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Determinants of plasma levels of von Willebrand factor and coagulation factor VIII

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Determinants of Plasma levels of von Willebrand Factor and Coagulation Factor VIII

Determinants of Plasma levels of von Willebrand Factor and Coagulation Factor VIII

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Table of Contents

Chapter 1	Introduction	9
Chapter 2	Plasma Coagulation Factor Levels in Venous Thrombosis	19
Chapter 3	Von Willebrand factor and its Propeptide: The Influence of Secretion and Clearance on Protein Levels and the Risk of Venous Thrombosis	43
Chapter 4a	Haplotypes Encoding the Factor VIII 1241Glu Variation, Factor VIII Levels and the Risk of Venous Thrombosis	63
Chapter 4b	Haplotypes Encoding the Factor VIII 1241Glu Variation and the Risk of Myocardial Infarction	83
Chapter 5	Beta 2 Adrenergic Receptor Polymorphisms: Association with Factor VIII and von Willebrand Factor Levels and the Risk of Venous Thrombosis	93
Chapter 6	Vasopressin 2 Receptor Gene Variations and the Regulation of Levels of von Willebrand Factor and Factor VIII and the Risk of Venous Thrombosis	101
Chapter 7	Plasma Levels of von Willebrand Factor Propeptide, von Willebrand Factor and Coagulation Factor VIII in Carriers and Patients with Nephrogenic Diabetes Insipidus	121
Chapter 8	Associations of Aquaporin 2 Gene Variations with Plasma Levels of von Willebrand Factor and Factor VIII and the Risk of Venous Thrombosis	143
Chapter 9	Associations of Blood Pressure and Antihypertensive Drugs with Plasma Levels of Coagulation Factor VIII	161
Chapter 10	Summary & Discussion	181
Chapter 11	Samenvatting	199
	Nawoord	207
	Curriculum Vitae	211

Chapter 1

Introduction

Introduction

Even though venous and arterial thrombosis are both defined by the occlusion of a blood vessel by a blood clot, the causes of venous and arterial thrombosis are usually different. In venous thrombosis, blood clots often form when blood flow is hindered and stasis occurs. Studies have shown that secondary hemostasis, the formation of insoluble fibrin fibers from the soluble plasma protein fibrinogen, plays an important role in venous thrombosis^{1,2}. In arteries, blood flow is generally high and thrombosis is usually induced by damage to the vessel wall, such as the development and possible rupture of an atherosclerotic plaque³. In contrast to venous thrombosis, primary hemostasis, the adhesion of activated platelets to the site of vascular damage and to each other, plays an important role in arterial thrombosis. Indeed, inhibitors of platelet aggregation are effective in the treatment and prevention of arterial thrombosis. Since the causes of venous and arterial thrombosis are different, most of the risk factors for developing either disease are different as well. However, both diseases also share several risk factors. For instance, the risks of both venous and arterial thrombosis are increased by elevated plasma levels of von Willebrand factor (VWF) and coagulation factor VIII (FVIII)⁴. VWF is a glycoprotein that circulates in the blood as large multimers⁵. VWF is synthesized by megakaryocytes and endothelial cells⁵. Its release into the circulation is constitutive, but large VWF multimers can be secreted from endothelial storage pools, known as Weibel Palade bodies (WPb), after a specific stimulus^{6,7}. When VWF is activated, it serves as 'glue' between platelets and a damaged vessel wall and between platelets themselves⁵. VWF therefore plays a vital role in primary hemostasis and elevated levels of VWF increase the risk of arterial thrombosis⁴. FVIII on the other hand is part of secondary hemostasis. It serves as a co-factor for activated FIX in the activation of FX, which in turn, together with activated FV, activates prothrombin to thrombin. Thrombin finally converts fibrinogen into fibrin. Factor VIII is synthesized mainly in the liver, but also in endothelial cells in the microvasculature of the lungs^{8,9}. Elevated levels of FVIII increase the risk of venous thrombosis¹⁰⁻¹⁶.

Even though VWF and FVIII are two separate proteins that have different functions, they are often studied together in the assessment of thrombosis risk. VWF is the carrier protein of FVIII and protects it against proteolytic cleavage^{5,8}. Without VWF, the half-life of FVIII is short and plasma levels remain low. Plasma levels of both proteins usually fluctuate together. Elevated levels of VWF and FVIII cluster within families¹⁷, which indicates that they are at least in part determined genetically. Furthermore, plasma levels of both VWF and FVIII are strongly influenced by the genetically determined ABO blood group; individuals with non-OO blood group genotypes have higher levels than individuals with the blood group OO genotype¹⁸. The mechanisms that underlie the substantial inter-individual variation in the general population however, remain poorly understood.

Thesis Outline

This thesis focuses on the determinants of plasma levels of VWF and FVIII. In general, plasma levels of a protein can be influenced by three processes; synthesis of the protein, secretion of the protein into the circulation and inactivation or clearance of the protein from the circulation. All three processes are discussed in this thesis, but the main focus is on the secretion of VWF (and FVIII) into the circulation.

In **Chapter 2**, the role of elevated levels of clotting factors in general in the risk of venous thrombosis is reviewed.

In **Chapter 3**, we look at the influence of both VWF secretion and VWF clearance on the risk of venous thrombosis. For this, we made use of the VWF propeptide. The VWF propeptide plays a role in the assembly of VWF multimers and also in the intra-cellular trafficking of VWF¹⁹⁻²². VWF propeptide is secreted in equimolar amounts with mature VWF, but has a separate lifespan and is cleared at a much faster rate than VWF^{5,23-25}. VWF propeptide can therefore be interpreted as a measure of the VWF secretion rate, and, together with molar plasma concentration of VWF, it can help estimate the clearance rate of VWF^{24,26-29}.

In **Chapters 4a and 4b**, we discuss variations in the gene encoding FVIII and their influence on levels of FVIII and on the risks of both venous (**4a**) and arterial (**4b**) thrombosis. We looked at one particular single nucleotide polymorphism (SNP), D1241E, which associates with plasma levels of FVIII^{30,31}. Because the SNP is present in at least three different haplotypes according to SeattleSNPs³², we studied effects of these three haplotypes separately to determine the functionality of D1241E.

In **Chapter 5**, we looked at the secretion of VWF and FVIII. Because adrenaline infusion can increase plasma levels of VWF and FVIII, we studied three known functional variations in the gene encoding the β 2 Adrenergic Receptor, ADBR2³³⁻³⁵.

In **Chapter 6**, we studied another G protein coupled receptor (GPCR), namely the arginine vasopressin 2 receptor (V2R). Upon stimulation, the V2R triggers renal water retention³⁶. However, stimulation of the V2R also leads to the release of WPb, which results in a rise in plasma levels of VWF and FVIII³⁷. Based on the results described in Chapter 6, we looked more carefully into the physiological processes of fluid homeostasis and blood pressure regulation. In **Chapter 7**, we studied families with Nephrogenic Diabetes Insipidus (NDI), a rare kidney disorder³⁶, as a model to investigate the effects of impaired renal water retention on the secretion of VWF and FVIII.

In **Chapter 8**, we studied this mechanism in a case control study on venous thrombosis. We looked at the effects of variations in the gene encoding aquaporin 2 (AQP2), a water channel of which the expression and function is regulated by the V2R³⁶.

Finally, in **Chapter 9**, we discuss the effects of blood pressure and antihypertensive medication, including diuretics, on plasma levels of FVIII.

Study Populations

For the research described in this thesis, we made use of several study populations, which are briefly described below.

The Leiden Thrombophilia Study (LETS) is a population based case control study which consists of 474 patients with a first episode of deep vein thrombosis and 474 healthy control subjects, frequency matched for sex and age to the patients. Plasma and DNA samples of the participants are available and blood pressure was measured. The design of this study has previously been described in detail^{38,39}.

The Study of Myocardial Infarctions Leiden (SMILE) is a population based case control study which consists of 560 male patients with a myocardial infarction and 646 male controls, frequency matched on age to the patients. As in the LETS, plasma and DNA samples of the participants were available. The design of this study has previously been described in detail⁴⁰.

The Risk of Arterial Thrombosis in Relation to Oral Contraceptives Study (RATIO) is a population based case control study on arterial thrombosis. The study includes female patients, younger than 50 years, with myocardial infarction, ischemic stroke or peripheral artery disease. A combined control group consisting of 925 women, younger than 50 years, with no history of coronary, cerebral or peripheral artery disease, was included. For the research described in this thesis, we looked only at 217 patients with myocardial infarction and 639 control subjects of whom plasma and DNA samples were available. The design of this study has previously been described extensively⁴¹.

The Cardiovascular Health Study (CHS) is a population based cohort study on cardiovascular disease. The CHS consists of two separate cohorts of 5201 and 687 individuals respectively. For the research described in this thesis, only the original CHS cohort of 5201 individuals was included. All participants were 65

years or older and came from four USA communities. The design of this study has previously been described in detail⁴².

The Factor Eight in Nephrogenic Diabetes Insipidus Study (FENDI) includes a large proportion of all Dutch families with NDI. Patients and their family members, including NDI carriers and unaffected individuals, were included, along with 48 healthy, unrelated control subjects. DNA and plasma samples are available of all participants. The design of this study is described in detail in Chapter 7 of this thesis.

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Chapter 2

Plasma Coagulation Factor Levels in Venous Thrombosis

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Summary

High plasma levels of several coagulation factors have been described to be associated with an increased risk of venous thrombosis. The mechanisms underlying these associations as well as the mechanisms involved in the regulation of plasma levels of coagulation factors however, are mostly unknown. Whether or not these factors should be included in the workup of patients with venous thrombosis remains to be determined.

In this review article we discuss the present knowledge on the effects of plasma levels of coagulation factors on the development of venous thrombosis.

Furthermore, we review recent findings and ideas on the mechanisms through which elevated plasma coagulation factor levels may influence thrombosis. And finally, we enter into the matter of the possible determinants of elevated plasma levels of coagulation factors.

Introduction

Venous thrombosis is a common disease with an incidence of one to two per 1000 individuals per year¹. Even though a venous thrombosis is rarely fatal, it is a disease with considerable morbidity. Deep venous thrombosis can lead to pulmonary embolization, which may have very severe consequences². A more common consequence of thrombosis is the development of post-thrombotic syndrome, of which the symptoms can vary from a mere discoloration of the skin to severe ulceration of the affected area³.

Thrombosis is a multicausal disease⁴. Many factors can contribute to shift the balance between coagulation and anticoagulation towards a state of hypercoagulability. Such 'risk factors' can be genetically determined, acquired or even a combination of both. Because of the complex pathophysiology of the disease, it is still not possible to predict whether or not an individual will develop venous thrombosis or not. To improve our ability to predict thrombotic disease, it is important to extend our understanding of the pathophysiological processes that lead to thrombosis and to identify new risk factors for the disease. In recent years, many risk factors for thrombosis have been discovered. An important group of risk factors is formed by elevated plasma levels of various

coagulation factors. In this review article, we will discuss the present knowledge on the association between plasma levels of coagulation factors and the risk of venous thrombosis, the mechanisms through which elevated coagulation factor levels influence risk and the determinants of elevated coagulation factor levels.

Fibrinogen

Fibrinogen is the precursor of the end-product of secondary hemostasis, fibrin. The soluble fibrinogen is cleaved by thrombin to form fibrin monomers which subsequently polymerize to form an insoluble fibrin clot. Factor XIII (FXIII) covalently links fibrin chains to each other to stabilize the fibrous network. Fibrinogen is comprised of dimers of three subunits, A α , B β and γ , which are encoded by three genes clustered together on chromosome 4.

In 1994, Koster et al⁵ first described the association between high levels of fibrinogen and the risk of a first venous thrombosis. In 199 patients and 199 healthy control subjects from the Leiden Thrombophilia Study (LETS), individuals with fibrinogen levels over 5 g/L had a nearly fourfold increase in risk of venous thrombosis. Furthermore, there was a dose-response relationship between levels of fibrinogen and the risk of thrombosis. In 2003, Van Hylckama Vlieg et al⁶ reanalyzed the LETS, now including 474 patients and 474 healthy controls. Again, an increase in the risk of thrombosis was observed for high levels of fibrinogen. However, after stratification on age, the risk remained only in the older participants of the LETS. In people younger than 45 years, no increase in risk was observed. After adjustment for other known risk factors, the increase in risk largely disappeared even in the elderly. The authors suggested that high levels of fibrinogen are not causal to the risk of thrombosis, but merely reflect other age-related risk factors. In 2000, Austin et al reported⁷ only a weak association between fibrinogen levels and the risk of thrombosis in a population of 91 African-American patients with a first or a recurrent thrombosis and 185 African-American controls. The risk observed was small. In individuals with fibrinogen levels over 5 g/L, the odds ratio was 1.5 and this finding was not statistically significant. Austin et al reported the absence of a

dose response relationship between fibrinogen levels and thrombosis risk. In addition, according to Tsai et al in 2002⁸ high levels of fibrinogen were not associated with venous thrombosis in the prospective Longitudinal Investigation of Thromboembolism Etiology (LITE). To our knowledge, the association between high fibrinogen levels and the risk of recurrent thrombosis has not been studied extensively.

Levels of fibrinogen are determined both environmentally and genetically⁹.

Fibrinogen is an acute phase protein which levels strongly increase after strenuous exercise, trauma and inflammation. It is very likely that a thrombotic event can increase levels of fibrinogen. However, Kamphuisen et al have shown that elevated levels of fibrinogen amongst cases of the LETS were not the consequence of an acute phase reaction induced by the thrombotic event¹⁰.

Gene polymorphisms in the fibrinogen β gene have been reported to be associated with levels of fibrinogen, however they appear not to be associated with the risk of venous thrombosis^{5,7,11}. In contrast, Uitte de Willige et al¹² recently reported a gene polymorphism in the fibrinogen γ gene which was not associated with levels of total fibrinogen but with reduced levels of fibrinogen γ' and with an increased risk of venous thrombosis in the LETS. They hypothesized that the single nucleotide polymorphism (SNP) c10034t, which is linked to several other variations in the fibrinogen α and β and γ genes, influences the splicing of fibrinogen γ pre-mRNAs, resulting in altered levels of the splicing variant γ' . Uitte de Willige et al reported that decreased levels of fibrinogen γ' and particularly decreased fibrinogen γ' /total fibrinogen ratios increase the risk of venous thrombosis in the LETS.

High levels of fibrinogen are associated with venous thrombosis, but it seems unlikely that they lie in the causal pathway. It is more likely that high levels of fibrinogen reflect the presence of other risk factors that lead to the development of thrombosis. However, the study performed by Uitte de Willige et al did show that changes in the γ' /total fibrinogen ratio do affect the risk of thrombosis¹². It is possible that changes in this ratio influence the functioning of fibrinogen or of the fibrin clots derived from it.

Prothrombin

Prothrombin is the precursor of thrombin which is the key player in secondary hemostasis. Thrombin can cleave fibrinogen to enable fibrin clot formation. Furthermore, thrombin functions as a positive feedback in the activation of secondary hemostasis and it activates platelets via interaction with and cleavage of protease-activated receptors (PARs). However, thrombin also exhibits anticoagulant and antifibrinolytic activities^{13,14}.

In 1996 Poort et al reported that elevated levels of prothrombin increase the risk of venous thrombosis in the LETS¹⁵. Levels in the highest quartile (>1.15 U/ml) increased the risk more than twofold compared to levels in the lowest quartile (<0.95 U/ml). The authors showed that there is a dose-response effect between levels and risk. These results have been confirmed in one other study¹⁶. Legnani et al reported in 2003 that in 152 pre-menopausal women with venous thromboembolism and 296 healthy female controls, prothrombin levels in the upper quartile (>1.11 IU/ml) increased the risk of thrombosis at least twofold. In combination with either the use of oral contraceptives or with a common SNP in the prothrombin gene, G20210A, which we will discuss below, the risk increased even further. In contrast, Folsom et al reported that in the prospective LITE study, high prothrombin antigen levels of prothrombin were not a risk factor of venous thrombosis¹⁷.

Poort et al also described the influence of a common SNP in the 3'UTR of the prothrombin gene, G20210A, on prothrombin levels and the risk of thrombosis¹⁵. Heterozygous carriers of the rare 20210A allele have increased levels of prothrombin compared to homozygous wild type carriers. Also, heterozygous carriers had a nearly threefold increase in the risk of venous thrombosis. These associations have been confirmed in several independent studies¹⁸⁻²². Also, the risk of thrombosis was increased in carriers of the prothrombin 20210A allele in the prospective LITE study¹⁷. The G20210A variation did not increase the risk of recurrent thrombosis^{23,24}.

Another SNP, A19911G, associated with slightly elevated prothrombin levels in the LETS was described in 2001 by Ceelie et al²⁵. Homozygous carriers of the G allele had 8 U/dl higher levels of prothrombin than homozygous carriers of the

A allele. A19911G alone did not affect the risk of venous thrombosis. However, in heterozygous carriers of the G20210A mutation, the odds ratio increased from 1.6 in 19911AA carriers to 4.7 in those with 19911G on the other allele (since 20210A is always linked to 19911A, 19911G must lie on the other allele). Several groups studied the mechanism through which these two prothrombin mutations could influence levels of prothrombin. It has been shown repeatedly that the 20210A variation leads to increased mRNA processing and an increased mRNA stability²⁶⁻²⁸. In 2004, Danckwardt et al showed that the 3'UTR of the prothrombin gene is highly susceptible to function-altering variations²⁹. The authors also described the functionality of another 3'UTR polymorphism, C20221T, which enhances mRNA processing. It is believed that 19911G increases pre-mRNA processing, by an increase in splicing efficiency³⁰. Soria et al showed in the GAIT study in 2000, that there was strong linkage between G20210A and prothrombin levels, indicating that 20210A is indeed directly responsible for increased prothrombin levels²¹.

Mechanisms through which high levels of prothrombin influence thrombosis risk have been studied, but not extensively. In 2001, Castaman et al³¹ studied resistance to activated protein C (APC) in 27 thrombosis families with the G20210A mutation. APC resistance increased with levels of prothrombin and with the presence of the 20210A allele. The increase in APC resistance in individuals heterozygous for G20210A and with high levels of prothrombin may help explain the increase in the risk of thrombosis. Furthermore, Kyrle et al³² reported that the endogenous thrombin potential, a measure of thrombin-forming capacity, was strongly increased in heterozygous carriers of 20210A and even more so in homozygous carriers of 20210A compared to controls. This was confirmed by Curvers et al in 2002³³. In addition, Koenen et al found that the APC- independent anticoagulant activity of protein S was reduced in carriers of the prothrombin 20210A allele and that this was due to the elevated prothrombin levels in these individuals³⁴. Finally, it was shown by Wolberg et al in 2003³⁵ that increasing levels of prothrombin can alter the structure of the fibrin clot formed. The authors speculated that this alteration in fibrin clot structure may contribute to the risk of thrombosis.

The literature has provided convincing evidence for the role of prothrombin gene variations associated with elevated plasma prothrombin levels in the development of thrombosis, even though additional data from prospective studies are needed. However, it remains unclear what the precise mechanism behind these effects is.

Factor VIII

Activated factor VIII (FVIII) is the cofactor for activated factor IX (FIX) in the activation of factor X (FX). The gene encoding FVIII is located on the X chromosome and mutations in the FVIII gene can lead to the bleeding disorder, hemophilia A. For stability in the circulation and protection from proteolytic cleavage by APC, FVIII depends strongly on its carrier protein von Willebrand Factor (VWF). FVIII levels in plasma are mainly determined by VWF levels, which in turn strongly depend on ABO blood group. Levels of VWF, and therefore of FVIII are higher in individuals with non-O blood groups³⁶. In 1995, Koster et al studied the influence of ABO blood group, levels of VWF and levels of FVIII on the risk of a first venous thrombosis in the first 301 patients and 301 controls of the LETS³⁷. In univariate analyses, all three parameters were associated with the risk of venous thrombosis. However in a multivariate analysis, FVIII proved to be the main determinant of risk. In individuals with FVIII levels over 150 IU/dl the risk of thrombosis increased nearly five-fold compared to individuals with levels lower than 100 IU/dl and a clear dose-response relationship was observed between risk and levels of FVIII. The risk increase caused by high levels of VWF disappeared completely in the multivariate analysis, for ABO blood group pheno- and genotype however, a small effect remained^{37,38}. The results from the LETS have been confirmed in a number of other independent studies³⁹⁻⁴⁶. Furthermore, in the prospective LITE study, high levels of FVIII proved to be a risk factor of thrombosis⁸. Besides increasing the risk of a first venous thrombosis, high levels of FVIII may also increase the risk of recurrences, even though the literature is not entirely consistent^{24,47,48}.

As mentioned above, VWF is the main determinant of levels of FVIII. However, other than ABO blood group, which explains up to 30 % of variability in VWF and FVIII levels, little is known about the determinants of high levels of VWF and FVIII. Since VWF and FVIII are acute phase proteins, it might be that the association observed between high levels of both proteins and the risk of thrombosis is merely a reflection of a post-thrombotic acute phase response. However, it has been shown in at least two studies that this is not the case^{10,49} and also, high levels of FVIII are predictive to a thrombosis and not only observed to be a risk factor after a thrombotic event⁸.

Several studies have indicated a genetic role in the regulation of FVIII levels^{36,50-52}, but no association between high levels of VWF and FVIII and variations in the VWF and FVIII genes has been found⁵³⁻⁵⁶. Recently, a haplotype in the FVIII gene was reported to be associated with a small decrease in FVIII levels and, correspondingly, with a decrease in risk^{57,58}. Surprisingly, the risk decrease associated with this haplotype could only partially be explained by the decrease in FVIII levels⁵⁸.

To identify the genetic determinants of increased levels of FVIII, proteins that influence secretion and clearance of FVIII and VWF are being studied.

Adrenalin infusion causes a rise in levels of VWF and FVIII. Since the rise in levels can be blocked by β blockers, this effect is believed to be mediated through the β adrenergic receptors⁵⁹. Therefore, it was hypothesized that variations in the genes encoding the β 1 adrenergic receptor (ADBR1) or the β 2 adrenergic receptor (ADBR2) could influence levels of VWF and FVIII.

However, even though the results are not completely consistent, it was shown that functional variations in these genes are not associated with levels of VWF and FVIII^{60,61}. Another gene that was studied for its association with FVIII levels encodes the LDL receptor related protein (LRP). It was shown in both in vitro and in vivo studies that LRP is involved in the plasma clearance of FVIII^{62,63}. However, again, no association was observed between LRP gene variations and FVIII levels⁶⁴.

The mechanism through which high FVIII levels could influence thrombosis risk has not been fully clarified. It has been reported that high levels of FVIII

associate with decreased APC sensitivity^{65,66}. In 2001 O'Donnell showed that high levels of FVIII can drastically increase thrombin generation ⁶⁷. Besides the effects of high levels of FVIII, the functionality of the protein may also play a role, since a reduction in thrombosis risk associated with a common FVIII gene haplotype could only partially be explained by a reduction in protein levels⁵⁸. Both in retrospective and in prospective studies, high levels of FVIII are a strong risk factor of thrombosis. Even though it cannot be excluded that FVIII levels are not causal to thrombosis but reflect for instance the amount of endothelial damage, high FVIII is one of the strongest predictors of venous thrombosis. The mechanisms that underlie high FVIII levels remain to be determined.

Factor IX

FIX is a vitamin K dependent glycoprotein. It is activated by activated factor XI (FXI) or activated factor VII (FVII) and together with its cofactor activated FVIII, activated FIX activates FX. Just like the FVIII gene, the gene encoding FIX is located on the X chromosome and mutations in the FIX gene can lead to the bleeding disorder hemophilia B. High levels of FIX however may increase the risk of venous thrombosis.

In 2000, Van Hylckama Vlieg et al reported that individuals with FIX levels over the 90th percentile (>129 U/dl) have a nearly threefold increase in thrombosis risk when compared to individuals with levels below this cutoff value in the LETS⁶⁸. The risk remained after adjustment for possible confounders. The risk of thrombosis appeared to increase linearly with FIX levels. The authors reported that the risk was slightly higher in women than in men and that the risk was highest in pre-menopausal women not using oral contraceptives. In the same year Lowe et al reported similar findings in a study consisting of 66 women with venous thromboembolism (VTE) and 163 controls between the ages of 45 and 64⁶⁹. After adjustment for hormone replacement therapy, women with high FIX levels (>150 IU/dl) had a 2.3 fold increase in the risk of VTE. In an Austrian study, it was found that high levels of FIX also increase the risk of recurrence after a previous thrombosis⁷⁰, however, in the

LETS, high levels of FIX were not associated with recurrent thrombosis²⁴.

Weltermann et al followed 546 patients after finishing anticoagulant treatment for a first spontaneous thrombosis. After three years of follow-up, the relative risk of recurrence for patients with levels of FIX in the highest quartile (>138 IU/dl) was 2.2. After adjustment for other risk factors, the relative risk was smaller, 1.6, but remained significant. Furthermore, high levels of FIX increased the risk of recurrence caused by high levels of FVIII from 2.7 to 6.6. To our knowledge, the effect of high levels of FIX on the risk of thrombosis has not yet been examined in a prospective study.

Little is known about the causes of elevated levels of FIX. It has been shown that FIX levels depend on age, increasing strongly during adolescence⁷¹. The age-dependent regulation of FIX levels has been linked to two age regulatory elements in the 5' upstream region and the 3' untranslated region (UTR) of the FIX gene. No common genetic variations associated with high levels of FIX have been reported. Very recently, Khachidze et al⁷² reported the results of a genome-wide scan in the GAIT study. No quantitative-trait loci were identified that were linked to levels of FIX.

Besides genetics, environmental factors affect levels of FIX as well. In 2001 Lowe et al showed that levels of FIX are increased in postmenopausal women on hormone replacement therapy⁶⁹. This finding is in agreement with earlier reports of elevated factor IX levels in oral contraceptive users⁷³.

Like FVIII, literature seems consistent on the role of FIX in the development of thrombosis. No evidence for direct causality has been provided, but in retrospective studies, high levels of FIX are associated with venous thrombosis. Again, like in the case of FVIII, the mechanisms that underlie high FIX levels remain to be determined.

Factor XI

FXI is a component of the contact system which is activated by FXII after contact with a thrombogenic surface, but can also be activated by thrombin^{74,75}. FXI plays a role in the formation of fibrin, but is indirectly also involved in the

inhibition of fibrinolysis, via a stimulatory effect on the activation of thrombin activatable fibrinolysis inhibitor (TAFI)^{76,77}.

There is only one report on the association between levels of FXI and the risk of venous thrombosis. In 2000, Meijers et al reported that high levels of FXI are a risk factor for thrombosis in the LETS⁷⁸. The risk increased twofold in individuals with FXI levels over the 90th percentile (>120.8%). The risk remained after adjustment for other known risk factors of thrombosis and after exclusion of known genetic risk factors. A dose response relationship was observed between FXI levels and thrombosis risk. In addition, Souto et al showed in the GAIT study that there is a large phenotypic correlation between FXI and thrombosis³⁹. To our knowledge, there are no data on FXI levels and thrombosis risk in prospective studies. Furthermore, it appears as if high FXI levels have no effect on the risk of recurrent thrombosis, even though Eichinger et al have reported that high FXI levels may facilitate an increase in this risk caused by high levels of TAFI^{24,79}.

It is unclear what factors influence levels of FXI. Gerdes et al sequenced the FXI gene in 22 patients from thrombophilic families lacking an established risk factor for thrombosis and nine patients with FXI levels higher than 140%⁸⁰. No variations were found that associated with increased levels of FXI.

The effect of high FXI levels on the risk of thrombosis has to be confirmed in other studies.

TAFI

TAFI is a procarboxypeptidase that can be activated by enzymes as trypsin, plasmin and thrombin/thrombomodulin. Activated TAFI can cleave off carboxy-terminal lysine residues of fibrin polymers that are important for the binding of plasminogen to fibrin and thus delays the fibrinolytic process^{81,82}.

In 2000, Van Tilburg et al studied the effects of TAFI levels on the risk of venous thrombosis in the LETS⁸³. The risk of thrombosis increased nearly 2 fold for TAFI levels over the 90th percentile (>122 U/dl). The risk remained after adjustment for possible confounders. However, there was no dose-response relation between risk and TAFI levels. High levels of TAFI also influence the

risk of recurrent thrombosis⁷⁹. The risk of recurrence was twice as high in patients with high TAFI levels compared to patients with lower levels. Patients with high TAFI levels also had higher levels of FVIII, FIX and FXI. Patients with both high levels of TAFI and high levels of FVIII had the highest risk of recurrence. Juhan-Vague et al studied the effect of known cardiovascular risk factors on the inter-individual variability of TAFI levels⁸⁴. The authors concluded that there is a high inter-individual variation in levels, but that this variation is poorly explained by lifestyle characteristics. In 2001, Henry et al identified seven new SNPs in the TAFI gene, five in the promoter and two in the 3' UTR. These SNPs explained up to 60% of the variability in TAFI levels⁸⁵. Several other studies have reported on the association between TAFI gene variations and protein levels⁸⁶⁻⁸⁸. However, the association between TAFI SNPs and the risk of thrombosis remains inconclusive⁸⁹⁻⁹³. A haplotype that is associated with reduced TAFI levels also encodes a protein which is more stable after activation⁸⁷. This complicates the analysis of association studies, where the result will depend on the actual TAFI assay used⁸⁷.

In contrast to other known risk factors of venous thrombosis, high levels of TAFI did not interact with Factor V Leiden^{83,90}. However, in the LETS⁸³ high TAFI and high FVIII levels appeared to interact in enhancing the risk of thrombosis. Besides this possible interaction with FVIII, it has been shown in a rabbit thrombosis model that TAFI interacts with FXI⁷⁶.

The effects of TAFI on thrombosis need further confirmation by other independent studies. The lack of a dose-response effect between TAFI levels and thrombosis risk and the lack of strong associations between TAFI gene variations and thrombosis risk indicates that TAFI may not be causally related to venous thrombosis.

Factors V, VII, X and XII

The associations between the risk of venous thrombosis and high levels of several coagulation factors, other than the factors discussed above, have been studied. However the results of these studies were either negative or inconclusive and further studies have not been performed.

Factor V (FV), which, when activated, can accelerate the conversion of prothrombin to thrombin by activated FX, is one of the most extensively studied coagulation factors with respect to the risk of thrombosis. A mutation in the FV gene, FV Leiden, causes APC resistance and is a strong risk factor for venous thrombosis⁹⁴. However, this is a functional change in the FV molecule, whereas neither high nor low levels of FV are associated with the risk of venous thrombosis⁹⁵.

Inconclusive results were observed for the protein involved in the initiation of the coagulation cascade, FVII. In the prospective LITE study, high levels of FVII were a possible determinant of venous thrombosis⁸, but in the LETS, no association between FVII and thrombosis risk was observed⁵.

High levels of FX are associated with risk of thrombosis, as shown by de Visser et al in the LETS, however, the association disappeared completely after adjustment for other vitamin K dependent factors⁹⁶.

Finally, levels of FXII, a component of the contact system proved not to associate with the risk of venous thrombosis in at least two studies^{97,98}.

Conclusions

Elevated levels of some, but not all coagulation factors are important risk factors of venous thrombosis. High levels of factors VIII and IX showed to be strong predictors of thrombosis on their own. Clustering of high levels of these factors contributes to an even higher risk of venous thrombosis than either factor alone^{70,99}. Intermediate dose dependent effects on thrombosis risk were observed for elevated prothrombin and factor XI levels. However more data, especially from prospective studies are needed to confirm these results. High levels of other factors, such as fibrinogen and FX, are associated with venous thrombosis, but it appears as if they merely reflect the presence of others risk factors and are not predictors of thrombosis on their own. For some factors, like FVII and TAFI more research is required to determine the true association between plasma levels and the risk of thrombosis. Finally, plasma levels of for instance FV and FXII do not associate with the risk of thrombosis at all.

2. Coagulation Factor Levels & Venous Thrombosis

Despite the extensive literature on the thrombotic risk of increased levels of several coagulation factors, it still remains uncertain whether these factors should be determined in thrombophilia screening. Current data do not support screening, as the presence or absence of increased factor levels do not determine the duration of anticoagulant treatment nor do they clearly determine the risk of recurrent venous thrombosis. Furthermore, the risk associated with increased factor levels is generally continuous and any cutoff value is arbitrary. Finally, the measurement of these factors is also greatly dependent on several clinical and (pre-)analytical conditions. At the time we think measurement of these factors is not to be recommended in routine thrombophilia screening. For all coagulation factors, however, it is important that the mechanisms that underlie elevated plasma levels and the mechanisms through which high levels may influence the development of thrombosis are studied further.

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2. Coagulation Factor Levels & Venous Thrombosis

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2. Coagulation Factor Levels & Venous Thrombosis

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2. Coagulation Factor Levels & Venous Thrombosis

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Chapter 3

Von Willebrand Factor and its Propeptide: The Influence of Secretion and Clearance on Protein Levels and the Risk of Venous Thrombosis

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Summary

Background and Objectives. Elevated levels of Factor VIII (FVIII) are associated with an increased risk of thrombosis. FVIII levels are determined mainly by von Willebrand Factor (VWF). We investigated the contribution of secretion and clearance rates to elevated VWF antigen (VWF:Ag) and to thrombosis risk. VWF is secreted in equimolar amounts with its propeptide which has a shorter half-life. VWF propeptide can be used as a measure of VWF secretion and allows estimation of VWF half-life.

Methods and Results. We have measured VWF propeptide, VWF:Ag, FVIII:Ag and FVIII activity (FVIII:C) in the Leiden Thrombophilia Study. In controls high VWF propeptide was associated with high VWF:Ag, FVIII:Ag and FVIII:C. In contrast to mature VWF:Ag, VWF propeptide was not influenced by blood groups. Using an ELISA based assay we showed that the VWF propeptide lacks ABO antigens. Levels were higher in men and increased with age. Long VWF half-life was also associated with high VWF:Ag, FVIII:Ag and FVIII:C. VWF half-life was influenced by blood group (10hrs in O versus 12hrs in non-O individuals), but not by sex and only slightly by age. VWF propeptide was higher in thrombosis patients than in controls. VWF half-life was similar in patients and controls (11.4 and 11.1 hrs, respectively).

Conclusions. Both secretion and clearance rates are important determinants of VWF and FVIII levels. However, mainly high VWF and FVIII levels caused by increased secretion seem associated with thrombosis. ABO blood group influences clearance rates of VWF rather than VWF secretion rates.

Introduction

Several studies have shown that elevated plasma levels of von Willebrand factor (VWF) and factor VIII (FVIII) are important risk factors for venous thrombosis¹⁻⁷. The risk of thrombosis seems affected mainly by FVIII, but because VWF is the main determinant of FVIII levels, understanding the regulation of VWF levels is important.

VWF is a glycoprotein that circulates in plasma as large multimers⁸. The protein functions as an adhesion molecule to bind platelets at sites of vascular damage.

Furthermore, VWF is the carrier protein of FVIII, which is a cofactor in secondary hemostasis. Binding of FVIII to VWF protects it from degradation in or premature clearance from the circulation. Plasma levels of FVIII depend strongly on levels of VWF. Therefore, cellular secretion as well as clearance rates of VWF may be important determinants of FVIII levels.

Levels of VWF and consequently levels of FVIII strongly depend on ABO blood group⁹. Individuals with non-O blood groups generally have higher levels of VWF and FVIII than individuals with blood group O. VWF carries ABO blood group antigens¹⁰. It is not exactly known how the presence of ABO blood group antigens influences the lifecycle of VWF. Blood group is believed to influence the clearance rate of VWF from the circulation, but may also play a role in the secretion of VWF¹¹⁻¹³.

VWF is synthesized by endothelial cells and megakaryocytes. The primary translation product undergoes a number of post-translational modifications, including glycosylation, multimerization and endoproteolytic cleavage of a propeptide. Both mature, highly polymerized VWF and the propeptide are targeted to Weibel-Palade bodies (WPb), endothelial cell-specific storage organelles. The contents of WPb are released into the circulation only after exposure of the endothelium to specific stimuli^{8,13-16}. In vitro studies have shown that partially processed, functionally incompetent VWF is released in a constitutive manner. The physiological significance of this pathway is poorly understood, however^{8,14}.

Upon stimulation mature VWF and propeptide are released in equimolar amounts^{8,17}. Once in the blood, mature VWF and its propeptide become completely dissociated and have a different life span^{8,17,18}. VWF propeptide is important for intracellular trafficking and processing of VWF^{19,20-22}, but it is not clear yet whether it has a separate function in the circulation as well⁸. The VWF propeptide is cleared from the circulation at a much faster rate than mature VWF²³, with a half-life of approximately 2 hours. Mature VWF has a half-life between 10 and 12 hours. It is not clear yet whether the VWF propeptide carries ABO antigens like the mature VWF and whether its levels are influenced by ABO blood group²⁴.

3. VWF Propeptide, VWF and FVIII Levels & Venous Thrombosis

Assuming that the inter-individual variation in its half-life is relatively small, plasma levels of VWF propeptide reflect the rate of VWF secretion at steady state^{23,25-27}. Measuring concentrations of VWF propeptide and mature VWF at steady state then allows an estimation of the clearance rate of mature VWF. To investigate the contribution of VWF secretion and clearance to VWF and FVIII levels, we measured VWF propeptide levels in the Leiden Thrombophilia Study (LETS). Levels of the VWF propeptide were interpreted as a measure of the rate of VWF secretion. Furthermore, we used the levels of mature VWF and the VWF propeptide to estimate the half-life of VWF, assuming a VWF propeptide half-life of two hours.

Using these variables, we studied the influence of VWF secretion and clearance on levels of VWF and FVIII. We also evaluated the contribution of both secretion and clearance of VWF to the risk of venous thrombosis.

Patients and Methods

Leiden Thrombophilia Study

The LETS consists of 474 consecutive patients and 474 controls. All patients were referred for anticoagulant treatment after a first objectively confirmed episode of deep vein thrombosis without an underlying malignancy. Pregnant women were excluded from the study. Use of medication, including oral contraceptives or hormone replacement therapy, was not an exclusion criterion. The controls were matched for sex and age and were acquaintances or partners of the patients. Mean age for both patients and control subjects was 45 years, ranging from 15 to 69 years for patients and 15 to 72 years for controls. Both groups consisted of 272 women (57.4%) and 202 men (42.6%). FVIII:C was measured in the plasma of all participants by a one-stage clotting assay¹. FVIII:Ag and VWF:Ag were measured by ELISA in the plasma of the first 301 patients and 301 controls¹. These 602 individuals were not selected, but merely the first 602 individuals to enter the study. Results are expressed as international units per ml (IU/ml). Blood samples from patients were drawn at least six months after the thrombotic event. The design of this study has previously been described in more detail^{28,29}.

Measuring VWF propeptide

VWF propeptide antigen levels were measured by ELISA as described previously²³. In brief, microtiter wells were coated overnight at 4°C with the antibody CLB-Pro 35²³. Subsequently, wells were washed and blocked with 1% Bovine Serum Albumin (BSA) at room temperature for 2 hours. Samples were diluted to three different concentrations and added to the wells after washing. Samples were incubated for two hours at 37°C to ensure protein binding. After incubation, wells were washed and the bound VWF propeptide was detected with the antibody CLB-Pro 14.3 coupled to peroxidase²³. Pooled normal plasma, which was calibrated against a plasma sample in which the absolute amount of VWF propeptide had previously been determined with purified recombinant VWF propeptide as a gold standard, was used as a standard. The normal pooled plasma had a VWF propeptide concentration of 6.13 nM (see below), which corresponds to one unit per ml (U/ml).

Estimation of VWF half-life

To estimate the half-life of the mature VWF, two important assumptions were made. First, we assume that VWF propeptide half-life is relatively invariable (2 hours) for the whole LETS population, compared to the half-life of mature VWF and that the clearance of both polypeptides follows first-order kinetics. It has been shown previously that these are reasonable assumptions^{23,26,27}. Second, we assume that both mature VWF and VWF propeptide levels were at steady state at the time of blood draw. Again, this is a reasonable assumption, even for cases, as it was shown previously that an acute phase reaction was absent in most LETS samples³⁰ and all LETS blood samples were drawn in resting state. When these conditions are met, steady state kinetics enable us to estimate VWF half-life for each individual³¹:

$$\text{Concentration Steady state} = \frac{\text{Infusion Rate}}{\text{Total Body Clearance}}$$

3. VWF Propeptide, VWF and FVIII Levels & Venous Thrombosis

In case of VWF, the infusion rate is the rate of secretion. To calculate the half-life, we use the following formula, in which Total Body Clearance is the apparent volume of distribution (V_D) times $\ln 2$ divided by the half-life.

$$\text{Secretion Rate} = \text{Concentration Steady state} * \frac{V_D * \ln 2}{\text{Half-life}}$$

Secretion Rate and V_D are equal for VWF and the VWF propeptide, which leads to the following:

$$\frac{[\text{VWF}]_{\text{Steady State}}}{\text{VWF half-life}} = \frac{[\text{Propeptide}]_{\text{Steady State}}}{\text{Propeptide half-life}} = \frac{[\text{Propeptide}]_{\text{Steady State}}}{2 \text{ hours}}$$

One unit per ml of VWF propeptide in the LETS corresponds to 6.13 nM. According to de Romeuf et al. one international unit of mature VWF per ml corresponds to 31 nM²⁴. After converting all VWF propeptide and mature VWF concentrations in the LETS from (international) units to molar concentrations, the VWF half-life could be estimated for each individual. Throughout this paper we will refer to these estimates as 'VWF half-life', but it should be noted that these are just estimates calculated from the VWF/propeptide ratio after making two important assumptions.

ABO Antigens on VWF propeptide

Wells were coated overnight at 4°C with the antibody CLB-Pro 35.

Subsequently, wells were washed and blocked with 1% Bovine Serum Albumin (BSA) at room temperature for 2 hours. Plasma was used from an all blood group A plasma pool, containing the plasma of 29 individuals and from a patient with acquired von Willebrand's disease (VWD) with blood group A. This patient had normal VWF propeptide levels (1.34 U/ml), but very low levels of mature VWF (0.09 IU/ml). The latter plasma was used to check for potentially falsely positive results caused by nonspecific binding of mature VWF. Wells were incubated with duplicates of seven dilutions of these plasmas (one in 20, one in 40 and so on until one in 1280) for two hours at 37°C to ensure VWF propeptide binding. We used four different secondary antibody combinations,

CLB-Pro 14.3 HRP to establish the presence of VWF propeptide, rabbit anti-VWF HRP to exclude the presence of mature VWF, murine anti-A/goat anti mouse HRP to determine the presence of A antigens on the VWF propeptide and anti-B/goat anti mouse HRP as a control in the blood group A plasma pool and the acquired VWD plasma. This experiment was repeated using plasma from a B plasma pool, containing the plasma of 5 individuals. Here, anti B/goat anti mouse HRP was used to determine the presence of B antigens on VWF propeptide. All experiments were repeated on blood group O plasma as well as a negative control.

Statistical Analysis

Protein levels and half-lives are presented as means with their 95% confidence intervals (CI95). The differences in levels between groups (Δ) are presented with their CI95s and were tested with ANOVA and Student's t-tests.

Associations between continuous variables were tested with linear regression. To evaluate the effects of VWF propeptide levels and VWF half-life on the risk of thrombosis, odds ratios (OR) and their corresponding CI95s according to Woolf were calculated³². Odds Ratios were adjusted for levels of FVIII using logistic regression models.

Results

Levels of VWF propeptide and VWF half-life in healthy controls

In the controls, high levels of VWF propeptide were associated with elevated levels of both VWF and FVIII (Table 1). Mean VWF:Ag and FVIII:Ag levels were 1.52 and 1.41 IU/ml respectively for the highest quartile of VWF propeptide (>1.26 U/ml) versus 0.93 and 0.84 IU/ml for the lowest VWF propeptide quartile (<0.94 U/ml). Regression analysis showed a linear association of the VWF propeptide with VWF:Ag (Pearson's correlation coefficient (R) = 0.61, regression coefficient (β) = 0.95 (CI95 0.81 to 1.09)), FVIII:Ag (R = 0.57, β = 0.91 (CI95 0.76 to 1.06)) and FVIII:C (R = 0.51, β = 0.61 (CI95 0.49 to 0.72)). Results were similar in cases. Stratification by ABO blood group did not influence the outcomes.

3. VWF Propeptide, VWF and FVIII Levels & Venous Thrombosis

We estimated the half-life of VWF, which ranged from 4.6 to 20.1 hours in the LETS. Longer VWF half-life, indicating decreased VWF clearance, was associated with elevated levels of both VWF and FVIII (Table 1). Mean VWF:Ag and FVIII:Ag levels were 1.56 IU/ml and 1.28 IU/dl respectively for the highest quartile of VWF half-life (>12.8 hours) versus 0.84 IU/ml and 0.87 IU/dl for the lowest half-life quartile (<9.2 hours). Again, regression analysis showed a linear association of the VWF half-life with VWF:Ag ($R = 0.71$, $\beta = 0.10$ (CI95 0.09 to 0.11)), FVIII:Ag ($R = 0.38$, $\beta = 0.05$ (CI95 0.04 to 0.07)) and FVIII:C ($R = 0.41$, $\beta = 0.04$ (CI95 0.03 to 0.05)).

Both secretion and clearance of VWF are determinants of levels of VWF and FVIII. However, secretion and clearance rates of VWF did not influence each other. Linear regression showed no association between levels of VWF propeptide and VWF half-life ($R = 0.096$, $\beta = -1.089$ (CI95 -2.372 to 0.195)).

Table 1. Quartiles of VWF propeptide and VWF half-life and VWF & FVIII levels in controls.

Quartiles of VWF	Mean VWF:Ag	Mean FVIII:Ag	Mean FVIII:C
1 (0.44-0.94)	0.93 (0.87-0.99)	0.84 (0.78-0.90)	0.96 (0.90-1.01)
2 (0.94-1.06)	1.12 (1.05-1.19)	0.97 (0.90-1.04)	1.04 (0.99-1.10)
3 (1.06-1.26)	1.32 (1.25-1.39)	1.14 (1.06-1.21)	1.18 (1.12-1.25)
4 (1.26-2.03)	1.52 (1.43-1.61)	1.41 (1.30-1.51)	1.33 (1.27-1.40)
Quartiles of VWF	Mean VWF:Ag	Mean FVIII:Ag	Mean FVIII:C
1 (4.6-9.2)	0.84 (0.79-0.90)	0.87 (0.80-0.95)	0.96 (0.90-1.01)
2 (9.2-11.0)	1.13 (1.07-1.20)	1.01 (0.93-1.09)	1.07 (1.01-1.13)
3 (11.0-12.8)	1.32 (1.25-1.40)	1.16 (1.08-1.25)	1.19 (1.13-1.25)
4 (12.8-20.1)	1.56 (1.48-1.63)	1.28 (1.18-1.38)	1.29 (1.22-1.35)

*Quartiles based on levels in controls only.

Levels of VWF propeptide and VWF half-life and known determinants of VWF and FVIII

The LETS controls with blood group O had lower levels of VWF and FVIII than those with non-O blood groups (Table 2). The half-life of VWF was also influenced by ABO blood group. Non-O individuals had a longer mean half-life of approximately 12 hours compared to O individuals, whose mean half-life was approximately 10 hours.

In contrast, levels of VWF propeptide were similar in both O and non-O controls, 1.08 and 1.13 U/ml respectively (Table 2), which is consistent with previous reports^{24,33}.

Table 2. Protein levels and VWF half life for ABO blood groups O versus non-O and women versus men in controls.

Mean Value	O	non-O	Mean Difference (Δ)	CI95 of Δ
VWF:Ag IU/ml	1.06	1.33	0.26	0.18 to 0.35
FVIII:Ag IU/ml	0.94	1.19	0.25	0.16 to 0.34
FVIII:C IU/ml	1.00	1.19	0.19	0.16 to 0.27
VWF propeptide U/ml	1.08	1.13	0.05	-0.003 to 0.11
VWF half-life Hrs.	10.02	11.98	1.96	1.36 to 2.57
Mean Value	Women	Men	Mean Difference (Δ)	CI95 of Δ
VWF:Ag IU/ml	1.26	1.34	0.08	0.02 to 0.15
FVIII:Ag IU/ml	1.15	1.27	0.12	0.05 to 0.19
FVIII:C IU/ml	1.21	1.18	-0.03	-0.07 to 0.01
VWF propeptide U/ml	1.14	1.21	0.07	0.02 to 0.11
VWF half-lifeHrs.	1.21	1.18	0.16	-0.28 to 0.60

The influence of sex, a known determinant of levels of VWF and FVIII, was studied as well among the controls. Results are shown in the second part of Table 2. Both VWF:Ag and FVIII:Ag were higher in men than in women, for FVIII:C no difference was observed. The VWF propeptide was also higher in men, indicating an increased VWF secretion in men compared to women. VWF half-life was not different between men and women.

Finally, we studied the effect of age on VWF secretion and clearance rates in healthy controls. Linear regression shows that both levels of VWF propeptide and VWF half-life increase with age. The association between age and VWF propeptide ($R = 0.30$, $\beta = 0.006$ (CI95 0.004 to 0.008)) however, was stronger than that between age and VWF half-life ($R = 0.12$, $\beta = 0.03$ (CI95 0.001 to 0.05)). The R^2 s for both variables indicate that almost 10 % of all variation in VWF secretion and only 1% of all variation in VWF clearance can be explained by age.

ABO blood group

In contrast to the association between VWF level and ABO blood group, we did not observe an association between VWF propeptide and ABO blood group (Table 2). The ABO dependence of the VWF level is thought to be related to the presence of ABO oligosaccharide structures on the N-linked oligosaccharide chains of VWF^{10,34}. We hypothesized that the lack of association between ABO blood group and VWF propeptide levels can be explained by the absence of ABO antigens on the VWF propeptide. However, there are no previous reports excluding the presence of ABO antigens on the VWF propeptide. Therefore, we have studied whether or not ABO antigens are present on VWF propeptide using an ELISA based method.

In both blood group A pooled plasma, normal blood group B plasma and in the plasma of a patient with blood group A and acquired VWD, we could measure normal amounts of VWF propeptide. A secondary antibody against mature VWF showed that no VWF had bound the primary anti-VWF propeptide antibody in either plasma sample (data not shown). Even though both the blood group A pooled plasma and the acquired VWD plasma samples came from blood group A individuals only, no A antigen was present on the VWF propeptide in either sample. Also, no B antigen was present on VWF propeptide in the blood group B plasma pool, indicating that ABO antigens are expressed only on mature VWF and not on the VWF propeptide. Both during these experiments and in the past, under similar conditions, the presence of A and B antigens could be demonstrated on mature VWF³⁵.

Levels of VWF propeptide and VWF half-life in cases versus controls

Patients had higher VWF propeptide levels than controls (Table 3a). In the LETS, patients had a mean VWF propeptide level of 1.22 versus 1.11 U/ml in controls. Stratification by ABO blood group did not influence this observation. In agreement with this observation, higher levels of VWF propeptide were associated with an increased risk of venous thrombosis (Table 3b). There was a clear dose-response relationship between VWF propeptide levels and thrombotic risk. Individuals with VWF propeptide levels in the highest quartile

had an increased risk of thrombosis (odds-ratio (OR) 2.6, 95% confidence-interval (CI95) 1.7-4.1) compared to those in the lowest quartile. A large proportion of this risk increase disappeared after adjustment for FVIII:Ag (OR 1.4, CI95 0.8-2.3).

Table 3a. VWF, FVIII, VWF propeptide & half-life in cases versus controls.

Mean Value	Cases	Controls	Mean Difference (Δ)	CI95 of Δ
VWF:Ag IU/ml	1.36	1.21	0.15	0.09 to 0.21
FVIII:Ag IU/ml	1.31	1.08	0.23	0.16 to 0.30
FVIII:C IU/ml	1.28	1.11	0.17	0.14 to 0.23
VWF propeptide U/ml	1.22	1.11	0.11	0.07 to 0.16
VWF half-life Hrs.	11.43	11.14	0.29	-0.14 to 0.72

VWF half-life was similar in patients and controls (11.43 and 11.14 hrs, respectively) (Table 3a). Stratification by ABO blood group did not influence this observation. Also, VWF half-life only marginally influenced thrombosis risk (OR 1.4, CI95 0.9-2.3 in the highest versus the lowest quartile) and no dose-response relationship was observed (Table 3b). The marginal increase in risk completely disappeared after adjustment for FVIII:Ag (OR 0.9, CI95 0.6-1.6).

Stratification by sex did not influence the effects of VWF propeptide levels or VWF half-life on the risk of thrombosis.

To ensure the latter findings were not influenced by VWF secretion, we looked at VWF half-life in cases and controls for each quartile of VWF propeptide separately. Only in the lowest VWF propeptide quartile was the mean VWF half-life slightly longer in cases than in controls, 12.5 versus 11.3 hrs. respectively ($\Delta = 1.2$ hrs (CI95 0.002 to 2.4)). For the higher three VWF propeptide quartiles, there was no difference in VWF half-life between cases and controls.

Table 3b. VWF propeptide quartiles and VWF half-life quartiles and the risk of venous thrombosis.

Quartiles of VWF propeptide* (range)	Cases	Controls	Odds Ratio (CI95)
1 (0.44-0.94)	54	80	1
2 (0.94-1.06)	41	71	0.9 (0.5-1.4)
3 (1.06-1.26)	80	79	1.5 (0.9-2.4)
4 (1.26-2.03)	126	71	2.6 (1.7-4.1)
Quartiles of VWF half-life* (range)	Cases	Controls	Odds Ratio (CI95)
1 (4.6-9.2)	47	75	1
2 (9.2-11.0)	91	75	1.9 (1.2-3.1)
3 (11.0-12.8)	97	76	2.0 (1.3-3.3)
4 (12.8-20.1)	66	75	1.4 (0.9-2.3)

*Quartiles based on half-lives in controls only.

Discussion

A novel aspect of this study is that we have assessed both the VWF secretion rate and VWF clearance rate on the basis of steady state VWF propeptide concentrations. The plasma level of VWF propeptide can be used as a tool to study the secretion of VWF, considering equimolar secretion of the mature VWF and its propeptide^{8,16,17}. In addition, the clearance of VWF can be evaluated using the molar concentrations of VWF propeptide, VWF and the half-life of the VWF propeptide. We measured VWF propeptide levels in the LETS to study the effects of VWF secretion and clearance on levels of VWF and FVIII and on the risk of venous thrombosis.

To study VWF clearance effects, we estimated VWF half-life. As described in the methods we had to make assumptions to allow this estimation. The assumptions that clearance of VWF and VWF propeptide follows first order kinetics and that both proteins were at steady state at time of blood draw are based on previous results from both the LETS and other studies. The most crucial assumption however, is that the half-life of the VWF propeptide is more or less constant in all LETS individuals. We believe that this assumption is

supported by previous data as well. VWF propeptide half-life has been shown to be relatively invariable before^{23,26,27}, although in relatively small groups of individuals, both men and women. Furthermore, in our study, VWF propeptide levels were independent of ABO blood group, which is a major determinant of VWF level variations. Nonetheless, there is no proof that VWF propeptide half-life is indeed constant, so therefore, the calculated VWF half-lives should be interpreted as estimations only.

In the controls of the LETS, high levels of VWF propeptide were associated with higher levels of VWF and FVIII. As could be expected, increased secretion of VWF indeed leads to higher plasma levels of both VWF and FVIII. Similarly, decreased clearance rates were associated with elevated levels of VWF and FVIII. The magnitude of the effects of both secretion and clearance of VWF on levels of VWF and FVIII was more or less the same (Table 1).

Even though secretion and clearance of VWF both influenced protein levels of VWF and FVIII, they did not affect each other. Elevated levels of VWF propeptide were not associated with either in- or decreased VWF half-life and vice versa. This makes the existence of a direct feedback system regulating VWF levels unlikely. Levels of VWF and FVIII are therefore influenced independently by both secretion and clearance of VWF.

Levels of VWF and FVIII are strongly influenced by ABO blood group. Blood group O individuals have lower levels than non-O individuals. We found that the estimated half-life of VWF was influenced by ABO blood group. VWF half-life in LETS controls with blood group O was estimated to be almost 2 hours shorter than in those with a non-O blood group. Most likely, the ABO antigens present on the mature VWF influence the clearance rate of the molecule^{12,13}.

Levels of VWF propeptide were not influenced by ABO blood group and were similar in O and non-O individuals. ABO antigens present on the mature VWF therefore are unlikely to influence the secretion rate of VWF, rather these structures influence the clearance of mature VWF. We did not detect A or B antigens on the VWF propeptide itself. The observation that VWF propeptide levels were not influenced by ABO blood group, supports our findings that these antigens are in fact absent from the VWF propeptide and restricted solely

to the mature VWF polypeptide.

Elevated levels of FVIII have been recognized as an important risk factor of venous thrombosis. Therefore, it can be expected that determinants of elevated FVIII levels also increase the risk of venous thrombosis. Levels of VWF propeptide, which are associated with levels of FVIII, were indeed higher in cases than in controls. There was a dose-response relationship between levels of VWF propeptide and the risk of venous thrombosis, just as there was a strong dose-response relationship between levels of VWF propeptide and levels of FVIII (and VWF). After adjustment for FVIII levels, the risk largely disappeared. This is a corroboration of previous findings in the LETS described by Koster et al¹, where FVIII and not its determinant VWF appeared responsible for increases in the risk of thrombosis.

However, for the VWF half-life, which was also associated with levels of FVIII, there was not such a dose response relationship with the risk of venous thrombosis. The risk appeared slightly elevated in the second and third quartiles of VWF half-life compared to both the lowest and highest quartile. However, only a marginal effect on risk was observed for the highest half-life quartile, which was associated with the highest mean FVIII levels. This marginal risk increase disappeared completely after adjustment for FVIII levels. It may seem that these results contradict the previous findings in the LETS described by Koster et al, however, before the measurement of the VWF propeptide, we were not able to make the distinction between high FVIII and VWF levels caused by increased secretion and those caused by decreased clearance. When taken high levels of FVIII as a whole, we of course observe the same increase in risk as described previously¹.

It seems contradictory that two determinants of elevated levels of VWF and FVIII, secretion and clearance of VWF, should have a different effect on thrombosis risk. However, blood samples from cases have been collected after a thrombotic event. Therefore, it is not possible to discriminate between high FVIII levels as a cause or a consequence of the thrombosis. It now appears that elevated levels of FVIII are mainly a risk factor of venous thrombosis if associated with increased secretion and less if associated with decreased

clearance. An obvious and credible explanation could be that in the LETS, where blood samples were collected several months after the thrombotic event, elevated levels of FVIII reflect mainly the consequence of thrombosis and less the cause of thrombosis. However, high FVIII has also been identified as an important risk factor for venous thrombosis in a large prospective study, in which blood samples were drawn well before a thrombotic event⁷. A more plausible explanation would then be that high FVIII is in the causative pathway but that the majority of the increased VWF and FVIII levels in LETS patients are the consequence of increased secretion of VWF due to post-thrombotic endothelial damage. Or, perhaps FVIII levels caused by increased VWF secretion merely reflect the condition of vascular endothelium and therefore associate with venous thrombosis. In this last scenario, increased levels of VWF and consequently of FVIII are merely epiphenomena of an increased thrombotic risk caused by endothelial damage. That would be consistent with the fact that high FVIII is a predictor of a recurrent thrombosis⁴. In summary, both secretion and clearance of VWF are important determinants of levels of both VWF and FVIII. However, it appears that mainly increased VWF secretion increases the risk of venous thrombosis, whereas decreased VWF clearance does not or less so. Secretion and clearance rates of VWF do not influence each other. ABO blood group influences levels of VWF and FVIII via the clearance of VWF, rather than via VWF secretion.

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3. VWF Propeptide, VWF and FVIII Levels & Venous Thrombosis

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Chapter 4a

Haplotypes Encoding the Factor VIII 1241Glu Variation, Factor VIII levels and the Risk of Venous Thrombosis

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Summary

Levels of Factor VIII (FVIII) are associated with the risk of venous thrombosis. The FVIII variation D1241E has been reported to be associated with decreased levels of FVIII. Our objective was to study whether D1241E is associated with levels of FVIII and the risk of venous thrombosis and whether this association is caused by D1241E or another linked variation. We analyzed the association of three FVIII gene haplotypes encoding 1241E (further denoted as HT1, HT3, and HT5) with FVIII levels and thrombosis risk. This analysis was performed in the Leiden Thrombophilia Study (LETS). The control populations of two case-controls studies on arterial thrombosis in men and women respectively, were used to confirm the effects observed on FVIII:C in the LETS. In men, HT1 was associated with a 6% reduction in FVIII:C and with a reduced risk of venous thrombosis (odds ratio 0.4 (CI95 0.2–0.8)). Logistic regression showed that the risk reduction was only partially dependent of the reduction in FVIII levels. HT1 showed no effects in women on either FVIII:C or risk of thrombosis. The number of carriers of HT3 and HT5 were too low to make an accurate estimate of the risk of venous thrombosis. Neither HT3 nor HT5 showed effects on levels of FVIII:C. When we consider that all three haplotypes encoding 1241E show different effects on FVIII:C and thrombosis risk, it is possible that D1241E is not the functional variation. However, FVIII gene variations do contribute to both levels of FVIII and the risk of thrombosis.

Introduction

Several studies have shown that elevated levels of coagulation factor VIII (FVIII) are an important risk factor of venous thrombosis. This risk remains after adjusting for the main determinants of FVIII levels, ABO blood group and von Willebrand Factor (VWF)¹⁻⁸. FVIII activity (FVIII:C) ≥ 150 IU/dL increases the risk of a first venous thrombosis fivefold compared to levels below 100 IU/dL. The frequency of FVIII:C ≥ 150 IU/dL among thrombosis patients is 25%. Since these levels are found in 10% of the population, the contribution of elevated FVIII levels to all thrombotic events in the population is considerable¹. Several lines of evidence support the idea that high FVIII levels are indeed

causative to thrombosis and not a consequence of the thrombotic event, such as a dose-dependent relationship with risk¹, persistence of elevated levels over time⁴, no indications for an acute phase response⁹, the induction of thrombosis by high FVIII in mouse models¹⁰, dependency on ABO blood group and the familial clustering of FVIII levels¹¹. The latter, familial clustering, supports the hypothesis that FVIII levels are, at least in part, determined genetically¹². So far, no genetic variations have been found in the gene encoding FVIII that are associated with an increase in levels and thrombotic risk^{6,13}. Recently however, a single nucleotide polymorphism (SNP), c94901g¹⁴, causing an amino acid change in the B-domain of FVIII, Asp1241Glu (D1241E), has been reported to be associated with a decrease in FVIII:C. Machiah *et al*¹⁵ found that this polymorphism accounts for approximately 5% of the total variation in FVIII:C in the families of the GAIT study. More recently, Scanavini *et al*¹⁶ reported that 1241E is associated with an 11% reduction in FVIII:C in 145 healthy women and 150 thrombotic women. Since high FVIII:C increases the risk of thrombosis, conditions associated with reduced FVIII:C levels are expected to be associated with a decrease in risk. However, Machiah *et al* did not report estimations on risk and Scanavini *et al* did not find significant differences in genotype distribution between cases and controls^{15,16}.

It is unclear through which mechanism the 1241E variant could influence levels of FVIII. Scanavini *et al* hypothesized that the variation might be involved in APC resistance, but could not demonstrate this¹⁶. It is very well possible that D1241E is not causal to the decrease in levels, but that c94901g, encoding D1241E is in linkage disequilibrium with the causal variation. On the website of SeattleSNPs¹⁴, SNP and haplotype data are presented for a wide variety of genes, including the gene encoding FVIII. These data are based on the re-sequencing of these genes in 23 subjects of European-American descent. In this population the SNP c94901g encoding 1241E is present in at least three different haplotypes of which possibly only one could be responsible for the reported effects on FVIII levels.

To investigate the influence on FVIII:C and on the risk of venous thrombosis we studied the effect of these three common haplotypes encoding the 1241E

variant in the Leiden Thrombophilia Study (LETS), a large population based case-control study on venous thrombosis. Associations found between these haplotypes and FVIII:C in the LETS were also evaluated in the control subjects of two large population-based case-control studies on arterial thrombosis, the Study of Myocardial Infarctions Leiden (SMILE), consisting of men under the age of 70 years and the Risk of Arterial Thrombosis In Relation to Oral Contraceptives Study (RATIO), consisting of women under the age of 50 years.

Patients and Methods

Study Populations

Leiden Thrombophilia Study (LETS)

The LETS consists of 474 consecutive patients and 474 controls. All patients were referred for anticoagulant treatment after a first objectively confirmed episode of deep vein thrombosis without an underlying malignancy. Controls were matched for sex and age and were acquaintances or partners of the patients. Mean age for both patients and control subjects was 45 years, ranging from 15 to 69 years for patients and 15 to 72 years for controls. Both groups consisted of 272 women (57.4%) and 202 men (42.6%). All patients and controls were of Dutch origin. DNA samples were available of 471 cases and 471 controls. FVIII:C was measured in the plasma of all participants by a one-stage clotting assay, levels are expressed as IU/dL¹. VWF:Ag was measured by ELISA in the plasma of the first 301 patients and 301 controls, levels are expressed as IU/mL¹. Pooled normal plasma, calibrated directly against the WHO standard for VWF and FVIII (91/666) was used as a reference. In each assay, two control plasmas were included for inter-assay quality assurance. Plasma samples had not been thawed and refrozen before these measurements. The design of this study has previously been described in more detail¹⁷.

Study of Myocardial Infarctions Leiden (SMILE)

The SMILE consists of 560 patients and 646 control subjects of which only the controls were included here. Cases were men below the age of 70, diagnosed with a first myocardial infarction. The control group consists of men who were

referred to the Leiden Anticoagulation Clinic for prophylactic anticoagulation therapy for a short period following minor orthopaedic interventions. Controls did not have a history of myocardial infarction and had not used anticoagulants for a period of at least six months prior to participating in this study. Controls were frequency matched to cases on 10-year age groups. All patients and controls were of Dutch origin. Plasma and DNA samples were available of all participants. Plasma FVIII:C was measured in two dilutions by a one-stage clotting assay with FVIII deficient plasma and automated activated partial thromboplastin time (APTT, Organon Teknika, Boxtel, the Netherlands) on a STA (Diagnostic Stago, Boehringer Mannheim, Mannheim, Germany). Levels are expressed as IU/dL. Pooled normal plasma, calibrated against an in-house standard which was calibrated against the WHO standard for VWF and FVIII (91/666) was used as a reference. In each assay, two control plasmas were included for inter-assay quality assurance. Plasma samples had not been thawed and refrozen before these measurements. Exclusion criteria for this study were renal disease, severe (neuro-)psychiatric problems or a life expectancy of less than one year. The design of this study has previously been described in more detail¹⁸.

Risk of Arterial Thrombosis in Relation to Oral Contraceptives Study (RATIO)

The RATIO is a case-control study with patients with different vascular events (ischemic stroke, peripheral vascular disease and myocardial infarction) and healthy controls, of which only the controls were included here. The control group consists of 925 women between the ages of 18 to 49 who had no history of coronary, cerebral, or peripheral arterial disease. Controls were selected via Random Digit Dialling. Only controls of which plasma samples were available (639 women) were included for the current study. 93% of the controls were Caucasian.

Plasma FVIII:C was measured in two dilutions in duplicate by a one-stage clotting assay with FVIII deficient plasma and automated activated partial thromboplastin time (APTT, Organon Teknika) on a STA (Diagnostic Stago, Boehringer Mannheim). Levels are expressed as IU/dL. VWF:Ag was measured

by ELISA, using polyclonal antibodies (Dako, High Wycombe, UK). Levels are expressed as IU/dL. Pooled normal plasma, calibrated against an in-house standard which was calibrated against the WHO standard for VWF and FVIII (91/666) was used as a reference. In each assay, two control plasmas were included for inter-assay quality assurance. Plasma samples had not been thawed and refrozen before these measurements. The design of this study has previously been described in more detail¹⁹.

Combined Control Populations

Because the FVIII gene is located on the X-chromosome, effects were studied in detail in men and women separately. Combining the different control populations, two large control groups were formed, all male and all female respectively. The first control group consisted of all male control subjects from the LETS and all control subjects from the SMILE. The second control group consisted of all female control subjects from the LETS and all control subjects from the RATIO. Because controls were selected stratified for age and because age is known to influence FVIII levels, all analyses on levels were repeated after adjusting for age using linear regression. VWF levels and blood group were not determined in all individuals, so overall correction for these factors was not possible. Analyses were also performed after normalization of levels per study.

Genetic Analysis

According to SeattleSNPs (April 2004), the SNP encoding the 1241E variant is present in at least three FVIII haplotypes, indicated throughout this paper as HT1, HT3 and HT5. Haplotypes are depicted in Table 1. In order to identify carriers of these haplotypes, c94901g (numbering according to SeattleSNPs, April 2004¹⁴; rs1800291), encoding D1241E was determined in all individuals. Subsequently, two haplotype tagging SNPs specific for HT3 and HT5, g24052a (rs6655259) and g27882c (rs-number pending) respectively, were determined in both hetero- and homozygous carriers of the 1241E encoding allele. Genotyping was performed using either polymerase chain reaction – restriction fragment length polymorphisms analyses (PCR-RFLP) or 5'nuclease/Taqman assays.

The polymerase chain reactions with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA) and for PCR-RFLP were performed on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany). Fluorescence endpoint reading for allelic discrimination of the 5'nuclease/Taqman assay was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA). PCR conditions, restriction endonucleases and the sequences of probes and primers used for genotyping are available on request. Genotyping was successful in all but 8 samples for g24052a (3 from the LETS, 4 from the SMILE and 1 from the RATIO) and 2 samples for g27882c (1 from the SMILE and 1 from the RATIO). There was no evidence for the existence of other haplotypes than the three proposed by SeattleSNPs.

Table 1. Haplotypes on the FVIII gene.

SNP \ Haplotype	*	*								*	1	1	1	1	1	1
	2	2	5	5	5	6	6	7	7	9	1	1	1	1	4	4
	4	7	4	6	8	4	9	0	1	4	3	5	7	8	2	2
	0	8	8	6	0	3	6	1	2	9	9	7	6	3	2	7
	5	8	8	6	6	8	8	0	5	0	2	9	3	7	4	3
Haplotype	2	2	4	7	5	4	5	5	9	1	7	4	8	4	2	7
All-Common	G	G	C	C	G	A	G	G	A	C	T	T	I	G	A	A
HT1	G	G	G	T	A	A	A	A	A	<u>G</u>	A	C	I	G	G	G
HT3	<u>A</u>	G	G	T	G	C	G	G	G	<u>G</u>	A	C	D	A	G	G
HT5	G	<u>C</u>	G	T	A	A	A	A	A	<u>G</u>	A	C	I	G	G	G

SNP numbering according to SeattleSNPs (April 2004) SNPs indicated with "*" are used to tag haplotypes (94901 encodes D1241E). SNP 94901 distinguishes HT1, HT3 and HT5 from the all-common form and 24052 and 27882 tag HT3 and HT5 respectively.

Sequencing

All exons and exon-intron boundaries of the FVIII gene were sequenced in DNA samples of nine individuals. Selection criteria for these individuals are described under "Results".

PCR Primers for all 26 exons of the FVIII gene were designed from the FVIII reference sequence (Accession number AH002692.1). The primers are extended either with an M13-forward or with an M13-reverse sequence. All primers were designed to include complete exon-intron transitions. Fragments for exon 1 and exon 14 were split into respectively 2 and 10 overlapping fragments.

PCRs were performed in a 25- μ l reaction volume, with a final MgCl₂ concentration of 1.5 mM except for exon 13. All PCR programs included an initial denaturation of 5 min by 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at either 55°C, 53°C, or 50°C and a final extension at 72°C was performed for 5 minutes. The PCR products were sequenced in both forward and reverse direction on an ABI Prism 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The raw sequence data were analyzed using SeqScape Software for comparative sequencing analysis (Applied Biosystems). All primers and PCR-conditions are available on request.

Statistical Analyses

FVIII:C levels are presented as means with their standard deviation (SD). To evaluate the difference in levels between groups Student's t test was used. The differences, together with the 95% confidence intervals (CI95) of the differences, are given. The differences in FVIII levels between groups were also studied after adjustment for age using linear regression. To evaluate the influence of the different geno- and haplotypes on the risk of thrombosis, odds ratios (OR) and their corresponding CI95 according to Woolf²⁰ were calculated. ORs were corrected for levels of FVIII using logistic regression.

Results

c94901g, encoding D1241E, was in Hardy-Weinberg equilibrium for the female controls of the LETS and RATIO. The haplotypes present in the LETS, SMILE and RATIO were in accordance with those reported by SeattleSNPs. Haplotype frequencies were slightly lower than previously reported¹⁴. They were approximately 14% for HT1 and 2% for both HT3 and HT5 in all three studies.

FVIII Haplotypes and levels of FVIII

Effects of the FVIII gene variations on levels of FVIII in controls of the separate studies are presented in Tables 2a and 2b. Results and analyses of the combined control groups are presented in Tables 3a and 3b.

Table 2a. FVIII:C (IU/dL) in male control subjects from LETS and SMILE separately.

Male Controls	Geno- / Haplotype (N)	LETS		SMILE	
		N	Mean FVIII:C	N	Mean FVIII:C
D1241E	D (701)	163	110.6 (28.8)	538	124.3 (33.2)
-	E (145)	38	101.9 (31.6)	107	119.2 (35.6)
HT1	HTx (726)	170	110.2 (29.4)	556	124.2 (33.1)
-	HT1 (118)	30	102.5 (29.8)	88	119.2 (36.4)
HT3	HTx (829)	196	109.1 (29.1)	633	123.5 (33.6)
-	HT3 (15)	4	109.0 (50.4)	11	125.8 (33.4)
HT5	HTx (835)	198	109.2 (29.3)	637	123.6 (33.6)
-	HT5 (10)	3	90.7 (36.5)	7	114.9 (30.2)

Table 2b. FVIII:C (IU/dL) in female control subjects from LETS and RATIO separately.

Female Controls	Geno- / Haplotype (N)	LETS		RATIO	
		N	Mean FVIII:C	N	Mean FVIII:C
D1241E	D / D (619)	184	111.0 (28.2)	435	114.5 (47.3)
-	D / E (245)	77	114.4 (33.5)	168	108.6 (39.9)
-	E / E (31)	9	117.4 (32.1)	22	125.4 (56.0)
HT1	HTx / HTx (673)	197	111.4 (28.7)	476	114.1 (46.2)
-	HTx / HT1 (197)	67	113.8 (33.0)	130	108.4 (41.7)
-	HT1 / HT1 (24)	6	118.5 (35.4)	18	127.6 (61.4)
HT3	HTx / HTx (865)	266	112.2 (29.8)	599	113.4 (46.3)
-	HTx / HT3 (29)	4	106.9 (39.1)	25	111.3 (32.1)
HT5	HTx / HTx (862)	258	111.8 (29.7)	604	113.5 (46.2)
-	HTx / HT5 (32)	12	120.7 (32.7)	20	109.5 (32.2)

For D1241E in all the controls combined, lower levels of FVIII:C were observed in the absence of the 1241D encoding allele. Heterozygous carriers and homozygous wildtype carriers had similar levels of FVIII.

In both the separate as well as the combined male control groups, hemizygous carriers of the 1241E encoding alleles had lower levels of FVIII (Tables 2a & 3a). In the combined control group, FVIII:C was reduced by 6%. Looking at the three separate haplotypes, HT1, HT3 and HT5, the reduction disappeared for carriers of HT3. For carriers of HT5, a reduction in FVIII:C was observed, however with wide confidence intervals. Also, correction for age made the reduction much smaller while confidence intervals remained wide. For HT1 however, the 6% reduction in FVIII:C remained (Table 3a).

Table 3a. FVIII:C (IU/dL) in all male control subjects from LETS and SMILE combined.

Male Controls	Geno- / Haplotype (N)	Mean FVIII:C (SD)	Δ (CI95)	Δ^* (CI95)
D1241E	D (701)	121.1 (32.7)	-	-
-	E (145)	114.7 (35.3)	6.4	6.0
HT1	HTx (726)	120.9 (32.8)	-	-
-	HT1 (118)	115.0 (35.5)	5.9	6.3
HT3	HTx (829)	120.1 (33.2)	-	-
-	HT3 (15)	121.3 (37.4)	-1.3	-2.6
HT5	HTx (835)	120.2 (33.2)	-	-
-	HT5 (10)	107.6 (32.3)	12.6	5.3

Δ is the difference between the means given with the corresponding CI95. Δ^* is the difference between the mean distances to the regression line after linear regression adjusting for age. HTx signifies all haplotypes but the one given.

In the female control groups, no clear effect of the 1241E encoding alleles on levels of FVIII was observed (Tables 2b & 3b). Also the separate haplotypes showed no consistent effects.

Table 3b. FVIII:C (IU/dL) in all female control subjects from LETS and RATIO combined.

Female Controls	Geno- / Haplotype (N)	Mean FVIII:C (SD)	Δ (CI95)	Δ^* (CI95)
D1241E	D / D (619)	113.5 (42.5)	-	-
-	D / E (245)	110.4 (38.0)	3.1	3.4
-	E / E (31)	123.1 (49.9)	-9.6	-11.2
HT1	HTx / HTx	113.3 (41.8)	-	-
-	HTx / HT1	110.2 (39.0)	3.1	3.3
-	HT1 / HT1	125.3 (55.5)	-12.0	-14.5
HT3	HTx / HTx	113.1 (41.9)	-	-
-	HTx / HT3	110.7 (32.4)	2.4	3.0
HT5	HTx / HTx	113.0 (42.0)	-	-
-	HTx / HT5	113.7 (32.3)	-0.7	-0.3

Δ is the difference between the means given with the corresponding CI95 calculated vs. the wildtype (D / D or HTx / HTx). Δ^* is the difference between the mean distances to the regression line after linear regression adjusting for age. HTx signifies all haplotypes but the one given.

In both the male and the female control group, VWF:Ag, which was measured in the RATIO and in part of the LETS only, was not influenced by either D1241E nor by the three haplotypes. In those subgroups where VWF and ABO blood group were measured, correction for VWF and blood group did not influence the outcomes.

FVIII haplotypes and the risk of venous thrombosis

The risk of venous thrombosis in the LETS (Table 4a)

Overall, men and women combined, 1241E appeared to slightly decrease the risk of venous thrombosis. Homozygous carriers of the E encoding alleles had an OR of 0.7 (CI95 0.4-1.1). When stratified for haplotypes, it became clear that this risk reduction was caused by HT1. Homozygous carriers of HT1 had an OR of 0.5 (CI95 0.3-0.9). For HT3 and HT5 no reduction in risk was observed.

Table 4a. Distribution of FVIII geno- and haplotypes in all patients with venous thrombosis and controls from LETS.

LETS	Geno- / Haplotype [†]	Cases	Controls	OR (CI95)
D1241E	D / D	363	347	1*
-	D / E	75	77	0.9 (0.7 to 1.3)
-	E / E	33	47	0.7 (0.4 to 1.1)
HT1	HTx / HTx	386	367	1*
-	HTx / HT1	65	67	0.9 (0.6 to 1.3)
-	HT1 / HT1	20	36	0.5 (0.3 to 0.9)
HT3	HTx / HTx	460	462	1*
-	HTx / HT3	9	4	2.3 (0.7 to 7.4)
-	HT3 / HT3	2	4	0.5 (0.1 to 2.8)
HT5	HTx / HTx	454	456	1*
-	HTx / HT5	11	12	0.9 (0.4 to 2.1)
-	HT5 / HT5	6	3	2.0 (0.5 to 8.1)

* reference group; [†] the homozygous genotypes also include hemizygotes.

The risk of venous thrombosis in men (Table 4b)

In men alone we found a more pronounced protective effect of the 1241E encoding alleles against venous thrombosis. Carriers of 1241E had an OR of 0.5 (CI95 0.3-0.9). After stratification for haplotypes this reduction in risk remained

mainly for HT1. It was even more pronounced for carriers of HT1 for which the OR was 0.4 (CI95 0.2-0.8).

Table 4b. Distribution of FVIII genotypes and haplotypes in all male patients with venous thrombosis and all male controls from LETS.

LETS Men	Geno- / Haplotype	Cases	Controls	OR (CI95)
D1241E	D	180	163	1*
-	E	22	38	0.5 (0.3 to 0.9)
HT1	HTx	188	170	1*
-	HT1	14	30	0.4 (0.2 to 0.8)
HT3	HTx	200	196	1*
-	HT3	2	4	0.5 (0.1 to 2.7)
HT5	HTx	196	198	1*
-	HT5	6	3	2.0 (0.5 to 8.2)

* reference group.

After adjustment for FVIII:C with logistic regression, the OR for HT1 in men was 0.5 (CI95 0.2-1.0). The reduction in FVIII levels accounted for part of the reduction of the risk of venous thrombosis, but HT1 seems to influence risk in a manner independent of levels as well. HT3 and HT5 were too rare to allow meaningful risk estimates.

Table 4c. Distribution of FVIII geno- and haplotypes in all female patients with venous thrombosis and all female controls from LETS.

LETS Women	Geno- / Haplotype	Cases	Controls	OR (CI95)
D1241E	D / D	183	184	1*
-	D / E	75	77	1.0 (0.7 to 1.4)
-	E / E	11	9	1.2 (0.5 to 3.0)
HT1	HTx / HTx	198	197	1*
-	HTx / HT1	65	67	1.0 (0.7 to 1.4)
-	HT1 / HT1	6	6	1.0 (0.3 to 3.1)
HT3	HTx / HTx	260	266	1*
-	HTx / HT3	9	4	2.3 (0.7 to 7.6)
HT5	HTx / HTx	258	258	1*
-	HTx HT5	11	12	0.9 (0.4 to 2.1)

* reference group.

The risk of venous thrombosis in women (Table 4c)

In women, virtually no effect on the risk of venous thrombosis was observed. For both hetero- and homozygous carriers of 1241E, the OR was approximately 1. Similar ORs were observed for heterozygous carriers of HT1 and HT5 and homozygous carriers of HT1. In heterozygous carriers of HT3, the risk appeared increased (OR 2.3), however, the confidence interval was wide (0.7-7.6).

Sequencing of the FVIII gene

The effects observed on levels of FVIII and the risk of thrombosis appeared to depend mainly on HT1. To identify haplotype-specific variations that may explain this effect, all exons and exon-intron boundaries of the FVIII gene were sequenced in nine male individuals, three per haplotype. Individuals were selected on phenotype for HT1. Three male controls from the LETS, hemizygous for HT1, were chosen with FVIII:C < 100 IU/dL, because of the protective effect on venous thrombosis in men and the reduction in FVIII:C associated with HT1. HT3 and HT5 appeared not to be associated with any particular phenotype. Therefore, for both HT3 and HT5 three hemizygous male carriers were selected without reference to FVIII levels.

Besides the SNPs already reported by SeattleSNPs, no haplotype-specific variations were found. Since none of these previously reported variations are likely to influence either protein levels or protein function, it is possible that functional variations lie in intronic regions of the FVIII gene.

Discussion

Elevated levels of FVIII are an important risk factor for venous thrombosis. There are strong indications that FVIII levels are, at least in part, determined genetically, beyond the well-known effect of ABO blood group. To assess whether the D1241E variation in the B-domain of FVIII, which in previous studies was linked to FVIII levels, is indeed causative to the previously reported effects on FVIII:C^{15,16}, we genotyped two additional SNPs to tag three haplotypes that all carry the minor allele of this variation.

We studied these three haplotypes separately in the LETS and in the control

populations of the SMILE and the RATIO studies. In general, FVIII:C was relatively high in all three studies. It was shown previously in the LETS that this is not a result of activation⁶, since FVIII:C were strongly associated with the actual protein concentration (FVIII:Ag). Nor were these high FVIII levels the result of an acute phase response⁹. The high mean FVIII levels were not the result of a shift in the distribution of ABO blood groups (LETS control group, 42.6% with blood group O). So there is no explanation for the overall slightly elevated FVIII levels in our study populations. However, this elevation does not affect analyses comparing effects of the different geno- and haplotypes within these populations.

We observed an association between 1241E and lower FVIII:C, but in men only. In men, a reduction of approximately 6% in FVIII:C was observed in those individuals with 1241E. After stratifying for haplotypes this effect appeared to be restricted to HT1. The data also imply a reductive trend of FVIII:C for HT5, which is genetically very similar to HT1. However if HT1 and HT5 are looked at as one haplotype, the effects are weaker than for HT1 alone.

In contrast to Scanavini *et al*⁶, who described an 11% reduction in FVIII:C in female carriers of the 1241E encoding alleles, we found no effects in female control subjects. Stratifying for haplotypes made no difference. There are few differences between the female controls from the LETS and the RATIO and the controls used by Scanavini *et al*⁶ that could explain these contrasting results.

Scanavini *et al*¹⁶ excluded users of oral contraceptives, whereas we did not.

However exclusion or inclusion of oral contraceptive users is highly unlikely to have influenced the results of D1241E in either study. Since prescription of oral contraceptives does not depend on genotype, there is no reason to assume that oral contraceptives users will be unequally distributed over the different geno- and haplotypes.

In summary, we showed that in our study populations, a reduction in FVIII:C is restricted to male carriers of HT1, indicating that there is another variation on this haplotype that influences FVIII:C in men only. There is no satisfactory explanation for the discrepancy in effects between the two sexes.

As expected, we observed decreases in risk of venous thrombosis in the group

where FVIII levels were decreased. In male carriers of 1241E and particularly carriers of HT1, who showed a 6% reduction in FVIII:C, a strong protection against venous thrombosis was observed. It is tempting to hypothesize that this reduction in risk is caused solely by the reduction in FVIII levels, however logistic regression showed that only a small part of this effect is caused by the reduction in FVIII:C. Nonetheless, similar to the role of ABO blood group, this again is an indication for a causative association between FVIII:C and the risk of venous thrombosis. Besides the small effect HT1 has on the risk of venous thrombosis via a reduction in FVIII:C, adjustment with logistic regression showed us that HT1 influences the risk of venous thrombosis in a manner independent of FVIII levels as well.

The data presented here suggest that the effects associated with D1241E are limited to HT1. Thus, D1241E is not likely to be the functional variation responsible for the effect on FVIII levels and thrombosis risk as it is present in all three haplotypes. Indeed, so far, no mechanisms for the functionality of D1241E have been identified. The B-domain of the FVIII molecule, in which D1241E is located, is not necessary for procoagulant activity²¹, but probably plays an important role in determining levels of FVIII in the circulation²². The B domain is heavily glycosylated. At least nineteen of the twenty-five N-linked glycosylation sites on FVIII are located in the B domain^{23,24}. It has been shown that these sites play an important role in the secretion of FVIII²⁵ and that they may also influence FVIII clearance from the circulation²⁶. The nearest N-linked glycosylation site to D1241E however, is 14 residues away. Although it cannot be excluded, the minor change in side group from aspartic acid to glutamic acid makes it highly unlikely that D1241E would influence glycosylation in the B domain.

After re-sequencing of the FVIII gene, we did not identify other possibly functional variations specific for HT1. However, we only sequenced exons and exon-intron boundaries, so additional variations undoubtedly exist within non-coding regions. So even though no functional variant was found in HT1 and numbers of carriers of the other two haplotypes, HT3 and HT5 remained very small, we should seriously consider the possibility of the existence of an as yet

unidentified functional mutation in HT1 other than D1241E. At the same time, the possibility also exists that as yet unidentified variations in HT3 and HT5 exist that counteract the effects of D1241E, masking its functionality in these two haplotypes.

In conclusion, we observed a reduction in FVIII:C that was restricted to male carriers of HT1. HT1 was also associated with a decrease in risk of venous thrombosis. A small part of this decrease in risk could be explained by the reduction in FVIII levels. In addition, HT1 had a protective effect against venous thrombosis, which could not be explained by the reduction in FVIII:C as well. Although this should be investigated further, it is possible that D1241E is not the main player in these effects, since both carriers of HT3 and HT5 apparently show no reduction in either FVIII:C or in the risk of venous thrombosis.

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Chapter 4b

Haplotypes Encoding the Factor VIII 1241Glu Variation and the Risk of Myocardial Infarction

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Elevated levels of coagulation Factor VIII (FVIII) are an important risk factor for venous thrombosis¹⁻⁴. FVIII activity (FVIII:C) ≥ 150 IU/dL increases the risk of a first venous thrombosis fivefold compared to levels below 100 IU/dL. There are also indications that elevated FVIII levels contribute to the risk of arterial thrombosis⁵⁻¹¹.

In 2003, Machiah *et al*/reported a missense single nucleotide polymorphism (SNP) in the X-chromosomal FVIII gene, c94901g, which causes an amino acid change in the B-domain of FVIII, Asp1241Glu (D1241E)¹². This amino acid change was associated with a decrease in FVIII:C. Machiah *et al* found that this polymorphism accounts for approximately 5% of total variation in FVIII:C in the population of the GAIT study¹². More recently, Scanavini *et al*/reported that 1241E was associated with an 11% reduction in FVIII:C in 145 healthy women and 150 women with venous thrombosis¹³.

It is unclear by which mechanism the 1241E variant influences the levels of FVIII. Scanavini *et al*/hypothesized that the variation was involved in APC resistance, but could not demonstrate this¹³. On the website of SeattleSNPs¹⁴, SNP and haplotype data are presented for a wide variety of genes, including the gene encoding FVIII. These data are based on the re-sequencing of these genes in 23 subjects of European-American descent. In this population the SNP encoding 1241E is present in at least three different haplotypes, HT1, HT3 and HT5, of which possibly only one is responsible for the reported effects on FVIII levels.

Recently, we investigated the effects of these three haplotypes on levels of FVIII and on the risk of venous thrombosis¹⁵. We confirmed a reduction in levels of FVIII, but this effect was restricted to only one of the three haplotypes, HT1, in men. In this group, men with HT1, a reduction in venous thrombosis risk was observed; however, the reduction in risk could only partially be explained through the reduction in FVIII levels¹⁵. This suggested that functional variations in the FVIII molecule may also contribute to the development of venous thrombosis.

To investigate whether these three FVIII gene haplotypes also affect the risk of arterial thrombosis, we studied the effects of these haplotypes in two population based case-control studies on myocardial infarction, the Study of Myocardial Infarctions Leiden (SMILE) and the Risk of Arterial Thrombosis In Relation to Oral Contraceptives Study (RATIO). Both studies have been described previously in more detail^{16,17}. In brief, the SMILE consists of 560 patients, all men below the age of 70, diagnosed with a first myocardial infarction. The control group consists of 646 men who did not have a history of myocardial infarction. Control subjects were frequency matched to patients on 10-year age groups. Plasma and DNA samples were available of all participants. The RATIO consists of three substudies for different vascular events: ischemic stroke, peripheral vascular disease and myocardial infarction. Only patients with myocardial infarction were included in the present study. Patients were 248 women between the ages of 18 to 49 who were hospitalized for a first myocardial infarction. Of 217 of these 248 women plasma and DNA samples were available and these 217 women were included in the analysis. The RATIO has one large control group for all three substudies. This group consists of 925 women between the ages of 18 to 49 who had no history of coronary, cerebral, or peripheral arterial disease. The controls were selected via Random Digit Dialing. The controls of whom plasma and DNA samples were available (639 women) were included in the current study.

In order to identify carriers of the three haplotypes HT1, HT3 and HT5, c94901g (numbering according to SeattleSNPs, April 2004¹⁴; rs1800291), encoding D1241E was determined in all individuals. Subsequently, two haplotype tagging SNPs specific for HT3 and HT5, g24052a (rs6655259) and g27882c (rs-number pending) respectively, were determined in both hetero- and homozygous carriers of the 1241E encoding allele. Genotyping was performed using either polymerase chain reaction – restriction fragment length polymorphisms analyses (PCR-RFLP) or 5'nuclease/Taqman assays. This has previously been described in detail¹⁵.

The haplotypes present in the SMILE and RATIO were in accordance with those reported by SeattleSNPs. Haplotype frequencies were approximately 14%

for HT1 and 2% for both HT3 and HT5 in the control groups of both studies. The distribution of geno- and haplotypes over case and controls of the SMILE and RATIO are depicted in Tables 1a and 1b.

Table 1a. Distribution of FVIII geno- and haplotypes in men with a first myocardial infarction and healthy control subjects.

SMILE	Geno- Haplotype [†]	Cases	Controls	OR (CI95)
D1241E	D	465	539	1*
	E	95	107	1.0 (0.8-1.4)
HT1	HTx	487	557	1*
	HT1	70	88	0.9 (0.7-1.3)
HT3	HTx	551	634	1*
	HT3	6	11	0.6 (0.2-1.7)
HT5	HTx	544	638	1*
	HT5	16	7	2.7 (1.1-6.6)

* reference group; [†] HTx signifies all haplotypes but the one given

In contrast to what we found for venous thrombosis¹⁵, neither the 1241E allele nor HT1 were associated with the risk of myocardial infarction in men. In the SMILE, the odds ratio (OR) for 1241E was 1.0 (95% confidence interval (CI95) 0.8-1.4) and for HT1 the OR was 0.9 (0.7-1.3). HT5 was rare, and an increase in risk for HT5 was observed, with an OR of 2.7 (CI95 1.1-6.6) in hemizygous carriers of HT5. HT3 was even less common and the risk estimate had wide confidence intervals.

In the RATIO, no clear effect of 1241E on the risk of myocardial infarction in young women was observed, although the results were compatible with a graded reduced risk, with an OR of 0.9 (CI95 0.6-1.3) for heterozygous 1241E carriers and an OR of 0.5 (CI95 0.2-1.5) for 1241E homozygotes. This risk reduction was most pronounced for HT1 carriers: for heterozygous women the OR was 0.8 (CI95 0.6-1.2) and for homozygous women the OR was 0.2 (0.02-1.2). In accordance with the increase in risk observed in hemizygous men for HT5, the risk of myocardial infarction appeared to be increased slightly in women heterozygous for HT5, with an OR of 1.5 (CI95 0.7-3.2).

Table 1b. Distribution of FVIII geno- and haplotypes in young women with a first myocardial infarction and healthy control subjects.

RATIO	Geno- Haplotype[†]	Cases	Controls	OR (CI95)
D1241E	D / D	149	439	1*
	D / E	52	169	0.9 (0.6–1.3)
	E / E	4	23	0.5 (0.2–1.5)
HT1	HTx / HTx	167	481	1*
	HTx / HT1	37	130	0.8 (0.5–1.2)
	HT1 / HT1	1	18	0.2 (0.02–1.2)
HT3	HTx / HTx	194	606	1*
	HTx / HT3	11	25	1.4 (0.7–2.8)
HT5	HTx / HTx	195	608	1*
	HTx HT5	10	21	1.5 (0.7–3.2)

* reference group; [†] HTx signifies all haplotypes but the one given

Previously we reported a reduction of risk of venous thrombosis in male carriers of the HT1 haplotype, which is one of the three haplotypes on which the D1241E variant is found, which is associated with reduced factor VIII plasma levels¹⁵. In the current analysis we find no association of this haplotype with the occurrence of myocardial infarction in men, while it was associated with a reduced risk of myocardial infarction in young women. It is surprising that an association between HT1 and the risk of myocardial infarction was observed in women and not in men, whereas for venous thrombosis the opposite is true. It is possible that in younger individuals, such as the women in the RATIO, coagulation plays a more important role in the development of arterial disease, whereas in older individuals such as most patients of the SMILE, arterial endothelial damage is the more important trigger of a myocardial infarction. However, this study again indicates an association between the FVIII gene HT1 and thrombotic disease. HT3 and HT5 were rare in our study populations. Because of wide confidence intervals, no firm conclusions can be drawn. However increases in the risk of myocardial infarction were observed in carriers of these 1241E encoding haplotypes. These results need to be confirmed in other studies.

Since effects were different between the three haplotypes, these results indicate that D1241E itself is not a functional variation but that in all likelihood, it is

merely linked to other functional FVIII gene variations. This observation is in agreement with the different effects that these three haplotypes have on levels of FVIII in the SMILE and RATIO and also in the Leiden Thrombophilia Study (LETS) as was reported previously. HT1 was associated with reduced levels of FVIII in men whereas HT3 and HT5 showed no effect on levels¹⁵.

In conclusion, this study indicates that FVIII gene variations may influence the risk of arterial thrombosis.

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Chapter 5

Beta 2 Adrenergic Receptor Polymorphisms: Association with Factor VIII and von Willebrand Factor Levels and the Risk of Venous Thrombosis

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Several studies have shown that elevated plasma levels of coagulation factor VIII (FVIII) are a risk factor of venous thrombosis. This risk remained after correction for the main determinants of FVIII levels, blood group and von Willebrand factor (VWF¹⁻³. FVIII activity levels (FVIII:C) ≥ 150 IU/dL increase the risk of a first venous thrombosis fivefold when compared to levels below 100 IU/dL. The prevalence of FVIII:C ≥ 150 IU/dL among thrombosis patients is 25%. Since these levels are found in 10 % of the population, the contribution of elevated FVIII levels to all thrombotic events in the population is considerable². Several lines of evidence support the idea that high FVIII levels are indeed causative to thrombosis and not a consequence of the thrombotic event, such as a dose-dependent relationship with risk², persistence of elevated levels over time³ and familial clustering⁴. The latter, familial clustering, supports the hypothesis that FVIII levels are, at least in part, determined genetically. Because no variations have been found in the FVIII and VWF genes that are associated with thrombosis^{1,5}, it is likely that genes encoding proteins regulating plasma levels of FVIII and VWF are involved.

A candidate regulator of FVIII levels is the $\beta 2$ adrenergic receptor ($\beta 2AR$). It is well known that adrenaline infusion causes a significant rise in FVIII levels. This effect can be blunted by prior administration of a β -blocker. Hoppener *et al.*⁶ showed that in patients with venous thromboembolism and FVIII levels > 175 IU/dL, FVIII:C levels could be effectively lowered by treatment with propranolol. FVIII:C returned to its initial elevated levels within two months after discontinuation of treatment. However, Schönauer *et al.*⁷ reported that in patients with venous thromboembolism and FVIII levels > 170 IU/dL, propranolol administration could not lower FVIII levels significantly. We approached this issue from a different angle by determining the possible association of single nucleotide polymorphisms (SNP) in the $\beta 2AR$ gene with FVIII and VWF levels and thrombotic risk.

We studied three coding $\beta 2AR$ SNPs, that have previously been implicated in clinically relevant effects⁸⁻¹⁰, in a large population based case-control study, the Leiden Trombophilia Study (LETS). The LETS consists of 474 consecutive

patients and 474 controls. All the patients were referred for anti-coagulant treatment after a first objectively confirmed episode of deep vein thrombosis. Patients with underlying malignancies were excluded. The controls were matched for sex and age. DNA samples are available of 469 cases and 470 controls. FVIII:C was measured in the plasma of all these participants by a one-stage clotting assay. VWF antigen and FVIII antigen (FVIII:Ag) were measured by ELISA in the plasma of 301 patients and 301 controls. The design of this study has previously been described in more detail¹¹. Using polymerase chain reaction – restriction fragment length polymorphism analyses the three β 2AR SNPs, Arg16Gly, Glu27Gln and Thr164Ile, were determined. The distributions of genotypes for the three studied SNPs were in Hardy-Weinberg equilibrium in the controls. The allele-frequencies in the controls were consistent with those found in previous studies^{12,13}. No differences in allelic distributions were observed between the cases and controls (Table 1). There was a protective effect on the occurrence of venous thrombosis for the rare allele of Thr164Ile, however this effect was weak with wide confidence limits around the odds ratio. Based on the frequencies of the SNPs, four different haplotypes were identified in our study-population (Table 1) with the help of special software, Arlequin¹⁴. These haplotypes corresponded to those reported in previous publications^{10,12}. No significant differences were found in the distribution of the haplotypes between cases and controls. The trend we observed for the rare allele of Thr164Ile was recovered in haplotype 4. In addition, homozygotes for haplotype 2 showed an odds ratio of 1.67 (95% confidence interval: 0.75-3.74). Combining this with the results for the individual SNPs, it is unlikely that the gene for the β 2AR contains a common polymorphism that is associated with the risk of venous thrombosis. No association was found between FVIII:C, FVIII:Ag and VWF levels and the different genotypes and haplotypes in the control group. Because ABO blood group strongly influences levels of FVIII and VWF, we also analysed the data stratified by blood group O and non-O in healthy individuals. Again, no effect was observed.

Table 1. Distribution of $\beta 2AR$ genotypes and haplotypes in patients with venous thrombosis and controls.

SNP	Genotype	Case	Control	OR*	CI95	Haplotype	Haplotype	Case	Control	OR*	CI95
16	Gly/Gly	183 (39%)	180 (38%)	1 [†]	0.77-1.34	1	HxHx [‡]	148 (32%)	154 (33%)	1 [†]	0.81-1.45
	Gly/Arg	219 (47%)	213 (45%)				H1Hx	225 (48%)	216 (46%)	1.08	
	Arg/Arg	66 (14%)	77 (16%)				H1H1	96 (20%)	100 (21%)	1.00	
	Total	468	470		0.57-1.24		Total	469	470		0.70-1.43
27	Glu/Glu	96 (20%)	100 (21%)	1 [†]	0.78-1.52	2 16Gly-27Gln-164Thr	HxHx [‡]	325 (69%)	340 (72%)	1 [†]	0.83-1.48
	Glu/Gln	225 (48%)	216 (46%)				H2Hx	127 (27%)	120 (26%)	1.11	
	Gln/Gln	148 (32%)	154 (33%)				H2H2	16 (3%)	10 (2%)	1.67	
	Total	469	470		0.70-1.43		Total	468	470		0.75-3.74
164	Thr/Thr	458 (98%)	453 (96%)	1 [†]	0.26-1.28	3 16Arg-27Gln-164Thr	HxHx [‡]	183 (39%)	180 (38%)	1 [†]	0.77-1.34
	Thr/Ile	10 (2%)	17 (4%)				H3Hx	219 (47%)	213 (45%)	1.01	
	Total	468	470				H3H3	66 (14%)	77 (16%)	0.84	
							Total	468	470		0.57-1.24
164	Thr/Thr	458 (98%)	453 (96%)	1 [†]	0.26-1.28	4 16Gly-27Gln-164Ile	HxHx [‡]	458 (98%)	453 (96%)	1 [†]	0.26-1.28
	Thr/Ile	10 (2%)	17 (4%)				H4Hx	10 (2%)	17 (4%)	0.58	
	Total	468	470				Total	468	470		

*Odds Ratio

†Reference Category

"x" indicates any haplotype other than the haplotype studied

O'Donnell *et al*¹³ recently reported a significant effect on FVIII:C levels for SNP Glu27Gln in healthy blood group O individuals. They found, that in a group of 59 healthy blood group O individuals, those with genotype Gln/Gln had lower levels of FVIII:C than those with genotype Glu/Glu. However, this effect was very small and only marginally significant. In our study, we could not confirm these results in a group of 201 healthy blood group O individuals. FVIII:C levels within this group were for Gln/Gln: 111.04 IU/dL (104.14-117.95), for Gln/Glu: 109.01 IU/dL (102.18-115.84) and for Glu/Glu: 109.54 IU/dL (99.50-119.57)).

In conclusion, genotypes and haplotypes of the β 2AR have no influence on either the occurrence of venous thrombosis or plasma-levels of FVIII and VWF. It remains to be determined what genetic variations are responsible for the familial clustering of elevated levels of FVIII.

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Chapter 6

Vasopressin 2 Receptor Gene Variations and the Regulation of Levels of von Willebrand Factor and Factor VIII and the Risk of Venous Thrombosis

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Summary

Objectives. Stimulation of the Vasopressin 2 Receptor (V2R) with vasopressin (AVP) results in increased von Willebrand factor (VWF) and factor VIII (FVIII) plasma levels. Elevated FVIII levels are a risk factor of thrombosis. We hypothesized that variations in the X-chromosomal V2R gene (*AVPR2*) influence VWF and FVIII levels and thrombosis risk.

Methods and Results. We genotyped a case-control study on venous thrombosis, the Leiden Thrombophilia Study, for four *AVPR2* variations: a-245c, G12E, L309L and S331S. Rare alleles of a-245c, G12E and S331S, which were in linkage disequilibrium, were associated with higher VWF, VWF propeptide and FVIII levels and a decreased thrombosis risk in men. Expression constructs of 12G-V2R and 12E-V2R, coupled to green fluorescent protein, were stably transfected in MDCKII cells, which lack endogenous V2R-expression. Both V2R variants were fully glycosylated and expressed on the basolateral membrane. In ³H-AVP saturation experiments, binding affinity was increased threefold in 12E-V2R-GFP cells. K_D was 4.5 nM (95% confidence interval (CI95) 3.6-5.4) for V2R-12E-GFP and 16.5 nM (CI95 10.1-22.9) for V2R-12G-GFP with similar B_{max} for both receptors.

Conclusions. The 12E-V2R variant has increased binding affinity for AVP and is associated with increased VWF, VWF propeptide and FVIII levels and decreased thrombosis risk in men.

Introduction

Several studies have shown that elevated plasma levels of coagulation factor VIII (FVIII) are a risk factor of venous thrombosis¹⁻⁷ and possibly also for arterial thrombosis⁸⁻¹⁴. The mechanisms that underlie the substantial inter-individual variations in FVIII levels in the general population are still poorly understood. There are strong indications that FVIII levels are at least in part determined genetically^{15,16}. Besides variations in the genes encoding FVIII and its carrier protein von Willebrand factor (VWF) and ABO blood group, genetic variations in proteins that regulate plasma levels of FVIII and VWF may result in increased levels of FVIII.

A candidate regulator of FVIII levels is the arginine vasopressin 2 receptor (V2R). The V2R is a G-protein coupled receptor consisting of 371 amino acids. The main function of the V2R is to maintain blood volume and pressure by stimulating water retention by the kidney. Upon binding of arginine vasopressin (AVP (also known as the antidiuretic hormone (ADH))) to the V2R in renal principal cells, a cAMP signalling cascade is initiated, resulting in increased expression of the water channel aquaporin 2 (AQP2) and its translocation to the apical membrane. This will result in the reabsorption of water from the pre-urine¹⁷. V2R is also expressed in vascular endothelial cells where its stimulation by AVP results in the release of Weibel Palade bodies (WPb) into the circulation, causing a sharp rise in the plasma levels of VWF and FVIII¹⁸. In fact, 1-desamino-8-d-arginine vasopressin (desmopressin or DDAVP), a synthetic analogue of AVP, is frequently used to treat patients with mild von Willebrand disease or Haemophilia A¹⁹. Altered functioning of the V2R could thus result in altered plasma levels of VWF and FVIII. Therefore, we were interested to see whether variations in the gene encoding the V2R, *AVPR2*, can influence levels of VWF propeptide, VWF and FVIII and also the risk of venous thrombosis. We hypothesized that gain of function mutations in *AVPR2* will lead to increased secretion of VWF, leading to increased levels of FVIII and an increased risk of venous thrombosis. To test this hypothesis, several single nucleotide polymorphisms (SNPs) were genotyped in a case control study on venous thrombosis, the Leiden Thrombophilia Study (LETS). Allelic distributions of these genotypes were used for association studies with levels and thrombosis risk. Resequencing and in vitro studies were used to investigate the functionality of these variations.

AVPR2 is located on the long arm of the X-chromosome on the same chromosomal band as the FVIII gene, Xq28. In between these two genes lies the gene encoding the renin binding protein (RBP)²⁰. Via inhibition of renin, RBP can influence the formation of angiotensin II, which stimulates the release of AVP.²¹ Therefore, we additionally genotyped the LETS for an *RBPSNP* that has been reported to be associated with plasma (pro-)renin levels²⁰.

Materials and Methods

Leiden Thrombophilia Study (LETS)

The LETS consists of 474 consecutive patients and 474 healthy controls. All patients were referred for anti-coagulant treatment after a first objectively confirmed episode of deep vein thrombosis. Controls were frequency-matched for sex and age and were acquaintances or partners of the patients. Mean age for both the patient and the control groups was 45 years, ranging from 15 to 69 for patients and 15 to 72 for controls. Both groups consisted of 272 women (57.4%) and 202 men (42.6%). All individuals with underlying malignancies were excluded. DNA samples are available of 471 cases and 471 controls. The design of this study has previously been described in more detail^{22,23}. FVIII antigen (FVIII:Ag), VWF antigen (VWF:Ag) and VWF propeptide were measured by ELISA in the plasma of the first 301 patients and 301 controls included in the study^{1,24}. Pooled normal plasma was used as a reference. Results for FVIII:Ag and VWF:Ag are expressed as international units per ml (IU/ml). Results for VWF propeptide are expressed as units per ml (U/ml), with one unit defined as the amount of VWF propeptide in one ml of the pooled normal plasma. The LETS was approved by the appropriate ethical committees and all participants gave informed consent.

Genotyping

Based on frequencies and prevalence in Caucasians, we initially selected three *AVPR2* SNPs: G12E, L309L and S331S (rs2071126, rs5201 & rs5202 respectively). The entire LETS was genotyped for these three SNPs using polymerase chain reaction – restriction fragment length polymorphism analysis (PCR-RFLP). The polymerase chain reactions and enzymatic digestions were performed on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany) and primers were purchased from Eurogentec (Seraing, Belgium). After resequencing *AVPR2* as described below, the LETS was genotyped for a fourth *AVPR2* variation, being a-245c (rs4898372). *AVPR2* a-245c and the *RBP* SNP t61c (rs2269372) were determined using 5' nuclease/Taqman assays. The polymerase chain reactions with fluorescent allele-specific oligonucleotide

probes (Assay-by-Design, Applied Biosystems, Foster City, CA) were also performed on the PTC-225 thermal cycler. Fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA).

PCR conditions, restriction endonucleases and the sequences of probes and primers used for genotyping are available on request.

Sequencing

The complete genomic region of *AVPR2*, including the 3 exons and introns and 5'- and 3'-UTR, was resequenced. A 3.3 kb long region was amplified in fragments using 5 sets of primers (available on request). PCR-products were purified using a QIAquick PCR Purification Kit (Qiagen Benelux, Venlo, the Netherlands). Sequence reactions and fragment analysis were performed by the Leiden Genome Technology Center (www.lgtec.nl, LGTC, Leiden, the Netherlands) on an ABI 3700 or ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

AVPR2 Constructs

The expression construct encoding the wildtype V2R tagged with green fluorescent protein at the C-terminus (12G-V2R-GFP)²⁵ was kindly provided by Dr. Alexander Oksche (FMP, Berlin, Germany). The expression construct encoding the 12E-V2R-GFP was made from 12G-V2R-GFP with site-directed mutagenesis, using a QuikChange II Site-Directed Mutagenesis Kit (Qiagen Benelux, Venlo, the Netherlands) and a 31 base long mutagenesis primer (Eurogentec, Seraing, Belgium).

Cell Culture

Polarized Madin Darby Canine Kidney type II Cells (MDCK II), which lack endogenous V2R expression (kindly provided by Dr. Alexander Oksche, FMP, Berlin, Germany), were cultured at 37°C under 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum, 1% non-essential amino acids, gentamycin, L-glutamine

and sodium carbonate. Cells were stably transfected with the 12G-V2R-GFP or 12E-V2R-GFP constructs as described previously²⁶ and colonies were selected based on V2R expression.

Immunoblotting and Immunocytochemistry

For immunoblotting, cells were lysed in Laemmli buffer containing 0.1 M DTT. High mannose or total N-linked sugar moieties were removed with endoglycosidase H (endoH) or protein N-glycosidase F (PNGase F) (both from New England Biolabs, Beverly, MA, USA) respectively, according to the manufacturer's protocol. Polyacrylamide gel electrophoresis, Western blotting, and immunodetection were performed as described elsewhere^{26,27}. For detection of V2R-GFP, 1:5,000 diluted rabbit anti-GFP serum was used (kindly provided by Dr. B. Wieringa, dept of Cell Biology, RUNMC, Nijmegen, the Netherlands). As secondary antibodies, horseradish peroxidase-coupled goat anti-rabbit IgGs (Sigma) were used. Immunocytochemistry, confocal laser-scanning microscopy (CLSM), and data quantification were performed as described elsewhere²⁵. As primary antibody, 1:100-diluted rat anti-E-cadherin (Sigma, St. Louis, MO) was used. As secondary antibody, 1:100-diluted goat anti-rat IgG coupled to Alexa 594, was used (Molecular Probes, Leiden, the Netherlands). Horizontal extended-focus images and vertical images were obtained with a Bio-Rad MRC-1000 laser scanning confocal imaging system using a $\times 60$ oil-immersion objective, a 32 Kalman collection filter, an aperture diaphragm of 2.5, and an axial resolution of 0.14 $\mu\text{m}/\text{pixel}$. The images were contrast-stretched using Adobe Photoshop.

Radioligand Binding Assay

Untransfected MDCKII cells and 12G-V2R-GFP or 12E-V2R-GFP expressing MDCKII cells were seeded at a density of 150.000 cells/cm² on Costar-filters (Corning, Corning, NY, USA) in 24 wells plates and grown to confluence over three to four days. Cells were washed twice in ice-cold PBS-CM (PBS with 1 mM MgCl₂ and 0.1 mM CaCl₂) and the plates were placed on ice, to prevent V2R internalization. The cells were incubated on the basolateral side with ³H-

AVP in concentrations ranging from 0 to 100 nM in PBS-CM for two hours on ice. Cells were washed with PBS-CM and subsequently, the filters were excised and added to 5 ml Filter-Count scintillation fluid (PerkinElmer, Waltham, MA, USA) and counted in a Tri-Card 1600 TR Liquid Scintillation Analyzer (PerkinElmer). The saturation curves of 12G-V2R-GFP and 12E-V2R-GFP were baseline corrected for values counted in untransfected cells to correct for non-specific binding. Three independent experiments were performed in duplicate.

Statistical Analysis

To evaluate the difference in levels of FVIII, VWF and VWF propeptide between genotypes, Student's t tests and linear regression modelling were used. The differences, together with the 95% confidence intervals (CI95) of the differences, are given. Differences in levels between groups were adjusted for age using linear regression modelling. To evaluate the influence of the different genotypes on the risk of thrombosis, odds ratios (ORs) and their corresponding CI95 according to Woolf²⁸ were calculated. For some genotypes, there were empty cells and when that was the case, CI95s were calculated according to Mehta *et al.*²⁹ Because both the *AVPR2* and the RBP genes are located on the X-chromosome, all analyses described above were stratified on sex. Data for the AVP-saturation experiments in MDCKII cells were analyzed with Graphpad Prism. The maximum binding capacity (B_{max}) and dissociation constant (K_D) were determined along with the corresponding CI95s.

Results

Sequencing

Initially, we genotyped the LETS for three *AVPR2* SNPs: G12E, L309L and S331S. As is described below, G12E and S331S were associated with both thrombosis risk and levels of VWF propeptide, VWF and FVIII. We resequenced the entire *AVPR2* genomic region in all male carriers of these two SNPs, to identify variations linked to G12E and S331S. We identified one other potentially functional polymorphism, namely a-245c. Therefore, we additionally genotyped the LETS for the a-245c SNP.

Genotyping

Genotyping was successful in all individuals except for *AVPR2* a-245c in two and *RBP* t61c in one individual. In the female controls, all SNPs genotyped were in Hardy-Weinberg equilibrium. The frequencies of three of the *AVPR2* SNPs, a-245c, G12E and S331S, were low (approximately 2.5%) in healthy controls. *AVPR2* L309L and *RBP* t61c were more common and had frequencies of 35% and 21% respectively in healthy controls. There was one male control subject, who was heterozygous for the X-chromosomal *AVPR2* SNP L309L. Y-chromosome markers were present in his genomic DNA, indicating that this individual has Klinefelter syndrome (karyotype 47 XXY). This person was excluded from the analyses.

The *RBP* SNP t61c was not linked to any of the *AVPR2* SNPs. Nor were any of the *AVPR2* and *RBP* SNPs linked to FVIII gene SNPs (D1241 (rs1800291), g24052a (rs6655259) and g27882c (rs-number pending)), which had been determined in the LETS previously³⁰. On the other hand, the *AVPR2* SNPs a-245c, G12E and S331S were strongly linked to each other. Furthermore, rare alleles of these three SNPs only occurred in individuals also carrying rare alleles of L309L.

AVPR2 and RBP SNPs and levels of VWF propeptide, VWF and FVIII

In men, associations with levels of VWF propeptide, VWF and FVIII were observed for three *AVPR2* SNPs, a-245c, G12E and S331S (Table 1a). These three SNPs were in strong linkage disequilibrium. Male carriers of the rare alleles of these SNPs had higher levels of VWF propeptide, VWF and FVIII than carriers of the common alleles. These differences remained after adjustment for age (data not shown). *AVPR2* L309L and *RBP* t61c were not associated with VWF propeptide, VWF and FVIII levels in male controls. The associations between a-245c, G12E and S331S and levels in men were not present in women (Table 1b). However, there were slightly increased VWF and FVIII levels in heterozygous carriers of these three SNPs. Furthermore, levels of all three proteins appeared to decrease in carriers of the *RBP* 61c-allele. These associations remained after adjustment for age (data not shown).

Table 1a. Mean levels of VWF propeptide, VWF and FVIII in healthy male controls.

Genotype	N	VWF propeptide (U/ml)	Δ	CI95	VWF:Ag (IU/ml)	Δ	CI95	FVIII:Ag (IU/ml)	Δ	CI95
a-245c	a'	1.14	0.31	0.08 to 0.53	1.27	0.28	-0.08 to 0.63	1.14	0.33	-0.03 to 0.69
	c	1.45			1.54			1.47		
G12E	g'	1.14	0.25	0.01 to 0.50	1.27	0.18	-0.21 to 0.58	1.14	0.26	-0.15 to 0.66
	a	1.39			1.46			1.40		
L309L	a'	1.15	0.00	-0.11 to 0.12	1.29	-0.09	-0.27 to 0.09	1.16	-0.05	-0.24 to 0.13
	g	1.15			1.21			1.11		
S331S	c'	1.14	0.31	0.08 to 0.53	1.27	0.28	-0.08 to 0.63	1.14	0.33	-0.03 to 0.69
	t	1.45			1.54			1.47		
RBP t61c	t'	1.15	0.01	-0.14 to 0.13	1.29	-0.11	-0.31 to 0.09	1.17	-0.13	-0.34 to 0.77
	c	1.15			1.18			1.04		

* Reference group

Δ represents the mean difference to the reference group

Table 1b. Mean levels of VWF propeptide, VWF and FVIII in healthy female controls.

Genotype	N	VWF		Δ	CI95	VWF:Ag (IU/ml)		Δ	CI95	FVIII:AG (IU/ml)	Δ	CI95
		Propeptide (U/ml)										
a-245c	aa*	1.08	-	-	-	1.17	-	-	-	1.03	-	-
	ac	1.01	-0.08	-0.25 to 0.09	-0.24 to 0.29	1.19	0.03	0.06	-0.22 to 0.34	1.09	0.06	-0.22 to 0.34
G12E	gg*	1.08	-	-	-	1.17	-	-	-	1.03	-	-
	ga	1.00	-0.08	-0.28 to 0.12	-0.21 to -0.40	1.27	0.10	0.19	-0.13 to 0.52	1.23	0.19	-0.13 to 0.52
L309L	aa*	1.10	-	-	-	1.20	-	-	-	1.08	-	-
	ag	1.06	-0.05	-0.12 to 0.03	-0.21 to -0.02	1.10	-0.10	-0.13	-0.25 to -0.01	0.95	-0.13	-0.25 to -0.01
	gg	1.04	-0.06	-0.11 to 0.12	-0.04 to 0.45	1.40	0.20	0.09	-0.16 to 0.35	1.17	0.09	-0.16 to 0.35
S331S	cc*	1.09	-	-	-	1.17	-	-	-	1.03	-	-
	ct	1.00	-0.08	-0.16 to 0	-0.21 to -0.40	1.27	0.10	0.19	-0.13 to 0.52	1.23	0.19	-0.13 to 0.52
RBP t61c	tt*	1.10	-	-	-	1.20	-	-	-	1.07	-	-
	tc	1.06	-0.04	-0.12 to 0.04	-0.21 to 0.02	1.11	-0.09	-0.11	-0.24 to 0.02	0.96	-0.11	-0.24 to 0.02
	cc	0.97	-0.13	-0.28 to 0.02	-0.29 to 0.24	1.17	-0.03	-0.12	-0.39 to 0.16	0.96	-0.12	-0.39 to 0.16

* Reference group

 Δ represents the mean difference to the reference group

AVPR2 and RBP SNPs and the risk of venous thrombosis

Distributions of the different genotypes over patients and controls, along with odds ratios and CI95s are given in Table 2.

Table 2. Genotype distributions over patients and healthy controls and ORs in both men and women.

LETS		Men				Women			
Genotype		Cases	Controls	OR	CI95	Cases	Control	OR	CI95
a-245c	aa*	201	193	1.0	-	258	258	1	-
	ac	-	-	-	-	11	12	0.9	0.4-2.1
	cc	0	6	0.0	0.0-0.8	-	-	-	-
G12E	gg*	202	195	1.0	-	259	261	1	-
	ga	-	-	-	-	10	9	1.1	0.5-2.8
	aa	0	5	0.0	0.0-1.1	-	-	-	-
L309L	aa*	155	153	1.0	-	131	146	1	-
	ag	-	-	-	-	109	103	1.2	0.8-1.7
	gg	47	47	1.0	0.6-1.6	29	21	1.5	0.8-2.8
S331S	cc*	202	193	1.0	-	255	260	1*	-
	ct	-	-	-	-	14	10	1.4	0.6-3.3
	tt	0	7	0.0	0.0-0.7	-	-	-	-
RBP t61c	tt*	170	171	1.0	-	173	186	1	-
	tc	-	-	-	-	85	70	1.3	0.9-1.9
	cc	31	29	1.1	0.6-1.9	11	14	0.8	0.4-1.9

* Reference Group

The most common *AVPR2* variation, L309L, had no effect on thrombosis risk in men. In women, the risk appeared to increase slightly with the presence of the rare allele of this SNP. The other three *AVPR2* SNPs, a-245c, G12E and S331S, were linked and consequently, showed similar effects on thrombosis risk.

Because these three SNPs were rare, there were no women homozygous for the rare alleles. Heterozygous women showed no difference in risk compared to women homozygous for the common allele. However, men who were hemizygous for the rare alleles of these three SNPs were protected against thrombosis; all male carriers of the rare alleles were healthy controls. For the *RBP* SNP, no effects on thrombosis risk were observed in either men or women.

Transfection of MDCKII cells

Of the three SNPs that are associated with plasma levels and thrombosis risk, G12E was the only coding, non-synonymous SNP. Therefore, it was most likely to have an effect on V2R functioning. MDCKII cells were successfully transfected with either 12G-V2R-GFP or 12E-V2R-GFP and stable cell-lines were formed. Immunocytochemistry showed that both 12G-V2R-GFP and 12E-V2R-GFP co-localized with the marker-protein E-cadherin on the basolateral membrane of the cells (Figure 1). In both cell-lines, late endosomal/lysosomal localization of V2R-GFP was also observed, indicating that receptor internalization occurred for both V2R variants (Figure 1). For the AVP binding experiments described below, colonies of both 12G-V2R-GFP and 12E-V2R-GFP were visually chosen with similar V2R-GFP expression levels. To test whether glycosylation was normal, we treated complete cell-lysates with Endo H and PNGase F to cleave off N-linked high mannose sugar groups (indicative for V2R localized to the endoplasmatic reticulum) or all N-linked sugar moieties, respectively. GFP immunoblotting of the cell lysates revealed that the extent and form of glycosylation were similar to that of wtV2R as shown previously²⁷, indicating that the processing of 12E-V2R is similar to that of 12G-V2R.

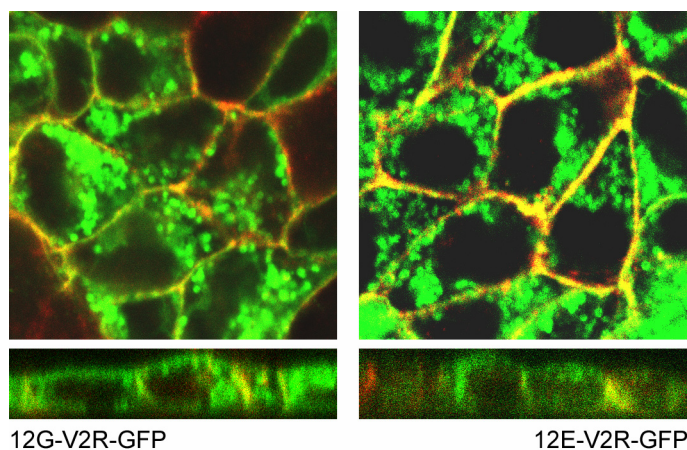


Figure 1. V2R-GFP expressed in MDCKII cells. Localization of both 12G- and 12E-V2R-GFP (both green) and co-localization (yellow) with E-cadherin (red) in stably transfected MDCKII cells visualized from above and from the side.

V2R Binding Affinity

Results of V2R saturation experiments with tritium-labeled AVP are presented in Figure 2. Both the 12G- and the 12E-V2R cell-lines show a similar $B_{\max}$ which is reached at an AVP concentration of approximately 50 to 60 nM. However, the saturation curve of the 12E-V2R is steeper than the 12G-V2R curve, indicating an increased binding affinity of 12E-V2R-GFP for AVP. Indeed, binding affinity for AVP was increased threefold in the MDCKII cells stably transfected with 12E-V2R-GFP compared to MDCKII cells stably transfected with 12G-V2R-GFP. The K_D was 4.5 nM (CI95 3.6-5.4) for 12E-V2R and 16.5 nM (CI95 10.1-22.9) for 12G-V2R with a similar $B_{\max}$ for both receptors.

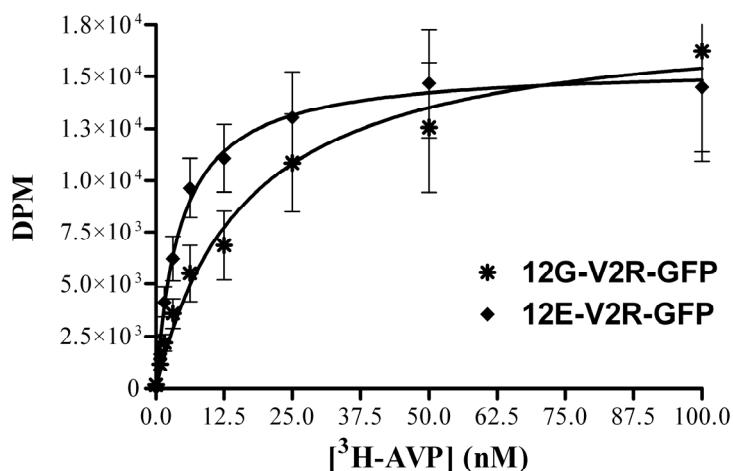


Figure 2. V2R-GFP binding affinity for AVP in MDCKII cells. Saturation curves of 12G- and 12E-V2R-GFP with tritium labeled AVP in stably transfected MDCKII cells. Both curves are baseline corrected for values counted in untransfected cells to correct for non-specific binding.

Discussion

We genotyped all LETS participants for four SNPs in *AVPR2* and for an additional SNP in the adjacent *RBP* gene. All five SNPs were in Hardy-Weinberg equilibrium in female controls. The *AVPR2* SNPs were not linked to

the *RBPSNP* t61c nor to any of the *FVIII* SNPs for which the LETS was genotyped previously³⁰. Three *AVPR2* SNPs, a-245c, G12E and S331S were in strong linkage disequilibrium and were associated with an increase in plasma levels of VWF propeptide, VWF and FVIII but contrastingly also with a decrease in thrombosis risk in men. Of these three SNPs, G12E was the most likely functional variation, as G12E is a coding non-synonymous SNP, located in the N-terminal extracellular tail that may influence ligand binding. Indeed, in stably transfected MDCKII cells, binding affinity for AVP was increased threefold for 12E-V2R compared to the wildtype 12G-V2R.

Many studies have shown that elevated levels of VWF and FVIII increase the risk of venous thrombosis¹⁻⁷. Surprisingly, three *AVPR2* SNPs that were associated with increased VWF and FVIII levels were simultaneously associated with a decrease in thrombosis risk in the LETS. These effects were observed in men only, not in women, which may be explained by the absence of women homozygous for the rare alleles of these SNPs. Heterozygous women appeared to have slightly increased VWF and FVIII levels. However, there was no effect on thrombosis risk in this group.

An explanation for these seemingly contradicting results may be found in the renin angiotensin system (RAS). The release of AVP by the hypothalamus-pituitary axis can be triggered by angiotensin II²¹. Angiotensin II is the end-product of the renin angiotensin system (RAS), which is an endocrine system that regulates vascular tone and blood pressure³¹. RAS has both procoagulant and antifibrinolytic properties. The angiotensin converting enzyme (ACE) suppresses the expression of tissue-type plasminogen activator via the inactivation of bradykinin and stimulates the expression of both tissue factor and plasminogen activator inhibitor type 1 via angiotensin II^{32,33}. Angiotensin II may also directly up-regulate the expression of the V2R³⁴. When the binding affinity of the V2R for AVP is increased, as we observed for 12E-V2R, the body can respond to lower plasma concentrations of AVP. This could lead to increased secretion of VWF from WPb and consequently higher plasma levels of VWF and FVIII. However, it could also lead to an early correction of blood volume and osmolality. Therefore, overall RAS activity would be kept low,

resulting in decreased procoagulant and antifibrinolytic activity. If this reduction in procoagulant and antifibrinolytic activity outweighs the effects of the increase in VWF and FVIII levels, this could explain the reduction in thrombosis risk that we observed. RAS, and ACE in particular, has been implicated to play a role in venous thrombosis before. An insertion/deletion polymorphism in the ACE gene associates with levels of ACE, t-PA and PAI-1. Several studies, including the LETS have shown an association with the risk of venous thrombosis as well, although results from these different studies are sometimes contradictory and not conclusive³⁵⁻⁴².

We conclude that *AVPR2* variations are associated with both plasma levels of VWF and FVIII and the risk of venous thrombosis. Larger studies are needed to confirm these associations, since the number of carriers of the rare alleles of these variations was low in the LETS. The *AVPR2* G12E variant is a gain-of-function mutation that leads to a normally expressed, fully functional V2R with increased binding affinity for AVP. This increased affinity for AVP may lead to an increase in VWF secretion from WPb, which explains the association of 12E and possibly of the two SNPs linked to it, a-245c and S331S, with high plasma levels of VWF and FVIII. Of course, this does not rule out possible functionality of and contribution to the observed effects by either a-245c or S331S. Possibly, the decrease in thrombosis risk associated with these three SNPs could be explained by an overall down-regulation of RAS activity, due to increased responsiveness of the kidney to AVP. Further research is necessary to confirm this hypothesis.

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Chapter 7

Plasma Levels of von Willebrand Factor Propeptide, von Willebrand Factor and Coagulation Factor VIII in Carriers and Patients with Nephrogenic Diabetes Insipidus

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Summary

Background & Objectives High von Willebrand factor (VWF) and factor VIII (FVIII) levels are a risk factor for thrombosis. Determinants of high VWF and FVIII levels remain poorly understood. Secretion of VWF from endothelial storage pools is regulated by vasopressin type-2 receptor (V2R). Nephrogenic Diabetes Insipidus (NDI) is characterized by renal insensitivity to vasopressin (AVP) caused by mutations in the genes encoding the V2R or the aquaporin 2 water channel (AQP2). We hypothesized that carriers of *AQP2* mutations compensate excess fluid loss by up-regulating AVP release and V2R expression, resulting in increased VWF secretion.

Patients Methods & Results We included 13 NDI families: 14 NDI patients (12 V2R- and 2 AQP2-linked), 14 carriers (9 V2R- and 5 AQP2-linked) and 25 unaffected individuals. We also included 48 unrelated controls. No differences were observed between patients, carriers and unaffected individuals in hematocrite, serum osmolality and blood pressure. Detectable AVP levels were elevated in all patients and carriers. AVP reached detectable levels in all carriers of *AQP2* mutations, compared to 27% and 56% in unrelated and related unaffected individuals, respectively. VWF propeptide, a measure of the VWF secretion rate, VWF antigen and FVIII activity were highest in carriers of *AQP2* mutations.

Conclusion Increased AVP levels in carriers of *AQP2* mutations are associated with increased VWF secretion. This study provides an important indication about the role of fluid homeostasis in the regulation of VWF and FVIII levels.

Introduction

Several studies have shown that elevated plasma levels of coagulation factor VIII (FVIII) are a risk factor of venous thrombosis¹⁻⁷. FVIII levels strongly depend on levels of its carrier protein von Willebrand factor (VWF) and plasma levels of both proteins usually fluctuate together. The mechanisms that underlie the substantial inter-individual variations in VWF and FVIII levels in the general population however are still poorly understood.

A regulator of VWF (and FVIII) secretion is the arginine vasopressin type-2 receptor (V2R), which is expressed in vascular endothelial cells, amongst others in the microvasculature of the lungs, and in epithelial cells of the principal collecting ducts in the kidney^{8,9}. In vascular endothelial cells, stimulation of the V2R with arginine vasopressin (AVP; also known as the anti-diuretic hormone ADH) leads to the exocytosis of Weibel Palade bodies (WPb)⁸. WPb are specialized endothelial storage organelles which contain amongst others ultra-large VWF multimers^{10,11}. In humans, administration of a synthetic analogue of AVP, 1-desamino-8-d-arginine vasopressin (desmopressin or DDAVP) leads to a sharp rise in plasma VWF and FVIII levels⁸. In fact, DDAVP is often used to treat patients with mild von Willebrand disease or hemophilia A¹².

In renal principal collecting duct cells, stimulation of the V2R leads to increased expression and relocalization of aquaporin 2 (AQP2) water channels to the apical membranes of the cells, rendering this plasma membrane permeable for water. Following an osmotic gradient of sodium and urea, water will then pass the apical membrane via AQP2 and will leave the cells on the basolateral side, into the circulation, via AQP3 and AQP4⁹. Genetic defects in either the V2R or AQP2 lead to Nephrogenic Diabetes Insipidus (NDI), a rare disorder in which the patient is unable to concentrate the pre-urine and produces large amounts of hypotonic urine, which can lead to severe dehydration and electrolyte imbalance¹³. The gene encoding the V2R, *AVPR2*, is located on the X-chromosome and V2R-linked NDI follows an X-linked recessive inheritance pattern⁹. The *AQP2* gene is located on chromosome 12. In general, AQP2-linked NDI follows an autosomal recessive inheritance pattern⁹. However, in order to be successfully relocated to the cell membrane, AQP2 needs to form tetramers¹⁴. Rare *AQP2* mutations that lead to impaired tetramer formation and/or mislocalization follow an autosomal dominant inheritance pattern^{9,14}. Before the diagnosis of NDI could be made based on genetics, a distinction between V2R- and AQP2-linked NDI was made based on a DDAVP test. In this test, NDI patients were injected with DDAVP. In patients with a normal V2R, WPb would be released in response to DDAVP and plasma levels of VWF, FVIII and tissue-type plasminogen activator (t-PA, co-stored with VWF in

WPb) would peak, indicating a defect in AQP2 instead of V2R. In patients with V2R-linked NDI, there would be no response to DDAVP. In older literature describing the outcomes of DDAVP tests in patients and carriers of NDI, basal VWF, FVIII and t-PA levels and also the peak height of all three proteins appeared highest in carriers of AQP2-linked NDI, even when compared to healthy non-carriers¹⁵⁻¹⁸.

Since the V2R is involved in the regulation of VWF and FVIII levels, we were interested to see whether changes in V2R mediated renal water retention can affect plasma levels of VWF and FVIII. Secretion of AVP is regulated amongst others by angiotensin II, the end product of the renin angiotensin system (RAS)¹⁹. RAS is activated when blood volume is low or blood osmolality is high. Decreased renal water retention will therefore lead to increased angiotensin II production and therefore increased AVP secretion. It has been shown in rodents that increased angiotensin II levels can also directly up-regulate V2R expression²⁰. In the present study, we choose NDI as an extreme model to study the effects of impaired renal water retention on secretion and plasma levels of VWF and FVIII. We hypothesized that *AQP2* mutations will cause an up-regulation of V2R expression and AVP release in an attempt to compensate for a decrease in total blood volume and an increase in blood osmolality, which causes increased VWF secretion particularly in carriers of AQP2-linked NDI. These effects will be less visible in NDI-patients and in carriers of V2R, as up-regulation of V2R expression and AVP release will not result in sufficient compensation for excess fluid loss in these groups.

Patients and Methods

Factor VIII & Nephrogenic Diabetes Insipidus Study (FENDI)

The FENDI study population consists of patients with NDI, either V2R- or AQP2-linked, their primary family members and a group of unrelated healthy controls. All participants gave informed consent and the study was approved by the ethical committees of both the Leiden University Medical Center (LUMC) and the Radboud University Nijmegen Medical Centre (RUNMC).

Inclusions of NDI patients and Family Members

Patients and their direct family members were informed about the FENDI study during an NDI patient information day, held in the RUNMC and asked to participate. Most participating families were included in either the RUNMC or the LUMC. Two families however were included at the families' homes. After completing a questionnaire, which included questions on medical history, current use of medication and family history of both NDI and thrombosis, blood samples were taken as described below and blood pressure was measured. Participants were in a fasting state until after the blood draw and blood pressure measurement. NDI patients however, were advised to drink water and take medication as normally. Individuals with manifest malignancies and pregnant women were excluded from the study.

Inclusions of Healthy Controls

Healthy controls were included in the FENDI study from a sub-group of the control population of the MEGA study^{21,22}, a case control study on venous thrombosis which was ongoing in the LUMC at the time of FENDI inclusions. Healthy controls were included in the MEGA study as all MEGA controls subjects, after being recruited via random digit dialing. As healthy controls, only individuals without history of venous thrombosis, manifest malignancies, current pregnancy or (family-) history of NDI were included in the FENDI study. Blood samples were taken as described below and blood pressure was measured after participants had filled out the MEGA questionnaires.

Blood Draw and Blood Pressure Measurements

Venous blood was drawn from all participants using 19G or 21G butterfly needles (Abbott, Illinois, USA) and a Lueradaptor (Sarstedt, Nümbrecht, Germany) in three different types of tubes, namely serum gel, trisodium citrate (0.1 volume of 0.106 M) or EDTA (1.6mg per 1 ml of blood) (all Monovette by Sarstedt).

Serum tubes were left to clot for 24 hours and then centrifuged at room temperature for 20 minutes at 2000 g. Serum was removed and directly used in

7. VWF & FVIII Levels in Nephrogenic Diabetes Insipidus

analyses. After measuring hematocrite in whole citrated blood, the citrate tubes were centrifuged at room temperature for 10 minutes at 2800 g within 2 hours of the blood draw. Plasma was pooled and then stored at -80° C in 1 ml aliquots. EDTA tubes were immediately placed on ice and then centrifuged at 4° C for 10 minutes at 2800 g within 30 minutes of the blood draw. EDTA plasma was pooled and stored in one aliquot at -80° C. After centrifugation, the pellets containing blood cells of both the citrated and EDTA blood were kept for DNA isolation from leucocytes as described previously²¹.

After the blood draw, the blood pressure of each participant was measured in sitting position on both upper arms. In case of different readings between both arms, the lowest value was used for analyses.

Laboratory Measurements

Blood osmolality was measured in serum using Osmostat OS-6030 (Menarini, Florence, Italy). The coefficient of variation (CV) was 3%. Results are expressed as mOsmol/kg.

Plasma AVP levels were measured by radioimmunoassay after extraction of 5 ml EDTA plasma on ODS-silica columns (Incstar, Stillwater, MN, USA).

Recovery ranges were between 92.7% and 97.6%. The intra-assay VC at several levels was between 7.1% and 9.3%, the inter-assay VC between 8.2% and 10.3%. Results are expressed as ng/l. The lower detection limit was 0.2 ng/l.

FVIII activity (FVIII:C) was measured in citrated plasma by one-stage clotting assay on the STA-R® analyzer (STA-R®, Diagnostica Stago, Asnières, France) using the STA®APTT reagent and STA®Unicalibrator. The intra-assay CV was 4.0% and the inter-assay CV was 6.7%. A single measurement was performed of all samples in a 1:40 dilution. Results are expressed as IU/dl.

FVIII antigen (FVIII:Ag) levels were measured by ELISA in two different dilution (1:20 and 1:40) in citrated plasma, using a monoclonal anti-FVIII IgG (CLB-Ajax) and monoclonal anti-FVIII IgG (CLB-117) conjugate. Pooled normal plasma, calibrated against the 3th WHO international standard FVIII/VWF plasma, was used as a reference. Except for one, all samples had a CV smaller than 10%. Results are expressed as IU/dl.

VWF antigen (VWF:Ag) levels were measured in citrated plasma on an automated coagulation analyzer (STA-R®, Diagnostica Stago, Asnières, France) using the STA Liatest von Willebrand Factor kit (Roche Diagnostic, Mannheim, Germany). A single measurement was performed for all samples in a 1:8 dilution. Results are expressed as IU/dl.

VWF propeptide levels, a measure of the VWF secretion rate²³⁻²⁵, were measured by ELISA as described previously^{23,26}. Pooled normal plasma, which was calibrated against a plasma sample in which the absolute amount of VWF propeptide had previously been determined using purified recombinant VWF propeptide as a gold standard, was used as a standard. The normal pooled plasma had a VWF propeptide concentration of 6.13 nM (see below), which corresponds to 100 units per dl. Results are expressed as U/dl.

Finally, t-PA levels were measured in citrated plasma by ELISA using the t-PA Antigen ELISA Reagent Kit (Technoclone, Vienna, Austria). All samples were measured in duplicate and pooled plasma was used as a control. The CV was smaller than 10%. Results are expressed as IU/dl.

ABO Blood Group

ABO blood group genotypes were determined in all participants. Three SNPs in the ABO blood group gene (rs8176719, rs8176749 and rs8176750) were genotyped using 5' nuclease/Taqman assays to distinguish ABO genotypes O₁, A₁, A₂ and B. The polymerase chain reactions with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA) were performed on a PTC-225 thermal cycler. Fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA).

AQP2 and AVPR2 Sequence Analyses

The entire genomic regions of both the *AVPR2* and *AQP2* genes, including 3' and 5' UTR and introns, were resequenced in all NDI patients and their participating family members, to establish the nature of the NDI causing mutations in patients and determine carriership in the family members.

7. VWF & FVIII Levels in Nephrogenic Diabetes Insipidus

For *AVPR2*, a 3.3 kb long region was amplified in fragments using 5 sets of primers. For *AQP2*, a 6.6 kb long region was amplified in fragments using 7 sets of primers. Primer sequences are available on request. PCR-products were purified using a QIAquick PCR Purification Kit (Qiagen Benelux, Venlo, the Netherlands). Sequence reactions and fragment analysis were performed by the Leiden Genome Technology Center (www.lgtc.nl, LGTC, Leiden, the Netherlands) on an ABI 3700 or ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

Statistical Analyses

To evaluate the difference in levels of FVIII, VWF and VWF propeptide between genotypes, Student's t tests and linear regression modeling were used. Mean values are presented, along with the differences to the reference group and the 95% confidence intervals (CI95) of the differences. Adjustments for parameters such as age, sex and ABO blood group were done with multiple linear regression modeling. Levels of one parameter, AVP, were below the detection limit in part of the participants. Besides calculating mean values and differences between groups, we calculated a χ^2 comparing the proportions of individuals per group in which AVP levels were higher than the detection limit.

Results

FENDI Study Population

In total, fourteen patients with NDI from thirteen families were included in the study, twelve patients with X-linked NDI and two patients with the more rare autosomal recessive form of NDI. 39 family members and 48 healthy unrelated controls were included. All FENDI participants are Caucasian. Baseline characteristics of the FENDI population are given in Table 1.

Table 1. Baseline characteristics of the FENDI population.

FENDI Participants		n	% women	mean age (range)	mean BMI	%ABO genotype OO	% ABO genotypes OO, OA2 & A2A2
non-carriers	unrelated	48	47.9	47.7	26.4	52.1	58.3
	related	25	52.0	43.0	24.6	36.0	52.0
NDI carriers	AVPR2	9	100.0	40.0	25.6	33.3	44.4
	AQP2	5	60.0	44.2	24.6	40.0	40.0
NDI patients	AVPR2	12	0.0	33.2	26.2	33.3	41.7
	AQP2	2	0.0	26.0	26.9	50.0	50.0

NDI Mutations

The entire genomic regions of both the *AVPR2* and *AQP2* genes were resequenced in all NDI patients and their family members. We identified fourteen carriers of NDI; nine of them had a mutation in the *AVPR2* gene and five in the *AQP2* gene. Two of the female carriers of *AVPR2* mutations suffer from multiple symptoms associated with NDI, probably caused by skewed X-inactivation. There were 25 non-carrier unaffected family members, all from X-linked NDI families. Eleven mutations in the *AVPR2* gene and one mutation in the *AQP2* gene were identified (Table 2). All but two of the *AVPR2* mutations have been described before and can be found, along with the references to the original articles describing them, on an online database

<http://www.medicine.mcgill.ca/nephros/default.htm>.

Two *AVPR2* mutations have not been described previously, namely 636A>T (Gln92Leu) and 1444C>A (Ala326Glu). Most *AVPR2* mutations, as well as the *AQP2* mutation, were missense mutations. We also identified two nonsense mutations and one deletion in the *AVPR2* gene. *AVPR2* mutations were found throughout the whole length of the coding region of the gene.

Table 2. NDI causing mutations in the AVPR2 and AQP2 genes in the FENDI population.

Gene	Nucleotide	Amino Acid	Domain*	Type	Consequence	N (patients)	N (carriers)
AVPR2	491C>T	Leu → Phe	TMI	Missense	non-functional receptor	1	-
	546T>C	Leu → Pro	CI	Missense	unknown	2**	1
	623G>A	Val → Met	TMII	Missense	misfolded receptor	1	1
	636A>T§	Gln → Leu	TMII	Missense	unknown	1	2
	818-824del	Stop at codon 161	CII	Deletion-frameshift	truncated protein	1	-
	861C>T	Ser → Leu	TMIV	Missense	misfolded receptor	-	1
	965C>T	Arg → Cys	EIII	Missense	possibly misfolded receptor	2	1
	972C>A	Thr → Asn	EIII	Missense	unknown	1	1
	1034C>T	Gln → Stop	TMV	Nonsense	unknown	1	1
	1444C>A§	Ala → Glu	TMVII	Missense	unknown	1	-
	1476C>T	Arg → Stop	CIV	Nonsense	misfolded receptor or impaired intracellular trafficking	1	1
AQP2	559C>T	Arg → Cys	EIII	Missense	unknown	2	5

* TM=transmembrane; C=cytoplasmic; E= extracellular; roman numbering indicates which TM; C or E domain is meant.

** Patients from the same family.

§ Novel mutation

Blood Pressure, Osmolality, Hematocrite & t-PA

In NDI, fluid homeostasis is impaired. We measured several markers of fluid homeostasis in all FENDI participants, namely hematocrite, serum osmolality and systolic and diastolic blood pressure. Results are shown in Table 3. After adjustment for age, there were no differences in hematocrite between the groups of control subjects, unaffected family members, carriers and patients, either AQP2- or V2R-linked.

Apart from AQP2-linked NDI patients, all groups had higher serum osmolality than the reference group of unrelated control subjects. However, osmolality was increased not only in NDI carriers and patients, but in unaffected family members as well.

All groups appeared to have lower systolic and diastolic blood pressure than the unrelated control group. However these differences disappeared after adjustment for age and sex. It should be noted that most NDI patients are treated with diuretics, which can reduce blood pressure in individuals unaffected by NDI. It may seem paradoxical, but diuretics, in combination with a reduction in salt intake, can reduce urine production in patients with NDI²⁷, as water resorption will be brought to a maximum in the proximal tubules in response to systemic sodium depletion.

In all participants, except for two, t-PA levels were below the detection limit. Therefore, t-PA levels have not been included in the analyses.

Plasma Levels of AVP

AVP levels were below the detection limit in a considerable proportion of the FENDI population. We could observe differences in mean detectable AVP levels. Non-carriers, both unrelated controls and unaffected family members had mean detectable AVP levels of approximately 0.9 ng/l. All patients and carriers had higher levels, even though confidence intervals of the differences to the reference group were wide, due to small groups. However, in this analysis, individuals with undetectably low AVP levels have been left out. Therefore we compared the proportion of individuals with detectable AVP levels per group. Compared to unrelated control subjects, the proportion of

Table 3. Homeostatic parameters in the FENDI population.

		HEM	Δ^* (CI95)	OSM	Δ (CI95)	SBP	Δ^{**} (CI95)	DPB	Δ^{**} (CI95)
Non carriers	unrelated§	0.39	-	284.7	-	130.2	-	80.0	-
	related	0.38	-0.01 (-0.02 to 0)	292	7.3 (4.4 to 10.2)	123.8	-3.7 (-11.4 to 3.9)	73.4	-4.0 (-9.2 to 1.1)
NDI carriers	AVPR2	0.37	0 (-0.01 to 0.02)	289.4	4.8 (1.3 to 8.2)	111.1	-15.9 (-26.9 to 5.0)	71.7	-5.5 (-12.4 to 1.4)
	AQP2	0.37	-0.02 (-0.07 to 0.04)	290.0	5.3 (0.8 to 9.8)	122.0	-6.4 (-20.5 to 7.6)	70.0	-7.9 (-16.8 to 1.0)
NDI patients	AVPR2	0.42	0.01 (-0.01 to 0.02)	289.2	4.5 (1.3 to 7.7)	119.2	-0.9 (-10.5 to 8.6)	72.5	-0.54 (-7.1 to 6.0)
	AQP2	0.39	-0.02 (-0.05 to 0.01)	286.5	1.8 (-5.4 to 9.0)	117.5	1.7 (-20.5 to 23.9)	75.0	4.5 (-9.7 to 18.7)

* Adjusted for sex

** Adjusted for age and sex

§ Reference Group

HEM: Hemotocrite; OSM: Osmolality (mOsmol/kg); SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure

detectable AVP levels was increased in all groups, except for the AQP2-linked NDI patients. Compared to the unaffected family members, the proportions of detectable AVP levels appeared increased only in the group of AQP2 carriers. Results are shown in Table 4.

Table 4. AVP concentration in the FENDI population.

		AVP (ng/l)	Δ	CI95	Ndetectable /Ntotal	% detectable	χ^2 1	χ^2 2
Non carriers	unrelated§	0.90	-	-	13/48	27	-	0.009
	related*	0.92	0.02	-0.49 to 0.53	14/25	56	0.009	-
NDI carriers	AVPR2	1.13	0.23	-0.50 to 1.0	7/9	78	0.002	0.249
	AQP2	1.12	0.22	-0.50 to 1.0	5/5	100	0.001	0.062
NDI patients	AVPR2	1.53	0.63	-0.25 to 1.51	10/12	83	0.000	0.103
	AQP2	2.20	1.30	-0.38 to 2.98	1/2	50	0.430	0.869

§ Reference group Δ (AVP) and χ^2 1

* Reference group χ^2 2

Plasma Levels of VWF Propeptide, VWF and FVIII

Mean VWF propeptide, VWF and FVIII levels are shown in Table 5a.

Differences between the groups and the corresponding CI95s have been adjusted for age, sex and non-O ABO blood groups. For the latter adjustment, we have divided FENDI participants into two groups, namely ABO genotype OO versus all other genotypes. However, VWF and FVIII levels in carriers of ABO genotypes OA₂ and A₂A₂ are comparable to carriers of the OO genotype²⁸, therefore, we repeated this adjustment with ABO genotypes OO, OA₂ and A₂A₂ versus all other genotypes, but no obvious differences in outcomes were observed.

The groups of NDI patients and carriers were too small to positively confirm differences in levels with the reference group of unrelated control subjects. For VWF propeptide, which is a measure of the VWF secretion rate, the highest levels were observed in carriers of AQP2 mutations. Indeed, mature VWF levels and FVIII:C were also highest in the group of AQP2 carriers. This trend was not followed for FVIII:Ag however.

Table 5a. VWF propeptide, VWF and FVIII levels in the FENDI population.

		VWF propeptide	Δ^* (CI95)	VWF:Ag	Δ^* (CI95)	FVIII:Ag	Δ^* (CI95)	FVIII:C	Δ^* (CI95)
non-carriers	unrelated	110.8	-	116.5	-	126.4	-	132.5	-
	related	116.7	6.7 (-14.5 to 27.9)	112.8	-6.4 (-25.1 to 12.2)	125.7	-3.1 (-23.1 to 16.8)	126.0	-10.8 (-39.3 to 17.8)
NDI carriers	AVPR2	116.6	10.4 (-22.3 to 43.1)	122.8	7.4 (-20.9 to 35.6)	125.6	0.1 (-28.9 to 29.1)	128.2	-7.9 (-54.1 to 38.2)
	AQP2	138.0	28.3 (-18.2 to 74.7)	123.8	5.8 (-31.8 to 43.5)	115.3	-12.5 (-51.1 to 26.1)	145.4	9.7 (-51.5 to 70.9)
NDI patients	AVPR2	118.3	10.4 (-20.1 to 40.9)	114.8	-2.4 (-28.3 to 23.6)	131.0	5.9 (-20.3 to 32.1)	132.3	-0.1 (-40.0 to 39.9)
	AQP2	115.0	12.6 (-55.5 to 80.7)	109.5	2.6 (-58.0 to 63.2)	108.8	-5.0 (-66.7 to 56.7)	121.5	0.5 (-96.6 to 97.6)

* Adjusted for sex, age and ABO blood group (OO versus all others)

Table 5b. VWF propeptide, VWF and FVIII levels with NDI families.

	n	VWF propeptide	Δ^* (CI95)	VWF: Ag	Δ^* (CI95)	FVIII: Ag	Δ^* (CI95)	FVIII: C	Δ^* (CI95)
AVPR2 families	unaffected	116.7	-	112.8	-	125.7	-	126.0	-
	carriers	116.6	-2.8 (-27.2 to 21.6)	122.8	8.3 (-14.3 to 30.8)	125.6	0.9 (-25.0 to 26.9)	128.2	0.2 (-24.8 to 25.2)
	patients	118.3	7.5 (-19.4 to 34.4)	114.8	7.2 (-16.2 to 30.6)	131.0	10.5 (-14.8 to 35.8)	132.3	8.0 (-14.7 to 30.7)
AVPR2 families§	unaffected	116.7	-	112.8	-	125.7	-	126.0	-
	carriers	105.7	-15.2 (-40.7 to 10.3)	119.4	2.5 (-22.5 to 27.5)	123.9	-4.1 (-24.3 to 16.1)	121.9	-8.1 (-35.0 to 18.8)
	patients	123.5	12.2 (-13.1 to 37.5)	117.6	10.2 (-11.6 to 32.1)	131.1	11.6 (-11.7 to 35.0)	134.9	11.1 (-10.5 to 32.6)
AQP2 families	carriers	138.0	-	123.8	-	115.3	-	145.4	-
	patients	115.0	-52.0 (-132.5 to 28.5)	109.5	-17.0 (-475.0 to 440.9)	108.8	-2.7 (-57.3 to 52.0)	121.5	-14.6 (-62.5 to 33.4)

* Adjusted for sex, age and ABO blood group (OO versus all others)

§ Symptomatic carriers classified as NDI patients

Because high levels of VWF and FVIII cluster within families²⁹, we also compared mean VWF propeptide, VWF and FVIII levels in carriers and patients with those of their family members. Within the V2R families, the unaffected family members were set as reference group. Because two female V2R carriers suffer from NDI-related symptoms, we repeated the analyses classifying them as patients instead of carriers. Within the AQP2 families, there were no unaffected family members and the group of carriers was set as reference group. Results of these comparisons are given in Table 5b.

Discussion

We are interested in determinants of elevated levels of VWF and FVIII as these are important risk factors for the development of thrombosis. Stimulation of the V2R with AVP results in the secretion of VWF from WPb. We hypothesized that *AQP2* mutations will cause an up-regulation of V2R expression and AVP release in an attempt to compensate for excess fluid loss, which causes increased VWF secretion particularly in carriers of AQP2-linked NDI. Up-regulation of AVP secretion and V2R expression could be effective in all NDI carriers. However, only in carriers of AQP2-linked NDI, where V2R is fully functional and normally expressed, could up-regulation lead to over-expression of the V2R, which could lead to excess secretion of VWF. In patients, up-regulation of AVP release and V2R expression could not lead to effective compensation of fluid loss and was therefore not expected.

In order to test our hypotheses, we included fourteen patients with NDI and their primary family members. We were able to include fourteen carriers of NDI, five of which were carriers of *AQP2* mutations. The number of patients and carriers included in this study is small, especially considering the fact that we were interested to observe differences mainly in the subgroup of AQP2-linked NDI carriers. However, NDI is a very rare disorder and a large proportion of the NDI families known in the Netherlands was included in this study.

The twelve V2R-linked NDI patients came from eleven families and indeed, eleven different V2R mutations were identified. Two of these mutations have

not been described previously. Both AQP2-linked NDI patients were homozygous for the same *AQP2* mutation. It is possible that both patients descend from a common ancestor.

When looking at several homeostatic variables, no clear differences were observed between the groups. There were no differences in hematocrite and blood pressure, systolic or diastolic, after adjustment for sex or age and sex respectively. Osmolality was increased in all groups compared to unrelated controls. However, osmolality was also elevated in the unaffected family members, which indicates that this was not an effect of NDI. Apparently, patients and carriers of NDI included in our study have normal blood pressure, blood osmolality and hematocrite. This may be explained by the fact that all families included in the study were very aware of the consequences of NDI, so all patients made sure to maintain hydrated and most patients were treated with diuretics in order to decrease their urinary excretion.

AVP levels did differ between groups. Mean levels were similar in both related and unrelated unaffected controls and were highest in both NDI patient groups. However, important is that AVP levels were below detection limits in a large proportion of FENDI participants, who were left out of this analysis. Therefore, we compared the proportions of detectable AVP levels per group. Compared to unrelated controls, this proportion was larger in every group, except for AQP2-linked NDI patients. However, compared to the related controls the proportion appeared higher only in carriers of *AQP2* mutations. This finding is in agreement with our hypothesis that AVP release is up-regulated in carriers of AQP2-linked NDI, in order to effectively compensate increased loss of fluids. Also in agreement with our primary hypothesis is the observation that carriers of *AQP2* mutations had the highest levels of VWF propeptide, which reflects an increased VWF secretion in this group. Indeed VWF:Ag and FVIII:C were also highest in this group. FVIII:Ag however was low among AQP2 carriers. A weakness in our study is that no unaffected family members of AQP2-linked NDI patients could be included. Because high VWF and FVIII levels cluster within families²⁹, we would have preferred to compare VWF and FVIII levels in AQP2 carriers to their own unaffected family members.

The FENDI population is relatively small for studying variables with very large inter-individual variations in plasma levels, such as VWF and FVIII. Differences in levels of VWF and FVIII between NDI carriers, patients and unaffected individuals have therefore not been confirmed, but this study does give an important indication about the role of fluid homeostasis in the regulation of VWF and FVIII. Further investigations into more common *AQP2* gene variations will have to confirm whether fluid homeostasis also plays a role in the regulation of VWF and FVIII and possibly in the risk of venous thrombosis in the general population.

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Chapter 8

Associations of Aquaporin 2 Gene Variations with Plasma Levels of von Willebrand Factor and Factor VIII and the Risk of Venous Thrombosis

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Summary

Background Aquaporin 2 (AQP2) transports water from the principal collecting ducts of the kidney back into the circulation. Expression of AQP2 is regulated by the Vasopressin 2 Receptor (V2R), which also regulates secretion of von Willebrand Factor (VWF), the carrier protein of Factor VIII (FVIII). Impaired function of AQP2 enhances secretion of vasopressin and may thus enhance VWF secretion. We hypothesized that AQP2 gene variations influence VWF and FVIII levels and the risk of venous thrombosis.

Methods We sequenced a 6.6 kb long genomic region around the AQP2 gene in 25 individuals selected from the Leiden Thrombophilia Study (LETS), consisting of 474 venous thrombosis patients and 474 control subjects. We identified 18 single nucleotide polymorphisms (SNPs), of which 16 were genotyped in the entire LETS.

Results Although reliable haplotypes could not be formed, the SNPs were linked within 5 clusters. For SNPs in clusters 2, 4 and 5, up to 2.5-fold increases in thrombosis risk were observed. For the same SNPs we observed associations with arterial blood pressure. None of the AQP2 SNPs were associated with VWF or FVIII levels in healthy controls. *Conclusions.* AQP2 gene variations are associated with the risk of venous thrombosis. However, the increase in thrombosis risk was not the result of increases in plasma levels of VWF and FVIII.

Introduction

Several studies have shown that elevated plasma levels of coagulation factor VIII (FVIII) are a risk factor of venous thrombosis¹⁻⁷. The mechanisms that underlie the substantial inter-individual variations in FVIII levels in the general population are still poorly understood. There are strong indications that FVIII levels are at least in part determined genetically^{8,9}. Besides variations in the genes encoding FVIII, its carrier protein von Willebrand factor (VWF) and ABO blood group, genetic variations in proteins that regulate plasma levels of FVIII and VWF may result in increased levels of FVIII.

In the present study, we investigated the role of variations in the aquaporin 2 (AQP2) gene in the regulation of VWF and FVIII levels and the risk of venous thrombosis. AQP2 is a water channel that is expressed in epithelial cells of the principal collecting ducts in the kidney¹⁰. Principal collecting duct epithelial cells are normally water impermeable. However, upon a specific stimulus, AQP2 tetramers are relocated from the Golgi to the apical membrane of the cell. In the membrane, AQP2 transports water from the pre-urine in the collecting ducts into the epithelial cells. Subsequently, water is transported back into circulation by AQP3 and AQP4¹⁰.

Relocation of AQP2 tetramers as well as AQP2 gene transcription are stimulated by intracellular cAMP¹⁰⁻¹², which is generated after activation of the arginine vasopressin 2 receptor (V2R) by arginine vasopressin (AVP, or anti-diuretic hormone (ADH))^{10,13}. The V2R is expressed on the basolateral membrane of the principal collecting duct epithelial cells, but also in vascular endothelial cells. In vascular endothelial cells, stimulation of the V2R leads to exocytosis of Weibel Palade bodies (WPb), which contain amongst others VWF. Administration of 1-desamino-8-d-arginine vasopressin (desmopressin or DDAVP), a synthetic analogue of AVP, leads to a sharp rise of plasma levels of VWF and FVIII in humans¹³.

Synthesis and secretion of AVP by the hypothalamus-pituitary axis is stimulated by angiotensin II¹⁴. It has been shown in rodents that angiotensin II can also directly up-regulate the expression of the V2R itself¹⁵. Angiotensin II is the final cleavage product of angiotensinogen in the renin angiotensin system (RAS). Angiotensinogen is secreted by the liver and is cleaved to angiotensin I by renin, which is secreted by the kidneys in case of low blood volume or high blood osmolality. Angiotensin I is subsequently cleaved by the angiotensin converting enzyme (ACE) to angiotensin II¹⁶.

Low expression or low activity of the AQP2 water channel could lead to a decrease in blood volume and increases in blood osmolality, renin secretion and angiotensin II levels and hence to increased AVP secretion and possibly V2R expression and consequently WPb release. Therefore, we hypothesized that functional variations in the AQP2 gene can influence the secretion of VWF and

therefore plasma levels of VWF and FVIII and the risk of venous thrombosis in the general population. Loss of function variations in the AQP2 gene, which will lead to decreased renal water resorption, leading to an increase in RAS activity and AVP secretion, will be associated with higher VWF and FVIII levels and possibly with an increase in thrombosis risk. Gain of function variations will have opposite effects.

To test this hypothesis, we sequenced the entire genomic region of the AQP2 gene in 25 individuals selected from the Leiden Thrombophilia Study (LETS), a large population-based case-control study on venous thrombosis. The entire LETS was genotyped for the variations found and associations of the variations with VWF antigen (VWF:Ag), FVIII antigen (FVIII:Ag) and VWF propeptide, a measure of the VWF secretion rate, as well as associations with the risk of venous thrombosis were studied.

Patients and Methods

Leiden Thrombophilia Study (LETS)

The LETS consists of 474 consecutive patients and 474 healthy controls. All patients were referred for anti-coagulant treatment after a first objectively confirmed episode of deep vein thrombosis. Controls were frequency-matched for sex and age and were acquaintances or partners of patients. Mean age for both the patient and the control groups was 45 years, ranging from 15 to 69 for patients and 15 to 72 for controls. Both groups consisted of 272 women (57.4%) and 202 men (42.6%). Individuals with underlying malignancies were excluded. DNA samples were available of 471 cases and 471 controls. The design of this study has previously been described in more detail^{17,18}.

FVIII antigen (FVIII:Ag), VWF antigen (VWF:Ag) and VWF propeptide were measured by ELISA in the plasma of the first 301 patients and 301 controls included in the study^{1,19}. Pooled normal plasma, calibrated directly against the WHO standard for VWF and FVIII (91/666), was used as a reference. Results for FVIII:Ag and VWF:Ag are expressed as international units per ml (IU/ml). Results for VWF propeptide are expressed as units per ml (U/ml), with one unit defined as the amount of VWF propeptide in one ml of the pooled normal

plasma. Systolic and diastolic blood pressure were measured on the upper-arm in resting position in all participants.

AQP2 Sequence Analyses

The entire genomic region of the AQP2 gene, including 5' and 3' UTRs and introns, was resequenced in a selection of 25 individuals from the LETS, from 1.4 kb upstream of the transcription start site to 0.4 kb downstream of the stop codon. We selected 25 individuals with the highest VWF:Ag and FVIII:Ag. Because increasing age and non-O ABO blood group are associated with increased VWF and FVIII levels, all 25 individuals selected were younger than 50 years and 20 of them had blood group O. A 6.6 kb long region was amplified in fragments using 7 sets of primers. Primer sequences are available on request. PCR-products were purified using a QIAquick PCR Purification Kit (Qiagen Benelux, Venlo, the Netherlands). Sequence reactions and fragment analysis were performed by the Leiden Genome Technology Center (www.lgtc.nl, LGTC, Leiden, the Netherlands) on an ABI 3700 or ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA).

AQP2 Genotyping

By sequencing, eighteen single nucleotide polymorphisms (SNPs) were identified within the AQP2 gene. The entire LETS was genotyped for sixteen of these SNPs. Genotyping for the last two SNPs failed. Fifteen SNPs were determined using 5' nuclease/Taqman assays. The polymerase chain reactions with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA) were performed on the PTC-225 thermal cyclers. Fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems).

One SNP, AQP2-19, failed to genotype by Taqman and was determined using polymerase chain reaction – restriction fragment length polymorphism analysis (PCR-RFLP). The polymerase chain reactions and enzymatic digestions were also performed on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany) and primers were purchased from Eurogentec (Seraing, Belgium).

PCR conditions, restriction endonucleases and the sequences of probes and primers used for genotyping are available on request.

Statistical Analyses

Levels of VWF propeptide, VWF:Ag and FVIII:Ag are presented as means, together with the differences from the reference group and the corresponding 95% confidence intervals (CI95). In SNP clusters where associations were observed, associations of individual SNPs with levels were adjusted for the other SNPs within the cluster using linear regression modeling. Associations between AQP2 SNPs and systolic and diastolic blood pressure were studied using linear regression modeling. To evaluate the influence of the different genotypes on the risk of thrombosis, odds ratios (OR) and their corresponding CI95 according to Woolf²⁰ were calculated. For one genotype, there was a zero in the equation. In this case, CI95s were calculated according to Mehta²¹. In SNP clusters where increases or decreases in thrombosis risk were observed, ORs for individual SNPs were adjusted for the other SNPs within the cluster using binary logistic regression modeling.

Results

Sequencing and Genotyping

We identified eighteen SNPs within the AQP2 gene in the 25 individuals selected from the LETS. The SNPs, with their rs-numbers and their location in the gene, are presented in Table 1. All sixteen SNPs were in Hardy-Weinberg equilibrium in the healthy control subjects of the LETS. The SNPs were linked to each other in five clusters of SNPs, as determined with Haploview Software²², however, recombination between the SNPs was too large to form reliable haplotypes. Therefore, the entire LETS population was genotyped for sixteen of the eighteen SNPs. Two SNPs failed to genotype. Sequencing data however, place these two SNPs, AQP2-16 and AQP2-18 in clusters 2 and 5 respectively.

Table 1. AQP2 gene variations in the LETS.

Cluster	SNP	rs-number	Nucleotide	Location
1	AQP2-1	rs3759125	a > c	upstream
	AQP2-5	rs461872	g > a	intron 1
2	AQP2-2	rs3759126	a > g	upstream
	AQP2NF10	rs3741559	g > a	intron 1
	AQP2-18*	rs371777	a > c	intron 3
	AQP2-19	rs403201	c > g	intron 3
3	AQP2-3	rs3782319	g > a	intron 1
	AQP2-10	rs11169225	t > a	intron 1
	AQP2-14	rs12372344	t > c	intron 2
	AQP2NF19	rs7299924	a > g	intron 3
4	AQP2-4	rs3782320	g > a	intron 1
	AQP2-7	rs3782322	g > a	intron 1
	AQP2-8	pending	g > a	intron 1
5	AQP2-6	rs467199	a > g	intron 1
	AQP2-13	rs426496	t > c	exon 2 (silent)
	AQP2-15	rs402813	c > t	intron 3
	AQP2-16*	rs439779	c > t	intron 3
	AQP2-20	rs457487	c > a	3'-UTR

* SNPs that failed to genotype in the LETS

AQP2 SNPs and the Risk of Venous Thrombosis

The distributions of the different genotypes over patients and controls in the LETS for the AQP2 SNPs, together with ORs and corresponding CI95s are presented in Table 2. Effects on thrombosis risk were observed for SNPs in three of the five clusters, namely clusters 2, 4 and 5. In cluster 2, there was no change in risk in heterozygous carriers of any of the three SNPs in the cluster, but the risk of venous thrombosis was increased 2.2 and 2.5-fold in homozygous carriers of the minor alleles of AQP2-2 and AQP2-19, respectively. In cluster 4, increases in thrombosis risk were also observed, although not as strong as in cluster 2. Hetero- and homozygous carriers of the minor alleles of all three SNPs in cluster 4 showed ORs of approximately 1.5. Finally in cluster 5, twofold increases in risk were observed for homozygous carriers of the minor alleles of all four SNPs in this cluster. No effects on risk were observed in heterozygous carriers.

Because the SNPs are in linkage disequilibrium within the five clusters, the effects described above may be caused by only one of the SNPs per cluster. Therefore, we used unconditional logistic regression modeling to identify the SNPs in each of the three clusters described above that is most likely to be responsible for the effects. In cluster 2, effects on risk disappeared for AQP2-2 and AQP2NF10, but appeared to remain for AQP2-19, after adjustment for the other SNPs within the cluster. The adjusted ORs were 1.5 (0.8-2.6) and 2.3 (0.6-9.2) for hetero- and homozygous carriers of AQP2-19, respectively. In cluster 5, effects on risk remained strongest for AQP2-20 after adjusting for the other SNPs within the cluster. The adjusted ORs were 1.3 (0.9-1.9) and 1.9 (0.9-4.1) for hetero- and homozygous carriers of AQP2-20, respectively. In cluster 4, effects disappeared for each of the three SNPs after adjustment for the other two SNPs within the cluster.

Table 2. Distribution of LETS patients and controls over AQP2 genotypes.

Cluster	SNP	Genotype	Cases	Controls	OR	CI95
1	AQP2-1	AA*	132	145	1	-
		AC	219	224	1.1	0.9-1.5
		CC	120	102	1.3	0.9-1.8
	AQP2-5	AA*	140	130	1	-
		AG	225	213	1.3	0.9-1.9
		GG	106	128	1.0	0.8-1.4
2	AQP2-2	AA*	325	333	1	-
		AG	129	129	1.0	0.8-1.4
		GG	17	8	2.2	0.9-5.1
	AQP2NF10	GG*	340	351	1	-
		GA	115	106	1.1	0.8-1.5
		AA	16	13	1.3	0.6-2.7
	AQP2-19	CC*	334	355	1	-
		CG	114	101	1.2	0.9-1.6
		GG	14	6	2.5	0.9-6.5
3	AQP2-3	GG*	291	295	1	-
		GA	152	144	1.1	0.8-1.4
		AA	22	28	0.8	0.4-1.4
	AQP2-10	TT*	301	293	1	-
		TA	151	153	1.0	0.7-1.3
		AA	18	25	0.7	0.4-1.3

3	AQP2-14	TT*	302	292	1	-
		TC	151	153	1.0	0.7-1.3
		CC	18	26	0.7	0.4-1.3
	AQP2NF19	AA*	293	284	1	-
		AG	153	149	1.0	0.8-1.3
		GG	16	25	0.6	0.3-1.2
4	AQP2-4	GG*	379	402	1	-
		GA	84	63	1.4	1.0-2.0
		AA	7	5	1.5	0.5-4.7
	AQP2-7	GG*	378	402	1	-
		GA	84	64	1.4	1.0-2.0
		AA	8	5	1.7	0.5-5.3
	AQP2-8	GG*	404	419	1	-
		GA	63	50	1.3	0.9-1.9
		AA	4	0	∞	0.7- ∞
5	AQP2-6	GG*	325	338	1	-
		GA	127	122	1.1	0.8-1.5
		AA	19	11	1.8	0.8-3.8
	AQP2-13	CC*	322	336	1	-
		CT	126	124	1.1	0.8-1.4
		TT	23	11	2.2	1.1-4.6
	AQP2-15	TT*	323	336	1	-
		TC	128	125	1.1	0.8-1.4
		CC	19	9	2.2	1.0-4.9
	AQP2-20	AA*	240	268	1	-
		AC	194	182	1.2	0.9-1.6
		CC	35	20	2.0	1.1-3.5

* Reference group

AQP2 SNPs and Blood Pressure

Because we hypothesized that AQP2 gene variations influence VWF and FVIII levels and thrombosis risk via fluid homeostasis and blood pressure regulation, we looked at the associations between the AQP2 SNPs and arterial blood pressure in the control subjects of the LETS. No clear associations between AQP2 SNPs and blood pressure were observed. However, linear regression appeared to show weak associations of the SNPs within clusters 2, 4 and 5 with both systolic and diastolic blood pressure in healthy LETS controls (Table 3).

Table 3. Associations of AQP2 SNPs with systolic and diastolic blood pressure in healthy controls of the LETS.

Cluster	SNP	Regression coefficient systolic blood pressure*	CI95	Regression coefficient diastolic blood pressure*	CI95
2	AQP2-2	1.79	-1.87 to 5.46	1.09	-0.87 to 3.05
	AQP2NF10	1.91	-1.67 to 5.49	1.04	-0.87 to 2.96
	AQP2-19	2.73	-1.25 to 6.71	1.14	-0.98 to 3.26
4	AQP2-4	-3.69	-8.4 to 1.05	-2.15	-4.69 to 0.38
	AQP2-7	-3.03	-7.75 to 1.69	-1.79	-4.31 to 0.73
	AQP2-8	-5.41	-11.38 to	-3.59	-6.77 to -0.40
5	AQP2-6	1.29	-2.28 to 4.85	0.22	-1.69 to 2.12
	AQP2-13	2.33	-1.22 to 5.88	0.41	-1.49 to 2.31
	AQP2-15	2.03	-1.60 to 5.67	0.99	-0.96 to 2.93
	AQP2-20	-0.09	-3.23 to 3.05	-0.54	-2.21 to 1.14

* Regression coefficients represent the mmHg rise in blood pressure with each minor allele

When adjusting for the SNPs within the clusters, associations with blood pressure in cluster 2 remained for AQP2-19. The adjusted regression coefficients were 5.00 (-1.75 to 11.75) and 1.71 (-1.89 to 5.31) for AQP2-19 on systolic and diastolic blood pressure respectively, meaning that systolic blood pressure rises 5 mmHg and diastolic blood pressure rises 1.71 mmHg with each minor allele. In cluster 4, associations with blood pressure remained for AQP2-4. The adjusted regression coefficients for AQP2-4 were -12.72 (-35.66 to 10.21) and -7.40 (-19.60 to 4.81) for systolic and diastolic blood pressure respectively. In cluster 5, associations with blood pressure remained for SNP AQP2-15, whereas the associations disappeared for AQP2-6, AQP2-13 and AQP2-20. For AQP2-15, the adjusted regression coefficients were 7.21 (-6.09 to 20.51) and 10.46 (3.39 to 17.53) on systolic and diastolic blood pressure respectively.

AQP2 SNPs and Plasma Levels of VWF propeptide, VWF and FVIII

As shown in Table 4, no associations of plasma levels of VWF propeptide, VWF or FVIII with any of the AQP2 SNPs were observed in the healthy control subjects of the LETS.

Table 4. Levels of VWF propeptide, VWF and FVIII in healthy controls from the LETS.

Cluster	SNP	Genotype	VWF propeptide (U/ml)	Δ	CI95	VWF:Ag (IU/ml)	Δ	CI95	FVIII:Ag (IU/ml)	Δ	CI95
1	AQP2-1	AA (94)*	1.10	-	-	1.21	-	-	1.09	-	-
		AC (143)	1.11	0.01	-0.05 to 0.08	1.22	0.01	-0.09 to 0.12	1.07	-0.03	-0.14 to 0.08
		CC (62)	1.13	0.03	-0.04 to 0.10	1.22	0.01	-0.11 to 0.13	1.10	0.01	-0.11 to 0.13
2	AQP2-5	AA (90)*	1.10	-	-	1.21	-	-	1.10	-	-
		AG (145)	1.11	0.01	-0.06 to 0.08	1.23	0.02	-0.09 to 0.13	1.08	-0.02	-0.13 to 0.09
		GG (64)	1.12	0.02	-0.05 to 0.09	1.20	-0.01	-0.12 to 0.11	1.08	-0.02	-0.14 to 0.11
	AQP2-2	AA (213)*	1.10	-	-	1.22	-	-	1.07	-	-
		AG (79)	1.15	0.05	-0.01 to 0.12	1.22	0.00	-0.10 to 0.10	1.12	0.05	-0.05 to 0.15
		GG (6)	1.05	-0.04	-0.16 to 0.07	1.12	-0.09	-0.41 to 0.22	1.11	0.04	-0.26 to 0.35
3	AQP2NF10	GG (225)*	1.09	-	-	1.21	-	-	1.07	-	-
		GA (68)	1.16	0.07	0.00 to 0.14	1.24	0.02	-0.08 to 0.13	1.11	0.04	-0.07 to 0.15
		AA (6)	1.05	-0.04	-0.16 to 0.07	1.21	-0.09	-0.41 to 0.23	1.11	0.04	-0.28 to 0.35
	AQP2-19	CC (224)*	1.10	-	-	1.22	-	-	1.07	-	-
		CG (67)	1.16	0.06	-0.01 to 0.13	1.22	0.00	-0.10 to 0.11	1.13	0.06	-0.05 to 0.17
		GG (4)	1.06	-0.04	-0.28 to 0.20	1.15	-0.06	-0.45 to 0.32	1.02	-0.06	-0.43 to 0.32
3	AQP2-3	GG (185)*	1.12	-	-	1.23	-	-	1.09	-	-
		GA (96)	1.10	-0.02	-0.08 to 0.04	1.19	-0.03	-0.13 to 0.06	1.08	-0.01	-0.11 to 0.09
		AA (14)	1.18	0.06	-0.07 to 0.20	1.34	0.11	-0.09 to 0.31	1.17	0.08	-0.15 to 0.31
	AQP2-10	TT (183)*	1.13	-	-	1.23	-	-	1.09	-	-
		TA (103)	1.07	-0.06	-0.12 to 0.00	1.18	-0.04	-0.14 to 0.05	1.06	-0.03	-0.13 to 0.06
		AA (13)	1.14	0.01	-0.13 to 0.15	1.33	0.10	-0.11 to 0.31	1.16	0.07	-0.17 to 0.31
3	AQP2-14	TT (185)*	1.13	-	-	1.23	-	-	1.09	-	-
		TC (101)	1.07	-0.06	-0.12 to 0.00	1.18	-0.05	-0.14 to 0.04	1.06	-0.04	-0.14 to 0.06
		CC (13)	1.14	0.01	-0.13 to 0.15	1.33	0.10	-0.11 to 0.31	1.16	0.07	-0.17 to 0.31
	AQP2NF19	AA (177)*	1.13	-	-	1.24	-	-	1.09	-	-
		AG (96)	1.07	-0.06	-0.12 to 0.00	1.18	-0.05	-0.15 to 0.04	1.06	-0.04	-0.14 to 0.06
		GG (13)	1.14	0.01	-0.13 to 0.15	1.33	0.09	-0.12 to 0.31	1.16	0.07	-0.18 to 0.31

4	AQP2-4	GG (257)* GA (42) AA (0)	1.10 1.15 -	- 0.05 -	- -0.02 to 0.12 -	1.21 1.22 -	- 0.00 -	- -0.12 to 0.13 -	1.10 0.99 -	- -0.11 -	- -0.22 to - -
	AQP2-7	GG (256)* GA (43) AA (0)	1.10 1.15 -	- 0.05 -	- -0.02 to 0.12 -	1.21 1.23 -	- 0.02 -	- -0.10 to 0.15 -	1.09 1.01 -	- -0.08 -	- -0.21 to 0.05 -
	AQP2-8	GG (269)* GA (30) AA (0)	1.11 1.13 -	- 0.03 -	- -0.04 to 0.10 -	1.22 1.21 -	- -0.00 -	- -0.15 to 0.14 -	1.09 0.99 -	- -0.10 -	- -0.26 to 0.05 -
5	AQP2-6	GG (213)* GA (79) AA (7)	1.09 1.15 1.05	- 0.06 -0.04	- -0.01 to 0.12 -0.14 to 0.05	1.21 1.24 1.12	- 0.03 -0.09	- -0.07 to 0.13 -0.39 to 0.20	1.06 1.13 1.09	- 0.07 0.03	- -0.04 to 0.17 -0.26 to 0.31
	AQP2-13	CC (214)* CT (78) TT (7)	1.10 1.14 1.07	- 0.04 -0.03	- -0.03 to 0.10 -0.21 to 0.15	1.21 1.22 1.19	- 0.01 -0.02	- -0.09 to 0.11 -0.31 to 0.27	1.07 1.10 1.12	- 0.03 0.04	- -0.08 to 0.13 -0.24 to 0.33
	AQP2-15	TT (215)* TC (78) CC (6)	1.10 1.14 1.09	- 0.05 -0.01	- -0.02 to 0.11 -0.20 to 0.18	1.21 1.22 1.22	- 0.00 0.01	- -0.10 to 0.10 -0.31 to 0.32	1.07 1.11 1.18	- 0.04 0.11	- -0.07 to 0.14 -0.19 to 0.42
	AQP2-20	AA (172)* AC (115) CC (11)	1.10 1.13 1.11	- 0.03 0.01	- -0.03 to 0.09 -0.14 to 0.16	1.21 1.21 1.25	- -0.00 0.04	- -0.10 to 0.09 -0.21 to 0.29	1.09 1.08 1.03	- -0.01 -0.06	- -0.11 to 0.08 -0.29 to 0.18
	* Reference group										

Discussion

We studied the associations of common SNPs in the AQP2 gene with plasma levels of VWF propeptide, VWF and FVIII and the risk of venous thrombosis in the LETS. We identified eighteen SNPs in the AQP2 gene in a selection of 25 LETS participants. We were able to genotype the entire LETS for sixteen of these SNPs. Reliable haplotypes could not be made, but the SNPs were linked together in five clusters. Increases in thrombosis risk were observed in three of the clusters. In these same three clusters, weak associations of the SNPs with arterial blood pressure were observed, although not all in the same direction. No associations of any of the AQP2 SNPs with plasma levels of VWF propeptide, VWF and FVIII were observed in healthy controls of the LETS. Increases in risk of venous thrombosis were observed in clusters 2, 4 and 5. Effects in cluster 4 were moderate but in clusters 2 and 5, two- to two and a half-fold increases in thrombosis risk were observed. The SNPs in these clusters however, were not associated with changes in levels of VWF and FVIII, so it is unlikely that these SNPs act on thrombosis risk via increased levels of VWF and FVIII. There were weak associations with arterial blood pressure in these clusters. This association was negative for cluster 4, but positive in clusters 2 and 5, in which the effects on risk were strongest. It is possible that one or more of these AQP2 SNPs influence renal water retention, which could lead to changes in overall RAS activity. RAS can influence coagulation in other ways than via VWF secretion. ACE has antifibrinolytic properties as it inactivates bradykinin, which normally stimulates the expression of tissue-type plasminogen activator (t-PA)²³. Furthermore, angiotensin II acts both antifibrinolytic and procoagulant by stimulating both plasminogen activator inhibitor type-1 (PAI-1) and tissue factor (TF)^{23,24}. Therefore, it is possible that the increases in risk associated with the AQP2 SNPs in clusters 2, 4 and 5 are accomplished through an increase in overall RAS activity due to loss of function variations in AQP2. It is surprising however, that this is not reflected in plasma levels of VWF propeptide, VWF and FVIII.

When we used regression modeling to identify the 'responsible' SNPs per cluster, consistent results were observed in cluster 2. Effects on thrombosis risk

remained strongest for AQP2-19 when adjusted for the other SNPs in the cluster. The same was the case for the associations with systolic and diastolic blood pressure. AQP2-19 is therefore most likely the effector in cluster 2. It should be noted however, that AQP2-18, which failed to genotype, also falls within this cluster and may be the actual effector instead of AQP2-19. In cluster 5, the results were inconsistent. Effects on risk remained strongest for AQP2-20, whereas associations with arterial blood pressure remained strongest for AQP2-15. AQP2-16, which failed to genotype, also falls within this cluster and may be the actual effector. In cluster 4, associations with blood pressure remained strongest for AQP2-4 but effects on thrombosis risk disappeared for all three SNPs in the cluster after adjustment for each other.

In conclusion, several common AQP2 gene variations are associated with increases in the risk of venous thrombosis and possibly with changes in arterial blood pressure. AQP2 SNPs were not associated with plasma levels of VWF propeptide, mature VWF or FVIII. Further studies are necessary to determine which of the AQP2 SNPs that we identified in the LETS population are responsible for the effects and associations we observed and to uncover the mechanism underlying these associations and determine the functionality of the individual SNPs.

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Chapter 9

Associations of Blood Pressure and Antihypertensive Drugs with Plasma Levels of Coagulation Factor VIII

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Summary

Introduction High factor VIII activity (FVIII:C) is a risk factor for thrombosis, but mechanisms underlying high FVIII:C are largely unknown. FVIII secretion is indirectly regulated by angiotensin II, which also regulates blood pressure. FVIII:C and blood pressure may be associated and antihypertensives may lower FVIII:C.

Methods We investigated two population-based studies, the Leiden Thrombophilia Study (LETS), a case-control study on venous thrombosis, and the Cardiovascular Health Study (CHS), a prospective cohort study on cardiovascular disease in elderly. Associations between FVIII:C, blood pressure and antihypertensives were studied using multivariate linear regression, excluding individuals with cardiovascular disease.

Results Blood pressure was positively associated with FVIII:C in LETS, but not in CHS. The regression coefficient was 0.15 (0.01-0.30) in LETS and -0.05 (-0.13-0.04) in CHS for systolic blood pressure and 0.38 (0.11-0.65) in LETS and -0.17 (-0.31--0.02) in CHS for diastolic blood pressure on FVIII:C, adjusting for age, sex and race. Antihypertensive users had higher FVIII:C than non-users. Mean differences were 7.2 (-2.9-17.3) and 4.4 (1.7-7.1), adjusting for age, sex and race in LETS and CHS respectively. This difference could only partially be explained by a difference in health between users and non-users. In CHS, we observed that especially loop diuretics were associated with lower FVIII:C than the reference group, calcium channel blockers.

Conclusions Regulation of blood pressure and regulation of FVIII:C are linked. High blood pressure associates positively with high FVIII:C in LETS, but not or negatively in CHS. Use of antihypertensives is associated with higher FVIII:C compared to non-use.

Introduction

Several studies have shown that elevated plasma levels of coagulation factor VIII (FVIII) are an important risk factor for the development of venous thrombosis¹⁻⁷ and possible also for the development of arterial thrombosis⁸⁻¹⁴.

Unfortunately, the mechanisms that underlie the substantial inter-individual variations in FVIII levels in the general population are still poorly understood. An important regulator of FVIII levels could be the vasopressin 2 receptor (V2R). Stimulation of this receptor with the anti-diuretic hormone arginin vasopressin (AVP) or its synthetic analogue 1-desamino-8-d-arginine vasopressin (desmopressin or DDAVP) results in a strong rise in plasma levels of FVIII by way of its carrier protein, von Willebrand factor (VWF)^{15,16}. Synthesis and release of AVP by the hypothalamus pituitary axis is stimulated amongst other factors by angiotensin II¹⁷. Furthermore, it has been shown in rodent models that angiotensin II can also directly up-regulate the expression of V2R¹⁸. Therefore, angiotensin II may play a role in the regulation of plasma levels of VWF and FVIII. Angiotensin II also plays an important role in the maintenance of vascular tone and blood pressure. As the end product of the renin angiotensin system (RAS), angiotensin II increases blood pressure by working as a vasoconstrictor and it helps to maintain blood volume and osmolality by stimulating the release of aldosterone and AVP¹⁹.

Since both plasma levels of FVIII and blood pressure are increased by increased RAS activity, blood pressure and plasma levels of FVIII may be positively associated. A positive association between blood pressure and VWF has been described previously²⁰, but the authors propose that this association is caused by increased vascular endothelial damage. If RAS can influence VWF and FVIII levels as well, it could be that the use of antihypertensive medication that interferes with either RAS or fluid homeostasis, will not only lower blood pressure, but can also influence plasma levels of FVIII, via suppression of VWF and FVIII secretion.

Therefore, we hypothesized that there is a positive association between systolic and diastolic blood pressure and plasma levels of FVIII. Furthermore, we hypothesized that antihypertensive drug use is associated with lowering of FVIII levels, especially those that act through inhibition of RAS. To test these hypotheses, we studied the cross-sectional relationships between blood pressure, FVIII levels and the use of different types of antihypertensive

medication in two large population-based studies, the Leiden Thrombophilia Study (LETS) and the Cardiovascular Health Study (CHS).

Patients and Methods

Leiden Thrombophilia Study

The LETS is a population based case-control study on venous thrombosis which consists of 474 consecutive patients with deep vein thrombosis and 474 healthy controls with no history of deep vein thrombosis. For the present analyses, we will include healthy controls only. The controls were matched for sex and age and were acquaintances or partners of the patients. Subjects with underlying malignancies were excluded from the study. Mean age for control subjects was 47 years, ranging from 15 to 72 years. The group consisted of 272 women (57.4%) and 202 men (42.6%). All participants were of Dutch descent and living in the Netherlands. All participants completed a questionnaire including questions about present use of medication and type of medication. After completing the questionnaire, blood pressure was measured on the upper-arm in resting position prior to a blood draw. FVIII:C was measured in the plasma of all participants by a one-stage clotting assay¹. Pooled normal plasma, which was calibrated against a WHO reference, was used as a standard. Results are expressed as international units per dl (IU/dl). The design of this study has previously been described in more detail²¹. Relevant baseline characteristics of LETS controls are shown in Table 1a.

Table 1a. General Characteristics of LETS controls, divided in non-users and users of antihypertensive drugs.

		All (%)	No antihypertensive treatment (%)	Antihypertensive treatment (%)
All		474	440	34
Sex	Female	272 (57.4)	256 (58.2)	16 (47.1)
	Male	202 (42.6)	184 (41.8)	18 (52.9)
Age	Mean age	46.6	45.7	58.6
FVIII:C	Mean levels	108.4	107.5	120.8

Cardiovascular Health Study (CHS)

The CHS is a prospective population based cohort study of cardiovascular disease in adults aged 65 years and older from four U.S. communities. The present cross-sectional analyses include the original cohort of CHS, consisting of 5201 individuals which were recruited to the study from 1989 to 1990. A second cohort consisting of 687 individuals who were recruited from 1992 to 1993 were not included in the present study, because FVIII levels were not measured in this group. The design of CHS has previously been described²². All participants underwent a baseline study visit which included clinical measures, medical history questionnaires and a medication inventory. Sitting blood pressure was measured twice on the right-arm and measures were averaged. Blood samples were drawn with the subject in a fasting state, as described previously²³. FVIII:C was measured using FVIII deficient plasma and partial thromboplastin. Pooled normal plasma, which was calibrated against a WHO reference, was used as a standard. Results are expressed as IU/dl. This procedure has been described previously²⁴. Medical history of all participants has been acquired through the questionnaires and hospital medical records. As part of the medical history questionnaire, participants were asked whether or not a physician had told them they had high blood pressure. The collection of medication data in CHS has been described previously²⁵. For the analyses described in this manuscript, we excluded all CHS participants with severe renal insufficiency and all participants missing data on renal function, hypertension status, use of antihypertensive medication or FVIII:C. We also excluded all participants with a history of venous thrombosis. Furthermore, we excluded all participants with current clinical cardiovascular disease as this can be an indication for treatment with antihypertensives and can also modify FVIII levels. In total, we excluded 2171 participants, leaving 3030 individuals. Relevant baseline characteristics of CHS participants are shown in Table 1b. CHS participants were here divided into normo- and hypertensives based on the question whether they had been diagnosed as hypertensive by a physician or not.

Table 1b. General Characteristics of eligible CHS participants, divided in non-users and users of antihypertensive drugs.

	All (%)	No antihypertensive treatment		Antihypertensive treatment	
		Normotensive (%)	Hypertensive (%)	Normotensive (%)	Hypertensive (%)
All	3030	1724 (56.9)	311 (10.3)	127 (4.2)	868 (28.6)
Sex	Female	1928 (63.6)	191 (61.4)	87 (68.5)	581 (66.9)
	Male	1102 (36.4)	655 (38.0)	40 (31.5)	287 (33.1)
Race	White	2887 (95.3)	1656 (96.1)	294 (94.5)	814 (93.8)
	Other	143 (4.7)	68 (3.9)	17 (5.5)	54 (6.2)
Age range (years)	65-69	1178 (38.9)	690 (40.0)	126 (40.5)	316 (36.4)
	70-74	1007 (33.2)	570 (33.1)	107 (34.4)	296 (34.1)
	75-79	547 (18.1)	290 (16.8)	48 (15.4)	179 (20.6)
	80+	298 (9.8)	174 (10.1)	30 (9.6)	77 (8.9)
Self-reported health status	Excellent	567 (18.7)	419 (24.3)	51 (16.4)	82 (9.4)
	Very good	887 (29.3)	519 (30.1)	92 (29.6)	242 (27.9)
	Good	1120 (37.0)	594 (34.5)	112 (36.0)	361 (41.6)
	Fair	407 (13.4)	171 (9.9)	50 (16.1)	163 (18.8)
Any subclinical CVD	Poor	43 (1.4)	19 (1.1)	4 (1.3)	18 (2.1)
	No	1505 (49.7)	949 (55.0)	132 (42.4)	364 (41.9)
FVIII:C (IU/dl)	Yes	1525 (50.3)	775 (45.0)	179 (57.6)	504 (58.1)
	Mean levels	118.3	116.7	116.4	121.8

CVD: Cardiovascular disease

Statistical Analyses

We tested the association between systolic and diastolic blood pressure and FVIII levels, using multivariate linear regression models among both LETS and CHS subjects who were not treated with antihypertensive medication for any indication at the time of the blood draw. Adjustments were made for sex and age in both LETS and CHS and additionally for race in CHS only. To correct for the effect health status may have on both blood pressure and FVIII:C, CHS individuals with fair or poor self-reported health were excluded. Coefficients are presented along with their 95% confidence intervals (CI95).

To evaluate the differences in FVIII levels between users and non-users of antihypertensive medication and between the different types of antihypertensive drugs, we used both Student's t testing and multivariate linear regression models. Findings were adjusted for sex and age in the LETS and CHS and additionally for race in CHS only and finally, CHS participants with fair or poor self-reported health were excluded. Mean FVIII:C per group is reported along with the mean differences to the reference group and their corresponding CI95.

Antihypertensive Medication

At the time of inclusion of participants in both LETS and CHS, six different classes and subclasses of antihypertensive drugs were widely available. We divided treated individuals into categories in two different manners. Our first approach was to create six groups of subjects based on the type of drug taken: ACE inhibitors (ACE-I), β -blockers (BB), diuretics divided into thiazide-like diuretics (HCT) and loop diuretics (LOOP), vasodilators (VASO) (including α -blockers) and calcium channel blockers (CCB). Mean FVIII levels per group were compared to the mean levels of the remaining five groups combined. Since the use of one type of drug does not exclude the use of another, these groups were not mutually exclusive. Therefore, our second approach was to create 13 mutually exclusive categories: 'ACE-I alone', 'BB alone', 'HCT alone', 'LOOP alone', 'VASO alone' and 'CCB alone', 'ACE-I & HCT', 'ACE-I & LOOP', 'ACE-I & 1 other' (no diuretics), 'HCT & 1 other' (no LOOP), 'LOOP & 1 other' (inc.

HCT), '2 others' (no ACE-I or diuretics) and finally '3 or more' (including ACE-I and diuretics). Mean FVIII:C per category was compared to the mean FVIII:C in the category 'CCB alone', because numbers in this category are relatively large and calcium channel blockers do not directly interfere with RAS or fluid homeostasis.

To study the effects of different drugs on FVIII levels, we used the CHS only. Numbers of treated individuals in the LETS were too small to create the categories described above.

Results

Blood pressure and FVIII levels

The associations between both systolic and diastolic blood pressure and FVIII levels in LETS controls not using antihypertensives are depicted in Table 2a.

Table 2a. Linear regression of blood pressure on FVIII:C (IU/dl) in the LETS control subjects.

	N	Regression Coefficient	CI95
Systolic blood pressure	440 [†]	0.15*	0.01 to 0.30*
Diastolic blood pressure		0.38*	0.11 to 0.65*

* Adjusted for age and sex

[†] Users of antihypertensive drugs are excluded

There was a positive association between both systolic and diastolic blood pressure and FVIII:C in the controls of the LETS. The regression coefficient was 0.15 (CI95 0.01 to 0.30), adjusting for age and sex, for systolic blood pressure on FVIII:C. For diastolic blood pressure, the regression coefficient was 0.38 (CI95 0.11 to 0.65), adjusting for age and sex.

The associations between both systolic and diastolic blood pressure and FVIII levels in eligible CHS participants not using anti-hypertensive medication are depicted in Table 2b. There was no association between systolic blood pressure and FVIII:C in the CHS. The regression coefficient was -0.05 (CI95 -0.12 to 0.03) after adjustment for age sex and race. For diastolic blood pressure, there was a negative association with FVIII:C. The regression coefficient was -0.14

(CI95 -0.27 to 0.0), adjusting for age, sex and race. After exclusion of all individuals with fair or poor health, results were similar. We repeated the analysis after exclusion of all participants older than 70 or with sub-clinical cardiovascular disease, to create a group that was more comparable to the LETS. There were no differences in outcomes in this selection compared to all eligible CHS participants not using antihypertensives.

Table 2b. Linear regression of blood pressure on FVIII:C (IU/dl) in the CHS.

	N	Regression Coefficient	CI95
Systolic blood pressure	2035**	-0.05*	-0.12 to 0.03*
Diastolic blood pressure		-0.14*	-0.27 to 0.00*
Systolic blood pressure	1787**	-0.05§	-0.13 to 0.04§
Diastolic blood pressure		-0.17§	-0.31 to -0.02§
Systolic blood pressure	550**	-0.07†	-0.23 to 0.10†
Diastolic blood pressure		-0.21†	-0.48 to 0.06†

* Adjusted for age, sex and race

§ Adjusted for age, sex and race; individuals with poor or fair health are excluded

† People aged 65-70, without sub-clinical cardiovascular disease and excellent, very good or good health, adjusted for age, sex and race

** Users of antihypertensive drugs are excluded

Use of antihypertensive medication and FVIII levels

Amongst LETS controls, there were 34 users of antihypertensive drugs. Individuals on antihypertensive medication had higher levels of FVIII than those not on antihypertensive medication, 120.8 vs. 107.4 IU/dl respectively (mean difference 13.3, CI95 3.2 to 23.4). After adjustment for age and sex, the difference became smaller, but the effect remained. Results are shown in Table 3a.

Table 3a. FVIII:C (IU/dl) in users and non-users of antihypertensive drugs in LETS control subjects

	N	FVIII:C	Δ	CI95	Δ*	CI95*
No antihypertensives	440	107.5	-	-	-	-
Antihypertensives	34	120.8	13.3	3.2 to 23.4	7.2	-2.9 to 17.3

Δ: Difference; * Adjusted for age and sex

In CHS, similar results were observed. There were 995 eligible users of antihypertensive drugs versus 2035 eligible non-users in CHS. As in the LETS, users had higher levels of FVIII than non-users, 121.8 vs. 116.6 IU/dl (mean difference 5.2, CI95 2.5 to 7.9). The effect became smaller but remained after adjustment for age, sex and race and after additional exclusion of individuals with fair or poor health. Results are shown in Tables 3b.

Table 3b. FVIII:C (IU/dl) in users and non-users of antihypertensive drugs in the CHS

	N	FVIII:C	Δ	CI95	Δ^*	CI95*
No antihypertensives	2035	116.6	5.2	2.5 to 7.9	4.4	1.7 to 7.1
Antihypertensives	995	121.8				
No antihypertensives	1787 [§]	115.9	4.4	1.5 to 7.3	3.9	1.0 to 6.8
Antihypertensives	787 [§]	120.3				

Δ : Difference; * Adjusted for age and sex;

[§] Individuals with poor or fair health are excluded

Associations of different antihypertensive drugs with FVIII levels

First we studied the differences in mean FVIII:C for the six categories of antihypertensive drugs taken by CHS participants, comparing every type of drug to all others. Among all eligible individuals, no differences in levels of FVIII were observed between the six categories of drugs (Table 4). All types of drugs were associated with increased FVIII levels compared with untreated individuals. However, the severity of the indication can influence both the choice of treatment and levels of FVIII. Therefore, we excluded all individuals with fair or poor self-reported health. In this selected group, it appeared as if individuals taking loop diuretics had slightly lower levels than individuals treated with other types of antihypertensives.

Table 4. FVIII:C (IU/dl) and different classes of antihypertensive drugs

	N	FVIII:C	Δ^*	CI95*
ACE-I	140	122.2	0.7	-6.0 to 7.3
BB	214	121.1	0.9	-4.8 to 6.6
HCT	527	123.0	2.0	-3.0 to 7.0
LOOP	51	120.7	-2.6	-13.0 to 7.0
VASO	201	121.8	0.6	-5.2 to 6.3
CCB	114	120.7	-1.3	-8.7 to 6.0
	N [§]	FVIII:C	Δ^*	CI95*
ACE-I	110 [§]	121.4	0.5	-7.0 to 7.9
BB	175 [§]	119.4	-0.5	-6.7 to 5.8
HCT	427 [§]	121.9	1.9	-3.7 to 7.6
LOOP	33 [§]	116.5	-4.9	-17.7 to 7.6
VASO	150 [§]	120.2	0	-6.6 to 6.6
CCB	86 [§]	122.5	1.8	-6.4 to 10.1

The reference group is made up of users of all other drugs than the type specified

Δ : Difference; * Adjusted for age, sex and race

[§] Individuals with poor or fair health are excluded

Second, we studied the differences in mean FVIII:C after all medication types were grouped into mutually exclusive categories (Table 5). After adjustment for age, sex and race only users of loop diuretic alone, users of thiazide-like diuretics in combination with an ACE inhibitor and users of a combination of two drugs, excluding ACE inhibitors or diuretics appeared to have lower FVIII:C than the reference group which consisted of users of calcium channel blockers alone. After exclusion of individuals with fair or poor self-reported health, the differences were even more pronounced. It should be noted however, that mean FVIII:C in this selection was relatively high in the reference group.

Table 5. FVIII:C (IU/dl) and mutually exclusive groups of antihypertensive drugs

	N	FVIII:C	Δ*	CI95*	N§	FVIII:	Δ *	CI95*
CCB alone†	58	124.2	-	-	45§	126.6	-	-
ACE-I alone	66	120.4	-2.8	-15.8 to 10.2	51§	119.2	-7.6	-22.3 to 7.1
BB alone	69	119.7	-2.1	-15.1 to 10.8	55§	118.6	-6.8	-21.3 to 7.7
VASO alone	57	120.4	-1.7	-15.2 to 11.9	42§	120.0	-5.2	-20.7 to 10.3
HCT alone	271	121.7	-2.6	-13.1 to 8.0	220§	121.5	-5.9	-17.7 to 6.0
LOOP alone	14	109.1	-17.3	-38.8 to 4.3	8§	97.8	-31.3	-58.9 to
ACE-I & HCT	28	116.8	-5.7	-22.4 to 11.0	26§	115.8	-9.5	-27.3 to 8.3
ACE-I & LOOP	6	128.7	1.2	-29.9 to 32.2	4§	120.0	-8.9	-46.4 to 28.7
ACE-I & 1 other	21	124.0	2.2	-16.3 to 20.6	15§	123.0	-2.5	-23.9 to 19.0
HCT & 1 other	194	125.7	2.2	-8.7 to 13.1	156§	123.0	-3.3	-15.5 to 8.9
LOOP & 1 other	21	124.2	1.6	-16.9 to 20.2	16§	120.5	-3.6	-24.7 to 17.5
2 others	18	107.5	-14.5	-34.0 to 5.0	15§	105.3	-19.2	-40.7 to 2.2
3 or more	45	119.6	-3.4	-17.9 to 11.0	32§	122.6	-2.9	-19.6 to 13.8

Δ: Difference; * Adjusted for age, sex and race

§ Individuals with poor or fair health are excluded

† Reference group

Discussion

We studied the associations between plasma levels of FVIII, blood pressure and use of antihypertensive drugs in two large population based studies, the LETS and the CHS. In LETS, but not in CHS blood pressure was positively associated with plasma levels of FVIII. Users of antihypertensive drugs had higher levels of FVIII than non-users in both studies. There were no differences in FVIII levels between users of different types of drugs in all eligible CHS participants.

However, after exclusion of individuals with fair or poor self-reported health status, users of Loop diuretics had lower FVIII levels than users of other types of antihypertensives.

We found a positive cross-sectional relationship between blood pressure and FVIII plasma levels in the LETS. This association with FVIII levels was present both for diastolic as well as for systolic blood pressure. This makes it unlikely that blood pressure and FVIII:C only associate due to a simultaneous acute rise in FVIII levels and systolic blood pressure caused by anxiety brought on by the blood draw awaiting the participant. However, in CHS, there was no association

between systolic blood pressure and FVIII:C and even a negative association between diastolic blood pressure and FVIII:C. In older individuals, a low diastolic blood pressure can be an indication of poor health and vascular disease²⁶. Since vascular damage, ageing and disease generally lead to increased levels of FVIII, this may explain the negative association between diastolic blood pressure and FVIII:C. However, after exclusion of all CHS participants older than 70 years or with both clinical and sub-clinical cardiovascular disease, the negative association between FVIII:C and diastolic blood pressure remained. Furthermore, in this selection there was still no positive association between FVIII:C and systolic blood pressure as observed in the controls of the LETS. Besides nationality and selection criteria, the most obvious difference between this last CHS selection and the controls from the LETS is mean age. Therefore, if both observations are true, age is most likely the factor that modifies the relationship between blood pressure and FVIII:C. Age is a known determinant of levels of FVIII. FVIII strongly increases over the years whereas diastolic blood pressure can decrease dramatically with older age. It should be noted however that systolic blood pressure can increase with age.

Both LETS and CHS give consistent results on levels of FVIII and the use of antihypertensive medication; individuals who use antihypertensive medication have higher levels of FVIII. Drugs that inhibit RAS, by inhibiting the conversion of angiotensin I into angiotensin II by ACE (ACE inhibitors) or the renal secretion of renin (β -blockers)^{27,28}, indirectly inhibit the release of vasopressin and therefore the release of VWF and FVIII. Therefore, ACE inhibitors and β -blockers were expected to be associated with lower levels of FVIII, not higher. Vasodilators and calcium channel blockers could influence RAS or fluid homeostasis by causing sodium and water retention. They may therefore also be linked to the regulation of plasma levels of FVIII, but only indirectly.

An obvious explanation for the observed effects would be that users of antihypertensives are simply less healthy than non-users which could explain their high FVIII levels. Indeed, in CHS, after exclusion of the least healthy individuals, the differences in FVIII levels between users and non-users became

smaller. However, the effects did remain. Therefore, it could be possible that the association with high FVIII:C is an actual effect of the antihypertensive medication and not merely a reflection of a poorer health amongst users compared to non-users. Possibly, the body tries to compensate the effects of the drugs, by up-regulating the release of hormones that elevate blood pressure and concentrate urine, resulting in increased secretion of VWF and FVIII. It has been shown previously for instance that the body can restore the conversion of angiotensin I to angiotensin II during chronic ACE inhibition in a clinically stable situation²⁹. Of course, this is a cross-sectional study, and no information on FVIII:C before the start of treatment is available. Levels may still be lower than they would have been without antihypertensive treatment.

When we compared different types of anti-hypertensive drugs to each other, no differences in FVIII levels were observed initially. However, after exclusion of the least healthy, individuals that take loop diuretics appeared to have lower levels, comparable to non-users. When we looked at mutually exclusive categories of antihypertensives, the same effect was observed. FVIII levels were also lower among users of thiazide-like diuretics in combination with an ACE inhibitor and among users of a combination of two drug-types, excluding diuretics or ACE inhibitors. Levels of FVIII appeared particularly high in the chosen reference group, users of calcium channel blockers. Lower levels of FVIII were most expected in users of drugs interacting with RAS or fluid homeostasis, namely ACE inhibitors, β -blockers and diuretics. Indeed, loop diuretics and also ACE inhibitors in combination with thiazide-like diuretics were associated with lower levels compared to calcium channel blockers that do not interfere with RAS. However, there was no clear effect or even a trend towards lower FVIII levels in all RAS or fluid homeostasis interfering antihypertensives compared with the other types of antihypertensives.

In conclusion, this study supports the hypothesis that the regulation of blood pressure and the regulation of plasma levels of FVIII are linked. Blood pressure, both systolic and diastolic is positively associated with levels of FVIII, but only in the LETS, not in CHS, which could be explained by the difference in mean age between both populations. In CHS, the association is absent for systolic

blood pressure and even negative for diastolic blood pressure. The use of antihypertensive drugs is associated with increased FVIII levels. This effect can partially be explained by the health status of treated individuals, but not completely. However, no information is available on levels before the start of treatment. Users of loop diuretics have lower levels of FVIII compared to users of other antihypertensives. A similar trend was observed for users of thiazide-like diuretics in combination with an ACE inhibitor and for users of a combination of two drug-types, excluding diuretics or ACE inhibitors.

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Chapter 10

Summary & Discussion

Background

Elevated levels of von Willebrand factor (VWF) and coagulation factor VIII (FVIII) are well-established risk factors for the development of thrombosis. Epidemiologic studies have shown that high levels of FVIII increase the risk of venous thrombosis¹⁻⁷, whereas elevated levels of VWF increase the risk of arterial thrombosis⁸. VWF is the carrier protein of FVIII and plasma levels of both proteins generally fluctuate together. Levels of both VWF and FVIII are highly dependent on ABO blood group. Individuals with non-O blood groups have higher levels of VWF and FVIII than individuals with blood group O. ABO blood group can explain up to 30% of total variability of VWF and FVIII in the population. Even though high plasma levels of VWF and FVIII cluster within families, only few genetic variations were found to be associated with VWF and FVIII levels, besides ABO blood group. Therefore, the mechanisms that underlie the substantial inter-individual variations in VWF and FVIII levels in the general population are still poorly understood.

The research described in this thesis aimed to identify the mechanisms underlying elevated plasma levels of VWF and FVIII. We have looked at effects of VWF secretion and clearance rates on plasma levels of VWF and FVIII and on the risk of venous thrombosis, we have looked at variations within the FVIII gene and we have studied genes and proteins involved in endocrine routes that possibly influence secretion of VWF and FVIII into the circulation. Before presenting and discussing the data of our studies however, we have first reviewed current knowledge and literature on the role of elevated clotting levels in general in the development of venous thrombosis in Chapter 2 of this thesis.

VWF Secretion versus VWF Clearance

In Chapter 3 of this thesis, we present the results of our study of the VWF propeptide in the Leiden Thrombophilia Study (LETS)^{9,10}. Here, we used the VWF propeptide as a tool to distinguish between VWF secretion and VWF clearance effects. VWF propeptide plays an important role in the intracellular trafficking of VWF and in the assembly of VWF multimers¹¹⁻¹³. VWF

propeptide is cleaved from the mature VWF before or directly after secretion into the circulation¹⁴⁻¹⁶. In the circulation, VWF propeptide has a different lifespan from mature VWF and is cleared at a much faster rate¹⁷. In the present and several previous studies, levels of VWF propeptide have been interpreted as a measure of the secretion rate of mature VWF¹⁷⁻¹⁹. After making the assumptions that VWF propeptide is cleared at a more or less constant rate with a half life of approximately 2 hours and that VWF propeptide and VWF levels are at steady state, the molar plasma concentrations of VWF propeptide and mature VWF could be used to estimate the half life of mature VWF in plasma. In the LETS, both increased VWF secretion and decreased VWF clearance were associated with increased plasma levels of VWF and FVIII, as expected. In contrast to mature VWF, VWF propeptide was not influenced by ABO blood group and we demonstrated that the propeptide does not carry ABO antigens. VWF clearance was influenced by ABO blood group. VWF half life was longer in individuals with non-O blood groups compared to individuals with blood group O. Increased VWF secretion was associated with an increased risk of venous thrombosis, but, surprisingly, decreased VWF clearance was not. An important outcome of this study is that the risk of venous thrombosis appears to increase with high VWF and FVIII levels mainly when these high levels are caused by increased secretion and less so when caused by decreased clearance. If high FVIII levels directly increase the risk of venous thrombosis, it should not matter whether these high FVIII levels are caused by increased VWF secretion or decreased VWF clearance. In our study, blood samples were drawn a few months after the thrombotic event. It is tempting to explain the discrepancy in effects between secretion and clearance by post-thrombotic vascular damage or acute phase response, resulting in increased VWF secretion and increased FVIII levels. However, previous studies have shown that that an acute phase reaction was absent in most LETS samples²⁰. Furthermore, high FVIII has been identified as a possible risk factor for venous thrombosis in a prospective study as well⁷. But still, it is likely that the associations between plasma levels and thrombosis risk may actually be caused by poor condition of vessel walls. After a thrombosis, the vascular endothelium can be damaged,

resulting in increased VWF release. But also before a thrombotic event, VWF and FVIII can be elevated because of poor condition of vessel walls. In that case it may actually be the endothelial damage and not the high FVIII levels themselves that lead to thrombosis. This hypothesis can also explain the associations between FVIII and thrombosis recurrences; when post-thrombotic endothelial damage is large, VWF and FVIII are high but the risk of a recurrence is also high. It should be noted however, that an increase in thrombosis risk remains associated with high FVIII levels after adjustment for VWF levels in the LETS. This remaining risk increase cannot be explained by endothelial damage. Perhaps, increased levels of FVIII are indeed causative to an increased risk of venous thrombosis, but maybe high levels of VWF and FVIII should be interpreted mainly as a marker of other underlying risk factors of venous thrombosis.

Variations in the FVIII Gene

Whether high levels of VWF and FVIII are directly causative to a thrombosis or not, the mechanisms that underlie the substantial inter-individual variations in levels in the general population remain an interesting research topic. In Chapters 4a and 4b of this thesis we presented the results of our studies on a FVIII variant, D1241E, of which the E allele was reported to associate with lower levels of FVIII^{21,22}. The SNP encoding D1241E is present in at least three FVIII gene haplotypes, according to SeattleSNPs²³. We genotyped the LETS, the Study of Myocardial Infarctions Leiden (SMILE)²⁴ and the Risk of Arterial Thrombosis In Relation to Oral Contraceptives Study RATIO²⁵, not only for D1241E itself, but also for two additional SNPs, allowing us to distinguish all three haplotypes, HT1, HT3 and HT5 respectively, that carry the 1241E allele. To summarize our findings, we too found that the E allele of this particular FVIII variation was associated with lower levels of FVIII, however, in men only, not in women. Furthermore, effects on levels differed between haplotypes, which indicates that D1241E may not be a functional variation itself. Besides effects on levels, we also looked at effects on the risk of venous and arterial thrombosis. HT1, which of all three haplotypes was most strongly

associated with decreased FVIII levels in men, was associated with a decreased risk of venous thrombosis in men and possibly with a decreased risk of myocardial infarction in women. When we adjusted the odds ratio for HT1 on venous thrombosis in men for FVIII levels, a decrease in risk remained. This indicates that the decrease in thrombosis risk associated with HT1 is accomplished in other ways than merely a reduction in FVIII levels. In contrast to HT1, HT5 was associated with increases in arterial thrombosis risk, both in men and in women. HT5 did not clearly influence FVIII levels however, so, as for HT1, the associations with thrombosis risk are likely to be accomplished in other ways than merely through changes in FVIII plasma levels.

These studies show that common FVIII gene variations indeed influence FVIII levels and the risk of venous and arterial thrombosis.

Besides mutations in the FVIII and VWF genes that cause either hemophilia A or von Willebrand disease (VWD), it is likely that there exist more common variations that have subtle effects on FVIII and VWF plasma levels. However, after many years of research on this topic by many research groups, only a small number of such variations have been identified. Furthermore, even the FVIII SNPs studied here showed only minor effects on FVIII levels and allele frequencies were relatively low. Considering this, genetic variations that influence VWF and FVIII levels substantially in larger proportions of the population are more likely to be found in genes encoding proteins that regulate plasma levels of VWF and FVIII than in the VWF and FVIII genes themselves.

Regulated Release of VWF and FVIII

We studied variations in the genes of two G-protein coupled receptors (GPCRs) that may be involved in the secretion of VWF and FVIII, namely the $\beta 2$ adrenergic receptor ($\beta 2AR$) and the arginine vasopressin 2 receptor (V2R), and their role in the regulation of VWF and FVIII levels. In Chapter 5, we showed the results of our study of three coding non-synonymous SNP in the gene encoding the $\beta 2AR$, ADBR2. Previous studies had shown that all three SNPs are most likely functional variations that associate with several different diseases, including asthma and obesity²⁶⁻²⁸. In the LETS however, none of the three SNPs

was associated with either plasma levels of VWF and FVIII, or the risk of venous thrombosis. Variations in the gene encoding the V2R, AVPR2, on the other hand, did show associations with both plasma levels and thrombosis risk in the LETS, as shown in Chapter 6.

We looked at four SNPs in the AVPR2 gene, of which three proved to be in strong linkage disequilibrium to each other, a-245c, G12E and S331S. The fourth SNP, L309L, showed no effects on VWF and FVIII levels and no effects on venous thrombosis risk. The three linked SNPs, however, did. The minor allele frequencies of the three SNPs were low and no women homozygous for the rare alleles of any of the three SNPs were identified. Effects on VWF and FVIII levels in heterozygous women were minor and effects on risk were absent. Because the AVPR2 gene is located on the X-chromosome, we did identify men hemizygous for the rare alleles of these three SNPs. In men we saw clear associations with levels of VWF and FVIII; levels were higher in hemizygous carriers of the minor alleles. Furthermore, VWF propeptide levels were higher too, suggesting that VWF secretion was increased. In contrast to what we would have expected to find for SNPs associated with higher levels of VWF and FVIII, the minor alleles of these three SNPs were associated with an absolute protection against venous thrombosis in men in the LETS. Contrasting as the results may seem, they appear to reflect true effects. Therefore we looked more closely into the full sequence of the AVPR2 gene in male carriers of the minor alleles of the three linked SNPs. After resequencing the entire genomic region of AVPR2 in male carriers of -245c, 12E and 331S, G12E remained our most likely functional candidate and expression constructs were made including V2R cDNA, C-terminally tagged with green fluorescent protein (GFP), 12G-V2R-GFP and 12E-V2R-GFP. We stably transfected these in polarized kidney epithelial cells that lack endogenous V2R expression, namely MDCKII cells. In vitro experiments showed that both wild-type 12G-V2R and mutant 12E-V2R were normally expressed, glycosylated and localized in MDCKII cells. However, the binding affinity for arginine vasopressin (AVP) was increased more than threefold for the mutant 12E-V2R, as compared to the wild-type 12G-V2R. In summary, the V2R variation G12E is a gain of function mutation that is

associated with increased secretion of VWF and a decreased risk of venous thrombosis.

Renin Angiotensin System

We believe that an explanation for these contradicting results may be found in the renin angiotensin system (RAS). Figure 1 shows a schematic overview of RAS. RAS is a hormone cycle that regulates vascular tone and blood pressure²⁹. RAS is activated by the release of renin by the kidneys when blood volume is low or blood osmolality is high. Renin converts angiotensinogen, which is secreted by the liver, into the still inactive deca-peptide angiotensin I. This is the rate-limiting step of RAS. The renin binding protein (RBP) can inhibit renin by covalently binding to it³⁰. A SNP in the RBP gene, t61c, which was reported to influence renin plasma levels³⁰, did not associate with plasma levels of VWF and FVIII or the risk of venous thrombosis. The angiotensin converting enzyme (ACE) cleaves angiotensin I into the active octa-peptide angiotensin II. ACE can simultaneously inactivate the vasodilator bradykinin. The end product of RAS, angiotensin II works as a potent vasoconstrictor and triggers the release of the hormones aldosterone and AVP, which stimulate sodium and water re-uptake from the glomerular filtrate respectively. Furthermore, angiotensin II counteracts effects of bradykinin. Increased RAS activity eventually leads to an increase in blood volume and a decrease in blood osmolality. However, RAS also influences coagulation and fibrinolysis. Because angiotensin II triggers the release of AVP, RAS directly influences the release of VWF from Weibel Palade bodies (WPb) in endothelial cells. RAS may indirectly influence VWF release as well, as it has been shown in rodents that angiotensin II can upregulate the expression of V2R³¹. Furthermore ACE acts both procoagulant and antifibrinolytic as it stimulates the expression of both tissue factor (TF) and plasminogen activator inhibitor type I (PAI-I) via angiotensin II and suppresses tissue-type plasminogen activator (t-PA) expression via the inactivation of bradykinin³²⁻³⁴.

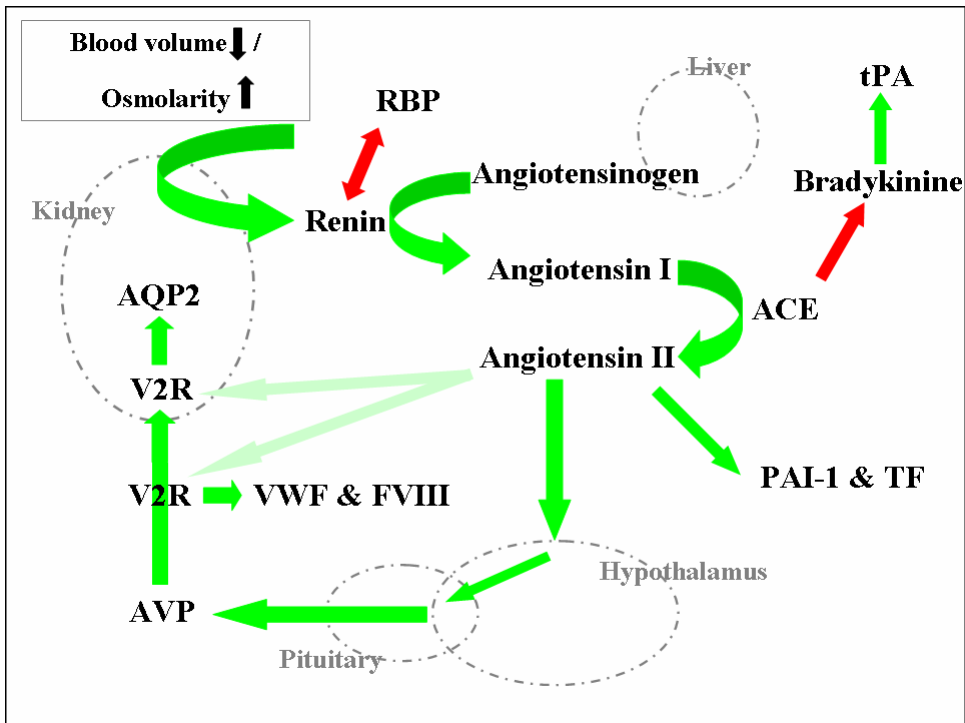


Figure 1. The Renin Angiotensin System and the Vasopressin 2 Receptor. The bright green arrows indicate stimulation or activation, whereas the red arrows indicate inhibition or inactivation. The light green arrows indicate possible upregulation. Abbreviations used: RBP is renin binding protein; tPA is tissue-type plasminogen activator; ACE is angiotensin converting enzyme; PAI-1 is plasminogen activator inhibitor; TF is tissue factor; AVP is arginine vasopressin; V2R is vasopressin 2 receptor; VWF is von Willebrand factor; FVIII is coagulation factor VIII; AQP2 is the aquaporin 2 water channel.

If the V2R can respond rapidly to changes in blood volume or osmolality, the overall activity of RAS will be kept low. That would result in less procoagulant and antifibrinolytic activity of angiotensin II and ACE and could lead to a decrease in the risk of venous thrombosis. A V2R variant with increased binding affinity for AVP, like 12E-V2R, will be activated at low plasma levels of AVP and therefore generate an early response to volume and osmolality changes. Such a variant would likely also lead to an increase in VWF secretion from WPb, which could then result in increased plasma levels of VWF and FVIII. We believe that such an effect is accomplished by the V2R 12E variant. Obviously, for this to be true, the decrease in thrombosis risk caused by

decreased RAS activity needs to be greater than the increase in thrombosis risk caused by the elevated levels of FVIII. If one believes that FVIII levels are generally more a marker of underlying risk factors instead of a strong risk factor for thrombosis itself, as discussed above, this may well be the case.

This line of thinking led to new research questions. If effects on VWF secretion and thrombosis risk are not solely accomplished by changes in V2R activity but more by changes in renal water retention and RAS activity as a whole, can other genes and proteins in this system then also influence VWF and FVIII levels and the risk of thrombosis?

Renal Water Retention and VWF and FVIII Levels

To address the issue of changes in renal water retention, we used the rare genetic kidney disorder, nephrogenic diabetes insipidus (NDI)³⁵ as an extreme model of impaired renal water retention as described in Chapter 7. V2R induced water retention is accomplished via increased expression and intra-cellular trafficking of the water channel aquaporin 2, effects that are counteracted via stimulation of bradykinin receptors^{35,36}. We hypothesized that AQP2 mutations would cause an up-regulation of V2R expression and AVP release in an attempt to compensate for a decrease in total blood volume and an increase in blood osmolality, which would result in increased VWF secretion particularly in heterozygous carriers of AQP2-linked NDI. These effects would be less visible in NDI-patients and in carriers of V2R mutations, as up-regulation of V2R expression and AVP release could not result in sufficient compensation for excess fluid loss in these groups. To test this hypothesis, we set up the Factor Eight in Nephrogenic Diabetes Insipidus study (FENDI), which includes 13 NDI families: 14 NDI patients (12 V2R- and 2 AQP2-linked), 14 carriers (9 V2R- and 5 AQP2-linked) and 25 unaffected individuals, as well as 48 unrelated controls.

In the FENDI, no differences were observed between NDI patients, carriers and unaffected individuals in markers for fluid homeostasis such as hematocrite, serum osmolality and blood pressure. AVP levels, however, were elevated in all

patients and carriers in which AVP was above the detection limit. AVP reached detectable levels in all carriers of AQP2 mutations, compared to 27% and 56% in unrelated and related unaffected individuals, respectively. VWF propeptide, a measure of the VWF secretion rate, VWF antigen and FVIII activity were also highest in carriers of AQP2 mutations.

Even though we included a large proportion of all Dutch families with NDI, numbers in the FENDI study remain very low. Nonetheless, the study does indicate a role for the AQP2 gene in the regulation of FVIII and VWF levels. To test whether AQP2 gene variations can also influence levels of VWF and FVIII and the risk of thrombosis in the general population, we looked at the AQP2 gene in the LETS. As described in Chapter 8, we sequenced a 6.6 kb long genomic region around the AQP2 gene in 25 individuals selected from the LETS based on VWF and FVIII levels, ABO blood group and age. We identified 18 single nucleotide polymorphisms (SNPs), of which 16 were genotyped in the entire LETS. Although reliable haplotypes could not be formed, the SNPs were linked within 5 clusters. In three of these clusters, 2, 4 and 5, up to threefold increases in thrombosis risk were observed. In these same clusters we observed associations of the AQP2 SNPs with arterial blood pressure. None of the AQP2 SNPs were associated with VWF or FVIII levels in controls. Because linkage between the SNPs within the clusters was high, it was not possible to determine the functional SNPs in each cluster with certainty.

The results described above indicate that the V2R and AQP2 and renal water retention in general may play a role in the regulation of VWF and FVIII levels and in the development of venous thrombosis. However these studies also raise more questions. What are the mechanisms that underlie the associations we observed? What role does RAS play in these observations? How is it possible that several AQP2 SNPs are associated with the risk of venous thrombosis, but not with levels of VWF and FVIII? These are questions that remain to be answered and may lead to very interesting research projects.

Blood Pressure Regulation and FVIII Levels

In order to gain more insight in the role of RAS in the regulation of FVIII levels, we studied the associations of systolic and diastolic blood pressure in both the LETS and the Cardiovascular Health Study (CHS)³⁷. Furthermore, we compared FVIII levels in users and non-users of different types of antihypertensive drugs, including ACE inhibitors and diuretics. Results of this study have been described in Chapter 9 of this thesis. In LETS control subjects, both systolic and diastolic blood pressure were positively associated with FVIII levels. However, in CHS, they were not and diastolic blood pressure was even negatively associated with FVIII levels. The main difference between the LETS and CHS populations lies in the age range and mean age of the participants. LETS control subjects range in age from 16 to 73, with a mean of 47 years. In CHS the age ranged from 65 to 98 with a mean of 72 years. At higher age, low diastolic blood pressure can reflect poor condition of artery walls. Poor vessel condition is generally thought to be associated with higher levels of VWF and FVIII. Therefore the age and health differences may have caused the discrepancy in results between LETS and CHS. Indeed, after exclusion of CHS participants with fair or poor health or over the age of 70 years, the negative association of diastolic blood pressure with FVIII levels disappeared. However, even in this selection of the healthiest CHS participants, the associations of systolic and diastolic blood pressure with FVIII did not become positive. Apart from the remaining difference in age between LETS and CHS participants, we have not been able to explain this observation.

When we compared FVIII levels between users and non-users of antihypertensive medication, both in LETS and CHS, users had higher levels than non-users. Adjustments for age, sex, race and health status resulted in smaller differences in levels in both studies, but differences remained. If increased RAS activity leads to increased VWF secretion, than drugs that inhibit RAS, such as ACE inhibitors, should lower VWF secretion and hence lower FVIII levels. This appeared not to be the case. An obvious explanation would be that users of antihypertensives are less healthy than non-users which would explain higher FVIII levels. When we excluded the less healthy CHS

participants, indeed differences in FVIII levels between users and non-users in CHS became smaller. However, a difference did remain. It could also be possible that FVIII levels were really increased because of the use of antihypertensive medication and that the higher FVIII levels did not merely reflect poor health amongst users compared to non-users. Possibly, the body tries to compensate the effects of the drugs, by up-regulating the release of hormones that elevate blood pressure and concentrate urine, resulting in increased secretion of VWF and FVIII. It has been shown previously for instance that the body can restore the conversion of angiotensin I to angiotensin II during chronic ACE inhibition in a clinically stable situation³⁸. However, because both LETS and CHS are cross-sectional studies, we had no information on FVIII levels in any of the LETS and CHS participants before the start of treatment. FVIII levels may still have been lower than they would have been without antihypertensive treatment.

In the LETS, numbers of antihypertensive users were too low to compare FVIII levels between different types of antihypertensives, but in CHS they were not. To make fair comparisons and prevent confounding effects of the severity of the medical condition of antihypertensive users, we excluded all CHS participants who were treated with antihypertensive drugs for any other indication than hypertension. No differences in FVIII levels between the different types of drugs were observed initially. However, after exclusion of less healthy participants, individuals that take loop diuretics appeared to have lower levels, comparable to non-users. FVIII levels also appeared lower among users of thiazide-like diuretics in combination with an ACE inhibitor and among users of a combination of two drug-types, excluding diuretics or ACE inhibitors. Levels of FVIII appeared particularly high in users of calcium channel blockers. Lower levels of FVIII were most expected in users of drugs interacting with RAS or fluid homeostasis, namely ACE inhibitors, β -blockers and diuretics. Indeed, loop diuretics and also ACE inhibitors in combination with thiazide-like diuretics were associated with lower levels compared to calcium channel blockers that do not interfere with RAS.

These observations suggest that RAS indeed plays a role in the regulation of FVIII levels and interference in RAS or fluid homeostasis with ACE inhibitors or diuretics appears to alter FVIII levels. A weakness of the study is that both LETS and CHS are cross-sectional studies and no information was available on FVIII before the start of treatment or after ceasing treatment. RAS is a complicated hormone route that influences and is influenced by many other endocrine systems. Furthermore, RAS has many positive and negative feedback loops and the research described in this thesis is not sufficient to elucidate the mechanisms causing the observed associations. The study could help formulate new research questions however and may form a good basis for future studies on the subject.

Conclusions

The research described throughout this thesis aims to gain better understanding of determinants of plasma levels of VWF and FVIII. Because high plasma levels of both proteins are well established risk factors for venous and arterial thrombosis, we also studied effects of these determinants on the risk of thrombosis where the study designs allowed it. Whether high FVIII levels are causative to an increase in the risk of venous thrombosis remains to be determined. The research described here was successful in identifying several determinants of plasma levels of VWF and FVIII, however many new questions were raised. It can safely be assumed that fluid homeostasis and RAS play a role in the regulation of VWF and FVIII levels. The exact routes and mechanisms through which effects on levels are accomplished are not understood yet. Furthermore, fluid homeostasis and RAS probably play a more complex role in coagulation than merely inducing VWF secretion from WPb and thus raising VWF and FVIII levels.

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Chapter 11

Samenvatting

Hoge plasmaspiegels van von Willebrand factor (VWF) en stollingsfactor VIII (FVIII) zijn geassocieerd met een verhoogd risico op het krijgen van zowel arteriële als veneuze trombose. VWF lijkt vooral een rol te spelen bij arteriële trombose, terwijl FVIII vooral geassocieerd is met veneuze trombose. Echter, VWF is het drager-eiwit van FVIII en zonder VWF is FVIII zeer instabiel en wordt het snel geklaard. Spiegels van VWF en FVIII fluctueren daarom over het algemeen in dezelfde richting en het is moeilijk één van beide eiwitten apart van het andere te bestuderen. Spiegels van VWF en FVIII variëren sterk tussen individuen. Uit familiestudies hebben we geleerd dat spiegels van VWF en FVIII, ten dele, erfelijk worden bepaald. De erfelijk bepaalde ABO bloedgroep kan ongeveer 30% van de variatie in VWF en FVIII spiegels verklaren. Veel andere erfelijke factoren die spiegels van VWF en FVIII kunnen beïnvloeden zijn echter nog niet gevonden. Het belangrijkste doel van het onderzoek dat in dit proefschrift wordt beschreven was inzicht te verkrijgen in mechanismen die kunnen leiden tot verhoogde plasmaspiegels van VWF en FVIII.

Het proefschrift begint met een korte algemene inleiding in **Hoofdstuk 1** en een overzicht van de huidige literatuur over de invloed van plasmaspiegels van verschillende stollingseiwitten op het risico op het krijgen van veneuze trombose in **Hoofdstuk 2**.

In **Hoofdstuk 3** hebben we gekeken naar de effecten van de secretie- en klaringssnelheid van VWF op plasmaspiegels van VWF en FVIII en op het risico voor veneuze trombose. Voor deze studie hebben we gebruik gemaakt van het VWF propeptide, dat in equimolaire hoeveelheden aan het bloed wordt afgegeven met het matuur VWF. Echter, omdat het propeptide veel sneller geklaard wordt dan matuur VWF kunnen spiegels van het propeptide geïnterpreteerd worden als een maat voor de secretiesnelheid van VWF. Bovendien, als de secretiesnelheid en de plasmaconcentraties van VWF en het propeptide bekend zijn en we aannemen dat de halfwaardetijd van het propeptide ongeveer 2 uur is, kan de halfwaardetijd van VWF uitgerekend worden. Zo hebben we een maat voor zowel de secretie- als de klaringssnelheid van VWF. Uit ons onderzoek bleek dat zowel een verhoogde secretie als een vertraagde klaring van VWF leiden tot hogere spiegels van VWF en FVIII.

Maar alleen verhoogde VWF secretie leidt tot een verhoogd trombose-risico. Dit is verassend en doet wellicht vermoeden dat de hoge VWF en FVIII spiegels een reflectie zijn van een verminderde conditie van de vaatwand, die de eigenlijke oorzaak van het verhoogde trombose-risico zou kunnen zijn. Een andere belangrijke vinding van deze studie is dat de secretiesnelheid van VWF niet afhankelijk was van ABO bloedgroep, maar de klaringssnelheid wel. ABO bloedgroep beïnvloedt VWF en FVIII spiegels dus waarschijnlijk via de klaringssnelheid van VWF.

In **Hoofdstukken 4a en 4b** hebben we gekeken naar een variatie in het FVIII gen, D1241E, die in twee verschillende studies geassocieerd bleek met verlaagde FVIII spiegels. Echter, in geen van beide studies was onderzocht of deze variatie ook geassocieerd was met een verminderd risico op het krijgen van trombose. Bovendien ligt deze variant in drie verschillende haplotypes. Het is goed mogelijk dat de eerdere observaties eigenlijk veroorzaakt zijn door een andere variatie die gelinked is aan D1241E. Wij hebben D1241E en twee andere 'single nucleotide polymorphisms' (SNPs), specifiek voor twee van de drie haplotypes, bestudeerd in drie grote studies, één naar veneuze trombose in zowel mannen als vrouwen, één naar arteriële trombose in mannen en één naar arteriële trombose in vrouwen. D1241E bleek ook in onze studie geassocieerd met lagere spiegels van FVIII. Maar als we apart naar de drie verschillende haplotypes keken bleek deze associatie alleen aanwezig voor één van de drie haplotypes, HT1, niet voor de andere twee. HT1 en één van de andere twee haplotypes, HT5, bleken ook geassocieerd met veranderingen in het risico op het krijgen van trombose. HT1 was geassocieerd met een verminderd risico voor veneuze trombose in mannen en arteriële trombose in vrouwen. HT5 was juist geassocieerd met een verhoogd risico voor arteriële trombose in zowel mannen als vrouwen en wellicht ook op veneuze trombose. Het laatste haplotype, HT3, was noch met spiegels, noch met risico geassocieerd. Omdat we verschillende resultaten vinden voor de drie haplotypes waar D1241E in ligt, is het onwaarschijnlijk dat D1241E zelf de oorzakelijke SNP is. Een verrassende bevinding in deze studie was dat als we de associatie tussen HT1 en het risico op veneuze trombose corrigeerden voor FVIII spiegels, de associatie weliswaar

kleiner werd, maar niet verdween. HT1 beïnvloedt het risico op veneuze trombose dus niet alleen via een verlaging van FVIII spiegels.

In **Hoofdstuk 5** hebben we gekeken naar associaties tussen VWF en FVIII spiegels en trombose-risico en drie variaties in het gen voor de $\beta 2$ adrenerge receptor. Uit eerder studies is gebleken dat de drie SNPs die we hebben bekeken geassocieerd zijn met verschillende ziektes zoals de ziekte van Parkinson en obesitas. Daarom lijkt het waarschijnlijk dat deze drie SNPs een invloed hebben op het functioneren van de receptor. Maar hoewel infusie van adrenaline, een ligand voor deze receptor, leidt tot verhoogde VWF en FVIII spiegels, waren de drie SNPs niet geassocieerd met VWF en FVIII spiegels en ook niet met het risico op veneuze trombose.

In de **Hoofdstukken 6, 7 en 8** wordt ons onderzoek beschreven naar de effecten van waterhuishouding in het lichaam op VWF en FVIII spiegels en het risico voor trombose. In **Hoofdstuk 6** staan de resultaten beschreven van onderzoek naar SNPs in het gen voor de vasopressine 2 receptor (V2R). Deze receptor stimuleert waterretentie in de nieren, maar is ook betrokken bij de afgifte van VWF uit vasculaire endotheel-cellen. Een variatie in het gen, G12E, bleek geassocieerd met verhoogde spiegels van VWF en FVIII maar met een verlaagd risico op veneuze trombose in mannen. Na onderzoek van deze variatie in een in vitro model bleek dat de 12E variant van de receptor een verhoogde bindingsaffiniteit voor de natuurlijke ligand, vasopressine, heeft ten opzichte van de 12G variant. De discrepantie tussen de effecten van de 12E variant op VWF en FVIII spiegels en op trombose risico kunnen waarschijnlijk verklaard worden via het renine angiotensine systeem (RAS). Verhoogde activiteit van de V2R leidt tot verhoogde afgifte van VWF, maar ook tot een snelle respons op een te lage bloeddruk of te hoge bloedosmolariteit. Omdat bloeddruk en osmolariteit gereguleerd worden door het RAS, zal een snelle correctie leiden tot verminderde RAS activiteit in het lichaam. RAS heeft naast een bloeddrukverhogende, ook een pro-trombotische werking. Daarom kan verminderde RAS activiteit leiden tot een lager trombose risico.

Omdat het lijkt dat waterhuishouding een rol kan spelen in de regulatie van VWF en FVIII spiegels hebben we gekeken naar VWF propeptide, VWF en

FVIII spiegels in patiënten en dragers van nefrogene diabetes insipidus (NDI). De resultaten hiervan staan beschreven in **Hoofdstuk 7**. NDI is een erfelijke aandoening, veroorzaakt door een defect in de V2R of in het door de V2R aangestuurde waterkanaal aquaporine 2 (AQP2). Wij vermoedden dat dragers van AQP2 defecten eventueel overmatig vochtverlies kunnen compenseren door opregulatie van V2R expressie. Dit zou echter ook leiden tot meer afgifte van VWF. In NDI patiënten met AQP2 defecten kan opregulatie niet compenseren voor overmatig vochtverlies en in patiënten en dragers met V2R defecten is opregulatie van V2R expressie niet of slechts gedeeltelijk mogelijk. Inderdaad bleek uit ons onderzoek dat AQP2-dragers hogere spiegels van VWF en FVIII en met name van VWF propeptide hebben vergeleken met AQP2-patiënten, V2R-dragers en V2R-patiënten en vergeleken met gezonde controlepersonen. Echter, omdat NDI een zeer zeldzame aandoening is waren de aantallen mensen in de studie te klein om definitieve conclusies te trekken. We hebben ook gekeken naar effecten van AQP2 gen variaties in een grote studie naar veneuze trombose, variaties die wellicht functioneel zijn, maar niet tot een ernstige aandoening als NDI leiden. De resultaten van dit onderzoek staan beschreven in **Hoofdstuk 8**. We hebben de complete sequentie van het AQP2 gen bekeken in een selectie van 25 mensen die hoge VWF en FVIII spiegels hadden, maar jonger waren dan 50 jaar. Bovendien hadden 20 van de 25 mensen bloedgroep O. De SNPs die we vonden in deze 25 mensen hebben we bekeken in de hele studiestudiepopulatie. Een deel van de SNPs bleek geassocieerd te zijn met een verhoogd risico op veneuze trombose en waarschijnlijk ook met veranderingen in arteriële bloeddruk. Echter, geen van de SNPs was geassocieerd met veranderingen in VWF en FVIII spiegels in gezonde personen. Tenslotte hebben we in **Hoofdstuk 9** onderzocht of er een verband bestaat tussen FVIII spiegels, arteriële bloeddruk en het gebruik van bloeddrukverlagende middelen. Wij vonden dat naarmate de bloeddruk hoger wordt, FVIII spiegels ook hoger worden. Dit geldt echter niet in mensen met een hoge leeftijd. Mensen die bloeddrukverlagende middelen gebruiken bleken hogere FVIII spiegels te hebben dan niet-gebruikers, maar het is niet duidelijk of dit een direct effect van de medicatie is of dat de FVIII spiegels een slechtere

gezondheid in de gebruikers weerspiegelen. Wij vonden geen duidelijke verschillen in FVIII spiegels tussen de verschillende soorten bloeddrukverlagende middelen.

Alle in dit proefschrift beschreven resultaten zijn samengevat en bediscussieerd in **Hoofdstuk 10**.

Nawoord

Zelden heb ik ergens zo tegen opgezien als tegen een promotieonderzoek en vier jaar op hetzelfde lab leken voor mij een eeuwigheid. Maar uiteraard waren de vier jaar zo voorbij en heb ik het, afgezien van een dipje in het midden, vier jaar lang erg naar mijn zin gehad. Natuurlijk heeft het geholpen dat mijn onderzoek goed liep, maar het waren vooral een heleboel mensen die deze periode zo bijzonder hebben gemaakt.

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Curriculum Vitae

De schrijfster van dit proefschrift werd op 29 maart 1978 geboren in Hattem. Na het behalen van haar gymnasium β diploma aan het Gymnasium Ceeleum in Zwolle, ging zij in 1996 Biofarmaceutische Wetenschappen studeren aan de Universiteit Leiden. Zij koos voor de afstudeerrichting Farmacochemie en deed een onderzoeksstage bij de afdeling Medicinale Fotochemie onder begeleiding van dr. Gerard Beijersbergen van Henegouwen, getiteld: New Derivatives of 5-Aminolevulinic Acid for Photo-Dynamic Therapy: N-Acetyl Derivatives. Verder liep zij gedurende zes maanden stage bij de afdeling 'Grundlagen Entwicklung Gesichtspflege' bij Beiersdorf AG in Hamburg, Duitsland en gedurende drie maanden bij de afdeling 'Personal Health Care' bij Procter & Gamble in Egham, Groot Brittannië. Zij voltooide haar studie in augustus 2002.

Na een korte periode van vrijwilligerswerk in Ecuador, ging zij op zoek naar een promotieplek en kwam terecht bij het 'Haemostasis and Thrombosis Research Center' van de afdeling Hematologie (inmiddels het 'Eindhoven Laboratory for Experimental Vascular Medicine', afdeling 'Thrombosis and Hemostasis') in het Leids Universitair Medisch Centrum. Vanaf juni 2003 deed zij gedurende 4 jaar en drie maanden onderzoek naar determinanten van plasma spiegels van von Willebrand factor en factor VIII onder begeleiding van Dr. Jeroen Eikenboom en Prof. Dr. Rogier Bertina. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Tijdens haar promotieonderzoek werkte zij samen met 2 afdelingen van de Radboud Universiteit Nijmegen en werkte zij gedurende enkele maanden bij de 'Cardiovascular Health Research Unit' van de Universiteit van Washington in Seattle, Verenigde Staten van Amerika.

Sinds september 2007 is zij werkzaam als onderzoeker in Kopenhagen, Denemarken, op een gezamenlijk project van de afdelingen 'Molecular Cardiology' en 'MicroRNA Research Group' van de Universiteit van Kopenhagen en 'Biochemistry, Pharmacology and Genetics' van de Universiteit van Zuid Denemarken in Odense.

