Fibrinolysis, inflammation and cardiovascular disease

Epidemiological studies on Plasminogen activator inhibitor-type 1 and C-reactive protein

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Proefschrift

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Stellingen

1. Het 4G/4G-genotype in het PAI-1 gen lijkt te beschermen tegen een herseninfarct. *(o.a. dit proefschrift)*

2. Personen met het 5G/5G-genotype hebben geen hoge PAI-1 niveaus in de ochtend, en daardoor mogelijk minder kans op een hartinfarct op dat moment. *(o.a. dit proefschrift)*

3. De PAI-1 respons in reactie op "triggers" is relevanter dan een enkele waarde gemeten in rust.

4. Het is nog te vroeg om CRP te verlagen als preventieve maatregel voor hart- en vaatziekten.

5. Het vermelden van financiële belangen van auteurs bij wetenschappelijke artikelen verlaagt de geloofwaardigheid voor de lezer.*(Chaudhry et al. BMJ 2002;325:1391-1392)*

6. De integratie van verstandelijk gehandicapten in de samenleving vereist meer aanpassingen van de samenleving dan van verstandelijk gehandicapten.

7. Vetbelasting zou de belasting van de volksgezondheid ten gevolge van vet kunnen verlagen.

Stellingen behorende bij het proefschrift:
'Fibrinolysis, inflammation and cardiovascular disease; Epidemiological studies on Plasminogen Activator Inhibitor-type 1 and C-reactive protein'

Tiny Hoekstra
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Abstract

Plasminogen activator inhibitor-type 1 (PAI-1) is the main inhibitor of fibrinolysis and a potential risk factor for cardiovascular disease. In addition to its regulating role in fibrinolysis, PAI-1 may have detrimental effects in the cardiovascular system also through other processes, e.g. inflammation. Although PAI-1 is generally elevated in the presence of cardiovascular disease, it is not yet clear whether it is a causal risk factor. A polymorphism in the promoter region of the PAI-1 gene, the 4G/5G-polymorphism, affects the transcription of the PAI-1 gene, yielding higher blood levels of PAI-1 for the 4G-allele. In case of a causal role of PAI-1 in cardiovascular disease, an increased cardiovascular risk would be expected for the 4G-allele.

The general objective of the studies in this thesis was to examine whether PAI-1 and the 4G/5G-polymorphism are associated with cardiovascular risk. Both associations with markers of atherosclerosis and clinical end points were studied. Because of the acute-phase properties of PAI-1, we additionally examined the role of C-reactive protein (CRP), a sensitive marker of inflammation.

In the Arnhem Elderly Study, comprising 641 subjects aged 65-84 years, we observed a strong daytime fluctuation in PAI-1 activity with peak levels in the early morning. The diurnal pattern was clearly present for the 4G/4G and 4G/5G-genotypes, but not for the 5G/5G-genotype. These findings raised the hypothesis that 5G-homozygotic persons may be relatively protected from the early morning peak incidence in cardiovascular events.

In a population of 208 smoking men the 4G/5G-polymorphism was not associated with two non-invasive markers of atherosclerosis, i.e. common carotid intima-media thickness and the ankle-brachial index. Neither did we observe consistent associations between the 4G/5G-polymorphism and coronary stenosis after pooling three similar case-control studies (n = 776) with angiographically determined coronary atherosclerosis.

In the Arnhem Elderly Study we investigated whether PAI-1 and the 4G/5G-polymorphism were associated with mortality and incidence of cardiovascular events during 7.8 years of follow-up. We observed that the 4G/4G-genotype was protective against incident stroke and transient ischemic attack (relative risks of 0.4 and 0.3 respectively), which is in contrast to the generally accepted hypothesis that the 4G-allele would increase cardiovascular risk. For PAI-1 levels, an increased risk of stroke and ischemic attacks was observed in the highest tertile.

In the Arnhem Elderly Study, PAI-1 and CRP were inter-related, but only in lean elderly. The associations between CRP and other components of the metabolic syndrome were also predominantly present in lean elderly. In elderly men, CRP was predictive for future cardiovascular events, but not in women.

Overall, PAI-1 and the 4G/5G-polymorphism do not appear to play a major role in atherosclerosis, but the effects on thrombosis are still controversial. Causality of PAI-1 was not supported by an increased risk of cardiovascular events for the 4G/4G-genotype. In contrast, subjects with the 4G/4G-genotype were relatively protected against stroke.

Contents

1

General introduction

Cardiovascular disease is the number one cause of death in Western countries. In the Netherlands, 36% of the deaths in 1999 were due to cardiovascular disease, with ischaemic heart disease and stroke as the most important causes within this category.[1] The development of cardiovascular disease is a multifactorial process, with both genetic and environmental factors involved. Interactions between genetic and environmental factors add to the complexity of the pathogenesis of the disease. Two main processes can be distinguished in the development of cardiovascular disease, i.e. atherosclerosis and thrombosis. Established biological and lifestyle cardiovascular risk factors, such as hypertension, hypercholesterolemia, and smoking, can only explain a part of the prevalence of cardiovascular disease and there is an ongoing search for new risk factors. A better understanding of factors involved in the disease process may provide opportunities for both primary and secondary prevention.

Fibrinolysis and inflammation are believed to play important roles in the cardiovascular disease process. Several factors from these systems are currently under investigation as potential risk factors for cardiovascular disease in epidemiological studies. Fibrinolysis is the system that breaks down a thrombus by degrading fibrin and is crucial in the thrombotic stage of the disease. In addition, fibrinolysis may also be involved in the earlier stage of atherosclerosis. The role of inflammation in the cardiovascular disease process is currently one of the major topics in the research on cardiovascular disease. Several fibrinolytic factors are also known as acute-phase proteins, indicating interactions between both fibrinolytic and inflammatory pathways at molecular level. Investigating both pathways simultaneously is therefore very relevant.

In this thesis, epidemiological studies on the association between Plasminogen Activator Inhibitor-type 1 (PAI-1) and C-reactive protein (CRP), two newly identified risk factors, and cardiovascular risk are presented. PAI-1 is the main inhibitor of fibrinolysis and in addition an acute-phase protein. CRP is a sensitive marker of inflammation.

PLASMINOGEN ACTIVATOR INHIBITOR-TYPE 1

After vessel trauma, both the coagulation system and fibrinolysis are activated. The end product of the coagulation cascade is fibrin, which forms the major component of a thrombus. Fibrinolysis is the process by which fibrin is degraded, leading to dissolution of the thrombus.[2] The balance between blood coagulation and fibrinolysis is crucial in maintaining haemostasis. A low fibrinolytic activity may lead to occlusion of arteries by excessive thrombus formation and thereby cardiovascular events. Over activation of the fibrinolytic system, on the other hand, may in rare situations lead to bleeding.[3,4] The active component of fibrinolysis is plasmin,[2] which breaks down fibrin into fibrin degradation products. Tissue-type plasminogen activator (t-PA) promotes the conversion of inactive plasminogen into the active plasmin. T-PA is in turn regulated by plasminogen activator inhibitor type 1 (PAI-1), which is the main and fast-acting inhibitor of fibrinolysis.

Next to its regulating function in fibrinolysis, PAI-1 is involved in various other mechanisms, which play a role in a range of physiological and pathophysiological processes.[5] For instance, there is a growing body of evidence for a causal role of PAI-1 in tumor growth, vascularisation, and metastasis.[5] PAI-1 is furthermore involved in the acute-phase response during inflammation,[6] although its exact role in inflammation is not fully understood.

In plasma, PAI-1 occurs to a major extent in its active, free form. A minor part of PAI-1 in plasma is present in complex with t-PA. Plasma PAI-1 can be measured either as PAI-1 activity or as PAI-1 antigen. PAI-1 antigen comprises all PAI-1 (free or in complex with t-PA), while PAI-1 activity is a measurement of the amount of t-PA that can be inhibited.[7] Both measures are highly intercorrelated[7] and are more or less interchangeable. In this thesis we will use the term 'level' to indicate plasma PAI-1 concentration irrespective of the method used (activity/antigen).

PAI-1 AND CARDIOVASCULAR DISEASE

Because of its inhibiting action on fibrinolysis, PAI-1 is considered a potential risk factor for cardiovascular disease. The role of PAI-1 in the thrombotic stage of cardiovascular disease has been studied predominantly, but evidence also exists that PAI-1 is involved in atherosclerosis. The evidence for a role of PAI-1 in cardiovascular disease will be evaluated separately for atherosclerosis and thrombosis.

PAI-1 and atherosclerosis

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries.[8] Atherosclerosis is regarded an inflammatory disease[9] that starts already at a very early age with the subendothelial accumulation of lipid-laden macrophages (i.e. foam cells). More advanced lesions consist of a lipid-rich core covered by a fibrous cap consisting of smooth muscle cells and extracellular matrix. Advanced atherosclerosis of the coronary artery causes angina pectoris and is a strong risk factor for myocardial infarction.

PAI-1 has been localized in the vessel wall, and shows an increased expression in human atherosclerotic plaques.[10-13] PAI-1 might stimulate the development and/or progression of atherosclerosis by inhibiting the clearance of fibrin within the atherosclerotic plaque. However, from animal studies it is known that PAI-1 is also involved in mechanisms other than fibrinolysis, which might affect the atherosclerotic process. Mice studies have shown that PAI-1 inhibits cellular migration and deposition of matrix components.[14] Although these mechanisms seem favorable, they may have adverse effects on plaque stability because decreased smooth muscle cell migration results in plaques with a large amount of lipid relative to smooth muscle cells and connective tissue, which are particularly prone to rupture.[15]

Increased PAI-1 levels are observed in subjects with symptomatic atherosclerosis compared to healthy subjects.[16,17] Two cross-sectional studies investigated the association between PAI-1 and markers of atherosclerosis in asymptomatic

populations.[18,19] Both in the ARIC[19] and in the NHLBI Family Heart Study[18] PAI-1 antigen was weakly positively associated with intima-media thickness, a non-invasive marker of generalized atherosclerosis. However, the observed associations were explained to a large degree by other risk factors (e.g. measures of obesity and cholesterol). It is furthermore possible that the observed associations are due to low-grade inflammation in response to the atherosclerotic process.

In summary, epidemiological studies show increased levels of PAI-1 in the presence of atherosclerosis, but whether this is causal is still under debate. From animal studies we know that PAI-1 is involved in various mechanisms within the atherosclerotic process, which both may have favorable and unfavorable results on atherosclerosis. How and to what extent these mechanisms influence plaque progression remains poorly understood.

PAI-1 and thrombotic outcomes

Atherosclerotic plaque disruption activates the coagulation cascade and thrombus formation at the site of rupture. A thrombus can cause an acute occlusion of the artery, leading to massive ischemia of the target tissue, e.g. myocardial infarction or ischemic stroke. If the thrombus does not lead to a complete occlusion of the artery, it will eventually be incorporated in the plaque, resulting in narrowing of the artery. It is furthermore possible that the thrombus is released, after which it can cause obstruction of an artery somewhere else in the circulatory system, i.e. embolism.

Because of its fibrinolysis inhibiting properties it is biologically plausible that increased PAI-1 levels increase thrombotic risk. In case-control studies indeed in patients with a history of a cardiovascular event increased PAI-1 levels were observed.[20,21] Whether or not PAI-1 is predictive for future cardiovascular events may be dependent on background risk. In populations with angina pectoris, PAI-1 levels have been shown to be predictive for future events[17,22-26] and also in populations with a history of a myocardial infarction PAI-1 was predictive for recurrent events. However, in prospective studies in general (healthy) populations PAI-1 is not an independent risk factor for cardiovascular events.[23,27-29] Only one prospective study specifically studied the association with future risk of stroke, in which PAI-1 had no predictive role. This study was performed in subjects initially free of cardiovascular disease.[30] Prospective data in elderly is scarce. As far as we know, the Cardiovascular Health Study[27] is the only study that investigated the relationship of PAI-1 with future cardiovascular risk in a population of elderly. In this population PAI-1 was not predictive for future cardiovascular events.

In summary, although PAI-1 levels are increased in the presence of cardiovascular disease, prospective studies are inconclusive and the results seem to depend on the presence of cardiovascular history. The association between PAI-1 and cardiovascular risk in elderly has insufficiently been studied. Especially the association between PAI-1 and the risk of stroke deserves more attention.

CAUSE OR CONSEQUENCE?

In epidemiological studies, the association between PAI-1 and cardiovascular disease (both atherosclerosis and thrombosis) may be confounded by many biological and environmental cardiovascular risk factors which are known to affect PAI-1 levels (e.g. obesity, cholesterol, see Chapter 2 of this thesis for a review). Alternatively, these risk factors may (partly) have harmful effects via PAI-1. Another complicating factor is the acute-phase response of PAI-1 during inflammation, which makes it plausible that PAI-1 levels increase in response to the ongoing cardiovascular disease process. PAI-1 can thus reflect the severity of the atherosclerotic processes in the vessel wall, which could underlie the observed association between PAI-1 levels and cardiovascular disease. It is therefore difficult to draw conclusions on the causality of PAI-1 in the cardiovascular disease process, based on either retrospective or prospective epidemiological studies. We studied the 4G/5G-polymorphism in stead of PAI-1 levels, which could be useful in answering the question on the causal role of PAI-1 in cardiovascular disease as explained below.

4G/5G-POLYMORPHISM

The 4G/5G-polymorphism is a common single base pair insertion/deletion polymorphism in the promoter region of the PAI-1 gene. The 4G-allele has a sequence of four guanosine, whereas the 5G-allele has a fifth guanosine inserted.[31,32] The 4G/5G polymorphism is common in the general population, with the two alleles having approximately equal frequencies. The 4G/5G polymorphism affects the binding of a nuclear protein to the PAI-1 promoter, thereby altering the expression of the PAI-1 gene.[31] The 4G/4G-genotype has been associated with higher PAI-1 levels compared to the 5G/5G-genotype, with the heterozygous genotype having intermediate levels.[33-38] Data of this polymorphism are therefore used as a proxy for long-term, habitual PAI-1 levels. However, it has recently become clear that the association between the 4G/5G-polymorphism and plasma PAI-1 levels may not be as strong as previously expected.[39-41] Although the 4G/5G-polymorphism may not be strongly associated with PAI-1 levels, it may be associated with PAI-1 responses after triggering (for example after vessel damage), and therefore it is still worthwhile to study the 4G/5G-polymorphism. An association between the 4G/5G-polymorphism and cardiovascular disease would contribute to evidence for a causal role of PAI-1 in cardiovascular disease.

4G/5G-POLYMORPHISM AND CARDIOVASCULAR DISEASE

4G/5G-polymorphism and atherosclerosis

The association between the 4G/5G-polymorphism and atherosclerosis has only marginally been studied. In a large case-control study a higher frequency of the 4G/4G-genotype was found in subjects with coronary stenosis compared to controls, although the 4G/5G-polymorphism was not associated with the number of affected

arteries.[42] However, in two other case-control studies the 4G/5G-polymorphism was not associated with the presence[37,43] or extent[37] of coronary disease. The association between the 4G/5G-polymorphism and non-invasive markers of atherosclerosis has not been described.

4G/5G-polymorphism and thrombotic outcomes

Most studies that examined the association between the 4G/5G-polymorphism and cardiovascular disease focused on the relation with myocardial infarction. In a recent meta-analysis of 9 case-control studies of the 4G/5G-polymorphism and myocardial infarction, including a total of 2,813 patients and 3,358 controls,[44] a small increased risk for the 4G/4G-genotype was observed (OR=1.20, 95%-CI: 1.04 – 1.39). Because of the very strict diagnostic and methodological inclusion criteria for the studies, 11 of 18 published studies were excluded from this meta-analysis. In two of the excluded studies a significantly increased risk of myocardial infarction was observed for the 4G/4G-genotype.[31,45] The authors suggested that the pooled odd ratio might be an overestimation of the actual risk, because of probable publication bias of small studies that did not observe an association.

Prospective studies do not support an association between the 4G/5G-polymorphism and risk of myocardial infarction. In the Physicians' Health Study the 4G/5G-polymorphism was not predictive for future myocardial infarction in men initially free of cardiovascular disease.[46] In postmenopausal women[47] and in elderly[48] the 4G/5G-polymorphism was not predictive for fatal myocardial infarction.

Recently, some studies investigated the association between the 4G/5G-polymorphism and stroke. A trend towards a protective effect of the 4G-allele was found in several studies,[47-51] but only in one study the association was strong enough to reach statistical significance.[47] In a case-control study in Korean subjects, on the contrary, the 4G-allele was associated with an increased risk of stroke.[52]

C-REACTIVE PROTEIN

C-reactive protein (CRP) is an acute-phase protein with normal values below 2 mg/L in general populations. Hepatic synthesis of CRP is regulated by various cytokines (e.g. interleukin-6, interleukin-1, tumor necrosis factor-α). CRP values above 10 mg/L are generally used to indicate clinically relevant inflammation. The exact function of CRP is unclear, but it may stimulate tissue factor production and activate complement when aggregated. Low-grade chronic inflammation (i.e. CRP <10 mg/L) has been shown to predict risk of cardiovascular events in both apparently healthy populations and in cardiovascular patients.[53,54] In the Physicians' Health Study an increased plasma concentration of CRP (≥2.10 mg/L) was associated with a twofold increased risk of stroke and a threefold increased risk of myocardial infarction compared to low CRP values (<0.55 mg/L).[55,56] In elderly populations results are less convincing. In the Iowa 65+ Rural Health Study, CRP was associated with mortality only in elderly who also had increased interleukin-6.[57] In the Caerphilly Prospective Heart Disease Study the association between CRP and future ischemic heart

disease disappeared after adjusting for other risk factors such as obesity and smoking.[58] In the Cardiovascular Health Study stronger associations were observed for elderly women than for elderly men, and the strongest associations for those with subclinical disease at baseline.[59] In the Women's Health Study, CRP was the single strongest predictor of future coronary events.[60]

A number of mechanisms by which CRP might directly promote vascular disease have been postulated, including activation of the classical complement system in the arterial wall.[61,62] Alternatively, the atherosclerotic process in the vascular wall may increase CRP synthesis. Despite the large amount of data, the causality of the relationship between CRP and cardiovascular risk is still under debate.

OUTLINE OF THE THESIS

The main goal of the epidemiological studies in this thesis is to provide insight into the role of PAI-1 in both atherosclerosis and thrombotic events. An increased cardiovascular risk for the 4G/4G-genotype of the 4G/5G-polymorphism in the PAI-1 gene would be in favor of a causal role of PAI-1 in the pathology of cardiovascular disease. Therefore, we additionally studied the association between the 4G/5G-polymorphism and cardiovascular disease. Besides its established role in fibrinolysis, PAI-1 is also an acute-phase protein, providing an alternative pathway by which PAI-1 might be associated with cardiovascular risk. We therefore studied in addition the role of CRP, a sensitive marker of inflammation, which enabled us to study the role of PAI-1 independent of inflammation. In the Arnhem Elderly Study we examined the relation between PAI-1 and CRP and we studied whether CRP itself was a risk factor for cardiovascular events. See Figure 1.1 for an overview of the associations studied.

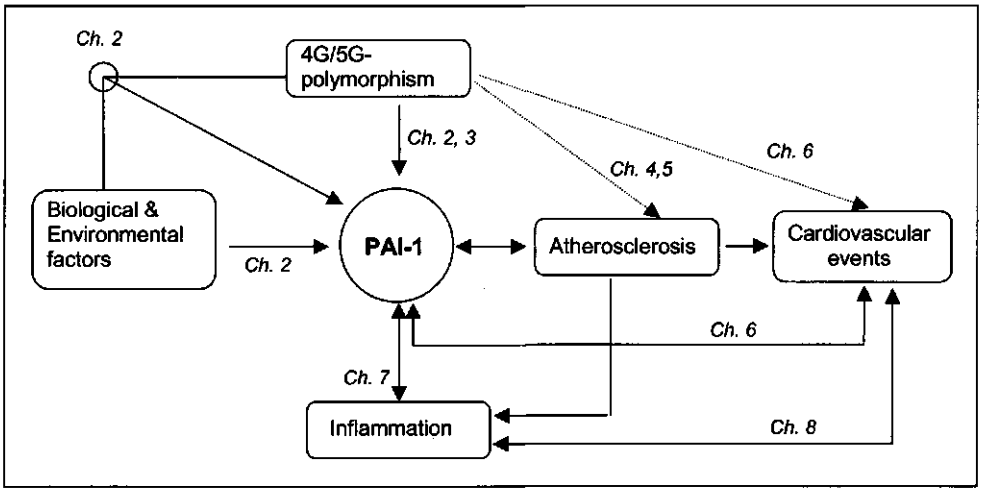

Figure 1.1. Overview of the role of PAI-1 in cardiovascular disease and the chapters in which the associations are described. Dashed lines indicate associations described in this thesis, but which are unlikely to be directly biologically related.

Research questions were:

1. *Which factors influence PAI-1 levels and should be treated as potential confounders in epidemiological studies on the association between PAI-1 and cardiovascular risk?*

First, in Chapter 2 a review is given on factors that affect PAI-1 concentrations. In this review special emphasis is given to interactions of metabolic and lifestyle factors with the 4G/5G-polymorphism. In Chapter 3 the diurnal variation in PAI-1 is described in an elderly population, also separately for the genotypes of the 4G/5G-polymorphism.

2. *Is the 4G/5G-polymorphism associated with atherosclerosis?*

In Chapter 4 we report on the association between the 4G/5G-polymorphism and two non-invasive markers of atherosclerosis, namely carotid wall thickness and ankle-brachial index, in a population of smoking men. In Chapter 5 three case-control studies were pooled to investigate the association of the 4G/5G-polymorphism with coronary stenosis, assessed by angiography.

3. *Are PAI-1 and the 4G/5G-polymorphism independent risk factors for cardiovascular events in the elderly, especially for stroke?*

We studied the associations between PAI-1 activity, the 4G/5G-polymorphism and risk of cardiovascular events in a prospective study, the Arnhem Elderly Study (Chapter 6). We investigated the relation between both plasma PAI-1 and the 4G/5G-polymorphism with several end points (all-cause and cardiovascular mortality, incidence of stroke and incidence of myocardial infarction).

4. *Are PAI-1 and CRP intercorrelated?*

Both PAI-1 and CRP are considered as components of the metabolic syndrome en we used the baseline data of the Arnhem Elderly study to explore the association of CRP with PAI-1 and other components of the metabolic syndrome (Chapter 7).

5. *Is CRP an independent risk factor for cardiovascular disease in elderly?*

In Chapter 8 we investigated whether CRP itself is an independent risk factor for future cardiovascular events in the Arnhem Elderly Study.

Finally, the main results are summarized, and implications and recommendations for future research are described in the General Discussion (Chapter 9).

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2

Plasma plasminogen activator inhibitor-type 1

A review of genetic, metabolic and lifestyle determinants

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INTRODUCTION

Plasminogen activator inhibitor-type 1 (PAI-1) is the main and fast-acting inhibitor of activation of fibrinolysis, which plays an important role in vascular disease prevention by removing thrombi from the vascular system. Plasmin, the active component of fibrinolysis, breaks down fibrin into its degradation products. Plasminogen and tissue-type plasminogen activator (t-PA) bind to the surface of fibrin where t-PA cleaves inactive plasminogen into active plasmin. T-PA is inhibited by PAI-1, which inactivates t-PA by forming an irreversible 1:1 complex.

Elevated PAI-1 is considered a potential risk factor for cardiovascular disease because of its regulating role in fibrinolysis. Apart from the well-documented function in fibrinolysis, PAI-1 is also involved in other processes, like cell migration and tissue remodeling. Importantly, PAI-1 is a strong reacting acute-phase reactant, reflected by the possibility of fast and large increase in plasma levels (up to >100 fold) during the acute-phase response.[1]

In this review we give an overview of the determinants of plasma PAI-1. We will give a broader introduction on PAI-1 and the epidemiological evidence for the association between PAI-1 and cardiovascular disease, followed by a description of genetic, metabolic and lifestyle factors that affect plasma PAI-1 levels.

PAI-1: CHARACTERISTICS AND REGULATION

In plasma, PAI-1 is present both in an active and in low concentrations of a latent form, which is cleared from the circulation at a high rate. Active PAI-1 can be stabilized by vitronectin, a protein that binds to PAI-1 in plasma. In addition, PAI-1 in plasma is present in complex with t-PA. PAI-1 in plasma can be measured either as PAI-1 activity or as PAI-1 antigen. PAI-1 antigen comprises all PAI-1 (free or in complex with t-PA), while PAI-1 activity is a measurement of the amount of t-PA that can be inhibited. The two measures are highly intercorrelated in most situations and both frequently used. We will from now on refer to plasma PAI-1 levels to indicate either activity or antigen, unless specified otherwise. Plasma PAI-1 undergoes a circadian pattern with peak plasma levels observed in the early morning.[2-6]

A second pool of PAI-1 is found in the α-granules of platelets, where PAI-1 is stored in mainly (~90%) the latent, free form.[7,8] Platelet PAI-1 is released after vessel trauma, and substantial amounts of active PAI-1 can accumulate from activated platelets at sites of arterial platelet-rich thrombi, protecting a thrombus clot from premature lysis.[7,9]

Regulation of plasma PAI-1 is a complex process, mainly occurring at the level of transcription of the PAI-1 gene. PAI-1 is produced by different cell types, including endothelial cells, hepatocytes, and adipocytes,[10] and multiple regulatory factors have been identified that play a role in PAI-1 transcription, e.g. growth factors (TGF-β), inflammatory cytokines (IL-1, TNF-α), and hormones, glucose, insulin, and glucocorticoids (reviewed by Irigoyen[11]). PAI-1 is cleared from the circulation mainly by the liver.[12] Activity of PAI-1 is in addition regulated by the spontaneous inactivation of PAI-1with a half-life of 1-2 hours.[13]

4G/5G-POLYMORPHISM

The 4G/5G polymorphism is a common single base pair insertion/deletion polymorphism in the promoter region of the PAI-1 gene that affects the gene transcription.[14] The 4G-allele has a sequence of four guanosines and the 5G-allele has a fifth guanosine inserted. The extra guanosine base creates an additional binding site for an inhibitor, resulting in an attenuated response to transcription factors for the 5G-allele.[14,15] Both PAI-1 and the 4G/5G-polymorphism have been studied in relation to cardiovascular risk. The nature of the polymorphism can be described as response polymorphism. It implies that the difference between 4G and 5G expresses more in the presence of the relevant environmental or disease factors and less so in healthy volunteers.

PLASMA PAI-1, THE 4G/5G-POLYMORPHISM AND CARDIOVASCULAR DISEASE

PAI-1, 4G/5G-polymorphism and coronary heart disease

In most population-based prospective studies, PAI-1 was not significantly associated with the occurrence of a first event of coronary heart disease.[16,17] In the few studies among healthy populations in which plasma PAI-1 was associated with risk of coronary events the strength of the association was strongly reduced after adjusting for cardiovascular risk factors.[18,19] The differences reported on the prognostic value of PAI-1 for coronary risk in epidemiological studies has been suggested to be at least in part attributed to the confounding variables controlled for.[20]

Furthermore, case-control and cross-sectional studies showed increased plasma PAI-1 levels in patients with existing coronary heart disease.[21,22] Also, PAI-1 levels predicted future (recurrent) coronary events in populations with angina pectoris[23] or a history of a myocardial infarction.[24,25]

An association between the 4G/5G-polymorphism and cardiovascular disease would contribute to evidence for the hypothesis that PAI-1 is a causal risk factor for cardiovascular disease. In a recent meta-analysis of 9 genetic studies, mainly case-control, a small increased risk for myocardial infarction was observed for the 4G/4G-genotype[26] (OR=1.20, 95% CI:1.04 - 1.39). Iwai and colleagues observed that the 4G/5G-polymorphism was associated with a faster progression to acute coronary syndromes after first anginal pain.[27] In postmenopausal women[28] and in elderly[29] the 4G/5G-polymorphism was not predictive for fatal myocardial infarction. In the Physicians' Health Study the 4G/5G-polymorphism was not predictive for future myocardial infarction in men initially free of cardiovascular disease.[30]

PAI-1, 4G/5G-polymorphism and stroke

Johansson and colleagues found that plasma PAI-1 was not significantly associated with incidence of (first) stroke in a population-based cohort (nested case-control design).[31] In stroke patients, increased PAI-1 levels have been observed compared to healthy controls, both in the acute-phase and still several months after the event.[21,32,33]

Remarkably, most studies observed a protective effect for stroke of the 4G-allele,[28,29,32,34,35] although only in one study the effect was strong enough to reach statistical significance.[28] In a Korean case-control study the 4G-allele was associated with an increased risk of ischemic stroke.[36] The genetic background of the study population may possibly explain this discrepant finding. A protective role of 4G in stroke, opposite to an increased risk of 4G in myocardial infarction, indicates a difference in pathogenesis of both entities. It can be suggested that in stroke the 4G-allele might be involved in increased PAI-1 in tissue, which might be protective.

Many cardiovascular risk factors are known to influence plasma PAI-1 and may thus confound or determine the association between plasma PAI-1, its genetic variability and cardiovascular risk in epidemiological studies. Therefore, a good understanding of the factors that influence plasma PAI-1 is needed when studying the association between PAI-1 and cardiovascular risk.

General characteristics and plasma PAI-1

PAI-1 levels tend to be higher in men than in women. This gender difference is known to diminish with increasing age.[37] Several studies showed an age-related rise in PAI-1 levels in women, whilst for men a negative correlation was observed between PAI-1 and advanced age.[38-40]

Genetic determinants of plasma PAI-1

Estimates on heritability of plasma PAI-1 levels range from 26% to 71%.[39,41-44] So far, nine different polymorphisms have been detected in the PAI-1 gene, which have recently been reviewed by Nordt and colleagues.[45] Three of them are located in the promoter region, one in intron 4, one in exon 8 and four in the 3'-untranslated region of the PAI-1 gene. The most extensively studied polymorphism is relation to PAI-1 levels is the already mentioned 4G/5G-polymorphism in the promoter region of the gene.

4G/5G-polymorphism and PAI-1 levels

The 4G/4G-genotype has been associated with higher PAI-1 levels compared to the 5G/5G-genotype, with the heterozygous genotype having intermediate levels.[7,46-50] The genotype-related differences in PAI-1 concentrations, however, were not present in all studies[42,51] and in studies in which it did, only a small part of the variance in PAI-1 was explained by the 4G/5G-polymorphism.[44,46] More recently, evidence is accumulating that, although this polymorphism may not strongly affect basal PAI-1 levels, it may affect PAI-1 responses. *In vitro* studies suggest for example strongest PAI-1 increases in response to interleukin-1,[14,52] and VLDL[53] for the 4G-allele. Further evidence for this hypothesis comes from clinical studies that showed a much stronger increase in PAI-1 in the acute-phase for patients with the 4G-allele than for those with the 5G-allele.

Other polymorphisms in the PAI-1 gene

In vitro studies in human endothelial cells showed that the HindIII-polymorphism in the 3'-untranslated region of the PAI-1 gene affects PAI-1 transcription in response to insulin and lipoproteins.[54, 55] The 1/1-genotype showed the strongest response in PAI-1 after stimulation with insulin and the 2/2 genotype after triggering with both VLDL or Lp(a).[55, 56] Linkage disequilibrium with small variations in the promoter region of the gene could possibly explain these results. No strong linkage disequilibrium exists between the HindIII-polymorphism and the 4G/5G-polymorphism.[45]

The inability to account for all observed heritability of plasma PAI-1 by polymorphisms in the PAI-1 gene may be related to an undiscovered genetic variation in promotor or distant enhancers. Alternatively, the heritability may originate in heritability of determinants of PAI-1 levels such as lipids, insulin resistance and chronic inflammation.

METABOLIC DETERMINANTS

The metabolic syndrome comprises a cluster of cardiovascular risk factors, including (abdominal) obesity, glucose intolerance/type II diabetes mellitus, dyslipidaemia and hypertension. No uniform definition of the metabolic syndrome is used, and it has been argued that PAI-1 should be considered part of it.[57,58] PAI-1 is associated with many of the components of the metabolic syndrome as will be discussed below in more detail.

Obesity

Obesity, especially central fat, is associated with increased PAI-1 levels,[59] and weight reduction has been shown to be effective in lowering PAI-1[60-65] The relationship between PAI-1 and obesity has recently been reviewed by Mutch *et al.*[66] PAI-1 is synthesized and secreted directly by adipose tissue,[67-70] but adipose tissue further increases plasma PAI-1 by increased hepatic PAI-1 production which is stimulated by adipocytes-derived cytokines (TNF-α en TGF-β).[68]

In vitro studies showed higher PAI-1 production for human visceral fat than for subcutaneous fat.[70,70-72] Recent data suggest that stromal cells, and thus not the adipocytes itself, are the most important source of PAI-1 within adipose tissue.[72] Visceral fat contains a higher amount of stromal cells than subcutaneous fat, which might thus explain the regional differences in PAI-1 production.[72] However, not all studies showed a higher PAI-1 expression in visceral than in subcutaneous fat[73] or even show opposite results.[74] The authors of the latter study[74] suggested that their unexpected finding might be explained by the adipose tissue in their study being derived from more obese subjects than in the other studies. They hypothesize that obesity *per se* may influence regional differences in PAI-1 secretion.

In addition, adipocytes from obese subjects produced more PAI-1 than adipocytes from lean subjects,[75,76] even after adjusting for adipocyte size.[76] In contrast with

these findings is the observation that PAI-1 expression in human subcutaneous adipose tissue increased after weight reduction.[65] The adipose secretion rates of PAI-1 in human abdominal subcutaneous adipose tissue were not different across the 4G/5G-genotypes.[77]

Results from studies on the association between measures of obesity and PAI-1 stratified for the 4G/5G-polymorphism do not provide consistent evidence for genotype-specific associations. One study showed a clear association between PAI-1 levels and the 4G/5G-polymorphism in obese but not in lean subjects.[59] In Pima Indians, body mass index (BMI) and PAI-1 were associated in both homozygous groups, but not in the 4G/5G-genotype.[78] In patients with angiographically determined coronary disease the association between PAI-1 and BMI was strongest for the 5G/5G genotype.[49]

Based on a study in PAI-1 knockout mice the hypothesis was raised that PAI-1 might also promote the evolvement of obesity.[79] A recent study in 505 humans showed that the prevalence of obesity was twofold higher in carriers of the 4G-allele than of the 5G-allele,[80] which is in agreement with this hypothesis. The mechanism by which PAI-1 might promote the development of obesity is not clear yet, but might involve effects of PAI-1 on cell migration and angiogenesis. Further studies on the potential effects of PAI-1 on the progression of obesity are needed.

In summary, it is evident that (central) obesity is an important determinant of plasma PAI-1 levels. The associations differ across the genotypes of the 4G/5G-polymorphism, but further influences independent of 4G/5G are also possible and this should be further unraveled. The hypothesis that PAI-1 might affect the development of obesity is only marginally investigated.

Blood lipids

PAI-1 is associated with cholesterol, LDL-cholesterol, VLDL and triglyceride levels, and negatively with HDL-cholesterol.[20] *In vitro*, VLDL has consistently been shown to induce a concentration-dependent increase in PAI-1 expression in endothelial,[81,82] and hepatic cells.[81,83] It has been shown that VLDL stimulation of PAI-1 expression in endothelial cells is mediated through transcriptional activation of the PAI-1 gene, and a VLDL-response element has been identified in the promoter region of the PAI-1 gene.[53] Besides effects of VLDL on PAI-1 gene transcription, VLDL might also affect the stability of the PAI-1 mRNA transcripts.[83] The effects of LDL-cholesterol are less consistent. Native-LDL generally does not induce PAI-1 synthesis *in vitro*, unless high concentrations are used or when LDL is oxidized, or glycated.

Chronic and acute hypertriglyceridemia have been associated with changes in plasma PAI-1.[84] However, acute hypertriglyceridemia induced by intravenous administration of a fat emulsion did not affect PAI-1 in healthy males.[85]

The association between triglyceride levels and PAI-1 was genotype specific, with a steeper slope in subjects with the 4G/4G-genotype in patients with coronary artery disease,[49] and in type 2 diabetics.[86]

Insulin resistance and diabetes

Type 2 diabetics have higher PAI-1 plasma levels than healthy subjects.[87] Bastard and colleagues have recently reviewed the role of plasma PAI-1 in insulin resistance.[57] Cross-sectional studies show positive associations between fasting insulin levels and PAI-1 both in subjects with normal[87-90] and impaired glucose tolerance[87,88] and also in type 2 diabetics.[87] Additional evidence for an association between PAI-1 and insulin is provided by *in vitro* studies, demonstrating that both insulin and proinsulin stimulate transcription of the PAI-1 gene in several tissues.[91-93] However, acute intravenous administration of insulin in humans did not increase PAI-1 levels.[94-97] An alternative explanation for increased levels of PAI-1 in the presence of diabetes could be that PAI-1 is elevated because of high serum glucose levels. Evidence for this hypothesis is provided by *in vitro* studies in which glucose stimulates PAI-1 expression in endothelial and vascular smooth muscle cells.[98,99] In a prospective study of 1,047 nondiabetic subjects, PAI-1 was an independent risk factor for the development of type 2 diabetes.[100] However, in healthy type I diabetics, PAI-1 is lower than in normal individuals arguing against glucose as a mediator.

Regulation of PAI-1 by the Angiotensin-system

The renin-angiotensin system (RAS) plays a key role in the regulation of blood pressure. Renin converts angiotensinogen to angiotensin 1, which in turn is converted to the vasoconstrictor angiotensin 2 by Angiotensin 1 converting enzyme (ACE). Inhibition of the RAS by ACE-inhibitors lowers blood pressure, a widely used therapy for hypertension. The fibrinolytic and the renin-angiotensin systems are linked by angiotensin II, which increased production of PAI-1 both *in vivo*[101] and *in vitro*.[102-104] Also its metabolite angiotensin IV stimulated PAI-1 production from human adipocytes in an *in vitro* study.[103] Vaughan has recently reviewed the link between the RAS and the fibrinolytic system.[105]

ACE levels have been correlated to PAI-1 concentrations and a polymorphism in the ACE-gene has been shown to be predictive for PAI-1 levels. Intervention studies with ACE-inhibitors have furthermore shown a decrease in PAI-1 levels,[106] but the effects appear to largely depend on type of ACE-inhibitor. Further evidence for a link between RAS and fibrinolysis is provided by the observation that during a period of activation of RAS by salt depletion, the concentration of PAI-1 significantly increased, both in normotensive subjects[106] as in subjects with essential hypertension.[107] The latter study furthermore showed that this effect was only present within subjects with the 4G/4G-genotype.[107]

HORMONAL INFLUENCES

Menopausal status and Hormone Replacement Therapy

In observational epidemiological studies it has consistently been observed that PAI-1 levels increase after menopause.[108-110] In the Framingham Offspring Study 32%

higher PAI-1 concentrations were observed in postmenopausal compared to premenopausal women.[108]

Observational studies show 15-50% lower PAI-1 levels in postmenopausal women using hormone replacement therapy (HRT) compared to non-users[111,112] In the Cardiovascular Health Study,[111] HRT-users were thinner and had less abdominal fat than non-users, which largely accounted for the observed difference in PAI-1 between users and non-users. However, estrogen dose was inversely associated with PAI-1 antigen but not with BMI in this study, which is in favor for a true effect of HRT on PAI-1.

Several randomized controlled trials with HRT have been conducted, which consistently show a decline in PAI-1.[113-118] In the large randomized HOPE-trial[115] a dose response association was observed for conjugated equine estrogen (CEE), which was weakened by progestogen. In general smaller effects on PAI-1 are observed for transdermal HRT, possibly because this does not first-pass the liver.[119,120]

Estrogen may directly decrease PAI biosynthesis and secretion or may increase the clearance rate.[121] The effect of sex hormones could furthermore be through effects on body composition and insulin resistance. Given the strong effects of sex hormones on PAI-1 these latter mechanisms may only explain a small part of the observed effects.

Two small studies[122,123] investigated the effects of HRT stratified by the 4G/5G-polymorphism. A trial with transdermal HRT in 38 postmenopausal women with coronary artery disease showed the strongest decrease in PAI-1 for the 4G/4G-genotype, while PAI-1 in the 5G/5G-genotype remained unchanged.[122] Brown and colleagues,[123] on the other hand, did not observe different effects of estrogen administration across the variants of the 4G/5G-polymorphism in a study among 19 postmenopausal women.

In summary, postmenopausal status and HRT are consistent determinants of plasma PAI-1, but data on interactions with the 4G/5G-polymorphism is limited.

Oral contraceptives

Use of oral contraceptives (OC) has consistently been associated with lower PAI-1 levels in cross-sectional studies.[124,125] Experimental data show that use of OC, both second and third generation, leads to a substantial decrease in PAI-1.[126-129] Estrogen is generally considered the compound responsible for this decrease, but also administration of progesteron-only pills lowered PAI-1, although not significantly.[130] The decrease in PAI-1 is already achieved after a short period of OC-use and after cessation PAI-1 returns to baseline levels within 8 days.[129] As far as we know, no studies have been reported on the effect of OC-use separately for the 4G/5G-polymorphism.

DIETARY FACTORS AND PLASMA PAI-1

For several dietary factors, the relation with plasma PAI-1 have been examined. Nutrients that have most intensively been studied are n-3 fatty acids and alcohol, but limited data is also available on other dietary factors.

N-3 Fatty acids

Several interventions with polyunsaturated n-3 fatty acids have been performed, with conflicting results. In most studies, supplementation with n-3 fatty acids was associated with an increase in PAI-1.[131-136] However, also interventions are known that lowered PAI-1 or in which PAI-1 was unaltered.[137,138] Most studies were however rather small and differences in (control) supplements and designs of the studies make comparisons difficult. Hansen and colleagues estimated, based on a total of 17 trials, that a 17.7% increase in PAI-1 could be attributed to n-3 fatty acids supplementation.[139] Overall, it can be concluded that n-3 fatty acids may lead to an increase in PAI-1 levels, but that these effects are only modest and depend on the type of fat. The mechanisms are still not fully understood.

Alcohol

In cross-sectional studies alcohol consumers consistently have higher levels of PAI-1 than alcohol-abstainers.[140-145] In the NHLBI Family Heart Study, a large population-based study, PAI-1 was only increased in subjects consuming more than 15 grams of alcohol per day.[142] The association between alcohol consumption and PAI-1 is dose-dependent (J-shaped).[144,145]

Several small intervention studies in male volunteers demonstrated an acute and strong increase in PAI-1 after alcohol consumption.[146-148] Intake of 40 grams of alcohol resulted in 12 times higher PAI-1 activity levels 5 hours after intake. This rise was independent of the type of beverage consumed (beer, wine, or spirits), suggesting that ethanol is the active compound.[146] In women, only one intervention study on the short-term effects of alcohol is performed,[149] showing a sharp increase in PAI-1 after consumption of wine at dinner in post-menopausal, but not in premenopausal women.[149]

Apart from the acute, transient effects of alcohol on PAI-1 levels also the long-term effects have been studied. Most long-term interventions show higher PAI-1 levels after a period of alcohol intake.[150-152] However, one intervention in which subjects were given about 20 grams of alcohol per day for a period of 30 days failed to increase PAI-1.[153] Also in the study of McConnell[151] a small increase was observed. The specific mechanisms, by which alcohol increases PAI-1 remain uncertain, but cannot be explained by a direct effect of alcohol on PAI-1 gene transcription. In contrast, *in vitro* studies demonstrated down-regulation of PAI-1 gene transcription in cultured human endothelial cells by ethanol.[154,155]

Other dietary factors

Antioxidants may attenuate the response to infection, and since PAI-1 is an acute phase reactant, antioxidants might decrease PAI-1 levels. The association between vitamin C and PAI-1 has only been studied in one cross-sectional study, in which an inverse association between serum ascorbate and PAI-1 was observed.[156] An experiment in obese men with administration of antioxidant vitamins did not change PAI-1 levels.[157] Administration of α-tocopherol led to a decrease in PAI-1 in type 2 diabetics.[158]

An experimental study in a porcine model of hypercholesterolemia demonstrated that vitamin C and vitamin E reduced local and systemic PAI-1.[159] Because the association between antioxidants and PAI-1 has only rarely been studied, no firm conclusions are allowed. Present data, however, suggest that a PAI-1 lowering effect of antioxidants is possible.

SMOKING

Data on the relationship between smoking and PAI-1 are scarce. Several cross-sectional studies showed higher PAI-1 levels for smokers than for former and never smokers.[160-163] However, in a study of monozygotic twins discordant for smoking no significant difference was observed and associations with cigarette dose were absent.[164] In the Caerphilly Study, PAI-1 levels increased gradually with the amount of tobacco, but PAI-1 levels were significantly increased only for the heaviest smokers.[144] In the Northern Sweden MONICA study no association was observed between smoking status and PAI-1 activity.[165] Apparently, smoking is not a major determinant of PAI-1. Triglycerides and insulin resistance may mediate the small effects observed in some of the studies. In an experimental study, transdermal nicotine administration did not affect plasma PAI-1.[166]

PHYSICAL ACTIVITY

Cross-sectional studies suggest that individuals that regularly exercise have reduced PAI-1 levels compared to sedentary subjects.[140,167-169] In the Northern Sweden MONICA study, a strong and dose-dependent association between PAI-1 and regular leisure time physical activity was observed both in men and women.[168] Both intervention studies on the short- and long-term effects of physical activity on PAI-1 have been performed, as outlined below.

Short-term effects of exercise

An exercise test generally results in a fall in PAI-1 levels, both in healthy populations and in populations with a history of cardiovascular disease.[170-174] Physical activity has been shown to increase the release of tissue-type plasminogen activator (t-PA) from the vascular endothelium. As a result of this increase in t-PA-antigen, PAI-1 activity is expected to decrease, because of the formation of complexes with t-PA. PAI-1 activity appears to respond faster to exercise than PAI-1 antigen,[172] as

expected from very rapid interaction between t-PA and PAI-1. Exercise-related changes in liver blood flow contribute to the rapid clearance of components such as t-PA-PAI-1 and possibly PAI-1 and constitute another influence on blood levels in the acute period. No studies were done on the 4G/5G-genotype specific short-term effects of exercise.

Training programs

Training programs generally result in decreases in PAI-1.[175-178] It is difficult to give an overall estimate of the strength of the effect on PAI-1 because of the large differences in populations (age, baseline condition) and exercise programs (intensity and duration) but the effects are notable. In men, a reduction of 80% was found after a 9 months training program and in women a reduction of 73%.[179] A study comparing training effects in younger and older subjects showed a stronger decline in older subjects.[180] In contrast, a 6-months intensive training program in elderly only led to a moderate decrease in PAI-1 antigen.[181] In the study of De Geus et al[182] the magnitude of effect clearly depended on baseline PAI-1 activity. It is expected that an effect of physical activity only lead to a fall in PAI-1 in subjects with a higher body weight or insulin resistance, probably by an improvement of insulin resistance.

In contrary to other studies, the Oslo Diet and Exercise Study did not show any decrease in PAI-1 values after a one-year exercise program focused on endurance training.[183] El-Sayed and colleagues[184] compared the effect of 12 weeks of low intensity exercise with high intensity exercise and found a significant difference in PAI-1 activity, only in the high intensity group. The effects of training programs on PAI-1 are likely to be mediated by changes in body weight and blood lipids.

We are aware of only one study that investigated the effects of physical training on PAI-1 levels stratified by the 4G/5G-polymorphism.[185] After three years of regular exercise, the largest decrease in PAI-1 was observed for the 4G/4G-genotype (-36% versus -5% for the other genotypes).[185] Baseline levels were (non-significantly) increased in the 4G/4G-genotype, which might partly explain the genotype-specific association.

Circadian pattern of plasma PAI-1

PAI-1 undergoes a circadian pattern with peak plasma levels observed in the early morning.[2-6] In the Rotterdam Study among a sub sample of 263 men and women aged 55 years and over, the morning/afternoon difference in PAI-1 antigen was also pronounced in persons with the 4G/4G-genotype than in the other genotypes.[186] We also observed this in a population of 599 elderly (see Chapter 3). A biological explanation for a genotype-specific diurnal variation in PAI-1 is provided by Maemura and colleagues, who identified a transcription factor (CLIF: cycle-like factor) that is involved in the circadian pattern of PAI-1.[187] The binding site of this transcription factor overlaps with the location of the 4G/5G-polymorphism, making an interaction between the 4G/5G-polymorphism and the diurnal pattern biological plausible.

PAI-1 AND THE ACUTE-PHASE RESPONSE

Evidence is accumulating that PAI-1 plays a crucial role in inflammatory conditions. PAI-1 is an acute-phase protein,[1] and proinflammatory cytokines, e.g. interleukin-1, TNF-α and TGF-β, have been shown to stimulate PAI-1 production.[11] The acute-phase response of PAI-1 may be affected by the 4G/5G-polymorphism. In transfected HepG2 cell lines it was demonstrated that the 4G-allele produced six times more mRNA than the 5G-allele in response to interleukin-1.[14] In severe trauma patients the 4G/4G-genotype showed the strongest response not only in PAI-1 but also in TNF-α and interleukin-1. Especially the difference in response in interleukin-1 was remarkably, with the largest difference across the genotypes six days after severe trauma.[188] Apparently, PAI-1 not only increases in response to proinflammatory cytokines, but also stimulates the synthesis of cytokines itself, suggesting a central role of PAI-1 in the acute-phase response. The stronger acute-phase response for the 4G/4G-genotype may have detrimental effects on prognosis in acute conditions. Recent studies indeed provide evidence for genetic aspects of this hypothesis. The 4G-allele has been associated with the development of septic shock after meningococcal infection,[189] a poor survival rate after severe trauma[188] and an increased mortality rate after aneurysm repair.[190]

SUMMARY

The habitual level of PAI-1 is influenced by many factors, of which obesity and insulin resistance are the most important. It is possible to reduce plasma PAI-1 by changes in life style, e.g. weight reduction and physical activity. Data on potential interactions between environmental and metabolic variables on one hand and the 4G/5G-polymorphism on the other hand are still scarce. It becomes more and more clear that PAI-1 may possibly not be a major (causal) factor in cardiovascular disease, but its role in inflammation deserves further attention. In the presence of the 4G-allele not only the PAI-1 response was more pronounced but also the response of other acute-phase reactants, which implies that the increases of these reactants are secondary to the increase in PAI-1. A myocardial infarction also provokes an acute phase response. It can thus be hypothesized that the 4G-allele might exacerbate tissue injury during the acute phase after a myocardial infarction and thereby negatively affect the prognosis.

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3

Diurnal variation in PAI-1 activity predominantly confined to the 4G-allele of the PAI-1 gene

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ABSTRACT

Background: We examined the diurnal pattern in Plasminogen Activator Inhibitor-type 1 (PAI-1) activity and Plasminogen activator (t-PA) in relation to the 4G/5G-polymorphism in the promoter of the PAI-1 gene.
Methods: The analyses were performed in the Arnhem Elderly Study, a population-based study of 598 elderly. A single blood sample was drawn and the time of blood sampling was recorded (between 8 a.m. and 5.30 p.m.).
Results: Plasma PAI-1 activity was strongly associated with time of blood sampling, showing the highest values in the early morning. The diurnal pattern was clearly present in the 4G/4G (n=184) and 4G/5G (n=275) genotypes, but not in the 5G/5G-genotype (n=139). T-PA antigen showed a weak diurnal variation, which did not differ across the variants of the 4G/5G-polymorphism.
Conclusion: Our findings raise the hypothesis that 5G-homozygotic persons may be relatively protected from diurnal variation in the occurrence of coronary events.

INTRODUCTION

The occurrence of acute coronary events peaks in the early morning.[1-3] This may partly be explained by an inability to cope with a thrombus at that time due to low fibrinolytic activity.[4] Plasminogen activator inhibitor-type1 (PAI-1) is the main inhibitor of fibrinolysis.[5] After its initial discovery, small studies have clearly shown a strong diurnal variation in PAI-1 activity.[6-13] Also for tissue-type plasminogen activator (t-PA) diurnal patterns have been observed.[6,7,9,10] However, both the diurnal variations in PAI-1 and t-PA have not been described in larger populations. The 4G/5G-polymorphism of the PAI-1 gene is an insertion/deletion polymorphism of the promoter region gene with four (4G-allele) or five guanosines (5G-allele) in a row. The extra guanosine base creates an additional binding site for an inhibitor, resulting in an attenuated response to transcription factors.[14,15] A meta-analysis of studies on the 4G/5G-polymorphism and myocardial infarction[16] indicated an increased risk for the 4G/4G-genotype (overall odds ratio versus 5G/5G-genotype: 1.20, 95%-confidence interval: 1.04-1.37).

It can be hypothesised that the diurnal variation in PAI-1 is more pronounced in the presence of the 4G-allele. This hypothesis is supported by the recent discovery of a transcription factor (CLIF), which may contribute to the diurnal PAI-1 pattern.[17] In vitro, this transcription factor up-regulates expression of the PAI-1 gene. The binding site for this transcription factor overlaps with the site of the 4G/5G-polymorphism.[17]

In the present study we investigated the diurnal variation in PAI-1 activity and t-PA antigen in a population of elderly and furthermore performed stratified analyses for the three variants of the 4G/5G-polymorphism.

METHODS

Study population

The Arnhem Elderly Study is a population-based cohort study. A random sample of non-institutionalized elderly men and women (aged 64-84 years) were invited to participate in a health survey, including home interviews (n=1012) and a physical examination (n=685). The sample was stratified for age and sex. The selection of participants is described elsewhere in detail.[18] A single non-fasting blood sample was available for 641 subjects. Because of technical reasons, data on PAI-1 activity and/or the 4G/5G-polymorphism was missing for 43 subjects, leaving 598 subjects for the analysis. Written informed consent was obtained from the participants before the physical examination. The ethical committee of Wageningen University approved the study.

Data collection

Trained interviewers visited the participants at home. Interview topics included smoking habits, health status, medication and demographic data. Smoking status was coded as current, former and never. Packyears of cigarette smoking were calculated for current and former smokers as the number of cigarettes smoked times the number of years divided by 20. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). A history of cardiovascular disease was considered present if the participant reported a diagnosis of heart disease and/or stroke ever in life. Subjects were considered on medical treatment for cardiovascular disease if ACE-inhibitors, β-blockers, thrombolytic agents, lipid-lowering medications and/or salicylates had been prescribed during the 3 months prior to the interview.

Blood sampling was performed between 8 a.m. and 5.30 p.m. and the time of blood sampling was recorded for every subject. Samples have been stored at -80°C. PAI-1 activity in plasma was determined using the Chromolize® kit (Biopool, Umeå, Sweden). T-PA antigen was measured using the Imulyse® kit (Biopool, Umeå, Sweden). DNA was isolated with standard techniques. The 4G/5G-genotyping was performed according to Margaglione,[19] with minor modifications. In brief, a mutated oligonucleotide was synthesized, which inserted a site for the BseLI enzyme within the product of amplification. PCR-products were digested at 55°C with the BseLI enzyme (MBI Fermentas, Vilnius, Lithuania). The fragments were fractionated by 4%-agarose-gel electrophoresis. Serum total cholesterol was determined by an enzymatic method (CHOD-PAP). HDL-cholesterol and LDL-cholesterol were measured directly (Dimension®HDL method and N-geneous®LDL respectively). Serum insulin was determined with an immunometric assay (Immulite®2000 insulin).

Statistical analysis

PAI-1 activities (IU/mL) showed a skewed distribution and were therefore log-transformed (natural log). To enable log-transformation, PAI-1 activities of 0 IU/mL (n=83) were replaced by 0.01 IU/mL (i.e., lowest measured value divided by 2). Spearman correlation coefficients (r_s) were computed to examine linear associations

among variables. For PAI-1 activity, geometric means and 95%-confidence intervals (95%-CI) were calculated per time interval by analysis of variance. For t-PA antigen, arithmetic mean values were calculated by time interval. Median times of blood sampling per time interval were included in a regression model to test for trend over the time intervals. Adjustments were made for potential confounders, i.e., age, sex, BMI, smoking status (current, former or never), packyears of smoking, alcohol consumption (yes/no), serum LDL and HDL-cholesterol, insulin, history of cardiovascular disease (yes/no) and use of cardiovascular medication (yes/no). Stratified analyses per genotype were performed. The relation between potential confounders and time of blood sampling was carefully evaluated within the three different genotypes. From this analysis, only smoking emerged as a potential confounder as the number of smokers was not equally distributed over the genotypes at all time intervals. Therefore, analyses were repeated after exclusion of smokers. Furthermore, analyses were repeated after exclusion of subjects with a history of cardiovascular disease. Analyses were performed with the SAS statistical package. A *P*-value of 0.05 was considered statistically significant.

RESULTS

The characteristics of the study population by PAI-1 genotype are shown in Table 3.1. The frequency distribution of the 4G/4G, 4G/5G, and 5G/5G variants was 31%, 46% and 24%, respectively. The median PAI-1 activity level in the total population was 1.96 IU/mL. Smoking was more often present in the 4G/4G-genotype than in the other two genotypes (31% versus 23% and 19% respectively).

PAI-1 activity was strongly correlated with t-PA antigen (r_s=0.63, *P*=0.0001). PAI-1 was furthermore associated with BMI, LDL-cholesterol and insulin (r_s ranging from 0.13-0.34). PAI-1 activity was negatively associated with age (r_s=-0.12, *P*=0.003) and HDL-cholesterol (r_s=-0.31, *P*=0.0001). Adjusting for time of blood sampling did not change the strength of the correlations. PAI-1 activity was higher in smokers than in non-smokers (geometric mean of 1.78 versus 1.01 for former smokers and 0.98 for never smokers, *P*=0.03), also after adjusting for time of blood sampling. No difference in PAI-1 activity was observed between men and women. T-PA antigen was positively associated with age, BMI, total cholesterol, LDL-cholesterol and insulin (r_s ranging from 0.09-0.31, all *P*< 0.05) and negatively with HDL-cholesterol (r_s=-0.28, *P*=0.0001).

T-PA antigen was higher in men than in women (11.03 versus 9.67 ng/mL) and higher in smokers than in non-smokers (11.28, 10.44 and 9.62 ng/mL for smokers, former and never smokers respectively, *P*=0.001).

Time of blood sampling was significantly associated with age (r_s=0.08, *P*=0.046) and with LDL-cholesterol (r_s=-0.10, *P*=0.02). Other characteristics were not significantly associated with the time of blood sampling.

Table 3.1. Characteristics of the study population (n=598) by the 4G/5G-polymorphism

	4G/4G (n=184)	4G/5G (n=275)	5G/5G (n=139)
Age (yr)	73.0 ± 5.7	74.0 ± 5.5	74.0 ± 5.8
BMI (kg/m^2)	25.4 ± 3.3	26.3 ± 4.2	25.9 ± 3.8
Men (%)	53.8	51.6	53.2
Smoking status (%)			
Current smokers	31	23	19
Former smokers	38	43	48
Never smokers	32	34	34
Alcohol consumers (%)	69	72	79
History of cardiovascular disease (%)	22	22	18
Use of cardiovascular medications (%)	18	20	21
Total cholesterol (mmol/L)	6.3 ± 1.2	6.2 ± 1.2	6.2 ± 1.3
HDL-cholesterol (mmol/L)	1.4 ± 0.5	1.4 ± 0.4	1.4 ± 0.3
LDL-cholesterol (mmol/L)	3.8 ± 1.1	3.7 ± 1.0	3.8 ± 1.0
Insulin (pmol/L)*	137 (97-207)	140 (99-230)	137 (91-222)
PAI-1 activity (IU/mL)*	2.1 (0.6-5.4)	2.2 (0.8-6.0)	1.4 (0.3 - 3.9)
tPA antigen (ng/mL)†	10.2 ± 3.7	10.8 ± 3.9	9.9 ± 3.7

Values are given as mean ± SD unless indicated otherwise. *Median with interquartile range in parentheses, because of skewed distribution. †T-PA levels were available for 565 subjects (4G/4G: n=175, 4G/5G: n=259, 5G/5G: n=131).

In Figure 3.1 the observed PAI-1 activities (geometric means) are reported as a function of the time of blood sampling, showing a steady decrease in PAI-1 activity during the day. The test for trend over the time intervals was highly significant (P=0.0001). In blood samples drawn before 9 a.m. the geometric mean of PAI-1 activity levels was about 13 times higher than in samples drawn after 4 p.m. (4.47 versus 0.35 IU/mL). Adjustment for potential confounders (age, sex, BMI, smoking status (current, former or never), packyears of smoking, alcohol consumption (yes/no), serum LDL and HDL-cholesterol, insulin, history of cardiovascular disease (yes/no) and use of cardiovascular medication (yes/no)) had only negligible effects (P-trend = 0.0001, data not shown). Diurnal variation in PAI-1 activity was present both in men (n=315) and women (n=283), and also both in smokers (n=147) and non-smokers (n=449) (all P-trend ≤ 0.001).

T-PA antigen levels also varied with the time of blood sampling (P-trend=0.0001), but the diurnal pattern was less pronounced than that of PAI-1 (Figure 3.2). Adjusting for the potential confounders did not alter the pattern.

In Figure 3.3 the diurnal variation in PAI-1 is shown separately for the three different genotypes using broader categories to obtain sufficient power. The percentage of subjects with the 5G/5G-genotype sampled before 10 a.m. was lower than for the other genotypes (11% versus 17% and 20%, respectively). The overall distribution of the 4G/5G-polymorphism over the different time intervals was however not statistically different (tested with χ^2, P=0.23).

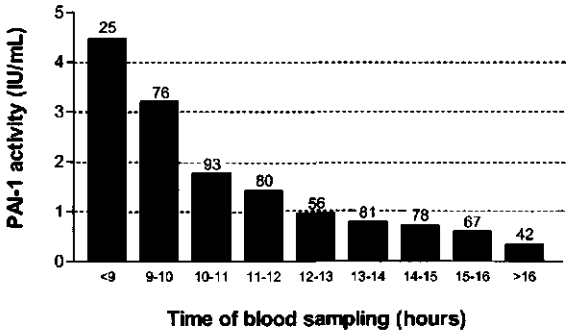

Figure 3.1. PAI-1 activity (geometric mean) by time of blood sampling. The numbers above the bars indicate the number of subjects in each group.

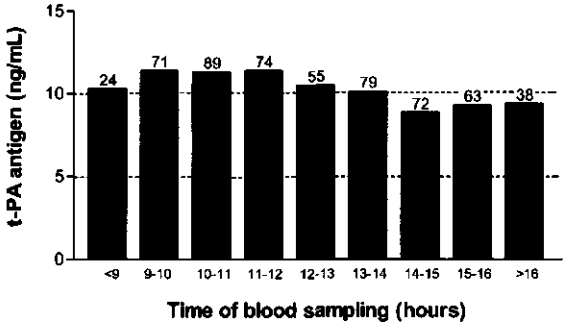

Figure 3.2. T-PA antigen (mean) by time of blood sampling. The numbers above the bars indicate the number of subjects in each group.

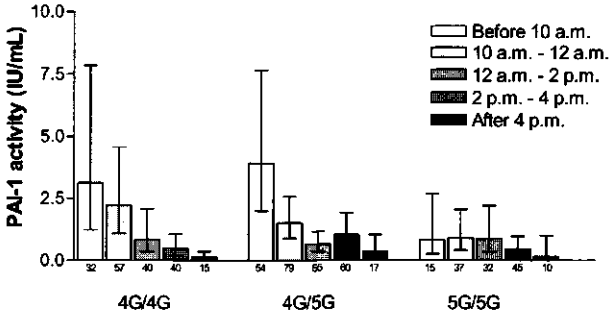

Figure 3.3. PAI-1 activity (geometric mean and 95%-confidence interval) by time of blood sampling separately for the 4G/5G-genotypes. The numbers below the bars indicate the number of subjects in each group.

We observed a diurnal variation in PAI-1 activity for both the 4G/4G and the 4G/5G-genotypes (*P*-trend=0.0001, for both genotypes), but not for the 5G/5G-genotype (*P*-trend=0.10). After adjustment for a large number of potential confounders, a significant trend in PAI-1 activity across the time intervals was observed for the 5G/5G-genotype (*P*=0.04), but this which was considerably weaker than for the other two genotypes (*P*=0.0001, both for 4G/4G and 4G/5G). Before 10 a.m. the geometric mean PAI-1 differed significantly (*P*=0.01) among the three genotypes, with the lowest geometric mean for PAI-1 activity observed for the 5G/5G-genotype (0.8 IU/mL compared to 3.5 and 5.1 IU/mL for the 4G/4G and 4G/5G-genotype respectively, *P*=0.01). This difference persisted after adjustment for potential confounders, i.e. age, sex, BMI, smoking status, packyears of smoking, alcohol consumption, serum LDL and HDL-cholesterol, insulin, history of cardiovascular disease and use of cardiovascular medications. The observed adjusted geometric means were 3.6, 4.8 and 0.7 IU/mL for the 4G/4G, 4G/5G and 5G/5G-genotypes respectively (*P*=0.008).

No significant differences in PAI-1 concentrations were observed among the three genotypes at any of the other time intervals, neither in crude nor in adjusted analyses. Exclusion of current smokers yielded essentially similar results (Both 4G/4G and 4G/5G: *P*-trend=0.0001, 5G/5G: *P*-trend=0.10). From this we conclude that the attenuated peak in PAI-1 activity before 10 a.m. in 5G/5G-subjects cannot be explained by smoking. Also, exclusion of subjects with a history of cardiovascular disease did not materially change the results (data not shown). The diurnal pattern of t-PA antigen was not different for the three variants of the 4G/5G-polymorphism (data not shown).

Discussion

Plasma PAI-1 activity showed a strong diurnal variation in a population of elderly. We furthermore demonstrated this diurnal variation to be predominantly confined to the 4G-allele. We conclude that the 4G-allele may be dominant in expressing the diurnal increase in the early morning, which would be in accordance with the data about the reduced capacity of the 4G-allele to respond to repression.[14,15]

Our observation of a strong diurnal variation in PAI-1 is in agreement with the findings of several small studies that performed serial PAI-1 determinations, showing increased levels in the early morning.[6-13]

Maemura *et al* recently discovered an endothelial derived transcription factor, CLIF (cycle like factor), which may contribute to the diurnal PAI-1 pattern.[17] CLIF in complex with another transcriptional factor (CLOCK) up-regulates the PAI-1 gene in endothelial cells.[17] The expression of the PAI-1 gene in adipose tissue also shows a circadian pattern, which may in part explain the diurnal variation in blood.[20]

Diurnal variations in plasma t-PA have also been observed.[6,7,10] In addition, circadian fluctuations have been reported in the efficacy of intravenous t-PA treatment in patients with acute myocardial infarction, with resistance to thrombolysis during the morning hours.[21,22] In our study, the diurnal variation in PAI-1 activity was much

stronger than the diurnal variation in t-PA, which is in agreement with other studies.[6,7] Therefore, the diurnal variation in PAI-1 is unlikely to be secondary to the variation in t-PA.

Our finding that the diurnal in PAI-1 is predominantly confined to the 4G-allele is in agreement with the findings of the Rotterdam Study, which showed a more pronounced morning/afternoon difference in PAI-1 antigen within the 4G/4G-genotype than in the other genotypes.[23] As far as we know, no other studies were performed on diurnal variation in PAI-1 activity in which 4G/5G genotyping of the PAI-1 gene has been performed.

The 4G/5G-polymorphism overlaps with one of the two binding sites for the CLIF-CLOCK complex,[17] which makes a differential diurnal pattern across the genotypes biologically plausible. It would be very informative to explore the potential differential binding of the CLIF-CLOCK complex to the 4G and 5G-allele, and potential interaction of the complex with the repressor protein that apparently binds to the 5G-allele and suppresses gene expression.

The Arnhem Elderly Study was not primarily designed to study the diurnal variation in PAI-1 activity, and therefore some limitations have to be considered. We could not investigate diurnal variation within subjects, because only a single blood sample per person was available. Differences among individuals may have contributed to the observed variation in PAI-1 activity. However, extensive adjustment for potential confounders did not alter the observed patterns. The analyses of diurnal variation in PAI-1 activity within strata of genotype are less prone to bias. Potential confounders within this respect are variables that are associated with time of blood sampling on one hand and the circadian pattern of PAI-1 activity (not activity itself) on the other hand. Few variables are likely to be related to both of these factors. We carefully studied the relation of potential confounders with time of blood sampling within the three different genotypes. From this analysis, only smoking emerged as a potential confounder as the number of smokers was not equally distributed at over the genotypes at all time intervals. Therefore, analyses were repeated after exclusion of smokers. This subgroup analysis yielded essentially similar results in genotype-specific associations. The proportion of 5G/5G-subjects sampled before 10 a.m. was lower than for the other genotypes. Since there is no plausible biological explanation for this phenomenon, we consider this mainly due to chance. It is important to note, however, that this unequal distribution does not affect the internal validity of the study but only the power within that stratum.

Small studies showed the peak in PAI-1 activity to occur between 3 and 5 a.m.[6,13] It can be hypothesized that the differences in PAI-1 concentrations among the three genotypes may be larger at this time of the day, but it is not feasible to examine this within a population-based study.

In a population of elderly the presence of diseases and medications may influence variability in PAI-1 activity, which complicates the study of genotype-specific associations. Nevertheless, we observed a genotype-specific diurnal variation in PAI-1 activity. The findings that we obtained from our population-based cohort

should be confirmed by clinical studies using serial blood samples, preferably in younger, non-smoking subjects.

Our findings suggest that it would be worthwhile to incorporate 4G/5G-genotyping in studies of diurnal variation in coronary event to evaluate whether early morning peaks are less pronounced in homozygotes for the 5G allele. Studies on the determinants of PAI-1 peak levels in the early morning and the possibilities to reduce this rise in PAI-1, especially in carriers of the 4G-allele, are also worth further investigation. Interventions that affect the action of the novel transcription factor CLIF,[17] thereby modulating diurnal variation in PAI-1. We think that our data, combined with the recent findings on CLIF by Maemura et al, raise the interesting hypothesis that the diurnal variation of PAI-1 varies among genotypes of the 4G/5G-polymorphism. Our findings should be confirmed in a study with serial PAI-1 determinations at different hours in three fixed groups of individuals with different genotypes.

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4

The 4G/5G-polymorphism in the PAI-1 gene is not associated with markers of atherosclerosis in male smokers

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ABSTRACT

Background: Plasminogen activator inhibitor-1 (PAI-1) is elevated in the presence of atherosclerosis, but it is not yet clear if this relationship is causal. PAI-1 levels are partly modulated by the 4G/5G-polymorphism in the PAI-1 gene.
Methods: We studied the association between the 4G/5G-polymorphism and atherosclerosis in 208 smoking men. Common carotid artery intima-media thickness (IMT) and the ankle-brachial index (ABI) were used as markers of atherosclerosis.
Results: The 4G-allele was not associated with a higher prevalence of atherosclerosis, neither for IMT nor ABI. Although not significantly, a high IMT (>1.07 mm) was even less frequent for the 4G/5G and 4G/4G-genotypes compared to the 5G/5G-genotype (odds ratios of 0.6 and 0.4 respectively, p-value for trend: 0.08).
Conclusion: Our findings cast doubt on a causal role for PAI-1 in the atherosclerotic process.

INTRODUCTION

Plasminogen activator inhibitor type 1 (PAI-1) is an inhibitor of fibrinolysis and proteolysis in the vessel wall and it has been suggested that high PAI-1 values increase the risk of cardiovascular disease.[1,2] The 4G/5G-polymorphism is an insertion/deletion polymorphism of the promoter region of the PAI-1 gene with four (4G-allele) or five guanosines (5G-allele) in a row. The extra guanosine base creates an additional binding site for an inhibitor, resulting in an attenuated response to transcription factors.[3,4] Previous studies have shown increased PAI-1 levels in the presence of the 4G-allele.[3-6] However, it can be hypothesized that the 4G/5G-polymorphism influences acute PAI-1 responses rather than PAI-1 levels, which may explain the lack of an association between the 4G/5G-polymorphism and PAI-1 concentrations in other studies.[7,8]

A meta-analysis of studies on the 4G/5G-polymorphism and myocardial infarction[9] indicated a slightly increased risk for the 4G-allele with a pooled odds ratio of 1.20 (95%-confidence interval: 1.04 - 1.37). Previously, it has been suggested that the association between the 4G/5G-polymorphism and myocardial infarction could be stronger in high-risk than in low-risk populations.[10,11] This suggests that the impact of the 4G/5G-polymorphism may vary according to the presence or absence of other cardiovascular risk factors that affect PAI-1 concentrations (e.g. smoking).

Increased levels of PAI-1 have been found in the presence of atherosclerosis.[12,13] Furthermore, PAI-1 has been localized in atherosclerotic arteries.[14-16] However, it is at present not clear whether PAI-1 elevation is a cause or consequence of atherosclerosis. An association between the 4G/5G-polymorphism and atherosclerosis would favor a causal role of PAI-1 in atherosclerosis.

In the present study among smoking men, we investigated the association between the 4G/5G-polymorphism and two non-invasive markers of atherosclerosis, *i.e.* carotid artery intima-media thickness (IMT) and the ankle-brachial index (ABI).

MATERIALS AND METHODS

Subjects and design

Baseline data of a trial of vitamin E supplementation and IMT in 218 male smokers were used. Recruitment of subjects has been described in more detail elsewhere.[17] For two persons no baseline IMT-measurement was available and for eight persons data on the 4G/5G-polymorphism were lacking because DNA isolation was not successful, leaving 208 subjects for the present study. Written informed consent had been obtained from all participants. The ethics committees of Wageningen University and Nijmegen University Hospital approved the study.

Intima-media thickness and ankle-brachial index

Intima-media thickness (IMT) of the distal 1.0cm of the common carotid artery was measured using high-resolution B-mode ultrasound with a 10 MHZ transducer. Details of the scanning and reading procedure have been described elsewhere.[18] The mean of the near and far wall both at the right and left side was used in the data-analyses.

The systolic blood pressure at the ankle (arteria tibiallis posterior) and at the arm (arteria brachialis) were both measured in supine position after a 5-minute rest using a 8 Mhz Doppler probe (Imex, Biomedic; Colorado) and a random-zero sphygmomanometer. The ankle-brachial index (ABI) was calculated as the systolic blood pressure at the ankle divided by the systolic blood pressure at the arm. The lower of two measurements was used in data-analyses.

Cardiovascular risk factors

Data on smoking, medical history, medication and family history of cardiovascular disease (CVD) were obtained by questionnaire. Pack-years of smoking were calculated by multiplying the number of years smoked by the daily number of cigarettes, divided by 20. History of CVD included self-reported myocardial infarction, stroke, coronary bypass grafting or current use of cardiovascular medication. Subjects were considered to have a positive family history of CVD if they reported at least one first-degree family member with CVD before the age of 60 years. Plasma cotinin (marker of short-term smoking) was measured by packed column gas-liquid chromatography.[19] Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Total plasma cholesterol was analyzed enzymatically (CHOD-PAP, no 237574, Boehringer Mannheim GmbH, FRG). HDL cholesterol was determined with the polyethylene glycol method.[20] LDL cholesterol was calculated by the Friedewald equation.[21] Plasma C-reactive protein (CRP) was measured with a highly sensitive ELISA procedure (Dako A/S, Glostrup, Denmark). Hypertension was defined as a blood pressure of 160/95 mmHg or above, or use of antihypertensive medication.

4G/5G-polymorphism

DNA was isolated from whole blood. Polymerase chain reaction (PCR) amplification of genomic DNA was performed using the following allele specific primers: insertion allele (5G) 5'-GTC TGG ACA CGT GGG GG-3'; deletion allele (4G) 5'-GTC TGG ACA CGT GGG GA-3' each in a separate PCR reaction together with a common downstream primer and a control upstream primer to verify the occurrence of DNA amplification in the absence of the allele on the genomic DNA. The PCR-conditions were as described elsewhere.[22] The amplified DNA fragments were fractionated by agarose gel electrophoresis and viewed under ultraviolet light after staining with ethidium bromide. Twenty randomly selected samples were also genotyped using a different method.[23] The two methods yielded fully identical results.

Statistical analyses

Analysis of variance and χ^2-testing were used to test for differences in general characteristics between the three genotypes. Deviations of the 4G/5G-polymorphism distribution from that expected for a population in Hardy-Weinberg equilibrium were analyzed using the χ^2–test. Spearman correlation coefficients (r_s) between markers of atherosclerosis and cardiovascular risk factors were calculated. Odds ratios (OR) for high IMT or low ABI according to 4G/5G genotype were obtained by logistic regression analysis. Subjects in the upper quartile of IMT (>1.07mm) or in the lower quartile of ABI (<1.03), respectively, were classified as cases. All other subjects were used as controls. Adjustments were made for age, BMI, hypertension, HDL-cholesterol, LDL-cholesterol and smoking (cotinin and pack-years)). Tests for trends were performed by including genotype as a categorical variable in the logistic model (0, 1 and 2 for the 5G/5G, 4G/5G and 4G/4G-genotypes, respectively). Data analysis was repeated after exclusion of 35 subjects who reported a history of CVD. The SAS statistical package (version 6.11) was used. P-values below 0.05 were considered statistically significant.

Results

Characteristics of the population by genotype are reported in Table 4.1. Thirty-three percent had the 4G/4G genotype, 54% the 4G/5G-genotype and 13% the 5G/5G-genotype. The overall allele frequencies were 0.60 and 0.40 for the 4G and 5G alleles, respectively. The genotype distribution fulfilled the criteria of a Hardy-Weinberg distribution (P>0.05). No significant differences in subject characteristics were observed across the genotypes.

IMT and ABI were inversely associated (r_s= -0.23, P<0.05). Age was the strongest predictor of both ABI (r_s=-0.26, P=0.0002) and IMT (r_s=0.46, P=0.0001). ABI was furthermore inversely correlated with systolic blood pressure (r_s=-0.19, P=0.01) and positively with BMI (r_s=0.19, P=0.01).

Table 4.1. Characteristics of 208 male smokers separately for the 4G/5G-polymorphism.

	4G/4G n=69 (33%)	4G/5G n=113 (54%)	5G/5G n=26 (13%)
General and lifestyle characteristics			
Age (yr)	59.8 ± 5.9	59.9 ± 6.2	62.4 ± 6.8
Body mass index (kg/m^2)	26.3 ± 3.7	25.8 ± 3.2	26.3 ± 3.1
Packyears of smoking	40.8 ± 23.5	35.2 ± 20.6	36.8 ± 18.1
Hypertension*	13 (19%)	28 (25%)	2 (8%)
Plasma concentrations			
Cholesterol (mmol/L)	6.0 ± 1.1	6.0 ± 1.1	5.7 ± 1.2
HDL-cholesterol (mmol/L)	1.2 ± 0.3	1.2 ± 0.4	1.1 ± 0.3
LDL-cholesterol (mmol/L)	4.0 ± 0.9	4.1 ± 0.9	4.1 ± 1.2
Triglycerides (mmol/L)	1.8 ± 1.2	1.7 ± 0.9	1.7 ± 1.0
CRP (ng/mL)	2.3 (1.2 - 4.8)	2.5 (1.0 - 4.7)	2.8 (1.7 - 4.6)
Cotinin (μg/mL)	235 ± 106	264 ± 129	250 ± 107
Cardiovascular history			
Family history of CVD†	16 (23%)	21 (19%)	3 (12%)
History of CVD‡	11 (16%)	21 (19%)	3 (12%)
Markers of atherosclerosis			
Intima-Media Thickness (mm)	0.94 ± 0.14	0.95 ± 0.18	0.98 ± 0.18
Ankle-Brachial Index	1.07 ± 0.06	1.07 ± 0.06	1.06 ± 0.09

Data are reported as mean (±SD) for continues variables and as n (%) for categorical variables. Because of a skewed distribution, CRP values are reported as median with an interquartile range.

* Hypertension: systolic blood pressure ≥ 160 mmHg and/or diastolic blood pressure ≥ 95 mmHg or use of antihypertensive medication.

† Family history of CVD: At least 1 first-degree family member with cardiovascular disease before the age of 60 years.

‡ History of CVD: history of MI, stroke or bypass or use of medication for CVD.

In addition, IMT was positively correlated with plasma total- and LDL-cholesterol, CRP, BMI, and diastolic and systolic blood pressure (r_s ranging from 0.14 to 0.40, all $P<0.05$).

The distributions of IMT and ABI are shown in Figure 4.1. Both for IMT and ABI, mean values were not significantly different across the genotypes. The 4G-allele was not associated with a higher prevalence of atherosclerosis, neither for IMT nor ABI (Table 4.2). Although not significantly, there was a trend towards a lower probability of a high IMT (>1.07mm) for both the 4G/5G and 4G/4G-genotypes compared to the 5G/5G-group (4G/5G: OR=0.6 (95%-CI: 0.2 - 1.6), 4G/4G: OR=0.4 (95%-CI: 0.1 - 1.1), *P*-trend = 0.08)). For low ABI (<1.03), OR of 0.5 (95%-CI: 0.2 - 1.2) was observed for the 4G/5G-genotype and 0.7 (95%-CI: 0.3 - 1.9) for the 4G/4G-genotype. Adjustments for possible confounders did not materially alter the results. Findings remained similar after exclusion of subjects with a history of CVD (data not shown).

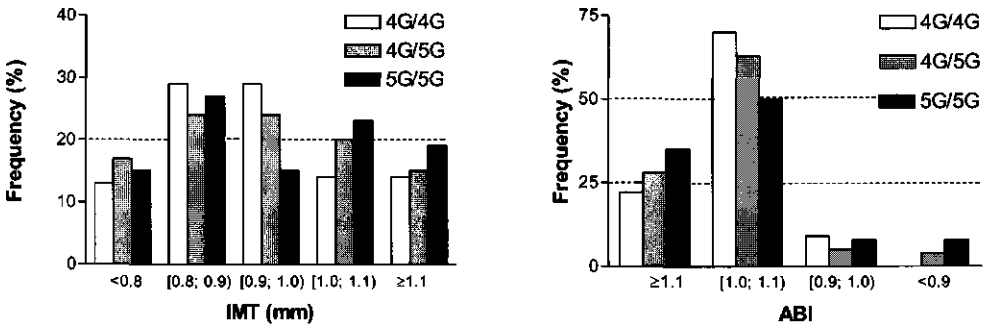

Figure 4.1. Distribution of intima-media thickness and ankle-brachial index by 4G/5G-genotype

Table 4.2. Association of the 4G/5G-polymorphism with indicators of atherosclerosis (IMT and ABI) in 208 male smokers.

	5G/5G*	4G/5G	4G/4G	*P* trend
Intima-Media Thickness				
Cases/controls† (n)	8/18	24/89	10/59	
Crude OR	1.0	0.6 (0.2 - 1.6)	0.4 (0.1 - 1.1)	0.08
Adjusted OR‡	1.0	0.9 (0.3 - 2.7)	0.5 (0.1 - 1.7)	0.18
Ankle-Brachial index				
Cases/controls† (n)	8/18	19/94	16/53	
Crude OR	1.0	0.5 (0.2 - 1.2)	0.7 (0.3 - 1.9)	0.81
Adjusted OR‡	1.0	0.5 (0.2 - 1.4)	0.9 (0.3 - 2.9)	0.64

The 95%-confidence intervals are presented in parentheses.

* Reference group

† Cases: subjects in upper quartile of IMT (>1.07mm) or in the lowest quartile of ABI (<1.03) respectively. Controls: subjects in any of the other three quartiles.

‡ Adjusted for age, BMI, hypertension, HDL-cholesterol, LDL-cholesterol, triglycerides and smoking (cotinin and packyears).

Discussion

In the present study no association was observed between the 4G/5G-polymorphism and two established markers of atherosclerosis, i.e. ABI and IMT. The 4G-allele is generally associated with higher PAI-1 concentrations and therefore one would expect an increased risk of atherosclerosis if PAI-1 were causally involved. Our findings, howerver, do not support this hypothesis.

The 4G/5G-polymorphism has been examined previously in relation to coronary atherosclerosis, as assessed by angiography.[11,24] Ossei-Gerning *et al* observed no association between the 4G/5G-polymorphism and the presence or extent of

coronary atherosclerosis.[24] In contrast, Gardemann *et al* found that the 4G/5G-polymorphism was associated both with the presence and severity of coronary atherosclerosis. The latter association, however, was only observed in high-risk subjects.[11]

In the ARIC-study and the NHLBI Family Heart Study, PAI-1 levels were positively associated with IMT[12,13] but these relationships disappeared after adjustment for other risk factors like age, BMI and smoking. In untreated hypertensives,[25] ABI was significantly and inversely associated with PAI-1 antigen and this association persisted after adjustment for other cardiovascular risk factors. However, in the Rotterdam Study, no association between ABI and PAI-1 antigen was observed.[26] It is possible that observed associations between PAI-1 and markers of atherosclerosis are due to PAI-1 elevations in response to the atherosclerotic process. Furthermore, possible confounding by cardiovascular risk factors make these results difficult to interpret. It has been argued that adjusting for cardiovascular risk factors should not be performed because of the possible intermediary role of PAI-1 in the biological pathway of these risk factors.[13] This may be avoided by examining the 4G/5G-polymorphism rather than PAI-1 values, as we did in the present study.

A drawback of our study is the limited number of subjects, which makes strong conclusions not justified. However, with the observed genotype distribution we were able to detect a difference in IMT of 0.098 mm (< 1 SD) and in ABI of 0.039 (≈ 0.5 SD) between the 4G/4G and 5G/5G-genotypes, which we consider clinically relevant.

IMT is considered a useful marker for generalized atherosclerosis and has been shown to predict future cerebrovascular and coronary events.[27] ABI, used for the diagnosis of peripheral arterial disease, has been associated with coronary and carotid artery disease and cardiovascular and overall mortality.[27] Only a few subjects in our study had an ABI below the conventional cut-off value of 0.9 and therefore the 20th percentile of the distribution in the study population (i.e. 1.03) was used as cut-off for low ABI in our analyses.

Evidence exists that the association of the 4G/5G-polymorphism with risk of cardiovascular disease is stronger in high-risk than low-risk populations.[10,11] Gardemann *et al* observed that the association between the 4G/5G-polymorphism and coronary stenosis was more pronounced in current and former smokers than in the population as a whole.[11] The lack of an association between the 4G/5G-polymorphism and markers of atherosclerosis in our population of male smokers suggests that previously reported associations might be due to PAI-1 elevation in response to the atherosclerotic process.

From our findings, we hypothesize that PAI-1 plays a role in a later (thrombotic) rather than an earlier (atherosclerotic) stage of the cardiovascular disease process. Furthermore, elevated PAI-1 may predispose to formation of plaques with a high amount of lipid compared to smooth muscle cells as a result of decreased smooth muscle cell migration. These types of plaques are particularly prone to rupture.[28] Thus, although our data do not support a causal effect of PAI-1 in the atherosclerotic

process, it may affect the nature of the atherosclerotic plaque thereby contributing to risk of thrombosis.

ACKNOWLEDGMENTS

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5

The 4G-allele of the PAI-1 gene is associated with a higher prevalence of coronary stenosis only in low-risk subjects

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ABSTRACT

Background: PAI-1 (plasminogen activator inhibitor-type 1) is an inhibitor of fibrinolysis and increased levels of PAI-1 may predict cardiovascular disease. The 4G-allele of the 4G/5G-polymorphism is associated with an increased transcription of the PAI-1 gene. We investigated whether the 4G-allele of the PAI-1 gene is associated with a higher risk of coronary stenosis and if this association is stronger in subgroups with a high background cardiovascular risk.

Methods: Data were obtained from three case-control studies. Cases had angiographically determined coronary stenosis (n=285). Both coronary controls (i.e. no substantial stenosis, n=293) and population-based controls (n=198) were included.

Results: A non-significant increased risk of coronary stenosis was observed for the 4G-allele relative to the 5G/5G-genotype when compared to coronary controls (4G/5G: OR=1.37 (95%-CI: 0.87 - 2.14), 4G/4G: OR=1.58 (95%-CI: 0.97 - 2.57)). No association was observed using the population-based control group. Stratification for background cardiovascular risk (based on Framingham Prediction Score) showed an increased risk of coronary stenosis only in the low-risk group (cases versus coronary controls, 4G/5G: OR=2.25 (95%-CI: 1.05 - 4.85), 4G/4G: OR=2.86 (95%-CI: 1.27 - 6.47)).

Conclusion: Our results do therefore not support the hypothesis that the effects of the 4G/5G-polymorphism are stronger in high-risk populations.

INTRODUCTION

Plasminogen activator inhibitor-type 1 (PAI-1) is a main regulator of fibrinolysis and proteolysis and increased levels of PAI-1 may predict cardiovascular disease.[1] Plasma PAI-1 levels are related to a common, single-base-pair guanosine insertion (5G)/ deletion (4G) polymorphism in the promoter region of the PAI-1 gene.[2] Both alleles bind a transcription activator, whereas only the 5G-allele also binds a repressor protein to an overlapping binding site.[2] The 4G-allele is associated with higher transcription of the gene and therefore the 4G/5G-polymorphism is an independent marker of increased plasma levels of PAI-1.[2-4] This association was confirmed in several,[5-10] but not all studies.[11,12]

Elevated PAI-1 levels were observed in patients with a history of a myocardial infarction[9,10] or with atherosclerotic disease.[13] This does not necessarily indicate that PAI-1 is a causal risk factor. It is also possible that PAI-1 levels are raised as result of the atherosclerotic process or in response to an acute cardiovascular event. Furthermore, the observed associations may be due to confounding by cardiovascular risk factors that are known to affect PAI-1 levels and are simultaneously independent risk factors for cardiovascular disease.[10,14] It is furthermore possible that some of these risk factors partly exert their effect on cardiovascular disease by an effect on PAI-1 concentration.

Genetic markers of increased PAI-1 expression are by definition not induced by atherosclerosis or by other external factors and therefore no discussion on the sequence of events exists. The meta-analysis by Boekholdt *et al* showed a slight increased risk of myocardial infarction for the 4G-allele.[15] It has furthermore been suggested that the effect of the 4G/5G-polymorphism might be stronger in populations with a high background cardiovascular risk than in low-risk populations.[16] This hypothesis is supported by the observation of Gardemann et al. that the association between the 4G/5G-polymorphism and coronary artery disease predominantly existed within high-risk subgroups.[17]

In the present paper, three case-control studies on angiographically defined coronary stenosis were combined to study whether the 4G-allele of the 4G/5G-polymorphism is associated with a higher risk of coronary stenosis and if this association is stronger in the subgroups with a higher background cardiovascular risk. The cases in this study were compared to two types of controls; namely coronary controls (i.e. showing no substantial stenosis) and population-based controls.

METHODS

Study population

Data were obtained from three case-control studies among subjects that underwent coronary angiography. These studies were described elsewhere in detail.[18-20] The first study was on the relation between coronary atherosclerosis and LDL oxidation.[18] The objective of study 2 was to investigate the association between plasma homocysteine and coronary atherosclerosis.[19] The third study was conducted to investigate the association between oxysterols and coronary stenosis.[20] These studies will be referred to as study 1, study 2 and study 3 respectively.

Case-control status

Cases and coronary controls were recruited from patients who underwent coronary angiography for suspected coronary atherosclerosis. Two of the three studies (study 1 and study 2) additionally selected population-based controls.

Cases

Initial selection criteria for cases differed slightly between the three studies. *Study 1:* At least 85% stenosis in one and at least 50% stenosis in a second artery. *Study 2:* At least 90% stenosis in one and at least 40% stenosis in one additional coronary artery. *Study 3:* More than 80% stenosis in at least one of the three major coronary vessels.

Coronary controls

Study 1: Less than 50% stenosis in no more than two of the three major coronary vessels. *Study 2:* Less than 50% stenosis in no more than one coronary artery. *Study 3:* Less than 50% stenosis in all three major coronary vessels.

Population-based controls

Study 1 and study 2 included population-based controls. *Study 1:* Controls were selected from participants in the Rotterdam Elderly Study[21] and through an advertisement in a newspaper. The controls had no plaques in the carotid artery as assessed by ultrasound echography. *Study 2:* A random sample was drawn from a register of about 10,000 men previously recruited for participation in a trial that was cancelled. Spouses of participants were also invited to participate.

Exclusion criteria

The major exclusion criteria for all three studies were a myocardial infarction during 2.5 (study 2) or 12 months (study 1 and 3) before the study; diabetes mellitus; chronic (gastro)intestinal diseases; alcohol or drug abuse. In study 1 and 3 only subjects who underwent their first angiography were included. Subjects under cardiac care for more than 2.5 years; with previous bypass surgery and subjects in whom more than two months had elapsed between angiography and case selection were excluded in study 3. Other exclusion criteria differed only slightly between the three studies. To improve the comparability between the studies, cases from study 3 with less than 50% stenosis in a second vessel and coronary controls from study 3 with stenosis in three vessels were excluded from the present analyses (n=33).

Data were available for 790 persons (study 1: n=345, study 2: n=311, study 3: n=134). For 14 subjects no data were available on the 4G/5G-polymorphism due to an insufficient amount of DNA. Thus, the final study population consisted of 776 subjects (cases: n=285, coronary controls: n=293, population-based controls: n=198).

Data collection

Blood concentrations of triglyceride, total cholesterol, LDL-cholesterol, HDL-cholesterol, and blood pressure, height and weight were measured as described elsewhere.[18-20] Hypertension was defined as systolic blood pressure above 160 mmHg or diastolic blood pressure above 95 mmHg or use of blood pressure lowering medication. Hypercholesterolemia was defined as total cholesterol above 6.5 mmol/L or use of cholesterol lowering medication. Other data such as alcohol use, smoking habits and family history of cardiovascular disease were obtained by questionnaires.

4G/5G-polymorphism

A 221 base-pair fragment in the promoter region of the PAI-1 gene with the 4G/5G-polymorphism was amplified using PCR[22] followed by dot blot and hybridization with antigen specific oligonucleotides.[23] The antigen specific oligonucleotide for the 4G allele was γ^{32}P-ACACGTGGGGAGTCAGC and for 5G was γ^{32}P-ACACGTGGGGGAGTCAGC. A subset of 129 samples (17%) was also genotyped using the method described by Margaglione with some modifications.[24] Both methods yielded complete identical results.

Statistical analysis

Characteristics of cases and the control groups were compared with ANOVA or the χ^2-test. The χ^2-test was used to test for deviation of genotype distribution from Hardy-Weinberg equilibrium. Odds ratios (OR's) were calculated by logistic regression to investigate the relation between the 4G/5G-polymorphism and coronary stenosis. Adjustments were made for coronary heart disease risk factors (family history, smoking, hypertension and hypercholesterolemia). Study was included as a dummy-variable in multivariate analyses to adjust for differences between the three studies. Stratified analysis was performed to detect potential effect modifiers. OR's were calculated in strata of gender, hypercholesterolemia, hypertension, obesity (BMI > 30 kg/m^2), family history of cardiovascular diseases, smoking status (current/former/never), age (cut-off value: 60 years), cholesterol (cut-off value: 6.5 mmol/L), LDL-cholesterol (cut-off value: 3.5 mmol/L), triglycerides (cut-off value: 2.0 mmol/L) and HDL-cholesterol (cut-off value: 1.1 mmol/L). The Framingham Prediction Score[25] was calculated as overall cardiovascular risk variable. The Framingham Prediction Score only considers smokers and non-smokers. A high percentage of the cases (63.5 %) were ex-smokers. Assuming that many of them may have recently stopped smoking we considered ex-smokers as smokers when calculating the scores. Sex-specific median risk scores (men: 9; women: 8) were used to divide the subjects into a low-risk and a high-risk group. Stratified analyses were performed for these two groups. A two-sided probability of less than 0.05 was considered statistically significant.

Results

The characteristics of cases and controls are shown in Table 5.1. Cases had higher total-cholesterol, LDL-cholesterol and triglycerides than both control groups, and lower HDL-cholesterol concentrations. Almost all cases were hypertensive (90.2%), against 65.9% of the coronary controls and 21.7% of the population-based controls. In cases the lowest percentage of current smokers (20.7%) and the highest percentage of ex-smokers (63.5%) was observed. A family history of cardiovascular disease was more common in cases (32.9%) than in coronary controls (24.2%), and population-based controls (7.6%).

Table 5.1. Characteristics of the study population (Mean ± SD)

	Cases (n=285)	Coronary Controls (n=293)	Population-based controls (n=198)
Age, years	56.5 ± 9.3	53.9 ± 9.8*	56.1 ± 9.4
Sex, % male	82.1	64.5*	78.8
BMI, kg/m^2	26.5 ± 2.8	26.0 ± 3.3†	26.2 ± 3.7
Total cholesterol, mmol/L	6.5 ± 1.3	6.1 ± 1.3*	6.1 ± 1.5*
LDL-cholesterol, mmol/L	4.5 ± 1.2	4.2 ± 1.2*	4.3 ± 1.4†
HDL-cholesterol, mmol/L	0.98 ± 0.27	1.2 ± 0.38*	1.1 ± 0.32*
Hypercholesterolemia, %	55.8	35.2*	35.4*
Triglycerides, mmol/L	2.1 ± 1.1	1.7 ± 0.91*	1.5 ± 0.87*
Systolic blood pressure, mm Hg	134.5 ± 16.1	132.3 ± 16.6	134.5 ± 16.0
Diastolic blood pressure, mm Hg	82.1 ± 8.5	80.9 ± 8.9	83.5 ± 8.8
Hypertension, %	90.2	65.9*	21.7*
Smokers, %	20.7	27.3*	29.3*
Ex-smokers, %	63.5	44.4*	45.5*
Family history of CVD‡, %	32.9	24.2†	7.6*

* $P<0.01$ for difference controls versus cases; † $P<.05$ for difference controls versus cases

‡ CVD=cardiovascular disease

The 4G-allele frequency was 0.58 for cases, 0.53 for coronary controls and 0.57 for population-based controls. The difference between the 4G-allele frequency of the cases and the coronary controls was not significant (P=0.09). Genotype distribution was in Hardy-Weinberg equilibrium in both cases and coronary controls but not in population-based controls (χ^2 = 6.93; 1 *df*; $P<0.04$) (Table 5.2).

Cases versus coronary controls

Although not significant, the 4G/4G-genotype showed an increased risk of coronary stenosis when cases were compared with coronary controls (Table 5.3). Relative to the 5G/5G-genotype the OR for the 4G/4G-genotype was 1.58 (95%-CI: 0.97 - 2.57) and 1.37 (95%-CI: 0.87 - 2.14) for the 4G/5G-genotype. Adjustments for possible confounders did not alter the results substantially. The results were not considerably different when the analyses were performed separately for the three studies (data not shown).

Cases versus population-based controls

No increased risk was found for carriers of the 4G-allele (Table 5.3) when comparing cases with population-based controls. The OR for the 4G/5G-group was 0.80 (95%-CI: 0.47 - 1.36) and for the 4G/4G-group 1.01 (95%-CI: 0.56 - 1.81) compared to the 5G/5G-group. After adjustment for possible confounding factors similar results were found. The results were not different across the studies (data not shown).

Table 5.2. Distribution of the 4G/5G-polymorphism of the PAI-1 gene in cases and controls

Genotype	Cases N (%)	Coronary controls N (%)	Population-based controls N (%)
5G/5G	44 (15)	61 (21)	27 (14)
4G/5G	149 (52)	151 (52)	115 (58)
4G/4G	92 (32)	81 (28)	56 (28)
Total	285	293	198

Table 5.3. Univariate and multivariate odds ratios (95%-CI) for cases (n=285) compared to coronary controls (n=293) and population-based controls* (n=198)

	Cases versus coronary controls		Cases versus population-based controls*	
	Univariate	Multivariate†	Univariate	Multivariate†
5G/5G	1.00	1.00	1.00	1.00
4G/5G	1.37 (0.87 -2.14)	1.24 (0.76-2.05)	0.80 (0.47-1.36)	0.73 (0.29-1.83)
4G/4G	1.58 (0.97-2.57)	1.55 (0.90-2.66)	1.01 (0.56-1.81)	0.79 (0.30-2.09)

* Population-based controls were only included in study 1 and 2

† Adjusted for family history, smoking, hypertension, hypercholesterolemia and study

‡ Reference

Effect modification

Cases versus coronary controls

Analyses stratified for several cardiovascular risk factors were performed (e.g. lipid profile, blood pressure, age, smoking habits). No clear pattern of effect modification was observed for the separate risk factors except for age. For subjects younger than 60 years an increased risk of coronary stenosis for the 4G/4G-genotype was observed (4G/4G: 2.09 (95%-CI: 1.15 - 3.78); 4G/5G: 1.64 (95%-CI: 0.95 - 2.81)) while no association was found for older persons (4G/4G: 0.75 (95%-CI: 0.30 - 1.89); 4G/5G: 0.83 (95%-CI: 0.35 - 1.96)). Analyses were also performed separately for the low-risk and high-risk groups (based on the Framingham Prediction Score). Of the cases 55.1% were classified as high-risk against 36.9% of the coronary controls. The association between the 4G/5G-polymorphism and coronary stenosis was present in the low-risk group but not in the high-risk group (Table 5.4). In the low-risk group the OR for the 4G/5G-genotype was 2.25 (95%-CI: 1.05 - 4.83) and for the 4G/4G-genotype 2.86 (95%-CI: 1.27 - 6.47).

Cases versus population-based controls

No effect modification by any of the separate cardiovascular risk factors was observed in the analyses with the population-based controls (data not shown). Of the

population-based controls 42.4% were classified as high-risk based on the Framingham Prediction Score. Also stratified analyses for the low-risk and the high-risk groups did not result in different OR's (Table 4).

Table 5.4. Multivariate* odds ratios (95%-CI) stratified over two groups based on their background cardiovascular risk as assessed with the Framingham risk score for the cases (n=285) compared to the coronary controls (n=293) and population-based controls† (n=198)

	Cases versus	Coronary controls	Cases versus	population-based controls†
	Low-risk group	High-risk	Low-risk	High-risk
5G/5G	1.00	1.00	1.00	1.00
4G/5G	2.25 (1.05-4.83)	0.75 (0.35-1.61)	0.97 (0.19-4.93)	0.57 (0.17-1.83)
5G/5G	2.86 (1.27-6.47)	0.96 (0.42-2.22)	1.69 (0.32-9.05)	0.45 (0.13-1.57)

* Adjusted for family history, smoking, hypertension, hypercholesterolemia and study

† Population-based controls were only included in study 1 and 2

‡ Reference

Discussion

In this study a non-significant increased risk of coronary stenosis was found for the 4G-allele comparing cases with coronary controls. This association was only observed for subjects younger than 60 years. Stratifying for overall background cardiovascular risk based on the Framingham Prediction Score showed only an association in the low-risk group. Comparing cases with population-based controls showed no association between the 4G/5G-polymorphism and coronary stenosis also not after stratification for cardiovascular risk factors.

Results clearly depended on the type of controls. Coronary controls underwent the same selection procedures as the cases and one could expect them to have the same accuracy in reporting information. Furthermore, we do not expect misclassification between cases and coronary controls because of the large contrast between these two groups. On the other hand, coronary controls may not be representative of all non-diseased persons in the source population. Therefore in study 1 and 2 also population-based controls without clinical symptoms of coronary artery disease were included. Despite absence of symptoms it cannot be ruled out that some of the population-based controls may have had substantial coronary narrowing, which could have led to dilution of the association. Only in study 1 population-based controls underwent carotid artery ultrasound echography which made substantial coronary atherosclerosis less likely.

The genotype distribution within the population-based controls was not in Hardy-Weinberg equilibrium. The 4G-allele frequency of this control group was high compared to other European control groups,[14] which might be the reason for the absence of an association between the 4G/5G-polymorphism and coronary stenosis when using this control group. This could be caused by an unknown kind of selection or by differential misclassification. The latter is not very likely because the

investigator who performed the genotyping was blinded for case or control status. A subset of 129 genotypings (17%) was also repeated with a different method and no discrepancies were observed.[17,23,26-28] The 4G-allele frequency of the coronary control group (0.53) was comparable to the frequency in the coronary control group of a comparable study (4G-allele frequency of 0.54).[17]

The criteria for the case-control status were not exactly identical among the three studies. We improved the comparability with respect to stenosis between studies 1 and 3, by excluding subjects from study 3 who did not fulfill the criteria of study 1. Other data collection methods (e.g. questionnaires, laboratory methods) also differed across the studies. We included "study" as explaining variable in all multivariate analysis to adjust for these differences. We furthermore performed analyses separately for the three studies, which gave essentially the same results. We therefore consider pooling of the three studies justified.

Our finding of a not statistically significant increased risk of coronary stenosis for carriers of the 4G-allele (cases versus coronary controls) is in agreement with Gardemann et al,[17] who found that the 4G/4G-genotype of the PAI-1 gene was associated with an increased risk of coronary stenosis (OR=1.31 (95%-CI: 1.04 - 1.65). The cases and the coronary controls were selected in the same way as in our study, but no population-based control group was included. Two studies did not observe an association between the 4G/5G-polymorphism and the presence[9, 29] or extent[9] of coronary disease.

Age was the only effect modifier, but only in the analysis using coronary controls. An increased risk for carriers of the 4G-allele on coronary stenosis was found for persons younger than 60 years (4G/4G: 2.09 (95%-CI: 1.15 - 3.78); 4G/5G: 1.64 (95%-CI: 0.95 - 2.81) but not for older subjects. Eriksson et al. found an increased prevalence of the 4G-allele in patients with myocardial infarction under the age of 45.[2] When the polymorphism is associated with myocardial infarctions at younger age, one can expect a lower frequency of the 4G-allele in older case groups. This would imply that an effect of the 4G/5G-polymorphism in older study populations would be hard to detect.

A priori we expected a stronger effect of the 4G/5G-polymorphism in high-risk groups than in low-risk groups. In a meta-analysis of Iacoveillo *et al.* was found that "the risk of myocardial infarction for carriers of the 4G-alelle in populations at higher risk of myocardial infarction is almost twice that of low-risk populations".[16] Similar results were found by Gardemann et al who observed that the association between the 4G/5G-polymorphism and coronary artery disease predominantly existed within high-risk subgroups.[17] Our results do not support these previous findings.

Genetic markers have the advantage of not being affected by other risk factors of cardiovascular disease. Since in this study no PAI-1 levels were measured there is no direct evidence that the 4G/5G-polymorphism was associated with PAI-1 levels. The 4G/5G-polymorphism has been associated with plasma PAI-1 in several studies,[5-8] although not observed in all studies.[11] In a study of Iwai et al it was suggested that the 4G/5G-polymorphism might have a stronger influence on the local production of PAI-1 at the site of plaque rupture than on plasma PAI-1 levels.[30]

In summary, we only observed a significant association between the 4G/5G-polymorphism and coronary stenosis in a low-risk subgroup and only when compared to coronary controls. Our results do therefore not provide strong evidence for the hypothesis that the 4G-allele of the PAI-1 gene is associated with increased risk of coronary stenosis and do not confirm previous observations that the associations are stronger in high-risk populations.

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6

The 4G/4G-genotype of the PAI-1 gene is associated with a reduced risk of stroke in the elderly

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ABSTRACT

Background: Plasminogen Activator Inhibitor-type 1 (PAI-1) inhibits tissue-type Plasminogen Activator (t-PA) and is thereby the main inhibitor of fibrinolysis. At elevated levels, PAI-1 may increase risk of cardiovascular disease. The 4G/5G-polymorphism affects the transcription of the PAI-1-gene and the 4G-allele has been associated with increased PAI-1 levels. We investigated whether plasma PAI-1, t-PA and the 4G/5G-polymorphism are associated with cardiovascular events at old age.
Methods: We studied the associations of PAI-1 activity, t-PA antigen and the 4G/5G-polymorphism with all-cause mortality, cardiovascular mortality, incident myocardial infarction, stroke and transient ischemic attack in a prospective study among 637 men and women aged 65-84 years.
Results: The mean follow-up time was 7.8 years. The 4G/4G-genotype was associated with a decreased risk of stroke (relative risk (RR)=0.4 (95%-CI: 0.2 - 0.9), adjusted for age and sex)), transient ischemic attack (RR=0.3 (95%-CI: 0.1 - 0.8), and cardiovascular mortality (RR=0.5 (95%-CI: 0.1 - 1.0). The 4G/5G-polymorphism was not associated with risk of myocardial infarction. PAI-1 activity in the highest tertile gave an increased risk of stroke (RR=3.3 (95%-CI: 1.5 - 7.1), cardiovascular mortality (RR=2.3 (95%-CI: 1.2 - 4.4)) and all-cause mortality (RR=1.5 (95%-CI: 1.1-2.1)), compared to the lowest tertile.
Conclusions: Our results suggest a protective effect of the 4G-allele against stroke, which is notable given the direct relationship with PAI-1 level. We propose that a local increase in tissue PAI-1 associated with the 4G-allele may stabilize plaques, thereby reducing the risk of stroke.

INTRODUCTION

Plasminogen Activator Inhibitor-type 1 (PAI-1) is the main inhibitor of fibrinolysis and a potential risk factor for cardiovascular disease.[1,2] PAI-1 forms a complex with tissue-type Plasminogen Activator (t-PA),[2] thereby inhibiting formation of plasmin, the active component of fibrinolysis. PAI-1 levels have been shown to predict future coronary events in populations with angina pectoris[3,4] or a history of myocardial infarction.[5,6] However, in prospective studies in general (healthy) populations, PAI-1 is not an independent risk factor for coronary events.[7-10]

Being an acute phase reactant,[11] an increased PAI-1 concentration may be a consequence rather than a cause of the cardiovascular disease process. Furthermore, many cardiovascular risk factors are associated with plasma PAI-1, which may confound the observed associations between PAI-1 and cardiovascular disease. Alternatively, it is possible that risk factors (e.g. overweight) partly exert their effect on cardiovascular disease through an effect on PAI-1. It is thus difficult to draw conclusions on the causality of PAI-1 based on observational epidemiological data.

The 4G/5G-polymorphism is a common polymorphism in the promoter region of the PAI-1 gene.[12] Both the 4G and 5G-allele have a binding site for an activator of

transcription. The 5G-allele has an additional binding site for a repressor, resulting in lower transcription rates of the PAI-1 gene compared to the 4G-allele.[13,14] In general, higher PAI-1 levels are observed for the 4G/4G-genotype than for the 5G/5G-genotype.[15-17] An association between the 4G/5G-polymorphism and cardiovascular disease would provide support for a causal role of PAI-1. A recent meta-analysis of 9 case-control studies on the association between the 4G/5G-polymorphism and risk of myocardial infarction showed a slightly increased risk for the 4G/4G-genotype (OR=1.20; (95% CI 1.04 - 1.39)).[18]

The associations of PAI-1 and the 4G/5G-polymorphism with stroke received far less attention than the association with coronary heart disease. In the Northern Sweden MONICA study PAI-1 was not predictive for first stroke.[19] Both case-control and prospective studies suggest a protective effect of the 4G-allele against stroke and transient ischemic attack.[20-24] No prospective study in a single population has been performed that focused on both PAI-1 activity and the 4G/5G-polymorphism in relation to stroke.

In the present longitudinal study we investigated whether PAI-1, t-PA and the 4G/5G-polymorphism could predict for the occurrence of cardiovascular events in an elderly population.

METHODS

Study population

The Arnhem Elderly Study is a population-based Dutch cohort study. In 1991/1992 a random sample of 1,793 non-institutional elderly men and women (aged 65-84 years) was invited to participate in a health survey. The study included home interviews (n=1,012) and a physical examination (n=685). The sample was stratified for age and sex, as described in more detail elsewhere.[25] A single non-fasting blood sample was available for 641 subjects. Because of technical reasons, data on both PAI-1 activity and the 4G/5G-polymorphism were missing for 4 subjects, leaving 637 subjects for the analysis. For 31 of these subjects PAI-1 activity was missing and for 8 subjects no 4G/5G-genotyping was available. Written informed consent was obtained from the participants before physical examination. The ethical committee of Wageningen University approved the study.

Data collection

Trained interviewers visited the participants at home for data collection on demographics, smoking habits, health status, and medication. Smoking status was coded as current, former or never. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). A history of cardiovascular disease was considered present if the participant reported a history of heart disease and/or stroke. Medication for cardiovascular disease was coded present in case of prescribed use of ACE-inhibitors, β-blockers, thrombolytic agents, lipid-lowering medications and/or salicylates during the 3 months prior to the interview.

Hypertension was defined as systolic blood pressure ≥ 160 mm Hg or diastolic blood pressure ≥ 95 mm Hg or use of blood pressure lowering medication.

Laboratory determinations

Blood sampling was performed between 8.00 a.m. and 5.30 p.m. and time of blood sampling was recorded for every subject. Samples were stored at -80°C. Plasma PAI-1 activity was determined using the Chromolize™ kit (Biopool, Umeå, Sweden). T-PA antigen was measured using the Imulyse® kit (Biopool, Umeå, Sweden). DNA was isolated with standard techniques. The 4G/5G-genotyping was performed with the method described by Margaglione[26] with some modifications. In brief, a mutated oligonucleotide was synthesized, which inserted a site for the BseLI enzyme within the product of amplification. PCR-products were digested at 55°C with the BseLI enzyme (MBI Fermentas, Vilnius, Lithuania). The fragments were fractionated by 4%-agarose-gel electrophoresis. Serum total cholesterol was determined by an enzymatic method (CHOD-PAP). HDL-cholesterol and LDL-cholesterol were measured directly (Dimension®HDL method and N-geneous®LDL respectively). Serum insulin was determined with an immunometric assay (Immulite®2000 insulin). C-reactive protein (CRP) was assessed using a highly sensitive ELISA procedure.[27]

Follow-up

Municipal registries provided data on mortality and migration of the study cohort at regular time intervals until February 2001. One person was lost to follow-up due to emigration. Data on morbidity and cause-specific mortality were obtained from general practitioners (GP), either directly or by one of the authors (T.H.) by means of a standard questionnaire. Follow-up data for incident cardiovascular events and cause-specific mortality were available for 518 subjects (81%). Reasons for missing data were: (1) subjects did not give permission at baseline (n=39, 6%), (2) the present GP could not be traced (n=31, 5%), (3) the GP refused participation (n=39, 8%), or (4) could not provide valid data (n=10, 2%). Baseline characteristics of elderly for whom data from medical records were missing, were similar to those included in the analysis, except for serum cholesterol (6.0 versus 6.3 mmol/L, P=0.02). No differences were observed for age and gender distribution.

Events were coded by a medical doctor (E.G.) according to the International Classification of Diseases, Tenth Revision (ICD-10). Endpoints used in the analysis comprised all-cause mortality, death due to major cardiovascular disease (ICD-codes: I00-I96), incidence of myocardial infarction (I21-I22, both fatal and non-fatal), incidence of stroke (I60-I69, fatal and non-fatal), and incidence of transient ischemic attack (G45). In case of recurrent events, only the first event was considered in the analyses.

Statistical analysis

Differences in subjects characteristics among the three 4G/5G genotypes were tested with ANOVA (continuous variables) or χ^2-testing (categorical variables). A χ^2-test was performed to evaluate whether the 4G/5G-polymorphism was in Hardy-

Weinberg equilibrium. Spearman's correlation coefficients (r_s) were calculated of PAI-1 with other cardiovascular risk factors. The Cox-proportional hazard model was used to calculate hazard rate ratios (subsequently referred to as relative risks, RR) for tertiles of PAI-1 and t-PA respectively, with the lower tertile as reference. Trend analyses were performed by including the median values per tertile in the model. For analyzing the trend across 4G/5G-genotypes the number of 4G-alleles was added to the model. The RRs were adjusted for age and sex ('Model 1'), and additionally for BMI, smoking status (current, former, never), history of cardiovascular disease, use of cardiovascular medication, hypertension, total cholesterol, LDL-cholesterol and CRP ('Model 2'). In the analyses of PAI-1 and t-PA additional adjustment for time of blood sampling was performed because of the known circadian variation of these variables. The SAS system was used for all statistical analyses. A *P*-value of 0.05 was considered statistically significant.

RESULTS

The baseline characteristics of the study population by 4G/5G genotype are presented in Table 6.1.

Table 6.1. Characteristics of the study population by the 4G/5G-polymorphism

	4G/4G (n=193)	4G/5G (n=287)	5G/5G (n=149)
Men (%)	54	52	54
Age (yr)	73.0 ± 5.7	74.0 ± 5.4	74.0 ± 5.9
BMI (kg/m^2)*	25.4 ± 3.3	26.2 ± 4.1	25.8 ± 3.7
Smoking status (%)			
Current smokers	31	24	21
Former smokers	37	43	46
Never smokers	33	33	34
Alcohol consumers (%)*	69	72	79
Diabetes mellitus (%)*‡	8	4	3
History of cardiovascular disease‡ (%)	21	22	19
Use of cardiovascular medications‡ (%)	18	20	21
Serum total cholesterol (mmol/L)	6.3 ± 1.2	6.2 ± 1.2	6.2 ± 1.3
Serum HDL-cholesterol (mmol/L)	1.4 ± 0.5	1.4 ± 0.4	1.4 ± 0.3
Serum LDL-cholesterol (mmol/L)	3.8 ± 1.1	3.7 ± 1.0	3.8 ± 1.0
Serum CRP (ng/mL)	2.0 (1.0 - 4.1)	2.3 (1.2 - 4.2)	2.0 (1.0 - 4.0)
Serum Insulin (pmol/L)	136 (95 - 213)	140 (97 - 228)	135 (89 - 218)
Plasma PAI-1 activity (IU/mL)†	2.2 (0.6 - 5.4)	2.2 (0.8 - 6.0)	1.4 (0.3 - 3.8)
Plasma t-PA antigen (ng/mL)*	10.2 ± 3.8	10.7 ± 3.9	9.9 ± 3.7

Continuous variables are presented as mean ± SD or as median (Q1-Q3) for skewed data.

* $P<0.10$; † $P<0.05$ for difference between the genotypes. Statistical testing was performed with ANOVA for continuous variables and the χ^2 test for categorical variables. For skewed variables log-transformed data were used; ‡ Self-reported, see method section.

The frequency distribution of the 4G/4G, 4G/5G, and 5G/5G variants was 31%, 46% and 24%, respectively. The 4G/5G-polymorphism distribution was not in Hardy-Weinberg equilibrium (χ^2=4.3; 1*df*, *P*<0.05). Median plasma PAI-1 activity was lower for the 5G/5G genotype compared to both other genotypes (1.4 versus 2.2 IU/mL, *P*=0.02). Only 1% of the variance in PAI-1 activity was explained by the 4G/5G-polymorphism. Plasma PAI-1 activity was positively associated with BMI, serum LDL-cholesterol, CRP and insulin (r_s ranging from 0.13 to 0.34, all *P*< 0.05). Age and HDL cholesterol were negatively associated with PAI-1 (r_s=-0.11, *P*=0.005 and r_s= -0.31, *P*<0.001, respectively). PAI-1 activity was strongly correlated with t-PA antigen (r_s=0.64, *P*<0.001).

Table 6.2. Relative risks (95%-CI) of fatal and non-fatal cardiovascular incidents and all-cause mortality with PAI-1 activity in 492 Dutch elderly men and women.

	Tertiles of PAI-1 activity (IU/mL)			
	< 0.9	0.9 - 3.9	> 3.9	P_{trend}
Median (IU/mL)	0.2	2.0	8.4	
Incident stroke				
Cases/Person-years	10/1,296	25/1,266	23/1,156	
RR, model 1*	1	2.8 (1.4 - 5.9)	3.3 (1.5 – 7.1)	0.016
RR, model 2†	1	4.9 (2.1 - 11.8)	6.1 (2.5 - 14.6)	0.001
Incident TIA				
Cases/Person-years	15/1,251	17/1,262	19/1,117	
RR, model 1*	1	1.1 (0.6 - 2.3)	1.8 (0.9 - 3.7)	0.09
RR, model 2†	1	1.4 (0.6 - 3.3)	2.2 (1.0 - 4.9)	0.06
Incident MI				
Cases/Person-years	8/1,294	10/1,280	12/1,132	
RR, model 1*	1	1.3 (0.5 - 3.3)	2.0 (0.8 - 5.2)	0.14
RR, model 2†	1	1.1 (0.4 - 3.0)	1.5 (0.6 - 4.3)	0.38
CVD mortality				
Cases/Person-years	18/1,309	30/1,314	26/1,185	
RR, model 1*	1	1.9 (1.1 - 3.5)	2.3 (1.2 - 4.4)	0.025
RR, model 2†	1	2.2 (1.1 - 4.4)	3.3 (1.6 - 6.7)	0.003
All-cause mortality‡				
Cases/Person-years	78 / 1,684	87 / 1,662	85 / 1,595	
RR, model 1*	1	1.2 (0.9 - 1.7)	1.5 (1.1 - 2.1)	0.025
RR, model 2†	1	1.2 (0.9 - 1.7)	1.8 (1.2 - 2.5)	0.002

TIA = transient ischemic attack; MI = myocardial infarction; CVD = cardiovascular disease.

* Model 1: adjusted for sex, age and time of blood sampling.

† Model 2: adjusted for sex, age, time of blood sampling, BMI, smoking status (current, former, never), history of cardiovascular disease, hypertension, total cholesterol, LDL-cholesterol, insulin and CRP.

‡ Survival analyses for all-cause mortality included 606 elderly.

Table 6.3. Relative risks (95%-CI) of fatal and non-fatal cardiovascular incidents, all-cause mortality with the 4G/5G-polymorphism in 518 Dutch elderly men and women

	4G/5G-polymorphism			
	5G/5G (reference)	4G/5G	4G/4G	P_{trend}
Incident stroke				
Cases/Person-years	18/925	32/1,747	9/1,177	
RR, model 1*	1	0.9 (0.5 - 1.6)	0.4 (0.2 - 0.9)	0.021
RR, model 2†	1	1.0 (0.5 - 1.8)	0.5 (0.2 - 1.1)	0.09
TIA				
Cases/Person-years	16/887	26/1,716	7/1,168	
RR, model 1*	1	0.8 (0.4 - 1.5)	0.3 (0.1 - 0.8)	0.015
RR, model 2†	1	0.8 (0.4 - 1.6)	0.3 (0.1 - 0.8)	0.011
Incident MI				
Cases/Person-years	6/938	16/1,737	9/1,167	
RR, model 1*	1	1.4 (0.6 - 3.6)	1.1 (0.4 - 3.2)	0.89
RR, model 2†	1	1.7 (0.6 - 4.8)	1.3 (0.4 - 3.9)	0.75
Mortality CVD				
Cases/Person-years	23/952	39/1,798	15/1,194	
RR, model 1*	1	0.9 (0.5 - 1.5)	0.5 (0.3 - 1.0)	0.043
RR, model 2†	1	0.9 (0.5 - 1.6)	0.5 (0.2 - 1.1)	0.07
All-cause mortality‡				
Cases/Person-years	64/1,212	130/2,311	71/1,607	
RR, model 1*	1	1.1 (0.8 - 1.4)	0.9 (0.6 - 1.2)	0.32
RR, model 2†	1	1.0 (0.8 - 1.4)	0.8 (0.6 - 1.2)	0.21

TIA = transient ischemic attack; MI = myocardial infarction; CVD = cardiovascular disease.

* Model 1: adjusted for sex and age.

† Model 2: adjusted for sex, age, BMI, smoking status (current, former, never), hypertension, history of cardiovascular disease, total cholesterol, LDL-cholesterol, insulin and CRP.

‡ Survival analyses for all-cause mortality included 629 elderly.

During the follow-up period 48% of the men and 35% of the women died. Variables predictive for all-cause mortality were sex, smoking status, hypertension, CRP and history of cardiovascular disease (all $P<0.05$). Measures of cholesterol (total, HDL and LDL) were not significantly associated with all-cause mortality (data not shown).

Data from medical records were available for 518 elderly (250 women and 268 men). The mortality rate of this sub-sample was 34% and 46% for women and men respectively. In men, 38% of the deaths were due to cardiovascular disease, and for women this percentage was 45%. Mean follow-up time was 7.8 years. During the follow-up period, 31 cases of first myocardial infarction (MI), 59 cases of first stroke and 51 cases of first transient ischemic attack (TIA) were recorded.

The results of the Cox-proportional hazard analyses on all-cause mortality, cardiovascular mortality, and incidence of cardiovascular events in tertiles of PAI-1

activity are presented in Table 6.2. PAI-1 activity in the upper tertile (> 3.9 IU/mL) gave an increased risk of stroke (RR=3.3 (95%-CI: 1.5 - 7.1)), cardiovascular mortality (RR=2.3 (95%-CI: 1.2 - 4.4)) and all-cause mortality (RR=1.5 (95%-CI: 1.1 - 2.1)). For these endpoints the trend across the tertiles was statistically significant ($P<0.05$), also after adjustment for cardiovascular risk factors (model 2, see method section). The significant trend across the PAI-1 tertiles persisted for incident stroke after exclusion of 129 subjects with a history of cardiovascular disease (data not shown). PAI-1 activity was also positively associated with incidence of MI and TIA, but the risk estimates did not reach statistical significance (RR=2.0 (95%-CI: 0.8 - 5.2) and RR=1.8 (95%-CI: 0.9 - 3.7), respectively).

Table 6.4. Relative risks (95%-CI) of fatal and non-fatal cardiovascular incidents, all-cause mortality with t-PA antigen in 464 Dutch elderly men and women.

	Tertiles of t-PA antigen (ng/mL)			
	< 8.5	8.5 - 11.7	> 11.7	P_{trend}
Median (ng/mL)	6.9	9.9	13.9	
Incident stroke				
Cases/Person-years	20/1,259	16/1,151	21/1,067	
RR, model 1*	1	0.8 (0.4 - 1.6)	1.3 (0.7 - 2.5)	0.36
RR, model 2†	1	0.8 (0.4 - 1.8)	1.4 (0.7 – 3.0)	0.28
TIA				
Cases/Person-years	20/1,225	15/1,119	15/1,040	
RR, model 1*	1	0.7 (0.4 - 1.4)	0.9 (0.4 - 1.8)	0.74
RR, model 2†	1	0.7 (0.3 - 1.5)	0.8 (0.4 - 1.8)	0.64
Incident MI				
Cases/Person-years	4/1,281	19/1,109	7/1,069	
RR, model 1*	1	5.2 (1.8 - 15.4)	1.9 (0.6 - 6.7)	0.54
RR, model 2†	1	4.2 (1.4 - 12.8)	1.1 (0.3 - 4.0)	0.56
Mortality CVD				
Cases/Person-years	24/1,284	16/1,184	33/1,094	
RR, model 1*	1	0.7 (0.4 - 1.3)	1.8 (1.0 - 3.1)	0.022
RR, model 2†	1	0.8 (0.4 - 1.5)	1.8 (1.0 - 3.4)	0.037
All-cause mortality‡				
Cases/Person-years	63/1,641	76/1,577	99/1,438	
RR, model 1*	1	1.2 (0.8 - 1.7)	1.7 (1.3 - 2.4)	0.001
RR, model 2†	1	1.2 (0.8 - 1.7)	1.7 (1.2 - 2.4)	0.003

TIA = transient ischemic attack; MI = myocardial infarction; CVD = cardiovascular disease.

* Model 1: adjusted for sex, age and time of blood sampling.

† Model 2: adjusted for sex, age, time of blood sampling, BMI, smoking status (current, former, never), history of cardiovascular disease, hypertension, total cholesterol, LDL-cholesterol, insulin and CRP.

‡.Survival analyses for all-cause mortality included 573 elderly.

In Table 6.3 RRs for all-cause mortality and cardiovascular events in strata of 4G/5G genotype are presented. After adjustment for age and sex, the 4G/4G-genotype was associated with a decreased risk of stroke (RR=0.4 (95%-CI: 0.2 - 0.9)), TIA (RR=0.3 (95%-CI: 0.1 - 0.8)), and cardiovascular mortality (RR=0.5 (95%-CI: 0.3 - 1.0)). Further adjustments (model 2) did not weaken these associations. Restricting the analysis to ischemic stroke yielded a similarly decreased risk for the 4G/4G-genotype (RR=0.2 (95%-CI: 0.04 - 1.0), model 2). No significant associations were observed between the 4G/5G-polymorphism and the other endpoints (P_{trend}>0.05).

Table 6.4 shows findings for t-PA antigen, which appeared to be predictive for all-cause and cardiovascular mortality (both *P*-trend < 0.05). These associations remained statistically significant after adjusting for cardiovascular risk factors. T-PA was associated with an increased risk of MI in the mid-tertile, but not in the upper tertile. T-PA antigen was not associated with incidence of stroke or TIA.

Discussion

In a general population of elderly we observed a protective effect of the 4G/4G-genotype against incident stroke and TIA. For PAI-1 activity, however, the opposite was observed, namely a higher risk of stroke and TIA at increasing levels. The associations between PAI-1 level and risk of MI and cardiovascular mortality did not reach statistical significance.

We are among the first to investigate PAI-1 activity and the 4G/5G-polymorphism simultaneously in relation to stroke in a prospective, population-based study. Our finding of a protective effect of the 4G-allele against stroke is in agreement with previous case-control and prospective studies[20-24] (see Figure 1). One Korean study yielded contrasting data.[28] The authors of this study suggested that this discrepancy might be due to racial differences. Johansson *et al.* found no significant association between PAI-1 antigen and risk of a (first) stroke in a population-based case-control study, but their estimate points into the same direction as ours (odds ratio of 1.3 (95%-CI: 0.7-2.6) for upper versus lower PAI-1 quartile).[19] Stroke as considered in the present study is a heterogeneous entity, comprising both ischemic and hemorrhagic subtypes. The majority was ischemic, and restricting the analyses to these events yielded similar results for the 4G/5G polymorphism, PAI-1 level and t-PA. The number of hemorrhagic strokes was too low for sub-group analyses.

The underlying mechanism explaining the protective effect of the 4G-allele against stroke is probably in local tissue processes and not in fibrinolysis. PAI-1 in the atherosclerotic plaque might protect the fibrous cap against degradation by metalloproteinases, and subsequently against rupture. Alternatively, PAI-1 may protect against laminin degradation and brain cell death.[29] Our results suggest that tissue proteolysis en stabilization of plaques are more important pathways in stroke than in acute MI.

High levels of PAI-1 have been shown to predict (recurrent) coronary events in populations with angina pectoris[3,4] or a history of MI,[6] but not in healthy populations.[8-10] In our Arnhem Elderly Study, a (non-significantly) increased risk of

MI was found at increased PAI-1 levels. Only one other prospective study on PAI-1 and cardiovascular disease in elderly has been performed, namely the Cardiovascular Health Study.[7] In this cohort of elderly without prevalent cardiovascular disease, PAI-1 was not associated with the occurrence of coronary events. In our study, 20% of the elderly had a history of cardiovascular disease. We adjusted both for prevalent cardiovascular disease and of use of cardiovascular medication, which did not alter the results. Similar findings were also obtained after adjustment for CRP, an acute-phase protein strongly associated with atherosclerosis.[30] Excluding subjects with a cardiovascular history affected our findings neither.

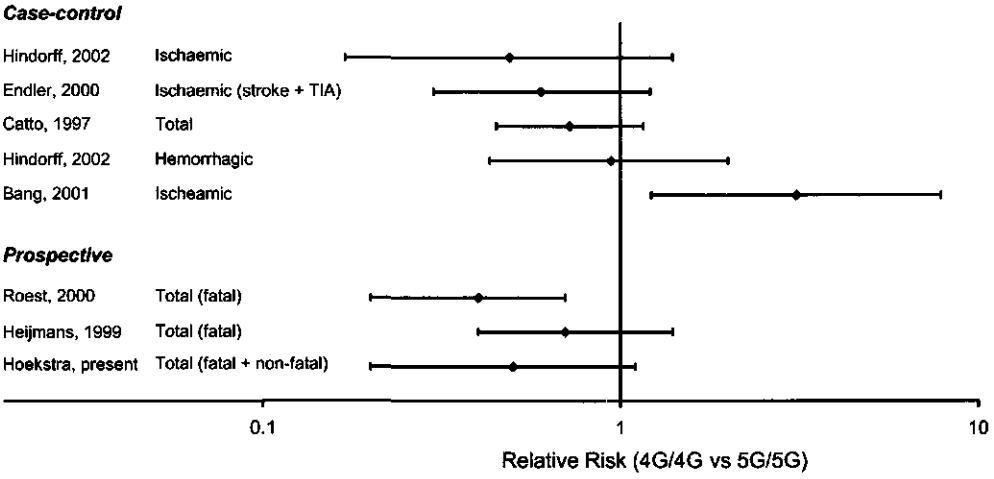

Figure 6.1. Overview of studies investigating the association between the 4G/5G-polymorphism and stroke. Data are presented as point estimates with corresponding 95%-confidence intervals. For as far as possible adjusted estimates were used.

If PAI-1 levels were causally associated with cardiovascular risk, one would expect to find also an increased risk for the 4G/4G-genotype. In our population, however, this was not the case, which is in agreement with results of previous prospective studies.[24,31] The weak association between genotype and phenotype might explain the lack of consistency between the results of PAI-1 levels and the 4G/5G-polymorphism. We could explain only 1% of the variance in PAI-1 activity by the 4G/5G-polymorphism. The association between the 4G/5G-polymorphism and plasma PAI-1 levels may not be as strong as previously suggested.[32-34] It is possible that the 4G/5G-polymorphism mainly determines the magnitude of PAI-1 response and does not strongly affect PAI-1 levels in resting conditions. Furthermore, plasma PAI-1 levels may not adequately reflect local PAI-1 levels, e.g. in vascular tissue.

A limitation of our study is that the study population was not in Hardy-Weinberg equilibrium. The reason for this is not known and unexpected for a random sample. The frequency of the 4G/5G-genotype is somewhat lower than expected on basis of allele-frequencies. The only suggestion that we have is that the restriction to non-institutionalized individuals might have caused selection related to the 4G/5G-polymorphism.

Due to comorbidity, it is sometimes difficult to accurately code the underlying cause of death, and misclassification is thus possible. However, we consider it unlikely that differential misclassification across the genotypes might have occurred. Data on cause-specific mortality and incidence of cardiovascular events were missing for 19% of the study population. However, loss to follow-up was on the level of the general practitioner rather than the individual, and it is therefore unlikely that loss to follow-up has largely influenced the observed associations for PAI-1 and the 4G/5G-polymorphism.

In conclusion, our results suggest a protective role of the 4G-allele in the development of stroke. Although the 4G-allele is generally associated with increased PAI-1 levels, we did not observe an inverse association between PAI-1 and stroke. More research is needed on the mechanisms that underlie the potential protective effect of the 4G-allele. More prospective studies on incident stroke including both PAI-1 level and the 4G/5G-polymorphism are needed to confirm our findings.

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7

Relationship of C-reactive protein with components of the metabolic syndrome in normal-weight and overweight elderly

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Submitted

Abstract

Background: Obesity plays a key role in the metabolic syndrome and it has consistently been shown that C-reactive protein (CRP) is associated with markers of obesity. We studied the associations of CRP with components of the metabolic syndrome in normal-weight and overweight elderly.

Methods: Data analyses were performed within the Arnhem Elderly Study, a population-based study of 605 free-living Dutch elderly aged 65-84 years. Data collection included anthropometry, plasma C-reactive protein (CRP), plasma plasminogen activator inhibitor 1 (PAI-1), serum insulin, serum lipids, blood pressure, lifestyle and medical history. Overweight was defined as a body mass index (BMI) of 25 kg/m^2, or above. CRP was log-transformed (ln-CRP) because of the skewed distribution. Associations of ln-CRP with components of the metabolic syndrome were studied by multivariate linear regression analysis, in strata of gender and overweight status. Adjustments were made for age, physical activity, and smoking.

Results: A total of 322 subjects (53%) were overweight. In normal-weight elderly women, ln-CRP was significantly associated with BMI, PAI-1, serum insulin, and HDL-cholesterol after adjustment for age, physical activity and smoking. In overweight women, however, these relationships were weak and no longer statistically significant. Also in men, the associations of ln-CRP with PAI-1 and serum insulin appeared to be modified by overweight status but interaction terms did not reach statistical significance.

Conclusion: In the elderly, CRP could be a more important component of the metabolic syndrome in women than in men. Furthermore, our data suggest only a minor role of CRP in the metabolic syndrome in the presence of overweight.

INTRODUCTION

C-reactive protein (CRP) is an acute-phase protein that is secreted by the liver in response to pro-inflammatory cytokines. Concentrations above 10 mg/L are generally used to indicate clinically relevant or acute inflammation. Low-grade chronic inflammation (CRP<10 mg/L) has been shown to predict risk of cardiovascular events in both apparently healthy populations and in cardiovascular patients.[1, 2] In the Physicians' Health Study an increased plasma concentration of CRP (≥2.10 mg/L) was associated with a twofold increased risk of stroke and a threefold increased risk of myocardial infarction compared to low CRP values (<0.55 mg/L).[3, 4] A number of mechanisms by which CRP might directly promote vascular disease have been postulated, including activation of the classical complement system in the arterial wall.[5, 6] Alternatively, the atherosclerotic process in the vascular wall may increase CRP synthesis. This hypothesis would imply that CRP is a marker of the extent and/or severity of the atherosclerotic process, rather than a causal agent. In addition, the observed association of CRP with cardiovascular disease may be due to confounding by smoking and other cardiovascular risk factors.

It has been suggested that CRP is involved in the metabolic syndrome.[7-9] Obesity is a key factor in the metabolic syndrome, with possibly a central role for cytokines

produced by adipose tissue.[10] Body mass index (BMI) has been shown to be an important correlate of CRP in both elderly and younger populations.[11-14] Examining the association of CRP with components of the metabolic syndrome by overweight status may provide more insight into the complex role of CRP in the development of cardiovascular disease. We addressed this research question in a general population of 605 Dutch elderly.

METHODS

Study population

From October 1991 until April 1992 a random sample of 1,793 non-institutionalized elderly residents of Arnhem, a Dutch city of approximately 133,000 inhabitants, were invited to participate in a study on lifestyle and health. The selection of participants is described in detail elsewhere.[15] A total of 1,012 elderly man and women between 65-84 years of age were interviewed, and 685 were physically examined. From 643 subjects a non-fasting blood sample was taken. Data from 605 participants for whom data on plasma CRP was available were included in the present analysis. The ethics committee of Wageningen University approved the study and written informed consent was obtained from all participants.

Data collection

Trained interviewers visited the participants at home and collected data on physical activity, food patterns, drinking and smoking habits, activities of daily living, chronic diseases, use of health care and medication. Physical activity was assessed by a validated questionnaire on household activities, sports and leisure time activities, developed for free-living elderly.[16] Activity scores were computed from the intensity and duration of specific activities. Two categories of physical activity level (low and high) were created, based on sex-specific median activity scores of the study population (i.e., 9.7 for men and 5.7 for women). The presence of chronic diseases was assessed by a questionnaire. Subjects reported for each disease whether they had visited a general practitioner or medical specialist in the past 3 months. Cardiovascular disease was considered present if the subject reported a history of coronary heart disease and/or stroke.

Non-fasting samples have been stored at -80°C. Plasma CRP was assessed using a highly sensitive ELISA procedure.[17] Serum total cholesterol was determined by an enzymatic method (CHOD-PAP). HDL-cholesterol and LDL-cholesterol were measured directly (Dimension® HDL method and N-geneous®LDL respectively). Serum insulin was determined with an immunometric assay (Immulite®2000 insulin). PAI-1 activity in plasma was determined using the Chromolize™ kit (Biopool, Umeå, Sweden). Systolic and diastolic blood pressure were measured twice in supine position with a random-zero sphygmomanometer. The mean of the two measurements was used in the analyses. Mean arterial pressure (MAP) was calculated as 'diastolic blood pressure + 1/3 [systolic blood pressure - diastolic blood pressure]'. Hypertension was defined as systolic blood pressure ≥160 mmHg and/or

diastolic blood pressure ≥95 mmHg and/or use of antihypertensive medication. Body weight was measured to the nearest 0.5 kilogram on a calibrated weighing scale with indoor clothing and without shoes. Height was measured to the nearest 0.5 cm using a wall-mounted measuring tape. Body mass index (BMI) was calculated as weight divided by squared height (kg/m^2).

Data analysis

Spearman correlation coefficients (r_s) were calculated to examine associations among cardiovascular risk factors, i.e., age, plasma CRP, serum insulin, PAI-1 activity, HDL-cholesterol, BMI and MAP. Spearman correlations were also obtained after stratification by gender and overweight, defined as a body mass index ≥25 kg/m^2. Plasma CRP values were log-transformed to improve normality (ln-CRP). Multivariate linear regression analysis was performed with ln-CRP as the dependent variable and cardiovascular risk factors as the independent variables, in men and women separately and in strata of overweight. Findings were additionally adjusted for age, physical activity ('high' *vs.* 'low') and smoking status (current, past or never). To test whether the observed associations differed between normal-weight and overweight elderly, an interaction term (overweight (0,1)*variable of interest) was added to the regression model. Analyses were repeated after exclusion of subjects with a self-reported history of cardiovascular disease and subjects with a plasma CRP value above 10 mg/L. Two-sided *P*-values below 0.05 were considered statistically significant.

Results

Characteristics of the population are given in Table 7.1. Median CRP levels were slightly higher in men than in women (2.4 mg/L *vs.* 2.1 mg/L). The percentage of current smokers was twice as high in men (33%) than in women (16%). Among men, 10% had never smoked compared to 59% in women. In 16 men (5%) and 4 women (1%) a CRP value above 10 mg/L was observed. Of the men, 50% were overweight (BMI ≥ 25 kg/m^2) compared to 57% of the women. The number of women using hormone replacement therapy was negligible (n=4, 1.4%).

BMI was the strongest correlate of CRP in women (r_s=0.39, *P*<0.001). The correlation between BMI and CRP in men was weak and did not reach statistical significance (r_s=0.09, *P*=0.11). CRP was positively correlated both with PAI-1 (r_s= 0.21 and r_s=0.24 for men and women, respectively, both *P*<0.001) and the mean arterial pressure (r_s= 0.12 (*P*=0.030) and r_s=0.15 (*P*=0.010) for men and women, respectively). CRP was negatively correlated with HDL-cholesterol (r_s= -0.24 and r_s= -0.28 for men and women, respectively, both *P*<0.001). CRP was furthermore correlated with total and LDL-cholesterol in men (both r_s=0.12, *P*=0.03) and with insulin in women (r_s=0.15, *P*=0.013). CRP was not correlated with age, neither in men nor in women. CRP was lower in physically active than in sedentary men (geometric means: 1.87 *vs.* 2.95 mg/L, respectively, *P*<0.001) and women (1.57 *vs.* 2.35 mg/L, respectively, *P*=0.008). No significant differences in CRP concentrations

were observed between current, former and never smokers, or between alcohol consumers and abstainers.

Table 7.1. Characteristics of a general population of 605 Dutch elderly

	Men (n = 315)	Women (n = 290)	*P*-value*
Age (yr)	73.2 ± 5.4	74.1 ± 5.9	0.06
BMI (kg/m^2)	25.4 ± 3.1	26.5 ± 4.5	0.001
Blood pressure (mmHg)			
Systolic	148 ± 20	153 ± 21	0.004
Diastolic	81 ± 11	82 ± 11	0.10
MAP	103 ± 13	106 ± 12	0.012
Hypertensive (%)	42	53	0.18
History of cardiovascular disease (%)†	25	17	0.001
Smoking status (%)			
Current	10	59	0.001
Past	57	26	
Never	33	16	
Alcohol consumers (%)	82	62	0.001
Physical activity score	9.7 (4.2 – 13.9)	5.7 (3.2 – 8.9)	<0.001
Serum total cholesterol (mmol/L)	6.0 ± 1.2	6.5 ± 1.2	<0.001
Serum HDL-cholesterol (mmol/L)	1.3 ± 0.3	1.5 ±0.4	<0.001
Serum LDL-cholesterol (mmol/L)	3.7 ± 1.0	3.8 ± 1.0	0.044
Serum insulin (pmol/L)	136 (96 – 222)	142 (98 – 225)	0.78
Plasma PAI-1 activity (IU/mL)	2.1 (0.6 – 5.4)	1.8 (0.5 – 5.5)	0.59
Plasma CRP (mg/L)	2.4 (1.2 – 4.7)	2.1 (1.0 – 3.8)	0.06

MAP: mean arterial pressure; Continuous variables are given as mean ± SD, and skewed data as median (Q1-Q3); * *P*-values for differences between men and women. Statistical testing was performed with the t-test for independent samples (continuous variables), Wilcoxon two-sample test (non-parametric variables) or χ^2 test (categorical variables); † Self-reported history of coronary heart disease and/or stroke.

In Table 7.2 results from multivariate regression analyses with ln-CRP as the dependent variable are presented. Age, BMI, physical activity ('high' *vs.* 'low'), smoking status (current, former and never), serum total cholesterol, serum HDL-cholesterol, mean arterial pressure (MAP), serum insulin, and plasma PAI-1 activity were simultaneously included as independent variables. In men, physical activity and HDL-cholesterol and MAP were the variables that contributed significantly to the model ($P<0.05$). In women, only BMI and HDL-cholesterol were significantly associated with ln-CRP. All variables together explained 11% of the variance in ln-CRP in men compared to 21% in women (adjusted R^2). In women BMI alone explained 15% of the variance in ln-CRP.

Table 7.2. Association between cardiovascular risk factors and log-transformed plasma CRP in elderly men and women, obtained by multivariate regression analysis

	β (95%-CI)[†]			
	Men (n=315)		Women (n=290)	
Age (yr)	0.02	(-0.01; 0.04)	0.01	(-0.02; 0.03)
BMI (kg/m^2)	-0.003	(-0.05; 0.04)	0.07	(0.03; 0.10)*
Physical activity ('high' *vs.* 'low')	-0.33	(-0.60; -0.07)*	-0.16	(-0.47; 0.16)
Smoking status[‡]				
Past	-0.26	(-0.69; 0.18)	0.22	(-0.13; 0.57)
Current	-0.20	(-0.67; 0.26)	0.26	(-0.14; 0.66)
Serum lipids (mmol/L)				
Total cholesterol	0.08	(-0.03; 0.20)	0.05	(-0.06; 0.16)
HDL cholesterol	-0.75	(-1.20; -0.30)*	-0.79	(-1.22; -0.36)*
MAP (per 10 mmHg)	0.01	(0.03; 0.24)*	0.11	(-0.02; 0.23)
Insulin (per 10 pmol/L)	0.001	(-0.01; 0.01)	-0.002	(-0.01; 0.005)
PAI-1 activity (IU/mL)	0.01	(-0.01; 0.02)	0.005	(-0.02; 0.03)
Adjusted R^2 (full model)	11%		21%	

* $P<0.05$ in the multivariate regression model; [†] Linear regression coefficient (β) denotes change in log-transformed plasma CRP (ln-CRP) per unit change in cardiovascular risk factor, with 95%-confidence interval in parentheses; [‡] Dummy variables for past and current smoking were entered into the regression model, with never-smoking as reference.

Stratification by BMI (<25 *vs.* ≥25 kg/m^2) was performed to examine the associations of CRP with components of the metabolic syndrome by overweight status, both in men (Table 7.3) and in women (Table 4). In normal-weight women a strong association between BMI and ln-CRP was observed, which appeared to be absent in overweight women (Figure 7.1). The interaction was statistically significant (P=0.005) also after adjustment for age, physical activity and smoking status (Table 7.5). BMI was not associated with ln-CRP in men, neither in normal-weight nor in overweight subjects. Further adjustment for other potential confounders did not materially alter the results (data not shown).

Table 7.3. Spearman correlation coefficients among components of the metabolic syndrome in 315 elderly men, stratified by overweight

	CRP (mg/L)	BMI (kg/m^2)	PAI-1 (IU/mL)	Insulin (pmol/L)	HDL-C (mmol/L)
BMI <25 kg/m^2					
Plasma CRP (mg/L)	--				
BMI (kg/m^2)	0.06	--			
PAI-1 activity (IU/mL)	0.30‡	0.23†	--		
Serum insulin (pmol/L)	0.18*	0.17*	0.26†	--	
HDL-C (mmol/L)	-0.33‡	-0.14	-0.29‡	-0.30‡	--
MAP (mmHg)	0.18*	0.19*	0.03	0.03	0.08
BMI ≥25 kg/m^2					
Plasma CRP (mg/L)	--				
BMI (kg/m^2)	0.03	--			
PAI-1 activity (IU/mL)	0.06	0.11	--		
Serum insulin (pmol/L)	-0.03	-0.03	0.24†	--	
HDL-C (mmol/L)	-0.11	-0.05	-0.16*	-0.09	--
MAP (mmHg)	0.07	0.11	0.12	-0.10	0.07

HDL-C: serum HDL-cholesterol; MAP: mean arterial pressure; * $P<0.05$; † $P<0.01$; ‡ $P<0.001$.

Table 7.4. Spearman correlation coefficients among components of the metabolic syndrome in 290 elderly women, stratified by overweight

	CRP (mg/L)	BMI (kg/m^2)	PAI-1 (IU/mL)	Insulin (pmol/L)	HDL-C (mmol/L)
BMI <25 kg/m^2					
Plasma CRP (mg/L)	--				
BMI (kg/m^2)	0.32‡	--			
PAI-1 activity (IU/mL)	0.24†	0.15	--		
Serum insulin (pmol/L)	0.15	0.13	0.16	--	
HDL-C (mmol/L)	-0.31‡	-0.11	-0.29†	-0.29†	--
MAP (mmHg)	0.12	0.17	0.003	0.03	-0.09
BMI ≥25 kg/m^2					
Plasma CRP (mg/L)	--				
BMI (kg/m^2)	0.13	--			
PAI-1 activity (IU/mL)	0.02	0.21†	--		
Serum insulin (pmol/L)	0.04	0.27‡	0.32‡	--	
HDL-C (mmol/L)	-0.10	-0.11	-0.30‡	-0.37‡	--
MAP (mmHg)	0.13	0.04	0.06	-0.03	0.09

HDL-C: serum HDL-cholesterol; MAP: mean arterial pressure;* $P<0.05$; † $P<0.01$; ‡ $P<0.001$.

Table 7.5. Associations of ln-CRP with components of the metabolic syndrome in elderly men and women, stratified by overweight

	β (95% CI)		
	BMI <25 kg/m^2	BMI ≥25 kg/m^2	*P*-value for interaction*
Men (n=315)			
BMI (kg/m^2)	0.07 (-0.07; 0.21)	0.02 (-0.06; 0.07)	0.38
PAI-1 activity (IU/mL)	0.02 (-0.01; 0.04)	0.004 (-0.02; 0.03)	0.09
Serum insulin (per 10 pmol/L)	0.01 (-0.003; 0.03)	0.001 (-0.01; 0.01)	0.08
HDL-C (mmol/L)	-0.55 (-1.21; 0.11)	-0.53 (-1.08; 0.01)	0.95
MAP (per 10 mmHg)†	0.13 (-0.02; 0.28)	0.07 (-0.07; 0.21)	0.52
Women (n=290)			
BMI (kg/m^2)	0.21 (0.10; 0.33)	0.04 (-0.009; 0.09)	0.005
PAI-1 activity (IU/mL)	0.07 (0.03; 0.10)	-0.01 (-0.03; 0.02)	0.002
Serum insulin (per 10 pmol/L)	0.02 (0.004; 0.04)	-0.001 (-0.01; 0.01)	0.020
HDL-C (mmol/L)	-1.32 (-1.95; -0.68)	-0.42 (-0.95; 0.11)	0.039
MAP (per 10 mmHg)	0.14 (-0.07; 0.34)	0.08 (-0.32; 0.56)	0.68

HDL-C = serum HDL-cholesterol; MAP = mean arterial pressure; Data are presented as linear regression coefficients (β) with 95% confidence intervals in parentheses. Adjustments were made for age, physical activity ('high' *vs.* 'low') and smoking status (never, former and current). * *P*-value for the interaction between overweight status and the variable of interest.

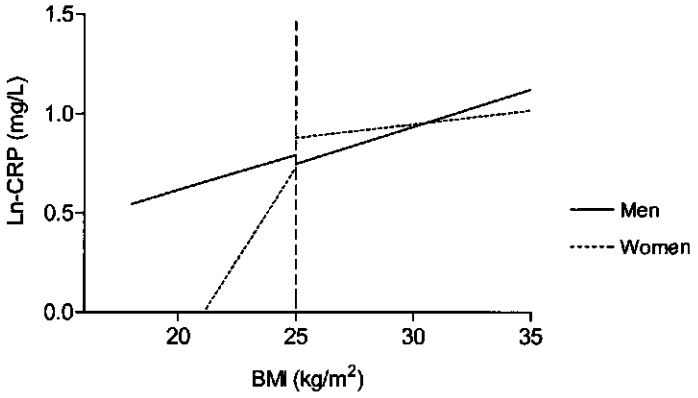
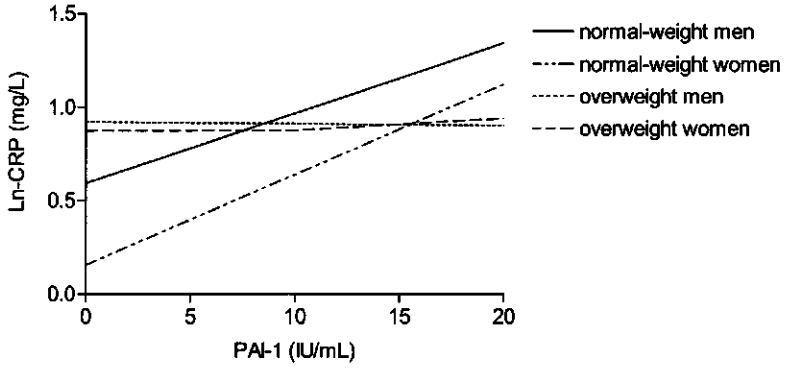


Figure 7.1. Association of ln-CRP with BMI in elderly men and women, stratified by overweight. Findings have been obtained by linear regression analysis; Overweight: BMI ≥25 kg/m^2.

A.



B.

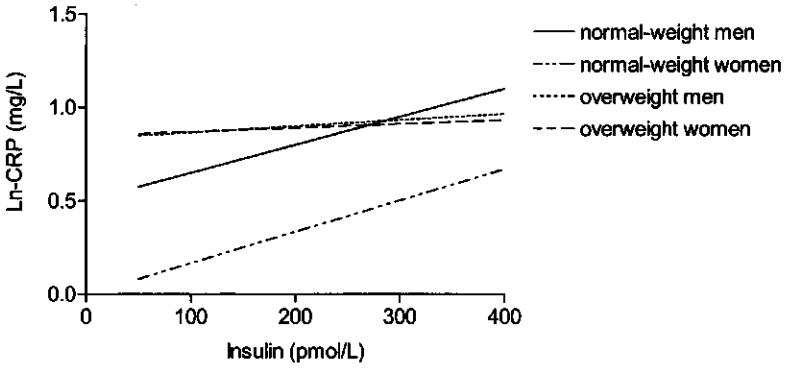


Figure 7.2. Association of ln-CRP with PAI-1 activity (A) and serum insulin (B) in elderly men and women, stratified by overweight. Findings have been obtained by linear regression analysis; Overweight: BMI ≥25 kg/m^2.

Ln-CRP was clearly associated with PAI-1 activity in normal-weight elderly, but this association was absent in overweight elderly (Figure 7.2). The interaction term for overweight status and PAI-1 was statistically significant both in men and in women (both $P<0.05$) in crude analysis. In men, the interaction term was no longer statistically significant after adjusting for age, physical activity and smoking status ($P=0.09$). Also for insulin, stronger associations with ln-CRP were observed in normal-weight than in overweight elderly (Figure 7.3), with a statistically significant interaction term ($P=0.020$) in women (Table 7.5). Furthermore, the association between ln-CRP and HDL-cholesterol was stronger in normal-weight than in overweight women ($P=0.039$). For men, no interaction between overweight and HDL-cholesterol was observed. The associations between ln-CRP and MAP were not influenced by overweight status.

Repeating the analyses after exclusion of subjects with a history of cardiovascular disease and subjects with CRP >10 mg/L yielded similar results (data not shown).

DISCUSSION

In our study among a general population of Dutch elderly, we observed that overweight modified the associations between plasma CRP and major components of the metabolic syndrome. CRP was stronger associated with body mass index, PAI-1 activity and serum insulin in normal-weight than in overweight elderly. Findings appeared to be more pronounced in women than in men. Associations of CRP with MAP were not modified by overweight. To avoid confounding by the severity of the atherosclerotic process or other conditions that could have caused increased CRP levels, the analyses were repeated after exclusion of subjects with a history of cardiovascular disease or CRP above 10 mg/L. This did not result in alterations of the observed associations.

Little is known about the underlying pathogenic mechanism of the metabolic syndrome, but (abdominal) obesity is believed to play a central role.[10] Adipose tissue not only has a function as energy storage, but is also important as secretory organ. Adipose tissue derived products are amongst others cytokines and PAI-1, which may contribute to the detrimental effects of obesity on cardiovascular risk. The metabolic functions of adipose tissue have recently been reviewed.[18]

In epidemiological studies it has consistently been shown that higher body weight is associated with increased levels of CRP.[11-14] In addition, it has been shown that weight reduction lowers CRP.[19, 20] The observed associations between markers of obesity and CRP are most likely mediated by interleukin-6 (IL-6), which is produced by adipose tissue and is known to regulate the hepatic synthesis of CRP.[21] In studies that stratified for gender stronger associations between CRP and BMI were observed for women than for men.[12, 22, 23] Our results are consistent with these observations. Some possible explanations for the gender-specific associations of CRP with BMI can be given. First, at equal BMI, women have significantly higher amounts of total body fat compared to men and also a different distribution of body fat.[24] Another explanation could be the involvement of sex steroids, which may influence the metabolic activity of adipose tissue.[18] In line with this hypothesis, a correlation of CRP with estradiol has been demonstrated in overweight women.[20]

We observed a strong association between CRP and BMI, but only in normal-weight women. The absence of an association between CRP and BMI at higher BMI suggests an upper limit in body weight-associated CRP levels. Because of the ability of the liver to produce extremely high levels of CRP during inflammation, we consider it unlikely that this upper limit in CRP is caused by a lower hepatic CRP production in response to interleukins. We hypothesize that the production of CRP inducing factors in adipose tissue (e.g. IL-6) may be diminished at higher body weight. The quantity of metabolites secreted by adipose tissue may depend on both total amount of fat mass and the distribution of fat over the different depots, which has indeed been demonstrated for PAI-1, TNF-α and other metabolites.[25] This adipose tissue heterogeneity might explain why we observed different associations with CRP in normal-weight and overweight elderly. *In vitro* studies showed a 3-fold higher release of IL-6 in omental than abdominal subcutaneous adipose tissue.[26] It is

not known whether IL-6 production differs between normal-weight and overweight elderly.

As far as we know, previous studies have not performed stratified analyses by overweight status. Mean values of CRP in strata of BMI and overall associations between CRP and components of the metabolic syndrome have been reported, but these results cannot be compared to our findings. In a population of middle-aged obese women, CRP was associated with BMI,[20] which is in contrast to our finding that in overweight elderly women. Possibly, the older age of our cohort provides an explanation for this discrepancy.

CRP was positively associated with plasma PAI-1 activity and serum insulin in our study. As for BMI, these associations predominantly existed within the population of normal-weight elderly, both for men and women. Adipose tissue is an important source of circulating PAI-1.[27-29] PAI-1 is directly secreted by adipose tissue and adipocytes-derived cytokines (TNF-α en TGF-β) furthermore stimulate hepatic PAI-1 production.[28] It has been observed that adipocytes in obese subjects could produce more PAI-1 than adipocytes in lean subjects[30], even after adjustment for adipocyte size.[31]

We observed no effect modification by overweight in the associations of CRP with MAP. Although blood pressure is also involved in the metabolic syndrome, the link between this variable and the metabolic syndrome may not be mediated by adipose tissue.

From our data in a general elderly population, we conclude that CRP is unlikely to play an important, independent role in the metabolic syndrome in the presence of overweight. This finding, however, needs to be confirmed in prospective epidemiological studies, both in the elderly and in younger populations. In future research, it may be worthwhile to include other indicators of fat mass and body fat distribution, and to examine whether weight reduction alters the relationship between CRP and components of the metabolic syndrome.

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8

High-sensitivity C-reactive protein is predictive for cardiovascular and all-cause mortality in elderly men, but not in elderly women

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ABSTRACT

Background: C-reactive protein (CRP) is a sensitive marker of inflammation. Recently, there has been a growing interest in small CRP elevations since chronic low-grade inflammation may play a role in cardiovascular disease.

Methods: We studied the associations of CRP with all-cause mortality, cardiovascular mortality, incidence of myocardial infarction, stroke and transient ischemic attack in a prospective study among 605 Dutch elderly men and women aged 65-84 years.

Results: The mean follow-up time was 7.7 years. In men, high CRP (>2.2 mg/L) was associated with an increased risk of all-cause mortality (RR=1.5 (95%-CI: 1.1 - 2.1)), CVD mortality (RR=2.2 (95%-CI: 1.2 - 4.2) and incidence of stroke (RR=2.7 (95%-CI 1.2 - 6.2)). The observed relationships remained similar after adjusting for potential confounders. In women, high CRP was not associated with any of the cardiovascular endpoints, but the number of events in women was small.

Conclusions: We conclude that increased levels of CRP, indicating low-grade inflammation, may contribute to cardiovascular risk in elderly men. Our finding that CRP is not associated with cardiovascular risk in elderly women, however, needs further confirmation in larger prospective epidemiological studies.

INTRODUCTION

C-reactive protein (CRP), a hepatically-derived acute phase reactant protein, is a highly sensitive marker of inflammation. Serum CRP concentrations above 10 mg/L are generally considered to indicate clinical inflammation. More recently, there has been a growing interest in small CRP elevations since chronic low-grade inflammation may play a role in cardiovascular disease.[1,2] CRP has been associated with worse prognosis in patients with coronary heart disease and stroke.[3-5]

However, CRP may also have an added value in the prediction of cardiovascular and all-cause mortality in general healthy populations, including the elderly.[6-12] We examined whether CRP predicted all-cause and cardiovascular mortality in the Arnhem Elderly Study, a prospective population-based study of Dutch elderly men and women with a mean follow-up of 7.7 years.

METHODS

Study population

The Arnhem Elderly Study is a prospective, population-based study among older inhabitants of Arnhem, a city in the Netherlands. Participants were randomly selected from the general population after stratification for age and sex, as described in more detail elsewhere.[13] A total of 1,012 non-institutionalized elderly men and women, aged 65-84 years, were enrolled in the health survey in 1991/1992. Physical examination was performed in 685 subjects (68%), and blood samples were available for 641 subjects (63%). CRP values were randomly missing for 36

subjects, leaving 605 subjects for the present analysis. Written informed consent for collection of follow-up data and approval by the ethical committee of Wageningen University were obtained.

Data collection

Trained interviewers visited the participants at home and collected data on smoking habits, health status, medication and demographics. Smoking status was coded as current, former and never. A history of cardiovascular disease was considered present if the participant reported a history of heart disease or stroke. Use of cardiovascular medication during the previous 3 months was recorded, which comprised use of ACE-inhibitors, beta-blockers, thrombolytic agents, lipid-lowering medications or salicylates. Physical examination included anthropometry, blood pressure measurement and blood sampling (non-fasting). Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Hypertension was defined as a systolic blood pressure ≥160 mmHg, or diastolic blood pressure ≥95 mmHg or use of antihypertensive medication.

Laboratory determinations

Serum aliquots were stored at -80°C. C-reactive protein (CRP) was assessed using highly sensitive ELISA.[14] Serum total cholesterol was determined by an enzymatic method (CHOD-PAP). Serum HDL and LDL cholesterol were measured directly (Dimension®HDL method and N-geneous®LDL respectively). Serum insulin was determined with an immunometric assay (Immulite®2000 insulin).

Follow-up

Municipal registries provided data on mortality and migration of the study cohort at regular time intervals until February 2001. Information on vital status at the end of follow-up was complete. Data on morbidity and cause-specific mortality were obtained from general practitioners (GP), either directly or by one of the authors (T.H.) by means of a standard questionnaire. Follow-up data for incident cardiovascular evens and cause-specific mortality were available for 490 subjects (81%). Reasons for missing data were: (1) subjects gave no permission (n=39, 6%), (2) the GP could not be traced (n=30, 5%), (3) the GP refused participation (n=38, 6%), or (4) could not provide valid data (n=8, 1%). Baseline characteristics of elderly, of whom data from medical records were missing, were comparable with those included in the analysis, except for serum cholesterol (6.0 versus 6.3, P=0.02). No differences were observed concerning age and gender distribution.

Endpoints were coded according to the International Classification of Diseases, tenth revision (ICD-10). Outcomes for the present analyses comprised all-cause mortality, death due to major cardiovascular disease (I00-I96), incidence of myocardial infarction (I21-I22), incidence of stroke (I60-I69), and incidence of transient ischemic attack (G45). In case of recurrent cardiovascular events, only the first event was considered in the analysis.

Data analysis

Spearman's correlation coefficients were calculated for the association between CRP, subject characteristics and cardiovascular risk factors. Based on the median CRP level of the total population (i.e., 2.2 mg/L) the study cohort was divided into a 'low' and 'high' CRP group. Cox survival analysis was used to estimate risk of all-cause mortality, cardiovascular mortality, incident myocardial infarction (both fatal and non-fatal), incident stroke (both fatal and non-fatal), and incident transient ischemic attacks for subjects with 'high' CRP (>2.2 mg/L) compared to subjects with 'low' CRP (≤2.2 mg/L). Hazard rate ratios (subsequently referred to as relative risk, RR) were adjusted for age ('Model 1'), and additionally for BMI, smoking status (current, former, never), history of cardiovascular disease, blood pressure (both systolic and diastolic), cholesterol and insulin ('Model 2'). The SAS system was used for all statistical analyses and *P*-values below 0.05 were considered statistically significant.

RESULTS

Baseline characteristics of the study population are shown in Table 8.1. CRP was significantly higher in men than in women (median of 2.4 vs. 2.1 mg/L, *P*=0.02). CRP levels exceeded 10 mg/L in 17 men (5%) and 4 women (1%).

Table 8.1. Characteristics of a general population of 605 Dutch elderly

	Men (n=315)	Women (n=290)
Age (yr)	73.2 ± 5.4	74.1 ± 5.9
Body mass index (kg/m^2)	25.4 ± 3.1	26.5 ± 4.5
Blood pressure (mmHg)		
Systolic	148 ± 20	153 ± 21
Diastolic	81 ± 11	82 ± 11
Smoking status (%)		
Current	33	16
Former	57	26
Never	10	59
Diabetes, self reported (%)	3	6
History of cardiovascular disease (%)*	25	17
Use of cardiovascular medication (%)†	20	19
Serum total cholesterol (mmol/L)	6.0 ± 1.2	6.5 ± 1.2
Serum HDL-cholesterol (mmol/L)	1.3 ± 0.3	1.5 ± 0.4
Serum LDL-cholesterol (mmol/L)	3.7 ± 1.0	3.8 ± 1.0
Serum CRP (mg/L)	2.4 (1.2 - 4.7)	2.1 (1.0 - 3.8)
Serum insulin (pmol/L)	136 (96 - 222)	142 (98 - 225)
Deceased during follow-up (%)	49	34

Continuous variables are presented as mean ± SD, or median with interquartile range (Q1-Q3) in case of skewed distribution.

* Includes self-reported heart disease or stroke; † Use of ACE-inhibitors, beta-blockers, thrombolytic agents, lipid-lowering medication or salicylates during the 3 months prior to the interview.

Table 8.2. Relative risks (95%-CI) of fatal and non-fatal cardiovascular events and all-cause mortality with CRP in a general population of Dutch elderly men and women

	Men (n = 250)†		Women (n = 240)†	
	CRP ≤2.2 mg/L	CRP >2.2 mg/L	CRP ≤2.2 mg/L	CRP >2.2 mg/L
Incident stroke				
Cases/person-years	8/968	20/867	15/1,008	16/857
RR, model 1*	1	2.7 (1.2 - 6.2)	1	1.3 (0.6 - 2.7)
RR, model 2†	1	2.7 (1.2 - 6.2)	1	1.0 (0.4 - 2.4)
Incident MI				
Cases/person-years	9/935	11/854	6/1,012	4/887
RR, model 1*	1	1.3 (0.5 - 3.2)	1	0.7 (0.2 - 2.5)
RR, model 2†	1	1.3 (0.5 - 3.2)	1	0.8 (0.2 - 3.4)
Incident TIA				
Cases/person-years	8/956	16/840	14/982	13/838
RR, model 1*	1	2.2 (0.9 - 5.2)	1	1.0 (0.5 - 2.2)
RR, model 2†	1	2.5 (1.0 - 6.2)	1	0.9 (0.4 - 2.1)
CVD mortality				
Cases/person-years	14/975	30/895	17/1,025	14/896
RR, model 1*	1	2.2 (1.2 - 4.2)	1	1.0 (0.5 - 2.0)
RR, model 2†	1	2.1 (1.1 - 4.0)	1	0.4 (0.2 -1.0)
All-cause mortality‡				
Cases/person-years	61/1,250	92/1,144	50/1,310	50/1,115
RR, model 1*	1	1.5 (1.1 - 2.1)	1	1.2 (0.8 - 1.7)
RR, model 2†	1	1.4 (1.0 - 2.0)	1	0.9 (0.5 - 1.4)

TIA = transient ischemic attack; MI = myocardial infarction; CVD = cardiovascular disease.

* Model 1: adjusted for age;† Model 2: adjusted for age, BMI, smoking status (current, former, never), history of cardiovascular disease, blood pressure, serum total cholesterol, and serum insulin.

‡ Survival analyses for all-cause mortality included 315 men and 290 women respectively

There was no significant correlation between age and serum CRP. In men, serum CRP was significantly correlated with LDL-cholesterol (r_s=0.12, P=0.03), HDL-cholesterol (r_s=-0.24, P<0.001), total cholesterol (r_s=0.12, P=0.03), and systolic blood pressure (r_s=0.13, P=0.02). BMI was a strong determinant of CRP in women (r_s=0.39, P<0.001) but not in men (r_s=0.09, P=0.11). In women, there were also significant associations of CRP with HDL-cholesterol (r_s= -0.28, P<0.001), insulin (r_s=0.15, P=0.01), and diastolic blood pressure (r_s=0.18, P=0.002). CRP was lower in physically active than in sedentary elderly, both in men (geometric means: 1.87 vs. 2.95 mg/L, respectively, P<0.001) and women (1.57 vs. 2.35 mg/L, respectively, P=0.008). No significant differences in CRP concentrations were observed among current, former and never smokers, or between alcohol consumers and abstainers.

Data from medical records were available for 490 elderly (240 women and 250 men). The mortality rate of this sub-sample was 34% of the women and 47% of the men. In men 38% of the deaths were due to cardiovascular disease and for women this

percentage was 45%. During the mean follow-up time of 7.7 years, 30 cases of myocardial infarction, 59 strokes and 51 TIA's were detected.

History of cardiovascular disease, and systolic blood pressure were significant predictors for all-cause mortality in both sexes. Measures of cholesterol, smoking, BMI, and presence of diabetes were not significantly related to all-cause mortality. RR for CRP and all-cause mortality and cardiovascular endpoints are presented in Table 8.2.

In men, high CRP was associated with an increased risk of all-cause mortality (RR=1.5 (95%-CI: 1.1 - 2.1)), CVD mortality (RR=2.2 (95%-CI: 1.2 - 4.2) and incidence of stroke (RR=2.7 (95%-CI: 1.2 - 6.2)). The observed relationships remained similar after adjusting for potential confounders (i.e., Model 2). In women, high CRP was not associated with any of the cardiovascular end points, but the number of events in women was small.

Discussion

In this cohort of elderly Dutch men and women, highest rates of cardiovascular events and mortality were observed in the high CRP group in men, but not in women. Multivariate analyses, with adjustment for age, blood pressure, BMI, serum total cholesterol, serum insulin, smoking status, and history of cardiovascular disease, did not substantially change these results. Findings in elderly men, therefore, broadly reflect the findings from other prospective studies.[3-8,10,11]

However, our results are not consistent with studies among elderly women that found a positive association between CRP and future coronary events,[5,9,12] stroke,[6,10,11] and all-cause mortality.[7,8] The Women's Health Study and the Women's Health Initiative Observational Study, two large prospective, nested case-control studies in postmenopausal women, showed high-sensitive CRP to be an important predictor of myocardial infarction or coronary heart disease mortality in healthy women.[9,12] The women included were younger (45 years and above and 50 to 79 years respectively) than the women of the Arnhem Elderly cohort.

A major shortcoming of the present study is the lack of power, which hampers the drawing of strong conclusions, especially for risk of myocardial infarction. The absence of an increased cardiovascular risk for high CRP in women may be due to chance. An alternative explanation might be that the determinants of serum CRP were different for men than for women, possibly resulting in a different predictive value. BMI is positively associated with cardiovascular risk in women, whereas CRP also increases with BMI. The latter may be explained by adipose tissue secreting interleukin-6, which subsequently may stimulate the hepatic synthesis of CRP.[15] Therefore, in the setting of obesity (especially in women), high CRP levels may not confer additional cardiovascular risk after adjusting for measures of obesity, because it is a marker of another cardiovascular risk factor. It might be that CRP in obese women is not a good marker for risk because it is mainly a reflection of the amount of adipose tissue. It is possible that in women over (roughly) 65 years of age, a detrimental effect of CRP would not be apparent, not only because of comorbidity

(e.g., obesity), but also because of competing causes of death associated with a low-grade inflammatory state.

Main causes of death are different for middle aged compared to elderly populations, e.g., the relative incidence of congestive heart failure, cerebrovascular disease, chronic obstructive pulmonary disease, pneumonia, and other infections increase with age, while the incidence of myocardial infarction decreases.[16] In line with this phenomenon, we observed a higher incidence of stroke compared to myocardial infarction in the cohort of the Arnhem Elderly Study. Furthermore, it may be hypothesized that an association between CRP and future coronary events and mortality may be stronger in elderly with a higher coronary risk. In our study the proportion of elderly with a history of cardiovascular disease was indeed higher in men than in women.

We conclude that increased levels of CRP, indicating low-grade inflammation, may contribute to cardiovascular risk in elderly men. Our finding that high CRP does not increase risk of cardiovascular disease in elderly women, however, needs further confirmation in larger prospective epidemiological studies.

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9

General discussion

The main goal of the epidemiological studies presented in thesis was to examine the relationship between PAI-1 and cardiovascular disease. Interpretation of observational, data on these associations is complicated because of potential confounding by a wide range of cardiovascular risk factors and other types of bias. The 4G/5G polymorphism in the promotor region of the PAI-1 gene is associated with serum PAI-1 levels. An increased cardiovascular risk for the 4G-allele would add evidence for a causal role of PAI-1 in the cardiovascular disease process. Therefore, in most studies we investigated the association of the 4G/5G-polymorphism with atherosclerotic and thrombotic outcomes.

In addition to its well-known function in fibrinolysis, PAI-1 is also an acute-phase protein, providing an alternative pathway by which PAI-1 might be associated with cardiovascular risk. We therefore also examined the role of CRP, a sensitive marker of inflammation, which enabled us to study the role of PAI-1 independent of inflammation.

This chapter provides an overview of the main findings of the studies described in this thesis and the consistency with existing scientific data, followed by some methodological considerations and suggestions for future research.

MAIN FINDINGS

PAI-1 showed a strong diurnal variation with an early morning peak, which appeared to be confined to the 4G-allele. This might lead to an increased risk of cardiovascular events especially in the early morning. Elderly with the 4G/4G-genotype were protected against stroke. The latter finding is in contrast to the generally accepted hypothesis that the 4G-allele (as marker for increased plasma PAI-1 levels) increases risk of cardiovascular events due to diminished fibrinolytic activity.

An overview of the characteristics and the main findings of the studies described in this thesis are given in Table 9.1. In the following paragraphs, we will discuss our findings on PAI-1 and CRP in more detail and make a comparison with reported scientific data.

Plasminogen activator inhibitor-type 1

Diurnal variation in PAI-1

In the population-based cohort of the Arnhem Elderly Study, plasma PAI-1 activity showed a strong diurnal variation with peak levels in the early morning. This finding is in agreement with the results of several small studies in which serial PAI-1 determinations were performed.[1-8] We furthermore demonstrated this diurnal variation to be primarily confined to the 4G-allele. In the Rotterdam Study among a sub sample of 263 men and women aged 55 years and over, the morning/afternoon difference in PAI-1 antigen was also more pronounced in persons with the 4G/4G-genotype than in persons with the other genotypes.[9]

Table 9.1. Studies described in this thesis and the main findings

Ch.	Design	Population	Exposure	Cardiovascular endpoint	Main results
3	Cross-sectional	Arnhem Elderly Study, 598 men and women, aged 64-84 years	PAI-1 activity, 4G/5G-polymorphism t-PA antigen	N.A.	**Overall**: Strong diurnal variation in PAI-1 with peak levels in the:early morning (before 9 a.m.13x higher than > 4 p.m. **Stratified for 4G/5G-polymorphism:** Diurnal variation for 4G/4G and 4G/5G (both *P*-trend=0.0001), but not for the 5G/5G-genotype (*P*-trend=0.10)
4	Cross-sectional	208 male smokers, mean age of 59 years	4G/5G-polymorphism	Markers of atherosclerosis: Intima media thickness (IMT), Ankle-brachial index (ABI)	4G/4G vs 5G/5G: IMT > 1.07mm: OR=0.5 (95%-CI: 0.1 - 1.7) ABI < 1.03: OR=0.9 (95%-CI: 0.3 - 2.9)
5	Case-control	Pooled data of three case-control studies: -285 cases -293 coronary controls -198 population-based controls	4G/5G-polymorphism	Coronary stenosis (assessed with coronary angiography)	**Coronary controls:** association between the 4G/5G-polymorphism and stenosis, but only for low-risk sub sample **Population-based controls:** no significant associations
6	Prospective	Arnhem Elderly Study, 637 men and women, aged 64-84 years	PAI-1 activity, 4G/5G-polymorphism, t-PA antigen	All-cause and cardiovascular mortality, incidence of MI, stroke and TIA	**PAI-1** (upper vs lower tertile): Predictive for incident stroke, TIA, cardiovascular and all-cause mortality **4G/5G-polymorphism** (4G/4G vs 5G/5G): protective against stroke (RR=0.4 (95%-CI: 0.2 - 0.9) and TIA (RR=0.3 (95%-CI: 0.1 - 0.8) **T-PA** (upper vs lower tertile): predictive for cardiovascular and all-cause mortality
7	Cross-sectional	Arnhem Elderly Study, 605 men and women	CRP, PAI-1 activity	N.A.	CRP was strongly associated with components of the metabolic syndrome, but predominantly within elderly without overweight
8	Prospective	Arnhem Elderly Study, 605 men and women	CRP	All-cause and cardiovascular mortality, incidence of MI, stroke and TIA	**Men**: high CRP (> 2.2 mg/L) predictive for stroke, TIA, cardiovascular and all-cause mortality **Women**: No significant associations

A biological explanation for a genotype-specific diurnal variation in PAI-1 is provided by Maemura *et al.*, who identified a transcription factor (CLIF: cycle-like factor) that is involved in the circadian pattern of PAI-1.[10] The binding site of this transcription factor overlaps with the location of the 4G/5G-polymorphism. The early morning peak in PAI-1 might partly be responsible for the high frequency of cardiovascular events at this time of the day and this may be different for the PAI-1 genotypes.

4G/5G-polymorphism and atherosclerosis

We observed no consistent association between the 4G/5G-polymorphism and advanced coronary stenosis in our pooled case-control study (Chapter 5, see Table). A non-significantly increased risk of coronary stenosis was observed for the 4G/4G-genotype when cases were compared with coronary controls, but not when compared with population-based controls. In a case-control study by Gardemann *et al.* of 2,565 subjects who underwent coronary angiography, the 4G/4G-genotype was associated with the presence of coronary stenosis, but not with the number of diseased vessels.[11] They observed the strongest associations between the 4G/5G-polymorphism and coronary stenosis in high-risk sub samples (e.g. smokers, subjects with high BMI, and hypertensives).[11] Stratification for background risk in our study (based on the Framingham Prediction Score), showed opposite results, i.e. a stronger association in low risk individuals. Two other studies did not observe any association between the 4G/5G-polymorphism and angiographically determined coronary artery disease.[12,13]

Non-invasive markers of the atherosclerotic process make it possible to study atherosclerosis at an earlier stage, when subjects are still free of symptoms. We investigated the association between the 4G/5G-polymorphism and both intima-media thickness (IMT) and ankle-brachial index (ABI) in male smokers and found no indication that the 4G-allele is associated with more advanced atherosclerosis than the 5G-allele. Although not statistically significant, a high IMT (>1.07 mm) was even less frequent for the 4G/5G and 4G/4G-genotypes compared to the 5G/5G-genotype (odds ratios of 0.6 and 0.4 respectively, *P*-value for trend: 0.08). Previous studies showed positive associations between PAI-1 antigen levels and intima-media thickness,[14,15] but data on 4G/5G-polymorphism in relation to non-invasive markers of atherosclerosis are lacking.

Plasma PAI-1, 4G/5G-polymorphism and coronary events

In the Arnhem Elderly Study, PAI-1 activity was strongly predictive for cardiovascular mortality, independent of other cardiovascular risk factors (including CRP). Also for incident myocardial infarction an increased risk was observed for elevated PAI-1 levels, although the association was not significant.

In previous studies in populations with angina pectoris or a history of a myocardial infarction, PAI-1 levels predicted future (recurrent) coronary events. However, in most previous population-based prospective studies PAI-1 was not significantly associated with the occurrence of a first event.[16,17] In the few studies among healthy populations in which plasma PAI-1 was associated with risk of coronary events the

strength of the association was strongly reduced after adjusting for cardiovascular risk factors.[18,19] Only one other prospective study on PAI-1 and cardiovascular risk in the elderly has been performed, i.e. the Cardiovascular Health Study,[16] in which no association was observed between PAI-1 and coronary risk. Elderly with known cardiovascular disease were excluded from this study, whereas in the Arnhem Elderly Study 20% of the population had a history of cardiovascular disease. Even in elderly without clinical evidence for cardiovascular disease, the atherosclerotic process is likely to be advanced, which might be the underlying reason for the association between PAI-1 and cardiovascular events.

We did not observe an increased coronary risk for the 4G/4G-genotype, which is in agreement with previous prospective studies. In the Physicians' Health Study the 4G/5G-polymorphism was not predictive for future myocardial infarction in men initially free of cardiovascular disease.[20] In postmenopausal women[21] and in elderly subjects[22] the 4G/5G-polymorphism was not predictive for fatal myocardial infarction.

In a recent meta-analysis of 9 studies, mainly case-control, a small increased risk for myocardial infarction was observed for the 4G/4G-genotype (OR=1.2 (95%-CI: 1.0 - 1.4)).[23]

Plasma PAI-1, 4G/5G-polymorphism and risk of stroke

In the Arnhem elderly the 4G/4G-genotype was protective for future stroke (RR= 0.4 (95%-CI: 0.2 - 0.9). Most previous studies on stroke also showed a protective effect for the 4G-allele (see Chapter 6, Figure 1),[21,22,24-26] but only in one study the effect was strong enough to reach statistical significance.[21] However, in a Korean case-control study the 4G-allele was associated with an increased risk of ischemic stroke.[27] Despite the protective effect of the 4G-allele, we observed a higher risk of stroke for higher PAI-1 levels. Johansson *et al.* found that PAI-1 antigen was non-significantly associated with incidence of first stroke in a population-based cohort (OR upper versus lower PAI-1 quartile: 1.3 (95%-CI: 0.7 - 2.6).[28] The population studied by Johansson was younger and did not include subjects with a history of cardiovascular disease. In the Arnhem Elderly Study, a positive association of PAI-1 with stroke persisted after exclusion of 20% of the subjects with a history of cardiovascular disease. The Arnhem Elderly Study is the first prospective study in which PAI-1 levels as well as the 4G/5G-polymorphism was examined in relation to risk of stroke within a single population.

C-REACTIVE PROTEIN

Role of CRP in the metabolic syndrome

Both PAI-1 and CRP have been suggested to be part of the metabolic syndrome. In the Arnhem Elderly Study we examined the associations between CRP, PAI-1, and other components of the metabolic syndrome (i.e. BMI, insulin, HDL-cholesterol, and blood pressure) stratified by gender and overweight status (BMI > 25 kg/m^2). In normal-weight women, CRP was associated with BMI, PAI-1, serum insulin and

HDL-cholesterol. In overweight women, however, these relationships were weak and not statistically significant.

CRP and risk of thrombotic events

In the Arnhem Elderly Study, CRP was predictive for future cardiovascular events in men, which is in agreement with a large number of previous epidemiological studies (see for review De Ferranti and Rifai[29]). In a meta-analysis on the association between CRP and coronary heart disease, including a total of 2,557 cases, an odds ratio of 1.9 (95%-CI: 1.5 - 2.3) was found.

The mechanisms responsible for associations between CRP and cardiovascular risk are not fully understood. CRP might directly promote vascular disease. Alternatively, CRP might be an indicator of other cardiovascular risk factors or of the extent of pre-existing disease.

In contrast to previous studies in women,[30,31] we did not observe a relationship between CRP and future cardiovascular events in elderly women. Our study may have had insufficient power to detect an association. An alternative explanation might be that the determinants of serum CRP were different for men than for women (see Chapter 7).

METHODOLOGICAL CONSIDERATIONS

Epidemiological studies like the ones reported in this thesis are prone to several forms of bias, which might have affected the internal validity of the results. Several sources of potential bias will be discussed, as well as several other methodological issues.

Selection bias

In case-control studies the selection of a proper control group is of major importance. The control group should be representative of those who, had they developed the disease, would have been selected as cases. In the pooled case-control study (Chapter 5), two types of controls were selected, i.e. coronary controls and population-based controls. Coronary controls were selected from subjects that had undergone coronary angiography in the same hospital as the cases. A major advantage of selecting coronary controls is ascertainment of the absence of stenosis. Furthermore, since these subjects underwent similar diagnostic procedures as the cases, one could expect them to have the same accuracy in reporting information. However, coronary controls may not be representative of the source population, and thus selection bias might have occurred. It is for example conceivable that subjects with a positive family history of cardiovascular disease are more likely to be referred to the hospital than subjects without such a history, which could have attenuated the associations.

The second control group comprised a sample from the general population (population-based controls). The distribution of the 4G/5G-polymorphism in the

population-based controls was not in Hardy-Weinberg equilibrium, which indicates selection based on the 4G/5G-polymorphism. The frequency of the 4G-allele was high in comparison to other European control populations. The estimates based on the analysis with population-based controls might thus be biased, which might explain the absence of an association. We therefore have more confidence in the comparison with coronary controls.

In prospective studies, like the Arnhem Elderly Study, two potential sources of selection bias have to be considered: non-response and loss to follow-up. Non-response will only lead to biased results if it is associated both with exposure, and with disease outcome independently of the exposure of interest, which is unlikely. Loss to follow-up may cause biased results if it is related to both exposure and outcome. For all-cause mortality, loss to follow-up was not an issue in the Arnhem Elderly Study, because only one person was lost to follow-up due to emigration. Data on cause-specific mortality and incidence of cardiovascular events, however, were missing for 19% of the study population. Reasons for this were as follows: no permission (6%), the general practitioner could not be traced (5%), refused participation (8%), or could not provide valid data (2%). Thus, loss to follow-up occurred at the level of the general practitioner rather than of the participants and is therefore unlikely to be related to exposure or disease outcome. Baseline characteristics of elderly subjects who were lost to follow-up were similar to those included in the analysis, except for serum cholesterol (6.0 versus 6.3, P=0.02). No differences were observed with regard to age and gender distribution. It is thus not expected that loss to follow-up has largely influenced the associations between exposures (PAI-1, 4G/5G-polymorphism, CRP) and cardiovascular events.

Information bias

Measurement of plasma PAI-1 activity

To minimize variance due to the circadian pattern of PAI-1, time of blood sampling should ideally be standardized. In the Arnhem Elderly Study blood sampling was performed between 8.00 a.m. and 5.30 p.m. Time of blood sampling was registered, making it possible to adjust for the diurnal variation in PAI-1 activity in all statistical analyses. We therefore do not expect this diurnal variation to have caused biased estimates of the risk associated with PAI-1 activity in the Arnhem Elderly Study.

Measurement and interpretation of plasma PAI-1 activity is furthermore complicated by spontaneous transformation of active PAI-1 into its latent form, by complex forming with t-PA and by the potential release of PAI-1 from platelets.[32] In the Arnhem Elderly Study plasma samples were cooled shortly after blood drawing and deep-frozen after a few hours, which will have prevented inactivation of PAI-1. We chose to measure PAI-1 activity and not PAI-1 antigen, because the latter is more affected by leakage of PAI-1 from platelets to plasma. The samples were non-fasting, but this has probably not had a major effect on variation in PAI-1.[32] Samples were stored at -80°C for a maximum of 7 years and sample degradation over this time period is not expected to have influenced PAI-1 to a large extent.[33]

Measurement of serum C-reactive protein

We used a high-sensitive method to measure serum CRP concentrations at baseline in the Arnhem Elderly Study. The variation of CRP concentrations in a healthy individual over time is substantial, but it has been shown that in stable individuals there is a constant low level of CRP with occasional outliers.[34] In order to precisely determine the habitual level of CRP in a healthy volunteer, one blood sample is regarded as sufficient.[35] To avoid confounding by transient acute-phase reactions we repeated all analyses of CRP after exclusion of 5% of men and 1% of women with CRP levels above 10 ng/mL, indicating clinically relevant inflammation.

Validation of 4G/5G genotyping

In general, quality control for genotyping methods receives insufficient consideration because of the assumption that errors in genotyping are unlikely to occur. However, as for all data collection methods, both systematic and random errors are possible.

We used three different methods to genotype the 4G/5G-polymorphism in the study of male smokers (Chapter 4), the pooled case-control study (Chapter 5) and in the Arnhem Elderly Study (Chapters 3 and 6).[21,36,37] The three methods were cross-validated by repeating the genotyping in a random sample of male smokers and the case-control population, using the method of the Arnhem Elderly Study. Completely identical results for the 4G/5G polymorphism were obtained. Some misclassification might have occurred due to errors in genotyping. However, the assessment was blinded, which strongly reduces the likelihood of differential misclassification. Therefore, we are confident that misclassification of 4G/5G-genotype in our studies did not bias the observed associations.

Endpoint assessment

We present several cardiovascular endpoints in the different chapters, both intermediate markers of the atherosclerotic process as well as clinical cardiovascular events. Measurement errors in markers of intima-media thickness or ankle-brachial index might have led to misclassification. However, differential misclassification across the genotypes of the 4G/5G-polymorphism is not likely and therefore the results will not be biased. In the pooled case-control study (Chapter 5) the large contrast in the extent of coronary stenosis precludes serious misclassification of cases and coronary controls. Despite the absence of symptoms, it cannot be ruled out that some of the population-based controls may have had substantial coronary narrowing, which could have led to dilution of the true association.

For the all-cause mortality data in the Arnhem Elderly Study, no large problems are expected, since data were obtained from municipal registries covering the total study population. However, the morbidity and cause-specific mortality data are more prone to errors. Especially in a population of elderly with a high prevalence of comorbidity, the true cause of death is difficult to establish. A medical doctor coded causes of death by the International Classification of Diseases, Tenth Revision (ICD-10). Only events with a high level of certainty (as judged by the treating general practitioner)

were included in the analysis. The researchers were furthermore unaware of exposure at the time of collection and coding of morbidity data. Thus, some non-differential misclassification in endpoints may have occurred, but differential misclassification is improbable.

Confounding

A wide range of cardiovascular risk factors, e.g. BMI, cholesterol, hypertension, insulin resistance and inflammation, have been associated with plasma PAI-1 levels and should be considered as potential confounders in our analyses. However, it is also possible that part of the harmful effects of these cardiovascular risk factors (i.e. body mass index, variables of the insulin resistance syndrome) may be mediated by effects on PAI-1 level. It is therefore debated whether or not one should adjust for these 'confounders' when studying the relationship between PAI-1 levels and cardiovascular risk in observational studies. In the Arnhem Elderly Study, the adjustments for confounders did not have a large effect on the estimates, indicating that the effects were independent of these risk factors. Potential confounders that were not measured in the Arnhem Elderly Study were for example blood levels of triglycerides and glucose. Furthermore, blood samples were drawn without an overnight fast, which will have led to inaccurate assessment of confounders such as serum cholesterol and insulin. Residual confounding can therefore not completely be ruled out.

In the analysis in which we studied the diurnal variation in PAI-1 (Chapter 3), confounding was a potential threat to validity. We obtained a single non-fasting blood sample and examined the diurnal variation in PAI-1 activity by categorizing subjects in groups according to the time of blood sampling, after which geometric mean PAI-1 levels between groups were compared. The observed diurnal pattern in PAI-1 might thus be confounded by differences between subjects. However, the circadian pattern was still clearly present after extensive adjustments (e.g. smoking status, cardiovascular history and BMI) and we therefore believe that the observed diurnal pattern in plasma PAI-1 is not explained by differences in characteristics between the groups measured at different time points.

Confounding is less likely for the relationship between 4G/5G-polymorphism and cardiovascular disease. Due to 'Mendelian randomization', it is likely that variables are equally distributed over the genotypes and will not confound the observed associations.[38] Only allelic variations that are in linkage disequilibrium with the 4G/5G-polymorphism would then be unevenly distributed over the genotypes and potentially confound the associations (both in case-control and in prospective studies).

A major disadvantage of the case-control design is the possibility that the variable of interest is influenced by disease status. One could argue that this is not an issue when studying a genetic factor, e.g. the 4G/5G-polymorphism. Because of the higher efficiency of the case-control design compared to prospective studies, it can even be argued that the case-control design is the preferred study design when the exposure of interest is a genetic factor.[38]

Power

The development of cardiovascular disease is a multifactorial process with many genes and environmental factors involved. Large study populations are required for risk estimations related to a single polymorphism, and the number needed increases exponentially for the detection of gene-environment interactions.[39] So, even if the 4G/5G-polymorphism is truly associated with cardiovascular risk, it may be missed in small studies.

In the Arnhem Elderly Study the power was sufficient to detect several significant associations between exposure (plasma PAI-1 activity, CRP, and the 4G/5G-polymorphism) and cardiovascular outcomes. However, the lack of an association between CRP and cardiovascular risk in women in the Arnhem Elderly Study may be explained by limited statistical power. In particular, the interpretation of the associations for incident myocardial infarction is hampered by the small number of events in women in our study population.

INFERENCE

Causality of PAI-1

The 4G/5G-polymorphism appeared not to be a strong determinant of atherosclerosis (Chapter 4 and 5), and these findings do thus not support a causal association between PAI-1 and the presence of atherosclerosis. The acute-phase properties of PAI-1 might be an alternative pathway by which PAI-1 could have detrimental effects in the cardiovascular system. Observed associations between atherosclerosis and PAI-1 might thus be due to increased PAI-1 synthesis, as a consequence of atherosclerosis. Even in elderly without clinical evidence for cardiovascular disease, the atherosclerotic process is likely to be advanced, which might be the underlying reason for the association between PAI-1 and cardiovascular events in the Arnhem Elderly Study.

We tried to study the role of PAI-1 in cardiovascular disease independent of inflammation by adjusting for baseline CRP concentrations. However, this may be have been insufficient since many uncertainties exist about the exact role of CRP in cardiovascular disease. In addition, little is known about the link between PAI-1 and CRP in the acute-phase response. In the Arnhem Elderly Study the correlation coefficient between PAI-1 and CRP was 0.21. Based on scientific literature, it is known that CRP shows a stronger inflammatory response than PAI-1 activity.[40] Our observational data, regrettably, do not allow strong conclusions on inflammatory properties of PAI-1 in the cardiovascular disease process. Larger studies taking into account both PAI-1 and CRP are therefore needed to compare their roles in the acute-phase response and potential interactions.

Level versus response

Fibrinolysis is a system with low activity but with a large potential to respond when activated, i.e. when the blood vessel wall is damaged. The ability of the system to respond to triggers might therefore be biologically more relevant than a PAI-1 measurement in resting conditions. Both venous occlusion of the forearm and exercise stress stimulate fibrinolysis and have been used to measure PAI-1 response.[41,42] However, these methods are not commonly used in large epidemiological studies. Before these measurements can be used on a large scale, their validity should be further explored and methods should be standardized.[41,42] In addition, PAI-1 measured in plasma is a systemic marker of fibrinolysis and may not be a good marker of fibrinolytic status locally, which may be more relevant in the cardiovascular disease pathology. It is, however, not possible to assess PAI-1 at the site of the vascular process.

4G/5G-polymorphism and PAI-1 levels

Under the assumption that the 4G-allele is associated with elevated PAI-1 levels, comparison of cardiovascular risk in subjects with respectively none, one, or two copies of the 4G-allele could be interpreted as an experiment of nature in which individuals have randomly been allocated to high or low PAI-1 concentrations.[38] An association between the 4G/5G-polymorphism and risk of cardiovascular disease would thus support the hypothesis that PAI-1 is a causal risk factor for cardiovascular disease. At the time the studies in this thesis were initiated it was believed that the 4G/5G-polymorphism and PAI-1 levels were consistently associated, with highest PAI-1 levels for the 4G/4G-genotype, intermediate for the 4G/5G and lowest for the 5G/5G-genotype.[12,43-46] More recently, however, a number of studies have failed to show an association between the 4G/5G-polymorphism and PAI-1 levels.[47] The association between the genotype and plasma levels may thus not be as strong as initially expected. In the Arnhem Elderly Study only 1% of the variance in PAI-1 was explained by the 4G/5G-polymorphism (see Chapter 6). Median plasma PAI-1 activity was lower for the 5G/5G genotype compared to both other genotypes (1.4 versus 2.2 IU/mL). The difference between median values in the highest versus the lowest PAI-1 tertile was much larger (8.4 versus 0.18 IU/mL). Unfortunately, the association between genotype and phenotype could not be studied in the other populations described in this thesis.

It is possible that the 4G/5G-polymorphism mainly determines the magnitude of PAI-1 response and does not have a strong effect on PAI-1 level, which could explain the lack of a consistent association between the 4G/5G-polymorphism and PAI-1. As discussed earlier, the magnitude of PAI-1 response to triggers may be of larger interest in the cardiovascular disease process than resting PAI-1 levels. Studying the 4G/5G-polymorphism could thus still provide valuable information in spite of the absence of a strong and consistent relation with plasma PAI-1. With regard to stroke, it is at present unclear whether plasma PAI-1 accurately represents PAI-1 cerebral

levels. Therefore, our findings on PAI-1 and stroke presented in Chapter 6 should be interpreted with caution.

Causality of CRP

Based on our findings we hypothesize that CRP could be a more important component of the metabolic syndrome in women than in men and that CRP only plays a minor role in the metabolic syndrome in the presence of overweight (Chapter 7). Our findings, however, need to be confirmed in other epidemiological studies, both in the elderly and in younger populations.

Possibly, a more advanced atherosclerotic process in men might explain a major part of the variance in CRP and, consequently, the observed association with cardiovascular endpoints (Chapter 8). In the Arnhem Elderly Study, no intermediate marker of atherosclerosis was available. It might be that CRP in women is not a good marker for risk because it is mainly a reflection of the amount of adipose tissue.

Overall conclusions

Although we observed an increased risk for stroke and cardiovascular mortality for elevated PAI-1 levels, it is still not clear whether PAI-1 is a causal risk factor. Taking into account also the lack of consistency in the scientific literature, the independent role of PAI-1 in cardiovascular disease remains subject to debate. Our findings on 4G/5G genotype and cardiovascular disease provide no support for a causal role of PAI-1. The 4G allele, generally associated with higher levels of PAI-1, was even associated with a lower risk of stroke. We confirmed the diurnal variation in PAI-1, previously observed by others, but also showed this circadian pattern to be confined to the 4G-allele, which may have implications for the early-morning peak in cardiovascular events.

Future research

The 4G/5G-polymorphism and diurnal PAI-1 variation

Our finding of a genotype-specific diurnal variation in PAI-1 (Chapter 3) needs to be confirmed in other populations. The most appropriate approach would be to perform multiple blood sampling during the day in a study population pre-stratified for the 4G/5G-polymorphism, using a standardized protocol with regard to meals and sleep/wake rhythm to reduce variation in PAI-1. If the diurnal variation in PAI-1 truly depends on the 4G/5G-polymorphism, a second research question should be answered, namely whether 4G-carriers have an increased risk specifically of cardiovascular events in the early morning. For this purpose, the 4G/5G-genotype distribution of patients with an acute event in the morning may be compared to that of patients with an event at another time of the day.

PAI-1 in acute inflammation: consequences for severity of a cardiovascular event?

An effect of the 4G/5G-polymorphism on the prognosis of trauma and other disorders with a strong acute-phase response has been demonstrated.[48,49] It is possible that PAI-1 has damaging effects itself and/or that PAI-1 launches the synthesis of other inflammatory reactants. The effect of PAI-1 reduction in acute conditions therefore deserves future investigation.

A myocardial infarction also provokes an acute phase response.[50] It can thus be hypothesized that the 4G/4G-genotype may result in a stronger inflammatory response after a myocardial infarction, thereby worsening the prognosis. It would therefore be worthwhile to include time of an event in clinical studies.

4G/5G-polymorphism and risk of stroke

We observed a protective effect of the 4G-allele against stroke, which is supported by several previous studies. Most studies were too small to detect significant differences and to examine ischemic and hemorrhagic stroke separately. A large case-control study with different, well-defined types of stroke would therefore be worthwhile. Also, more prospective studies are needed to confirm our finding that plasma PAI-1 levels and the 4G/5G-polymorphism have contrasting effects on risk of stroke.

PAI-1 and atherosclerosis

PAI-1 might influence both progression and vulnerability of an atherosclerotic plaque.[51] Magnetic resonance imaging can be used specifically to determine plaque morphology; it displays the different components of plaque, which allows the identification of unstable plaques.[52] Correlating both PAI-1 levels and the 4G/5G-polymorphism with these markers would provide more insight in the role of PAI-1 in atherosclerosis. However, this new method is still considered experimental and remains a costly procedure, limiting its applicability in large epidemiological studies.

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Summary

Plasminogen activator inhibitor-type 1 (PAI-1) is the main inhibitor of fibrinolysis and thereby a potential risk factor of cardiovascular disease. In addition to its regulating role in fibrinolysis, PAI-1 is also involved in other processes by which PAI-1 may have detrimental effects on the cardiovascular disease process. For example, PAI-1 is involved in inflammation and in processes like cell proliferation and cell migration. Many biological and lifestyle factors are known that influence PAI-1 levels, which make it difficult to estimate whether PAI-1 is a causal and independent risk factor for cardiovascular disease.

A polymorphism in the promoter region of the PAI-1 gene, the 4G/5G-polymorphism, has also extensively been studied in relation to cardiovascular risk. This polymorphism affects the transcription of the PAI-1 gene in response to several triggers, for example proinflammatory cytokines. The 4G-allele results in higher transcription rates than the 5G-allele and has been associated with higher plasma PAI-1 levels. The 4G/4G-genotype has been studied as potential risk factor for cardiovascular disease, but the results are not yet conclusive.

The general objective of the studies described in this thesis was to evaluate whether PAI-1 and the 4G/5G-polymorphism are associated with risk of cardiovascular disease. PAI-1 is potentially involved in atherosclerosis and in the thrombotic stage of the cardiovascular disease process. We therefore studied the associations both with markers of atherosclerosis and with clinical end points. PAI-1 is furthermore an acute-phase reactant and we therefore additionally studied C-reactive protein, a sensitive marker of inflammation.

Determinants of plasma PAI-1

In Chapter 2 the scientific literature on determinants of plasma PAI-1 is reviewed. There is strong evidence for a substantial influence of obesity and the metabolic syndrome on plasma PAI-1. PAI-1 can be reduced by lifestyle interventions such as weight reduction and physical activity. Interactions between metabolic and lifestyle factors and the 4G/5G-polymorphism have only rarely been studied.

PAI-1 is known to follow a diurnal pattern with highest levels in the early morning. It has been suggested that this early morning peak in PAI-1 may partly be responsible for the increased number of cardiovascular events at this time of the day. In Chapter 3 we studied this diurnal variation in plasma PAI-1 and we examined whether the patterns were different for the genotypes of the 4G/5G-polymorphism. The analyses were performed in the Arnhem Elderly Study, a population-based study of 598 elderly. A single blood sample was drawn and the time of blood sampling was recorded (between 8 a.m. and 5.30 p.m.). We observed a strong diurnal pattern in PAI-1 activity, with the highest values observed in the early morning, The diurnal pattern was clearly present in the 4G/4G (n=184) and 4G/5G (n=275) genotypes, but not in the 5G/5G-genotype (n=139). These findings raised the hypothesis that 5G-

homozygotic persons may be relatively protected from the early morning peak incidence in cardiovascular events.

PAI-1, THE 4G/5G-POLYMORPHISM AND CARDIOVASCULAR DISEASE

The association between the 4G/5G-polymorphism and markers of atherosclerosis is described in Chapter 4 and 5. In Chapter 6 findings are presented on PAI-1 activity, the 4G/5G-polymorphism and incidence of cardiovascular events in elderly.

4G/5G-polymorphism and atherosclerosis

We observed no association between the 4G/5G-polymorphism and two non-invasive markers of atherosclerosis, i.e. common carotid artery intima-media thickness (IMT) and the ankle-brachial index (ABI) in a population of 208 male smokers (Chapter 4). The 4G-allele was not associated with a higher prevalence of atherosclerosis, neither for IMT nor ABI. Although not statistically significant, a high IMT (>1.07 mm) was even less frequent for the 4G/5G and 4G/4G-genotypes compared to the 5G/5G-genotype (odds ratios (ORs) of 0.6 and 0.4 respectively, *P*-value for trend: 0.08). In Chapter 5 we investigated the association between the 4G/5G-polymorphism and coronary stenosis by pooling the data of three similar case-control studies. In these studies the distribution of the 4G/5G-polymorphism in 285 cases with angiographically determined coronary stenosis was compared with the distributions in two control groups: a coronary control group (n=293) and a population-based control group (198). The coronary controls were selected by the same procedure as the cases, but did not have advanced coronary stenosis. A non-significantly increased risk of coronary stenosis was observed for the 4G-allele relative to the 5G/5G-genotype when compared to coronary controls (4G/4G-genotype: OR=1.58 (95%-CI: 0.97 - 2.57)). No association was observed in comparison to the population-based control group. Stratification for background cardiovascular risk (based on Framingham Prediction Score) showed an increased risk of coronary stenosis only in the low-risk group (cases versus coronary controls, 4G/4G-genotype: OR=2.86 (95%-CI: 1.27 - 6.47)).

PAI-1, the 4G/5G-polymorphism and coronary events

In Chapter 6 a longitudinal study is described (Arnhem Elderly study) in which the predictive value of PAI-1 activity, t-PA antigen and the 4G/5G-polymorphism for cardiovascular events was studied. The most important finding was that the 4G/4G-genotype protected against incident stroke and transient ischemic attack. This observation is in contrast to the generally accepted hypothesis that the 4G-allele would increase cardiovascular risk. However, these results are in agreement with some other studies. The mechanism is probably not part of the fibrinolytic system. For PAI-1 levels, an increased risk of stroke and transient ischemic attack was observed in the highest tertile.

C-REACTIVE PROTEIN

In Chapter 7 the role of C-reactive protein (CRP) in the metabolic syndrome is cross-sectionally examined in a population of 605 elderly men and women (Arnhem Elderly Study). CRP was strongly associated with components of the metabolic syndrome (BMI, PAI-1, HDL-cholesterol and blood pressure), but these associations were predominantly present in lean elderly (BMI < 25 kg/m^2). The data suggest that in elderly CRP may not be an important component of the metabolic syndrome in the presence of overweight. PAI-1 and CRP were inter-related, but only in lean subjects. In Chapter 8 the predictive value of CRP for future cardiovascular events in the elderly was studied, showing that CRP does have an added value in this population for men but not for women. Including CRP in cardiovascular risk algorithms for older men may thus improve their predictive value.

DISCUSSION AND CONCLUDING REMARKS

In Chapter 9, our results are methodologically considered and placed in the context of other scientific literature in this field. Overall, PAI-1 and the 4G/4G-genotype do not appear to be associated with the presence of atherosclerosis, but the effects on thrombosis are still controversial. Although we observed an increased risk for several cardiovascular events for elevated PAI-1 levels, it is still not clear whether PAI-1 is a causal risk factor. Causality of PAI-1 was not supported by an increased risk of cardiovascular events for the 4G/4G-genotype. In contrast, the 4G/4G-genotype was associated with a lower risk of stroke. More research is needed on the mechanisms responsible for the protective effect for the 4G/4G-genotype against stroke and ischemic attack.

The role of PAI-1 and the 4G/5G-polymorphism in acute inflammatory situations may be of clinical relevance. This should be further studied, also in relation to the acute-phase response in myocardial and cerebrovascular infarction. In addition, our finding of a genotype-specific diurnal variation in PAI-1 might have clinical implications and should be further elucidated.

Samenvatting

De vertaling van de titel van het proefschrift in het Nederlands:

Fibrinolyse, ontsteking en hart- en vaatziekten. Epidemiologisch onderzoek naar plasminogen activator inhibitor-type 1 en C-reactief proteïne

ACHTERGROND VAN HET ONDERZOEK

Hart- en vaatziekten

In westerse landen zijn hart- en vaatziekten nog steeds de belangrijkste doodsoorzaak. In Nederland was in 2000 het aandeel van de hart- en vaatziekten in de totale sterfte 36%. Onder de verzamelnaam hart- en vaatziekten vallen onder meer hartinfarct en beroerte. Van veel verschillende factoren is inmiddels bekend dat ze het risico op hart- en vaatziekten verhogen, bijvoorbeeld roken en een verhoogd cholesterol gehalte in het bloed. Daarnaast spelen waarschijnlijk ook andere factoren een rol waar we nog minder van af weten. In de studies die beschreven zijn in dit proefschrift heb ik twee minder bekende risicofactoren onderzocht, namelijk Plasminogen Activator Inhibitor-type 1 (PAI-1) en C-reactief Proteïne (CRP).

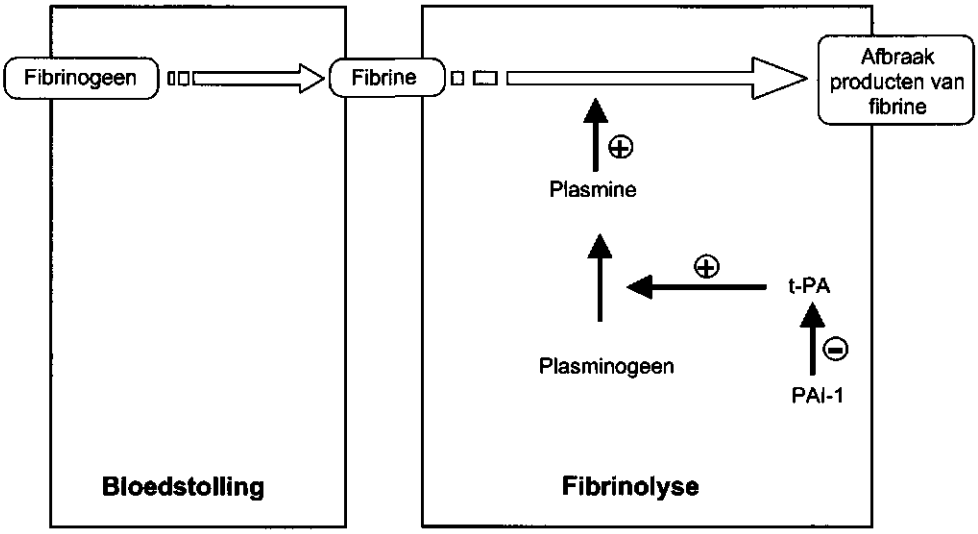
Hartinfarct
Een hartinfarct is het gevolg van een afsluiting van de kransslagader(s). Dit begint met een kleine beschadiging van de gladde binnenwand van het bloedvat. Het lichaam probeert die beschadiging te herstellen; er klonteren bloedplaatjes samen op de beschadigde plaats en witte bloedcellen dringen de vaatwand binnen. Die witte bloedcellen nemen cholesterol op, wat een brijachtige massa geeft waar zich later ook kalk op afzet. Hierdoor worden de kransslagaders steeds nauwer en kan het zuurstofrijke bloed de hartspier moeilijker bereiken. Dit noemen we slagaderverkalking of atherosclerose. Wanneer een bloedstolsel een vernauwde kransslagader plotseling helemaal afsluit, ontstaat een hartinfarct. Door die afgesloten kransslagader krijgt het bijbehorende gedeelte van de hartspier geen zuurstof meer en sterft af. De ernst van een hartinfarct hangt af van de grootte van de beschadiging en welk deel van de hartspier is aangedaan.

Cerebrovasculair accident
Als de bloedvoorziening naar de hersenen plotseling ergens onderbroken wordt, spreken we van een cerebrovasculair accident (afgekort tot CVA, ook wel beroerte genoemd). Meestal gaat het bij een CVA om een herseninfarct (bij 80% van de getroffenen). Dit is vergelijkbaar met een hartinfarct, maar dan in het hoofd. Een herseninfarct ontstaat doordat een bloedstolsel een slagader in het hoofd afsluit. Een deel van de hersencellen krijgt daardoor geen zuurstof meer en sterft af. De vernauwing in de slagader is vaak het gevolg van slagaderverkalking of atherosclerose. Een hersenbloeding ontstaat doordat een zwakke plek in het

bloedvat openbarst waardoor er hersenweefsel beschadigd raakt. De verschijnselen die optreden zijn vergelijkbaar als bij een herseninfarct.

Fibrinolyse

Als een bloedvat beschadigd raakt, wordt er een bloedstolsel gevormd om de bloeding te stoppen. Een bloedstolsel bestaat voor een belangrijk deel uit fibrine, dat later weer wordt afgebroken (fibrinolyse). Plasmine is de werkzame stof van de fibrinolyse. De hoeveelheid actief plasmine dat in het bloed voorkomt, wordt bepaald door diverse eiwitten die elkaar beïnvloeden (remmen of activeren). Zie figuur 1 voor een overzicht van de fibrinolyse. Het eiwit Plasminogen Activator Inhibitor-type 1 (afgekort tot PAI-1) is de belangrijkste remmer. Het evenwicht tussen enerzijds de bloedstolling en anderzijds de fibrinolyse is van cruciaal belang. Een hoge concentratie PAI-1 in het bloed zorgt ervoor dat bloedstolsels minder efficiënt worden opgeruimd en zou daarom mogelijk een risicofactor kunnen zijn voor het ontstaan van hart- en vaatziekten.



Figuur 1. Schema van de bloedstolling en de fibrinolyse.

De regulerende rol in de fibrinolyse is de meest bekende functie van PAI-1, maar daarnaast is PAI-1 ook betrokken bij andere processen, waaronder ontsteking. Dit heeft mogelijk een bijkomende ongunstige invloed op het proces van hart- en vaatziekten. De concentratie van PAI-1 in het bloed wordt bepaald door veel verschillende factoren. In hoofdstuk 2 wordt een overzicht gegeven van de meest belangrijke genetische, biologische en leefstijlfactoren. Hieruit blijkt dat met name lichaamsgewicht en het insulineresistentiesyndroom een belangrijke invloed hebben op PAI-1.

Het 4G/5G-polymorfisme

Het genetisch materiaal (DNA) is opgebouwd uit een grote hoeveelheid bouwstenen. Een gen is een stuk genetisch materiaal dat codeert voor een eiwit. In het PAI-1 gen komt een kleine genetische variatie (polymorfisme) voor met twee verschillende vormen. De ene vorm (de 4G-vorm) heeft één bouwsteen minder heeft dan de andere vorm (5G-vorm). Deze kleine variatie heeft een effect op de hoeveelheid PAI-1 die het lichaam produceert. Beide vormen komen ongeveer evenveel voor in de algemene bevolking. Aangezien iedereen van elk gen twee copien heeft (van beide ouders één), komen er drie combinaties (genotypes) voor, namelijk het 4G/4G-genotype, het 4G/5G-genotype en het 5G/5G-genotype. Over het algemeen worden de hoogste bloed PAI-1 waarden gevonden voor het 4G/4G-genotype en de laagste voor het 5G/5G-genotype.

DOEL VAN HET ONDERZOEK

Met ons onderzoek beoogden we na te gaan of bij ouderen de hoeveelheid PAI-1 in het bloed en het 4G/5G-polymorfisme voorspellend is voor het ontstaan van hart- en vaatziekten. Hiervoor hebben we gebruik gemaakt van de gegevens van het Arnhemse Ouderen Onderzoek. Bovendien hebben we in een tweetal andere populaties onderzocht of het 4G/5G-polymorfisme iets te maken heeft met de aanwezigheid van atherosclerose (aderverkalking). Ook hebben we gekeken naar de rol van ontsteking

Eerst zullen de opzet en de belangrijkste resultaten van het Arnhemse Ouderen Onderzoek besproken worden, waarna de resultaten van de onderzoeken naar de relatie tussen het 4G/5G-polymorfisme en atherosclerose aan bod zullen komen.

OPZET VAN HET ARNHEMSE OUDEREN ONDERZOEK

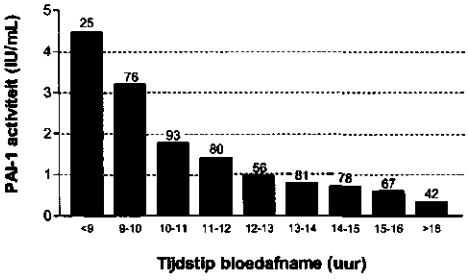
In 1991/1992 is er in Arnhem een onderzoek gedaan naar lichamelijke activiteit in relatie tot diverse gezondheidskenmerken bij 1012 zelfstandig wonende ouderen van 65 tot en met 84 jaar. Er zijn gegevens verzameld over leefwijze (zoals lichamelijke activiteit, voedingsgewoonten, roken) en gezondheid. Bij 641 personen is verder lichamelijk onderzoek uitgevoerd en bloed afgenomen. In het bloed zijn onder andere het cholesterolgehalte en de hoeveelheid PAI-1 bepaald. Ook is het polymorfisme in het PAI-1 gen bepaald.

We hebben de personen uit het onderzoek gevolgd in de tijd. Gegevens over verhuizingen en sterfte tot 1 februari 2001 zijn verkregen via de bevolkingsadministraties van de gemeente Arnhem en andere gemeenten in Nederland. Verder zijn we met hulp van huisartsen en verpleeghuizen nagegaan welke personen hart- en vaatziekten hebben gekregen. We hebben het risico op totale sterfte, sterfte ten gevolge van hart- en vaatziekten, hartinfarcten en beroertes bestudeerd in relatie tot PAI-1 en het 4G/5G-polymorfisme.

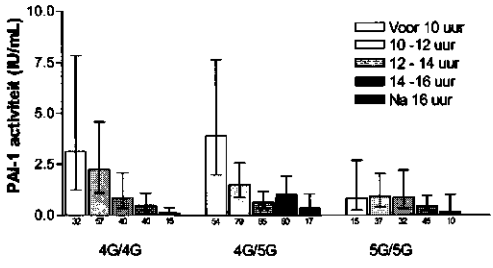
RESULTATEN

4G/5G-polymorfisme en dagvariatie in PAI-1 activiteit

Veel van de hartinfarcten vinden vroeg in de ochtend plaats. De redenen hiervoor zijn nog onbekend, maar er wordt gedacht dat de variatie over de dag heen van factoren uit de fibrinolyse hier mogelijk iets mee te maken hebben. Uit kleinschalig onderzoek was al bekend dat de concentratie PAI-1 in het bloed sterk afhankelijk is van het moment op de dag, met de hoogste waarden vroeg in de ochtend. Deze dagvariatie in PAI-1 was nog niet bestudeerd in grotere (epidemiologische) onderzoeken. In het Arnhemse Ouderen Onderzoek vonden we een duidelijk dagritme (zie figuur 2). De gemeten PAI-1 activiteit in bloedmonsters die voor 9 uur 's ochtends waren afgenomen was ongeveer 13 maal zo hoog als in monsters afgenomen na 4 uur 's middags. Verder hebben we onderzocht of deze variatie over de dag verschillend was voor de drie varianten van het 4G/5G-polymorfisme. In de groepen met het 4G/4G en het 4G/5G-genotype was een duidelijke variatie in PAI-1 activiteit over dag aanwezig. Deze was echter nagenoeg afwezig in de groep met het 5G/5G-genotype (zie figuur 2). Dit zou kunnen betekenen dat personen met het 5G/5G-genotype relatief beschermd zijn tegen een hartinfarct in de vroege ochtend. Deze resultaten staan beschreven in hoofdstuk 3 van het proefschrift.



Figuur 2. Dagvariatie in PAI-1 activiteit



Figuur 3. Dagvariatie in PAI-1 activiteit per genotype

4G/5G-polymorfisme en atherosclerose

Er bestaan aanwijzingen dat PAI-1 al vroeg in het ziekteproces effecten zou kunnen hebben, namelijk bij het ontstaan van atherosclerose. In twee verschillende studies zijn we nagegaan of het 4G/4G-genotype vaker gepaard gaat met (een meer ernstige vorm van) atherosclerose. Deze studies staan beschreven in hoofdstuk 4 en 5.

De mate van atherosclerose kun je op verschillende manieren meten. Zo is in een populatie van 208 rokende mannen de dikte van de vaatwand van de halsslagader gemeten (hoofdstuk 4). Hierbij is een dikke vaatwand een indicatie van ernstige atherosclerose. We vonden geen duidelijk verband tussen het 4G/5G-polymorfisme en de dikte van de vaatwand. De 4G-vorm ging niet gepaard met een dikkere

vaatwand. Tegen de verwachting was het percentage mannen met een dikke vaatwand zelfs hoger voor het 5G/5G-genotype (31% voor 5G/5G; 21% voor 4G/5G; 14% voor 4G/4G).

In hoofstuk 5 hebben we patiënten met een vernauwing van de kransslagaderen vergeleken met gezonde controles. Of iemand een vernauwing heeft, kan in het ziekenhuis worden bepaald door middel van angiografie. Dit is een onderzoek waarbij een katheter onder plaatselijke verdoving in de bloedvaten wordt geschoven, waarna contrastvloeistof wordt ingespoten. Met behulp van röntgenstralen kunnen de bloedvaten in beeld worden gebracht. De onderzoeksgroep bestond uit 285 personen met een vernauwing van de kransslagader, 293 controles die ook een angiografie hadden ondergaan, maar geen vernauwing bleken te hebben en 198 controles uit de algemene bevolking. We vonden geen duidelijke verbanden met het 4G/5G-polymorfisme. Het percentage personen met het 4G/4G-genotype was wel het hoogst in de groep patiënten, maar de verschillen met de groepen controles waren niet significant.

PAI-1 activiteit, het 4G/5G-polymorfisme en het risico op hart- en vaatziekten

In het Arnhemse Ouderen Onderzoek vonden we geen groot verschil in PAI-1 concentraties in het bloed tussen de verschillende genotypes. De gemiddelde PAI-1 waarde in het bloed was iets lager in de groep met het 5G/5G-genotype (1.4 IU/mL) dan in de andere twee groepen (beide 2.2 IU/mL). Op basis van de PAI-1 concentratie in hun bloed werden de ouderen ingedeeld in drie groepen. We hebben onderzocht of ouderen in de groep met de hoogste PAI-1 concentraties aan het begin van het onderzoek vaker last kregen van hart- en vaatziekten dan groep met lage waarden. Ouderen met de hoogste PAI-1 waarden (hoger dan 3.9 IU/mL) hadden een hoger risico op hart- en vaatziekten dan ouderen in de groep met lage PAI-1 waarden (lager dan 0.93 IU/mL). De kans op sterfte ten gevolge van hart- en vaatziekten bleek ongeveer drie maal zo hoog te zijn voor de groep met hoge PAI-1 waarden in vergelijking met de groep die een lage PAI-1 concentratie in het bloed hadden. Ook voor het optreden van hartinfarct en CVA werden verhoogde risico's gevonden (drie- en viermaal verhoogd).

Van te voren verwachtten we ook een hoger risico op hart- en vaatziekten voor het 4G/4G-genotype te zullen vinden, maar dit bleek niet het beval te zijn. Tegen onze verwachting in vonden we dat ouderen met het 4G/4G-genotype relatief beschermd waren tegen het ontstaan van een CVA (5 maal zo laag risico). Ook andere recente onderzoeken hebben dergelijke bevindingen laten zien. Dat personen met het 4G/4G-genotype blijkbaar beschermd zijn tegen CVA's kan mogelijk verklaard worden door mechanismen die buiten de fibrinolyse liggen.

C-reactief proteïne

We hebben in de laatste twee hoofdstukken van het proefschrift gekeken naar CRP (C-reactive proteïne). Dit is een eiwit dat verhoogd is als iemand een ontsteking heeft. De concentratie kan dan wel 1000 maal verhoogd zijn ten opzichte van

normaal. Heel licht verhoogde CRP-waarden blijken in diverse onderzoeken gerelateerd te zijn aan het risico op hart- en vaatziekten. Ook PAI-1 is verhoogd in de aanwezigheid van een ontsteking. In het Arnhemse Ouderen Onderzoek hebben we beide factoren met elkaar gecorreleerd en hieruit bleek dat ze alleen in personen zonder overgewicht geassocieerd waren. In hoofdstuk 8 hebben we onderzocht of CRP ook in het Arnhemse Ouderen Onderzoek voorspellend was voor het ontstaan van hart- en vaatziekten. Bij mannen bleek dit wel het geval te zijn, maar niet bij de vrouwen.

DISCUSSIE EN CONCLUSIE

Uit het onderzoek is naar voren gekomen dat PAI-1 en het 4G/5G-polymorfisme geen grote invloed te hebben op atherosclerose. De relatie met acute vaatincidenten (zoals een hartinfarct en CVA) is nog niet duidelijk. Aan de ene kant vonden we een verhoogd risico voor verhoogde PAI-1 waarden, maar dit werd niet ondersteund door een verhoogd risico voor het 4G/4G-genotype. We vonden zelfs een beschermend effect van het 4G/4G-genotype voor CVA. Er is meer onderzoek nodig naar de mechanismen die hierbij betrokken zijn. Onze bevinding dat het dagritme in PAI-1 verschillend is voor de verschillende genotypes kan mogelijk van klinisch belang zijn en moet verder worden onderzocht.

Dankwoord

Het heeft even geduurd, maar het is nu toch echt af. Langs deze weg wil ik iedereen bedanken die, op welke manier dan ook, heeft bijgedragen aan dit proeschrift.

Allereerst wil ik mijn (co-) promotoren van harte bedanken. In de eerste plaats voor het vertrouwen dat jullie steeds in mij gesteld hebben. Evert, jouw deur stond altijd voor mij open. Jouw kritische opmerkingen zijn mijn werk zeker ten goede gekomen. Frans, ondanks dat je wat meer op de achtergrond aanwezig was, was jouw inbreng zeer waardevol. Je hebt een positieve manier om tegen de zaken aan te kijken en op de juiste momenten ben jij het die knopen doorhakt. Ook bedankt voor de peptalks van de laatste tijd, die waren hard nodig. Marianne, jij bent pas later bij mijn project betrokken geraakt en ik moet zeggen dat ik er een geweldige begeleidster bij heb gekregen. Vooral je enthousiasme en je bereidheid om steeds met me mee te denken heb ik zeer gewaardeerd. Al die vakantiedagen die je de laatste tijd voor mij hebt opgeofferd, daar kan ik je niet genoeg voor bedanken!

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De PAI-1 activiteitsbepalingen heb ik uitgevoerd op het lab van TNO-gaubius in Leiden. Piet Meijer en Riet Kreft, bedankt voor jullie hulp en gastvrijheid. Met name toen we de eerste methode maar niet op de rails konden krijgen, heb ik veel gehad aan jullie expertise.

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Het was niet makkelijk om van de deelnemers van de Arnhemse Ouderen Studie de medische gegevens te verzamelen over ziekte en sterfte. Hierbij was ik erg afhankelijk van de hulp van de verschillende huisartsen. Ik wil alle huisartsen die hebben meegewerkt dan ook van harte bedanken voor hun onmisbare hulp en zeker ook alle assistenten die mij steeds behulpzaam waren.

Zonder vrijwilligers is het niet mogelijk om epidemiologisch onderzoek te doen en daarom ben ik dan ook dank verschuldigd aan iedereen die aan één van de

onderzoeken beschreven in dit proefschrift heeft meegedaan. Zonder hen was dit proefschrift niet mogelijk geweest.

Ik heb het ontzettend naar mijn zin gehad op de afdeling Humane Voeding en Epidemiologie en dat kwam zeker niet in de laatste plaats door mijn collega's. Jane, geweldige kamergenoot, ik vond het erg gezellig dat ik een kamer met je mocht delen. Ik vond het jammer dat je er de laatste periode niet was, maar je had ten slotte een hele goede reden! Mariska, Alida en Annet, buren van 623, bedankt dat ik jullie kamer mocht gebruiken als koffiekamer en als extra werkkamer (nog meer ruimte om een bende van te maken...), en natuurlijk ook voor alle gezelligheid en steun. Mariska, die dagelijkse massages zal ik zeker gaan missen! Ook alle andere (ex-)collega's wil ik bedanken voor alle gezelligheid en steun, met name Judith, Machteld, Ingeborg, Maud, Margreet, Edine, Hans, Ans, Lucy, Julia, Elvina, en Romain, maar ook alle anderen. Machteld en Sander, bedankt dat ik een tijdje op sabatical mocht komen in Schotland, dat heeft me zeker goed gedaan. Julia, you were a great room mate. Thank you for all the fun we had!

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Alle medewerkers van de afdeling Humane Voeding en Epidemiologie wil ik heel hartelijk bedanken voor hun belangstelling en steun in welke vorm dan ook gedurende de afgelopen jaren. Speciaal wil ik noemen het secretariaat (Marie en Eva), IT (Dirk en Ben) en Jan Burema voor statistische adviezen.

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About the author

Tiny (Trijntje) Hoekstra was born on June 9, 1973 in Damwoude, the Netherlands. In 1991, she passed secondary school (VWO) at the 'Chr. Scholengemeenschap Oostergo' in Dokkum. In the same year she started the study "Nutrition and Dietetics at the "Hanzehogeschool" in Groningen for which she graduated in 1995. From 1995 to 1997 she studied "Human Nutrition" at Wageningen University. As part of that she study she conducted a research project focused on the bioavailablity of carotenoids from spinach. In September 1997 she received her MSc degree. In October 1997 she started with a PhD-project entitled 'Physical activity, PAI-1 and cardiovascular risk in elderly: a study on gene-environment interactions' at the Division of Human Nutrition & Epidemiology at Wageningen University, of which the main results are described in this thesis. In 1999 she attended the annual New England Epidemiology Summer Program of the New England Epidemiology Institute in Boston, USA. She joined the education program of the Graduate School VLAG. In 2002 she was appointed at Wageningen University for a period of three months to develop computer-based assignments for the MSc-course 'Epidemiology and Public Health'. She was a member of the daily board of the committee for temporary scientific staff members within the Division (1998-2000). Furthermore, she was a member of the PhD-council of the Graduate School VLAG (2000-2001).

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