The *4G5G* polymorphism in the gene for PAI-1 and the circadian oscillation of plasma PAI-1

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Plasminogen activator inhibitor type I (PAI-1) antigen concentrations follow a circadian oscillation peaking in the morning. Some individuals show no apparent circadian rhythm, while others show up to a 10-fold variation in PAI-1 over 24 hours. Results from experimental studies suggest that a polymorphism in the promoter of the gene for PAI-1 *(4G5G)* directly influences the circadian expression of the PAI-1 gene. We studied whether the diurnal variation of PAI-1 antigen differs for the genotypes of the *4G5G* polymorphism. A population-based, cross-sectional study was performed among 263 subjects selected from the Rotterdam Study, a population-based cohort of 7983 men and women aged 55 years and older. The *4G* allele was associated with a more pronounced circadian expression of PAI-1 antigen. Morning PAI-1 antigen concentrations were 79 ng/mL (95% confidence interval [CI], 68-92) in subjects homozygous for *4G,* 62 ng/mL (95% CI, 54-72) in heterozygous subjects, and 59 ng/mL (95% CI, 49-71) in subjects homozygous for *5G.* While respective PAI-1 antigen concentrations in the afternoon were 40 ng/mL (95% CI, 33-49), 41 ng/mL (95% CI, 37-47), and 40 ng/mL (95% CI, 49-71). These findings suggest that the morning increase in PAI-1 antigen concentration is more pronounced among subjects homozygous for the *4G* allele compared with the morning increase among the other genotypes. Additionally, these findings show that homozygosity for the *4G* allele is associated with increased PAI-1 levels during the morning only. (Blood. 2003;101:1841-1844)

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Introduction

Although acute cardiovascular events occur throughout the day, it has been shown that acute myocardial infarction, sudden cardiac death, silent ischemia, and stroke have a circadian pattern of occurrence with a peak in the morning.[1-4] A meta-analysis suggested that 9% of all myocardial infarctions are attributable to the morning excess incidence of cardiac events.[5] The morning excess of cardiac events may result in part from the circadian variation of fibrinolytic activity. Plasminogen activator inhibitor type 1 (PAI-1) is the major component of inhibitors of fibrinolytic activity. PAI-1 shows a clear circadian oscillation peaking in the morning.[6-8]

Recently, a novel transcription factor, CLIF (cyclelike factor), has been identified that regulates the circadian oscillation of PAI-1 gene expression.[9] In the PAI-1 promoter, 2 E-box elements *(CACGTG)* are responsible for the activation of PAI-1 by CLIF; one of the E-boxes is located at −677 to −672. This overlaps with the sequence of the *4G5G* polymorphism of the PAI-1 promoter. The *4G5G* polymorphism is a common single base-pair polymorphism located 675 bp upstream of the start of transcription of the PAI-1 gene. This polymorphism has been associated with increased plasma PAI-1 concentrations[10] and with a slightly increased risk for myocardial infarction.[11-13] We examined whether the circadian oscillation pattern of PAI-1 antigen differs for subjects with the different genotypes of the *4G5G* polymorphism in the PAI-1 gene.

Patients and methods

Population

A cross-sectional study was performed among subjects selected from the Rotterdam Study. The Rotterdam Study is a prospective population-based study of 7983 subjects. The rationale and design of the study have been described elsewhere.[14] In short, between March 1990 and July 1993 all subjects aged 55 years and older, living in a suburb of Rotterdam, the Netherlands, were invited to participate. The overall response rate was 78%. The study has been approved by the Medical Ethics Committee of Erasmus University and written informed consent was obtained from all participants. The present study utilizes data from an earlier case-control study that was set up to examine the association of hemostatic and fibrinolytic parameters with cardiovascular diseases.[15,16] Cases of myocardial infarction were 150 subjects with a history of myocardial infarction.[17,18] Controls were 150 subjects without a history of cardiovascular disease (ie, no history of myocardial infarction, angina pectoris, or stroke; a normal ECG; and no peripheral arterial disease [ankle/arm index > 0.9]) who were randomly drawn from the same 5-year age strata as that of the myocardial infarction cases. We excluded subjects using anticoagulant drugs.

Clinical investigation

Information on current health status, medical history, drug use, and smoking behavior was obtained by a questionnaire. The home interview was followed by 2 visits to the research center, between 8 AM and 4 PM.

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Table 1. Characteristics of the study population according to whether blood samples of the subjects were collected before or after noon

	Morning	Afternoon
No. of subjects	146	117
Age, y	72 (9.8)	73 (9.4)
Women, yes (%)	73 (50)	62 (53)
Systolic blood pressure, mmHg	138 (23)	143 (22)
Diastolic blood pressure, mmHg	74 (13)	75 (12)
Serum total cholesterol, mM	6.39 (1.17)	6.38 (1.16)
Serum HDL cholesterol, mM	1.28 (0.33)	1.32 (0.37)
Ankle/arm blood pressure ratio, ratio	1.07 (0.22)	1.06 (0.18)
History of myocardial infarction, yes (%)	81 (55)	51 (44)
PAI-1 antigen, ng/L*	67	41

Values are numbers (%) or means (standard deviation [SD]).

*Mean back-transformed from logarithmic scale.

Patients were not asked to fast or to refrain from smoking. During these visits several cardiovascular risk indicators were determined. Sitting blood pressure was measured at the right upper arm with a random zero sphygmomanometer.

Laboratory investigations

Blood sampling and storage have been described elsewhere.[19] Blood samples were collected during the first visit to the research center between 8 AM and 4 PM, using CTAD vacutainers (0.11M citrate, 15 mM theophylline, 3.7 mM adenosine, and 0.198 mM dipyridamole; Diatube H, Becton and Dickinson, Mylan, Cedex, France).[20] Serum total and high-density lipoprotein (HDL) cholesterol were determined with an automated enzymatic procedure. PAI-1 antigen concentration was measured in CTAD plasma using the Innotest PAI-1 (Innogenetics, Zwijndrecht, Belgium).[21] Genomic DNA was isolated from blood cells and PAI-1 genotype was determined by allele-specific PCR amplification.[22]

Statistical analysis

The distribution of the plasma concentration of PAI-1 antigen was positively skewed, therefore statistical analysis was carried out on log-transformed values, but the presented mean values and 95% confidence intervals (CIs) have been transformed back into the original scale. For each genotype, mean PAI-1 antigen concentration for subjects who visited the research center before noon was compared with that in subjects who visited the center after noon. Linear regression analysis with log-transformed PAI-1 concentrations as dependent variable was used to compare PAI-1 levels between subjects visiting the research center before or after noon, adjusted for systolic blood pressure, serum HDL cholesterol, and body mass index. Back transformation of the regression coefficients has been performed by taking the exponent of the coefficient and the exponent of upper and lower values of the confidence interval. The result is a multiplicative factor.

This is a relatively small study with relatively little power to test for interaction. We noticed that the morning/afternoon difference of heterozygous subjects was similar to that of subjects homozygous for *5G*. We therefore decided to combine these 2 groups. Interaction between the effect of homozygosity for the *4G* allele and the diurnal effect were tested with the product of an indicator variable for *4G4G* and an indicator variable for visit before noon, together with both indicators as independent variables in a linear regression model.

Results

Characteristics of the study population

After exclusion of those with missing data on either the *4G5G* polymorphism (n = 22) or PAI-1 antigen concentration (n = 29), or time of blood collection (n = 4), the study population consisted of 263 subjects (132 cases of myocardial infarction and 131 control subjects). We compared the observed distribution of genotypes among the subjects without cardiovascular disease with that expected for a population according to a Hardy Weinberg equilibrium. The frequency of the *4G* allele was 53% and that of the *5G* allele was 47%, and the distribution of genotypes was in Hardy-Weinberg equilibrium; 39 control subjects (30%) were found to be homozygous for the *4G* allele, whereas 62 (47%) control subjects were heterozygous, and 30 (23%) control subjects were homozygous for the *5G* allele.

Table 1 presents characteristics of the study population according to time of blood collection.

4G5G genotypes and PAI-1 levels

Homozygosity for the *4G* allele was associated with increased levels of PAI-1 antigen. In subjects homozygous for the *4G* allele PAI-1 antigen concentration was 62 ng/mL (95% CI, 54-70), in those heterozygous it was 50 ng/mL (95% CI, 46-56), and in those homozygous for the *5G* allele it was 50 ng/mL (95% CI, 43-58).

Diurnal pattern of PAI-1

PAI-1 antigen levels showed a diurnal pattern with higher values in the morning. Figure 1 presents PAI-1 levels according to the time of blood collection. Between 8 and 9 AM, 28 subjects had a median PAI-1 of 63 ng/mL; between 9 and 10 AM, 69 subjects had a median PAI-1 of 64 ng/mL; between 10 and 11 AM, 44 subjects had a median PAI-1 of 65 ng/mL; between 11 AM and noon, 5 subjects had a median PAI-1 of 62 ng/mL; between noon and 1 PM, blood was drawn in one subject with a PAI-1 of 43 ng/mL; between 1 and 2 PM, 58 subjects had a median PAI-1 of 40 ng/mL; between 2 and 3 PM, 44 subjects had a median PAI-1 of 38 ng/mL; between 3 and 4 PM, 14 subjects had a median PAI-1 of 36 ng/mL. The figure illustrates that high peaks of PAI-1 antigen are predominantly found during the morning hours.

Diurnal pattern of PAI and *4G5G* genotypes

Differences in PAI-1 expression levels according to time of blood collection appeared to depend somewhat on genotype (test for interaction, $P = .057$), and for this reason separate analyses were conducted according to genotype. In subjects with the *4G4G* genotype the morning/afternoon difference was larger than in the other genotypes (Table 2). Results from univariate linear regression analyses suggest that PAI-1 antigen was 1.95 (95% CI, 1.54-2.51) times higher in the morning among subjects homozygous for *4G*; for heterozygous subjects it was 1.50 (95% CI, 1.23-1.82) times

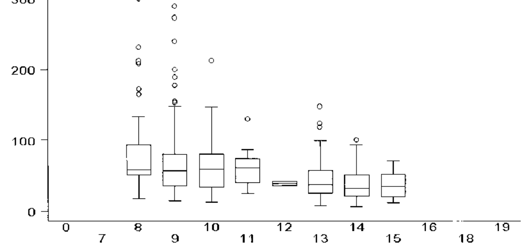

Figure 1. PAI-1 antigen concentrations according to the time of day. Box and whisker plot showing median values, interquartile ranges (box), and extreme values (whiskers) of plasma PAI-1 antigen concentrations among all 263 subjects according to the time their blood was collected.

Table 2. Plasma concentration of PAI-1 antigen in samples collected before and after noon for the different genotypes

	4G4G	*4G5G*	*5G5G*
Subjects visiting before noon, n	57	57	32
PAI-1 (95% CI) ng/mL	79 (68-92)	62 (54-72)	59 (49-71)
Subjects visiting after noon, n	33	61	23
PAI-1 (95% CI) ng/mL	40 (33-49)	41 (37-47)	40 (32-51)

Values (adjusted for age and sex) are numbers or means (95% confidence intervals) calculated on log-transformed data, back-transformed into the original scale.

4G4G indicates homozygous for the *4G* allele; *4G5G*, heterozygous; and *5G5G*, homozygous for the *5G* allele.

higher in the morning; and for subjects homozygous for *5G* it was 1.47 (95% CI, 1.08-1.97) times higher in the morning. Similar statistically significant differences between morning and afternoon PAI-1 levels were found after adjustment for the confounders (potential confounders are presented in Table 1). After adjustment for systolic blood pressure, HDL cholesterol, and a history of myocardial infarction, PAI-1 antigen was 1.92 (95% CI, 1.61-2.41) times higher in the morning among subjects homozygous for the *4G* allele; 1.58 (95% CI, 1.32-1.90) times higher in the morning among heterozygous subjects; and 1.65 (95% CI, 1.26-2.16) times higher in the morning among those homozygous for *5G*.

History of myocardial infarction and diurnal PAI-1

There was no statistically significant difference between the morning effect on PAI-1 among subjects with a history of myocardial infarction compared with those without a history of cardiovascular diseases. PAI-1 antigen was 1.54 (95% CI, 1.28-1.85) times higher in the morning among subjects with a history of myocardial infarction; it was 1.75 (95% CI, 1.43-2.13) times higher in the morning among subjects without a history of cardiovascular diseases.

Discussion

We assessed whether the circadian oscillation pattern of PAI-1 antigen differs for the genotypes for the *4G5G* polymorphism in the PAI-1 gene. Homozygosity for the *4G* allele was associated with increased PAI-1 levels only during the morning hours. All 3 genotypes showed higher PAI-1 antigen concentrations in the morning, but this morning effect was more pronounced among subjects homozygous for the *4G* allele (test for interaction, $P = .057$).

We believe that our findings provide a valid estimate of the effect of the *4G* allele of the *4G5G* polymorphism on the circadian oscillation pattern of PAI-1 antigen. PAI-1 antigen levels were measured in a selected group of subjects who participated in a large population-based cohort study. Half of the study population had a history of myocardial infarction and the other half did not have a history of cardiovascular diseases. The proportion of patients with a history of myocardial infarction was slightly higher in the morning, which of course explains higher PAI-1 levels in the morning.[23] However, the fact that roughly half the studied population had a history of myocardial infarction does not totally account for the observed differences in PAI-1. History of myocardial infarction was adjusted for in the models, and this adjustment did not qualitatively alter the conclusion. Moreover, the morning effect among subjects with myocardial infarction was not more pronounced than among patients without myocardial infarction. Increased PAI-1 levels have been associated with increased blood pressure, increased body mass index, and decreased HDL cholesterol.[16,24] Systolic blood pressure and HDL cholesterol were also related to time of blood collection in our study. Therefore, these factors may also be an alternate explanation for our findings. Yet, adjustment for these factors did not notably change our findings.

This study is the first to show an association between the *4G5G* polymorphism and the circadian pattern of plasma PAI-1 antigen concentrations. Experimental work had already suggested that the *4G5G* polymorphism might influence the circadian oscillation of PAI-1 antigen.[9] The observation that the *4G5G* polymorphism is associated with the circadian pattern of PAI-1 supports the view that the *4G5G* polymorphism may affect the circadian expression of PAI-1.

Our findings shed an interesting light on the current knowledge about PAI-1 as a risk factor for myocardial infarction. The role of PAI-1 as a risk factor for cardiovascular diseases has been questioned.[23] The finding that the *4G* allele is associated with increased plasma levels of PAI-1 in the morning confirms that the *4G5G* polymorphism in the PAI-1 gene is of functional importance in regulating the expression of the PAI-1 gene. Additionally, homozygosity for the *4G* allele is associated with a slightly increased risk for myocardial infarction.[12] And, subjects with 1 or 2 *4G* alleles had a faster progression from first anginal symptoms to acute coronary syndromes than *5G5G* subjects.[25] The present study suggests that the morning increase in PAI-1 is more pronounced in *4G4G* subjects. The morning peak corresponds to the diurnal variation of the time of onset of myocardial infarction. It is tempting to suggest that the *4G4G* genotype, maybe through the morning increase in PAI-1, plays a role in the excess number of cases of myocardial infarction in the morning. This would confirm a role of PAI-1 as a risk factor for myocardial infarction.

In conclusion, our findings support the view that the *4G5G* polymorphism in the promoter of the gene for PAI-1 may affect the circadian expression of the PAI-1 gene.

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