Haemostasis and cardiovascular disease

Anske van der Bom

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Voor mijn ouders

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JG van der Bom, Bots ML, Haverkate F, Grobbee DE, Kluft C. Fibrinolytic capacity and risk of myocardial infarction. *Fibrinolysis* 1996;10(Suppl 2):9-12.

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1 Introduction

The term 'coronary thrombosis' was derived from clinical and pathological observations on myocardial infarction made at least 80 years ago[1] and perhaps even earlier.[2,3] It was probably not until the 1930s that clinically manifest ischaemic heart disease in the form of myocardial infarction became a familiar condition rather than the unusual occurrence or even the rarity it had hitherto been. When the growing epidemic of clinical ischaemic heart disease prompted an increasing and concerted research programme after the Second World War, interest largely centred on the lipid nature of the atheromatous plaque and the contributions dietary fat intake and blood cholesterol levels make to it.

Thrombotic Occlusion

Two developments in the early and mid 1970s signalled a renewed interest in the thrombotic component of ischaemic heart disease. One was the first results of randomized controlled trials of aspirin in the secondary prevention of myocardial infarction.[4] The other development was a debate, mainly between pathologists, as to whether thrombosis is a cause or a consequence of myocardial infarction. It was not until 1980 that the point was settled when angiographic studies showed the high frequency of thrombotic occlusion during myocardial infarction.[5] In the past decade developments in thrombolytic treatment of evolving transmural myocardial infarction, improved image quality on angiography, and the introduction of angioscopy have contributed to finally establish the significance of thrombosis in the pathogenesis of myocardial infarction, sudden death, unstable angina pectoris and cerebrovascular disease.

Atherosclerosis

In the past century there have been two major hypotheses for the pathogenesis of atherosclerosis: the 'encrustation' theory and the 'lipid' theory. The encrustation theory of Rokitansky[2], later modified by Duguid and Robertson[6,7,8], suggests that intimal thickening results from fibrin deposition, with subsequent organisation by fibroblasts and secondary lipid accumulation. The lipid hypothesis of Virchow[9] depends on the inference that lipid in the arterial wall represents a transudation of blood lipid, which subsequently forms complexes with acid mucopolysaccharides. Ross integrated these two hypotheses into the now common view on atherosclerosis, the response-to-injury hypothesis.[10] According to this theory the earliest lesion of atherosclerosis is the fatty streak. It is considered an inflammatory response to changes in the arterial wall. These changes may somehow be associated with the various risk factors for atherosclerosis. Intimal smooth muscle proliferation and 'platelet' derived growth factor play a major role in the development of advanced lesions of atherosclerosis, the fibrous plaque.

It is increasingly apparent that fibrin is involved in the initiation and development of atherosclerosis.[11,12] Fibrin appears to be intervening at virtually all stages of lesion development in atherosclerosis. Fibrin and microthrombus deposition on normal intima is associated with endothelial disruption and intimal edema[13,14,15], and edema is a primary characteristic of early proliferative lesions.[16,17,18] Fibrin strands on or in the intima promote smooth muscle cell migration and proliferation, and contribute to the growth of plaques.[19] Fibrin also provides a continuing source of fibrin degradation products, that have mitogenic activity which may sustain smooth muscle cell proliferation in growing plaques[17,20], and act as chemoattractants for blood leucocytes. Accumulation of the lipid core in fibrous plaques may be influenced by fibrin which appears to bind lipoprotein(a) with high affinity, thereby immobilizing its lipid moiety within the lesion[10].

Hypercoagulable State

Traditionally, the thrombotic event was considered as the endpoint of the atherosclerotic process, a local reaction on damaged endothelium. In the last two decades, however, the hypothesis that haemostasis is involved in thrombotic processes has gained renewed interest. Hypercoagulability and impaired fibrinolytic function have been suggested to

predispose to arterial thrombosis by promoting the formation of occlusive thrombi on fissured atherosclerotic plaques. The finding that the levels of some haemostatic factors were different in subjects who have or will develop clinical cardiovascular disease as compared to subjects without cardiovascular disease, led to the concept of hypercoagulable state.[21,22,23,24,25] It has been suggested that increased levels of haemostatic factors directly increase the risk for myocardial infarction.

The observation that acute myocardial infarction and sudden cardiac death are more frequent in winter and in the morning indicates that the onset of these cardiovascular events is not random, and provides a clue to hte underlying mechanism.[26,27] It appears that an atherosclerotic plaque is exposed to systemic physiologic processes that could increase the likelihood of plaque rupture and thrombosis. Diurnal and seasonal variations in haemostasis may play a role in the temporal variation in the incidence of cardiovascular events.

Research Question

This thesis is concerned with examining the role of the haemostatic system in arterial disease.

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2 Temporal changes

2.1 Seasonal variation of fibrinogen

In most western countries the number of deaths rises considerably in winter.[1] In the Netherlands the average excess number of deaths each winter is approximately 2.5% of the yearly number of deaths.[2] Winter excess morbidity and mortality are for a great part due to cardiovascular and respiratory disease. For the Netherlands 66% of the excess winter mortality is due to cardiovascular disease mortality. It is unlikely that the seasonal variation in cardiovascular disease mortality is due to a seasonal variation in degree of atherosclerosis. Thrombotic tendency, on the other hand, may very well be influenced by the seasons. The currently, best identified marker for increased thrombotic tendency is fibrinogen. Fibrinogen has been suggested to show a cyclic seasonal variation. A seasonal variation of fibrinogen has been found in subjects aged 65 and older in two studies in the United Kingdom.[3,4] In men and women aged 45 to 64 living in the USA, little evidence for seasonal variation of fibrinogen was found.[5] Some have suggested that the seasonal variation of fibrinogen is due to temperature changes[4,6], and others proposed that it is merely due to winter infections.[3] In the present study we set out to assess whether fibrinogen shows a seasonal pattern in subjects aged 55 years and older in the Netherlands and whether it differs with age. Furthermore, we examined the role of outdoor temperature in this variation.

Methods

Population

The Rotterdam Study is a population based study of 7,983 subjects aged 55 years and over. Between March 1990 and July 1993 all 10,275 subjects aged 55 years and older, living in Ommoord, a suburb of Rotterdam, The Netherlands, were invited to participate.

The overall response rate was 78%. The aim of the Rotterdam Study is to investigate the incidence of and risk factors for chronic disabling diseases. Its rationale and design have been described elsewhere.[7] The study has been approved by the Medical Ethics Committee of the Erasmus University and written informed consent was obtained from all participants.

The present study population consisted of all 2,511 subjects that visited the research centre between the beginning of the study in March 1990 and January 1992. Blood samples were available from 2,414 of them. We excluded all 89 subjects that used anticoagulant drugs, since our method for measuring fibrinogen, a prothrombin time derived method, is less valid in case of an increased prothrombin time. Finally, the study population consisted of 2,325 men and women.

Measurements

Subjects were all visited at home. Information on current health status, medical history, drug use, and smoking behaviour was obtained by a computerized questionnaire, which included the Dutch version of the Rose cardiovascular questionnaire.[8] The home interview was followed by two visits at the research centre. During those visits several cardiovascular risk indicators were determined. Height and weight were measured, and body mass index was calculated (in kilograms per meter squared). Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer. The average of two measurements obtained at one occasion was used. Blood sampling and storage have been described previously.[9] In short, blood samples were drawn after minimal stasis. Platelet poor plasma was snap frozen in liquid nitrogen and stored at -80°C until determination. Fibrinogen was measured in all subjects that visited the research centre before January 1992. Fibrinogen levels were derived from the clotting curve of the prothrombin time assay using Thromborel S as reagent on an Automated Coagulation Laboratory (ACL 300, Instrumentation Laboratory). This method is in good correlation with the frequently used method as described by Von Clauss.[10,11] In our laboratory the coefficient of variation was 5 to 6 percent. The actual measurements of fibrinogen were performed between January 1991 and May 1992, the storage time of plasma varied from 3 to 10 months. Serum total cholesterol and high density lipoprotein (HDL) cholesterol were determined with an automated enzymatic procedure.[12] Information on average daily outdoor temperatures was obtained from The Netherlands Royal Meteorologic Institute and measured at the Rotterdam Airport Zestienhoven, located nearby the research centre.

Statistical analysis

Associations of fibrinogen and its potential determinants were evaluated with linear regression analysis. Seasonal variation was modelled assuming that the outcome variable follows a sinusoidal curve with a period of one year (Figure 2.1.1). The curve can be described mathematically in terms of three parameters, *a* the annual mean, and the seasonal difference, which is a function of *b* and *c*. The null hypothesis of no seasonal variation corresponds to both *b* and *c* being zero and can be tested by a Wald test.[13] The expected value for the outcome variable on day t of the year is given by:

$a + b^*\sin(2\pi(t-1)/365) + c^*\cos(2\pi(t-1)/365)$

To evaluate whether temperature played a role in the seasonal variation of fibrinogen the association between fibrinogen and temperature was adjusted for seasonal variation by entering temperature, first as a linear and second as a quadratic term, in the regression function.

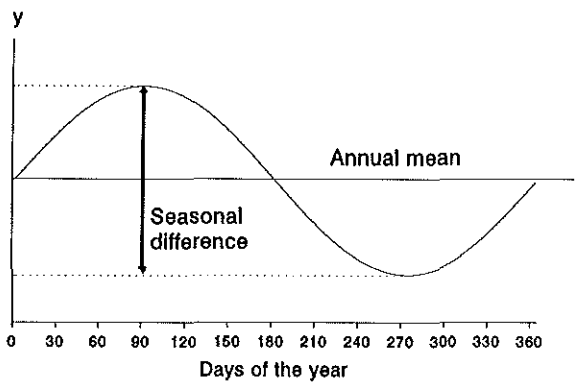

Figure 2.1.1 Seasonal curve for a hypothetical outcome variable.

Results

Mean fibrinogen levels were 0.12 g/l (95 % confidence interval (CI) 0.06,0.17) lower in men than in women; means were 2.71 g/l (SD 0.74) and 2.83 g/l (0.66) for men and women respectively. An increase in one decade of age was associated with an increase in fibrinogen of 0.20 g/l (CI 0.17,0.23) in men and of 0.18 g/l (0.14,0.22) in women. The following associations were adjusted for age, gender and season. In smokers the mean fibrinogen level was 0.29 g/l (CI 0.23,0.35) higher than in non-smokers. Fibrinogen was associated with body mass index (BMI); an increase in BMI of one kg/m^2 was associated with an increase in fibrinogen of 0.013 g/l (CI 0.006,0.020). An increase in systolic blood

pressure of 10 mmHg was associated with an increase in fibrinogen of 0.014 g/l (CI 0.001,0.026). An increase in neutrophil count of 10^9 /l was associated with an increase in fibrinogen of 0.05 g/l (CI 0.04,0.06). A decrease in serum HDL cholesterol of one mmol/l was associated with an increase in fibrinogen of 0.22 g/l (CI 0.14,0.30). Fibrinogen was not associated with plasma total cholesterol or diastolic blood pressure. Table 2.2.1 shows the distribution of age and gender over the year and the seasonal variation of other cardiovascular disease risk factors. It shows that body mass index tended to be slightly higher in winter. Neutrophil count was lowest in March/April.

In univariate regression analysis an increase in outside temperature of 10°C was associated with a decrease in fibrinogen of 0.09 g/l (CI 0.04,0.13). After adjustment for season the association was attenuated severely (0.02 g/l (CI -0.06,0.10) per 10°C).

Figure 2.1.2 shows mean fibrinogen values for periods of two months for 1990 and 1991 separately, together with the fitted seasonal curve. The seasonal difference for fibrinogen was 0.34 g/l (CI 0.29, 0.39). Men showed a more marked seasonal variation than women and the seasonal variation was more pronounced in subjects aged over 75 compared to those aged 75 and younger (Table 2.1.2). Additional adjustment for body mass index, systolic and diastolic blood pressure, and total and HDL cholesterol did not materially change the results. Adjustment for temperature (both linearly and as a quadratic term) did not change the results; the seasonal variation of fibrinogen was 0.31 g/l (CI 0.24,0.37).

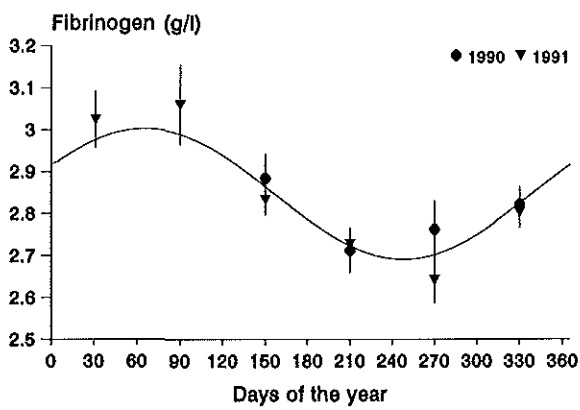

Figure 2.1.2. Seasonal variation of plasma fibrinogen.

Table 2.1.1. Seasonal variation of cardiovascular disease risk factors measured between March 1990 and December 1991. (When appropriate, results are adjusted for age, gender and current smoking.)

	January/ February	March/ April	May/ June	July/ August	September/ October	November/ December	p*
Number	84	172	439	495	565	570	
Female (%)	68	73	65	59	63	68	0.15
Age (yrs)	69	79	71	68	68	73	<0.001
Body mass index (kg/m^2)	27.3	26.2	26.6	26.1	26.4	26.7	0.02
Systolic blood pressure (mmHg)	137	135	139	138	137	137	0.17
Diastolic blood pressure (mmHg)	69	71	72	72	71	71	0.11
Total cholesterol (mmol/l)	6.7	6.4	6.5	6.7	6.9	6.6	<0.001
HDL cholesterol (mmol/l)	1.37	1.30	1.33	1.33	1.38	1.36	0.08
Erythrocyte sedimentation rate mm/1st hr	11	12	13	12	13	13	0.88
Neutrophil count ($*10^9/l$)	6.6	6.3	7.0	6.9	7.1	6.9	0.008
Current smoking (%)	14	19	22	26	27	19	0.10
Fibrinogen (g/l)	2.96	3.01	2.85	2.69	2.71	2.80	<0.01

Values are proportions or means.

* p value for F test.

Table 2.1.2 Seasonal difference of fibrinogen.

	Seasonal difference (g/l)	95% Confidence interval
Crude difference	0.47	0.42,0.51
Adjusted difference*	0.34	0.29,0.39
Men*	0.47	0.38,0.56
Women*	0.27	0.21,0.33
Subjects ≤ 75 years*	0.29	0.24,0.35
Subjects >75 years*	0.43	0.34,0.52
Adjusted for temperature*	0.31	0.24,0.37

* adjusted for age, gender and smoking.

Discussion

The findings in our study showed a seasonal variation of plasma fibrinogen in men and women aged 55 years and over, which was independent of changes in outside temperature. The seasonal difference in fibrinogen concentration in this study (0.31 g/l) was higher than that reported by Woodhouse and coworkers[3] (0.13 g/l) and lower than that observed by Stout and Crawford[4] (0.71 g/l). Differences in the study populations and environmental situations may explain these differences. The seasonal difference was higher in men. The effect of season on the plasma fibrinogen concentrations was stronger in the subjects aged over 75 years, compared to those aged 55 to 75 years. This would agree with the finding that the seasonal effect on mortality is higher in older than in relatively younger people.[14] This may also explain why in a study of subjects aged 45 to 64 little evidence for seasonal variation was found.[5]

We studied fibrinogen levels in an unselected population of 2,325 men and women that visited us during a period of more than one and a half year. The curves of the two years were similar, this makes assay drift as a possible explanation unlikely. The measurements were not performed in one series of subjects followed over the year, but in subsequent subjects. The advantage of this cross-sectional design is that all measurements are independent of each other.

Care is needed in trying to explain the seasonal variation of fibrinogen in terms of

other seasonal variables such as temperature. Any two variables that vary seasonally will be correlated simply because they have similar time dependencies. A better approach is to look at the association between the two variables after adjustment for seasonal effect, i.e. all other parameters that vary with the seasons except temperature. Following that analysis we saw that temperature and fibrinogen were not associated. These results suggest that the apparent association between fibrinogen and temperature is confounded by an other factor that varies with the seasons. The nature of this determinant of fibrinogen with a seasonal pattern is unknown. Although smoking, diet, obesity and chronic infections[15] have been related to raised plasma values, the determinants of fibrinogen are poorly understood. In our study both smoking and obesity could not be identified as explanatory for the seasonal variation of fibrinogen. Chronic infections such as Helicobacter Pylori and Chlamydia Pneumoniae have seasonal patterns that may explain the seasonal variation of fibrinogen.[16] Crawford et al found no association between the rise in fibrinogen in winter and infections.[17] We had no specific measure for chronic infection, but using a crude measure, neutrophil count, we found no evidence for a role of infection in the seasonal pattern of fibrinogen.

The range of daily mean outside temperatures in the present study is typically representative for a mild sea climate. The variance observed over the year is presumably enough to give a valid estimate of the association between temperature and fibrinogen in that range. However it must be emphasized that the findings may not be extrapolated to temperatures outside the range we studied. Extremely high or low temperatures may have different effects on plasma fibrinogen concentration.

Previous studies have consistently shown that an increase in fibrinogen concentration is associated with an increased risk of cardiovascular disease.[18,19,20] In the Northwick Park Heart Study an increase in fibrinogen of 0.6 g/l was associated with an increase in risk of coronary events of 60%.[21] A rise in fibrinogen of 0.3 g/l, as was seen in our study population, may explain an increase in cardiovascular disease events of 30%.

In conclusion, in men and women fibrinogen is higher in winter than in summer. This is more pronounced in the elderly. Although low outside temperature appeared to be associated with increased levels of fibrinogen, the seasonal variation of fibrinogen could not be attributed to differences in temperature. The seasonal variation of fibrinogen may explain the increased mortality in winter.

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2.2 Circadian variation of plasminogen activator inhibitor

The incidence of acute myocardial infarction follows a circadian rhythm with a peak in the morning and a trough in the evening. Muller et al[1] found a threefold increase in the frequency of myocardial infarction at 9 AM compared to 11 PM. A similar diurnal variation in the time of onset has been found for a number of other disorders associated with arterial thrombosis, including sudden cardiac death, angina at rest, and stroke.[2,3]

A possible explanation for the circadian variation of arterial thrombosis could be alterations in blood components that depress the rate of thrombus removal. Lysis of fibrin clots is initiated by tissue-type plasminogen activator (t-PA). The concentration of t-PA activity is regulated by plasmin activator inhibitor type 1 (PAI-1). T-PA and PAI-1 concentrations show a circadian pattern with a peak in the morning.[4] Some individuals appear to show a trivial circadian rhythm, while others show up to a tenfold variation in PAI-1 over 24 h.[5] At present it is not yet established whether these differences in PAI-1 throughout the day confer an increased risk of cardiovascular disease. Nore are underlying mechanisms elucidated. A recently detected common single base pair polymorphism in the promoter region of the PAI-1 gene, -675 (4G/5G), has been associated with increased plasma PAI-1 concentrations.[6,7] We examined whether the circadian variation of PAI-1 antigen differs for the different genotypes for the 4G/5G polymorphism in the PAI-1 gene.

Methods

Population

A cross-sectional study was performed in subjects selected from the Rotterdam Study. The Rotterdam Study is a prospective population based study of 7983 subjects. The rationale and design of the study have been described elsewhere.[8] In short, between March 1990 and July 1993 all subjects aged 55 years and older, living in Ommoord, a suburb of Rotterdam, the Netherlands, were invited to participate. The overall response rate was 78%. The study has been approved by the Medical Ethics Committee of the

Erasmus University and written informed consent was obtained from all participants.

Subjects for the present study (n=450) were selected for a case-control study examining the association of the 4G5G polymorphism and PAI-1 antigen with the risk for myocardial infarction. 150 subjects had a history of myocardial infarction, based on the presence of an infarction pattern on the ECG, using the diagnostic classification system of the Modular ECG Analysis System,[9,10] independent of a history of chest pain and 300 subjects without a history of cardiovascular disease (i.e. no history of myocardial infarction, angina pectoris, stroke, a normal ECG and no peripheral arterial disease (ankle/arm index > 0.9)) were drawn from the same five year age strata, where the cases of myocardial infarction were from. We excluded subjects using anticoagulant drugs.

Clinical investigations

All subjects were first visited at their home. Information on current health status, medical history (including myocardial infarction and stroke), drug use, and smoking behaviour was obtained by a computerized questionnaire, which included the Dutch version of the Rose cardiovascular questionnaire.[11] The home interview was followed by two visits at the research centre, between 8 AM and 4 PM. Patients were not asked to fast or to refrain from smoking. During those visits several cardiovascular risk indicators were determined. Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer. The average of two measurements obtained at one occasion was used. Systolic blood pressure at the ankles (posterior tibial artery) was measured in supine position with an adult size regular cuff just above the malleoli, and a 8 MHz Doppler transducer.[12] The ankle/arm index is the ratio of the systolic blood pressure at the ankle to the arm systolic pressure. Peripheral arterial disease was defined as a right or left ankle/arm index lower than or equal to 0.9.

Laboratory investigations

Blood sampling and storage have been described elsewhere.[13] Blood samples were collected using CTAD vacutainers (0.11M citrate, 15 mM theophylline, 3.7 mM adenosine and 0.198 mM dipyridamole; Diatube H, Becton and Dickinson, Mylan, Cedex, France)[14]. Serum total and HDL cholesterol were determined with an automated enzymatic procedure. PAI-1 antigen concentration was measured in CTAD plasma using the Innotest PAI-1 (Innogenetics, Zwijndrecht, Belgium).[15] Genomic DNA was isolated from blood cells and PAI-1 genotype was determined by allele specific PCR amplification.[16]

Statistical analysis

After exclusion of those with missing data on either the 4G/5G polymorphism or PAI-1 antigen concentration, the study population consisted of 397 subjects. The distribution of the plasma concentration of PAI-1 antigen was positively skewed, therefore statistical analysis was carried out on logtransformations, but the mean values and 95% confidence intervals (CI) presented, have been transformed back into the original scale. All analyses have been performed first for subjects with a history of myocardial infarction and subjects without a history of cardiovascular disease separately. As there were no major differences we combined all subjects in one group. Presented data are adjusted for age, gender and caseness (i.e. presence or absence of a history of myocardial infarction). For each genotype mean PAI-1 antigen concentration for subjects who visited the research centre before 12 AM were compared to that in those that visited the centre after 12 AM.

Results

To check proper selection from the source population, we compared the observed distribution of genotypes in the group without cardiovascular disease to that expected for a population according to a Hardy Weinberg equilibrium. The frequency of the 4G allele was 53 percent and that of the 5G allele was 47 percent and the distribution of genotypes was in Hardy-Weinberg equilibrium; 77 subjects (29%) were found to be homozygous for the 4G allele, whereas 129 (49%) were heterozygous, and 59 (22%) were homozygous for the 5G allele (Table 2.2.1).

Possession of the 4G allele was associated with increased concentration of PAI-1 antigen. In subjects homozygous for the 4G allele PAI-1 antigen concentration was 63 ng/ml (CI 57,70), in those heterozygous it was 52 ng/ml (47,56), and in those homozygous for the 5G allele it was 47 ng/ml (41,53). Table 2.2.2 presents plasma PAI-1 concentrations for the genotypes in the groups with and without cardiovascular disease.

Table 2.2.1. Distribution of genotypes among case and control subjects (4G4G = homozygous for the 4G allele; 4G5G = heterozygous; 5G5G = homozygous for the 5G allele).

	5G5G	4G5G	4G4G
Subjects with a history of MI	25 (19%)	56 (42%)	51 (39%)
Control subjects	59 (22%)	129 (49%)	77 (29%)

Table 2.2.2 Plasma concentration of PAI-1 antigen (ng/ml) in the different genotypes among subjects with and without a history of myocardial infarctions (5G5G=homozygous for the 5G allele; 4G5G=heterozygous; 4G4G= homozygous for the 4G allele).

	5G5G	4G5G	4G4G
Controls	46 (39,54)	51 (46,57)	61 (53,70)
History of MI	48 (38,59)	54 (47,63)	65 (56,75)

Values are means and (95% confidence intervals) adjusted for age and gender.

Table 2.2.3 Plasma concentration of PAI-1 antigen in samples collected before and after noon for the different genotypes (Adjusted for age, sex and caseness; 4G4G=homozygous for the 4G allele; 4G5G=heterozygous; 5G5G= homozygous for the 5G allele).

	5G5G	4G5G	4G4G
Subjects visiting before noon (n)	47	87	82
PAI-1 (95% CI) ng/ml	57 (49,67)	65 (58,73)	77 (69,87)
Subjects visiting after noon (n)	37	96	45
PAI-1 (95% CI) ng/ml	36 (30,43)	42 (38,47)	43 (37,51)

To investigate the diurnal variation of PAI-1 antigen and whether this differs between genotypes, we compared PAI-1 concentrations measured in blood samples collected in the morning with samples collected in the afternoon in the genotypes. Overall the mean concentration of PAI-1 antigen was higher before noon, age and sex adjusted means were 68 (95% CI 63,183) and 41 (38,44), respectively. In subjects with the 4G4G genotype the morning/afternoon difference was more pronounced than in the other genotypes (Table 2.2.3).

Discussion

We assessed whether the diurnal variation of PAI-1 concentration is different for the genotypes of the 4G/5G polymorphism at the PAI-1 gene locus. Homozygosity for the 4G

allele was associated with increased PAI-1 concentrations mainly in the morning. All three genotypes showed higher PAI-1 antigen concentrations in the morning, but the increase tended to be more pronounced in subjects homozygous for the 4G allele.

A unique feature of our study is that we assessed the diurnal rhythm of PAI-1 antigen not by repeated measurements in a number of individuals, but in plasma from different subjects, at different hours of the day. Thus, the concentrations are independent of each other and provide an independent estimate of PAI-1 concentration at these hours of the day. Besides, our subjects were not asked to fast nor to refrain from smoking, therefore PAI-1 antigen concentrations in the present study are a reflection of PAI-1 antigen concentrations during 'real' life circumstances.

We are cautious to draw firm conclusions from the differences in morning increase in PAI-1 concentrations between the genotypes, because, most likely due to the limited number of subjects and the relatively large variation of PAI-1 antigen, they were not significantly different from one another. Nevertheless, our findings shed an interesting light on the current knowledge about PAI-1 as a risk factor for myocardial infarction. Increased PAI-1 concentrations have been associated with an increased risk of myocardial infarction in some reports[17], but not in others.[18,19] Experimental studies have suggested that the 4G/5G polymorphism in the PAI-1 gene is of functional importance in regulating the expression of the PAI-1 gene.[6] This is confirmed by the finding that the 4G allele is associated with increased plasma concentrations of PAI-1. Additionally, homozygosity for the 4G allele is associated with an increased risk for myocardial infarction,[7] which appeared to be largely confined to smokers.[20] The present study suggests that the morning increase in PAI-1 may be more pronounced in 4G4G subjects. The morning peak corresponds to the diurnal variation of the time of onset of myocardial infarction.[1] It is temptative to suggest that the 4G4G genotype plays a role in the excess number of cases of myocardial infarction in the morning. This would confirm a role of PAI-1 as a risk factor for myocardial infarction.

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3 Atherosclerosis

3.1 Hypercoagulable state

Recognition of the central role of thrombosis in the pathogenesis of myocardial infarction, cerebrovascular, and peripheral arterial disease has prompted growing interest in the association of haemostatic variables with arterial disease. The observation that the mean concentration of some haemostatic factors was higher or the concentration of coagulation inhibitors was lower, in subjects who have or develop clinical coronary heart disease led to the concept of a hypercoagulable state in this disease.

There are two main types of measurement in the investigation of hypercoagulable state.[1] The first type of measurement is that of concentrations of haemostatic factors. In these studies, the most promising factors identified are fibrinogen, factor VIII, von Willebrand factor and tissue-type plasminogen activator.[2,3,4,5] The second type of measurement is that of activation products of the haemostatic system.[6] A variety of peptides is released with the activation of haemostatic factors in vivo. They can be termed 'activation markers', because they indicate that activation of the haemostatic system has taken place. For example, the degradation product of prothrombin, fragment 1 + 2 (F1 + 2), arises from conversion of prothrombin to thrombin. These peptides have a short half life in the circulation, from 10 to 90 minutes, so that their presence in the circulation means that ongoing haemostatic activity is taking place. Furthermore, concentrations of thrombin/antithrombin (TAT) complexes express the amount of thrombin present in the circulation and are therefore also considered to be indicative for hypercoagulability.[7] Similarly for fibrinolysis, if plasmin is generated in the circulation, the potent inhibitor of plasmin, α2-antiplasmin immediately inhibits its biological activity, and measurement of plasmin/antiplamsmin (PAP) complexes has been shown to be an adequate approach to assess the amount of plasmin formed in vivo.[8]

All individuals have a measurable amount of activation markers in their circulation, confirming the original Astrup hypothesis that low grade coagulation is a continuous process of wear and tear in normal individuals.[9] Studies of activation markers F 1+2, TAT and PAP in patients with cardiovascular disease revealed inconsistent findings.[10,11,12,13,14,15]

The present cross-sectional study was undertaken to investigate hypercoagulable states, as measured by markers of thrombin ánd plasmin generation, together with a marker for degradation of fibrin, D-Dimer in subjects with different manifestations of arterial disease. We assessed concentrations of F1+2, TAT, PAP complexes and D-Dimer in patients with coronary, cerebrovascular and peripheral arterial disease.

Methods

Population

A case-control study was performed among subjects from the Rotterdam Study, a prospective study of a cohort of 7,983 men and women aged 55 years and over. The aim of the Rotterdam Study is to investigate the incidence of and risk factors for chronic disabling diseases. Its rationale and design have been described previously.[16] Between March 1990 and July 1993 all 10,275 men and women aged 55 years and over, living in Ommoord, a district of Rotterdam, the Netherlands, were invited to participate. The study consisted of an initial home interview by a trained research assistant and a series of medical examinations made during two visits at the research centre. The study has been approved by the Medical Ethics Committee of the Erasmus University and written informed consent was obtained from all participants. The overall response rate was 78%.

Selection of cases and controls

A sample of 150 subjects with a history of myocardial infarction were selected based on the presence of an infarction on the ECG, using the diagnostic classification system of the Modular ECG Analysis System [17,18], independent of a history of chest pain. A sample of 75 subjects with a definite or probable history of transient ischaemic attack (TIA) (n=75) were drawn based on a positive medical history of TIA obtained in two stages. First four screening questions on presence of temporal visual, locomotor, sensory or speech disturbances were asked and when affirmative, a detailed history of the symptoms was obtained, in addition to general information, such as date of event, onset, duration, and associated symptoms. Based on this information, symptoms were classified

of 75 subjects with a history of stroke (n = 75) were selected based on a medical history of stroke based on the question 'Did you ever suffer from stroke, diagnosed by a physician'. Cerebrovascular disease was defined as either a stroke or a transient ischaemic attack or a combination of both. Subjects with peripheral arterial disease (n = 150) were selected based on an ankle to arm systolic blood pressure ratio lower than 0.9.[20] Control subjects (n = 300) were drawn from those without arterial disease, i.e. with no history of myocardial infarction, stroke or transient ischemic attack, a normal ECG and with an ankle to arm systolic pressure ratio above 0.9; control subjects were drawn from the same 5-year-age strata where the cases of myocardial infarction were from. Subjects using anticoagulant drugs at the time of examination were excluded.

Measurements

Subjects were all visited at home. Information on current health status, medical history, drug use, and smoking behaviour was obtained by a computerized questionnaire. The home interview was followed by two visits at the research centre. Height and weight were measured, and body mass index was calculated (in kilograms per meter squared). Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer, and the average of two measurements obtained at one occasion was used. Blood pressure at the ankles (posterior tibial artery) was measured in supine position just above the malleoli, with an 8 MHz Doppler transducer. The ankle arm pressure ratio is the ratio of the systolic blood pressure at the ankle to the arm systolic pressure.[20] ECG was recorded and coded using the Means computerized coding system.[17,18] Blood sampling procedures and storage have been described elsewhere.[21] Blood was collected in tubes containing 0.129 mol/l sodium citrate. All tubes were stored on ice before and after blood sampling. Platelet poor plasma was obtained by a two stage centrifugation: firstly at 1,600 g, at 4°C for 10 minutes and after a careful transfer of the plasma midlayer a second centrifugation was performed at 10,000 g, at 4°C for 10 minutes. Plasma was immediately frozen in liquid nitrogen and stored at -80°C. Mean storage time was two years. Concentrations of thrombin/antithrombin, plasmin/antiplasmin, prothrombin fragment 1+2 and D-Dimer were determined with commercially available test from Behringwerke Diagnostica (Marburg, Germany). Serum total and high density lipoprotein (HDL) cholesterol were determined with an automated enzymatic procedure.

Statistical analysis

The distributions of prothrombin fragment 1+2, thrombin/antithrombin, plasmin/antiplasmin complexes and D-Dimer were all positively skewed. All statistical

analyses were carried out on logtransformed data, but the mean values and 95% confidence intervals presented have been transformed back into the original scales of measurement.

All data were available for 127 cases with a history of myocardial infarction, 66 cases with a history of transient ischaemic attack, 67 cases with a history of stroke, 131 subjects with peripheral arterial disease and for 263 control subjects. Nine subjects were selected for two case groups: they were cases with a history of stroke as well as cases with a history of transient ischemic attack, therefore the group of subjects with a history of cerebrovascular disease consisted of 124 subjects.

Multiple linear regression analysis was used to assess the association between prothrombin fragment 1+2, thrombin/antithrombin, plasmin/antiplasmin and D-Dimer and the cardiovascular disease risk factors, adjusted for the other cardiovascular disease risk factors and for the presence of coronary, cerebrovascular and peripheral arterial disease (three dummy variables in the model).

F1+2, TAT and PAP were categorized according to quartiles of their distributions. The associations of F1+2, TAT and PAP with D-Dimer were examined with analysis of variance, after adjustment for age, sex and the presence of coronary, cerebrovascular or peripheral arterial disease. The p-value for the F-test is presented.

Analysis of variance was used to calculate means and 95 % confidence intervals adjusted for age and sex for the cases with a history of myocardial infarction, cerebrovascular or peripheral arterial disease and for controls.

After inspection of the distribution of D-Dimer, cutpoints between 0 μg/l and the maximum value of D-Dimer with steps of 20 μg/l were chosen. Logistic regression was used to further examine the association of categories of D-Dimer with cerebrovascular disease and myocardial infarction.

Among patients with a history of myocardial infarction, stroke and transient ischemic attack there were several patients with peripheral arterial disease. To obtain a more precise estimate of the association between peripheral arterial disease and the determinants under study, we analyzed the associations between prothrombin fragment 1+2, thrombin/antithrombin, plasmin/antiplasmin and D-Dimer with peripheral arterial disease in all subjects. All 645 subjects were classified according to their ankle to arm blood pressure ratio in categories from lower than 0.8 to above 1.2, with steps of 0.2. For these categories the mean concentration of the determinants (prothrombin fragment 1+2, thrombin/antithrombin, plasmin/antiplasmin and D-Dimer) were compared. To make sure that these findings were not different for subjects with singular peripheral arterial disease as compared to those with peripheral and coronary or cerebrovascular disease, we performed separate analyses for these groups. No material differences were

found (data not presented).

To examine potential effect modification of the association between atherosclerosis and D-Dimer concentrations by F1 + 2, TAT and PAP, we stratified the study population in quartiles of F1 + 2, TAT and PAP. Three dummy variables were made to indicate the second, third and highest quartiles in comparison to the lowest quartile. Statistical testing for effect modification was performed by adding the products of each of the three dummy variables and ankle to arm pressure index to the regression model.

Results

General characteristics of the study population are presented in Table 3.1.1.

Table 3.1.1. Characteristics of the study population.

	Myocardial infarction	Cerebrovascular disease	Peripheral arterial disease	Controls
Age (yrs)	73 (10)	73 (9)	71 (10)	72 (9)
Female (%)	47	56	63	62
BMI (kg/m^2)	27 (4)	27 (4)	26 (3)	26 (4)
Systolic BP (mmHg)	144 (24)	145 (21)	150 (26)	140 (21)
Diastolic BP(mmHg)	75 (14)	74 (12)	74 (12)	74 (11)
Total chol.(mmol/l)	6.3 (1.1)	6.5 (1.2)	6.9 (1.4)	6.5 (1.1)
HDL chol. (mmol/l)	1.2 (0.4)	1.3 (0.4)	1.3 (0.3)	1.4 (0.4)
Current smoking (%)	28	26	27	13

Values are percentages or means (standard deviation).
BMI = body mass index
BP = blood pressure

Associations with cardiovascular disease risk factors

Associations between the established cardiovascular risk factors and the concentrations of F1 + 2, TAT, PAP and D-Dimer are shown in Table 3.1.2. With increasing age all four parameters increased. In women F1 + 2 was higher than in men; 2.1 nmol/l (95% CI 2.0,2.2) and 1.8 nmol/l (1.7,1.9), respectively. TAT, and PAP complexes, and D-Dimer were not significantly different for men and women. The concentration of PAP

complexes was inversely associated with body mass index and positively with HDL cholesterol. Smokers had higher concentrations of D-Dimer compared to non-smokers, 54 μg/l (47,62) and 42 μg/l (39,45) respectively. F1 + 2, TAT, and PAP complexes were not increased in smokers.

Increased concentrations of D-Dimer were observed in subjects with high concentrations of prothrombin fragment 1 + 2, thrombin/antithrombin, and plasmin/antiplasmin. The associations of D-Dimer with F1 + 2, TAT complexes and PAP complexes are presented in Table 3.1.3.

Associations with arterial disease

There was no notable difference in the concentrations of F1 + 2, TAT, or PAP complex between the subjects with a history of either myocardial infarction or cerebrovascular or peripheral arterial disease and control subjects. Only D-Dimer concentrations were increased in cases (Table 3.1.4). The ratio of the concentration of TAT complexes, as well as that of F1 + 2, to the concentration of PAP complexes was not associated with the disease status.

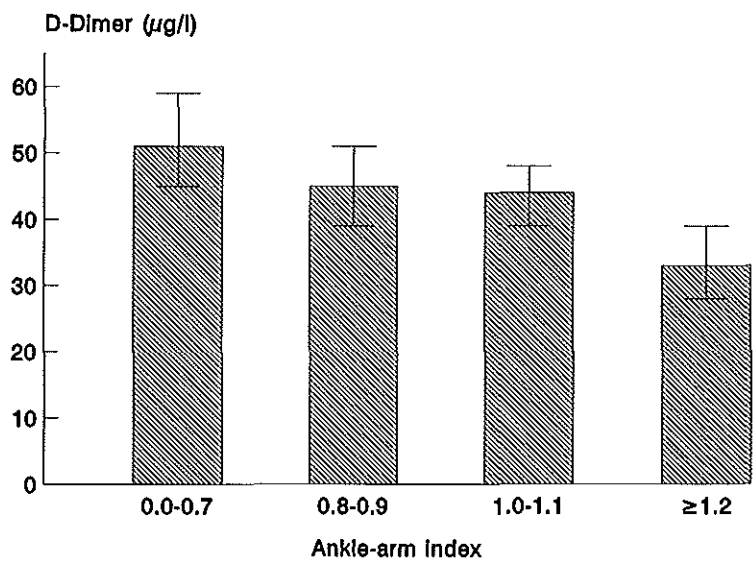

Figure 3.1.1. Concentrations of D-Dimer (μg/l) and 95% confidence intervals, according to degree of peripheral arterial disease as assessed by the ankle to arm blood pressure ratio (ankle-arm index).

There was a gradual increase in the prevalence of both myocardial infarction and cerebrovascular disease for increasing concentrations of D-Dimer. The prevalences and odds ratios of cases of myocardial infarction and cerebrovascular disease as compared to control subjects are presented in Table 3.1.5. Concentrations of D-Dimer increased gradually with decreasing ankle to arm blood pressure ratio (Figure 3.1.1).

Stratification in four subgroups according to quartiles of the distribution of F1+2 showed that the association between atherosclerosis and D-Dimer differed between these subgroups. In the lowest quartile, D-Dimer was hardly increased with increased atherosclerosis, whereas in the highest quartile D-Dimer increased markedly with increasing atherosclerosis (Figure 3.1.2). Testing for effect modification revealed a borderline significant p-value, p=0.06. For strata of quartiles of TAT and PAP similar increasing trends were observed, but not statistically significant.

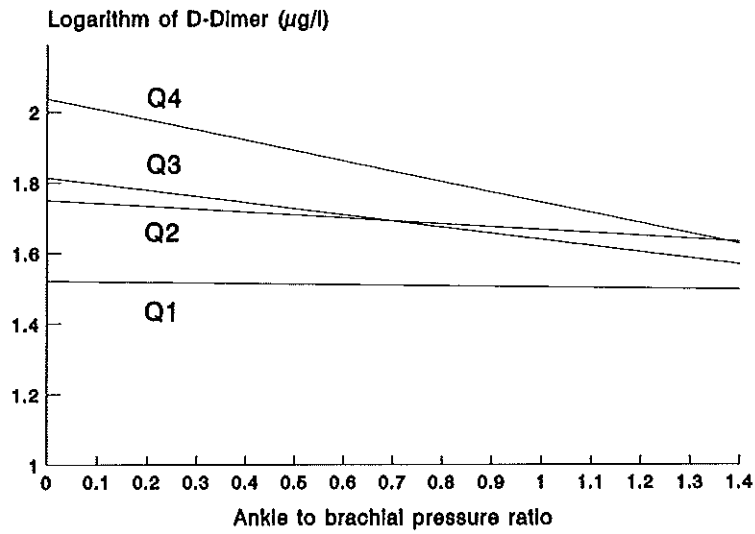

Figure 3.1.2. Regression lines for subgroups consisting of subjects in the 4 quartiles (Q1, Q2, Q3, and Q4) of the distribution of F1+2 (Cutpoints 1.54,1.90,2.39 µg/l), adjusted for age and sex.

Table 3.1.2. Multiple regression coefficients of plasma F1+2, TAT, PAP and D-Dimer with established cardiovascular risk factors.*

Independent variables	F1+2 (nmol/l)	TAT (μg/l)	PAP (μg/l)	D-Dimer (μg/l)
Age (per 10 year)	0.04 (0.01;p<0.01)	0.06 (0.02;p<0.01)	0.066 (0.012;p<0.01)	0.26 (0.02;p<0.01)
Women vs men	0.07 (0.02;p<0.01)	/	/	/
Body mass index (per kg/m^2)	/	/	-0.017 (0.003;p<0.01)	/
Systolic BP (per 10 mmHg)	/	0.014 (0.009;p=0.09)	/	-0.025 (0.008;p<0.01)
Diastolic BP (per 10 mmHg)	/	/	/	0.045 (0.015;p<0.01)
Total chol. (per mmol/l)	/	/	/	/
HDL chol. (per mmol/l)	/	/	0.127 (0.028;p<0.01)	-0.072 (0.040;p=0.08)
Current vs non smokers	/	/	/	0.126 (0.041;p<0.01)

* Values are coefficients (standard error, p-value) of multiple linear regression with the logarithm of F1+2, TAT, PAP or D-Dimer as dependent variables and the cardiovascular risk factors as independent variables, adjusted for presence of cardiovascular disease.
/ values are not shown if p-value ≥ 0.10
BP=blood pressure

Table 3.1.3. Concentration of D-Dimer (mean and 95% confidence interval) according to quartiles (Q1, Q2, Q3, and the upper quartile Q4) of F1+2, TAT and PAP.*

	Q1	Q2	Q3	Q4	p+
Prothrombin fragment 1+2	29 (27,35)	46 (41,51)	46 (41,51)	59 (53,66)	<0.001
Thrombin/antithrombin	28 (25,31)	40 (36,44)	65 (58,73)	55 (50,62)	<0.001
Plasmin/antiplasmin	36 (32,41)	42 (37,48)	55 (48,61)	46 (40,52)	<0.001

* Cutpoints for quartiles were 1.54, 1.90 and 2.39 nmol/l for F1+2, 2.21,3.05, and 4.62 μg/l for TAT and 324,489, and 799 μg/l for PAP.
\+ p value for F-test
Values are adjusted for age, sex and case-control status.

Table 3.1.4. Mean (95% CI) of prothrombin fragment 1+2, thrombin/antithrombin, plasmin/antiplasmin complexes and D-Dimer for subjects with a history of myocardial infarction, cerebrovascular disease (stroke/TIA), peripheral arterial disease and for controls.

	F1+2 (nmol/l)	TAT (μg/l)	PAP (nmol/l)	D-Dimer (μg/l)
Controls	2.0 (1.9,2.1)	3.9 (3.5,4.3)	525 (492,562)	40 (36,44)
Myocardial infarction	1.9 (1.8,2.1)	4.2 (3.6,4.8)	497 (450,548)	53 (47,61)
Cerebrovascular disease	2.0 (1.9,2.1)	3.4 (2.9,4.0)	475 (430,524)	51 (45,58)
Peripheral arterial disease	1.9 (1.7,2.0)	3.2 (2.8,3.7)	503 (457,555)	44 (38,50)

Values are adjusted for age and sex

Table 3.1.5. Odds ratios (95% CI) of cerebrovascular disease (stroke and TIA) and myocardial infarction by concentrations of D-Dimer.

	≤19 μg/l	20-39 μg/l	40-59 μg/l	60-79 μg/l	>80 μg/l
Cerebrovascular disease					
Crude risk	1.0*	1.8 (0.9,3.4)	1.7 (0.8,3.7)	1.9 (0.7,4.7)	2.4 (1.2,4.8)
Adjusted for age and sex	1.0*	1.9 (0.9,3.8)	1.9 (0.8,4.3)	2.1 (0.8,5.7)	2.6 (1.2,5.8)
and for smoking	1.0*	1.8 (0.9,3.7)	1.8 (0.8,4.3)	2.0 (0.7,5.7)	2.4 (1.1,5.4)
Myocardial infarction					
Crude risk	1.0*	0.92 (0.5,1.8)	1.3 (0.6,2.7)	1.6 (0.7,3.8)	1.8 (0.9,3.4)
Adjusted for age and sex	1.0*	1.04 (0.5,2.0)	1.4 (0.6,3.1)	2.0 (0.8,5.0)	2.1 (1.0,4.4)
and for smoking	1.0*	0.97 (0.5,1.9)	1.2 (0.5,2.7)	1.8 (0.7,4.7)	1.8 (0.8,3.8)

* Reference risk

Discussion

In the present study, ongoing haemostatic activity as measured by the activation markers for thrombin and plasmin production, F1+2, TAT and PAP, was not increased in subjects with coronary, cerebral or peripheral arterial disease. There were, however, signs of increased formation and lysis of fibrin in all three manifestations of arterial disease, as reflected by increased concentrations of a degradation product of fibrin, D-Dimer. Additionally, concentrations of F1+2, TAT and PAP appeared to modify the strength of the association between D-Dimer and arterial disease.

Although several cross-sectional studies have examined the associations between activation products of the haemostatic system and arterial disease, subgroups with different concentrations of activation markers have not been studied before. In agreement with our findings, increased concentrations D-Dimer have consistently been reported in subjects with arterial disease.[11,12,13,22] Findings from studies on the association of F1+2, TAT and PAP with arterial disease were less consistent. No association between F1+2 and peripheral arterial disease was found in 929 male patients admitted to a coronary rehabilitation unit.[15] Similar results were obtained by De Buyzere et al.[23] F1+2 and TAT concentrations were found to be increased in 225 patients with angina pectoris, but no graded association with the severity of atherosclerosis was observed.[10] Peripheral arterial disease was associated with increased TAT complexes in a smaller group of 40 consecutive patients with symptomatic peripheral arterial disease.[13] The difference between our study and the studies that reported a positive association between arterial disease and increased concentration F1+2 and TAT is that the patients in the former studies were symptomatic at the moment of participation in the studies. Measurements in our study were performed in non-symptomatic phases.

Subjects with arterial disease are supposed to have a hypercoagulable state. Based on F1+2, TAT and PAP concentrations, we found no evidence of increased prothrombin activation or plasmin activity. Nevertheless, the observed increase in D-Dimer concentrations verifies the presence of locally increased activities of both thrombin and plasmin. Additionally, stratification of the present study population suggests that, although F1+2, TAT and PAP concentrations were not increased in subjects with arterial disease, the latter mentioned concentrations did play a role in the relation between haemostasis and arterial disease. At different concentrations of F1+2, TAT and PAP, the association between D-Dimer concentrations and arterial disease differed. At relatively low F1+2 concentration, and possibly also at low concentrations of TAT and PAP the association between arterial disease and D-Dimer was absent or very weak. Thus, subjects with a low F1+2 concentration appeared not to increase their D-Dimer in the presence of peripheral arterial disease. This suggests that subjects with arterial disease who have, due to other factors, relatively low F1+2 concentrations, form and lyse less fibrin than those with arterial disease and higher F1+2 concentrations.

The finding that F1+2, TAT and PAP concentrations appear to modify the association between arterial disease and concentrations of D-Dimer makes it interesting to study determinants

of F1+2, TAT and PAP concentrations. Information about determinants of F1+2, TAT and PAP is scarce. In agreement with others we found that all three increased with age.[24,25] Additionally, we observed that some of the cardiovascular disease risk factors are associated with F1+2, TAT and PAP.

Whether the concentrations of F1+2, TAT and PAP and their effect on the associations between D-Dimer and arterial disease have implications for future risk of cardiovascular disease is unknown. It is conceivable that subjects with higher PAP concentrations are better able to lyse fibrin and have, therefore, a lower risk for thromboembolic events. A longitudinal study may answer this. Some studies have suggested a positive association between increased D-Dimer concentrations and future risk of ischemic events,[12,11,26] but we know of no longitudinal study on the association of F1+2, TAT and PAP with future cardiovascular disease risk.

In conclusion, plasma concentrations of F1+2, TAT and PAP are not altered in arterial disease. In patients with arterial disease, increased formation and lysis of fibrin take place. Concentrations of F1+2, and possibly also concentrations of TAT and PAP, partly determine the strength of the association between arterial disease and the amount of fibrin that is formed and lysed.

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3.2 Fibrinolytic activity

The importance of thrombus formation in triggering coronary heart disease events is now widely accepted.[1] Progress has been made in understanding the haemostatic mechanisms which may contribute to arterial thrombus formation. Several markers of fibrinolysis were found to be associated with the risk for myocardial infarction. Tissue type plasminogen activator (t-PA) antigen has been positively associated with risk for myocardial infarction.[2,3] Although less consistently, increased plasminogen activator inhibitor-1 (PAI-1) and decreased t-PA activity were associated with an increased risk for myocardial infarction, too.[3,4,5,6,7] D-dimer is a breakdown product formed when plasmin acts on cross-linked fibrin and is, therefore, considered as a marker of plasmin activity.[8] Relatively little data exist on the association between D-dimer and the risk for myocardial infarction, but increased D-Dimer concentrations appear to predict thrombotic events.[9,10]

The association between atherosclerosis and fibrinolytic parameters has also been studied. Normal[11,12,13], as well as elevated concentrations of PAI-1 antigen and activity, t-PA antigen and D-Dimer have been found in patients with coronary artery disease.[14,15,16,17,18] D-Dimer was related to few of the cardiovascular disease risk factors[19] and the finding of a positive associations of D-dimer and atherosclerosis appears to be consistent. The plasma elevations of PAI-1 and t-PA, however, may have been due to the presence of other risk factors in patients, such as a higher body mass index, higher triglycerides, lower HDL cholesterol or diabetes. Concentrations of fibrinolytic parameters are strongly associated with these risk factors.[16,20] In the Edinburgh Artery Study t-PA antigen and PAI-1 activity were increased in patients with peripheral arterial disease, but after controlling for a number of cardiovascular risk factors there was no remaining independent relationship between either t-PA or PAI-1 concentrations and disease.[21,22] Similarly, in the Atherosclerosis Risk in Communities (ARIC) Study, adjustment for cardiovascular disease risk factors removed the positive association of PAI-1 antigen and t-PA antigen with carotid atherosclerosis.[23,11]

As increased D-dimer concentrations were observed jointly with increased PAI-1 activities, it was suggested that increased D-dimer concentrations may be associated with impaired fibrinolytic activity.[24] However, a direct association between PAI-1 antigen or PAI-1 activity and D-dimer could not be demonstrated.[23,]

We, therefore, evaluated whether PAI-1 and t-PA are independently associated with atherosclerosis. Furthermore, we explored the role of fibrinolysis in the atherosclerotic process, by studying the association between D-dimer and atherosclerosis in subjects with different concentrations of t-PA antigen, t-PA activity, and PAI-1 antigen.

Methods

Population

The study population consisted of subjects that participated in a cross-sectional case-control study initially set up to assess the association between fibrinolytic function and myocardial infarction, performed among participants of the Rotterdam Study. The Rotterdam Study is a prospective population based study of 7,983 subjects. Between March 1990 and July 1993 all subjects aged 55 years and older, living in Ommoord, a suburb of Rotterdam, the Netherlands, were invited to participate. The overall response rate was 78%. The study has been approved by the Medical Ethics Committee of the Erasmus University and written informed consent was obtained from all participants. The rationale and design of the Rotterdam Study have been described previously.[25] Cases (n = 150) were selected from the cohort, based on the presence of an infarction pattern on the ECG, using the diagnostic classification system of the Modular ECG Analysis System[26,27], irrespective of a history of chest pain. Control subjects (n = 300) were drawn from the same five-year-age strata where the cases of myocardial infarction were from and constituted a sample of study participants who had no history of cardiovascular disease, i.e. no history of myocardial infarction, angina pectoris, stroke, a normal ECG and no peripheral arterial disease (ankle to brachial pressure index > 0.9).[28] Subjects using anticoagulant drugs were excluded from the study population. Findings on the associations between fibrinolytic factors and risk for myocardial infarction have been described elsewhere.[29,30]

Measurements

All subjects were first visited at their home. Information on current health status, medical history (including myocardial infarction and stroke), drug use, and smoking behaviour was obtained by a computerized questionnaire, which included the Dutch version of the Rose cardiovascular questionnaire.[31] The home interview was followed by two visits at the research centre. During those visits several cardiovascular risk indicators were determined. Height and weight were measured, and body mass index was calculated (in kilograms per meter squared). Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer. The average of two measurements obtained at one occasion was used. Systolic blood pressure at the ankles (posterior tibial artery) was measured in supine position with an adult size regular cuff just above the malleoli, and a 8 MHz Doppler transducer.[32] The ankle to brachial pressure index (ABPI) is the ratio of the systolic blood pressure at the ankle to the systolic pressure of the arm. Peripheral arterial disease was defined as a right or left ankle to brachial pressure index lower than or equal to 0.9.[28] Blood sampling and storage have been described elsewhere.[33] In

short, a non-fasting blood sample was taken, applying minimal stasis, with a 21 gauge butterfly needle with tube. Collection of Stabilyte plasma was started from July 1992 onwards. Consequently there was no baseline Stabilyte plasma available for 56 subjects (32 control and 24 case subjects) that were selected for the present study and had visited the research centre before that date. For them we measured t-PA activity in Stabilyte plasma collected in the first follow-up examination of the Rotterdam Study. The mean time period between their first and second examinations was 2.5 years. T-PA activity was measured in Stabilyte® plasma using the Chromolize®t-PA bio-immunoassay (Biopool, Umeå, Sweden). T-PA antigen was measured in CTAD plasma using the Immulyse® t-PA enzyme-immunoassay (Biopool, Umeå, Sweden). PAI-1 antigen was determined in CTAD plasma using the Innotest PAI-1 (Innogenetics, Zwijndrecht, Belgium). D-Dimer was determined using the Enzygnost D-Dimer micro from Behringwerke Diagnostica (Marburg, Germany).

Statistical analysis

As the distributions of PAI-1 antigen and D-Dimer were positively skewed, statistical analyses were carried out after log-transformation. The mean values and 95% confidence intervals presented have been transformed back into the original scales of measurement. After verifying the linearity of the associations between the fibrinolytic parameters and their potential correlates, linear regression was used to describe the associations of the t-PA antigen and activity, PAI-1 antigen and D-Dimer concentrations with other cardiovascular disease risk factors and with ankle to brachial pressure index. R^2 of these regression equations was used to estimate the percentage of variation of the dependent variable due to variation in the independent variables. Direction and magnitude of the regression coefficients were not materially different for cases as compared to controls, therefore we present the analyses for the study population as a whole. Separate analyses were performed for t-PA activity in the blood samples collected at baseline and the other (n = 56) blood samples collected in the second phase of the study. No major differences were found (data not shown).

To examine potential effect modification on the association between atherosclerosis and D-Dimer by the fibrinolytic factors, we stratified the study population in 'normal' (n = 140), 'intermediate' (n = 96), and 'high' (n = 89) PAI-1 antigen concentrations, arbitrarily choosing cutpoints at 50 ng/ml and 75 ng/ml and performed linear regression with the logarithm of D-Dimer as dependent variable and ankle to brachial pressure index as independent variable. Similarly, analyses were performed after stratification for t-PA antigen by choosing 10 ng/ml and 15 ng/ml (n = 167, n = 103 and n = 55, respectively) as cutpoints and for t-PA activity by choosing 1.0 IU/ml and 1.5 IU/ml

(n=145, n=86, and n=94, respectively) as cutpoints. Statistical testing for effect modification was performed by adding the product of ankle to brachial pressure index and each of the two dummy variables that were created as indicators for the three categories of PAI-1 antigen, t-PA antigen and t-PA activity to the regression model.

Results

After exclusion of the subjects with missing data the study population consisted of 325 subjects. Distribution of fibrinolytic parameters for the whole population is presented in Table 3.2.1. There was no difference in the concentrations of t-PA antigen, t-PA activity, PAI-1 antigen or D-Dimer between men and women. Characteristics of the study population divided in subjects with peripheral atherosclerosis, i.e. an ankle to brachial pressure index below 0.9, subjects with an intermediate ankle to brachial pressure index, between 0.9 and 1.1 and subjects without peripheral atherosclerosis in either of the legs, i.e. a lowest ankle to brachial pressure index of 1.1 or above, are shown in Table 3.2.2. Most of the established cardiovascular disease risk factors showed the expected associations with peripheral atherosclerosis.

Table 3.2.1. Distribution of fibrinolytic parameters for the study population.

	Mean	SD	Smallest	Largest
PAI-1 antigen (ng/ml)	54*		11.2	611
T-PA antigen (ng/ml)	10.5	5.8	0.5	36.8
T-PA activity (IU/ml)	1.17	0.67	0.00	3.24
D-Dimer (μg/l)	43*		5	691

* Back transformation from logarithmic mean.

Association with cardiovascular disease risk factors

Associations of t-PA antigen, t-PA activity, PAI-1 antigen and D-Dimer with established cardiovascular risk factors and with one another are presented in Table 3.2.3. T-PA activity increased with age in men by 0.2 IU/ml per decade (SE 0.06, $p<0.01$) and in women with 0.1 IU/ml per decade (0.05, $p=0.01$). Thirty two percent of the variation in D-Dimer (R^2) was explained by age differences. D-Dimer was not notably associated with any of the other cardiovascular disease risk factors, nor with the other fibrinolytic parameters. Thirty percent of the variation in t-PA activity (R^2) could be attributed to variation in PAI-1. The magnitude of the association between t-PA activity and body mass

index decreased by 40 %, when adjusted for PAI-1 antigen concentration. The association of t-PA activity with HDL cholesterol was no longer present after adjustment for PAI-1. The variation in t-PA antigen was for 19 % explained by the variation in PAI-1 antigen. The variation in PAI-1 antigen was for 12 % explained by body mass index and for 7 % by HDL cholesterol.

Table 3.2.2. Baseline characteristics of the study population for subjects with atherosclerosis (ankle to brachial pressure index (ABPI) < 0.9), an intermediate group of subjects (ABPI 0.9 - 1.1) and subjects without atherosclerosis (ABPI ≥ 1.1).

	ABPI < 0.9	ABPI 0.9-1.1	ABPI ≥ 1.1	p*
Number	42	123	160	
Age (years)	78.4 (1.33)	72.7 (0.8)	70.5 (0.7)	<0.01
Women (%)	60	65	49	<0.01
Systolic BP (mmHg)	150 (3)	144 (2)	136 (2)	<0.01
Diastolic BP (mmHg)	79 (2)	74 (1)	73 (1)	0.03
Body mass index (kg/m^2)	26.6 (0.6)	26.6 (0.3)	26.2 (0.3)	0.37
Current smokers (%)	30	19	11	0.01
Total cholesterol (mmol/l)	6.5 (0.2)	6.4 (0.1)	6.4 (0.1)	0.91
HDL cholesterol (mmol/l)	1.25 (0.06)	1.35 (0.03)	1.39 (0.03)	0.11
Log (PAI-1 antigen (ng/ml))	1.73 (0.04)	1.72 (0.02)	1.74 (0.02)	0.73
T-PA antigen (ng/ml)	11.0 (0.84)	10.2 (0.47)	10.3 (0.42)	0.68
T-PA activity (IU/ml)	1.41 (0.11)	1.16 (0.06)	1.11 (0.05)	0.05
Log (D-Dimer (μg/l))	1.75 (0.05)	1.65 (0.03)	1.59 (0.03)	0.03

Values are means (standard error) or proportions, adjusted for age and sex.

* p value obtained with linear regression analysis.

Table 3.2.3. Association of t-PA antigen, t-PA activity, PAI-1 antigen and D-Dimer with cardiovascular risk factors.

	t-PA antigen (ng/ml)		t-PA activity (IU/ml)		PAI-1 antigen (ng/ml)		D-Dimer (μg/l)	
Independent variable	Coeff. (SE)	p	Coeff. (SE)	p	Coeff. (SE)	p*	Coefficient(se)	p*
Age (10 years)	0.04 (0.03)	0.25	0.17 (0.04)	<0.01	-4.9 (3.0)	0.28	0.26 (0.02)	<0.01
Women (yes)	-0.21 (0.59)	0.73	-0.04 (0.07)	0.59	9.04 (5.48)	0.94	6.46 (10.0)	0.52
Systolic BP (10 mmHg)	0.36 (0.14)	0.01	-0.01 (0.01)	0.88	1.2 (1.3)	0.01	-0.08 (0.23)	0.72
Diastolic BP (10 mmHg)	0.42 (0.24)	0.09	-0.03 (0.03)	0.33	2.5 (2.3)	0.29	0.53 (0.40)	0.24
Body mass index (kg/m^2)	0.38 (0.08)	<0.01	-0.05 (0.01)	<0.01	3.84 (0.71)	<0.01	-0.27 (1.19)	0.31
Current smoking (yes)	0.65 (0.75)	0.38	0.16 (0.09)	0.07	11.8 (6.9)	0.27	0.27 (13.3)	0.29
Total cholesterol (mmol/l)	0.48 (0.27)	0.07	-0.05 (0.03)	0.11	3.4 (2.5)	0.18	-1.55 (4.67)	0.94
HDL Cholesterol (mmol/l)	-1.77 (0.82)	0.03	0.35 (0.10)	<0.01	-35.9 (7.5)	<0.01	-22.5 (13.5)	0.16
T-PA ag (ng/ml)	1		-0.013 (0.006)	0.04	2.80 (0.46)	<0.01	1.03 (0.93)	0.05
T-PA c (IU/ml)	-0.9 (0.4)	0.04	1		-35.3 (3.63)	<0.01	-5.7 (7.32)	0.79
PAI-1 (ng/ml)	0.03 (0.01)	<0.01	-0.006 (0.001)	<0.01	1		0.02 (0.09)	0.59
D-Dimer (μg/l)	0.004 (0.003)	0.05	-0.000 (0.000)	0.79	0.009 (0.034)	0.35	1	

Coefficients (SE) and p values, from linear regression analysis with t-PA antigen, t-PA activity, PAI-1 antigen en D-dimer as dependent variables, adjusted for age and gender.

* p-value obtained from linear regression analysis with logarithm of PAI-1 or D-dimer as dependent variable.

BP = bllod pressure

Table 3.2.4. Association of t-PA antigen (t-PA ag), t-PA activity (t-PA c), PAI-1 antigen (PAI-1 ag) and D-Dimer with peripheralarterial disease as assessed by an increase in ankle to brachial pressure ratio.

	t-PA antigen (ng/ml)		t-PA activity (IU/ml)		PAI-1 antigen (ng/ml)		D-Dimer (μg/l)	
Independent variable	Coeff. (SE)	p	Coeff. (SE)	p	Coeff. (SE)	p*	Coeff.(SE)	p*
ABPI (unit)⊥	-2.9 (1.7)	0.09	-0.38 (0.20)	0.06	3.90 (15.6)	0.34	-68.4 (27.2)	<0.01
adjusted for t-PA ag	-		-0.46 (0.20)	0.02	12.6 (15.1)	0.53	-66.9 (27.3)	<0.01
adjusted for t-PA c	-3.5 (1.7)	0.04	-		-11.0 (14.3)	0.10	-72.1 (27.4)	<0.01
adjusted for PAI-1 ag	-2.4 (1.6)	0.13	-0.45 (0.17)	0.01	-		-68.4 (27.3)	<0.01

Regression coefficients with standard errors and p values adjusted for age and gender.

* p value from regression analyses with the logarithm of PAI-1 or D-dimer as a dependent variable.

⊥ ABPI = ankle to brachial pressure index; a decrease in ABPI represents an increase in atherosclerosis.

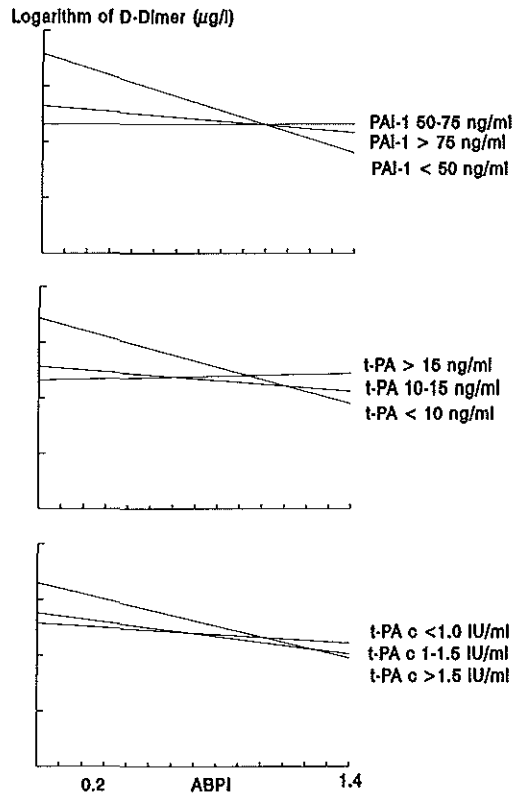

Figure 3.2.1. **Upper)** Regression lines representing the association between D-Dimer and ankle to brachial pressure index for subjects with normal, intermediate and high PAI-1 antigen concentrations. **Middle)** Regression lines representing the association between D-Dimer and ankle to brachial pressure index for subjects with normal, intermediate and high t-PA antigen concentrations. **Lower)** Regression lines representing the association between D-Dimer and ankle to brachial pressure index for subjects with normal, intermediate and high t-PA activity concentrations.

Association with atherosclerosis

The associations of t-PA antigen, t-PA activity, PAI-1 antigen and D-Dimer with atherosclerosis are shown in Table 3.2.4. Atherosclerosis (lower ankle to brachial pressure index) was associated with increased concentrations of t-PA antigen, t-PA activity and D-Dimer. PAI-1 antigen concentration was not related to the ankle to brachial pressure index. After adjustment for smoking, body mass index, systolic and diastolic blood pressure, and total and HDL cholesterol, the magnitude of the association

between t-PA antigen and ankle to brachial pressure index attenuated (regression coefficient 2.01 ng/ml (SE 1.78, p = 0.26) per unit decrease in ankle to brachial pressure index), whereas the association between t-PA activity and ankle to brachial pressure index did not materially change (0.37 IU/ml (SE 0.19, p = 0.06) per unit decrease in ankle to brachial pressure index). When the association between t-PA activity and peripheral atherosclerosis was adjusted for t-PA antigen, it became stronger (Table 3.2.4).

The study population was stratified in groups with 'normal', 'intermediate' and 'high' PAI-1 antigen concentration, by choosing 50 ng/ml and 75 ng/ml as cutpoints. Only in subjects with PAI-1 concentrations below 50 ng/ml, atherosclerosis was associated with increased D-Dimer concentrations (Figure 3.2.1 upper). Statistical testing for effect modification showed coefficients of interaction terms that were both significantly different from zero; p = 0.01 and p = 0.04. Stratification by t-PA antigen and t-PA activity showed a positive association between atherosclerosis and D-Dimer in subjects with 'normal' t-PA antigen and 'high' t-PA activity (Figure 3.2.1 middle, lower). The coefficients of interaction terms for t-PA antigen were (borderline) significant, p = 0.06 and p = 0.02, and those for t-PA activity were not significant, p = 0.72 and p = 0.17.

Discussion

In the present study, subjects with peripheral arterial disease had increased concentrations of D-Dimer, in particular when concentrations of PAI-1 and t-PA antigen were relatively low, and t-PA activity relatively high. Subjects with peripheral arterial disease did not have increased PAI-1 antigen concentrations. T-PA activity and, to a lesser extent, also t-PA antigen were elevated in subjects with arterial disease, independent of one another and of the established cardiovascular disease risk factors.

Conventional thinking about the role of coagulation and fibrinolysis in thrombosis implies that hypercoagulation and hypofibrinolysis promote the occurrence of cardiovascular disease. In apparent contrast to this view is our finding that increased concentrations of D-Dimer, a marker of fibrin production and plasmin activity, are associated with arterial disease, with its progression and with future cardiovascular events.[9,34] To explain this seeming discrepancy, increased D-Dimer has been suggested to represent increased fibrin 'turnover', with increased but inadequate fibrinolysis. The present study provides new information with respect to the association between fibrinolytic parameters and arterial disease. Categorization of our study population in subgroups of different PAI-1 concentrations showed that among subjects with arterial disease increased concentrations of D-Dimer were only found in those who had 'low' PAI-1 concentrations. This is in agreement with an earlier study among subjects with clinical arterial disease where the highest concentrations of D-Dimer were found in subjects with the lowest concentrations of PAI-1.[35] Because our study subjects with arterial disease and low PAI-1 concentrations had increased D-Dimer and those with higher PAI-1 antigen concentrations had normal D-Dimer concentrations, we propose

that high D-Dimer in subjects with arterial disease represents higher fibrinolytic activity.

Longitudinal studies suggest that increased D-Dimer is associated with an increased risk for myocardial infarction.[9,10,24] It is difficult to combine the presence of increased fibrinolytic activity, indicated by increased D-Dimer, with increased future risk for cardiovascular events. As arterial disease is associated with increased D-Dimer, one inherently finds that increased D-Dimer is associated with an increased risk for thromboembolic events. With the aid of subgroup analyses, we showed that among subjects with arterial disease increased D-dimer concentrations may indicate a higher fibrinolytic activity. A longitudinal study among subgroups of subjects with different concentrations of PAI-1 will offer the opportunity to differentiate those with normal from those with impaired fibrinolytic capacity.

Findings from experimental studies suggest that high PAI-1 concentrations promote fibrin deposition.[36,37] Furthermore, if fibrin deposits are not sufficiently removed, incorporation of fibrin into the vessel wall may cause progression of arterial disease.[38] Therefore, subjects with higher PAI-1 concentrations are expected to have more arterial disease. This is, however, not confirmed by our finding that subjects with atherosclerosis have normal PAI-1 concentrations. Additionally, we showed that among subjects with atherosclerosis those with relatively high PAI-1 antigen concentration appear to have a decreased ability to lyse fibrin deposits (lower D-dimer). This suggests that PAI-1 antigen concentration may influence the degree of lysis of fibrin without affecting the progression of atherosclerosis.

T-PA antigen and activity were increased in subjects with atherosclerosis. Whether these increased t-PA activities and t-PA antigen concentrations are cause or consequence of the atherosclerotic process can not be concluded from a cross-sectional study. However, with respect to the increased t-PA activities, we consider a causal link to atherothrombosis unlikely. Increased t-PA antigen concentrations have been suggested to be marker of endothelial injury.[2] Our findings are in good agreement with that view. Taken together, we tend to conclude that increased concentrations of t-PA antigen and activities in subjects with atherosclerosis are more likely to be consequences than causes of the atherosclerotic process.

In contrast to earlier findings with different methodology,[5,6] we observed increased t-PA activities in subjects with atherosclerosis. T-PA activities recorded in acidified plasma are 10 to 20 times higher than otherwise.[39] The observed positive association between atherosclerosis and t-PA activity may reflect a proper antithrombotic defence mechanism in response to the atherosclerotic process and supports the hypothesis that atherosclerosis is associated with increased fibrinolytic activity.

Increased t-PA antigen, the activator of plasminogen, has been shown to be a stronger and more consistent predictor for coronary events than PAI-1. It is commonly speculated that the positive association between t-PA antigen and the risk of coronary events is due to the close association of t-PA antigen with its inhibitor PAI-1.[39,40]

Nevertheless, we showed that only nineteen percent of the variation in t-PA antigen was due to the variation in PAI-1. PAI-1 en t-PA antigen were jointly associated with several risk factors responsible for the insulin resistance syndrome, such as body mass index, hypertension, and decreased HDL cholesterol. Involvement of the fibrinolytic system in this syndrome has been suggested repeatedly.[41,42,43] T-PA antigen, however, has characteristics distinct from those that it shares with PAI-1. T-PA antigen is, and PAI-1 antigen is not, increased in subjects with atherosclerosis.

For clinical practice our findings have the following implications. Low D-dimer concentrations might not necessarily indicate a lesser degree of atherosclerosis. Low D-dimer in combination with low PAI-1 is indeed associated with a better disease status, but if the PAI-1 concentration is high, D-dimer is no longer indicative of the presence or absence of arterial disease.

In conclusion, increased concentrations of D-dimer reflect ongoing lysis of fibrin in peripheral arterial disease. Although PAI-1 is not associated with peripheral arterial disease, its concentration influences an individuals ability to lyse fibrin and it may, therefore, blur the predictive value of D-dimer.

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3.3 Antithrombin

The increase in procoagulatory factors has been associated with cardiovascular disease.[1,2] Antithrombin is a potent inhibitor of coagulation. Familial deficiency of antithrombin increases the risk of venous thrombosis[3,4,5], and possibly of arterial thrombosis.[6,7] However, studies on the relation between antithrombin and arterial thrombosis have shown contrasting results. Both low[8,9] and high[10,11,12] activity of antithrombin have been associated with ischaemic heart disease, and stroke, whereas in subjects with an increased risk of cardiovascular disease (diabetes mellitus, post-menopausal women, hypercholesterolemic subjects, smokers) antithrombin activity was increased.[9,13,14,15,16] In a longitudinal study among healthy men, cardiac mortality was higher in both the low and high tertiles of the antithrombin distribution compared to the middle tertile.[17] In a longitudinal study of men and women with pre-existing coronary, cerebral, or peripheral arterial disease, occurrence of cardiovascular events tended to be reduced in the highest quintiles of the antithrombin distribution.[18]

We examined the nature of the association of antithrombin activity with atherosclerosis in order to explore some of the discrepant previous observations.

Methods

Population

The Rotterdam Study is a cohort study of subjects aged 55 years and over. Between March 1990 and July 1993 all residents of a suburb of Rotterdam were invited to participate in the study, which was approved by the Medical Ethics Committee of the Erasmus University.[19] Written informed consent was obtained from all participants. The response rate was 78%, and 7983 men and women participated in the study. The rationale of the study has been described elsewhere. In short, the Rotterdam Study aims to clarify the determinants of chronic disabling disease in the aging population. For cardiovascular disease, the study focuses on the contribution of thrombogenic factors to atherosclerotic disease and on the presence and progression of atherosclerosis and its determinants.

Measurements

Information on current health status, medical history (including myocardial infarction and stroke), drug use, and smoking was obtained with a computerized questionnaire, which included the Dutch version of the Rose questionnaire for angina pectoris and intermittent claudication.[20] Height and weight were measured, and body mass index (in kilograms per meter square) was calculated. Sitting blood pressure was measured in the right upper arm with a random zero sphygmomanometer, the average of two measurements was used. Systolic blood pressure at the ankles (posterior tibial artery) was measured in half lying (±45°) position with a regular cuff just above the malleoli, and a 8 MHz Doppler transducer.[21] The ankle/arm index (AAI) is the ratio of the ankle systolic blood pressure to the arm systolic blood pressure. Participants were categorized according to the lowest of the left or right ankle/arm index.

Blood handling has been described previously.[22] Antithrombin activity was determined by a chromogenic method as heparin cofactor (Coatest, Chromogenix). Factor VII activity was determined with Tromborel S (Hoechst-Behring, Germany) as reagent, a sensitive thromboplastin preparation from human placenta and factor VII deficient plasma. Fibrinogen was measured as derived fibrinogen of the prothrombin time assay using Tromborel S as reagent on an Automated Coagulation Laboratory (ACL 300, Instrumentation Laboratory, IJsselstein, The Netherlands).[23] Serum total cholesterol was determined with an automated enzymatic procedure.[24] High density lipoprotein (HDL) cholesterol concentration was derived similarly after precipitation using the phosphotungstate method[25], with a minor modification as described by Grove.[26]

Data analysis

The present study was based on information of the first 1656 subjects that visited the research centre. Subjects of whom measurment of ankle arm index was missing (n = 140) and those using anticoagulant drugs (n = 89) were excluded from the analysis. The number of people on whom final analyses was performed was 1427. The association of antithrombin with ankle/arm index and with each cardiovascular risk factor was evaluated with simple and multiple linear regression analysis. The quadratic association was evaluated using linear regression analysis of antithrombin on the ankle/arm index, combined with ankle/arm index squared. Analysis of covariance was used to estimate mean antithrombin activity in groups with increasing ankle/arm index. When appropriate, adjustments were made for age and gender.

Results

Baseline characteristics of the study population are presented in Table 3.3.1.

Antithrombin activity ranged from 49% to 161%, with a mean of 108 % (SD 16%). An antithrombin activity below 75% was found in 44 (2.5%) subjects. Women had a higher mean activity of antithrombin than men: 110% (SE 0.5) and 105% (SE 0.7), respectively ($p<0.01$). For men and women mean antithrombin activity decreased with age, 4% (SE 0.8, $p<0.01$) and 3% (SE 0.6, $p<0.01$) per decade, respectively (Figure 3.3.1).

Table 3.3.1 Selected baseline characteristics of the study population

	Men		Women	
	Mean	SD	Mean	SD
Number	517		910	
Age (years)	70	8.4	71	8.0
Body mass index (kg/m^2)	26	3.1	27	4.2
Current smoking (%)	30		19	
Systolic blood pressure (mmHg)	136.3	19.5	137.7	21.1
Diastolic blood pressure (mmHg)	72.2	10.6	71.2	10.9
Total cholesterol (mmol/l)	6.31	1.17	6.85	1.25
HDL cholesterol (mmol/l)	1.20	0.40	1.42	0.38
Fibrinogen (g/l)	2.76	0.76	2.90	0.70
Factor VIIc (%)	103	19	113	20
Ankle/arm index	1.10	0.22	1.06	0.21
Antithrombin (%)	105	16	110	17
History of MI (%)	16		5	
History of stroke (%)	6		4	

Values are percentages or means and standard deviations.

Mean antithrombin activity (adjusted for age) in men and women across groups of ankle/arm index are shown in Figure 3.3.2. In men the association between antithrombin and ankle/arm index was quadratic; regression coefficient for antithrombin (%) on AAI (per 0.1 increase) and AAI^2 were 3.0 (SE 1.5, $p=0.04$) and -1.7 (SE 0.7, $p=0.02$) respectively. In women a linear association between between antithrombin and ankle/arm index was observed: a decrease in ankle/arm index with 0.1 was associated with an increase of antithrombin of 0.7% (SE 0.3, $p=0.01$). When the square of AAI was added to the model, coefficients for antithrombin (%) on AAI (per 0.1 increase)

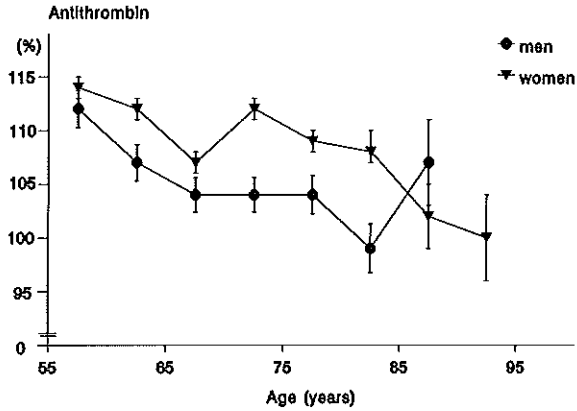

Figure 3.3.1 Antithrombin activity (%) (mean and SE) by age and gender (test for linear trend for men and women $p < 0.01$).

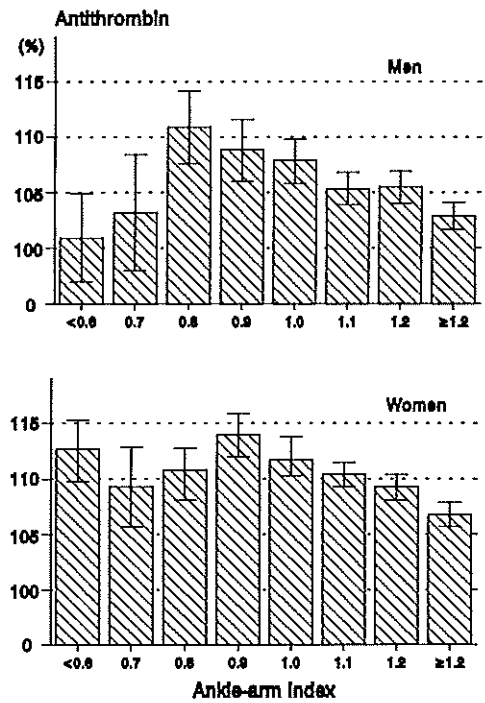

Figure 3.3.2 Antithrombin activity (%) (mean and SE, adjusted for age) by ankle/arm index for men and women.

and AAI[2] were 0.7 (SE 1.1, p=0.53) and -0.8 (SE 0.6, p=0.19) respectively.

In men and women antithrombin was linearly associated with serum total cholesterol, factor VIIc, fibrinogen and smoking (Table 3.3.2). Antithrombin was not related to HDL cholesterol, body mass index, systolic or diastolic blood pressure. Additional adjustment for smoking, body mass index, serum lipids, fibrinogen, and factor VIIc did not substantially change the association between antithrombin and AAI.

Subjects with a history of myocardial infarction (n=129) or stroke (n=67) and those free from symptomatic cardiovascular disease had similar antitrombin levels, 109% (SE 1.4), 112% (SE 2.0) and 108% (SE 0.4) respectively.

Table 3.3.2 Antithrombin in relation to smoking, total cholesterol, fibrinogen, and factor VIIc in men and women.

	Men		Women	
	Coefficient*	95% CI	Coefficient*	95% CI
Current smoking (yes)	2.5	-0.3, 5.4	1.9	-0.8, 4.6
Total cholesterol (per mmol/l)	1.9	0.8, 2.9	1.8	1.0, 2.7
Fibrinogen (per g/l)	3.5	1.8, 5.2	2.3	0.8, 3.9
Factor VIIc (per 10%)	1.4	0.7, 2.2	1.0	0.4, 1.5

* Coefficients of bivariate linear regression analysis, adjusted for age.

Discussion

We found a quadratic association between antithrombin and atherosclerosis in men. Antithrombin activity is higher in men with moderate peripheral arterial atherosclerosis, compared to those without. In men with more severe atherosclerosis antithrombin shows no further increase; if anything antithrombin is decreased. In women antithrombin increased with decreasing ankle/arm pressure index.

The present study was based on a large population based cohort of men and women aged 55 years and over. Response rates were high in the study, the subgroup on whom the present study was based consisted of the first subjects that visited the research centre and of whom data on the ankle/arm pressure index were available. Laboratory analysis were performed blinded.

The ankle/arm index is a reliable measure for presence and severity of atherosclerosis, and a low ankle/arm index is strongly related to atherosclerotic

abnormalities in other vascular beds.[27] Moreover, low ankle/arm index is a strong predictor of cardiovascular disease and all-cause mortality,[28,29] and shows an inverse dose-response relation with risk factors for cardiovascular disease.[30] The ankle/arm index provides a graded marker for arterial lesions, which enables to investigate non-linear associations.

We found no difference in antithrombin activity between those with a history of myocardial infarction or stroke and those without such a history. Earlier findings on the association between antithrombin and cardiovascular disease are conflicting: low[31] and high[32] antithrombin activity have been associated with its presence. To compare subjects with cardiovascular disease to subjects free from symptomatic disease may indeed give conflicting results if the association under study is not linear. We therefore used ankle/arm index as a marker for atherosclerosis, rather than focusing on presence or absence of a positive history of cardiovascular disease.

Our findings on the association between antithrombin activity and ankle/arm pressure ratio may explain discrepant results reported from other studies. In the Northwick Park Heart Study (NPHS), a study among men initially free of clinically manifest ischemic heart disease, a higher ischaemic heart disease mortality in the highest and in lowest tertiles of antithrombin was observed.[16, 17] This suggests that in asymptomatic subjects both *high and low* activity of antithrombin indicate an increased risk of cardiovascular events. In the Progreto Lombardi Aterotrombosi (PLAT) study population, consisting of 953 subjects with pre-existing coronary, cerebral, or peripheral ischemic disease, a higher ,though not significant, incidence of cardiovascular events in the lowest fifths of antithrombin was found.[18] Furthermore, the mean activity of antithrombin was lower in those with peripheral arterial disease who subsequently developed atherothrombotic events.[33] The PLAT findings suggest that in patients with symptomatic cardiovascular disease a *low* activity of antithrombin reflects an increased risk of subsequent cardiovascular thrombotic events. Combining the PLAT, NPHS and our results a possible explanation might be that subjects with active atherosclerosis have increased levels of antithrombin as a result of a protective mechanism against pro-coagulatory influences. Whereas in case of severe atherosclerosis the capacity of the defence mechanism is limited and antithrombin is not increased further. Rather, a decrease may follow increased consumption of antithrombin in subclinical thrombotic processes. The increased consumption of antithrombin might lower circulating antithrombin despite its increased production, as has been recognized in other conditions.[34] Consequently, increased as well as decreased levels of antithrombin may indicate increased risk and vascular damage.

Apart from the finding at <0.6 ankle/arm pressure ratio, women show a similar

pattern for the association between the ankle/arm pressure ratio and antithrombin to that found in men. Nevertheless in linear regression analyses the association between the two is linear. This could support that the association between antithrombin and peripheral atherosclerosis is modified by gender. Further studies are needed to clarify this possible difference between men and women.

Antithrombin activity was influenced by age and gender. Men had lower values than women and in both men and women antithrombin decreased with age. Previous investigations have not agreed upon the correlation of antithrombin with age and gender. Several previous studies did not demonstrate age or gender differences[35], but others did.[36] In the ARIC study antithrombin also decreased with age in men, but in contrast to our findings, it increased with age in women.[16] The women included in the ARIC study are aged 45 to 65 years. The age difference could explain why antithrombin rises with age in the ARIC study and falls in the women in our study.

In conclusion, our findings suggest that increased cardiovascular disease risk is associated with increased concentrations of antithrombin. Likewise, in response to the atherosclerotic process antithrombin increases, but with increasing severity of the disease antithrombin tends to decrease. Such changes may reflect a protective mechanism in response to atherosclerosis combined with an increased consumption in proportion to the severity of this. Changes in antithrombin over time might be useful in predicting the risk of cardiovascular disease and the progression of atherosclerosis.

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4 Cardiovascular events

4.1 Tissue type plasminogen activator

It is now widely accepted that blood clot formation in coronary arteries is the key event in myocardial infarction.[1] Intervention studies have demonstrated that in patients with myocardial infarction, activation of the fibrinolytic system by infusion of tissue type plasminogen activator (t-PA), results in rapid clot dissolution, and improved case fatality.[2] It has been hypothesized that people with a decreased endogenous fibrinolytic capacity are at increased risk for a myocardial infarction. Yet, to measure the fibrinolytic capacity is a major challenge. Some have suggested to measure plasma levels of t-PA months or years prior to a myocardial infarction. A *de*creased level of t-PA activity in patients with a history of myocardial infarction was directly associated with an increased risk of a recurrent myocardial infarction.[3,4,5] In contrast, in patients with angina pectoris t-PA activity level was not associated with subsequent myocardial infarction.[6] In the latter study *in*creased levels of t-PA antigen were associated with an increased risk of myocardial infarction. This finding had been reported earlier.[7,8,9] Whether the increased t-PA antigen levels represent decreased or increased fibrinolytic activity is still subject of debate.[10]

A different approach to seek for differences between subjects that do develop a myocardial infarction and those that do not, is to look for differences at the DNA level. Unlike plasma concentrations of proteins, DNA is not influenced by the event of a myocardial infarction, nor by preclinical atherosclerosis, nor by its risk factors. The gene for t-PA has been sequenced[11] and mapped to chromosome 8p12-p11.2.[12] Recently, an Alu repeat insertion/deletion (I/D) polymorphism, was found in intron h of this gene.[13]

The present study was set up to further explore whether plasma levels of t-PA antigen

and activity are associated with myocardial infarction, and whether the Alu I/D polymorphism could serve as a genetic marker for coronary heart disease in survivors of a myocardial infarction.

Methods

Population

A cross-sectional case-control study was performed in subjects selected from the Rotterdam Study. The Rotterdam Study is a prospective population based study of 7983 subjects. Between March 1990 and July 1993 all subjects aged 55 years and older, living in Ommoord, a suburb of Rotterdam, the Netherlands, were invited to participate. The overall response rate was 78%. The study has been approved by the Medical Ethics Committee of the Erasmus University and written informed consent was obtained from all participants. The rationale and design of the study have been described elsewhere[14].

Case patients (n = 150) were selected from the cohort, based on the presence of an infarction pattern on the ECG, using the diagnostic classification system of the Modular ECG Analysis System,[15,16] independent of a history of chest pain. Two control subjects per case were drawn from the same five-year-age strata where the cases of myocardial infarction were from and constituted a sample of study participants who had no history of cardiovascular disease, i.e. no history of myocardial infarction, angina pectoris, stroke, a normal ECG and no peripheral arterial disease (ankle/arm index > 0.9). We excluded subjects using anticoagulant drugs.

Measurements

All subjects were first visited at their home. Information on current health status, medical history (including myocardial infarction and stroke), drug use, and smoking behavior was obtained by a computerized questionnaire, which included the Dutch version of the Rose cardiovascular questionnaire.[17] The home interview was followed by two visits at the research center, between 8 AM and 4 PM. Patients were not asked to fast or to refrain from smoking. During those visits several cardiovascular risk indicators were determined. Height and weight were measured, and body mass index was calculated (in kilograms per meter squared). Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer. The average of two measurements obtained at one occasion was used. Systolic blood pressure at the ankles (posterior tibial artery) was measured in supine position with an adult size regular cuff just above the malleoli, and

a 8 MHz Doppler transducer.[18] The ankle/arm index is the ratio of the systolic blood pressure at the ankle to the arm systolic pressure. Peripheral arterial disease was defined as a right or left ankle/arm index lower than or equal to 0.9. Blood sampling and storage have been described elsewhere.[19] Blood samples were collected using CTAD vacutainers (0.11M citrate, 15 mM theophylline, 3.7 mM adenosine and 0.198 mM dipyridamole; Diatube H, Diagnostica Stago, France) and Stabilyte® vacutainers (Biopool, Umeå, Sweden)[20]. Stabilyte plasma was for the first time collected in subjects visiting the research center in July 1992. Consequently no baseline Stabilyte plasma was available for 56 subjects (32 control and 24 case subjects) that were selected for the present study and had visited the research centre before that date. For them we measured t-PA activity in Stabilyte plasma collected in the second phase of the Rotterdam Study (1993-1994). The mean time period between the first and the second center visit with blood collection was 2.5 years.

T-PA antigen was measured in CTAD plasma using the Immulyse® t-PA enzyme immuno-assay of Biopool (Umeå, Sweden).[21] T-PA activity was measured in Stabilyte® plasma using the Chromolize® t-PA bio-immunoassay of Biopool (Umeå, Sweden). Serum total and high density lipoprotein cholesterol were determined with an automated enzymatic procedure.

After the DNA was isolated from blood cells, an insertion/deletion (I/D) polymorphism resulting from the presence/absence of an Alu repeat in the 8th intron of the t-PA gene was identified in all subjects. The amplification of the 967/655-bp fragments of the t-PA gene was performed essentially as previously described[13] using a 5′ primer 5′-TCCGTAACAGGACAGCTCA-3′ (PR-TPAOL1;nt25,216-25,234[12]) and as 3′ primer 5′-ACCGTGGCTTCAGTCATGGA-3′ (PR-TPAOL2; nt 26181-26162). Conditions: 50 μl of a mixture containing 20 mM Tris-HCL, pH 8.4, 50 mM KCl, 1 mM $MgCl_2$, 0.05% (v/v) detergent (polyoxyethylene ether), 0.05 % (v/v) DMSO, 0.2 mM of each nucleoside triphosphate, 100 ng of each primer, 100 ng DNA and 1 U Taq polymerase were subjected to denaturation for 4 min at 94 °C, followed by 32 cycles of 94 °C (1 min), 58 °C (1 min), 72 °C (2 min), finally followed by 4 min at 72 °C. Some 25 μl of the PCR products were separated on a 2.5% agarose gel.

Statistical analysis

Means and proportions for baseline cardiovascular risk factors were computed for the cases and the controls. The relative risk of myocardial infarction (estimated as the odds ratio) for those hetero- and homozygous for the insertion compared to those homozygous for the deletion, and for the quartiles of t-PA antigen and t-PA activity compared to the lowest quartile, were calculated using logistic regression. Results are

presented with a corresponding 95% confidence interval (95% CI). Means and proportions of potential confounders were calculated for the three genotypes and differences between the genotypes were tested with analysis of variance. Adjusted relative risks were calculated further by logistic regression models that controlled for age, gender and systolic blood pressure. To assess the possible influence of other cardiovascular disease risk factors on the association between the t-PA antigen concentration and myocardial infarction, adjusted estimates of risk were obtained with logistic regression models which controlled for age and gender, total and HDL cholesterol as well as systolic and diastolic blood pressure, body mass index and current smoking. The association between t-PA antigen and t-PA activity was evaluated using multivariate linear regression analyses. Separate analyses were performed for t-PA activity in blood collected at baseline and that collected in the second phase of the study. No material differences were found. (Data not shown.)

Table 4.1.1 Baseline characteristics of the study population

	Controls n=250	Cases n=121
Age (years)*	72 (9)	73 (10)
Women (%)	60	43
Systolic blood pressure (mmHg)	139 (21)	143 (24)
Diastolic blood pressure (mmHg)	73 (11)	76 (13)
Body mass index (kg/m^2)	26 (4)	27 (4)
Current smoking (%)	15	24
Total cholesterol (mmol/l)	6.5 (1.1)	6.2 (1.1)
HDL cholesterol (mmol/l)	1.4 (0.3)	1.2 (0.3)
T-PA antigen (ng/ml)	10.1 (5.5)	11.2 (5.6)
T-PA activity (IU/ml)	1.12 (0.64)	1.21 (0.75)

Values are unadjusted percentages or means and standard deviations.

* This variable was used as matching criterium.

Results

Of the 450 subjects selected, blood cells were available for 421 subjects; CTAD plasma was available for 433 subjects; Stabilyte plasma was available for 411 subjects. After exclusion of those with missing data, the study population consisted of 121 cases, and 250 controls. Characteristics of the study population are shown in Table 4.1.1. As expected there were more men among the cases than among the controls, and subjects with a history of myocardial infarction had a higher prevalence of conventional cardiovascular risk factors than controls. Serum total cholesterol was higher in controls as compared to cases.

Table 4.1.2 Distribution of t-PA genotypes in case and control subjects and relative risk of myocardial infarction (odds ratio (95%CI) associated with the three genotypes. (D/D = homozygous for the deletion; I/D = heterozygous for the insertion/deletion; I/I = homozygous for the insertion polymorphism).

	D/D*	D/I	I/I
Cases of myocardial infarction (n)	14	58	49
Control subjects (n)	48	127	75
Crude risk	1	1.57 (0.80,3.07)	2.24 (1.11,4.50)
Adjusted for age and gender	1	1.53 (0.77,3.03)	2.16 (1.06,4.39)
Adjusted risk†	1	1.45 (0.71,2.96)	2.13 (1.02,4.43)

* Reference risk (1)

† Adjusted for age, gender and systolic blood pressure

The I/D polymorphism

Among the 250 controls 48 (19%) were found to be homozygous for the deletion allele, whereas 127 (51%) were heterozygous and 75 (30%) were homozygous for the insertion allele; the observed distribution of genotypes was consistent with that predicted by the Hardy-Weinberg equilibrium.

The genotypes characterized by either one or two insertion alleles (genotypes I/D and I/I) were associated with an excess number of cases of myocardial infarction as compared to the D/D genotype. The 'crude' relative risks (odds ratios) of non-fatal myocardial

infarction for the different genotypes are presented in Table 4.1.2. To examine whether the results of our study were confounded by other risk factors for myocardial infarction, we evaluated whether these cardiovascular risk factors were associated with the different genotypes. Table 4.1.3 presents means and proportions of these risk factors for the different genotypes. No significant differences in cardiovascular risk factors between the genotypes were found. Only the systolic blood pressure was somewhat higher in the I/I genotype. Additionally, we adjusted the relative risks of non-fatal myocardial infarction for the different genotypes for age, gender and systolic blood pressure (Table 4.1.2). There was no difference in mean plasma t-PA activity or t-PA antigen concentration for the three genotypes (Table 4.1.3). Further adjustment for plasma concentrations of t-PA antigen and activity did not alter the associations.

Table 4.1.3. Potential confounders of the association between the polymorphism and myocardial infarction were evaluated for the different genotypes, when appropriate adjusted for age, gender and caseness (D/D = homozygous for the deletion; I/D = heterozygous for the insertion/deletion; I/I = homozygous for the insertion polymorphism).

	D/D (17%)	I/D (50%)	I/I (33%)	p*
Age (years)	71	73	72	0.77
Women (%)	56	57	51	0.56
Systolic blood pressure (mmHg)	133	141	142	0.03
Diastolic blood pressure (mmHg)	75	74	74	0.76
Body mass index (kg/m^2)	27	26	26	0.63
Current smoking (%)	18	19	17	0.99
Total cholesterol (mmol/l)	6.3	6.5	6.4	0.51
HDL cholesterol (mmol/l)	1.3	1.3	1.3	0.90
T-PA antigen (ng/ml)	10.0	10.7	10.5	0.70
T-PA activity (IU/ml)	1.14	1.18	1.12	0.70

Values are percentages or means.

* p for trend obtained from linear regression analysis

Plasma concentrations of t-PA antigen and activity

Cases had a higher mean concentration of t-PA antigen than controls. The difference adjusted for age and gender was 1.08 ng/ml (SE 0.63, p=0.09). The t-PA activity levels were slightly and not significantly higher in cases compared to controls; age and gender adjusted difference 0.07 IU/ml (SE 0.07, p=0.36). There was no difference in the levels of t-PA antigen or activity between men and women.

T-PA antigen was positively associated with the risk of myocardial infarction; compared to subjects with a concentration in the lowest quartile of the t-PA antigen distribution, the relative risk for myocardial infarction increased in the second, third and upper quartile , 1.7 (95% CI 0.9,3.3), 2.3 (1.2,4.4), and 2.0 (1.0,3.8) respectively. When adjusted for HDL and total cholesterol, body mass index, systolic and diastolic blood pressure, and current smoking the risk attenuated, compared to that in the lowest quartile, the relative risks in the second, third and upper quartiles were, 1.7 (95% CI 0.8,3.6), 1.7 (0.8,3.5) and 1.3 (0.6,2.8), respectively.

The relative risks for myocardial infarction in the second, third, and upper quartiles compared to the lowest quartile of the t-PA activity distribution, were 1.1 (95% CI 0.6,2.2), 1.1 (0.6,2.2) and 1.3 (0.7,2.6), respectively. When adjusted for HDL and total cholesterol, body mass index, systolic and diastolic blood pressure, and current smoking the risk for myocardial infarction associated with t-PA activity increased; compared to that in the lowest quartile, the relative risks in the second third and upper quartiles were, 1.2 (95% CI 0.6,2.3), 1.3 (0.6,2.5) and 1.8 (0.9,3.7), respectively.

There was an inverse association between t-PA antigen and activity. After adjustment for age and gender, an increase in t-PA antigen with 1 ng/ml was associated with a decrease in t-PA activity of 0.014 IU/ml (SE 0.006, p=0.02). However, after additional adjustment for body mass index, systolic blood pressure, total and HDL cholesterol the association was no longer present (regression coefficient -0.001 IU/ml (SE 0.006, p=0.86)).

Discussion

The objective of the present study was to assess the association of the I/D polymorphism of the t-PA gene and the plasma concentration of t-PA antigen and activity with myocardial infarction. The insertion allele of the t-PA gene was associated with an increased risk of non-fatal myocardial infarction, independent of plasma concentrations of t-PA and of other known risk factors. Consequently, this polymorphic marker offers

an independent predictor for non-fatal myocardial infarction. T-PA antigen concentration was positively associated with the risk of myocardial infarction, but after adjustment for other cardiovascular disease risk factors this association markedly attenuated. Increased t-PA activity, usually interpreted as the main determinant of clot dissolving capacity of the haemostatic system, showed a tendency to be associated with an increased risk for myocardial infarction.

Concerning the design of the study, several aspects need to be discussed. First, it is unlikely that population heterogeneity can explain our results. Cases and controls were drawn from one single-center population-based study. All subjects participating in our study were caucasians and allele frequencies did not differ from those observed by others.[22] Second, by virtue of its design, a cross-sectional study is limited to cases of non-fatal myocardial infarctions. In order to investigate whether the I/D polymorphism is also a marker for fatal myocardial infarction, a longitudinal study is required. Third, a source of bias in a cross-sectional study may be a changed risk profile after the myocardial infarction. As for plasma levels of t-PA antigen and activity, this type of bias can not be excluded, hence we are cautious to interpret the findings concerning plasma levels as causal. As for the association between the I/D polymorphism and the risk of myocardial infarction no such bias is expected. Fourth, blood samples were taken at various times during the day and subjects were not asked to fast. Therefore, the between subject variability of t-PA levels in our study population may be larger than in some other studies.[23] This may influence the precision of the estimated difference between cases and controls. However, the point estimate of the difference in t-PA antigen level between cases and controls was 10%, which is similar to that reported by others.[6,9] Finally, in previous studies t-PA activity was measured without measures against complex formation with the main inhibitor of t-PA, plasminogen activator inhibitor 1 (PAI-1), which led to relatively low levels of t-PA activity. In our study an acid blood collection and more adequate methodology was used to measure the free t-PA activity.[20]

The theory underlying this research is that people with a decreased fibrinolytic capacity are at increased risk for an acute myocardial infarction. Several methods to assess 'fibrinolytic capacity' have been proposed. Plasma concentrations of the fibrinolytic factors have been associated with the risk for myocardial infarction.[3,4,5,6,7,8,9] We chose to assess the thus far only identified polymorphism in the gene for t-PA. Assessment of genetic parameters has the advantage that intersubject variability can be measured irrespective of where and when the fibrinolytic capacity should be at its highest, namely in the coronary arteries at the moment of thrombus formation. The nature of the I/D polymorphism, an insertion of an *Alu* repeat in an intron, a non-translated region, makes a direct functional effect of the I allele on the t-PA protein

unlikely, but not impossible.[24] In this respect, the *Alu* insertion in the t-PA gene shows a similarity with the *Alu* insertion/deletion polymorphism present in the gene for angiotensin-converting enzyme (ACE).[25] Here, the deletion allele is strongly associated with plasma concentrations of ACE.[26,27] Despite intensive sequence effort spanning the entire coding region of the ACE-gene, no apparent functional polymorphism has been found.[28] It was suggested that the *Alu* insertion/deletion event can alter mRNA stability and/or splicing. In contrast to the ACE I/D polymorphism, the *Alu* polymorphism in the t-PA gene was not associated with t-PA plasma concentrations. Furthermore, the basal endothelial t-PA synthesis was reported not to be influenced by the t-PA *Alu* polymorphism.[29] However, these findings do not exclude the existence of an association between this polymorphism and the endogenous fibrinolytic capacity. As already indicated above, the circulating levels of t-PA as measured in an asymptomatic period might not reflect the fibrinolytic capacity at the moments and site of thrombus formation. Additionally, the *Alu* repeat insertion may be closely linked to a mutation, at or near the t-PA gene, that produces a functional effect (impaired fibrinolytic capacity?), and may cause an increased risk for myocardial infarction.

The heterozygous genotype seemed to have an intermediately increased risk for myocardial infarction, which is suggestive for a gradually increased risk for myocardial infarction across the genotypes. This suggests a dose related response and supports a causal relation. It may be speculated that a relative risk of two is relatively small for a genetic disorder. Yet, cardiovascular disease is a multifactorial disease. The complex process of atherothrombosis is influenced by many, partly competitive determinants. Interaction of known and unknown cardiovascular disease risk factors may influence the impact of these factors on the course of the disease, resulting in a relatively low relative risk.

Interpretation of the finding that increased plasma concentrations of t-PA antigen and possibly also increased t-PA activity were associated with increased risk for myocardial infarction, against a background of a supposedly decreased fibrinolytic activity is difficult. Increased t-PA antigen concentration in subjects at higher risk for myocardial infarction are in agreement with findings in other studies,[6,8,9] and has been suggested to reflect predominantly t-PA/PAI-1 complex.[30] As PAI-1 concentration in plasma is much higher than t-PA antigen concentration and the t-PA antigen assay measures both free and complexed t-PA, increased concentration of t-PA antigen is supposed to indicate a reduced rather than enhanced fibrinolytic activity. In the present study, increased t-PA antigen was indeed associated with a decreased t-PA activity. This supports the notion that the increased risk of myocardial infarction in those with increased t-PA antigen concentrations is due to a decreased t-PA activity.[31] The observation that the association

between t-PA antigen and myocardial infarction is attenuated when cardiovascular risk factors are taken into account indicates that t-PA antigen is associated with these risk factors. Whether t-PA antigen is associated with the risk for myocardial infarction as an intermediate factor in the same causal chain, or in concert with the conventional cardiovascular disease risk factors remains to be established. An increased t-PA activity tended to be associated with an increased risk for myocardial infarction. As we are the first to assess the association between myocardial infarction and t-PA activity as measured in Stabilyte plasma, and as the association was not statistically significant, it is at this moment difficult to judge the relevance of this finding.

In conclusion, the insertion allele of the *Alu*-repeat insertion/deletion polymorphism of the t-PA gene is independently associated with non-fatal myocardial infarction. This polymorphism consequently appears to be an independent genetic indicator for increased risk of non-fatal myocardial infarction. The increased risk associated with the I allele is not reflected in t-PA plasma concentrations.

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4.2 Plasminogen activator inhibitor

The risk for myocardial infarction may be increased in case of a reduced fibrinolytic activity.[1,2] Plasminogen activator inhibitor type 1 (PAI-1) is a potent inhibitor of thrombolytic activity.[3] Increased concentrations of circulating PAI-1 were associated with an increased risk for myocardial infarction in some studies,[4,5] but not in others.[6,7,8,9] As plasma concentrations of PAI-1 are strongly associated with several cardiovascular disease risk factors, it has been difficult to assess whether PAI-1 plays an independent role in the pathogenesis of cardiovascular disease. Recently progress was made at the genetic level of PAI-1. A common polymorphism was discovered in the promoter region 675 base-pairs upstream of the start of transcription of the PAI-1 gene, where one allelic variant carries four guanosines (4G) and the other five (5G).[10] The 4G allele was found to be associated with increased plasma PAI-1 concentrations,[11] and with an increased risk for myocardial infarction.[12] These findings suggest a causal role for PAI-1 in the pathogenesis of myocardial infarction. However, the association of the 4G/5G polymorphism with myocardial infarction was not confirmed by Ye and coworkers.[13] As is has been suggested that genes and invironment may influence one another[14], we hypothesized that these discrepancies may be due to the influence of extraneous factors, such as age, gender, or smoking habits, on the strength of the association between the genotype and the risk for myocardial infarction.

The present study was designed to explore whether the 4G/5G polymorphism in the PAI-1 gene is associated with myocardial infarction and whether the strength of the association is modified by age, sex, smoking, body mass index, blood pressure and serum cholesterol.

Methods

Population

A cross-sectional case-control study was performed in subjects selected from the Rotterdam Study. The Rotterdam Study is a prospective population based study of 7,983 subjects. Between March 1990 and July 1993 all subjects aged 55 years and older, living in Ommoord, a suburb of Rotterdam, the Netherlands, were invited to participate. The overall response rate was 78%. The study has been approved by the Medical Ethics Committee of the Erasmus University and written informed consent was obtained from all participants. The rationale and design of the study have been described elsewhere.[15]

Case patients (n = 150) were selected from the cohort, based on the presence of an infarction pattern on the ECG, using the diagnostic classification system of the Modular ECG Analysis System,[16,17] independent of a history of chest pain. Two control subjects per case were randomly drawn from the same five-year-age strata where the cases of myocardial infarction were from and constituted a sample of study participants who had no history of cardiovascular disease, i.e. no history of myocardial infarction, angina pectoris, stroke, a normal ECG and no peripheral arterial disease (ankle/arm index >0.9). We excluded subjects using anticoagulant drugs.

Clinical investigations

All subjects were first visited at their home. Information on current health status, medical history, drug use, and smoking behaviour was obtained by a computerized questionnaire, which included the Dutch version of the Rose cardiovascular questionnaire.[18] The home interview was followed by two visits at the research centre, between 8 AM and 4 PM. Subjects were not asked to fast or to refrain from smoking. During those visits several cardiovascular risk indicators were determined. Height and weight were measured, and body mass index was calculated (in kilograms per meter squared). Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer. The average of two measurements obtained at one occasion was used. Systolic blood pressure at the ankles (posterior tibial artery) was measured in supine position with an adult size regular cuff just above the malleoli, and a 8 MHz Doppler transducer.[19] The ankle/arm index is the ratio of the systolic blood pressure at the ankle to the arm systolic pressure. Peripheral arterial disease was defined as a right or left ankle/arm index lower than or equal to 0.9.

Laboratory investigations

Blood sampling and storage have been described elsewhere.[20] Blood samples were collected using CTAD vacutainers (0.11M citrate, 15 mM theophylline, 3.7 mM adenosine and 0.198 mM dipyridamole; Diatube H, Becton and Dickinson, Mylan, Cedex, France).[21] Serum total and HDL cholesterol were determined with an automated enzymatic procedure. PAI-1 antigen concentration was measured in CTAD plasma using the Innotest PAI-1 (Innogenetics, Zwijndrecht, Belgium).[22] Genomic DNA was isolated from blood cells and -675(4G/5G) PAI-1 genotype was determined by allele specific PCR amplification.[23]

Statistical analysis

After exclusion of those with missing data on either the 4G/5G polymorphism or PAI-1

antigen concentration, the study population consisted of 132 cases and 265 controls. As the distribution of the plasma concentration of PAI-1 antigen was positively skewed, statistical analyses were carried out on log-transformed data. The mean values and 95% confidence intervals (CI) presented were transformed back into the original scale.
Logistic regression was used to calculate the relative risk (estimated as the odds ratio) for myocardial infarction with increasing concentrations of PAI-1 antigen by steps of 50 ng/ml and with the different 4G/5G genotypes. Adjusted relative risks were calculated by logistic regression models that controlled for age, sex, current smoking, body mass index, systolic blood pressure, total and HDL cholesterol. To examine the modifying effect of age, sex, smoking, body mass index, serum cholesterol and systolic blood pressure, on the strength of the association between the 4G/5G polymorphism and risk of myocardial infarction, we stratified the study population in men and women, in two age categories (cutpoint 75 years), in current smokers (subjects that answer the question 'Do you smoke?' with 'yes') and non-smokers (past and never smokers), according to increasing levels of body mass index (cutpoints 25 kg/m^2 and 27 kg/m^2), systolic blood pressure (cutpoints 130 mmHg and 145 mmHg), total cholesterol (cutpoints 5.5 mmol/l, and 7.0 mmol/l) and HDL cholesterol (cutpoints 1.0 mmol/l and 1.5 mmol/l). The cutpoints for categories of continuous variables with logical steps were, arbitrarily, chosen after exploration of the distribution of these variables in our study population.

Results

In the study population the frequency of the 4G allele was 53% and the distribution of the genotypes was in Hardy Weinberg equilibrium: 77 subjects (29%) were found to be homozygous for the 4G allele, 129 (49%) were heterozygous, and 59 (22%) were homozygous for the 5G allele (Table 4.2.1).

Table 4.2.1 Distribution of genotypes among case and control subjects. (4G4G = homozygous for the 4G allele; 4G5G = heterozygous; 5G5G = homozygous for 5G allele)

	5G5G	4G5G	4G4G
Cases of myocardial infarction	25 (19%)	56 (42%)	51 (39%)
Control subjects	59 (22%)	129 (49%)	77 (29%)

Table 4.2.2 Population characteristics of the sample of 397 men and women investigated.

	Mean	SD	Smallest	Largest
Sex (% women)	55			
Age (years)	72	9	55	95
Systolic blood pressure (mmHg)	140	22	89	212
Diastolic blood pressure (mmHg)	74	12	43	123
Body mass index (kg/m^2)	26	4	17	50
Current smoking (%)	18			
Total cholesterol (mmol/l)	6.41	1.12	3.5	9.4
HDL cholesterol (mmol/l)	1.34	0.36	0.6	2.9
PAI-1 (ng/ml)*	54		11.5	611

Values are proportions or means with standard deviations and ranges.

* Back-transformed logarithmic mean.

Baseline characteristics of the study population are presented in Table 4.2.2. Overall, PAI-1 antigen plasma concentrations were slightly lower in men than in women; means adjusted for age were 51 ng/ml (95% CI 47,56) and 56 ng/ml (52,61), respectively. There was no clear difference in PAI-1 concentrations between current smokers and non-(current)smokers; age and sex adjusted means were 58 ng/ml (50,67) and 53 ng/ml (50,57), respectively. The 4G allele was associated with higher PAI-1 antigen concentrations. In controls, those homozygous for 4G had the highest PAI-1 antigen concentration 61 ng/ml (53,70); heterozygous subjects had intermediate PAI-1 concentrations, 51 ng/ml (46,57), and subjects homozygous for the 5G allele had the lowest PAI-1 concentrations, 46 ng/ml (39,54). In cases, a similar trend was present, 65 ng/ml (56,75) in subjects with 4G4G, 54 ng/ml (47,63) in those with 4G5G, and 48 ng/ml (38,59) in subjects with 5G5G.

The risk for non-fatal myocardial infarction tended to increase with increasing concentrations of PAI-1 antigen. In comparison to those with a PAI-1 antigen concentration lower than 50 ng/ml, subjects with values between 50 and 100 ng/ml had

a relative risk (odds ratio) for myocardial infarction of 1.11 (95% CI 0.70,1.77) and subjects with PAI-1 of 100 ng/ml and above had a relative risk of 1.64 (0.86,3.11). After adjustment for smoking, body mass index, systolic blood pressure, serum total and HDL cholesterol, risks were no longer increased (odds ratios 0.82 (0.48,1.39) and 1.00 (0.49,2.05), respectively).

The relative risk (odds ratios) for myocardial infarction was increased in subjects with the 4G4G genotype as compared to those homozygous for the 5G allele (Table 4.2.3). Additional adjustment for plasma concentrations of PAI-1 antigen did not alter the association (Table 4.2.3).

Table 4.2.3 Relative risk (odds ratio (95% CI)) for myocardial infarction for heterozygous subjects and subjects homozygous for the 4G allele compared to subjects homozygous for the 5G allele.

	4G5G	4G4G
Crude risk	1.02 (0.58,1.80)	1.56 (0.89,2.82)
Adjusted for age and sex	0.96 (0.54,1.71)	1.42 (0.78,2.59)
Adjusted for age, sex and smoking	0.96 (0.54,1.72)	1.41 (0.77,2.58)
Adjusted for age, sex and [PAI-1]	0.94 (0.53,1.68)	1.31 (0.71,2.42)
Adjusted for age, sex and other factors*	1.04 (0.58,1.90)	1.50 (0.80,2.83)

* Adjusted for age, sex, smoking, body mass index, systolic and diastolic blood pressure, serum HDL and total cholesterol and PAI-1 antigen concentration.

Smoking was significantly differently distributed among the genotypes. Among controls, the proportion of smokers was 8 percent in those homozygous for the 4G allele as compared to 16 percent in those heterozygous and 21 percent in those homozygous for the 5G allele. In contrast, among cases the proportion of smokers in subjects with the 4G4G genotype was much higher; for the 5G5G, 4G5G and 4G4G the percentages of smokers were 16, 25 and 32 respectively. These findings suggested effect modification by smoking on the association between the polymorphism and myocardial infarction. Stratification for smoking showed that the increased risk of myocardial infarction associated with the 4G allele was considerably higher in smokers. In smokers, the relative risk for myocardial infarction for heterozygous subjects was 1.47 (0.39,5.52) and for those homozygous for the 4G allele it was 5.33 (1.24,22.9), both in comparison to subjects homozygous for the 5G allele. In non-smokers, these relative risks were 0.82

(0.43,1.57) and 0.89 (0.45,1.77), respectively. Stratification for age, sex, body mass index, systolic blood pressure and for total and HDL cholesterol, after exclusion of smokers, showed no difference in the increased risk of myocardial infarction associated with the 4G allele for the different strata (data not shown).

The difference in the strength of the association between the polymorphism and myocardial infarction for smokers compared to non-smokers raised the question whether the cardiovascular risk of smoking differed for the various genotypes. In the study population as a whole smokers had a doubled risk for myocardial infarction as compared to non-(current)smokers (odds ratio 2.0 (95% CI 1.2,3.5)). To assess whether the relative risk of smoking for myocardial infarction was different for the various genotypes we stratified the population according to the three genotypes. In subjects homozygous for the 4G allele the association between smoking and the risk for myocardial infarction was stronger than in those with the other genotypes (Table 4.2.4). Additional adjustment for plasma concentration of PAI-1 antigen did not alter the findings.

Table 4.2.4 Relative risk (odds ratio (95% CI) adjusted for age and sex) for myocardial infarction of current smokers compared to non-current smokers, for the whole population and for the three genotypes separately.

	Odds ratio (95% confidence interval)
Whole population	2.0 (1.2,3.5)
Subjects with 5G5G	1.1 (0.3,3.7)
Subjects with 4G5G	1.5 (0.7,3.3)
Subjects with 4G4G	4.9 (1.8,13)

Discussion

The main finding of the present study is that smoking increased the risk for myocardial infarction associated with the 4G allele of the PAI-1 gene. Additionally, the cardiovascular risk of smoking differed markedly for the three genotypes of the 4G/5G polymorphism. These associations were independent of peripheral venous plasma concentrations of PAI-1 antigen and of other known risk factors.

Our study has certain limitations. First, the case group comprised only non-fatal cases

of myocardial infarction. The unequal distributions of smokers among cases and controls suggest that many smokers with the 4G allele have either developed a non-fatal myocardial infarction or have died. Therefore, we presume that similar results would have been obtained for fatal cases of myocardial infarction. Second, a source of bias in a cross-sectional study may be a changed risk profile of the cases after the event of interest. This may be important for the plasma concentration of PAI-1, but our main interest was in the influence of the 4G/5G polymorphism on the risk for myocardial infarction. In this respect no such bias is expected, because genotypes do not change in case of a myocardial infarction. With regard to smoking, subjects that have had a myocardial infarction may have quit smoking afterwards. It is unlikely that the proportion of those who quit smoking, is different for the different genotypes. Therefore, the relative risk for myocardial infarction associated with the different genotypes among smokers provides a valid estimate of the 'true' association between polymorphism and risk. In the group of non-smokers, the relative risk may be overestimated due to the presence of ex-smokers in this category. Furthermore, the changed risk profile of the cases of myocardial infarction with respect to smoking may diminish the increase in risk for myocardial infarction associated with smoking. Therefore, one has to be cautious to interpret our findings on the risk for myocardial infarction associated with smoking. Nevertheless, the finding that current smoking was associated with a doubled risk for myocardial infarction is in agreement with findings from prospective studies.[24,25]

The association between the 4G/5G polymorphism and myocardial infarction has been studied before. An increased risk for myocardial infarction in subjects with the 4G4G genotype was first found in a Swedish case control study among men aged 45 years or less.[12] It could not be confirmed in two other studies.[13,26] Differences between study populations in distributions of risk modifiers, such as smoking, may very well explain the different findings. The proportion of smokers among a highly selected group of healthy physicians participating in the Physicians Health Study, an intervention trial of aspirin and beta-carotene, was 11 percent at baseline, in 1983.[27] In view of our findings, this may explain why among these physicians, the 4G4G genotype did not seem to increase the incidence of myocardial infarction. In the ECTIM Study, a case-control study among men and women aged 35-64 years living in Belfast and France, the proportion of smokers should be sufficiently high to find an association between the 4G/5G polymorphism and the risk of myocardial infarction. However, in the ECTIM study population, the distribution of genotypes in the control group differed from that predicted in a Hardy Weinberg equilibrium. This may be due to inappropriate selection from the source population. Therefore, as the authors proposed, their failure to detect an association may have been due to selection bias as a result of the relatively low response

rates (60% in Belfast and 50% in France).[28,29]

Our findings shed new light on the possible mechanism of the association between the 4G/5G polymorphism and the risk of myocardial infarction. Experimental studies suggested that the 4G/5G polymorphism in the PAI-1 gene is of functional importance in regulating the expression of the PAI-1 gene.[11] The deletion of one guanine at -675 in the PAI-1 promoter region may have a direct effect in an increased PAI-1 transcription in subjects with the 4G allele.[30] This is confirmed by the finding that the 4G allele is associated with increased plasma concentrations of PAI-1. Additionally, it has been suggested that components of the acute phase response may increase PAI-1 expression preferentially in individuals homozygous for the 4G allele compared with individuals of other genotypes.[10] We showed that homozygosity for the 4G allele is associated with an increased risk for myocardial infarction, at least in smokers. The latter association, however, seems to be independent of circulating concentrations of PAI-1. Moreover, despite the strong association between the 4G/5G polymorphism and the risk for myocardial infarction in smokers, smoking does not seem to influence plasma PAI-1 concentrations. Apparently, the polymorphism in the PAI-1 gene influences the risk of myocardial infarction through a mechanism that is not reflected in circulating concentrations of PAI-1 antigen, but conceivably at a more local level. Otherwise, the polymorphism may be associated with a greater variability in plasma PAI-1 concentrations. These greater variabilities may then predispose an individual to a thrombotic event at the peak of plasma levels.[14] The 4G/5G polymorphism itself, but also a genetic mutation in linkage disequilibrium with the polymorphic locus within or in the vicinity of the PAI-1 gene may influence the thrombotic risk. Even more intriguing is the biological background of how smoking can induce such an increase in risk. Experimental studies are needed to answer this.

The present findings have important implications for counselling individuals with respect to their cardiovascular disease risk. Information on the, easily detectable, 4G allele significantly adds to the cardiovascular risk profile of smokers.

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4.3 Fibrinogen

To date, it is not clear whether elevated plasma fibrinogen itself increases the risk for myocardial infarction (risk factor), or whether the elevated level is merely a reflection of the presence of preclinical atherosclerosis, or of an association with a true risk factor (risk indicator).

To elucidate whether high plasma fibrinogen per se increases the risk for myocardial infarction, the association between the risk for myocardial infarction and increased plasma fibrinogen independent of other factors related to the risk for myocardial infarction should be studied. When such 'independently' raised fibrinogen is associated with an increased risk for myocardial infarction, then this indicates that plasma fibrinogen is causally related to this risk. Unfortunately no intervention to specifically influence plasma fibrinogen level is known. Drugs known to influence fibrinogen levels, such as fibric acid derivates, also alter many other factors related to the risk for myocardial infarction.[1]

Yet, an alternative approach is to study genetic markers that are associated with elevated fibrinogen. Several polymorphisms in the genes for fibrinogen have been associated with increased plasma fibrinogen, such as the -455G/A polymorphism.[2] An individual's genetic status is neither changed by presence of disease, nor by risk factors for disease. We propose that if a certain genotype for fibrinogen is associated with an increased plasma fibrinogen, and an increased plasma fibrinogen causes an increased risk for myocardial infarction, then that genotype should also be associated with an increased risk for myocardial infarction.

The A allele of the -455G/A polymorphism in the ß-fibrinogen gene has been associated with increased levels of plasma fibrinogen.[3] Whether possession of the A allele is associated with an increased risk for myocardial infarction is not clear. In the ECTIM Study, the -455G/A polymorphism was not associated with the risk for myocardial infarction, but the authors reported that bias in selection of subjects, or the weak association between the genotype and plasma fibrinogen level may explain the absence of an association.[4]

The present study was conducted to explore whether the A allele of the -455G/A polymorphism in the ß-fibrinogen gene is associated on the one hand with raised fibrinogen levels and on the other hand with an increased risk for myocardial infarction.

Methods

Population

A cross-sectional case-control study was performed among subjects selected from the Rotterdam Study. The Rotterdam Study is a prospective population based study of 7983 subjects. Between March 1990 and July 1993 all subjects aged 55 years and older, living in Ommoord, a suburb of Rotterdam, the Netherlands, were invited to participate. The overall response rate was 78%. The study has been approved by the Medical Ethics Committee of the Erasmus University and written informed consent was obtained from all participants. The rationale and design of the study have been described elsewhere.[5]

Cases (n = 150) were selected from the cohort, based on the presence of an infarction pattern on the ECG, using the diagnostic classification system of the Modular ECG Analysis System,[6,7] independent of a history of chest pain. Two controls per case were drawn from the same five-year-age strata where the cases of myocardial infarction were from and constituted a sample of study participants who had no history of cardiovascular disease, i.e. no history of myocardial infarction, angina pectoris, stroke, a normal ECG and no peripheral arterial disease (ankle/arm index > 0.9). We excluded subjects using anticoagulant drugs.

Measurements

All subjects were first visited at their home. Information on current health status, medical history, drug use, and smoking behaviour was obtained by a computerized questionnaire, which included the Dutch version of the Rose cardiovascular questionnaire.[8] The home interview was followed by two visits at the research centre. Subjects were not asked to fast or to refrain from smoking. During those visits several cardiovascular risk indicators were determined. Height and weight were measured, and body mass index was calculated (in kilograms per meter squared). Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer. The average of two measurements obtained at one occasion was used. Systolic blood pressure at the ankles (posterior tibial artery) was measured in supine position with an adult size regular cuff just above the malleoli, and a 8 MHz Doppler transducer.[9] The ankle/arm index is the ratio of the systolic blood pressure at the ankle to the arm systolic pressure. Peripheral arterial disease was defined as a right or left ankle/arm index lower than or equal to 0.9. Blood sampling and storage have been described elsewhere.[10] Blood samples were collected using CTAD vacutainers (0.11M citrate, 15 mM theophylline, 3.7 mM adenosine and 0.198 mM dipyridamole; Diatube H, Becton and Dickinson, Mylan, Cedex, France)[11]. Fibrinogen was measured as described by Von Clauss.[12] The -

455G/A polymorphism restriction fragment length polymorphism of the ß-fibrinogen gene was determined by amplification of the polymorphic region by polymerase chain reaction, followed by digestion with the restriction enzyme *Hae*III as described by Thomas et al.[3]

Statistical analysis

Means and proportions of cardiovascular risk factors for men and women were calculated. As homozygosity for the A allele was rare and heterozygosity for the -455G/A polymorphism was also associated with increased levels of fibrinogen, we combined both genotypes in one group. Subjects with a genetically increased fibrinogen, i.e. those carrying the A allele were categorized as the exposed group and compared with those homozygous for the G allele (G/G genotype), the non-exposed.

We examined whether increased plasma fibrinogen level is a risk indicator in our study by comparing the risk for myocardial infarction for different levels of fibrinogen with a logistic regression model with fibrinogen level as a continuous variable. The number of cases and controls for the exposed and non-exposed were determined and logistic regression was used to calculate the relative risk (estimated as the odds ratio) for myocardial infarction in the exposed compared to the non-exposed. To examine the presence of confounding in the study population we compared means and proportions of cardiovascular risk factors for exposed and non-exposed subjects. Differences were tested with t-test and Pearson's chi-square. Additional adjustments for the established cardiovascular disease risk factors that may confound the association were made by adding them to the logistic model.

To examine the potentially modifying effect of age, sex, smoking, body mass index, serum total and HDL cholesterol and systolic and diastolic blood pressure, on the association between the -455G/A polymorphism and the risk for myocardial infarction, we stratified the study population in men and women, in age categories (cutpoint 75 years), in current smokers (subjects that answer the question 'do you smoke' with 'yes') and non-smokers (past and never smokers) and according to increasing levels of body mass index (cutpoints 25 kg/m^2 and 27 kg/m^2), systolic blood pressure (cutpoints 130 mmHg and 145 mmHg), total cholesterol (cutpoints 5.5 mmol/l and 7.0 mmol/l) and HDL cholesterol (cutpoints 1.0 mmol/l and 1.5 mmol/l).

Table 4.3.1 Baseline characteristics of the study population.

	Men	Women
Number	206	259
Age (years)	70 (8)	74 (9)
Systolic blood pressure (mmHg)	137 (21)	142 (12)
Diastolic blood pressure (mmHg)	74 (12)	74 (12)
Body mass index (kg/m^2)	26 (2.7)	27 (4.2)
Current smoking (%)	28	13
Total cholesterol (mmol/l)	6.30 (1.11)	6.63 (1.13)
HDL cholesterol (mmol/l)	1.25 (0.34)	1.42 (0.35)
Fibrinogen (g/l)	3.57 (0.83)	3.72 (0.84)

Values are percentages or means (standard deviations).

Results

After exclusion of those with missing data, the study population consisted of 139 cases and 287 controls. General characteristics are presented in Table 4.3.1. Frequency of the A allele was 0.20 and the distribution of the genotypes was in Hardy Weinberg equilibrium, both in cases and controls.

Plasma fibrinogen level was higher in the subjects carrying one or two A alleles as compared to those homozygous for the G allele, 3.8 (95% CI 3.6,3.9) and 3.6 (3.5,3.7) respectively. The association between fibrinogen and the presence of the A allele was restricted to controls and non-smokers (Table 4.3.2). Fibrinogen level was associated with the risk for myocardial infarction; the risk was increased with 45 % for each increase in 1 g/l in fibrinogen level (odds ratio 1.45 (95% CI 1.12,1.88).

Among the 139 cases, 86 subjects (62%) were homozygous for the G allele. Similarly, among the 287 controls, 183 subjects (64%) were homozygous for the G allele. The risk for myocardial infarction was not different for A allele carriers as compared to non-A allele carriers (Table 4.3.3). To check whether the genetic status was associated with other cardiovascular disease risk factors (confounding), we compared these risk factors across genetic categories (Table 4.3.4). The G/G genotype was associated with somewhat

higher total serum cholesterol levels, with higher HDL cholesterol levels and possibly also with a lower proportion of smokers, a somewhat higher systolic and diastolic blood pressure. Additional adjustment for smoking, total and HDL cholesterol, systolic and diastolic blood pressure did not substantially change the association between the polymorphism and the risk for myocardial infarction (Table 4.3.3).

As the association between presence of the A allele and plasma fibrinogen level was restricted to non-smokers, we examined the association between presence of A allele and myocardial infarction in non-smokers (102 case and 241 control subjects). The association did not substantially differ from the overall results; odds ratio, adjusted for age and sex 0.93 (95% CI 0.56,1.53).

No evidence for effect modification of the association between the -455G/A polymorphism and the risk for myocardial infarction by age, sex, body mass index, serum total cholesterol, serum HDL cholesterol, systolic blood pressure, or diastolic blood pressure was observed. Results for age, sex and smoking are presented in Table 4.3.5.

Table 4.3.2 Plasma fibrinogen (g/l) in the GG and the GA/AA genotypes for cases, control subjects, current smokers and non-current smokers. (GG = homozygous for the G allele; GA/AA = carriers of the A allele).

	GG	GA/AA	p
Cases of myocardial infarction[¬]	3.83 (3.65,4.01)	3.82 (3.59,4.05)	0.94
Control subjects[¬]	3.47 (3.36,3.58)	3.72 (3.57,3.87)	0.009
Current smokers[§]	3.97 (3.70,4.24)	3.96 (3.65,4.28)	0.79
Non-current smokers[§]	3.50 (3.40,3.60)	3.71 (3.57,3.84)	0.02

[¬] Values are means (95% confidence interval) adjusted for age, sex and smoking.

[§] Values are means (95% confidence interval) adjusted for age, sex and caseness.

Table 4.3.3. Relative risk (odds ratio (95% confidence interval (CI))) for myocardial infarction associated with the GA and AA genotypes as compared to the GG genotype.

	Relative risk	95% CI
Crude risk	1.08	(0.71,1.65)
Adjusted for age and gender	1.01	(0.66,1.55)
Adjusted for age, gender and smoking	0.95	(0.62,1.47)
,, and HDL cholesterol	0.87	(0.55,1.35)
,, and systolic and diastolic blood pressure	0.97	(0.61,1.52)

Table 4.3.4. Means and proportions of cardiovascular disease risk factors for the different genotypes. (GG = homozygous for the G allele, GA/AA = heterozygous or homozygous for the A allele)

	GG	GA/AA	p
Number	269	157	
Age (years)	71.2 (0.5)	73.0 (0.7)	0.14
Women (%)	58	50	0.16
Systolic blood pressure (mmHg)	140 (1.3)	138 (1.7)	0.19
Diastolic blood pressure (mmHg)	74 (0.7)	73 (0.9)	0.20
Body mass index (kg/m^2)	26.4 (0.2)	26.4 (0.3)	0.93
Current smoking (%)	17	22	0.18
Total cholesterol (mmol/l)	6.53 (0.07)	6.40 (0.09)	0.26
HDL cholesterol (mmol/l)	1.38 (0.02)	1.31 (0.03)	0.04
Fibrinogen (g/l)	3.59 (0.05)	3.76 (0.07)	0.03

Values are percentages or means (standard error).

Adjusted for caseness and when appropriate adjusted for age and sex.

Table 4.3.5 Risk for myocardial infarction (odds ratio adjusted for age and sex) for the GA and AA genotypes as compared to the GG genotype, stratified for age, sex, and smoking.

Group	Relative risk for myocardial infarction	95% confidence interval
All study participants	1.01	(0.66,1.55)
Age < 75 years	0.80	(0.47,1.36)
Age > 75 years	1.31	(0.69,2.50)
Men	0.85	(0.48,1.50)
Women	1.09	(0.61,1.97)
Smokers	1.01	(0.42,2.41)
Non-smokers	0.94	(0.59,1.50)

Discussion

The main finding of this study is that the A allele of the -455G/A polymorphism in the ß-fibrinogen gene, was associated with increased plasma fibrinogen, but not with the risk for non-fatal myocardial infarction.

To appreciate these findings some aspects of the study design need to be discussed. To address our etiologic question we performed a population-based case-control study. Cases of non-fatal myocardial infarction and controls were drawn from one large single-center study. All subjects participating in the study were caucasians and allele frequencies were similar to those reported by others.[3] The observed distribution of genotypes was identical to the expected distribution (Hardy Weinberg equilibrium) in cases as well as controls. Also, allele frequencies for each genotype were similar in case and control groups. This confirms that case and control subjects originated from the same source population, and provides support for the absence of an association between the -455G/A polymorphism and myocardial infarction. A source of bias in a cross-sectional design might be that the risk profile of cases has changed after their myocardial infarction. As the aim of the present study was primarily to investigate the influence of possession of the A allele on the risk for myocardial infarction this potential source of

bias does not play a role. Plasma fibrinogen level may be different after a myocardial infarction, but genotypes are not changed by an infarct. Finally, by virtue of its design, a cross-sectional study is limited to non-fatal cases of myocardial infarction. If the A allele would be associated with an increased proportion of fatal cases of myocardial infarction, then we would underestimate the true risk for myocardial infarction associated with possession of the A allele. To examine whether the A allele is associated with an increased proportion of fatal cases of myocardial infarction, a longitudinal study is needed.

Assessment of causality of the association between fibrinogen and risk for myocardial infarction has been difficult. Fibrinogen, as an acute phase protein, is strongly associated with (preclinical) atherosclerosis and also with a number of cardiovascular risk factors, notably smoking.[13,14,15] Therefore the link between fibrinogen and cardiovascular events may have been due to the association of fibrinogen with these other factors. Examining the association between a determinant of fibrinogen that is independent of these other factors, and the risk for myocardial infarction provides an estimate of the association between fibrinogen and myocardial infarction that is unbiased with respect to the other determinants of fibrinogen. Our "independent determinant" of plasma fibrinogen was the -455G/A polymorphism. Nevertheless, the question remains whether fibrinogen measurement with the clotting rate based method as described by von Clauss is specific enough to determine the association between plasma fibrinogen and the risk for myocardial infarction. At least three molecular forms of fibrinogen have been identified which may have different effects on the risk for myocardial infarction.[16] Recent studies on fibrates and ticlopidin show reductions in fibrinogen as measured with a functional clotting rate based method, and unchanged fibrinogen molecular mass.[17] We can not exclude the possibility that the increase in fibrinogen level due to the -455G/A polymorphism reflects another form than the 'risk carying' form of fibrinogen.

Quantitative studies in humans are by their nature limited in precision, and it is never possible to achieve the degree of control possible in a laboratory. Perhaps the most persuasive evidence to support a judgement of cause-effect relationship arises when a number of studies show similar results. The association between the -455G/A polymorphism and myocardial infarction has also been studied in the ECTIM study.[2,4] The findings in this study are similar to ours. The authors suggest that selection bias and/or the relatively weak association between the -455G/A genotypes and plasma fibrinogen levels may explain the lack of association between the -455G/A polymorphism and the risk for myocardial infarction. As discussed earlier, we find no reasons to assume selection bias in our study. Furthermore, in the Northwick Park Heart Study a rise in plasma fibrinogen level of one standard deviation (0.59 g/l) increased the risk for (future)

non-fatal ischemic heart disease by 60%.[18] From this estimate the difference in fibrinogen levels between the genotype with at least one A allele compared to that without an A allele (0.25 g/l) would be associated with a 25% increase in risk (we did not use the risk estimate from the present study, because of its cross-sectional design). In the ECTIM study and the present study the risk for myocardial infarction in subjects with the A allele did not even show a trend to an increase.

Stratified analyses did not support the presence of modification of the strength of the (absent) association for any of the factors mentioned. It remains possible that some unknown factor modifies the effect of possession of the A allele on the risk for myocardial infarction. Still, one expects an overall trend to an increase in risk for myocardial infarction. As the risk for non-fatal myocardial infarction did not even tend to be increased in the A allele carriers, we presume that possession of the A allele is not associated with an increased risk for non-fatal myocardial infarction.

The association between the -455G/A polymorphism and plasma fibrinogen was not found in current smokers and not in cases with a history of myocardial infarction. A possible explanation for this is that in smokers and in cases other factors increase the fibrinogen level and thereby obscure the contribution of the A allele. In the ECTIM study the association between the -455G/A polymorphism and fibrinogen level was, however, more pronounced in smokers and in cases.[2] A difference between the ECTIM Study and our study is that in our study smoking status and fibrinogen level were assessed at the same time, whereas in the ECTIM study blood samples were taken 3 to 9 months after the myocardial infarction and cigarette consumption was defined as the daily consumption just before the event.

For clinical practice the question of causality is important. The role of fibrinogen as an indicator for an increased risk for myocardial infarction is well established. Our finding suggests that lowering of fibrinogen levels directly may not decrease the risk for myocardial infarction. Increased plasma fibrinogen level may, however, change following reductions in risk by other measures.

In conclusion, the finding that the A allele of the -455G/A polymorphism in the ß-fibrinogen gene is associated with increased plasma fibrinogen levels, but not with an increased risk for myocardial infarction, does not support the view that this increased plasma fibrinogen level is causally related to the risk for myocardial infarction.

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4.4 Response to activated protein C

The resistance to activated protein C (APC)[1] is a recently described abnormality of the coagulation that is associated with a 3 to 10 fold increased risk of venous thromboembolism[2,3,4,5]. APC is the key component in a physiologically important anticoagulant system that cleaves and inactivates the prethrombotic factors Va and VIIIa. Resistance to APC is frequently associated with a point mutation in the factor V gene, the mutation affects the site of cleavage of factor Va by activated protein C, rendering it relatively resistant to inactivation which leads to an increased thrombotic tendency.

Thrombotic occlusion of the coronary arteries is the general cause of myocardial infarction. In addition to local vessel wall characteristics, the balance of procoagulant and anticoagulant factors undoubtedly plays a critical part in determining the occurrence, extent, and stability of the occlusive coronary thrombus and hence the risk of myocardial infarction.[6,7] Similarly, thromboembolism in the cerebral arteries is a frequent cause of stroke and transient ischaemic attacks. Some have suggested that APC resistance may be associated with increased risk of arterial thrombosis.[8,9] Others did not find support for the view that factor V mutation is associated with increased risk of myocardial infarction or cerebrovascular events.[10,11,12,13]

An extremely low response to APC, is often due to the single base mutation of the factor V gene, arginine 506 to glutamine.[14,15,16] Accordingly, a functional test for APC response is usually used as a screening test for the factor V mutation. The level of the threshold is based on an arbitrary cutoff point in the APC distribution in healthy subjects. Subjects with values below a certain threshold are considered potential carriers of the mutation. However, not all low values are caused by the factor V mutation. We aimed at establishing other determinants of the APC response level. We evaluated whether the APC response, as a continuous variable, is associated with arterial thromboembolism by comparing APC response and prevalence of the factor V mutation in patients with and without a history of stroke, transient ischemic attack or myocardial infarction.

Methods

Population

A case-control study was performed among subjects from the Rotterdam Study, a prospective study of a cohort of 7,983 men and women aged 55 years and over. The aim of the Rotterdam Study is to investigate the incidence of and risk factors for chronic disabling diseases. Its rationale and design have been described previously ([17]). Between March 1990 and July 1993 all 10,275 men and women aged 55 years and older, living

in Ommoord, a district of Rotterdam, the Netherlands, were invited to participate. The study consisted of an initial home interview by a trained research assistant and a series of medical examinations made during two visits at the research center. The study has been approved by the Medical Ethics Committee of the Erasmus University and written informed consent was obtained from all participants. The overall response rate was 78%.

Selection of cases and controls

Subjects with a history of myocardial infarction (n = 115) were selected based on the presence of an infarction on the ECG, using the diagnostic classification system of the Modular ECG Analysis System,[18,19] independent of a history of chest pain. Subjects with a definite or probable history of transient ischemic attack (TIA) (n = 55) were drawn based on a positive medical history of TIA obtained in two stages. First four screening questions on presence of temporarily visual, locomotor, sensory or speech disturbances and when affirmative, in addition to general information, such as date of event, onset, duration, and associated symptoms, a detailed history of the symptoms was obtained. Based on this information, symptoms were classified by a neurologist (PJ Koudstaal) as definite TIA, probable TIA, and no TIA. Subjects with a history of stroke (n = 62) were selected based on a medical history of stroke based on the question 'Did you ever suffer from stroke, diagnosed by a physician'. Five subjects had a history of TIA as well as one of stroke. Control subjects (n = 222) were drawn from those without arterial disease, i.e. with no history of myocardial infarction, stroke or transient ischemic attack, a normal ECG and with an ankle to arm systolic pressure ratio above 0.9;[20] control subjects were frequency matched to the cases of myocardial infarction (they were selected from the 5-year-age strata where the cases of myocardial infarction were found). Cerebrovascular disease was defined as either a stroke or a transient ischemic attack. Subjects using anticoagulant drugs at the time of examination were excluded.

Measurements

Subjects were all visited at home. Information on current health status, medical history, drug use, and smoking behavior was obtained by a computerized questionnaire. The home interview was followed by two visits at the research center. During those visits several cardiovascular risk indicators were determined. Height and weight were measured, and body mass index was calculated (in kilograms per meter squared). Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer, and the average of two measurements obtained at one occasion was used. Blood pressure at the ankles (posterior tibial artery) was measured in supine

position just above the malleoli, with an 8 MHz Doppler transducer. The ankle arm pressure ratio is the ratio of the systolic blood pressure at the ankle to the arm systolic pressure (20). ECG was recorded and coded using the Means computerized coding system (18,19). Blood sampling procedures and storage have been described elsewhere.[21] Blood was collected in tubes containing 0.129 M sodium citrate. All tubes were stored on ice before and after blood sampling. Platelet poor plasma was obtained by a two stage centrifugation: firstly at 1600 g, at 4°C for 10 minutes and after carefully transferring the plasma midlayer a second centrifugation was performed at 10000 g, at 4°C for 10 minutes. Plasma was immediately frozen in liquid nitrogen and stored at -80°C. Mean storage time was two years. Plasma from 30 apparently healthy volunteers was centrifuged for 30 minutes at 2000 g, at 4°C and pooled to serve as reference plasma for the APC response test. The APC response in the pooled plasma was 3.27.

The response of the plasma activated partial thromboplastin time (APTT) to APC was determined in platelet poor plasma that was collected in 0.129M sodium citrate, using the Coatest APC resistance test of Chromogenix (kit no. 0548-51) and expressed as the ratio of APTT with and without addition of APC. Serum total and high density lipoprotein (HDL) cholesterol were determined with an automated enzymatic procedure.[22]

For each case and control subject the whole blood collected and stored at baseline was thawed and underwent DNA extraction. Genotype assay using the polymerase chain reaction (PCR) technique was performed by an investigator (P.E.S.) and laboratory personnel who were unaware of each subject's status as a case or control subject. The G1691A mutation was detected by amplification of a 220-bp fragment of exon 10/intron 10 of the factor V gene, followed by digestion with the restriction enzyme *Mnl* I. The primers and conditions that were used were described elsewhere.[14,23] The 220-bp polymerase chain reaction (PCR) product of a normal factor V allele was cleaved by *Mnl* I in fragments of 116, 67 and 37 bp, whereas the 1691A allele resulted in fragments of 153 and 67 bp.

Statistical analysis

Means and proportions of potential determinants of APC response, such as gender, age, body mass index, systolic and diastolic blood pressure, serum total and HDL cholesterol, APTT, factor V mutation and current smoking were calculated for five categories of APC response and adjusted for caseness, i.e. a history of myocardial infarction or cerebrovascular disease (two dummy variables in the regression model) using linear regression analysis.

Logistic regression was used to assess the association of cerebrovascular disease and myocardial infarction to APC response and the factor V mutation. The odds ratio as

estimated from the logistic model was used as the measure of association and is referred to as relative risk (RR). With myocardial infarction or cerebrovascular disease (stroke and transient ischemic attack) as outcome variable we compared the levels of APC response and genotypes of the factor V mutation adjusted for age (numerical variable) and gender. By adding current smoking, total cholesterol and APTT as covariates in the logistic regression model we evaluated whether these potential confounding factors affected the estimates of the relative risks. No blood cells were available for 5 subjects, therefore the factor V mutation is missing for those subjects. In the regression models with factor V as a confounder the indicator method for missing data was used.[24]

Results

Activated protein C response

APC response ranged from 1.5 to 9.5. The mean APC response was 4.3 (SD 1.1) among control subjects, 3.9 (1.0) for subjects with a history of cerebrovascular disease and 4.3 (1.1) for those with a history of myocardial infarction. Mean APC response was 2.5 (0.6) for subjects with the mutation and 4.3 (1.0) for those without the mutation. Mean APC response was higher in men (n=202) than in women (n=247); 4.5 (95% confidence interval (CI) 4.4, 4.7) and 3.9 (CI 3.8, 4.1) respectively. Several cardiovascular risk factors that may influence the APC response were compared across five levels of APC response (Table 4.4.1). In men, but not in women, APC response decreased with age with 0.18 (CI 0.01, 0.35) per decade. Smoking men had a higher APC response than non-smoking men, mean difference 0.47 (CI 0.16, 0.78). APC response in smoking women was not different from that in non-smoking women. Again in men, but not in women, increased levels of cholesterol were associated with decreased levels of APC response. An increase of cholesterol of 1 mmol/l was associated with a decrease in APC response of 0.14 (CI 0.01, 0.27). A high APTT was associated with a slightly increased APC response (Table 4.4.1).

The relative risk of cerebrovascular disease (stroke and TIA) increased gradually, with 43% for each decrease in APC response by one unit (relative risk 1.43 (CI 1.12, 1.81), after adjustment for age and gender. Analyses for stroke and transient ischemic attack separately revealed no material differences; relative risks 1.32 (CI 0.99, 1.77) and 1.56 (CI 1.14, 2.14) respectively. The relative risk of cerebrovascular disease according to the different levels of APC response is presented in Figure 4.4.1, and in Table 4.4.2 crude and adjusted risks are presented. Adjustment for presence of the factor V mutant allele did not substantially change the results; the adjusted relative risk of cerebrovascular disease for each decrease in APC response of one unit was 1.43 (CI 1.12, 1.81).

APC response was not associated with the risk of myocardial infarction (RR 1.10 (CI 0.89, 1.37). In Table 4.4.3 crude and adjusted risks are presented.

Factor V mutation

In control subjects the prevalence of heterozygosity for the factor V mutation was five percent (11/222). The prevalence of heterozygosity for the mutation was not different in cases; in cases of cerebrovascular disease it was 6 percent (6/107) and in cases of myocardial infarction it was 4 percent (4/114).

The relative risk of myocardial infarction for those with the mutation relative to those without was not increased, (relative risk 0.77, (CI 0.24, 2.55)). The relative risk of cerebrovascular disease for those with the mutation compared to those without was also not increased (relative risk 1.12 (CI 0.40, 3.15)).

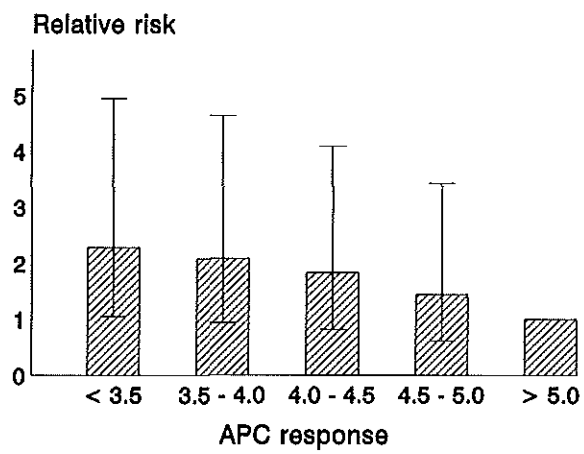

Figure 4.4.1 Relative risk of stroke and TIA according to level of APC response (adjusted for age and gender).

Table 4.4.1 Cardiovascular risk factors in strata of APC response.*

Activated protein C response	<3.5	3.5-3.9	4.0-4.4	4.5-4.9	≥5	p(trend)
Number	108	96	95	67	83	
Age (years)	73	73	71	73	72	0.33
Women (%)	69	68	52	45	32	0.0002
Body mass index (kg/m^2)■	26	27	26	27	27	0.33
Systolic blood pressure (mmHg)■	140	140	143	142	142	0.81
Diastolic blood pressure (mmHg)■	73	74	74	74	75	0.42
Total cholesterol (mmol/l)■	6.6	6.5	6.5	6.3	6.3	0.07
HDL cholesterol (mmol/l)■	1.3	1.4	1.3	1.2	1.3	0.56
Current smoking (% yes)■	20	11	17	29	26	0.03
APTT (s)■	30	30	30	31	31	0.02

* Values are adjusted for case-control status.
■ Adjusted for age and gender.

Table 4.4.2 Relative risk of cerebrovascular disease (stroke and TIA) by levels of APC response.

Activated protein C response	<3.5	3.5-3.9	4.0-4.4	4.5-4.9	≥5
Cerebrovascular disease (n)	32	28	21	16	15
Control subjects (n)	48	50	43	33	48
Crude risk	2.27 (1.09,4.70)	1.66 (0.79,3.53)	1.56 (0.71,3.42)	1.55 (0.67,3.58)	1*
Adjusted for age and gender	2.43 (1.13,5.22)	1.83 (0.84,3.98)	1.78 (0.80,3.97)	1.61 (0.69,3.74)	1*
Adjusted for other confounders▪	2.57 (1.17,5.65)	2.15 (0.96,4.38)	2.02 (0.89,4.61)	1.51 (0.63,3.59)	1*

* Reference risk

▪ Adjusted for age, gender, current smoking and serum total cholesterol (mmol/l)

Table 4.4.3 Relative risk of myocardial infarction by levels of APC response.

Activated protein C response	<3.5	3.5-3.9	4.0-4.4	4.5-4.9	≥5
Myocardial infarction (n)	24	22	29	20	20
Control subjects (n)	48	50	43	33	48
Crude risk	1.20 (0.56,2.46)	1.06 (0.51,2.18)	1.62 (0.78,3.28)	1.45 (0.68,3.13)	1*
Adjusted for age and gender	1.53 (0.72,3.24)	1.36 (0.64,2.91)	1.84 (0.90,3.78)	1.55 (0.72,3.38)	1*

* Reference risk

Discussion

We assessed APC response and factor V mutation in subjects with and without a history of cardiovascular disease, in particular stroke, transient ischaemic attack and myocardial infarction. A decreased APC response was associated with an increased risk of non-fatal stroke and transient ischemic attack, which was independent of the factor V mutation. APC response was not associated with myocardial infarction. Our findings did not show an increased risk of myocardial infarction or cerebrovascular disease in subjects with the factor V mutation.

The present study was based on a large population based cohort of caucasian men and women aged 55 years and over. Response rates were high in the study, controls were randomly sampled and laboratory analysis were performed blinded. Some limitations need to be discussed. First, because of its cross-sectional design, the study was restricted to non-fatal cases of cerebrovascular disease and myocardial infarction, and the APC response has been measured after the events (myocardial infarction, stroke, transient ischemic attack). To evaluate whether APC response changes after events, and whether the association between an increased risk of cerebrovascular disease and a decreased APC response is different for fatal compared to non-fatal cerebrovascular disease, a longitudinal study is needed. Second, the cases of stroke were based solely on a history of stroke. No confirmation of strokes by a neurologist was available. Yet, the possible misclassification of cases of stroke is probably random, i.e. not associated with the APC response level, and if so, this will result in an underestimation of the true association. Third, no information was available on the nature of the strokes (haemorrhage or infarction). In the Netherlands haemorrhagic strokes constitute about 15% of the total number of strokes.[25] Therefore, under the assumption that a decreased APC is associated with increased risk of ischemic stroke, our empirical risk estimate is again more likely to be an underestimation of the true association. The information on transient ischemic attacks and myocardial infarction is obtained with a higher level of accuracy, therefore misclassification of disease status is less likely.

APC response levels in our study are higher than reported by others. This may partly be the result of our methods of processing the blood. We evaluated APC response in double centrifuged platelet poor plasma. Absence of blood platelets in plasma is associated with an increased APC response.[26]

Antiphospholipid antibodies (APA) are antibodies that inhibit in vitro phospholipid dependent coagulation reactions, such as the APTT. Presence of APA in plasma may cause a decreased response to APC. Also APA have been associated with increased risk of arterial thrombosis.[27] Therefore, APA may confound the association between APC

response and cerebrovascular disease. The prevalence of APA is unknown. An APTT longer than 40 s is indicative of presence of APA in plasma. In our study 5 subjects had an APTT longer than 40 s, only one of them had a decreased APC response, 1.53. Therefore we presume that the role of APA in our study is of negligible importance.

A unique feature of the present study is that we chose to analyze APC response as a continuous parameter. Decreased APC response, when below an arbitrary cutoff point that is chosen from the distribution of APC response in healthy controls, called APC resistance, is only partly determined by presence of the factor V Leiden mutation. In addition to the mutation, the APC response test may recognize other mechanisms, including hitherto unknown components of the protein C/protein S system. In our study a decrease in APC response was associated with a gradual increase in the risk of cerebral thromboembolism, independent of the factor V mutation. Our findings suggest that the APC response may be used as a continuous measure for risk of cerebrovascular disease. Decreased APC response indicates an increased risk of cerebrovascular disease but not of myocardial infarction. Seemingly, the brain is more sensitive to an imbalance of the protein C/protein S system. It may be hypothesized that the protein C/protein S system indeed has different pathophysiologic mechanisms in the brain, because thrombomodulin, an endothelial cofactor protein for thrombin mediated protein C activation, is present in all human tissues except the brain.[28]

In the Physicians' Health Study, a longitudinal study among 14,916 apparently healthy men, the risks of myocardial infarction and stroke in men with the mutation were not increased (10). We also did not find an increased risk of cerebrovascular disease or myocardial infarction in patients with the factor V Leiden mutation. These findings strengthen the hypothesis that the risks of myocardial infarction and stroke are not increased in subjects with the factor V Leiden mutation.

Until now non-genetic determinants of APC response levels have hardly been recognized. Our finding that APC response is determined by other factors but the factor V mutation, such as age, gender, smoking and serum total cholesterol, may have important implications for the use of the APC response test as a screening test for the factor V mutation. Men have higher values than women and the APC response levels decrease with age in men. Halbmeyer and colleagues proposed that APC resistance predisposes to stroke (8). They defined a normal APC response on the basis of their findings in healthy controls of whom 24 percent was female. From their cases of stroke 18 out of 30 (60%) were female. The fact that women have lower levels of APC response and are differently distributed among case and control subjects may in part explain their findings.

In conclusion a decreased APC response is associated with an increased risk of

cerebrovascular disease, but not with myocardial infarction. This association is independent of the factor V mutation. Our findings do not show an increased risk of cerebral or myocardial thromboembolism in subjects with the factor V Leiden mutation in a population based study of older men and women. The APC response is influenced by gender, age, smoking and serum total cholesterol.

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5 General discussion

Thrombus formation at arterial sites is assumed to be dependent on at least three factors: (1) the depth of vessel wall injury and the thrombogenicity of the exposed plaque[1,2]; (2) hemodynamic factors that promote vasoconstriction and stasis of blood flow; and (3) the thrombophylic tendency in the blood. Thrombophylic tendency may be reflected in plasma levels of haemostatic parameters. The present thesis addresses the question whether plasma levels of haemostatic parameters in peripherally collected blood are associated with the risk for arterial thrombus formation. As a thrombophylic tendency may be associated with variations in the genes coding for haemostatic factors, we additionally studied the association of selected polymorphisms in genes for haemostatic parameters with the risk for thrombus formation.

Haemostasis plays a role in arterial disease

The evidence that an individuals' haemostatic function is, under particular circumstances, causally related to thromboembolic arterial disease has been available from several sources. It has been shown that subjects with haemophilia have a lower risk for cardiovascular death. In subjects with factor VIII activity below 0.40 IU/ml, the risk to die from cardiovascular disease was 80 percent lower than in subjects without haemophilia (relative risk 0.2 (95% CI 0.0,0.7).[3] Remarkable in this study was also that subjects with haemophilia appeared to have as much atherosclerosis as others. This suggests that haemostatic function plays a role in the acute phase of occlusion of arteries, but not in the atherosclerotic process. However, one has to take into account that

subjects with haemophilia have extraordinarily low levels of specific haemostatic factors (factor VIII or factor IX). It is therefore disputable whether it may be inferred from these findings that an intermediately decreased factor VIII level is also protective for cardiovascular disease or even that increased factor VIII levels increase the risk for cardiovascular disease.

Furthermore, the use of drugs that influence vitamine K dependent clotting factors has clearly been shown to reduce the risk for arterial thrombo-embolic events.[4] Again, these drugs induce such an extreme disturbance of haemostasis that these findings may not give us direct information as to whether haemostatic function in subjects without medication also determines the risk for arterial disease. This inspires to examine the role of haemostasis in cardiovascular disease.

Plasma levels of haemostatic parameters

Previous studies

The association between haemostatic parameters and the risk for thrombus formation at arterial sites has been studied in several longitudinal and cross-sectional studies. The number and type of haemostatic parameters measured vary by study. In order to provide an overview of the knowledge that has been obtained from the various studies, design and main findings from longitudinal studies on the association between levels of haemostatic (coagulation and fibrinolytic) parameters and risk for coronary heart disease are summarized in Table 5.1 and Table 5.2. As there are generally less sources of bias in studies with a longitudinal design, only the longitudinal studies on the association between haemostatic parameters and the incidence of ischemic heart disease that have so far published their results are included. The first nine columns of Table 5.2 show findings from studies among apparently healthy subjects. The tenth column summarizes longitudinal findings from the Rotterdam Study. The 11th to 15th columns show findings from studies among subjects that already suffered from cardiovascular disease at inclusion into the study. As preclinical atherosclerosis may be important to explain (part of) an association between haemostatic parameters and risk of thrombo-embolic events, the last column presents the associations between the haemostatic parameters and atherosclerosis, as measured by the ankle to arm blood pressure index in the Rotterdam Study.

Table 5.1. Methods of the longitudinal studies that stuied the incidence of ischemic heart according to baseline levels of haemstatic parameters.

Study	Population (age range)	Follow-up (years)	End points
NPHS[25,26]	1511 men (40-64)	11.0	109 first coronary events
Göteborg **1913**[29]	792 men (54)	13.5	92 cases of MI 60 deaths due to CVD
Leigh[31]	384 men (40-69)	7.3	40 cases of MI
Framingham[32]	554 men, 761 women (47-79)	14	312 ischemic heart disease events
Caerphilly[28]	4860 men	5.1/3.2	251 coronary events
PROCAM[33]	2116 men (40-65)	6	82 coronary events
Göteborg **1933**[30]	1016 men (50)	9	27 non-fatal MI 9 deaths due to CHD
GRISP[34]	5239 men (40-60)	5	107 cases of MI
PHS[12]	22071 men	5	216 cases of MI
Rotterdam Study	7,983 men and women (55 and over)	3	150 cases of MI
Edinburgh[5]	617 CI pts*	1	36 coronary heart disease events
PLAT[6,7]	953 pts with arterial disease	2	80 end points of ischemic disease
ECAT[11]	2960 AP pts+		106 definite coronary events
Hamsten[8]	109 MI└	3	16 reinfarctions
Jansson[10]	213 AP pts	7	28 death due to cardiovascular disease

* CI pts = patients with intermittent claudication
\+ AP pts = patients with angina pectoris
└ MI = non fatal cases of myocardial infarction in men aged 45 years or less

Table 5.2. Results of the longitudinal studies on the association between plasma levels of haemostatic parameters and the risk for cardiovascular disease.

	NPHS	1913	Leigh	Fram	Caerph.	PROC	1933	GRISP	PHS	RS	Edinb.	PLAT	ECAT	Hamsten	Jenssen		AABPI
Coagulation																	
APTT										^			=				=
F1+2																	=
Fibrinogen	^	^	^	^	^	^	^		^	^	^	^					^
FPA											=		=				
D-Dimer									^		^	^					^
V	=																
VIIc	^				*	^						*					=
vWF					*					^	=	^	^				=
VIIIc	=	=				*				^		^	=				^
ATIIIc	U											*	=				
TAT										=							=
Fibrinolysis																	
Plasminogen		=											=				
alfa2-AP													=				
PAI-1 ag									=	=			=	^	^		=
PAI-1 c													=		=		
t-PA ag									^	^			^		^		^
t-PA c													=				^
PAP										=							=
WBCLT	=	=															
ECLT													=				

^ positive association between level of haemostatic factor and risk for myocardial infarction

= no association between level of haemostatic factor and risk for myocardial infarction

U U-shaped association between level of haemostatic factor and risk for myocardial infarction

* level of heamostatic factor is measured, but data on association with disease not reported (yet)

APTT activated partial thromboplastin time

F1+2 prothrombin fragment 1+2

FPA fibrinopeptide A

V factor V

VIIc factor VII activity

vWF von Willebrand factor

VIIIc factor VIII activity

ATIIIc antithombin activity

TAT thrombin/antithrombin complex

alfa2AP α2-antiplasmin

PAI-1ag plasminogen activator inhibitor type 1 antigen

PAI-1c plasminogen activator inhibitor type 1 activity

t-PA ag tissue type plasminogen activator antigen

t-PA c tissue type plasminogen activator activity

PAP plasmin/antiplasmin complex

WBCLT whole blood clot lysis time, ECLT = euglobulin clot lysis time

AABPI ankle to arm blood pressure index

Reasons for absence of an association between plasma levels and risk

In longitudinal studies on haemostatic factors and the risk for thromboembolic events the time interval between measurement of the parameters and the event of interest varies from several months to about 16 years (Table 5.1). The half life of active circulating proteins ranges from seconds to several hours. Whether the levels at one point in time are related to levels several years later, at the time of the myocardial infarction, is not known. Therefore, even if plasma levels may be important risk indicators for thromboembolic events, the time interval between the moment of collection of the blood and the myocardial infarction may limit discovery of this fact.

In addition, it is conceivable that not the plasma levels of the haemostatic parameters per se, but rapid or excessive changes in levels trigger thromboembolic events. This may, for example, explain why it has been so difficult to disclose an independent association between plasma PAI-1 and risk for myocardial infarction in observational studies, while data from experimental studies suggest that increased plasma PAI-1 levels directly increase the risk for arterial thrombus formation.[9] The 4G allele of the -675(4G/5G) polymorphism at the PAI-1 gene locus is associated with increased plasma levels of PAI-1 and it appears to be associated with a higher morning increase in plasma PAI-1. This 4G allele is also associated with an increased risk for myocardial infarction. In our study, as well as in others,[10,11,12] plasma PAI-1 was not independently associated with the risk for myocardial infarction. This suggests that marked increases of plasma PAI-1 levels, but not the circulating levels of PAI-1 per se, may be associated with an increased risk for myocardial infarction.

Reasons for artefactual associations between plasma levels and risk

Any association between plasma levels of haemostatic factors and arterial disease may be due to confounding. Two main sources of confounding have to be considered;

(1) The concentration of the haemostatic parameters may be a direct consequence of pre-existing disease, in particular (pre-clinical) atherosclerosis. As atherosclerosis is associated with an increased risk for thromboembolic events, this may lead to the incorrect conclusion that the concentration of the haemostatic parameter is positively associated with the risk for thromboembolic events. From several studies it has become clear that pre-clinical atherosclerosis is associated with the concentration of haemostatic variables, possibly through inflammatory mechanisms.[13,14,15,16,17] Associations between haemostatic parameters and atherosclerosis as observed in the Rotterdam Study, presented in Table 5.2 are consistent with these findings.

(2) The association between the haemostatic parameters and the increased risk for thromboembolic events may reflect the association between the haemostatic parameters

and other true risk factors, such as smoking, increased serum cholesterol, and blood pressure. Haemostatic parameters have been associated with many of the established cardiovascular risk factors.[18,19,20] Figure 5.1 summarizes the potential associations between plasma levels of haemostatic parameters, other risk factors for thromboembolic events, and the presence of (pre-clinical) atherosclerosis.

As plasma levels of haemostatic risk indicators are associated with preclinical atherosclerosis and with cardiovascular risk factors, and as it is impossible to exactly quantify atherosclerosis, the exact exposure to most of the established cardiovascular risk factors and the plasma levels of the haemostatic parameters exactly at the moment of interest, it is virtually impossible to demonstrate a truly independent association between levels of haemostatic parameters and the risk for thromboembolic events.

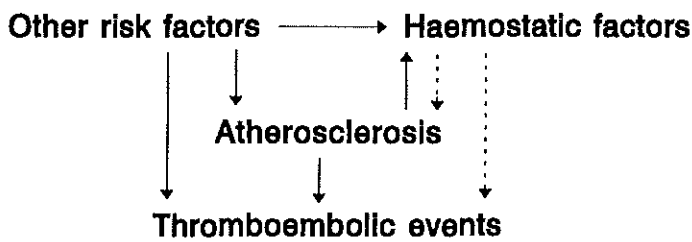

Figure 5.1 Potential associations between haemostatic parameters, (pre-clinical) atherosclerosis, other risk factors for thrombo-embolic events, and the risk for thrombo-embolic events.

Inferences

From Table 5.2 it is clear that for investigators fibrinogen has, so far, been the most rewarding risk indicator for the risk for ischaemic heart disease. Besides fibrinogen, D-Dimer, von Willebrand factor, factor VIII, and tissue type plasminogen activator antigen have repeatedly and consistently been associated with an increased risk for ischemic heart disease. Von Willebrand factor was positively associated with clinical cardiovascular disease, but not with asymptomatic carotid atherosclerosis in other studies, which may indicate that von Willebrand factor is more involved in the acute thrombotic component of cardiovascular disease than in atherogenesis.[11,21,22] Besides von Willebrand factor, all other indicators for an increased risk of ischaemic heart disease are also increased with atherosclerosis. This may suggest that plasma levels of the haemostatic parameters directly cause atherosclerosis. Alternatively, it may suggest that

the increased levels of haemostatic parameters may be the result of presence of (preclinical) atherosclerosis and that haemostasis is affected by the presence of atherosclerosis. A possible scenario is a vicious circle starting with vessel wall alteration by chronic stimuli resulting in a mild acute-phase reaction with fibrinogen elevation and local fibrin deposition with thrombus formation. Activation of fibrinolysis then produces split products which attract cells leading to lipid accumulation and plaque growth. Fissure of the established plaque again triggers an acute-phase response with repetition of the cycle. The end result of several such cycles may be occlusion of the vessel and occurrence of symptomatic cardiovascular disease (Figure 5.2).

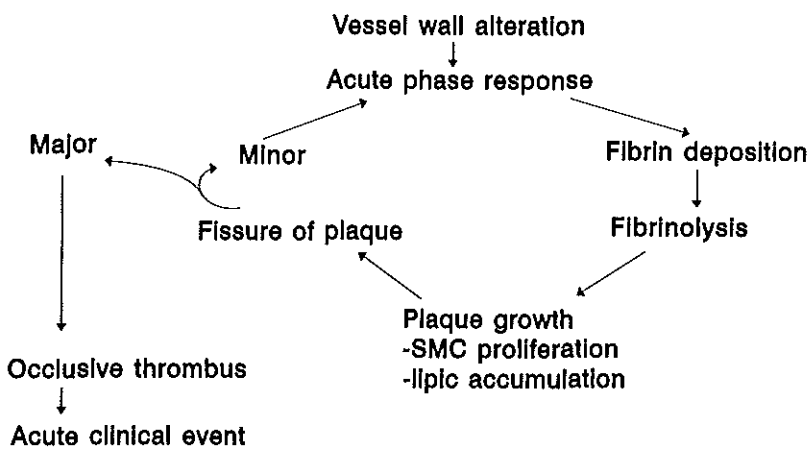

Figure 5.2 Vicious circle leading from plaque to symptomatic disease.

Genetic parameters

In assessing plasma levels of haemostatic parameters as risk factors for thromboembolic events, three major disadvantages were identified: (1) plasma levels have a short half life and may differ markedly in time (circadian, and seasonal variation), (2) plasma levels may be influenced by presence of preclinical atherosclerosis, and (3) by other cardiovascular risk factors, such as serum total cholesterol. In contrast to plasma levels, genetic parameters are constant. Genes are not affected by atherosclerosis or cardiovascular disease risk factors. Therefore, confounding of the associations by these factors is unlikely.

However, associations between genetic parameters and risk for disease may also be

the result of bias. If a genotype is associated with mortality, this may lead to misleading results. Care is needed in particular in the interpretation of associations found in cross-sectional studies, but also in longitudinal studies this problem may occur.

In addition, an association of risk for cardiovascular disease with a marker polymorphism does not directly disclose the underlying mechanism. A polymorphism may lead to changes in blood concentrations of the haemostatic parameters, to changes of the induction pattern after stimuli, or to local dysfunction in tissue remodelling. It is not a priori clear which aspect of the effect is essential for the pathogenetic effect studied. Furthermore, a polymorphism in the gene for a haemostatic factor may be a marker for a mutation in a another gene located nearby the gene for the haemostatic factor that has no bearing on the haemostatic function whatsoever.

Contributions from the Rotterdam Study

Arterial disease is not only associated with a hypercoagulable but also with a higher fibrinolytic state

In patients with coronary, cerebrovascular and peripheral arterial disease we found an increased concentration of fibrin degradation products, as measured by D-Dimer. The blood level of D-Dimer is determined by two main factors: the amount of fibrin formed (providing the amount of substrate for formation of degradation products) and the activity of the fibrinolytic system.

We were the first to show that in order to increase D-Dimer levels in the presence of atherosclerosis, the concentration of plasminogen activator inhibitor (PAI-1) needs to be normal or relatively low. As PAI-1 is a potent inhibitor of fibrinolytic activity, we inferred that the fibrinolytic activity at loci of fibrin deposition is not impaired. In contrast, fibrinolytic activity must be relatively high in order to increase D-Dimer. As the presence of atherosclerosis itself does not increase PAI-1 levels, we put emphasis on 'relatively higher', instead of using the term 'increased' fibrinolytic activity.

Tissue type plasminogen activator activity (measured in acidified plasma) may be positively associated with the risk for myocardial infarction.

From findings in the Northwick Park Heart Study an association between low fibrinolytic activity and risk of ischemic heart disease had been suggested. An overall estimate of the blood fibrinolytic capacity, measured as lysis of clots from diluted blood, showed an association between a low activity and the risk of myocardial infarction.[26] Further development of laboratory techniques allowed in subsequent studies to study tissue type

plasminogen activator (t-PA) activity. A decreased t-PA activity, usually interpreted as the main determinant of clot-dissolving capacity of the haemostatic system, was found in patients with a history of myocardial infarction and was associated with an increased risk of second myocardial infarction.[23,24]

In the present study t-PA activity was measured in acidified plasma, which has the advantage that at the moment of blood collection, complex formation of t-PA and plasminogen activator inhibitor (PAI-1) is halted. T-PA activity was found to be positively associated with peripheral arterial disease. Moreover, subjects with an increased t-PA activity tended to have a higher risk for myocardial infarction. The latter finding was not statistically significant and should therefore be interpreted with caution. Nevertheless, this finding confirms the finding that atherosclerosis is associated with a relatively higher fibrinolytic activity.

Fibrinogen as a risk indicator rather than a risk factor

It was first observed in the Northwick Park Heart Study, among white men aged 40 to 64 years that fibrinogen is a strong and valid indicator for an increased risk of ischaemic heart disease.[25,26] Approximately half of all the coronary events occurred in the highest tertile of fibrinogen. Seven other longitudinal studies have so far confirmed a positive association between fibrinogen and cardiovascular disease, notably myocardial infarction and stroke, which has lead to the conclusion that fibrinogen is an independent risk factor for myocardial infarction.[27,28,29,30,31,32,33,34]

The mechanism by which increased levels of plasma fibrinogen cause an increased risk for myocardial infarction is largely unknown. Blood rheology,[35] platelet aggregation,[36] and endothelial function have been suggested to play a role. An increased concentration of fibrinogen causes an increased plasma viscosity which on its term may induce microcirculatory disorders, like reduced oxygen release in post-stenotic vascular segments.[37]

Plasma fibrinogen is associated with very early stages of preclinical atherosclerosis.[38] Furthermore, plasma fibrinogen concentration appears to be associated with virtually all other determinants of cardiovascular disease.[19] To determine an independent role of plasma fibrinogen with respect to the risk for cardiovascular disease, it is, therefore, of major importance to adjust the crude associations for all potential confounders of this association. As described earlier, it is virtually impossible to adjust accurately for all confounders. For example the classification of 'smoking' is always only an approximate of a subjects' real smoking behaviour that, of course, changes by the day or even by the hour.

We showed that fibrinogen, when increased due to the -455G/A polymorphism, is

not associated with an increased risk for myocardial infarction. This suggests that plasma fibrinogen is not an independent risk factor for myocardial infarction, but merely an indicator of increased cardiovascular disease risk.

Polymorphisms in the genes for haemostatic factors
We were the first to show an association between an *Alu* repeat insertion/deletion polymorphism in the gene for tissue-type plasminogen activator (t-PA) and the risk for myocardial infarction.

The -675(4G/5G) polymorphism at the PAI-1 gene locus had been associated with the risk for myocardial infarction, but findings were not confirmed in two other study populations. We propose that an explanation for this inconsistency may reside in the characteristics of the study populations. In our study the association was confined to smokers.

Suggestions for future research

Non-experimental research
(1) Etiognostic research
It is clear that haemostasis somehow plays a causal role in atherothrombotic disease. In addition, plasma levels of several of the haemostatic parameters are associated with the incidence of cardiovascular events. Whether circulating levels in plasma are causally related to the incidence of cardiovascular events is not clear. It appears that measurement of levels of haemostatic parameters at one occasion, with follow-up of cardiovascular events in the study population for several years, may not give us direct information with respect to causality of the association. To establish causality of the association, a different design of non-experimental studies may be considered:
(i) In non-experimental studies measurement of plasma levels of haemostatic parameters are commonly measured in subjects that have fasted and refrained from smoking for a certain amount of hours, at a specified moment in time. In future research advantages and disadvantages of these, commonly suggested to be the most ideal conditions, should be carefully considered. Our findings on a circadian variation in PAI-1 suggest a causal relation between the amount of increase of plasma PAI-1 in the morning (associated with homozygosity for the 4G allele of the -675(4G/5G) polymorphism at the PAI-1 gene locus) and risk for myocardial infarction. Had we measured the levels of plasma PAI-1 in all subject after fasting and at one similar time point, this information would have remained hidden.

(ii) Besides the time of blood collection also frequency of blood collection may lead to new perspectives. So far, little attention has been payed to changes in plasma levels of haemostatic parameters in individuals. Observational studies with repeated measurements of haemostatic parameters to examine changes in haemostatic parameters in time (24-hours, seasons), may help to answer these questions. Similarly, subjects might be identified that have a more pronounced seasonal variation or that have a higher increase in a certain haemostatic parameter in response to extraneous factors, such as smoking.
(iii) Besides plasma levels of haemostatic parameters, other measures to assess the role of haemostasis with respect to the risk for cardiovascular disease, such as genetic markers, and changes in plasma levels associated with genetic markers, may add substantially to the current knowledge.

(2) Prognostic research
Apart from their potentially causal role in cardiovascular disease, plasma levels of haemostatic parameters may be used to estimate absolute risks for cardiovascular events in individuals. Development of prognostic models with haemostatic and non-haemostatic risk indicators in subjects with and without cardiovascular disease may supply valuable tools for assessment of an individuals' cardiovascular prognosis.

The contribution of genetic parameters in determining absolute risks may be substantial. For example homozygosity for the 4G allele of the -675(4G/5G) polymorphism markedly increased the absolute risk for myocardial infarction in smokers.

(3) Diagnostic research
Thus far, plasma levels of several of the haemostatic parameters have been identified as indicators for the presence of (pre-clinical) atherosclerosis. Similar to its role in etiognostic and prognostic research, also in diagnostic research repeated measurements of the haemostatic parameters may be useful. We presume that assessment of changes in haemostatic indicators for atherosclerosis in time may help to diagnose worsening or amelioration of atherosclerosis.

Experimental research
For clarification of the mechanism involved in the association between haemostatic function and cardiovascular disease, experimental research is essential. In vivo studies in which response of the haemostatic system to extraneous factors, such as smoking, alcohol comsumption, (lipid rich) diet, or physical exercise is determined, will add to the understanding of the association. Additionally, experimental testing at cellular and

molecular level is needed for disclosure of the pathological mechanisms.

Implications for clinical practice

Over the years several modifiable risk factors have been identified that are associated with an increased risk for coronary heart disease or cerebrovascular disease. For example, intervention studies showed that treatment of mild to moderate hypertension reduces the risk of coronary heart disease and cerebrovascular disease. Also, recent trials demonstrated that lowering of serum cholesterol in subjects with elevated cholesterol results in a reduction of coronary heart disease risk. However, it has repeatedly been shown that subjects at the highest absolute risk of future cardiovascular events benefit much more from treatment than those at an intermediately increased risk.[39,40,41,42]

Practising physicians regularly have to decide whether a particular patient with certain cardiovascular characteristics should be put on drug treatment. In this decision the potential benefits and hazards of the drug treatment should be carefully weighted. To provide a 'tailored care', a number of guidelines has been issued in which the start of treatment in an individual patient is related to the individuals' absolute risk of future coronary heart disease and cerebrovascular disease.[43] So far, haemostatic risk indicators have not been included in these guidelines. It has, however, been shown that in patients with an elevated cholesterol level, fibrinogen could be used to identify those at particularly high risk for coronary events.[11,44] Fibrinogen and the other haemostatic risk indicators may be used to improve prognostic models.

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6 Summary

Developments in thrombolytic treatment of evolving transmural myocardial infarction, improved image quality on angiography, and the introduction of angioscopy have contributed to establish the significance of thrombosis in the pathogenesis of myocardial infarction, sudden death, unstable angina pectoris, and ischemic stroke. Hypercoagulability and impaired fibrinolytic function have been suggested to predispose to arterial thrombosis by promoting the formation of occlusive thrombi on fissured atherosclerotic plaques. It has been suggested that high levels of haemostatic factors directly increase the risk for myocardial infarction. This thesis is concerned with examining the role of the haemostatic system in arterial disease.

The observation that acute myocardial infarction and sudden cardiac death are more frequent in winter and in the morning indicates that the onset of cardiovascular events is not random, and provides a clue to a mechanism. It appears that an atherosclerotic plaque is exposed to systemic physiologic processes that could increase the likelihood of plaque rupture and thrombosis. Diurnal and seasonal variations in haemostasis may play a role in the temporal variation in the incidence of cardiovascular events. *Chapter 2* describes the seasonal variation of fibrinogen and the diurnal variation of plasminogen activator inhibitor type 1 (PAI-1).

Chapter 2.1 describes a population based cross-sectional study that was performed among participants of the Rotterdam Study, a cohort of 7,983 men and women, aged 55 years and over. Fibrinogen levels were measured by the prothrombin time derived method in the first 2,325 participants of the study. Fibrinogen levels were considerably higher in winter. The seasonal difference was 0.34 g/l (95 % confidence interval 0.29, 0.39) and was more pronounced in subjects aged 75 years and over than in subjects aged 55 to 75 years, 0.43 g/l (0.34, 0.52) and 0.29 g/l (0.24, 0.35), respectively. Additional adjustment for body mass index, systolic and diastolic blood pressure, and total and HDL cholesterol did not materially change the findings. Low outside temperatures were associated with increased levels of fibrinogen. Yet, after adjustment

for season, outside temperature was no longer related to fibrinogen. Adjustment for outside temperature did not change the seasonal variation of fibrinogen, seasonal difference 0.31 g/l (0.24,0.37). In conclusion, fibrinogen levels are highest in winter. The seasonal variation of fibrinogen is more pronounced in the elderly. Outside temperature plays a minor role in the seasonal variation of fibrinogen. Seasonal variation of fibrinogen may partly explain the increased cardiovascular disease mortality in winter.

Chapter 2.2 focuses on PAI-1 antigen that is known to follow a diurnal rhythm with a peak in the morning and a trough in the evening. The cause of this is unknown. A polymorphism in the promoter region of the gene for PAI-1 (4G/5G) partly determines PAI-1 antigen concentrations. We studied whether the diurnal variation of PAI-1 antigen concentration differs for the genotypes of the 4G/5G polymorphism. In 132 subjects with a history of myocardial infarction and 265 without a history of cardiovascular disease, all selected from the Rotterdam Study cohort, in which blood samples were drawn at different times of the day (between 8.00 AM and 16.00 PM), PAI-1 antigen concentration was measured. Possession of the 4G allele was associated with increased levels of PAI-1 antigen. In subjects homozygous for the 4G allele PAI-1 antigen concentration was 63 ng/ml (CI 57,70), in those heterozygous it was 52 ng/ml (47,56), and in those homozygous for the 5G allele it was 47 ng/ml (41,53). These differences were more pronounced in the morning. Homozygosity for the 4G allele (n = 128) was associated with an 78 percent (95% CI 45,119) higher PAI-1 antigen concentration in the morning as compared to that in the afternoon, whereas in heterozygous subjects it was 56 percent (33,82) higher in the morning and in those homozygous for the 5G allele it was 65 percent (31,107) higher in the morning. Respective PAI-1 antigen concentrations were 77 ng/ml (69,87), 65 ng/ml (58,73) and 57 ng/ml (49,67) in the morning, and 43 ng/ml (37,51), 42 ng/ml (38,47) and 36 ng/ml (30,43) in the afternoon. We concluded that the morning increase in PAI-1 antigen concentration seems to be more pronounced in subjects homozygous for the 4G allele. The relative contribution of the 4G allele in determining PAI-1 antigen concentration is higher in the morning.

Earlier findings that the levels of some haemostatic factors were different in subjects who have or will develop clinical cardiovascular disease as compared to subjects without cardiovascular disease, led to the concept of hypercoagulable state.

In *chapter 3.1* the presence of a 'hypercoagulable state' as assessed by indices of thrombin and plasmin generation and of the amount of fibrin that is lysed, in patients with coronary, cerebral and peripheral arterial disease was studied. In 127 subjects with a history of myocardial infarction, in 124 with a history of stroke and/or transient ischemic attack, in 131 patients with peripheral arterial disease and in 263 control subjects in the same age group without arterial disease were selected. Subjects using

anticoagulant drugs were not selected. Levels of prothrombin fragment 1+2 (F1+2), thrombin/antithrombin (TAT) complexes, plasmin/antiplasmin (PAP) complexes and D-Dimer (DD) were determined in all subjects. F1+2, TAT, and PAP were neither associated with a history of cardiovascular events, nor with peripheral arterial disease. In contrast, positive associations were found for D-Dimer. Mean D-Dimer level was 40 μg/l (95% CI 35,44) in control subjects; 53 μg/l (47,61) in subjects with a history of myocardial infarction and 51 μg/l (45,58) in subjects with a history of stroke and or transient ischemic attack. D-Dimer increased gradually with increasing severity of peripheral atherosclerosis; a decrease in ankle/arm systolic blood pressure ratio of 0.1 was associated with an increase in D-Dimer of 3.9 μg/l ($p<0.01$). This was more pronounced in subjects with higher F1+2, TAT and PAP concentrations. In conclusion, as concentrations of D-Dimer are increased in patients with coronary, cerebral and peripheral arterial disease, it appears that arterial disease is associated with increased formation and lysis of fibrin. This process is more pronounced in subjects with high F1+2, TAT and PAP, which mark the onset of coagulation and fibrinolysis respectively.

Increased concentrations of plasminogen activator inhibitor type 1 (PAI-1) and of D-Dimer have jointly been found in subjects with cardiovascular disease. To understand this apparent paradox of increased fibrinolytic activity (high D-Dimer) and increased fibrinolytic inhibition (high PAI-1), we examined in *chapter 3.2* the relation between D-Dimer, PAI-1 and the activator of fibrinolysis, tissue type plasminogen activator (t-PA) in subjects with varying severity of peripheral atherosclerosis. The study population was that of the same cross-sectional case-control study initially set up to elucidate the association between fibrinolytic factors and the risk for myocardial infarction. The ankle to brachial systolic blood pressure ratio, t-PA antigen and activity, PAI-1 antigen and D-Dimer were measured in all 325 subjects. T-PA antigen and t-PA activity were, independent of each other, increased with atherosclerosis; t-PA antigen increased with 3.5 ng/ml (SE 1.7, $p=0.04$) and t-PA activity with 0.46 IU/ml (0.20, $p=0.02$) per unit decrease in ankle to brachial pressure ratio (i.e. increase in atherosclerosis). PAI-1 antigen was not associated with atherosclerosis. Atherosclerosis was associated with increased D-Dimer, mainly in subgroups with PAI-1 antigen below 50 ng/ml, t-PA antigen below 10 ng/ml, or t-PA activity above 1.5 IU/ml. Concentration of PAI-1 and t-PA activity were not associated with D-Dimer concentration. A slight positive association was observed between t-PA antigen and D-Dimer. We concluded that PAI-1 antigen is not associated with D-Dimer. In subjects with peripheral atherosclerosis PAI-1 antigen is not increased, and low PAI-1 levels (and possibly also low levels of t-PA antigen and high levels of t-PA activity) appear to be required to increase D-dimer concentrations in these subjects. This suggests that increased D-dimer concentrations in subjects with atherosclerosis are not associated

with increased inhibition, but rather reflect higher activity of fibrinolysis.

Antithrombin is a potent inhibitor of thrombotic tendency. Whether atherosclerotic disease is associated with high or low antitrombin is unclear. Studies of the relation between antithrombin and presence of arterial disease have shown contrasting results. In *chapter 3.3* the association between atherosclerosis and antithrombin was evaluated. The ankle to arm pressure ratio is a graded marker for atherosclerosis, and provides the opportunity to investigate non-linear associations. In the first 1516 participants of the Rotterdam Study that did not use anticoagulants both the ankle to arm blood pressure ratio and antithrombin were measured. In men the association between the two was quadratic: antithrombin activity was increased in men with moderate peripheral arterial atherosclerosis compared to those without, whereas in men with more severe atherosclerosis it was decreased. In women the association was linear, a decreased ankle to arm pressure index was associated with an increased antithrombin activity. These associations were independent of smoking, body mass index, serum lipids, fibrinogen, and factor VIIc. We propose that the activity of antithrombin rises in response to increased risk of cardiovascular disease and also to the presence of atherosclerosis, while with increasing severity of the atherosclerotic process antithrombin may decrease in men. This may explain the contrasting results found in previous studies. Changes in antithrombin over time might be useful in predicting the risk of cardiovascular disease and progression.

In chapter 4 associations of plasma levels and genetic variants in the genes for several haemostatic factors with the risk for thrombo-embolic events are described. In *chapter 4.1* the association of a polymorphism in the gene for t-PA and of plasma concentrations of t-PA (antigen and activity) with the prevalence of myocardial infarction, was studied. We determined t-PA antigen and t-PA activity in plasma and genotyped for the *Alu* repeat insertion/deletion polymorphism in intron h of the t-PA gene in 121 subjects with a history of myocardial infarction and 250 controls. Homozygosity for the insertion was associated with twice as many cases of myocardial infarction compared to homozygosity for the deletion (odds ratio 2.24 (95% CI 1.11, 4.50). T-PA antigen was positively associated with the risk of myocardial infarction; compared to that in the lowest quartile, the relative risks (odds ratio) in the second, third and upper quartile were 1.7 (CI 0.9,3.3), 2.3 (1.2,4.4) and 2.0 (1.0,3.8), respectively. When adjusted for body mass index, HDL and total cholesterol, systolic and diastolic blood pressure and current smoking, the risk associated with t-PA antigen concentration attenuated. Increased concentrations of t-PA activity tended to be associated with an increased risk of myocardial infarction. In conclusion, this study provided evidence for an independent association of the insertion allele of the insertion/deletion polymorphism in the t-PA gene

with non-fatal myocardial infarction. Increased t-PA antigen is associated with an increased risk of myocardial infarction. However, this association was not independent of cardiovascular disease risk factors.

The 4G/5G polymorphism in the plasminogen activator inhibitor (PAI-1) gene appears to be related to the risk for myocardial infarction, but findings are inconsistent. We hypothesized that environmental factors, such as smoking may influence the association between the genetic variant and the risk of myocardial infarction. In the study described in *chapter 4.2* we determined the -675(4G/5G) polymorphism at the PAI-1 gene locus in blood cells of 132 subjects with a history of myocardial infarction and of 265 controls. Homozygosity for the 4G allele (4G4G) was associated with a non-significantly increased risk for myocardial infarction compared to homozygosity for the 5G allele (5G5G) (odds ratio 1.6 (CI 0.9,2.8)). This association varied for smokers compared to non-smokers. The risk for myocardial infarction was fivefold higher in 4G4G smokers than in 5G5G smokers (odds ratio 5.3 (95% CI 1.2,23)); among non-smokers, the 4G allele was not associated with an increased risk for myocardial infarction. Overall, smokers had a doubled risk for myocardial infarction compared to non-smokers (odds ratio 2.0 (95% CI 1.2,3.5)). Among 4G4G subjects, smokers had a five times higher risk for myocardial infarction than non-smokers (odds ratio 4.9 (1.8,13)). Among 5G5G subjects, smoking did not notably increase the risk for myocardial infarction (odds ratio of 1.1 (0.3,3.7)). Adjustment for PAI-1 antigen levels did not alter the findings. We concluded that the increase in the risk for myocardial infarction associated with the 4G allele of the PAI-1 gene is largely confined to smokers. Additionally, in subjects homozygous for the 4G allele smoking appears to induce a much stronger increase in the risk for myocardial infarction than in subjects homozygous for the 5G allele.

An association between increased plasma fibrinogen and an increased risk for myocardial infarction is well established, but the nature of this association is subject to debate. In *chapter 4.3* our aim was to shed light on the potentially causal nature of this association. We examined whether increased plasma fibrinogen, due to a condition that is independent of cardiovascular events, also increases the risk for myocardial infarction. The genotype of the -455G/A polymorphism in the ß-fibrinogen gene was determined in 139 subjects with a history of myocardial infarction and 287 controls, using PCR. Functional plasma fibrinogen levels were determined according to von Clauss. Plasma level of fibrinogen was significantly higher in subjects with one or two A alleles as compared to subjects with the GG genotype, 3.8 (95% CI 3.6,3.9) g/l and 3.6 (3.5,3.7) g/l, respectively. With increasing plasma fibrinogen level the risk for myocardial infarction increased gradually, a rise in fibrinogen of 1 g/l was associated with a 45

percent increased risk (odds ratio adjusted for age, gender and smoking 1.45 (95% CI 1.12,1.88)). There was no association between the genotypes of the -455G/A polymorphism and the risk for myocardial infarction. Thus, the -455G/A polymorphism is associated with increased plasma fibrinogen levels, but not with an increased risk for myocardial infarction. These findings indicate that an increased plasma fibrinogen, due to this genetic factor, does not increase the risk for myocardial infarction.

In *chapter 4.4* we investigated the association of activated protein C (APC) response and of the factor V Leiden mutation with myocardial infarction, stroke and transient ischemic attack. APC response was determined in double centrifuged platelet poor plasma of 115 subjects with a history of myocardial infarction, 112 with a history of stroke and/or transient ischemic attack and 222 age matched control subjects without arterial disease. All subjects were genotyped for the G1691A mutation in the factor V gene. Results are adjusted for age and gender. The risk of cerebrovascular disease gradually increased with decreasing APC response. An APC response lower than 3.5 was associated with more than twice as many cases of cerebrovascular disease compared to an APC response above 5 (odds ratio 2.4 (95% CI 1.1,5.2)). Additional adjustment for the factor V mutation did not change these findings. APC response was not associated with myocardial infarction. We concluded that the risks of cerebrovascular disease and of myocardial infarction were not increased in subjects with the factor V mutation. A decrease in APC response is associated with an increased risk of cerebrovascular disease, but not of myocardial infarction. The risk was independent of the factor V G1691A mutation. The factor V mutation is not associated with cerebrovascular disease or with myocardial infarction.

In *chapter 5* we summarized the results from longitudinal studies that have been performed to study the association of plasma levels of haemostatic parameters with the risk for cardiovascular events. We discussed the difficulties in the assessment of the association and in the interpretation of findings from such studies. Additionally, we discussed advantages and disadvantages of the use of genetic parameters. Finally suggestions for future research and clinical applicability of the findings from our study were discussed.

7 Samenvatting

Dankzij ontwikkelingen op het gebied van trombolyse bij hartinfarcten, van beeldkwaliteit in de angiografie en de introductie van angioscopie, is duidelijk geworden dat bloedstolsels een belangrijke rol spelen in de pathogenese van hart-en vaatziekten. Een verhoogde stollingsneiging zou de kans op de stolselvorming en dus het risico op hart- en vaatziekten kunnen verhogen. Aangezien personen met verhoogde plasmaconcentraties van bepaalde stollingsfactoren een verhoogd risico hebben op hart- en vaatziekten, neemt men aan dat deze plasmaconcentraties zouden kunnen wijzen op een verhoogde stollingsneiging. In het onderzoek beschreven in dit proefschrift worden verschillende aspecten van het verband tussen haemostase en hart- en vaatziekten onderzocht.

Dat hartinfarcten en plotselinge hartdood vaker plaatsvinden in de ochtenduren en in de winter, doet vermoeden dat sommige risicofactoren voor deze aandoeningen tijdsafhankelijk zijn. Concentraties van enkele stollingsfactoren vertonen dag- en/of seizoensvariaties en zouden de temporele variatie in de incidentie van hart- en vaatziekten kunnen verklaren. *Hoofdstuk 2.1* beschrijft de seizoensvariatie van fibrinogeen in de eerste 2.325 deelnemers van de Rotterdam Study, een cohortstudie onder 7,983 mannen en vrouwen van 55 jaar en ouder. Plasma fibrinogeen werd gemeten met de protrombine-tijd-afgeleide methode. Fibrinogeen was hoger in de winter. Het verschil in fibrinogeen in het plasma tussen zomer en winter was gemiddeld 0,34 g/l (95%BI 0,29;0,39). Dit verschil was groter in ouderen; het was 0,43 g/l (0,34;0,52) voor mannen en vrouwen van 75 jaar oud en ouder; en het was 0,29 g/l (0,24;0,35) voor mannen en vrouwen van 55 tot 75 jaar. Correctie voor quetelet index, systolische en diastolische bloeddruk, en totaal en HDL cholesterol veranderde weinig aan deze getallen. Lage buitentemperatuur leek samen te hangen met een hoger fibrinogeen, maar na correctie voor 'tijdstip in het jaar' bleek dat fibrinogeen niet langer verband hield met

buitentemperatuur. Correctie voor temperatuur veranderde weinig aan het verband tussen 'tijdstip in het jaar' en fibrinogeen, de seizoensvariatie van fibrinogeen was 0,31 g/l (0,24;0,37). We concludeerden dat fibrinogeen het hoogste is in de wintermaanden. Het seizoensverschil is groter bij mensen ouder dan 75 jaar oud. De buitentemperatuur heeft weinig invloed op de seizoensvariatie van fibrinogeen. De seizoensvariatie van fibrinogeen zou gedeeltelijk de hogere indicentie van hart-en vaatziekten in de winter kunnen verklaren.

In *hoofdstuk 2.2* besteden we aandacht aan de variatie van plasminogeen activator inhibitor type 1 (PAI-1) waarvan de plasma concentratie bijna twee keer zo hoog is in de ochtend als in de avond. Een verklaring hiervoor is niet bekend. Personen met het 4G allel van -675(4G5G) polymorfisme in het promotorgebied van het gen voor PAI-1 hebben in het algemeen een hoger PAI-1 dan mensen zonder dit allel. Wij onderzochten of de dagvariatie in PAI-1 verschillend is voor de verschillende genotypen. We maten plasmaconcentraties van PAI-1 antigeen en genotypen voor het -675(4G5G) polymorfisme in 132 personen die eerder een hartinfarct doormaakten en in 265 personen vrij van hart- en vaatziekten. We vonden dat het hebben van een 4G allel inderdaad samengaat met een hogere concentratie PAI-1 antigeen in het plasma. Homozygoten voor het 4G allel hadden een gemiddelde PAI-1 antigeen concentratie van 63 ng/l (57;70); heterozygoten hadden gemiddeld 52 ng/l (47;56) en in homozygoten voor het 5G allel was het gemiddelde 47 ng/l (41;53). Deze verschillen waren groter in de ochtend. Het ochtend/middag verschil was 78% (45;119) in de 4G homozygoten, 56% (33;82) in de heterozygoten en 65% (31;107) in de 5G homozygoten. De respectievelijke PAI-1 plasma concentraties waren 77 ng/ml (69;87), 65 ng/l (58;73) en 57 ng/l (49;67) in de ochtend en 43 ng/l (37;51), 42 ng/l (38;47) en 36 ng/l (30,43) in de middag. We concludeerden dat de ochtendpiek van PAI-1 antigeen meer uitgesproken aanwezig lijkt in personen die homozygoot zijn voor het 4G allel en dat de bijdrage van het 4G allel in het bepalen van PAI-1 plasma waarde hoger is in de ochtend.

Het feit dat personen met hart- en vaatziekten andere plasma concentraties van bepaalde haemostatische factoren hebben dan personen zonder hart- en vaatziekten leidde tot het begrip 'verhoogde stollingsstatus'. In de studie beschreven in *hoofdstuk 3.1* onderzochten we de 'stollingsstatus' van 127 personen die een hartinfarct (MI) en 124 personen die een beroerte of TIA (CVD) hadden doorgemaakt, van 131 personen met atherosclerose in de benen (PAD) en van 263 personen vrij van hart- en vaatziekten (controle), door bij hen verschillende maten voor de vorming van trombine, plasmine en voor de fibrinolyse (de plasmaconcentraties van protrombine fragment 1 + 2 (F1 + 2), trombine/antitrombine complex (TAT), plasmine/antiplasmine (PAP) complex en D-Dimeer) te meten. F1 + 2, TAT en PAP waren in alle groepen hetzelfde. D-Dimeer was

verhoogd in alle drie de groepen patiënten; het was 53 µg/l (47;61) in de MI patiënten, 51 µg/l (45;58) in de CVD patiënten en 40 µg/l (35;44) in de controle groep. De D-Dimeer concentratie liep geleidelijk op met toenemende atherosclerose in de benen; een afname in de enkel/arm systolische bloeddruk ratio van 0.1 ging gepaard met een toename in D-Dimeer van 3.9 µg/l ($p<0.01$). Dit was sterker in personen met een relatief hoger F1+2, TAT en PAP. We concludeerden dat aangezien arterieel lijden in coronaire, cerebrale en perifere slagaders gepaard gaan met een verhoogde D-Dimeer concentratie, al deze vormen van vaatlijden ook gepaard gaan met een verhoogde vorming en lyse van fibrine.

In patiënten met hart- en vaatziekten werden verhoogde D-Dimeer concentraties gevonden tezamen met verhoogde concentraties van PAI-1, een belangrijke remmer van de fibrinolyse. Om deze schijnbare paradox van verhoogde fibrinolytische activiteit (hoog D-Dimeer) en toegenomen remming van de fibrinolyse (hoog PAI-1) te onderzoeken, deden we de studie beschreven in *hoofdstuk 3.2*. We bestudeerden de relatie tussen PAI-1, D-Dimeer, en de activator van de fibrinolyse, weefsel plasminogeen activator (t-PA) in 325 personen met normale tot ernstige atherosclerotische beenarteriën. In de gehele studiepopulatie werden concentraties van PAI-1 antigeen, t-PA antigeen en activiteit en D-Dimeer en de mate van perifere vaatlijden (systolische bloeddruk aan de arm gedeeld door de systolische bloeddruk aan de benen (enkel/arm index)) gemeten. T-PA antigeen en t-PA activiteit waren, onafhankelijk van elkaar, verhoogd met toenemende atherosclerose; t-PA antigeen steeg met 3,5 ng/ml (SE 1,7; $p=0,04$) en t-PA activiteit met 0,46 IU/ml ($0.20;p=0.02$) per eenheid afname in enkel/arm index. PAI-1 was ongewijzigd bij atherosclerose. D-Dimeer steeg geleidelijk met toename van atherosclerose, vooral in personen met een PAI-1 antigeen concentratie lager dan 50 ng/ml, een t-PA antigeen concentratie lager dan 10 ng/ml en een t-PA activiteit boven 1,5 IU/ml. D-Dimeer bleek onafhankelijk van PAI-1 antigeen en t-PA activiteit; en leek enigszins verhoogd bij verhoogde t-PA antigeen concentraties. We concludeerden dat de concentraties PAI-1 antigeen en D-Dimeer onafhankelijk zijn van elkaar en dat ondanks het feit dat patiënten met perifere atherosclerose geen verhoogde PAI-1 antigeen concentratie hebben, hun PAI-1 wel van belang lijkt voor het verhogen van D-Dimeer.

Antitrombine is een belangrijke remmer van de stolling. Het is onduidelijk of atherosclerose geassocieerd is met een hoge of juist een lage antitrombine concentratie. De resultaten van eerdere studies die dit onderzochten zijn tegenstrijdig. In *hoofdstuk 3.3* beschrijven we onze bevindingen ten aanzien van de relatie tussen antitrombine en atherosclerose. Zoals eerder in hoofdstukken 3.1 en 3.2 gebruikten we de verhouding van de systolische bloeddruk gemeten aan de arm tot die gemeten aan de benen, als maat voor perifere atherosclerose. Dit gaf ons de gelegenheid een potentieel niet lineair

verband tussen antitrombine en atherosclerose te onderzoeken. In de eerste 1516 deelnemers van de Rotterdam Studie, die geen anticoagulantia gebruikten, werden zowel de enkel/arm index als de antitrombine activiteit gemeten. In mannen vonden we een kwadratisch verband tussen beide: antitrombine activiteit was verhoogd in mannen met matige atherosclerose in vergelijking tot mannen zonder duidelijke perifere atherosclerose, en in mannen met ernstige atherosclerose was de antitrombine activiteit lager dan in die met matige atherosclerose. In vrouwen vonden we een lineair verband; bij hen was een toename van atherosclerose geassocieerd met een toename van antitrombine activiteit. Deze verbanden waren onafhankelijk van roken, quetelet index, serum totaal en HDL cholesterol, fibrinogeen en factor VIIc. Dit doet vermoeden dat antitrombine activiteit stijgt in reactie op atherosclerose, terwijl bij zeer ernstige toename van atherosclerose, deze activiteit weer afneemt. Dit zou de tegenstrijdige resultaten uit eerdere studies kunnen verklaren. De veranderingen van antitrombine activiteit zouden een rol kunnen spelen als indicatoren voor de ernst van de atherosclerose en dus als risico-indicatoren voor hart-en vaatziekten.

Hoofdstuk 4 bevat onze studies naar het verband tussen trombo-embolische aandoeningen (hartinfarcten, beroertes en TIA's) en genetische varianten van de genen voor haemostatische factoren en plasmaconcentraties van haemostatische factoren. In *hoofdstuk 4.1* beschreven we hoe t-PA antigeen, t-PA activiteit en een polymorfisme in het gen voor t-PA verband houden met het risico op een hartinfarct. In 121 personen met een voorgeschiedenis van een hartinfarct en in 250 personen zonder hart-en vaatziekten (controle personen) maten we t-PA antigeen, t-PA activiteit en het *Alu* repeat insertie/deletie polymorfisme in intron h van het gen voor t-PA. Van de personen die homozygoot waren voor het insertie allel had een dubbel zo groot aantal een voorgeschiedenis van een hartinfarct dan onder de personen die homozygoot waren voor het deletie allel (odds ratio 2,24 (95% BI 1,11;4,50). T-PA antigeen had een positief verband met het risico op een hartinfarct; ten opzichte van het risico in de groep met een t-PA antigeen concentratie in het laagste kwartiel van de verdeling, was het risico op een hartinfarct in de drie volgende kwartielen van de verdeling van t-PA respectievelijk 1,7 (0,9;3,3), 2,3 (1,2;4,4) en 2,0 (1,0;3,8). Dit verhoogde risico daalde aanzienlijk na correctie voor quetelet index, HDL en totaal cholesterol, systolische en diastolische bloeddruk, en roken. Tegen de verwachting in leek ook een verhóógde t-PA activiteit verband te houden met een verhoogd risico op hartinfarcten. In dit onderzoek vonden we dus een nieuwe risico-indicator voor (niet fatale) hartinfarcten, namelijk het insertie allel van het *Alu* repeat polymorfisme in intron h van het gen voor t-PA. Verder bevestigden we een positief verband tussen t-PA antigeen en het risico op hartinfarcten en hadden we aanwijzingen dat een verhoogde t-PA activiteit een verhoogd risico op

hartinfarcten zou kunnen inhouden.

Het 4G/5G polymorfisme in het gen voor PAI-1 lijkt verband te houden met het risico op hartinfarcten, maar resultaten van eerdere studies zijn niet consistent. Wij dachten dat deze inconsistente bevindingen misschien het resultaat waren van de verschillen tussen de populaties waarin het risico van het 4G/5G polymorfisme onderzocht werd. Dit onderzochten we in de studie beschreven in *hoofdstuk 4.2*, waarin we het -675(4G/5G) polymorfisme in het gen voor PAI-1 bepaalden in bloedcellen van 132 personen met een voorgeschiedenis van een hartinfarct en in 265 personen vrij van hart- en vaatziekten. Homozygoten voor het 4G allel hadden een hoger, maar niet statistisch significant verhoogd, risico op een hartinfarct dan de homozygoten voor het 5G allel (odds ratio 1,6 (95% BI 0,9;2,8)). Voor de rokers, echter, was dit risico veel hoger dan voor de niet-rokers, odds ratio's 5,3 (1,2;23) en 1,1 respectievelijk. In het algemeen verdubbelde roken het risico op een hartinfarct (odds ratio 2,0 (1,2;3,5)). In personen die homozygoot waren voor het 4G allel, vervijfvoudigde roken het risico op een hartinfarct (odds ratio 4,9 (1,8;13)). Terwijl in zij die homozygoot waren voor het 5G, roken nauwelijks enige invloed had op het risico op het krijgen van een infarct (odds ratio 1,1 (0,3;3,7)). Dit alles was onafhankelijk van de PAI-1 plasmaconcentraties. We concludeerden dat de toename in het risico van een hartinfarct door dragerschap van een 4G allel, eigenlijk alleen gevonden wordt in personen die roken. Anderzijds verhoogd roken het risico op een hartinfarct eigenlijk vooral in personen die homozygoot zijn voor het 4G allel.

Dat een verhoogd plasma fibrinogeen samenhangt met een verhoogd risico op een hartinfarct is intussen duidelijk aangetoond, maar de eventuele causale aard van dit verband staat nog steeds ter discussie. In *hoofdstuk 4.3* beschrijven we een studie waarin we nader zijn ingegaan op dit probleem. We deden dit door te bekijken of plasma fibrinogeen, wanneer het verhoogd is door een factor die zelf onafhankelijk is van hart- en vaatziekten, ook samenhangt met een verhoogd risico op hartinfarcten. We genotypeerden 139 personen met een voorgeschiedenis van een hartinfarct en 287 personen vrij van hart- en vaatziekten, voor het -455G/A polymorfisme in het gen voor beta-fibrinogeen. Functioneel fibrinogeen in het plasma werd bepaald met behulp van de von Clauss methode. In vergelijking met de personen die homozygoot waren voor het G allel, was het fibrinogeen hoger in personen met één of twee A allelen. Hoe hoger het plasmafibrinogeen, hoe hoger het risico op een hartinfarct; een toename in fibrinogeen van 1 g/l gaf een stijging in het risico op een infarct van 45 % (odds ratio 1,45 (95% BI 1,12;1,88). De verschillende genotypen hadden allen hetzelfde risico op een hartinfarct. Aangezien het -455G/A polymorfisme wel verband hield met plasmafibrinogeen en niet met de kans op een hartinfarct, heeft plasmafibrinogeen,

indien verhoogd door het dragerschap van een A allel, geen invloed op de kans om een hartinfarct te krijgen. Dit doet vermoeden dat verhoogd plasmafibrinogeen niet causaal gerelateerd is aan de kans op het krijgen van een hartinfarct.

In *hoofdstuk 4.4* wordt een studie beschreven waarin we onderzochten of de respons op geactiveerd proteïne C (APC response) en de factor V Leiden mutatie verband houden met het risico op hartinfarcten en cerebrovasculaire ziekten (beroertes en TIA's). Aanwezigheid van de G1691A mutatie (factor V Leiden) en APC respons van dubbel gecentrifugeerd plaatjesarm plasma werden bepaald in 115 personen met een voorgeschiedenis van een hartinfarcten, 112 personen met een voorgeschiedenis van een beroerte en/of TIA, en in 222 personen met ongeveer dezelfde leeftijd zonder hart- en vaatziekten. Het risico op cerebrovasculaire ziekten steeg geleidelijk met een afname van de APC response. De personen met een APC response lager dan 3,5 hadden een twee keer zo hoog risico op cerebrovasculaire ziekten dan diegenen met een APC respons hoger dan 5,0 (odds ratio 2,4 (95% BI 1,1;5,2)). De aan- of afwezigheid van de factor V Leiden mutatie had geen invloed op dit verband. In tegenstelling tot cerebrovasculaire ziekten was het risico op een hartinfarct niet verschillend voor de verschillende niveaus van APC response. Het risico op hartinfarcten, noch het risico op cerebrovasculaire ziekten was verhoogd in personen met de factor V Leiden mutatie. We concludeerden dat een lage APC response samenhangt met een verhoogd risico op cerebrovasculaire ziekten, maar niet met het risico op een hartinfarct. Dit risico is onafhankelijk van de aanwezigheid van de factor V Leiden mutatie. De factor V Leiden mutatie blijkt niet gerelateerd te zijn aan de kans op cerebrovasculaire ziekten, noch aan die op het krijgen van een hartinfarct.

In *hoofdstuk 5* vatten we de resultaten van de tot op heden gepubliceerde longitudinale studies naar het verband tussen plasmaniveaus van haemostatische factoren en het risico op hartinfarcten samen. We bespreken de beperkingen aan deze vorm van onderzoek. Daarnaast bespreken we de meest belangrijke voor- en nadelen van het gebruik van genetische parameters. We besluiten met aanbevelingen voor verder onderzoek en de klinische toepasbaarheid van de huidige opvattingen.

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