Search for novel genetic risk factors for venous thrombosis : a dual approach

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Chapter 1

General introduction

Thrombosis

Blood performs many important functions within the body, including the transport of oxygen, the supplement of nutrients, and the removal of waste products. An undisturbed blood flow is essential for the function of organs and tissues. When a blood vessel is damaged, a series of reactions is needed to stop bleeding from the damaged vessel on one hand, and to maintain blood flow within the vessel on the other hand. This process is called hemostasis. Abnormalities in hemostasis can result in either bleeding (haemorrhage) or thrombosis. Thrombosis is the formation of a blood clot (thrombus) within a blood vessel, which partially or completely obstructs the blood flow. Thrombosis can occur both in veins (venous thrombosis) and arteries (arterial thrombosis).

Venous thrombosis

Venous thrombosis is a common disease with an annual incidence of one to three per thousand individuals in the general population, ranging from one per one hundred thousand individuals per year in childhood to one per hundred individuals per year in the eldery.[1,2] Thrombi can form in any vein within the body. The most common clinical manifestation of venous thrombosis is deep venous thrombosis, which mainly affects the veins in the lower legs. More rare events of venous thrombosis are deep venous thrombosis of the arm, superficial thrombophlebitis and thrombotic events in the brain, eye, liver and mesentery. When an unstable thrombus breaks free, it can travel through the bloodstream (embolize) and obstruct the arteries of the lungs (pulmonary embolism). Obstruction of a large pulmonary artery or many small pulmonary arteries can be fatal. Other major complications of venous thrombosis are recurrences[3-5] and the development of a post-thrombotic syndrome.[3,5,6] Furthermore, venous thrombosis has a negative impact on the quality of life of thrombosis patients.[7,8]

Arterial thrombosis

Arterial thrombosis is the formation of a thrombus in the arterial system.[9] The most common risk factor for the formation of an arterial thrombus is atherosclerosis.[10] Atherosclerosis is the process in which a lipid- and calcium-rich plaque is formed on the artery wall. Formation of these so-called "fatty streaks" already starts in childhood and continues to build up during life. Eventually, atherosclerotic plaques can rupture, leading to arterial thrombus formation and obstruction of the arterial circulation. Inflammation plays a prominent role in formation and rupture of the atherosclerotic plaque.[11] When arterial thrombosis occurs in the coronary arteries, it can lead to myocardial infarction (heart attack). When it occurs in the cerebral circulation, it can lead to stroke or a transient ischemic attack (TIA).

Risk factors for venous thrombosis

As early as 1856, Virchow postulated, in his nowadays famous "Virchow's triad", that the pathogenesis of thrombosis is the result of at least one of the following factors: alterations in blood flow (stasis), alterations in the composition of the blood and damage to the vascular endothelium.[12] Arterial thrombosis is mainly caused by factors contributing to the development of vascular lesions. Risk factors for venous thrombosis mainly contribute to changes in blood flow (stasis) and the composition of the blood (hypercoagulability). Risk factors for venous thrombosis are categorized in genetic ones and non-genetic or environmental ones. Venous thrombosis is considered to be a multifactorial disease in which risk factors of both groups are involved. A thrombotic event can occur when the "thrombosis potential", the resultant of genetic and environmental factors and their interactions, exceeds a certain threshold.[13,14]

Environmental risk factors

Environmental risk factors, sometimes called acquired risk factors or non-genetic risk factors, are characteristics in a person's life that can influence his or her risk of getting a disease. Environmental risk factors for venous thrombosis include increasing age, malignancy and its treatment, trauma, surgery, lupus anticoagulant, pregnancy, puerperium, the use of female hormones (hormone replacement therapy and oral contraceptive pill) and immobilization because of e.g. long-distance travel, bed rest for an extended period of time, or plaster cast.[15] A family history of venous thrombosis also increases the risk of venous thrombosis.[16,17] Further, a previous thrombotic event increases the risk of a recurrent event.[4,18,19]

Genetic risk factors

Genetic risk factors are entirely the result of a person's genetic make-up, and are inherited from one's parents. Over the years, several genetic risk factors for venous thrombosis have been identified (see Table 1). The first genetic risk factor for venous thrombosis, antithrombin deficiency,[20,21] was discovered in 1965, followed by reports on deficiencies of protein C[22,23] and protein S[24,25] in the 1980s. These deficiencies of the natural anticoagulant proteins are relatively rare in the general population and show a high allelic heterogeneity. Mutations in the genes coding for these three proteins all result in absence or reduced function of the protein, so-called "loss of function" mutations. The factor V Leiden (Arg506Gln)[26] and the prothrombin 20210G/A[27] mutations, two risk factors that were discovered in the 1990s, are examples of "gain of function" mutations, which enhance the concentration or activity of the protein. Both mutations are relatively common in the general population, with large geographical differences in frequency.[28-30] Another genetic risk factor for venous thrombosis is ABO blood group non-O.[31-33] Blood group non-O carriers have an increased risk of venous

thrombosis compared to blood group O carriers, who are lacking either the enzyme glycosyltransferase (blood group A) or galactosyltransferase (blood group B).[34] In the last decade, several other genetic variants have been reported which influence the risk of venous thrombosis, e.g. prothrombin 19911A/G variation and fibrinogen 10034C/T mutation (reviewed in reference 35). However, not all these findings have been replicated (yet). Finally, elevated levels of many hemostasis-related proteins increase the risk of venous thrombosis.[36] These proteins include fibrinogen,[37,38] factors II,[27] VIII,[39] IX[40] and XI,[41] homocysteine[42] and thrombin activatable fibrinolysis inhibitor (TAFI).[43] The genetic determinants of these plasma phenotypes are still poorly understood.

Table 1
Prevalence and relative risk of known genetic risk factors for venous thrombosis

Risk factor	Prevalence in general population (%)	Prevalence in consecutive patients (%)*	Relative risk	References
Deficiencies of				
Protein C‡	0.3 - 0.8	3	4[a] - 8[b]	44[a], 45, 46[b]
Protein S‡	0.03 - 1	1	1[a] - 8[b]	44[a], 46[b]
Antithrombin‡	0.02	1	5[a] - 10[b]	44[a], 46, 47[b]
Mutations				
Factor V Leiden‡	3	20	7	48
Prothrombin 20210A‡	2	6	3	27
Blood group non-O	57	71	2	32
Elevated levels (>P90) of §				
Fibrinogen	9	18	2	37
Factor II	10	17	2	27
Factor VIII	10	23	3	39
Factor IX	10	20	2	40
Factor XI	10	19	2	41
Homocysteine	10	16	2	42
TAFI	9	14	2	43

* As found in the Leiden Thrombophilia Study (LETS).[49,50]
‡ For heterozygotes.
§ Reviewed in reference 36.
P90: 90th percentile as measured in healthy controls.
(a) As found in case-control studies.
(b) As found in family studies.

Genetic risk factors are missing
Using family and twin-based studies, the heritability of venous thromboembolism was estimated between 50 and 60%.[51-53] In addition, about 20-30% of consecutive thrombosis patients report one or more first-degree relatives with venous thrombosis.[17,54] Together this indicates that genetic components play an important

role in de pathogenesis of venous thrombosis. Venous thrombosis also has the tendency to cluster within families. This is called familial thrombophilia. In contrast to monogenetic disorders (e.g. Hemophilia and Huntington's disease) in which the disease is caused by a single gene defect, familial thrombophilia is considered to be an oligogenetic disorder in which at least two genetic defects segregate in the family.[55-58] However, in only 13% of these thrombophilia families, two or more of the known genetic defects are found (apart from ABO blood group non-O). In the majority of these families, only one (60%) or none (27%) of the known genetic defects are found, indicating that unknown genetic risk factors are segregating within these families.[14]

Most of the previously mentioned hemostasis-related plasma phenotypes, which are associated with thrombotic risk, show a relatively high heritability.[59-61] However, at present, little information is available on the genetic variants that contribute to the inter-individual variation of these plasma phenotypes. All together, we hypothesize that genetic determinants of venous thrombosis exist, which have not been identified so far.

Aim of this thesis

The aim of this thesis was the identification of novel genetic risk factors for venous thrombosis. The key objective was the identification of genes or genomic regions that contribute to the susceptibility to venous thrombosis. More extensive knowledge of genetic risk factors for venous thrombosis, their interaction with other risk factors and the molecular basis of these interactions, will lead to a better understanding of the pathogenesis of venous thrombosis. This can eventually lead to a better diagnosis, treatment and prevention of venous thrombosis.

Genetic approaches

There are two different approaches to identify novel genetic risk factors for complex diseases such as venous thrombosis: the hypothesis-based candidate gene approach and the more discovery-based genome-wide approach. In this thesis both approaches were used.

Candidate gene approach

The candidate gene approach has been used in family studies to study the association between a phenotype and a disease. When a phenotype was associated with the disease, the gene responsible for the phenotype was selected as candidate gene and screened for causal variant(s). In thrombosis research, this approach has successfully been used to find the many genetic variants segregating in thrombophilia families and causing the deficiencies of antithrombin,[20,62] protein C[22,63] and protein S.[24,64]

Nowadays, the candidate gene approach usually uses large association (case-control) studies to test whether a genetic variant is associated with a phenotype or disease on a population scale. This is a popular and widely used approach because of its advantages, e.g. a (large) study population is relatively easy to collect (compared to selected families), and the design has sufficient power to find modest effects.[65]

Candidate genes are usually selected on the basis of theoretical knowledge of the function of the protein encoded by the gene. There is a fairly well-established knowledge of the biochemistry of blood coagulation,[66-68] making it easy to select candidate genes for venous thrombosis based on the function of the protein and their hypothetical effect on fibrin formation or fibrinolysis. Nowadays, however, most of these thrombosis candidate genes have been extensively studied.[35,36,69] Alternatively, candidate genes can be selected from positions on the genome (linkage regions) that were identified using genome-wide scans.

The most frequently studied genetic variants in association studies are single nucleotide polymorphisms (SNPs). A SNP itself can be a functional (i.e. disease causing) variant or it can be in linkage disequilibrium (LD) with the functional variant. SNPs occur about each 300 bases along the 3-billion-base human genome, making it labor-intensive to genotype all SNPs (all genetic variations) within a single gene (on average 10-15 kilobases). Therefore we have used a haplotype-based candidate gene approach in this thesis. A haplotype is a combination of alleles of different genetic markers that are located closely together on the same chromosome and tend to be inherited together (not easily separable by recombination). These markers are usually SNPs. Since it was shown that the genome can be divided into long segments of strong LD (haplotype blocks),[70,71] we genotyped only those SNPs that were unique for a haplotype. These so-called haplotype-tagging SNPs (htSNPs) serve as a proxy for the other SNPs within the haplotype. So by genotyping only a few htSNPs, we could capture most of the common variations within a gene, without genotyping all SNPs.[72] It was demonstrated before that the factor V Leiden mutation would have been found when the gene coding for factor V was selected as candidate gene in a haplotype-based approach.[73] This indicates that this approach can be a successful strategy to find genetic variants which influence the risk of venous thrombosis.

Genome-wide approach

A genome-wide approach can be used to discover locations on the genome that contain genes that contribute to the development of the disease. New developments in high-throughput genotyping technologies and the publication of the sequence of the human genome[74,75] have made it possible to perform association studies on a genome-wide scale. In these so-called genome-wide association (GWA) studies,[76]

hundred thousands or even millions of SNPs across the genome are selected from online databases (e.g. dbSNP and HapMap) and genotyped in a large case-control study population. Unlike "regular" association studies, in which candidate genes are selected, no assumptions about the genomic location of the causal variants are made in GWA studies. A disadvantage of GWA studies are the costs of genotyping the large number of SNPs.

Linkage analysis can also follow a genome-wide approach. In this strategy, several hundreds of genetic markers (usually microsatellites) are genotyped across the genome. The underlying idea is that, within a study population of related individuals, a genetic marker, which can be linked to a functional variant, is segregating together with a trait. This trait can be either a disease (venous thrombosis in our case) or an intermediate phenotype (e.g. factor VIII levels). Usually a study population of families (extended pedigrees) or affected sibling pairs is used.

In thrombosis research, a genome-wide approach was previously used in a large French-Canadian protein C deficient pedigree (kindred Vermont II) to identify a second genetic defect which, together with protein C deficiency, explains the high frequency of venous thrombosis in this extended family.[77,78] Screening of 34 candidate genes, involved in hemostasis and inflammation, did not provide support for the hypothesis that one of these genes was the second genetic defect.[79] The genome scan, however, revealed three regions (chromosomes 11q23, 10p12 and 18p11.2-q11.2) that might contain a new genetic risk factor.[78] The latter two regions were also found in the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project in a genome scan for quantitative trait loci (QTL), influencing plasma factor XII levels and activated protein C resistance (APCR), respectively.[80,81] The only candidate gene found in the three regions was platelet-activating factor acetylhydrolase, located at 11q23. However, additional research excluded this gene as a risk factor for venous thrombosis.[82] The Spanish GAIT study contains extended pedigrees, both with and without thrombosis. They mainly searched for genetic determinants of plasma levels of hemostasis-related proteins,[51] including levels of fibrinogen,[83] factors VII,[84] VIII,[81] IX[85] and XII,[80] protein C[86] and S,[87] homocysteine,[88] von Willebrand factor,[89] tissue factor pathway inhibitor[90] and APCR.[81] The GAIT genome scans yielded some candidate genes (e.g. the *NAD(P)H-:menadione oxidoreductase 1* (*NQO1*) gene on chromosome 16q23 in the protein C levels genome scan[86]) that could explain part of the variation in levels. However, replications are needed to confirm these findings.

In our genome-wide scan for venous thrombosis we used affected sibling pairs instead of extended families. We tested to what extent affected siblings share alleles identical by descent (IBD). On average siblings share half of their genetic material:

there is a prior probability of 0.25 of sharing 2 alleles IBD, a probability of 0.50 of sharing 1 allele IBD and a probability of 0.25 of sharing 0 alleles IBD. We expect to find novel thrombosis susceptibility genes at those genomic regions where affected siblings share more alleles IBD than expected a priori. In this thesis we have performed a non-parametric linkage analysis. This analysis has some advantages over the parametric linkage analysis, mainly used for Mendelian disorders (e.g. cystic fibrosis); it does not require knowledge about the mode of inheritance, disease allele frequencies and disease genotype penetrances.

Outline of this thesis

Chapter 2 presents the results of the candidate gene approach. In **Chapter 2.1**, we assessed whether haplotypes of the genes coding for interleukin-1 (IL-1) beta, IL-1 receptor antagonist, IL-1 receptor type 1 and IL-1 receptor type 2 (*IL1B*, *IL1RN*, *IL1R1* and *IL1R2*) are associated with the risk of venous thrombosis. The proteins coded by these four genes are part of the IL-1 signaling system and were selected as candidate genes because it had been suggested that the overall effect of the proinflammatory cytokine IL-1 on coagulation and fibrinolysis is prothrombotic.[91-93] Using this approach we identified a haplotype in *IL1RN* which was associated with an increased risk of venous thrombosis. In **Chapter 2.2**, the role of *IL1RN* haplotypes, mRNA levels of *IL1RN* and the risk of myocardial infarction was investigated.

A second candidate gene that we selected was *F9*, the gene coding for coagulation factor IX. Factor IX plays an important role in the coagulation cascade by activating factor X. This eventually leads to thrombin and clot formation.[94,95] Elevated levels of factor IX increase the risk of venous thrombosis (see Table 1).[40] The molecular basis of these elevated factor IX levels is unknown. All together this makes factor IX an interesting candidate gene. In **Chapter 2.3**, we investigated whether sequence variants and haplotypes of *F9* are associated with factor IX levels and the risk of venous thrombosis.

In **Chapter 2.4**, we investigated whether a relative common functional polymorphism (called *Marburg I*) in the factor VII-activating protease (FSAP) gene (*HABP2*) is associated with venous thrombosis. FSAP is a protease that can promote both coagulation and fibrinolysis by activating factor VII and single-chain plasminogen activators.[96,97] The *Marburg I* variant of FSAP was reported to have an impaired potential to activate pro-urokinase, whereas it could still activate factor VII.[98]

Chapter 3 presents the results of the genome-wide linkage approach. In **Chapter 3.1**, we describe the collection of the Genetics in Familial Thrombosis (GIFT) study population and the characteristics (e.g. prevalence of known genetic risk factors

for venous thrombosis) of this population. The results of the genome-wide scan for venous thrombosis in the affected sibling pairs of the GIFT study are presented in **Chapter 3.2**. Using this approach we aimed at finding novel susceptibility regions for venous thrombosis. In this chapter we also describe the fine mapping of two regions, which might contain novel candidate genes for venous thrombosis. From these two regions, we selected eleven candidate genes, which were further studied in **Chapter 3.3**. A large number of htSNPs in these candidate genes were genotyped and added to the linkage analysis. Furthermore, we compared the allele frequencies of these SNPs between the affected siblings and a panel of healthy subjects. Finally, a combined linkage association analysis was performed to investigate to what extent the SNPs contribute to the observed linkage signals.

In the final chapter, **Chapter 4**, the results presented in this thesis are summarized and discussed.

Study populations used in this thesis

LETS

The Leiden Thrombophilia Study (LETS), a large population-based case-control study on the causes of venous thrombosis, was used in **Chapters 2.1**, **2.3** and **2.4** to study the effect of genetic variations on the risk of venous thrombosis. The design of the LETS has previously been described in detail.[49,50] Between January 1988 and December 1992, 474 consecutive patients were included, selected from Anticoagulation Clinics located in three cities in the Netherlands (Leiden, Amsterdam and Rotterdam). These clinics monitor all patients treated for venous thrombosis within a well defined geographical area. All patients had an objectively confirmed first episode of deep vein thrombosis and were younger than 70 years. Individuals with active malignancies were excluded. Four hundred and seventy-four control subjects were included. Control subjects were partners or acquaintances of patients, frequency matched for sex and age and without a history of venous thrombosis and malignancies. Patients and control subjects were inhabitants of the same geographic area and were all of Caucasian descent.

SMILE

In **Chapter 2.2** we used the population-based case-control Study of Myocardial Infarctions Leiden (SMILE) to investigate the effects of genetic variations on the risk of myocardial infarction. The design of the SMILE study has previously been described in detail.[99] The patient population consisted of 560 men, consecutively diagnosed with an objectively confirmed first episode of myocardial infarction, who were hospitalized in Leiden (the Netherlands) between January 1990 and January 1996. Control subjects were 646 men, frequency matched to the patients by 10-year

age groups, who underwent an orthopedic intervention between January 1990 and May 1996 and had received prophylactic anticoagulants for a short period after the intervention. The control subjects were selected from the records of the Leiden Anticoagulation Clinic, and did not have a history of myocardial infarction. Patients and control subjects were inhabitants of the same geographical area and all born in the Netherlands.

GIFT

The Genetics in Familial Thrombosis (GIFT) study was used in **Chapter 3** in the search for novel genetic risk factors for venous thrombosis. In the GIFT study we collaborated with 29 Anticoagulation Clinics throughout the Netherlands. Approximately 6600 young patients (≤45 years at the time of the thrombotic event) who were referred to these clinics for the treatment of venous thrombosis between January 2001 and January 2005 were approached. The thrombotic event could have been a deep vein thrombosis of the leg or arm, a pulmonary embolism, a superficial thrombophlebitis or a rare presentation of venous thrombosis (e.g. in brains, eye or mesentery). The event could have been a first episode or a recurrency. Patients with one or more siblings who also had developed venous thrombosis were asked to participate together with their affected sibling(s). In total, 460 affected siblings (287 sibling pairs) of Caucasian descent with at least one objectively confirmed venous thromboembolic event were included in the study. Parents were also asked to participate in the GIFT study. When parents were deceased or not willing to participate, unaffected siblings were asked to participate. In total 355 relatives were included: 105 fathers, 133 mothers and 117 unaffected siblings.

Healthy subjects

We used a control group of healthy subjects to perform an association analysis and a combined linkage-association analysis. This control group has previously been used in a case-control study on the causes of recurrent venous thrombosis.[100,101] The control group was recruited through a general practice in The Hague (the Netherlands). Two thousand eight hundred twelve individuals, aged 20-90 years, were approached to take part in a health survey on risk factors of cardiovascular disease. In total, 532 individuals agreed to take part in the study. From the currently available DNA samples, we selected 331 individuals of Caucasian descent, all without a history of venous thrombosis and cardiovascular disease.

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Chapter 2

Candidate genes

Chapter 2.1

Haplotypes of *IL1B*, *IL1RN*, *IL1R1* and *IL1R2* and the risk of venous thrombosis

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Summary
Objective: It has been suggested that the overall effect of the major proinflammatory cytokine interleukin-1 (IL-1) on coagulation and fibrinolysis is prothrombotic. The aim of this study was to investigate whether common variations in *IL1B*, *IL1RN*, *IL1R1* and *IL1R2* influence the risk of venous thrombosis.
Methods and Results: In a case-control study on the causes of deep venous thrombosis, the Leiden Thrombophilia Study (LETS), we genotyped eighteen single nucleotide polymorphisms (SNPs) in *IL1B*, *IL1RN*, *IL1R1* and *IL1R2*, enabling us to tag a total of 25 haplotype groups. Overall testing of the haplotype frequency distribution in patients and controls indicated that a recessive effect was present in *IL1RN* (p=0.031). Subsequently, the risk of venous thrombosis was calculated for each haplotype of *IL1RN*. Increased thrombotic risk was found for homozygous carriers of haplotype 5 (H5, tagged by SNP 13888T/G, rs2232354) of *IL1RN* (Odds ratio (OR)=3.9; 95% confidence interval (CI): 1.6-9.7; p=0.002). No risk was associated with haplotype 3 of *IL1RN*, which contains the frequently examined allele 2 variant of the intron 2 VNTR.
Conclusions: We found that *IL1RN*-H5H5 carriership increases the risk of venous thrombosis.

Introduction
Interleukin-1 (IL-1) is a multifunctional proinflammatory cytokine that can be produced by nearly all cell types, including monocytes, activated macrophages and endothelial cells.[1] IL-1 plays, in synergy with tumor necrosis factor alpha (TNF-α), a key role in autoimmune and inflammatory diseases by activating the expression of genes associated with the innate and adaptive immune response.[2] IL-1 synthesis can be induced by bacterial endotoxins, viruses, antigens and by other cytokines such as TNF-α and the interferons.[3] IL-1 can cause fever, inflammation and tissue damage. The margin between benefit for resistance and toxicity in humans is extremely narrow.[3]

The IL-1 superfamily comprises the agonists IL-1α and IL-1β (predominant form in humans), and their antagonist IL-1Ra.[4] Both IL-1 agonists can bind to IL-1 receptor type 1 (IL-1R1) and the "decoy" receptor IL-1 type 2 (IL-1R2).[5] High affinity binding is only established if bound IL-1α or IL-1β is also bound to the IL-1 receptor accessory protein (IL-1R AcP).[6] Complex formation of IL-1α or IL-1β with both IL-1R1 and IL-1R AcP is required for IL-1 induced signaling.[7] IL-1Ra also functions as ligand for the IL-1R1 receptor, however signal transduction does not occur because IL-1Ra lacks the binding site for IL-1R AcP.[4] IL-1α, IL-1β and IL-1Ra also bind to IL-1R2. However, this receptor is not capable of signal transduction, because it lacks the toll-like region in the cytoplasmic domain.[8] By binding IL-1, IL-1R2 controls the

amount of IL-1, which is free to bind the IL-1R1 receptor.

Several studies have provided insight in the molecular events that link inflammation to thrombosis.[9,10] IL-1 can affect the coagulation system in various ways. Tissue factor expression is up-regulated by proinflammatory cytokines like IL-1, TNF-α and IL-6.[11] Because tissue factor plays a central role in the initiation of coagulation, this suggests a strong link between inflammation and hypercoagulability. IL-1 also promotes coagulation by down-regulating the expression of thrombomodulin and endothelial cell protein C receptor, two important components of the protein C anticoagulant pathway.[9] Furthermore, IL-1 influences fibrinolysis by increasing the production of plasminogen activator inhibitor and decreasing the production of tissue-type plasminogen activator.[9,12] Together this suggests an overall prothrombotic effect for IL-1. This would explain the finding that elevated levels of proinflammatory cytokines, including IL-1β, are associated with the risk of venous thrombosis.[13] It is also possible, however, that the inflammatory reaction seen in patients with a history of venous thrombosis represents a post-thrombotic phenomenon, since no association was observed in a prospective study.[14]

We hypothesized that common variations in the genes coding for IL-1β, IL-1Ra, IL-1R1 and IL-1R2 (*IL1B*, *IL1RN*, *IL1R1* and *IL1R2*) influence the risk of venous thrombosis by modulating the IL-1 pathway. To test this hypothesis we genotyped eighteen single nucleotide polymorphisms (SNPs) in these genes, which together tag 25 haplotype groups, in all patients and control subjects of a case-control study on the causes of deep venous thrombosis, the Leiden Thrombophilia Study (LETS).

Methods

Study population

The design of the Leiden Thrombophilia Study has previously been described in detail.[15] We included 474 consecutively diagnosed patients with an objectively confirmed first episode of deep vein thrombosis and 474 controls, frequency matched for sex and age. Individuals with active cancer were excluded. All patients and controls were of Caucasian descent. The mean age for both groups was 45 years (range 15-69 for patients, 15-72 for controls). Both groups consisted of 272 (57.4%) women and 202 (42.6%) men. Venous blood was collected into 0.1 volume of 0.106 mol/L trisodium citrate. High molecular weight DNA was isolated from leukocytes by standard methods. DNA samples were available from 471 patients and 471 controls. Plasma samples were available from 473 patients and 474 controls.

Genetic analysis

IL1B, *IL1RN*, *IL1R1* and *IL1R2* were re-sequenced by SeattleSNPs in 23 subjects of

European-American descent.[16] This resulted in the identification of 23 SNPs in *IL1B*, 83 in *IL1RN*, 68 in IL1R1 and 87 in *IL1R2*. For each gene, haplotypes were constructed using the unphased SNP data from the 46 chromosomes and the software program PHASE 2.[17] We identified the most common haplotype groups of these four genes and the eighteen SNPs needed to tag these 25 haplotype groups (Table 1). All patients and controls were genotyped for these eighteen haplotype tagging (ht) SNPs. Besides the eighteen htSNPs, an additional polymorphism in *IL1RN* (17163C/T, rs4252041) and an 86-bp variable number of tandem repeats (VNTR) in intron 2 of *IL1RN* [18] were genotyped in selected individuals.

Table 1
Allele frequency distribution in patients and controls for haplotype tagging SNPs (htSNPs) used in this study

Gene	GenBank Accession number	SNP*	Reference SNP ID	Minor allele frequency	
				Patients	Controls
IL1B	AY137079	794C/T‡	rs16944	0.331	0.341
		2766T/del	rs3917354	0.202	0.209
		5200G/A§	rs1143633	0.363	0.348
		8546C/T	rs2853550	0.082	0.089
IL1RN	AY196903	12602G/A	rs3181052	0.118	0.139
		13760T/C	rs419598	0.266	0.266
		13888T/G	rs2232354	0.195	0.173
		16857T/C	rs315952	0.299	0.307
		19327G/A	rs315949	0.397	0.380
IL1R1	AF531102	12544C/G	rs2228139	0.064	0.082
		12974C/T	rs3917290	0.385	0.418
		23657A/G	rs3917318	0.277	0.247
		23772A/C	rs3917320	0.055	0.050
		27421T/A	rs3917332	0.188	0.172
IL1R2	AY124010	740T/C	rs719248	0.473	0.494
		5590T/C	rs3218874	0.127	0.108
		18072A/G	rs3218977	0.138	0.160
		19891A/G	rs2072472	0.261	0.242

* SNP numbering according to SeattleSNPs,[16] minor allele underlined.
‡ In literature referred to as -511C/T.[29]
§ In literature referred to as 5810G/A.[30]

Genotyping
The 13888T/G and 17163C/T SNPs in *IL1RN*, were genotyped by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis. The 86-bp VNTR was genotyped by PCR followed by gel electrophoresis. All other polymorphisms were genotyped using a 5'-nuclease/TaqMan assay.[19] PCRs with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied

Biosystems, Foster City, CA, USA) were performed in 96 wells plates (Greiner Bio-One, the Netherlands) on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany) and fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA, USA).

Fibrinogen and C-reactive protein levels
Plasma levels of the inflammatory biomarkers fibrinogen and C-reactive protein (CRP) were measured as described before.[20]

Statistical analysis
In the healthy controls, Hardy-Weinberg equilibrium for each htSNP was tested by the χ^2-statistic. To estimate the degree of linkage disequilibrium (LD) in our study population, we calculated D' and r^2 (measures for LD) between SNPs in *IL1B* and *IL1RN* and between SNPs in *IL1R1* and *IL1R2* using Haploview.[21] A Pearson χ^2-test was performed to detect differences in SNP allele frequency distribution between patients and controls.

TagSNPs (Version 2)[22] was used to estimate the frequency of the haplotypes present in the LETS population. R^2_h values (measure of the uncertainty in the prediction of haplotypes based on the selected htSNPs) were calculated using the SNP genotypes and the program TagSNPs. Haplotypes with R^2_h>0.95 were considered to be derived without uncertainty. Subsequently, haplotypes (H) were constructed for each individual (Figure 1A). When for an individual more than one haplotype combination was possible, haplotypes were only assigned to that individual when the haplotype combination had a probability >95% based on the results of the TagSNPs program; e.g., heterozygotes for *IL1R1* haplotypes 1 and 2 (H1H2) and heterozygotes for *IL1R1* haplotype 3 and 7 (H3H7) have the same genotype (Figure 1A), but the TagSNPs results indicated that H1H2 is much more likely (probability=99%).

For further analyses we excluded carriers of haplotypes with a R^2_h<0.95 and subjects in whom the haplotype combination could not be assigned with a probability >95%. In addition all carriers of rare haplotypes were excluded. This resulted in exclusion of 27/471 patients and 44/471 controls for *IL1B*, 70/471 patients and 74/471 controls for *IL1RN* and 3/471 patients and 2/471 controls for *IL1R2*. For *IL1R1* no individuals were excluded from the analyses.

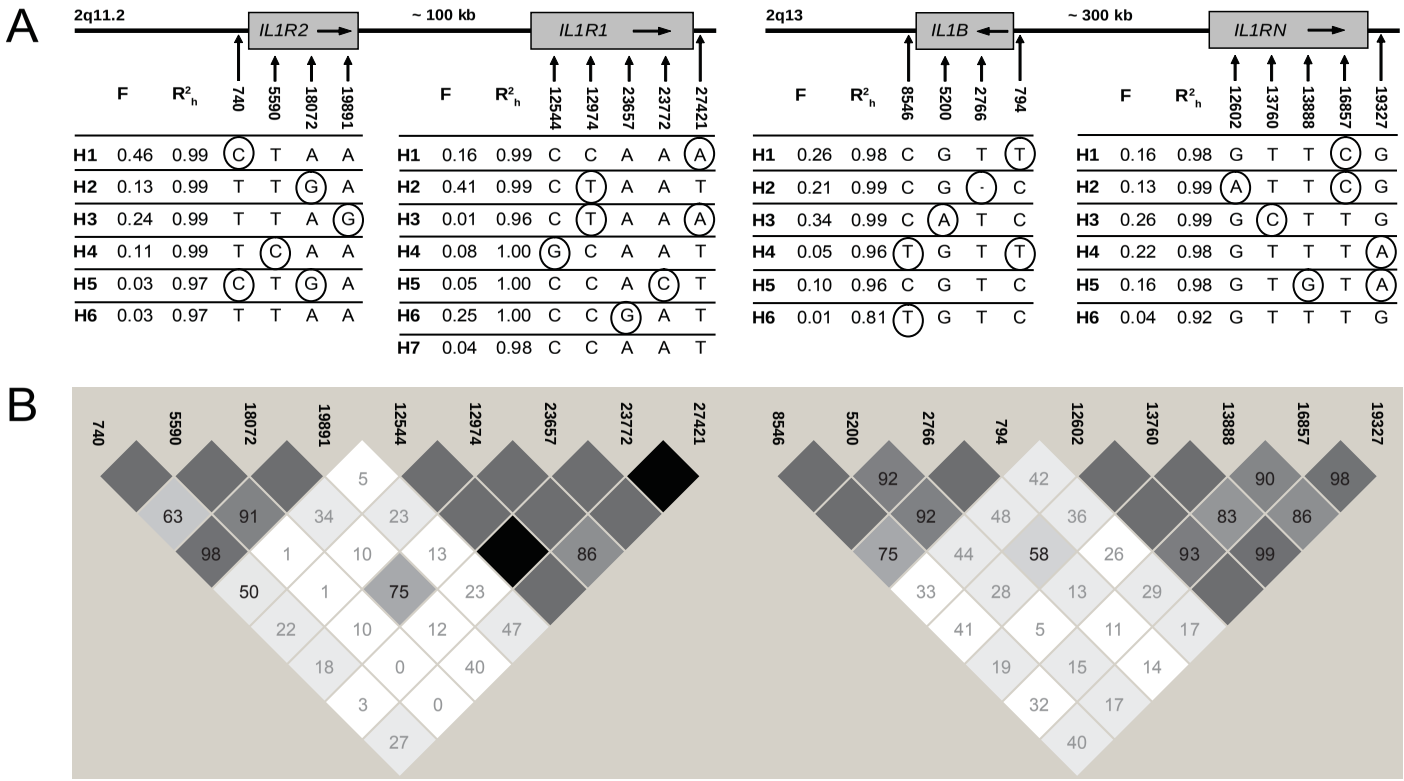

Figure 1

A: Haplotype groups and htSNPs (rare alleles circled) of *IL1R2*, *IL1R1*, *IL1B* and *IL1RN*. One SNP can be tagging for more than one haplotype and some haplotypes are tagged by a combination of two SNPs. SNP numbering according to SeattleSNPs.[16] F: allele frequency in control subjects of LETS. R^2_h: squared correlation between true and predicted haplotype dosage (zero, one or two copies). Arrows give the location of the SNPs: SNPs within a box indicate intron or exon SNPs, whereas SNPs outside a box indicate SNPs in the promoter region, 5′ or 3′ UTR, or flanking regions.

B: Haploview LD plot between *IL1R2* and *IL1R1* (left) and *IL1B* and *IL1RN* (right). LD is ranging from zero (D′=0, white) to hundred (D′=1, dark grey (if LOD≥2) or black (if LOD<2)).

A Pearson χ^2-test was performed to compare haplotype frequencies between patients and controls. This test will detect additive and dominant effects. Since this test has no power to detect recessive effects, we also performed a Pearson χ^2-test on the m+1 by 2 (patients and controls) table of m categories of homozygous carriers (H1H1, H2H2….,HmHm) and a category containing all heterozygous haplotypes (H1Hx + H2Hx …..+ HmHx)

To investigate whether SNPs or haplotypes were associated with venous thrombosis, odds ratios (ORs) and 95% confidence intervals (95%CI) according to Woolf[23] were calculated as measure of the relative risk of thrombosis for carriers of the exposure category (e.g. H4 carriers) compared to the reference category (e.g. non-H4 carriers).

For the risk calculations different reference groups were used for each haplotype. Therefore two additional models were tested to analyze the effect of *IL1RN* haplotypes on the risk of venous thrombosis; model one was a logistic regression model containing homozygous carriers (excluding H3H3 carriers) and a reference group consisting of H3H3 carriers (H3 is the most common haplotype of *IL1RN*) and all heterozygous carriers. Model two was a logistic regression model containing all fifteen *IL1RN* haplotype combinations (H1H1……,H1H5, H2H3……,H5H5) and H3H3 as a reference group.

We have in addition performed an overall recessive test without assigning haplotypes to individuals using the software program Chaplin.[24] The effect of the *IL1RN* haplotypes in a recessive model was also assessed using the program Haplo. stats,[25] which also does not assign haplotypes to individuals. H3H3 was used as reference group in this analysis.

For *IL1B*, *IL1R1* and *IL1R2*, the same haplotype analyses were performed as described above for *IL1RN*. None of the haplotypes of these three genes were associated with venous thrombosis.

For the analysis of the association of haplotypes with fibrinogen and CRP levels, levels were logarithmically transformed. For each haplotype means with 95% CI were calculated.

Results

Haplotype tagging SNPs

From the data of SeattleSNPs, we selected eighteen SNPs (Table 1) which together tag the six most common haplotype groups of *IL1B*, the six most common haplotype

groups of *IL1RN*, the seven most common haplotype groups of *IL1R1* and the six most common haplotype groups of *IL1R2* (Figure 1A). For all htSNPs the distribution of genotypes among control subjects was in Hardy-Weinberg equilibrium, except for SNP 12974C/T (p=0.049). Previous studies indicated LD between SNPs in *IL1B* and *IL1RN*.[26,27] However, Haploview analysis showed that in our population the degree of linkage disequilibrium (measured as D') was low between SNPs in *IL1B* and *IL1RN* and between SNPs in *IL1R1* and *IL1R2* (Figure 1B). D' values were high within the genes (Figure 1B), indicating that recombination events are rare in these genes. This confirmed the validity of our approach to construct haplotypes over a complete gene. We found low r^2 values between the selected SNPs in *IL1B* (r^2 ranging from 0.02 to 0.24), *IL1RN* (r^2 ranging from 0.03 to 0.30), *IL1R1* (r^2 ranging from 0.02 to 0.30) and *IL1R2* (r^2 ranging from 0.004 to 0.24), indicating that the SNPs are indeed haplotype specific.

Table 1 shows the allele frequency distribution in patients and controls for all eighteen SNPs. For all SNPs, no significant difference in allele frequency between patients and controls was found (data not shown). The risk of venous thrombosis was calculated for all 18 SNPs (supplemental Tables I to IV). An increased risk of venous thrombosis was found for homozygous allele A carriers of the *IL1B* intron 4 SNP 5200G/A (OR=1.4; 95% CI: 0.9-2.1; p=0.13), homozygous allele A carriers of the *IL1R1* 3' flanking SNP 27421T/A (OR=2.1; 95% CI: 0.9-4.9; p=0.10), and homozygous allele G carriers of the *IL1RN* intron 2 SNP 13888T/G (OR=2.8; 95% CI: 1.3-6.1; p=0.007). No effect on venous thrombosis risk was found for heterozygous carriers of these three SNPs. Odds ratios less than 1 were found for carriers (heterozygous + homozygous) of the rare alleles of the *IL1RN* intron 1 SNP 12602G/A (OR=0.8; 95% CI: 0.6-1.0; p=0.07), of the *IL1R1* exon 3 SNP 12544 C/G (OR=0.7; 95% CI: 0.5-1.0; p=0.08), of the *IL1R1* intron 3 SNP 12974 C/T (OR=0.8; 95% CI: 0.6-1.0; p=0.09) and of the *IL1R2* intron 6 SNP 18072 A/G (OR=0.8; 95% CI: 0.6-1.0; p=0.11).

Haplotypes

In total 25 common haplotype groups (Figure 1A) were expected on basis of SeattleSNPs data. TagSNPs analysis showed that, in addition to these 25 haplotype groups, three rare haplotypes in *IL1B* (frequency ranging from 0.07% to 1.0%; R^2_h<0.79), eight rare haplotypes in *IL1RN* (frequency ranging from 0.04% to 1.4%; R^2_h<0.89) and two rare haplotypes in *IL1R2* (frequency 0.13% and 0.16%; R^2_h<0.89) were predicted based on the genotypic data. No additional haplotypes in *IL1R1* were present in our population. Analysis in the control subjects of LETS showed that haplotype frequencies (Figure 1A) differed only slightly from those reported by SeattleSNPs. This can be explained by the relatively small size of the group studied by SeattleSNPs (46 alleles) compared to our group (1884 alleles).

Haplotypes were constructed from genotype data and assigned to each of the patients and control subjects. All common haplotypes, except H6 of both *IL1B* (R^2_h=0.81) and *IL1RN* (R^2_h=0.92), had a high R^2_h value (Figure 1A), indicating that the assignment of haplotypes to individuals was performed with sufficiently high certainty.

Table 2
Frequency distribution in patients and controls for haplotypes of *IL1B*, *IL1RN*, *IL1R1*, and *IL1R2*

Gene	Haplotype	Patients*	Controls*	Pearson χ^2 p-value (additive)	Pearson χ^2 p-value (recessive)
IL1B	H1	0.263	0.273	0.956	0.200
	H2	0.208	0.210		
	H3	0.368	0.351		
	H4	0.064	0.064		
	H5	0.096	0.102		
IL1RN	H1	0.171	0.166	0.439	0.031
	H2	0.127	0.149		
	H3	0.273	0.268		
	H4	0.226	0.243		
	H5	0.203	0.174		
IL1R1	H1	0.175	0.162	0.368	0.176
	H2	0.373	0.409		
	H3	0.013	0.010		
	H4	0.064	0.082		
	H5	0.055	0.050		
	H6	0.277	0.247		
	H7	0.044	0.040		
IL1R2	H1	0.449	0.464	0.527	0.891
	H2	0.112	0.132		
	H3	0.259	0.241		
	H4	0.126	0.107		
	H5	0.026	0.029		
	H6	0.029	0.027		

* Individuals included: *IL1B* (444 patients, 427 controls), *IL1RN* (401 patients, 397 controls), *IL1R1* (471 patients, 471 controls) and *IL1R2* (468 patients, 469 controls).

Overall test of association of haplotypes with thrombosis

Table 2 shows the frequency distribution in patients and controls for the haplotypes of *IL1B*, *IL1RN*, *IL1R1*, and *IL1R2*. For all four genes, two global tests were performed to provide an overall test of association. The additive model showed no significant difference in haplotype frequencies between patients and controls for all four genes (Table 2). However, for the recessive model, a significant difference between patients and controls was observed for *IL1RN*. To investigate the cause of this difference, odds ratios were calculated for the most common haplotype groups of *IL1RN* (Table 3).

IL1RN haplotypes

An almost four-fold increased risk of venous thrombosis (OR=3.9; 95% CI: 1.6-9.7; p=0.002) was found for homozygous carriers of H5 (H5H5) (Table 3). No increased risk was found for heterozygous carriers of H5 (H5Hx).

Table 3
Thrombosis risk for haplotypes of *IL1RN*

Haplotype (H)	Patients (%) n=401	Controls (%) n=397	OR	95% CI
H1				
HxHx	279 (69.6)	278 (70.0)	1*	
H1Hx	107 (26.7)	106 (26.7)	1.0	0.7-1.4
H1H1	15 (3.7)	13 (3.3)	1.2	0.5-2.5
H2				
HxHx	310 (77.3)	286 (72.0)	1*	
H2Hx	80 (20.0)	104 (26.2)	0.7	0.5-1.0
H2H2	11 (2.7)	7 (1.8)	1.5	0.6-3.8
H3				
HxHx	210 (52.4)	216 (54.4)	1*	
H3Hx	163 (40.6)	149 (37.5)	1.1	0.8-1.5
H3H3	28 (7.0)	32 (8.1)	0.9	0.5-1.5
H4				
HxHx	237 (59.1)	227 (57.2)	1*	
H4Hx	147 (36.7)	147 (37.0)	1.0	0.7-1.3
H4H4	17 (4.2)	23 (5.8)	0.7	0.4-1.4
H5				
HxHx	261 (65.1)	265 (66.8)	1*	
H5Hx	117 (29.2)	126 (31.7)	0.9	0.7-1.3
H5H5	23 (5.7)	6 (1.5)	3.9	1.6-9.7

* Reference category; Hx: all haplotypes but the one given.

For these risk calculations a different reference group was used for each haplotype. Therefore we analyzed the effect of *IL1RN* haplotypes on venous thrombosis also with two additional models (see Methods section). For both models, only H5H5 carriership showed an effect on the risk of venous thrombosis (model one: OR=4.0; 95% CI: 1.6-9.9; p=0.003; model two: OR=4.4; 95% CI: 1.6-12.3; p=0.005).

To demonstrate that the results were not biased by the assignment of haplotypes to individuals, we also performed an analysis using software programs not requiring haplotype assignments to individuals. An overall recessive test, using the program Chaplin[24] and all genotypic data, showed a significant difference in haplotype distribution between patients and controls for *IL1RN* (p=0.005). The effect of the *IL1RN* haplotypes was tested in a recessive model using the program Haplo.stats.[25]

An increased risk was found for H5H5 carriers (OR=4.0; 95% CI: 1.6-9.9; p=0.003). This effect is similar to the risk calculated for H5H5 when haplotypes were assigned to individuals (Table 3).

According to SeattleSNPs,[16] one prevalent subhaplotype (31%) is present in the H5 group. Because we found an increased risk in the H5H5 carriers of *IL1RN*, we determined the prevalence of this subhaplotype in all H5H5 carriers by genotyping the 3' UTR 17163C/T SNP. The rare T allele was found in 3/46 H5 alleles in patients (frequency=0.06) and 2/12 H5 alleles in control subjects (frequency=0.16). Because of its low frequency we did not genotype the entire study population for this polymorphism.

Heterozygous carriers of H2 had a slightly reduced risk of venous thrombosis (OR=0.7; 95% CI: 0.5-1.0; p=0.043), which was not influenced by stratification for age or sex.

IL1RN intron 2 VNTR

The 86-bp intron 2 VNTR is a well-known and frequently genotyped polymorphism in *IL1RN*. The rare allele, allele 2, has been found to be associated with a broad range of inflammatory diseases.[28] To identify the *IL1RN* haplotype(s) in which this allele is located, we genotyped the VNTR in all homozygous carriers of each of the six haplotype groups (n=177). Allele 2 was found in 117/120 H3 alleles and in one H5H5 carrier, being heterozygous for the VNTR. Allele 2 was not present in carriers of H1H1, H2H2, H4H4 and H6H6. These results indicate that allele 2 of the VNTR is part of *IL1RN* H3.

Markers of inflammation

Fibrinogen and CRP are markers of inflammation that are expected to be increased in subjects with high IL-1 levels. Fibrinogen and CRP levels were slightly higher in patients than in control subjects.[20] In the control subjects, none of the haplotypes had an effect on the fibrinogen or CRP levels (data not shown).

Discussion

IL-1 is a proinflammatory cytokine, which plays an important role in inflammation by activating the expression of acute phase proteins. IL-1 signaling involves the receptors IL-1R1 and IL-1R2, the antagonist IL-1Ra and the accessory protein IL-1R AcP. IL-1 influences both coagulation and fibrinolysis, suggesting an overall prothrombotic effect. Whereas others studied association of single IL-1 SNPs with disease, we used a haplotype-based approach to investigate whether common variations in *IL1B*, *IL1RN*, *IL1R1* and *IL1R2* influence the risk of venous thrombosis. Global testing using a recessive model showed a difference in haplotype frequency

between patients and controls for *IL1RN* (p=0.031). While for most haplotypes no or at most marginal effects were observed, homozygous carriers of H5 of *IL1RN* had an increased risk of venous thrombosis (OR=3.9; 95% CI: 1.6-9.7). Caution is needed when interpreting these results since the number of H5H5 carriers (23 patients and 6 controls) and 13888GG carriers (tagging SNP of H5; 25 patients and 9 controls) is low. Therefore, subsequent studies will be needed to determine the validity of this finding.

Although we found an increased risk of venous thrombosis for H5H5 carriers, the functional SNP causing this risk still has to be identified. *IL1RN* H5 is tagged by the combination of 13888G and 19327A (see Figure 1). It is unlikely that the functional SNP is 19327G/A, because no association between H4 (tagged by 19327A) and thrombosis risk was found. An obvious candidate for being the functional SNP is 13888T/G, which is unique for H5 and is itself also associated with an increased risk of venous thrombosis. The 13888T/G SNP is located in intron 2 of *IL1RN* in a highly polymorphic region. This region does not contain any obvious regulatory elements which would predict that 13888T/G is a functional variant. It is also possible that the functional SNP is not 13888T/G, but a SNP in linkage disequilibrium with 13888T/G or a SNP forming a subhaplotype of H5. *IL1RN* H5 contains a number of subhaplotypes, but the frequencies were too low to investigate their effect on the risk of venous thrombosis in LETS. Future re-sequencing of H5H5 carriers from LETS may also help to identify candidate functional SNPs in *IL1RN* H5.

Apart from H5 a rare *IL1RN* haplotype exists (frequency in control subjects=0.35%, R^2_h=0.81) which is tagged by 13888G (not listed in Figure 1). This haplotype was too rare to study its effect on venous thrombosis risk in LETS.

H6 carriers of both *IL1B* and *IL1RN*, and carriers of thirteen rare haplotypes were excluded from our haplotype analysis. Inclusion of these haplotypes did not importantly change the global additive and recessive p-values (Table 2) or the haplotype associated thrombotic risk of *IL1RN* (Table 3).

Although IL-1β levels were previously measured in our study population,[13] we did not include these in our analyses because with the assay approach used, only 64 out of 942 individuals had detectable IL-1β levels. Instead, we used the inflammatory biomarkers fibrinogen and CRP. Fibrinogen and CRP levels were not associated with any of the haplotypes.

Few studies have been reported on the association of polymorphisms in *IL1R1* and *IL1R2* with disease, whereas numerous studies report on effects of polymorphisms in *IL1B* and *IL1RN*. We genotyped two well known SNPs in *IL1B*, 794C/T (-511C/T in literature[29]) and 5200G/A (5810G/A in literature[30]). Although others did observe risks associated with both SNPs in a broad range of inflammatory diseases,[31] we only found a slight increase in venous thrombosis risk associated with 5200G/A, whereas no such association was found for 794C/T. Another extensively studied polymorphism is the intron 2 VNTR in *IL1RN*.[18] We found that allele 2 of this VNTR is part of H3 of *IL1RN*. Although allele 2 of the VNTR has been associated with many different diseases,[28] we did not find an association between H3 of *IL1RN*, which contains allele 2, and venous thrombosis risk. Interestingly, H3 of *IL1RN* contains apart from allele 2 of the VNTR about 50 haplotype tagging SNPs, which will make it very hard to identify the functional SNP in this haplotype.

Our haplotype-based approach was limited to the most common haplotype groups of the four genes (Figure 1A). Rare haplotypes found by SeattleSNPs were not tagged by their own haplotype specific SNP in our study, but instead these haplotypes were incorporated into one of the 25 haplotype groups listed in Figure 1A. Therefore, we cannot exclude a risk associated with one of these rare haplotypes.

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Supplemental Tables

Table I
Thrombosis risk for SNPs in *IL1B*

SNP	Patients (%) n=471	Controls (%) n=471	OR	95% CI
C794T				
CC	209 (44.4)	204 (43.3)	1*	
CT	212 (45.0)	213 (45.2)	1.0	0.7-1.3
TT	50 (10.6)	54 (11.5)	0.9	0.6-1.4
CT+TT	262 (55.6)	267 (56.7)	1.0	0.7-1.2
T2766-				
TT	298 (63.3)	296 (62.8)	1*	
T-	154 (32.7)	153 (32.5)	1.0	0.8-1.3
--	19 (4.0)	22 (4.7)	0.9	0.5-1.6
T- + --	173 (36.7)	175 (37.2)	1.0	0.8-1.3
G5200A				
GG	198 (42.0)	191 (40.6)	1*	
GA	204 (43.3)	232 (49.3)	0.8	0.6-1.1
AA	69 (14.6)	48 (10.2)	1.4	0.9-2.1
GA+AA	273 (58.0)	280 (59.4)	0.9	0.7-1.2
C8546T				
CC	395 (83.9)	391 (83.0)	1*	
CT	75 (15.9)	76 (16.1)	1.0	0.7-1.4
TT	1 (0.2)	4 (0.8)	0.2	0.03-2.2
CT+TT	76 (16.1)	80 (17.0)	0.9	0.7-1.3

* Reference category; Numbering according to SeattleSNPs.[16]

Table II
Thrombosis risk for SNPs in *IL1RN*

SNP	Patients (%) n=471	Controls (%) n=471	OR	95% CI
G12602A				
GG	371 (78.8)	347 (73.7)	1*	
GA	89 (18.9)	117 (24.8)	0.7	0.5-1.0
AA	11 (2.3)	7 (1.5)	1.5	0.6-3.8
GA+AA	100 (21.2)	124 (26.3)	0.8	0.6-1.0
T13760C				
TT	252 (53.5)	255 (54.1)	1*	
TC	187 (39.7)	181 (38.4)	1.1	0.8-1.4
CC	32 (6.8)	35 (7.4)	0.9	0.6-1.5
TC+CC	219 (46.5)	216 (45.9)	1.0	0.8-1.3
T13888G				
TT	312 (66.2)	317 (67.3)	1*	
TG	134 (28.5)	145 (30.8)	0.9	0.7-1.2
GG	25 (5.3)	9 (1.9)	2.8	1.3-6.1
TG+GG	159 (33.8)	154 (32.7)	1.1	0.8-1.4
T16857C				
TT	235 (49.9)	232 (49.3)	1*	
TC	190 (40.3)	189 (40.1)	1.0	0.8-1.3
CC	46 (9.8)	50 (10.6)	0.9	0.6-1.4
TC+CC	236 (50.1)	239 (50.7)	1.0	0.8-1.3
G19327A				
GG	177 (37.6)	177 (37.6)	1*	
GA	214 (45.4)	230 (48.8)	0.9	0.7-1.2
AA	80 (17.0)	64 (13.6)	1.3	0.8-1.8
GA+AA	294 (62.4)	294 (62.4)	1.0	0.8-1.3

* Reference category; Numbering according to SeattleSNPs.[16]

Table III
Thrombosis risk for SNPs in *IL1R1*

SNP	Patients (%) n=471	Controls (%) n=471	OR	95% CI
C12544G				
CC	414 (87.9)	395 (83.9)	1*	
CG	54 (11.5)	75 (15.9)	0.7	0.5-1.0
GG	3 (0.6)	1 (0.2)	2.9	0.3-27.6
CG+GG	57 (12.1)	76 (16.1)	0.7	0.5-1.0
C12974T				
CC	174 (36.9)	149 (31.6)	1*	
CT	231 (49.0)	250 (53.1)	0.8	0.6-1.1
TT	66 (14.0)	72 (15.3)	0.8	0.5-1.2
CT+TT	297 (63.1)	322 (68.4)	0.8	0.6-1.0
A23657G				
AA	254 (53.9)	274 (58.2)	1*	
AG	173 (36.7)	161 (34.2)	1.2	0.9-1.5
GG	44 (9.3)	36 (7.6)	1.3	0.8-2.1
AG+GG	217 (46.1)	197 (41.8)	1.2	0.9-1.5
A23772C				
AA	419 (89.0)	426 (90.4)	1*	
AC	52 (11.0)	43 (9.1)	1.2	0.8-1.9
CC	0	2 (0.4)	-	-
AC+CC	52 (11.0)	45 (9.6)	1.2	0.8-1.8
T27421A				
TT	310 (65.8)	317 (67.3)	1*	
TA	145 (30.8)	146 (31.0)	1.0	0.8-1.3
AA	16 (3.4)	8 (1.7)	2.1	0.9-4.8
TA+AA	161 (34.2)	154 (32.7)	1.1	0.8-1.4

* Reference category; Numbering according to SeattleSNPs.[16]

Table IV
Thrombosis risk for SNPs in *IL1R2*

SNP	Patients (%) n=471	Controls (%) n=471	OR	95% CI
T740C				
TT	129 (27.4)	115 (24.4)	1*	
TC	238 (50.5)	247 (52.4)	0.9	0.6-1.2
CC	104 (22.1)	109 (23.1)	0.9	0.6-1.2
TC+CC	342 (72.6)	356 (75.6)	0.9	0.6-1.2
T5590C				
TT	356 (75.6)	374 (79.4)	1*	
TC	110 (23.4)	92 (19.5)	1.3	0.9-1.7
CC	5 (1.1)	5 (1.1)	1.1	0.3-3.7
TC+CC	115 (24.4)	97 (20.6)	1.3	0.9-1.7
A18072G				
AA	350 (74.3)	328 (69.6)	1*	
AG	112 (23.8)	135 (28.7)	0.8	0.6-1.0
GG	9 (1.9)	8 (1.7)	1.1	0.4-2.8
AG+GG	121 (25.7)	143 (30.4)	0.8	0.6-1.0
A19891G				
AA	262 (55.6)	272 (57.7)	1*	
AG	172 (36.5)	170 (36.1)	1.1	0.8-1.4
GG	37 (7.9)	29 (6.2)	1.3	0.8-2.2
AG+GG	209 (44.4)	199 (42.3)	1.1	0.8-1.4

* Reference category; Numbering according to SeattleSNPs.[16]

Chapter 2.2

Haplotypes of the interleukin-1 receptor antagonist gene, interleukin-1 receptor antagonist mRNA levels and the risk of myocardial infarction

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Summary

Background: The overall effect of the major proinflammatory cytokine interleukin-1 (IL-1) on coagulation and fibrinolysis is prothrombotic. We recently found that haplotype 5 (H5) of the gene (*IL1RN*) coding for the interleukin-1 receptor antagonist (IL-1Ra), the natural inhibitor of IL-1, is associated with an increased risk of venous thrombosis. It is unclear whether variations in *IL1RN* affect the risk of myocardial infarction.

Objectives: The aim of this study was to investigate the effect of the five most common haplotype groups of *IL1RN* on the risk of myocardial infarction and on *IL1RN* mRNA levels.

Patients/Methods: We genotyped five single nucleotide polymorphisms (SNPs) in *IL1RN* in 560 male patients and 646 male control subjects of a population-based case-control study on myocardial infarction, enabling us to tag the five common haplotype groups of *IL1RN*. For all haplotype groups the relationship with the risk of myocardial infarction and *IL1RN* mRNA levels was determined.

Results: An increased risk of myocardial infarction was found for H3 carriers (tagged by SNP 13760T/C, odds ratio=1.3; 95% confidence interval: 1.1-1.7) compared to non-H3 carriers. No effect on myocardial infarction risk was found for the other haplotypes. H3 carriers had decreased *IL1RN* mRNA levels compared to non-H3 carriers ($p<0.01$), whereas mRNA levels were higher in H2 carriers compared to non-H2 carriers ($p<0.01$).

Conclusions: We found that H3 carriership increases the risk of myocardial infarction. This effect could be explained by the reduced *IL1RN* expression in H3 carriers, which is expected to result in reduced levels of IL-1Ra, the principal antagonist of IL-1.

Introduction

Interleukin-1 (IL-1) is a multifunctional pro-inflammatory cytokine that plays an important role in autoimmune and inflammatory diseases by activating the expression of genes associated with the innate and adaptive immune response.[1] The IL-1 superfamily comprises the agonists IL-1α and IL-1β (the predominant form in humans) and the IL-1 receptor antagonist IL-1Ra, all capable of binding to the functional IL-1 receptor type I (IL-1R1) or the non-signaling receptor type II (IL-1R2). IL-1Ra functions as a natural inhibitor of IL-1 activity by binding to the IL-1R1 receptor and thereby blocking IL-1 signaling.[2]

Several studies have provided insight into the mechanisms that link inflammation with cardiovascular events.[3,4] Inflammatory cytokines, including IL-1, can affect coagulation by up-regulation of tissue factor expression[5] and down-regulation of the expression of thrombomodulin and the endothelial protein C receptor.[6] IL-1 also influences fibrinolysis by increasing the production of plasminogen activator inhibitor

and decreasing the production of tissue-type plasminogen activator.[6,7] Overall, this suggests a prothrombotic effect for IL-1. In addition, pro-inflammatory cytokines like tumor necrosis factor-α and IL-1 can also stimulate the endothelial surface to increase the expression of leukocyte adhesion molecules,[8] thereby promoting atherosclerosis.[9] Rupture of the atherosclerotic plaque can lead to thrombus formation, eventually causing arterial thrombosis and myocardial infarction.[9]

Recently, we investigated whether common variations in the genes coding for IL-1β (*IL1B*), IL-1Ra (*IL1RN*), IL-1R1 (*IL1R1*) and IL-1R2 (*IL1R2*) affect venous thrombosis risk.[10] We showed that homozygous carriers of a certain haplotype (H5) of *IL1RN* have an almost 4-fold increased risk of venous thrombosis. It is unclear whether variations in *IL1RN* also affect the risk of myocardial infarction. Studies on the relationship between the often studied intron 2 variable number of tandem repeat (VNTR) of *IL1RN* and cardiovascular diseases have yielded contradictory results.[11-14] However, the development of lethal arterial inflammation[15] and severely fatty liver[16] in IL-1Ra deficient mice emphasizes the importance of IL-1Ra in controlling the pro-inflammatory effects of IL-1. In the present study, we investigated the effect of the five most common haplotype groups of *IL1RN* on the risk of myocardial infarction and on *IL1RN* mRNA levels in the "Study of Myocardial Infarctions Leiden".

Methods

Study population and data collection

The design of the population-based case-control Study of Myocardial Infarctions Leiden (SMILE) has previously been described in detail.[17] Patients consisted of 560 men, consecutively diagnosed with an objectively confirmed first episode of myocardial infarction. Control subjects were 646 men, frequency matched to the patients by 10-year age groups. All patients and controls were born in the Netherlands. The mean age of patients was 56.2 years (range 32.1-70.1) and of control subjects 57.3 years (range 27.2-74.8). All individuals completed a questionnaire about the presence of cardiovascular risk factors. Venous blood was collected into 0.106 mol/L trisodium citrate. This blood was used for the preparation of plasma and the isolation of RNA and high molecular weight DNA. Plasma levels of the inflammatory biomarkers fibrinogen and C-reactive protein (CRP) were measured as described before.[18,19] High molecular weight DNA was isolated from leukocytes by standard methods. RNA was isolated from whole citrated blood using a silica-based method.[20] DNA and plasma samples were available for all patients and control subjects. RNA samples were available for 524 patients and 628 control subjects.

Genetic analysis

We previously found five common (frequency in controls >5%) haplotype groups

in *IL1RN* in the Leiden Thrombophilia Study (LETS).[10] Since both LETS and SMILE recruited individuals from the same geographical region, we tested the same five haplotype groups in the SMILE. To tag these five common haplotype (H) groups, we genotyped all patients and control subjects for five haplotype tagging single nucleotide polymorphisms (htSNPs): 12602G/A (rs3181052), 13760T/C (rs419598), 13888T/G (rs2232354), 16857T/C (rs315952) and 19327G/A (rs315949) (numbering according to SeattleSNPs[21]). SNP 13888T/G was genotyped using a polymerase chain reaction followed by restriction fragment length polymorphism analysis. All other SNPs were genotyped using a 5'-nuclease/TaqMan assay.[22] PCRs with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA, USA) were performed in 96 wells plates (Greiner Bio-One, the Netherlands) on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany) and fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA, USA). For one patient genotyping failed for all SNPs. In addition, genotyping for 13888T/G failed for two controls subjects.

IL1RN mRNA levels

Messenger RNA (mRNA) levels of *IL1RN* were measured as described before.[23] *IL1RN* mRNA levels were expressed relative to the mRNA levels of a control gene (*CDKN1A*). Both were measured by multiplex ligation-dependent probe amplification (MLPA). The used probe set measures all *IL1RN* transcript isoforms.

Statistical analysis

In control subjects, Hardy-Weinberg equilibrium for each htSNP was tested by the χ^2-statistic. Haplotypes were constructed for each individual as described before.[10] When for an individual more than one haplotype combination was possible, haplotypes were only assigned to that individual when the haplotype combination had a probability >95% based on the haplotype frequency estimations of the TagSNPs program[24]; e.g., heterozygotes for haplotypes 2 and 4 (H2H4) and heterozygotes for haplotype 1 and the rare haplotype 10 (H1H10) have the same genotype, but the TagSNPs results indicated that H2H4 is much more likely (probability=99%). Haplotypes could not be assigned to 15 patients and 22 controls because of uncertainty in haplotype assignment (probability<95%). These individuals were excluded from the haplotype analyses.

For each haplotype odds ratios (ORs) and 95% confidence intervals (95% CI) according to Woolf [25] were calculated as a measure of the relative risk of myocardial infarction for carriers of the exposure category (e.g. H4 carriers) compared to the reference category (e.g. non-H4 carriers). Analyses were stratified for age (<50 and

≥50 years), smoking or metabolic risk factors. A metabolic risk factor was defined as having obesity, diabetes, hypertension or hypercholesterolemia.[17]

For the analysis of the association of haplotypes with *IL1RN* mRNA levels, mRNA medians were calculated for each haplotype. A Mann-Whitney test was performed to test for differences in medians between two haplotype groups (e.g. H1H1 and H1Hx, in which Hx is all haplotypes but the one given). A Kruskal-Wallis test was performed to test for differences in medians across haplotype groups (e.g. H1H1, H1Hx and HxHx). For the analysis of the association of haplotypes with CRP levels, CRP levels were logarithmically transformed. For each haplotype, geometric mean CRP and mean fibrinogen levels with 95% CI were calculated.

Table 1
The risk of myocardial infarction in men for the five *IL1RN* SNPs

SNP	Patients (%) n=559	Controls (%) n=646*	OR	95% CI
12602G/A				
GG	407 (72.8)	479 (74.1)	1‡	
GA	145 (25.9)	156 (24.1)	1.1	0.8-1.4
AA	7 (1.3)	11 (1.7)	0.7	0.3-2.0
GA+AA	152 (27.2)	167 (25.9)	1.1	0.8-1.4
13760T/C				
TT	284 (50.8)	370 (57.3)	1‡	
TC	238 (42.6)	238 (36.8)	1.3	1.0-1.7
CC	37 (6.6)	38 (5.9)	1.3	0.8-2.1
TC+CC	275 (49.2)	276 (42.7)	1.3	1.0-1.6
13888T/G				
TT	367 (65.7)	415 (64.4)	1‡	
TG	176 (31.5)	206 (32.0)	1.0	0.8-1.2
GG	16 (2.9)	23 (3.6)	0.8	0.4-1.5
TG+GG	192 (34.3)	229 (35.6)	0.9	0.7-1.2
16857T/C				
TT	254 (45.4)	305 (47.2)	1‡	
TC	253 (45.3)	258 (39.9)	1.2	0.9-1.5
CC	52 (9.3)	83 (12.8)	0.8	0.5-1.1
TC+CC	305 (54.6)	341 (52.8)	1.1	0.9-1.4
19327G/A				
GG	228 (40.8)	235 (36.4)	1‡	
GA	255 (45.6)	315 (48.8)	0.8	0.7-1.1
AA	76 (13.6)	96 (14.9)	0.8	0.6-1.2
GA+AA	331 (59.2)	411 (63.6)	0.8	0.7-1.1

* n=644 for 13888T/G.
‡ Reference category.

Results

Haplotype tagging SNPs

For all htSNPs the distribution of genotypes in control subjects was in Hardy-Weinberg equilibrium. The risk of myocardial infarction was calculated for all five SNPs (Table 1). SNP 13760T/C appeared to be associated with an increase in risk of myocardial infarction for both heterozygous (OR=1.3; 95% CI: 1.0-1.7) and homozygous 13760C carriers (OR=1.3; 95% CI: 0.8-2.1) compared to homozygous 13760T carriers. No effect on myocardial infarction risk was found for the other SNPs.

IL1RN haplotypes

In total five common (frequency>5%) haplotype groups were expected based on our previous findings.[10] TagSNPs analysis showed that, in addition to these five haplotype groups, six rare haplotypes (frequency ranging from 0.04-2%) were predicted based on the genotypic data. The risks of myocardial infarction for the five common haplotype groups of *IL1RN* are listed in Table 2. H3 carriers (tagged by 13760C) had an increase in myocardial infarction risk (OR=1.3; 95% CI: 1.1-1.7) compared to non-H3 carriers. Most of the myocardial infarction patients were of older age. Because it is expected that the contribution of genetic risk factors to the development of myocardial infarction is higher in young patients, we stratified for age. Both in men younger than 50 years (146 patients and 158 controls) and older than 50 years (398 patients and 466 controls) H3 showed an effect on myocardial infarction risk (<50 years: OR=1.2; 95% CI: 0.8-1.9; ≥50 years: OR=1.4; 95% CI: 1.1-1.8) similar to that in the overall population. Smoking is a well known risk factor for myocardial infarction. We observed a slightly higher risk of myocardial infarction for H3 carriers (OR=1.5; 95% CI: 1.1-2.1) among non-smokers (204 patients and 413 controls) than among smokers (356 patients and 233 controls; OR=1.2; 95% CI: 0.9-1.7). When comparing subjects without a metabolic risk factor (360 patients and 455 controls) to those with a metabolic risk factor (200 patients and 191 controls), H3 carriers without a metabolic risk factor had an increased risk of myocardial infarction compared to non-H3 carriers (OR=1.6; 95% CI: 1.2-2.1), whereas the risk was absent in H3 carriers with a metabolic risk factor (OR=1.0; 95% CI: 0.6-1.4). All other haplotypes did not influence myocardial infarction risk, neither in the overall population, nor in the various subgroups.

Markers of inflammation

We studied the association between the five *IL1RN* haplotype groups and the inflammatory biomarkers fibrinogen and CRP. In the control subjects, none of the haplotype groups were associated with fibrinogen or CRP levels (data not shown).

Table 2
The risk of myocardial infarction in men for the five common haplotypes of *IL1RN*

Haplotype (H) [SNPs]*	Patients (%) n=544	Controls (%) n=624	OR	95% CI
H1 [GTTCG]				
HxHx	375 (68.9)	427 (68.4)	1‡	
H1Hx	156 (28.7)	173 (27.7)	1.0	0.8-1.3
H1H1	13 (2.4)	24 (3.8)	0.6	0.3-1.2
H1Hx+H1H1	169 (31.1)	197 (31.6)	1.0	0.8-1.3
H2 [ATTCG]				
HxHx	399 (73.3)	464 (74.4)	1‡	
H2Hx	138 (25.4)	149 (23.9)	1.1	0.8-1.4
H2H2	7 (1.3)	11 (1.8)	0.7	0.3-1.9
H2Hx+H2H2	145 (26.7)	160 (25.6)	1.1	0.8-1.4
H3 [GCTTG]				
HxHx	285 (52.4)	371 (59.5)	1‡	
H3Hx	224 (41.2)	218 (34.9)	1.3	1.1-1.7
H3H3	35 (6.4)	35 (5.6)	1.3	0.8-2.1
H3Hx+H3H3	259 (47.6)	253 (40.5)	1.3	1.1-1.7
H4 [GTTTA]				
HxHx	349 (64.2)	378 (60.6)	1‡	
H4Hx	170 (31.3)	216 (34.6)	0.9	0.7-1.1
H4H4	25 (4.6)	30 (4.8)	0.9	0.5-1.6
H4Hx+H4H4	195 (35.8)	246 (39.4)	0.9	0.7-1.1
H5 [GTGTA]				
HxHx	379 (69.7)	429 (68.8)	1‡	
H5Hx	155 (28.5)	177 (28.4)	1.0	0.8-1.3
H5H5	10 (1.8)	18 (2.9)	0.6	0.3-1.4
H5Hx+H5H5	165 (30.3)	195 (31.2)	1.0	0.7-1.2

* Order of SNPs: 12602G/A, 13760T/C, 13888T/G, 16857T/C and 19327G/A, tagging SNPs underlined.
‡ Reference category.
Hx: all haplotypes but the one given.

IL1RN mRNA levels

As we reported before, normalized *IL1RN* mRNA levels were slightly higher in patients (median=1.9) than in control subjects (median=1.7, p<0.001).[23] *IL1RN* mRNA levels in control subjects are listed in Table 3 for the five common haplotype groups of *IL1RN*. H1 carriers appeared to have a decreased *IL1RN* expression compared to non-H1 carriers, although the effect was not significant (p>0.10). H2 carriers had an increased *IL1RN* expression. *IL1RN* mRNA levels were increased for both heterozygous H2 carriers (H2Hx, median=1.86, p<0.01) and homozygous H2 carriers (H2H2, median=2.42, p<0.05) compared to non-H2 carriers (median=1.63). H3 carriers showed a decrease in *IL1RN* expression across the genotypes (p<0.01). H3Hx carriers

(median=1.63, p<0.05) and especially H3H3 carriers (median=1.37, p<0.01) showed a decrease in *IL1RN* mRNA levels compared to non-H3 carriers (median=1.76). Similar trends in *IL1RN* expression were found for H2 and H3 in patients (data not shown). No effect of H4 and H5 on *IL1RN* expression was found.

Table 3
IL1RN haplotypes and *IL1RN* mRNA levels

Haplotype (H) [SNPs]*	Controls (%) n=606‡	*IL1RN* median mRNA level§	Kruskal-Wallis P-value
H1 [GTTCG]			
HxHx	415 (68.5)	1.72	
H1Hx	170 (28.1)	1.65	
H1H1	21 (3.5)	1.46	>0.10
H2 [ATTCG]			
HxHx	488 (80.5)	1.63	
H2Hx	147 (24.3)	1.86	
H2H2	11 (1.8)	2.42	<0.01
H3 [GCTTG]			
HxHx	362 (59.7)	1.76	
H3Hx	210 (34.7)	1.63	
H3H3	34 (5.6)	1.37	<0.01
H4 [GTTTA]			
HxHx	369 (60.9)	1.64	
H4Hx	208 (34.3)	1.77	
H4H4	29 (4.8)	1.74	>0.10
H5 [GTGTA]			
HxHx	414 (68.3)	1.69	
H5Hx	175 (28.9)	1.71	
H5H5	17 (2.8)	1.70	>0.10

* Order of SNPs: 12602G/A, 13760T/C, 13888T/G, 16857T/C and 19327G/A, tagging SNPs underlined.

‡ Exclusion because of uncertainty in haplotype assignment (n=22) and failed mRNA analysis (n=18).

§ Normalized to *CDKN1A*.

Hx: all haplotypes but the one given.

Discussion

We found that haplotype 3 (H3) of the gene (*IL1RN*) coding for the interleukin-1 receptor antagonist (IL-1Ra), the natural inhibitor of the proinflammatory cytokine IL-1, was associated with an increased risk of myocardial infarction (OR=1.3; 95% CI: 1.1-1.7). This risk was even somewhat stronger in non-smokers (OR=1.5; 95% CI: 1.1-2.1) and those without a metabolic risk factor (OR=1.6; 95% CI: 1.2-2.1). Furthermore, H3 carriers showed a decreased *IL1RN* mRNA expression compared to non-H3 carriers. A decrease in *IL1RN* mRNA levels is expected to result in less IL-1Ra protein to compete with IL-1 to bind to the IL-1R1 receptor. Therefore it is

likely that the inflammatory effect, but also the prothrombotic effect, of IL-1 will increase when the IL-1Ra plasma level is lower than normal. This would subsequently result in an increased risk of myocardial infarction. H5H5 carriership, which was previously found to be a risk factor for venous thrombosis,[10] did not increase the risk of myocardial infarction, nor did it affect *IL1RN* mRNA levels.

In our study 13760T/C was used as tagging SNP for H3. This polymorphism is a synonymous SNP in exon 2 of *IL1RN*. It is not known whether 13760T/C itself is the functional polymorphism. Our finding that 13760T/C is associated with the risk of myocardial infarction, was not confirmed in a recent nested case-control study.[26] Besides 13760T/C, H3 contains about 50 polymorphisms that are unique to this haplotype,[21] which will make it difficult to identify the functional SNP. A promising candidate is the *IL1RN* intron 2 VNTR, which is strongly linked to 13760T/C.[10] It has been suggested that each repeat of the VNTR contains several transcription factor binding sites.[27] Because the rare allele of the VNTR (allele 2) consists of two repeat units, compared to the four repeat units of the common allele 1, one would expect a decreased transcriptional activity for this variant. Such a hypothesis would be supported by our finding that H3 carriers have a decreased *IL1RN* mRNA expression. However, in other studies no association between allele 2 of the intron 2 VNTR and acute myocardial infarction[12,13] and coronary artery disease[14] has been found, whereas this allele is associated with many inflammatory diseases (reviewed in reference 28). Francis *et al*. reported an association of allele 2 of the intron 2 VNTR with single-vessel coronary disease, but not with multivessel coronary disease.[11] Their finding was, however, not confirmed in a subsequent study.[12] This makes the *IL1RN* intron 2 VNTR less likely to be the functional polymorphism.

Our finding that carriers of H3 (harboring the intron 2 VNTR) have decreased *IL1RN* mRNA levels is in line with the results of Tolusso *et al*. who found that in 178 healthy blood donors homozygous allele 2 carriers of the intron 2 VNTR have decreased plasma levels of IL-1Ra compared to non-allele 2 carriers.[29] However, Hurme *et al*. found in 200 healthy blood donors that allele 2 of the intron 2 VNTR was associated with a 1.2-fold increase in IL-1Ra plasma level.[30] This finding was confirmed by Rafiq *et al*., who reported that haplotype C (tagged by rs579543), which is strongly linked to the intron 2 VNTR, is associated with a 1.15-fold higher plasma level of IL-1Ra.[31] Also, in vitro experiments using stimulated leukocytes, indicated higher IL-1Ra production in allele 2 carriers of the intron 2 VNTR.[32,33] Clearly, more information is needed on how variations in *IL1RN* relate to transcriptional activity, IL-1Ra production in various tissues and plasma levels of IL-1Ra. Other studies are needed to confirm our findings.

For H2 carriers we observed an increase in *IL1RN* mRNA expression. If the increased risk of myocardial infarction for H3 carriers is indeed caused by lower *IL1RN* mRNA expression, one would expect a decreased risk of myocardial infarction for H2 carriers. The point estimate indeed indicated a decreased risk only for H2H2 carriers (OR=0.7; 95% CI: 0.3-1.9), but the confidence interval was wide due to low numbers. Additional studies are needed to assess whether H2H2 is associated with a decreased risk of myocardial infarction. Rafiq *et al*. found no clear effect of their haplotype B, specifically tagged by 16857C, which in our study is tagging for H1 and H2 (the latter one in combination with 12602G/A), on IL-1Ra levels.[31] In our study we observed no effect on *IL1RN* expression for carriers of H4 (tagged by 19327A) compared to non-H4 carriers. This is in contrast with the results of Rafiq *et al*., who found that their most frequent haplotype (Haplotype A) was strongly associated with lower IL-1Ra levels.[31] Haplotype A is tagged by rs4251961 (SeattleSNPs: 1018C[21]), which is in strong linkage disequilibrium with 19327A (rs315949).[21,31] It should be noted that these studies were relatively small. In addition, the haplotype definitions of the various studies do often not match completely.

Our haplotype-based approach was limited to the most common haplotype groups of *IL1RN*.[10] Rare haplotypes found by SeattleSNPs were not tagged by their own haplotype specific SNP. Instead, these haplotypes were incorporated into one of the five common haplotypes. Therefore, we cannot exclude a risk associated with one of these rare haplotypes. Larger studies will be needed to investigate the effect of these haplotypes on the risk of myocardial infarction.

In conclusion, H3 carriership increased the risk of myocardial infarction. This effect could be explained by the reduced *IL1RN* expression in H3 carriers, which is expected to result in reduced levels of IL-1Ra. Lower IL-1Ra levels are likely to promote the inflammatory and prothrombotic effect of IL-1, because less IL-1Ra is available to function as antagonist of IL-1.

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Chapter 2.3

Sequence variants and haplotypes of the factor IX gene and the risk of venous thrombosis

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Summary
Background: Elevated plasma levels of factor IX (FIX) increase the risk of venous thrombosis. The molecular basis of these elevated FIX levels is unknown.
Objectives: We investigated whether variants of the FIX gene (*F9*) could explain elevated FIX levels and thrombosis risk.
Patients/Methods: Large parts of *F9* were sequenced in male subjects with an isolated elevated FIX level, selected from the Leiden Thrombophilia Study (LETS), a large case control study on the causes of deep venous thrombosis (DVT). In addition, all subjects from LETS were genotyped for six single nucleotide polymorphisms (SNPs), together tagging the eight most common haplotype groups of *F9*. The association of *F9* haplotypes with DVT risk and FIX levels was investigated.
Results: Sequencing of male subjects with an isolated elevated FIX level revealed two rare variations in *F9*; -816G/A (minor allele frequency (MAF)=1.7%) and 32781G/A (MAF=0.4%). In the whole LETS population, both SNPs were not associated with FIX levels or DVT. A two-fold decreased DVT risk was found for men carrying haplotype 6 (OR=0.5, 95% CI: 0.3-0.9). No association was found between FIX levels and the *F9* SNPs in men and women, and between FIX levels and *F9* haplotypes in men.
Conclusions: Variations in *F9* may affect the risk of DVT. However, these variations do not explain FIX levels.

Introduction
Venous thrombosis is a multicausal disease that is caused by the interaction of both genetic and acquired risk factors.[1,2] Acquired risk factors include immobilization, surgery, malignancy and the use of female hormones.[3] Genetic factors include the factor V Leiden mutation,[4] the prothrombin 20210A mutation,[5] deficiencies of protein C,[6] protein S,[7] and antithrombin[8] and ABO blood group non-O.[9,10] Further, it has been reported that elevated levels of several hemostasis-related proteins (e.g. fibrinogen, factors VIII, IX and XI, and homocysteine) increase the risk of venous thrombosis.[11-15] Genetic effects account for a large proportion of the variation in these phenotypes.[16-18] However, little information is available on the genetic variants that contribute to the interindividual variation of these phenotypes.

Factor IX (FIX) is a vitamin K dependent glycoprotein that plays a key role in hemostasis, as shown by the bleeding tendency of patients with FIX deficiency (hemophilia B).[19] After activation by the tissue factor:factor VIIa complex[20] or by factor XIa,[21] FIX activates factor X, which eventually leads to thrombin and clot formation.[22,23] FIX plasma levels above the 90th percentile of the distribution as measured in healthy subjects (>129 U/dl), increase the risk of deep venous thrombosis 2 to 3-fold.[13] Adjustment for variables known to affect FIX levels such as age,[24,25] sex and oral contraceptive use,[25] and for genetic factors and plasma phenotypes

that influence venous thrombosis risk, only marginally affected the risk estimates for high FIX levels.[13] The molecular basis of these elevated FIX levels is unknown. Although FIX levels have an estimated heritability of 20% to 39%,[16,26,27] no major genetic determinants of FIX levels were found in a genome wide scan searching for genetic determinants of FIX levels.[28]

In the present study, we used a dual approach to identify variants of the factor IX gene that are associated with elevated factor IX levels and thrombosis risk. First, we sequenced large parts of the FIX gene (*F9*) in men with an isolated elevated plasma FIX level. When a novel sequence variation was found, we subsequently investigated the association of this variant with FIX levels and thrombotic risk in a large case-control study on the causes of deep venous thrombosis, the Leiden Thrombophilia Study (LETS). Secondly, we used a haplotype-based approach to investigate the effects of all common variants in *F9*. Smith *et al*. previously used this approach in postmenopausal women. They found that their haplotype 4 (tagged by 12806T/A, rs4149755) was associated with venous thrombosis.[29] In the present study, we genotyped six single nucleotide polymorphisms (SNPs) in *F9*, which together tag the eight most common haplotype groups of *F9*, in all patients and control subjects of the LETS.

Methods

Study population

The design of the Leiden Thrombophilia Study has previously been described in detail.[30] We included 474 consecutively diagnosed patients with an objectively confirmed first episode of deep vein thrombosis and 474 healthy subjects, frequency matched for sex and age. Individuals with active cancer were excluded. All patients and controls were of Caucasian descent. The mean age for both groups was 45 years (range 15-69 for patients, 15-72 for controls). Both groups consisted of 272 (57.4%) women and 202 (42.6%) men. Venous blood was collected into 0.1 volume of 0.106 mol/L trisodium citrate. High molecular weight DNA was isolated from leukocytes by standard methods. DNA and plasma samples were available from 471 patients (269 women, 202 men) and 472 (271 women, 201 men) controls.

Factor IX measurement

FIX antigen levels were measured previously by an enzyme-linked immunosorbent assay (ELISA).[13] Levels were expressed in units (U) per deciliter (dL), where 100 U correspond to the FIX antigen in one deciliter of pooled normal plasma. Forty-nine individuals (48 patients and 1 control) were on long term coumarin treatment. These were excluded from analyses in which FIX levels were involved.

Sequencing

Forty-eight men (29 patients and 19 controls) had a plasma FIX level above 129 U/dl. From these 48 men, we selected 19 individuals (9 patients and 10 controls) with an isolated elevated FIX antigen level, i.e. FIX/prothrombin-, FIX/factor X-, FIX/protein C- and FIX/antithrombin- ratio all higher than 1.2. Prothrombin activity,[5] factor X antigen,[31] protein C activity[32] and antithrombin activity[33] were measured as previously described. Coding regions, splice junctions, the 5'- and 3'-untranslated regions (UTRs; nucleotide (nt) -29 to -1 and 31370 to 32758 (numbering according to Yoshitake *et al*.[34])) and the 5'- and 3'-flanking regions (nt -899 to -30 and nt 32759 to 33501) of *F9* were sequenced using the CEQ Dye Terminal Cycle Kit (Beckman Coulter, Fullerton, CA, USA) and the CEQ 2000 DNA Sequence Analyser System. Primer sequences and polymerase chain reaction (PCR) conditions are available upon request. To obtain a first estimate of the frequencies of observed sequence variations in the general population, we used DNA samples of 64 healthy volunteers recruited from hospital personnel.

Haplotype-based approach

F9 was re-sequenced by SeattleSNPs in 23 subjects of European-American descent, which resulted in the identification of 32 SNPs.[35] Haplotypes were constructed using the unphased SNP data from the 46 chromosomes and the software program PHASE 2.[36] We identified the most common haplotype groups of *F9* (frequency>1%) and the six SNPs (-793G/A (rs411017), 2627T/C (rs371000), 9410C/T (rs392959), 20422A/G (rs6048), 32056G/A (rs440051) and 33566C/G (rs434144)) needed to tag these eight haplotype groups.

Genotyping

The -816G/A, -793G/A, 2627T/C, 32056G/A and 32781G/A SNPs were genotyped by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis. The other polymorphisms (9410C/T, 20422A/G and 33566C/G) were genotyped using a 5'-nuclease/TaqMan assay.[37] PCRs with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA, USA) were performed in 96 wells plates (Greiner Bio-One, the Netherlands) on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany) and fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA, USA). Genotyping failed for one male patient for -793G/A, one male patient for 2627T/C, two control subjects (one woman, one man) and one female patient for 9410C/T, five control subjects (three woman, two men) and five patients (one woman, four men) for 20422A/G, and three control subjects (one woman, two men) and four patients (one women, three men) for 33566C/G.

Statistical analysis

In healthy control subjects, Hardy-Weinberg equilibrium for each SNP was tested by χ^2-statistic. For men, haplotypes were assigned manually. Since men are hemizygous for the X-chromosome, there was no uncertainty in assigning haplotypes. For women, the software program Haplo.stats[38] was used to investigate the effect of *F9* haplotypes on the risk of venous thrombosis. Haplotypes frequencies were estimated by Haplo.stats, without assigning haplotypes to individuals. Analyses were performed using an additive or dominant model.

To investigate whether SNPs or haplotypes were associated with venous thrombosis, odds ratios (ORs) and 95% confidence intervals (95% CI) according to Woolf[39] were calculated as measure of the relative risk of thrombosis for carriers of the exposure category (e.g. haplotype 4 carriers) compared to the reference category containing all carriers of haplotype 1, which is the most frequent haplotype. For women, allelic ORs were calculated by Haplo.stats.

To analyze the association of SNPs and haplotypes with FIX levels, means (U/dl) with 95% CI were calculated. The association between haplotypes and FIX levels was investigated in men, because individual haplotypes could easily be assessed.

Results

Sequencing

We selected 19 men with an isolated elevated FIX level, in whom the exons, the exon-intron boundaries, the 5′- and 3′-UTRs and the 5′- and 3′-flanking regions of *F9* were sequenced. These regions were expected to contain most of the sequence variations that might contribute to interindividual variation in plasma concentration and/or activity of FIX. Sequencing revealed two rare sequence variants in *F9*: a G to A change at nucleotide position -816 in the 5′-flanking region was found in one control subject and an A to G change at nucleotide position 32781 was identified in the 3′-flanking region in one patient. Both variants were not listed in FIX online databases.[35,40] Apart from these two variants, eight known polymorphisms were identified: -793G/A (rs411017) and -698C/T (rs378815), both located in the 5′-flanking region and completely linked to each other with a minor allele frequency (MAF) of 42%; 20002C/A (rs422187) at the 3′-end of intron 5 and 20422A/G (rs6048, Ala148Thr) in exon 6, both completely linked to each other with a MAF of 26%; 29532C/T (rs370713) and 29648G/A (rs413536) at the 3′-end of intron 6, both completely linked to each other with a MAF of 26%; and 32056G/A (rs440051) and 32207-8 Ins/Del GT in the middle part of the 3′-UTR, again completely linked to each other in the 19 selected men with a MAF of 26%. These findings were in line with data from SeattleSNPs[35]and HapMap.[41]

-816G/A and 32781A/G variants, FIX levels, and thrombotic risk

The eight known polymorphisms found in our sequences were similarly distributed in the 19 men with an isolated elevated FIX level and the 64 healthy subjects, recruited from hospital personnel. None of the 64 healthy subjects carried the -816A allele or the 32781G allele. To study the effect of the -816A and the 32781G allele on FIX levels and the risk of venous thrombosis, we extended our genetic analysis to all individuals of the LETS. Among controls, the frequencies of -816A and 32781G were 1.7% and 0.4%, respectively. Homozygous (female) carriers were not found. There were no individuals carrying both the -816A and the 32781G allele. Table 1 summarizes the relationship between the -816G/A and 32781A/G variations and FIX levels and the risk of venous thrombosis. Because the allele frequency of both variations is low, we decided to analyze men who were hemizygous for the rare allele (-816A and 32781G) together with women who were heterozygous for the rare allele (-816GA and 32781AG). No effect on FIX antigen levels and the risk of venous thrombosis was found for -816GA+A and 32781AG+G carriers (see Table 1).

Table 1
-816G/A and 32781A/G genotypes, FIX levels, and the risk of DVT

SNP	Patients (%) n=471	Controls (%) n=472	OR	95% CI	Mean FIX (U/dl)‡	95% CI
-816G/A						
GG + G	457 (97.0)	459 (97.2)	1*		103	101-105
GA + A	14 (3.0)	13 (2.8)	1.1	0.5-2.3	116	97-136
32781A/G						
AA + A	466 (98.9)	469 (99.4)	1*		103	101-105
AG + G	5 (1.1)	3 (0.6)	1.7	0.4-7.1	87	69-104

* Reference category.

‡ Based on FIX levels of 471 control subjects.

Haplotype tagging SNPs

From the data of SeattleSNPs, we selected six SNPs, which together tag the eight most common haplotype groups of *F9* (Table 2). For all SNPs the distribution of genotypes among control women was in Hardy-Weinberg equilibrium. The effect on the risk of venous thrombosis was calculated for all six SNPs (supplemental Tables I and II). For men, a decreased risk of venous thrombosis was found for allele A carriers of 5'-flanking SNP -793G/A (OR=0.7, 95% CI: 0.4-1.0), allele G carriers of exon 6 SNP 20422A/G (OR=0.6, 95% CI: 0.4-0.9), allele A carriers of 3'-UTR SNP 32056G/A (OR=0.6, 95% CI: 0.4-1.0) and allele G carriers of 3'-flanking SNP 33566C/G (OR=0.5, 95% CI: 0.3-0.8). For women, a decreased risk of venous thrombosis was found for allele C carriers (TC+CC) of intron 1 SNP 2627T/C (OR=0.7, 95% CI: 0.5-1.0). No effect on the risk of venous thrombosis was found for the other SNPs.

Table 2

Association of *F9* haplotypes with DVT risk and FIX levels

H*	Haplotype tagging SNPs						Men				Women		
							Frequency				Frequency		
	-793	2627	9410	20422	32056	33566	Patients n=197	Controls n=198	OR (95% CI)	FIX levels‡ (95% CI)	Patients n=268	Controls n=268	OR (95% CI)
H1	G	C	C	A	G	C	0.33	0.25	1§	103 (98-108)	0.28	0.28	1§
H2	G	T	C	A	G	C	0.27	0.24	0.9 (0.5-1.5)	102 (98-107)	0.30	0.23	1.2 (0.8-1.7)
H3	A	T	T	A	G	C	0.07	0.05	1.1 (0.4-2.8)	110 (98-121)	0.03	0.04	0.8 (0.4-1.5)
H4	G	C	C	G	G	C	0.06	0.08	0.6 (0.3-1.4)	99 (89-110)	0.08	0.08	0.9 (0.5-1.5)
H5	A	C	C	A	G	C	0.06	0.08	0.6 (0.3-1.4)	99 (92-106)	0.05	0.08	0.6 (0.3-1.1)
H6	A	T	C	G	A	G	0.12	0.19	0.5 (0.3-0.9)	110 (103-118)	0.17	0.17	1.0 (0.7-1.4)
H7	A	T	C	G	G	C	0.03	0.03	0.6 (0.2-2.2)	127 (90-163)	0.03	0.03	0.8 (0.4-1.7)
H8	G	C	T	A	G	C	0.02	0.02	0.6 (0.1-2.6)	126 (81-172)	0.01	0.02	0.5 (0.2-1.6)

* H=haplotype; exclusion because of incomplete genotype: eight men (three control subjects, five patients) and four women (three control subjects, one patient). Carriers of rare haplotypes (men: n=24, women: n=19) are not indicated in the table. Shaded cells indicate minor alleles.

‡ Mean FIX levels (U/dl) in controls. One control on long term coumarin treatment was excluded.

§ Reference category.

F9 haplotypes

In total eight common (frequency>1%) haplotype groups (Table 2) were expected on basis of SeattleSNPs data. In addition to these eight haplotype groups, we found eleven rare haplotypes (frequency ranging from 0.3-0.8%) in men. In women nineteen rare haplotypes (frequency ranging from 0.1-0.9%) were predicted based on the genotypic data. All male carriers (n=24) of rare haplotypes were excluded from the haplotype analyses, whereas for the haplotype analysis in women the program Haplo.stats combined all rare haplotypes in one haplotype group.

Table 2 shows the frequency distribution in patients and controls for the eight common *F9* haplotypes and the thrombotic risk associated with these haplotypes. For men, a decreased risk of venous thrombosis was found for carriers of haplotypes 4 to 8. This effect was especially pronounced for haplotype 6, which was associated with a 2-fold decreased thrombotic risk (OR=0.5, 95% CI: 0.3-0.9). Interestingly, all men carrying the -816A (n=5) or 32781G (n=2) allele were carriers of the same haplotype 6. For women, the results for an additive model are shown. The results for a dominant model (data not shown) were similar to that of the additive model. Several haplotypes showed a slightly decreased risk of venous thrombosis, although these effects were not significant (see Table 2). No effect on the risk of venous thrombosis was found for the other haplotypes of *F9*.

F9 SNPs, haplotypes and FIX levels

The association between the six tagging SNPs and the eight common haplotypes and FIX antigen levels was assessed in male control subjects. In women, only the association between the SNPs and FIX antigen levels was assessed. None of the SNPs had an effect on FIX levels in men (supplemental Table III) and women (supplemental Table IV). The relationship between FIX antigen levels and *F9* haplotypes in men is shown in Table 2. Haplotype 6 carriers have a slightly higher FIX level (mean=110 U/dl, 95% CI: 103-118) compared to haplotype 1 (mean=103 U/dl, 95% CI: 98-108). However, this effect is not significant (p=0.06). No effect on FIX levels was observed for the other *F9* haplotypes.

Discussion

High FIX levels (>129 U/dl, above the 90th percentile as measured in healthy control subjects) increase the risk of venous thrombosis 2 to 3-fold compared to individuals having FIX levels below 129 U/dl.[13] At present, the genetic determinants of the interindividual variation in FIX levels are still unknown. We have used a dual approach to identify variants in *F9* which are associated with elevated FIX levels or an increased thrombotic risk.

Sequencing of *F9* in individuals with an isolated elevated FIX level revealed, apart from 8 known polymorphisms, two rare variants, -816G/A (MAF=1.7%) and 32781A/G (MAF=0.4%). Both variants were recently reported by Khachidze *et al*. in subjects of the GAIT study.[28] The prevalences of -816G/A and 32781A/G in the GAIT (MAFs 1.4% and 0.7%, respectively) are similar to those in our study. Neither the -816A nor the 32781G allele was associated with FIX antigen levels or with the risk of venous thrombosis. In the GAIT study no association was found between these two variants and FIX activity. Since the prevalences of the SNPs are low, much larger studies are needed to draw definite conclusions.

The rare -816G/A variant is located in the upstream region of *F9*. The variation is located close to a critical age-regulatory element (AE5', nt -770 to -802) which is required for age-stable expression of *F9*.[42] The 32781A/G variation is located in the 3'-flanking region of *F9* in a region originally described to contain a consensus sequence (CATTG, nt 32780 to 32784) that may be involved in cleavage and polyadenylation of pre-mRNA.

Adams *et al*. previously reported that female carriers of -698C, which is completely linked to -793G, had higher FIX levels than -698T carriers.[43] They suggested that this could be explained by the presence of a polymorphic oestrogen response element (ORE) in the *F9* promoter. The -698C allele shows a closer homology to ORE than -698T, binding oestrogen receptor alpha more strongly. Since -698C is completely linked to -793G, we could study the effect of -698C/T by investigating -793G/A. In our study no effect of -793G/A on FIX levels (see supplemental Table IV) and thrombotic risk (see supplemental Table II) was found in women.

We identified several SNPs that seem to decrease the risk of venous thrombosis (see supplemental Tables I and II). The only coding SNP among these SNPs is 20422A/G that causes a Thr148Ala change in exon 6 of *F9* (also known as FIX Malmö[44]). This polymorphism is located in the activation peptide of FIX. We found a decreased risk of venous thrombosis for male and female carriers of the G allele. The same SNP was identified in the LETS population as associated with venous thrombosis as part of a large discovery study including 18.000 SNPs predicted to affect gene function.[45]

We found a slightly decreased risk of venous thrombosis for several haplotypes of *F9* for both men and women, haplotype 6 being the most promising one (OR=0.5, 95% CI: 0.3-0.9 in men). The functional SNP causing this two-fold decreased thrombotic risk still has to be identified. Haplotype 6 is not tagged by a single SNP, but by a combination of SNPs (see Table 2). These SNPs also individually decrease the risk of venous thrombosis (see supplemental Table II). An obvious candidate for being

the functional SNP would be SNP 20422G/A. This SNP is also present in haplotypes 4 and 7, both showing a similar effect on venous thrombosis risk as haplotype 6. SNPs 32056G/A and 33566C/G are both unique for haplotype 6. They are located in the 3'-UTR of *F9*. The regions around both SNPs, however, do not contain any obvious regulatory elements, which would predict that these SNPs are functional variants. It is also possible that the actual functional SNP is a SNP in LD with one of the tagging SNPs of haplotype 6. SNP 33685C/G (rs434447) is a polymorphism in LD with 32056G/A and 33566C/G and is also unique for haplotype 6. Like 32056G/A and 33566C/G, 33685C/G is located in the 3'-UTR of *F9*. Besides SNP 33685C/G, SeattleSNPs and HapMap data report about 10 other SNPs in LD with 32056G/A and 33566C/G, all not unique for haplotype 6. According to SeattleSNPs data, there is one prevalent (frequency=40%) subhaplotype present in haplotype 6, which was not identified by HapMap. It is possible that the effect of haplotype 6 is caused by this subhaplotype.

Recently, Smith *et al*. investigated the effect of *F9* haplotypes on venous thrombosis risk in postmenopausal women.[29] Their haplotype 2 (our haplotype 6) was not associated with venous thrombosis risk in postmenopausal women. They reported that their haplotype 4 (tagged by 12806A/T (rs4149755)), was associated with a slightly increased risk of venous thrombosis (OR=1.4, 95% CI: 1.0-2.1). This haplotype is a subhaplotype (frequency=30%) of our haplotype 1, which we used as the reference haplotype in the analyses. To compare our results with the data of Smith *et al*., we calculated the risk of haplotype 1, compared to the group of all other haplotypes. An increased risk for haplotype 1 was found in men (OR=1.5, 95% CI: 1.0-2.3), which is similar to the risk of haplotype 4 in postmenopausal women in the study of Smith *et al*.[29] However, no effect on the risk of venous thrombosis was found in women. Furthermore, none of the haplotypes had an effect on the risk of venous thrombosis in the subgroup of postmenopausal LETS women (n=170). Whether the increased risk for haplotype 1 in men is a recessive effect, not seen in women because of the low number of homozygous haplotype 1 carriers, remains to be investigated in larger studies.

Neither the haplotypes nor the SNPs were associated with FIX antigen levels in men. In women, only the effect of SNPs on FIX levels was studied. In the genome-wide scan for genetic determinants of FIX levels in men and women of the GAIT study, no major determinants were found in or outside the FIX gene. It is possible that a set of genetic determinants with small or modest effects, not located at the *F9* locus, together contribute to high FIX levels.

Because of the complex haplotype structure of *F9*, extensive genotyping of SNPs is required to tag all the haplotypes of *F9*. We limited our haplotype-based approach to the most common haplotype groups of *F9*. Rare haplotypes found by SeattleSNPs were not tagged by their own haplotype specific SNP in our study, but instead these haplotypes were incorporated into one of the eight common haplotype groups. Therefore, we cannot exclude a risk associated with one of these rare haplotypes. Larger studies will be needed to investigate the effect of these haplotypes on the risk of venous thrombosis.

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Supplemental Tables

Table I
Thrombosis risk in men for the six tagging SNPs in *F9*

SNP	Patients* (%)	Controls* (%)	OR	95% CI
-793G/A				
G	143 (71.1)	125 (62.2)	1‡	
A	58 (28.9)	76 (37.8)	0.7	0.4-1.0
2627T/C				
T	101 (50.2)	109 (54.2)	1‡	
C	100 (49.8)	92 (45.8)	1.2	0.8-1.7
9410C/T				
C	185 (91.6)	183 (91.5)	1‡	
T	17 (8.4)	17 (8.5)	1.0	0.5-2.0
20422A/G				
A	155 (78.3)	136 (68.3)	1‡	
G	43 (21.7)	63 (31.7)	0.6	0.4-0.9
32056G/A				
G	171 (84.7)	153 (76.1)	1‡	
A	31 (15.3)	48 (23.9)	0.6	0.4-1.0
33566C/G				
C	172 (86.4)	148 (74.4)	1‡	
G	27 (13.6)	51 (25.6)	0.5	0.3-0.8

* -793G/A and 2627T/C (201 patients, 201 controls), 9410C/T (202 patients, 200 controls), 20422A/G (198 patients, 199 controls), 32056G/A (202 patients, 201 controls) and 33566C/G (199 patients, 199 controls).
Numbering according to Yoshitake *et al*.[34]
‡ Reference category.

Table II

Thrombosis risk in women for the six tagging SNPs in *F9*

SNP	Patients* (%)	Controls* (%)	OR	95% CI
-793G/A				
GG	126 (46.8)	115 (42.4)	1‡	
GA	120 (44.6)	122 (45.0)	0.9	0.6-1.3
AA	23 (8.6)	34 (12.5)	0.6	0.3-1.1
GA+AA	143 (53.2)	156 (57.6)	0.8	0.6-1.2
2627T/C				
TT	85 (31.6)	65 (24.0)	1‡	
TC	131 (48.7)	150 (55.4)	0.7	0.4-1.0
CC	53 (19.7)	56 (20.7)	0.7	0.4-1.2
TC+CC	184 (68.4)	206 (76.0)	0.7	0.5-1.0
9410C/T				
CC	239 (89.2)	234 (86.7)	1‡	
CT	29 (10.8)	34 (12.6)	0.8	0.5-1.4
TT	0	2 (0.7)	-	-
CT+TT	29 (10.8)	36 (13.3)	0.8	0.5-1.3
20422A/G				
AA	136 (50.7)	121 (45.1)	1‡	
AG	103 (38.4)	123 (45.9)	0.7	0.5-1.1
GG	29 (10.8)	24 (9.0)	1.1	0.6-2.0
AG+GG	132 (49.3)	147 (54.9)	0.8	0.6-1.1
32056G/A				
GG	178 (66.2)	174 (64.2)	1‡	
GA	78 (29.0)	85 (31.4)	0.9	0.6-1.3
AA	13 (4.8)	12 (4.4)	1.1	0.5-2.4
GA+AA	91 (33.8)	97 (35.8)	0.9	0.6-1.3
33566C/G				
CC	175 (65.3)	171 (63.3)	1‡	
CG	77 (28.7)	86 (31.9)	0.9	0.6-1.3
GG	16 (6.0)	13 (4.8)	1.2	0.6-2.6
CG+GG	93 (34.7)	99 (36.7)	0.9	0.6-1.3

* -793G/A, 2627T/C and 32056G/A (269 patients, 271 controls), 9410C/T and 33566G/A (268 patients, 270 controls) and 20422A/G (268 patients, 268 controls).

Numbering according to Yoshitake *et al*.[34]

‡ Reference category.

Table III

FIX levels in men controls for the six tagging SNPs in *F9*

SNP	n*	Mean FIX (U/dl)	95% CI
-793G/A			
G	124	103	100-106
A	76	108	103-113
2627T/C			
T	109	107	103-111
C	91	103	99-106
9410C/T			
C	183	104	102-107
T	16	113	102-124
20422A/G			
A	135	103	100-106
G	63	108	102-115
32056G/A			
G	153	103	100-106
A	47	110	103-116
33566C/G			
C	148	104	101-107
G	50	109	103-115

* Exclusion because of coumarin treatment (n=1). -793G/A, 2627T/C and 32056G/A: n=200, 9410C/T: n=199, 20422A/G and 33566C/G: n=198.

Numbering according to Yoshitake *et al*.[34]

Table IV
FIX levels in women controls for the six tagging SNPs in *F9*

SNP	n*	Mean FIX (U/dl)	95% CI
-793G/A			
GG	115	101	96-105
GA	122	102	98-106
AA	34	103	95-111
2627T/C			
TT	65	105	99-110
TC	150	100	97-104
CC	56	101	95-108
9410C/T			
CC	234	102	99-105
CT	34	98	91-105
TT	2	92;156	-
20422A/G			
AA	121	100	96-104
AG	123	103	99-107
GG	24	103	93-112
32056G/A			
GG	174	100	97-103
GA	85	103	98-108
AA	12	115	97-132
33566C/G			
CC	171	100	97-103
CG	86	103	98-108
GG	13	112	95-129

* -793G/A, 2627T/C and 32056G/A: n=271, 9410C/T and 33566G/A: n=270, 20422A/G: n=268.
Numbering according to Yoshitake *et al.*[34]

Chapter 2.4

The *Marburg I* polymorphism of factor seven-activating protease is not associated with venous thrombosis

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Hans L. Vos, Rogier M. Bertina and Frits R. Rosendaal

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In a recently published paper in *Blood*,[1] Hoppe *et al*. showed in 213 patients and 213 controls that carriers of the *Marburg I* polymorphism of the Factor Seven-Activating Protease (FSAP)[2] had an increased risk of venous thrombosis (Odds ratio (OR): 3.5, 95% confidence interval (CI): 1.2-10.0).

FSAP is a serine protease, which has two functions in hemostasis. It activates factor VII, thereby promoting coagulation,[3] but it can also activate single chain plasminogen activators, thus promoting fibrinolysis.[4] Recently, a single nucleotide polymorphism (1601 G/A) was discovered in the gene coding for FSAP, which results in the substitution of glycin 511 by glutamic acid (FSAP *Marburg I*) and which is present in 2-9% of the Caucasian population.[1,5,6] The *Marburg I* variant has an impaired pro-urokinase activating potency, whereas it can still activate factor VII normally.[7]

If true, the finding of Hoppe *et al*. would support the hypothesis that reduced fibrinolysis contributes to the risk of venous thrombosis. However, the authors indicated that their control group might be biased due to the exclusive inclusion of healthy blood donors, and that larger studies were needed to validate their results. Therefore we determined the *Marburg I* polymorphism in 471 consecutive patients with a first episode of deep venous thrombosis (DVT) and 471 sex- and age-matched healthy controls of the Leiden Thrombophilia Study (LETS), a case-control study on the causes of venous thrombosis.[8]

We determined the *Marburg I* 1601 G/A polymorphism with a 5′ nuclease/TaqMan assay (Assay-by-Design, Applied Biosystems, Foster City, CA, USA).[7,9] Nucleotide sequences of primers and probes are available on request. An odds ratio with 95% CI was calculated as a measure of the relative risk of thrombosis for carriers of the *Marburg I* allele (homozygous or heterozygous) compared to homozygous wildtype allele carriers. Factor VII activity was measured previously using Thromborel S® reagent and factor VII deficient plasma.[10]

In Table 1, the risk of venous thrombosis is shown for the *Marburg I* polymorphism. *Marburg I* was found in 30 controls (allele frequency=0.034) and 27 cases (allele frequency=0.030). No association between *Marburg I* and venous thrombosis was found. Similar results were obtained when the analysis was stratified by sex or age (<45 and ≥45 years). Factor VII activity was not influenced by the presence of *Marburg I*.

Our results indicate, in contrast with the finding of Hoppe *et al*., that the *Marburg I* allele of FSAP is not a risk factor for venous thrombosis. This difference may be explained by the frequency of the *Marburg I* allele in the control population studied

by Hoppe *et al*., which was considerably lower (0.012) than that reported in other studies, including ours (0.023-0.043).[5,6] In thrombosis patients the frequency of the *Marburg I* allele was similar in the study of Hoppe *et al*. (0.039) and ours (0.030). So, the low prevalence in the control group (5/426) seems the explanation for the findings by Hoppe *et al*., either because these were blood donors, in which case the mutant allele would be infrequent in a group selected on health, or because the control group was relatively small. On the other hand we cannot exclude small geographical differences in the *Marburg I* frequency.

Table 1
Marburg I polymorphism and the risk of venous thrombosis

FSAP nt 1601	Patients (%) n=471	Controls (%) n=471	OR	95% CI
GG	444 (94.3)	441 (93.6)	1*	
GA	26 (5.5)	28 (6.0)	0.9	0.5 – 1.6
AA	1 (0.2)	2 (0.4)	0.5	0.05 – 5.5
GA + AA	27 (5.7)	30 (6.4)	0.9	0.5 – 1.5

*Reference category.

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Chapter 3

The Genetics In Familial Thrombosis (GIFT) Study

Chapter 3.1

Genetics In Familial Thrombosis study: sample collection and description of study population

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Hans L. Vos and Rogier M. Bertina

Summary
Venous thromboembolism is a multicausal disorder with an annual incidence of one to three per thousand individuals. Both acquired and genetic risk factors are involved in the development of the disease. The reported heritability is about 50-60% and at present few genetic risk factors are known. However, in most thrombophilic families these currently known genetic risk factors cannot explain the familial clustering of thromboses, suggesting that there must be genetic determinants of venous thromboembolism that have not yet been identified. In order to search for these novel genetic risk factors we recruited a panel of affected sibling pairs with venous thromboembolism at a young age (Genetics In Familial Thrombosis study, GIFT). Here we present the recruitment of the study population and we draw up an inventory of the classical genetic and acquired risk factors in this selected panel of brothers and sisters with venous thromboembolism. In total 211 families were included consisting of 213 sibships with two, three or four affected siblings with at least one objectively confirmed venous thromboembolic event. A high prevalence of the common genetic risk factors factor V Leiden (36.5%) and ABO blood group non-O (82.9%) was found. Together with the high percentage of recurrences (45.9%) and the observation that nearly half of the sibships had at least one parent who also had developed a venous thromboembolic event, these data suggest that genetics play an important role in the development of venous thromboembolism in these families. Since in more than 90% of this panel of small thrombophilia families none or only one of the classical genetic risk factors is found this panel seems very suitable for the discovery of novel genetic risk factors for venous thrombosis.

Introduction
Venous thromboembolism is a common disorder with an annual incidence of one to three per thousand individuals.[1-3] Overall it occurs more often in men than in women, but the incidence rate is somewhat higher in young women because of risk factors associated with reproduction.[4] The most frequent clinical manifestations are superficial and deep vein thrombosis (DVT) of the leg and pulmonary embolism (PE). Rarely, thrombus formation occurs at other locations (upper extremities, liver, cerebral sinus, retina, mesenteric). Major outcomes of venous thromboembolism are death, recurrence, post-thrombotic syndrome, and major bleeding due to anticoagulant treatment. The disease may also impair quality of life, particularly after development of the post-thrombotic syndrome.[5,6] An extensive list of genetic and acquired risk factors exists.[7] This illustrates that venous thromboembolism should be considered as a multicausal disease,[8] in which one risk factor is seldom sufficient to cause the disease and in which both genetic and environmental risk factors are involved and interact.

Acquired risk factors include advanced age, immobilisation, surgery, trauma, pregnancy, puerperium, lupus anticoagulants, malignancy, and use of female hormones.[7] The observation that about 20 to 30% of consecutive patients with thrombosis report at least one first-degree relative with venous thromboembolism,[9,10] suggests that genes might play an important role in the development of the disease. Family- and twin-based studies indeed showed that venous thromboembolism is highly heritable (heritability 50-60%).[11-13] Many families exist with a clear tendency to develop venous thromboembolism. This so-called familial thrombophilia is considered to be an oligogenetic disease, where at least two genetic defects segregate in the family.[14-16] The main genetic risk factors known at present are mutations in the genes coding for the natural anticoagulants antithrombin,[17] protein C[18] and protein S,[19,20] that all lead to "loss of function", and the "gain of function" variants factor V Leiden[21,22] and prothrombin 20210A.[23] Furthermore, ABO blood group non-O is a very common and well established genetic risk factor for venous thromboembolism.[24-26] Screening for these classical genetic risk factors (however not taking into account ABO blood group) in the index patients of thrombophilia families showed the presence of at least two genetic risk factors in 13% of the families, whereas in 60% of the families only one of these genetic risk factors was found and in 27% none of the known risk factors was found.[27] Because of the belief that these families carry multiple genetic defects, these data suggest that genetic risk factors are missing in these families.

The idea that genetic risk factors are missing is further supported by the observation that plasma levels of many hemostasis-related proteins both correlate with thrombosis (e.g. elevated levels of factor VIII, factor IX and factor XI all increase thrombosis risk),[28-30] and show a high degree of heritability.[11,31-33] However, at present no variations in the genes coding for these proteins have been identified that influence their levels. Furthermore, a large genetic component was found for a number of coagulation activation markers (e.g. prothrombin fragment 1+2, D-dimer, thrombin-antithrombin complex).[34]

Based upon these observations we hypothesize that there must be genetic determinants of venous thromboembolism that have not yet been identified. Most of the genes known to be involved in coagulation have been investigated extensively.[35,36] Therefore we think that there must be other genes that play a direct or indirect role in hemostasis and that contribute to the susceptibility to venous thromboembolism. Knowledge of these genes, especially in combination with a better insight in the interaction between genetic and environmental factors, may improve individualized risk profiling for venous thromboembolism.

In order to search for these missing genetic risk factors we recruited a panel of Dutch affected sibling pairs with venous thromboembolism at a young age (Genetics In Familial Thrombosis study, GIFT). By performing a genome-wide linkage scan we aim at finding novel thrombosis susceptibility genes in the future. In the present study we draw up an inventory of the classical genetic and acquired risk factors in this selected panel of brothers and sisters with venous thromboembolism.

Subjects and Methods

Recruitment of study population and inclusion

A flowchart of the recruitment and inclusion of the GIFT study population is shown in Figure 1. The recruitment of the study population was performed in collaboration with 29 Anticoagulation Clinics spread throughout the Netherlands. In our country these clinics monitor coumarin treatment for patients within a well defined geographical area. Virtually all patients with a diagnosis of venous thromboembolism are treated with oral anticoagulants, which is always controlled by an area-specific Anticoagulation Clinic. All young patients (≤45 years at the time of the thrombotic event) who were referred to one of the participating clinics for the treatment of their venous thromboembolism between 1 January 2001 and 1 January 2005 were contacted (n=6624). The thrombotic event could have been a deep venous thrombosis (DVT, thrombosis in leg or arm), a pulmonary embolism (PE), a superficial thrombophlebitis (STP) or a rare presentation of venous thrombosis (e.g. in brains, eye or mesentery). The event could have been a first episode or a recurrence. The chosen age limit of 45 years was based on the experience that the majority of patients from thrombophilic families develop their thrombosis before this age.[37,38] All patients received written information about the study. It was explained to them that they could participate in a study on genetic risk factors for venous thromboembolism. Patients with a sibling (brother or sister) who also had developed a venous thromboembolic event were eligible to join the GIFT affected sibling pair study, together with their affected sibling. All patients were asked to send back a reply coupon on which they could indicate whether they had family members, in particular siblings, who also had developed a venous thromboembolic event. When a patient did not respond to the letter a reminder was sent after one month (n=2545). Two hundred and ninety-five individuals were excluded because of the following reasons: deceased, address unknown, emigration, not capable of speaking Dutch, serious illness, no venous thromboembolism. About 80% of the subjects replied to our letter and 4351 of them were interested in study participation.

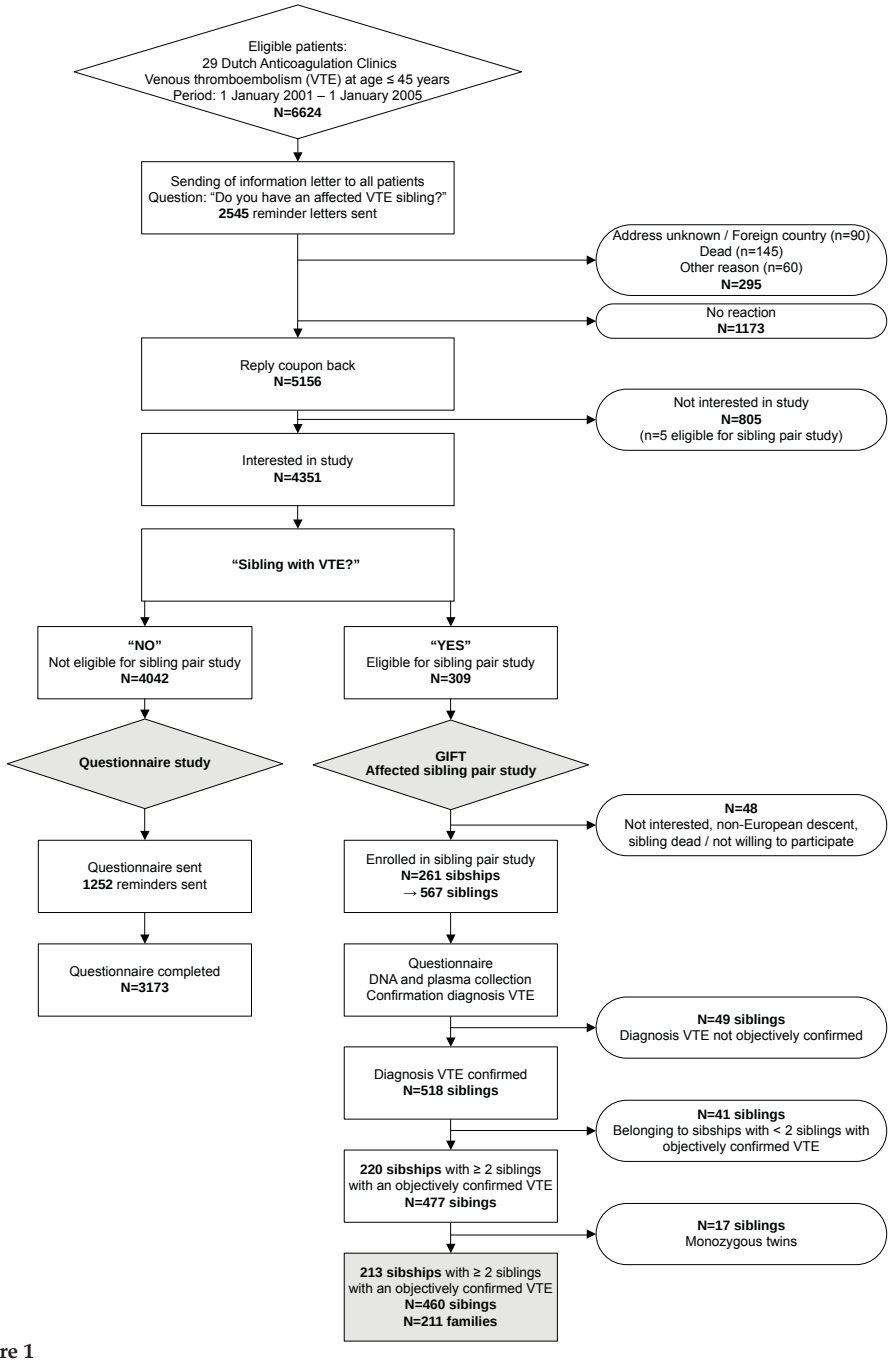

Figure 1

Flowchart of patient inclusion. VTE=venous thromboembolism.

The individuals (n=4042) that did not report a sibling with venous thromboembolism were not eligible for the affected sibling pair study. They were asked to complete a standard questionnaire, which contained among other things questions about their venous thromboembolic event(s) and about the presence of acquired risk factors before or at the time of the thrombotic event(s). As acquired risk factors we considered surgery, hospitalisation without surgery, prolonged at home immobilisation for more than four days, plaster cast, pregnancy and post-partum period, all present in the three months preceding the venous thromboembolic event. Furthermore, malignancies and use of oral contraceptives or hormone replacement therapy at the time of the thrombotic event were considered as acquired risk situations. About 80% (n=3173) of all individuals who received the questionnaire completed it.

Of all index patients who replied to our initial letter 7% (n=309) reported at least one sibling with venous thromboembolism. After exclusion of 48 patients (reasons: index patient or sibling not interested in participation, sibling deceased, non-European descent) 261 index patients entered the affected sibling pair study together with their 306 affected siblings. All participants completed the abovementioned questionnaire. In addition DNA and plasma were collected (see "Blood collection and DNA and plasma preparation"). Use of oral anticoagulants, hormones (oral contraceptives or hormone replacement therapy) and other medication at the time of venapuncture was documented. The diagnosis venous thromboembolism could be objectively confirmed in 518 subjects (see "Confirmation of diagnosis venous thromboembolism"). After exclusion of sibships with only one objectively diagnosed sibling 220 sibships with at least two siblings with a confirmed event remained. Familial relationships were verified with the software program GRR (Graphical Representation of Relationships)[39] using genotype data of the genome-wide linkage scan. GRR analysis showed that in the 220 sibships one half-sibling pair and ten monozygous twin pairs were present. Because monozygous twins are genetically identical, these twins were excluded for the genome-wide linkage analyses. Of three monozygous twin pairs only one twin was excluded because an additional sibling with venous thromboembolism was available. Furthermore GRR analysis revealed that in our sibling pair population two extended families were present, each consisting of two sibships. Eventually we included in the GIFT sibling pair study 211 families consisting of 213 sibships with two (n=185, including one half-sibling pair), three (n=22) or four (n=6) siblings with at least one objectively confirmed venous thromboembolic event. Of these sibships 42% consisted of only women, 13% consisted of only men and 45% of the sibships was mixed. In total 460 individuals (211 index patients and 249 non-index patients) were included.

This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands). All participants gave written informed consent.

Confirmation of diagnosis venous thromboembolism

The clinical diagnosis of venous thromboembolism, especially of PE, suffers from a large number of false-positive diagnoses.[40] To minimize misclassification we included in the GIFT affected sibling pair study only those individuals in whom objective tests had confirmed at least one venous thromboembolism diagnosis. Information on the tests that had been performed to diagnose the venous thromboembolic event(s) was obtained by requesting discharge letters and radiology reports from general practitioners and hospitals. The provided information was reviewed independently by two physicians. A DVT was objectively confirmed when ultrasonography (compression ultrasound or echo-Doppler/duplex), contrast venography, impedance phlethysmography (IPG) or computed tomography (CT) had been performed. The diagnosis PE was confirmed by ventilation/perfusion (V/Q) scan, spiral CT, thorax CT, pulmonary angiography or post-mortem examination. Objective confirmation of a STP was based on the judgement of patient's general practitioner or medical specialist or on ultrasonography. Mesenteric and portal vein thrombosis were objectively diagnosed by ultrasound or laparotomy. A venous sinus thrombosis was confirmed by magnetic resonance imaging (MRI), magnetic resonance angiography (MRA) or CT. In 518 of the 567 affected siblings at least one venous thromboembolic event could be objectively confirmed (see Figure 1). When the diagnostics were not convincing (e.g. low probability mismatch for V/Q scan) an event was considered as not objectively confirmed. A few events were not confirmed because the thrombosis turned out to be arterial instead of venous. Some events could not be confirmed because no discharge letter or radiology report was available (e.g. because the event was too long ago or because the patient was treated abroad).

Blood collection and DNA and plasma preparation

Venous blood was collected into four different Sarstedt Monovette® tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany); a serum tube (S-Monovette® 2.6 ml, Serum-Gel), a citrate tube (S-Monovette® 10 ml, Coagulation 9 NC, containing 0.1 volume 0.106 M trisodium citrate), an acidified buffered citrate tube (S-Monovette® 5 ml, Stabilyte™) and an EDTA tube (S-Monovette® 4 ml, EDTA KE, containing 1.6 mg potassium EDTA/ml blood). Within two hours after venapuncture plasma was prepared by centrifugation for 10 min at 2800 g at room temperature. The serum tube was left at room temperature overnight before centrifugation at 2000 g at room temperature. Plasma and serum samples were snap-frozen and stored at -80°C. High molecular weight DNA was isolated from leukocytes by standard methods

and stored at -80°C. For some individuals no venapuncture was performed and consequently no plasma samples are available. DNA samples of these subjects were obtained from buccal swabs. Plasma samples were available for 434 (198 index and 236 non-index patients) of the 460 included affected siblings. At the time of venapuncture 70 (35.4%) index patients and 73 (30.9%) non-index patients were using oral anticoagulants (vitamin K antagonists).

Laboratory analyses

Protein C antigen (Ag) levels and total protein S Ag levels were measured by enzyme linked immunosorbent assay (ELISA). All antibodies were obtained from Dako (Dako A/S, Glostrup, Denmark). Wells were coated overnight at 4°C with rabbit anti-human protein C (5 μg/ml in buffer A containing 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 9) or anti-human protein S (4 μg/ml in buffer A). Subsequently three independently diluted citrate plasma samples (1:400, 1:800, 1:1600 for protein C and 1:2000, 1:4000, 1:8000 for protein S) were added and the plate was incubated for two hours (or overnight for protein S) at room temperature. Samples were diluted in buffer B (50 mM TEA, 100 mM NaCl, 10 mM EDTA, 0.1% Tween, pH 7.5). After washing horseradish peroxidase-conjugated rabbit anti-human protein C or protein S (both 1:1000 diluted) was added to the wells. After two hours incubation at room temperature, the plate was incubated with substrate, which was prepared by dissolving one 10 mg tablet of ortho-phenylenediamine (OPD) dihydrochloride (Sigma P-8287, Sigma-Aldrich, St. Louis, MO, USA) in 25 ml of citrate-phosphate buffer (22 mM citric acid, 51 mM NaH_2PO_4, 0.03% H_2O_2, pH 5.0). After 20 minutes (for protein C) or 25 minutes (for protein S) the colour reaction was stopped by adding 1 M H_2SO_4 and the plate was read spectrophotometrically at 492 nm. Between all incubation steps wells were washed five times with buffer B. A calibration curve was obtained using 1:200 to 1:12800 dilutions of pooled normal plasma for protein C and 1:1000 to 1:64000 dilutions for protein S. Protein C and total protein S levels were expressed in units per deciliter (U/dl). By definition 1 dl of pooled normal plasma contains 100 units. The interassay coefficient of variation (CV) was 1.6% (n=26) for protein C and 2.3% (n=24) for total protein S. The criteria for the diagnosis of protein deficiencies were plasma levels below the lower limit of normal combined with normal values of prothrombin (to exclude a vitamin K deficiency), which was measured by an ELISA using commercial polyclonal affinity purified sheep anti-human prothrombin IgG as capture antibody and polyclonal affinity purified sheep anti-human prothrombin IgG conjugated to horseradish peroxidase as detection antibody (Cedarlane Laboratories Ltd, Burlington, Canada). An individual was considered protein C deficient when the plasma level was below 33 U/dl for users of vitamin K antagonists and below 65 U/dl for non-users.[41] For total protein S deficiency the criteria were ≤32 U/dl for users of vitamin K antagonists (mean minus 2 standard deviations (SD), n=26

subjects using vitamin K antagonists) and ≤72 U/dl (mean-2 SD, n=2562 controls) for non-users. Because oral contraceptive use and pregnancy lead to a reduction in protein S levels,[42-44] no judgements concerning protein S deficiency were made for women using oral contraceptives and pregnant women.

Free protein S Ag was determined in citrate plasma by an enzyme-linked ligandsorbent assay (ELSA) according to Giri *et al*.[45] with some modifications as described before.[46] Microtiter plates were coated overnight at 4-8°C with purified C4b-BP (Hyphen-Biomed, Neuville-Sur-Oise, France; 3.5 μg/ml in buffer A). After removing the C4b-BP, wells were left empty for 10 min at room temperature, then washed four times with buffer C (0.05 M Tris-HCl, 0.1 M NaCl, 0.1% Tween, 0.05% ovalbumin, pH 7.5) and incubated with buffer C containing 2.5% ovalbumin for 1 hour at 37°C to reduce background absorbance. After four washes with buffer D (0.05 M Tris-HCl, 0.1 M NaCl, 0.1% Tween, 0.05% ovalbumin, 0.005 M $CaCl_2$, 0.01 M benzamidine-HCl, pH 7.5) the dilutions of calibrator (1:10-1:640) and test samples (1:20 and 1:40) were added and incubated for 15 min at room temperature. After four washes with buffer D, horseradish peroxidase-conjugated anti-human protein S antibody (0.0325 μg/mL; Dako A/S, Glostrup, Denmark) was added and incubated for one hour at 37°C followed by four washes with buffer D. Subsequently, tetramethylbenzidine (TMB, 2 mg/ml) and H_2O_2 (0.01%) were added and incubated for 15 min at room temperature, after which the reaction was stopped by adding 2 M H_2SO_4 and the absorbance at 450 nm was measured. Supernatant of pooled normal plasma supplemented with an equal volume of 10% PEG 6000 was used as calibrator. This plasma contained 28.4 U/dl of protein S total, which is all free protein S. All sample dilutions were prepared within 10 minutes of starting the assay. The interassay CV was 11.2% (n=44). A sample was considered deficient in free protein S when levels were ≤6 U/dl for users of vitamin K antagonists (mean-2SD, n=20 subjects using vitamin K antagonists) and ≤23 U/dl (mean-2 SD, n=30 controls) for non-users. As mentioned above, women using oral contraceptives and pregnant women were not taken into account when assigning protein S deficiencies. Subjects were classified into two phenotypic subtypes using the classification as proposed at the meeting of the Scientific Subcommittee of the International Society on Thrombosis and Haemostasis in 1992 (Munich, Germany). Subjects were classified as protein S type I deficient when both total and free protein S levels were below the lower limit of normal. Individuals with normal total protein S levels but low free protein S levels were classified as protein S type III deficient.

Antithrombin activity was determined by a chromogenic assay (Coamatic® Antithrombin, Chromogenix-Instrumentation Laboratory, Milan, Italy) on a STA-R coagulation analyzer (Diagnostica Stago, Asnières-sur-Seine, France), according to

the manufacturer's protocol. Results were the mean of two measurements (1:40 and 1:80 dilutions) and were expressed in U/dl. The interassay CV was 7.8% (n=21) at a level of about 112 U/dl and 6.5% (n=21) at a level of about 50 U/dl. An individual sample was considered antithrombin deficient when the citrate plasma level of antithrombin was below 80 U/dl.[47]

Factor VIII Ag levels were measured by ELISA using two monoclonal antibodies directed against the light chain of factor VIII.[48] Wells were coated overnight with CLB-Cag A (1.3 μg/ml in buffer A). After adding of the diluted citrate plasma samples (1:20 and 1:40 dilutions) the plate was incubated for two hours at room temperature. Subsequently the tagging antibody (in buffer B) CLB-Cag 117 conjugated to horseradish peroxidase was added. After two hours of incubation substrate (prepared as described for protein C and S ELISA) was added and after fifteen minutes the reaction was stopped by adding 2 M H_2SO_4. The absorbance was measured at 450 nm. Between all incubations wells were washed 4 times with buffer B. Monoclonal anti-FVIII antibodies were kindly provided by Dr. J. van Mourik (CLB, Sanguin Blood Supply Foundation, Amsterdam, the Netherlands). Pooled normal plasma (1:5-1:320), calibrated against the WHO standard (91/666) for factor VIII Ag, was used as a reference. Factor VIII Ag levels were expressed in IU/dl. The interassay CV was 5.4% (n=17).

The presence of anti-β_2-glycoprotein I (anti-β_2-GPI) IgG antibodies was measured in citrate plasma as described before.[49,50] A positive patient sample was used for calibration and the results for this sample were arbitrarily set at 100 arbitrary units (AU). A sample was considered positive when the measurement was higher than 42 AU (mean of 40 controls + 3 SD).

Genotyping

The single nucleotide polymorphisms (SNP) factor V Leiden (rs6025), prothrombin 20210G/A (rs1799963), fibrinogen gamma (FGG) 10034C/T (rs2066865, specific for FGG haplotype 2)[51] and three SNPs (rs8176719 (261G/delG), rs8176749 (930G/A) and rs8176750 (1061C/delC)) in the ABO blood group gene, discriminating the genotypes O, A^1, A^2 and B, were genotyped using a 5'-nuclease/TaqMan assay.[52] Polymerase chain reactions with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA, USA) were performed on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany) and fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA, USA). Methylenetetrahydrofolate reductase (MTHFR) 677C/T (rs1801133) was genotyped by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, using the Sequenom MassARRAY®

Platform (Sequenom, San Diego, CA, USA), according to the iPLEX™ assay protocol.

Statistical analysis

Frequencies were compared by Pearson's chi-square test. To analyze combinations of the classical genetic risk factors (factor V Leiden, prothrombin 20210A, deficiencies of antithrombin, protein C, protein S type I and type III) subjects were selected in whom all risk factors could be tested (373 individuals (155 men and 218 women); 172 index patients and 201 non-index patients). For these analyses subjects without plasma samples and women who were pregnant or using oral contraceptives at the time of venapuncture (no assignment of protein S deficiencies) were excluded.

Results

Characteristics GIFT affected sibling pairs

Some characteristics of the 460 subjects who were included in the GIFT affected sibling pair study are shown in Table 1. Data are specified for the 211 index patients and their 249 siblings (non-index patients). The study population consists of more women than men and the frequency of women is higher in the non-index group compared to the index group. About 40% of the individuals (41.0% of index and 38.3% of non-index patients) was overweight (body mass index (BMI) ≥25 & <30 kg m^{-2}) and about 23% (22.0% of index and 23.9% of non-index patients) was obese (BMI ≥25 kg m^{-2}). These percentages are similar to the ones recently reported for venous thromboembolism patients of a large Dutch patient control study.[53] Age at the first venous thromboembolic event, as self-reported, is 34 years (range 15-45) for the index patients and 33 years (range 16-64) for the non-index patients. Approximately 60% of the subjects reported a DVT (in 97% of cases of the leg, in 3% of the arm) as their first venous thromboembolic event. For about 20% the first event was a PE and about 9% had a PE in combination with a DVT. Most subjects whose first event was a STP (9%) suffered from another type of venous thromboembolism later in life. Three index patients and nine non-index patients were only diagnosed with STP (seven subjects had one event, four subjects had two events and one subject had four events). In about 1.5% of individuals the first venous thromboembolic event occurred at a more rare location (brain, mesentery, vena porta). Almost half of the patients presented with more than one venous thromboembolic event (two events: 33%, three events: 9%, four events: 3%, five events: 0.7%, six events: 0.2%). For all 460 included siblings at least one diagnosis was confirmed by objective tests. In total we objectively confirmed 618 (82%) of the 754 venous thromboembolic events that were reported by the included siblings. Almost half of the sibships had at least one parent who also had experienced a venous thromboembolic event. In 44% one parent was affected and in nearly 5% of families both parents were affected.

Table 1
Characteristics of GIFT population

	Index patients n=211	Non-index patients n=249
Number of women (%)	121 (57.3)	174 (69.9)
Mean body mass index, kg m^{-2} (SD)	27.0 (5.0)	26.9 (4.9)
Mean age at first VTE, years (SD)*	34.2 (8.1)	33.1 (10.1)
Mean age at blood or buccal swab collection, years (SD)	40.3 (6.4)	43.3 (9.0)
Type of first VTE (%)*		
Deep vein thrombosis (DVT, leg or arm)	129 (61.1)	148 (59.4)
Pulmonary embolism (PE)	40 (19.0)	52 (20.9)
DVT + PE	20 (9.5)	22 (8.8)
Superficial thrombophlebitis	20 (9.5)	22 (8.8)
Other presentation‡	2 (0.9)	5 (2.0)
Mean number of VTE events (range)*	1.6 (1-5)	1.6 (1-6)
Number of patients with recurrency (%)*	99 (46.9)	112 (45.0)
Both parents VTE	10 (4.7)	
One parent VTE	93 (44.1)	

* Based on questionnaire data.
‡ Sinus thrombosis (n=2 index patients and n=1 non-index patient), portal vein thrombosis (n=2 non-index patients) and mesenteric venous thrombosis (n=2 non-index patients).
VTE=venous thromboembolism.

Presence of acquired risk situations at first venous thromboembolism

Table 2 gives an overview of the acquired risk factors that were present in the GIFT individuals before or at the time of their first venous thromboembolic event. In 34% of men and 91% of women the first venous thromboembolic event was provoked, i.e. occurred in the presence of at least one acquired risk factor. About 11% developed their first venous thromboembolic event after surgery. More than 65% of women used oral contraceptives at the time of their first event. This number is similar to the number of 70% that was reported for premenopausal women of the Leiden Thrombophilia Study (LETS), including unselected patients (only patients older than 70 years and patients with malignancies were excluded) with a first DVT.[54] Eighteen percent of women got their first venous thromboembolic event while they were pregnant (2.7%) or after delivery (15.3%). In young women of the LETS study this prevalence was somewhat lower (pregnancy 5.0%, post-partum period 8.2%).[55] In the GIFT population, consisting of young patients, no hormone replacement therapy was reported. Immobilisation (p=0.006), due to other causes than the post-partum period, and malignancies (p=0.01) were more present in men than women.

Table 2

Presence of acquired risk factors before or at first venous thromboembolic event

	GIFT men (%) n=165	GIFT women (%) n=295
Surgery	21 (12.7)	29 (9.8)
Immobilisation, hospitalisation, plaster cast	34 (20.6)	33 (11.2)
Malignancy	8 (4.8)	3 (1.0)
Pregnancy		8 (2.7)
Post-partum period		45 (15.3)
Oral contraceptive use		194 (65.8)
Acquired risk factor present	56 (33.9)	269 (91.2)

Prevalence of other risk factors for venous thromboembolism

Prevalences of other non acquired risk factors for venous thromboembolism in GIFT index patients are presented in Table 3. The frequencies of the risk factors in the non-index patients did not differ significantly from the frequencies in the index patients. Compared to unselected patients with a first DVT the GIFT population is enriched for the genetic risk factors factor V Leiden ($p=2.1x10^{-6}$) and ABO blood group non-O ($p=8.5x10^{-4}$). Furthermore, high factor VIII levels (≥150 IU/dl) are much more prevalent in GIFT individuals than in unselected DVT patients ($p=9.0x10^{-6}$). The prothrombin 20210A mutation and homozygous carriership for MTHFR 677T and FGG 10034T are as frequent in the GIFT population as in unselected DVT patients. Anti-β_2-GPI antibodies are present in 6.6% of the index patients, which is also similar to the frequency in unselected patients, and they are more prevalent in index women (8.3%) than men (3.3%). In one family two sisters are positive for anti-β_2-GPI antibodies.

Many different mutations in the genes encoding protein C, S and antithrombin can cause a protein deficiency. In the present study protein levels were used as a surrogate for genetic defects. Based on the analysis of a single plasma sample 4% and 5% of GIFT patients have a laboratory outcome that corresponds with a deficiency of antithrombin and protein C, respectively. These frequencies resemble the frequencies in unselected patients with a first DVT (4.2% and 4.6%) when the assignment of a deficiency in these patients was also based on a single measurement.[47] According to our criteria 7.6% of index patients have plasma protein S levels corresponding with a type I protein S deficiency and more than 10% of patients was diagnosed as a type III protein S deficiency. These prevalences are higher than those found in the unselected DVT patients of LETS. No difference in prevalence of antithrombin, protein C and protein S deficiency was found between men and women.

Table 3

Prevalence of known risk factors for venous thromboembolism in GIFT index patients, unselected patients and healthy controls

	GIFT index patients (%) n=211	GIFT patients (%) 1 VTE n=249	GIFT patients (%) >1 VTE n=211	Unselected DVT patients (%)	General Population (%)	Reference
Genetic factors						
Factor V Leiden	36.5	30.5	40.8	19.5	3.0	62
Prothrombin 20210A	6.6	9.2	5.2	6.2	2.3	23
Antithrombin deficiency	4.0*	6.6	3.4	1.1-4.2§	0.2-1.9§	47
Protein C deficiency	5.1*	3.5	6.8	2.7-4.6§	0.4-1.5§	47
Protein S deficiency type I	7.6‡	5.0	8.6	1.2	0.7	47
Protein S deficiency type III	10.5‡	10.1	10.9	1.9	1.4	ℓ
ABO blood group non-O	82.9	81.1	80.6	70.9	57.1	25
MTHFR 677T (homozygous)	14.5	12.7	14.8	10.0	9.9	78
FGG 10034T (homozygous)	15.2	12.4	15.2	12.2	6.0	51
Laboratory phenotypes						
High factor VIII Ag	48.5*	47.6	48.8	28.9	13.0	69
Anti-β_2-GPI antibodies	6.6*	6.1	7.8	7.5	3.4	50

* n=198; no plasma available for thirteen index patients.

‡ n=172; no plasma available for thirteen index patients, and 26 women who were pregnant or using oral contraceptives were excluded.

§ Frequency ranges due to different criteria used: A single measurement or two measurements (antithrombin); A single measurement, two measurements, or a single measurement and the presence of a mutation (for protein C).

ℓ Leiden Thrombophilia Study, unpublished results.

VTE=venous thromboembolism; MTHFR=methylenetetrahydrofolate reductase; FGG=fibrinogen gamma; Anti-β_2-GPI=anti-β_2-glycoprotein I.

Number of classical genetic risk factors in GIFT families

In about 40% of GIFT index patients none of the classical genetic risk factors (factor V Leiden, prothrombin 20210A, deficiencies of protein C, S and antithrombin) was present (Table 4). Approximately 50% had one genetic risk factor and about 7.5% had two or three genetic risk factors. These frequencies were similar in men and women. ABO blood group non-O was equally distributed among carriers of no, one, or more than one classical genetic risk factor with frequencies of 83%, 86% and 69%, respectively.

Table 4

Overview of classical genetic risk factors in GIFT sibships

N genetic risk factors (%)	Factor V Leiden	Prothrombin 20210A	Antithrombin deficiency	Protein C deficiency	Protein S deficiency type I	Protein S deficiency type III	N (%) (n=172)*
0 (41.3%)	-	-	-	-	-	-	71 (41.3)
1 (51.2%)	+	-	-	-	-	-	50 (29.1)
	-	+	-	-	-	-	6 (3.5)
	-	-	+	-	-	-	4 (2.3)
	-	-	-	+	-	-	5 (2.9)
	-	-	-	-	+	-	11 (6.4)
	-	-	-	-	-	+	12 (7.0)
2 (6.4%)	+	+	-	-	-	-	2 (1.2)
	+	-	+	-	-	-	1 (0.6)
	+	-	-	+	-	-	1 (0.6)
	+	-	-	-	+	-	1 (0.6)
	+	-	-	-	-	+	3 (1.7)
	-	+	+	-	-	-	1 (0.6)
	-	+	-	+	-	-	1 (0.6)
	-	-	-	+	-	+	1 (0.6)
3 (1.2%)	+	+	-	-	-	+	2 (1.2)

* 172 index patients in whom all risk factors were tested.

Classical genetic risk factors and the presence of acquired risk factors

Three hundred and seventy-three individuals were tested for all classical genetic risk factors. In the group of subjects without a classical genetic risk factor (n=151) more often an acquired risk factor was present at the time of the first venous thromboembolic event (74.2% compared to 62.2% in 222 subjects with a genetic risk factor, p=0.015). Stratification for sex showed that this effect was only seen in men. The first venous thromboembolic event was provoked in 89.7% of women without a genetic risk factor (n=87) and in 90.8% of women with a genetic risk factor (n=131). Of the 64 men without a genetic risk factor 53.1% reported the presence of an acquired risk factor at their first venous thromboembolic event. In the 91 men with a genetic risk factor this frequency was 20.9% ($p=3.1 \times 10^{-5}$). Examination of the individual

genetic risk factors demonstrated that the prevalence of all genetic risk factors was lower in the group of men with a provoked first event compared to men with an unprovoked first event. This difference was only significant for the most frequent risk factor, factor V Leiden, which is present in 39.4% of men with an unprovoked first venous thromboembolic event and in 16.1% of men with a provoked first venous thromboembolic event (p=0.002). In women these analyses were not performed, because the subgroup of women with an unprovoked first venous thromboembolic event was too small.

In 39 individuals (10.5%), 30 men (19.4%) and 9 women (4.1%), the first venous thromboembolic event was unprovoked and no genetic risk factor was present. In all five men with at least two genetic risk factors the first venous thromboembolic event was unprovoked, while in the 25 women with at least two genetic risk factors all first events were provoked.

Recurrent venous thromboembolism

At the time of recruitment 211 (45.9%) of the 460 siblings had developed more than one venous thromboembolic event (Table 1), which is very high for this population of relatively young patients. Previous reports on recurrence percentages vary depending on the selection of the patient population; a cumulative recurrence percentage of approximately 16.5% in 7 years was reported for DVT patients without malignancies,[56] whereas a higher rate of about 30% in 8 years was reported for an older patient population which included patients with malignancies.[57] We found that the mean age at recruitment was somewhat higher for individuals with more than one venous thromboembolic event (mean age: 42.9 years) compared to individuals with a single venous thromboembolic event (mean age: 41.1 years). The percentage recurrences was similar in men (47.3%) and women (45.1%), whereas most studies report that recurrences occur more frequent in men than women.[56,58,59]

Whereas it has been shown consistently that patients with a first provoked venous thromboembolic event have a lower risk of recurrence than patients with an unprovoked event,[56,57,60] we observed in the GIFT population, that for both men and women the percentage recurrences was not higher in individuals with an unprovoked first venous thromboembolic event (recurrence percentage: 46.8% in men and 50.0% in women) compared to individuals with a provoked event (recurrence percentage: 48.2% in men and 44.6% in women). One exception were women who were immobilised before their first event. Of these 33 women only six (18.2%) experienced a recurrent event.

The prevalence of other (non acquired) risk factors for venous thromboembolism did not differ between the group with a single venous thromboembolic event and the group with at least one recurrent event, except for factor V Leiden (Table 3). The prevalence of factor V Leiden was higher in individuals who experienced a recurrency (40.8%) compared to individuals with a single venous thromboembolic event (30.5%, p=0.02).

In the GIFT individuals who were tested for all classical genetic risk factors the occurrence of recurrences was investigated. In the 222 subjects with at least one genetic risk factor the percentage recurrences was higher (51.4%) than in the 151 subjects without a genetic risk factor (39.7%, p=0.027). Stratification for sex showed that this effect was seen both in men and women, but was only significant in women (p=0.018). The percentage recurrences in subjects with at least one genetic risk factor did not differ between men (percentage recurrences: 50.5%) and women (51.9%). However, the recurrence percentage for subjects without a genetic risk factor was higher in men (45.3%) than in women (35.6%).

Monozygous twins

Ten monozygotic twin pairs were present in the GIFT population. In all twins at least one venous thromboembolic event could be objectively confirmed. For the genome-wide linkage analyses these identical twins were excluded, because they are genetically indistinguishable. In the GIFT population also two dizygotic twin pairs were present (one brother-brother pair and one brother-sister pair). In Table 5 some characteristics of the monozygous twins are shown. The monozygous twin population consists of seven female and three male pairs. Age at first venous thromboembolic event (mean 32.7 years, range 18-44), number of venous thromboembolic events (mean 1.55, range 1-3), and the percentage of individuals with more than one event (45%) are the same as in the whole GIFT population. Two twin pairs (#4 and #10) have ABO blood group O and the other eight twin pairs have ABO blood group non-O. This frequency is similar to the frequency reported in the entire GIFT study (Table 3). One male and four female twin pairs did not carry any of the known classical genetic risk factors. Of the three male twin pairs one pair was heterozygous for factor V Leiden and another pair had a protein S type I deficiency. In the six male twins no acquired risk factors were accompanying their first venous thromboembolic event. Two female twin pairs were heterozygous for factor V Leiden and one female twin pair had a protein S type III deficiency. The latter twin pair (#5) was also positive for anti-β_2-GPI antibodies. In most females the first venous thromboembolic event occurred in the presence of an acquired risk factor, predominantly oral contraceptive use.

Table 5

Characteristics of monozygous twins

Twin pair	Sex	First VTE		n VTE events	Risk factors	
		Age	Type		Acquired (first VTE)	Genetic
#1	M	44.9	DVT	1	-	Factor V Leiden
		42.4	DVT	2	-	
#2	M	36.7	STP	1	-	Protein S deficiency type I
		36.9	DVT	2	-	
#3	M	42.4	PE	1	-	-
		38.9	DVT + PE	2	-	
#4	F	18.5	DVT	3	OC	Factor V Leiden
		32.1	DVT	1	Post-partum period	
#5	F	28.5	DVT	2	Pregnancy	Factor V Leiden
		24.6	PE	1	OC	
#6	F	35.4	PE	1	-	Protein S deficiency type III
		29.2	DVT	2	OC	
#7	F	35.3	DVT + PE	2	Immobilisation + OC	-
		38.7	DVT	1	OC	
#8	F	28.0	DVT	2	Immobilisation + OC	-
		31.5	DVT	1	OC	
#9	F	20.5	DVT	3	OC	-
		23.9	DVT	1	OC	
#10	F	44.3	DVT	1	Surgery	-
		21.1	DVT	1	OC	

VTE=venous thromboembolism; OC=oral contraceptive use.

Discussion

The aim of the Genetics In Familial Thrombosis (GIFT) study is to identify novel genetic risk factors for venous thromboembolism. This study was set up because we believe that at present genetic risk factors for venous thromboembolism are missing. For our study we selected sibships with two, three or four siblings with at least one objectively confirmed venous thromboembolic event at a young age. By this approach we aimed at recruiting a sample of small families with genetic defects which make them more susceptible to venous thromboembolism. In the present study we have drawn up an inventory of the classical genetic and acquired risk factors in this selected population. The expected enrichment for genetic risk factors was demonstrated by the high prevalences of the two most common genetic risk factors for venous thromboembolism, factor V Leiden and ABO blood group non-O. Furthermore, the high recurrence percentage and the observation that nearly half of the sibships had at least one parent who also had developed a venous thromboembolic event suggest that genetics play an important role in the development of venous thromboembolism in these families. Since in a large part of this panel of small thrombophilia families none or only one of the classical genetic

risk factors is found this panel seems very suitable for the discovery of novel genetic risk factors for venous thrombosis.

Among families with a tendency to venous thromboembolism the prevalence of heritable risk factors for venous thromboembolism is much higher than among unselected consecutive patients.[27,61] In the GIFT families we found a prevalence of 36.5% for the most common classical genetic risk factor for venous thromboembolism, factor V Leiden. This prevalence is much higher than among unselected Dutch DVT patients (19.5%)[62] and seems more comparable with the prevalences of 40-60% that were reported in small panels of large thrombophilic families.[27,63] In another Dutch patient population, including PE patients in addition to DVT patients, about 16% of patients carried factor V Leiden.[64,65] In a Dutch panel of patients with recurrent venous thromboembolism factor V Leiden was present in 27.5% of patients.[66]

Deficiencies of the natural anticoagulants protein C, S and antithrombin were assigned by phenotypic testing of a single plasma sample, so results should be interpreted cautiously. Furthermore, especially the laboratory diagnosis of protein S deficiency is known to be extremely difficult.[67] It is complicated by a large overlap between protein S levels in heterozygous protein S deficient and in normal individuals, by fluctuation of levels over time, and by the influence on levels of sex, age, pregnancy and use of hormones.[42-44] The prevalence of deficiencies in the GIFT population is probably overestimated, as it was already demonstrated before that the prevalence of deficiencies decreases when more stringent criteria are used, like testing of a second plasma sample and genetic testing.[47] Previously it was reported that deficiencies of the main coagulation inhibitors occur in about 15% of thrombophilic families.[27] Future genetic analyses of protein C, protein S and antithrombin are needed to draw definite conclusions about the prevalence of hereditary deficiencies in the GIFT families. In some GIFT sibships both protein S type I and type III deficiency are present. The coexistence of these two different subtypes of protein S deficiency in one family has been reported before and seems to be explained by the observation that total protein S levels increase with increasing age, whereas free protein S levels are not influenced by age.[68]

High factor VIII levels (≥150 IU/dl) were present in almost half of the GIFT population compared to 29% of unselected DVT patients of LETS.[69] This difference becomes even more striking when comparing with the subgroup of young LETS patients, since high factor VIII levels were less frequent in young patients than in older patients.[70] An elevated level of factor VIII is a well established risk factor for venous thromboembolism.[36] Factor VIII levels are determined to a large extent by levels of Von Willebrand factor (VWF), its carrier protein. Several studies have reported

clustering of high factor VIII levels within families[71-73] and it was suggested that genetic factors largely contribute to the variation in factor VIII and VWF levels.[31,32,74,75] However, apart from ABO blood group, which explains about 30% of the variation in factor VIII and VWF levels,[74] little is known about these genetic determinants. The high prevalence of high factor VIII levels in the GIFT population may partly be contributed to the high frequency of ABO blood group non-O (83%). This prevalence is higher than among unselected DVT patients (71%) and among healthy controls (57%).[25] To our knowledge none of the studies on thrombophilic families did include ABO blood group in their thrombophilia screening, although ABO blood group non-O is a well established very common risk factor for venous thromboembolism, as recently demonstrated in a review and meta-analysis,[26] and interaction with factor V Leiden has been reported.[25,76] Currently, ABO blood group typing is not included in the panel of tests used to identify those considered at particular risk of venous thromboembolism. Whether testing for ABO blood group should be added to this thrombophilia screening needs to be further explored. Additional research on genetic determinants of high factor VIII and VWF levels in the GIFT study will be performed in the future.

In the GIFT population ten monozygous twin pairs and two dizygotic twin pairs were present. In the Netherlands about 18-19 twins are born per thousand births and about 30-40% of twins is monozygous.[77] The high number of monozygous twins (ten monozygous twins per 465 births) points to an important contribution of genetic factors in the development of their venous thromboembolism. Of all seven female twin pairs at least one twin of the pair used oral contraceptives at the time of her first venous thromboembolic event. Three female twin pairs without any known classical genetic risk factor developed their first venous thromboembolic event while they both used oral contraceptives. Further search for genetic variants that interact with oral contraceptive use seems therefore warranted.

In more than 90% of GIFT families none (41%) or only one (51%) classical genetic risk factor was found. These percentages are very similar to those previously reported in a smaller sample of thrombophilic families, where in only 13% of families at least two genetic risk factors were present.[27] Since we believe that familial thrombophilia is a multigenic disorder,[14-16] this means that in a large proportion of families genetic risk factors are missing.

We have drawn up an inventory of the classical genetic and acquired risk factors in the selected GIFT population of small thrombophilic families. Investigation of the number of classical genetic risk factors that are present in these families suggests that genetic risk factors are still missing. This finding supports our assumption that

novel genetic risk factors can be discovered in this population.

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Chapter 3.2

Genome-wide scan in affected sibling pairs reveals two novel susceptibility regions for venous thromboembolism: The Genetics In Familial Thrombosis (GIFT) study

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Summary
Venous thromboembolism is a common disorder with an annual incidence of one to three per thousand individuals. It is considered to be a multicausal disease in which both acquired and genetic risk factors are involved (heritability 50-60%). Several genetic risk factors are known: the factor V Leiden mutation, the prothrombin 20210A mutation, deficiencies of protein C, protein S, and antithrombin and ABO blood group non-O. However, in most thrombophilic families these currently known genetic risk factors cannot explain the clustering of thrombosis, indicating that genetic risk factors are missing. The Genetics In Familial Thrombosis (GIFT) study aims at identifying novel thrombosis susceptibility alleles using an affected sibling pair approach. Via 29 Dutch Anticoagulation Clinics, 287 affected sibling pairs (belonging to 211 families) with an objectively confirmed VTE at a young age (≤45 years) were recruited. We performed a genome-wide linkage scan in all affected siblings using 402 microsatellites and 5 single nucleotide polymorphisms (SNPs): factor V Leiden, prothrombin 20210A and 3 ABO blood group SNPs. Multipoint non-parametric linkage analysis, using the S_{all} statistic, was performed with MERLIN for the autosomes and with MINX for the X chromosome. Suggestive linkage was found at chromosomal regions 7p (LOD score=2.23, p=0.0007) and Xq (LOD score=1.70, p=0.003). Both regions were followed up with extra markers, that were also typed in 355 parents and unaffected siblings. The linkage results support the presence of novel thrombosis susceptibility regions at 7p21.3 (LOD score=3.09, p=0.00008) and on Xq25-q26.3 (LOD score=1.86, p=0.002).

Introduction
Venous thromboembolism is a disorder in which a blood clot (thrombus) is formed in a vein, which partially or completely obstructs the blood flow. It is a common disease, with an annual incidence of one to three per thousand individuals in the general population.[1,2] The most frequent clinical manifestations of venous thromboembolism are deep vein thrombosis (DVT) of the leg and pulmonary embolism (PE). Other forms of venous thromboembolism include deep vein thrombosis of the arm, superficial thrombophlebitis and rare thrombotic events in the veins of e.g. the brain, eye or abdomen. Major complications of venous thromboembolism are death of PE (1.4-2.5% of all thrombotic events), development of a post-thrombotic syndrome (30-40%) and recurrences (~30%).[3,4] Venous thromboembolism is considered to be a multicausal disease in which both acquired and genetic risk factors are involved and interact.[5,6] Acquired risk factors include immobilization, trauma, surgery, malignancy, pregnancy, puerperium, lupus anticoagulant and the use of female hormones.[7] Genetic risk factors include the factor V Leiden mutation,[8] the prothrombin 20210A mutation,[9] deficiencies of the natural anticoagulants protein C,[10] protein S,[11] and antithrombin[12] and ABO blood group non-O.[13-15] Using family

and twin-based studies, the heritability of venous thromboembolism was estimated between 50 and 60%.[16-18]

Familial thrombophilia, the clustering of venous thromboembolism within families, is considered to be an oligogenetic disorder in which at least two genetic defects segregate in the family.[19-21] However, in only 13% of these families, two known genetic defects are found (apart from ABO blood group non-O). In the majority of these families, only one (60%) or none (27%) of the known genetic defects are found, indicating that unknown genetic risk factors are segregating within these families.[6] Apart from the aforementioned genetic risk factors for venous thromboembolism, several plasma phenotypes have been reported that increase thrombosis risk. Elevated levels of hemostasis-related proteins, such as fibrinogen, factors VIII, IX and XI, increase the risk of venous thromboembolism.[22-25] Genetic effects account for a large proportion of the variation in these phenotypes.[26-28] However, at present, little information is available on the genetic variants that contribute to the inter-individual variation of these phenotypes.

We hypothesized that genetic determinants of venous thromboembolism exist, which have not been identified so far. Most of the previously identified genetic risk factors for venous thromboembolism have been discovered by studying genes known to be involved in hemostasis (candidate gene approach). After finding of a risk-associated plasma phenotype in thrombophilic families or large patient control studies, the main genetic determinant of this phenotype was subsequently identified. Many hemostasis-related genes have been extensively studied using this candidate gene approach.[29,30] Besides the candidate gene approach one can also use a genome-wide linkage approach, in which the genome is systematically scanned for genes or genomic regions that contribute to the susceptibility to venous thromboembolism. In thrombosis research, two studies have previously used this approach.[16,31,32] Both studies used extended thrombosis families for their analyses. The aim of the Vermont study was to identify a second genetic defect which, together with protein C deficiency, explained the high frequency of venous thrombosis in a large protein C deficient pedigree of French-Canadian descent (kindred Vermont II).[31,32] The Spanish GAIT (Genetic Analysis of Idiopathic Thrombophilia[16]) study mainly searched for genetic determinants of plasma levels of hemostasis-related proteins. These two genome scans both identified 10p12-p13 and 18p11.2-q11.2 as regions that might contain thrombosis susceptibility alleles.[32,33,34]

In the present study, we report the results of the first genome-wide linkage scan for venous thromboembolism using affected sibling pairs. We aimed at identifying genomic regions containing novel thrombosis susceptibility genes. We recruited 460

siblings from 211 families constituting 287 affected sibling pairs (185 duos, 22 trios and 6 quartets), who all developed venous thromboembolism at a young age, and performed a non-parametric linkage analysis using 402 microsatellite markers.

Subjects and methods

Study population

The design of the Genetics In Familial Thrombosis (GIFT) study is described in detail in Chapter 3.1. In the GIFT study we collaborated with 29 Anticoagulation Clinics throughout the Netherlands. Such clinics monitor all patients treated for venous thromboembolism within a well defined geographical area. Approximately 6600 young patients (≤45 years at the time of the thrombotic event) who were referred to these clinics for the treatment of venous thromboembolism between January 2001 and January 2005 were approached. The thrombotic event was a DVT of the leg or arm, a PE, a superficial thrombophlebitis or a rare presentation of venous thrombosis (e.g. in brain, eye or abdomen). The event was either a first episode or a recurrent event. The age limit of 45 years was chosen based on the experience that the majority of patients from thrombophilic families develop their thrombosis before this age.[35,36] All patients were asked to complete a questionnaire on environmental risk factors for venous thromboembolism and the nature and circumstances of the thrombotic event. Patients reporting one or more siblings who also had developed venous thromboembolism (~7%) were asked to participate in the GIFT study together with their affected sibling(s). In total, 261 sibships (567 individuals) of Caucasian descent were included in the study. Parents of the affected siblings were also asked to participate in the study. When parents were deceased or not willing to participate, unaffected siblings were asked to participate. The GIFT study was approved by the Medical Ethics Committee of the Leiden University Medical Center. Written informed consent was obtained from all participants, according to the Principles of the Declaration of Helsinki.

Sample collection

Blood from affected siblings was obtained by venapuncture and collected into tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany) containing 0.1 volume of 0.106 mol/L trisodium citrate. Genomic DNA was isolated from leukocytes (n=533) or buccal swabs (n=34) by standard methods. Genomic DNA of parents and unaffected siblings was isolated from buccal swabs. DNA concentration was determined by absorption at 260 nm using a standard spectrophotometer. DNA samples were stored at -80°C.

Phenotypic evaluation
To objectively confirm the thrombotic events, a letter of discharge or radiology report was requested from General Practitioners or hospitals for each individual. This information was reviewed independently by two physicians using a standardised approach, resulting in a final diagnosis (see Chapter 3.1). In 220 sibships the diagnosis venous thromboembolism could be objectively confirmed in at least two affected siblings. Sibships with only one objectively diagnosed sibling were excluded.

DNA markers
A genome-wide scan was performed in all affected siblings using 402 microsatellite markers of the ABI Prism Linkage Mapping Set MD-10 (n=380) or MD-5 (n=22) (Applied Biosystems, Foster City, CA, USA). The average spacing of the markers was 9.3 centiMorgan (cM). The average heterozygosity was 0.79 (range 0.58-0.91). In addition, all individuals (affected siblings, parents, unaffected siblings) were genotyped for five single nucleotide polymorphisms (SNPs): the factor V Leiden mutation (rs6025),[8] the prothrombin 20210A mutation (rs1799963),[9] and three SNPs (rs8176719 (261G/delG), rs8176749 (930G/A) and rs8176750 (1061C/delC)) in the ABO blood group gene, discriminating the genotypes O, A^1, A^2 and B.[37] During the fine mapping of interesting regions, affected siblings, parents and unaffected siblings were genotyped for nineteen additional microsatellites of the ABI Prism Linkage Mapping Set HD-5 version (twelve chromosome X markers, six chromosome seven markers and D1S452 on chromosome 1 near *F5,* the gene coding for coagulation factor V).

Genotyping
Markers were amplified using standard conditions and reagents, with the exception that some polymerase chain reactions (PCR) were optimized to amplify two markers simultaneously. PCR products were pooled according to size and fluorescent tag (FAM, VIC, NED) and measured using an ABI Prism DNA Analyzer 3700 or 3730 (Applied Biosystems, Foster City, CA, USA). Genotypes were analyzed using the software program Genemapper Version 3.0 (Applied Biosystems, Foster City, CA, USA). All genotypes were independently checked (and corrected if necessary) by two operators. In total, 5% of the samples were genotyped in duplicate as part of genotyping quality control. As monozygous (MZ) twins should have identical genotypes, we considered the average discordance rate of 0.2% (range 0-0.5%) between MZ twin pairs (n=10) to be an approximation of the genotyping error rate. All SNPs were genotyped using a 5'-nuclease/TaqMan assay.[38] PCRs with fluorescent allele-specific oligonucleotide probes (Assay-by-Design, Applied Biosystems, Foster City, CA, USA) were performed on a PTC-225 thermal cycler (Biozym, Hessisch Oldendorf, Germany) and fluorescence endpoint reading for allelic discrimination

was done on an ABI 7900 HT (Applied Biosystems, Foster City, CA, USA).

Analyzed genotypic data were stored in a locally developed SQL database. This database was further used to compare genotypes of repeated samples, to calculate success rates and to generate files for linkage analysis. The location of the markers was taken from an integrated genetic map with interpolated genetic map positions.[39] The position of the markers is given in deCODE cM, estimated via locally weighted linear regression (lo(w)ess) from the physical map positions of Build 35.1 and from published deCODE and Marshfield genetic map positions. The average success rate per marker was 94.5% for the markers in the initial scan and 98.2% for the fine mapping markers. The average success rate per sample was 97.6% in the initial scan and 98.6% during fine mapping.

Data analysis

Mendelian inconsistencies were identified with the software program PEDSTATS.[40] Unlikely double recombinants were identified with the software program MERLIN using the default settings and erroneous genotypes were removed with PEDWIPE.[41] Familial relationships were verified using the software program GRR (Graphical Representation of Relationships).[42] GRR analysis resulted in the identification of one half-sibling pair and ten MZ twin pairs. In three families with an affected MZ twin pair and an additional affected sibling, the twin with the lowest genotyping success rate was excluded. The other seven MZ twin pairs were excluded from further analysis. For the half-sibling pair, an extra (dummy) father was added. GRR analysis further revealed that four families were linked into two three-generation families. For these two families, extra family relationships (e.g. aunt-nephew) were taken into account in MERLIN.

Linkage analysis

Non-parametric linkage (NPL) analysis based on the S_{all} statistic[43] was performed with MERLIN for all autosomes, whereas the X chromosome was analyzed with MINX, an X-specific version of MERLIN.[41] The genome-wide significance level was estimated by performing a simulation analysis in 10,000 random datasets. Random datasets were generated with MERLIN using the same marker allele frequencies, missing data, marker spacing and family structures as used in the actual analyses. Subsequently, each dataset was analyzed with MERLIN. The probability (with 95% confidence interval (CI)) of observing a linkage signal equal to or higher than our maximum LOD score was calculated, i.e. genome-wide p-value=n/10,000, in which n is the total number of simulations with a signal equal to or higher than the observed LOD score.

Results

Characteristics of GIFT study population

The characteristics of the GIFT siblings are shown in Table 1. After exclusion of MZ twins, 211 families (460 individuals) with at least two siblings with an objectively confirmed venous thromboembolic event were included in this study. Two of these 211 families were three-generation families. The total number of affected sibling pairs was 287 (185 duos, 22 trios and 6 quartets). The GIFT study population was enriched for factor V Leiden (36.5% in GIFT index patients vs. 19.5% in consecutive thrombosis patients and 3% in the general population)[44] and ABO blood group non-O (82.9% vs. 70.9% and 57%).[14] The frequency of the prothrombin 20210A mutation in GIFT siblings (6.6%) was similar to that of consecutive thrombosis patients (6.2%), but higher compared to the general population (2%).[9]

Table 1
Characteristics of the 460 affected siblings of the GIFT study

Characteristics	Index patients n=211*	Non-index patients n=249*
No. (%) women	57.3	69.9
Mean age (years) at thrombotic event (±SD) ‡	34.2 (±8.1)	33.1 (±10.1)
First venous thrombotic event (%)‡		
DVT	61.1	59.4
PE	19.0	20.9
DVT + PE	9.5	8.8
Thrombophlebitis	9.5	8.8
Other presentation§	0.9	2.0
Recurrences (%)‡	46.9	45.0
Known genetic risk factors (%)		
FVL mutationℓ	36.5	34.1
Prothrombin 20210A mutationℓ	6.6	8.0
ABO blood group non-O	82.9	79.1
Protein C deficiency	5.1	5.1
Protein S deficiency type I	7.6	6.0
Protein S deficiency type III	10.5	10.4
Antithrombin deficiency	4.0	5.9

* Protein C and antithrombin deficiencies: 198 index patients and 236 non-index patients (excluded 13 index patients and 13 non-index patients with no plasma available); protein S deficiency: 172 index patients and 201 non-index patients (excluded 13 index patients and 13 non-index patients with no plasma available, and 26 index women and 35 non-index women who were pregnant or using oral contraceptives).

‡ Self-reported.

§ E.g. sinus, vena porta and mesentery venous thrombosis.

ℓ Heterozygotes + homozygotes.

Initial genome-wide scan

The initial genome-wide scan for venous thromboembolism yielded three linkage signals with a LOD score higher than one (Table 2, Figure 1). The highest linkage signal (LOD score=2.23, p=0.0007) was found on chromosome 7p22.2. The second linkage signal was located on chromosome Xq25 (LOD score=1.70, p=0.003) and the third on chromosome 8q12.1 (LOD score=1.32, p=0.007).

Table 2
Chromosomal regions with a LOD score>1.0 as observed in the initial genome-wide scan

Chromosomal region	Position maximum LOD score (cM)	LOD score	Nominal p-value	LOD-1 interval (cM)
7p22.2	13.5	2.23	0.0007	0 - 32.3
8q12.1	68.1	1.32	0.007	43.7 - 109.7
Xq25	134.2	1.70	0.003	123.2 - 149.2

cM=centiMorgan, LOD=logarithm of odds.

Finemapping

The two most promising linkage results, at chromosomal regions 7p22.2 and Xq25, were followed up by genotyping of extra markers in these regions, not only in the 460 affected siblings but also in their parents (105 fathers, 133 mothers) and unaffected siblings (n=117). In both regions, the average spacing between markers decreased from 8-9 cM to about 4 cM. The information content increased from 0.4-0.6 to about 0.8 for the 7p22.2 region and from 0.5-0.8 to 0.7-0.9 for the Xq25 region. After fine mapping, both linkage signals increased (Table 3, Figures 2 and 3). The LOD score on chromosome 7 increased to 3.09 (p=0.00008). The maximum LOD score was located at position 14.9 cM with a LOD-1 support interval of 12.9 cM. The maximum LOD score of 3.09 was genome-wide significant, since a LOD score of 3.09 or higher was observed only in 294/10,000 simulations (p=0.029, 95% CI: 0.026-0.032). The linkage peak on chromosome X shifted about 5 cM towards the q-telomere. The maximum LOD score increased to 1.86 (p=0.002) and was located at position 139.3 cM with a LOD-1 support interval of 22.7 cM. Genome-wide significance was not reached as in 10,000 simulations 4018 times a LOD score equal to or greater than 1.86 was observed (p=0.402, 95% CI: 0.392-0.412). Interestingly, the gene coding for coagulation factor IX (*F9*) is located just below the top of the linkage peak at 144.7 cM.

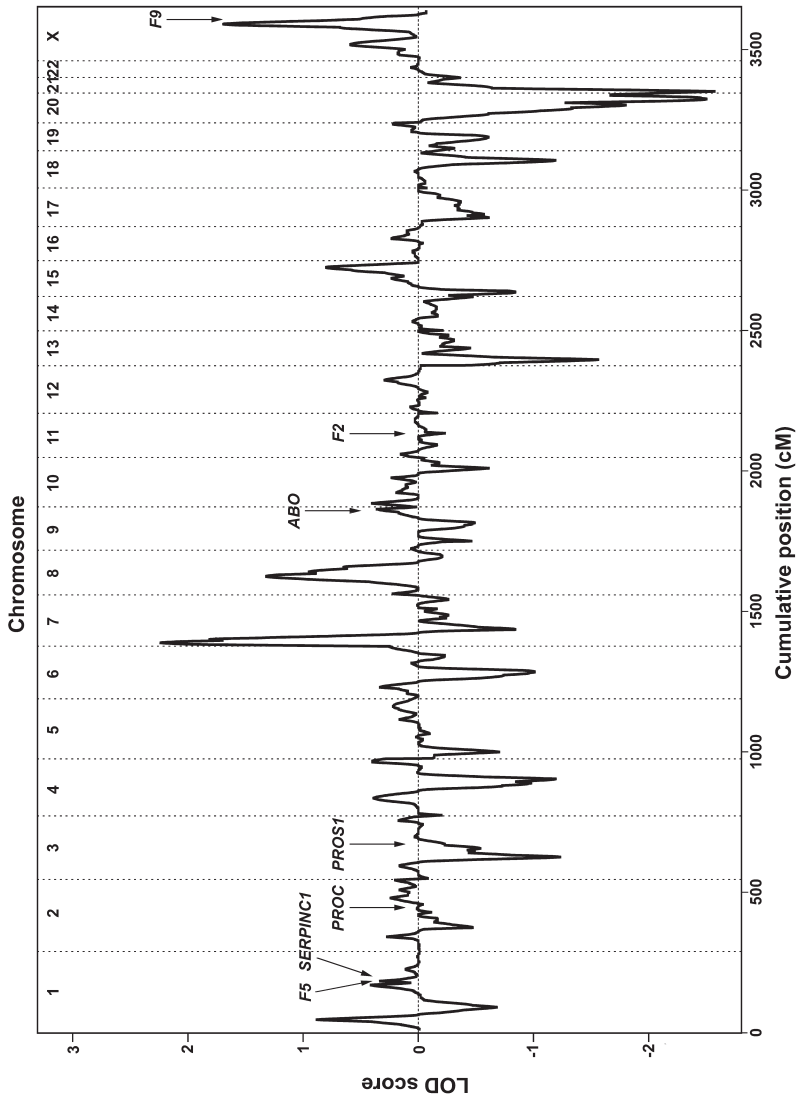

Figure 1
Graphical overview of the initial genome-wide scan in the GIFT study. Chromosome numbers on top. cM=centiMorgan, LOD=logarithm of odds. The locations of the factor IX gene (*F9*) and the genes of the known genetic risk factors for venous thromboembolism are indicated with arrows: *F5, F2, ABO, PROC, PROS1* and *SERPINC1.*

Table 3
Chromosome 7 and X regions after fine mapping

Chromosomal region	Position maximum LOD score (cM)	LOD score	Nominal p-value	Genome-wide p-value*	LOD-1 interval (cM)
7p21.3	14.9	3.09	0.00008	0.029	6.7 - 19.6
Xq26.3	139.3	1.86	0.002	0.402	126.0 - 148.7

* = n/10,000, in which n is the total number of simulations with a signal equal or higher than the observed LOD score.
cM=centiMorgan, LOD=logarithm of odds.

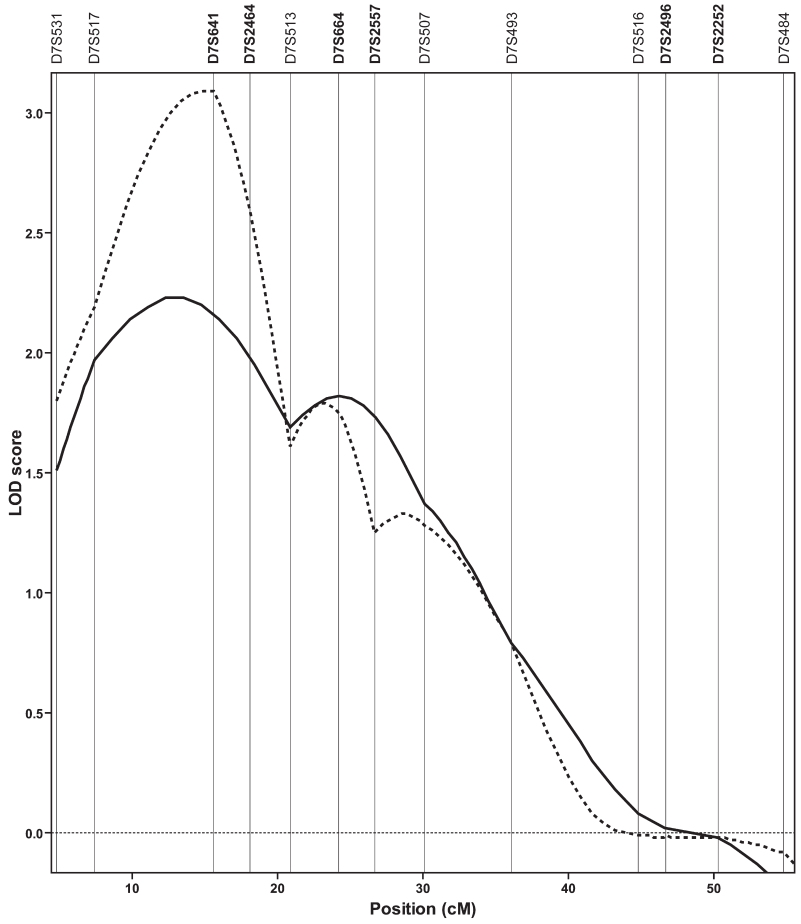

Figure 2
Fine mapping of the chromosomal region 7p. Solid line: results of the initial genome scan. Dashed line: results of the initial genome scan + fine mapping markers. Fine mapping markers are indicated in bold.
cM=centiMorgan, LOD=logarithm of odds.

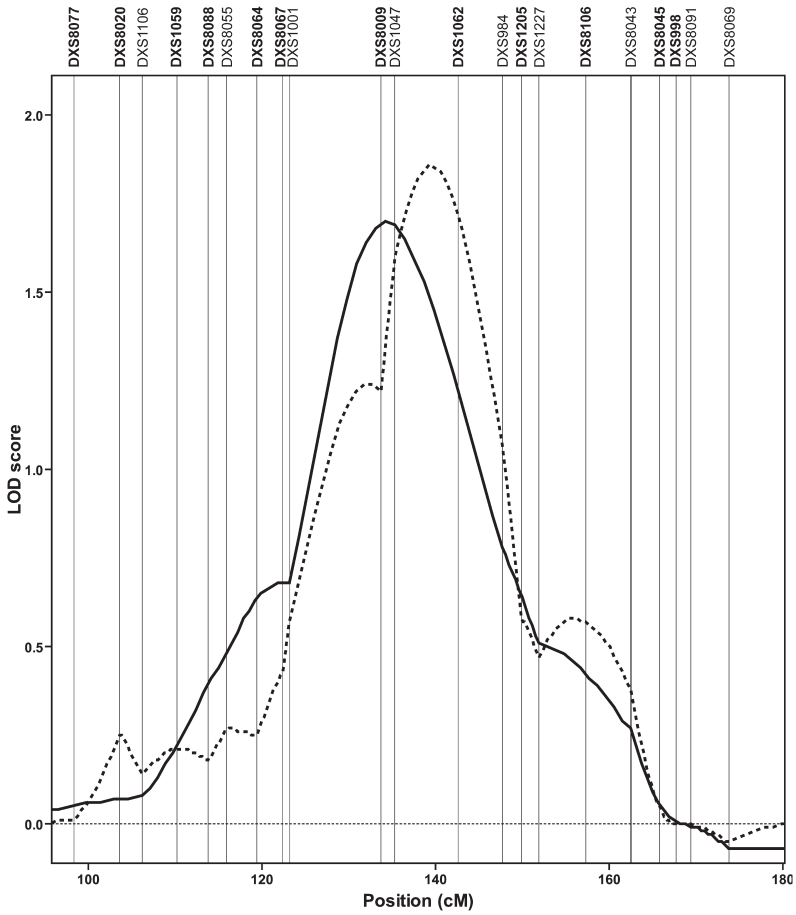

Figure 3

Fine mapping of the chromosomal region Xq. Solid line: results of the initial genome scan. Dashed line: results of the initial genome scan + fine mapping markers. Fine mapping markers are indicated in bold. cM=centiMorgan, LOD=logarithm of odds.

Known genetic risk factors

The prevalences of the known genetic risk factors for venous thromboembolism in the GIFT siblings are shown in Table 1. A high prevalence of factor V Leiden and ABO blood group non-O was found. However, linkage analysis, including genotype data of affected siblings, parents and unaffected siblings for the factor V Leiden SNP and D1S452, indicated a LOD score of 0.34 (p=0.10) at the location of the factor V gene (*F5*) on chromosome 1q24.1. Similar to the *F5* locus, no clear linkage signals were found at the locations of the other known genetic risk factors for venous thromboembolism: prothrombin (*F2*), protein C (*PROC*), protein S (*PROS1*), antithrombin (*SERPINC1*)

and ABO blood group (*ABO*) (see Figure 1). Except at the *F9* locus, no evidence for linkage was found at the locations of other genes encoding proteins known to be involved in coagulation.

Discussion

We report the results of the first genome-wide linkage scan in affected sibling pairs with venous thromboembolism. In total, 287 affected sibling pairs with an objectively confirmed venous thromboembolism at young age (index patients ≤45 years) were included in the Genetics In Familial Thrombosis (GIFT) Study. The high prevalence of factor V Leiden (36.5%) and ABO blood group non-O (82.9%) illustrates the important contribution of genetic factors to the development of the disease in the GIFT panel. Linkage was found at chromosomal regions 7p and Xq. The addition of six markers to the chromosome 7p region and twelve markers to the chromosome Xq region strengthened the support for linkage in both regions. However, only the chromosome 7p linkage signal was genome-wide significant. For both linkage signals, many families positively contributed to the LOD score. There were no families that contributed significantly more than other families (regardless of the number of siblings within a family). Both three-generation families did not contribute to the linkage signals.

Besides *F9*, no genes coding for known coagulation proteins are located in the 7p and Xq regions. *F9* codes for coagulation factor IX, which after activation by factor XIa or factor VIIa/tissue factor activates factor X, a process eventually leading to thrombin and clot formation.[45,46] Elevated plasma levels of factor IX were found to dose-dependently increase the risk of venous thrombosis in a large case-control study for venous thrombosis, the Leiden Thrombophilia Study (LETS). Individuals with factor IX levels above the 90th percentile of the distribution in healthy subjects have a 2 to 3-fold increased risk of thrombosis compared to individuals having factor IX levels below this cutoff point.[24] This finding was confirmed in a second study.[47] We previously studied whether common haplotypes of *F9* could explain elevated factor IX levels and thrombosis risk (see Chapter 2.3).[48] We found that haplotype 6 was associated with a two-fold decreased risk of venous thrombosis in men (OR=0.5, 95% CI: 0.3-0.9), however, no association with factor IX levels was found. It should be noted that a haplotype analysis is difficult to perform for *F9*, because of its complex haplotype structure.

The 7p and Xq regions contain about 150 and 250 genes, respectively. Future studies are required to screen these genes as possible candidate genes for venous thromboembolism. Obviously, also replication studies are needed to validate our findings.

Two previous genome scans for venous thrombosis did not show evidence for linkage at the chromosome 7p region.[16,32] Both studies did not include the X chromosome in their linkage analysis. The Vermont genome scan revealed three regions (chromosomes 11q23, 10p12 and 18p11.2-q11.2) that might contain novel thrombosis susceptibility genes.[32] The latter two regions were also found in the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project in a genome scan for quantitative trait loci (QTL) influencing plasma factor XII levels and activated protein C resistance, respectively.[33,34] The only serious candidate gene found in these three regions was the gene coding for the alpha(2) subunit of platelet-activating factor acetylhydrolase 1b (*PAFAH1B2*), which is located at 11q23. However, a subsequent study excluded this gene as risk factor for venous thromboembolism.[49] In our genome scan, no evidence for linkage with venous thromboembolism was found in these three regions.

Factor V Leiden is a relatively common mutation in the general population (carrier frequency=3%) and is associated with an increased risk of venous thrombosis (relative risk for heterozygous carriers≈7).[44] The GIFT study population is enriched for the factor V Leiden mutation (frequency=36.5%) when compared with consecutive patients with deep venous thrombosis (frequency=19.5%).[44] It is not surprising that, although the GIFT study population is enriched for factor V Leiden, only a weak linkage signal was found at the location of *F5* (1q24.1, LOD score=0.34). There is a number of sibling pairs carrying the factor V Leiden mutation that share no allele(s) identical-by-descent (IBD=0) because of the presence of more than one factor V Leiden allele in their parents (two heterozygous parents or one homozygous parent). These families do negatively contribute to the linkage signal. Furthermore, twelve percent of the sibling pairs is discordant for the factor V Leiden mutation (i.e. one sibling carries the mutation, whereas the other sibling does not carry the mutation) and these pairs do not (IBD=1) or negatively (IBD=0) contribute to the LOD score. This observation may predict that novel genetic risk factors which are as common as factor V Leiden will probably also not be found in the GIFT population with a genome-wide linkage scan. The GIFT study population is also enriched for ABO blood group non-O (frequency=82.9%). A weak linkage signal (LOD score=0.35) was found at the ABO gene locus (9q34.2). Similar to the factor V Leiden mutation, ABO blood group non-O is likely to be too common to be detected as a risk factor in our genome scan. Furthermore, because of the different genotypes A and B, blood group non-O carriers not necessarily share alleles IBD.

The genome-wide linkage approach using affected sibling pairs is a proper method to identify novel susceptibility regions for a complex disease. For example, chromosome 19p was found as a novel susceptibility region for celiac disease in a genome-wide linkage scan.[50] Subsequent research identified a common variant in

the myosin IXB gene, which was associated with celiac disease ($p=2.1x10^{-6}$) in two independent cohorts and increased the risk of celiac disease by 2.3-fold.[51] Similarly, a major susceptibility locus for type 2 diabetes mellitus was found on chromosome 2p in a genome-wide linkage scan.[52] Positional cloning showed that variants in the calpain-10 gene, located in the chromosome 2p region, were associated with type 2 diabetes mellitus.[53]

The average distance between the markers used for this genome-wide scan was about 9 cM. However, there are several gaps in the genome coverage that are larger than 9 cM and which might cause false negative results. In our study, thrombotic events with different clinical symptoms (e.g. DVT, PE) were grouped together into a single group. Therefore, the results of the genome-wide scan do not provide data about regions that might contain novel genetic risk factors for an individual clinical symptom of venous thromboembolism.

In conclusion, we have identified two novel susceptibility regions for venous thromboembolism, 7p21.3 and Xq25-q26.3. Future identification of the gene(s) and their functional variants, which are responsible for the linkage signals, will give better insights in the molecular genetics of familial thrombophilia and might be important for the diagnosis, treatment and prevention of venous thromboembolism.

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CHAPTER 3.3

Screening of eleven candidate genes from the 7p21.3 and Xq25 q26.3 linkage regions for association with venous thromboembolic risk: results from The Genetics In Familial Thrombosis (GIFT) Study

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Summary
The Genetics In Familial Thrombosis (GIFT) study aims at identifying novel genetic risk factors for venous thromboembolism. We previously identified two novel thrombosis susceptibility regions (7p21.3 and Xq25-q26.3) using a genome-wide linkage scan in affected sibling pairs. In the present study, we screened eleven candidate genes, selected from these regions. In total, 106 haplotype tagging single nucleotide polymorphisms (SNPs) were genotyped in these candidate genes in the GIFT population (460 affected siblings, 238 parents, 117 unaffected siblings) and in a panel of healthy controls (n=331). Addition of these SNPs to the linkage analysis did only marginally change the linkage signals on chromosomes 7p and Xq. SNPs in *GPR30, LFNG* and *RAC1* were found to be associated with venous thromboembolism. Further analysis revealed that haplotype 4 of *GPR30*, tagged by rs10262070 together with rs10235056, was associated with an increased risk of venous thromboembolism (OR=1.6; 95% CI: 1.0-2.5). In addition, haplotype 1 of *RAC1*, which is tagged by the C allele of rs836480, was associated with a 1.3-fold increased risk of venous thromboembolism (p=0.04). An association given linkage analysis indicated that rs836480 in *RAC1* contributes to the linkage signal on chromosomal region 7p. Further studies are needed to identify additional genetic variants contributing to the two linkage peaks on chromosome 7p and Xq.

Introduction
We hypothesize that, besides the currently known genetic risk factors (e.g. factor V Leiden), genetic determinants for venous thromboembolism exist which have not been identified so far. The Genetics In Familial Thrombosis (GIFT) study aims at identifying these novel genetic risk factors for venous thromboembolism. Previously, we identified two susceptibility regions for venous thromboembolism (chromosomes 7p21.3 and Xq25-q26.3) in a genome-wide linkage scan using affected sibling pairs (see Chapter 3.2). Both regions were not found in two previous genome scans for thrombosis in extended pedigrees, a large protein C deficient pedigree of French-Canadian descent (kindred Vermont II)[1] and a panel of Spanish extended families (Genetic Analysis of Idiopathic Thrombophilia (GAIT)[2]).

In the present study, we investigated whether genetic variants in eleven selected candidate genes, eight in the 7p region and three in the Xq region, contribute to the linkage signals found in our genome scan for venous thromboembolism. We genotyped 106 haplotype tagging single nucleotide polymorphisms (htSNPs) in the selected candidate genes in the GIFT study population (all affected siblings and their parents and unaffected siblings) and in a panel of healthy controls.

Subjects and methods

Study population

The design of the Genetics In Familial Thrombosis (GIFT) study has previously been described in detail (see Chapter 3.1). In the GIFT study, we collaborated with 29 Dutch Anticoagulation Clinics throughout the Netherlands. Approximately 6600 young patients (≤45 years at the time of the thrombotic event), who were referred to these clinics for the treatment of venous thromboembolism between January 2001 and January 2005, were approached. All patients were asked to complete a questionnaire on environmental risk factors for venous thromboembolism and the nature and circumstances of the thrombotic event. Patients with one or more siblings, who also had developed venous thromboembolism, were asked to participate together with their affected sibling(s). In total, 460 affected siblings (287 sibling pairs) of Caucasian descent with at least one objectively confirmed venous thromboembolic event were included in the study. Parents and unaffected siblings were also asked to participate in the study. In total 355 individuals (105 fathers, 133 mothers and 117 unaffected siblings) were included in the study. Genomic DNA of affected siblings was isolated from leukocytes (n=435) or buccal swabs (n=25) by standard methods. Genomic DNA of parents and unaffected siblings was isolated from buccal swabs. The GIFT study was approved by the Medical Ethics Committee of the Leiden University Medical Center. Written informed consent was obtained from all participants.

A control group of healthy subjects was used in combination with the GIFT thrombosis patients to compare SNP allele frequencies and to perform an association given linkage analysis. This control group has previously been used in a case-control study on the causes of recurrent venous thromboembolism.[3,4] The control group was recruited through a general practice in The Hague (the Netherlands). Two thousand eight hundred twelve individuals, aged 20-90 years, were approached to take part in a health survey on risk factors of cardiovascular disease. In total, 532 individuals agreed to take part in the study. For the current study, 331 individuals of Caucasian descent were available, all without a history of venous thromboembolism and cardiovascular disease.

Selection of candidate genes

Candidate genes were selected from the LOD-1.5 support intervals. For the chromosome 7p linkage peak (LOD score=3.09), this interval is 25.3 cM wide and stretches from 0-15.2 Mb on this chromosome (NCBI database build 36.1). This region contains about 150 (predicted) genes. For the chromosome Xq linkage peak (LOD score=1.86), this interval is 41.7 cM or 26.9 Mb wide, ranging from 117.2-144.1 Mb, and contains about 250 (predicted) genes. We used the online system Biomart, a data-mining tool that extracts information from the Ensembl databases,[5] to collect

information about these 400 genes. In our query, we included the position of the gene and links to the Uniprot[6] and the HUGO Gene Nomenclature[7] databases, which provided more detailed information about the function of the protein encoded by the gene, similarity to other genes or proteins, tissue specificity, association with diseases and studies in knock-out mice. All genes were independently screened by four investigators. During a consensus meeting, eleven candidate genes were selected which, for various reasons, might influence the risk of venous thromboembolism: *PDGFA*, *GPR30*, *CHST12*, *LFNG*, *GNA12*, *RAC1*, *C1GALT1* and *SCIN* from the chromosomal region 7p and *HS6ST2*, *CD40LG* and *F9* from the chromosomal region Xq. Selection criteria were based on a previously reported role in coagulation (*GNA12*, *RAC1* and *F9*), glycosylation (*CHST12*, *LFNG*, *C1GALT1* and *HS6ST2*) or platelet or endothelial related functions (*PDGFA*, *GPR30*, *SCIN* and *CD40LG*). Only for *F9* the association with thrombosis was previously extensively investigated.[8-11]

Selection of single nucleotide polymorphisms

We used the online Genome Variation Server (GVS)[12] to graphically display the linkage disequilibrium (LD) structure[13] of the SNPs present in the eleven genes and their flanking regions. Data from the dbSNP build 127 database were used. Genotypic data from Caucasians (CEU) from the HapMap project[14] were used. For *RAC1* and *F9*, haplotype information was also available from SeattleSNPs.[15] Haplotypes were identified for each gene using $r^2>0.8$ as criterion for linkage among SNPs. Subsequently, tagging SNPs were selected for each haplotype. In total, 115 haplotype tagging SNPs (htSNPs) were selected: twelve in *GNA12*, eighteen in *CHST12*, ten in *GPR30*, seven in *RAC1*, sixteen in *SCIN*, five in *C1GALT1*, eight in *CD40LG*, nineteen in *F9* and fourteen in *HS6ST2* (see also Tables 3 and 4). For *LFNG* we selected the only two SNPs tested in HapMap individuals (rs13234810 and rs2895). No SNPs were determined in *PDGFA* in HapMap individuals. Therefore, four previously reported SNPs (rs1129401/rs1741566, rs1800814, rs1800815 and an intron 4 SNP)[16,17] were selected in this gene.

Genotyping

SNPs were genotyped in affected siblings, parents, unaffected siblings and control subjects by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, using the Sequenom MassARRAY® Platform (Sequenom, San Diego, CA, USA). The iPLEX™ assay protocol was followed.[18] Genotypes were analyzed using the Typer version 4.0 software program (Sequenom, San Diego, CA, USA). About 7% of the samples were genotyped in duplicate as part of genotyping quality control. Analyzed genotypic data were stored in a locally developed SQL database. This database was further used to compare genotypes of repeated samples, to calculate success rates and to generate files for linkage and association analysis.

For the linkage analysis, the location of the repeat markers and SNPs was taken from an integrated genetic map with interpolated genetic map positions.[19] The position is in deCODE cM, estimated via locally weighted linear regression (lo(w)ess) from the physical map positions of Builds 34.3 and 35.1 and from published deCODE and Marshfield genetic map positions. In total, nine SNPs failed to genotype correctly. For the remaining 106 SNPs, the average success rate per SNP and per sample was >97%. For all SNPs the distribution of genotypes among control subjects (for X chromosome SNPs only in women) was in Hardy-Weinberg equilibrium, except for SNPs rs3808354 (p=0.011), rs953770 (p=0.035), rs2906166 (p=0.025), rs208356 (p=0.005), rs709280 (p=0.037) rs2527686 (p=0.049), rs3823602 (p=0.015) and rs1182179 (p=0.027). An extra check of the genotypic data of these SNPs did not indicate a systematic error in the iPLEX™ assays used.

Data analysis

Mendelian inconsistencies were identified using the software program PEDSTATS.[20] Unlikely double recombinants were identified with the software program MERLIN using the default settings and erroneous genotypes were removed with PEDWIPE.[21] Non-parametric linkage (NPL) analysis, based on the S_{all} statistic[22] and taking into account the linkage disequilibrium (LD) between SNPs,[23] was performed by MERLIN for the chromosomal region 7p. The chromosomal region Xq was analyzed with MINX, an X-specific version of MERLIN.[21] To investigate whether a SNP was associated with venous thromboembolism, we used a Cochran Armitage trend test to test for differences in allele frequencies between the affected siblings and healthy controls. When computing the variance of the statistic, we took into account that genotypes of siblings are correlated.[24] An association given linkage analysis using an in-house written score statistic[25] was performed to test whether one of the SNPs was in LD with a putative disease locus. When the identical-by-descent (IBD) distribution explains part of the variance of the SNP genotypes (i.e. when the effect comes from the sibling pairs with IBD 2), this test is more powerful than the Cochran Armitage trend test. The software program Haplo.stats[26] was used to investigate the effect of haplotypes on the risk of venous thromboembolism in GIFT index patients and healthy controls. Haplotype frequencies were estimated by Haplo.stats, without assigning haplotypes to individuals. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated by Haplo.stats under an additive model for each haplotype and compared to the most frequent haplotype. The haplo.score function of Haplo.stats was used to calculate the effect of one haplotype compared to a reference group of all other haplotypes.

Results

Characteristics of study population

The characteristics of the GIFT population and the group of healthy subjects are shown in Table 1. There was no difference in sex distribution (p=0.36) between the GIFT index patients and the control subjects. The frequency of ABO blood group non-O and the factor V Leiden and prothrombin 20210A mutations was similar in GIFT index patients (82.9%, 36.5% and 6.6%) and GIFT non-index patients (79.1%, 34.1% and 8.0%), but as expected much lower in the control group (60.2%, 6.6% and 3.9%). Frequencies in the control group were comparable with those found in the control group of the Leiden Thrombophilia Study (57%, 3% and 2%, respectively).[27-29]

Table 1
Characteristics of the GIFT population and control subjects

Characteristics	Index patients n=211	Non-index patients n=249	Control subjects n=331
Women (%)	57.3	69.9	61.3
Mean age (years) at thrombotic event (±SD)	34.2 (±8.1)	33.1 (±10.1)	-
Known genetic risk factors (%)			
Factor V Leiden mutation*	36.5	34.1	6.6
Prothrombin 20210A mutation*	6.6	8.0	3.9
ABO blood group non-O	82.9	79.1	60.2

* heterozygotes + homozygotes.

Linkage analysis

Genotypic data of the 106 SNPs were added to our previous linkage analysis on chromosomal regions 7p and Xq (see Chapter 3.2) to explore whether these SNPs could further improve evidence for linkage. Both linkage signals slightly changed when SNPs were added to the analysis (Table 2, Figures 1 and 2). The LOD score of the linkage peak on chromosome 7 decreased from 3.09 to 2.87 and the width of the LOD-1 support interval decreased from 12.9 cM to 10.4 cM. Addition of SNPs to the linkage analysis did not change the evidence for linkage on chromosome X (1.86 versus 1.85) nor did it change the width of the LOD-1 support interval (22.7 cM versus 23.1 cM). For both regions, the information content (0.8-0.9) remained unchanged.

Table 2
Linkage analysis of chromosomal regions 7p and Xq after adding 106 SNPs

Chromosomal region	Position maximum LOD score (cM)	LOD-1 interval (cM)	LOD score	Nominal p-value
7p21.3	15.6	9.5 - 19.9	2.87	0.00014
Xq26.3	141.1	126.0 - 149.1	1.85	0.002

cM=centiMorgan, LOD=logarithm of odds.

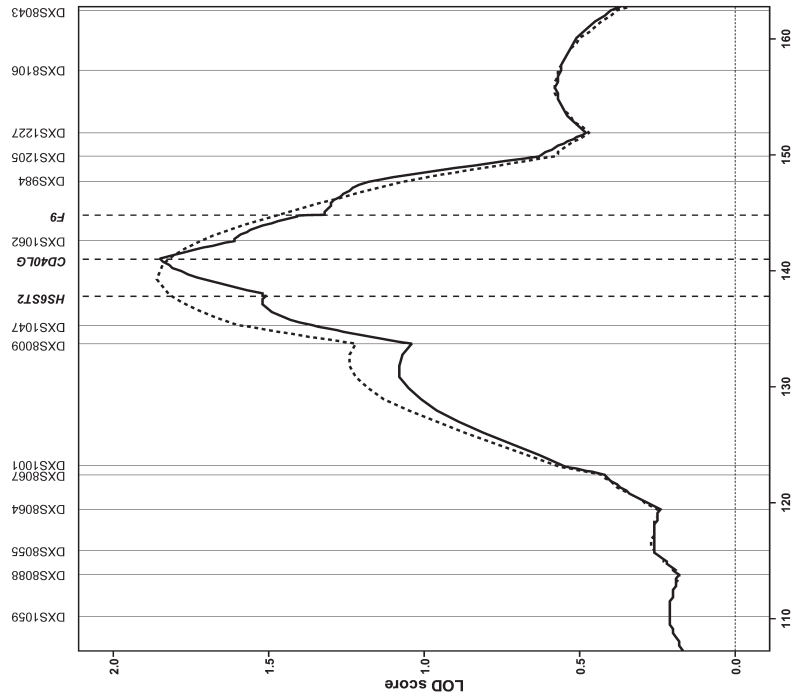

Figure 2
Linkage analysis of chromosomal region Xq. Dashed line: results linkage analysis without SNPs. Solid line: results linkage analysis with SNPs. Markers and candidate genes (in bold) are indicated at the top of the figure. cM=centiMorgan, LOD=logarithm of odds.

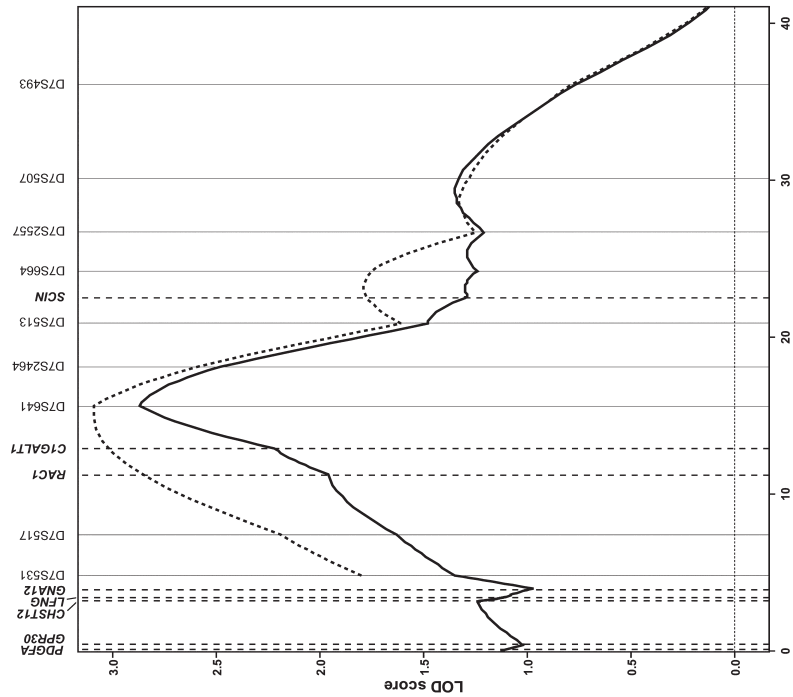

Figure 1
Linkage analysis of chromosomal region 7p. Dashed line: results linkage analysis without SNPs. Solid line: results linkage analysis with SNPs. Markers and candidate genes (in bold) are indicated at the top of the figure. cM=centiMorgan, LOD=logarithm of odds.

Association analysis

An association analysis was performed to test whether any of the SNPs was associated with venous thromboembolism. The results are shown in Table 3 for the chromosome 7 SNPs and in Table 4 for the SNPs on the X chromosome. SNPs associated with venous thromboembolism were located in *GPR30* (five SNPs, especially rs10262070, p=0.002), *LFNG* (rs13234810, p=0.007), *GNA12* (rs208358, p=0.049 and rs709280, p=0.040) and *RAC1* (rs836480, p=0.038). No association with venous thromboembolism was found for SNPs in *PDGFA*, *SCIN*, *C1GALT1* and *CHST12*. On the X chromosome, rs5975351 in *HS6ST2* in men (p=0.024), rs593097 and rs3092949 in *CD40LG* in women (p=0.026 and p=0.041, respectively) and *F9* SNPs rs6048 (factor IX Malmö) in men (p=0.024) and 32781A/G in women (p=0.041) were associated with venous thromboembolism. The overall sex-adjusted association test, however, did not indicate an association with venous thromboembolism for any of the tested X chromosome SNPs.

Table 3

Association analysis for 66 SNPs in the chromosomal region 7p

Gene	SNP	Alleles	Minor allele frequency		p-value
			Affected siblings	Healthy controls	
PDGFA	rs1800814	C/T	0.483	0.484	0.971
PDGFA	rs28420971	C/T	0.122	0.125	0.886
PDGFA	Intron 4	C/T	0.261	0.258	0.924
PDGFA	rs1129401	T/C	0.263	0.261	0.962
GPR30	rs1997243	A/G	0.181	0.138	**0.042**
GPR30	rs10269151	G/A	0.027	0.041	0.179
GPR30	rs10235056	G/A	0.124	0.087	**0.043**
GPR30	rs3808353	G/A	0.183	0.162	0.333
GPR30	rs3808354	G/T	0.117	0.170	**0.013**
GPR30	rs1133043	G/C	0.342	0.371	0.296
GPR30	rs13242034	G/A	0.18	0.178	0.932
GPR30	rs10262070	C/T	0.304	0.222	**0.002**
GPR30	rs12113381	C/T	0.132	0.171	0.059
GPR30	rs4236375	C/T	0.448	0.488	**0.032**
CHST12	rs953770	C/T	0.453	0.421	0.239
CHST12	rs884564	C/T	0.275	0.236	0.125
CHST12	rs1010300	C/T	0.189	0.187	0.928
CHST12	rs4719524	G/A	0.267	0.229	0.141
CHST12	rs7782956	A/G	0.196	0.177	0.399
CHST12	rs1476614	A/G	0.481	0.457	0.392
CHST12	rs17793630	A/G	0.062	0.069	0.628
CHST12	rs2270005	T/G	0.110	0.117	0.699
CHST12	rs10245377	G/C	0.151	0.161	0.645
CHST12	rs2969078	T/C	0.254	0.275	0.436

Table 3, continued

Gene	SNP	Alleles	Minor allele frequency		p-value
			Affected siblings	Healthy controls	
CHST12	rs10267898	C/T	0.082	0.084	0.911
CHST12	rs11772554	C/A	Failed		
CHST12	rs2906173	T/C	Failed		
CHST12	rs11486794	C/T	0.170	0.157	0.533
CHST12	rs11768384	A/G	0.400	0.381	0.508
CHST12	rs2906168	G/A	0.275	0.298	0.400
CHST12	rs2906166	C/T	0.323	0.307	0.535
CHST12	rs11764837	C/T	0.219	0.212	0.782
LFNG	rs13234810	A/G	0.284	0.358	**0.007**
LFNG	rs2895	T/C	0.098	0.078	0.227
GNA12	rs11354	C/T	0.433	0.391	0.144
GNA12	rs798532	C/T	0.290	0.298	0.768
GNA12	rs41304915	T/A	0.006	0.002	0.261
GNA12	rs208358	G/A	0.229	0.183	**0.049**
GNA12	rs208356	A/T	0.326	0.284	0.141
GNA12	rs709280	A/C	0.401	0.340	**0.040**
GNA12	rs208343	A/G	0.110	0.096	0.411
GNA12	rs7790322	C/T	Failed		
GNA12	rs2527686	A/G	0.489	0.465	0.427
GNA12	rs757788	A/G	0.126	0.138	0.538
GNA12	rs3823602	G/C	0.197	0.211	0.565
GNA12	rs1182179	A/G	0.290	0.287	0.917
RAC1	rs10951982	G/A	0.207	0.242	0.156
RAC1	rs836480	C/A	0.426	0.486	**0.038**
RAC1	rs702484	C/G	0.263	0.284	0.405
RAC1	rs6942723	A/G	Failed		
RAC1	rs6463554	G/C	0.106	0.140	0.072
RAC1	rs836547	G/T	0.070	0.095	0.099
RAC1	rs6954996	G/A	0.042	0.047	0.685
C1GALT1	rs13226799	T/C	0.056	0.079	0.105
C1GALT1	rs10487590	G/T	0.227	0.203	0.299
C1GALT1	rs3807862	T/C	Failed		
C1GALT1	rs2190936	C/T	0.162	0.192	0.166
C1GALT1	rs1047763	G/A	0.159	0.136	0.254
SCIN	rs6967031	T/C	0.479	0.492	0.655
SCIN	rs10268615	A/G	Failed		
SCIN	rs849783	T/C	0.057	0.056	0.940
SCIN	rs849785	C/T	Failed		
SCIN	rs1404519	A/C	0.144	0.133	0.572
SCIN	rs702477	T/C	0.350	0.330	0.458
SCIN	rs886890	G/A	0.462	0.449	0.651

Table 3, continued

Gene	SNP	Alleles	Minor allele frequency		p-value
			Affected siblings	Healthy controls	
SCIN	rs17357392	A/G	0.242	0.263	0.391
SCIN	rs2708656	A/G	0.204	0.214	0.659
SCIN	rs17166250	T/C	0.058	0.063	0.715
SCIN	rs10244231	G/T	0.141	0.128	0.510
SCIN	rs849763	A/G	0.304	0.319	0.563
SCIN	rs2691815	A/G	0.423	0.456	0.225
SCIN	rs2691814	C/G	0.323	0.293	0.258
SCIN	rs1180397	A/C	Failed		
SCIN	rs2033604	G/A	0.331	0.361	0.269

P-values<0.05 indicated in bold. Failed: failed to genotype correctly.

Table 4

Association analysis for 40 SNPs in the chromosomal region Xp

Gene	SNP	Alleles	Minor allele frequency				p-value		
			Affected siblings		Healthy controls		Men	Women	Overall*
			Men	Women	Men	Women			
HS6ST2	rs5933172	A/G	0.135	0.115	0.129	0.132	0.901	0.485	0.695
HS6ST2	rs243440	C/G	0.152	0.121	0.117	0.141	0.553	0.641	0.986
HS6ST2	rs243444	G/A	0.209	0.142	0.157	0.158	0.184	0.526	0.743
HS6ST2	rs17317084	C/T	0.117	0.148	0.135	0.138	0.563	0.652	0.997
HS6ST2	rs5930575	A/G	0.019	0.034	0.032	0.022	0.629	0.468	0.791
HS6ST2	rs7877499	G/A	0.141	0.135	0.141	0.139	0.970	0.850	0.916
HS6ST2	rs5975351	C/T	0.019	0.056	0.047	0.084	**0.024**	0.415	0.076
HS6ST2	rs5975352	A/G	0.148	0.134	0.143	0.138	0.812	0.825	0.985
HS6ST2	rs12844906	G/A	0.075	0.060	0.048	0.055	0.330	0.640	0.415
HS6ST2	rs17390	T/C	0.372	0.351	0.360	0.399	0.807	0.330	0.439
HS6ST2	rs17324216	C/T	0.132	0.145	0.157	0.148	0.582	0.799	0.643
HS6ST2	rs4830252	C/T	0.327	0.328	0.299	0.376	0.690	0.587	0.567
HS6ST2	rs2872900	C/T	0.155	0.157	0.137	0.126	0.476	0.281	0.280
HS6ST2	rs6638047	C/T	0.037	0.049	0.079	0.073	0.119	0.122	0.053
CD40LG	rs5930970	C/G	0.428	0.474	0.372	0.412	0.531	**0.026**	0.070
CD40LG	rs17341637	G/A	0.123	0.142	0.141	0.111	0.991	0.403	0.573
CD40LG	rs3092949	G/A	0.335	0.308	0.406	0.356	0.407	**0.041**	0.071
CD40LG	rs1126535	T/C	0.173	0.196	0.127	0.175	0.658	0.138	0.225
CD40LG	rs5930973	G/A	0.055	0.067	0.055	0.057	0.965	0.537	0.696
CD40LG	rs715762	C/T	0.105	0.107	0.079	0.096	0.602	0.416	0.450
CD40LG	rs7050168	A/G	0.025	0.024	0.000	0.022	0.402	0.285	0.270
CD40LG	rs3092923	T/C	0.080	0.096	0.04	0.096	0.828	0.272	0.401
F9	rs6528633	A/T	0.331	0.346	0.287	0.341	0.817	0.429	0.517
F9	-816	G/A	0.012	0.02	0.008	0.035	0.391	0.700	0.470
F9	rs411017	G/A	0.273	0.341	0.387	0.352	0.053	0.473	0.133

Table 4, continued

Gene	SNP	Alleles	Minor allele frequency				p-value		
			Affected siblings		Healthy controls		Men	Women	Overall*
			Men	Women	Men	Women			
F9	rs401597	C/T	0.241	0.286	0.352	0.371	0.058	0.198	0.061
F9	rs371000	T/C	0.425	0.411	0.371	0.433	0.751	0.963	0.842
F9	rs392959	C/T	0.055	0.044	0.071	0.059	0.701	0.211	0.297
F9	rs4149755	T/A	0.093	0.067	0.063	0.058	0.203	0.680	0.346
F9	rs408567	G/A	0.207	0.229	0.223	0.236	0.563	0.927	0.714
F9	rs6048	A/G	0.228	0.302	0.349	0.321	**0.024**	0.367	0.072
F9	rs4149759	T/C	0.05	0.089	0.103	0.067	0.235	0.651	0.775
F9	rs4149730	C/T	0.025	0.038	0.023	0.039	0.640	0.734	0.992
F9	rs4149762	G/A	0.031	0.058	0.048	0.052	0.340	0.615	0.897
F9	rs4149751	A/C	Failed						
F9	rs440051	G/A	0.217	0.21	0.190	0.240	0.935	0.705	0.768
F9	32781	A/G	0.000	0.017	0.000	0.005	0.500	**0.041**	0.180
F9	rs434144	C/G	0.214	0.215	0.234	0.255	0.437	0.282	0.257
F9	rs17340148	C/T	0.098	0.067	0.032	0.067	0.080	0.430	0.154
F9	rs4825206	C/T	0.453	0.474	0.472	0.498	0.493	0.691	0.530
F9	rs5907573	T/A	0.462	0.497	0.472	0.495	0.556	0.900	0.821

* Sex-adjusted p-value. P-values<0.05 indicated in bold. Failed: failed to genotype correctly.

GPR30 haplotypes

Because almost half of the *GPR30* SNPs were associated with venous thromboembolism, we also assessed the effect of *GPR30* haplotypes on the risk of venous thromboembolism. Haplo.stats analysis indicated that eight common (frequency>1%) haplotypes were present in *GPR30* (Table 5). Haplotype 4, tagged by the rare alleles of rs10262070 and rs10235056, was associated with an increased risk of venous thromboembolism (OR=1.6; 95% CI: 1.0-2.5). Haplo.stats also indicated a haplotype which was tagged only by the rare allele of rs10262070. This haplotype was rare (frequency=0.8%) and was not associated with the risk of venous thromboembolism (OR=1.0; 95% CI: 0.2-4.2). All other *GPR30* haplotypes were not associated with the risk of venous thromboembolism. This includes haplotype 2, which is tagged by the rare alleles of rs10262070 and rs1997243 (OR=1.4; 95% CI: 0.9-2.0).

Table 5

Risk of venous thromboembolism for the eight common haplotypes of *GPR30*

H	Haplotype tagging SNPs										Frequency		OR	95% CI
	rs1997243	rs10269151	rs10235056	rs3808353	rs3808354	rs1133043	rs13242034	rs10262070	rs12113381	rs4236375	Index patients n=191*	Controls n=305*		
H1	A	G	G	G	G	C	G	C	C	T	0.35	0.37	1‡	
H2	G	G	G	G	G	G	G	T	C	C	0.17	0.13	1.4	0.9 - 2.0
H3	A	G	G	A	G	G	A	C	C	C	0.15	0.13	1.2	0.8 - 1.8
H4	A	G	A	G	G	G	G	T	C	C	0.13	0.09	1.6	1.0 - 2.5
H5	A	G	G	G	T	G	G	C	T	T	0.09	0.13	0.7	0.5 - 1.2
H6	A	A	G	G	G	G	A	C	C	C	0.02	0.04	0.6	0.3 - 1.3
H7	A	G	G	A	G	G	G	C	T	C	0.03	0.03	0.9	0.4 - 1.9
H8	A	G	G	G	T	G	G	C	C	C	0.01	0.03	0.6	0.2 - 1.7

Minor allele frequency				
Index patients§	Controls§	ORℓ	95% CI	
0.17	0.14	1.3	0.9 - 1.8	
0.03	0.04	0.7	0.3 - 1.4	
0.13	0.09	1.5	1.0 - 2.3	
0.17	0.16	1.1	0.8 - 1.5	
0.11	0.17	0.4	0.3 - 0.6	
0.36	0.37	0.9	0.7 - 1.2	
0.18	0.18	0.7	0.7 - 1.4	
0.29	0.22	1.5	1.1 - 1.9	
0.13	0.17	0.7	0.5 - 1.0	
0.45	0.51	0.8	0.6 - 1.0	

* Exclusion because of incomplete genotype: 20 patients and 26 controls.

‡ Reference category.

§ rs1997243 and rs1133043 (208 patients, 329 controls), rs10269151 (209 patients, 326 controls), rs10235056 (210 patients, 329 controls), rs3808353 (209 patients, 324 controls), rs3808354 (201 patients, 330 controls), rs13242034 (209 patients, 320 controls), rs10262070 (210 patients, 322 controls), rs12113381 (208 patients, 327 controls) and rs4236375 (200 patients, 329 controls).

ℓ Allelic odds ratio.

RAC1 haplotypes

Because *RAC1* is the closest gene to the position of the maximum LOD score of the chromosome 7p linkage peak showing some evidence for association with venous thromboembolism, we further investigated this gene. Haplo.stats analysis indicated the presence of eight common (frequency>1%) haplotypes in *RAC1* (Table 6). Haplotype 4 was associated with a 3.3-fold decreased risk of venous thromboembolism (OR=0.3; 95% CI: 0.2-0.7; p=0.003) when compared to the most common (reference) haplotype

1. The A allele (frequency=49%) of rs836480, which was found to be associated with a reduced risk of venous thromboembolism (p=0.038), is present in all haplotypes except in the reference haplotype, haplotype 1. This suggests that the haplotype 1, harbouring the C allele (frequency=51%) of rs836480, itself might be associated with an increased risk of venous thromboembolism. To investigate this, we compared haplotype 1 with the group of all other haplotypes. Using this approach, we found that haplotype 1 was associated with an increased risk of venous thromboembolism (OR=1.3; 95% CI: 1.0-1.7; p=0.04).

Table 6
Risk of venous thromboembolism for the eight common haplotypes of *RAC1*

H	Haplotype tagging SNPs						Frequency		OR	95% CI
	rs10951982	rs836480	rs702484	rs6463554	rs836547	rs6954996	Index patients n=197*	Controls n=318*		
H1	G	C	C	G	G	G	0.56	0.50	1‡	
H2	A	A	G	G	G	G	0.16	0.17	0.8	0.6 - 1.2
H3	G	A	C	C	G	G	0.08	0.11	0.7	0.4 - 1.0
H4	A	A	G	G	T	G	0.02	0.06	0.3	0.2 - 0.7
H5	G	A	C	G	G	G	0.04	0.04	0.8	0.5 - 1.6
H6	G	A	C	G	G	A	0.05	0.04	1.0	0.5 - 1.9
H7	G	A	G	G	T	G	0.03	0.03	1.0	0.5 - 2.1
H8	G	A	G	C	G	G	0.03	0.02	1.6	0.7 - 3.7

* Exclusion because of incomplete genotype: 14 patients and 13 controls.
‡ Reference category.

To test whether rs836480, which tags haplotype 1 of *RAC1*, contributes to the linkage signal on chromosome 7p, we performed an association given linkage analysis. The significance level of the association analysis increased when linkage was taken into account; p=0.038 for the association analysis versus p=0.014 for the association given linkage analysis. This indicates that rs836480 contributes to the linkage signal on chromosomal region 7p.

Discussion

In our search for novel genetic risk factors for venous thromboembolism, eleven candidate genes were screened as possible novel susceptibility genes for venous thromboembolism. These genes were selected from two thrombosis susceptibility regions, previously identified using a genome-wide linkage scan in affected sibling pairs. In total, 106 haplotype tagging SNPs were genotyped in these candidate genes in all GIFT individuals and in a panel of healthy controls.

We found that inclusion of SNP genotypes to our previous linkage analysis only slightly changed the linkage signals in chromosomal regions 7p and Xq, supporting the linkage evidence at both regions. Several SNPs were associated with venous thromboembolism, rs10262070 in *GPR30* (p=0.002) and rs13234810 in *LFNG* (p=0.007) showing the strongest effects.

GPR30 codes for the G-protein-coupled receptor 30,[30] a member of the G-protein-coupled receptor superfamily (GPCRs) that acts as an estrogen receptor.[31] GPCRs are membrane-bound proteins that, upon activation, induce a cellular response by activation of a G-protein.[32] Some GPCRs are involved in hemostasis.[33,34] *GPR30* expression has been reported in several tissues, including the liver.[35] This makes *GPR30* an interesting candidate gene. Haplotype analysis indicated that haplotype 4 of *GPR30* was associated with a 1.6-fold increased risk of venous thromboembolism. The minor allele frequencies of rs10262070 and rs10235056 (tagging SNPs for haplotype 4) in GIFT index patients (frequency=29% and 13%) were similar to that of HapMap Europeans (frequency=29% and 12%).[14] Our control group showed a somewhat lower frequency (22% and 9%). Future haplotype analysis in population-based case-control studies will be needed to confirm our findings. Haplotype 4 is tagged by the rare alleles of rs10262070 and rs10235056. Rs10262070 is located in the 3'-flanking region of *GPR30* in a region that does not contain any obvious regulatory elements that could indicate that this SNP is a functional variant. There are, however, several SNPs which are in LD with rs10262070. This includes rs1133122 in *ZFAND2A*, a gene that encodes a facultative subunit of the proteasome. Rs1133122 is located in the 3'-untranslated region, six nucleotides upstream of the polyadenylation signal of *ZFAND2A*. Rs10235056 is located in the 3'-untranslated region of *GPR30*. Another gene, *MGC11257*, is present at the complementary strand at the location of *GPR30* and encodes the hypothetical protein LOC84310. To our knowledge, neither rs10262070 nor rs10235056 have previously been reported as being associated with disease. Even though variants of *GPR30* may be associated with venous thromboembolism, it is unlikely that these variants contribute to the chromosome 7p linkage signal because *GPR30* is located 7.6 cM (~15.2 Mb) from the maximum LOD score of the linkage peak in a region with a LOD score of 1.03.

RAC1 encodes the protein Rac1,[36] a member of the Rho family of the Ras superfamily of small GTP-binding proteins. Rho GTPases are involved in a broad range of cellular events, including regulation of the cytoskeleton, cell proliferation and gene expression.[37] The finding that *RAC1* deficient mice have diminished platelet secretion and aggregation as well as prolonged bleeding time made *RAC1* a promising candidate gene.[38] The C allele of rs836480 in *RAC1,* which tags haplotype 1, the most common haplotype of *RAC1,* was found to be associated with an increased risk of

venous thromboembolism (OR=1.3; 95% CI: 1.0-1.7; p=0.04). Both alleles of rs836480 have about the same frequency (~0.5), indicating that common alleles can influence the risk of venous thromboembolism. An association given linkage analysis indicated that rs836480 explains part of the linkage signal on chromosome 7p. Rs836480 is located in intron 2 of *RAC1* in a region that does not contain any obvious regulatory elements which could indicate that rs836480 is a functional variant. To our knowledge, no association with disease has previously been reported for rs836480.

F9 is the only selected candidate gene that encodes a known coagulation factor, namely factor IX. This made *F9* a strong and obvious candidate gene. Rs6048, also known as factor IX Malmö,[39] was associated with a decreased risk of venous thromboembolism in GIFT men. Factor IX Malmö is a coding SNP in exon 6 (Thr148Ala) of *F9* and is located in the activation peptide of factor IX. We previously found in the Leiden Thrombophilia Study (LETS) that rs6048 was associated with a decreased risk of venous thrombosis in men (see Chapter 2.3).[11] In a large discovery study, the same SNP was associated with a decreased risk of venous thrombosis in a combined study population of LETS and the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis study (MEGA-1).[10] However, that finding was not replicated in MEGA-2.[10] Both rs6048 and 32781A/G did not contribute to the linkage signal on chromosomal region Xq. This indicates that variants in other genes are causing the linkage signal on the X chromosome. We were unable to perform a haplotype analysis for *F9*, because of its complex haplotype structure. Haplo.stats analysis indicated that more than 90 *F9* haplotypes (23 haplotypes with frequency>1%) were present in GIFT women and about 50 haplotypes (22 haplotypes with frequency>1%) in GIFT men.

In conclusion, we screened eleven candidate genes as novel susceptibility genes for venous thromboembolism. Although several SNPs were associated with venous thromboembolism, only rs836480 in *RAC1* explains a part of the chromosomal 7p linkage peak. Therefore, variants in genes, other than those screened in the present study, might exist that explain the observed linkage signals on chromosome 7p and Xq. Additional studies are needed to select and screen these genes as susceptibility genes for venous thromboembolism.

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Chapter 4

General discussion and summary

Background

Venous thrombosis is a common disorder in which a blood clot (thrombus) is formed in a vein, most commonly affecting the deep veins of the leg. It is a major health concern in the Western world, with an annual incidence of one to three per thousand individuals.[1,2] Venous thrombosis is considered to be a multifactorial disease in which both acquired (e.g. use of female hormones, increasing age, immobilization)[3] and genetic risk factors are involved and interact to cause the disease.[4,5] Genetic risk factors include the deficiencies of protein C,[6,7] protein S,[8] and antithrombin,[9,10] the factor V Leiden[11] and prothrombin 20210A mutations,[12] and ABO blood group non-O.[13-15] A heritability of 50-60% was estimated for venous thrombosis,[16-18] suggesting that genetic factors play an important role in the pathogenesis of venous thrombosis. Familial thrombophilia, the clustering of venous thrombosis within families, is considered to be an oligogenetic disease in which at least two genetic defects segregate in the family.[19-22] However, in the majority of these thrombophilic families none or only one of the known genetic risk factors is found, indicating that additional genetic risk factors remain to be identified.[5] Furthermore, several plasma phenotypes (e.g. elevated levels of factors VIII and IX) have been reported that influence thrombosis risk.[12,23-29] The putative genetic determinants of these plasma phenotypes are still poorly understood.

Aim and approach

The aim of the studies described in this thesis was to identify novel genes or genomic regions that contribute to the susceptibility to venous thrombosis. This will contribute to a better understanding of the development of venous thrombosis, and can eventually lead to a better diagnosis, treatment and prevention of venous thrombosis.

In this thesis we used two different approaches to identify novel susceptibility genes for venous thrombosis: the hypothesis-based candidate gene approach and the discovery-based genome-wide approach.

In the candidate gene approach the association between a variant in a candidate gene (e.g. a single nucleotide polymorphism (SNP)) and a phenotype (e.g. factor VIII levels) or a disease (venous thrombosis in our case) is investigated. This approach was previously successfully used to identify genetic variants segregating in thrombophilia families and causing the deficiencies of antithrombin,[9,30] protein C[6,31] and protein S.[8,32] A few years ago, it was shown that the human genome can be divided into haplotype blocks of strong linkage disequilibrium.[33,34] This finding made it possible to capture most of the common variations within a gene without genotyping all the genetic variants; the so-called haplotype-based candidate gene approach.[35] In

this strategy, haplotype-tagging SNPs, serving as a proxy for other SNPs within the haplotype, are genotyped in a large case-control study to test whether haplotypes are associated with a phenotype or disease. The haplotype-based candidate gene approach was used in **Chapter 2** of this thesis. We selected both coagulation-related candidate genes and genes outside the classical coagulation pathways that might influence the risk of venous thrombosis.

Genome-wide linkage analysis is the other main approach used in this thesis. This approach can be used to screen the whole genome for susceptibility regions for a disease. This screening is usually performed by genotyping several hundreds of genetic markers across the genome. The underlying idea is that, within a study population of related individuals (e.g. sibling pairs, extended families) a genetic marker is segregating together with the trait. This trait can either be a disease (venous thrombosis) or an intermediate phenotype (e.g. factor VIII levels). We have performed a non-parametric genome-wide linkage analysis using affected sibling pairs in **Chapter 3** of this thesis.

Interleukin-1

Interleukin-1 (IL-1) is a pro-inflammatory cytokine that plays an important role in autoimmune and inflammatory diseases (e.g. inflammatory bowel disease) by stimulating the expression of genes involved in the innate and adaptive immune response.[36] The IL-1 family includes the agonists IL-1α and IL-1β (which is the predominant form in humans), and the IL-1 receptor antagonist (IL-1Ra).[37] All three proteins can bind to the functional IL-1 receptor type I (IL-1R1) or to the non-signaling receptor type II (IL-1R2).[38] IL-1Ra acts as a natural inhibitor of IL-1α and IL-1β by binding to the IL-1R1 receptor and blocking IL-1 signaling.[37]

Inflammation is thought to play a role in the development of thrombosis by influencing both coagulation and fibrinolysis.[39,40] The pro-inflammatory cytokines IL-6, IL-1 and tumor necrosis factor (TNF) can up-regulate tissue factor expression in monocytes, thereby promoting coagulation.[41] The latter two cytokines can also promote coagulation by down-regulating the expression of thrombomodulin and the endothelial protein C receptor on endothelial cells.[39] Furthermore, TNF and IL-1 can influence fibrinolysis by decreasing the production of tissue-type plasminogen activator and increasing the production of plasminogen activator inhibitor in endothelial cells.[39,42]

In **Chapter 2.1**, we investigated whether genetic variants of *IL1B*, *IL1RN*, *IL1R1* and *IL1R2*, respectively encoding IL-1β, IL-1Ra, IL-1R1 and IL-1R2, were associated with the risk of venous thrombosis. These four genes were selected as candidate

genes because of the overall prothrombotic effect of IL-1.[39-41] We hypothesized that variants in these four genes influence the risk of venous thrombosis by modulating the IL-1 pathway. To test this hypothesis we genotyped all subjects of the Leiden Thrombophilia Study (LETS)[43,44] for 18 haplotype-tagging SNPs in *IL1B*, *IL1RN*, *IL1R1* and *IL1R2*. This approach enabled us to detect a total of 25 haplotypes in these genes. An overall test of association under a recessive model indicated a significant difference in the haplotype frequency distribution between patients and healthy controls for *IL1RN*. Subsequent analyses showed that homozygous carriers of haplotype 5 had an almost 4-fold increased risk of venous thrombosis. This finding needs to be confirmed in other studies, especially because the number of homozygous haplotype 5 carriers in the LETS study was low. If this finding is replicated, the question arises which variant in haplotype 5 causes the increase in risk of venous thrombosis. There is no clear candidate for a functional SNP in haplotype 5. The SNP tagging haplotype 5 (rs2232354), which itself is also associated with an increased risk of venous thrombosis, is located in intron 2 of *IL1RN* in a highly polymorphic region that does not contain any obvious regulatory elements. Sequencing of homozygous haplotype 5 carriers might help to identify the functional SNP(s), because it might reveal a more obvious candidate SNP in (part of) the homozygous haplotype 5 carriers. We further observed that allele 2 of the frequently studied intron 2 variable number of tandem repeats (VNTR) of *IL1RN* is part of haplotype 3. This VNTR is interesting, because allele 2 of this variant has been associated with many different diseases.[45] However, no association was found between haplotype 3 of *IL1RN* and the risk of venous thrombosis.

Inflammation is strongly implicated in the pathogenesis of atherosclerosis.[46-49] IL-1 promotes atherosclerosis by stimulating the endothelial surface to increase the expression of leukocyte adhesion molecules.[50] Rupture of the atherosclerotic plaque can lead to thrombus formation, causing arterial thrombosis and myocardial infarction.[46,47] It is not clear whether variations in *IL1RN* also affect the risk of myocardial infarction. Reports on the relationship between the intron 2 VNTR of *IL1RN* and cardiovascular diseases have been conflicting.[51-54] In **Chapter 2.2** we therefore investigated the role of *IL1RN* haplotypes, mRNA levels of *IL1RN* and the risk of myocardial infarction in the Study of Myocardial Infarctions Leiden (SMILE). We found that haplotype 3 carriers had decreased *IL1RN* mRNA levels and an increased risk of myocardial infarction. Reduced *IL1RN* expression is expected to result in decreased levels of IL-1Ra. Lower IL-1Ra levels are likely to promote the inflammatory and prothrombotic effect of IL-1, because less IL-1Ra is available to function as an antagonist of IL-1.

Haplotype 3 was tagged by rs419598, a synonymous SNP in exon 2 of *IL1RN*. It is unknown whether rs419598 itself is a functional polymorphism. Rs419598 was not associated with myocardial infarction in the Physician's Health Study.[55] In this prospective study, seven SNPs in the IL-1 cluster (one in *IL1A*, five in *IL1B* and rs419598 in *IL1RN*) were genotyped. SeattleSNPs data indicated that about 50 polymorphisms are unique for haplotype 3 of *IL1RN*,[56] making it difficult to identify the functional SNP. However, a promising candidate is the intron 2 VNTR, which is strongly linked to rs419598.[57] It has been suggested that each repeat of the VNTR contains several transcription factor binding sites.[58] Because the rare allele of the VNTR (allele 2) consists of two repeat units, while the common allele 1 contains four repeat units, one would expect a decreased transcriptional activity for this variant. Such a hypothesis would be supported by our finding that haplotype 3 carriers have a decreased *IL1RN* mRNA expression. However, in most other studies no association was found between allele 2 of the intron 2 VNTR and vascular disease.[52-54] There are conflicting results in the literature about the effect of allele 2 of the intron 2 VNTR on production and plasma levels of IL-1Ra.[59-63] More information is needed on how variations in *IL1RN* relate to transcriptional activity, IL-1Ra production in various tissues and plasma levels of IL-1Ra.

Although we have selected four prominent proteins of the IL-1 signaling system, variants in other proteins involved in this system may also effect inflammation and thereby the risk of venous thrombosis or myocardial infarction. There are several additional candidate genes in the IL-1 signaling pathway. One of these is IL-1α, another member of the IL-1 family.[37] IL-1α is generally not found in the circulation, because most IL-1α is transported to the plasma membrane or the cell nucleus. The role of membrane bound IL-1α in the pathogenesis of disease remains unclear. Membrane bound IL-1α can be released in the circulation from dying cells or by calpain-mediated cleavage.[64] In hypercholesterolemic monkeys, IL-1α mRNA expression was found in several types of arterial cells, including vascular smooth muscle cells (VSMC).[65] Cellular IL-1α also promotes the proliferation of VSMC.[66] These findings suggest that IL-1α might contribute to the pathogenesis of atherosclerosis. Another candidate is the IL-1 receptor accessory protein, IL-1R AcP. This protein forms a complex together with IL-1R1 and IL-1α or IL-1β, and is essential for IL-1 signaling.[67] IL-18 is also a member of the IL-1 family that seems to play a key role in atherosclerosis.[68] Variations in the IL-18 gene may influence IL-18 levels and the risk of coronary artery disease.[69] Besides IL-18, there are several other relatively newly found members of the IL-1 family (IL-1F5 to IL-1F11)[70] and IL-1 receptor family (IL-1R4 to IL-1R9).[71] The roles of these proteins in inflammation are not yet completely understood. Whether genetic variants in IL-1α, IL-1R AcP and other members of the IL-1 family and IL-1 receptor family influence the risk of

venous thrombosis and/or myocardial infarction remains largely to be investigated.

In contrast to the hypothesis that inflammation influences the risk of venous thrombosis, it is also possible that inflammation is a result of venous thrombosis rather than a cause.[72] This would explain the finding that elevated levels of pro-inflammatory cytokines, measured after the thrombotic event, are associated with the risk of venous thrombosis,[73] whereas elevated levels of these cytokines were not associated with venous thrombosis in a prospective study.[74]

In conclusion, variations in the IL-1 receptor antagonist gene seem to play a role in both the risk of venous thrombosis and myocardial infarction. However, replication studies are needed to confirm these findings. If replicated, further research is needed to unravel the mechanisms that underlie these increased risks.

Factor IX

Factor IX (FIX) is a vitamin K dependent glycoprotein that plays a key role in hemostasis by activating factor X, a process which eventually leads to thrombin and clot formation.[75,76] Individuals with FIX plasma levels above the 90th percentile (>129 U/dl), as measured in healthy subjects, have a 2 to 3-fold increased risk of deep venous thrombosis compared to individuals with levels below this cutoff value.[27] Adjustment for confounders only marginally affected the risk estimates for high FIX levels.[27,77,78] The mechanisms that underlie elevated FIX levels are unknown. No major quantitative trait loci for FIX were found in a genome wide scan searching for genetic determinants of plasma FIX levels.[79]

In **Chapter 2.3**, we aimed at identifying genetic variants in the FIX gene (*F9*) that could explain elevated FIX levels and thrombosis risk. We sequenced the coding regions, splice junctions, the 5'- and 3'-untranslated regions and the 5'- and 3'-flanking regions of *F9* in nineteen men from the LETS study, all with an isolated elevated plasma FIX antigen level. Two rare variants were found, -816G/A and 32781G/A (minor allele frequencies 1.7% and 0.4%, respectively). Both variants were not associated with FIX levels and thrombosis risk in the LETS population. These two variants were recently also reported in subjects of the GAIT study, with similar frequencies.[79] In the GAIT study no association was found between both variants and FIX activity. Because of the low frequencies of -816G/A and 32781G/A, very large studies are needed to study the association of these variants with FIX levels.

As a second approach, we genotyped all LETS subjects for six SNPs, together tagging the eight most common haplotype groups of *F9*, and investigated the relationship between *F9* SNPs, *F9* haplotypes, FIX levels and the risk of venous thrombosis. Several

SNPs seemed to decrease the risk of venous thrombosis. The most interesting SNP was the exon 6 SNP 20422A/G (also known as FIX Malmö), which is located in the activation peptide of factor IX.[80] After completion of our study, this polymorphism was also identified in the LETS population as associated with venous thrombosis as part of a large discovery study involving 19,682 SNPs predicted to affect gene function.[81] In our study, no effect on FIX levels and thrombotic risk was observed for -793G/A. In contrast with this finding, female carriers of -698C, which is completely linked to -793G, were recently reported to have higher FIX levels than -698T carriers.[82] The authors explained their observation by a closer homology to a polymorphic oestrogen response element in the *F9* promoter for -698C than -698T.

We observed a two-fold decreased risk of venous thrombosis for men carrying haplotype 6. The functional polymorphism of this haplotype still has to be identified. Haplotype 6 is tagged by a combination of four SNPs. These SNPs also individually decrease the risk of venous thrombosis. The only coding polymorphism among these SNPs is the FIX Malmö SNP. This polymorphism is also present in haplotypes 4 and 7, which both show a similar but not significant effect on venous thrombosis risk as haplotype 6, most likely because of the low number of haplotype 4 and haplotype 7 carriers. 32056G/A and 33566C/G are the only two polymorphisms in our study that are unique for haplotype 6. The regions around these SNPs, however, do not contain any obvious regulatory elements, which would predict that these SNPs are functional variants. Another possibility is that the actual functional SNP is a SNP which is linked to one of the tagging SNPs of haplotype 6. Obviously, the effect of haplotype 6 needs to be validated in other studies. In a recent study, no association was found between our haplotype 6 and the risk of venous thrombosis in postmenopausal women.[83] In the same study, a subhaplotype of our haplotype 1 (which we used as the reference haplotype in the analyses) was associated with a 1.5-fold increased risk of venous thrombosis. To compare this finding with our data, we calculated the risk of haplotype 1 compared to the group of all other haplotypes. We found a 1.5-fold increased risk for haplotype 1 in LETS men. However, no effect on the risk of venous thrombosis was found in women. None of the haplotypes had an effect on the risk of venous thrombosis in the subgroup of postmenopausal LETS women.

No clear association was found between FIX levels and the *F9* SNPs in men and women, and between FIX levels and *F9* haplotypes in men. There seems to be a trend towards higher FIX levels for haplotype 6 carriers (110 U/dl) compared to haplotype 1 carriers (103 U/dl), but this effect was not significant (p=0.06). In a genome-wide scan for genetic determinants of FIX levels in men and women of the GAIT study, no major determinants were found in or outside the FIX gene.[79] It is possible that a

set of genetic determinants with small or modest effects, not located in the *F9* locus, together contribute to high FIX levels.

Factor VII-activating protease

Factor VII-activating protease (FSAP) is a plasma serine protease that was first described by Choi-Miura *et al*. in 1996.[84] This Japanese group isolated a plasma protein that showed a high affinity for hyaluronan, a non-sulfated glycosaminoglycan,[85] and therefore named the protein Plasma Hyaluronan Binding Protein (PHBP). Later, this protein was called FSAP, because of its ability to activate factor VII (FVII).[86] Besides promoting coagulation by activating FVII, FSAP can also promote fibrinolysis by activating single chain plasminogen activators.[87] In 2002, a SNP (1601G/A) was discovered in the gene coding for FSAP, which results in a glycine to glutamic acid substitution in the protease domain (G511E). The 1601A variant of FSAP, also known as *Marburg I*, is a weak activator of pro-urokinase, whereas it can still activate FVII normally.[88] In **Chapter 2.4**, we challenged the conclusion of Hoppe *et al*., who reported that *Marburg I* carriers had an 3.5-fold increased risk of venous thrombosis.[89] We did not confirm their finding as we found no association between FSAP *Marburg I* and venous thrombosis in the LETS.[90] A possible explanation for this discrepancy is the low frequency of the *Marburg I* allele in the control group used by Hoppe *et al*. Later, Hoppe *et al*. re-analyzed their data using a different control group and found that the association between FSAP *Marburg I* and venous thrombosis was limited to idiopathic cases.[91] However, in the meantime several other studies have confirmed our findings.[92-96] Although current studies do not support an effect of FSAP *Marburg I* on the risk of venous thrombosis, very large studies might be necessary to draw definitive conclusions.

Besides its effect on coagulation and fibrinolysis, FSAP may have more important functions outside hemostasis. It was shown that FSAP is present in atherosclerotic lesions, especially in unstable atherosclerotic plaques,[97] and is an inhibitor of platelet-derived growth factor BB-induced proliferation and migration of vascular smooth muscle cells[98] and of basic fibroblast growth factor/epidermal growth factor-dependent proliferation of human umbilical vein endothelial cells.[99] Using a vascular injury mouse model, it was demonstrated that FSAP also acts as a potent inhibitor of neointima formation.[100] FSAP *Marburg I* is a weaker inhibitor of neointima formation,[100] which might explain the observed association between FSAP *Marburg I* and carotid stenosis[101] and cardiovascular disease.[102] FSAP can also stimulate the release of the vasodilator bradykinin by cleavage of high molecular weight kininogen.[103] Furthermore, FSAP may inhibit angiogenesis *in vivo*.[104]

Although the current research suggests that FSAP is a regulatory factor in the vascular system, future studies will be needed to further unravel the function of FSAP in hemostasis and cardiovascular diseases. Furthermore, it is possible that FSAP plays a role in other (regulatory) systems/pathways than those studied to date.

Genetics In Familial Thrombosis (GIFT) study

In **Chapter 3**, we used a genome-wide linkage approach to systematically scan the genome for genes or genomic regions that contribute to the susceptibility to venous thromboembolism. This approach was previously used in two other thrombosis-related studies, the Vermont study and the GAIT (Genetic Analysis of Idiopathic Thrombophilia) study.[16,105,106] The aim of the genome-wide scan in the Vermont study was to identify a second genetic defect which, together with protein C deficiency, would explain the high frequency of venous thrombosis in a large protein C deficient pedigree of French-Canadian descent (kindred Vermont II).[105,106] The Spanish GAIT study mainly searched for quantitative trait loci (QTL) influencing plasma levels of hemostasis-related proteins and other factors.[16] These plasma levels include, among others, factor XII,[107] activated protein C resistance,[108] von Willebrand factor,[109] free protein S,[110] protein C,[111] fibrinogen,[112] homocysteine,[113] tissue factor pathway inhibitor,[114] factor VII[115] and factor IX.[79] Both the Vermont and GAIT study used extended families for their analyses. In the Genetics In Familial Thrombosis (GIFT) study, we used affected sibling pairs to perform a genome-wide linkage scan for venous thromboembolism.

Detailed characteristics of the GIFT study are described in **Chapter 3.1**. We collaborated with 29 Anticoagulation Clinics throughout the Netherlands to approach siblings with venous thromboembolism. After data cleaning, 211 families of Caucasian descent (460 individuals, 287 affected sibling pairs) with at least two siblings with an objectively confirmed venous thromboembolic event at a young age (≤45 years) were included in the GIFT study. Almost half of the GIFT patients (47.3% of men, 45.1% of women) developed a second venous thromboembolic event later in life. About 44% of the sibships reported one parent who also developed a venous thromboembolic event during his or her life, whereas 5% of the sibships had two parents with venous thromboembolic disease. The GIFT study population was enriched for factor V Leiden (36.5% in GIFT index patients versus 19.5% in consecutive thrombosis patients and 3% in the general population)[116] and ABO blood group non-O (82.9% versus 70.9% and 57%).[14] In 34% of men and 91% of women at least one acquired risk factor was present at the time of their first venous thromboembolic event. The most frequent acquired risk factor for women was oral contraceptive use (65%).

In **Chapter 3.2**, we reported the results of the genome-wide linkage scan in the GIFT study that was performed using 402 microsatellite markers. The two highest linkage signals were found on chromosome 7p and chromosome Xq. Both regions were followed up with extra markers that were also typed in parents and unaffected siblings. The linkage results support the presence of novel thrombosis susceptibility regions at 7p21.3 (LOD score=3.09, p=0.00008) and on Xq25-q26.3 (LOD score=1.86, p=0.002). However, only the chromosome 7p linkage signal was genome-wide significant (p=0.029). No evidence for linkage at the chromosome 7p region was found in the Vermont and GAIT studies.[16,106] Both studies excluded the X chromosome in their linkage analysis. In the Vermont study, three regions were identified (11q23, 10p12 and 18p11.2-q11.2) that might contain novel thrombosis susceptibility genes.[106] The latter two regions were also found in the GAIT project in a genome scan for QTLs influencing plasma levels of factor XII and activated protein C resistance, respectively.[107,108] No strong (i.e. obviously coagulation-related) candidate genes were found in the chromosomal regions 10p12 and 18p11.2-q11.2. The gene (*PAFAH1B2*) coding for the alpha(2) subunit of platelet-activating factor acetylhydrolase isoform 1b (PAFAH1B2) was selected in the Vermont study as candidate gene from the 11q23 region. PAFAH1B2 is a downregulator of the inflammatory activity of PAF.[117] Deficiency of PAFAH1B2 might therefore promote inflammation, thereby also promoting thrombosis. However, a subsequent study excluded PAFAH1B2 as risk factor for venous thromboembolism.[118] In our genome scan, no evidence for linkage with venous thromboembolism was found in these three regions.

Although the GIFT population was enriched for factor V Leiden and ABO blood group non-O, low linkage signals were found at the locations of the genes coding for factor V Leiden (*F5*) and ABO blood group (*ABO*). This finding can be explained by the observation that a number of sibling pairs carrying factor V Leiden that share no allele(s) identical-by-descent (IBD=0) because of the presence of more than one factor V Leiden allele in their parents (two heterozygous parents or one homozygous parent). These kind of families do negatively contribute to the LOD score. Furthermore, twelve percent of the sibling pairs is discordant for factor V Leiden (i.e. one sibling carries the mutation, whereas the other sibling does not carry the mutation) and these pairs do not (IBD=1) or negatively (IBD=0) contribute to the LOD score. This observation may predict that novel genetic risk factors which are as common as factor V Leiden probably will not be found in the GIFT population using a genome-wide linkage scan. Similar to factor V Leiden, ABO blood group non-O is likely too common to be detected as a risk factor in our linkage analysis. Furthermore, because of the existence of the allelic variants A and B, blood group non-O carriers not necessarily share alleles IBD.

The chromosomal regions 7p and Xq contain about 150 and 250 genes, respectively. Besides *F9*, no genes coding for known coagulation proteins are located in these regions. In **Chapter 2.3**, we selected *F9* as a candidate gene for our haplotype-based approach. We found that haplotype 6 was associated with a two-fold decreased risk of venous thrombosis in men, however, no association with factor IX levels was found. In **Chapter 3.3**, we investigated whether genetic variants in eleven selected candidate genes, eight in the 7p region (*PDGFA*, *GPR30*, *CHST12*, *LFNG*, *GNA12*, *RAC1*, *C1GALT1* and *SCIN*) and three in the Xq region (*GNA12*, *RAC1* and *F9*), contributed to the linkage signals found in our genome scan for venous thromboembolism. Therefore, 106 haplotype tagging SNPs in these eleven genes were genotyped in the GIFT study population (all affected siblings and their parents and unaffected siblings) and in a panel of healthy controls.

Both the chromosome 7p and Xq linkage signals only marginally changed when the genotypic data of the 106 SNPs were added to our previous linkage analysis. This finding supports the linkage evidence at both regions. An association analysis was performed to investigate whether any of the SNPs were associated with venous thromboembolism. SNPs in several genes were found to be associated with venous thromboembolism. Among these SNPs were five SNPs in *GPR30*. The strongest effects were found for rs10262070 in *GPR30* (p=0.002) and rs13234810 in *LFNG* (p=0.007). *LFNG* encodes the protein lunatic fringe β-1,3-N-acetylglucosaminylt ransferase, a glycosyltransferase that modifies fucose residues of Notch receptors by adding N-acetylglucosamine (GlcNac).[119,120] *GPR30* encodes G-protein-coupled receptor 30, a member of the G-protein-coupled receptor superfamily (GPCRs).[121] G-protein-coupled receptor 30 primarily acts as an estrogen receptor[122] and was selected as candidate gene because GPCRs are known to be involved in hemostasis[123,124] and *GPR30* expression was reported in the liver.[125] Because almost half of the *GPR30* SNPs were associated with venous thromboembolism, we also assessed the effect of *GPR30* haplotypes on the risk of venous thromboembolism. We found that one of the eight *GPR30* haplotypes, tagged by the rare allele of SNPs rs10262070 and rs10235056, was associated with a 1.6-fold increased risk of venous thromboembolism. Even though variants of *GPR30* might be associated with venous thromboembolism, it is highly unlikely that these variants contribute to the chromosome 7p linkage signal, because *GPR30* is located 7.6 cM (~15.2 Mb) away from the maximum LOD score of the linkage peak in a region with a much lower LOD score (1.03) than that of the maximum LOD score (2.87).

RAC1 is the closest gene to the position of the maximum LOD score of the chromosome 7p linkage peak showing some evidence for association with venous thromboembolism, i.e. rs836480 in *RAC1* was associated with thromboembolic

risk (p=0.038). *RAC1* encodes the Rac1 protein[126] and was selected as candidate gene because of the finding that *RAC1*-deficient mice have diminished platelet secretion and aggregation as well as a prolonged bleeding time.[127] Therefore, we further investigated this gene. We found that the C allele of rs836480 was only present in haplotype 1 (frequency in controls=50%). This haplotype was associated with a 1.3-fold increased risk of venous thromboembolism. An association given linkage analysis indicated that rs836480 in *RAC1* contributes to the linkage signal on chromosome 7p. Rs836480 is located in intron 2 of *RAC1* in a region that does not contain any obvious regulatory elements which could indicate that rs836480 is a functional variant.

In conclusion, two novel thrombosis susceptibility regions (7p21.3 and Xq25-q26.3) were found using a genome-wide linkage scan in affected sibling pairs. Screening of eleven candidate genes, selected from both regions, revealed that only rs836480 in *RAC1* could explain at least a part of the observed linkage signal on chromosome 7p. Therefore, variants in genes, different from those screened in the present study, should exist that explain the observed linkage signals on chromosome 7p and Xq.

Different approaches can be followed to identify the genetic variants responsible for our observed linkage signals. One approach is to select another set of genes and perform an analysis as was done in **Chapter 3.3**. During the screening of the eleven candidate genes, one novel gene was mapped to the chromosomal 7p21.3 region, about 1.3 Mb from the location of the maximum LOD score. This gene, *COL28A1*, encodes the collagen XXVIII alpha 1 chain.[128] The involvement of collagen in the binding of von Willebrand factor (vWF) to the vascular endothelium[129] makes *COL28A1* an interesting candidate gene. In fact, collagen XXVIII contains a vWF-like A-domain itself. Apart from *COL28A1*, there are no obvious candidate genes in the chromosomal regions 7p21.3 and Xq25-q26.3. Another option is to select candidate genes purely based on their location on the chromosome, e.g. *NXPH1* can be selected as candidate gene, because it is located right at the location of the maximum LOD score on chromosome 7. A 2.2 Mb gene desert (a region containing no known genes) is located between markers D7S641 (location maximum LOD score) and D7S513.

Furthermore, candidate genes can also be selected from other linkage signals besides those on chromosome 7 and X, even though it is less likely that these peak regions contain novel thrombosis genes because of the lower LOD scores. The chromosomal region 8q12.1 contains a linkage signal with the highest LOD score (1.32) after chromosome 7 and X. The most logical candidate to select in this region is *PLAT*, which encodes tissue-type plasminogen activator (t-Pa). t-Pa is a serine protease that converts plasminogen to plasmin, thereby promoting fibrinolysis.[130] Increased

activity of t-Pa causes hyperfibrinolysis, which leads to bleeding, whereas a decreased t-Pa activity causes hypofibrinolysis, possibly leading to thrombus formation.

Screening of a large number of candidate genes might be required to find the gene(s) responsible for the linkage signals. This makes this approach labor-intensive and time-consuming. Another approach would be the genotyping of thousands of SNPs in peak regions without prior selection of candidate genes. This approach can provide dense information about genetic variants in the peak region that might contribute to the linkage signals. A disadvantage of this approach is the relatively high cost of genotyping such a large number of SNPs. Because SNP chips for Genome Wide Association (GWA)[131] scans are getting cheaper and cheaper, it might therefore be more cost-effective to perform a complete GWA scan in the GIFT population and in the healthy subjects. Such a scan might also yield genetic variants associated with venous thromboembolism that are located in regions on the genome that were not identified in our genome-wide linkage scan.

Because advances in technology have made it possible to sequence the whole genome of an individual,[132,133] whole genome sequencing might be another technique to identify genetic variants in the GIFT population, which might be associated with venous thromboembolism. However, to date this approach is still prohibitively expensive to perform on a large number of subjects. Furthermore, it is an ongoing challenge to efficiently screen the large amounts of genomic data for functional variants. It may also raise ethical questions about the handling of genomic data of an individual.[134]

Besides searching for novel susceptibility genes for venous thromboembolism, the GIFT population is also suitable for searching for genetic determinants of intermediate phenotypes. Similar as was done in the GAIT project, the GIFT population can be used to search for QTLs influencing plasma levels of hemostasis-related proteins. Although the GAIT study has already identified some candidate genes in QTL genome scans of hemostasis related phenotypes (e.g. *NQO1* as determinant of protein C levels[111]), replications are needed to confirm these findings. Furthermore, the genetic determinants of several plasma phenotypes remain unclear (e.g. the genetic determinants of factor VIII levels[115]). Because 33 percent of the GIFT individuals was using vitamin K antagonists at the time of venapuncture, QTL genome scans in the GIFT population are probably limited to those phenotypes not influenced by these antagonists.

Furthermore, it is possible to perform linkage analyses including covariates like factor V Leiden or oral contraceptive use. By including factor V Leiden in the

analysis the genome can be scanned for genes that interact with factor V Leiden (gene-gene interaction). Genetic variations that interact with oral contraceptive use (gene-environment interaction) may be detected by adding oral contraceptive use as a covariate. This might help to unravel the mechanism by which oral contraceptives increase the risk of venous thrombosis.

Since detailed questionnaires (including questions about thromboembolic events, presence of genetic and environmental risk factors, family history, etc…) are available from the affected sibling pairs and from 3173 young thrombosis patients without an affected sibling (see also **Chapter 3.1**), future analyses can be performed to gain better insight in the mechanism and conditions that lead to (recurrent) venous thromboembolism.

Candidate gene approach versus genome-wide approach

In this thesis we have used two approaches to identify unknown genetic risk factors for venous thrombosis: the candidate gene approach (**Chapter 2**) and the genome-wide approach (**Chapter 3**). Both approaches have their advantages and disadvantages. The candidate gene approach has the advantage that only limited funding and resources are required to investigate whether a genetic variant in a candidate gene is associated with a disease. Another advantage of a candidate gene approach compared to the genome-wide approach is the greater power to detect genetic variants with small effects. One of the major drawbacks of the candidate gene approach is that it is limited by how much is known of the biology of the disease being investigated (venous thrombosis in our case); it is only useful to select candidate genes known to be involved in the disease, or in pathways that might influence the pathogenesis of the disease. Therefore, the candidate gene approach will not lead to the identification of entirely new genes or pathways involved in venous thrombosis. It can, however, be used to investigate the role of pathways (e.g. inflammation) for which it is hypothesized that they are involved in the disease.

Nowadays, most coagulation genes have already been extensively studied,[135-137] making it difficult to select novel candidate genes, which is one of the reasons we turned to a genome-wide approach. The major advantage of this approach is the ability to identify novel genes or pathways involved in a disease. Unfortunately, this approach is expensive and labor-intensive. Genome-wide linkage scans identify chromosomal regions of several cM rather than directly identifying novel genes. Association analyses are needed to investigate whether genetic variants, in genes located in these chromosomal regions, are associated with the disease and explain the linkage signal(s). Therefore, the genome-wide approach and candidate gene approach can be used in tandem to identify novel genetic variants. After identification of the

causal variant, functional studies are needed to unravel the biological mechanism underlying the causal mutation.

Genome-wide linkage analysis has mainly been successful in identifying genes in Mendelian disorders (e.g. Huntington disease[138] and cystic fibrosis[139-141]). For most complex diseases, linkage analysis has yielded limited results.[142] However, there are several success stories in which causal variants were identified using a genome-wide linkage scan followed by an association study. For example, using a genome-wide linkage scan, chromosome 19p was identified as a novel susceptibility region for celiac disease.[143] A common variant in the myosin IXB gene, located in the chromosome 19p region, was later identified as being associated with a 2.3-fold increased risk of celiac disease.[144] Similarly, variants in the calpain-10 gene were found to be associated with type 2 diabetes mellitus.[145] This gene was selected as candidate gene from the chromosome 2p susceptibility region.[146]

Conclusion

More extensive knowledge of genetic risk factors for venous thrombosis, their interaction with other risk factors and the molecular basis of these interactions, will contribute to a greater understanding of the pathogenesis of venous thrombosis. This can eventually lead to improved laboratory diagnosis of venous thrombosis, more efficient treatment strategies and a better prevention of venous thrombosis. The results described in this thesis might contribute to the first step of this process: identification of novel genetic risk factors for venous thrombosis.

In this thesis we showed that also variants in genes (e.g. *IL1RN*) outside hemostasis can affect the risk of venous thrombosis. This finding indicates that future candidate genes can be selected from the inflammatory pathways. As a result, more insight might be obtained on how inflammation and coagulation interact in the development of venous thrombosis. We further used the GIFT population to scan the genome for novel thrombosis susceptibility genes. This approach yielded two regions that might contain novel thrombosis genes. Although the gene(s) and the functional variant(s) causing the linkage signals have not yet been identified, additional research should result in the identification of novel genetic risk factors for venous thrombosis.

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Samenvatting

Inleiding

Veneuze trombose is een aandoening waarbij een stolsel (bloedprop) gevormd wordt in één van de venen (aderen) van de bloedsomloop. Jaarlijks worden één tot drie op de duizend personen getroffen door deze aandoening. Veneuze trombose kan in het hele lichaam optreden, maar meestal gebeurt dit in de diepe venen van de benen (trombosebeen). Als een stukje stolsel losbreekt, kan dit zich via de bloedsomloop verplaatsen naar de longen. Hier kan het stolsel de slagaderen van de longen blokkeren, waardoor een longembolie ontstaat met mogelijk fatale gevolgen.

Veneuze trombose is een zogenaamde complexe ziekte. Dit betekent dat bij het ontstaan van veneuze trombose zowel verworven factoren (omgevingsfactoren) alsmede genetische (erfelijke) factoren betrokken zijn. Verworven risicofactoren voor veneuze trombose zijn onder andere het gebruik van vrouwelijke hormonen (anticonceptiepil, hormoongebruik tijdens overgang), gevorderde leeftijd, immobilisatie, operatie, zwangerschap en kraambed. Bekende genetische risicofactoren zijn de deficiënties (tekorten) van de antistollingseiwitten proteïne C, proteïne S en antitrombine en de factor V Leiden en protrombine 20210A mutaties. Verder hebben personen met ABO bloedgroep non-O (A, B of AB) een hogere kans op het krijgen van veneuze trombose.

Dat genetische factoren een belangrijke rol spelen bij het ontstaan van veneuze trombose blijkt ondermeer uit het feit dat 20 tot 30% van de trombosepatiënten tenminste één eerstegraads familielid heeft met veneuze trombose. Veneuze trombose kan ook clusteren binnen een familie. Dit wordt familiaire trombofilie genoemd en wordt gezien als een ziekte waarbij tenminste twee genetische afwijkingen voorkomen binnen een familie. In een meerderheid van deze families wordt echter maar één of zelfs geen bekende genetische risicofactor voor veneuze trombose gevonden. Dit suggereert dat er nog onbekende genetische risicofactoren zijn. Verder is bekend dat verhoogde plasmaspiegels van verschillende stollingseiwitten, bijvoorbeeld de factoren VIII and IX, het risico op veneuze trombose verhogen en deze verhoogde spiegels lijken ook over te erven in families. De genetische determinanten van deze plasmaspiegels zijn echter nog onbekend.

Doel en benadering

Het doel van het onderzoek, zoals beschreven in dit proefschrift, is het identificeren van nieuwe tot nog toe onbekende genetische risicofactoren voor veneuze trombose. Het streven was om genen of genomische gebieden te identificeren die bijdragen aan het ontstaan van veneuze trombose. Identificatie van nieuwe genetische risicofactoren voor veneuze trombose is belangrijk voor het in kaart brengen van hoe veneuze trombose precies ontstaat. Verder kan het bijdragen aan een betere

diagnose, behandeling en preventie van veneuze trombose.

In dit proefschrift is gekozen voor twee verschillende benaderingen om de ontbrekende genetische risicofactoren voor veneuze trombose te identificeren: de kandidaatgen benadering en de genoomwijde benadering.

De kandidaatgen benadering is een methode waarbij wordt onderzocht of een genetische variant in een kandidaatgen geassocieerd is met een fenotype (in ons geval veneuze trombose). In dit proefschrift (zie **Hoofdstuk 2**) werden twee verschillende soorten genetische varianten onderzocht: SNPs (single nucleotide polymorphism, variatie van één DNA nucleotide) en haplotypes (combinatie van allelen op een chromosoom). Voor de kandidaatgen benadering is gebruik gemaakt van de "Leiden Erfelijke Trombose Studie" (LETS), een patiënt-controle onderzoek naar de oorzaken van veneuze trombose met 474 opeenvolgende patiënten met een eerste diep veneuze trombose en 474 gezonde controles.

De genoomwijde benadering kan gebruikt worden om het complete genoom (alle chromosomen) te screenen op chromosomale gebieden die de vatbaarheid voor een ziekte (mede) kunnen verklaren. Het screenen gebeurt meestal door middel van het genotyperen van honderden genetische markers verspreid over het genoom. Een genetische marker is een stukje DNA met een variabele lengte waarvan de overerving gevolgd kan worden. De achterliggende gedachte is dat, binnen een studiepopulatie van verwante personen (bijvoorbeeld broer-zus paren, families), een genetische marker, die gelinkt (gekoppeld) is met een functionele variant, samen met een ziekte overerft. Een genoomwijde linkage (koppelings) analyse met broer-zus paren (broer-broer, zus-zus of broer-zus) met veneuze trombose werd uitgevoerd in **Hoofdstuk 3** van dit proefschrift. Broer-zus paren delen gemiddeld 50% van hun DNA. Chromosomale gebieden die meer dan gemiddeld gedeeld worden door broer-zus paren met veneuze trombose bevatten mogelijk genen die een rol spelen bij het ontstaan van veneuze trombose.

Interleukine-1
Interleukine-1 (IL-1) is een pro-inflammatoire (ontstekingsbevorderende) cytokine die een belangrijke rol speelt bij auto-immuun en inflammatoire ziekten doordat het de expressie van genen die betrokken zijn bij het immuunsysteem kan activeren. De IL-1 familie omvat de agonisten IL-1α en IL-1β (meest voorkomende vorm bij mensen), en de IL-1 receptor antagonist (IL-1Ra), die alle drie kunnen binden aan de functionele IL-1 receptor type I (IL-1R1) of aan de "dummy" receptor type II (IL-1R2). IL-1Ra fungeert als een remmer van IL-1α en IL-1β door de IL-1R1 receptor te binden en daardoor IL-1 signaaltransductie te blokkeren.

Inflammatie (ontsteking) speelt waarschijnlijk een rol bij trombosevorming door zowel de stolling als de fibrinolyse (afbraak van stolsel) te beïnvloeden. IL-1 bevordert de stolling door de expressie van weefselfactor (tissue factor) te verhogen en door de expressie van trombomoduline en endotheel proteïne C receptor te verlagen. IL-1 beïnvloedt de fibrinolyse door de productie van weefsel-type plasminogeen activator te verlagen en de productie van plasminogeen activator inhibitor te verhogen.

In **Hoofdstuk 2.1** is bekeken of genetische varianten in de genen van IL-1β, IL-1Ra, IL-1R1 en IL-1R2 (*IL1B*, *IL1RN*, *IL1R1* en *IL1R2*) geassocieerd waren met het risico op veneuze trombose. Onze hypothese was dat genetische varianten in de vier IL-1 genen een effect hebben op het risico op veneuze trombose doordat ze de IL-1 signaaltransductie beïnvloeden. Om deze hypothese te testen, zijn alle LETS personen gegenotypeerd voor 18 SNPs in *IL1B*, *IL1RN*, *IL1R1* en *IL1R2*, om zodoende 25 haplotypes in deze genen te kunnen toewijzen. Uit een globale associatietest bleek dat er alleen voor *IL1RN* een significant verschil was in haplotype frequentieverdeling tussen patiënten en controles. Verdere berekeningen lieten zien dat homozygote dragers van *IL1RN* haplotype 5 een vier keer verhoogde kans op veneuze trombose hebben. De functionele variant in haplotype 5 is echter nog niet bekend. Een frequent onderzochte genetische variant in *IL1RN* is de intron 2 "variable number of tandem repeats" (VNTR). Deze VNTR is interessant omdat allel 2 van deze variant geassocieerd is met verschillende ziekten. Wij vonden dat allel 2 van de VNTR onderdeel is van haplotype 3. Haplotype 3 was echter niet geassocieerd met het risico op veneuze trombose.

Inflammatie heeft ook invloed op het ontstaan van atherosclerose. IL-1 bevordert de vorming van een atherosclerotische plaque (vetafzetting aan de vaatwand) door endotheelcellen te stimuleren om meer leukocyten adhesie moleculen tot expressie te brengen. Als een dergelijke atherosclerotische plaque scheurt, kan dit leiden tot arteriële (slagaderlijke) trombose of een myocardinfarct (hartaanval). Het was onduidelijk of varianten in *IL1RN* een effect hebben op het risico op een myocardinfarct. Daarom is in **Hoofdstuk 2.2** het effect van *IL1RN* haplotypes op *IL1RN* mRNA spiegels en op het risico op myocardinfarct onderzocht. Hiervoor is gebruikt gemaakt van het "Stolling en MyocardInfarct onderzoek LEiden" (SMILE), een patiënt-controle onderzoek met 560 mannen met een eerste myocardinfarct en 646 gezonde controles. Haplotype 3 dragers hadden significant verlaagde *IL1RN* mRNA spiegels ($p<0.01$) en een 1.3-voudig verhoogde kans op een myocardinfarct. Wij verwachten dat een verlaagde *IL1RN* expressie zal leiden tot verlaagde IL-1Ra spiegels. Lagere IL-1Ra spiegels leiden weer tot een versterking van het protrombotisch effect van IL-1, omdat minder IL-1Ra aanwezig is om te fungeren als antagonist van IL-1.

De functionele variant van haplotype 3 is nog niet bekend. Het is mogelijk dat de intron 2 VNTR de functionele variant is. Omdat allel 2 van de VNTR minder bindingsplaatsen heeft voor transcriptiefactoren dan het meest voorkomende allel 1, wordt een lagere transcriptieactiviteit voor deze variant verwacht. Deze hypothese is in overeenstemming met onze bevinding dat haplotype 3 dragers een lagere *IL1RN* mRNA expressie hebben. In de literatuur zijn echter tegenstrijdige resultaten te vinden over de associatie tussen allel 2 van de VNTR en verschillende cardiovasculaire ziekten. Bovendien is het onduidelijk of allel 2 van de VNTR geassocieerd is met verhoogde of juist verlaagde productie van IL-1Ra. Toekomstig onderzoek is nodig om duidelijkheid te scheppen in de relatie tussen *IL1RN* varianten en *IL1RN* mRNA expressie, IL-1Ra productie en IL-1Ra plasmaspiegels.

Factor IX

Factor IX (FIX) is een vitamine K afhankelijk stollingseiwit dat een belangrijke rol speelt in de activering van factor X. Dit proces leidt uiteindelijk tot de vorming van een stolsel. Personen met FIX plasmaspiegels boven het 90ste percentiel (>129 U/dl), zoals bepaald in gezonde personen, hebben een twee tot drie keer hogere kans op diep veneuze trombose vergeleken met personen met FIX plasmaspiegels onder het 90ste percentiel. Het biologisch mechanisme dat verantwoordelijk is voor de hoge FIX spiegels is nog onbekend.

Het onderzoek in **Hoofdstuk 2.3** had als doel het identificeren van genetische varianten in het FIX gen (*F9*), dat gelegen is op het X chromosoom, die hoge FIX spiegels en tromboserisico zouden kunnen verklaren. Eerst zijn we op zoek gegaan naar onbekende genetische varianten in *F9* door middel van het sequencen van grote gedeelten van *F9* in 19 LETS mannen met een geïsoleerd verhoogde FIX antigeen plasmaspiegel. Twee zeldzame varianten werden gevonden: -816G/A en 32781A/G. Beide varianten waren niet geassocieerd met FIX spiegels en tromboserisico. Voor de tweede benadering werd gekeken naar het effect van *F9* SNPs en *F9* haplotypes op FIX spiegels en het risico op veneuze trombose. Hiervoor werden alle LETS personen voor zes SNPs gegenotypeerd zodat er in totaal acht haplotypes vastgesteld konden worden. We vonden dat mannelijke dragers van haplotype 6 een twee keer verlaagde kans hadden op veneuze trombose. De enige coderende SNP in dit haplotype is de exon 6 SNP 20422A/G. Deze SNP, ook wel FIX Malmö genoemd, bevindt zich in het activeringspeptide van FIX. FIX Malmö laat bij zowel mannen als vrouwen een beschermend effect zien op het risico op veneuze trombose, vergelijkbaar met dat van haplotype 6. *F9* SNPs hadden geen invloed op FIX spiegels in mannen en vrouwen en *F9* haplotypes hadden geen effect op FIX spiegels in mannen. Dragers van haplotype 6 leken wel hogere FIX spiegels te hebben dan haplotype 1 (het referentie haplotype) dragers, maar dit effect was niet significant. In een recente

studie werd geen effect van haplotype 6 op de kans op veneuze trombose gevonden in een groep van postmenopausale vrouwen. In diezelfde studie werd echter wel een 50 procent hoger risico op veneuze trombose gevonden voor een subhaplotype van haplotype 1. In de LETS studie hebben wij het effect van haplotype 1 bepaald door het te vergelijken met de groep van alle andere haplotypes. Wij vonden een licht verhoogd risico voor haplotype 1 in mannen. In vrouwen zagen we echter geen effect van haplotype 1. Dit laatste was ook het geval voor de subgroep van postmenopausale vrouwen in de LETS.

Factor VII-activerend protease

Factor VII-activerend protease (FSAP) is een plasma protease (enzym dat eiwitten afbreekt) dat de stolling kan bevorderen door factor VII te activeren. FSAP kan ook de fibrinolyse beïnvloeden door plasminogeen activatoren te activeren. In 2002 is een SNP (1601G/A) ontdekt in het FSAP gen die resulteert in een glycine naar glutaminezuur verandering in het protease domein. De 1601A variant van FSAP, ook bekend als FSAP *Marburg I,* is een slechte activator van plasminogeen activatoren. Factor VII kan echter nog gewoon geactiveerd worden. In een recente Duitse studie werd beweerd dat *Marburg I* dragers een 3.5-voudig verhoogde kans op veneuze trombose hadden. In **Hoofdstuk 2.4** hebben wij dit resultaat gecontroleerd in de LETS studie. Wij vonden geen associatie tussen FSAP *Marburg I* en het risico op veneuze trombose. Een mogelijke verklaring voor de tegenstrijdige resultaten is de lage frequentie van het *Marburg I* allel in de controlegroep van de Duitse studie. Als reactie op onze bevindingen werd de data in de Duitse studie opnieuw geanalyseerd met een andere controlegroep. Hieruit kwam naar voren dat de eerder gevonden associatie tussen FSAP *Marburg I* en veneuze trombose alleen geldt voor trombosepatiënten waarin de trombose niet verklaard kon worden door de aanwezigheid van bekende risicofactoren (idiopathische trombose). In verschillende andere studies werd echter ook geen associatie gevonden tussen FSAP *Marburg I* en (idiopathische) veneuze trombose.

Genetica In Familiare Trombose (GIFT) studie

In **Hoofdstuk 3** hebben we een genoomwijde linkage benadering gebruikt om het genoom systematisch te scannen op genomische gebieden of genen die bijdragen aan het ontstaan van veneuze trombose. Deze benadering is eerder gebruikt in twee andere trombose studies: de Vermont studie en de GAIT (Genetische Analyse van Idiopathische Trombofilie) studie. Het doel van de Vermont studie was het identificeren van een tweede genetisch defect dat samen met proteïne C deficiëntie de hoge frequentie van veneuze trombose in een grote proteïne C deficiënte familie zou kunnen verklaren. In de GAIT studie wordt voornamelijk gezocht naar locaties op het genoom die plasmaspiegels van hemostase-gerelateerde eiwitten kunnen

beïnvloeden. Zowel de Vermont studie als de GAIT studie gebruiken uitgebreide families met trombose voor de analyses. In de Genetica In Familiare Trombose (GIFT) studie hebben we voor de genoomwijde linkage scan gebruikt gemaakt van broer-zus paren met trombose (affected sibling pairs).

In de GIFT studie hebben wij samengewerkt met 29 trombosediensten, verspreid over heel Nederland, om jonge (≤45 jaar) broer-zus paren met veneuze trombose te benaderen (**Hoofdstuk 3.1)**. Uiteindelijk zijn in de GIFT studie 211 Europese families geïncludeerd met tenminste één broer-zus paar met een objectief vastgestelde veneuze trombose. In totaal zijn 460 broers en zussen geïncludeerd die samen 287 aangedane broer-zus paren vormen. Van al deze broers en zussen werd DNA verzameld en van 434 personen ook bloedplasma. Van de ouders en niet-aangedane broers en zussen werd DNA verzameld. Vergeleken met opeenvolgende patiënten uit de LETS studie, bleek de GIFT populatie verrijkt te zijn voor factor V Leiden en voor ABO bloedgroep non-O. De protrombine 20210A mutatie kwam in vergelijkbare frequentie voor in de GIFT studie en in de LETS.

In **Hoofdstuk 3.2** werden de resultaten van de genoomwijde linkage scan in de GIFT studie beschreven. Verspreid over het gehele genoom werden 402 genetische markers bepaald in alle aangedane broer-zus paren. Uit de analyses kwamen twee gebieden, één op chromosoom 7p en één op chromosoom Xq, naar voren waar zich mogelijk genen bevinden die betrokken zijn bij het ontstaan van veneuze trombose. Om dit resultaat te bevestigen werden extra markers bepaald, niet alleen in de aangedane broer-zus paren maar ook in hun ouders en niet-aangedane broers en zussen. Deze extra analyse versterkte onze bevinding aangezien beide linkage pieken aanwezig bleven. De linkage signalen op chromosoom 7p21.3 en chromosoom Xq25-q26.3 hadden respectievelijk een LOD score van 3.09 (p=0.00008) en 1.86 (p=0.002). De LOD score (Logarithm Of Odds; logaritme van de kans) geeft de mate van waarschijnlijkheid aan. Een LOD score van bijvoorbeeld drie geeft aan dat het duizend keer meer waarschijnlijk is dat zich op die locatie genen bevinden die betrokken zijn bij de ziekte dan ergens anders op het genoom. Uit een simulatieanalyse bleek echter dat alleen het chromosoom 7p linkage signaal genoomwijd significant was (p=0.029). In de Vermont en GAIT studies werd geen bewijs voor linkage met trombose gevonden op chromosoom 7. Beide studies hebben het X chromosoom niet meegenomen in hun genoomscan. In de Vermont studie werden drie gebieden (11q23, 10p12 en 18p11.2-q11.2) gevonden waar zich mogelijk genen bevinden die betrokken zijn bij het ontstaan van trombose. De laatste twee regionen werden in de GAIT studie ook gevonden in een genoomscan voor genetische determinanten die respectievelijk plasmaspiegels van factor XII of ongevoeligheid voor geactiveerd proteïne C (APC) beïnvloeden. In alle drie de gebieden werden echter geen duidelijke kandidaatgenen

gevonden. In de GIFT genoomscan vonden wij geen aanwijzingen voor linkage in deze drie gebieden.

De chromosomale gebieden 7p en Xq bevatten respectievelijk ongeveer 150 en 250 genen. Onder deze genen bevinden zich, behalve *F9* dat in **Hoofdstuk 2.3** werd bestudeerd, geen genen die coderen voor stollingseiwitten. In **Hoofdstuk 3.3** hebben we onderzocht of genetische varianten in elf geselecteerde kandidaatgenen, acht in het chromosoom 7p gebied (*PDGFA*, *GPR30*, *CHST12*, *LFNG*, *GNA12*, *RAC1*, *C1GALT1* en *SCIN*) en drie in het chromosoom Xq gebied (*GNA12*, *RAC1* and *F9*), bijdroegen aan de linkage signalen zoals gevonden in onze genoomwijde linkage scan voor veneuze trombose. Hiervoor zijn 106 haplotype specifieke SNPs in de elf kandidaatgenen geselecteerd en vervolgens gegenotypeerd in de GIFT studie populatie (alle aangedane broer-zus paren, hun ouders en hun niet-aangedane broers en zussen) en in een groep van 331 gezonde controles.

De chromosoom 7p en Xq linkage signalen bleven nagenoeg gelijk na toevoeging van de genotype data van de 106 SNPs aan de eerdere linkage analyse. Dit ondersteunt het bewijs voor linkage in beide regionen. Uit een associatie-analyse bleek dat SNPs in verschillende genen geassocieerd waren met veneuze trombose. Onder deze SNPs bevonden zich vijf SNPs in *GPR30*. De sterkste effecten werden gevonden voor rs10262070 in *GPR30* (p=0.002) en rs13234810 in *LFNG* (p=0.007). *GPR30* codeert voor de G-proteïne-gekoppelde receptor 30, een eiwit dat voornamelijk functioneert als een estrogeen receptor. Aangezien ongeveer de helft van de *GPR30* SNPs geassocieerd was met veneuze trombose, is ook het effect van *GPR30* haplotypes op het risico op veneuze trombose onderzocht. We vonden dat één van de *GPR30* haplotypes geassocieerd was met een 1.6 keer verhoogde kans op veneuze trombose. Het is echter zeer onwaarschijnlijk dat genetische varianten in *GPR30*, ook al zijn ze geassocieerd met veneuze trombose, bijdragen aan het chromosoom 7p linkage signaal, aangezien *GPR30* 7.6 cM (~15.2 Mb) verwijderd is van de maximale LOD score van de linkage piek. Er is ook gekeken naar het effect van *RAC1* haplotypes op het risico op veneuze trombose, omdat *RAC1* van de elf geselecteerde genen het dichtst bij de maximale LOD score van de chromosoom 7p linkage piek ligt én omdat rs836480 in *RAC1* geassocieerd was met tromboserisico (p=0.038). We vonden dat het C allel van rs836480, specifiek voor haplotype 1 (het meest voorkomende *RAC1* haplotype), geassocieerd was met een 1.3 keer verhoogde kans op veneuze trombose. Door middel van een associatie gegeven linkage analyse kon bepaald worden dat rs836480 in *RAC1* bijdraagt aan het chromosoom 7p linkage signaal. Toekomstig onderzoek zal moeten uitwijzen welke genetische varianten verantwoordelijk zijn voor de twee linkage signalen op chromosoom 7p en Xq. Deze varianten bevinden zich waarschijnlijk buiten de elf genen die in **Hoofdstuk 3.3** gescreend zijn.

Conclusie

Meer kennis van de genetische risicofactoren voor veneuze trombose, hun interactie met andere genetische en verworven risicofactoren en de moleculaire achtergrond van die interacties, is belangrijk voor een beter inzicht in het ontstaan van veneuze trombose. Dit kan uiteindelijk bijdragen aan een verbeterde laboratoriumdiagnose van veneuze trombose, efficiëntere behandelingsstrategieën en een verbeterde preventie van veneuze trombose. De resultaten beschreven in dit proefschrift dragen mogelijk bij aan de eerste stap van het in deze alinea beschreven proces: het identificeren van de nog onbekende genetische risicofactoren voor veneuze trombose.

In dit proefschrift hebben we aangetoond dat ook varianten in genen (bijvoorbeeld *IL1RN*) buiten de stolling een effect kunnen hebben op het risico op veneuze trombose. Deze bevinding geeft aan dat het zinvol kan zijn om toekomstige kandidaatgenen te selecteren uit biologische mechanismen die indirect betrokken zijn bij het ontstaan van veneuze trombose, in plaats van het selecteren van de toch al frequent onderzochte trombosekandidaatgenen. In onze tweede benadering hebben we in de GIFT studie een genoomwijde linkage scan uitgevoerd om zodoende nieuwe kandidaatgenen voor veneuze trombose te vinden. Dit heeft geresulteerd in de identificatie van twee gebieden waar zich mogelijk nieuwe trombosegenen bevinden. Aanvullend onderzoek in de GIFT studie zal hopelijk leiden tot de identificatie van de functionele varianten die de linkage signalen veroorzaken.

Nawoord

Het in dit proefschrift beschreven onderzoek was zeker niet gelukt zonder de hulp van een aantal personen die ik hierbij graag persoonlijk wil bedanken voor hun inzet.

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Curriculum Vitae

De auteur van dit proefschrift werd op 8 juni 1979 geboren te Rheden. Na het behalen van zijn HAVO diploma in 1996 aan het Christelijk Lyceum te Arnhem, begon hij datzelfde jaar aan het Hoger Laboratorium Onderwijs (HLO) aan de Hogeschool van Utrecht. Hier werd gekozen voor de afstudeerrichting Biochemie en werden twee stages uitgevoerd te Long Beach in de Verenigde Staten. De eerste stage werd gedaan bij de afdeling Diagnostic & Molecular Health Care van het Veterans Affairs Medical Center Long Beach onder begeleiding van Dr. Martin Jadus, en was getiteld "Macrophage colony stimulating factor, recognition and destruction of tumor cells". De tweede stage werd gedaan bij de afdeling Chemistry and Biochemistry van de California State University Long Beach onder begeleiding van Dr. Roger Acey, en was getiteld "Isolation and purification of metallothionein expressed in tobacco leaves".

Na het behalen van zijn HLO diploma, is hij in 2000 begonnen met een verkorte opleiding Scheikunde aan de Universiteit van Utrecht. Bij de onderzoeksgroep Biochemie van Membranen (tegenwoordig onderdeel van de onderzoeksgroep Chemische Biologie en Organische Chemie) werd onder begeleiding van Drs. Anja Rebber en Prof. dr. Ben de Kruijff een stage uitgevoerd, getiteld "Structural role of Arg-52 in KcsA".

Na zijn afstuderen in 2003, is hij datzelfde jaar begonnen aan het door NWO gesubsidieerde onderzoek "Search for novel genetic risk factors for venous thrombosis: a dual approach", waarvan de resultaten in dit proefschrift beschreven staan. Dit project werd, onder begeleiding van Prof. dr. Rogier M. Bertina en Dr. Marieke C.H. de Visser, uitgevoerd in het Hemostase en Trombose Onderzoekscentrum (inmiddels het Einthoven Laboratorium voor Experimentele Vasculaire Geneeskunde, subafdeling Trombose en Hemostase) van de afdeling Hematologie in het Leids Universitair Medisch Centrum.

Sinds november 2008 is hij werkzaam als klinisch moleculair geneticus i.o. in het DNA-diagnostiek laboratorium van de afdeling Klinische Genetica van het Erasmus Medisch Centrum te Rotterdam.