

## Thrombophilia

Issue or non-issue in clinical practice?

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Issue or non-issue in clinical practice?

**Proefschrift**

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## TABLE OF CONTENTS

Chapter 1	General introduction and outline of the thesis	9
<b>Part I: Hyperhomocysteinemia</b>		
Chapter 2	The risk of venous and arterial thrombosis in hyperhomocysteinemia is low and mainly depends on concomitant thrombophilic defects. <i>Thromb Haemost</i> 2007;98:457-463.	21
Chapter 3	Methionine-loading and random homocysteine tests have no added value in risk assessment for venous and arterial thrombosis. <i>J Thromb Haemost</i> 2007;5:614-616.	37
Chapter 4	The risk of venous and arterial thrombosis in Hyperhomocysteinemic subjects may be a result of elevated factor VIII levels. <i>Haematologica</i> 2007;92:1703-1706.	47
Chapter 5	Hyperhomocysteinemia is not a risk factor for venous and arterial thrombosis, and is associated with elevated factor VIII levels. <i>Thromb Res</i> 2008; (in press).	57
<b>Part II: Infections</b>		
Chapter 6	Mesenteric vein thrombosis associated with primary cytomegalovirus infection: a case report. <i>Blood Coagul Fibrinolysis</i> 2007;18:509-511.	73
Chapter 7	Possible contribution of cytomegalovirus infection to the high risk of (recurrent) venous thrombosis after renal transplantation. <i>Thromb Haemost</i> 2008;99:127-132.	81
Chapter 8	Absolute risk of venous and arterial thrombosis in HIV-infected patients and effects of combination antiretroviral therapy. <i>J Thromb Haemost</i> 2006;4:1928-1930.	95

Chapter 9	The relationship between progression to AIDS and thrombophilic abnormalities in HIV infection. <i>Clin Chem</i> 2008; (in press).	103
<b>Part III:</b>	<b>Thrombophilia testing</b>	
Chapter 10	Selective testing for thrombophilia in patients with first venous thrombosis. Results from a retrospective family cohort study on absolute thrombotic risk for currently known thrombophilic defects in 2479 relatives. <i>Submitted.</i>	123
Chapter 11	A higher risk of recurrent venous thrombosis in men is due to hormonal risk factors in women in thrombophilic families. <i>Submitted.</i>	143
	Summary and discussion	159
	Samenvatting en discussie	167
	Dankwoord (acknowledgements)	177

# Chapter 1

**General introduction  
and  
outline of the thesis**



## GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

The overall incidence of venous thrombosis is 1-3 per 1000 persons each year and rises exponentially from < 0.005% in children to 1% per year in the elderly [1]. Most venous thromboses start in the calf veins, from where they may extend and cause proximal deep vein thrombosis (DVT), and subsequently pulmonary embolism (PE) [2]. Patients with DVT or PE remain at risk for recurrent venous thrombosis. This risk is most pronounced in the first months after the acute episode and declines slowly over subsequent years [3]. Risk factors for venous thrombosis are presented in Table 1. Despite anticoagulant treatment for 3-6 months, the recurrence rate of venous thrombosis is 25% within 5 years, while the immediate death rate due to pulmonary embolism is 2.6% [4] and the post-thrombotic syndrome occurs in up to 50% of patients [5]. Recurrent venous thrombosis usually leads to life-long anticoagulant treatment, which increases long term health care costs substantially and has serious potential side effects as major bleeding. Therefore, it is pivotal to try to decrease the risk of first venous thrombosis as well as recurrence, rather than to start with indefinite anticoagulant treatment after a first episode of venous thrombosis. Prevention will probably be more profitable if it becomes possible both to identify more precisely those persons who are at risk of venous thrombosis and to quantify the risk to which they are exposed.

Hypercoagulability of the blood is one of the factors that are involved in the pathology of thrombosis [6]. It is due to abnormalities of coagulation and fibrinolysis. These abnormalities may be inherited or acquired, and are classified thrombophilic defects, while their clinical expression is known as thrombophilia. Originally, thrombophilia was characterized by first and recurrent venous thrombosis at young age. It was associated with rare hereditary thrombophilic defects that showed to be strong risk factors for venous thrombosis [7]. Since then, many other prevalent thrombophilic defects have been recognized as mild risk factors. Whether patients should be tested for the presence of one or more thrombophilic defects depends on the clinical implications of positive tests in patients with venous thrombosis, and in relatives when the defects are inherited. When a thrombophilic defect is associated with a high risk of recurrence, extended anticoagulant treatment after a first episode of venous thrombosis should be considered. Primary thromboprophylaxis becomes an option when a thrombophilic defect is associated with a high risk of first venous thrombosis, provided that the

**Table 1.** Risk factors for venous thrombosis

<b>Inherited</b>	<b>Acquired</b>
- Antithrombin deficiency	- Age
- Protein C deficiency	- Malignancy
- Protein S deficiency	- History of venous thrombosis
- Factor V Leiden	
- Prothrombin G20210A	
<b>Environmental</b>	<b>Mixed or not well established</b>
- Surgery and major trauma	- High factor VIII
- Pregnancy and puerperium	- High factor IX
- Oral contraceptives	- High factor XI
- Hormone replacement therapy	- High TAFI
- Prolonged immobilization	- Hyperhomocysteinemia
	- Infection
	- Sex

risk-benefit ratio of thromboprophylaxis is favourable. However, for most thrombophilic defects little is known about their absolute risk of venous thrombosis. Moreover, the risk of venous thrombosis may be influenced by interactions between thrombophilic defects. Furthermore, thrombophilic defects may be acquired as result of numerous conditions and diseases [8]. Their contribution may vary from transient to long-lasting, dependently of the underlying condition or disease.

Primary objective of this thesis was the absolute risk for currently known thrombophilic defects, either single or many combinations. Secondary objective was the absolute risk of venous thrombosis associated with hyperhomocysteinemia and infectious diseases, and the contribution of thrombophilic defects to this risk.

### **Hyperhomocysteinemia**

Mild hyperhomocysteinemia (homocysteine levels up to 100  $\mu\text{mol/L}$ ) has been associated with an increased risk of first and recurrent venous and arterial thrombosis in many case-control studies and meta-analyses; relative risk ranged from 2.5 to 3.0 [9-11]. To explain this observation, the “homocysteine hypothesis” was postulated at the end of the 1990’s [12,13]. It was based on experimental studies. Most of these experimental studies were done at homocysteine concentrations which were at least 10 times higher than those in patients with mild hyperhomocysteinemia, and should therefore be interpreted with caution. They sug-

gested that homocysteine promotes atherogenesis by oxidative arterial injury, damages the vascular matrix, and augments proliferation of vascular smooth muscle. Oxidative injury of the endothelium by homocysteine may contribute to thrombosis by altering procoagulant properties of the blood, and impairing endothelium-dependent vasomotor regulation [12,13]. Hyperhomocysteinemia is defined as an increased plasma level of homocysteine after a 10 hour fast and/or after methionine loading. According to the Dutch Heart Foundation, a random homocysteine sample may simplify the procedure for diagnosing hyperhomocysteinemia. As homocysteine levels are reduced with vitamins B6, B11 (folic acid) and B12 in the large majority of cases [14], this has led to a straight forward view that the risk of both venous and arterial thrombosis might be reduced with vitamin-B supplementation. Prospective placebo-controlled clinical trials that studied the effect of B-vitamins on the risk of recurrent venous and arterial thrombosis were started in 1996, but it took more than 8 years before the results were published [15-19]. All showed no improved clinical outcome in patients with prior venous or arterial thrombosis. The negative results of these studies can be explained by a low absolute risk of thrombosis in subjects with mild hyperhomocysteinemia, because it will then be difficult to demonstrate a clinical benefit of homocysteine lowering treatment. However, a low absolute risk of thrombosis in hyperhomocysteinemia might also be a result of testing homocysteine levels after random or methionine-loading assignment. Random and particularly methionine-loading homocysteine tests may have no clinical relevance in subjects with normal fasting homocysteine levels when they do not change the risk of thrombosis. Finally, an associated condition rather than hyperhomocysteinemia itself could be the thrombotic culprit. This could also explain the negative findings of the B-vitamin trials. Hyperhomocysteinemia would then be an epiphenomenon rather than a causal risk factor for venous and arterial thrombosis.

### **Infections**

Although several studies have focused on inflammatory and infectious diseases as possible causes of arterial thrombosis [20,21], less is known about their role in the development of venous thrombosis. According to Virchow's trias, infections could affect blood flow, damage the vessel wall or increase coagulability of the blood [6,22]. Parallels with arterial thrombosis suggest that damage of the vessel wall might not be limited to physical damage, but could also impair endothelial function. Inflammation influences endothelial function in both arteries and

veins [23,24], and a link between infection and venous thrombosis via endothelial activation has been suggested [25]. Thus, there are several mechanisms by which infections can increase the risk of venous and arterial thrombosis. However, as many infections are transient or not noted, it will be difficult to demonstrate whether hypercoagulability in patients with infections results in an increased risk of thrombosis. In this respect, patients with HIV-infection and renal transplant recipients might be of special interest. Many cases have been reported in whom cytomegalovirus (CMV) infection and HIV-infection were associated with thrombosis. These have recently been reviewed [26,27]. Patients with HIV-infection are, obviously, chronically infected. CMV infection in renal transplant recipients is a very common complication, that can easily reactivate due to immunosuppressive drugs [28,29]. Therefore, one would expect a persistent hypercoagulable state especially in these patients and consequently an increased risk of thrombosis. This assumption, however, needs to be clarified.

### **Thrombophilia testing**

Since 1965, an increasing number of abnormalities of coagulation and fibrinolysis have been identified as risk factors for venous thrombosis. These thrombophilic defects include hereditary deficiencies of antithrombin, protein C and protein S, factor V Leiden, prothrombin G20210A, elevated levels of factors VIII, IX, XI, homocysteine and thrombin activatable fibrinolysis inhibitor (TAFI) [30-38]. Together, their prevalence is approximately 25% in the normal population and more than 60% in subjects with venous thrombosis [39]. Venous thrombosis is a multicausal disease, often involving acquired or environmental risk factors as well as genetic predisposition [39]. Whether patients with venous thrombosis should be tested for thrombophilic defects is still a matter of debate, which is highlighted by two recent prospective studies on this issue [40,41]. These studies reported a similar risk of recurrence in patients with a thrombophilic defect, compared to patients without this defect, and concluded that testing for thrombophilic defects has no clinical value. However, the thrombotic potency of different thrombophilic defects were quantified as equal, while it would be more appropriate to weigh defects according to predefined risk estimates. Moreover, both studies were population studies, that are not suitable for risk assessments in subjects with strong, but rare hereditary thrombophilic defects, as numbers are too small. To compare the absolute risk of first and recurrent venous thrombosis for single and combined thrombophilic defects, sufficient numbers of particularly subjects with rare defects

are required. These can more easily be obtained in large family cohort studies. The results of such studies are urgently awaited for.

### **Sex differences**

Recent studies showed that men have a 2-4-fold higher risk of recurrent venous thrombosis than women [41-43]. For this reason, it was suggested to continue anticoagulant treatment after first venous thrombosis in men for a longer time than women [42,43]. Although this treatment probably will prevent most recurrences, it is associated with major bleeding in 2-3% of patients per year [44,45]. Moreover, the reason why men would be at higher risk of recurrent venous thrombosis than women is unknown. It could be explained by an imbalance of environmental risk factors for venous thrombosis in men compared to women, for example, due to hormonal risk factors to which only women are exposed. In previous studies, which reported that men had an increased risk of recurrent venous thrombosis compared to women, men were older at first venous thrombosis [42,43]. As increasing age is associated with an increased risk of venous thrombosis [3], and follow-up in (prospective) studies were relatively short (maximum 2-8 years) [41,42], another explanation for this difference could be older age in combination with short follow-up times. It will be obvious that the observed difference in risk of recurrence between men and women must be established and needs to be clarified.

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# **Part I      Hyperhomocysteinemia**



# Chapter 2

## **The risk of venous and arterial thrombosis in hyperhomocysteinemia is low and mainly depends on concomitant thrombophilic defects**

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**ABSTRACT**

As homocysteine-lowering treatment has not reduced the risk of recurrent thrombosis in recent clinical trials, we hypothesized that mild hyperhomocysteinemia is an epiphenomenon or associated with a low absolute risk of thrombosis. In this retrospective study, we enrolled 478 evaluable first degree relatives of consecutive patients with venous thrombosis or premature atherosclerosis, and hyperhomocysteinemia. Absolute risks of thrombosis and effects of concomitant thrombophilic defects were compared. Relative risks were adjusted for clustering in families, age, sex, and atherosclerotic risk factors, where appropriate. Annual incidence of venous thrombosis was 0.16% (95% confidence interval [CI], 0.08-0.30) in hyperhomocysteinemic relatives versus 0.11% (95% CI, 0.05-0.20) in normohomocysteinemic relatives; adjusted relative risk 1.6 (95% CI, 0.6-4.5). Annual incidences of arterial thrombosis were 0.34% (95% CI, 0.21-0.52) and 0.24% (95% CI, 0.15-0.37) in hyperhomocysteinemic and normohomocysteinemic relatives, respectively; adjusted relative risk 1.5 (95% CI, 0.6-3.5). Concomitance of multiple thrombophilic risk factors increased the risk of venous thrombosis in hyperhomocysteinemic relatives 20 fold, but a comparable effect was demonstrated in normohomocysteinemic relatives. We conclude that hyperhomocysteinemia is associated with a low absolute risk of venous and arterial thrombosis. Concomitant thrombophilic defects are probably main determinants on the risk of venous thrombosis, rather than hyperhomocysteinemia itself.

## INTRODUCTION

Homocystinuria is a rare autosomal recessive inborn error of metabolism that results in homocysteine plasma levels often exceeding 400  $\mu\text{mol/L}$ . It is associated with a high risk of venous and arterial thrombosis [1]. Since the early 1990's mild hyperhomocysteinemia has been identified as a risk factor for venous thrombosis, as well as arterial thrombotic events [2-5]. As vitamin B6, vitamin B12 and folic acid can decrease homocysteine plasma levels [6], this has led to a straightforward view that treatment with these supplements would reduce the risk of venous and arterial thrombosis. Recently, however, large prospective clinical trials that reported on the effects of lowering homocysteine levels with vitamin B6, vitamin B12 or folic acid therapy showed no improved clinical outcome in patients with prior ischemic stroke [7], myocardial infarction [8] or venous thrombosis [9]. Other clinical trials to obtain evidence that this treatment of hyperhomocysteinemia is clinically beneficial are still awaited.

We hypothesize that the risk of venous thrombosis in hyperhomocysteinemic subjects predominantly depends on coexistence with other thrombophilic defects. Hyperhomocysteinemia itself might be an epiphenomenon rather than a risk factor. Alternatively, the negative results of above mentioned clinical trials may be due to a low absolute risk of thrombosis in these subjects. Data on the absolute risk of thrombosis associated with hyperhomocysteinemia has not been reported previously. Therefore, we performed a large retrospective family cohort study to assess the absolute risk of venous and arterial thrombosis in hyperhomocysteinemic subjects, and considering the multicausality of thrombosis.

## METHODS

### Subjects

Between January 2000 and January 2004 first-degree relatives, who were 15 years of age or older, of consecutive patients (proband) with documented venous thrombosis or any documented arterial thrombotic event before the age of 50 years and hyperhomocysteinemia were enrolled in the study. Patients were referred by their general physicians to the thrombosis outpatient clinics of three participating university hospitals, either to confirm clinically suspected venous thrombosis, or to evaluate symptomatic premature atherosclerosis. Relatives were enrolled after

informed consent was obtained. Detailed information about previous episodes of venous and arterial thrombosis, exposure to exogenous risk factors for thrombosis and anticoagulant treatment was collected by physicians at the outpatient clinics, using a validated questionnaire [10] and reviewing medical records. Clinical data was collected prior to laboratory testing. Additional thrombophilia tests included deficiencies of antithrombin, protein C and protein S, factor V Leiden, the prothrombin G20210A mutation, and increased plasma levels of factor VIII. The study was approved by the institutional review boards of the participating hospitals.

### **Laboratory studies**

Levels of homocysteine were measured by high-performance liquid chromatography after overnight fasting and 6 h after an oral methionine-load of 0.1 g/kg bodyweight, during a low-protein diet (minimum, 5 g and maximum, 10 g) [11]. Hyperhomocysteinemia was defined as a fasting homocysteine level  $> 18 \mu\text{mol/L}$  or as a postload homocysteine level  $> 48 \mu\text{mol/L}$ , which are above the 95<sup>th</sup> percentile of normal in the Dutch population [12]. Activity of antithrombin (Coatest, Chromogenix, Mölndal, Sweden) and protein C (Berichrom Protein C; Behring, Marburg, Germany) were measured by chromogenic substrate assays, protein C and protein S antigen levels by Enzyme Linked Immuno Sorbent Assay (ELISA) (DAKO, Glostrup, Denmark). Antithrombin deficiency was defined by decreased levels of antithrombin activity ( $< 65 \text{ IU/dL}$ ), protein C deficiency by decreased levels of either protein C antigen ( $< 65 \text{ IU/dL}$ ) and/or activity ( $< 65 \text{ IU/dL}$ ) and protein S deficiency by decreased total protein S antigen levels ( $< 65 \text{ IU/dL}$ ), corresponding with plasma levels below the under limit of their normal ranges. Factor V Leiden and prothrombin G20210A were demonstrated by polymerase chain reactions [13,14]. Factor VIII:C was measured by one-stage clotting assays (Amelung GmbH, Lemgo, Germany) and was increased at levels above the 75<sup>th</sup> percentile in the Dutch population ( $150 \text{ IU/dL}$ ) [15]. These levels have been identified to give an increased risk of both venous and arterial thrombosis [15,16]. If relatives were on long-term anticoagulant treatment with acenocoumarol, a short acting vitamin K antagonist, blood samples were taken after treatment had been interrupted for at least two weeks; in the meantime nadroparin was given subcutaneously.

**Definitions**

Venous thrombosis was considered established if deep vein thrombosis was confirmed by compression ultrasound or venography, and pulmonary embolism by ventilation and perfusion lung scanning, spiral CT scanning or pulmonary angiography, or when the patient had received full dose heparin and a vitamin K antagonist for at least 3 months without objective testing at a time when these techniques were not yet available. Secondary venous thrombosis was defined if it had occurred at or within 3 months after exposure to exogenous risk factors including surgery, trauma, immobilization for more than 7 days, pregnancy, post-delivery period, the use of oral contraceptives or hormonal replacement therapy, or malignancy. In the absence of these risk factors venous thrombosis was considered to be primary.

Coronary and peripheral arterial disease had to be symptomatic and angiographically proven, while myocardial infarction was diagnosed according to clinical, enzymatic and electrocardiographic criteria. Ischemic stroke was defined as the onset of rapidly developing symptoms and signs of loss of cerebral function which lasted at least 24h and had an apparent vascular cause, as demonstrated by computed tomography (CT) or magnetic resonance imaging (MRI). If a cerebral event completely resolved within 24h without cerebral lesions at scanning, it was classified as transient ischemic attack (TIA). Risk factors for atherosclerosis included known diabetes mellitus, hyperlipidemia, hypertension and active smoking.

**Statistical analysis**

We analyzed the absolute risk of first venous and arterial thrombosis in relatives, comparing those who did or did not have hyperhomocysteinemia. Probands were excluded from analysis to avoid bias. Observation time was defined as the period from the age of 15 years until the first thrombotic episode or until the end of the observation period, i.e. the day when hyperhomocysteinemia was established or withdrawn. Annual incidences of venous and arterial thrombosis were calculated by dividing the number of events by the number of observation years. When calculating the annual incidence of venous thrombosis, the occurrence of arterial thrombosis was ignored and vice versa. Assuming an annual incidence of thrombosis of 0.5% in relatives with hyperhomocysteinemia, the sample size was calculated to be 15000 observation years to demonstrate a difference, compared to normohomocysteinemic relatives with 95% probability.

**Table 1.** Characteristics of 478 relatives of probands with hyperhomocysteinemia

	Hyperhomocysteinaemia		
	Present (n=183)	Absent (n=295)	P
Male	84 (46)	136 (46)	1.00
Homocysteine levels (µmol/L)			
Fasting	16.8 (8.6-91.0)	11.3 (0.8-17.9)	
Loading	61.0 (11.5-248.9)	36.5 (11.8-47.9)	
Age at enrollment (years)	48 (15-82)	44 (15-80)	< 0.001
15-30 years	17 (9)	59 (20)	
30-45 years	59 (32)	98 (34)	
45-60 years	60 (33)	83 (28)	
Older than 60 years	47 (26)	53 (18)	
Concomitant thrombophilic defects			
Antithrombin deficiency	2 (1)	2 (1)	0.64
Protein C deficiency	2 (1)	7 (2)	0.49
Protein S deficiency	5 (3)	4 (1)	0.31
Factor V Leiden	25 (14)	31 (11)	0.31
Prothrombin G20210A	5 (3)	8 (3)	1.00
Factor VIII > 150 IU/dL	48 (27)	67 (23)	0.38
Venous thrombosis			
Age at onset (years)	51 (26-61)	41 (17-70)	0.65
Classification			
Deep venous thrombosis	5 (50)	7 (78)	
Pulmonary embolism	5 (50)	2 (22)	
Primary	6 (60)	5 (56)	0.74
Secondary to			
Surgery, trauma, immobilization	2 (20)	3 (33)	
Oral contraception	1 (10)	0 (0)	
Pregnancy, puerperium	1 (10)	1 (11)	
Malignancy	0 (0)	0 (0)	
Arterial thrombosis			
Age at onset (years)	63 (36-80)	56 (25-79)	0.18
Classification			
Myocardial infarction	9 (43)	12 (60)	
Ischaemic stroke or TIA	7 (33)	4 (20)	
Peripheral arterial thrombotic event	5 (24)	4 (20)	
Risk factors			
Active smoking	73 (40)	126 (43)	0.57
Hyperlipidemia	36 (20)	40 (14)	0.09
Hypertension	49 (27)	51 (17)	0.02
Diabetes mellitus	10 (5)	13 (4)	0.66

Continuous variables denoted as median (range), categorical variables as number (%).

With a Cox proportional hazards regression model we adjusted relative risks for clinically relevant covariates, including age and sex, and for arterial thrombosis also for hypertension, hyperlipidemia, active smoking, and diabetes mellitus. To account for clustering within families, outcome rates were analyzed by using random-effects logistic regression with Gaussian distribution in Stata, version 9.1 (Stata Corp., College Station, Texas).

Continuous variables were expressed as median values and ranges; categorical data as counts and percentages. Differences between groups were evaluated by the Student t test or Mann-Whitney U test, depending on the normality of data for continuous data and by Fisher exact test for categorical data. A two-tailed p-value of less than 0.05 indicated statistical significance. The 95% confidence intervals (95% CI) around the incidence rates were calculated under the Poisson distribution assumption.

Statistical analyses were performed using SAS software, version 9.1 (SAS-Institute inc., Cary, North Carolina).

## RESULTS

Our family cohort contained 170 probands, with a total number of 1125 relatives, identified by pedigree analysis, who were 15 years of age or older. Of these relatives, 242 had died before the start of this study. Another 382 relatives did not participate because of various reasons, including refusal or inability to give informed consent and residence outside the Netherlands. Twenty three relatives

**Table 2.** Annual incidences and relative risks of venous and arterial thrombosis in 478 relatives

	Observation Years	Relatives with event	Annual incidence,% (95% CI)	Crude relative risk (95% CI)	Adjusted* relative risk (95% CI)
<b>Venous thrombosis</b>					
Normohomocysteinemia	8413	9	0.11 (0.05-0.20)	Reference	Reference
Hyperhomocysteinemia	6172	10	0.16 (0.08-0.30)	1.5 (0.7-3.8)	1.6 (0.6-4.5)
<b>Arterial thrombosis</b>					
Normohomocysteinemia	8418	20	0.24 (0.15-0.37)	Reference	Reference
Hyperhomocysteinemia	6132	21	0.34 (0.21-0.52)	1.4 (0.8-2.9)	1.5 (0.6-3.5)

\* Adjusted for clustering in families, age and sex, and, for arterial thrombosis, also for hypertension, hyperlipidemia, active smoking, and diabetes mellitus.

were not evaluable because of missing laboratory data. The remaining 478 relatives were analyzed. Median follow up time was 30 years (range, 0-67). Their clinical characteristics are summarized in Table 1. Males and females were equally distributed. The median age at enrollment in hyperhomocysteinemic relatives was 48 years (range, 15-82) and was higher than in normohomocysteinemic relatives (44 years; range 15-80,  $P < 0.001$ ). Concomitant thrombophilic defects were demonstrated in 42% of relatives with hyperhomocysteinemia and in 34% of relatives with normohomocysteinemia ( $P=0.08$ ). Of these, factor V Leiden was more often demonstrated in both groups than expected from its prevalence in the general population (i.e. 5%) [17]. Venous thrombosis had occurred in ten hyperhomocysteinemic relatives (5%) and in nine normohomocysteinemic relatives (3%,  $P=0.23$ ). Median age at onset of the first episode of venous thrombosis was 51 years (range, 26-61) in relatives with hyperhomocysteinemia and 41 years (range, 17-70) in relatives with normohomocysteinemia ( $P=0.65$ ). First arterial thrombotic events were documented in 21 relatives (11%) with hyperhomocysteinemia and in 20 relatives (7%) with normohomocysteinemia ( $P=0.09$ ). Hypertension was more common in hyperhomocysteinemic relatives (27%) versus normohomocysteinemic relatives (17%,  $P=0.02$ ). Hyperlipidemia tended to be more common in hyperhomocysteinemic relatives (20%) than in normohomocysteinemic relatives (14%,  $P=0.09$ ). Other characteristics were not statistically different in both groups.

Annual incidences of venous thrombosis were 0.16% (95% CI, 0.05-0.20) in relatives with hyperhomocysteinemia and 0.11% (95% CI, 0.05-0.20) in relatives without hyperhomocysteinemia; adjusted relative risk 1.6 (95% CI, 0.6-4.5) (Table 2). In hyperhomocysteinemic relatives, annual incidence of arterial thrombosis was 0.34% (95% CI, 0.21-0.52) compared to 0.24% (95% CI, 0.15-0.37) in normohomocysteinemic relatives; adjusted relative risk 1.6 (95% CI, 0.6-4.5).

In Table 3, annual incidences of venous and arterial thrombosis are shown for both fasting and methionine-loading homocysteine levels, each stratified into quartiles. No clear relationship between annual incidence of venous thrombosis and height of fasting and methionine-loading homocysteine levels, respectively, was seen. The lowest annual incidence of venous thrombosis was observed in relatives with fasting homocysteine levels lower than 10.5  $\mu\text{mol/L}$ ; 0.06% (95% CI, 0.08-0.23). The highest annual incidence of venous thrombosis was observed in relatives with methionine-loading homocysteine levels above 55.3  $\mu\text{mol/L}$ ; 0.22% (95% CI, 0.10-0.43). For arterial thrombosis, there was a tendency of increasing annual incidences with increasing fasting and methionine-loading homocysteine levels,

**Table 3.** Annual incidences of venous and arterial thrombosis in relatives of probands hyperhomocysteinemia, stratified in quartiles of homocysteine levels

Homocysteine levels ( $\mu\text{mol/L}$ )		Relatives with event	Annual incidence, % (95% CI)
<b>Venous thrombosis</b>			
Fasting	< 10.5	2	0.06 (0.08-0.23)
	10.5-12.9	8	0.21 (0.09-0.42)
	12.9-16.4	3	0.07 (0.02-0.22)
	>16.4	6	0.15 (0.06-0.33)
Loading	< 34.8	2	0.06 (0.01-0.22)
	34.8-42.0	6	0.19 (0.07-0.41)
	42.0-55.3	2	0.05 (0.01-0.18)
	> 55.3	9	0.22 (0.10-0.43)
<b>Arterial Thrombosis</b>			
Fasting	< 10.5	5	0.16 (0.05-0.37)
	10.5-12.9	9	0.23 (0.11-0.44)
	12.9-16.4	12	0.30 (0.16-0.52)
	>16.4	16	0.41 (0.23-0.66)
Loading	< 34.8	8	0.25 (0.11-0.49)
	34.8-42.0	7	0.22 (0.09-0.45)
	42.0-55.3	12	0.31 (0.16-0.55)
	> 55.3	12	0.30 (0.15-0.52)

There were no statistically significant differences between quartiles.

**Table 4.** The contribution of concomitance of thrombophilic factors to the risk of venous thrombosis in relatives of probands with hyperhomocysteinemia

Number of concomitant thrombophilic defects*	Observation Years	Relatives with event	Annual incidence,% (95% CI)	Crude relative risk (95% CI)
<b>Normohomocysteinemia</b>				
0	5438	2	0.04 (0.005-0.13)	Reference
1	2280	4	0.18 (0.05-0.45)	4.8 (0.8-37.2)
$\geq 2$	594	3	0.51 (0.10-1.48)	13.7 (2.0-115.5)
<b>Hyperhomocysteinemia</b>				
0	3008	3	0.10 (0.02-0.29)	2.7 (0.4-22.8)
1	2610	4	0.15 (0.04-0.39)	4.2 (0.7-32.5)
$\geq 2$	407	3	0.74 (0.15-2.15)	20.0 (3.0-168.5)

\* Including antithrombin protein C and protein S deficiency, factor V Leiden, prothrombin G20210A, and elevated factor VIII levels.

ranging from 0.16% (95% CI, 0.05-0.37) in relatives with fasting homocysteine levels lower than 10.5  $\mu\text{mol/L}$ , to 0.41% (95% CI, 0.23-0.66) in relatives with fasting homocysteine levels above 16.4  $\mu\text{mol/L}$  (crude relative risk 2.6; 95% CI, 0.9-6.6), and from 0.25% (95% CI, 0.11-0.49) to 0.30% (95% CI, 0.15-0.52) in relatives with methionine-loading levels lower than 34.8  $\mu\text{mol/L}$  and higher than 55.3  $\mu\text{mol/L}$ , respectively (crude relative risk 1.2; 95% CI, 0.5-3.1).

Annual incidence of venous thrombosis was 0.10% (95% CI, 0.02-0.29) in relatives who had hyperhomocysteinemia without concomitant thrombophilic defects, 0.15% (95% CI, 0.04-0.39) in combination with one thrombophilic defect, and 0.74% (95% CI, 0.15-2.15) in the presence of two or more concomitant defects (Table 4). In normohomocysteinemic relatives, annual incidences of venous thrombosis were 0.04% (95% CI, 0.005-0.13), 0.18% (95% CI, 0.05-0.45) and 0.51% (95% CI, 0.10-1.48), respectively. Only the concomitance of two or more thrombophilic defects was associated with an increased risk of venous thrombosis in both hyperhomocysteinemic relatives (crude relative risk 20.0; 95% CI, 3.0-168.5) and normohomocysteinemic relatives (crude relative risk 13.7; 95% CI, 2.0-115.5). Differences between hyperhomocysteinemic and normohomocysteinemic relatives were not significant, comparing relatives with one thrombophilic defect (crude relative risk 0.9; 95% CI, 0.2-3.5) or with two or more thrombophilic defects (crude relative risk 1.5; 95% CI, 0.3-7.2).

## DISCUSSION

Although the risk of venous thrombosis in hyperhomocysteinemic relatives was 1.6 fold higher than in normohomocysteinemic relatives, the difference was not statistically significant. More important, the absolute risk in hyperhomocysteinemia was low and comparable with the annual incidence of venous thrombosis in the general population (i.e. 0.1-0.3%) [18,19]. Only concomitance of two or more thrombophilic defects revealed an increased absolute risk of venous thrombosis in hyperhomocysteinemic relatives, but a similar association was demonstrated in normohomocysteinemic relatives. Our findings seem inconsistent with those from a previous case-control study that suggested that hyperhomocysteinemia was an independent risk factor for venous thrombosis [20]. However, in that study hyperhomocysteinemic patients who had an episode of venous thrombosis were in 4% of the cases also antithrombin deficient, in 6% protein C deficient, and in 3% protein

S deficient, which is far more prevalent than reported in the general population (i.e. 0.02-0.4%, each) [21]. Moreover, hyperhomocysteinemia was associated with factor V Leiden, but this result was not further analyzed because of small numbers. Thus, the increased risk of venous thrombosis in that study was possibly influenced by concomitance of these thrombophilic defects. Patients were not screened for other thrombophilic defects such as prothrombin G20210A and elevated levels of factor VIII, as we did in our study.

Our observed adjusted relative risk for venous thrombosis in relatives with hyperhomocysteinemia of 1.6 is lower than reported in previous studies. Two meta-analyses found overall odds ratios of 2.5 and 2.9 for the risk of venous thrombosis in hyperhomocysteinemic subjects, respectively, which were statistically significant [2,5]. However, these studies did not exclude publication bias by funnel plot analysis, which could have resulted in a higher relative risk compared to our study. Furthermore, although both studies used a clear definition for hyperhomocysteinemia (i.e. homocysteine levels above the 95th percentile or mean plus two standard deviations calculated from the distribution in control groups), fasting hyperhomocysteinemia (when mentioned) ranged as homocysteine levels between  $> 14.1 \mu\text{mol/L}$  and  $> 22.2 \mu\text{mol/L}$  and methionine-loading hyperhomocysteinemia between  $> 29.9 \mu\text{mol/L}$  and  $> 79.0 \mu\text{mol/L}$ . Although this was insurmountable, as no definition has ever been postulated of which homocysteine levels are considered as hyperhomocysteinemia, a meta-analysis may not be a proper statistical tool to identify hyperhomocysteinemia as a risk factor for venous or arterial thrombosis. In our study, we had the same difficulty to define a cut-off level for hyperhomocysteinemia. Therefore, we stratified relatives into quartiles according to increasing homocysteine levels. For venous thrombosis, we did not find a relationship between annual incidence and increasing homocysteine levels. Apparently, there is no sharp cut-off point for homocysteine levels to identify subjects at risk of venous thrombosis. On the other hand, for arterial thrombosis we did see an increasing annual incidence with increasing fasting or methionine-loading homocysteine levels. Nevertheless, the annual incidence of arterial thrombosis remained low, even in the highest quartiles of fasting and methionine-loading homocysteine levels, i.e. 0.41 and 0.30%, respectively. These annual incidences are comparable with the annual incidence of arterial thrombosis in the Framingham study (i.e. 0.1-0.4%) [22]. Thus, the absolute risk of arterial thrombosis at highest homocysteine levels did not exceed the risk in the normal population. Although there is evidence that homocysteine influences the

development of atherosclerosis [23-25], this effect may be too small in subjects with mild hyperhomocysteinemia to result in a clinically relevant increase in risk of arterial thrombotic events. As a consequence of the low absolute risk of arterial thrombosis, as well as venous thrombosis, it will be difficult to demonstrate a benefit of homocysteine lowering treatment with vitamin B6, vitamin B12 and folic acid on clinical outcome [7-9]. Possible clinical implications of our findings may be that there is no need to screen patients with venous or arterial thrombosis for mild hyperhomocysteinemia, nor the relatives of patients in whom mild hyperhomocysteinemia has been demonstrated. Homocysteine lowering treatment seems not to be justified, as the potential absolute risk reduction with this treatment is small and might, according to other studies, even be harmful [8,27].

Some aspects of our study warrant comment. First, the centers that included probands were all university hospitals, implying a potential for bias. Also, due to the retrospective design of the study, a number of relatives had venous thrombosis which were not established by objective techniques because these were not available yet. This implies the possibility of overtreatment which might have overestimated our annual incidences. However, the annual incidences of both venous and arterial thrombosis did not exceed the incidences in the general population, making such bias less likely [18,19,22]. Second, the response rate of living relatives was not optimal, despite maximum efforts to include as many first degree relatives as possible. This does clearly demonstrate the intricacy of performing homocysteine tests in which individuals have to return to a clinic after fasting in a procedure that takes approximately 6 hours, whereas the methionine-loading test may have side effects, such as nausea and malaise [28]. As it is likely that asymptomatic subjects are less willing to undergo these tests, this would have resulted in an overestimation of annual incidences of thrombosis in our study cohort. Still, annual incidences of venous and arterial thrombosis in hyperhomocysteinemic subjects were low and comparable to normohomocysteinemic subjects. Third, we assessed the effects of the number of concomitant thrombophilic defects, assuming that the venous thrombotic potency of individual thrombophilic defects was equal. It might have been more appropriate to weigh defects according to predefined risk estimates, but this could not be done as many combinations were found and the numbers of subjects with specific combinations were too small. Fourth, factor V Leiden was more often observed in the study cohort than in the general population. Because we studied first degree relatives of patients with either venous thrombosis or premature atherosclerosis with hyperhomocysteinemia, we

might hereby have introduced selection bias. Relatives could be *a priori* at higher risk of venous thrombosis because factor V Leiden is more frequently found in patients with venous thrombosis [21]. For this reason, we used a random-effects model to adjust for clustering in families. Therefore, our results can be extrapolated to the general population. Finally, it could be argued that our study was underpowered to detect differences in risk of thrombosis that were observed. In our sample size calculation, annual incidences of venous and arterial thrombosis in hyperhomocysteinemic subjects were presumed to amount 0.5% each. As these were actually lower and even were within the ranges of the normal population, a larger size of the study population might reveal statistical significance, but differences would not become clinically relevant.

We conclude that the absolute risk of venous and arterial thrombosis in subjects with hyperhomocysteinemia is low. For venous thrombosis, concomitant thrombophilic defects are probably main determinants of this risk, rather than hyperhomocysteinemia itself.

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# Chapter 3

## **Methionine-loading and random homocysteine tests have no added value in risk assessment for venous and arterial thrombosis**

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**ABSTRACT**

**Background:** Hyperhomocysteinemia is a risk factor for venous and arterial thrombosis. To identify subjects at risk of thrombosis, measurements of fasting and methionine-loading homocysteine levels are usually recommended. Alternatively, random homocysteine measurements may simplify the procedure. In this approach, random levels  $< 10$  and  $> 20$   $\mu\text{mol/L}$  indicate normohomocysteinemia and hyperhomocysteinemia, respectively, while consecutive fasting and methionine-loading tests remain required at levels 10-20  $\mu\text{mol/L}$ . We performed a study to determine the most accurate and suitable diagnostic strategy in a large cohort of families with hereditary (index) deficiencies of protein S, protein C or antithrombin.

**Methods:** Random, fasting and methionine-loading homocysteine samples were measured in 713 relatives. According to predefined cut-off levels hyperhomocysteinemic and normohomocysteinemic relatives were identified and their absolute risks of thrombosis were compared.

**Results:** Relatives with random homocysteine levels  $> 20$   $\mu\text{mol/L}$  were not at risk of venous or arterial thrombosis compared to relatives with levels  $< 10$   $\mu\text{mol/L}$ ; relative risk (RR) 0.9 (95% CI, 0.4-2.3) and 1.7 (95% CI, 0.5-5.7), respectively. Random levels between 10-20  $\mu\text{mol/L}$  were measured in 63% of relatives. Fasting hyperhomocysteinemia was associated with an increased risk of both venous thrombosis; RR 2.6 (95% CI, 1.3-4.8) and arterial thrombosis; RR 3.7 (95% CI, 1.5-8.4). Relatives with normal fasting homocysteine levels, but methionine-loading hyperhomocysteinemia were not at risk; RR 0.8 (95% CI, 0.2-1.9) for venous thrombosis and 1.1 (95% CI, 0.2-3.9) for arterial thrombosis. The relative risk estimates were independent of index deficiencies.

**Conclusion:** Only fasting homocysteine levels are sufficient to identify hyperhomocysteinemic subjects at risk of thrombosis.

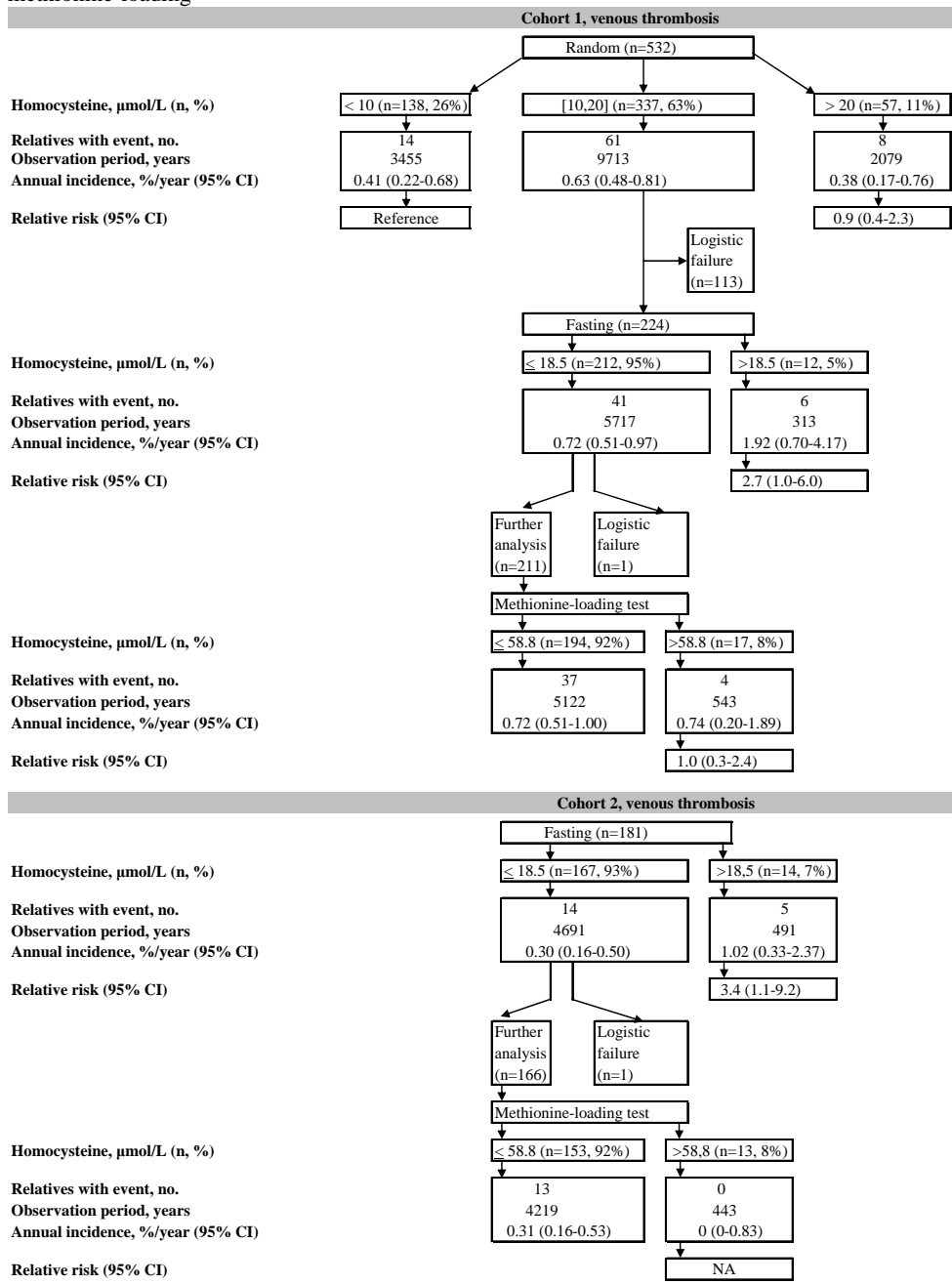
## INTRODUCTION

Hyperhomocysteinemia (HHcy) is a risk factor for both venous and arterial thrombosis [1-2]. To identify subjects at risk of thrombosis, measurements of fasting and methionine-loading (postload) homocysteine (Hcy) levels are usually recommended. Alternatively, random-Hcy measurements may simplify the procedure. In this approach, random-Hcy levels  $<10$  and  $>20$   $\mu\text{mol/L}$  indicate normohomocysteinemia (NHcy) and HHcy, respectively, while succeeding measurements of fasting and postload-Hcy remain required at levels 10-20  $\mu\text{mol/L}$ . Previous reports did show an increased risk of venous and arterial thrombosis in subjects with postload-HHcy [3-5]. However, both fasting and postload-Hcy were measured, independent whether subjects had fasting-HHcy or not. We performed a retrospective study to accurately identify HHcy, associated with an increased risk of venous or arterial thrombosis, by comparing different diagnostic strategies. Postload-Hcy was not measured when relatives already had fasting HHcy because it would not change their classification as HHcy.

## METHODS

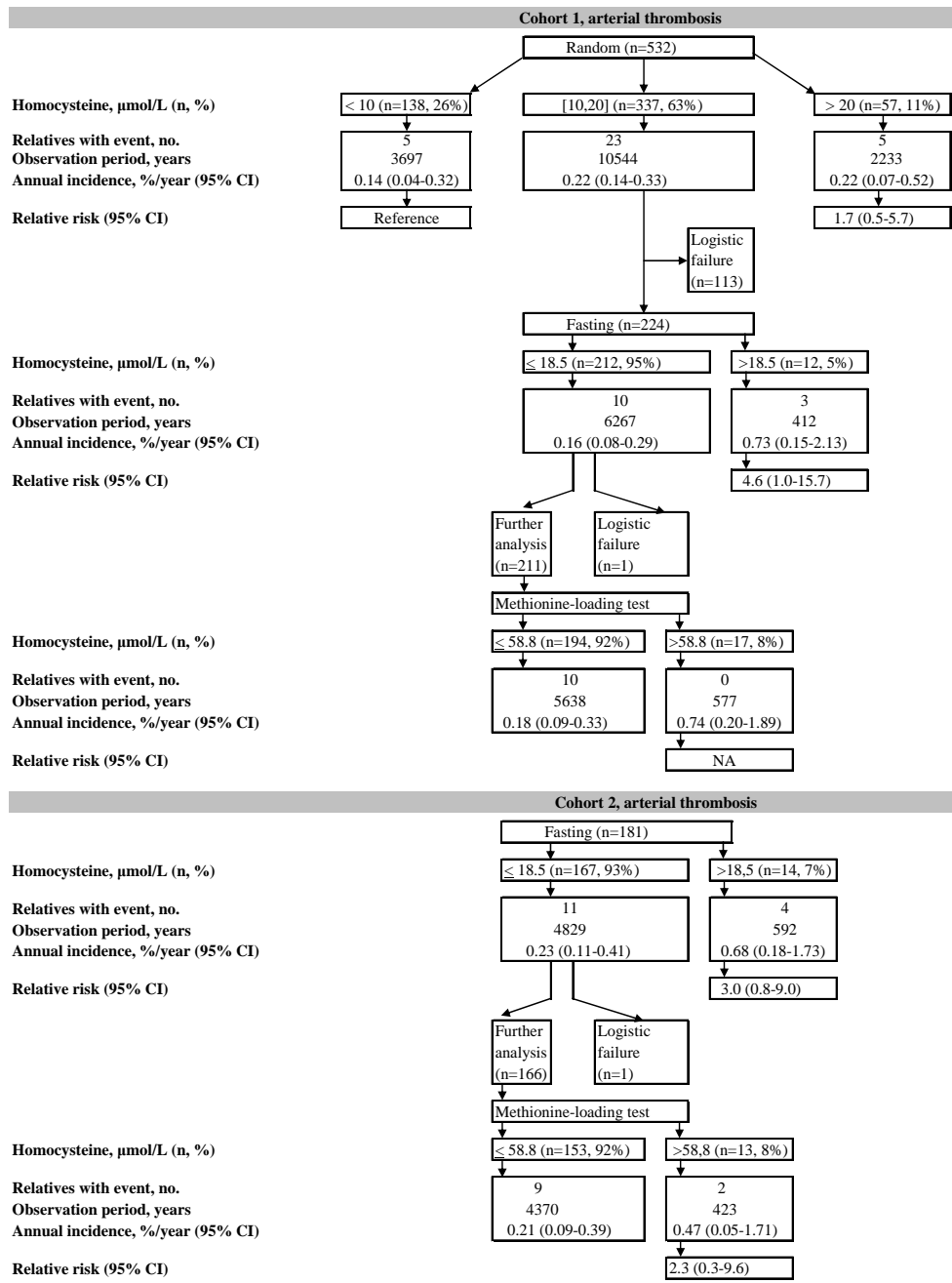
Subjects were derived from a family study, which has been described in detail previously [6,7]. Briefly, 713 relatives (15 years or older, response rate 90%) were enrolled to evaluate the concept of multicausality of venous thrombosis. Proband were consecutive patients with documented venous thrombosis, in whom an inherited antithrombin, protein C or protein S (index) deficiency was recognized. Relatives were tested for all currently known thrombophilic defects and HHcy in addition to these deficiencies. Clinical data and blood-sampling was collected at date of enrollment. Follow-up time started at age 15 years and ended at the date of enrollment or at the date of thrombosis. Therefore, our study was retrospective and relatives were not treated for HHcy with B-vitamins. According to predefined cut-off levels, HHcy and NHcy relatives were identified and their absolute risks of thrombosis were compared.

**Figure 1.** Cohort 1 (relatives tested for hyperhomocysteinemia after July 2001) and cohort 2 (relatives tested for hyperhomocysteinemia before July 2001), showing their distribution according to their homocysteine levels after application of random cut-off levels, after fasting, and after methionine-loading



NA denotes not applicable.

Figure 1. continued



NA denotes not applicable.

## RESULTS

Women and men were equally distributed. Median age at enrollment (i.e. end of study) was 42 years (range, 15-92). Venous thrombosis had occurred in 102 relatives (14%) at a median age of 31 years (range, 16-80). Arterial thrombosis had occurred in 48 relatives (7%) at a median age of 57 years (range, 26-80). Median time interval between venous or arterial thrombosis and homocysteine measurement was 14 years (range, 0-47) and 5 years (range, 0-37), respectively. Median random, fasting and postload-Hcy levels were 11.9  $\mu\text{mol/L}$  (range, 5.5-62.7), 12.3  $\mu\text{mol/L}$  (range, 5.6-42.7) and 38.3  $\mu\text{mol/L}$  (range, 17.5-110.7), respectively. Since its implementation in July 2001, random-Hcy testing was performed in 538 relatives (Figure 1). Another 181 relatives were tested for HHcy with only fasting and postload-Hcy measurements (before July 2001). Comparing relatives with random-Hcy  $>20 \mu\text{mol/L}$  versus  $< 10 \mu\text{mol/L}$ , annual incidences of venous thrombosis were 0.38% (95% CI, 0.17-0.76) and 0.41% (95% CI, 0.22-0.68), respectively; relative risk (RR) 0.9; 95% CI, 0.4-2.3. For arterial thrombosis, these were 0.22% (95% CI, 0.07-0.52) versus 0.14% (95% CI, 0.04-0.32); RR 1.7; 95% CI, 0.5-5.7. Relatives with random-Hcy between 10 and 20  $\mu\text{mol/L}$  (63%) had successively fasting and postload-Hcy tested, as had relatives who were screened before July 2001. Of these, 113 were lost due to logistic problems. Of the remaining 405 relatives, 26 (6%) had fasting HHcy ( $>18.5 \mu\text{mol/L}$ ) and 30 (8%) postload HHcy ( $>58.8 \mu\text{mol/L}$ ). Antithrombin, protein C and protein S deficiencies and other thrombophilic defects, and classical risk factors for arterial thrombosis (diabetes mellitus, hyperlipidemia, hypertension and smoking) were equally distributed over NHcy relatives and relatives with fasting/postload HHcy.

In Table 1, annual incidences of venous and arterial thrombosis in relatives with NHcy and HHcy are shown. Fasting HHcy revealed an increased risk of venous thrombosis (RR 2.6; 95% CI, 1.3-4.8) and arterial thrombosis (RR 3.7; 95% CI, 1.5-8.4). In contrast, relatives with NHcy and postload HHcy showed no differences in risks of venous or arterial thrombosis; RR 0.8 (95% CI, 0.2-1.9) and 1.1 (95% CI, 0.2-3.9), respectively. Because our study cohort was built up with a high risk thrombophilic population, we repeated our analysis after excluding all deficient relatives. The annual incidence of venous thrombosis in non-deficient relatives without fasting HHcy was 0.20% (95% CI, 0.12-0.33), which increased to 1.13% (95% CI, 0.45-2.33) in non-deficient relatives with fasting HHcy; RR 5.5 (95% CI, 2.1-13.1). In non-deficient relatives with postload HHcy, the annual inci-

**Table 1.** Annual incidences of venous and arterial thrombosis in relatives with hyperhomocysteinemia versus normohomocysteinemic relatives

	<b>Observation years*</b>	<b>Relatives with event</b>	<b>Incidence/year,% (95% CI)</b>	<b>Relative risk (95% CI)</b>
<b>Venous thrombosis</b>				
Fasting homocysteine				
No hyperhomocysteinemia	10408	55	0.53 (0.40-0.69)	Reference
Hyperhomocysteinemia	804	11	1.37 (0.68-2.45)	2.6 (1.3-4.8)†
Methionine-loading test§				
No hyperhomocysteinemia	9341	50	0.54 (0.40-0.71)	Reference
Hyperhomocysteinemia	986	4	0.41 (0.11-1.04)	0.8 (0.2-1.9)**
<b>Arterial thrombosis</b>				
Fasting homocysteine				
No hyperhomocysteinemia	11096	21	0.19 (0.12-0.29)	Reference
Hyperhomocysteinemia	1004	7	0.70 (0.28-1.44)	3.7 (1.5-8.4)
Methionine-loading test§				
No hyperhomocysteinemia	10008	19	0.19 (0.11-0.30)	Reference
Hyperhomocysteinemia	1000	2	0.20 (0.02-0.72)	1.1 (0.2-3.9)

\* Period from age 15 years or older until the first episode of thrombosis or the end of follow-up.

† After exclusion of relatives with antithrombin, protein C or protein S deficiency, the relative risk was 5.5 (95% CI, 2.1-13.1).

§ Methionine-loading test performed in relatives with no fasting hyperhomocysteinemia.

\*\* After exclusion of relatives with antithrombin, protein C or protein S deficiency, the relative risk was 1.4 (95% CI, 0.2-5.5).

dence of venous thrombosis was 0.27% (95% CI, 0.03-0.97), compared to 0.19% (95% CI, 0.10-0.31) in non-deficient relatives without postload HHcy; RR 1.4 (95% CI, 0.2-5.5).

## DISCUSSION

Our study shows that fasting Hcy levels are sufficient to identify subjects at risk of venous or arterial thrombosis, due to HHcy, as only fasting HHcy was a risk factor for thrombosis. Postload HHcy as well as random Hcy levels of >20  $\mu\text{mol/L}$  were not associated with venous or arterial thrombosis. Although our study cohort was built up with a high-risk thrombophilic population, we demonstrated that fasting HHcy remained a risk factor for venous thrombosis after exclusion of relatives with deficiencies of antithrombin, protein C or protein S, which is in accordance with another study [8], while methionine-loading HHcy was still not a risk factor.

Moreover, the remaining annual incidence of venous thrombosis in non-deficient relatives without fasting HHcy (0.20%) or postload HHcy (0.27%) was comparable to the general population, i.e. 0.1-0.3% per year [9,10]. Therefore, our results can be extrapolated to the general population.

Some methodological aspects of our study warrant comment. First, we incorporated two different strategies in our analysis. This, however, was insurmountable as facts and recommendations of homocysteine measurements have changed over the years, making it difficult for us to keep up with one strategy. One could oppose that due to the random-Hcy testing strategy we missed all relatives who had a random-Hcy level of  $<10 \mu\text{mol/L}$  or  $>20 \mu\text{mol/L}$  when calculating fasting/postload HHcy risk estimates. Bias, however, is unlikely as these cut-off values did not enable us to distinguish an increased and decreased risk of thrombosis. Second, as our study was retrospective, we were unable to measure homocysteine at time of thrombosis. This has probably not influenced our results as we found no correlation between age and Hcy levels at time of enrollment ( $r^2 = 0.025$  for random-Hcy,  $r^2 = 0.012$  for fasting-Hcy and  $r^2 = 0.049$  for postload-Hcy, respectively, data not further shown). The retrospective design of our study should also be addressed to relatives who were treated with heparin and vitamin K antagonists for more than three months at a time when there was no objective testing available yet. These were classified as having experienced venous thrombosis, which might have overestimated our annual incidences. Third, despite maximum efforts, many relatives were lost to perform a methionine-loading test after we obtained random homocysteine. This does, however, clearly demonstrate the intricacy of performing a postload-Hcy test for which individuals have to return after fasting in a procedure that takes approximately 6 hours, whereas the result would not have clinical implications. This might have influenced our results, as it is likely that subjects without a history of thrombosis were less willing to undergo the time-consuming postload-Hcy test with its possible side effects (nausea and malaise). Thus, one would expect a higher risk of venous thrombosis in hyperhomocysteinemic relatives. In fact, annual incidence of venous thrombosis was 0.38% (95% CI, 0.20-0.63) in 113 relatives who were lost to follow-up after a random test of Hcy. Nevertheless, postload HHcy relatives were still not at higher risk of venous thrombosis. A final point of thought is whether fasting HHcy is relevant in thrombophilia screening. Although our study showed that fasting HHcy is a risk factor for venous and arterial thrombosis, the unanswered question remains whether mildly elevated Hcy levels are truly causal of thrombosis or are either a

consequence or just a marker of other diseases. B-vitamins may decrease homocysteine levels, but up till date clinical trials that reported about the effect of lowering Hcy with vitamin therapy, showed no improved clinical outcome in patients with arterial thrombosis [11,12] or venous thrombosis [13]. However, questions about this matter lay beyond the scope of our study. We aimed to determine which tests would be clinical relevant and feasible to identify Hcy levels associated with an increased risk of thrombosis. We conclude that only fasting-Hcy suffices to screen for HHcy.

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# Chapter 4

## **The risk of venous and arterial thrombosis in hyperhomocysteinemic subjects may be a result of elevated factor VIII levels**

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**ABSTRACT**

In a large retrospective study of thrombophilic families, we analyzed 405 relatives of patients, hypothesizing that hyperhomocysteinemia and elevated factor VIII levels are closely related. Median factor VIII levels in hyperhomocysteinemic relatives were 169 IU/dL, compared to 136 IU/dL in normohomocysteinemic relatives ( $P=0.007$ ), and were more often elevated ( $>150$  IU/dL;  $P=0.006$ ). Hyperhomocysteinemia was associated with an increased risk of venous and arterial thrombosis; relative risk (RR) 2.6 (95% CI, 1.3-4.8) and 3.7 (95% CI, 1.5-8.4), respectively. Relatives with elevated FVIII were also at risk; RR 2.3 (95% CI, 1.4-4.0) for venous thrombosis and 2.3 (95% CI, 1.0-5.1) for arterial thrombosis. After excluding all relatives with elevated factor VIII, RR for hyperhomocysteinemia and venous thrombosis dropped to 1.3 (95% CI, 0.2-9.8) and nil relatives had arterial thrombosis. We conclude that it is likely that the increased risk of venous and arterial thrombosis in hyperhomocysteinemia is mainly related to elevated FVIII levels.

## INTRODUCTION

Hyperhomocysteinemia is a disorder of methionine metabolism. Since McCully made the clinical observation that elevated plasma homocyst(e)ine levels are linked with vascular disease in 1969 [1], many clinical and epidemiological studies have demonstrated that hyperhomocysteinemia is a risk factor for both arterial and venous thrombosis [2,3]. Experimental studies suggest that the thrombogenic propensity associated with hyperhomocysteinemia results from endothelial dysfunction and injury [4]. Still, it remains unclarified whether homocysteine itself or a related metabolite or cofactor is primarily responsible for the thrombogenic effects of hyperhomocysteinemia. Especially the recent observation that lowering homocysteine levels with vitamin B6, vitamin B12 and folic acid did not reduce the risk of venous and arterial thrombosis in large prospective randomized clinical trials [5-7], supports the assumption that an associated condition, rather than hyperhomocysteinemia itself, is the thrombotic culprit. As high factor VIII levels are associated with both venous and arterial thrombosis, and with endothelial injury as well [8-10], we hypothesize that hyperhomocysteinemia and factor VIII levels are closely related to each other. In a retrospective study, we assessed the contribution of hyperhomocysteinemia and elevated factor VIII levels to the absolute risk of venous and arterial thrombosis.

## METHODS

Subjects were derived from a previous large single center family cohort study, designed to evaluate the risk of thrombosis associated with hereditary deficiencies of antithrombin, protein C or protein S [11,12]. Briefly, probands in that study were consecutive patients with documented venous thrombosis, in whom one of these deficiencies was established. Relatives, 15 years of age or older, were identified by pedigree analysis and enrolled after informed consent was obtained. Clinical data was collected prior to blood sampling [11,12]. Clinical outcome events were classified by objective criteria. Relatives were tested for all currently known thrombophilic defects [12]. Levels of homocysteine were measured by high-performance liquid chromatography [13]. Between May 1995 and July 2001 homocysteine samples were collected after overnight fasting. Hyperhomocysteinemia was defined as a fasting level above 18.5  $\mu\text{mol/L}$ , as described in the

**Table 1.** Clinical characteristics of 405 relatives with or without hyperhomocysteinemia derived from probands with an antithrombin, protein C or protein S deficiency

	Hyperhomocysteinemia		<i>P</i>
	Present (n=26)	Absent (n=379)	
Women, n (%)	18 (69)	194 (51)	0.10
Median age at enrollment (range), yrs	41 (20-80)	43 (15-85)	0.59
Venous thrombosis, n (%)	11 (42)	55 (15)	< 0.001
Median age at onset (range), yrs	35 (23-80)	31 (16-68)	0.22
Spontaneous, n (%)	5 (45)	25 (45)	1.0
Secondary to, n (%)	6 (55)	30 (55)	1.0
Oral contraceptives, n	0	12	
Pregnancy, n	3	8	
Surgery, trauma, immobilization, n	3	10	
Arterial thrombosis, n (%)	7 (27)	21 (5)	< 0.001
Median age at onset (range), yrs	61 (32-76)	54 (26-72)	0.31
Classification			
Myocardial infarction, n (%)	1 (4)	6 (2)	0.37
Transient ischemic attack, n (%)	2 (8)	7 (2)	0.11
Ischemic stroke, n (%)	4 (15)	5 (1)	< 0.001
Peripheral arterial thrombosis, n (%)	0 (0)	3 (1)	1.0
Classical risk factors			
Hypertension, n (%)	5 (19)	61 (16)	0.59
Hyperlipidemia, n (%)	5 (19)	49 (13)	0.37
Diabetes mellitus, n (%)	0 (0)	14 (4)	1.0
Smoking present, n (%)	5 (19)	118 (31)	0.27
Venous and arterial thrombosis, n (%)	4 (15)	6 (2)	0.002

Dutch population [14]. The Dutch Heart Foundation provided a guideline in 2001, which stated that random homocysteine samples (i.e. obtained not after an overnight fasting) could exclude hyperhomocysteinemia at levels below 10  $\mu\text{mol/L}$  and definitely establish hyperhomocysteinemia when levels were above 20  $\mu\text{mol/L}$  ([www.hartstichting.nl/Uploads/Brochures/Hartstichting176.pdf](http://www.hartstichting.nl/Uploads/Brochures/Hartstichting176.pdf)). From that time until the end of study (July 2004), we screened relatives accordingly. Only when they had a random homocysteine level between 10 and 20  $\mu\text{mol/L}$ , they would return to our clinic for a fasting and methionine-loading homocysteine measurement. Recently, we have shown that random homocysteine levels above 20  $\mu\text{mol/L}$  were not associated with an increased risk of venous or arterial thrombosis [15].

Increased fasting levels were appropriate to identify subjects who were at risk of thrombosis, associated with hyperhomocysteinemia. Therefore, we excluded relatives from the present analysis if fasting levels were not measured. Factor VIII:C was measured by one-stage clotting assay (Amelung GmbH, Lemgo, Germany) and was increased at levels above 150 IU/dL, as these levels have been identified to be an independent risk factor for both venous and arterial thrombosis [8,9]. Annual incidences of venous and arterial thrombosis were calculated by dividing the number of first events and the number of observation years, censored for either arterial or venous thrombosis. Observation time was defined as the period from the age of 15 years until the first thrombotic event or until the end of study. As recently a potential link between venous and arterial thrombosis has been made [16], we additionally calculated annual incidences of successive venous and arterial thrombosis. Observation time in that analysis was defined as the period from the age of 15 years until the second thrombotic event or until the end of study.

## RESULTS

The original family cohort comprised 172 probands, with a total number of 1266 relatives. Of these relatives, 70% (n=891) were eligible (i.e. living and aged 15 years or older). Eighty eight relatives did not participate due to inability to obtain informed consent. Another 308 relatives were excluded from analysis as they were only screened by random homocysteine tests, and could not be classified by additional measurements of fasting homocysteine levels. Relatives who were not evaluable because of other missing laboratory data were also excluded (n=90). The remaining 405 relatives were analyzed. Their clinical characteristics are summarized in Table 1. Median time interval between venous thrombosis and thrombophilia testing was 12 years (range, 0-43). For arterial thrombosis this was 6 years (range, 0-33). Median factor VIII levels in hyperhomocysteinemic relatives were higher than in normohomocysteinemic relatives (169 IU/dL versus 136 IU/dL,  $P=0.007$ ). Elevated factor VIII levels ( $> 150$  IU/dL) were also more often observed in hyperhomocysteinemic relatives versus normohomocysteinemic relatives (65% versus 38%,  $P=0.006$ ), while other thrombophilic defects were equally divided (Table 2).

**Table 2.** Prevalence of concomitant thrombophilic defects in 405 relatives of probands with an antithrombin, protein S or protein C deficiency

Variable, n (%)	Hyperhomocysteinemia		<i>P</i>
	Present (n=26)	Absent (n=379)	
Antithrombin deficiency	4 (15)	32 (8)	0.27
Protein C deficiency	2 (8)	34 (9)	1.0
Protein S deficiency	3 (12)	37 (10)	0.73
Elevated factor VIII	17 (65)	144 (38)	0.006
Factor V Leiden	3 (12)	50 (13)	1.0
Prothrombin G20210A	2 (8)	35 (9)	1.0

Hyperhomocysteinemia was associated with an increased risk of venous and arterial thrombosis each, or both; relative risks were 2.6 (95% CI, 1.3-4.8) for venous thrombosis, 3.7 (95% CI, 1.5-8.4) for arterial thrombosis, and 7.3 (95% CI 2.1-25.9) for both events (Table 3). Relatives with elevated factor VIII levels were also at risk; relative risks were 2.3 (95% CI, 1.4-4.0) for venous thrombosis, 2.3 (95% CI, 1.0-5.1) for arterial thrombosis, and 9.6 (95% CI, 1.2-76.1) for both. Excluding relatives with elevated factor VIII levels, relative risk of venous thrombosis in hyperhomocysteinemic relatives dropped to 1.3 (95% CI, 0.2-9.8), and nil hyperhomocysteinemic relatives had arterial thrombosis, or both venous and arterial thrombosis. Excluding relatives with normal factor VIII levels, relative risks in hyperhomocysteinemic relatives were 2.6 (95% CI, 1.3-5.2), 4.0 (95% CI, 1.6-9.9), and 5.4 (95% CI, 1.4-20.0), respectively.

## DISCUSSION

Although our results should be handled with caution, because of small numbers, this study suggests that the absolute risk of venous and arterial thrombosis in hyperhomocysteinemic subjects was not a result of hyperhomocysteinemia itself, but depended on elevated factor VIII levels. Hyperhomocysteinemia did not influence the risk when factor VIII levels were normal. However, the already higher absolute risk in relatives with elevated factor VIII levels was reinforced by hyperhomocysteinemia, suggesting an interaction. These findings are in agreement with large prospective randomized clinical trials that showed no effect of homocysteine lowering therapy with B-vitamins on the risk of venous and arterial thrombosis [5-7]. Although it has been known for quite some time that

**Table 3.** Annual incidences of first episodes of venous and arterial thrombosis

	<b>Observation years</b>	<b>Relatives with event</b>	<b>Incidence/year,% (95% CI)</b>	<b>Relative risk (95% CI)</b>
<b>Venous thrombosis</b>				
Hyperhomocysteinemia				
Absent	10408	55	0.53 (0.40-0.69)	Reference
Present	804	11	1.37 (0.68-2.45)	2.6 (1.3-4.8)
Elevated factor VIII*				
Absent	5889	21	0.36 (0.22-0.55)	Reference
Present	5133	43	0.84 (0.60-1.13)	2.3 (1.4-4.0)
Hyperhomocysteinemia in relatives with normal factor VIII levels				
Absent	5674	20	0.35 (0.21-0.54)	Reference
Present	215	1	0.47 (0.01-2.59)	1.3 (0.2-9.8)
Hyperhomocysteinemia in relatives with elevated factor VIII levels				
Absent	4592	20	0.72 (0.49-1.01)	Reference
Present	541	1	1.85 (0.89-3.40)	2.6 (1.3-5.2)
<b>Arterial thrombosis</b>				
Hyperhomocysteinemia				
Absent	11096	21	0.19 (0.12-0.29)	Reference
Present	1004	7	0.70 (0.28-1.44)	3.7 (1.5-8.4)
Elevated factor VIII*				
Absent	6196	9	0.15 (0.07-0.28)	Reference
Present	5712	19	0.33 (0.20-0.52)	2.3 (1.0-5.1)
Hyperhomocysteinemia in relatives with normal factor VIII levels				
Absent	5976	9	0.15 (0.06-0.29)	Reference
Present	220	0	0 (0-1.68)	NA
Hyperhomocysteinemia in relatives with elevated factor VIII levels				
Absent	4978	12	0.24 (0.12-0.46)	Reference
Present	734	7	0.95 (0.38-1.96)	4.0 (1.6-9.9)
<b>Venous and arterial thrombosis</b>				
Hyperhomocysteinemia				
Absent	11251	6	0.05 (0.02-0.11)	Reference
Present	1025	4	0.39 (0.11-1.00)	7.3 (2.1-25.9)
Elevated factor VIII*				
Absent	6249	1	0.02 (0.004-0.09)	Reference
Present	5835	9	0.15 (0.07-0.29)	9.6 (1.2-76.1)
Hyperhomocysteinemia in relatives with normal factor VIII levels				
Absent	6029	1	0.35 (0.21-0.54)	Reference
Present	220	0	0.47 (0.01-2.59)	NA
Hyperhomocysteinemia in relatives with elevated factor VIII levels				
Absent	5080	5	0.72 (0.49-1.01)	Reference
Present	755	4	1.85 (0.89-3.40)	5.4 (1.4-20.0)

Factor VIII levels missing in 4 relatives. NA; not applicable.

hyperhomocysteinemia and elevated factor VIII levels are closely related to endothelial injury [4,10,17], it is surprising that none of previous studies which identified hyperhomocysteinemia as an independent risk factor for venous or arterial thrombosis adjusted the risk of thrombosis for elevated factor VIII levels [2,3,14]. Also interesting is our finding that a history of both venous and arterial thrombosis was associated with elevated levels of factor VIII and hyperhomocysteinemia. This supports the assumed relationship between both diseases [16].

As our study cohort was built up with a high-risk thrombophilic population, annual incidences of venous thrombosis were higher than reported in the normal population (i.e. 0.1-0.3%) [18,19]. However, antithrombin, protein C and protein S deficiencies were equally divided among hyperhomocysteinemic and normohomocysteinemic subjects, and survival analysis showed that non-deficient hyperhomocysteinemic relatives remained at increased risk of venous thrombosis compared to non-deficient, normohomocysteinemic relatives (data not shown). Hence, it is less likely that these inherited deficiencies alone explained the higher risk of venous thrombosis in hyperhomocysteinemic subjects, which is in accordance with another study [14].

Some aspects of our study warrant comment. First, as our study was retrospective, we did not measure fasting homocysteine at time of thrombosis. Still, we are confident that this has not influenced our results as we found no correlation between age and homocysteine levels at time of enrollment ( $r^2=0.012$ , data not further shown). Second, despite maximum efforts, approximately 10% of subjects were not enrolled due to missing fasting homocysteine values. This does demonstrate the intricacy of homocysteine tests in which individuals have to return to a clinic in a fasting state, and also have to undergo a methionine-loading test, which demands six hours and has possible side effects such as nausea and malaise [20]. Considering that asymptomatic subjects are less willing to undergo these tests, this would have resulted in an overestimation of annual incidences of thrombosis in our study cohort. Still, hyperhomocysteinemic relatives were not at risk of venous and arterial thrombosis when factor VIII levels were normal. Third, relatives who were only tested for hyperhomocysteinemia with random samples and had homocysteine levels  $< 10 \mu\text{mol/L}$  or  $> 20 \mu\text{mol/L}$  were excluded from analysis. Their exclusion probably did not influence our results as we previously showed that these subjects were at similar risk of venous and arterial thrombosis [15]. Fourth, classical cardiovascular risk factors were not taken into account because a proper analysis was not

possible due to the small number of relatives with hyperhomocysteinemia. Finally, our overall event number was small and for that reason our findings may be due to a type II error. Because we did a post-hoc analysis of data, we were not able to retrieve missing data which would enlarge our sample size and possibly could exclude this statistical error. We emphasize that our findings should be seen as hypothetical. Although pathophysiologically plausible, they need to be established in further studies.

We conclude that it is likely that the increased risk of venous and arterial thrombosis in hyperhomocysteinemia is mainly related to elevated factor VIII levels.

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# Chapter 5

## **Hyperhomocysteinemia is not a risk factor for venous and arterial thrombosis and is associated with elevated factor VIII levels**

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**ABSTRACT**

As prospective trials failed to establish a reduced risk of venous and arterial thrombosis in hyperhomocysteinemic subjects after supplementation with B-vitamins, we hypothesized that an associated condition, rather than hyperhomocysteinemia itself, is the thrombotic culprit. We assessed the contribution of elevated factor VIII levels to the risk of thrombosis in hyperhomocysteinemia in a retrospective family-cohort study. Relatives of consecutive probands, who had venous thrombosis or premature atherosclerosis, and hyperhomocysteinemia, elevated factor VIII levels, or both, were enrolled. 1052 relatives were analyzed. Hyperhomocysteinemic relatives had more often elevated factor VIII levels than normohomocysteinemic relatives (38% versus 28%,  $P=0.024$ ). Other thrombophilic defects were equally divided. Hypertension and smoking were more often found in hyperhomocysteinemic relatives than in normohomocysteinemic relatives (28% versus 48%;  $P=0.027$ , and 19% versus 38%;  $P=0.029$ ). After adjusting for these confounders, the relative risk of venous thrombosis in hyperhomocysteinemic relatives was 0.8 (95% confidence interval [CI] 0.3-1.7) and 0.9 (95% CI, 0.5-1.6) for arterial thrombosis. The absolute risk of venous thrombosis in hyperhomocysteinemia was 0.14% (95% CI, 0.06-0.30) and 0.31% (95% CI, 0.18-0.52) for arterial thrombosis. Hyperhomocysteinemia had no effect on the risk of venous thrombosis or arterial thrombosis in relatives with elevated factor VIII levels; relative risks 1.0 (95% CI, 0.4-2.3) and 1.1 (95% CI, 0.5-2.5), respectively. We conclude that hyperhomocysteinemia is not a risk factor for venous and arterial thrombosis, and is associated with elevated factor VIII levels.

## INTRODUCTION

In 1969, McCully first proposed that elevated homocysteine levels causes cardiovascular disease [1]. His hypothesis was based on the finding of atherosclerotic plaques at autopsies of people with homocystinuria, a rare metabolic disease which results in extremely high homocysteine levels. Later, experimental studies showed that high levels of homocysteine were associated with endothelial damage and oxidative stress [2,3]. Since the early 1990's McCully's hypothesis was modified positing that mild hyperhomocysteinemia was also a risk factor for both venous and arterial thrombosis, which has been supported by numerous epidemiologic studies [4-7]. As vitamin B6, vitamin B12 and folic acid can decrease homocysteine levels [8], it was considered plausible that treatment with these supplements would reduce the risk of venous and arterial thrombosis. Recently, however, large prospective clinical trials that reported on the effects of lowering homocysteine levels with vitamin B6, vitamin B12 or folic acid therapy showed no improved clinical outcome in patients with prior ischemic stroke [9], myocardial infarction [10] or venous thrombosis [11,29]. The negative results of these studies might be explained by a low absolute risk of thrombosis in subjects with mild hyperhomocysteinemia, as we recently demonstrated [12]. Another explanation might be that an associated condition, rather than hyperhomocysteinemia itself, is the thrombotic culprit. We previously observed that the risk of thrombosis in hyperhomocysteinemic subjects strongly depended on coexistence with other thrombophilic defects [12], which supports this assumption. As high factor VIII levels are associated with venous and arterial thrombosis, and with endothelial injury [13-15], we hypothesize that these are associated.

In the present study, we assessed the contribution of elevated factor VIII levels to the risk of venous and arterial thrombosis in mild hyperhomocysteinemia.

## METHODS

### Subjects

We pooled the subjects from two previous studies that reported on the risk of venous and arterial thrombosis in hyperhomocysteinemic subjects, and subjects with elevated factor VIII. These studies had an identical design, which has been described in detail elsewhere [12,14]. In short, between September 1997 and July

2004 first-degree relatives, who were 15 years of age or older, of consecutive patients (proband) with documented venous thrombosis or any documented arterial thrombotic event before the age of 50 years and hyperhomocysteinemia, elevated levels of factor VIII, or both, were enrolled. Patients were referred by their general physicians to the thrombosis outpatient clinics of three participating university hospitals, either to confirm clinically suspected venous thrombosis, or to evaluate symptomatic premature atherosclerosis. Relatives were enrolled after informed consent was obtained. Detailed information about previous episodes of venous and arterial thrombosis, exposure to exogenous risk factors for thrombosis and anticoagulant treatment was collected by physicians at the outpatient clinics, using a validated questionnaire [16] and reviewing medical records. Clinical data was collected prior to laboratory testing. Additional thrombophilia tests included deficiencies of antithrombin, protein C and protein S, factor V Leiden, and the prothrombin G20210A mutation. Approval was obtained by the institutional review boards of the participating hospitals.

### **Laboratory studies**

Levels of homocysteine were measured by high-performance liquid chromatography [17] after overnight fasting. Hyperhomocysteinemia was defined as a fasting homocysteine level  $> 18 \mu\text{mol/L}$ , which is above the 95<sup>th</sup> percentile of normal in the Dutch population [18]. Although clinical guidelines recommended to perform a methionine-loading test to identify hyperhomocysteinemia at time of enrollment, the results of this test were not analyzed in the study after we and others have recently shown that this test does not contribute to the assessment of a subject's thrombotic risk [27,28]. Factor VIII:C was measured by one-stage clotting assays (Amelung GmbH, Lemgo, Germany) and was increased at levels above the 75<sup>th</sup> percentile in the Dutch population (150 IU/dL) [13]. These levels have been identified to be an independent risk factor for both venous and arterial thrombosis [13,14]. Activity of antithrombin (Coatest, Chromogenix, Mölndal, Sweden) and protein C (Berichrom Protein C, Behring, Marburg, Germany) were measured by chromogenic substrate assays, protein C and protein S antigen levels by Enzyme Linked Immuno Sorbent Assay (ELISA) (DAKO, Glostrup, Denmark). Antithrombin deficiency was defined by decreased levels of antithrombin activity ( $< 65 \text{ IU/dL}$ ), protein C deficiency by decreased levels of either protein C antigen ( $< 65 \text{ IU/dL}$ ) and/or activity ( $< 65 \text{ IU/dL}$ ) and protein S deficiency by decreased total protein S antigen levels ( $< 65 \text{ IU/dL}$ ), corresponding with plasma levels below

the under limit of their normal ranges. Factor V Leiden and prothrombin G20210A were demonstrated by polymerase chain reactions [19,20]. If relatives were on long-term anticoagulant treatment with acenocoumarol, a short acting vitamin K antagonist, blood samples were taken after treatment had been interrupted for at least two weeks; in the meantime nadroparin was given subcutaneously.

**Table 1.** Clinical characteristics of 1052 relatives of probands with hyperhomocysteinemia and/or elevated levels of FVIII

	Hyperhomocysteinemia		<i>P</i>
	Present (n=132)	Absent (n=920)	
Women	71 (54)	541 (59)	0.30
Age at enrollment (years)	52 (16-86)	46 (15-87)	< 0.001
Venous thrombosis	7 (5)	45 (5)	0.83
Age at onset (years)	54 (19-80)	45 (17-85)	0.35
Primary	4 (57)	20 (44)	0.69
Secondary to:			
Oral contraception	2 (29)	9 (20)	
Pregnancy, puerperium	0 (0)	4 (9)	
Surgery, trauma, immobilization	1 (14)	12 (26)	
Malignancy	0 (0)	0 (0)	
Arterial thrombosis	15 (11)	69 (8)	0.12
Age at onset (years)	61 (35-80)	54 (25-80)	0.18
Classification			
Myocardial infarction	2 (13)	32 (46)	
Transient ischemic attack	2 (13)	6 (9)	
Ischemic stroke	4 (27)	14 (20)	
Peripheral arterial thrombotic event	7 (47)	17 (25)	
Classical risk factors			
Hypertension	37 (28)	177 (19)	0.027
Hyperlipidemia	19 (14)	143 (16)	0.80
Diabetes mellitus	10 (8)	50 (5)	0.32
Smoking	64 (48)	352 (38)	0.029
Concomitant thrombophilic defects			
Antithrombin deficiency	1 (0.8)	3 (0.3)	0.42
Protein C deficiency	1 (0.8)	9 (1)	1.00
Protein S deficiency	0 (0)	17 (2)	0.25
Elevated factor VIII	50 (38)	257 (28)	0.024
Factor V Leiden	15 (11)	122 (13)	0.69
Prothrombin G20210A	4 (3)	22 (2)	0.56

Continuous variables denoted as median (range), categorical variables as number (%).

**Definitions**

Venous thrombosis was considered established if deep vein thrombosis was confirmed by compression ultrasound or venography, and pulmonary embolism by ventilation/perfusion lung scanning, spiral CT scanning or pulmonary angiography, or when the patient had received full dose heparin and a vitamin K antagonist for at least 3 months without objective testing at a time when these techniques were not yet available. Secondary venous thrombosis was defined if it had occurred at or within 3 months after exposure to exogenous risk factors including surgery, trauma, immobilization for more than 7 days, pregnancy, post-delivery period, the use of oral contraceptives or hormonal replacement therapy, or malignancy. In the absence of these risk factors venous thrombosis was considered to be primary.

Coronary and peripheral arterial disease had to be symptomatic and angiographically proven, while myocardial infarction was diagnosed according to clinical, enzymatic and electrocardiographic criteria. Ischemic stroke was defined as the onset of rapidly developing symptoms and signs of loss of cerebral function which lasted at least 24h and had an apparent vascular cause, as demonstrated by CT or magnetic resonance imaging. If a cerebral event completely resolved within 24h without cerebral lesions at scanning, it was classified as transient ischemic attack (TIA). Risk factors for atherosclerosis included known diabetes mellitus, hyperlipidemia, hypertension and smoking.

**Statistical analysis**

We analyzed the absolute risk of first venous and arterial thrombosis in relatives, comparing those who did or did not have hyperhomocysteinemia. Probandes were excluded from analysis to avoid bias. Observation time was defined as the period from the age of 15 years until the first thrombotic episode or until the end of the observation period. Our study was retrospective and relatives were not treated for hyperhomocysteinemia with B-vitamins. Annual incidences of venous and arterial thrombosis were calculated by dividing the number of events by the number of observation years. When calculating the annual incidence of venous thrombosis, the occurrence of arterial thrombosis was ignored and vice versa. With a Cox proportional hazards regression model we adjusted relative risks for clinically relevant covariates, which showed statistical significance at a level of  $P < 0.10$  in univariate analysis.

Continuous variables were expressed as median values and ranges; categorical data as counts and percentages. Differences between groups were evaluated by the

Student t test or Mann-Whitney U test, depending on the normality of data for continuous data and by Fisher exact test for categorical data. A two-tailed p-value of less than 0.05 indicated statistical significance. The 95% confidence intervals (95% CI) around the incidence rates were calculated under the Poisson distribution assumption. Statistical analyses were performed using SAS software, version 9.1 (SAS-Institute inc., Cary, North Carolina, USA).

## RESULTS

Our family cohort contained 361 probands, with a total number of 2389 relatives, identified by pedigree analysis, who were 15 years of age or older. Of these relatives, 515 had died before the start of this study. Another 717 relatives did not participate because of various reasons, including refusal or inability to give informed consent and residence outside the Netherlands. Another 105 relatives were not evaluable because of missing laboratory data. The remaining 1052 relatives were analyzed. Their clinical characteristics are summarized in Table 1. Median age at enrollment of hyperhomocysteinemic relatives was 52 years (range, 16-86) and was higher than in normohomocysteinemic relatives (46 years; range 15-87,  $P < 0.001$ ). Venous thrombosis had occurred in seven hyperhomocysteinemic relatives (5%) and in 45 normohomocysteinemic relatives (5%,  $P = 0.83$ ). Median age at onset of the first episode of venous thrombosis was 54 years (range, 19-80) in relatives with hyperhomocysteinemia and 45 years (range, 17-85) in relatives with normohomocysteinemia ( $P = 0.35$ ). Arterial thrombotic events were documented in 15 relatives (11%) with hyperhomocysteinemia and in 69 relatives (8%) with normohomocysteinemia ( $P = 0.12$ ). Hypertension was more common in hyperhomocysteinemic relatives (28%) versus normohomocysteinemic relatives (19%,  $P = 0.027$ ), as was smoking (48% versus 38%,  $P = 0.029$ ). Other classical risk factors for atherosclerosis were not statistically different in both groups. Of concomitant thrombophilic defects, factor V Leiden was more often demonstrated than expected from its prevalence in the general population (i.e. 5%) [21]. Elevated factor VIII levels were more often observed in hyperhomocysteinemic relatives than in normohomocysteinemic relatives (38% versus 28%,  $P = 0.024$ ), while other thrombophilic defects were equally divided.

**Table 2.** Annual incidences of venous and arterial thrombosis in 1052 relatives with correction of concomitant factors

	Observation years	Relatives with event	Incidence/year, % (95% CI)	Relative risk (95% CI)
<b>Venous thrombosis</b>				
Hyperhomocysteinemia				
Absent	27663	45	0.16 (0.12-0.22)	Reference
Present	4840	7	0.14 (0.06-0.30)	0.9 (0.4-2.0)*
<b>Arterial thrombosis</b>				
Hyperhomocysteinemia				
Absent	27672	69	0.25 (0.19-0.32)	Reference
Present	4795	15	0.31 (0.18-0.52)	1.3 (0.7-2.2) <sup>†</sup>

\* Adjusted for elevated levels of FVIII the relative risk was 0.8 (95% CI, 0.3-1.7).

<sup>†</sup> Adjusted for elevated levels of factor VIII, hypertension and smoking the relative risk was 0.9 (95% CI, 0.5-1.6).

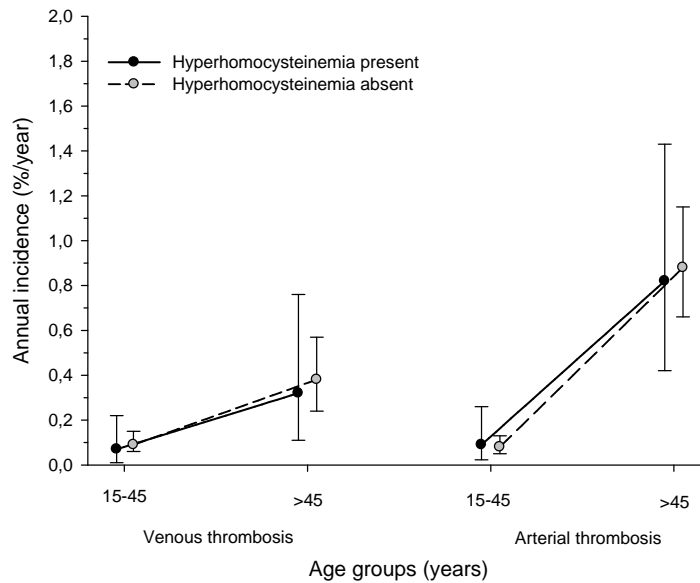
**Table 3.** Contribution of hyperhomocysteinemia on the risk of venous and arterial thrombosis in relatives with elevated FVIII

	Observation years	Relatives with event	Incidence/year, % (95% CI)	Relative risk (95% CI)
<b>Venous thrombosis</b>				
Elevated FVIII				
Absent	21228	23	0.11 (0.07-0.16)	Reference
Present	11056	29	0.26 (0.18-0.38)	2.4 (1.4-4.2)
With normohomocysteinemia	8679	23	0.26 (0.17-0.40)	Reference
With hyperhomocysteinemia	2377	6	0.25 (0.09-0.55)	1.0 (0.4-2.3)
<b>Arterial thrombosis</b>				
Elevated FVIII				
Absent	21125	48	0.22 (0.17-0.30)	Reference
Present	11123	35	0.32 (0.22-0.44)	1.4 (0.9-2.1)
With normohomocysteinemia	8840	27	0.31 (0.20-0.44)	Reference
With hyperhomocysteinemia	2283	8	0.35 (0.15-0.69)	1.1 (0.5-2.5)

In Table 2, annual incidences of first venous and arterial thrombotic events are presented. Annual incidences of venous thrombosis were 0.14% (95% CI, 0.06-0.30) in relatives with hyperhomocysteinemia and 0.16% (95% CI, 0.12-0.22) in relatives with normohomocysteinemia; adjusted relative risk 0.8 (95% CI, 0.3-1.7).

In hyperhomocysteinemic relatives, annual incidence of arterial thrombosis was 0.31% (95% CI, 0.18-0.52) compared to 0.25% (95% CI, 0.19-0.32) in normohomocysteinemic relatives; adjusted relative risk 0.9 (95% CI, 0.5-1.6). Only one relative with hyperhomocysteinemia and venous thrombosis had a normal factor VIII level, and none of the relatives who had hyperhomocysteinemia and arterial thrombosis had a normal factor VIII level, were non smokers and had normotension. Annual incidences were 0.04% (95% CI, 0.01-0.23) and 0.25% (95% CI, 0.09-0.55) for venous thrombosis in hyperhomocysteinemic relatives with normal and elevated factor VIII levels, respectively (relative risk 6.2; 95% CI, 0.7-51.6). For arterial thrombosis these were 0% (95% CI, -0.60) versus 0.37% (95% CI, 0.12-0.85). On the contrary, hyperhomocysteinemia had no additional effect on the risk of thrombosis in relatives with elevated FVIII levels (Table 3). Relative risks were 1.0 (95% CI 0.4-2.3) for venous thrombosis and 1.1 (95% CI 0.5-2.5) for arterial thrombosis, respectively. Figure 1 shows annual incidences of venous and arterial thrombosis in hyperhomocysteinemic and normohomocysteinemic relatives according to their age. These increased with age in both groups ( $P=0.02$  for venous thrombosis,  $P=0.003$  for arterial thrombosis), but differences between groups were not statistically significant. To see whether a different cut-off point for hyperhomo-

**Figure 1.** Age specific annual incidences of venous and arterial thrombosis in relatives of probands with hyperhomocysteinemia and/or elevated FVIII



cysteinemia would change the results, we stratified homocysteine levels into quartiles (Table 4). No clear relationship between annual incidence of either venous or arterial thrombosis and height of fasting homocysteine levels was observed.

## DISCUSSION

Our study shows a low absolute risk of venous and arterial thrombosis in hyperhomocysteinemic relatives, comparable with the annual incidence of venous and arterial thrombosis in the normal population (i.e. 0.1-0.3% for venous thrombosis and 0.1-0.4% for arterial thrombosis) [22-24]. Elevated factor VIII levels, hypertension, and active smoking were more often present in hyperhomocysteinemic subjects than in normohomocysteinemic subjects. Of relatives who had venous thrombosis and hyperhomocysteinemia, only one case had a normal factor VIII level (14%), while 23 of 45 relatives with normohomocysteinemia also had normal FVIII levels (51%). Although this last result involves small numbers, it supports the hypothesis that factor VIII levels and hyperhomocysteinemia are associated with each other, also because more hyperhomocysteinemic subjects had elevated factor VIII levels. A similar result was found in a previous study, but then in a high

**Table 4.** Annual incidences of venous and arterial thrombosis in relatives of probands with hyperhomocysteinemia and/or elevated FVIII stratified in quartiles

<b>Fasting homocysteine level (µmol/L)</b>	<b>Observation years</b>	<b>Relatives with event</b>	<b>Incidence/year, % (95% CI)</b>
<b>Venous thrombosis</b>			
< 9.9	7095	10	0.14 (0.07-0.26)
9.9-12.1	7910	13	0.16 (0.09-0.28)
12.1-15.2	8896	15	0.17 (0.09-0.28)
> 15.2	9119	14	0.15 (0.08-0.26)
<b>Arterial thrombosis</b>			
< 9.9	7094	10	0.14 (0.07-0.26)
9.9-12.1	7920	25	0.31 (0.20-0.47)
12.1-15.2	8938	23	0.26 (0.16-0.39)
> 15.2	9010	29	0.32 (0.22-0.46)

risk thrombophilic population where hyperhomocysteinemia only increased the risk of venous and arterial thrombosis when factor VIII levels were elevated [25]. Our findings may explain why lowering of homocysteine levels with B-vitamins has not resulted in a decrease of venous and arterial thrombosis in large prospective randomized clinical trials [9-11,29], assuming that elevated factor VIII levels are not decreased by these vitamins. However, it is not known whether or not elevated factor VIII levels are decreased by B-vitamins.

Our results are inconsistent with meta-analyses that reported that hyperhomocysteinemia was a risk factor for venous and arterial thrombosis [4,7]. However, these meta-analyses did not exclude publication bias by funnel plot analysis, which could have resulted in a higher relative risk compared to our study. Furthermore, a meta-analysis may not be a proper statistical tool to identify hyperhomocysteinemia as a risk factor for venous or arterial thrombosis, as a clear cut-off level for hyperhomocysteinemia has never been postulated. We stratified relatives into quartiles according to increasing homocysteine levels, but did not find a relationship between annual incidence of thrombosis and increasing homocysteine levels. This is in accordance with another study [12]. It must also be pointed out that the prove of an association between hyperhomocysteinemia and thrombosis is based mostly on the results of large-scale retrospective studies, and a causal relationship from prospective studies. However, intervention studies failed to show a causal relationship [9-11,29]. Our negative findings are in line with the latter studies.

Some aspects of our study warrant comment. First, as our study was retrospective, we did not measure fasting homocysteine at time of thrombosis. Still, we are confident that this has not influenced our results as we found no correlation between age and homocysteine levels at time of enrollment ( $r^2=0.014$ , data not further shown). Second, despite maximum efforts, our response rate was not optimal due to missing homocysteine values in a number of subjects. This does demonstrate the intricacy of performing homocysteine tests in which individuals have to return to a clinic in a fasting state, and also have to undergo a methionine-loading test, which takes place six hours later and has possible side effects such as nausea and malaise [26]. As it is likely that asymptomatic subjects are less willing to undergo these tests, this would have resulted in an overestimation of annual incidences of thrombosis in our study cohort. Still, hyperhomocysteinemic relatives were not at increased risk of venous and arterial thrombosis. Third, due to the retrospective design of our study, a number of relatives had venous thrombosis which was not established by objective techniques because these were not available at that

time. This implies the possibility of overdiagnosis. The annual incidence of venous thrombosis, however, did not exceed the annual incidence in the normal population. Fourth, although clinical guidelines recommended to perform a methionine-loading test to identify hyperhomocysteinemic subjects during the study period, this test was not implied because we and others have shown that this test does not contribute to the assessment of a subject's thrombotic risk [27,28]. Fifth, the absolute risk of thrombosis in hyperhomocysteinemia was low. Consequently, the number of events in our study cohort was small. A power problem, however, seems not likely as the upper confidence interval of annual incidence of venous thrombosis in hyperhomocysteinemic relatives was 0.30% per year in our study, which is still normal. For arterial thrombosis, this upper confidence interval was slightly higher than normal. We explain this as hyperhomocysteinemic subjects were more often active smokers, or had hypertension. Finally, factor V Leiden was more often observed in the study cohort than in the general population [21]. Because we studied first degree relatives of patients with either venous thrombosis or premature atherosclerosis with hyperhomocysteinemia or elevated levels of factor VIII, we might hereby have introduced selection bias. Relatives could be *a priori* at higher risk of venous thrombosis because factor V Leiden is more frequently found in patients with venous thrombosis [21], but the effect of selection bias proved to be minimal as the annual incidence of both venous and arterial thrombosis in our study cohort was normal.

In conclusion, this study failed to show an association between hyperhomocysteinemia and venous or arterial thrombosis. Hyperhomocysteinemia was related to elevated factor VIII levels.

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# **Part II**

# **Infections**



# Chapter 6

## **Mesenteric vein thrombosis associated with primary cytomegalovirus infection: a case report**

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## **ABSTRACT**

In the past few years several studies supported an interplay between cytomegalovirus (CMV) infections and a prothrombotic state. We describe a case of primary CMV infection in an immunocompetent adult that was complicated with mesenteric vein thrombosis. Transient protein C deficiency, lupus anticoagulant and APC resistance were found, in combination with a heterozygous prothrombin G20210A mutation. We discuss the possible mechanisms of CMV related venous thrombosis.

## INTRODUCTION

Cytomegalovirus (CMV) infection is usually a self-limiting disease in healthy adults, although significant complications may occur, including pneumonia, pericarditis, colitis, hepatitis and hemolytic anemia [1]. Venous thrombosis has been described in association with CMV reactivation in the context of immunosuppression after organ transplantation, but only very rarely in immunocompetent individuals [2]. We report here the case of an immunocompetent adult who presented with mesenteric vein thrombosis complicating a primary CMV infection, and describe the possible mechanisms of CMV related venous thrombosis.

## CASE REPORT

A 35-year-old female with a 14-day history of abdominal pain and diarrhoea, was hospitalized in July 2006. No visits abroad or food-poisoning were noted, nor exposure to external risk factors for venous thrombosis, such as trauma, surgery, immobility or pregnancy. Her medical history revealed axillary vein thrombosis in 1991, while she used oral contraceptives and was heterozygous for the prothrombin G20210A mutation (revealed in July 2004 at thrombophilia screening, shortly before a planned pregnancy); she received anticoagulants for 6 months and oral contraception was stopped. On examination she was in discomfort, febrile (39.5°C), normotensive, had pressure pain in epigastrio, and splenomegaly. Hemoglobin level was 6.7 mmol/L, WBC count  $4.8 \times 10^9/L$ , platelet count  $101 \times 10^9/L$ , CRP 42 mg/L, lactate dehydrogenase (LDH) 741 IU/L (normal range 0-250 IU/L), alanine aminotransferase (ALAT) 111 IU/L and aspartate aminotransferase (ASAT) 94 IU/L (normal ranges 0-40 IU/L and 0-45 IU/L, respectively). After blood cultures were taken, the patient started with analgesics and intravenous broad spectrum antibiotics with a working diagnosis of bacterial gastroenteritis. As her clinical situation deteriorated, the following day a computed tomography (CT) of the abdomen was taken and showed thrombosis of the superior mesenteric vein and lienal vein. With laparoscopy no necrosis or ischemia was seen. Antibiotics were withdrawn, and anticoagulant treatment was introduced after blood was collected for thrombophilia screening (techniques and definitions described in [3,4]) and testing for CMV serology and antigenemia. Serological tests for CMV (ELISA) was positive for IgM antibodies ( $> 100$  IU/mL, whereas  $< 5$  IU/mL is a

**Table 1.** Thrombophilia results pre- and post cytomegalovirus infection in a patient with mesenteric vein thrombosis

	Cytomegalovirus		
	Absent	Present	Normal
Antithrombin	105	93	80-120%
Protein C antigen	121	59	65-145%
Protein C activity	116	57	65-145%
Protein S antigen	96	94	65-145%
Factor VIII	NA	223	50-150%
Lupus anticoagulant	Negative	Positive	Negative
Plasminogen	105	110	70-130%
nAPC-SR	0.88	0.61	0.82-1.22
Factor V Leiden	Wild type		Wild type
Prothrombin G20210A	Heterozygous		Wild type
INR	1.0	1.2	0.8-1.3

nAPC-SR indicates normalized activated protein C sensitivity ratio; NA, not available.

negative result) and IgG antibodies (115 IU/mL, whereas < 2 IU/mL is a negative result), as was a CMV pp65 antigenemia assay (1 copy/ 50.000 granulocytes, whereas 0 copies/ 50.000 granulocytes is a negative result). A second serological test after two weeks showed an increase of CMV IgG antibodies to 145 IU/mL. After 16 days the patient had completely recovered, blood cultures remained negative, hemoglobin, platelet count and liver enzymes returned to normal, and she was discharged from the hospital. Thrombophilia tests revealed protein C deficiency, elevated level of factor VIII, lupus anticoagulant and APC resistance (Table 1). Interestingly, protein C deficiency, lupus anticoagulant and APC resistance were absent in 2004.

## DISCUSSION

CMV infection is common and comprises the whole lifespan of humans, with a seroprevalence rate of approximately 50% at the age of 50 in the Western world [5]. In immunocompetent patients it is generally asymptomatic or reveals symptoms similar to those of infectious mononucleosis: fever, lymphadenopathy, hepatitis, and sometimes hemolytic anemia and thrombocytopenia [1]. Our patient had CMV antigenemia together with a high CMV IgM level and a positive clinical history,

which is very suggestive for primary CMV infection. The CMV antigen level of our patient was however low, probably due to the presence of high CMV IgM and IgG levels and the fact that the first symptoms appeared two weeks before. To date, between 20-30 cases have been reported of CMV infection associated with venous thrombosis, and most of these have recently been reviewed [2,6]. To our knowledge, only three of these were associated with mesenteric vein thrombosis [7-9]. Due to the rarity of reports of primary CMV infection and venous thrombosis, one has to assume that CMV infection itself is not a single causative factor for the development of venous thrombosis. Indeed, venous thrombosis is a multicausal disease, often involving acquired or environmental risk factors as well as genetic predisposition [10]. Our patient was heterozygous for the prothrombin G20210A mutation and had a history of axillary vein thrombosis. Both conditions are associated with an increased risk of (recurrent) venous thrombosis [11,12], and therefore possibly have contributed to mesenteric vein thrombosis in our patient. However, as there were acquired thrombophilic changes at time of primary CMV infection in our patient, together with the case reports that suggested a relation between CMV infection and venous thrombosis [2,6], CMV may be a concomitant contributing factor to develop venous thrombosis.

Several mechanisms have been proposed to explain CMV-related venous thrombosis. CMV infection can cause vascular cell damage and expression of adhesion proteins leading to increased platelet and leukocyte adhesion, lupus anticoagulant and enhanced FVIII synthesis or secretion [2]. Since elevated factor VIII levels and lupus anticoagulant are associated with an increased risk of venous thrombosis [13,14], CMV infection might increase this risk indirectly. Low protein C levels have been reported in various infections, possibly due to consumption as it has anti-inflammatory properties [15]. This could have caused the protein C deficiency in our patient. Although inherited protein C deficiency is a strong risk factor for venous thrombosis, when acquired this risk is unknown. Nevertheless, it is likely that this obvious deficiency contributed to the development of thrombosis.

Spontaneous resolutions of venous thrombosis after primary CMV infection have been observed in patients who did not receive anticoagulant treatment [16], which also suggests that primary CMV infection is a transient trigger for venous thrombosis. Still, we decided to continue anticoagulant therapy in our patient for a longer term, also considering her previous episode of axillary vein thrombosis, the heterozygosity for the prothrombin G20210A mutation and that a definitive answer may not be drawn on the basis of a single case. This and prior case reports on this

issue do however reinforce the need for seroepidemiological or pathology-based studies to establish the supposed association of primary CMV infection with apparently spontaneous venous thrombosis.

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# Chapter 7

## **Possible contribution of cytomegalovirus infection to the high risk of (recurrent) venous thrombosis after renal transplantation**

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**ABSTRACT**

Renal transplant recipients are at increased risk of venous thrombosis, which has been regarded as a postoperative complication, although it may persist afterwards. As numerous case reports showed that active cytomegalovirus (CMV) infection can be found at time of onset of venous thrombosis and is frequently found in renal transplant recipients, we hypothesized that one might be the result of the other. To calculate the risk of (recurrent) venous thrombosis in renal transplant recipients, and to see whether CMV infection influenced this risk, we retrospectively analyzed 606 living consecutive renal transplant recipients. CMV status at time of transplantation and at time of enrollment was determined. Absolute risks of first venous thrombosis and recurrence were compared with CMV status, and were corrected for surgery related venous thrombosis, age, and anticoagulant treatment. Annual incidence of venous thrombosis was 0.88% (95% CI, 0.65-1.15) in all recipients and 0.59% (95% CI, 0.41-0.83) corrected for surgery related venous thrombosis. CMV positive and seroconverted recipients tended to have an increased risk of venous thrombosis compared to CMV negative recipients; corrected relative risks were 2.0 (95% CI, 0.9-5.2) and 1.7 (95% CI, 0.6-4.7), respectively. The cumulative 10-years recurrence rate of venous thrombosis in CMV seronegative, seroconverted, and seropositive recipients was 10%, 51% and 59%, respectively. We conclude that CMV infection tended to be associated with an increased risk of (recurrent) venous thrombosis. Prospective studies are warranted to establish this observation, which suggests that CMV infection influences the high risk of (recurrent) venous thrombosis in renal transplant recipients.

## INTRODUCTION

Renal transplant recipients are at increased risk to develop venous thrombosis, with its highest incidence within the first three months after transplantation [1]. Whether they remain at increased risk after three months is largely unknown. Recently, however, two reports showed a recurrence rate of up to 60% within six years after stopping initial anticoagulant treatment [2,3]. The high risk of venous thrombosis in renal transplant recipients has been attributed to surgery, long term immunosuppressive drugs and antiphospholipid antibodies [4,5]. Active cytomegalovirus (CMV) infection might be interesting as another contributing factor, because it is a very common complication in renal transplant recipients, and CMV has a lifelong latency after initial infection [6]. Due to immunosuppressive drugs CMV can easily reactivate [7]. CMV infects endothelial cells, causing vascular cell damage [8,9], induces lupus anticoagulant [10], and enhances factor VIII synthesis or secretion [11,12]. Furthermore, vascular damage has been associated with high levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) [13,14]. Since elevated levels of factor VIII, and sVCAM-1, and lupus anticoagulant are all associated with an increased risk of venous thrombosis [14-16], and, obviously, all recipients require prolonged immunosuppressive therapy, active CMV infection might increase this risk indirectly. This assumption is supported by numerous case reports of venous thrombosis during CMV infection, which recently have been reviewed [17,18]. However, it is not clear whether subjects with primary CMV infection or CMV reactivation indeed have a higher risk of venous thrombosis compared to CMV negative subjects. Only a few seroepidemiological studies have reported about this possible association, but were inconclusive, possibly as these studies did not include CMV infection in their primary objectives [2,3]. Recipients with chronic rejection might be an interesting subgroup as it has been associated with CMV infection in allograft recipients [25].

We performed a study to assess the absolute risk of first venous thrombosis and recurrence, respectively in renal transplant recipients. We estimated this risk in recipients who either had CMV infection prior to transplantation or developed CMV infection after transplantation, compared to CMV seronegative renal transplant recipients to ascertain the contribution of CMV infection.

## **METHODS**

### **Subjects**

Between October 2001 and November 2005 all renal transplant recipients who were operated in our center since 1968 and who survived with a functioning allograft were asked to participate in the study at their visit to the outpatient clinic (date of enrollment). Patients who had received a combined transplantation (i.e. kidney and pancreas or kidney and liver) were invited to participate as well. Between 1968 and 1989, after transplantation, patients received a combination of prednisolone and azathioprine as immunosuppressive therapy, between 1989 and 1997 a combination of ciclosporine and low dose prednisolone, and after 1997 a combination of ciclosporine, mycophenolate mofetil and low dose prednisolone. No CMV prophylaxis was given throughout the study period. Induction therapy was not given until Januari 2000. After this date recipients were standardly given IL-2 receptor blocker (daclizumab) on the date of transplantation, and 14 days after transplantation. A total of 606 out of 847 (72%) renal transplant recipients signed written informed consent.

Relevant donor, recipient and transplant characteristics were extracted from the Groningen Renal Transplant Database. This database holds information of all renal transplantations, that have been performed at our center since 1968. Extracted from the database were primary renal disease, type and date of transplantation, and CMV status at time of transplantation. Information about previous episodes of venous thrombosis and anticoagulant treatment was collected by reviewing medical records. The study was approved by the institutional review board of our hospital.

### **Laboratory studies and definitions**

Blood was drawn at enrollment to determine serum creatinine levels, platelet counts, IgG antibodies against CMV and sVCAM-1 levels. Serum creatinine levels were determined using the Jaffé method. Twenty-four hour creatinine was determined from a 24-h urine sample. CMV IgG antibodies were determined by enzyme immunoassay as previously described [17]. Patients were classified as CMV seropositive when they had a CMV IgG titer above 1 IU/mL at time of transplantation. CMV seroconversion was defined when patients were seronegative at time of transplantation, but had a CMV IgG titer above 1 IU/mL at date of enrollment. All other patients were classified as seronegative for CMV. CMV IgG levels > 250 IU/mL at time of enrollment were classified as active CMV infection

as previously described [11]. Soluble VCAM-1 levels were measured in EDTA plasma by ELISA (Bender MedSystems, Vienna, Austria).

Venous thrombosis was considered established if deep vein thrombosis was confirmed by compression ultrasound or venography, and pulmonary embolism by ventilation and perfusion lung scanning, spiral CT scanning or pulmonary angiography.

### **Statistical analysis**

We analyzed the absolute risk of first venous thrombosis after transplantation in all renal transplant recipients. CMV seropositive and CMV seroconverted renal transplant recipients were compared with seronegative CMV renal transplant recipients. Age, sex and rejection of the graft were also taken into account to assess whether these variables had an additional effect on the risk of venous thrombosis. Annual incidence of venous thrombosis was calculated by dividing the number of events by the number of observation years. Observation time was defined as the period from transplantation until the first venous thrombotic episode or until the end of the observation period. Relative risks were corrected for renal transplant surgery related venous thrombosis by subtracting the first three months after transplantation from the observation time in all recipients, and by excluding recipients from analysis who had venous thrombosis within the first three months after transplantation. Correction for anticoagulant treatment was done by subtracting the treatment time (227 years) from observation time. As a consequence, patients who were on life-long anticoagulant treatment at time of transplantation for any reason were excluded from analysis, and patients in whom life-long anticoagulant treatment became indicated after transplantation only the remaining period at risk was evaluated. These patients had either recurrent venous thrombosis prior to transplantation (n=4), or prosthetic heart valves (n=11), atrial fibrillation (n=12), chronic heart failure (n=5), peripheral arterial occlusive disease (n=4) or vasculitis (n=4) before or after transplantation. Correction for age was done by using Mantel-Haenszel methods, stratifying recipients in aged younger than 50 years, and aged 50 years or older.

Freedom of first venous thrombosis and recurrence was analyzed by the Kaplan-Meier method. The cumulative recurrence rate was calculated over the period from the end of anticoagulant treatment after the first episode of venous thrombosis until either the date of first recurrence or the end of the observation period.

Continuous variables are expressed as median values and ranges; categorical data as counts and percentages. Differences between groups were evaluated by the Student t test or Mann-Whitney U test, depending on the normality of data for continuous data and by Fisher exact test for categorical data. A two-tailed p-value of less than 0.05 indicated statistical significance. The 95% confidence intervals (95% CI) around the incidence rates were calculated under the Poisson distribution assumption. Statistical analyses were performed using SPSS software, version 14.0 (SPSS Inc., Chicago, IL).

**Table 1.** Clinical characteristics

	CMV negative	CMV positive	CMV sero- conversion	Total
No. of patients	169	285	152	606
Women, no. (%)	68 (40)	139 (49)	67 (44)	274 (45)
Median age at enrollment, year (range)	51 (23-78)	56 (24-83)	55 (24-83)	55 (23-83)
Age at transplantation, year (range)	40 (18-66)	47 (17-77)	41 (15-73)	44 (15-77)
Cause of end stage renal disease, no. (%)				
Primary glomerular disease	44 (26)	71 (25)	55 (36)	170 (28)
Glomerular disease of vascular/ autoimmune origin	9 (5)	23 (8)	7 (5)	39 (6)
Tubular interstitial disease	31 (18)	36 (13)	27 (18)	94 (16)
Polycystic renal disease	28 (17)	61 (21)	18 (12)	107 (18)
Dysplasia and hypoplasia	12 (7)	6 (2)	3 (2)	21 (3)
Renovascular disease	9 (5)	17 (6)	7 (5)	33 (5)
Diabetes mellitus	6 (4)	12 (4)	4 (3)	23 (4)
Other or unknown cause	30 (18)	58 (20)	31 (20)	119 (20)
First venous thrombosis, no. (%)	13 (8)	23 (8)	16 (11)	52 (9)
Median age at onset, year (range)	52 (21-65)	51 (31-69)	43 (28-65)	51 (21-69)
Renal transplant surgery induced venous thrombosis	8 (5)	6 (2)	5 (3)	19 (3)
DVT ipsilateral from kidney transplantate	7 (4)	9 (3)	5 (3)	21 (3)
DVT contralateral from kidney transplantate*	4 (2)	7 (2)	2 (1)	13 (2)
Pulmonary embolism	2 (1)	7 (2)	9 (6)	18 (3)
History of transplant rejection, no. (%)	11 (7)	35 (12)	18 (12)	64 (11)
sVCAM-1 (ng/mL), mean ( $\pm$ SD)	896 (343)	1088 (451)	1091 (416)	1035 (432)
CMV IgG > 250 IU/mL	NA	50 (18)	25 (16)	75 (12)
Serum creatinine ( $\mu$ mol/L), mean ( $\pm$ SD)	146 (52)	165 (85)	163 (100)	159 (82)
24-h creatinine clearance, mean ( $\pm$ SD)	58 (20)	53 (25)	52 (22)	53 (23)
Thrombocytopenia, <100 X 10 <sup>9</sup> /L, no. (%)	3 (2)	3 (1)	2 (1)	8 (1)

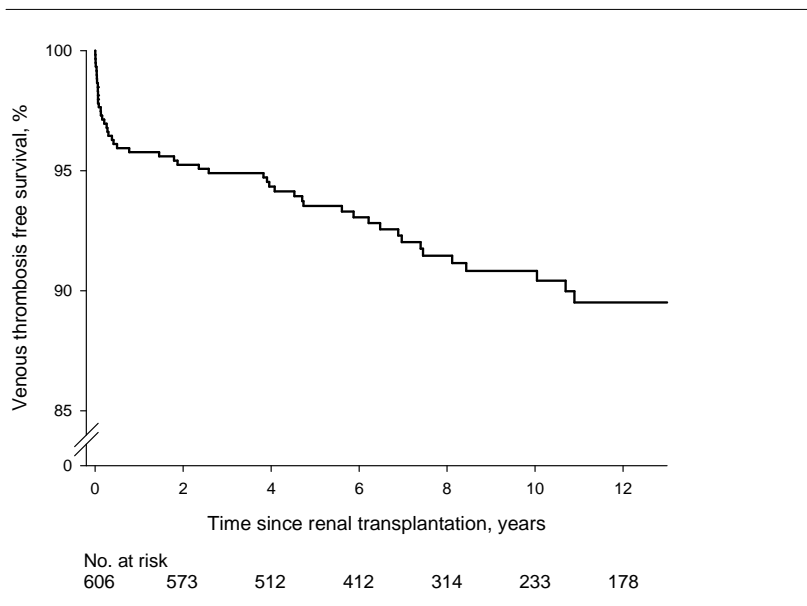
\* Two renal transplant recipients had axillary vein thrombosis. SD, denotes standard deviation; NA, not applicable.

**RESULTS**

Clinical characteristics of 606 renal transplant recipients who were enrolled are summarized in Table 1. Forty-five percent were women. Median age at enrollment was 55 years (range, 23-83 years), and 44 years at transplantation (range, 15-77 years). All recipients routinely received perioperative thromboprophylaxis.

Venous thrombosis had occurred in 52 recipients (9%) after transplantation. Median age at onset was 51 years (range, 21-69 years). Of these patients, 37% had venous thrombosis within the first three months, 40% had deep vein thrombosis on the ipsilateral side of the kidney transplant, 25% had deep vein thrombosis on the contralateral side of the transplant, and 35% had pulmonary embolism. Venous thrombotic event-free survival showed that the highest risk of venous thrombosis was in the first six months, but patients remained at risk over the next 10 years (Figure 1). Recipients received oral anticoagulants because of their first venous thrombosis for a median time of 6 months (range, 3-156 months). Seven of them still received oral anticoagulants for this reason at date of enrollment and were excluded from analysis of recurrent venous thrombosis.

**Figure 1.** First venous thrombosis event-free survival in renal transplant recipients



Annual incidence of venous thrombosis was 0.88% (95% CI, 0.65-1.15) in all recipients (Table 2). It was 0.59% (95% CI, 0.41-0.83) when excluding the first three months after renal transplant surgery from observation time. Recipients who were 50 years or older tended to have a higher risk of venous thrombosis than younger recipients; corrected relative risk 1.7 (95% CI, 0.9-3.5). Annual incidence of venous thrombosis in females was 0.75% (95% CI, 0.46-1.14) compared to 1.00% (95% CI, 0.68-1.41) in males; corrected relative risk 0.9 (95% CI, 0.4-1.6). In CMV seronegative recipients annual incidence of venous thrombosis was 0.81% (95% CI, 0.11-0.82), in CMV seropositive recipients 0.98% (95% CI, 0.62-1.46), and in CMV seroconverted recipients 0.81% (95% CI, 0.46-1.31); corrected relative risks compared to CMV seronegative recipients were 2.0 (95% CI, 0.9-5.2) and 1.7 (95% CI, 0.6-4.7), respectively.

In recipients who were CMV seropositive, mean sVCAM-1 levels were 1088 ng/mL and in CMV seroconverted recipients 1035 ng/mL, which were both higher compared to CMV seronegative recipients (mean sVCAM level 896 ng/mL;  $P < 0.001$ ). In addition, recipients with elevated CMV IgG antibodies had also increased sVCAM-1 levels (mean level 1174 ng/ml vs 1015 ng/ml;  $P < 0.001$ ). In recipients who experienced graft rejection, soluble VCAM-1 levels were higher than in patients who had no history of graft rejection (mean level 1921 ng/mL vs 1148

**Table 2.** Risk of first venous thrombosis associated with age, sex and CMV status

	Observation time (years)	Pt. with event	Annual incidence, % (95% CI)	Corrected annual incidence, %* (95% CI)	Crude relative risk (95% CI)	Corrected relative risk* (95% CI)
<b>Age (years)</b>						
All	5941	52	0.88 (0.65-1.15)	0.59 (0.41-0.83)	-	
15-50	3251	25	0.77 (0.50-1.14)	0.45 (0.25-0.76)	Reference	Reference
≥ 50	2690	27	1.00 (0.66-1.46)	0.78 (0.47-1.22)	1.3 (0.8-2.2)	1.7 (0.9-3.5)
<b>Sex</b>						
Male	3115	31	1.00 (0.68-1.41)	0.63 (0.37-0.99)	Reference	Reference
Female	2826	21	0.75 (0.46-1.14)	0.55 (0.31-0.92)	0.8 (0.4-1.3)	0.9 (0.4-1.6)
<b>CMV status</b>						
Seronegative	1597	13	0.81 (0.43-1.39)	0.33 (0.11-0.77)	Reference	Reference
Seropositive	2359	23	0.98 (0.62-1.46)	0.78 (0.45-1.25)	1.2 (0.6-2.4)	2.0 (0.9-5.2)
Seroconversion <sup>†</sup>	1985	16	0.81 (0.46-1.31)	0.59 (0.29-1.05)	1.0 (0.5-2.1)	1.7 (0.6-4.7)

\* Annual incidence and relative risk corrected for anticoagulation use, renal transplant surgery induced venous thrombosis, and age, where appropriate.

† Of 152 CMV seroconverted recipients, 146 received a CMV seropositive kidney.

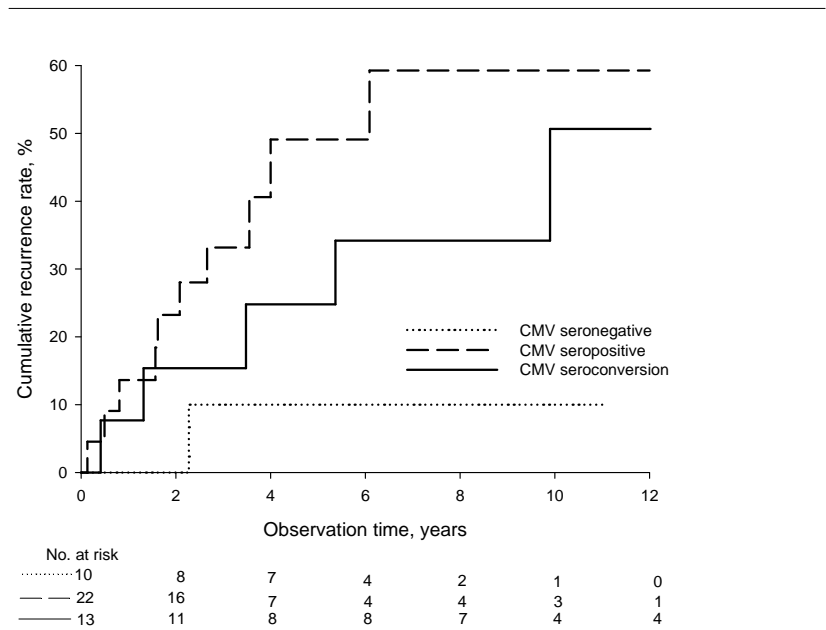
ng/mL;  $P=0.024$ ). Fifty-three of the 64 recipients (83%) with a history of graft rejection were CMV seropositive at time of enrollment, compared to 384 (71%) recipients who had no history of graft rejection ( $P=0.044$ ). Crude relative risk of venous thrombosis in recipients with graft rejection versus no graft rejection was 1.6 (95% CI, 0.7-3.4).

Within five years after withdrawal of oral anticoagulant treatment, 36% of recipients had recurrent venous thrombosis, and within ten years 45% of recipients. Subgroup analysis showed that 10% of CMV seronegative recipients had a recurrence within ten years, while in CMV seroconverted and seropositive recipients this risk was 51% and 59%, respectively (Figure 2).

## DISCUSSION

In this cohort of renal transplant recipients, our most notable findings were a high incidence of first venous thrombosis and a high risk of recurrence. Overall, the absolute risk of first venous thrombosis was 8.8 times higher than reported in the

**Figure 2.** Cumulative recurrent rate of venous thrombosis in renal transplant recipients



general population (i.e. 0.1%) [18]. Although renal transplant surgery had a considerable effect on the absolute risk of venous thrombosis, it remained 6 times higher compared to the general population after correction for the first three postoperative months. Patients remain at increased risk of venous thrombosis for 6 weeks after other major surgical procedures, like hip and knee arthroplasty [27]. Therefore, it is unlikely that the persisting high risk more than three months after renal transplantation is attributable to surgery. Despite our finding that aging is a possible confounder, recipients younger than 50 years were still at increased risk of venous thrombosis. The prevalence of 9% of venous thrombosis in our study cohort is in agreement with two previous studies [2,3], whereas another retrospective cohort study found an annual incidence of 0.29% of venous thrombosis [23]. In the latter study, however, diagnosis of venous thrombosis relied on Medicare claims, and the follow-up of recipients in that study was limited to 1.5 to 3 years after transplantation, which may have caused an underestimation of the annual incidence of venous thrombosis. To our knowledge, no other studies have reported annual incidences of venous thrombosis in renal transplant recipients. Recipients that died before the date of enrollment, were not included in the study. Thus even though annual incidences of venous thrombosis were high in our study, it is possible that these risks are underestimated, especially when considering that pulmonary embolism is an important cause of death in renal transplant recipients [24].

Although CMV seroconverted recipients and CMV seropositive recipients in our study had a 1.7 and 2.0 fold increased risk of first venous thrombosis compared to CMV negative patients, respectively, the differences were not statistically significant. It is possible that this is a consequence of relatively small numbers of patients in this analysis of subgroups. Another explanation might be that the risk of CMV induced venous thrombosis in our population was diluted, assuming that CMV is mainly thrombogenic at time of primary infection or reactivation [19,20]. Unfortunately, this information was not available in our patients. As venous thrombosis within three months after transplantation was considered as postoperative, CMV induced venous thrombosis in this period was possibly missed. However, 5% of CMV seronegative versus 3% of CMV seroconverted, and 2% of CMV seropositive renal transplant recipients had venous thrombosis within the first three months postsurgery, making it less likely that this has influenced our results. The hypothesis that CMV increases the risk of venous thrombosis does however seem likely when considering the high rate of recurrent venous thrombosis in our patients, i.e. 51% and 59% in CMV seroconverted and seropositive recipients, com-

pared to 10% in CMV seronegative recipients. These findings, however, have to be handled with caution, as numbers were small. Elevated CMV IgG antibodies were associated with higher sVCAM-1 levels compared to recipients with low CMV IgG antibodies, while graft rejection was associated with elevated sVCAM-1 levels and tended to be more common in patients who had CMV infection and venous thrombosis. These findings support the hypothesis that CMV infection is associated with vascular damage, and that vascular damage during CMV infection is enhanced if patients experienced graft rejection, as postulated in another study [25]. This might result in an increased risk of venous thrombosis. Since we only had information on CMV IgG levels at time of enrollment and not on CMV IgM levels or antigenemia, we may have incorrectly included recipients as having a non-active CMV infection while they had an active CMV infection. As other seroepidemiological studies which primarily addressed the effect of cytomegalovirus on the risk of venous thrombosis are lacking, it is difficult to compare our results with those from other studies. However, one other study reported a prevalence of 34% of active CMV infection in renal transplant recipients who had first venous thrombosis [2]. This result was not further discussed by the authors, but seems in line with our findings. Another study failed to show CMV-viraemia in renal transplant recipients with recurrent venous thrombosis compared to recipients without recurrence [3]. This might be explained as the diagnosis of active CMV infection in that study was made with a PCR method [21], from which it is known that it only detects viraemia levels for a short time, while a patient or doctor delay for diagnosing venous thrombosis is not uncommon in clinical practice. Unfortunately, they did not measure CMV antibodies, which remain increased up till months after initial infection or reactivation and are lifelong detectable. Hence, that study might have missed active CMV infection at time of diagnosing recurrent venous thrombosis. In the absence of a controlled prospective study and because our results of the role of CMV infection in the development of venous thrombosis are not statistically significant, we cannot claim a cause-and-effect relationship of cytomegalovirus and the risk of venous thrombosis.

The risk of 36% to develop recurrent venous thrombosis within five years and 45% within ten years is in agreement with others, who found a risk of 46-60% of recurrent venous thrombosis in renal transplant recipients over a similar time-period [2,3]. In the general population the risk of recurrent venous thrombosis is 18% within ten years [27], and in antithrombin, protein C, or protein S deficient patients the risk of recurrence is 23% within five years [28]. This shows that the

risk of recurrent venous thrombosis in renal transplant recipients is excessive, and at least demands the consideration whether renal transplant recipients should have prolonged anticoagulant treatment after their first venous thrombotic episode. On the other hand, our results may have been overstated, as none of our patients were screened for thrombophilia. We cannot rule out whether these patients had inherited thrombophilia. However, deficiencies of antithrombin, protein C or protein S are rare, even in patients who had venous thrombosis [29]. Other inheritable thrombophilic risk factors are less likely to have contributed to the high risk of recurrence, as it appears that they do not influence this risk [30]. Although other studies showed that recurrences of venous thrombosis in renal transplant recipients were often spontaneous [2,3], a comment on this is difficult as medical charts often did not provide sufficient information to classify recurrences as spontaneous or secondary to external risk factors. It seems likely that most recurrences were spontaneous, because our recipients usually received thromboprophylaxis at exposure to risk factors such as surgery, pregnancy/puerperium, trauma or immobilization, whereas the use of oral contraceptives and hormonal replacement therapy were strongly discouraged after a first episode of venous thrombosis.

In conclusion, renal transplant recipients are at high risk of first venous thrombosis and recurrence. CMV infection tended to be associated with an increased risk of (recurrent) venous thrombosis. Controlled prospective studies are warranted to establish this observation, which suggests that CMV infection contributes to the high risk of (recurrent) venous thrombosis in renal transplant recipients.

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Chapter **8**

**Absolute risk of venous and arterial thrombosis  
in HIV-infected patients and effects  
of combination antiretroviral therapy**

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## ABSTRACT

**Background:** Several reports noticed an increased risk of venous and arterial thrombosis in HIV-infected patients, who received combination antiretroviral therapy.

**Methods:** Medical records of 519 HIV-infected patients, who were registered in our hospital from January 1989 through December 2004, were reviewed, to assess the absolute risk of venous and arterial thrombosis in HIV-infected patients and the effect of antiretroviral therapy.

**Results:** Annual incidences of venous and arterial thrombosis were 0.65% (95% CI, 0.39-0.92) and 0.45% (95% CI, 0.24-0.77), respectively. In patients who received combination antiretroviral therapy, the annual incidence of venous thrombosis was 0.72% (95% CI, 0.39-1.29), versus 0.58% (95% CI, 0.25-1.14) in patients who did not receive these drugs. For arterial thrombosis, annual incidences were 0.46% (95% CI, 0.18-0.95) and 0.43% (95% CI, 0.16-0.95); relative risk (RR) 1.1 (0.4-3.1). In patients who used a protease inhibitor, annual incidence of venous and arterial thrombosis increased to 0.80% (95% CI, 0.40-1.43) and 0.51% (95% CI, 0.20-1.05), respectively; RR's were 1.4 (95% CI, 0.6-3.4) for venous thrombosis and 1.2 (0.4-3.5) for arterial thrombosis.

**Conclusion:** The absolute risk of venous and arterial thrombosis in HIV-infected patients was 2-6 times higher compared to the general population, irrespective of antiretroviral therapy use. This suggests a pathophysiological role for HIV infection in the risk development of venous and arterial thrombosis which needs to be further unraveled.

Several reports noticed an increased risk of venous thrombosis and arterial thrombosis in HIV-infected patients, who received combination antiretroviral therapy [1-3]. Whether HIV-infected patients, who are not on combination antiretroviral therapy are also at risk of thrombosis is largely unknown.

To assess the absolute risk of venous and arterial thrombosis in HIV-infected patients and the effect of antiretroviral therapy, we reviewed medical records of 519 HIV-infected patients, who were registered in our hospital from January 1989 through December 2004. HIV infection was documented by HIV-1 antibody ELISA and confirmatory Western blot analysis or by PCR-detectable HIV load. Venous thrombosis included deep vein thrombosis, pulmonary embolism and vein thrombosis at other sites, established by objective techniques [4]. Arterial thrombosis was diagnosed when a patient had either myocardial infarction, ischemic stroke, transient ischemic attack (TIA), or symptomatic peripheral artery occlusive disease, in accordance with earlier described diagnostic criteria [4].

For each patient with a thrombotic event, we collected detailed information regarding history of malignancies, opportunistic infections, smoking, family history of thrombosis, medication history, cholesterol and CD4 counts. Patients with venous thrombosis were tested for thrombophilia, including deficiencies of protein S, protein C, antithrombin, factor V Leiden, prothrombin G20210A mutation and elevated levels (>150%) of factors VIII:C, IX:C and XI:C as previously described [4].

A total of 519 consecutive patients were enrolled; 77% were male. Median age at HIV diagnosis was 35 years (range, 12-77). Venous thrombosis had occurred in 19 patients (4%); 12 had deep vein thrombosis, 5 pulmonary embolism and 2 cerebral vein thrombosis. Median age at onset of venous thrombosis was 46 years (range, 17-64). Arterial thrombosis had occurred in 13 patients (3%). Their median age was 45 years (range, 37-56). Five patients had symptomatic peripheral artery occlusive disease, 4 ischemic stroke, 3 myocardial infarction and one patient had a TIA. The median time from HIV diagnosis to venous thrombosis was 1 year (range, 0-12) and to arterial thrombosis 5 years (range, 0-9). Median CD4 counts at time of venous thrombosis was 130 cells/ $\mu$ L (range, 2-630) and at time of arterial thrombosis 252 cells/ $\mu$ L (range, 4-465).

Several risk factors for thrombosis were identified. Of 19 patients with venous thrombosis, 2 had their event postpartum, 1 had a family history with venous thrombosis in a first-degree relative, 2 had malignancies. Of these patients, 11 were tested for thrombophilia. Abnormal findings were observed in 9 patients, including

free protein S deficiency (n=7), elevated factor VIII:C levels (n=9) and factor V Leiden (n=2). Of 13 patients with arterial thrombosis, 10 were active smokers, 3 had a family history, 6 hypercholesterolemia, and 3 hypertension.

Annual incidences of venous and arterial thrombosis were 0.65% (95% CI, 0.39-0.92) and 0.45% (95% CI, 0.24-0.77), respectively (Table 1). Patients who were 45 years or older had a higher risk of venous or arterial thrombosis than younger patients. Although females showed lower annual incidences of venous and arterial thrombosis compared to males, the differences were not statistically sig-

**Table 1.** Risk of venous and arterial thrombosis associated with age, sex and drug therapy

	<b>Observation time* (years)</b>	<b>Pt no. with event</b>	<b>Annual incidence (per 100 patient years)</b>	<b>Relative Risk (95% CI)</b>
<b>Venous thrombosis</b>				
Age (years)				
All	2911	19	0.65 (0.39-0.92)	-
12-45	2136	9	0.42 (0.19-0.80)	Reference
≥ 45	775	10	1.29 (0.62-2.37)	3.1 (1.2-7.5)
Sex				
Male	2355	17	0.72 (0.42-1.16)	Reference
Female	556	2	0.36 (0.04-1.30)	0.5 (0.1-2.2)
Drug therapy				
No combination ART	1383	8	0.58 (0.25-1.14)	Reference
Combination ART	1528	11	0.72 (0.36-1.29)	1.3 (0.5-3.1)
Any PI combination	1381	11	0.80 (0.40-1.43)	1.4 (0.6-3.4)
<b>Arterial thrombosis</b>				
Age (years)				
All	2905	13	0.45 (0.24-0.77)	-
12-45	2144	5	0.23 (0.07-0.54)	Reference
≥ 45	761	7	0.92 (0.37-1.89)	4.0 (1.3-12.6)
Sex				
Male	2350	11	0.47 (0.23-0.84)	Reference
Female	555	2	0.36 (0.04-1.30)	0.8 (0.2-3.5)
Drug therapy				
No combination ART	1380	6	0.43 (0.16-0.95)	Reference
Combination ART	1525	7	0.46 (0.18-0.95)	1.1 (0.4-3.1)
Any PI combination	1377	7	0.51 (0.20-1.05)	1.2 (0.4-3.5)

\* Period from HIV-diagnosis or start of combination ART until the first episode of thrombosis or the end of follow-up.

ART denotes antiretroviral therapy; PI, protease inhibitor.

nificant. In patients who received combination antiretroviral therapy, the annual incidence of venous thrombosis was 0.72% (95% CI, 0.39-1.29), versus 0.58% (95% CI, 0.25-1.14) in patients who did not receive these drugs. For arterial thrombosis, annual incidences were 0.46% (95% CI, 0.18-0.95) and 0.43% (95% CI, 0.16-0.95); relative risk (RR) 1.1 (95% CI, 0.4-3.1). In patients who used a protease inhibitor, annual incidence of venous and arterial thrombosis increased to 0.80% (95% CI, 0.40-1.43) and 0.51% (95% CI, 0.20-1.05), respectively; RR's were 1.4 (95% CI, 0.6-3.4) for venous thrombosis and 1.2 (95% CI, 0.4-3.5) for arterial thrombosis.

Overall, the absolute risk of in our patient group was 6.5 times higher than reported in the general population (i.e. 0.1%) [5] and comparable with heterozygous carriers of factor V Leiden [6]. Despite our finding that ageing is an important confounder, median age at onset of venous thrombosis was 16 years less than the median age at onset of venous thrombosis in the community [7]. Hence, our patients were at higher risk of venous thrombosis, irrespective of their age.

Decreased free protein S levels were demonstrated in 7 of 11 tested patients with venous thrombosis. This finding is in agreement with previous studies [8,9]. Moreover, 9 of these patients had elevated levels factor VIII:C. Because we do not routinely screen for thrombophilia in HIV-infected patients it remains unclarified whether abnormal levels of these proteins contributed to the risk of venous thrombosis.

In the Framingham study, age related annual incidences of arterial thrombosis in men were 0.07% (aged < 45 years), 0.15% (45-54 years), 0.26% (55-64 years), and 0.39% (65-74 years) [10]. The risk of arterial thrombosis in our patients younger than 45 years was 3 fold higher, whereas it was 2-6 fold higher in patients aged 45 years or older.

Males almost had a 2 fold increased risk of venous thrombosis, compared to females. We have no explanation for this difference, which was also reported from a much larger study [1].

We observed a higher risk of venous and arterial thrombosis in patients who were on combination antiretroviral therapy. This was more pronounced when combination antiretroviral therapy contained a protease inhibitor. Thus, our data suggests an additional risk of venous and arterial thrombosis in patients who used combination antiretroviral therapy, particularly when it contained a protease inhibitor. Although not significant in our population, previous sufficiently powered studies showed a similar mild effect of combination antiretroviral therapy on the

risk of venous and arterial thrombosis [1,3]. The absolute risk of venous thrombosis in patients not using combination antiretroviral therapy was still approximately 6 times higher compared to the general population [5]. This result suggests a pathophysiological role for HIV infection in the risk development of venous thrombosis, which needs to be further unraveled. As most of our patients with arterial thrombosis were active smokers and almost half of them had hypercholesterolemia, the estimated risk of arterial thrombosis should be adjusted for these classical risk factors. However, regression analyses could not be done, as information about these risk factors was often not complete in medical charts. Other studies are needed to show whether this association is fully explained by HIV-infection itself.

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# Chapter 9

## **The relationship between progression to AIDS and thrombophilic abnormalities in HIV infection**

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## ABSTRACT

**Background:** HIV-infected patients are at increased risk of venous and arterial thrombosis. We hypothesized that acquired thrombophilic abnormalities that could predispose to thrombosis are most pronounced in patients with advanced stages of HIV.

**Methods:** A total of 109 consecutive HIV-infected patients were included and were tested twice for currently known thrombophilic abnormalities with between-test intervals of at least three months (median 3 months; range 3-12 months). Detailed information about date of HIV diagnosis, HIV treatment and previous episodes of venous and arterial thrombosis was collected.

**Results:** Following diagnosis of HIV, 16% of patients had symptomatic thrombosis (10% venous, 6% arterial). Protein C deficiency was established by repeated measurements in 9% of patients, elevated factor VIII levels in 41%, high fibrinogen levels in 22%, and free protein S deficiency in 60%. Median factor VIII levels were higher in patients with AIDS (CD4 counts <200 cells/ $\mu$ L) compared to non-AIDS-defining illness (226 IU/dL versus 149 IU/dL;  $P < 0.001$ ), while median free protein S levels were lower (45 IU/dL versus 58 IU/dL;  $P < 0.001$ ). Developing AIDS was associated with increasing factor VIII levels and with decreasing free protein S levels. Increasing factor VIII levels were correlated with increasing fibrinogen levels and decreasing free protein S levels.

**Conclusions:** Multiple acquired and persistent thrombophilic abnormalities are more frequently observed in HIV-infected patients compared to the normal population. The frequencies of these thrombophilic abnormalities increase with progression to AIDS. These findings may contribute to the high prevalence of venous and arterial thrombosis in HIV-infected patients.

## INTRODUCTION

Several reports have documented an increased risk of venous thrombosis and arterial thrombosis in HIV-infected patients [1-4]. Patients with AIDS as documented by CD4 counts < 200 cells/ $\mu$ L had a higher risk of thrombosis than HIV-infected patients with a more robust immune system [5-7]. Why HIV-infected patients are at higher risk of thrombosis is largely unknown. A link between infection and thrombosis via endothelial activation has been suggested [8,9]. The same cytokines responsible for endothelial activation are upregulated during the course of HIV infection [10,11]. These cytokines, including tumor necrosis factor  $\alpha$ , interleukin 1, and interleukin 6, activate coagulation and down-regulate the expression of fibrinolytic proteins [12,13]. Elevated levels of procoagulant proteins and decreased levels of anticoagulant proteins have been identified as risk factors for venous and arterial thrombosis [14-18]. Of these, factor VIII and fibrinogen are acute phase proteins, [15,16] that become risk factors when their levels remain elevated for a prolonged time [16,17]. Low levels of protein C have been reported in various infections, possibly due to the consumption of protein C as an anti-inflammatory mediator [19]. Inherited protein C deficiency is a strong risk factor for venous thrombosis [18], but it is unknown whether acquired deficiency is also a risk factor. Inherited protein S deficiency is another risk factor for venous thrombosis, and possibly for arterial thrombosis as well [16,18]. Approximately 60% of protein S is bound to complement C4b-binding protein, while only free protein S is active as anticoagulant [20]. During infections, the level of C4b-binding protein increases up to 400% of its normal level [20]. Some small studies, showed decreased levels of both protein S and protein C in HIV-infected patients [21-23]. Recently, a larger study of 94 HIV-infected women showed that advancing HIV-infection was associated with high factor VIII levels and a decrease of protein S activity [24]. However, in this study all women were tested only once.

We studied a group a HIV-infected patients primarily to ascertain whether progression to AIDS was associated with increased frequency and/or severity of thrombophilic abnormalities and secondarily to determine the overall risk of venous and arterial thrombosis in HIV-infected patients.

## METHODS

### Patients

Between May 2006 and December 2006, 120 consecutive patients with HIV treated at the outpatient clinic of our university hospital were asked to participate in our study. Detailed information about date of HIV diagnosis, HIV status, HIV treatment, previous episodes of venous and arterial thrombosis, exposure to risk factors for thrombosis and anticoagulant treatment was retrospectively collected by physicians at the outpatient clinic by using a questionnaire and reviewing medical records. In women, the use of oral contraceptives and their obstetric history were also documented, considering that oral contraceptives and pregnancy are risk factors for venous thrombosis, and may be associated with thrombophilic abnormalities. Clinical data were collected prior to laboratory testing to avoid bias in assessing clinical outcome events. To determine whether HIV status was correlated with thrombophilic abnormalities, blood samples were collected simultaneously for measurements of CD4 cell counts and HIV-RNA, and for thrombophilia testing. Thrombophilia tests included deficiencies of antithrombin, protein C, total protein S and free protein S, factor V Leiden, the prothrombin G20210A mutation, increased levels of fibrinogen and factor VIII, and lupus anticoagulant. Anticardiolipin antibodies were also measured. To assess effects of acute phase inflammatory reactions, CRP was also measured. All tests were repeated in a second blood sample collected after a time interval of at least 3 months (median 3 months; range, 3-12 months) to confirm the levels of proteins and numbers of CD4 cells obtained for the first set of measurements.

### Laboratory studies

Lymphocyte subsets (CD3, CD4, CD8) were analyzed within 24 hours of collection, using standard flow cytometry techniques. Plasma levels of HIV-RNA were measured with the Nuclisens HIV RNA assay (Organon Teknika) with a lower detection limit of 40 copies/mL. CRP was determined by nephelometry (BNIIN, Dade Behring). CRP levels  $\geq 5$  mg/L were used to identify inflammatory acute phase reactions. Activity of antithrombin (Coatest, Chromogenix, Mölndal, Sweden) and protein C (Berichrom Protein C; Behring, Marburg, Germany) were measured by chromogenic substrate assays, protein C and protein S antigen levels by Enzyme Linked Immuno Sorbent Assay (ELISA) (DAKO, Glostrup, Denmark). Antithrombin deficiency was defined by decreased levels of antithrombin activity

(< 65 IU/dL), protein C deficiency by decreased levels of protein C antigen (< 65 IU/dL) and/or activity (< 65 IU/dL), protein S deficiency by decreased levels of total protein S antigen (< 65 IU/dL) and/or decreased free protein S antigen levels (< 65 IU/dL), corresponding with plasma levels below the limit of their reference intervals [18]. Factor VIII:C was measured by one-stage clotting assays (Amelung GmbH, Lemgo, Germany) and was considered increased at a level above 150 IU/dL, as this level has been identified to give an increased risk of both venous and arterial thrombosis [15]. Fibrinogen levels were measured according to the Clauss method (Baxter, Miami, Fla) and were considered increased at levels higher than 3.5 g/L. Reference intervals were determined in healthy volunteers who had no personal or family history of venous thrombosis, were not pregnant, and had not used oral contraceptives during the preceding 3 months. Factor V Leiden and prothrombin G20210A were demonstrated by polymerase chain reactions [25,26]. Screening for lupus anticoagulant was performed by 3 different phospholipid-dependent coagulation tests: DRVVT (dilute Russell viper venom time), APTT (activated partial thromboplastin time) and TTI (tissue thromboplastin inhibition) [27]. Abnormal tests were repeated on a 1:1 mixture of patient plasma with normal plasma to exclude deficiencies of coagulation factors. If the test remained abnormal, phospholipid dependency was demonstrated by a phospholipid neutralization test. DRVVT was performed using reagents (LA-screen and LA-confirm) from Gradipore. APTT was performed using actin FSL (Dade Behring). TTI was performed using Thromboplastin IS (Dade Behring) in 2 dilutions (1:50 and 1:500). Anticardiolipins were measured by enzyme-linked immunosorbent assay (ELISA) in samples diluted 1:100 in phosphate-buffered saline (PBS)/10% fetal calf serum (FCS). Duplicate measurements of nine calibrators for immunoglobulin G (IgG) and IgM anticardiolipin antibodies (Louisville APL Diagnostics, Louisville, KY) were used to prepare a calibration curve according to the manufacturer's instructions. Levels  $\geq 40$  IU/mL were considered positive [27]. If patients were on long-term anticoagulant treatment with vitamin K antagonists, blood samples were taken after treatment had been interrupted; in the meantime nadroparin was given subcutaneously.

### **Definitions**

Patients were classified into three groups according to their HIV status [28]. Patients classified as asymptomatic HIV infection had a CD4 cell count of more than 500 cells/ $\mu$ L at repeated measurements; patients classified as early symptoma-

tic HIV disease had CD4 cell counts between 200 and 500 cells/ $\mu$ L; and patients with CD4 cell counts less than 200 cells/ $\mu$ L were classified as having AIDS. Venous thrombosis was considered established if deep vein thrombosis was confirmed by compression ultrasound or venography, and pulmonary embolism by ventilation and perfusion lung scanning, spiral CT scanning, or pulmonary angio-

**Table 1.** Clinical characteristics of 109 HIV-infected patients

	<b>Total</b>
No. of patients	109
Men, n (%)	72 (66)
Age at study entry, years, median (range)	41 (19-76)
Age at diagnosis of HIV, years, median (range)	34 (16-73)
Venous thrombosis, n (%)	11 (10)
Age at onset, years, median (range)	45 (22-56)
Deep vein thrombosis, n (%)	6 (6)
Pulmonary embolism, n (%)	5 (5)
Primary, n (%)	6 (6)
Secondary to	
Surgery, trauma, immobilization, n (%)	3 (3)
Oral contraception, n (%)	0 (0)
Pregnancy, puerperium, n (%)	1 (1)
Malignancy, n (%)	1 (1)
Arterial thrombosis, n (%)	6 (6)
Age at onset, years, median (range)	53 (44-59)
Myocardial infarction, n (%)	2 (2)
Ischemic stroke or TIA, n (%)	2 (2)
Peripheral arterial thrombotic event, n (%)	2 (2)
Both venous and arterial thrombosis, n (%)	2 (2)
Atherosclerotic risk factors	
Smoking, n (%)	48 (44)
Hypertension, n (%)	12 (11)
Hyperlipidemia, n (%)	17 (16)
Diabetes mellitus, n (%)	3 (3)
Highly active antiretroviral therapy, n (%)	82 (75)
HIV status	
First CD4 cell count/ $\mu$ L, median (range)	430 (20-1220)
Second CD4 cell count/ $\mu$ L, median (range)	400 (30-1220)
Difference between first and second CD4 cell count/ $\mu$ L, median, (range)	60 (0-340)
First viral load, copies/mL, median (range)	< 40 (< 40, > 10e6)
Second viral load, copies/mL, median (range)	< 40 (< 40, > 10e6)

graphy. Coronary and peripheral arterial disease had to be symptomatic and angiographically proven, whereas myocardial infarction was diagnosed according to clinical, enzymatic and electrocardiographic criteria. Ischemic stroke was defined as the onset of rapidly developing symptoms and signs of loss of cerebral function which lasted at least 24h and had an apparent vascular cause, as demonstrated by computed tomography (CT) or magnetic resonance imaging (MRI). If a cerebral event completely resolved within 24h without cerebral lesions at scanning, it was classified as a transient ischemic attack (TIA). Risk factors for atherosclerosis included known diabetes mellitus, hyperlipidemia, hypertension and active smoking.

### **Statistical analysis**

Continuous variables are expressed as median values and ranges, and categorical data as counts and percentages. Differences between groups were evaluated by the Student t test or Mann-Whitney U test, depending on the normality of data for continuous data and by Fisher exact test for categorical data. A two-tailed p-value of less than 0.05 indicated statistical significance. Medians and interquartile ranges for protein levels were calculated by group. Interquartile range included the 25th and 75th percentile values, representing the extent of variability in the sample without undue emphasis on extremes, which can occur when data are highly skewed. In box plots, whiskers extended to 1.5 times the interquartile range. Annual incidences of venous and arterial thrombosis were calculated by dividing the number of events by the number of observation years. Observation time was defined as the period from the age at HIV diagnosis until the first thrombotic episode or until the end of the observation period. When calculating the annual incidence of venous thrombosis, the occurrence of arterial thrombosis was ignored and vice versa. The 95% confidence intervals (95% CI) around the incidence rates were calculated assuming a Poisson distribution.

Because two blood samples were collected, we categorized our results as “single abnormality”, indicating a specific result in at least one blood sample, and as “confirmed abnormality”, indicating a specific result in both blood samples.

Statistical analyses were performed using SAS software, version 9.1 (SAS-Institute Inc).

## RESULTS

A total of 120 consecutive HIV-infected patients were asked to participate in the study. Of these patients, 2 failed to provide informed consent, 4 refused collection of a second blood sample, 2 died (one from liver cell carcinoma and another had Non Hodgkin lymphoma in combination with deep vein thrombosis), and 3 were lost to follow-up due to geographical reasons. The remaining 109 patients were analyzed. The median interval between HIV diagnosis and date of study entry was 5 years (range, 0-20). The clinical characteristics of the patient population are summarized in Table 1. Sixty six percent were men, and the median age at HIV diagnosis was 34 years (range, 16-73). Eleven patients (10%) were discovered to have venous thrombosis while they were HIV positive, and arterial thrombosis was found in 6 patients (6%). Seventy five percent of patients received highly active anti-retroviral therapy (HAART). The median CD4 cell count at first blood sampling was 430 cells/ $\mu$ L (range, 20-1220) and 400 cells/ $\mu$ L (range, 30-1220) at second blood sampling, with an individual median difference of 60 cells/ $\mu$ L (range, 0-340) indicating stable CD4 counts during the study period. The median actual interval between the blood collections was 3 months (range, 3-12 months).

The results of thrombophilia tests are presented in Table 2. Protein C deficien-

**Table 2.** Thrombophilic abnormalities in 109 HIV-infected patients

	First sample, n (%)	Second sample, n (%)	Single, n (%)	Confirmed, n (%)
Antithrombin deficiency	2 (2)	1 (1)	2 (2)	1 (1)
Protein C deficiency	14 (13)	11 (10)	15 (14)	10 (9)
Total protein S deficiency	3 (3)	0 (0)	3 (3)	0 (0)
FV Leiden	6 (6)	-	-	-
Prothrombin G20210A	1 (1)	-	-	-
Elevated factor VIII	51 (47)	60 (55)	66 (61)	45 (41)
Excluding CRP levels > 5 mg/L	40 (37)	45 (41)	51 (47)	34 (31)
Elevated fibrinogen levels	32 (29)	32 (29)	40 (37)	24 (22)
Excluding CRP levels > 5 mg/L	20 (18)	25 (23)	29 (27)	16 (15)
Free protein S deficiency*	72 (66)	74 (68)	81 (74)	65 (60)
Lupus anticoagulant	9 (8)	7 (6)	12 (11)	4 (4)
Anticardiolipin antibodies	2 (2)	2 (2)	2 (2)	2 (2)

\* Women neither used oral contraceptives, nor were pregnant.

Single indicates an abnormal result in at least one blood sample; confirmed indicates an abnormal result in two blood samples.

cy was confirmed in 9% of patients, elevated factor VIII levels in 41% of patients and elevated fibrinogen levels in 22% of patients. Excluding patients with CRP levels  $\geq 5$  mg/L, elevated factor VIII and fibrinogen levels were confirmed in 31% and 15% of patients, respectively. Free protein S deficiency was demonstrated in 74% of patients (confirmed in 60%). This result was not confounded by oral contraceptive use or pregnancy. None of the female patients used oral contraceptives, or were pregnant or within 6 months of delivery. Oral contraception was discouraged, considering that interactions with antiretroviral therapy make oral contraceptives less reliable. The most frequent thrombophilic abnormalities were further analyzed. Over the whole study period, median fibrinogen, factor VIII and free protein S levels were 3.6 g/L (range, 1.9-5.5), 226 IU/dL (range, 116-370), and 45 IU dL (range, 20-61) in patients with AIDS-defining illness (CD4 count  $< 200$  cells/ $\mu$ L) versus 2.9 g/L (range, 1.9-5.8;  $P= 0.062$ ), 149 IU/dL (range, 48-392;  $P< 0.001$ ), and 58 IU/dL (range, 13-121;  $P< 0.001$ ) in patients with non-AIDS-defining illness (CD4 count  $\geq 200$  cells/ $\mu$ L) (Table 3). Prevalences of persistently elevated fibrinogen levels ( $P= 0.006$ ), elevated factor VIII levels ( $P< 0.001$ ) and free protein S deficiency ( $P< 0.001$ ) were higher in patients with AIDS than in non-AIDS-defining illness.

With more advanced stages of HIV infection (CD4 counts  $> 500$  cells/ $\mu$ L vs 200-500 cells/ $\mu$ L vs  $< 200$  cells/ $\mu$ L), factor VIII levels were significantly higher, whereas free protein S levels were significantly lower (Figure 1). For fibrinogen

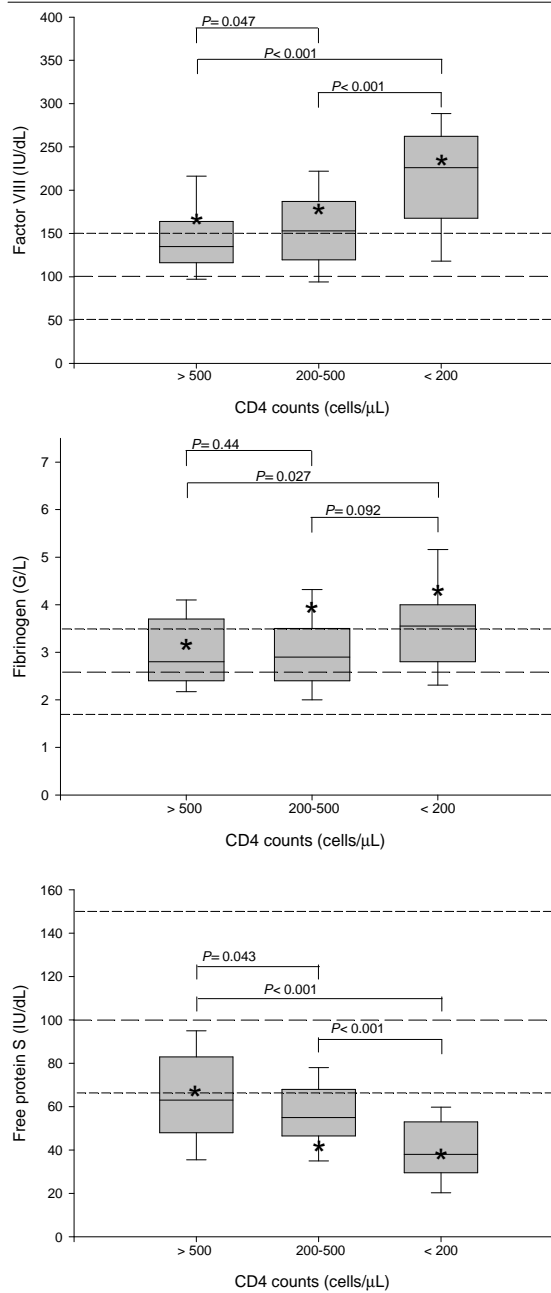
**Table 3.** Thrombophilic abnormalities related to HIV status

	AIDS defined illness (n=13)	Non-AIDS defined illness (n=90)	<i>P</i>
Male, n%	10 (77)	58 (64)	0.54
Median age at study entry, years	47 (31-64)	40 (19-76)	0.13
<b>Thrombophilia</b>			
Fibrinogen, g/L, median (range)	3.6 (1.9-5.5)	2.9 (1.9-5.8)	0.062
Confirmed elevated fibrinogen levels, n (%)	7 (54)	15 (17)	0.006
Factor VIII, IU/dL, median (range)	226 (116-370)	149 (48-392)	$< 0.001$
Confirmed elevated factor VIII, n (%)	10 (77)	33 (37)	0.013
Free protein S, IU/dL, median (range)	45 (20-61)	58 (13-121)	$< 0.001$
Confirmed free protein S deficiency, n (%)	13 (100)	48 (53)	$< 0.001$

HIV status established by repeated measurements of CD4 cell counts.

Confirmed indicates a positive result in two blood samples.

**Figure 1.** Factor VIII, fibrinogen and free protein S levels related to CD4 counts in HIV-infected patients

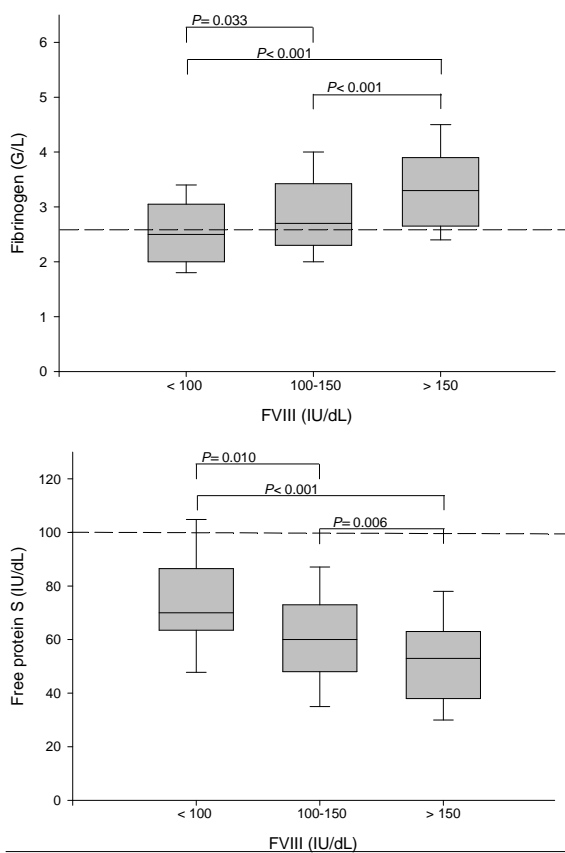


Dashed lines represent the mean reference levels with upper and lower reference interval limits. Exclusion of results from patients with thrombosis (median levels indicated with \*) did not alter the results.

levels the difference was less pronounced and only significant when asymptomatic HIV patients were compared with patients who had AIDS ( $P=0.027$ ). A positive relation was observed between increasing factor VIII levels and fibrinogen levels, while an inverse relationship was observed between increasing factor VIII levels and declining free protein S levels (Figure 2).

The overall annual incidence of venous thrombosis was 1.61% (95% CI, 0.81-2.89) and of arterial thrombosis 0.87% (95% CI, 0.32-1.88). The median age at time of the first event was 45 years (range, 22-56) for venous thrombosis and 53 years (range, 44-59) for arterial thrombosis. The median time interval between on-

**Figure 2.** Fibrinogen and free protein S levels in HIV-infected patients related to factor VIII levels



Dashed lines indicate mean reference (normal) levels.

Excluding patients with thrombosis did not alter the results.

set of venous and arterial thrombosis and first blood collection were 3.2 years (range, 0-11), and 5.0 years (range, 1.7-14.1), respectively. In univariate analysis, smoking, hyperlipidemia, hypertension and diabetes mellitus were not associated with thrombophilic abnormalities.

## **DISCUSSION**

Protein C deficiency was confirmed in 9% of HIV-infected patients, elevated factor VIII levels in 41%, elevated fibrinogen levels in 22% and free protein S deficiency in 60%. After excluding patients with high CRP levels, these prevalences remained high compared to the normal population which exhibits a prevalence rate of protein C deficiency <0.4%, a 10% rate of elevated factor VIII or fibrinogen levels, and an unknown prevalence of free protein S deficiency [14,17]. Conversely, lupus anticoagulant and anticardiolipin antibodies were not observed as frequently as in previous studies that reported lupus anticoagulant in 60% and anticardiolipin antibodies in 90% of HIV-infected patients [33,34]. However, these studies had small populations and did not follow current strict guidelines to classify anticardiolipin antibody levels and lupus anticoagulant as positive [27]. Our finding is in agreement with a more recent study of HIV-infected patients none of whom was demonstrated to have lupus anticoagulant [24].

We observed a clear relationship between advancing HIV disease and an increase of thrombophilic abnormalities. Patients with AIDS more often had elevated factor VIII levels, elevated fibrinogen levels, and free protein S deficiency than patients with non-AIDS-defining illness. These differences maybe due to HIV disease itself, considering that the same cytokines that activate the coagulation system have been described in the setting of progressive HIV [10,11]. Both elevated factor VIII and fibrinogen levels have been associated with an increased risk of venous and arterial thrombosis [15-17]. For acquired deficiencies of protein C and free protein S such an association has not been reported. It is remarkable, however, that the combination of high factor VIII and fibrinogen levels, and decreased free protein S levels have been reported in patients with systemic lupus erythematosus [35] and other auto-immune diseases [36], in patients with cytomegalovirus infections [37], and in patients with the nephrotic syndrome [38], conditions which are all associated with an increased risk of venous and arterial thrombosis [35-38]. These thrombophilic abnormalities might explain a possible

link between venous and arterial thrombosis, which has recently been suggested [39]. Because of small numbers of observations, we could not perform a proper multivariate analysis on classical cardiovascular risk factors. However, our patients often had hyperlipidemia or hypertension, considering their young median age, and a striking number were smokers, which is in accordance with other studies [4,5].

Sixteen percent of our cohort of 109 consecutive patients with HIV experienced thrombosis during a median follow-up period of five years; venous events were documented in 10%, arterial events in 6% of patients. Annual incidences of venous thrombosis (1.61%) and arterial thrombosis (0.87%) were 5-16-fold higher and 2-8 fold higher, respectively, than in the normal population (i.e. 0.1-0.3% and 0.1-0.4%) [29-31]. The median age at onset of venous thrombosis was 45 years, 17 years earlier than the median age of onset for venous thrombosis in non-HIV-infected patients [32], and was 53 years for onset of arterial thrombosis, a decade earlier than the median age of onset for arterial thrombosis in the Framingham study [31]. Although these results should be interpreted cautiously, because the population studied is small, they suggest that HIV-infected patients are at high risk of venous and arterial thrombosis, as also shown in other studies [1-4].

Although our study comprised a relatively small number of patients, it is the largest to date that analyzed acquired thrombophilic abnormalities in HIV-infected patients. Our finding that the development of AIDS was associated with increasing thrombophilic abnormalities may have clinical relevance. HAART is used for (long term) immunologic reconstitution, which may improve these thrombophilic abnormalities, leading to a decreased risk of venous and arterial thrombosis. Indeed, one study showed a decreased risk of arterial thrombosis or death in more than 36,000 HIV-infected patients who received HAART [4]. Another study reported a decrease in levels of von Willebrand factor, carrier of factor VIII, after HIV-infected patients started on HAART [40]. Larger prospective studies that address endothelial activation markers and thrombophilic abnormalities in HIV-infected patients may clarify the relation between HIV infection and venous thrombosis, and an association between venous and arterial thrombosis. Our data suggest that such a link in HIV-infected patients is plausible. Because it is not common practice to screen for thrombophilia in HIV-infected patients, medical charts often did not provide sufficient information about CD4 counts in patients at time of thrombosis, and we are, therefore, unable to comment on CD4 counts and/or thrombophilic abnormalities at time of thrombosis in our study. Our small numbers did not enable us to compare the risk of thrombosis in subgroups. Other studies, however, have shown

the risk of venous thrombosis to be highest in patients with AIDS, with an odds ratio of 29.9 (95% CI, 3.6-246.3) in patients with AIDS versus non-AIDS-defining illness [5-7], whereas free protein S deficiency and elevated factor VIII levels have been reported in 78-100% of HIV-infected patients at time of venous thrombosis [3], in apparent agreement with our findings. A further limitation of our data is that patients with HIV are a heterogeneous group with higher rates of co-infections compared to the normal population, which might have contributed to thrombophilic abnormalities as well. An analysis of these parameters was beyond the scope of our study.

We conclude that HIV-infected patients have a higher prevalence of thrombophilic abnormalities, and more persistent thrombophilic abnormalities than found in the normal population. These abnormalities increase with the development of AIDS and may contribute to the high prevalence of venous and arterial thrombosis in HIV-infected patients.

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# **Part III      Thrombophilia testing**



# Chapter 10

## **Selective testing for thrombophilia in patients with first venous thrombosis. Results from a retrospective family cohort study on absolute thrombotic risk for currently known thrombophilic defects in 2479 relatives**

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## ABSTRACT

**Background:** Screening for thrombophilia in subjects with venous thrombosis is a controversial issue.

**Methods:** In a retrospective family cohort, where probands had thrombosis and a thrombophilic defect, 2479 relatives were tested for hereditary antithrombin, protein C and protein S deficiency, factor (F)V Leiden, prothrombin G20210A, and in addition high levels of FIX, FXI, TAFI, and homocysteine.

**Findings:** In antithrombin, protein C, and protein S deficient relatives annual incidences of venous thrombosis were 1.77% (95% confidence interval [CI], 1.14-2.60), 1.52% (95% CI, 1.06-2.11) and 1.90% (95% CI, 1.32-2.64), respectively, at a median age of 29 years and a positive family history >20% symptomatic relatives. In relatives with FV Leiden, prothrombin G20210A or high FVIII levels, these were 0.49% (95% CI, 0.39-0.60), 0.34% (95% CI, 0.22-0.49) and 0.49% (95% CI, 0.41-0.51). High FIX, FXI, TAFI and hyperhomocysteinemia were not independent risk factors. Annual incidence of major bleeding in antithrombin, protein C or protein S deficient relatives on oral anticoagulant treatment was 0.29% (95% CI, 0.03-1.04). Cumulative recurrence rates in relatives with antithrombin, protein C or protein S deficiency were 19% at 2 years, 40% at 5 years and 55% at 10 years. In relatives with FV Leiden, prothrombin G20210A or high levels FVIII, these were 7%, 11% and 25%, respectively.

**Interpretation:** Considering its clinical implications, testing for thrombophilia may be restricted to hereditary deficiencies of antithrombin, protein C and protein S in patients with first venous thrombosis at young age and/or a family history in >20% of their relatives.

## INTRODUCTION

Since 1965, an increasing number of coagulation disorders have been identified as risk factors for venous thrombosis. These thrombophilic defects include hereditary deficiencies of antithrombin, protein C and protein S, factor (F) V Leiden, prothrombin G20210A, high levels of FVIII, FIX, FXI and thrombin activatable fibrinolysis inhibitor (TAFI) [1-8]. In addition, hyperhomocysteinemia showed to be a metabolic thrombophilic defect [9]. Together, their prevalence is approximately 25% in the normal population and more than 60% in subjects with venous thrombosis [10]. Venous thrombosis is now considered a multicausal disease [10]. Gene-gene interactions and environmental risk factors increase the risk of venous thrombosis. Whether patients with venous thrombosis should be tested for thrombophilic defects is still a matter of debate. Previous studies mainly addressed the relative risk of thrombosis for single thrombophilic defects [2-9]. However, clinical implications of thrombophilic defects depend on the absolute risk of first venous thrombosis and recurrence rather than the relative risk. Moreover, the absolute risk varies for different thrombophilic defects and may be increased by concomitance of other defects [1,11]. To compare the absolute risk for single and combined thrombophilic defects, sufficient numbers of particularly subjects with rare defects are required.

We performed a retrospective study in a large series of families to assess the absolute risk of first venous thrombosis and recurrence for currently known thrombophilic defects, either as single or combined defects. We also took into account whether events were idiopathic or provoked.

## METHODS

We pooled data of individual subjects from five large retrospective family cohort studies with various thrombophilic index defects, which have previously been described [1,2,5,12-15]. These studies had the same design and were performed by three university hospitals. The first study was a single center study and contained first degree relatives of consecutive patients (proband) with documented venous thrombosis and established hereditary deficiencies of either antithrombin, protein C, or protein S [1,12]. They were enrolled between April 1999 and July 2004. Three studies were multicenter studies of first degree relatives of consecutive

patients with venous thrombosis or premature atherosclerosis (< 50 years of age) and the presence of either the prothrombin G20210A mutation, high levels of FVIII at repeated measurements, or hyperhomocysteinemia [3,5,13]. Enrollment started in May 1998 and was completed in July 2004. The fifth study was a multicenter study of first degree relatives of consecutive patients with venous thrombosis and FV Leiden enrolled between May 1995 and July 1998 [2,14]. Approval was obtained from the institutional review boards of the three participating hospitals.

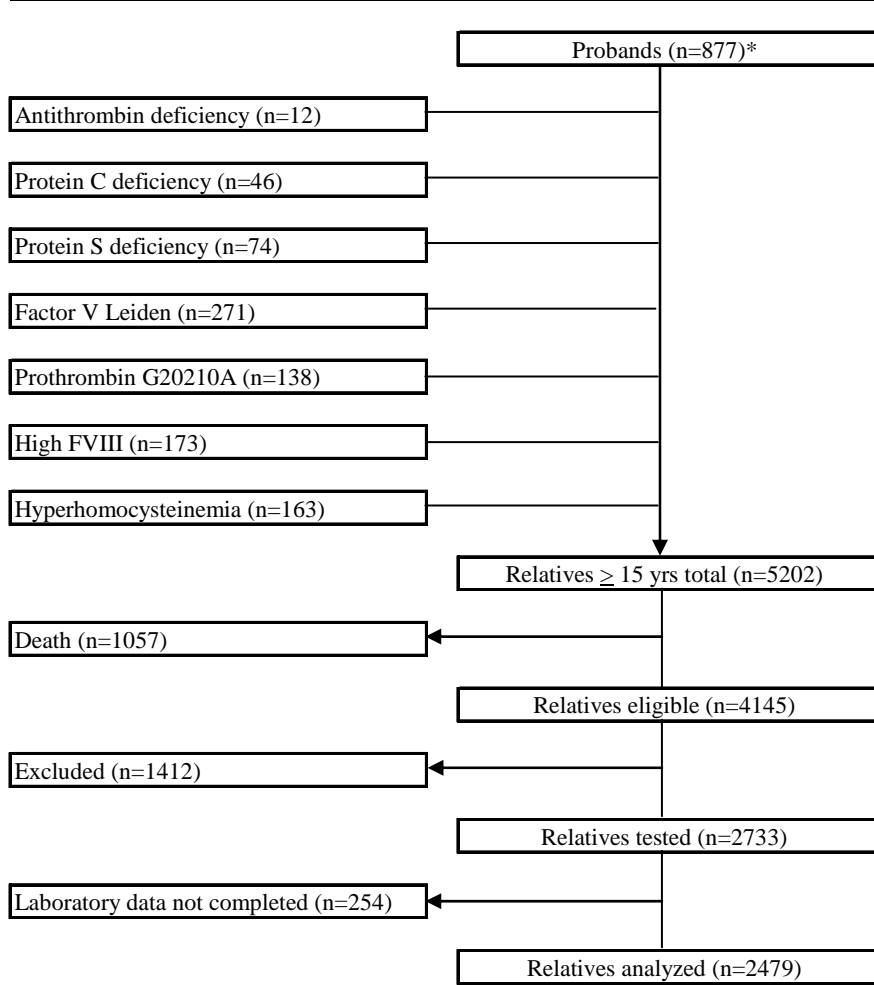
### **Subjects**

All relatives, identified by pedigree analysis, were 15 years of age or older and were contacted through the probands. All participants provided written informed consent. Physicians at the thrombosis outpatient clinics collected detailed information about previous episodes of venous thrombosis, exposure to exogenous risk factors for venous thrombosis, and anticoagulant treatment using a validated questionnaire [15], and by reviewing medical records. Clinical data was collected prior to laboratory testing. Relatives were tested for hereditary deficiencies of antithrombin, protein C and protein S, FV Leiden, prothrombin G20210A, and high levels of FVIII, regardless their index defects. In addition, high levels of FIX, FXI and TAFI, and hyperhomocysteinemia were measured in most relatives as well, but not all due to shortage of stored plasma or the inability to perform homocysteine tests for which relatives were asked to return to our outpatient clinic in a fasting state.

### **Laboratory studies**

Activity of antithrombin (Chromogenix, Mölndal, Sweden) and protein C (Behring, Marburg, Germany) were measured by chromogenic substrate assays, protein C and protein S antigen levels by Enzyme Linked Immuno Sorbent Assay (ELISA) (DAKO, Glostrup, Denmark). Antithrombin deficiency was defined by decreased levels of antithrombin activity (< 65 IU/dL), protein C deficiency by decreased levels of either protein C antigen (< 65 IU/dL) and/or activity (< 65 IU/dL), and protein S deficiency type I by decreased total protein S antigen levels (< 65 IU/dL), corresponding with plasma levels below the lower limit of their normal ranges [1]. Strict criteria for inheritance of deficiencies were used [1]. Protein S type III deficiency was not considered because it was not a risk factor for venous thrombosis in a recent study [12]. FV Leiden and prothrombin G20210A were demonstrated by polymerase chain reactions [16,17]. Factors VIII:C, IX:C, and XI:C

**Figure 1.** Flow diagram of the family cohort



\* Probands were classified according to their index defect. In case of co-segregation, the index defect was chosen in the following order: antithrombin deficiency, protein C deficiency, protein S deficiency, FV Leiden, prothrombin G20210A, high factor VIII levels, hyperhomocysteinaemia.

were measured by one-stage clotting assays (Amelung GmbH, Lemgo, Germany) and were increased at levels above 150 IU/dL [4-7]. TAFI activity was measured by chromogenic substrate assay (Pentafarm, Basel, Switzerland). TAFI levels above the 95<sup>th</sup> percentile were defined as increased (> 125 U/dL). Levels of homocysteine were measured by high-performance liquid chromatography after

overnight fasting [19]. Hyperhomocysteinemia was defined as a fasting homocysteine level  $> 18.5 \mu\text{mol/L}$  [9]. Lupus anticoagulant and anticardiolipin antibodies, using previously described tests [1], were rarely demonstrated and therefore not evaluated. If relatives were on treatment with acenocoumarol, a short acting vitamin K antagonist, blood samples were taken after treatment had been interrupted for at least two weeks; meanwhile nadroparin was given subcutaneously.

### Clinical endpoints

Venous thrombosis was considered established if deep vein thrombosis was confirmed by compression ultrasound or venography, and pulmonary embolism by ventilation and perfusion lung scanning, spiral CT scanning or pulmonary angiography, or when the patient had received full dose heparin and a vitamin K antagonist for at least 3 months without objective testing at a time when these techniques were not yet available. Provoked venous thrombosis was defined if it had occurred at or within 3 months after exposure to exogenous risk factors including surgery, trauma, immobilization for more than 7 days, pregnancy, puerperium, the use of oral contraceptives or hormonal replacement therapy, or malignancy. In the absence of these risk factors venous thrombosis was considered idiopathic.

From relatives with an antithrombin, protein C or protein S deficiency, we retrieved information on bleeding events associated with the treatment with vitamin K antagonists. Clinically overt bleedings, which required hospitalization or blood transfusion, were intracranial or retroperitoneal, or if they led directly to death were classified as major.

**Table 1.** Risk of first venous thrombosis associated with thrombophilic defects\*

Index defect	Observation years	Relatives with event	Annual incidence, % (95% CI)	Adjusted Relative risk <sup>†</sup> (95% CI)
Antithrombin deficiency (n=60)	1416	25	1.77 (1.14-2.60)	28.2 (13.5-58.6)
Protein C deficiency (n=91)	2301	35	1.52 (1.06-2.11)	24.1 (13.7-42.4)
Protein S deficiency (n=94)	1846	35	1.90 (1.32-2.64)	30.6 (26.9-55.3)
High FVIII (n=776)	26315	130	0.49 (0.41-0.51)	7.1 (4.3-11.8)
Factor V Leiden (n=649)	18237	89	0.49 (0.39-0.60)	7.5 (4.4-12.6)
Prothrombin G20210A (n=288)	8324	28	0.34 (0.22-0.49)	5.2 (2.8-9.7)

\* As concomitance of defects occurred frequently, relatives could be counted twice or more.

<sup>†</sup> Adjusted for age, sex and clustering in families and compared to relatives with none of the mentioned defects (annual incidence 0.05%; 95% CI, 0.02-0.08).

### Statistical analysis

We assessed the absolute risk of venous thrombosis in relatives with deficiencies of antithrombin, protein C, or protein S, FV Leiden, prothrombin G20210A or high levels of FVIII. Relatives with more than one thrombophilic defect were assigned to each of the corresponding subgroups, to calculate the absolute risk for each defect separately, either as single defect or as combined defects. Relatives were excluded when the laboratory set of these six thrombophilic defects was not completed. Probands were excluded from analysis to avoid bias. Possible interactions between high FVIII levels and high levels of FIX, FXI or TAFI, or hyperhomocysteinemia were analyzed by comparing the risk of venous thrombosis for each of the latter defects separately and in combination with high FVIII levels to the risk in relatives without these defects. Observation time was defined as the period from the age of 15 years until the first thrombotic episode or the end of study. Clinical data and blood samples were collected at the end of the observation period. Hence, treatment and clinical outcome were not influenced by the results of thrombophilia testing.

Event free survival for first venous thrombosis and recurrence, respectively were analyzed by the Kaplan-Meier method. Cumulative recurrence rates were calculated over the period from the end of anticoagulant treatment after the first episode of venous thrombosis until either the date of first recurrence or the end of study. We pooled data when cumulative incidences of first venous thrombosis in relatives with different thrombophilic index defects were comparable, to assess the effects of exogenous risk factors. Relative risks were adjusted for age, sex and clustering of defects within families using a Cox regression model with venous thrombosis as dependent variable and thrombophilic defects as independent variables, including interaction terms.

Continuous variables are expressed as median values and ranges; categorical data as counts and percentages. Differences between groups were evaluated by Student t test or Mann-Whitney U test, depending on the normality of data for continuous data, and by Fisher exact test for categorical data. A two-tailed *P*-value < 0.05 indicates statistical significance. The 95% confidence intervals (95% CI) around the incidence rates were calculated under the Poisson distribution assumption.

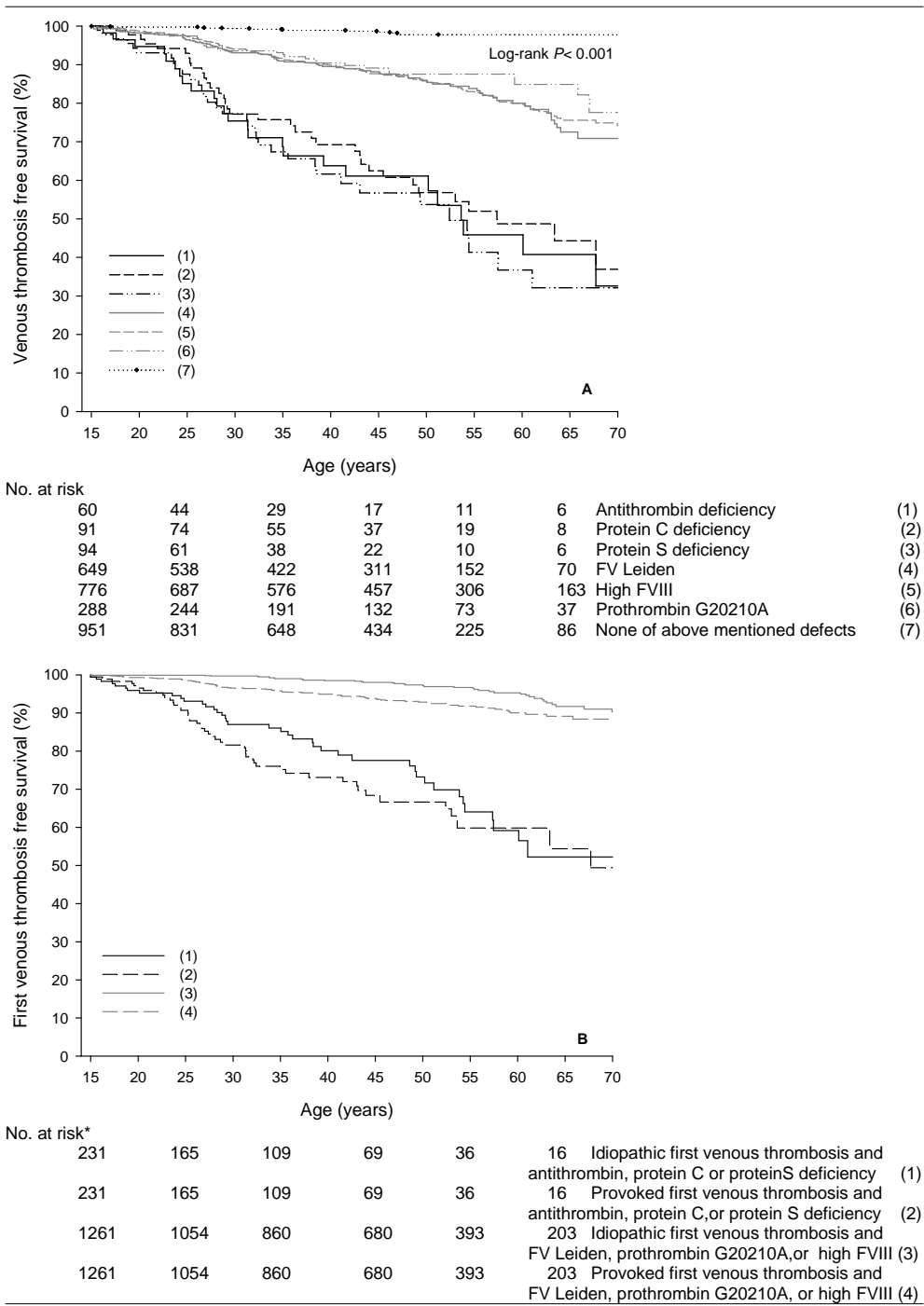
Statistical analyses were performed using SAS software, version 9.1 (SAS-Institute inc., Cary, North Carolina).

## RESULTS

Our study cohort contained 877 probands with 5202 relatives, who were 15 years of age or older (Figure 1). Of probands, median age at enrollment was 46 years (range, 9-89) and median age at onset of first venous thrombosis was 34 years (range, 8-88). Median age at onset of first venous thrombosis was 28 years (range, 12-68) in probands with antithrombin, protein C or protein S deficiency and 35 years (range, 8-88) in probands with FV Leiden, prothrombin G20210A or elevated FVIII levels. Prevalence of venous thrombosis in relatives of probands with antithrombin, protein C or protein S deficiency was 83/400 (21%), which was higher than in relatives of probands with FV Leiden, prothrombin G20210A, or high factor VIII levels (118/1431; 8%,  $P < 0.001$ ) or in relatives of probands with none of these thrombophilic abnormalities (28/648; 4%,  $P < 0.001$ ). To avoid selection bias, probands were not further analyzed. Of relatives, 1057 (20%) had died before the start of the study; 21% of relatives of probands with deficiencies of antithrombin, protein C or protein S, and 20% of relatives of probands with other defects. Another 1412 relatives did not participate because of various reasons, including refusal or inability to give informed consent or residence outside the Netherlands, and 254 relatives were not evaluable because the set of thrombophilic tests was not performed completely. Forty-five percent were male. Median age at enrollment was 46 years (range, 15-92). Median observation period was 30 years (range, 0-77). Venous thrombosis had occurred in 229 relatives (9%). Of these events, 91 (40%) were idiopathic, 31 (14%) were associated with oral contraceptives (in women, 22%), 40 (17%) with pregnancy/puerperium (in women, 29%), 65 (28%) with surgery, trauma or immobilization and 1 (0.4%) with malignancy. Median age at onset of the first episode of venous thrombosis was 35 years (range, 15-84). Relatives with antithrombin, protein C, or protein S deficiency had venous thrombosis at younger age than relatives with FV Leiden, prothrombin G20210A, or high FVIII levels (median 29 years vs. 40 years;  $P < 0.001$ ), either idiopathic (median 34 years vs. 53 years;  $P < 0.001$ ) or provoked (median 27 vs. 34 years;  $P = 0.022$ ).

Annual incidences of venous thrombosis in relatives with antithrombin, protein C or protein S deficiency, possible concomitance of other thrombophilic defects not taking into account, were 1.77% (95% CI 1.14-2.60), 1.52% (95% CI, 1.06-2.11), and 1.90% (95% CI, 1.32-1.64), respectively, and in relatives with FV Leiden, prothrombin G20210A, or high FVIII levels 0.49% (95% CI, 0.39-0.60), 0.34% (95% CI, 0.22-0.49) and 0.49% (95% CI, 0.41-0.51), respectively (Table 1).

**Figure 2.** Event free survival of first venous thrombosis in relatives with thrombophilic defects



\* When analyzing idiopathic events, provoked events were censored and vice versa.

Including the 254 relatives who were not completely tested for these six thrombophilic defects did not substantially change risk estimates. Survival analysis showed that relatives with these defects were at continuous risk of venous thrombosis as compared to relatives in whom none of these defects were demonstrated ( $P < 0.001$ ), (Figure 2A). Because annual and cumulative incidences of first venous thrombosis were similar in antithrombin, protein C or protein S deficient relatives, as were these in relatives with FV Leiden, prothrombin G20210A, or high FVIII levels, relatives were pooled in two groups to compare provoked versus idiopathic venous thrombosis (Figure 2B). Provoked events were observed more frequently at age 20-35 years. This difference was more pronounced in the group of relatives with anticoagulant deficiencies. Overall, lifetime risk of first venous thrombosis was comparable whether the first episode was provoked or not.

Relatives with high levels of FIX, FXI, or TAFI, or hyperhomocysteinemia were only at risk of venous thrombosis when they also had high FVIII levels (Table 2). Adjusted relative risks were 1.5 (95% CI, 0.9-2.3), 2.4 (95% CI, 1.5-3.8), 1.8 (95% CI, 1.0-3.6), and 2.9 (95% CI, 1.6-5.3), respectively, compared to relatives with normal levels. Excluding relatives with high FVIII levels, these were 0.3 (95% CI, 0.1-0.8), 0.8 (95% CI, 0.3-2.1), 0.5 (95% CI, 0.2-1.3), and 0.6 (95% CI, 0.2-1.4), respectively. As the risk of thrombosis associated with these defects was apparently due to high FVIII levels, they were not further considered.

The absolute risks of venous thrombosis for single thrombophilic defects and combinations are summarized in Table 3. Compared to the annual incidence of venous thrombosis in relatives with none of these defects (0.05%; 95% CI, 0.02-0.08), annual incidences were elevated in relatives with single heterozygous prothrombin G20210A (0.19%; 95% CI, 0.08-0.38), high FVIII levels (0.23%; 95% CI, 0.16-0.33), and heterozygous FV Leiden (0.22%; 95% CI, 0.13-0.33). In relatives with a single deficiency of protein C, antithrombin or protein S these were 0.54% (95% CI, 0.15-1.39), 1.15% (95% CI, 0.42-2.50), and 1.45% (95% CI, 0.66-2.75), respectively. Concomitance of one or more other defects increased the absolute risk of venous thrombosis markedly. This effect of concomitance decreased in relatives with FV Leiden, prothrombin G20210A or high FVIII levels when relatives with concomitant deficiencies of antithrombin, protein C or protein S were excluded. Double heterozygosity of FV Leiden and prothrombin G20210A or/and homozygosity of these mutations was demonstrated in 81 relatives; 23 homozygotes of FV Leiden (8 events, annual incidence 1.30%; 95% CI, 0.56-2.56); 8 homozygotes of prothrombin G20210A, (no events); 50 double heterozygotes of

these mutations (7 events, annual incidence 0.48%; 95% CI, 0.19-0.99), 6 homozygotes of FV Leiden who also were heterozygotes of prothrombin G20210A (1 event); and 3 homozygotes of prothrombin G20210A who were also heterozygotes of FV Leiden (no events). Relatives with these genotypes are hereafter indicated as 'double heterozygous/homozygous.'

After their first episode of venous thrombosis, relatives received anticoagulant

**Table 2.** Annual incidences of first episodes of venous thrombosis in relatives with high levels of FIX, FXI, or TAFI or hyperhomocysteinemia\*

	<b>Observation years</b>	<b>Relatives with event</b>	<b>Annual incidence, % (95% CI)</b>	<b>Adjusted Relative risk<sup>†</sup> (95% CI)</b>
<b>High FIX levels</b>				
Absent (n=1684)	48702	124	0.25 (0.21-0.30) <sup>‡</sup>	Reference
Present (n=280)	9751	24	0.25 (0.16-0.37)	1.0 (0.5-1.5)
With normal FVIII levels (n=136)	4400	3	0.07 (0.02-0.20)	0.3 (0.1-0.8)
With high FVIII levels (n=144)	5321	21	0.39 (0.24-0.60)	1.5 (0.9-2.3)
<b>High FXI levels</b>				
Absent (n=2192)	64350	179	0.28 (0.24-0.32) <sup>§</sup>	Reference
Present (n=148)	4798	25	0.52 (0.33-0.77)	2.2 (1.3-3.5)
With normal FVIII levels (n=62)	1858	4	0.22 (0.06-0.55)	0.8 (0.3-2.1)
With high FVIII levels (n=86)	2940	21	0.71 (0.44-1.09)	2.4 (1.5-3.8)
<b>High TAFI levels</b>				
Absent (n=1824)	53044	149	0.28 (0.24-0.33) <sup>¶</sup>	Reference
Present (n=203)	7257	21	0.29 (0.18-0.44)	1.0 (0.6-1.6)
With normal FVIII levels (n=121)	4161	6	0.14 (0.05-0.31)	0.5 (0.2-1.3)
With high FVIII levels (n=82)	3095	15	0.49 (0.27-0.80)	1.8 (1.0-3.6)
<b>Hyperhomocysteinemia</b>				
Absent (n=1642)	48075	127	0.26 (0.22-0.31) <sup>  </sup>	Reference
Present (n=190)	6507	25	0.38 (0.25-0.57)	1.7 (1.0-2.9)
With normal FVIII levels (n=112)	3380	5	0.14 (0.05-0.34)	0.6 (0.2-1.4)
With high FVIII levels (n=78)	3077	20	0.65 (0.40-1.00)	2.9 (1.6-5.3)

\* Numbers of relatives tested for levels of FIX, FXI and TAFI, and homocysteine were 1964, 2340, 2027, and 1832, respectively.

<sup>†</sup> Adjusted for age, sex, and clustering of thrombophilic defects in families.

<sup>‡</sup> Annual incidence in relatives with normal FIX and FVIII levels 0.17% (95% CI, 0.13-0.22).

<sup>§</sup> Annual incidence in relatives with normal FXI and FVIII levels 0.18% (95% CI, 0.15-0.23).

<sup>¶</sup> Annual incidence in relatives with normal TAFI and FVIII levels 0.18% (95% CI, 0.14-0.23).

<sup>||</sup> Annual incidence in relatives with normal homocysteine and FVIII levels 0.19% (95% CI, 0.15-0.24).

**Table 3.** Risk of first venous thrombosis associated with co-segregation

	Relatives with event	Annual incidence, % (95% CI)	Corrected Annual incidence, % (95% CI)*
<b>Prothrombin G20210A</b>			
Single defect (n=155)	8	0.19 (0.08-0.37) <sup>†</sup>	0.19 (0.08-0.37)
One other defect (n=95)	13	0.45 (0.24-0.77)	0.40 (0.21-0.71)
Two or more defects (n=38)	7	0.59 (0.24-1.23)	0.37 (0.04-1.32)
<b>High FVIII</b>			
Single defect (n=431)	36	0.23 (0.16-0.33)	0.23 (0.16-0.33)
One other defect (n=275)	69	0.79 (0.61-1.00)	0.49 (0.35-0.68)
Two or more defects (n=70)	25	1.13 (0.73-1.67)	0.90 (0.45-1.62)
<b>Factor V Leiden</b>			
Single defect (n=369)	25	0.25 (0.16-0.37) <sup>‡</sup>	0.25 (0.16-0.37)
One other defect (n=229)	43	0.62 (0.45-0.84)	0.53 (0.38-0.73)
Two or more defects (n=54)	21	1.54 (0.95-2.35)	0.77 (0.21-1.99)
<b>Protein C deficiency</b>			
Single defect (n=33)	4	0.54 (0.15-1.39)	-
One other defect (n=35)	19	2.02 (1.22-3.16)	-
Two or more defects (n=23)	12	1.92 (0.99-3.35)	-
<b>Antithrombin deficiency</b>			
Single defect (n=25)	6	1.15 (0.42-2.50)	-
One other defect (n=20)	10	1.89 (0.91-3.48)	-
Two or more defects (n=15)	9	2.47 (1.13-4.68)	-
<b>Protein S deficiency</b>			
Single defect (n=37)	9	1.45 (0.66-2.75)	-
One other defect (n=40)	16	1.81 (1.04-2.94)	-
Two or more defects (n=17)	10	2.92 (1.40-5.38)	-

Dotted gray area represents annual incidence of venous thrombosis in relatives without any defect (0.05%), with its 95% confidence interval (0.02-0.08%).

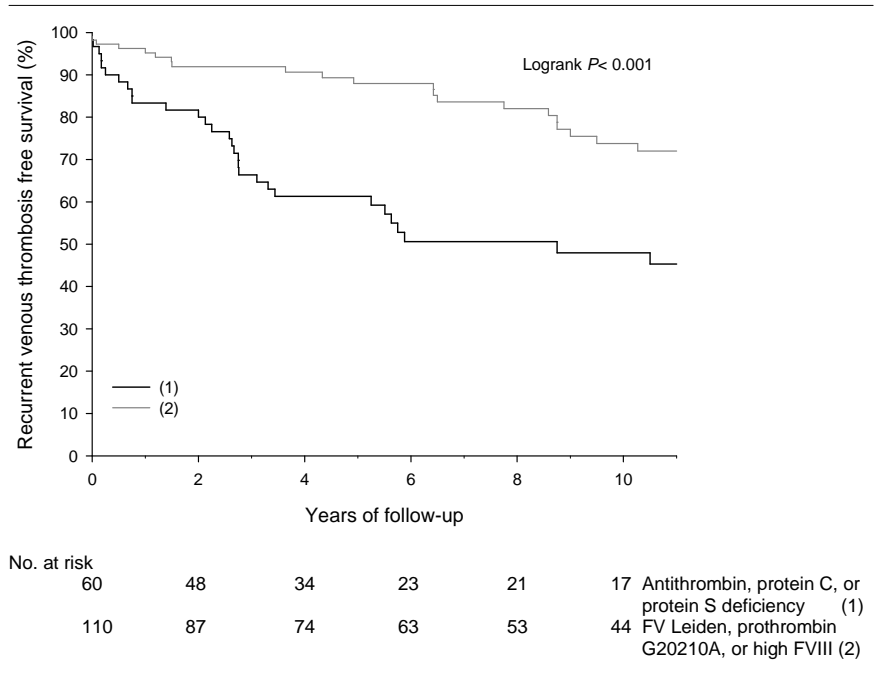
\* Excluding relatives with hereditary antithrombin, protein C, or protein S deficiency.

<sup>†</sup> 151 single heterozygous prothrombin G20210A, annual incidence 0.19% (95% CI, 0.08-0.38).

<sup>‡</sup> 359 single heterozygous Factor V Leiden, annual incidence 0.22% (95% CI, 0.13-0.33).

treatment for a median time of 6 months (range, 3-256). At enrollment, 42 relatives still received anticoagulant treatment. None of them experienced a recurrence. Of the remaining 187 relatives, 60 (32%) had antithrombin, protein C or protein S deficiency, and 110 (59%) had FV Leiden, prothrombin G20210A or high FVIII levels. Cumulative recurrence rates in relatives (not on continuing anticoagulant treatment) with antithrombin, protein C or protein S deficiency, were 19% after 2 years, 40% after 5 years and 55% after 10 years. Median age at recurrence was 36 years (range, 20-75), annual incidence 6.23% (95% CI, 4.31-8.70). Log rank test revealed no statistical differences whether first event was idiopathic or provoked (52% vs. 59%;  $P= 0.93$ ). In relatives with FV Leiden, prothrombin G20210A, or high FVIII levels, recurrence rates were 7%, 11%, and 25%, respectively at a median age of 43 years (range, 21-85) and an annual incidence of 2.25% (95% CI, 1.52-3.21) (Figure 3). Recurrence rates were 29% after an idiopathic first event and 24% after a provoked first event ( $P= 0.91$ ). Numbers of double heterozygous-/homozygous carriers of FV Leiden or/and prothrombin G20210A were too small to obtain accurate estimates of recurrence rates.

**Figure 3.** Event free survival of recurrent venous thrombosis in relatives of probands with thrombophilic defects



Major bleeding was observed in 2 relatives with antithrombin, protein C or protein S deficiency while they were treated with vitamin K antagonists, including relatives on long term treatment; annual incidence was 0.29% (95% CI, 0.34-1.04).

## DISCUSSION

This study shows that thrombophilic defects can be classified as strong and mild risk factors for venous thrombosis. Strong risk factors included hereditary deficiencies of antithrombin, protein C and protein S, whereas heterozygous FV Leiden, heterozygous prothrombin G20210A and high FVIII levels were mild risk factors. Although high levels of FIX, FXI, and TAFI, and hyperhomocysteinemia were identified as risk factors for venous thrombosis as reported before [4-9], the associated risk was due to concomitance of high factor VIII levels, as suggested in previous studies [6,8,14,19]. When concomitance of other thrombophilic defects was not taken into account, the risk was 15-19-fold higher in relatives with a strong thrombophilic defect and 3-5-fold higher in relatives with a mild thrombophilic defect compared to the community [20,21].

Because combinations of thrombophilic defects were frequently observed (i.e. 60% of relatives), we also estimated the absolute risk for single and combined defects. The annual incidence in relatives with a single strong thrombophilic defect ranged from 0.54 to 1.45% and increased to 1.92-2.92% when it was combined with two or more other defects. In relatives with a single mild defect, annual incidence ranged from 0.19% to 0.25% and increased to 0.59%-1.54%. Annual incidence was 0.05% in relatives without any of these defects. These results support the concept of multicausality [10,11]. The higher risk in relatives with a combination of a mild defect and another defect was partly due to concomitance of a strong thrombophilic defect.

Exogenous risk factors had an additional effect on the risk of first venous thrombosis, particularly in relatives with a strong thrombophilic defect at age 35 years or younger. This effect was mainly due to the use of oral contraceptives and pregnancy/puerperium, being the most prevalent exogenous risk factors at young age. However, exogenous risk factors did not influence lifetime risk of first venous thrombosis.

Strong and mild thrombophilic defects could also be classified with respect to the risk of recurrent venous thrombosis. Recurrence rates at 5 and 10 years ranged

from 40% to 55% and from 11% to 25%, respectively, compared to 22% to 30% in the community [22]. This finding is in agreement with previous studies that did not demonstrate an increased risk of recurrence in subjects with mild thrombophilic defects [23,24].

Our data shows that only strong thrombophilic defects may have clinical implications in patients with venous thrombosis and their relatives. Since the risk of recurrence remained high over at least 5-10 years after the first episode of venous thrombosis in relatives with a strong thrombophilic defect, it could be considered to extend this treatment for at least 5-10 years. Although the risk of prolonged anticoagulant treatment includes major bleeding, it was only 0.29% per year in these relatives. This risk is lower than we previously reported in unselected patients with venous thrombosis, who were treated with vitamin K antagonists (2.8% per year) [25]. Maybe, strong thrombophilic defects protect against bleeding during anticoagulant treatment. However, it may also be due to the younger age of these relatives at time of their thrombotic event. Nevertheless, the low risk of major bleeding will diminish the reluctance to extend anticoagulant treatment. It should, however, be noted that antithrombin, protein C or protein S deficiencies are rare, even in patients with venous thrombosis [10]. A positive family history of venous thrombosis (> 20% of first degree relatives) could be used in our study to identify these subjects. Young age was another predictor of a strong thrombophilic defect, considering that the median age at onset of first venous thrombosis was 29 years in relatives with these deficiencies, compared to 62 years in the community [21].

The risk of first venous thrombosis and recurrence in double heterozygous/homozygous relatives could not be accurately estimated because numbers were too small. Homozygosity of FV Leiden was the strongest risk factor for venous thrombosis in this subgroup. Previous studies on these risk factors contained small numbers as well (n=17, 7 and 20, respectively) [24,26,27]. Although these studies consistently reported an increased risk of first venous thrombosis and recurrence in heterozygous/homozygous patients, we think that the results of our study and previous studies are not conclusive.

Our findings seem inconsistent with two recent prospective studies on this issue [23,28]. These studies reported a similar risk of recurrence in patients with a thrombophilic defect, compared to patients without a defect. However, follow-up after first venous thrombosis was only 2 years in one study [28], while the other study contained only 25 patients with deficiencies of antithrombin, protein C or protein S [23]. Moreover, the thrombotic potency of different thrombophilic

defects was quantified as equal, while it would be more appropriate to weigh defects according to predefined risk estimates. A comparison with our findings is hampered because age at onset of first venous thrombosis in these studies (mean 51 and 67 years, respectively) [23,28], was markedly higher than in our study (median 35 years). This difference emphasizes that strong thrombophilic defects are risk factors at young age. It also explains why only 1% of first episodes of venous thrombosis in our study was associated with malignancy, compared to 4-20% in the normal population [20,29].

Some methodological aspects of our study warrant comment. The results may have been influenced by its design of a family cohort study. However, population studies are not suitable for risk assessments in subjects with strong, but rare thrombophilic defects [23,28]. Second, unknown inheritable thrombophilic defects may have influenced our results. This is not likely, as the annual incidence of venous thrombosis in relatives without any of tested thrombophilic defects was only 0.05%, and relative risks were adjusted for clustering within families. Third, events were not always confirmed by objective techniques, because these techniques were not available at the time of event. Therefore, incidences of venous thrombosis may have been overestimated. Since these were compared to incidences from population studies that used the same classification of venous thrombosis [20-22], relative risk estimates would not change. Fourth, referral bias may have been introduced by the university hospital setting, but was probably reduced by testing consecutive patients with venous thrombosis. Finally, an excess of fatal events in relatives with a strong thrombophilic defect might have underestimated the risk of venous thrombosis. However, these defects were not associated with a reduced life expectancy in previous studies [30,31], whereas our study did not show a difference in death rate between relatives of probands with a strong thrombophilic defect and relatives of probands with another defect.

In conclusion, of thrombophilic defects, hereditary deficiencies of antithrombin, protein C and protein S are associated with an excessively high absolute risk of first and recurrent venous thrombosis. Considering its possible clinical implications, thrombophilia testing may be restricted to these deficiencies in patients with first venous thrombosis. A positive family history of venous thrombosis (> 20% of relatives of probands) or young age could be used in our study to identify these rare subjects.

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Chapter **11**

**A higher risk of recurrent venous thrombosis  
in men is due to hormonal risk  
factors in women in thrombophilic  
families**

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## ABSTRACT

**Background:** Why men appear to have an increased risk of recurrent venous thrombosis compared to women is unknown.

**Methods:** In a retrospective thrombophilic family cohort study (n= 6079) life-time risk of recurrent venous thrombosis was assessed in men and women to clarify a possible difference.

**Results:** Of 816 subjects with first venous thrombosis, 337 had a recurrence. Adjusted relative risk of recurrence was 1.6 (95% confidence interval [CI], 1.3-2.0) in men compared to women. Women were younger at time of their first event (mean 34 years vs 44 years,  $P < 0.001$ ) and at time of recurrence (40 years vs 48 years,  $P < 0.001$ ). After excluding provoked first and recurrent venous thrombosis, adjusted relative risk was 1.2 (95% CI, 0.8-1.7), while mean age at recurrence was comparable in men and women (50 years vs 49 years,  $P = 0.595$ ). Women revealed recurrence after a longer period than men ( $P = 0.003$ ). In women with a hormonal first event, median interval between first event and recurrence was 10.4 years vs 2.7 years in men ( $P < 0.001$ ). This difference was not observed when only idiopathic events were considered ( $P = 0.938$ ).

**Conclusion:** The difference in life-time risk of recurrent venous thrombosis between men and women in thrombophilic families can be explained by a younger age of women at time of first venous thrombosis due to hormonal risk factors, and a longer interval between a provoked first episode of venous thrombosis and recurrence in women.

## INTRODUCTION

Venous thrombosis is a major health problem. Annual incidence of first venous thrombosis ranges from 0.1% to 0.3% in the normal population [1,2], while first recurrence rate is 3% to 5% per year, with a peak within the first two years after stopping anticoagulant treatment [3-5]. The duration of anticoagulant treatment after a first episode of venous thrombosis depends on the estimated risk of recurrence. Recent studies showed that men have a 1.6-3.6-fold higher risk of recurrent venous thrombosis than women [6-9]. For this reason, it was proposed to continue anticoagulant treatment in men for a longer time than women [6,7,9]. Although this treatment probably will prevent most recurrences, it is associated with major bleeding in 2-3% of patients per year [10,11]. Therefore, this finding must be established and needs to be clarified. It is likely to assume a relationship between the risk of venous thrombosis and the hormonal state to explain the difference in risk of recurrent venous thrombosis between men and women. Because thrombophilic subjects are at higher risk of venous thrombosis and reveal venous thrombosis at younger age than the normal population [12], hormonal effects on the risk of first and recurrent venous thrombosis may be enhanced in these subjects.

We performed a retrospective study in a large series of families with established thrombophilic defects (n= 6079), enabling a long follow-up time, to assess the life-time risk of recurrent venous thrombosis in men and women and to clarify a possible difference in risk between men and women.

## METHODS

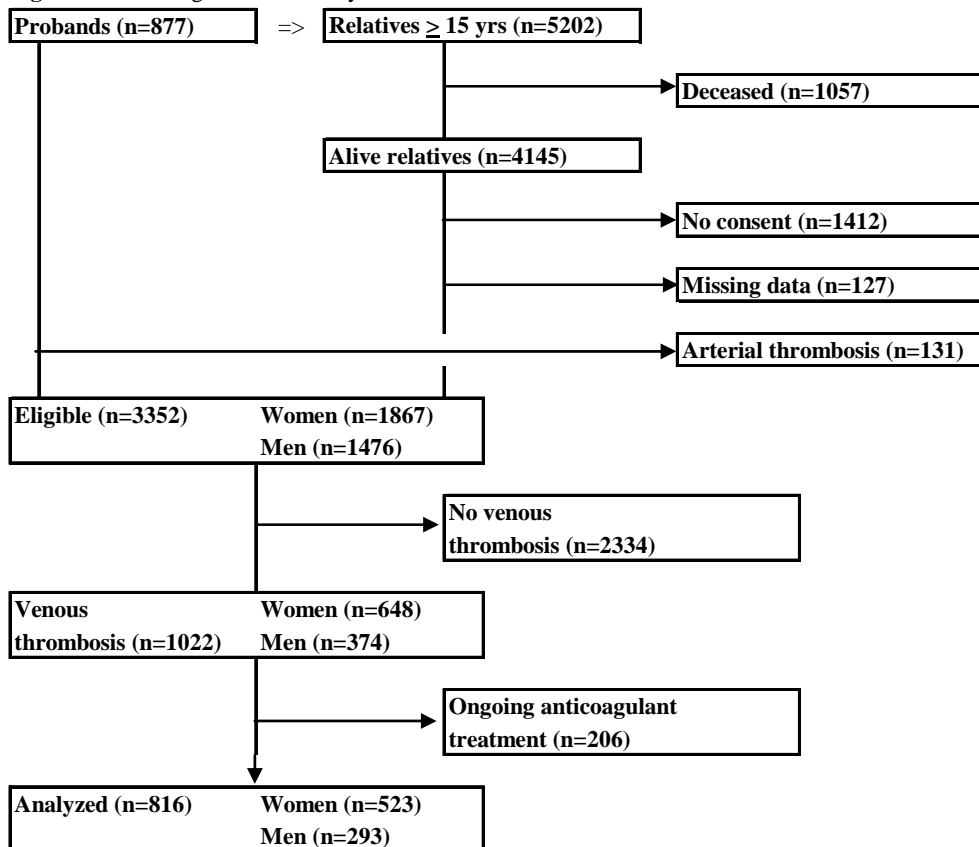
We pooled data of individual subjects from five large family cohort studies, performed to assess the absolute risk of venous thrombosis associated with various thrombophilic defects as previously described [13-19]. The design of these studies was identical. One study was single center and contained first degree relatives of patients (proband) with documented venous thrombosis and established hereditary deficiencies of either antithrombin, protein C, or protein S [13,14]. They were enrolled between April 1999 and July 2004. Three studies were multicenter studies of first degree relatives of patients with venous thrombosis or arterial thrombosis at age < 50 years and the presence of either the prothrombin 20210A mutation, high FVIII levels, or hyperhomocysteinemia [15-17]. Enrollment started in May 1998

and was completed in July 2004. The fifth study was a multicenter study of first degree relatives of patients with venous thrombosis and factor V Leiden who were enrolled between May 1995 and July 1998 [18,19]. Approval was obtained by the institutional review boards of the three participating university hospitals.

### Subjects

All relatives, identified by pedigree analysis, were 15 years of age or older and were contacted through the probands. All participants provided written informed consent. As the objective of this study was recurrent venous thrombosis, all probands and relatives with venous thrombosis, who were 15 years of age or older, were included without introducing bias. Physicians at the thrombosis outpatient clinics of the participating centers collected detailed information about previous

**Figure 1.** Flow diagram of the study cohort



episodes of venous thrombosis, exposure to exogenous risk factors for venous thrombosis, and anticoagulant treatment using a validated questionnaire [20], and by reviewing medical records. Clinical data was collected prior to laboratory testing. Proband and relatives were tested for currently known thrombophilic defects, including hereditary antithrombin, protein C and protein S type I and type III deficiency, factor V Leiden, prothrombin G20210A, elevated levels of factors VIII, IX, XI and TAFI, and hyperhomocysteinemia. Laboratory tests and definitions of abnormal results have been described in detail elsewhere [13,14,17]. If subjects were on long-term anticoagulant treatment with vitamin K antagonists, blood samples were taken after treatment had been interrupted, meanwhile nadroparin was given subcutaneously.

**Definitions**

The first episode of venous thrombosis was considered established if deep vein thrombosis was confirmed by compression ultrasound or venography, and pulmonary embolism by ventilation/perfusion lung scanning, spiral CT scanning or pulmonary angiography, or when the patient had received full dose heparin and a

**Table 1.** Characteristics of men and women with a first episode of venous thrombosis

	Women (n=523)	Men (n=293)	P
<b>First venous thrombosis</b>			
Mean age at onset, (SD), yrs	34 (15)	44 (15)	< 0.001
Idiopathic, n (%)	133 (25)	217 (74)	< 0.001
Provoked, n (%) by:			
- Oral contraceptives, n (%)	114 (22)		
- Pregnancy/puerperium, n (%)	121 (23)		
- Surgery, trauma, immobilization, n (%)	148 (28)	74 (25)	0.39
- Malignancy, n (%)	7 (1)	2 (1)	0.50
<b>Prevalence thrombophilic defects*</b>			
Antithrombin deficiency, n (%)	22 (4)	11 (4)	0.89
Protein C deficiency, n (%)	49 (11)	38 (15)	0.14
Protein S deficiency, n (%)	58 (13)	34 (13)	0.85
FV Leiden, n (%)	249 (48)	127 (43)	0.26
FVIII > 150 IU/dL, n (%)	281 (61)	166 (64)	0.39
Prothrombin G20210A, n (%)	88 (17)	48 (17)	1.00

\* Numbers of women/men tested for antithrombin, protein C, protein S, factor V Leiden, elevated FVIII, prothrombin G20210A were 513/288, 452/254, 461/254, 517/290, 462/258; and 506/281, respectively.

vitamin K antagonist for at least 3 months without objective testing at a time when these techniques were not yet available. Superficial phlebitis was not classified as a thrombotic event. If recurrence of deep vein thrombosis at the same site was suspected, but objective tests were not conclusive, it was diagnosed when the patient revealed pronounced signs and symptoms of recurrence without preceding postthrombotic syndrome, or when pulmonary embolism was objectively demonstrated. If these criteria were not fulfilled, anticoagulant treatment was withheld and the event was not classified as recurrent venous thrombosis. Venous thrombosis was defined provoked if it had occurred at or within 3 months after exposure to exogenous risk factors including surgery, trauma, immobilization for more than 7 days, pregnancy, post-delivery period, the use of oral contraceptives or hormonal replacement therapy, or malignancy. In the absence of these risk factors venous thrombosis was classified idiopathic. Venous thrombosis was considered to be associated with hormonal risk factors when women were pregnant, in the post-delivery period, used oral contraceptives or were on hormonal replacement therapy at time of or within 3 months before onset of venous thrombosis.

### Statistical analysis

The absolute risk of recurrent venous thrombosis was calculated over the period from the end of anticoagulant treatment after the first episode of venous thrombosis

**Table 2.** Recurrence rates of venous thrombosis in men and women

	Overall venous thrombosis*		Idiopathic venous thrombosis**	
	Women	Men	Women	Men
No. of subjects	523	293	122	207
No. of recurrences	198	139	46	90
Mean age at onset of recurrence	40	48†	49	50††
Observation period, yrs	3896	1499	615	998
Annual incidence, % (95% CI)	5.1 (4.4-5.8)	9.3 (7.8-10.9)	7.5 (5.5-10.0)	9.0 (7.3-11.1)
Crude relative risk (95% CI)	Reference	1.7 (1.4-2.1)	Reference	1.2 (0.8-1.7)
Adjusted relative risk (95% CI)#	Reference	1.6 (1.3-2.0)	Reference	1.2 (0.8-1.7)

\* Overall indicates any first venous thrombosis and any recurrence.

\*\* Idiopathic indicates idiopathic first venous thrombosis and idiopathic recurrence.

†  $P < 0.001$ , ††  $P = 0.595$ .

# Adjusted for antithrombin, protein C and protein S deficiency.

CI, confidence interval.

until either the date of first recurrence or the end of study. We compared the risk of recurrence between men and women; the risk of idiopathic recurrence after an idiopathic first event between men and women; and the risk of recurrence in women after a first event associated with or without hormonal risk factors at time of the first thrombotic event. Incidences and 95% confidence intervals (95% CI) were calculated under the Poisson distribution assumption. Freedom of recurrent venous thrombosis was analyzed by the Kaplan-Meier method. Relative risks were calculated using a multivariable Cox regression model adjusting for hereditary antithrombin, protein C, or protein S type I deficiency, including interaction terms, with venous thrombosis as dependent variable and deficiencies as independent variables, because we previously demonstrated that only these hereditary deficiencies increase the risk of recurrence [12].

Continuous variables were expressed as mean values and standard deviations; categorical data as counts and percentages. Differences between groups were evaluated by the Student t test or Mann-Whitney U test, depending on the normality of data for continuous data and by Fisher exact test for categorical data. A two-tailed p-value of less than 0.05 indicated statistical significance.

Statistical analyses were performed using SAS software, version 9.1 (SAS-Institute inc., Cary, North Carolina).

**Table 3.** Recurrence rates of venous thrombosis in women with a non-hormonal vs hormonal first event\*

	<b>Non-hormonal first venous thrombosis</b>	<b>Hormonal first venous thrombosis</b>
No. of subjects	273	201
Mean age at onset of first event, yrs	40	30†
No. of recurrences**	81	68
Mean age onset recurrent event, yrs	47	40††
Observation period, yrs	1604	2074
Annual incidence, % (95% CI)	5.1 (4.0-6.3)	3.3 (2.5-4.2)
Crude relative risk (95% CI)	Reference	0.6 (0.5-0.9)
Adjusted relative risk (95% CI)#	Reference	0.7 (0.5-0.97)

\* Hormonal event indicates venous thrombosis associated with oral contraception, pregnancy or puerperium, or hormonal replacement therapy.

\*\* Recurrences associated with hormonal risk factors were excluded.

†  $P < 0.001$ , ††  $P = 0.003$ .

# Adjusted for antithrombin, protein C and protein S deficiency.

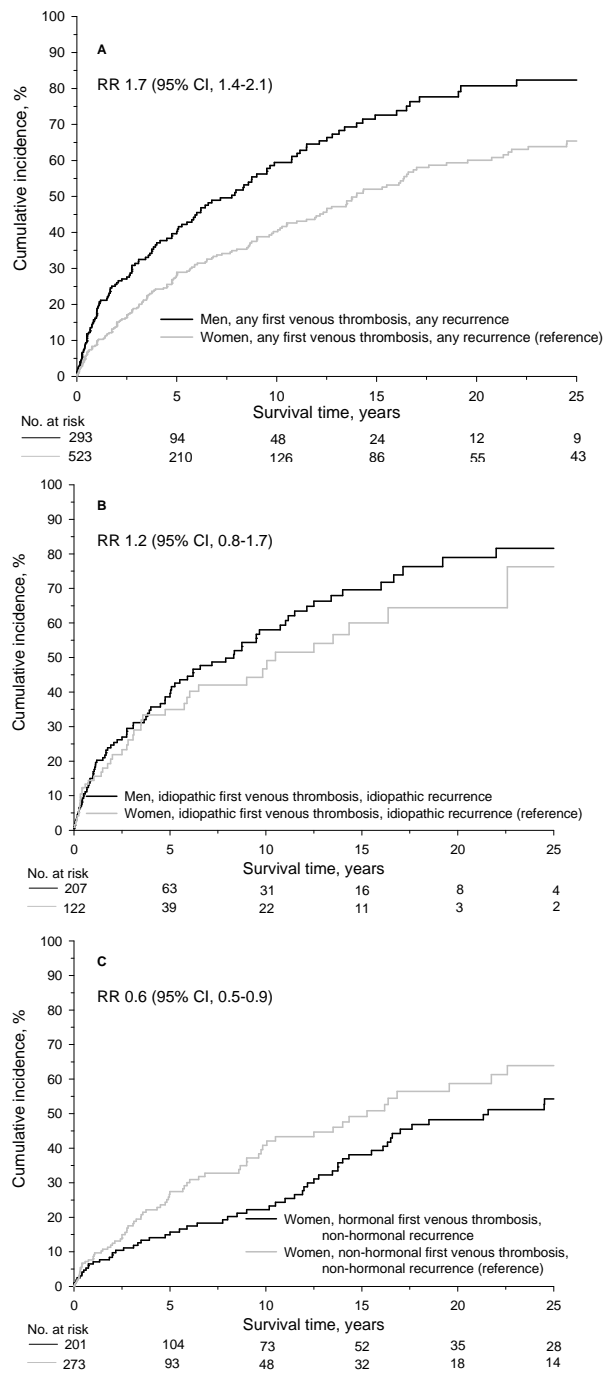
CI, confidence interval.

## RESULTS

Our study cohort contained 877 probands, with a total number of 5202 relatives, who were 15 years of age or older (Figure 1). Of relatives, 1057 deceased before the start of the study, whereas another 1412 relatives did not participate because of various reasons, including refusal or inability to give informed consent or residence outside the Netherlands. Another 131 probands were excluded because they were probands who were enrolled with an arterial event and 127 subjects had missing data. Of the remaining 3352 subjects, 1022 had experienced a first venous thrombosis of whom 206 were still on oral anticoagulant treatment for this reason at date of enrollment. Clinical characteristics of 816 evaluable subjects (523 women and 293 men) are shown in Table 1. Women were younger at time of their first event than men (mean age 34 years vs 44 years,  $P < 0.001$ ). Of first events, 133 (25%) were idiopathic in women and 217 (74%) in men ( $P < 0.001$ ); 121 (22% in women) were associated with oral contraceptives or hormonal replacement therapy; 114 (23% in women) were associated with pregnancy or puerperium; and 148 (28%) were associated with surgery, trauma or immobilization in women vs 74 (25%) in men ( $P = 0.39$ ). Thrombophilic defects were equally divided between men and women.

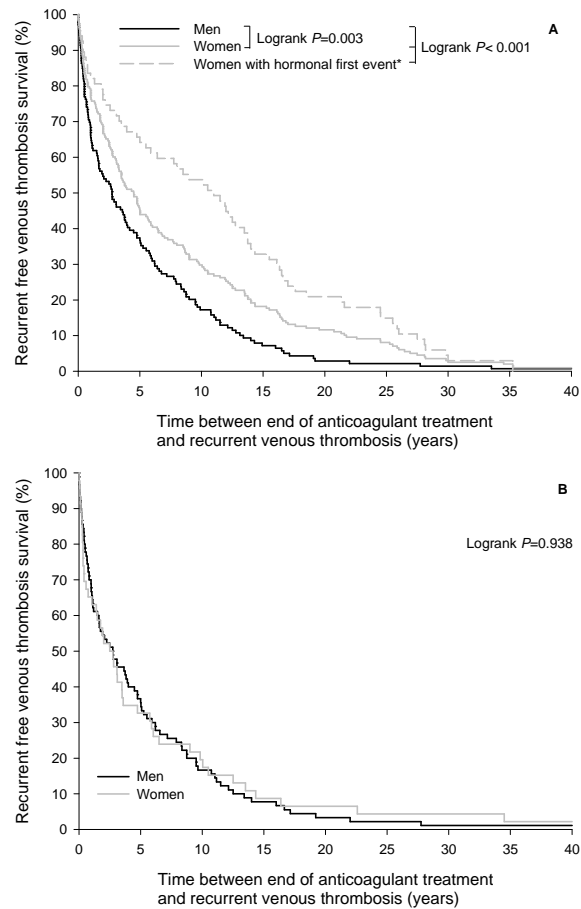
Venous thrombosis recurred in 337 subjects. Mean age at recurrence was 42 years (SD, 17), 227 recurrences (67%) were idiopathic. Women were younger at time of recurrence than men (mean 40 years vs 48 years,  $P < 0.001$ ) (Table 2). Overall, absolute risk of recurrence was 5.1% per year (95% CI, 4.4-5.8) in women and 9.3% per year (95% CI, 7.8-10.9) in men. Crude relative risk of recurrence was 1.7 (95% CI, 1.4-2.1) in men compared to women, adjusted for thrombophilic deficiencies it was 1.6 (95% CI, 1.3-2.0). In subjects with idiopathic first venous thrombosis and recurrence, the crude and adjusted relative risk was 1.2 (95% CI, 0.8-1.7), while mean age at time of first event (46 years vs 44 years,  $P = 0.378$ ) and recurrence (49 years vs 50 years,  $P = 0.595$ ) was comparable in men and women. In women, crude relative risk of recurrence after first venous thrombosis associated with hormonal risk factors (oral contraceptives, and pregnancy or puerperium) compared to women with non-hormonal first venous thrombosis was 0.6 (95% CI, 0.5-0.9), and adjusted for thrombophilic deficiencies it was 0.7 (95% CI, 0.5-0.97) (Table 3). Cumulative recurrence rates over 25-years follow-up are shown in Figure 2.

**Figure 2.** Cumulative recurrence rates of venous thrombosis in men and women



RR indicates relative risk.  
Hormonal first event indicates first venous thrombosis associated with oral contraception, pregnancy or puerperium, or hormonal replacement therapy.

**Figure 3.** Time between end of anticoagulant treatment for first venous thrombosis and recurrence



A, any first venous thrombosis, any recurrence.

B, idiopathic first venous thrombosis, idiopathic recurrence.

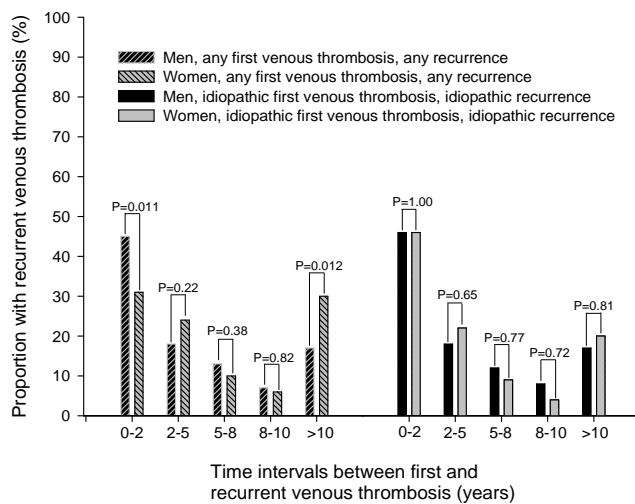
\* Hormonal first event indicates first venous thrombosis associated with oral contraception, pregnancy or puerperium, or hormonal replacement therapy.

Women revealed recurrence after a longer time period than men ( $P=0.003$ ), which was mostly due to hormonal risk factors ( $P<0.001$ ) (Figure 3A). In women with a hormonal first event, median interval between end of anticoagulant treatment for first event and recurrence was 10.4 years vs 2.7 years in men ( $P<0.001$ ). These time differences were not observed when only idiopathic events were con-

sidered ( $P= 0.938$ ) (Figure 3B). After stratifying time intervals between end of the initial anticoagulant treatment and onset of recurrence, men had a higher recurrence rate within the first two years than women (45% vs 31%,  $P= 0.011$ ), while 30% of women had a recurrent event more than 10 years after the end of anticoagulant treatment compared to 17% in men ( $P= 0.012$ ) (Figure 4). These differences were not observed when provoked first and recurrent venous thrombotic events were excluded from analysis.

To account for possible misclassification, as not all events were confirmed by objective techniques because these were not available at the time of event onset, we repeated the analysis in men and women who had first venous thrombosis and recurrence diagnosed with objective techniques. In this analysis, crude relative risk of recurrence was 2.0 (95% CI, 1.5-2.8) in men compared to women, while it was 1.0 (95% CI, 0.7-1.6) for idiopathic first and recurrent events.

**Figure 4.** Time intervals between end of anticoagulant treatment for first venous thrombosis and recurrence



## DISCUSSION

Men had a higher risk of recurrent venous thrombosis than women. They were of older age at onset of first event and had recurrence after a shorter time-interval.

This finding is in line with other studies that reported on this issue [6-9]. However, no differences in risk of recurrence, age at onset of first and recurrent venous thrombosis, respectively, and time interval between first and recurrent event were demonstrated between men and women when provoked events were excluded from analysis. Therefore, our study shows that the higher risk of recurrence in men is likely due to provoked events rather than an unknown thrombophilic abnormality. Of all first events in women, 28% were related to surgery, trauma and immobilization, compared to 25% in men, while 45% were associated with oral contraception (22%) or pregnancy or puerperium (23%). This explains the younger age of women at onset of first venous thrombosis, as women are mainly exposed to these hormonal risk factors at reproductive age. Women had recurrences after a longer time interval than men. Of women in whom first venous thrombosis was associated with hormonal risk factors, more than 50% had a recurrence 10.4 years or longer after the end of the initial period of anticoagulant treatment, while this interval was less than 2.7 years in men. Because oral contraceptives were discouraged and thromboprophylaxis was recommended during puerperium and pregnancy after prior venous thrombosis, age became the main determinant of recurrence, thus clarifying the longer interval. Overall, men were older at time of first venous thrombosis and recurrence. The cumulative recurrence rate in men was 20% higher than in women after 25 years of follow-up, while they had a 1.7-fold higher risk of recurrence compared to women. This is in agreement with a meta-analysis that showed a 1.6-fold higher risk of recurrent venous thrombosis in men compared to women [9]. Because this meta-analysis could not account for age with multivariate analysis, the risk in men may have been overestimated [9]. When we excluded provoked events from analysis, we observed that the risk of recurrence in men compared to women dropped to 1.2 (95% CI, 0.8-1.7), while differences in age at onset of first venous thrombosis and recurrence, respectively were no longer seen. Pengo and Prandoni reported a similar relative risk of recurrence in men compared to women (1.21; 95% CI, 0.95-1.55), excluding provoked venous thrombosis [21]. Recurrence rates may be influenced by thromboprophylaxis at exposure to risk factors for venous thrombosis after prior venous thrombosis. In spite of guidelines on applications for thromboprophylaxis, its dosage and duration are still a matter of debate [22]. Moreover, implementation of guidelines in clinical practice varies widely [23]. As a consequence, provoked recurrent venous thrombosis is prone to confounding [24], which we reduced by excluding these events from analysis.

A previous Austrian study showed a 3.6-fold higher risk of recurrence (95% CI, 2.3-5.8) in men compared to women, including women who had first venous thrombosis associated with oral contraceptives (39% of the female study population) [6]. In an accompanying editorial comment, it was discussed that this study had a potential weakness because men were older than women [25]. After adjustment for age, the relative risk was 1.2 (95% CI, 1.1-1.4). In our study, women in whom first venous thrombosis was associated with hormonal risk factors had a 40% decreased risk of recurrence, and were 10 and 7 years younger at time of first event and recurrence, respectively, compared to women in whom first venous thrombosis was not associated with hormonal risk factors. Moreover, cumulative recurrence rate in these women was 55% after 25 years, while it was 76% in women who had idiopathic first venous thrombosis and recurrence. This suggests that the higher risk of recurrence in men compared to women in the Austrian study [6], could be due to oral contraceptive use in younger women with first venous thrombosis.

Our study showed that 31% of women experienced recurrent venous thrombosis more than 10 years after the end of anticoagulant treatment compared to 17% of men. These women might have been missed in prospective studies, that suggested that men had an increased risk of recurrent venous thrombosis, because maximum follow-up time in these studies amounted to 2 to 8 years [6-8].

Some methodological aspects of our study warrant comment. Because the study was performed in thrombophilic families, we cannot exclude that male gender is a risk factor for recurrent venous thrombosis in non-thrombophilic families. However, in a previous study we demonstrated that only subjects with hereditary deficiencies of antithrombin, protein C or protein S are at increased risk of recurrence [12]. Adjustment for these deficiencies did not change relative risk estimates. Moreover, as thrombophilic defects were equally divided among men and women, it is less likely that relative risk estimates in men compared to women were influenced by the study design. Nevertheless, absolute risk estimates and cumulative recurrence rates in this study cannot be extrapolated to the normal population. Second, referral bias may have been introduced by the setting of university hospitals, but it was probably reduced by testing consecutive patients with thrombosis for thrombophilic defects. Third, we cannot provide detailed information on mortality in our study population. However, an excess of fatal events in relatives with antithrombin, protein C or protein S deficiency is not likely as these deficiencies were not associated with a reduced life expectancy in previous

studies [26,27], while the overall mortality due to recurrent venous thrombosis is low [28,29]. Finally, subjects with clinical suspicion of venous thrombosis, who were treated with heparin and vitamin K antagonists for more than three months at a time when objective testing was not available yet, might have overestimated our recurrence rates. However, one should expect a similar effect in men and women. Moreover, our results were not influenced when only objectively confirmed events were considered. Hence, it is less likely that primary outcomes were confounded by events that could not be confirmed by objective techniques.

We conclude that the difference in life-time risk of recurrent venous thrombosis between men and women in thrombophilic families can be explained by a younger age of women at time of first venous thrombosis due to hormonal risk factors, and a longer interval between a provoked first episode of venous thrombosis and recurrence in women.

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## **Summary and discussion**

## SUMMARY

**Chapter 1** describes the outline of this thesis and the rationale to subdivide this thesis into three parts: hyperhomocysteinemia, infections and thrombophilia testing.

### **Part I: Hyperhomocysteinemia**

**Chapter 2** describes a retrospective study, wherein 478 evaluable first degree relatives of consecutive patients with venous thrombosis or premature atherosclerosis, and hyperhomocysteinemia were enrolled. Absolute risks of venous and arterial thrombosis were compared. Annual incidence of venous thrombosis was 0.16% (95% confidence interval [CI], 0.08-0.30) in hyperhomocysteinemic relatives versus 0.11% (95% CI, 0.05-0.20) in normohomocysteinemic relatives; adjusted relative risk 1.6 (95% CI, 0.6-4.5). Annual incidences of arterial thrombosis were 0.34% (95% CI, 0.21-0.52) and 0.24% (95% CI, 0.15-0.37) in hyperhomocysteinemic and normohomocysteinemic relatives, respectively; adjusted relative risk 1.5 (95% CI, 0.6-3.5). Concomitance of multiple thrombophilic risk factors increased the risk of venous thrombosis in hyperhomocysteinemic relatives, but a comparable effect was demonstrated in normohomocysteinemic relatives. This study shows that hyperhomocysteinemia is associated with a low absolute risk of venous and arterial thrombosis. This risk is probably too low to result in a clinically relevant risk reduction of thrombosis with B-vitamin therapy.

In **Chapter 3**, three different strategies for diagnosing hyperhomocysteinemia are compared. Random, fasting and methionine-loading homocysteine samples were measured in 713 relatives of probands with hereditary (index) deficiencies antithrombin, protein C or protein S. According to predefined cut-off levels, hyperhomocysteinemic and normohomocysteinemic relatives were identified and their risks of thrombosis were compared. Relatives with random homocysteine levels  $> 20 \mu\text{mol/L}$  were not at risk of venous or arterial thrombosis compared to relatives with levels  $< 10 \mu\text{mol/L}$ ; relative risk 0.9 (95% CI, 0.4-2.3) and 1.7 (95% CI, 0.5-5.7), respectively. Fasting hyperhomocysteinemia was associated with an increased risk of both venous thrombosis (relative risk 2.6; 95% CI, 1.3-4.8) and arterial thrombosis (relative risk 3.7; 95% CI, 1.5-8.4). Relatives with normal fasting homocysteine levels, but methionine-loading hyperhomocysteinemia were not at risk; relative risk 0.8 (95% CI, 0.2-1.9) for venous thrombosis and 1.1 (95%

CI, 0.2-3.9) for arterial thrombosis. The relative risk estimates were independent of index deficiencies. It was concluded that only fasting homocysteine levels are sufficient to identify hyperhomocysteinemic subjects at risk of thrombosis.

In **Chapters 4 and 5**, a relation between hyperhomocysteinemia and elevated factor VIII levels is hypothesized. In a retrospective study of thrombophilic families where probands had an antithrombin, protein C or protein S deficiency, 405 relatives were analyzed (**Chapter 4**). Median factor VIII levels in hyperhomocysteinemic relatives were 169 IU/dL, compared to 136 IU/dL in normohomocysteinemic relatives ( $P=0.007$ ), and were more often elevated ( $>150$  IU/dL;  $P=0.006$ ). Hyperhomocysteinemia was associated with an increased risk of venous and arterial thrombosis; relative risk 2.6 (95% CI, 1.3-4.8) and 3.7 (95% CI, 1.5-8.4), respectively. Relatives with elevated FVIII were also at risk; relative risk 2.3 (95% CI, 1.4-4.0) for venous thrombosis and 2.3 (95% CI, 1.0-5.1) for arterial thrombosis. After excluding all relatives with elevated factor VIII levels, relative risk for hyperhomocysteinemia and venous thrombosis dropped to 1.3 (95% CI, 0.2-9.8) and nil relatives had arterial thrombosis.

In **Chapter 5**, 1052 relatives of consecutive probands, who had venous thrombosis or premature atherosclerosis, and hyperhomocysteinemia, elevated factor VIII levels, or both, were enrolled. Hyperhomocysteinemic relatives had more often elevated factor VIII levels than normohomocysteinemic relatives (38% versus 28%,  $P=0.024$ ). Other thrombophilic defects were equally divided. Hypertension and smoking were more often found in hyperhomocysteinemic relatives than in normohomocysteinemic relatives (28% versus 48%;  $P=0.027$ , and 19% versus 38%;  $P=0.029$ ). After adjusting for these confounders, the relative risk of venous thrombosis in hyperhomocysteinemic relatives was 0.8 (95% CI, 0.3-1.7) and 0.9 (95% CI, 0.5-1.6) for arterial thrombosis.

These results may explain why lowering of homocysteine levels with B-vitamins has not resulted in a decrease of venous and arterial thrombosis in large prospective randomized clinical trials, assuming that elevated factor VIII levels are not decreased by these vitamins.

## **Part II: Infections**

**Chapter 6** describes a case of acute CMV infection in an immunocompetent adult that was complicated with mesenteric vein thrombosis. Transient protein C deficiency, lupus anticoagulant and APC resistance were found, in combination

with a heterozygous prothrombin G20210A mutation. This case provides evidence that active CMV infection might be related with a thrombophilic state and venous thrombosis, which is the hypothesis of **Chapter 7**. As acute infection and reactivation of CMV is a very common complication in renal transplant recipients, one would expect to find an increased risk of venous thrombosis in this patient group, which should be associated with CMV. In a retrospective study, the risk of (recurrent) venous thrombosis in renal transplant recipients, and CMV status was analyzed in 606 living consecutive renal transplant recipients. Annual incidence of venous thrombosis was 0.59% (95% CI, 0.41-0.83) corrected for surgery related venous thrombosis. CMV positive and seroconverted recipients tended to have an increased risk of venous thrombosis compared to CMV negative recipients; corrected relative risks were 2.0 (95% CI, 0.9-5.2) and 1.7 (95% CI, 0.6-4.7), respectively. The cumulative 10-years recurrence rate of venous thrombosis in CMV seronegative, seroconverted, and seropositive recipients was 10%, 51% and 59%, respectively. Although not statistically significant, these findings suggest that CMV infection is associated with an increased risk of (recurrent) venous thrombosis in renal transplant recipients.

Although many previous studies reported an increased risk of venous and arterial thrombosis in HIV-infected patients, these generally reported on the possible effect of antiretroviral therapy on thrombosis rather than giving overall absolute risk estimates. In **Chapter 8**, the absolute risk of venous and arterial thrombosis in HIV-infected patients and the effect of combination antiretroviral therapy was assessed in 519 HIV-infected patients. Annual incidences of venous and arterial thrombosis were 0.65% (95% CI, 0.39-0.92) and 0.45% (95% CI, 0.24-0.77), respectively. In patients who received combination antiretroviral therapy, the annual incidence of venous thrombosis was 0.72% (95% CI, 0.39-1.29), versus 0.58% (95% CI, 0.25-1.14) in patients who did not receive these drugs. For arterial thrombosis, annual incidences were 0.46% (95% CI, 0.18-0.95) and 0.43% (95% CI, 0.16-0.95). Overall, the absolute risk of venous and arterial thrombosis was 2-6 times higher than reported in the general population. Patients were at higher risk of thrombosis irrespective of their age and whether or not they had used combination antiretroviral therapy. These results suggest a pathophysiologic role for HIV infection on the risk of thrombosis which is further unraveled in **Chapter 9**. In this chapter, it is hypothesized that advanced stages of HIV are associated with increased acquired thrombophilic abnormalities that could predispose to

thrombosis. A total of 109 consecutive HIV-infected patients were included and were tested twice for currently known thrombophilic abnormalities with a time interval of at least three months. Sixteen percent of patients revealed symptomatic thrombosis during HIV infection (10% venous, 6% arterial). Protein C deficiency was established by repeated measurements in 9% of patients, elevated factor VIII levels in 41%, high fibrinogen levels in 22%, and free protein S deficiency in 60%. Median factor VIII levels were higher in patients with AIDS compared to non AIDS defining illness (226 IU/dL versus 149 IU/dL;  $P < 0.001$ ), while median free protein S levels were lower (45 IU/dL versus 58 IU/dL;  $P < 0.001$ ). Advanced stages of HIV disease was associated with increased factor VIII levels and with decreased free protein S levels. Therefore, HIV-infected patients did reveal multiple acquired and persistent thrombophilic abnormalities compared to the normal population, which extend in the progression to AIDS. This may clarify the high prevalence of venous and arterial thrombosis in these patients.

### **Part III: Thrombophilia testing**

In **Chapter 10**, 2479 relatives of patients with venous thrombosis and a thrombophilic defect were retrospectively analyzed to assess the absolute risk of first venous thrombosis and recurrence for currently known thrombophilic defects. In antithrombin, protein C, and protein S deficient relatives annual incidences of venous thrombosis were 1.77% (95% CI, 1.14-2.60), 1.52% (95% CI, 1.06-2.11) and 1.90% (95% CI, 1.32-2.64), respectively, at a median age of 29 years and a positive family history >20%. In relatives with factor V Leiden, prothrombin G20210A or elevated factor VIII levels, these were 0.49% (95% CI, 0.39-0.60), 0.34% (95% CI, 0.22-0.49) and 0.49% (95% CI, 0.41-0.51). Elevated factor IX, XI, TAFI and hyperhomocysteinemia were not independent risk factors. Cumulative recurrence rates in relatives with antithrombin, protein C or protein S deficiency were 19% at 2 years, 40% at 5 years and 55% at 10 years. In relatives with factor V Leiden, prothrombin G20210A or high levels of factor VIII, these were 7%, 11% and 25%, respectively. This study shows that hereditary deficiencies of antithrombin, protein C and protein S are associated with a high absolute risk of first and recurrent venous thrombosis. Considering its possible clinical implications, thrombophilia testing may be restricted to these deficiencies in patients with first venous thrombosis. A positive family history of venous thrombosis or young age could be used in our study to identify these rare subjects.

In the final part of this thesis (**Chapter 11**), a retrospective study in a large series of families with established thrombophilic defects (n=6079) was performed to clarify a possible difference between men and women on their risk of recurrent venous thrombosis. Of 816 subjects with first venous thrombosis, 337 had a recurrence. Overall, relative risk of recurrence was 1.7 (95% CI, 1.4-2.1) in men compared to women; adjusted for hereditary deficiencies of natural anticoagulants 1.6 (95% CI, 1.3-2.0). Women were younger at time of their first event (mean 34 years versus 44 years,  $P < 0.001$ ) and at time of recurrence (40 years vs. 48 years,  $P < 0.001$ ). After excluding provoked first and recurrent venous thrombosis, adjusted relative risk was 1.2 (95% CI, 0.8-1.7), while mean age at recurrence was comparable in men and women (50 years versus 49 years,  $P=0.595$ ). Women revealed recurrence after a longer period than men ( $P=0.003$ ), which was 7.7 years longer in women with first venous thrombosis associated with hormonal risk factors compared to men ( $P < 0.001$ ), but not when only idiopathic events were considered ( $P=0.938$ ). These findings show that the difference in life-time risk of recurrent venous thrombosis is not explained by gender itself. A difference can be observed when the follow-up period is relatively short and is explained by a younger age of women at time of first venous thrombosis, due to hormonal risk factors, and a longer interval between provoked first episode and recurrence in women.

## **DISCUSSION**

In recent years, numerous epidemiological studies have provided a better insight in the pathophysiology of venous thrombosis. A growing amount of hemostatic abnormalities have been identified as risk factors for venous thrombosis. Initially, these were hereditary defects of natural anticoagulant proteins, which showed to be strong risk factors. Later, some point mutations of genes that code for procoagulant proteins, and finally elevated plasma levels of procoagulant proteins, probably based on a mixture of genetic and acquired origin. Last categories showed to have a high prevalence within the normal population, but were associated with only a mild increased risk of thrombosis. Mild hyperhomocysteinemia, which is a metabolic defect, was also identified as a mild risk factor for thrombosis.

A number of the studies that have been described in this thesis addressed the clinical impact of all these thrombophilic defects in individual patients. In these studies, the absolute risk of thrombosis was calculated, both for single thrombophilic defects and combinations which were frequently observed in families with a thrombophilic defect. By pooling these family cohorts, the intended population of subjects was obtained. This design made it possible to analyze also rare thrombophilic defects. Only the latter defects, heritable deficiencies of antithrombin, protein C and protein S turned out to be strong risk factors for venous thrombosis, which may have implications, or can get implications in the future. The remaining thrombophilic defects were mild risk factors, which seem to be of little clinical relevance for the individual patient.

Although aggregation of thrombophilic defects resulted in a higher absolute risk of venous thrombosis, the clinical impact of this finding appears small. In subjects with a strong thrombophilic defect, this defect alone seems to determine the strategy for primary prevention and duration of anticoagulant treatment after a first episode of venous thrombosis or recurrence. On the other hand, the risk of venous thrombosis for several combinations of mild thrombophilic defects did not reach a level at which it might have consequences. Double heterozygosity or homozygosity for factor V Leiden or prothrombin G20210A, and the combination of heterozygosity for one of these mutations and homozygosity for the other one, are possible exceptions. However, the prevalence of these combined defects was too low to enable reliable estimates of the associated risk of venous thrombosis. Simple clinical criteria were defined to identify subjects in whom selective testing on strong thrombophilic defects might have a high yield. These criteria need to be validated in prospective studies.

In this thesis, it was shown that some of the known thrombophilic abnormalities were not independent risk factors. These contained high plasma levels of factor IX, XI, and TAFI, and mild hyperhomocysteinemia as well. The higher risk of venous thrombosis associated with these thrombophilic abnormalities was actually due to co-existence of high factor VIII plasma levels. Regarding combinations of high plasma levels of factor VIII, IX and XI, a laboratory artefact should be considered. Strongly increased factor VIII levels may shorten APTT-values in one-stage assays of factor IX and factor XI, respectively, and consequently result in falsely high levels of factors IX and XI.

The negative findings of clinical intervention studies on the effects of vitamin-B supplementation in symptomatic patients with mild hyperhomocysteinemia are

in agreement with the here presented finding that mild hyperhomocysteinemia is not an independent risk factor for venous (and arterial) thrombosis. Assuming that mild hyperhomocysteinemia results in a high factor VIII level due to endothelial damage, one might speculate that repair of endothelium and, hence a decrease of factor VIII levels require many years of supplementation with B-vitamins. However, this hypothesis does not clarify the observation that the absolute risk of venous and arterial thrombosis in subjects with mild hyperhomocysteinemia was not increased compared to the normal population.

Numerous exogenous risk factors for venous thrombosis are associated with acquired thrombophilic abnormalities, like pregnancy and the use of oral contraceptives. Two studies provided evidence of a relationship between infections of CMV and HIV and an increased risk of venous thrombosis, and, between HIV infection and arterial thrombosis as well. Moreover, HIV-infected patients showed many thrombophilic abnormalities, which were related to progression of HIV infection. These findings warrant future studies to the role of these and other infections to the development of venous and arterial thrombosis.

Currently, testing for thrombophilia is a clinical non-issue, excepted in a small group of real thrombophiliacs. However, it remains a scientific main issue to further unravel the pathophysiology of thrombosis.

## **Samenvatting en discussie**

## SAMENVATTING

In **Hoofdstuk 1** wordt de vraagstelling van dit proefschrift uiteengezet en de ratio om deze vraagstelling in drie delen op te splitsen, namelijk hyperhomocysteinemie, infecties en trombofiele testen.

### **Deel I: Hyperhomocysteinemie**

**Hoofdstuk 2** beschrijft een retrospectieve studie waarin 478 eerstegraadsfamilieleden van opeenvolgende patiënten met veneuze trombose of premature atherosclerose en hyperhomocysteinemie werden geïncludeerd. Het absolute risico op veneuze en arteriële trombose werd bepaald voor familieleden met en zonder hyperhomocysteinemie. De jaarlijkse incidentie van veneuze trombose was 0.16% (95% betrouwbaarheidsinterval [BI], 0.08-0.30) voor familieleden met hyperhomocysteinemie en 0.11% (95% BI, 0.05-0.20) voor familieleden zonder; gecorrigeerd relatief risico 1.6 (95% BI, 0.6-4.5). De jaarlijkse incidentie van arteriële trombose bedroeg 0.34% (95% BI, 0.21-0.52) voor hyperhomocysteinemische familieleden en 0.24% (95% BI, 0.15-0.37) voor normohomocysteinemische familieleden; gecorrigeerd relatief risico 1.5 (95% BI, 0.6-3.5). Het risico op veneuze trombose was verhoogd in aanwezigheid van meerdere trombofiele afwijkingen, maar dit betrof zowel familieleden met als zonder hyperhomocysteinemie. De jaarlijkse incidentie van zowel veneuze als arteriële trombose voor familieleden met hyperhomocysteinemie was laag en vergelijkbaar met die voor de normale bevolking. Hierdoor lijkt de klinische relevantie van hyperhomocysteinemie als risicofactor voor trombose gering te zijn.

In **Hoofdstuk 3** worden drie verschillende methoden voor het vaststellen van hyperhomocysteinemie met elkaar vergeleken. Homocysteïne monsters werden afgenomen op een willekeurig tijdstip, in nuchtere toestand, en na een orale methionine belastingtest bij 713 familieleden van patiënten met veneuze trombose en een erfelijke (index) deficiëntie van antitrombine, proteïne C of proteïne S. Op basis van vooraf vastgestelde afkapwaarden werden hyperhomocysteinemische en normohomocysteinemische familieleden geïdentificeerd en werd hun risico op trombose vergeleken. Familieleden met homocysteïne waarden  $> 20 \mu\text{mol/L}$ , gemeten op een willekeurig tijdstip hadden geen verhoogd risico op veneuze of arteriële trombose in vergelijking met familieleden die homocysteïne waarden  $< 10 \mu\text{mol/L}$  hadden; relatief risico respectievelijk 0.9 (95% BI, 0.4-2.3) en 1.7 (95% BI,

0.5-5.7). Nuchter gemeten hyperhomocysteïnemie was geassocieerd met een verhoogd risico van zowel veneuze trombose (relatief risico 2.6; 95% BI, 1.3-4.8) en arteriële trombose (relatief risico 3.7; 95% BI, 1.5-8.4). Familieleden met normale nuchtere homocysteïne waarden, maar met hyperhomocysteïnemie na de orale methionine belastingtest hadden geen verhoogd risico; relatief risico 0.8 (95% BI, 0.2-1.9) voor veneuze trombose en 1.1 (95% BI, 0.2-3.9) voor arteriële trombose. Het relatief risico was niet afhankelijk van de index deficiënties. Geconcludeerd werd dat alleen nuchter gemeten homocysteïne waarden toereikend zijn om het risico op veneuze en arteriële trombose in te schatten bij personen met hyperhomocysteïnemie.

In **Hoofdstuk 4 en 5** worden 2 studies naar een veronderstelde relatie tussen hyperhomocysteïnemie en verhoogde factor VIII spiegels beschreven. In de eerste, retrospectieve studie werden 405 familieleden van patiënten met veneuze trombose en een antitrombine, proteïne C of proteïne S deficiëntie onderzocht (**Hoofdstuk 4**). De mediane factor VIII spiegels waren 169 IU/dL in familieleden met hyperhomocysteïnemie en 136 IU/dL in familieleden met normohomocysteïnemie ( $P=0.007$ ), en waren vaker verhoogd ( $>150$  IU/dL;  $P=0.006$ ). Hyperhomocysteïnemie was geassocieerd met een verhoogd risico op veneuze en arteriële trombose; relatief risico respectievelijk 2.6 (95% BI, 1.3-4.8) en 3.7 (95% BI, 1.5-8.4). Familieleden met verhoogde factor VIII spiegels hadden ook een verhoogd risico; relatief risico 2.3 (95% BI, 1.4-4.0) voor veneuze trombose en 2.3 (95% BI, 1.0-5.1) voor arteriële trombose. Nadat familieleden met verhoogde factor VIII spiegels uit de analyse waren geëxcludeerd, daalde het relatief risico voor hyperhomocysteïnemie en veneuze trombose naar 1.3 (95% BI, 0.2-9.8) terwijl geen van de familieleden arteriële trombose had.

In **Hoofdstuk 5** wordt de tweede, retrospectieve studie beschreven, waarin 1052 familieleden werden geïncludeerd van opeenvolgende patiënten met veneuze trombose of premature atherosclerose in combinatie met hyperhomocysteïnemie en/of verhoogde factor VIII spiegels. Familieleden met hyperhomocysteïnemie hadden vaker een verhoogde factor VIII spiegel dan familieleden met normohomocysteïnemie (38% versus 28%,  $P=0.024$ ). Andere trombofiele defecten waren gelijk verdeeld over beide groepen. Hypertensie en roken kwamen vaker voor bij hyperhomocysteïnemische familieleden dan bij normohomocysteïnemische familieleden (28% versus 48%;  $P=0.027$ , en 19% versus 38%;  $P=0.029$ ). Na correctie voor deze factoren was het relatief risico voor veneuze trombose in hyper-

homocysteïnemische familieleden 0.8 (95% BI, 0.3-1.7) en 0.9 (95% BI, 0.5-1.6) voor arteriële trombose.

De bevinding dat het tromboserisico bij personen met hyperhomocysteïnemie afhankelijk is van gelijktijdig verhoogde factor VIII spiegels zou kunnen verklaren waarom in placebo gecontroleerde interventiestudies geen effect van vitamine B-therapie op het risico op trombose werd gevonden, aannemende dat factor VIII spiegels niet verlaagd worden door B-vitamines. Hyperhomocysteïnemie lijkt veeleer een risicomarker of epifenomeen te zijn dan een risicofactor voor trombose.

## **Deel II: Infecties**

In **Hoofdstuk 6** wordt een immunocompetente patiënt beschreven bij wie ten tijde van een acute CMV infectie veneuze mesenteriaal trombose ontstond. Een tijdelijke proteïne C deficiëntie, de aanwezigheid van lupus anticoagulans en APC resistentie werden bij trombofilieonderzoek gevonden, naast heterozygotie van de protrombine G20210A mutatie. Deze casus laat zien dat acute CMV infectie geassocieerd kan zijn met verworven trombofiele veranderingen, die kunnen bijdragen aan het optreden van veneuze trombose. Verder onderzoek naar de associatie van CMV infectie en veneuze trombose wordt beschreven in **Hoofdstuk 7**. Als een CMV infectie geassocieerd is met een verhoogd risico op veneuze trombose, zou deze associatie bij niertransplantatiepatiënten aantoonbaar moeten zijn, omdat een acute CMV infectie en reactivatie frequent voorkomen bij deze patiënten. In een retrospectieve studie werd het risico op een eerste en recidiverende veneuze trombose bij 606 niertransplantatiepatiënten berekend en hun relatie met CMV status geanalyseerd. De jaarlijkse incidentie van veneuze trombose, gecorrigeerd voor chirurgie gerelateerde trombose, bedroeg 0.59% (95% BI, 0.41-0.83). CMV positieve en CMV geseroconverteerde transplantanten hadden mogelijk een hoger risico op veneuze trombose dan CMV negatieve transplantanten; gecorrigeerde relatieve risico's waren respectievelijk 2.0 (95% BI, 0.9-5.2) en 1.7 (95% BI, 0.6-4.7). Het cumulatieve risico op recidiverende veneuze trombose over een periode van 10 jaar na transplantatie bij CMV negatieve, geseroconverteerde en positieve transplantanten was respectievelijk 10%, 51% en 59%. Hoewel deze uitkomsten niet statistisch significant waren, suggereren zij dat CMV infectie geassocieerd is met een verhoogd risico op (recidiverende) veneuze trombose bij niertransplantatiepatiënten.

In het verleden bleek uit meerdere studies dat patiënten met HIV een verhoogd risico op veneuze en arteriële trombose hadden. Deze studies werden uitgevoerd om een al dan niet aanwezige associatie van antiretrovirale therapie en trombose aan te tonen, terwijl informatie over absolute risico's ontbrak. In **Hoofdstuk 8** wordt het absolute risico op veneuze en arteriële trombose in 519 HIV-geïnfecteerde patiënten berekend, alsmede het mogelijke effect van effectieve antiretrovirale therapie op dit risico. De jaarlijkse incidentie van veneuze en arteriële trombose was respectievelijk 0.65% (95% BI, 0.39-0.92) en 0.45% (95% BI, 0.24-0.77). Patiënten die effectieve antiretrovirale therapie kregen hadden een jaarlijkse incidentie van 0.72% (95% BI, 0.39-1.29) voor veneuze trombose versus 0.58% (95% BI, 0.25-1.14) voor patiënten die deze medicijnen niet ontvingen. Voor arteriële trombose was dit risico respectievelijk 0.46% (95% BI, 0.18-0.95) en 0.43% (95% BI, 0.16-0.95). Het totale risico op trombose, hetzij veneus, hetzij arterieel, was bij HIV-geïnfecteerde patiënten 2 tot 6-voudig verhoogd ten opzichte van de normale populatie. Het tromboserisico bij deze patiënten bleef verhoogd na correctie voor leeftijd en effectieve antiretrovirale therapie. Deze resultaten suggereren dat HIV een pathofysiologische rol speelt in het ontstaan van trombose, wat verder wordt onderzocht in **Hoofdstuk 9**. In dit hoofdstuk wordt verondersteld dat verslechterde HIV-status, gebaseerd op het aantal CD4 positieve lymfocyten, geassocieerd is met toegenomen trombofiele afwijkingen. In totaal werden 109 opeenvolgende HIV-geïnfecteerde patiënten geïncludeerd. De patiënten werden twee keer getest op de aanwezigheid van trombofiele afwijkingen met een minimum tijdsinterval van 3 maanden tussen beide bloedafnames. Vanaf het moment dat bij deze patiënten HIV was gediagnosticeerd, was bij 16% symptomatische trombose opgetreden. Een geconfirmeerde proteïne C deficiëntie werd vastgesteld in 9% van de patiënten, terwijl verhoogde factor VIII spiegels bij 41%, verhoogde fibrinogeen spiegels bij 22%, en een vrij proteïne S deficiëntie bij 60% van de patiënten werden bevestigd bij herhaalde metingen. De mediane factor VIII spiegel was hoger in patiënten met AIDS, vergeleken met die in patiënten met als non-AIDS gedefinieerde ziekte (226 IU/dL versus 149 IU/dL;  $P < 0.001$ ), terwijl de mediane vrij proteïne S spiegel lager was (45 IU/dL versus 58 IU/dL;  $P < 0.001$ ). Een verslechtering van de HIV-status was geassocieerd met een toename van factor VIII spiegels en een afname van vrij proteïne S spiegels. Geconcludeerd werd dat bij HIV-geïnfecteerde patiënten meerdere verworven en persisterende trombofiele afwijkingen voorkomen in een hogere frequentie dan in de normale populatie. De ernst hiervan neemt toe bij verslechtering van de HIV-status. Mogelijk wordt de

hoge prevalentie van veneuze en arteriële trombose in deze patiëntengroep mede hierdoor verklaard.

### **Deel III: Testen van trombofilie**

**Hoofdstuk 10** beschrijft een retrospectieve studie, waarin 2479 familieleden van opeenvolgende patiënten met veneuze trombose of premature atherosclerose en een trombofiele afwijking werden geïnccludeerd. Het doel van deze studie was om het absolute risico op een eerste veneuze trombose en recidief veneuze trombose te berekenen voor verschillende trombofiele afwijkingen. De jaarlijkse incidentie van veneuze trombose voor familieleden met een antitrombine, proteïne C of proteïne S deficiëntie was 1.77% (95% BI, 1.14-2.60), 1.52% (95% BI, 1.06-2.11) en 1.90% (95% BI, 1.32-2.64). De mediane leeftijd ten tijde van de eerste episode van veneuze trombose was 29 jaar. Van alle familieleden was meer dan 20% symptomatisch. Voor familieleden met factor V Leiden, protrombine G20210A of verhoogde factor VIII spiegels, was deze incidentie respectievelijk 0.49% (95% BI, 0.39-0.60), 0.34% (95% BI, 0.22-0.49) en 0.49% (95% BI, 0.41-0.51). Verhoogde spiegels van factor IX, XI, TAFI en homocysteïne bleken geen onafhankelijke risicofactoren te zijn voor veneuze trombose. Het absolute risico op een eerste veneuze trombose steeg aanzienlijk wanneer de gevonden trombofiele afwijkingen gecombineerd voorkwamen. Het cumulatieve risico op recidiverende veneuze trombose voor familieleden met een antitrombine, proteïne C of proteïne S deficiëntie was 19% na 2 jaar, 40% na 5 jaar en 55% na 10 jaar. Voor familieleden met factor V Leiden, protrombine G20210A of verhoogde factor VIII spiegels, was dit respectievelijk 7%, 11% en 25%. Deze studie toont aan dat in het bijzonder erfelijke deficiënties van antitrombine, proteïne C of proteïne S geassocieerd zijn met een hoog absoluut risico op veneuze trombose en recidief. Gelet op de klinische implicaties kan het testen op trombofiele afwijkingen beperkt blijven tot deze deficiënties bij patiënten met een eerste veneuze trombose. Een positieve familieanamnese en veneuze trombose op jonge leeftijd konden in deze studie gebruikt worden ter identificatie van deze zeldzame patiënten.

Het laatste hoofdstuk van dit proefschrift (**Hoofdstuk 11**) beschrijft een retrospectieve studie waarin meerdere familiestudies werden samengevoegd (n=6079). Het doel van deze studie was om het recent beschreven verschil in risico op recidiverende veneuze trombose tussen mannen en vrouwen te analyseren. Van 816 personen met een eerste veneuze trombose, trad bij 337 een recidief op. Voor de

gehele populatie was het relatief risico op recidief veneuze trombose 1.7 (95% BI, 1.4-2.1) in mannen vergeleken, met vrouwen. Gecorrigeerd voor erfelijke trombofiele deficiënties van antitrombine, proteïne C en proteïne S was dit 1.6 (95% BI, 1.3-2.0). Vrouwen waren jonger ten tijde van de eerste trombose (gemiddeld 34 jaar versus 44 jaar,  $P < 0.001$ ) en ten tijde van het eerste recidief (40 jaar versus 48 jaar,  $P < 0.001$ ). Na exclusie van eerste en recidiverende veneuze trombose, die geassocieerd waren met uitlokkende risicofactoren, was het relatief risico 1.2 (95% BI, 0.8-1.7), terwijl de gemiddelde leeftijd van mannen en vrouwen ten tijde van het recidief vergelijkbaar was (50 jaar versus 49 jaar,  $P = 0.595$ ). Bij vrouwen trad een recidief op na een langer interval dan bij mannen ( $P = 0.003$ ). Dit interval was 7.7 jaar langer bij vrouwen na een eerste veneuze trombose, welke geassocieerd was met hormonale risicofactoren (orale anticonceptie, zwangerschap, en kraambed) ( $P < 0.001$ ). Dit verschil was niet aanwezig wanneer mannen en vrouwen met idiopathische trombose werden vergeleken ( $P = 0.938$ ). Uit deze bevindingen blijkt dat het ogenschijnlijke verschil in risico op recidiverende veneuze trombose tussen mannen en vrouwen wordt verklaard doordat secundaire veneuze trombose bij vrouwen op jongere leeftijd optreedt als gevolg van hormonale risicofactoren, terwijl het interval van eerste veneuze trombose tot het optreden van recidief bij deze vrouwen langer is dan bij mannen.

## DISCUSSIE

Tallose epidemiologische studies tijdens de afgelopen jaren hebben ertoe geleid dat meer inzicht is verkregen in de pathofysiologie van veneuze trombose. Een toenemend aantal hemostatische afwijkingen werd geïdentificeerd als risicofactoren voor veneuze trombose. Aanvankelijk betrof dit erfelijke defecten van natuurlijke anticoagulante eiwitten, die sterke risicofactoren bleken te zijn. Daarna enkele puntmutaties van genen, die coderen voor procoagulante eiwitten en ten slotte verhoogde plasmaspiegels van procoagulante eiwitten, mogelijk op basis van een gecombineerde genetische en verworven oorsprong. De laatstgenoemde categorieën bleken een hoge prevalentie te hebben in de normale bevolking, maar geassocieerd te zijn met een mild verhoogd risico. Ten slotte bleek milde hyperhomocysteinemie een frequent voorkomende metabole stoornis te zijn, die eveneens als een milde risicofactor voor veneuze trombose werd geclassificeerd.

Een deel van de studies in dit proefschrift was gericht op de betekenis van al deze trombofiele afwijkingen voor de individuele patiënt. In deze studies werd het absolute risico berekend, zowel voor de afzonderlijke trombofiele afwijkingen, als voor de talrijke combinaties hiervan, die in families met een trombofiele afwijking frequent voorkwamen. Door samenvoeging van afzonderlijke familiecohorten werd een studiepopulatie verkregen, die de beoogde doelgroep vormde. Door deze opzet was ook een analyse mogelijk van zeldzame trombofiele afwijkingen. Alleen de laatstgenoemde afwijkingen, erfelijke deficiënties van antitrombine, proteïne C en proteïne S bleken sterke risicofactoren voor veneuze trombose te zijn, waarvan de aanwezigheid implicaties kan hebben of in de toekomst mogelijk kan krijgen. De overige trombofiele afwijkingen waren milde risicofactoren, die voor de individuele patiënt van beperkte klinische betekenis lijken te zijn.

Weliswaar resulteerde aggregatie van trombofiele afwijkingen in een hoger absoluut risico op veneuze trombose, maar de klinische impact hiervan is waarschijnlijk gering. Bij personen met een sterke trombofiele afwijking lijkt deze reeds bepalend te zijn voor het beleid inzake primaire preventie en de behandelingsduur van anticoagulante behandeling na een eerste episode van veneuze trombose of een recidief. Anderzijds is het hogere risico op veneuze trombose in personen met combinaties van milde trombofiele afwijkingen onvoldoende om hieraan consequenties te verbinden. Een mogelijke uitzondering wordt gevormd voor dubbele heterozygotie of homozygotie voor factor V Leiden en protrombine G20210A, evenals gecombineerde heterozygotie en homozygotie van deze mutaties. Echter, de frequentie van deze gecombineerde defecten was te laag om betrouwbare schattingen te kunnen maken van het geassocieerde risico op veneuze trombose. Op basis van de beschikbare gegevens werden eenvoudige klinische criteria gedefinieerd, waarmee de personen kunnen worden geïdentificeerd waarbij selectief testen op sterke trombofiele afwijkingen een hoge opbrengst heeft. De waarde hiervan zal moeten blijken in prospectieve studies.

Enkele van de bekende trombofiele afwijkingen bleken geen onafhankelijke risicofactoren te zijn voor veneuze trombose. Het betreft hoge plasmaspiegels van factor IX, XI en TAFI, evenals milde hyperhomocysteinemie. Het met deze trombofiele afwijkingen geassocieerde hogere risico op veneuze trombose bleek in feite te worden bepaald door de gelijktijdige aanwezigheid van een hoge factor VIII plasma spiegel. Waar het de combinatie van hoge plasmaspiegels van factor VIII, IX, en XI betreft, moet de mogelijkheid van een laboratoriumartefact worden overwogen. Dit zou veroorzaakt kunnen worden door beïnvloeding van APTT-

metingen bij de bepaling van factor IX en factor XI spiegels door sterk verhoogde factor VIII waarden.

De negatieve bevindingen van klinische interventiestudies naar de effecten van vitaminesuppletie bij symptomatische patiënten met milde hyperhomocysteïnemie zijn in overeenstemming met de hier gepresenteerde bevinding dat milde hyperhomocysteïnemie geen onafhankelijke risicofactor voor veneuze (en arteriële) trombose is. Enige nuancering is gerechtvaardigd. Het is denkbaar dat milde hyperhomocysteïnemie resulteert in een hoge factor VIII spiegel als gevolg van endotheelschade. Mogelijk treedt een daling van de factor VIII spiegel pas op na jarenlange vitamine suppletie. Resteert de waarneming dat het absolute risico op veneuze en arteriële trombose bij personen met milde hyperhomocysteïnemie niet hoger was dan het risico hierop in de normale bevolking.

Meerdere exogene risicofactoren voor veneuze trombose zijn geassocieerd met verworven trombofiele afwijkingen. Zwangerschap en het gebruik van orale anti-conceptiva vormen voorbeelden hiervan. In twee studies werd aannemelijk dat infecties van CMV en HIV risicofactoren zijn voor veneuze trombose en HIV-infectie eveneens voor arteriële trombose. Bij de laatste patiëntencategorie werden trombofiele afwijkingen gevonden, waarvan de omvang gerelateerd bleek aan het stadium van de HIV-infectie. Deze bevindingen vormen een aanknopingspunt voor verder onderzoek naar de relatie van deze en andere infecties en het optreden van veneuze, alsmede arteriële trombose.

Weliswaar is er voor het testen op trombofiele afwijkingen in de klinische praktijk slechts een beperkte plaats, dit geldt allesbehalve voor wetenschappelijk onderzoek naar de betekenis van hemostatische veranderingen bij het ontstaan van trombose.



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