Genetically determined coagulation disorders in ischemic stroke

Mary-Lou van Goor

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Genetically Determined Coagulation Disorders in Ischemic Stroke

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Veur pap en mam

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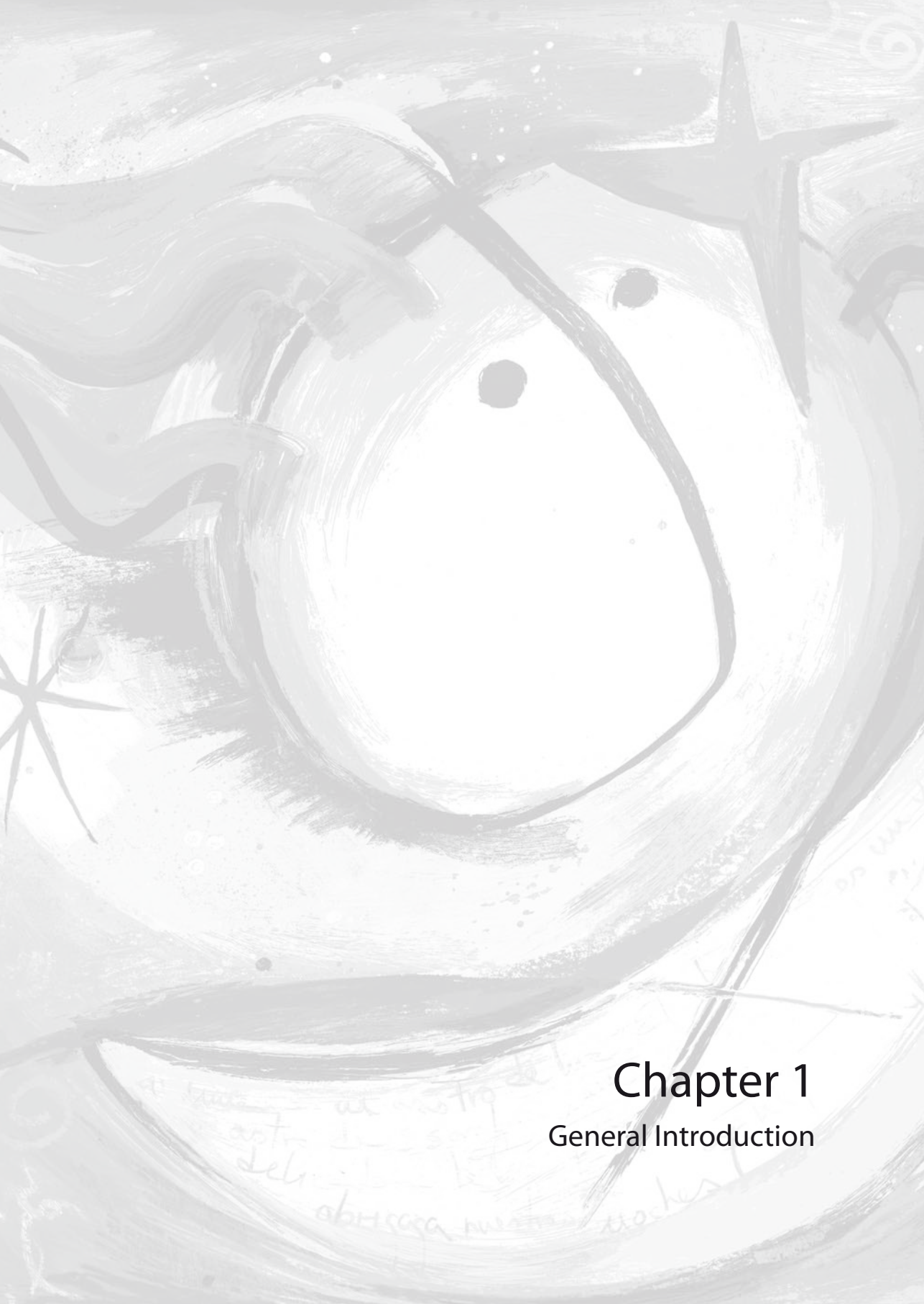

Chapter 1

General Introduction

INTRODUCTION

About 30,000 patients per year suffer a stroke in the Netherlands. Twenty five percent of these patients die within 4 weeks after the event and 60% are still disabled 6 months after the stroke. In the western world, stroke is the third cause of death, and the leading cause of disability. The overall risk of stroke, myocardial infarction or vascular death in patients with a TIA or non-disabling stroke is about 7% per year.[1] The most well known risk factors for these vascular complications are age, male sex, diabetes, hypertension, hyperlipidemia, and smoking, but there are many others such as cerebral infarction or white matter lesions on CT scan of the brain, severe stenosis of carotid or vertebral arteries, and cardiac arrhythmia or other abnormalities on ECG and echocardiography. With this large number of risk factors, the occurrence of a vascular event can be predicted by means of multiple regression models based on data from large studies.[2-4] However, these prognostic factors are not clustered, and rather weak. Furthermore, especially among the younger patients, often no risk factors are detected at all.[5] This is why patients with a high risk cannot be adequately identified.[6] It is therefore important to try and identify new risk factors for the occurrence of stroke and major vascular events. In this respect, genetically determined coagulation disorders are of particular interest. In the past decade, several of these disorders have been identified and linked to thrombotic complications, especially to venous thrombosis.[7-9] Their role in arterial thrombosis, including ischemic stroke, is less clear.

The aim of the research described in this thesis was to investigate the role of genetically determined coagulation disorders in ischemic stroke. We therefore performed several retrospective studies and one prospective case-control study of patients with recent ischemic stroke (the COCOS study). We studied the relationship between ischemic stroke and various coagulation disorders as well as their phenotypic expressions.

CONTENTS OF THIS THESIS

Chapter 2 describes the results of a retrospective case-control study among young patients with ischemic stroke and healthy controls. In chapter 2.1 the association between the G20210A prothrombin gene variant, prothrombin level and the occurrence of ischemic stroke is described. In chapter 2.2, we studied the same association for glycoprotein receptor IIb/IIIa gene polymorphism. Chapter 3 covers the rationale, background and design of the COntrolled study of genetically determined COagulation disorders in patients with a recent TIA or ischemic Stroke (COCOS). Chapter 4 contains the association between prothrombotic coagulation disorders and ischemic stroke in the COCOS population. Chapter 4.1 provides a review of the literature concerning the Factor V Leiden (FVL) mutation and the G20210A prothrombin gene variant as risk factors for ischemic stroke combined with the results of these coagulation disorders within the COCOS study. Chapter 4.2 and 4.3 describe the role of the -148C/T fibrinogen gene polymorphism and the protein Z G79A gene polymorphism and their phenotypic expressions in ischemic stroke. Chapter 5 focuses on the association between fibrinolysis related genetically determined coagulation disorders in the COCOS population. In chapter 5.1 the association between 4G/5G PAI-1 gene polymorphism and PAI-1 levels and ischemic stroke is studied. Chapter 5.2 describes the association between the annexin A5 Kozak sequence (-1C/T) polymorphism

and ischemic stroke. Chapter 5.3 addresses the recently identified polymorphisms of the TAFI gene and their phenotypic expression in relation to ischemic stroke. In Chapter 6 all genetically determined coagulation disorders of the COCOS study are combined to assess whether the risk of ischemic stroke changes with the presence of more than one coagulation disorder in comparison with an isolated mutation. Finally, chapter 7 and 8 provide a general discussion and summary of the results of the studies presented in this thesis.

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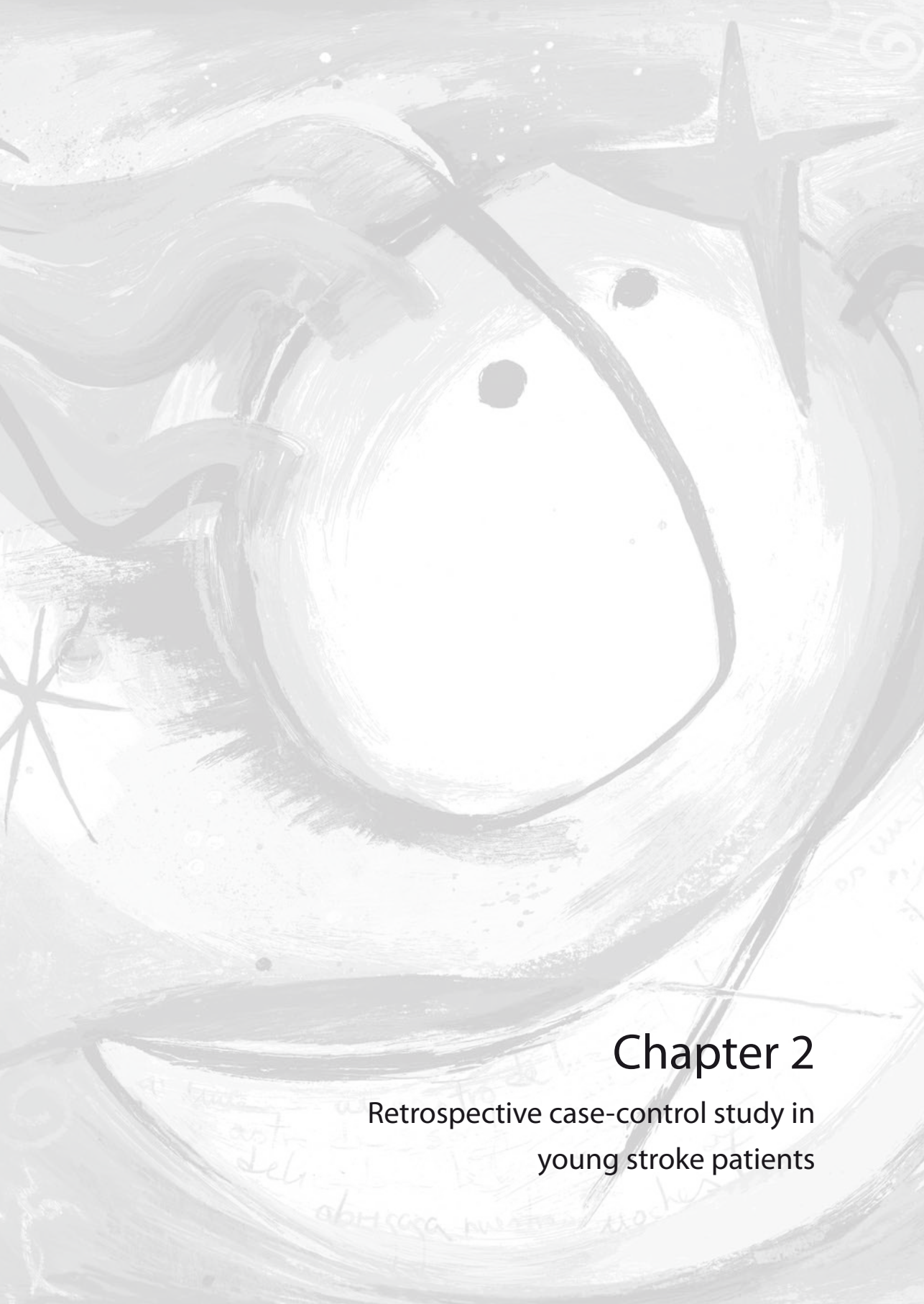
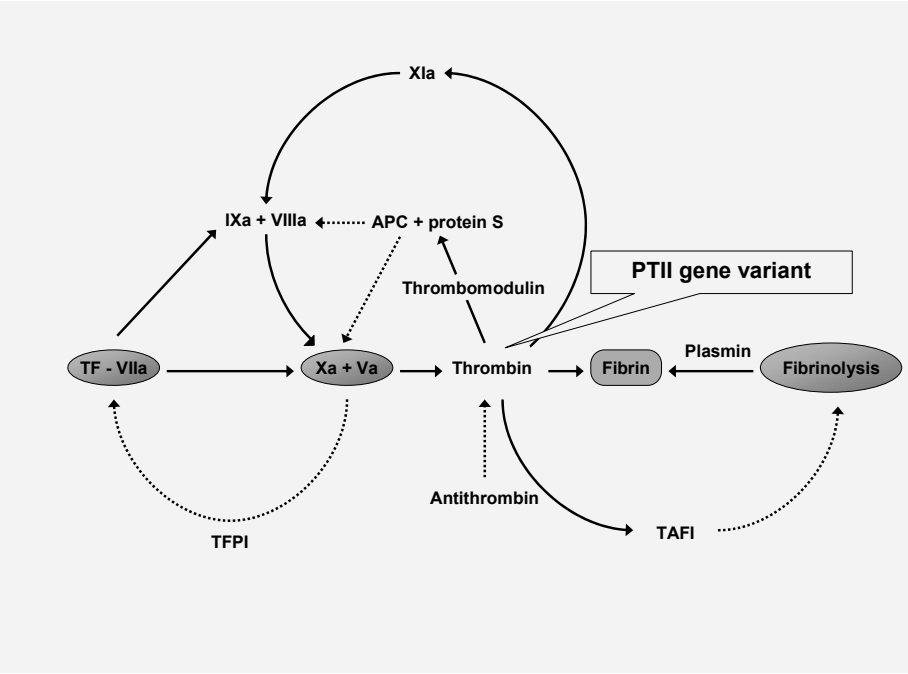


Chapter 2

Retrospective case-control study in young stroke patients

Chapter 2.1

Elevated prothrombin is a risk factor for cerebral arterial ischemia in young adults



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ABSTRACT

Background: The prevalence of elevated prothrombin in the absence of the G20210A prothrombin gene mutation has not been studied in patients with cerebral ischemia.

Methods: We carried out a case-control study of the G20210A prothrombin gene variant and prothrombin activity in 49 adult patients aged 45 years or less, with TIA or ischemic stroke without cardiac embolism or large vessel disease, and 87 controls from a group of blood donors.

Results: Five patients were heterozygous for the A-allele (OR = 2.3, 95% CI: 0.6 to 8.0). Even after exclusion of individuals with the prothrombin gene variant, the prothrombin activity was significantly higher in patients than in controls (1.11 vs 0.97, p=0.0003). The relative risk of cerebral ischemia in patients within the fourth quartile of prothrombin activity (1.10 U/ml or higher), was 3.2 fold (95% CI: 1.03 to 9.96), than in patients whose level of prothrombin activity was in the second or third quartile.

Conclusion: We conclude that, although the G20210A prothrombin gene variant may be a weak risk factor for TIA and ischemic stroke in young patients, increased prothrombin activity, which is more frequent than the mutation, appears to be more strongly related to cerebral ischemia.

INTRODUCTION

Although cerebral ischemia occurs predominantly at older age, about 10% of the cases appear in individuals under 45 years of age. Unlike their older counterparts in whom it is mostly attributed to atherosclerosis and cardioembolism, the etiology of cerebral ischemia in the young remains unknown in a large proportion of patients. In 1996, Poort et al.[1] reported that a G to A transition in the 3'- untranslated region of the prothrombin (PT) gene confers an enhanced risk of venous thromboembolism (VTE). This mutation is associated with high PT levels. More recently, it has also been shown that the prothrombin gene variant is frequently associated with cerebral venous thrombosis, especially in women who take oral contraceptives.[2,3] Whether this 20210A PT variant is also a relevant genetic risk factor for arterial thrombosis and for cerebral ischemia in particular, remains controversial.[4-8] High PT activity is much more frequent than the mutation [1,4,9], and has been shown to be an independent risk factor in patients with VTE.[1] The cause of elevated PT in the absence of the 20210A allele is unknown. None of the studies performed in patients with cerebral ischemia have reported on the PT levels.

The aim of the study reported here was to estimate the relative risk of focal cerebral ischemia, related to the PT 20210A allele and to elevated PT activity, in a series of adult patients aged 45 years or less who had had a TIA or ischemic stroke of unknown cause.

PATIENTS AND METHODS

Patients

Adult patients, aged 45 years or less, with ischemic stroke or a TIA who were diagnosed and treated at the Department of Neurology of the University Hospital Rotterdam, were enrolled. Ischemic stroke was defined as the acute onset of focal cerebral dysfunction due to cerebral ischemia with symptoms lasting more than 24 hours. Symptoms lasting less than 24 hours and without persistent deficit were classified as TIA. Diagnosis was confirmed with CT-scan. Clinical stroke types were classified according to Bamford et al[10], and etiologic classification was based on the TOAST-criteria.[11] Patients with a known cause according to the TOAST classification, i.e. large-artery atherosclerosis, cardioembolism or vasculopathy were excluded. All patients were thoroughly evaluated for cardiovascular and metabolic risk factors. A total of 54 patients fulfilled the initial entry criteria, i.e. adult patients between 18 and 45 years, with focal cerebral ischemia and without a known cause according to the TOAST criteria. Twenty seven patients had a lacunar stroke and the same number a stroke of undetermined cause. Of these 54 patients, 22 had had a TIA and 32 an ischemic stroke. The patients' mean age was 36 years, and 31 (57%) were female. Seven (13%) of the patients had a history of angina pectoris, myocardial infarction, stroke or peripheral arterial disease and 4 (8%) of venous thrombosis. A family history of either arterial or venous thrombosis was present in 17 (46%) and 3 (7%) patients, respectively. Smoking was de most frequent risk factor (29 patients, 54%), followed by oral contraceptive use (14 patients, 52% of the women). Almost half of them had hyperlipidemia (n=21) and eight (15%) suffered from hypertension. Five out of the 54 patients were excluded from this study due to unavailability of DNA and plasma.

Control subjects

A group of 87 controls with a similar age and sex distribution (mean age: 37, 51% female) and without a history of cerebral ischemia were recruited from a pool of blood donors at the blood bank in Rotterdam. Due to privacy regulations, no detailed clinical information regarding the blood donors is available.

Ethical considerations

Informed consent was given by patients and by blood donors (control group) for drawing of blood for the measurement of the coagulation parameters necessary for this study.

METHODS

Laboratory assays

The PT 20210G/A alleles and Factor V Leiden mutation were identified simultaneously, using a multiplex PCR method previously described.[12] DNA sample was available from 49 patients. Prothrombin activity was evaluated from blood samples collected within 1 year after the event. The activity was measured by a chromogenic assay performed on an ACL-300R (Instrumentation Laboratory, IJsselstein, The Netherlands) automated coagulometer. The assay was performed according to the manufacturers' instructions, modification of the original technique[13], and includes the use of Echis carinatus venom (Sigma, Zwijndrecht, The Netherlands) as activator and S-2238 (Chromogenix, Nodia, Amsterdam, The Netherlands) as substrate. Prothrombin activity was measured in 39 of the 49 patients. The remaining 10 patients were excluded because they were either on oral anticoagulant treatment, the value of the thrombotest was below normal or no plasma was available for assessment of PT activity.

Statistical methods

We estimated the relative risk of cerebral ischemia according to the PT levels and in the presence of PT variant. The study population was divided into four quartiles according to the distribution of the PT activity. The relative risk of stroke in each quartile was then estimated. Odds ratios with 95% confidence interval (CI), χ^2, Fisher's exact test and Student's t were used to compare subgroups, where appropriate.

RESULTS

A total of 49 patients were selected, i.e. adult patients between 18 and 45 years, without a known cause for the focal cerebral ischemia according to TOAST criteria and from whom material was available for laboratory studies.

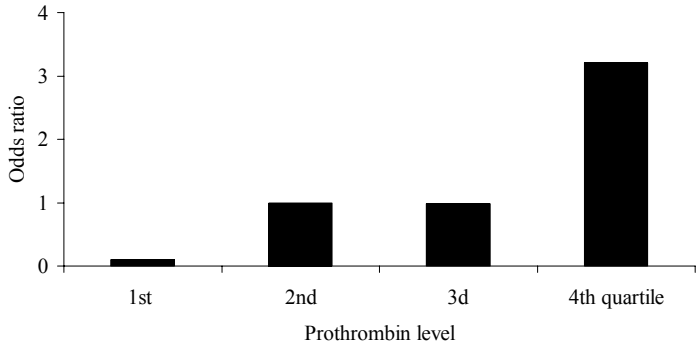
Prothrombin gene variant

Five (10.2%) patients, of whom two had a TIA and three an ischemic stroke, and four (4.7%) controls were heterozygous for PT 20210A. The relative risk (OR) of cerebral ischemia in these patients is 2.3 (95% CI: 0.6-8.3). No homozygotes for the 20210A variant were identified. The

clinical features of the five patients who were heterozygous for PT 20210A did not differ from the others. All but one had at least one other vascular risk factor (data not shown).

Prothrombin activity

The mean PT activity, measured in three of the heterozygous patients, was significantly higher than in the patients without the variant: 1.49 ± 0.18 vs. 1.11 ± 0.19 U/ml, respectively (Student's t, p=0.002). The mean PT activity in the patient group as a whole was 1.14 ± 0.21 U/ml, and in the controls it was 0.98 ± 0.14 U/ml (p=0.0001). Even after exclusion of all individuals with the PT gene variant, the PT activity was significantly higher in the patients than in the controls (1.11 ±.19 vs. 0.97 ± 0.14 U/ml, p=0.0003). Figure 1 shows how the risk of cerebral ischemia is related with the PT activity in a dose-dependent manner. The relative risk of stroke (odds ratio) in the fourth quartile of PT activity (mean PT level: 1.19 U/ml) was 3.2 times higher (95% CI: 1.03 to 9.96) than in the second and third quartile, where the relative risk was approximately 1 (95% CI: 0.3 to 3.0).



Score test for trend of odds: chi2 (1df)=17.1, p<.00001

Figure 1: Prothrombin activity and risk of cerebral ischemia. Relative risk (odds ratios) for cerebral ischemia according to PT activity . Patients and controls were stratified according to PT level: first quartile <0.91, second quartile 0.91 to 1.00, third quartile 1.00 to 1.10 and fourth quartile 1.10 U/ml or higher. Mean PT values in each quartile are indicated on the X-axis. Odds ratios were computed for patients in the first, third and fourth quartile, as compared to the second quartile. The 95% confidence interval of the odds ratio for the first quartile was .01 to .97, for the third quartile .31 to 3.04, and for the fourth quartile 1.03 to 9.96.

We investigated whether other clinical features could influence PT activity (Table 1). Female patients had a significantly higher PT level than males (1.20 U/ml vs. 1.06 U/ml, p=0.04). Among controls, the PT level in women did not differ from the PT level in men (0.99 vs. 0.97 U/ml, p=0.9). Age, type of ischemic event (TIA vs. ischemic stroke; lacunar stroke vs. stroke of unknown cause) and a positive history of arterial or venous thrombosis did not influence PT activity.

Table 1: Relationship of prothrombin level with clinical characteristics in the patients

Risk factor or clinical characteristic	YES	NO	p (t test)
Demographics			
Male sex	1.06	1.20	0.04
Age over 37 yrs	1.12	1.16	N.S.
Type of event			
Ischemic stroke	1.14	1.14	N.S.
Small vessel disease	1.13	1.15	N.S.
Previous event			
AP, MI, stroke or PAD	1.26	1.13	N.S.
Family history			
MI stroke or PAD	1.13	1.06	N.S.
Venous thrombosis*	1.00	1.15	N.S.

*Venous thrombosis includes deep venous thrombosis and pulmonary embolism AP= angina pectoris, MI=myocardial infarction, PAD=peripheral arterial disease.
Results expressed as mean PT activity (U/ml).

DISCUSSION

This study shows that PT levels in patients with focal cerebral ischemia are often high, and that high PT levels by themselves are associated with an increased stroke risk, even in the absence of the PT G20210A mutation. The association with ischemic stroke has not been described before. The etiology of increased PT activity in the majority of our cases remains unclear. Our results show that PT is not an acute-phase reactant protein, as shown by the fact that it is found elevated long after the thrombotic episode, as well as in control individuals, a finding also reported by Poort et al.[1] The PT levels in these cases might be elevated due to either other genetic variations in the PT or other (coagulation) genes and/or to acquired, metabolic or exogenous, factors. In order to gain insight into the pathogenesis of increased PT activity, we correlated the PT level with the clinical features of the patients. Prothrombin activity was not influenced by age, neither did it correlate with the type of ischemic event or with a personal or familial history of arterial or venous thrombosis. Gender appeared as the only variable which correlated with PT activity, being higher in women than in men. Oral contraceptives have been reported to cause changes in several proteins involved in blood coagulation and fibrinolysis, with increased levels of PT among them.[14] However, among our female patients oral contraceptives were already stopped at the time blood was drawn for PT analysis. In addition, there was no difference in the PT activity between women who at the time of event were using OC and those who did not (1.24 vs. 1.10 U/ml, p= 0.7, data not shown). Finally, another argument against a relevant effect of OC in PT activity is that among controls no difference was found between men and women and, although we do not have information about the percentage of female blood donors who used OC we can assume based on their age that at least some were using them.

Other metabolic or acquired risk factors (smoking, hypertension, hyperlipidemia), probably did not influence PT levels either for the same reason, since they were already being treated at the time blood was drawn for PT analysis.

In conclusion, our results suggest that increased PT activity is a risk factor in young patients with cerebral ischemia of unknown cause. Large (nested) case-control studies are needed before a definitive conclusion can be reached about the role of elevated PT, and its interactions with other risk factors.

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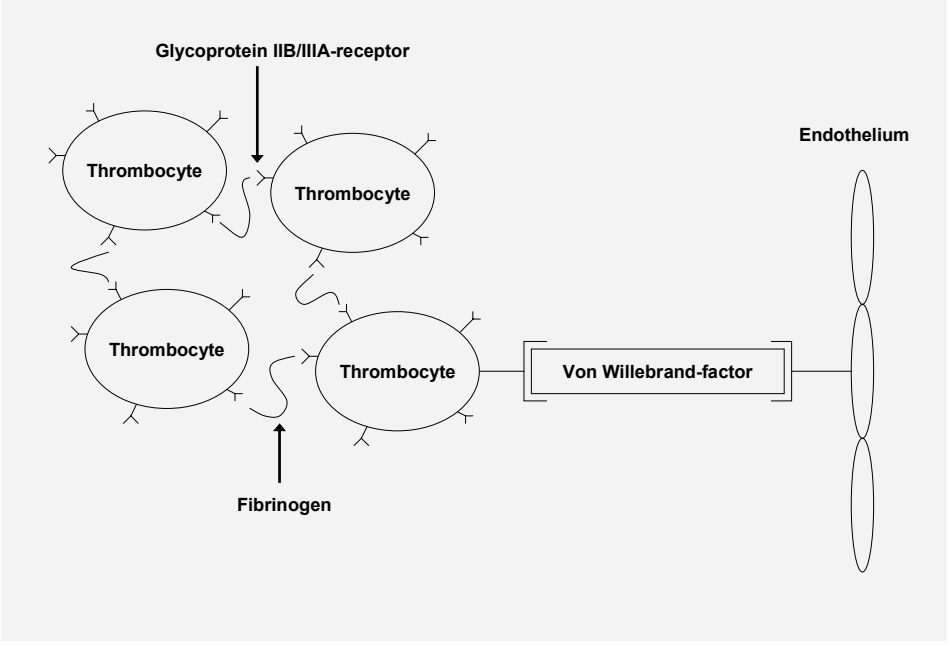
Chapter 2.2

PLA1/A2 polymorphism of the platelet glycoprotein receptor IIb/IIIa in young patients with cryptogenic TIA or ischemic stroke.



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ABSTRACT

Background: The relationship between the PLA2 allele of the Leu^{33}Pro polymorphism of glycoprotein IIb/IIIa receptor (GPIIb/IIIa) and ischemic stroke is uncertain. The purpose of this study was to investigate a possible association between the GPIIb/IIIa PLA1/A2 polymorphism and the occurrence of cryptogenic stroke in young patients.

Methods: From a consecutive series of 80 patients aged 45 or less with a recent ischemic stroke or TIA, we selected 45 patients with stroke due to small vessel occlusion or stroke of undetermined etiology (according to the TOAST criteria). Controls were 60 healthy blood donors with a similar age distribution. All patients underwent CT of the brain and were screened for cardiovascular risk factors, cardiac disorders and large vessel disease. The frequency of the PLA2 allele was determined by PCR and Msp1 restriction analysis.

Results: Eight patients (16%) and 16 controls (27%) were heterozygous for PLA2 allele. Two patients (4%) were homozygous for PLA2.The relative risk of ischemic stroke associated with PLA2 allele was estimated at 0.8 (95% CI: 0.3-1.9).

Conclusion: This study does not support the association between the PLA1/A2 polymorphism and cryptogenic stroke or TIA in patients aged 45 or less.

INTRODUCTION

Although acute ischemic stroke is more common among elderly persons, 10 percent of all cerebral infarctions occur among patients aged 45 years or less. Several conditions may contribute to the occurrence of ischemic stroke in this age-group, including abnormalities of hemostasis.[1]

Platelet membrane glycoprotein IIb/IIIa is a platelet membrane receptor and member of the integrin family of adhesive molecules that, by binding fibrinogen and von Willebrand factor, promotes platelet aggregation and thrombosis.[2] The gene encoding this glycoprotein is polymorphic at exon 2. The more common allele encodes a leucine (PLA1) and the less common allele encodes a proline (PLA2).[3] The wild type leucine is found in approximately 85% of the white population, whereas the proline is present in 15%.[4]

Recent studies have unravelled the mechanism by which the PlA2 polymorphism could contribute to arterial thrombosis. The polymorphism has been shown to increase binding to immobilized fibrinogen, a greater extent of polymerized actin and cell spreading and greater fibrin clot retraction. In platelets this may result in a difference in platelet function, which strengthens the putative role of the polymorphism in cardiovascular disease.[5]

The PlA2 allele of glycoprotein IIb/IIIa receptor has been associated in some case-control studies with coronary heart disease[6], especially among young white patients.[7-9] However, a meta-analysis of published studies between 1996 and 1999 showed no evidence of a relationship between the PlA1/A2 polymorphism and myocardial infarction.[10]

Studies in stroke patients have also reported contradictory results. Five case-control studies in patients of all ages[11-15] failed to show a relationship between PlA1/A2 polymorphism and the occurrence of ischemic stroke. Three studies described a weak association in specific subgroups, like in young women with a clinically identified probable cause of their stroke[16] or with several cardiovascular risk factors[17], and in non smokers who were younger than 50 years.[18]

The purpose of our study was to further investigate the evidence on the association between GPIIIa PlA1/A2 polymorphism and the occurrence of cryptogenic stroke in young patients. We focused on young patients with cryptogenic stroke, because we expected that specific etiologic factors such as a hypercoagulable state as a result of a genetically determined disorder like PlA1/A2 polymorphism would be more common in this group.

MATERIALS AND METHODS

Patients with a stroke due to small vessel occlusion, and patients with a stroke of undetermined origin and with a negative diagnostic evaluation, were considered to have had cryptogenic stroke, and could be included in this study. This etiological classification was based on the TOAST criteria.[19] All patients were admitted to the department of Neurology of the Erasmus Medical Center Rotterdam between June 1997 and October 1998 and they were selected from a consecutive series of 80 patients aged 45 or less with a recent ischemic stroke. Ischemic stroke was defined as the acute onset of focal cerebral dysfunction due to cerebral ischemia, with symptoms lasting more than 24 hours. Symptoms lasting less than 24 hours and leaving no persistent deficit were classified as TIA. We used CT or MRI to rule out hemorrhage and uncommon causes of stroke-like syndromes. All patients were screened for cardiovascular risk

factors, such as previous (cardio)-vascular events, smoking habit, hyperlipidemia, use of oral contraceptives, history of hypertension, diabetes mellitus or venous thrombosis and family history of cardiovascular risk factors and venous thrombosis.

All patients were also screened for cardiac abnormalities by a cardiologist who routinely ordered 24 hours ECG monitoring and transthoracic and/or transoesophageal echocardiography. Screening for large vessel disease included duplex ultrasound and, if indicated, angiography of the carotid or vertebral arteries. Occlusion or stenosis in the extracranial part of > 50% of a symptomatic artery were considered to be indicative of large vessel disease.

In the patients with cryptogenic stroke, venous blood samples were collected for routine laboratory measurements, including platelet count, hemoglobin, hematocrit, lipid profile, renal and liver function tests. The frequency of the PLA2 allele was determined by PCR and Msp1 restriction analysis.

Sixty healthy blood donors served as controls. Only the frequency of the PlA2 allele was determined in these patients. Risk factors and other clinical data apart from age and sex were not assessed, because of privacy regulations.

Informed consent was given by patients and controls for drawing of blood for measurements of coagulation parameters necessary for this study.

We estimated the relative risk of stroke (odds ratio with 95% confidence interval) in the presence of PlA2 allele by comparing the prevalence of the PlA2 allele in both groups. Assuming a prevalence of the PlA2 allele of 15%, we estimated that in a study with 45 cases and 60 controls a minimal odds ratio of 4.0 could be detected with a significance level of 0.05 and a power of 80%.

RESULTS

Forty-five patients with stroke due to small vessel occlusion (n=24, 53%) or stroke of undetermined etiology (n=21, 47%) were selected from a consecutive cohort of 80 young stroke patients. Patients had a mean age of 36 years (range 20-45 years) and controls of 37 years (range 21-45 years). Twenty-three patients (51%) were women, 22 (49%) were men. Sixteen patients (36%) had a TIA, 29 (64%) had an ischemic stroke, all confirmed by CT-scan. Twenty-seven patients (60%) smoked, 7 (16%) had hypertension and 16 patients (38%) had hyperlipidemia. Three patients (7%) had a history of venous thrombosis and 3 others had a family history of venous thrombosis. Oral contraceptives were used by 10 (43%) of the women. None had the ischemic event during pregnancy or puerperium.

Eight patients (16%) and 16 controls (27%) were heterozygous for PlA2 allele. From these, 3 had small vessel occlusion and 5 had a stroke of undetermined etiology. Two patients (4%) were homozygous for PlA2, but none of the controls. Among the homozygous patients one had small vessel occlusion, the other a stroke of undetermined etiology. For the purpose of this analysis they were both included with the heterozygotes.

The relative risk of ischemic stroke in the presence of PlA2 allele was estimated at 0.8 (95% CI:0.3-1.9). There were no clinically or statistically significant differences in the distribution of the cardiovascular risk factors mentioned above, between patients with and without the PlA1/A2 polymorphism.

DISCUSSION

The cause of a stroke in young patient remains often unclarified. Especially in this age-group, in which atherosclerosis is less frequent[1,20], genetically determined coagulation disorders could provide an additional explanation for the occurrence of the stroke. However, in the present study the PlA1/A2 polymorphism of the GPIIIa receptor was not associated with the occurrence of cryptogenic stroke at young age.

Before our results can be accepted, some methodological issues should be discussed. First, a limitation of our study is the small sample size and hence, a low power in detecting a weak risk factor, with odds ratios typically around 1.5 to 2.5. Nevertheless, due to a higher prevalence of the GPIIIa PlA2 polymorphism in our study than we initially expected, the confidence interval surrounding our estimate of the relative risk is rather tight. Second, the GPIIIA PlA2 polymorphism may interact with determinants of atherosclerosis rather than be the single cause of stroke in some young stroke victims. For example, one study[17] described an almost 5-fold increased risk of ischemic stroke in women with diabetes, hypertension or elevated plasma homocysteine levels and the PlA1/A2 polymorphism. In another study[16], the PlA2 allele appeared to be associated with stroke in a subgroup of young female patients with an underlying cause of the stroke, such as cardiac embolism or ipsilateral carotid stenosis. However, both reports applied post-hoc sub-group analyses among a small number of patients. Furthermore, no consistent pattern of risk factors was emerged.

Finally, ethnic differences in the prevalence of genetically determined disorders exist.[21] We could however, not adjust for ethnic origin because we didn't have these data on the control subjects at our disposal.

We conclude that the GPIIIA PlA2 polymorphism does not appear to be a strong risk factor for cryptogenic stroke in young patients. The results of our study, taken together with previous case-control studies[22] suggest that an association between the GPIIIa PlA2 polymorphism and ischemic stroke is not likely at all.

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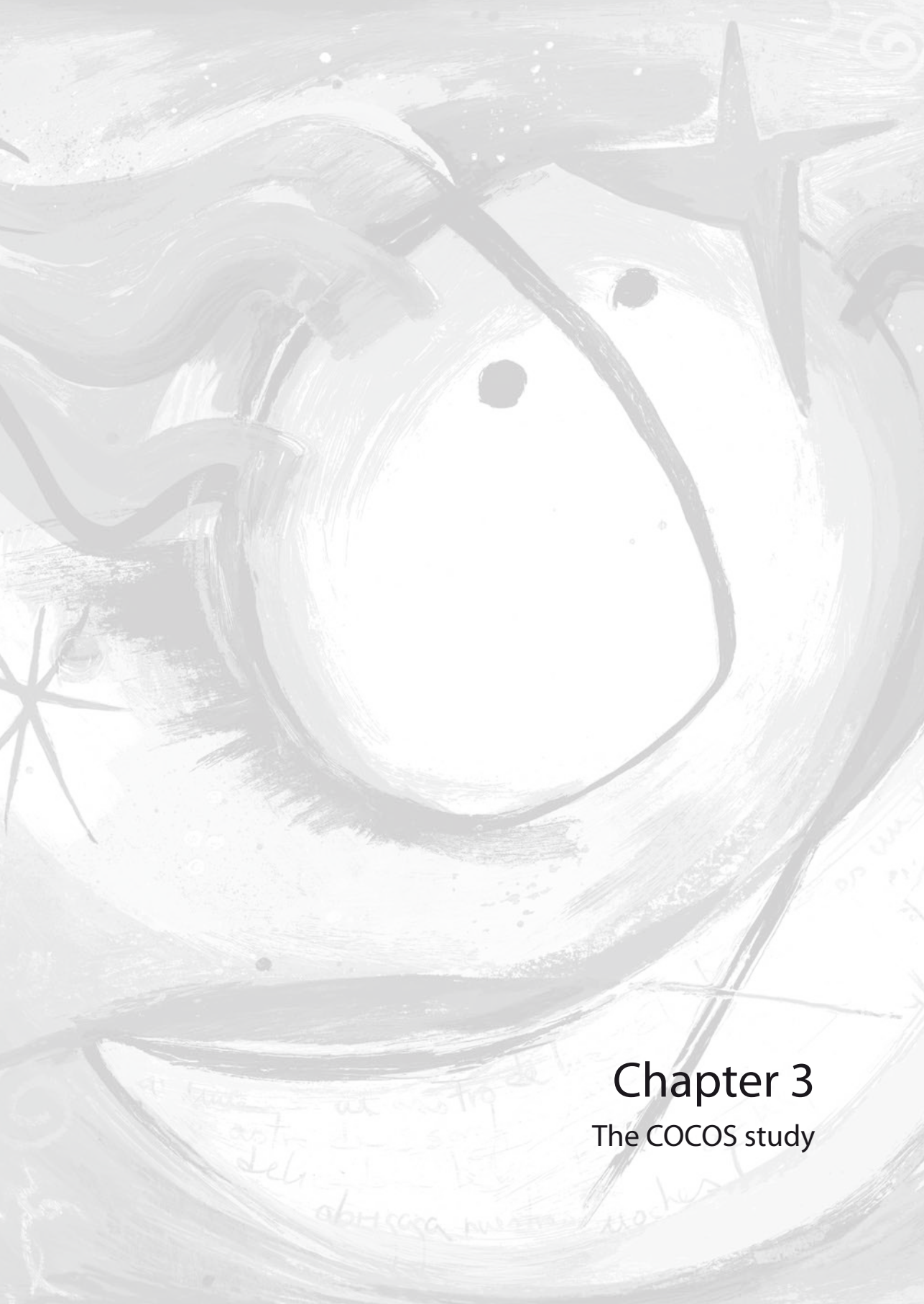


Chapter 3

The COCOS study

INTRODUCTION

Before discussing the background and design of the Controlled study of genetically determined coagulation disorders in patients with TIA or ischemic stroke (COCOS), I will first briefly review the basic principles of hemostasis and the clotting cascade.

HEMOSTASIS

The adequate control of flow and viscosity of the blood requires a precise balance between activation and inhibition of coagulation factors, fibrinolytic factors, platelets and tissue factors. Hemostasis involves the process of blood clotting and subsequent dissolution of the clot. Any disturbance of the balance between procoagulant and anticoagulant factors may lead to an increased risk of thrombosis or an increased bleeding risk. The following figure provides a simple representation of hemostasis.

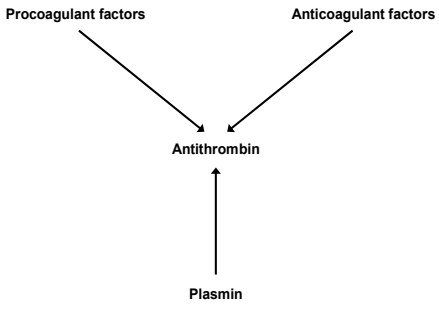


Figure 1: Schematic presentation of hemostasis

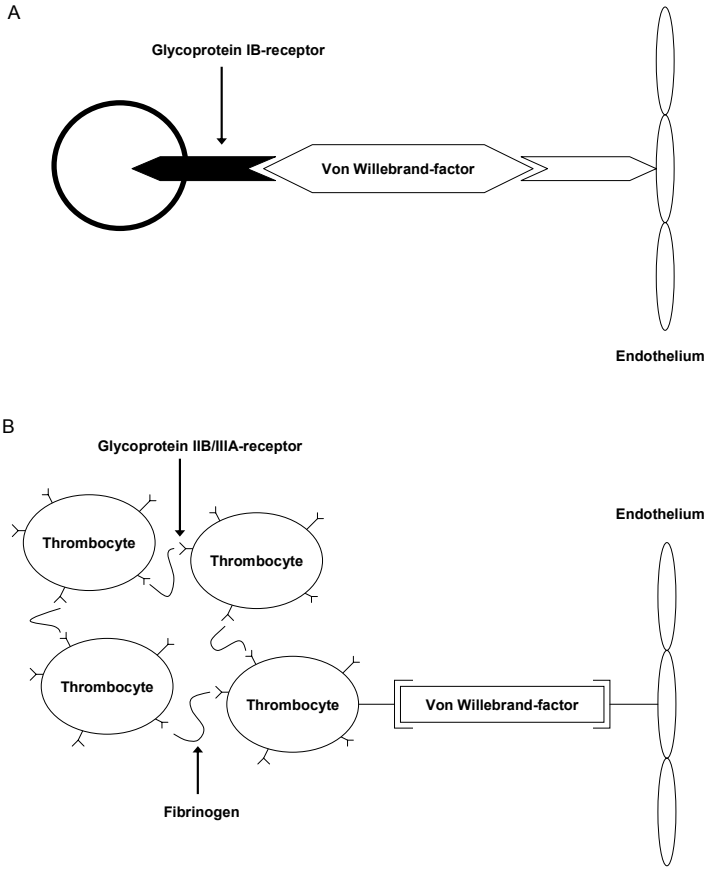
In the process of hemostasis three stages can be distinguised: 1) primary hemostasis, 2) formation of fibrin clot and 3) after some time the breakdown of the fibrin clot, called fibrinolysis.

Primary hemostasis

Primary hemostasis starts when subendothelial structures, especially collagen, come into direct contact with the blood as a result of a defect in the blood vessel wall. Collagen activates platelets by binding to the platelet receptors Ia/IIa and IV on the surface. Adhesion of activated platelets to the vessel wall is achieved by binding of another platelet receptor, the glycoprotein receptor Ib, to the Von Willebrand factor, that in its turn binds to collagen in the vessel wall. This results in a direct connection between the vessel wall and the platelet, with the Von Willebrand factor as linkage (Figure 2a). As a consequence of the adhesion, platelets are further activated and express the glycoprotein IIb/IIIa receptor on their surface. Fibrinogen binds to these receptors and provides connections between individual platelets, a process called platelet aggregation (figure 2b). During the platelet activation several biochemical reactions in the platelets take place. An

example is the formation of thromboxane-A2 from arachidonic acid. Thromboxane-A2 activates other platelets contributing to the formation of the platelet clot. The enzyme cyclo-oxygenase (COX), which is responsible for the formation of thromboxane, is completely inhibited by aspirin. There are 2 isoforms of cyclo-oxygenase, COX-1 and COX-2. COX-1 is considered the constitutive isoform and is responsible for basal prostaglandin production for tissue homeostasis. It is found in many tissues, including the stomach, kidney and platelets, where it catalyzes the synthesis of prostaglandins involved in gastric cytoprotection, renal blood flow regulation and platelet function. COX-2 is the inducible isoform. It is not present at baseline but increases in response to inflammation. Aspirin acts as an almost selective COX-1 inhibitor at low dosis. At higher doses it also inhibits COX-2 activity. It needs further investigation if simultaneously inihibition of COX-1 and COX-2 provides better secondary prevention for ischemic stroke than COX-1 inhibition alone.

Figure 2a and 2b: Platelet activation and adhesion



Formation of fibrin clot

During this phase the clotting factors become activated resulting in the formation of the fibrin clot. Traditionally, the conversion of prothrombin into thrombin was regarded as the result of two different clotting pathways, i.e. the intrinsic- and extrinsic pathway. Recently, however, it was shown that this view merely represents the clotting in vitro. The in vivo clotting process is much simpler. Coagulation starts at a site of injury with the release of tissue factor, which activates factor VII. Subsequently, the clotting cascade sets off which means that each clotting factor is activated by a former clotting factor in the cascade. The process of activation requires enzymatic cleavage of the factor into a peptide and an active clotting factor, which can then activate the following clotting factor. Each step takes place on a negatively charged surface of phospholipids provided by activated platelets. The tissue factor/factor VIIa complex cleaves factor X into factor Xa and forms the tissuefactor/factor VIIa/factor Xa complex. The latter complex is also called "tenase complex". The tenase complex activates, with assistance of factor V, prothrombin (factor II) into thrombin (factor IIa). Thrombin, in turn, converts fibrinogen into fibrin and at that moment the blood starts to clot (figure 3). Apart from this main pathway in the clotting cascade, two other loops can enhance coagulation. The tissue factor/factor VIIa complex can indirectly activate factor X by activation of factor IX to factor IXa. Together with factor VIII, factor IXa can activate factor X to Xa. A second enhancing loop involves activation of factor XI by thrombin. Factor XIa can activate factor IX which results in further activation of factor X by the first loop. In that way thrombin can stimulate its own formation. Almost all clotting factors are synthesized by the liver. Factor II, VII, IX and X are vitamin K-dependent.

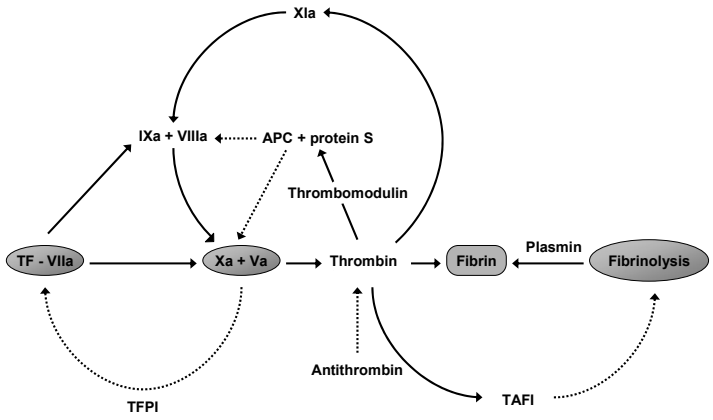


Figure 3. The clotting cascade. The black arrows represent stimulating steps, the dotted arrows represent inhibitory steps. For further details see text.

Inhibitors of coagulation

In order to prevent hypercoagulation, there are 3 physiologically inhibitory mechanisms of the clotting cascade. The first and most powerful inhibitor is antithrombin III. This protein inhibits factor Xa and factor IIa almost completely. Another is activated protein C that degrades, in the presence of protein S, factor Va and factor VIIIa, thereby limiting the activity of these factors in the clotting cascade. The tissue factor pathway inhibitor (TFPI) inhibits the influence of the tissue factor/factor VIIa complex.

Fibrinolysis

Another way of controlling the amount of fibrin is the degradation of fibrin clots by plasmin, a process called fibrinolysis (figure 4). Tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) convert plasminogen to plasmin that consecutively degrades the fibrin to fibrin degradations products (FDP). The fibrinolysis has just as the coagulation system regulatory loops. The release of plasminogen and plasmin is followed by rapid inactivation by their respective inhibitors, plasminogen- activator-inhibitors type 1 (PAI-1) and type 2 (PAI-2). At last, any free circulating plasmin is inhibited by α_2-antiplasmin.

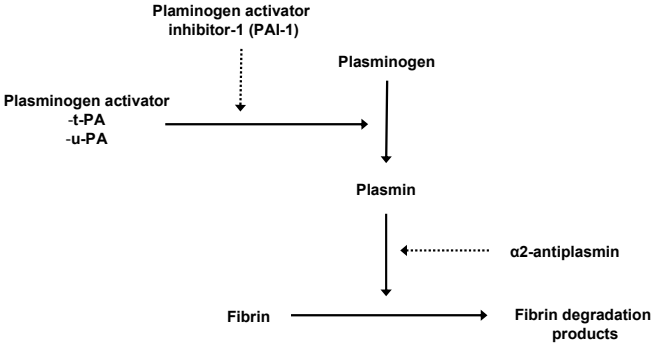


Figure 4: schematic presentation of the fibrinolysis.

BACKGROUND OF THE COCOS STUDY

As discussed above the continuous in vivo process of clotting and subsequent dissolution of the clot leads to a certain viscosity of the blood. Changes in function or in the plasma level of a clotting factor immediately lead to a disturbance of the fine balance in the clotting cascade. This results in an increased risk of thrombosis or in an increased bleeding risk, depending on whether the factor concerning has procoagulant or anticoagulant features. The function and the plasma level of a clotting factor are genetically determined. Mutations or polymorphisms in the clotting genes can give rise to changes in function of clotting factors or to modified plasma levels of the proteins. Both changes lead to a hypercoagulable state which might result in ischemic stroke, either as a result of increased thrombus formation or as a result of increased blood viscosity. Furthermore, damage of the vessel wall by atherosclerosis may lead to an increased activation of the clotting cascade resulting in an increased thrombosis risk.

In the past decade several specific genetically determined disorders of coagulation that increase the risk of venous thrombo-embolic complications, have been identified. In case-control and family studies it has been demonstrated that deficiencies of antithrombin, protein C and protein S, and –more recently- Factor V Leiden and G20210A prothrombin gene variant predispose to venous thrombosis.[1-3] Whether these genetically determined coagulation disorders are also associated with an increased risk of arterial thrombosis, especially ischemic stroke, is less clear. Within the group of genetically determined coagulation disorders, factor V Leiden and prothrombin gene variant have been studied most extensively in stroke. However, the majority of the studies was small and therefore underpowered. Moreover, some studies also included hemorrhagic stroke[4,5] or did not determine stroke subtype reliably.[6-9] Furthermore, there were many differences in ethnic origin of the participants of the studies. Before starting COCOS, we performed a systematic review of the literature concerning the association between FVL mutation, prothrombin gene variant and ischemic stroke, that is further described in chapter 4.

In the general population a correlation has been found between 4G/5G polymorphism and PAI-1 level and the fibrinogen polymorphism and the level of this protein.[10,11] Studies in patients with arterial thrombosis have yielded contradictory results on whether there is an association[12-14]or not.[15-18] Increased levels of fibrinogen and of plasminogen activator inhibitor-1 (PAI-1) have been reported in patients with myocardial infarction or stroke. Most of these factors are acute phase reactants. It is not clear whether some of these increased levels of coagulation factors are in fact genetically determined. It is also unknown whether these elevated levels are linked to polymorphisms or are regulated by other genetic or environmental mechanisms.[19-21]

Recently, polymorphisms in the protein Z gene, TAFI (thrombin activatable fibrinolysis inhibitor) gene and AnxA5 (Annexine V) gene have been reported.[22-25] Their role in ischemic stroke, together with that of their phenotypic expressions in plasma, needs further investigation.

In the following figure of the clotting cascade the possible genetic mutations that are studied within COCOS are indicated with arrows.

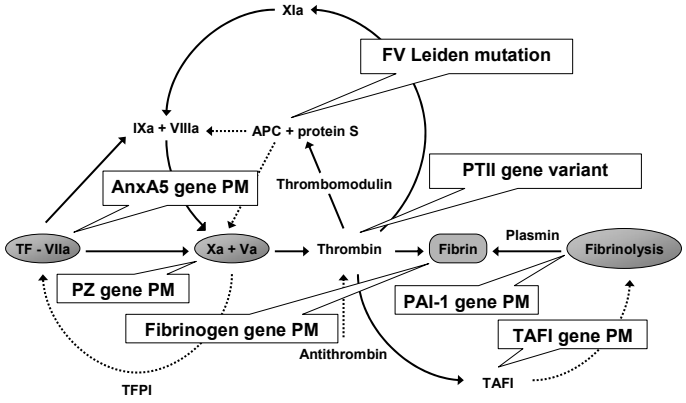


Figure 5. PM=polymorphism, AnxA5=Annexine V, vWF=von Willenbrand factor, FVL=factor V Leiden, PTII=prothrombin, PZ=protein Z, TAFI=thrombin activatable fibrinolysis inhibitor, PAI-1=plasminogen activator inhibitor-1

AIM OF THE STUDY

The aim of the COCOS-study is to answer the question whether genetically determined coagulation disorders are associated with the occurrence of ischemic stroke. This leads to the following more detailed sub-questions:

1. Are the most common genetically determined hemostatic risk factors for venous thrombosis, i.e. FV-L mutation and the G20210A prothrombin gene variant and their phenotypic expressions associated with the occurrence of ischemic stroke?
2. Are reported polymorphisms of fibrinogen, vWF and PAI-1 and their phenotypic expression associated with the occurrence of ischemic stroke?
3. Are there new polymorphisms that are associated with the occurrence of ischemic stroke?

DESIGN OF THE STUDY

COCOS is a case-control study with prospective inclusion of the participants. A case-control study aims, just like a prospective follow-up study, to identify features that influence the frequency of an illness. In a follow-up study, individuals exposed or not to a risk factor are followed and continuously screened for the development of relevant diseases, wheras in a case-control study individuals that have developed the disease (cases) are compared with individuals who are free of the disease (controls) at a certain time regarding the presence of potential risk factors. Consequently, a follow-up study measures and compares frequencies of illness and a case-control study measures and compares frequencies of exposure. From the incidences in a follow-up study the relative risk can be calculated. In 1951 Cornfield introduced the theory that the ratio from the incidences of which the relative risk is estimated, can be estimated also from a case-control study. This theory, "the rare disease assumption", was further described by Mantel and Haenszel in 1959, by Miettinen in 1976 and finally by Thomas and Greenland in 1982.[26-29] It implies that if the disease is rare enough, the odds ratio of a case-control study can be interpreted as a relative risk.

A case-control study can be summarized as follows:

	Cases (disease +)	Controls (disease -)
Determinant +	a	b
Determinant -	c	d

and the odds ratio (OR) can be calculated as:

OR = ad/bc

An advantage of the case-control design with prospective inclusion is that detailed information of the participants concerning for instance the neurological complaints, medical history, medication use, family history, can be gathered. Another advantage is the relatively fast inclusion within a few years. However, an important disadvantage is the lack of the determination of the variable beforehand. For this, a nested case-control study is needed.

Probably the most discussed and controversial principle of the case-control study is the choice of the controls. In an ideal world, the controls must reflect the same population and the same exposure category as where the cases stem from. Frequently chosen control populations are hospital controls, i.e. patients with another disease than the cases, healthy blood donors or population controls from the same geographical area as the cases. An advantage of hospital donors is that they are easy to find and quality of medical information is usually high. A disadvantage is that it cannot be excluded that the exposure has some relation to the illness. Furthermore, especially in case of participants of specialized hospitals, hospital controls do not well reflect the geographical aspects of the cases and other variables such as social economic status. Using healthy blood donors carries the advantage of the easy and speed of composing a control group. A disadvantage is the lack of sufficient medical information and the relatively healthy character of the controls. Population based controls from the same geographical area are the most valid choice, as they are the best representatives of the population at risk from which the cases originate. A relative disadvantage is that it is time consuming to find them. In COCOS we have chosen for population controls matched for sex and age, with a range of 5 years.

Confounding is a main problem of epidemiological research and means that the effect of a determinant is partly or completely determined by another variable. This problem can be partly overcome by matching cases and controls for possible confounding variables, such as age and sex. Another way of dealing with this problem is adjustment for confounding variables during the statistical analysis.

Essential in epidemiology is the matter of causation and causal interference. Since almost all diseases are multifactorially induced various attempts have been made to organize this complex process in order to gain a better understanding of the occurrence of diseases. A rather simple example is the host-agent-environment theory in which the occurrence of a disease is the result of the presence of an agent which causes the disease in a susceptible person, given certain environmental circumstances. A probably more realistic but more complex view of disease etiology is the web of causation. In this concept all predisposing factors for a disease and their complex relations both with each other and with the disease are considered. Hill has suggested that the following aspects of an association must be considered when attempting to distinguish causal from noncausal associations: (1) strength, (2) consistency, (3) specificity, (4) temporality, (5) biologic gradient, (6) plausibility, (7) coherence, (8) experimental evidence and (9) analogy. Rothman proposed the concept of sufficient cause and component causes.[30] A sufficient cause is defined as a set of minimal conditions and events (component causes) that inevitably produce disease. Minimal implies that none of the conditions or events is superfluous. A given disease is considered to have a fixed number of sufficient causes. If a component cause is member of all sufficient causes it becomes a necessary cause. In disease etiology, the completion of a sufficient cause may be considered equivalent to the onset of disease. In COCOS, we tried to reduce the occurrence of the stroke to one sufficient cause, i.e. the coagulation disorder, by adjusting for other possible component causes such as smoking.

Inclusion and exclusion criteria

Cases were consecutively recruited patients with first-ever ischemic stroke, admitted to the department of Neurology of the Erasmus MC between January 1999 and December 2001. We used population controls, i.e. partners, friends or neighbours of the patients. They were age-en sex-matched (range 5 years), did not have a history of stroke and were not related to the patient. Patients, controls and their parents should be born in Northern Europe and be of the Caucasian race, because substantial differences in the prevalence of genetically determined coagulation disorders have been reported between people from different geographical regions or race.[31] Patients with a definite non-atherosclerotic cause for the stroke such as a mechanical heart valve, endocarditis, DIC or carotid dissection were excluded, because we considered these conditions as a likely and sufficient cause of the stroke. Other exclusion criteria were age over 75 years, the use of oral anticoagulants and the absence of written informed consent.

Definitions and measurements

Ischemic stroke was defined as the acute onset of focal cerebral dysfunction due to cerebral ischemia with symptoms lasting more than 24 hours. Patients with TIA (symptoms lasting less than 24 hours) were included only if the neurological deficit in the acute phase was observed by a neurologist. In all patients, a CT of the brain was made within three days from the onset of symptoms to confirm the diagnosis of ischemic stroke and to rule out hemorrhagic stroke or uncommon causes. Clinical stroke subtypes were classified according to the OCSP criteria, modified by the results of CT.[32] Etiologic stroke types were classified according to the TOAST criteria.[33] Stroke severity was assessed with the NIH scale and Barthel scale.[34,35]

In patients as well as controls, we collected detailed information about cardiovascular risk factors, such as previous cardiovascular events, smoking habit, hyperlipidemia, use of oral contraceptives, history of hypertension, diabetes mellitus, and venous thrombosis. Hypertension was defined as a systolic blood pressure above 160 mmHg and a diastolic blood pressure above 90 mm Hg 5 days after the event, or the use of medication because of hypertension at inclusion. Hyperlipidemia was defined as fasting cholesterol level above 5.0 mmol/l or the use of medication because of hyperlipidemia at inclusion. Diabetes mellitus was defined as the use of antidiabetic medication at assessment or glyco-Hb > 6.5 %. Ischemic changes on ECG or a history of angina pectoris combined with an increasing level of CK was scored as myocardial infarction. Deep venous thrombosis was scored as "definite" when there was evidence of a venous thrombosis on phlebographic or duplex investigation. In case of clinical signs of deep venous thrombosis without support from the ancillary investigations, this was scored as "possible" deep venous thrombosis. In the same way, pulmonary embolism was defined as "definite" in case of positive findings on a VP-scan, CT-thorax or arteriography and as "possible" when there were only clinical signs of pulmonary embolism. Intermittent claudication was scored "positive" in case of clinical symptoms or signs of arterial vascular insufficiency in the legs. Peripheral arterial disease was defined as the presence of a bifemoral bypass, a documented arterial occlusion in the past, amputation of a leg above the ankle or a known arterial stenosis (femoral, brachial).

Blood samples and procedures

One week after the ischemic stroke, venous blood samples were taken under strictly standardized conditions. This standardization ensured that differences between participants were real and

not the result of circadian rhythm, physical exercise or different food intake. The participant was in fasting condition, with no exposure to tobacco or alcohol for at least 8 hours and the drawing of blood took place after 15 minutes of rest. The blood extraction was performed at one week after the stroke as we want to bypass the first acute phase response. In the venous blood samples routine laboratory measurements such as platelet count, hemoglobin and hematocrit, lipid profile, renal and liver function tests were carried out. Secondly, the specific DNA analysis and protein assays for the genetic mutations or polymorphisms and their phenotypic expression were performed. The exact procedures of the specific tests will be described in the corresponding chapters of this thesis.

All patients were screened for cardiac abnormalities by means of standard twelve lead ECG examination in order to find a possible cardiac cause for the ischemic stroke. A cardiologist was consulted in female patients aged 55 years or less, in male patients aged 45 years or less and in patients with ECG abnormalities or a clinical suspicion of cardiac disease. In these patients, 24 hours ECG monitoring, transthoracic and/or transesophageal echocardiography was carried out. We did not perform complete cardiac work-up in the older patients, because in this patient group the chance of finding a relevant cardiac cause for the stroke is very low, given a normal ECG and the absence of clinical cardiac symptoms.[36] Screening for large vessel disease included duplex ultrasound or, if indicated, angiography of the carotid or vertebral arteries.

Sample size estimation

Assuming a prevalence of the factor V Leiden mutation of 5% in the control group[37], inclusion of 116 cases and 116 controls would allow us to estimate an odds-ratio of 4.0 with a power of 80% at a significance level of 5%. The number of patients and controls was rounded to 125.[38,39] For the studied polymorphisms the prevalence in the general population is higher than 5%, so the minimally detectable odds ratio would be lower.

Statistical analysis

The relationship between each hemostatic risk factor and ischemic stroke was expressed by odds-ratios with a 95% confidence interval. Multiple logistic regression analysis will be used to adjust for possible confounders. Adjustments were made for known risk factors for ischemic stroke, such as hypertension, smoking, diabetes mellitus.

Follow-up

If possible the patient was seen at more than three months after the onset of symptoms to assess stroke outcome according to modified Rankin and Barthel score. A new venous blood sample was gathered at that time and the measurements of fibrinogen, prothrombin, CRP, cholesterol and glyco-Hb were repeated. The blood extraction took place under the same standardized conditions as during the first blood sample.

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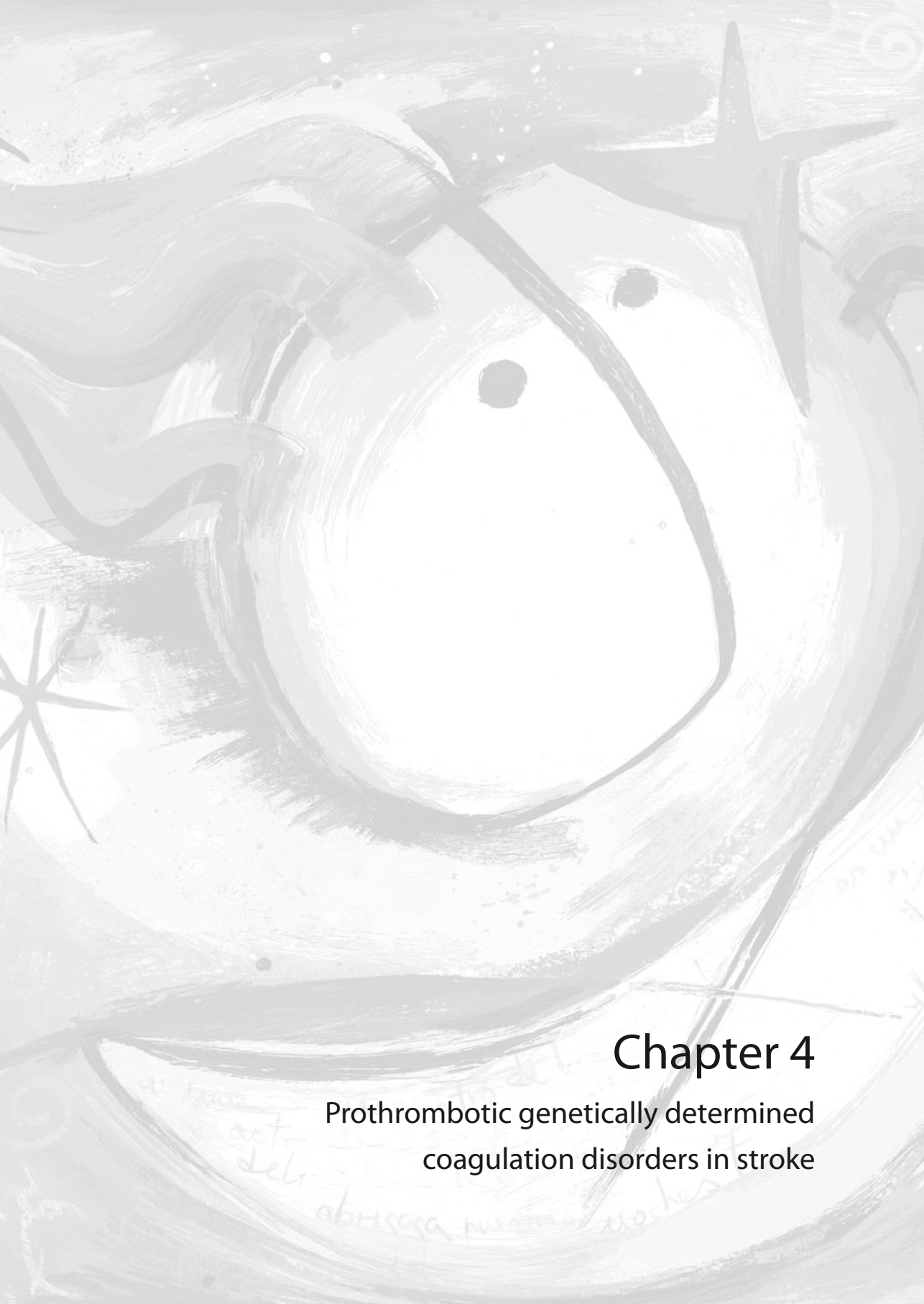
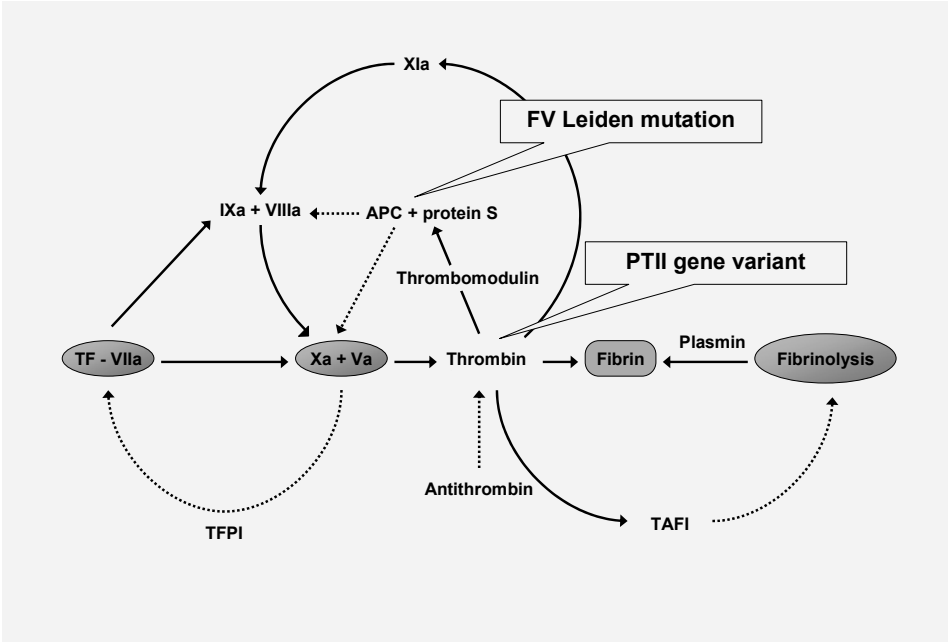


Chapter 4

Prothrombotic genetically determined coagulation disorders in stroke

Chapter 4.1

Factor V Leiden mutation and prothrombin 20210A gene variant in ischemic stroke.



M.P.J. van Goor, E.B. Gómez García, F.W.G. Leebeek, G.J. Brouwers, P.J. Koudstaal, D.W.J. Dippel. Factor V Leiden mutation and prothrombin 20210A gene variant as risk factors for ischemic stroke: a systematic review. Submitted.

ABSTRACT

Background: Whether carriership of the factor V Leiden mutation or the prothrombin 20210A gene variant predispose to arterial thrombosis, and in particular to stroke, is unclear.
Methods: We performed a Medline search of case-control studies of the prothrombin (PT) 20210A gene variant or factor V Leiden (FVL) mutation as a risk factor for ischemic stroke, published between 1995 and 2000. We used predefined criteria for inclusion of the studies and selected patients with ischemic stroke as a subgroup, if possible. We made a summary estimate of the relative risk of ischemic stroke, in relation to either FVL mutation or the prothrombin gene variant by computing the Mantel-Haenszel (M-H) odds ratio. In addition, we performed a case-control study in a well defined ischemic stroke population and population based controls of the same ethnic origin to further establish a possible association between ischemic stroke and FVL mutation or PT gene variant. Furthermore, the association between prothrombin levels and ischemic stroke was studied.
Results: The M-H odds ratio associated with ischemic stroke in the presence of FVL mutation was 1.35 (95% CI: 1.11-1.65), and of prothrombin gene variant 1.32 (95% CI: 0.95-1.84). When only studies were considered in which brain imaging was performed in more than 95% of the patients, the M-H odds ratio changed to 1.52 (95% CI: 1.22-1.90) for FVL mutation, and to 1.36 (95% CI: 0.96-1.93) for prothrombin gene variant. However, when the studies that also included children were excluded, the M-H odds ratio dropped to 0.89 (95% CI: 0.67-1.19) for FVL mutation, and to 1.05 (95% CI: 0.63-1.74) for prothrombin gene variant. Our own case-control study also provided no evidence for an increased risk of ischemic stroke in the presence of the FVL mutation or G20210A prothrombin gene variant. In contrast to the genotype, prothrombin level was associated with ischemic stroke. The adjusted OR associated with ischemic stroke in the highest quartile of prothrombin was 2.2 (95% CI: 0.96-4.90)
Conclusion: An association between ischemic stroke and the FVL mutation or the prothrombin 20210A gene variant is not likely. However, elevated prothrombin levels in the absence of the G20210A prothrombin gene variant may be associated with an increased risk of ischemic stroke.

INTRODUCTION

Several case-control studies have been performed concerning the relationship between prothrombotic conditions, such as carriership of the factor V Leiden (FVL) mutation or the prothrombin 20210A gene variant, and thrombotic events. FVL mutation results in resistance of FVa to activated protein C which is known to be associated with a prothrombotic phenotype.[1] Prothrombin gene (PT) variant is characterized by G to A mutation at position 20210 at the 3' end of the prothrombin gene and is associated with increased prothrombin levels in plasma.[2] It has been well established that both FVL mutation and prothrombin gene variant predispose to venous thrombosis.[1, 3-5] Whether these genetically determined disorders of hemostasis also predispose to arterial thrombosis, and in particular to stroke, is still unclear. The majority of studies was small and therefore underpowered. Moreover, in some studies stroke type was not reliably determined in all patients. Hemorrhagic strokes are relatively common among young patients. Since it is unlikely that FVL mutation or the prothrombin gene variant are associated with hemorrhage, this may have further decreased the power of these studies.

To make a more reliable and precise estimate of the association between either the FVL mutation or the prothrombin 20210A gene variant and the occurrence of ischemic stroke, we combined the evidence from all published case-control studies in a systematic review. Special attention was paid to critical aspects of study-design, such as verification of stroke type, choice of control subjects and age of participants.

In addition, we studied the association between ischemic stroke and FVL mutation and prothrombin gene variant within a well defined case-control population of Caucasian participants (COCOS study). In case of the prothrombin gene variant, we investigated to what extent a possible association could be explained by the phenotypic expression, i.e. the prothrombin level.

METHODS

Systematic review

We performed a Medline search of published case-control studies between 1995 and 2000 of the prothrombin gene variant or FVL mutation as a risk factor for ischemic stroke, using the key words prothrombin gene mutation, G20210A, factor V Leiden, stroke or arterial thrombosis. The design of each case-control study was further characterized as prospective or retrospective, depending on whether the data were collected according to a predefined protocol, and as "nested" or not. In a nested case-control study both cases and controls are derived from the same longitudinal population-based cohort and therefore the exposure distribution is the same for both groups. In such a design, exposure, i.e. the coagulation disorder, may be determined at the start of the cohort-study, and will not be affected by the event under study, i.e. stroke, which may not necessarily be a problem in genetic studies. Cases had to be patients with acute ischemic stroke, preferably confirmed by brain imaging study (CT or MRI) and by a neurologist. Controls and cases had to stem from a well-defined dynamic population.[6] Controls were defined as population based, when some method of aselective population sampling was carried out, for example by random digit dialling. Hospital-based controls were patients who were admitted

for a condition that was presumably not related to a prothrombotic condition or stroke, such as head injury or multiple sclerosis. If studies included cases of cerebral venous sinus thrombosis or either hemorrhagic or ischemic stroke, we extracted the latter subgroup if possible, and computed the odds-ratio on the basis of these figures. We estimated the relative risk of stroke in relation to FVL mutation and prothrombin gene variant in each study by computing the odds ratio OR= (IS,E/CO,E) / (IS,NE/CO,NE), IS=patients with ischemic stroke, E=exposed, i.e. presence of a coagulation disorder, CO=controls, NE=not exposed, i.e. absence of a coagulation disorder. The odds ratio gives an estimate of the relative risk of stroke in relation to the presence of a genetically determined coagulation disorder. A mean odds-ratio was computed with the Mantel-Haenszel technique by weighing the odds ratio of each study with the inverse of its variance. The meta-analysis was done in STATA version 6.0 with the metan command.[7]

COCOS study

The design of the study has been described extensively in chapter 3 of this thesis. In short, COCOS is a case-control study with prospective inclusion of Caucasian participants with first ever ischemic stroke, confirmed by CT. Controls are population based, age-and sex-matched and of the Caucasian race. Blood samples for genotype and phenotype were drawn under strictly standardized conditions one week after the event and in almost half of the patients also after three months. For the determination of FVL mutation and prothrombin G20210A gene variant high molecular weight genomic DNA was isolated from the white cell fraction of citrated blood according to the high-salt concentration standard procedure.[8] Simultaneous screening of FVL mutation and prothrombin 20210A gene variant was performed by PCR amplification followed by digestion with restriction enzymes Mnl-I and Hind-III[9] and visualization on an agarose electrophoresis gel. Prothrombin activity was measured with a chromogenic method which uses Echis carinatus venom as activator and S-2238 as substrate, according to a previously described protocol.[10]

The relationship between either the FVL mutation or the prothrombin gene variant and ischemic stroke was analysed using logistic regression analysis and expressed as an odds ratio with a 95% confidence interval (95% CI). The prothrombin levels were divided into quartiles and the relationship between prothrombin level and ischemic stroke was estimated as odds ratio of each quartile against the lowest quartile, and for the lowest quartile versus the higher three quartiles. We used logistic regression analysis to adjust for possible confounders, such as oral contraceptive use, as this vascular risk-factor is known to affect prothrombin level.

RESULTS

Systematic review

Twenty-six studies were identified. Three studies were excluded from this review. These excluded studies are discussed below.

In de Lucia et al.[11] the FVL mutation was found in 19 cases (38%!) and in none of the controls. The authors suggest that they had selected young patients with familial defects in the protein C pathway. This study was not included in our systematic review because of obvious selection bias. In the study of Bentolila et al.[12] three patients had venous sinus thrombosis. It is not clear whether these patients had the prothrombin 20210A gene variant or the FVL mutation. If so,

Table 1. Review of included case-control studies

Author (refno.)	Country	Study design	Cases (N)	Controls (N)	Age (yr) (mean, range)	Males	Caucasians	CT confirmation	Arterial Ischemic Stroke	Comments
Ridker [31]	USA	Nested	209	704	63 (40-84)	100%	100%	>95%	>95%	Physicians' Health Study
Catto [26]	UK	Prospective	386	247	74 (65-80)	UNK	UNK	100%	90%	38 patients with intracerebral hemorrhage
Kontula [14]	Finland	Retrospective	236	87	48 (SD:±9)	58%	UNK	UNK	UNK	controls from two different geographical areas
Press [27]	USA	Prospective	161	170	64 (55 – 73)	91%	UNK	UNK	UNK	
Markus [35]	UK	UNK	180	70	65 (35-87)	63%	100%	100%	100%	
v.d. Bom [28]	NL	Nested	112	222	> 55	UNK	UNK	UNK	UNK	The Rotterdam Study
Albucher [29]	France	Prospective	30	75	33 (18-45)	UNK	UNK	100%	100%	ill-defined patient group
Landi [15]	Italy	Retrospective	95	189	33 (5-44)	45%	UNK	100%	100%	2 children (5 and 6 yrs.) included, none with genetic mutation
Martinelli [16]	Italy	Retrospective	155	155	43 (SD ±13)	55%	UNK	0%	UNK	
Corral [17]	Spain	Retrospective	104	104	66 (24 – 88)	52%	UNK	0%	UNK	
Sanchez [18]	Spain	Retrospective	66	66	40 (6-61)	74%	UNK	100%	100%	
Halbmayer [30]	Vienna	Prospective	112	70	45 (34-56)	45%	UNK	100%	100%	
Longstreth [19]	USA	Retrospective	104	388	37 (18 – 44)	0%	90%	UNK	40%	54 hemorrhagic stroke, 2 venous stroke, 9 dissection
de Stefano [20]	Italy	Retrospective	72	198	34 (2 – 50)	49%	UNK	100%	100%	unknown number of children
Reuner [21]	Germ.	Retrospective	131	354	51 (21 – 78)	58%	UNK	100%	100%	
Cushman [36]	USA	Nested	216	495	75 (69 – 71)	46%	UNK	UNK	UNK	Cardiovascular Health Study
Nabavi [22]	Germ.	Retrospective	225	200	35 (14 – 45)	48%	UNK	100%	100%	subgroup of unknown aetiology higher prevalence of FVL mutation
Lalouschek [34]	Austria	UNK	96	96	64 (28-91)	60%	UNK	100%	100%	
Lalouschek [23]	Austria	Retrospective	81	81	65 (28-89)	60%	UNK	100%	100%	
Ridker [32]	USA	Nested	259	1774	63 (40-84)	100%	100%	>95%	>95%	Physicians' Health Study
Margaglione [24]	Italy	Retrospective	202	1036	39 (3-50)	52%	UNK	100%	100%	no differences in results after multiple logistic regression
Gaustadnes [25]	Denmark	Retrospective	207	4188	45 (0.5-71)	41%	100%	100%	51%	consecutive children born during 1 week as control group
Voetsch [33]	Brazil	UNK	114*	119	33 (15-45)	40%	100%	100%	100%	caucasian and african patients and controls

* Caucasian individuals only, UNK=Unknown

they account for almost half of the cases with the prothrombin gene variant. Since venous sinus thrombosis is frequently associated with prothrombin gene variant[4], this study was excluded from this review. In Ferraresi et al.[13] the inclusion criteria for 65 of 105 cases were not mentioned, they probably had asymptomatic carotid stenosis. The data from this study are therefore not included in our review.

The remaining 23 studies were case-control studies. Three studies concerned the prothrombin gene variant only, 13 studied the FVL mutation, and 7 both prothrombotic conditions. In Table 1 the main characteristics of each study are shown. In Table 2 and 3 the results of each individual study with regard to the presence of FVL mutation and prothrombin gene variant are listed.

Table 2. Results: Factor V Leiden mutation

Author (refno.)	IS,E	IS,NE	CO,E	CO,NE	OR	95% CI
Ridker [31]	9	200	42	662	0.7	0.3-1.5
Catto [26]	15	333	14	233	0.8	0.4-1.6
Kontula [14]	9	227	1	86	7.4*	0.9-58.7
Press [27]	4	157	4	166	1.1	0.3-4.3
Markus [35]	15	165	5	65	1.2	0.4-3.4
Van der Bom [28]	6	101	11	211	1.1	0.4-3.2
Albucher [29]	3	27	1	74	8.2	0.8-82.5
Landi [15]	4	91	3	187	2.7	0.6-12.5
Martinelli [16]	5	150	2	153	2.6	0.5-13.3
Sanchez [18]	3	63	3	63	1.0	0.2-5.1
Halbmayer [30]	8	104	6	65	0.8	0.3-2.5
Longstreth [19]	0	40	16	372	0.3	0.2-4.7
de Stefano [20]	5	67	6	192	2.4	0.7-8.1
Cushman [36]	13	192	35	460	0.9	0.5-1.7
Nabavi [22]	19	206	12	188	1.4	0.7-3.1
Lalouschek [34]	5	91	8	88	1.7	0.5-5.3
Lalouschek [23]	10	71	4	77	2.7	0.8-9.0
Margaglione [24]	30	172	43	993	4.0	2.5-6.6
Gaustadnes [25]	23	184	276	3912	1.8	1.1-2.8
Voetsch [33]	5	109	7	112	0.7	0.2-2.3

IS=ischemic stroke, E=exposed/FVL mutation, NE=non-exposed/ FVL mutation negative, CO=controls, OR=odds ratio, 95% CI=95% confidence interval; *odds ratio calculated with control group of same area as cases, odds ratio with all controls in study 1.32 (95% CI: 0.4-4.36)

Table 3. Results: Prothrombin 20210A gene variant

Author (refno.)	IS,E	IS,NE	CO,E	CO,NE	OR	95% CI
Martinelli [16]	6	149	5	150	1.2	0.4-4.0
Corral [17]	1	103	2	102	0.5	0.1–5.6
Longstreth [19]	1	39	6	376	1.6	0.2-13.7
De Stefano [20]	9	63	5	193	5.5	1.8-17.1
Reuner [21]	3	128	8	346	1	0.3-3.9
Lalouschek [34]	5	91	5	91	1.0	0.3-3.6
Ridker [32]	11	248	69	1705	1.1	0.6-2.1
Margaglione [24]	10	192	43	993	1.2	0.6-2.4
Gaustadnes [25]	4	203	10	490	1.0	0.3-3.1
Voetsch [33]	6	108	3	116	2.2	0.5-8.8

IS=ischemic stroke, E=exposed/prothrombin gene variant, NE=non-exposed/ normal prothrombin gene, CO=controls, OR=odds ratio, 95% CI= 95% confidence interval

Twelve studies had a retrospective design[14-25] seven studies were prospective.[26-32] Three studies did not mention whether the data were collected prospectively or retrospectively.[33-35]

The estimated M-H odds ratio, based on the results of all 23 included studies of FVL mutation was 1.35 (95% CI: 1.11-1.65) and of prothrombin gene variant 1.32 (95% CI: 0.95-1.84), (Figure 1).

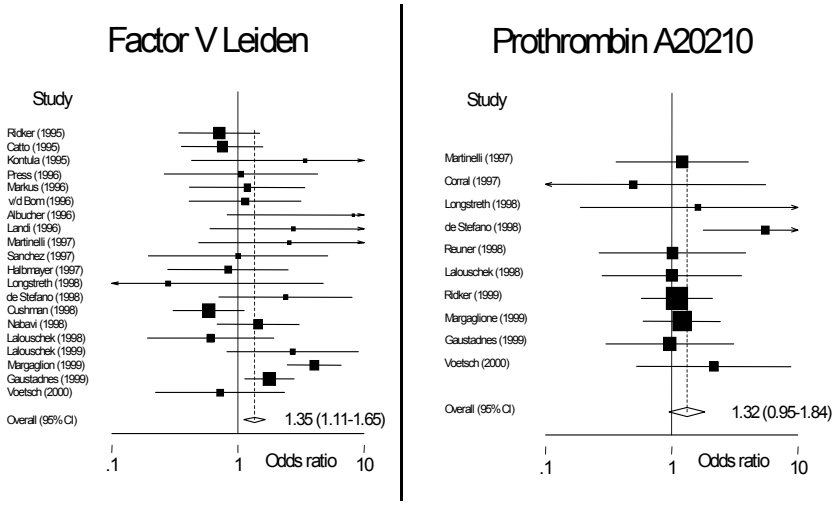


Figure 1: Graphical representation of a meta-analysis of case-control studies of factor V Leiden (left) and Prothrombin 20210A gene variant (right) as a possible risk factor for stroke.

In six studies, however, no brain imaging studies were performed and hence, no certain distinction between hemorrhagic and ischemic stroke was made.[14, 16, 17, 27, 28, 36] In two studies CT or MRI showed hemorrhagic stroke[19, 26] or even venous stroke[19], but we considered the ischemic stroke cases only. Taking into account only those studies in which brain imaging was performed in more than 95% of the patients, the M-H odds ratio changed from 1.35 to 1.52 (95% CI: 1.22-1.90) for FVL mutation and from 1.32 to 1.36 (95% CI: 0.96-1.93) for prothrombin gene variant respectively (Table 4).

Table 4. Results of the meta-analysis: Odds ratio and 95% confidence interval for factor V Leiden mutation and Prothrombin 20210A gene variant in subgroups

	Factor V Leiden mutation		Prothrombin gene variant	
Subgroup	N of studies	OR (95% CI)	N of studies	OR (95% CI)
Brain imaging in > 95% of patients	14	1.51 (1.22-1.90)	7	1.36 (0.96-1.93)
Age > 17 years	12	0.89 (0.67-1.19)	5	1.05 (0.63-1.74)
Use of population controls	12	1.15 (0.84-1.57)	4	1.05 (0.49-2.27)

OR=odds ratio, 95% CI=95% confidence interval

Most studies included adults only, seven also included children.[15, 18, 20, 22, 24, 25, 33] Considering only the studies with patients aged 18 years or more, the M-H odds ratio was 0.89 (95% CI: 0.67-1.19) for FVL mutation and 1.05 (95% CI: 0.63-1.74) for prothrombin gene variant (Table 4).

All studies used different criteria for the selection of controls, and in some they were poorly defined.[20] Most studies used population based controls[14-19, 22, 23, 27-29, 34-36] others used blood bank donors[18, 21, 26, 30] or healthy physicians[26, 31, 32] and other hospital workers[24, 33] as control subjects. One study used a cohort of new-born babies as control group.[25] A subanalysis of the studies in which only population-based control subjects were used, showed a change in M-H odds ratio for FVL from 1.35 to 1.15 (95% CI: 0.84-1.57) and from 1.32 to 1.05 (95% CI: 0.49-2.27) for prothrombin gene variant (Table 4).

Although ethnic origin can be an important confounder when studying genetically determined coagulation disorders, it was mentioned in only six studies.[19, 25, 31-33, 35] Because of this small sample size, we did not perform a subgroup analysis of the relationship between ischemic stroke and ethnic origin.

COCOS study

Five patients (4%) and 4 controls (3%) were heterozygous for the FVL mutation. None of the participants were homozygous for the FVL mutation. The OR of ischemic stroke associated with the FVL mutation was 1.3 (95%CI: 0.27-6.6). Two patients (2%) and 5 controls (4%) had the G20210A prothrombin gene variant. Again no homozygous mutations were found in cases or controls. The OR of ischemic stroke associated with the prothrombin 20210A gene variant was 0.4 (95% CI: 0.04-2.49). The OR did not change after adjustment for smoking, use of contraceptives, hypertension or diabetes for both coagulation disorders.

The mean prothrombin activity was significantly higher in participants with the G20210A prothrombin gene variant than in participants without the mutation (1.08 U/ml versus 1.32 U/ml, p<0.0001, student's t). The mean prothrombin activity in patient group as a whole was 1.09 ± 0.16 U/ml and in controls it was 1.10 ± 0.16 U/ml, (p=0.6). After exclusion of all individuals with the prothrombin gene variant, the mean prothrombin activity in controls slightly changed to 1.09 ±0.15 U/ml. In the convalescent phase, the mean prothrombin activity in patients was 1.11 ± 0.19 U/ml compared with 1.10 ± 0.16 U/ml in controls. Females had significantly higher prothrombin levels than males 1.12± 0.15 U/ml versus 1.06 ± 0.12 (p=0.0007). The prothrombin activity was also significantly higher in participants with hypercholesterolemia. Age, hypertension, smoking and type of stroke did not influence the prothrombin level. The OR of ischemic stroke in the fourth quartile of prothrombin activity was 1.03 (95%CI: 0.58-1.85) during the first blood examination. Three months later the OR in the highest quartile versus the lower was 2.0 (95 % CI: 0.90-4.37). After adjustment for oral contraceptives the OR slightly increased to 2.2 (95% CI: 0.96-4.90) (Figure 2a and 2b).

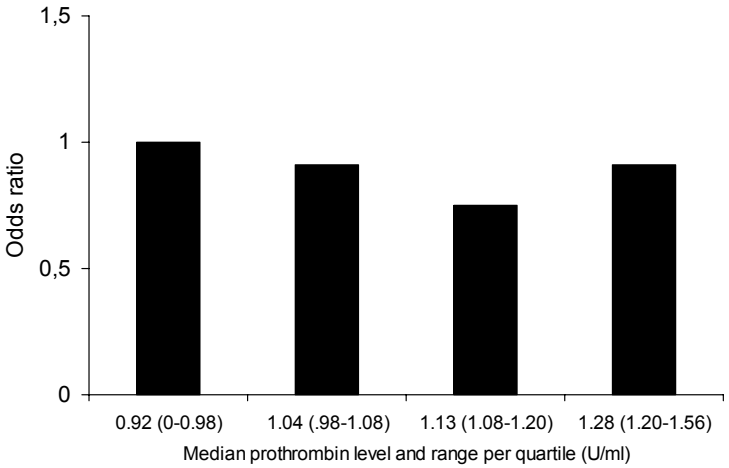


Figure 2a: Relative risk (odds ratios) for ischemic stroke according to prothrombin (PT) level during the first blood sample in the acute phase. Patients and controls were stratified according to PT level: first quartile <0.98, second quartile 0.98-1.08, third quartile 1.08-1.20, fourth quartile 1.20 U/ml or higher. Mean PT level in each quartile are indicated on the x-axis. Odds ratios (OR) were computed for patients in the second, third and fourth quartile as compared with the first quartile. The 95% confidence interval of the odds ratio for the second quartile was 0.45-1.85, for the third quartile 0.37-1.51, for the fourth quartile 0.45-1.86.

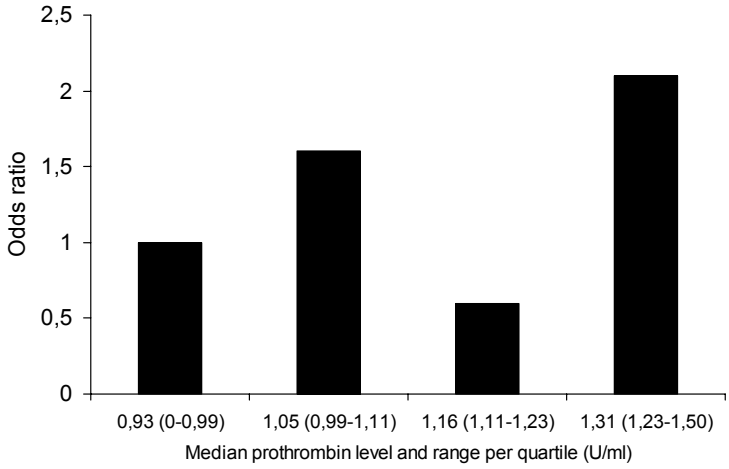


Figure 2b: Relative risk (odds ratios) for ischemic stroke according to prothrombin (PT) level during the second blood sample in the convalescent phase. Patients and controls were stratified according to PT level: first quartile <0.99, second quartile 0.99-1.18, third quartile 1.18-1.23, fourth quartile 1.23 U/ml or higher. Mean PT level in each quartile are indicated on the x-axis. Odds ratios (OR) were computed for patients in the second, third and fourth quartile as compared with the first quartile. The 95% confidence interval of the odds ratio for the second quartile was 0.59-4.36, for the third quartile 0.19-1.97, for the fourth quartile 0.76-5.67.

DISCUSSION

Systematic review

In this study, we systematically reviewed case-control studies that addressed the association between prothrombin 20210A gene variant or FVL mutation and the occurrence of ischemic stroke. The M-H odds ratio for the association with ischemic stroke was 1.35 (95% CI: 1.11-1.65) for FVL mutation and 1.32 (95% CI: 0.95-1.84) for prothrombin gene variant. The results of this meta-analysis, based on studies published between 1995 and 2000, suggest that FVL is a weak risk factor for stroke. Although the effect of the prothrombin gene variant is probably of similar direction and magnitude, it was just not statistically significant.

Some studies included patients with hemorrhagic stroke although the aim was to investigate the relationship between ischemic stroke and genetically determined coagulation disorders.[19,26] Hemorrhagic events are unlikely to be caused by FVL mutation or prothrombin gene variant. Furthermore, in some studies, the diagnosis of ischemic stroke was not reliable because no imaging studies were done at all[16,17,36] or only in a proportion of the patients.[13] Both for FVL mutation and the prothrombin gene variant the relative risk of stroke seemed to increase when we excluded the studies without a high stroke-type verification rate through brain imaging. This finding emphasizes the importance of valid inclusion criteria.[37-39]

Some studies included children with stroke.[15, 18, 20, 24] In children, FVL may be a stronger risk factor than in adults.[40-42] We could not assess the effect of age on the possible relationship between prothrombotic conditions and ischemic stroke, as we could not re-analyse the data from each study. Therefore, we could assess the influence of age only on study level. After excluding those

studies with at least one patient who was younger than 18 years, the possible relationship between ischemic stroke and FVL mutation or prothrombin gene variant disappeared. This suggests that these genetically determined prothrombotic conditions may be especially important at a relatively young age. However, these prothrombotic conditions may also interact with the atherosclerotic process, especially in high risk patients. This interaction will be missed if not specifically sought for, in particular if the study is restricted to young patients with stroke of undetermined (i.e. non-atherosclerotic) origin.

Ethnic origin was not mentioned in the majority of studies.[11,13,15-21, 23, 24, 26, 27, 29, 34, 36] Geographic and demographic differences in prevalence of FVL mutation and prothrombin gene variant are well know.[38,43] We did not analyze the studies in which ethnic origin was mentioned separately because of their small number. Although an unequivocal definition of race is difficult to give[44], future studies should control for ethnicity either by matching, or by modifying their inclusion criteria for patients as well as for controls.

None of the studies assessed the relative risk that was associated with the phenotype that corresponds to the genetic defect, in this case resistance to activated protein C, or prothrombin level in plasma. This could be of importance, as more than one genetic defect could cause the phenotype, which means that the association between the occurrence of stroke and the phenotype could be stronger. On the other hand, assessment of phenotype could make the associations more plausible.

Publication bias may severely distort the results of a meta-analysis. In general, papers with positive results are more likely to become published. We performed a funnel plot in STATA with the funnel command[45] to qualitatively assess the effect of bias in these analyses. In a funnel plot the trials' effect estimates are plotted against the sample size. The funnel plot is based on the fact that precision in estimating the underlying association will increase as the sample size of the studies increases. Our funnel plot did not suggest bias, because the plot resembles a symmetrical inverted funnel (Figure 3).[46]

It has been suggested that, as the randomized clinical trial is the paradigm of occurrence research, it would be wise to use similar criteria of inclusion for case-control studies as would have been used to include patients for a large (population-based) cohort study.[47] In this light, the choice of population controls is easier to defend than that of hospital controls. Interestingly, both the association of FVL mutation and prothrombin gene variant with stroke disappeared when only studies with population-based controls were considered. However, these large studies concerned mostly elderly subjects without confirmation of the ischemic stroke by means of CT. In this meta-analysis, CT confirmation, age and type of controls were important confounding factors. In our opinion, future studies of FVL mutation, prothrombin 20210A gene variant or similar potential risk factors for ischemic stroke should include young stroke patients, confirm the ischemic nature of the stroke by brain imaging, use population based controls, and assess the corresponding phenotypes.

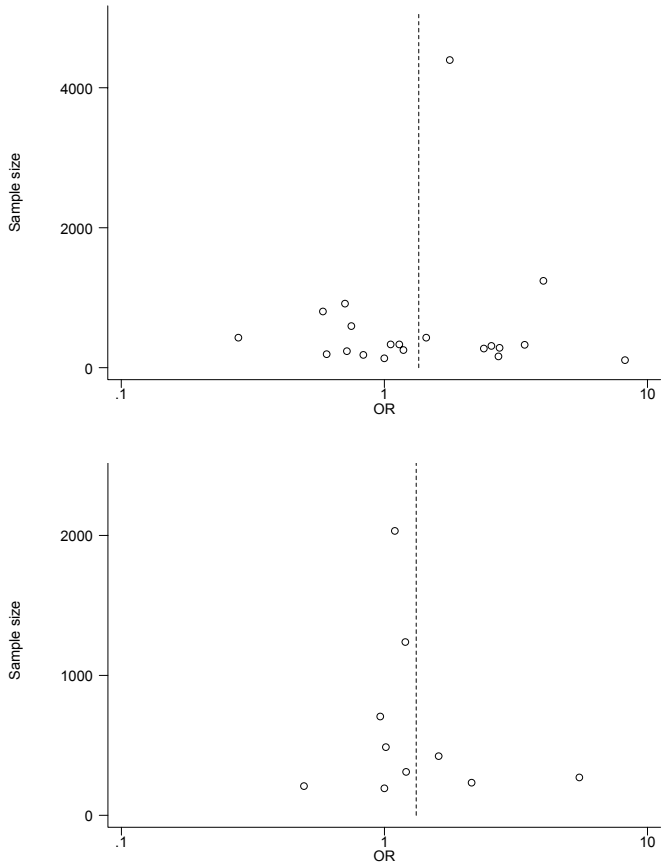


Figure 3a and 3b: Funnel plot of effect size (x-axis) versus sample size (y-axis) in case-control studies of factor V Leiden mutation (3a, upper) and Prothrombin 20210A gene variant (3b, lower) as a possible risk factor for stroke.

COCOS study

Although our systematic review of the literature suggested a weak association between ischemic stroke and FVL mutation and G20210A prothrombin gene variant, we cannot corroborate an association by means of the results of the COCOS study. However, high prothrombin levels in the absence of the G20210A prothrombin gene variant seemed to be a risk factor for the occurrence of ischemic stroke.

A strength of this study is that cases were prospectively and consecutively included and that all patients underwent neuro-imaging to rule out hemorrhage. We were able to include population controls, thus avoiding the biases induced by "hospital controls". We collected detailed information about cardiovascular risk factors, medical history and family history from patients as well as controls. All participating subjects were of the Caucasian race and the allele frequencies of the polymorphism in the control group were in Hardy-Weinberg equilibrium, indicating that we studied an unselected group.

Our findings are in agreement with several larger population studies and case-control studies, which were performed after the year 2000.[48-52] These studies all reported a relative risk of around 1.0 with a narrow 95% confidence interval excluding a strong association between both of the mutations with the occurrence of ischemic stroke.

Some authors suggested that FVL mutation or G202120A prothrombin gene variant might be a risk factor in subgroups of patients, for example patients with patent foramen ovale[53-55], with cryptogenic stroke[56] or stroke at young age[20] whereas others could not support these findings.[36,48, 49, 58, 59] We cannot comment on subgroups, as subgroup analysis in our population was not possible because of the low prevalence of both mutations and the sample size of the study.

In our study, elevated prothrombin levels were associated with an increased stroke risk. This finding is in agreement with that of one other retrospective case-control study in young ischemic stroke patients described in chapter 2.1.[60] It is unlikely that prothrombin merely acted as an acute phase reactant since it was elevated long after the acute event as well as in controls, a finding that was also reported by Poort et al.[2] The association between prothrombin levels in the absence of the G20210A prothrombin gene variant and the occurrence of ischemic stroke has not been reported before. Prothrombin may be elevated due to other genetic variations within the prothrombin gene or due to metabolic or endogenous factors. Gender was correlated with prothrombin activity that was higher in women than in men. As the patients and controls were sex-matched, gender is not a possible confounder in our study. Higher prothrombin levels were measured in participants with hypercholesterolemia. However, the frequency of hypercholesterolemia was not significantly different between patients and controls. High prothrombin levels are reported in combination with the use of oral contraceptives.[61] After adjustment for this factor, our results did not change essentially.

In conclusion, an association between the FVL mutation and G20210A prothrombin gene variant and the occurrence of ischemic stroke in the Caucasian is highly unlikely. Consequently, there is no indication for routinely screening of these genetic coagulation disorders in these patients. Future studies should focus on carefully selected subgroups of patients such as ischemic stroke patients with patent foramen ovale or patients of specific geographic area or race, for instance the Mediterranean origin. Furthermore, elevated prothrombin levels seem to be associated with an increased risk of ischemic stroke. This association as well as its underlying pathophysiological mechanism, needs to be further investigated in larger studies.

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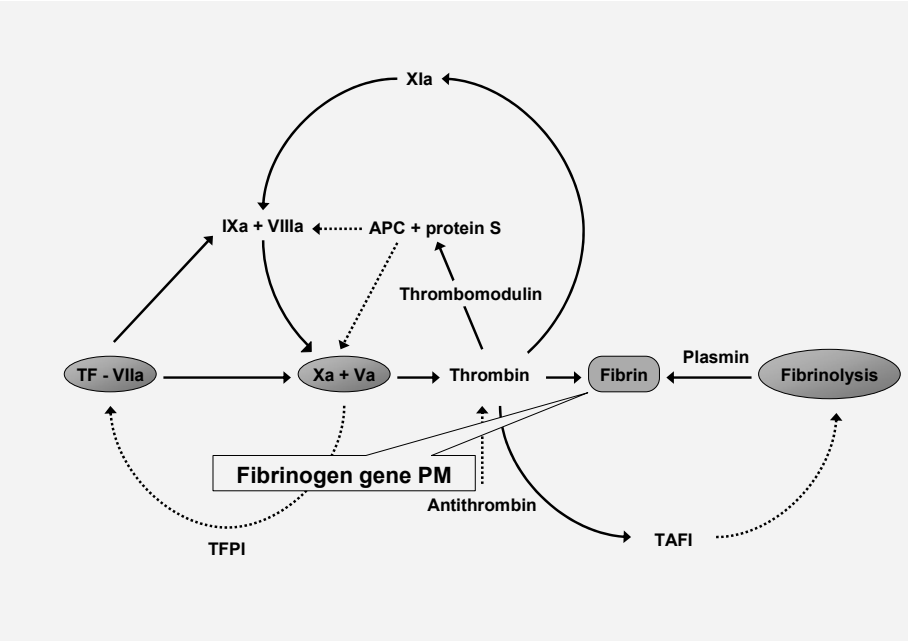
Chapter 4.2

-148 C/T Fibrinogen gene polymorphism and fibrinogen levels in ischemic stroke



M.P.J. van Goor, E.B. Gómez García, F.W.G. Leebeek, G.J. Brouwers, P.J. Koudstaal, D.W.J. Dippel. The -148 C/T fibrinogen gene polymorphism and fibrinogen levels in ischemic stroke: a case-control study. JNNP 2004 (in press)

ABSTRACT

Background: Increased fibrinogen level is a risk factor for ischemic stroke. Fibrinogen promoter gene polymorphisms are associated with fibrinogen levels. The aim of this study was to study whether the –148C/T fibrinogen gene promoter polymorphism increases stroke risk by modifying fibrinogen level.

Methods: We performed a case-control study among patients with first ever ischemic stroke, confirmed by CT. All patients were screened for cardiovascular risk factors, cardiac disorders and large vessel disease. Patients with a definite non-atherosclerotic cause of the stroke, oral anticoagulants or age over 75 years were excluded. Venous blood samples were collected for fibrinogen level and routine coagulation tests one week after the stroke. The measurements could be repeated after 3 months in about half of the patients. Population controls were age -and sex-matched, and did not have a history of stroke. The -148C/T fibrinogen polymorphism was determined by PCR followed by digestion with restriction enzymes HindIII/AluI.

Results: We included 124 patients and 125 controls. The mean age was 56 years (range 18 to 75). Thirty four patients (27%) and 41 (33%) controls were heterozygous for the -148 C/T fibrinogen polymorphism and 6 (5%) patients and 5 (4%) controls had the T/T genotype. The odds ratio of ischemic stroke associated with CC -homozygotes versus T-carriers was 0.8 (95% CI: 0.5 to 1.4). The relative risk of ischemic stroke associated with fibrinogen levels in the highest quartile was 3.9 (95% CI: 1.9 to 8.4) at one week, but it decreased to 1.4 (95% CI: 0.6 to 3.3) at 3 months after the stroke.

Conclusion: Fibrinogen levels are associated with stroke, but the -148 C/T fibrinogen gene polymorphism is not a strong risk factor for ischemic stroke. High fibrinogen levels most likely represent an acute phase response.

INTRODUCTION

Fibrinogen is a well-known risk factor for myocardial infarction and stroke. Several meta-analyses of (nested) case-control studies have confirmed that high fibrinogen levels are associated with an increased risk of cardiovascular disease and stroke.[1-4] Whether this relationship between fibrinogen level and stroke marks the inflammatory status of the vessel wall or causally contributes to the occurrence of ischemic stroke, is a much debated question.[5-11]

Higher fibrinogen levels have been reported in individuals carrying the rare allele of the fibrinogen β-promoter gene polymorphisms in the general population.[12-16] These genetic features could therefore increase stroke risk. An alternative view is that fibrinogen is merely a marker of acute phase reactions, and hence an innocent bystander rather than causative agent in stroke. However, a combination of both hypothesis is also possible, i.e. that the presence of the allele of the promoter gene polymorphism which causes higher levels, could also mediate the fibrinogen increase as part of the acute phase response after stroke.

Fibrinogen is a 340 kDa glycoprotein consisting of 2 sets of 3 polypeptide chains, known as α-, β-, γ-chains, linked by disulfide bonds. The 3 polypeptide chains are encoded by three different genes clustered on chromosome 4 in region q28.[17] The β-chain gene has been most extensively been studied because the β-chain synthesis is the limiting step in the production of fibrinogen. Several promoter polymorphisms of the β-chain gene are described, such as –455G/A, –148 C/T and –854 G/A. These polymorphisms are in almost complete linkage disequilibrium.[18] The –148 C/T polymorphism is located close to the consensus sequence of the IL-6 responsive element and expression studies show that this polymorphism is functional and determines the response to an IL-6 stimulation.[19]

A few case-control studies focused on the possible association between the fibrinogen promoter gene polymorphisms and stroke. The published results are not in agreement.[20-23]The –455G/A polymorphism, has been reported to be a risk factor for stroke in subgroups only, such as patients with lacunar stroke[22], with large vessel disease[21] or in the japanese race.[23] However, in a prospective cohort study the -148 C/T fibrinogen gene polymorphism was not associated with an increased risk of stroke.[20] In this study only genotype and not phenotype was described. Because the -455 G/A and -148 C/T fibrinogen polymorphisms are in complete allelic association[18], the results of these studies are contradictory.

To better understand to what extent the higher risk of stroke as a result of elevated fibrinogen levels is genetically determined or an acute phase response, it would be necessary to study the polymorphism and fibrinogen levels simultaneously, as well as a sequential measurement of the levels of fibrinogen.

We therefore designed a case-control study, firstly to investigate the possible association between the –148 C/T fibrinogen polymorphism and ischemic stroke and secondly, the extent to which this association could be explained by the fibrinogen level.

METHODS

Study design

The design of the study has been described extensively in chapter 3 of this thesis. In short, COCOS is a case-control study with prospective inclusion of Caucasian participants with first

ever ischemic stroke, confirmed by CT. Controls are population based, age-and sex-matched, of the Caucasian race and not related to the patients. Blood samples for genotype and phenotype were drawn under strictly standardised conditions one week after the event and in almost half of the patients also after three months.

Blood samples and procedures

Fibrinogen levels, cholesterol, glyco-Hb, and CRP levels were determined in the blood samples. CRP was used as a control parameter of the acute phase response. Fibrinogen was measured as described by von Clauss.[24] Genomic DNA was isolated from the white cell fraction of citrated blood, according to the high-salt concentration standard procedure.[25] The –148 C/T fibrinogen polymorphism was detected by PCR followed by digestion with restriction enzymes HindIII/ AluI.[18]

In patients as well as in controls, we collected detailed information about cardiovascular risk factors, such as smoking habit, hyperlipidemia, hypertension, diabetes, use of oral contraceptives, and about medical history and family medical history. Patients were screened for cardiac abnormalities by means of standard twelve lead ECG examination. A cardiologist was consulted in female patients aged 55 years or less, in male patients aged 45 years or less and in patients with ECG abnormalities or a history of cardiac disease. In these patients, 24 hours ECG monitoring, transthoracic and/or transesophageal echocardiography was carried out. We did not perform complete cardiac work-up in the older patients, because in this patient group, the chance of finding a relevant cardiac cause for the stroke is very low, given a normal EEG and the absence of cardiac symptom. Screening for large vessel disease included duplex ultrasound or, if indicated, angiography of the carotid or vertebral arteries.

Statistical aspects

For the -148 C/T mutation with an expected prevalence of the T allele of about 20% in the controlgroup[26], the minimal detectable odds ratio would be 2 to 3.

The relationship between the –148 C/T fibrinogen polymorphism and ischemic stroke was expressed as an odds ratio with a 95% confidence interval. The fibrinogen levels were divided into quartiles and the relationship between fibrinogen level and ischemic stroke was estimated by odds ratio per quartile, and for the highest quartile versus the lower three quartiles. Multiple logistic regression analysis was used to adjust for possible confounders, such as smoking, hypertension, as these vascular risk-factors are known to affect fibrinogen level as well. Furthermore, the relationship between genotype and stroke severity was explored by relating genotype to a dichotomized Barthel score.

RESULTS

During the study period 1034 patients with acute stroke were admitted to our hospital. Twenty percent of the patients had an intracerebral hemorrhage, 13% died within a few days as a result of the stroke, 28% had a recurrent ischemic stroke. Of the remaining patients, 38% were of Caribbean or Mediterranean origin and were therefore not included. One patient was excluded later, after she was found to have DIC, induced by a pancreatic malignancy. The final study population consisted of 124 patients and 125 controls. In 123 patients and 123 controls, blood samples were available for detection of the fibrinogen polymorphism and fibrinogen level.

Table 1 shows the baseline demographics and vascular risk factors in patients and controls. Patients were more often smokers and more often had hypertension, diabetes or were known to have cardiovascular disease compared with controls.

In half of the cases no cause for the ischemic stroke was found according to the TOAST classification (Table 2). In 2 young patients the evaluation was not complete because both refused echocardiography. One patient was excluded later on, because she appeared to have suffered from DIC, resulting from a pancreatic malignancy.

Table 1. Baseline characteristics

	Cases (n=124)	Controls (n=125)
Demographics		
Age, yr (sd)	56 (±12)	56 (±12)
Female sex	58 (47%)	59 (47%)
Index event		
Stroke: TIA	115: 9	NA
Risk factors		
Smoking	61 (49%)	37 (30%)
Hypertension	60 (48%)	24 (19%)
Diabetes	18 (14%)	5 (4%)
Hypercholesterolemia	78 (63%)	84 (67%)
Oral contraceptives	11 (19%)	8 (14%)
Positive family history for cardiovascular disease	75 (61%)	56 (45%)
Previous DVT	6 (5%)	0 (0%)

Table 2. Division of stroke subtypes in cases according to the TOAST criteria

TOAST	N (%)
Large vessel disease	11 (9%)
Cardiac embolism	4 (3%)
Small vessel disease	46 (37%)
Other etiology	0 (0%)
Undetermined etiology	61 (49%)
Incomplete evaluation	2 (2%)

Thirty four (27%) patients and 41 (33%) controls were heterozygous for –148 C/T fibrinogen gene polymorphism and 6 (5%) patients and 5 (4%) controls were homzygous for the T allele. The genotype frequencies in controls were in a Hardy-Weinberg equilibrium. The odds ratio (OR) of ischemic stroke associated with the -148C/T genotype was 0.8 (95% CI:0.4-1.3) and with the –148 T/T genotype 1.1 (95% CI: 0.5-1.4, Table 3).

We also estimated the relative risk of stroke associated with the -148 C/T fibrinogen gene polymorphism in several subgroups of patients. There were no indications that the polymorphism was a risk factor in patients who smoked or had hypertension or diabetes. Furthermore, there

were no differences in stroke risk for the fibrinogen gene polymorphism in patients with small vessel disease, cardiac embolism or large vessel disease. There was no relationship between stroke severity and genotype (data not shown).

Table 3. Genotype frequencies of the –148C/T polymorphism among patients and controls and the odds ratio (with 95% confidence interval) of ischemic stroke associated with the CC homozygotes versus T-allele carriers

	Cases (n=123)	Controls (n=123)	OR (95%CI)
CC	83 (67%)	77 (62%)	--
CT / TT	40 (33%)	46 (37%)	0.8 (0.5-1.4)

In the stroke patients, the highest fibrinogen levels were found in combination with the TT genotype and the lowest in combination with the CC genotype (table 4). In controls, there were no differences in fibrinogen level according to genotype.

The mean fibrinogen level was higher in patients than in controls (3.7 g/l vs. 3.4 g/l, $p<0.02$ student's t). The blood sample could be repeated at least three months after the ischemic stroke in 64 patients. At that time, the mean fibrinogen level had decreased to 3.3 g/l, which did not significantly differ from the level in controls.

Table 4. Mean fibrinogen levels (g/l) and standard deviation (g/l) according to genotype in controls and in patients one week after the stroke(1st blood sample) and three months after the stroke (2nd blood sample) n=number of patients or controls, sd=standard deviation

	Controls			Patients 1st sample			Patients 2nd sample		
Genotype	mean	sd	n	mean	sd	n	mean	sd	n
CC	3.45	0.6	70	3.62	1.1	81	3.25	0.8	42
CT	3.34	0.5	38	3.82	0.9	34	3.43	0.6	19
TT	3.38	0.4	4	3.75	0.6	6	4.07	0.7	3

We divided the fibrinogen levels in quartiles and estimated the OR for ischemic stroke per quartile fibrinogen (Table 5). The odds ratio for ischemic stroke in the highest quartile versus the lower three quartiles was 3.9 (95% CI: 1.9-8.5). After adjustment for smoking and hypertension the OR was 3.5 (95% CI:1.7-7.2). The OR based on fibrinogen levels in the second blood samples, taken three months after the index event, dropped to 1.4 (95% CI: 0.6-3.3).

Table 5. Odds ratio (OR) for ischemic stroke per quartile of fibrinogen level (g/l) 1st blood sample was taken at 1-2 weeks after stroke, 2nd blood sample was taken at 3 months after stroke.

	1 week after stroke	3 months after stroke
Quartile (fibrinogen range)	OR (95% CI)	OR (95% CI)
1 (1.9-3.0)	Ref	ref
2 (3.1-3.5)	0.5 (0.2-1.1)	0.5 (0.2-1.3)
3 (3.6-3.9)	0.6 (0.3-1.4)	0.7 (0.3-1.7)
4 (>3.9)	2.6 (1.1-6.5)	1.01 (0.4-2.7)
4 vs 1-3	3.9 (1.9-8.5)	1.4 (0.6-3.3)

The mean CRP level was 16 mg/l (median 5mg/l, range 1-215 mg/l) in patients and 4 mg/l (median 3 mg/l, range 1-21 mg/l) in controls one week after the stroke. After three months the CRP values were equal in both groups and lower than during the first blood examination. CRP level was strongly related to fibrinogen early after the stroke (r=0.56).

DISCUSSION

We studied the association of the -148 C/T fibrinogen gene polymorphism and first-ever ischemic stroke in a case-control study among Caucasian subjects. Our results showed no association between ischemic stroke and the –148 C/T fibrinogen promoter gene polymorphism.

Internal validity

Cases were prospectively and consecutively included. All patients underwent neuro-imaging to rule out hemorrhage. We were able to include population controls, thus avoiding the biases induced by "hospital controls". We collected detailed information about cardiovascular risk factors, medical history and family history from patients as well as controls. All subjects participating were of the Caucasian race and allele frequencies of the polymorphism in the control group were in Hardy-Weinberg equilibrium, indicating that we studied a representative group.

Because the primary aim of the study was to investigate the influence of a genetic feature, the T allele of the fibrinogen polymorphism, on the risk of stroke, bias does not play a role. Although the phenotypic expression, i.e. fibrinogen level, may vary, we can assume that genotypes are not changed by the occurrence of ischemic stroke. As a consequence of its design our study is limited to nonfatal cases of stroke. If the T allele was associated with fatal cases of stroke, we would underestimate the true risk for ischemic stroke associated with the presence of the T allele.

External validity

One could argue that our study is not large enough to exclude an association between ischemic stroke and the –148 C/T fibrinogen gene polymorphism. The OR in our study was 0.8 with a narrow 95% confidence interval of 0.5 to 1.4. Although we did not reach statistical significance for the risk, we think we can conclude that the 95% confidence interval indicates that even if the polymorphism is a risk factor, it cannot be a strong one. One other (nested case-control) study confirms our findings in that it did not find evidence for an increased stroke risk in the presence of the polymorphism.[20]

We found a statistically significant difference between fibrinogen levels in patients and controls one week after the stroke. This finding confirms the already accepted association between fibrinogen level and cardiovascular events.[1-4] In several nested case-controls studies higher fibrinogen levels at baseline were found among patients who developed a stroke during the follow-up time with OR's varying between 1.3 and 2.6 in the highest quartile.[2,9,27,28] Our OR of 1.4 (95%CI: 0.6-3.3) for the highest quartile three months after the stroke is compatible with previous studies.

The fibrinogen level in controls was not related to genotype in our study. Two other cross-sectional cohort studies did not find increased fibrinogen levels in individuals with a CT or TT genotype at –148 position of the fibrinogen promoter gene as well.[15,29]

Discussion

There are several explanations for the differences in fibrinogen levels between stroke patients and controls that were highly significant shortly after the event but had mostly disappeared after 3 months.

Fibrinogen levels are subject to biologic variation, with elevated circulating levels as a consequence of an acute phase reaction. This might explain the elevated fibrinogen levels in patients one week after the stroke. This is supported by concomitant changes in CRP level, an established acute phase glycoprotein. Secondly, other cardiovascular risk factors such as hypertension, smoking and diabetes, are associated with higher fibrinogen levels and advanced atherosclerosis.[30] The lower fibrinogen levels at three months after the stroke could in theory have resulted from rigorous treatment of these risk factors, particularly smoking. However, only 5 patients (4 %) stopped smoking. This could therefore only partly explain our observations.

Apart from the cardiovascular risk factors, Il-6 elevates the fibrinogen level and also the CRP level.[2,28,31] In one case-control study, the –174 G/C polymorphism of the IL-6 gene was associated with higher IL-6 and fibrinogen levels but not with cardiovascular disease.[32] In contrast, in a recent case-control study the polymorphism was associated with lacunar stroke.[33] It may be that this or another polymorphism of the IL-6 gene, rather than the known fibrinogen promoter gene polymorphisms, are associated with the occurrence of ischemic stroke. Since the -148 C/T polymorphism lies close to the putative IL-6 responsive element in the fibrinogen gene, one could hypothesize that this polymorphism is also within the IL-6 responsive region, and therefore its intrinsic effect on the fibrinogen levels, determined by the presence of a C or T allele, is additionally modulated by IL-6. This could explain why the fibrinogen levels were associated with the -148C/T fibrinogen promoter polymorphic alleles only in patients, but not in the group of controls.

In conclusion, the –148 C/T fibrinogen gene polymorphism is not associated with an increased risk of ischemic stroke in Caucasian patients. The high fibrinogen levels shortly after the stroke most likely represent a nonspecific acute phase response, although a causative or prognostic role cannot be excluded. Future studies should focus on other polymorphisms, and their phenotypic expression, that may be related to increased acute phase proteins.

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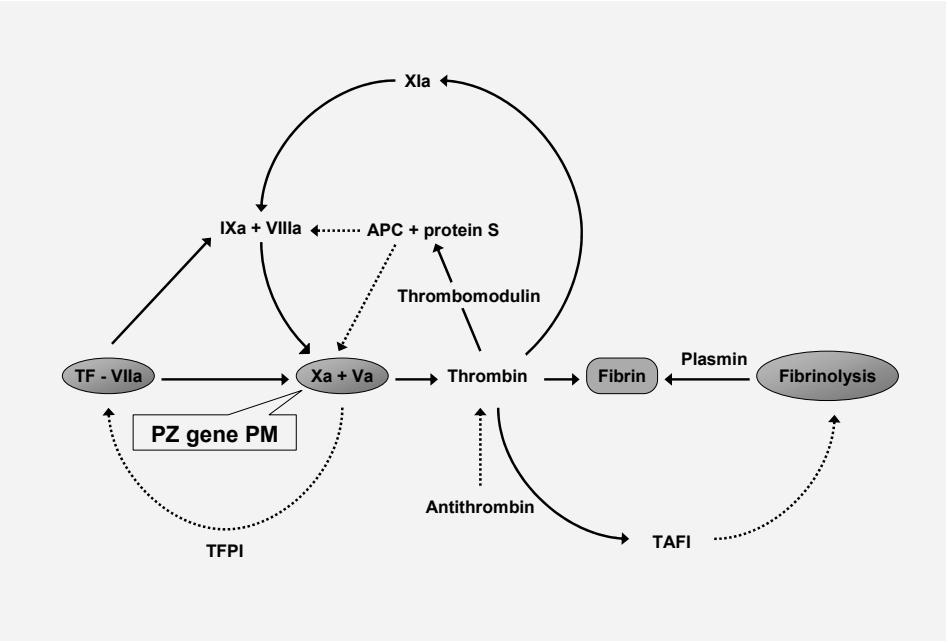
Chapter 4.3

Protein Z gene polymorphism and protein Z levels in ischemic stroke



M.P.J. van Goor, D.W.J. Dippel, K.S-G. Jie, M.P.M. de Maat, P.J. Koudstaal, F.W.G. Leebeek. Low protein Z levels but not the protein Z gene G79A polymorphism are a risk factor for ischemic stroke. Submitted.

ABSTRACT

Background: Protein Z (PZ) is a vitamin K-dependent plasma protein that plays a role in dampening coagulation. It acts as a cofactor in the inhibition of factor Xa by PZ-dependent protease inhibitor. The exact role of PZ in arterial thrombosis and ischemic stroke is still unknown. The risk of ischemic stroke may be under genetic influence, through modulation of plasma levels of PZ. The aim of this study was to determine the association between the intron F G79A PZ gene polymorphism, PZ level and ischemic stroke.

Methods: We performed a case-control study in 124 Caucasian patients with first ever ischemic stroke confirmed by CT, and 125 age-and sex-matched controls The population controls were unrelated to the patients and did not have a history of stroke. Patients with a definite non-atherosclerotic cause of the stroke, use of oral anticoagulants or age over 75 years were excluded. Venous blood samples for PZ level were collected one week and 3 months after the ischemic stroke. The G79A PZ gene polymorphism was determined by polymerase chain direction and restriction enzyme digestion and PZ levels were determined using a commercially available enzyme-linked immunosorbent assay.

Results: Mean age was 56 years (range 18 to 75). The relative risk of ischemic stroke associated with PZ levels in the lowest quartile was 1.9 (95% CI: 1.0 to 3.8) at one week, but it increased to 2.9 (95% CI: 1.3 to 6.7) at 3 months after the stroke. Thirty-nine patients (33%) and 32 (28%) controls were heterozygous for the G79A PZ gene polymorphism and 4 (3%) patients and 4 (4%) controls had the AA-genotype. The PZ levels were significantly lower in subjects with the AA-genotype and intermediate in heterozygote subjects. The odds ratio of ischemic stroke associated with A-allele carriers versus GG-homozygotes was 1.2 (95% CI: 0.7 to 2.1). All associations were adjusted for differences in frequency of cardiovascular risk factors between cases and controls.

Conclusion: Low PZ levels are independently associated with an increased stroke risk. However, the G79A PZ gene polymorphism is not a strong risk factor for ischemic stroke. We suggest that previous studies were confounded by acute phase reactions.

INTRODUCTION

Protein Z (PZ) is a vitamin K dependent single-chain glycoprotein of 62 kDa synthesized in the liver. Its amino-terminal sequence is highly homologous to the vitamin K-dependent coagulation factors II, VII, IX, X and protein C.[1,2] Although PZ was already purified from bovine plasma in 1977[3] and later found in human plasma[1], its precise physiological function remains unclear.

Although initial data suggested that PZ deficiency was associated with a bleeding tendency,[4] subsequent clinical studies and a PZ null mice model failed to confirm these observations.[5-7] More recently, PZ was found to act as a cofactor in the inhibition of factor Xa by a plasma protein called Z-dependent protease inihibitor (ZPI).[2] It therefore seems more likely that low PZ levels are associated with a procoagulant rather than an anticoagulant state. Vasse et al[8] were the first to report a significant association between PZ deficiency and arterial (ischemic stroke) rather than venous thrombosis. Further support for a role of low PZ levels and arterial thrombosis comes from two additional studies in patients with ischemic stroke and acute coronary syndromes[9,10] Several other clinical case-control studies, mainly among younger patients, have yielded contradictory results (Table 1). Two studies reported a significant association between high (instead of low) PZ levels and the risk of ischemic stroke[11,12], whereas other authors found no differences in PZ level between patients and controls.[13] Two studies have investigated the PZ levels in the acute as well as the convalescent phase, with conflicting results.[10,12] Whereas the first[10] found lower PZ levels in cases, the second[12] reported a strong association between elevated PZ levels and ischemic stroke in the acute phase, that was no longer present 3 months after the stroke.

Table 1. Results of previous case-control studies on protein Z level, PZ gene polymorphism and ischemic stroke

Author	year	N cases/controls	Timing of sample	PZ levels	OR (95%CI) for IS and PM
Protein Z level					
Vasse[8]	2001	169 / 88	convalescent	↓	NA
Kobelt[11]	2001	157 / 192	convalescent	↑	NA
Wuillemin[14]	2001	157 / 192	convalescent	↑	NA
Lopaciuk[13]	2002	99 / 100	convalescent	=	NA
Heeb[10]	2002	154 / 206	convalescent	↓	NA
McQuillan[12]	2003	173 / 186	acute & convalescent	↑	NA
Intron F G79A PZ gene polymorphism					
Lichy[17]	2004	200 / 199	convalescent	NA	0.58 (0.39-0.86)

↑ increased in patients compared to controls; ↓ decreased in patients compared to controls; = no difference between patients and controls.

The gene encoding for PZ has been characterized and several common single nucleotide polymorphisms in the gene have been reported.[15,16] Recently, the A allele of the intron F polymorphism G79A in the PZ gene was described to be protective for ischemic stroke in the young.[17] In this study the PZ level was determined only in controls and not in patients.

To further clarify the role of PZ level in ischemic stroke and to determine in howfar this role is genetically determined, it is necessary to study the polymorphism and PZ plasma level simultaneously, as well as a sequential measurement of the PZ level. We therefore studied the association between the PZ G79A promoter gene polymorphism, PZ level and ischemic stroke among first ever ischemic stroke patients and population controls of the same genetic area.

METHODS

Study design

We performed a case-control study with prospective inclusion of the participants. Cases were consecutively recruited patients with first ever acute ischemic stroke, admitted to the department of Neurology of a university hospital, between January 1999 and December 2001. This hospital is an urban area hospital without specific selection criteria for the admission of stroke patients. However, young stroke patients are referred more frequently to this center than to the non-academic centers in the region. Fifty percent of the urban population consists of Caribbean or Mediterranean habitants. We used population controls, i.e partners, friends or neighbours of the patients. They were age-and sex matched, of the same race, without a history of stroke and not related to the patient. The local ethics board approved the study and informed consent was obtained form all subjects.

Inclusion and exclusion criteria

Patients, controls and their parents were born in Northern Europe and of the Caucasian race, because substantial differences in the prevalence of genetically determined coagulation disorders between people from different geographical regions or race have been reported.[16] Patients with a definite non-atherosclerotic cause for the stroke like mechanical heart valve, endocarditis or carotid dissection were excluded. Other exclusion criteria were age above 75 years and the use of oral anticoagulants.

Definitions and measurements

Ischemic stroke was defined as the acute onset of focal cerebral dysfunction due to cerebral ischemia with symptoms lasting more than 24 hours. Patients with TIA (symptoms lasting less than 24 hours) were included only if the neurological deficit in the acute phase was observed by a neurologist. In all patients a CT scan of the brain was made within three days after the onset of symptoms to confirm the diagnosis of ischemic stroke and to rule out hemorrhagic stroke.

Clinical stroke subtypes were classified according to the OCSP criteria, adjusted for CT findings.[18] Etiologic stroke type was classified according to the TOAST criteria.[19] We defined large artery atherosclerosis as a stenosis of presumed atherosclerotic origin in the symptomatic extracranial carotid or in the basilar artery of more than 50%. Stroke severity was assessed with the Barthel index at inclusion.[20]

Hypertension was defined as systolic blood pressure above 160 mm Hg or diastolic blood pressure above 90 mmHg 5 days after the event, or the use of antihypertensive medication. Hyperlipidemia was defined as fasting cholesterol level above 5.0 mmol/l or use of lipid lowering drugs. Diabetes mellitus was defined as the use of antidiabetic medication or glycosylated hemoglobin level over 6.5%. Myocardial infarction was defined as typical chest pain with documented elevated cardiac enzymes or confirmation by ECG. Deep venous thrombosis had

to be confirmed by compression ultrasonography or phlebology and pulmonary embolism by ventilation-perfusion scan or computed tomography of the chest.

Blood samples and procedures

One week and 3 months after the stroke, venous blood samples were taken under strictly standardized conditions as the patients were in fasting condition, with no exposure to cigarette smoking or alcohol for at least the preceding 8 hours. Venapuncture was performed after 15 minutes of rest. We measured PZ levels, cholesterol, glyco-Hb, and CRP levels. CRP was used as a control parameter of the acute phase response. PZ was measured using a commercially available Elisa (Asserachrom® protein Z, Roche Diagnostics, Almere, the Netherlands). The interassay- and intra-assay variation were 6.6% and 2.9% respectively. Genomic DNA was isolated from the white cell fraction of citrated blood, using a standard salting out procedure.[21] The PZ gene polymorphism was detected by amplification of a 320 bp sequence. Each 30 µl PCR reaction contained 20ng genomic DNA, 12.5 pmol of each primer (5'-TAA CAC CAT AGA CAG AGT CCG ATA TTC GC-3' and 5'-ATG AAC TCG GCA TTA GAA CAT GGT TGG AA-3'.), 150 nmol of each dNTP, 0.75 U Taq polymerase (Amersham) en PCR buffer (Amersham). The reaction components were incubated at 94ºC for 4 min, followed by 32 cycles of 94ºC for 1 min, 57ºC for 1 min and 74ºC for 1 min. Digestion for at least 1 hour at 37ºC with Hpal yielded 2 products of 221 and 99bp in length in the presence of the A allele, whereas the G allele was not digestible. The digestion products were separated by electrophoresis through a 2% agarose gel and visualized under UV-light.[17]

In both patients and controls, we collected detailed information about cardiovascular risk factors, such as smoking habit, hyperlipidemia, hypertension, diabetes, use of oral contraceptives, and about medical history and family medical history. Patients were screened for cardiac abnormalities by means of standard twelve lead ECG examination. A cardiologist was consulted in female patients aged 55 years or less, in male patients aged 45 years or less, and in patients with ECG abnormalities or a history of cardiac disease. In these patients, 24 hours ECG monitoring, transthoracic and/or transesophageal echocardiography was performed. Screening for large vessel disease included duplex ultrasound or, if indicated, angiography of the carotid or vertebral arteries.

Statistical aspects

For the PZ gene G79A polymorphism with an expected prevalence of the A allele of about 20% in the controlgroup[16] with a power of 80% and a significance level of 5%, the minimal detectable odds ratio would be 2.3

The relationship between the G79A PZ gene polymorphism and ischemic stroke was analysed using logistic regression analysis and expressed as an odds ratio with a 95% confidence interval. The PZ levels were divided into quartiles and the relationship between PZ level and ischemic stroke was estimated as odds ratio of each quartile against the lowest quartile, and for the lowest quartile versus the higher three quartiles. The logistic regression analysis was used to adjust for possible confounders, such as smoking, hypertension, diabetes and oral contraceptive use, as these vascular risk-factors are known to affect PZ level as well as cardiovascular risk. Furthermore, the relationship between genotype and stroke severity was explored by relating genotype to a dichotomized Barthel score (0-19 vs. 20 pts).

RESULTS

During the study period 1034 patients with acute stroke were admitted to our hospital. Twenty percent of the patients had an intracerebral hemorrhage, 13% died within a few days, 28% had a recurrent ischemic stroke. Of the remaining patients, 38% were of Caribbean or Mediterranean origin and were therefore excluded. One patient was excluded later, after she was found to have diffuse intravascular coagulation, induced by a pancreatic malignancy. The final study population consisted of 124 patients and 125 controls. Because in several participants some blood samples were not collected for logistical reasons, blood samples were available in 118 patients and 113 controls for detection of the PZ gene polymorphism and in 102 patients and 107 controls for the PZ level. In about half of the patients the blood sample could be repeated more than three months after the index event. In the other half the blood sample could not be repeated because patients stayed in a nursing-home outside the area of our hospital, used oral anticoagulants or had died. None of the subjects had a significant vitamin K deficiency or liver dysfunction, since the FX levels varied between 75% and 164% of normal values.

Table 2 shows the baseline demographics and vascular risk factors in patients and controls. Patients were more often smokers and more often had hypertension, diabetes or were known to have cardiovascular disease compared with controls.

In half of the cases no cause was found according to the TOAST classification. Eleven patients (9%) suffered from large vessel disease and 46 (37%) from small vessel disease. In four patients (3%) a probable source of cardio embolism was detected. In two young patients (2%) the evaluation was not complete because both refused echocardiography.

Table 2. Baseline characteristics

	Cases (n=124)	Controls (n=125)
Demographics		
Age, yr (sd)	56 (±12)	56 (±12)
Female sex	58 (47%)	59 (47%)
Index event		
Stroke: TIA	115: 9	NA
Risk factors		
Smoking	61 (49%)	37 (30%)
Hypertension	60 (48%)	24 (19%)
Diabetes	18 (14%)	5 (4%)
Hypercholesterolemia	78 (63%)	84 (67%)
Oral contraceptive	11 (19%)	8 (14%)
Family history of cardiovascular disease	75 (61%)	56 (45%)
Previous DVT	6 (5%)	0 (0%)

TIA=transient ischemic attack, DVT=deep venous thrombosis

In the acute phase the mean PZ level in patients and controls was almost equal (1.64 ± 0.6 μg/ml ±vs. 1.77 ± 0.6 μg/ml respectively, p=0.11 student's t). In the convalescent phase the mean PZ level in patients dropped to 1.47 ± 0.6 μg/ml, which was significantly lower than during the acute phase (p<0.008, student's t), and significantly lower than in controls as well (p<0.005, student's t) (figure 1).

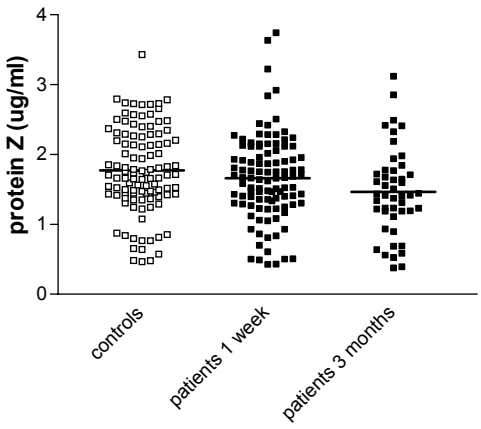


Figure 1. Protein Z levels in controls and patients 1 week and 3 months after the ischemic stroke

We divided the PZ levels in quartiles and estimated the relative risk for ischemic stroke in the lowest quartile versus the higher three quartiles. During the acute phase, the OR for ischemic stroke associated with low PZ level was 1.7 (95% CI: 0.9-3.2). After adjustment for smoking, hypertension, diabetes and oral contraceptives the OR changed to 1.9 (95% CI: 1.0-3.8). Exclusion of patients who did not have a second sample did not affect the relationship of PZ levels in the acute phase with stroke occurrence. In the convalescent phase, three months later, the OR for lowest quartile of PZ level versus the higher three was 3.4 (95% CI: 1.6-7.3) and remained highly significant after adjustment, i.e. 2.9 (95% CI: 1.3-6.7) (Table 3). Odds ratio's increased monotonously with each quartile of protein Z level (p for trend = 0.006)

Table 3. Adjusted odds ratio (OR) for ischemic stroke per quartile and for lowest quartile versus higher three quartiles during the acute and convalescent phase. 95% CI=95% confidence interval; #p for trend=0.12; +p for trend=0.006

	1st blood sample	2nd blood sample
Quartile	OR (95% CI)#	OR (95% CI)+
4	Ref	Ref
3	1.8 (0.8-3.3)	2.6 (1.0-6.7)
2	1.1 (0.5-2.5)	3.5 (1.3-9.9)
1	2.5 (1.2-5.2)	5.0 (1.8-12.5)
1 vs. 2-4	1.9 (1.0-3.8)	2.9 (1.3-6.7)

In patients as well as controls, those with diabetes mellitus had higher PZ levels ($p<0.05$, student's t). In patients older than 45 years, the PZ levels in the acute phase as well as in the convalescent phase were significantly lower than in age-matched controls (1.82 µg/ml vs. 1.61 µg/ml, $p<0.02$, student's t, and 1.82 µg/ml vs 1.53 µg/ml, $p<0.02$, student's t, respectively)

The mean CRP level was 16 mg/l (median 5mg/l, range 1-215 mg/l) in patients and 4 mg/l (median 3 mg/l, range 1-21 mg/l) in controls one week after the stroke. After three months the mean CRP values were equal in both groups and lower than during the first blood examination. There was no correlation between CRP levels and PZ levels in both blood samples (Spearman' rho 0.02, $p=0.83$).

In controls, the highest PZ levels were found in combination with the GG genotype and the lowest in combination with the AA genotype. In patients also the highest levels were found in the GG genotype in the acute phase as well as in the convalescent phase. The presence of the A-allele was associated with lower levels, but there were no statistically significant differences between the heterozygous and homozygous A-genotype in patients (Table 4).

Table 4. Mean protein Z levels (g/l) and standard deviation (g/l) according to genotype in controls and in patients one week after the stroke(1st blood sample) and three months after the stroke (2nd blood sample) n=number of patients or controls, sd=standard deviation

	Controls			Patients 1st sample			Patients 2nd sample		
Genotype	mean	sd	n	mean	sd	n	mean	sd	n
GG	1.90	0.61	68	1.79	0.51	65	1.6	0.65	27
GA	1.56	0.46	28	1.43	0.64	30	1.2	0.49	18
AA	0.81	0.04	3	1.61	0.49	3	-	-	0
Total	1.77	0.61	107	1.64	0.59	102	1.46	0.62	47

Thirty nine (33%) patients and 32 (28%) controls were heterozygous for G79A PZ gene polymorphism and 4 (3%) patients and 4 (4%) controls were homozygous for the A-allele. The genotype frequencies in controls were in Hardy-Weinberg equilibrium. The odds ratio (OR) of ischemic stroke associated with A-allele carriers versus GG-homozygotes was 1.2 (0.7-2.1). After exclusion of patients who did not have a second blood sample, the association between genotype and the occurrence of ischemic stroke was not different from that of the group as a whole.

We also estimated the relative risk of stroke associated with the PZ gene polymorphism in several subgroups of patients. There were no indications that the polymorphism was a risk factor in patients who smoked or had hypertension or diabetes. Furthermore, no differences in stroke risk for the PZ gene polymorphism in patients with small vessel disease, cardiac embolism or large vessel disease was found. Neither was there a relationship between stroke severity and genotype (data not shown).

DISCUSSION

We studied the association between PZ gene polymorphism and PZ levels with ischemic stroke in a case-control study among Caucasian subjects. We found a trend towards lower levels of PZ in patients compared with controls shortly after the stroke. Three months after stroke onset, the PZ levels in patients were significantly lower than in controls. Furthermore, we observed a strong correlation between genotype and PZ levels. The genotype, however, was not associated with the occurrence of ischemic stroke.

Internal validity

A strength of this study is that cases were prospectively and consecutively included and that all patients underwent a detailed assessment, including neuro-imaging to rule out hemorrhage. We were able to include population controls, thus avoiding the biases induced by "hospital controls". We collected detailed information about cardiovascular risk factors, medical history and family history from patients as well as controls. All participating subjects were of the Caucasian race and the allele frequencies of the polymorphism in the control group were in Hardy-Weinberg equilibrium, indicating that we studied an unselected group.

A limitation of this study is the inclusion of mainly nonfatal cases of stroke. If the A-allele would be associated with fatal stroke, we underestimate the true risk for ischemic stroke associated with the presence of the A-allele.

A further limitation was the small number of patients in whom we were able to obtain a blood sample 3 months after the onset of stroke, which raises the possibility of selection bias. However, the similar age, sex and genotype distribution does not support that. Moreover, our results did not change after exclusion of patients with only one blood sample.

We did find a relationship of protein Z level with presence of the A allele in controls, and a relationship between decreased protein Z level and stroke. One would therefore expect therefore that more stroke patients would have the A allele than controls, but this was not the case. The most likely explanation is in our view the existence of a yet unknown polymorphism that is alsoassociated with decreased protein Z levels. A systematic laboratory error that could explain differences in prot Z level is excluded, because all samples were assessed in one batch, by one laboratory assistant, who was not aware of the status of the samples.

Other studies

Our findings are in accordance with two other case-controls studies[8,10] that reported an increased risk of stroke in subjects with low PZ levels. Furthermore, our results are in agreement with recent pathophysiological studies that described a much higher relevance of PZ in humans as a mediator of factor X inhibition than as a procoagulant agent.[2,5] Our results are in discordance with two previously reported studies, investigating PZ level in the acute phase and the convalescent phase together.[12] One study[10] found no differences in PZ levels measured within 4 days and 2 months after the stroke. The other study[12] reported significantly higher PZ levels in the acute phase, whereas that difference had disappeared three months later. In our study, during the first sample the PZ levels were slightly lower in patients than in controls and significantly higher than in the convalescent phase. The different findings of PZ can be possibly explained by the moment the blood samples were taken. We measured the PZ level 7 to 14 days after the event, whereas McQuillan et al[12] took blood samples within the first week,

when the acute phase response is more pronounced. The presence of an acute phase response in our study was supported by concomitant changes in CRP level, an established acute phase glycoprotein. The difference between the two studies in PZ levels in the convalescent phase cannot be explained by influence of a mild vitamin K deficiency as FX antigen level were normal in cases as well as controls. The use of oral anticoagulant therapy does not provide an explanation as this was an exclusion criterion for our study. Another reason for the difference could be that we included by chance only patients with the AA-genotype, which is associated with low PZ levels, during the convalescent phase. However, the distribution of the genotype frequencies was not different from that during the acute phase. Moreover, the OR for low PZ levels and ischemic stroke did not change when only the patients in whom blood samples were taken during both phases were considered. Furthermore, our results during the convalescent phase disagree with those from two case-controls studies[11-13], but are supported by the majority of the pathophysiological[1,2,5] and clinical studies.[8-10, 13]

One could argue that our study is not large enough to exclude an association between ischemic stroke and the G79A PZ gene polymorphism. The OR in our study was 1.2 with a narrow 95% confidence interval of 0.7 to 2.1, which indicates that even if the polymorphism is a risk factor, it cannot be a strong one. One other study that addressed the relationship between PZ gene and stroke reported a protective role of genetically determined low PZ levels in juvenile cerebral ischemia.[17] In our population, there were no differences in the genotype distibution of the PZ G79A gene polymorphism in the study as a whole or in the young participants. We cannot explain this difference since the allele frequencies in controls in both studies were in Hardy-Weinberg equilibrium, and the participants in both studies represented a genetically homogeneous group. In the same study[17], the PZ level was determined only in a part of the control group. The authors inferred that high PZ levels could be a risk factor for ischemic stroke because the A-allele, that is associated with lower PZ levels, was more frequent in the control group. As the PZ levels in patients were not determined, we think it is premature to conclude that high PZ levels are associated with ischemic stroke, particularly since our results and those of three other studies suggest the opposite.[8,10,11]

The first aim of our study was not to unravel the pathophysiological mechanism of PZ in ischemic stroke. However, as low PZ levels in the convalescent phase were independently associated with an increased risk of ischemic stroke, a causative role for PZ in ischemic stroke seems likely. This is supported by our finding that the lowest PZ levels were measured in patients older than 45 years, in whom also other cardiovascular risk factors were more prevalent. It may be that an (atherosclerotic) vascular lesion is necessary for PZ deficiency to affect the risk of stroke. This view is supported by one study that showed that PZ is present in human atherosclerotic lesions, suggesting involvement of PZ in the process of atherogenesis.[22] The exact role of PZ in the pathogenesis of atherothrombosis clearly needs further study.

Our study demonstrates an independent association between low blood plasma levels of PZ and an increased risk of ischemic stroke in Caucasian patients. Further studies are necessary to confirm this association and to elucidate the possible causal nature of this association. Such studies should focus on PZ gene polymorphisms and PZ level simultaneously in stroke populations of different ethnicity and age.

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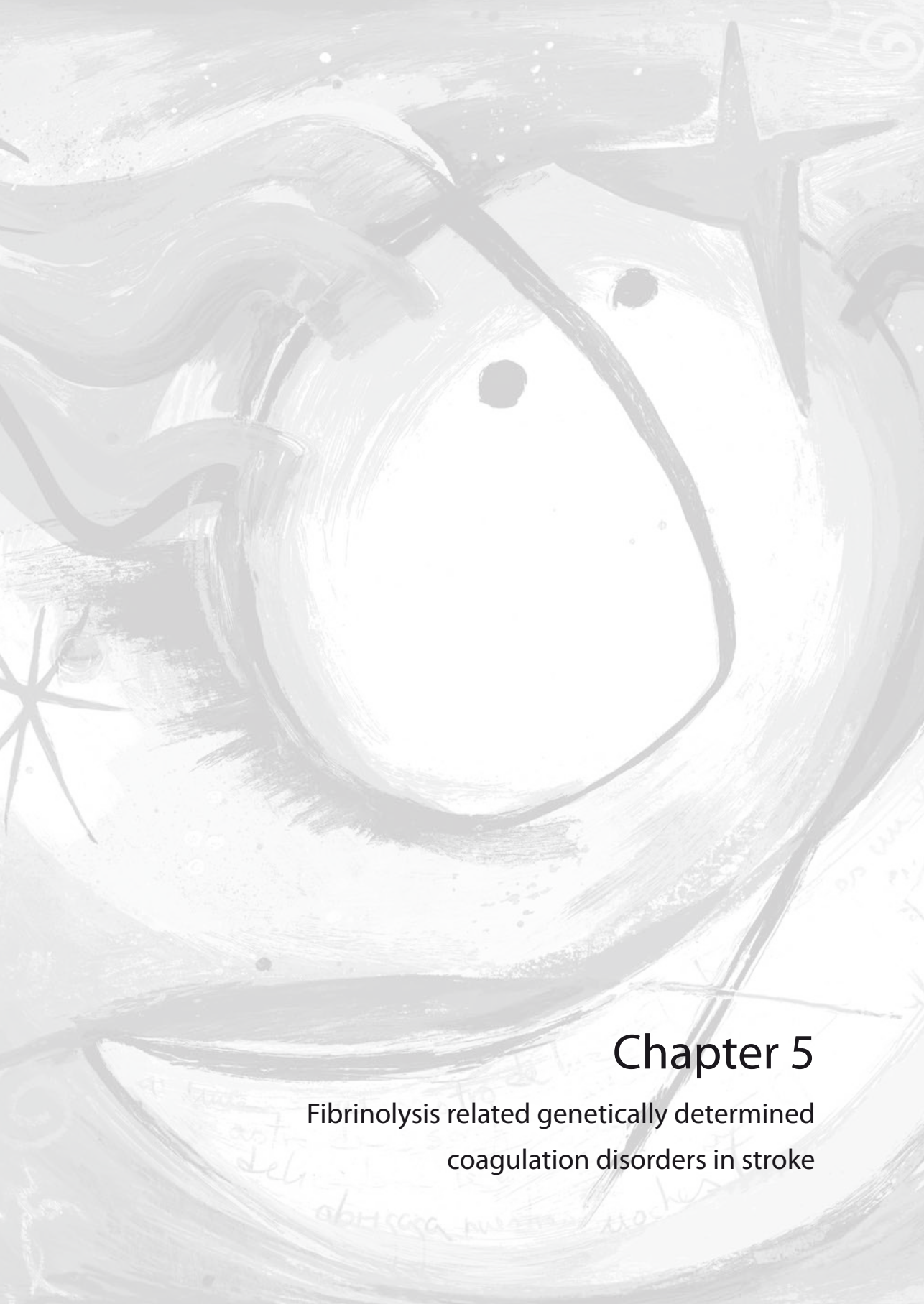
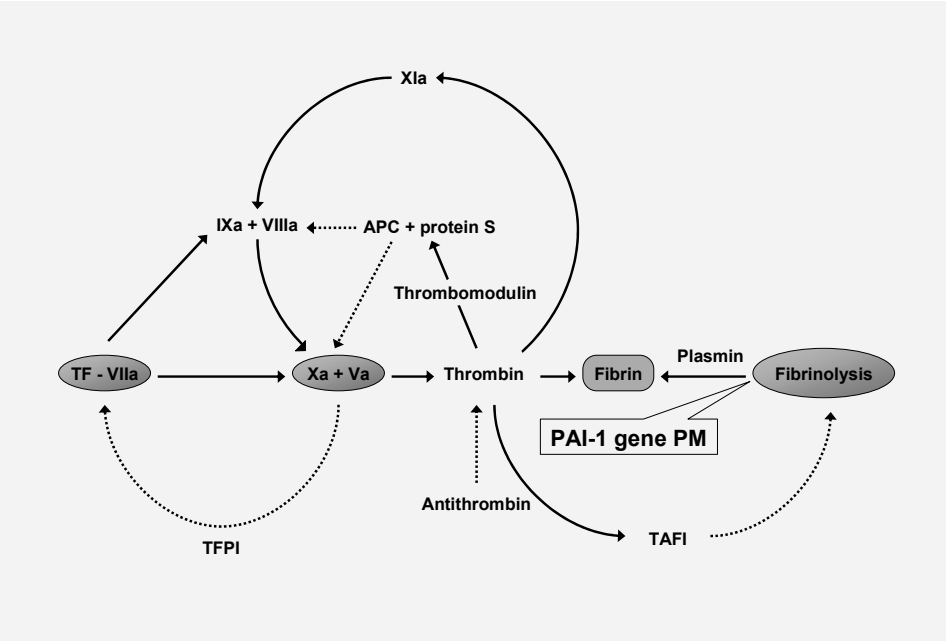


Chapter 5

Fibrinolysis related genetically determined coagulation disorders in stroke

Chapter 5.1

The 4G/5G PAI-1 gene polymorphism and PAI-1 levels in ischemic stroke



M.P.J. van Goor, E.B. Gómez García, F.W.G. Leebeek, G.J. Brouwers, P.J. Koudstaal, D.W.J. Dippel. The Plasminogen Activator Inhibitor (PAI-1) 4G/5G promoter polymorphism and PAI-1 levels in ischemic stroke: a case-control study. Submitted.

ABSTRACT

Background: High levels of plasminogen activator inhibitor type 1 (PAI-1) have been implicated as a risk factor for cardiovascular disease, but its precise role remains controversial. The 4G allele of the PAI-1 4G/5G promoter polymorphism is associated with higher levels of PAI-1. We studied the relationship between ischemic stroke and the PAI-1 4G/5G polymorphism and PAI-1 antigen levels.

Methods: We performed a case-control study among patients aged 18-75 years with first ischemic stroke, confirmed by CT. All patients were screened for cardiovascular risk factors, cardiac disorders and large vessel disease. We excluded patients with a definite non-atherosclerotic cause of the stroke and patients using oral anticoagulants. Population-controls were age -and sex-matched, without a history of stroke, and of the Caucasian race. The PAI-1 4G/5G polymorphism was detected by allele-specific PCR amplification. Venous blood samples were taken for PAI-1 level one week after the stroke, under strictly standardized conditions.

Results: We included 124 patients and 125 controls. Mean age was 56 yrs (range 18 to 75 yrs). Sixty one patients (50%) and 58 (47%) controls were heterozygous for the PAI-1 4G/5G polymorphism. The homozygous 4G/4G genotype was found in 33 patients (27%) and also in 36 controls (29%). The odds ratio of ischemic stroke associated with 4G-carriers versus 5G/5G homozygotes was 1.0 (95% CI: 0.6-1.8). The relative risk of ischemic stroke associated with the level of PAI-1 in the upper quartile was 0.73 (95%CI: 0.4 to 1.4).

Conclusion: Neither the PAI-1 4G/5G polymorphism nor the PAI-1 antigen level is a strong risk factor for ischemic stroke.

INTRODUCTION

Increased levels of the plasminogen activator inhibitor-1 (PAI-1) have been reported in patients with myocardial infarction and stroke.[1-3] Whether these increased levels are a result of a genetic variation of the PAI-1 gene, and whether they increase the risk of ischemic stroke is not yet clear.

PAI-1 is a glycoprotein with a molecular weight of approximately 50 KD that serves as the major physiological inhibitor of tissue plasminogen activator (t-PA), thereby attenuating fibrinolysis.[4,5] The human PAI-1 gene is located on chromosome 7 and several polymorphisms within this gene are associated with PAI-1 level in plasma.[6,7] A common polymorphism, located in the promoter region, consists of a single guanosine insertion/deletion variation (4G or 5G), resulting in two alleles containing either 4 or 5 guanosines in a row. In the general population, an association has been found between the 4G/5G polymorphism and the PAI-1 level, with individuals homozygous for the 4G allele having a significantly higher PAI-1 concentration in plasma.[7] Thus, the 4G allele could increase the risk of ischemic stroke through its association with high PAI-1 levels, that inhibit endogenous fibrinolysis.

The association of PAI-1 level and the 4G/5G polymorphism has been more extensively studied in myocardial infarction than in stroke. A recent meta-analysis of nine studies showed a significant 20% increased risk of myocardial infarction for the 4G/4G genotype.[8] In stroke patients, studies have yielded conflicting results. Several case-control studies failed to find an association, but they often considered selected patients, such as women, patients with TIA and minor stroke or even hemorrhagic stroke.[2,9,10] Furthermore, most studies reported on genotype only instead of genotype and phenotype simultaneously. Recently, the 4G allele was reported to be protective for ischemic stroke in the elderly.[11] In the same age group in women, the allele has been associated with a reduced mortality from stroke.[12] In contrast, in another case-control study, the 4G/4G genotype was found to be an independent risk factor for atherothrombotic stroke.[13] Finally, a recent and large prospective follow-up study could not support an association between the PAI-1 polymorphism and stroke in the elderly.[14]

To clarify whether a higher risk of stroke as a result of elevated PAI-1 levels is genetically determined, it would be of interest to study the PAI-1 polymorphism and its phenotypic expression simultaneously in a well defined population.

We therefore designed a case-control to investigate the possible association between 4G/5G PAI-1 gene promoter polymorphism and the occurrence of ischemic stroke and secondly, to study the role of PAI-1 level.

METHODS

Study design

The design of the study has been described extensively in chapter 3 of this thesis. In short, COCOS is a case-control study with prospective inclusion of Caucasian participants with first ever ischemic stroke, confirmed by CT. Controls are population based, age-and sex-matched, of the Caucasian race and not related to the patients. Blood samples for genotype and phenotype were drawn under strictly standardised conditions one week after the event and in almost half of the patients also after three months.

Blood samples and procedures

Blood for the detection of PAI-1 concentration was drawn using the vacutainer system and collected in CTAD- tubes, containing citrate and platelet stabilizing agents (Beckton Dickenson, Plymoth,UK). Blood was centrifuged for 20 min at 4ºC and plasma was stored using small aliquots at -80ºC until use. PAI-1 level, cholesterol and glyco-Hb were determined in the blood samples. PAI-1 antigen levels were measured using a commercially available ELISA (TintElize®PAI-1, Biopool, Umea, Sweden) and were expressed in ng/ml. Genomic DNA was isolated from the white cell fraction of citrated blood, according to the high-salt concentration standard procedure.[15] The PAI-1 4G/5G gene polymorphism was detected by allele-specific PCR amplification.[16]

Statistical aspects

The relationship between the 4G/5G PAI-1 gene polymorphism and ischemic stroke was expressed as an odds ratio with a 95% confidence interval. The PAI-1 levels were divided into quartiles and the relationship between PAI-1 level and ischemic stroke was estimated by odds ratio for the highest quartile versus the lower three quartiles. Multiple logistic regression analysis was used to adjust for possible confounders, such as smoking, hypertension, as these vascular risk-factors are known to affect PAI-1 level as well. Furthermore, we assessed the relationship between genotype and stroke severity by relating genotype to a dichotomized Barthel score (0-19 vs. 20 points).[17]

RESULTS

During the study period 1034 patients with acute stroke were admitted to our hospital. Twenty percent of the patients had an intracerebral hemorrhage, 13% died within a few days as a result of the stroke, 28% had a recurrent ischemic stroke. Of the remaining patients, 38% were of Caribbean or Mediterranean origin and were therefore not included. One patient was excluded later, after she was found to have DIC, induced by a pancreatic malignancy. The final study population consisted therefore of 124 patients and 125 controls. In 123 patients and 123 controls, blood samples were available for detection of the PAI-1 polymorphism and PAI-1 level.

Population characteristics

Table 1 shows the baseline demographics and vascular risk factors in patients and controls. Patients were more often smokers and more often had hypertension, diabetes or previous cardiovascular disease than controls.

In half of the cases no cause for the stroke was found according to the TOAST classification. Eleven patients (9%) suffered from large vessel disease and 46 (37%) from small vessel disease. Four patients (3%) had a probable cardiac source of embolism. In two young patients (2%) the evaluation was not complete because both refused echocardiography.

Genotype distribution

The distribution of PAI-1 genotypes in patients was: 4G/4G, 33 (27%), 4G/5G, 61 (49%) and 5G/5G 29 (24%). When compared with the matched controls, whose genotype distribution was, 4G/4G, 36 (29%), 4G/5G, 58 (47%) and 5G/5G 29 (24%), there were no significant differences. The allele

frequencies in patients were 0.52 for 4G and 0.48 for 5G, compared with 0.53 for 4G and 0.47 for 5G in controls. The genotype frequencies in controls were in Hardy-Weinberg equilibrium. The odds ratio (OR) of ischemic stroke associated with the 4G-allele versus 5G/5G-homozygotes was 1.0 (95% CI: 0.6-1.8). There was no difference in relative risk of ischemic stroke between heterozygous and homozygous 4G-genotype (Table 2).

Table 1. Baseline characteristics

	Cases (n=124)	Controls (n=125)
Demographics		
Age, yrs (sd)	56 (12)	56 (12)
Female sex	58 (47%)	59 (47%)
Index event		
Stroke: TIA	115: 9	NA
Risk factors		
Smoking	61 (49%)	37 (30%)
Hypertension	60 (48%)	24 (19%)
Diabetes	18 (14%)	5 (4%)
Hypercholesterolemia	78 (63%)	84 (67%)
Oral contraceptives	11 (19%)	8 (14%)
Family history of cardiovascular disease	75 (61%)	56 (45%)
Previous DVT	6 (5%)	0 (0%)

Table 2. Frequencies of 4G/5G PAI-1 gene polymorphism among patients and controls and the odds ratio (with 95% confidence interval) of ischemic stroke associated with the PAI-1 gene polymorphism

	Cases (n=123)	Controls (n=123)	OR (95%CI)
5G/5G	29	29	reference
4G/5G	61	58	1.1 (0.6-2.0)
4G/4G	33	36	0.9 (0.5-1.8)
Frequency of 5G allele	0.48	0.47	
Frequency of 4G allele	0.52	0.53	

PAI-1 levels

The mean PAI-1 level was lower in patients than in controls, 22.9 ng/ml vs. 25.1 ng/ml respectively, but this difference was not statistically significant (student's t $p>0.22$). We divided the PAI-1 level in quartiles and the relative risk for stroke in the highest quartile versus the lower three quartiles was 0.74 (95% CI: 0.4-1.4). In patients as well as in controls, the highest PAI-1 levels were associated with the 4G/4G genotype (Table 3).

Table 3. Mean PAI-1 level (ng/ml) and standard deviation (ng/ml) according to genotype in controls and in patients one week after the stroke
n=number of patients or controls, sd=standard deviation

	Controls			Patients		
Genotype	mean	sd	n	mean	sd	n
4G/4G	28.3	16.8	35	25.3	17.5	31
4G/5G	25.6	14.6	57	21.5	12.5	58
5G/5G	20.3	12.7	28	22.7	10.4	28

Subgroups

We estimated the relative risk of stroke associated with the PAI-1 promoter gene polymorphism in several subgroups of patients. There were no statistically significant differences in the distribution of genotypes according to gender, smoking habits, hypertension, diabetes or stroke type. There was no relationship between stroke severity and genotype (data not shown).

In controls, the mean PAI-1 level was higher in subjects with diabetes (38.1 vs. 24.5, $p<0.04$). In patients, we found no differences in PAI-1 level in those with or without diabetes (24.5 vs.22.6, $p>0.59$). Similarly, the mean PAI-1 level was significantly higher in controls with hyperlipidemia (27.3 vs.20.7, $p<0.02$) than in those without, whereas in patients no differences were found in PAI-1 levels in those with or without hyperlipidemia (24,6 vs. 20.1, $p=0.08$).

DISCUSSION

Internal validity

The strength of our study is the prospective and consecutive inclusion of patients by a neurologist. Furthermore, all patients underwent neuro-imaging to rule out hemorrhage. We were able to include population controls, thus avoiding the biases induced by "hospital controls". We collected detailed information about cardiovascular risk factors, medical history and family history from patients as well as controls. All subjects participating were of the Caucasian race and allele frequencies of the polymorphism in the control group were in Hardy-Weinberg equilibrium, indicating that we studied a representative group.

Because the primary aim of the study was to investigate the influence of a genetic feature, the 4G allele of the PAI-1 polymorphism, on the risk of stroke, confounding by other variables is unlikely. Although the phenotypic expression, i.e. PAI-1 level, may vary, it is unlikely that genotype is affected by confounding variables or by the occurrence of ischemic stroke. As a consequence of its design our study is limited to nonfatal cases of stroke. If the 4G allele was associated with fatal cases of stroke, we would underestimate the true risk for ischemic stroke associated with the 4G allele.

PAI-1 levels in patients were not increased in comparison with controls. The blood samples in patients and controls were collected according to ECAT procedures[18] and taken under strictly standardized conditions, i.e. in fasting condition, with no exposure to alcohol or nicotine and after 15 minutes of rest. By doing so, we were able to exclude the confounding influence on the PAI-1 level by smoking, diabetes or physical exercise. Therefore, we think that the measurements of the PAI-1 level and the lack of difference in PAI-1 level between patients and controls are valid. These

results are in agreement with one other case-control study.[19] In some other studies that reported on PAI-1 level and stroke, non-fasting blood samples were taken.[1,2,11] This might explain the higher PAI-1 levels in patients, especially since cardiovascular risk factors that increase the PAI-1 activity like smoking, diabetes and hyperlipidemia were more prevalent in the patient group. Furthermore, in these studies tubes containing citrate were used instead of CTAD-tubes containing platelet stabilizing agents. This might further explain the differences in PAI-1 level.

External validity

A limitation of our study is its small size. One could argue that our study is not large enough to exclude an association between ischemic stroke and the PAI-1 4G/5G polymorphism. The OR in our study was 1.0 with a narrow 95% confidence interval of 0.6 to 1.8. Our study indicates that even if the polymorphism is a risk factor, it cannot be a strong one. This observation is in agreement with several other case-controls studies that also found no evidence for an increased stroke risk in the presence of the polymorphism.[2,14,19,20] Our results are not consistent with a recent prospective study that described a protective effect against stroke for the 4G allele in the elderly.[11] In that study the population was not in Hardy-Weinberg equilibrium, which may suggest selection bias. Other studies that reported the 4G allele to be protective for stroke also concerned subgroups of patients, like young women[10] or patients with minor stroke.[9] On the other hand, one other case-control study concluded that the 4G allele is a significant risk factor in a selected patient group of atherothrombotic stroke.[13] Selection of patients might partly explain the heterogeneous results of the studies concerning the 4G/5G polymorphism of the PAI-1 gene and stroke.

In our study, the highest PAI-1 levels were associated with 4G allele in patients as well as controls, which is in agreement with the accepted relation between PAI-1 level and the 4G allele.[16,21] Elevated plasma PAI-1 level is a core feature in the insulin resistance syndrome (IRS). This syndrome is defined as a cluster of abnormalities, which includes obesity, glucose intolerance and dyslipidemia and which is strongly related with low grade inflammation.[22] In our control population, PAI-1 levels were higher in those having diabetes and hyperlipidemia. An explanation for this might be that these cardiovascular risk factors are better treated in the patient group. However, only a small percentage of the patients suffered from diabetes and there was no difference in the prevalence of hyperlipidemia between patients and controls. This does not provide enough explanation why the PAI-1 level was not increased in our patients.

The relationship between elevated PAI-1 levels and myocardial infarction (MI) seems to be more straightforward than the relationship between PAI–1 and ischemic stroke. An explanation for this could be the difference in pathophysiological mechanism between MI and ischemic stroke. In MI patients, rupture of atherosclerotic plaques is considered the most important underlying mechanism. Atherosclerotic plaques are associated with elevated PAI-1 levels.[23] In stroke patients, small vessel disease and thromboembolism without significant large vessel disease are more common causes of cerebral ischemia than carotid and aortic arch atherosclerosis.[24] This may, at least partially, explain the lack of association.

In conclusion, our results indicate that PAI-1 promoter gene polymorphism is not a risk factor for ischemic stroke. Although our results confirm the association between plasma PAI-1 level and genotype, they do not support the hypothesis that increased PAI-1 levels are an important risk factor for ischemic stroke.

Future studies should focus on other promoter gene polymorphisms and their phenotypic expression, to further examine to which extent genetically determined coagulation disorders contribute to the occurrence of ischemic stroke.

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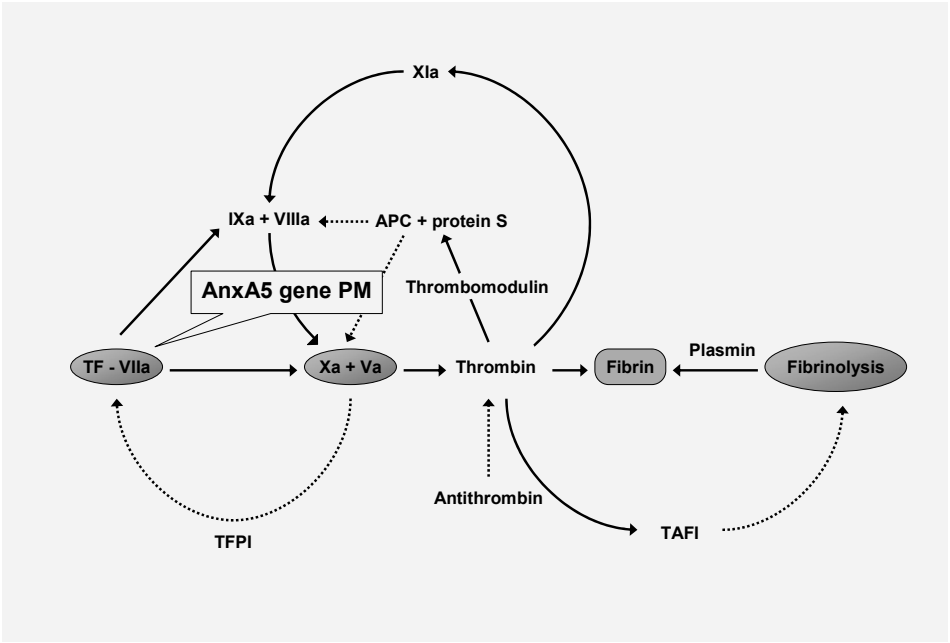
Chapter 5.2

Annexin A5 -1C/T polymorphism in ischemic stroke



M.P.J. van Goor, W.L. van Heerde, S. Schoormans, P.J. Koudstaal, D.W.J. Dippel, F.W.G. Leebeek. The Annexin A5 -1C/T polymorphism in ischemic stroke: a case-control study. J Thromb Haemostasis (in press).

ABSTRACT

Background: The -1C/T polymorphism of the annexin A5 (AnxA5) gene has been associated with myocardial infarction. Whether this polymorphism is also a risk factor for ischemic stroke is unknown.

Methods: We performed a case-control study among patients with first ischemic stroke, confirmed by CT. Population-controls were age-and sex-matched, and did not have a history of stroke. The -1C/T AnxA5 polymorphism was detected by PCR of exon 2 of the AnxA5 gene, followed by restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme.

Results: We included 124 patients and 125 controls. Mean age was 56 yrs (range 18 to 75 yrs). Thirty (25%) patients and 23 (24%) were heterozygous for the -1C/T AnxA5 gene polymorphism and 1 (1%) patient and 1(1%) control were homozygous for the T-allele. The odds ratio of ischemic stroke associated with T-allele carriers versus CC-homozygotes was 1.1 (95%CI: 0.6-2.0).

Conclusion: The -1C/T AnxA5 gene polymorphism is not a strong risk factor for ischemic stroke.

INTRODUCTION

It has been suggested that annexin A5 (AnxA5) has a regulatory role in hemostasis, by regulating the exposure of negatively charged phospholipids ions and prolonging the phospholipid-dependent coagulation reactions.[1] AnxA5 has various antithrombotic properties. It prevents formation of prothrombinase and tenase complexes and inhibits platelet aggregation and thrombus formation both in vitro as in vivo.[2,3]

The AnxA5 gene has been described in 1994[4], followed by the discovery of a polymorphism (-1C/T) in the Kozak sequence of the gene.[5] The -1C/T polymorphism has been shown to play a protective role against myocardial infarction in young patients and it has been suggested that this is due to an increased translation efficiency resulting in increased levels of AnxA5.[6] In the same study, no association was found in other groups of patients such as patients with spontaneous intracranial hemorrhage, deep venous thrombosis or coronary heart disease.[6] On the other hand, a recent case-control study reported a slight non significant increase in the risk of myocardial infarction in men in the presence of the -1C/T polymorphism.[7] Furthermore, in a control population the levels of AnxA5 were not affected by the –C/T polymorphism.[8]

To our knowledge, the association between the AnxA5 Kozak sequence (-1C/T) polymorphism and ischemic stroke has not been studied before. The aim of this study was to investigate the role of the -1C/T polymorphism of the AnxA5 gene in ischemic stroke in a well-defined Caucasian population.

METHODS

Study design

The design of the study has been described extensively in chapter 3 of this thesis. In short, COCOS is a case-control study with prospective inclusion of Caucasian participants with first ever ischemic stroke, confirmed by CT. Controls are population based, age-and sex-matched, of the Caucasian race and not related to the patients. Blood samples for genotype and phenotype were drawn under strictly standardised conditions one week after the event and in almost half of the patients also after three months.

Blood samples and procedures

One week after the stroke, venous blood samples were taken. Genomic DNA was isolated from the white cell fraction of citrated blood, according to the high-salt concentration standard procedure.[9] The –1C/T AnxA5 polymorphism was detected by PCR of exon 2 of the AnxA5 gene, followed by restriction fragment length polymorphism (RLPF) analysis using the restriction enzyme NcoI.[8]

Statistical aspects

For the -1 C/T mutation with an expected prevalence of the T allele of about 12% in the control group[6], the minimal detectable odds ratio would be 2.7 (β=20%, α=5%). The relationship between the AnxA5 –1 C/T polymorphism and ischemic stroke was expressed as an odds ratio with a 95% confidence interval. Furthermore, the relationship between genotype and stroke severity was assessed, by relating genotype to a dichotomized Barthel score (0-19 vs. 20 pts).

RESULTS AND DISCUSSION

Our study population consisted of 124 patients and 125 controls. In 118 patients and 97 controls, blood samples were available for detection of the AnxA5 gene polymorphism.

Table 1 shows the baseline demographics and vascular risk factors in patients and controls. Patients were more often smokers and more often had hypertension, diabetes or were known to have cardiovascular disease compared with controls.

In half of the cases no cause was found according to the TOAST classification. Eleven patients (9%) suffered from large vessel disease and 46 (37%) from small vessel disease. In four patients (3%) a probable source of cardio embolism was detected. In two young patients (2%) the evaluation was not complete because both refused echocardiography.

Table 1. Baseline characteristics

	Cases (n=124)	Controls (n=125)
Demographics		
Age, yr (sd)	56 (±12)	56 (±12)
Female sex	58 (47%)	59 (47%)
Index event		
Stroke: TIA	115: 9	NA
Risk factors		
Smoking	61 (49%)	37 (30%)
Hypertension	60 (48%)	24 (19%)
Diabetes	18 (14%)	5 (4%)
Hypercholesterolemia	78 (63%)	84 (67%)
Oral contraceptives	11 (19%)	8 (14%)
Family history of cardiovascular disease	75 (61%)	56 (45%)
Previous DVT	6 (5%)	0 (0%)

Thirty (25%) patients and 23 (24%) controls were heterozygous for –1 C/T AnxA5 gene polymorphism and 1 (0.9%) patient and 1 (1.0%) control were homozygous for the T allele. The genotype frequencies in controls were in Hardy-Weinberg equilibrium. The odds ratio (OR) of ischemic stroke associated with the CC-homozygotes versus T-allele carriers was 1.1 (95% CI: 0.6-2.0). The allelic frequencies are comparable to the results published in other control populations and patients with myocardial infarction.[6,7,10]

We estimated the relative risk of stroke associated with the -1C/T AnxA5 gene polymorphism in several subgroups of patients. There were no statistically significant differences in the distribution of genotypes according to gender, smoking habits, hypertension, diabetes or stroke subtype according to TOAST. Furthermore, there was no relationship between stroke severity and genotype (data not shown).

A strength of our study is that cases were prospectively and consecutively included by a neurologist. Furthermore, all patients underwent neuro-imaging to rule out hemorrhage. We were able to include population controls, thus avoiding the biases induced by "hospital controls". We collected detailed information about cardiovascular risk factors, medical history and family history from patients as well as controls. All subjects participating were of the Caucasian race and allele frequencies of the polymorphism in the control group were in Hardy-Weinberg equilibrium, indicating that we studied a representative group.

Table 2. Genotype frequencies of the AnxA5 –1C/T gene polymorphism among patients and controls and the odds ratio (with 95% confidence interval) of ischemic stroke associated with the CC homozygotes versus T-allele carriers

	Cases (n=118)	Controls (n=97)	OR (95%CI)
CC	87 (74%)	73 (75%)	reference
CT/ TT	31 (26%)	24 (25%)	1.1 (0.6-2.0)

A limitation of this study is the inclusion of mainly nonfatal cases of stroke. If the T-allele would be associated with fatal cases of stroke, we underestimate the true risk for ischemic stroke associated with the presence of the T allele. Because the primary aim of this study was to investigate the influence of a genetic feature, the T allele of the AnxA5 –1C/T gene polymorphism, on the risk of stroke, bias does not play a role here.

This is the first study on the association between ischemic stroke and the AnxA5 -1C/T gene polymorphism. We conclude that the polymorphism is not a strong risk for ischemic stroke. Although the T-allele has been associated with myocardial infarction, this study does not provide evidence for an important role of the AnxA5 -1C/T gene polymorphism in ischemic stroke.

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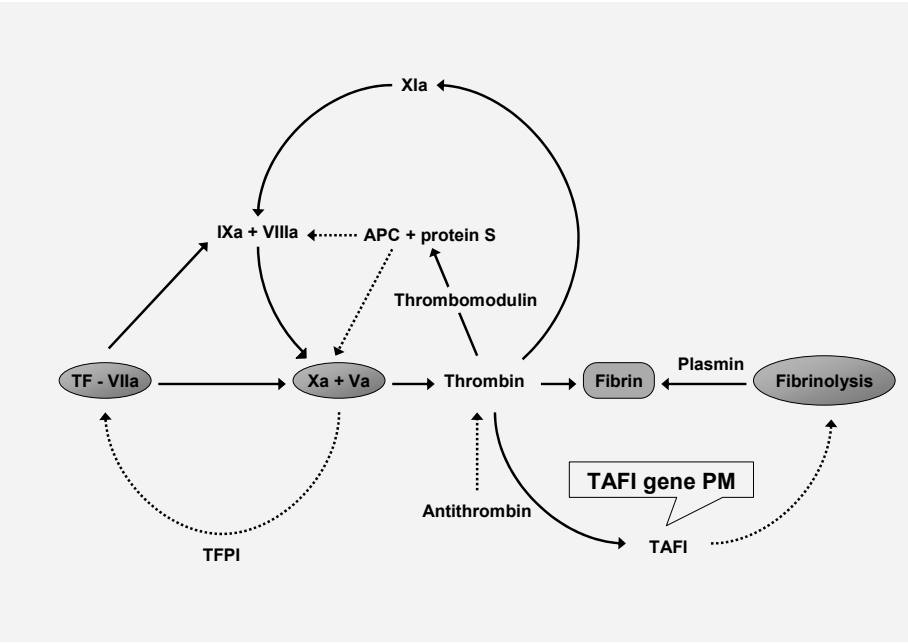
Chapter 5.3

TAFI gene polymorphism and TAFI levels in ischemic stroke



F.W.G. Leebeek, M.P.J. van Goor, A.H.C. Guimaraes, G.J. Brouwers, D.W.J. Dippel, D.C. Rijken. High functional levels of Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) are associated with an increased risk of first ischemic stroke. Submitted.

ABSTRACT

Background: Thrombin-activatable fibrinolysis inhibitor (TAFI) is a recently described fibrinolysis inhibitor that attenuates fibrinolysis by cleaving the C-terminal lysines of partially degraded fibrin, thereby reducing plasminogen activation. Increased levels of TAFI have been associated with an increased risk of venous thrombosis. A role in the pathogenesis of arterial thrombosis, such as acute myocardial infarction, has been suggested in several studies, but results have been contradictory.

Methods: We studied TAFI antigen levels, functional TAFI levels using a new clot lysis based assay, and three TAFI gene polymorphisms in 125 patients with a recent ischemic stroke, and compared this with an age- and sex- matched control group.

Results: Clot lysis times were significantly increased in patients compared to controls (clot lysis time 47.3 ± 3.8 min vs. 44.1 ± 3.7 min, $p<0.00001$). Functional TAFI levels, defined as TAFI-related retardation, the difference in clot lysis time in the absence or presence of a specific TAFI inhibitor potato carboxypeptidase inhibitor (PCI), were significantly higher in patients than controls (19.5 ± 4.2 min vs. 17.7 ± 3.7 min, $p <0.005$). Individuals with clot lysis times and TAFI-related retardation in the highest quartile had a markedly increased risk of ischemic stroke compared with the lowest quartile (OR 8.2, CI 3.2-21.3, and 4.0, CI 1.6-9.8 respectively). No difference was found between patients and controls with respect to TAFI antigen levels and TAFI –438A/G, 505G/A and 1040C/T genotype.

Conclusion: We conclude that an increase of functional TAFI levels, resulting in a decreased fibrinolysis, is associated with a marked increased risk of ischemic stroke.

INTRODUCTION

Several studies have indicated that reduced fibrinolysis, for instance due to increased PAI-1 levels, may be associated with the risk of acute ischemic stroke.[1-4] Thrombin-activatable fibrinolysis inhibitor (TAFI) is a more recently described fibrinolysis inhibitor of which the role in the pathogenesis of ischemic stroke is not yet clear. The known physiological role of activated TAFI is the inhibition of fibrinolysis by removing C-terminal lysine and arginine residues of partially degraded fibrin, thereby slowing the rate of tissue plasminogen activator-induced plasminogen activation.[5-7] TAFI also inhibits plasminogen activation induced by other plasminogen activators.[8] A role of TAFI in arterial thrombosis, including unstable angina pectoris and myocardial infarction, has been suggested, but is still controversial.[9-13] In patients with symptomatic coronary heart disease (CHD) contradictory results concerning TAFI antigen levels have been reported, some studies showing elevated levels in CHD[9-11], whereas others found decreased levels.[12,13] This may be caused by a difference in study populations, but also due to a difference in assay characteristics, for instance antibody specificity.[14,15]

One recent study found increased TAFI activity levels in patients with a history of ischemic stroke.[16] In addition, one other small study showed increased TAFI antigen levels shortly after the occurrence of ischemic stroke.[17] So far at least 14 polymorphisms in the TAFI gene have been identified, and some of these are strongly associated with plasma TAFI antigen levels.[18-20] Therefore, it is of interest to study the allele frequencies of TAFI gene polymorphisms also in patients with ischemic stroke.

We determined TAFI gene polymorphisms, as well as functional TAFI levels and TAFI antigen levels in patients with a recent acute ischemic stroke to further explore the possible association between TAFI and ischemic stroke.

METHODS

Study design

We performed a case-control study with prospective inclusion of the participants. Cases were consecutively recruited patients with first ever acute ischemic stroke, admitted to the department of Neurology of a university hospital, between January 1999 and December 2002. We used population controls, i.e partners, friends or neighbours of the patients. They were age- and sex matched, did not have a history of stroke and were not related to the patient.

Inclusion and exclusion criteria

Patients, controls and their parents should be born in Northern Europe and be of the Caucasian race, because substantial differences in the prevalence of genetically determined coagulation disorders have been reported between people from different geographical regions or race. Patients with a definite non-atherosclerotic cause of the stroke, such as a mechanical heart valve, endocarditis or carotid dissection were excluded. Other exclusion criteria were age above 75 years and the use of oral anticoagulants.

Definitions and measurements

Ischemic stroke was defined as the acute onset of focal cerebral dysfunction due to cerebral ischemia with symptoms lasting more than 24 hours. Patients with TIA (symptoms lasting less than 24 hours) were included only if the neurological deficit in the acute phase was witnessed by a neurologist. In all patients a CT of the brain was made within three days from the onset of symptoms to confirm the diagnosis of ischemic stroke and to rule out hemorrhagic stroke. Clinical stroke subtypes were classified according to the OCSP criteria, modified by CT.[21] Etiologic stroke type was classified according to the TOAST criteria.[22] We defined large artery atherosclerosis as a stenosis of presumed atherosclerotic origin in the symptomatic cervical artery of more than 50%. Stroke severity was assessed with the Barthel index.[23] Hypertension was defined as systolic blood pressure above 160 mm Hg or diastolic blood pressure above 90 mm Hg 5 days after the event, or the use of antihypertensive medication. Hypercholesterolemia was defined as fasting cholesterol level above 5.0 mmol/l or use of lipid lowering drugs. Diabetes mellitus was defined as the use of antidiabetic medication or glyco-Hb >6.5%. Myocardial infarction was defined as typical chest pain with documented elevated cardiac enzymes, or confirmation by ECG. Deep venous thrombosis had to be confirmed by compression ultrasonography or phlebology and pulmonary embolism by ventilation-perfusion scan or spiral computed tomography of the chest.

In patients as well as in controls, we collected detailed information about cardiovascular risk factors, such as smoking habit, hyperlipidemia, hypertension, diabetes, use of oral contraceptives, and about medical history and family medical history. Patients were screened for cardiac abnormalities by means of standard twelve-lead ECG examination. A cardiologist was consulted in female patients aged 55 years or less, in male patients aged 45 years or less and in patients with ECG abnormalities or a history of cardiac disease. In these patients, 24 hours ECG monitoring, transthoracic and/or transesophageal echocardiography was carried out. Screening for large vessel disease included duplex ultrasound or, if indicated, angiography of the carotid or vertebral arteries.

Blood samples and procedures

Between 7-14 days after the stroke, venous blood samples were taken under strictly standardized conditions. The patients were in fasting condition, with no exposure to cigarette smoking or alcohol for at least the preceding 8 hours and the drawing of blood took place after 15 minutes of rest. Blood was collected in citrate (0.105 M) using a Vacutainer system (Beckton Dickinson, Plymouth, UK). The blood was centrifuged (2000x g for 30 min at 4°C) and the collected plasma was stored in aliquots at -70°C until use. Genomic DNA was isolated from the white cell fraction of citrated blood, according to the high-salt concentration standard procedure.[24]

Analysis of TAFI gene polymorphisms

Determination of the -438A/G, 505A/G (Ala147Thr) and 1040 C/T (Thr325Ile) genotype was performed using polymerase chain reaction (PCR) and subsequent restriction analysis. The -438A/G and 1040 C/T (Thr325Ile) polymorphism were analyzed by duplex PCR and digestion analysis. The PCR conditions have been previously described.[12] The end volume of the PCR mixture was 25 μl and contained: 50 ng genomic DNA, 37.5 pM of each primer, 1.5 mM of each dNTP (Pharmacia), 2.5 μL buffer (15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl, pH 8.3) and 1.25 U Taq DNA polymerase (Boehringer Mannheim). The PCR conditions used for all PCR reactions

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 37°C and analyzed on a 2.5% agarose gel.

TAFI Functional Assay

TAFI functional assay based on clot lysis was performed as described elsewhere (Guimarães et al., manuscript in preparation). Briefly, 100 μl of diluted plasma samples (20-fold dilution of individual plasmas in TAFI-depleted plasma) were added to the wells of a microtitre plate containing 25 μl of a reaction mix composed of thrombin, thrombomodulin, $CaCl_2$ and r-tPA (concentrations in the final mixture: 3.3 NIH units/ml, 0.6 units/ml, 20 mM, and 0.10 μg/ml respectively), after which clots were formed. A control with addition of potato carboxypeptidase inhibitor (PCI) was tested for each sample (concentration in the final mixture 30 μg/ml). The wells were immediately covered with paraffin oil and the plate was placed in a pre-warmed incubation chamber (37°C, TECAN Sunrise Microplate-reader). 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 lysis time in the absence and in the presence of PCI, ($LT_{(-PCI)}$ - $LT_{(+PCI)}$).

Recombinant tPA (Actilyse) was kindly supplied by Boehringer Ingelheim (Ingelheim, Germany). Human thrombin and PCI were purchased from Sigma (St Louis, MO, USA) and Calbiochem (La Jolla, CA, USA), respectively. Rabbit lung thrombomodulin, with a specific activity towards thrombin of 1.2 units/μg, was acquired from American Diagnostica Inc (Greenwich, CT, USA).

Plasma TAFI Antigen level determination

Plasma TAFI Ag levels were determined by means of a sandwich ELISA consisting of an affinity-purified sheep anti-TAFI IgG raised against TAFI purified from plasma as a capture antibody, and HRP-conjugated affinity-purified sheep anti-TAFI IgG for detection (Affinity Biologicals, Kordia, Leiden, The Netherlands). This assay measures total TAFI antigen, including TAFI, activated TAFI (TAFIa) and inactivated TAFIa. All the necessary buffers and reagents were prepared according to the manufacturer's instructions. Normal plasma from Chromogenix was used as reference material arbitrarily defined to contain 100% TAFI antigen.

Statistical aspects

For the TAFI 1040C/T (Thr325Ile) polymorphism with an expected prevalence of the TT genotype of 10% in the control group, the minimal detectable odds ratio associated with the TT genotype would be 2.8 (with an α=0.05, β=0.80). The relationship between the TAFI gene polymorphisms and ischemic stroke was expressed as an odds ratio (OR) with a 95% confidence interval (CI). The clot lysis times (LT), TAFI-related retardation (RT), and TAFI antigen levels were divided into quartiles, based on the measurements in both cases and controls. The relationship between LT, RT, and TAFI antigen levels and ischemic stroke was estimated by odds ratio per quartile. Multiple logistic regression analysis was used to adjust for possible confounders, such as smoking, hypertension, age, as these vascular risk-factors may be associated with variations in TAFI levels.

RESULTS

Patient population

The baseline characteristics of patients and controls are given in Table 1. Stroke subtypes were classified according to the TOAST criteria and are also given in Table 1. Smoking, hypertension and diabetes occurred more frequently in the patients compared to controls.

Table 1. Baseline characteristics

	Patients (n=124)	Controls (n=125)
Demographics		
Age, yr (sd)	56 (±12)	56 (±12)
Female sex	58 (47%)	59 (47%)
Index event		
Stroke: TIA	115: 9	
Stoke subtypes (TOAST criteria)		
Large vessel disease	11 (9%)	NA
Cardiac embolism	4 (3%)	
Small vessel disease	46 (37%)	
Undetermined etiology	61 (49%)	
Incomplete evaluation	2 (2%)	
Risk factors		
Smoking	61 (49%)	37 (30%)
Hypertension	60 (48%)	24 (19%)
Diabetes	18 (14%)	5 (4%)
Hypercholesterolemia	78 (63%)	84 (67%)
Oral contraceptives	11 (19%)	8 (14%)
Previous DVT	6 (5%)	0 (0%)

TAFI activity and antigen levels in ischemic stroke

Functional TAFI levels were measured by means of a clot lysis assay in TAFI-depleted plasma. Clot lysis time ($LT_{(-PCI)}$) was significantly increased in patients with ischemic stroke compared with controls 47.3 ± 3.8 min vs. 44.1 ± 3.7 min ($p<0.00001$, Table 2), which indicates less efficient fibrinolysis in patients with ischemic stroke compared to controls. Clot lysis times were also determined in the presence of a specific TAFI inhibitor, potato carboxypeptidase inhibitor (PCI). TAFI-related retardation (RT), defined as the difference in clot lysis time (LT) measured in the absence ($LT_{(-PCI)}$) and presence ($LT_{(+PCI)}$) of PCI ($LT_{(-PCI)} - LT_{(+PCI)}$), was increased in patients compared with controls, which reflects higher functional TAFI levels in ischemic stroke (Table 2). The results of $LT_{(-PCI)}$ and RT of the individual patients and controls are shown in figure 1.

We subdivided the individuals according to quartiles of clot lysis time ($LT_{(-PCI)}$) and RT, of which the results are given in figure 2. A significant association between $LT_{(-PCI)}$ and RT and

the risk of ischemic stroke was found. Individuals with the highest $LT_{(-PCI)}$ (4th quartile) had a markedly increased risk of ischemic stroke: odds ratio (OR) of 8.2, confidence interval (CI) 3.2-21.3 compared to the lowest quartile (figure 2). This was also found when the highest quartile was compared with the lower three quartiles (OR 5.0, CI 2.1-11.0). This finding reflects the relative increase of the number of patients compared with controls in the upper quartile of $LT_{(-PCI)}$ (43 patients in the 4th quartile vs 13 controls). Similar findings were obtained when the TAFI-related retardation (RT) were divided into quartiles (OR 4.0, CI 1.6-9.8, highest quartile vs lowest quartile, Figure 2).

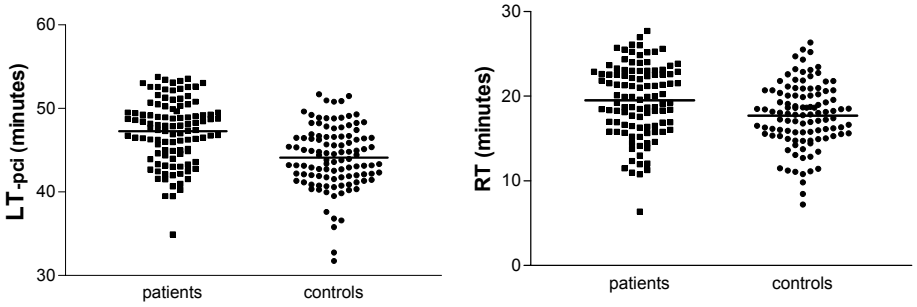


Figure 1. Clot lysis times (LT_{-PCI}) (left figure) and TAFI-related retardation ($RT = LT_{-PCI} - LT_{+PCI}$) (right figure) in patients with ischemic stroke and controls. The horizontal lines indicate mean value.

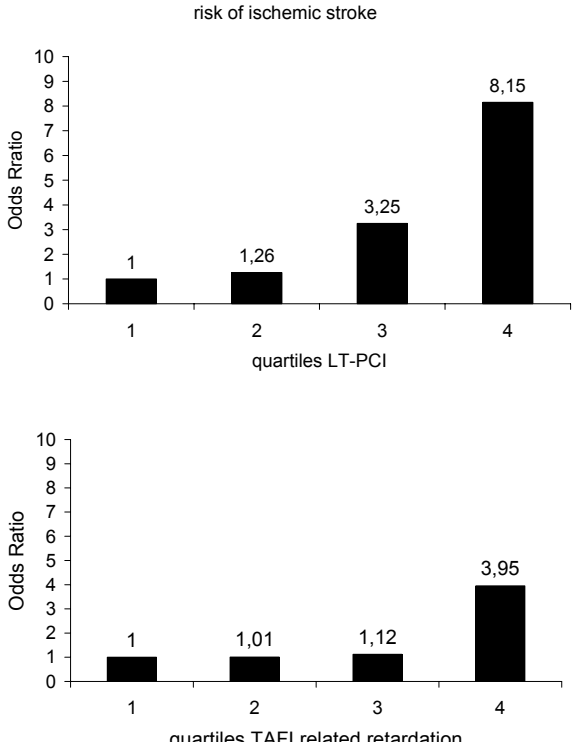


Figure 2. Risk of ischemic stroke expressed in odds ratio's for every quartile of both clot lysis time (LT_{-PCI}) (upper figure) and TAFI related retardation ($RT = LT_{-PCI} - LT_{+PCI}$) (lower figure). Odds ratios are given above the bars.

TAFI antigen levels were not significantly different between patients and controls, with mean values of 103 % and 101.5 % respectively (OR 1.0) (table 2). After dividing TAFI antigen levels into quartiles no increased risk of stroke was seen in individuals with the highest TAFI antigen levels (highest quartile vs. lowest quartile, OR 1.3, CI 0.6-3.0).

Table 2: TAFI antigen levels and functional TAFI in patients and controls

	Patients (n=124)	Controls (n=125)	P value*	Odds Ratio (95% CI)
TAFI ag (%)	103.0± 31.3	101.5 ± 34.5	0.74; ns	1.00 (0.99-1.01)
LT_{-PCI} (min)	47.3 ± 3.8	44.1± 3.7	<0.00001	1.25 (1.15-1.36)
LT_{+PCI} (min)	27.8 ± 2.5	26.4 ± 2.5	<0.0001	1.25 (1.11-1.41)
RT (min)	19.5 ± 4.2	17.7 ± 3.7	0.0012	1.12 (1.04-1.20)

Data are given as mean ± SD; ag = antigen; LT –PCI = clot lysis time in the absence of potato carboxypeptidase inhibitor (PCI); LT+PCI = clot lysis time in the presence of PCI; RT = TAFI-related retardation (LT-PCI – LT+PCI); *two-sample t-test with equal variance

Stroke severity and TAFI levels.

We did not detect a difference between TAFI antigen levels or clot lysis times in different types of stroke, subdivided as presented in table 1 (data not shown).

TAFI gene polymorphisms in relation to ischemic stroke.

We could not detect an association between the three studied polymorphisms in the TAFI gene and the risk of ischemic stroke. The 1040C/T polymorphism, which codes for the Ile325Thr is especially important, since this is a functional polymorphism resulting in a difference in half-life and activity. Also this polymorphism in the TAFI gene was not associated with functional TAFI levels: OR CT + TT vs. CC (reference) is 0.9 (CI 0.6-1.5).The results of all three studied polymorphisms are shown in Table 3.

TAFI gene polymorphisms in relation to functional TAFI and TAFI antigen levels.

TAFI antigen levels were strongly correlated with all three studied polymorphisms (table 4). This was found for both the control group and the patient group. Functional TAFI levels, as measured by the TAFI related retardation (RT), were not associated with the three polymorphisms studied (table 5). In our population TAFI antigen levels were not correlated with functional TAFI levels (TAFI ag vs RT: r = 0.09, ns).

Table 3: Associations between TAFI genotype and risk of ischemic stroke

Genotype	Genotype numbers and allele frequencies patients (n=124)	Genotype numbers and allele frequencies controls (n=125)	OR (95% CI)
1040 C/T			
CC	55 (44%)	51 (42%)	reference
CT	51 (41%)	58 (48%)	0.82 (0.48-1.39)
TT	18 (15%)	12 (10%)	1.39 (0.62-3.13)
CT+TT	69 (56%)	70 (58%)	0.91 (0.55-1.51)
C	0.65	0.66	
T	0.35	0.34	
505 G/A			
GG	54 (45%)	48 (41%)	reference
GA	53 (44%)	51 (44%)	0.93 (0.54-1.59)
AA	13 (11%)	18 (15%)	0.64 (0.29-1.43)
GA+AA	66 (55%)	69 (59%)	0.85 (0.51-1.42)
G	0.67	0.63	
A	0.33	0.37	
-438A/G			
GG	61 (49%)	61 (50%)	reference
GA	48 (38%)	50 (41%)	0.96 (0.56-1.63)
AA	16 (13%)	10 (8%)	1.60 (0.67-3.80)
GA+AA	64 (51%)	60 (49%)	1.07 (0.64-1.75)
G	0.68	0.71	
A	0.32	0.29	

Table 4: Association between TAFI antigen levels and TAFI gene polymorphisms

		TAFI antigen levels (%)			
		Patients n		Controls n	
TAFI 1040	CC	124.8 ± 22.5	46	123.8 ± 27.1	44
	CT	89.6 ± 18.2	43	92.6 ± 29	52
	TT	76.5 ± 40.2	16	52.5 ± 15	10
TAFI 505	GG	95.1 ± 34.4	47	89.1 ± 34.1	42
	GA	108.7 ± 23.6	45	105.4 ± 30.5	45
	AA	122.6 ± 31.6	9	132.5 ± 23.2	16
TAFI –438	GG	122.0 ± 22.7	51	123.1 ± 26.7	50
	AG	90.0 ± 26.0	41	86.9 ± 29.3	47
	AA	70.2 ± 26.5	14	60.5 ± 22.0	9

Data given as mean ± SD. TAFI antigen levels were not significantly different between patients and controls for each genotype.

Functional TAFI levels and base line characteristics

We also studied the association between various baseline characteristics and functional TAFI levels in the total study group. TAFI related retardation was not different between males and females, individuals with and without diabetes and smokers and non-smokers. Individuals with hypertension, hypercholesterolemia and of higher age (>45 years) had increased functional TAFI levels (table 6). The results of clot lysis times, LT(+pci), LT(-pci), and TAFI-related retardation (RT), shown in table 2 were similar after adjustment for these confounding variables including hypertension, diabetes, smoking, age and hypercholesterolemia (data not shown).

Table 5: Association between clot lysis time (LT_{-PCI}) and TAFI-related retardation ($LT_{-PCI} - LT_{+PCI}$) and TAFI gene polymorphisms

		Clot lysis time (LT_{-PCI}) (minutes)				TAFI-related retardation (minutes)			
		Patients n		Controls n		Patients n		Controls n	
TAFI 1040	CC	47.1 ± 4.2	46	43.6 ± 4.0	43	18.7 ± 4.3	46	16.9 ± 4.1	43
	CT	47.8 ± 3.3	43	44.6 ± 3.2	52	20.8 ± 4.1	43	18.3 ± 3.0	52
	TT	46.5 ± 3.8	16	43.2 ± 4.9	10	18.5 ± 4.0	16	16.9 ± 4.6	10
TAFI 505	GG	46.8 ± 4.0	46	43.4 ± 3.7	41	18.8 ± 4.2	46	17.1 ± 3.7	41
	AG	47.9 ± 3.6	45	44.0 ± 3.9	45	20.8 ± 4.3	45	17.9 ± 3.4	45
	AA	46.3 ± 4.0	10	46.0 ± 3.2	16	17.1 ± 3.9	10	18.3 ± 4.4	16
TAFI –438	GG	47.4 ± 4.4	51	44.1 ± 3.9	49	19.2 ± 4.6	51	17.4 ± 4.2	49
	AG	47.4 ± 2.9	41	44.4 ± 3.1	46	20.1 ± 3.8	41	18.0 ± 2.9	46
	AA	46.5 ± 4.0	14	42.3 ± 5.4	9	18.8 ± 4.3	14	16.9 ± 4.8	9

Data given as mean ± SD

Table 6: Effects of metabolic and environmental variables on functional TAFI levels (expressed as TAFI-related retardation LT (-PCI) – LT(-PCI) in minutes) in the total study group.

Variable	Yes (n)	No (n)	P value
Smoking	18.6 ± 4.3 (85)	18.6 ± 4.0 (117)	0.92
Hypercholesterolemia	19.2 ± 3.7 (136)	17.6 ± 4.5 (76)	0.0058
Age >45 years	19.1 ± 4.0 (170)	16.7 ± 4.0 (42)	0.0005
Diabetes	18.1 ± 4.2 (21)	18.7 ± 4.1 (191)	0.563
Hypertension	20.0 ± 3.8 (66)	18.0 ± 4.1 (146)	0.0007
Male sex	19.0 ± 4.0 (114)	18.2 ± 4.2 (98)	0.18

Data are given as mean ± SD

DISCUSSION

In this study, we found increased clot lysis times, indicative of impaired fibrinolysis in patients with a recent ischemic stroke compared with healthy controls. The risk of ischemic stroke was strongly associated with fibrinolysis, as was demonstrated by the odds ratio of 8.2 for individuals within the highest quartile of clot lysis times compared to the lowest quartile. This was also seen, though to a lesser extent, for functional TAFI levels, defined as TAFI-related retardation (RT, $LT_{(-PCI)} - LT_{(-PCI)}$). This indicates that increased functional levels of TAFI are associated with an increased risk of ischemic stroke.

Only two previous studies on TAFI levels in stroke patients have been reported so far. Montaner et al. showed in a small study including 30 patients that TAFI antigen levels were increased in patients with ischemic stroke.[17] Plasma samples were taken within 24 hours of onset of the symptoms. Therefore the high TAFI antigen levels may be caused by an acute phase response, and may not reflect a causative role of TAFI in the development of ischemic stroke.[9,25] Santamaria et al. performed a case-control study, comparable with our study design.[16] The patients were included at least one month after the event, thereby excluding the influence of a potential acute phase response. They measured functional TAFI levels using a synthetic substrate based assay. They found that increased functional TAFI levels were associated with a 6-fold increased risk of ischemic stroke. The study population consisted of patients with at least one previous ischemic event, which may result in a selection of patients with recurrent thrombotic events, whereas our patients had a recent first episode of ischemic stroke. Despite these differences in patient population our study confirms the finding that increased functional TAFI levels are associated with an increased risk of ischemic stroke.

The mechanism by which increased functional TAFI levels may result in ischemic stroke is still unknown. A decreased fibrinolytic potential may result in more stable thrombi, that are less prone to lysis. Our findings are in concordance with previous studies on other fibrinolysis inhibitors, such as plasminogen activator inhibitor type I (PAI-1), of which increased levels are also suggested to predispose to arterial thrombosis.[2,4]

Earlier studies have shown that elevated TAFI antigen levels are associated with venous thrombosis.[18,26] Patients with TAFI antigen levels above the 90th percentile had a two-fold increased risk of deep venous thrombosis.[26] Also TAFI gene polymorphisms that are related to high TAFI antigen levels are associated with an increased risk of deep venous thrombosis.[18] The role of TAFI in arterial thrombosis is, however, still unresolved. Several studies have shown that reduced TAFI antigen levels are found in patients with a history of acute myocardial infarction or unstable angina pectoris (UAP).[12,13] We have previously shown in patients with UAP that lower TAFI antigen levels were present in patients who were refractory to medical treatment (more severe cases) compared to non-refractory patients.[12] In contrast, other studies found increased levels of TAFI antigen in patients with arterial thrombosis.[9-11] A major difference between the above mentioned studies was the method of determination of TAFI antigen levels. It has become evident that some antigen assays have a variable antibody reactivity towards the different isoforms of TAFI.[14,15] The discrepancy between the studies may therefore in part be attributed to the various assays that have been used in the past. In our study we measured TAFI antigen levels by means of an ELISA that is more specific for the 325Thr isoform (1040C/C genotype).[15] It would be interesting to use TAFI antigen assays, which are not sensitive to TAFI genotypes, to measure TAFI antigen levels in new prospective studies in arterial thrombotic disorders. In

our study no difference was observed in TAFI levels of patients with ischemic stroke compared with a population based control group. Because of the problems arising with TAFI antigen level measurement, and the difficulties of interpretation of the various results, measurement of functional TAFI levels seems to be of utmost importance, as is also shown in our study.

Relatively few studies have been performed on TAFI activity levels in arterial thrombosis.[17,27] The tests used in these studies were based on synthetic substrates. A clot lysis based assay is a more physiological method to study the impact of TAFI on fibrinolysis. In addition, this assay has to be specific for TAFI. In the clot lysis based assay used in this study, patient plasma was diluted 20-fold in TAFI deficient plasma. The clot lysis time (LT) was also measured in the presence of a specific TAFI inhibitor PCI. TAFI-related retardation, defined as $LT_{(-PCI)} - LT_{(+PCI),}$ is a specific parameter for functional TAFI. Several TAFI activity assays have been developed.[28,29] Also activity assays based on clot lysis times that are TAFI dependent have previously been described[28], however this assay may be influenced by other plasma components.[30] The method that we have used to study functional TAFI levels is used for the first time in a patient population. The finding that both the clot lysis time without addition of PCI, and the TAFI-related retardation are significantly higher in patients compared to controls, indicates that functional TAFI levels are an important risk factor for ischemic stroke. However, also clot lysis times in the presence of PCI are significantly higher in patients with ischemic stroke (table 2). This indicates that other variables that influence clot lysis time probably also contribute to the increased risk of ischemic stroke. We adjusted for other potential confounders in our study population, such as hypercholesterolemia, age, and smoking, but these did not influence the association of clot lysis times with the risk of ischemic stroke. In the total study group, TAFI-related retardation was higher in individuals with hypercholesterolemia, higher age and hypertension. We did not find a good correlation between TAFI antigen levels and TAFI-related retardation. This may be caused by the ELISA antigen assay used in our study, of which the results are strongly dependent upon TAFI isoforms in plasma.[15] Therefore the measured differences in TAFI antigen levels between the various genotypes may be exaggerated.[15] TAFI antigen levels and functional TAFI levels or clot lysis times may correlate better using different antigen assays[15,31], some of which are known to be less sensitive for TAFI isoforms.[15] Our findings are consistent with a recent study in young patients with acute myocardial infarction, in which TAFI activity was significantly higher in patients with AMI compared to controls.[27] In that study a discrepancy was found between TAFI activity and antigen levels, without a correlation in both patients and controls

It is well known that TAFI genotype is correlated with TAFI antigen levels. We confirmed that three polymorphisms (-438A/G, 505G/A and 1040C/T) are associated with TAFI antigen levels, both in patients and controls. We could not demonstrate a difference in the risk of ischemic stroke between the various genotypes. Previous studies showed that the 505G/A SNP (Ala147Thr) in the TAFI gene is associated with the risk of acute myocardial infarction.[13] Individual carrying the "TAFI antigen decreasing allele" (505G, Ala147) had an increased risk of acute myocardial infarction in a combined analysis of three case-control studies.[13] In our study we could not find this association between the TAFI 505G/A gene polymorphism and the risk of ischemic stroke. We also could not detect an association between the 1040 C/T polymorphism (Ile325Thr) and the risk of ischemic stroke, in agreement with two previous studies on the risk of AMI.[27,32]

One of the limitations of our study is the size of the study population, especially, as already mentioned above, in assessing the association between TAFI gene polymorphisms and ischemic stroke. Based on the confidence intervals, however, TAFI gene variations will probably not be a strong risk factor for ischemic stroke.

We conclude that reduced fibrinolysis, as shown by a prolongation of clot lysis times in patients with a first ischemic stroke, results in a significantly increased risk of ischemic stroke. This is mainly due to an increase of functional TAFI, but also other factors influencing the fibrinolytic system may be involved. Additional studies involving larger patients groups are necessary to study both functional levels and non-genotype dependent antigen levels as well as TAFI genotype.

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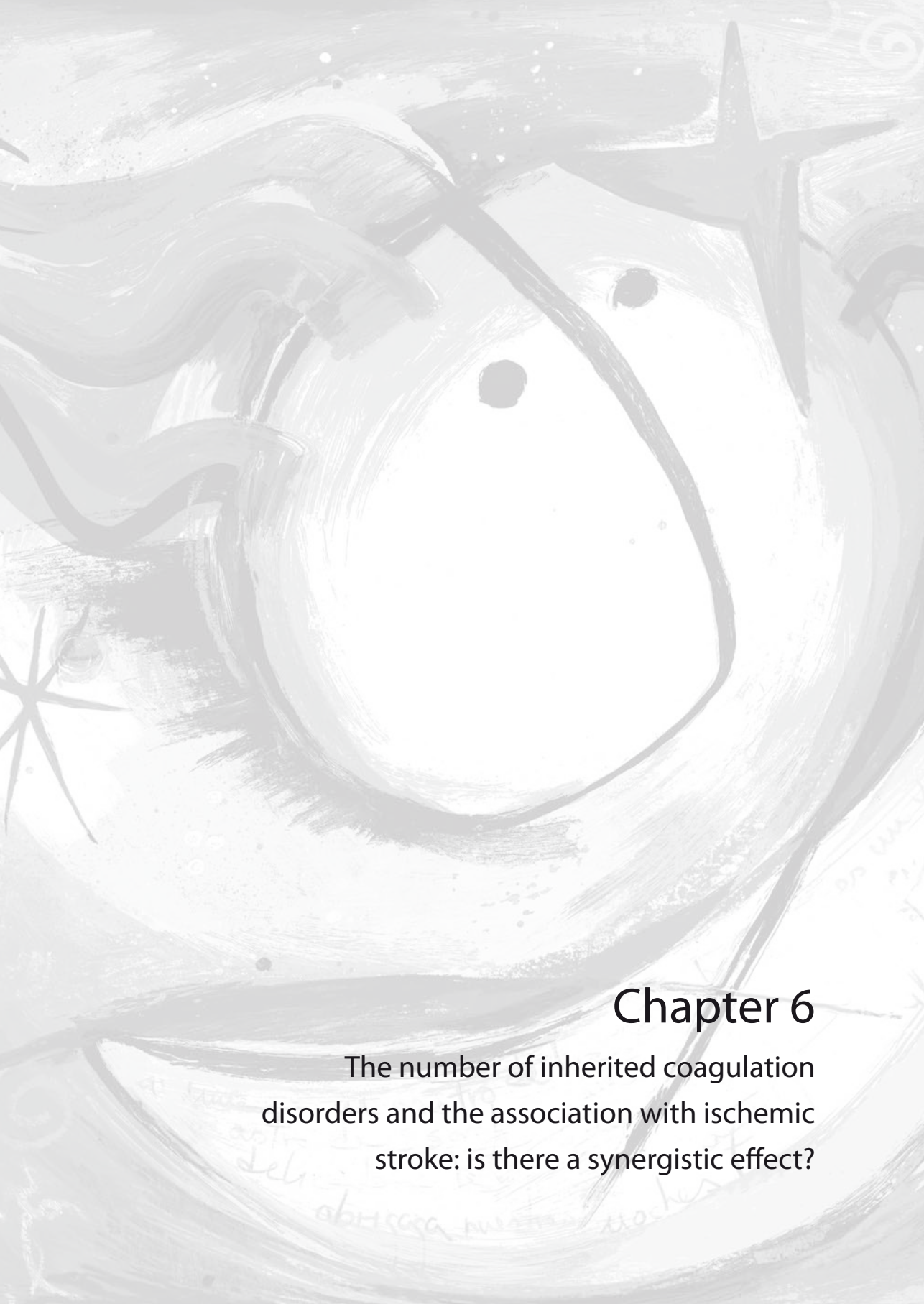

Chapter 6

The number of inherited coagulation disorders and the association with ischemic stroke: is there a synergistic effect?

INTRODUCTION

Ischemic stroke is the third cause of death and the first cause of disability in the Western World. In the majority of ischemic stroke patients rather weak risk factors such as age, male sex, hypertension and smoking can be found. In young patients often no risk factors are found at all. As the chance of recurrence of cardiovascular events is an important issue for the patient as well as for the clinician, identification of new and stronger risk factors is needed. Inherited coagulation disorders are important risk factors for venous thrombosis and are thereforeobvious targets for investigation in arterial thrombosis, including ischemic stroke. The difficulty in clinical practice is generally not making the diagnosis of underlying thrombophilia, as it is often revealed by routine blood tests, but in assessing whether the thrombophilia is a risk factor for the ischemic stroke. Chapter 4 and 5 of this thesis address this issue. We studied the role of several inherited coagulation disorders in the occurrence of ischemic stroke. In conclusion, none of the studied coagulation disorders was associated with the occurrence of ischemic stroke. This finding is in agreement with other larger case-control studies.[1-5]

As our results provide no evidence for a higher risk for ischemic stroke in the presence of a single genetically determined coagulation disorder, it seems obvious to investigate the influence of clustered coagulation disorders on stroke risk. We hypothesized that there could be a synergistic effect on stroke risk in the presence of more than one inherited coagulation disorder. We used our case-control study to investigate if the number of genetically determined coagulation disorders was associated with the occurrence of ischemic stroke.

METHODS

The design of the study has been extensively described in chapter 3. In short, COCOS is a case-control study with prospective inclusion of Caucasian participants with first ever ischemic stroke, confirmed by CT. Controls are population based, age-and sex-matched, of the Caucasian race and not related to the patients. Blood samples for genotype were drawn under strictly standardised conditions one week after the event. The studied coagulation disorders were FVL mutation, G20210A prothrombin gene variant and polymorphisms of the -148 C/T fibrinogen gene, 4G/5G PAI-1 gene, -1C/T AnxA5 gene, G79A PZ gene and 1040C/T, 505G/A, -438A/G TAFI gene. The methods for isolating DNA and detection of the separate mutations and polymorphisms have been described in chapters 4 and 5. In order to test the hypothesis that the number of genetically determined coagulation disorders is associated with the occurrence of ischemic stroke, additional statistical analyses within the COCOS population were performed. We estimated the odds ratio (OR) associated with ischemic stroke in the presence of more than one inherited coagulation disorder for each number of disorders separately. The OR for the presence of any coagulation disorder was also estimated. In second instance, the coagulation disorders were divided in two groups. One consisted of coagulation disorders that mainly relate to fibrinolytic part of the clotting cascade, i.e. PAI-1 gene polymorphism, 1040 C/T TAFI gene polymorphism, 505 G/A TAFI gene polymorphism, -438 A/G TAFI gene polymorphism and the -1C/T AnxA5 gene polymorphism. The second group consisted of coagulation disorders that mainly relate to the prothrombotic part of the clotting cascade, i.e. the FVL mutation, the G20210A prothrombin gene variant, the -148 C/T fibrinogen gene polymorphism and the G79A

PZ gene polymorphism. For both groups the OR associated with ischemic stroke in the presence of more than 1 coagulation disorder of that group was estimated. Multiple logistic regression analysis was used to adjust for possible confounding variables, such as smoking, hypertension and use of oral contraceptives.

RESULTS

The study population consisted of 124 patients and 125 controls. The mean age was 56 years (range 18-75 years). Forty-seven percent of the participants were female. Patients were more often smokers and more often had hypertension, diabetes or were known to have cardiovascular disease compared with controls. The determination of the PZ gene polymorphism and the AnxA5 gene polymorphism was performed in second instance. At that time, in only 83% of the patients and controls DNA was available for genotyping. In the other participants, there was no remainder of the blood sample for technical reasons. Table 1 summarizes the distribution of the genotypes in patients and controls and the relative risk associated with the occurrence of ischemic stroke in the presence of each individual coagulation disorder.

Table 1 Prevalences of inherited thrombophilia in patients and controls and associated odds ratio with 95% confidence interval (95%CI). PM=polymorphism

Coagulation disorder	Genotype	Cases (N=124)	Controls (N=125)	OR (95% CI)
FVL mutation	GG GA+AA	115 5 + 0	119 4 + 0	1.3 (0.4-4.6)
PTII gene variant	GG GA+AA	118 2 + 0	118 5 + 0	0.4 (0.1-1.8)
-148C/TFibrinogen gene PM	CC CT+TT	83 34 + 6	77 41 + 5	0.8 (0.5-1.4)
4G/5G PAI-1gene PM	5G/5G 4G/5G+4G/4G	29 61 + 33	29 58 +36	1.0 (0.6-1.8)
-1C/T AnxA5 gene PM	CC CT+TT	87 30 + 1	73 23 + 1	1.1 (0.6-2.0)
TAFI -1040 gene PM	CC CT+TT	55 51 + 18	51 58 + 12	0.9 (0.6-1.5)
TAFI 505 gene PM	GG GA+AA	54 53 + 13	48 51 + 18	0.9 (0.5-1.4)
TAFI -438 gene PM	AA AG+GG	61 48 + 16	61 50 + 10	1.1 (0.6-.8)
G79A PZ gene PM	GG GA+AA	75 39 + 4	77 32 + 4	1.2 (0.7– 2.1)
Any inherited disorder	absent present	3 121	5 119	1.7 (0.4-6.6)

None of these genetically determined coagulation disorders were individually associated with an increased risk of ischemic stroke. The OR for ischemic stroke in the presence of more than 1 coagulation disorder was 1.2 (95% CI: 0.5-2.7) and the OR for the presence of any coagulation disorders was 1.7 (95% CI: 0.4-6.6). Table 2 shows that the relative risk was not different for the groups with a fixed number of genetic variants. After separating the coagulations disorders in procoagulant and fibrinolysis related disorders, again no convincing association was found (Table 3 and 4). The OR for ischemic stroke in the presence of any fibrinolysis related coagulation disorder was 0.9 (95% CI: 0.5-1.5). For the group of disorders related to the procoagulant part of the clotting cascade the OR was 1.0 (95% CI: 0.3-3.0). After adjustment for smoking, hypertension and use of oral contraceptives the results remained the same in all groups.

Table 2 Distribution of the number of coagulation disorders in patients and controls with associated odds ratio and 95% confidence interval (95% CI); NA=not applicable

Number of coagulation disorders	Cases (n=124)	Controls (n=125)	OR (95%CI)
0	3	5	0.6 (0.1-2.5)
1	8	8	1.0 (0.4-2.6)
2	26	20	1.3 (0.7-2.3)
3	33	37	0.9 (0.6-1.4)
4	28	31	0.9 (0.5-1.5)
5	22	19	1.2 (0.6-2.1)
6	3	4	0.8 (0.2-3.4)
7	1	0	NA

Table 3. Distribution of the number of fibrinolysis related coagulation disorders in patients and controls with associated odds ratios and 95% confidence interval (95% CI); NA=not applicable

Number of coagulation disorders	Cases (n=124)	Controls (n=125)	OR (95%CI)
0	6	7	0.9 (0.3-2.6)
1	15	21	0.7 (0.4-1.4)
2	38	30	1.3 (0.8-2.0)
3	39	40	1.0 (0.6-1.5)
4	22	25	0.9 (0.5-1.6)
5	4	2	2.0 (0.4-10.9)

Table 4. Distribution of the number of coagulation disorders related to the procoagulant part of the clotting cascade in patients and controls with associated odds ratios and 95% confidence interval (95% CI); NA=not applicable

Number of coagulation disorders	Cases (n=124)	Controls (n=125)	OR (95%CI)
0	53	50	1.1 (0.7-1.6)
1	51	57	0.9 (0.6-1.3)
2	18	17	1.1 (0.5-2.1)
3	1	0	NA

DISCUSSION

Internal validity

A strength of this study is the well defined population as we collected detailed information about cardiovascular risk factors, medical history and family history in consecutively included patients as well as in controls. The ischemic nature of the stroke was determined by CT scan of the brain and patients with TIA were only included if the neurological deficit was observed by a neurologist. The insurance of a well defined ischemic stroke group is important as the studied prothrombotic coagulation disorders are not likely to cause hemorrhagic stroke. All participants were of the Caucasian race and the allele frequencies of the polymorphism in the control group were in Hardy-Weinberg equilibrium, indicating that we studied a representative group. Because we only studied survivors, we cannot exclude that the presence of the variant allele of one of the studied coagulation disorders is associated with fatal ischemic stroke. However, an association with fatal stroke is not very likely, since genotype was not related to stroke severity in our population. Furthermore, the majority of deaths within a few days after stroke is caused by pneumonia or other (non-hematological) complications.

External validity

Our results show that inherited coagulation disorders are a frequent finding in ischemic stroke patients, but that it is unlikely that they are relevant in the pathogenesis of the stroke. This is in agreement with three similar studies[6-8] that failed to find an association between ischemic stroke and the presence of any genetically determined coagulation disorder. In the same studies also no association was reported for pathogenic subgroups of patients such as those with large or small vessel disease or cardio-embolic stroke[6] or younger stroke patients.[7] On the other hand, an increased stroke risk in patients with patent foramen ovale in combination with the FVL mutation or prothrombin gene variant was reported by several case-control studies.[9-11] The prothrombin gene variant has also been associated with an increased stroke risk in selected population of Mediterranean origin.[12,13] In our population we were not able to perform subgroup analysis because of the small sample size and the low prevalence of especially the FVL mutation and the prothrombin gene variant.

In conclusion, in our study of Caucasian individuals the number of genetically determined coagulation disorders was not associated with an increased risk of ischemic stroke. A synergistic effect of combinations of coagulation disorders is also not likely. Therefore, in Caucasian patients with ischemic stroke, routinely performed coagulation tests to screen for an inherited coagulation disorder are not indicated. These findings have also implications for ischemic stroke patients with coincidently found genetic thrombophilia. These patients are often treated empirically with oral anticoagulants, based on the assumptions that the association between stroke and the coagulation disorder is likely to be causal and that anticoagulants are the most effective therapy. However, neither of these assumptions is evidence-based and the most appropriate treatment for secondary prevention in these patients is still antiplatelet therapy. Further studies are needed to investigate the role of genetically determined coagulation disorders in selected stroke populations such as patients with PFO or patients of Mediterranean origin.

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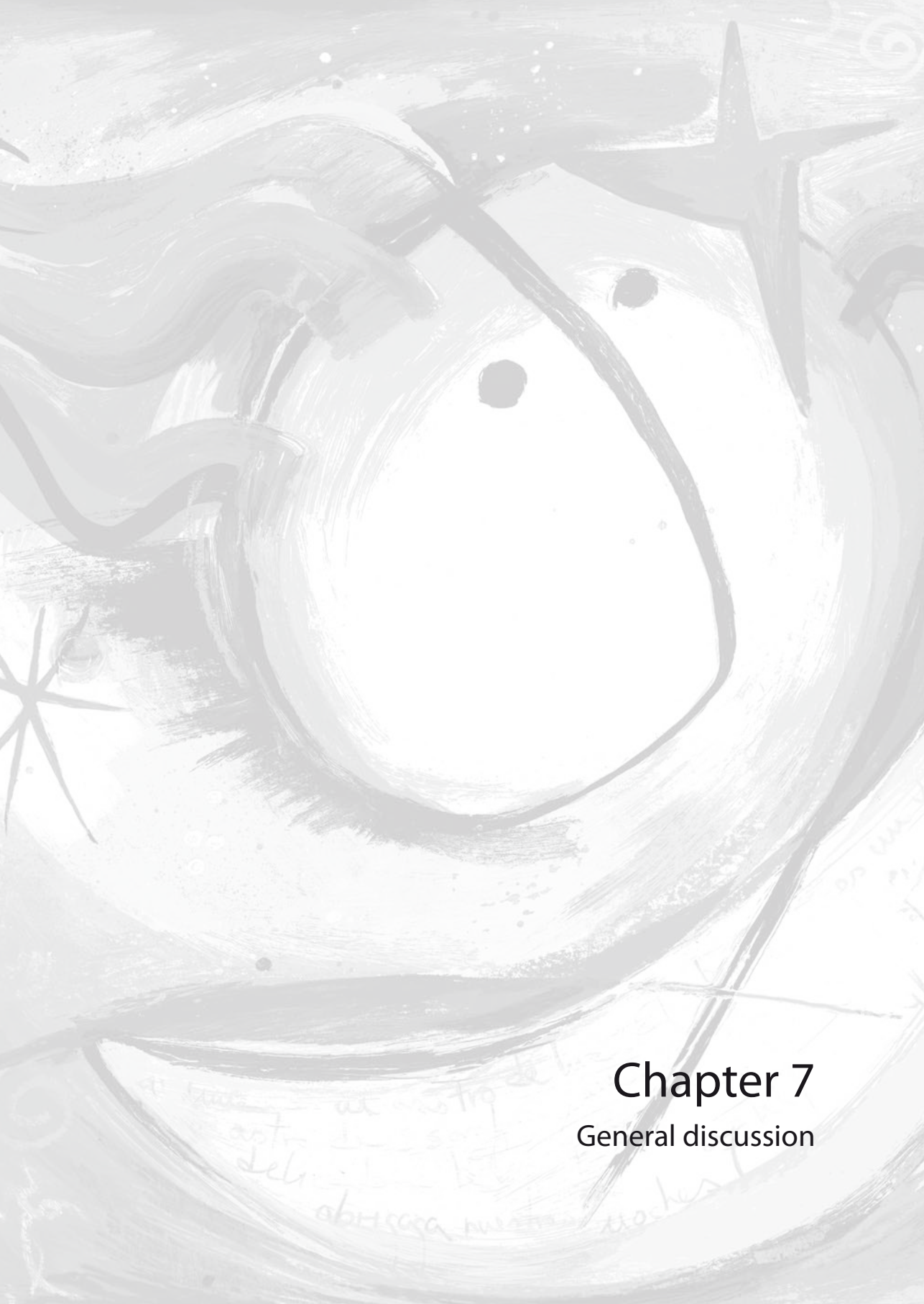

Chapter 7

General discussion

The objective of this thesis was to gain more insight in the association between genetically determined coagulation disorders and the occurrence of ischemic stroke. The COCOS study was performed as a co-operation between the departments of Neurology and Hematology of the Erasmus Medical Center. Detailed clinical information about the participants, as well as the remainder of the blood samples was stored in a database. This provides an easy accessible database of DNA and blood plasma of a well-defined case-control population. In case new polymorphisms, a possible association with ischemic stroke can be studied rapidly.

We have studied potent risk factors for venous thrombosis but also some more recently described polymorphisms of the genes encoding for coagulation factors. In this chapter the main findings will be summarized and some methodological aspects will be considered. The clinical implications will be discussed and finally suggestions for further research will be made.

MAIN FINDINGS

The main negative associations of this thesis are:

- The FVL mutation and the G20210A prothrombin gene variant were not associated with an increased risk of ischemic stroke in the Caucasian
- The -148C/T fibrinogen gene polymorphism, the 4G/5G PAI-1 gene polymorphism, nor the PLA1/PLA2 polymorphism were associated with an higher risk of ischemic stroke in the Caucasian
- The more recent described polymorphism in the PZ gene, AnxA5 gene and the TAFI gene were not associated with an increased risk of ischemic stroke in the Caucasian
- The number of genetically determined coagulation disorders is not related to the occurrence of ischemic stroke. Therefore, a synergistic effect is not likely

The main positive associations of this thesis are:

- The phenotypic expression of the polymorphism, i.e. the protein plasma level was clearly related to genotype
- High fibrinogen levels in stroke patients shortly after the event are mainly the result of an acute phase response
- Low PZ levels and high prothrombin levels may be associated with the occurrence of ischemic stroke in the Caucasian
- High functional level of TAFI are associated with an increased risk of ischemic stroke in the Caucasian

The comparison of these findings with those of other studies is described in the previous chapters and will not be repeated here.

METHODOLOGICAL ISSUES

In this section, I will discuss some general issues, which pertain to all studies performed within the COCOS population.

Study design

The COCOS study was a case-control study among Caucasian patients with first ever ischemic stroke. In 125 patients and 125 controls blood sample were taken to determine genotype

concerning 9 different coagulation factor genes and their corresponding phenotypic expression one week after the event. In 68 patients the blood sample for plasma protein level was repeated in the convalescent phase, more than 3 months later. The design of the study provides fast inclusion of the participants as well as detailed information about the participants concerning cardiovascular risk factors, medical history and family history. Hemorrhagic stroke was excluded by CT scan of the brain and patients with TIA were only included if the neurological deficit was observed by a neurologist. This ensures a well defined ischemic stroke group. This is necessary as we were studying prothrombotic risk factors, which are unlikely to cause hemorrhagic stroke. As the blood extraction was standardized, different findings in plasma protein levels between participants as a result of food intake, smoking, physical exercise or circadian rhythm, were ruled out.

The design of the study has three important disadvantages. The first is that the exposure, i.e. the plasma protein level, was determined after the occurrence of the index event. We partly dealt with this issue by repeating the measurements of the protein levels in the convalescent phase. However, this could be performed in only about 50% of the patients. Secondly, we cannot exclude that the studied mutations or polymorphisms are associated with fatal stroke, as we included only survivors of ischemic stroke. Again, for answering this question population based prospective studies are more suitable than clinical case-control studies. However, an association with fatal stroke is not very likely, since genotype was not related to stroke severity in our population. Furthermore, the majority of deaths within a few days after stroke is caused by pneumonia or other (non-hematological) complications. Finally, as a result of the study design we were not able to further unravel the pathophysiological mechanism of the occurrence of ischemic stroke. In our patients, we found elevated levels of fibrinogen during the acute phase. Whether these increased levels are a cause or consequence of ischemic stroke is not yet clarified. Nested case-control studies can only partly contribute to this question as the risk factor is determined long before the occurrence of ischemic stroke, comparable with the repeated measurement after three months in our study. Animal studies with continuous measurements of the protein levels can give more information about the pathophysiological mechanism of stroke occurrence.

Ethnicity

In order to create a genetic homogenous group, only participants of the "Caucasian race" were included. Caucasian was defined as "the participant and his or her parents has to be born in Northern Europe with the river Rhône as Southern border for Northern Europe". Such division was needed as in Rotterdam about 50% of the urban population is foreign and originates from Caribbean or Mediterranean areas. However, the assumption that ethnicity is an isolated epidemiological variable delineating clinically distinct disease groups is controversial.[1] Ethnic classification may vary from one community to another, as the perception of an ethnic group may be different across countries. Ethnicity as a variable may be greatly influenced by cultural attitude. As Caldwell and Popenoe[2] put it, "what is black to someone from the United States may be white to a Brazilian or a Caribbean islander". Ethnicity is not a dichotomous variable, such as gender. How black is black or how white is white? Defining disease on the basis of ethnicity might perhaps hide socioeconomic differences that are the real cause of disease in some ethnic groups.[1] Within COCOS, I think we have adequately dealt with the issue of ethnicity. Because of the sample size of 125 and the aim to study genetically determined coagulation disorders, it is necessary to choose participants of the same genetic area. Keeping this in mind,

the group of blacks seems to be more heterogeneous than the group of whites. Furthermore, the FVL mutation and G20210A prothrombin gene variant were almost exclusively found in whites.[3;4] We used population based controls of the same genetic area as the patients in order to generate a control group of comparable socioeconomic status. Finally, we did not compare between groups of presumed different origin.

Generalizability

When studying the association between a genetically determined coagulation disorder and the occurrence of ischemic stroke, two underlying mechanisms can be assumed. First, the coagulation disorder itself can be an isolated risk factor for ischemic stroke and its presence is enough to cause the ischemic event. In that case, the study population is limited to patients in whom no other cause for the stroke is found. On the other hand, the presence of the coagulation disorder could increase the risk of ischemic stroke in combination with other cardiovascular risk factors. We have chosen to include all consecutive patients with first ever ischemic stroke, either with or without an established cause. The relationship between the coagulation disorder and ischemic stroke could hence be studied for the group as a whole as well as for subgroups. This enabled us to test both hypotheses. The baseline characteristics of our study were comparable with other stroke populations.[5] Patients were more often smokers and more often had hypertension, diabetes and were more often known to have cardiovascular disease. In 49% of the cases no cause for the ischemic stroke was found according to the TOAST classification.[5] Nine percent suffered from large vessel disease, 37% from small vessel disease and in 3 % of the patients a probable source of cardiac embolism was detected. This breakdown of possible causes is comparable with previous studies, except for the lower rate of cardiac embolism in our study. In general, in ischemic stroke populations in 15-20% a cardiac cause for the ischemic event is found. There are several explanations for the difference in prevalence of cardiac disorders. We classified only the probable and not the possible causes of the TOAST classification as positive cardiac embolism. Furthermore, only in the young patients (47%) transthoracal echocardiography was performed. In 44% of them this investigation was followed by transesopageal echocardiography, which is more sensitive in detecting small cardiac abnormalities or abnormalities in the left atrial appendage. A final explanation may be the use of oral anticoagulants. In general stroke populations, about 50% of the patients with cardiac embolism were already known to suffer from atrial fibrillation at the time of the ischemic stroke and therefore treated with oral anticoagulants. As this therapy was an exclusion criterion in our study, this might also contribute to the lower prevalence of cardiac embolism in the COCOS study.

PATHOGENESIS

Hypercoagulability: Venous thrombosis versus arterial thrombosis

The studied coagulation disorders in COCOS, especially the FVL mutation and the G20210A prothrombin gene variant, are strong risk factors for venous thrombosis.[6-8] New treatment strategies by means of anticoagulant therapy have lead to prognostic improvement of patients with venous thrombosis, but also to improvement of primary and secondary prevention.[9] However, in arterial thrombosis, the role of the genetically determined coagulation disorders appears to be less rectilinear and more complicated. In the studies presented in this thesis,

none of the studied genetically determined coagulation disorders appeared to be a risk factor for ischemic stroke, which is supported by several other (nested) case-control studies. The explanation for the different influence of coagulation disorders between venous and arterial thrombosis can only be explained by different underlying pathogenetic mechanisms. In venous thrombosis three factors are important, i.e. blood stasis, hypercoagulability and vessel damage, collectively known as the Virchow 's triad. There is no need for all these components to be present in order for thrombosis to occur. As a matter of fact, venous thrombosis may even occur in the presence of only one of these conditions, wherein clotting changes and blood flow are seen to play the major role. In recent years, remarkable advances in the understanding of the concept of hypercoagulability have been made, with special emphasis to genetically determined thrombophilic abnormalities.[10-12] These give rise to disturbances of the balanced clotting cascade, leading to increased activation of the procoagulant factors of hemostasis, leading to increased thrombus formation. Venous thrombi are mainly composed of fibrin and red blood cells and usually are formed in areas of stasis. In arterial thrombosis the underlying mechanism is based on atherosclerosis. Atherosclerosis is increasingly viewed as a chronic inflammatory disease that, over many years leads to the development of atherosclerotic plaques. Rupture of local vulnerable plaques is considered the most important underlying mechanism of myocardial infarction.[13-16] In ischemic stroke small vessel disease and thromboembolism without significant large vessel disease are more common causes. Arterial thrombi are primarily composed of platelets and are formed in areas of rapid blood flow. Formation of local thrombus as a result of a hypercoagulable state like in venous thrombosis is, because of higher flow of blood in the larger arteries, not very likely. The pathogenesis of arterial thrombosis is a complex process in which environmental and genetic factors seem to interact.[16-19]

CLINICAL IMPLICATIONS

In none of the studies described in this thesis we found support for an important causal role for genetically determined coagulation disorders. Hence, we face the paradox that polymorphisms within certain genes do correlate with plasma levels of the respective proteins, and that certain laboratory parameters correlate consistently with ischemic stroke, but that positive associations between genotype and ischemic stroke are absent. Therefore, there is no indication to routinely determine the presence of these coagulation disorders in patients with ischemic stroke.[20] Also, there is no need to treat ischemic stroke patients with coincidently detected genetic coagulation disorders, differently from ischemic stroke patients without these disorders. Increased plasma levels of procoagulant factors are most likely the result of an acute phase response, leading to a similar approach of treatment as for the genotype. Decreased protein Z levels might be an exception as these were in our population almost similar in patients and controls in the acute phase, but significantly lower in patients in the reconvalescent phase. Protein Z has been reported to act as cofactor in the inhibition of FXa, leading to a more procoagulant than anticoagulant state in case of low protein Z levels. However, the exact role of protein Z in ischemic stroke needs to be clarified. As yet, decreased protein Z levels have no clinical consequences.

In childhood stroke, genetically determined coagulation disorders are a well known risk factor.[21-24] Therefore, the only category of ischemic stroke patients with an indication for coagulation screening are children with ischemic stroke. Another group of interest is that of patients with suspected antiphospholipid syndrome. Antiphospholipid syndrome is defined as

recurrent arterial or venous thromboses, sometimes combined with miscarriage, in the presence of antiphospholipid antibodies (anticardiolipin IgG or IgM and/or lupus anticoagulant).[25] It is obviously indicated to perform coagulation tests in these patients because of their high risk of recurrent thrombotic events. In case of presence of lupus anticoagulant (LA) or elevated anticardiolipin IgG or IgM (ACL), oral anticoagulant therapy is often started. However, there is no evidence for an effect of this treatment on recurrent thrombotic events in stroke patients with positive APL. We performed a follow-up study among 128 young ischemic stroke patients, of whom 17% had positive aPL.[26] The relative risk of any thrombotic event in patients with aPL was 0.9 (95% CI: 0.3-2.4) and for recurrent ischemic stroke or TIA 0.7 (95% CI: 0.3-2.2). These findings were in agreement with the recent results of WARRS/APASS[27], a large prospective cohort study, that not indicated an effect of treatment with low intensity (INR 1.4-2.8) oral anticoagulants in patients with aPL. Two further remarks on this subject need to be made. APL, especially ACL, are liable to changes, for instance as a result of medication use or acute phase reactions. Repeated measurements are absolutely mandatory to show consistency of the findings and to minimize the possibility that increased ACL levels occurred by chance. Second, the studies that reported an increased thrombotic risk in patients with aPL mostly concerned very high ACL levels, i.e above 100 U.[28-31] In patients with mildly elevated ACL the relationship is less clear. Therefore, only in patients with very high ACL the measurements must be repeated. In these patients systemic lupus erythomatodes (SLE) is frequently detected.

FUTURE STUDIES

The COCOS population is a well designed database for future research concerning coagulation disorders in ischemic stroke patients. In my opinion, it is unlikely that a genetically determined coagulation disorder in itself plays a causative role in the occurrence of ischemic stroke. Future studies should focus on the subgroups of patients in whom interaction of a coagulation disorder and another minor cause such as patent foramen ovale (PFO) can be studied. PFO has been associated with cryptogenic stroke[32-34] and several studies showed a further increase of stroke risk in patients with PFO combined with FVL mutation or G20210A prothrombin gene variant.[35-37] Although defining ethnicity is controversial, future studies should be performed in stroke patients of different ethnic regions. The prothrombin gene variant has been repeatedly reported as a significant risk factor for cryptogenic ischemic stroke in Mediterranean populations[38,39] and recently PFO was reported to be significantly more frequent in whites compared with blacks.[40]

For the FXIII Val34Leu polymorphism, a protective effect on the development of venous thrombosis and MI has been shown in several studies [41-44]. The results in ischemic stroke are contradictory[42,45,46] and need further investigation.

Finally, the exact role of protein Z and TAFI in ischemic stroke needs to be further clarified. For TAFI a new study has already started, the ATTACK study, a co-operation between the departments of Hematology, Neurology, Cardiology and Vascular Surgery of the Erasmus MC. Patients with ischemic stroke, myocardial infarction and peripheral vascular diseases will be included. Again genotype and phenotype will be studied in relation to the disease. When this reveals an association of protein levels in the convalescent phase and the occurrence of ischemic stroke, searching for a responsible genotype will be of renewed interest.

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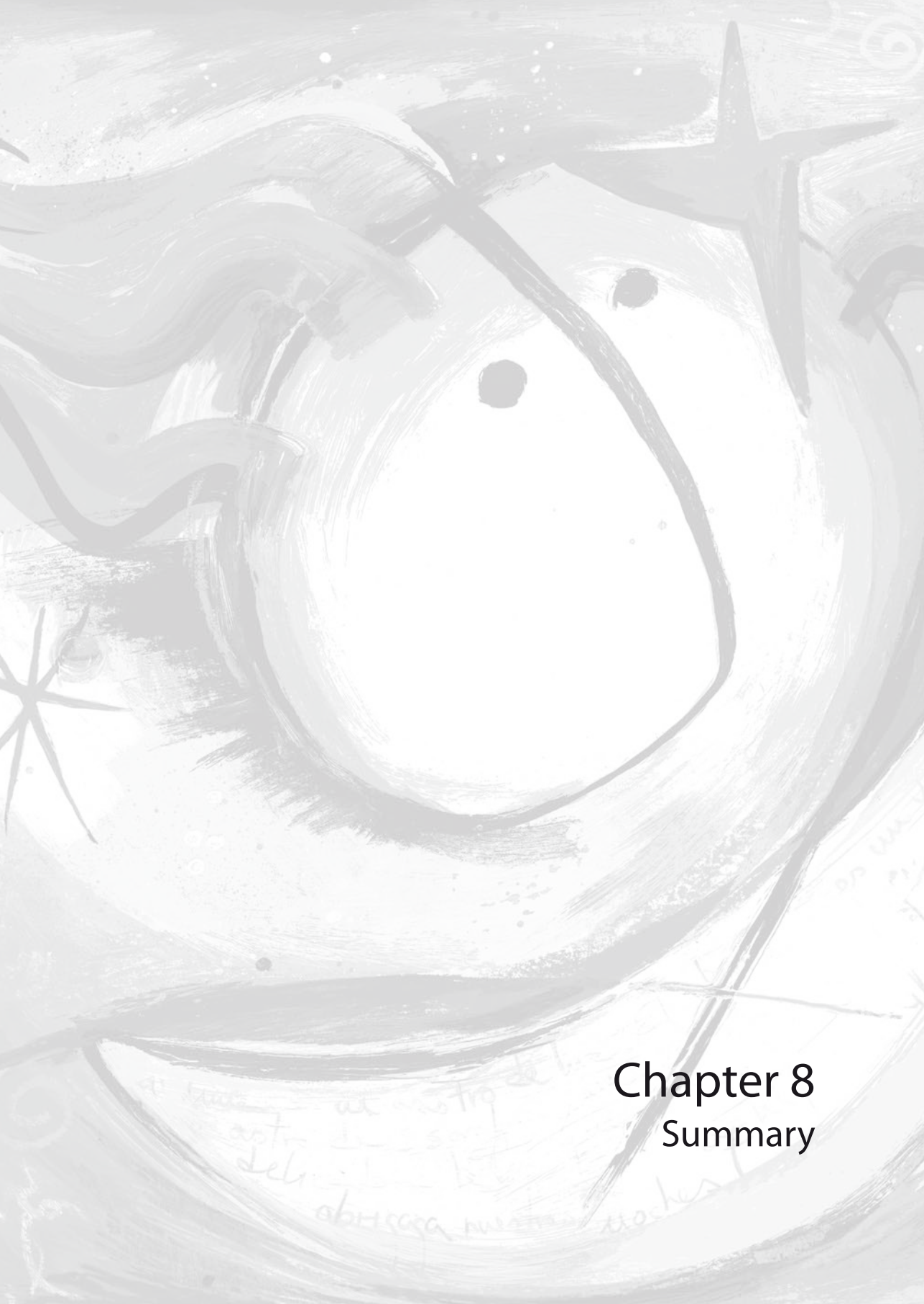

Chapter 8
Summary

SUMMARY

Stroke is the main cause of disability and the third cause of death in the Western world. The known cardiovascular risk factors as smoking, hypertension and diabetes are often weak and not clustered. A search for new risk factors is necessary, as it is important to identify patients with high risk for (recurrent) vascular events. Genetically determined coagulation disorders are strong risk factor for venous thrombosis. Their role in arterial thrombosis, like ischemic stroke, is less clear.

In this thesis, I describe our research concerning the role of genetically determined coagulation disorders in the occurrence of ischemic stroke. We studied the relationship between ischemic stroke and various mutations and polymorphisms of coagulation related genes as well as their phenotypic expressions in order to identify a genetic mutation as an important risk factor for ischemic stroke. For this purpose, we performed two case-control studies in patients with recent ischemic stroke.

In chapter 2, the results of a retrospective case-control study in 49 young patients with ischemic stroke and 87 healthy controls are described. Chapter 2.1 adresses the association between the G20210A prothrombin gene variant, the prothrombin level and ischemic stroke. Although the prothrombin gene variant was only a weak risk factor for ischemic stroke, increased prothrombin activity, which was more frequent than the mutation, appeared to be more strongly related to cerebral ischemia. Chapter 2.2 shows the association between ischemic stroke and the PLA1/A2 gene polymorphism in the same study population. The polymorphism was not associated with an increased risk of stroke.

Chapter 3 covers the rationale, background and design of the COntrolled study of genetically determined COagulation disorders in ischemic Stroke (COCOS). The COCOS study was a case-control study with prospective inclusion of 125 patients with first ever ischemic stroke. Controls were population based, age-and sex-matched and not related to the patients. All participants were of the Caucasian race. Blood samples were taken one week after the ischemic stroke and repeated after three months in about half of the patients. Genotype and phenotype of several genetically determined coagulation disorders were determined. Because of the repeated measurements, we were able to compare protein levels in the acute phase and the convalescent phase.

Chapter 4 concerns the association in the COCOS population between ischemic stroke and the presence of a prothrombotic genetically determined coagulation disorder. Chapter 4.1 provides a review of the literature concerning the Factor V Leiden mutation and the G20210A prothrombin gene variant as risk factors for ischemic stroke combined with the results of these coagulation disorders within the COCOS study. Based on our own results and those in the literature, an association between one of these mutations and the occurrence of ischemic stroke is highly unlikely. Consequently, there is no indication for routinely performing these coagulation studies in ischemic stroke patients. The influence of both mutations in subgroups, such as patients with patent foramen ovale of patients of Mediterranean origin, is not clear yet and needs further investigation. Elevated protrombin levels, however, were associated with an increased risk of ischemic stroke. This finding is comparable to our results in young stroke patients described in chapter 2.1. Chapter 4.2 describes the role of the -148C/T fibrinogen gene polymorphism and fibrinogen level in ischemic stroke. The polymorphism was not associated with an increased stroke risk. Fibrinogen levels were significantly higher in patients compared

with controls one week after the stroke, but this difference had mostly disappeared after three months. High fibrinogen levels most likely represent an acute phase response. In Chapter 4.3 the association between G79A protein Z gene polymorphism and protein Z levels is studied. Again, the studied polymorphism was not associated with the occurrence of ischemic stroke. Protein Z levels, however, were inversely related to ischemic stroke. The relative risk of ischemic stroke associated with protein Z levels in the lowest quartile was 3.4 (95% CI: 1.3-7.3) during the convalescent phase. Therefore, a causative role for protein Z in the occurrence of ischemic stroke seems likely.

Chapter 5 focuses on the association between fibrinolysis related genetically determined coagulation disorders in the COCOS population. In chapter 5.1 and chapter 5.2 the association between 4G/5G PAI-1 gene polymorphism and PAI-1 levels and the annexin A5 Kozak sequence (-1C/T) polymorphism and ischemic stroke is studied. Neither the polymorphisms nor the PAI-1 level were associated with the occurrence of ischemic stroke. Chapter 5.3 addresses three recently identified polymorphisms of the TAFI gene, TAFI antigen levels and functional TAFI levels in relation to ischemic stroke. None of the TAFI gene polymorphisms were associated with a higher stroke risk. However, an increase of functional TAFI levels, which results in a decreased fibrinolysis, was associated with an increased risk of ischemic stroke.

In Chapter 6 all genetically determined coagulation disorders of the COCOS study are combined to assess whether the risk of ischemic stroke changes with the presence of more than one coagulation disorder in comparison with an isolated mutation. The number of genetic mutations was not associated with the occurrence of ischemic stroke and we found no evidence for a synergistic effect of certain combination of coagulation disorders.

In chapter 7, our findings are discussed and reviewed in a broader context. Furthermore, this chapter contains methodological issues and describes suggestions for further research.

SAMENVATTING

Het herseninfarct is de belangrijkste oorzaak van invaliditeit en de derde doodsoorzaak in de westerse wereld. Bekende cardiovasculaire risicofactoren, zoals roken, hypertensie en diabetes, zijn niet geclusterd en hun afzonderlijke invloed is vaak relatief klein. Het identificeren van nieuwe risicofactoren voor het optreden van een herseninfarct is van belang om patiënten met een hoog (recidief) risico op te sporen. Genetisch bepaalde stollingstoornissen zijn een belangrijke risicofactor voor het optreden van veneuze trombose. Hun rol in het ontstaan van arteriële trombose, zoals het herseninfarct, is minder duidelijk.

In dit proefschrift beschrijf ik de resultaten betreffende de rol van genetisch bepaalde stollingsstoornissen bij het optreden van een herseninfarct. De relatie tussen het herseninfarct en verschillende mutaties en polymorfismen in de stollingsgenen en hun fenotypische expressies wordt bestudeerd met als mogelijk doel een van de onderzochte stollingsstoornissen als risicofactor voor het herseninfarct te identificeren. Hiervoor werden 2 case-control studies onder patiënten met een recent doorgemaakt herseninfarct verricht.

In hoofdstuk 2 beschrijf ik de resultaten van een retrospectieve case-control studie onder 49 jonge patiënten met een doorgemaakt herseninfarct en 87 gezonde controles. Hoofdstuk 2.1 bestudeert de associatie tussen de G20210A protrombine gen variant, het protrombine level en het herseninfarct. Hoewel de protrombine gen variant hooguit een zwakke risicofactor was, bleek een verhoogd protrombine level, dat frequenter was dan de mutatie, sterker gerelateerd aan het optreden van het herseninfarct. Hoofdstuk 2.2 laat het verband zien tussen het herseninfarct en het PLA1/A2 gen polymorfisme in dezelfde studie populatie. Het polymorfisme was niet geassocieerd met een verhoogd risico op het optreden van een herseninfarct.

Hoofdstuk 3 bevat de rationale, achtergrond en design van de "COntrolled study of genetically determined COagulation disorders in ischemic Stroke" (COCOS). De COCOS studie is een case-control studie met prospectieve inclusie van 125 patienten met een eerste doorgemaakt herseninfarct. Controles zijn afkomstig uit de normale bevolking, zijn gematched voor leeftijd en geslacht en niet verwand aan de patienten. Alle deelnemers zijn van het caucasische ras. De bloedmonsters werden afgenomen 1 week na het herseninfarct en herhaald na 3 maanden bij ongeveer de helft van de patiënten. Het genotype en het fenotype van verschillende genetisch bepaalde stollingsstoornissen werden bepaald. Door de bloedafname te herhalen, was het mogelijk de eiwit concentraties in de acute fase en de herstelfase te vergelijken.

In Hoofdstuk 4 beschrijf ik de associatie in de COCOS populatie tussen het optreden van een herseninfarct in aanwezigheid van een meer protrombotisch genetisch bepaalde stollingsstoornis. Hoofdstuk 4.1 toont de resultaten van een review van de literatuur betreffende de Factor V Leiden mutatie en de G20210A protrombine gen variant als risicofactor voor het herseninfarct, gevolgd door de resultaten van deze mutaties binnen de COCOS studie. Een associatie tussen een van deze mutaties en het optreden van een herseninfarct is op grond van onze resultaten en die in de literatuur erg onwaarschijnlijk. Daarom is er geen indicatie voor het routinematig uitvoeren van dit stollingsonderzoek bij patiënten met een eerste herseninfarct. De invloed van beide mutaties in subgroepen van patiënten, zoals patiënten met een persisterend foramen ovale of patiënten van mediterrane afkomst, is nog niet goed duidelijk en dient verder onderzocht te worden. Verhoogde protrombine levels, daarentegen, waren geassocieerd met een verhoogd risico of een herseninfarct. Deze bevinding is vergelijkbaar met onze resultaten in jonge patiënten met een herseninfarct beschreven in hoofdstuk 2.1. Hoofdstuk 4.2 beschrijft

de rol van het -148C/T fibrinogeen gen polymorfisme en fibrinogeen level in het optreden van een herseninfarct. Het polymorfisme was niet geassocieerd met een verhoogd risico op een herseninfarct. Fibrinogeen levels waren significant hoger in patiënten dan in controles 1 week na het herseninfarct, maar 3 maanden later was dit verschil grotendeels verdwenen. Hoge fibrinogeen levels zijn meest waarschijnlijk het gevolg van een acute fase reactie. In hoofdstuk 4.3 wordt de associatie tussen het G79A proteïne Z gen polymorfisme en proteïne Z levels bestudeerd. Het polymorfisme was opnieuw niet geassocieerd met het optreden van een herseninfarct. Proteine Z levels, echter, waren omgekeerd evenredig geassocieerd met het herseninfarct. Gedurende de convalescente fase was het relatieve risico op het optreden van een herseninfarct geassocieerd met proteine Z levels in het laagste quartiel 3.4 (95% CI: 1.3-7.3). Daarom lijkt een causale rol voor proteine Z in het ontstaan van het herseninfarct waarschijnlijk.

Hoofdstuk 5 betreft de associatie tussen meer fibrinolyse gerelateerde genetisch bepaalde stollingsstoornissen binnen de COCOS populatie en het optreden van een herseninfarct. In hoofdstuk 5.1 en hoofdstuk 5.2 bestudeerden wij de associatie tussen het 4G/5G PAI-1 gen polymorfisme en PAI-1 levels en het annexin A5 Kozak sequence (-1C/T) polymorfisme en het herseninfarct. Noch de polymorfismen noch het PAI-1 level waren geassocieerd met het optreden van een herseninfarct. Hoofdstuk 5.3 betreft drie recent geïdentificeerde polymorfismen van het TAFI gen, TAFI antigeen levels en functionele TAFI levels in relatie tot het herseninfarct. Geen van de TAFI gen polymorfismen was geassocieerd met een hoger risico op een herseninfarct. Een toename van functionele TAFI levels, welke resulteren in een afgenomen fibrinolyse, is daarentegen wel geassocieerd met een verhoogd risico op een herseninfarct.

In hoofdstuk 6 worden alle genetisch bepaalde stollingsstoornissen binnen COCOS gecombineerd om te zien of het risico op een herseninfarct verandert in aanwezigheid van meer dan één stollingsstoornis in vergelijking met 1 geïsoleerde mutatie. Het aantal stollingsstoornissen was niet geassocieerd met het optreden van het herseninfarct en we vonden geen aanwijzingen voor de aanwezigheid van een synergistisch effect van bepaalde combinaties van stollingsstoornissen.

In hoofdstuk 7 worden onze bevindingen besproken en in een bredere context geplaatst. Tevens komen in dit hoofdstuk de methodologische aspecten aan de orde en doe ik suggesties voor verder onderzoek.

DANKWOORD

De afgelopen jaren hebben velen geholpen bij de tot standkoming van dit proefschrift. Een aantal wil ik met naam noemen.

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Lieve Eline, hoe dubbel dit ook klinkt, maar zonder jou had ik niet zo hard kunnen werken. Mama is nu echt klaar met typen. Lieverds, ik hou van jullie. We zijn weer met z'n drietjes!

CURRICULUM VITAE

De schrijfster van dit proefschrift werd in 1972 geboren in Swalmen. Na een middelbare schoolopleiding Gymnasium B, verliet zij het mooie Limburgse land om Geneeskunde te gaan studeren aan de Erasmus Universiteit in Rotterdam. In 1995 werd het doctoraal examen met goed gevolg afgelegd en 2 jaar later het arts-examen. Aansluitend begon zij haar medische carrière als AGNIO Neurologie op de afdeling neurologie van het Erasmus MC te Rotterdam. Daar startte zij in augustus 1998 met haar opleiding tot neuroloog (opleiders: Prof. dr. F.G.M. van der Meche, Prof. dr. P.J. Koudstaal, Prof. dr. P.A.E. Sillevis Smitt). Gedurende deze opleiding kreeg zij door middel van een AGIKO constructie ruimte voor het verrichten van klinisch wetenschappelijk onderzoek. Dit onderzoek, dat een samenwerkingsverband met de afdeling hematologie van het Erasmus MC betrof, was de basis voor dit proefschrift. Momenteel is zij nog steeds werkzaam als AGIO ter voltooiing van haar opleiding tot neuroloog. Ze is getrouwd en heeft een prachtige dochter van 2 jaar.

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