Venous and arterial thrombosis : associations and risk factors

Roshani, S.

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Author: Sare Roshani
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Promotores:	Prof. dr P.H. Reitsma
	Prof. dr S. Middeldorp (Universiteit van Amsterdam)
Overige leden:	prof. dr. G.P.M. Luyten
	prof. dr. P.E. Slagboom
	prof. dr. J.C.M. Meijers (Universiteit van Amsterdam)

Contents

General introduction and outline of the thesis

Venous thromboembolism, risk factors and prophylaxis

Venous thrombosis, encompassing the clinical spectrum of deep vein thrombosis (DVT) and pulmonary embolism (PE), poses a significant clinical and economic burden on Western societies. It occurs annually in 1 to 2 per 1000 inhabitants, and has a steep age gradient, with an incidence of up to 1 per 100 in individuals older than 80 years. [1]. Individuals with venous thrombosis constitute 0.64% of all hospital admissions [2], and two-thirds have DVT as their primary manifestation, while the remaining one-third has PE. The overall mortality rate within 30 days of an event is nearly 6%. [3] A recurrence occurs in 7 to 14% of patients within the first year after the initial event. [4] The recurrence rate is even as high as 30% in the first 10 years, and remains high lifelong. [4] Other long-term complications include post-thrombotic syndrome in nearly one-third to half of DVT patients [5], and pulmonary hypertension, which has been reported to occur in nearly 4% of patients within the first two years after the first PE.[6] These epidemiological characteristics of DVT and PE underline the importance of understanding the etiology of these events, as such understanding would promote our ability to predict and prevent risk.

Venous thrombosis is a multi-factorial disease that is influenced by genetic determinants as well as acquired risk factors such as major trauma, prolonged immobilization, surgery, oral contraceptive use, hormone replacement therapy, pregnancy and puerperium. Thrombophilia, a term coined by Nygaard and Brown[7], is used to describe the inherited tendency toward venous thrombosis. Mutations underlying thrombophilia vary from rare 'loss-of-function' mutations in natural anticoagulants to common 'gain-of-function' mutations such as factor V Leiden and prothrombin G20210A. [8] Twenty-five to 35% of individuals experiencing a first episode of DVT or PE are heterozygous or homozygous for at least one of these mutations. Classifying the risk factors as 'genetic' or 'acquired' is not always straightforward. Examples are elevated factor VIII levels and hyperhomocysteinemia that are known to increase the risk of both venous and arterial thrombosis, and that are genetically and environmentally determined. [9]

The risk factors underlying thrombophilia have a varying clinical penetrance, and the presence of a thrombophilic defect *per se* does not always result in a thrombosis. Moreover, it is well documented that individuals carrying more than

one thrombophilic defect are at higher risk than those with a single inherited risk factor. [10] However, approximately one-third of familial venous thromboembolic events remain unexplained.[11]

Appropriate prophylaxis can significantly reduce venous thrombosis related mortality and morbidity.[12] Without prophylaxis, the incidence of hospital-acquired DVT is 10 to 40% among medical or general surgical patients and 40 to 60% following major orthopedic surgery.[13;14] Certain issues remain unresolved surrounding appropriate prophylaxis, including optimal dosing in specific patient populations with respect to efficacy, safety and patient compliance. [15] For instance, the presumed lower risk of significant bleeding by low dose prophylaxis with low-molecular-weight heparin during pregnancy may be outweighed by an unacceptable high risk of venous thrombosis recurrence. [16;17]

Arterial and venous thrombosis association

Arterial thrombotic events, i.e. myocardial infarction, stroke and peripheral artery disease have long been considered an entity separate from venous thrombosis. This distinction was supported by differences in the blood clot composition, underlying risk factors, and prophylactic as well as therapeutic measures. More recent evidence indicates that these two types of thrombosis might share at least some common risk factors [18;19], and experiencing one type of thrombotic event appears to predispose to the development of the other.[20] For example, a consistent finding in several cohort studies [21-23] was that patients with a previous venous thrombotic episode had an about 50% higher risk to develop arterial thrombotic events in subsequent years than individuals without prior venous thrombosis. However, the underlying mechanisms, particularly the role of multiple thrombophilic defects and classical cardiovascular risk factors in this association have not been elucidated.

In this thesis, we address several unresolved questions. First, can we identify new hereditary thrombophilic defects in a large family with an unexplained thrombotic tendency? What are the potential clinical implications of thrombophilia testing? Is our strategy of thrombosis prophylaxis in pregnant women with thrombophilia adequate? Second, are there common risk factors that may explain the association of arterial and venous thrombosis?

Study populations

The investigations described in this thesis were performed in three previously described studies with exception of those in chapter 5 and 8. The earlier studies were the Beethoven study (chapter 6 and 9), the GENES study (chapter 2 and 3) and the Leiden Thrombophilia Study (LETS) (chapter 7, 2 and 3).

The Beethoven study

The Beethoven study consists of three prospective cohorts of thrombophilic families which were identified by probands with documented DVT, PE, or premature arterial cardiovascular diseases (any arterial thrombotic event before 50 years of age), and either hyperhomocysteinemia, prothrombin G20210A, or persistently elevated levels of factor VIII. Subjects were recruited between August 1997 and May 2004 from three academic hospitals: Academic Medical Center, Amsterdam, University Medical Center, Groningen and Academic Hospital Maastricht. Details of these studies have been published previously. [24-26] Various other thrombophilic defects were tested in all participants. Information on previous episodes of venous thrombosis, arterial cardiovascular disease, exposure to exogenous risk factors for thrombosis, anticoagulant treatment, and the presence of cardiovascular risk factors was collected by validated questionnaire and by reviewing medical records at baseline. Also, every 6 months until April 2006, all participants provided a detailed questionnaire focusing on new episodes of venous thrombosis, arterial cardiovascular diseases, exposure to risk factors, and medication use.

The GENES study

The pedigree studied for this thesis was drawn from the GENES study [27] in which Dutch thrombophilic families were included with the purpose of discovering new genetic risk factors of venous thrombosis. The probands had at least one first-degree or two second-degree family members with the same diagnosis and did not carry one of the known thrombophilic defects, i.e. factor V Leiden, prothrombin G20210A and deficiencies of antithrombin, protein C and protein S. One pedigree was selected for further investigation because it showed the highest heritability of ‘endogenous thrombin potential’ (ETP) levels (68%). Subsequently,

a genome wide linkage analysis was performed in the selected pedigree for several coagulation factor levels and global coagulation determinants using the 'Sequential Oligogenic Linkage Analysis Routines' (SOLAR) program.

The Leiden Thrombophilia Study (LETS)

LETS is a population based case-control study, originally meant to investigate new risk factors of venous thrombosis. Between January 1988 and December 1992, 474 patients younger than 70 years from anticoagulation clinics in Leiden, Amsterdam and Rotterdam with a first DVT of the leg or arm were included in LETS. An unrelated control for each case was selected matched on age and sex. Participants did not have overt malignancy. All participants filled out a standard questionnaire regarding risk factors of venous thrombosis.

The follow-up part of the LETS was performed to investigate the risk factors for recurrent venous thrombosis. Cases were followed as described previously [28] after anticoagulation cessation until January 2000. Information during follow-up on the occurrence of risk situations, use of anticoagulation treatment, and recurrent events was collected by repeated mailed questionnaires. Patients were interviewed by telephone when they responded positively to any item of the questionnaire or when they did not return it.

Outline of the thesis

The studies presented in this thesis follow two objectives, each presented in a separate section. The first section focuses on investigations performed in a selected pedigree from GENES that were aimed at discovering a genetic explanation for the significant quantitative trait loci that were found in a genome wide linkage study. In the other section, we examined the implications of thrombophilia testing, and safety of high doses of low-molecular-weight heparins as prophylaxis during pregnancy.

Part I

Chapter 2: We studied the linkage observed in the selected pedigree on chromosome 20 for the levels of protein C by evaluating the association between protein C levels and polymorphisms of three candidate genes encoding

thrombomodulin, endothelial protein C receptor and forkhead-box A2. In addition we assessed the association of the levels of protein C with the levels of soluble endothelial protein C receptor. To investigate the external validity of our observations, we confirmed the results in the control population of LETS.

Chapter 3: We scrutinized the linkage signals for the levels of factor (F) V and prothrombin on chromosome 16 by studying the association of haplotypes of *NQO1* (candidate gene) with the levels of FV and prothrombin in the pedigree from GENES. We performed similar analyses in the control population of the LETS. Furthermore, the associated risk of venous thrombosis with each *NQO1* haplotype was calculated in the LETS. In **chapter 4,** we studied the risk of venous thrombosis and the levels of vitamin K dependent coagulation factors for different haplotypes of the enzymes (*VKORC1*, *GGCX and NQO1*) involved in the vitamine K cycle.

Chapter 5: The current knowledge of hereditary and acquired thrombophilic defects and the associated risk of venous thrombosis are reviewed in this chapter and the clinical implications for thrombophilia testing are addressed.

Chapter 6: We discussed the risk of major bleeding around the delivery, known as post-partum hemorrhage, in women who received high doses of low-molecular-weight heparin to prevent venous thrombosis.

Part II of this thesis deals with the association between arterial and venous thrombosis.

Chapter 7: We intended to confirm the increased risk of arterial cardiovascular diseases after an episode of venous thrombosis in the Beethoven study. More importantly, we investigated whether the presence of multiple cardiovascular risk factors and thrombophilic defects could explain this increased risk.

Chapter 8: In this chapter the risk of venous thrombosis recurrence, in the LETS follow-up study, in patients with low and elevated levels of cytokines as compared with patients with undetectable levels of cytokines, are presented. In addition, we investigated the influence of high D-dimer (>250 ng/ml) and CRP (>3 mg/L) on recurrence risk of venous thrombosis.

Chapter 9: Here we report the role of established thrombophilic defects, fibrinolysis markers, and polymorphisms in genes encoding platelet receptors in the pathogenesis of idiopathic retinal vein thrombosis (RVO) by studying a

large number of patients without known risk factors for RVO and a sex and age matched control group.

Chapter 10: The risk of arterial thrombosis in double heterozygous or homozygous carriers of factor V Leiden or prothrombin G20210A is compared with single heterozygous carriers of one of these mutations. This analysis was done in the relatives on the Beethoven study and was recalculated by excluding all relatives who had another co-inherited thrombophilic defect.

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Part I

High levels of protein C are determined by *PROCR* haplotype 3

Sara Roshani, Maria Carolina Pintao, Marieke C.H. de Visser, Chris Tieken, Michael W.T. Tanck, Iris M. Wichers, Joost C.M. Meijers, Frits R. Rosendaal, Saskia Middeldorp and Pieter H. Reitsma

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Abstract

Background
Genetic determinants of plasma levels of protein C (PC) are poorly understood. Recently, we identified a locus on chromosome 20 determining high PC levels in a large Dutch pedigree with unexplained thrombophilia. Candidate genes in the LOD-1 support interval included *FOXA2*, *THBD* and *PROCR*.
Objectives
To examine these candidate genes and their influence on plasma levels of PC.
Patients/Methods
Exons, promoter and 3'UTR of the candidate genes were sequenced in twelve family members with normal to high PC levels. Four haplotypes of *PROCR*, common in the European population, were examined in the family, and critical SNPs encountered during resequencing were genotyped in the family and in a large group of healthy individuals (the Leiden Thrombophilia Study (LETS) controls). Soluble endothelial protein C receptor (sEPCR) and soluble thrombomodulin (sTM) plasma levels were measured in the family.
Results
PROCR haplotype 3 (H3) and *FOXA2* rs1055080 were associated with PC levels in the family but only *PROCR H3* was also associated with plasma levels in the healthy individuals. Carriers of both variants had higher PC levels than carriers of only *PROCR H3* in the family but not in healthy individuals, suggesting that a second determinant is present between *FOXA2* and *PROCR*. Plasma levels of PC and sEPCR were associated in both studies, contrasting to sTM which was not associated with variations of *THBD* or with PC levels.
Conclusion
Chromosome 20 harbors a locus influencing PC and sEPCR plasma levels and a detailed analysis of candidate genes suggests that *PROCR H3* is responsible.

Introduction

Protein C (PC) is a vitamin K-dependent plasma glycoprotein that circulates at a concentration of ~40-80 nM and plays a major role in the control of the coagulation cascade. Upon activation by the thrombin-thrombomodulin complex, activated PC (APC) inactivates FVa and FVIIIa, consequently reducing thrombin formation[1]. Binding of PC to the endothelial protein C receptor (EPCR) increases the activation rate of PC 20-fold [2]. Individuals with abnormalities in components of the PC pathway (such as PC or protein S deficiency) have an increased risk of venous thrombosis[3].

Determinants of variation in PC levels are poorly known but, given the high heritability of about 50%, genetic determinants are likely to be important [4,5]. The PC gene (*PROC*) is located on chromosome 2 and around 6% of the variability in PC levels has been attributed to polymorphisms in the promoter region of the gene [6,7]. The GAIT (Genetic Analysis of Idiopathic Thrombosis) study estimated that additive effects of genes outside of the *PROC* structural locus cause approximately half of the phenotypic variation in PC levels [4]. The authors identified a major quantitative trait locus (QTL) for PC levels on chromosome 16, where *NQO1*, a gene encoding a quinone reductase involved in the metabolism of vitamin K, is located. Subsequently they found that variations in this gene were associated with PC levels [5].

Variations in the *PROCR* gene (encoding the EPCR) were associated with a moderate increase in levels of PC in the Leiden Thrombophilia Study (LETS) [8] and in the Cardiovascular Health Study (CHS) [9]. While this work was in progress, a genome-wide association scan (GWAS) in the Atherosclerosis Risk in Communities (ARIC) study claimed four loci associated with PC levels, that included *PROC*, *PROCR* and three other genes (*EDEM2*, *GCKR* and *BAZ1B*) [10].

The GENES study was designed to search for novel hereditary risk factors for venous thrombosis in families with unexplained thrombophilia [11]. Previously, a genome-wide linkage analysis was performed. In one particular family, a QTL influencing PC levels was found on chromosome 20 (chr 20), with a log-odds (LOD) score of 4.8 at 51cM [12]. In the 1-LOD support interval (38-64cM), three candidate genes encoding components potentially influencing PC levels are

present, namely forkhead box A2 (*FOXA2,* previously known as hepatic nuclear factor 3ß), thrombomodulin (*THBD*), and the endothelial protein C receptor (*PROCR*).

FOXA2 is part of the forkhead box family and encodes a transcription factor (FOXA2) for a large number of genes, including *PROC*. Two binding sites for FOXA2 in the promoter region of *PROC* have been described and mutations in this region were associated with type I PC deficiency [13].

THBD encodes thrombomodulin (TM), a transmembrane protein that, in complex with thrombin, enhances thrombin-mediated PC activation by more than 1000-fold [1].

PROCR encodes the already mentioned EPCR. Besides the membrane-anchored form, a soluble form of EPCR (sEPCR) lacking the transmembrane and cytoplasmic domain is present in human plasma. Like EPCR, sEPCR binds PC and APC with similar affinity [14]. Binding of sEPCR to APC inhibits its anticoagulant activity by impairing the inactivation of FVa, and binding to PC prevents PC activation by thrombin-TM complexes [15]. Four haplotypes of *PROCR* are present in the European population [8]. Haplotype 3 (H3) is tagged by a missense variation that leads to the Ser219Gly variant (rs867186). The Gly219 variant is associated with increased levels of sEPCR which can be explained by an increased sensitivity of the protein to sheddases such as metalloprotease ADAM17 16 and by the expression of an alternatively spliced mRNA that lacks the sequence encoding the transmembrane domain [17].

In this study, we investigated whether genetic variations in the three candidate genes on chr 20 influence the plasma level of PC.

Patients and methods

Subjects

GENES. The pedigree analyzed in the present study is one of the 22 families originally included in GENES, a study of Dutch families with unexplained thrombophilia [11]. Probands with personal and/or family history of venous thrombosis (defined as at least one first degree or two second degree relatives with venous thrombosis) but with none of the known inherited thrombophilic defects (i.e. PC-, protein S-, antithrombin deficiency, factor V Leiden or prothrombin

G20210A variation) were recruited together with their extended pedigree, including spouses. A standardized history was taken and for most individuals, plasma and DNA samples were obtained for coagulation tests and genotyping [12]. This analysis focuses on the largest pedigree in GENES, consisting of 185 individuals distributed over five generations. In this family, four individuals had a history of thrombosis of whom two had experienced more than one event. Detailed information about these individuals is given in table 1.

Leiden Thrombophilia Study (LETS). For replication of the results obtained in the family, relevant DNA variations were genotyped in healthy (i.e., non-thrombotic) individuals, who were the controls in a population-based case-control study for venous thrombosis (LETS). The design of this study has been described before [18]. Briefly, 474 consecutive patients with a first episode of deep vein thrombosis and 474 sex- and age-matched healthy controls were included. All patients were younger than 70 years and had no overt malignancy. Controls were healthy acquaintances and partners brought by the patients, accordingly to pre-established criteria. Mean age for patients and controls was 45 years (range 15-69 for patients and 15-72 for controls). Standardized questionnaire, DNA and plasma samples were obtained from all the participants. GENES and LETS were approved by the Central Committee on Research Involving Human Subjects (CCMO) and all participants have provided informed consent.

Table 1. Characteristics of family members with venous thrombosis.

	First episode of VT			Second episode of VT			PC	*PROCR*	*FOXA2*
Sex	Type	Age (y)	Risk factor	Type	Age (y)	Risk factor	(%)	genotype	rs1055080
Female	PE	32	Pregnancy	-	-	-	-	H1H2	CC
Male	DVT	41	Trauma	-	-	-	82	H2H2	CC
Male	DVT	4	Trauma	DVT	28	Idiopathic	114	H2H3	CT
Female	DVT	40	Idiopathic	DVT	44	Idiopathic	178	H3H3	CT

Abbreviations - VT: venous thrombosis; PE: pulmonary embolism; DVT: deep vein thrombosis. PC levels are presented as percentage of reference pooled plasma. *FOXA2* rs1055080: NM_021784.4:c.*50C>T or NM_153675.2:c.*50C>T.

Resequencing of candidate genes

Three genes in the linkage region (LOD-1 support interval) on chr 20 were selected for further analysis.

FOXA2 has two known mRNA splicing variants. The first variant (NM_021784) contains 2 exons and covers 2422 bases. Alternative splicing at the 5'-end results in a second mRNA variant (NM_153675) in which translation initiation starts 6 amino acids later than in variant 1. Variant 2 also has an additional (untranslated) 5'-exon, leading to a total mRNA length of 2410 bases. *THBD* is transcribed from an intron-less gene as a 4109 bases long mRNA (NM_000361). *PROCR* has four exons with transcription length of 1449 bases (NM_006404).

To investigate genetic variations associated with PC levels in the family, we selected twelve family members based on their PC levels: (a) three with normal levels (72, 75 and 82%); (b) three with intermediate high levels (114, 116 and 128%); and (c) six with the highest levels (range: 166 – 212%). Three out of four patients with thrombosis were included in this panel (table 2). PC plasma level was not available for the fourth individual.

Exons and their flanking regions, 5' and 3' UTRs, and 1000 bp upstream to the initiation codon were resequenced. For *FOXA2*, the DNA sequence covering both splicing variants and 1000 bp upstream to exon 1 of both isoforms was analyzed. Primers and PCR conditions are available on request.

After amplification, the PCR product was sequenced using an ABI Prism® 3730 DNA Analyzer (Applied Biosystems, Carlsbad, California, USA). The results were analyzed using vector NTI® software version 10 (Invitrogen, Paisle, UK). Potentially interesting variations found by sequencing were investigated in all family members and in the healthy individuals using single nucleotide polymorphism (SNP) genotyping assays.

SNP genotyping assays

All SNPs were determined using TaqMan SNP genotyping assays (Applied Biosystems, California, USA). PCR reactions were performed in 384-well plates using the GeneAmp PCR System 9700 (Applied Biosystems, California, USA) and fluorescent endpoints were read on a 7900 HT Real-Time PCR System (Applied Biosystems, California, USA).

Common haplotypes of *PROCR* were tested in all family members with DNA available. Three haplotype tagging SNPs were chosen: rs2069952 (H1, pre-designed assay), rs867186 (H3, pre-designed assay) and rs2069951 (H4, custom

assay). The presence of the rare allele determines the mentioned haplotype and the presence of three common genotypes determines H2 [8].
Two common variations in *FOXA2* (rs1055080 and rs2277764) that were identified during sequencing analysis in individuals with high levels of PC were investigated in all family members and/or in healthy individuals using a custom TaqMan genotyping SNP assay.

Plasma assays
Protein C. In both the family and healthy individuals, blood was collected in tubes containing 0.106 mol L^{-1} trisodium citrate. Plasma was prepared by centrifugation at 2000 g for 10 min at room temperature and stored at -70°C [12,18]. PC levels were determined using a chromogenic assay (Chromogenix, Mölndal, Sweden). Levels were expressed as percentage of the level in a reference pooled plasma. Measurements in GENES and LETS were performed in different laboratories, several years apart, using different reference pooled plasmas.
Plasma soluble EPCR (sEPCR) levels were determined in the family using the Asserachrom sEPCR ELISA kit (Diagnostica Stago, Asnières, France) according to the manufacturer's instructions. Samples were tested in duplicate and plasmas were diluted 1/26 prior to the assay.
Plasma soluble TM (sTM) levels were measured in the family using the CD141 ELISA kit (Diaclone, Besançon, France) according to the manufacturer's instructions. Samples were tested in triplicate in non-diluted plasmas.

Linkage analysis
To assess the influence of the investigated genetic variations and plasma measurements of sEPCR and sTM on the QTL on chr 20 for PC levels, linkage analyses were performed using SOLAR [12]. The effects of the genetic variations were assessed by adding them to the marker set or by adding them as covariate to the linkage model (conditional analyses). Effects of sEPCR and sTM levels were assessed by adding them as covariates to the linkage model. In addition, LOD scores for sEPCR and sTM levels were determined on chromosome 20. Following Lander and Kruglyak [19], and correcting for two phenotypes, we used thresholds of 3.6 for genome wide significance and 2.2 for suggestive linkage.

Statistical analysis

Mean and 95% confidence interval (CI95) were used to compare continuous variables with normal distribution (i.e., PC and sEPCR). Patients using vitamin K antagonists at the time of venapuncture were excluded. Median and range were used to describe sTM because of the skewed distribution in the family. Linear regression analysis was used to analyze the correlation between sEPCR and PC levels. All calculations were performed using PASW Statistics 17.0 (IBM Corporation, Somers, USA).

Results

Genetic analysis of candidate genes PROCR, FOXA2 and THBD

Resequencing of candidate genes yielded five variations in *FOXA2*, four variations in *THBD* and five variations in *PROCR*. All non-synonymous variations are summarized in table 2. For *PROCR*, variations were within the expected for the haplotypes and only the haplotypes are shown. All SNPs have been previously reported. One variation in *PROCR* (rs867186, a tagging SNP for H3) and two variations in *FOXA2* (rs1055080, in the 3'- UTR and rs2277764, in the 5'-UTR) were associated with higher levels of PC. Other SNPs found in *FOXA2* were rs1800847, rs1203910 and rs1212275, all leading to synonymous amino acid substitutions (not shown). Relevant variations found in *THBD* were: rs1042579 (p.Val473Ala), rs1042580, rs3176123 and rs1962 (last three in the 3'-UTR) but none of these was associated with PC plasma levels. This excludes *THBD* as a likely determinant of PC levels.

The co-inheritance of the rare variations of *PROCR* (rs817186) and *FOXA2* (rs1055080 and rs2277764) in individuals with high PC plasma levels suggests that *PROCR* and *FOXA2* SNPs are inherited as a single haplotype in these individuals. In an attempt to distinguish which gene is actually responsible, we genotyped all members of the family for *FOXA2* rs1055080 and for the tagging SNPs of common *PROCR* haplotypes (table 3). Genotyping of *PROCR* showed 52 heterozygotes and two homozygotes for rs817186 minor allele, whereas for *FOXA2* rs1055080, thirty four individuals were heterozygotes and no homozygote for the minor allele was present. Except for two individuals, all

Table 2. Polymorphisms detected in twelve family members with normal, intermediate and high PC levels. Only non-synonymous variations are shown in the table.

Sample	PC (%)	PROCR haplotype	FOXA2 rs1055080	rs2277764	THBD rs1962	rs3176123	rs1042580	rs1042579
36049	72	H2H4	1	1	1	1	3	1
29640	75	H1H2	1	1	2	2	1	2
29448*	82	H2H2	1	1	1	1	2	1
29494*	114	H2H3	2	2	1	1	1	1
29599	116	H1H3	1	1	2	2	1	2
29495	128	H2H3	2	2	1	1	1	1
29600	166	H2H3	2	2	1	2	1	2
29680	166	H1H3	2	2	2	1	1	1
29552	169	H3H3	2	2	1	2	1	2
29678	170	H1H3	2	2	2	1	1	1
29529*	178	H3H3	2	2	1	2	1	2
29687	212	H2H3	2	2	2	1	1	1

*individuals with thrombosis; 1 homozygous for the common allele, 2 heterozygous and 3 homozygous for the rare allele. PC levels are presented as percentages of the reference pooled plasma.

carriers of the *FOXA2* rs1055080 minor allele were also carriers of *PROCR* H3, again suggesting co-inheritance in the family.

To answer the question whether *PROCR* H3 or FOXA2 rs1055080 is responsible for PC levels variation, we investigated these SNPs in LETS controls. *FOXA2* rs1055080 was determined in 465 healthy individuals, out of whom thirty seven carried the minor allele, two in homozygous state (table 3). Only eight carriers of the minor *FOXA2* rs1055080 allele also carried *PROCR* H3, which suggests that, in this population-based study, *PROCR* and *FOXA2* are not inherited together, reinforcing the idea that the co-inheritance of the rare variations is particular to this family.

FOXA2 rs2277764 was also determined in LETS controls but because of its tight linkage with *FOXA2* rs1055080 (r^2=0.98), this variation was not analyzed further.

PROCR H3 is associated with high levels of PC in the family and in healthy individuals

Table 3 shows the mean plasma levels of PC and 95% confidence intervals (CI95) for the different genotype groups. Levels of PC cannot be compared directly between the family and healthy individuals because different pooled plasmas were used as a reference. In the healthy individuals, levels of PC were systematically lower than the levels in the family. In the family, mean PC level was higher in *PROCR* H3 carriers (mean: 131%; CI95: 123-138), than in non-carriers (mean: 106%; CI95: 102-110). In two individuals from the family who were homozygous for H3, PC levels were 169% and 178%. In healthy individuals, mean PC level was higher in H3 heterozygotes (mean: 113%; CI95: 110-117) than in non-carriers of H3 (mean: 99%; CI95: 97-100). Mean PC level in homozygotes for H3 was 127% (CI95: 109-144) which is somewhat higher than the levels in heterozygote carriers, but the confidence intervals overlap.
Carriers of the *FOXA2* rs1055080 minor allele in the family had increased levels of PC (mean: 139%; CI95: 128-150) in comparison with non-carriers (mean: 109%; CI95: 105-113). This was not seen for heterozygous carriers and non-carriers in the healthy individuals (mean: 100% (CI95: 93-106) and 103% (CI95: 101-104), respectively). Two individuals were homozygous for the minor allele and PC levels were 92 and 123%. This indicates that the minor allele of *FOXA2* rs1055080 is not associated with plasma level of PC in the population.
We further examined whether family members carrying both rare variants in *PROCR* and *FOXA2* had increased levels of PC in comparison to carriers of only one rare variant (table 3). Mean level of PC was higher in carriers of both *PROCR* H3 and *FOXA2* rs1055080 rare variants (mean: 142%; CI95: 131-153) than in carriers of *PROCR* H3 only (mean: 120%; CI95: 111-129). This indicates that, in this family, a second gene variation (other than *PROCR* H3) may determine protein C levels. Since in the healthy individuals no effect of *FOXA2* rs1055080 on PC levels was found, this second gene variation is not *FOXA2* rs1055080.

PROCR H3 is associated with elevated sEPCR levels in the family and in the healthy individuals

In the family, sEPCR was increased in carriers of *PROCR* H3 (mean: 261 ng/ml; CI95: 240-281) in comparison to non-carriers (mean: 103 ng/ml; CI95: 97-108). Carriers of the *FOXA2* rs1055080 minor allele also had increased sEPCR in the family but not in healthy individuals (table 3). No difference in the level

of sEPCR was observed between carriers of the rare allele for both variations and carriers of *PROCR* H3 only, either in the family or in the health individuals. sEPCR and PC levels were correlated in both the family (r^2=0.18) and healthy individuals (r^2=0.15).

sTM concentration is not different between PROCR H3 carriers and non-carriers

Median sTM plasma level was 1.2 ng/ml (range: 0.1 to 4.0) in the family. Median levels were not different between *PROCR* H3 carriers (median: 1.3 ng/ml; range: 0.3 to 4.0) and non-carriers (median: 1.2 ng/ml; range: 0.1 to 4.0). sTM was not measured in LETS.

Linkage analysis

Finally, we (re)examined linkage between PC, sEPCR and sTM levels and genetic markers on chr 20, now including *PROCR* H3 and *FOXA2* rs1055080. For PC levels, the addition of new genetic markers did not change the LOD-score (Fig. 1a). When the analysis was performed conditional on *PROCR* H3 and *FOXA2*, the LOD score for PC went down to <2.0, suggesting that most of the variation of PC levels can be attributed to these genetic markers (Fig. 1b).

For sEPCR, the linkage analysis performed with the initial genetic markers yielded a LOD score of 6.2. Adding *FOXA2* rs1055080 to the analysis increased the LOD score to 7.7 and addition of *PROCR* H3 increased it further to 9.3, reinforcing the idea that this haplotype is largely responsible for sEPCR plasma level variation (Fig. 1c). For sTM, the LOD score remained <1.0 for any model (Fig. 1d).

Table 3. PC and sEPCR plasma levels for *PROCR* H3 and *FOXA2* rs1055080 carriers and non-carriers in the family and in the healthy individuals from LETS.

			Protein C				Soluble EPCR			
Genotype	Family (n)	LETS (n)	Family (n)	Mean (CI95)	LETS (n)	Mean (CI95)	Family (n)	Mean (CI95)	LETS (n)	Mean (CI95)
PROCR H3										
HxHx	106	360	85	106 (102-110)	360	99 (97-100)	96	103 (97-108)	360	94 (91-96)
H3Hx	52	100	47	131 (123-138)	100	113 (110-117)	47	261 (240-281)	99	258 (248-269)
H3H3	2	10	2	169; 178*	10	127 (109-144)	1	337*	10	439 (399-478)
Total	160	470	134	-	470	-	144		469	-
FOXA2										
CC	126	428	104	109 (105-113)	428	103 (101-104)	114	131 (118-144)	427	138 (129-146)
CT	34	35	30	139 (128-150)	35	100 (93-106)	30	249 (216-282)	35	117 (96-139)
TT	-	2	-	-	2	92;123*	-	-	2	54;176*
Total	160	465	134	-	465	-	144	-	464	-
PROCR H3 + *FOXA2*										
HxHx +CC	104	326	83	106 (102-110)	326	99 (97-100)	94	103 (98-109)	326	94 (92-96)
HxHx +CT/TT	2	29	2	87;117*	29	97 (90-104)	2	66;81*	29	90 (81-98)
H3Hx/H3H3+CC	22	102	21	120 (111-129)	102	115 (111-119)	20	263 (235-291)	101	279 (265-294)
H3Hx/H3H3+CT/TT	32	8	28	142 (131-153)	8	111 (101-121)	28	262 (232-292)	8	217 (168-265)
Total	160	465	134	-	465	-	144	-	464	-

Abbreviations - (n): number of individuals per group; PC levels are presented as percentages of the reference pooled plasma and sEPCR as ng/ml. *only one or two measurements were available and therefore, mean and CI95 were not calculated. Genotype for *FOXA2* rs1055080 (NM_021784.4:c.*50C>T or NM_153675.2:c.*50C>T) was performed in 465 controls from LETS with DNA available. PC measurement was not available for 26 individuals from the family and sEPCR measurement was not available for 16 individuals from the family and for 1 control from LETS.

Discussion

In this study, we provide evidence for the association of a common haplotype (H3) of *PROCR* with high plasma levels of PC. Resequencing of *PROCR* did not provide proof for additional determinants of plasma PC levels. Furthermore, we excluded that two other genes involved in the PC anticoagulant pathway, i.e. *FOXA2* and *THBD*, both located on chr 20 close to the *PROCR* gene, determined the plasma level of PC, both at the level of common variations as well as on the level of rare sequence variations. Finally, variations in *THBD* did not influence the levels of sTM (data not shown) nor were these levels associated with the level of PC. This contrasted with the clear relationship between levels of PC and sEPCR.

Our results regarding the role of chr 20 in determining PC levels are in agreement with previous reports [8-10,20]. Most notably, a recent study by Tang et al. that was published while this work was in progress, came to very similar findings using a radically different approach [10]. In that study, two neighboring genes, i.e. *PROCR* and *EDEM2*, were found as determinants of variations in PC levels. In the family, carriers of *PROCR* H3 who also carried the rare allele of *FOXA2* rs1055080 had higher PC levels than carriers of H3 alone. The fact that this effect was absent in the healthy individuals from LETS indicates that a second determinant is present between the *FOXA2* and *PROCR* genes, which could well be *EDEM2*.

When we genotyped the family and the healthy individuals from LETS for *EDEM2* rs6120846 and rs3746429 and analyzed the association to PC plasma levels, PC levels were lower in carriers of one or two minor alleles in both the family and healthy controls (unpublished observations), which is in accordance with the data from Tang et al [10]. In addition, we observed that sEPCR levels were also lower in carriers of the minor alleles of *EDEM2* which suggests that, also in this case, PC levels are influenced through EPCR. This is supported by the fact that we could not see any relationship between the minor alleles and levels of other coagulation proteins (FII, FV, FVII, FVIII, FIX, FX or FXI).

The precise mechanisms underlying the association of *PROCR* and PC levels are not known, and there might be an important role for sEPCR. It is known that PC (and APC) has a comparable affinity (Kd≈30nM) for membrane bound EPCR and sEPCR [14]. Therefore, complex formation between PC and sEPCR in plasma

is to be expected and thus, high levels of sEPCR might drive high levels of PC. The problem with this explanation is that, in general, PC levels are much higher than sEPCR levels, even in the presence of *PROCR* H3. PC levels in carriers of H3 in the family is estimated to range from 63 to 163 nM whereas sEPCR levels in the same individuals is estimated to range from 4 to 18 nM. Based on these estimations it is difficult to simply explain the relationship between PC and sEPCR levels. Perhaps, higher sEPCR levels are associated with low levels of EPCR on the endothelial membrane, thus leading to a redistribution of PC between the membrane-bound compartment and a soluble compartment. There is indeed evidence that the H3 haplotype associated Gly219 involves increased EPCR shedding from the endothelium [16], but evidence that this leads to lower density on the endothelial membrane is not available. In preliminary studies we have analyzed blood-originated endothelial cells (BOECs) from carriers and non-carriers of *PROCR* H3 (three individuals of each) by flow cytometry, but the data suggests that the expression of EPCR on the membrane is not different between the groups (unpublished data). It has also been hypothesized that the local concentration of sEPCR at the endothelial surface is higher than the concentrations measured in the plasma, possibly exceeding the Kd of PC interaction [20], but this also does not readily explain the increased plasma PC levels. Future research might solve this problem.

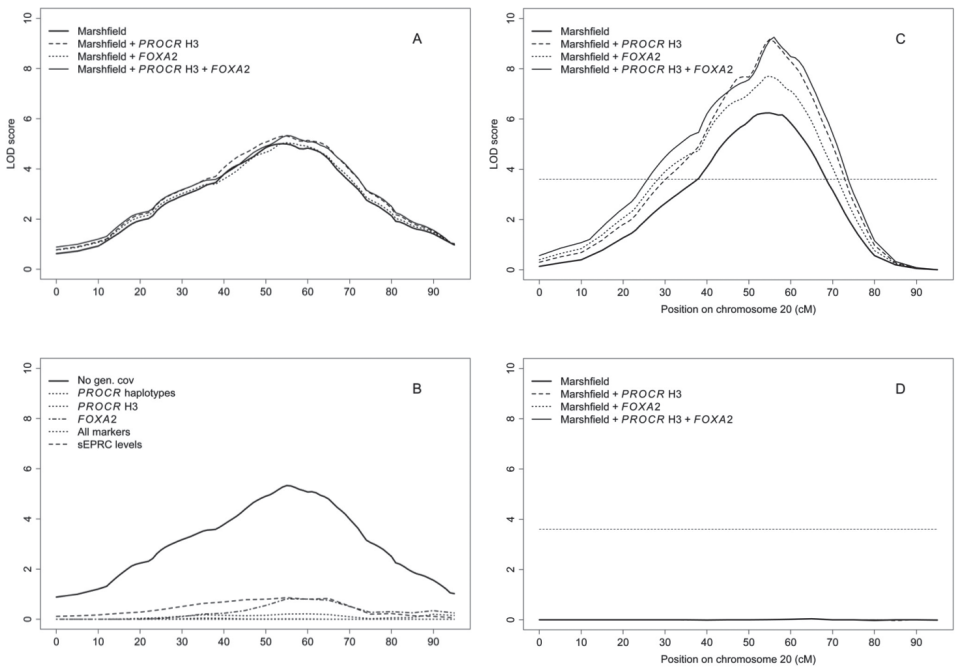
There are claims that H3 not only influences PC levels but also the level of other coagulation proteins, e.g. FVII [21,22]. In the present GENES study or in the healthy individuals from LETS, this association was not confirmed (data not shown), neither was H3 associated with levels of FII, FV, FVIII, FIX or FXI [8].

Since low levels of PC, as in individuals with inherited PC deficiency, are a risk factor for venous thrombosis, it is tempting to assume that high levels of PC are protective, but there is no evidence that the latter is indeed the case. Thus, it is also reasonable to assume that the H3 haplotype, from the perspective of PC levels, would protect against venous thrombosis. This does not seem to be the case: in the family, two of the H3 carriers who had high levels of PC experienced recurrent venous thrombosis, but this family study obviously does not have sufficient power to determine the relationship between H3 and venous thrombosis. Population-based case-control studies have not been conclusive. Some authors report increased risk of venous [20,23] and arterial thrombosis [24] in H3

carriers, and others claim no association [8,9,25,26]. It seems fair to conclude though, that the markedly elevated levels of PC associated with H3 do not protect against thrombotic disease.

In conclusion, our data provide new evidence for the association of *PROCR* H3 and sEPCR with plasma levels of PC and suggest that *FOXA2* and *THBD*, two other genes on chr 20, are not involved. Further studies are necessary to elucidate the mechanisms underlying this association.

Figure 1. LOD-scores for Protein C levels using additional markers (a) or additional covariates (b), and for sEPCR (c) and sTM (d) levels using additional markers. The horizontal line indicates the genome-wide significance threshold.



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NQO1: Candidate gene in a quantitative trait locus affecting factor V and prothrombin levels

Sara Roshani, Marieke C.H. de Visser, Michael W.T. Tanck, Iris M. Wichers, Joost C.M. Meijers, Saskia Middeldorp, Frits R. Rosendaal and Pieter H. Reitsma

Abstract

Background

Earlier genome-wide linkage analyses of coagulation factor levels in a large Dutch pedigree (GENES Family 8) yielded suggestive linkage signals on chromosome (chr) 16 for Factor (F) V (LOD score: 3.9 at 110 cM) and FII levels (LOD score: 3.4 at 97 cM).

Objectives

NQO1, which encodes an enzyme involved in vitamin K metabolism, is a candidate gene in the LOD-1 region of the linkage signals on chr16 and therefore we evaluated the influence of five *NQO1* haplotypes on coagulation factor levels (i.e. protein C, protein S, FII, FV, FVII, FVIII and FIX) in the pedigree and in controls of a population-based case-control study on venous thrombosis, the Leiden Thrombophilia Study (LETS). We also assessed the risk of venous thrombosis for each haplotype in the LETS.

Results

NQO1 haplotype 4 (H4) carriers in Family 8 had lower FV levels than non-carriers while H4 had no effect on FV levels in the LETS controls. Each H4 copy was associated with 11.8 U/dl decrease in FV level (95% CI: -21.4, -2.3) in the family. In the LETS controls, H4 was associated with lower levels of vitamin K-dependent coagulation factors. The strongest association was observed with the levels of FII (-2.6; 95% CI: -5.2, -0.1) and total protein S (-4.3; 95% CI: -7.6, -1.0). We did not observe a similar pattern in the Family 8. NQO1 haplotypes do not influence the risk of thrombosis in the LETS.

Conclusions

Haplotype 4 carriers of *NQO1* have lower levels of vitamin K-dependent coagulation factors especially lower FII and total protein S. None of the haplotypes affect the risk of venous thrombosis.

Introduction

The levels of many coagulation factors are in part genetically determined [1]. However, the genes influencing the levels are not all known.

Previously, we conducted genome-wide linkage analyses of coagulation factor levels and of the outcome of global coagulation assays in a large Dutch pedigree (Family 8) from the GENES study [2]. Four statistically significant linkage signals were observed: on chromosome (chr) 20 for protein C levels (LOD score: 4.8), on chr 17 for prothrombin time (LOD score: 3.8) and on chr 16 for factor (F) V (LOD score: 3.9) and FII (LOD score: 3.4) levels. The protein C linkage signal and the signal on chr 17 will be presented elsewhere (in preparation). In the current study, we set out to explore explanations for the positive linkage signal for factor V and prothrombin (FII) on chr 16 (figure 1).

Candidate genes were searched in the LOD-1 region using Biomart (www.biomart.org). Only one gene, *NQO1,* was identified with a plausible role in blood coagulation factor synthesis. NQO1 is a cytosolic enzyme, which is expressed in the liver and converts vitamin K to hydroquinone vitamin K, the co-enzyme of gamma-glutamyl carboxylase (GGCX) (figure 2). Gamma-carboxyl modification enables proteins such as blood coagulation factors (i.e. protein C, S and Z, FII, FVII, FIX and FX), proteins involved in calcium homeostasis (osteocalcin and matrix Gla protein), cell growth (Gas6) and signal transduction (RPGP1 and RPGP2) to bind to calcium and thereby to be physiologically active [3]. Although factor V is not itself carboxylated, one can not exclude an indirect influence of NQO1. The precedent for this, is protein S that influences the level of tissue factor pathway inhibitor and the level of C4BP [4;5].

In the present study we investigated the association between haplotypes of *NQO1* and the levels of FV and FII and various other factors in GENES Family 8. To verify our findings we performed similar analyses in the control population of the Leiden Thrombophilia Study (LETS), a population based case-control study on venous thrombosis. Furthermore, we evaluated the risk of venous thrombosis for each *NQO1* haplotype in the LETS.

Figure 1: linkage results for FV and FII on chromosome 16

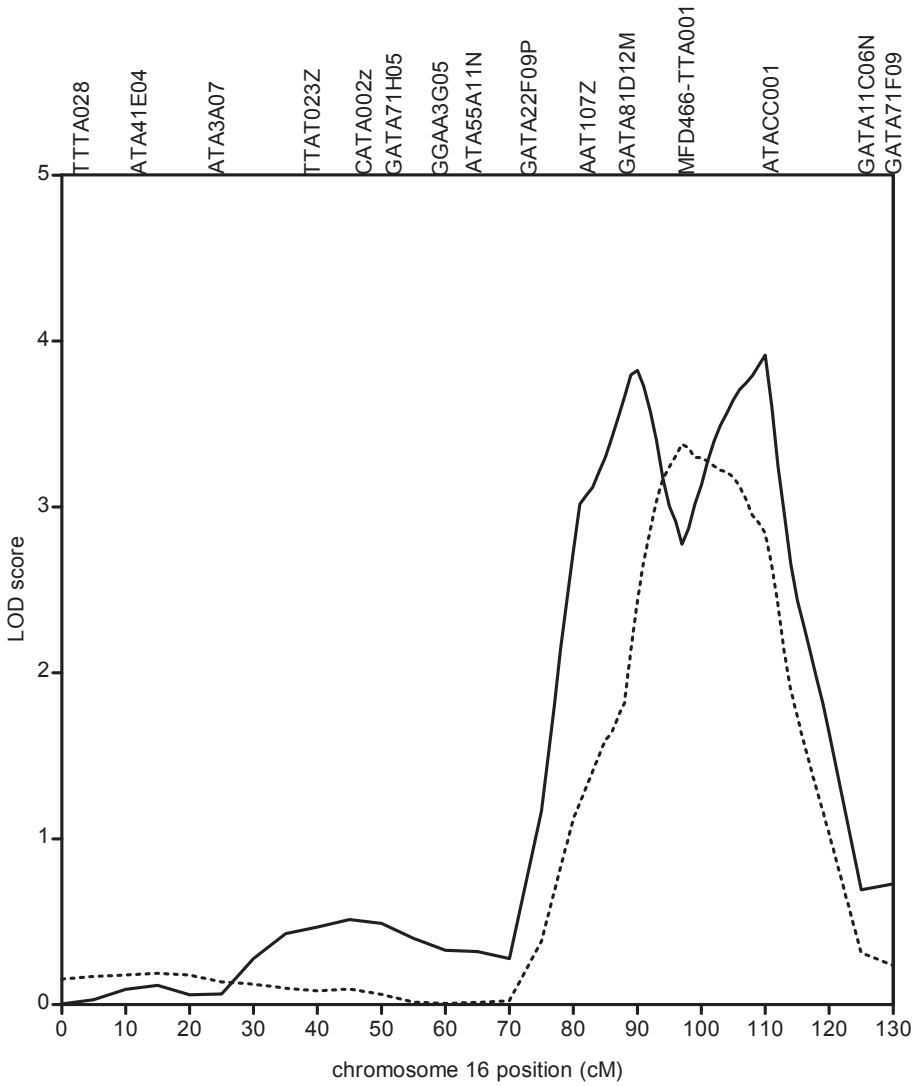
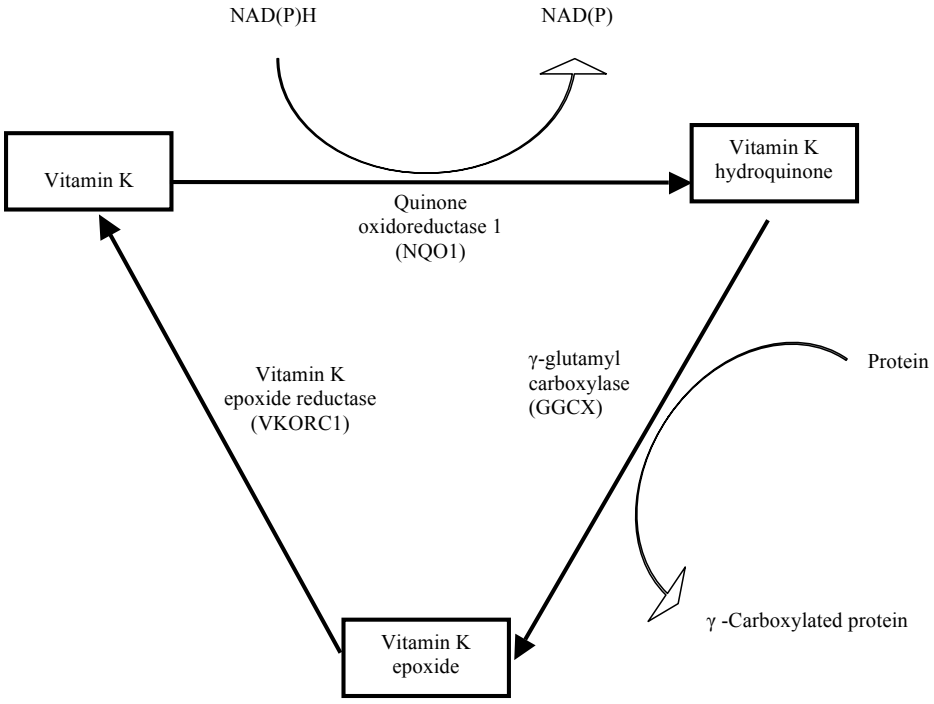


Figure 2: vitamin K cycle: NQO1 converts vitamin K to hydroquinone vitamin K, the co-enzyme of gamma-glutamyl carboxylase (GGCX). After gamma carboxylation vitamin K transforms to vitamin K epoxide which then by vitamin K epoxide reductase complex subunit 1 (VKORC1), the rate limiting enzyme in the vitamin K cycle, is reduced to vitamin K



Subjects and methods

Family 8 and the GENES study

Family 8 is one of the thrombophilic families included in a larger study called the GENES study. GENES is described in a previous publication [2]. In short, 43 families which were ascertained through probands with venous thromboembolism (VTE) and a strong family history of VTE participated in this study. Family history of VTE was defined as at least one first degree or two second degree family members affected by VTE. As the purpose of GENES was to identify new genetic risk factors for VTE, none of the probands should have any of the known thrombophilic defects: factor V Leiden (FVL), prothrombin G20210A or deficiencies of antithrombin, protein C and protein S. The diagnosis of VTE was established based on a standardized questionnaire or on documented medical records. Blood and DNA samples were available for the participants of GENES. The GENES study was approved by the Central Committee on Research Involving Human Subjects (CCMO) and all subjects provided an informed consent.

Leiden Thrombophilia Study

Details of the Leiden Thrombophilia Study (LETS) have been published previously [6]. Briefly, 474 patients younger than 70 years of age with a first deep vein thrombosis were recruited from anticoagulation clinics in Leiden, Amsterdam and Rotterdam (the Netherlands) between January 1988 and December 1992. None of the participants had overt malignancy. As controls, partners or friends of the patients who did not have venous thrombosis were included. Levels of FII [7], FV [8], FVII [9], FIX [10], FVIII [11], protein C and protein S [12] have been determined before and were expressed in U/dl.

Linkage analysis in Family 8

Using the Sequential Oligogenic Linkage Analysis Routines (SOLAR) program probability of identity by descent and variance components linkage analysis in Family 8 members were previously performed for several intermediate phenotypes such as prothrombin F1+2, thrombin generation time, endogenous thrombin potential, clot lysis time, activated protein C sensitivity ratio, prothrombin time, activated partial thromboplastin time, activity of FII, FVII, FVIII, FIX, FXI and

antithrombin, and antigen concentrations of total and free protein S, protein C, FV and tissue factor pathway inhibitor [2]. Genotyping was conducted by the NHLBI Mammalian Genotyping Service at the Marshfield Medical Foundation (Marshfield, WI, USA, Weber and Broman, 2001) using the 10 cM spaced short tandem repeat polymorphism screening set 16 [2]. In the current study, linkage analyses for FV and FII levels were performed adjusted for *NQO1* haplotypes to investigate whether these haplotypes influenced the linkage signals on chr 16.

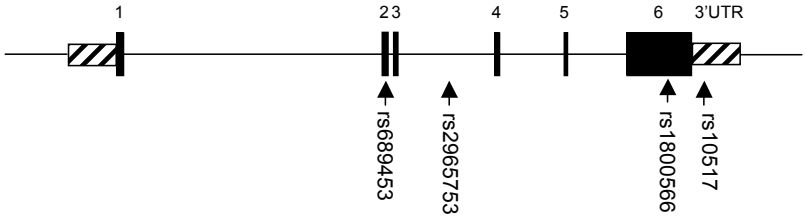
SNP genotyping in Family 8 and LETS

We searched for haplotype tagging (ht) SNPs in *NQO1* in the European Hapmap population (CEU) using the Genome Variation Server (http://gvs.gs.washington.edu/GVS). A htSNP is a polymorphism whose minor allele is specific to one haplotype (H). Four htSNPs (rs689453, rs10517, rs1800566 and rs2965753) were identified, which together tag five haplotypes (figure 3). We used TaqMan® SNP genotyping assays to determine the htSNPs. Fluorescent allele-specific oligonucleotide probes (Applied Biosystems, CA, USA) were used for PCR amplification and fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems).

Statistical analysis

We used linear regression analysis in Family 8 as well as in the LETS control population to investigate the association between the haplotypes of *NQO1* and vitamin K-dependent (protein C and S, FII, FVII and FIX) and independent (FV and FVIII) coagulation factor levels. The regression coefficient (β) represents the mean difference of the levels related to each haplotype copy. Furthermore, to investigate whether carrying a certain haplotype increases the risk of deep venous thrombosis, we computed the odds ratios (OR) and 95% confidence intervals (CI), as an estimate of relative risk of thrombosis for subjects heterozygous or homozygous for each *NQO1* haplotype compared with non-carriers (reference group). The latter analyses were also performed in the LETS.

Figure 3: Five haplotypes of *NQO1* and their frequencies in Family 8 and controls of the Leiden Thrombophilia Study



	Frequency (%)					
	LETS	Family 8				
H1	9	8	A	A	C	C
H2	62	55	G	A	C	C
H3	2	1	G	A	C	T
H4	17	18	G	A	T	C
H5	11	18	G	G	C	T

Results

Family 8 characteristics

Family 8 comprises 218 individuals in 5 generations. Plasma and DNA samples are available for 161 members. The mean (range) age of the members is 46 (15-87) years and 80 (50%) of them are men. Four members experienced thrombosis of whom two had a recurrent event. The linkage signals for FV and FII levels were located at chromosomal regions 16q23 (location maximum LOD score: 110 cM; LOD-1 support interval: 98-114 cM) and 16q22 (location 97 cM; LOD-1 support interval: 89-113 cM), respectively (figure 1).

NQO1 haplotype tagging SNPs

Four htSNPs identified five haplotypes of *NQO1* (figure 3). Haplotypes could not be assigned to three members of Family 8 and 14 controls and 15 patients of the LETS because of missing genotypes due to technical failure in SNP genotyping. Haplotypes and their frequencies in Family 8 and in LETS controls are shown in figure 3. Haplotype 5 (H5) was tagged by two SNPs and H2 consisted of the common alleles of all htSNPs.

Association of NQO1 haplotypes with coagulation factor levels in Family 8 and LETS controls

The regression coefficients (β) for the association of each *NQO1* haplotype with coagulation factor levels are summarized in table 1. Members of Family 8 who inherited H4 had lower FV levels than H4 non-carriers while H4 had no effect on FV levels in the LETS controls. Each H4 copy was associated with 11.8 U/dl decrease in FV level (95% CI: -21.4, -2.3). No other haplotypes showed significant association with FV level, neither in Family 8 nor in LETS controls. In the LETS controls, H4 was consistently associated with lower levels of vitamin K-dependent coagulation factors. The strongest association of H4 was with the levels of factor II (95% CI: -5.2, -0.1) and total protein S (95% CI: -7.6, -1.0). We did not observe similar pattern in the Family 8.

Association of NQO1 haplotypes with thrombosis risk

Table 2 shows the thrombosis risks for homozygous and heterozygous carriers of each *NQO1* haplotype as compared with non-carriers of that particular haplotype in the LETS. H1 carriers (homozygotes and heterozygotes) had a 30% lower risk of thrombosis (95% CI: 0.5-1.1) than non-carriers of H1. Contrarily, H4 carriers (homozygotes and heterozygotes) had a 1.3 times higher thrombosis risk than non-carriers (95% CI: 0.96-1.7). Other haplotypes of *NQO1* were not associated with thrombosis risk.

Linkage analysis with adjustment for NQO1 haplotypes

Adjusting the linkage analyses of FV and FII levels for *NQO1* haplotypes did not reduce the linkage signal on chr16 noticeably. The LOD score after adjustment was reduced mildly.

Table 1: Association of *NQO1* haplotypes with levels of coagulation factors in Family 8 and in controls of the Leiden Thrombophilia Study

NQO1 haplotype	FII		FVII		FIX		FV		FVIII		PC		tPS	
	LETS	Family	LETS	Family	LETS	Family	LETS	Family	LETS	Family	LETS	Family	LETS	Family
H1	0.5	-2.9	0.4	1.6	-4.3	-1.9	-1.8	1.8	-5.8	-0.8	-1.0	-0.0	1.1	-6.7
H2	0.7	2.7	2.2	-1.3	7.4	1.1	-1.2	2.1	-1.6	3.8	1.2	-0.02	1.9	1.9
H3	0.2	2.9	-3.9	18.7	29.8	8.5	-4.6	16.5	-28.4	13.6	-1.1	4.1	2.5	-12.7
H4	**-2.6**	-3.7	-2.6	2.1	-15.8	0.9	2.7	**-11.8**	6.1	-3.6	-0.7	4.4	**-4.3**	-1.1
H5	1.7	0.05	-1.1	-3.2	3.9	-2.8	1.0	4.5	2.1	-4.5	-0.7	-4.6	0.7	2.9

Regression coefficients β are shown. The direction of the regression coefficient represents the effect of each extra copy of the haplotype (i.e. a positive regression coefficient means that the haplotype increases phenotype mean).
The bold typed ciphers are statistically significant.

Table 2: The risk of thrombosis for *NQO1* haplotypes in the Leiden Thrombophilia Study

Haplotype	Patients (%) N=459	Controls (%) N=460	OR	95% CI
H1 (rs689453)				
HxHx	399 (87)	382 (83)	1*	
H1Hx	55 (12)	76 (16)	0.7	0.5-1.0
H1H1	5 (1)	2 (0.4)	2.4	0.5-12.4
H1Hx/ H1H1	60 (13)	78 (17)	0.7	0.5-1.1
Frequency H1	7	9		
H2 (all common)				
HxHx	61 (13)	68 (15)	1	
H2Hx	239 (52)	216 (47)	1.2	0.8-1.8
H2H2	159 (35)	176 (38)	1.0	0.7-1.5
H2Hx/ H2H2	398 (87)	392 (85)	1.1	0.8-1.6
Frequency H2	61	62		
H3 (rs10517)				
HxHx	447 (97)	447 (97)	1	
H3Hx	12 (3)	12 (3)	1.0	0.4-2.3
H3H3	-	1 (0.2)	0.9	0.4-2.0
H3Hx/ H3H3	12 (3)	13 (3)		
Frequency H3	1	2		
H4 (rs1800566)				
HxHx	293 (64)	318 (69)	1	
H4Hx	155 (34)	126 (27)	1.3	1.0-1.8
H4H4	11 (2)	16 (4)	0.7	0.3-1.6
H4Hx/ H4H4	166 (36)	142 (31)	1.3	1.0-1.7
Frequency H4	19	17		
H5 (rs10517 and rs2965753)				
HxHx	361 (79)	368 (80)	1	
H5Hx	89 (19)	84 (18)	1.1	0.8-1.5
H5H5	9 (2)	8 (2)	1.1	0.4-3.0
H5Hx/ H5H5	98 (21)	92 (20)	1.1	0.8-1.5
Frequency H5	12	11		

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

Discussion

We studied the effect of *NQO1* haplotypes on the levels of several coagulation factors in a large family and in a population based case-control study on venous thrombosis, the Leiden Thrombophilia Study. We observed that H4 carriers had lower levels of FV in the family but were unable to confirm this difference in LETS controls. In the LETS controls, H4 appears to be associated with lower levels of vitamin K-dependent coagulation factors. It is worth mentioning that coagulation factor levels can not easily be compared between Family 8 and LETS because assays were performed in different laboratories at different time points and sometimes other assays were used (e.g. for FV levels).

The effect of *NQO1* polymorphisms on the levels of coagulation factors is poorly studied and the results remain controversial. Rs1800566, which tags the haplotype 4, results in a proline to serine substitution. This amino acid change was reported to cause rapid degradation of the enzyme [13] and thereby lower or undetectable enzymatic activity in heterozygous and homozygous carriers respectively [14]. This postulates that H4 carriers have lower levels of vitamin K-dependent coagulation factors. Similar to our observation in the LETS, a significant correlation between rs1437135 in *NQO1* (a SNP in complete linkage disequilibrium with rs1800566) and the levels of protein C, protein S and FII but not FVII, FIX and FX is reported in a genome-wide linkage study of Spanish families (Genetic Analysis of Idiopathic Thrombophilia (GAIT)) [15]. However, no relation was evident between the levels of protein C and S and rs1800566 (the htSNP of H4) in a study in the Japanese population [16]. Unfortunately, the Spanish authors did not consider FV levels in their analysis, probably because vitamin K is not deemed essential for FV biosynthesis. In both genome-wide linkage studies (GAIT and Family 8), adjusting for *NQO1* polymorphisms did not attenuate the linkage signal suggesting the presence of other genetic variation on chr 16 that is responsible for the linkage peaks. Since identity by descent probabilities are already quite accurate in Family 8, the possibility to narrow down the linkage signal by fine mapping is limited. We assessed the risk of venous thrombosis in the LETS for each *NQO1* haplotype. Heterozygous carriers of haplotype 4 were at almost significantly 30% higher risk of venous thrombosis as compared with

non-carriers. However, as the effect was not dose-dependent we presume it would lose its significance by enlarging the sample size.

In conclusion, we observed that haplotype 4 carriers of *NQO1* gene have lower levels of vitamin K-dependent coagulation factors especially lower FII and total protein S, in the control population of the Leiden Thrombophilia Study. None of the haplotypes seem to affect the risk of venous thrombosis. One *NQO1* haplotype was associated with FV levels in Family 8. However, linkage analyses adjusted for *NQO1* haplotypes showed that variations in *NQO1* could not explain the linkage peaks on chromosome 16 for the levels of FV and FII.

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Haplotypes of VKORC1, NQO1 and GGCX, their effect on activity levels of vitamin K-dependent coagulation factors, and the risk of venous thrombosis

4

Marieke C.H. de Visser, Sara Roshani, Julie W. Rutten, Astrid van Hylckama Vlieg, Hans L. Vos, Frits R. Rosendaal and Pieter H. Reitsma

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Dear Sirs,

Vitamin K antagonists, e.g. warfarin, acenocoumarol, and phenprocoumon, are widely used as treatment for individuals with increased thrombosis risk. The target of these drugs is the vitamin K epoxide reductase complex subunit 1 (VKORC1), a key enzyme in the vitamin K cycle. A reduced form of vitamin K (vitamin K hydroquinone) serves as a cofactor for gamma-carboxylase (GGCX) in the posttranslational carboxylation of vitamin K-dependent proteins, such as the coagulation proteins factor II, VII, IX and X, protein C, protein S, and protein Z. This carboxylation of glutamate residues is essential for full activation of these proteins. During gamma-carboxylation vitamin K epoxide is generated, which must be rapidly reduced again because of limited availability of reduced vitamin K. Via VKORC1 and NAD(P)H dehydrogenase [quinone] 1 (NQO1), vitamin K epoxide is recycled to its active form vitamin K hydroquinone. Binding of vitamin K antagonists to VKORC1 inhibits recycling of vitamin K, resulting in the formation of inactive, non-carboxylated proteins.

Whereas data on NQO1 and GGCX are scarce, genetic variation (single nucleotide polymorphisms, SNPs) in the *VKORC1* gene was repeatedly reported to influence the individual response of patients to vitamin K antagonists. Carriers of a specific *VKORC1* haplotype (A), consisting of several SNPs in complete linkage disequilibrium, were found to require a lower maintenance dose of vitamin K antagonists compared with haplotype B carriers [1].

Elevated plasma levels of vitamin K-dependent coagulation factors II, VII, IX, and X were previously found to be associated with an increased risk of venous thrombosis and they seem to have a significant genetic component [2]. However, only a few genetic determinants of these plasma levels have been identified. Furthermore, clustering of the levels of vitamin K-dependent proteins has been reported [3], suggesting that a common modifier gene exists. Genetic variation in *VKORC1*, *NQO1,* and *GGCX* might affect plasma activity levels of vitamin K-dependent proteins, and thereby thrombosis risk.

Several studies investigated the association between *VKORC1* variation and venous thrombosis risk [4-7], mainly by genotyping a SNP distinguishing haplotypes A and B. Lacut et al. [5] reported that haplotype A protected against thrombosis, whereas the other studies did not show any association. In a recent German study no association between SNPs in *VKORC1*, *NQO1,* and *GGCX* and activity levels

of vitamin K-dependent coagulation factors was found [8], whereas a Spanish study reported an association between an *NQO1* SNP with protein C levels [9].

Until now the association between genetic variation in *NQO1* and *GGCX* and venous thrombosis risk has not been studied. Furthermore, most previous studies only studied one SNP per gene, thereby not taking into account all common haplotypic variation. In the present study we investigated *VKORC1*, *NQO1,* and *GGCX* haplotypes and their association with activity levels of vitamin K-dependent coagulation proteins and venous thrombosis risk.

For our investigation we used the Leiden Thrombophilia Study (LETS), a population-based case-control study on venous thrombosis, including 474 consecutive patients aged 18-70 years with a first deep-vein thrombosis and 474 age- and sex-matched healthy controls [10]. Venous blood was collected into tubes containing 0.1 volume 0.106 mol/L trisodium citrate. Plasma was prepared by centrifugation for 10 minutes at 2000 g at room temperature and stored at -70°C. High molecular weight DNA was isolated from leukocytes and stored at 4°C. Measurements of factor II [11], factor VII [12], and protein C activity [13] have been described before.

Haplotype tagging SNPs in the *VKORC1* (n=4), *NQO1* (n=4), and *GGCX* (n=6) genes were identified in either the Caucasian Seattle PGA panel (*NQO1*, *GGCX*) or the Caucasian (CEU) Hapmap panel (*VKORC1*) using the Genome Variation Server (GVS, http://gvs.gs.washington.edu/GVS). Minor allele frequencies (MAF) were above 3%. All SNPs were genotyped using a 5'-nuclease/TaqMan assay (Applied Biosystems, Foster City, CA, USA). For all fourteen SNPs, the distribution of genotypes among control subjects was in Hardy-Weinberg equilibrium (tested using the χ^2-statistic). Tagging SNPs and haplotypes are shown in Table 1A. For *VKORC1* haplotypes, Geisen's nomenclature was used [14]. Analyses were performed with PLINK v1.06 software (http://pngu.mgh.harvard.edu/purcell/plink/) [15]. Haplotypes for the three genes were inferred in subjects without missing genotypes and haplotype allele frequencies were compared between patients and controls (Table 1A).

None of the haplotypes of the three genes affected venous thrombosis risk. Our results on *VKORC1* are in agreement with most previous findings. We could not confirm the finding that homozygous carriers of *VKORC1* haplotype A (tagged by rs2359612) are protected against thrombosis [5]. Individual SNPs were also not associated with the risk of venous thrombosis.

Linear regression analysis was used to test for an association between haplotypes and activity levels of vitamin K-dependent coagulation proteins in the LETS control population. Table 1B shows the regression coefficients β. Only two regression coefficients were significantly different from zero ($p<0.05$). *NQO1* H4 was associated with reduced factor II activity. Each copy of *NQO1* H4 was associated with a reduction in factor II activity of 2.68 % (i.e., linear regression coefficient $\beta= -2.68$; $p=0.04$). This reduction was consistent as also factor VII and protein C activity were reduced in *NQO1* H4 carriers. *NQO1* H4 is tagged by rs1800566 (p.Pro187Ser). The proline to serine substitution was found to be associated with loss of NQO1 protein and NQO1 activity [16], which may explain the observed reduction in levels. The rs1800566 SNP is in complete linkage disequilibrium with rs1437135 (http://www.hapmap.org) which was previously reported to be associated with protein C levels in the GAIT study [9]. Individual analysis of all fourteen SNPs also showed an association between rs1800566 and factor II activity. This finding was the only significant result in the single SNP analysis. The second significant result in the haplotype analysis was the association between *GGCX* H1 and reduced factor II activity ($\beta= -1.90$; $p=0.048$), but this result may be spurious. The reduction was not consistent as factor VII activity was not reduced. Rieder et al. previously showed that *VKORC1* haplotype A (combination of VKORC1*2A and VKORC1*2B) is associated with a reduced expression of VKORC1 in the liver [1]. In LETS controls a trend towards lower activity of factor II, factor VII and protein C was observed in haplotype A carriers, which is in accordance with Rieder's report.

In conclusion, we did not find an association between haplotypes of *VKORC1*, *NQO1* and *GGCX* and venous thrombosis risk. *NQO1* H4 does possibly have a small influence on activity levels of vitamin K-dependent proteins. However, these changes are too subtle to noticeably change thrombosis risk.

Table 1A. Haplotypes and tagging SNPs of *VKORC1*, *NQO1* and *GGCX*

	Tagging SNPs rs number (SeattleSNPs numbering[1])						Frequency LETS	
VKORC1 haplotypes[2] (clusters)[3]	rs2884737 (5808)	rs17708472 (6009)	rs2359612 (7566)	rs7294 (9041)			Patients n=469	Controls n=466
VKORC1*2A (A)	T	C	**T**	G			13.2	14.5
VKORC1*2B (A)	**G**	C	**T**	G			26.9	26.3
VKORC1*4 (B)	T	**T**	C	G			22.0	21.7
VKORC1*3 (B)	T	C	C	**A**			36.8	36.2
VKORC1*1 (B)	T	C	C	G			1.2	1.3
NQO1 haplotypes	rs689453 (1910)	rs2965753 (2898)	rs1800566 (9144)	rs10517 [4]			Patients n=461	Controls n=462
NQO1 H1	**A**	A	C	C			7.1	8.7
NQO1 H2	G	A	C	C			60.7	61.8
NQO1 H3	G	A	C	**T**			1.2	1.4
NQO1 H4	G	A	**T**	C			19.2	17.1
NQO1 H5	G	**G**	C	**T**			11.9	11.0
GGCX haplotypes	rs6738645 (7475) [5]	rs699664 (10067)	rs10179904 (10496)	rs11676382 (12970)	rs17026447 (13031)	rs2028898 (13333)	Patients n=465	Controls n=461
GGCX H1	A	G	C	G	T	C	43.6	41.6
GGCX H2	A	G	C	**C**	T	C	9.8	11.5
GGCX H3	C	A	C	G	**G**	C	2.9	2.7
GGCX H4	**C**	**A**	C	G	T	**T**	30.6	30.2
GGCX H5	**C**	G	**T**	G	T	C	10.9	10.5
GGCX H6	**C**	G	C	G	T	C	2.2	3.4

Minor alleles in bold and underlined [1] http://pga.mbt.washington.edu/, [2]According to Geisen et al (14), [3]According to Rieder et al (1), [4]Not determined in SeattleSNPs panels, [5]in Hapmap CEU population A is minor allele

Table 1B. Association of *VKORC1*, *NQO1* and *GGCX* haplotypes with activity of vitamin K-dependent coagulation proteins in controls

	Factor II (%)	Factor VII (%)	Protein C (%)
VKORC1 haplotypes			
VKORC1*2A	1.57	0.81	1.60
VKORC1*2B	-1.11	-1.46	-1.17
VKORC1*4	0.02	2.36	-0.96
VKORC1*3	0.07	-0.36	0.98
VKORC1*1	-1.63	-5.67	-4.12
VKORC1 haplotype A[1]	-0.07	-0.73	-0.16
NQO1 haplotypes			
NQO1 H1	1.18	1.21	-0.31
NQO1 H2	0.75	2.28	1.35
NQO1 H3	0.85	-4.31	-2.05
NQO1 H4	**-2.68***	-2.85	-1.03
NQO1 H5	1.47	-1.21	-0.92
GGCX haplotypes			
GGCX H1	**-1.90***	0.94	-2.02
GGCX H2	1.76	-2.71	-1.40
GGCX H3	2.32	-0.16	-0.18
GGCX H4	1.21	0.95	2.29
GGCX H5	-0.95	-2.09	-0.59
GGCX H6	-0.70	4.15	2.78

Regression coefficients β are shown. The direction of the regression coefficient represents the effect of each extra copy of the haplotype (i.e. a positive regression coefficient means that the haplotype increases phenotype mean). Activity in pooled normal plasma is 100%.

[1]According to Rieder et al (1)

* $p<0.05$

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Thrombophilia and Venous Thromboembolism: Implications for Testing

5

Sara Roshani, Danny M. Cohn and Saskia Middeldorp

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Abstract

In the last decades, the knowledge on the etiology of venous thromboembolism (VTE) has increased tremendously. In approximately half of patients presenting with VTE, one or more thrombophilic defects can be identified. This has led to widespread testing for thrombophilia, despite the fact that, at present, it is unclear whether this should have therapeutic consequences. Here we review the currently established hereditary and acquired thrombophilic defects, and focus on the pros and cons of testing in the setting of VTE. Thrombophilia is defined as a disorder associated with an increased tendency to venous thromboembolism (VTE). Thrombophilia can be acquired, such as in patients with cancer, or congenital, in which case a defect in the coagulation system is hereditary. Egeberg was the first to use the term thrombophilia in 1965, when he described a Norwegian family that had a remarkable tendency to VTE, based on a deficiency of antithrombin.[1] Since then, various laboratory abnormalities, both hereditary and acquired, have been discovered that increase the risk of VTE. This article reviews the currently established thrombophilic abnormalities and discusses the potential usefulness and implications of testing for thrombophilia.

Coagulation cascade and regulatory mechanisms

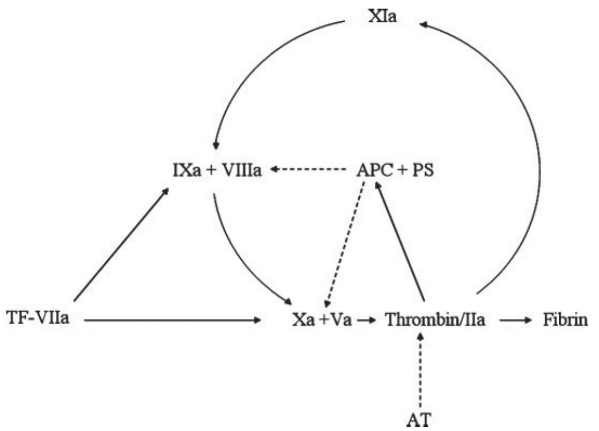
Well-established hereditary thrombophilia can be categorized into abnormalities of the natural anticoagulant system, elevation of plasma levels of coagulation factors, abnormalities in the fibrinolytic system, and miscellaneous hereditary conditions.

Fig. 1 depicts the current, simplified insight into the regulation of the coagulation system. Coagulation is initiated by a tissue factor (TF)/factor (F) VIIa complex that can activate FIX or FX. At high TF concentrations, FX is activated primarily by the TF/FVIIa complex, whereas at low TF concentrations, the contribution of the FIXa/FVIIIa complex to the activation of FX becomes more pronounced. Coagulation is maintained through the activation of FXI by thrombin. The coagulation system is regulated by the protein C pathway. Thrombin activates protein C. With protein S as a cofactor, activated protein C (APC) inactivates FVa and FVIIIa, which results in a downregulation of thrombin generation and consequently in an upregulation of the fibrinolytic system. Antithrombin is the other important natural anticoagulant that inhibits not only thrombin but also FXa and other coagulation factors by forming irreversible complexes. Furthermore, tissue factor pathway inhibitor (TFPI) inhibits the initiation of coagulation. Thrombin-activatable fibrinolysis inhibitor (TAFI) inhibits fibrinolysis, thereby protecting a formed thrombus from lysis in the presence of large amounts of thrombin.

Abnormalities of the anticoagulant system

Most defects in the natural anticoagulant systems increase the tendency toward thrombosis. In the following paragraphs, deficiencies of the well-established thrombophilic abnormalities (i.e., deficiencies of protein C, protein S, and antithrombin) are discussed, as well as the evidence on less well-known candidates.

Figure 1. Regulation of blood coagulation. Coagulation is initiated by a tissue factor (TF)/factor (F) VIIa complex that can activate FIX or FX. At high TF concentrations, FX is activated primarily by the TF/FVIIa complex, whereas at low tissue factor concentrations the contribution of the FIXa/FVIIIa complex to the activation of FX becomes more pronounced. Coagulation is maintained



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Protein C Deficiency

Protein C deficiency appears to be quite rare, with a prevalence of 0.2% in the general population and 2.5 to 6% in patients with first VTE.[2,3] Numerous mutations in the gene coding for protein C lead to several types of protein C deficiency and are summarized in a published database.[4] In type I deficiency, levels of both antigen and activity are reduced, whereas in type II deficiency, antigen levels are normal but one or more functional defects lead to a decreased activity. Abnormalities in type II protein C deficiency can occur on sites of substrate binding, thrombomodulin interaction, or calcium binding. Most patients have heterozygous protein C deficiency with a protein C level 50% of normal. Homozygous protein C–deficient patients who completely lack the natural anticoagulant protein are extremely rare. These patients will develop a syndrome characterized by diffuse VTE with accompanying skin necrosis shortly after birth, known as neonatal purpura fulminans, which is fatal unless protein C is given in the form of protein C concentrate, fresh frozen plasma,[5] or FIX concentrate (which contains a large amount of protein C and S) together with heparin.[6] Homozygous or compound heterozygous patients with mutations leading to severely reduced but not absent levels of protein C do not have this clinical syndrome but may present with VTE in adulthood or will develop skin necrosis when exposed to vitamin K antagonists (VKA). These patients have protein C levels less than 20% of normal.[7]

Protein S Deficiency

The prevalence of protein S deficiency in the general population is estimated between 0.026% and 0.13% in a Scottish study among healthy blood donors, and at 1 to 2% patients with first VTE episode[8,9] . Protein S, in addition to being a cofactor to protein C, independently inhibits the prothrombinase and tenase complexes.[10] The pattern of inheritance is autosomal dominant. One third of patients with protein S deficiencies will have VTE by the age of 60 years. Homozygosity is associated with neonatal purpura fulminans.[5] Warfarin skin necrosis has been reported in protein S–deficient patients.[11]

Antithrombin Deficiency

Antithrombin directly inhibits thrombin and activated FIX, FX, and FXI by forming a covalent complex, a process that is accelerated 1000-fold by heparin and endogenous heparin-like substances. Antithrombin deficiency occurs in 0.02% of the general population and in 0.5 to 7.5% of patients presenting with a first VTE.[12] Traditionally, antithrombin-deficient patients are considered at higher risk for thrombosis than patients with other congenital thrombophilic states, although this observation may be caused by selection of severely thrombophilic families in the early days of thrombophilia testing, when only antithrombin deficiency was known. However, the rarity of the deficiency may point to a more severe clinical expression. The inheritance pattern is autosomal dominant, and almost all patients are heterozygous.

Activated Protein C Resistance and FV Leiden

FV Leiden (FVL) is responsible for at least 90% of APCresistant conditions. It is the most prevalent thrombophilic defect that occurs in 5 to 12% of the general population, with variations throughout the world.[13] APC, together with its cofactor protein S, regulates the coagulation cascade by inactivating FVa and FVIIIa. APC cleaves FVa in three sites, but cleaving the first site at amino acid position 506 is necessary for better access to the other two sites. The mutation in the first site is known as FVL, and in the other site is described as FV Cambridge (at position 306)[14] and FV Hong Kong.[15] In carriers of FVL, FVa is inactivated approximately 10 times more slowly than normal,[16] whereas the implications of the other mutations on APC resistance are not as strong.[17] No mutations at cleavage sites in FVIII were found that cause APC resistance.[18] Recently, through genome-wide scans in family studies, a locus on chromosome 18 was described that appeared to influence normal variation in APC resistance and FVIII levels, as well as susceptibility to thrombosis.[19] However, to date, this has not been confirmed by other groups. Other mechanisms for APC resistance are antiphospholipid antibody syndrome (APS),[16] high concentration of FVIII,[20] and reduced levels of protein S.[21]

Prothrombin G20210A Mutation

The second most prevalent form of thrombophilia is prothrombin G20210A. This mutation is found in 1% of the population, in 4 to 8% of patients with a first VTE.[22] The proposed mechanism for thrombosis is elevation in the levels of normal prothrombin.[23] A prothrombin level higher than 115% (90th percentile) results in a 2.1-fold increased VTE risk also in the absence of the prothrombin 20210A mutation.[23,24] However, carriers and noncarriers cannot be distinguished using the prothrombin level because there is a high degree of overlap between the groups.[24]

Elevated Levels of Coagulation Factors

As is the case for prothrombin (FII), increased levels of coagulation FVIII, FIX, FXI, and fibrinogen increase the risk of VTE.[25–28] In several studies, elevated FVIII levels were shown to be quite prothrombotic, with a dose-dependent 5- to 10-fold relative risk increase.[25,29,30] For each 10% increase of FVIII, the VTE risk increases by 10% (95% confidence interval, 0.9 to 21.0),[30] and this is independent of an acute-phase role of FVIII.[29,31] Although the etiology of persistently elevated FVIII is not clear, it appears to be determined in part genetically in some patients,[30,32] but not all studies confirm this.[33] Elevated FIX was also shown to increase the risk of VTE 2.5-fold, independent of several potential confounders and other genetic defects.[26] Elevated FXI is associated with a 2-fold risk increase for VTE.[27] The likely mechanism for an elevation of FXI is excessive thrombin generation, which in turn leads to more thrombin deposition and downregulation of fibrinolysis by activation of TAFI.[34] Finally, fibrinogen levels >500 mg/dL are associated with approximately a 4-fold risk increase of VTE,[28] which was not explained by acute-phase reactions as measured by C-reactive protein.[31] Elevated levels of FV,[35] FVII,[28] and FX,[36] have not shown to increase the risk for thrombosis.

Miscellaneous (candidate) Risk Factors

Hyperhomocysteinemia

Severe hyperhomocysteinemia or homocystinuria is a rare condition that is associated with strongly elevated levels of homocysteine (>100 mmol/L), premature arterial and VTE, mental retardation, and a Marfan-like stature.[37] It is caused by homozygosity or compound heterozygosity of mutations in cystathionine-b-synthase, whereas heterozygosity results in mild to moderate homocysteinemia. More than 90 mutations in this gene are known to cause homocystinuria.[38] The second gene that may be involved is methylenetetrahydrofolate reductase (MTHFR). Several mutations are known to increase the levels of homocysteine; homozygosity leading to MTHFR deficiency, however, is rare. Contrary to homocystinuria, mild hyperhomocysteinemia is common and, depending on chosen cutoff values, occurs in 5 to 10% of the population. A common thermolabile C677T polymorphism results in mild hyperhomocysteinemia in homozygotes who are low in folate, vitamin B12, or vitamin B6.[39] It has remained a matter of debate whether mild homocysteinemia is a cause or consequence of VTE. The current opinion is that the association is mild; a 5 mmol/L increase in plasma level increases the risk for VTE by 1.27 in prospective studies, and by 1.60 in retrospective studies.[40] The presence of the homozygous TT677 MTHFR polymorphism increases the risk 1.20-fold, with no observed effect in studies in North America. It was hypothesized that this differential effect is explained by the higher folate intake in this part of the world.

Other natural anticoagulants

TFPI inhibits the coagulation process during its earliest phase. Levels below the 10th percentile of values in control subjects were associated with a 1.7-fold risk increase for VTE in one study.[41]

Fibrinolytic system

Generally, impaired overall fibrinolysis appears to be associated with an increased risk for VTE, although the role of the individual components is not very clear.[42] No relationship has been found between deficiencies of plasminogen, levels of polymorphisms in the gene, and levels of polymorphisms in the gene coding for

tissuetype plasminogen activator, whereas increased levels of tissue plasminogen activator inhibitor type 1 have shown conflicting results.[42] High levels of TAFI have been shown consistently to increase the risk for VTE.[43–45]

Miscellaneous

Although it has been hypothesized that deficiencies in the contact coagulation pathway, in particular FXII, may increase the risk of thrombosis because of a reduced fibrinolytic capacity, several studies have indicated that FXII deficiency does not increase the risk of VTE.[46,47]

Acquired Thrombophilia

Antiphospholipid antibody syndrome

APS is an autoimmune disorder defined as venous and/ or arterial thrombosis or recurrent pregnancy loss in the presence of persistent antibodies against phospholipids or phospholipid-binding proteins, and is categorized into primary and secondary status based on presence of autoimmune diseases such as systemic lupus erythematosus.[48] The syndrome is not common in consecutive patients with VTE, although the prevalence of isolated antiphospholipid antibodies measured on a single occasion in healthy subjects is not uncommon, and is only a weak risk factor for VTE.[46] The presence of lupus anticoagulant, however, is associated with a 5- to 16- fold increased risk for VTE.[49]

Implications of testing for thrombophilia

Given that, at present, a thrombophilic defect can be demonstrated in more than 50% of the patients who present with a VTE, the tendency to test patients for thrombophilia has increased tremendously. However, the usefulness and cost effectiveness of testing is a matter of debate.[51,52] The (dis)advantages and implications of thrombophilia testing are discussed in the following section. Reasons for testing for thrombophilia might be clarification of the cause, the possibility to adjust therapeutic regimes of VTE in thrombophilic patients for the optimal prevention of recurrence, and the possibility to track asymptomatic family members (and subsequently take preventive measures). Conversely, testing

for thrombophilia might lead to needless expenses, anxiety, and social problems.

Reasons to Test for Thrombophilia

It is often argued that patients and their doctors would like to have an explanation for the episode of VTE, although this has never been explicitly studied. It should be realized however, that the existence of a thrombophilic defect does not exclude other reasons for a prothrombotic state. For example, a 60-year-old male presenting with an idiopathic deep VTE of the leg might have an occult cancer as well as a thrombophilic defect. An important argument in favor of testing for thrombophilia would be the possibility to adjust therapeutic measures for treatment of a VTE (by means of intensity or duration of treatment). The optimal therapy for VTE depends on the risk of recurrence, the (dis)- comfort of the therapy and the risk of side effects, such as (major) bleeding. The estimated risk of recurrence for VTE, in general, is 5% per year,[52,53] although idiopathic episodes tend to recur more frequently (20% in the first 2 years) compared with provoked episodes.[54] Standard therapy for patients with a first VTE includes anticoagulant treatment with VKAs for 3 to 6 months, with international normalized ratios between 2 and 3.[55] This therapy ensues an annual bleeding risk of 0.25% for fatal bleeding and 1.0% for life-threatening bleeding.[56,57] A different approach to thrombophilic patients, compared with nonthrombophilic patients, is only justified if the former have a different risk of recurrence. Even though thrombophilia has shown to increase the risk of a first VTE, it is to date still controversial whether thrombophilia also increases the risk of recurrent VTE. The estimated relative risk of recurrence in patients with thrombophilia is small, compared with patients without thrombophilia (Table 1). The estimated odds ratios (ORs) for the natural anticoagulant deficiencies, as described mainly in retrospective analyses, were 2.5.[58–60] In one prospective study, the follow- up of the Leiden Thrombophilia Study (LETS), the risk of recurrence appeared even more moderate, with an OR of 1.8.[61] Two meta-analyses studied the risks of recurrence in patients with the common thrombophilias: FVL and the prothrombin G20210A mutation. The risk of recurrence was found consistently to be 1.3- to 1.4-fold higher in patients with FVL and 1.4- to 1.7-fold higher in patients with the prothrombin mutation.[62,63] For the other thrombophilic defects, less data are available. Three studies assessed the risk of recurrence in patients with high levels of FVIII coagulant

activity (FVIII:c) compared with patients with normal levels. One case-control study showed that elevated levels of FVIII:c above 150% were associated with an approximate 2-fold increased risk of recurrent VTE, compared with patients with a single episode.[30] In two cohort studies, the estimated relative risk of recurrent VTE was 6-fold increased in those with FVIII:c levels above the 90th percentile, corresponding to 234% and 294%, respectively.[64,65] These results could not be reproduced in the LETS follow-up study, in which an OR of only 1.3 was found.[61] The data on estimated risk of recurrence for elevated levels of FIX and FXI are scarce, but their impact on recurrence seems negligible.[61] The attribution of mild hyperhomocysteinemia in terms of risk of recurrence appears low (1.8 to 2.7),[66–68] and treating hyperhomocysteinemia did not show a decrease in the number of recurrences. [67] The risk of recurrence in antiphospholipid or anticardiolipin antibodies was investigated in four studies. [69–72] The outcomes regarding relative risk for recurrence range between 2- and 6-fold. These results are difficult to interpret, given that in these studies the antiphospholipid and anticardiolipin antibodies or lupus anticoagulant were not tested repetitively (as suggested by international guidelines[48]). Moreover, duration of anticoagulant treatment differed substantially. Adjustment of anticoagulant treatment in thrombophilic patients after a first VTE has only been addressed for a difference in intensity. This has not shown to be beneficial in patients with VTE, regardless of thrombophilia. Reducing the intensity of VKAs below 2.0 led to an increase of recurrence risk (1.9 versus 0.6%),[73,74] whereas major bleeding complications did not differ between a low-intensity and regular-intensity treatment (0.96 versus 0.93%).[73] A higher intensity of VKA in patients with antiphospholipid antibodies showed no reduction in the risk of recurrence, but led to an increase in the bleeding risk.[75,76] Whether clinical outcome of patients with VTE and thrombophilia improves with prolongation of anticoagulant has never been investigated. Current trials focus on whether such an intervention outweighs the bleeding risk, given that it is known that oral anticoagulant medication prevents VTE by more than 90%, as long as it is used.[77] Finally, a potential advantage of testing patients with VTE for thrombophilia may be the identification of asymptomatic family members. These individuals have a 2- to 10-fold increased risk for VTE as compared with noncarriers.[78–81] Regardless of this increased relative risk, the overall absolute risk remains low (Table 2). It is often argued that asymptomatic family members with

thrombophilia may benefit from targeted prevention in high-risk situations (e.g., pregnancy, puerperium, surgery,immobilization, and trauma), and the avoidance of acquired risk factors, most notably oral contraceptives. It is clear from Table 2 that bleeding risk associated with continuous anticoagulant treatment outweighs the risk of VTE. It is notable that the figures considering surgery, trauma, and immobilization, as shown in Table 2, have been collected for the larger part in times before standard prophylaxis was routine patient care. For pregnancy, 80% of the episodes occur in the postpartum period. Whether this should lead to administration of prophylaxis in the postpartum period is a matter of physician and patient preference, given that the number needed to treat is 25 in case of a deficiency in the natural anticoagulants and approximately twice as high in patients with the common thrombophilias. Finally, it is clear from data in Table 2 that the use of oral contraceptives should be weighed against the disadvantages of other contraceptive methods.

Table 1. Estimated Relative Risk of VTE Recurrence in Patients with Thrombophilia

Type of Thrombophilia	Relative Risk
Natural anticoagulant deficiencies	1.8-2.5[58-61]
FVL	1.3-1.4[62,63]
Prothrombin 20210A	1.4-1.7[62,63]
Elevated FVIII:c	1.3-6[61,64,65]
Elevated levels of FIX	1.2[61]
Elevated levels of FXI	0.6[61]
Mild hyperhomocysteinemia	1.8-2.7[66-68]
Antiphospholipid antibodies	2-6[69-72]

VTE, venous thromboembolism; FVL, factor V Leiden; FVIII:c, factor VIII coagulation activity; F, factor.

Table 2. Absolute Risk of VTE in Asymptomatic Carriers of Thrombophilia

Type of Thrombophilia	Overall Risk (%/year)	Surgery, Trauma, or Immobilization (%/episode)	Pregnancy (%/pregnancy)	Oral Contraceptive Use (%/year of use)
Natural anticoagulant deficiencies	0.4-4.0[78,87-91]	8.1[78]	4.1[78]	4.3[78]
FVL	0.1-0.7[78,80,81,87,90,92,94]	1.8-2.4[81]	1.9-2.1[78,81]	0.5-2.0[78,81]
Prothrombin 20210A	0.1-0.4[94-97]	2.0[95]	2.8[95]	0.2[95]
Elevated FVIII:c	0.3[97]	1.2[97]	1.3[97]	0.6[97]
Mild hyperhomocysteinemia	0.2[98]	0.9[98]	0.5[98]	0.1[98]

VTE, venous thromboembolism; FVL, factor V Leiden; FVIII:c, factor VIII coagulant activity; F,factor.

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Reasons Not to Test for Thrombophilia

Disadvantages of testing patients with a VTE for thrombophilia might be the cost of testing, which is approximately *s*500.[51] Several sophisticated studies focused on the cost effectiveness of testing for thrombophilia. [82,83] These studies focused on selected patient groups, because universal testing was considered less cost effective. It is of note that the external validity of the results may be distorted by the fact that the findings were based on a range of various assumptions. One study, by Marchetti et al,[82] assessed the cost effectiveness of testing for double heterozygosity of FVL and the prothrombin mutation, and subsequently prolonging anticoagulant therapy in those tested positive for both common thrombophilias. This strategy was considered cost effective, given that testing all patients with VTE provided one additional day of life at the cost of $13.624/quality-adjusted life-years.[82] The Thrombosis: Risk and Economic Assessment of Thrombophilia Screening study assumed that testing for thrombophilia might be efficacious in high-risk situations.[83] The cost effectiveness of four different testing scenarios was calculated: (1) testing all women prior to prescription of oral contraceptives and restricting prescription only to those tested negative for thrombophilia; (2) testing all women prior to prescribing hormone replacement therapy and restricting prescription only to those tested negative for thrombophilia; (3) testing women at the onset of pregnancy and prescribing prophylaxis to those tested

positive for thrombophilia; and (4) testing all patients prior to major elective orthopedic surgery and prescribing extended thromboprophylaxis to those tested positive for thrombophilia. It was concluded in this study that the second scenario would be most cost effective, compared with the other scenarios. Nevertheless, selective screening based on the presence of previous personal or family history of VTE was considered to be more cost effective than universal testing in the four different scenarios.

Furthermore, the psychological impact and consequences of knowing that one is a carrier of a (genetic) thrombophilic defect could be regarded as a drawback of testing. Most studies that focused on impact of testing for thrombophilia showed that patients had experienced low psychological distress following thrombophilia testing. [84,85] Nevertheless, qualitative studies described several negative effects. In the study by Bank et al,[86] it is mentioned that parents were worried that their children "would be negatively influenced by factor V Leiden" and that some carriers "felt stigmatized." Finally, a disadvantage of testing for thrombophilia could be its potential social consequences (for instance, problems with health insurance or life insurance), although little data are available on this issue.

Conclusions

The discovery of newer thrombophilic defects has led to a deeper insight in the development of VTE during the last decades. However, testing patients with VTE, with respect to its advantages and disadvantages, has so far no direct consequences in terms of different treatment strategies. Given the absence of clear benefits, thrombophilia testing should be performed with restraint.

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Incidence of postpartum hemorrhage in women receiving therapeutic doses of low-molecular-weight heparin: results of a retrospective cohort study

6

Sara Roshani, Danny M. Cohn , Alexander C. Stehouwer, Hans Wolf, Joris A. M. van der Post, Harry R. Büller, Pieter W. Kamphuisen and Saskia Middeldorp

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Abstract

Background
Low-molecular-weight heparin (LMWH) is the drug of choice to prevent venous thrombosis in pregnancy, but the optimal dose for prevention while avoiding bleeding is unclear. We investigated whether therapeutic doses of LMWH increase the incidence of postpartum hemorrhage in a retrospective controlled cohort study.
Methods
We identified all pregnant women who received therapeutic doses of LMWH between 1995 and 2008 in the Academic Medical Center, Amsterdam, The Netherlands. The controls were women registered for antenatal care in the same hospital who did not use LMWH during pregnancy, matched by random electronic selection for age, parity and delivery date to LMWH users. We compared the incidence of PPH (blood loss> 500 mL), incidence of severe PPH (blood loss> 1000 mL) and the median blood loss in two cohorts of LMWH users and non-users.
Results
The incidence of PPH was 18% in LMWH users (N=95) and 22% in non-users (N=524) (RR 0.8; 95%CI 0.5 to 1.4). The incidence of severe PPH was 6% in both groups (RR 1.2; 0.5 to 2.9). Median amount of blood loss differed only in normal vaginal deliveries. It was 200 mL in LMWH users and 300 mL in non-users (difference -100 mL; 95%CI -156 to -44).
Conclusion
We observed that therapeutic doses of LMWH in pregnancy was not associated with clinically meaningful increase in the incidence of PPH or severe PPH in women delivered in our hospital although this observation may be confounded by differential use of strategies to prevent bleeding. A randomized controlled trial is necessary to provide a definite answer about the optimal dose of LMWH in pregnancy.

Introduction

Low-molecular-weight heparin (LMWH) is the drug of choice in pregnant women requiring prophylaxis or treatment for venous thrombosis. However, the optimal dose with respect to efficacy and safety is uncertain.[1] LMWH has the disadvantage that its anticoagulant effect can only be partially antagonized. This is of particular importance with respect to its use in high doses and raises concerns about an increased risk of bleeding, most notably postpartum hemorrhage (PPH), when used in pregnant women.

PPH is defined by the World Health Organization (WHO) as postpartum blood loss in excess of 500 mL.[2] However, since other definitions have been suggested,[3] we classified blood loss more than 1000 mL as severe PPH. PPH has an incidence of 19% in nulliparous deliveries in the Netherlands.[4] The diagnosis encompasses excessive blood loss from uterus, cervix, vagina and perineum. The commonest cause of primary PPH (PPH < 24 hours following delivery) is uterine atony.[5] In order to limit the risk of PPH, current guidelines recommend discontinuation of LMWH 12 to 24 hours prior to delivery.[1;6] However, as labour can commence spontaneously, timely discontinuation cannot be guaranteed. The risk of PPH associated with use of LMWH has been assessed in several studies.[3;7-13] These studies either included a small or an unknown number of women treated with therapeutic doses of LMWH [3;7-10] or they lacked a control group of women who did not use LMWH.[7;9-11;13] Only two studies report the bleeding risk associated with antepartum therapeutic doses of LMWH: a prospective multicenter survey in the UK and Ireland and a systematic review of studies about LMWH use in pregnancy.[11;13] Blood loss more than 500 mL was observed in 6/126 (4.8%) and 3/174 (1.7%) of women who were treated with therapeutic doses of LMWH in these two studies respectively. On the other hand, significant failure rates have been observed despite prophylaxis with low-dose LMWH in pregnancy.[14-16] In our hospital, pregnant women whom we judge to require anticoagulant prophylaxis are treated with therapeutic doses of LMWH. This protocol was based on a systematic review that we performed in 1998.[14] In this review of several cohorts of women, recurrent venous thromboembolism (VTE) occurred in 2.0% (3/149) of pregnant women, all of whom were treated with prophylactic or intermediate doses of LMWH. Similar findings were reported in another large cohort study in

which 7 of 8 recurrent episodes of VTE occurred in women on prophylactic or intermediate doses of enoxaparin.[15]
We performed a controlled cohort study in our hospital to assess the risk of PPH associated with therapeutic doses of LMWH in pregnant women.

Material and methods

Identification of study cohorts
By hospital protocol, anti-Xa levels were measured at one-month intervals in women who were treated with therapeutic doses of LMWH or heparinoid during pregnancy. Thus, our study cohort was identified by collection of hospital ID numbers in whom anti-Xa measurements were performed between mid-August 1995 and mid-February 2008. We reviewed charts to assess whether the anti-Xa measurements were performed during pregnancy. Inclusion criteria were: therapeutic doses of LMWH, pregnancy duration of at least 25 weeks gestation, and delivery in the Academic Medical Center (AMC).
The control cohort consisted of women who had been registered for antenatal care in the AMC before 24 weeks gestational age, delivered in the AMC and did not use LMWH during their pregnancy. Women treated with LMWH and controls were matched by random electronic selection for age (±2 years), parity (nulliparous or multiparous) and date of delivery (±1 year) in a 1:6 ratio. This study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam.

Intervention
The hospital protocol was to base LMWH doses on body weight prior to pregnancy, in which the therapeutic dose of LMWH was prescribed according to the manufacturer (Table 1).
All women were seen at the outpatient clinic of the Department of Vascular Medicine with regular intervals in which measurements of anti-Xa levels were performed. Dose-adjustments were only done if peak anti-Xa activity was lower than 0.4 or higher than 1.2 anti-Xa units on repeated occasions. A multidisciplinary team of obstetricians and vascular medicine experts discussed patients at regular intervals. Women were advised to discontinue LMWH as soon

as either contractions started, membranes ruptured or administer the last injection the morning prior to the day that induction of labour or a cesarean section was planned. Also women were informed that epidural or spinal anesthesia was contraindicated within 24 hours after the last dose of LMWH. Management of postpartum hemorrhage was performed at the attending obstetrician's discretion.

Table 1. Types of LMWH administered and the median and range of the doses per day

LMWH type	N	Median*	Range	Weight range
Enoxaparin, mg	16	120	60 to 200	53 to 116
Dalteparin, IU anti-Xa	9	15000	10000 to 20000	64 to 115
Nadroparin, IU anti-Xa	64			
<75 kg	33	11400	11400 to 15200	48 to 74
≥75 kg	31	15200	11400 to 20900	75 to 117
Danaparoid, IU anti-Xa	3	4000	3000 to 4500	55 to 66
Tinzaparin, IU anti-Xa	3	18000	14000 to 28000	75 to 82

* Doses are presented in mg for enoxaparin and IU for other LMWHs

Outcomes

The primary outcomes were PPH and severe PPH defined as the amount of blood loss estimated by the attending obstetrician or midwife of more than 500 mL and more than 1000 mL respectively, within 24 hours of delivery. Secondary outcomes were the estimated amount of blood loss in mL, blood transfusions in the first week postpartum, and recurrent VTE.

Statistical analysis

We calculated the incidence of PPH and severe PPH for LMWH users and non-users. Relative risks (RR) of PPH and severe PPH and their 95%CI in pregnant women treated with therapeutic doses of LMWH compared to non-users were calculated. Non-normally distributed data are presented as medians. We calculated the median blood loss difference between two cohorts of women and its 95%CI. Furthermore, we compared the median blood loss of both groups in

strata of a priori defined other risk factors, if kwn (i.e. type of vaginal delivery [normal versus assisted] or cesarean section [elective versus emergency], perineal laceration degree and ethnicity) to investigate their interaction with LMWH on the incidence of PPH. Blood transfusion in the first 24 hours of delivery was compared between two groups of the study using the X^2 test.

Results

We identified 95 women who used therapeutic doses of LMWH during pregnancy for various indications (see Figure 1 for case selection) and 524 women as control cohort who did not use LMWH in their pregnancy. Baseline characteristics of the study groups are shown in Table 2. Median gestational age (range) was 39 (26-44) weeks in LMWH users and 39 (25-43) in non-users. In both cohorts, almost 93% of vaginal deliveries proceeded spontaneously (normal vaginal delivery) and 7% needed assistance. Almost one-quarter (23 %) of the women treated with LMWH delivered by cesarean sections; half of these were elective, i.e. planned before onset of labour. In the control cohort 10% of the women underwent cesarean sections, most were emergency cesarean sections (90%).

Table 3 demonstrates the outcomes of the study, some stratified for types and subtypes of delivery. PPH occurred in 18% of women who used therapeutic doses of LMWH and in 22% of controls (RR for PPH: 0.8; 95%CI: 0.5 to 1.4). The incidence of severe PPH (6%) was the same in two groups of LMWH users and non-users (RR for severe PPH: 1.2; 95%CI: 0.5 to 2.9). The risk of PPH and severe PPH after vaginal or cesarean section delivery was not statistically significant different between two groups of women.

Median blood loss after vaginal delivery was 250 (range, 50 to 4000) and 300 (20 to 3600) mL in LMWH users and non-users respectively (median difference -50; 95%CI: -102 to 2). After cesarean section, it was 425 (200 to 2000) mL in LMWH users and 400 (100 to 2000) mL in non-users (25; -153 to 203). Median blood loss stratified for subtypes of delivery differed between LMWH users and non-users only after normal vaginal deliveries (200 (range, 50 to 4000) and 300 (20 to 3600) mL in LMWH users and non-users respectively.

Median blood loss did not differ between groups after stratification for ethnicity and perineal laceration degree (data not shown).

Blood transfusion was given, at the discretion of the attending obstetrician, in 5% of LMWH users and 3% of non-users after delivery (OR 1.6; 95%CI: 0.6 to 4.3). In terms of efficacy, recurrent VTE was suspected in one woman (1.2%, 95%CI 0.6-5.8) despite the use of therapeutic doses of LMWH. However, a recurrent episode was not confirmed as ventilation/perfusion scintigraphy revealed a perfusion defect on the same localization as the previous PE.

Figure 1. inclusion flowchart of women treated with LMWH.

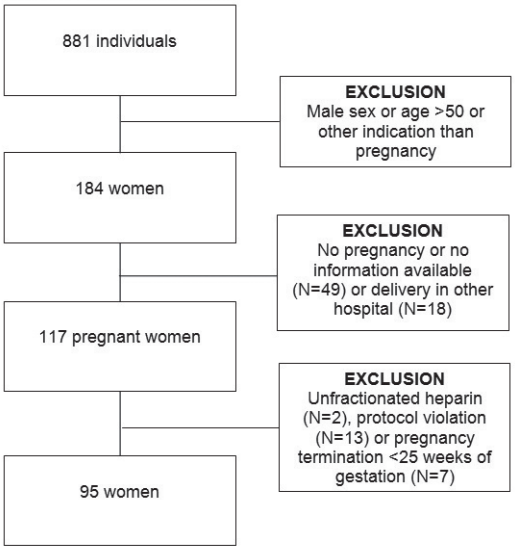

Table 2. Baseline characteristics of the two study groups

	Women who used therapeutic dose of LMWH (N=95)	Women who did not use LMWH (N=524)
Age, years Median (range)	32 (21-43)	31 (18-44)
Ethnicity N (%)		
Caucasian	67 (70)	264 (50)
African	14 (15)	167 (32)
Others/unknown*	14 (15)	93 (18)
Gestational age, weeks Median (range)	39 (26-44)	39 (25-43)
Delivery route		
Vaginal N (% of all women)	73 (77)	472 (90)
Normal delivery, (% of vaginal deliveries)	67 (92)	437 (93)
Assisted delivery, (% of vaginal deliveries)	6 (8)	35 (7)
Cesarean section N (% of all women)	22 (23)	52 (10)
Primary cesarean section, (% of cesarean sections)	11 (50)	5 (10)
Emergency cesarean section, (% of cesarean sections)	11 (50)	47 (90)
Perineal laceration degree N (% of vaginal deliveries)		
1st degree	7 (10)	43 (9)
2nd degree, Episiotomy	12 (16)	59 (12)
2nd degree, Spontaneous rupture	24 (33)	100 (22)
3rd degree	0 (0)	7 (1)
No laceration	29 (40)	263 (56)
Unknown	1 (1)	-
Birth weight, grams Median (range)	3150 (365-4290)	3235 (555-5035)
Indication for LMWH administration N (% of all women)		
History of VTE	15 (16)	
History of VTE and thrombophilia	52 (55)	
Current VTE†	11 (12)	
Current VTE† and thrombophilia	2 (2)	
Recurrent thrombophlebitis and thrombophilia	1 (1)	
Antiphospholipid syndrome	4 (4)	
Pre-eclampsia	1 (1)	
Prosthetic heart valve	7 (7)	
Prosthetic heart valve+ current heart thrombosis	1 (1)	
Current CVA	1 (1)	

*Data on ethnicity for 2 cases was missing, †VTE during current pregnancy

Table 3. Incidence of PPH, severe PPH and median (range) of blood loss stratified for types of deliveries and blood transfusion rate in two groups of the study

	Women who used therapeutic doses of LMWH (N=95)	Women who did not use LMWH (N=524)	RR	Median difference	95%CI of RR or median difference
PPH events N (%)	17 (18)	113 (22)	0.8		0.5 to 1.4
Vaginal delivery	9 (12)	100 (21)	0.5		0.3 to 1.1
Cesarean section	8 (36)	13 (25)	1.7		0.6 to 5.0
Severe PPH events N (%)	6 (6)	29 (6)	1.2	-	0.5 to 2.9
Vaginal delivery	4 (5)	27 (6)	0.9		0.3 to 2.8
Cesarean section	2 (9)	2 (4)	2.5		0.3 to 18.9
Blood loss Median (range)					
Vaginal delivery	250 (50 to 4000)	300 (20 to 3600)	-	-50	-102 to 2
Normal vaginal delivery	200 (50 to 4000)	300 (20 to 3600)	-	-100	-156 to -44
Assisted vaginal delivery	350 (250 to 550)	400 (100 to 2500)	-	-50	-217 to 117
Cesarean section	425 (200 to 2000)	400 (100 to 2000)	-	25	-153 to 203
Primary cesarean section	450 (200 to 1200)	200 (100 to 400)	-	250	-15 to 515
Emergency cesarean section	400 (200 to 2000)	400 (100 to 2000)	-	0	-225 to 225
Blood transfusion N (%)	5 (5)	18 (3)	1.6	-	0. 6 to 4.3

Discussion

We observed that the incidence of severe bleeding during delivery was not increased by using therapeutic doses of LMWH during pregnancy, though a non-statistically significant increase in the risk of severe PPH was noticed.
Similar to our finding, a previous study reported no difference in the risk of PPH (5.7%) in women who delivered vaginally and used LMWH (doses not specified) and those who did not use LMWH (OR 1.0; 95%CI: 0.2 to 4.7).[3] However, the absolute risk of PPH in our study cohorts (12% in LMWH users and 21% in non-LMWH users) was relatively higher. Although the incidence of PPH in our control group appears to be higher as compared to other studies that assessed PPH in the general population,[17-19] a previously performed population-based cohort study in the Netherlands also observed an incidence of PPH of 19%.[4] An explanation could be the difference in blood loss estimation and in treatment regimens. In the Netherlands, an active management in the third stage of delivery (such as prophylactic administration of oxytocics, immediate cord clamping or controlled cord traction) is not routinely performed, although oxytocics administered in the

third stage of delivery have been shown to reduce the amount of blood loss.[20] Therefore we hypothesize that withholding oxytocics might have led to a higher incidence of PPH in our control cohort, whereas this was not observed in the treated women since LMWH use warranted an active management of the third stage of delivery according to the hospital protocol. Furthermore, as our hospital is a tertiary referral center, the observed high incidence of blood loss more than 500 mL in the control cohort may be explained by comorbidities that increase the risk of a complicated delivery.

For cesarian section, the incidence of severe PPH may be more relevant to evaluate since blood loss between 500 and 1000 mL is not considered uncommon during surgery. Severe PPH risk was 2.5 times higher (95%CI: 0.3 to 18.9) in women who used LMWH as compared to those who did not, although the certainty of this estimate is limited by the small number of individuals in this stratum. In another study where the doses of the administered LMWH was not specified, the risk of severe PPH for LMWH users (5%) in cesarean sections was surprisingly stated half of the controls (12.5%) (OR 0.4; 95%CI: 0.04 to 3.4).[3]

Although this is the largest cohort of pregnancies treated with high doses of LMWH, its power to calculate the risk of PPH is limited and is at most 44% in calculating the relative risk of PPH in vaginal deliveries. Therefore we compared the median of blood loss between cohorts of LMWH users and non-users considering that median is less sensitive to outliers. The only difference in median blood loss was found in the subgroup of normal vaginal deliveries where it was lower in the LMWH users.

Some issues warrant comment. First, although this was a controlled cohort study, it is likely that strategies to decrease the risk of PPH differed between women who were treated with LMWH and controls. Given the observational study design, our study does not exclude an increased risk of PPH by use of therapeutic LMWH if similar obstetric measures are taken. Second, we have not measured anti-Xa levels shortly prior to delivery, since this was not part of the hospital protocol. However, the advice given to all women reflects a real life situation (i.e. to discontinue LMWH when contractions started, membranes ruptured or the evening before the planned induction of labour or cesarean section). Furthermore, evidence about the association between this duration and the risk of PPH is conflicting.[8;9;21] Third, blood loss was estimated rather than measured

which may have led to higher estimates.[22] This was done similarly in women treated and untreated with LMWH. If any, it is more likely that blood loss would be overestimated rather than underestimated in women who used LMWH than in women without LMWH.

In conclusion, we observed that therapeutic doses of LMWH administered in pregnancy was not associated with clinically meaningful increase in the incidence of PPH or severe PPH in women who delivered in our hospital. Although this observation may be confounded by differential use of strategies to prevent bleeding, it is unlikely that LMWH levels in blood at the time of delivery can cause PPH knowing the routine recommendations to stop the injections when signs of labor start. A randomized controlled trial to assess the safety of therapeutic doses of LMWH to prevent venous thromboembolism in pregnant women is necessary to provide a definite answer about the optimal dose of LMWH in this population.

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Part II

Risk factors of arterial cardiovascular complications in patients with prior venous thromboembolism

7

S. Roshani, W.M. Lijfering, M. Coppens, K. Hamulyák, M.H. Prins, H.R. Büller and S. Middeldorp

Neth J Med. 2011

Abstract

Background

The effect of cardiovascular risk factors (CVRs) and thrombophilic defects on the risk of arterial cardiovascular complications in patients with prior venous thromboembolism (VTE) is unclear.

Objective

We investigated whether the risk of arterial cardiovascular complication is increased after VTE and whether CVRs and thrombophilic defects influence this risk.

Methods

Subjects were selected from 3 family cohorts of probands with VTE or arterial cardiovascular complication before the age of 50 and thrombophilic defects (i.e. hyperhomocysteinemia, prothrombin G20210A or elevated FVIII). For this analysis, probands with arterial cardiovascular complications before inclusion and their relatives as well as relatives without the studied thrombophilic defects were excluded. We calculated the incidence of arterial cardiovascular complications (e.g. myocardial infarction, ischemic stroke, transient ischemic attack or peripheral arterial disease) in subjects with and without VTE and adjusted the relative risk for at least one CVR, two or more thrombophilic defects and quintiles of a propensity score (considering risk factors conditional to VTE history).

Results

861 subjects were included, of whom 399 had experienced VTE before inclusion. 12 arterial cardiovascular complications occurred in subjects with and 9 in subjects without VTE history. Hence the annual incidence was 1.0 (95%CI, 0.5-1.7) and 0.7 (0.3-1.2) in subjects with and without VTE (RR 1.5, 0.6-3.6). Adjusting for possible confounders did not change this relative risk.

Conclusion

The mildly elevated risk of arterial cardiovascular complications in patients with prior VTE appears to be independent of cardiovascular risk factors and thrombophilic defects.

Introduction

Following the observation of higher prevalence of subclinical atherosclerosis in patients with previous idiopathic venous thromboembolism (VTE) in 2003,[1] several studies have investigated the association between venous and arterial thrombosis. A mildly increased risk of arterial cardiovascular complications in patients with previous VTE has been demonstrated consistently[2-6]. A plausible explanation of such an association might be the presence of shared risk factors between VTE and arterial cardiovascular complication[7-9]. However, two large cohort studies were unable to establish a link between atherosclerosis at baseline and venous thrombosis during follow-up.[10;11] As the study populations in various published cohorts differ, we intended to confirm the increased risk of arterial cardiovascular complications after an episode of VTE in three prospective cohorts of thrombophilic families. More important, we aimed to investigate whether the presence of multiple conventional cardiovascular risk factors and thrombophilic defects are able to explain the risk increase.

Materials and methods

Study population

The study subjects were selected from three cohorts of thrombophilic families which were identified by probands with documented VTE or premature arterial cardiovascular complications (any event before 50 years of age) and either hyperhomocysteinemia, prothrombin G20210A or persistently elevated levels of factor VIII. Subjects were recruited between August 1997 and May 2004 from three academic hospitals: Academic Medical Center, Amsterdam, University Medical Center, Groningen and Academic Hospital Maastricht. Details of these studies have been published previously.[12-14] The study was approved by the institutional review boards of the participating hospitals. Additional thrombophilia tests for factor V Leiden and deficiencies of antithrombin, protein S and protein C were performed in all participants. Detailed information about previous episodes of VTE and arterial cardiovascular complication, exposure to exogenous risk factors for thrombosis and anticoagulant treatment was collected by validated questionnaire and by reviewing medical records at baseline. Also, cardiovascular

risk factors namely smoking, diabetes mellitus, hyperlipidemia and hypertension were recorded at inclusion.

Outcome

The outcome of this analysis was the first arterial cardiovascular complication as myocardial infarction (MI), ischemic stroke, transient ischemic attack (TIA) or peripheral arterial disease. Coronary and peripheral arterial disease had to be symptomatically and angiographically proven while MI was diagnosed according to clinical, enzymatic and electrocardiographic criteria. Ischemic stroke was defined as the onset of rapidly developing symptoms and signs of cerebral function loss which lasted at least 24 hours and had an apparent vascular cause, as demonstrated by computed tomography scan or magnetic resonance imaging. If a cerebral event completely resolved within 24 hours without cerebral lesions at scanning, it was classified as TIA.[12] We contacted subjects every 6 months until April 2006, with a detailed questionnaire to identify new episodes of VTE and arterial cardiovascular complication, exposure to risk factors and medication use.

Statistical analysis

To evaluate whether the risk of arterial cardiovascular complication is higher in subjects with history of VTE than those without, we excluded probands who had had arterial cardiovascular complication prior to the enrollment, as well as their relatives, because of higher risk of a recurrent event or the possible hereditary inclination to develop an event. Similarly, relatives with arterial cardiovascular complication before baseline were excluded. Furthermore, in order to compare subjects with comparable genetic background regarding thrombophilic defects, we excluded relatives with none of the three thrombophilic defects originally qualifying for inclusion.

The annual incidence (95% confidence interval [95%CI]) of the outcome was computed for two groups of subjects, with and without history of VTE. The follow-up period was defined as years between the inclusion date and the date of death, last contact visit or when an arterial cardiovascular complication occurred. The relative risk of arterial cardiovascular complication was computed by dividing the incidences of two groups. Potential confounders for the observed relative risk were considered as the presence of conventional cardiovascular risk factors

(i.e. smoking, diabetes mellitus, hyperlipidemia and hypertension and obesity (BMI≥25 kg/m2)) and thrombophilic defects. We computed the Mantel-Haenszel adjusted relative risk for the presence of at least one cardiovascular risk factor and two or more thrombophilic defects. We also developed a propensity score which is the probability of experiencing VTE, based on individual characteristics (i.e. age, cardiovascular risk factors and thrombophilic defects) using binary logistic regression model and subsequently computed the Mantel-Haenszel adjusted relative risk for the quintals of the propensity score.[15] Finally, to exclude the protective effect of anticoagulation on the development of arterial thrombosis, we subtracted periods of anticoagulation treatment from the follow-up period.

Results

A total of 861 subjects met the inclusion criteria for this analysis (Figure 1): 399 subjects (317 probands and 82 relatives) had experienced VTE prior to enrollment and 462 subjects (all relatives) had not. During follow-up, 21 subjects experienced an arterial cardiovascular event, of whom 12 had a history of VTE and 9 did not. The median follow-up duration was 3 years (range: 0.1-7) and did not differ between subjects with and without VTE. The annual incidence rate of arterial cardiovascular events was 1.0 (95%CI 0.5-1.7) in subjects with previous VTE, and 0.7 (0.3-1.2) in those without past VTE (RR 1.5; 95%CI 0.6-3.6). Table 1 shows the baseline characteristics of the two groups. Sex and age were balanced between both groups as were classical cardiovascular risk factors except for obesity which was more prevalent in subjects with a history of VTE (22% versus 16%). The thrombophilic defects that were qualified for inclusion, i.e. hyperhomocysteinemia, prothrombin G20210A mutation and elevated levels of FVIII, were present in 30%, 33% and 58% of subjects with a history of VTE, and in 39%, 35% and 49% in the subjects without previous VTE. Overall, the prevalences of co-inherited thrombophilic defects were somewhat higher in the group with a history of VTE, i.e. 24% versus 15% for the FV Leiden mutation, 3% versus 1% for protein S deficiency, 2% versus 1% for protein C deficiency and 2% versus 0.2% for antithrombin deficiency.

Table 2 shows the distribution of cardiovascular risk factors and thrombophilic defects of the subjects who experienced arterial cardiovascular events during

follow-up, stratified for history of VTE. Adjusting the observed relative risk for arterial cardiovascular events in subjects with a history of VTE versus those without previous VTE for the presence of at least one cardiovascular risk factor and for two or more thrombophilic defects did not changes the relative risk estimate (1.5 ; 95%CI 0.7-3.3 and 1.5; 0.7-3.3 respectively). Likewise, the adjusted relative risk for quintiles of the propensity score was 1.4 (95%CI 0.5-3.5). The relative risk of arterial cardiovascular events adjusted for quintiles of propensity score after subtracting periods of anticoagulation use from the follow-up was 1.7 (0.7-4.1).

Figure1: Applied selection for current analysis

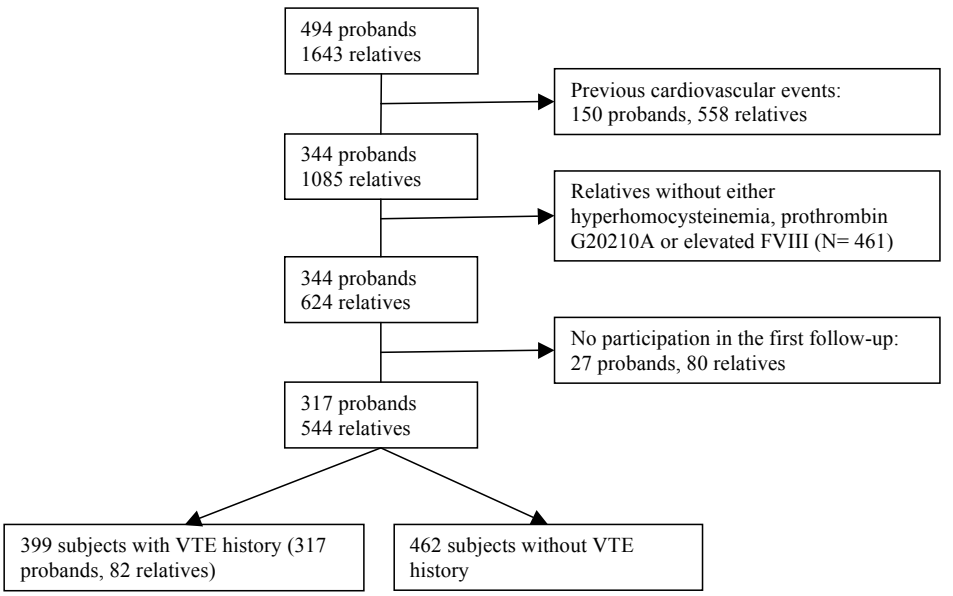

Table1: Baseline characteristics and number of arterial cardiovascular outcome stratified for history of venous thromboembolism

	Subjects without VTE history (N=462)	Subjects with VTE history (N=399)
Sex, M/ F (%)	40/ 60	36/ 64
Age, year (Mean± SD)	47±17	49±16
Hypertension (%)	82 (18)	82 (21)
Hyperlipidemia (%)	47 (10)	45 (11)
Diabetes mellitus (%)	16 (4)	15 (4)
Obesity BMI≥30 (%)	72 (16)	86 (22)
Smoking (%)	177 (38)	132 (33)
Hyperhomocysteinemia (%)	178 (39)	120 (30)
Prothrombin G20210A (%)	160 (35)	131 (33)
Factor VIII elevation (%)	225 (49)	232 (58)
FVL (%)	68 (15)	97 (24)
Protein S deficiency (%)	6 (1)	13 (3)
Protein C deficiency (%)	4 (1)	6 (2)
Antithrombin deficiency (%)	1 (0.2)	8 (2)
Number of arterial cardiovascular events N	9	12
Number of person-years of follow-up year	1367	1199
Annual incidence of arterial cardiovascular events (%) (95%CI)	0.7 (0.3-1.2)	1.0 (0.5-1.7)
Relative risk of arterial thrombotic events in subjects with VTE compared with those without (95%CI)	Ref	1.5 (0.6-3.6)

Table 2: Cardiovascular risk factor and thrombophilic defect distributions in subjects who developed arterial cardiovascular events stratified for VTE history

	Subjects without VTE history (N=9)	Subjects with VTE history (N=12)
Sex M/ F (%)	67/ 33	67/ 33
Age year (Mean± SD)	53±18	68±11
Hypertension (%)	1 (11)	4 (33)
Hyperlipidemia (%)	1 (11)	0 (0)
Diabetes mellitus (%)	0 (0)	1 (8)
Obesity BMI≥30 (%)	1 (11)	0 (0)
Smoking (%)	4 (44)	3 (25)
Hyperhomocysteinemia (%)	3 (33)	3 (25)
Prothrombin G20210A (%)	4 (44)	5 (42)
Factor VIII elevation (%)	3 (33)	8 (67)
FVL (%)	2 (22)	4 (33)
Protein S deficiency (%)	0 (0)	0 (0)
Protein C deficiency (%)	0 (0)	0 (0)
Antithrombin deficiency (%)	0 (0)	0 (0)

Discussion

In this prospective analysis of subjects from three prospective family cohort studies we observed that patients with previous VTE have a 1.5 times higher risk of developing arterial cardiovascular complications than their first degree relatives who do not have a history of VTE. The estimated relative risk did not alter by adjusting for cardiovascular risk factors or the presence of thrombophilic defects. To our knowledge, this analysis is the first that evaluated simultaneously the effect of cardiovascular risk factors and thrombophilic defects on the risk of arterial cardiovascular complications in patients with previous VTE.

Three other cohort studies of patients with either unprovoked and provoked venous thrombosis or pulmonary embolism also have confirmed the increased

risk of arterial cardiovascular complications after VTE.[6;16;17] Among which, one study adjusted the risk for age and cardiovascular risk factors where they did not notice a difference by adjustment.[16]

Our study is different from the previous ones because we included first-degree relatives of the patients with a history of VTE as the control cohort, implicitly expressing the highest possible similarity between the exposed (probands and relatives with VTE) and the control cohort for the environmental variables such as lifestyle and known and unknown genetic variables that are burdensome to adjust for and can produce residual confounding in any association under study. On the other hand, having strict inclusion criteria resulted in a small number of arterial cardiovascular complications. Hence, we could not investigate whether type of VTE (unprovoked versus provoked) modulates the risk of arterial cardiovascular complications. This may have led to underestimation of the observed increased risk as some but not all studies have shown that only subjects with unprovoked VTE had an increased risk of subsequent arterial cardiovascular complications.[2;3;6;17] Furthermore our results are only applicable in a highly selected cohort of thrombophilic families.

In conclusion, conventional cardiovascular risk factors and multiple thrombophilic defects do not seem to explain the mildly increased risk for arterial cardiovascular complication in subjects with a history of VTE.

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Inflammation markers, D-dimer and the risk of recurrent venous thrombosis

8

Sara Roshani, Carla Y. Vossen, Sverre C. Christiansen, Frits R. Rosendaal, Pieter H. Reitsma, Mary Cushman and Suzanne C. Cannegieter

Abstract

Several studies have suggested an association between inflammation and coagulation. Whether the risk of recurrent venous thrombosis is increased with high levels of cytokines is not fully elucidated. We investigated the associations of cytokine levels (TNF-α, IL-1β, IL-6, IL-8, IL-12p70 and IL-10), CRP and D-dimer with recurrent venous thrombosis in the Leiden Thrombophilia Study (LETS). We included 399 LETS participants who were followed for a mean of 8 years after cessation of treatment for their first venous thrombosis. We compared the recurrence risk for low or high cytokine levels (>10 pg/ml for TNF-α, IL-6 and IL-10, >20 pg/ml for IL-1β and IL-8 and >50 pg/ml for IL-12p70) versus undetectable cytokine levels, for CRP >3 versus ≤3 mg/L and D-dimer >250 versus ≤250 ng/ml. The blood draw took place 19 (range 6-68) months after the initial event. Sixty patients experienced a recurrence (19 per 1000 person years, 95% CI 14-24). Recurrence rates were not higher in individuals with low or high levels of cytokines compared to those with undetectable levels. The adjusted risk for age, sex and BMI was 2.2 (95% CI 1.3-3.8) for elevated CRP and 1.7 (0.9-3.4) for elevated D-dimer. Individuals with either elevated D-dimer or CRP and those with both elevated CRP and D-dimer had higher risk as compared to patients with low CRP and D-dimer levels (HR 1.9; 95% CI 1.1-3.5 and 3.1; 1.4-7.2 respectively).

In conclusion, high levels of CRP and D-dimer are associated with thrombosis recurrence while high levels of pro-inflammatory cytokines are not.

Introduction

Venous thrombosis is an important morbidity and mortality cause in Western society. Each year about 2 per 1000 individuals in Western populations develop venous thrombosis [1;2], 7% (range 3-13%) experience a recurrence in the first year and 12-25% recur over 5 years [3;4]. The risk profile for recurrent venous thrombosis appears to be different from that for a first event [5]. In contrast to the first events, age and most prothrombotic abnormalities do not appear to play an important role in provoking a recurrence in the general population [6;7] while male sex is a risk factor for recurrence but not for the first event [5]. Rare deficiencies of natural anticoagulants and antiphospholipid syndrome at most have a moderate effect on the risk of recurrent venous thrombosis [8-11]. In line with the first event, cancer, continued oestrogen use and obesity are known as determinants of recurrence risk [6;7;12-15]. Hemostatic activation, assessed by D-dimer level, appears to be an important determinant of the risk of recurrent venous thrombosis, although not all studies agree [16-20]. The effect of elevated levels of FVIII and FIX on the risk of recurrent event are either controversial or little discussed [7;21-23].

Several, mainly laboratory studies have suggested a link between coagulation and inflammation [24-26]. Studies evaluating the relation of the risk of first VTE with a promoter polymorphism (-174, G>C) of IL-6, known to influence IL-6 levels [27], or haplotypes of IL1RN failed to prove an association [28;29]. Further, a population-based study in which cytokine levels were measured prior to the first thrombosis showed no association between cytokine levels and the risk of first thrombosis [30]. These findings challenge earlier results that suggested an association between cytokines and the development of a first VTE [31;32]. Data on association of cytokine levels with risk of recurrent venous thrombosis are scarce. One study observed associations of IL-8, IL-6 and MCP-1 levels and recurrent events of venous thrombosis, however it is not mentioned whether the blood was drawn before or after the recurrences [33]. In contrast, the Prevention of Recurrent Venous Thromboembolism trial (PREVENT) study reported no association between genetic variants in 86 candidate genes involved in the inflammatory process and recurrent venous thrombosis [34]. Results of studies investigating the relation between CRP (C-reactive protein) and venous thrombosis are contradictory [35-41]. Altogether, the role of cytokines and CRP in the recurrence of venous thrombosis

is not yet clarified. In this study we aimed to evaluate the risk of recurrent venous thrombosis associated with plasma levels of the inflammatory markers, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12p70, CRP and D-dimer.

Materials and methods

Study population

This study is comprised of all patients who participated as cases in a population-based case-control study; the Leiden Thrombophilia Study (LETS). Details of this study have been published previously [42]. Briefly, 474 patients younger than 70 years of age with a first deep vein thrombosis were recruited from anticoagulation clinics in Leiden, Amsterdam and Rotterdam (the Netherlands) between January 1988 and December 1992. Participants had no overt malignancy. Blood was drawn at least 3 months after discontinuation of oral anticoagulant treatment unless treatment was prescribed indefinitely and could not be stopped (n=48). The median time interval between the first venous thrombosis and the blood draw was 19 (range 6-68) months.

Patients were followed as described previously [7] from the end of initial anticoagulation treatment period until January 1st 2000. Information during follow-up on the occurrence of risk situations, use of anticoagulation treatment, and recurrent events was gathered by repeated mailed screening questionnaires. Patients were interviewed by telephone when they responded positively to any item of the questionnaire or when they did not return it. Information on recurrent events and risk situations was confirmed by contacting patients' physicians. Recurrent events were confirmed by objective diagnostic tests, i.e. ultrasound, venography or impedance plethysmography for deep vein thrombosis and positive perfusion lung scan (at least one segmental perfusion defect), ventilation-perfusion lung scan (intermediate or high probability) or computerized tomography scan for pulmonary thrombosis. Venous events occurring within 90 days of the first event were considered a progression of the initial event. A provoked event was defined as occurring during pregnanacy or puerperium (period of 6 weeks after delivery), during (or within 30 days after the cessation of) oral contraceptive use, during immobilization (period of less than 3 month being immobilized due

to hospitalization or due to being bedridden at home) or within a period of 30 days after major trauma or surgery. The initial and follow-up phases of the LETS were approved by the medical ethics committee of the Leiden University Medical Center and all patients signed an informed consent for the participation in the follow-up phase.

Cytokines, CRP and D-dimer measurement

Blood (0.9 vol) was collected into Sarstedt Monovette tubes containing 0.106 M of trisodium citrate (0.1 vol), prepared by centrifugation for 10 minutes at 2000x*g* and stored in aliquots at –70 °C until assayed. Plasma was available for 470 patients with a first venous thrombosis. TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 were measured simultaneously using a commercially available multiplex cytometric bead assay (BD Biosciences, Alphen aan den Rijn, The Netherlands), as described earlier [31]. The detection limit of each of the cytokines was 2.5 pg/ml. CRP was measured by a sandwich enzyme immunoassay (Kordia, Leiden, The Netherlands) based on two polyclonal rabbit antibodies against CRP. D-dimer was measured using ELISA, as described earlier [43].

Statistical Analysis

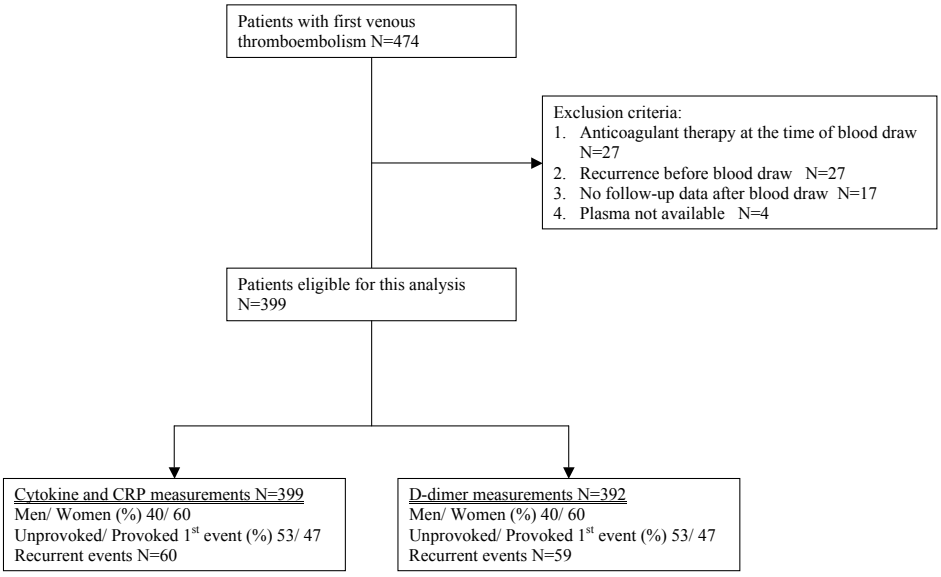
To evaluate the association between cytokine levels CRP, D-dimer and the risk of thrombosis recurrence we excluded the individuals who experienced recurrent venous thrombosis before the blood draw (n=27), who were taking oral anticoagulation therapy at the time of the blood draw (n=27) and those for whom we did not have follow-up information (n=17) from the analysis. (Figure 1)

We compared recurrence rates of thrombosis in patients with low (detectable but not high) or high levels of cytokines with patients with undetectable cytokine levels. We applied the same cut-off levels as used earlier in the LETS to discriminate low and high cytokine levels [31]. These levels are approximately equivalent to the 95th percentile in healthy individuals: above 10 pg/ml for TNF-α, IL-6 and IL-10, above 20 pg/ml for IL-1β and IL-8 and above 50 pg/ml for IL-12p70. The 95th percentile of D-dimer levels (250 ng/ml) in the LETS control population and the clinical cardiovascular risk cut-off level (3 mg/L) for CRP [44] were used as the cut-off points for high CRP and D-dimer levels. Follow-up time was defined from 90 days after the initial event until the recurrent event, death, or the last date

of follow-up, whichever occurred first. As cut-off points might be arbitrary, we also addressed other cut-off points for CRP and D-dimer; 50th, 67th, 80th and 90th percentiles of the control group distributions. Patients with CRP and D-dimer levels below the 50th percentile level in the LETS controls served as the reference group in this sensitivity analysis. Besides, we compared the risk of recurrence in patients with both elevated D-dimer and CRP or patients with either of them to patients whose D-dimer and CRP were below the cut-off.

Recurrence rates were calculated by dividing the number of recurrent events by the sum of the follow-up years. Ninety-five percent confidence intervals (95% CIs) were calculated according to Poisson distribution for the number of events [45]. Hazard ratios (HR), as estimation of the relative risk, were calculated using Cox regression. We adjusted hazard ratios for possible confounders like age, sex and body mass index (BMI; kg/m^2) and stratified the adjusted hazard ratios according to recurrence location (i.e. contralateral vs ipsilateral) and type of first or recurrent event (unprovoked vs provoked). To calculate the risk of unprovoked recurrences, we subtracted periods of exposure to risk factors (i.e. oral contraceptive use, pregnancy, trauma, surgery and immobilization) and the use of oral anticoagulants from the follow-up duration. Moreover, because of the broad range of time between the initial event and the blood draw, we computed the recurrence risks for high D-dimer or CRP stratifying by time elapsed between the first venous thrombosis and the blood draw (in tertiles)

Figure 1. Flow-chart of selection of patients for the analysis



8

Results

A total of 399 LETS patients were eligible for this analysis (figure 1). The mean age of the patients was 45 (range 14-69) years at the start of follow-up and 160 (40%) of them were men. Approximately half (n=210, 53%) of the first thromboses occurred in the absence of known risk factors for venous thrombosis. The mean follow-up duration was 8 years (range 1-12). In the 399 patients 60 (15%) recurrent events occurred with an incidence of 19 per 1000 person years (95% CI 14-24). The recurrences were located in the leg (n=47), arm (n=3), lung (n=7), leg and lung (n=2) and Budd-Chiari syndrome with extension into the vena cava (n=1).

Table 1 shows the mean, median and range of cytokines, CRP and D-dimer levels for individuals with detectable levels. Few patients had high levels of the cytokines (1 to 4% for the various cytokines). Elevated CRP (>3 mg/l) was seen in 107 patients (27%) and high D-dimer (>250 ng/ml) in 49 patients (13%).

The incidence rate of recurrent venous thrombosis was similar in individuals with high pro-inflammatory cytokines (i.e. all cytokines except IL-10) (n=38; 20 per 1000 person years; 95% CI 7-47) and those with undetectable pro-inflammatory levels (n=301; 22 per 1000 person years; 95% CI 16-30). The recurrence rate in individuals with detectable but not high levels (n=60) of at least one pro-inflammatory cytokine was 30 per 1000 person years (95% CI 15-54). The adjusted relative risks of recurrent venous thrombosis for age, sex and BMI in individuals with high and detectable (but not high) levels of at least one pro-inflammatory cytokine compared to those with undetectable levels were also not increased; hazard ratios were 1.0 (95% CI 0.6-1.5) for individuals with high levels and 1.3 (95% CI 0.7-2.4) for those with detectable but not high levels (Table 2). Similarly, no increased risks were found for each separate cytokine (Tables 3 and 4). None of the four individuals with high or low levels of the anti-inflammatory cytokine IL-10 had recurrent events.

The incidence of recurrent event in patients with elevated CRP (n=107) was 37 per 1000 person years (95% CI 24-55), which was higher than the incidence in patients with low CRP (n=292, 18 per 1000 person years; 95% CI 13-25). The corresponding hazard ratio for recurrence with elevated CRP, adjusted for age, sex and BMI, was 2.2 (95% CI 1.3-3.8). Elevated CRP was most predictive when

blood was drawn within 14 months after the index event (i.e. the first tertile of the period between the first venous thrombosis and blood draw) (HR 2.5, 95% CI 1.1-5.8). The HRs in de second (blood draw between 14 and 24 month of the first event) and third tertile (blood draw after 24 month of the first event) were 1.8 (95% CI 0.7-4.9) and 2.0 (95% CI 0.7-5.5).

The recurrence rate for levels of D-dimer above 250 ng/ml was 38 per 1000 person years (95% CI: 19-68), which was higher than the recurrence rate in patients with levels below 250 ng/ml (21 per 1000 person years; 95% CI 16-28). The adjusted hazard ratio was 1.7 (95% CI 0.9-3.4) for patients with high D-dimer levels compared to those with low levels. Similar to CRP, high levels of D-dimer were only associated with higher risk of recurrence when blood was drawn within 14 months after the index event (HR 5.8, 95% CI 2.1-16.2). The HRs in de second (blood draw between 14 and 24 month of the first event) and third tertile (blood draw after 24 month of the first event) were 1.2 (95% CI 0.3-4.1) and 0.7 (95% CI 0.1-3.1). Compared to patients with low levels of CRP and D-dimer, the recurrence risk was increased for individuals with either elevated D-dimer or CRP (HR 1.9; 95% CI 1.1-3.5) and for subjects with both elevated D-dimer and CRP (HR 3.1; 95% CI 1.4-7.2).

The adjusted hazard ratios did not substantially change when stratifying for the location of the recurrent event (contralateral versus ipsilateral), except for D-dimer (Table 4). Patients with elevated D-dimer showed a higher risk for recurrence in the contralateral leg (HR 3.1; 95% CI: 1.2-8.3) than in the ipsilateral leg (HR 0.9; 95% CI: 0.3-3.2).

Hazard ratios for the cytokines and CRP did not differ when stratifying for the type of recurrent event, i.e. unprovoked vs. provoked (data not shown). High D-dimer and CRP levels seemed to increase the risk of recurrence especially in those with provoked first events (HR 3.5; 95% CI 1.3-9.6, HR 3.6; 95% CI 1.5-8.7 respectively for D-dimer and CRP) compared to those with unprovoked first events (HR 1.2; 95% CI 0.5-2.9, HR 1.7; 95% CI 0.9-3.4). Evaluation of different cutpoints defining elevated CRP or D-dimer did not reveal a threshold that might perform better in identifying risk status. (Table 5)

Table 1. Mean, median and range of inflammatory markers and D-dimer levels in individuals with detectable levels

Analyte	N (detectable)	Mean	Median	Range	5th-95th percentile range
TNF-α*	26	28	14	5-212	5-160
IL-1β*	33	32	12	5-161	6-131
IL-6*	19	25	18	7-81	7-81
IL-8*	57	21	10	5-120	5-80
IL-10*	6	22	18	7-44	7-44
IL-12p70*	42	53	21	5-259	6-218
CRP#	399	3.8	1.4	0.0-142.3	0.2-15.8
D-dimer$	392	168	109	11-1946	35-671

Abbreviations: TNF=tumor necrosis factor, IL=interleukin, CRP=C-reactive protein.
Unites: * pg/ml, # mg/l, $ ng/ml

Table 2. Incidence and risk of recurrent thrombosis in individuals with undetectable, low and high levels of at least one pro-inflammatory cytokines

	Total N	Number of recurrences (%)	Incidence of recurrent thrombosis per 1000 person years (95% CI)	Adjusted HR** (95% CI)
Undetectable level of at least one pro-inflammatory cytokine	301	44 (15)	22 (16-30)	Ref.
Low level of at least one pro-inflammatory cytokine	60	11 (18)	30 (15-54)	1.3 (0.7-2.4)
High level of at least one pro-inflammatory cytokine	38	5 (13)	20 (7-47)	1.0 (0.6-1.5)

**Adjusted for age, sex and BMI

Table 3. Incidence of recurrent events associated with inflammatory markers and D-dimer

	N	Recurrent events N (%)	Incidence per 1000 person-year (95% CI)
TNF-α*			
Non-detectable	373	57 (15)	23 (18-30)
0-10	9	1 (11)	17 (0.4-95)
>10	17	2 (18)	18 (2-66)
IL-1β*			
Non-detectable	366	54 (15)	23 (17-29)
0-20	21	4 (19)	32 (9-81)
>20	12	2 (17)	25 (3-90)
IL-6*			
Non-detectable	380	56 (15)	23 (17-29)
0-10	6	1 (17)	23 (0.6-127)
>10	13	3 (23)	40 (8-116)
IL-8*			
Non-detectable	342	50 (15)	22 (16-29)
0-20	43	9 (21)	37 (17-70)
>20	14	1 (7)	10 (0.3-58)
IL-10*			
Non-detectable	393	59 (15)	23 (18-30)
0-10	2	1 (50)	108 (3-599)
>10	4	0 (0)	0 (0.0-132)
IL-12p70*			
Non-detectable	357	54 (15)	23 (17-30)
0-50	31	4 (13)	20 (5-50)
>50	11	2 (18)	30 (3-99)
CRP#			
≤ 3	292	36 (12)	18 (13-25)
> 3	107	24 (22)	37 (24-55)
D-dimer$			
≤ 250	343	48 (14)	21 (16-28)
> 250	49	11 (22)	38 (19-68)

Abbreviations: BD=blood draw, TNF=tumor necrosis factor, IL=interleukin, CRP=C-reactive protein. Unites: * pg/ml, # mg/l, $ ng/ml

Table 4. Hazard ratios of recurrent events associated with inflammatory markers and D-dimer level

Marker	Crude HR	Adjusted HR**				
		All recurrences N=60	Contralateral recurrences N=21	Ipsilateral recurrences N=27	Unprovoked 1st event N=210	Provoked 1st event N=189
TNF-α*						
0-10	0.8 (0.1-5.6)	0.8 (0.1-5.5)	0.8 (0.3-2.3)	1.0 (0.4-2.3)	0.9 (0.1-6.6)	***
>10	0.8 (0.2-3.4)	0.6 (0.2-2.6)	0.8 (0.1-5.7)	0.8 (0.1-5.7)	0.8 (0.2-3.3)	***
IL-1β*						
0-20	1.4 (0.5-4.0)	1.4 (0.5-3.7)	1.4 (0.6-3.1)	1.2 (0.5-2.7)	1.4 (0.4-4.7)	1.2 (0.2-8.8)
>20	1.1 (0.3-4.6)	1.0 (0.2-4.2)	1.4 (0.2-10.5)	1.2 (0.2-8.7)	0.7 (0.1-4.8)	3.2 (0.4-27.5)
IL-6*						
0-10	1.0 (0.1-7.0)	0.6 (0.1-4.6)	1.3 (0.5-3.2)	1.5 (0.7-3.3)	0.7 (0.1-5.3)	***
>10	1.9 (0.6-6.0)	1.8 (0.6-5.7)	1.6 (0.2-11.6)	3.2 (0.7-13.7)	1.5 (0.4-6.4)	3.1 (0.3-28)
IL-8*						
0-20	1.7 (0.9-3.6)	1.7 (0.8-3.5)	0.9 (0.4-2.2)	1.2 (0.5-2.5)	1.8 (0.8-3.9)	1.2 (0.2-9.1)
>20	0.5 (0.1-3.6)	0.5 (0.1-3.4)	***	1.1 (0.1-8.5)	0.6 (0.1-4.5)	***
IL-10*						
0-10	5.0 (0.7-36.0)	5.0 (0.7-37.1)	***	1.0 (0.2-4.0)	7.5 (0.9-58.1)	***
>10	***	***	***	***	***	***
IL-12p70*						
0-50	0.9 (0.3-2.4)	0.8 (0.3-2.3)	1.2 (0.5-2.8)	0.8 (0.3-2.1)	0.9 (0.3-3.1)	0.5 (0.1-4.1)
>50	1.2 (0.3-4.9)	1.0 (0.2-3.9)	1.4 (0.2-10.6)	1.0 (0.1-7.2)	0.6 (0.1-4.2)	5.4 (0.6-50.5)
CRP#						
> 3	2.0 (1.2-3.4)	2.2 (1.3-3.8)	2.6 (1.1-6.3)	2.2 (0.9-4.8)	1.7 (0.9-3.4)	3.6 (1.5-8.7)
D-dimer$						
> 250	1.8 (0.9-3.4)	1.7 (0.9-3.4)	3.1 (1.2-8.3)	0.9 (0.3-3.2)	1.2 (0.5-2.9)	3.5 (1.3-9.6)

The reference group was patients with non-detectable levels for cytokines, £3 mg/l for CRP and £250 ng/ml for D-dimer .***No recurrent events occurred

Table 5. Hazard ratios of recurrent events for different cut-off levels of CRP and D-dimer

	Cut-off level*#	N	Recurrent events N (%)	Crude HR	Adjusted HR**
CRP					
< 50 %		169	21 (14)	Ref	Ref
> 50 %	1.1	230	39 (20)	1.5 (0.9-2.5)	1.6 (0.9-2.8)
> 67 %	2.0	148	27 (22)	1.6 (0.9-2.8)	1.7 (1.0-3.2)
> 75 %	2.5	117	25 (27)	1.9 (1.1-3.4)	2.2 (1.2-4.0)
> 80 %	3.0	105	24 (30)	2.1 (1.2-3.7)	2.3 (1.2-4.3)
> 90 %	5.1	64	13 (26)	1.9 (0.9-3.7)	2.0 (1.0-4.0.0)
> 95 %	9.7	42	9 (27)	1.9 (0.9-4.2)	1.9 (0.8-4.5)
D-dimer					
< 50 %		128	17 (15)	Ref	Ref
> 50 %	74.0	264	42 (19)	1.3 (0.7-2.2)	1.4 (0.8-2.6)
> 67 %	103.0	204	31 (18)	1.2 (0.7-2.2)	1.4 (0.7-2.6)
> 75 %	121.4	171	27 (19)	1.3 (0.7-2.4)	1.5 (0.8-2.8)
> 80 %	136.3	145	24 (20)	1.4 (0.7-2.5)	1.6 (0.8-3.1)
> 90 %	185	91	16 (21)	1.5 (0.7-2.9)	1.7 (0.8-3.5)
> 95 %	250	49	11 (22)	1.8 (0.9-3.4)	1.7 (0.9-3.4)

* The cut-off levels are applied from the LET's controls.
# mg/l for CRP and ng/ml for D-dimer. **Adjusted for age, sex and BMI

Discussion

In this prospective study, we observed that "low detectable" or high levels of pro-inflammatory cytokines (i.e. TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70) do not increase the risk of a second thrombosis. Elevated levels of CRP (> 3 mg/d) were associated with an increased risk of recurrent venous thrombosis (adjusted HR 2.2; 95% CI 1.3-3.6), as well as D-dimer levels above 250 ng/ml (adjusted HR 1.7; 0.9-3.4).

To our best knowledge this is the first study evaluating the association of inflammatory markers and the risk of thrombosis recurrence. Several studies have

suggested that increased levels of inflammatory factors might be a result and not a cause of venous thrombosis. [46-48] This may explain why we could not demonstrate an association between cytokines and recurrent disease. It may also explain the small percentage of patients showing elevated levels of the various cytokines in our study (range 1-4%), as blood from all patients was drawn at least six months after the initial event. We did find a 2-fold increased risk of recurrence though in patients with elevated CRP levels. The clinical implication of this finding needs further investigation. The association we observed between high D-dimer levels and thrombosis recurrence (HR 1.7, 95% CI: 0.9-3.4), confirms previous studies, which found a 2 to 3-fold higher risk of recurrence associated with D-dimer levels more than 250 ng/ml [49-51].

A systemic increase in inflammatory markers in patients with venous thrombosis might reflect locally damaged veins and might therefore predict ipsilateral recurrent events. However, we did not find such an association, which is consistent with studies showing that residual vein thrombosis also correlates with contralateral recurrent events [50;52]. It can, however, not be excluded that plasma concentrations of inflammatory markers do not reflect the situation at the thrombus site. Interestingly, we have observed that increased recurrence risk for elevated CRP and D-dimer was present only in patients who had their blood drawn within 14 months after the initial event. However, these results should be interpreted with caution because few recurrent events happened in the second and third tertile of the time between the initial event and the blood draw especially for D-dimer risk estimation.

Our study has several limitations. Firstly, the measurements were only done once which precluded the evaluation of the association of persistent elevation of cytokines on the risk of thrombosis recurrence. Also, since the average time interval between the blood draw and the index thrombotic event was 19 months, we were unable to calculate the recurrence risk within the first year of thrombotic events when the absolute risk of recurrence is the highest.

In conclusion, elevated levels of CRP and D-dimer appear to increase the risk of venous thrombosis recurrence while high levels of pro-inflammatory cytokine do not. Furthermore, individuals with simultaneous elevated CRP and D-dimer have the most risk of recurrence compared to those with none of them elevated.

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Risk factors for idiopathic retinal vein occlusion

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Sara Roshani, Marieke C.H. de Visser, Joost C.M. Meijers, Pieter H. Reitsma, Irakli Goglidze, Harry R. Büller, Marc D. de Smet and Saskia Middeldorp

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Abstract

Risk factors for idiopathic retinal vein occlusion (RVO) are not well defined. We investigated the role of thrombophilia, fibrinolysis and platelet receptors in idiopathic RVO in a case-control study. Cases were 101 patients (55% men) with angiographically confirmed RVO, recruited from four Dutch academic ophthalmic centers. None of them had ocular disease or systemic risk factor predisposing to RVO. Controls (n=94, 52% men) were either brought by cases or selected from hospital contact lens services.

We compared the mean level of endogenous thrombin potential, antithrombin, protein C and S, Factor VIII and homocysteine as well as clot lysis time and thrombin activatable fibrinolysis inhibitor (TAFI) between the two groups. We calculated the odds ratio (OR) of RVO for antithrombin, protein C and S deficiency, hyperhomocysteinemia, factor V Leiden, prothrombin G20210A, lupus anticoagulant, anticardiolipin antibodies, *TAFI* and protein C receptor (*PROCR*) haplotypes (H) and platelet receptor polymorphisms. Increased ORs were observed for *PROCR* H1 (OR: 1.5, 95%CI: 0.8-2.8) and H4 (1.8, 0.6-5.5), elevated TAFI activity (1.5, 0.7-3.3), *TAFI* H1 (1.4, 0.8-2.7) and platelet receptor polymorphisms (rs5918; 1.8, 0.9-3.4 and in a recessive model rs1062535; 1.8, 0.8-3.9 and rs1126643; 1.9, 0.9-4.1) but neither increase reached significance.

None of the established thrombophilia and clot lysis time showed an association with the risk of idiopathic RVO. These results should be interpreted with caution due to limited power to detect small effects.

Introduction

Retinal vein occlusion (RVO) has a prevalence of 5.2 per 1000 individuals in the general population and constitutes the second most common retinal vascular disease after diabetic retinopathy [1]. The four year recurrence rate in the opposite eye is estimated to be 7% [2]. Despite the importance of RVO, the underlying etiology is not fully elucidated.

According to Virchow's triad, a thrombus can be formed following a change in one or more of three elements of vessel wall integrity, hemodynamics or blood coagulability. Risk factors that are correlated with arterial thrombosis, such as hypertension, hyperlipidemia and diabetes, and which are known to affect vessel wall integrity, predispose to RVO [3-5]. This is in line with studies reporting an increased risk of other manifestations of atherosclerosis such as stroke and myocardial infarction in patients with previous RVO [6-8]. The role of thrombophilic defects in the development of RVO is controversial except for elevated levels of homocysteine [9;10], although an association is not established for the C677T dimorphism in the gene of methylentetrahydrofolate reductase (MTHFR) [9;11]. Two meta-analyses showed that the Factor V Leiden (FVL) mutation increases the risk of RVO by about 50 to 60% [9;12], whereas other prothrombotic defects, i.e. prothrombin G20210A and deficiencies of antithrombin, protein C and S are not associated with RVO[9] . The role of lupus anticoagulant and anticardiolipin antibodies in RVO is uncertain [9;10;13]. Elevated TAFI levels are a risk factor for venous thrombosis [14;15] but its role in idiopathic RVO has not been investigated. Likewise, the relation between fibrinolysis and RVO has not received much attention [10;16;17]. Since RVO occurs at arterio-venous crossings where the blood flow is locally turbulent, changes in the platelet reactivity due to polymorphisms in platelet receptors may be important. The few available studies evaluating the role of platelets in RVO are of limited size making this association at best controversial [18-22].FVL (or activated protein C resistance) was found to be more prevalent in younger RVO patients [23;24], which has led to the hypothesis that risk factors other than hypertension and hyperlipidemia are of importance in RVO in young patients. We aimed to investigate the role of thrombophilia, fibrinolysis and platelet function in RVO by comparing their prevalence in RVO patients without known risk factors and in a control group.

Materials and Methods

Study population and data collection

Cases were RVO patients, selected from fluorescein angiography databases of four academic ophthalmic centers in the Netherlands: Academic Medical Center Amsterdam, University Hospital Maastricht, University Hospital Groningen and Eye Hospital in Rotterdam, for whom the diagnosis was established between January 1st 1970 and May 1st 2000. We recruited these patients only if they did not have ocular diseases or risk factors predisposing to RVO, namely open-angle glaucoma, optic nerve head drusen, hypermetropia, ocular inflammation or periphlebitis, hypertension, diabetes, hyperlipidemia, hematological disorders, renal insufficiency, hepatic disease and vasculitis. Controls were brought by the cases but if cases were unable to bring a volunteer, an appropriate healthy control was chosen from the hospital contact lens service that also services the same community. Controls were matched for age (± 3 years) to cases and were not genetically related to each other or to the cases. All participants provided written informed consent and the study was approved by the Medical Ethics Committee of Academic Medical Center Amsterdam, Medical Ethics Committee of University Hospital Maastricht, Medical Ethics Committee of University Hospital Groningen and Medical Ethics Committee of the Rotterdam Eye Hospital.

Patients and controls were subjected to a complete ocular examination including visual acuity measurement, biomicroscopy, intraocular pressure (IOP) measurement and a dilated fundus examination allowing confirmation of the RVO events in the cases, and excluding ocular abnormalities in the controls. Citrated blood was collected and centrifuged within 25 minutes after withdrawal at 4°C (3000 RPM for 10 minutes) and stored at -80°C until assayed.

Endogenous thrombin potential and established thrombophilia

The Endogenous Thrombin Potential (ETP) is derived from a global coagulation assay that measures tissue factor triggered thrombin generation in platelet-poor plasma and provides an estimate of the clotting potential. Coagulation was triggered by recalcification in the presence of 5 pM recombinant human tissue factor (Innovin®, Siemens, Marburg, Germany), 4 μM phospholipids, and 417 μM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf,

Switzerland). The ETP was determined with a calibrated automated thrombograph using a microtiter plate reading fluorometer (Fluoroskan Ascent, ThermoLab systems, Helsinki, Finland) and thrombinoscope software (Thrombinoscope BV, Maastricht, the Netherlands).

Antithrombin activity was determined with the Berichrom Antithrombin assay (Siemens Healthcare Diagnostics, Marburg, Germany) on a Behring Coagulation System (Siemens Healthcare Diagnostics). Protein C activity (normal values ≥ 70%) was assayed with the Coamatic protein C kit (Chromogenix, Mölndal, Sweden). Total protein S antigen (normal values ≥ 65%) was determined by ELISA (DAKO, Glostrup, Denmark). Free protein S (normal values ≥ 26%) was measured in the supernatant after precipitating the C4b-bound fraction of protein S with polyethylene glycol.

DNA was extracted from peripheral leukocytes. FVL and prothrombin 20210A polymorphisms were detected by routine PCR and restriction enzyme techniques. Factor (F) VIII levels were measured in a one-stage clotting assay with FVIII deficient plasma. The plasma levels were expressed as percentages of pooled normal plasma obtained from more than 150 hospital workers. Total homocysteine levels were measured using a high performance liquid chromatographic assay as reported previously [25]. Normal ranges for fasting and post-loading levels were 6-15 and 18-51 mmol/L for premenopausal women and were 6-19, 25-69, 8-18 and 25-54 mmol/L for postmenopausal women and men, respectively.

Two tests were performed to detect lupus anticoagulant: a diluted prothrombin time (dPT) using 200-fold diluted Innovin (Siemens Healthcare Diagnostics), and a diluted Russel's Viper Venom test (dRVVT, LAC Screen, Gradipore, North Ryde, Australia). dPT and dRVVT were considered prolonged when the ratio of patient plasma to normal plasma was >1.2. In case of a prolonged dRVV screening test, a mixing test with normal plasma (1:1) was performed, and the sample was considered positive when the clotting time of the dRVV confirmation test was more than 20% reduced compared to the dRVV screening test. A patient sample was considered positive for lupus anticoagulant with either a prolonged dRVVT or dPT.

The presence of anticardiolipin antibodies (ACA) IgG and IgM was tested by ELISA (IMTEC, Kordia, Leiden, The Netherlands). In case of anticoagulation therapy (n=2), protein C, S and lupus anticoagulant were not measured.

Using the Genome Variation Server (http://gvs.gs.washington.edu/GVS) common haplotypes were identified in the protein C receptor gene (*PROCR*) in the European Hapmap population. Three tagging single nucleotide polymorphisms (htSNPs; rs867186, rs2069952 and rs2069951) together tag four *PROCR* haplotypes. A htSNP is a polymorphism whose minor allele is specific to one haplotype (H). We used TaqMan SNP genotyping assays to determine the htSNPs. In this assay, fluorescent allele-specific oligonucleotide probes (Applied Biosystems, CA, USA) were used for PCR amplification and fluorescence endpoint reading for allelic discrimination was done on a ABI 7900 HT (Applied Biosystems).

Fibrinolysis parameters and TAFI polymorphisms

Lysis of a tissue-factor induced clot by exogenous tissue-type plasminogen activator was studied by monitoring changes in turbidity during clot formation and the subsequent lysis [26]. The Clot Lysis Time (CLT) was defined as the time from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing the clot lysis.

Thrombin-Activable Fibrinolysis Inhibitor (TAFI) activity was measured in plasma as described [27], and TAFI antigen was detected in plasma by ELISA as described before [28]. The measurements were done in duplicate. Plasma TAFI levels were expressed as percentage of pooled normal plasma.

We determined four major *TAFI* haplotypes by identifying three htSNPs (rs2146881, rs3742264 and rs1926447) in the European population and subsequently performing TaqMan SNP genotyping assays.

Platelet receptors' polymorphisms

Several SNPs were determined in *glycoprotein (GP) IB* (rs2243039 (Kozak polymorphism) and rs6065), *GPVI* (rs1613662), *GPIa* (rs1126643 and rs1062535), *GPIIB* (rs5911) and *GPIIIA* (rs5918) by TaqMan SNP genotyping assays or PCR and restriction enzyme technique *(*for *GPIa* rs1126643).

Statistical analysis

Student's t-test was used to evaluate the difference in the means of continuous variables between cases and controls. Odds ratios (OR) and 95% confidence

intervals (95%CI) were computed to assess the effect of discrete variables on RVO risk. Odds ratios were calculated to estimate RVO risk for the second and third tertiles of ETP, FVIII, CLT, TAFI activity and antigen level compared with the first tertile. The tertile stratification was done based on the levels in the controls. To investigate the effect of polymorphisms in platelet receptors and different haplotypes of *PROCR* and *TAFI* on the risk of RVO, we calculated odds ratios of RVO in heterozygous and homozygous subjects for the minor allele as compared to homozygous subjects for the common allele (reference group).

Results

Characteristics of the study population

We recruited 101 patients (56 men, 55.4%) with angiographically established RVO as cases and 94 (49 men, 52.1%) healthy subjects as controls. Mean age (±SD) was 48 (±11) and 46 (±10) in cases and controls respectively. The majority of the participants was younger than 60 years (84% of cases and 89% of controls) and of Caucasian origin (83% of cases and 88% of control).

Endogenous thrombin potential and established thrombophilia

Table 1 lists the results of ETP measurements and thrombophilia tests for both study groups. Mean ETP was 106% in both cases and controls and did not show an effect on the risk of RVO.

None of the cases or controls had antithrombin deficiency. Mean antithrombin, protein C activity and total protein S antigen level did not differ between cases and controls, but mean free protein S antigen level was higher in cases than in controls (mean difference: 6%, 95% CI: -10.4 to -2.5). Mildly decreased protein C levels, isolated low levels of free protein S and both total and free low protein S were observed in one case (protein C activity: 68%), two controls (free protein S: 20, 21%) and one control (total protein S antigen: 50% and free protein S: 24%), respectively. These results were based on single measurements. The prevalence of FVL, prothrombin G20210A, hyperhomocysteinemia, lupus anticoagulant and anticardiolipin antibodies as well as the mean FVIII antigen level were not different between cases and controls. Similarly, the risk of RVO was not increased in subjects with higher level of FVIII or homocysteine as compared to those having lower levels. Tertile classification of homocysteine was performed

separately for fasting and post-loading measurements and stratified for sex (data not shown) and showed no association.
In Table 2, the RVO risk associated with each *PROCR* haplotype is shown. Carriers of one or two alleles of H1 or H4 had an increased risk of RVO (OR: 1.5, 95% CI: 0.8 to 2.8 and 1.8, 0.6 to 5.5), although these estimates did not reach statistical significance.

Fibrinolysis parameters and TAFI polymorphisms
Table 3 describes CLT measurements and TAFI antigen and activity levels in cases and controls. Mean CLT was shorter in cases than in controls (mean difference: -7 %, 95% CI: -12.6 to -0.1). Since the distribution of CLT was slightly skewed, we also compared the medians and did not observe a difference between the two groups (71 (range: 40 to 127) in cases and 71 (45 to 150) in controls). No difference in the mean levels of TAFI antigen and activity between cases and controls was detected. However, subjects with TAFI activity in the highest tertile had a 1.5-fold (95% CI: 0.7 to 3.3) increased risk of developing RVO as compared to those in the lowest tertile.
Table 4 lists RVO risks associated with each *TAFI* haplotype. We were not able to assign a haplotype to 10 subjects (eight cases and two controls). Subjects carrying one or two H1 alleles had a slightly higher RVO risk (OR: 1.4, 95% CI: 0.8 to 2.7) than subjects without a H1 allele, although this was not statistically significant. H1 appeared to be associated with increased TAFI activity level.

Platelet receptor polymorphisms
Table 5 presents the association of six SNPs in four platelet receptor genes with the risk of RVO. Carriers of the rs5918 (in *GPIIIa* gene) minor allele appeared to have an increased risk of RVO in a dose-dependent manner (OR in heterozygotes: 1.7, 95% CI: 0.8 to 3.3, OR in homozygotes: 2.8, 0.5 to 15.9). Homozygotes for the minor alleles of the tested SNPs in *GPIa* (i.e. rs1062535 and rs1126643) showed a higher RVO risk (OR: 1.7, 95% CI: 0.7 to 4.1 and 1.8, 0.8 to 4.2 respectively). These SNPs were in strong linkage disequilibrium (r^2=0.82). In a recessive model (i.e. the risk in homozygotes for the minor allele compared with others), the odds ratios were 1.8 (95% CI: 0.8 to 3.9) for rs1062535 and 1.9 (95% CI: 0.9 to 4.1) for rs1126643.

Table 1. Endogenous thrombin potential and various established thrombophilic risk factors in RVO patients and controls

	Patients N= 101	Controls N= 94	Mean difference/ OR	95%CI
ETP, mean levels (%)	106	106	0	-5.9 to 6.3
<99 N (%)	26 (38)	28 (33)	1*	-
99-112 N (%)	18 (26)	29 (34)	0.7	0.3 to 1.5
>112 N (%)	25 (36)	28 (33)	1.0	0.5 to 2.1
Antithrombin, mean levels (%)	103	105	-2	-1.7 to 4.7
Antithrombin deficiency N	0	0	-	-
Protein C, mean levels (%)	109	106	3	-8.9 to 2.1
Protein C deficiency N (%)	1 (1)	0	-	-
Total protein S, mean levels (%)	103	102	1	-6.8 to 3.6
Free protein S, mean levels (%)	50	44	6	-10.4 to -2.5
Protein S deficiency N (%)	0	1 (1)	-	-
FVL N (%)	5 (5)	4 (4)	1.2	0.3 to 4.5
Prothrombin G20210A N (%)	2 (2)	0	-	-
FVIII, mean levels (%)	103	100	3	-9.9 to 5.3
<91 N (%)	27 (31)	32 (34)	1*	-
91-108 N (%)	34 (40)	31 (33)	1.3	0.6 to 2.6
>108 N (%)	25 (30)	31 (33)	0.5	0.5 to 1.9
Hyperhomocysteinemia N (%)	15 (16)	18 (19)	0.8	0.4 to 1.7
LAC N (%)	3 (3)	0	-	-
IgG ACA N (%)	1 (1)	1 (1)	0.9	0.1 to 15.1
IgM ACA N (%)	4 (4)	2 (2)	1.9	0.3 to 10.6
LAC or ACA N (%)	7 (7)	3 (3)	2.3	0.6 to 9.1

Abbreviations: endogenous thrombin potential (ETP), factor V Leiden (FVL), factor VIII (FVIII), lupus anticoagulant (LAC) and anticardiolipin antibody (ACA).
Missing measurements: 31 patients and 9 controls for ETP, 2 patients and 2 controls for FVL, 1 patient for prothrombin G20210A, 15 patients for FVIII, 6 patients for homocysteine, 5 patients and 1 control for protein C, 2 patients and 2 cases for total protein S, 23 patients for free protein S and 7 patients for antithrombin. * Reference category.

Table 2. Common haplotypes of the *PROCR* gene in RVO patients and controls

Haplotype (htSNP)	Patients (%) N= 77	Controls (%) N= 90	OR	95% CI
H1 (rs2069952)				
HxHx	28 (36)	41 (46)	1*	-
H1Hx	41 (53)	40 (44)	1.5	0.8 to 2.9
H1H1	8 (11)	9 (10)	1.3	0.5 to 3.8
H1Hx/ H1H1	49 (64)	49 (54)	1.5	0.8 to 2.8
Frequency H1	37.0	32.2		
H2 (all common)				
HxHx	22 (29)	20 (22)	1*	-
H2Hx	37 (48)	47 (52)	0.7	0.3 to 1.5
H2H2	18 (23)	23 (26)	0.7	0.3 to 1.7
H2Hx/ H2H2	55 (71)	70 (88)	0.7	0.3 to 1.4
Frequency H2	47.4	51.7		
H3 (rs867186)				
HxHx	63 (82)	68 (76)	1*	-
H3Hx	14 (18)	21 (23)	0.7	0.3 to 1.5
H3H3	0 (0)	1 (1)	-	-
H3Hx/ H3H3	14 (18)	22 (24)	0.7	0.3 to 1.5
Frequency H3	9.1	12.8		
H4 (rs2069951)				
HxHx	68 (88)	84 (93)	1*	-
H4Hx	8 (11)	6 (7)	1.6	0.5 to 4.9
H4H4	1 (1)	0 (0)	-	-
H4Hx/ H4H4	9 (12)	6 (7)	1.8	0.6 to 5.5
Frequency H4	6.5	3.3		

* Reference category, Hx: all haplotypes except the one given, htSNP: haplotype tagging SNP, OR: odds ratio, 95% CI: 95% confidence interval

Table 3. Fibrinolysis parameters and TAFI polymorphisms in RVO patients and controls

	Patients N= 101	Controls N= 94	Mean difference/ OR	95%CI
CLT, mean	70	77	-7	-12.6 to -0.1
<93 N (%)	29 (40)	31 (34)	1*	-
93-103 N (%)	23 (32)	31 (34)	0.8	0.4 to 1.6
>103 N (%)	20 (28)	30 (32)	0.7	0.3 to 1.5
TAFI antigen, mean	106	110	6	-0.3 to 8.0
<103 N (%)	35 (51)	33 (36)	1*	-
103-115 N (%)	12 (18)	28 (31)	0.4	0.2 to 0.9
>115 N (%)	21 (31)	30 (33)	0.7	0.3 to 1.7
TAFI activity, mean	102	101	2	-7.4 to 3.8
<93 N (%)	21 (31)	32 (36)	1*	-
93-103 N (%)	19 (28)	28 (32)	1.0	0.5 to 2.3
>103 N (%)	28 (41)	28 (32)	1.5	0.7 to 3.3

Abbreviations: clot lysis time (CLT) and thrombin activatable fibrinolysis inhibitor (TAFI).
Missing measurements: 28 patients and 2 controls for CLT, 32 patients and 3 controls for TAFI antigen, 33 patients and 6 controls for TAFI activity measurements.
* Reference category.

Table 4. Common haplotypes of the *TAFI* gene in RVO patients and controls

Haplotype (htSNP)	Patients (%) N= 69	Control (%) N= 88	OR	95% CI	Mean TAFI (95% CI)**
H1 (rs3742264)					
HxHx	29 (42)	45 (51)	1*	-	97.8 (94.1 to 101.5)
H1Hx	33 (48)	30 (34)	1.5	0.4 to 4.9	100.1 (94.9 to 105.3)
H1H1	7 (10)	13 (15)	0.7	0.1 to 7.3	114.6 (99.7 to 129.5)
H1Hx/ H1H1	40 (58)	43 (49)	1.4	0.8 to 2.7	104.5 (98.5 to 110.5)
Frequency H1	34.1	31.8			
H2 (rs2146881and rs1926447)					
HxHx	39 (57)	51 (58)	1*	-	103.8 (98.3 to 109.4)
H2Hx	27 (39)	33 (38)	0.9	0.3 to 2.8	98.0 (93.4 to 102.6)
H2H2	3 (4)	4 (4)	1.0	0.1 to 13.5	96.5 (89.2 to 103.7)
H2Hx/ H2H2	30 (43)	37 (42)	1.1	0.6 to 2.0	97.8 (93.7 to 102.0)
Frequency H2	23.9	23.3			
H3 (all common)					
HxHx	25 (36)	33 (38)	1*	-	105.1 (98.2 to 112.1)
H3Hx	37 (54)	39 (44)	1.1	0.3 to 4.3	99.4 (94.4 to 104.5)
H3H3	7 (10)	16 (18)	0.6	0.1 to 5.8	96.9 (91.8 to 101.9)
H3Hx/ H3H3	44 (64)	55 (62)	1.1	0.5 to 2.0	98.7 (94.8 to 102.6)
Frequency H3	36.9	40.3			
H4 (rs1926447)					
HxHx	62 (90)	80 (91)	1*	-	101.0 (97.2 to 104.9)
H4Hx	7 (10)	8 (9)	1.1	0.4 to 3.3	104.2 (95.2 to 113.2)
H4H4	0	0	-	-	-
H4Hx/ H4H4	7 (10)	8 (9)	1.1	0.4 to 3.3	104.2 (95.2 to 113.2)
Frequency H4	5.1	4.6			

** Mean TAFI activity level and 95% confidence interval in the controls, * Reference category, Hx: all haplotypes except the one given, htSNP: haplotype tagging SNP, OR: odds ratio, 95% CI: 95% confidence interval

Table 5. RVO risk for different genotypes of polymorphisms in four platelet receptors

Gene SNP	Genotype	Patients N=77(%)	Control N= 90 (%)	OR	95% CI
GPIba					
rs6065	CC	66 (86)	72 (80)	1*	-
	CT	11 (14)	18 (20)	0.7	0.3 to 1.5
	TT	0	0	-	-
	CT/ TT	11 (14)	18 (20)	0.7	0.3 to 1.5
rs2243093	TT	59 (77)	65 (72)	1*	-
(Kozak)	CT	18 (23)	25 (28)	0.8	0.4 to 1.6
	CC	0	0	-	-
	CT/ CC	18 (23)	25 (72)	0.8	0.4 to 1.6
GPIa					
rs1062535	GG	27 (35)	33 (37)	1*	-
	AG	32 (42)	44 (49)	0.9	0.5 to 1.8
	AA	18 (23)	13 (14)	1.7	0.7 to 4.1
	AG/ AA	50 (65)	57 (63)	1.1	0.6 to 2.0
rs1126643	CC	27 (35)	34 (38)	1*	-
	CT	30 (39)	42 (47)	0.9	0.5 to 1.8
	TT	20 (26)	14 (15)	1.8	0.8 to 4.2
	CT/ TT	50 (65)	56 (62)	1.1	0.6 to 2.1
GPIIIa					
rs5911	AA	30 (39)	25 (28)	1*	-
	AC	34 (44)	45 (50)	0.6	0.3 to 1.3
	CC	13 (17)	20 (22)	0.5	0.2 to 1.3
	AC/ CC	47 (61)	65 (72)	0.6	0.3 to 1.2
rs5918	TT	48 (62)	67 (74)	1*	-
	TC	25 (33)	21 (23)	1.7	0.8 to 3.3
	CC	4 (5)	2 (2)	2.8	0.5 to 15.9
	TC/ CC	29 (38)	23 (25)	1.8	0.9 to 3.4
GPVI					
rs1613662	AA	51 (66)	62 (69)	1*	-
	AG	25 (33)	25 (28)	1.2	0.6 to 2.4
	GG	1 (1)	3 (3)	0.4	0.0 to 4.0
	AG/ GG	26 (34)	28 (31)	1.1	0.6 to 2.2

* Reference category, OR: odds ratio, 95% CI: 95% confidence interval

Discussion

In this relatively large study on idiopathic RVO, we were unable to detect a significant association between established thrombophilia factors, fibrinolysis parameters or platelet receptor polymorphisms and idiopathic RVO. However, we observed non-significant risk increases for two haplotypes of *PROCR* (H1 and H4), for higher TAFI activity, for *TAFI* H1 and for several platelet receptor polymorphisms.

The endothelial protein C receptor (EPCR) has a crucial role in the protein C anticoagulant system by reinforcing protein C activation by thrombin [29]. In one study, higher circulating levels of this receptor (soluble EPCR, sEPCR) were associated with the risk of RVO in general [13]. Accordingly, we expected H3 *PROCR* carriers to have higher risk of RVO since H3 *PROCR* carriers are reported to have higher sEPCR levels than other haplotypes [30]. Since our data did not support this hypothesis and also the effect of *PROCR* haplotypes on VTE risk is not clear [30;31], we suggest further investigations before drawing a definite conclusion.

Two small-sized studies did not show an association between TAFI activity and RVO in general [10;17]. We observed that *TAFI* H1, possibly through higher TAFI activity [32], showed a trend toward higher risk of idiopathic RVO. However, CLT did not differ between cases and controls, suggesting that impaired fibrinolysis is not a major contributor in the pathogenesis of idiopathic RVO. Moreover, as higher CLT and ETP, both established risk factors for venous thrombosis [33;34], did not differ between patients and controls, coagulation and fibrinolysis are probably balanced in idiopathic RVO. In contrast to our observation in idiopathic RVO patients, two small studies have reported significant P-values for the association of high Euglobulin Clot Lysis Time and the risk of RVO in general [16;17].

Interestingly, we observed an association between some SNPs in platelet receptor genes and the risk of developing RVO. Functional studies have demonstrated that polymorphisms (rs1062535, rs1126643) in the GPIa component of the GPIa/IIb complex - the receptor for collagen on platelets - lead to a higher density of the complex and subsequently to enhanced adhesiveness to collagen [35]. However, their role in RVO is uncertain; several studies have shown no effect of rs1126643 [19;21;22] while in one study both SNPs were correlated with RVO [20]. Also several

studies have indicated that rs5918 in GPIIIa component of the GPIIb/IIIa complex - the receptor for fibrinogen and von Willebrand factor and the most abundant receptor on platelets - is not associated with RVO [18;19;22].

A limitation of our study is that despite a relative large number of idiopathic RVO patients, there was not sufficient power to detect modest risk increases. For instance the power to detect the observed 1.8 point estimate with our sample size for rs5918 was approximately 50%. For the same reason, we were unable to stratify our analysis for the types of RVO. Also, we did not adjust for multiple testing, which may lead to finding false positive associations.

In conclusion, our data suggest an association between *PROCR* H1 and H4, elevated TAFI activity, *TAFI* H1 and platelet receptor polymorphisms (rs5918, rs1062535 and rs1126643) and idiopathic RVO. We did not observe a role for established thrombophilia factors and CLT, although these results should be interpreted with caution due to the limited power to detect small effects.

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Risk of cardiovascular disease in double heterozygous carriers and homozygous carriers of factor V Leiden and prothrombin G20210A; a retrospective family cohort study

10

Rachel E.J. Roach, Sara Roshani, Karina Meijer, Karly Hamulyák, Willem M. Lijfering, Martin H Prins, Harry R Büller and Saskia Middeldorp

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F5 R506Q (Factor V Leiden) and *F2* G20210A (prothrombin G20210A) are risk factors for venous thrombosis.[1] The results of studies as to whether these mutations also increase the risk of cardiovascular disease (CVD) are inconsistent. A meta-analysis of 191 studies (N>150.000) calculated a 30% increased risk of CVD among single heterozygous *F5* R506Q or *F2* G20210A carriers compared to non-carriers.[2] Despite its large size, this study included too few double heterozygous and homozygous carriers to estimate the risk of CVD in individuals with these genetic traits. Therefore, we performed a post hoc analysis in a retrospective family cohort that contained a fairly large number of relatives who were double heterozygous or homozygous for *F5* R506Q and *F2* G20210A (n=52).

Details of our study have been published previously.[3-5] Briefly, 1641 first-degree relatives, aged 15 years or older, of consecutive patients (probands) with documented venous thrombosis or CVD before the age of 50 years and *F2* G20210A, high FVIII or hyperhomocysteinaemia, were enrolled after informed consent was obtained. Information on CVD and exposure to classical cardiovascular risk factors was collected by using a standardized questionnaire and reviewing medical records. All patients were screened for *F5* R506Q and *F2* G20210A. Information was obtained without knowledge of the genetic status.

Observation years were defined as the years from the age of 15 until the date of inclusion or until the date of the first thrombotic event. Incidences and 95% confidence intervals (95% CIs) were calculated under the Poisson distribution assumption. Relative risks and 95% CIs of cardiovascular disease were calculated in the double heterozygous/homozygous group, using single heterozygous carriers as a reference group. As we studied a thrombophilic cohort, we only compared single heterozygous *F5* R506Q or *F2* G20210A carriers to double heterozygous and homozygous carriers. The *a-priori* CVD risk for other relatives was deemed too high for them to be included as a reference group. To avoid bias, we excluded probands from the analysis.

To prevent the risk of CVD being based on relatives with cardiovascular risk factors, we intended to repeat the analysis after excluding all relatives with overweight/obesity, smoking, hypertension, dyslipidemia or diabetes mellitus. Unfortunately, as all relatives had at least one of these risk factors at the time of CVD, we were unable to do this. For similar reasons, another analysis was performed after excluding all relatives with concomitant thrombophilias.

The pedigrees of 500 probands (373 patients with objectively documented venous thrombosis, 107 patients with CVD, and 20 patients with both venous thrombosis and CVD) disclosed 1641 first-degree relatives aged 15 years or older. Of these relatives, 45 were not evaluable because of missing laboratory data and 1149 were found to be non-carriers of *F5* R506Q or *F2* G20210A (8% of whom had a cardiovascular event). The remaining 447 relatives were analyzed: 175 were single heterozygous for *F5* R506Q , 220 were single heterozygous for *F2* G20210A, 37 were double heterozygous for *F5* R506Q and *F2* G20210A, 8 were homozygous for *F5* R506Q and 7 were homozygous for *F2* G20210A. The clinical characteristics are summarized in Table 1. Males and females were distributed equally. The median age at inclusion was 47 years (range, 15-91). Concomitant thrombophilias were found in approximately 60% of all relatives.

During the observation period a total of 33 arterial thrombotic events occurred: 7% in single heterozygous *F5* R506Q or *F2* G20210A carriers and 12% in double heterozygous and homozygous carriers. The median age at onset was similar in both groups (53 years; range, 26-78). The annual incidence of CVD was 0.23% (95% CI, 0.13-0.79) in single heterozygous *F5* R506Q or *F2* G20210A carriers and 0.36% (95% CI, 0.13-0.79) in double heterozygous and homozygous carriers; relative risk 1.6 (95% CI 0.7-3.9) (Table 2). After exclusion of relatives with concomitant thrombophilias, the relative risk of CVD increased to 5.1 (95% CI, 1.3-22.9) in double heterozygous or homozygous *F5* R506Q and *F2* G20210A carriers compared to single *F5* R506Q or *F2* G20210A carriers.

In our study, double heterozygous and homozygous *F5* R506Q and *F2* G20210A carriers had a 1.6 fold (95% CI, 0.7-3.9) increased risk of CVD compared to single *F5* R506Q or *F2* G20210A carriers. These results did not reach statistical significance, however, they further the findings of two previous studies that found a modest association between single heterozygous *F5* R506Q or *F2* G20210A and CVD [2;6], but included too few subjects to calculate, as we did, a risk in homozygous and double heterzogygous carriers.

It is unknown why, in *F5* R506Q and *F2* G20210A carriers, the risk of CVD appears to be more than 10 times weaker than the risk of venous thrombosis. A plausible explanation is that atherosclerosis plays a major role in CVD and a smaller role in venous thrombosis.[7] Atherosclerosis is known to be associated with increased endothelial damage (of which hyperhomocysteinaemia is a marker),

procoagulant changes (such as high levels of FVIII)[8] and natural anticoagulant deficiencies.[9] This could explain the observed increased relative risk of 5.1 (95% CI, 1.2-22.9) found after exclusion of relatives with concomitant thrombophilias. However, as we did not investigate whether atherosclerosis was actually less common in these relatives (e.g with intima media thickness), these results should be treated with caution.

Some aspects of our study warrant comment. A strength of our study is that, due to the large cohort of thrombophilic families, it was possible to estimate the effect of double heterozygosity or homozygosity for *F5* R506Q and *F2* G20210A on CVD for the first time. A weakness is the retrospective design and that, despite its fairly large size, the number of double heterozygous or homozygous *F5* R506Q and *F2* G20210A carriers was too low to provide risk estimates with narrow confidence intervals. The number was also too low to make a subdivision of arterial thrombotic event types for additional sensitivity analyses. Another potential limitation of our study is referral bias, as this study was performed in 3 university hospitals. However, as we tested consecutive patients (probands), we probably reduced such a bias.

In conclusion, it is likely that double heterozygous or homozygous carriers of *F5* R506Q and *F2* G20210A have a higher risk of CVD than single *F5* R506Q or *F2* G20210A carriers. However, due to small numbers, further research is required to verify our findings.

Table 1. Clinical characterist

	Heterozygous FVL-Ptmut or Homozygous FVL or Ptmut	Single heterozygous FVL or Ptmut carriers	Total
Number	52 (12)	395 (88)	447
Male	21 (40)	185 (47)	206 (46)
Age at enrollment (years)	51 (16-85)	47 (15-91)	47 (15-91)
Arterial thrombosis	6 (12)	27 (7)	33
Age at onset	54 (41-78)	53 (26-78)	53 (26-78)
Classification			
Myocardial infarction	2 (4)	12 (3)	14 (3)
Ischemic stroke	1 (2)	8 (2)	9 (1)
Transient ischemic attack	2 (4)	5 (1)	7 (1)
Peripheral arterial thrombotic event	1 (2)	2 (0.5)	3 (2)
Relatives with no other thrombophilic defects*			
	21 (40)	153 (39)	174(39)

* No antIthrombin, protein C or protein S deficiency, hyperhomocysteinemia or high factor VIII levels.
FVL denotes factor V Leiden; Ptmut, prothrombin mutation.
Continous variables denoted as median (range), categorical variables as number (%).

Table 2. Risk of cardiovascular disease in relatives of probands with a thrombophilic defect

	Observation years	Relatives with event	Annual incidence % (95% CI)	Crude relative risk (95% CI)
All evaluable relatives				
Single heterozygous *F5* R506Q or *F2* G20210A carriers	11854	27	0.23 (0.15-0.33)	Reference
Double heterozygous or homozygous *F5* R506Q or *F2* G20210A	1646	6	0.36 (0.13-0.79)	1.60 (0.66-3.88)
Relatives with concomitant thrombophilias excluded				
Single heterozygous *F5* R506Q or *F2* G20210A carriers	4199	4	0.10 (0.03-0.24)	Reference
Double heterozygous or homozygous *F5* R506Q or *F2* G20210A	614	3	0.49 (0.10-1.43)	5.13 (1.15-2.92)

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Summary and discussion

This chapter summarizes the results of the investigations presented in this thesis.

Part I

In part I, the aim was to search for new risk factors of venous thrombosis by analyzing the genetic linkage signals for venous thrombosis and for intermediate phenotypes that were observed in the GENES study (**chapters 2, 3 and 4**). Furthermore, we aimed to evaluate the implications of the findings for diagnosis and prevention of venous thrombosis (**chapters 5 and 6**).

Novel risk factors of venous thrombosis

Venous thrombosis is a multifactorial disease that demands individually tailored prognostic and diagnostic procedures. The tailoring is impeded by the fact that almost half of the genetic risk factors are unknown. Progress in DNA technology in the last decades is enabling the identification of new genetic risk factors by genome-wide linkage studies and genome-wide association studies, methods that each has their own limitations. Genome-wide linkage studies are hampered by the heterogeneity in genetic variations predisposing to a single disease, meaning that findings in one family might not have validity for other families or for the general population. Furthermore, the statistical power of this type of study is dictated by the number of affected individuals in a family which is not always large. Population-based genome-wide association studies do not have this limitation, as individuals of the study group are not related to each other. However, confounding by population admixture, false positive associations and inability to study rare genetic variations are among its disadvantages.

Following the result of a previously conducted genome-wide linkage study, in **chapter 2** we evaluated the effect of genetic variations in three genes (*PROCR, THBD* and *FOXA2*) on the levels of protein C (PC) in a large pedigree and also in the control population of LETS (Leiden Thrombophilia Study). Haplotype 3 (H3) of *PROCR* was associated with higher levels of PC in the pedigree and also in the LETS controls. This finding is in line with the result of a recently published genome-wide association study [1]. Increased levels of sEPCR, as reported before in H3 carriers [2], could not explain the higher levels of PC because, although the affinity of PC to bind to circulating EPCR is the same as the affinity for

endothelial EPCR, the PC levels in H3 carriers are much higher than that of sEPCR. We hypothesized that the amount of endothelial EPCR in H3 haplotype carriers was decreased and consequently the pool of PC bound to this fraction decreased, leaving more PC to circulate in the blood. An analysis of blood-originated endothelial cells of *PROCR* H3 carriers and non-carriers with flow cytometry did not confirm this hypothesis as we observed no difference in the expression of EPCR between cells from H3 carriers and non-carriers. Hence, higher levels of PC in H3 carriers remain to be explained from a biochemical viewpoint. Interestingly, opposite to our expectation, H3 carriership with its inherent high PC levels does not protect against venous thrombosis [2].

In **chapter 3**, the effect of different haplotypes of *NQO1* on the levels of factor (F) V and FII was investigated. We observed a negative association between FV and H4 in the pedigree, but we did not find such an association in the controls of the LETS, indicating the presence of other genetic variations on chr16 to be responsible for the linkage peaks that were first observed in the GENES study [3]. In the LETS controls, H4 carriers had lower levels of vitamine K dependent coagulation factors, especially FII and total protein S, which is plausible knowing that H4 carriers have lower or undetectable activity of the NQO1 enzyme.

We studied the risk of venous thrombosis and the levels of vitamin K dependent coagulation factors for different haplotypes of the enzymes (*VKORC1*, *GGCX and NQO1*) involved in the vitamine K cycle in **chapter 4**. Similar to other studies, we noticed no association between haplotypes of *VKORC1* and the risk of venous thrombosis in LETS [4-6]. No association existed either with haplotypes of *GGCX* and *NQO1*. The levels of a panel of coagulation factors (protein C, protein S, protein Z, FII, FVII, FIX and FX) were reduced in carriers of the VKORC1*2B haplotype which is probably due to lower expression of VKORC1 in the liver of the carriers. The strongest effect was on FIX levels; each copy of VKORC1*2B haplotype was associated with a reduction of 3.26 U/dl.

Clinical aspects of thrombophilia

In **chapter 5**, we reviewed hereditary and acquired thrombophilia and the indications for thrombophilia testing. These were discussed in relation to the impact of test results on primary and secondary prevention settings or as a family

screening tool. Routine thrombophilia screening does not seem to be justified as most individuals with thrombophilia will not develop venous thrombosis. The usefulness of thrombophilia testing for secondary prevention of venous thrombosis depends on its impact on clinical management regarding dosis or duration of anticoagulation treatment and the risk-benefit balance of prophylaxis in high-risk situations. Although some authors suggest thrombophilia testing for patients with thrombosis before 50 years of age, recurrent events, family history of venous thrombosis and thrombosis in unusual sites [7;8] a recent evidence-based guideline recommends against thrombophilia testing [9]. Family screening remains also questionable because, although the risk of thrombosis in first degree relatives of patients with thrombophilia is two- to ten-fold increased, the absolute risk of venous thrombosis is low, even in high-risk situations [10-12].

Pregnancy is associated with a 5-fold increased risk of venous thrombosis, and the risk is even higher postpartum (±20-fold) [13]; a quarter of these events is a recurrence. Since a significant decrease in recurrence rate is observed with prophylaxis, [14;15] pregnant women with a history of thrombosis are generally advised to use of anticoagulation. Thrombophilia is not an indication for prophylaxis during pregnancy or postpartum in women without a history of venous thrombosis with the possible exception of antithrombin deficiency, homozygosity for factor V Leiden or prothrombin G20210A mutations, or combined heterozygosity for both mutations [12;16]. The optimal doses of LMWH in pregnancy with respect to thrombosis recurrence risk and the risk of postpartum bleeding is not clear. The increasing number of reports of the failure of low dose prophylaxis [17-19] indicates the need for randomized clinical trials to demonstrate the safety of high doses of LMWH for prophylactic measures in pregnancy.

In **chapter 6**, we observed that postpartum hemorrhage (PPH) did not occur more often in women who were given therapeutic doses of LMWH (RR: 0.8, 95% CI: 0.5-1.4). For women who delivered vaginally, this risk estimate of no increase was firm (RR: 0.5, 95%CI: 0.3-1.1), whereas for those women who delivered by cesarean section the risk of PPH (for cesarion section a priori defined as more than 1000 mL blood loss) appeared increased but due to the low number of women the confidence interval is very wide (RR: 2.5, 95% CI: 0.3-18.9). The median blood loss was found to be similar in treated and untreated women, except for the subgroup of normal vaginal deliveries where it was lower in the LMWH users

(median difference: -100, 95% CI: -156 to -44). A likely explanation for this observation is differential use of oxcytocics in LMWH users.

Part II

In part II we addressed the relationship between venous and arterial thrombosis. We aimed to test the hypothesis that the two conditions are related by the presence of shared risk (**chapters 7, 9 and 10**). In **chapter 8**, inflammatory markers were studied as risk factors for recurrence of venous thrombosis, since several lines of evidence have indicated that inflammation promotes the development of atherosclerosis and cardiovascular disease [20;21].

Several previous studies have questioned the distinction between venous and arterial thrombosis. An increased risk of arterial thrombosis [22-25] among individuals who have had previous venous thrombosis was established in three cohort studies [26-28]. Whether this association was based on "shared risk factors" is unlikely since the corresponding risk did not differ by adjusting for age and established cardiovascular risk factors [26]. In an analysis of the Beethoven study, a large cohort study of thrombophilic families in **chapter 7**, the same modestly increased risk of arterial thrombosis in individuals with previous venous thrombosis was observed, although it did not reach statistical significance. The risk did not change by adjusting for "shared risk factors" separately and simultaneously by using a propensity score considering age, cardiovascular risk factors and presence of one ore more thrombophilic defects conditional to venous thrombosis history. Therefore we concluded that "shared risk factors" alone can not explain this association.

Generally speaking, conventional cardiovascular risk factors have, if any, a mild effect on the development of venous thrombosis [29]. It should be noted that atherosclerosis also does not raise the risk of future venous thrombotic events [30;31]. Likewise, the role of thrombophilia in pathogenesis of arterial thrombosis remains obscure, especially for the rare thrombophilic defects such as antithrombin deficiency [32;33], protein C deficiency [34;35] or protein S deficiency [34;35]. A borderline effect on myocardial infarction though has been attributed to FVL and prothrombin G20210A [36]. The effect of double heterozygosity or homozygosity for FVL or prothrombin mutations was not studied because of the low prevalence of these mutations. In a post-hoc analysis of the Beethoven study (**chapter 8**), we

observed that relatives who were double heterozygous or homozygous for FVL or the prothrombin mutation were at a nonstatistically significant 1.6 times higher risk of arterial thrombosis as compared to heterozygotes for either mutation. The risk after excluding relatives with concomitant thrombophilia was 5.1 (95% CI: 1.2-22.9). In conclusion, double heterozygosity or homozygosity for FVL or the prothrombin mutation seemed to increase the risk of arterial thrombosis.

In the context of the relation of venous and arterial thrombosis, retinal vein occlusion (RVO) is an interesting disease, since risk factors for this venous occlusion mainly are established arterial risk factors, such as hypertension, hyperlipidemia and diabetes, whereas the relationship with thrombophilia is controversial [37]. In **chapter 10**, we evaluated the role of established thrombophilic defects, of assays indicating procoagulant state as well as of platelet receptor polymorphisms that are known to increase the thrombosis tendency [38-40], in patients with idiopathic RVO. The only suggestive association was found for platelet receptor polymorphism rs5918 with a dose-dependent effect on the risk of idiopathic RVO (OR for heterozygotes: 1.7, 95% CI: 0.8-3.3 and for homozygotes: 2.8, 0.5-15.9). No association was observed for established thrombophilia and clot lysis time.

Inflammation initiated by thrombosis in the veins can possibly contribute to higher risk of arterial thrombosis after venous thrombotic events. The formed thrombin triggers inflammation in the endothelium by activating neutrophils and inducing the production of selectins, cytokines and cellular adhesion molecules [41;42]. Dysfunctional endothelium not only lacks its normal antithrombotic and fibrinolytic activity but also becomes more thrombogenic by expressing higher amounts of von Willebrand factor, tissue factor, plasminogen activator inhibitor and factor V [43]. Chronically increased CRP and IL6, however, do not seem to influence the development of new venous thrombosis [44;45]. On the contrary, acute inflammatory diseases are known to increase the risk of venous and arterial thrombosis for a short period [46;47].

In **chapter 9**, the association between high levels of inflammatory biomarkers and D-dimer and the risk of recurrent venous thrombosis was evaluated in the case population of the LETS. The risk of recurrence, adjusted for age, sex and BMI was about 2.2 times higher during ongoing inflammation, indicated by CRP levels above 3 mg/L (95% CI: 1.3-3.8). No association was noted between

cytokine levels and the risk of recurrence, probably because of the higher detection limit of beads assays compared to ELISA assays which could have led to fewer individuals with detectable levels. As previously shown, higher D-dimer levels were associated with a higher risk of recurrence (HR: 1.7, 95% CI: 0.9-3.4). Furthermore, we observed an additive effect between D-dimer and CRP. Therefore, individuals with either elevated D-dimer or CRP and those with both elevated CRP and D-dimer had a higher recurrence risk compared to patients with low CRP and D-dimer levels (HR 1.9; 95% CI 1.1-3.5 and 3.1; 1.4-7.2 respectively).

Future perspectives

Despite advances in prediction of venous thrombosis, its incidence has not been changed which indicates the need to identify new risk factors for first and recurrent venous thrombosis. In this context, genome wide association studies seem promising in finding new candidate genetic risk factors especially for particular types of thrombosis such as retinal vein thrombosis where there are still ambiguities surrounding risk factors. Interestingly, sometimes the results of these studies are in line with genome wide linkage studies, like the one mentioned about the role of variations in chromosome 20 and the levels of protein C.

The risk of arterial thrombosis rises modestly after an episode of venous thrombosis. Whether shared risk factors or chronic inflammation triggered by venous thrombosis explains this risk remains unclear. There are indications, though not strong enough, that traditional cardiovascular risk factors can not solely justify this association.

At last, as mentioned above, a randomized clinical trial study between high and prophylactic doses of LMWH will answer which dose in pregnancy is optimal with respect to efficacy and bleeding risk during pregnancy and postpartum.

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Samenvatting

Dit hoofdstuk vat het onderzoek beschreven in dit proefschrift samen.

Deel I

In deel I hebben we gezocht naar nieuwe risicofactoren voor veneuze trombose door analyse van de genetische linkage signalen voor veneuze trombose en voor intermediaire fenotypen die waargenomen zijn in de GENES studie (**hoofdstuk 2, 3** en **4**). Verder worden de implicaties van trombofilieonderzoek voor de diagnose en de preventie van veneuze trombose besproken (**hoofdstukken 5** en **6**).

Nieuwe risicofactoren voor veneuze trombose

veneuze trombose is een multifactoriële ziekte die een geïndividualiseerde prognostische en diagnostische aanpak nodig heeft. Dit wordt bemoeilijkt door het feit dat nog lang niet alle genetische risicofactoren bekend zijn. Vooruitgang in DNA-technologie in de laatste decennia heeft de identificatie van nieuwe genetische risicofactoren door genoom-wijde linkage studies en genoom-wijde associatie studies vergemakkelijkt, alhoewel elk van deze onderzoeksvormen zijn eigen beperkingen heeft. Genoom-wijde linkage studies worden belemmerd wanneer er veel heterogeniteit is in de genetische variaties die tot ziekte predisponeren. Dit betekent dat de bevindingen in de ene familie misschien niet toepasbaar zijn in andere families, laat staan in de algemene bevolking. Bovendien wordt de statistische power van dit soort onderzoeken mede bepaald door het aantal aangedane personen in een familie, en dit aantal is vaak niet groot. Populatie-gebaseerde genoom-wijde associatie studies hebben deze beperkingen niet doordat studie deelnemers geen bloedverwanten van elkaar zijn. Echter, confounding door een ongelijke verdeling van etniciteiten over patiënten en controles, vals-positieve associaties, en het onvermogen om zeldzame genetische variaties te bestuderen, behoren tot de beperkingen van genoom-wijde associatie studies.

Naar aanleiding van het resultaat van een eerder uitgevoerd genoom-wijd linkage onderzoek, wordt in **hoofdstuk 2** het effect van genetische variaties in drie genen (*PROCR*, *THBD* en *FOXA2*) op de plasmaspiegel van proteïne C (PC) in een grote stamboom, en ook in de controlepopulatie van LETS (Leiden Trombofilie Studie) ,bestudeerd. Er werd een verband gevonden tussen haplotype 3 (H3)

van *PROCR* en hogere spiegels van PC in leden van de stamboom en ook in de LETS controles. Deze bevinding is in overeenstemming met het resultaat van een recent verschenen genoom-wijde associatie studie. Hogere circulerende EPCR spiegels bij H3 dragers, iets dat ook eerder beschreven is, kunnen de hogere PC spiegels niet verklaren. Hoewel de affiniteit van PC om te binden aan circulerende EPCR hetzelfde is als de affiniteit voor endotheel-gebonden EPCR, is de absolute PC spiegel in molairen in H3 dragers veel hoger dan de sEPCR spiegel. We veronderstelden dat de hoeveelheid endotheel-gebonden EPCR in H3 dragers misschien laag is en als gevolg hiervan de hoeveelheid PC gebonden aan deze fractie kleiner is, zodat er meer PC voor de circulatie beschikbaar is Flowcytometrie van uit bloed gekweekte endotheelcellen bevestigde deze hypothese niet. We zagen geen verschil in de expressie van EPCR tussen de cellen van de H3 dragers en niet-dragers. Kortom, een hogere PC spiegel in H3 dragers moet nog vanuit biochemisch oogpunt verklaard worden. In tegenstelling tot onze verwachtingen biedt H3 dragerschap met zijn inherente hoge PC spiegel geen bescherming tegen veneuze trombose.

In **hoofdstuk 3** is het effect van verschillende haplotypes van *NQO1* op de spiegel van factor (F) V en FII onderzocht. We zagen een negatieve associatie tussen FV en *NQO1* H4 in de stamboom maar niet in controles van de LETS, hetgeen wijst in de richting van een andere genetische variatie op chr16 die verantwoordelijk is voor de linkage signalen in de GENES studie. In de LETS controles hadden H4 dragers een lagere spiegel van vitamine K afhankelijke stollingsfactoren, in het bijzonder trombine en totaal proteïne S, wat aannemelijk is wetende dat H4 dragers een lagere of niet detecteerbare activiteit van het enzym NQO1 hebben.

In **hoofdstuk 4** is de relatie bestudeerd tussen het risico op veneuze trombose of de spiegel van vitamine K-afhankelijke stollingsfactoren met verschillende haplotypes van de genen die coderen voor enzymen die betrokken zijn bij de vitamine K-cyclus (VKORC1, GGCX en NQO1). In overeenstemming met eerdere observaties vonden wij geen verband tussen haplotypes van *VKORC1* en het risico op veneuze trombose in LETS. Tevens was er geen verband met haplotypes van *GGCX* en *NQO1*. De plasmaspiegel van een panel van stollingsfactoren (proteïne C, proteïne S, proteïne Z, FII, FVII, FIX en FX) was lager in dragers van het *VKORC1**2B haplotype, hetgeen waarschijnlijk te wijten is aan de lagere expressie van *VKORC1* in de lever van de dragers. Het sterkste

effect betrof de FIX spiegel met een daling van 3.26 U / dl voor elk allel van *VKORC1**2B.

Klinische aspecten van trombofilie

In **hoofdstuk 5** worden erfelijke en verworven vormen van trombofilie en de indicaties voor trombofilie onderzoek besproken. Het accent lag daarbij op de invloed van de testresultaten op primaire en secundaire preventie van trombose en op familie screening. Routinematige trombofilie screening is niet gerechtvaardigd, aangezien de meeste mensen met trombofilie geen veneuze trombose zullen ontwikkelen. Het nut van trombofilie onderzoek bij secundaire preventie van veneuze trombose is deels afhankelijk van de invloed van de uitslagen op het klinische management wat betreft dosis en duur van behandeling met anticoagulantia, en deels afhankelijk van de afweging tussen risico en baten van profylaxe in risicovolle situaties. Sommige auteurs hebben voorgesteld om trombofilie onderzoek alleen te verrichten bij patiënten met trombose voor hun vijftigste levensjaar, bij recidiverende trombose, bij een positieve trombose familiegeschiedenis of bij trombose op ongebruikelijke plaatsen. Een recente 'evidence-based' richtlijn adviseert om geen trombofilie onderzoek te verrichten in bovengenoemde situaties. Familie onderzoek is omstreden omdat het risico op trombose in eerste graads familieleden van patiënten met trombofilie weliswaar twee tot tien keer verhoogd is, maar het absolute risico op veneuze trombose laag blijft, zelfs in hoog-risico situaties.

Zwangerschap is geassocieerd met een vijfmaal verhoogd risico op veneuze trombose. Het risico is nog sterker verhoogd (± 20-maal) in de 6 tot 12 weken postpartum. Een kwart van de gevallen betreft recidief trombose. Omdat het recidief risico sterk daalt door profylaxe met anticoagulantia, wordt aan zwangere vrouwen met een voorgeschiedenis van trombose geadviseerd profylaxe te gebruiken in de postpartum periode. Trombofilie op zichzelf is geen indicatie voor profylaxe tijdens de zwangerschap of postpartum bij vrouwen zonder een voorgeschiedenis van veneuze trombose, met als mogelijke uitzondering, patiënten met een positieve familiegeschiedenis voor trombose die homozygoot zijn voor factor V Leiden of protrombine G20210A mutaties of gecombineerde heterozygositeit voor beide mutaties. De optimale dosering van LMWH tijdens de

zwangerschap ter vermijding van recidief trombose, gebalanceerd tegen het risico van postpartum bloeding is niet duidelijk. Het toenemende aantal meldingen van falen van lage dosering profylaxe onderstreept de noodzaak van gerandomiseerde klinische studies om de effectiviteit en veiligheid van een hoge dosis LMWH als profylaxe tijdens zwangerschap aan te tonen.

Hoofdstuk 6 toont aan dat postpartum bloeding (PPH) niet vaker voorkomt bij vrouwen die worden behandeld met therapeutische doses LMWH dan bij vrouwen die niet met LMWH werden behandeld (RR: 0.8, 95% CI: 0.5 tot 1.4). Het mediane bloedverlies bleek vergelijkbaar te zijn tussen behandelde en onbehandelde vrouwen behalve in de subgroep met een normale vaginale bevalling waar de hoeveelheid bloedverlies iets lager was in de LMWH gebruikers (mediaan verschil: -100, 95% CI: -156 tot -44). Een mogelijke verklaring hiervoor is het preferentiële gebruik van oxytocine in LMWH gebruikers.

Deel II

In deel II hebben we de relatie tussen veneuze en arteriële trombose bestudeerd. We hebben getracht om de "shared risk factors" hypothese te testen en dit komt aan bod in **hoofdstukken 7, 9** en **10**. Verschillende studies hebben aangetoond dat ontsteking de ontwikkeling van atherosclerose en hart- en vaatziekten bevordert. In **hoofdstuk 8** werden inflammatoire markers bestudeerd als risicofactor voor recidief veneuze trombose.

Mensen die veneuze trombose hebben gehad hebben een verhoogd risico op arteriële trombose. Of deze associatie uitsluitend gebaseerd is op 'shared risk factors' is onwaarschijnlijk, omdat het bijbehorende risico niet afhankelijk is van leeftijd of aanwezige cardiovasculaire risicofactoren. Een analyse in de Beethoven studie, een grote cohort studie van families met trombofilie (**hoofdstuk 7**), toonde eenzelfde verhoogd risico voor arteriële trombose bij mensen met eerdere veneuze trombose laten zien, hoewel het effect niet statistisch significant was. Wij onderzochten of gemeenschappelijke risicofactoren dit verband konden verklaren, door het berekenen van een zogenaamde propensity score gebaseerd op leeftijd, de aanwezigheid van cardiovasculaire risicofactoren en de aanwezigheid van één of meer trombofilie afwijkingen. Op grond van dit onderzoek kwamen wij tot de conclusie dat 'shared risk factors' het verband tussen veneuze trombose en arteriële hart- en vaatziekten niet voldoende kunnen verklaren.

In het algemeen hebben conventionele cardiovasculaire risicofactoren een gering effect op de ontwikkeling van veneuze trombose. Ook atherosclerose verhoogt het risico op toekomstige veneuze trombose niet. Tevens blijft de rol van trombofilie in de pathogenese van arteriële trombose klein. Dit geldt vooral voor de rol van zeldzame trombofilie afwijkingen zoals antitrombine deficiëntie, proteïne C deficiëntie of proteïne S deficiëntie. Echter, een klein effect op myocardinfarct is toegeschreven aan FVL en protrombine G20210A. Het effect van gecombineerde heterozygositeit of homozygositeit voor FVL of protrombine G20210A was vanwege de lage prevalentie van deze mutaties niet bekend. In een post-hoc analyse van de Beethoven studie (**hoofdstuk 8**) zagen we dat familieleden die dubbel heterozygoot of homozygoot voor FVL of protrombine mutatie G20210A waren een 1.6 maal hoger risico (niet significant) op arteriële trombose hadden in vergelijking met heterozygoten voor de afzonderlijke mutaties. Na uitsluiting van familieleden met andere trombofilie factoren bedroeg het relatieve risico voor dubbel heterozygoten of homozygoten zelfs 5.1 (95% CI: 1.2 tot 22.9) ten opzichte van heterozygoten voor iedere mutatie afzonderlijk.

Wat de relatie tussen veneuze en arteriële trombose betreft, is veneuze occlusie in het netvlies (Retinal Vein Occlusion: RVO) een interessante ziekte. Risicofactoren voor RVO betreffen voornamelijk dezelfde als voor arteriële hart- en vaatziekten zoals hypertensie, hyperlipidemie en diabetes mellitus, terwijl de rol van trombofilie omstreden is. In **hoofdstuk 10** hebben we de rol van trombofilie afwijkingen, bloedplaatjes receptor polymorfismen en fibrinolyse bij RVO bestudeerd in een patiënt-controle onderzoek van 101 patiënten met idiopathische RVO en gezonde controle personen. De enige suggestieve associatie die gevonden werd was voor polymorfisme rs5918 in een bloedplaatjes receptor met een dosisafhankelijk effect op het risico van idiopathische RVO (OR voor heterozygoten: 1.7, 95% CI: 0.8 tot 3.3 en voor de homozygoten: 2.8, 0.5-15.9). Er werd geen verband waargenomen tussen RVO en trombofilie of clot lysis tijd.

Ontsteking, geïnitieerd door veneuze trombose, kan mogelijk bijdragen aan het risico op arteriële trombose. Het gevormde trombine induceert ontsteking van het endotheel door het activeren van neutrofielen en het induceren van de productie van selectines, cytokines en cellulaire adhesiemoleculen. Ontstoken endotheel heeft verminderde antitrombotische en fibrinolytische eigenschappen en het wordt nog meer trombogeen door de uitscheiding van Von Willebrand-factor,

tissue factor, plasminogeen activator inhibitor en factor V. Het lijkt er niet op dat chronisch verhoogde spiegels van CRP of IL6 het risico op eerste veneuze trombose beïnvloeden. In **hoofdstuk 9** is het verband tussen inflammatoire biomarkers en D-dimeer en het risico op recidief veneuze trombose in de LETS geëvalueerd. Het risico op recidief trombose, gecorrigeerd voor leeftijd, geslacht en BMI, was 2.2 (95% CI: 1.3 tot 3.8) keer toegenomen bij een CRP spiegel hoger dan 3 mg/L. Er kon geen verband vastgesteld worden tussen cytokine spiegels en het risico op recidief trombose. De reden hiervoor is mogelijk de hogere detectielimiet van beads assays ten opzichte van ELISA assays wat heeft geleid tot een lager aantal mensen met een detecteerbare spiegel. Zoals eerder aangetoond hangt een D-dimeer spiegel boven 250 ng/ml samen met een hoger risico op recidief trombose (HR: 1.7, 95% CI: 0.9 tot 3.4). Verder was er een additief effect tussen de spiegels van D-dimeer en CRP. Dus mensen met een hoge D-dimeer of CRP en mensen met zowel hoge CRP als D-dimeer hadden een hoger recidief risico in vergelijking met patiënten met een lage CRP en D-dimeer spiegels (HR 1.9, 95% CI 1.1 tot 3.5 en 3.1, 1.4 tot7.2 respectievelijk).

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Na een episode van veneuze trombose stijgt het risico van arteriële trombose. Het is onduidelijk of het “shared risk factors” of chronische ontsteking zijn, die hieraan ten grondslag liggen.

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Curriculum vitae and publications

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Sare Roshani werd geboren op 16 september 1979 in Mashhad (Iran). Na drie jaar profiel wiskunde op de middelbare school gehad te hebben, raakte ze geïnteresseerd in de biologie en uiteindelijk is ze met profiel biologie in 1997 van de Saadi middelbare school in Mashhad cum laude afgestudeerd. In 2004 behaalde zij cum laude haar artsexamen aan het Mashhad University of Medical Science (1998-2004). Na haar afstuderen heeft ze 19 maanden als basis arts op spoedeisende hulp gewerkt. In deze periode besloot ze om ervaring op te doen in het wetenschappelijke aspect van de geneeskunde. Om die reden kwam ze in 2006 naar Nederland en begon aan haar promotieonderzoek over de associatie van arteriële en veneuze trombose waarvan de resultaten in dit proefschrift beschreven staan. Ze begon haar onderzoek eerst aan het Academisch Medisch Centrum in Amsterdam. Na een periode van 6 maanden, verhuisde ze naar het Leids Universitair Medisch Centrum en zette haar project op de afdeling Klinische Epidemiologie verder onder leiding van prof. S.Middeldorp en prof. P.H.Reitsma. Ondertussen leerde ze de Nederlandse taal en behaalde ze de Nederlandse taaldiploma (programma II). Tevens nam ze deel aan een assessment om haar artsendiploma te laten valideren en zich kunnen inschrijven in het BIG-register. Gedurende haar promotie-onderzoek, volgde ze verschillende cursussen over statistiek en epidemiologie, zoals klinische epidemiologie op Schiermonnikoog en lezingen van Kenneth Rothman. Ook heeft zij verschillende (inter)nationale congressen en symposia bezocht waar zij een poster heeft gepresenteerd of een mondelinge presentatie heeft gehouden. Zij zal in februari 2012 aan het Leids Universitair Medisch Centrum als basisarts afstuderen en zich verder specialiseren.

Roshani S, Cohn DM, Stehouwer AC , Wolf H , van der Post JAM, Büller HR, Kamphuisen PW, Middeldorp S. Incidence of postpartum hemorrhage in women receiving therapeutic doses of low-molecular-weight heparin: results of a retrospective cohort study. BMJ Open. 2011 Nov 14;1(2):e000257.

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