

**Original article:**

**LACK OF ASSOCIATION BETWEEN SER<sup>413</sup>/CYS POLYMORPHISM OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 2 (PAI-2) AND PREMATURE CORONARY ATHEROSCLEROTIC DISEASE**

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**ABSTRACT**

Plasminogen activator inhibitor type-2 (PAI-2) is a serine protease inhibitor of the fibrinolytic system produced predominantly by the macrophages and monocytes. It has been demonstrated that fibrinolysis regulation has a great importance in the pathogenesis of atherosclerotic plaques. Thus in the current investigation, we sought to determine whether Ser<sup>413</sup>/Cys polymorphism (rs6104) of PAI-2 gene could be associated with atherosclerosis and cardiovascular risk factors. Ser<sup>413</sup>/Cys polymorphism was determined by PCR-RFLP technique using *Mwo I* restriction enzyme for 184 men under 50 years of age and 216 women less than 55 years of age who underwent diagnostic coronary angiography. Data on the history of familial myocardial infarction or other heart diseases, hypertension, and smoking habit were collected by a simple questionnaire. Fasting levels of blood sugar, triglycerides, total cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol levels were also measured by enzymatic methods. Frequencies of the Ser<sup>413</sup> and Cys<sup>413</sup> alleles were 0.760 and 0.240 in the whole population, respectively. The PAI-2 gene variant analyzed was not significantly associated with either the prevalence of premature CAD or the classical risk factors of CAD development such as diabetes, serum cholesterol, triglycerides, low-density lipoprotein and high-density lipoprotein cholesterol, body mass index, hypertension, familial history of heart dysfunction or smoking.

**Keywords:** PAI-2, atherosclerosis, premature CAD, polymorphism

**INTRODUCTION**

Atherosclerotic coronary artery disease is a complex trait that accounts for the leading cause of premature death universally. There are some well recognized lifestyle risk factors which in combination with genetic factors play a key role in the pathogenesis of CAD, especially early-onset form of the disease known as premature

coronary artery disease. Meanwhile, fibrinolytic cascade and coagulation system involvements in atherosclerosis and its cardiovascular complications have been demonstrated by several studies (Ballantyne and Abe, 1997; Grant, 1997; Cambien, 1999; Juhan-Vague et al., 1999; Endler and Mannhalter, 2003; Nicholl et al., 2006; Ajjan and Ariens, 2009). The fibrinolytic cascade by generation of plasmin from pro-

teolysis of its zymogen helps to maintain the vascular endothelial homeostasis. This conversion is mainly catalyzed by Plasminogen activators (PAs), including urokinase (uPA) and tissue-type (tPA). Two specific serine protease inhibitors, plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2) have inhibitory effects on PAs and thus possess anti-fibrinolytic properties (Keeton et al., 1993).

PAI-2 belongs to the serine protease inhibitor (serpin) superfamily and is the major fibrinolytic inhibitor produced by the monocytes and macrophages while its presence has also been detected in the smooth muscle and vascular endothelial cells (Palafox-Sanchez et al., 2009). Human PAI-2 is a single chain protein consisting of 415 amino acids encoded by a 1900 bp transcript (Medcalf and Stasinopoulos, 2005). The PAI-2 gene is located on chromosome 18q21.3 and three polymorphisms have been identified on its coding region so far including Asn120/Asp, Asn404/Lys and Ser<sup>413</sup>/Cys (Kruithof et al., 1995). PAI-2 naturally has reversible conformational plasticity and can exist in both monomeric form and polymeric configuration as well; the latter being stabilized by disulfide bonds (Medcalf and Stasinopoulos, 2005). It seems that the additional Cys residue resulting from Ser<sup>413</sup>/Cys polymorphism (rs6104) somehow contributes to the formation of large molecular PAI-2 complexes (Mikus et al., 1993; Kruithof et al., 1995; Buyru et al., 2003; Medcalf and Stasinopoulos, 2005). Nevertheless, functional and conformational outcomes of this substitution have not been resolved well. But, since the reduction of the fibrinolytic activity has been proposed to be effective in atherosclerotic plaque formation (Cesari and Rossi, 1999) and PAI-2 has an important regulatory role in protective mechanisms against thrombosis, there might be some kind of association between this polymorphism and arterial occlusion. Hence in the present study, there was an attempt to determine if Ser<sup>413</sup>/Cys PAI-2 polymorphism has any important association with premature CAD in Southern Iran.

## MATERIALS AND METHODS

### *Study population*

The study population comprised 200 patients (92 females and 108 males) and 200 control subjects (124 females and 76 males) who underwent coronary angiography because of either symptoms of suspected premature CAD or unrelated conditions such as valvular heart disease or cardiomyopathy. The studied women (aged 32-55; mean  $49.26 \pm 6.32$ ) and men (aged 21-50; mean  $47.80 \pm 7.09$ ) were all under 55 and 50 years of age respectively. People were designated as premature CAD patients if they had  $\geq 50\%$  stenosis in at least one coronary artery while the control subjects were those who had  $< 30\%$  stenosis in all their major vessels. All the premature CAD patients (aged 28-55; mean  $49.48 \pm 7.02$ ) and control subjects (aged 21-55; mean  $50.15 \pm 6.67$ ) were from the same geographical region (Southern Iran) and had a similar socioeconomic background. All the cases were informed on the aims of the study and all the participants provided an informed consent. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences. Information on cardiovascular risk factors including hypertension (those with systolic/diastolic blood pressures higher than 140/90 mmHg or subjects who were using antihypertensive medications), smoking habits (cigarette and/or hookah), and family history of heart disease (incidence of coronary artery disease, myocardial infarction (MI) and aortocoronary bypass surgery in at least one of their first or second degree relatives) were also collected by a standardized questionnaire. These data were registered as a two level variables. The subjects who smoked at least one cigarette or hookah per day or were ex-smokers were considered as smokers while non-smokers were defined as those who had never smoked. Height and weights of the subjects were measured in light clothing, and body mass index (BMI) was calculated as mass in kilograms divided by standing height in meters squared.

### Biochemical measurements

Venous blood samples for biochemical analyses (Fasting blood sugar (FBS), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglyceride (TG)) were drawn from each subject after a twelve-hour fasting. Standard enzymatic assay (Pars Azmoon, diagnostic kits, Iran) was used to measure the biochemical parameters.

### DNA isolation and polymorphism analysis

Genomic DNA was extracted from leukocyte nuclei by salting out method (Miller et al., 1988). The analysis of the Ser<sup>413</sup>/Cys polymorphism in the PAI-2 gene was performed by PCR-RFLP method. The region surrounding the supposed mutation site (a 123 bp fragment) was obtained using the following primers: forward: 5'-ACA GTT TGT GGC AGA TCA TCC-3'; reverse: 5'-AAA AAT CTT TTG CAG AAG CAG C-3'. PCR amplification was carried out in a final volume of 20 µl containing 100 ng total DNA, 1.5 mM MgCl<sub>2</sub>, 1X PCR buffer, 10 pmol each primers, 2.5 mM of each dNTP and 1 Units Taq DNA polymerase. The reaction conditions were as follows: initial denaturation at 94 °C for 3 minutes and 40 subsequent cycles of amplification at 94 °C for 40 S, 60 °C for 40 S, and 72 °C for 40 S. Finally a 5 min step at 72 °C was used for ending the extension. The PCR products were digested with *MwoI* restriction endonuclease (Fermentas) by overnight incubation at 37 °C and then were size-separated by gel electrophoresis using 4 % (w/v) agarose. Ser<sup>413</sup>/Ser allele was identified as 108 and 15 bp fragments while the Cys<sup>413</sup>/Cys genotype was characterized by 78, 30 and 15 bp fragments.

### Statistical analysis

Statistical analyses were performed by SPSS (Statistical Package for Social Sciences) software, ver. 17.0 (SPSS Inc, Chicago, USA). Two-sided P values less than 0.05 were considered statistically significant for all the analyses. Pearson's Chi-square test was used for Hardy-Weinberg equilibrium assessments and also for the comparison of qualitative variables. Meanwhile two-tailed Student's t-test and analysis of variance (ANOVA) were used to compare quantitative data. The normality of distribution of the investigated variables was assessed using the Kolmogorov-Smirnov criterion and, where skewed (FBS and TG both in the whole population and in each of the two case/control sub-groups and Age, TC, HDL and LDL only in the case/control sub-groups), logarithmically transformed to reduce kurtosis.

## RESULTS

A total of 413 participants were included in this study, among which 400 individuals were genotyped successfully. The genotype frequencies of Ser<sup>413</sup>/Ser, Ser<sup>413</sup>/Cys, and Cys<sup>413</sup>/Cys were 58.75 %, 34.75 %, and 6.5 %, respectively in the whole population. The distribution of PAI-2 genotypes was found to occur in Hardy-Weinberg proportion (P=0.3788) (Table 1).

Allelic frequency distribution of Ser<sup>413</sup>/Cys polymorphism in male/female sub-groups indicated that the Ser<sup>413</sup> allele was the most prevalent allele type in patients (0.75) and controls (0.77). The patient and control groups were not significantly different with each other regarding the allelic and genotypic frequencies (P>0.05). The distribution of genotypes between genders was not statistically significant as well (Table 2).

**Table 1:** Hardy-Weinberg equilibrium test of PAI-2 Ser<sup>413</sup>/Cys polymorphism

Genotype	Observed Count	Expected Count	Genotype Frequency	Allele	Observed Count	Allele Frequency	X <sup>2</sup>	P
Ser <sup>413</sup> /Ser	235	231.8	0.5875	Ser <sup>413</sup>	609	0.760	0.775	0.3788
Ser <sup>413</sup> /Cys	139	145.4	0.3475					
Cys <sup>413</sup> /Cys	26	22.8	0.0650	Cys <sup>413</sup>	191	0.240		
Total	400				800			

**Table 2:** Distribution of the PAI-2 genotypes among case-control and male-female subjects

Genotype	Patients, N (%)	Control, N (%)	X <sup>2</sup>	P	Male, N (%)	Female, N (%)	X <sup>2</sup>	P
Ser <sup>413</sup> /Ser	113 (56.5)	122 (61.0)	1.369	0.504	112 (60.9)	123 (56.9)	0.654	0.721
Ser <sup>413</sup> /Cys	75 (37.5)	64 (32.0)			61 (32.1)	78 (36.1)		
Cys <sup>413</sup> /Cys	12 (6.0)	14 (7.0)			11 (6.0)	15 (6.9)		
Total	200 (100)	200 (100)			184 (100)	216 (100)		

The characteristics of the study population are presented at Table 3. As expected, most of the conventional risk factors had a significantly higher frequency or distribution in the premature CAD patients compared with the control group. The two groups were matched for age. Table 4 shows the distributions of the studied risk factors between various genotypes. As the P

values indicate, none of these variables had a significant association with PAI-2 Ser<sup>413</sup>/Cys polymorphism.

Table 5 and Table 6 summarize the biological parameters with different PAI-2 genotypes in patient and control groups respectively. Distributions of these variables were almost similar to those observed in the whole population group (Table 4).

**Table 3:** Conventional risk factors in premature CAD patients and control subjects

	Patients	Controls	P Value
Age [Yr], mean (SD)	49.48 (7.02)	50.15 (6.67)	0.258
Weight [Kg], mean (SD)	70.46 (10.96)	71.52 (12.24)	0.405
Hypertension N (%)	83 (41.50)	59 (29.50)	0.016
Smoker N (%)	103 (51.50)	75 (37.50)	0.007
Familial History N (%)	46 (23.00)	37 (18.50)	0.324
BMI [Kg/m <sup>2</sup> ], mean (SD)	25.28 (3.40)	25.71 (4.33)	0.311
log <sub>10</sub> FBS [mg/dl], mean (SD)	2.05 (0.17)	2.02 (0.17)	0.022
log <sub>10</sub> TG [mg/dl], mean (SD)	2.15 (0.27)	2.08 (0.27)	0.019
TC [mg/dl], mean (SD)	167.67 (38.23)	158.01 (42.27)	0.032
HDL [mg/dl], mean (SD)	35.35 (9.52)	37.27 (10.32)	0.076
LDL [mg/dl], mean (SD)	100.10 (28.04)	91.75 (32.12)	0.016

Abbreviation: FBS = Fasting blood sugar, HDL = High density lipoprotein, LDL = Low density lipoprotein, mg/dl = Milligram per Deciliter, SD = Standard deviation, TC = Total cholesterol, TG = Triglyceride

**Table 4:** Risk factor profile of the studied population according to the PAI-2 different genotypes

	Ser <sup>413</sup> /Ser	Ser <sup>413</sup> /Cys	Cys <sup>413</sup> /Cys	P value
Age [Yr], mean (SD)	49.11 (6.22)	50.01 (6.31)	50.23 (6.83)	0.460
Weight [Kg], mean (SD)	70.80 (11.07)	70.97 (12.25)	72.09 (12.34)	0.884
Hypertension N (%)	91 (38.72)	44 (31.65)	7 (26.92)	0.247
Smoker N (%)	107 (45.53)	55 (39.57)	16 (61.54)	0.104
Familial History N (%)	45 (19.15)	30 (21.58)	8 (30.77)	0.366
BMI [Kg/m <sup>2</sup> ], mean (SD)	25.38 (4.00)	25.41 (3.72)	26.26 (3.21)	0.593
log <sub>10</sub> FBS [mg/dl], mean (SD)	2.04 (0.17)	2.02 (0.16)	2.10 (0.19)	0.155
log <sub>10</sub> TG [mg/dl], mean (SD)	2.08 (0.27)	2.11 (0.26)	2.17 (0.33)	0.212
TC [mg/dl], mean (SD)	158.26 (45.52)	156.31 (46.02)	160.32 (48.06)	0.783
HDL [mg/dl], mean (SD)	36.30 (10.50)	35.79 (9.20)	37.90 (8.34)	0.647
LDL [mg/dl], mean (SD)	95.03 (33.77)	90.54 (31.40)	107.27 (36.5)	0.090

Abbreviation: FBS = Fasting blood sugar, HDL = High density lipoprotein, LDL = Low density lipoprotein, mg/dl = Milligram per Deciliter, SD = Standard deviation, TC = Total cholesterol, TG = Triglyceride

As shown in Table 5, there was no significant difference among premature CAD patients with different genotypes for any of the investigated variables except a higher prevalence of hypertensives in the wild type homozygotes (Ser<sup>413</sup>/Ser). Different genotypes of the control group were also statisti-

cally similar for the listed factors. Here, the only exception was the number of smokers which showed a significant difference between the mutant homozygotes (Cys<sup>413</sup>/Cys) and the heterozygous group (Ser<sup>413</sup>/Cys) (Table 6).

**Table 5:** Risk factor profile of the premature CAD patients according to the PAI-2 different genotypes

	Ser <sup>413</sup> /Ser (N=113)	Ser <sup>413</sup> /Cys (N=75)	Cys <sup>413</sup> /Cys (N=12)	P value
log <sub>10</sub> Age [Yr], mean (SD)	1.69 (0.82)	1.70 (0.78)	1.69 (0.91)	0.843
Weight [Kg], mean (SD)	69.97 (10.01)	72.75 (12.19)	70.00 (11.20)	0.424
Hypertension N (%)	56 (49.56)*	25 (33.33)	2 (16.67)	0.017
Smoker N (%)	58 (51.33)	38 (50.67)	7 (58.33)	0.884
Familial History N (%)	23 (20.35)	19 (25.33)	4 (33.33)	0.496
BMI [Kg/m <sup>2</sup> ], mean (SD)	25.04 (2.99)	25.55 (4.00)	25.41 (3.05)	0.627
log <sub>10</sub> FBS [mg/dl], mean (SD)	2.04 (0.18)	2.04 (0.13)	2.09 (0.19)	0.212
log <sub>10</sub> TG [mg/dl], mean (SD)	2.12 (0.30)	2.13 (0.25)	2.17 (0.30)	0.757
log <sub>10</sub> TC [mg/dl], mean (SD)	2.22 (0.12)	2.21 (0.11)	2.23 (0.09)	0.918
log <sub>10</sub> HDL [mg/dl], mean (SD)	1.55 (0.12)	1.52 (0.10)	1.56 (0.11)	0.371
log <sub>10</sub> LDL [mg/dl], mean (SD)	2.00 (0.17)	1.97 (0.15)	2.03 (0.22)	0.458

Abbreviation: FBS = Fasting blood sugar, HDL = High density lipoprotein, LDL = Low density lipoprotein, mg/dl = Milligram per Deciliter, SD = Standard deviation, TC = Total cholesterol, TG = Triglyceride

\* Significantly different from Ser<sup>413</sup>/Cys and Cys<sup>413</sup>/Cys by Pearson's chi-square

**Table 6:** Risk factor profile of the control subjects according to the PAI-2 different genotypes

	Ser <sup>413</sup> /Ser (N=122)	Ser <sup>413</sup> /Cys (N=64)	Cys <sup>413</sup> /Cys (N=14)	P value
log <sub>10</sub> Age [Yr], mean (SD)	1.69 (0.81)	1.70 (0.84)	1.71 (0.85)	0.337
Weight [Kg], mean (SD)	71.71 (12.13)	68.88 (12.39)	73.18 (11.72)	0.107
Hypertension N (%)	35 (28.69)	19 (29.69)	5 (35.71)	0.861
Smoker N (%)	49 (40.16)	17 (26.56)	9 (64.29)*	0.019
Familial History N (%)	22 (18.03)	11 (17.19)	4 (28.57)	0.597
BMI [Kg/m <sup>2</sup> ], mean (SD)	25.49 (4.86)	25.21 (3.32)	27.11 (3.27)	0.408
log <sub>10</sub> FBS [mg/dl], mean (SD)	2.03 (0.16)	2.00 (0.18)	2.09 (0.13)	0.073
log <sub>10</sub> TG [mg/dl], mean (SD)	2.03 (0.25)	2.07 (0.28)	2.15 (0.36)	0.069
log <sub>10</sub> TC [mg/dl], mean (SD)	2.20 (0.14)	2.19 (0.12)	2.18 (0.13)	0.538
log <sub>10</sub> HDL [mg/dl], mean (SD)	1.56 (0.13)	1.56 (0.11)	1.58 (0.08)	0.628
log <sub>10</sub> LDL [mg/dl], mean (SD)	1.96 (0.18)	1.93 (0.17)	2.01 (0.14)	0.059

Abbreviation: FBS = Fasting blood sugar, HDL = High density lipoprotein, LDL = Low density lipoprotein, mg/dl = Milligram per Deciliter, SD = Standard deviation, TC = Total cholesterol, TG = Triglyceride

\* Significantly different from Ser<sup>413</sup>/Cys by Pearson's chi-square

## DISCUSSION

Coronary atherosclerosis is a complex disease, in which environmental parameters, genetic factors, and interactions among them are all effective in its pathogenesis. Meanwhile, epidemiologic studies have revealed the overwhelming importance of hereditary predisposition in a way that at least 50 % of susceptibility to CAD is estimated to be hereditary (Chan and Boerwinkle, 1994; Roberts, 2008). Since obstructive coronary artery disease is caused by the deposition of atherosclerotic plaque and superimposition of thrombosis on the coronary artery wall, understanding the role of the involved genes in coagulation cascade and fibrinolytic system has a major importance in this field (Boekholdt et al., 2001). Fibrinolysis regulation is dependent mainly on a balance between plasminogen activators like u-PA and t-PA and their inhibitors such as PAI-1 and PAI-2. Up to now, multiple experimental studies have suggested a pathogenetic role for PAI-1 in the development of various complications of coronary heart disease (Carmeliet et al., 1993; Dawson et al., 1993; Jansson et al., 1993; Biemond et al., 1995; Mansfield et al., 1995; Ye et al., 1995; Fay et al., 1997; Devaraj et al., 2003) although this relationship has not been confirmed by some other researches (Dawson et al., 1993; Ye et al., 1995; Ridker et al., 1997; Hubacek et al., 2010). To the best of our knowledge, there were only two studies which have performed an association study between PAI-2 Ser<sup>413</sup>/Cys polymorphism and coronary artery atheroma and MI in the literature. Foy and Grant (1997) assessed the association between PAI-2 variants and the extent of coronary atheroma in 333 Caucasian MI patients compared with 286 healthy volunteers, but no significant association has been reported by them. Nevertheless, in the other report Buyru and colleagues (2003) have found a statistically significant association between Ser<sup>413</sup>/Ser genotype and MI in a case-control study consisting of 45 MI patients and 20 healthy subjects. Moreover, McCarthy et al. (2004) conducted a high-

throughput SNP association study to identify the target genes associated with premature coronary artery disease. They screened 210 different polymorphisms among 110 candidate genes from 418 premature CAD patients and found significant associations only with SNPs in thrombospondin-2 (THBS2), thrombospondin-4 (THBS4) and plasminogen activator inhibitor-2.

The present study was conducted on 400 Caucasian participants from Southern Iran but the results obtained here failed to demonstrate an association between rs6104 SNP of the PAI-2 gene and the premature CAD. The discrepancy observed between the result of the current investigation and that of the Buyru et al.'s (2003) study might have partly resulted from the differences existed between the designs of these two studies. Unlike the studies of Foy and Grant (1997) and Buyru et al. (2003) which had a comparison group instead of a true control group, our study possessed objective angiographic information for not only CAD patients but also for control subjects. Lack of objective angiographic information may cause to observe an unreal association between the mutation and the disease (Girelli et al., 1998). Additionally, Buyru and colleagues (2003) included only MI survivors as the patient group. Limiting the patient group to such a phenotype is too selective and rules out those who have not survived from MI. This could contribute to underrepresentation of the mutation effect (Girelli et al., 1998). To reduce the probability of spurious results, a control group with angiographically documented absence of premature CAD is provided in our study. Finally Buyru et al. (2003) found a significant association between MI and PAI-2 polymorphism by analyzing a quite low sample size (45 patients and 20 controls). The total number of subjects studied here (400 patients and controls) had the same problem statistically but in a lesser extent. Thus, a larger sample population should be examined to confirm the relationship between PAI-2 polymorphisms and the premature CAD. However, variations in the genetic backgrounds of the investigated popu-

lations may also account for this inconsistency.

In addition to the low sample size, the present study had other limitations. For example, the effects of genetic variants of PAI-2 on protein function and its circulating level or production have not been investigated. Moreover this is a retrospective case-control study which assessed only one polymorphism of PAI-2. Investigations of other SNPs by prospective studies are needed to evaluate more accurately the role of PAI-2 polymorphisms in the pathogenesis of premature coronary artery disease.

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