



## The Association between PAI-1 Gene Promoter Polymorphism and Serum Serpin E1, MDA, and Hs-CRP Levels in Coronary Artery Disease

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### ABSTRACT

**Background:** Coronary artery disease (CAD) caused by atherosclerosis. Studies have shown that there are a number of factors which are closely related to the development and progression of CAD that include Cellular binding molecules like Plasminogen Activator Inhibitor-1(PAI-1), Lipid peroxidation, inflammation and hemostasis.

**Objectives:** The present case-control study aimed to evaluate the association between Plasminogen Activator Inhibitor-1 (PAI-1) 4G/5G polymorphism and oxidative stress markers and Coronary Artery Disease (CAD).

**Patients and Methods:** Blood was drawn and DNA was extracted from 90 subjects (46 patients with angiographically diagnosed CAD and 44 age- and sex-matched healthy controls). The 4G/5G polymorphism of PAI-1 was detected by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis. Besides, the risk factors, serpin E1, Malondialdehyde (MDA), high-sensitivity C-Reactive Protein (hs-CRP), and lipid profile serum levels were measured by standard methods and were compared between the two study groups using independent samples t-test, one-way ANOVA, and Mann-Whitney U test as appropriated.

**Results:** Results: The frequency of 4G/4G genotype of PAI-1 gene was higher in the CAD patients than in the controls (28/46 (60.87%) vs. 8/44 (18.18%),  $P < 0.01$ ). Additionally, the serpin E1 plasma level was significantly higher in the CAD group carrying the 4G allele compared to those homozygous for the 5G allele ( $P = 0.016$ ). Besides, a significant difference was found between the 4G/4G and 5G/5G subjects of the CAD group regarding plasma High-Density Lipoprotein (HDL) ( $P < 0.01$ ). Also, significant differences were observed among the three genotypes of both groups concerning the plasma levels of cholesterol, triglyceride, and Low-Density Lipoprotein (LDL). However, no significant correlation was found between PAI-1 gene polymorphism and MDA serum level, hs-CRP, and risk of CAD.

**Conclusion:** The findings of this study suggested that 4G/4G PAI-1 polymorphism was associated with cholesterol, triglyceride, LDL, and HDL levels and could be regarded as a biomarker for risk of CAD.

#### ► Implication for health policy/practice/research/medical education:

Our findings suggested that the 4G/5G polymorphism of the PAI-1 gene seems to be a useful marker of fibrinolytic activity. Besides, this polymorphism might contribute to defective fibrinolytic activity due to elevated PAI-1 plasma level, eventually increasing the risk of coronary artery disease. Moreover, the study results demonstrated that this genetic variation might be important in correlation with some other factors, such as increased or decreased triglyceride, cholesterol, and LDL and HDL levels. However, no significant correlation was observed between PAI-1 4G/5G gene polymorphism and serum levels of stress oxidative factors, such as MDA and hs-CRP.

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## 1. Background

Atherosclerotic Coronary Artery Disease (CAD) remains one of the major health problems around the world. It appears as a multifactorial process caused by interaction of environmental risk factors with multiple predisposing genes. CAD has been ranked as primary cause of mortality and is the major cause of morbidity in the world. It has been estimated that between 1990 and 2020, ischemic heart disease alone will increase by 29% in men and 48% in women in developed countries and by 120% in women and 127% in men in developing countries (1). Several factors, including blood pressure, high plasma lipids, smoking, diabetes mellitus, and age, are involved in development of CAD and treatment of these risk factors reduces the rate of mortality (2-4).

Serpin E1, the protein product of Plasminogen Activator Inhibitor-1 (PAI-1) gene, originates from vascular endothelium and platelets. Serpin E1 plays an important role in intravascular thrombosis and high levels of serpin E1 in CAD are associated with the risk of premature atherosclerosis (2). It has also been reported that high levels of serpin E1 are associated with vascular wall impairment and endothelial dysfunction. Moreover, a cross-sectional case-control study indicated that elevated plasma serpin E1 activity was related to intima-media thickness of carotid arteries (4). Human PAI-1 gene is located at chromosome 7q22, and various genetic polymorphisms have been identified in its promoter region (4). A common insertion/deletion of guanine has been detected in this region (2). Deletion of the 4G allele was associated with increased serpin E1 plasma level due to inability of transcriptional repressor protein for binding to this polymorphic sequence (5). C-Reactive Protein (CRP) is a member of Pentraxin family, which plays a key role in the innate immune response. CRP is the most sensitive acute reactant protein and its concentration increases rapidly during inflammatory processes (6-8). This protein has been shown to have prognostic value in patients with acute coronary syndromes and plays different roles in pathogenesis of atherosclerosis (7).

Malondialdehyde (MDA) is a marker, which is commonly used to evaluate lipid peroxidation. Additionally, its assessment is considered to be an independent marker of oxidative damage. Therefore, MDA could be a useful indicator for identification of patients with CAD (7).

## 2. Objectives

The present study aims to evaluate the association between PAI-1 gene promoter polymorphism and serum serpin E1, MDA, and hs-CRP levels in CAD.

## 3. Materials and Methods

### 3.1. Subjects

The present case-control study was conducted on 90 subjects, including 46 patients with CAD and 44 age- and sex-matched healthy controls. Written informed consents were obtained from all the patients.

CAD was diagnosed based on examination of the results of coronary arteriography (> 50% stenosis of at least one major vessel), clinical symptoms, electrocardiogram, echocardiography, and treadmill exercise test. On the other

hand, the healthy controls had referred to Tabriz blood transfusion center for voluntary blood donation and none of them reported any past heart problems or family history of CAD. The individuals with the history of heart disease, lung disorder, liver dysfunction, renal disease, and cancer were excluded from the study.

### 3.2. Blood Sampling

Fasting venous blood samples (10 mL) were collected. The blood samples were centrifuged at 1500 g at room temperature for 10 minutes and then, the cells and sera were stored at -70°C until analysis.

### 3.3. Measurement of Biochemical Parameters

Serum level of serpin E1 was determined using Enzyme-Linked Immunosorbent Assay (ELISA) (eBioscience co. Ltd Cat. No. BMS2033). Besides, MDA serum level was measured based on reaction with Thiobarbituric Acid (TBA) and extraction with normal butanol using the method suggested by Buege and Aust (9). Absorption measurement was also made by a spectrophotometer at 535 nm and the Optical Density (OD) values were compared to the standard curve. Moreover, serum concentration of hs-CRP was measured by high-sensitivity turbidimetry method using Biosystems kit (Barcelona Spain COD 31927). The assay was analyzed on a semi-auto analyzer (Alcyon 300 made in USA). Lipid profile was also assessed through routine laboratory methods using an automated chemical analyzer. Finally, Low Density Lipoprotein (LDL)-cholesterol level was measured using Friedewald formula (10).

### 3.4. 4G/5G polymorphism of PAI-1 Gene

Genomic DNA was extracted from peripheral blood leukocytes with DNG-Plus (Sinaclon, Iran). Then, the common guanosine insertion/deletion polymorphism at position -675 of the PAI-1 gene was determined by Polymerase Chain Reaction (PCR) method. For amplification of the promoter region containing the 4G/5G polymorphism, a couple of primers were designed by Gene Runner software (version 3.05) as follows: 5' CACAGAGAGAGTCTGGCCACGT3' (forward) and 5' GGCCCAACAGAGGACTCTT3' (reverse). Each DNA amplification was performed in a total volume of 25 µL (24 µL PCR master mix plus, 1 µL of template DNA). PCR amplification was carried out in a thermal cycler with the following parameters: initial denaturation at 95°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 62°C for 60 seconds, extension at 72°C for 30 seconds, with a final extension at 72°C for 3 minutes. The PCR products were digested with *BesL1* (Thermosience) at 55°C overnight. The digestion products were analyzed by electrophoresis on 3% agarose gel in a tris-acetat-EDTA (TAE) buffer at 94 volts alongside 50 bp DNA ladder (Fermentas, Lithuania). The gel was finally stained with ethidium bromide and was visualized using the gel documentation system.

### 3.5. Statistical Analysis

Statistical analysis was performed using the SPSS statistical software, version 16. Continuous parameters

were compared using independent samples t-test, one-way ANOVA, and Mann-Whitney U test where appropriated. On the other hand, the categorical data were expressed as numbers and percentages and were compared using Chi-square test. Independent samples t-test was also used to compare the two groups regarding the study parameters. Finally, Tukey's post-hoc test was used to compare the three genotypes (4G/4G, 4G/5G, and 5G/5G) of PAI-1 gene concerning the study factors. P values less than 0.05 were considered to be statistically significant.

**4. Results**

The characteristics of the study subjects have been summarized in Table 1. As expected, the established cardiovascular risk factors (e.g., hypertension, diabetes, and smoking) were more prevalent in the CAD group than in the controls. A significant difference was also observed between the two groups with respect to the main factors, including PAI-1, hs-CRP, MDA, and blood sugar level. Lipid-profile analysis also showed a significant difference between the case and control groups concerning LDL,

cholesterol, and triglyceride concentrations. In contrast, the concentrations of High Density Lipoprotein (HDL) were lower in the CAD population than in the controls.

Distributions of genotypes and allelic frequencies of the polymorphisms of the PAI-1 promoter gene have been presented in Tables 2 and 3, respectively. Accordingly, the prevalence of the 4G/4G genotype was significantly higher in the case group compared to the controls. A significant difference was also found between the two groups regarding the frequency of the 4G allele.

In order to assess the relationship between PAI-I polymorphisms and CAD progression, linear regression analysis models were employed. The results of the analysis for the 4G allele (4G/4G, 4G/5G, and 5G/5G) revealed that the unadjusted Odds Ratio (OR) was 2.351 (95% CI: 3.12 - 35.31, P < 0.001) for 4G/4G and 0.69 (95% CI: 0.61 - 6.49, P = 0.249) for 4G/5G.

The associations between PAI-1 promoter gene polymorphisms and serpin E1 lipid profiles, MDA, and hs-CRP levels The plasma level of serpin E1 was significantly higher in the case group carrying the 4G allele compared

**Table 1.** General Characteristics and Biochemical Parameters of the Study Participants

Variable	Control(n = 44)	Case(n = 46)	P value
Age, Years	60.54 ± 5.29	62.00 ± 9.62	0.375 <sup>a</sup>
Sex	Male (n, %)	29, 65.9	33, 71.7
	Female (n, %)	15, 34.1	13, 28.3
DM	Positive (n, %)	4, 9.1	11, 23.9
	Negative (n, %)	40, 90.9	3, 76.10
Smoking	Positive (n, %)	10, 22.7	23, 51.1
	Negative (n, %)	34, 77.3	23, 49.9
Hypertension	Positive (n, %)	17, 38.6	31, 67.4
	Negative (n, %)	27, 61.4	15, 32.6
MDA	4.80 ± 0.78	6.10 ± 1.16	< 0.001 <sup>a</sup>
Hs-CRP	2.61 ± 1.64	5.33 ± 2.02	< 0.001 <sup>a</sup>
Cholesterol (mg/dL)	155.7 ± 25.7	195.2 ± 48.15	< 0.001 <sup>a</sup>
Triglyceride (mg/dL)	137.4 ± 43.44	198.4 ± 57.9	< 0.001 <sup>a</sup>
HDL (mg/dL)	36.75 ± 4.09	32.04 ± 3.76	< 0.001 <sup>a</sup>
LDL (mg/dL)	105.6 ± 40.7	129.3 ± 34.01	< 0.001 <sup>a</sup>
PAI-1 (ng/mL)	48.19 ± 12.32	84.34 ± 9.78	< 0.001 <sup>a</sup>
Blood sugar (mg/dL)	98.27 ± 31.71	140.1 ± 75.55	< 0.001 <sup>a</sup>

Abbreviations: DM, diabetic mellitus

\* P value based on ANOVA; \*\* P value based on chi-square test

**Table 2.** Comparison of the Control and Case Groups Regarding the Genotype and Allele Frequencies of PAI-1 Promoter Gene

Groups	Polymorphism			P value
	4G/4G	4G/5G	5G/5G	
Control	8	18	18	< 0.001 <sup>*</sup>
	18.18%	40.91%	40.91%	
Case	28	12	6	< 0.001 <sup>*</sup>
	60.87%	26.09%	13.04%	

\* P value based on chi-square test

**Table 3.** Allelic Frequencies of PAI-1 Promoter Gene

Allele	Allele Frequency (n = 180)
4G (n, %)	102 (57)
5G (n, %)	78 (43)

Abbreviations: DM, diabetic mellitus

\* P value based on ANOVA; \*\* P value based on chi-square test

to those homozygous for the 5G allele [4G/4G ( $87.85 \pm 9.21$ ) vs. 4G/5G ( $80.17 \pm 8.81$ ), ng/mL,  $P = 0.041$ ; 4G/4G ( $87.85 \pm 9.21$ ) vs. 5G/5G ( $76.33 \pm 6.65$ ), mg/dL,  $P = 0.016$ ]. Additionally, a significant difference was observed among the three genotypes in the CAD group with regard to plasma HDL levels [4G/4G ( $29.89 \pm 2.65$ ) vs. 4G/5G ( $35.50 \pm 2.77$ ), mg/dl,  $P < 0.001$ ; 4G/4G ( $29.89 \pm 2.65$ ) vs. 5G/5G ( $35.16 \pm 2.48$ ), mg/dL,  $P < 0.001$ ]. Also, significant differences were observed among the three genotypes in the control group regarding LDL and cholesterol levels [LDL: 4G/4G ( $132.1 \pm 31.9$ ) vs. 5G/5G ( $89.2 \pm 35.3$ ), mg/dL,  $P = 0.031$ ; cholesterol: 4G/4G ( $180.3 \pm 19.1$ ) vs. 5G/5G ( $147.6 \pm 23.1$ ), mg/dL,  $P = 0.006$ ]. However, no other significant differences were detected among the three PAI-1 genotypes with respect to the measured parameters.

## 5. Discussion

Studies have shown that CAD has a significant genetic basis in individuals under the age of 75 years (11). Indeed, several studies have made efforts to show the genetic factors involved in predisposition to CAD. Identification of these factors improves our knowledge about the pathophysiology of CAD, eventually playing a critical role in directing preventative and therapeutic efforts (10). Generally, a reduction in plasma fibrinolytic activity has been observed in patients with different stages of CAD and it has been suggested that genetic variability may be responsible for this phenomenon. Reduction of fibrinolytic potential is commonly related to increased plasma levels of serpin E1. Elevated serpin E1 concentrations in turn predict subsequent myocardial infarction instable angina pectoris and are associated with progressive CAD in young men with a history of myocardial infarction (12).

The present study findings indicated a remarkable association between 4G/5G promoter polymorphism of the PAI-1 gene and risk of CAD. Our findings and those of many meta-analyses have also demonstrated a direct connection between this polymorphism and severity of CAD (13, 14). The results of our study showed that PAI-1 was significantly elevated in the CAD patients in comparison to the controls and was significantly associated with severity of the disease.

Our study results also revealed that the plasma level of serpin E1 was significantly higher in the 4G/4G genotype compared to the 4G/5G and 5G/5G genotypes in the case group. This was consistent with the results of the studies conducted by T. Hokestra et al. (15) and JG Van Derbom et al. (16). In general, both alleles (4G/4G and 5G/5G) bind a transcriptional factor, but only 5G/5G binds a repressor protein (17). This implies that PAI-1 transcription is increased in the presence of 4G/4G polymorphism. In the same line, several researches have indicated that 4G/4G polymorphism of PAI-1 carried by many Caucasian and Asian populations affected the plasma levels of serpin E1, and high levels of serpin E1 were associated with several components of the atherothrombotic process, including endothelial cell, Smooth Muscle Cell (SMC), and platelets (18-20). PAI-1 reduced SMC content of the developing atherosclerotic plaque. Furthermore, production of collagen and other extracellular matrix proteins decreased in plaques. In this situation, plaques are prone to rupture and

this mechanism is a potential explanation for the increased risk of myocardial infarction and CAD associated with overexpression of serpin E1 (21).

Considering the lipid profile, PAI-1 was positively associated with cholesterol, triglyceride, and LDL, but negatively associated with HDL. Nonetheless, the molecular mechanism of this association is not clear. The 4G allele is known to be associated with a higher PAI-1 antigen level, which is in turn correlated to lipid profiles. This inverse association between PAI-1 promoter gene polymorphism and lipid profile has already been described by Chen et al. (22) and I Juhan-Vague et al. (23). This association is influenced by the genotype in the region of the PAI-1 promoter gene (24). In the present study, the subject with 4G/4G polymorphism in the CAD group had higher cholesterol and LDL levels and lower HDL levels compared to other genotypes. Moreover, serum cholesterol and LDL levels were higher in the control subjects with 4G/4G polymorphism, whereas lower HDL levels were observed in the case subjects with 4G/4G polymorphism. This may be related to consumption of lipid lowering drugs, such as gemfibrozil and statins, by the CAD subjects. Lipid lowering drugs may not only reduce cholesterol and triglyceride levels, but can also decrease serpin E1 plasma levels and PAI-1 mRNA expression (25). The PAI-1 mRNA concentration and secretion rate appears to be high in subcutaneous adipose tissue (26). Clinical studies have shown that significant weight loss reduced plasma serpin E1 levels in obese humans (27, 28). Generally, obesity is an important component of the Insulin Resistance Syndrome (IRS). Combined effect of insulin-dependent mechanism and adipose tissue might have a stronger influence on serpin E1 plasma levels (29). Furthermore, a large body of literature demonstrated that free radicals have been entangled in cardiac ischemic injury. These free radicals are produced in the body by reduction of oxygen. In normal conditions, they are removed by different antioxidative defense systems present in blood and tissues. In case of myocardial ischemia, which can lead to myocardial infarction, excessive free radicals may be generated (30), and measurement of MDA is a good marker of radical stress during reperfusion of the ischemic myocardium (31). To evaluate the oxidative stress status in the CAD patients in the current study, MDA level was assessed and the results showed significantly increased concentrations of MDA, as an index of lipid peroxidation, in the CAD patients compared to the controls, which was correlated to development of the disease. However, no significant association was found between PAI-1 gene polymorphism and serum level of MDA and risk of CAD. Evidence has indicated that inflammation played an important role in development of CAD. Previous studies also suggested that inflammation was not only an important trigger mechanism of coronary syndrome related to plaque rupture, but also participated in atherosclerosis promotion directly and actively (32, 33). Moreover, researches have disclosed that hs-CRP was a sensitive systemic marker of inflammation and a potent predictive marker of future cardiovascular risk (31, 34). In our study, a significant difference was observed between the patients and the controls regarding the value of

hs-CRP. Besides, there was an association between hs-CRP and CAD. hs-CRP was also correlated to lipids profile. This finding suggested a relationship between hs-CRP and LDL-cholesterol level and metabolic syndrome. Many studies have shown that change in the concentration of plasma lipids in CAD patients definitely contributed to development of vascular disease (7, 31, 35). In the present study, however, no significant association was found between PAI-1 gene polymorphism, and serum level of hs-CRP and risk of CAD.

### 5.1. Study Limitations

One of the main limitations of the present study was its small sample size. Additionally, other factors might have affected the development of cardiovascular disease. Thus, further studies with larger sample sizes are recommended to be conducted to evaluate the effect of this polymorphism on other risk factors of cardiovascular disease, such as plasminogen and oxidase-LDL.

### 5.2. Conclusion

Our findings suggested that the 4G/5G polymorphism of the PAI-1 gene was a useful marker of fibrinolytic activity, which might contribute to defective fibrinolytic activity due to elevated serpin E1 plasma level and, consequently, increase the risk of CAD. The results also demonstrated that this genetic variation might be important in association with some other factors, such as increased or decreased triglyceride, cholesterol, LDL, and HDL levels. However, no significant correlation was observed between the serum levels of stress oxidative factors, such as MDA and hs-CRP, and PAI-1 4G/5G gene polymorphism. The precise mechanisms of the inter-relationship between oxidative stress and fibrinolysis is not known yet and requires future in-depth studies.

**Conflicts of interest:** None declared.

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### Authors' Contribution

Ansar Karimian and Fatemeh Khaki Khatibi developed the original idea and the protocol, Anrsar Karimian wrote the manuscript, Safar Farajnia and Ansar Karimian contributed to development of the PCR-RFLP protocol, and Morteza Ghojzadeh did the final analysis of the data.

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We have no financial interests related to the material in the manuscript.

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