The Role of 4G/5G Genetic Polymorphism of Plasminogen Activator Inhibitor-1 Gene in Myocardial Infarction among Egyptians

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Abstract: Objectives: To assess weather the common polymorphic allele (4G) of plasminogen activator inhibitor-1 (PAI-1) gene is associated with myocardial infarction (MI) and with PAI-1 enzyme level in Egyptian patients. Methods: Fifty consecutive patients who presented with acute MI and 48 normal control subjects were included. Clinical features were examined, PAI-1 4G/5G gene polymorphism was detected using polymerase chain reaction and PAI-1 levels with other risk factors were determined in all subjects. Results: Patient age averaged 51 (±SD 10.4) years, 68% were men and 46% had a family history of MI. Overall frequency of 4G allele was 60.4% among patients versus 51.0% among normal controls. There was no significant difference in genotype distribution (4G/4G, 4G/5G and 5G/5G), (P=0.34) and allele frequency (P=0.191) between patients with myocardial infarction and controls. Neither carriage of 4G allele (OR=1.46; 95% CI: 0.83-2.59; p=0.191) nor 4G/4G homozygosity (OR= 1.73; 95% CI: 0.684-4.36; p=0.245) was associated with MI. There was a significant increase of PAI-1enzyme level (p<0.001) among patients than the control group and the plasma levels of the enzyme were highest in myocardial infarction patients who were homozygous for the 4G allele (4.3±3.5) with a stepwise decrease in levels as the number of 4G alleles decreased; (3.6±2.3) for 4G/5G and (2.6±1.8) for 5G/5G; however the difference was not statistically significant (F=0.82, P=0.45); and even the increased mean level of PAI-1enzyme in patients with 4G/4G genotype than in patients with 5G/5G genotype; was not statistically significant (P=0.568). The study revealed also increased frequency of smoking (P= 0.001), family history of myocardial infarction (P=0.03) and hypertension (P = 0.016) among patients than controls. In multivariate analyses, risk factors associated with MI were smoking; hypertension and high level of PAI-1 enzyme. Conclusion: The common PAI-1 polymorphism (4G) was not associated with MI in Egyptian population, however modest risk (i.e., OR, =1.46) could not be excluded. Increased level of PAI-1enzyme, smoking and hypertension are significant risk factors for myocardial infarction among Egyptians.
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1. Introduction

Plasminogen activator inhibitor-1 (PAI-1) plays a central role in modulating intravascular thrombosis and thrombolysis. It may contrast plaque growth but also promote thrombosis and plaque vulnerability provoking acute myocardial infarction (MI). It serves as the main physiological inhibitor of endogenous fibrinolytic activity by inhibiting both tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Kohler et al, 2000 and Agirbasli, 2005). Impaired fibrinolysis due to high PAI-1 activity has been shown to be associated with an increased risk of thrombotic events (Smith, 1998). PAI-1 over-expression may also promote development of weak plaques with thin fibrous caps by inhibiting both u-PA receptor and integrin-mediated cell adhesion and migration (Cortellaro et al, 1993). Therefore, over-expression of PAI-1 may play a critical role in the development of MI, by impairing both thrombolysis and plaque stability (Hamsten et al, 1985). Since PAI-1 has also an inhibitory effect on the smooth muscle cell (SMC) migration and neointima formation it is possible that low PAI-1 levels may promote development of high grade coronary stenosis. On the other hand, by inhibiting cellular migration and neointima formation PAI-1 over-expression may have a protective effect against plaque growth (Collet et al, 2003).

High triglyceride levels were possibly connected with a predisposition to thrombosis through a coexisting high level of plasminogen activator inhibitor enzyme. Boncoraglio and his colleagues (2006), found that the 4G/4G genotype of PAI-1 was significantly associated with high cholesterol, but not with triglycerides. Chronic kidney disease (KCD) was also strongly associated with an increased risk of MI in the general population. The underlying mechanism behind this relationship is unclear but seems to be

independent from other common risk factors (Meisinger et al, 2006).

PAI-1 gene is located on chromosome 7 and contains eight introns and nine exons (Deng et al, 2001). A single guanosine insertion/deletion (4G/5G) polymorphism in the promoter region of PAI-1 gene at position –675 bp, may play an important role in the regulation of PAI-1 expression (Sobel, 1999). Moreover, morning increase in PAI-1 activity may be determined by 4G/4G genotype (Schneider et al., 2004). PAI-1 4G/5G polymorphism was associated with an increased (Libby et al, 2005; Onalan et al, 2008 and Isordia-Salas et al., 2009) or decreased risk of MI (Junker et al., 1998) in some studies, while no significant association was found in others (Sugano et al., 1998; Doggen et al., 1999 and Crainich et al., 2003).

The purpose of this study was to investigate the association between the deletion allele of the PAI-1 gene (4G) with myocardial infarction as well as with the plasma levels of PAI-1 enzyme among Egyptian patients.

2. Patients and Methods:

This case control study included 50 consecutive Egyptian patients (34 males and 16 females); their ages ranged from 33 to 80 years old having myocardial infarction admitted to the cardiovascular intensive care unit in Kasr El Eny Hospital. Patients had no diabetes mellitus or obesity and 48 unrelated healthy, age and sex matched volunteers (control group); their exclusion criteria were: diabetes mellitus, obesity, signs of ischemic changes on electrocardiogram and ischemic changes during maximal stress exercise test. Clinical information was obtained by history, physical examination and routine laboratory analyses. Participants were genotyped for 4G/5G polymorphism using the polymerase chain reaction analysis, and their plasma PAI-1 enzyme levels were measured. Informed consent was obtained from all participants after a clear explanation of potential risk of the study.

Blood sampling

Specimen Collection:

Venous blood samples (10ml) were withdrawn from each subject and divided into three parts:

- Two ml was collected in a tube containing ethylenediamine tetraacetate (EDTA) as an anticoagulant and submitted to genomic DNA extraction from peripheral blood leucocytes using QIA amp DNA mini kit (QIAGEN, Inc., Germany).
- Six ml was collected in a clean dry centrifuge tube. Blood was allowed to clot at 37 ° C water bath. Clot was separated and centrifuged for 10 minutes at 3000 g. Serum was divided into aliquots and stored at – 20ºC until analyzed. Samples were assayed for measurements of cholesterol, triglycerides, urea and creatinine.
- The rest of the blood sample was used for detection of serum levels of PAI-1 enzyme.

Methods

Molecular detection of 4G/5G polymorphism genotyping

To identify the plasminogen activator inhibitor-1 (PAI-1) genotypes, polymerase chain reaction amplification of promoter region containing the 4G/5G polymorphism was done. The PCR reaction used an upstream control primer (5`- AAG CTT TTA CCA TGG TAA CCC CTG GT- 3`), an allele specific primer 4G (5`-GTC TGG ACA CGT GGG GA-3`) or 5G (5`-GTC TGG ACA CGT GGG GG-3`) and a common downstream primer (5`-TGC AGC CAG CCA CGT GAT TGT CTA-3`).

Two PCR reactions were run per a sample (one for 4G allele and the other for 5G allele); i.e. each reaction contained (upstream primer, downstream primer and one primer for 4G or 5G).

The 4G allele-specific PCR reaction mixture with a total volume of 25µl contained 50-100 ng DNA, 1unit of Taq polymerase, 1x PCR buffer Taq, 0.8mmol/l $MgCl_2$, 50 µmol/L dNTPs, 200 nmol/L upstream primer and 400 nmol/L allele specific and downstream primers. The 5G allele-specific reaction was identical except $MgCl_2$ which was 1.1 mmol/l. The 4G allele thermal cycling conditions were 32 cycles of denaturation at 94◦C for 35s, annealing 65◦C for 35s and extension 72◦C for 70s. The 5G allele thermal cycling conditions were the same except that the number of cycles was 22 cycles and annealing at 58◦C. The PCR product was electrophoresed on 1.5% agarose gel stained with ethidium bromide. A 257bp control band resulted from the upstream and downstream primers. The 4G or5G allele specific primer and downstream primer generated 139bp fragment. Patients who showed amplification products with only 4G primers were 4G/4G, and with only 5G primers were 5G/5G and patients who had amplification products with both primers were 4G/5G heterozygote (Figures, 1and 2).

Biochemical analyses:

The serum levels of PAI-1 enzyme were measured by immunoenzymatic ELISA method using the Hyphen Bio-Med kit. The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for PAI-1 has been pre-coated onto a micro-plate. Standards and samples were pipetted into the wells and any PAI-1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-

linked polyclonal antibody specific for PAI-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of PAI-1 bound in the initial step (Declerck et al 1998).

-Serum total cholesterol was estimated according to Röschlau et al, 1974 and triglycerides according to Fossati and Prencipe, 1982. Urea and creatinine were measured using enzymatic methods (Faweet and Scott, 1960).

Statistical analysis:

The data were analyzed using the statistical package SPSS (version 15). They were expressed as mean± standard deviation for quantitative variables and number and percentage for qualitative values. Statistical differences between categorical data like gender, genotype distribution, smoking, family history of myocardial infarction and hypertension of patients and control groups were tested using Chi Square test. For qualitative variables, independent sample t test and ANOVA (analysis of variance) with post Hoc Bonferroni test were used for normally distributed variables, while Nonparametric Mann -Whitney test and kruskal-Wallis test were applied for variables which were not normally distributed (PAI-1 level, urea and creatinine). Associations were assessed as OR and 95% confidence intervals (CI). Logistic regression analysis was used to test for significant risk factors for myocardial infarction. Values less than or equal to 0.05 were considered statistically significant.

3. Results:

Patient age averaged 51 (±SD 10.4) years, 68% were men, and 46% had a family history of MI. There were no statistically significant differences between patients and controls as regards to age and gender, however there was a significant increased frequency of smoking (X^2=11.83, P=0.001), family history of myocardial infarction (X^2=4.7, P=0.03), hypertension (X^2=5.76, P=0.016) and increased PAI-1 level (Z=-4.42, p<0.001) among patients with myocardial infarction than controls as shown in table (1). Only 48 patients were genotyped (two of the cases had DNA amplification failure). There was no significant difference in genotype distribution (4G/4G, 4G/5G and 5G/5G) (X^2=2.16 and P=0.34) between the two groups and in spite of the 4G allele occurred more frequently than the 5G allele in the patient group (60.4% of patients vs. 51.0% of control) (OR=1.46; 95% CI: 0.83-2.59) the difference was not statistically significant (p=0.191). Regression analysis showed no significant differences between patients and controls as regard 4G4G vs. 4G5G+5G5G (OR 1.73, 95% CI: 0.684-4.361and p=0.245) and 4G4G+4G5G vs. 5G5G genotype (OR=1.99, 95% CI: 0.612-6.433 and p=0.247) (Table 2).

The plasma levels of plasminogen activator inhibitor-1 enzyme were the highest in myocardial infarction patients who were homozygous for the 4G allele (4.3±3.5) with a stepwise decrease in levels as the number of 4G alleles decreased (3.6±2.3) for 4G/5G and (2.6±1.8) for 5G/5G but the difference was not statistically significant (F=0.82, P=0.45). Cholesterol, triglycerides, urea and creatinine, showed no statistically significant difference in patients having the three different genotypes (p values were = 0.98, 0.98, 0.56 and 0.41, respectively) (Table 3) and even between patients which were homozygous to 4G/4G alleles and 5G/5G alleles, the only significant difference was detected in creatinine level (P=0.039). Logistic regression was done to test for significant predictors of MI**.** Male gender, age, smoking, family history, hypertension, PAI-1 level, 4G allele were involved in the regression model while only smoking, hypertension and PAI-1 level were found to be significant predictors (Table 4).

Table (1): General characteristics of patients and controls

Characteristic	Patients no= 50	Controls no= 48	Test of significance	p value
Gender Male, no (%) Female, no (%)	 34 (68%) 16 (32%)	 31 (64.6%) 17(35.4%)	X^2 = 0.128	0.721
Age (years) Range Mean ± SD	 (33-80) 51±10.4	 (35-79) 50.65±10	t= 0.162	0.871
Smoking	33(66%)	15 (31.3%)	X^2 = 11.83	0.001*
Family history of myocardial infarction	23 (46%)	12 (25%)	X^2 = 4.7	0.03*
Hypertension	23 (46%)	11(22.9%)	X^2 = 5.76	0.016*
PAI-1 level IU/mL	3.75±2.69	1.81 ± 0.45	Z= - 4.42	<0.001*

*p≤0.05 is significant.

Table (2): 4G 5G genotypic distribution and allelic frequencies; and their associations with myocardial infarction among Egyptians:

	Patients (n=48)	Control (n=48)	OR	95%CI	P value
Genotype, n (%)					
4G4G	15 (31.3%)	10 (20.8%)			0.34
4G5G	28 (58.3%)	29 (60.4%)			
5G5G	5 (10.4%)	9 (18.8%)			
4G4G vs. 4G5G+5G5G	15 (31.3%) 33(68.8%)	10 (20.8%) 38(79.2%)	1.73	0.684- 4.361	0.245
4G4G+4G5G vs.5G5G	43 (89.6%) 5 (10.4%)	39(81.3%) 9(18.8%)	1.99	0.612-6.433	0.247
Allelic frequency, n (%)					
4G	58 (60.4%)	49 (51.0%)	1.46	0.83-2.59	0.191
5G	38 (39.6%)	47 (49.0%)			

OR: odds ratio; CI: confidence interval.

Table (3): Characteristics of Egyptian patients with MI by 4G/5G polymorphism:

Parameter	4G/4G (n=15)	4G/5G (n=28)	5G/5G (n=5)	p value
Age	48.5±11.1	52±10.4	49.2±6.6	0.54
PAI-1 level	4.3±3.5	3.6±2.3	2.6±1.8	0.45
Cholesterol (mg/dl)	188±53.8	185.6±45.4	184±23	0.98
Triglycerides (mg /dl)	224.3±63.6	226.1±61.3	221±67.7	0.98
Urea (mg /dl)	44±9.1	45± 20.3	36± 14.8	0.56
Creatinine (mg/dl)	3± 2.1	2.2±3.2	1.2±0.5	0.41

Table (4): Multivariate logistic regression analysis for myocardial infarction:

	B	p-value	Exp(B)	95.0% C.I. for Exp(B)
Gender (Males)	0.479	0.360	1.615	0.578-4.509
Age	- 0.019	0.426	0.981	0.935-1.029
Smoking	-1.537	0.001*	4.650	1.878-11.516
Family history	0.705	0.105	2.025	0.863-4.751
Hypertension	1.822	<0.001*	6.182	2.436-15.866
PAI-1 level	1.292	<0.001*	3.641	2.177-6.088
4G allele	0.112	0.780	1.118	0.510-2.455

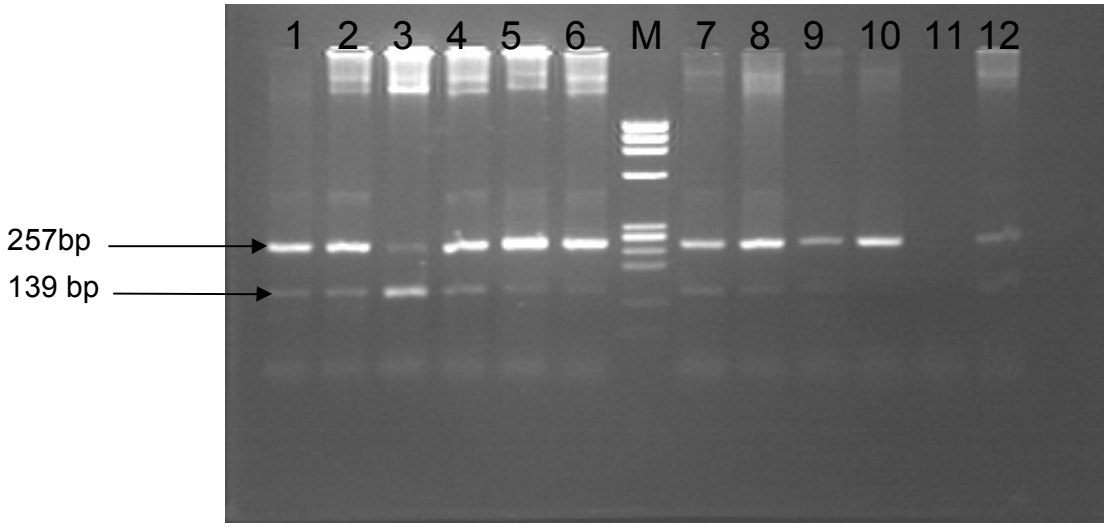

Fig (1): Example of polymorphic genotyping using 1.5% agarose gel electrophoresis of PCR amplification products. The 275 bp band corresponds to the control band and 139bp fragment to 4G band. Lane M is ØX174 HaeIII molecular weight marker. Lanes, 1, 2, 3, 4, 5, 6,7,8,9 and 12 showed 4G amplification bands. Lane 10 had no 4G band. Lane 11 showed neither band for control nor for amplified 4G (failure of DNA amplification).

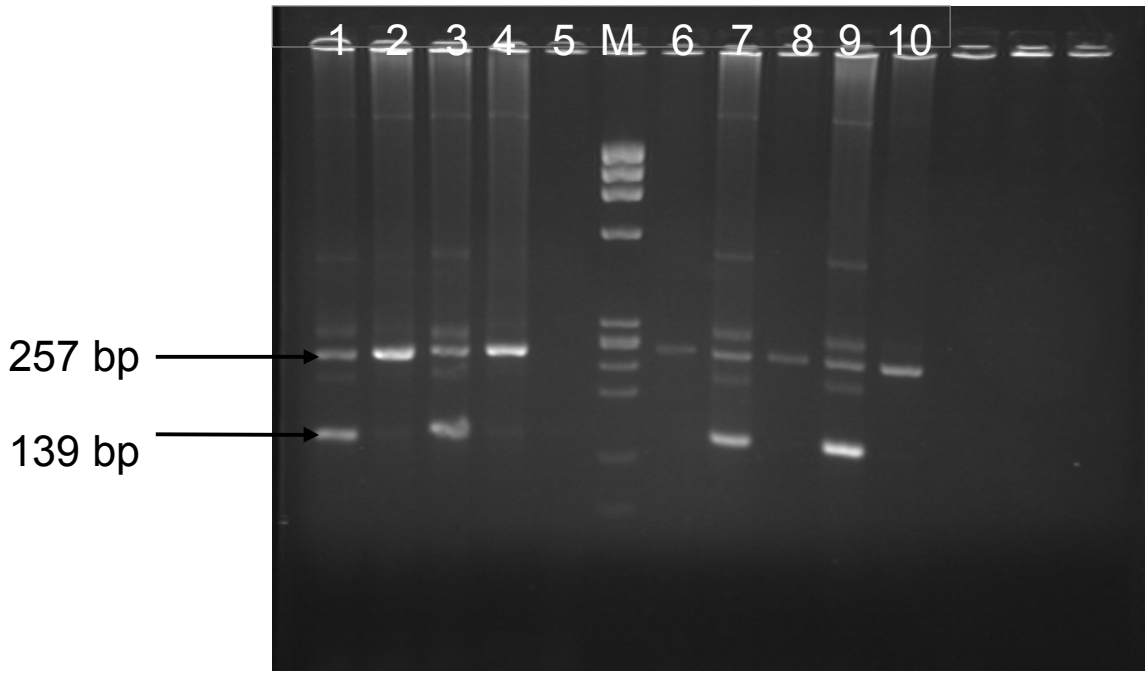

Fig (2): Example of polymorphic genotyping using 1.5% agarose gel electrophoresis of PCR amplification products. The 257 bp band corresponds to the control band and 139bp fragment to 5G band. Lane M is ØX174 HaeIII molecular weight marker. Lanes 1, 2, 3, 4, 7and 9 shows 5G amplification bands. Lane 5 shows neither band for control nor for amplified 5G (failure of DNA amplification). Lanes 6 and 8 had no 5G bands. Lane 10 shows 5G amplification band but faint in density.

4. Discussion

The etiology of cardiovascular disease is multifactorial and strongly involves genetic and environmental factors. PAI-1 is a noteworthy factor in the plasminogen activation/plasmin cascade and its level is usually related to cardiovascular disease. Evaluation of inter-individual variation of the PAI-1 level is important for the assessment of an individual's risk for thrombotic disorders (Mei et al, 2003).

Both the 4G and 5G alleles in the promoter region of PAI-1 gene; has a binding site of a common transcription activator. However the 5G allele has an additional binding site for a repressor, leading therefore to lower transcription rates and less PAI-1 activity. Thus, the transcription activity of the 4G allele is higher than that of the 5G allele (Tsantes et al, 2008).

In the present study; in spite of the frequency of PAI-1 4G/4G genotype was found to be higher in Egyptian patients with myocardial infarction than the control group, it did not show significant difference in genotype distribution (4G4G, 4G5G and5G5G) (X^2 =2.16 and P = 0.34) and also, the 4G allele occurred more frequently than the 5G allele in the patient group (60.4% of patients vs. 51.0% of control) (OR= 1.46; 95% CI: 0.83-2.59) but the difference between them was still, not statistically significant (p= 0.191). Moreover we found about two-fold increased risk of myocardial infarction associated with 4G4G +4G5G (OR=1.99) (95% CI: 0.612- 6.433) compared with 5G5G. However, the p value was still not significant (p= 0.247) and also 4G4G vs. 4G5G+5G5G (OR, 1.73; 95% CI: 0.684- 4.361; p= 0.245). However modest risk of the 4G allele (OR= 1.46) and 4G4G genotype (OR= 1.73) for MI could not be excluded.

No significant association between PAI-1, 4G and MI was also reported by Crainich and his colleague (2003), who documented the lack of association of the plasminogen activator inhibitor-1 4G/5G promoter polymorphism with cardiovascular disease in the elderly. Also; Atherosclerosis, Thrombosis, and Vascular Biology Italian Study Group(2003), found no evidence of association between PAI-1 gene polymorphisms and the development of acute myocardial infarction at a young age (under the age of 45years). Sugano et al (1998), documented that plasminogen activator inhibitor-1 promoter 4G/5G genotype is not a risk factor for myocardial infarction in a Japanese population and Ding et al (2006), found that plasminogen activator inhibitor type 1 gene polymorphisms were associated with plasma plasminogen activator inhibitor type 1 levels but not with myocardial infarction. Despite of our insignificant result this study adjusted OR of 1.46 for the 4G allele vs to the 5G allele and of 1.73 for 4G/4G vs the other two genotypes and these observations are consistent with that PAI-1, 4G exerting at most a modest independent effect on athrothrombotic events occurring late in disease progression as the polymorphism probably require interaction with other genetic and environmental factors. Also, our study was retrospective with respect to MI events, raising the possibility of changes in prevalence of PAI-1, 4G among cases compared with controls due to differential survival rates after MI based on PAI-1, 4G carrier status. Nevertheless, prospective, matched case-control studies would be of interest.

We examined the relationship between the 4G/5G gene polymorphisms and plasma PAI-1 level among myocardial infarction patients and the control group in Egyptians. The 4G/4G homozygous subjects showed the highest plasma concentrations of PAI-1, the lowest were seen in subjects with the 5G/5G genotype, and intermediate concentrations were recorded in heterozygotes; however the difference was not statistically significant (F=0.82, P= 0.45). This may be due to wide variability of the level of PAI-1 enzyme in our sample and we recommend increased sample size in future studies to decrease this variability.

By using logistic regression analysis only smoking; hypertension and PAI-1 level were found to be associated with myocardial infarction among Egyptians.

Variability in PAI-1 plasma concentrations has been reported in different ethnic groups around the world. In some cases this appears to be governed by 4G/5G polymorphism (Dawson et al., 1991; Ye et al., 1995; Nordt et al., 2001). While in others environmental factors such as smoking are involved (Eliasson et al., 1995). Smoking by patients carrying the 4G allele may have an important impact on the frequency of MI. Anti-tobacco campaigns aimed at this group should therefore be intensified and the screening of such individuals should be contemplated. However, interaction with other, traditional risk factors is almost certainly involved in the development of MI and it is important to identify them for primary prevention early in life. The association between hypertension and MI may be related to the relevance of the Ca2+ -dependent potassium channel in the control of human blood pressure and its impact on cardiovascular disease which was evidenced by Tomás et al (2008), who found that two polymorphisms in the pore-forming alpha subunit gene (KCNMA1) were risk factors for severe essential hypertension and myocardial

infarction**.** We also studied the biochemical characteristics of the Egyptian patients with MI and we found only positive significance difference in creatinine level between patients with 4G/4G and those with 5G/5G polymorphisms (P=0.039), Merino and his colleagues (2000), documented that a mild to moderate elevation of serum creatinine level is an independent risk factor for stroke and MI in patients with carotid artery stenosis.

In conclusion: The pathogenesis of MI is complex and multifactorial, with multiple interacting environmental and genetic determinants. The common PAI-1 polymorphism (4G) was not associated with MI in Egyptian population however modest risk (i.e., OR=1.46) could not be excluded. PAI-1variants might affect risk only in concert with other specific environmental and genetic factors. Hence, further research on coagulation- related genetic factors is warranted, including prospective studies of 4G/5G and other PAI-1polymorphisms in large populations at risk for atherothrombotic events. Increased level of PAI-1enzyme**,** smoking and hypertension are significant predictors of myocardial infarction among Egyptians.

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