely contributor to the variation in VWF:Ag levels.

von Willebrand Factor and the risk of arterial thrombosis

Considering the important role of VWF in primary haemostasis, VWF is expected to be an important protein in the pathogenesis of arterial thrombosis and subsequent coronary heart disease (CHD) or ischemic stroke. Indeed, it has been well established that elevated VWF:Ag levels are a risk factor for CHD[7, 46-48] and stroke[10, 49]. However, it remains unclear whether VWF is causally related to the occurrence of CHD.

VWF is a marker of endothelial dysfunction, which precedes atherosclerosis[50]. In turn, atherosclerosis is the major cause of CVD. Therefore VWF may be primarily a marker of the underlying pathology of CVD, namely atherosclerosis, rather than an actual causal mediator (reverse causation). Also, the association between VWF and CHD can be influenced by other environmental factors that increase both VWF levels and cardiovascular risk (confounders). Confounding and reverse causation entangle current observational studies in proving causality. However, as VWF levels are to a great extent genetically determined and genetic variations are present from gestation, genetics can be used to study the non-confounded effect of VWF on the risk of CHD. If VWF is a pathogenic mediator in the occurrence of CHD, genetically elevated VWF:Ag levels should increase the risk of CHD to same extent as predicted by the direct effect of VWF:Ag levels on CHD[51].

Identifying VWF as an actual causal risk factor for CVD is important for cardiovascular risk prediction models, primary and secondary prevention, and new drug development.

AIM AND OUTLINE OF THIS THESIS

The primary aim of this thesis is to obtain new insights in the role of VWF in the occurrence of CVD. In addition, we aim to uncover new genetic determinants of VWF:Ag levels and to investigate their effect on VWF:Ag levels in patients with cardiovascular disease, in patients with von Willebrand Disease, and in healthy individuals.

First, we review all available literature on known genetic determinants of VWF:Ag levels and VWF activity in relation to the risk of CVD **(chapter 2)**. Next, we investigate in a large population-based study of elderly individuals, the Rotterdam Study, the relationship between common genetic variations in the VWF gene, VWF:Ag levels and the risk of incident CHD **(chapter 3)**. With this design we aim to obtain novel insights in the causality of VWF in the occurrence of CHD. In the Rotterdam study, we also investigate the relationship between genetic variations in other candidate genes for VWF (*THBS1*, *THBS2*, *THBS4*), VWF:Ag levels and the risk of incident CHD **(chapter 4)**. Next, we study the effect of genetic variations in two novel candidate genes, which have been associated with VWF:Ag levels in the general population (*STXBP5* and *STX2*), on VWF:Ag levels and the risk of CVD **(chapter 5)**. This study is executed in a unique case-control study of young patients with a first cardiovascular event and healthy controls: the "Genetic risk factors for Arterial Thrombosis at young age: the Role of TAFI and other Coagulation factors" (ATTAC) study.

Chapter 6 focuses on prognostic markers of recurrent cardiac events in young patients with premature CHD. For this follow-up study we use all patients with CHD of the ATTAC study in whom we study the association between VWF:Ag levels, but also CRP and fibrinogen levels, and the risk of recurrence of cardiovascular events.

Low VWF levels are associated with an increased bleeding tendency. Besides variations in the VWF gene itself it is still unknown what other genes are involved in the occurrence of low VWF:Ag levels. To this end, we perform a meta-analysis of genome-wide association studies in order to uncover new genetic determinants of low VWF:Ag levels **(chapter 7)**. Subsequently, we investigate the effect of common genetic variations in *STXBP5* and *STX2* on VWF levels and bleeding phenotype in patients with von Willebrand Disease (VWD), the most common inherited bleeding disorder **(chapter 8)**. The encoding proteins of *STXBP5* and *STX2* are involved in the regulated secretion pathway of VWF molecules, which can be initiated by endothelial cell activation that occurs for example during heavy exercise. In order to improve our understanding of the involvement of genetic variations in *STXBP5* and *STX2* in the regulated release of VWF molecules, we study the effect of these genetic variations on the release of VWF during heavy exercise in healthy individuals **(chapter 9)**.

VWF is considered an important target of new drug development for patients with acute thrombotic events and for patients with stable CHD undergoing percutaneous

coronary intervention. In **chapter 10**, we study the in vitro effect of a new antithrombotic drug candidate ALX-0081, which is directed to the A1 domain of VWF, on blood samples of patients undergoing percutaneous coronary intervention.

In the final chapter **(chapter 11),** the most important findings of this thesis will be discussed in a broader perspective. Also, the methodological considerations will be addressed. Finally, the possible clinical implications and future directions are discussed.

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JE van Loon*, MC van Schie*, MPM de Maat, FWG Leebeek.
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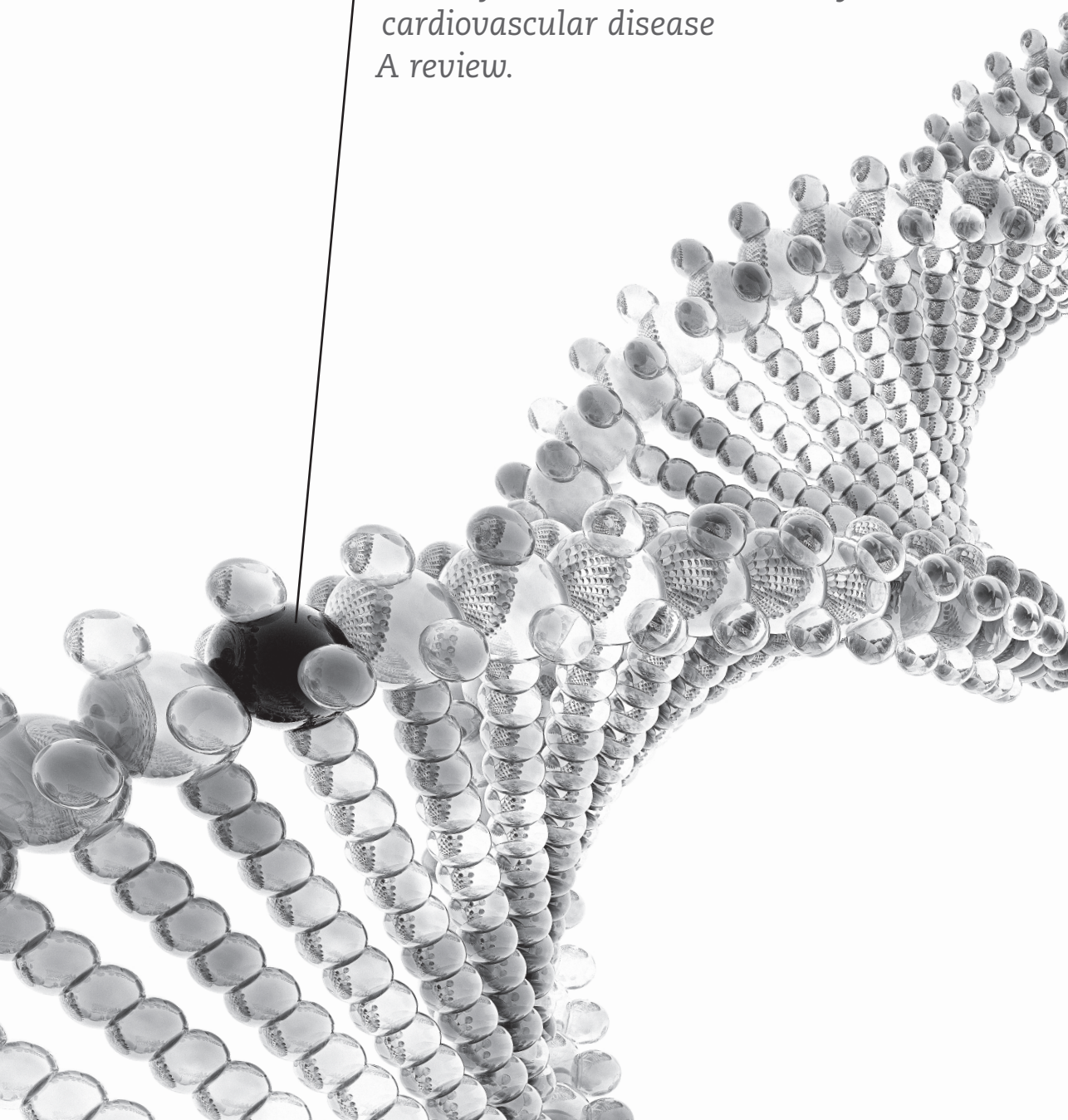
** these authors have contributed equally to the manuscript*

CHAPTER 2

Genetic determinants of von Willebrand Factor levels and activity in relation to the risk of cardiovascular disease
A review.



ABSTRACT

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. Since VWF plasma levels are to a great extent genetically determined, many genetic 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. Also, genetic variations in other regulators of VWF, such as ABO blood group, ADAMTS13, thrombospondin-1 and the recently identified SNARE protein genes, have been investigated. In this narrative review we present the available literature. Studying the associations between genetic variations and the risk of arterial thrombosis may help elucidating the role of VWF in the pathogenesis of arterial thrombosis. However, as current studies differed in study design, study population, and study endpoint, and were often underpowered, it is still 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 in 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) has a key role in arterial thrombus formation, since this large multimeric glycoprotein facilitates platelet adhesion and aggregation at sites of vascular injury[1]. VWF is mainly 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 ABO blood group, but also single nucleotide polymorphisms (SNPs) in the VWF gene (*VWF*) affect VWF levels[10-11]. Since 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 has already been described by Mendel's second law, which briefly 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].

Not only high VWF plasma levels may be relevant in the development of arterial thrombosis, but also VWF activity that depends strongly on its multimer size may be an important mediator. ADAMTS13 *("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 ADAMTS13 gene (ADAMTS13) and TSP-1 (THBS1) gene may also influence the association between VWF levels and the risk of arterial thrombosis[19-21].

In the last two decades, numerous studies on genetic determinants of VWF plasma levels, VWF activity and the risk of arterial thrombosis have been conducted. In the present paper we will review these studies comprehensively and will 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 in 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]. Also, statins may 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].

von Willebrand Factor promoter region

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

Thus far, 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 have been caused by 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]. Since 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 variations 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 another 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

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 Controls	525 451	Caucasian	62 ± 9.2 61.3 ± 9	rs7954855 rs7965413	-2661A>G -2527G>A	Haplotype 0.37	NA	AG haplotypes 0.93 [0.77;1.22] MI	[49]
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 Controls	352 736	Caucasian	66.5 ± 4.9 65.2 ± 5.6	rs7966230	-3268G>A	0.38	NA	2.6 [1.0;6.8] CG+GG vs.CC CHD (advanced atherosclerosis)	[51]

Table 1 • Association between polymorphisms in the promoter region of the VWF gene, VWF levels and activity, and arterial thrombosis (table continued)

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	NA	[9]
							1.96 AG		
							1.99 AA		
							p=0.31		
Stroke patients	124	Caucasian	56 ± 12	rs7966230	-3268G>A	0.30	NS	0.9 [0.5;1.5] CG vs. CC	[24]
Controls	125		56 ± 12					0.7 [0.3;1.6] GG vs. CC	
Healthy subjects	394	Unknown	39 ± 11	rs7966230	-3268G>A	Haplotype 0.37	0.95 1/1	NA	[48]
				rs7964777	-2709C>T		0.97 1/2		
				rs7954855	-2661A>G		0.99 2/2		
				rs7965413	-2527G>A		p=0.70		

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

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, though 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].

von Willebrand Factor coding regions

During screening of patients with von Willebrand disease for mutations, several polymorphisms in the coding regions of *VWF* have been identified. These have been summarized in table 2. Most studies have focused on exon 18 and 28, since 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 FVIII. 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 strong and 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

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	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
type 1 diabetes				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

Table 2 • Association between polymorphisms in the coding regions of the VWF gene, VWF levels and activity, and arterial thrombosis. (table continued)

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	124	Caucasian	56 ± 12	rs1800386	A/G	exon 28	Tyr1584Cys	0.016	NA	NS (data not shown)	[58]
Controls	125										
AT patients	374	Caucasian	43 ± 7	rs1800386	A/G	exon 28	Tyr1584Cys	0.003	NA	NS (data not shown)	[58]
controls	332										
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	[62]
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

(VWF:CB) activity and VWF:RCo activity than the common Tyr/Tyr phenotype (p<0.001) [58]. In addition, VWF proteins containing cysteine 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 points out the importance of considering ABO blood group as effect modifier in the association between SNPs and VWF plasma levels.

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 been associated with VWF:Ag levels previously[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.

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 ADAMTS13 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 ADAMTS13 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.

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]. 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, 90]. This finding was replicated in at least two other studies[91-92]. 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[91]. 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 the present paper 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 studying all performed studies, one of our main observations is that only a limited number has investigated the association between genetics, VWF levels and the risk of arterial thrombosis in one study. In addition, studies differed in study design, study population (e.g. different age or ethnic background), and study 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, but 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, ADAMTS13, 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 part of the variability in VWF levels. It has 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 thrombo-

sis[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[93-94].

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 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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JE van Loon, M Kavousi, FWG Leebeek, JF Felix, A Hofman, JCM Witteman, MPM de Maat

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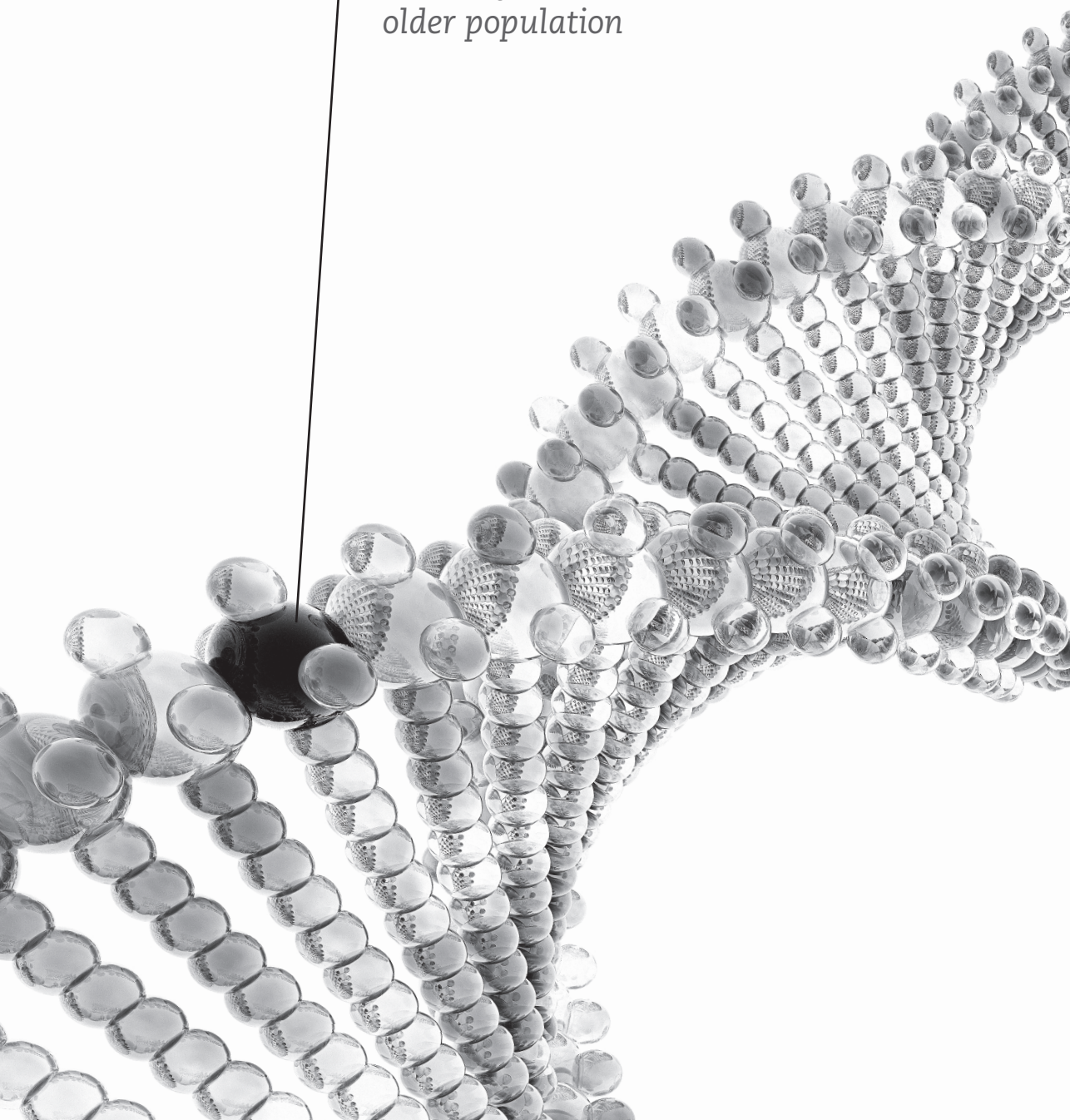
CHAPTER 3 *Von Willebrand Factor plasma levels, genetic variations, and coronary heart disease in an older population*



ABSTRACT

Background. High von Willebrand Factor (VWF) levels are associated with an increased risk of coronary heart disease (CHD). However, it remains unclear whether VWF is causally related to the occurrence of CHD or primarily mirrors endothelial dysfunction, which predisposes to atherosclerosis and subsequent CHD.

Objectives. Since VWF is largely determined by genetic factors, we investigated whether VWF antigen levels (VWF:Ag) and the risk of CHD are affected by common variations in the VWF gene.

Methods. We included 7002 participants (≥55years) from the large prospective population-based Rotterdam Study in the discovery cohort. The extension cohort of the Rotterdam Study, consisting of 3011 participants, was used as replication cohort. We determined VWF:Ag levels and genotype data of 38 single-nucleotide polymorphisms (SNPs) in *VWF*. Subsequently, hazard ratios for CHD were calculated and genetic analyses were performed to assess the relationship between SNPs, VWF:Ag levels and CHD risk.

Results. We identified and replicated three SNPs that were associated with VWF:Ag: *rs*216321 (β=0.10 [95%CI 0.06;0.13]) (Ala852Gln), *rs*1063856 (β=0.05 [95%CI 0.03;0.07]) (Thr789Ala), and rs2283333 (β=0.09 [95%CI 0.05;0.21]) (intron 15). However, genetic polymorphisms in the VWF gene were not associated with the risk of CHD.

Conclusions. In this study we have shown that genetic variations in *VWF* strongly affect VWF plasma levels, but are not associated with the risk of CHD. Our findings therefore do not support a strong causal relationship between VWF and CHD in elderly individuals of ≥ 55 years, but suggest that VWF is primarily a marker of CHD.

INTRODUCTION

For decades von Willebrand Factor (VWF) is considered an important protein in the pathogenesis of arterial thrombosis and subsequent coronary heart disease (CHD) due to its twofold function in haemostasis and thrombus formation. VWF initiates adherence of platelets to the injured vessel wall and subsequent platelet aggregation, especially at sites of high shear stress[1-2]. In addition, VWF protects coagulation factor VIII (FVIII) from clearance[3].

It has been well established that elevated VWF plasma levels are a risk factor for CHD[4-7]. However, it remains unclear whether VWF is causally related to the occurrence of CHD or primarily mirrors endothelial dysfunction, which predisposes to atherosclerosis and subsequent cardiovascular events.

In general, plasma concentrations of VWF in healthy subjects are characterized by a large variation. This can be partly explained by a number of lifestyle and environmental factors, such as age, smoking, hypertension, cholesterol and diabetes, which are known to influence plasma VWF concentration[8]. In addition, approximately 60% of the variability of VWF plasma levels can be ascribed to genetic factors, such as ABO blood group (*ABO*) and variations in the VWF gene (*VWF*)[9]. A recent meta-analysis of genome-wide association studies of the CHARGE consortium further confirmed involvement of common variations in these genes and discovered novel gene loci that determine VWF plasma levels[10].

To date, it has been recognized that single nucleotide polymorphisms (SNPs) in the VWF gene contribute to the variability in VWF concentration[10-12]. However, reports on the association between VWF polymorphisms and the risk of CHD in current literature are incomplete and inconsistent[13-17]. Also, most studies investigated single SNPs only without covering the total common genetic variation in the VWF gene[18].

We aimed to investigate comprehensively the effect of common genetic variations in *VWF* on VWF plasma concentration and the occurrence of CHD in a large prospective population-based cohort of elderly individuals, the Rotterdam Study. The size of our study population, the large number of events, and the long follow-up will provide a better understanding of the potential causal relationship between VWF and CHD.

METHODS

Study design and study population

For this study we included participants from the Rotterdam Study (RS), an ongoing prospective population-based cohort study among individuals of 55 years and older living in a suburb in the city of Rotterdam in the Netherlands[19]. The study started in

1990 with 7,983 participants (out of 10,215 invitees) (RS–I) and was extended in 1999 to an additional 3,011 individuals (out of 4,472 invitees), who had turned 55 years or had moved into the study district since the start of the study (RS–II). In the current study, the original cohort (RS–I) was used as the discovery cohort (RS–I) and the extension cohort (RS–II) was used as replication cohort.

For the discovery cohort, all participants with a history of CHD at baseline (n = 981) were excluded from the analysis. As expected, these subjects were older (72.2 ± 0.3) and more often male (58.2%) compared to participants free from CHD at baseline. Also, classical cardiovascular risk factors were more prevalent among the excluded participants.

In total 7002 individuals were free from CHD at baseline and eligible for participation. Within this discovery cohort, we used three different subgroups for our analysis; for the genetic analysis of the association between VWF polymorphisms and incident CHD, we used all participants for whom genotype data were available (N = 5194); for the analysis of the association between VWF:Ag and incident CHD, we selected participants of the third examination cycle (1997-1999) of the original cohort (RS-I-3). We additionally excluded all participants (N = 157) who had a CHD event between the start of the study and the time of blood sampling for VWF:Ag measurements; for the analysis of the association between VWF polymorphisms and VWF:Ag levels, we used participants of the third examination cycle, for whom genotype data were available.

In order to replicate our findings of the genetic analyses in the discovery cohort (RS–I), we selected participants of the first examination cycle (2000-2001) of the extension cohort (RS–II). The replication cohort consisted of 3011 individuals, of whom 2849 were free from CHD at baseline. Of these genotype data were available for 1994 individuals.

The Rotterdam Study has been approved by the medical review board at Erasmus University Medical Center in Rotterdam. Written informed consent was obtained from all participants.

Assessment coronary heart disease

History of CHD was determined during the baseline interview and verified in medical records. History of CHD was defined as a myocardial infarction (MI) or a revascularization procedure, including percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery (CABG).

After enrollment in the Rotterdam Study, participants were continuously monitored for incident CHD, which was defined as a MI or cardiac death, 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.

Participants were followed from baseline until an event of CHD, until death or until the last health status update in which they were known to be free of CHD or until January 1st,

2007. For the analysis of CHD, we censored participants who were diagnosed with CHD at the date of the event. Follow-up was complete until January 1st, 2007.

Baseline measurements

At baseline, a detailed interview was taken from all participants, as well as an extensive set of examinations, including a physical examination and blood sampling. Participants were followed for a variety of diseases. Examinations of characteristics that change over time were repeated every 3-4 years. Clinical measurements were obtained during visits to the research center. Blood pressure was calculated as the mean of two measurements using a random-zero sphygmomanometer at the right brachial artery while the subject was in a sitting position. Hypertension was defined as a diastolic blood pressure (DBP) of ≥ 90 mm Hg and/or a systolic blood pressure (SBP) 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)[20]. Total cholesterol and high-density lipoprotein cholesterol were measured using an automated enzymatic procedure. Diabetes mellitus was defined as the use of blood glucose-lowering medication and/or a fasting serum glucose level ≥ 7.0 mmol/L. Body mass index was calculated as the weight (in kilograms) divided by the square of the height (in meters). Smoking behavior and current medication use were assessed during the interview at home. Blood group antigen phenotypes were reconstructed by haplotypes analysis of 4 single nucleotide polymorphisms, rs687289, rs507666, rs8176704, and rs8176749, which collectively serve as tagging SNPs for the O, A1, A2, and B allele[21].

Blood sampling procedure and von Willebrand factor plasma measurement

Fasting venous blood samples were collected in citrated tubes and plasma was stored at –80°C. VWF antigen (VWF:Ag) was determined in blood samples obtained from the discovery and replication cohorts with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies and horseradish-peroxidase-conjugated anti-human VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging, respectively. All samples were measured in one batch in order to avoid differences in storage time. The intra-assay coefficient of variation was 5.8% and the inter-assay coefficient of variation was 7.8%. For DNA isolation, blood was collected in tubes containing ethylene diaminetetraacetic acid (EDTA; Beckton Dickinson), and extracted using a Hamilton STAR pipetting platform and AGOWA magnetic bead technology. Isolated DNA was stored in Matrix 2D-barcode tubes in a 96-well format until genetic analysis.

Genetic analysis

The VWF gene spans 176 kbp and is located on the p13.3 region of chromosome 12. For the genetic analysis we determined genotypes of SNPs in the VWF gene (*VWF*) using the Illumina 550K assay (supplementary table 1). SNPs were excluded for minor allele frequency (MAF) ≤ 1%, Hardy-Weinberg equilibrium (HWE) $P < 10^{-6}$, or SNP call rate ≤ 98%. In addition, we studied the linkage disequilibrium (LD) structure based on the R^2 and D' between the selected SNPs with Haploview software (version3.11; www.broad.mit.edu/mpg/haploview/index.php) and constructed haplotypes blocks with a haplotype frequency of > 1% , in order to select non-redundant SNPs only (supplementary figure 1). In total 38 SNPs in *VWF*, including 2kb of the 3'-region and 2kb of the 5'-region were genotyped.

Statistical analysis

Data on baseline characteristics are presented as means and standard deviations for continuous variables and as counts and percentages for categorical data. Since VWF:Ag levels were skewed, these data were natural logarithmically transformed (lnVWF:Ag) and presented as geometric mean and geometric standard deviation. We used Cox proportional hazards regression models to assess the association between VWF:Ag and CHD. VWF:Ag levels were divided into quartiles: ≤0.94 IU/mL, 0.94-1.23 IU/mL, 1.23-1.62 IU/mL, >1.63 IU/mL. The first quartile was used as the reference category. In addition, cumulative event-free survival curves per quartile of VWF:Ag were constructed using the Kaplan-Meier (KM) method. In order to compare the KM-curves we used a Log-rank test.

Allele frequencies of the *VWF* polymorphisms were calculated by genotype counting. For each SNP, the deviation from the Hardy-Weinberg equilibrium was tested by means of a Chi-squared test with one degree of freedom. We used linear regression analyses with additive genetic models to determine the association between genetic variations in *VWF* and lnVWF:Ag levels. Beta-coefficients represent the increase in lnVWF:Ag levels per coded allele. To assess the association between genetic variations in *VWF* and CHD we used Cox proportional hazards regression with additive genetic models. Hazard ratios represent the increase in risk of CHD per coded allele.

All analyses were adjusted for age and sex, and additionally for classical cardiovascular risk factors (SBP, DPB, anti-hypertensive medication with the indication hypertension, cholesterol, HDL, anti-lipid drugs, BMI, smoking and diabetes), and ABO blood group. Missing values of these covariates were imputed five times using a multiple imputation method including age, sex, and VWF:Ag as predictors. Statistical analyses were performed on each of the five datasets and subsequently pooled into one final result using SPSS software. Statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS Inc, Chicago, USA). A two-sided value of $P < 0.05$ was considered statisti-

cally significant. For the genetic association analysis a two-sided value of $P < 0.001$ was considered statistically significant after Bonferroni correction.

RESULTS

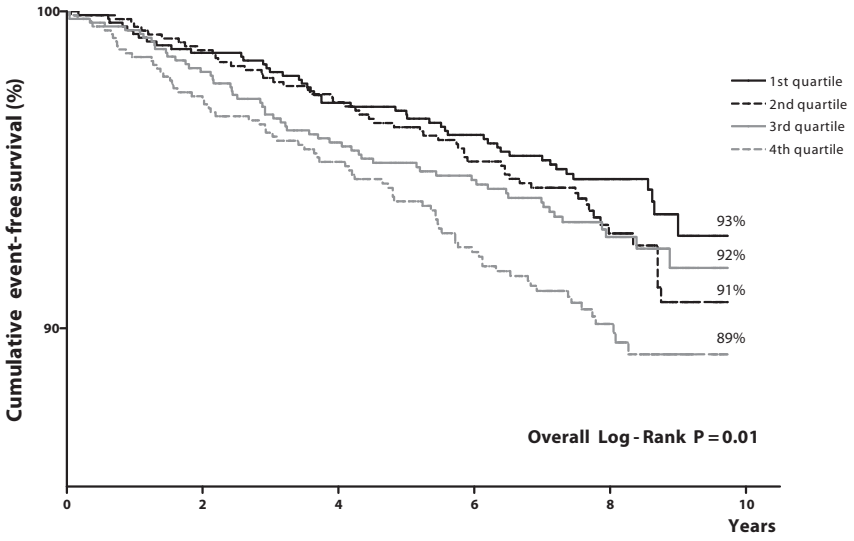
For the discovery cohort (RS–I) the total follow-up time was 75,298 person years (mean 10.8 years), during which 738 CHD events occurred. Follow-up data were available on 6,972 participants and genotyping data were available on 5,194 participants. The total follow-up time in RS-I-3 was 24,140 person years (mean 7.2 years), during which 231 CHD events occurred. For RS-I-3 VWF:Ag levels were available on 3,363 individuals. Of these, genotype data were available on 2,914 participants (202 CHD events).

For the replication cohort (RS–II), VWF antigen level measurements were available on 2,427 participants and genotyping data were available on 1,994 participants. The total

Table 1 • Baseline characteristics of the study population

	Discovery Cohort		Replication Cohort
	RS-I (N = 5194)	RS-I-3 (N = 3363)	RS-II (N = 1994)
Age (years)	69.1 ± 9.2	72.1 ± 9.2	64.5 ± 7.9
Female sex (%)	3229 (62%)	2064 (61%)	1122 (56%)
Blood pressure (mmHg)			
Systolic	139.0 ± 22.2	135.5 ± 20.7	143.1 ± 21.4
Diastolic	73.9 ± 11.3	73.6 ± 10.8	79.1 ± 10.9
Antihypertensive medication with indication hypertension (%)	860 (17%)	777 (23%)	426 (21%)
Diabetes Mellitus (%)	446 (9%)	437 (13%)	210 (11%)
Smoking (%)			
Current	1173 (23%)	694 (20%)	398 (20%)
Former	2012 (39%)	1433 (43%)	971 (49%)
Cholesterol (mmol/L)			
Total	6.6 ± 1.2	6.7 ± 1.2	5.8 ± 1.0
High-density lipoprotein	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
Lipid reducing agents (%)	88 (2%)	68 (2%)	222 (11%)
Body Mass Index (kg/m2)	26 ± 4	27 ± 4	27 ± 4
Blood group			
O (%)	2336 (45%)	1332 (40%)	908 (46%)
Non-O (%)	2856 (55%)	1581 (47%)	1086 (55%)

Table presents baseline characteristics of the discovery cohort for whom genotype data were available, of the discovery cohort for whom VWF:Ag levels were available, and for the replication cohort for whom genotype data were available. Summary statistics for continuous variables are presented as mean ± standard deviation. Categorical data are summarized as percentages.



No. at risk					
1st quartile	844	818	792	734	358
2nd quartile	828	793	754	698	338
3rd quartile	844	809	749	680	361
4th quartile	843	770	705	616	315

Figure 1 • Kaplan-Meier curve for the cumulative event-free survival per quartile of VWF:Ag. Cut-off points (IU/mL) for quartiles were: ≤0.94 IU/mL, 0.94-1.23 IU/mL, 1.23-1.62 IU/mL, and >1.63 IU/mL.

follow-up time for this cohort was 14,030 person years (mean of 5.8 years), during which 125 CHD events occurred.

The baseline characteristics of RS–I, RS-I-3, and RS–II are shown in table 1. Participants with incident CHD had significantly higher levels of VWF:Ag (mean 1.51 ± 0.9 IU/mL) than individuals without incident CHD (mean 1.34 ± 0.6) ($P < 0.0001$). In figure 1 it is clearly shown that the cumulative event-free survival is better in individuals with VWF:Ag levels in the lowest quartile, than in individuals with VWF:Ag levels in the upper quartiles (log-rank test p = 0.01).

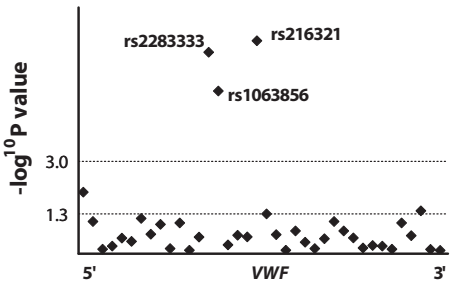


Figure 2 • $-\text{Log}^{10}$ of the p-values of the associations between *VWF* SNPs and VWF:Ag in the discovery cohort (RS-I-3). Thresholds are set at 1.3 ($-\text{Log}^{10}$ P = 0.05) and at 3.0 ($-\text{Log}^{10}$ P = 0.001) after Bonferroni correction.

Table 2. Significant associations between *VWF* polymorphisms and VWF:Ag levels

	Discovery Cohort RS-I-3			Replication Cohort RS-II		
rs-number	MAF*	Beta-coefficient (95% CI)	P	MAF*	Beta-coefficient (95% CI)	P
rs216321	0.09	0.10 (0.06;0.13)	$1.2x10^{-7}$	0.09	0.07 (0.03;0.11)	0.002
rs1063856	0.34	0.05 (0.03;0.07)	$5.1x10^{-6}$	0.35	0.07 (0.04;0.09)	$4.2x10^{-8}$
rs2283333	0.10	0.09 (0.05;0.21)	$2.8x10^{-7}$	0.10	0.05 (0.01;0.09)	0.01

Linear regression analysis with an additive genetic model adjusted for age and sex. Beta-coefficient represents the increase in lnVWF:Ag per coded allele.
** MAF is minor allele frequency*

We investigated 38 SNPs in the VWF gene in relation to VWF:Ag levels (fig.2, supplementary table 2) and the risk of CHD (fig.3, supplementary table 3). Six SNPs were associated with VWF:Ag levels. After Bonferroni correction to adjust for multiple testing, three SNPs still had a significant effect on VWF:Ag levels: *rs*216321 (Ala852Gln) (β = 0.10 [95%CI 0.06;0.13]), *rs*1063856 (Thr789Ala) (β = 0.05 [95%CI 0.03;0.07]), and rs2283333 (intron 15) (β = 0.09 [95%CI 0.05;0.21]). All of these SNPs replicated significantly in the RS-II cohort, as shown in table 2. The VWF:Ag levels per genotype of these SNPs are displayed in figure 4. Additional adjustment for cardiovascular risk factors and ABO blood group (data not shown) did not influence the effect sizes. As shown in figure 4 none of these SNPs nor any other SNPs was associated with CHD risk.

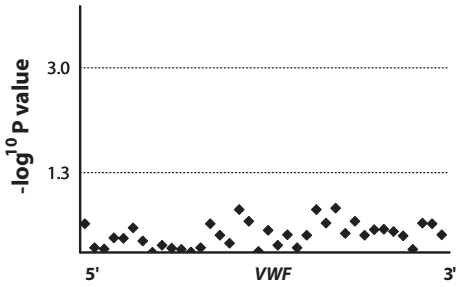


Figure 3 • -Log[10] of the p-values of the associations between *VWF* SNPs and CHD risk in the discovery cohort (RS-I). Thresholds are set at 1.3 (–Log[10] P = 0.05) and at 3.0 (–Log[10] P = 0.001) after Bonferroni correction.

DISCUSSION

In this large prospective population-based cohort study among elderly individuals of ≥ 55 years we identified three SNPs in *VWF* that were strongly associated with VWF:Ag levels: *rs*216321, *rs*1063856, and *rs*2283333. However, genetic variation in *VWF* had no effect on the risk of CHD.

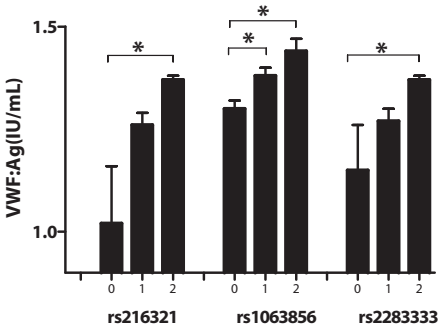


Figure 4 • VWF:Ag levels (IU/mL) (mean ± SE) per genotype of the identified SNPs. Genotypes are presented as the number of VWF:Ag levels increasing alleles. We used the genotype with no VWF:Ag levels increasing alleles as reference category.
* represents a p-value of < 0.05.

These findings contribute to the discussion whether VWF plays a causal role in the pathogenesis of cardiovascular disease. If VWF is a pathogenic mediator in the occurrence of CHD, genetically elevated VWF plasma levels should increase the risk of CHD to same extent as predicted by the direct effect of VWF plasma levels on CHD, the so-called Mendelian Randomization approach[22-23]. However, the absence of an association between *VWF* polymorphisms and CHD risk in our study does not provide evidence for this hypothesis. Conversely, our data suggest that VWF is primarily a marker of CHD.

Important strengths of the present study are the prospective design, the long and nearly complete follow-up and the large number of cardiovascular events. In addition, our study is exceptional in that it investigated the relationship between the total common genetic variation in *VWF*, VWF plasma concentration and cardiovascular risk in one study, unlike other prospective studies, which only investigated the effect of VWF:Ag levels on the CHD risk without incorporating genetics[6, 24-25].

The association between VWF:Ag levels and CHD has been established comprehensively in the past[4-7]. We also show a clear relationship with similar effect sizes between VWF:Ag and incidence CHD in elderly subjects[6, 25].

A potential limitation of our study is that we excluded prevalent CHD at baseline (i.e. early onset disease) and therefore possibly failed to find an association between *VWF* polymorphisms and CHD. One of the reasons to exclude prevalent CHD was to avoid survivor bias. However, there was no association between *VWF* polymorphisms and prevalent CHD in our study (data not shown). In addition, the allele frequencies in our population were high and similar to those reported by dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), making selection bias unlikely.

Another explanation for the lack of association between *VWF* polymorphisms and CHD may be the high age of our study population, because the contribution of genetics to disease is expected to decrease with increasing age[26]. This hypothesis is substanti-

ated by our previous findings in a well-defined case-control study of young individuals (mean age 43.2 years) with a first event of arterial thrombosis and healthy control subjects (mean age 39.6 years)[27-28]. In that study we did observe an association between a *VWF* polymorphism, the synonymous SNP *rs*1063857 (Y/Y795), and the risk of arterial thrombosis[11]. *Rs*1063857 is in complete and perfect LD with the non-synonymous SNP *rs*1063856 of the present study. SNP *rs*1063856 has recently been identified as a strong genetic determinant of VWF:Ag levels in a meta-analysis of genome wide association studies of the CHARGE consortium[10]. In our present study we confirmed the association between *rs*1063856 and VWF:Ag levels. However, we did not observe a relationship with CHD.

ABO blood group is an important determinant of VWF levels, because the presence of blood group A and B antigens on VWF molecules leads to a decreased clearance of VWF molecules. Consequently, individuals with blood group O have 25% lower VWF plasma concentrations than individuals with blood group non-O[29]. Furthermore, ABO blood group has been previously suggested to be associated with the risk of CHD[30-32] and may therefore be a confounder in our analysis. In our genetic analyses additional adjustment for ABO blood group did not change the effect sizes. Also, *VWF* and *ABO* are located on different chromosomes. Hence, the association between genetic variations in *VWF* and VWF plasma concentration is likely unrelated to ABO blood group.

During our search for genetic variations that may affect the risk of CHD, we should keep in mind that we are looking for very modest effects. Previous large genome-wide association studies on CHD have not identified SNPs in coagulation-related genes[33-35]. Therefore, we were unlikely to find SNPs with large effects. In addition, although we identified three SNPs in *VWF* that were highly significantly associated with VWF plasma concentration, these SNPs explain only a small part of the variation in VWF plasma concentration. Other large genetic association studies also found that only a small variance was explained by the identified SNPs[36-37]. This observation is in agreement with previous findings of Van Hylckema Vlieg et al., suggesting that the genetic basis for the plasma levels of haemostasis factors may lie partly outside the genes coding for these factors[38]. Hence, variations in for example the ABO blood group gene or in the recently identified genes by the CHARGE consortium[10] may be far more important than genetic variations in the VWF gene itself. Also, the upcoming next-generation sequencing studies will enable us to find more rare variants, but with much greater effects.

In the absence of an association between VWF polymorphisms and the risk of CHD, the hypothesis that high VWF:Ag levels are causative for CHD can be questioned. However, a large number of studies showed the substantial predictive value of VWF for CHD incidence[8]. Furthermore, the fact that VWF has a role in the occurrence of CHD is illustrated by numerous fundamental studies. VWF is detected at sites of platelet accumulation in coronary artery thrombi[39-40]. Both in vitro studies and in vivo studies showed

that antibodies targeting VWF inhibit platelet aggregation and reduce coronary artery thrombosis in animal model[41-42]. These findings demonstrate that VWF has a role in the interplay of mechanisms leading to CHD.

At this point we have no evidence to strengthen the hypothesis of a strong causal relationship between elevated VWF plasma concentrations and the occurrence of CHD in elderly individuals. However, our findings do not exclude the possibility that VWF is a causal mediator. In the present study we have only investigated the association with VWF plasma concentration, while for example VWF activity or VWF multimer size may also be intermediates in the pathogenesis of CHD. In addition, we should not neglect the involvement of environmental factors and their complex life-long interactions with multiple genes. Consequently, future research on the causal role of VWF should have a broader view in order to deal with these complex interactions.

In conclusion, genetic variability in the VWF gene affects VWF plasma concentration, but is not associated with the risk of CHD in elderly individuals. However, VWF levels are associated with CHD risk in this population. These findings suggest that VWF is primarily a marker of endothelial dysfunction and atherosclerosis, which underlie CHD. Whether VWF is in addition a causal risk factor for CHD must be elucidated by future fundamental research.

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SUPPLEMENTARY TABLES

Supplementary table 1 • Common genetic variations in VWF

rs#	Position	Location	Allele*	MAF†
*rs*933407	5915522	3′ UTR	C>T	0.39
*rs*2885517	5920581	3′ UTR	C>T	0.45
*rs*2270151	5931221	Intron 50	G>A	0.17
*rs*2286646	5931752	Intron 49	G>A	0.22
*rs*12317523	5933403	Intron 47	C>T	0.28
*rs*12369177	5943781	Intron 47	G>T	0.38
*rs*11063961	5944277	Intron 47	A>G	0.22
*rs*917857	5952085	Intron 43	A>G	0.43
*rs*917859	5952374	Intron 43	G>A	0.35
*rs*4764521	5954584	Intron 43	G>A	0.19
*rs*216856	5956526	Intron 42	C>T	0.34
*rs*216867	5961261	Intron 42	G>A	0.12
*rs*216872	5963801	Intron 40	T>C	0.30
*rs*2070887	5964213	Intron 40	T>C	0.07
*rs*216883	5967918	Intron 38	C>T	0.25
*rs*216309	5997188	Intron 28	T>C	0.45
*rs*216312	5999245	Intron 27	C>T	0.43
*rs*216313	5999525	Intron 27	A>G	0.07
*rs*11611917	6006895	Intron 22	G>A	0.24
*rs*216321	6014245	Exon 20	C>T	0.09
*rs*12810426	6014399	Intron 19	G>A	0.21
*rs*216338	6018587	Intron 18	G>A	0.35
*rs*216340	6020060	Intron 18	G>A	0.21
*rs*1063856	6023795	Intron 18	C>T	0.35
*rs*2283333	6033113	Intron 15	C>T	0.10
*rs*980130	6039288	Intron 13	C>T	0.30
*rs*980131	6039459	Intron 13	C>T	0.40
*rs*7139057	6039584	Intron 13	T>G	0.18
*rs*4764482	6039994	Intron 13	C>T	0.47
*rs*1800378	6042463	Exon 13	T>C	0.34
*rs*22283332	6044990	Intron 10	G>A	0.09
*rs*3782711	6053442	Intron 7	T>C	0.12
*rs*2238104	6057926	Intron 6	G>T	0.46
*rs*2239144	6066444	Intron 6	C>A	0.12
*rs*2239140	6070704	Intron 6	C>T	0.47
*rs*11064024	6072310	Intron 6	G>A	0.38
*rs*7306706	6085895	Intron 5	G>A	0.42
*rs*10849387	6108740	5′UTR	C>T	0.33

** Nucleotide change, first allele is the coded allele*

† Minor allele frequency

Supplementary table 2 • Association between VWF polymorphisms and VWF:Ag levels

rs#	**Beta-coefficient (95% CI)**	***P-value***
*rs*933407	0.003 (-0.02, 0.03)	0.78
*rs*2885517	0.004 (-0.02, 0.03)	0.72
*rs*2270151	0.03 (0.001, 0.06)	0.04
*rs*2286646	-0.01 (-0.04, 0.01)	0.26
*rs*12317523	-0.02 (-0.04, 0.004)	0.10
*rs*12369177	-0.004 (-0.03, 0.02)	0.70
*rs*11063961	-0.01 (-0.03, 0.02)	0.56
*rs*917857	-0.01 (-0.03, 0.01)	0.54
*rs*917859	-0.01 (-0.03, 0.02)	0.64
*rs*4764521	0.01 (-0.01, 0.04)	0.30
*rs*216856	-0.02 (-0.04, 0.01)	0.18
*rs*216867	-0.03 (-0.06, 0.004)	0.09
*rs*216872	0.01 (-0.01, 0.03)	0.32
*rs*2070887	-0.01 (-0.05, 0.03)	0.67
*rs*216883	0.01 (-0.01, 0.03)	0.42
*rs*216309	0.01 (-0.01, 0.04)	0.18
*rs*216312	0.003 (-0.02, 0.02)	0.76
*rs*216313	-0.03 (-0.09, 0.02)	0.24
*rs*11611917	-0.03 (-0.05, 0.00)	0.05
*rs*216321	0.10 (0.06, 0.13)	1.2×10^{-7}
*rs*12810426	-0.01 (-0.04, 0.01)	0.28
*rs*216338	0.01 (-0.01, 0.03)	0.25
*rs*216340	0.01 (-0.02, 0.03)	0.51
*rs*1063856	0.05 (0.03, 0.07)	5.1×10^{-6}
*rs*2283333	0.09 (0.05, 0.21)	2.8×10^{-7}
*rs*980130	0.01 (-0.01, 0.03)	0.29
*rs*980131	-0.003 (-0.02, 0.02)	0.75
*rs*7139057	-0.02 (-0.05, 0.004)	0.10
*rs*4764482	-0.004 (-0.03, 0.02)	0.68
*rs*1800378	0.02 (-0.004, 0.04)	0.11
*rs*22283332	-0.02 (-0.06, 0.01)	0.23
*rs*3782711	-0.03 (-0.06, -0.003)	0.07
*rs*2238104	0.01 (-0.01, 0.03)	0.40
*rs*2239144	-0.02 (-0.05, 0.02)	0.31
*rs*2239140	0.01 (-0.02, 0.03)	0.56
*rs*11064024	-0.004 (-0.03, 0.02)	0.70
*rs*7306706	-0.02 (-0.04, 0.003)	0.09
*rs*10849387	0.03 (0.01, 0.05)	0.01

Linear regression analysis with an additive genetic model adjusted for age and sex. Beta-coefficient represents the increase in lnVWF:Ag per coded allele.

Supplementary table 3 • Association between VWF polymorphisms and the risk of coronary heart disease

rs#	Hazard ratio (95%CI)	*P-value*
*rs*933407	1.07 (0.94, 1.22)	0.34
*rs*2885517	1.01 (0.89, 1.15)	0.83
*rs*2270151	0.99 (0.83, 1.17)	0.88
*rs*2286646	1.04 (0.90, 1.22)	0.58
*rs*12317523	1.04 (0.90, 1.20)	0.59
*rs*12369177	1.06 (0.93, 1.21)	0.40
*rs*11063961	1.04 (0.89, 1.21)	0.65
*rs*917857	1.00 (0.88, 1.14)	0.99
*rs*917859	1.02 (0.90, 1.17)	0.75
*rs*4764521	1.02 (0.87, 1.19)	0.86
*rs*216856	0.99 (0.87, 1.13)	0.90
*rs*216867	1.00 (0.83, 1.22)	0.97
*rs*216872	0.99 (0.86, 1.13)	0.84
*rs*2070887	1.14 (0.87, 1.48)	0.34
*rs*216883	0.96 (0.83, 1.10)	0.53
*rs*216309	0.98 (0.86, 1.11)	0.71
*rs*216312	0.92 (0.81, 1.05)	0.20
*rs*216313	0.84 (0.61, 1.17)	0.31
*rs*11611917	1.00 (0.86, 1.15)	0.95
*rs*216321	0.92 (0.74, 1.14)	0.44
*rs*12810426	0.98 (0.84, 1.13)	0.75
*rs*216338	0.96 (0.84, 1.09)	0.51
*rs*216340	1.02 (0.80, 1.29)	0.84
*rs*1063856	1.04 (0.92, 1.19)	0.52
*rs*2283333	0.88 (0.72, 1.07)	0.20
*rs*980130	0.94 (0.82, 1.07)	0.33
*rs*980131	0.92 (0.81, 1.04)	0.19
*rs*7139057	0.95 (0.81, 1.10)	0.49
*rs*4764482	0.94 (0.83, 1.06)	0.31
*rs*1800378	0.96 (0.83, 1.10)	0.52
*rs*22283332	0.92 (0.74, 1.14)	0.43
*rs*3782711	0.92 (0.76, 1.12)	0.42
*rs*2238104	1.05 (0.93, 1.19)	0.46
*rs*2239144	0.94 (0.78, 1.14)	0.54
*rs*2239140	0.99 (0.88, 1.13)	0.90
*rs*11064024	0.94 (0.82, 1.07)	0.33
*rs*7306706	1.06 (0.94, 1.21)	0.34
*rs*10849387	1.05 (0.91, 1.20)	0.51

Cox' proportional hazard regression analysis with an additive genetic model adjusted for age and sex. Hazard ratios with 95% confidence interval represent the estimated relative risk of CHD per coded allele.

JE van Loon, MPM de Maat, A Hofman, JCM Witteman, FWG Leebeek
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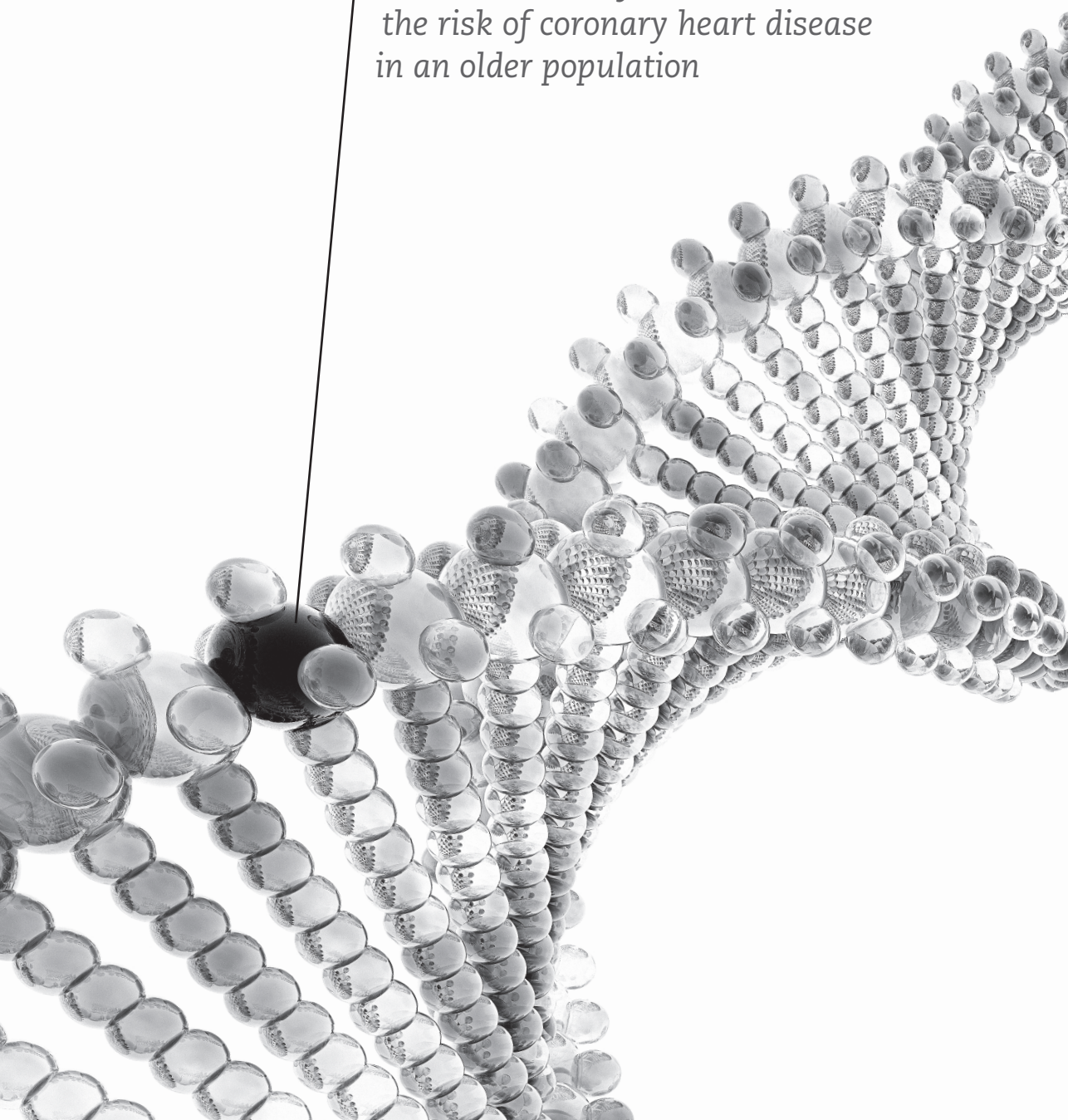
CHAPTER 4

Relationship between thrombospondin gene variations, von Willebrand factor levels and the risk of coronary heart disease in an older population



ABSTRACT

Background. The proposed relationship between thrombospondins and coronary heart disease (CHD) is possibly mediated by high von Willebrand Factor (VWF) levels. Therefore, we investigated the effect of polymorphisms in *THBS1*, *THBS2*, and *THBS4* on VWF antigen levels and the risk of CHD in a prospective setting.

Methods. 6454 participants, free from CHD at baseline, were included from the large prospective population-based Rotterdam study. VWF:Ag levels were determined in 5801 individuals. Genotype data of 30 single nucleotide polymorphisms (SNPs) in *THBS1*, *THBS2*, and *THBS4* were obtained using the Illumina 550K assay (N = 5143). To determine the association between genetic variation in the thrombospondin genes and VWF:Ag levels we used linear regression analysis. To assess the association between genetic variations in the thrombospondin genes and CHD we used Cox proportional hazards regression.

Results. In *THBS1*, two SNPs were associated with VWF:Ag levels: rs2618164 (intronic) (β = 0.03 IU/mL per coded allele 95%CI [0.003;0.06]) and rs2228262 (Asn700Ser) (β = 0.04 IU/mL per coded allele 95%CI [0.01;0.08]) In *THBS2*, rs11756439 (intronic) was associated with VWF:Ag levels (β = 0.03 IU/mL per coded allele 95%CI [0.003;0.05]). After adjustment for multiple testing the associations between thrombospondin variations and VWF:Ag levels did not maintain statistical significance. None of the genetic polymorphisms in *THBS1*, *THBS2*, or *THBS4*, were significantly associated with the risk of CHD

Conclusions. We found no evidence to support the relationship between genetic variation in thrombospondin genes and the risk of CHD. However, genetic variations in *THBS1* and *THBS2* are weakly involved in the regulation of VWF:Ag levels.

INTRODUCTION

The relationship between elevated von Willebrand Factor (VWF) plasma levels and an increased risk of coronary heart disease (CHD) has been well established[1-2]. In recent years several genetic determinants of VWF antigen (VWF:Ag) levels have been identified[3-5]. In addition, specific regulators of VWF, such as ADAMTS13, have gained interest because of their possible role in the development of CHD[6].

One other example of these interesting regulatory factors is Thrombospondin-1 (TSP-1), which can control VWF multimer size both directly via cleavage of the VWF linking disulfide bonds, and indirectly by competing with ADAMTS13[7-8]. In this way, TSP-1 is able to regulate the thrombotic potential of VWF molecules and may therefore be involved in the pathogenesis of CHD.

TSP-1 is part of the thrombospondin family, which consists of five extracellular matrix proteins that are involved in cell-matrix and cell-cell interactions[9]. The possible relationship between thrombospondins and CHD became apparent when Topol et al. identified genetic polymorphisms in thrombospondin genes (*THBS1*, *THBS2*, and *THBS4*) that were associated with the risk of myocardial infarction (MI)[10]. However, replication studies that followed showed contradictory results, possibly due to small population samples and retrospective study design[11-14]. Consequently, no consensus has emerged on the actual relationship between genetic variations in thrombospondin genes and the risk of CHD yet. In addition, there are only limited studies available on the effect of these genetic polymorphisms on VWF levels[14]. Therefore, we aimed to investigate the effect of genetic polymorphisms in *THBS1*, *THBS2*, and *THBS4* on VWF antigen (VWF:Ag) levels and the risk of CHD in a prospective setting.

METHODS

Study population

For this study we included participants from the Rotterdam Study (RS), an ongoing prospective population-based cohort study among individuals of ≥ 55 years living in a suburb in the city of Rotterdam in the Netherlands[15]. We selected 6454 participants, who were free from CHD at baseline, which was defined as a previous myocardial infarction (MI) or a revascularization procedure, including percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery (CABG). History of CHD was determined during the baseline interview and verified in medical records[16]. From baseline, participants have been continuously monitored for incident CHD, which was defined as a MI, revascularization procedure or cardiac death, 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.

Laboratory measurements

We determined VWF:Ag levels in 5801 individuals of whom plasma was available with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies and horseradish-peroxidase-conjugated anti-human VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging, respectively.

Genetic analysis

For the genetic analysis we determined genotype data of common single nucleotide polymorphisms (SNPs) in *THBS1* (chromosome 15), *THBS2* (chromosome 6), and *THBS4* (chromosome 5) using the Illumina 550K assay. SNPs were excluded when minor allele frequency (MAF) ≤ 1%, Hardy-Weinberg equilibrium (HWE) $P < 10^{-6}$, or SNP call rate ≤ 98%. In total, seven SNPs in *THBS1*, fourteen SNPs in *THBS2*, and nine SNPs in *THBS4*, including 2kb of the 3'-region and 2kb of the 5'-region, were genotyped and included in our analysis. Genotyping was successful in 5143 participants.

Statistical analysis

We used linear regression analysis, adjusted for age and sex, with additive genetic models to determine the association between genetic variants in the thrombospondin genes and VWF:Ag levels. Beta-coefficients represent the increase in VWF:Ag levels (IU/mL) per coded allele. To assess the association between genetic variations in the thrombospondin genes and CHD we used Cox proportional hazards regression with additive genetic models. Hazard ratios represent the increase in risk of CHD per coded allele. Additional adjustment for classical cardiovascular risk factors did not influence the effect sizes (data not shown). To correct for multiple testing we calculated the false discovery rate (q-value) for each SNP[17].

RESULTS AND CONCLUSIONS

The mean follow-up time was 6.4 years (41,363 person years), during which 473 CHD events occurred. The mean age of the participants was 68.7 ± 8.7 years and 60.2% were female. As expected, most subjects had one of more cardiovascular risk factors. The mean blood pressure was 139 ± 22 mmHg systolic and 77 ± 11 mmHg diastolic. The mean total cholesterol was 6.32 ± 1.2 mmol/L and the mean BMI was 26.7 ± 3.9. Over 20% of the participants currently smoke and 12% have diabetes.

Participants with incident CHD had a mean VWF:Ag level of 1.42 ± 0.8 IU/mL versus 1.30 ± 0.6 IU/mL in individuals without incident CHD. Subjects with VWF:Ag levels in the highest quartile had an increased risk of CHD adjusted for age, sex and cardiovas-

cular risk factors relative to subjects with VWF:Ag levels in the lowest quartile (HR 1.39 [0.98;1.76]).

In *THBS1*, two SNPs were associated with VWF:Ag levels: rs2618164 (intronic) (β = 0.03 IU/mL per coded allele 95%CI [0.003;0.06]) and rs2228262 (Asn700Ser) (β = 0.04 IU/mL per coded allele 95%CI [0.01;0.08]) (figure 1-A). Rs2228262 is a missense mutation that encodes an amino acid substitution of asparagine into serine at position 700. Carriers of the G allele had 4% higher VWF:Ag levels than carriers of the A allele. Interestingly, the G allele has also been associated with an increased risk of myocardial infarction previously[10]. In *THBS2*, rs11756439 (intronic) was associated with VWF:Ag levels (β = 0.03 IU/mL per coded allele 95%CI [0.003;0.05]). After adjustment for multiple testing the associations between thrombospondin variations and VWF:Ag levels did not maintain statistical significance. This observation is in line with the recent findings of the CHARGE consortium that did not identify SNPs for VWF:Ag levels that were located in or close to thrombospondin genes[4]. Yet, current large genome-wide association studies can only detect common SNPs with relatively large effects and therefore overlook genetic factors

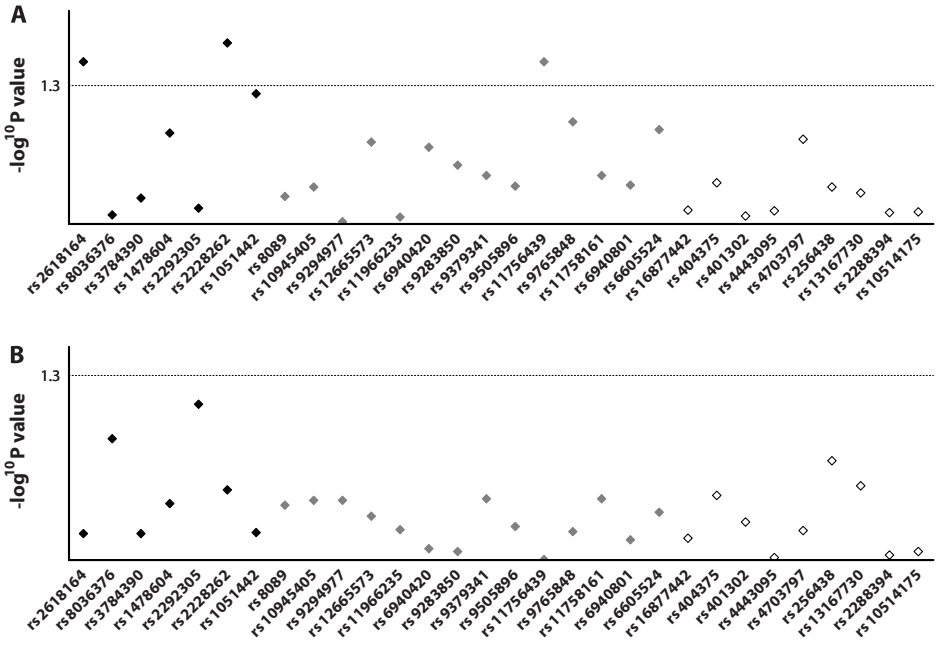


Figure 1 • (A) -Log[10] of the p-values of the associations between SNPs in thrombospondin genes and VWF:Ag. (B) -Log[10] of he p-values of the associations between SNPs in thrombospondin genes and CHD risk. In both figures the threshold is set at 1.3, which the -Log[10] p-value of 0.05. The black checks represent the SNPs in *THBS1*, the grey checks represent the SNPs in *THBS2*, and the white checks represent the SNPs in *THBS4*.

with smaller effects, such as thrombospondin genes, which still may be relevant in the regulation of VWF plasma levels.

None of the genetic polymorphisms in *THBS1*, *THBS2*, or *THBS4*, were significantly associated with the risk of CHD (figure 1-B). These findings are in line with most case-control replication studies that followed the GeneQuest study of Topol et al.[11, 13, 18-19]. Also, the meta-analysis of Koch et al. showed that SNPs in *THBS1*, *THBS2* and *THBS4* were not associated with CHD[12].

Important strengths of the present study are the prospective design, the long and nearly complete follow-up and the large number of cardiovascular events. However, our study may still have had limited statistical power, since we expect that the effect of genetic variation in thrombospondin genes on VWF:Ag levels is small and therefore the possible contribution of thrombospondins in the development CHD will also be small. Conversely, considering the diverse functions of thrombospondins in for example atherosclerosis, inflammation, and thrombus formation it can be argued that the link between thrombospondins and CHD is not explained by the regulation of VWF levels, but is actually independent of VWF.

In conclusion, we found no evidence to support the relationship between genetic variants in thrombospondin genes and the occurrence of CHD. However, we have shown that genetic variations in *THBS1* and *THBS2* are weakly involved in the regulation of VWF:Ag levels.

FUNDING SOURCES

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SUPPLEMENTARY TABLES

Supplementary table • Effect of genetic polymorphisms in thrombospondin genes on VWF:Ag levels and the risk of CHD.

rs#	Gene	Position	Allele	VWF:Ag	p-value	q-value	CHD	p-value	q-value
				β (95%CI)			HR (95%CI)		
rs2618164	*THBS1*	37651164	C>T	0.03 [0.003, 0.06]	0.03	0.30	1.04 [0.86, 1.27]	0.65	0.89
rs8036376	*THBS1*	37655919	A>C	-0.004 [-0.04, 0.03]	0.82	0.89	0.85 [0.70, 1.05]	0.14	0.89
rs3784390	*THBS1*	37658372	G>A	-0.01 [-0.05, 0.03]	0.57	0.81	0.95 [0.75, 1.20]	0.65	0.89
rs1478604	*THBS1*	37660613	T>C	0.02 [-0.01, 0.04]	0.14	0.57	0.94 [0.80, 1.09]	0.40	0.89
rs2292305	*THBS1*	37668114	A>G	-0.01 [-0.04, 0.03]	0.71	0.89	0.83 [0.68, 1.02]	0.08	0.89
rs2228262	*THBS1*	37669470	A>G	0.04 [0.01, 0.08]	0.02	0.30	1.12 [0.89, 1.41]	0.32	0.89
rs1051442	*THBS1*	37674941	C>T	0.03 [-0.002, 0.06]	0.06	0.45	1.05 [0.86, 1.27]	0.64	0.89
rs8089	*THBS2*	169435358	C>A	0.01 [-0.02, 0.03]	0.55	0.81	0.93 [0.79, 1.10]	0.41	0.89
rs10945405	*THBS2*	169438768	C>T	0.01 [-0.01, 0.03]	0.45	0.75	0.94 [0.81, 1.08]	0.38	0.89
rs9294977	*THBS2*	169441804	A>G	-0.001 [-0.02, 0.02]	0.95	0.95	0.94 [0.81, 1.09]	0.38	0.89
rs12665573	*THBS2*	169443422	A>G	0.03 [-0.01, 0.07]	0.17	0.57	1.10 [0.84, 1.43]	0.49	0.89
rs11966235	*THBS2*	169444462	C>T	0.002 [-0.02, 0.03]	0.86	0.89	0.96 [0.82, 1.13]	0.61	0.89
rs6940420	*THBS2*	169449539	G>A	0.02 [-0.01, 0.05]	0.19	0.57	0.98 [0.82, 1.17]	0.83	0.97
rs9283850	*THBS2*	169455088	G>A	-0.01 [-0.04, 0.01]	0.28	0.75	0.99 [0.86, 1.14]	0.87	0.97
rs9379341	*THBS2*	169461317	T>C	0.01 [-0.01, 0.04]	0.35	0.75	1.07 [0.92, 1.25]	0.37	0.89
rs9505896	*THBS2*	169464552	A>G	0.01 [-0.02, 0.04]	0.44	0.75	0.96 [0.82, 1.12]	0.58	0.89
rs11756439	*THBS2*	169465662	T>C	0.03 [0.003, 0.05]	0.03	0.30	1.00 [0.86, 1.17]	0.99	0.99
rs9765848	*THBS2*	169467459	C>T	-0.03 [-0.06, 0.01]	0.11	0.57	1.05 [0.86, 1.29]	0.63	0.89
rs11758161	*THBS2*	169468976	C>T	0.014[-0.02, 0.04]	0.35	0.75	1.09 [0.90, 1.32]	0.37	0.89
rs6940801	*THBS2*	169473299	A>G	-0.01 [-0.04, 0.02]	0.43	0.75	1.04 [0.85, 1.26]	0.72	0.90
rs6605524	*THBS2*	169473752	A>G	-0.02 [-0.04, 0.01]	0.13	0.57	1.06 [0.91, 1.23]	0.46	0.89
rs16877442	*THBS4*	79380374	T>C	-0.01 [-0.04, 0.03]	0.74	0.89	1.05 [0.82, 1.34]	0.70	0.90
rs404375	*THBS4*	79384515	A>G	0.01 [-0.01, 0.03]	0.41	0.75	0.94 [0.81, 1.08]	0.35	0.89
rs401302	*THBS4*	79391227	C>T	0.003 [-0.03, 0.03]	0.84	0.89	1.06 [0.88, 1.28]	0.54	0.89
rs443095	*THBS4*	79398306	G>A	-0.01 [-0.08, 0.06]	0.75	0.89	0.99 [0.63, 1.56]	0.96	0.99
rs4703797	*THBS4*	79400765	G>A	0.02 [-0.01, 0.04]	0.16	0.57	0.96 [0.83, 1.12]	0.62	0.89
rs256438	*THBS4*	79402005	G>T	0.01 [-0.01, 0.03]	0.45	0.75	0.91 [0.78, 1.05]	0.20	0.89
rs13167730	*THBS4*	79406003	G>T	0.01 [-0.03, 0.05]	0.51	0.81	1.15 [0.89, 1.48]	0.30	0.89
rs2288394	*THBS4*	79409559	C>T	0.004 [-0.02, 0.03]	0.78	0.89	1.01 [0.85, 1.19]	0.92	0.99
rs10514175	*THBS4*	79411987	G>A	0.003 [-0.02, 0.03]	0.77	0.89	0.99 [0.85, 1.15]	0.87	0.97

Linear regression analysis with an additive genetic model adjusted for age and sex. Beta-coefficient (β) represents the increase in VWF:Ag per coded allele. Cox proportional hazard regression analysis with an additive genetic model adjusted for age and sex. Hazard ratios with 95% confidence interval represent the estimated relative risk of CHD per coded allele.

JE van Loon, FWG Leebeek, JW Deckers, DWJ Dippel, D Poldermans, DP Strachan, W Tang, ChJ O'Donnell, NL Smith, MPM de Maat

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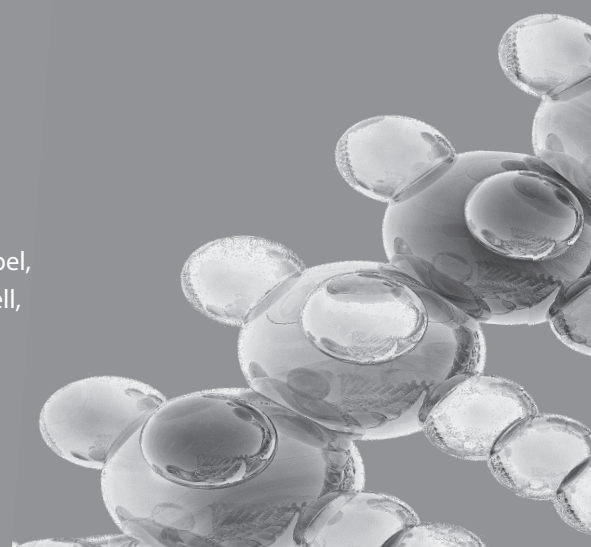
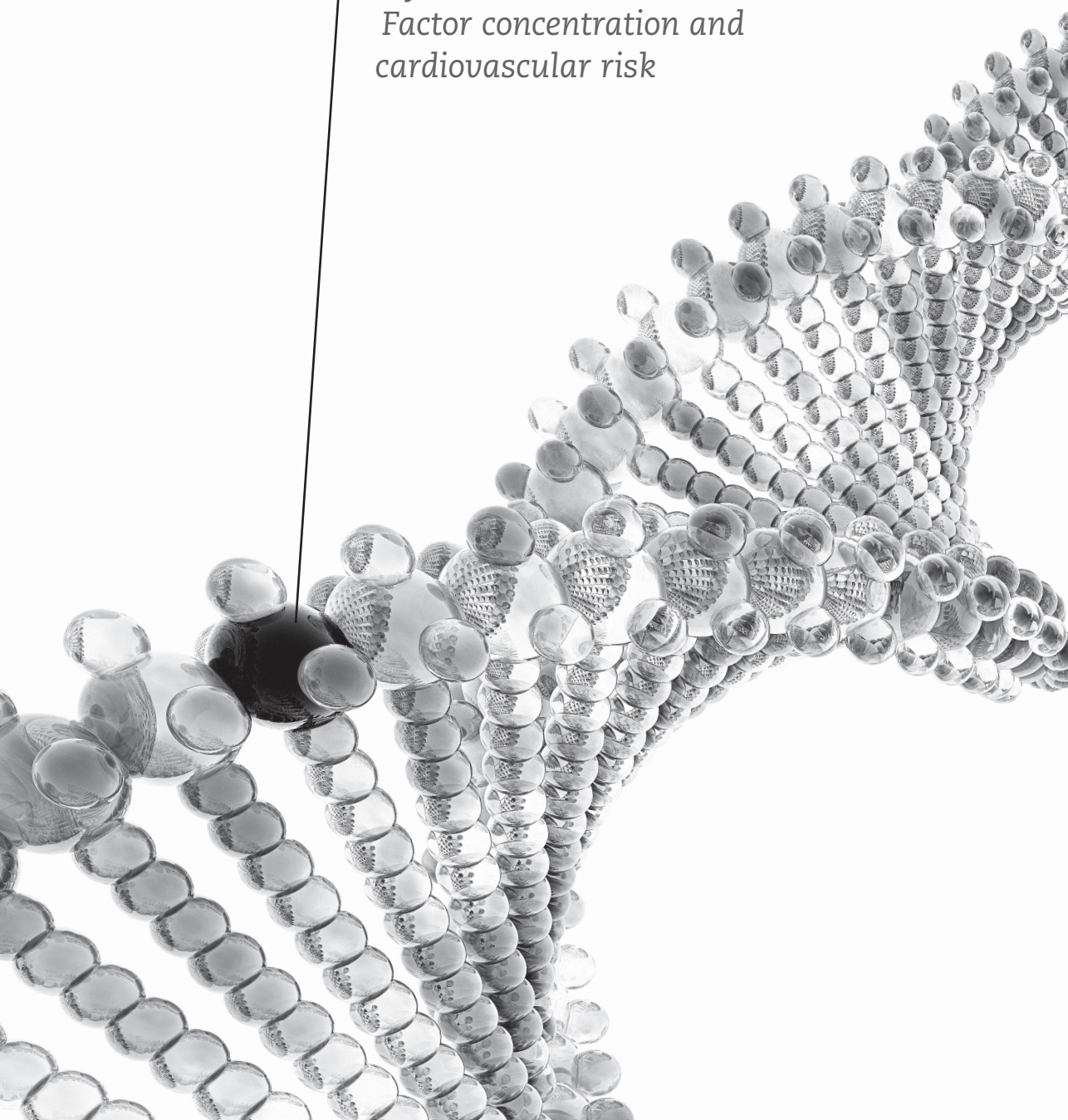


CHAPTER 5 *Effect of genetic variations in Syntaxin Binding Protein-5 and Syntaxin-2 on von Willebrand Factor concentration and cardiovascular risk*



ABSTRACT

Background. Elevated von Willebrand Factor (VWF) plasma levels are associated with an increased risk of cardiovascular disease. A meta-analysis of genome wide association studies on VWF identified novel candidate genes, i.e. syntaxin-binding protein 5 (*STXBP5*) and syntaxin 2 (*STX2*), which are possibly involved in the secretion of VWF. We investigated whether VWF antigen levels (VWF:Ag), VWF collagen-binding activity (VWF:CB), and the risk of arterial thrombosis are affected by common genetic variations in these genes.

Methods and Results. In 463 young Caucasian subjects (males ≤ 45 years, females ≤ 55 years), who were included one to three months after a first event of arterial thrombosis, and 406 controls, we measured VWF:Ag and VWF:CB. Nine haplotype tagging SNPs of *STXBP5* and *STX2* were selected and subsequently analysed using linear regression with additive genetic models adjusted for age, sex and ABO blood group. The minor alleles of *rs*9399599 and *rs*1039084 in *STXBP5* were associated with lower VWF plasma levels and activity, whereas the minor allele of *rs*7978987 in *STX2* was associated with higher VWF plasma levels and activity. The minor alleles of the SNPs in *STX2* were associated with a reduced risk of arterial thrombosis (*rs*1236:OR 0.73 [95%CI 0.59, 0.89], *rs*7978987:OR 0.81 [95%CI 0.65, 1.00], *rs*11061158:OR 0.69 [95%CI 0.55, 0.88]).

Conclusions. Genetic variability in *STXBP5* and *STX2* affects both VWF concentration and activity in young individuals with premature arterial thrombosis. Furthermore, in our study genetic variability in *STX2* is associated with the risk of arterial thrombosis. However, at this point the underlying mechanism remains unclear.

INTRODUCTION

Since elevated von Willebrand Factor (VWF) plasma levels are associated with an increased risk of cardiovascular disease (CVD), elucidating determinants of VWF plasma levels is of great interest[1-2]. It is already known that VWF plasma levels can be influenced by both genetic[3-4] and non-genetic factors. These factors have their effect on different stages during the lifetime of the VWF molecules, which involve many biological mechanisms. However, determinants that have been identified previously - heritability estimates for VWF levels are on average 50% - are not sufficient to explain the entire variability of VWF levels[3].

VWF has a twofold function in primary haemostasis: it initiates adherence of platelets to the injured vessel wall and subsequent platelet aggregation, especially at sites of high shear[5-6]. VWF is mainly produced by endothelial cells, but also for about 5-10% by megakaryocytes. The majority of the newly synthesized VWF proteins is directly secreted into the circulation via the constitutive pathway[7]. Large and ultra-large VWF multimers are stored in Weibel Palade bodies (WPBs) of endothelial cells and alpha-granules of platelets [8-9]. These storage granules release VWF multimers upon a variety of physiologic agonists, such as hypoxia[10], epinephrine, histamine, thrombin, fibrin and vasopressin[11].

Recently, the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium conducted a meta-analysis of genome wide association studies (GWAS) in five large population-based cohort studies to identify new genetic determinants of VWF levels[12]. Besides confirmation of previously indentified candidate genes, such as the ABO blood group gene and the VWF structural gene itself, the CHARGE consortium identified and replicated novel associations with six genetic loci, among which the *STXBP5* and the *STX2* genes. Syntaxin 2 (*STX2*) is a binding substrate for syntaxin binding protein 5 (*STXBP5*) and is a member of the Soluble N-ethylmaleimide-sensitive factor (NSF) Attachment protein Receptor (SNARE) protein family. These proteins drive vesicle exocytosis by fusion of granules and target membranes, a process involved in the regulation of numerous secretory events[13], such as WPB exocytosis. WPBs and alpha-granules release large amounts of VWF after endothelial cell activation, for example in atherosclerosis and subsequent arterial thrombosis. Moreover, these storage granules secrete not only VWF molecules, but also other substances, including pro-inflammatory factors, such as P-selectin, eotaxin and interleukine-8. Hence, considering the involvement of *STXBP5* and *STX2* in secretion of VWF and other pro-thrombotic and pro-inflammatory factors, which may in turn lead to development of atherosclerosis, these candidate genes may have a direct effect on the risk of CVD as well.

We aimed to further expand previous findings of the CHARGE consortium in an independent case-control study. Our study population is unique, since it consists of specifically young individuals with a first event of arterial thrombosis. In addition, the influence

of genetics is generally more pronounced in younger individuals than in older individuals who have been exposed to potential cardiovascular risk factors for a longer period of time[14]**.** Consequently, we have investigated the effect of common genetic variations in *STXBP5* and *STX2*, including the 3'and 5' UTR-regions, on VWF plasma concentration, VWF collagen binding activity, and the risk of arterial thrombosis.

METHODS

Patients

The 'Genetic risk factors for Arterial Thrombosis at young age: the role of TAFI and other Coagulation factors' (ATTAC) study is a single-center, case-control study, described in more detail previously[15-16]. Briefly, the cases (n = 463) were defined as patients with a first event of arterial thrombosis, which were consecutively recruited one to three months after the event at the departments of cardiology, neurology and vascular surgery at the Erasmus University Medical Center. Patients were eligible for inclusion when they were 18-45 years for males and 18-55 years for females at the time of diagnosis. The cases consist of three subgroups: (1) patients with coronary heart disease (CHD), defined as either an acute myocardial infarction (AMI) or unstable angina pectoris (UAP), (2) patients with either ischemic stroke (IS) or a transient ischemic attack (TIA) and (3) patients with peripheral arterial disease (PAD). The control group (n = 409) consists of neighbours or friends of the patient, fulfilling the same age criteria and without a history of cardiovascular events. For the current study we included Caucasian individuals only.

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

Blood sampling

Blood was drawn one to three months after the ischemic event by venipuncture in the antecubital vein using Vacutainer system (Becton-Dickinson, Plymouth, UK). Blood for coagulation measurements was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within 1 hour at 2000 x g for 10 min at 4°C. Plasma was additionally centrifuged at 20 000 x g for 10 minutes at 4°C and stored in aliquots at -80°C. For DNA isolation blood was collected in tubes containing ethylene diaminetetraacetic acid (EDTA; Beckon Dickinson) and genomic DNA was isolated according to standard salting-out procedures and stored at 4°C for genetic analysis.

Laboratory measurements

VWF antigen (VWF:Ag) was determined with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase conjugated anti-human VWF

antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging, respectively. VWF collagen binding (VWF:CB) was measured with an in-house ELISA using type I collagen (Sigma, St. Louis, USA) for catching and horseradish peroxidase conjugated anti-human VWF antibodies for tagging. The intra-assay variation coefficients of VWF:Ag and VWF:CB were 5.7% and 5.9%, respectively.

Genotyping

The *STXBP5* gene spans 182 kbps and is located in the q24 region of chromosome 6. The *STX2* gene spans 50 kbp and is located in the q24.3 region of chromosome 12. We obtained data from the International HapMap project (phase II November 2008 http://www.hapmap.org) on the linkage disequilibrium (LD) pattern and selected haplotype-tagging single-nucleotide polymorphisms (ht-SNPs) using Haploview software (version 3.11; www.broad.mit.edu/mpg/haploview/index/php). For both genes blocks of haplotypes with a frequency of ≥ 0.03 were defined in order to select these ht-SNPs. We took potential functionality into consideration by preferentially selecting non-synonymous ht-SNPs or SNPs that are located in known regulatory elements. In this study, we considered only SNPs that were present in a Caucasian population. We selected 6 ht-SNPs in *STXBP5* and 3 ht-SNPs in *STX2*, which were genotyped using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of the primers and probes used for the assay are available upon request. Endpoint fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, CA, USA) and clustered according to genotype using SDS 2.1 software (Applied Biosystems, Foster City, CA, USA). Genotyping was successful for each SNP in on average 96% of all subjects. Baseline characteristics of missing individuals were similar to those who were genotyped successfully.

Statistical analysis

Allele frequencies were calculated by genotype counting. For each SNP the deviation from the Hardy-Weinberg equilibrium was tested in controls by means of a Chi-squared test with one degree of freedom. The data of VWF:Ag and VWF:CB levels are approximately normally distributed and presented as mean and standard deviation (SD). We used linear regression on untransformed VWF:Ag and VWF:CB measures using additive genetic models adjusted for age, sex and ABO blood group (data are shown as unstandardized beta-coefficients representing the change in VWF:Ag or VWF:CB per minor allele with a 95% confidence interval). The relative risks of arterial thrombosis for all polymorphisms were assessed by means of a logistic regression analysis using additive genetic models adjusted for age, sex and ABO blood group (data are shown as odds ratios representing the increase in risk per minor allele with a 95% confidence interval). Statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS Inc, Chicago, USA). A two-sided value of $p < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics

We included 463 cases and 406 controls of Caucasian origin, of which 188 (41%) in the arterial thrombosis group and 149 (36%) in the control group were men (table 1). Mean age was 43.1 ± 6.7 years in patients and 39.3 ± 7.7 years in controls. The arterial thrombosis group consisted of 271 (59%) patients with either AMI or UAP, 148 (32%) cases with either IS or TIA and 44 (9%) cases with PAD. As expected the prevalence of known classical cardiovascular risk factors, including positive family history, high body mass index (BMI), hypertension, diabetes, statin use and current smoking, was significantly higher in patients than in controls (table 1). The distribution of ABO blood group was similar in cases and controls (P = 0.66).

Mean levels of VWF:Ag and VWF:CB were significantly higher in cases (mean ± SD, 1.28 ± 0.54 IU/mL and 1.40 ± 0.57 IU/mL, respectively) than in controls (1.09 ± 0.37 IU/mL; *P* < 0.0001 and 1.25 ± 0.42 IU/mL; *P* = < 0.0001, respectively). The allele frequency distributions of all polymorphisms in controls did not deviate from Hardy-Weinberg equilibrium.

Table 1 • ATTAC population baseline characteristics

	Cases (n = 463)	Controls (n = 409)	P-value
Demographics			
Sex (% men)	188 (41%)	149 (36%)	
Age (years)	43.1 ± 6.7	39.3 ± 7.7	
Diagnosis			
UAP/ AMI	271 (59%)		
TIA/IS	148 (32%)		
Peripheral Arterial disease	44 (9%)		
Risk Factors			
Positive family history (%)	280 (61%)	129 (32%)	<0.0001
Body Mass Index (kg/m^2)	26.6 ± 4.7	25.5 ± 4.3	<0.0001
Hypertension (%)	136 (29%)	24 (6%)	<0.0001
Diabetes (%)	39 (8%)	3 (1%)	<0.0001
Statin use (%)	372 (81%)	5 (1%)	<0.0001
Current smoker (%)	187 (41%)	92 (23%)	<0.0001
ABO blood group			
O (%)	202 (44%)	186 (45%)	
Non-O (%)	257 (56%)	123 (55%)	

Data are presented as mean ± SD or percentage for categorical variables

Genetic variation and VWF antigen levels

In patients, two ht-SNPs of *STXBP5* showed an association with VWF:Ag: *rs*9399599, which is located in intron 25, and *rs*1039084, which is a missense mutation that encodes an amino acid substitution of asparagine into serine at position 436 (table 2). Both SNPs are in high linkage disequilibrium with *rs*9390459, which had the highest genome wide significance level for VWF plasma levels in the meta-analysis of the CHARGE consortium (D' = 1.00, R^2 = 0.87 for *rs*9399599 and D'= 0.96, R^2 = 0.86 for *rs*1039084) (phase II November 2008 http://www.hapmap.org).

In the arterial thrombosis group the minor alleles of *rs*9399599 and *rs*1039084 were associated with lower VWF:Ag levels, also after Bonferroni correction. In the subgroup of patients with CHD only, *rs*9399599 was borderline significantly associated with VWF concentration (β = -0.08 [-0.15, 0.002], *P* = 0.06). Interestingly, the strength of association for *rs*1039084 increased even further (β = -0.12 [-0.20, -0.04], *P* = 0.004). By contrast, neither of the polymorphisms remained related to VWF:Ag levels in the subgroup of patients with IS or TIA. In the control group the effect of *rs*9399599 and *rs*1039084 on VWF:Ag levels was only borderline significant.

In *STX2* only one ht-SNP showed an effect on VWF plasma levels: *rs*7978987, which is located in intron 9 and had a p-value of 3.82×10^{-11} in the meta-analysis of the CHARGE consortium GWAS (table 2). In patients, *rs*7978987 was associated with higher VWF:Ag levels. After Bonferroni correction rs7978987 remained borderline significantly associated with VWF:Ag levels. In controls none of the ht-SNPs of *STX2* were associated with VWF plasma levels.

In the linear regression analyses we also used an age and sex adjusted model (data not shown). The effect sizes of the polymorphisms in this model were similar to those obtained using a model which was in addition adjusted for ABO blood group.

VWF collagen binding activity

VWF collagen binding activity (VWF:CB) levels are displayed in table 2 for cases and controls, separately. Interestingly, all *STXBP5* polymorphisms that were associated with VWF:Ag showed similar associations with lower VWF:CB levels in patients. None of these SNPs were associated with the VWF:CB/VWF:Ag ratio (data not shown). By contrast, *rs*7978987 of *STX2* was associated with higher VWF:CB levels and VWF:CB/VWF:Ag ratio (β = 0.18 [0.01, 0.36], *P* = 0.03). In controls VWF:CB is not influenced by genetic variability in *STXBP5* and *STX2*.

Risk of arterial thrombosis

In the total population, *rs*1236 and *rs*11061158 of *STX2* had a strong and significant relationship with the risk of arterial thrombosis, independent of ABO blood group and after correction for multiple testing (table 3). The minor alleles of *rs*1236 and *rs*11061158

Table 2 • Effect of *STXBP5* and *STX2* polymorphisms on VWF:Ag and VWF:CB

				Controls				Cases			
Gene	rs-number	Allele	MAF*	VWF:Ag β (95% CI)	P	VWF:CB β (95% CI)	P	VWF:Ag β (95% CI)	P	VWF:CB β (95% CI)	P
STXBP5	rs9399599	T>A	0.45	-0.05 [-0.09, -0.002]	0.04	-0.03 [-0.09, 0.02]	0.22	-0.09 [-0.15, -0.02]	0.007	-0.10 [-0.17, -0.03]	0.006
	rs1039084	G>A	0.44	-0.05 [-0.09, 0.001]	0.06	-0.03 [-0.08, 0.03]	0.33	-0.09 [-0.15, -0.03]	0.006	-0.10 [-0.17, -0.03]	0.008
	rs693850	T>C	0.17	-0.02 [-0.08, 0.04]	0.59	-0.02 [-0.09, 0.05]	0.47	-0.08 [-0.16, -0.002]	0.05	-0.11 [-0.19, -0.02]	0.02
	rs497704	G>A	0.04	0.02 [-0.11, 0.15]	0.75	0.02 [-0.13, 0.18]	0.77	-0.06 [-0.25, 0.14]	0.58	-0.05 [-0.27, 0.17]	0.66
	rs127173905	C>T	0.25	0.04 [-0.01, 0.10]	0.10	0.02 [-0.04, 0.09]	0.55	0.02 [-0.06, 0.09]	0.68	0.10 [0.01, 0.18]	0.03
	rs1765028	C>T	0.43	0.04 [-0.01, 0.08]	0.14	0.03 [-0.03, 0.08]	0.34	0.02 [-0.04, 0.09]	0.46	0.06 [-0.01, 0.13]	0.09
STX2	rs1236	T>A	0.47	-0.01 [-0.05, 0.04]	0.70	-0.001 [-0.06, 0.05]	0.98	0.05 [-0.02, 0.12]	0.19	0.06 [-0.02, 0.14]	0.14
	rs7978987	G>A	0.37	-0.02 [-0.07, 0.03]	0.42	0.001 [-0.06, 0.06]	0.98	0.08 [0.01, 0.16]	0.03	0.10 [0.02, 0.18]	0.02
	rs11061158	G>T	0.26	0.002 [-0.05, 0.05]	0.92	0.03 [-0.03, 0.09]	0.30	0.02 [-0.06, 0.10]	0.66	0.03 [-0.06, 0.12]	0.54

*Linear regression analysis with an additive genetic model, adjusted for age, sex, and blood group. P-value is the probability that the null-hypothesis, beta-coefficient is zero after adjustment, is true. Data are represented as beta-coefficients (β) per minor allele with a 95% confidence interval (CI). *MAF = minor allele frequency*

Table 3 • Association between *STXBP5* and *STX2* polymorphisms and the risk of arterial thrombosis

Gene	rs-number	Allele	Total Group (OR [95% CI])	CHD subgroup (OR [95% CI])	IS/TIA subgroup (OR [95% CI])
STXBP5	rs9399599	T>A	1.11 [0.91, 1.34]	1.12 [0.88, 1.42]	1.04 [0.79, 1.37]
	rs1039084	G>A	0.99 [0.81, 1.21]	1.02 [0.80, 1.29]	0.87 [0.66, 1.14]
	rs693850	T>C	1.02 [0.79, 1.31]	0.93 [0.68, 1.26]	1.04 [0.74, 1.45]
	rs497704	G>A	0.88 [0.49, 1.56]	0.82 [0.40, 1.66]	0.89 [0.41, 1.96]
	rs127173905	C>T	0.99 [0.79, 1.25]	0.96 [0.73, 1.26]	1.13 [0.84, 1.54]
	rs1765028	C>T	1.02 [0.84, 1.25]	1.08 [0.85, 1.37]	1.06 [0.81, 1.40]
STX2	rs1236	T>A	0.72 [0.59, 0.89]	0.64 [0.49, 0.82]	0.76 [0.57, 1.00]
	rs7978987	G>A	0.81 [0.65, 1.00]	0.70 [0.54, 0.91]	0.81 [0.60, 1.10]
	rs11061158	G>T	0.69 [0.55, 0.88]	0.67 [0.50, 0.89]	0.72 [0.52, 0.99]

Logistic regression analysis with an additive genetic model adjusted for age, sex, and blood group. Data are represented as the increase in odds ratio per minor allele with a 95% confidence interval. OR = odds ratio. CI = confidence interval. CHD = coronary heart disease. IS/TIA = ischemic stroke or transient ischemic attack.

had a protective effect on the occurrence of arterial thrombosis (figure 1). Interestingly, in the subgroup analysis of patients with CHD the minor alleles of the three polymorphisms that cover the entire genetic variation of STX2 were associated with a decreased risk of CHD. In the subgroup of patients with ischemic stroke or TIA this effect was less apparent. Genetic variants in the *STXBP5* gene were not associated with the risk of arterial thrombosis.

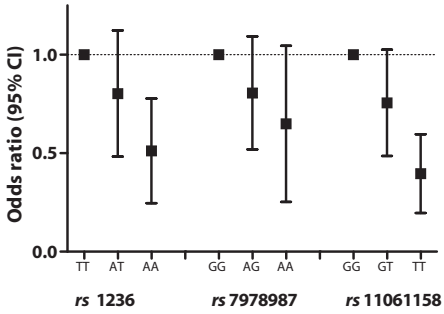


Figure 1 • *STX2* genotypes and the risk of arterial thrombosis. Odds ratios per genotype of *STX2* polymorphisms. Homozygous carriers of the common allele are used as reference category.

DISCUSSION

In this manuscript, we show that genetic variations in *STXBP5* and *STX2* affect both VWF concentration and VWF collagen binding activity in a population of young individuals with a first event of arterial thrombosis. Whereas the minor alleles of *rs*9399599 and *rs*1039084 in *STXBP5* were associated with lower VWF:Ag and VWF:CB levels, the minor allele of *rs*7978987 in *STX2* was associated with higher VWF:Ag and VWF:CB levels. These findings are a further exploration of the results of the meta-analysis of the CHARGE consortium. Interestingly, the effects of the minor alleles on VWF concentration are similar to those demonstrated by the CHARGE consortium (β-coefficient -0.048 for *rs*9390459 and 0.033 for *rs*7978987 per dosage allele). As well as confirming the association with VWF plasma levels, we also found an association between these genetic variants and VWF activity. In addition, *rs*7978987 in *STX2* was associated with a higher VWF:CB/VWF:Ag ratio, which indicates that the secreted VWF molecules are functionally more active. Since ultra large VWF multimers, which have the most haemostatic potential, are stored in WPB and alpha-granules, this finding came up our expectations.

It is noteworthy that the relationship between genetic variability in *STXBP5* and *STX2* and VWF plasma levels was seen especially in patients with arterial thrombosis and that this relationship was less clear in healthy controls. In healthy individuals, VWF plasma levels are determined mainly by the activity of the constitutive pathway, because the endothelium is not triggered to release the VWF molecules. Since *STX2* and *STXBP5* encode proteins that may be involved in the regulated secretion pathway of VWF molecules, which is only stimulated after endothelial cell activation, one would expect to find an effect of these polymorphisms not in healthy subjects, but particularly in patients who have CVD. In addition, it is known that at a higher age more atherosclerosis is present, which brings the endothelium into a mild state of activation and leads to chronic low-level stimulation of the regulatory pathway of VWF release. This may explain why a relationship between VWF:Ag levels and genetic polymorphisms in STXBP and STX2 was found in the older (mean age 60.0 years), but relatively healthy subjects of the CHARGE cohorts.

In the CHD subgroup the associations between polymorphisms and VWF levels seem more pronounced than in the IS/TIA subgroup. As ischemic stroke is a heterogeneous phenotype, caused by multiple and sometimes unknown underlying factors, the contribution of genetic variation is hard to study. While heterogeneity might be reduced by analysis by etiologic subtype, such analysis was not possible in our ATTAC study because of the limited number of patients in the subgroup of stroke[17].

Unexpectedly, the minor alleles of the polymorphisms in *STX2* were strongly associated with a decreased risk of arterial thrombosis. This effect seemed strongest in the CHD subgroup, where the minor alleles of all three SNPs gave a protective effect. Yet,

the precise mechanisms by which these polymorphisms influence the risk of arterial thrombosis are unclear, because the minor alleles of the polymorphisms in STX2 tended to be associated with higher VWF plasma levels. As well as VWF molecules, WPBs and alpha-granules contain numerous other substances, such as P-selectin, angiopoeitin-2, osteoprotegerin, and eotoxin-3[13]. It has also been hypothesized that not all components of the storage granules have to be present at all times *per se* and can even be segregated into different subgroups of substances[18]. We therefore propose that the risk of CVD is reduced not by VWF itself but by other substances that are secreted by the Weibel Palade Bodies.

In conclusion, this study shows that genetic variations in *STXBP5* and *STX2* affect VWF antigen plasma levels and VWF collagen binding activity in young patients with premature arterial thrombosis. It remains unclear whether altered VWF levels are caused by dysfunction of the VWF secretion pathway or by another, unknown, mechanism. We also observed that genetic variability in *STX2* is associated with the risk of arterial thrombosis in young individuals. Future research is required to study the functionality of the polymorphisms in more detail and to improve our understanding of the possible importance of the secretion pathway of VWF in the pathogenesis of arterial thrombosis.

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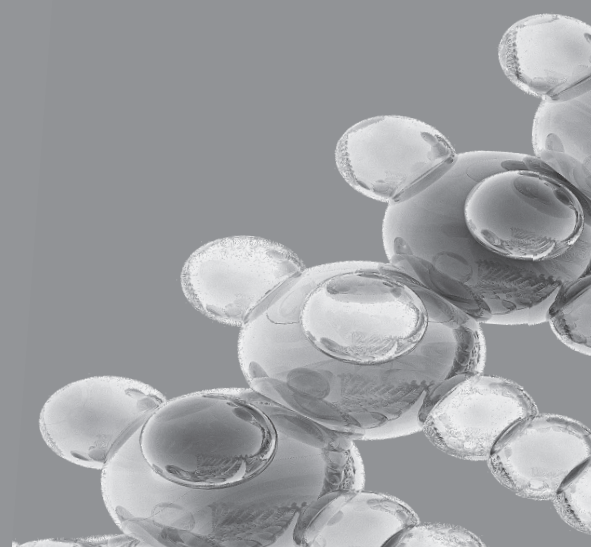
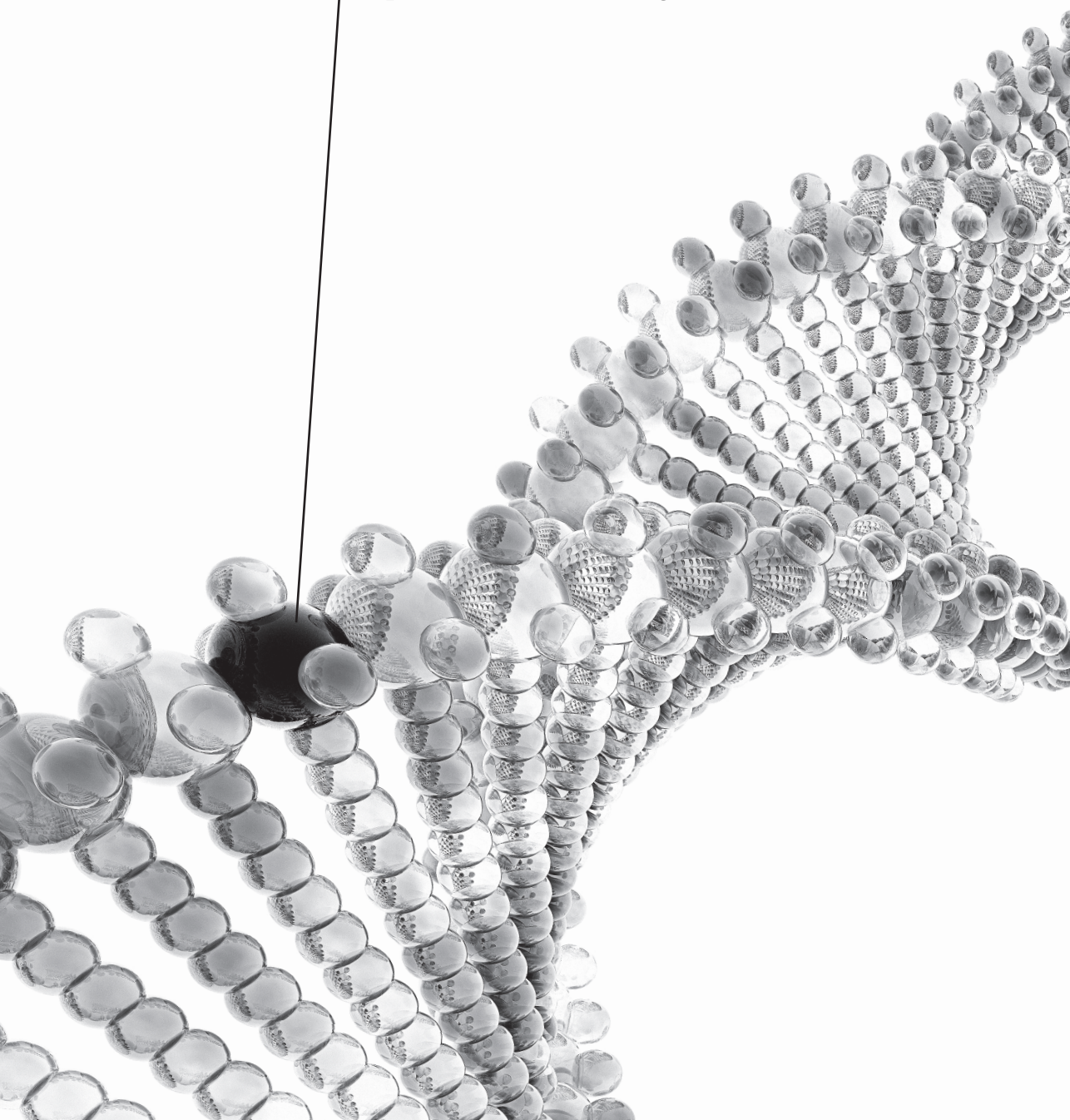


CHAPTER 6 *Prognostic markers in young patients with premature coronary heart disease*



ABSTRACT

Objectives. To evaluate the survival and prognostic implications of cardiovascular, inflammatory and prothrombotic risk factors in young patients with premature coronary heart disease (CHD).

Methods. Follow-up data were obtained from 353 young patients with a first cardiac event (men ≤ 45 years and women ≤ 55 years). Baseline characteristics on traditional risk factors were collected at the time of the first event, and plasma levels of C–reactive protein (CRP), von Willebrand Factor (VWF), and fibrinogen were measured one to three months after the first event to exclude an acute phase response. We performed age and sex adjusted Cox regression analyses to assess the relationship between these factors and recurrent events with three different endpoints: all cause mortality, recurrent cardiac event (myocardial infarction or revascularisation procedure), and any recurrent event (cardiac event, cerebrovascular event or all cause mortality).

Results. During a total follow-up time of 1483 person years (mean 4.2 years), 11 patients died (3%), 42 patients had a recurrent cardiac event (12%), and 53 patients had any recurrent event (15%). CRP was associated with an increased risk of any recurrent event (HR 1.28[95%CI 1.02-1.59] per unit increase in lnCRP). Also, both CRP (5.00[1.04-24.04] and fibrinogen (5.04[1.05-24.23]) were associated with all cause mortality when levels were above the 50th percentile.

Conclusions. Fifteen percent of young patients with a first cardiac event have a recurrent event or die within a median follow-up of 4.2 years. In these young patients we have shown that, independently of cardiovascular risk factors, high CRP levels contribute to the risk of recurrent events, including all cause mortality, and high fibrinogen levels are associated with all cause mortality.

INTRODUCTION

Despite many improvements in medical treatment, coronary heart disease (CHD) is still a major health concern in today's clinical practice. Only a very small percentage (< 10%) of all patients with myocardial infarction is below the age of 45 years[1-2]. However, the number of young individuals with coronary atherosclerosis is probably much larger than can be currently estimated. Autopsies have shown that about 50% of young individuals have coronary atherosclerosis[3]. Few studies have accomplished to include sufficiently large groups of young CHD patients to investigate their risk profiles and prognosis. However, there is a growing need to identify those at risk for recurrent events, since especially young CHD patients comprise an interesting group for preventive cardiology.

Generally, young subjects with CHD have multiple traditional risk factors and have a different risk profile than older patients[4-5]. Also, the occurrence of recurrent symptoms and events is surprisingly common. Reported long-term event rates including mortality are as high as 50%[6-7]. The main predictors of long-term recurrent events and mortality in young subjects that have been established over the years are diabetes, a low ejection fraction, atrial fibrillation, use of antiarrhythmic drugs, continued smoking, and plasma plasminogen activator inhibitor (PAI-1) concentration[5-7]. Moreover, there is a growing believe that prognostic risk factors differ between men and women.

In the last few decades novel risk factors for CHD have been identified in previously healthy subjects. Examples of these are inflammatory markers, such as C-reactive protein (CRP), and prothrombotic markers, including von Willebrand Factor (VWF) and fibrinogen[8-11]. However, information on their predictive value for recurrent cardiovascular events in especially young patients is very scarce.

Considering the necessity to recognize young CHD patients at risk for a recurrent event and to improve prevention strategies in this special group of patients, we aimed to evaluate the prognostic implications of traditional risk factor, CRP, VWF, and fibrinogen in a unique and relatively large cohort of young patients with a first acute coronary syndrome.

METHODS

Patients

This follow-up study is a sub-study of the 'Genetic risk factors for Arterial Thrombosis at young age: the role of TAFI and other Coagulation factors' (ATTAC) study. The ATTAC study is a single-center, case-control study, described in more detail previously[12]. For this sub-study we obtained follow-up data from all cardiac patients (N = 385), who were consecutively recruited one to three months after their first event (acute myocardial

infarction or unstable angina pectoris) at the department of Cardiology at the Erasmus Medical Center Rotterdam between 2001 and 2010. Patients were eligible for inclusion when they were 18-45 years for males and 18-55 years for females at the time of diagnosis. The follow-up study was approved by the medical research board at Erasmus University Medical Center and written informed consent was obtained from all participants at inclusion.

After all cardiac patients were asked to participate in the follow-up study, data on their current health status were obtained via a telephone interview and verified in medical records. We used three different endpoints: all cause mortality, recurrent cardiac event, which was defined as a myocardial infarction, percutaneous coronary intervention or coronary artery bypass graft surgery, and any recurrent event, including a cardiac event (myocardial infarction or revascularisation procedure), cerebrovascular event (CVA or TIA) or all cause mortality.

Of all cardiac patients, 22 patients were lost to follow-up, mainly because of emigration or because their contact information had changed and could not be traced down. Ten patients gave no permission for the follow-up study. We could include 353 cardiac patients in our analyses. Between the first event and the start of the follow-up study eleven patients died. Since we could not obtain informed consent of these patients to investigate their cause of death, we classified these patients as deaths of any cause.

Blood sampling

Blood was drawn one to three months after the first ischemic event by venipuncture in the antecubital vein using the Vacutainer system (Becton-Dickinson, Plymouth, UK). Blood for coagulation measurements was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within 1 hour at 2000 x g for 10 min at 4°C. Plasma was additionally centrifuged at 14 000 x g for 10 minutes at 4°C and stored in aliquots at –80°C.

Laboratory measurements

CRP was determined in serum using Rate Near Infrared Particle Immunoassay (Immage*w* Immunochemistry System, Beckman Coulter, USA). This system measures concentrations ranging from 0.2 to 1440 mg/L, with a within-run precision <5.0%, a total precision <7.5%, and a reliability coefficient of 0.995.

VWF antigen (VWF:Ag) was determined at baseline with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase conjugated anti-human VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging, respectively. The intra-assay coefficient of variation was 5.7%, and the interassay coefficient of variation was 7.8%.

Plasma fibrinogen was measured as described by von Clauss[13] on the Sysmex CA 1500 coagulation analyzer (Dade Behring, Leusden, Netherlands). The within-day variation was 1.7% and the between-day variation 6.3%.

Cholesterol and HDL were determined on Modular AnalyticsÒ (Roche Diagnostics, Mannheim, Germany). The total assay variation was 3% and 2% for cholesterol and HDL, respectively.

Statistical analysis

Data on population characteristics are presented as means and standard deviations for continuous variables and as counts and percentages for categorical data. Since CRP and VWF:Ag levels were skewed, these data were natural logarithmically transformed (lnCRP and lnVWF:Ag, respectively) and presented as geometric mean and standard deviation. We used Cox regression analyses adjusted for age and sex to assess the relationship between the selected markers and the risk of recurrent events. For the association between traditional cardiovascular risk factors and the risk of recurrent events a sex-specific analysis was performed additionally. Since CRP, VWF, and fibrinogen are associated with cardiovascular risk factors, all associations were adjusted additionally for cardiovascular risk factors present at inclusion (family history of cardiovascular disease, hypertension, diabetes, cholesterol, high-density lipoprotein, hypercholesterolemia, BMI and smoking). The analyses with all cause mortality as endpoint were not adjusted for sex or diabetes, since only one of all deaths was female and none had diabetes. Levels of CRP, VWF and fibrinogen were divided into two groups: below and above the 50th percentile. Cut-off level for CRP was 0.87 mg/L, for VWF 1.23 IU/mL, and 3.4 g/L for fibrinogen.

Cumulative survival curves and cumulative event-free survival curves were constructed using the Kaplan-Meier (KM) method. In order to compare the KM-curves, we used a Log-rank test.

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

RESULTS

Our study population consists of 353 patients with a total follow-up of 1483 person years (mean ± SD, 4.2 ± 2.6 years). Baseline characteristics are shown in table 1, as well as the reference values and the baseline characteristics of 487 control subjects that were included in the ATTAC study. Of all cardiac patients, 299 had a myocardial infarction as first event and 54 had unstable angina pectoris as first event. Most patients received a drug-eluting stent (91%) and had single vessel disease (76%). Left ventricular ejection

Table 1. Baseline characteristics

	Cardiac patients	Controls ATTAC study	Reference values
	N = 353	N = 487	
Age (years)	43.8 ± 5.9	38.8 ± 7.8	
Female sex, N (%)	156 (44%)	293 (63%)	
First event			
Myocardial infarction	299 (85%)		
NSTEMI	232 (89%)		
STEMI	30 (11%)		
Unstable angina pectoris	54 (15%)		
Risk factors			
Family history of cardiovascular disease, N (%)	173 (49%)	112 (23%)	
Hypertension, N (%)	88 (25%)	30 (7%)	
Diabetes, N (%)	35 (10%)	8 (2%)	
Smoking, current, N (%)	133 (38%)	123 (25%)	
Smoking, former, N (%)	165 (47%)	129 (27%)	
Total cholesterol (μmol/L)	4.2 ± 0.9	5.0 ± 0.9	2.9 – 6.5
HDL (μmol/L)	1.2 ± 0.4	1.5 ± 0.4	> 1.55
Hypercholesterolemia, N (%)	172 (49%)	167 (42%)	
BMI (kg/m^2)	27 ± 5	25 ± 4	
Medication at inclusion			
Bèta-blockers, N (%)	291 (82%)	14 (3%)	
ACE-inhibitors, N (%)	210 (60%)	4 (1%)	
Diuretics, N (%)	22 (6%)	8 (2%)	
Calcium antagonists, N (%)	31 (9%)	2 (0.4%)	
Angiotensin-II receptor antagonists, N (%)	14 (4%)	7 (2%)	
Statins, N (%)	336 (95%)	8 (2%)	
Antiplatelet drugs, N (%)	347 (98%)	6 (1%)	
CRP (mg/L)	0.78 ± 2.1	0.65 ± 1.9	0.2–7.3
VWF:Ag (IU/mL)	1.19 ± 0.7	1.04 ± 0.4	0.60 – 1.40
Fibrinogen (g/L)	3.45 ± 0.7	ND	1.5 – 3.6

Summary statistics for continuous variables are presented as mean ± standard deviation. Categorical data are summarized as percentages.
ND = not determined.

fraction was available in 109 subjects: good (> 55%) in 73 subjects, moderate (40-55%) in 32 subjects, and poor (<40%) in 4 subjects.

During follow-up, 11 patients died of any cause (3%), 42 patients had a recurrent cardiac event (12%), and two patients had a cerebrovascular event (<1%). The mean age of the total follow-up cohort was 43.8 years and 156 (44%) patients were female.

Of all patients, 98% was on anti-platelet therapy (aspirin and/or clopidogrel), 95% used statins, and 94% used any blood pressure lowering drugs, including bèta-blockers, ACE-inhibitors, calcium antagonists, diuretics or angiotensin-II receptor antagonists. In the total group, both age and sex did not differ between patients who had a recurrent event and patients without reinfarction.

Classical cardiovascular risk factors were not associated with recurrent cardiac events (Table 2). However, in a sex-specific analyses diabetes was associated with any recurrent event (2.66 [95% CI 1.02-6.94]) and a recurrent cardiac event (4.11 [95% CI 1.50-11.27]) in men. In women, current smoking and former smoking were significantly associated with both recurrent cardiac event and any recurrent event. Current smoking had a HR of 6.61 [95% CI 1.52-28.78] for any recurrent event and a HR of 12.3 [95% CI 1.63-93.45] for a recurrent cardiac event. Former smoking had a HR of 5.96 [95% CI 1.16-30.71] for any recurrent event and a HR of 9.82 [95% CI 1.10-87.88] for a recurrent cardiac event.

CRP levels were higher in patients who died of any cause (1.04 mg/L), in patients with a recurrent cardiac event (1.02 mg/L), and in patients with any recurrent event (1.06 mg/L) than in patients without reinfarction (0.73 mg/L). The age and sex adjusted hazard ratios per unit increase in lnCRP levels were 1.56 [95% CI 0.92-2.63] for all cause mortality, 1.23 [95% CI 0.96-1.59] for a recurrent cardiac event, and 1.28 [95% CI 1.02-1.59] for any recurrent event (table 3). In a multivariate model including all traditional risk factors the hazard ratios per unit increase in lnCRP levels were 1.80 [95% CI 0.95-3.39] for all

Table 2 • Association between traditional cardiovascular risk factors and the risk of recurrent events

	All cause mortality HR (95% CI)	Recurrent cardiac event HR (95% CI)	Any recurrent event HR (95% CI)
Age	0.98 (0.89-1.08)	0.98 (0.93-1.03)	0.99 (0.94-1.03)
Female sex	-	1.26 (0.68-2.33)	0.98 (0.57-1.67)
Family history of cardiovascular disease	0.69 (0.19-2.43)	0.96 (0.52-1.78)	0.91 (0.53-1.56)
Hypertension	0.26 (0.03-2.04)	0.59 (0.26-1.34)	0.56 (0.27-1.14)
Diabetes	-	1.88 (0.84-4.25)	1.39 (0.63-3.07)
Smoking, current	0.40 (0.09-1.67)	2.07 (0.96-4.43)	1.42 (0.76-2.66)
Smoking, former	1.34 (0.32-5.60)	1.96 (0.73-5.28)	1.66 (0.74-3.69)
Total cholesterol	1.47 (0.77-2.81)	1.11 (0.79-1.56)	1.17 (0.88-1.57)
HDL	1.31 (0.29-6.01)	1.20 (0.55-2.59)	1.29 (0.66-2.51)
Hypercholesterolemia	1.72 (0.44-6.68)	1.01 (0.54-1.91)	1.10 (0.63-1.92)
BMI	0.94 (0.82-1.08)	0.99 (0.93-1.06)	0.98 (0.93-1.04)

Cox regression analysis to assess the relationship between traditional cardiovascular risk factors and the risk of recurrent events. Recurrent cardiac event was defined as a myocardial infarction or revascularisation procedure. Any recurrent event was defined as a cardiac event, a cerebrovascular event or all cause mortality. Data are presented as hazard ratios with 95% confidence intervals. Analyses with the endpoint all cause mortality were not performed for female sex (number of event = 1) and diabetes (number of event = 0).

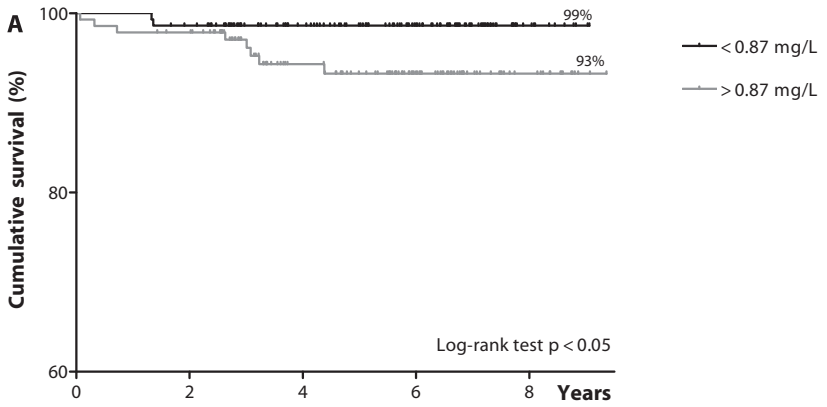
Table 3 • Adjusted association between inflammatory and prothrombotic markers and the risk of recurrent events

Variable	Model 1	Model 2
	HR (95% CI)	HR (95% CI)
All cause mortality		
lnCRP	1.56 (0.92-2.63)	1.80 (0.95-3.39)
lnVWF:Ag	0.90 (0.22-3.64)	1.33 (0.21-8.23)
Fibrinogen	1.85 (0.91-3.80)	1.79 (0.80-3.97)
Recurrent cardiac event		
lnCRP	1.23 (0.96-1.59)	1.21 (0.90-1.63)
lnVWF:Ag	1.12 (0.53-2.36)	1.10 (0.48-2.56)
Fibrinogen	0.87 (0.53-1.40)	0.88 (0.52-1.50)
Any recurrent event		
lnCRP	1.28 (1.02-1.59)	1.35 (1.03-1.77)
lnVWF:Ag	1.06 (0.54-2.05)	1.20 (0.55-2.62)
Fibrinogen	1.05 (0.70-1.57)	1.15 (0.74-1.77)

Cox regression analysis to assess the relationship between selected biomarkers (CRP, von Willebrand Factor, and fibrinogen) and the risk of a recurrent event. lnCRP and lnVWF:Ag are the natural logarithmically transformed CRP and vWF:Ag levels. Recurrent cardiac event was defined as a myocardial infarction or revascularisation procedure. Any recurrent event was defined as a cardiac event, a cerebrovascular event or all cause mortality. Data are presented as hazard ratios with 95% confidence intervals per unit increase in lnCRP, lnVWF, and fibrinogen, respectively. Model 1 is adjusted for age and sex. Model 2 is additionally adjusted for family history of cardiovascular disease, hypertension, diabetes, cholesterol, high-density lipoprotein, hypercholesterolemia, BMI and smoking. The analyses with all cause mortality as endpoint were not adjusted for sex or diabetes.

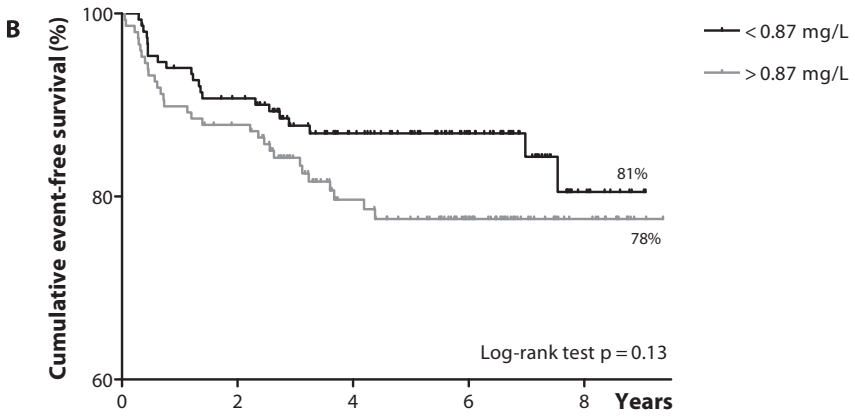
cause mortality, 1.21 [95% CI 0.90-1.63] for a recurrent cardiac event, and 1.35 [95% CI 1.03-1.77] for any recurrent event. Patients with CRP levels above the 50th percentile had a 5-fold increased risk of all cause mortality (HR 5.00 [95%CI 1.04-24.04]). Moreover, it is clearly shown that subjects with the highest CRP levels have a lower cumulative survival (93%) than subjects with the lowest CRP levels (99%, log-rank test $p < 0.05$) (Figure 1A). Also, the cumulative event-free survival was lower in subjects with the highest CRP levels (78%) than in subjects with the lowest CRP levels (81%, log-rank test $p = 0.13$), though just below the statistical significance level (figure 1B).

The mean fibrinogen levels were 3.8 ± 0.7 g/L in patients who died of any cause, 3.4 ± 0.9 g/L in patients with a recurrent cardiac event, 3.4 ± 0.9 g/L in patients with any recurrent event, and 3.5 ± 0.7 g/L in patients without reinfarction. High fibrinogen levels were weakly correlated with an increased risk of all cause mortality only (table 3). Subjects with fibrinogen levels above the 50th percentile had a significantly increased risk of all cause mortality (HR 5.04 [95% CI 1.05-24.23])), also after adjustment for classical cardiovascular risk factors (HR 10.41 [95% CI 1.18-91.80]). The cumulative survival of patients



No. at risk

< 50th percentile	144	141	103	68	13
> 50th percentile	140	136	91	55	19



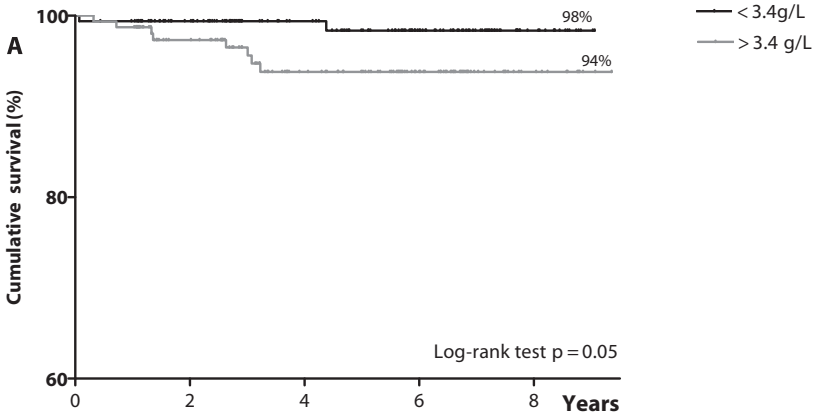
No. at risk

< 50th percentile	152	135	93	21	13
> 50th percentile	148	128	77	33	19

Figure 1 • Kaplan-Meier curve for cumulative survival (A) and cumulative event-free survival (B) for CRP levels above and below the 50th percentile. The cut-off value was 0.87 mg/L.

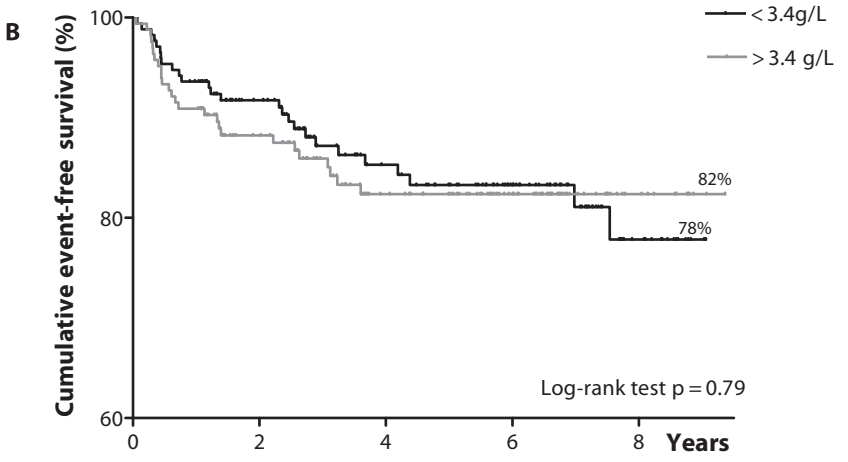
with the highest fibrinogen levels was 94% compared to 98% in patients with the lowest fibrinogen levels (Log-rank test p = 0.05) (Figure 2).

The mean VWF:Ag levels were 1.1 ± 1.0 IU/mL in patients who died of any cause, 1.2 ± 0.5 IU/mL in patients with a recurrent cardiac event, 1.2 ± 0.6 IU/mL in patients with any recurrent event, and 1.2 ± 0.7 IU/mL in patients without reinfarction. VWF:Ag levels were not associated with recurrent events (Table 3).



No. at risk

< 50th percentile	164	141	99	63	17
> 50th percentile	158	130	92	60	15



No. at risk

< 50th percentile	173	136	86	57	17
> 50th percentile	165	122	82	56	15

Figure 2 • Kaplan-Meier curve for cumulative survival (A) and cumulative event-free survival (B) for fibrinogen levels above and below the 50th percentile. The cut-off value was 3.4 g/L.

DISCUSSION

In this follow-up study of young patients with premature CHD fifteen percent had a recurrent cardiac event or died of any cause within a median follow-up period of 4.2 years. In addition, high CRP levels contributed to the risk of recurrent events and all cause mortality. High fibrinogen levels were associated with all cause mortality only.

The independent relationship between CRP levels and all cause mortality or any recurrent event was yet unknown in young patients with CHD. Since we measured CRP levels one to three months after the first event, our findings are less influenced by an acute phase response that follows infarction. This is in contrast to many other studies that measured CRP levels at the onset of a first myocardial infarction. Some studies showed that CRP levels during acute myocardial infarction predict 30-day and long-term mortality[14-15], whereas other studies did not show this relationship[16-18]. CRP levels measured at the time of an acute myocardial infarction may reflect the severity of disease at that time point and may therefore not be useful for long-term cardiovascular assessment. Harb et al. measured CRP levels two months after a myocardial infarction. Although they showed that CRP levels tended to be higher in patients with recurrent events, CRP was not an independent marker for recurrent events in a multivariate model[19].

In addition, we found a significant association between high fibrinogen levels and all cause mortality, even after adjustment for cardiovascular risk factors. Studies on the predictive value of fibrinogen for recurrent cardiovascular events are scarce. One study reported an association between fibrinogen levels and the risk of coronary events in patients with stable coronary artery disease[20]. Also, a study of Pineda et al. in young subjects with coronary artery disease (median age 41 years) showed that fibrinogen was predictive for cardiovascular events, though only in the unadjusted analysis[21].

No relation was found between VWF levels and the risk of recurrent events. VWF:Ag levels have been associated with reinfarction and/or mortality risk in patients with CHD by numerous studies[22]. However, these patients were generally much older than our current study population. Pineda et al. investigated the prognostic implications of VWF:Ag levels in young patients with a first myocardial infarction[21]. In the current study there was also no association between VWF:Ag levels and cardiovascular events observed.

Since the inflammatory markers CRP and fibrinogen, but not VWF, were associated with recurrent events or all cause mortality, our results suggest that inflammation is an important determinant of prognosis. Indeed, there is a strong cross-talk between inflammation and thrombosis[23]. Inflammation has been associated with the occurrence of myocardial infarction[24-25]. The absence of an association in young CHD patients between VWF:Ag levels and recurrent events in our study and in previous studies is an interesting and unexpected finding, since VWF is a well established risk factor for CHD in older subjects. Levels of VWF:Ag mirror the extent of endothelial dysfunction and thereby the

atherosclerosis burden in the body. Since CRP and fibrinogen, but not VWF, were associated with recurrent events or all cause mortality, we hypothesize that at young age inflammation, as reflected by the CRP and fibrinogen levels, is more important than the extent of atherosclerosis for prognosis. However, considering the number of recurrent events in our study our results should be interpreted with care.

Surprisingly, traditional cardiovascular risk factors were not associated with recurrent events in the total group. However, since it is suspected that there are strong gender differences in prognostic markers we performed a sex-specific analysis. In this analysis diabetes was associated with recurrent events in men. In women both former smoking and current smoking were associated with recurrent events. However, considering the small numbers in this sub-analysis larger studies are required to investigate this in more detail.

In our study, 98% of all cardiac patients were on anti-platelet therapy, 95% used statins, and 94% used any blood pressure lowering drug. It is interesting that despite optimal medical therapy these young patients experience this many recurrent events (12%), although the survival after a first cardiac event is excellent (97%). Recently, in a large group of young patients (≤ 50 years) undergoing PCI it has been shown that the long-term outcome has not changed in the last 30 years despite improved secondary prevention[26]. These findings imply a negative trend in risk profiles of young individuals or that current treatment strategies are possibly still not adequate.

Although our study comprises a considerable large group of young subjects with premature CHD followed in time, our study was still limited by the number of patients and the number of recurrent events. Also, since we included patients after one to three months, we possibly missed patients who had a recurrent event before inclusion or patients who died shortly after the first event. Finally, CRP and fibrinogen were significantly associated with respectively a recurrent event and all cause mortality, though their effect sizes were modest. Nevertheless, the findings of the current study clearly illustrate that the risk profiles in young CHD patients are different than the risk profiles at older age and that the prevention strategies should be adapted to this.

In conclusion, we show that although the survival after a first cardiac event in young patients is good, the recurrent event rate is quite high despite optimal medical therapy. In addition, in these young patients high CRP levels contribute to the risk of recurrent events and high fibrinogen levels are associated with all cause mortality independently of cardiovascular risk factors.

FUNDING SOURCES

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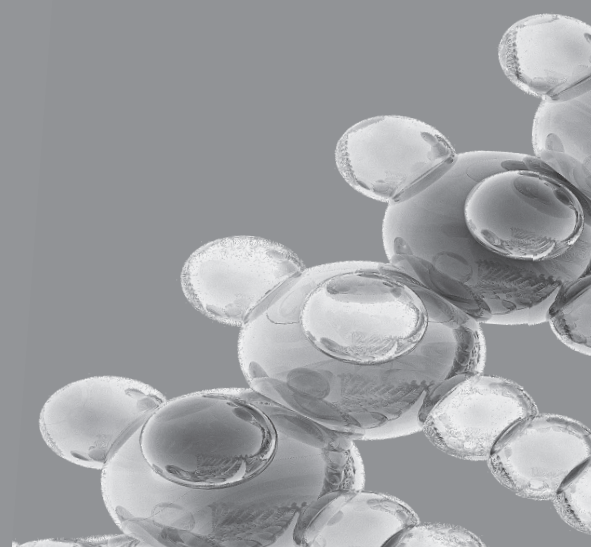
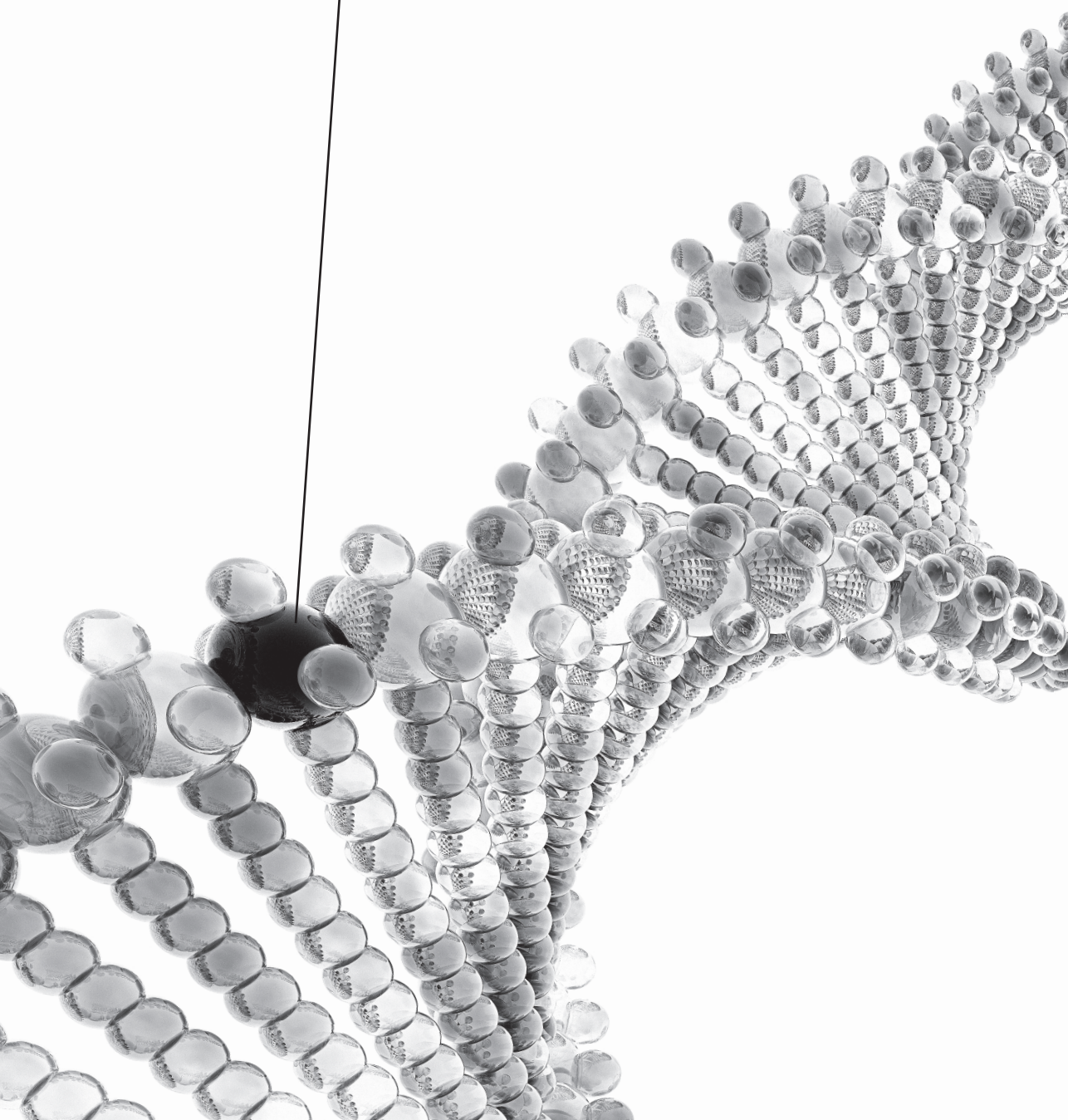
JE van Loon, CHARGE consortium

submitted



CHAPTER 7

Genome-wide association studies identify genetic loci for low von Willebrand Factor levels



ABSTRACT

Background. von Willebrand Factor (VWF) levels are highly variable and are largely determined by genetic factors. Low VWF levels are associated with bleeding symptoms and are a diagnostic criterion for von Willebrand Disease, the most common inherited bleeding disorder. To date, it is unknown which genetic loci besides cis-variation in *VWF*, are associated with reduced VWF levels. Therefore, we conducted a meta-analysis of genome-wide association studies to identify genetic loci associated with low VWF levels.

Methods and results. For this meta-analysis we included 31,149 participants of European ancestry from eleven community-based studies. From all participants VWF antigen (VWF:Ag) measurements and genome-wide single nucleotide polymorphisms (SNPs) scans were available. Each study conducted analyses of all directly genotyped and imputed SNPs using logistic regression on dichotomized VWF:Ag measures (lowest 5% for blood group O and non-O) with an additive genetic model adjusted for age and sex. An inverse-variance weighted meta-analysis was performed for VWF:Ag levels and summary p-values and β-coefficients were calculated. A total of 86 SNPs exceeded the genome-wide significance threshold of $5x10^{-8}$ and comprised five loci on four different chromosomes: 6q24 (smallest p-value $8.8x10^{-10}$), 9q34 ($2.1x10^{-60}$), 12p13 ($8.3x10^{-22}$), 12q23 ($4.3x10^{-8}$), 13q13 ($2.6x10^{-8}$). All loci were within or close to genes, including *STXBP5* (6q24), *STAB5* (12q23), *ABO* (9q34), *VWF* (12p13), and *UFM1* (13q13).

Conclusions. In addition to ABO blood group and *VWF*, three other genetic loci (*STXBP5*, *STAB2*, and *UFM1*) are associated with low VWF levels. These findings point to novel mechanisms for the occurrence of low VWF levels.

INTRODUCTION

Von Willebrand Factor (VWF) is a multifunctional glycoprotein, which is almost exclusively secreted by endothelial cells and released upon endothelial cell activation. VWF initiates adherence of platelets to the injured vessel wall and subsequent platelet aggregation facilitating adequate haemostasis[1-2].

Normal plasma levels of VWF antigen (VWF:Ag) are characterized by a large inter-individual variation and range from 0.60 to 1.40 IU/mL[3]. Various environmental and lifestyle factors affect VWF:Ag levels, but a large part of the variability in VWF:Ag levels can be explained by genetic factors[4].

The necessity of maintaining normal VWF levels in the circulation is illustrated by two clinical manifestations that may occur when VWF exceeds its normal range. High VWF antigen (VWF:Ag) levels have been associated with an increased risk of venous thrombosis and arterial thrombosis[5-8]. Conversely, low VWF antigen levels are associated with an increased bleeding tendency and are a characteristic of von Willebrand Disease (VWD). VWD is the most common inherited bleeding disorder in humans and is caused by a quantitative deficiency of von Willebrand Factor (VWF) (type 1 and 3 VWD) and/or a qualitative defect of VWF molecules (type 2 VWD)[9].

Most severe forms of type 1 VWD are caused by dominant-negative mutations in the VWF gene (*VWF*) and are highly heritable[10-13]. However, in individuals with moderately decreased VWF levels *VWF* mutations are often not found and linkage with the VWF locus is rarely seen [10-13]. Hence, it is difficult to differentiate between subjects with physiologically low VWF levels and subjects with low VWF levels because of VWD[14]. However, since VWF levels are strongly genetically determined, it is expected that genetic variations in other genes than *VWF* are likely to be involved in the etiology of type 1 VWD. Therefore, we performed a meta-analysis of genome-wide association studies (GWAS) in eleven large population-based cohort studies of the Cohorts for Heart and Aging Research of Genomic Epidemiology (CHARGE) Consortium to identify genetic loci that are associated with low VWF:Ag levels.

METHODS

Study populations

This meta-analysis was conducted in the CHARGE Consortium[15], which includes data from several population-based cohort studies. VWF antigen (VWF:Ag) measurements were available in four of these; the Rotterdam Study (RS) I and II , the Framingham Heart Study (FHS), and the Atherosclerotic Risk in Communities (ARIC) study. In addition, we included data from seven other studies that had VWF:Ag measurements and genome-

wide data available: the British 1958 Birth cohort (B58C) study, the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER), the Prevention of Renal and Vascular Endstage Disease (PREVEND) study, Lothian Birth Cohort 1921 and 1936, Vis Croatia Study (CROATIA-Vis) and ORKNEY complex Disease Study (ORCADES). The design of the studies have been described previously[16-28].

Genome-wide scans and VWF:Ag measurements were available for analysis in 31,149 individuals. Eligible participants were not using a coumarin-based anticoagulant at the time of VWF:Ag measurement and were of European ancestry by self report. All studies were approved by institutional review committees. In addition, written informed consent was obtained from all participants, as well as permission to use their DNA for research purposes.

Table 1 • Characteristics of the study participants per cohort

	ARIC	B58C-WTCCC	B58C-T1DGC	FHS
Count	9257	1461	2484	2806
Age, y (SD)	54.3 (5.7)	44.9 (0.4)	45.3 (0.3)	54.2 (9.7)
Men, (%)	47.1	50.0	48.3	45.4
Current smoker, (%)	24.6	22.9	23.0	18.6
Body mass index, kg/m2 (SD)	27.0 (4.8)	27.4 (4.94)	27.4 (4.9)	27.4 (5.0)
Waist circumference, cm (SD)	96.1 (13.3)	92.2(13.3)	91.9 (13.6)	92.4 (14.2)
Systolic blood pressure, mm Hg (SD)	118.4 (17.0)	126.7(15.3)	126.7 (14.7)	125.5 (18.6)
Total/HDL cholesterol, ratio(SD)	4.7 (1.7)	4.0 (1.1)	4.0 (1.2)	4.4 (1.5)
Fasting glucose, mg/dL(SD)	106.9 (30.8)	N/A	N/A	100.0 (26.6)
Triglycerides, mg/dL (SD)	137.2 (92.4)	183.3 (132.5)	183.3 (152.6)	145.7 (113.1)
Diabetes, (%)	8.4	1.9	1.3	7.5
Hypertension, (%)	26.8	4.2	4.8	15.9
Lipid treatment, (%)	3.4	N/A	N/A	6
Hormone replacement (women), (%)	21.4	2.3	2.5	17
Prevalent cardiovascular disease, (%)	4.8	N/A†	N/A††	5.3
Median VWF:Ag, % (IQR)	105 (81-134)	117 (93-146)	116 (93-144)	121 (92-156)
No. of subjects with low VWF:Ag	476	72	123	141
Blood group O, N (%)	3916 (42)	643 (44)	1150 (46)	1327 (47)

Table presents baseline characteristics of all subjects per cohort. Summary statistics for continuous variables are presented as means and standard deviations (SD), unless otherwise specified. Categorical data are summarized as percentages.

† In B58C-WTCCC 4.2% has medication for heart disease or high blood pressure

†† In B58C-T1DGC 4.75% has medication for heart disease or high blood pressure

‡ Percentage of prevalent myocardial infarction or cerebrovascular accident.

Baseline measurements and von Willebrand Factor measures

Baseline measures of clinical and demographic characteristics were obtained at the time of cohort entry for ARIC, CROATIA-Vis, ORCADES, PROSPER, PREVEND and RS, and at the time of phenotype measurements for B58C, LBC1921, LBC1936, and FHS. Measures were obtained using standardized methods as specified by each study and included in-person measures of height and weight, as well as self-reported treatment of diabetes and hypertension, current alcohol consumption, and prevalent cardiovascular disease (history of myocardial infarction, angina, coronary revascularisation, stroke, or transient ischemic attack). Blood group antigen phenotypes (O and non-O) were reconstructed using genotype data of *rs*687289, which is a marker for the O allele[29].

Von Willebrand Factor antigen (VWF:Ag) was measured in all cohorts using different enzyme-linked immunosorbent assays (ELISA) (supplementary table 1).

LBC 1921	LBC 1936	ORCADES	PROSPER	PPREVEND	RS I	RS II	CROATIA-VIS
176	757	677	5047	3621	5974	1895	766
86.6 (0.4)	72.5 (0.7)	53.5 (15.3)	75.4 (3.4)	49.7 (12.4)	72.3 (7.0)	64.9 (8.1)	56.6(15.6)
45.5	52.4	46.4	47.9	51.1	42.8	45.7	41.4
2.8	7.9	8.5	26.5	35.6	16.7	20.3	28.0
26.1 (4.2)	27.9 (4.2)	27.6 (4.8)	26.8 (4.2)	26.1 (4.3)	26.8 (3.9)	27.3 (4.2)	27.4 (4.4)
N/A	N/A	93.8 (14.0)	N/A	89.0 (13.3)	93.5 (11.5)	94.0 (11.6)	95.7 (11.7)
157.0 (23.2)	147.1 (18.3)	130.2 (19.3)	154.5 (21.8)	129.1 (19.8)	143.5 (21.0)	142.9 (20.9)	137.4 (24.2)
N/A	3.8 (1.1)	3.6 (0.9)	4.7 (1.3)	74.2 (9.8)	4.5 (1.3)	4.5 (1.3)	3.9(0.9)
N/A	N/A	97.9 (17.6)	N/A	88.34 (20.0)	6.0 (1.5)	6.0 (1.7)	102.8 (25.3)
N/A	145.84 (72.5)	118.8 (59.0)	155 (71.0)	127.8 (87.4)	1.52 (0.74)	1.61 (0.89)	137.4 (80.9)
6.3	11.2	3.0	10.3	1.6	14.5	11.1	6.7
47.7	49.3	21.2	62.2	30.0	42	28.5	25
N/A	N/A	11.5	0	4.4	16.1	13.3	3.2
N/A	16.4	N/A	N/A	24.5	11.1	N/A	N/A
24.4	29.3	8.60	44.6	4.0‡	11	5.2	13.8
147 (119-180)	120 (95-149)	110 (86-140)	136 (109-168)	65 (36-110)	125 (95-166)	114 (89-148)	131 (103-160)
8	40	37	254	181	163	100	40
96 (55)	419 (55)	297 (44)	2703 (53)	1644 (45)	1547 (45)	954 (46)	289 (38)

Genotyping

For the genotyping, DNA was collected from phlebotomy from all studies except B58C, which used cell lines. Genome-wide assays of SNPs were conducted independently in each cohort using various Affymetrix and Illumina panels (Supplementary table 2). Each study conducted genotype quality control and data cleaning, including assessment of Hardy-Weinberg equilibrium and variant call rates. Details on genotyping assays have been described in detail previously and are provided in supplementary table 2[15].

For this analysis we investigated genetic variation in the 22 autosomal chromosomes[30]. Genotypes were coded as 0, 1, and 2 to represent the number of copies of the coded alleles for all chromosomes[30]. Each study independently imputed its genotype data to the ≈ 2.6 million SNPs indentified in the HapMap Caucasian (CEU) sample from the Centre d'Etude du Polymorphisme Humain[31-33]. Imputation software, including MACH, BIMBAM, or IMPUTE, were used to impute unmeasured genotypes with SNPs that passed quality-control criteria based on phased haplotypes observed in HapMap. Imputation results were summarized as an "allele dosage", which was defined as the expected number of copies of the minor allele of that SNP (a continuous value between 0 and 2) for each genotype. Each cohort calculated the ratio of observed to expected variance of the dosage statistics for each SNP. This value, which generally ranges from 0 to 1 (i.e. poor to excellent), reflects imputation quality.

Table 2 • Genome-wide Significant Association of 5 Loci with low VWF levels

SNP								
Most significant	**Region**	**Position**	**Variant***	**MAF**	***P***	**OR [95% CI]†**	**# Hits**	**Closest gene**
rs8176704	9q34	13512373	A>G	0.07	2.4×10^{-64}	2.83 [2.52;3.18]	40	0.0 kb from ABO (intron)
rs216303	12p13	6029306	T>C	0.09	5.3×10^{-22}	0.57 [0.51;0.64]	20	0.0 kb from VWF (intron)
rs1221638	6q24	147576998	A>G	0.43	5.8×10^{-10}	1.28 [1.19;1.39]	35	0.0 kb from STXBP5 (intron)
rs4981022	12q23	102674004	A>G	0.32	1.2×10^{-08}	0.79 [0.73;0.85]	1	0.0 kb from STAB2 (intron)
rs17057285	13q13	37635821	A>ϖC	0.005	2.6×10^{-08}	0.42 [0.30;0.56]	1	200 kb from UFM1

Abbreviations used in this table are: SNP for single nucleotide polymorphism, MAF for minor allele frequency, P for statistical significance level and OR for odds ratio.

* *The first allele is the coded allele and the second allele the reference allele.*

† *Odds Ratio (OR) represents the risk of having VWF:Ag levels in the lowest 5% relative to the upper 95%.*

Statistical analysis

Genotype-phenotype data were analyzed independently by each study. VWF:Ag measurements were used as dichotomous variable (low versus normal) with low VWF defined as the lowest 5% within blood groups, i.e. blood group O and non-O. All studies conducted analyses of all directly genotyped and imputed SNPs using logistic regression on dichotomized VWF:Ag measures with an additive genetic model adjusted for

age and sex. ARIC and PROSPER adjusted for field site, additionally. B58C adjusted for sex, date and time of sample collection, postal delay, and the nurse who performed the inclusion, which also adjusts for the region of residence. Age adjustment was not necessary in B58C, since all cohort members were born in one week.

An inverse-variance weighted meta-analysis was performed with genomic control correction being applied at cohort level[34]. All meta-analysis were conducted using METAL software (http://www.sph.umich.edu/csg/abecasis/Metal/index.html).

The a priori threshold of genome-wide significance was set at a p-value of 5.0×10^{-8}. When more than one SNP clustered at a locus, the SNP with the smallest p-value was selected to represent the locus.

RESULTS

For this meta-analysis 31,149 participants of European ancestry were included. The sample size and participant characteristics from each cohort are displayed in table 1. The mean age ranged from 45 years in B58C to 87 years in LBC1921 and on average 48% of the participants was female.

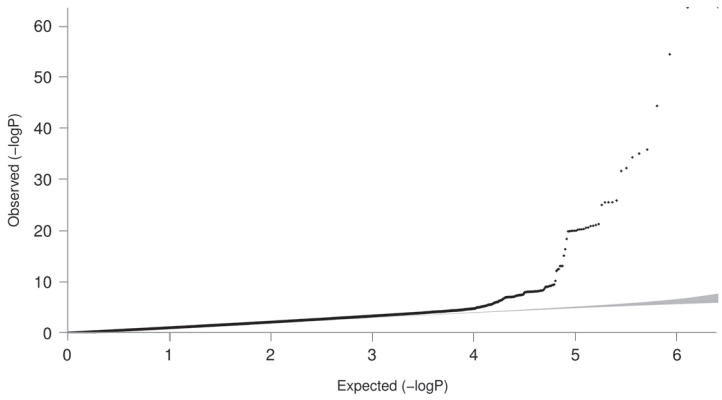


Figure 1 • Quantile-quantile plot of the observed and expected distribution of p-values for all ~2.6 million SNPs and their association with low VWF levels based on meta-analyzed data.

A quantile-quantile plot of the observed p-value from meta-analysis against expected p-value distribution is shown in figure 1. Figure 2 illustrates the primary findings from the meta-analysis and presents p-values for each of the interrogated SNPs across the 22 autosomal chromosomes. A total of 97 SNPs exceeded the genome-wide significance

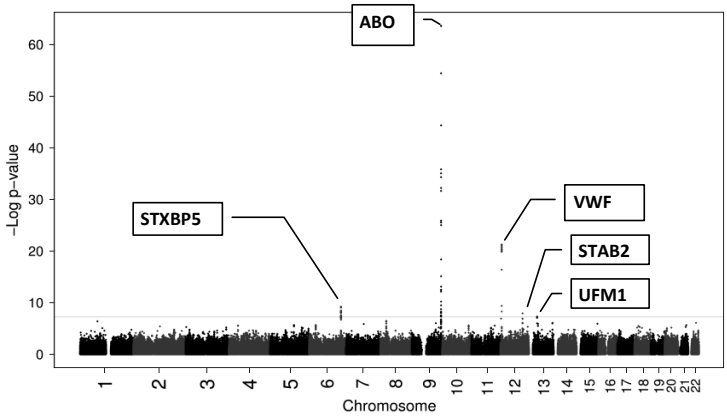
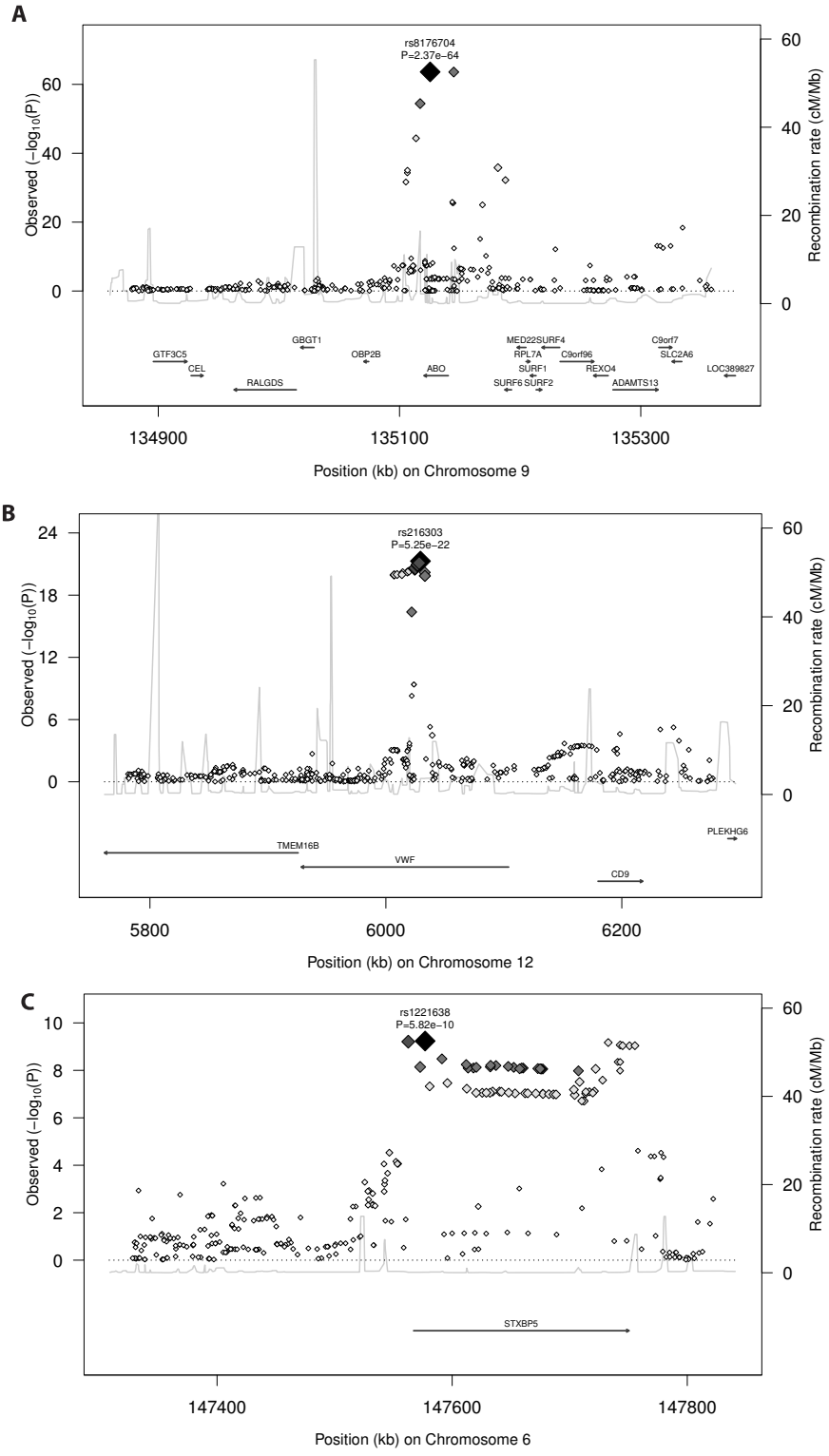


Figure 2 • –Log[10] p-values for each of the ~2.6 million tests performed as part of the GWA analysis of low VWF levels. The grey dashed horizontal line marks the 5 x 10^{-8} p-value threshold of genome-wide significance.

threshold of 5 x 10^{-8} and clustered around five genetic loci on four different chromosomes (figure 3). The SNP with the strongest statistical significance was *rs*8176704, which is located at 9q34 (intron) of the ABO blood group gene (P = 2.4 x 10^{-64}). The odds ratio (OR) for having VWF levels in the lowest 5% was 2.83 [95% CI 2.52;3.18]. The second most significant locus was marked by *rs*216303, which is located at 12p13 (intron) of the VWF gene (OR 0.57 [95% CI 0.51;0.64], P = 7.5 x 10^{-12}). The third genome-wide significant signal at chromosomal position 6q24 (intron) was within *STXBP5* (Syntaxin Binding Protein 5). Rs1221638 was associated with the smallest p-value (5.8 x 10^{-12}) in this region (OR 1.28 [95% CI 1.19;1.39]). The fourth statistical significant signal was marked by rs4981022, which is located at 12q23 (intron) of *STAB2* (stabilin-2) (OR 0.79 [95% CI 0.73;0.85], P = 1.2 x 10^{-8}). The final genome-wide significant locus was marked by rs17057285 (OR 0.41 [95% CI 0.30;0.56], P = 2.6 x 10^{-8}), which is close to *UFM1* (ubiquitin-fold modifier 1).

In addition to our five genome-wide significant loci, five other loci demonstrated multiple-SNP hits with p-values below 1.0 x 10^{-6}: rs10848820 (P = 1.2 x 10^{-7}) within *TSPAN9* (tetraspanin 9), rs4276643 (P = 3.4 x 10^{-7}) within *SCARA5* (scavenger receptor class A, member 5), rs17398299 (P = 4.1 x 10^{-7}) close to 1 gene, *LPHN2* (latrophilin 2), rs5995441 (P = 8.3 x 10^{-7}) within *CARD10* (caspase recruitment domain family, member 10), and rs3750450 (P = 9.6 x 10^{-7}) within *EPB41L4B* (erythrocyte membrane protein band 4.1 like 4B).


A
rs8176704
P=2.37e−64
Observed (−log10(P))
Recombination rate (cM/Mb)
GTF3C5
CEL
RALGDS
GBGT1
OBP2B
ABO
MED22SURF4
RPL7A
SURF1
SURF6 SURF2
C9orf96
REXO4
C9orf7
SLC2A6
ADAMTS13
LOC389827
134900
135100
135300
Position (kb) on Chromosome 9
B
rs216303
P=5.25e−22
Observed (−log10(P))
Recombination rate (cM/Mb)
TMEM16B
VWF
CD9
PLEKHG6
5800
6000
6200
Position (kb) on Chromosome 12
C
rs1221638
P=5.82e−10
Observed (−log10(P))
Recombination rate (cM/Mb)
STXBP5
147400
147600
147800
Position (kb) on Chromosome 6

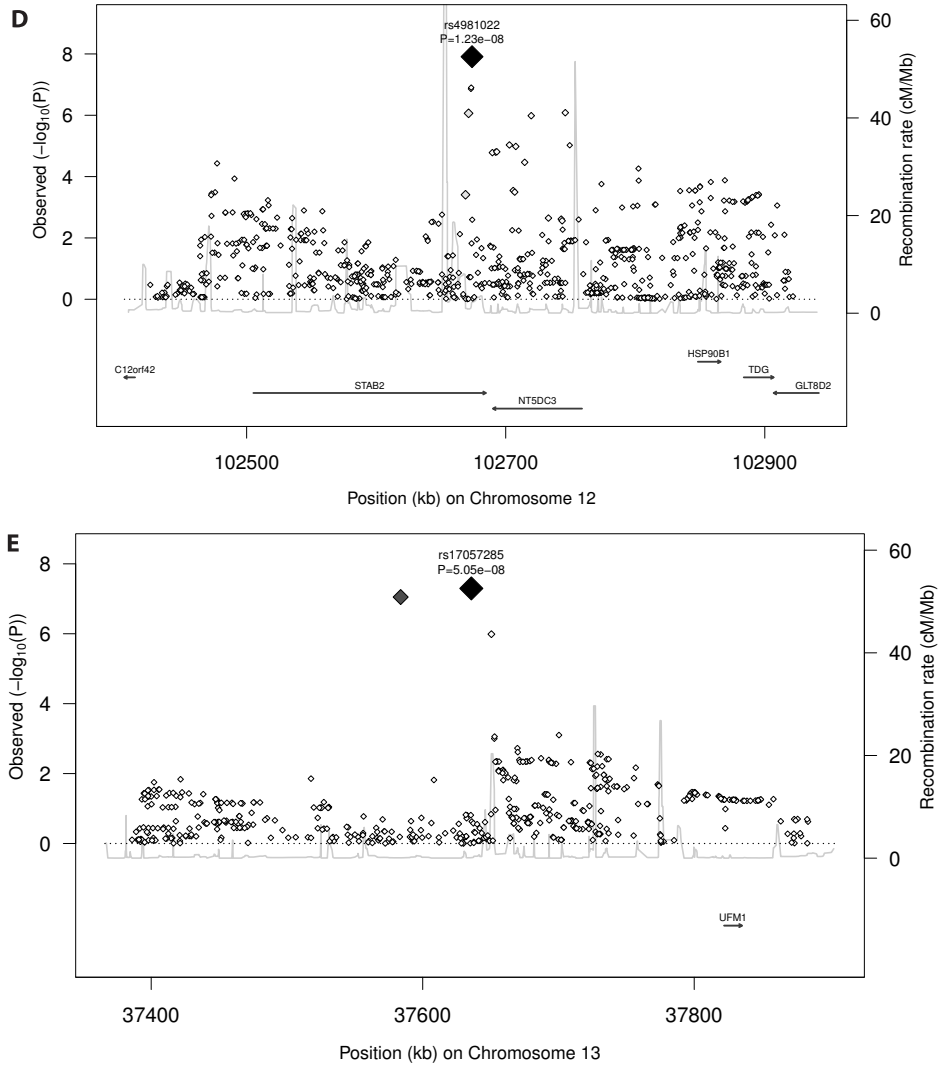


Figure 3 • Regional plots of top marker loci associated with low VWF levels. A through E: The association p-values (–log[10] transformed, indicated by the left *y* axis) for SNPs in a 60-kb region of each of the five loci (*ABO*, *VWF*, *STXBP5, STAB2, UFM1*) are plotted against their chromosome positions (NCBI build 3) on x-axis. The top SNPs are presented as a large diamond in red font and neighbouring variants are presented in different colours based on linkage disequilibrium based on HapMap Caucasian data: red: $1 \leq r^2 > 0.8$; orange: $0.8 \geq r^2 > 0.6$; yellow: $0.6 \geq r^2 > 0.3$; green: $0.3 \geq r^2 > 0.1$; blue: $0.1 \geq r^2 > 0.05$; light blue: $0.05 \geq r^2 > 0.0$. The left y-axis is the p-value on the -log[10] p-value scale and the gray line marks the threshold of genome-wide significance ($P = 5 \times 10^{-8}$). Shown in light blue are the estimated recombination rates in HapMap with values indicated by the right y-axis. Regional genes and their direction of transcription are depicted with green arrows.

DISCUSSION

In this meta-analysis of GWA data from eleven population-based cohorts comprising 31,149 individuals of European ancestry we identified five genetic loci that are associated with low VWF levels: *ABO*, *VWF*, *STXBP5, STAB2*, and *UFM1*.

The most significant signal in our study came from a well known determinant of VWF:Ag levels, the ABO locus. The presence of blood group A and B antigens on VWF molecules leads to a decreased clearance of VWF molecules. Consequently, individuals with blood group O have 25% lower VWF plasma concentrations than individuals with blood group non-O[35]. Although we used a different cut-off point for low VWF levels for blood group O and non-O separately to adjust for the effect of blood group, the ABO locus still reached a very high level of statistical significance. This implies that having either A or B antigens or both leads to different VWF levels. Indeed, carriers of the B antigen have higher VWF levels than carriers of the A antigen and carriers of both antigens have the highest VWF levels[36].

The second locus is within the VWF gene. It has been well established that common genetic polymorphisms in the VWF gene contribute to the variability in VWF:Ag levels[37-39]. The most significant SNP that marked the VWF locus was rs216303, which is located within an intronic region. Until recently intronic polymorphisms seemed irrelevant for disease development and regulating protein levels in plasma. However, there is now an increasing recognition that intronic variants do contribute by for example influencing the form and efficacy of gene splicing and mRNA stability[39]. Another possibility is that SNPs in the intronic regions are in high LD with other functional SNPs in adjacent regions.

The third locus is within the STXBP5 gene, which encodes the syntaxin binding protein 5. STXBP5 can bind to Soluble N-ethylmaleimide-sensitive factor (NSF) Attachment protein Receptor (SNARE) proteins, among which syntaxin-2 and syntaxin-4. Syntaxin-4 has been shown to be involved in Weibel Palade Body exocytosis[40], the well known mechanism for the secretion of VWF molecules from endothelial cells. We have previously shown in a well defined cohort of young patients with a first event of arterial thrombosis that genetic variation in *STXBP5* is associated with VWF:Ag levels[41]. The LD between rs1221638 and the SNP that had the highest significance in the previous meta-analysis is $D' = 0.90$ and $R^2 = 0.67$.

The fourth locus was marked by rs4981022, which is located in the STAB2 gene. Stabilin–2 is a transmembrane receptor protein and is primarily expressed in liver and spleen sinusoidal endothelial cells. STAB2 can bind various ligands, such heparin, LDL, bacteria, and advanced glycosylation products, and subjects them to endocytosis[42]. STAB2 has not shown to be involved in the regulation of VWF:Ag levels previously. However, if STAB2 is important in the regulation of VWF levels it would most likely involve the clearance of VWF molecules.

The final genome-wide significant locus was marked by rs17057285, which is close to the UFM1 gene. *UFM1* encodes the ubiquitin-fold modifier 1, which has been recently identified as a novel protein conjugating system[43]. UFM1 is highly expressed in the pancreatic islets of Langerhans and has a role in the development of type 2 diabetes. UFM1 has not yet been linked to VWF so far.

Four of the identified loci for low VWF levels (i.e. *ABO*, *VWF*, *STXBP5*, and *STAB2*) have previously shown to be involved in the regulation of VWF:Ag levels in general[44]. Although this finding was not entirely surprising, we expected to discover a number of other genetic loci as well, since mechanisms that lead to low VWF levels may differ from those that regulate the entire range of VWF levels. However, not all of the previously identified genetic loci came up in the current analysis and we found one additional novel locus. Indeed this suggests that mechanisms that lead to low VWF levels partly differ from those that regulate VWF levels in general, but also share some attributes.

UFM1 is the only novel genetic locus associated with low VWF levels. However, rs17057285, the SNP with the highest P-value that marked this locus, has a very small minor allele frequency of about 0.5% only. Therefore this finding should be interpreted with care and should await replication in independent samples.

Our results are one of the first steps towards improvement of diagnosis and treatment of individuals with low VWF levels. In today's clinical practice it is hard to distinguish between physiologically low VWF levels and VWF levels due to von Willebrand Disease (VWD), because VWF levels and bleeding symptoms are highly variable and occur both quite frequently in the general population. Until recently, it was believed that low VWF levels and VWD are caused by mutations in the VWF gene only. However, now it has been shown that 35% of type 1 (partial quantitative deficiency of VWF) VWD patients have no apparent *VWF* mutations[11-12, 45]. This suggests that genetic variations in genes other than *VWF* may lead to low VWF levels. Indeed, current findings show that next to ABO blood group and *VWF*, other genetic loci are involved in the occurrence of low VWF levels.

In the current study we have not included a replication cohort. Generally, it has been recommended to include all cohorts in the discovery panel to maximize statistical power, rather than use some of the cohorts for replication. In addition, the identified genetic loci comprise extremely small p-values and were previously discovered in the meta-analysis using VWF:Ag as a continuous measure. For these reasons it is very unlikely that our findings are false-positive or came out by chance.

In conclusion, we identified five genetic loci that are associated with low VWF levels: *ABO*, *VWF*, *STXBP5, STAB2*, and *UFM1*. Our findings confirm the hypothesis that genes other than *VWF* lead to low VWF levels. Further research is warranted in order to elucidate whether these genetic loci also contribute to the incidence of bleeding symptoms and VWD.

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SUPPLEMENTARY TABLES

Supplementary table 1 • VWF antigen assay used in each cohort

Cohort	Assay description
ARIC	Antigen was determined by an ELISA kit from American Bioproducts Co (Parsippany, NJ). The reliability coefficient obtained from repeated 39 testing of individuals over several weeks was 0.68, and the method CV was 18.5%. No reference range available.
B58C	Antigen was measured by ELISA assays that used a double-antibody sandwich (DAKO, Copenhagen, Denmark). The standard curve was constructed using the 9th British standard for Blood Cogulation Factors from National Institute for Biological Standards and Controls (NIBSC), South Mimms, Herefordshire UK, and the results were expressed as International units/decilitre (IU/dl). As a control, the pooled plasma of 20 healthy middle-aged persons was run on each ELISA plate. The intra-assay CV was 6%, the inter-assay CV was 8%, and reference range was 50 to 200 IU/dl.
FHS	The von Willebrand Factor was assessed using ELISA. In our laboratory, the intra-assay coefficient of variation was 8.8%. No reference range available.
LBC	The VWF antigen method is an in-house ELISA using reagents from Dako, High Wycombe, UK. The intra-assay coefficient of variation was 3.2% and inter-assay was 4.2%.
ORKNEY	Same as B58C
PROSPER	Von Willebrand factor antigen was measured with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies (DAKO, Copenhagen, Denmark). No reference range available.
PREVEND	Antigen was determined by an ELISA kit from Dade Behring of vWf antigen (Ag) was measured by immunoturbidimetric determination using the Dade Behring vWF:Ag test kit (Dade Behring Marburg GmbH, Marburg, Germany) using EDTA anticoagulated plasma.
RS	Von Willebrand factor antigen was measured with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies (DAKO, Copenhagen, Denmark). The intra-assay CV was 1.9%, inter-assay CV was 6.3%, and the reference range was 0.60-1.40 IU/ml.
VIS	Same as B58C

Supplemental Table 2 • Genotyping and imputation methods for autosomal chromosomes by study

	ARIC	B58C-WTCCC	B58C-T1DGC	FHS	LBC1921	LBC1936	ORCADES	PROSPER	PREVEND	RS	VIS
N	9257	1461	2484	2806	176	757	677	5047	3621	5558	766
Platform	Affymetrix	Affymetrix	Illumina	Affymetrix	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina
Chip	6.0	500K	550K (v3)	500K, MIPS 50K	Human 610_ Quadv1	Human 610_ Quadv1	HumanHap300 (v2)	660k (quad)	CytoSNP12 v2	V3 Illumina Infinium II HumanHap550	HumanHap300 (v1)
SNP exclusion criteria:											
MAF	< 1%	None	None	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%	< 1%
HWE p-value	$< 1.0 \times 10^{-5}$	None	None	$< 1.0 \times 10^{-6}$	$< 1.0 \times 10^{-3}$	$< 1.0 \times 10^{-3}$	$< 1.0 \times 10^{-6}$	$< 1.0 \times 10^{-5}$	$< 1.0 \times 10^{-3}$	$< 1.0 \times 10^{-5}$	$< 1.0 \times 10^{-6}$
Call rate	< 0.95	None	None	< 0.97	≤ 0.98	≤ 0.98	≤ 0.98	≤ 0.975	≤ 0.95	≤ 0.90	≤ 0.98
Total number SNPs	597,357	419,829	539,458	378,163	542,050	542,050	285,491	557,192	232,571	530,683	305,068
Imputation software	MACH	IMPUTE	MACH	MACH	MACH	MACH	MACH	MACH	Beagle	MACH	MACH
Imp. software version	1.0.16	0.2.0	1.0.16	1.0.15	1.0.16	1.0.16	1.0.16	1.0.15	3.3.2	1.0.15	1.0.15

Abbreviations used in this table are MAF for minor allele frequency and HWE for Hardy-Weinberg equilibrium

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

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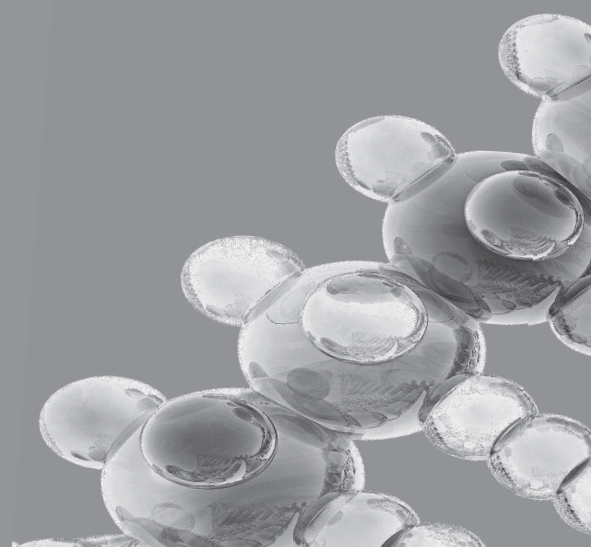
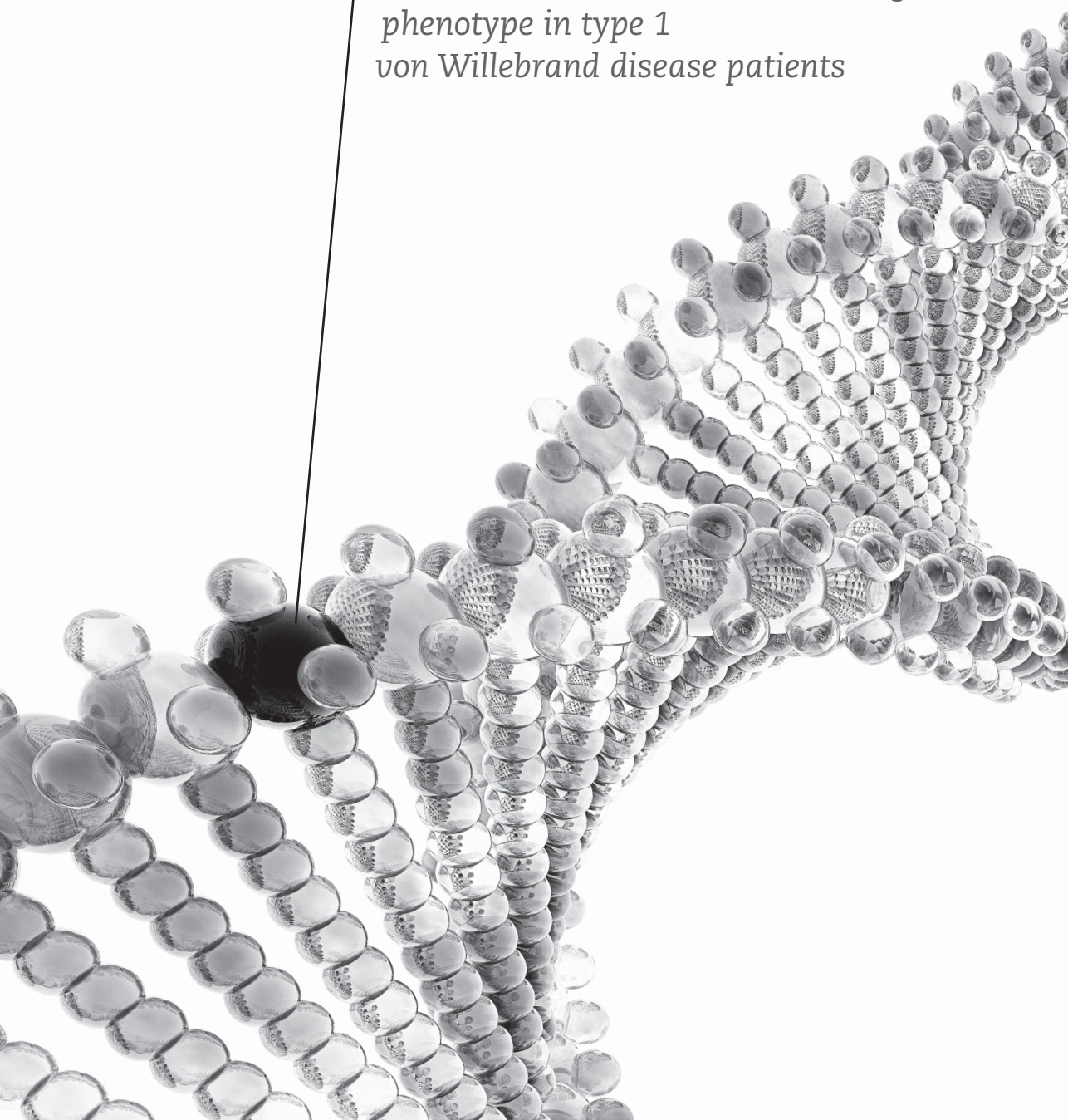


CHAPTER 8

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



ABSTRACT

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

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

Results. In *STX2*, *rs*7978987 was significantly associated with VWF:Ag levels (bèta-coefficient (β)=–0.04 IU/mL per allele, [95%CI –0.07;–0.001], p=0.04) and VWF:CB activity (β=–0.12 IU/mL per allele, [95%CI –0.17;–0.06], p<0.0001). For *rs*1039084 in *STXBP5* a similar trend with VWF:Ag levels was observed: (β=–0.03 IU/mL per allele [95% CI –0.06;0.003], p=0.07). In women, homozygous carriers of the minor alleles of both SNPs in *STXBP5* had a significantly higher bleeding score than homozygous carriers of the major alleles. (Rs1039084 p=0.01 and rs9399599 p=0.02).

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

INTRODUCTION

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

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

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

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

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

METHODS

Study Population

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

Ethics Statement

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

Laboratory measurements

Blood was drawn by venipuncture in the antecubital vein using Vacutainer system (Becton-Dickinson, Plymouth, UK). Blood for coagulation measurements was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within 1 hour at 2000 x g for 10 min at 4°C. Plasma was additionally centrifuged at 14,000 x g for 10 minutes at 4°C and stored in aliquots at –80°C. For DNA isolation blood was collected in tubes containing ethylene diaminetetraacetic acid (EDTA; Beckon Dickinson) and genomic DNA was isolated according to standard salting-out procedures and stored at 4°C for genetic analysis.

VWF:Ag was determined with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase conjugated anti-human VWF antibodies (DakoCytomation, Glostrup, Denmark) for catching and tagging, respectively. For our analyses we used the lowest VWF:Ag level that was ever measured in a patient (historical VWF:Ag measurement).

VWF collagen binding (VWF:CB) was measured with an in-house ELISA using type I collagen (Sigma, St. Louis, USA) for catching and horseradish peroxidase conjugated anti-human VWF antibodies for tagging.

VWF:RCo was assayed with formalin-fixed platelets using a PAP4 Model Aggregometer (Bio-Data Corp. Hatboro, Pennsylvania) according to Macfarlane et al[8].

Bleeding score

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

Genotyping analysis

The *STXBP5* gene spans 182 kbps and is located in the q24 region of chromosome 6. The *STX2* gene spans 50 kbp and is located in the q24.3 region of chromosome 12. Initially, we obtained data from the International HapMap project (phase II November 2008 http://www.hapmap.org) on the linkage disequilibrium (LD) pattern and selected haplotype-tagging single-nucleotide polymorphisms (ht-SNPs) using Haploview software (version 3.11; www.broad.mit.edu/mpg/haploview/index/php). For both genes blocks of haplotypes with a frequency of ≥ 3% were defined in order to select ht-SNPs. We took potential functionality into consideration by preferentially selecting non-synonymous ht-SNPs or SNPs that are located in known regulatory elements. We considered only SNPs that were present in a Caucasian population. Of these ht-SNPs, three were significantly associated with VWF:Ag levels in our previous study among young patients with arterial thrombosis and healthy controls[7]. Therefore, we selected and genotyped only these three SNPs in *STXBP5* (*rs*1039084 and *rs*9399599) and in *STX2* (*rs*7978987) for our current study using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The polymorphisms in *STXBP5*, rs1039084 and rs9399599, are in high linkage disequilibrium with *rs*9390459, which had the highest genome wide significance level for VWF plasma levels in the meta-analysis of the CHARGE consortium ($D' = 1.00$, $R^2 = 0.87$ for *rs*9399599 and $D' = 0.96$, $R^2 = 0.86$ for *rs*1039084) (phase II November 2008 http://www.hapmap.org). Also, rs7978987 in *STX2* had a highly significant *P* value of 3.82×10^{-11} in this meta-analysis. Endpoint fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, CA, USA) and clustered according to genotype using SDS 2.1 software (Applied Biosystems, Foster City, CA, USA). Genotyping was successful for each SNP in on average 96% of all subjects.

Statistical analysis

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

The effect of genetic variation on the bleeding score has been assessed by ANCOVA adjusted for the age at the time of completing the questionnaire. Since the bleeding score differs significantly between men and women, we stratified this analysis by sex additionally.

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

RESULTS

Baseline characteristics

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

Table 1 • Baseline characteristics of the study population

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

Summary statistics for continuous variables are presented as mean ± standard deviation. Categorical data are summarized as percentages. Abbreviations used in this table are VWF for Von Willebrand Factor and VWD for Von Willebrand Disease.
The P value represents the difference between sexes for each variable.

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

In *STX2*, *rs*7978987 (intron 9) was significantly associated with VWF:Ag levels (beta-coefficient (β) = –0.04 IU/mL per allele, [95%CI –0.07;–0.001], p = 0.04) and VWF:CB activity (β = –0.12 IU/mL per allele, [95%CI –0.17;–0.06], p < 0.0001), also after adjustment for blood group (p = 0.04 for VWF:Ag and p < 0.0001 for VWF:CB) (table 2). Interestingly, the frequency of the minor allele (MAF) of rs7978987, which corresponded with higher VWF levels, was lower among our patients with type 1 VWD (MAF = 0.29) than reported

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

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

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

by dbSNP (http://www.ncbi.nlm.nih.gov/ projects/SNP/) (MAF = 0.38) and in the meta-analysis of the CHARGE consortium (MAF = 0.35)[4].

For *rs*1039084 in *STXBP5*, which is a missense mutation that encodes an amino acid substitution of asparagine into serine at position 436 a similar trend with VWF:Ag levels was observed: (β = –0.03 IU/mL per allele [95% CI –0.06;0.003], p = 0.07) (table 2). *Rs*9399599 in STXBP5, which is located in intron 25, was not associated with VWF:Ag levels nor with VWF:CB, though carriers of the minor allele had lower VWF:Ag levels and VWF:CB activity as was also observed for rs1039084. Rs1039084 and rs9399599 have a strong linkage disequilibrium of D' = 0.88 and R^2 = 0.71. Haplotype analysis showed that both SNPs contribute to the variation in VWF:Ag levels (figure 1).

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

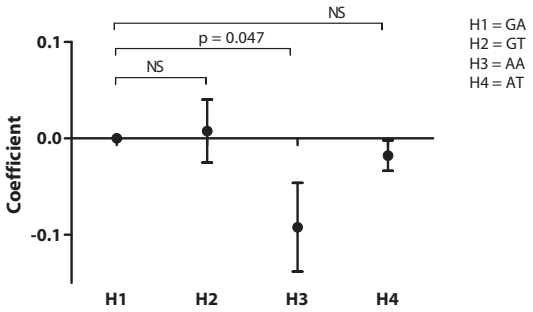


Figure 1 • Haplotype analysis for rs1039084 and rs9399599
Graph presents the coefficients with standard error per haplotype. Haplotype 1 was used as reference haplotype.
NS = not significant

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

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

DISCUSSION

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

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

SNPs in *STXBP5* and *STX2* have been previously identified in the meta-analysis of the CHARGE consortium as determinants of VWF levels in the general population. The effect sizes we obtained were comparable with previous studies[4,7]. In our study, the selected SNPs are the same or are in high linkage disequilibrium with those identified in the CHARGE meta-analysis. Though the selected SNPs in *STXBP5* are in high LD (D' = 0.88 and R^2 = 0.71) the effect estimate of rs1039084 was slightly higher than for rs9399599.

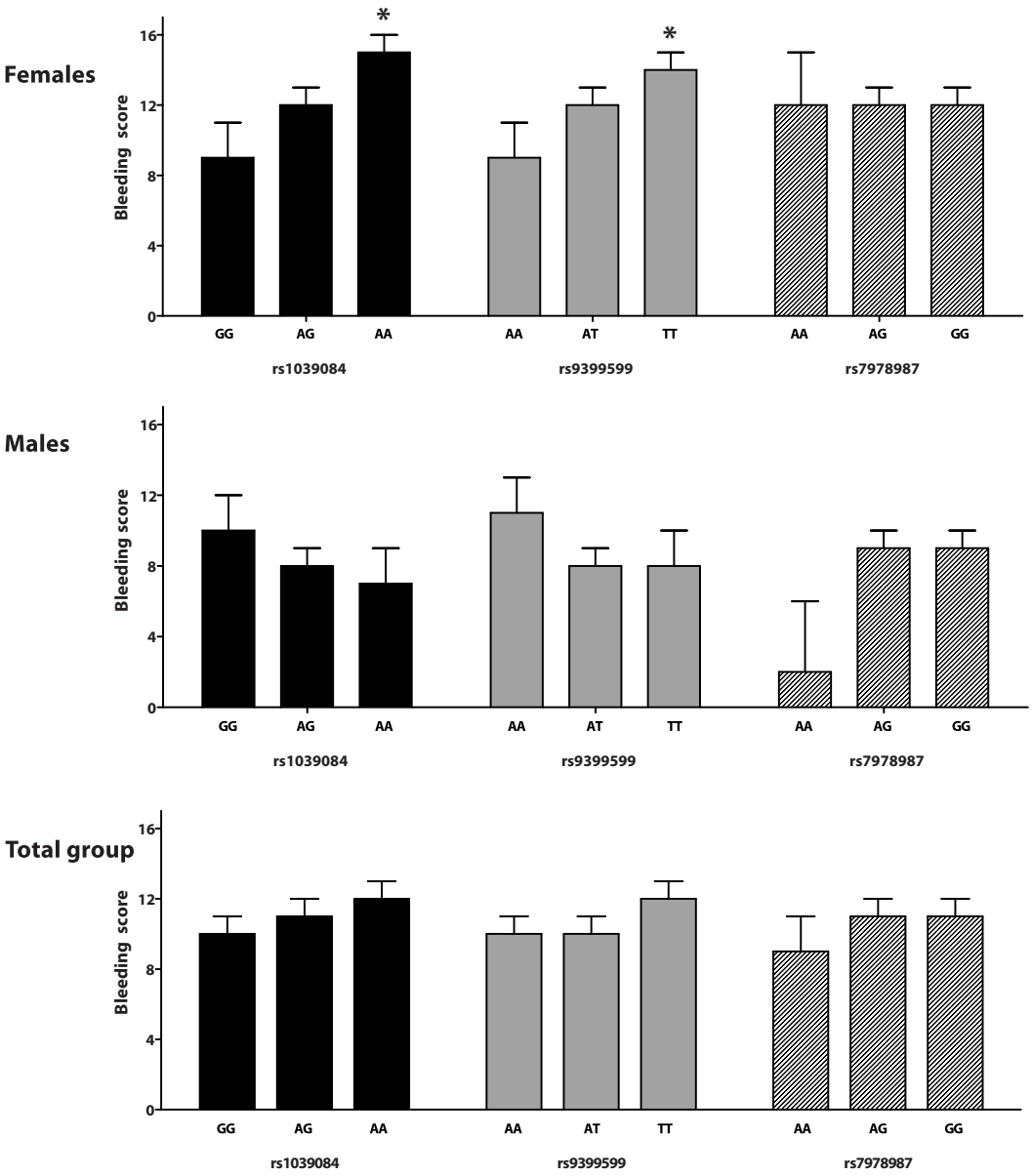


Figure 2 • Bleeding score.
Bleeding score per genotype for each SNP for the total group and stratified by sex.
* $P < 0.05$

Haplotype analysis showed that both SNPs have an independent effect on VWF:Ag levels. It can be anticipated that since rs1039084 is a non-synonymous SNP and rs9399599 is intronic, rs1039084 may be the actual causal variant with a greater effect.

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

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

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

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

In today's clinical practice diagnosis of type 1 VWD is difficult, because of the high variability in VWF plasma levels and the incomplete penetrance of the phenotype. In

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

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

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

ACKNOWLEDGEMENTS

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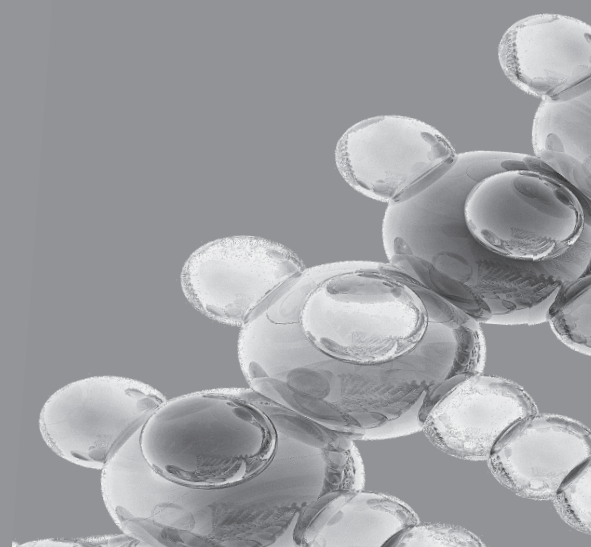
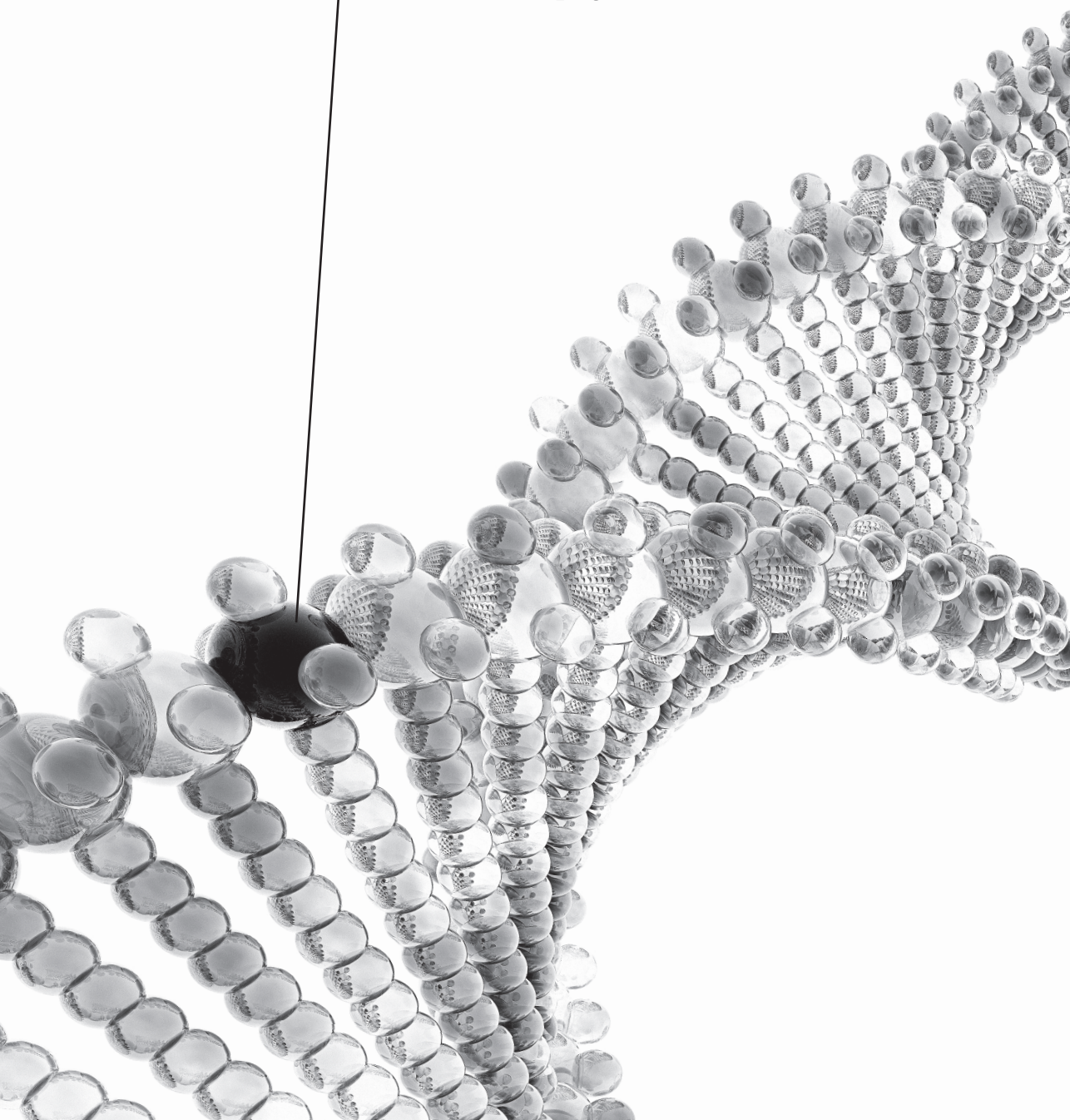
JE van Loon, MAH Sonneveld, S Praet,
MPM de Maat, FWG Leebeek

submitted



CHAPTER 9 *Physical fitness determines the von Willebrand Factor response to exhaustive physical exercise*



ABSTRACT.

Background. Stress triggers the endothelium to release von Willebrand Factor (VWF) from the Weibel Palade bodies. Since VWF is a risk factor for arterial thrombosis, it is of great interest to discover important determinants of VWF response to stress. We aimed to investigate the effect of heavy physical exercise on VWF levels and to identify important mediators herein.

Methods. 105 healthy individuals (18-35 years) free from cardiovascular risk factors were included in this study. Each participant performed an incremental exhaustive exercise test on a cycle ergometer. Gas exchange measurements were obtained and the cardiac function was continuously monitored. Blood was drawn at baseline and directly after exhaustion. VWF antigen levels (VWF:Ag) were determined with an in-house ELISA. DNA was isolated for genotyping of common genetic variations in two important genetic determinants of VWF:Ag levels: Syntaxin Binding Protein-5 (*STXBP5*) and Syntaxin-2 (*STX2*) genes.

Results. The median VWF:Ag level at baseline was 0.94 IU/mL [IQR 0.8-1.1] and increased with 47% [IQR25-73] after exhaustive exercise to a median maximum VWF:Ag of 1.38 IU/mL [IQR1.1-1.8] ($p<0.0001$). The strongest performance related determinants ($p<0.0001$) were the peak power output per kg (β=0.6 [95%CI 0.4;0.7]), the ratio between the power output at the ventilatory threshold and the peak power output (β=-2.6 [95%CI -4.00;-1.2]), VO_2 max per kg (β=0.05 [95%CI 0.03;0.06])and the maximum respiratory exchange ratio (β=4.3 [95%CI 2.4;6.0]). We observed a gender difference in VWF:Ag response to exercise (females 1.2 IU/mL [1.1;1.6] and males 1.7 IU/mL [1.2;2.1], p=0.001), which was caused by a difference in performance. Genetic variations in *STXBP5* and *STX2* were not associated with VWF:Ag levels at baseline, at exhaustion, nor associated with the absolute VWF:Ag increase.

Conclusions. The increase of VWF:Ag levels upon exhaustive exercise is strongly determined by physical fitness and the intensity of the exercise, rather than by genetic variations in *STXBP5* and *STX2*. Our findings further strengthen the hypothesis that regular physical exercise leads to a beneficial phenotype that may protect against cardiovascular disease.

INTRODUCTION

Blood coagulation changes in response to physical exercise[1-2]. One of the major players in blood coagulation is von Willebrand factor (VWF), a multifunctional glycoprotein that initiates primary haemostasis. It is well known that levels of VWF increase steeply upon intense physical exercise[3]. To date it is not fully understood what mediators, both non-genetic and genetic, affect VWF response to stress. However, it is of great interest to discover new determinants of the excretion mechanism of VWF molecules, since high VWF levels have been associated with venous thrombosis[4] and arterial thrombosis[5-7].

VWF is mainly synthesized by endothelial cells and marks endothelial cell activation[8-9]. The majority of the freshly synthesized VWF molecules are constitutively released into the circulation. A small part of especially large VWF multimers – harbouring the greatest haemostatic potential – is stored in Weibel Palade Bodies of endothelial cells[10-13]. Numerous agonists initiate the release from these storage granules, including hypoxia, epinephrine, histamine, thrombin, fibrin, and vasopressin[14-15].

Plasma VWF levels have a wide biological variation, since numerous lifestyle factors environmental factors, and genetic factors continuously influence VWF levels in the circulation[15]. Previous studies among human twins have demonstrated that more than half of the variability in VWF levels is caused by genetic variations in the genome[16-17]. The most important genetic determinant is ABO blood group[18]. In addition, recently six new genetic loci have been discovered using a hypothesis-free approach with genome-wide association studies[19]. Two of the newly identified genetic loci, Syntaxin Binding Protein-5 (*STXBP5*) and Syntaxin-2 (*STX2*), are of specific interest, since their encoding proteins interact with SNARE complex proteins, such as SNAP23 and syntaxin-4, which have been shown to be involved in Weibel Palade Body (WPB) exocytosis, a well known mechanism for the secretion of VWF molecules by endothelial cells[20].

We aimed to identify important mediators, including lifestyle factor, environmental factors, and common genetic variations in the *STXBP5* and *STX2* genes, of VWF response to incremental exhaustive exercise in a large group of young healthy individuals.

METHODS

Study participants

For the "RESPOnse" (Role of SNARE protein genes in the regulation of Von Willebrand Factor concentration and other coagulation factors) study we included 105 healthy individuals, who were between 18 to 35 years of age and of North-European ancestry. All participants were either students or employee at the Erasmus University Medical Center Rotterdam. Exclusion criteria were known cardiovascular risk factors, including

hypertension, hypercholesterolemia, diabetes, obesity (BMI > 30 kg/m^2), and a positive family history of cardiovascular disease. In addition, participants never had a thrombotic event or coagulation disorder, were non-smokers, had no known malignancies, no liver or renal dysfunction, did not use medication that may influence VWF levels and were not pregnant. Oral contraceptives use was allowed in this study. Subjects were requested to abstain from caffeinated beverages twelve hours prior to the test and to avoid heavy exercise on the day of the test. The study was approved by the medical research board at Erasmus University Medical Center and written informed consent was obtained from all participants at inclusion.

Baseline measurements

At baseline all patients received a questionnaire on current health status and physical condition. We measured height, length, and blood pressure. Also, before the start of the cycle ergometer test, we performed a rest electrocardiogram (ECG) to exclude abnormalities in electric conduction true the heart, arrhythmias etc. All participants declared to be in good health and none of them had medical contraindications for participation in the study.

Cycle ergometer maximal test

Each participant performed an incremental exercise test until exhaustion, performed on a cycle ergometer (Ergoline, ER800, Lode, the Netherlands) using a linearly increasing (12 or 18.5 W min-1) ramp protocol. These slopes were chosen to achieve exhaustion within 8-12 minutes as recommended by Zhang et al[21]. Participants started with a warming-up phase of 4 minutes without resistance. They were instructed to pedal at a frequency between 60 and 80 rotations per minute (rpm). The loaded phase was terminated when pedalling frequency dropped below 60 rpm and was followed by cooling-down for at least 2 minutes.

Cardiac function was monitored using a 12-lead electrocardiogram with heart rate (HR) being recorded continuously. Gas exchange measurements were performed continuously by using a computerized metabolic cart (Oxycon Pro, Carefusion, the Netherlands) that was calibrated before each test. Maximal whole-body oxygen uptake capacity was defined in the present study as VO_2 remaining unchanged or increasing less than 1 ml/min/kg for 30 sec or more despite an increment in work load[22]. VO_2 is the capacity to transport and use oxygen during exercise and is a measure for physical fitness. The ventilatory threshold (VT1) was determined by an increase in ventilation (Ve)/VO_2 but without a concomitant increase in Ve/VCO_2. The ventilatory threshold represents the moment at which metabolism changes from aerobic to anaerobic. Two experienced exercise physiologists reviewed the plots averaged over 30 sec of the Ve/VO_2 and Ve/VCO_2 and determined VT1 values. In case of disagreement, the opinion of

a third investigator was sought. The power output (W) was assessed to establish the workload capacity of the participant. Power output is dependent on physical fitness, but also on talent. Finally, maximum respiratory exchange ratio (RER) was determined. The RER is a ratio between the amount of carbon dioxide (CO_2) exhaled and the amount of oxygen (O^2) inhaled per breath. The maximum RER is a measure for the intensity of the test.

Blood sampling

Blood was drawn before and directly after exhaustion (within 1 minute) via a cannula in the antecubital vein using a Vacutainer system (Becton-Dickinson, Plymouth, UK). Blood for coagulation measurements was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within 1 hour at 3500 rpm for 10 min at 4°C. Plasma was additionally centrifuged at 14 000 rpm at room temperature and stored in aliquots at –80°C. For DNA isolation we stored the buffy coats of the remaining citrated blood at –20°C until use. Genomic DNA was isolated according to standard salting-out procedures and stored at 4°C for genetic analysis.

Laboratory measurements

VWF antigen (VWF:Ag) was determined with an in-house ELISA with polyclonal rabbit anti-human VWF antibodies and horseradish peroxidase conjugated anti-human VWF antibodies (DakoCytomation, Glostrop, Denmark) for catching and tagging, respectively.

Genotyping

The STXBP5 gene spans 182 kbps and is located in the q24 region of chromosome 6. Initially, we obtained data from the International HapMap project (phase II November 2008 http://www.hapmap.org) on the linkage disequilibrium (LD) pattern and selected haplotype-tagging single-nucleotide polymorphisms (ht-SNPs) using Haploview software (version 3.11; www.broad.mit.edu/mpg/haploview/index/php). For both genes blocks of haplotypes with a frequency of ≥ 3% were defined in order to select these ht-SNPs. We took potential functionality into consideration by preferentially selecting non-synonymous ht-SNPs or SNPs that are located in known regulatory elements. We considered only SNPs that were present in a Caucasian population. Of these ht-SNPs, three were significantly associated with VWF:Ag levels in our previous study among young patients with arterial thrombosis and healthy controls[23]. Therefore, we selected and genotyped only these three SNPs in *STXBP5* (rs1039084 and rs9399599) and in *STX2* (rs7978987) for our current study using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The polymorphisms in *STXBP5*, rs1039084 and rs9399599, are in high linkage disequilibrium with rs9390459, which had the highest genome wide significance level for VWF plasma levels in the meta-analysis of the CHARGE consortium

(D' = 1.00, R^2 = 0.87 for rs9399599 and D'= 0.96, R^2 = 0.86 for rs1039084) (phase II November 2008 http://www.hapmap.org). Also, rs7978987 in STX2 had a highly significant P value of 3.82 x 10^{-11} in this meta-analysis[19]. Endpoint fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, CA, USA) and clustered according to genotype using SDS 2.1 software (Applied Biosystems, Foster City, CA, USA). Genotyping was successful for each SNP in on average 98% of all subjects.

Statistical Analysis

Data on baseline characteristics are presented as means and standard deviations for continuous variables and as counts and percentages for categorical data. Since VWF:Ag levels were skewed, these data were natural logarithmically transformed (lnVWF:Ag) and presented as median and interquartile range (IQR). Mann-Whitney tests were used for unpaired two-group comparisons of non-parametric data.

The association between baseline characteristics and performance related determinants with VWF:Ag increase were assessed with linear regression models. Beta-coefficients represent the increase of lnVWF:Ag per unit increase of the selected variable. Also, logistic regression was used to assess the relationship between VWF:Ag response and baseline characteristics, using VWF:Ag response as categorical variable. VWF:Ag increase was divided into two categories: low response with a VWF:Ag increase below the median (<0.40 IU/mL) and high response with a VWF:Ag increase above the median (≥ 0.40 IU/mL). Gender differences in performance-related determinants were assessed with univariate analysis of variance (ANOVA). The power output and the VO_2 were adjusted for weight and presented as the value per kg.

Allele frequencies of the VWF polymorphisms were calculated by genotype counting. For each SNP, the deviation from the Hardy-Weinberg equilibrium was tested by means of a Chi-squared test with one degree of freedom. We used linear regression analyses with additive genetic models to determine the association between genetic variations in VWF and lnVWF:Ag levels. We had a power of 0.80 to detect a difference of 0.2 between carriers of the minor allele and carriers of the common allele, assuming a minor allele frequency of 0.40. Beta-coefficients represent the increase in lnVWF:Ag levels per coded allele. Statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS Inc, Chicago, USA). A two-sided value of $p < 0.05$ was considered statistically significant.

RESULTS

In de current study 105 healthy individuals were included. Baseline characteristics are shown in table 1. The mean age was 24 years and 61% were female. Blood pressure (mean 114/70 mmHg) and BMI (mean 23 kg/m^2) were within the normal range. Of all

Table 1 • Baseline characteristics

	N = 105
Age (years)	24.3 ± 4.4
Female sex, N (%)	64 (61%)
Systolic blood pressure (mmHg)	114 ± 12
Diastolic blood pressure (mmHg)	70 ± 9
Heart rate (bpm)	76 ± 10
BMI (kg/m^2)	23 ± 2
Oral contraceptives	33 (52%)
Blood group O, N (%)	54 (51%)
VWF:Ag baseline (IU/mL), median (IQR)	0.94 (0.77-1.12)
VWF:Ag maximum (IU/mL), median (IQR)	1.38 (1.10-1.84)
VWF:Ag absolute increase (IU/mL), median (IQR)	0.40 (0.23-0.70)
VWF:Ag relative increase (%), median (IQR)	47 (25-73)

Summary statistics for continuous variables are presented as mean ± standard deviation. Categorical data are summarized as percentages. VWF:Ag levels are presented as median and interquartile range (IQR).

subjects, 86 (82%) used alcoholic beverages of whom 25 subjects (24%) drink five or more glasses per week, and 5 subjects (5%) drink fifteen or more glasses per week. Of all female participants 33 women (52%) used oral contraceptives, seven women (11%) had an intra-uterine device (MirenaÒ), and two women (3%) had a Nuva ringÒ .

The median VWF:Ag level at baseline was 0.94 IU/mL and increased with 47% after exhaustive exercise to a median maximum VWF:Ag of 1.38 IU/mL ($p < 0.0001$) (table 1). Baseline characteristics were not associated with baseline VWF:Ag levels (table 2). As expected, blood group non-O was associated with higher baseline VWF:Ag levels

Table 2 • Association between baseline characteristics and VWF:Ag levels

	Baseline VWF:Ag	**P-value**	**Absolute VWF :Ag increase**	**P-value**
	β [95% CI]		β [95% CI]	
Age	1.01 [1.00; 1.02]	0.18	0.97 [0.94;1.01]	0.14
Male sex	0.98 [0.87;1.11]	0.73	2.20 [1.72;2.83]	< 0.0001
Systolic blood pressure	1.00 [0.99;1.01]	0.93	1.02 [1.00;1.03]	0.02
Diastolic blood pressure	1.00 [0.99;1.00]	0.21	1.02 [1.00;1.03]	0.04
BMI	1.00 [0.97;1.02]	0.90	1.02 [0.95;1.08]	0.60
Alcohol consumption	0.90 [0.77;1.05]	0.18	0.70 [0.48;1.01]	0.06
Blood group non-O	1.32 [1.19;1.48]	< 0.0001	0.90 [0.68;1.21]	0.48
Oral contraceptives	0.96 [0.81;1.15]	0.67	0.79 [0.55;1.15]	0.22

Univariate linear regression analysis, beta-coefficient (β) represents the increase of VWF:Ag levels with 95% confidence interval per unit increase of the selected variable.

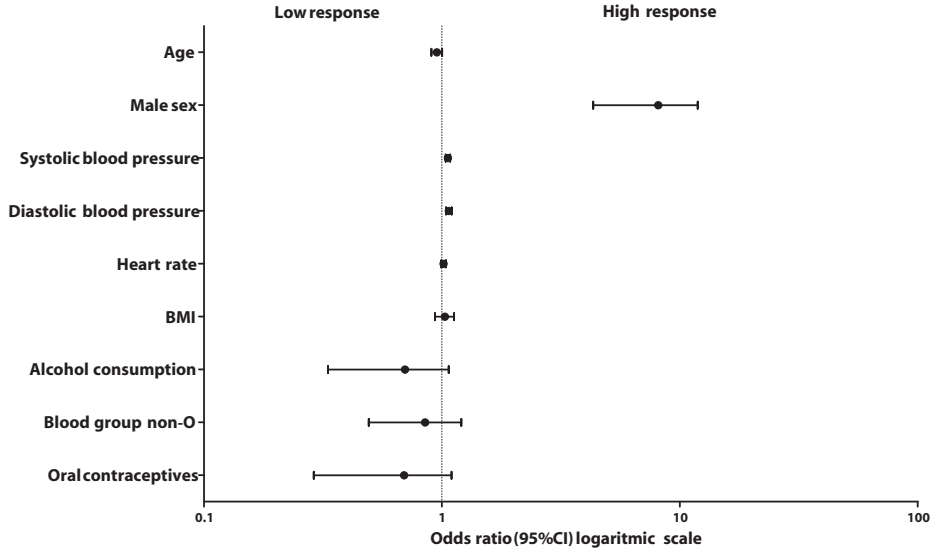


Figure 1 • Odds ratios with 95% confidence intervals for having a high von Willebrand Factor response (≥ 0.40 IU/mL) for each variable on the y-axis.

(geometric mean of VWF:Ag for blood group O: 0.82 ± 0.03 IU/mL and for non-O: 1.08± 0.03 IU/mL). Of all baseline characteristics, sex, systolic blood pressure, and diastolic blood pressure were significantly associated with the VWF:Ag level response (table 2 and figure 1). Alcohol consumption, defined as any amount of glasses per week, was borderline significantly associated with a lower VWF:Ag response ($p = 0.06$). The strongest determinant was sex. In a multivariate model including sex, blood pressure, and alcohol consumption, only sex remained significantly associated with VWG:Ag levels (beta-coefficient for males 2.29 [95% CI 1.70;3.10], $p < 0.0001$).

At baseline we observed no difference between males and females (median [IQR]: females 0.95 IU/mL [0.78;1.12] and males 0.96 IU/mL [0.74;1.11], $p = 0.50$). At exhaustion males had significantly higher VWF:Ag levels than females (females 1.22 IU/mL [1.06;1.58] and males 1.66 IU/mL [1.24;2.05], $p = 0.001$). OAC use was not associated with the VWF:Ag level response to exercise.

Next, we investigated the association between performance related determinants and the VWF:Ag response (table 3). All performance-related determinants were significantly associated with the increase in VWF:Ag levels, but not with the baseline VWF:Ag levels. The strongest performance related determinants ($p < 0.0001$) were the peak power output per kg, the ratio between the power output at VT1 and the peak power output, VO_2 max per kg and the maximum RER. In addition, a multivariate regression was performed. However, strong multicollinearity was observed between all performance-related variables. Therefore, we selected variables that showed the least correlation with each other

Table 3 • Performance-related determinants of VWF:Ag increase

	Univariate β [95% CI]	Multivariate Model I β [95% CI]	Multivariate Model II β [95% CI]
Peak Power Output per kg	0.57 [0.42;0.72]*		0.51 [0.36;0.65]*
Watts per kg VT1	0.42 [0.17;0.66]†		
Watts VT1/ Watts max	-2.57 [-3.95;-1.20]*	-1.02 [-2.36;0.33]	
VO_2 max per kg	0.05 [0.03;0.06]*	0.04 [0.03;0.06]*	
VO_2 VT1 per kg	0.03 [0.01;0.05]†		
VO_2 VT1 / VO_2 max	-2.14 [-3.55;-0.72]†		-0.41 [-1.66;0.83]
Max Respiratory Exchange Ratio	4.29 [2.40;6.00]*	2.52 [0.79;4.25]†	2.49 [0.90;4.07]†
Test duration	0.09 [0.03-0.15]†		

Linear regression analysis with natural log VWF:Ag as dependent. Beta-coefficient represents the increase in VWF:Ag per unit increase of the selected variable. $p < 0.0001$, † $p < 0.01$, ‡ $p < 0.05$.*

for the multivariate model. To this end, we used two models with different combinations of variables. Nevertheless, multicollinearity could not be excluded completely, whereby the results of the multivariate regression analysis should still be interpreted with care. Next, we added sex to model I and model II, which resulted in the lost of the significant association between sex and VWF:Ag increase (Model I: β = 1.31 [95% CI 0.96;1.79], p = 0.09; model II: β = 1.26 [95% CI 0.92;1.72], p = 0.15). In table 4 the performance-related values are presented for males and females separately. Physical fitness, as represented by the VO_2 VT1/max and power output VT1/max, was slightly higher in females than in males. The intensity of the test and the endurance of the participant, as represented by the VO2 max, peak power output, and the maximum RER, were higher in males than in females. There was no difference in test duration between males and females.

Table 4 • Gender differences in performance-related determinants

	Males N = 41	Females N = 64	P-value
Peak power output per kg (Watts/kg)	4.4 ± 0.1	3.4 ± 0.1	< 0.0001
Power output per kg VT1 (Watts/kg)	2.8 ± 0.1	2.3 ± 0.1	< 0.0001
Power output VT1 / max (%)	63 ± 2	67 ± 1	0.04
VO_2 max per kg (mL/min/kg)	50.2 ± 1.0	39.2 ± 0.8	< 0.0001
VO2 VT1 per kg (mL/min/kg)	33.9 ± 0.9	27.9 ± 0.7	< 0.0001
VO_2 VT1 / max (%)	68 ± 2	72 ± 1	0.051
Maximum respiratory exchange ratio	1.18 ± 0.01	1.13 ± 0.01	0.001
Test duration	18.1 ± 0.4	18.2 ± 0.3	0.91

Univariate analysis. Data are presented as means ± standard error.

Finally, we investigated the association between genetic variations in *STXBP5* and *STX2* on VWF:Ag response to exercise (table 5). There was no significant association between genetic variations and VWF:Ag levels at baseline, at exhaustion, nor an association with the absolute VWF:Ag increase. Also, ABO blood group, the most important genetic determinant of VWF:Ag levels, was not associated with the VWF:Ag level response to exercise.

Table 5 • VWF:Ag levels per genotype of polymorphisms in *STXBP5* and *STX2*

	N	Baseline (IU/mL)	Exhaustion (IU/mL)	Absolute difference (IU/mL)
rs1039084				
GG	33	0.94 ± 0.06	1.45 ± 0.08	0.52 ± 0.06
AG	44	1.02 ± 0.05	1.56 ± 0.07	0.54 ± 0.05
AA	25	0.96 ± 0.06	1.42 ± 0.10	0.46 ± 0.06
P for trend		0.44	0.44	0.67
rs9399599				
AA	32	0.89 ± 0.06	1.40 ± 0.09	0.51 ± 0.06
AT	45	1.05 ± 0.05	1.59 ± 0.07	0.54 ± 0.05
TT	25	0.96 ± 0.06	1.41 ± 0.10	0.45± 0.06
P for trend		0.11	0.18	0.56
rs7978987				
GG	48	0.98 ± 0.05	1.43 ± 0.07	0.45 ± 0.05
AG	45	0.98 ± 0.05	1.55 ± 0.07	0.57 ± 0.05
AA	12	0.94 ± 0.09	1.42 ± 0.14	0.47 ± 0.09
P for trend		0.94	0.43	0.18

DISCUSSION

In this large study among 105 healthy young subjects, VWF:Ag levels increased significantly upon incremental exhaustive exercise with a median increase of 47%. The VWF:Ag response was highly variable and was dependent on performance-related and physical fitness-related determinants. Neither baseline characteristics nor the studied genetic determinants of VWF:Ag levels affected the extent of VWF:Ag response to physical exercise.

For a long time it has been anticipated that regular physical exercise has a favourable effect on many biological mechanisms, thereby improving health and fitness. Regular physical exercise is associated with a decreased all-cause mortality and with a reduced cardiovascular risk[24]. The positive effects of physical exercise on cardiovascular disease development may be induced by alterations in haemostasis. Paradoxically, the short-term effect of exercise is associated with hypercoagulability, though especially in

sedentary individuals, whereas the chronic effect of exercise leads to a hypocoagulable state. This hypocoagulable state is achieved by a compensatory exhaustion of platelets in physically active individuals and underlines the beneficial effects of exercise on long-term prevention of cardiovascular disease[25]. Indeed, in our study, the VWF:Ag levels increase upon exercise was the highest in individuals with the lowest physical fitness and in individuals with the most intensive exercise. The baseline VWF:Ag levels were not associated with physical fitness or intensity of the test. This observation is in line with previous findings that showed that levels of coagulation factor VII (FVII), VWF, and FVIII are not increased in professional athletes at rest[26]. The lower VWF:Ag response in well-trained individuals underlines the hypothesis that frequent physical exercise has a positive effect on cardiovascular risk.

STXBP5 and *STX2* are two novel genetic loci that have been associated with VWF:Ag levels in the general population[19]. In addition, genetic variation within these genes affects VWF:Ag levels in young patients with a first event of arterial thrombosis[23]. Considering the involvement of the *STXBP5* and *STX2* encoding proteins in the regulated secretion of VWF molecules, our hypothesis was that genetic variants within these genes would affect the release of VWF molecules, but not the steady state levels, which are determined by the constitutive pathway. To this end, we included young individuals below the age of 35 years to exclude the presence of extensive atherosclerosis, which is related to endothelial dysfunction and consequent higher VWF:Ag levels. Their baseline VWF:Ag levels therefore represent a steady state situation. To provoke release of VWF molecules from Weibel Palade Bodies in our study, all participants performed heavy physical exercise, which induces beta-adrenergic receptor activation[27] and subsequent endothelial cell activation. In line with our expectation, genetic variation in *STXBP5* and *STX2* was not associated with VWF:Ag levels at baseline, though we had sufficient power to detect the previously observed effect of these genetic variants. However, the VWF:Ag increase upon exercise was also not affected by genetic variation in *STXBP5* and *STX2* . This finding was in contrast to our hypothesis. Possibly genetic variations in *STXBP5* and *STX2* are indeed not involved in WPB exocytosis or their effect was smaller than we could detect with our sample size. Furthermore, we observed that the VWF:Ag increase was highly variable and strongly dependent on physical fitness and the intensity of the exercise performed. Consequently, the effect of environmental factors may have been stronger than the genetic effect. Finally, detecting a difference in VWF:Ag increase is difficult per definition, since this variable is subjected to regression to the mean.

Another important genetic determinant of VWF:Ag levels is blood group. Individuals with blood group O have 25% lower VWF:Ag levels than individuals with blood group non-O, because the presence of blood group A and B antigens on VWF molecules leads to a decreased clearance of VWF molecules[28]. Furthermore, blood group non-O has been associated with an increased risk of CHD[29-31]. Ribeiro et al. observed that males

with blood group non-O (N = 8) had higher post-exercise VWF:Ag levels than males with blood group O (N = 8), though the delta VWF:Ag was not statistically significantly different between the groups[32]. In our study, subjects with blood group non-O had higher levels at exhaustion (median [IQR], 1.60 IU/mL [1.2-2.0]), than subjects with blood group O (1.29 IU/mL [1.1-1.6], p = 0.03). However, blood group was not associated with the VWF:Ag increase upon exercise (0.49 IU/mL [0.3-0.7] in non-O versus 0.46 [0.2-0.7] in O, p = 0.34). Assuming that exercise induces the release of VWF molecules from its storage granules and the clearance does not change during exercise, it was expected that subjects with blood group O and subjects with blood group non-O had a similar increase after exercise.

In conclusion, we have shown in a large and homogeneous group of young healthy individuals that VWF:Ag levels increase strongly upon exhaustive physical exercise and is primarily dependent on physical fitness and the intensity of the exercise performed. Genetic variations in *STXBP5* and *STX2* that have previously been identified as important genetic determinants of VWF:Ag levels were not associated with the VWF:Ag response to physical exercise. Also, ABO blood group, the most important genetic determinant of VWF:Ag levels was not associated with the VWF:Ag increase. These findings suggest that environmental factors may be superior to genetic factors in determining the VWF:Ag response to stress. Finally, the results of this study further strengthen the hypothesis that regular physical exercise leads to a beneficial phenotype that may protect against cardiovascular disease.

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JE van Loon, PPT de Jaegere, H Ulrichts,
HHDM van Vliet, MPM de Maat, PhG de Groot,
ML Simoons, FWG Leebeek

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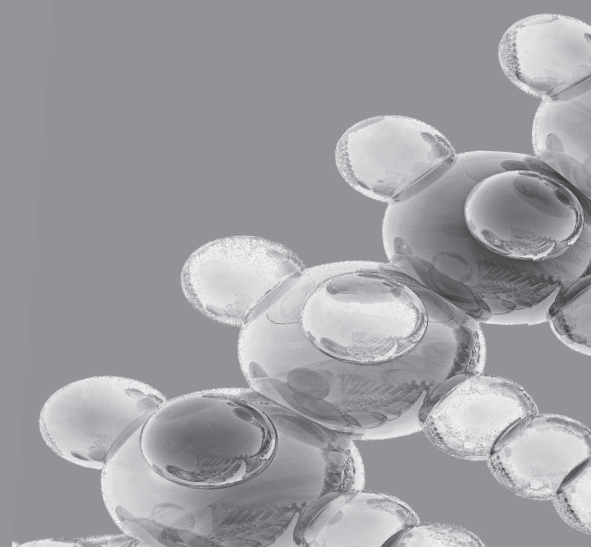
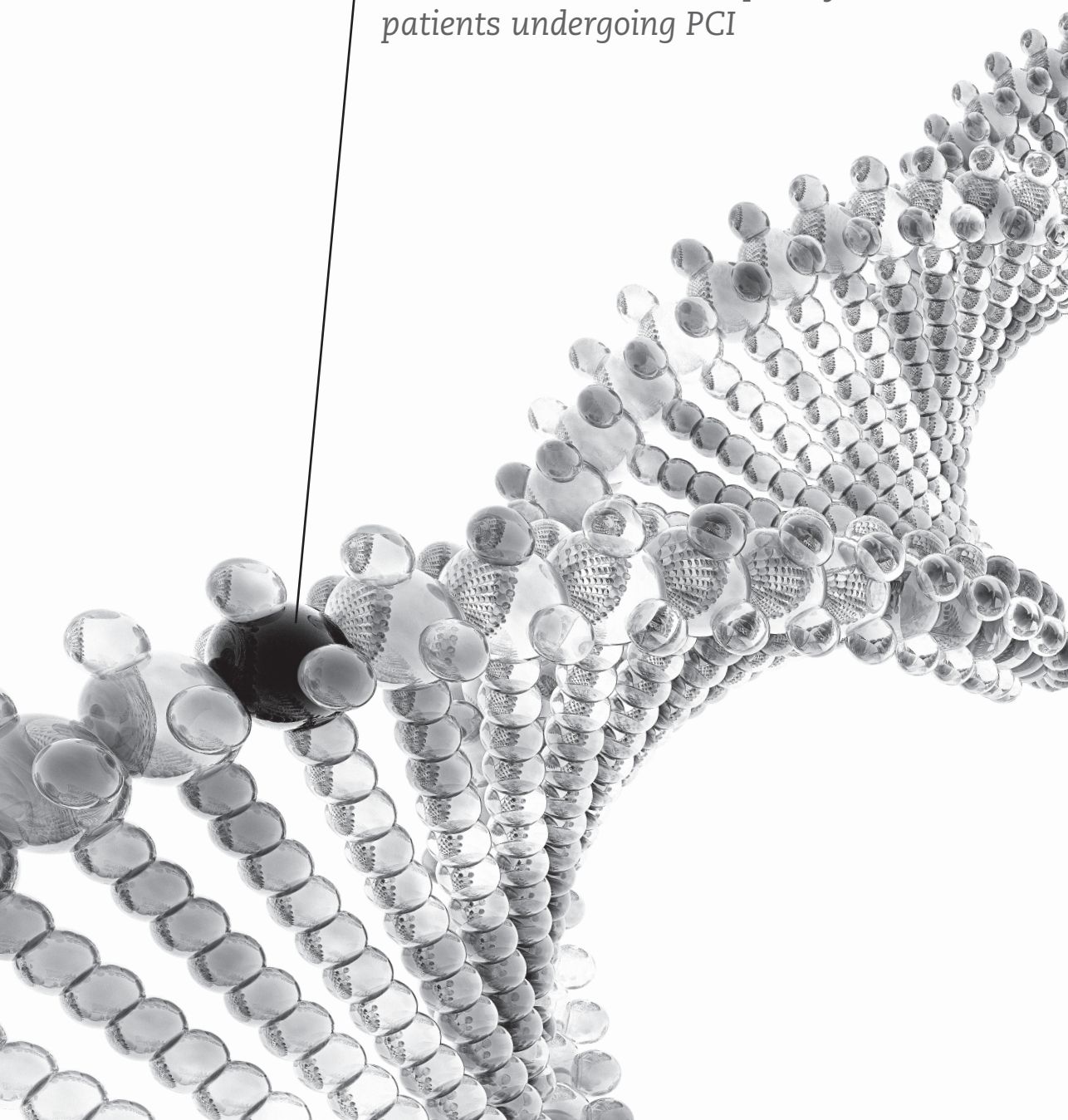


CHAPTER 10 *The in vitro effect of the new antithrombotic drug candidate ALX-0081 on blood samples of patients undergoing PCI*



ABSTRACT

Background. Compound ALX-0081 is a bivalent humanized Nanobody® that binds the A1-domain of von Willebrand Factor (VWF) with high affinity. Consequently, it can block the interaction between VWF and its platelet-receptor-GPIb, which leads inevitably to formation of arterial thrombi.

Objectives. Assessing the *in vitro* effects of ALX-0081 on platelet adhesion and aggregation in coronary artery disease (CAD) patients to determine the optimal concentration of ALX-0081 and the effect of co-medication.

Methods. We included nine CAD patients, who were scheduled for elective PCI, and eleven healthy volunteers. At admission all patients received aspirin, clopidogrel and heparin. Blood was drawn 24 hours before and one hour after start of the PCI procedure and was subsequently spiked with different concentrations of ALX-0081 or buffer. The efficacy of ALX-0081 was assessed by *in vitro* experiments: flow chamber experiments, ristocetin-induced platelet aggregation (RIPA), and the platelet function analyzer (PFA-100™).

Results. VWF levels in CAD patients were significantly higher than in healthy controls. During PCI VWF levels did not rise. In all *in vitro* experiments, ALX-0081 led to complete inhibition of platelet adhesion and aggregation. However, the required effective concentration was higher in patients than in controls and was related to plasma VWF levels.

Conclusions. ALX-0081 is able to completely inhibit *in vitro* platelet adhesion and aggregation in CAD patients scheduled for elective PCI. The efficacy of ALX-0081 is not influenced by PCI or co-medication. However, due to higher VWF levels in CAD patients a higher effective concentration of ALX-0081 was required than in healthy individuals.

INTRODUCTION

Nowadays patients with stable coronary artery disease (CAD) or an acute coronary syndrome (ACS) undergoing a percutaneous coronary intervention (PCI) - with or without stent implantation - are treated by the dual antiplatelet therapy of acetylsalicylic acid (aspirin) and clopidogrel[1]. Periprocedural medication is further optimized by administration of unfractionated heparin (UFH). The additional use of GPIIbIIIa inhibitors, such as abciximab, tirofiban and eptifibatide, has been recommended for PCI in high risk patients and primary PCI, since these expensive GPIIbIIIa antagonists improve outcome, but also increase the risk of hemorrhagic complications substantially[2-4]. Although current adjunctive medication for PCI is effective in reducing the risk of short-term and long-term complications, development of other more potent and cost-effective antithrombotic drugs is a research topic of interest.

Von Willebrand Factor (VWF) is a multifunctional glycoprotein, which is almost exclusively secreted by endothelial cells and released upon endothelial cell activation. VWF initiates adherence of platelets to the injured vessel wall and subsequent platelet aggregation leading inevitably to the formation of arterial thrombi[5-6]. Besides this strong pro-thrombotic function, high plasma VWF levels are associated with an increased risk of acute arterial thrombosis[7-8]. Furthermore, VWF levels have important prognostic implications, including second cardiac event or cardiac death[9-10]. Hence, VWF is considered as an important target of new drug development for patients with acute thrombotic events and for patients with stable CAD undergoing PCI.

Compound ALX-0081 is a bivalent humanized Nanobody® that contains the structural and functional properties of naturally occurring heavy-chain antibodies[11-13]. ALX-0081 is able to block the interaction between VWF and its platelet receptor glycoprotein (GP) Ib by binding the A1 domain of the VWF molecule with high affinity. Considering the attractive antithrombotic function of this Nanobody®, it can be used to develop a complete drug to treat patients with stable CAD or acute thrombotic events, who will undergo a PCI procedure.

We assessed the *in vitro* effects of ALX-0081 on platelet adhesion and aggregation in patients with CAD, who were scheduled for an elective PCI procedure. In patients with CAD it has been well established that VWF plasma levels are elevated[4]. Moreover, since we expect that plasma VWF levels may rise even further during a PCI procedure, we performed these experiments with blood obtained before and during the PCI procedure. Subsequently, we investigated whether VWF concentration affects the optimal concentration regimen of ALX-0081. In addition, we studied the effect of co-medication on the efficacy of ALX-0081. Finally, we compared three independent laboratory techniques (perfusion chamber studies, platelet function analyser, and ristocetin-induced platelet aggregation test) to determine which biomarker-assay assesses the proximal pharmacodynamic effect of ALX-0081 in PCI patients.

METHOD

Study Design

For this pilot study, as a preparation of a Phase IIa study of ALX-0081, we included nine patients, who were scheduled for an elective PCI procedure, and eleven healthy control subjects for the determination of VWF concentration. All patients received 80 mg aspirin before the start of the study, 600 mg clopidogrel the night before the procedure and UFH (5000 U - 20000 U) during the procedure. Exceptions were patient 4, who received the GpIIbIIIa antagonist abciximab during the PCI procedure and patient 7, who took clopidogrel already before the onset of the study.

At inclusion, demographics of the patients were assessed, as well as their history of coronary risk factors and any antecedents of thrombotic complications. Blood of the patients was analyzed for classical hematological parameters (e.g. hemoglobin, hematocrit, thrombocyte count, leucocyte count) and chemical parameters (e.g. ALT, AST, creatinine) at time of admission.

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

Blood sampling

Blood was drawn 24 hours before the PCI procedure (baseline) and 1 hour after the start of the PCI by venipuncture in the antecubital vein using Vacutainer system (Becton-Dickinson, Plymouth, UK). Blood was collected in 3.2% trisodium citrate (9:1 vol/vol). For VWF measurements citrated blood was centrifuged within 1 hour at 2000 x g for 10 min at 4°C. Plasma was additionally centrifuged at 20 000 x g for 10 minutes at 4°C.

At baseline all patients were administered with aspirin. The second blood sample represents the condition where all patients were in addition loaded with clopidogrel and heparin.

Laboratory measurements

VWF plasma level assays

Total VWF concentration (VWF:Ag) was determined with an in-house ELISA with polyclonal rabbit anti-human VWF and horseradish peroxidase conjugated anti-human VWF (DakoCytomation, Glostrop, Denmark) for catching and tagging, respectively. VWF collagen binding activity (VWF:CB) was measured by an in-house ELISA using type I collagen (Sigma, St. Louis, USA) for catching and horseradish peroxidase conjugated anti-human VWF for tagging. In addition, VWF activity (VWF:Act) was measured using a fully automated immunoassay for the quantitative determination. It is based on turbid-

ity measurement of latex particles immobilized with a monoclonal anti-VWF antibody that recognizes a functional epitope of VWF (HemosIL, Instrumentation Laboratory Company, Lexington, MA, USA)[14-15].

Platelet adhesion to collagen under high shear

Citrated blood was pre-warmed at 37°C for 5 minutes with or without addition of ALX-0081 at various concentrations (varying from 0.1 to 5.0 µg/mL) and then circulated through the chamber for five minutes over a collagen-coated surface at a wall shear of 1600 s^{-1} using a Havard infusion pump (pump 22, model 2400-004, USA) [16]. Platelet adhesion was quantified as the mean percentage surface coverage of ten independent randomly picked images per coverslip using light microscopy (Leitz Diaplan, Leica, Rijswijk, the Netherlands) and computer-assisted analysis with Optimas 6.0 software (DVS, Breda, the Netherlands).

Ristocetin-induced platelet aggregation

Blood samples of healthy controls and PCI patients were used to prepare platelet rich plasma (PRP), by centrifugation of whole blood at 1300 rpm for 7 minutes. Subsequently, PRP was pre-incubated with various concentrations of ALX-0081 or buffer after which aggregation was induced by 1.5 mg/mL ristocetin (ristocetin-induced platelet aggregation test (RIPA)). The antibiotic ristocetin is able to facilitate the non-physiological activation of the A1-domain of VWF [17]. The amplitude of the aggregation response was determined five minutes after ristocetin addition and used as an indicator for the degree of platelet aggregation.

Platelet Function Analyzer (PFA-100™)

Blood was taken 24 hours before and during the PCI procedure and was incubated with Phosphate buffered saline (PBS) buffer or various concentrations of ALX-0081 (0.1 µg/mL, 0.2 µg/mL, 0.4 µg/mL, 0.8 µg/mL, 1.6 µg/mL and 3 µg/mL) in buffer. Blood samples were aspirated at high shear rates (5000-6000 s^{-1}) through a capillary in the instrument cartridge of the PFA-100™ (DadeBehring, GmbH, Marburg, Germany) and encountered a membrane coated with collagen and adenosine diphosphate (ADP) [18]. The membrane triggered platelet activation and aggregation leading to occlusion of a central aperture and termination of the blood flow, which was measured as closure time (CT).

Activated partial thromboplastin time and platelet aggregation

To evaluate the efficacy of the currently used standard antithrombotic therapy, activated partial thromboplastin time (aPTT) and platelet aggregation induced by arachidonic acid or ADP was determined (Chrono-log aggregometer, Kordia, Leiden, the Netherlands). For platelet aggregation blood was taken 24 hours before and during the PCI proce-

dure and used for PRP preparation. Platelet aggregation was induced by either 1mM arachidonic acid or 10µM ADP. The amplitude of the aggregation response (percentage aggregation) was measured five minutes after aggregation induction before and after the PCI procedure.

Statistical analysis

All VWF plasma level measurements are displayed as means ± standard error. The Mann-Whithney U test was used to assess the difference of VWF antigen levels between patients and controls. The Wilcoxon Singed Ranks test was used to assess the difference in patients before and during PCI procedure. To assess a difference in the effective concentration of ALX-0081 before and during the PCI procedure we used a Wilcoxon Signed Ranks test on all laboratory assays.

RESULTS

Patient characteristics

Baseline characteristics of the patients are displayed in table 1. For this study we included seven male and two female patients. Age ranged from 57 to 90 years (median 74 years). None of the patients showed biochemical abnormalities that are known to affect platelet function. In addition no concurrent liver dysfunction or kidney insufficiency was observed.

Table 1 • Characteristics PCI patients and history of thrombotic complications.

	N=9
Age (mean)	71.9 ± 12 years
Gender (% males)	77.8% (8)
BMI (kg/m2)	27.8 ± 3.5
History	
CVA/TIA	-
AMI	22.2%(2)
PCI	44.4% (4)
CABG	11.1% (1)
Renal failure	22.2% (2)
PVD	-
COPD	11.1% (1)
Inflammatory disease	11.1% (1)

Data are presented as mean ± SD or percentage (exact number) for categorical variable. Abbreviations used in this table are CVA for cerebrovascular accident, TIA for transient ischemic attack, AMI for acute myocardial infarction, PCI for percutaneous intervention, CABG for coronary artery bypass grafting, PVD for peripheral vascular disease, and COPD for chronic obstructive pulmonary disease.

VWF measurements

The mean level of VWF:Ag was 0.92 ± 0.06 IU/mL in the healthy volunteers (table 2). VWF:Ag levels of patients before and during the PCI procedure were 1.37 ± 0.14 IU/mL and 1.29 ± 0.17 IU/mL, respectively. These values were higher compared to the VWF:Ag levels in the healthy volunteers (before PCI $P = 0.01$ and during PCI $P = 0.11$). By contrast, there was no statistically significant increase of VWF:Ag levels during the procedure compared to the baseline value before PCI.

Table 2 • VWF measurements in healthy volunteers and patients elected for PCI procedure.

	VWF:Ag (IU/dL)	VWF:CB/VWF:Ag (IU/dL)	VWF:Act/VWF:Ag (IU/dL)
Healthy volunteers	0.92 ± 0.06	1.39 ± 0.08	1.17 ± 0.04
Before PCI	1.37 ± 0.14	1.24 ± 0.06	0.97 ± 0.04
During PCI	1.29 ± 0.17	1.39 ± 0.07	1.01 ± 0.01
P – value	0.31	0.05	0.24

P-value represents difference between VWF:Ag levels in patients before and during PCI, as well as for the VWF:CB/VWF:Ag and VWF:Act/VWF:Ag ratios.

Platelet adhesion to collagen under shear

Perfusion of blood of healthy volunteers over the collagen coated surface resulted in a rapid and extended platelet deposition on the collagen surface (Fig 1A). Using blood of patients obtained before PCI, platelet aggregate size was remarkably smaller and platelet spreading seemed to be reduced (Fig 1B). Using blood obtained during PCI, the platelet aggregate size was reduced even further (Fig 1C) and surface coverage was remarkably lower, as expected due to co-medication (aspirin and clopidogrel). Addition of ALX-0081 completely and concentration-dependently inhibited platelet adhesion under shear conditions in blood samples taken before and during PCI-procedure.

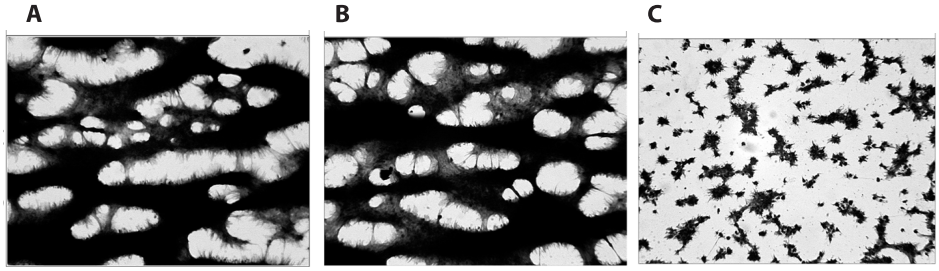


Figure 1 • In vitro perfusion studies

In vitro perfusion studies with blood from healthy individuals (A) and blood from PCI patients obtained before (B) and during PCI (C). Citrated blood was incubated with buffer and perfused for 5 min over a collagen-coated surface at a wall shear rate of 1600 s^{-1}. Adhered platelets were fixated with glutaraldehyde and stained with May-Grünwald.

For all patients, complete inhibition was observed when 0.8 µg/mL ALX-0081 was present in the blood. The effective ALX-0081 concentration, i.e. concentration of ALX-0081 resulting in less than 3% surface coverage, ranged from 0.4 µg/mL to 0.8 µg/mL before PCI and from 0.2 µg/mL to 0.8 µg/mL during PCI procedure ($P = 0.66$, n = 5) (Fig 2A). Only five patients could be incorporated into the statistical analysis, due to technical problems, difficulties during the analysis of the blood perfusion assay, and administration of abxicimab in one patient during the PCI procedure.

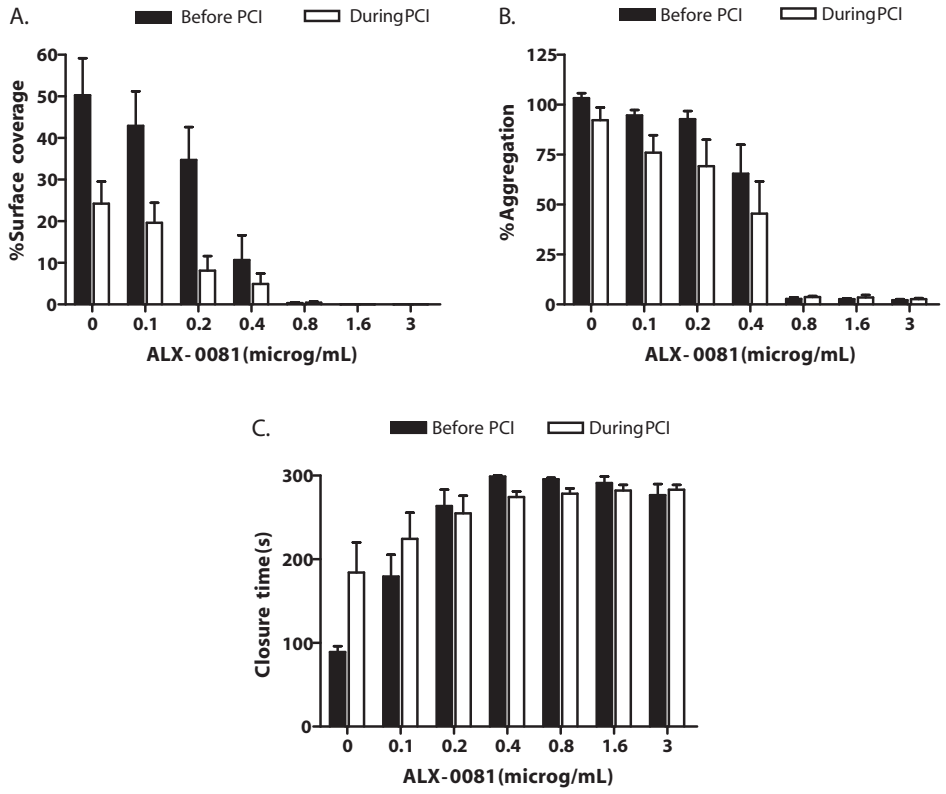


Figure 2 • Effect of ALX-0081 on platelet adhesion, aggregation and closure time.
A. Effect of ALX-0081 on in vitro platelet adhesion in blood obtained from PCI patients under flow conditions. Results of platelet adhesion of blood spiked with various concentrations of ALX-0081 are represented as mean plus standard error of the mean. The average % surface coverage obtained with blood from all patients is shown. B. Effect of ALX-0081 on platelet aggregation of PCI patients under flow conditions. The mean ristocetin-induced aggregation responses (% aggregation) of 9 patients before and during PCI are represented (mean plus standard error of the mean). C. Effect of ALX-0081 on closure time in PFA-100ä. Mean closure times obtained with blood from 9 patients before and during PCI using collagen/ADP cartridges are represented (mean plus standard error of the mean).

In blood samples incubated with buffer only, the surface coverage for each patient was significantly lower in blood samples obtained during PCI compared to blood samples obtained before PCI ($P = 0.04$, n = 7).

Ristocetin-Induced Platelet Aggregation

In healthy individuals complete inhibition of ristocetin-induced platelet aggregation (RIPA) was observed with 0.4 µg/mL ALX-0081 (n = 10). By contrast, complete inhibition in patients was only observed with an ALX-0081 concentration of 0.8 µg/mL (Fig 2B). In addition, the effective concentration of ALX-0081, i.e. concentration of ALX-0081 resulting in less than 10% aggregation, ranged from 0.4 µg/mL to 0.8 µg/mL and was not significantly different in the blood samples taken before and during PCI ($P = 0.16$, n = 9).

In the blood samples incubated with buffer a similar aggregation response was observed ($P = 0.37$, n = 9), demonstrating that the medication administered to the patients during PCI has no effect on the read out of the RIPA.

Platelet Function Analyzer

Addition of ALX-0081 to the blood samples led concentration-dependently to an increase of the closure-time, i.e. CT more than 300 s (fig 2C), using platelet function analyzer (PFA-100™). By contrast to the results in perfusion chamber and RIPA, non-closure was already observed with an ALX-0081 concentration of <0.1-0.4 µg/mL. The effective concentration of ALX-0081, i.e. concentration of ALX-0081 resulting in a CT of more than 250 s, ranged from less than 0.1 µg/mL to 0.4 µg/mL and was not significantly different before or during PCI ($P = 0.50$, n = 5). Four patients were excluded from the analysis with PFA-100™, since the effective concentration could not be ascertained due to a CT of more than 250 in the blood samples obtained during the PCI-procedure and incubated with buffer. Closure time of buffer samples, was borderline significantly prolonged during PCI compared to before PCI ($P = 0.09$, n = 9).

Platelet Aggregation Induced by Arachidonic Acid or by ADP

As expected, all patients who were administered with aspirin already before the onset of this study showed a strong inhibition of the arachidonic acid aggregation both before and during the PCI procedure ($P = 0.89$, n = 9). The ADP response varied widely between the patients. During PCI, when all patients were administered with clopidogrel, the ADP response was reduced ($P = 0.09$, n = 9). Interestingly, three patients out of nine still displayed an important ADP aggregation (more than 65%), despite the administration of antiplatelet drugs, suggesting an incomplete antithrombotic protection in these patients.

As expected, aPTT values were significantly prolonged (mean aPTT was >240 seconds) during PCI procedure as all patients were administered with unfractionated heparin at the start of the PCI.

Relation between VWF:Ag levels and the effective concentration of ALX-0081

There was an association between VWF:Ag levels and the effective concentration assessed in perfusion studies (Fig 3A) and RIPA (Fig 3B), but not for the PFA-100™ (Fig 3C). Suggesting a linear association an effective ALX-0081 concentration of approximately 0.4 µg/mL is suggested for plasma VWF:Ag levels of 1.00 IU/mL for both perfusion studies and RIPA.

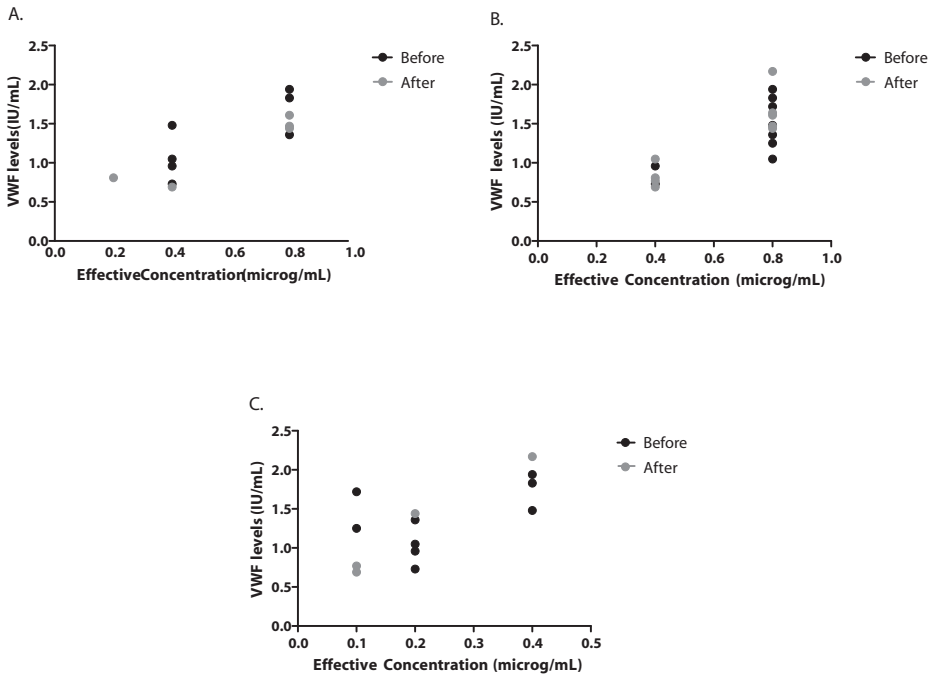


Figure 3 • Relationship between VWF:Ag levels and the effective concentration of ALX-0081
Relationship between VWF:Ag levels using blood obtained pre and post PCI and the effective concentration of ALX-0081 as determined in perfusion chamber studies (A), RIPA (B) and PFA-100™(C).

DISCUSSION

In this study we assessed the effect of ALX-0081 on platelet adhesion and aggregation in blood obtained from patients scheduled for an elective PCI procedure, in order to determine the optimal concentration for patients with CAD in future clinical trials.

ALX-0081 completely abolished platelet adhesion to collagen under flow conditions (1600 s^{-1}) and in the RIPA assay in both controls and patients. In patients, complete inhibition required a higher concentration of ALX-0081 than in controls. In the PFA-100™, ALX-0081 led concentration dependently to prolongation of the closure time with non-closure observed in all patients at 0.4 µg/mL ALX-0081.

We are aware that our study is limited by a small sample size and that our findings should be interpreted with care. However, we believe that our findings show thoroughly that ALX-0081 could be effective in inhibiting platelet adhesion and aggregation. Therefore, more extensive research on ALX-0081 is warranted.

The apparent lower required effective concentration in healthy volunteers in the perfusion and RIPA studies has been established previously in *in vitro* experiments performed with blood of healthy individuals and animal models [11-12]. This may be explained by the higher VWF:Ag levels observed in our patients. Indeed, patients with stable CAD tend to have elevated plasma VWF levels due to endothelial cell activation [4]. Therefore, we studied whether the efficacy of ALX-0081 was related to the VWF levels present in plasma of patients. We observed an association between the plasma VWF:Ag levels and the effective concentration of ALX-0081 as determined in RIPA and flow studies. However, this association should be interpreted with care considering the number of subjects in the analysis.

It is noteworthy that in all assays no difference in effective concentration was observed before and during PCI procedure, indicating that modification of ALX-0081 dosage for PCI procedure is not required.

Another question that was addressed in this study is the effect of co-medication on the efficacy of ALX-0081. In all 3 experimental set-ups, no difference in effective concentration of ALX-0081 was observed 24 hours before or during PCI, suggesting that co-administration of clopidogrel and heparin has no influence on the efficacy of ALX-0081. This can be explained by the fact that these antiplatelet drugs block different consecutive steps in thrombus formation. ALX-0081 interferes with the initial step of platelet adhesion to the injured vessel wall under high shear, whereas clopidogrel and aspirin affect platelet activation, a process which occurs after the platelet adhesion has occurred. Abciximab and other GPIIbIIIa blockers inhibit platelet-platelet interactions, thereby hampering platelet aggregation. Heparin is an anticoagulant that hinders stabilisation of the developing thrombus.

Platelet activation by thromboxane A_2, as measured in arachidonic acid aggregration, was inhibited for all patients as expected due to the aspirin intake. Remarkably, in 3 of the 9 patients, ADP aggregation was not significantly reduced - using blood obtained during PCI - suggesting an incomplete antiplatelet protection despite clopidogrel administration. Indeed, several studies describe a high incidence of clopidogrel resistance, which is partially explained by genetic variations affecting the function of the cytochrome 450 protein that converts the clopidogrel pro-drug to its active metabolite in the liver [19-22].

Besides ALX-0081, there are several other novel drugs that specifically interact with VWF molecules [23]. ARC1779 is an anti-VWF aptamer, which binds the A1 domain of activated VWF and inhibits its interaction the GPIb receptor on platelets [24]. This drug has been shown to be effective in inhibiting VWF activity and VWF dependent platelet function in patients with an acute myocardial infarction. Interestingly, they did not observe a difference in effective concentration between patients and healthy volunteers[25]. Also, in patients with von Willebrand Disease and thrombotic thrombocytopenic purpura ARC1779 showed to be effective[26-27].

Finally, we compared three independent laboratory techniques to find a biomarker assay for assessment of ALX-0081 efficacy in PCI patients. In perfusion chamber studies the platelet deposition during PCI was significantly reduced due to the antiplatelet regimen, thereby hampering analysis of these coverslips. Accordingly, surface coverage of buffer samples (without ALX-0081) was also significantly reduced during PCI, indicating that the administration of antiplatelet drugs influences the read-out of perfusion studies[28-29]. These observations disfavor perfusion studies as biomarker assay in PCI-patients. In addition, although studies in perfusion chambers are very useful in research settings, they are not adequately adapted for routine based analysis, because they are labor-intensive and not standardized adequately.

By contrast, the PFA-100™ provides a simple, rapid and fully automated assessment of high shear-dependent platelet function. In addition, it has been shown previously that the PFA-100™ device can predict myocardial damage and outcome in patients with acute myocardial infarction [30-31]. However, during PCI-procedure 4 out of 9 patients already displayed a closure time of more than 250 s in buffer samples (without ALX-0081) and closure time of buffer samples was significantly prolonged during PCI. This suggests that the administration of antiplatelet drugs has an important impact on the read-out of the PFA-100™. This has previously been shown by other research groups [32-34]. As the read-out is not solely VWF-platelet dependent, the PFA-100™ device is disregarded as potential biomarker for ALX-0081 efficacy.

Finally, the use of RIPA as a biomarker was assessed. RIPA is a generally acquainted and validated technique in most hematology laboratories using PRP of patients. RIPA in buffer samples was not significantly changed during PCI-procedure, implying that

clopidogrel and heparin have no effect on the read-out of this assay. Therefore, RIPA is preferred to be used as biomarker-assay for assessment of ALX-0081 efficacy in patients undergoing PCI procedure. However, since RIPA is a labor-intensive assay, new techniques should be explored to study the efficacy of ALX-0081 in larger studies.

In conclusion, ALX-0081 is able to inhibit *in vitro* platelet adhesion and aggregation completely in patients undergoing a PCI procedure. The efficacy of ALX-0081 is not influenced by co-medication, such as aspirin and clopidogrel. In addition, the effective concentration of ALX-0081 was similar using blood obtained before and during PCI. However, due to high VWF plasma levels in patients undergoing PCI, a higher effective concentration of ALX-0081 was required compared to healthy individuals. This first proof of principle study requires future studies to investigate the pharmacokinetics, safety and efficacy of this new and promising anti-thrombotic drug candidate.

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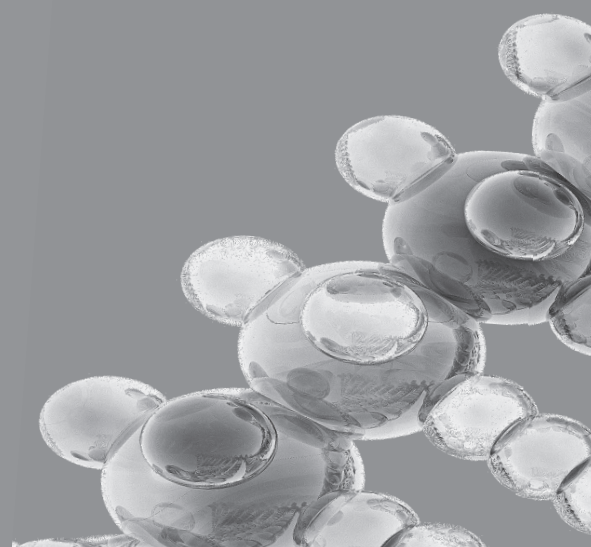
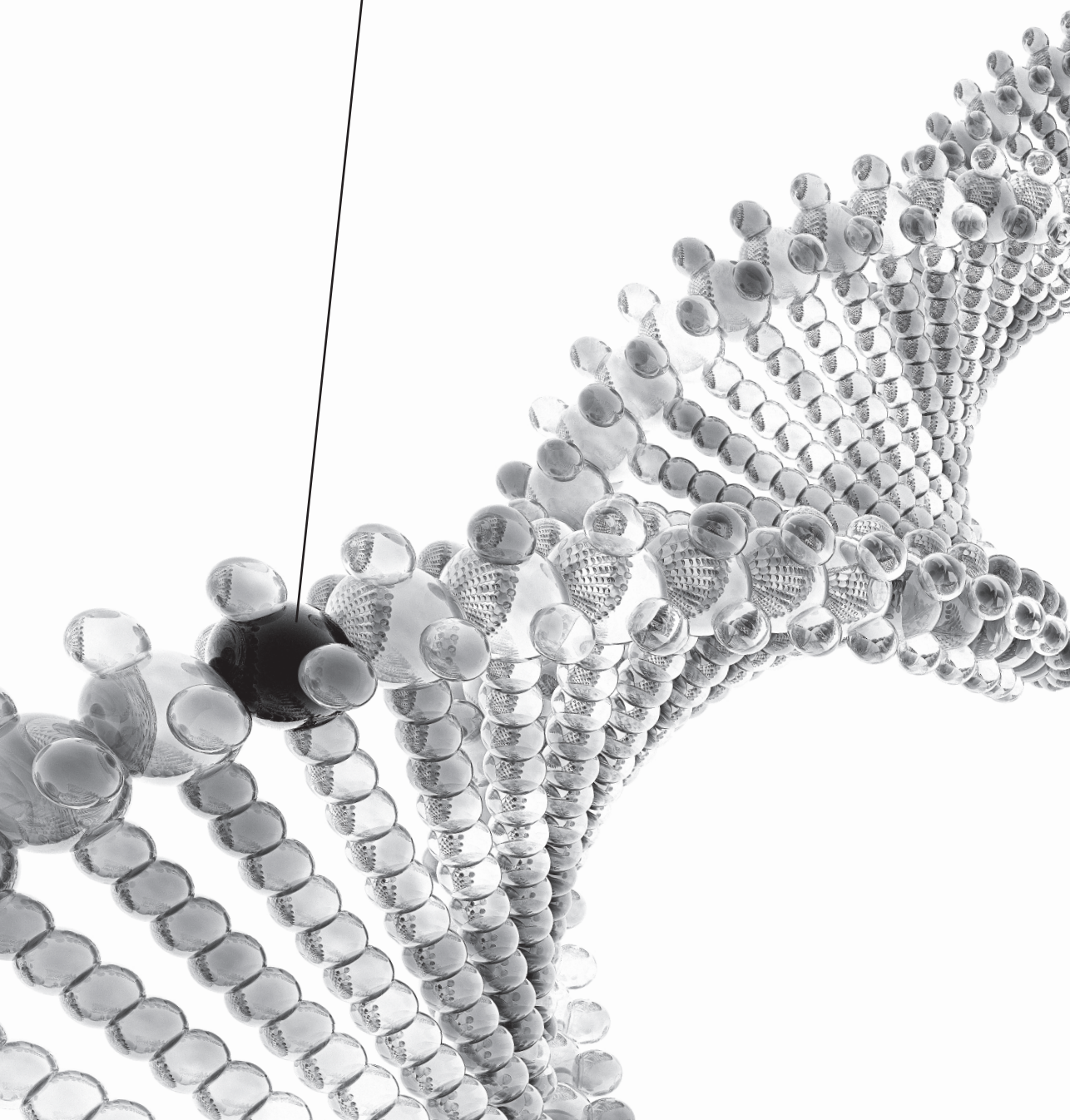


CHAPTER 11 *General Discussion*



Despite previous comprehensive research on the variation of von Willebrand Factor (VWF) antigen (VWF:Ag) levels and the association between VWF:Ag levels and the occurrence of arterial thrombosis, there remain many ambiguities in this research area. The primary aim of this thesis was to unravel the role of VWF in the pathophysiology of arterial thrombosis. Secondary, we aimed to identify novel genetic determinants of VWF:Ag levels. Both research aims have led to a better understanding of the variability in VWF:Ag levels and the possible causal relationship between VWF and the occurrence of arterial thrombosis.

In this final chapter the main findings of this thesis will be discussed and interpreted. In addition, the most important methodological considerations will be addressed, as well as possible clinical implications and future directions.

VWF AND THE RISK OF ARTERIAL THROMBOSIS

Candidate gene approached studies

Since high VWF:Ag levels increase the risk of coronary heart disease (CHD)[1-4] and stroke[5-6], and VWF:Ag levels are to a high degree genetically determined[7-8], it is of great interest to discover genetic determinants of VWF:Ag levels. Uncovering genetic determinants of VWF:Ag levels is of importance for the explanation of the large variability in VWF:Ag levels. In addition, genetic determinants, which are present from gestation, can also be used to investigate the non-confounded effect of VWF on the risk of cardiovascular disease in genetic association studies. With this research strategy, explained in more detail under "methodological considerations", it would be possible to draw conclusions about the possible causal relationship between VWF and occurrence of cardiovascular disease.

To date, many studies have investigated genetic determinants of VWF:Ag levels and VWF activity, and the relationship between these genetic determinants and the risk of arterial thrombosis. Initially, interest was only focussed on solitary genetic polymorphisms within genes encoding proteins known to be involved in the regulation of VWF:Ag levels, i.e. the candidate gene approach. The first identified genetic determinants of VWF:Ag levels were localized within the promoter region of the VWF gene (*VWF*), which regulates gene transcription[9-10]. Also, in the coding regions of *VWF* several genetic polymorphisms were discovered[11]. Later on, genetic variations in other, VWF-related, candidate genes were investigated, including ABO blood group, *ADAMTS13*, and thrombospondin genes. Some of these genetic variants were also associated with the risk of arterial thrombosis[12-15]. By reviewing the literature on genetic determinants of VWF:Ag levels and their relationship with cardiovascular risk[11], one of our main observations was that only a limited number of studies combined the investigation of the association between genetic variation, VWF:Ag levels and the risk of arterial thrombosis in one study. Furthermore,

the studies differed in study design, study population, and study endpoint, and were often underpowered. Clearly, there was need for large prospective studies to further establish the role of VWF in the occurrence of arterial thrombosis. Consequently, we investigated the relationship between genetic variations across the VWF gene, VWF:Ag levels and the risk of incident coronary heart disease (CHD) in a large population-based study, the Rotterdam study, among patients aged 55 years and over (N = 7002)[16]. We identified three non-redundant SNPs (rs216321, rs1063856, and rs2283333) in *VWF* that were highly significantly associated with VWF:Ag levels. However, genetic variation in *VWF* had no effect on the risk of CHD in this older population, from which we concluded that in the elderly common genetic variants in *VWF* that determine VWF:Ag levels do not directly increase the risk of CHD. In contrast to the results of this prospective study, we did observe an association between a *VWF* polymorphism, the synonymous SNP rs1063857, and the risk of arterial thrombosis in a well-defined case-control study of young individuals (mean age 43.2 years) with a first event of arterial thrombosis and healthy control subjects (mean age 39.6 years)[17]. Rs1063857 is in complete and perfect LD with the non-synonymous SNP rs1063856, which was identified in the Rotterdam study as determinant of VWF:Ag levels. This suggests that age may mediate the effect of genetics on cardiovascular risk. Indeed, it has been shown previously that the effect of genetic variations on a specific trait is larger at young age[18]. Another explanation for the absence of an association between *VWF* variants and CHD risk in the Rotterdam study is that the identified SNPs may have smaller effects on CHD risk than we can detect with our sample size and number of incident cases. Also, rare genetic variants with large effects were not covered within the current analysis. So, although an indirect approach with common genetic variants has not proven a causal relationship, it is still possible that VWF has a role in the occurrence of CHD that can be identified with other research strategies.

Another strategy was to investigate other candidate genes for VWF:Ag levels. To this end we selected the thrombospondin genes, *THBS1* (chromosome 15), *THBS2* (chromosome 6), and *THBS4* (chromosome 5)[19]. Thrombospondin-1 (TSP-1) is able to control VWF multimer size both directly via cleavage of the VWF linking disulfide bonds, and indirectly by competing with ADAMTS13[20-21]. TSP-1 is part of the thrombospondin family, which consists of five extracellular matrix proteins that are involved in cell-matrix and cell-cell interactions[13]. It was previously shown that genetic variants in thrombospondin genes were associated with the risk of myocardial infarction[15]. However, it remained unclear whether the increased risk was mediated via increased levels of VWF:Ag or yet by another mechanism. In addition, the replication studies that followed showed contradictory results[22-25]. Therefore, we investigated common genetic variations within the thrombospondin genes and their relationship with VWF:Ag levels and incident CHD in the same elderly population of the Rotterdam study[19]. Genetic variation in

thrombospondin genes contributed only weakly to the variation in VWF:Ag levels and had no direct relationship with the risk of CHD. Since the effect of genetic variation in thrombospondin genes on VWF:Ag levels was very small in this study, we expected the possible contribution of thrombospondins to the risk of CHD also to be very small. So we may have had insufficient statistical power to detect these small effect sizes. Conversely, considering the diverse functions of thrombospondins in for example atherosclerosis, inflammation, and thrombus formation it can be argued that the previously suggested link between thrombospondins and CHD is not explained by the regulation of VWF:Ag levels, but is actually independent of VWF[19].

From these candidate gene studies it has become clear that we are looking for very modest effects of genetic variation on cardiovascular risk. The role of VWF in the interplay leading to cardiovascular disease must be therefore much more complex, than was previously anticipated. Also, genetic determinants of VWF:Ag levels that were identified in candidate gene studies explain only a few percent of the variability in VWF:Ag levels[16]. This suggests either that there are genetic determinants with greater effects to be discovered in the genome or that there are hundreds of SNPs, which explain each a small fraction of the variation in VWF:Ag levels. In the latter scenario it will be difficult to detect significant effects on cardiovascular risk, because this requires very large sample sizes. In any case, the role of VWF in the pathophysiology of cardiovascular disease is not very straightforward and the question remains whether epidemiologic studies are the right way to prove causality between VWF and cardiovascular disease. This will be discussed in more detail under "methodological considerations".

Genome-wide based studies

From 2001 onwards, genetic association studies have made tremendous progress in the efficiency of detecting novel genetic determinants of for example VWF:Ag levels. Modern technologies facilitated investigation of the whole genome for SNPs that may be associated with VWF:Ag levels. This resulted among others in the landmark article of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium, which uncovered six new genetic loci (*STXBP5*, *SCARA5*, *STAB2*, *STX2*, *TC2N*, and *CLEC4M)* for VWF:Ag levels alongside already known loci, including *VWF* and the ABO blood group gene[26].

Genome-wide association studies enable us to investigate associations between genetic polymorphisms across the genome and a specific trait without a prior hypothesis. Hypothesis-free testing facilitates the discovery of genes outside known biological pathways of the regulation of VWF:Ag levels. However, these genes often encode proteins that play a role within unknown or unexpected pathways. Designing new studies that connect these novel pathways to the regulation of certain phenotypes and eventually cardiovascular disease development is challenging.

Two of the new genetic loci for VWF:Ag levels identified by the CHARGE consortium, *STX2* and *STXBP5*, are possibly involved in the regulated secretion pathway of VWF molecules by endothelial cells and are therefore of particular interest[27]. We expanded the results of the CHARGE meta-analysis by investigating the effect of genetic polymorphisms, which cover the total genetic variation in *STX2* and *STXBP5*, on VWF:Ag levels, VWF collagen binding (VWF:CB) activity levels and the risk of arterial thrombosis. This was performed in a case-control study among young patients with a first event of arterial thrombosis and healthy controls: the "Genetic risk factors for Arterial Thrombosis at young age: the Role of TAFI and other Coagulation factors" (ATTAC) study[28]. We found that rs9399599 and rs1039084 in *STXBP5* and rs7978987 in *STX2* were associated with both VWF:Ag levels and VWF:CB activity levels[29]. Both rs1039084 and rs9399599 in *STXBP5* are in high linkage disequilibrium with rs9390459, which had the highest genome wide significance level for VWF plasma levels in the meta-analysis of the CHARGE consortium ($D' = 1.00$, $R^2 = 0.87$ for rs9399599 and $D' = 0.96$, $R^2 = 0.86$ for *rs*1039084). Also, rs7978987 in *STX2* had a highly significant *P* value of 3.82×10^{-11} in this meta-analysis.

We found three levels of evidence for the involvement of the STXBP5 and STX2 proteins in Weibel Palade Body exocytosis of VWF molecules in the ATTAC study. First, we replicated the findings for VWF:Ag levels of the CHARGE consortium and found novel associations between these SNPs and VWF:CB activity levels. Rs7978987 in *STX2* was associated with a higher VWF:CB/VWF:Ag ratio, which indicates that the secreted VWF molecules are functionally more active. Indeed, Weibel Palade bodies contain (ultra) large VWF molecules that are the most haemostatically effective. Secondly, the relationship between genetic variability in *STXBP5* and *STX2* and VWF:Ag levels was especially seen in patients with arterial thrombosis and was less clear in the control subjects. In healthy individuals, VWF:Ag levels are determined mainly by the activity of the constitutive pathway, because the endothelium is not triggered to release the VWF molecules. Since *STX2* and *STXBP5* encode proteins that may be involved in the regulated secretion pathway of VWF molecules, which is only stimulated after endothelial cell activation, one would expect to find an effect of these polymorphisms not in healthy subjects, but particularly in patients who have arterial thrombosis. Thirdly, the minor alleles of the polymorphisms in *STX2* were strongly associated with a decreased risk of arterial thrombosis. Yet, the precise mechanisms by which these polymorphisms exhibit their effect on the risk of arterial thrombosis are unclear, because the minor alleles of the polymorphisms in *STX2* tended to be associated with higher VWF:Ag levels. It is possible that other substances secreted by Weibel Palade bodies, such as P-selectin, angiopoeitin-2, osteoprotegerin, and eotoxin-3, alter the risk of arterial thrombosis, rather than VWF[30].

To further explore the role of *STXBP5* and *STX2* in the regulation of VWF:Ag levels, we investigated whether genetic variants within *STXBP5* and *STX2* affect the VWF:Ag response to incremental exhaustive exercise in young healthy volunteers. Our hypothesis

was that genetic variation in *STXBP5* and *STX2* would only influence the VWF:Ag levels after exhaustive exercise, since under steady state conditions in young and healthy individuals VWF:Ag levels are determined by the constitutive pathway. Indeed, we observed no effect of the selected SNPs on VWF plasma levels at baseline. However, these genetic variants were also not associated with VWF:Ag levels after exhaustive exercise nor with the increase of VWF:Ag levels. This finding suggests that genetic variations in *STXBP5* and *STX2* do either not contribute to the VWF:Ag level increase upon exhaustive physical exercise or implies that their effect was smaller than we could detect with our sample size. Furthermore, the VWF:Ag increase was highly variable and strongly dependent on physical fitness and the intensity of the exercise performed. Consequently, the effect of environmental factors may have been stronger than the genetic effect in this particular study.

VWF AS PROGNOSTIC MARKER

VWF is a risk factor for arterial thrombosis in healthy subjects, but also in patients with already prevalent atherosclerotic disease, including CHD[7]. In young patients with CHD little is known on the prognostic value of VWF and other new biomarkers, including CRP and fibrinogen. However, it is very important to recognize young CHD patients at risk for a recurrent event, since young patients form an excellent group for preventive cardiology. Therefore, we studied the recurrent event rate and the prognostic value of traditional, inflammatory, and prothrombotic risk factors in young patients of the ATTAC study with a first event of CHD. Interestingly, fibrinogen and C-reactive protein, but not VWF were associated with recurrent events. This was in contrast to our expectations, since VWF:Ag levels have been associated with reinfarction and/or mortality risk in patients with CHD by numerous studies[7]. However, the patients in former studies were generally much older than our current study population and may have had more extensive atherosclerosis for that reason. Another study in young patients has not identified an association between VWF:Ag levels and a first myocardial infarction[31]. Hence, at younger age inflammation seems an important determinant of prognosis in patients with CHD.

LOW VWF PLASMA LEVELS

Uncovering new genetic determinants of VWF:Ag levels is not only important in the scope of arterial thrombosis, but also for individuals with low VWF:Ag levels and bleeding symptoms. Low VWF:Ag levels are a diagnostic criterion for von Willebrand disease (VWD), the most common inherited bleeding disorder. According to the ISTH classifica-

tion guidelines, individuals with VWD have clinically relevant bleeding symptoms, low VWF:Ag levels and a positive family history of bleeding symptoms[32]. Diagnosis of type 1 VWD – characterized by a partial quantitative deficiency of VWF molecules – is difficult, because both VWF:Ag levels and bleeding symptoms are highly variable. In addition, low VWF:Ag levels may only be a risk factor for bleeding and therefore do not necessarily coincide. Hence, since both clinical bleeding symptoms and low VWF:Ag levels are highly frequent in the general population, these clinical manifestations may occur together by chance. If VWF gene mutations are found in subjects with low VWF levels and bleeding symptoms, the diagnosis type 1 VWD can be made with more certainty. However, three large studies in Europe, the UK and Canada have shown that 35% of the type 1 VWD patients have no apparent *VWF* mutations. This was surprising, since the genetic component is evident in most patients because of a strong family history of bleeding[33-35]. Therefore, there is now a growing expectation that mutations in other genes than *VWF* may be involved in the pathogenesis of type 1 VWD or at least in individuals with low VWF:Ag levels.

First, we investigated whether genetic variations in *STXBP5* and *STX2* are also involved in the regulation of VWF:Ag levels in patients with type 1 VWD. We found that genetic variation in *STX2* was associated with VWF:Ag levels in patients previously diagnosed with type 1 VWD. In addition, genetic variation in *STXBP5* was associated with the bleeding phenotype, as assessed by the bleeding score described by Tosetto et al[36], in female type 1 VWD patients. In the total population and in men we did not observe this association. This can be explained by the fact that women experience generally more challenges to the haemostatic system during life, whereby the bleeding score in women may be a better reflector of clinically relevant bleeding tendency, than in men. Indeed, the menorraghia item of the bleeding score mainly drove the association between genetic variation in STXBP5 and the bleeding score.

Next, we investigated SNPs across the whole genome for associations with low VWF:Ag levels without a prior hypothesis in a meta-analysis of genome-wide association data from eleven population-based cohorts comprising 31.149 individuals of European ancestry. Five genetic loci were identified that are associated with low VWF:Ag levels (lowest 5%): *ABO*, *VWF*, *STXBP5, STAB2*, and *UFM1*. Four of these were already reported in the meta-analysis of the CHARGE consortium using continuous VWF antigen levels as phenotype[26]. SCARA5, TC2N, and CLEC4M, which were previously identified as important new genetic loci for continuous VWF levels, were not identified in this meta-analysis. This suggests that mechanisms that lead to low VWF levels partly differ from those that regulate VWFAg levels, but also share some attributes. Furthermore, our findings in this meta-analysis confirm the hypothesis that genes other than *VWF* lead to low VWF:Ag levels.

GENERAL METHODOLOGICAL CONSIDERATIONS

Causality and Mendelian Randomization

Causal discovery is one of main topics in clinical epidemiology that has engaged researchers over time. A cause of a specific disease is defined as: "A cause of a disease event is an event, condition, or characteristic that preceded the disease event and without which the disease event either would not have occurred at all or would not have occurred until some time later".[37] However, in complex diseases more factors lead to the disease together, that is a single factor is neither sufficient nor necessary for the disease event to occur. Cardiovascular disease is a classical example of a disease that is caused by multiple factors. Investigating environmental factors for causation in multifactorial diseases such as cardiovascular disease is challenging, since factors act in concert to lead eventually to the disease. For example, it is possible that a particular factor is only correlated with environmental factors that affect the probability of disease (confounding). Also, some of these factors may only be a consequence of the disease (reverse causation). In both cases, the factor under investigation may be wrongfully implicated as a pathogenic mediator. These two problems of confounding and reverse causation hinder current observational studies in their attempt to prove causation.

This issue applies also to the relationship between VWF and arterial thrombosis. VWF:Ag levels increase upon endothelial cell activation and through endothelial dysfunction. Endothelial dysfunction precedes atherosclerosis development, which is one of the pathologic components leading to arterial thrombosis[38]. In case of reverse causation, occurrence of arterial thrombosis leads to higher VWF:Ag levels. So, although the biological function of VWF makes a causal role for VWF in the development of arterial thrombosis plausible, high VWF:Ag levels may rather be a consequence of the disease.

A method for causal discovery with observational studies is the Mendelian Randomization approach. In case of a causal relationship we expect to observe that genetically elevated VWF:Ag levels increase the risk of arterial thrombosis to same extent as predicted by the direct effect of VWF:Ag levels on the risk of arterial thrombosis[39-40]. Since genetic variants are generally not associated with environmental factors and are not influenced by the onset of disease, genetic association studies are not hindered by confounding and reverse causation.

We have applied this method in our study within the Rotterdam study, in which we investigated common genetic variants in the VWF gene and their association with VWF:Ag levels and CHD risk[16]. Given the observed effect of VWF:Ag levels on the risk of CHD and the observed effect of genetic variations on VWF levels in this study, we should have detected a hazard ratio of 1.03 in order to prove causality. We had insufficient power to detect such a small hazard ratio. In addition, the size of this hazard ratio suggests that there are many other factors involved in the pathway leading to CHD with greater effects.

Even in case we would have found a significant association between a genetic variant and CHD risk of this effect size, it remains questionable whether this effect would add to current risk prediction. Indeed, it has been recently shown that adding VWF:Ag levels to the Framingham risk score has no incremental value beyond classical cardiovascular risk factors[41]. So, although Mendelian Randomization seems an excellent method to bypass confounding and reverse causation, it is limited by study power, heterogeneous effects, complexity of the associations under investigation, and lack of suitable genetic variants with large effects[42].

Genetic Association Studies

Genetic association studies have been designed to detect associations between genetic polymorphisms and a specific trait, including quantitative characteristics or diseases. With these studies multiple genetic loci have been discovered for e.g. cardiovascular disease, dementia, and diabetes, but also for several quantitative characteristics, including cholesterol, C-reactive protein, and several haemostatic factors. Despite these successes, genetic association studies comprise also several limitations, which will be addressed here. First, genetic variants that are highly significantly associated with VWF levels explain only a few percent of the variability. Even in the meta-analysis of the CHARGE consortium the identified genetic loci accounted together for only 10% of the variation in VWF levels[26]. Where are the remaining genetic determinants? One hypothesis is that rare polymorphisms with large effects will explain this missing heritability. The rapid development of next-generation sequencing will be the next step in genetic epidemiology and will focus on the discovery of rare variants with large effects in order to resolve the missing heritability.

Second, current genetic association studies often fall short on sufficient statistical power. This is particularly a problem when using the Mendelian Randomization approach. Often the genetic variant explains only a small percentage of the variation of the phenotype under investigation and therefore can only have a small effect on disease risk. In order to increase statistical power large population-based cohort studies have started a few years ago to collaborate and pool their DNA samples.

As a consequence of the rapidly evolving innovations of genotyping techniques it is now possible to study a million SNPs at the same time. With these techniques genetic association studies shifted from a candidate gene approach with a strong prior hypothesis to a hypothesis-free approach in genome-wide association studies. Since it is likely to find novel associations by chance when investigating this high number of SNPs, genome-wide association studies have adjusted the level of statistical significance to a p-value of 5×10^{-8}. For other studies that selected multiple SNPs, either for replication or for a candidate gene approach, correction for multiple testing is also required. Mainly the p-value of 0.05 was divided by the number of SNPs under investigation, i.e. Bonferroni

correction. However, as Bonferroni correction is a quite conservative method, the majority of the associations found did not remain statistically significant after adjustment for multiple testing. The question is whether Bonferroni is a too conservative method in studies that are supported by a prior hypothesis or in studies based on previously observed strong findings by genome-wide association studies. For example in chapter 5 of this thesis, we investigated common genetic variations in *STXBP5* and *STX2*, which were previously identified by the CHARGE consortium as novel genetic loci for VWF levels. Since the encoding proteins of these genes are possibly involved in the regulated secretion machinery of VWF molecules, we had a good prior hypothesis to execute this study. So, although we used Bonferroni correction in this study, a statistical significance level of $p = 0.05$ would have been justified.

Lastly, genetic association studies often do not identify the actual causal variant, but only a proxy that is in high LD with the causal variant. In addition, some anticipate that the effect of the discovered SNP is an underestimation of the true effect of the causal variant. For these reasons, it is difficult to find out what the precise mechanism of a newly discovered genetic locus is. In order to elucidate the underlying mechanism for the *STXBP5* and *STX2* loci, we investigated in chapter 9 whether genetic variation in *STXBP5* and *STX2* affects the exercise driven increase of VWF:Ag levels in healthy individuals. Unfortunately, our findings did not confirm our hypothesis that the *STXBP5* and *STX2* encoding proteins are involved in the regulated secretion pathway of VWF molecules.

CLINICAL IMPLICATIONS

The findings we have obtained by performing the studies described in this thesis provide a few novel insights, but have no direct clinical implications at this point. Our ultimate goal was to determine whether a true causal mechanism underlies the association between high VWF:Ag levels and an increased risk of CVD. This would not only lead to a better understanding of the pathophysiology of CVD, but also have implications for preventive and therapeutic strategies. Another aim was to identify genetic determinants of VWF:Ag levels in order to use these genetic variants for genetic profiling and eventually personalized medicine.

Unfortunately, the studies we have performed show that the relationship between VWF and CVD is not straightforward, but involves many other factors, which act in concert with VWF to eventually lead to the occurrence of arterial thrombosis. Another problem that we came across is that the genetic variants under investigation explain only a small part of the variation in VWF:Ag levels. Consequently, these variants exhibit only small effects – when even detectable – on cardiovascular risk. This last problem is actually the main problem of current genetic association studies that use common

genetic variants. It is questionable whether we should continue with genetic association studies, when genetic testing may not improve the prediction of disease beyond classical risk factors or new biomarkers. Some believe that combining large numbers of SNPs may explain a larger fraction of the genetic variance and make more accurate risk prediction possible. However, others stress that the predictive value of genetic profiling will never be high enough so that it can be incorporated in clinical decision making [43]. Nevertheless, by expanding our knowledge on the genetic architecture of common diseases and quantitative traits, we will gain more insights in the etiology of cardiovascular disease.

In the absence of an association between *VWF* polymorphisms and the risk of CHD, the hypothesis that high VWF:Ag levels are causative for CHD can be questioned. However, a large number of studies showed the substantial predictive value of VWF for CHD incidence[7]. Furthermore, the fact that VWF has a role in the occurrence of CHD is illustrated by numerous fundamental studies. VWF is detected at sites of platelet accumulation in coronary artery thrombi[44-45]. Both in vitro studies and in vivo studies showed that antibodies targeting VWF inhibit platelet aggregation and reduce coronary artery thrombosis in animal model[46-47]. Finally, patients with VWD have a lower prevalence of CVD than the general population. These findings demonstrate that VWF has a role in the interplay of mechanisms leading to CHD.

Apart from whether VWF is a causal mediator in the pathophysiology of CVD, VWF is considered as an important target of new drug development for patients with acute arterial thrombosis. In chapter 10, we show that ALX-0081, a bivalent humanized Nanobody®, is able to inhibit *in vitro* platelet adhesion and aggregation completely in patients undergoing a percutaneous coronary intervention (PCI) procedure[48-51]. Considering the attractive antithrombotic function of this Nanobody®, it can be used to develop a complete drug to treat patients with stable coronary artery disease or acute thrombotic events, who will undergo a PCI procedure. *In vivo* experiments with a baboon model for unstable angina pectoris have shown that ALX-0081 has a high efficacy and a better safety profile than currently marketed antithrombotics[52]. At this moment a phase 2 trial is ongoing in high-risk PCI patients comparing the efficacy and safety of ALX-0081 to abciximab.

RECOMMENDATIONS FOR FUTURE DIRECTIONS

Next-generation sequencing will be one of the most important subsequent steps in genetic epidemiology. Sequencing of the whole exome or whole genome will facilitate the detection of genetic variants with a low frequency, but with large effect sizes. It has

been anticipated that these genetic variants will explain the missing heritability. Large consortia, including the CHARGE consortium, are currently working on these sequencing efforts and will present their data in de following year. Possibly these variants will have value for risk prediction models.

Another important research direction will be the discovery of the exact functional variant. Current genetic association studies that use linkage disequilibrium between SNPs identify SNPs that are in high LD with the causal variant. This may not only have led to an underestimation of the true effect, but also does not provide information on how a gene may modulate VWF levels or disease risk. More fundamental research is necessary to investigate this in more detail.

Finally, as VWF is an important target for new antithrombotic drug development, we should proceed with current studies that investigate compounds such as ALX-0081 and ARC1779. In the near future large randomized-controlled trials will be set up to investigate the efficacy and safety of these new drugs. This type of research may be the ultimate and most effective way to investigate causality between VWF and CVD.

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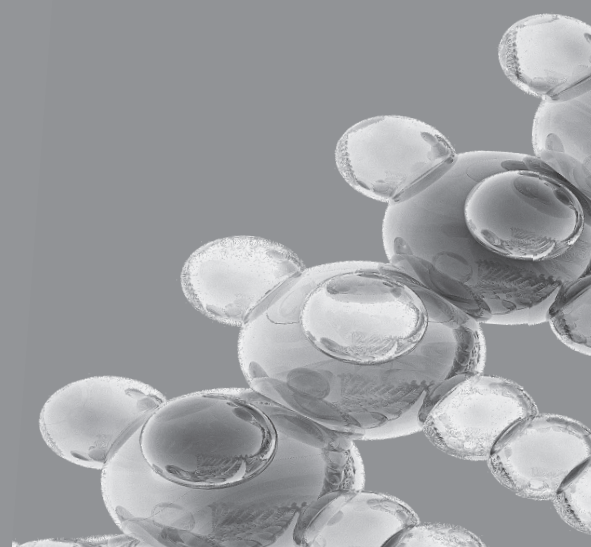
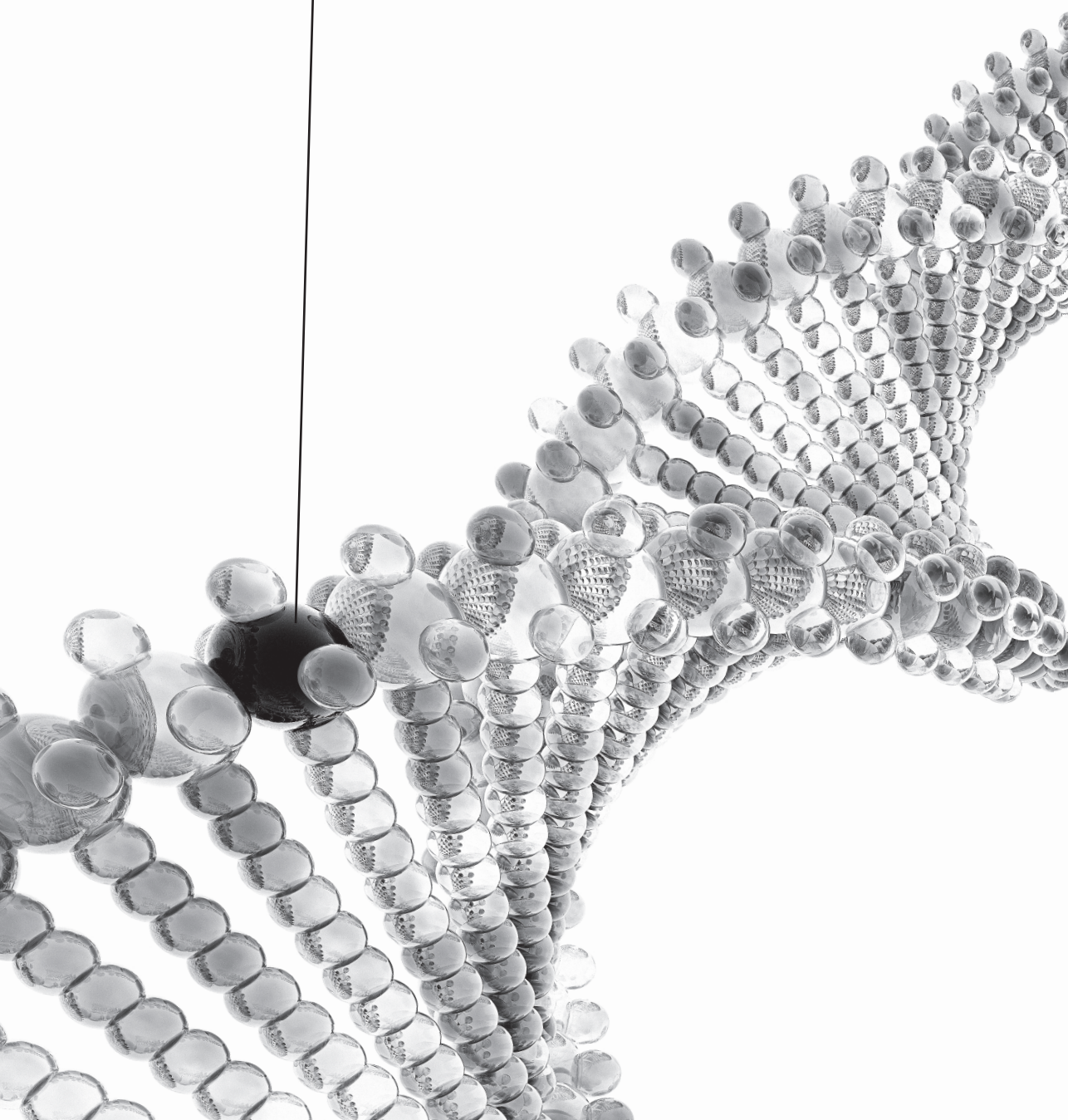


SUMMARY



Von Willebrand Factor (VWF) is an important player in blood coagulation, facilitating platelet adhesion and aggregation in order to maintain normal haemostasis. VWF antigen (VWF:Ag) levels have a large biological variation. This variation between and within individuals is caused by the effect of lifestyle factors and environmental factors, but most importantly by the effect of genetic factors. However, to date only part of the variability in VWF:Ag levels has been explained.

High VWF:Ag levels have been associated with an increased risk of arterial thrombosis, including coronary heart disease (CHD). However, it is still unknown whether VWF is an actual causal mediator in arterial thrombosis or whether high VWF levels primarily mirror endothelial dysfunction, which predisposes to atherosclerosis and subsequent arterial thrombosis. Since VWF levels are to a great extent genetically determined, genetic association studies can be used to investigate the non-confounded effect of VWF on the risk of CHD. The primary aim of this thesis was to unravel the role of VWF in the pathophysiology of arterial thrombosis. Secondly, we aimed to identify novel genetic determinants of VWF:Ag levels.

So far, many genetic association studies have been performed to assess the effect of genetic variations in the VWF gene *(VWF)* on VWF:Ag levels and VWF activity levels, and on the risk of arterial thrombosis. Also, we investigated genetic variations in other regulators of VWF, such as ABO blood group, ADAMTS13, thrombospondin-1 and the recently identified SNARE protein genes, *STXBP5 and STX2*. In **chapter 2**, we reviewed all available literature on this subject. One of our main observations was that only a limited number of studies had investigated the association between genetic variation, VWF:Ag levels or VWF activity, and the risk of arterial thrombosis in one study. In addition, the reviewed studies differed in study design, study population, and study endpoint, and were often underpowered. Nevertheless the studies provided interesting results that suggest that VWF is a causal mediator of arterial thrombosis, though without a definite answer. From this review it became clear that large prospective studies are required to further establish the role of VWF in the occurrence of arterial thrombosis. Therefore, we investigated the relationship between genetic variations across *VWF*, VWF:Ag levels and the risk of incident CHD in a large population-based study, the Rotterdam study, among patients aged 55 years and over (**chapter 3**). We identified three non-redundant single nucleotide polymorphisms (SNPs) in *VWF* that were highly significantly associated with VWF:Ag levels. Also, the association between high VWF:Ag levels and CHD risk was confirmed. However, the risk of CHD in this older population was not affected by these SNPs nor by any other genetic variant in *VWF*. Consequently, these findings did not strengthen the hypothesis of a strong causal relationship between high VWF:Ag levels and the occurrence of CHD in elderly individuals, but suggest that VWF is primarily a marker of CHD.

Next to *VWF*, numerous other candidate genes have been identified to play a role in the regulation of VWF:Ag levels. Therefore, we selected the thrombospondin genes and investigated the effect of genetic polymorphisms in *THBS1*, *THBS2*, and *THBS4* on VWF:Ag levels and CHD risk in the Rotterdam study (**chapter 4**). Genetic variation in *THBS1* and *THBS2* were weakly involved in the regulation of VWF levels. However, no evidence was found to support the relationship between genetic variation in thrombospondin genes and the occurrence of CHD, suggesting that the proposed link between thrombospondins and CHD is likely independent of VWF.

Recent developments in genetic epidemiology gave rise to a new era of genetic association studies in which the whole genome in relation to complex diseases and phenotypes can be investigated without a prior hypothesis. Recently, we participated in a meta-analysis of genome-wide association studies of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium that identified new genetic determinants of VWF levels, which were located within genetic regions than were not known to be involved in the regulation of VWF:Ag levels. Two of these novel loci were the syntaxin-binding protein 5 (STXBP5) gene and the syntaxin 2 (STX2) gene. Their encoding proteins interact specifically with SNARE complex proteins, such as SNAP23 and syntaxin-4, which have been shown to be involved in Weibel Palade Body (WPB) exocytosis, the well known mechanism for the secretion of VWF molecules by endothelial cells. In **chapter 5**, we expanded the previous findings of the CHARGE consortium in an independent case-control study among young patients with a first event of arterial thrombosis and healthy controls, the ATTAC study. In this study it was shown that genetic variations in *STXBP5* and *STX2* affect VWF:Ag levels and VWF collagen binding activity in young patients with premature arterial thrombosis. In addition, we observed that genetic variability in *STX2* is associated with the risk of arterial thrombosis in young individuals, though not via elevated VWF:Ag levels or activity levels. How genetic variation in STX2 modulates arterial thrombosis risk remains yet unknown and the underlying mechanisms should be elucidated.

VWF is primarily a risk factor for arterial thrombosis in healthy subjects, but also predicts cardiovascular risk in patients with already prevalent atherosclerotic disease, including CHD. In young patients with CHD little is known on the prognostic value of VWF. However, it is very important to recognize young CHD patients at risk for a recurrent event, since young patients form an excellent group for preventive cardiology. In **chapter 6,** we evaluated the prognostic implications of traditional risk factors, C-reactive protein (CRP), fibrinogen, and VWF in a unique and relatively large cohort of young patients with a first acute coronary syndrome. We show that although the survival after a first cardiac event in young patients is excellent (97%), the recurrent event rate (12%) after a median follow-up time of 4.2 years is quite high despite optimal medical therapy. In addition, high levels of CRP and fibrinogen, but not VWF, contribute independently

of classical cardiovascular risk factors to the risk of recurrent events in these young patients, suggesting that inflammation may be important for the prognosis of young patients with premature CHD.

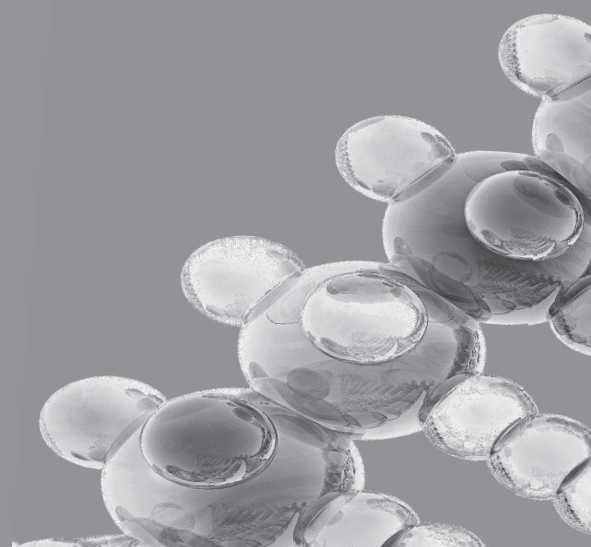
Uncovering new genetic determinants of VWF:Ag levels is not only important in the scope of arterial thrombosis, but also for individuals with low VWF:Ag levels. Low VWF:Ag levels are a diagnostic criterion for von Willebrand disease (VWD), the most common inherited bleeding disorder. In current medical practice it is hard to distinguish between subjects with physiologically low VWF:Ag levels and subjects with VWD, since subjects with moderately low VWF:Ag levels often have no mutations in *VWF*. Hence, considering the strong genetic component in VWF:Ag level regulation, there is a growing expectation that genetic variation in other genes than *VWF* must be involved in the pathogenesis of VWD. Therefore, we tested SNPs across the whole genome for associations with low VWF:Ag levels without a prior hypothesis in a meta-analysis of genome-wide association data from eleven population-based cohorts comprising 31.149 individuals of European ancestry (**chapter 7**). Five genetic loci were identified: *ABO*, *VWF*, *STXBP5, STAB2*, and *UFM1*. Four of these were also reported in the meta-analysis of the CHARGE consortium using the total, continuous VWF antigen levels as phenotype. This suggests that mechanisms that lead to low VWF levels partly differ from those that regulate VWF levels in general, but also share some attributes. Furthermore, our findings in this meta-analysis confirm the hypothesis that genes other than *VWF* may lead to low VWF:Ag levels. In line with the previous chapter, we aimed to assess the relationship between genetic variation in *STXBP5* and *STX2*, VWF:Ag levels, and the bleeding phenotype in patients previously diagnosed with type 1 VWD (**chapter 8**). Indeed, genetic variation in *STX2* was associated with VWF:Ag levels. Interestingly, genetic variation in *STXBP5* was associated with the bleeding phenotype in female type 1 VWD patients. These findings may explain part of the variation in VWF:Ag levels and bleedings symptoms in patients with type 1 VWD. Also, alongside known VWF mutations, genetic variations in *STXBP5* and *STX2* may help to diagnose individuals with low VWF:Ag levels in the future.

Since the encoding proteins of *STXBP5* and *STX2* are involved in the regulated secretion of VWF molecules, which is among others initiated by physical exercise, we investigated in **chapter 9** the effect of genetic variation in *STXBP5* and *STX2* on VWF:Ag level response to incremental exhaustive exercise in young and healthy volunteers. In addition, we aimed to identify other important mediators herein. Genetic variations in *STXBP5* and *STX2* were not associated with VWF:Ag increase upon physical exercise. To the contrary, performance-related determinants, such as physical fitness, appeared to be the most important factors in the VWF response to exercise.

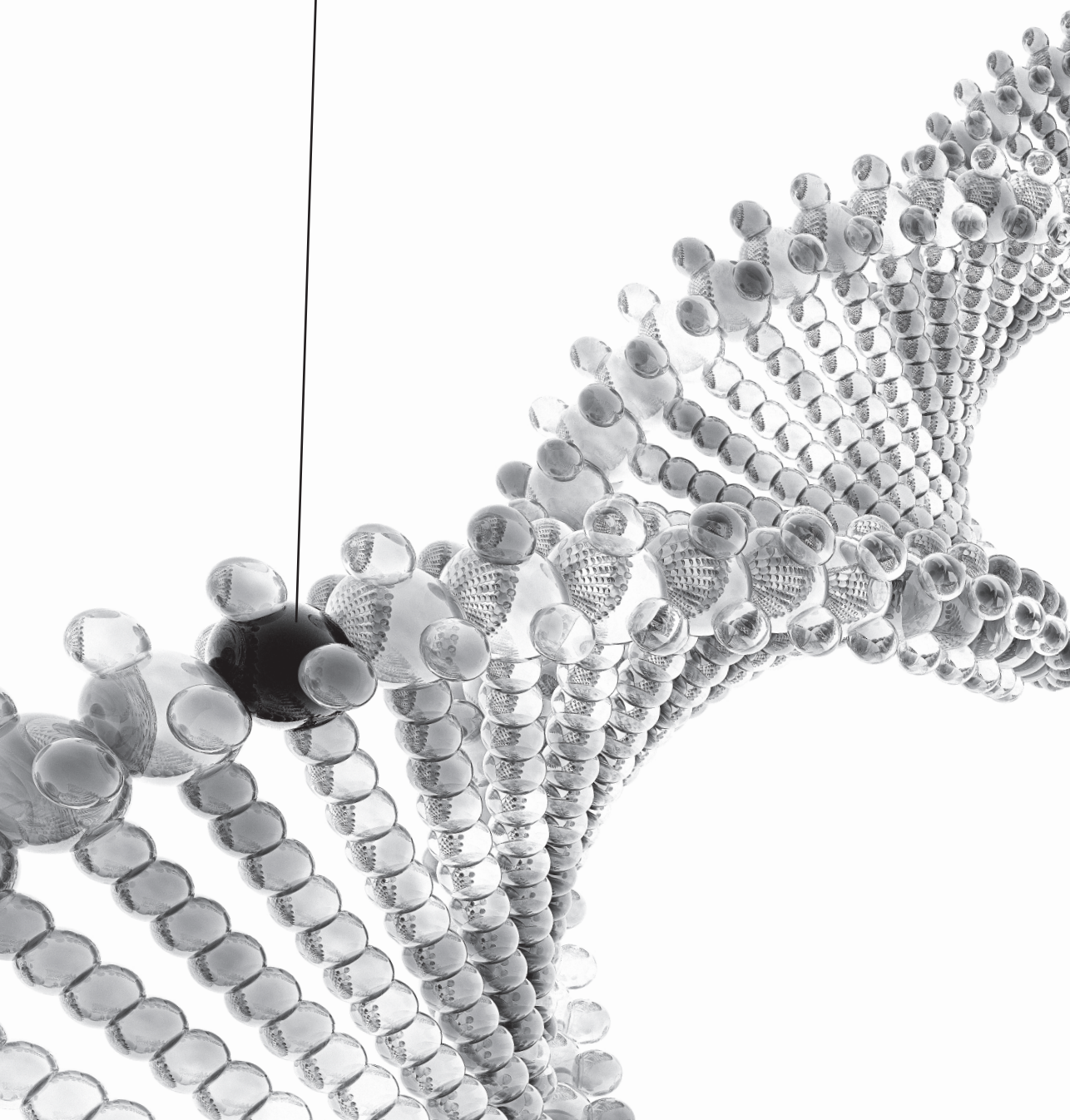
Apart from whether VWF is a causal mediator in the pathophysiology of arterial thrombosis, VWF is considered as an important target of new drug development for patients with acute arterial thrombosis. In **chapter 10**, we investigate ALX-0081, which

is a bivalent humanized Nanobody® that is able to block the interaction between VWF and its platelet receptor glycoprotein (GP) Ib by binding the A1 domain of the VWF molecule with high affinity. ALX0081 is able to completely inhibit *in vitro* platelet adhesion and aggregation in patients undergoing a percutaneous coronary intervention (PCI) procedure. This first proof of principle study requires future studies to investigate the pharmacokinetics, safety and efficacy of this new and promising anti-thrombotic drug candidate.

In conclusion, the executed studies described in this thesis have led to a better understanding of the genetic variability in VWF:Ag levels and the possible causal relationship between VWF and the occurrence of arterial thrombosis.



NEDERLANDSE SAMENVATTING



von Willebrand Factor (VWF) speelt een belangrijke rol in de bloedstolling. Als de vaatwand beschadigd raakt, zorgt VWF voor stolselvorming door initiatie van plaatjesadhesie en –aggregatie. VWF antigeen (VWF:Ag) gehalten in het bloed hebben een grote biologische variatie. Deze variatie wordt deels veroorzaakt door leefstijl- en omgevingsfactoren, maar voornamelijk door genetische factoren. Tot op heden kan nog maar een deel van de variatie in VWF gehalten verklaard worden.

Hoge VWF gehalten zijn geassocieerd met een verhoogd risico op arteriële trombose, zoals coronair hartlijden. Het is echter onbekend of VWF daadwerkelijk een causale rol speelt in de ontstaanswijze van arteriële trombose. Mogelijk weerspiegelen de hoge VWF gehalten alleen endotheel dysfunctie, wat ten grondslag ligt aan atherosclerose. Atherosclerose is de belangrijkste oorzaak van arteriële trombose. Om deze vraag te kunnen beantwoorden worden genetische associatiestudies gebruikt. Aangezien genetische variaties in het genoom al vanaf de conceptie aanwezig zijn, worden deze niet beïnvloed door leefstijl- en omgevingsfactoren. Daarom is het mogelijk om met genetische associatiestudies het directe effect van VWF op het risico van arteriële trombose te onderzoeken - zonder de invloed van andere factoren- en een oorzakelijk (causaal) verband aan te tonen.

Het belangrijkste doel van dit proefschrift was het ontrafelen van de rol van VWF in de pathofysiologie van arteriële trombose. Daarnaast had dit proefschrift als doel nieuwe genetische determinanten van VWF gehalten te identificeren.

In de afgelopen jaren zijn veel genetische associatie studies gedaan naar de relaties tussen genetische variaties in het VWF gen (*VWF*), VWF:Ag gehalten en de VWF activiteit, en het risico op arteriële trombose. Ook werden genetische variaties in andere genen onderzocht, zoals AB0 bloedgroep, *ADAMTS13*, *TSP-1*, *STXBP5* en *STX2*. In **hoofdstuk 2** staat een overzicht van de huidige literatuur met dit onderwerp. Opvallend is dat slechts een beperkt aantal studies de relaties tussen genetica, VWF:Ag gehalten of VWF activiteit, en het risico op arteriële trombose in één studie hebben onderzocht. Daarnaast verschillen de besproken studies in studieopzet, studiepopulatie en studie-eindpunt. Ook was de omvang van de meeste studies niet toereikend. Ondanks deze tekortkomingen laten de huidige studies aanwijzingen zien voor een causale rol van VWF in de ontstaanswijze van arteriële trombose. Echter, de resultaten van de besproken studies gaven hier nog geen definitieve conclusie over. Er was dus een dringende vraag naar grote prospectieve studies om de rol van VWF in het ontstaan van arteriële trombose verder te onderzoeken. Daarom hebben wij in een grote prospectieve cohort studie, de Rotterdam studie, de relatie onderzocht tussen genetische variaties in *VWF*, VWF:Ag gehalten en het risico op coronair hartlijden (**hoofdstuk 3**). In deze studie onder personen van 55 jaar en ouder werden drie genetische variaties in *VWF* geïdentificeerd die sterk statistisch significant geassocieerd zijn met VWF:Ag gehalten. Ook werd de relatie tussen hoge VWF gehalten en een verhoogd risico op coronair hartlijden bevestigd. Echter, het risico op coronair

hartlijden in deze populatie werd niet beïnvloed door genetische variaties in *VWF*. Deze resultaten steunen dus niet de hypothese dat er een sterke causale relatie is tussen VWF and coronair hartlijden, maar suggereren dat VWF primair een marker is van het risico voor coronair hartlijden.

Naast het VWF-gen zijn er ook andere kandidaat genen betrokken bij de regulatie van VWF gehalten, zoals de trombospondine-genen. In de Rotterdam studie werd het effect van polymorfismen in de trombospondine genen, *THBS1*, *THBS2* en *THBS4* op VWF:Ag gehalten en op het risico voor coronair hartlijden onderzocht (**hoofdstuk 4**). Genetische variatie in *THBS1* en *THBS2* waren zwak geassocieerd met VWF:Ag gehalten. Er werd geen bewijs gevonden voor een relatie tussen genetische variaties in trombospondine genen en het voorkomen van coronair hartlijden. Deze bevinding suggereert dat de mogelijke link tussen trombospondines en coronair hartlijden niet verklaard wordt door hogere VWF gehalten.

Recente ontwikkelingen in het veld van de genetische epidemiologie hebben aanleiding gegeven tot een nieuwe generatie genetische associatiestudies. Deze studies kunnen de relatie tussen genetische variaties in het hele genoom met complexe aandoeningen en fenotypen onderzoeken zonder een a priori hypothese. Wij hebben recent meegewerkt aan een meta-analyse van genoombrede associatie studies van het CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology) consortium. Met deze meta-analyse werden nieuwe genetische determinanten van VWF gehalten ontdekt. Deze genetische determinanten waren gelegen in genetische regionen die eerder nog niet gerelateerd waren aan de regulatie van VWF gehalten. Twee van de nieuwe genetische determinanten zijn *STXBP5* (syntaxin-binding protein 5) en *STX2* (syntaxin-2). Deze genen coderen voor eiwitten die een interactie kunnen aangaan met de zogenaamde SNARE complex eiwitten, zoals SNAP23 en syntaxin-4, die betrokken zijn bij de Weibel Palade Body (WPB) exocytose. WPB exocytose is het mechanisme waardoor opgeslagen VWF moleculen door het endotheel worden afgegeven aan de circulatie. In **hoofdstuk 5** worden de bevindingen van het CHARGE consortium gerepliceerd in een onafhankelijke case-control studie onder patiënten met een eerste arteriële trombose en gezonde controle personen, de ATTAC studie. In deze studie hebben wij laten zien dat genetische variaties in *STXBP5* en *STX2* invloed hebben op VWF gehalten en VWF collageen bindingsactiviteit. Daarnaast hebben wij aangetoond dat genetische variatie in *STX2* is geassocieerd met het risico op arteriële trombose. Dit wordt echter niet veroorzaakt door verhoogde VWF gehalten of VWF activiteit. Op welke manier genetische variatie in *STX2* het risico op arteriële trombose kan beïnvloeden blijft tot nu toe onduidelijk en moet verder opgehelderd worden.

Een hoog VWF gehalte is allereerst een risicofactor voor arteriële trombose in gezonde personen, maar voorspelt ook het cardiovasculair risico bij patiënten met al aanwezig atherosclerotisch lijden, zoals coronair hartlijden. Bij jonge personen met prematuur

atherosclerotisch lijden is de prognostische waarde van VWF nog onbekend. Het is echter belangrijk om jonge personen met coronair hartlijden die een verhoogd risico hebben op een recidief te identificeren, omdat jonge personen een goede doelgroep zijn voor preventie. In **hoofdstuk 6** wordt de prognostische waarde van traditionele risicofactoren, C-reactive protein (CRP), fibrinogeen en VWF geëvalueerd in een unieke en grote groep van jonge personen met prematuur atherosclerotisch lijden. In deze studie vinden we dat de overleving na een eerste arteriële trombose uitstekend is (97%). Echter 12% van de patiënten krijgt een recidief na een mediane follow-up tijd van 4.2 jaar ondanks optimale medicamenteuze therapie. Daarnaast zijn hoge gehalten van CRP en fibrinogeen onafhankelijk van cardiovasculaire risicofactoren geassocieerd met het recidief risico. VWF gehalten waren niet geassocieerd met het recidief risico. Deze bevinding suggereert dat inflammatie belangrijk is bij de prognose van jonge personen met prematuur atherosclerotisch lijden.

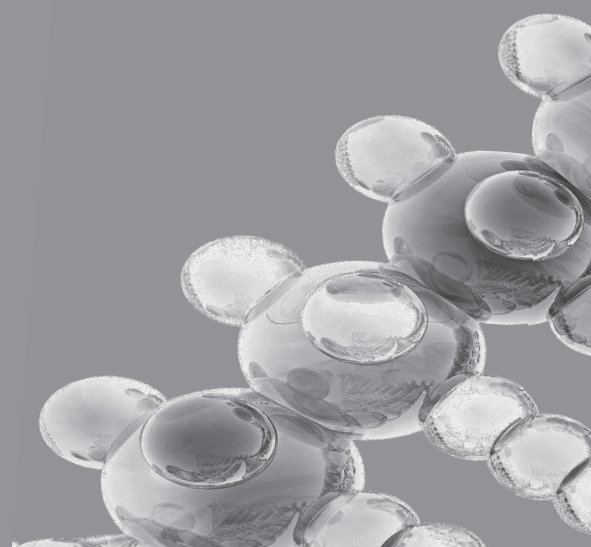
Het identificeren van nieuwe genetische determinanten van VWF gehalten in niet alleen belangrijk om de pathofysiologie van arteriële trombose op te helderen, maar ook voor personen met lage VWF gehalten. Lage VWF gehalten zijn een diagnostisch criterium voor de von Willebrand ziekte, de meest voorkomende bloederziekte. In de praktijk is het moeilijk om te differentiëren tussen personen met fysiologisch lage VWF gehalten en personen met lage VWF gehalten passend bij von Willebrand ziekte, omdat niet alle personen met lage VWF gehalten mutaties hebben in het VWF gen. Echter gezien de sterke genetische component in de regulatie van VWF gehalten in het bloed wordt er gedacht dat genetische variaties in andere genen dan het VWF gen betrokken zijn bij het ontstaan van de von Willebrand ziekte. Daarom hebben wij de relatie tussen genetische variaties in het gehele genoom met lage VWF gehalten onderzocht in een meta-analyse van elf genoombrede associatie studies bestaande uit 31.149 personen van Europese afkomst (**hoofdstuk 7**). Vijf genetische regionen werden geïdentificeerd: *ABO, VWF, STXBP5, STX2* en *UFM1*. Vier van deze genetische regionen werden al eerder gerapporteerd door het CHARGE consortium en waren significant geassocieerd met continue VWF gehalten. Deze bevinding suggereert dat de mechanismen die specifiek leiden tot lage VWF gehalten deels verschillen van de mechanismen die VWF gehalten reguleren onder normale omstandigheden, maar ook veel overeenkomsten hebben. Daarnaast bevestigen onze resultaten de hypothese dat genetische variaties in andere genen dan het VWF gen kunnen leiden tot lage VWF gehalten. In vervolg op het vorige hoofdstuk hebben wij de relatie tussen genetische variatie in *STXBP5* en *STX2*, VWF gehalten en het bloedingsfenotype bij patiënten met de von Willebrand ziekte type I onderzocht (**hoofdstuk 8**). In deze studie vonden we ook dat genetische variatie in *STX2* was geassocieerd met VWF gehalten. Een andere interessante bevinding is de relatie tussen genetische variatie in *STXBP5* en bloedingssymptomen bij patiënten met von Willebrand ziekte type I. Deze bevindingen verklaren deels de variatie in VWF gehalten

en bloedingssymptomen bij patiënten met von Willebrand ziekte. In de toekomst kunnen de genetische variaties in *STXBP5* en *STX2* naast de al bekende *VWF* mutaties helpen bij het stellen van de diagnose van personen met lage VWF gehalten.

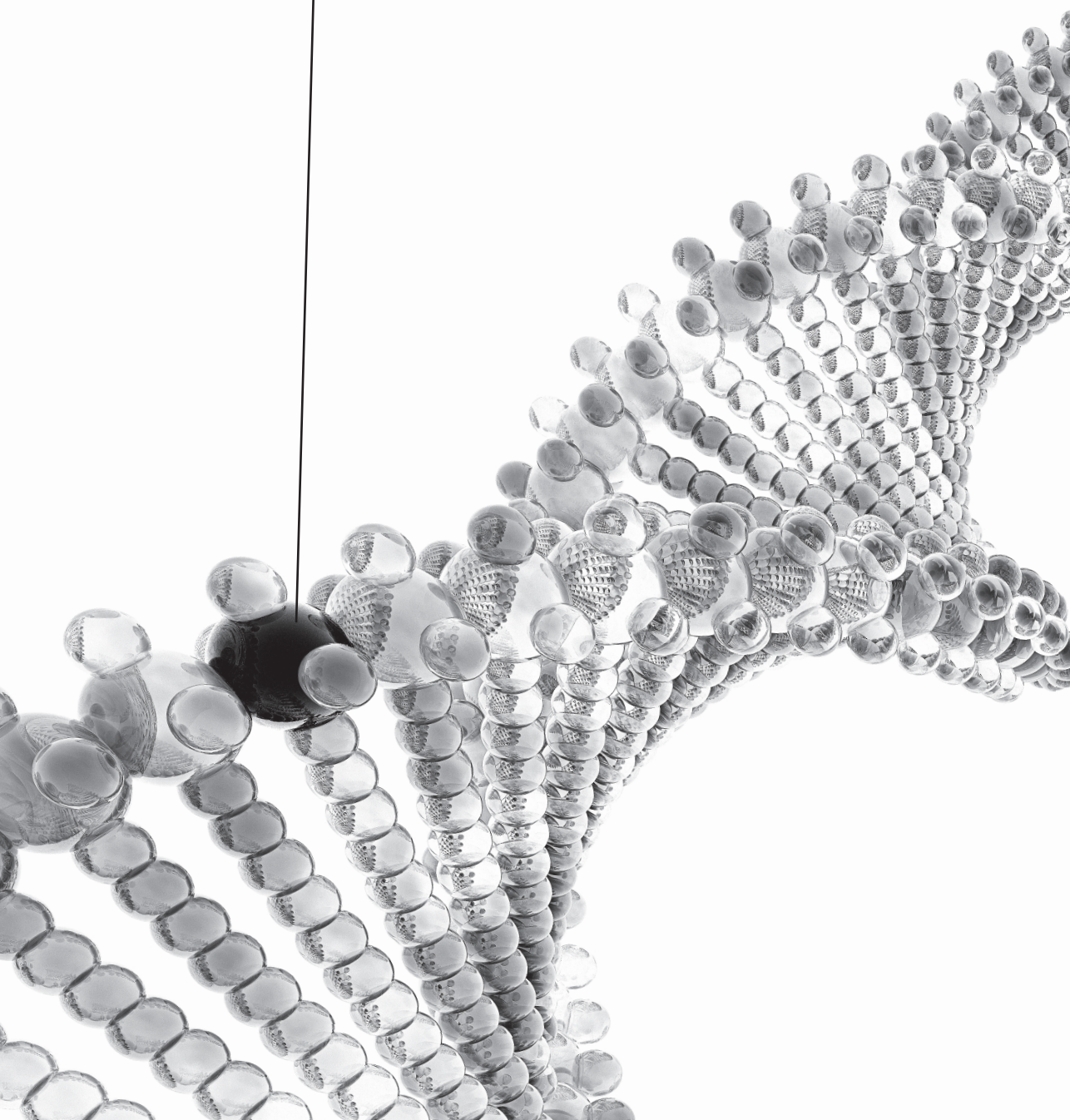
STXBP5 en *STX2* coderen voor eiwitten die betrokken zijn bij de gereguleerde afgifte van VWF moleculen door endotheelcellen. Deze gereguleerde afgifte kan onder andere geactiveerd worden door lichamelijke inspanning. In **hoofdstuk 9** onderzoeken we bij jonge gezonde vrijwilligers het effect van genetische variaties in *STXBP5* en *STX2* op VWF gehalten na intensieve lichamelijke inspanning. Daarnaast had deze studie als doel belangrijke determinanten van de VWF gehalte stijging als gevolg van lichamelijke inspanning te vinden. In deze studie zagen we dat genetische variaties in *STXBP5* en *STX2* niet geassocieerd waren met de VWF gehalte stijging als gevolg van lichamelijke inspanning. Getraindheid, lichamelijke conditie en de intensiteit van de inspanningstest waren daarentegen sterk bepalend voor stijging van het VWF gehalte.

VWF kan gebruikt worden als aangrijpingspunt voor de ontwikkeling van nieuwe antistollingsmedicijnen voor bijvoorbeeld patiënten met acute arteriële trombose. In **hoofdstuk 10** wordt ALX-0081 onderzocht, dat de interactie tussen VWF en de plaatsjesreceptor GPI kan blokkeren door aan het A1 domein van VWF te binden met hoge affiniteit. ALX-0081 kan *in vitro* plaatjes adhesie en aggregatie volledig remmen in bloed van patiënten die een percutane coronaire interventie ondergaan. Deze voorlopige resultaten laten zien dat ALX-0081 een veelbelovende kandidaat is voor de ontwikkeling van een nieuw antistollingsmedicijn. Echter, er zijn meer studies nodig om de farmacokinetiek, veiligheid en effectiviteit van ALX-0081 te onderzoeken.

Concluderend hebben de studies beschreven in dit proefschrift geleid tot een uitbreiding van de kennis over de genetische variabiliteit van VWF gehalten en de mogelijke causale relatie tussen VWF en het ontstaan van arteriële trombose.



LIST OF PUBLICATIONS



MANUSCRIPTS BASED ON STUDIES DESCRIBED IN THIS THESIS

Chapter 2

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

Chapter 3

von Willebrand Factor plasma levels, genetic variations, and Coronary Heart Disease in an older population. *(**Van Loon JE**, Kavousi M, Leebeek FWG, Felix JF, Hofman A, Witteman JCM, De Maat MPM. J Thromb Haemost. 2012;10(7):1262-9)*

Chapter 4

Relationship between Thrombospondin gene variations, von Willebrand Factor levels and the risk of coronary heart disease in an older population. *(**Van Loon JE**,De Maat MPM, Hofman A, Witteman JCM, Leebeek FWG. J Thromb Haemost. 2011;9(7):1415-7)*

Chapter 5

Effect of genetic variations in Syntaxin Binding Protein-5 and Syntaxin-2 on von Willebrand Factor concentration and cardiovascular risk. *(**Van Loon JE**, Leebeek FWG, Deckers JW, Dippel DWJ, Poldermans D, Strachan DP, Tang W, O'Donnell ChJ, Smith NL, De Maat MPM. Circ Cardiovasc Genet. 2010;3(6):507-12.)*

Chapter 6

Prognostic markers in young patients with premature coronary heart disease *(**Van Loon JE**, De Maat MPM, Deckers JW, Van Domburg RT, Leebeek FWG. Atherosclerosis. 2012;epub.)*

Chapter 7

Genome-wide association studies identify genetic loci for low von Willebrand Factor levels. *(**Van Loon JE** et al. Submitted)*

Chapter 8

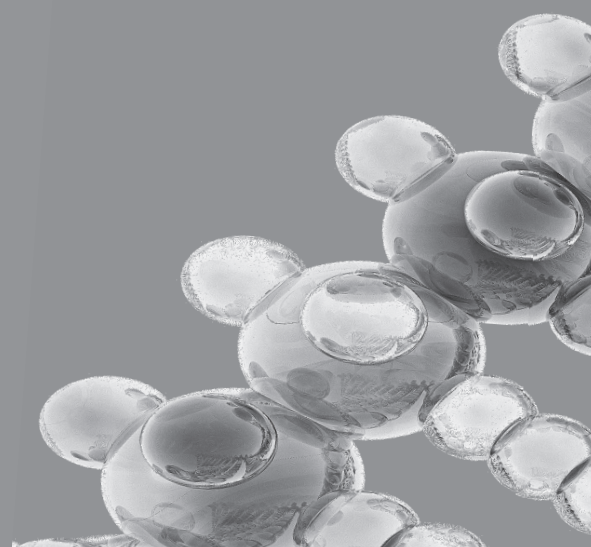
Genetic variations in SNARE protein genes determine von Willebrand Factor plasma levels and bleeding phenotype in patients with type 1 von Willebrand Disease. *(**Van Loon JE**, Sanders YV, De Wee, EM, Kruip MJHA, De Maat MPM, Leebeek FWG. PlosOne.2012;7(7):e40624)*

Chapter 9

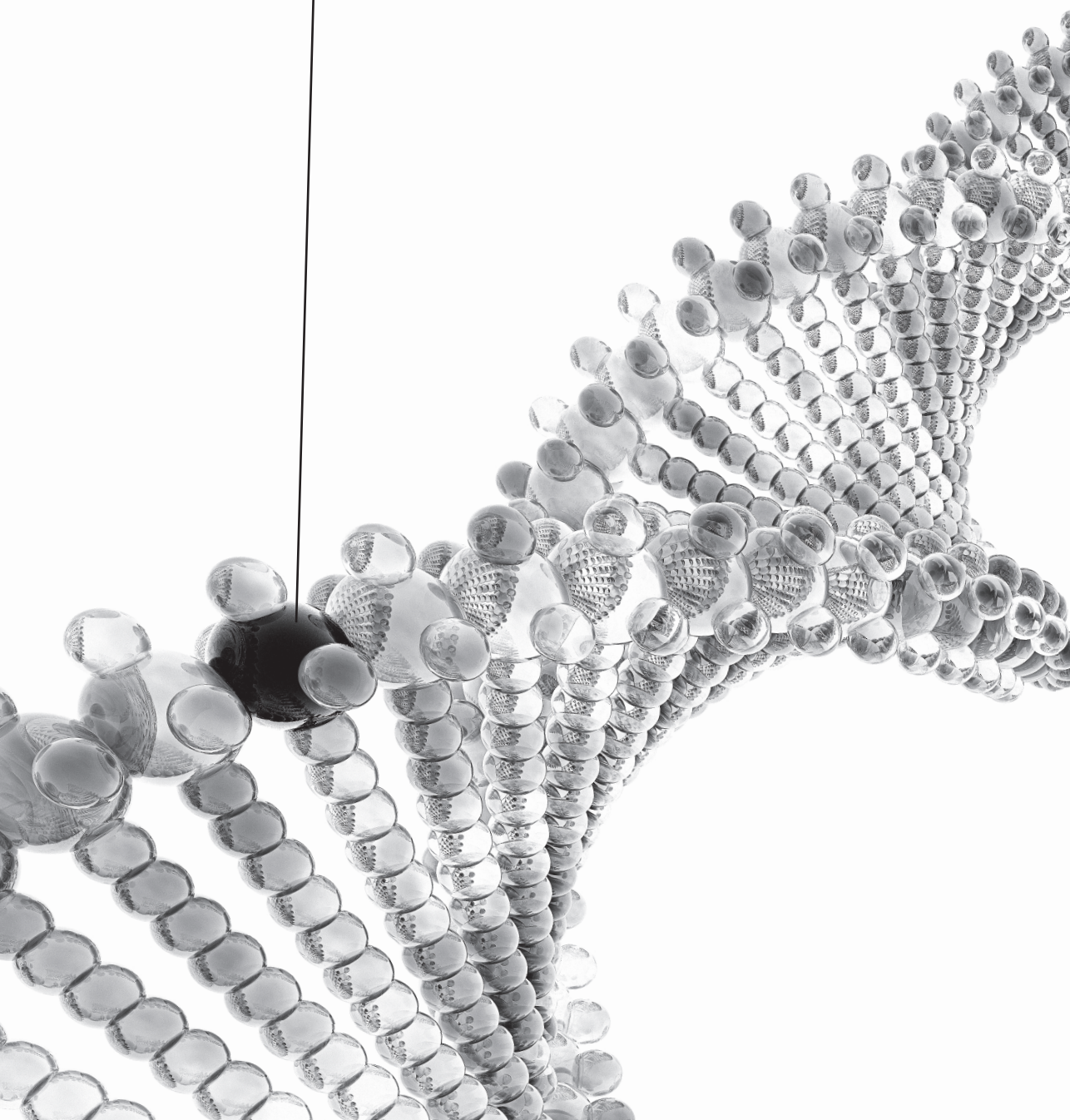
Physical Fitness determines the von Willebrand Factor Response to Exhaustive Physical Exercise. ***(Van Loon JE,*** *Sonneveld MAH, Praet S, De Maat MPM, Leebeek FWG. In preparation)*

Chapter 10

The in vitro effect of the new antithrombotic drug candidate ALX-0081 on blood samples of patients undergoing PCI. *(**Van Loon JE**, De Jaegere PPT, Ulrichts H, Van Vliet HHDM, De Maat MPM, De Groot PhG, Simoons ML, Leebeek FWG. Thromb Haemost. 2011;106(1):165-71)*



SCIENTIFIC SESSIONS / AWARDS AND PRICES



SCIENTIFIC SESSIONS

2012 **Symposium Dutch Society of Thrombosis and Haemostasis (NVTH), Koudekerke, the Netherlands**

· Oral presentation: *"Von Willebrand Factor response to incremental exhaustive exercise"*

2012 **Atherosclerosis, Thrombosis and Vascular Biology Scientific sessions, Chicago, United States**

· Poster presentation: *"Prognostic markers in patients with premature coronary heart disease".*
(Travel grant Dr. W. Stiggelboutprogramma Dutch Heart Foundation)

2011 **International Society of Thrombosis and Haemostasis XXIII, Kyoto, Japan**

· Oral presentation: *"Genetic variations in SNARE protein genes determine von Willebrand factor plasma levels in patients with type I von Willebrand disease".*

· Oral presentation: *"Genome-wide association studies identify new determinants of low von Willebrand factor plasma levels".*
(Travel grant Dr. W. Stiggelboutprogramma Dutch Heart Foundation)

2010 **European Society of Cardiology scientific sessions, Stockholm, Sweden**

· Poster presentation: *"Von Willebrand Factor, Genetics, and Coronary Heart Disease: New insights in their relationship".*
(Travel grant Dr. W. Stiggelboutprogramma Dutch Heart Foundation)

2010 **Symposium Dutch Society of Thrombosis and Haemostasis (NVTH), Noordwijkerhout, the Netherlands**

· Oral presentation: *"High von Willebrand Factor concentration: a causal risk factor for Coronary Heart Disease?"*

2009 **International Society of Thrombosis and Haemostasis XXII, Boston, United States**

· Poster presentation: *"Von Willebrand Factor does not predict outcome in unstable angina".*

AWARDS AND PRICES

2012 **Award of excellence**
Abstract award, annual symposium Dutch Society of Thrombosis and Haemostasis (NVTH)

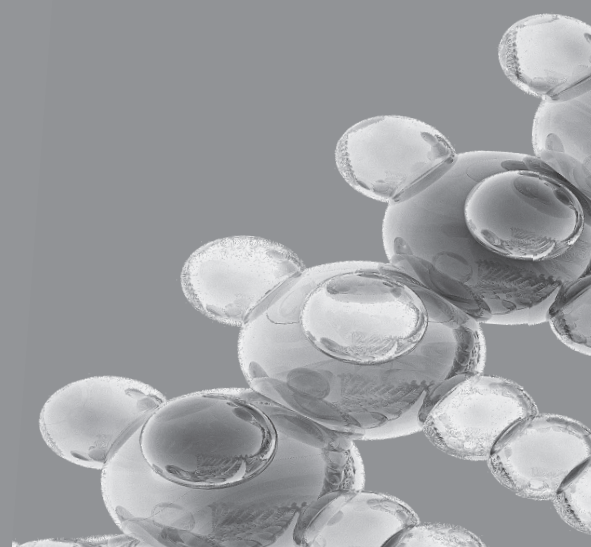
2011 **Young Investigator Award**
International Society of Thrombosis and Haemostasis XXIII, Kyoto, Japan

2011 **CSL Behring Prof. Heimburger Award 2011**

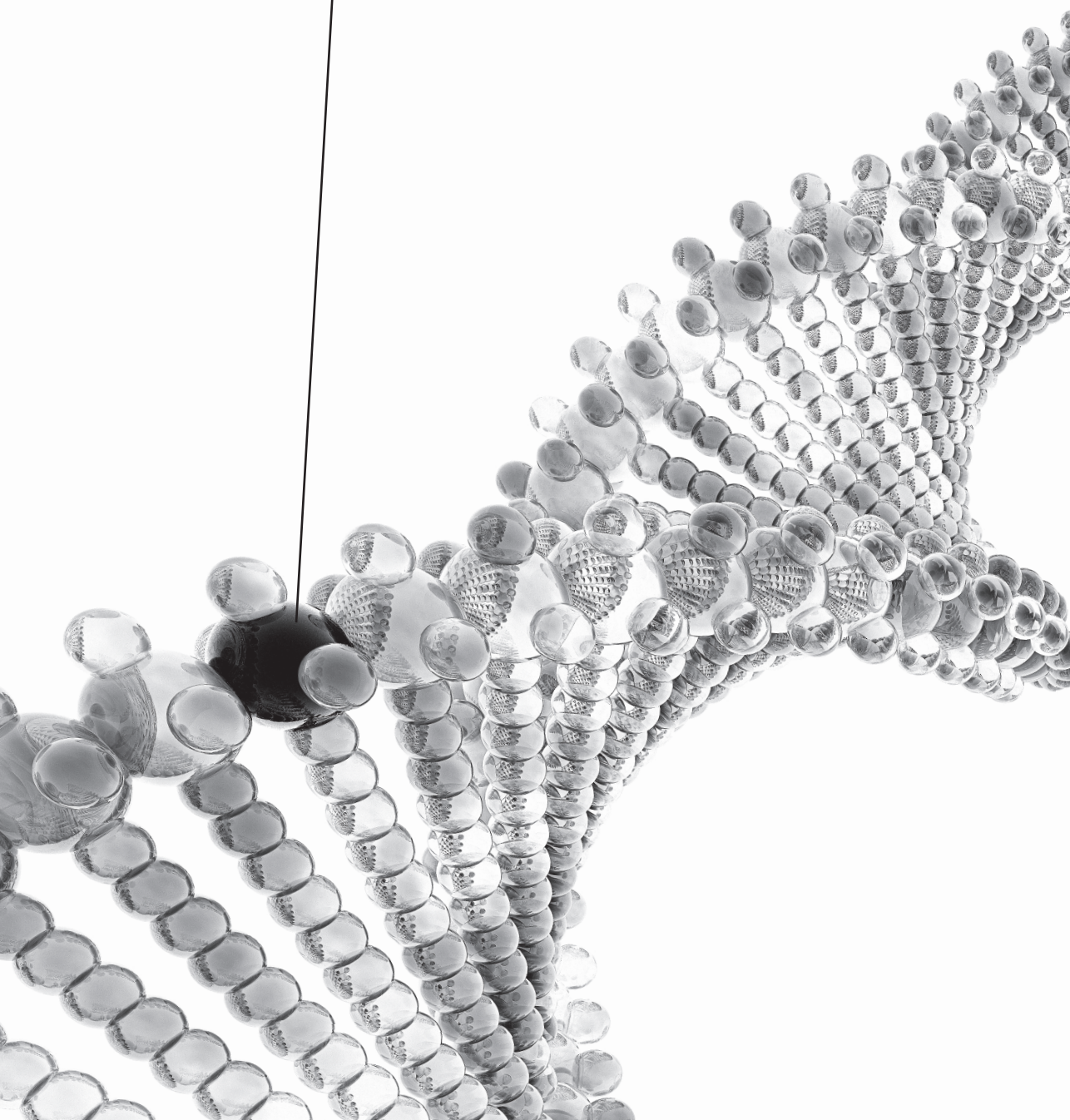
2010 **Dr. J. Stibbe Bokaal**
Best oral presentation, annual symposium Dutch Society of Thrombosis and Haemostasis (NVTH)

2007 **Gerrit Jan Mulder Award**
Best research project 2006.

2006 **Erasmus University Medical Centre Rotterdam Scholarship**
Funding for a research project at the University of Maryland, Baltimore, United States

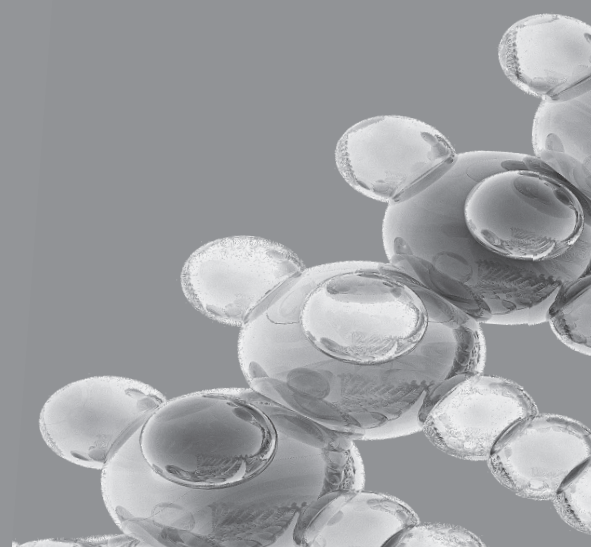


CURRICULUM VITAE

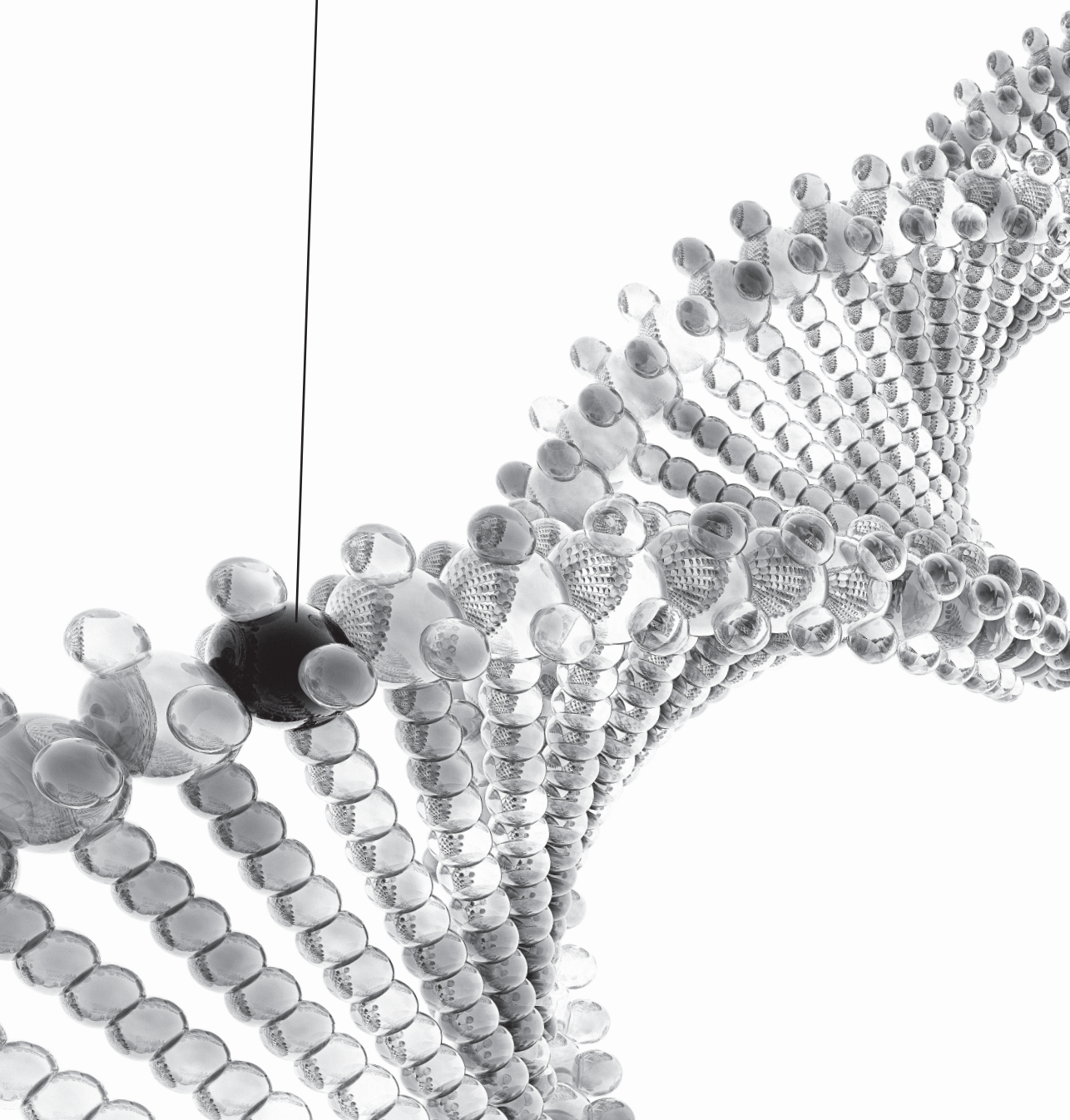


CURRICULUM VITAE

Janine van Loon werd geboren op 4 juni 1984 te Rotterdam. Zij volgde het voorgezet wetenschappelijk onderwijs aan het Emmauscollege te Rotterdam, alwaar zij in 2002 haar gymnasium diploma behaalde. In 2002 werd zij ingeloot voor de studie geneeskunde, die zij volgde aan de Erasmus Universiteit Rotterdam. Tijdens haar studie werd zij in 2006 geselecteerd om met een Erasmus MC-beurs vijf maanden wetenschappelijk onderzoek te doen aan de University of Maryland in Baltimore (VS), onder leiding van Prof. dr. A. Shuldiner. Voor dit onderzoek ontving zij in 2007 de Gerrit Jan Mulder prijs. Na haar terugkomst uit de Verenigde Staten vervolgde zij haar medische opleiding en behaalde in 2008 haar artsexamen. In oktober 2008 startte zij haar promotieonderzoek op de afdeling hematologie, onder supervisie van Prof. dr. Leebeek en dr. de Maat. Het promotieonderzoek resulteerde in het proefschrift dat nu voor u ligt. Per 1 juli 2012 is zij begonnen met de opleiding tot Cardioloog in het Erasmus MC (opleider dr. F.J. ten Cate). Momenteel volgt zij haar vooropleiding interne geneeskunde in het Sint Franciscus Gasthuis te Rotterdam (opleider dr. A.P. Rietveld).



DANKWOORD



Mijn promotietijd was een fantastische tijd! Het was echter nooit zo'n succes geworden zonder de hulp, begeleiding en steun van een aantal personen. In het hiernavolgende wil ik deze personen graag bedanken.

In de eerste plaats wil ik alle deelnemers aan de verschillende wetenschappelijke onderzoeken die genoemd worden in dit proefschrift hartelijk danken voor hun deelname. Wetenschappelijk onderzoek is simpelweg niet mogelijk zonder personen die zich hiervoor beschikbaar stellen. "Dank u wel!"

Ik bedank de kleine commissie, Prof. dr. Zijlstra, Prof. dr. Meijers en Prof. dr. Sonneveld voor het kritisch lezen van mijn proefschrift en de bereidheid zitting te nemen in de oppositie.

Prof. dr. Leebeek, beste Frank, ik denk dat ik mij gelukkig mag prijzen met jou als promotor. Je hebt me geleerd zeer zelfstandig te werken, wat soms veel motivatie vergde, maar wat mij heeft gevormd tot de onderzoeker die ik nu ben. Ondanks je drukke bestaan maakte je tijd voor vragen, beslissingen, discussies of gewoon voor een handtekening. Je bent een bedreven wetenschapper en clinicus, maar in de eerste plaats een gezinsman. Ik kijk uit om in de toekomst met je samen te werken!

Dr. de Maat, beste Moniek, ik had me geen betere "side-kick" kunnen wensen. Jouw biochemische blik op mijn klinische stukken was onmisbaar. Ook het snelle nakijkwerk hield de vaart erin, met alle projecten waar ik soms tegelijkertijd mee bezig was. Ik heb jouw begeleiding als zeer waardevol ervaren, dank daarvoor.

De stollingsgroep. Dick, ik bewonder je kennis en bedank je voor je uitstekende vragen tijdens de werkbesprekingen. Marieke, ook jij gaf een kritische noot aan de werkbesprekingen. Ik zal je zeker in consult vragen! Shirley: eindelijk hebben we er een pittige, maar ook gezellige, post-doc bij. Ik heb er vertrouwen in dat je ver gaat komen. Joyce, wat zou onze werkgroep of liever gezegd de hele afdeling hematologie, zonder jou moeten? Ik heb genoten van je verhalen en je zelfgebakken taarten. Michelle, ik ben blij met jou als "opvolgster." Je bent echt een leuke meid en een waardevolle aanvulling voor de groep. Yvonne, ik heb bewondering voor het enthousiasme waarmee je WIN te lijf gaat en ik weet zeker dat het allemaal gaat lukken. Carina, de jongste van het stel, maar nu al zo gedreven, jij komt er wel. Lieve stollingsmeiden, ik vond het supergezellig met jullie (misschien nog een keer een restaurantje uitproberen?).

De oudgedienden: Goran, Tamara, Marjolein, Marianne, Elim, Eva, Jasper en Reinilde. ATTAC- inclusie, leuke congressen en cursussen, samenwerking en hulp op het lab: in vele opzichten bedankt. Verder wil ik iedereen van het diagnostiek stollingslaboratorium bedanken en dan met name Hans, Jan en Audrey: dank voor jullie hulp tijdens de ATTAC- en de RESPONSE-studie.

Ee1389. Jasper, wie had gedacht dat de "hardrockuR" en de "doktuR" het zo goed zouden vinden. Ik vond het ontzettend leuk om drie jaar naast jou te zitten. Je flauwe grappen en slechte Duits waren een welkome afwisseling van mijn fulltime computerwerk. We

zien elkaar vast nog op "gezichtsboek" of bij een leuke cabaretvoorstelling. Natalie, ik bewonder je doorzettingsvermogen met je drukke labwerk en tussendoor ook nog even huisje, boompje, beestje. Ik wens je heel veel geluk met je gezinnetje! François, onze serieuze werker, maar op z'n tijd in voor een geintje. Annelieke, gezellig dat je bij ons kwam zitten. Rowan en Erdogan succes met de afronding van jullie promoties. Rogier, ik heb echt met je gelachen. Ik hoop dat je die VENI te pakken hebt. Ex-kamergenoten, Irene, Kerim, Zorica en Jurgen, ook jullie zorgden op tijd voor de nodige afleiding.

Hema13. Onno, ik heb genoten van ons slap geouwehoer bij de koffie. Ik hoop dat je Brazilië-avontuur gaat lukken. Nicole, jij was wel mijn meest enthousiaste collega. Succes met alles waar je nu mee bezig bent! Isabel, in voor een kwebbel op de gang. Merel en Roel veel geluk in de liefde en, bijna net zo belangrijk, met de proefschriften. Ans en Leenke, dank voor de goede (administratieve) zorgen. To all my other fellow PhD students: I wish you all the best!

Lydi en JW, jullie zijn mij al voor gegaan en hoe! We hebben al een hoop mooie dingen met elkaar meegemaakt: de studententijd, het Amerika-avontuur, de mijlpalen. Ik kijk uit naar onze gezamelijke etentjes waar we onze medische perikelen uitwisselen en proefkonijnen zijn voor nieuwe kookkunsten.

Emma en Egbert, jullie gaan een spannende tijd tegemoet als aanstaande ouders. Maar we blijven gewoon doorgaan met onze meer dan gezellige etentjes. Wellicht hebben jullie nog goede opvoedkundige adviezen voor ons (al dan niet over katten).

Peter, ik vind het ontzettend leuk dat jij mijn paranimf bent. Je was mijn maatje bij Ragnar, die goede oude tijd. Nu zit je alweer ruim anderhalf jaar in Luxemburg en zien we elkaar eigenlijk te weinig. Dit is een uitstekende gelegenheid om je weer even terug te halen naar Nederland en mij als niet-medico bij te staan bij mijn verdediging.

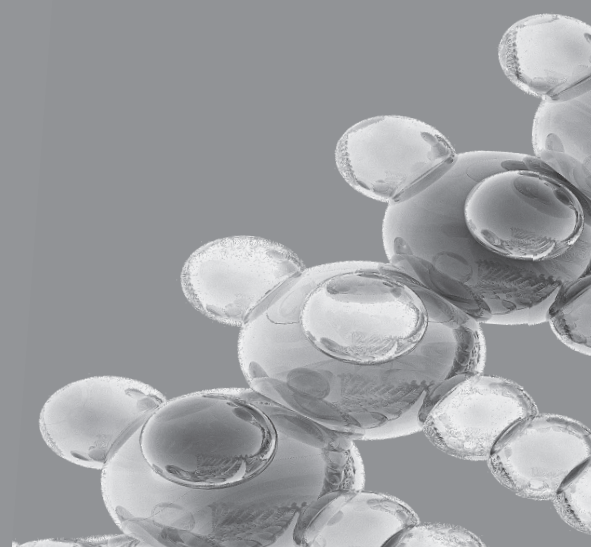
Simone, vanzelfsprekend ben jij mijn paranimf. Wat ben ik blij dat ik zo iemand als jij had tijdens mijn promotie! Jouw gezellig was onmisbaar. Ik kon altijd bij je komen spuien als dat nodig was of even koffie met je drinken als de letters over mijn scherm dansten. We waren roommates tijdens alle cursussen en congressen, waar we (oke door mijn geklets) nauwelijks aan slapen toekwamen. Ik wens je heel veel succes met alles wat komen gaat, ik heb er het volste vertrouwen in!

Heike en Ron, lief stel. Eigenlijk was het bij jullie al vanaf het begin als een warm bad voor mij. Jullie hartelijkheid is "one of a kind!" Bedankt voor jullie meeleven tijdens de studie, promotie en inmiddels bij alle ziekenhuisperikelen. Ik geniet van de doordeweekse roti, en ook het lekkere bijkomen in Frankrijk. Joie de vivre!

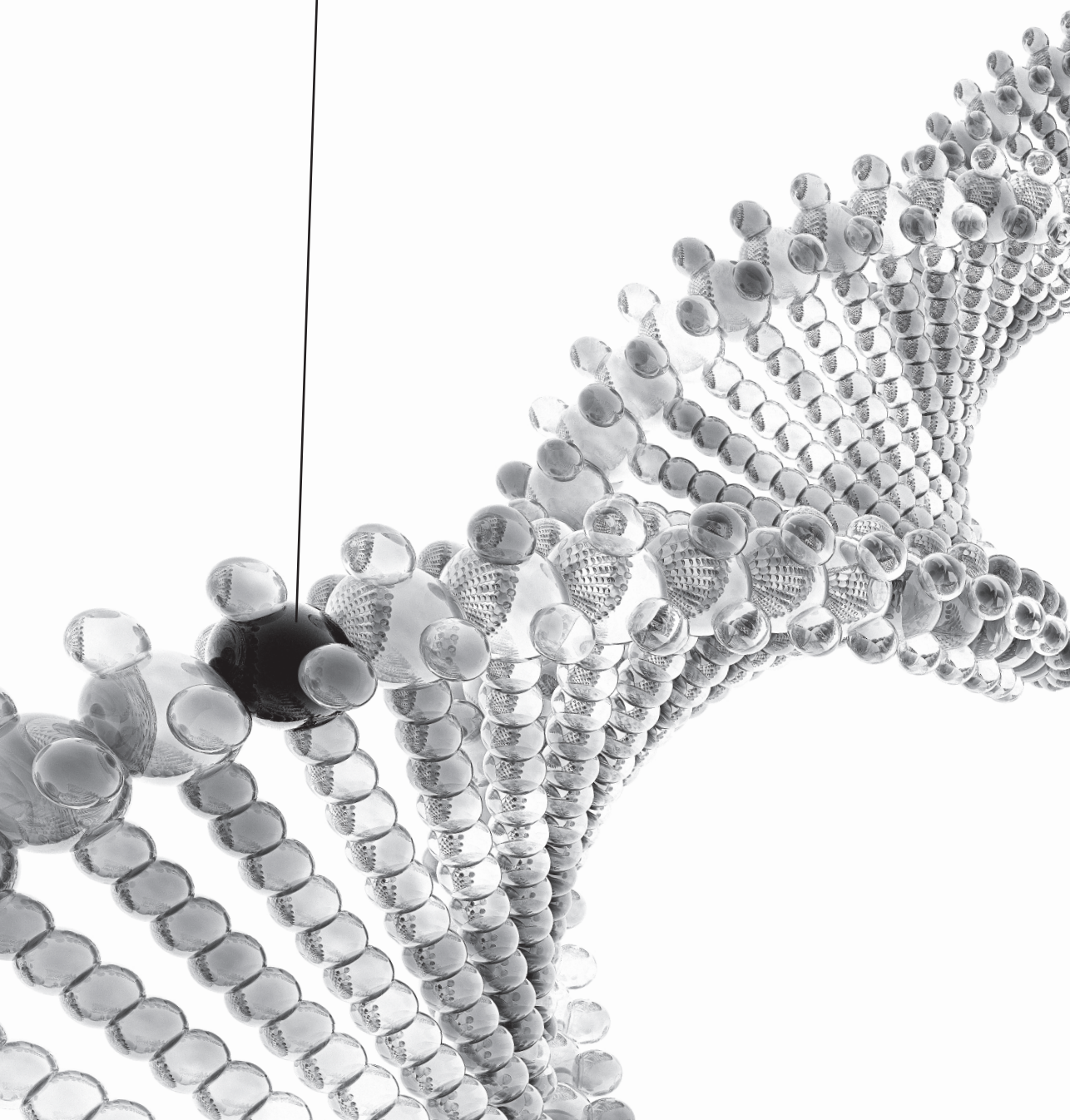
Lieve Hans en Rosie, papa en mama, jullie zijn mijn grootste fans. Door jullie liefde en steun ben ik gekomen tot waar ik nu ben. Ik bewonder het feit dat jullie altijd met me meeleven en zelfs mijn soms onnavolgbare onderzoeken proberen te volgen. Jullie staan voor me klaar, in het weekend met ouderwetse shoarma, op Schiphol om me op te halen na een of ander congres of gewoon doordeweeks aan de telefoon. Ik ben trots

dat ik zulke fantastische ouders heb! Ik kijk ernaar uit dat jullie me nog lang aan de zijlijn toejuichen.

Tot slot mijn lieve Gijs. Wat jij voor mij betekent is niet in woorden uit te drukken, zeker niet als ik van dit dankwoord geen sentimenteel epos wil maken. Ik ga hier dus niet vertellen dat ik je geweldig vind, dat je een uitstekend huisarts zult worden, dat jij er altijd voor me bent, dat je gewoon een knappe vent bent waar ik altijd mee kan lachen en die zelfs mijn "gemiep" af en toe aanhoort, dat je een levensgenieter bent waar ook ter wereld, maar ook gewoon in je hangmat op de Broersveld en dat jij degene bent die samen met mij aan een slotje hangt in Parijs, want dan zou ik je tekort doen. Ik hou simpelweg van je en ik heb zin in de rest van ons leven!



PhD PORTFOLIO



PHD PORTFOLIO SUMMARY

ERASMUS UNIVERSITEIT ROTTERDAM

Summary of PhD training and teaching activities

Name of PhD student: J.E. van Loon	PhD period: October 2008 – June 2012
Erasmus MC Department: Haematology	Promotor: Prof. dr. F.W.G. Leebeek
Research School: C.O.E.U.R.	Co-promotor: Dr. M.P.M. de Maat

1. PhD training

	Year	Workload Hours/ECTS
General academic skills		
Biomedical English Writing and Communication	2010	4.0
Research skills		
Introduction to clinical research (NIHES)	2009	0.9
Biostatistics for clinicians (NIHES)	2009	1.0
Modern statistical methods (NIHES)	2009	4.3
Genetic analysis in clinical research (NIHES)	2009	1.9
SNP's and human diseases (MolMed)	2009	1.9
In-depth courses (e.g. Research school, Medical Training)		
7x COEUR courses on molecular biology in atherosclerosis and cardiovascular research, vascular medicine, pathophysiology of ischemic heart disease, clinical cardiovascular epidemiology, neurovascular and peripheral vascular disease, arrhythmia research methodology, and intensive care research	2009-2010	10.5
3x AIO cursus of Ned. Ver. Trombose en Hemostase	2009-2011	6.0
Oral presentations		
2x Oral communication Internation Society of Thrombosis and Haemostasis Scientific Sessions	2011	0.2
2x Oral communicationSymposium Ned. Ver. Trombose en Hemostase	2010-2012	0.2
3x Poster presentation ISTH 2009, ESC 2010, ATVB 2012	2009-2012	0.3
International conferences		
2x International Society of Thrombosis and Haemostasis Scientific Sessions	2009-2011	3.0
European Cardiology Society Scientific Sessions	2010	0.9
Atherosclerosis, Thrombosis and Vascular Biology	2012	0.9

Seminars and workshops		
12x COEUR research seminar	2008-2012	4.8
2. Teaching activities		
Lecturing		
2x College keuzeonderwijs tweedejaars geneeskunde	2011-2012	0.2
Supervision HLO student (20 weeks)	2010	1.5
Totaal		42.5

von Willebrand Factor (VWF) is an important player in blood coagulation, facilitating platelet adhesion and aggregation in order to maintain normal haemostasis. VWF levels have a large biological variation. High VWF levels have been associated with an increased risk of arterial thrombosis, including coronary heart disease. However, it is still unknown whether VWF is an actual causal mediator in arterial thrombosis or whether high VWF levels primarily mirror endothelial dysfunction, which predisposes to atherosclerosis and subsequent arterial thrombosis. The studies described in this thesis will provide new insights in the relationship between genetic variants, VWF levels and the risk of arterial thrombosis.

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