

Assessment of the role of plasma nitric oxide levels, T-786C genetic polymorphism, and gene expression levels of endothelial nitric oxide synthase in the development of coronary artery disease

Khalil Mahmoodi, Mohammad Soleiman Soltanpour¹, Koorosh Kamali²

Department of Cardiology, School of Medicine, Zanjan University of Medical Sciences, ¹Department of Medical Laboratory Sciences, School of Paramedical Sciences, Zanjan University of Medical Sciences, ²Department of Public Health, School of Public Health, Zanjan University of Medical Sciences, Zanjan, Iran

Background: Reduced bioavailability of nitric oxide (NO) and the T-786C polymorphism of endothelial nitric oxide synthase (eNOS) gene have been reported as risk factors for the development of coronary artery disease (CAD) with conflicting results. We investigated the association of plasma NO levels, T-786C genetic polymorphism, and gene expression levels of eNOS with CAD risk in an Iranian subpopulation. **Materials and Methods:** Studied population included 100 patients with angiographically verified CAD and 100 ethnically matched controls. Analysis of T-786C genetic polymorphism and gene expression levels of eNOS was conducted by polymerase chain reaction (PCR) restriction fragment length polymorphism and real-time reverse transcription-PCR methods, respectively. Plasma levels of NO were measured using Griess method. **Results:** The CC genotype distribution (15% vs. 6%, $P = 0.011$) and minor C allele frequency (36.5% vs. 21.5%, $P = 0.001$) of eNOS T-786C polymorphism differed significantly between CAD patients and control. Furthermore, eNOS T-786C polymorphism was more common among smoker than nonsmoker CAD patients (27.7% vs. 7.8%, $P = 0.044$). The association of the eNOS T-786C polymorphism with the severity of CAD (number of diseased vessel) was significant ($P < 0.05$). The gene expression levels of eNOS were significantly lower in the heterozygote (0.49 ± 0.1 , $P = 0.023$) and mutant homozygote (0.36 ± 0.09 , $P = 0.011$) genotypes than that of wild-type genotype ($P < 0.05$). In addition, NO levels were significantly lower in CAD patients compared with control subjects (42.62 ± 12.26 vs. 55.48 ± 16.57 , $P = 0.002$) and showed intergenotypic variation in the CAD patients. **Conclusion:** Our study indicated that reduced NO levels and eNOS T-786C genetic polymorphism are significant risk factors for the development and severity of CAD in the Iranian population.

Key words: Coronary artery disease, endothelial nitric oxide synthase, eNOST-786C polymorphism, polymerase chain reaction-restriction fragment length polymorphism

How to cite this article: Mahmoodi K, Soltanpour MS, Kamali K. Assessment of the role of plasma nitric oxide levels, T-786C genetic polymorphism, and gene expression levels of endothelial nitric oxide synthase in the development of coronary artery disease. *J Res Med Sci* 2017;22:34.

INTRODUCTION

Coronary artery disease (CAD) is the leading cause of morbidity and mortality in many countries including Iran. The high prevalence of CAD occurrence in Iranian population and its related complication is one of the most pressing health problems.^[1] Identification of

new risk factors seems to be of great importance for prediction and prevention of coronary events. Several endothelium-derived relaxing factors such as nitric oxide (NO) are involved in the regulating the function of endothelial cells.^[2] It has been widely accepted that endothelial dysfunction occurs in response to numerous cardiovascular risk factors and precedes the development of atherosclerosis.^[3] One of the main regulators of vascular endothelial cells functions is the NO that acts

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

Access this article online	
Quick Response Code: 	Website: www.jmsjournal.net
	DOI: 10.4103/1735-1995.202144

Address for correspondence: Dr. Mohammad Soleiman Soltanpour, Department of Medical Laboratory Sciences, School of Paramedical Sciences, Zanjan University of Medical Sciences, Parvin-e Etesami Boulevard, Zanjan 45157863, Iran. E-mail: soltanpourm@zums.ac.ir

Received: 02-07-2016; **Revised:** 09-10-2016; **Accepted:** 20-12-2016

as an important atheroprotective and vasodilation factor.^[4] NO inhibits several key step of atherogenesis including platelet aggregation, leukocyte adhesion to vascular endothelium, and migration and proliferation of vascular smooth muscle cells.^[5] NO is produced by endothelial nitric oxide synthase (eNOS) from L-arginine in vascular tissues. The gene for eNOS located on chromosome 7q35-q36 and consisted of 26 exons.^[6] A single-nucleotide polymorphism, namely, T-786C (rs2070744), has been identified in the promoter region of eNOS gene and was shown to reduce promoter activity by approximately 50%.^[7,8] The T-786C polymorphism has been proposed as a risk factor for CAD occurrence with conflicting results.^[9-13] Moreover, studies investigated that the association of this common polymorphism with intracellular NO production is limited.^[14] The aim of the present study was to investigate the association of T-786C polymorphism with CAD risk in an Iranian population. Furthermore, the plasma levels of NO, intergenotypic variation of NO levels, and gene expression levels of eNOS were investigated.

MATERIALS AND METHODS

Study population

Our case-control study included 100 patients with a diagnosis of CAD and 100 healthy matched controls. The CAD diagnosis was made by angiography conducted by an expert cardiovascular specialist. The CAD patients (56 male and 44 female) were included in the study when presenting with $\geq 50\%$ stenosis in at least one major coronary vessel. Patients with previous myocardial infarction history showing positive angiogram result were also included in the study. The severity of CAD was determined based on the number of stenotic vessel showing more than 50% stenosis. Accordingly, patients were classified as single-, double-, and triple-vessel stenosis patients. The percentage of stenosis was estimated by visual estimation of luminal diameter in area of stenosis relative to area with normal diameter (without stenosis) for all stenosis. We used a quantitative system for grading of stenosis including normal: absence of plaque/no luminal stenosis; minimal: plaque with $<25\%$ stenosis; mild: 25%–49% stenosis; moderate: 50%–69% stenosis; and severe (occluded) 70%–99% stenosis. Measurement of the left ventricular ejection fraction (LVEF) was done in some patients with echocardiography. Based on the protocol established in our center, the LVEF values $\geq 55\%$ were considered as severely depressed systolic functions while LVEF values $\leq 30\%$ were considered normal.

Patients showing fewer than 50% stenosis were excluded from the study. Patients who suffered from valvular heart disease, cardiomyopathy, inflammatory disease, and other organ failures such as heart failure, renal disease, rheumatoid

arthritis, or cancer were excluded from the study. Clinical assessment of CAD patients revealed that 12% of them had a positive familial history of CAD, 21% had hypertension, 36% had smoking habit, 23% had diabetes mellitus, 44% had hypercholesterolemia, and 46% had hypertriglyceridemia, which were determined based on some published criteria.^[15]

All of the control subjects were selected after careful inspection of a cardiovascular specialist. Control subjects (51 male and 49 female) were selected based on normal angiography results and the absence of any personal or family history of CAD or other reasons to suspect CAD. Furthermore, control subjects suffering from concomitant diseases such as malignant diseases, organ failure, and febrile conditions were excluded. For all subjects, a complete medical history including questions about smoking habits, history of hypertension and diabetes, and family history of heart disease was obtained by questionnaire. The mean age of CAD patients and controls was 59.4 ± 23.5 and 56.7 ± 29.5 , respectively. Both patients and controls were selected from Zanjan population with an Azerian ethnic background. Furthermore, matching of case and controls for age and sex was not performed in this study as the distribution of age and sex was not statistically different between groups.

The estimation of study sample size was based on the previous studies.^[14,16] The minimum required sample size according to Tangurek *et al.*'s^[16] study (CC + TC frequency in case and control group were 60.4% and 40.4%, respectively) was calculated by 97 persons in each group. The calculation of study sample size was done by OpenEpi version 2.2 software (Informer Technologies, Inc. Atlanta, USA) (free online statistical software available at: www.openepi.com) and the following formula

$$n = \frac{\left(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta} \right)^2 (P_1[1-P_1] + P_2[1-P_2])}{(P_1 - P_2)^2} \text{ assuming } \alpha = 0.05, \beta = 0.2, P_1 = 40.4\%, P_2 = 60.4\%.^{[16]}$$

All of the study subjects participated voluntarily in the study and written informed consent was obtained from all participants. The study was approved by the Ethic Committee of Zanjan University of Medical Sciences (Ethical code: ZUMS.REC.1394.344), Zanjan, Iran.

Blood samples collections

From each participant, 5 ml fasting blood was collected in ethylenediaminetetraacetic acid-containing tubes and instantly centrifuged. Plasma fraction was separated and stored at -40°C , and the cellular fraction was used for DNA and RNA extraction.

Endothelial nitric oxide synthase T-786C polymorphism analysis

Genomic DNA was purified from blood leukocytes using a commercial kit (GG2001, Viogene, Poland). The eNOS T-786C polymorphism was genotyped by amplifying the polymorphic site using the following primers forward 5'-CAG ATG CCC AGC TAG TGG-3' and reverse 5'-GGA CCT CTA GGG TCA TGC-3 at an annealing temperature of 62°C. Restriction digestion was performed on 8 µL of polymerase chain reaction (PCR) product using 2 units of MspI restriction enzyme (EURx Ltd., Poland) for 5 h at 37°C. The digested PCR products were electrophoresed on a 2.5% agarose gel and stained with SYBR Green dye. After digestion, the 506 bp amplicon cleaves into 285 and 221 bp in the presence of T allele whereas the C allele produces 285, 177, and 44 bp fragments.

Endothelial nitric oxide synthase gene expression analysis

Total RNA was extracted from blood leukocytes using TRIZOL reagents (Invitrogen, Carlsbad, CA, USA). A commercially available kit (dART RT, EURx Ltd., 80-297 Gdansk, Poland) was used for cDNA construction in a total volume of 20 µl according to the kit instruction. Quantitative real-time reverse transcription (RT)-PCR was performed in an ABI 7300 instrument using high ROS SYBR Green PCR Master Mix Kit (Ampliqon, Denmark). Thermal cycling conditions were as follows: initial activation of the polymerase enzyme at 95°C for 15 min and 35–40 cycles of denaturation at 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s. To normalize target gene expression, β2M was amplified as an internal control in parallel to eNOS gene. The sequence of real-time RT-PCR primers for eNOS gene were as follows: forward: 5'-GTGGCTGGTACATGAGCACT-3', reverse: 5'-GTCTTTCCACAGGGACGAGG-3' and for β2M were forward: 5'-TCTTTCTGGCCTGGAGGCTATC-3', reverse: 5'-CGGATGGATGAAACCCAGACAC-3'. Calculation of fold changes in gene expression levels among different samples was performed by 2-ΔΔCT method.

Biochemical analysis

Determination of NO in plasma samples was performed using the Griess reagent kit (Promega Corporation, Madison, USA). Briefly, plasma samples were deproteinized with 30% zinc sulfate solution. Then, 100 µl of deproteinized plasma samples and 100 µl of appropriate standard

solutions were incubated with 100 µl Griess reagent at 37°C for 30 min. Then, the absorbance of reactions was measured on a microplate reader at 543 nm, and the concentration of NO was determined using a standard curve. Plasma total cholesterol (TC), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-C), and fasting glucose levels were measured using commercially available enzyme assay kits (Pars Azmoon kit, Tehran, Iran) using Mindray Auto Analyzer (BS-200).

Statistical analysis

Numerical variables presented as mean ± standard deviation and were compared using Student's *t*-test for normally distributed samples and Mann-Whitney U-test for nonnormally distributed samples. The Shapiro-Wilk test was used to assess whether the numerical data were normally distributed. Categorical variables were compared with Chi-square test. Furthermore, in case with small sample size [such as those in Table 1], the comparisons of categorical variables were conducted using Fisher's exact test. Logistic regression analysis was used to detect the independent effect of each risk factor in CAD. The deviation of genotype distribution from the Hardy-Weinberg equilibrium was assessed in both patients and controls by a free online calculator. The statistical significance was set at $P < 0.05$ and confidence intervals (CIs) were set at 0.95. All statistical analyses were performed using SPSS 16 software (SPSS Inc., Chicago, IL, USA).

RESULTS

The comparison of clinical and biochemical parameters of the CAD patients and control subjects revealed no significant differences in the mean ages, sex distribution, and TG levels between the two groups ($P > 0.05$). However, CAD patients had significantly higher plasma levels of TC and low-density lipoprotein-cholesterol (LDL-C) and lower plasma levels of HDL-C when compared with the control group. However, the CAD patients had significantly higher prevalence of diabetes, hypertension, and smoking habit compared with control group [Table 2].

The mean plasma levels of NO was significantly lower in the CAD group than the control group (42.62 ± 12.26 vs. 55.48 ± 16.57 , $P = 0.002$) [Table 2]. In addition, the plasma

Table 1: Number of coronary artery disease patients with 1, 2, and 3 diseased vessel according to various genotypes of eNOST-786C polymorphism

eNOST-786C polymorphism	DV			P (2 vs. 1)	P (3 vs. 1)
	1 (n=37), n (%)	2 (n=41), n (%)	3 (n=22), n (%)		
TT	21 (46.3)	17 (47.1)	4 (54.2)	-	-
TC	13 (44.8)	20 (42.4)	10 (29.2)	0.26	0.042
CC	3 (8.9)	4 (10.5)	8 (16.6)	0.54*	0.002*
CC+TC	16 (53.7)	24 (52.9)	18 (45.8)	0.179	0.005

*Fisher's exact test. DV=Diseased vessel; TT=Wild type; TC=Heterozygote; CC=Homozygote; eNOS=Endothelial nitric oxide synthase

levels of NO were significantly lower in heterozygote and mutant homozygote genotypes of eNOST-786C polymorphism than that of wild-type genotype ($P < 0.05$). However, plasma levels of NO did not differ significantly between heterozygote and mutant homozygote genotypes ($P = 0.323$) [Figure 1]. Furthermore, mean plasma levels of NO was significantly lower in smoker compared with nonsmoker CAD patients (36.6 ± 10.8 vs. 45.8 ± 8.7 , $P = 0.001$). However, no significant association was seen between plasma NO levels and TC levels, TG levels, HDL-C and LDL-C levels ($P > 0.05$).

As presented in Table 3, the distribution of TC heterozygote genotype (43% vs. 31%; odds ratio [OR] = 2.1, 95% CI = 1.13–3.80, $P = 0.017$), CC homozygote genotype (15% vs. 6%; OR = 3.75, 95% CI = 1.34–10.44, $P = 0.011$), and C allele (36.5% vs. 21.5%; OR = 2.1, 95% CI = 1.34–3.27, $P = 0.001$) of eNOST-786C polymorphism was significantly different between CAD patients and control subjects ($P < 0.05$). Furthermore, the genotype distribution of

eNOS T-786C polymorphism was in the Hardy–Weinberg equilibrium in both CAD patients ($P = 0.469$) and control subjects ($P = 0.414$), indicating the absence of selection bias in our study. In addition, this polymorphism significantly increased the risk of CAD by 2.76-fold and 2.35-fold under recessive or dominant genetic model, respectively [Table 3]. Furthermore, statistical analysis using Chi-square test demonstrated that –786CC genotype is more common among smoker compared with nonsmoker CAD patients (27.7% vs. 7.8%; OR = 3.56, 95% CI = 1.12–11.22, $P = 0.044$). However, no significant differences in the genotype frequency of T-786C polymorphism were observed between hypertensive and nonhypertensive CAD patients ($P = 0.08$) and also between diabetic and nondiabetic CAD patients ($P = 0.95$). Moreover, the association of eNOS T-786C polymorphism with the severity (number of diseased vessels) of CAD revealed statistically significant differences in the genotype distribution of eNOS T-786C polymorphism between patients with one- and

Table 2: Clinical characteristics of the coronary artery disease patients and the control subjects included in our study

Variables	CAD patients (n=100)	Controls (n=100)	P
Age (years)*	59.4±23.5	56.7±29.5	0.475
Sex (male/female)†	56/44	51/49	0.478
TG (mg/dl)*	180.4±95.6	169.4±75.7	0.395
Total cholesterol (mg/dl)*	192.8±65.4	167.3±49.5	0.002
HDL-C (mg/dl)*	38.6±10.5	43.3±14.8	0.010
LDL-C (mg/dl)*	101.3±56.8	85.5±43.2	0.028
Hypertension, n (%)‡	21 (21)	9 (9)	0.017
Diabetes, n (%)‡	23 (23)	11 (11)	0.024
Smoking, n (%)‡	36 (36)	12 (12)	0.002
Nitric oxide (µmol/l)‡	42.62±12.26	55.48±16.57	0.002

*Student's t-test, †Chi-square test, ‡Mann–Whitney U-test. TG=Triglyceride; HDL-C=High-density lipoprotein-cholesterol; LDL-C=Low-density lipoprotein-cholesterol; CAD=Coronary artery disease

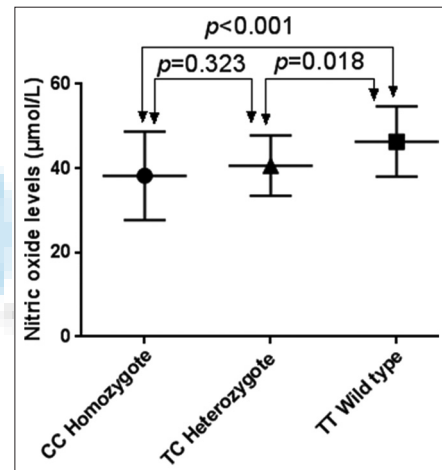


Figure 1: The association between different genotypes of endothelial nitric oxide synthase T-786C polymorphism and mean plasma levels of nitric oxide in coronary artery disease patients

Table 3: Genotype and allele frequency of eNOST-786C polymorphism in coronary artery disease patients and control subjects

eNOST-786C polymorphism	CAD patients (n=100), n (%)	Controls (n=100), n (%)	OR	95% CI	P
Genotype					
TT	42 (42)	63 (63)	Reference	-	-
TC	43 (43)	31 (31)	2.1	1.13-3.80	0.017
CC	15 (15)	6 (6)	3.75	1.34-10.44	0.011
Dominant model					
TT	42 (42)	63 (63)	Reference	-	-
TC + CC	58 (58)	37 (37)	2.35	1.33-4.14	0.003
Recessive model					
TT + TC	85 (85)	94 (94)	Reference	-	-
CC	15 (15)	6 (6)	2.76	1.02-7.45	0.044
Allele					
T	127 (63.5)	157 (78.5)	Reference	-	-
C	73 (36.5)	43 (21.5)	2.1	1.34-3.27	0.001

TT=Wild type; TC=Heterozygote; CC=Homozygote; CAD=Coronary artery disease; eNOS=Endothelial nitric oxide synthase; OR=Odds ratio; CI=Confidence interval

three-diseased vessel but not between patients with one- and two-diseased vessel [Table 1].

Moreover, investigating the gene expression levels of eNOS in CAD patients revealed that mRNA expression levels of eNOS were significantly lower in -786CC genotype than -786TT genotype ($P = 0.011$) [Figure 2]. However, gene expression levels of eNOS did not differ significantly between -786TC genotype and -786CC genotype ($P = 0.076$).

Finally, as indicated in Table 4, logistic regression analysis revealed that the TC, HDL-C, LDL-C, plasma glucose levels, plasma NO levels, TC heterozygote genotype, and CC homozygote genotype were independent risk factors for CAD ($P = 0.001$, $P < 0.001$, $P < 0.001$, $P = 0.002$, $P = 0.037$, $P < 0.001$, $P = 0.003$, $P = 0.009$, respectively), whereas TG ($P = 0.067$), age ($P = 0.296$), and sex ($P = 0.317$) were not independent risk factors for CAD.

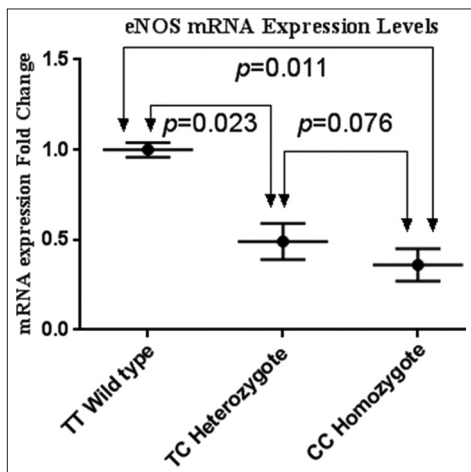


Figure 2: The effects of various genotypes of endothelial nitric oxide synthase T-786C polymorphism on endothelial nitric oxide synthase gene expression levels in coronary artery disease patients

Table 4: Multiple logistic regression analysis of association between the eNOST-786C genotypes and risk of coronary artery disease

Variable	B	SE	Wald	df	Significance
Age	0.016	0.015	1.093	1	0.296
Sex	0.405	0.405	1.001	1	0.317
TG	0.315	0.023	1.109	1	0.067
Total cholesterol	0.020	0.005	17.373	1	$P < 0.001$
HDL-C	-0.083	0.020	17.033	1	$P < 0.001$
LDL-C	0.019	0.006	9.401	1	0.002
GLC	0.018	0.009	4.371	1	0.037
NO	-0.089	0.020	19.587	1	$P < 0.001$
TC genotype	-2.513	0.960	11.377	1	0.003
CC genotype	-0.733	0.820	6.859	1	0.009

TG=Triglyceride; HDL-C=High-density lipoprotein-cholesterol; LDL-C=Low-density lipoprotein-cholesterol; NO=Nitric Oxide; CAD=Coronary artery disease; GLC=Glucose; SE=Standard error

DISCUSSION

The main findings obtained from our study were as follows: (i) reduced plasma levels of NO are risk factors for the development of CAD, (ii) eNOS T-786C polymorphism is a significant risk factor for development and severity of CAD in the Iranian population, (iii) eNOS T-786C polymorphism is associated with decreased gene expression levels of eNOS and acts as a reducing factor for plasma NO levels.

Results of the present study indicated that genotype and allele frequencies of eNOST-786C polymorphism differed significantly between CAD patients and control subjects that were in accordance with some previously published study.^[8-10,13,17] Furthermore, the increased risk of CAD posed by this polymorphism was observed at heterozygote, homozygote, allelic, dominant, and recessive genetic models that confirmed the strong association of this genetic variation with development and severity of CAD.

However, our study was inconsistent with some other studies reporting no association between this polymorphism and CAD risk.^[18-20] The reasons behind these inconsistent results remain to be determined. Yet, numerous factors such as variation in study design, different selection criteria to define CAD patients and control subjects, differences in sample size, numerous gene-gene and gene-environmental interactions, and ethnic background of studied populations may explain the heterogeneity of association studies.^[21] Indeed, a recently published study which investigated the association of the eNOST-786C polymorphism with CAD risk at different populations reported inconsistent results in different ethnicities.^[10] The C allele frequency of eNOS T-786C polymorphism in our study was 36.5% in CAD patients that were similar to reported frequency of C allele in Turkish population^[16] (37.5%), Saudi population^[22] (40%), and Brazilian population^[23] (36%) and were much higher than that reported for Korean population^[24] (13.3%) and China population^[13] (23.1%).

The results of our study are in line with the results of some previously published studies including a meta-analysis study by Rai *et al.* and a case-control study by Salimi *et al.*^[8,14] Although similar results with Rai *et al.* and Salimi *et al.* studies were obtained in our study, neither of the theses studies did not investigate the gene expression levels of eNOS in their study. Furthermore, the effect of T-786C polymorphism on eNOS gene expression levels was not investigated in their study. Our study for the first time investigated the eNOS gene expression levels in relation to eNOS T-786C polymorphism and demonstrated decreased gene expression levels of eNOS gene in carriers of C allele

relative to carriers of T allele. Hence, based on gene expression analysis results in our study, the decreased plasma levels of NO in carriers of TC and CC genotypes relative to TT genotype can be explained with decreased eNOS gene expression levels in carriers of TC and CC genotypes. Since plasma NO levels are determined by three different isoforms (NOS1, NOS2, NOS3 [eNOS]) of NO synthase, our recent finding clearly provides evidence for direct involvement of eNOS T-786C polymorphism in modulating the plasma levels of NO by altering the gene expression levels of eNOS. Consequently, without gene expression analysis, we cannot attribute any change in plasma NO levels to eNOS T-786C polymorphism.

Several lines of evidence suggest that reduced bioavailability of NO may cause endothelial dysfunction and atherosclerosis.^[17,25] The results of the present study also indicated reduced bioavailability of NO as a common finding in the CAD patients. This result may be attributable to the increased frequency of C allele in the CAD patients compared to the control subjects. Moreover, reduced NO levels in atherosclerotic vessels may be caused by reactive oxygen species (ROS) that is generated by oxidative stress in the CAD patients. ROS scavenge NO, thereby reducing NO bioavailability.^[5,26] Our study indicated that eNOS T-786C polymorphism is associated with decreased levels of NO in the CAD patients that were in accordance with some previously published studies.^[17] According to some studies, eNOS-786C allele has been linked with a significant reduction in the promoter activity of eNOS gene that causes decreased gene expression levels of eNOS as well as reduced biosynthesis of NO.^[27,28] Our results also showed lower gene expression level of eNOS in carriers of eNOS-786C allele and provided a causal relationship between this polymorphism and plasma NO levels.

Moreover, a significantly higher prevalence of the eNOS-786C allele observed among smoker compared with nonsmokers CAD patients. This result may be explained by Nohria *et al.*'s study that demonstrated increased Rho kinase (ROCK) activity in smokers. The activation of ROCK leads to reduced biosynthesis of NO through suppression of eNOS in atherosclerotic vessels.^[29] Thus, the synergistic interaction between eNOS and -786CC genotype and smoking leads to reduced biosynthesis of NO that in turn attenuates endothelial function and predisposes patients to increased CAD risk.^[12] Therefore, this result may provide an evidence for a gene-environment interaction in modulating predisposition to the CAD risk. Similar results were reported by Rios *et al.* in a study of 715 patients with CAD in Brazilian population.^[30] Furthermore, eNOS T-786C polymorphism was found to be more prevalent in CAD patients with triple-vessel involvement when compared to patients with single-vessel involvement. This finding

demonstrated that eNOS T-786C polymorphism contributes to the severity of disease in CAD patients, a finding which has been shown in some other studies.^[9]

CONCLUSION

Reduced plasma level of NO is associated with increased risk of CAD in our population. Moreover, eNOST-786C polymorphism is a significant risk factor for development and severity of CAD. Furthermore, eNOST-786C polymorphism is a contributing factor for both decreased gene expression levels of eNOS and reduced plasma levels of NO.

Acknowledgment

We would like to thank all of the volunteers for their participation in the present study.

Financial support and sponsorship

The current research was supported by funds (Grant number: A-12-836-3.1506) provided by Zanjan University of Medical Sciences, Zanjan, Iran.

Conflicts of interest

The authors have no conflicts of interest.

AUTHORS' CONTRIBUTION

- KM contributed in the conception and design of the work, drafting the work and revising it, approval of the final version of the manuscript and agreed for all aspects of the work.
- MSS contributed in the conception and design of the work, conducting the study, analysis or interpretation of data, revising the draft, approval of the final version of the manuscript and agreed for all aspects of the work
- KK contributed in the data analysis and statistical analysis, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work.

REFERENCES

1. Ebrahimi M, Kazemi-Bajestani SM, Ghayour-Mobarhan M, Ferns GA. Coronary artery disease and its risk factors status in Iran: A review. *Iran Red Crescent Med J* 2011;13:610-23.
2. Rahimmanesh I, Shahrezaei M, Rashidi B High blood pressure and endothelial dysfunction: Effects of high blood pressure medications on endothelial dysfunction and new treatments. *J Res Med Sci* 2012;17:S298-311.
3. Gutiérrez E, Flammer AJ, Lerman LO, Elízaga J, Lerman A, Fernández-Avilés F. Endothelial dysfunction over the course of coronary artery disease. *Eur Heart J* 2013;34:3175-81.
4. Soyduñç S, Çelik A, Demiryürek S, Davutoğlu V, Tarakçıoğlu M, Aksoy M. The relationship between oxidative stress, nitric oxide, and coronary artery disease. *Eur J Gen Med* 2007;4:62-6.
5. Tousoulis D, Kampoli AM, Tentolouris C, Papageorgiou N, Stefanadis C. The role of nitric oxide on endothelial function. *Curr*

- Vasc Pharmacol 2012;10:4-18.
6. Marsden PA, Heng HH, Scherer SW, Stewart RJ, Hall AV, Shi XM, *et al.* Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem* 1993;268:17478-88.
 7. Saini V, Bhatnagar MK, Bhattacharjee J. Endothelial nitric oxide synthase Glu298Asp (G894T) gene polymorphism in coronary artery disease patients with type 2 diabetes mellitus. *Diabetes Metab Syndr* 2012;6:106-9.
 8. Rai H, Parveen F, Kumar S, Kapoor A, Sinha N. Association of endothelial nitric oxide synthase gene polymorphisms with coronary artery disease: An updated meta-analysis and systematic review. *PLoS One* 2014;9:e113363.
 9. Narne P, Ponnaluri KC, Singh S, Siraj M, Ishaq M. Association of the genetic variants of endothelial nitric oxide synthase gene with angiographically defined coronary artery disease and myocardial infarction in South Indian patients with type 2 diabetes mellitus. *J Diabetes Complications* 2013;27:255-61.
 10. Liu D, Jiang Z, Dai L, Zhang X, Yan C, Han Y. Association between the -786T>C 1polymorphism in the promoter region of endothelial nitric oxide synthase (eNOS) and risk of coronary artery disease: a systematic review and meta-analysis. *Gene* 2014;545:175-83.
 11. Trimèche-Othmani T, Kammoun M, Callebert J, Abid K, Maatouk F, Mebazaa A, *et al.* Association between genetic polymorphisms of ACE, eNOS, DDAH-2 and ADMA, SDMA and NOx with coronary artery disease in Tunisian population. *Int J Clin Exp Med* 2016;9:4119-24.
 12. Ragia G, Nikolaidis E, Tavridou A, Arvanitidis KI, Kanoni S, Dedoussis GV, *et al.* Endothelial nitric oxide synthase gene polymorphisms -786T>C and 894G>T in coronary artery bypass graft surgery patients. *Hum Genomics* 2010;4:375-83.
 13. Han Y, Xu W, Zhang W, Liu N, Ji Y. T-786C polymorphism in the endothelial nitric oxide synthase gene is associated with increased risk of coronary artery disease in a Chinese population. *Pharmacology* 2010;85:211-6.
 14. Salimi S, Naghavi A, Firoozrai M, Zand H, Tavilani H, Nakhaee A, *et al.* Association of plasma nitric oxide concentration and endothelial nitric oxide synthase T-786C gene polymorphism in coronary artery disease. *Pathophysiology* 2012;19:157-62.
 15. Tabatabaei-Malazy O, Qorbani M, Samavat T, Sharifi F, Larijani B, Fakhrzadeh H. Prevalence of dyslipidemia in Iran: a systematic review and meta-analysis study. *Int J Prev Med* 2014;5:373-93.
 16. Tangurek B, Ozer N, Sayar N, Terzi S, Yilmaz H, Dayi SU, *et al.* The relationship between endothelial nitric oxide synthase gene polymorphism (T-786 C) and coronary artery disease in the Turkish population. *Heart Vessels* 2006;21:285-90.
 17. Saini V, Bhatnagar MK, Bhattacharjee J. Association of endothelial dysfunction with endothelin, nitric oxide and eNOS Glu298Asp gene polymorphism in coronary artery disease. *Dis Markers* 2011;31:215-22.
 18. Abdelhedi R, Kharrat N, Bouayed NA, Abid L, Abdelmouleh W, Sahnoun IT, *et al.* Lack of association of NOS3 and ACE gene polymorphisms with coronary artery disease in Southern Tunisia. *Biochem Genet* 2013;51:92-100.
 19. Sung JH, Lee BE, Kim JO, Jeon YJ, Kim SH, Lim SW, *et al.* Association between eNOS polymorphisms and risk of coronary artery disease in a Korean population: a meta-analysis. *Genet Mol Res* 2015;14:16508-20.
 20. Kincl V, Máchal J, Drozdová A, Panovský R, Vašku A. The relation between eNOS -786 C/T, 4 a/b, MMP-13 rs640198 G/T, eotaxin 426 C/T, -384 A/G, and 67 G/A polymorphisms and long-term outcome in patients with coronary artery disease. *Dis Markers* 2015;2015:232048.
 21. Gorroochurn P, Hodge SE, Heiman GA, Durner M, Greenberg DA. Non-replication of association studies: "pseudo-failures" to replicate? *Genet Med* 2007;9:325-31.
 22. Alkharfy KM, Al-Daghri NM, Al-Attas OS, Alokail MS, Draz HM, Hussain T. Endothelial nitric oxide synthase gene polymorphisms (894G>T and -786T>C) and risk of coronary artery disease in a Saudi population. *Arch Med Res* 2010;41:134-41.
 23. da Costa Escobar Piccoli J, Manfredini V, Hamester FI, Bandinelli JB, Turkienicz IM, Chies JA, *et al.* Interaction between endothelial nitric oxide synthase gene polymorphisms (-786T>C, 894G>T and intron 4 a/b) and cardiovascular risk factors in acute coronary syndromes. *Arch Med Res* 2012;43:205-11.
 24. Kim IJ, Bae J, Lim SW, Cha DH, Cho HJ, Kim S, *et al.* Influence of endothelial nitric oxide synthase gene polymorphisms (-786T>C, 4a4b, 894G>T) in Korean patients with coronary artery disease. *Thromb Res* 2007;119:579-85.
 25. Mudau M, Genis A, Lochner A, Strijdom H. Endothelial dysfunction: The early predictor of atherosclerosis. *Cardiovasc J Afr* 2012;23:222-31.
 26. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007;87:315-424.
 27. Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, *et al.* T-786→C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. *Circulation* 1999 8;99:2864-70.
 28. Doshi AA, Ziolo MT, Wang H, Burke E, Lesinski A, Binkley P. A promoter polymorphism of the endothelial nitric oxide synthase gene is associated with reduced mRNA and protein expression in failing human myocardium. *J Card Fail* 2010;16:314-9.
 29. Nohria A, Grunert ME, Rikitake Y, Noma K, Prsic A, Ganz P, *et al.* Rho kinase inhibition improves endothelial function in human subjects with coronary artery disease. *Circ Res* 2006;99:1426-32.
 30. Rios DL, D'Onofrio LO, Souza JK, Queiroz AM, Raduy-Marón L, Silva-Neto N, *et al.* Smoking-dependent and haplotype-specific effects of endothelial nitric oxide synthase gene polymorphisms on angiographically assessed coronary artery disease in Caucasian- and African-Brazilians. *Atherosclerosis* 2007;193:135-41.