The role of the fibrinolytic system in arterial and venous thrombosis

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The role of the fibrinolytic system in arterial and venous thrombosis

De rol van het fibrinolytisch systeem in arteriële en veneuze trombose

(met een samenvatting in het Nederlands)

Proefschrift

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1

General Introduction

MYOCARDIAL INFARCTION AND VENOUS THROMBOSIS

Thrombotic disease is a leading cause of morbidity and mortality in Western countries. The incidence of myocardial infarction is 3.5 per 1000 persons per year.[1] For venous thrombosis the incidence is 2 per 1000 persons per year.[2] Incidences show a sharp rise with increasing age. The development of both myocardial infarction and venous thrombosis is a multicausal process. Epidemiological studies have identified several risk factors for myocardial infarction and venous thrombosis. Established risk factors for myocardial infarction include smoking, hypercholesterolemia, hypertension, diabetes, and obesity.[3] Important risk factors for venous thrombosis, often divided in environmental and genetic factors, are cancer, hormone use, surgery, immobility, factor V Leiden, the prothrombin 20210A mutation and deficiencies of the natural anticoagulants antithrombin, protein C and protein S.[4]

The etiology of venous thrombosis and myocardial infarction displays similarity but the development of the diseases also involves distinct pathways. In both venous thrombosis and myocardial infarction a blood vessel is occluded with a blood clot. In venous thrombosis a fibrin-rich blood clot is formed in the veins. The occlusion of a coronary artery with a platelet-rich thrombus usually occurs after the rupture of an atherosclerotic plaque.[5] While thrombus formation is a rather acute process, the development of atherosclerosis is a chronic process.

HEMOSTASIS, FIBRINOLYSIS, AND RISK OF THROMBOSIS

Hemostasis, the complex process which causes the bleeding from an injured blood vessel to stop, requires the combined activity of vascular, platelet, and coagulation factors to form a hemostatic plug. The coagulation pathway involves several procoagulant and anticoagulant factors. The last step in the coagulation pathway is the cleavage of fibrinogen into fibrin monomers by thrombin. Fibrin monomers polymerize spontaneously to form a fibrin clot. The fibrinolytic system enables removal of the fibrin clot when a damaged vessel wall has been restored and involves multiple proteins for activation and regulation. A simplified scheme of the fibrinolytic system is depicted in Figure 1. The principal activator of plasminogen in plasma is tissue plasminogen activator (t-PA). t-PA is released from the endothelial cells into the circulation upon stimulation. It circulates mostly in an inactive complex with its main inhibitor plasminogen activator inhibitor-1 (PAI-1).[6] Cleavage of plasminogen by t-PA forms the active enzyme plasmin. The latter is the primary enzyme to

dissolve a fibrin clot. Plasmin is inhibited by its fast inhibitor α2-antiplasmin.[7] α2-Antiplasmin can be cross-linked to fibrin by coagulation factor XIIIa, facilitating local inhibition of fibrinolysis. Thrombin activatable fibrinolysis inhibitor (TAFI), when activated by thrombin or the thrombin-thrombomodulin complex, removes the C-terminal lysines from fibrin, thereby preventing the binding of t-PA and plasmin to fibrin which results in a decreased lysis of the clot.[8]

Hemostatic abnormalities can lead to excessive bleeding or thrombotic complications. Individuals with a decreased coagulation or an increased fibrinolytic capacity suffer from a bleeding tendency, as is seen in individuals with deficiencies in procoagulant proteins factor VIII and factor IX or fibrinolytic inhibitors PAI-1 and α2-antiplasmin.[9-11] Increased coagulation potential increases the risk of venous thrombosis and, albeit to a lesser extent, the risk of myocardial infarction.[12,13] Therefore it is plausible that decreased fibrinolytic potential also would increase thrombotic risk. Studies investigating hypofibrinolysis as risk factor for venous and arterial thrombosis are, however, scarce and yielded inconsistent results.

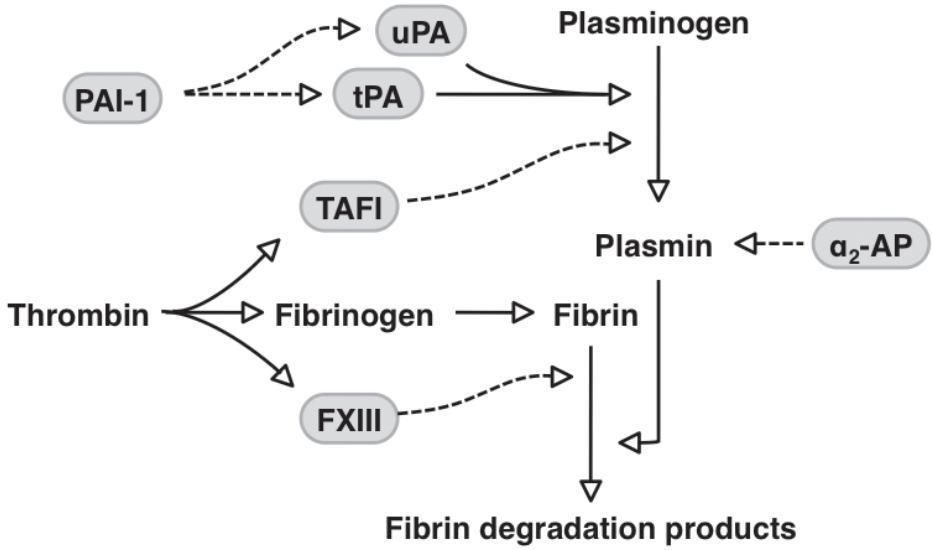

Figure 1 *A simplified representation of the fibrinolytic system.*
Solid arrows denote stimulation and activation, and dotted arrows denote inhibition. α2-ap α2-antiplasmin; PAI-1 plasminogen activator inhibitor; TAFI thrombin activatable fibrinolysis inhibitor; t-PA tissue-type plasminogen activator; u-PA urokinase plasminogen activator (adapted from [14]).

The individual fibrinolytic components have biological properties in processes besides lysis of fibrin clots, including inflammation, tissue remodeling, and the metabolic syndrome. A possible association of the fibrinolytic system with arterial thrombosis may therefore involve mechanisms beyond fibrin lysis, as these processes are involved in and related to atherosclerosis. Extensive reviews on the fibrinolytic proteins, their role in and association with venous and arterial thrombosis are presented in chapter 2 and 3 of this thesis.

STUDY POPULATIONS

The results described in this thesis have been based on four different studies: the Study of Myocardial Infarctions LEiden (SMILE) (chapter 4, 5, and 6), the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis study (MEGA study) (chapter 7 and 8), Vermont kindred II (chapter 9), and the Leiden Thrombophilia Study (LETS) (chapter 10).

Study of Myocardial Infarctions LEiden (SMILE)

The SMILE was designed to investigate the causal role of coagulation abnormalities in the development of myocardial infarction by exploring the association between polymorphisms in genes encoding coagulation factors and risk of myocardial infarction. The SMILE was conducted between July 1994 and February 1997. Patients (n=560) were men below 70 years of age. They were hospitalized with a first myocardial infarction between January 1990 and January 1996 in a general or university hospital in Leiden and identified in medical records. Control subjects (n=646) were men who had undergone an orthopedic intervention between January 1990 and May 1996 and had received prophylactic anticoagulants for a short period following the intervention. The control subjects were identified in the records of the Leiden anticoagulation clinic and frequency-matched to the cases on 10 year age groups. All participants completed a questionnaire concerning the presence of cardiovascular risk factors and a morning fasting blood sample was drawn. Medication use and history of diabetes were ascertained in an interview with controls and retrieved from discharge letters for the cases. Details of the SMILE have been described previously.[15]

Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA study)

The MEGA study was designed to study combinations of genetic and environmental

risk factors for venous thrombosis. The MEGA study included consecutive patients between 18 and 70 years with a first pulmonary embolism or a venous thrombosis of the leg or arm. Between March 1999 and May 2002, patients were identified at the anticoagulation clinics of Leiden, Amsterdam, Den Haag, Utrecht, or Amersfoort and were asked to bring their partners as control subject. From January 2002 to September 2004, a second control group was selected from the same geographical area as the patients by random digit dialing and these control subjects were frequency-matched for sex and age to the patients. The MEGA study included 3257 patients, 1908 partner controls, and 3000 random controls. Participants filled in a standard questionnaire on potential risk factors for venous thrombosis. Three months after discontinuation of the anticoagulant therapy, a blood sample from patients and their partners was taken in the anticoagulation clinic. Of patients who received prolonged anticoagulant therapy (>1 year), blood was drawn one year after the event. The random control subjects were invited to the clinic for a blood draw after returning their questionnaire. Details of the MEGA study have been described previously.[16,17]

Expanded Vermont kindred II

Vermont kindred II includes all living descendants and their spouses of a couple born in the 1830s. This French Canadian pedigree suffers from a high risk of venous thrombosis, partially attributable to type I protein C deficiency due to a 3363 inserted C mutation in exon 6 of the protein C gene.[18] Initial data collection started in 1985. The original pedigree consisted of seven generations, with clinical data available from the most recent five. In the 1990s and again from 2002 onwards information was updated with later generations and descendants of a sister of the woman of the original couple. Expanded Kindred Vermont II includes an additional eight pedigrees from Quebec, Canada, and New Hampshire who share a common ancestor with the original kindred. All participating subjects completed questionnaires and were interviewed regarding their medical history in general, risk factors for thrombosis, and thrombosis history, and blood samples were drawn. In the final dataset, 528 pedigree members were included.

The increased risk of venous thrombosis in the kindred is not completely explained by the protein C deficiency and there is evidence that the protein C mutation interacts with a second major genetic defect. When mutations in several candidate genes were ruled out as the co-occurring mutation, family members were genotyped for 375 autosomal markers to perform a genome wide linkage analysis. Details of Vermont Kindred II have been described in detail previously.[18,19]

Leiden Thrombophilia Study (LETS) follow-up

The LETS is a case-control study investigating risk factors for venous thrombosis, including 474 consecutive patients with a first venous thrombosis of the leg or arm identified at the anticoagulation clinics of Leiden, Amsterdam, and Rotterdam between January 1988 and December 1992. Patients were below 70 years of age and without malignancies. Between October 1990 and January 1994 and at least 3 months after discontinuation of oral anticoagulant treatment for the first thrombotic event, blood samples were drawn and an interview on risk factors for venous thrombosis was performed. When patients had an indication for prolonged anticoagulation, a blood sample was taken during treatment. The follow-up study included all patients of the LETS and aimed to investigate risk factors for recurrent venous thrombosis. Information about recurrent events and risk factors was collected by four repeated questionnaires and telephone interviews. Follow-up started 90 days after the date of the initial thrombotic event and ended on January 1, 2000. Details of the LETS follow-up have been described previously.[20]

OUTLINE OF THE THESIS

The aim of the research presented in this thesis is to provide insight into the role of the fibrinolytic system in myocardial infarction and venous thrombosis.

In **chapter 2** the results of epidemiological studies investigating the association between fibrinolysis and venous and arterial thrombosis are reviewed. The pleiotropic effects of the fibrinolytic factors are described in **chapter 3**. Fibrinolytic proteins have properties apart from their function in clot lysis and play roles in for example insulin resistance, inflammation and stability of the atherosclerotic plaque. In this chapter these properties are reviewed and linked to the inconsistent results of studies on arterial disease. **Chapter 4** describes the association between increased clot lysis time (CLT) as measured with an overall plasma-based clot lysis assay, and risk of myocardial infarction in the SMILE. **Chapter 5** evaluates plasma levels of four of the fibrinolytic factors, i.e. plasminogen, α2-antiplasmin, PAI-1, and t-PA, as risk factors for myocardial infarction in the SMILE. The association between TAFI levels and risk of myocardial infarction is reported in **chapter 6**. As TAFI levels are in part genetically regulated, four single-nucleotide polymorphisms (SNPs) of which three are associated with TAFI levels, were also evaluated as risk factors for myocardial infarction in the SMILE. The risk of venous thrombosis associated with overall plasma hypofibrinolysis, i.e., prolonged CLT, alone and in combination

with established risk factors associated with hypercoagulability, is described in **chapter 7** using data from the MEGA study. Determinants of CLT in the general population have never been investigated before and the association between plasma levels of fibrinolytic proteins and risk of venous thrombosis is unclear. Therefore in **chapter 8**, the influence of plasma levels of fibrinolytic factors (plasminogen, α2-antiplasmin, TAFI, PAI-1, and t-PA) and several coagulation factors on CLT has been studied and the association between the fibrinolytic proteins and venous thrombosis has been investigated in the MEGA study. In **chapter 9** genetic loci influencing CLT are examined by performing a genome wide quantitative trait loci (QTL) analysis in Vermont kindred II. Furthermore, we studied hypofibrinolysis as risk factor for venous thrombosis in this large protein C deficient family. Increased CLT, increased TAFI levels and TAFI genotypes were previously found to be associated with a first venous thrombosis in the LETS. In **chapter 10** we aimed to investigate these factors as risk factors for recurrent venous thrombosis in the follow-up study of the LETS. In **chapter 11** the results presented in the previous chapters are discussed in a broader context, including what we have learned from these results about the etiology of both arterial and venous thrombosis, the clinical relevance, methodological considerations and suggestions for further research.

2

Fibrinolysis and the risk of venous and arterial thrombosis

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ABSTRACT

Purpose of review
The fibrinolytic system is often regarded as just an innocent bystander in the pathogenesis of venous and arterial thrombosis, while (hyper)coagulation as a risk factor has been studied extensively. In this review, we evaluated studies that investigated the association between fibrinolysis and thrombosis.

Recent findings
There is some evidence for an association between impaired overall fibrinolytic activity and increased risk of venous or arterial thrombosis. Plasminogen levels were found not to be related to thrombosis. Plasma levels of tissue-type plasminogen activator were related to arterial thrombosis in a number of studies but not to venous thrombosis. Thrombin activatable fibrinolysis inhibitor levels appeared to be associated with venous thrombosis. Studies on the association between thrombin activatable fibrinolysis inhibitor or plasminogen activator inhibitor-1 and arterial thrombosis had conflicting results.

Summary
Current evidence on an association between fibrinolysis and thrombosis is inconclusive. Although overall assays point to an association, not all individual factors have an association with thrombosis. Most importantly, plasminogen deficiency is not related to thrombosis, which suggests that the fibrinolytic system as a whole is unimportant in the occurrence of thrombosis. Certain components of the fibrinolytic system, however, appear to be involved in processes unrelated to fibrin degradation but related to other processes important in the development of thrombosis.

INTRODUCTION

Fibrinolysis is often regarded simply as a secondary phenomenon responsive to the coagulation cascade, and not much attention has been given to the fibrinolytic system as a risk factor for thrombotic disease. In contrast, the role of (hyper)coagulation as a risk factor for thrombosis has been studied extensively.[4] It is plausible, however, that defective fibrinolysis is also associated with thrombosis. In recent years, the association between impaired fibrinolytic function and thrombotic disease has gained attention, in part stimulated by the discovery of a new regulator of fibrinolysis: thrombin activatable fibrinolysis inhibitor (TAFI).[8]

In this review, we will discuss the role of the fibrinolytic system in venous and arterial thrombosis. We will consider the individual components of the system and we will discuss the role of hypofibrinolysis in venous and arterial thrombosis.

PLASMINOGEN

Plasminogen is the precursor of plasmin, the proteolytic enzyme thought to be vital for the dissolution of fibrin clots.

Venous thrombosis

Despite several case reports, there is no evidence of an association between plasminogen deficiency and venous thrombosis. The most common clinical manifestation in individuals with homozygous and compound heterozygous plasminogen deficiency is ligneous conjunctivitis, that is a nonthrombotic disorder in which extra-vascular deposition of fibrin within the eye occurs (for a review, see Brandt[21]). A recent study on 50 patients from 44 families with severe type 1 plasminogen deficiency showed that 80% of the patients suffered from ligneous conjunctivitis, but none had experienced venous thrombosis.[22] In a population-based case-control study, the prevalence of plasminogen deficiency was similar between patients with venous thrombosis and age and sex-matched controls.[23]

Arterial thrombosis

Patients with homozygous or compound heterozygous plasminogen deficiency do not appear to have an increased risk of arterial thrombosis. The association between plasminogen deficiency and arterial thrombosis, however, has not been studied extensively, since the numbers of plasminogen-deficient patients are small.

Among 19 Japanese patients with homozygous plasminogen deficiency and very low plasminogen levels (10% of normal), three patients developed stroke, four myocardial infarction, and three patients had angina pectoris. The disease manifestations in this uncontrolled series, however, occurred at advanced age, which does not necessarily point to a causal role for homozygous plasminogen deficiency.[23] In a case–control study in a Japanese population, the prevalence of plasminogen deficiency in a group of patients with cardioembolic stroke was not different from in the control group.[23]

TISSUE-TYPE PLASMINOGEN ACTIVATOR

Plasminogen is converted to the active enzyme plasmin by tissue-type plasminogen activator (t-PA) released from endothelial cells. The activity of t-PA is markedly enhanced in the presence of fibrin.

Venous thrombosis

In several recent studies no relation was found between t-PA levels and risk of venous thrombosis. Circulating levels of t-PA did not predict a first venous thrombosis in a nested case–control study within the Physicians' Health Study cohort including 55 patients and controls, after a mean follow-up of 5 years.[24] In another prospective cohort study including 303 participants who had suffered from venous thrombosis, baseline plasma t-PA levels did not predict recurrence.[25]

In addition to studies focussing on t-PA levels, several researchers investigated the relation between the t-PA insertion/deletion (I/D) polymorphism and venous thrombosis. This polymorphism results from the presence or absence of an Alu repeat in the eighth intron of the t-PA gene. No difference was found between patients with venous thrombosis and controls with respect to the different genotypes in a Turkish study.[26] African-Americans with the DD genotype, however, had a 2.5-fold increased risk of venous thrombosis compared with those with the II genotype [95% confidence interval (CI) 1.1–6.0].[27] In the latter study, individuals with plasma t-PA levels in the highest quartile had a 3.2-fold increased risk of venous thrombosis compared with individuals in the first quartile (95% CI 1.4–7.3), while the different genotypes were not found to be related to t-PA plasma levels.[26,27]

Arterial thrombosis

Several studies investigating t-PA antigen levels and arterial thrombosis have

reported a positive association. In the Northern Sweden Health and Disease Cohort,[28,29] t-PA antigen levels in the highest quartile increased the risk of acute myocardial infarction approximately six-fold in both men and women relative to the lowest quartile. These results were confirmed in a number of case–control studies.[30,31] In yet another study,[32] t-PA activity in the lowest quartile was found to increase the risk of a first myocardial infarction, which seems to be in disagreement with the results of the antigen levels [odds ratio (OR) 10.7; 95% CI 1.3–89.6]. Some evidence exists for a positive association between t-PA levels and ischemic stroke. In the Swedish cohort studies mentioned above,[33] baseline t-PA antigen levels in the highest quartile increased the risk of ischemic stroke 2.3-fold (95% CI 1.1–4.9). In a large Swedish case–control study[34] including 600 patients with stroke and 600 controls, the median t-PA antigen level was slightly increased in patients compared with controls. Although t-PA activity in patients was moderately increased over the first 10 days after the event, 3 months after the event the difference with the controls disappeared. Conversely, in the Caerphilly study,[35] a prospective study among 2208 men, t-PA antigen did not have any predictive value on either cardiovascular disease, ischemic stroke, or coronary heart disease after a mean follow-up of 13 years.

Several nucleotide sequence changes have been identified in the t-PA gene, but no clear association between these changes and arterial thrombosis has been found. An association between the –7531 C/T polymorphism in the upstream enhancer of the t-PA gene and forearm vascular release rate of t-PA (reflecting local secretion of t-PA) was demonstrated,[36] but the polymorphism did not seem to influence the level of circulating plasma t-PA.[34,36-38] Only a few studies have been performed on the relation between the 7531C/T variant and stroke or myocardial infarction.[34,37,39] In one study,[37] the –7531C/T polymorphism appeared to be related to a first myocardial infarction. For stroke, contradictory results have been found.[34,39]

The observations that increased levels of t-PA are associated with an elevated risk of arterial thrombosis initially seemed paradoxical. Circulating t-PA levels, however, are dependent on the plasminogen activator inhibitor-1 (PAI-1) concentration. Therefore, as PAI-1 levels are usually in excess of t-PA levels, the t-PA measured in these studies is inactive as a result of the rapidly formed t-PA/PAI-1 complex and does not represent the release after cellular stimulation (reviewed by Booth[40]). Thus, the positive association found in the aforementioned studies presumably reflects an association of high PAI- 1 levels and arterial thrombosis, which is mechanistically more plausible. Surprisingly, however, the association between PAI-1 levels and arterial thrombosis is much less convincing (see below).

PLASMINOGEN ACTIVATOR INHIBITOR-1

PAI-1 is thought to be the principal inhibitor of t-PA. PAI-1 is secreted from endothelial cells, but is also released from platelet a granules following platelet activation.

Venous thrombosis

Levels of circulating PAI-1 do not appear to modulate the risk of venous thrombosis. In the Physicians' Health Study, no differences in PAI-1 antigen levels at baseline were found between participants who did and who did not suffer from venous thrombosis.[24] This was confirmed by a nested case–control study within the longitudinal investigation of thromboembolism etiology (LITE) cohort among 308 patients and 640 controls.[41] In another cohort study no association between PAI-1 activity and antigen levels and a recurrence of venous thrombosis was found.[25] Several case–control studies,[42-44] however, did find higher levels of PAI-1 antigen or activity in patients with (recurrent) venous thrombosis than in the controls.

The 4G/5G I/D polymorphism at position -675 in the promoter region of the PAI-1 gene is the most frequently studied polymorphism of PAI-1. The 4G allele is associated with increased gene transcription and elevated PAI-1 antigen levels compared with the 5G variant.[42,45,46] Indeed, in an Italian population it was found that the prevalence of 5G homozygous carriers was lower in 70 patients with deep venous thrombosis (10%) compared with 100 healthy controls (26%) and there was a trend towards higher levels of PAI-1 in patients.[42] This association, however, was not found in an African-American population including 91 patients and 185 controls.[27]

Arterial thrombosis

Results of studies on the relation between PAI-1 and arterial thrombosis have been conflicting and unconvincing. In several studies, high levels of PAI-1 activity or antigen did not predict a stroke or a first or recurrent myocardial infarction.[30,33,47,48] In other studies high PAI-1 activity or antigen levels were found to increase the risk of arterial thrombosis in crude analyses, but the predictive value of PAI-1 disappeared after taking insulin resistance markers into account.[29,31,32,37] This suggests that the metabolic syndrome is directly linked to PAI-1 levels (reviewed by Alessi and Juhan-Vague).[49] It is thought that besides a role in fibrinolysis, PAI-1 affects insulin signaling and adipocyte differentiation, and promotes the development of diabetes. In this way PAI-1 could play a part in the development of arterial cardiovascular disease.[49]

In one prospective study, 249 patients with angina pectoris were followed for 4 years and increased PAI activity was a risk factor for subsequent coronary events in

patients with or without diabetes, also after adjustment for potential confounders.[50] This was confirmed by the Caerphilly study including 2208 men, in whom high PAI-1 activity (third tertile) resulted in an approximately 50% increase in risk of either cardiovascular disease, ischemic stroke, or coronary heart disease.[35]

A meta-analysis[51] based on nine studies published from 1995 to March 1998, including 1299 patients and 1717 controls, found only a minor increase in the risk of myocardial infarction in individuals with the 4G/4G genotype compared with the 5G/5G (OR 1.2; 95% CI 1.0–1.5). In a subgroup analysis with two studies in high-risk populations, that is patients with diabetes and who were admitted for routine angiography for investigation of chest pain, the risk of myocardial infarction was clearly increased for those with the 4G/4G genotype relative to those with 5G/5G (OR 2.3; 95% CI 1.3–3.8).[51] A more recent meta-analysis[46] including 11 763 patients and 13 905 controls from 37 case–control studies yielded an OR of 1.06 (95% CI 1.0–1.1) for coronary disease and an OR of 1.04 (95% CI 1.0–1.1) for myocardial infarction for individuals with the 4G/4G genotype compared with those with 5G/5G. There was an indication of publication bias, however, indicating that even this small increase in risk that was found may well be overestimated.

The relation between the 4G allele and stroke is indecisive. In some studies, no association was found,[34,52-54] and in others an increased risk of ischemic stroke was associated with the 4G allele.[55] Several studies have shown a protective role for the 4G/4G genotype of the PAI-1 gene for stroke.[54,56-59] The protective effect may not be related to the function of PAI-1 in fibrinolysis, but to the inhibitory effect of PAI-1 on activation of matrix-degrading enzymes in the atherosclerotic plaque, and in that way preventing cardiac rupture followed by an ischemic embolism.[60] High levels of PAI-1 may also be protective due to the inhibition of t-PA, which may cause local damage in the neurological tissue after an ischemic stroke (reviewed by Melchor and Strickland[61]).

THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR

TAFI circulates in plasma as a proenzyme and is converted to its active form by thrombin or the thrombin–thrombomodulin complex. TAFIa removes C-terminal lysine or arginine residues from partially degraded fibrin, thereby preventing t-PA and plasminogen binding, which is required for efficient activation of plasminogen.

Venous thrombosis

High TAFI levels appear to be associated with venous thrombosis. In the Leiden Thrombophilia Study (LETS), a population-based case–control study including 474 patients and 474 controls, TAFI levels above the 90th percentile of the controls were found to be associated with an almost two-fold increased risk of a first deep vein thrombosis of the leg, compared with levels below the 90th percentile (OR 1.7; 95% CI 1.1–2.7).[62] In another case–control study including 60 patients and 62 controls a four-fold increased risk of venous thrombosis was found for TAFI levels above the 90th percentile (95% CI 1.4–10.9).[63] Moreover, in a prospective cohort study including 600 patients who already had a venous thrombosis, TAFI levels above the 75th percentile of the controls were found to be associated with an almost two-fold increased risk of a recurrence (OR 1.7; 95% CI 1.1–2.7).[64] Surprisingly, in the LETS, the risk of deep venous thrombosis for carriers of the 505A allele, who have higher TAFI antigen levels compared with 505G allele carriers, was decreased (OR 0.7; 95% CI 0.4–1.0).[65]

Arterial thrombosis

The association between TAFI levels and arterial thrombosis is not yet clear. High levels of TAFI antigen and activity were found to be associated with an increased risk of arterial thrombosis in multiple studies. In the PRIME study, a cohort study set up to explain the difference in incidence in coronary heart disease between France and Northern Ireland, individuals with baseline TAFI antigen levels in the highest tertile had a five-fold higher risk of angina pectoris than those in the lowest tertile (95% CI 1.4–18.6).[66] In a case–control study including 124 patients with a first ischemic stroke and 125 controls, TAFI activity in the highest quartile was associated with a four-fold increased risk (95% CI 1.6–9.0).[67] TAFI levels were also higher in young patients with a first myocardial infarction than in controls.[31]

Due to the large interindividual variability in plasma TAFI antigen levels, which is poorly explained by lifestyle characteristics, it has been suggested that genetic variability is the major determinant of plasma variability. Indeed, several polymorphisms have been identified which are related to TAFI levels.[68-70] The 1040C/T polymorphism in the coding region results in the Thr325Ile substitution. The (rare) TT allele has been associated with reduced TAFI antigen levels,[31,65,71] although this was not confirmed in a small study.[63] Moreover, the Ile325 allele possesses both increased TAFIa half life and antifibrinolytic potency in comparison with the Thr325 allele.[72] In a case–control study, however, the distribution of the Thr325Ile polymorphism did not differ between patients and controls, although the

Ile325 variant exhibited lower TAFI antigen levels.[31]

In the PRIME study,[73] the Ala147Thr polymorphism was associated with coronary heart disease. This haplotype was also associated with increased TAFI levels. Surprisingly, this haplotype gave an increased risk of coronary heart disease in France (OR 1.5; 95% CI 1.1–2.2) but not in Northern Ireland (OR 0.8; 95% CI 0.6–1.1). Other polymorphisms or haplotypes in the TAFI gene were not related to coronary heart disease.[73] Similar results were found for the risk of angina pectoris in the PRIME study.[66] A protective role for the Ala147Thr polymorphism was suggested in a case-control study with male survivors of a first myocardial infarction aged less than 60 years in Sweden, the UK, France and Italy (OR 0.8; 95% CI 0.6–1.0).[74] TAFI levels above the 90th percentile were also protective for myocardial infarction (OR 0.6; 95% CI 0.3–0.9). These studies contradict the idea that increased TAFI levels increase the risk of thrombosis by enhancing the inhibition of fibrinolysis. There is a major difference, however, between the various studies in the assays that were used to measure TAFI levels. It has become clear that some antigen assays are genotype dependent, which may have led to the discrepancies between studies.[75] Thus the results of the studies, including those on venous thrombosis (see above), should be interpreted with care. In addition, besides inhibiting fibrinolysis, TAFI has an inhibitory effect on inflammation[76] and as inflammatory processes play an important role in the development of cardiovascular disease, this could explain the protective effect of TAFI on arterial thrombosis (reviewed by Bouma and Mosnier[77]).

OVERALL FIBRINOLYTIC POTENTIAL

Several assays have been developed to measure overall fibrinolytic potential. The most frequently used assays are the euglobulin clot lysis time (ECLT) and dilute whole-blood clot lysis time (DWBCLT). A major disadvantage of these fibrinolytic assays is that the results do not reflect a true global fibrinolytic potential. The assays are either performed with selected plasma fractions (ECLT) or in the presence of citrate (DWBCLT), which excludes the interplay between coagulation and fibrinolysis through TAFI and factor XIII. Recently, a plasma-based tissue factor-induced clot lysis assay has been developed, in which a fibrin clot is lysed by exogenously added t-PA. The clot lysis time (CLT), as measured with this assay, was shown to be influenced by levels of other proteins involved in fibrinolysis and thrombin generation, including TAFI, plasminogen, a2-antiplasmin, PAI-1, and antithrombin.[78] A pitfall of this assay still is the absence of blood cells, which also

contribute to fibrinolytic capacity. New whole blood fibrinolysis assays are under development.[79] These whole blood fibrinolysis assays, however, have to be measured immediately after the blood draw, which complicates their use in large studies.

Venous thrombosis

Fifteen years ago, the relationship between impaired fibrinolytic activity and venous thrombosis was also reviewed.[80] The authors reported that the evidence on the association was inconclusive. While impaired fibrinolytic activity did not seem to predict overall risk of venous thrombosis, some evidence for an association between decreased fibrinolytic potential and postoperative thrombosis was found. The fibrinolytic activity in the studies included in the review was measured by the ECLT or DWBCLT assays with the drawbacks mentioned above. Recently, only a few additional studies have investigated the overall fibrinolytic potential and the risk of venous thrombosis.

In a prospective cohort study, ECLT did not predict recurrent venous thrombosis.[25] In contrast, we have shown that reduced overall fibrinolytic potential as measured with a plasma-based tissue factor-induced clot-lysis assay increased the risk of first venous thrombosis in the LETS.[81] A two-fold increased risk of deep vein thrombosis of the leg was found in patients with CLTs above the 90th percentile of the values found in controls compared with individuals with CLTs below this cut-off point (OR 1.9; 95% CI 1.3–2.9). In the MEGA study,[82] a population-based case–control study including almost 2500 patients and 3000 controls, this relation between hypofibrinolysis and the risk of venous thrombosis could be confirmed and we showed a clear dose response for CLTs and risk of deep vein thrombosis of the leg and pulmonary embolism. Using quartiles of CLT based on the values found in the controls, we found that individuals with CLTs in the highest quartile had a two-fold increased risk of venous thrombosis compared with those in the first quartile of CLT.

Arterial thrombosis

There is some evidence that impaired overall fibrinolysis is involved in the risk of arterial thrombosis. An association between fibrinolytic potential and arterial occlusions was found in the Northwick Park Heart Study.[83] In this follow-up study, a difference of one standard deviation in fibrinolytic activity (DWBCLT) was associated with an increase of about 40% in ischemic heart disease risk in men aged 40–54 years at entry over a mean follow-up of 16.1 years. In older men (54–64 years), this relation was absent. In the same study, postmenopausal women who died of ischemic heart disease had lower fibrinolytic activity at baseline than those who remained

alive.[84] In a more recent case–control study,[31] young (<51 years) patients with a first myocardial infarction also showed decreased fibrinolytic potential (ECLT) compared with controls.

CONCLUSION

In this paper, we reviewed the literature on the role of hypofibrinolysis in venous and arterial thrombosis. There is support for a relation between decreased overall fibrinolytic potential and increased risk of venous thrombosis, and assays measuring part of the fibrinolytic system (i.e. DWBCLT and ECLT) seem to correlate with arterial disease. The role of the individual components of the fibrinolytic system is far from clear. Surprisingly, plasminogen levels do not seem to be important to the risk of thrombosis, as plasminogen-deficient patients do not have an increased risk of venous and arterial thrombosis, suggesting that fibrinolysis as a whole cannot be important in the development of thrombotic disease. Both overall fibrinolytic potential and levels of certain individual factors, however, do appear to be related to thrombosis. At present, it cannot be excluded that components of the fibrinolytic system not only generate plasmin, but that they also have other functions besides plasmin generation, which are responsible for the thrombotic risk. Examples of plasmin-independent functions of fibrinolytic proteins include t-PA-induced neurotoxicity in the brain,[61] and the role of TAFI in vascular inflammation.[76] Which fibrinolytic factors contribute to thrombotic risk, and whether this risk is explained by reduced plasmin generating capacity, or by mechanisms not relating to fibrin breakdown awaits further investigation.

3

The impact of the fibrinolytic system on the risk of venous and arterial thrombosis

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ABSTRACT

In this review we discuss the association of overall hypofibrinolysis and individual fibrinolytic protein levels with venous and arterial thrombosis. Decreased overall fibrinolytic potential and high plasma levels of thrombin-activatable fibrinolysis inhibitor have been consistently associated with risk of venous thrombosis, whereas little evidence exists for a role of plasminogen, α2-antiplasmin, tissue plasminogen activator, and plasminogen activator inhibitor 1. Overall fibrinolytic potential has been associated with arterial thrombosis in young individuals, but studies on the individual components gave conflicting results. These inconsistent results could be a consequence of nonfibrinolytic properties of fibrinolytic proteins, including roles in inflammation, vascular remodeling, atherosclerosis, and the metabolic syndrome. The nonfibrinolytic properties of these proteins may have opposing effects on development of arterial disease as compared with the lytic properties, which may explain opposite results in different studies with slightly different population characteristics. These properties may be more relevant in arterial than in venous thrombosis.

INTRODUCTION

It has now been well established that hypercoagulability increases the risk of a first venous thrombosis. Although hypercoagulability has also been shown to increase the risk of arterial thrombosis, the association is not as strong as for venous thrombosis, and results from arterial studies are inconsistent.[13]

We have recently demonstrated that in addition to hypercoagulability, a hypofibrinolytic state is associated with an increased risk for a first venous thrombosis and myocardial infarction as well.[81,85] Furthermore, the presence of both hypercoagulability and hypofibrinolysis in a single individual substantially increases risk of venous thrombosis.[82] In this review we discuss the association of hypofibrinolysis with arterial and venous thrombosis.

FIBRINOLYTIC SYSTEM

In normal hemostasis, the fibrinolytic system enables removal of a fibrin clot when the damaged vessel wall is restored. Activation and regulation of fibrinolysis occurs by multiple proteins and results in the generation of plasmin, as indicated in Figure 1. Plasminogen may be activated by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). uPA binds to a cellular receptor (urokinase plasminogen activator receptor [uPAR]) resulting in enhanced activation of cell-bound plasminogen and its main role is the induction of pericellular proteolysis.[86] tPA is the most important activator of plasminogen in plasma and the main regulator of fibrinolysis. After stimulation, tPA is locally released into the circulation from the endothelial cells where it is produced.[6] tPA-mediated plasminogen activation is facilitated by a fibrin surface, which restricts fibrinolysis to the site of thrombus formation.[87] Furthermore, when bound to fibrin, tPA is protected from inhibition by plasminogen activator inhibitor 1 (PAI-1), its principal inhibitor in plasma.[88,89]

The level of PAI-1 in blood usually exceeds that of tPA; thus, in general, no active tPA circulates in plasma.[90] α2-Antiplasmin is the primary physiological inhibitor of plasmin.[7] Plasmin is rapidly inhibited by α2-antiplasmin in plasma, but it is partly protected from α2-antiplasmin when plasmin is bound to fibrin.[91] During thrombus formation, α2-antiplasmin is cross-linked to fibrin by factor XIIIa, facilitating local inhibition of fibrinolysis.[92] Thrombin-activatable fibrinolysis inhibitor (TAFI) directly connects coagulation and fibrinolysis. It can be activated by thrombin, but its activation is over 1000-fold enhanced by the thrombin-

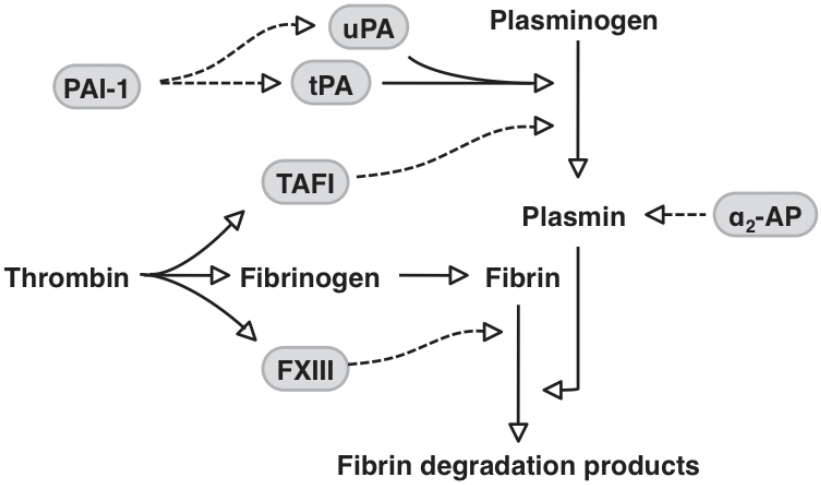

Figure 1 *A simplified representation of the fibrinolytic system.*
Solid arrows denote stimulation and activation, and dotted arrows denote inhibition. α2-ap α2-antiplasmin; PAI-1 plasminogen activator inhibitor; TAFI thrombin activatable fibrinolysis inhibitor; t-PA tissue-type plasminogen activator; u-PA urokinase plasminogen activator.

thrombomodulin complex. Activated TAFI removes carboxyl-terminal lysine residues from partially degraded fibrin. Consequently, the binding of plasminogen and tPA to the fibrin clot are decreased, which attenuates clot lysis.[93]

OVERALL HYPOFIBRINOLYSIS AS A RISK FACTOR FOR VENOUS THROMBOSIS

Until recently, no clear evidence for a role of decreased overall fibrinolytic potential in venous thrombosis, apart from a possible increase in risk found in patients undergoing surgery, existed (reviewed in[80]). In older studies the euglobulin clot lysis time, performed with plasma fractions, and the dilute whole blood clot lysis time, which excludes the interplay between coagulation and fibrinolysis through TAFI and factor XIII, were used to assess overall fibrinolytic potential. However, a drawback of these two assays is that they test only part of the fibrinolytic system. Prolonged clot lysis time (CLT) as measured with a more recently developed plasma-based assay, which is influenced by plasma levels of TAFI, plasminogen, α2-antiplasmin,

and PAI-1,[78] was associated with venous thrombosis. Individuals with CLTs >90th percentile had a twofold increased risk.[81] In the Multiple Environmental and Genetic Assessment (MEGA) of risk factors for venous thrombosis,[82] which includes >2000 patients and >2500 controls, we found a clear dose-response relation between CLT and risk of venous thrombosis (Table 1). Individuals with hypofibrinolysis who also had risk factors associated with hypercoagulability, such as factor V Leiden and immobilization, had a higher risk than would be expected based on the sum of the individual risks. Women younger than 50 years with the combination of hypofibrinolysis and oral contraceptives had the most pronounced increase in risk. Those with hypofibrinolysis, defined as CLT >75th percentile, taking oral contraceptives had a 20-fold increased risk of venous thrombosis, whereas hypofibrinolysis or oral contraceptive use alone increased the risk of venous thrombosis about twofold.

OVERALL HYPOFIBRINOLYSIS AS A RISK FACTOR FOR ARTERIAL THROMBOSIS

Subsequently, we determined whether hypofibrinolysis as determined by our new plasma-based assay was also a risk factor for a first arterial event. We determined CLTs in the Study of Myocardial Infarction Leiden (SMILE), a case-control study including 421 men with myocardial infarctions and 642 control subjects. In men <50 years of age, increased CLT (>75th percentile) was associated with a threefold (odds ratio [OR], 3.2; 95% confidence interval [CI] 1.5 to 6.7) increased risk of myocardial infarction compared with men with a short CLT (<25th percentile). The risk was reduced (OR 1.8; 95% CI, 0.7 to 4.8) after adjusting for several cardiovascular risk factors (Table 1).[85] No association between CLT and myocardial infarction was found in men >50 years of age. These results were confirmed in a study that showed prolonged CLTs as measured with the same assay constituted a risk factor for coronary artery disease, ischemic stroke, and peripheral arterial disease in young men and women.[94] Similar results were found in the Northwick Park Heart Study. In this study including 1382 men 40 to 64 years of age, fibrinolytic activity was measured by dilute whole blood clot lysis time at study entry.[83] During a mean follow-up period of 16 years, 179 men experienced an episode of ischemic heart disease. In those 40 to 54 years of age at entry, an increase of one standard deviation in dilute whole blood CLT was associated with an increase of ~40% in the risk of ischemic heart disease. Again this association was not found in older men. In the same study, mean dilute

Table 1 *Risk of Venous Thrombosis (MEGA Study) and Myocardial Infarction (SMILE) According to Quartiles of CLT*.*

Quartile of CLT	**1 (ref.)**	**2**	**3**	**4**
Venous thrombosis (MEGA study)				
No. of cases	340	491	558	701
No. of control subjects	641	641	641	641
OR_{adj} (95% CI)†	1	1.5 (1.3-1.8)	1.8 (1.5-2.2)	2.4 (2.0-2.8)
Myocardial infarction (SMILE study)				
<50 y of age				
No. of cases	14	23	34	47
No. of control subjects	46	39	31	42
OR_{adj} (95% CI)‡	1	1.7 (0.7-3.7)	3.1 (1.4-6.9)	3.2 (1.5-6.7)
OR_{adj} (95% CI)**§**	1	1.2 (0.5-3.1)	2.1 (0.8-5.2)	1.8 (0.7-4.8)
≥50 y of age				
No. of cases	75	75	75	78
No. of control subjects	114	122	130	118
OR_{adj} (95% CI)‡	1	0.9 (0.6-1.4)	0.8 (0.5-1.2)	0.9 (0.6-1.3)
OR_{adj} (95% CI)**§**	1	0.9 (0.6-1.5)	0.8 (0.5-1.2)	0.7 (0.4-1.1)

MEGA, Multiple Environmental and Genetic Assessment; SMILE, Study of Myocardial Infarctions Leiden; CLT, clot lysis time; OR, odds ratio; CI, confidence interval; ref., reference group.

** Data of the MEGA study are reproduced from reference 82, and data of the SMILE study are reproduced from reference 85.*

† OR adjusted for age and sex.

‡ OR adjusted for age.

§ *OR adjusted for age, log-transformed triglycerides, body mass index, log-transformed C-reactive protein, high-density lipoprotein cholesterol, total cholesterol, systolic and diastolic blood pressure, alcohol use, smoking status, and presence of diabetes.*

whole blood CLT was higher in women who died of ischemic heart disease than in survivors. No results were reported on age groups, probably because of the small overall patient group.[84] In a case-control study on patients <51 years of age with

a first myocardial infarction, patients had a ~30% increased euglobulin clot lysis time compared with controls.[31] And finally, in the European Concerted Action on Thrombosis and Disabilities (ECAT) study, a large cohort study including 2806 patients with angina pectoris, the number of patients with an euglobulin clot lysis time >300 minutes was slightly higher in the event group than in those who did not suffer from a coronary event (relative risk [RR] 1.30; 95% CI, 0.83 to 2.06).[95] In the Progetto Lombardo Atero-Trombosi (PLAT) study, however, a cohort including patients with myocardial infarction, angina pectoris, or transient ischemic attack, fibrinolytic activity expressed as area lysed by the euglobulin fraction on a fibrin film, either before or after venous occlusion, was similar in those who suffered from a coronary event during follow-up and in those who did not.[96] In the ECAT or PLAT study, results were only presented for the overall group, not for separate age groups.

The finding that overall hypofibrinolysis seems to be a risk factor primarily in young subjects is in agreement with other studies that found coagulation factor levels or polymorphisms in genes of coagulation factors to associate stronger with myocardial infarction in younger than in older subjects.[97,98]

In summary, multiple studies confirm an association of elevated CLT with arterial disease, predominantly in younger individuals. Is has to be noted, however, that the majority of these studies did not employ true "overall" clot lysis assays, but used assays that test only part of the fibrinolytic system.

PLEIOTROPIC EFFECTS OF FIBRINOLYTIC COMPONENTS AND ASSOCIATION WITH ARTERIAL DISEASE

The individual fibrinolytic components have biological properties in processes besides lysis of fibrin clots, including inflammation, tissue remodeling, and the metabolic syndrome. A possible association of the fibrinolytic system with arterial thrombosis may thus involve mechanisms beyond fibrin lysis. In the next paragraphs we discuss the pleiotropic effects of individual proteins and the association with arterial disease to indicate that both fibrinolytic and nonfibrinolytic properties of fibrinolytic proteins may contribute to the risk of arterial disease. Furthermore, we compare the risk of venous and arterial thrombosis associated with levels of individual fibrinolytic proteins.

Nonfibrinolytic Properties of Plasmin

Plasmin, the activated form of plasminogen, plays a key role in vascular remodeling

by degrading a variety of basement membrane proteins.[86,99] Plasmin also activates matrix metalloproteases and thereby facilitates degradation of matrix proteins.[100] Matrix degradation in advanced atherosclerotic lesions is thought to contribute to destabilization and the eventual rupture of the plaques.[101] In line with this, plasminogen knockout mice had delayed removal of necrotic tissue and fibrin, impaired wound healing, and reduced inflammatory cell and smooth muscle cell accumulation compared with wild-type mice after electric injury of the femoral artery.[102] Plasminogen deficiency greatly accelerates the formation of intimal lesions in apoprotein (Apo)E knockout mice.[103]

Furthermore, plasmin is involved in inflammatory processes by releasing interleukin-1 from macrophages and activating transforming growth factor-β,[104,105] and it plays an important role in the dissemination of various pathogens. Compared with wild-type mice, plasminogen-deficient mice seem to be more resistant to several infections, such as plague, *Borrelia* fever, and streptococci.[106] Because various pathogens have been implicated in atherosclerosis development, this nonfibrinolytic property of plasmin might be linked to arterial disease as well.[107]

Plasminogen and α2-antiplasmin and the Risk of Venous and Arterial Disease

So far, no evidence indicates that levels of plasminogen or α2-antiplasmin are associated with risk of venous thrombosis. Homozygous deficiency of α2-antiplasmin results in severe hemorrhagic tendencies, and heterozygosity does not appear to have a clinical phenotype.[108] Several studies on the incidence of venous thrombosis in plasminogen-deficient families have been performed, but evidence suggests, at best, a modest increase in risk (reviewed in[21]). Patients with plasminogen deficiency do suffer from ligneous conjunctivitis, which is a rare nonthrombotic manifestation of fibrin-rich, pseudomembranous lesions.[21] A few studies have investigated the association between homozygous plasminogen deficiency and arterial disease in Japanese subjects. In a screening for plasminogen deficiency, it was found that of 19 deficient patients with plasminogen levels of ~10% of normal, 10 had suffered from arterial complications (stroke [n = 3], myocardial infarction [n = 4], and angina pectoris [n = 3]). The age at onset of the events was >50 years in all patients, and the absence of controls makes it difficult to conclude that the arterial events were causally related to plasminogen deficiency.[23] In a case-control study, the prevalence of plasminogen deficiency in a group of Japanese patients with cardioembolic stroke was not different from the control group.[36] A small number of cohort and case-control studies investigated levels of plasminogen and α2-antiplasmin and the risk of arterial disease in the general population. Plasminogen and α2-antiplasmin

circulate at relatively high concentration in plasma so are generally not the limiting factors in fibrinolysis.[90] In one case-control study on myocardial infarction, there was some evidence for higher levels of α2-antiplasmin in patients than in controls.[109] In the ECAT study, levels of α2-antiplasmin were not associated with risk of coronary events after 2 years of follow-up.[95] No controlled studies have shown decreased levels of plasminogen to increase the risk of arterial disease. In two studies, increased plasminogen levels were associated with an increased risk of arterial disease.[95,110] These findings were unexpected considering the role of plasminogen in fibrinolysis. Possibly, elevated plasminogen reflected an increased inflammatory state because plasminogen transcription is found to be increased in response to interleukin-6.[111]

Association of PAI-1 with Cardiovascular Risk Factors and Nonfibrinolytic Properties of PAI-1 and uPA

PAI-1 levels are associated with several risk factors for cardiovascular disease, such as inflammation, atherosclerosis, and insulin resistance, and they are even considered to be a true component of the metabolic syndrome. For this reason PAI-1 has been studied extensively in relation to arterial disease.

In vivo and in vitro experiments have shown PAI-1 to be involved in insulin resistance.[112] In adipocytes of insulin-resistant mice, insulin stimulated PAI-1 gene expression.[113] Tumor necrosis factor (TNF)-α, which interferes with insulin signaling,[114] also increases PAI-1 mRNA in murine adipose tissue in vivo.[115] Furthermore, PAI-1 induction by TNF-α in adipocytes is augmented by insulin itself.[116] Epidemiological studies have indeed found PAI-1 levels to be increased in patients with insulin resistance.[112] Moreover, PAI-1 levels independently predict diabetes in patients with insulin resistance.[117]

In addition to a role in insulin resistance, PAI-1 is also associated with inflammation and development of obesity. PAI-1 levels are associated with visceral fat mass, which may be attributed to production of PAI-1 in ectopic adipose tissues[118] and in macrophages present in visceral fat.[119] PAI-1 knockout mice put on a high-fat diet were protected against fat accumulation,[119] and also when PAI-1 knockouts were crossed with ob/ob mice, PAI-1 deficiency reduced adiposity as compared with wild-type mice.[120] Also, the degree of inflammation was reduced in adipose tissue of PAI-1 deficient mice.[119] Furthermore, several factors involved in inflammation have been found to upregulate PAI-1 expression and secretion, such as angiotensin II[121] and cytokines.[122]

Evidence indicates that levels of PAI-1 and tPA are influenced by the renin-angiotensin and bradykinin systems, which regulate blood pressure. While

angiotensin II promotes PAI-1 synthesis in vitro and bradykinin increases tPA, angiotensin-converting enzyme (ACE) inhibitors decrease PAI-1 levels and augment bradykinin-induced tPA release (reviewed in[123]).

The underlying mechanisms involved in these processes are rather complex and causality remains to be established. It may be that PAI-1 adds to the increased risk of arterial disease in individuals with metabolic syndrome, insulin resistance, or increased inflammatory state and contributes to the development of cardiovascular disease.

Although PAI-1 mRNA was found to be elevated in human atherosclerotic vessels,[124,125] which may enhance vessel wall damage, in other in vivo studies PAI-1 deficiency was not associated with atherosclerosis or PAI-1 even protected against it.[126,127] It was indeed suggested that PAI-1 may limit plaque growth, prevent abnormal matrix remodeling,[128] and consequently reduce the risk of arterial disease by inhibiting uPA. uPA is thought to enable smooth muscle cell proliferation and macrophage infiltration as well as to mediate matrix degradation in macrophage-rich vulnerable regions of the plaques, which makes them more susceptible to rupture.[86,129] uPA is locally expressed in the atherosclerotic plaque, and the level is positively correlated with the presence and severity of atherosclerotic lesions.[129] Furthermore, uPA induces chemotaxis, which is regulated by PAI-1. PAI-1 forms a complex with uPA and subsequently binds to and internalizes the uPA receptor (uPAR).[130]

PAI-1 and tPA and the Risk of Venous and Arterial Disease

PAI-1 antigen, PAI-1 activity, and tPA antigen

Although overall hypofibrinolytic potential is a clear risk factor for venous thrombosis,[81,82] plasma levels of tPA and PAI-1 do not appear to be associated with venous thrombosis.[131] There might be an association with tPA and PAI-1 levels and arterial disease, although inconsistent and conflicting results have been reported. A problem concerning the measurement of both tPA and PAI-1 levels is that the levels have a strong diurnal variation, which could influence the results if measurements are not performed following standardized procedures.[132]

Most population-based studies found increased PAI-1 antigen or activity levels to be associated with increased risk of arterial disease in unadjusted models or models only adjusted for age and sex,[28,30,32,35,109,110,133-136] as did cohort studies including patients with angina or other preexisting arterial disease.[95,96,137] No association between PAI-1 and arterial events during follow-up was found in two prospective studies on patients with coronary heart disease[138] or severe angina pectoris.[139]

Opposite results were found in the Cardiovascular Health Study, in which high PAI-1 levels, if anything, were protective against cardiovascular disease in the healthy elderly (RR, 0.5; 95% CI, 0.2 to 1.2 for the fourth quartile compared with the first).[48] An effect in the same direction was found in a case-control study with ~400 patients who had a myocardial infarction and 200 controls (OR, 0.97; 95% CI, 0.96 to 0.98 for 1 ng/mL increase).[140]

Similar to PAI-1, most studies have found increased levels of tPA antigen to be associated with an increased risk of arterial events in population-based subjects[24,28,30,35,37,110,140-143] and in subjects with preexisting arterial disease[95,137,139] when no or minimal adjustments were made for cardiovascular risk factors. However, in either a cohort of patients with cardiovascular disease (PLAT study)[96] or coronary heart disease, no association was found between tPA antigen and risk of subsequent cardiovascular disease.

It has become apparent that the selection of potential confounders accounted for is of major influence on the results of studies investigating levels of tPA and PAI-1 as a risk factor for arterial thrombosis. This is nicely illustrated by the results of the ECAT study, a prospective cohort study including 2806 patients with angina pectoris who were followed for 2 years for coronary events. After adjusting for clusters of confounders, the prognostic role of PAI-1 was shown to be primarily related to insulin resistance, whereas the association of high levels of tPA with future arterial disease could be explained by different mechanisms, including insulin resistance, inflammation, and endothelial cell damage.[95] Therefore, comparing results of studies on tPA or PAI-1 is difficult and largely depends on adjustments made. Although associations have been found between tPA[24,28,30,95,141,143-145] or PAI-1[28,35,95] and arterial disease, in other studies the increased risk in individuals with high levels of tPA[35,110,139,140,142] or PAI-1[109,110,136,145] disappeared after adjusting for cardiovascular risk factors. Thus, it remains questionable whether tPA or PAI-1 levels are indeed causally related to arterial disease or just a reflection of the presence of other risk factors.

tPA activity and t-PA release after stimulation

The biological relevance of circulating levels of tPA antigen is unclear. Increased tPA might be expected to be associated with a decreased risk of arterial disease because of increased fibrinolytic activity. However elevated tPA antigen levels do not represent increased fibrinolytic activity because most of the tPA antigen in plasma is inactive due to complex formation with PAI-1. The contribution of tPA to fibrinolysis could better be assessed by monitoring the release of tPA following stimulation or by

measuring tPA activity. An inverse relationship between tPA antigen and tPA activity has even been found.[142,146]

Evidence of an association between tPA activity or tPA release after stimulation and venous thrombosis is scarce and inconsistent.[25,80] Several studies on arterial disease have also yielded conflicting results. In a nested case-control study of the Rotterdam study, increased tPA activity was not associated with myocardial infarction in crude analysis but was associated with an increased risk after adjusting for cardiovascular risk factors.[142] However, two cohort studies and two case-control studies have found elevated tPA activity to decrease the risk of arterial disease.[32,134,137,138]

In the PLAT study, tPA release after venous stasis at baseline was increased in patients with a major atherothrombotic event, compared with those who remained free of events after 2 years of follow-up.[96] However, in another cohort study on patients with coronary heart disease, patients with a decreased tPA release after venous occlusion had a higher risk of myocardial infarction, ischemic stroke, or unstable angina during follow-up of 42 months. [138] In a case-control study of young patients with myocardial infarction, the tPA release after venous occlusion was reduced in patients compared with controls.[109] In the ECAT study, the percentage of patients with zero activity after venous occlusion was 6.9% higher in those who suffered from a myocardial infarction or died of cardiovascular disease than in those who did not have an event, whereas tPA antigen levels after venous occlusion were not associated with risk.[95] So, sophisticated measures of tPA also are not consistently associated with risk of arterial disease.

Nonfibrinolytic Properties of TAFI

Although TAFI was first recognized as an attenuator of fibrinolysis,[8] more recently multiple nonfibrinolytic properties of TAFI have been described.[147] Potentially, the anti-inflammatory function of TAFI is relevant for arterial disease. TAFI downregulates the inflammatory response via inactivation of bradykinin and the anaphylatoxins C3a and C5a, which are substrates for TAFI both in vitro and in vivo.[76,148] Furthermore, TAFI modulates functions of plasminogen in cell migration. In a model of peritoneal inflammation in mice expressing half-normal amounts of plasminogen, TAFI deficiency increased leukocyte migration compared with mice with normal TAFI levels.[149] In another study, TAFI was found to protect against cobra venom factor, which is a potent nonspecific activator of complement. TAFI-deficient mice challenged with cobra venom factor showed increased mortality after priming by lipopolysaccharide.[150] TAFI itself has also been found to be an acute phase protein

in mice.[151] The role of TAFI in wound healing[152] may also be of importance for arterial disease, but no experimental data to support this hypothesis are yet available.

TAFI and the Risk of Venous and Arterial Thrombosis

High TAFI levels have been repeatedly shown to be a risk factor for a first venous thrombosis.[62,64,153] However, results of studies on the association between TAFI levels and arterial disease have been contradictory. This might be due to the pleiotropic effects of TAFI. Increased TAFI levels could increase the risk of venous or arterial thrombosis by decreasing the fibrinolytic potential. Alternatively, decreased TAFI levels could increase the risk of thrombosis through a decreased potential to counteract inflammation, which plays a role in arterial thrombosis.

Several case-control studies have found increased TAFI levels to increase the risk of arterial diseases such as angina pectoris,[154] myocardial infarction,[31] acute coronary disease,[155] or coronary artery disease.[156] In one case-control study TAFI antigen levels were decreased in patients with myocardial infarction but activity levels of TAFI were increased.[31] In a case-control study (321 patients and 645 controls) nested within the Prospective Epidemiological Study of Myocardial Infarction (PRIME) cohort, a large prospective study set up to investigate potential reasons for the difference in incidence of coronary heart disease in France and Northern Ireland, no clear association was found between baseline TAFI antigen levels and angina pectoris or myocardial infarction and coronary death.[73]

Conversely, we have found a strong inverse association between TAFI levels and risk of a first myocardial infarction in men in a case-control study (SMILE). Those with TAFI levels <25th percentile had an over threefold increased risk compared with those with levels >75th percentile (OR, 3.4; 95%CI, 2.3 to 5.1). Furthermore, we found an approximately ninefold increased risk of myocardial infarction in smokers or in those with high C-reactive protein levels compared with nonsmokers or subjects with low C-reactive protein levels and high TAFI levels, suggesting a role of TAFI in inflammation.[157] These results are in line with the Hypercoagulability and Impaired Fibrinolytic Function Mechanisms (HIFMECH) study, a European multicenter case-control study in which elevated TAFI levels also appeared to be associated with a decreased risk of myocardial infarction. The TAFI antigen assay used, however, was later found to be dependent on a specific TAFI genotype, which makes these results less reliable.

CONCLUSION

Decreased overall plasma fibrinolytic potential and high plasma levels of TAFI are consistently associated with the risk of venous thrombosis, whereas so far little evidence for a role of plasma levels of plasminogen, α2-antiplasmin, tPA, and PAI-1 in the risk of venous thrombosis exists. It is thus unclear whether the association between overall plasma fibrinolytic potential and venous thrombosis truly reflects the combined effects of all fibrinolytic proteins on risk, the effect of TAFI alone, or that the outcome of the plasma-based CLT assay is associated with venous thrombosis by mechanisms other than fibrin clot lysis.

Whereas decreased plasma fibrinolytic potential appears consistently associated with arterial thrombosis in young individuals, studies on the association of levels of plasminogen, α2-antiplasmin, tPA, PAI-1, and TAFI give conflicting results. Although a role for each protein in development of arterial disease cannot be fully excluded at present, evidence exists that the associations between levels of tPA and PAI-1 are indirect and just reflect the presence of other risk factors such as insulin resistance or inflammation.

The inconsistent results between studies on the individual proteins could be a result of the properties of fibrinolytic components in processes other than the dissolution of a blood clot, such as inflammation, vascular remodeling, atherosclerosis, and the metabolic syndrome, which may be more important in arterial than in venous thrombosis. Individual components of the fibrinolytic system can have multiple functions both promoting and inhibiting arterial disease, which may explain opposite results in different studies with slightly different population characteristics. Whether the consistent association of overall CLT with risk of arterial disease reflects a role of fibrinolysis in arterial disease or rather reflects nonfibrinolytic properties of the fibrinolytic proteins is at present not clear.

In conclusion, plasma hypofibrinolysis is likely to affect venous and arterial thrombosis. However, levels of individual fibrinolytic components seem to play a more prominent role in arterial than in venous disease, which may be explained by nonfibrinolytic functions of these proteins that have a role in development of arterial, but not or to a lesser extent of venous thrombosis.

4

Reduced plasma fibrinolytic capacity as a potential risk factor for a first myocardial infarction in young men

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ABSTRACT

Studies on the relationship between fibrinolysis and arterial thrombosis have been conflicting. Previously, we demonstrated that hypofibrinolysis, as measured by a plasma-based assay, increased the risk of venous thrombosis. The present study investigated increased clot lysis time (CLT) measured with the same assay as a risk factor for myocardial infarction in a case-control study including 421 men with a first myocardial infarction and 642 controls below 70 years. CLT was strongly associated with body-mass index, lipid levels, blood pressure and C-reactive protein. Overall, risk of myocardial infarction was 1.4-fold (95% confidence interval (CI) 1.0-1.9) increased for CLT in the fourth quartile (longest CLT) compared with the first quartile. After adjusting for cardiovascular risk factors this risk disappeared (OR 1.0, 95%CI 0.6-1.5). In men aged <50 years the association was pronounced (OR 3.2, 95%CI 1.5-6.7). After adjustment for cardiovascular risk factors the risk was nearly twofold increased (OR 1.8, 95%CI 0.7-4.8). In men aged ≥50 years, no clear association between CLT and risk of myocardial infarction was found. Our study suggests that hypofibrinolysis increases the risk of a first myocardial infarction in young men, although the causality of this association remains to be determined.

INTRODUCTION

The role of the fibrinolytic system on the risk of myocardial infarction is unclear. Although individual factors of the fibrinolytic system have been associated with myocardial infarction, results have been inconclusive and contradictory (for a review, see[131]). Increased plasminogen activator inhibitor-1 (PAI-1) activity or antigen has been found to be associated with an increased risk of first and recurrent arterial diseases.[35,50] However, other studies found no association[30,47] or the risk associated with increased PAI-1 levels disappeared after adjustment for markers of insulin resistance.[32,37] Several studies have shown a positive association between tissue-type plasminogen activator (t-PA) and the risk of myocardial infarction,[28,30,31] although this could not be confirmed in the Caerphilly study, a large prospective study.[35] The role of thrombin activatable fibrinolysis inhibitor (TAFI) as a risk factor for cardiovascular disease also is still not clear. Increased levels of TAFI were found to increase the risk of arterial disease in some studies, and to decrease it in others (reviewed in[147]).

Global assays, such as the euglobulin clot lysis time (CLT) and the dilute whole-blood CLT, were associated with cardiovascular disease in some, but not all studies (reviewed in[131]). However, both assays do not reflect true global fibrinolytic potential. In the euglobulin clot lysis test, plasma inhibitors of fibrinolysis are largely removed, and the interaction of fibrinogen, plasminogen and plasminogen activators is assayed. The dilute whole-blood CLT excludes the interplay between coagulation and fibrinolysis through TAFI and factor XIII as the test is performed in the presence of citrate, which prohibits thrombin generation. A previously described plasma-based assay appears to reflect true global plasma fibrinolytic potential, because the outcome of this assay is influenced by levels of plasminogen, alpha2-antiplasmin, plasminogen activator inhibitor-1 and thrombin activatable fibrinolysis inhibitor.[78] We recently showed in two independent case-control studies that increased CLT, as measured with this assay, was associated with an increased risk of venous thrombosis.[81,82]

In the present study, we report the association between arterial risk factors and CLT and CLT as a risk factor for myocardial infarction in the Study of Myocardial Infarctions Leiden (SMILE), a large case-control study in men.

METHODS AND MATERIALS

Subjects

The design of the SMILE has been described previously.[15] Patients were 560 male survivors of a first myocardial infarction between 1990 and 1996, below the age of 70 at the time of myocardial infarction. Patients using oral anticoagulants at time of blood drawing were excluded, leaving 426 patients available for the present study. Control subjects consisted of 646 men without a history of myocardial infarction. They had undergone a minor orthopaedic intervention between January 1990 and May 1996 and had received prophylactic anticoagulants for a short period after the intervention. They had not received anticoagulants in the 6-months prior to participation in the study. Control subjects were frequency matched on 10-years age groups to the patients.

Every participant completed a questionnaire on cardiovascular risk factors. Questions referred to current and former smoking habits and alcohol use, presence of diabetes, and current use of medication. In addition, for patients, the presence of diabetes prior to myocardial infarction was retrieved from discharge letters. Blood pressure was measured after a rest of at least 10 min with the person sitting in an upright position. Body mass index (BMI) was derived by dividing weight (in kilograms) by squared height (in metres). Of five patients and four control subjects, plasma was not available or the CLT (see Laboratory analysis) could not be measured as plasma was too turbid leaving 421 patients and 642 control subjects in the present analysis.

All participants gave informed consent. Approval for this study was obtained from the Medical Ethics Committee of the Leiden University Medical Centre, Leiden, the Netherlands.

Laboratory analysis

Fasting blood samples were drawn from the antecubital vein in Sarstedt Monovette tubes (Sarstedt, Nümbrecht, Germany) and were obtained between July 1994 and February 1997. Bloods were mainly drawn in the morning (median 9.30 h, 95% before 11.00 h), without a systematic difference between patients and controls. The time between myocardial infarction and blood drawing ranged from 88 days to 6 years with a median of 2.6 years.

Blood in the first tube was allowed to clot, and serum was used for measuring total cholesterol, high-density lipoprotein (HDL)-cholesterol and triglyceride levels. Blood taken in 0.106 mol/l trisodium citrate was centrifuged for 10 min at 3000

g at room temperature. The citrated plasma was aliquoted in multiple tubes and immediately stored at −80°C. Total cholesterol and triglyceride concentrations were measured using enzymatic assays adapted to a Hitachi 747 analyzer (Boehringer Mannheim, Mannheim, Germany), and HDL-cholesterol concentration was measured on a Hitachi 911 analyzer (Boehringer Mannheim). Plasma C-reactive protein (CRP) levels were measured using an enzyme linked immunosorbent assay (CRP EIA HS assay; Kordia, the Netherlands).

Citrated plasma samples which had not been previously thawed were used for this study. Lysis of a tissue factor-induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis as described previously.[78] Briefly, 50 µl plasma was pipetted into the wells of a 96-well microtitre plate. Subsequently, 50 µl of a mixture containing phospholipid vesicles (40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine and 40% L-α-dioleoylphosphatidylethanolamine, final concentration 10 µmol/l), t-PA (final concentration 56 ng/ml), tissue factor (final dilution 1/1000) and $CaCl_2$ (final concentration 17 mmol/l) diluted in HEPES buffer [25 mmol/l HEPES (N-2-hydroxytethylpiperazine-N'-2-ethanesulfonic acid), 137 mmol/l NaCl, 3.5 mmol/l KCl, 3 mmol/l $CaCl_2$, 0.1% bovine serum albumin (BSA), pH 7.4], was added using a multichannel pipette. After thorough mixing, the plate was incubated at 37°C in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation, Menlo Park, CA), and the optical density (OD) at 405 nm was monitored every 20 s, resulting in a clot-lysis turbidity profile. The CLT was derived from this clot-lysis profile and defined as the time from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing the lysis of the clot. The intra-assay coefficient of variation was 5.5% (n = 99) and the inter-assay coefficient of variation was 6.6% (n = 90).

Statistical analysis

To study the association between arterial risk factors with CLT in the control group, mean CLTs with 5^{th} and 95^{th} percentiles were calculated. Differences in mean CLT between categories of discrete variables in the control subjects were calculated and confidence intervals were constructed for the difference based on a t-distribution. Multiple linear regression analysis was performed with age, total cholesterol, HDL cholesterol, BMI and systolic and diastolic blood pressure entered in one model as continuous variables to determine the independent effect of these variables on CLT in the control subjects. CRP levels and triglyceride levels were entered into this

model in quartiles as the distribution was skewed. To study the effect of CLT on risk of myocardial infarction, CLTs were grouped into quartiles based on the distribution among the control subjects, taking the lowest quartile as the reference group for the odds ratio (OR). A 95% confidence interval (CI) was calculated according to the method of Woolf.[158] Unconditional logistic regression was performed to adjust for age and other potential confounders. In the logistic regression model lipid levels, BMI, CRP level and blood pressure were included as continuous variables. Triglyceride and CRP levels used in the model were 10 log-transformed as these variables were not normally distributed. Subgroups were made according to age. An arbitrary cut-off at age 50 years was chosen in accordance to previous publications on this study.[97,159,160] The Statistical Package for the Social Sciences (spss), version 14.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses.

Results

Characteristics of 421 patients who had a myocardial infarction and 642 control subjects are described in Table 1. Mean age of patients was 56.0 years and of control subjects 57.4 years. Mean CLT in 642 control subjects was 77.6 min (5^{th}-95^{th} percentile 57.5-108.4) and 79.5 min (5^{th}-95^{th} percentile 58.1-118.4) in 421 patients (mean difference 1.9; 95%CI −0.6-4.4).

Table 2 shows the association between established arterial disease risk factors and CLT in control subjects. No clear association between age and CLT was observed although men above 70 years appeared to have shorter CLT than younger individuals. No apparent difference in CLT between smokers and non-smokers was found (mean difference 1.4 min; 95% CI −2.0-4.8), between diabetics and non-diabetics (mean difference 3.7 min; 95% −5.1-12.5) or between regular users of alcohol and no/occasional users (mean difference 3.3 min; 95% CI −1.4-8.1). Clot lysis time increased with increasing BMI. Furthermore, CLT clearly increased with increasing total cholesterol, triglycerides and CRP level, and decreased with increasing HDL-cholesterol levels (Table 2). In multiple regression analysis, triglycerides (β 3.8 min/quartile; 95% CI 2.1-5.5), BMI (β 1.7 min/(kg/m^2); 95% CI 1.3-2.2), diastolic blood pressure (β 0.3 min/mmHg; 95% CI 0.1-0.5), systolic blood pressure (β −0.1 min/mmHg; 95% CI −0.2—0.003) and CRP (β 1.7 min/quartile; 95% CI 0.4-3.1) were independently associated with CLT. Total cholesterol (β 1.1 min/(mmol/l); 95% CI −0.4-2.5), HDL-cholesterol (β −3.9 min/(mmol/l); 95% CI −9.1-1.3) and age (β −0.1 min/year; 95% CI −0.2-0.1) were no longer clearly associated with CLT in the multiple regression analysis. Within the patient group, no effect of time between event and blood draw on CLT [−0.01 min per week increase (95%CI −0.04-0.02)]

was found, suggesting the absence of a post-hoc effect of the event on CLT.

Table 1 *Characteristics of patients with myocardial infarction and control subjects.*

Characteristics*	**Overall**		**<50 years**	
	Patients (*n* = 421)	**Control subjects (*n* = 642)**	**Patients (*n* = 118)**	**Control subjects (*n* = 158)**
Mean age, years (SD)	56.0 (9.0)	57.4 (10.8)	44.4 (4.3)	42.5 (6.9)
Smoking tobacco use, *n.* (%)				
No	162 (38.5)	429 (66.8)	29 (24.6)	94 (59.5)
Yes	259 (61.5)	213 (33.2)	89 (75.4)	64 (40.5)
Alcohol use, *n.* (%)				
Never	61 (14.5)	64 (10.0)	8 (6.8)	15 (9.5)
Occasionally	17 (4.0)	21 (3.3)	4 (3.4)	5 (3.2)
Regularly	343 (81.5)	557 (86.8)	106 (89.8)	138 (87.3)
BMI†, *n.* (%)				
<20 kg/m²	4 (1.0)	10 (1.6)	2 (1.7)	4 (2.5)
20–24 kg/m²	114 (27.1)	186 (29.0)	27 (22.9)	57 (36.1)
25–29 kg/m²	232 (55.2)	342 (53.4)	63 (53.4)	78 (49.4)
≥30 kg/m²	70 (16.7)	103 (16.1)	26 (22.0)	19 (12.0)
Diabetes, *n.* (%)				
Absent	409 (97.1)	620 (96.6)	116 (98.3)	157 (98.1)
Present	12 (2.9)	22 (3.4)	2 (1.7)	3 (1.9)
Total cholesterol‡, mmol/l				
<5.17	87 (20.8)	163 (25.4)	22 (28.6)	52 (32.9)
5.17–5.81	109 (26.0)	159 (24.8)	30 (25.4)	32 (20.3)
5.82–6.59	111 (26.5)	159 (24.8)	26 (22.0)	41 (25.9)
≥6.60	112 (26.7)	160 (25.0)	40 (33.9)	33 (20.9)
Diastolic blood pressure§, mmHg				
≤80	131 (31.4)	200 (31.4)	36 (30.8)	61 (39.1)
85	80 (19.2)	93 (14.6)	20 (17.1)	25 (16.0)
90	111 (26.6)	168 (26.4)	30 (25.6)	43 (27.6)
≥95	95 (22.8)	176 (27.6)	31 (26.5)	27 (17.3)

Characteristics*	Overall		<50 years	
	Patients (*n* = 421)	Control subjects (*n* = 642)	Patients (*n* = 118)	Control subjects (*n* = 158)
Systolic blood pressure§, mmHg				
≤125	158 (24.8)	109 (26.1)	43 (36.8)	74 (47.4)
130–135	114 (17.9)	90 (21.6)	32 (27.4)	37 (23.7)
140–150	204 (32.0)	129 (30.9)	34 (29.1)	31 (19.9)
≥155	161 (25.3)	89 (21.3)	8 (6.8)	14 (9.0)

BMI, body mass index.

** Data for patients refer to the period prior to myocardial infarction for age, smoking, alcohol use, and diabetes. Data for patients refer to time of blood draw for body mass index, total cholesterol, diastolic blood pressure, and systolic blood pressure.*

† Information about BMI was unavailable for 1 patient and 1 control subject.

‡ Information about total cholesterol was unavailable for two patients and one control subject.

§ Information about diastolic and systolic blood pressure was unavailable for four patients and five control subjects.

Using the case group, similar trends were seen for the association between these risk factors and CLT (data not shown).

The association between CLT and the risk of myocardial infarction is shown in Table 3. In the overall study, a weak dose-response relationship between CLT and the risk of myocardial infarction was found. The risk of myocardial infarction was 1.4-fold (95%CI 1.0-1.9) increased for individuals with hypofibrinolysis, i.e. CLTs in the highest quartile, compared with the first quartile after adjusting for age. After further adjustment for triglycerides (model 2), this association disappeared (OR 0.9; 95%CI 0.6-1.3). Additional adjustment for BMI, CRP, total and HDL-cholesterol, and systolic and diastolic blood pressure (model 3) did not change the results, nor did additional adjustment for smoking, alcohol use and diabetes (model 4). In individuals <50 years of age, an age-adjusted OR of 3.2 (95%CI 1.5-6.7) was found in those with hypofibrinolysis. Adjusting for triglycerides attenuated this risk (OR 2.0; 95%CI 0.9-4.5). Further adjustment resulted in ORs of 1.6 (95%CI 0.6-4.1) (model 3) and 1.8 (95%CI 0.7-4.8) (model 4). In men aged ≥50 years, hypofibrinolysis was not associated with the risk of myocardial infarction (age-adjusted OR 0.9; 95%CI 0.6-1.3). Adjusting for triglycerides moderately decreased the risk (OR 0.5; 95%CI

Table 2 *Association between cardiovascular risk factors and clot lysis time in control subjects.*

Cardiovascular risk factor	Control subjects n. (%) (N = 642)	Mean CLT (5^{th}–95^{th} percentile)
Age at time of blood draw, years		
<50	158 (24.6)	77.5 (56.0–124.9)
50–59	180 (28.0)	78.2 (60.0–106.5)
60–69	247 (38.5)	79.3 (59.5–111.0)
70–75	57 (8.9)	68.7 (55.1–82.6)
Smoking		
No	429 (66.8)	78.1 (57.8–108.2)
Yes	213 (33.2)	76.7 (56.0–110.4)
Alcohol use		
Never	64 (10.0)	74.6 (56.8–101.5)
Occasionally	21 (3.3)	75.0 (51.5–92·6)
Regularly	557 (86.8)	78.1 (57.5–110.3)
BMI*, kg/m²		
<20	10 (1.6)	66.5 (53.6–90.7)
20–24	186 (29.0)	69.4 (55.5–86.9)
25–29	342 (53.4)	77.5 (59.5–104.9)
≥30	103 (16.1)	93.9 (60.5–169.0)
Diabetes		
Absent	620 (96.6)	77.7 (57.7–108.0)
Present	22 (3.4)	74.0 (55.9–133.1)
Total cholesterol*, mmol/l		
<5.17	163 (25.4)	72.0 (55.4–102.2)
5·17–5.81	159 (24.8)	76.6 (56.8–96.3)
5·82–6.59	159 (24.8)	79.4 (60.3–105.1)
≥6.60	160 (25.0)	82.5 (60.9–124.0)
HDL-cholesterol†, mmol/l		
<1.10	159 (24.9)	85.1 (56.0–158.2)
1.10–1.28	159 (24.9)	78.7 (58.0–115.1)
1.29–1.53	163 (25.5)	75.5 (56.0–105.1)
≥1.54	158 (24.9)	71.2 (57.0–89.5)

Cardiovascular risk factor	Control subjects n. (%) (N = 642)	Mean CLT (5th–95th percentile)
Triglycerides*, mmol/l		
<0.90	162 (25.3)	69.0 (55.5–87.1)
0.90–1.24	160 (25.0)	73.5 (56.0–92.6)
1.25–1.82	161 (25.1)	77.2 (58.3–109.9)
≥1.83	158 (24.6)	91.0 (64.0–158.2)
CRP, mg/L		
<0.78	161 (25.1)	71.8 (55.4–93.2)
0.78–1.55	162 (25.2)	77.5 (56.9–109.8)
1.56–3.42	160 (24.9)	78.1 (58.8–106.6)
≥3.43	159 (24.8)	83.1 (60.3–143.7)
Diastolic blood pressure‡, mmHg		
≤80	200 (31.4)	72.7 (56.2–92.8)
85	93 (14.6)	77.6 (58.7–127.3)
90	168 (26.4)	75.9 (56.0–104.7)
≥95	176 (27.6)	85.1 (60.6–144.2)
Systolic blood pressure‡, mmHg		
≤125	158 (24.8)	74.1 (56.1–102.1)
130–135	114 (17.9)	76.4 (59.8–101.9)
140–150	204 (32.0)	79.8 (56.4–129.5)
≥155	161 (25.3)	79.5 (58.1–111.7)

CLT, clot lysis time; BMI, body mass index; CRP, C-reactive protein.
** Information about BMI, total cholesterol and triglycerides was unavailable for one control subject.*
† Information about HDL-cholesterol was unavailable for three control subjects.
‡ Information about diastolic and systolic blood pressure was unavailable for five control subjects.

0.3-0.9) and full adjustment (model 4) slightly increased this risk again (OR 0.7; 95%CI 0.4-1.1).

Table 3 *Risk of myocardial infarction according to quartiles of clot lysis time.*

Quartile of CLT	**1***	**2**	**3**	**4**
Lysis time at cut-off, min		66.4	73.4	83.0
Overall study				
No. cases	89	98	109	125
No. control subjects	160	161	161	160
OR (95% CI)†	1	1.1 (0.8–1.6)	1.2 (0.9–1.7)	1.4 (1.0–1.9)
OR (95% CI)‡	1	1.0 (0.7–1.4)	0.9 (0.6–1.4)	0.9 (0.6–1.3)
OR (95% CI)§	1	1.0 (0.7–1.5)	1.0 (0.7–1.4)	0.9 (0.6–1.4)
OR (95% CI)¶	1	1.0 (0.7–1.5)	1.0 (0.7–1.6)	1.0 (0.6–1.5)
<50 years				
No. cases	14	23	34	47
No. control subjects	46	39	31	42
OR (95% CI)†	1	1.7 (0.7–3.7)	3.1 (1.4–6.9)	3.2 (1.5–6.7)
OR (95% CI)‡	1	1.5 (0.6–3.4)	2.3 (1.0–5.3)	2.0 (0.9–4.5)
OR (95% CI)§	1	1.2 (0.5–2.8)	1.9 (0.8–4.7)	1.6 (0.6–4.1)
OR (95% CI)¶	1	1.2 (0.5–3.1)	2.1 (0.8–5.2)	1.8 (0.7–4.8)
≥50 years				
No. cases	75	75	75	78
No. control subjects	114	122	130	118
OR (95% CI)†	1	0.9 (0.6–1.4)	0.8 (0.5–1.2)	0.9 (0.6–1.3)
OR (95% CI)‡	1	0.8 (0.5–1.3)	0.7 (0.4–1.0)	0.5 (0.3–0.9)
OR (95% CI)§	1	0.9 (0.6–1.4)	0.7 (0.4–1.1)	0.6 (0.4–1.0)
OR (95% CI)¶	1	0.9 (0.6–1.5)	0.8 (0.5–1.2)	0.7 (0.4–1.1)

CLT, clot lysis time; OR, odds ratio; CI, confidence interval.

** Reference group.*

† Odds ratio adjusted for age (model 1).

‡ Odds ratio adjusted for age and log-transformed triglycerides (model 2).

§ Odds ratio adjusted as in model 2 plus BMI, log-transformed C-reactive protein, HDL-cholesterol, total cholesterol, systolic and diastolic blood pressure (model 3).

¶ Odds ratio adjusted as in model 3 plus alcohol use, smoking status, and presence of diabetes (model 4).

DISCUSSION

This case-control study showed that hypofibrinolysis, as measured with an overall plasma-based assay, was associated with a twofold increased risk of myocardial infarction in men aged <50 years. CLT was strongly associated with BMI, levels of triglycerides, CRP levels and, to a lesser extent, with blood pressure. However, these arterial risk factors could not fully explain the association between CLT and risk of myocardial infarction. The overall effect was weak, and there was no association in older men.

Clot lysis time increased with increasing BMI, which confirms our previous findings of the Moet zijn: Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA-study), a population based case-control study on venous thrombosis.[82] This association may be explained by increased levels of PAI-1, which is considered to be a biomarker of visceral fat storage.[112] TAFI may also explain this association, as levels have been associated with lipid levels and BMI,[154,161] although not in all studies.[162] Recently a link between PAI-1 and the metabolic syndrome has been described.[49] The metabolic syndrome consists of a cluster of metabolic abnormalities, including obesity, insulin resistance, elevated triglyceride levels, and low HDL cholesterol concentration, so possibly also explaining the association between lipid levels and CLT. Using lipid levels and BMI in one model resulted in an independent effect of only triglycerides and BMI on CLT.

The positive association between CRP levels, diastolic or systolic blood pressure and CLT may also be mediated via increased TAFI or PAI-1 levels.[154,162-164]

After adjusting for age, we found a threefold increase in risk of myocardial infarction for the highest quartile of CLT in men <50 years of age. After further adjustment for several arterial risk factors this risk was attenuated. The risk, however, remained twofold increased although confidence intervals were wide.

It could, however, be argued whether these adjustments are justified in this study. For example, the increased risk of myocardial infarction in individuals with increased BMI may be attributable (in part) to increased PAI-1 levels, which directly cause longer CLT values.

This is the first study regarding the association of fibrinolytic capacity and myocardial infarction, using this overall assay. So far, only a few studies have investigated the effect of overall fibrinolytic activity on the risk of arterial disease. In the Northwick Park Heart Study, a large prospective cohort study of 1382 Caucasian men, decreased fibrinolytic potential, i.e., one standard deviation increase in dilute whole blood CLT, increased the risk of ischemic heart disease in men aged 40-54

years with about 40%, but not in men between 54-64 years.[83] In the same study it was found that women who died of ischaemic heart disease (n = 19) also had increased dilute whole blood CLT compared to those who survived (n = 621).[84] In a case-control study on young patients (below 51 years of age) with a first myocardial infarction, patients had about 30% increased euglobulin CLT compared with controls.[31] In another case-control study including 33 patients with myocardial infarction and 33 control subjects, patients had slower lysis rates than the controls.[165]

Our results are in line with the concept that hypercoagulability, for example as a result of the F5 R506Q (Factor V Leiden) or F2 G20210A (prothrombin G20210A) mutation, increases the risk of arterial thrombosis.[13,46] Thus, not only an increased capacity to form a clot, but also a decreased capacity for clot lysis precipitates arterial clot formation. As was found for these abnormalities too, effects were generally weaker in arterial than venous disease. Whether a combination of hypercoagulability and hypofibrinolysis synergistically increases the risk of myocardial infarction as it does for venous thrombosis[82] remains to be studied. The present study was insufficiently powered to investigate this hypothesis.

We found hypofibrinolysis only to be a risk factor for myocardial infarction in younger men, which is in agreement with other studies that found coagulation factor levels or polymorphisms in genes of coagulation factors to associate more strongly with myocardial infarction in younger than in older subjects.[83,97,98,159]

A consequence of using a case-control design is that increased CLT could be a result rather than a cause of a myocardial infarction and that the levels do not represent the preinfarction levels. Nevertheless, the blood samples were drawn at least half a year and, in most of the patients, several years after the myocardial infarction. Within the patient group, we did not find an effect of time between event and blood draw on CLT suggesting that long CLTs are not a consequence of the myocardial infarction or an acute phase reaction.

We cannot exclude an interaction of cardiovascular medication use with CLT. In this study we were unable to reliably investigate whether these interactions exist, and whether they affect risk estimates, because most patients use medication against hypertension, hypercholesterolemia, or diabetes, whereas control subjects do not. However, as this medication might increase clot lysis[166] and therefore decrease CLT, the risk estimates may be underestimated.

As in nearly all case-control studies, we studied patients who survived a first myocardial infarction. In theory, this could have led to survival bias, if CLT affected survival. The main factors affecting survival are size of the infarction, and delay in effective assistance, which affect the time frame from onset of symptoms to

the start of interventions, such as thrombolytic therapy.[167,168] It seems unlikely that hypofibrinolysis would affect mortality to such an extent to affect the results.

In conclusion, decreased overall fibrinolytic potential, which is strongly associated with serum lipid levels, is associated with an increased risk of myocardial infarction in young men. This association is not found in men ≥50 years of age.

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5

Plasma levels of fibrinolytic proteins and the risk of myocardial infarction in men

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ABSTRACT

Hypofibrinolysis as measured with overall clot lysis assays is associated with risk of arterial thrombosis. Individual components of the fibrinolytic system, however, have not been studied extensively in relation to arterial disease or results of studies were inconsistent. The relation between plasminogen and α2-antiplasmin levels and cardiovascular risk factors and the association between plasminogen, α2-antiplasmin, tissue-plasminogen activator (t-PA), and plasminogen activator inhibitor-1 (PAI-1) and risk of myocardial infarction was investigated in the Study of Myocardial Infarctions Leiden (555 men with a first myocardial infarction and 635 controls). α2-Antiplasmin was associated with age and lipid levels while plasminogen correlated with lipids, C-reactive protein and smoking. Increased levels of all fibrinolytic factors were associated with myocardial infarction. Age-adjusted odds ratios (OR) (95% confidence interval) for quartile 4 compared with 1 were 1.7 (1.2-2.3) for plasminogen, 1.9 (1.3-2.6) for α2-antiplasmin, 1.7 (1.2-2.3) for t-PA, and 1.7 (1.2-2.4) for PAI-1. After adjusting for cardiovascular risk factors, only α2-antiplasmin levels remained associated with risk [OR 1.4 (1.0-2.0)]. t-PA and PAI-1 levels predominantly reflected lipid levels whereas plasminogen reflected the inflammatory state. Concluding, elevated α2-antiplasmin levels are independently associated with risk of myocardial infarction. t-PA, PAI-1, and plasminogen levels appear to reflect other cardiovascular risk factors.

INTRODUCTION

Decreased fibrinolytic potential as measured with overall clot lysis assays has been found to be associated with increased risk of arterial thrombosis, especially in young individuals, in several studies.[83,85,94] Surprisingly, plasma levels of individual components of the fibrinolytic system have either not been studied extensively in the context of arterial thrombosis or were not consistently associated with arterial thrombosis. In particular, population-based studies on the role of α2-antiplasmin and plasminogen in risk of arterial thrombosis are scarce. In the European Concerted Action on Thrombosis and Disabilities (ECAT) study, a cohort study including patients with angina pectoris, no association was found between levels of α2-antiplasmin and risk of myocardial infarction or sudden death.[95] Unexpectedly, increased levels of plasminogen were associated with an increased risk. In the Atherosclerosis Risk in Communities (ARIC) study, a population-based cohort study on subjects between 44 and 65 years at baseline, a positive association between plasminogen levels and coronary heart disease was also found.[110]

The results of studies on levels of tissue type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) have been conflicting. Both t-PA and PAI-1 levels were associated with arterial disease in multiple studies. However, whether increased levels of t-PA and PAI-1 independently increase the risk remained to be elucidated.[14] PAI-1 is now recognized as a true component of the metabolic syndrome,[49] which is strongly associated with arterial thrombosis. In several studies the predictive value of PAI-1 and t-PA disappeared after adjusting for cardiovascular risk factors such as body mass index (BMI), insulin resistance, inflammation and lipid levels (reviewed in Meltzer *et al.* [14]). This may indicate that levels of these fibrinolytic factors are rather a reflection of underlying disease than a direct cause of arterial thrombosis.

Results of studies on the association of plasma levels of Thrombin Activatable Fibrinolysis Inhibitor (TAFI) with risk of arterial thrombosis are also contradictory. Several studies have found increased levels of TAFI to be associated with an increased risk of arterial disease[156,169] while others found no association.[170] We have recently shown increased functional TAFI levels to be associated with a decreased risk of myocardial infarction in the Study of Myocardial Infarctions LEiden (SMILE), a large case-control study in men.[157] In the present study, we investigate associations between levels of plasminogen, α2-antiplasmin, PAI-1, and t-PA and risk of myocardial infarction in the SMILE. Furthermore, as knowledge on determinants of plasma levels of α2-antiplasmin and plasminogen is scarce, the

association between established cardiovascular risk factors and these two fibrinolytic factors was also studied.

METHODS AND MATERIALS

Subjects

The design of the SMILE has been described previously.[171] Patients were 560 men with a first myocardial infarction between 1990 and 1996, below the age of 70 at the onset of myocardial infarction. Two of the following three characteristics had to be identifiable in the discharge report or hospital care record to confirm acute myocardial infarction: typical chest pain, electrocardiographical changes indicative of evolving myocardial infarction or a transient rise in cardiac enzymes to more than twice the upper limit of normal.

The control group consisted of 646 men without a history of myocardial infarction. They had undergone a minor orthopedic intervention between January 1990 and May 1996 for which they had received prophylactic anticoagulants for a short period. They had not received anticoagulants in the 6-months prior to participation in the study. Control subjects were frequency matched on 10-year age groups to the patients. Every participant completed a questionnaire on cardiovascular risk factors including questions on current and former smoking habits and alcohol use, presence of diabetes, and current use of medication. In addition, for patients, presence of diabetes prior to myocardial infarction was retrieved from discharge letters. A person was classified as hypertensive or hypercholesterolemic when he was taking prescription drugs for these conditions. Blood pressure was measured after a rest of at least 10 minutes with the person sitting in an upright position. BMI was derived by dividing weight (in kilograms) by squared height (in meters). All participants gave informed consent. Approval for this study was obtained from the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands.

Laboratory analysis

Fasting blood samples of patients and control subjects were drawn from the antecubital vein in Sarstedt Monovette tubes (Sarstedt, Nümbrecht, Germany) and were obtained between July 1994 and February 1997. Blood samples were primarily drawn in the morning (median 9.30 h, 95% before 11.00 h), without a systematic difference between patients and control subjects. Time between myocardial infarction and blood draw ranged from 88 days to 6 years with a median of 2.6 years.

Serum and plasma samples were aliquoted in multiple tubes and immediately stored at –80°C. Plasma levels of von Willebrand factor (VWF) and C-reactive protein (CRP) and serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride levels were measured as described previously.[171,172]

Plasma levels of fibrinolytic factors were measured in citrated plasma. α2-Antiplasmin and plasminogen activity was measured using chromogenic assays (STA Stachrom antiplasmin and STA Stachrom plasminogen from Diagnostica Stago, Asnières, France) and were performed on a STA-R coagulation analyzer using a commercial calibration standard (Diagnostica Stago, Asnières, France) and expressed as a percentage of normal. PAI-1 antigen levels were measured with a Technozym PAI-1 enzyme-linked immunosorbent assay (ELISA) reagent kit (Kordia, Biopool, the Netherlands) and were expressed in ng/ml. Antigen levels of t-PA were assessed by ELISA using a commercially available mouse anti-t-PA antibody (Nuclilab BV, Ede, The Netherlands) as capture, and a biotine-labelled rabbit anti human t-PA antibody (Nuclilab BV, Ede, The Netherlands) as detecting antibody. Bound detecting antibody was visualised using biotin-labeled streptavidine, followed by Tetramethylbenzidine (TMB) staining. A calibration curve was constructed using purified t-PA (Nuclilab BV, Ede, The Netherlands), and results were expressed as ng/ml.

The intra-assay coefficients of variation were 1.7% for plasminogen, 4.8% for α2-antiplasmin, 7.6% for PAI-1 and 11.4% for t-PA and the inter-assay coefficients of variation were 1.6% for plasminogen, 4.6% for α2-antiplasmin, 5.0% for PAI-1 and 8.1% for t-PA.

In 5 patients and 11 control subjects fibrinolytic protein levels were not measured as available plasma was not sufficient, leaving 555 patients and 635 control subjects in the analyses.

Statistical analysis

The association between cardiovascular risk factors and plasma levels of α2-antiplasmin and plasminogen were studied in the control group. Mean α2-antiplasmin and plasminogen levels were calculated with 5th and 95th percentiles for categories of cardiovascular risk factors. Quartiles of blood pressure, total cholesterol, HDL cholesterol, triglyceride, VWF and CRP were defined based on the distribution among control subjects. Multiple linear regression was used to investigate which factors were independently associated with levels of α2-antiplasmin and plasminogen. As the associations between triglycerides, VWF, and CRP with α2-antiplasmin and plasminogen were not linear these variables were entered in the model divided into

quartiles resulting in a regression coefficient for a quartile increase of the independent variable. For reasons of comparability systolic blood pressure, HDL cholesterol and total cholesterol were also included in the model as quartiles as were levels of α2-antiplasmin when studied as determinants of plasminogen and vice versa. Using 10log-transformation for the not normally distributed variables instead of quartiles did not considerably change the results. However, as a regression coefficient of a 10log-transformed variable is more difficult to interpret than a regression coefficient for each quartile increase we present the latter.

To study the effect of fibrinolytic factors on risk of myocardial infarction, levels of α2-antiplasmin, plasminogen, PAI-1, and t-PA were grouped into quartiles based on the distribution among the control subjects, taking the lowest quartile as the reference group for the odds ratio (OR). A 95% confidence interval (CI) was calculated according to the method of Woolf.[158] Unconditional logistic regression was performed to adjust for age and other potential confounders (see below). In the logistic regression model age, BMI, blood pressure, VWF, CRP, and lipid levels were included as continuous variables. Triglycerides, VWF and CRP levels were included in the model after 10log-transformation as these variables were not normally distributed. Subgroups were made according to age. An arbitrary cut-off at age 50 years was chosen in accordance to previous publications on the SMILE study.[85,97,159] SPSS 16.0 (SPSS, Chicago, IL, USA) was used for statistical analyses.

Confounders

Available literature on factors influencing plasma levels of α2-antiplasmin and plasminogen is limited. Associations between established cardiovascular risk factors and levels of α2-antiplasmin or plasminogen were studied in our own data and potential confounders were selected from these analyses and included in the statistical model.

Several studies investigated determinants of t-PA and PAI-1. Therefore, confounding variables in the association between t-PA or PAI-1 and myocardial infarction were chosen from these studies and included in the models, i.e. diabetes, BMI, lipid levels, plasma levels of VWF and CRP, and blood pressure.[49,95] Indeed, these factors were also associated with t-PA and PAI-1 levels in our own data, with the exception of VWF, which was not associated with PAI-1 in our data (data not shown). The analyses on PAI-1 and t-PA and risk of myocardial infarction were also mutually adjusted for each other.

RESULTS

Mean age of the 555 patients with myocardial infarction was 56.3 years (5^{th}-95^{th} percentiles 40.0-68.8 years) and mean age of 635 control subjects was 57.4 (5^{th}-95^{th} percentiles 34.7-72.1 years). Risk factors for arterial disease such as smoking, obesity, diabetes, hypertension, and hypercholesterolemia were more prevalent in patients than in control subjects (Table 1).

Cardiovascular risk factors and plasma levels of α2-antiplasmin and plasminogen

The association between risk factors for myocardial infarction and α2-antiplasmin levels in control subjects is shown in Table 2. α2-Antiplasmin was negatively associated with age, HDL cholesterol, VWF, and systolic blood pressure level, and positively associated with total cholesterol, triglyceride and plasminogen levels. α2-Antiplasmin also increased with BMI although the small group of men (n=10) with BMI<20 kg/m² did not have low levels. To determine the independent effect of these factors on α2-antiplasmin levels, age, BMI, HDL cholesterol, VWF, systolic blood pressure, total cholesterol, triglyceride and plasminogen were simultaneously included in a multiple linear regression model. Age (β=-0.3%/year; 95%CI -0.4;-0.2) and HDL cholesterol (β=-1.2%/quartile; 95%CI -2.2;-0.3) were both negatively associated with α2-antiplasmin. BMI (β=0.3%/kg*m^{-2}; 95%CI 0.0;0.6) and total cholesterol (0.8%/quartile; 95%CI -0.1;1.8) were positively associated with α2-antiplasmin. Plasminogen was strongly related to α2-antiplasmin (β=2.5%/quartile; 95%CI 1.8;3.4). Systolic blood pressure (β=-0.5%/quartile; 95%CI -1.5;0.4), triglycerides (β=-0.2%/quartile; 95%CI -1.3;0.8), and VWF (β=0.0%/quartile) were not associated with α2-antiplasmin. So, HDL cholesterol, total cholesterol and plasminogen were the strongest determinants of α2-antiplasmin.

Plasminogen levels increased with levels of triglycerides, total cholesterol, and CRP and were increased in smokers (Table 2). Also alcohol use was associated with plasminogen although not in a dose dependent manner as the occasional drinkers had the lowest levels of plasminogen. Similar to α2-antiplasmin, plasminogen increased with BMI but the small group of underweight subjects (BMI < 20 kg/m²) had high levels. We performed a multiple regression including age, BMI, triglycerides, total cholesterol, CRP, alcohol use, smoking, and α2-antiplasmin levels. Except for age, all variables were independently associated with plasminogen. The regression coefficients were 1.6%/quartile (95%CI 0.9;2.4) for total cholesterol and 1.2%/quartile (95%CI 0.2;1.9) for triglycerides.

Table 1 *Characteristics of men with first myocardial infarction and control subjects.*

Characteristics	Patients	Control subjects
N	**555**	**635**
Mean age, y (5^{th}-95^{th} percentile)	56.3 (40.0-68.8)	57.4 (34.7-72.1)
Current smoking, no. (%)		
No	208 (37.5)	426 (67.1)
Yes	347 (62.5)	209 (32.9)
Alcohol use, no. (%)		
Never	86 (15.5)	64 (10.1)
Occasionally	24 (4.3)	20 (3.1)
Regularly	445 (80.2)	551 (86.8)
BMI, * no. (%)		
Less than 20 kg/m^2	4 (0.7)	10 (1.6)
20-24 kg/m^2	152 (27.4)	182 (28.7)
25-30 kg/m^2	305 (55.1)	341 (53.8)
At least 30 kg/m^2	93 (16.8)	101 (15.9)
Diabetes, no. (%)		
Absent	529 (95.3)	614 (96.7)
Present	26 (4.7)	21 (3.3)
Hypertension, no. (%)		
Absent	449 (80.9)	529 (83.3)
Present	106 (19.1)	106 (16.7)
Hypercholesterolemia, no. (%)		
Absent	544 (98.0)	623 (98.1)
Present	11 (2.0)	12 (1.9)

BMI, body mass index.

Data for patients refer to the period prior to myocardial infarction, apart from BMI.

** For 1 patient and 1 control subject BMI was missing.*

Table 2 *Cardiovascular risk factors among 635 control subjects and the association with α2-antiplasmin and plasminogen levels.*

Cardiovascular risk factor	n	mean α2-antiplasmin level (%) (5^{th}-95^{th} percentile)	mean plasminogen level (%) (5^{th}-95^{th} percentile)
age (year)			
27-39	54	104 (84-122)	93 (74-107)
40-49	103	98 (80-121)	94 (77-113)
50-59	178*	97 (77-115)	97 (78-114)
60-69	243	93 (74-114)å	94 (77-114)
70-75	57	90 (78-108)	91 (72-115)
BMI ($kg*m^{-2}$)†			
Less than 20	10	97 (74-115)	99 (79-115)
20-24	182	94 (74-112)	92 (72-112)
25-29	341	96 (78-116)	94 (78-112)
At least 30	101*	98 (78-123)	98 (80-116)
diabetes			
no	614*	96 (77-116)	94 (77-113)
yes	21	95 (78-123)	92 (70-113)
diastolic blood pressure (mmHg)‡			
less than 80	199	96 (76-119)	93 (77-115)
85	90*	96 (81-116)	96 (76-114)
90	167	94 (74-114)	93 (74-114)
at least 95	174	97 (78-116)	96 (78-112)
systolic blood pressure (mmHg)‡			
less than 125	157	98 (75-121)	94 (76-113)
130-135	114	95 (77-116)	94 (80-109)
140-150	200*	96 (78-115)	94 (75-115)
at least 155	159	93 (78-112)	95 (76-115)
triglycerides (mmol/L)†			
less than 0.90	157	94 (74-115)	90 (72-109)
0.90-1.24	159	95 (76-113)	94 (76-111)
1.25-1.82	161	96 (78-117)	96 (80-115)
at least 1.83	157	98 (81-118)	98 (81-116)

Cardiovascular risk factor	n	mean α2-antiplasmin level (%) (5th-95th percentile)	mean plasminogen level (%) (5th-95th percentile)
total cholesterol (mmol/L)†			
less than 5.17	161	94 (75-113)	91 (72-111)
5.17-5.81	156	95 (76-117)	93 (76-111)
5.82-6.59	157	97 (80-119)	94 (79-110)
at least 6.60	160	98 (80-118)	99 (84-116)
HDL-cholesterol (mmol/L)§			
less than 1.1	159	98 (78-116)	95 (76-116)
1.1-1.28	156	97 (80-119)	94 (76-113)
1.29-1.53	162	94 (73-114)	94 (76-112)
at least 1.54	155	95 (78-117)	94 (78-115)
C-reactive protein (mg/L)			
less than 0.78	159	95 (74-113)	89 (74-108)
0.78-1.55	161	96 (78-116)	93 (78-107)
1.56-3.42	159*	96 (78-119)	95 (76-119)
at least 3.43	156	96 (78-116)	101 (85-118)
smoking			
no	426*	96 (78-117)	93 (76-112)
yes	209	95 (75-115)	98 (80-116)
alcohol use			
no	64	96 (79-117)	92 (74-111)
occasionally	20	95 (55-123)	89 (70-106)
yes	551*	96 (77-116)	95 (77-114)
VWF (%)‖			
less than 97	159	97 (77-117)	92 (74-108)
98-124	151	97 (81-118)	94 (78-114)
125-158	145	96 (74-114)	96 (77-115)
at least 159	152	93 (75-114)	96 (77-117)

Cardiovascular risk factor	n	mean α2-antiplasmin level (%) (5th-95th percentile)	mean plasminogen level (%) (5th-95th percentile)
plasminogen (%)*			
less than 87	171	91 (74-109)	
87-93	152	95 (76-114)	
94-100	155	97 (81-116)	
at least 101	156	100 (81-120)	
α2-antiplasmin (%)			
less than 87	163		91 (72-109)
87-94	163		93 (76-112)
9 5-104	153		95 (76-113)
at least 105	155		99 (84-116)

BMI, body mass index; VWF, von Willebrand factor.

** For 1 control subject plasminogen levels were missing.*

† For 1 control subject BMI was missing.

‡ For 5 control subjects systolic and diastolic blood pressure was missing.

§ For 3 control stubjects HDL cholesterol was missing.

|| For 28 control subjects WVF was missing.

Plasminogen increased 2.9% (95%CI 2.2-3.6) with each quartile increase of CRP and 2.2% (95%CI 1.5-2.9) per quartile increase in α2-antiplasmin. Compared to occasional drinkers of alcohol, regular drinkers had 5.7% higher levels (95%CI 1.5-10.0), and abstainers had 3.4% higher levels (95%CI -1.4;8.2). Smoking increased plasminogen levels with 3.6% (95%CI 1.9-5.2) compared to not smoking. So, plasminogen was strongly associated with variables related to inflammation.

Plasma levels of fibrinolytic proteins and risk of myocardial infarction

α2-antiplasmin

Mean α2-antiplasmin level in patients was 99% (median 98%; 5th-95th percentile 81-119%) and 96% in controls (median 95%; 5th-95th percentile 77-116%). Levels of α2-antiplasmin were associated with risk of myocardial infarction in a dose dependent manner. In men with the highest levels of α2-antiplasmin, the age-adjusted risk was approximately two-fold increased (model 1: OR 1.9; 95%CI 1.3-2.6; 4th quartile (Q) compared with 1st) (Table 3). As lipid levels were the strongest determinants of α2-

antiplasmin levels, apart from plasminogen levels, we first adjusted for HDL and total cholesterol (model 2).

Table 3 *Risk of myocardial infarction with increasing quartiles of α2-antiplasmin.*

Quartile	**1***	**2**	**3**	**4**
cut-off point (%)	86	93	100	
patients†	96	128	152	178
control subjects†	163	163	154	155
model 1: age	1	1.3 (0.9-1.9)	1.6 (1.2-2.3)	1.9 (1.3-2.6)
model 2: Model 1 + HDL and total cholesterol	1	1.2 (0.8-1.7)	1.5 (1.0-2.1)	1.5 (1.0-2.2)
model 3: Model 2 + BMI	1	1.2 (0.8-1.7)	1.5 (1.0-2.1)	1.5 (1.0-2.2)
model 4: Model 3 + plasminogen	1	1.2 (0.8-1.7)	1.4 (1.0-2.0)	1.4 (1.0-2.0)

BMI, body mass index.

** Reference group.*

† For 1 patient α2-antiplasmin levels were missing.

This reduced the OR but high levels of α2-antiplasmin were still associated with an increased risk of myocardial infarction (OR 1.5; 95%CI 1.0-2.1). Further adjustment for BMI (model 3; OR 1.5; 95%CI 1.1-2.2) and additional adjustment for plasminogen did not reduce the OR further (model 4; OR 1.4; 95%CI 1.0-2.0). The same analysis in men below 50 years of age resulted in an age-adjusted OR of 2.6 (95%CI 1.2-5.9; Q4 vs. Q1) and 1.6 (95%CI 0.7-3.8) after adjusting for age, HDL and total cholesterol, BMI and plasminogen (model 4). In men older than 50 these age-adjusted ORs were 1.7 (95%CI 1.2-1.7) and 1.4 (95%CI 0.9-2.2) after extensive adjustment.

Plasminogen

Mean plasminogen level in patients was 96% (median 96%; 5^{th}-95^{th} percentile 79-115%) and 94% in controls (median 94%; 5^{th}-95^{th} percentile 77-113%). The risk of myocardial infarction increased with each increasing quartile of plasminogen (Table 4). The risk in men with the highest levels of plasminogen was 1.7-fold (95%CI 1.2-2.3; Q4 vs Q1) increased after adjusting for age (model 1). As plasminogen levels were strongly associated with variables related to inflammation, we adjusted for CRP and smoking (model 2) reducing the OR to no effect (OR 1.1 (95%CI 0.7-1.5). Adjusting for age and CRP or age and smoking separately yielded similar results as simultaneous adjustment for age and both CRP and smoking (data not shown).

Adding triglycerides, total cholesterol and alcohol use marginally changed the odds ratio (OR 0.9; 95%CI 0.06-1.3; model 3) as did additional adjustment for α2-antiplasmin levels (OR 0.8; 95%CI 0.5-1.2; model 4). Similar results were found when analyses were performed separately in men below 50 years of age and in men of 50 years and older. Although the age-adjusted risk in young men, was higher (OR 2.6; 95%CI 1.3-5.0; Q4 vs Q1) than in the older (OR 1.3; 95%CI 0.9-1.9; Q4 vs Q1), the increased risks disappeared after further adjustment for smoking, alcohol use and levels of triglycerides, total cholesterol, and α2-antiplasmin (model 4) (OR 0.8; 95%CI 0.3-1.8 in men <50 years of age and 0.7; 95%CI 0.4-1.1 in men >50 years).

Table 4 *Risk of myocardial infarction with increasing quartiles of plasminogen.*

Quartile	**1***	**2**	**3**	4
cut-off point (%)	**86**	**94**	**104**	
patients†	112	125	144	174
control subjects†	171	152	155	156
model 1: age	1	1.2 (0.9-1.7)	1.4 (1.0-1.9)	1.7 (1.2-2.3)
model 2: Model 1 + CRP and smoking	1	1.0 (0.7-1.5)	1.1 (0.8-1.5)	1.1 (0.7-1.5)
Model 3: Model 2 + triglycerides, total cholesterol, and alcohol use	1	1.0 (0.7-1.4)	1.0 (0.7-1.5)	0.9 (0.6-1.3)
Model 4: Model 3 + α2-antiplasmin	1	0.9 (0.6-1.3)	0.9 (0.6-1.3)	0.8 (0.5-1.2)

CRP, C-reactive protein.

** Reference group.*

† For 1 control subject plasminogen levels were missing.

PAI-1

Mean PAI-1 level in patients was 107.4 ng/ml (median 69.7 ng/ml; 5^{th}-95^{th} percentile 14.7-316.9 ng/ml) and 88.8 ng/ml in controls (median 54.9 ng/ml; 5^{th}-95^{th} percentile 13.0-302.7 ng/ml). Those with high PAI-1 levels had an increased risk of myocardial infarction (OR 1.7; 95%CI 1.2-2.3; Q4 vs Q1) but no dose response relation was found after adjusting for age (model 1) (Table 5). As PAI-1 is a marker of the insulin resistance syndrome, we first adjusted for triglycerides, HDL and total cholesterol, BMI and diabetes (model 2) resulting in an OR of 1.1 (95%CI 0.8-1.6), with the largest effect after adjusting for triglycerides and HDL cholesterol (data not shown). Adjusting only for age and CRP to determine the role of inflammation in the association between PAI-1 and myocardial infarction only slightly decreased the OR

to 1.5 (95% 1.1-2.1; Q4 vs Q1). Further adjustments for other potential confounders (CRP, VWF, systolic and diastolic blood pressure; model 3) did not change the results, neither did additional adjustment for t-PA (model 4). Analysing men above and below 50 years of age separately gave similar results (data not shown).

Table 5 *Risk of myocardial infarction with increasing quartiles of PAI-1.*

Quartile	**1***	**2**	**3**	**4**
cut-off point (ng/ml)	32.9	54.9	99.0	
patients†	118	109	121	202
control subjects†	158	159	159	158
Model 1: age	1	0.9 (0.7-1.3)	1.0 (0.7-1.4)	1.7 (1.2-2.3)
Model 2: Model 1 + HDL and total cholesterol, triglycerides, BMI and diabetes	1	0.8 (0.6-1.2)	0.8 (0.6-1.2)	1.1 (0.8-1.6)
Model 3: Model 2 + CRP, VWF, systolic and diastolic blood pressure	1	0.8 (0.6-1.2)	0.8 (0.6-1.2)	1.1 (0.8-1.7)
Model 4: Model 3 + t-PA	1	0.8 (0.6-1.2)	0.8 (0.6-1.2)	1.1 (0.8-1.7)

BMI, body mass index; CRP, C-reactive protein; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue plasminogen activator; VWF, von Willebrand factor.

** Reference group.*

† For 1 control subject and 5 patients PAI-1 levels were missing.

t-PA

Mean t-PA level in patients was 9.2 ng/ml (median 8.9 ng/ml; 5^{th}-95^{th} percentile 5.0-14.6 ng/ml) and 8.8 ng/ml in controls (median 8.1 ng/ml; 5^{th}-95^{th} percentile 4.5-15.3 ng/ml).

The risk of myocardial infarction was increased in men with t-PA levels above the median (age-adjusted OR 2.0; 95%CI 1.4-2.7 (Q3) and OR 1.7; 95%CI 1.2-2.4 (Q4 vs Q1)) (Table 6). Additional adjusting for lipid levels, diabetes and BMI (model 2) attenuated the risk (OR 1.5; 95%CI 1.1-2.2 (Q3) and OR 1.1; 95%CI 0.8-1.6 (Q4)), again with the largest reduction in risk after adjustment for triglycerides or HDL cholesterol (data not shown). In contrast, adjusting for age and CRP had only little effect on risk, reducing the OR for Q3 to 1.8 (95% 1.3-2.6) and Q4 to 1.6 (95%CI 1.1-2.2) and similar results were found after adjusting for age and VWF as marker of endothelial activation (OR 1.9; 95%CI 1.3-2.6 (Q3) and OR 1.6; 95%CI 1.2-2.3 (Q4)). Including all potential confounders (lipid levels, diabetes, BMI, VWF, CRP, systolic

and diastolic blood pressure) reduced the risk slightly more (OR 1.4; 95%CI 1.0-2.0 (Q3); OR 1.0; 95%CI 0.7-1.5 (Q4) model 3). Adding PAI-1 to the statistical model did not influence the risk (model 4). Results were similar in men of 50 years and older (data not shown). In younger men the age-adjusted risk was slightly decreased in Q2 (OR 0.8; 95%CI 0.4-1.4) and over two-fold increased in the upper two quartiles (OR 2.5; 95%CI 1.3-1.6 (Q3) and OR 2.8; 95%CI 1.4-5.4 (Q4)). These ORs decreased to 0.5 (95%CI 0.2-1.0) for Q2, 1.7 (95%CI 0.8-3.4) for Q3 and 1.6 (95%CI 0.7-3.6) when using multivariate model 4.

Table 6 *Risk of myocardial infarction with increasing quartiles of t-PA.*

Quartile	**1***	**2**	**3**	**4**
cut-off point (ng/ml)	6.3	8.1	10.6	
patients†	104	104	185	160
control subjects†	158	159	159	158
Model 1: age	1	1.1 (0.8-1.5)	2.0 (1.4-2.7)	1.7 (1.2-2.4)
Model 2: Model 1 + HDL and total cholesterol, triglycerides, BMI and diabetes	1	0.9 (0.6-1.3)	1.5(1.1-2.2)	1.1 (0.8-1.6)
Model 3: Model 2 + CRP, VWF, systolic and diastolic blood pressure	1	0.9 (0.6-1.4)	1.4 (1.0-2.0)	1.0 (0.7-1.5)
Model 4: Model 3 + PAI-1	1	0.9 (0.6-1.3)	1.4 (1.0-2.0)	1.0 (0.6-1.5)

BMI, body mass index; CRP, C-reactive protein; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue plasminogen activator; VWF, von Willebrand factor.

** Reference group.*

† For 1 control subject and 2 patients t-PA levels were missing.

DISCUSSION

In this study we have shown that increased levels of α2-antiplasmin are associated with a two-fold increased risk of a first myocardial infarction in men. After adjusting for several potential confounders the risk was still 40% increased as compared to individuals with low levels of α2-antiplasmin. Risk of myocardial infarction was also increased in men with elevated levels of plasminogen, PAI-1 and t-PA in age-adjusted models, but this increased risk was not independent of other risk factors.

The present study is the first to show increased α2-antiplasmin levels to be

associated with an increased risk of myocardial infarction. This is consistent with established findings on the association between hypofibrinolysis as measured with overall clot lysis assays and an increased risk of arterial thrombosis[85,94] and with the bleeding tendency observed in patients with α2-antiplasmin deficiency.[108] The ECAT study is the only other large study that examined the relation between α2-antiplasmin levels and risk of arterial disease.[95] In this cohort study, which consisted of approximately 2600 patients with angina pectoris at baseline and 97 events after two years of follow-up, no association between α2-antiplasmin levels and myocardial infarction and cardiovascular death was found. This difference in study population may explain the divergent results in the ECAT study and the study presented in this paper.

Epidemiological literature on α2-antiplasmin in relation to risk factors for arterial thrombosis is limited. Here we show that plasma levels of α2-antiplasmin are only marginally influenced by established cardiovascular risk factors. Although levels of the majority of haemostatic factors increase with age, α2-antiplasmin levels decreased with age, which is in agreement with results of the ECAT study.[173] There was also a weak negative association between α2-antiplasmin and HDL cholesterol and a positive association with BMI and total cholesterol. Although the association between lipid levels and α2-antiplasmin has been found before,[174] to our knowledge studies on the mechanism linking lipid levels to levels of α2-antiplasmin are at present lacking. There is no obvious explanation for the strong correlation between plasminogen and α2-antiplasmin plasma levels. It is, however, a common finding that several coagulation and fibrinolytic factors cluster together and correlate possibly via a common regulatory mechanism.[175-177]

We found elevated plasminogen levels to be associated with risk of myocardial infarction, although the risk disappeared after adjustment for potential confounders. The ECAT [95] and ARIC study[110] also found a positive association between plasminogen levels and risk of arterial disease, which is contradictory considering the role of plasminogen in fibrinolysis. In the present study the association disappeared after adjusting for lipid levels, CRP, smoking, and alcohol use. In the ECAT study no adjustments were made apart from study center, age and sex, and the risk of myocardial infarction or sudden death remained only slightly increased after these adjustments.[95] In the ARIC study adjustments were made for several cardiovascular risk factors, although not for triglycerides and CRP, and substantially increased risks were found even after these adjustments.[110] The differences in results between studies may thus be explained by differences in confounding factors considered in the analyses.

We found that regular drinkers of alcoholic beverages had increased plasminogen levels. In a study on hepatocyte cell lines it has indeed been shown that alcohol increases plasminogen gene expression and a moderate dose of alcohol also increased plasmin levels in mice.[178] Similar to α2-antiplasmin, the association between plasminogen and lipid levels was shown previously but the mechanism behind the association has not been described.[110] In contrast, various pathways relating plasminogen to inflammation have been described. Elevated plasma levels of plasminogen may reflect an increased inflammatory state as plasminogen transcription is increased by interleukin-6.[111] This may also explain the association between plasminogen and smoking, as smoking leads to an increased inflammatory state.[179] Conversely, there is evidence that plasminogen induces an inflammatory response. When bound to several plasminogen receptors, shown for example for the receptors enolase-1 and histone H2B, plasminogen facilitates transmigration and basement membrane degradation and aids in the degradation of the extracellular matrix and recruitment of macrophages.[180] Thus, increased inflammation can cause increased plasminogen levels and, vice versa, increased plasminogen levels can increase the inflammatory state. Consequently, as inflammation increases the risk of myocardial infarction,[111,180] the positive association between plasminogen and myocardial infarction can be either indirect and just reflecting the inflammatory state or it can indeed be causal. However, after adjusting for smoking alone, which, in contrast to high CRP levels, can only cause and not result from an increased inflammatory state or high plasminogen levels, we find that the association between plasminogen and myocardial infarction disappears. This provides evidence against a causal role for plasminogen in myocardial infarction.

Both t-PA and PAI-1 were not independently associated with myocardial infarction in our study. Previous studies on t-PA and PAI-1 levels gave conflicting results. While some studies have found increased t-PA and PAI-1 levels to be associated with an increased risk of arterial disease,[28,30,35] several studies have found no association,[110,140,145] and in two studies even a trend towards a decreased risk was found in subjects with elevated PAI-1.[48,140] These inconsistent results have been ascribed to the adjustments made for confounders in the analyses as these vary across studies.[14,95] In the ECAT study, the prognostic value of t-PA and PAI-1 was studied after adjustment for clusters of confounding variables.[95] Our results on t-PA and PAI-1 are to a large extent in agreement with this study. In the ECAT study the risk of arterial disease associated with increased PAI-1 levels disappeared after adjustment for parameters associated with insulin resistance (BMI, triglycerides, HDL cholesterol, systolic blood pressure, diabetes). In our study these factors,

and particularly lipid levels, could also explain the association between PAI-1 and myocardial infarction. In accordance with results of the ECAT study, the effect of t-PA on arterial disease risk was explained by the combination of the markers of insulin resistance, and although to a lesser extent, to CRP as marker of inflammation and VWF as indicator of endothelial activation. Our results are also, at least in part, in agreement with two meta-analyses conducted on the association between t-PA and PAI-1 and coronary heart disease.[143] PAI-1 was not associated with coronary heart disease in this meta-analysis including 6 prospective cohort studies. In the meta-analysis including 12 prospective cohort studies on t-PA and coronary heart disease an OR of 1.48 was found for the third tertile of t-PA compared with the first. There was, however, evidence of publication bias, showing a tendency to more extreme ORs in the smaller studies and no adjustments were made for inflammation or VWF suggesting that this risk estimate was overestimated.

Besides differences in statistical models used, the roles of the fibrinolytic proteins other than in clot dissolution may also have attributed to the inconsistency of results of different studies (reviewed in Meltzer *et al.* [14]). Besides its role in clot lysis, plasminogen possibly contributes to destabilisation of atherosclerotic plaques independent of fibrin proteolysis. Plasmin activates several matrix metalloproteinases (MMPs)[86] and it has been consistently shown that MMPs increase atherosclerosis progression and plaque instability (reviewed by Newby [181]).
Furthermore, PAI-1 can both promote and suppress vascular remodeling by mechanisms not directly related to clot lysis. Local PAI-1 levels have been shown to be associated with severity of atherosclerosis[182] and PAI-1 deficient mice were found to be protected against atherosclerosis progression in the carotid artery.[127] In contrast PAI-1 may also protect against abnormal matrix remodeling in advanced atherosclerotic plaques as well as plaque rupture and destabilisation of a plaque caused by urokinase plasminogen activator.[60,128] Consequently, heterogeneity in atherogenic state within and between study populations may hamper direct comparison of study results. Future research may gain more insight in the role of fibrinolytic factors in arterial disease by taking this into account.

While decreased overall fibrinolytic potential as measured with an overall plasma clot lysis assay has been shown to be associated with an increased risk of myocardial infarction in the SMILE,[85] only elevated α2-antiplasmin levels were associated with risk, whereas increased TAFI levels even strongly protected against myocardial infarction.[157,183] An explanation for our findings is that CLT measures the combination of the individual factors, also taking the interplay between them into account. The complex interplay between proteins in the fibrinolytic process is

not taken into account when plasma levels of the individual factors are measured. Indeed, we recently showed that the clot lysis time is sensitive for fibrinolytic factors, but that the outcome of the assay appears to be determined by factors beyond plasma levels of fibrinolytic proteins.[183]

A limitation of the case-control study design is that levels of the fibrinolytic proteins are measured after the event, and that post-event levels may not reflect levels prior to the myocardial infarction. Blood samples were drawn several months or even years after the event by which we attempted to minimize the likelihood that an acute phase reaction was responsible for the plasma levels of the fibrinolytic proteins. Furthermore, we made adjustments for CRP levels, which is an acute phase protein. Caution is, however, needed in interpreting the results and replication of our results is required using studies with other study designs, preferably adequately powered prospective studies.

From our study we conclude that increased levels of α2-antiplasmin are associated with an increased risk of myocardial infarction in men. PAI-1, t-PA and plasminogen are no independent risk factors for myocardial infarction. Plasminogen is primarily a marker of inflammation, while high PAI-1 and t-PA levels predominantly reflect increased lipid levels and to a lesser extent inflammation. In addition, endothelial activation may in part explain the association between elevated levels of t-PA and myocardial infarction.

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6

Low thrombin activatable fibrinolysis inhibitor activity levels are associated with an increased risk of a first myocardial infarction in men

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ABSTRACT

Background
Studies on the relation between thrombin activatable fibrinolysis inhibitor (TAFI) and arterial thrombosis have produced conflicting results.TAFI regulates fibrinolysis, but other roles of this inhibitor, including anti-inflammatory properties, have also been demonstrated.

Design and methods
We investigated the association between TAFI activity and the risk of myocardial infarction. Additionally, we studied the association of common single nucleotide polymorphisms in the TAFI gene with levels of the TAFI protein and risk of myocardial infarction.We included 554 men under 70 years old with a first myocardial infarction and 643 controls participating in the Study of Myocardial Infarctions Leiden (SMILE), a case-control study.

Results
We found odds ratios (95% confidence intervals) of a first myocardial infarction of 2.4 (1.6–3.6), 3.2 (2.1–4.7) and 3.4 (2.3–5.1) for subjects whose TAFI levels were in the third, second and first quartiles (lowest TAFI levels), respectively, compared with the fourth quartile, after adjusting for arterial disease risk factors. The rare -438A and 1040T alleles were associated with lower, and the rare 505G allele with higher TAFI levels than the common alleles. Carriers of the -438A allele had an increased risk of myocardial infarction (odds ratio 1.6 (1.0–2.5) for AA; odds ratio 1.2 (0.9–1.5) for AG compared with GG). The other single nucleotide polymorphisms were not associated with myocardial infarction.

Conclusions
Low TAFI activity levels are associated with increased risk of a first myocardial infarction in men. The results on the association between TAFI single nucleotide polymorphisms and myocardial infarction were inconsistent.

INTRODUCTION

Thrombin activatable fibrinolysis inhibitor (TAFI) forms the molecular link between the coagulation and fibrinolytic systems.[93] Various animal models have demonstrated that TAFI regulates both venous and arterial fibrinolysis *in vivo*.[184-187] Furthermore, epidemiological studies have shown elevated TAFI levels to be a mild risk factor for the development of a first or recurrent venous thrombosis.[62,64,153] However, studies on the association between single nucleotide polymorphisms (SNP) that are associated with TAFI levels and the risk of venous thrombosis have given conflicting results.[65,70,188]

Epidemiological data on the association between TAFI levels and the risk of arterial disease are inconsistent. Several case-control studies have shown high TAFI levels to be associated with the risk of coronary artery disease.[154-156] However, elevated TAFI levels, or polymorphisms in the *TAFI* gene associated with increased TAFI levels, have been related to a *decreased* risk of myocardial infarction.[74] In one case-control study, high TAFI antigen decreased but high TAFI activity increased the risk of myocardial infarction in young patients.[31] Furthermore, in a prospective study, decreased TAFI levels were associated with refractiveness to medical treatment in patients with unstable angina pectoris.[189]

Recently, biological functions of TAFI distinct from fibrinolysis regulation have been described, including regulation of inflammation, blood pressure, cell migration, and wound healing.[147] These functions may depend on substrates of TAFI other than fibrin, for instance bradykinin, the anaphylatoxins C3a and C5a, annexin II, and osteopontin.[76,190]

The contradictory results from epidemiological studies may be explained by two opposite effects of TAFI on the development of arterial thrombosis. While the association between high levels of TAFI and arterial thrombosis may be the result of a hypofibrinolytic state, the association between low TAFI levels and arterial thrombosis may be explained by a defective regulation of inflammation.

Another explanation of the contradictory results could be the use of different methods for measuring TAFI. Several different antigen assays as well as distinct activity assays have been used in epidemiological studies. With the discovery that some enzyme linked immunosorbent assays of TAFI antigen are dependent on the 1040C/T polymorphism, revealing a lack of reactivity with the 1040T variant,[75] even more caution is required in the interpretation of results.

The -438G/A SNP in the promoter region of the *TAFI* gene,[68] the 505G/A SNP (which encodes an Ala to Thr substitution at position 147),[68] and the 1040C/T SNP

(encoding a Thr to Ile substitution at position 325)[69] are known to be associated with TAFI plasma antigen levels. In Caucasians, three main haplotypes can be identified (www.hapmap.org). The -438G/A and 1040C/T are generally located in the same haplotype, but are not in complete linkage disequilibrium.[191] The 505A/G is typical to a second haplotype. A third haplotype is characterized by a fourth SNP in intron 4 (i4 + 164 A/C). In the Study of Myocardial Infarctions Leiden (SMILE), a case-control study of men with a first myocardial infarction, we investigated determinants of the level of TAFI. In addition we studied the effect of plasma TAFI levels, as determined by a functional assay, and the -438G/A, 505A/G, 1040C/T, and i4 + 164A/C SNP in the *TAFI* gene on the risk of a first myocardial infarction.

DESIGN AND METHODS

Selection of participants

The design of SMILE has been described previously.[171] In brief, the recruited patients were men who had a first myocardial infarction between January 1990 and January 1996, who were below the age of 70. Control subjects were frequency matched on 10-year age groups to the patients and were men without a history of myocardial infarction, without renal disease or severe (neuro)psychiatric problems, with a life expectancy of more than 1 year, and who had not taken any oral anticoagulants in the 6-month period prior to participation in the study. All participants completed a questionnaire and an interview took place prior to collection of blood samples. Questions referred to present and former smoking habits and alcohol use, diabetes, and current use of medications. Blood pressure was measured after a rest of at least 10 min with the person sitting in an upright position. In addition, for patients, information on diabetes and medication use prior to myocardial infarction was retrieved from discharge letters. A person was classified as hypertensive or hypercholesterolemic when he was taking prescription drugs for these conditions. All participants signed an informed consent form. Approval for this study was obtained from the the Medical Ethics Committee of the Leiden University Medical Center, Leiden, The Netherlands.

Blood collection and laboratory analysis

Fasting blood samples were drawn from the antecubital vein into Sarstedt Monovette tubes (Sarstedt, Nümbrecht, Germany) and were obtained between July 1994 and February 1997. Most blood samples were collected in the morning (at a median of

9:35 am, with 95% before 11:00 am). The time between myocardial infarction and blood sampling ranged from 88 days to 5.8 years with a median of 2.6 years.

Total cholesterol, HDL-cholesterol, triglyceride, and C-reactive protein (CRP) concentrations were measured as described previously.[171]

Plasma TAFI activity levels were determined in citrated plasma with a chromogenic assay (Pefakit TAFI, Pentapharm Ltd., Basel, Switzerland) by converting TAFI into its active form using a reagent containing thrombinthrombomodulin and subsequently measuring carboxypeptidase activity. Measurements were run on a BCS coagulation analyzer (Dade Behring Inc., Marburg, Germany). TAFI activity was expressed in percentages using a calibration curve of pooled normal plasma (pool of samples from more than 200 healthy volunteers). The inter-assay coefficients of variation were 5.8% (n=32) at ~100% TAFI concentration and 8.0% (n=31) at ~28% TAFI concentration. For six patients and three control subjects, plasma was unavailable or reliable results were not obtained; therefore 554 patients and 643 control subjects were included in the analysis.

The -438G/A (rs2146881), i4 + 164 A/C (rs3818477), 505G/A (rs3742264), and 1040C/T (rs1926447) polymorphisms were determined by a 5'nuclease assay (Taqman; Applied Biosystems, Foster City, CA) using a standard polymerase chain reaction (PCR) mix (Eurogentec, Seraing, Belgium) and allele-specific fluorescent probes equipped with a minor groove binding moiety (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Mean TAFI levels were calculated with 5th and 95th percentiles for categories of arterial risk factors. Quartiles of continuous variables (blood pressure, total cholesterol, HDL-cholesterol, triglyceride, and CRP) were defined based on the distribution among control subjects. A multiple linear regression analysis was performed among the control subjects to determine the independent effect of arterial risk factors on TAFI levels. As the association between lipid levels or CRP and TAFI is not linear, these variables were entered in the model using quartiles.

The association between TAFI levels and the risk of a first myocardial infarction was investigated by calculating odds ratios (OR), as an approximation of relative risks, and 95% confidence intervals (CI). TAFI levels were grouped into quartiles based on the distribution among the control subjects. The highest quartile (highest TAFI levels) was taken as the reference group. OR adjusted for age and other potential confounders were calculated using multivariate unconditional logistic regression. Age, levels of triglycerides, cholesterol, CRP, and body mass index

(BMI) were used as continuous variables in the model. Levels of triglycerides and CRP were entered into the model after 10log transformation because these variables were not normally distributed. Genotypic frequencies for each of the four loci were determined from the observed genotypic counts, and X^2-analysis was used to check for departures from the Hardy-Weinberg equilibrium. The association between the four TAFI SNP and risk of myocardial infarction was also assessed by means of unconditional logistic regression. The homozygous genotype of the common allele was taken as the reference group. SPSS 14.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses.

RESULTS

The mean age of the 554 patients with myocardial infarction was 56.4 years (5^{th}–95^{th} percentiles, 40.4–68.8 years) and that of the 643 control subjects was 57.3 (5^{th}–95^{th} percentiles, 34.7–72.1 years). Arterial risk factors such as smoking, obesity, diabetes, hypertension, and hypercholesterolemia were more prevalent in patients than in control subjects (Table 1). The mean TAFI level in patients was 84% (5^{th}–95^{th}, percentile 56–112%) and 90% in controls (5^{th}–95^{th}, percentile 58–128%). Age, BMI, presence of diabetes, smoking, or alcohol use had no or little effect on TAFI levels (Table 2). Cholesterol and triglyceride levels were positively associated with TAFI levels. TAFI levels were 83% in subjects with low cholesterol levels (first quartile) and 98% in those with high cholesterol levels (4^{th} quartile). Similarly, TAFI levels increased from 84 to 94% when comparing individuals with low and high triglyceride levels. TAFI levels also increased with CRP levels (85% for those with low and 96% for individuals with high CRP). No apparent association was present with HDL cholesterol or blood pressure. Multiple linear regression was performed to determine the independent effect of CRP, total cholesterol, and triglycerides on TAFI levels. With each increase in quartile of CRP, TAFI increased 2.8% (β =2.8%/quartile of CRP; 95% CI 1.4–4.4). For total cholesterol this was 3.8%/quartile (95% CI 2.3–5.3) and 1.7% (95% CI 0.2–3.2) for triglycerides. Adding smoking to the model did not change the coefficients (*data not shown*).

The association between TAFI levels (in quartiles) and the risk of myocardial infarction is shown in Table 3. The fourth quartile (highest TAFI levels) was taken as the reference group. The risk of myocardial infarction increased with each decreasing quartile. Men with the lowest TAFI levels (first quartile) had a 2.8-fold age-adjusted increased risk of myocardial infarction compared with men with TAFI levels in the

fourth quartile (OR 2.8; 95% CI 1.9–4.0). Adjusting for other factors found to be associated with TAFI in the control subjects (CRP, triglycerides, and cholesterol), resulted in similar or slightly higher odds ratios (OR 3.4; 95% CI 2.3–5.1). Further adjustment for BMI, smoking, alcohol use, and diabetes (full model) did not substantially change the estimates.

Table 1 *Characteristics of men with a first myocardial infarction and control subjects.*

Characteristics*	**Patients (*n* = 554)**	**Control subjects (*n* = 643)**
Mean age, years (5^{th}-95^{th} percentile)	56.4 (40.4-68.8)	57.3 (34.7-72.1)
Current smoking, *n.* (%)		
No	209 (37.7)	429 (66.7)
Yes	345 (62.3)	214 (33.3)
Alcohol use, *n.* (%)		
Never	85 (15.3)	64 (10.0)
Occasionally	23 (4.2)	21 (3.3)
Regularly	446 (80.5)	558 (86.8)
Body mass index†, *n.* (%)		
<20 kg/m²	4 (0.7)	10 (1.6)
20–24 kg/m²	151 (27.3)	186 (29.0)
25–29 kg/m²	306 (55.3)	340 (53.0
≥30 kg/m²	92 (16.6)	106 (16.5)
Diabetes, *n.* (%)		
Absent	528 (95.3)	621 (96.6)
Present	26 (4.7)	22 (3.4)
Hypertension, *n.* (%)		
Absent	450 (81.2)	537 (83.5)
Present	104 (18.8)	106 (16.5)
Hypercholesterolemia, *n.* (%)		
Absent	543 (98.0)	632 (98.3)
Present	11 (2.0)	11 (1.7)

** Data for patients refer to the period prior to myocardial infarction, apart from body mass index.*

† Body mass index was missing for one patient and one control subject.

Table 2 *Arterial risk factors among control subjects and the association with TAFI levels.*

	N. of control subjects (total=643)	**Mean TAFI% (5^{th}-95^{th} pecentile)**
Age (years)		
27-39	56	85 (36-124)
40-49	104	91 (40-138)
50-59	179	95 (68-133)
60-69	247	89 (58-124)
70-75	57	85 (52-112)
Body mass index (kg/m^2) *		
less than 25	196	87 (58-125)
25-29	340	92 (59-130)
at least 30	106	92 (59-126)
Diabetes		
Absent	621	91 (60-128)
Present	22	84 (50-128)
Current smoking		
No	429	89 (58-124)
Yes	214	93 (60-133)
Alcohol use		
Never	64	87 (54-121)
Occasionally	21	91 (64-127)
Regularly	558	91 (58-129)
Diastolic blood pressure (mmHg)†		
less than 80	199	89 (60-125)
85	93	92 (63-128)
90	169	90 (59-131))
at least 95	177	91 (53-133)
Systolic blood pressure (mmHg)†		
less than 125	158	88 (58-123)
130-135	113	89 (63-129)
140-150	205	93 (60-137)
at least 155	162	89 (57-125)

	N. of control subjects (total=643)	Mean TAFI% (5^{th}-95^{th} pecentile)
Triglycerides (mmol/L)*		
less than 0.90	162	84 (55-122)
0.90-1.24	162	89 (59-120)
1.25-1.82	160	95 (66-129)
at least 1.83	158	94 (58-133)
Total cholesterol (mmol/L)*		
less than 5.17	164	83 (54-122)
5.17-5.81	159	89 (58-128)
5.82-6.59	160	91 (64-126)
at least 6.60	159	98 (64-139)
HDL cholesterol (mmol/L)‡		
less than 1.1	160	87 (53-124)
1.1-1.28	159	93 (58-130)
1.29-1.53	163	91 (65-124)
at least 1.54	158	90 (57-130)
C-Reactive protein (mg/L)		
less than 0.78	161	85 (58-118)
0.78-1.55	161	89 (58-130)
1.56-3.42	160	92 (64-133)
at least 3.43	161	96 (62-129)

** Information about body mass index, total cholesterol, and triglycerides was unavailable for one control subject.*

† Information about diastolic and systolic blood pressure was unavailable for five control subjects.

‡ Information about HDL-cholesterol was unavailable for 3 control subjects.

Excluding individuals who were using aspirin at the time of blood sampling (127 patients and 35 control subjects) did not change the results, nor did excluding the 132 patients who used oral anticoagulants at the time of blood collection (*data not shown*).

We also investigated the effect of the combination of low TAFI levels and smoking or increased CRP levels on the risk of myocardial infarction. Table 4 shows the risk of myocardial infarction for quartiles of TAFI in smokers and non-smokers. The reference group was formed of the non-smokers with the highest levels (4^{th}

quartile) of TAFI, who had the lowest risk of myocardial infarction.

Table 3 *Risk of myocardial infarction with decreasing quartile of TAFI level.*

Quartile of TAFI-activity level	**4 (ref.)**	**3**	**2**	**1**
TAFI-activity (range in %)	102-161	89-101	76-88	19-75
N. of cases	65	132	172	185
N. of control subjects	156	157	168	162
OR_{adj}^{1} (95%CI)	1	2.1 (1.4-3.0)	2.5 (1.8-3.6)	2.8 (1.9-4.0)
OR_{adj}^{2} (95%CI)	1	2.3 (1.6-3.4)	3.0 (2.1-4.4)	3.5 (2.4-5.1)
OR_{adj}^{3} (95%CI)	1	2.4 (1.6-3.6)	3.2 (2.1-4.7)	3.4 (2.3-5.1)

CI, confidence interval; ref., reference group; OR_{adj}^{1}, Odds ratio adjusted for age; OR_{adj}^{2}, Odds ratio adjusted for age, C-Reactive Protein, triglycerides, and total cholesterol; OR_{adj}^{3}, Odds ratio adjusted for age, C-Reactive Protein, triglycerides, total cholesterol, body mass index, smoking, alcohol use, and diabetes.

Within the non-smokers, decreased TAFI levels (1st quartile) were associated with an almost two-fold increased risk of myocardial infarction (OR 1.9; 95% CI 1.1–3.2). In men who smoked and had high TAFI levels (4th quartile) an OR of 2.1 was found (95% CI 1.1–3.7). Smokers with TAFI levels in the 1st quartile had a risk of myocardial infarction that was increased 9.4-fold (95% CI 5.4–16.4). The odds ratios were 2.8 (95% CI 1.8–4.4) for men with low TAFI levels and a CRP level below the 75th percentile, 1.9 (95% CI 1.0–3.5) for those with CRP levels above the 75th percentile and high TAFI levels, and the risk of myocardial infarction was increased 8.2-fold (95% CI 4.4–15.2) in men with high CRP levels and low TAFI levels, all compared with men with low CRP and high TAFI levels.

Table 5 shows the risk of myocardial infarction associated with four SNP in the *TAFI* gene as well as mean TAFI level for each genotype. The observed frequency distribution of the *TAFI* genotypes in the control group did not deviate from that predicted for the four SNP under the Hardy-Weinberg equilibrium. The SNP at position -438, 505, and 1040 were strongly associated with TAFI levels. The mean TAFI level in those subjects carrying the -438GG genotype was 94%, while the mean TAFI level in carriers of the -438AA genotype was 76%. Mean TAFI level was 82% for carriers of the 505GG genotype and 107% for those with the 505AA genotype. Individuals with the 1040CC genotype had a mean TAFI level of 94%, which was higher than that of subjects with the 1040TT genotype (mean TAFI level 75%). The

association between the i4 + 164A/C SNP and TAFI levels was less clear. Carriers of the -438AA genotype, who had low TAFI levels, had an increased risk of myocardial infarction (OR 1.6; 95% CI 1.0–2.5) compared with carriers of the -438GG genotype. The odds ratio for carriers of the -438AG genotype was 1.2 (0.9–1.5). The other SNPs appeared not to be associated with risk.

Table 4 *Combined risk of myocardial infarction by quartiles of TAFI and smoking status.*

	TAFI (%, range)	**N. of cases**	**N. of controls**	**OR_{adj} (95%CI)**
Smoking				
No	102-161	25	88	1 (reference)
	89-101	47	112	1.5 (0.8-2.6)
	76-88	75	113	2.3 (1.4-4.0)
	19-75	62	116	1.9 (1.1-3.2)
Yes	102-161	40	68	2.1 (1.1-3.7)
	89-101	85	45	6.6 (3.7-11.8)
	76-88	97	55	6.2 (3.6-10.8)
	19-75	123	46	9.4 (5.4-16.4)
CRP				
$<75^{th}$ percentile	102-161	35	104	1 (reference)
	89-101	84	110	2.4 (1.5-3.8)
	76-88	112	130	2.7 (1.7-4.3)
	19-75	125	138	2.8 (1.8-4.5)
$>75^{th}$ percentile	102-161	30	52	1.9 (1.0-3.5)
	89-101	48	47	3.4 (1.9-5.9)
	76-88	60	38	5.3 (3.0-9.3)
	19-75	60	24	8.2 (4.4-15.2)

OR_{adj}, Odds ratio adjusted for age; CI, confidence interval.

Results of several studies on the association between levels of TAFI and risk of myocardial infarction were, in retrospect, considered not reliable as assays depended on the 1040C/T genotype. To verify whether the association between low TAFI activity and myocardial infarction was independent of the 1040T allele, the association of TAFI levels and myocardial infarction was determined only in those men who carried the 1040CC genotype. Among men carrying the 1040CC genotype,

the age-adjusted odds ratio for myocardial infarction among those with TAFI levels in the first quartile was 2.0 (95% CI 1.2–3.3), compared with those with TAFI levels in the fourth quartile. Using the full model, the odds ratio was 2.2 (95% CI 1.2–3.9).

Table 5 *Effect of TAFI SNP on functional TAFI levels and on risk of myocardial infarction.*

SNP	N. of cases (%) (N=554)	N. of controls (%) (N=643)	mean TAFI%* (5^{th}-95^{th} percentile)	OR_{adj} (95%CI)
438				
GG	297 (53.6)	378 (58.8)	94 (66-131)	1 (reference)
AG	215 (38.8)	231 (35.9)	86 (58-120)	1.2 (0.9-1.5)
AA	42 (7.6)	34 (5.3)	76 (58-98)	1.6 (1.0-2.5)
i4+164				
AA	203 (36.6)	237 (36.9)	93 (60-137)	1 (reference)
AC	274 (49.5)	301 (46.8)	88 (57-124)	1.1 (0.8-1.4)
CC	77 (13.9)	105 (16.3)	89 (68-118)	0.9 (0.6-1.2)
505				
GG	264 (47.7)	297 (46.2)	82 (55-111)	1 (reference)
AG	236 (42.6)	282 (43.9)	95 (68-127)	0.9 (0.7-1.2)
AA	54 (9.7)	64 (10)	107 (39-153)	0.9 (0.6-1.4)
1040				
CC	269 (48.6)	310 (48.2)	94 (66-133)	1 (reference)
CT	233 (42.1)	285 (44·3)	88 (58-123)	0.9 (0.7-1.2)
TT	52 (9.4)	48 (7.5)	75 (54-99)	1.2 (0.8-1.9)

OR_{adj}, Odds ratio adjusted for age; CI, confidence interval.
**TAFI levels in control subjects.*

DISCUSSION

This case-control study shows that individuals with low TAFI levels, as measured with a functional assay, had an increased risk of a first myocardial infarction compared with those with high TAFI levels. In accordance with these results, individuals carrying the A allele of the -438A/G SNP in the promoter region of the *TAFI* gene, who have low TAFI levels, had an increased risk of myocardial infarction, compared with

individuals carrying the GG genotype. The 505G/A and 1040C/T polymorphisms, which are also associated with levels of TAFI activity, were not associated with the risk of myocardial infarction.

The increased risk of myocardial infarction in men with lower TAFI levels remained after adjusting for several arterial risk factors, some of which (i.e., triglycerides and total cholesterol) were shown to be strongly associated with TAFI levels. The increased risk was found to be independent of the 1040T allele. A dose-response association was found between TAFI levels and myocardial infarction, but the risk difference between the third and fourth quartile was most pronounced while the odds ratios for the first, second and third quartiles were similar. In other words, there might be a threshold, above which levels of TAFI protect against myocardial infarction.

The results of our study partly contrast those of previous studies and are not in line with what would be expected based on the role of TAFI in the regulation of fibrinolysis. Several case-control studies have found increased TAFI levels to increase the risk of coronary artery disease or myocardial infarction[31,155,156] or did not find any association between TAFI and arterial thrombosis.[170] Explanations for these differences could be different outcome measures,[155,156,170] inclusion of men and women,[31,155,156,170] restriction to a relatively young population,[31] or blood sampling during the acute phase of the disease.[156] In some studies comparisons were only made between mean TAFI levels of patients and controls and thus extreme values were not studied and adjustments for possible confounders that could have influenced the results were not performed.[31,156] In one case-control study TAFI antigen levels were decreased in patients with myocardial infarction but activity levels of TAFI were increased, a difference that could not be explained by the 1040C/T SNP.[31] In the PRIME-study, a large prospective cohort study set up to investigate potential reasons for the difference in incidence of coronary heart disease in France and Northern Ireland, no clear association was found between baseline TAFI levels and angina pectoris or myocardial infarction and coronary death. However, in this study TAFI was measured with an antigen and not activity assay. Surprisingly, the 505A allele, which is associated with increased TAFI levels, was associated with an increased risk of coronary heart disease in France but with a decreased risk in Northern Ireland.[73] The HIFMECH study, a European multicenter case-control study, found elevated TAFI levels to be associated with a decreased risk of myocardial infarction, confirmed by a higher frequency of the 505A allele in patients than in controls.[74] The TAFI antigen assay used in the HIFMECH study was later found to be dependent on the 1040C/T genotype.

Although TAFI has been shown to inhibit fibrinolysis *in vivo*, recent studies suggest that TAFI plays an important role in regulating complement-mediated vascular inflammation *in vivo*. TAFI inhibits inflammation *in vivo* by inactivating the inflammatory mediators bradykinin and the anaphylatoxins C3a and C5a.[76] Furthermore, TAFI$^{-/-}$ plasminogen$^{+/-}$ mice expressed increased leukocyte migration compared with their wild-type counterparts in a model of peritoneal inflammation.[149] In another study, TAFI-deficient mice, primed by lipopolysaccharide, showed increased mortality after infusion of cobra venom factor, which is a potent non-specific activator of complement proteins.[150] Based on these results, one might expect that TAFI-deficient mice would have a more rapid progression of atherosclerosis, in which inflammation plays a pivotal role. However, to our knowledge, no data on atherosclerosis progression in TAFI knockout mice are available. As inflammation plays an important role in the development of atherosclerosis and arterial thrombosis,[192] it is not implausible that increased TAFI levels decrease the risk of myocardial infarction by decreasing the inflammatory response. These two counteracting effects may also explain the conflicting study results on TAFI and arterial thrombosis, and, because inflammation is less important in venous disease, the consistent relation between high TAFI levels and venous thrombosis risk. There is also evidence that TAFI binds to collagen resulting in reduced platelet adhesion to collagen under flow conditions,[193] which could also contribute to the protective effect of TAFI.

The high risk of myocardial infarction found in smokers with low TAFI levels is also in line with an excessive inflammatory response. It is known that smoking leads to an inflammatory state,[179] a fact also supported by increased CRP levels in smokers compared to non-smokers in this study.[171] It is possible that the effect of smoking on risk of myocardial infarction is most pronounced in those individuals with decreased TAFI levels, who have reduced potential to neutralize this inflammatory response. This hypothesis is also supported by the 8.2-fold increased risk in men with high levels of CRP, indicating elevated inflammatory state, and low TAFI levels. In addition to smoking and increased CRP levels, hypercholesterolemia is also associated with an increased inflammatory state.[194] A large number of patients, however, were using lipid-lowering drugs at the time of blood sampling, making an analysis on the combination of cholesterol and TAFI levels unreliable. The data on hypercholesterolemia based on the use of lipid-lowering drugs prior to myocardial infarction (Table 1) were insufficient for such an analysis.

We found a higher risk in individuals carrying the -438A allele than in those carrying the -438G allele, which supports our finding that decreased TAFI levels

increase the risk of myocardial infarction. No clear increase in risk was found for those carrying the 1040T allele compared with the 1040C, while both the -438A and 1040T alleles appeared to decrease TAFI levels. It is known that the 1040 T for C substitution results in a TAFIa species with a prolonged half-life at 37°C.[72] Possibly, this increased stability of TAFIa compensates for the decreased levels of the protein.

Surprisingly, the 505G/A SNP (or Ala147Thr), which has the largest effect on TAFI levels, was not associated with the risk of myocardial infarction. It could be that this SNP has an unknown function that counteracts the protective effect of the increased levels. The TAFI crystal structure shows that the amino acid at position 147 is solvent exposed.[195] Thus, theoretically, this residue, which is located close to the Arg145 that is known to be one of the essential residues for substrate peptide anchoring,[196] might contribute to ligand binding and the 505G/A polymorphism might affect this process. Although the 505G/A polymorphism does not affect TAFI fibrinolytic activity,[72] the effect of the polymorphism on the catalytic activity towards other molecular substrates such as bradykinin and anaphylatoxins has, to our knowledge, not yet been tested. A decreased activity of the A variant towards TAFI substrates involved in inflammation could plausibly explain why no association is found between the 505G/A SNP and the risk of myocardial infarction. Another possibility is that it is not the 505G/A SNP itself that is the functional variant but that this SNP is in strong linkage disequilibrium with another functional SNP. It has been suggested that the 505G/A SNP is not directly related to TAFI levels but that the 1583T/A SNP, which is in strong linkage disequilibrium with the 505G/A SNP, is actually the functional variant.[191] Similarly, the -438G/A might itself not be functional.[197] However, the linkage between SNP in the *TAFI* gene is so strong that the results of the SNP measured in our study are reliable indicators of the effects of the putative functional SNP.

It is possible that reduced TAFI levels are not causally linked to an increased risk of myocardial infarction, but that TAFI levels are reduced by another causal factor preceding the myocardial infarction. We are not aware of any risk factor for myocardial infarction that would reduce TAFI levels. The only disease state that could lead to low TAFI levels would be liver disease, which was only present in two cases and three controls, who had normal TAFI levels. Furthermore, TAFI levels were found to increase with risk factors for myocardial infarction, such as lipid levels and CRP, rather than decrease. As TAFI is also suggested to be an acute phase protein[151] it is not likely that decreased TAFI levels are a result of some other underlying factor. Samples were frozen and stored for approximately 10 years. This could have influenced the levels of TAFI. However, if TAFI activity decreases during

storage this effect would be equal in patients and controls so no bias of the results is expected, or at most an attenuation of the observed effects.

In conclusion, our study provides evidence that decreased TAFI levels are associated with an increased risk of a first myocardial infarction in men. The results on the association between TAFI SNPs and myocardial infarction were inconsistent.

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Synergistic effects of hypofibrinolysis and genetic and acquired risk factors on the risk of a first venous thrombosis

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ABSTRACT

Background

Previously, we demonstrated that hypofibrinolysis, a decreased capacity to dissolve a blood clot as measured with an overall clot lysis assay, increases the risk of venous thrombosis. Here, we investigated the combined effect of hypofibrinolysis with established risk factors associated with hypercoagulability.

Methods and findings

Fibrinolytic potential was determined with a plasma-based clot lysis assay in 2,090 patients with venous thrombosis and 2,564 control participants between 18 and 70 y of age enrolled in the Multiple Environmental and Genetic Assessment (MEGA) of risk factors for venous thrombosis study, a population-based case-control study on venous thrombosis. Participants completed a standardized questionnaire on acquired risk factors. Hypofibrinolysis alone, i.e., clot lysis time (CLT) in the fourth quartile (longest CLT) (in absence of the other risk factor of interest) increased thrombosis risk about 2-fold relative to individuals with CLT in the first quartile (shortest CLT). Oral contraceptive use in women with CLT in the first quartile gave an odds ratio (OR) of 2.6 (95% confidence interval [CI] 1.6 to 4.0), while women with hypofibrinolysis who used oral contraceptives had an over 20-fold increased risk of venous thrombosis (OR 21.8, 95% CI 10.2 to 46.7). For immobilization alone the OR was 4.3 (95% CI 3.2 to 5.8) and immobilization with hypofibrinolysis increased the risk 10.3-fold (95% CI 7.7 to 13.8). Factor V Leiden alone increased the risk 3.5-fold (95% CI 2.3 to 5.5), and hypofibrinolysis in factor V Leiden carriers gave an OR of 8.1 (95% CI 5.3 to 12.3). The combination of hypofibrinolysis and the prothrombin 20210A mutation did not synergistically increase the risk. All ORs and 95% CIs presented are relative to individuals with CLT in the first quartile and without the other risk factor of interest.

Conclusions

The combination of hypofibrinolysis with oral contraceptive use, immobilization, or factor V Leiden results in a risk of venous thrombosis that exceeds the sum of the individual risks.

INTRODUCTION

A hypercoagulable state, an increased capacity to form thrombin, is known to be associated with an increased risk of venous thrombosis.[4] However, the role of the fibrinolytic system in the development of venous thrombosis has not yet been extensively investigated.

We have shown that reduced fibrinolytic potential, as measured by a plasma-based clot lysis assay, increases the risk of a first deep vein thrombosis (DVT).[81] An almost 2-fold increased risk of DVT was found in individuals with clot lysis times (CLTs) above the 90th percentile of the values found in control participants compared with individuals with CLTs below this cut-off point.

Venous thrombosis is a multicausal disease, and several genetic and acquired risk factors associated with venous thrombosis have already been described.[4] The occurrence of multiple risk factors within a single patient may be associated with a risk of thrombosis that exceeds the sum of the individual risks. Such an increase is, for example, found in women with the factor V Leiden mutation who also use oral contraceptives. While carriers of the factor V Leiden mutation have a 5-fold increased risk of venous thrombosis, and oral contraceptive use increases the risk about 3-fold, the combination of these risk factors leads to a 20- to 30-fold increased risk (reviewed in[198]). A synergistic effect is also found for the combination of the factor V Leiden mutation and malignancy or air travel.[16,199] We hypothesized that the combination of hypofibrinolysis and hypercoagulability would synergistically enhance the risk of venous thrombosis. This hypothesis was investigated in the Multiple Environmental and Genetic Assessment (MEGA) of risk factors for venous thrombosis study, which is a large population-based case-control study on venous thrombosis. First, we studied hypofibrinolysis as a risk factor for thrombosis, and investigated the joint effect of hypofibrinolysis and several established risk factors for venous thrombosis. Second, we studied the influence of several genetic and acquired factors on the outcome of the clot lysis assay.

METHODS

Study Design

The design of the MEGA study has been described previously.[16] Between March 1999 and May 2002, consecutive patients aged 18 to 70 y, with a first DVT of the leg or a first pulmonary embolism (PE), were identified at six anticoagulation clinics in

The Netherlands. In these clinics, the anticoagulant therapy of all patients in a well-defined geographical area is monitored, which gave us the opportunity to identify consecutive and unselected patients with venous thrombosis.

Partners of participating patients were invited to take part as control participants. From January 2002 to September 2004, an additional control group was selected from the same area by random digit dialing using the Mitofsky-Waksberg method[200] and was frequency matched to the patients for age and sex. This matching was on group level in which random controls were selected in numbers proportional to the number of patients within strata of sex and 5-y age groups.

Patients or control participants with severe psychiatric problems or those who could not speak Dutch were excluded.

For the MEGA study, 4,131 patients were eligible. Of those patients, 194 died soon after the venous thrombosis. Of the remaining 3,937 patients, 51 were in the end stage of a disease and 629 were unable or refused to participate, leaving 3,257 patients in the study who filled in a questionnaire (83%). Of the total of 3,257 patients of the MEGA study, 2,360 had an eligible partner. One partner died soon after the request for participation and, of the remaining 2,359 partners, 1,908 participated (81%). Of the nonparticipating partners, 15 were in the end-stage of a disease and 436 refused, were unable to participate, or could not be located. Of the 4,350 eligible random digit dialing control participants, four died before they were able to participate, 15 were in the end stage of a disease, and 1,331 refused to participate or could not be located, leaving 3,000 participants in the study (69%).

All participants signed a written informed consent form. Approval for this study was obtained from the Medical Ethics Committee of the Leiden University Medical Center, Leiden, The Netherlands.

Validation of Thrombosis Diagnosis

Written informed consent to obtain medical records was given by 95% of the patients. Information regarding the diagnostic procedure was obtained via hospital records and general practitioners. For 10% of these patients the information regarding the diagnostic procedure could not be obtained. A PE was considered “definite” when diagnosed with a high-probability ventilation-perfusion scan (VQ scan), positive spiral computed tomography (CT), or angiogram. A PE was considered “probable” when diagnosed with a low- or intermediate-probability VQ scan, inconclusive spiral CT, or angiogram. A deep venous thrombosis was considered definite when a (Doppler) ultrasound showed the presence of a thrombus in the deep veins. When no information regarding the diagnostic procedure was available or when a patient

was registered at the anticoagulation clinic with a different or additional diagnosis to the one that had been objectively confirmed, the diagnosis by which the patient was registered at the anticoagulation clinic was added. A registration of a PE was considered probable while a DVT registration was considered definite. Only those patients were included in the study when their diagnosis was definite or probable. Of those patients in whom diagnostic information was available, only 2% was excluded as they did not have a venous thrombotic event according to the medical records.

Data Collection and Current Analysis

All participants were asked to complete a standardized questionnaire on acquired risk factors for venous thrombosis, such as surgery, plaster cast, confinement to bed, injury, oral contraceptive use, hormonal replacement therapy, pregnancy, and malignancy. Body mass index (BMI in kg/m^2) was calculated from self-reported weight and height. All items in the questionnaire referred to the period before the index date. The date of diagnosis of thrombosis as reported by the participant was used as the index date for the patients. For the control participants, the date they filled in the questionnaire was used as index date. Idiopathic venous thrombosis was defined as venous thrombosis in patients who had never had malignancies and without surgery, injury, plaster cast, or confinement to bed in the year prior to the thrombosis or oral contraceptive use or hormone replacement therapy at the time of the event.

When participants were unable to fill in the questionnaire, questions were asked by telephone, using a standardized mini-questionnaire. Three months after discontinuation of the anticoagulant therapy, a blood sample was taken from patients and participating partners in the anticoagulation clinic. In patients who received prolonged anticoagulant therapy (>1 y), blood was drawn 1 y after the event. The random control participants were invited to the clinic for a blood draw after returning their questionnaire. All participants were interviewed regarding current anticoagulant use.

In the current analyses only patients who had their blood drawn were included (n = 2,420). Users of oral anticoagulants at time of blood draw were excluded (n = 290) as well as 38 patients who, according to the medical records did not have venous thrombosis, leaving 2,092 patients available for this study.

Of these 2,092 patients, 1,328 partner controls participated in the MEGA study. Blood was drawn of 1,138 partner controls and seven were excluded because they used anticoagulants, leaving 1,131 partner controls available for this study. Of the 3,000 random control participants, 1,460 had their blood drawn and 1,440 were

not using oral anticoagulants.

Blood Collection and Laboratory Analysis

Blood samples were drawn into vacuum tubes containing 0.106 M trisodium citrate. Plasma was obtained by centrifugation at 2,000*g* for 10 min at room temperature and stored in aliquots at −80 °C. Samples that were not previously thawed were used for this study. Lysis of a tissue factor–induced clot by exogenous tissue-type plasminogen activator (t-PA) was studied by monitoring changes in turbidity during clot formation and subsequent lysis as described previously.[81] In short, 50 µl plasma was pipetted in a 96-well microtiter plate. Subsequently, 50 µl of a mixture containing phospholipid vesicles (40% L-α-dioleoylphosphatidylcholine, 20% L-α-dioleoylphosphatidylserine, and 40% L-α-dioleoylphosphatidylethanolamine, final concentration 10 µM), t-PA (final concentration 56 ng/ml), tissue factor (final dilution 1:1000), and $CaCl_2$ (final concentration 17 mM), diluted in HEPES buffer (25 mM HEPES [*N*-2-hydroxytethylpiperazine-*N*'-2-ethanesulfonic acid], 137 mM NaCl, 3.5 mM KCl, 3 mM $CaCl_2$, 0.1% bovine serum albumin, pH 7.4), was added using a multichannel pipette. After thorough mixing, the plate was incubated at 37 °C in a Spectramax 340 kinetic microplate reader (Molecular Devices Corporation), and the optical density at 405 nm was monitored every 20 s, resulting in a clot-lysis turbidity profile. The CLT was derived from this clot-lysis profile and defined as the time (minutes) from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing the lysis of the clot. The intra-assay coefficient of variation was 5.5% (n = 99) and the inter-assay coefficient of variation was 6.6% (n = 90).

A detailed description of DNA extraction and DNA analysis for the factor V Leiden (G1691A) mutation and the prothrombin (G20210A) mutation in the MEGA study has been published previously.[16]

Of two patients (without participating partner), three partners, and four random control participants, no citrated plasma was available or the CLT could not be measured as the plasma was too turbid.

The DNA analyses and the clot lysis assays were performed by staff who had no knowledge of whether the sample was from a patient or a control participant.

Statistical Analysis

We investigated whether elevated CLT is a risk factor for venous thrombosis by calculating odds ratios (ORs), as an approximation of relative risks, and 95% confidence intervals (CIs). CLTs were grouped into quartiles or deciles based on the

distribution among all control participants. The lowest quartile or decile was taken as the reference group. ORs adjusted for age (continuous) and sex (categorical) (OR_{adj}) were calculated using multivariate logistic regression. Analyses in subgroups by sex were adjusted only for age. Analyses in subgroups of age were adjusted for sex and for age (continuous) within each stratum of age. Adjustment for age as a dummy variable (five categories) did not change the results compared to models with age as a continuous covariate, and the latter adjustment is presented for all analyses.

A patient and his or her partner control may be more similar than a random pair selected from the population would be, e.g., on (measured and unmeasured) lifestyle factors that could influence fibrinolytic potential. Therefore, we performed conditional logistic regression to adjust for all matching factors in the analyses with partners as control group,[199] including 1,128 patient-partner pairs. In the analyses with the random control group, unconditional logistic regression including all patients and random control participants were performed. To obtain a final estimate, results of unmatched and matched analyses were pooled. As both estimates used the same subset of 1,128 patients, the estimates are correlated. Therefore these estimates were pooled using analyses taking this correlation into account.[17]

When subgroup analyses were performed on men and women separately, only an unmatched analysis with the random control group was performed, as a matched analysis was not possible because the patient and matched control were nearly always of the opposite sex.

We assessed the joint effect of quartiles of CLT with other risk factors for venous thrombosis by calculating ORs. Participants in the first quartile of CLT and without the other risk factor of interest were used as the reference category, and dummy variables were used for the seven other categories.

To study the association between clotting abnormalities and acquired (risk) factors with CLT in the control group (partners and random controls), mean CLTs of different groups were calculated and confidence intervals were constructed for the difference based on a *t*-distribution. The association between age and BMI with CLT was assessed using linear regression. Linear regression was also used to determine the association between the time interval between the thrombotic event and blood draw and CLT in the patients. Longer CLTs in those with a small time interval than in those with a large time interval could indicate that increased CLT is rather an acute-phase reaction than a risk factor for venous thrombosis.

Cases and controls with missing data were excluded in the analyses. SAS 9.1 (SAS Institute) was used for all statistical analyses.

RESULTS

In this study, 2,090 patients, 1,128 partner controls, and 1,436 random controls were included. Mean age at time of blood draw of patients was 49 y (range 19–71 y) and mean age of all control participants was 48 y (range 18–71 y). Of the patient group 926 participants (44%) were men, whereas in the control group 1,226 participants (48%) were men. Of the 2,090 patients, 650 were diagnosed with an isolated PE, 1,245 with an isolated DVT of the leg, and 195 of all the patients were diagnosed with both a PE and a DVT of the leg.

Hypofibrinolysis as a Risk Factor for Venous Thrombosis

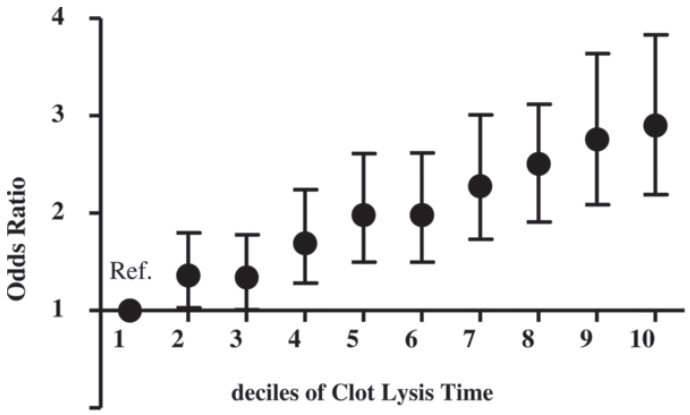
The association between CLT and the risk of venous thrombosis is shown in Figure 1. Using deciles of CLT based on the values found in all control participants, we found a clear dose-response relation between CLT and the risk of venous thrombosis. The OR_{adj} for individuals in the tenth decile of CLT compared with individuals in the first decile was 2.9 (95% CI 2.2 to 3.8). In Table 1 the risk of venous thrombosis is presented in quartiles. Individuals with hypofibrinolysis, i.e., CLTs in the highest quartile, had an increased relative risk of venous thrombosis of 2.4 (95% CI 2.0 to 2.8) compared with individuals in the lowest quartile of CLT. Adjustment for age and sex did not change this result (OR_{adj} 2.4, 95% CI 2.0 to 2.8). Using only the random control group yielded an OR_{adj} of 2.5 (95% CI 2.1 to 3.1), and the matched analysis with only partner controls resulted in a similar OR_{adj} of 2.0 (95% CI 1.5 to 2.6) for those with hypofibrinolysis.

Hypofibrinolysis was associated with a 3.2-fold increased risk of a simultaneous DVT of the leg and a PE (OR_{adj} 3.2, 95% CI 1.9 to 5.3) (Table 2). Hypofibrinolysis conferred a 2.1-fold increased risk of an isolated PE (OR_{adj} 2.1, 95% CI 1.6 to 2.8). For an isolated DVT of the leg an OR_{adj} of 2.6 was found (95% CI 2.1 to 3.2).

The relative risk of venous thrombosis in individuals with hypofibrinolysis was most pronounced in women below age 50 y (OR_{adj} 3.2, 95% CI 2.2 to 4.6) (Table 3). As blood of these women was drawn after the event and most patients but not the control participants had ceased oral contraceptive use before time of blood draw, this difference could have influenced the risk found in younger women. For that reason we also calculated the risk in women below age 50 y who did not use oral contraceptives at time of the index date and still did not use oral contraceptives at time of blood draw. In those women, an OR_{adj} of 2.3 (95% CI 1.3 to 4.1) was found. In women over age 50 y, the OR_{adj} was 2.8 (95% CI 1.7 to 4.6). The relative risk in men

below age 50 y with hypofibrinolysis was slightly lower (OR_{adj} 2.2, 95% CI 1.4 to 3.5) than in men over age 50 y (OR_{adj} 2.8, 95% CI 1.8 to 4.3).

The risk of idiopathic thrombosis in those with hypofibrinolysis was approximately 3-fold increased (OR_{adj} 3.1, 95% CI 2.2 to 4.4).



n_p	119	157	154	185	216	212	237	243	279	288
n_c	256	258	255	256	257	257	256	257	250	262

Figure 1 *Risk of venous thrombosis for deciles of clot lysis time.*
The odds ratios are adjusted for age and sex. Error bars, 95% confidence interval; n_c, number of control participants (all); n_p, number of patients; Ref., reference category.

Table 1 *Relative risk of venous thrombosis according to quartiles of clot lysis time.*

Quartile of clot lysis time	**1 (Ref.)**	**2**	**3**	**4**
Lysis time at cut-off (min)	-	57.9	65.6	75.9
Cases (n)	340	491	558	701
Controls (all, n)	641	641	641	641
OR (95% CI)	1	1.5 (1.3 to 1.8)	1.8 (1.5 to 2.2)	2.4 (2.0 to 2.8)
OR_{adj} (95% CI)	1	1.5 (1.3 to 1.8)	1.8 (1.5 to 2.2)	2.4 (2.0 to 2.8)

CI, confidence interval; OR, crude odds ratio; OR_{adj}, odds ratio adjusted for age and sex; ref. reference group.

Table 2 *Relative risk of venous thrombosis according to quartiles of clot lysis time in subgroups of venous thrombosis.*

Quartile of clot lysis time	1 (Ref.)	2	3	4
DVT of the leg				
Cases (n) 200	285	340	420	
Controls (all, n)	542	545	504	514
OR_{adj} (95% CI)	1	1.5 (1.2 to 1.8)	2.1 (1.6 to 2.6)	2.6 (2.1 to 3.2)
DVT of the leg + PE				
Cases (n)	24	50	53	68
Controls (all, n)	428	407	339	358
OR_{adj} (95% CI)	1	2.1 (1.2 to 3.4)	2.6 (1.6 to 4.4)	3.2 (1.9 to 5.3)
PE				
Cases (n)	116	156	165	213
Controls (all, n)	481	467	426	425
OR_{adj} (95% CI)	1	1.4 (1.1 to 1.8)	1.6 (1.2 to 2.1)	2.1 (1.6 to 2.8)

CI, confidence interval; DVT, deep vein thrombosis; OR_{adj}, odds ratio adjusted for age and sex; PE, pulmonary embolism, ref., reference group.

Hypofibrinolysis in Combination with Other Established Risk Factors

Table 4 shows the risk of venous thrombosis for individuals with hypofibrinolysis (i.e., the highest quartile of CLT) in combination with established risk factors. The reference group is the group with the lowest CLTs (i.e., in the first quartile) and in absence of the other risk factor. To study the joint effect of hypofibrinolysis and oral contraceptive use on venous thrombosis, only women below the age of 50 y were included. Women with the lowest CLTs who were taking oral contraceptives had a 2.6-fold increased risk of venous thrombosis (OR_{adj} 2.6, 95% CI 1.6 to 4.0) compared with women with the lowest CLTs who did not take oral contraceptives. In those with the highest CLTs who did not take oral contraceptives, an OR_{adj} of 1.9 (95% CI 1.1 to 3.3) was found compared with the reference group. The combination of hypofibrinolysis and oral contraceptive use gave a 22-fold increased risk (OR_{adj} 21.8, 95% CI 10.2 to 46.7).

The risk of venous thrombosis in individuals with a combination of hypofibrinolysis and immobilization (defined as either plaster cast, confinement to bed for more than 4 d, or surgery) was also assessed (Table 4).

Table 3 *Relative risk of venous thrombosis according to quartiles of clot lysis time.*

Quartile of clot lysis time	1 (Ref.)	2	3	4
Women < 50 years				
Cases (n)	175	219	172	152
Random controls (n)	186	131	85	51
OR_{adj} (95% CI)	1	1.8 (1.3 to 2.4)	2.2 (1.5 to 3.0)	3.2 (2.2 to 4.6)
Women < 50 years not taking oral contraceptives*				
Cases (n)	47	49	50	43
Random controls (n)	92	74	56	41
OR_{adj} (95% CI)	1	1.4 (0.8 to 2.3)	1.9 (1.1 to 3.2)	2.3 (1.3 to 4.1)
Women ≥ 50 years				
Cases (n)	35	85	126	200
Random controls (n)	56	72	79	117
OR_{adj} (95% CI)	1	1.9 (1.1 to 3.2)	2.6 (1.6 to 4.4)	2.8 (1.7 to 4.6)
Men < 50 years				
Cases (n)	53	65	98	113
Random controls (n)	91	90	65	80
OR_{adj} (95% CI)	1	1.2 (0.7 to 1.9)	2.4 (1.5 to 3.9)	2.2 (1.4 to 3.5)
Men ≥ 50 years				
Cases (n)	77	122	162	236
Random controls (n)	72	96	85	80
OR_{adj} (95% CI)	1	1.2 (0.8 to 1.8)	1.8 (1.2 to 2.7)	2.8 (1.8 to 4.3)

CI, confidence interval; OR_{adj}, odds ratio adjusted for age, ref., reference group.
** At index date and at time of blood draw.*

The OR_{adj} for individuals with hypofibrinolysis and no immobilization was 2.4 (95% CI 1.9 to 2.9), and for individuals with CLT in the lowest quartile who were immobilized it was 4.3 (95% CI 3.2 to 5.8). Those who were immobilized and who had hypofibrinolysis had a 10-fold increased risk of venous thrombosis compared with individuals without these risk factors (OR_{adj} 10.3; 95% CI 7.7 to 13.8). When plaster cast, confinement to bed, and surgery were analyzed separately in combination with hypofibrinolysis, the same trends were found as when these three risk factors were combined into one variable (unpublished data).
In individuals with hypofibrinolysis without the factor V Leiden mutation an OR_{adj} of

Table 4 *Hypofibrinolysis, presence of established genetic and acquired risk factors, and relative risk of venous thrombosis.*

Risk Factor	Present?	Quartile of CLT	Cases (n)	Controls (n)*	OR_{adj} (95% CI)
Oral contraceptive use at index date†‡	No	1	50	93	1 (reference)
		4	44	42	1.9 (1.1 to 3.3)
	Yes	1	125	93	2.6 (1.6 to 4.0)
		4	107	9	21.8 (10.2 to 46.7)
Immobilization‡§	No	1	194	550	1 (reference)
		4	415	550	2.4 (1.9 to 2.9)
	Yes	1	142	88	4.3 (3.2 to 5.8)
		4	282	85	10.3 (7.7 to 13.8)
Factor V Leiden	No	1	288	605	1 (reference.)
		4	590	608	2.4 (2.0 to 2.9)
	Yes	1	52	36	3.5 (2.3 to 5.5)
		4	111	33	8.1 (5.3 to 12.3)
Prothrombin 20210A	No	1	332	636	1 (reference)
		4	661	620	2.3 (2.0 to 2.8)
	Yes	1	8	5	2.4 (0.7 to 8.1)
		4	40	21	4.4 (2.5 to 7.6)

CI, confidence interval; OR_{adj}, odds ratio adjusted for age (in analyses on hypofibrinolysis and oral contraceptive use) or age and sex (in other analyses).

** In analyses on hypofibrinolysis and oral contraceptive use, random controls are used. In the other analyses all controls are used.*

† Analyses in women below the age of 50 years.

‡ Information about oral contraceptive use was unavailable for one patient and information about immobilization was unavailable for eight cases and nine control participants.

§ Immobilization was defined as plaster cast, confinement to bed for more than 4 d, or surgery.

2.4 (95% CI 2.0 to 2.9) was found (Table 4). Carriers of the factor V Leiden mutation with the lowest CLTs had a risk of venous thrombosis that was 3.5-fold (95% CI 2.3 to 5.5) increased. The risk of venous thrombosis increased 8.1-fold (95% CI 5.3 to 12.3) for individuals with hypofibrinolysis and the factor V Leiden mutation. Similar analyses for the prothrombin 20210A mutation gave an OR_{adj} of 2.3 (95% CI 2.0 to

2.8) for hypofibrinolysis only, 2.4 (95% CI 0.7 to 8.1) for the prothrombin 20210A mutation only, and 4.4 (95% CI 2.5 to 7.6) for the combination. When patients with pulmonary emboli were excluded, the same trend in risks was found, but somewhat higher ORs were found for the risk of factor V Leiden alone and the combination of factor V Leiden with hypofibrinolysis (unpublished data).

In each of the above analyses of combinations of risk factors, the risks of venous thrombosis in the 2nd and 3rd quartile of CLT (in the presence or absence of the other risk factor) showed a consistent trend. With each increasing quartile of CLT, the risk of venous thrombosis also increased (unpublished data).

Determinants of Clot Lysis Time

All control participants were used to study genetic and acquired factors as determinants of CLTs (Table 5). Mean CLT in the control group was 69.8 min (5th to 95th percentile, 49.4 to 105.1). CLT increased with age (3.1 min per decade, 95% CI 2.6 to 3.6). In men a plateau was reached at age 60. Overall, mean CLT in men (70.9 min, 5th to 95th percentile 50.2 to 106.2) was slightly higher than mean CLT in women (68.7 min, 5th to 95th percentile 48.6 to 103.0), although the difference was small (2.2 min, 95% CI 0.7 to 3.7). CLTs substantially increased with increasing BMI. The increase in CLT per 1 kg/m^2 increase in BMI was 2.0 min (95% CI 1.8 to 2.1). When we used conventional classification of BMI as underweight (<20 kg/m^2), normal (20–24 kg/m^2), overweight (BMI 25–29 kg/m^2), and obese (BMI ≥ 30 kg/m^2), mean CLT was 58.0 min (5th to 95th percentile 44.8 to 73.2) in those who were underweight, 63.5 min (5th to 95th percentile 48.0–84.3) in those who had normal BMI, 73.8 min (5th to 95th percentile 52.3 to 109.2) in individuals who were overweight, and 83.9 min (5th to 95th percentile 56.3 to 131.0) in obese individuals. In women not using oral contraceptives, the mean CLT was 5.4 min longer (95% CI 3.0 to 7.7) than in women taking oral contraceptives. Diabetes was associated with a 17% increase in CLT (11.7 min, 95% CI 7.2 to 16.2) compared with individuals without diabetes. In carriers of the prothrombin 20210A mutation, mean CLT was 12% increased (8.1 min, 95% CI 2.3 to 13.8) compared with noncarriers. Carriers of the factor V Leiden mutation had similar CLTs to noncarriers (difference 2.1 min, 95% CI –1.2 to 5.5).

It is known that BMI is associated with plasminogen activator inhibitor-1 (PAI-1) levels[49] and the prothrombin 20210A mutation was found to be associated with increased thrombin-activatable fibrinolysis inhibitor (TAFI) activation in a study that used a clot lysis assay similar to that used in our study.[201] We adjusted the ORs in Table 2 for BMI and the prothrombin 20210A mutation to see whether the

Table 5 *Clot lysis times in all control participants.*

Group	Subgroup	Participants (n)	Clot lysis time, Mean (5^{th} to 95^{th} Percentile)
Age at blood draw (year), men			
	All	1,226	70.9 (50.2 to 106.2)
	18–29	96	63.6 (44.5 to 98.8)
	30–39	228	68.1 (49.5 to 102.9)
	40–49	286	72.9 (52.5 to 106.5)
	50–59	345	73.8 (50.5 to 108.3)
	60–71	271	70.2 (50.0 to 110.4)
Age at blood draw (year), women			
	All	1,338	68.7 (48.6 to 103.0)
	18–29	117	57.7 (44.5 to 73.8)
	30–39	242	61.9 (47.1 to 81.9)
	40–49	331	66.2 (47.9 to 100.3)
	50–59	410	73.9 (52.0 to 114.2)
	60–71	238	75.6 (52.4 to 110.8)
Use of oral contraceptives in women aged <50 years			
	Yes	266	60.0 (46.2 to 76.3)
	No	424	65.3 (48.2 to 96.3)
Diabetes*			
	Yes	71	81.1 (54.2 to 140.7)
	No	2,389	69.3 (49.3 to 102.5)
BMI (kg/m^2)†			
	<20	119	58.0 (44.8 to 73.2)
	20–24	1,117	63.5 (48.0 to 84.3)
	25–29	949	73.8 (52.3 to 109.2)
	≥30	312	83.9 (56.3 to 131.0)
Factor V Leiden mutation			
	AA or AG	132	71.8 (48.5 to 118.2)
	GG	2,432	69.7 (49.4 to 104.0)

Group	Subgroup	Participants (n)	Clot lysis time, Mean (5th to 95th Percentile)
Prothrombin 20210A mutation			
	AG	43	77.7 (54.5 to 124.9)
	GG	2,521	69.6 (49.4 to 104.9)

** Information about diabetes was unavailable for 95 control participants.*
† Information about BMI was unavailable for 67 control participants.

increased risk of venous thrombosis in individuals with hypofibrinolysis could (in part) be explained by the increase in these fibrinolytic factors. Adjustment for BMI and the prothrombin 20210A mutation only moderately decreased the association between hypofibrinolysis and venous thrombosis (ORs [95% CIs] for quartile 2, 3, and 4: 1.4 [1.2 to 1.7], 1.6 [1.3 to 1.9], and 1.8 [1.5 to 2.1]).

Within the patient group, we studied the association between the time interval between the event and the day of blood draw and CLT. The 5th to 95th percentile of this time interval was 188–502 d, with a mean of 318 d (median 301 d). No association was found between the time interval and CLT (0.004 min decrease in CLT/d increase; 95% CI −0.006 to 0.014).

DISCUSSION

In this large population-based case-control study, including over 4,500 individuals, we have shown that decreased fibrinolytic activity, as measured with a plasma-based assay, was associated with an increased risk of a first venous thrombosis. The combination of hypofibrinolysis with immobilization, factor V Leiden, and especially oral contraceptive use, resulted in high relative risks. CLTs were associated with age, BMI, diabetes, oral contraceptive use, and the prothrombin 20210A mutation.
The risk of venous thrombosis for individuals with hypofibrinolysis was 2.4-fold increased compared with those with CLTs in the first quartile. Furthermore, there was a clear dose-response relation between CLT and the risk of venous thrombosis. Our findings support the results of the Leiden Thrombophilia Study (LETS).[81] In this case-control study, CLTs above the 90th percentile of the levels found in the control group were associated with an almost 2-fold increased risk of venous thrombosis.

Because hypercoagulation is a well-established risk factor for venous

thrombosis,[4,16] we hypothesized that hypercoagulation and hypofibrinolysis have synergistic effects on venous thrombosis. First, we studied the risk of venous thrombosis in individuals with hypofibrinolysis combined with established acquired risk factors. We found a 22-fold increase in risk in women with hypofibrinolysis who were taking oral contraceptives compared with women with neither risk factor. Although the thrombosis risk associated with oral contraceptive use is well known, the mechanisms behind the increased risk of venous thrombosis are still incompletely elucidated.[198] Oral contraceptive use induces complex changes in the hemostatic system, including increased levels of several procoagulant factors, reduction in levels of anticoagulant factors, and activated protein C resistance. In addition, oral contraceptive use results in complex changes in fibrinolytic variables suggestive of increased fibrinolytic capacity. However, the net effect of these changes appears to be prothrombotic. The combination of oral contraceptive use and factor V Leiden was previously shown to result in a thrombosis risk larger than the sum of the individual risks (reviewed in[198]). This effect is presumably due to further exacerbation of thrombin-generating capacity induced by both risk factors. The substantially increased risk associated with hypofibrinolysis and oral contraceptive use possibly relates to a concomitant presence of excessive thrombin-generating capacity and defective clot breakdown.

The combination of immobilization (confinement to bed, surgery, or plaster cast) and hypofibrinolysis resulted in a synergistic effect, possibly via a similar mechanism because immobilization leads to an increase of thrombin formation. The increased risk of venous thrombosis during stasis may be caused by endothelial cell activation and expression of P-selectin, allowing tissue factor–bearing microvesicles to initiate coagulation and thrombosis.[202] Furthermore, increased thrombin generation occurs after surgery, as demonstrated, for example, by elevated levels of thrombin–antithrombin complexes.[203,204] Although no other studies have investigated the combined risk of immobilization and hypofibrinolysis, in a review it was concluded that there was some evidence for an association between decreased fibrinolytic potential (measured using different assays to the one used in the present study) and postoperative thrombosis.[80] Furthermore, it is found that, among hospitalized patients, the prevalence of asymptomatic thrombi is high, up to about 40% (reviewed in[205]). Although most asymptomatic thrombi resolve without causing clinical consequences, it is plausible that those individuals with a decreased capacity to remove these thrombi have a higher risk of eventually developing a symptomatic venous thrombosis. Similarly, the risk of an unprovoked (idiopathic) thrombosis was also increased in individuals with hypofibrinolysis, which may be explained by

defective breakdown of asymptomatic thrombi, as 1% of a healthy population was found to have such an asymptomatic thrombus.[206]

Second, we evaluated the effect of hypofibrinolysis in combination with established genetic risk factors, i.e., factor V Leiden or the prothrombin 20210A mutation. The joint presence of factor V Leiden and hypofibrinolysis resulted in a large increase in risk of DVT. Individuals with both factor V Leiden and hypofibrinolysis had an 8.1-fold increased risk, compared with the absence of both risk factors. This joint effect can also be explained by the presence of both an increased procoagulant state and a decreased fibrinolytic potential.

The combination of hypofibrinolysis and the prothrombin 20210A mutation did not lead to a large increase in risk. A difficulty in evaluating the combination of these two risk factors is the apparent effect of prothrombin 20210A on CLT. In accordance with results previously reported,[201] we found that individuals with the prothrombin 20210A mutation had increased CLTs, conceivably related to increased activation of TAFI.

We found shorter CLTs in women using oral contraceptives compared with women not taking oral contraceptives. This is in contrast with the results of a crossover study showing no difference in CLT before and during oral contraceptive use.[207] In that study, t-PA activity, plasminogen, and plasmin-α_2-antiplasmin complexes were increased during oral contraceptive use, and levels of PAI-1 antigen and activity and t-PA antigen were decreased. It was suggested that this increased fibrinolytic activity is counteracted by the increased TAFI levels that were also found. However, a direct comparison of the studies is complicated, because the CLT assays used were slightly different. In the crossover study, the plasma was clotted with thrombin and t-PA, while in our assay tissue factor and t-PA is used.

We found a positive relation between BMI and CLT and increased CLTs in individuals with diabetes. It is plausible that PAI-1 plays an important role in these associations, as it is known that PAI-1 is overexpressed in patients with diabetes and obesity (reviewed in[49]).

In our previous study, we showed that the risk of venous thrombosis in individuals with hypofibrinolysis as measured with our clot lysis assay did not disappear after adjustment for several coagulation factors shown to influence CLTs in the control population.[81] This result suggests that the CLTs are mostly determined by fibrinolytic factors. It is also supported by the association between BMI and CLT and the increased CLTs in individuals with diabetes and those with the prothrombin 20210A mutation. Which fibrinolytic factor or which combination of factors is important for this increased risk is yet to be investigated.

A drawback of using case-control studies for finding associations between the risk of venous thrombosis and blood parameters is that the blood of the patients is drawn after the thrombotic event. In this way decreased fibrinolysis could be a consequence of the disease rather than a cause. However, when CLT would increase as a result of the event, one would expect such an acute-phase effect to attenuate with time after the thrombotic event. In this study no relation was found between CLT and the number of days between the event and the blood draw.

We conclude that the risk of venous thrombosis is increased in individuals with decreased fibrinolytic potential, as measured by an overall plasma-based assay. The combination of hypofibrinolysis with established genetic and acquired risk factors associated with hypercoagulation has a synergistic effect on the thrombosis risk. This joint effect is observed in individuals with hypofibrinolysis who use oral contraceptives, are immobilized, or are carriers of the factor V Leiden mutation, but not in patients who carry the prothrombin 20210A mutation.

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Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1

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ABSTRACT

Elevated plasma clot lysis time (CLT) increases risk of venous and arterial thrombosis. It is unclear which fibrinolytic factors contribute to thrombosis risk. In 743 healthy controls we investigated determinants of CLT. By comparison with 770 patients with a first venous thrombosis we assessed plasma levels of fibrinolytic proteins as risk factors for thrombosis. Plasminogen activator inhibitor-1 (PAI-1) levels were the main determinants of CLT, followed by plasminogen, thrombin activatable fibrinolysis inhibitor (TAFI), prothrombin, and α2-antiplasmin. Fibrinogen, factor VII, X, and XI contributed minimally. These proteins explained 77% of variation in CLT. Levels of the fibrinolytic factors were associated with thrombosis risk (odds ratios, highest quartile versus lowest, adjusted for age, sex, and BMI: 1.6 for plasminogen, 1.2 for α2-antiplasmin, 1.6 for TAFI, 1.6 for PAI-1, 1.8 for tissue plasminogen activator (t-PA)). Adjusting for acute-phase proteins attenuated the risk associated with elevated plasminogen levels. The risk associated with increased t-PA nearly disappeared after adjusting for acute-phase proteins and endothelial activation. TAFI and PAI-1 remained associated with thrombosis after extensive adjustment. Concluding, CLT reflects levels of all fibrinolytic factors except t-PA. Plasminogen, TAFI, PAI-1, and t-PA are associated with venous thrombosis. However, plasminogen and t-PA levels may reflect underlying risk factors.

INTRODUCTION

Decreased fibrinolytic potential as measured with a plasma-based assay has consistently been shown to be a risk factor for venous and arterial thrombosis.[81,82,85,94] Furthermore, we recently showed that the combination of hypofibrinolysis and risk factors associated with hypercoagulation resulted in a substantially higher risk than expected based on the individual risks conferred by these factors.[82] The clot lysis assay used in these studies determines time to half maximal lysis of a plasma clot initiated with tissue factor. To induce fibrinolysis, tissue Plasminogen Activator (t-PA) is added to the plasma prior to clot formation. The clot lysis time (CLT) calculated from the turbidity profile of the clot formation and clot lysis is thought to represent overall plasma fibrinolytic capacity. In *in vitro* experiments, we previously investigated the effect of changes in levels of a single fibrinolytic factor on CLT. CLT increased with the addition of active plasminogen activator inhibitor-1 (PAI-1) to pooled normal plasma and with the addition of pooled normal plasma to plasma of a patient with severe α2-antiplasmin deficiency. Furthermore, CLT increased when thrombin activatable fibrinolysis inhibitor (TAFI) was added to immuno-depleted normal plasma. When purified plasminogen was added to immuno-depleted plasminogen deficient plasma, CLT decreased.[78] In these experiments, a linear relation between TAFI and α2-antiplasmin levels and CLT was found, whereas the relation between plasminogen and CLT was biphasic and between PAI-1 and CLT S-shaped. The relative contribution of the plasma concentration of different fibrinolytic factors to CLT or the association between these factors and CLT in the general population is, however, not known. The procoagulant capacity of plasma has been shown to contribute to some extent to CLT. For example, individuals with the prothrombin G20210A mutation have longer CLTs as a result of increased thrombin generation resulting in increased TAFI activation.[201]

Based on the experiments with purified proteins, we surmised CLT will be explained by a combination of plasma levels of plasminogen, α2-antiplasmin, TAFI, and PAI-1. As a high concentration of t-PA is added to test plasma to induce fibrinolysis, the role of plasma t-PA levels in CLT is probably minor. In addition, we hypothesized that low levels of plasminogen, and high levels of α2-antiplasmin, TAFI, and PAI-1 constitute a risk factor for venous thrombosis and explain the association between CLT and risk of venous thrombosis.

Studies on the association between plasma levels of individual fibrinolytic factors and risk of venous thrombosis are either lacking or gave inconclusive results. Surprisingly, the association between plasma levels of plasminogen and

risk of venous thrombosis has not yet been investigated. Studies on plasminogen deficient individuals, however, have not provided any evidence for a causal role of plasminogen in venous thrombosis risk, but were of limited power to detect an effect.[22,23] Studies investigating levels of α2-antiplasmin and the risk of venous thrombosis are also scarce and have failed to show an association, possibly due to low patient numbers.[208,209] Increased TAFI levels were more consistently associated with risk of venous thrombosis[62,64,153] although not in thrombophilic families.[170]

The role of PAI-1 and t-PA in venous thrombosis is controversial. In the Longitudinal Investigation of Thromboembolism Etiology (LITE) study, a large population-based prospective study on venous thrombosis in middle-aged and elderly individuals no association was found between levels of PAI-1 or t-PA/PAI-1 complex and the risk of venous thrombosis.[41] Although several other studies also failed to show an association between t-PA and PAI-1 and the risk of venous thrombosis, others did find an association (extensively reviewed by Prins and Hirsh[80]). In this review it was concluded that there is evidence that increased plasma levels of t-PA and PAI-1 are associated with postoperative thrombosis.

The aim of the present study was to investigate the determinants of CLT in the general population. Furthermore, the association between plasma levels of plasminogen, α2-antiplasmin, TAFI, PAI-1, and t-PA and risk of venous thrombosis was studied.

SUBJECTS AND METHODS

Study design, study population, and data collection

For this study patients and control subjects of the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study were used. The design of the MEGA-study has been described extensively.[82] Between March 1999 and May 2002, consecutive unselected patients aged 18 to 70 years, with a first deep vein thrombosis of the leg or a first pulmonary embolism were identified at six anticoagulation clinics in the Netherlands. Information on the diagnostic procedure was obtained from hospital records and general practitioners. A deep venous thrombosis was confirmed with Doppler ultrasonography. A pulmonary embolism was confirmed by a ventilation perfusion lung scan, spiral computed tomography, or angiogram. From January 2002 to September 2004, control subjects were selected from the same geographical area as the patients by random digit dialing (RDD) using the Mitofsky-Waksberg method[200] and were frequency matched for sex and age to

the patients. This matching was performed on group level in which random controls were selected in numbers proportional to the number of patients within strata of sex and 5-year age groups. Patients or control subjects with severe psychiatric problems or those who could not speak Dutch were excluded. Participation rate was 83% among patients and 69% among control subjects. All participants signed an informed consent form. Approval for this study was obtained from the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands.

All participants were asked to complete a standardized questionnaire on acquired risk factors for venous thrombosis. Body mass index (BMI in kg/m^2) was calculated from self-reported weight and height. All items in the questionnaire referred to the period before the index date, which was for the patients the date of diagnosis of thrombosis and for the controls the date they filled in the questionnaire. When participants were unable to fill in the questionnaire, questions were asked by telephone, using a standardized mini-questionnaire. Three months after discontinuation of the anticoagulant therapy, patients were invited to the anticoagulation clinic for a blood sample. Of patients who received prolonged anticoagulant therapy (>1 year), blood was drawn under treatment with vitamin K antagonists. The control subjects were invited to the clinic for a blood draw after returning their questionnaire. All participants were interviewed regarding present anticoagulant use.

For the current study we analyzed all patients included in the MEGA-study who were presented at the anticoagulation clinic between March 1st 2001 and May 31st 2002 (n=770) and all RDD controls invited for participation from January 1st 2002 until December 31st 2003 (n=743) who provided a blood sample, which can be considered random samples of all patients and RDD controls of whom blood samples were available.

As validation population to study determinants of clot lysis time in, the control group of the Study of Myocardial Infarctions Leiden (SMILE) was used, which included 630 men between 18 and 70 years of age. Of these participants, measurements of all fibrinolytic variables were available. These men were without a history of myocardial infarction, without renal disease or severe (neuro)psychiatric problems, with a life expectancy of more than a year, and who had not taken any oral anticoagulants in the 6-month period prior to participation in the study.[85]

Blood collection and laboratory analysis

Blood samples were primarily drawn in the morning (median 9.40 h, 95% before 11.00 h), without a systematic difference between patients and control subjects.

Time between thrombosis and blood draw ranged from 95 days to 877 days with a median of 299 days and 74% of all patients provided a blood sample between 6 and 12 months after the thrombosis. Blood samples were drawn into vacuum tubes containing 0.106 M trisodium citrate. Plasma was obtained by centrifugation at 2000 g for 10 minutes at room temperature and stored in aliquots at -80°C. Lysis of a tissue factor-induced clot by exogenous tissue-type plasminogen activator (t-PA) was studied by monitoring changes in turbidity during clot formation and subsequent lysis as described previously.[78] In short, 50 µl plasma was pipetted in a 96-well microtitre plate. Subsequently, a 50 µl mixture containing phospholipid vesicles, t-PA (final concentration 56 ng/mL), tissue factor, and $CaCl_2$ diluted in HEPES was added using a multichannel pipette. In a kinetic microplate the optical density at 405 nm was monitored every 20 seconds, resulting in a clot-lysis turbidity profile. The CLT was derived from this clot-lysis profile and defined as the time from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing the lysis of the clot. α2-Antiplasmin and plasminogen activity were measured using chromogenic assays (STA Stachrom α2-antiplasmin and STA Stachrom plasminogen from Diagnostica Stago, Asnières, France) and were performed on a STA-R coagulation analyzer using a commercial calibration standard (Diagnostica Stago, Asnières, France). PAI-1 antigen levels were measured with a Technozym PAI-1 enzyme-linked immunosorbent assay (ELISA) reagent kit (Kordia, Biopool, the Netherlands). Plasma TAFI activity levels were determined with a chromogenic assay (Pefakit TAFI, Pentapharm LTD, Basel, Switzerland) by converting TAFI into its active form using a reagent containing thrombin-thrombomodulin and subsequently measuring the carboxypeptidase activity. Measurements were run on a BCS coagulation analyser (Dade Behring Inc., Marburg, Germany). Antigen levels of t-PA were assessed by ELISA using a commercially available mouse anti-t-PA antibody (Nuclilab BV, Ede, The Netherlands) as capture, and a biotin-labelled rabbit anti human t-PA antibody (Nuclilab BV, Ede, The Netherlands) as detecting antibody. Bound detecting antibody was visualised using horseradish peroxidase-labelled streptavidin, followed by Tetramethylbenzidine (TMB) staining. A calibration curve was constructed using purified t-PA (Nuclilab BV, Ede, The Netherlands). The inter-assay coefficients of variation were 6.6% for CLT, 1.6% for plasminogen, 4.6% for α2-antiplasmin, 5.8% for TAFI, 7.2% for PAI-1 and 8.1% for t-PA.

Measurements of antithrombin and protein C levels were performed with a chromogenic assay and prothrombin (factor II) activity, factor VII activity, factor VIII activity, factor X activity, and factor XI activity were measured with a mechanical clot

detection method on a STA-R coagulation analyzer following the instructions of the manufacturer (Diagnostica Stago, Asnieres, France). Total protein S levels and levels of factor IX antigen were determined by ELISA (Diagnostica Stago). Fibrinogen activity was measured on the STA-R analyzer according to methods of Clauss.[210] Von Willebrand factor (VWF) antigen was measured with the immunoturbidimetric method, using the STA Liatest kit, following the instructions of the manufacturer (Diagnostica Stago). Total homocysteine, total cysteine and methionine (as sum methionine + methioninesulfoxide) were measured using liquid chromatography-mass spectrometry.

The abovementioned parameters were all expressed in percentages relative to pooled normal plasma, except t-PA and PAI-1 levels that were expressed in ng/mL, fibrinogen levels that were expressed in g/L, and the sulphur amino acids that were expressed in μmol/L.

All laboratory measurements were performed without knowledge of whether the sample was from a patient or a control subject.

Statistical analysis

To study determinants of CLT in the general population, simple and multiple linear regression with CLT as dependent variable were performed in the control group. As the use of vitamin K antagonists influences CLT, these analyses were restricted to subjects not taking oral anticoagulants in whom all investigated parameters (plasminogen, α2-antiplasmin, TAFI, PAI-1, t-PA, prothrombin, factor VII, factor VIII, factor IX, factor X, factor XI, protein S, protein C, antithrombin, fibrinogen, VWF and CLT) were measured (N=733). Nine subjects had missing values for the sulphur containing amino acids (homocysteine, cystein and methionine) and were excluded from the analyses including these three factors. As the distribution of CLT is skewed, CLT was 10log-transformed. t-PA, PAI-1, factor VIII and factor XI, homocysteine and fibrinogen also had skewed distributions and were entered after a 10log-transformation for a better fit of the model. The R^2 was used as a measure of explained variance. To compare the relative strength of the various determinants within the model, all variables (CLT and other plasma factors) were standardized by calculating Z-scores. The Z-score for an observation of a subject is calculated by subtracting the mean from the observed value and dividing the residual by the standard deviation (SD). Simple or multiple linear regression analysis was performed with the standardized variables. The resulting standardized regression coefficient (β) for a factor indicates the increase in SDs of log-CLT, when that particular factor increases with 1 SD and all other variables in the model are unchanged. The fit of the

resulting regression models was examined by plotting the observed CLT versus the predicted CLT and versus the independent variables in the model. If this plot showed a non-linear relationship for any of the independent variables, higher order terms were added to the model.

We estimated the unexplained variance ($\sigma^2_{unknown}$) by subtracting an estimate of variance due to the measurement error of CLT ($\sigma^2_{measurement\ error}$) from the residual variance of the regression analysis. The variance due to measurement error of CLT was estimated from the inter assay variation. Ninety normal pooled plasma samples were measured on ninety 96-well plates yielding a SD of the 10log-transformed CLT measurement of 0.028.

To study the effect of fibrinolytic factors and CLT on risk of venous thrombosis, levels of plasminogen, α2-antiplasmin, TAFI, PAI-1, t-PA, and CLT were grouped into quartiles based on the distribution among the control subjects. Odds Ratios (ORs) with 95% confidence intervals (CIs) were calculated taking the lowest quartile as the reference group for the OR. Unconditional logistic regression was performed to adjust for age, sex and other potential confounders. In the logistic regression model BMI, levels of factor VIII, fibrinogen, VWF and fibrinolytic factors when used as covariates were included as continuous variables. VWF, fibrinogen, t-PA and PAI-1 were included in the model after 10log-transformation. Entering these factors as categorical variables did not change the results. Participants on oral anticoagulants (7 controls and 92 patients) were excluded in the analyses concerning CLT. SPSS 16.0 (SPSS, Chicago, IL, USA) was used for statistical analyses.

RESULTS

Determinants of clot lysis time

To examine determinants of CLT in the general population, 733 control subjects of the MEGA-study were studied, including 374 men and 359 women with a mean age of 46 years (range 18-70). Mean CLT was 65.3 minutes (median 61.7; range 35.0-204.7).

Linear regression was performed to investigate the association between factors of the coagulation and fibrinolytic system and CLT, using the log-transformed CLT as dependent variable. In Table 1 the standardized regression coefficients (βs) of the analyses including plasminogen, α2-antiplasmin, TAFI, PAI-1, and t-PA are shown. In simple linear regression analyses, all fibrinolytic factors except plasminogen were associated with CLT. The strongest association was found

between PAI-1 and CLT, with a regression coefficient of 0.63 (95%CI 0.58-0.69), indicating that with every SD increase in PAI-1, CLT increases with 0.63 SD (PAI-1 and CLT log-transformed). This model had an R^2 of 0.63^2=0.40, denoting that with PAI-1 as independent factor, 40% of the variation in CLT was explained. Including all fibrinolytic factors (plasminogen, α2-antiplasmin, TAFI, PAI-1, and t-PA) in a multiple regression model increased the explained variance to 53% (Table 1).

Table 1 *Mean change in clot lysis time* with 1 standard deviation increase in fibrinolytic factor.*

	Simple models†		**Multiple Model‡**	
	β (95%CI)	**R^2**	**β (95%CI)**	**R^2**
plasminogen	0.04 (-0.03-0.12)	0	-0.10 (-0.158--0.045)	0.53
α2-antiplasmin	0.26 (0.19-0.33)	0.07	0.20 (0.14-0.25)	
TAFI	0.40 (0.33-0.47)	0.16	0.28 (0.23-0.34)	
PAI-1*	0.63 (0.58-0.69)	0.40	0.54 (0.48-0.59)	
t-PA*	0.39 (0.32-0.45)	0.15	0.08 (0.03-0.14)	

PAI-1, plasminogen activator inhibitor 1; t-PA, tissue plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor.

** Clot lysis time, PAI-1 and t-PA were 10log-transformed.*

† five different models. In each model clot lysis time was the dependent variable and only one of the fibrinolytic factors as independent variable.

‡ Clot lysis time as dependent variable and all five fibrinolytic factors simultaneously as independent variables in the model.

Next, we used a separate model without fibrinolytic variables, but including all coagulation factors and sulphur containing amino acids (prothrombin, factor VII, VIII, IX, X, and XI, protein C, protein S, antithrombin, fibrinogen, VWF, cystein, methionine, and homocysteine) resulting in an R^2 of 0.29 (regression coefficients not shown). Combining all coagulation and fibrinolytic factors in one overall multiple model yielded a R^2 of 0.58 (regression coefficients not shown). Table 2 (model A) shows a reduced model from a backward selection procedure with a similar R^2 (0.58), which included the variables which remained significant at α=0.10. PAI-1 remained the strongest determinant of CLT (β = 0.49 (95%CI 0.43-0.54)).

Table 2 *Multiple linear regression on clot lysis time* without (model A) and with (model B) a squared and cubic term for PAI-1.*

	Model A		Model B	
	β (95%CI)	**R^2**	**β (95%CI)**	**R^2**
prothrombin	0.15 (0.08-0.22)	0.58	0.16 (0.11-0.22)	0.69
factor VII	0.05 (-0.09-0.11)		0.07 (0.02-0.12)	
factor IX	0.06 (-0.01-0.12)			
factor X	0.07 (-0.00-0.13)		0.06 (0.00-0.12)	
factor XI*			0.05 (0.01-0.10)	
protein C	0.07 (0.00-0.13)			
antithrombin	-0.05 (-0.11-0.00)			
fibrinogen	0.08 (0.02-0.14)		0.10 (0.05-0.15)	
plasminogen	-0.26 (-0.33--0.20)		-0.29 (-0.34--0.23)	
α2-antiplasmin	0.14 (0.09-0.20)		0.16 (0.11-0.21)	
TAFI	0.21 (0.15-0.26)		0.23 (0.18-0.28)	
PAI-1*	0.49 (0.44-0.54)		0.28 (0.22-0.35)	
PAI-1 (squared)*			0.27 (0.22-0.32)	
PAI-1 (cubic)*			0.22 (0.14-0.29)	

PAI-1, plasminogen activator inhibitor 1; TAFI, thrombin activatable fibrinolysis inhibitor.
** Clot lysis time, factor XI, and PAI-1 were log-transformed.*

To obtain an estimation of the accuracy of the model, the predicted log-CLT Z-score was calculated for each study participant, using model A of table 2 and back transformed to the original CLT scale. Figure 1a shows the observed CLT plotted against the predicted CLT. The fit of the model seems adequate for shorter CLT, but the longest CLTs are underestimated using this linear model. The reason for this is the nonlinear or cubic association between PAI-1 and CLT as shown in Figure 1b. Therefore, to better fit the model, a squared and cubic term for PAI-1 were added to the overall model including all coagulation and fibrinolytic factors. Table 2 model B shows the reduced model from a backward selection procedure. CLT predicted with regression coefficients from a multiple regression model including the variables of model B plotted against the observed CLT is shown in Figure 1c.

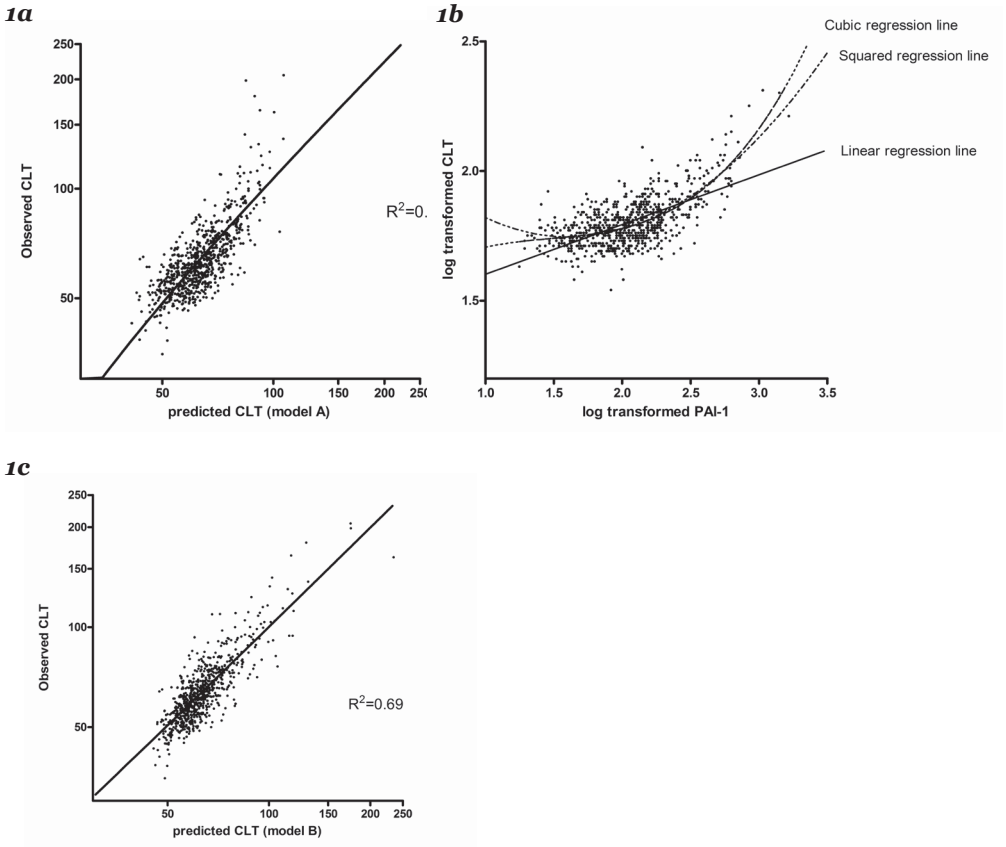

Figure 1a *Observed clot lysis time (CLT) plotted against the predicted CLT using regression coefficients derived from a linear regression model including levels of prothrombin, factor VII, factor IX, factor X, protein C, antithrombin, fibrinogen, plasminogen, α2-antiplasmin, TAFI and PAI-1 (Table 2; model A).* ***1b*** *Association between PAI-1 and CLT.* ***1c*** *Observed CLT plotted against predicted CLT using regression coefficients derived from a linear regression including levels of prothrombin, factor VII, factor X, factor XI, fibrinogen, plasminogen, α2-antiplasmin, TAFI, PAI-1 and a squared and cubic term for PAI-1 (Table 2; model B).*

The R^2 of this model increased to 0.69, meaning that the residual variation in log-CLT was 31%. The estimated residual variance was equal to 0.0028. The estimated variance due to measurement error was 0.028^2=0.00077, because our inter assay SD was equal to 0.028. This is equal to 27% of the residual variance. Consequently, 8% (27% of 31%) of the total variation in CLT is due to measurement error and 23%

(73% of 31%) of the variation in CLT remains unexplained. Accordingly, 77% of the variation in CLT could be explained.

Figure 2 illustrates the effect of the combination of levels of fibrinolytic factors (plasminogen, α2-antiplasmin, TAFI, and PAI-1) on CLT in control subjects. Each fibrinolytic factor was divided in three groups (low, intermediate, and high level, using the mean + or -$^{2}/_{3}$*SD as arbitrary cut-off points), resulting in 81 categories. Mean clot lysis time for each category was calculated. The mean CLTs were classified as low (below -$^{1}/_{3}$*SD), intermediate (-$^{1}/_{3}$*SD to $^{1}/_{3}$*SD) , high ($^{1}/_{3}$*SD to 2*SD) and very high (>2*SD). Groups of similar mean CLT are indicated with the same color, with the more intense colors representing higher mean CLT. Thus, confirming data from the analyses in tables 1 and 2, the figure shows a clear increase in CLT with increasing plasma levels of PAI-1, TAFI, and α2-antiplasmin and decreasing levels of plasminogen. Remarkable is the individual in the upper right corner of the figure with low levels of plasminogen and high levels of α2-antiplasmin, TAFI, and PAI-1 with a CLT 3.5*SD above mean CLT.

To validate the model, we performed an additional regression analysis in the control group of the Study of Myocardial Infarctions LEiden (SMILE) (n=630) using the variables of the model of Table 1. Prothrombin levels were not measured in SMILE and we were thus not able to use the model as presented in table 2. Regression coefficients in this analysis were similar to those found in MEGA: -0.01 for plasminogen, 0.23 for α2-antiplasmin, 0.17 for TAFI, and 0.53 for PAI-1. The R^2 of this model was 0.39.

Fibrinolytic proteins and risk of venous thrombosis

Next, the association between plasma levels of the individual fibrinolytic components and risk of venous thrombosis was investigated. In these analyses, 770 patients and 743 control subjects were included. Mean age at time of blood draw of patients was 49 year (range 19-71) and mean age of control participants was 46 year (range 18–70). In the control group 379 participants (51%) were men and in the patient group 347 participants (45%) were men. Of all patients 215 (28%) were diagnosed with an isolated pulmonary embolism, 475 (62%) with an isolated deep vein thrombosis of the leg, and 80 (10%) with both a pulmonary embolism and a deep vein thrombosis of the leg.

Increased levels of each of the individual fibrinolytic proteins were associated with an increased risk of venous thrombosis after adjustment for age and sex (Table 3). As levels of fibrinolytic factors increase with increasing BMI,[95,110] risks were further adjusted for BMI. After adjusting for BMI, the ORs were 1.6 (95%CI

1.2-2.2) for plasminogen, 1.6 (95%CI 1.2-2.1) for TAFI, 1.6 (95%CI 1.1-2.1) for PAI-1, and 1.8 (95%CI 1.3-2.6) for t-PA for the highest quartile compared with the lowest. α2-Antiplasmin was no longer associated with risk of venous thrombosis (OR 1.2; 95%CI 0.9-1.7).

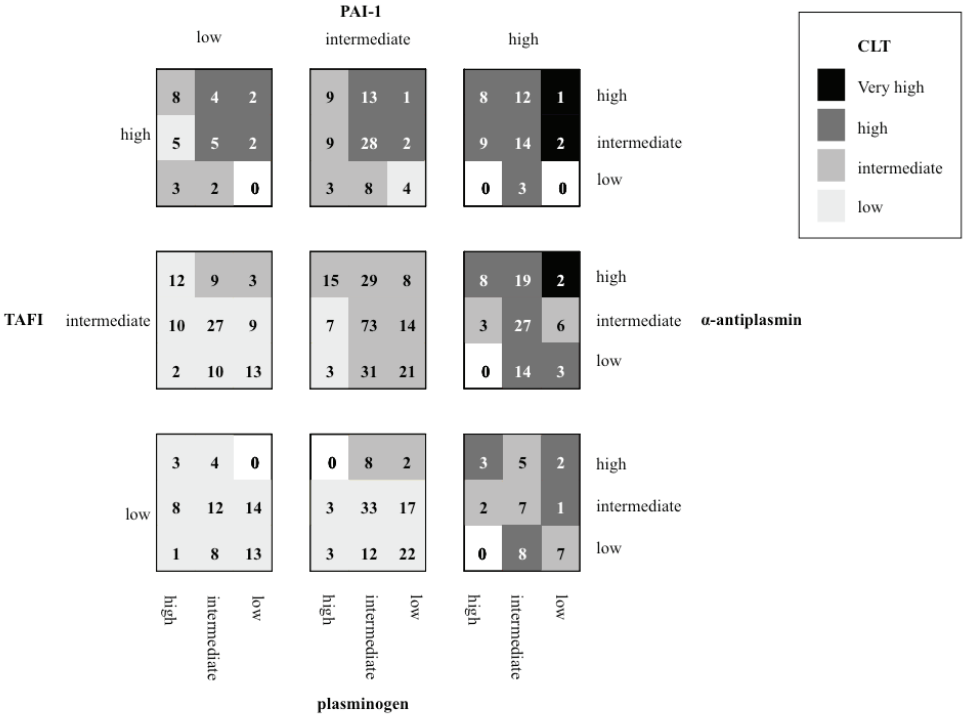

Figure 2 *CLT for different combinations of levels of fibrinolytic factors.*
Numbers in the squares indicate the number of control subjects included in each category. CLT, clot lysis time; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin activatable fibrinolysis inhibitor.
Cut-off points for fibrinolytic factors (μ=population mean; SD=standard deviation): low:<μ-2/3SD; intermediate: μ-2/3SD to μ+2/3SD; high: >μ+2/3SD. Cut-off for CLT low:<μ-1/3 SD; intermediate: μ-1/3SD to μ+1/3SD; high: μ+1/3SD to μ+2SD; very high: >μ+2SD. Cut-offs for CLT and PAI-1 were based on 10log transformed values.

Previously it has been shown that plasminogen, PAI-1, and t-PA are markers of inflammation.[95,111] Indeed the risks of thrombosis somewhat decreased after adjustment for markers of inflammation. The risk of venous thrombosis, adjusted

for age, sex, BMI, and levels of the acute-phase proteins fibrinogen and factor VIII, decreased to 1.3 (95%CI 0.9-1.8) for the highest quartile of plasminogen. After adjusting for the same factors the risk for the highest quartile of PAI-1 was still 1.7-fold increased (95%CI 1.2-2.3) and for the highest levels of t-PA, the adjusted OR decreased to 1.5 (95%CI 1.0-2.1), all compared with the first quartile.

t-PA can also be seen as a marker of endothelial activation,[6] so we further adjusted for plasma levels of VWF. The risk of venous thrombosis for individuals with the highest levels of t-PA reduced to 1.3 (95% 0.9-1.9) after adjusting for age, sex, BMI, fibrinogen, factor VIII and VWF. Similar adjustments in the analyses of the other fibrinolytic factors did not considerably change the results. Adjusting for age, sex, fibrinogen, factor VIII, and VWF resulted in ORs for the 4th quartile of 1.4 (95%CI 0.9-2.0) for plasminogen, 1.0 (95%CI 0.7-1.5) for α2-antiplasmin, 1.4 (95%CI 1.0-2.0) for TAFI, and 1.6 (95%CI 1.1-2.2) for PAI-1. Finally we adjusted the risks for each fibrinolytic variable additionally for the other fibrinolytic factors. The risk for the 4th quartile of plasminogen adjusted for age, sex, BMI, factor VIII, fibrinogen, VWF, and for plasma levels of α2-antiplasmin, PAI-1, t-PA and TAFI was 1.3 (95%CI 0.9-2.0). Similar analysis for the other fibrinolytic factors gave an OR of 1.0 (95%CI 0.7-1.4) for α2-antiplasmin, 1.4 (95%CI 1.0-2.0) for TAFI, 1.5 (95%CI 1.0-2.1) for PAI-1, and 1.1 (95%CI 0.7-1.6) for t-PA. Hence PAI-1 and TAFI were still associated with venous thrombosis in these models.

To investigate whether CLT increases the risk of venous thrombosis also independently of the fibrinolytic factors, risk of venous thrombosis for quartiles of CLT was estimated after adjustment for age and sex, and fibrinolytic factors. Age and sex adjusted ORs were 1.5 (95%CI 1.1-2.2) for individuals in the 2nd quartile of CLT, 2.7 (95%CI 1.9-3.7) for the 3rd and 3.4 (95%CI 2.5-4.9) for the 4th quartile, all compared with the lowest. After further adjustment for plasma levels of PAI-1 the risk of venous thrombosis remained 1.5-fold (95%CI 1.1-2.2), 2.5-fold (95%CI 1.8-3.6), and 3.0-fold (95%CI 2.1-4.5) increased for the 2nd, 3rd, and 4th quartile respectively. Entering squared and cubic terms for PAI-1 into the model did not change these results (data not shown). Further adjustment for levels of plasminogen, α2-antiplasmin, TAFI, t-PA, and prothrombin gave ORs of 1.4 (95%CI 1.0-2.0) for the 2nd, 2.6 (95%CI 1.8-3.8) for the 3rd and 3.2 (95%CI 2.0-5.2) for the 4th quartile of CLT.

Table 3 *Fibrinolytic factors and risk of venous thrombosis.*

Quartile of fibrinolytic factor	1 (ref.)	2	3	4	Median (5^{th}-95^{th} percentile)
Plasminogen*					
cut off in %	88	96	105		
patients	131	180	209	250	99 (80-126) %
control subjects	188	187	185	182	96 (77-128) %
$OR_{age\ \&\ sex}$	1	1.3 (1.0-1.8)	1.5 (1.1-2.0)	1.8 (1.4-2.5)	
$OR_{age,\ sex\ \&\ BMI}$	1	1.2 (0.9-1.7)	1.3 (1.0-1.8)	1.6 (1.2-2.2)	
α2-antiplasmin*					
cut off in %	95	102	110		
patients	175	179	205	211	104 (83-123) %
control subjects	197	197	183	165	102 (86-120) %
$OR_{age\ \&\ sex}$	1	1.0 (0.8-1.3)	1.2 (0.9-1.7)	1.4 (1.0-1.9)	
$OR_{age.\ sex\ \&\ BMI}$	1	1.0 (0.7-1.3)	1.1 (0.8-1.5)	1.2 (0.9-1.7)	
TAFI*					
cut off in %	106	117	127		
patients	143	195	166	266	121 (93-155) %
control subjects	199	180	179	184	117 (92-147) %
$OR_{age\ \&\ sex}$	1	1.4 (1.1-1.9)	1.2 (0.9-1.7)	1.8 (1.3-2.4)	
$OR_{age,\ sex\ \&\ BMI}$	1	1.3 (1.0-1.8)	1.1 (0.8-1.6)	1.6 (1.2-2.1)	
PAI-1*					
cut off in ng/mL	55	62	71		
patients	148	169	120	332	146 (40-552) ng/mL
control subjects	185	186	186	185	113 (36-401) ng/mL
$OR_{age\ \&\ sex}$	1	1.1 (0.8-1.5)	0.8 (0.6-1.1)	2.2 (1.6-2.9)	
$OR_{age,\ sex\ \&\ BMI}$	1	1.1 (0.8-1.4)	0.7 (0.5-0.9)	1.6 (1.1-2.1)	
t-PA*					
cut off in ng/mL	4.8	6.1	7.8		
patients	134	135	209	292	7.0 (3.8-12.0) ng/mL
control subjects	185	186	186	185	6.1 (3.4-10.9) ng/mL
$OR_{age\ \&\ sex}$	1	1.0 (0.8-1.4)	1.6 (1.2-2.2)	2.3 (1.7-3.3)	
$OR_{age,\ sex\ \&\ BMI}$	1	0.9 (0.7-1.3)	1.3 (0.9-1.8)	1.8 (1.3-2.6)	

BMI, body mass index; PAI-1, plasminogen activator inhibitor 1; ref., reference group; t-PA, tissue plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor.
** PAI-1 levels were not measured in one patient; TAFI, plasminogen, α2-antiplasmin, t-PA, and PAI-1 levels were not measured in one control subject.*

DISCUSSION

Increased clot lysis time as measured with an overall plasma-based assay is associated with an increased risk of venous and arterial thrombosis.[81,82,85,94,211] However, the factors influencing CLT in the general population were unknown, which prompted us to look for determinants of CLT in a large group of healthy individuals derived from the MEGA-study. In this study we could explain 77% of the variation in CLT. Plasma levels of PAI-1 explained the majority of the variance in CLT, followed by TAFI levels, which is in line with the observation from the present study that elevated levels of PAI-1 and TAFI are independent risk factors for venous thrombosis. Plasma levels of α2-antiplasmin and plasminogen were associated with CLT to similar extent as TAFI. α2-Antiplasmin was not associated with venous thrombosis. Surprisingly, plasminogen was positively associated with venous thrombosis. Levels of t-PA did not determine CLT but were associated with risk of venous thrombosis. Plasminogen and t-PA, however, may be just markers of other risk factors such as inflammation and endothelial activation. Besides fibrinolytic factors, prothrombin is an important determinant of CLT. Fibrinogen, factor VII, X, and XI contributed to the variation in CLT to a lesser extent.

Determinants of clot lysis time

Our results are in agreement with previous *in vitro* experiments in which CLT increased with increasing levels of α2-antiplasmin, TAFI, and PAI-1, and with decreasing levels of plasminogen.[78] Moreover, we could replicate the associations found in the MEGA-study to a large extent in the control group of the SMILE, an independent study population. In our CLT assay t-PA is added to initiate fibrinolysis, mimicking the *in vivo* release of endothelial t-PA after stimulation.[6] Consequently, plasma levels of t-PA do not influence the CLT. Elevated prothrombin levels increase CLT, probably via increased TAFI activation, which is in line with studies showing that individuals with the prothrombin 20210A mutation had longer CLTs than those without the mutation, and with studies showing that the lysis time of prothrombin-enriched plasma clots was prolonged proportionally to the amount of added prothrombin in a TAFI-dependent manner.[82,201] Finally, levels of fibrinogen or the sulphur containing amino acids which also have been found to be associated with stiffer or denser clots that are more resistant to fibrinolysis[212] were minimally or not associated with CLT.

With a statistical model including all factors associated with CLT, we could explain 77% of the variation in CLT. The 23% unexplained variation may be due to

factors not measured in our study, but known to influence fibrinolysis, or by yet unknown factors. Possible candidates are coagulation factor XIII and Tissue Factor Pathway Inhibitor (TFPI), which were, although not strongly, previously shown to be associated with CLT using an age-adjusted model in the control group of the LEiden Thrombophilia Study (LETS).[81] Other proteins known to influence fibrinolysis are for instance lipoprotein(a),[213] which competes with plasminogen for binding to fibrinogen, vitronectin,[214] which binds to PAI-1 and stabilizes it, histidine-rich glycoprotein which binds to plasminogen and modulates plasminogen bioavailability for plasmin generation,[215] and the plasmin inhibitors α_2-macroglobulin, α_1-antitrypsin, and C1 inhibitor.[216] We are currently examining possible genetic factors influencing CLT through Quantitative Trait Loci (QTL) analysis in an extended thrombophilic pedigree.[211] Part of the unexplained variation may be the result of lack of fit of the model as the linear regression model assumes a linear relation between the proteins and CLT which may not always be completely accurate. Additionally, only plasma antigen or activity levels of the fibrinolytic factors are included. Consequently the functionality or stability of the factors and the interplay between the factors in the coagulation and fibrinolytic cascade are not taken into account. Plasma levels of plasminogen and α2-antiplasmin are presumably not the limiting factors in fibrinolysis as they circulate at high concentrations in healthy subjects. The amount of plasmin formed and subsequent lysis of the clot may therefore depend more on regulating steps prior to final activation of plasminogen than on the total amount of plasminogen or α2-antiplasmin present in plasma.[40]

Although CLT could largely be explained by the levels of the fibrinolytic factors and prothrombin, the association between CLT and venous thrombosis remained after adjusting for the fibrinolytic factors measured. This suggests that indeed CLT is not fully explained by these factors, and that additional proteins are involved. Alternatively, a complex interplay between fibrinolytic factors and prothrombin, not fully accounted for by the statistical models is responsible for the association between elevated CLT and venous thrombosis.

Fibrinolytic proteins and risk of venous thrombosis

To our knowledge this is the first large study on levels of plasminogen and α2-antiplasmin and risk of venous thrombosis. Although plasminogen levels were negatively associated with CLT, unexpectedly plasminogen was positively associated with risk of venous thrombosis. Plasma levels of plasminogen are strongly linked to plasma levels of other coagulation and fibrinolytic factors (see supplemental table), which may mask a protective effect of elevated plasminogen levels on venous

thrombosis. The role of plasminogen in thrombosis risk, however, has always been unclear. Although plasmin is thought to be the key enzyme responsible for fibrin degradation, plasminogen deficient subjects do not appear to suffer from thrombotic events but primarily from ligneous conjunctivitis, a rare form of chronic conjunctivitis characterized by the development of firm fibrin-rich lesions mainly on the tarsal conjunctivae (reviewed by Brandt[21]). In contrast, population-based studies have found increased plasminogen levels to be associated with an increased risk of arterial thrombosis.[95,110] One explanation for this association is that plasminogen levels are increased by inflammatory processes.[111] We have previously demonstrated that the positive association between plasma levels of plasminogen and myocardial infarction disappears after adjustment for markers of inflammation.[217] In the current study, adjustment for the acute-phase proteins fibrinogen and factor VIII attenuated the association between plasminogen and venous thrombosis, indeed suggesting that plasminogen is a marker of inflammation. However, plasminogen could play a role in venous thrombosis through alternative pathways. Plasmin has other substrates besides fibrin, such as protease-activated receptor-1 (PAR-1), the extracellular matrix, TFPI, and factor V, and plasmin could induce endothelial damage, all potentially important in thrombosis risk.[86,218-221]

Although α2-antiplasmin was positively associated with CLT, no association was found between α2-antiplasmin and risk of venous thrombosis after adjusting for BMI. This is in agreement with two small studies in which levels of α2-antiplasmin were not associated with postoperative thrombosis.[208,209] As stated previously, α2-antiplasmin normally circulates at high levels and may therefore not be a limiting factor.

TAFI defines the molecular connection between the coagulation and fibrinolytic cascades.[222,223] We find TAFI levels to be associated with an increased risk of venous thrombosis. This is in accordance with previous studies investigating the association between TAFI levels and first thrombosis, either in a general population[62] or in factor V Leiden carriers,[153] and with a study on TAFI and recurrent venous thrombosis.[64] In these studies, TAFI levels above the 90th percentile of the control group were associated with a 2 to 4-fold increased risk of venous thrombosis.

The role of t-PA and PAI-1 in venous thrombosis is controversial. Although several studies have found a positive relation with venous thrombosis others have not. In an extensive review it was concluded that PAI-1 and t-PA may be important in venous thrombosis especially in individuals undergoing surgery.[80] In present study, elevated levels of PAI-1 and t-PA were associated with venous thrombosis. Adjustment for acute-phase proteins and VWF attenuated the increased risk found

in individuals with high t-PA levels. As most of the t-PA antigen in plasma is bound to PAI-1 and therefore inactive it is plausible that t-PA is just a marker of underlying processes, such as inflammation and endothelial activation. This observation is in agreement with two studies investigating the association between t-PA and arterial thrombosis.[95, 217] Increased PAI-1 levels were still associated with venous thrombosis, even after extensive adjustment, which suggests that hypofibrinolysis caused by elevated PAI-1 levels indeed increases thrombosis risk.

It should be noted that the adjustments made for plasma proteins in all analyses presented must be considered with caution. Adjustments are justified for factors that are risk factors for venous thrombosis and that influence the fibrinolytic factor of interest but are not influenced by the fibrinolytic factor themselves.[224] As coagulation factors and fibrinolytic factors tend to cluster and correlate and possibly share genetic regulation, causal inference and justification of the adjustments is difficult.[175-177]

In conclusion, variation in clot lysis time could be explained for 77%. PAI-1 was the principal determinant of CLT, followed by plasminogen, TAFI, prothrombin, and α2-antiplasmin. Increased plasma levels of plasminogen, TAFI, PAI-1, and t-PA were associated with an increased risk of venous thrombosis, although plasminogen and t-PA may be markers of other risk factors for venous thrombosis.

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Supplemental table *Coagulation and fibrinolytic factors associated with levels of plasminogen.**

quartiles of coagulation or fibrinolytic factor	N	mean plasminogen level (%)(5^{th}-95^{th} percentile)	Regression coefficient† (95%CI)
fibrinogen (g/L)			
<2.77	189	89 (72-108)	
2.77-3.17	180	97 (75-126)	
3.17-3.65	183	100 (81-134)	
>3.65	181	109 (89-145)	9.78 (8.36-11.21)
prothrombin (%)			
<102	196	89 (71-107)	
102-109	175	95 (79-111)	
109-119	196	102 (81-129)	
>119	166	110 (86-151)	0.63 (0.56-0.71)
factor VII (%)			
<91	191	96 (76-126)	
91-106	193	96 (75-126)	
106-123	179	100 (76-131)	
>123	170	104 (82-136)	0.15 (0.10-0.20)
factor VIII (%)			
<88	187	95 (75-127)	
88-108	180	98 (74-128)	
108-133	186	99 (79-130)	
>133	180	101 (81-136)	0.07 (0.04-0.10)
factor IX (%)			
<91	183	90 (72-114)	
91-102	183	95 (77-121)	
102-113	184	100 (77-127)	
>113	183	109 (86-149)	0.43 (0.37-0.49)
factor X (%)			
<103	185	89 (74-106)	
103-116	190	96 (76-114)	
116-129	192	101(79-129)	
>129	166	110 (84-150)	0.45 (0.40-0.51)

quartiles of coagulation or fibrinolytic factor	N	mean plasminogen level (%)(5^{th}-95^{th} percentile)	Regression coefficient† (95%CI)
factor XI (%)			
<87	196	94 (72-127)	
87-98	183	96 (77-121)	
98-110	180	99 (78-128)	
>110	174	106 (81-138)	0.29 (0.23-0.35)
Protein C (%)			
<101	196	95 (71-133)	
101-115	194	99 (79-131)	
115-129	166	100 (81-130)	
>129	177	100 (81-126)	0.28 (0.22-0.33)
Antithrombin (%)			
<100	196	95 (71-133)	
100-106	194	99 (79-131)	
106-112	166	100 (81-130)	
>112	177	99 (81-126)	0.19 (0.07-0.30)
α2-antiplasmin (%)			
<95	194	91 (72-115)	
95-102	192	96 (76-126)	
102-110	182	101 (83-132)	
>110	165	107 (82-149)	0.58 (0.48-0.69)
TAFI (%)			
<106	198	93 (72-125)	
106-117	177	96 (76-120)	
117-127	178	100 (78-131)	
>127	180	106 (82-148)	0.32 (0.26-0.38)

CI, Confidence Interval, TAFI, thrombin activatable fibrinolysis inhibitor.
** Protein S, tissue plasminogen activator, and plasminogen activator inhibitor-1 were not associated with plasminogen.*
† in % increase in plasminogen/1 unit increase of the independent variable.

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Genome scan of clot lysis time and its association with thrombosis in a protein C deficient kindred

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ABSTRACT

Previously we found increased clot lysis time (CLT), as measured with a plasma-based assay, to increase the risk of venous thrombosis in two population-based case-control studies. Genes influencing CLT are as yet unknown. We tested CLT as risk factor for venous thrombosis in 346 members of Kindred Vermont II, a pedigree suffering from a high thrombosis risk, partially attributable to a type I protein C deficiency. Furthermore we tested for quantitative trait loci for CLT using variance component linkage analysis. Protein C deficient family members had shorter CLT than non-deficient members (median CLT 67 versus 75 minutes). One standard deviation increase in CLT increased risk of venous thrombosis 2.4-fold (95% confidence interval (CI) 1.4-3.2) in individuals with normal protein C levels. Protein C deficiency without elevated CLT increased risk 6.9-fold (95%CI 4.2-11.5). The combination of both risk factors increased thrombosis risk 27.8-fold (9%CI 11.7-66.0). Heritability of CLT was 42-52%. We found suggestive evidence of linkage on chromosome 11 (62 cM), which was partly explained by the prothrombin 20210A mutation, and on chromosome 13 (52 cM). Thrombin Activatable Fibrinolysis Inhibitor (TAFI) genotypes associated with TAFI levels did not explain any variation in CLT.

In conclusion, hypofibrinolysis appears to increase risk of venous thrombosis in this family. The combination of hypofibrinolysis and protein C deficiency leads to a high risk of thrombosis. Protein C deficient family members have shorter CLT than those without the deficiency. CLT is in part genetically regulated. Suggestive quantitative trait loci were found on chromosome 11 and 13.

INTRODUCTION

Decreased fibrinolytic potential, as measured with an overall plasma-based assay has consistently been shown to be associated with an increased risk of venous and arterial thrombosis.[81,82,85,94] This clot lysis assay is initiated with tissue factor and tissue plasminogen activator (t-PA). Clot lysis time (CLT) is predominantly influenced by plasma levels of the key players in the fibrinolytic system, i.e., plasminogen activator inhibitor type 1 (PAI-1), thrombin activatable fibrinolysis activator (TAFI), α2-antiplasmin, and plasminogen but also by levels of coagulation factors, notably factor II (prothrombin).[78,183]

CLT is in part determined by genetic factors as shown by heritability analyses in families with unexplained thrombophilia.[225] Apart from the prothrombin 20210A mutation, however, which is associated with increased prothrombin levels and increased CLT,[82,201] the nature of genetic variation influencing CLT is unknown. CLT is a complex trait resulting from a dynamic process of the interplay between several individual fibrinolytic and coagulation factors and may therefore be more informative in relation to thrombotic disease risk than the levels of the individual factors. The identification of genetic loci influencing CLT could increase our understanding of the fibrinolytic system, the interaction between the factors involved, and consequently our understanding of thrombotic disease itself.

Thrombosis is a complex disease with multiple genetic and environmental risk factors and it is commonly accepted that the presence of multiple risk factors in a single person is necessary for the development of thrombosis.[4] Vermont Kindred II is a large pedigree suffering from a high risk of venous thrombosis, partially attributable to a type I protein C deficiency due to a 3363 inserted C mutation in exon 6 of the protein C gene.[18] The increased risk of venous thrombosis in this family, however, is not completely explained by protein C deficiency, suggesting that other genetic variants contribute to thrombosis risk. Recently, it was demonstrated that in addition to the protein C mutation a mutation in the Cell Adhesion Molecule 1 gene may contribute to thrombosis risk.[19] It is plausible that additional genetic or environmental risk factors contribute to thrombosis risk in this family.

The aims of the present study were to assess hypofibrinolysis as risk factor for venous thrombosis in this protein C deficient kindred, determine the heritability of CLT, and perform a genome scan of CLT to identify genetic loci causing variation in CLT.

SUBJECTS AND METHODS

Participants and inclusion criteria

Kindred Vermont II and Expanded Kindred Vermont II have been described in detail previously.[18,19] Kindred Vermont II includes 7 generations of descendants of a couple born in the 1830s. Pedigree members were of French Canadian and Abenaki American Indian origin, living primarily in Vermont. Data of the most recent 5 generations have been collected between 1985 and 1988 and between 1994 and 2001. From 2002 to 2008, data of later generations and descendants of a sister of the wife of the original couple were collected. Expanded Kindred Vermont II includes an additional 8 pedigrees from Quebec, Canada, and New Hampshire. These pedigrees span 2 to 4 generations, with 2 to 26 members. All subjects were interviewed on history of venous thrombosis, general medical history, and risk factors for thrombosis. History of venous thrombosis was classified as verified when subjects were hospitalized and treated for venous thrombosis with an objectively diagnosed deep vein thrombosis or pulmonary embolism. All participants or their legal guardians gave informed consent.

Blood samples for the clot lysis measurement of a total of 403 pedigree members were available. Of the 403 members, 3 had missing data on age and 23 were taking oral anticoagulants (Coumadin derivatives) at the time of sampling and were excluded, leaving 377 pedigree members available for the analyses (100 protein C deficient and 277 protein C normal). For the analyses investigating increased CLT as risk factor for venous thrombosis, 31 subjects with unknown history of thrombosis or subclinical venous thrombosis were excluded (13 protein C deficient and 18 protein C normal). For the heritability and linkage analysis, 32 subjects with missing body mass index (BMI) measurement were excluded. As the year of blood draw was not always the same year as the year of BMI measurement, we also excluded 5 subjects of whom information on BMI was available from childhood and the blood draw was done in adulthood or vice versa. Furthermore, 9 subjects with missing genotype data were excluded. In total 10 protein C deficient members and 36 members with normal protein C levels were excluded from the heritability and linkage analyses leaving 331 subjects available for the analyses (90 protein C deficient and 241 protein C normal). This study was approved by the Human Experimentation Committees of the University of Vermont College of Medicine, Burlington (VT, USA).

Blood collection and laboratory analysis

Peripheral blood was collected into glass Vacutainer tubes containing 3.8% buffered

citrate solution (Beckton Dickinson, Franklin Lakes, NJ). Platelet poor plasma was produced within 1 hour by centrifugation at 3000*g* for 10 minutes at room temperature and stored at -70°C.

Lysis of a tissue factor-induced clot by exogenous t-PA was studied by monitoring changes in turbidity during clot formation and subsequent lysis as described previously.[82] In short, 50 µl plasma was pipetted in a 96-well microtitre plate. Subsequently, a 50 µl mixture containing phospholipid vesicles, t-PA, tissue factor, and $CaCl_2$ diluted in HEPES-buffered saline was added using a multichannel pipette. In a kinetic microplate the optical density at 405 nm was monitored every 20 seconds, resulting in a clot-lysis turbidity profile. The CLT was derived from this clot-lysis profile and defined as the time from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing the lysis of the clot. The inter-assay coefficient of variation was 6.0% and the intra-assay coefficient of variation was 3.0%.

The presence of the His107Pro protein C mutation (resulting from a 3363C insertion) was determined by amplifying target genomic DNA using, as one of the primers, a mutagenic oligonucleotide primer that, in concert with the inserted C mutation, creates a Bg1I cleavage site. The product was digested with Bg1I and analyzed on a 2% agarose gel. The prothrombin G20210A allele was also detected by amplification of genomic DNA with a mutagenic primer resulting in a HindIII cleavage site if the less frequent A allele was present.[226]

Individuals were genotyped for 375 autosomal markers by the NHLBI Mammalian Genotyping Service at the Marshfield Medical Research Foundation[227] using Screening Set version 10 as previously described.[228] Additional markers for fine mapping were genotyped using an ABI310 or 3100 (Applied Biosystems Inc, Foster City, CA, USA) at the Vermont Cancer Center DNA analysis facility, and selected markers were run by Decode Genetics Inc. (Reykjavik, Iceland). The probability of identity by descent (IBD) was estimated using the linkage analysis package Loki.[229] In addition, we genotyped four Single Nucleotide Polymorphisms (SNPs) in the TAFI gene (*CPB2*): -438G/A (rs2146881), i4 + 164 A/C (rs3818477), 505G/A (rs3742264 causing an Ala 147Thr variation), and 1040C/T (rs1926447 causing a Thr325Ile variation). The -438G/A and 1040C/T are generally located in the same haplotype, but are not in complete linkage disequilibrium.[191] The 505A/G is typical for a second haplotype. These three SNPs are associated with TAFI levels in the general population. A third haplotype is characterized by a fourth SNP in intron 4 (i4 + 164 A/C). This SNP is not associated with TAFI levels. An additional 8 SNPs for fine mapping on chromosome 11 were genotyped. Genotyping was done at the

Laboratory for Clinical Biochemistry Research, Genotyping Core Laboratory at the University of Vermont College of Medicine. TacqMan Assays by Design (Applied Biosystems Inc) was used for SNP genotyping under standard conditions. (TaqMan SNP Genotyping Asssays Protocol, Rev B, Part #4332856b, Applied Biosystems Inc). Manual scoring was used for end point fluorescence scoring using the SDS software (Applied Biosystems).

Statistical Analysis

To reduce skewness and kurtosis, we applied an inverse-normal transformation on CLT to obtain a normal distribution. We used likelihood analysis[230] to estimate the effect and to statistically test the effect, as an odds ratio, of a 1 standard deviation (sd) increase in CLT on risk of venous thrombosis. Likelihood analysis, as implemented in jPAP,[231] was used for all other analyses. Parameters were estimated as the values that maximized the likelihood. The 95% confidence interval (CI) around the odds ratio (OR) was computed using the standard error of the estimated logarithm of the OR, after adjusting for age and sex. The difference in CLT and in onset age between individuals with and without protein C deficiency was statistically tested taking family relations into account using a polygenic variance component method in SOLAR.[232] Heritability, the proportion of the phenotypic variance attributed to polygenes, and common household effect, the proportion of the variance attributed to environmental factors shared within a household, were estimated for CLT using the variance component method in SOLAR.[232] The distribution of CLT within the pedigree was assumed to be multivariate normal with the variance-covariance matrix given by: covariance (one person to another person) = $h^2K+c^2H+e^2I$, where K is derived from the kinship matrix, H from the household matrix, and I from the identity matrix. The h^2 (heritability, proportion of phenotypic variance attributable to additive genetic factors), c^2 (household effect or common environment) and e^2 (residual or environmental variance) were estimated by maximum likelihood, while simultaneously estimating the effect of protein C deficiency and of covariates that were previously found to be associated with CLT (age, sex, oral contraceptive use, BMI, and the prothrombin 20210A mutation[82]). Linkage between the autosomal markers or the selected SNPs for finemapping on chromosome 11 and quantitative levels of CLT was assessed using standard multipoint variance components linkage methods, implemented in SOLAR.[232] This method compares the likelihood of a model in which the variance resulting from a QTL is restricted to 0 (no linkage) to the likelihood of an unrestricted model, computing the log of odds (LOD) scores. A LOD score >2.0 was considered as suggestive linkage and a LOD score>3.3 was

considered as significant linkage. The 95% confidence interval (CI) of a locus was determined by the points on the curve defined by dropping the LOD score by one unit. Covariates used were age, sex, BMI, the prothrombin 20210A mutation, protein C deficiency, and the TAFI SNPs. To statistically test the effect of TAFI genotypes on heritability and linkage of CLT, we considered an additive genetic model in which the genotype was coded as 0, 1, or 2, depending on whether the subject was homozygous for the major allele (genotype=0), heterozygous (genotype=1), or homozygous for the minor allele (genotype=2). Analyses were performed in the overall family and members with normal protein C levels separately. The group of protein C deficient members was too small to allow separate analyses.

RESULTS

Mean age of the 377 family members at the time of the blood draw was 35 year (range 3-86 year). Forty-four % (n=164) were male and 27% (n=100) were carriers of the 3363C insertion in the protein C gene.

CLT and risk of venous thrombosis

Table 1 presents the pedigree members stratified according to protein C deficiency status and their history of venous thrombosis. There was a trend towards a higher age of onset of venous thrombosis in non-protein C deficient family members (mean onset age 47 years), compared with protein C deficient members (mean onset age 36 years) (p-value for difference in onset age=0.24). Furthermore, CLT was longer in pedigree members who did not carry the mutation than in carriers (median CLT respectively 75 minutes and 67 minutes in the event free individuals; p-value for difference in transformed CLT=0.01)

Table 1 *Characteristics of Kindred Vermont II.*

protein C deficiency	Venous thrombosis	N	mean age (years) (range)	mean onset age (years) (range)	median clot lysis time (minutes) (range)
no	no	255	32 (3-76)		75 (46-300)
no	yes	4	66 (40-77)	47 (27-70)	99 (70-150)
yes	no	78	37 (11-86)		67 (41-300)
yes	yes	9	49 (18-74)	36 (19-69)	87 (66-300)

Table 2 presents the risk of venous thrombosis associated with increased CLT in individuals with and without protein C deficiency. An increase of 1 sd of transformed CLT was associated with an increased risk of venous thrombosis in non-deficient family members (OR 2.6; 95%CI 1.6-4.2) as well as in deficient family members (OR 3.4; 95%CI 2.1-5.5).

Next, we analyzed the data a different way taking the family members with normal protein C levels and normal CLT as the reference group. ORs were calculated for 1 sd in CLT increase and no protein C deficiency, for protein C deficiency but no increase in CLT, and for the combination of increased CLT and protein C deficiency. While an increase of 1 sd in CLT in individuals with normal protein C levels increased risk of venous thrombosis 2.4-fold (95%CI 1.4-3.2) and protein C deficiency without elevated CLT increased risk 6.9-fold (95%CI 4.2-11.5), the combination of both risk factors increased risk of venous thrombosis 27.8-fold (95%CI 11.7-66.0).

Table 2 *Protein C deficiency, increased CLT and risk of venous thrombosis.*

Protein C deficiency	1 SD increase in CLT	OR (95% CI)	OR (95% CI)
no	no	1 (reference)	1 (reference)
no	yes	2.6 (1.6-4.2)*	2.4 (1.4-3.2)*
yes	no	1 (reference)	6.9 (4.2-11.5)*
yes	yes	3.4 (2.1-5.5)†	27.8 (11.7-66.0)*

CI, confidence interval; CLT clot lysis time; OR odds ratio; SD standard deviation.

** reference category: family members without protein C deficiency and no increased CLT.*

† reference category: protein C deficient family members with no increased CLT.

Heritability and household estimates of CLT

Table 3 shows the heritability and variance explained by covariates in the overall study and in the non-deficient family members using different models. Heritability ranged from 42-52%, indicating a significant contribution of additive genetic factors to variance in CLT. No household effect was present in either analysis. Excluding family members with a history of verified venous thrombosis yielded similar results (data not shown).

Linkage analysis

The results of the multipoint linkage analysis, over the 22 autosomal chromosomes for CLT levels, are shown in Table 4. In the overall study, two suggestive linkage

Table 3 *Proportion of phenotypic variance explained by covariates and heritability (h2).*

Model	N	Covariates*	h^2 (sd)†
Overall study			
Model 1	331	17%	43% (9%)
Model 2	331	19%	42% (9%)
protein C deficient members excluded			
Model 1	241	20%	52% (12%)
Model 2	241	23%	50% (12%)

**proportion of total variance.*
†heritability is the proportion of variance after adjustment for covariates.
Model 1: Protein C deficiency (only in the overall study), age at time of blood draw, sex, oral contraceptive use, and BMI.
Model 2: Model 1 + prothrombin 20210A mutation.

peaks were found. One suggestive linkage signal was found on chromosome 11 (LOD score 2.5) at 63 cM (markers located within the 95%CI of this linkage peak were D11S1993, D11S2365 D11S1985, D11S4459, D11S2363, D11S2006, and D11S2371). Further adjustment for the prothrombin 20210A mutation, which is located at 58 cM and added significantly to the model (p=0.048), reduced the LOD score to 1.8. Also on chromosome 13 a suggestive linkage peak was found (LOD score 2.0 at 52 cM; LOD score 2.2 after adjusting for the prothrombin 20210A mutation; markers located within the 95%CI of this linkage peak were D13S1807 and D13S800). As protein C deficient members of the family had shorter CLT than those with normal protein C levels, we excluded the individuals with protein C deficiency to obtain a more homogeneous group. These analyses yielded a LOD score of 2.4 at chromosome 11 at 65 cM (LOD score 1.7 at 58 cM after adjusting for the prothrombin 20210A mutation) and a LOD score of 2.3 at chromosome 13 at 52 cM (3.1 at 51 cM after adjusting for the prothrombin 20210A mutation). Also a significant linkage peak on chromosome 11 appeared at 18 cM (LOD score 3.2; markers located within the 95%CI of this linkage peak were D11S1999 and D11S1981). After adjusting for the prothrombin 20210A mutation this LOD score increased to 3.8.

As the linkage peak on chromosome 11 at 18 cM was rather narrow and therefore prone to being a false-positive finding as a result of genotyping errors, we genotyped 8 extra SNPs to this region (rs973108, rs4757993, rs4628658, rs6578886, rs4758249, rs10839963, rs7114039, rs10769903 all located between 12 and 14 cM on

Table 4 *Variance component linkage analysis results for locations on autosomal chromosomes showing a log of odds (LOD) score above 2.0 in the initial scan.*

Model	Chromosome	Location peak (95% CI)	LOD
Overall study			
model 1	11	17 (12-28) cM	1.8
model 2	11	17 (13-27) cM	2.0
protein C deficient members excluded			
model 1	11	18 (14-25) cM	3.2
model 2	11	17 (14-22) cM	3.8
model 2: after finemapping on chromosome 11	11	18 (15-31) cM	2.4
Overall study			
model 1	11	63 (57-73) cM	2.5
model 2	11	62 (56-74) cM	1.8
protein C deficient members excluded			
model 1	11	65 (54-79) cM	2.4
model 2	11	58 (52-85) cM	1.7
Overall study			
model 1	13	52 (46-62) cM	2.0
model 2	13	52 (45-61) cM	2.2
protein C deficient members excluded			
model 1	13	52 (47-59) cM	2.3
model 2	13	51 (47-55) cM	3.1

CI, confidence interval.
Model 1: Protein C deficiency (only in the overall study), age at time of blood draw, sex, oral contraceptive use, and BMI.
Model 2: Model 1 + prothrombin 20210A mutation.

the Marshfield map). We merged them with our other markers and recomputed the IBD probabilities. The addition of these SNPs to a genuine linkage peak is expected to increase the evidence of linkage. Including these SNPs, however, decreased the linkage peak to 2.4.

The TAFI gene is an obvious candidate gene located at 43 cM, near the peak at chromosome 13 (52 cM). We genotyped 4 polymorphisms in the TAFI gene, -438G/A, i4 + 164A/C, 505G/A, and 1040C/T. The genotype analyses revealed no significant association of any of these 4 SNPs with CLT and including these SNPs as covariates in the model did not change the LOD scores (data not shown). Table 5 shows the differences in CLT for the different genotypes of these TAFI SNPs and the prothrombin 20210A mutation.

Excluding the thrombosis cases from the analyses did not considerably change the results.

Table 5 *Median CLT in minutes for the prothrombin and TAFI genotypes.*

Genotype	protein C deficient		normal protein C	
	N	Median CLT	N	Median CLT
prothrombin				
20210GG	75	69.5	218	75.3
20210GA	15	73.7	24	87.3
TAFI				
1040CC	33	69.9	77	76.1
1040CT	40	70.9	117	76.8
1040TT	13	73.9	45	76.3
TAFI				
-438GG	33	70.8	88	75.3
-438GA	45	69.5	122	77.0
-438AA	9	77.3	31	77.4
TAFI				
505GG	43	73.3	137	76.6
505GA	38	70.8	93	76.2
505AA	6	66.8	10	82.6
TAFI				
i4 + 164AA	33	70.1	98	77.4
i4 + 164AC	45	69.5	106	76.2
i4 + 164CC	6	71.8	33	74.9

CLT, clot lysis time; TAFI, thrombin activatable fibrinolysis inhibitor.

DISCUSSION

In this large protein C deficient family, we showed that hypofibrinolysis was associated with an increased risk of venous thrombosis. The combination of a 1 sd increase in CLT and protein C deficiency increased risk of venous thrombosis 28-fold, compared with family members with normal protein C levels and no prolonged CLT. Furthermore, we showed that additive genetic effects or heritability accounted for approximately 40 to 50% of the variability in CLT after adjusting for age, sex, oral contraceptive use, and BMI. Multipoint linkage analysis revealed two loci, on chromosome 11 and 13, which influenced CLT. Part of the linkage on chromosome 11 could be explained by the prothrombin 20210A variant. SNPs in the TAFI gene located on chromosome 13 did not explain any of the variability in CLT.

In several population based case-control studies we have shown increased CLT to be associated with an increased risk of venous thrombosis and arterial thrombosis.[81,82,85,94] Also in this study we show hypofibrinolysis to be associated with an increased risk of venous thrombosis and a synergistic effect on thrombosis risk of the combination of hypofibrinolysis and protein C deficiency. Although the number of confirmed venous thrombosis cases was small, these results again provide evidence for an important role of hypofibrinolysis in thrombosis.

A remarkable finding was the much shorter CLT in the protein C deficient family members compared to those without the mutation. The difference in CLT was approximately 8 minutes. By way of comparison, the difference in median CLT between the group of patients with a first venous thrombosis and the control group in a large population based case-control study was 4 minutes.[82] Protein C has profibrinolytic properties as it decreases TAFI activation by decreasing thrombin formation. Protein C is transformed to its active form by thrombin-mediated cleavage. Effective activation of protein C by thrombin, however, requires the transmembrane glycoprotein, thrombomodulin as a cofactor, an event further amplified by the endothelial cell protein C receptor.[233] Therefore protein C will be minimally activated in this plasma-based clot lysis assay and no difference in CLT based on protein C status is expected. The association between protein C and CLT is probably explained by the association between protein C with other coagulation or fibrinolytic factors. Protein C has been found to be associated with other coagulation factors[176] and genes can influence plasma levels of interrelated coagulation factors.[234] Furthermore, we have studied plasma levels of fibrinolytic and coagulation markers as determinants of CLT in a healthy population and, although there was a positive association between protein C and CLT, this association disappeared when we

adjusted for other fibrinolytic and coagulation factors.[183]

This is the first time a QTL-analysis has been performed on CLT as measured with this assay. We show that the prothrombin 20210A mutation on chromosome 11 significantly explains part of the variation in CLT. This is in accordance with a study showing that this mutation increases CLT via increased TAFI activation[201] and with our previous work exploring determinants of CLT in which we show that increased prothrombin levels are associated with prolonged CLT.[183] Linkage did not drop to zero after adding the prothrombin 20210A mutation as covariate which means that this polymorphism does not explain all of the linkage signal in this chromosomal region. There is still unexplained variability in CLT at this locus, possibly by other mutations in the prothrombin gene or in other genes in this region.

In two QTL analyses on TAFI levels, the location on chromosome 13 where the TAFI gene is located (13q14.11) has been identified as a QTL for TAFI antigen and TAFI activity.[235,236] Although the TAFI gene was located just outside the 95% CI of the identified linkage peak on chromosome 13, this was an obvious candidate gene. We tested whether TAFI SNPs that have been previously shown to be associated with TAFI levels could explain this linkage peak, but these TAFI genotypes were not associated with CLT and did not show an effect on linkage.

Both peaks on chromosome 11 and chromosome 13 do not harbor any other structural genes encoding coagulation or fibrinolytic factors, or genes which might be otherwise related to clot lysis. The strongest linkage signal was found on chromosome 11 at 18 cM. After finemapping with 8 more SNPs, however, this peak was shown to be a false positive as the LOD score decreased from 3.8 to 2.4.

The heritability of the CLT was estimated at approximately 40 to 50% and no household effect was found. In a study including thrombophilic families, heritability of 26% and a household effect of 17% was reported for CLT as measured with the same assay.[225] Only age and sex were taken into account while the inclusion of BMI in our model may have decreased our estimated household effect. Heritability estimates, however, cannot be compared between different populations and should be evaluated in a qualitative manner,[237] so we can conclude that CLT is to a large extent genetically determined. Two other studies investigated genetic involvement of fibrin clot phenotypes or clot lysis. One family study investigated heritability of clot lysis profiles,[238] and one twin study assessed heritability and QTL of clot lysis parameters.[239] Direct comparison of both studies with the analyses presented in our current manuscript is difficult as the assay used to measure lysis time differed from these studies. We used a higher concentration of t-PA and added tissue factor to initiate coagulation, whereas the clot lysis assay used in the previous studies was

initiated by low-dose thrombin. Furthermore, only age, sex, and country of origin were included as covariates in the published QTL study as these were the most important vascular risk factors in that study. BMI, however, is a strong environmental determinant of fibrinolysis and taking this into account in the analyses may have influenced the results as it increases power.[240]

In conclusion, increased CLT is associated with an increased risk of venous thrombosis in a large protein C deficient pedigree, and the combination of protein C deficiency and prolonged CLT leads to high risk of thrombosis. Protein C deficiency was associated with decreased CLT. Heritability of CLT was approximately 40 to 50%. We found evidence suggestive of linkage on chromosome 11 at 62 cM, which could partly be explained by the prothrombin 20210A mutation located at 58 cM, and on chromosome 13 at 52 cM.

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Hypofibrinolysis as a risk factor for recurrent venous thrombosis; results of the LETS follow-up study

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Recurrence of venous thrombosis occurs in 12–28% of patients within 5 years after anticoagulation therapy is discontinued.[20,241] Prolongation of treatment could prevent these recurrences, but has the drawback of major hemorrhages, and therefore may only be beneficial in patients at high risk of recurrence.[242] Few factors are known that predict recurrence, apart from male sex and an idiopathic first thrombotic event. Specifically, thrombophilic defects, which are strongly associated with a first venous thrombosis, do not appear to be associated with recurrence risk, or only very weakly.[20] We have recently shown that hypofibrinolysis is a risk factor for a first venous and arterial thrombotic event, and hypothesized that hypofibrinolysis may also be associated with recurrence risk.[81,82,85] Data on fibrinolysis and recurrence risk are scarce. In a cohort study, no differences were found in mean euglobulin clot lysis time (ECLT), tissue-type plasminogen activator (t-PA) or plasminogen activator inhibitor-1 between patients who did and did not suffer from recurrent thrombosis.[25] In another prospective study, thrombin-activatable fibrinolysis inhibitor (TAFI) levels above the 75th percentile increased the risk of recurrence [relative risk (RR) 1.7; 95% confidence interval (CI) 1.1–2.7].[64] In the Leiden Thrombophilia Study (LETS), a population-based case–control study, both clot lysis time (CLT), measured by a global plasma-based assay, and TAFI levels above the 90th percentile were associated with a two-fold increased risk of a first venous thrombosis.[62,81] The aim of the current study was to investigate increased CLT, increased TAFI levels and single-nucleotide polymorphisms (SNPs) in the TAFI gene (–438G/A, 505G/A and 1040C/T) as risk factors for recurrent venous thrombosis in the LETS population.

The study population consisted of 474 consecutive patients below 70 years of age and without malignancies, diagnosed with a first deep venous thrombosis between January 1988 and December 1992.[20,243] At least 3 months after discontinuation of oral anticoagulant treatment for the first thrombotic event, blood samples were drawn and an interview on risk factors for venous thrombosis was performed. Forty-eight patients had an indication for prolonged anticoagulation, and in these patients a blood sample was taken while they were using oral vitamin K antagonists. Information about recurrent events and risk factors was collected by four repeated questionnaires and telephone interviews, as described elsewhere.[20] Initial and self-reported events were confirmed with information from discharge letters. Plasma CLT was determined with a tissue factor-induced and t-PA-induced clot lysis assay, as described previously.[81] TAFI antigen levels were determined using the Laurell method.[62] TAFI –438G/A (rs2146881), 505G/A (rs3742264) and 1040C/T (rs1926447) were determined using standard PCR conditions (available upon request) and digestion of the products with pyCH4IV, Fnu4HI and SpeI,

respectively.[189] Three patients had missing data on TAFI SNPs and five on CLT. Forty-eight patients using oral anticoagulants at the time of venipuncture were excluded from the analysis on CLT.

As cut-off points, we used percentiles (70, 80 and 90) of CLT and TAFI level measured in the control subjects of the LETS in accordance with previous reports.[62,81] A Cox proportional hazards model was used to calculate hazard ratios (HRs) and 95% CIs adjusted for age and sex. Follow-up started 90 days after the first event and ended at the date of first recurrence, death, emigration, or end of the study (1 January 2000), whichever occurred first. Time at risk was defined as the time between discontinuation of anticoagulation and end of follow-up.

The study population consisted of 272 women and 202 men. The mean follow-up time was 7.3 years (standard deviation 2.7). Complete follow-up was obtained for 447 patients (94%). Ninety patients (57 men and 33 women) suffered from a recurrent event. No association was found between CLT and risk of recurrence. TAFI levels were also not associated with recurrent venous thrombosis (Table 1). Additional analyses for CLT and TAFI, using the 50th percentile as cut-off point, using quartiles, including only those patients with a first idiopathic thrombosis, or using only idiopathic recurrences, yielded similar results. Also, further adjustment for body mass index or oral contraceptive use at time of blood draw did not influence the results.

The common –438GG genotype was associated with increased TAFI levels, as compared with the other variants. The HR (95% CI) for the –438AG genotype was 1.8 (1.2–2.8) and that for the –438AA genotype was 1.3 (0.5–3.2), as compared with the –438GG variant. The 1040C/T SNP was associated with TAFI levels and risk in a similar pattern, but HRs were somewhat lower. The rare 505A allele was associated with increased TAFI levels, relative to the 505G allele. The HR for the 505AA genotype as compared with the 505GG genotype was 0.8 (95% CI 0.3–1.9).

This is the first study to report on the association between this overall clot lysis assay and risk of recurrent venous thrombosis. One follow-up study on ECLT, including 303 patients of whom 41 suffered from a recurrence,[25] is in accordance with our results, as ECLT was similar in those with and without a recurrence. Our results for TAFI are unclear. We found no association between TAFI levels and recurrence. This is in contrast to the findings of another follow-up study including 600 patients with a first idiopathic thrombosis of the leg or pulmonary embolism, and 83 recurrences in which high TAFI levels were associated with an increased risk of a recurrent event.[64] An explanation for the discrepancy may be a difference in study population, as in the LETS both idiopathic and provoked thrombotic events of

Table 1 *Risk of recurrence of venous thrombosis according to clot lysis time and thrombin-activatable fibrinolysis inhibitor (TAFI) level.*

Cut-off percentile	Clot lysis time at cut-off (min)	Total number of patients	Number of events	HR (95% CI)
< 70	65.4	263	43	1 (reference)
> 70		158	28	1.2 (0.7–2.0)
< 80	68.7	302	52	1 (reference)
> 80		119	19	1.0 (0.6–1.7)
< 90	74.2	346	58	1 (reference)
> 90		75	13	1.1 (0.6–2.1)
Cut-off percentile	**TAFI levelt at cut-off (U dL^{-1})**	**Total number of patients**	**Number of events**	**HR (95% CI)**
< 70	113	312	59	1 (reference)
> 70		162	31	1.2 (0.7–1.8)
< 80	117	368	72	1 (reference)
> 80		106	18	0.9 (0.6–1.6)
< 90	122	408	79	1 (reference)
> 90		66	11	1.0 (0.5–1.9)
TAFI SNP	**Mean TAFI levels (5th–95th percentile) (U dL^{-1})**	**Total number of patients**	**Number of events**	**HR (95% CI)**
–438				
GG	110 (87–131)	240	34	1 (reference)
AG	105 (85–129)	202	51	1.8 (1.2–2.8)
AA	95 (76–113)	29	5	1.3 (0.5–3.2)
505				
GG	102 (83–126)	263	49	1 (reference)
AG	110 (88–132)	198	31	0.8 (0.5–1.2)
AA	115 (94–136)	37	6	0.8 (0.3–1.9)
1040				
CC	110 (89–131)	219	35	1 (reference)
CT	106 (85–132)	218	50	1.4 (0.9–2.2)
TT	97 (76–119)	34	5	0.9 (0.4–2.4)

CI, confidence interval; HR, hazard ratio; SNP, single-nucleotide polymorphism.
HR adjusted for age at blood draw and sex.

arm and leg were included, whereas patients with pulmonary embolism or active cancer were excluded. This difference in study population is also reflected by other results from the study by Eichinger *et al.*,[64] who also reported associations between levels of factor VIII, factor IX and factor XI and risk of recurrent venous thrombosis, whereas in the LETS these associations were not present.[20] Nevertheless, for both of the estimates for CLT and TAFI, CIs ranged from approximately 0.5 to 2.0, indicating that a small effect of these factors on recurrence risk cannot be excluded. Increased TAFI antigen levels were associated with an increased risk of a first venous thrombosis in the LETS, but the 505A allele, associated with higher TAFI levels, was associated with a decreased risk.[62,65] Surprisingly, we again find that an allele associated with decreased TAFI levels (–438A) increases the risk of recurrence, although patients homozygous for the –438A allele had a lower risk than those with the heterozygous genotype. Furthermore, if anything, there was a reduced HR for recurrence in carriers of the 505A allele, but CIs were wide. Whether these associations are real or represent a chance finding due to low patient numbers remains to be elucidated. To be noted is our recent finding that both low functional TAFI levels and the –438A allele were associated with an increased risk of myocardial infarction, which we ascribed to the anti-inflammatory properties of TAFI.[147,157] Whether inflammation also plays a role in first or recurrent venous thrombosis is uncertain.[244]

Variation in fibrinolytic capacity, as measured by CLT or TAFI antigen levels, does not appear to affect recurrence risk. The results for TAFI SNPs may indicate that low TAFI levels increase the risk of recurrence.

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Summary and general discussion

The aim of this thesis was to investigate the role of the fibrinolytic system in the development of both arterial thrombosis, in particular myocardial infarction, and venous thrombosis. In two case-control studies we investigated the association between overall hypofibrinolysis, or increased clot lysis time (CLT) as measured with an overall plasma based assay, and risk of a first myocardial infarction (chapter 4) and venous thrombosis (chapter 7). Furthermore, plasma levels of five fibrinolytic proteins, i.e., plasminogen, α2-antiplasmin, thrombin activatable fibrinolysis inhibitor (TAFI), tissue plasminogen activator (t-PA), and plasminogen activator inhibitor-1 (PAI-1), were studied in relation to myocardial infarction (chapter 5 and 6) and venous thrombosis (chapter 8). We also investigated these fibrinolytic proteins as determinants of CLT (chapter 8) and performed a quantitative trait loci (QTL) analysis in a large protein C deficient pedigree to investigate whether genetic variation affects CLT (chapter 9). Finally, increased CLT and TAFI levels as risk factor for recurrent venous thrombosis were studied in a cohort study (chapter 10). In the current chapter, we elaborate on our results. We describe what we have learned from these results about the etiology of both arterial and venous thrombosis, their clinical relevance, methodological considerations, remaining questions, and suggestions for further research.

MAIN FINDINGS PRESENTED IN THIS THESIS

In this thesis, we primarily describe the results of two case-control studies: the Study of Myocardial Infarctions LEiden (SMILE) and the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis study (MEGA study). We hypothesized that hypofibrinolysis as measured with an overall plasma based assay was associated with an increased risk of myocardial infarction and venous thrombosis and that CLT in the general population was determined by the combination of plasma levels of the different fibrinolytic factors. Furthermore, we hypothesized that levels of individual fibrinolytic factors, in particular the factors influencing the clot lysis assay, would also be associated with risk of arterial and venous thrombosis.

Although the results described in this thesis to a large extent corroborated with our original hypotheses (see Table 1), not all results led to straightforward conclusions. Results regarding myocardial infarction were partly similar to the results on venous thrombosis but also clear differences became apparent. Increased CLT was a risk factor for a first myocardial infarction and for a first venous thrombosis. In adjusted models, CLT remained associated with venous thrombosis, and with

myocardial infarction in men below 50 years. In men above 50 years of age the association disappeared. Except for elevated TAFI levels, which were associated with a decreased risk of myocardial infarction, each fibrinolytic protein was positively associated with myocardial infarction and venous thrombosis in age or age and sex adjusted models. In models adjusted for other potential confounders only α2-antiplasmin levels were positively associated with myocardial infarction. High TAFI levels remained associated with a decreased risk for myocardial infarction in these adjusted models. Elevated PAI-1 and TAFI levels were both associated with an increased risk of venous thrombosis after further adjustments. In the control group of the MEGA-study, we identified PAI-1 levels as the main determinant of CLT. Plasminogen, α2-antiplasmin, TAFI and prothrombin levels were also associated with CLT. Plasma levels of fibrinogen, and coagulation factors VII, X, and XI were minor determinants of CLT. Our results clearly indicate a difference between the role of the fibrinolytic system in myocardial infarction and its role in venous thrombosis and also emphasize the difference in etiology of the two diseases. As described in chapter 3, an explanation for the difference in results between venous and arterial thrombosis may be that the primary function of the fibrinolytic proteins in venous thrombosis is the dissolution of the fibrin clot, while in arterial thrombosis other roles of fibrinolytic proteins, such as in inflammation, plaque stabilization, and insulin resistance appear more important.

CLT and TAFI levels did not constitute risk factors for recurrent venous thrombosis, but TAFI genotypes associated with increased TAFI levels, if anything, suggested a decreased risk, which is contrary to expectations based on the role of TAFI in fibrinolysis and to the association found between high TAFI levels and an increased risk of a first venous thrombosis.

We showed that increased CLT is also a risk factor for a first venous thrombosis in a large kindred exhibiting type I protein C deficiency. Furthermore, protein C deficiency was associated with decreased CLT. By means of a QTL analysis two suggestive linkage peaks on chromosome 11 and 13 were identified that may point to genetic variation influencing CLT. No obvious candidate genes were located at or near these peaks

METHODOLOGICAL CONSIDERATIONS AND A DELIBERATION ON CAUSALITY

Confounding and causal inference

The fibrinolytic proteins have functions apart from their role in fibrinolysis (discussed in chapter 3). In addition, the fibrinolytic factors are influenced by a wide range of cardiovascular risk factors which should all be considered as possible confounders. In this paragraph some methodological issues concerning causal pathways and justification of adjustments for confounders performed in this thesis are addressed. We put emphasis on the association between PAI-1, plasminogen, and CLT and myocardial infarction and venous thrombosis.

PAI-1

In Figure 1 a simplified scheme of different pathways linking PAI-1 to thrombosis is shown. The majority of the evidence for these relationships is derived from animal and *in vitro* models (reviewed in [49,112,245,246]). Factors drawn left of PAI-1 with an arrow pointed to PAI-1 are putative confounding factors. These factors modulate PAI-1 levels and are themselves independent risk factors for myocardial infarction or venous thrombosis via other pathways than via PAI-1 as depicted by the direct arrow from these factors to myocardial infarction or venous thrombosis. Factors drawn right of PAI-1, with an arrow directed from PAI-1 to these factors and an arrow from these factors to myocardial infarction or venous thrombosis, indicate possible causal pathways by which PAI-1 could increase or decrease risk of myocardial infarction or venous thrombosis. As shown in the figure, several factors that influence PAI-1 levels are also factors that are themselves influenced by levels of PAI-1. Taking adipose tissue as an example: adipose (and in particular visceral fat) tissue is a major PAI-1 producing organ, but the role of PAI-1 in adipose tissue development remains controversial. PAI-1 deficient mice have been shown to either accelerate or inhibit growth of adipose tissue mass compared to their wild-type counterparts.[120,247,248] Reasons for these discrepancies are unclear and suggest that PAI-1 may stimulate or inhibit fat deposition depending on the model used.

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Table 1 *Main results of this thesis: associations between clot lysis time, individual fibrinolytic factors and venous and arterial thrombosis. Number in brackets is the chapter in this thesis in which results are presented.*

outcome	**myocardial infarction***		**first venous thrombosis***		**recurrent venous thrombosis***		**clot lysis time**
statistical model adjusted for	age	multi-variable	age and sex	multi-variable	age and sex	multi-variable	several fibrinolytic and coagu-lation factors
CLT	+ (4)	+ (4)§	+ (7)	+ (7)	= (10)	= (10)	
plasminogen	+ (5)	= (5)	+ (8)	= (8)	n.m.	n.m.	- (8)
α2-antiplasmin	+ (5)	+ (5)	+ (8)	= (8)	n.m.	n.m.	+ (8)
PAI-1	+ (5)	= (5)	+ (8)	+ (8)	n.m.	n.m.	+ (8)
t-PA	+ (5)	= (5)	+ (8)	= (8)	n.m.	n.m.	= (8)
TAFI	- (6)	- (6)	+ (8)	+ (8)	= (10)	= (10)	+ (8)
TAFI SNPs	-¶ (6)	n.a.	n.m.	n.m.	-¶ (10)	n.a.	= (11)

PAI-1, plasminogen activator inhibitor-1; SNP, single nucleotide polymorphism; t-PA, tissue plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor.

** logistic regression model.*

† linear regression model.

+ positive association; - negative association; = no association; n.a. not applicable; n.m. variable not measured in that study.

§ association only found in men younger than 50 years.

¶ TAFI genotypes associated with increased levels were associated with a decreased risk (1 out of 3 SNPs that were associated with levels was associated with myocardial infarction, 1 out of 3 SNPs was associated with recurrent venous thrombosis, the other two SNPs were not clearly associated but showed a trend).

Results may depend on the obesity model used, for example dietary induced obesity versus genetically determined obesity models (*ob/ob* mice) or, in the nutritionally induced models, on composition and timing of the diet or specific genetic background. By adjusting for body mass index (BMI), as surrogate marker for adipose tissue, the confounding effect of BMI will be taken into account but at the same time adjustments are potentially made for a factor that is in the causal pathway between PAI-1 and myocardial infarction or venous thrombosis. Therefore it is debatable whether adjustments for these factors are completely justified. As a result the risk estimates for increased PAI-1 levels on thrombosis in the fully adjusted models as presented in chapter 5 and 8 may be either under- or overestimated, depending on direction and strength of the associations.

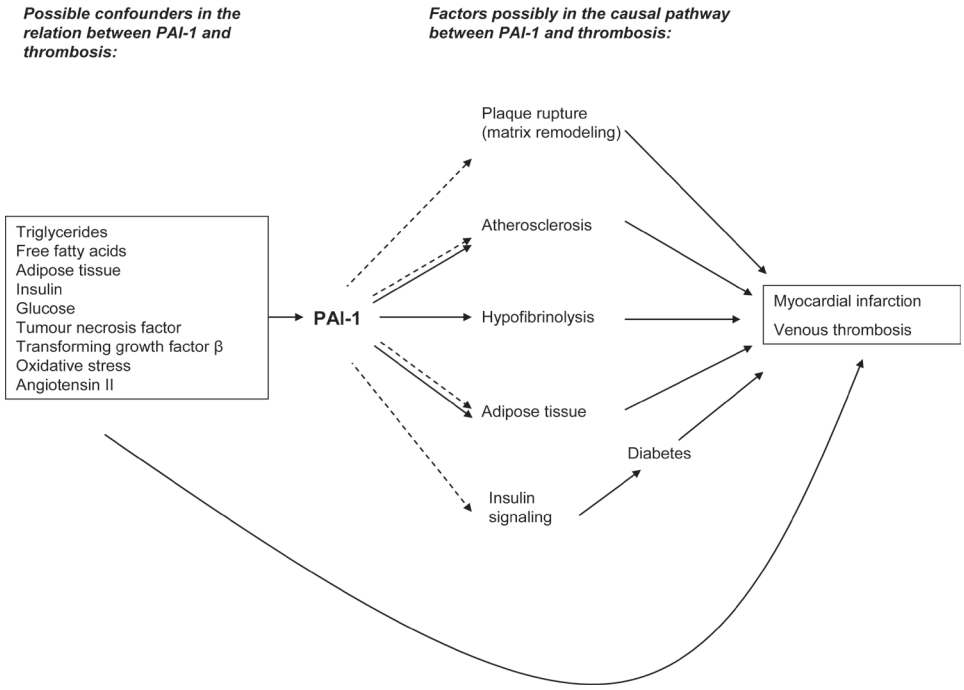

Figure 1 *Pathways linking PAI-1 with thrombosis.*
Solid arrows denote stimulation and activation, and dotted arrows denote inhibition.

Plasminogen

Plasmin is the proteolytic enzyme that dissolves the fibrin clot. Surprisingly, increased plasminogen levels were associated with an increased risk of myocardial

infarction and venous thrombosis although after adjustment for inflammatory markers this risk disappeared. The role of plasminogen in thrombosis risk has always been controversial. One question, however, remains: How could prolonged CLT, i.e., the time necessary to break down a fibrin clot in plasma by plasmin, increase risk of arterial and venous thrombosis, while there is no evidence that plasminogen levels are itself important in this risk? One can only speculate about this. Firstly, earlier studies investigating plasminogen deficient individuals have been small or did not include a control group so no firm conclusions can be drawn based on these studies.[21,23,249] Secondly, individuals with homozygous plasminogen deficiency do not completely lack plasminogen but usually still have approximately 10% of normal activity left which may be sufficient to prevent thrombosis.[23] Levels of plasminogen in healthy subjects are high and are usually not the limiting factor in clot lysis.[21] Therefore, although we show in chapter 8 that plasminogen levels are a determinant of CLT, the level of plasminogen that is activated may depend mostly on prior steps. In an *in vitro* model, plasminogen influenced CLT only when the level fell below 40%.[78] Another explanation is that, besides plasmin there are other proteins that can digest fibrin clots, such as leukocyte-derived cathepsins or elastase.[250] A second alternative concerns internalization and degradation of fibrin by monocytes, a process shown to be mediated by MAC-1.[251] Support for these alternative pathways of fibrin degradation comes from the observation that clot lysis in plasminogen knockout mice is not absent, but just delayed compared with wild-type mice.[252]

Figure 2 shows a simplified representation of the possible relation between plasminogen and thrombosis via different pathways. There is evidence that plasminogen levels are increased in response to inflammation,[111] implicating that inflammation is a confounder, but several roles of plasminogen have been described which may causally link plasminogen levels to thrombosis.[180,253] In the SMILE (chapter 5) the association between plasminogen and myocardial infarction disappeared after adjustment for smoking status providing no evidence for a causal role of plasminogen. In the MEGA-study, the risk of venous thrombosis in individuals with elevated levels of plasminogen did not change when adjustment was made for smoking (data not shown). Adjustment for acute phase proteins, however, did attenuate the risk. Again, all adjustments have to be interpreted with caution. We show in chapter 8 that plasminogen is associated with the majority of coagulation factors and with lipid levels. To our knowledge, the underlying mechanism for these associations has not yet been investigated. Therefore causes and consequences, and also confounding factors and factors in the causal pathway, cannot be discriminated

at this point (indicated in the graph with the bidirectional arrow). Although it cannot be excluded that plasminogen increases risk of thrombosis, it is possible that elevation of plasminogen levels is rather a consequence than a cause, a factor secondary to the disease or to other risk factors and, although predictive for thrombosis risk, not causally associated. We compared the strength of the association between all fibrinolytic factors (plasminogen, α2-antiplasmin, PAI-1, t-PA, and TAFI) and markers of inflammation (CRP in the SMILE, and factor VIII and fibrinogen in the MEGA-study). Indeed plasminogen is the protein most strongly associated with inflammation (data not shown) which may point to plasminogen levels as markers of underlying risk factors and more an acute phase protein than a risk factor by itself.

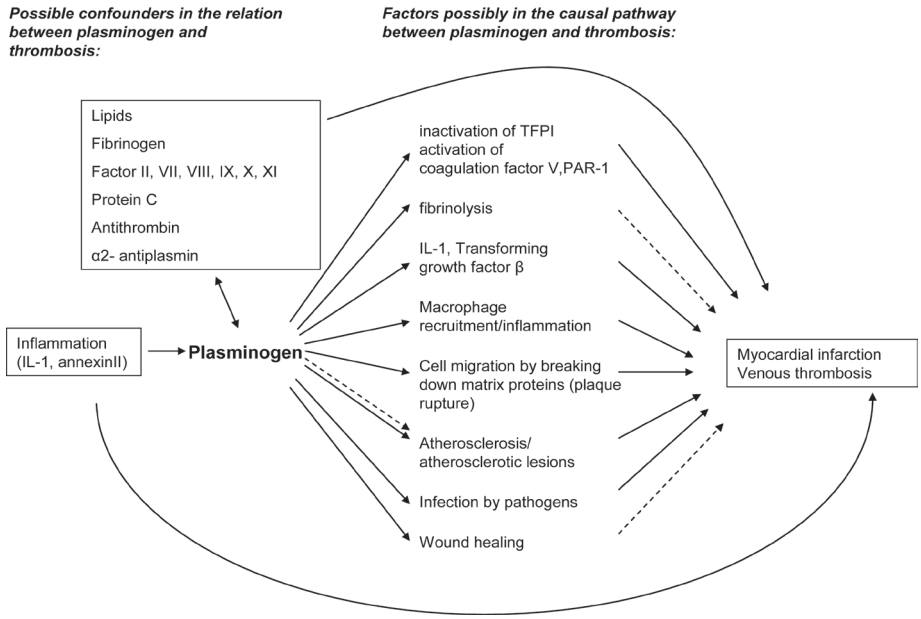

Figure 2 *Pathways linking plasminogen with thrombosis.*
Solid arrows denote stimulation and activation, and dotted arrows denote inhibition.

CLT

In Figure 3, CLT and its determinants, known and unknown confounders, risk of myocardial infarction and venous thrombosis and the relation between them is depicted. Seventy-seven percent of the variation in CLT is explained by plasma levels of PAI-1, plasminogen, α2-antiplasmin, TAFI, prothrombin, fibrinogen,

factor VII, factor X, and factor XI. In chapter 4 and 7 we show that increased CLT is associated with an increased risk of myocardial infarction and venous thrombosis. We hypothesized that the factors influencing CLT would affect the risk of thrombosis by their effect on CLT indicated by the arrow from the fibrinolytic proteins via CLT to myocardial infarction and venous thrombosis. For example, PAI-1 increases CLT and by increasing CLT PAI-1 increases risk of thrombosis. Of course, as described previously, the fibrinolytic proteins have pleiotropic effects so risk may also be affected via other pathways indicated by the arrow from the fibrinolytic proteins via the question mark to myocardial infarction and venous thrombosis. We suggest, based on the results described in chapter 6, that this is true for the association between TAFI levels and myocardial infarction: Elevated TAFI levels were associated with a decreased risk of myocardial infarction even though high TAFI levels increased CLT (chapter 8).

In chapter 8 we show that of the fibrinolytic factors influencing CLT, only PAI-1 and TAFI were associated with risk of venous thrombosis, after adjustment for several confounding factors. To investigate whether PAI-1 and TAFI increase risk of venous thrombosis by decreasing fibrinolytic potential (or by increasing CLT) we adjusted the risk associated with increased PAI-1 and TAFI levels for CLT. For this analysis individuals taking oral anticoagulants at time of blood draw were excluded. The odds ratio for the highest quartile of PAI-1 was 1.4 after adjusting for age, sex, and BMI compared with the lowest quartile. Further adjustment for CLT reduced the odds ratio to 1.1. For TAFI levels similar results were found, indeed suggesting that elevated PAI-1 and TAFI levels increase risk of venous thrombosis via increased CLT, of course assuming there is no bias and all confounding is taken into account.

In chapter 5 we describe that only α2-antiplasmin was associated with risk of myocardial infarction. In contrast to the previous analyses on PAI-1 and TAFI and the risk of venous thrombosis, the increased risk of myocardial infarction in individuals with elevated α2-antiplasmin levels did not attenuate after adjusting for CLT (data not shown). This may indicate that high levels of α2-antiplasmin increase risk of myocardial infarction via another pathway than via hypofibrinolysis. If α2-antiplasmin is involved in processes that are important in the etiology of myocardial infarction but not so much of venous thrombosis, this also explains why α2-antiplasmin is associated with myocardial infarction, but not with risk of venous thrombosis.

Seventy-seven percent of the variation in CLT could be explained, primarily by the levels of the fibrinolytic factors and prothrombin. As elucidated in chapter 8, the

remaining 23% of the variation may be the result of lack of fit of the statistical model which assumes linear relations between the fibrinolytic and coagulation factors and CLT. As the model includes plasma levels of the proteins, it also does not take into account the interplay between the factors. Furthermore, there are probably other proteins that influence CLT but are not included in the model (indicated in Figure 3 as the unknown factors). The age and sex adjusted risk of venous thrombosis was approximately 3-fold increased in individuals with CLT in the highest quartile compared with the first. Further adjustment for the determinants of CLT measured in our study (PAI-1, TAFI, α2-antiplasmin, plasminogen and prothrombin) only minimally changed this risk (chapter 8). This suggests that indeed CLT is not fully explained by these factors, and that additional proteins are involved. Theoretically the association between CLT and venous thrombosis can still be explained by unmeasured (or unknown) confounding factors. When unmeasured determinants of

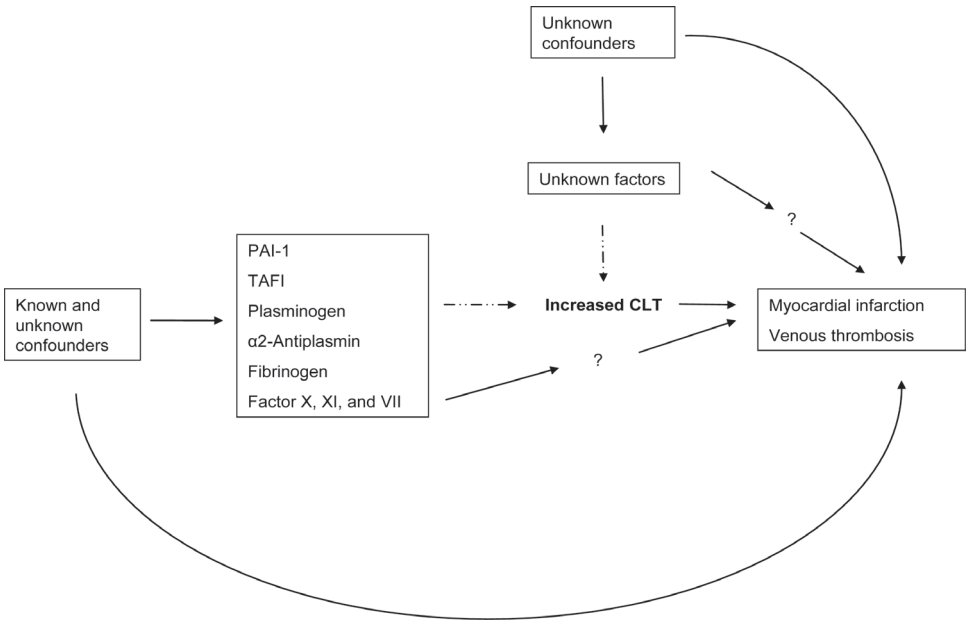

Figure 3 *CLT and its determinants, known and unknown confounders, risk of myocardial infarction and venous thrombosis and the association between them.*
Solid arrows denote an effect (either positive or negative). The boxes from which dotted arrows point towards 'Increased CLT' include the determinants of CLT. The question marks indicate effects of the determinants of CLT, other than on fibrinolysis, on processes that influence risk of thrombosis (e.g. inflammation).

CLT are influenced by a risk factor for venous thrombosis, i.e., a confounder, the risk of venous thrombosis associated with prolonged CLT is also confounded by this risk factor, as is depicted in Figure 3. However, this hypothetical unknown confounding factor would need to have extraordinarily strong associations with both venous thrombosis and CLT to increase an estimate from null (or no association) to a 3-fold increased risk. Previously, an illustrative paper was published exploring how strong an unmeasured confounder has to be to fully explain an observed finding.[254] For a confounding factor with a prevalence of 20%, the relative odds of both outcome and exposure would have to be 4- to 5-fold increased before a relative risk of 1.0 would be increased to 1.57. So to increase a relative risk from 1.0 to 3.0, these associations have to be even stronger. In our study, we believe that plausible confounding factors with such a high prevalence and high levels of associations are unlikely to be present.

Survival bias

The SMILE and MEGA study included individuals who survived a myocardial infarction or venous thrombosis at least 6 months or even several years. If either *hyper-* or *hypo*fibrinolysis would predominantly cause fatal myocardial infarction or venous thrombosis, this could have influenced the distribution of CLT in the patient groups of the SMILE or MEGA study and consequently influenced our results. When those with *hypo*fibrinolysis more often die because of a more massive infarction or venous clot than other patients with thrombosis, this would result in an underestimation of patients with hypofibrinolysis in our studies. Survival of venous thrombosis appears to be strongly influenced by external factors, such as age, malignancy, and patient location at onset (community, hospital or nursing home).[255] Indeed, in the MEGA-study approximately 75% of the eligible patients who died before they could enter the study died of cancer and not of the venous thrombosis itself. Survival of myocardial infarction appears to depend on patient delay in seeking medical care and delay in providing effective assistance determining the time between onset of infarction and start of intervention.[168] Infarct size, however, may also play a role.[167] If indeed infarct size has influenced survival, this could have resulted in an underestimation of hypofibrinolysis as risk factor for thrombosis.

Theoretically it is also possible that patients with *hyper*fibrinolysis who suffered from a deep vein thrombosis of the leg, had faster lysis of the clot and therefore an increased risk of embolizing of the clot to the lung compared with patients with hypofibrinolysis. As individuals have a higher risk of dying after suffering from a pulmonary embolism than after an isolated deep vein thrombosis of the leg, this would result in an underestimation of the number of patients with a short CLT

and therefore an overestimation of the risk of venous thrombosis associated with hypofibrinolysis. One might expect, however, to see a similar trend between CLT and venous thrombosis in those who did not die, i.e., a stronger association between CLT and deep vein thrombosis of the leg than between CLT and pulmonary embolism. When we split the analyses to subtypes of venous thrombosis, we do not find such a difference (chapter 7) so this type of survival bias seems unlikely.

Selection bias

One could argue whether those individuals who provided a blood sample in the MEGA study are a representative sample of all participants of the MEGA. Of the 3257 patients, 2420 patients provided a blood sample. Of the partner control group and the control subjects recruited using random digit dialing approximately 50% of the participants had their blood drawn. An individual may have several reasons for not being willing or not being able to come to the hospital for a blood draw. For example, sick persons may not be able to make a visit to the hospital. Also individuals with lack of time because of a fulltime job and thus those who are generally healthy may not want to provide a blood sample. Either way, there can be some sort of selection. When this selection differs for patients and control subjects and participation was also related to fibrinolytic variables, this could have influenced our risk estimates, e.g., when the unhealthy patients with long CLT and the healthy control subjects with short CLT provided a blood sample. There is, however, no reason to believe that the selection was different for patients and control subjects conditional on CLT.

CLINICAL RELEVANCE

CLT as clinical marker

Cardiovascular diseases are the main cause of morbidity and mortality in industrialized countries. Accurate prediction of cardiovascular risk is important for clinical decision making and the search for novel risk markers gains interest.[256] Although it has been suggested that it is feasible to determine CLT in a routine hemostasis laboratory[257] at this moment there is no ground to support such implementation.

To be useful as a clinical risk marker it has to meet several criteria. As stated by the American Heart Association,[258] a risk marker has to:

1. Differ between individuals with and without the disease
2. Predict development of future outcomes in prospective cohort or nested case-control studies

3. Add predictive information to established standard risk markers
4. Change predicted risk sufficiently to change recommended therapy
5. Improve clinical outcomes especially when tested in a randomized trial
6. Improve clinical outcomes sufficiently to justify additional costs of testing and treatment

At present, CLT has been shown to only satisfy the first criterion as CLT is associated with both arterial and venous thrombosis in case-control studies.

CLT as clinical marker for myocardial infarction

In the general population, the incidence of myocardial infarction is too low to make screening for hypofibrinolysis cost-effective. In clinical practice, a patient group who could benefit from a new clinical marker would consist of patients with an intermediate risk of thrombosis because of their medical condition and who are, as a group, just above or just below the threshold of prophylactic treatment. For CLT to be of any value it should discriminate in this intermediate risk group between those who will develop the disease and those who will not; consequently testing for hypofibrinolysis would be used to guide treatment. We have shown in a subset of men below 50 years of age that risk of myocardial infarction is approximately 2-fold increased for those with long CLT, when other risk factors are taken into account. Although we have not specifically tested whether CLT adds any predictive value to established standard risk markers, i.e. age, smoking, blood pressure, total cholesterol, HDL-cholesterol, and diabetes (the markers used to calculate the Framingham risk score), it does not seem very likely that it will. Previously, in the Framingham Heart Study, the usefulness of 10 biomarkers from distinct biologic pathways for predicting cardiovascular risk has been evaluated. In addition to the standard risk factors the inclusion of multiple biomarkers, even though each was individually associated with cardiovascular events, only added minimally to overall prediction of risk.[259] One of the reasons for a marker to discriminate poorly even when relative differences between those who did and those who did not suffer from an event are present, is that the distribution of the marker within the patient group overlaps with the distribution of the marker within the control group, which is true for CLT. Also markers that correlate with other factors in the model may not provide a substantial increase in predictive value.[260] Except for smoking, we found CLT to associate with all factors of the Framingham risk score suggesting that indeed CLT would not enhance the amount information obtained from traditional indicators.

CLT as clinical marker for a first venous thrombosis

As described in chapter 7, the risk of venous thrombosis in the general population

is approximately two-fold increased in those with hypofibrinolysis. Similar to myocardial infarction, however, the incidence of venous thrombosis is too low to support screening for hypofibrinolysis in the general population. Furthermore, there is no evidence yet that for example prophylactic treatment with anticoagulants for individuals with hypofibrinolysis would prevent a first venous thrombosis.

The combination of hypofibrinolysis with oral contraceptives resulted in a twenty-fold increased risk. One could question whether it is worthwhile to keep women with hypofibrinolysis off oral contraceptives. With an incidence of venous thrombosis in young women of ~2 per 10,000 women in the age between 14 and 49 years, screening for hypofibrinolysis in young women starting oral contraceptives is, however, also far from advisable, considering the tens of thousands of women who have to be denied oral contraceptives to prevent one death. Nevertheless, it may be possible that there are high risk groups in which screening for hypofibrinolysis is cost-effective. The incidence of venous thrombosis in patients with plaster cast or braces or patients with knee surgery for example is approximately 5% in the absence of prophylaxis.[261,262] We show that in those with hypofibrinolysis in combination with immobilization (plaster cast, surgery, and confinement to bed), risk of venous thrombosis is ~10-fold increased indicating that hypofibrinolysis may have some discriminating value and testing for it may guide prophylactic treatment with anticoagulants in this group of patients. Further research, however, is needed to explore increased CLT as possible predictive marker for venous thrombosis in high risk populations.

CLT as clinical marker for a recurrent venous thrombosis

Recurrence of venous thrombosis occurs in 12-28% of patients within five years after anticoagulation therapy is discontinued.[20,241] Few factors are known to predict recurrence, apart from male sex and an idiopathic first thrombotic event. Identification of those individuals with an increased risk of recurrent venous thrombosis may result in better treatment and prevention of these recurrences. In chapter 10 we show that elevated CLT is not associated with risk of recurrent venous thrombosis suggesting that screening for hypofibrinolysis in patients with a first venous thrombosis would be of no value in the prevention of a recurrence.

Therapeutics

Thrombolytic therapy is used in a variety of thrombotic disorders such as myocardial infarction and acute ischemic stroke although for venous thrombosis the benefit of thrombolytic therapy is not yet demonstrated.[263] The most commonly used drug

for thrombolytic therapy is t-PA. Current research is exploring the possibilities of other profibrinolytic therapeutics, i.e. TAFIa inhibitors or PAI-1 inhibitors. The main drawback of currently used thrombolytics and anticoagulants is the high risk of major bleeding. Both TAFI deficient and PAI-1 deficient mice, and also PAI-1 deficient patients have only a mild phenotype and do not display spontaneous bleeding.[10,264,265] Therefore, TAFIa and PAI-1 may be promising antithrombotic drugs. It was found that the efficacy of thrombolytic therapy was decreased in stroke patients with high TAFIa levels compared with patients with lower TAFIa levels.[266] Furthermore, inhibition of TAFIa has been shown to promote thrombolysis in animal models[187] as well as to inhibit fibrin deposition in sepsis models.[267] A phase II trial has been performed investigating a TAFIa inhibitor in addition to low molecular weight heparin in the acute phase of pulmonary embolism.[268] The manufacturer has, however, stopped the development of this drug for unknown reasons. Also PAI-1 inhibitors had favorable effects on clot lysis in animal models.[269,270] Taking in mind the pleiotropic effects of PAI-1 and TAFI, however, the inhibition of PAI-1 and TAFIa may not only be beneficial. By inhibiting these proteins, not only fibrinolytic capacity may be increased but also the favorable properties of PAI-1 and TAFI may be inhibited. Our finding described in chapter 6 that low TAFI levels are associated with an increased risk of myocardial infarction also suggests that inhibiting of TAFIa could have unintentional side effects. Another potential limitation of some of the TAFIa inhibitors is the paradoxical enhancement of TAFIa activity at low doses, which could hamper optimal dosing.[271]

FUTURE STUDIES

Fibrinolytic system and atherosclerotic plaque stability

Epidemiological studies on the fibrinolytic system and arterial thrombosis have yielded inconsistent results. As described in chapter 3, a reason for this could be the pleiotropic effects of the fibrinolytic proteins. The fibrinolytic system plays a role in the degradation of the extracellular matrix and could therefore contribute to the stability of atherosclerotic plaques. Activators of the fibrinolytic system may therefore increase fibrinolytic capacity, which is beneficial but may also have harmful effects by increasing proteolysis of the extracellular matrix and subsequent plaque rupture through activation of matrix metalloproteinases. Conversely, elevated levels of inhibitors of fibrinolysis could lead to diminished clot breakdown, but also to stabilization of the plaque. We show in chapter 4 that decreased overall fibrinolytic

potential was associated with an increased risk of a first myocardial infarction, but only in the subset of men below 50 years of age. These results do not necessarily mean that in the older population the fibrinolytic system is not important in the risk of myocardial infarction. While at younger age the role of the fibrinolytic system in clot lysis may be most important in the etiology of myocardial infarction, the roles of the fibrinolytic proteins in other processes like plaque stability may become more important with age, as the prevalence of atherosclerosis strongly increases with age. Theoretically beneficial and harmful effects of a protein could level out and the net effect of the fibrinolytic factors may differ depending on the state of atherogenesis. Within and between study populations, participants are probably in different stages of plaque formation. While in those without atherosclerosis the role in clot lysis of the fibrinolytic proteins might be most important, the properties in plaque stability may gain importance with the formation of atheromatous plaques, but this has never been thoroughly investigated. Future studies may provide more insight in these processes when taking a measure of atherosclerosis into account, for example, the intima-media thickness.

TAFI and atherosclerosis

In chapter 6 we show that elevated TAFI levels are associated with a decreased risk of myocardial infarction, which was the opposite to what was expected based on the inhibitory role of TAFI in fibrinolysis. We hypothesized that TAFI decreases the inflammatory response and therefore is protective against the formation of atherosclerosis. Although several groups have developed TAFI knock-out mice to study the effect of TAFI on different processes,[264] to our knowledge the effect of TAFI deficiency on development of atherosclerosis has never been studied. As $ApoE^{-/-}$ mice are prone to atherosclerosis, the comparison of $ApoE^{-/-}/TAFI^{-/-}$ with $ApoE^{-/-}/TAFI^{+/+}$ mice may elucidate whether indeed TAFI protects against the formation of atherosclerotic lesions.

Circulating t-PA versus releasable t-PA activity upon stimulation

The results of the association between plasminogen and t-PA levels were similar in relation to myocardial infarction and venous thrombosis. Increased t-PA antigen level appears rather a reflection of underlying risk factors such as inflammation and endothelial activation than a risk factor for arterial and venous thrombosis by itself. In healthy individuals, PAI-1 is present in fivefold excess over t-PA so most of the t-PA circulates in an inactive form as t-PA-PAI-1 complex. t-PA antigen levels may therefore not represent fibrinolytic capacity. t-PA is excreted from the endothelial

cells upon stimulation. The ability of the system to respond to triggers may therefore better represent the fibrinolytic potential of an individual. A few small studies have investigated the association between t-PA release after venous occlusion in relation to arterial and venous thrombosis with inconclusive results.[80,272] Obviously this method is rather invasive and therefore not appealing to perform on a large scale but it is possibly biologically the most relevant.

Determinants of CLT

In chapter 8 we show that 77% of the variation in CLT could be explained by plasma levels of PAI-1, TAFI, plasminogen, α2-antiplasmin, prothrombin, and to a lesser extent by levels of fibrinogen and coagulation factors VII, X and XI. In chapter 9 a search for genetic variation influencing CLT is performed by means of a linkage analysis. Two suggestive linkage peaks were found on chromosome 11 and 13, indicating that genetic variation at these loci may influence CLT. None of the genes coding for the proteins identified as determinants of CLT are located at these positions. These locations may contain a gene encoding a protein influencing levels of these factors or a protein that itself directly influences CLT. No obvious candidate genes are located at these positions but further investigation and fine-mapping of this region could result in discovery of novel genes and proteins involved in regulation of fibrinolysis.

Flow model

Veins have a slower flow rate than arteries. Explanations for the dissimilarity in results for the fibrinolytic proteins and risk of arterial and venous thrombosis may therefore not only be caused by the pleiotropic effects of the fibrinolytic proteins. The fibrinolytic proteins may differ in activity under a different flow rate. With *in vitro* flow models using preformed fibrin clots the effect of purified fibrinolytic proteins under different flow rates could be studied.

CONCLUSIONS

In this thesis we show that prolonged CLT, as measured with an overall plasma based assay, is associated with an increased risk of myocardial infarction and first venous thrombosis. This association has been consistently found by us and others in several independent case-control studies. The role of the individual fibrinolytic proteins in arterial and venous thrombosis is, however, less clear possibly in part

due to pleiotropic effects of these factors. Nevertheless, we have provided evidence for a role of TAFI and PAI-1 in venous thrombosis, and for TAFI and α2-antiplasmin in arterial disease. We have given more insight into several aspects of the fibrinolytic system in relation to thrombotic disease and hope that this thesis provides valuable information that will be used in future research.

11

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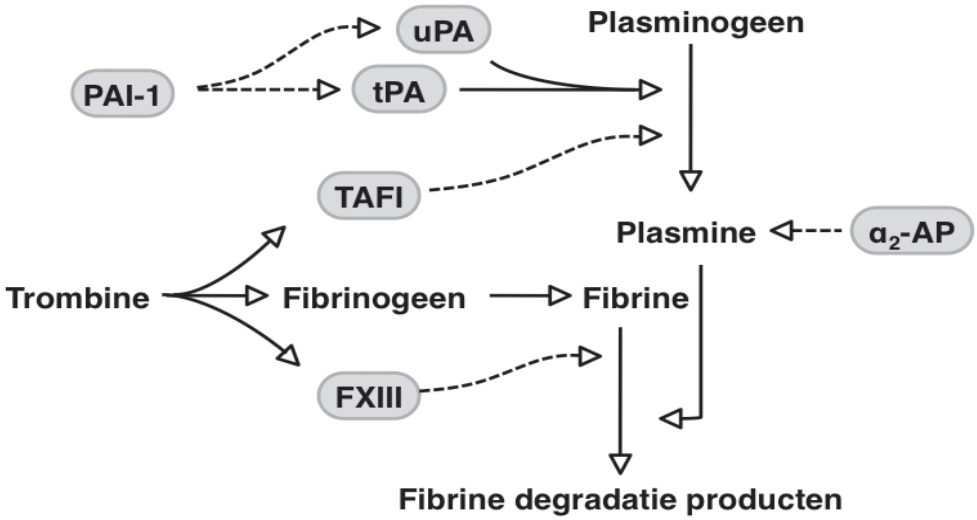
Nederlandse samenvatting

ACHTERGROND

Veneuze en arteriële trombose zijn twee van de belangrijkste oorzaken van ziekte en sterfte in Westerse landen. Met behulp van epidemiologische studies zijn er vele risicofactoren voor hartinfarct en veneuze trombose geïdentificeerd. De etiologie van een hartinfarct en van veneuze trombose is deels gelijk, maar er zijn ook duidelijke verschillen. Bij beide ziekten is er een bloedvat verstopt met een bloedstolsel. Bij veneuze trombose wordt er een fibrinerijk stolsel in de venen gevormd. De afsluiting van een coronaire arterie met een bloedplaatjesrijke trombus gebeurt meestal na de ruptuur van een atherosclerotische plaque. Trombus formatie is een relatief acuut proces terwijl de ontwikkeling van atherosclerose een chronisch proces is.

Hemostase is het complexe proces dat er voor zorgt dat een bloeding uit een beschadigd bloedvat stopt. Een belangrijk onderdeel hierin is het vormen van een bloedstolsel. De laatste stap in de stollingscascade is het knippen van fibrinogeen door trombine tot fibrine monomeren die polymeriseren tot een fibrinestolsel.



Figuur 1 *Een vereenvoudigd model van het fibrinolytisch system.*
Pijlen met een doorgetrokken lijn geven activering aan en stippellijnen geven remming aan.
PAI-1 plasminogeen activator inhibitor; u-PA urokinase plasminogeen activator; t-PA tissue-type plasminogeen activator; TAFI thrombin activatable fibrinolysis inhibitor; α2-ap α2-antiplasmine.

Het fibrinolytisch systeem zorgt voor het afbreken van het fibrinestolsel als het beschadigde bloedvat weer gerepareerd is. Een schema van dit systeem is getekend in figuur 1. De primaire activator van plasminogeen in plasma is tissue plasminogeen activator (t-PA). t-PA komt na stimulatie vrij vanuit de endotheelcellen in het plasma. In de circulatie is t-PA voornamelijk inactief doordat het snel een complex vormt met de belangrijkste inactivator, plasminogeen activator inhibitor type 1 (PAI-1). t-PA knipt plasminogeen tot het actieve plasmine. Plasmine is het enzym dat het fibrine stolsel oplost. Het wordt geïnactiveerd door α2-antiplasmine. Thrombin activatable fibrinolysis inhibitor (TAFI) wordt geactiveerd door trombine en voorkomt de activering van plasminogeen door t-PA waardoor de afbraak van het fibrine stolsel geremd wordt.

Afwijkingen in het stollingssysteem kunnen leiden tot trombose. Een verhoogde stollingsneiging verhoogt het risico op veneuze trombose en, hoewel in mindere mate, ook op hartinfarct. Daarom is het plausibel dat een verlaagde fibrinolytische capaciteit, ook wel hypofibrinolyse genoemd, het risico op veneuze en arteriële trombose ook verhoogt. Studies waarin de associatie onderzocht werd tussen het fibrinolytisch systeem en het risico op trombose zijn echter schaars en gaven inconsistente resultaten.

DIT PROEFSCHRIFT - RESULTATEN

Het doel van het onderzoek beschreven in dit proefschrift is het verkrijgen van een beter inzicht in de rol van het fibrinolytisch systeem in het ontstaan van hartinfarct en veneuze trombose.

Hoofdstuk 2 en 3 beschrijven de literatuur op het gebied van fibrinolyse en arteriële en veneuze trombose. Hypofibrinolyse zoals gemeten met een overall test die in een plasmamonster de tijd meet die nodig is om een stolsel af te breken (de zogenaamde ‘clot lysis tijd’ (CLT)) blijkt in verschillende studies consequent met het risico op veneuze trombose geassocieerd te zijn. Ook een verhoogde TAFI concentratie in het bloed is geassocieerd met een verhoogd risico op veneuze trombose. Er is weinig bewijs voor een rol voor plasminogeen, α2-antiplasmine, t-PA of PAI-1 in veneuze trombose. Een verlengde CLT is ook geassocieerd met een verhoogd risico op arteriële trombose maar studies naar de individuele factoren gaven inconsistente resultaten. De fibrinolytische factoren hebben tevens functies buiten de fibrinolyse,

zoals functies in ontsteking, atherosclerose en insuline resistentie. Een voorbeeld hiervan is TAFI dat de fibrinolyse remt, wat dus het trombose risico zou kunnen verhogen, maar ook de ontstekingsreactie tegengaat wat weer goed zou zijn voor het voorkomen van hartinfarcten. Deze tegengestelde effecten op arteriële en veneuze trombose zouden de oorzaak kunnen zijn van de tegenstrijdige resultaten van de verschillende epidemiologische studies.

Hoofdstuk 4, 5, en 6 beschrijven de resultaten van de Study of Myocardial Infarctions LEiden (SMILE). De SMILE is een patiënt-controle onderzoek waarin 560 mannelijke patiënten onder de 70 jaar zijn geïncludeerd die een eerste hartinfarct hebben gehad tussen januari 1990 en januari 1996 en hiervoor opgenomen waren in een ziekenhuis in Leiden. De controle groep bestond uit 646 mannen die een kleine orthopedische ingreep hebben ondergaan tussen januari 1990 en mei 1996 en hiervoor kortdurend antistollingsmedicatie moesten gebruiken. Ze werden geïdentificeerd in de medische statussen van de trombosedienst Leiden.
In **hoofdstuk 4** hebben we gekeken naar hypofibrinolyse als risico factor voor een eerste hartinfarct. Dit was de eerste studie waarin gekeken werd of de CLT gemeten met de overall fibrinolyse test in plasma geassocieerd was met hartinfarct. Wij vonden dat inderdaad het risico verhoogd was in de mensen met de langste CLT. Echter, dit was alleen zo in de mannen onder de 50 jaar. In de mannen boven de 50 jaar vonden we deze associatie niet. **Hoofdstuk 5** beschrijft de associatie tussen plasma concentraties van plasminogeen, α2-antiplasmine, t-PA en PAI-1 en het risico op een eerste hartinfarct. Verhoogde concentraties van al deze 4 factoren waren geassocieerd met een verhoogd risico op hartinfarct. Echter, plasminogeen, t-PA en PAI-1 bleken meer een weerspiegeling te zijn van andere risicofactoren zoals ontsteking en endotheelschade dan dat ze zelf het risico verhoogden. Alleen α2-antiplasmine was geassocieerd met hartinfarct ook nadat er gecorrigeerd was voor andere risicofactoren. In **hoofdstuk 6** beschrijven we de relatie tussen plasma spiegels van TAFI en het risico op hartinfarct. Wij vonden dat mannen met een lage TAFI concentratie in plasma een hoger risico hadden op een hartinfarct dan mensen met een hoge TAFI concentratie. Dit resultaat was onverwacht gezien de remmende werking van TAFI op de fibrinolyse. Echter, TAFI heeft ook een anti-inflammatoire werking die wellicht beschermt tegen het ontstaan van atherosclerose en dus beschermt tegen het krijgen van een hartinfarct. Het zou ook kunnen dat de lage TAFI concentratie niet het risico op een hartinfarct zelf verhoogt, maar dat lage TAFI spiegels een gevolg zijn van de ziekte of van andere risicofactoren. TAFI spiegels worden voor een groot deel bepaald door genetische variatie in het

TAFI gen. Daarom hebben we 3 'single nucleotide polymorfismen' (SNPs), d.w.z. 3 verschillende varianten in het TAFI gen die geassocieerd zijn met TAFI spiegels bepaald in de studiepopulatie. Als SNPs die geassocieerd zijn met verlaagde levels ook een verhoogd risico geven op een hartinfarct zou dit extra bewijs zijn voor een causale rol van TAFI omdat genen de TAFI concentratie kunnen beïnvloeden en zo de ziekte zouden kunnen beïnvloeden, maar de ziekte kan niet de genen beïnvloeden. Eén van de drie varianten was inderdaad ook met het krijgen van een hartinfarct geassocieerd, maar de twee anderen niet. Concluderend kunnen we zeggen dat lage TAFI spiegels geassocieerd zijn met een verhoogd risico op een eerste hartinfarct, wellicht door het verlagen van de ontstekingsreactie. De associatie met TAFI SNPs is inconsistent.

In hoofdstuk 7 en 8 worden de resultaten beschreven van de 'Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA studie)'. De MEGA studie is een groot patiënt-controle onderzoek waarin alle opeenvolgende patiënten tussen de 18 en 70 jaar met een eerste veneuze trombose tussen 1999 en 2004 uit 6 verschillende trombosediensten in de Randstad werden geïncludeerd. Controles bestonden uit partners van de patiënten terwijl een tweede controlegroep willekeurig was gekozen uit de algemene populatie. **Hoofdstuk 7** beschrijft de relatie tussen CLT en het risico op veneuze trombose in 2090 patiënten en 2564 controle personen. We vinden dat hoe langer de CLT, hoe hoger het risico op veneuze trombose is. Bovendien hebben we gekeken of de combinatie van hypofibrinolyse en risicofactoren die geassocieerd zijn met een verhoogde stollingsneiging leidt tot nog een extra verhoogd risico, dus een risico dat meer is dan de som van de individuele risico's. Dit was inderdaad het geval. Vooral de combinatie van hypofibrinolyse en het gebruik van de anticonceptiepil gaf een hoog risico. Vrouwen die niet aan de pil waren maar wel hypofibrinolyse hadden, hadden een 2x verhoogd risico op veneuze trombose en de pil in mensen met korte CLT gaf een 2,6x verhoogd risico. De combinatie van hypofibrinolyse en pil verhoogde het risico op veneuze trombose meer dan twintig maal (vergeleken met vrouwen met korte CLT en zonder anticonceptiepil). Aangezien niet bekend is welke van de individuele factoren de lengte van CLT bepalen en daarmee het risico op veneuze trombose hebben we in **hoofdstuk 8** in een random subgroep van de controles van de MEGA studie determinanten van CLT bepaald en in een willekeurig gekozen subgroep van patiënten en controles de associatie tussen plasminogeen, α2-antiplasmine, t-PA, PAI-1 en TAFI en het risico op veneuze trombose. De belangrijkste determinant van CLT was de concentratie van PAI-1, gevolgd door spiegels van plasminogeen,

TAFI, protrombine, en α2-antiplasmine. Fibrinogeen en de stollingsfactoren VII, X, en XI droegen minimaal bij. In totaal kon met deze factoren 77% van de variatie in CLT verklaard worden. Verhoogde spiegels van plasminogeen, α2-antiplasmine, t-PA, PAI-1 en TAFI waren alle vijf geassocieerd met een verhoogd risico op veneuze trombose. Echter, na correctie voor andere risicofactoren bleken alleen TAFI en PAI-1 nog geassocieerd met trombose risico. De concentratie van plasminogeen lijkt een reflectie te zijn van de inflammatoire status, de concentratie van α2-antiplasmine voornamelijk van lipidenspiegels en de concentratie van t-PA van ontsteking en endotheelschade.

In **hoofdstuk 9** zijn we op zoek gegaan naar genetische variatie die de lengte van CLT beïnvloedt. Dit hebben we gedaan in Vermont Kindred II, een grote familie met een verhoogd risico op veneuze trombose dat gedeeltelijk verklaard wordt doordat een deel van de familieleden een deficiëntie hebben van proteïne C; een belangrijk enzym dat de stolling tegen gaat. Door in het genoom van elk individu op verschillende plaatsen een marker ('vlaggetje') te meten, kan bijvoorbeeld worden bepaald of bepaalde markers bij mensen met een lange CLT horen. Met deze methode vonden we aanwijzingen dat genetische variatie op chromosoom 11 en chromosoom 13 een deel van de variatie in CLT kon verklaren. Aangezien het TAFI gen op chromosoom 13 ligt hebben we gekeken of die variatie verklaard kon worden door TAFI SNPs die geassocieerd zijn met TAFI spiegels, maar dit was niet het geval. Een deel van de variatie in CLT kon verklaard worden door een genetische mutatie in het protrombine gen dat gelegen is op chromosoom 11. Ook hebben we in deze familie gekeken of CLT geassocieerd is met tromboserisico. Dit is inderdaad het geval en er is bovendien synergie tussen hypofibrinolyse en proteïne C deficiëntie welke samen tot een 28x verhoogd risico op veneuze trombose leidde.

De Leiden Thrombophilia Study (LETS) is een patiënt-controle onderzoek met 474 patiënten met een eerste veneuze trombose en 474 controle personen. Dit was de eerste studie waarin was gevonden dat een verlengde CLT het risico op veneuze trombose verhoogt. Bovendien was in de LETS aangetoond dat ook een verhoogde concentratie van TAFI geassocieerd was met het risico op trombose. Ongeveer 20% van de patiënten krijgt een tweede trombose binnen vijf jaar na de eerste. Er zijn nog maar weinig risicofactoren voor een tweede trombose bekend, terwijl het identificeren van patiënten met een hoog risico hierop de preventie van een tweede trombose zou kunnen verbeteren. In de vervolgstudie van de LETS zijn alle patiënten gemiddeld 7.3 jaar gevolgd om te bepalen of ze een tweede trombose

kregen en om risicofactoren hiervoor te onderzoeken. **Hoofdstuk 10** beschrijft de associatie tussen CLT, TAFI en TAFI SNPs en het risico op een tweede trombose. Wij vinden echter geen relatie tussen de lengte van CLT of TAFI spiegels en het risico op een tweede trombose. Onze data suggereren echter dat wellicht TAFI SNPs die geassocieerd zijn met verlaagde TAFI spiegels een verhoogd risico op een tweede trombose geven.

CONCLUSIE

In dit proefschrift tonen we aan dat een verlengde CLT, zoals gemeten in plasma met een globale test, geassocieerd is met een verhoogd risico op hartinfarct en veneuze trombose. Deze associatie is consistent gevonden door ons en door anderen in verschillende patiënt-controle onderzoeken. De rol van de individuele fibrinolytische factoren in arteriële en veneuze trombose is minder duidelijk, mogelijk deels omdat de factoren niet alleen een rol hebben in de fibrinolyse, maar ook in andere processen die belangrijk zijn voor het ontstaan van trombose. Desalniettemin hebben wij aanwijzingen gevonden voor een rol voor TAFI en α2-antiplasmine in hartinfarct en voor TAFI en PAI-1 in veneuze trombose.

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Wat is epidemiologie zonder goed datamanagement? Ingeborg en Inge, dankjewel voor jullie hulp!

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Curriculum Vitae

and

Publications

Mirjam Meltzer werd geboren op 18 juni 1978 te Den Haag. Haar VWO diploma heeft ze behaald op het Rijnlands Lyceum in Wassenaar. In 1998 is ze begonnen aan de studie Voeding & Gezondheid aan de Wageningen Universiteit, met o.a de specialisatie epidemiologie. Binnen deze opleiding heeft ze een afstudeeronderzoek gedaan bij het International Center for Diarrhoeal Disease Research, Bangladesh (ICDDRB) en op de afdeling Maatschappelijke Gezondheidszorg aan de Katholieke Universiteit van Leuven, België. In 2005 werkte ze acht maanden aan de Universiteit van Wageningen mee aan het wetenschappelijke rapport 'Food, Nutrition, Physical Activity and the Prevention of Cancer: a Global Perspective' van het internationale World Cancer Research Fund. In maart 2006 begon ze haar promotieonderzoek waarvan de resultaten beschreven staan in dit proefschrift. Het onderzoek was een samenwerking tussen de afdeling Klinische Chemie en Hematologie van het Universitair Medisch Centrum Utrecht, waar ze begeleid werd door dr. T. Lisman en prof. dr. Ph.G. de Groot, en de afdeling Klinische Epidemiologie van het Leids Universitair Medisch Centrum, waar ze begeleid werd door dr. C.J.M. Doggen en prof. dr. F.R. Rosendaal. Gedurende dit project volgde ze verschillende cursussen op het gebied van stolling en epidemiologie en gaf ze onderwijs op het vlak van de epidemiologie aan studenten geneeskunde en biomedische wetenschappen. Mirjam Meltzer hield presentaties op nationale en internationale congressen. In 2007 ontving ze de Wetenschapsprijs van de Nederlandse Vereniging voor Trombose en Hemostase, in 2007 en 2009 ontving ze een young investigators award van de International Society of Thrombosis and Haemostasis en in 2008 een reisbeurs van de European Hematology Association. In 2008 verbleef ze 2,5^{e} week in Salt Lake City, VS, voor een project in samenwerking met de universiteit van Utah en Vermont op het gebied van genetische epidemiologie.

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