

## PAI-1 4G/5G Polymorphism and Plasma Levels Association in Patients with Coronary Artery Disease

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### Abstract

**Background:** Type-1 plasminogen activator inhibitor (PAI-1) 4G/5G polymorphism may influence the PAI-1 expression. High plasma levels of PAI-1 are associated with coronary artery disease (CAD).

**Objective:** This study investigated the influence of PAI-1 4G/5G polymorphism on plasma PAI-1 levels and its association with CAD assessed by coronary angiography.

**Methods:** Blood sample of 35 individuals with angiographically normal coronary arteries, 31 individuals presenting mild/moderate atheromatosis, 57 individuals presenting severe atheromatosis and 38 healthy individuals (controls) were evaluated. In patients and controls, the PAI-1 4G/5G polymorphism was determined by PCR amplification using allele-specific primers. Plasma PAI-1 levels were quantified by ELISA assay (American Diagnostica).

**Results:** No difference was found between groups regarding age, gender and body mass index. Plasma PAI-1 levels and 4G/4G genotype frequency were significantly higher in the severe atheromatosis group compared to the other groups ( $p < 0.001$ ). Furthermore, patients with 4G/4G genotype ( $r = 0.28$ ,  $p < 0.001$ ) had significantly higher plasma PAI-1 levels than those with 5G/5G genotype ( $r = 0.02$ ,  $p = 0.4511$ ). In addition, in a multiple logistic regression model, adjusted for all the other variables, PAI-1 was observed to be independently associated with CAD  $> 70\%$  ( $p < 0.001$ ).

**Conclusion:** The most important finding of this study was the association between 4G/4G genotype, high plasma PAI-1 levels and coronary stenosis higher than 70% in Brazilian individuals. Whether high plasma PAI-1 levels are a decisive factor for atherosclerosis worsening or it is a consequence remains to be established. (Arq Bras Cardiol 2011;97(6):462-467)

**Keywords:** Plasminogen activator inhibitor 1/genetics; polymorphism, genetic; coronary disease.

### Introduction

The main inhibitor of fibrinolysis is the type-1 plasminogen activator inhibitor (PAI-1), which is the primary inhibitor of the physiological plasminogen activator (t-PA). Increased plasma PAI-1 levels have been reported in survivors of myocardial infarction (MI) compared with the general population, and this increase is correlated with the recurrence of MI<sup>1,2</sup>. Impaired fibrinolytic function has been associated with coronary artery disease (CAD) and MI.

The insertion or deletion of a nucleotide in the promoter region of the PAI-1 gene (-675 4G/5G) was identified in 675 base pairs before the onset of the transcript<sup>3</sup>. Homozygous individuals for the 4G/4G deletion have high plasma PAI-1 levels, carriers of 5G/5G have reduced levels, and 4G/5G heterozygous individuals have intermediate levels<sup>4,5</sup>. The inability to bind a transcriptional repressor

protein has been associated with the 4G allele, resulting in higher PAI-1 mRNA expression and higher circulating PAI-1 levels<sup>6</sup>.

Plasma PAI-1 levels may be influenced by age, sex, obesity, hypertension, smoking, hypercholesterolemia, and genetic polymorphisms, including polymorphism -675 4G/5G<sup>6,7</sup>. Increased plasma PAI-1 levels may undermine the fibrinolytic system and promote the survival of the fibrin clot. Therefore, the genotyping of this polymorphism could be relevant to assess the performance of the fibrinolytic system in patients with established diagnosis of CAD.

There are limited and controversial data regarding the impact of 4G/5G polymorphism of the PAI-1 gene in the extent of CAD. This polymorphism is involved in CAD, with the highest risk in 4G/4G homozygotes in some populations<sup>8</sup>, but not in others<sup>9</sup>. The role of PAI-1 polymorphism in Brazilian patients suffering from CAD has not been established so far. The distribution frequency of the PAI-1 gene was studied to test the hypothesis that the 4G/4G genotype favors the CAD severity in individuals submitted to coronary angiography.

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## Methods

### Individuals and study design

The approval for the study was granted by the Hospital Socor and Ethical Research Committee of the Universidade Federal de Minas Gerais. Informed consent was obtained from all participants, and the relevant clinical details for each individual were recorded. These details included personal and demographic data, family history, risk factors and coronary angiographic results that were initially recorded by three independent cardiologists (Table 1). A total of 123 male and female individuals attending the coronary angiography clinic in the Hemodynamic Department of Hospital Socor of Belo Horizonte, Minas Gerais, Brazil, with ages ranging from 42 to 69 years, were studied. Based on coronary angiography results, the participants were classified into three groups: angiographically normal arteries ( $n = 35$ ), mild/moderate atheromatosis ( $n = 31$ ) and severe atheromatosis ( $n = 57$ ). The severe atheromatosis group was also subdivided according to the number of affected arteries into severe atheromatosis in one vessel (SA-1,  $n = 16$ ), two vessels (SA-2,  $n = 13$ ) and three or more affected blood vessels (SA-3,  $n = 28$ ). All patients had a history of stable angina. However, none had a recent myocardial infarction or unstable angina (at least within the last three months), congestive heart failure, coagulation disorders, renal problems, hepatic or auto-immune diseases or cancer or were on warfarin treatment or undergoing lipid-modifying therapy. In the same period, 38 healthy individuals were selected from the community in general (control group), with mean age and body mass index (BMI) similar to the average of three groups of patients. However, this study was not intended to develop a case-control study type.

### Angiography

Coronary angiography was performed via percutaneous transfemoral approach. The images were digitally recorded and all angiograms were analyzed by three experienced cardiologists who were not aware of the patients' clinical details. Only patients who had three identical awards were selected. The extent and the progression of angiographically documented CAD were classified as follows:

1. **Normal** coronary arteries (No angiographic stenosis)
2. **Mild** (Up to 30% stenosis of the luminal diameter in one or more coronary arteries)
3. **Moderate** (30 to 70% stenosis of the luminal diameter in one or more coronary arteries)
4. **Severe** (Greater than 70% stenosis of the luminal diameter in one or more coronary arteries)

### Sample collection and laboratory determinations

Fasting peripheral venous blood samples were collected from each participant prior to angiography. Four and a half mL of blood was collected into vacutainer tubes containing 3.8% trisodium citrate anticoagulant (Becton-Dickinson) using a 21-gauge needle. Additionally, 5mL was collected in a tube without anticoagulant and 5mL with EDTA. Samples were

immediately centrifuged for 15 minutes at 1100g. Plasma and serum were immediately isolated and aliquots were stored at  $-70^{\circ}\text{C}$  for batch-wise analysis. The EDTA blood samples were submitted to genomic DNA extraction using the Wizard purification system (Promega, Inc.). The PAI-1 dosage was performed using plasma aliquots with the IMUBIND<sup>®</sup> Plasma PAI-1 ELISA (American Diagnostica<sup>®</sup> Inc. – USA) kit diagnostics according to instructions from manufacturer.

The 4G/5G polymorphism was determined using allele-specific polymerase chain reaction (PCR), as previously described<sup>2</sup>. PCR reactions were performed in a PT100 PCR thermocycler (MJ Research, Waltham, USA) using 1 pMol of each primer (Invitrogen<sup>®</sup>, São Paulo, SP), 0.2 mM of dNTPs (GIBCO BRL<sup>®</sup>, São Paulo, SP), and 1 unit of Taq polymerase (Phonetrria<sup>®</sup>-Belo Horizonte, Minas Gerais, Brazil). PCR reactions were submitted to 15 cycles consisting of 1 min at  $95^{\circ}\text{C}$  for denaturing, 1 min at  $59^{\circ}\text{C}$  for primer annealing and 1 min at  $72^{\circ}\text{C}$  for primer extension, preceded by an initial step of the denaturing at  $95^{\circ}\text{C}$  for 5 minutes, followed by 20 cycles with girdling at  $52^{\circ}\text{C}$  and finishing with a pitch of  $72^{\circ}\text{C}$  for 5 minutes. DNAs from previously typed individuals were included in each sample set in order to control enzyme activity. Samples were then analyzed by silver stained acrylamide gel electrophoresis.

### Statistical considerations

Data were analyzed by a Sigma Stat version 1.0 software system using one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks followed by the Tukey test. Categorical variables (risk factors and 4G/5G polymorphism) were analyzed using the Chi-square test. Spearman's test was used for correlation between polymorphism status (categorical variable) and plasma PAI-1 (continuous variable) and between CAD (categorical variable) and PAI-1 (continuous variable). An  $X^2$  goodness-of-fit test was used to confirm whether the observed genotype frequencies agreed with those expected under the hypothesis of Hardy-Weinberg. In addition, multiple logistic regression was performed since the variables cannot be considered independent. In this model,  $\text{CAD} > 70\%$  was considered as a dependent variable. The sample size was calculated considering the average values measured and standard deviations for PAI-1 based on a pilot study that included 50 samples. The alpha error level or confidence level was 5% corresponding to a 95% Confidence Interval. The beta error level or statistical power [ $1 - \beta$ ] was 20%. A calculated statistical power (type II or beta error), with alpha error level 5% was 97.3%. Accordingly, a minimum satisfactory sample size for plasma levels could be 15 individuals in each group. Since the severe atheromatosis group was subdivided, the minimum sample size was defined using the coefficient of variation previously described in the literature, considering a ten percent variation for the average and a minimum number of eleven individuals per group. Statistical differences with 5% of significance level were found. For genotypes of PAI-1 4G5G polymorphism, considering the variation observed of the allele and genotype frequencies from different studies, the sample size calculated was a minimum of 29 individuals in each group with an alpha error level of 5%, 95% confidence interval and 80% of statistical power ( $1 - \beta$ ).

## Results

The characterization of groups, including sex and age, as well as the risk factors associated with atherosclerosis, number of individuals and percentage of each variable are shown in Table 1. The four groups studied presented homogeneity with respect to age, sex and body mass index. A significant difference was observed for the smoking parameter for the severe atheromatosis group compared to the angiographically normal group ( $p = 0.02$ ). Regarding a sedentary lifestyle, mild/moderate and severe atheromatosis groups were significantly different from the control and angiographically normal group ( $p < 0.05$ ). Conventional lipid profile parameters are also presented in Table 1 as the means and standard deviations. No significant differences were found between groups for total cholesterol, HDL and LDL. Only triglyceride levels were significantly lower in the control group compared to the others.

The results for plasma PAI-1 and 4G/5G polymorphism are shown in Table 2. The plasma PAI-1 levels were significantly higher in the severe atheromatosis group than in the others ( $p < 0.001$ ). The severe atheromatosis group also appear to have higher frequency of 4G/4G genotype and lower frequency of 4G/5G compared to the other groups. No significant difference was seen in the 5G/5G genotype between groups. No deviation from the Hardy-Weinberg equilibrium was observed. When the severe atheromatosis group was subdivided, participants with one affected blood vessel presented higher PAI-1 levels compared with two and three affected blood vessels (Figure 1).

A linear regression showed that PAI-1 can be predicted from a linear combination of CAD ( $r = 0.47$ ;  $p < 0.0001$ ). Indeed, since these results were analyzed by using a binary logistic regression model, adjusted for all the other variables, PAI-1 seems to be independently associated with CAD  $> 70\%$  ( $p < 0.001$ ). This observation was confirmed by the odds

ratio obtained for PAI-1 (2.358; 95%; CI = 1.085 – 9.432;  $p < 0.001$ ). In addition, plasma PAI-1 levels showed a positive and significant association with 4G/4G genotype ( $r = 0.28$ ,  $p < 0.001$ ).

## Discussion

Several authors have demonstrated the association of high PAI-1 levels with the presence of CAD. These results support the concept of the contribution of fibrin in intravascular atherothrombogenesis<sup>10</sup>. Decreased fibrinolytic activity, mainly caused by the increase in plasma PAI-1 concentration, has been associated with CAD in several studies<sup>11</sup>. In this study, plasma PAI-1 levels were significantly higher in the severe atheromatosis group compared to the other groups (Table 2). The averages obtained for SA-1, SA-2, and SA-3 subgroups were also significantly high compared to the control and angiographically normal group (Figure 1). These data show an association between the plasma PAI-1 concentration and CAD severity, considering the percentage of stenosis of more than 70%, regardless of the number of affected arteries. After adjusting for risk factors and other variables, the results of the multivariate analysis be inferred about an independent association between plasma PAI-1 levels and the presence of coronary artery stenosis greater than 70% ( $p < 0.001$ ). The SA-1 subgroup also showed significant difference compared to the mild/moderate atheromatosis group ( $p = 0.004$ , Figure 1).

Another finding of this study was the association between 4G/4G genotype, increased plasma PAI-1 levels ( $r = 0.28$ ,  $p < 0.001$ ) and coronary artery stenosis with obstruction greater than 70%, suggesting that the presence of 4G allele may have influenced the increase in plasma PAI-1 levels, and that this increase may have affected the fibrinolytic

**Table 1 – Characterization of the groups studied**

	Controls	Angiographically Normal Arteries	Mild/Moderate Atheromatosis	Severe Atheromatosis	p
n	38	35	31	57	---
Men	18 (47%)	16 (46%)	17 (55%)	31 (54%)	0.341
Age (years)	59 ± 6	59 ± 7	59 ± 9	60 ± 8	0.299
BMI (Kg/m <sup>2</sup> )	26 ± 4	25 ± 4	26 ± 5	25 ± 3	0.204
Current Smoker	0	6 (17%)	8 (25%)	23 (40%)*	0.020
Hypertension	0	31 (88%)	25 (80%)	48 (84%)	0.543
Sedentary Lifestyle	20 (52%)	33 (94%)	23 (74%)*	43 (75%)*	<0.01
Family History	9 (23%)	14 (40%)	18 (58%)	29 (50%)	0.124
Diabetes mellitus	0	5 (14%)	7 (22%)	8 (14%)	0.354
Total cholesterol (mg/dL)	179 ± 40	206 ± 49	203 ± 56	201 ± 44	0.821
HDL (mg/dL)	47 ± 8	43 ± 9	43 ± 9	42 ± 9	0.734
LDL (mg/dL)	113 ± 36	129 ± 45	125 ± 46	127 ± 39	0.811
Triglycerides (mg/dL)	96 ± 43	167 ± 73*	170 ± 82*	159 ± 66*	<0.001

Significant differences between the groups are indicated by the symbol: \* vs controls (ANOVA after log-transformation); BMI - body mass index.

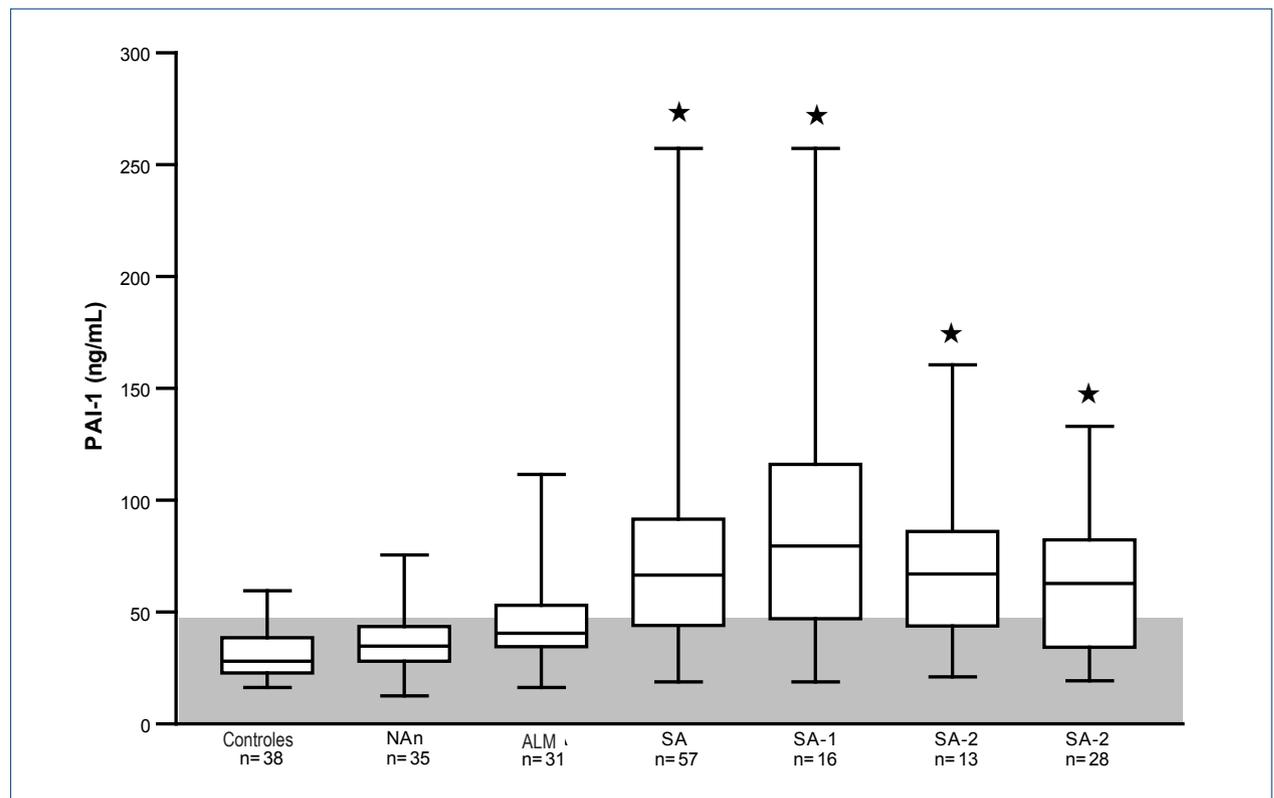
system, being thus a decisive factor for the progression of atherosclerosis in the individuals evaluated. Previous studies had demonstrated the association between 4G allele, high PAI-1 concentrations and CAD<sup>12-16</sup>. Although this may be a promising correlation, it may not be demonstrated in all populations<sup>17-19</sup>. This is the first study to demonstrate this correlation in the Brazilian population. Plasma PAI-1 levels

may be influenced by several factors<sup>20</sup>, among which genetic factors. Hong et al<sup>21</sup> showed that 42% of the variations found in plasma PAI-1 levels were due to genetic factors, in a study involving the Swedish population. Naram et al<sup>11</sup>, examining the South African population, observed that lower plasma PAI-1 levels were obtained for the black population compared with Caucasians. The authors

**Table 2 – Plasma PAI-1 levels according to PAI-1 genotype**

	Controls	Angiographically Normal Arteries	Mild/Moderate Atheromatosis	Severe Atheromatosis	p
n	38	35	31	57	---
PAI-1 (ng/mL)	28.1 (22.9 – 38.7)	34.9 (27.9 – 42.4)	40.5 (34.2 – 51.8)	66.6 (44.7 – 91.6)*	<0.001
4G/4G PAI-1 (ng/mL)	12 (31%) 27.9 (23.6 – 38.4)	10 (28%) 31.0 (25.4 – 36.2)	9 (29%) 33.8 (31.8 – 50.9)	27 (43%)* 45.8 (28.4 – 74.6)*	<0.001 0.002
5G/5G PAI-1 (ng/mL)	14 (36%) 32.0 (19.9 – 39.1)	12 (34%) 38.3 (23.4 – 52.6)	11 (35%) 49.9 (39.1 – 55.2)	20 (35%) 74.4 (47.7 – 95.6)*	0.434 0.005
4G/5G PAI-1 (ng/mL)	12 (31%) 27.2 (23.8 – 37.6)	13 (37%) 34.9 (31.1 – 39.1)	11 (35%) 39.6 (36.9 – 46.9)	10 (22%)* 84.1 (64.1 – 102.5)*	<0.001 <0.001

PAI-1 levels are expressed as median (interquartile range) and percentage presence of the 4G/5G polymorphism in the groups studied. (\*) and (†) indicates significant difference vs all the other groups, (\*) Kruskal-Wallis, (†) Chi-square test.



**Figure 1 – Distribution of plasma PAI-1 levels for controls, angiographically normal arteries (NAn), mild/moderate atheromatosis (MMA) and severe atheromatosis (SA) groups with one (SA-1), two (SA-2) and three or more affected vessels (SA-3). Each box represents the median and interquartile range of values, with the I bars (whiskers) extended to the minimum and maximum values. The gray area represents the reference values (0 - 47ng/mL). (★) Indicates significant differences compared to controls, NAn and MMA groups (p<0.01).**

concluded that the main reason for the detection of lower PAI-1 levels in black individuals was the low prevalence of 4G allele in this group of individuals. Although ethnicity influences plasma PAI-1 levels, this study showed no significant difference in PAI-1 levels for the different ethnic groups. The study design could justify such finding, because the group studied in this work (n=161) was composed of 30.9% of Caucasians, 26.8% of blacks and 42.3% of mestizos, but uniformly distributed in the three groups, showing no statistically significant differences between them for different ethnicities. The frequency of 4G and 5G alleles described for the Brazilian population, which presents a variety of ethnic groups, has not yet been fully established in literature. In this study, 51% of participants (n= 161) had 4G allele, while 49% had 5G allele.

Whether an increase in plasma PAI-1 levels is a decisive factor for worsening of atherosclerosis or its increased levels are a consequence of this injury still remains to be clarified. PAI-1 has been studied in different settings with thrombotic pathophysiology. PAI-1 also plays an important role in signal transduction, cell adherence, and migration<sup>1</sup>. In a diseased artery environment there is a heterogeneous population of cells, all of which may be synthesizing PAI-1<sup>17</sup>. These observations suggest that PAI-1 may be involved in CAD as a result of the initialization and progression of atheromatosis with enhanced fibrin accumulation, either by a fibrinolysis impaired effect or by enhancing the feedback synthesis response.

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## Conclusion

The most important finding of this study was the association between 4G/4G genotype, high plasma PAI-1 levels and coronary stenosis higher than 70% in Brazilian individuals. The relationship observed with severe atheromatosis was irrespective of smoking, arterial hypertension, sedentary lifestyle, family history and lipid profile. These data support the hypothesis that PAI-1 plays a role in the pathogenesis of atherosclerosis or its major clinical manifestation, CAD. Whether high plasma PAI-1 levels are a decisive factor for atherosclerosis worsening or it is a consequence of it, remains to be established.

## Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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