THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR

IN

VENOUS AND ARTERIAL THROMBOSIS

Thrombin Activatable Fibrinolysis Inhibitor in Venous and Arterial Thrombosis

© Emile Lucien Emanuel de Bruijne

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THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR IN VENOUS AND ARTERIAL THROMBOSIS

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Te weten wat men weet,
en te weten wat men niet weet,
dat is kennis.
Confucius (551-479 v.Chr.)

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Introduction

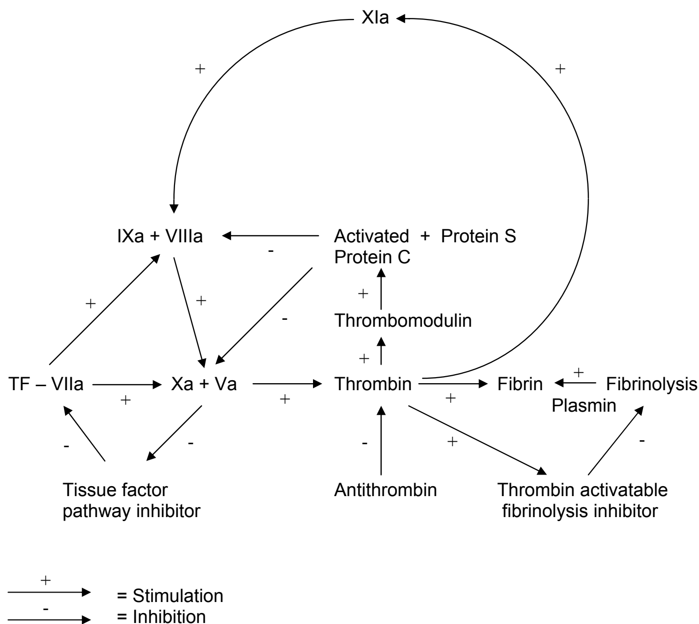
Venous and arterial thromboses are major causes of morbidity and mortality. Venous thrombosis is the result of pathological occlusive clot formation in the veins. It occurs mainly in the deep veins of the leg (deep vein thrombosis), from which parts of the clot frequently embolize to the lungs (pulmonary embolism). Less than 5% of all venous thromboses occur at other sites (e.g. arm or cerebral veins). Rarely occurring, but often life-threathening venous thrombosis is splanchnic vein thrombosis, including hepatic vein thrombosis, Budd Chiari syndrome (BCS) and portal vein thrombosis (PVT). Venous thrombosis often occurs spontaneously, but it also frequently accompanies medical and surgical conditions, both in the community and the hospital. Many risk factors for venous thrombosis are known, most of them related either to immobilization or to hypercoagulability [1]. The symptoms of venous thrombosis are non-specific, and therefore the clinical diagnosis is difficult and requires objective testing by imaging. Major complications of thrombosis include a disabling post-thrombotic syndrome and death due to fatal pulmonary embolism. Treatment with anticoagulants should be prompt and adequate.

Arterial thrombosis is the formation of a thrombus within an artery. Most arterial thrombi are superimposed on disrupted atherosclerotic plaque because plaque rupture exposes thrombogenic material in the plaque core to the blood, and is therefore referred to as atherothrombosis. This material then triggers platelet aggregation and fibrin formation, which results in the generation of a platelet-rich thrombus that can temporarily or permanently occlude blood flow. Arterial thrombosis is the most common cause of acute myocardial infarction, ischemic stroke, and limb gangrene. In arterial thrombosis at young age traditional risk factors such as gender, hypertension, smoking, diabetes mellitus, obesity and hypercholesterolemia do not fully explain the cardiovascular risk [2-4]. It is suggested that in these young patients other pathogenetic mechanisms than the classical risk factors may be of importance than in elderly patients. Therefore, additional factors must be present that determine the development of arterial thrombosis [5]. The genetic component in the pathogenesis of cardiovascular disease is stronger in young patients. In contrast, in elderly patients the contribution of the classical age-related cardiovascular risk factors is more important [4].

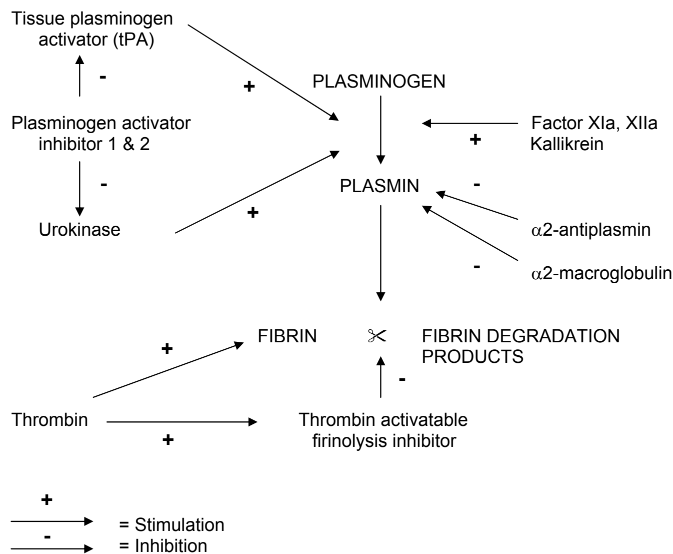
Arterial and venous thrombi are composed of platelets and fibrin, but the proportions differ. Arterial thrombi are rich in platelets because of the high shear in the injured arteries. In contrast, venous thrombi, which form under low shear conditions, contain relatively few platelets and are predominantly composed of fibrin and trapped red cells.

Coagulation and fibrinolysis

The hemostatic system has as main function the cessation of blood loss from a damaged vessel, wherein the damaged blood vessel wall will be covered by a platelet and fibrin-containing clot to stop bleeding and initiate repair of the damaged vessel. Historically hemostasis has been subdivided in primary hemostasis, which involves mainly platelet plug formation and secondary hemostasis, or coagulation, a complex process of activation of plasma coagulation factors, ultimately resulting in the formation of fibrin. As soon as the endothelial lining of a blood vessel wall is damaged, platelets immediately form a platelet plug at the site of injury, due to the interaction of platelets with the subendothelial collagen fibers, mediated by von Willebrand factor. Exposure of blood to tissue factor initiates the plasmatic coagulation cascade by the activation of coagulation factor VII. Other coagulation factors are activated in turn and finally prothrombin is activated to thrombin that converts fibrinogen into fibrin, the end product of coagulation. Fibrin fibers are necessary to strengthen the platelet plug. The coagulation process is tightly controlled by inhibitors of coagulation factors, including antithrombin and the protein C system. Fibrinolysis is the process by which a fibrin clot, the endproduct of the coagulation cascade, is degraded. The main enzyme is plasmin, which degrades fibrin, resulting in the formation of circulating fibrin degradation products. Recently, new proteins that regulate fibrinolysis have been discovered, including thrombin activatable fibrinolysis inhibitor (TAFI).



Coagulation figure



Fibrinolysis figure

TAFI

Thrombin activatable fibrinolysis inhibitor (TAFI) [6], also known as plasma procarboxypeptidase R [7], procarboxypeptidase U [8] and procarboxypeptidase B [9], is the precursor of a carboxypeptidase that potently attenuates fibrinolysis. The protein was called TAFI because it could be activated by thrombin and, when active, inhibits fibrinolysis. TAFI is synthesized in the liver and released into the circulation as a single-chain glycoprotein of 60 kDa. The pro-enzyme TAFI can be activated by thrombin, plasmin or the thrombin/thrombomodulin complex by a single proteolytic cleavage at Arg-92. This results in the activation peptide (TAFI-AP) and in the exposure of the substrate binding site of activated TAFI (TAFIa) of 36 kDa. TAFIa is thermolabile (half-life of 8–15 min at 37 °C) as a result of a spontaneous conformational conversion into an inactive form [TAFIa(i)], which is prone to further proteolytic cleavage. Activated TAFI (TAFIa) suppresses fibrinolysis by removing carboxy-terminal lysine and arginine residues from partially degraded fibrin, thereby reducing the binding of the fibrinolytic components plasminogen and tissue-type plasminogen activator [10, 11]. Using this mechanism, TAFI stabilizes the fibrin clot and makes the clot more resistant to lysis.

The TAFI gene (CPB2) was mapped to chromosome 13q14.11 [12, 13] and consists of 11 exons, spanning about 48 kb of genomic DNA [14]. Plasma levels of TAFI are partly

(25%) determined by genetic factors. Several SNPs in the promoter, coding and 3'-untranslated regions of the TAFI gene have been reported that may explain part of this genetic component [15].

TAFI is the link between coagulation and fibrinolysis. TAFI not only shifts the haemostatic balance to a more hypofibrinolytic state, but may also dampen the excessive inflammatory reaction by cleaving inflammatory mediators and inflammatory peptides [16, 17].

Disorders of fibrinolysis and venous and arterial thrombosis

In the past decades several studies have shown that a delicate balance exists between coagulation and fibrinolysis [18]. Disorders of coagulation can lead to an increased risk of bleeding (hemorrhage), for instance in patients with hemophilia due to factor VIII of IX deficiency, or obstructive clotting (thrombosis), for instance in patients with antithrombin deficiency.

Disorders resulting in enhanced or decreased fibrinolysis may also have clinical implications. Enhanced fibrinolysis may be associated with increased bleeding, as exemplified by patients with inherited or acquired alfa2-antiplasmin deficiency [19, 20]. On the other hand it is still disputed whether reduced fibrinolysis is associated with an increased thrombotic tendency. Patients with a hereditary plasminogen deficiency and venous thrombosis have been described, however other studies revealed that a complete absence of plasminogen does not result in a thrombotic tendency [21, 22]. For arterial thrombosis a pathogenetic role of fibrinolysis is also still unresolved. Although previous studies have reported that elevated plasminogen activator inhibitor-1 (PAI-1) levels which result in hypofibrinolysis are associated with arterial thrombosis, these studies did not use state of the art fibrinolysis tests [23-25]. In recent years, new assays have become available to study the fibrinolytic potential, which makes it possible to further evaluate the potential pathogenetic role of the fibrinolytic system in arterial and venous thrombosis. Because of its fibrinolysis inhibiting function, TAFI is anticipated to contribute to the pathogenesis of arterial thrombotic disease. This is supported by observations that show an association between TAFI levels and (unstable) angina pectoris, coronary artery disease and ischemic stroke [26-32]. However, such associations could not be confirmed in other studies [33-35]. So far only limited studies have been performed to study TAFI in thrombotic disorders and further studies are required to establish its potential pathogenetic role, both in venous and arterial thrombosis.

Outline of the thesis

The aim of this thesis is to further investigate the role of fibrinolysis and in particular TAFI in arterial and venous thrombosis. Therefore we have assessed fibrinolysis parameters, TAFI antigen and activity levels and variations in the TAFI gene, in well-defined study cohorts of patients with arterial or venous thrombosis. In addition we studied the role of TAFI in the outcome of a severe prothrombotic condition, meningococcal sepsis. The role of TAFI in venous thrombosis is explored in **Chapter 2** and **Chapter 3**. In **Chapter 2** we investigate the association between genetic variants in the TAFI gene on TAFI-antigen levels, clot lysis time and the risk of venous thrombosis. This will be investigated within "The Leiden Thrombophilia Study" (LETS), a case-control study investigating risk factors for venous thrombosis, including 474 consecutive patients with a first venous thrombosis of the leg or arm [36]. We study the association between genetic variation in TAFI and the risk of splanchnic vein thrombosis including patients with hepatic vein thrombosis (Budd Chiari syndrome, BCS) and non-cirrhotic portal vein thrombosis (PVT) in **Chapter 3**. Splanchnic vein thrombosis has been associated with a hypercoagulable state, which may be amplified by TAFI as an inhibitor of the fibrinolysis. This study is carried out in a large cohort of patients included in the "Liver and Thrombosis study group". In this case-control study a total of 118 patients, 39 patients with BCS and 85 patients with PVT of whom six patients had both BCS and PVT, are enrolled and compared with a matched control group [37]. We study the role of TAFI in arterial thrombosis in **Chapter 4, 5 and 6**. In **Chapter 4** we investigate the pathogenetic role of TAFI levels and genotypes in young patients with arterial thrombosis with a recent first-ever event of coronary heart disease (CHD) or cerebrovascular disease (ischemic stroke). These patients are included in the ATTAC study, "The Genetic risk factors for Arterial Thrombosis at a young age: the role of TAFI and other Coagulation factors (ATTAC)' study". This is an ongoing single-center, case–control study to explore the role of TAFI levels and genetic variation in the TAFI gene on the incidence of arterial thrombosis, including coronary heart disease, ischemic stroke and peripheral arterial disease, at a young age (18–45 years for males and 18–55 years for females). The study population includes 374 consecutive patients and 332 age- and sex matched controls. Patients are included 1–3 months after the event in order to avoid a possible influence of the event or an acute phase response on plasma levels of TAFI and TAFI activity. In **Chapter 5** the role of TAFI in young patients with a first manifestation of peripheral arterial disease (PAD) will be studied and compared with a population-based control group. The cases are young patients with PAD that are enrolled in the ATTAC study. Previous studies have suggested that hypofibrinolysis may be associated with increased risk of arterial thrombotic

disease. The evaluation of the plasma fibrinolytic potential using a recently developed and validated assay might provide stronger evidence that hypofibrinolysis is associated to arterial thrombosis than evaluation of the individual fibrinolytic factors. In **Chapter 6** we evaluate the contribution of the fibrinolytic system in arterial thrombosis by determining the global plasma fibrinolytic potential of the survivors of a first arterial thrombosis at young age in either the cerebral, cardiac or peripheral vascular system in the previously mentioned population-based case–control study (ATTAC). In **Chapter 7**, thrombin activatable fibrinolysis inhibitor levels and genotypes are investigated to elucidate its role as a link between coagulation and fibrinolysis in children with severe meningococcal sepsis, which is known to be associated with a severe prothrombotic state. A total of 112 children consecutively admitted to the paediatric intensive care unit (PICU) of the Erasmus University Medical Centre-Sophia (Rotterdam, the Netherlands) who participated previously in Rotterdam-based meningococcal sepsis studies between 1988 and 2005 will be studied [38]. Finally, in **Chapter 8** the results of our studies will be summarized and discussed.

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CHAPTER 2

THE EFFECT OF GENETIC VARIANTS IN THE THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI) GENE ON TAFI-ANTIGEN LEVELS, CLOT LYSIS TIME AND THE RISK OF VENOUS THROMBOSIS

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Summary

Thrombin activatable fibrinolysis inhibitor (TAFI) is an important inhibitor of fibrinolysis. High TAFI antigen levels are associated with an increased risk of deep venous thrombosis (DVT). Because TAFI levels are partly determined genetically, we assessed the association between three TAFI gene polymorphisms (-438 G/A, 505 A/G and 1040 C/T), TAFI antigen levels and clot lysis times and the risk of DVT. Carriers of the 505G allele, which is associated with lower TAFI antigen levels than the 505A allele, showed an increased risk of DVT. This indicates that the relationship between TAFI and venous thrombosis is more complex than previously suggested.

Thrombin activatable fibrinolysis inhibitor (TAFI) is an important inhibitor of fibrinolysis, which acts by inhibiting the assembly of fibrinolytic factors on the fibrin surface [1]. Recently, it was shown that elevated TAFI antigen levels are a mild risk factor for the occurrence of deep venous thrombosis (DVT) [2]. TAFI levels increase with age, mainly in women, and are elevated in oral contraceptive users [2].

Thrombin activatable fibrinolysis inhibitor levels are also genetically determined and the -438 G/A TAFI single nucleotide polymorphism (SNP) in the promoter region [3] and the 505 G/A SNP [3] and 1040 C/T SNP in the coding region [4] are associated with TAFI

Table 1. The risk of developing a venous thrombotic event and the effect of SNPs on TAFI antigen levels and clot lysis time.

SNP	Cases (%) (n = 471)	Controls (%) (n = 472)	OR (95% CI) mean (95% CI)	TAFI Ag (U/dl)* mean (95% CI)	CLT (min)* mean (95% CI)
-438					
GG	240 (51.0)	257 (54.4)	1	110 (108–111)	59.6 (58.4-60.8)
AG	202 (42.9)	185 (39.2)	1.17 (0.89-1.53)	104 (102–106)	63.0 (61.3-64.7)
AA	29 (6.1)	30 (6.4)	1.04 (0.60-1.78)	96 (92–100)	61.5 (56.0-67.1)
505					
GG	236 (50.1)	211 (44.7)	1	102 (101–103)	61.6 (59.9-63.2)
AG	198 (42.0)	210 (44.5)	0.84 (0.64-1.10)	108 (107–110)	60.7 (59.4-62.0)
AA	37 (7.9)	51 (10.8)	0.65 (0.41-1.03)	119 (115–122)	60.3 (56.9-63.6)
1040					
CC	219 (46.5)	215 (45.6)	1	110 (108–111)	59.1 (57.8-60.3)
CT	218 (46.3)	212 (44.9)	1.01 (0.77-1.32)	105 (104–107)	62.8 (61.2-64.3)
TT	34 (7.2)	45 (9.5)	0.74 (0.46-1.20)	98 (95–102)	62.3 (58.0-66.6)

CLT, clot lysis time.
Linear regression analysis showed a mean decrease of TAFI of 6.3 U/dl (95% CI 4.5–8.0) per additional A-allele at -438, a mean increase of 7.6 U/dl (95% CI 6.1–9.2) per additional A-allele at 505, and a decrease of 5.2 U/dl (95% CI 3.5–6.8) at 1040.
*TAFI and CLT levels in control subjects.

plasma antigen levels. The -438 G/A polymorphism was reported to be associated with an increased risk of developing venous thrombosis [5, 6].

Recently, the effect of TAFI polymorphisms on TAFI antigen levels has been debated since variable antibody reactivity towards TAFI isoforms (in particular the 1040C/T polymorphism) leads to artefacts in TAFI antigen levels [7].

We analysed data from the Leiden Thrombophilia Study (LETS) to assess the risk of developing DVT associated with three SNPs of the TAFI gene [-438 G/A (rs no. 2146881), 505 A/G (rs no. 3742264) and 1040 C/T (rs no. 1926447)] in 471 patients with a first DVT, aged 18–70 years (patients with cancer excluded) and 472 sex- and age-matched control subjects. The risk of venous thrombosis associated with each polymorphism was expressed as an odds ratio (OR) with a corresponding 95% confidence interval (95% CI). In addition, the TAFI multilocus haplotype effects on TAFI antigen levels were estimated using weighted linear regression as described by Tanck et al [8]. After modification of the method, (weighted) logistic regression was used to investigate the association between TAFI haplotypes and the risk of thrombosis. Furthermore, the effect of the three SNPs on the TAFI antigen levels and clot lysis time was assessed in the control group. TAFI antigen levels were measured by the Laurell method, which is insensitive to TAFI genotype artefacts [2, 7]. Moreover, TAFI antigen levels may not represent the functional activity of TAFI, which is measured by the rate of cleavage of a small substrate after activation with thrombin thrombomodulin [9]. Mosnier et al [10] described a plasma based clot lysis assay, which was initially developed to study TAFI-related processes. It was shown that clot lysis times were associated with both TAFI antigen and activity levels in a group of 20 healthy volunteers. However, recently we showed in a much larger group of healthy subjects (n = 469), which is the control group of the study we report on here, that the association between the clot lysis time and TAFI antigen levels was, at most, very weak (0.188 increase in clot lysis time (in min) per 1 U/dl increase in TAFI, after age-adjustment) [11].

In the total study population, 403 (43%) were men, 540 (57%) women and the mean age in patients and control subjects was 45.0 years (range 15–69 years) and 44.7 years (15–72 years), respectively. The distribution of the three TAFI SNPs in the control subjects did not deviate from Hardy–Weinberg equilibrium.

All three SNP were associated with TAFI antigen levels, which was more pronounced in homozygotes than in heterozygotes (Table I). For the -G438A and C1040T variants, the rare alleles were associated with lower levels than common alleles, while for the G505A the rare allele was associated with higher TAFI levels than the common allele. The effect on TAFI levels was most striking for the 505 genotypes, with 17% higher TAFI levels in

carriers of 505AA than 505GG. These findings are in agreement with earlier observations [4, 12].

These associations between genotypes and levels would predict an increased risk of thrombosis for the 505A, -438G and 1040C allele. A clear and graded relationship between genotype and risk of thrombosis was, however, only observed for the 505A genotype, with lower risks for the rare A-allele. Because there is a high level of linkage disequilibrium between these TAFI polymorphisms, haplotype analysis was necessary to analyse the effects of a single polymorphism while excluding the effects of associated polymorphisms. Haplotype analysis confirmed the association of the G505A polymorphism and risk of venous thrombosis. The 505G allele was associated with a mildly increased risk of venous thrombosis [OR 1.3 (1.0–1.6); Fig 1B]. The 505G allele was associated with reduced TAFI levels, both by the single genotype and by the haplotype analysis (Fig 1A). The analysis, therefore, suggested that a direct, functional role is more likely for the G505A polymorphism than for the other studied TAFI polymorphisms. We have now found a reduced risk of DVT with the allele that is associated with higher TAFI levels and lower clot lysis times, which was in contrast to the hypothesis on which we based on our previous findings [2]. A possible explanation, besides chance findings, is that moderately elevated TAFI antigen levels do not alter the risk of venous thrombosis. Previously, we only found an increased risk when TAFI antigen levels exceeded 122 U/dl [2], whereas none of the genotypes we studied were associated with such high levels of TAFI antigen. However, it may also be that

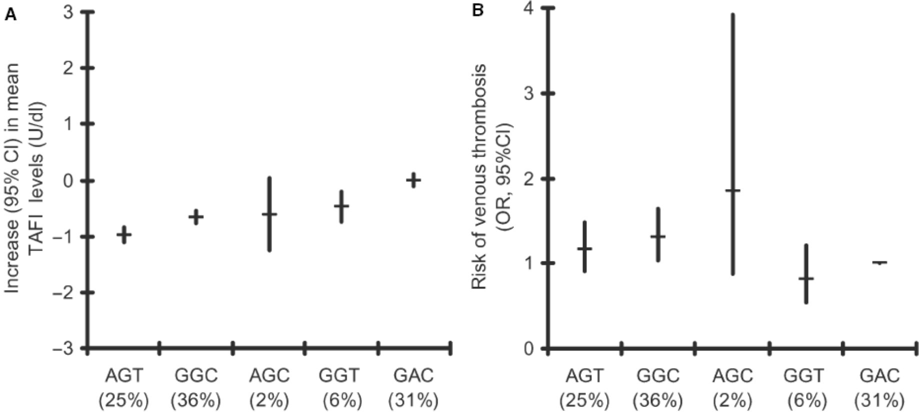

Fig 1. (A) The effect of TAFI haplotypes on plasma TAFI antigen levels in the LETS study population (levels are expressed as mean and 95% CI). (B) The effect of TAFI haplotypes on risk of venous thrombosis in patients vs controls expressed as odds ratios. The alleles in the haplotype are given in the following polymorphism order: -438G/A. 505G/A (Ala147Thr) and 1040C/T (Thr325Ile). GAC as reference.

the elevated levels of TAFI we previously observed in patients who had suffered venous thrombosis compared with healthy controls were a consequence rather than a cause of thrombosis.

All three SNPs were associated with TAFI antigen levels, but only a relationship between TAFI 505A polymorphism and clot lysis times was observed (Table I). We have recently shown in these same individuals that TAFI antigen levels and clot lysis times were only weakly associated [11]. The present study showed an increased risk of DVT in carriers of the 505 G allele. The 505 G-allele is associated with low TAFI antigen levels, which indicates that there is complex relationship between TAFI and the risk of venous thrombosis.

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CHAPTER 3

GENETIC VARIATION IN THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR (TAFI) IS ASSOCIATED WITH THE RISK OF SPLANCHNIC VEIN THROMBOSIS

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Summary

Background and Aims: Splanchnic vein thrombosis (SVT) has been associated with a hypercoagulable state. Thrombin-activatable fibrinolysis inhibitor (TAFI) may contribute to a hypercoagulable state, and therefore we were interested in the role of TAFI in SVT. Since the disease is frequently associated with liver insufficiency, which affects plasma levels of TAFI, we studied the role of variation in the TAFI gene in SVT.

Patients and Methods: In a multicenter case-control study on 118 patients with SVT (39 Budd-Chiari syndrome and 85 portal vein thrombosis) and 118 population-based controls, the relationship of SVT with single nucleotide polymorphisms (SNPs) and haplotypes in the TAFI gene (−438G/A, Ala147Thr, Thr325Ile and 1583A/T) was determined.

Results: The risk for SVT was decreased (OR 0.2, 95% CI 0.1-0.7) in 147Thr/Thr homozygotes and slightly, but not significantly, increased in carriers of the 325Ile allele (OR 1.6, 95% CI 0.9-2.7). Haplotype analysis confirmed that the Ala147Thr SNP has the strongest association with risk of SVT.

In conclusion: Genetic variation in the TAFI gene is associated with risk of SVT, suggesting a role for TAFI in the pathogenetic mechanism of SVT.

Introduction

Splanchnic vein thrombosis (SVT) is thrombosis of the hepatic veins and of the portal venous system. It includes the Budd-Chiari syndrome (BCS), which is characterized by occlusion of hepatic outflow at the level of hepatic venules, large hepatic veins or inferior caval vein [1] and portal vein thrombosis (PVT) [2]. PVT often occurs in conditions leading to decreased portal flow, such as cirrhosis or malignancies, abdominal surgery and infections. Both BCS and PVT have been observed in association with acquired or hereditary hypercoagulable conditions, such as myeloproliferative disorders and antiphospholipid syndrome or factor V Leiden mutation and prothrombin mutation [2-4].

Thrombin-activatable fibrinolysis inhibitor (TAFI) [5], also known as plasma procarboxypeptidase R [6], procarboxypeptidase U [7] and procarboxypeptidase B [8], is the precursor of a carboxypeptidase that potently attenuates fibrinolysis. Activated TAFI (TAFIa) suppresses fibrinolysis by removing carboxy-terminal lysine residues from partially degraded fibrin polymers, preventing the binding of the fibrinolytic components plasminogen and tissue-type plasminogen activator [9, 10]. By this mechanism, TAFI stabilizes the fibrin clot and makes the clot more resistant to lysis. Indeed, elevated plasma TAFI antigen levels are associated with a mildly increased risk for venous thrombosis [11, 12] and for recurrent venous thromboembolism [13]. The TAFI concentration in plasma is partly genetically determined, and several genetic variants have been reported that are associated with plasma TAFI levels, such as the –438G/A (rs#2146881) [14, 15] single nucleotide polymorphism (SNP) in the TAFI promoter region which is of interest because promoter SNPs may affect the transcription rate and because this SNP is associated with risk of venous thrombosis [14]. Also SNPs that result in amino acid changes and may affect the TAFI function are of interest, such as the Thr325Ile (1040C/T, rs#1926447) which results in a TAFI isoform with altered antifibrinolytic activity [16] and the Ala147Thr (505G/A, rs#3742264). These SNPs have also been associated with TAFI levels and venous thrombosis risk [14]. Also the 1583A/T (rs#1087) has a strong association with TAFI plasma levels [17]. However, the associations between these SNPs and risk of splanchnic vein thrombosis are still unknown.

In patients with liver disease, the role of TAFI in determining the risk of SVT can not be clearly determined, since plasma levels are strongly affected by the disease. Therefore, we focused on studying genetic variation, which is an important determinant of TAFI levels and not affected by the disease. SVT is frequently seen in patients with pre-existent liver disease, but may also result in a reduced liver synthesis capacity, as is seen in patients with BCS. Therefore, we studied the role of TAFI in SVT by determining the contribution of

variation in the TAFI SNPs that have been reported to be functional on SVT in a multicenter case-control study.

Materials and methods

Subjects

A total of 118 patients, 39 patients with BCS and 85 patients with PVT of whom six patients had both BCS and PVT, were enrolled in the study. These patients, identified in seven academic hospitals in the Netherlands between July 1997 and February 2003, underwent a full screening for thrombogenic disorders. Details of the procedure have been published previously [3]. Part of this previously reported patient population (n = 105) is included in this study. Briefly, diagnostic criteria for BCS and PVT were partial or complete obstruction of hepatic outflow or the portal vein, respectively, as documented by appropriate radiographic abdominal imaging (Doppler ultrasonography, computed tomography, magnetic resonance imaging or venography) or laparotomy. Patients with veno-occlusive disease or with hepatic outflow obstruction caused by congestive heart failure were excluded. Patients alive were asked to visit the hospital in which they were registered for blood sampling, at the same time enabling investigators to complete the questionnaire with information on previous thrombotic events, familial thrombosis, acquired risk factors of thrombosis, and the use of anticoagulants at the time of venipuncture. The population-based control group consisted of 118, sex- and age matched (≤ 5 years difference) healthy subjects who had no history of venous thromboembolism and did not use coumarin derivatives, and were partners or friends of the patients. The study was approved by the ethical committee of each participating hospital and the participants gave their informed consent before entering the study.

Methods

We selected four SNPs in the TAFI gene that comprehensively describe the DNA sequence variation of the TAFI gene, including the promoter and 3’ UTR region. The four haplotype-tagging SNPs were selected on the basis of the linkage disequilibrium map of the TAFI locus provided by the SeattleSNP project (http://pga.gs.washington.edu/) and the HAPMAP project (www.hapmap.org) to study the total common genetic variation. Since there are several SNPs that tag a specific haplotype, we selected SNPs based on their potential functionality as described in the introduction. Blood was collected in tubes containing 3.2% trisodium citrate (9:1 vol/vol) using a Vacutainer system (Becton Dickinson, Plymouth,

UK). The blood was centrifuged for 30 min at 2000g at 4° C and genomic DNA was isolated from the white cell fraction using standard procedures. Analysis of the SNPs in the TAFI gene (–438G/A (dbSNP rs#2146881), Ala147Thr (505G/A, dbSNP rs#3742264), Thr325Ile (1040C/T, dbSNP rs#1926447) and 1583A/T dbSNP rs#1087)) was performed using polymerase chain reaction (PCR) with subsequent restriction enzyme digestions as described previously [18]. Briefly: the PCR mixture (25 µL) contained: 50 ng genomic DNA, 37.5 pM of each primer, 1.5 mM of each dNTP (Pharmacia), 2.5 µL buffer (15 mM MgCl2 500 mM KCl 100 mM TrisHCl, pH 8.3) and 1.25 U Taq DNA polymerase (Boehringer Mannheim). The PCR conditions were: 4 min 95° C of initial denaturation, followed by 32 cycles of 1 min of denaturation at 94° C, 1 min annealing at 58 and 62° C resp and 2 min of elongation at 72° C. The PCR products were digested with their specific restriction enzymes for 180 min at 37ºC and analyzed on a 2.5% agarose gel. The laboratory staff was not aware of the patient or control status.

Statistical analysis

Patients with both BCS and PVT were included in the analyses of both diseases. The data are presented as means ± standard deviation (SD). Allele frequencies were calculated by gene counting and for each SNP the deviation from Hardy-Weinberg equilibrium was tested in controls using a χ^2 test with one degree of freedom. The association between TAFI SNPs and SVT was investigated by logistic regression, using the genotypes with the highest frequency (–438GG, 147Ala/Ala, 325Thr/Thr and 1583A/A) as reference groups [14].

Haplotypes present in the population were inferred by means of the haplo.em function of the program Haplo Stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html), which computes maximum likelihood estimates of haplotype probabilities [19, 20]. Haplotype reconstruction resulted in seven haplotypes with frequencies > 2%. The most common haplotype (G-Ala-Thr-A) served as the reference category.

The association between TAFI gene haplotypes and SVT was investigated by (weighted) logistic regression using the haplo.glm function of the program Haplo Stats [19-21]. The probability for each haplotype pair in each individual was assigned and then an individual's phenotype was directly modeled as a function of each inferred haplotype pair, weighed by their estimated probability, to account for haplotype ambiguity. Using haplo.score, we computed simulation P-values for each haplotype to account for multiple testing. Details on the background and theory of score statistics can be found in Schaid et al. [21]. The number of simulations was set at 1000.

A value of P < 0.05 was considered statistically significant. Except for the haplotype analyses, all statistical analyses were performed using the Statistical Package for Social Science for windows, version 10.1 (SPSS Inc. Chicago, Illinois, USA).

Results

The characteristics of the 118 patients with SVT (39 patients with BCS and 85 patients with PVT) and 118 controls are summarized in table 1. Fifty-four (46%) of the SVT patients and controls were male and the mean age of SVT patients and controls was 51 years.

The distributions of the –438G/A, Ala147Thr, Thr325Ile and 1583A/T TAFI SNPs were in Hardy-Weinberg equilibrium in the control group.

Subjects with the 147Thr/Thr genotype had a lower risk of SVT (OR 0.2, 95% CI 0.1-0.7) compared with the reference group of subjects with the 147Ala/Ala genotype, with an intermediate risk (OR 0.9, 95% CI 0.5-1.5) for the heterozygotes (table 2). Subjects with the 325Thr/Ile genotype had a higher risk of SVT (OR 1.8, 95% CI 1.0-3.0) than subjects

Table 1. Characteristics of patients with Splanchnic Vein Thrombosis (SVT), Budd-Chiari syndrome (BCS), Portal vein thrombosis (PVT) and healthy controls.

	SVT	BCS	PVT	Controls
	n = 118	n = 39	n = 85	n = 118
Age (y)	51 (18-81)	43 (18 - 66)	53 (24 – 81)	51 (17 - 77)
Male	54 (46%)	14 (36%)	42 (49%)	54 (46%)
Oral anticoagulation	47 (40%)	30 (77%)	21 (25%)	-
Inherited thrombophilia	27 (23%)	11 (28%)	17 (20%)	NA
Myeloproliferative disorders	27 (23%)	13 (33%)	15 (18%)	-
PNH	3 (3%)	1 (3%)	2 (2%)	NA
Lupus anticoagulant	4 (3%)	4 (10%)	-	NA
Anti-cardiolipin antibodies	5 (4%)	3 (8%)	2 (2%)	NA
Biopsy documented cirrhosis	15 (13%)	6 (15%)	10 (12%)	NA
History of pancreatitis	9 (8%)	-	9 (11%)	-
Infection	15 (13%)	3 (9%)	14 (16%)	-
Inflammatory bowel disease	3 (3%)	2 (5%)	2 (2%)	-
Autoimmune disease	10 (8%)	5 (13%)	5 (6%)	-
Previous abdominal surgery	39 (33%)	10 (26%)	30 (35%)	NA
Cancer	2 (2%)	-	2 (2%)	-
Oral contraceptives	24 (38%)	14 (56%)	14 (33)	12 (19)
Antithrombin activity levels (%)	92.1 ± 22.5	99.4 ± 23.8	91.0 ± 18.7	104.2 ± 13.5

Data are presented as mean (range). Inherited thrombophilia is defined as Factor V Leiden mutation, Prothrombin mutation, Protein C deficiency, Protein S deficiency or Antithrombin deficiency. Myeloproliferative disorders include Polycythemia vera, Essential thrombocythemia, Myelofibrosis, MPD Unclassified. Infection at time of diagnosis: liver viral, liver bacterial, cholecystitis/cholangitis, omphalitis, intestinal with(out) abcess, elsewhere abdominal, extra-abdominal, sepsis eci. Previous abdominal surgery: Splenectomy, Cholecystectomy, and other abdominal surgery. Oral contraceptive use % of women at time of diagnosis. PNH: paroxysmal nocturnal hemoglobinuria, NA: not available.

with the 325Thr/Thr genotype. Carriers of the 325Ile allele also had a slightly increased risk (OR 1.6; 95% CI 0.9-2.7). Subjects with the –438GA genotype showed an OR of 1.6 (95% CI 0.9-2.7) as compared to subjects with the –438GG genotype. The 1583A/T polymorphism was not associated with risk of SVT (table 2).

Table 2. Associations between TAFI genotypes and risk of SVT.

	Genotype distribution patients (n=118)		Genotype distribution controls (n=118)		OR (95% CI)
-438G/A*					
GG	48	(42%)	60	(52%)	1
GA	58	(51%)	46	(40%)	1.6 (0.9-2.7)
AA	8	(7%)	10	(9%)	1.0 (0.4-2.7)
GA & AA	66	(58%)	56	(48%)	1.5 (0.9-2.5)
A-allele (freq)	0.32		0.28		
Ala147Thr*					
Ala/Ala	58	(50%)	48	(41%)	1
Ala/Thr	54	(46%)	50	(43%)	0.9 (0.5-1.5)
Thr/Thr	5	(4%)	18	(16%)	0.2 (0.1-0.7)
Ala/Thr & Thr/Thr	59	(51%)	68	(59%)	0.7 (0.4-1.2)
Thr-allele (freq)	0.27		0.37		
Thr325Ile*					
Thr/Thr	40	(34%)	53	(45%)	1
Thr/Ile	69	(59%)	52	(44%)	1.8 (1.0-3.0)
Ile/Ile	9	(8%)	13	(11%)	0.9 (0.4-2.4)
Thr/Ile & Ile/Ile	78	(66%)	65	(55%)	1.6 (0.9-2.7)
Ile-allele (freq)	0.37		0.33		
1583 A/T*					
AA	47	(41%)	48	(58%)	1
AT	60	(52%)	45	(45%)	1.4 (0.8-2.4)
TT	8	(7%)	7	(7%)	1.2 (0.4-3.5)
AT & TT	68	(60%)	52	(52%)	1.3 (0.8-2.3)
A-allele (freq)	0.33		0.30		

*Genotype with highest TAFI antigen level was considered as reference. TAFI, thrombin-activatable fibrinolysis inhibitor; SVT, splanchnic vein thrombosis; OR, odds ratio; CI, confidence interval

In the subgroup of BCS subjects with the 147Thr/Thr genotype had also somewhat, although not significantly, lower risk of vein thrombosis with an OR of 0.2 (95% CI 0.02-1.3) compared with subjects with the 147Ala/Ala genotype, and an OR of 1.2 (95% CI 0.6-2.5) for the heterozygotes. The associations between the other TAFI SNPs and BCS was similar to those in the total group. Subjects with the 325Thr/Ile genotype had an OR for BCS of 1.3 (95% CI 0.6-2.9) when compared with subjects with the 325Thr/Thr genotype.

Subjects with the –438GA genotype showed an OR of 1.5 (95% CI 0.7-3.3) when compared with the subjects with the –438GG genotype. Subjects with the 1583AT genotype showed an OR of 1.4 (95% CI 0.7-3.0) when compared with the subjects with the 1583AA genotype.

In the PVT subgroup, the associations between the TAFI SNPs and risk of vein thrombosis were similar to those in the total SVT group. Subjects with the 147Thr/Thr genotype had a lower risk of PVT (OR 0.3, 95% CI 0.1-0.8) than subjects with the 147Ala/Ala genotype, with an intermediate risk (OR 0.9, 95% CI 0.5-1.6) for the heterozygotes. Subjects with the 325Thr/Ile genotype showed an OR of 1.7 (95% CI 1.0-3.1) when compared with subjects with the 325Thr/Thr genotype. In subjects with the –438GA genotype the OR for PVT was 1.4 (95% CI 0.8-2.5) when compared with subjects with the –438GG genotype. Subjects with the 1583AT genotype showed an OR of 1.5 (95% CI 0.8-2.9) when compared with the subjects with the 1583AA genotype.

Since the TAFI SNPs are in high linkage disequilibrium, we also analyzed the association between TAFI haplotypes and risk of SVT, using the most common haplotype (G-Ala-Thr-A) as reference (figure 1). The haplotype analysis confirmed that the Ala147Thr SNP has the strongest association with risk of SVT. The risk of SVT was significantly lower for the G-Thr-Thr-A haplotype (-438G/147Thr/325Thr/1583A, OR 0.1, 95% CI 0.03-0.6) and also somewhat, although not significantly, lower for the A-Thr-Thr-T haplotype (OR 0.3, 95%

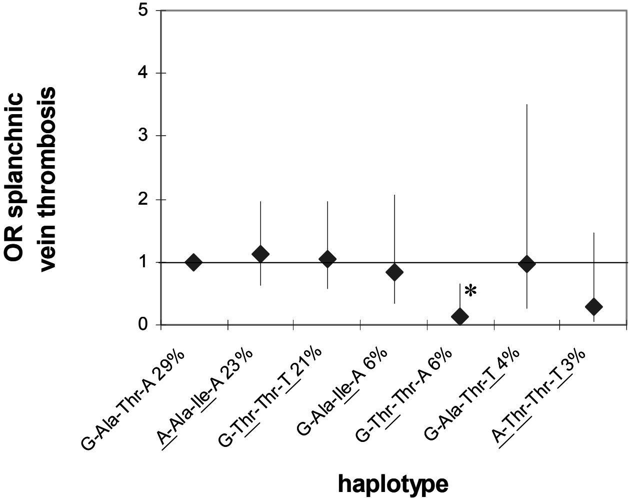

Figure 1. The effect of thrombin-activatable fibrinolysis inhibitor (TAFI) haplotypes on risk of splanchnic vein thrombosis (SVT) in patients versus controls expressed as odds ratio's. The alleles in the haplotype are given in the following polymorphism order: -438G/A, Ala147Thr (505G/A), Thr325Ile (1040C/T) and 1583A/T. Asterisk indicate haplotype, whose average effect is significantly different from the group with the most common haplotype (G-Ala-Thr-A) which served as the reference category.

CI 0.06-1.5), when compared with the common G-Ala-Thr-A haplotype. There was a clear SNP-SNP interaction, since no association with risk was seen for the G-Thr-Thr-T haplotype. In the BCS and PVT subgroups a similar trend, although not significantly, was observed for the G-Thr-Thr-A haplotype as compared to the common G-Ala-Thr-A haplotype.

Discussion

Our study indicates that SNPs in the TAFI gene are associated with the risk of SVT. Both the Ala147Thr and Thr325Ile SNPs are associated with risk of SVT.

We selected four haplotype-tagging SNPs in the TAFI gene to study the total common genetic variation (covering 92% of the genetic variation). Since there are several options for SNPs tagging a haplotype, we selected SNPs based on their potential functionality in order to learn more about the different mechanisms that may underlie associations with SVT. The -438G/A and 1583A/T were selected because they are associated with the TAFI concentration, and the –438G/A is located in the promoter region and may therefore affect synthesis regulation [16, 17]. The Ala147Thr SNP was also selected because of its association with TAFI concentration and with the risk of arterial thrombosis and because the amino acid substitution may affect functionality of TAFI [22, 23]. The Thr325Ile SNP was selected because this SNP encodes for two different isoforms of TAFI, with a difference in activity and half-life and because it also correlates with TAFI concentration [16].

We considered BCS and PVT as one entity (splanchnic vein thrombosis) as has been done in previous studies [24, 25] because a common pathway, that leads to a hypercoagulable condition, is expected.

In our study, the 147Thr/Thr genotype was significantly associated with decreased risk of SVT and the risk for SVT was slightly, but not significantly, increased in carriers of the 325Ile allele (OR 1.6, 95%CI 0.9-2.7). Previously, the Ala147Thr and theThr325Ile SNPs were reported to be strongly associated with TAFI antigen levels [15, 26]. However, from previous findings it has become clear that this association is mostly due to the genotype-dependent antibodies that are used in these assays [26]. When using assays that did not have this problem, it has been shown that the 147Ala and 325Ile alleles are still associated with lower TAFI antigen levels but that the relationship is less strong [15]. It is therefore expected that the genotypes that are associated with SVT in our study are the genotypes that are associated with the lowest TAFI antigen levels. A recent study by Martini and coworkers [14] reported also that carriers of the 505G (147Ala) allele have an increased risk of DVT. In contrast, previous studies reported an association between high levels of TAFI and risk of deep venous thrombosis [11] which suggests that the relation between genotypes

and risk may not be via the TAFI concentration in plasma per se, but may be related to a change in functional aspects of TAFI.

TAFI can circulate in different isoforms, and the activated form of TAFI of the 325Ile isoform has a half-life of 15 min while that of the 325Thr variant is 8 min. Furthermore, the antifibrinolytic potential of the 325Ile isoform was 30-60% greater than the 325Thr variant [27]. The ability of TAFIa 325Ile variant to release twice as much lysine during fibrinolysis as the 325Thr variant may shift the balance towards coagulation instead of fibrinolysis in these patients albeit the lower antigen levels. This may partially explain the increased risk of SVT in carriers of the 325Ile-allele.

Because there is a high degree of linkage disequilibrium, it is not possible to estimate the contribution of the individual SNPs by analyzing the SNPs separately in the univariate analysis. Therefore, we also studied the effects of haplotypes, in order to estimate the effects of the SNPs combined in one model. This haplotype analysis indicates that the Ala147Thr SNP has the strongest effect on risk of SVT. Mechanistic studies have so far shown little effect of the Ala147Thr SNP on the antifibrinolytic potential of TAFIa [27]. A possible explanation could be that there is linkage disequilibrium between the Ala147Thr with a yet unknown regulatory factor of TAFI.

The haplotype analysis assumes additivity of the effects of the alleles. We see some, but not strong, gene-dose effects and therefore it is not completely clear whether this assumption is completely valid. Larger studies are needed to elucidate more detailed the interaction between the alleles.

The strength of our study is that we determined TAFI genetic variations, performed haplotype analysis to assess the pathogenetic importance of TAFI in SVT and that our study is therefore not affected by an effect of the disease on TAFI levels. In addition, this study is performed in a group of patients with SVT that is unique and large considering that SVT is a rare disease. Our study had a case-control design, which means that only patients who survived the SVT were included, and since BCS is fatal in 20% of the cases within 2 years when treated in specialized centers [28] the true association between TAFI and SVT may have been underestimated.

In conclusion, the present study describes a relationship between TAFI gene SNPs, including haplotype analysis and risk of SVT, where subjects carrying TAFI “lowering alleles” had a higher risk of SVT which suggests that TAFI plays a role in the development of splanchnic vein thrombosis.

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CHAPTER 4

THE ROLE OF THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR IN ARTERIAL THROMBOSIS AT A YOUNG AGE: THE ATTAC STUDY

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Summary

Background and objectives: Thrombin activatable fibrinolysis inhibitor (TAFI) attenuates fibrinolysis and may therefore contribute to the pathophysiology of arterial thrombosis. The aim of the present study was to elucidate the pathogenetic role of TAFI levels and genotypes in young patients with arterial thrombosis.

Patients and methods: In a case-control study, 327 young patients with a recent first-ever event of coronary heart disease (CHD subgroup) or cerebrovascular disease (ischemic stroke subgroup) and 332 healthy young controls were included. TAFI levels (intact TAFI, activation peptide (TAFI-AP) and (in)activated TAFI (TAFIa(i))) and TAFI activity were measured and genetic variations in the TAFI gene (-438G/A, 505G/A and 1040C/T) were determined.

Results: In the total group of patients, TAFIa(i) levels were higher (145.1 ± 37.5%) than in controls (137.5 ± 31.3%, P = 0.02). Plasma levels of intact TAFI, TAFI-AP and TAFI activity were similar in patients and controls. In the CHD subgroup (n = 218) intact TAFI levels were higher (109.4 ± 23.0%) than in controls (102.8 ± 20.7%, P=0.02). In 325Ile/Ile homozygotes, lower TAFI levels and a decreased risk of arterial thrombosis were observed (OR 0.58, 95% CI 0.34-0.99) compared with patients with the common 325Thr/Thr genotype. This association was most evident in CHD patients (OR 0.48, 95% CI 0.26-0.90). Haplotype analyses supported a role for the Thr325Ile polymorphism.

Conclusions: TAFIa(i) levels were higher in patients with cardiovascular disease. Furthermore, the TAFI 325Thr/Ile polymorphism was associated with lower TAFI levels and with the risk of cardiovascular disease in young patients, especially in CHD.

Introduction

The fibrinolytic system appears to play an important role in the pathogenesis of arterial thrombosis as illustrated by the associations between increased levels of plasminogen activator inhibitor (PAI-1) and tissue plasminogen activator (t-PA) with an increased risk of myocardial infarction (MI) [1-3]. Thrombin activatable fibrinolysis inhibitor (TAFI) is another fibrinolytic factor that may play a role in arterial thrombosis [4]. TAFI is a carboxypeptidase that circulates in an inactive form and it can be activated by thrombin, plasmin or the thrombin/thrombomodulin complex.

Activated TAFI (TAFIa) potently attenuates fibrinolysis by removing carboxy-terminal lysine residues from partially degraded fibrin polymers, thereby reducing the binding of the fibrinolytic components plasminogen and tissue-type plasminogen activator [5-7]. Using this mechanism, TAFI stabilizes the fibrin clot and makes the clot more resistant to lysis.

Because of its function, TAFI is anticipated to contribute to the pathogenesis of arterial thrombotic disease. This is supported by observations that show an association between TAFI levels and unstable angina pectoris, coronary artery disease and ischemic stroke [8-11]. However, such associations could not be confirmed in other studies [12, 13]. Part of this inconsistency is the result of the use of unsuitable assays that have been used in most of these previous studies. Recently, several well-characterized and specific new assays for TAFI antigen and activity have become available [14, 15]. Application of these assays will elucidate the role of TAFI in the pathophysiology of arterial thrombosis.

The plasma levels of TAFI antigen are partly (25%) determined by genetic factors and several single nucleotide polymorphisms (SNPs) in the TAFI gene (CPB2) have been reported that may explain part of this genetic component [16]. The –438G/A (rs#2146881) SNP in the TAFI promoter region is associated with occurrence of venous thrombosis, possibly by affecting the transcription rate and consequently the plasma levels [17, 18]. Also, SNPs that result in amino acid changes and may affect the TAFI function are of interest, such as the Thr325Ile (1040C/T, rs#1926447) which determines the stability of the TAFIa molecule and thus affects its antifibrinolytic activity [19] and the Ala147Thr (505G/A, rs#3742264). These SNPs have also been associated with TAFI antigen levels [20] and arterial thrombosis for the Ala147Thr SNP [21]. Furthermore, the Ala147Thr (rs#3742264) is in high linkage disequilibrium (LD) with rs#9526136, which was recently reported to be highly associated with intact TAFI and TAFI activation peptide (TAFI-AP) in the SAHLSIS study [11].

As in young cardiovascular patients the contribution of genetic components in the development of arterial thrombosis is much more pronounced than in older patients [22], the aim of our study was to investigate variations of the TAFI gene on risk of arterial thrombosis in

a group of young patients with a first event. In addition, it was investigated whether plasma levels of TAFI and TAFI activity between patients and controls were different and to what extent plasma TAFI levels were influenced by the genetic variations in the TAFI gene.

Patients and Methods

Patients

The 'Genetic risk factors for **A**rterial **T**hrombosis at a young age: the role of **TA**FI and other **C**oagulation factors (ATTAC)' study is a single-center, case-control study to explore the role of TAFI levels and genetic variation in the TAFI gene on the incidence of arterial thrombosis at a young age. Cases included in the present study were consecutively recruited patients with a first-ever acute ischemic cardiac or focal cerebral event at the departments of Cardiology or Neurology of the Erasmus Medical Center Rotterdam in the Netherlands. Patients were eligible for inclusion if they were 18 - 45 years for males and 18 - 55 years for females. The study group consists of two subgroups: (i) patients with acute myocardial infarction (AMI) or unstable angina pectoris (UAP) [coronary heart disease (CHD) subgroup] and (ii) ischemic stroke (IS) or a transient ischemic attack (TIA) (IS subgroup). Patients were included one to three months after the event in order to avoid a possible influence of the event or an acute phase response on plasma levels of TAFI and TAFI activity.

A control group was obtained by asking the patients to bring a friend, neighbour or partner fulfilling the same age criteria but without a history of arterial thrombosis. Patients' relatives were not permitted. A detailed clinical history and physical examination was performed in both patients and controls. Information was obtained on cardiovascular risk factors, including smoking, hypercholesterolemia, diabetes mellitus, hypertension and family history of cardiovascular disease.

Eligible participants received oral and written information about the goals and methodology of the study. Written informed consent was obtained on enrolment from each participant. The study was approved by the Medical Ethics Committee of Erasmus MC and conducted according to the procedures of the Declaration of Helsinki.

Definitions

AMI was defined as typical chest pain, with elevated cardiac markers (CK MB/troponin T), and/or characteristic electrocardiographic findings. Unstable angina pectoris (UAP) was defined as typical chest pain while at rest. Transient ischemic attack (TIA) was defined as

the acute onset of focal cerebral dysfunction, which could not be explained otherwise than by focal cerebral ischemia, diagnosed by a neurologist. Symptoms had to be temporary and last < 24 h after onset. Ischemic stroke (IS) was defined as the acute onset of focal cerebral dysfunction as a result of cerebral ischemia, with symptoms lasting longer than 24 h. Brain imaging by CT-scan or MRI-scan had to be compatible with the diagnosis and was applied to rule out haemorrhagic stroke. Smoking status was defined as never, previous or current smoker. Hypercholesterolemia was defined as total cholesterol level > 5.0 mmol L^{-1} or receiving lipid-lowering treatment on day of the ischemic event. Patients with a medical history of diabetes or using either oral anti-diabetic medication or insulin therapy on day of the event were considered to be diabetics. Hypertension was defined by a systolic blood pressure ≥ 140 mmHg, and/or diastolic blood pressure ≥ 90 mmHg or the use of anti-hypertensive drugs. A positive family history was noted if the patient had a first-degree relative with a history of cardiovascular disease before the age of 60.

Laboratory assays

Blood collection

Blood was drawn under strictly standardized conditions [23] by venipuncture in the antecubital vein using the Vacutainer system (Becton Dickinson, Plymouth, UK). Blood for coagulation studies was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within 1 hour at 2,000 x g for 10 min at 4˚C. Plasma was additionally centrifuged at 20,000 x g for 10 min at 4˚C and stored at -80˚C until analysis. For DNA isolation, blood was collected in tubes containing ethylene diaminetetraacetic acid (EDTA; Becton Dickinson) and genomic DNA was extracted according to standard salting-out procedures and stored at 4°C for genetic analysis.

TAFI antigen levels

Plasma levels of intact TAFI, TAFI activation peptide (TAFI-AP) and (in)activated TAFI [TAFIa(i)] were measured using three sandwich-type ELISAs as reported previously [14] i.e. MA-T12D11/MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP, respectively. All levels of TAFI and TAFI fragments are expressed relative to the levels of pooled human plasma. The assay variability of the ELISAs was evaluated using four different plasma samples each assayed four times on four occasions. The intra-assay and inter-assay coefficients of variation for MA-T12D11/MA-T30E5-HRP were 6.2% and 8.3%, respectively, for MA-T12D11/MAT18A8-HRP 3.1% and 7.3%, respectively and for

MA-T30E5/MA-T17D7-HRP 3.3% and 6.4%, respectively. The interdilution coefficients of variation were 8.2%, 5.1% and 7.3% for MA-T12D11/MAT30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP, respectively. The detection limit of all three ELISAs was 1.6%. TAFI-depleted plasma revealed no detectable response in any ELISA.

TAFI activity assay

The TAFI functional assay was performed as described previously [15]. Briefly, plasma samples were diluted in TAFI-depleted plasma and clotted by a reaction mix composed of thrombin, Solulin [recombinant thrombomodulin which was a gift from PAION GmbH, Aachen, Germany (by courtesy of Dr. H. Brohmann)], $CaCl_2$, and recombinant tissue plasminogen activator (tPA) (Actilyse). Each sample was analysed in the presence and the absence of potato carboxypeptidase inhibitor (PCI). The optical density at 405 nm was monitored at 37°C for 150 min. Lysis time was defined as the time-point corresponding to a 50% decrease in optical density. Functional TAFI levels were calculated as TAFI-related retardation (RT), defined as the difference between the lysis times in the absence and in the presence of PCI. Using this assay, the intra- and inter-assay variability of the TAFI-related retardation of pooled normal plasma (twentyfold diluted) were 7% and 13%, respectively (n = 30; using several batches of TAFI-depleted plasma) [15].

Genetic analysis

Analysis of the SNPs in the TAFI gene [–438G/A (dbSNP rs#2146881), Ala147Thr (505G/A, dbSNP rs#3742264) and Thr325Ile (1040C/T, dbSNP rs#1926447)] was performed using polymerase chain reaction (PCR) with subsequent restriction enzyme digestions as described previously [8]. Briefly, the PCR mixture (25 µL) contained: 50 ng genomic DNA, 37.5 pM of each primer, 1.5 mM of each dNTP (Pharmacia, Uppsala, Sweden), 2.5 µL buffer (15 mM $MgCl_2$ 500 mM KCl 100 mM TrisHCl, pH 8.3) and 1.25 U Taq DNA polymerase (Boehringer Mannheim, Almere, The Netherlands). The PCR conditions were: 4 min 95°C of initial denaturation, followed by 32 cycles of 1 min of denaturation at 94°C, 1 min annealing at 58°C and 62°C, respectively, and 2 min of elongation at 72°C. The PCR products were digested with their specific restriction enzymes for 180 min at 37ºC and analyzed on a 2.5% agarose gel. The laboratory staff was not aware of the patient or control status.

Statistical analysis

The data are presented as means ± standard deviation (SD). To compare the TAFI plasma measurements from the controls with the patients and with genotypes, analysis of variance (ANOVA) was used, with adjustment for age and gender. We also performed logistic regression for each measurement of TAFI (as a continuous variable and divided in quartiles based on the distribution in the control group), with adjustment for age and gender. Allele frequencies were calculated by gene counting, and for each SNP the deviation from Hardy-Weinberg equilibrium was tested in controls using a Chi-squared test with 1 df. The association between TAFI SNPs and arterial thrombosis was investigated by logistic regression, with adjustment for age and gender, using the genotypes with the highest frequency (–438GG, 147Ala/Ala and 325Thr/Thr) as reference groups [17]. Haplotypes present in the population were inferred by means of the haplo.em function of the program Haplo Stats (http://cran.r-project.org/web/packages/haplo.stats/index.html), which computes maximum likelihood estimates of haplotype probabilities [24, 25]. Haplotype reconstruction resulted in five haplotypes with frequencies ≥ 3%. The most common haplotype (G-Ala-Thr) served as the reference category. The association between TAFI gene haplotypes and arterial thrombosis was investigated by (weighted) logistic regression using the haplo.glm function of the program Haplo Stats [24-26]. The probability for each haplotype pair in each individual was assigned, and then an individual's phenotype was directly modelled as a function of each inferred haplotype pair, weighed by their estimated probability, to account for haplotype ambiguity. Using haplo.score, we computed simulation p-values for each haplotype to account for multiple testing. Details on the background and theory of score statistics can be found in Schaid et al. [26]. The number of simulations was set at 1,000. A value of $p < 0.05$ was considered statistically significant. Except for the haplotype analyses, all statistical analyses were performed using the Statistical Package for Social Science for Windows, version 10.1 (SPSS Inc., Chicago, IL, USA).

Results

Patient population

In this study, we included 327 patients with arterial thrombosis and 332 controls of which 149 (46%) were male in the arterial thrombosis group and 122 (37%) in the control group (Table 1). Mean age of the patients and controls was 43 ± 7 years (range 21-55 years) and 39 ± 8 years (range 18-56), respectively. As expected, traditional risk factors were much more frequent in patients than in controls. Approximately 80% of both patients and controls were Caucasian and 20% were a mixture of African, Asian, Turkish, or unknown.

Table 1. Characteristics of arterial thrombosis patients and healthy controls.

	Patients (*n* = 327)	Controls (*n* = 332)	*P*-value*
Demographics			
Age (years)	42.7 ± 6.9	38.5 ± 8.0	< 0.0001
Male gender – n (%)	149 (46)	122 (37)	< 0.0001
Ethnical background – no. (%)			
Caucasian	257 (78.6)	283 (85.2)	0.03
African	13 (3.9)	7 (2.1)	ns
Asian	33 (10.1)	17 (5.1)	0.02
Turkish	11 (3.4)	14 (4.2)	ns
Unknown	13 (4.0)	11 (3.3)	ns
Risk Factors			
Smoking† – n (%)	253 (77)	162 (49)	< 0.0001
Hypercholesterolemia – n (%)	147 (45)	15 (5)	< 0.0001
Hypertension – n (%)	93 (28)	22 (7)	ns
Diabetes mellitus – n (%)	23 (7)	5 (2)	0.018
Clinical aspects			
BMI (kg m^{-2})	26.5 ± 4.6	25.1 ± 4.2	0.006
Specified diagnosis of first arterial thrombosis – n (%)			
Acute Myocardial Infarction	169 (52)	-	-
Unstable Angina Pectoris	49 (15)	-	-
Ischemic Stroke	52 (16)	-	-
Transient Ischemic Attack	57 (17)	-	-
Time between event and blood sampling (days)	84.3 ± 55.7	-	-

BMI, body mass index; ns, not significant. *P-value for difference to control group. †Current and former smoker

Plasma TAFI antigen levels and activity in arterial thrombosis

In patients with arterial thrombosis the levels of (in)activated TAFI [TAFIa(i)] were higher (145.1 ± 37.5%; mean ± SD) than in controls (137.5 ± 31.3%, p=0.02). Plasma levels of intact TAFI, TAFI activation peptide (TAFI-AP) and TAFI activity were similar in patients and controls (Table 2). Subgroup analysis of Caucasians alone showed similair TAFI levels and TAFI activity (data not shown). The levels of intact TAFI in the subgroup of patients with CHD were higher (109.4 ± 23.0%) than in controls (102.8 ± 20.7%, p=0.02) (Table 2). We also observed a trend towards higher levels of TAFIa(i) in the CHD subgroup (144.7 ± 38.8%), although this was not significant (p=0.06). The levels of TAFIa(i) in the subgroup of patients with ischemic stroke or TIA were higher (145.8 ± 34.8%) than in controls (137.5 ± 31.3%, p=0.04). The levels of intact TAFI or other TAFI variables between patients and controls did not differ (Table 2). Similar associations with arterial thrombosis were observed for each of the TAFI levels in the logistic regression models (data not shown). We did not observe significant associations between the three TAFI

measures with risk factors [hypertension, diabetes, hypercholesterolemia, body mass index (BMI) and smoking] or with medication (such as statins, aspirin and other platelet function inhibitors, ACE-inhibitors and ß-blockers). The time interval between the event and blood sampling was not associated with any of the TAFI measures (Intact TAFI, r = -0.083 and P = 0.137; TAFI-AP, r = -0.018 and P = 0.749; TAFIa(i), r = -0.019 and P = 0.740; TAFI retardation, r = -0.002 and P = 0.983).

Table 2. TAFI levels and TAFI activity in arterial thrombosis patients and healthy controls.

	Control group	Total patient group	P-value*	CHD subgroup	P-value*	IS or TIA subgroup	P-value*
	n=332	*n*=327		*n*=218		*n*=109	
TAFI antigen							
Intact TAFI	102.8 ± 20.7	107.3 ± 21.9	*0.12*	109.4 ± 23.0	*0.02**	103.1 ± 18.7	*0.73*
TAFI-AP	139.5 ± 54.7	144.0 ± 58.1	*0.69*	146.9 ± 52.6	*0.26*	138.1 ± 67.8	*0.40*
TAFIa(i)	137.5 ± 31.3	145.1 ± 37.5	*0.02**	144.7 ± 38.8	*0.06*	145.8 ± 34.8	*0.04**
TAFI activity	*n*=154	*n*=205		*n*=141		*n*=64	
TAFI Retardation (RT)	16.5 ± 2.2	16.3 ± 2.6	*0.34*	16.4 ± 2.7	*0.46*	16.2 ± 2.5	*0.22*

Data were given as mean ± SD. TAFI antigen levels were expressed in percentage. TAFI retardation was expressed in minutes. TAFI, thrombin activatable fibrinolysis inhibitor; TAFI-AP, TAFI activation peptide; TAFI a(i), (in)activated TAFI; CHD, coronary heart disease; IS, Ischemic Stroke; TIA, transient ischemic attack. *P-value for difference to control group, adjusted for age and gender.

TAFI gene polymorphisms and arterial thrombosis

The distribution of –438G/A, Ala147Thr and Thr325Ile TAFI SNPs in controls did not deviate from Hardy-Weinberg equilibrium. Also different ethnic subgroups did not deviate from Hardy-Weinberg equilibrium. The Thr325Ile polymorphism, which is a determinant of TAFIa stability, was associated with arterial thrombosis. In 325Ile/Ile homozygotes a decreased risk was observed (OR 0.58, 95% CI 0.34-0.99) compared with subjects with the common 325Thr/Thr genotype (Table 3). This association was especially seen in the CHD subgroup where patients with the 325Ile/Ile genotype had an odds ratio (OR) of 0.48 [95% confidence interval (CI 0.26-0.90)] compared with the reference group, with an intermediate risk (OR 0.78, 95% CI 0.54-1.13) for the heterozygotes (Table 3). In the subgroup of patients with ischemic stroke or TIA the –438G/A, Ala147Thr and Thr325Ile polymorphisms were not associated with a risk of arterial thrombosis. Neither were the –438G/A and Ala147Thr polymorphisms associated with risk of arterial thrombosis (Table 3). In Caucasians alone similar risk estimates were obtained, although these were not significant which is probably explained by the smaller group size (data not shown).

Table 3. Associations between TAFI genotypes and risk of arterial thrombosis.

	Genotype distribution patients (*n*=327)	Genotype distribution controls (*n*=332)	Total patient group OR (95% CI) (*n*=327)	CHD subgroup OR (95% CI) (*n*=218)	IS or TIA OR (95% CI) (*n*=109)
-438G/A†					
GG	160 (51%)	147 (49%)	1	1	1
GA	129 (42%)	131 (44%)	0.91 (0.65-1.26)	0.88 (0.61-1.27)	0.99 (0.61-1.58)
AA	22 (7%)	22 (7%)	0.92 (0.49-1.73)	0.91 (0.45-1.84)	0.96 (0.38-2.37)
A-allele (freq)	0.28	0.29			
505G/A†					
GG (Ala/Ala)	119 (39%)	127 (42%)	1	1	1
GA (Ala/Thr)	155 (50%)	147 (48%)	1.13 (0.80-1.58)	1.27 (0.87-1.86)	0.90 (0.56-1.46)
AA (Thr/Thr)	35 (11%)	31 (10%)	1.21 (0.70-2.08)	1.44 (0.79-2.61)	0.84 (0.37-1.90)
A-allele (freq)	0.36	0.34			
1040C/T†					
CC (Thr/Thr)	155 (50%)	141 (46%)	1	1	1
CT (Thr/Ile)	130 (42%)	126 (41%)	0.94 (0.67-1.31)	0.78 (0.54-1.13)	1.40 (0.87-2.26)
TT (Ile/Ile)	26 (8%)	41 (13%)	0.58 (0.34-0.99)	0.48 (0.26-0.90)	0.86 (0.40-1.87)
T-allele (freq)	0.29	0.34			

TAFI, thrombin activatable fibrinolysis inhibitor; OR, odds ratio; CI, confidence interval; CHD, coronary heart disease; IS, Ischemic Stroke; TIA, transient ischemic attack. †The most common genotype was considered as reference.

As the TAFI SNPs are in high linkage disequilibrium, combining the TAFI SNPs in haplotypes enables the identification of the SNP with the strongest association. We analysed the association between TAFI haplotypes and risk of arterial thrombosis, using the most common haplotype (G-Ala-Thr) as a reference. In the total group, none of the haplotypes was significantly associated with the risk of arterial thrombosis but the risk estimates confirmed a role for the 325Thr/Ile polymorphism (Figure 1A and Table 4). Slightly decreased risk estimates were observed for the G-Ala-Ile haplotype [OR 0.67, 95% CI (0.39-1.20)] in the total group and in the CHD subgroup [OR 0.67, 95% CI (0.37-1.21)], when compared with the common G-Ala-Thr haplotype.

The effect of TAFI gene polymorphisms and haplotypes on plasma TAFI antigen levels

Clear associations were observed between the genetic variation in the TAFI gene and TAFI levels, both in cases and in controls. These associations were observed for the different measures of TAFI, and both at the genotype and the haplotype level (table 5 and figure 1B-D). The three TAFI polymorphisms each showed a clear association with plasma

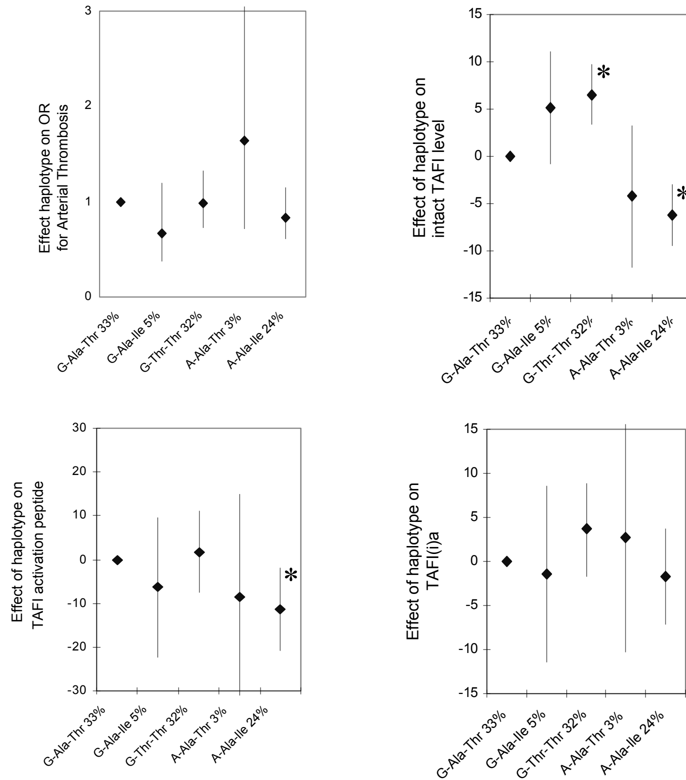

Figure 1A

The effect of thrombin activatable fibrinolysis inhibitor (TAFI) haplotypes on risk of arterial thrombosis in patients vs. controls expressed as odds ratio's. The alleles in the haplotype are given in the following polymorphism order: -438G/A, 505G/A (Ala147Thr) and 1040C/T (Thr325Ile). The group with the most common haplotype (G-Ala-Thr) served as the reference category.

Figure 1B-D

The effect of thrombin activatable fibrinolysis inhibitor (TAFI) haplotypes on respectively intact TAFI levels (figure B), TAFI activation peptide (figure C) and (in)activated TAFI (figure D) in arterial thrombosis in the total patient group [levels are expressed as mean (U mL-1) and 95% CI]. The alleles in the haplotype are given in the following polymorphism order: -438G/A, 505G/A (Ala147Thr) and 1040C/T (Thr325Ile). The group with the most common haplotype (G-Ala-Thr) served as the reference category.

TAFI antigen levels in patients and in controls (table 5). Subjects homozygous for the rare –438AA and 1040TT genotypes in the TAFI gene had lower plasma TAFI antigen levels than subjects with the common homozygous genotypes (-438GG and 1040CC, respectively) while heterozygotes had intermediate levels. Subjects homozygous for the 505AA genotype had higher plasma TAFI antigen levels than subjects homozygous for the common 505GG genotype while heterozygotes had intermediate levels.

Table 4. Relationship between TAFI haplotypes and arterial thrombosis risk.

	Haplotype frequency	Total patient group OR (95% CI)	CHD subgroup OR (95% CI)	IS or TIA subgroup OR (95% CI)
G-ALA-THR	0.33	1	1	1
G-ALA-ILE	0.05	0.67 (0.39-1.20)	0.67 (0.37-1.21)	0.81 (0.36-1.84)
G-THR-THR	0.32	1.00 (0.74-1.35)	0.98 (0.72-1.34)	0.74 (0.36-1.84)
A-ALA-THR	0.03	1.44 (0.63-3.31)	1.64 (0.71-3.82)	0.79 (0.19-3.35)
A-ALA-ILE	0.24	0.86 (0.63-1.18)	0.83 (0.61-1.15)	0.84 (0.53-1.32)

The most common haplotype (G-Ala-Thr) served as the reference category, minor alleles are underlined. The alleles in the haplotype are given in the following polymorphism order: –438G/A, Ala147Thr (505G/A) and Thr325Ile (1040C/T). CHD, coronary heart disease; IS, Ischemic Stroke; TIA, transient ischemic attack; OR, odds ratio.

The effects of the five TAFI haplotypes on respectively intact TAFI levels, TAFI activation peptide and (in)activated TAFI in arterial thrombosis in the total patient group are shown in figure 1B-D. Haplotype analyses showed a trend for a decreased risk of arterial thrombosis in the G-Ala-Ile and A-Ala-Ile haplotypes, which supported a role for the Thr325Ile SNP that was already suggested by the single polymorphism analysis.

Discussion

In this study, we observed a relationship between TAFI and arterial thrombosis in young subjects. In the total group of patients with premature arterial thrombosis, the levels of TAFIa(i) were significantly higher than in controls. Plasma levels of intact TAFI, TAFI activation peptide (TAFI-AP) and TAFI activity were similar in both patients and controls. In the subgroup of patients with CHD the levels of intact TAFI were significantly higher than in controls. We also observed an association between the TAFI 325Thr/Ile polymorphism and arterial thrombosis, especially in the subgroup of patients with CHD. This association was confirmed in the haplotype analysis.

Young patients with arterial thrombotic events will have, on average, less extensive atherosclerosis than elderly patients. This gives young patients with a history of MI a relatively favourable prognosis compared with older patients [27]. Because of the limited role of atherosclerosis, studying young patients gives the opportunity to explore other mechanisms that predispose to cardiovascular events. In the past, increased levels of several coagulation factors, leading to a hypercoagulable state, have been associated with premature arterial thrombosis. A decreased fibrinolytic activity as a result of increased levels of PAI-1 has been reported in patients under 45 years with a MI [1]. An increased level of the PAI-1 antigen is also a risk factor for reinfarction in men whose first MI occurred before the age of 45 years [2].

Table 5. Association between TAFI gene polymorphisms, TAFI, clot lysis time, and TAFI-related retardation.

	Intact TAFI (%)		TAFI-AP (%)		TAFIa(i) (%)		TAFI Retardation (RT)	
Genotype	Patients (n=327)	Controls (n=332)	Patients (n=327)	Controls (n=332)	Patients (n=327)	Controls (n=332)	Patients (n=205)	Controls (n=154)
TAFI –438								
GG	110.9 ± 21.4	109.0 ± 20.5	147.5 ± 59.1	147.9 ± 59.7	146.3 ± 36.5	140.1 ± 34.5	16.2 ± 2.6	16.4 ± 2.4
GA	104.4 ± 21.3	100.9 ± 19.4	137.2 ± 55.9	135.4 ± 55.0	144.6 ± 38.5	135.7 ± 30.4	16.5 ± 2.7	16.5 ± 2.2
AA	98.1 ± 23.4	83.6 ± 17.9	145.3 ± 63.9	114.5 ± 24.2	145.3 ± 42.8	133.2 ± 21.4	17.0 ± 3.2	18.7 ± 3.3
	P = 0.004	P < 0.0001	P = 0.362	P = 0.012	P = 0.966	P = 0.404	P = 0.600	P = 0.080
TAFI 505								
GG	101.5 ± 21.6	95.5 ± 18.3	136.0 ± 59.7	131.6 ± 43.9	141.4 ± 37.1	134.5 ± 31.2	16.7 ± 2.8	16.5 ± 2.5
GA	109.8 ± 21.5	107.9 ± 20.8	145.8 ± 56.1	146.0 ± 67.0	148.5 ± 37.9	141.7 ± 33.5	16.0 ± 2.6	16.6 ± 2.3
AA	115.6 ± 20.0	112.2 ± 21.2	150.7 ± 54.0	139.6 ± 38.5	151.3 ± 37.4	135.6 ± 24.0	16.5 ± 2.2	16.3 ± 1.6
	P < 0.0001	P < 0.0001	P = 0.281	P = 0.066	P = 0.247	P = 0.126	P = 0.254	P = 0.889
TAFI 1040								
CC	110.4 ± 22.1	106.8 ± 18.8	148.9 ± 63.0	146.9 ± 58.4	147.6 ± 37.4	140.1 ± 32.3	16.1 ± 2.6	16.3 ± 2.3
CT	105.1 ± 21.5	103.3 ± 21.4	135.7 ± 52.4	139.2 ± 57.2	144.1 ± 37.1	135.4 ± 32.4	16.5 ± 2.7	16.3 ± 2.1
TT	99.8 ± 18.9	91.1 ± 20.8	144.5 ± 53.9	117.8 ± 34.8	144.6 ± 42.1	136.1 ± 28.0	17.3 ± 2.8	18.3 ± 2.4
	P = 0.017	P < 0.0001	P = 0.206	P = 0.013	P = 0.787	P = 0.498	P = 0.276	P = 0.002

Data given as mean ± SD. P-value for patient and control group for each TAFI polymorphism, adjusted for sex and age. TAFI, thrombin activatable fibrinolysis inhibitor; TAFI-AP, TAFI activation peptide; TAFIa(i), (in)activated TAFI; TAFI retardation was expressed in min.

TAFI is an important inhibitor of fibrinolysis, by cleaving the carboxy-terminal lysine residues of partially degraded fibrin, which interferes with the interaction of plasminogen with fibrin. High TAFI levels may therefore result in a decreased fibrinolytic activity [5, 7]. Several studies have been performed to assess the relationship between TAFI levels and risk of arterial thrombosis. In previous studies, we and others have found that TAFI activity is significantly associated with the risk of a first ischemic stroke [10, 28]. TAFI antigen levels have also been associated with ischemic stroke, (unstable) angina pectoris and AMI [8, 21, 29, 30]. Previous studies were hampered by the fact that most antigen assays applied were highly sensitive to the TAFI genotype, which influenced the outcome of some of the early studies (reviewed in [31]). In the present study, TAFI concentrations were analysed by three newly developed immunologic assays with distinct reactivities, recognizing either intact TAFI, the released activation peptide (AP) or TAFIa(i) [14]. In contrast to previously used methods these new assays are genotype-independent. Our study is the first to measure levels of intact TAFI, released AP (a marker of the extent of TAFI activation) and TAFIa(i) in patients with arterial thrombosis at a young age. We also studied functional TAFI levels, defined as the difference in clot lysis time in the absence or presence of a specific activated TAFI inhibitor [potato carboxypeptidase inhibitor (PCI)]. It seems that TAFIa(i) plasma levels are better predictors than levels of the other TAFI variables. Our results are in line with those recently published by Tregouet et al. [32] on the predictive ability of TAFIa(i) plasma levels in individuals with CAD. TAFIa(i) levels could be a marker of thrombin generation as is suggested in the AtheroGene study. In our study, plasma levels of TAFI-AP were similar in patients and controls which is in contrast to the results of Ladenvall et al [11]. A possible explanation could be the inclusion criteria of the patients (< 70 years, only stroke patients and only white patients). An additional explantion could be a different clearance of the TAFI-AP and TAFIa(i), as discussed by Tregouet et al. [32].

DNA analysis was performed to identify SNPs in the TAFI gene that were expected to directly influence the plasma TAFI levels, and may thereby be associated with risk of arterial thrombosis. The advantage of studying DNA is that the results are not affected by the arterial thrombotic event, as is the case with TAFI levels, and that the influence is present at least since birth. We selected SNPs based on their potential functionality in order to learn more about the different mechanisms that may underlie associations with arterial thrombosis. The –438G/A SNP was selected because it is associated with the TAFI concentration, and as the –438G/A SNP is located in the promoter region it is expected to affect synthesis regulation [19, 33]. The Ala147Thr SNP was also selected because of its association with TAFI concentration and has previously been reported to be associated with a risk of arterial

thrombosis and because the amino acid substitution may affect functionality of TAFI [10, 21]. The Thr325Ile SNP was selected because this SNP encodes for two different isoforms of TAFI, with a difference in both activity and half-life and because it also correlates with TAFI concentration [19]. Together, these SNPs identify all haplotypes with frequency > 5%, as determined using Hapmap and Haploview.

We observed associations between TAFI SNPs and TAFI levels and also an association between the 325Thr/Ile polymorphism and a risk of arterial thrombosis. Carriers of the 325Ile-allele, associated with the lowest TAFI antigen levels, appeared to have a lower risk of arterial thrombosis. A previous study reported that decreased TAFI levels are associated with a lower restenosis rate after percutaneous coronary intervention [34]. Our results suggest that carriers of the 325Ile-allele in the TAFI gene are associated with lower levels and a risk of arterial thrombosis.

A strong point of our study is that consecutive cases were included and that the diagnosis of cardiovascular disease has always been confirmed by a cardiologist or a neurologist. Furthermore, all neurological patients underwent neuroimaging to rule out cerebral haemorrhage. We included population controls, thus avoiding the biases induced by ‘‘hospital controls’’. Detailed information about cardiovascular risk factors, medical history, and family history was collected from patients and controls. A limitation of our study is that the subgroup of patients with ischemic stroke or TIA was relatively small for assessing a possible association between TAFI gene polymorphisms, TAFI levels and ischemic stroke or TIA. In addition, ischemic stroke or TIA is a more heterogeneous disorder than CHD. Therefore, larger studies are needed to study the relationship between TAFI and the subgroup of ischemic stroke or TIA [11]. The size of this patient group was also somewhat small for haplotype analysis, which may explain why the polymorphisms that were significantly associated with TAFI levels or risk of disease did not result in significant associations in the haplotype analysis. Also here, there is a need for larger populations.

In conclusion, TAFIa(i) levels were higher in patients with cardiovascular disease. Furthermore, the 325Ile-allele both lowers TAFI levels and was protective against arterial thrombosis in this group of young patients, especially against coronary heart disease.

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CHAPTER 5

HIGH THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR LEVELS ARE ASSOCIATED WITH AN INCREASED RISK OF PREMATURE PERIPHERAL ARTERIAL DISEASE

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Summary

Background: Previous studies suggested that hypofibrinolysis is associated with increased risk of peripheral arterial disease. Thrombin activatable fibrinolysis inhibitor (TAFI) has been identified as an important inhibitor of fibrinolysis. The aim of our study was to assess the role of TAFI in young patients with peripheral arterial disease.

Methods: In a single-center case-control study we measured plasma TAFI antigen levels and functional TAFI in consecutive young patients (men 18-45 years and women 18-55 years) with a first manifestation of peripheral arterial disease and compared these with a population-based control group.

Results: A total of 47 peripheral arterial disease patients and 141 controls (mean age 43) were included. Intact TAFI antigen levels were significantly higher in patients with peripheral arterial disease (112.4 ± 21.1%) than in controls (104.9 ± 19.9%, p=0.03). The risk of peripheral arterial disease increased with 18% (OR 1.18; CI 1.01-1.34) per 10% increase of TAFI antigen. Functional TAFI levels were slightly higher in patients compared to controls, however this difference was not significant. For individuals with the highest functional TAFI levels, above the 90th percentile, the increased risk for peripheral arterial disease was most pronounced (OR 3.1; CI 1.02-9.41).

Conclusion: High TAFI levels are associated with increased risk of premature peripheral arterial disease.

Introduction

Previous studies have reported associations between hypofibrinolysis and an increased risk of peripheral arterial disease (PAD) [1, 2]. Most studies on hypofibrinolysis focussed on elevated plasminogen activator inhibitor-1 (PAI-1) levels and showed association between PAI-1 and PAD [3, 4] or symptoms of PAD [5]. Also the functional PAI-1 -675(4G/5G) polymorphism was associated with early thrombotic reocclusions [6].

Thrombin activatable fibrinolysis inhibitor (TAFI) has also been identified as an important inhibitor of fibrinolysis [7, 8]. Activated TAFI (TAFIa) exerts its anti-fibrinolytic function by removing the carboxy-terminal lysine residues of partially degraded fibrin. This results in a decreased binding and activation of plasminogen and thereby attenuates fibrinolysis [9, 10]. TAFI is activated by thrombin, plasmin or the thrombin/thrombomodulin complex by a single cleavage at Arg-92. This results in the release of the activation peptide (TAFI-AP) and exposure of the substrate binding site of TAFIa. TAFIa is thermolabile due to a spontaneous conformational conversion into an inactive form (TAFIa(i)), which is sensitive to further proteolytic cleavage.

Several studies have been performed to study TAFI levels in arterial thrombosis. It has been shown that increased functional TAFI levels and/or TAFI antigen levels are associated with ischemic stroke [11, 12]. The relationship between TAFI levels and the risk of coronary heart disease is less clear, since several conflicting studies have been reported [13-15]. So far no studies have been performed to assess the role of TAFI in peripheral arterial disease.

Therefore, the aim of our study was to investigate both antigen and functional TAFI-levels, including the activation markers TAFI-AP and TAFIa(i) in young patients with a first clinical manifestation of peripheral arterial disease.

Patients

The “Genetic risk factors for **A**rterial **T**hrombosis at young age: the role of **TA**FI and other **C**oagulation factors (ATTAC)” study is a single-center, case-control study to explore the role of TAFI levels and the incidence of arterial thrombosis at young age. Cases included in this study were consecutively recruited patients with a first-ever ischemic event due to peripheral arterial disease at the department of Vascular Surgery of the Erasmus Medical Center Rotterdam in the Netherlands. The diagnosis PAD was defined as peripheral arterial stenosis resulting in ischemia, classified according to the Rutherford criteria [16]. Patients were eligible for inclusion if they were 18 - 45 years for males and 18 - 55 years for females. Patients were included one to three months after the event in order to avoid a

possible influence of the event or an acute phase response on plasma levels of TAFI and TAFI activity.

A control group was obtained by asking the patients to bring a friend, neighbour or partner fulfilling the same age criteria but without a history of arterial thrombosis. Relatives of patients were not permitted. A detailed clinical history and physical examination, including arterial pulsations of upper and lower extremities, was performed in both patients and controls. For this study, 3 age- and sex-matched controls were randomly selected for each patient. Information was obtained on cardiovascular risk factors, including smoking, hypercholesterolemia, diabetes mellitus, hypertension and family history of cardiovascular disease. Smoking status was defined as never, previous or current smoker. Hypercholesterolemia was defined as total cholesterol level > 5.0 mmol/l or receiving lipid-lowering treatment on day of the ischemic event. Patients with a medical history of diabetes or using either oral anti-diabetic medication or insulin therapy on day of the event were considered to be diabetics. Hypertension was defined by a systolic blood pressure ≥ 140 mm Hg, and/or diastolic blood pressure ≥ 90 mmHg or the use of anti-hypertensive drugs. A positive family history was noted if the patient had a first-degree relative with a history of cardiovascular disease before the age of 60.

Written informed consent was obtained on enrollment from each participant, and the study was approved by the Medical Ethics Committee of Erasmus MC and conducted according to the procedures of the Declaration of Helsinki.

Methods

Blood was drawn under strictly standardized conditions [17], by venipuncture in the antecubital vein using the Vacutainer system (Becton Dickinson, Plymouth, UK). Blood for coagulation studies in both patients and controls was collected in 3.2% trisodium citrate (9:1 vol/vol). Citrated blood was centrifuged within one hour at 2,000 g for 10 min at 4˚C. Plasma was additionally centrifuged at 20,000 g for 10 min at 4˚C and stored at -80˚C until analysis.

TAFI antigen levels

Three recently developed sandwich-type ELISAs (i.e. MA-T12D11/MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP) were used to quantify the plasma levels of TAFI, TAFI activation peptide (TAFI-AP) and (in)activated TAFI (TAFIa(i)), respectively [18]. To obtain pooled human plasma, blood samples (n = 21 blood donors) were collected in 4% citrate according to the guidelines of the blood transfusion

centre (Red Cross, Leuven, Belgium) and plasma was pooled. Either non-activated pooled human plasma (MA-T12D11/MA-T30E5-HRP ELISA) or activated pooled human plasma (MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP ELISAs) was used as a standard (1:40 dilution of plasma in PTAE buffer (PBS pH 7.4 containing 0.002% Tween 80, 1g/l BSA and 5 mM EDTA), followed by serial two-fold dilutions up to 1:2560). For preparation of the standard for the MA-T12D11/MA-T18A8-HRP and MA-T30E5/ MA-17D7-HRP ELISAs, pooled human plasma was incubated with thrombin (20 nM), thrombomodulin (5 nM) and $CaCl_2$ (17 mM) in Hepes buffer at 37 °C for 15 minutes. The reaction was stopped by addition of H-D-Phe-L-Prolyl-L-arginine chloromethylketone (PPACK, 30 µM final concentration).

Plasma samples derived from patients were diluted 1:160 (MA-T12D11/MA-T30E5-HRP ELISA), 1:80 (MA-T12D11/MA-T18A8-HRP ELISA) and 1:80 (MA-T30E5/MA-17D7-HRP ELISA). All values of TAFI and TAFI activation markers are expressed relative to the levels in the pooled human plasma.

The assay variability of the ELISAs was evaluated using 4 different plasma samples each assayed 4 times on 4 occasions. The intra-assay and inter-assay coefficients of variation for MA-T12D11/MA-T30E5-HRP were 6.2% and 8.3%, respectively, for MA-T12D11/ MA-T18A8-HRP 3.1% and 7.3%, respectively and for MA-T30E5/MA-T17D7-HRP 3.3% and 6.4%, respectively.

The interdilution coefficients of variation were 8.2%, 5.1% and 7.3% for MA-T12D11/ MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-T17D7-HRP, respectively. The detection limit of all three ELISAs was 1.6%. TAFI-depleted plasma revealed no detectable response in any ELISA.

TAFI activity assay

The TAFI functional assay was performed essentially as described elsewhere [19]. Briefly, 100 µL of diluted plasma samples (20-fold dilution of individual plasmas in TAFI-depleted pooled normal plasma) were added to the wells of a microtitre plate containing 25 µL of a reaction mix composed of thrombin, Solulin (recombinant thrombomodulin which was a gift from PAION GmbH, Aachen, Germany (by courtesy of Dr. H. Brohmann)), $CaCl_2$, and recombinant tissue plasminogen activator (tPA) (Actilyse) (final concentrations in the assay: 3.3 NIH units mL^{-1}, 2.0 µg/ml, 20 mM, 20 mM, and 0.10 µg mL^{-1}, respectively), forming a plasma clot. A control with addition of potato carboxypeptidase inhibitor (PCI) was performed for each sample (final concentration 30 µg mL^{-1}). The wells were immediately covered with paraffin oil and the plate was placed in a pre-warmed (37ºC) incubation

chamber of a microplate reader (Victor^{3,TM} multilabel counter, Perkin Elmer, Turku, Finland). The optical density at 405 nm was monitored for 150 min. Lysis time (LT) was defined as the time-point corresponding to a 50% decrease in optical density. Functional TAFI levels were calculated as TAFI-related retardation (RT), defined as the difference between the LT in the absence and in the presence of PCI (LT_{-PCI} - LT_{+PCI}).

Using this assay the intra- and inter-assay variability of the TAFI-related retardation of pooled normal plasma (20-fold diluted) were 7% and 13%, respectively (n = 30; using several batches of TAFI-depleted plasma) [19]. No influence was detected of the use of oral anticoagulant therapy with vitamin K antagonists on functional TAFI assay (data not shown).

Fibrinogen, CRP and vWF

Fibrinogen levels were determined according to the prothrombin (PT)-derived method (Dade Thrombin Reagent, Siemens Diagnostics, Leusden, The Netherlands) on a Sysmex CA-1500 automated coagulation analyzer (Siemens Diagnostics, Leusden, the Netherlands). When the PT or APTT was prolonged in patients, for instance due to the use of oral anticoagulants, fibrinogen concentrations were determined according to the von Clauss method on an ACL-300 [HemosIL (Fibrinogen-C); Instrumentation Laboratory, Breda, the Netherlands]. CRP levels were measured by means of an in-house high-sensitive ELISA with polyclonal rat anti-human CRP antibodies (DAKO, Glostrup, Denmark) and a CRP-calibrator (DAKO). VWF antigen (vWF:Ag) was determined with an in-house ELISA with polyclonal rabbit anti-human vWF antibodies and horseradish peroxidase conjugated anti-human vWF (DakoCytomation, Glostrup, Denmark) for catching and tagging, respectively. The intra-assay variation coefficient for vWF:Ag was 5.7%.

Statistical analysis

The data are presented as means and standard deviations (SD) for continuous variables and as counts and percentages for categorical variables. To compare the plasma levels of TAFI variables between the controls and the PAD patients an analysis of variance (ANOVA) was used, with adjustment for age and gender. Spearman's correlation coefficient was calculated to study the associations between the various TAFI antigen levels. The association between TAFI antigen levels and PAD was investigated by logistic regression with TAFI antigen as a continuous variable, with adjustment for age and gender. In addition, we used a cut-off approach, in which the risk of PAD in patients with high TAFI levels (top 10% based on distribution of controls) was compared with risk of PAD in the remaining 90% of the

population, with adjustment for age and gender. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the Statistical Package for Social Science for windows, version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

The patient group consisted of 47 patients with a mean age of 43.2 ± 7.9 years (range 21-55 years). The characteristics of the patients and of the 141 individuals who served as a population-based control group are summarized in table 1. Most patients were current or previous smokers (93.6%) and had a positive family history of arterial thrombosis (63.8%) as well as other traditional risk factors. BMI in patients was not significantly higher in patients (25.8 ± 4.8 $Kg.m^{-2}$) than in controls (25.5 ± 4.5 $Kg.m^{-2}$). All patients had objectively diagnosed PAD and could be subclassified according to the Rutherford criteria: 27 (57.4%) patients with claudication (grade 2), 15 (31.9%) patients with ischemic rest pain (grade 3) and 5 (10.6%) patients with tissue loss (grade 4).

Table 1. Characteristics of PAD patients and healthy controls.

	Patients (n=47)	Controls (n=141)	p value
Demographics			
Age – years			
Mean	43.2 ± 7.9	43.0 ± 7.6	0,85
Male sex – n (%)	13 (27.7)	39 (27.7)	0.94
Oral anticoagulant use – n (%)	13 (27.7)	2 (1.4)	< 0.0001
Risk Factors			
Family history of premature cardiovascular disease – n (%)	30 (63.8)	47 (33.3)	< 0.0001
Hypercholesterolemia – n (%)	22 (46.8)	6 (4.3)	< 0.0001
Hypertension – n (%)	18 (38.3)	10 (7.1)	< 0.0001
Diabetes mellitus – n (%)	10 (21.3)	1 (0.7)	0.001
Smoking status – n (%)			
Never smoked	3 (6.4)	60 (42.6)	< 0.0001
Former smoker	21 (44.7)	46 (32.6)	< 0.0001
Current smoker	23 (48.9)	35 (24.8)	< 0.0001
Clinical aspects			
Body-mass index (kg/m^2)	25.8 ± 4.8	25.5 ± 4.5	NS
Rutherford criteria – n (%)			
Asymptomatic (Grade 1)	0	141	
Claudication (Grade 2)	27 (57.4)	-	
Ischemic rest pain (Grade 3)	15 (31.9)	-	
Tissue loss (Grade 4)	5 (10.6)	-	

NS = not significant

Intact TAFI antigen levels were significantly higher in patients with PAD (112.4 ± 21.1%; mean ± SD) versus healthy controls (104.9 ± 19.9%; p=0.03) (figure 1). The risk of PAD increased with 18% (OR 1.18; CI 1.01-1.35) per 10% increase of TAFI antigen. TAFI activation peptide, a marker for TAFI activation in vivo, was not significantly different from the controls, nor were the levels of activated and inactivated TAFI (TAFIa(i)) (table 2).

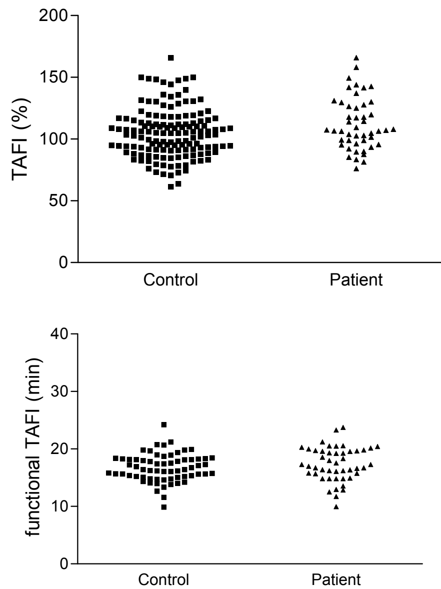

Figure 1. TAFI antigen levels and functional TAFI levels in patients with PAD and healthy controls.

Table 2. Laboratory parameters in PAD patients and healthy controls.

	Patients (n=47)	Controls (n=141)	p value
TAFI parameters			
TAFI antigen (%)	112.4 ± 21.1	104.9 ± 19.9	0.03
TAFI activation peptide (%)	140.9 ± 38.0	145.3 ± 52.5	0.58
TAFI a(i) (%)	139.3 ± 36.3	137.1 ± 34.2	0.71
Functional TAFI [TAFI-related retardation] (min)*	17.4 ± 3.0	16.7 ± 2.4	0.28
Haemostatic/inflammatory parameters			
Fibrinogen (g/L)	4.07 ± 0.85	3.39 ± 0.96	< 0.001
vWF:Ag (U/mL)	1.47 ± 0.52	1.03 ± 0.38	< 0.001
hsCRP (mg/L)	3.29 ± 4.58	1.56 ± 3.58	0.006

*The TAFI functional assay was measured as TAFI-related retardation and expressed in minutes (mean ± SD)

Functional TAFI was measured as TAFI-related retardation in a plasma clot lysis based assay. Functional TAFI was not significantly higher in patients compared to controls (17.4 ± 3.0 min vs. 16.7 ± 2.4 min; p=0.28, respectively) (figure 1). To further investigate whether increased functional TAFI levels are a risk factor for PAD we calculated odds ratios using different cut-off percentiles of functional TAFI, based on the levels in the control group. Individuals with functional TAFI levels in the highest 10% percentile showed an increased risk of PAD compared to individuals in the lowest 90% percentile (OR 3.1; 95% CI 1.02-9.41).

No relationships between TAFI activation peptide and TAFIa(i) with PAD were observed in our study (table 2). Functional TAFI was significantly correlated with TAFI activation peptide (r=0.19, p=0.047), but not with TAFI antigen levels and TAFIa(i) (table 3). The strongest correlation was seen between TAFI antigen levels and TAFI activation peptide levels (r=0.39, p<0.0001) and between TAFI activation peptide levels and TAFIa(i) (r=0.26, p<0.0001).

Table 3. Relationship between the various TAFI antigen levels in PAD patients and healthy control individuals.

	TAFI Ag	TAFI AP	TAFI a(i)	TAFI-related retardation	Fibrinogen	vWF:Ag	hsCRP
TAFI Ag		0.39	0.15	0.16	0.17	0.13	0.06
p value		< 0.0001	0.04	0.10	0.07	0.07	0.43
TAFI AP			0.26	0.19	0.05	0.03	0.05
p value			< 0.0001	< 0.05	0.61	0.67	0.50
TAFI a(i)				-0.02	-0.09	0.05	-0.01
p value				0.80	0.32	0.50	0.93
TAFI-related retardation					0.17	0.10	0.17
p value					0.07	0.30	0.08

Correlations (non-parametric, Spearman 2-tailed)

Fibrinogen, hsCRP and vWF antigen levels were significant higher in patients with PAD versus healthy controls. Mean fibrinogen levels were 4.07 ± 0.85 g/L in PAD patients versus 3.39 ± 0.96 g/L (p<0.001) in healthy controls. Mean hsCRP levels were 3.29 ± 4.58 mg/L in PAD patients versus 1.56 ± 3.58 mg/L (p=0.006) in healthy controls. Mean vWF:Ag levels were 1.47 ± 0.52 U/ml in PAD patients versus 1.03 ± 0.38 U/ml (p<0.001) in healthy controls.

Discussion

We studied for the first time TAFI levels in a well defined group of patients with peripheral arterial disease at young age. We showed that high TAFI levels are associated with increased risk of PAD. This is in line with previous reports that increased TAFI levels are associated with other forms of arterial thrombosis, i.e. ischemic stroke [11, 12].

Previous studies have indicated that hypofibrinolysis may be associated with increased risk of arterial thrombosis including coronary heart disease, ischemic stroke and PAD, although this was not confirmed in all studies [3, 4, 20-25]. This increased risk has mainly been attributed to increased levels of plasminogen activator inhibitor-1 [4]. In addition, it has been suggested that the PAI-1 -675(4G/5G) polymorphism is associated with the outcome of PAD [6].

TAFI is an important inhibitor of fibrinolysis, by cleaving the lysine binding sites of partially degraded fibrin, which interferes with the interaction of plasminogen with fibrin. High TAFI levels may therefore result in a decreased fibrinolytic activity [9, 10]. Several studies have been performed to assess the relationship between TAFI levels and the risk of arterial thrombosis. In previous studies, we and others have found that functional TAFI levels are significantly associated with the risk of first ischemic stroke [11, 26]. Also TAFI antigen levels have been associated with ischemic stroke, (unstable) angina pectoris and acute myocardial infarction [11, 13, 27-29]. Previous studies were hampered by the fact that most antigen assays are highly sensitive to the TAFI genotype, which influences the outcome of some of the early studies (reviewed in [22]). Recently, we have developed genotype-independent TAFI antigen assays, and we were able to analyze TAFI antigen levels using three newly developed immunological assays with distinct reactivities towards TAFI and TAFI fragments [18]. These assays measure intact TAFI, the released activation peptide (TAFI-AP) and the total of activated and inactivated TAFI. So far these assays have only been used to study TAFI levels in acute ischemic stroke, coronary heart disease and severe meningococcal infection [12, 15, 30, 31]. In acute ischemic stroke, it was shown that both intact TAFI and the activation peptide were increased in patients compared to healthy controls [12]. We previously showed that TAFIa(i) levels were higher in young patients with cardiovascular disease in our ATTAC study [15]. In patients with coronary artery disease in the AtheroGene study the amount of activated TAFI, measured by TAFIa/TAFIai ELISA was independently associated with increased risk of cardiovascular death [30]. In severe meningococcal infection in children intact TAFI and the activation peptide were also significantly increased [31].

We have studied young patients with PAD, because it is suggested that in these young patients other pathogenetic mechanisms than the classical risk factors may be of importance [4, 32]. Patients were included one to three months after the first ischemic event to exclude an acute phase response, although previous studies have suggested that the relationship between TAFI levels and arterial thrombosis is both seen in the acute phase and at three-months follow-up [11, 12]. In our study, TAFI antigen levels were significantly higher in PAD patients than in the healthy controls. TAFI-AP levels and TAFIa(i) levels were not higher in PAD patients. Since TAFI-AP is a marker of the extent of TAFI activation, this suggests that TAFI activation is not systemically increased in PAD patients.

We measured functional TAFI levels using a previously described clot lysis based method. By measuring clot lysis time with and without a specific inhibitor of TAFI (PCI) the functional activity of TAFI can be determined. Furthermore, a clot lysis based assay is a more physiological method than assays using chromogenic substrates to study the impact of TAFI on fibrinolysis [19]. A trend was seen for higher functional TAFI activity in PAD patients compared to controls, but this did not reach statistical significance. Individuals with functional TAFI levels above the 90th percentile however had an increased risk of PAD.

We also studied other haemostatic variables (Fibrinogen and vWF:Ag levels) and a marker of inflammation (hsCRP), which are of importance as risk factors in the development of PAD [33], in relation to TAFI. Fibrinogen, hsCRP and vWF were all significantly elevated in PAD patients in comparison to the healthy controls. However, the correlation between TAFI antigen with fibrinogen, vWF Ag and CRP was very weak. This suggests that the association between TAFI and PAD is independent of an inflammatory status or endothelium damage. Prospective studies are needed to investigate the association between TAFI levels and cardiovascular disease as, on the one hand, high TAFI levels may facilitate the development of cardiovascular comorbidity by shifting the haemostatic balance to a more hypofibrinolytic state and, on the other hand, TAFI levels may relate to the body's mechanisms dampening the excessive inflammatory reaction, such as previously found in patients with rheumatoid arthritis [34, 35]. Growing evidence suggests that inflammation, oxidative stress and hypofibrinolysis may have a pivotal role in the high prevalence of cardiovascular disease [36].

Our study has some important strengths. We measured TAFI antigen levels using three recently developed TAFI assays that are independent of TAFI genotypes. These assays make it possible to determine not only intact TAFI levels, but also provide information on TAFI activation. In addition, we have measured functional TAFI activity. A limitation is the

small size of our group of PAD patients. Only patients with a first clinical manifestation of peripheral arterial disease at young age were included. The statistically significant difference that we observed does not automatically mean that our findings are also of clinical relevance. Whether there is a role for TAFI in determining the outcome of PAD was not the aim of our study and needs to be studied in large, prospective studies. We selected our patients and controls from the same population (controls were friends of the patients), which eliminates part of the potential patient-control differences, but that remaining differences between patients and controls may contribute to our findings.

In conclusion, our study, although carried out in a small group of patients, shows for the first time that increased levels of TAFI are associated with risk of premature peripheral arterial disease.

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CHAPTER 6

HYPOFIBRINOLYIS IS A RISK FACTOR FOR ARTERIAL THROMBOSIS AT YOUNG AGE

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Summary

The relationship between defective fibrinolysis and arterial thrombosis is uncertain. The evaluation of the plasma fibrinolytic potential might provide stronger evidence linking fibrinolysis to arterial thrombosis than the evaluation of the individual fibrinolytic factors. We determined the plasma fibrinolytic potential of 335 young survivors of a first arterial thrombosis, including coronary artery disease (n = 198), ischaemic stroke (n = 103) and peripheral artery disease (n = 34), enrolled in a population-based case–control study and of 330 healthy individuals. Patients had significantly higher clot lysis times (CLTs) than the controls. Odds ratios (ORs) were calculated as a measure of relative risk. The OR for arterial thrombosis was determined in these subjects who had a CLT above the 60th, 70th, 80th, 90th and 95th percentiles of the values found in the control subjects. We found a progressive increase in risk of arterial thrombosis in subjects with hypofibrinolysis (OR: 1.7, 2.0, 2.3, 2.3 and 2.9, respectively). Relative risk estimates obtained in the whole group were comparable those obtained in the event-subgroups. In conclusion, a low plasma fibrinolytic potential, found in 10% of the population, increases the relative risk of arterial thrombosis twofold. This points to an important contribution of hypofibrinolysis to the burden of arterial thrombosis.

Introduction

In arterial thrombosis at young age, traditional risk factors such as gender, smoking, diabetes mellitus, obesity and hypercholesterolemia do not fully explain the cardiovascular risk. Therefore, additional factors must be present that contribute to the likelihood of developing arterial thrombosis [1]. The preponderance of the current evidence supports that a hypercoagulable state contributes to the risk of thrombosis, whereas fibrinolysis is often regarded as a secondary process [2, 3]. The contribution of individual proteins involved in fibrinolysis to the risk of arterial thrombosis has been previously investigated [4-6] and has led, for instance, to the classification of plasma apolipoprotein(a), which shares several homologous domains with plasminogen and thus potentially inhibits fibrinolysis, as an 'emerging' risk factor for arterial thrombosis [2, 7]. In addition, significant associations with arterial thrombosis have also been reported for two inhibitors of fibrinolysis, i.e. plasminogen activator inhibitor-1 (PAI-1) [8-10] and thrombin activatable fibrinolysis inhibitor (TAFI) [11-13]. However, the interpretation of the role of the individual components of the fibrinolytic system is far from clear and evidence for the involvement of the fibrinolytic system in the development of arterial thrombosis is still scarce. Moreover, the fibrinolytic component in a multifactorial disease, such as arterial thrombosis, is expected to be the consequence of multiple inter-linked factors and combined defects and might be therefore better evaluated with a global fibrinolytic assay. Some previous studies support the involvement of impaired fibrinolysis, i.e. hypofibrinolysis in the risk of arterial thrombosis [4, 5, 13-15] but the assays employed do not really reflect a global determination [4, 6].

This study evaluated the contribution of the fibrinolytic system in arterial thrombosis by determining the global plasma fibrinolytic potential [16] of the survivors of a first arterial thrombosis at young age in either the cerebral, cardiac or peripheral vascular system.

Methods

Study population

The **A**rterial **T**hrombosis at young age: the role of **TA**FI and other **C**oagulation factors study (ATTAC) is a single-centre case–control study, which included 374 consecutive patients with arterial thrombotic disease at young age and 332 age and sex-matched controls. Briefly, patients were eligible for inclusion if they were aged between 18 and 45 years for men or between 18 and 55 years for women and suffered a first acute ischaemic complication in one of the vascular organ systems (cardiac, cerebral or peripheral vascular system). Patients were subdivided into: (i) coronary heart disease (CHD), which included patients

with acute myocardial infarction (AMI) or unstable angina pectoris (UAP); (ii) ischaemic stroke (IS), which also included patients with transient ischaemic attacks (TIA) and (iii) patients who suffered from peripheral arterial disease (PAD). Patients were included 1–3 months after the event, to avoid an acute phase response. Controls were friends, neighbours or partners of the patients fulfilling the same age criteria but without a history of arterial thrombosis. The study protocol was in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of Erasmus MC. Written informed consent was obtained from each participant. A total of 41 individuals (two controls and 39 patients) were excluded from our analysis because they were using oral anticoagulants at the time of blood collection. The 39 excluded patients originated from the three event sub-groups (six IS, 20 CHD and 13 PAD patients).

Definitions

AMI was defined as typical chest pain, with elevated cardiac markers (creatinine kinase isoenzyme MB, troponin T) or characteristic electrocardiographic findings. UAP was defined as typical chest pain, while resting. TIA was defined as suddenly occurring focal cerebral deficit, which could not be explained otherwise than as local cerebral ischaemia. Symptoms had to be temporary and last less than 24 h after onset. IS was defined as suddenly occurring cerebral deficit, which cannot be explained otherwise than as local cerebral ischaemia with symptoms lasting longer than 24 h. Brain imaging by computed tomography or magnetic resonance imaging was carried out in all patients and had to be compatible with the initial diagnosis. The diagnosis PAD was defined as peripheral arterial stenosis resulting in ischaemia, classified according to the Rutherford criteria [17]. Clinical data were collected using a standard medical questionnaire concerning the presence of cardiovascular risk factors such as smoking habits, and research physicians performed physical examination. Smoking status was defined as never, previous or current smoker. Patients using either anti-diabetic medication or insulin on the day of inclusion were considered diabetics. Hypertension was defined as a systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg, or use of anti-hypertensive medication on the day of inclusion. A positive family history was noted if the patient had a firstdegree relative with a history of cardiovascular disease before the age of 60 years.

Blood collection

Blood was drawn under strictly standardized conditions by venipuncture in the antecubital vein using the Vacutainer system (Beckton Dickinson, Plymouth, UK). Citrated

platelet-poor plasma was prepared by a two-step centrifugation of blood, set at 2,000 g for 10 min, 4°C and at 20,000 g for 10 min, 4°C. Aliquots were stored at -80°C until use.

Plasma fibrinolytic potential

Lysis of a tissue factor-induced plasma clot by exogenous tissue-type plasminogen activator (tPA) was studied essentially as described previously with some minor modifications [16]. The final concentrations in the clotted plasma were tissue factor (1000 times diluted), $CaCl_2$ (17 mmol/l); tPA (25 ng/ml; Actilyse, Boehringer Ingelheim, Germany) and phospholipid vesicles (10 μmol/l). After mixing the diluted plasma and the reaction mixture, each clot was covered with paraffin oil (50 μl; Merck – No.107162) and the plate was placed immediately in a prewarmed (37°C) incubation chamber of a microplate reader (Victor^{TM} multilabel counter; Perkin Elmer, Turku, Finland). The optical density at 405 nm was monitored every minute for 400 min. The clot lysis time (CLT) was defined as the time from the midpoint of clear to maximum turbidity transition, which characterizes clot formation, to the midpoint of maximum turbid to clear transition, which represents clot lysis. Three individual plasma samples (two CHD patients and one control subject) did not reach the midpoint of maximum turbidity to clear transition within 400 min and were attributed a CLT of 400 min. A prolonged CLT in comparison to the control group CLTs is indicative of hypofibrinolysis while a shortened CLT is indicative of hyperfibrinolysis. The intra- and inter-assay variation coefficients were 3.5% (n = 48) and 6.5% (n = 42), respectively.

Statistical methods

Data are presented as means and standard deviations for continuous variables and as counts and percentages for categorical variables. The plasma fibrinolytic potential, given as CLT, presented a skewed distribution and required therefore logarithmic transformation before analysis. CLT values have been summarized as median and interquartile ranges (IQR). The contribution of elevated CLTs to the risk of arterial thrombosis was analysed by calculating crude odds ratios (OR) as estimates of the relative risk (RR) using binary logistic regression. Unconditional logistic regression was used to adjust for age and other cardiovascular risk factors. The increase in CLTs in the patient's subgroups, which points to hypofibrinolysis, was compared against the control group using one-way analysis of variance (ANOVA) followed by the Dunnett's test as post-test. Statistical analyses were performed both for the entire group of patients and within the subgroups of patients with CHD, IS and PAD.

All analyses were performed with the Statistical Package for the Social Sciences (SPSS) version 11.0.1 using two-sided tests at the 5% significance level.

Results

Plasma fibrinolytic potential in the ATTAC study population

The ATTAC study population as analysed in this study was composed of 335 patients and 330 controls of which 43% of the patients and 37% of the control group were male (Table 1). The mean age of the patients was higher than that of the controls (patients, 43 ± 7 years versus controls, 38 ± 8 years, P = 0.004). The body mass index of the patients was higher than that of the control group (i.e. 25.0 ± 4.2 vs. 26.4 ± 4.4 kg/m^2, P = 0.0001). Also, the prevalence of known classical cardiovascular risk factors, e.g. diabetes mellitus, was significantly higher in the patient group than in the control population (8% in the patient group vs. 1.5% in the control group). The vast majority of the patients had other vascular risk factors as well. Among the 335 patients, 198 (59%) had CHD, 103 (31%) had IS and 34 (10%) had PAD.

Table 1. Characteristics of patients and control subjects.

	Patients (n = 335)	Controls (n = 330)	*P*-value
Mean age, years	43 ± 7	38 ± 8	0.004
Mean BMI, kg/m^2*	26.4 ± 4.4	25.0 ± 4.2	0.0001
Gender, male	144 (43%)	122 (37%)	NS
Diabetes mellitus	27 (8%)	5 (1.5%)	<0.001
Smoking†	270 (81%)	167 (51%)	<0.001
Positive family history	222 (66%)	118 (36%)	0.0001

Values are given as n (%) or as mean ± SD.
*BMI, body mass index.
†Smoking status includes previous and current smokers.

The CLTs in the patient group were significantly higher than in the control group [median (IQR) respectively 94.1(27.8) vs. 87.4 (22.3), P = 0.001], indicative of hypofibrinolysis in arterial thrombosis patients. In the event subgroups (CHD, IS, PAD – Fig 1), the CLTs were elevated in comparison to the control group but the difference was only statistically significant for the CHD subgroup [97.5 (30.7) vs. 87.4 (22.3), P = 0.0001], whereas there was a trend for ischaemic stroke [90.8 (22.3) vs. 87.4 (22.3), P = 0.07].

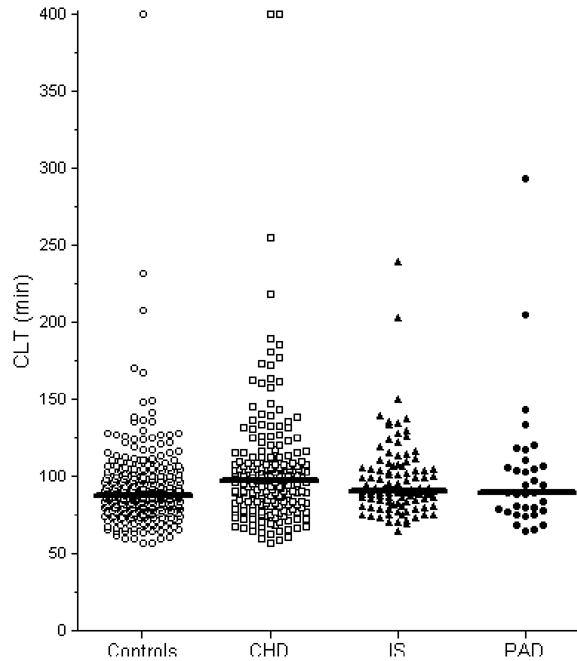

Figure 1. Hypofibrinolysis and Arterial Thrombosis.
Distribution of plasma fibrinolytic potential (CLT) in the ATTAC study population. The study population was subdivided into the control group (n = 330) and the event subgroups, namely, coronary artery disease (CHD, n = 198), ischaemic stroke (IS, n = 103) and peripheral artery disease (PAD, n = 34). An elevated CLT is indicative of hypofibrinolysis in arterial thrombosis patients. The horizontal line indicates the median.

Association of classical cardiovascular risk factors with the plasma fibrinolytic potential

The effect of a series of cardiovascular risk factors on the CLT values in the control individuals is shown in Table 2. CLTs were not significantly associated with age or oral contraceptive use. Furthermore, no associations were found in control subjects between CLTs and gender, smoking status, alcohol use, blood pressure or family history of CHD. Obese individuals had elevated CLTs (P = 0.001) and a linear trend was observed between the CLTs and the body mass index in control subjects (P = 0.0004). Control subjects with diabetes exhibited higher CLTs values (P = 0.001) than controls without diabetes.

Hypofibrinolysis as a risk for arterial thrombosis

In order to determine the contribution of elevated CLTs to the risk of arterial thrombosis, cut-off levels of CLTs in the control group were set at 60th, 70th, 80th, 90th and 95th

Table 2. Cardiovascular risk factors among control subjects and the association with the plasma fibrinolytic potential.

Risk factor	*N* controls	Median CLT (IQR), min	*P*-value
Age, years			
<25	19	85.4 (14.2)	0.3
25 to <35	85	86.6 (21.8)	
35 to <45	164	88.5 (20.7)	
45 to 55	62	91.4 (25.7)	
Gender			
Male	122	87.3 (21.1)	0.3
Female	208	87.5 (23.2)	
Oral contraceptives			
No	121	90.0 (23.9)	0.1
Yes	67	82.1 (18.9)	
Smoking			
Never	163	87.4 (22.8)	0.6
Former	79	86.7 (19.4)	
Current	88	89.3 (25.2)	
Alcohol use			
No	221	88.9 (22.1)	0.3
Yes	109	86.4 (22.1)	
Body mass index			
<20 kg/m^2	24	82.6 (21.0)	0.001
20–25 kg/m^2	162	85.8 (20.1)	
25–30 kg/m^2	113	89.3 (23.4)	
At least 30 kg/m^2	30	105.0 (17.0)	
Diabetes			
No	322	87.1 (21.2)	0.001
Yes	5	109.0 (73.3)	
Family history of CHD			
No	207	88.4 (21.9)	0.6
Yes	118	85.9 (21.6)	

P-values derived using logarithmically transformed CLTs values by either unpaired t-test or by one-way ANOVA.
IQR, inter-quartile range.

percentiles. A gradual increase of the ORs for arterial thrombosis in subjects with CLTs above the cut-off levels was observed (Table 3). The values below the cut-off were used as reference group. Among the subjects with an arterial thrombosis, 69 patients (i.e. 21% of the patients) had a CLT above the 90th percentile cut-off point. It follows that a CLT above (i.e. the 90th percentile) was associated with a 2.3-fold increased risk of arterial thrombosis [OR 2.3, 95% confidence interval (CI) 1.5–3.6]. This OR was comparable to

Table 3. Risk of arterial thrombosis according to the plasma fibrinolytic potential (CLTs).

Cut-off (percentile)	CLTs at cut-off (min)	*N* patients (%)	*N* controls (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
60	92.0	176 (53%)	132 (40%)	1.7 (1.2–2.3)	1.3 (1.0–1.8)
70	97.5	153 (46%)	99 (30%)	2.0 (1.4–2.7)	1.6 (1.1–2.2)
80	103.9	122 (36%)	65 (20%)	2.3 (1.6–3.3)	2.0 (1.4–2.9)
90	113.7	69 (21%)	33 (10%)	2.3 (1.5–3.6)	2.1 (1.3–3.3)
95	127.4	43 (13%)	16 (5%)	2.9 (1.6–5.2)	2.1 (1.1–3.9)

Cut-off levels of CLTs in the control group were set at the 60th, 70th, 80th, 90th and 95th percentiles and ORs for the development of arterial thrombosis were calculated by binary logistic regression. Adjusted ORs have been corrected for age and gender.

the OR obtained for the presence of diabetes (OR 2.5, 95%CI 1.3–4.8) in the ATTAC-study population (not shown). Adjustment for either gender or age alone produced no significant effect on the OR while the combination of age and gender resulted in a slight decrease in the OR for the 90th percentile [2.1 (95%CI 1.3–3.3)]. Further adjustment for body mass index (BMI), resulted in a decrease in the OR for the 90th percentile, but was still significant [1.9 (95%CI 1.2–3.0)]. Full adjustment for age, gender, BMI, smoking status, diabetes, total cholesterol levels, blood pressure and homocysteine levels did not alter the OR, which remained elevated at an OR for the 90th percentile of 1.9 (95%CI 1.1–3.3).

Of the 69 patients who had a CLT above the 90th percentile cut-off point, 44 patients belonged to the CHD event subgroup, 18 patients to the IS subgroup and seven to the PAD subgroup. Relative risk estimates for CLTs above the 90th percentile cut-off point were comparable for the three event subgroups [i.e. OR 2.6 (95%CI 1.6–4.2), OR 1.9 (95%CI 1.0– 3.6) and OR 2.3 (95%CI 0.94–5.8) for CHD, IS and PAD, respectively].

Discussion

Evidence for a relationship between a fibrinolytic deficit and the risk of arterial thrombosis is still controversial [4, 5, 18]. In the past, assessment of the fibrinolytic capacity using global tests pointed to a role for fibrinolysis in arterial thrombosis [15]. However, the above-mentioned global fibrinolysis assays (e.g. the diluted blood clot lysis time and the euglobulin clot lysis did not accurately reflect an overall determination time [13, 14, 19, 20]. Subsequent research focused on the determination of plasma levels of individual components of the fibrinolytic pathway (reviewed in [4-6]), which has renewed the ongoing discussion of whether fibrinolysis is involved in the aetiology of arterial thrombosis. Recently, a new plasma-based clot lysis assay, in which a tissue factor-induced plasma clot

is lysed by exogenous tPA has been described [16]. This plasma fibrinolytic potential was shown to reflect an overall determination as it was influenced by levels of several proteins involved in fibrinolysis (e.g. PAI-1 and antiplasmin) [21].

We therefore used this assay to investigate the contribution of fibrinolysis in arterial thrombosis. Our results show that patients with arterial thrombosis displayed a reduced plasma fibrinolytic potential. The risk of arterial thrombosis was higher for individuals with elevated CLTs. In fact, individuals with high CLT, defined as a value above the 90th percentile of the distribution of values in the control subjects, presented a relative risk of 2.3 (95%CI, 1.5 to 3.6) as compared to individuals with lower CLTs. This relative risk was comparable to the relative risk of diabetes (2.5, 95% CI 1.3–4.8) in the ATTAC-study population and also similar to the reported relative risk associated with elevated factor VIII levels [i.e. 2.3 (95%CI 1.0–5.1)] [22].

Our findings could not be ascribed to effects related to age or gender. Previously, Lisman et al observed an effect of gender and age on the CLT values in the control subjects [16]. In our study, no differences were observed on the CLTs between male and female while a trend was observed for age but this association did not reach statistical significance. This may be due to the fact that the control subjects in the study by Lisman et al were aged between 14 and 72 years while in the ATTAC-study the range was from 18 to 55 years [16].

In classic case–control studies, such as in the ATTAC study, there is always a possibility of reversed causality, i.e. that the outcome has caused the determinant, instead of the other way around. An important argument against reversed causality is the time (1–3 months) that has elapsed between the index event and the moment of blood sampling and assessment of CLT.

We observed a decrease in the relative risk after adjustment for BMI (besides age and gender). In our study population, the BMI was positively associated with age and with CLTs. In addition, CLTs were also associated with the occurrence of diabetes. These results suggest the involvement of PAI-1 in the observed effects. PAI-1 levels correlated with advancing age, body mass, plasma insulin levels and the presence of diabetes, which compose a cluster of metabolic abnormalities denominated as metabolic syndrome [9].

Further adjustment for known risk factors (such as smoking, diabetes, systolic and diastolic blood pressure and homocysteine levels) besides age, gender and BMI, did not further explain the increased risk of arterial thrombosis associated with high CLTs, i.e. with hypofibrinolysis (fully adjusted RR 1.9, 95%CI 1.1–3.3). This leads us to conclude that hypofibrinolysis is an important contributor to the overall burden of arterial thrombosis.

In the event subgroups, only the coronary artery disease patients displayed a significant increase in CLTs. As the CHD subgroup constituted a large section of the patient population, it may result in a more accurate estimation of the effects. However, similar relative risk estimates were found for the three event subgroups for CLT levels above the 90th percentile of the distribution in the control subjects, which agree well with the doubling of the relative risk observed for the entire group.

In conclusion, a low plasma fibrinolytic potential, which is indicative for hypofibrinolysis, constitutes a risk factor for arterial thrombosis in young individuals. These findings justify the further evaluation of the causality of hypofibrinolysis in the arterial thrombosis, by determining the plasma fibrinolytic potential in prospective studies in different populations.

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Conflicts of interest

The authors have no conflicts to disclose.

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CHAPTER 7

THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR IS ASSOCIATED WITH SEVERITY AND OUTCOME OF SEVERE MENINGOCOCCAL INFECTION IN CHILDREN

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Summary

Background and objectives: In pediatric meningococcal sepsis, an imbalance between coagulation and fibrinolysis and proinflammatory action play major roles. We hypothesized that thrombin activatable fibrinolysis inhibitor (TAFI) and/or TAFI activation markers are involved in the pathogenesis of meningococcal sepsis.

Patients and methods: Children with severe meningococcal sepsis (n = 112) previously included in Rotterdam-based trials participated in this study. Clinical and laboratory parameters and severity scores were assessed. TAFI and TAFI activation markers were determined: TAFI activation peptide (TAFI-AP) and (in)activated TAFI [TAFIa(i)]. The –438G/A, Ala147Thr, and Thr325Ile polymorphisms were genotyped.

Results: TAFI levels were significantly decreased in patients with meningococcal disease at admission compared to the convalescence state. TAFI was decreased in patients with septic shock vs. those with no shock. TAFI-AP levels were increased in patients with disseminated intravascular coagulation (DIC) vs. patients without DIC. TAFI-AP and TAFIa(i) were significantly increased in non-survivors vs. survivors. TAFI-AP levels and the TAFI-AP/TAFI ratio were also strongly correlated to severity scores and laboratory parameters. The TAFI 325Ile/Ile genotype was overrepresented in patients with DIC.

Conclusions: Activation markers of TAFI were associated with the occurrence of DIC and mortality in meningococcal sepsis patients. A determination of TAFI, TAFI-AP, and TAFIa(i) is required to enable coherent interpretation of the role of TAFI in disease.

Introduction

The observed clinical phenotype in meningococcal infection is thought to be determined by the activation of inflammatory, coagulation, and fibrinolysis pathways. While meningococcal infection results in a 'mild' sepsis with petechiae in some patients, others develop septic shock and/or disseminated intravascular coagulation (DIC). This, in combination with ischemia, may result in multi-organ failure. Although improved treatment possibilities have resulted in a decreased mortality over the past 15 years, up to 2% of meningococcal infections are still lethal in developed countries [1]. A significant number of patients suffer from long-term sequelae, including minor or major amputations. Thrombin activatable fibrinolysis inhibitor (TAFI) has been described as a potent inhibitor of fibrinolysis in vitro [2,3]. TAFI is a glycoprotein that is present in the plasma as a proenzyme and is activated by thrombin/thrombomodulin or plasmin by a single cleavage at Arg-92. This results in the activation peptide (TAFI-AP) and in the exposure of the substrate binding site of activated TAFI (TAFIa). TAFIa is thermolabile (half-life of 8–15 min at 37 °C) as a result of a spontaneous conformational conversion into an inactive form [TAFIa(i)], which is prone to further proteolytic cleavage.

TAFIa exerts its antifibrinolytic function by removing the carboxy-terminal lysine residues of partially degraded fibrin. These lysine residues are required for binding of plasminogen and tissue-type plasminogen activator (t-PA) and efficient plasmin formation. Removal of the lysine residues results in decreased binding and activation of plasminogen and induces inhibition of fibrinolysis [4,5]. Still, although plasma of TAFI-deficient mice fails to prolong clot lysis time in vitro, no differences are observed in in vivo markers of hemostasis and endotoxin-induced DIC between wild-type and TAFI-deficient mice [6,7].

Exuberant activation of TAFI could result in decreased degradation of thrombi and therefore in exacerbation of multiorgan failure. In addition, in vitro and animal studies have revealed that TAFIa also has an anti-inflammatory role by inactivating C5a, resulting in decreased vascular permeability and risk of septic shock [8,9]. Furthermore, TAFI-deficient mice appear to be protected from sepsis-induced liver injury [7]. Which of these functions dominates is currently unknown. In studies on adults with sepsis, TAFI levels were decreased [10,11]. The cause of this decrease, activation or consumption of TAFI was not revealed, because TAFI activation markers were not determined [10]. Knoefler et al. [12] observed no differences in TAFI levels in children of different age groups with and without hematological malignancies.

A variety of single nucleotide polymorphisms (SNPs) have been described for the TAFI gene. However, it remains rather unclear to what extent genetic variations in TAFI contribute to the disease severity [13].

In this study we investigated the levels of TAFI, the activation markers TAFI-AP and TAFIa(i), and TAFI polymorphisms in a cohort of pediatric patients with severe meningococcal infection. We analyzed whether these parameters were associated with survival, the presence of shock as a marker for inflammatory activity, and the presence of DIC as a marker for a possible involvement in fibrinolysis. We also analyzed the possible correlations of TAFI/TAFI activation markers with other coagulation and fibrinolysis related factors as well as inflammatory and endocrine factors.

Materials and methods

Participants

Patients consecutively admitted to the pediatric intensive care unit (PICU) of the Erasmus University Medical Center-Sophia (Rotterdam, the Netherlands), who previously participated in Rotterdam-based meningococcal sepsis studies between 1988 and 2005, were eligible for the current study (n = 279) [14–19]. Children were enrolled in the study after obtaining approval of the medical ethical committee and informed consent from the parents or guardians. The 112 patients from whom DNA and/or citrate blood were available were included. Clinical inclusion criteria were presentation with tachycardia, tachypnea, body temperature <36 °C or >38.5 °C (rectal) and petechiae [20]. Prospective data on all patients were collected at various time points. Both laboratory parameters and disease severity scoring systems, such as the PRISM score, the predicted death rate based on the Rotterdam score, and the presence of DIC, were selected as markers of severity of disease [21–23]. DIC was scored on admission and during the first 24 h, as some patients do not meet the criteria at first, but do develop DIC during the first hours in the PICU.

All laboratory parameters were obtained at baseline from an arterial blood sample that was collected within 4 h after admission to the PICU (t = 0, n = 67). Additionally, blood was sampled at t = 1 day for TAFI measurements (n = 58). For some of the patients, blood was also drawn 0.5 days after admission (n = 28) and 90 days after admission (n = 13). Differences in the number of patients at different time points are the result of inclusion of patients in several different studies, which did not all include a blood sample being drawn at t = 0.5 days. Additionally, no more blood samples were drawn when an arterial line was no longer available, when a patient was transported back to the referral hospital, or when

a patient died. At t = 90 days, according to regulations of the medical ethical committee, blood could only be drawn from cooperative children.

Laboratory tests

N. meningitidis serotyping

The *Neisseria meningitidis* serogroup was determined in cultured isolates at the Netherlands Reference Laboratory for Bacterial Meningitis Amsterdam using immunodiffusion with polyclonal antisera [24].

TAFI concentration

Citrate blood was drawn and the plasma stored at -80 °C until TAFI concentrations were determined without prior thawing. Three sandwich-type ELISAs (i.e. MA-T12D11/MA-T30E5-HRP, MA-T12D11/MA-T18A8-HRP, and MA-T30E5/MA-17D7-HRP) were used to quantify the plasma levels of TAFI, TAFI-AP, and TAFIa(i), respectively [25]. To obtain pooled human plasma, blood samples (n = 21 blood donors) were taken on 4% citrate according to the guidelines of the blood transfusion centre (Red Cross, Leuven, Belgium) and plasma was pooled. Either non-activated pooled human plasma (MA-T12D11/MA-T30E5-HRP ELISA) or activated pooled human plasma (MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP ELISAs) was used as a standard: 1:40 dilution of plasma in PTAE buffer (PBS pH 7.4 containing 0.002% Tween 80, 1 g L^{-1} BSA and 5 mM EDTA), followed by serial 2-fold dilutions up to 1:2560. For preparation of the standard for the MA-T12D11/MA-T18A8-HRP and MA-T30E5/MA-17D7-HRP ELISAs, pooled human plasma was incubated with thrombin (20 nM), thrombomodulin (5 nM) and $CaCl_2$ (17 mM) in HEPES buffer at 37 °C for 15 min. The reaction was stopped by addition of H-D-Phe-L-Prolyl-L-arginine chloromethylketone (PPACK, 30 μM final concentration).

Plasma samples derived from patients were diluted 1:160 (MA-T12D11/MA-T30E5-HRP ELISA), 1:80 (MA-T12D11/MA-T18A8-HRP ELISA) and 1:80 (MA-T30E5/MA-17D7-HRP ELISA). All values of TAFI and TAFI activation markers are expressed relative to the levels in the pooled human plasma.

Determination of levels of cytokines, coagulation factors and endocrinological factors

Plasma or serum levels of different parameters were determined previously [14,15,18,26].

DNA isolation and genotyping

DNA isolation from whole blood was performed with column methods using standard protocols (Qiagen, Inc., Valencia, CA, USA). Bi-allelic discrimination with *Taq*man analysis was used to determine the TAFI -438 G/A (rs2146881), Ala147Thr (505G/A, rs3742264), and Thr325Ile (1040C/T, rs1926447) genotypes in Caucasian patients only ($n = 82$). Primer sequences are available upon request from the corresponding author.

Statistical analysis

Statistical analysis was performed using SPSS 11.0. Binomial variables were analyzed using Pearson's chi-square test (2df) or Fisher's exact test when appropriate. For continuous variables, the Mann–Whitney U-test was used. Spearman's correlation was determined to assess the relation between TAFI levels and severity scores or laboratory parameters. TAFIa(i) and TAFI-AP levels were log transformed, resulting in a near normal distribution. These log transformed levels were analyzed in relation to different outcome variables, severity scores, and genotypes using the Student's *t*-test. Geometric mean and 95% CIs are depicted [27]. A comparison of genotype frequencies was made between outcome variables such as survival, need of ventilation, and presence of DIC. Probability (P) values < 0.05 were considered to be statistically significant. No correction was made for multiple testing.

Results

In total, 112 patients were included in this study. Sixty-nine (62%) were males and the median (range) age was 2.9 years (0.1–16.1). Septic shock was observed in 96 patients (87%). The median (range) PRISM score was 19 (1–43). Twelve patients did not survive (11%; Table 1). On admission, 60 patients (54%) met the criteria for DIC, whereas an additional 16 patients (14%) fulfilled the DIC criteria during the first 24 h of their stay in the PICU. In 95 patients (85%), blood cultures were positive for *N. meningitidis*. Serotype B was found in 72 of these, while 16 had serotype C. In the remaining seven, no serotype was determined. Additionally, *N. meningitidis* specific polymerase chain reaction (PCR) was positive in two patients. The remaining 15 patients had possible meningococcal infection based on clinical criteria [28].

TAFI levels and survival

Plasma at $t = 0$ was available for 57 survivors and 10 non-survivors. At $t = 0.5$ and 1 day, plasma was available from three and two non-survivors, respectively. Other patients died in the period between blood sampling. At $t = 0.5$ and 1 day, plasma was available from 25 and 56

survivors, respectively. At admission, levels of TAFI and TAFIa(i) were strongly decreased in the total group of patients with meningococcal infection (Table 2). TAFI-AP levels were normal at admission, but decreased to about 75% of normal reference levels during the first day. In survivors, TAFI levels returned to normal 90 days after admission (Table 2).

Table 1. Patient characteristics.

	Non-survivor *n* = 12	Survivor *n* = 100	*P**
Male gender (%)	9 (75.0)	60 (60.0)	0.37
Median age (min–max)	0.83 (0.46–9.43)	3.57 (0.12–16.11)	0.000
Presence of shock (%) *n* = 111	12 (100)	84 (84.8)	0.36
Mechanical ventilation (%) *n* = 110	12 (100)	55 (56.1)	0.003
Presence of DIC (%)	12 (100)	48 (48)	<0.001
PRISM score† (min–max) *n* = 100	31.5 (23–43)	18.0 (1–37)	0.000
Predicted death rate‡ (min–max) *n* = 104	95.9 (55.1–99.9)	5.7 (0.0–96.3)	0.000
Meningococcal serogroup			
B (%)	9 (90.0)	63 (80.8)	0.68
C (%)	1 (10.0)	15 (19.2)	

*Fisher's exact test or Mann–Whitney U-test were performed when appropriate.
†Related to the first six hours. ‡Based on the Rotterdam score. DIC, disseminated intravascular coagulation.

Table 2. Thrombin activatable fibrinolysis inhibitor (TAFI), TAFI activation peptide (TAFI-AP) and (in)activated TAFI [TAFIa(i)] levels in plasma of pediatric patients with meningococcal sepsis.

Time point (days)	0 *n* = 67	0.5 *n* = 28	1 *n* = 58	90 *n* = 13
TAFI*	26.5 (10.6)	36.5 (13.5)	40.1 (11.0)	91.1 (16.9)
TAFI-AP†	100 (80–126)	84 (61–115)	77 (66–90)	123 (100–150)
TAFIa(i)†	61 (53–70)	73 (60–90)	59 (52–67)	137 (108–173)
TAFI-AP/TAFI‡	2.9 (0.8–47.5)	2.1 (0.9–19.4)	1.7 (0.8–8.6)	1.4 (0.9–3.1)
TAFIa(i)/TAFI‡	2.6 (0.7–11.0)	2.2 (0.7–4.7)	1.5 (0.5–3.6)	1.6 (0.8–2.6)

*Mean (SD) % relative to pooled normal plasma. †Geometric mean (95% CI) % relative to pooled normal plasma. ‡Ratio, median (range).

In subgroup analysis, non-survivors and survivors had similar levels of TAFI at all time points (Fig. 1A). The TAFI-AP levels were significantly higher in non-survivors than in survivors on admission ($P < 0.001$; Fig. 1B). In addition, TAFI-AP levels were three to six times increased at other time points in non-survivors compared to survivors and no overlap was present; however, because of the small number of patients in the non-survivor group, no statistical analysis could be performed. In survivors, TAFI-AP levels were similar to the normal reference levels at all time points. On admission, the TAFIa(i) levels were significantly higher ($P = 0.047$) in non-survivors than in survivors, corroborating an enhanced TAFI activation in the non-survivor group (Fig. 1C).

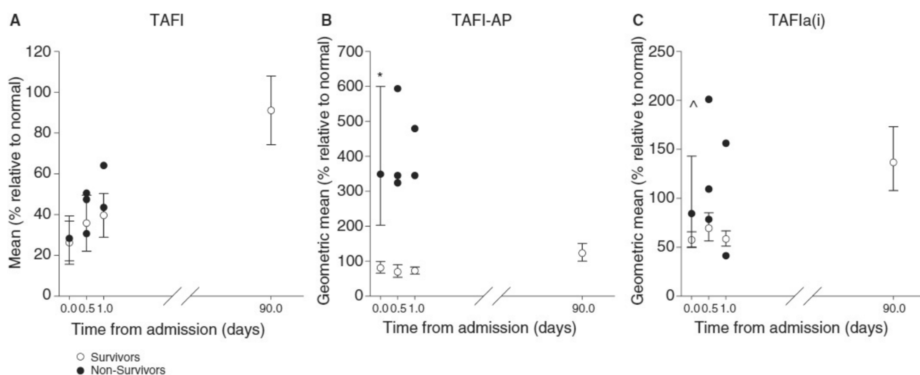

Figure 1. Plasma thrombin activatable fibrinolysis inhibitor (TAFI) and activation marker levels in survivors and non-survivors. Geometric mean or mean plasma TAFI levels (% relative to normal controls) on admission and after 0.5, 1, and 90 days as listed. Error bars represent SD for TAFI and 95%CI for TAFI activation peptide (TAFI-AP) and (in)activated TAFI [TAFIa(i)]. The numbers of survivors at these time points are 57, 25, 56, and 13, respectively. The numbers of non-survivors at these time points are 10, 3, and 2, respectively. As only very few non-survivors were left at t = 0.5 and 1 day, individual levels are depicted for these patients, and statistical analysis was performed only on admission. (A) TAFI levels. (B) TAFI-AP levels. (C) TAFIa(i) levels. *P < 0.001; ^P = 0.047.

TAFI levels and DIC and septic shock

Patients with septic shock showed lower TAFI levels compared to the small group of patients without septic shock, while no differences were observed for the TAFI activation markers (Figs. 2A–C). On admission, TAFI levels were slightly lower in patients with DIC than in patients without DIC (Fig. 3A). TAFI-AP levels were significantly increased at $t = 0$, 0.5 and 1 day in patients with DIC vs. no DIC on admission, while no difference was observed on admission with regard to the TAFIa(i) level (Figs. 3B,C). When the presence of DIC was defined within the first 24 h after admission, similar results were obtained (data not shown).

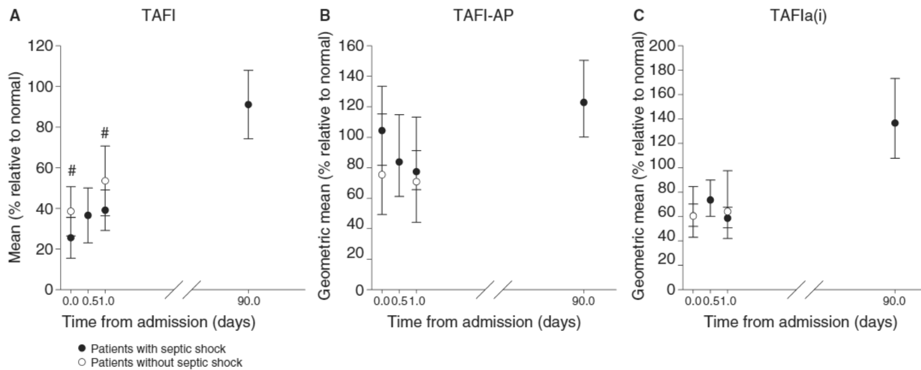

Figure 2. Plasma thrombin activatable fibrinolysis inhibitor (TAFI) and activation marker levels in patients with and without shock. Geometric mean or mean plasma TAFI levels (% relative to normal controls) on admission and after 0.5, 1, and 90 days as listed. Error bars represent SD for TAFI and 95% CI for TAFI activation peptide (TAFI-AP) and (in)activated TAFI [TAFIa(i)]. The numbers of patients with shock at these time points are 61, 28, 54, and 13, respectively. The numbers of patients without shock at the time points 0 and 1 day are 5 and 4, respectively. Data regarding shock were present for 66 out of 67 patients for whom TAFI and activation levels were determined at t = 0. (A) TAFI levels; #P < 0.01. (B) TAFI-AP levels. (C) TAFIa(i) levels.

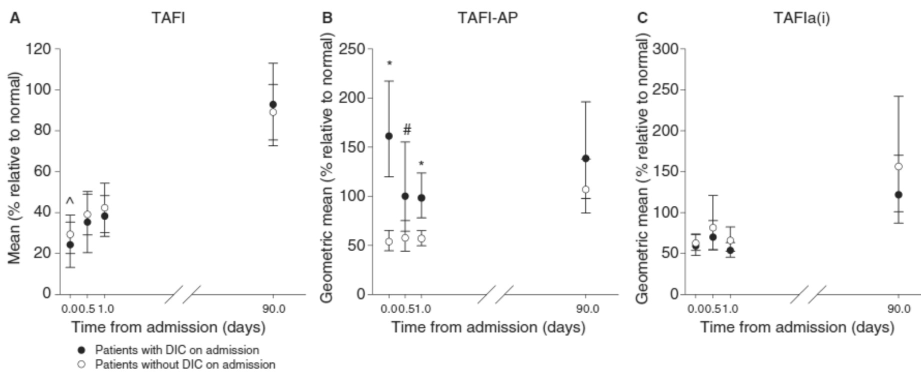

Figure 3. Plasma thrombin activatable fibrinolysis inhibitor (TAFI) and activation marker levels in patients with and without DIC on admission. Geometric mean or mean plasma TAFI levels (% relative to normal controls) on admission and after 0.5, 1, and 90 days as listed. Error bars represent SD for TAFI and 95% CI for TAFI activation peptide (TAFI-AP) and (in)activated TAFI [TAFIa(i)]. The numbers of patients with DIC at these time points are 38, 19, 32, and 7, respectively. The numbers of patients withoutDIC at these time points are 29, 9, 26, and 6, respectively. (A) TAFI; P = 0.05 at t = 0. (B) TAFI-AP levels. (C) TAFIa(i) levels. *P < 0.001; #P < 0.01; ^P = 0.05.

Some of the patients received protein C concentrate after $t = 0$ ($n = 26$, 21, 19, and 9 of the patients for whom TAFI levels are available at $t = 0$, 0.5, 1, and 90 days, respectively) because they participated in a randomized clinical trial. Because this may have

influenced the results, analyses were also performed excluding these patients. The only difference observed was for the TAFIa(i) levels one day after admission. TAFIa(i) levels were significantly higher in the patients receiving protein C concentrate when compared to those receiving placebo [geometric mean (95% CI) 72.1% (56.2–92.6%) and 54.4% (32.4–91.2%), respectively; $P = 0.03$], but this difference was already present on admission when no study medication had yet been administered [83.1% (66.9–103.3%) vs. 49.8% (42.6–58.1%), respectively; $P < 0.001$]. When comparing placebo groups and groups who received different dosages of protein C concentrate (low, moderate, or high), only at $t = 0$ was a significant difference observed between the patients receiving high dosages of protein C and the placebo group ($P = 0.004$, after Bonferroni correction). At this point, however, no protein C concentrate had been administered to any of the patients.

The TAFI 325Ile/Ile genotype was overrepresented in patients with DIC on admission when compared to the 325Thr/Thr genotype [crude odds ratio (OR) (95% CI) 10.5 (1.3–88), adjusted for age OR (95% CI) 13.7 (1.5–123)]. No other significant differences or associations were observed in TAFI genotype distributions.

TAFI activation markers: correlation with other clinical parameters

No correlation was observed between TAFI-AP and TAFIa(i) at any of the time points. On admission, TAFI-AP was negatively correlated with age ($n = 67$, Spearman's rho –0.44, $P < 0.001$). No correlation was observed between age and convalescent TAFI-AP levels three months after admission. In contrast, TAFIa(i) convalescent levels positively correlated with age ($n = 13$, Spearman's rho 0.747, $P = 0.003$), while no correlation was observed on admission. TAFI levels showed no correlation with age at any time point. TAFI-AP levels were positively correlated with PRISM score ($n = 67$, Spearman's rho 0.529, $P < 0.001$) and the predicted death rate calculated from the Rotterdam score ($n = 66$, Spearman's rho 0.584, $P < 0.001$).

We hypothesized that the ratio between activation markers such as TAFI-AP and TAFI might give a more proper indication of TAFI activation. TAFI was considerably more decreased in the acute phase than its activation markers, as reflected by the increased TAFI-AP/TAFI and TAFIa(i)/TAFI ratios (Table 2). However, a large variation between patients was found. The TAFI-AP/TAFI ratio was significantly increased in non-survivors compared to survivors at all time points: median (min–max) survivors 2.8 (0.8–47.5), 2.0 (0.9–8.8), and 1.7 (0.8–8.6) at $t = 0$, 0.5, and 1 day; non-survivors 15.8 (2.6–44), 7.3 (6.4–19.4), and 7.7 (7.5–7.9) at $t = 0$, 0.5, and 1 day ($P < 0.001$, $P = 0.008$, and $P = 0.02$, respectively). A

trend for higher TAFI-AP/TAFI ratio was observed for patients with septic shock compared to those without septic shock. Patients with DIC on admission had increased TAFI-AP/TAFI ratios at t = 0, 0.5, and 1 day: median (min–max) with DIC 7.4 (1.5–47.5), 2.5 (1.1–19.4), and 2.4 (1.2–8.6) at t = 0, 0.5, and 1 day, and without DIC 1.8 (0.8–6.9), 1.5 (0.9–2.5), and 1.3 (0.8–2.6) at t = 0, 0.5 and 1 day ($P < 0.001$ for t = 0 and 1 day and $P = 0.005$ for t = 0.5 day). No significant difference was observed in the ratios in the convalescence period. In addition, the TAFI-AP/ TAFI ratio was negatively correlated with age (Spearman's rho –0.474, P < 0.001), while it was positively correlated with PRISM score (Spearman's rho 0.622, $P < 0.001$) and the predicted death rate calculated from the Rotterdam score (Spearman's rho 0.691, $P < 0.001$).

Table. 3 Spearman's correlation coefficients of thrombin activatable fibrinolysis inhibitor (TAFI) levels with other laboratory parameters on admission.

		Spearman's rho (*P*)			
Laboratory parameter	*n*	TAFI	TAFI-AP	TAFIa(i)	TAFI-AP/TAFI
General factors					
Lactate	67	–	0.454 (<0.001)	–	0.391 (0.001)
C-reactive protein	66	–	–0.363 (0.003)	–	–0.390 (0.001)
Procalcitonin	35	–	0.542 (0.001)	–	0.596 (<0.001)
Cholesterol	29	0.603 (0.001)	–	0.569 (0.001)	–0.401 (0.031)
Cytokines					
IL-1β	29*	–	0.755 (<0.001)	0.466 (0.01)	0.638 (<0.001)
IL-1RA	36	–	0.801 (<0.001)	–	0.803 (<0.001)
TNF-α	23*	–	0.698 (<0.001)	0.589 (0.003)	0.637 (0.001)
sTNFR	36	–	0.660 (<0.001)	–	0.675 (<0.001)
IL-6	35	–	0.860 (<0.001)	–	0.836 (<0.001)
IL-8	35	–	0.783 (<0.001)	–	0.774 (<0.001)
IL-10	36	–	0.485 (0.003)	–	0.539 (0.001)
MIF	66	–	0.580 (<0.001)	–	0.667 (<0.001)
Coagulation/fibrinolyis					
Platelet count	67	0.246 (0.045)	–0.490 (<0.001)	–	–0.626 (<0.001)
Prothrombin	36	0.655 (<0.001)	–	0.501 (0.002)	–0.422 (0.01)
TAT	35	–	0.658 (<0.001)	0.331 (0.052)	0.674 (<0.001)
Protein C	36	0.638 (<0.001)	–	–	–0.422 (0.010)
Activated protein C	36	–	0.764 (<0.001)	–	0.742 (<0.001)
PAI-1	35	–	0.684 (<0.001)	–	0.727 (<0.001)
D-dimer	36	–	0.699 (<0.001)	–	0.796 (<0.001)
PAPc	35	–	0.687 (<0.001)	–	0.758 (<0.001)
sTM	36	–	0.530 (0.001)	–	0.560 (<0.001)
Factor V	14	–	–0.662 (0.01)	–	–0.741 (0.002)
Adrenal function					
ACTH	59	–	0.618 (<0.001)	–	0.604 (<0.001)
Cortisol	59	–	–0.356 (0.006)	–	–0.484 (<0.001)
Cortisol/ACTH ratio	57	–	–0.611 (<0.001)	–	–0.637 (<0.001)
Glucose	67	–	–0.372 (0.002)	–	–0.404 (0.001)

*Only patients for whom cytokine levels exceeded the detection limit of 5 pg mL^{-1} were included in the analysis. MIF, macrophage migration inhibitory factor; TAT, thrombin–antithrombin III complexes; PAI-1, plasminogen activator inhibitor-1; PAPc, plasmin-2antiplasmin complexes; sTM, soluble thrombomodulin.
A '–' indicates non-significant (Spearman's correlation coefficients).

TAFI levels in relation to other laboratory parameters

Strong correlations were observed between TAFI-AP and the TAFI-AP/TAFI ratio and other laboratory parameters, including cytokines and other coagulation factors. TAFI-AP levels were positively correlated with lactate and procalcitonin levels, while they were negatively correlated with platelet counts and levels of coagulation factor (F) V and C-reactive protein (CRP; Table 3). In addition, TAFI-AP levels were positively correlated with cytokine levels and most coagulation/fibrinolysis markers. With regard to adrenal function as a measure for anti-inflammatory action, a positive correlation was observed with adrenocorticotropic hormone (ACTH), while a negative correlation was observed between TAFI-AP and cortisol, cortisol/ACTH ratio, and glucose. TAFIa(i) level was positively correlated with prothrombin, thrombin–antithrombin III complexes (TAT), cholesterol and cytokines TNF-α and IL-1β. The TAFI-AP/TAFI ratio was correlated to the different laboratory markers in a similar way as TAFI-AP (Table 3).

Discussion

In our study we observed strongly decreased TAFI levels in both survivors and non-survivors of meningococcal sepsis in a pediatric cohort. Both markers of activation, and specifically TAFI-AP, were strongly increased in non-survivors compared to survivors. It must be noted that most non-survivors, as expected, died within the first 12 h after admission, leaving only a few patients in the non-survivor group at 0.5 and one day. For this reason, results after t = 0 should be interpreted with caution. TAFI levels were slightly more decreased in patients with DIC compared to those without DIC. TAFI-AP levels were increased in patients with DIC. In contrast, TAFIa(i) levels were similar for patients with and without DIC. This may be explained by the differences in the half-life of TAFI and its fragments in the circulation, which may prohibit the detection of differences between these fragments for the various clinical groups, specifically in meningococcal sepsis, which is known for its rapid onset and deleterious course. TAFI-AP may therefore be a more stable marker of activation. Also, the TAFI-AP/TAFI ratio, reflecting the relative activation, was increased in both patients with DIC and non-survivors. All levels were expressed as a percentage of pooled human plasma from healthy adult blood donors. No pediatric healthy controls were available. In our study TAFI levels on admission were not correlated with age. This confirms the observation of Knoefler *et al.* [12], who showed that TAFI levels were not significantly different between children of different age groups. In addition, TAFI levels at t = 90 days, when patients were fully recovered, normalized to approximately 100% of reference samples.

The association of TAFI-AP with DIC was illustrated by the negative correlation with platelet count and FV, which are decreased in DIC because of extensive consumption, and by the positive correlation with plasminogen activator inhibitor-1 (PAI-1), TAT complexes, activated protein C and D-dimer. Both TAFI and PAI-1 are known to inhibit fibrinolysis as a result of interference with plasmin formation [2–4,29,30]. Exuberant activation of TAFI and increase of PAI-1 might result in decreased degradation of thrombi and could therefore increase multi-organ failure by promoting formation of multiple microthrombi. Our results indicate that increased activation of TAFI is indeed associated with a more severe clinical disease state reflected by the low survival rate in patients with the highest level of TAFI-AP. However, the cause or effect of that increased severity remains to be established.

Another major feature of meningococcal septic shock is an increased vascular permeability, resulting in edema and persistent hypovolemia and organ dysfunction [31]. In animal models, TAFIa was shown to inactivate C3a and C5a, factors responsible for the increased vascular leaking that is seen in septic shock patients [8,9]. As the most severely ill patients have the highest C3a levels, the decrease of TAFI levels in our study in patients with septic shock compared to those without may also result from an increased activation [32]. However, no differences in TAFI-AP or TAFIa(i) levels were observed. When only TAFI was considered, one might reason that activation of TAFI was even increased in patients with septic shock in contrast to what would be expected from the animal studies described above. It must be noted, however, that the patients in our study admitted to a PICU were only included if an arterial line was *in situ* because of the blood needed for the analyses. This implies that these patients were severely ill and the use of inotropic medication was at least considered and in most cases administered. Indeed, data on TAFI levels were only available in five and four patients without shock at $t = 0$ and 1 day, respectively.

Supportive treatment of meningococcal sepsis has changed over the time period in which patients were included (more swift treatment because of public awareness, different inotropic agents and increased use of corticosteroids) [33]. Because mortality from meningococcal sepsis has decreased with improved treatment possibilities, this may have influenced the results of our study with regard to survival. Results with regard to the presence of shock or DIC, or correlations with the markers of severity, are not likely influenced, as these was scored on admission.

TAFI-AP levels were positively correlated with markers for severity of disease such as the PRISM score, the predicted death rate calculated from the Rotterdam score, lactate, procalcitonin, cytokine levels, and ACTH, while a negative correlation was found with

CRP and cortisol. Low CRP levels on admission are associated with a rapid onset of disease and are associated with a poor prognosis [21].

In our study, different results have been obtained for TAFI-AP and TAFIa(i) with regard to outcome and correlation with other disease severity markers. It should be noted that all other studies published so far have only described either intact TAFI levels or TAFIa levels upon full activation of intact TAFI, thus indirectly measuring intact TAFI levels [10,11,34]. The difference in levels of TAFI, TAFIa(i) and TAFI-AP and the ratio of individual markers may partially result from differences in the half-life or proteolytic degradation of the various fragments. Moreover, TAFIa converts spontaneously to TAFIa(i), which is prone to further cleavage into two degradation products that are not detectable in the TAFIa(i) ELISA [25]. Our results stress the importance of studying both TAFI levels and TAFI activation markers at the same time.

Genetic polymorphisms in *TAFI* were previously associated with TAFI levels and its activation. Previous studies showed that the 325 Ile variant showed an extended half-life of TAFIa *in vitro* to 15 min at 37 °C, while the activation had not changed. Moreover, the antifibrinolytic activity of the 325 Ile variant was increased compared to the 325 Thr variant, and it was therefore thought to be a more potent enzyme than the 325 Thr variant [35,36]. The TAFI 325Ile/Ile genotype was overrepresented in patients with DIC. This genotype was also reported to be overrepresented in the parents of patients who died of meningococcal disease, while in survivors of meningococcal disease the genotype frequency was decreased compared to the general population [37].

Given the association of TAFI-AP with mortality and DIC during the course of meningococcal sepsis, and the fact that the increase of TAFI-AP is not limited to the first couple of hours, as in cytokine expression, this molecule might serve as a prognostic marker. However, as increased TAFI-AP levels are indicative of an increased activation of TAFI, the current data also suggest that prevention of TAFI activation or pharmaco-logical interaction with TAFIa might be a useful therapeutic intervention [38].

In conclusion, activation markers of TAFI are associated with mortality and presence of DIC in meningococcal sepsis patients. In addition, an association with severity scores and laboratory parameters reflecting severity was observed. A determination of TAFI, TAFI-AP, and TAFIa(i) is required to enable coherent interpretation of the role of TAFI in disease, while the role of genetic polymorphisms and possibilities for therapeutic intervention require further investigation.

Addendum

M. Emonts and J. A. Hazelzet included the patients, collected blood and clinical data, were involved in data management and wrote the manuscript. M. Emonts performed statistical analyses. J. A. Hazelzet was the principal investigator for, and coordinated, the clinical studies. E. L. E. de Bruijne participated in the interpretation of results. A. H. C. Guimarães provided information on TAFI kinetics and participated in the interpretation of results. P. J. Declerck provided information on the TAFI assays and participated in the interpretation of the results. F. W. G Leebeek, M. de Maat, and D. C. Rijken participated in the interpretation of results and initiated the investigation of TAFI. A. H. C. Guimarães and A. Gils coordinated the investigation of TAFI levels. All authors approved the final version of the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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CHAPTER 8

GENERAL DISCUSSION AND SUMMARY

The main objective of this thesis was to investigate the role of fibrinolysis, in particular TAFI in arterial and venous thrombosis. For that purpose, several studies were initiated that investigated fibrinolysis parameters and TAFI antigen and activity levels in well-defined study cohorts of patients with arterial or venous thrombosis. Furthermore we studied the role of variations in the TAFI (CBP2) gene and the relationship with TAFI levels and risk of thrombosis. In addition, we studied the role of TAFI in the outcome of meningococcal sepsis, a severe life-threatening prothrombotic condition in children.

A study on the effect of genetic variants in the TAFI gene on TAFI-antigen levels, clot lysis time and the risk of venous thrombosis was presented in **Chapter 2**. It has been reported in several recent publications that TAFI is associated with venous thromboembolism [1, 2]. Several studies have indicated that single nucleotide polymorphisms (SNPs) in the TAFI gene determine plasma antigen levels [3-5]. Therefore, it is hypothesized that these genetic variations may also be associated with venous thrombosis risk. However the results of these studies on the influence of the SNPs on the risk of venous thrombosis, including deep vein thrombosis, pulmonary embolism and cerebral venous thrombosis, are conflicting [3, 6-9]. We found in our study that the 505G allele, which is associated with lower TAFI antigen levels than the 505A allele, increases the risk of DVT slightly [OR 1.3 (1.0–1.6)]. All three studied SNPs were associated with TAFI antigen levels, but only the 505A polymorphism was associated with clot lysis times. Haplotype analysis confirmed the association of the G505A polymorphism and risk of venous thrombosis. The analysis, therefore, suggested that a direct, functional role is more likely for the G505A polymorphism than for the other studied TAFI polymorphisms. Remarkably, we observed a reduced risk of DVT with the allele that is associated with higher TAFI levels and shorter clot lysis times, which was in contrast to our hypothesis, that was based on previous findings [1]. This indicates that the relationship between TAFI and venous thrombosis is more complex than previously suggested.

More recent studies have shown that hypofibrinolysis is associated with a higher risk of venous thrombosis. A reduced overall fibrinolytic potential, measured with a plasma-based tissue factor-induced clot-lysis assay, increased the risk of first venous thrombosis in the “The Leiden Thrombophilia Study” (LETS) [10]. The relationship between hypofibrinolysis and the risk of venous thrombosis was confirmed in “the Multiple Environmental and Genetic Assessment” (MEGA) study, a population-based case-control study including almost 2500 patients and 3000 controls [11]. In a subgroup of patients in the MEGA study the relationship between clot lysis times and several fibrinolysis proteins was studied in more detail

[12]. These studies revealed that the fibrinolytic potential was mainly determined by PAI-1 levels, and to a lesser extent by TAFI and alpha2-antiplasmin levels. Individuals within the highest quartile of TAFI levels had a significant higher risk than individuals with low TAFI levels (HR 1.6). This study confirms other recent studies that showed that elevated plasma TAFI antigen levels were associated with an almost two- to four-fold increased risk of venous thrombosis (deep vein thrombosis and pulmonary embolism) [1, 12-14] and of recurrent venous thromboembolism [2].

The role of genetic variation in TAFI in the pathogenesis of splanchnic vein thrombosis (SVT) was evaluated in patients with hepatic vein thrombosis (Budd Chiari syndrome, BCS) and non-cirrhotic portal vein thrombosis (PVT) in **Chapter 3**. We considered BCS and PVT as one entity, splanchnic vein thrombosis (SVT), as has been done in previous studies because a common pathway leading to a hypercoagulable condition is expected [15]. The risk for SVT was decreased (OR 0.2, 95% CI 0.1-0.7) in 147Thr/Thr homozygotes and slightly, but not significantly, increased in carriers of the 325Ile allele (OR 1.6, 95%CI 0.9-2.7). Haplotype analysis confirmed that the Ala147Thr SNP has the strongest association with risk of SVT. Our study therefore indicates that SNPs in the TAFI gene are associated with the risk of SVT. Previously, the Ala147Thr and theThr325Ile SNPs were reported to be strongly associated with TAFI antigen levels [4, 16]. However, from previous findings it has become clear that this association was mostly due to the genotype-dependent antibodies that were used in the TAFI assays. When using assays that overcome this problem, it has been shown that the 147Ala and 325Ile alleles are still associated with lower TAFI antigen levels but that the relationship is less strong [17]. Although levels of TAFI were not assessed in this study, it is evident that the genotypes that are associated with SVT in our study are the genotypes that are associated with reduced TAFI antigen levels. In contrast, previous studies reported an association between high levels of TAFI and risk of deep venous thrombosis. This may suggests that the relationship between TAFI genotypes and risk is not mediated solely by TAFI concentrations in plasma, but may also be related to changes in functional aspects of TAFI. This may partially explain the increased risk of SVT in carriers of the 325Ile-allele. We also studied the effects of haplotypes, in order to estimate the effects of the SNPs combined in one model. This haplotype analysis indicated that the Ala147Thr SNP has the strongest effect on risk of SVT. A possible explanation could be that there is linkage disequilibrium between the Ala147Thr with a yet unknown regulatory factor of TAFI. Recent studies showed that there are also clear differences in the underlying risk factors for Budd-Chiari syndrome and for portal vein thrombosis,

suggesting site specificity of thrombosis even within the splanchnic venous system [15]. These clear differences in underlying risk factors provide leads for further research on the site specificity of venous thrombosis and the development of thrombosis at these distinct sites. Recently we have studied in a larger cohort of 101 patients included in the EN-Vie study the role of fibrinolysis in the development of BCS [18]. This study showed that reduced fibrinolysis, measured using a plasma-based clot lysis assay, was a risk factor for BCS. This reduced fibrinolytic potential seemed to be particularly caused by elevated plasminogen activator inhibitor-1 (PAI-1) levels. Patients with BCS had significantly lower levels of TAFI antigen than matched healthy controls. This is in line with our findings in Chapter 2 where individual carrying TAFI-lowering alleles had a higher risk of SVT. However in the EN-Vie study we observed no significant differences in BCS risk between two studied TAFI alleles (C1040T and T1583A). Other possible explanations for lower TAFI levels in the BCS patients of the EN-Vie study are the occurrence of liver dysfunction as a result of the recent hepatic vein thrombosis and other associated disorders in the BCS patients, including myeloproliferative neoplasm's [18, 19].

Because of its fibrinolysis inhibiting function, TAFI is anticipated to contribute to the pathogenesis of arterial thrombotic disease. This is supported by observations that show an association between TAFI levels and (unstable) angina pectoris, coronary artery disease and ischemic stroke [5, 20-25]. However, such associations could not be confirmed in other studies [16, 26, 27].

The pathogenetic role of TAFI in arterial thrombosis, including coronary heart disease or cerebrovascular disease was further investigated in **Chapter 4**. In contrast to the previously reported studies, we studied young patients (males below the age of 45 and females below the age of 55) with arterial thrombosis, because it is suggested that in these individuals other pathogenetic mechanisms than the classical risk factors may be of importance, especially genetic background [28]. In this study, we used recently developed TAFI assays to study the TAFI mechanism in more detail. TAFI levels (intact TAFI, activation peptide (TAFI-AP) and (in)activated TAFI (TAFIa(i))) and TAFI activity were measured and genetic variations in the TAFI gene (-438G/A, 505G/A and 1040C/T) were determined. We observed a significant relationship between TAFI and arterial thrombosis in young subjects. In the total group of patients with premature arterial thrombosis, the levels of TAFIa(i) were significantly higher than in controls. Plasma levels of intact TAFI, TAFI activation peptide (TAFI-AP) and TAFI activity were similar in both patients and controls. In the subgroup of patients with CHD the levels of intact TAFI were significantly higher than in controls.

We also observed an association between the TAFI 325Thr/Ile polymorphism and arterial thrombosis, especially in the subgroup of patients with CHD. In 325Ile/Ile homozygotes, lower TAFI levels and a significant decreased risk of arterial thrombosis were observed (OR 0.58) compared with patients with the common 325Thr/Thr genotype. This association was most evident in CHD patients (OR 0.48). Haplotype analyses supported a role for the Thr325Ile polymorphism. The 325Ile-allele both lowers TAFI levels and was protective against arterial thrombosis in this group of young patients, especially against coronary heart disease. In conclusion, our study indicates that in young individuals high TAFI levels were associated with an increased risk of coronary heart disease and ischemic stroke. Our findings supports the earlier studies on elderly patients with ischemic stroke, including our own COCOS study, and with coronary heart disease [22, 23, 25, 29]. In a more recent study Meltzer et al. found remarkably different results in a large study on TAFI in an elderly group of males with a history of first myocardial infarction [30]. They observed that low TAFI levels, measured with a functional assay, are associated with an increased risk of MI. In addition they showed that carriers of the -438A allele, which was associated with low functional TAFI levels, had a significant increased risk of MI. The reason for this discrepancy might be that this study included patients several years after the event, whereas we included patients 1-3 months after the event. In addition only males were included in the SMILE study, whereas in our study we also included females. Furthermore we studied young individuals with arterial thrombosis.

Peripheral arterial thrombosis is another clinical manifestation of arterial thrombosis. The pathogenetic role of plasma TAFI antigen levels (intact TAFI, activation peptide (TAFI-AP) and (in)activated TAFI (TAFIa(i))) and functional TAFI in young patients with premature peripheral arterial disease (PAD) was investigated in **Chapter 5**. Intact TAFI antigen levels were significantly higher in patients with PAD than in healthy controls. TAFI-AP levels and TAFIa(i) levels were not higher in PAD patients. The risk of PAD increased with 18% (OR 1.18) per 10% increase of TAFI antigen. A trend was seen for higher functional TAFI activity in PAD patients compared to controls, but this did not reach statistical significance. For individuals with the highest functional TAFI levels, above the 90th percentile, the increased risk for PAD was most pronounced (OR 3.1). We also studied other haemostatic variables (fibrinogen and vWF:Ag levels) and a marker of inflammation (CRP), which have previously been considered risk factors in the development of PAD. Fibrinogen, CRP and vWF were all significantly elevated in PAD patients in comparison to the healthy controls. However, the correlation of TAFI antigen with fibrinogen, vWF and CRP was very weak.

This suggests that the association between TAFI and PAD is independent of an inflammatory status or endothelium damage. Our study, although carried out in a small group of patients, shows for the first time that increased levels of TAFI are associated with risk of premature peripheral arterial disease. So far no other studies on TAFI in PAD have been carried out. Only a few historical studies have investigated fibrinolysis as a risk factor for PAD. These studies also showed a possible pathogenetic role of hypofibrinolysis, mainly mediated by high PAI-1 levels in PAD [31-33]. Further studies in larger cohorts are needed to establish a causative role of hypofibrinolysis in PAD.

In the past only a few studies have been performed to study fibrinolysis in arterial thrombosis. This is partly due to the lack of adequate and easy to perform assays to measure overall fibrinolysis. But there was also a lack of interest, because changes in fibrinolysis were not considered to be a risk factor of thrombosis. Therefore the relationship between fibrinolysis and arterial thrombosis is still uncertain [34-37]. Recently plasma clot lysis assays have become available [17, 38], that are capable of studying the fibrinolytic potential in plasma. These tests have successfully been used to study fibrinolysis in various disease states accompanied with bleeding or thrombosis [18, 19]. So far no studies have been performed on the fibrinolytic potential in patients with arterial thrombosis. The evaluation of the plasma fibrinolytic potential might provide stronger evidence linking fibrinolysis to arterial thrombosis than the evaluation of the individual fibrinolytic factors. Therefore we evaluated in **Chapter 6** the contribution of the fibrinolytic system in arterial thrombosis by determining the global plasma fibrinolytic potential in survivors of a first arterial thrombosis at young age in either the cerebral, cardiac or peripheral vascular system and in a control group of healthy individuals in a population-based case–control study (ATTAC). We used a new plasma-based clot lysis assay, in which a tissue factor-induced plasma clot is lysed by exogenous tPA [10]. This plasma fibrinolytic potential was shown to reflect an overall determination as it was influenced by levels of several proteins involved in fibrinolysis (e.g. PAI-1 and alpha2-antiplasmin) [19]. We studied clot lysis times in 335 young survivors of a first arterial thrombosis, including coronary artery disease, ischemic stroke and peripheral artery disease and in 330 healthy individuals. Patients had significantly higher clot lysis times than the controls. We found an increase in risk of arterial thrombosis in subjects with hypofibrinolysis. Relative risk estimates obtained in the whole group were comparable to those obtained in the event-subgroups. This leads us to conclude that a low plasma fibrinolytic potential, which is indicative for hypofibrinolysis, constitutes a risk factor for

arterial thrombosis in young individuals. It is found in 10% of the population and increases the relative risk of arterial thrombosis two-fold.

The results of our study were recently confirmed by Meltzer et al [39]. In men under the age of 50 years an increased clot lysis time (CLT), indicative of hypofibrinolysis, was associated with a threefold increased risk of myocardial infarction compared with men with a short CLT. This analysis was performed within the "the Study of Myocardial Infarction Leiden" (SMILE), a case-control study including 421 men with myocardial infarctions and 642 control subjects [39]. In addition they showed in a subsequent study that individual fibrinolysis proteins may also be associated with cardiovascular risk. Increased levels of alpha2-antiplasmin, t-PA and PAI-1 are associated with risk, although only alpha2-antiplasmin remains significantly associated after correction for other cardiovascular risk factors. Both the study of Meltzer and our study indicate that especially in young individuals hypofibrinolysis may be a risk factor for arterial thrombosis, including myocardial infarction and ischemic stroke.

In **Chapter 7** the role of TAFI was studied in a severe prothrombotic condition in children with severe meningococcal infection. In pediatric meningococcal sepsis, an imbalance between coagulation and fibrinolysis and proinflammatory action play major roles. We hypothesized that TAFI is involved in the pathogenesis of meningococcal sepsis. Children with severe meningococcal sepsis participated in this study. We observed strongly decreased TAFI levels in both survivors and non-survivors of meningococcal sepsis in a pediatric cohort. TAFI levels were significantly decreased in patients with meningococcal disease at admission compared to the convalescence state. TAFI was decreased in patients with septic shock versus those with no shock. TAFI-AP levels were increased in patients with disseminated intravascular coagulation (DIC) compared to patients without DIC. TAFI-AP and TAFIa(i) were significantly increased in non-survivors versus survivors. TAFI-AP levels and the TAFI-AP/TAFI ratio were also strongly correlated to severity scores and laboratory parameters. Our results indicate that increased activation of TAFI is indeed associated with a more severe clinical disease state reflected by the low survival rate in patients with the highest level of TAFI-AP. However, no differences in TAFI-AP or TAFIa(i) levels were observed. It should be noted that all other studies published so far have only described either intact TAFI levels or TAFIa levels upon full activation of intact TAFI, thus indirectly measuring intact TAFI levels. Our results stress the importance of studying both TAFI levels and TAFI activation markers at the same time. The TAFI 325Ile/Ile genotype was overrepresented in patients with DIC. This genotype was also reported

to be overrepresented in the parents of patients who died of meningococcal disease, while in survivors of meningococcal disease the genotype frequency was decreased compared to the general population [40]. Given the association of TAFI-AP with mortality and DIC during the course of meningococcal sepsis, and the fact that the increase of TAFI-AP is not limited to the first couple of hours, as in cytokine expression, this molecule might serve as a prognostic marker. However, as increased TAFI-AP levels are indicative of an increased activation of TAFI, the current data also suggest that prevention of TAFI activation or pharmacological interaction with TAFIa might be a useful therapeutic intervention. In conclusion, activation markers of TAFI are associated with mortality and presence of DIC in meningococcal sepsis patients. A determination of TAFI, TAFI-AP, and TAFIa(i) is required to enable coherent interpretation of the role of TAFI in disease, while the role of genetic polymorphisms and possibilities for therapeutic intervention require further investigation.

Also others have investigated the role of TAFI in sepsis. Several investigators have shown that TAFI is strongly activated in both experimental sepsis models as in individuals with sepsis [41, 42]. The reason for decrease of TAFI levels observed in sepsis patients [43, 44] was yet unknown, We also observed strongly decreased levels, which was associated with an increase in TAFI-AP levels, indicative of an increased activation of TAFI. Since TAFI-AP levels were associated with outcome, i.e. higher TAFI-AP levels were associated with mortality and DIC, pharmacological interventions to reduce TAFI activation might be a potential therapy in sepsis patients. In a recent experimental sepsis model in rats, Muto et al showed that EF6265, an inhibitor of TAFIa, protects against sepsis-induced tissue damage through regulation of fibrinolysis and inflammation [45]. The mechanism of action by which TAFI is involved in mediating sepsis severity and outcome is still unknown. Remarkably Renckens at al have shown that TAFI knockout mice are protected from hepatic necrosis in experimental abdominal sepsis compared to wild-type mice, however this was not reflected by any difference in plasma markers of coagulation or fibrinolysis [46]. This suggests a more important role of TAFI in the regulation of inflammation in this disease state than of coagulation and fibrinolysis as was previously suggested [47].

Recommendations for further study

The studies presented in this thesis and studies recently reported in the literature indicate that hypofibrinolysis is a risk factor for both venous and arterial thrombosis. This does not seem to be limited to specific sites of thrombosis. For venous thrombotic disease hypofibrinolysis is associated with deep vein thrombosis, pulmonary embolism, hepatic vein thrombosis,

portal vein thrombosis, and central nervous system thrombosis. For arterial thrombotic disease hypofibrinolysis is associated with acute myocardial infarction, ischemic stroke and peripheral arterial disease. This is partly mediated by increased TAFI levels, but is also linked to an increase of PAI-1 and alpha2-antiplasmin, as has recently been shown by Meltzer et al [48]. The studies on the role of TAFI in venous and arterial thrombosis are still contradictory. Whereas deep vein thrombosis is associated with increased TAFI levels, splanchnic vein thrombosis is associated with reduced TAFI levels. This may indicate that not only its fibrinolysis inhibitory function, but also its influence on inflammatory response is of importance in the pathogenesis of this rarely occurring thrombosis. This is further substantiated by the fact that in our study TAFI lowering alleles were more common in patients with splanchnic vein thrombosis [49]. Future studies should therefore also take in account the influence of TAFI on the inflammatory response. Another observation that deserves further attention are the contradictory results on the role of TAFI in patients with myocardial infarction, because opposite effects have been reported in the literature. This cannot be fully explained by differences in study populations or differences in assays used in these various studies.

What can be the clinical implication of our findings? The fact that a risk factor can be identified for thrombosis is only valuable if this can lead to interventions that can be used as primary or secondary prevention. For instance does hypofibrinolysis increase the risk of thrombosis to that extent that primary prophylaxis is warranted? At this moment the answer is no. First of all, the pro-thrombotic effect seems to be modest (OR maximum 2 fold) and, secondly, no pharmacological intervention is currently available to increase fibrinolysis in these individuals. With regard to TAFI, specific inhibitors of TAFIa, for instance potato-carboxypeptidase inhibitor and EF6265 have been shown to potentiate fibrinolysis in vitro and reduce tissue factor-induced thrombosis in rats [45, 50, 51]. However these inhibitors are not clinically available.

For secondary prevention we have to know the risk of recurrence associated with hypofibrinolysis, or more specifically with increased TAFI levels. Only a few studies have been performed so far. Eichinger et al. showed a modest increased risk of recurrence of venous thrombosis in individuals with high TAFI levels [2]. A relative risk of recurrence of 2.9 was observed in individuals with highest levels compared with those with low TAFI levels. However in a more recently performed study, within the LETS study population, no relationship between hypofibrinolysis nor TAFI levels and the risk of recurrent thrombosis was observed [52].

In conclusion, in this thesis it is shown that increased TAFI levels and genetic variation in TAFI are associated with arterial thrombosis. Hypofibrinolysis is a rather frequently occurring risk factor for arterial thrombosis. Also an association between TAFI and venous thrombosis, occurring at various sites (DVT, SVT) was found, although this relationship is more complex and findings are sometimes contradictory. TAFI levels are also associated with the outcome of the severe prothrombotic condition meningococcal sepsis. The potential clinical implications of these findings deserve more attention in future studies.

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Discussie en samenvatting

De belangrijkste doelstelling van dit proefschrift was om de rol van de fibrinolyse, in het bijzonder Trombine Activeerbaar Fibrinolyse Inhibitor (TAFI), in arteriële en veneuze trombose te onderzoeken. Hiervoor werden verschillende studies geïnitieerd met goed gedefinieerde cohorten van patiënten met arteriële of veneuze trombose waarin we fibrinolyse parameters en TAFI antigeen en activiteit spiegels onderzochten. Verder bestudeerden we de relatie van variaties in het TAFI (CBP2) gen met TAFI spiegels en met het risico op trombose. Daarnaast bestudeerden we de rol van TAFI in de uitkomst van meningokokken sepsis, een ernstige levensbedreigende protrombotische aandoening bij kinderen.

Een studie over het effect van genetische varianten in het TAFI gen op TAFI-antigeen spiegels, stolsellysis tijd (CLT) en het risico op veneuze trombose werd gepresenteerd in **hoofdstuk 2**. In diverse recente publicaties werd gevonden dat TAFI geassocieerd was met veneuze trombo-embolie. Daarnaast hebben verscheidene studies aangetoond dat nucleotide polymorfismen (SNPs) in het TAFI-gen de plasma-antigeen spiegel bepalen. Daarom werd verondersteld dat deze genetische variaties ook kunnen worden geassocieerd met veneuze trombose risico. Echter, de resultaten van deze studies met betrekking tot de invloed van de SNPs op het risico van veneuze trombose, inclusief diepe veneuze trombose (DVT), longembolie en cerebrale veneuze trombose, zijn tegenstrijdig. Wij vonden in onze studie dat het 505G allel, dat wordt geassocieerd met lagere TAFI antigeen spiegels dan het 505A allel, het risico op DVT licht verhoogt [OR 1.3 (1.0-1.6)]. De drie SNPs die in dit proefschrift zijn bestudeerd, waren allen geassocieerd met TAFI antigeen spiegels, maar alleen het 505A polymorfisme was tevens geassocieerd met stolsellysis tijden. Haplotype analyse bevestigde de associatie van het G505A polymorfisme en het risico van veneuze trombose. De analyse suggereerde dus dat een directe, functionele rol meer waarschijnlijk is voor het G505A polymorfisme dan voor de andere onderzochte TAFI polymorfismen. We observeerden een opmerkelijk verminderd risico op DVT met het allel dat wordt geassocieerd met hogere TAFI spiegels en kortere stolsellysis tijden, wat in tegenstelling is tot onze hypothese, die gebaseerd was op eerdere bevindingen. Dit geeft aan dat de relatie tussen TAFI en veneuze trombose veel complexer is dan eerder was voorgesteld.

Meer recente studies hebben aangetoond dat hypofibrinolyse geassocieerd is met een hoger risico op veneuze trombose. Een verminderde totale fibrinolytische capaciteit, gemeten met een plasmagebaseerde weefselfactor geïnduceerde stolsellysis assay (onderzoek of test), verhoogde het risico op een eerste veneuze trombose in de "De Leidse Trombofilie Studie" (LETS). De relatie tussen hypofibrinolyse en het risico op veneuze trombose werd

bevestigd in de “Multiple Environmental and Genetic Assessment” (MEGA) studie, een populatiegebaseerde case-control studie onder bijna 2500 patiënten en 3000 controles. In een subgroep van patiënten in de MEGA studie werd de relatie tussen stolsellysis tijden en verschillende fibrinolyse eiwitten in meer detail bestudeerd. Deze studies toonden aan dat de fibrinolytische capaciteit vooral werd bepaald door plasminogeen activator inhibitor-1 (PAI-1) spiegels, en in mindere mate door TAFI en alfa2-antiplasmine spiegels. Individuen in het hoogste kwartiel van TAFI spiegels hadden een significant hoger risico dan personen met lage TAFI spiegels (HR 1.6). Deze studie bevestigt andere recente studies waaruit bleek dat verhoogde plasma TAFI antigeen spiegels in verband werden gebracht met een twee- tot viervoudig verhoogd risico van veneuze trombose (diepe veneuze trombose en longembolie) en van recidiverende veneuze trombo-embolie.

De rol van genetische variatie in TAFI in de pathogenese van splanchnische veneuze trombose (SVT) werd geëvalueerd bij patiënten met hepatische venetrombose (Budd Chiari syndroom, BCS) en niet-cirrotische portale veneuze trombose (PVT) in **hoofdstuk 3**. Omdat er een gemeenschappelijke weg wordt verwacht die leidt tot een hypercoagulante conditie, beschouwden wij zoals ook eerdere studies, het BCS en PVT als één entiteit, splanchnische veneuze trombose (SVT). Het risico van SVT was afgenomen (OR 0.2, 95% CI 0.1-0.7) in 147Thr/Thr homozygoten en licht, maar niet significant, gestegen in dragers van het 325Ile allel (OR 1.6, 95%CI 0.9-2.7). Haplotype analyse bevestigde dat de Ala147Thr SNP de sterkste associatie heeft met het risico van SVT. Onze studie geeft dus aan dat SNPs in het TAFI gen geassocieerd zijn met het risico van SVT. Eerder werd gerapporteerd dat de Ala147Thr en deThr325Ile SNPs sterk geassocieerd zijn met TAFI antigeen spiegels. Echter, uit eerdere bevindingen is duidelijk geworden dat deze associatie vooral te verklaren was door het gebruik van genotype-afhankelijke antilichamen in de TAFI assays. Bij het gebruik van assays waarbij dit probleem was opgelost, is aangetoond dat de 147Ala en 325Ile allelen nog steeds geassocieerd zijn met lagere TAFI antigeen spiegels, maar dat de relatie minder sterk is. Hoewel TAFI spiegels niet werden gemeten in onze studie, is het duidelijk dat genotypes die geassocieerd zijn met SVT in onze studie geassocieerd zijn met verlaagde TAFI antigeen spiegels. Daarentegen rapporteerden eerdere studies een associatie tussen hoge TAFI spiegels en het risico van diepe veneuze trombose. Dit kan suggereren dat de relatie tussen TAFI genotypen en risico niet uitsluitend gemedieerd wordt door de TAFI concentraties in plasma, maar ook kan worden bepaald door veranderingen in functionele aspecten van TAFI. Dit kan gedeeltelijk het verhoogde risico van SVT verklaren bij dragers van de 325Ile-allel. We hebben ook de effecten van de haplotypen onderzocht om

de effecten van de SNPs, gecombineerd in één model, te schatten. Deze haplotype-analyse gaf aan dat de Ala147Thr SNP het sterkste effect heeft op risico van SVT. Een mogelijke verklaring zou kunnen zijn dat er een 'linkage disequilibrium' is tussen het Ala147Thr met een nog onbekende regulerende factor van TAFI. Recente studies tonen dat er ook duidelijke verschillen zijn in de onderliggende risicofactoren voor het Budd-Chiari syndroom en voor portale veneuze trombose, wat plaatsspecifieke trombose suggereert, zelfs binnen het splanchnische veneuze systeem. Deze duidelijke verschillen in onderliggende risicofactoren bieden aanknopingspunten voor verder onderzoek naar plaatsspecifieke veneuze trombose en de ontwikkeling van trombose op deze verschillende plaatsen. Recent bestudeerden we in een groter cohort van 101 patiënten, geïncludeerd in de EN-Vie studie, de rol van de fibrinolyse bij het ontwikkelen van het BCS. Deze studie toonde aan dat een verminderde fibrinolyse, gemeten met behulp van een plasmagebaseerde stolsellysis assay, een risicofactor was voor het BCS. Deze verminderde fibrinolytische capaciteit leek met name te worden veroorzaakt door verhoogde plasminogeen activator inhibitor-1 spiegels. Patiënten met het BCS hadden significant lagere TAFI antigeen spiegels dan gematchte gezonde controles. Dit is in lijn met onze bevindingen in hoofdstuk 2 waar individuen die drager zijn van TAFI-verlagende allelen een hoger risico van SVT hadden. In de EN-Vie studie zagen we echter geen significante verschillen in BCS risico tussen twee bestudeerde TAFI allelen (C1040T en T1583A). Andere mogelijke verklaringen voor lagere TAFI spiegels in de BCS patiënten van de EN-Vie studie zijn het optreden van lever disfunctie als een gevolg van recente hepatische veneuze trombose en andere verwante stoornissen in de BCS-patiënten, inclusief myeloproliferatieve ziekten.

Vanwege zijn fibrinolyseremmende functie, wordt van TAFI verwacht dat het bijdraagt aan de pathogenese van arteriële trombotische ziekte. Dit wordt ondersteund door observaties die een associatie tonen tussen TAFI spiegels en (instabiele) angina pectoris, coronaire hartziekte en ischemische beroerte. Echter, zulke associaties kunnen niet worden bevestigd in andere studies.

De pathogenetische rol van TAFI in arteriële trombose, zoals coronaire hartziekte of cerebrovasculaire ziekte werd verder onderzocht in **hoofdstuk 4**. In tegenstelling tot de eerder gerapporteerde studies, bestudeerden wij jonge patiënten (mannen onder de leeftijd van 45 jaar en vrouwen onder de leeftijd van 55 jaar) met arteriële trombose, omdat gedacht wordt dat in deze personen andere pathogenetische mechanismen dan de klassieke risicofactoren, vooral de genetische achtergrond, van groter belang kunnen zijn dan in oudere patiënten. In deze studie, gebruikten we recent ontwikkelde TAFI assays om het TAFI mechanisme in

meer detail te bestuderen. TAFI spiegels (intact TAFI, activatie peptide (TAFI-AP) en (in) actief TAFI (TAFIa(i))) en TAFI activiteit werden gemeten en genetische variaties in het TAFI gen (-438G/A, 505G/A en 1040C/T) werden bepaald. We zagen een significante relatie tussen TAFI en arteriële trombose bij jonge patiënten. In de totale groep van patiënten met vroegtijdige arteriële trombose, waren de spiegels van TAFIa(i) significant hoger dan in de controlegroep. Plasmaspiegels van intact TAFI, TAFI activatie peptide (TAFI-AP) en TAFI activiteit waren vergelijkbaar in patiënten en controles. In de subgroep van patiënten met coronaire hartziekten waren de spiegels van het intacte TAFI significant hoger dan in de controlegroep. We hebben ook een associatie gezien tussen het TAFI 325Thr/Ile polymorfisme en arteriële trombose, vooral in de subgroep van patiënten met coronaire hartziekten. In 325Ile/Ile homozygoten werden lagere TAFI spiegels en een significant lager risico van arteriële trombose waargenomen (OR 0.58) in vergelijking met patiënten met het veel voorkomende 325Thr/Thr genotype. Deze associatie was het meest duidelijk in patiënten met coronaire hartziekten (OR 0.48). Haplotype analyse ondersteunde een rol voor het Thr325Ile polymorfisme. Het 325Ile-allel verlaagt zowel TAFI spiegels als dat het beschermend is tegen arteriële trombose in deze groep van jonge patiënten, met name tegen coronaire hartziekte. Concluderend geeft onze studie aan dat jonge mensen met een hoge TAFI spiegel een verhoogd risico van coronaire hartziekte en ischemische beroerte hadden. Onze bevindingen ondersteunen de eerdere studies in oudere patiënten met ischemische beroerte, inclusief onze eigen COCOS studie, en met coronaire hartziekten. In een meer recente studie vonden Meltzer et al opvallend verschillende resultaten in een grote studie over TAFI in een oudere groep mannen met een eerste myocardinfarct in de voorgeschiedenis. Zij observeerden dat lage TAFI spiegels, gemeten met een functionele assay, geassocieerd zijn met een verhoogd risico van myocardinfarct. Daarnaast lieten ze zien dat dragers van het -438A allel, dat was geassocieerd met lage functionele TAFI spiegels, een significant verhoogd risico van myocardinfarct hadden. De reden voor dit verschil zou kunnen zijn dat deze studie patiënten verscheidene jaren na het event includeerde, terwijl wij patiënten 1-3 maanden na het event includeerden. Daarnaast werden alleen mannen geïncludeerd in de SMILE studie, terwijl we in onze studie ook vrouwen hebben geïncludeerd. Bovendien onderzochten wij jonge mensen met arteriële trombose.

Perifere arteriële trombose is een andere klinische manifestatie van arteriële trombose. De pathogenetische rol van plasma TAFI antigeen spiegels (intact TAFI, activatie peptide (TAFI-AP) en (in)actief TAFI (TAFIa (i))) en functioneel TAFI bij jonge patiënten met prematuur perifeer arterieel vaatlijden (PAD) werd onderzocht in **hoofdstuk 5**. Intacte TAFI

antigeen spiegels waren significant hoger bij patiënten met perifeer arterieel vaatlijden dan bij gezonde controles. TAFI-AP spiegels en TAFIa(i) spiegels waren niet hoger in de perifeer arterieel vaatlijden patiënten dan in de controles. Het risico van perifeer arterieel vaatlijden steeg met 18% (OR 1,18) per 10% toename van TAFI antigeen. Een trend werd gezien voor een hogere functionele TAFI activiteit in perifeer arterieel vaatlijden patiënten vergeleken met controles, maar dit was niet statistisch significant. Voor personen met de hoogste functionele TAFI spiegels, boven de 90 ^{ste} percentiel, was het verhoogde risico voor perifeer arterieel vaatlijden het meest uitgesproken (OR 3.1). We hebben ook andere hemostatische variabelen (fibrinogeen en vWF:Ag spiegels) onderzocht en een marker van ontsteking (CRP), die eerder in aanmerking zijn genomen als risicofactoren in de ontwikkeling van perifeer arterieel vaatlijden. Fibrinogeen, CRP en vWF waren allemaal significant verhoogd in perifeer arterieel vaatlijden patiënten in vergelijking met de gezonde controles. Echter, de correlatie van TAFI antigeen met fibrinogeen, vWF en CRP was erg zwak. Dit suggereert dat de associatie tussen TAFI en perifeer arterieel vaatlijden onafhankelijk is van een inflammatoire status en van endotheel schade. Onze studie, hoewel uitgevoerd in een kleine groep patiënten, toont voor de eerste keer aan dat verhoogde TAFI spiegels worden geassocieerd met het risico van prematuur perifeer arterieel vaatlijden. Tot dusver zijn geen andere studies over TAFI in perifeer arterieel vaatlijden uitgevoerd. Slechts een paar oudere studies hebben fibrinolyse onderzocht als een risicofactor voor perifeer arterieel vaatlijden. Deze studies toonden ook een mogelijke pathogenetische rol aan van hypofibrinolysis, voornamelijk gemedieerd door hoge PAI-1 spiegels in perifeer arterieel vaatlijden. Verdere studies in grotere cohorten zijn nodig om een oorzakelijke rol vast te stellen van hypofibrinolysis in perifeer arterieel vaatlijden.

In het verleden zijn slechts enkele studies uitgevoerd om fibrinolyse in arteriële trombose te bestuderen. Dit is deels te wijten aan het ontbreken van adequate en gemakkelijk uit te voeren assays om de totale fibrinolyse te meten. Maar er was ook een gebrek aan belangstelling, omdat veranderingen in de fibrinolyse niet werden beschouwd als een risicofactor van trombose. Daarom is de relatie tussen de fibrinolyse en arteriële trombose nog onduidelijk. Onlangs zijn plasma stolsellysis assays beschikbaar gekomen, die in staat zijn om de fibrinolytische capaciteit in het plasma te meten. Deze testen zijn met succes gebruikt om de fibrinolyse te bestuderen in verschillende ziektebeelden welke gepaard gaan met bloeden of trombose. Er waren nog geen studies uitgevoerd op de fibrinolytische capaciteit in patiënten met arteriële trombose. De evaluatie van de plasma fibrinolytische capaciteit zou sterker bewijs kunnen bieden in het verbinden van de fibrinolyse aan arteriële trombose

dan door de beoordeling van de individuele fibrinolytische factoren. Daarom hebben we in **hoofdstuk 6** de bijdrage van het fibrinolytische systeem in arteriële trombose geëvalueerd door het bepalen van de globale plasma fibrinolytische capaciteit in overlevenden van een eerste arteriële trombose op jonge leeftijd in ofwel het cerebrale, cardiale of perifere vasculaire systeem en in een controlegroep van gezonde personen in een populatie gebaseerde case-control studie (ATTAC). We gebruikten een nieuwe plasmagebaseerde stolsellysis assay, waarbij een weefsel factor-geïnduceerd plasma stolsel werd gelyseerd door exogeen weefsel plasminogeen activator (t-PA). Deze plasma fibrinolytische capaciteit is een algemene bepaling en werd beïnvloed door spiegels van verschillende eiwitten die betrokken zijn bij de fibrinolyse (bv. PAI-1 en alfa2-antiplasmine). We bestudeerden stolsellysis tijden in 335 jonge patiënten na een eerste arteriële trombose, inclusief coronair vaatlijden, ischemische beroerte en perifeer arterieel vaatlijden en in 330 gezonde personen. De patiënten hadden significant hogere stolsellysis tijden dan de controles. We vonden een toename van het risico van arteriële trombose bij personen met hypofibrinolyse. Relatief risico schattingen verkregen in de hele groep waren vergelijkbaar met die in de subgroepen. Dit leidt ons tot de conclusie dat een lage plasma fibrinolytische capaciteit, wat een aanwijzing is voor hypofibrinolyse, een risicofactor vormt voor arteriële trombose bij jonge mensen. Het wordt gevonden in 10% van de bevolking en verhoogt het relatieve risico van arteriële trombose tweevoudig.

De resultaten van onze studie werden onlangs bevestigd door Meltzer et al. Bij mannen onder de leeftijd van 50 jaar was een verhoogde stolsellysis tijd, indicatief voor hypofibrinolyse, geassocieerd met een drievoudig verhoogd risico op een myocardinfarct in vergelijking met mannen met een korte CLT. Deze analyse werd uitgevoerd in “the Study of Myocardial Infarction Leiden” (SMILE), een case-control studie onder 421 mannen met hartinfarcten en 642 personen in de controlegroep. Bovendien toonden zij in een volgende studie dat individuele fibrinolyse eiwitten ook kunnen worden geassocieerd met cardiovasculair risico. Verhoogde spiegels van alfa2-antiplasmine, t-PA en PAI-1 zijn geassocieerd met risico, hoewel alleen alfa2-antiplasmine significant geassocieerd blijft na correctie voor andere cardiovasculaire risicofactoren. Zowel de studie van Meltzer als onze studie tonen aan dat vooral bij jonge individuen hypofibrinolyse een risicofactor kan zijn voor arteriële trombose, inclusief myocardinfarct en ischemische beroerte.

In **hoofdstuk 7** werd de rol van TAFI onderzocht in een ernstige protrombotische toestand bij kinderen met ernstige meningokokken infectie. Bij pediatrische meningokokken sepsis, speelt een disbalans tussen stolling en fibrinolyse en pro-inflammatoire actie een belangrijke

rol. Onze hypothese was dat TAFI betrokken is bij de pathogenese van meningokokken sepsis. Kinderen met ernstige meningokokken sepsis namen deel aan deze studie. Wij zagen sterk afgenomen TAFI spiegels in zowel overlevenden als niet-overlevenden van meningokokken sepsis in een pediatrisch cohort. TAFI spiegels waren significant verlaagd bij patiënten met meningokokken ziekte bij opname in vergelijking met de herstelde patiënten. TAFI was verlaagd bij patiënten met septische shock in vergelijking met patiënten zonder shock. TAFI-AP spiegels waren verhoogd bij patiënten met diffuse intravasale stolling (DIS) in vergelijking met patiënten zonder DIS. TAFI-AP en TAFIa(i) waren significant hoger bij niet-overlevenden versus overlevenden. TAFI-AP spiegels en de TAFI-AP/TAFI ratio waren ook sterk gecorreleerd met de ernst van de klinische scores en met laboratorium parameters. Onze resultaten geven aan dat toegenomen activatie van TAFI inderdaad geassocieerd is met een meer ernstiger klinisch ziekte stadium, weerspiegeld door de lage overlevingskans van patiënten met de hoogste TAFI-AP spiegels. Echter, er werden geen verschillen waargenomen in TAFI-AP of TAFIa(i) spiegels. Er moet opgemerkt worden dat alle tot nu toe gepubliceerde studies ofwel alleen intacte TAFI spiegels of TAFIa spiegels beschreven bij volledige activatie van intact TAFI, zodat dus indirect intacte TAFI spiegels werden gemeten. Onze resultaten benadrukken het belang van het bestuderen van zowel TAFI spiegels als TAFI activatie markers op hetzelfde moment. Het TAFI 325Ile/Ile genotype was oververtegenwoordigd bij patiënten met DIS. Van dit genotype werd ook gerapporteerd oververtegenwoordigd te zijn in de ouders van patiënten die overleden aan meningokokken ziekte, terwijl in overlevenden van meningokokken ziekte de genotype frequentie gedaald was vergeleken met de algemene bevolking. Gezien de associatie van TAFI-AP met sterfte en DIS in de loop van meningokokken sepsis, en het feit dat de toename van TAFI-AP niet beperkt is tot de eerste paar uren, zoals in cytokine expressie, kan dit molecuul dienen als een prognostische marker. Echter, zoals verhoogde TAFI-AP spiegels indicatief zijn voor een toegenomen activatie van TAFI, suggereren de huidige gegevens ook dat preventie van TAFI activatie of farmacologische interactie met TAFIa een nuttige therapeutische interventie kan zijn. Concluderend zijn activatie markers van TAFI geassocieerd met sterfte en aanwezigheid van DIS in patiënten met meningokokken sepsis. Een bepaling van TAFI, TAFI-AP, en TAFIa(i) is nodig om een samenhangende interpretatie van de rol van TAFI in ziekte mogelijk te maken, terwijl de rol van genetische polymorfismen en mogelijkheden voor therapeutische interventie nader onderzoek vereist.

Ook anderen hebben de rol van TAFI in sepsis onderzocht. Verschillende onderzoekers hebben aangetoond dat TAFI sterk geactiveerd wordt in zowel experimentele sepsis modellen als bij personen met sepsis. De reden voor afname van TAFI spiegels, waargenomen bij

patiënten met sepsis, was nog onbekend. Wij hebben ook sterk gedaalde spiegels waargenomen, welke geassocieerd werden met een toename in TAFI-AP spiegels, indicatief voor een verhoogde activatie van TAFI. Sinds TAFI-AP spiegels werden geassocieerd met uitkomst, dat wil zeggen hogere TAFI-AP spiegels werden geassocieerd met sterfte en DIS, kunnen farmacologische interventies om TAFI activatie te verminderen een potentiële therapie zijn bij patiënten met sepsis. In een recent experimenteel sepsis model in ratten toonde Muto et al dat EF6265, een remmer van TAFIa, beschermt tegen sepsis-geïnduceerde weefsel beschadiging door middel van regulatie van fibrinolyse en ontsteking. Het werkingsmechanisme waardoor TAFI betrokken is in het mediëren van ernst van sepsis en uitkomst is nog onbekend. Renckens et al toonden aan dat TAFI knock-out muizen opmerkelijk beschermd zijn tegen levernecrose in experimentele abdominale sepsis, in vergelijking met wild-type muizen, maar dit kwam niet tot uiting door enig verschil in plasma markers van stolling of fibrinolyse. Dit suggereert een belangrijkere rol voor TAFI in de regulatie van ontsteking in dit ziektebeeld dan van stolling en fibrinolyse zoals eerder werd gesuggereerd.

Aanbevelingen voor verdere studie

De gepresenteerde studies in dit proefschrift en de studies die onlangs gemeld zijn in de literatuur wijzen erop dat hypofibrinolysis een risicofactor is voor zowel veneuze als arteriële trombose. Dit lijkt niet te worden beperkt tot specifieke gebieden van trombose. Voor veneuze trombotische ziekte is hypofibrinolyse geassocieerd met diepe veneuze trombose, longembolie, hepatische veneuze trombose, vena porta trombose, en trombose van het centraal zenuwstelsel. Voor arteriële trombotische ziekte is hypofibrinolyse geassocieerd met een acuut hartinfarct, herseninfarct en perifeer arterieel vaatlijden. Dit is deels gemedieerd door verhoogde TAFI spiegels, maar is ook gekoppeld aan een verhoging van PAI-1 en alfa2-antiplasmine, zoals onlangs is aangetoond door Meltzer et al. De studies over de rol van TAFI in veneuze en arteriële trombose zijn nog steeds tegenstrijdig. Terwijl diepe veneuze trombose geassocieerd is met verhoogde TAFI spiegels, is splanchnische veneuze trombose daarentegen geassocieerd met verlaagde TAFI spiegels. Dit kan erop wijzen dat niet alleen de fibrinolyse remmende functie, maar ook de invloed op de inflammatoire respons van belang is in de pathogenese van deze zeldzaam voorkomende trombose. Dit wordt verder onderbouwd door het feit dat in onze studie TAFI verlagende allelen vaker voorkwamen bij patiënten met splanchnische veneuze trombose. Toekomstige studies moeten daarom ook rekening houden met de invloed van TAFI op de inflammatoire respons. Een andere observatie die verdere aandacht verdient zijn de tegenstrijdige resultaten over de rol van TAFI in patiënten met een myocardinfarct, omdat tegengestelde effecten in de

literatuur zijn gemeld. Dit kan niet volledig worden verklaard door verschillen in studie populaties of verschillen in gebruikte assays in deze verschillende studies.

Wat kan de klinische implicatie van onze bevindingen zijn? Het feit dat een risicofactor voor trombose kan worden geïdentificeerd is alleen waardevol als dit kan leiden tot interventies die kunnen worden gebruikt als primaire of secundaire preventie. Verhoogd hypofibrinolyse bijvoorbeeld het risico van trombose in die mate dat primaire profylaxe gerechtvaardigd is? Op dit moment is het antwoord nee. Allereerst lijkt het protrombotisch effect bescheiden te zijn (OR maximaal 2-voudig) en, in de tweede plaats is er momenteel geen farmacologische interventie beschikbaar om de fibrinolyse te verhogen bij deze individuen. Met betrekking tot TAFI hebben specifieke remmers van TAFIa, bijvoorbeeld potatocarboxypeptidase-remmer en EF6265, aangetoond de fibrinolyse in vitro te stimuleren en weefselfactor-geïnduceerde trombose bij ratten te verminderen. Maar deze remmers zijn niet klinisch beschikbaar.

Voor secundaire preventie moeten we het risico van herhaling weten in associatie met hypofibrinolyse, of meer specifiek met verhoogde TAFI spiegels. Slechts een paar studies zijn tot nu toe uitgevoerd. Eichinger et al toonde een bescheiden verhoogd risico van herhaling van veneuze trombose bij personen met hoge TAFI spiegels. Een relatief risico van herhaling van 2,9 werd waargenomen bij personen met de hoogste spiegels in vergelijking met die met lage TAFI spiegels. In een meer recent uitgevoerde studie, binnen de LETS studie populatie, werd echter geen relatie tussen hypofibrinolyse noch TAFI spiegels en het risico van recidief trombose waargenomen.

Concluderend is in dit proefschrift getoond dat verhoogde TAFI spiegels en genetische variatie in TAFI zijn geassocieerd met arteriële trombose. Hypofibrinolyse is een vrij veel voorkomende risicofactor voor arteriële trombose. Ook werd een associatie tussen TAFI en veneuze trombose gevonden (voorkomend op verschillende plaatsen zoals DVT en SVT), hoewel deze relatie complex is en bevindingen soms tegenstrijdig zijn. TAFI spiegels zijn ook geassocieerd met de uitkomst van de ernstige protrombotische meningokokken sepsis. De mogelijke klinische implicaties van deze bevindingen verdienen meer aandacht in toekomstige studies.

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 15 april 1970 te Piershil. Na het behalen van het VWO diploma in 1989 aan de Guido de Brès te Rotterdam, studeerde hij Geneeskunde aan de Erasmus Universiteit Rotterdam. Hij behaalde zijn artsexamen in 1995, waarna hij als arts-assistent ging werken in het IKAZIA ziekenhuis te Rotterdam. In 1998 startte hij in dit ziekenhuis met de opleiding tot internist (opleider dr. R.J. Th. Ouwendijk), welke in 2001 werd voortgezet in het Erasmus MC te Rotterdam (opleiders prof. dr. S.W.J. Lamberts en prof. dr. H.A.P. Pols). De registratie als internist vond plaats in 2004, de registratie als internist-vasculair geneeskundige in 2005 (opleider dr. A.H. van den Meiracker). Vanaf september 2005 is hij werkzaam als internist-vasculair geneeskundige in het Rode Kruis Ziekenhuis te Beverwijk. Per 1 juli 2011 zal hij zijn werk als internist-vasculair geneeskundige in het IJsselland Ziekenhuis te Capelle aan den IJssel voortzetten. Gedurende een deel van de opleiding tot internist en vervolgens als internist-vasculair geneeskundige heeft hij aan de studies beschreven in dit proefschrift gewerkt onder supervisie van prof. dr. F.W.G. Leebeek en dr. M.P.M. de Maat. Hij is gehuwd met Willeke de Bruijne-IJzerman en samen hebben zij 1 zoon, Tom en 1 dochter, Lara.

List of Abbreviations

AMI	acute myocardial infarction
ATTAC	**A**rterial **T**hrombosis at a young age: the role of **TA**FI and other **C**oagulation factors
BCS	Budd-Chiari syndrome
BMI	body mass index
CHD	coronary heart disease
CLT	clot lysis time
DIC	disseminated intravascular coagulation
DVT	deep venous thrombosis
IS	ischemic stroke
LETS	Leiden Thrombophilia Study
MI	myocardial infarction
OR	odds ratio
PAD	peripheral arterial disease
PAI-1	plasminogen activator inhibitor-1
PCI	potato carboxypeptidase inhibitor
PICU	paediatric intensive care unit
PVT	portal vein thrombosis
SD	standard deviation
SNP	single nucleotide polymorphism
SVT	splanchnic vein thrombosis
TAFI	thrombin activatable fibrinolysis iInhibitor
TAFIa	activated TAFI
TAFI-AP	activation peptide
TAFIa(i)	(in)activated TAFI
TIA	transient ischemic attack
t-PA	tissue plasminogen activator
UAP	unstable angina pectoris