Diurnal Variation in PAI-1 Activity Predominantly Confined to the 4G-allele of the PAI-1 Gene

Tiny Hoekstra, Johanna M. Geleijnse, Evert G. Schouten, Cornelis Kluft

1Division of Human Nutrition & Epidemiology, Wageningen University, Wageningen, The Netherlands; 2Gaubius Laboratory, TNO-PG, Leiden, The Netherlands

Keywords
Fibrinolysis, diurnal variation, PAI-1, t-PA, 4G/5G-polymorphism

Summary
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. 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.). 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. 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-type 1 (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/m²). 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. 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

Statistical Analysis
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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 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 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 ($-0.31$, $p = 0.0001$). 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 ($-0.28$, $p = 0.0001$). 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.

In Fig. 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,

### Table 1 Characteristics of the study population (n = 598) by the 4G/5G-polymorphism

<table>
<thead>
<tr>
<th></th>
<th>4G/4G</th>
<th>4G/5G</th>
<th>5G/5G</th>
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<tbody>
<tr>
<td>(n=184)</td>
<td>(n=275)</td>
<td>(n=139)</td>
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<tr>
<td>Age (yr)</td>
<td>73.0 ± 5.7</td>
<td>74.0 ± 5.5</td>
<td>74.0 ± 5.8</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>25.4 ± 3.3</td>
<td>26.3 ± 4.2</td>
<td>25.9 ± 3.8</td>
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<tr>
<td>Men (%)</td>
<td>53.8</td>
<td>51.6</td>
<td>53.2</td>
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<tr>
<td>Smoking status (%)</td>
<td></td>
<td></td>
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<tr>
<td>Current smokers</td>
<td>31</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>Former smokers</td>
<td>38</td>
<td>43</td>
<td>48</td>
</tr>
<tr>
<td>Never smokers</td>
<td>32</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>Alcohol consumers (%)</td>
<td>69</td>
<td>72</td>
<td>79</td>
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<tr>
<td>History of cardiovascular disease (%)</td>
<td>22</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Use of cardiovascular medications (%)</td>
<td>18</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>6.3 ± 1.2</td>
<td>6.2 ± 1.2</td>
<td>6.2 ± 1.3</td>
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<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.4 ± 0.5</td>
<td>1.4 ± 0.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.8 ± 1.1</td>
<td>3.7 ± 1.0</td>
<td>3.8 ± 1.0</td>
</tr>
<tr>
<td>Insulin (pmol/L)³</td>
<td>137 (97-207)</td>
<td>140 (99-230)</td>
<td>137 (91-222)</td>
</tr>
<tr>
<td>PAI-1 activity (IU/mL)³</td>
<td>2.1 (0.6-5.4)</td>
<td>2.2 (0.8-6.0)</td>
<td>1.4 (0.3-3.9)</td>
</tr>
<tr>
<td>T-PA antigen (ng/mL)³</td>
<td>10.2 ± 3.7</td>
<td>10.8 ± 3.9</td>
<td>9.9 ± 3.7</td>
</tr>
</tbody>
</table>

*Values are given as mean ± SD unless indicated otherwise. ³Median with interquartile range in parentheses, because of skewed distribution. ⁴T-PA levels were available for 565 subjects (4G/4G: n = 175, 4G/5G: n = 259, 5G/5G: n = 131).*
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 (Fig. 2). Adjusting for the potential confounders did not alter the pattern.

In Fig. 3 the diurnal variation in PAI-1 is shown separately for the three different genotypes using broader categories to increase 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 χ²; p = 0.23). 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 or 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 adjusting 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 sub-group analysis yielded essentially similar results in genotype-specific associations. The proportion of 5G/5G-subjects sampled before 10 am 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 highest 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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References


