Original Article

Nitric Oxide Synthase 3 Gene Polymorphisms and Their Association with Acute Myocardial Infarction and Chronic Stable Angina: A Case–Control Study from Northern India

Abstract

Background: Coronary artery disease (CAD) that encompasses acute myocardial infarction (AMI), chronic stable angina (CSA), and unstable angina (UA) has numerous known risk factors. Genetic predispositions contribute as major risk in the development of CAD and the genes regulating atherosclerosis are important for disease prevention. Nitric oxide synthase 3 (*NOS3*) gene responsible for nitric oxide (NO) production is of special importance. **Aim:** To evaluate the role of three *NOS3* polymorphisms (-786C/T, 894G/T, and 4a4b) in patients with CAD, particularly in AMI and CSA and their comparison with healthy controls. **Materials and Methods:** One hundred patients in each AMI and CSA group and 100 controls were included and were typed for three *NOS3* polymorphisms (-786C/T, 894G/T, and 4a4b) by polymerase chain reaction–restriction fragment length polymorphism. Plasma NO metabolites (NOx) were also evaluated. **Results:** A significant association of 894G/T polymorphism with AMI in dominant model (P = 0.052) and with CSA in dominant and codominant models was detected (P = 0.008 and P = 0.006, respectively). Plasma NO levels were found to be significantly higher (P < 0.0001) in healthy controls (43.80 ± 6.28) compared to AMI and CSA patients (37.05 ± 6.75 and 38.67 ± 5.61). No significant association of -786C/T and 4a4b polymorphism with AMI and CSA risk under recessive, dominant, and codominant models was detected. **Conclusion:** Our study revealed a significant association of 894G/T polymorphism with AMI and independent association of NOx levels with CAD, indicating high risk of CAD in the North Indian population. Our findings will be helpful in identifying the genetic risk factors associated with CAD and better management of the diagnostic as well as therapeutic measures.

Keywords: *Atherosclerosis, coronary artery disease, heart attack, nitric oxide, polymerase chain reaction–restriction fragment length polymorphism, variable number of tandem repeats*

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Introduction

Cardiovascular disease, a global health concern, affects more than half a billion people across the world. Cardiovascular diseases accounted for approximately one-third of all the deaths occurred globally in 2021, 80% of them being in developed countries.[1] The greater proportion of the aforesaid deaths is due to coronary artery disease (CAD), of which acute myocardial infarction (AMI) is a major manifestation, others being chronic stable angina (CSA) and unstable angina (UA). Since it is difficult to measure the prevalence of CAD in a population, the incidence of AMI can be used as a surrogate marker to determine the same, provided a consistent definition is used when different populations, countries, or continents are being compared.[2]

CAD that encompasses CSA, UA, and AMI has numerous known genetic and environmental risk factors. Genetic predispositions contribute as a major risk in the development of CAD. Genetic dysfunctions and the associated vascular anomalies can lead to the development of CAD, and the genes regulating atherosclerosis are important for disease prevention.[3] Among various such genes, nitric oxide synthase 3 (*NOS3*) gene responsible for nitric oxide (NO) production is of special importance.[4] NO is an important determinant in sustaining vascular homeostasis and integrity.[5,6] It inhibits atherosclerosis by reducing platelet aggregation and adhesion, leukocyte adhesion to the endothelium, and oxidation of atherogenic low-density lipoprotein (LDL).[7] Several known single-nucleotide polymorphisms (SNPs) in *NOS3* gene have

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been documented to have involvement in the pathogenesis of various vascular diseases including CAD and AMI.[7-9]

With extensive studies carried out, and ample data available on the importance of endothelial NO in vascular disease, it is imperative to investigate the association of NO metabolites (NOx) level and *NOS3* gene polymorphisms with AMI and CSA in the North Indian population.

With the above background, the present study aimed to evaluate the role of three *NOS3* gene polymorphisms (-786C/T, 894G/T, and 4a4b) in patients with CAD, particularly in AMI and CSA and their comparison with healthy controls. The association of smoking, alcohol, dietary patterns, and gender with level of NO metabolites (NOx) among the patients and healthy controls was also evaluated.

Materials and Methods

Study site and study subjects

This prospective study was conducted at a 2500-bedded tertiary care hospital and national referral center of Northern India. Patients were recruited from the outpatient departments and wards of the department of cardiology. The study was approved by the Institutional Ethics Committee (Ref. No. IESC/T-252/03.06.2011) and was in accordance with the Declaration of Helsinki. The study protocol was explained to the participants, and prior to their enrollment, their written informed consent was obtained.

We enrolled 100 nonrelated, consecutive, AMI patients (male:female [M:F] = 82:18; age range = 18–75 years) referred as AMI group and 100 nonrelated, consecutive, CSA patients (M:F = 82:18; age range = 18–75 years) referred as CSA group. Diagnosis of definite AMI and CSA was based on the American College of Cardiology/American Heart Association criteria.[10] Patients exclusively of North Indian origin were included in the study. Patients on oral anticoagulant or on any medication (antibiotics, aspirin, contraceptives, or steroids) were included in the study only if they were off the medication for ≥14 days (from the time of sampling). Patients with malignancy, unstable angina, stroke, hematological disorder, patients who underwent surgery or patients who had suffered trauma within the past 30 days, and pediatric patients were excluded from the study. A disease-free control population (n = 100) of nonrelated, sex and age matched as closely as feasible were also included in the study (control group). Hospital employees, trainees, and unrelated healthy patient attendants served as controls. Controls taking any form of medication or having had surgery or suffered trauma in the past 30 days were excluded from the study. Controls with a history of bleeding, thrombotic, or cardiac disorders were also excluded from the study.

The demographic details, relevant clinical history, associated risk factors, and comorbidities of the study participants were noted prospectively as part of patient clinical history. Briefly, the subjects were questioned on the use of drugs, cigarettes, and alcohol and also if they had a history of heart-related disorders, hypertension, abortions, and thrombosis-related complications (personal or family history). Subjects with a blood pressure over 140/90 mmHg were considered hypertensive. Criteria for diabetes were set as an abnormal fasting blood glucose level >125 mg/dl or having taken hypoglycemic agents. Alcohol intake was also recorded as present or absent. Individuals were considered smokers if they were previous or current smokers of >5 cigarettes per day. A positive family history was defined as the presence of at least one first- or second-degree relative history of CAD.

Sample collection

All study participants had 10 ml of their venous blood drawn into siliconized glass tubes containing 3.2% sodium citrate solution. Within 45 min of collection, plasma was extracted and was stored at −70°C for all nonmolecular tests. Peripheral blood leukocytes were also stored at −20°C for extracting DNA and performing the molecular tests.

Lipid profile, vasculitic profile (antinuclear antibody and dsDNA), blood sugar profile, electrocardiogram/echocardiography, renal function tests, and liver function tests were done for the study participants wherever necessary.

DNA extraction and quantification

Genomic DNA was extracted from peripheral blood leukocytes by spin column-based nucleic acid extraction method using QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

The usual yield of DNA from 10 ml of normal blood is approximately 350 µg. 10 µl of dissolved DNA was diluted to 1 ml using TE buffer and vortexed. The optical density (OD) of the diluted DNA was recorded at wavelengths of 260 λ and 280 λ using a UV spectrophotometer (GeneQuant, Pharmacia, USA). Protein-free DNA sample gave a ratio of 1.75–2.0 reading at 260 λ/280λ. The concentration of DNA was calculated using the formula: OD at 260 λ X dilution factor × 50 (one OD is equal to 50 µg of dsDNA).

Detection of nitric oxide synthase 3 gene polymorphisms

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) was used for detecting the two *NOS3* gene polymorphisms: -786C/T, 894G/T, and the intron 4a/4b variable number tandem repeat (VNTR) polymorphism.

The-786C/T polymorphism was detected by PCR-RFLP, using the following primers: forward, 5'-GAGTCTGGCCAACACAAATCC-3'; and reverse, 5'-GACCTCTAGGGTCATGCAGGT-3'. The amplified products were digested by HpaII and separated on a

2% agarose gel. The -786C allele was visualized as 327 + 284 + 46 bp bands and the -786T allele was visualized as 373 + 284 bp bands [Figure 1].

The 894G/T polymorphism was also detected by PCR-RFLP, using the following primers: forward, 5'-TCCCTGAGGAGGGCATGAGGC-3'; and reverse, 5'-TGAGGGTCACACAGGTTCCT-3'. The amplified products were digested by BanII and separated on a 2% agarose gel. The 894G allele was visualized as 320 + 137 bp bands and the 894T allele was visualized as the 457 bp fragment [Figure 2].

The 4a/4b VNTR polymorphism was detected using PCR with the following primers: forward, 5'-AGGCCCTATGGTAGTGCCTTT-3'; and reverse, 5'-TCTCTTAGTGCTGTGCTCAC-3', separated on a 2% agarose gel. The 4a allele was visualized as a 393 bp band and the 4b allele as a 420 bp fragment [Figure 3].

Measurement of nitric oxide

NO levels were evaluated indirectly by measuring its metabolites (NOx) in plasma samples. We measured total nitrite by converting nitrate to nitrite using total NO/nitrite/nitrate kits (R & D system, Minneapolis, USA). Briefly, the NO concentrations were determined based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction was followed by colorimetric detection of nitrite as an azo dye product of the Griess reaction, a two-step diazotization reaction in which acidified NO2 produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion was then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540–570 nm. The minimal detectable dose/sensitivity for the total NO/nitrite/nitrate kits was 0.25 µmol/L.

Statistical analysis

The data were analyzed using Statistical Package for the Social Sciences (SPSS) version 18.0 (IBM, Chicago, IL, USA). Differences in characteristics between cases and controls were examined using the Chi-square test for proportions and independent Student's *t*-test for continuous variables. Descriptive data were expressed as mean value ± standard deviation for continuous variables. Chi-square test was used for comparing genotype and allele frequencies for statistical significance between patients and controls. Odds ratios with corresponding 95% confidence intervals were determined to assess the strength of association of -786C/T, 894G/T, and 4a/4b VNTR polymorphisms with AMI and CSA risk. $P \leq 0.05$ was considered statistically significant.

Results

Demographic and clinical details of the study participants

The study participants among AMI group, CSA group, and control group had an average age of 55.99 ± 10.33 years, 56.38 ± 10.55 years, and 52.07 ± 14.44 years, respectively. With M:F ratio of 4:1 in both AMI and CSA groups and 3:1 in the control group, a clear male preponderance was noted among the study participants. The presence of smoking and alcohol consumption was found to be significantly higher among the patients who suffered from AMI and CSA than in controls ($P < 0.0001$). Most of the patients in the AMI

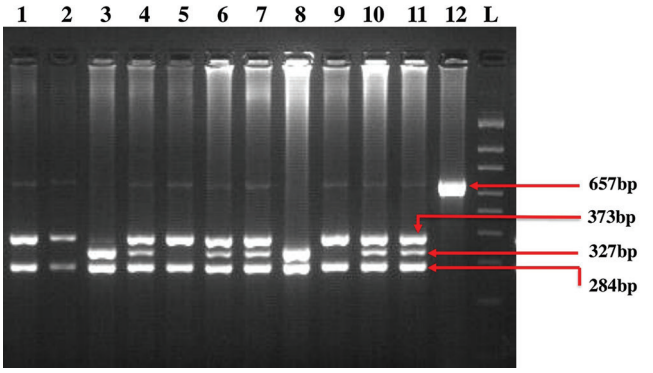

Figure 1: Polymorphism (-786C/T). L: 100 bp ladder; Lane 1, 2, 5, 9: Homozygous normal (TT); Lane 3,8: Homozygous variant (CC); Lane 4, 6, 7, 10, 11: Heterozygous variant (CT); Lane 12: polymerase chain reaction product

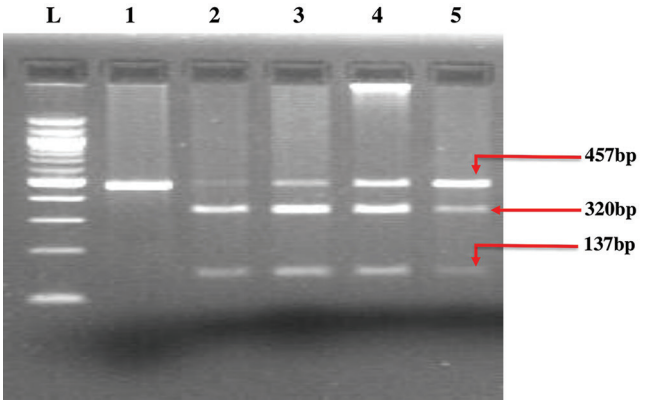

Figure 2: Polymorphism (894G/T). L: 100 bp ladder; Lane 1: Homozygous normal (TT); Lane 2: Homozygous variant (GG); Lane 3-5: Heterozygous variant (GT)

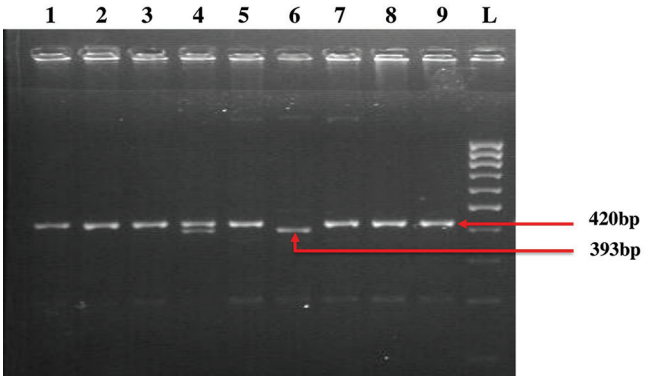

Figure 3: Polymorphism (intron 4, VNTR). L: 100 bp ladder; Lane 1-3, 5, 7-9: Homozygous for 4b VNTR; Lane 6: Homozygous for 4a VNTR; Lane 4: Heterozygous for 4a/4b VNTR

group were nonvegetarian (61; 61.0%), as opposed to in case of CSA and control groups, and the association was found to be statistically significant (P = 0.028). The mean high-density lipoprotein (HDL) levels were observed to be higher in the control group as compared to the AMI and CSA groups. Whereas, the mean LDL levels were higher in the AMI and CSA groups as compared to the control group. Comparison among the groups with regard to HDL and LDL level was found to be statistically significant (P < 0.0001). Diabetes mellitus, hypertension, and family history of CAD were presented only in patients groups and were seen in 24 (24.0%) and 37 (37.0%); 54 (54.0%) and 49 (49.0%); and 21 (21.0%) and 23 (23.0%), respectively, in AMI and CSA patients. The mean body mass index value among the study groups was observed almost similar and therefore not associated significantly (P = 0.452) with increased risk of CAD.

Demographic and clinical details of the study participants are depicted in Table 1.

Association of -786C/T polymorphism among the cases (acute myocardial infarction and chronic stable angina) and controls

The distribution of genotypic and allelic frequency for -786 C/T polymorphism in AMI cases vs. controls and CSA cases vs. controls is enlisted in Tables 2 and 3, respectively.

The distribution of -786 T/T, -786 T/C, and-786 C/C genotypes was 60; 60.0%, 36; 36.0%, and 04; 4.0% in AMI cases, 65; 65.0%, 33; 33.0%, and 02; 2.0% in CSA cases and 67; 67.0%, 32; 32.0%, and 01; 1.0% in controls, respectively. With reference to TT genotype, the frequency of CT and CC genotype was higher in AMI cases than the control group (P = 0.448 and P = 0.186, respectively) as well as higher in CSA cases than the control group (P = 0.840 and P = 0.558, respectively); however, in either of the comparison, it did not show any significant association. The frequency of C allele was higher in AMI cases when compared to controls (44; 22.0% vs. 34; 17.0%) and was also higher in CSA cases when compared to controls (37; 18.5% vs. 34; 17.0%). No significant relationship was observed between -786 C/T polymorphism with both AMI and CSA risk under the dominant, codominant and recessive genotype models (AMI: P =0.304, P = 0.550, P = 0.208; CSA: P =0.765, P = 0.880, P = 0.568) [Tables 2 and 3].

Association of 894G/T polymorphism among the cases (acute myocardial infarction and chronic stable angina) and controls

The distribution of genotype and allelic frequency for 894G/T polymorphism in AMI cases vs. controls and CSA cases vs. controls is enlisted in Tables 4 and 5, respectively.

The distribution of 894 G/G, 894 G/T and 894 T/T genotypes were 60; 60.0%, 33; 33.0%, and 07; 7.0% in AMI cases, 55; 55.0%, 41; 41.0%, and 04; 4.0% in CSA cases and 73; 73.0%, 23; 23.0%, and 04; 4.0% in controls, respectively. With reference to GG genotype, although the frequency of GT and TT genotype was higher in AMI cases than in the control group (P = 0.084 and P = 0.245, respectively), it did not show a statistically significant association. The frequency of T allele was higher in AMI cases than in controls (47; 23.5% vs. 31; 15.5%). A statistically significant relationship was observed between 894G/T polymorphism and AMI risk under the dominant (P = 0.052) genotype model; however, no significant relationship between the same was observed under the codominant (P = 0.550) and recessive genotype models (P = 0.116).

Table 1: Demographic and clinical details of the study participants among the various groups (*n*=100/group)

Characteristic	AMI group	CSA group	Control group	*P*
Age, mean±SD	55.99±10.33	56.38±10.55	52.07±14.44	0.635
Gender (%)				
Male	82	80	78	0.863
Female	18	20	22	
Hypertension (%)	54	49	0	<0.0001*
Diabetes (%)	24	37	0	<0.0001*
Smoker (%)	36	41	11	<0.0001*
Alcoholic (%)	26	26	7	<0.0001*
Food habits (%)				
Vegetarian	39	53	57	0.028*
Nonvegetarian	61	47	43	
Family history (%)	21	23	0	<0.0001*
Total cholesterol, mean±SD (mg/dL)	168.1±4.02	169.59±3.80	163.9±3.98	0.836
HDL, mean±SD (mg/dL)	30.18±2.61	30.55±2.74	41.31±2.59	<0.0001*
LDL, mean±SD (mg/dL)	109.49±4.78	109.84±5.19	99.96±3.22	<0.0001*
BMI, mean±SD	23.60±3.53	23.97±3.97	22.39±3.91	0.452

*P<0.05 was considered statistically significant. AMI: Acute myocardial infarction; CSA: Chronic stable angina; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; BMI: Body mass index; SD: Standard deviation

Table 2: Genotypic, allelic distribution, and association analysis of -786C/T gene polymorphism and risk of acute myocardial infarction under different genetic models (*n*=100/group)

Genotype/allele	AMI group, *n* (%)	Control group, *n* (%)	OR (95% CI)	*P*
TT (wild)	60 (60)	67 (67)	Reference	Reference
CT	36 (36)	32 (32)	1.25 (0.69–2.26)	0.448
CC	4 (4)	1 (1)	4.46 (0.48–41.0)	0.186
Recessive model				
CC	4	1	4.12 (0.45–37.5)	0.208
CT + TT	96	99		
Dominant model				
CT + CC	40	33	1.35 (0.75–2.41)	0.304
TT	60	67		
Codominant model				
CT	36	32	1.19 (0.66–2.14)	0.550
TT + CC	64	68		
Allele				
T	156 (78)	166 (83)	1.37 (0.83–2.26)	0.208
C	44 (22)	34 (17)		

*P<0.05 was considered statistically significant. AMI: Acute myocardial infarction; CI: Confidence interval; OR: Odds ratio

Table 3: Genotypic, allelic distribution, and association analysis of -786C/T gene polymorphism and risk of chronic stable angina under different genetic models (*n*=100/group)

Genotype/allele	CSA group, *n* (%)	Control group, *n* (%)	OR (95% CI)	*P*
TT (wild)	65 (65)	67 (67)	Reference	Reference
CT	33 (33)	32 (32)	1.06 (0.58–1.92)	0.840
CC	2 (2)	1 (1)	2.06 (0.18–23.2)	0.558
Recessive model				
CC	2	1	2.02 (0.18–22.6)	0.568
CT + TT	98	99		
Dominant model				
CT + CC	35	33	1.09 (0.60–1.96)	0.765
TT	65	67		
Codominant model				
CT	33	32	1.04 (0.57–1.89)	0.880
TT + CC	67	68		
Allele				
T	163 (81.5)	166 (83)	1.10 (0.66–1.85)	0.694
C	37 (18.5)	34 (17)		

*P<0.05 was considered statistically significant. CI: Confidence interval; CSA: Chronic stable angina; OR: Odds ratio

With reference to GG genotype, the frequency of GT genotype was significantly higher in CSA cases than in the control group (P = 0.006); however, the frequency of TT genotype was same in both CSA cases and the controls (P = 0.697). A statistically significant difference was observed in genotypic frequency of GT, but no significant difference was observed in genotypic frequency of TT. The frequency of T allele was significantly higher in CSA cases than in controls (P < 0.05). A statistically significant relationship was observed between 894G/T polymorphism and CSA risk under the dominant (P = 0.008) and codominant genotype models (P = 0.006); however, no significant relationship between the same was observed under the recessive genotype model (P = 1.0) [Tables 4 and 5].

Association of intron 4a4b polymorphism among the cases (acute myocardial infarction and chronic stable angina) and controls

The distribution of genotype and allelic frequency for intron 4a/4b polymorphism in AMI cases vs. controls and CSA cases vs. controls is enlisted in Tables 6 and 7, respectively.

The distribution of 4b/b, 4a4b, and 4a/a genotypes was 72; 72.0%, 26; 26.0%, and 02; 2.0% in AMI cases, 69; 69.0%, 30; 30.0%, and 01; 1.0% in CSA cases and 79; 79.0%, 20; 20.0%, and 01; 1.0% in controls, respectively. With reference to 4b/b genotype, the frequency of 4a4b and 4a/a genotype was higher in AMI cases than in the control group (P = 0.295 and P = 0.524, respectively) as well as was higher in CSA cases than in the control group (P = 0.103 and P = 0.924, respectively); however, the statistical analysis of observed genotypic frequencies did not show any significant association in either of the comparison. The frequency of 4a allele was higher in AMI cases when compared to controls (30; 15.0% vs. 22; 11.0%) and was also higher in CSA cases when compared to controls (32; 16.0% vs. 22; 11.0%). No statistically significant relationship was observed between 4a4b VNTR polymorphism with both AMI and CSA risk under the dominant, codominant, and recessive genotype models (AMI: P =0.251, P = 0.314, and P = 0.568; CSA: P =0.108, P = 0.104, and P = 1.0) [Tables 6 and 7].

Nitric oxide level among the study groups and its association with various risk factors

The mean plasma NO levels (NOx) among the various study groups are depicted in Table 8. NOx levels were found to be significantly higher among the healthy controls when compared to AMI and CSA patients (P = 0.0001). No significant difference was observed between NOx levels in AMI and CSA patients (P = 0.202). NOx levels were found to be comparatively lower among the smokers, alcoholics, and nonvegetarian individuals (in all the three study groups) when compared to nonsmokers, nonalcoholics, and vegetarian individuals [Table 8].

Discussion

Previous studies of our group have shown that various *NOS3* gene polymorphisms are associated with deep vein

Table 4: Genotypic, allelic distribution, and association analysis of 894G/T gene polymorphism and risk of acute myocardial infarction under different genetic models (*n*=100/group)

Genotype/allele	AMI group, *n* (%)	Control group, *n* (%)	OR (95% CI)	*P*
GG (wild)	60 (60)	73 (73)	Reference	Reference
GT	33 (33)	23 (23)	1.74 (0.92–3.28)	0.084
TT	7 (7)	4 (4)	2.12 (0.59–7.62)	0.245
Recessive model				
TT	7	4	1.80 (0.51–6.37)	0.358
GT + GG	93	96		
Dominant model				
GT + TT	40	27	1.80 (0.99–3.27)	0.052*
GG	60	73		
Codominant model				
GT	33	23	1.64 (0.88–3.08)	0.116
GG + TT	67	77		
Allele				
G	153 (76.5)	169 (84.5)	1.67 (1.01–2.77)	0.044*
T	47 (23.5)	31 (15.5)		

*P<0.05 was considered statistically significant. AMI: Acute myocardial infarction; CI: Confidence interval; OR: Odds ratio

Table 5: Genotypic, allelic distribution, and association analysis of 894G/T gene polymorphism and risk of chronic stable angina under different genetic models (*n*=100/group)

Genotype/allele	CSA group, *n* (%)	Control group, *n* (%)	OR (95% CI)	*P*
GG (wild)	55 (55)	73 (73)	Reference	Reference
GT	41 (41)	23 (23)	2.36 (1.27–4.39)	0.006*
TT	4 (4)	4 (4)	1.32 (0.31–5.54)	0.697
Recessive model				
TT	4	4	1.0 (0.24–4.11)	1.0
GT + GG	96	96		
Dominant model				
GT + TT	45	27	2.21 (1.22–3.99)	0.008*
GG	55	73		
Codominant model				
GT	41	23	2.32 (1.26–4.29)	0.006*
GG + TT	59	77		
Allele				
G	151 (75.5)	169 (84.5)	1.76 (1.07–2.91)	0.025*
T	49 (24.5)	31 (15.5)		

*P<0.05 was considered statistically significant. CI: Confidence interval; CSA: Chronic stable angina; OR: Odds ratio

thrombosis and ischemic stroke.[4,11] In the recent study, we have evaluated the role of plasminogen activator inhibitor-1 gene polymorphisms in the occurrence of CAD and revealed the significant association of 4G/5G

Table 6: Genotypic, allelic distribution, and association analysis of intron 4 variable number tandem repeat gene polymorphism and risk of acute myocardial infarction under different genetic models (*n*=100/group)

Genotype/allele	AMI group, *n* (%)	Control group, *n* (%)	OR (95% CI)	*P*
4b/b (wild)	72 (72)	79 (79)	Reference	Reference
4a4b	26 (26)	20 (20)	1.42 (0.73–2.77)	0.295
4a/a	2 (2)	1 (1)	2.19 (0.19–24.71)	0.524
Recessive model				
4a/a	2	1	2.02 (0.18–22.6)	0.568
4a4b + 4b/b	98	99		
Dominant model				
4a4b + 4a/a	28	21	1.46 (0.76–2.80)	0.251
4b/b	72	79		
Codominant model				
4a4b	26	20	1.40 (0.72–2.72)	0.314
4b/b + 4a/a	74	80		
Allele				
4b	170 (85)	178 (89)	1.42 (0.79–2.57)	0.235
4a	30 (15)	22 (11)		

*P<0.05 was considered statistically significant. AMI: Acute myocardial infarction; CI: Confidence interval; OR: Odds ratio

Table 7: Genotypic, allelic distribution, and association analysis of intron 4 variable number tandem repeat gene polymorphism and risk of chronic stable angina under different genetic models (*n*=100/group)

Genotype/allele	CSA group, *n* (%)	Control group, *n* (%)	OR (95% CI)	*P*
4b/b (wild)	69 (69)	79 (79)	Reference	Reference
4a4b	30 (30)	20 (20)	1.71 (0.89–3.29)	0.103
4a/a	1 (1)	1 (1)	1.14 (0.07–18.6)	0.924
Recessive model				
4a/a	1	1	1.0 (0.06–16.2)	1.0
4a4b + 4b/b	99	99		
Dominant model				
4a4b + 4a/a	31	21	1.69 (0.89–3.20)	0.108
4b/b	69	79		
Codominant model				
4a4b	30	20	1.71 (0.89–3.28)	0.104
4b/b + 4a/a	70	80		
Allele				
4b	168 (84)	178 (89)	1.54 (0.86–2.75)	0.145
4a	32 (16)	22 (11)		

*P<0.05 was considered statistically significant. CI: Confidence interval; CSA: Chronic stable angina; OR: Odds ratio

allele polymorphism with high risk of AMI in the Indian population.[12] The present study was conducted to ascertain the role of *NOS3* gene polymorphisms and their plasma level in the occurrence of CAD including AMI and CSA in the North Indian population. We particularly concentrated on three *NOS3* gene polymorphisms (-786C/T, 894G/T,

Table 8: Mean plasma nitric oxide level among the study groups and its association with gender and various risk factors (*n*=100/group)

	Study groups			*P*
	AMI group (µmol/L), mean±SD	CSA group (µmol/L), mean±SD	Control group (µmol/L), mean±SD	
Mean NOx level	37.05±6.75	38.67±5.61	43.80±6.28	<0.0001*
Risk factors				
Smokers	31.19±5.09	37.17±6.23	37.00±8.27	<0.0001*
Nonsmokers	40.34±5.17	39.71±4.93	44.64±5.49	0.0526
Alcoholics	31.76±6.87	35.53±5.08	33.00±6.37	0.0788
Nonalcoholics	38.90±5.68	39.88±5.36	44.61±5.50	<0.0001*
Vegetarian	39.02±6.42	39.07±5.18	45.61±6.49	<0.0001*
Nonvegetarian	35.78±6.71	38.21±6.10	41.39±5.14	<0.0001*
Male	37.04±6.82	38.53±5.73	43.05±6.52	<0.0001*
Female	37.05±6.05	39.20±5.21	46.45±4.55	<0.0001*

*P<0.05 was considered statistically significant. AMI: Acute myocardial infarction; CI: Confidence interval; CSA: Chronic stable angina; NOx: Nitric oxide levels; SD: Standard deviation

and 4a4b) and their influence on development of AMI and CSA. Among our study participants, a clear male preponderance was observed in each group, a finding which is in agreement with previous studies, suggesting that CAD is predominantly a disease of men.[4,11-14]

Atherothrombotic changes are one of the major causes of cardiovascular morbidity and mortality. The underlying process that leads to atheroma formation and coronary thrombosis is complex, multifactorial and involves multiple interrelated systems that regulate hemostasis, vascularity, fibrinolytic pathways, adhesion molecules, and their ligands.[9] Many different genes are known to dictate the regulation of aforesaid traits. Among various such genes, *NOS3* gene responsible for NO production is of special importance.[4] NO, synthesized by *NOS3* gene, is a well-characterized vasodilator that has an important role in maintaining the vascular tone in the systemic and cardiac circulation. Endothelial NO limits platelet activation, adhesion, and aggregation and inhibits atherosclerosis.[4,15,16] Production of NO may be impaired because of *NOS3* gene variations or polymorphisms and evidence suggests that decreased NO production may play an important role in the development of CAD.[11] Since NO has pivotal role in maintaining vascular homeostasis, the *NOS3* becomes an important candidate gene to see if any genetic variation will modulate the development of CAD.

Several known SNPs in *NOS3* gene have been documented to have involvement in the pathogenesis of various vascular diseases including CAD and AMI. We studied three polymorphisms (-786C/T, 894G/T, and 4a4b) of *NOS3* gene to find their association with NO levels in AMI and CSA patients. When compared with controls, we did not find any significant association of these polymorphisms with AMI patients; however, a significant association of 894G/T polymorphism was observed with CSA patients.

Although there are numerous studies reporting either the role of NO level or *NOS3* gene variation in the development of AMI and CSA,[17] very few studies have reported the role of *NOS3* gene variation in the determining NO level and their association with the CAD. Due to the extremely short half-life of NO, plasma NO levels were evaluated indirectly by measuring its metabolites (NOx). When compared with controls, we observed a significant decrease in plasma NOx levels in AMI and CSA ($P < 0.05$), a finding that is in concordance with previous studies.[17] However, some researchers have reported contradictory observations with high NO levels in CAD patients when compared with controls.[18] The contradictory findings may probably be due to the differences in the study population, study designs, environmental factors, ethnicity, hereditary patterns, and lifestyles which can affect the NO metabolism.

Dietary habits can have a significant effect on mortality due to CAD. Various studies have shown a significantly reduced risk of CAD and associated mortality among the individuals consuming vegetarian diet, probably due to lower cholesterol concentrations in vegetarian food than in nonvegetarian food.[19] Similarly, our cohort also showed a significantly increased risk of AMI among the nonvegetarians; however, no significant association was found for the development of CSA among the nonvegetarian individuals. Although hypertension and diabetes are known to be associated with the occurrence of CAD, we failed to obtain any significant difference in the presence of CAD in hypertensive vs. nonhypertensive participants and diabetic vs. nondiabetic participants. We found AMI and CSA to be more often among the individuals who smoke and consume alcohol, a finding that is concordant with previous studies.[13,14,20] Dyslipidemia poses a risk to the development of CAD and we also observed significantly increased levels of serum LDL and total cholesterol and low levels of serum HDL among the patients with CSA and AMI when compared to controls.

The present study has some limitations. First, the small sample size of our study. Due to the different sociocultural

traditions, India has a diverse population; hence, further studies with larger sample size are needed to confirm our findings. Second, as the current study was conducted at the single center, we realize that data extrapolated from our study may not be representative of the whole Indian scenario and must be interpreted cautiously.

A detailed multicentric research study in this aspect would surely give a better insight about the genetic variations and their association with the risk of developing AMI and CSA. Nevertheless, our findings can serve as a template for others to implement the positive aspects of our study and we do feel that the present data can surely be of use for meta-analysis in the future.

Conclusion

Our study findings revealed that out of three NOS3 gene polymorphisms (-786C/T, 894G/T, and 4a4b), only 894G/T polymorphism showed a significant association with AMI under the dominant genotype model and with CSA under dominant and codominant genotype models. Plasma NOx levels were also found to be independently associated with CAD patients.

Such significant association suggests high risk of CAD in the North Indian population. Our study findings will be helpful in identifying the genetic risk factors associated with CAD and better management of the diagnostic as well as therapeutic measures.

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Ethical statement

The study was approved by the Institutional Ethics Committee of All India Institute of Medical Sciences, New Delhi (Approval No. - IESC/T 252/03.06.2011).

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Conflicts of interest

There are no conflicts of interest.

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