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Prothrombotic gene polymorphisms and plasma factors in young north Indian survivors of acute myocardial infarction

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Abstract The aim of this study was to evaluate the association of prothrombotic gene polymorphisms [factor V Leiden (*FVL*) 1691GA, factor VII (*FVII*) 10976GA, *FVII* HVR4, platelet membrane glycoproteins *GP1BA* 1018CT, *GP1BA* VNTR, integrin *ITGB3* 1565TC, integrin *ITGA2* 807CT and methylenetetrahydrofolate reductase (*MTHFR)* 677C/T], plasma factors (fibrinogen and homocysteine) and traditional risk factors with acute myocardial infarction (AMI) in 184 patients ≤40 years of age and 350 controls (≤40 years) from north India. Multiple logistic-regression analysis showed that hypertension (OR 1.9, 95 % CI 1.1–3.8, $p = 0.042$), diabetes mellitus (OR 10.5, 95 % CI 2.0–56.7, $p = 0.006$), smoking (OR 7.1, 95 % CI 3.7–13.6, $p < 0.001$), low socio-economic status (OR 13.5, 95 % CI 2.3–78.4, $p = 0.004$), high waist-hip ratio (OR 35.6, 95 % CI 11.1–53.7, $p < 0.001$) and *FVL 1691GA* (OR 6.0, 95 % CI 1.2–13.4, $p = 0.03$) were independent risk predictors of AMI in young. Elevated plasma fibrinogen also showed association with increased AMI risk. *ITGA2* 807C/T polymorphism showed protection against AMI in univariate analysis only, while *GP1BA* VNTR-ac (OR 0.4, 95 % CI 0.2–0.9, $p = 0.033$) showed significant protection even after adjusting for age and sex. Multinominal logistic-regression analysis showed gene–gene (*GP1BA* 1018C/T with *GP1BA* VNTR and *ITGA2* 807C/T with *ITGB3* 1565T/C polymorphisms) and gene-environment interactions (gene polymorphisms with smoking) operating in the occurrence of AMI in young. In conclusion, the role of inherited predisposition to thrombosis in complex, polygenic and multifactorial disease like AMI is limited to certain genetic factors, in combination with environmental factor like smoking.

Keywords Acute myocardial infarction · Fibrinogen · Gene polymorphisms · Homocysteine · Smoking

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Introduction

Coronary artery disease (CAD) resulting in acute myocardial infarction (AMI) is a leading cause of death worldwide. The incidence of CAD in the young has been reported to be 12–16 % in Indians [1]. Reports on CAD in Indians from different parts of the world have shown that Asian Indians are at 3–4 times higher risk of CAD than white Americans, six times higher than Chinese and 20 times higher than Japanese [2, 3]. This high burden of CAD is of particular concern to India as it affects the productive age group. Globalization has led to opportunities worldwide and therefore many young Indians have ventured to the West. Hence, uncontrolled CAD in young Indians may cause increased burden on the health system of various countries. Adequate knowledge of risk factors which selectively operate in Indians can help to formulate the line of investigations in patients and immediate family members.

AMI is a complex, multifactorial and polygenic disease that involves interaction between genetic predisposition and environmental influences. Clinical and epidemiological studies have documented several prothrombotic risk factors which contribute significantly in the pathogenesis of AMI in a number of ancesteral populations [4–14]. However,

there is paucity of systematic data from India in this context, including the role of factor VII (*FVII)* HVR4, integrin *ITGA2* 807C/T, integrin *ITGB3* 1565T/C, platelet glycoprotein *GP1BA* HPA-2 (1018C/T) and *GP1BA* VNTR polymorphisms in the occurrence of AMI. This study was undertaken to study the role of such genetic factors and their interaction with traditional risk factors in our population.

Materials and methods

Study design and data collection

This case–control study was undertaken in the Department of Hematology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. The sample size for the study was determined using EPI INFO statistical package. A total of 184 patients (≤40 years) with definite AMI as per WHO criteria were recruited [15]. These were consecutive patients at the Department of Cardiology, PGIMER, Chandigarh. Controls ($n = 350$) were random unrelated young (≤40 years) healthy subjects from north India, matched with patients in terms of geographic origin and ethnicity. All the controls were recruited from normal controls that were required by the department for coagulation tests. None of the individuals gave history of any cardiac event or hospitalization. The patients and controls were enrolled between July 2006 and December 2009. The Ethics Committee of the Institute approved the study, and the subjects gave their informed written consent.

The collected data included age, sex, traditional and lifestyle factors such as hypertension, diabetes mellitus, family history of CAD, obesity, smoking, alcohol intake, socio-economic status (SES, high/middle/low) and lifestyle (rural/urban). Hypercholesterolemia was not included in the study as the patients were already on statins which lower the cholesterol levels. The participants were considered to have hypertension if elevated blood pressure (>140/90 mmHg) was measured or if they were already being treated with antihypertensive agents. They were defined as having diabetes mellitus if they had a fasting blood glucose level >140 mg/dl or were already being treated for diabetes. A positive family history was defined as the presence of at least one first or second degree relative with a history of CAD. In terms of smoking and alcohol consumption, the subjects were classified on the basis of self-report. Body mass index (BMI) was categorized according to WHO Expert Consultation 2004 as normal weight (18.5–22.9 kg/m^2), overweight (23–27.5 kg/m^2), obese (27.6–40 kg/m^2) and morbidly obese (>40 kg/m^2) [16]. Normal Waist-Hip Ratio (WHR) for males and females were ≤0.8 and ≤0.95, respectively.

Blood sampling was performed at least 4 weeks after the onset of coronary event when the patient had been hemodynamically stabilized. Aseptic venous blood samples were obtained after overnight fasting and collected in EDTA for DNA analysis and plasma homocysteine (Hcy) and in 3.2 % tri-sodium citrate (9:1 v/v) for plasma fibrinogen estimation.

Estimation of biochemical parameters

STA-Fibrinogen kits intended for use with STA-analyzers by clotting method of von Clauss [17] were used for plasma fibrinogen estimation. A calibration curve was prepared using clotting time of diluted plasma calibrators to calculate the levels of plasma fibrinogen. Normal plasma fibrinogen level in the adult population ranges from 2–4 g/l.

Total plasma Hcy levels were determined by using BioRad Microplate Enzyme Immunoassy Homocysteine kits (BioRad, Oslo, Norway) on Tecan Absorbance Reader (Tecan Austria GmbH, Austria, Europe) using Magellan CE software. Homocysteine control set was used as a control for increasing accuracy of measurement of plasma Hcy level. The limit of quantification for plasma Hcy was 1.0 μmol/l and coefficient of variation was less than 20 % as per the kit. Normal plasma Hcy level in the adult population ranges from 5-15 μmol/l.

Genetic analysis

Genomic DNA was extracted from peripheral blood leucocytes using standard phenol–chloroform method and ethanol precipitation [18]. Factor V Leiden (*FVL)* 1691G/A (rs6025) [7], platelet glycoprotein GpIIIa or CD61 or *ITGB3* 1565T/C (rs5918) [8], GpIbα or CD42-alpha or *GP1BA* HPA-2 (1018C/T) (rs6065) [9], *FVII* R353Q (10976G/A) (rs6046) [10] and *MTHFR* 677C/T (rs1801133) [11] polymorphisms were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) using site-specific restriction enzymes. GpIa or CD49B or *ITGA2* 807C/T (rs1126643) [12] polymorphism was detected by allele-specific PCR using internal controls. *GP1BA* VNTR [9] and *FVII* HVR4 [10] polymorphism were determined by PCR amplification and polyacrylamide gel electrophoresis.

Statistical methods

Descriptive data was expressed as mean ± standard deviation (SD) for continuous and proportions (%) for categorical variables. A comparison among patients and controls was performed by means of analysis of variance (ANOVA) with Bonferroni correction for BMI and WHR. Mann–Whitney test was used to compare plasma levels of fibrinogen and Hcy among patients and controls. Comparison of traditional risk factors, genotype frequencies, deviation of allele frequencies

from Hardy–Weinberg equilibrium (HWE) was done using Chi-square (χ^2) test. To adjust for the confounding effects of traditional coronary risk factors, multiple logistic-regression analysis was performed adjusted for age and sex. Risk factors that appear to be potentially significant predictors in single-variable analysis ($p < 0.05$) were included in the multiple logistic-regression analysis. Mann–Whitney test and Kruskal–Wallis test were used to investigate the correlation between two or more variables respectively. Multinominal logistic-regression analysis was used to study synergistic effects of any two factors (gene–gene and gene-environment interactions) in the occurrence of AMI. The strength of association of risk factors with the occurrence of AMI was estimated by calculation of odds ratio (OR) and Cornfield method for calculation of 95 % confidence intervals (CI). A p value of <0.05 was considered statistically significant. All the computations were carried out using Statistical Package for the Social Sciences (SPSS), version 13.0, Chicago for windows.

Results

Demographic characteristics and traditional coronary risk factors

The present study was carried out on 350 controls (mean age 31.1 ± 6.0 years, 75.7 % males) and 184 cases of AMI ≤ 40 years (mean age 36.4 ± 4.5 years, 96.2 % males). Comparison of traditional risk factors among cases and controls is shown in Table 1. Cases showed significant association with hypertension, diabetes mellitus, smoking, family history of CAD, alcohol intake, rural lifestyle, low SES, high BMI and high WHR in univariate analysis.

Plasma fibrinogen

Mean plasma fibrinogen was significantly higher in cases (3.8 ± 1.0 g/l) than controls (3.0 ± 0.6 g/l), showing association ($p < 0.001$) with risk of AMI in young.

Plasma homocysteine

Mean plasma Hcy was high in controls (27.2 ± 25.2 μm/l) as well as cases (24 ± 23.9 μm/l). The prevalence of hyperhomocysteinemia was lower in cases than controls (54.9 vs. 68.6 %, OR 0.6, 95 % CI 0.4–0.8, $p = 0.02$).

Genetic risk factors

All alleles were present in HWE in the study population. Comparison of genetic risk factors among cases and controls is shown in Table 2. *FV* 1691GA genotype had fourfold increased risk of AMI. The prevalence of *ITGA2* 807CT and *GP1BA* VNTR-ac genotype was significantly lower in cases than controls, showing protective effect against AMI. *GP1BA* 1018CT, *ITGB3* 1565TC, *FVII*

Table 1 Traditional risk factors among patients with acute myocardial infarction ≤40 years and controls

Characteristics	Controls $n = 350$ (%)	Cases $n = 184$ (%)	OR (95 % CI)	p value
Mean age (years)	31.1 ± 6.0	36.4 ± 4.5	–	<0.001[a]
Males	265 (75.7 %)	177 (96.2 %)	–	<0.001[a]
Hypertension	39 (11.1 %)	73 (39.7 %)	5.2 (3.4–8.2)	<0.001[a]
Diabetes mellitus	2 (0.6 %)	26 (14.1 %)	28.6 (6.7–122.1)	<0.001[a]
Smoking	73 (20.9 %)	113 (61.4 %)	6.0 (4.1–8.9)	<0.001[a]
Family history of CAD	49 (14.0 %)	43 (23.4 %)	1.9 (1.2–3.0)	0.009[a]
Alcohol intake	92 (26.3 %)	79 (42.9 %)	2.1 (1.4–3.1)	<0.001[a]
Rural lifestyle	116 (33.1 %)	80 (43.5 %)	1.6 (1.1–2.2)	0.019[a]
Low SES	86 (24.6 %)	71 (38.6 %)	1.9 (1.3–2.8)	<0.001[a]
BMI (kg/m^2)				
Mean ± SD	23.3 ± 2.8	24.8 ± 5.3	–	<0.001[a]
BMI > 23	180 (51.4 %)	127 (69 %)	2.1 (1.4–3.1)	<0.001[a]
WHR				
Mean ± SD	0.88 ± 0.05	0.95 ± 0.04	–	<0.001[a]
WHR > 0.95, M	6/265 (2.3 %)	79/177 (44.6 %)	34.8 (14.7–82.4)	<0.001[a]
WHR > 0.8, F	58/85 (68.2 %)	7/7 (100 %)	–	<0.001[a]

Data are expressed as mean ± SD or n (%)

BMI body mass index, *CAD* coronary artery disease, *CI* confidence interval, *F* females, *M* males, *OR* odds ratio, *SD* standard deviation, *SES* socioeconomic status, *WHR* waist hip ratio

[a] $p < 0.05$, significant

10976GA, *FVII* HVR4 and *MTHFR* 677CT gene polymorphisms were not associated ($p > 0.05$) with cases.

Multiple logistic regression analyses

Hypertension, diabetes mellitus, smoking, low SES, high WHR and *FVL* 1691GA were independent risk predictors of AMI in cases (Table 3). *GP1BA* VNTR-ac genotypes showed significant protection against AMI in cases. *ITGA2* 807CT genotype lost significance after adjusting for age and sex.

Gene–gene and gene-environment interactions

Multinominal logistic-regression analysis showed protective effect against AMI in cases with combined genotypes

Table 2 Genetic risk factors among patients with acute myocardial infarction ≤40 years and controls

Genotypes	Controls $n = 350$ (%)	Cases $n = 184$ (%)	OR (95 % CI)	p value
FVL 1691GA				
GG	343 (98 %)	171 (92.9 %)		
GA	7 (2 %)	13 (7.1 %)	3.7 (1.5–9.5)	0.003[a]
ITGA2 807CT				
CC	112 (32 %)	77 (41.8 %)		
CT	217 (62 %)	94 (51.1 %)	0.6 (0.4–0.9)	0.050[a]
TT	21 (6 %)	13 (7.1 %)		
ITGB3 1565TC (HPA-1)				
TT	300 (85.7 %)	155 (84.2 %)		
TC	48 (13.7 %)	27 (14.7 %)		
CC	2 (0.6 %)	2 (1.1 %)	1.9 (0.3–13.7)	0.764
GP1BA 1018CT (HPA-2)				
CC	316 (90.3 %)	173 (94 %)		
CT	34 (9.7 %)	11 (6 %)	0.6 (0.3–1.2)	0.14
GP1BA VNTR				
Aa	203 (58 %)	114 (62 %)		
Ad	74 (21.1 %)	57 (31 %)		
Ac	73 (20.9 %)	13 (7.1 %)	0.3 (0.2–0.5)	<0.001[a]
FVII 10976GA (R353Q)				
GG	182 (52 %)	93 (50.5 %)		
GA	146 (41.7 %)	69 (37.5 %)		
AA	22 (6.3 %)	22 (12 %)	1.9 (1.0–3.8)	0.071
FVII HVR4				
H6H6	93 (26.6 %)	55 (29.9 %)		
H6H7	175 (50 %)	85 (46.2 %)		
H7H7	77 (22 %)	38 (20.7 %)	0.9 (0.6–1.4)	0.613
H6H8	2 (0.6 %)	2 (1.1 %)		
H7H8	1 (0.3 %)	2 (1.1 %)		
H7H9	0 (0 %)	1 (0.5 %)		
H5H6	1 (0.3 %)	1 (0.5 %)		
H6H9	1 (0.3 %)	0 (0 %)		
MTHFR 677CT				
CC	250 (71.4 %)	120 (65.2 %)		
CT	91 (26 %)	55 (29.9 %)		
TT	9 (2.6 %)	9 (4.9 %)	1.9 (0.8–5.0)	0.195

Data are expressed as n (%)

CI confidence interval, *FVL* factor V Leiden, *FVII* factor VII, *GP* platelet membrane glycoproteins, *ITG* integrin, *OR* odds ratio, *MTHFR* methylenetetrahydrofolate reductase

[a] $p < 0.05$, significant

Table 3 Risk factors for acute myocardial infarction following adjustment for age and sex in patients ≤40 years and controls

Risk Factors	OR (95 % CI)	*p* value
Hypertension	1.9 (1.1–3.8)	0.042[a]
Diabetes mellitus	10.5 (2.0–56.7)	0.006[a]
Smoking	7.1 (3.7–13.6)	<0.001[a]
Family history of CAD	1.7 (0.9–3.6)	0.126
Alcohol intake	0.6 (0.3–1.1)	0.113
Rural lifestyle	0.5 (0.3–1.1)	0.062
Low SES	13.5 (2.3–78.4)	0.004[a]
High BMI	1.4 (0.8–2.5)	0.231
High WHR	35.6 (11.1–53.7)	<0.001[a]
FVL 1691GA	6.0 (1.2–13.4)	0.03[a]
ITGA2 807CT genotype	0.8 (0.5–1.4)	0.496
GP1BA VNTR-ac genotype	0.4 (0.2–0.9)	0.033[a]

BMI body mass index, *CAD* coronary artery disease, *CI* confidence interval, *FVL* factor V Leiden, *GP* platelet membrane glycoproteins, *ITG* integrin, *OR* odds ratio, *SES* socioeconomic status, *WHR* waist hip ratio

[a] $p < 0.05$, statistically significant

of *GP1BA* 1018CC and *GP1BA* VNTR-ac (OR 0.3, 95 % CI 0.2–0.6, $p < 0.001$) as well as *ITGA2* 807CT + TT and *ITGB3* 1565CC (OR 0.6, 95 % CI 0.4–0.8, $p = 0.004$). Multinominal logistic-regression analysis also showed synergistic effect of smoking and certain gene polymorphisms in the occurrence of AMI in cases. In comparison with non-smokers non-carriers, smoker cases carrying *ITGB3* 1565TC + CC genotype (OR 9.8, 95 % CI 3.9–24.3, $p < 0.001$), *GP1BA* VNTR-aa genotype (OR 9.6, 95 % CI 5.6–16.3, $p < 0.001$), *GP1BA* 1018CT + TT genotype (OR 9.6, 95 % CI 2.5–37.3, $p = 0.001$), *ITGA2* 807C*C* genotype (OR 6.5, 95 % CI 3.4–12.2, $p < 0.001$), *FVII* 353RQ + QQ genotype (OR 6.3, 95 % CI 3.6–11.0, $p < 0.001$), *FVII* H6H7 genotype (OR 6.4, 95 % CI 3.3–12.3, $p < 0.001$) and *MTHFR* 677CT + TT genotype (OR 8.8, 95 % CI 4.7–16.5, $p < 0.001$) had increased risk of AMI in young.

Mann–Whitney test showed that there was no significant difference in mean fibrinogen in controls ($p = 0.732$) and young patients ($p = 0.157$) with/without smoking status. Mean Hcy in controls ($p = 0.834$) and young patients ($p = 0.179$) showed no significant difference with/without *MTHFR* 677CT polymorphism using Kruskal–Wallis test.

Discussion

This is the first study to investigate the association of prothrombotic gene polymorphisms and plasma factors with AMI in young survivors of Northern Indian ancestry. We demonstrated that *FVL 1691GA* genotype was an independent risk factor for AMI whereas the *GP1BA* VNTR-ac genotype was protective, independent of traditional CAD risk factors. Additionally, we found a number of novel gene–gene and gene-environment interactions.

Except for *FVL* 1691GA, none other gene polymorphisms were independently associated with the risk of AMI in cases. Although majority of the studies have shown that there is an association of *ITGB3* PlA1/A2 (1565 T/C), *GP1BA* HPA-2 (1018 C/T), *FVII* 10976 G/A (R353Q), *FVII* HVR4 and *MTHFR* 677 C/T polymorphism with risk for AMI, there are reports to the contrary [6, 8–11]. These gene polymorphisms are unique to particular ethnic groups.

Our study is similar to studies from India and abroad. Khare et al. [13] showed that the prevalence of *FVL* 1691GA was higher in AMI cases as compared to controls ($p = 0.038$). This study was consistent with previous study which showed that *FVL* 1691GA was associated with risk of AMI [19]. Ranjith et al. [20] found very low frequency of *FVL* 1691GA in South African Indian population (<1 %) and failed to find its increased prevalence in their patients. Carriership of *FVL 1691GA* was associated with increased risk of AMI in a meta-analysis of 66,155 cases and 91,307 controls [21].

GP1BA VNTR-ac genotype had significant protective effect against AMI in young. In a case–control study of 101 Caucasian patients by Gonzalez-Conejero et al. [9], *GP1BA* VNTR-cb genotype was associated with increased risk of MI. Mikkelsson et al. [22] found that the haplotype defined by carriership of *GP1BA* HPA-2 Met and VNTR-b allele was associated with AMI in 80 Caucasian Finnish men. Our study is similar to the ARIC study by Afshar-Kharghan et al. [23] which showed protective effect of *GP1BA* VNTR-cc genotype. The addition of repeats to macroglycopeptide region of GpIbα protein, determined by VNTR polymorphism, increases the distance between ligand-binding domains of GpIbα and platelet plasma membrane. Higher or lower number of repeats, thus results in greater or smaller overall length of the macroglycopeptide region of GpIbα, would have a prothrombotic or antihemostatic effect, respectively. VNTR-c allele, associated with lower number of repeats has lesser prothrombotic effect, and showed greater protection. No studies from India are there to compare our results.

ITGA2 807CT genotype showed protective effect against AMI in young in univariate analysis but lost significance after adjustment for age and sex. Benze et al. [24] found that frequency of *ITGA2* 807T allele carriers was similar among 287 male AMI patients and 138 healthy controls from Germany [54.6 vs. 62.3 %; 0.73 (0.47–1.12)]. In a large case–control study by Zotz et al. [25], prevalence of *ITGA2* 807CT genotypes did not differ significantly between patient groups with CAD or MI and controls or blood donors. There is no study from India

evaluating the importance of *ITGA2* 807C/T polymorphism in the occurrence of AMI to compare our results.

In the present study, cases with *GP1BA* 1018CC and VNTR-ac combined genotype had protection against AMI. However, in a study by Gonzalez-Conejero et al. [9], c/b genotype of VNTR and 1018TT genotypes of *GP1BA* were associated with increased risk of CHD. Protection against AMI in young was observed with combined genotypes of *ITGA2* 807CT + TT and *ITGB3* 1565CC, which is in contradiction to previous studies [24, 25]. Synergistic effect of smoking and genotypes (*ITGB3* 1565TC + CC, *GP1BA* VNTR-aa, *GP1BA* 1018CT + TT, *ITGA2* 807CC, *FVII* 353RQ + QQ, *FVII* H6H7 and *MTHFR* 677CT + TT) in the occurrence of AMI was observed in our study. Ardissino et al. found a 14-fold increase in the occurrence of AMI in smoker carriers with *ITGB3* 1565C allele [26]. Smoking had no effect on fibrinogen in our study, which is in contradiction previous studies [27]. There was no interaction of Hcy with *MTHFR* 677CT polymorphism, which is in agreement with several studies [28].

Elevated plasma fibrinogen was associated with risk of AMI in cases in our study which is in concordance to studies from West India [13, 14], prospective studies [29–31] and meta-analysis [32]. We found high mean plasma Hcy in both cases and controls. This may be due to inappropriate dietary habits leading to deficiencies of Vitamin B_6, B_{12} and folic acid. It is possible that the general north Indian population is prone to high incidence of CAD. With this background level of hyperhomocysteinemia in controls, additional risk in patients may not be demonstrated and interaction with other confounding risk factors could be possible [33]. Our patients were recruited at least 1 month after the occurrence of an acute event and during this period patients may have adopted healthy dietary pattern along with the intake of nutrient supplements. This may have lowered the levels of plasma Hcy in the patients as compared to the controls. The increased risk due to hyperhomocysteinemia appears to be different in Indians as compared to Europeans. Population studies have shown that plasma Hcy levels are higher in immigrant ethnic Indians compared to North Americans and European Whites [34, 35]. A study from Singapore showed no increase in plasma Hcy in Indians as compared to the Malays and Chinese [36]. Our study is similar to the findings from several studies that plasma Hcy is not a major risk factor for AMI in Indians [14, 33, 37–39]. A study by Kumar et al. [39] on 50 young AMI patients (<40 years) and 27 controls showed hyperhomocysteinemia to be highly prevalent in patients as well as controls from North India and levels of Hcy decreased in 83.5 % of patients after three months of vitamin supplementation. Ghosh et al. [40] found hyperhomocysteinemia to be common amongst young AMI patients from western India, the major cause of which was folic acid deficiency. Hypertension, diabetes mellitus, smoking, low SES and high WHR were associated with increased risk of AMI in young.

Conclusions and implications

Since *FVL* 1691GA is independently associated with increased risk of AMI in young, it should be tested for in Indians. If found positive, a close monitoring of the patient for recurrence of AMI or venous thromboembolism is indicated. *ITGA2* 807C/T polymorphism was protective against AMI in univariate analysis, while *GP1BA* VNTR showed significant protection even after adjusting for age and sex. This analysis reiterates the association of traditional risk factors and AMI in young. Therefore, lifestyle management and control of diseases like hypertension and diabetes mellitus should be advised. More studies from India with long term follow up are indicated.

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Conflict of Interest None declared.

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