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Endothelial Nitric Oxide Synthase Gene Polymorphisms (894G>T and −786T>C) and Risk of Coronary Artery Disease in a Saudi Population

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Background and Aims. Endothelial nitric oxide synthase gene polymorphisms, either independently or through gene environmental interactions, are associated with cardiovascular diseases in multiple ethnic populations. However, no information is available with regard to such associations in a Saudi population despite a high incidence of cardiovascular abnormalities. We studied the associations of 894G>T and −786T>C polymorphisms of endothelial nitric oxide synthase gene with coronary artery disease in Saudi population.

Methods. Variants 894G>T and −786T>C were studied in 142 coronary artery disease patients and 145 normal controls by PCR-restriction fragment length polymorphism analysis and allele specific PCR, respectively.

Results. Carriers of GT and TT genotypes of 894G>T polymorphism were significantly high ($p < 0.0001$) in patients (47.2 and 7%, respectively) than in controls (27.6 and 4.8%, respectively). Likewise, carriers for TC and CC genotypes of −786T>C polymorphism were significantly high ($p < 0.001$) in patients (50 and 32% respectively) than in controls (34.5 and 22.5% respectively). Both 894G>T [OR (95% CI); 4.39 (1.69−11.42)] and −786T>C [OR (95% CI); 2.74 (1.02−7.32)] variants were independently associated with the disease status. Genotype distributions of 894G>T and −786T>C polymorphisms in the diseased and control populations matched with those found in Caucasian populations.

Conclusion. This study, for the first time, suggests an independent association of 894G>T and −786T>C polymorphisms of endothelial nitric oxide synthase gene with coronary artery disease in a Saudi population.

Key Words: Endothelial nitric oxide synthase gene, Polymorphisms, Coronary artery disease, Genotype distribution.

Introduction

Coronary artery disease (CAD) is the leading cause of cardiovascular-related deaths worldwide. Multiple risk factors including age, sex, smoking, hypertension, diabetes and genetic predisposition influence the onset of CAD (1,2). Atherosclerosis, a prerequisite for the development of CAD, results from a defective endothelial function, which is attributed mainly to an altered production of nitric oxide (NO), a vasodilator and atheroprotective molecule (3). NO is synthesized via a reaction that includes the conversion of L-arginine to L-citruline catalyzed by endothelial nitric oxide synthase (eNOS), which is one of the three isoforms of the enzyme (4). eNOS is the product of *eNOS* gene, which is 21 kb in size and consists of 26 exons (5). Additionally, promoter region of the *eNOS* gene harbors several transcription factor binding sites, regulating gene expression (6).

Because eNOS availability is regulated at transcriptional and posttranscriptional levels and owing to its role in the production of NO, *eNOS* gene is considered to be a potential candidate for cardiovascular diseases (7). Accordingly,

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several *eNOS* gene variants including single nucleotide polymorphisms (SNPs), a variable number of tandem repeats in the intron 4 and a cytosine adenine (CA) repeat microsatellite marker in the intron 13 have been described (8−10). Additionally, sequence variations have also been reported in the promoter region of the *eNOS* gene (11). Of the reported variants of the *eNOS* gene, single nucleotide polymorphism (SNP) at −786 position (786T > C) in the promoter region and G to T transversion at 894 position in exon 7 (894G > T), which results in the incorporation of aspartate in place of glutamate (Glu298Asp), are widely studied and found to be associated with low plasma NO concentrations and reduced vascular reactivity, emphasizing their importance in the onset of CAD (12).

A number of studies have found 894G > T and −786T > C polymorphisms of *eNOS* gene to be associated with the risk of developing cardiovascular diseases either independently or through gene/environmental interactions (8,11,13−20), whereas in contrast, several studies have found the lack of such associations (21−27). To the best of our knowledge, no information is available with regard to the distribution of −786T > C and 894G > T polymorphisms of *eNOS* gene or the association of these gene variants with the risk of developing CAD, despite its high incidence in a Saudi population (28). Thus, we examined the distribution of 786T > C and 894G > T polymorphisms of *eNOS* gene in Saudi CAD patients and normal controls. Additionally, we studied the association of these polymorphisms with the incidence of CAD.

Materials and Methods

Subjects

This study was conducted as a part of an ongoing biomarker screening program at the Diabetes and Endocrine Research Laboratory, Department of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia and includes a total of 142 CAD patients. CAD in the patients was confirmed by angiography with at least one major coronary artery showing > 50% of stenosis. Control subjects included 145 individuals attending the above-referred hospital for routine health checkups. Subjects with a history of myocardial infarction and cerebrovascular disease were excluded from the control cohort. The possibility of subclinical CAD in the control subjects was ruled out by the structured questionnaire and electrocardiogram (ECG). Informed consents were obtained from all subjects prior to their inclusion into the study. A standard questionnaire was obtained from all subjects collecting the information on ethnicity, medical history, smoking habits, presence or absence of hypertension, diabetes, dyslipidemia and current medications. Ex-smokers who had quit cigarette smoking within 1 year of the commencement of the study were excluded. All studied subjects were Saudi Arabian citizens. The study was conducted in accordance with the guidelines set by the Ethics Committee of the Research Center, College of Science, King Saud University, Riyadh, Saudi Arabia.

Clinical and Biochemical Measurements

Clinical and biochemical parameters were measured by standard laboratory procedures. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Subjects with systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 90 mmHg, measured at different time intervals and currently taking anti-hypertensive medication, were considered to be hypertensive. Fasting blood glucose (FBG), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HLD-C) lipid profile was assessed using an autoanalyzer (Konelab, Finland) and concentrations of low-density lipoprotein cholesterol (LDL-C) were calculated using Friedewald's formula. Diabetes in subjects was defined as FBG levels > 126 mg/dl (7 mmol/L) and included patients with history of using antidiabetic medication. Smoking indicates current smokers. Individuals were defined as dyslipidemic if their TC concentration was > 220 mg/dL and/or their TG concentration was > 200 mg/dL.

Genetic Analysis

Genomic DNA was extracted from peripheral blood using Blood Genomic Prep Mini-Spin Kit (GE Health Care, Buckinghamshire, UK). Allele-specific PCR was used with PureTaq Ready-to-Go PCR beads (GE Health Care) for screening −786T > C polymorphism, whereas the 894G > T polymorphism was studied by PCR followed by restriction enzyme digestion of the amplified products.

eNOS *gene −786T > C Polymorphism*

The *eNOS* gene −786T > C polymorphism was examined using the allele-specific primers: 5′-TTTCTCCAGCCCCTCA GATG-3′ (sense); 5′-GGCAGAGGCAGGGTCAGACG-3′ (sense allele-specific T); 5′-CATCAAGCTCTTCCCTGT CT-3′ (antisense allele-specific C); and 5′-AGGCCCAG CAAGGATGTAGT-3′ (antisense). In the presence of C allele, the antisense allele-specific C primer and sense primer will amplify a 176-bp DNA, whereas in the presence of T allele, sense allele-specific T primer and antisense primer will amplify 250-bp DNA. Sense and antisense primers will amplify a 387-bp common product. CC genotype will produce 176- and 387-bp products after amplification and TT genotype will produce 250- and 387-bp products, whereas TC genotype will generate 176-, 250- and 387-bp products. PCR conditions include an initial denaturation at 95°C for 4 min followed by 35 amplification cycles, each consisting of denaturation at 94°C for 30 sec, annealing at

60°C for 30 sec and extension at 72°C for 30 sec. Amplified products were analyzed by agarose gel electrophoresis.

eNOS *gene 894G>T polymorphism*

Forward and reverse primers used to screen 894G>T polymorphism were 5′-CATGAGGCTCAGCCCCAGAAC-3′ and 5′-AGTCAATCCCTTTGGTGCTCAC-3′. Thermocycling conditions consisted of initial denaturation at 95°C for 4 min followed by 35 cycles of amplification, each including denaturation for 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 2 min. PCR products were digested by MboI restriction endonuclease (New England Biolabs, UK) for 4 h at 37°C and resolved by electrophoresis on a 2.5% agarose gel. The 206-bp PCR product was cleaved into 119- and 87-bp fragments in the presence of a T at nucleotide 894, which corresponds to Asp298 but not in its absence.

Statistical Analysis

Frequency is expressed as percentage with 95% confidence interval (CI) and continuous variables are expressed as mean ± standard deviation. Cross tabulation analysis was utilized to determine differences in frequencies. Multinomial logistic regression analysis using the presence of CAD as dependent variable and other risk factors as independent variables was used to determine odd ratios. Significance was set at $p < 0.05$. Data were analyzed using SPSS v. 11.5 (SPSS Inc, Chicago, IL).

Results

Demographic and clinical parameters of 142 CAD patients and 145 control subjects are presented in Table 1. Among the studied cohort of subjects, patients were significantly older than control subjects (59.5 ± 9.8 vs. 43.1 ± 12.9, $p < 0.0001$). No significant gender difference was found between patients and control, whereas hypertension (70.4 vs. 8.3%, $p < 0.0001$), diabetes (31 vs. 0%, $p < 0.0001$) and dyslipidemia (69.7 vs. 8.9%, $p < 0.0001$) were significantly higher in patients than controls. Additional details on anthropometric, clinical and biochemical parameters are provided in Table 1. Nonsmokers and smokers were comparable between the patients and controls. Among the CAD cases, 50.7% were positive for family history for CAD, whereas 29.7% of control subjects had positive family history.

Distribution and allele frequencies of 894G>T and −786T<C polymorphisms among the patients and controls are shown in Figures 1 and 2, respectively. Agarose gel electrophoresis of samples for 894G>T and −786T<C polymorphisms is shown in Figures 3A and 3B. Distribution of genotypes and allele frequencies of both polymorphisms in patients and controls satisfied the Hardy-Weinberg equilibrium. Genotype distribution of 894G>T polymorphism in patients and controls were 45.8 vs. 67.6% (GG), 47.2 vs. 27.6% (GT) and 7 vs. 4.8% (TT), respectively, and was found to be statistically significant ($p < 0.0001$). Allele frequencies of 894G>T polymorphism in patients and controls were 69.4 vs. 80.5% (G allele) and 30.6 vs. 19.5% (T allele), respectively ($p = 0.002$). The genotype distribution of −786T>C polymorphism in patients and controls was 18.0 vs. 43.0% (TT), 50.0 vs. 34.5% (TC) and 32.0 vs. 22.5% (CC), respectively, and was found to be significant ($p = 0.001$). Allele frequencies of −786T>C polymorphism in patients and controls were

Table 1. Demographic and clinical characteristics of the study population

	CAD cases (n = 142)	Controls (n = 145)	p value
Mean (SE) age, years	59.5 ± 9.8	43.1 ± 12.9	<0.0001
BMI (kg/m^2)	30.7 ± 6.2	29.4 ± 5.1	0.09
Male sex, n (%)	83 (58.4)	74 (51)	0.19
Hypertension, n (%)	100 (70.4)	12 (8.3)	<0.0001
Diabetes, n (%)	44 (31.0)	0 (0)	<0.0001
Stroke, n (%)	14 (9.9)	0 (0)	<0.0001
Dyslipidemia, n (%)	99 (69.7)	13 (8.9)	<0.0001
Smoking, n (%)	27 (19.0)	30 (20.7)	0.58
Family history of CAD, n (%)	72 (50.7)	43 (29.7)	0.001
SBP (mmHg)	137.2 ± 18.3	118.9 ± 18.2	<0.0001
DBP (mmHg)	80.8 ± 12.6	77.6 ± 10.3	0.06
HDL-C (mmol/l)	0.98 ± 0.3	0.97 ± 0.4	0.91
LDL-C (mmol/l)	3.2 ± 0.9	2.7 ± 1.0	<0.0001
TC (mmol/l)	4.5 ± 1.0	4.8 ± 1.0	0.02
TG (mmol/l)	1.8 ± 1.0	1.0 ± 0.7	<0.0001
FBG (mmol/l)	8.9 ± 3.8	5.8 ± 2.1	<0.0001

CAD, coronary artery disease; SE, standard error; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides, FBG, fasting blood glucose.

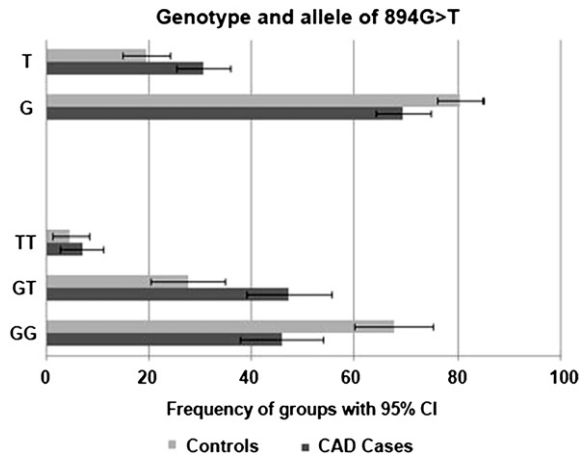

Figure 1. Genotype and allele frequencies of 894G>T polymorphisms of the *eNOS* gene in coronary artery disease (CAD) patients and controls.

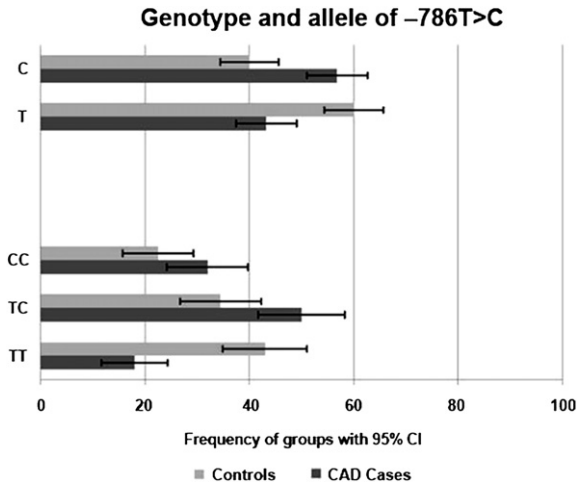

Figure 2. Genotype and allele frequencies of −786T>C polymorphisms of the *eNOS* gene in CAD patients and controls.

43.2 vs. 60.0% (T allele) and 56.8 vs. 40.0% (C allele), respectively (p <0.0001). The combined frequency of 894G>T and −786T<C polymorphisms in the patient and control population was found to be 2.8%.

Associations of 894G>T and −786T<C polymorphisms with CAD are presented in Table 2. Significant associations were found between −786T>C polymorphism and the incidence of CAD, with odds ratios (OR) of 3.31 (TT vs. CC, 95% CI; 1.73−6.32, p <0.001), and 3.41 (TT vs. TC+CC, 95% CI; 1.98−5.87, p <0.001), whereas no significant association was found between TT vs. TC genotypes with OR of 0.95 (95% CI; 0.53−1.7, p = 0.45). Similarly, significant associations were found between 894G>T polymorphism and risk of developing CAD with OR of 2.52 (GG vs. GT, 95% CI 1.53−4.17; p <0.001) and 2.47 (GG vs. GT + TT, 95% CI; 1.53−3.99, p <0.001), whereas no significant association was found between GG vs. TT genotypes with OR of 2.15 (95% CI 0.78−5.94, p = 0.133). Additionally, adjusted odds ratios for cardiovascular risk factors including age, male gender, hypertension, diabetes mellitus (T2DM), dyslipidemia, and smoking for *eNOS* gene 894G>T genotype in CAD patients are given in Table 3. CAD patients with higher age, diabetes, hypertension, dyslipidemia, and smoking history had a significantly high OR of 894GT/894TT genotypes combined. Similarly, CAD patients with higher age, male gender, diabetes, hypertension, dyslipidemia, and smoking history had a significantly higher OR of −786CC genotype and −786CC/−786TC genotypes combined (Table 4).

Discussion

In this study we found that CAD and control subjects differed significantly in genotype and allele frequencies of both 894G>T and −786T<C polymorphisms of *eNOS* gene. Additionally, both these variants are independently associated with CAD even after adjusting for several potential CAD risk factors. Furthermore, genotype frequencies of 894G>T and −786T<C polymorphisms in the studied population are inclined towards Caucasian rather than Asian population.

Owing to the central role played by the eNOS in the synthesis of NO, *eNOS* gene has been extensively studied as a potential candidate for cardiovascular diseases. Accordingly, several polymorphisms in the *eNOS* gene were identified and found to be associated with eNOS production and/or function, ultimately affecting NO production. It has been shown that eNOS protein containing aspartate at position 298 as a result of 894G>T polymorphism is subjected to selective proteolytic cleavage in endothelial cells and vascular tissues, implying that cleaved fragments would be

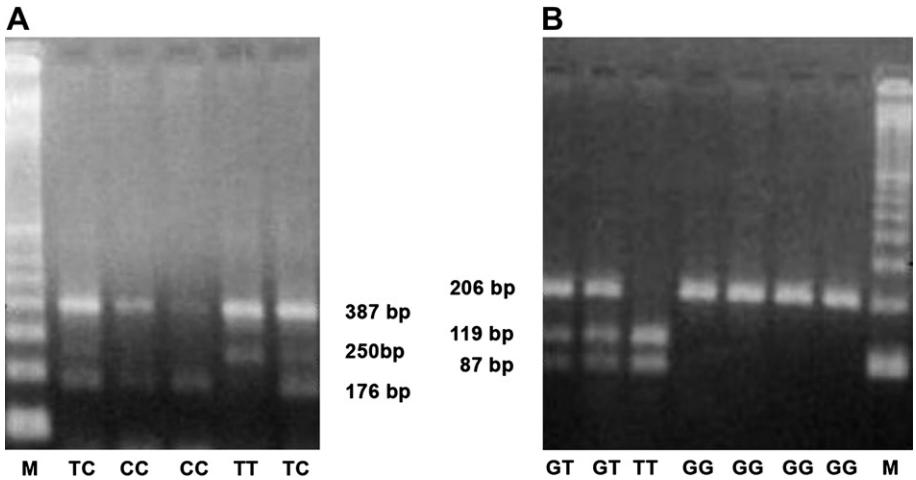

Figure 3. Agarose gel electrophoresis of samples showing the −786T>C and 894G>T polymorphisms of *eNOS* gene. (A) Allele-specific PCR amplified samples showing the TT, TC and CC genotypes of −786T>C polymorphism. M; 100-bp molecular weight marker. (B) PCR-restriction fragment length polymorphism analysis of samples showing GG, GT and TT genotypes of 894G>T polymorphism. M; 100-bp molecular weight marker.

Table 2. Odds ratios (OR) for CAD among carriers (n = 287) of the −786T>C and 894G>T polymorphisms

Genotype	Reference group	OR (95% CI)[a]	p value
CC	TT	3.31 (1.73–6.32)	<0.001
TC	TT	0.95 (0.53–1.7)	0.45
CC+TC	TT	3.41 (1.98–5.87)	<0.001
TT	GG	2.15 (0.78–5.94)	0.133
GT	GG	2.52 (1.53–4.17)	<0.001
TT + GT	GG	2.47 (1.53–3.99)	<0.001

[a]CI, confidence interval.

expected to lack eNOS activity (29–32). The −786T>C polymorphism in the promoter region of *eNOS* gene is associated with lower eNOS mRNA and serum nitrite/nitrate levels (33). Reporter gene assays and the promoter binding activities have confirmed the effect of −786T>C polymorphism on eNOS activity (11,34).

Association of 894G>T *eNOS* gene variant with cardiovascular diseases has been examined in different ethnic populations. The 894G>T polymorphism is significantly and independently associated with the occurrence and severity of CAD in an Italian population (14,35). Similarly, in a Japanese population, 894G>T polymorphism is significantly correlated with coronary spasm, myocardial infarction and acute coronary syndrome (20,36,37). The 894G>T polymorphism was found to be a major risk factor for CAD in a UK population (8) and with systolic dysfunction in U.S. subjects (38). Likewise, 894G>T polymorphism is significantly associated with premature CAD in a Turkish population (16,39). Importantly, the meta-analysis of the 894G>T polymorphism including 42 studies and comprising 13,876 CAD cases and 13,042 controls revealed an OR of 1.17 (95% CI 1.07–1.28, p <0.001) (12). The significant association of 894G>T polymorphism of *eNOS* gene with CAD in the studied population is consistent with the above data and suggests an increased risk for CAD in individuals harboring this variant. Additionally, the association of 894G>T polymorphism with CAD risk remained significant even after adjusting for potential risk factors, indicating a direct link with the incidence of CAD. Contrastingly however, several studies have found the lack of association of 894G>T polymorphism with cardiovascular diseases (13,15,22,23,25,40–45).

Several studies have also demonstrated a positive association between the presence of eNOS −786T>C polymorphism and the development of cardiovascular diseases (11,13–15,17,35,46,47). Similarly, a meta-analysis, which is based on 20 articles and comprising 11236 cases and 13562 controls, revealed the per allele odds ratio of 1.17 (95% CI 1.07–1.28, p <0.001) for 786T>C (12). In agreement with the above data, the −786T>C genotype is significantly associated with the incidence of CAD in the studied population. Additionally, the association between −786T>C polymorphism and CAD risk sustained significance even after adjusting for the potential risk factors, suggesting that this polymorphism can independently influence the onset of disease etiology. In contrast to association between −786T>C polymorphism and CAD, several studies have found the absence of such association (21–24). Ambiguity in these association studies involving 894G>T and −786T>C polymorphisms and cardiovascular diseases may be attributed to differences in ethnicity, sample size of the studied subjects, gene/environmental interactions or the inclusion and exclusion criteria of the subjects. In the studied population, both 894G>T and −786T>C polymorphisms exhibited significant interactions with several CAD risk factors and showed considerable shift in the ORs, indicating that these risk factors can significantly affect overall outcome of the disease pathology in a positive or negative manner. Although the associations of 894G>T and −786T>C

Table 3. ORs for cardiovascular risk factors by 894G>T genotype distribution in patients (n = 142) and controls (n = 145)

	GT	TT	TT + GT
Crude OR	2.52 (1.53–4.17)[b]	2.15 (0.78–5.94)	2.47 (1.53–3.99)[b]
Age, years (A)	1.23 (0.31–4.86)	4.15 (1.07–16.12)[a]	3.48 (1.72–7.02)[b]
Males (G)	0.94 (0.27–3.26)	2.63 (0.80–8.62)	2.77 (1.53–5.01)[b]
T2DM	0.79 (0.21–3.03)	2.83 (0.75–10.65)	3.45 (1.79–6.68)[b]
Hypertension (H)	0.86 (0.19–3.90)	2.06 (0.47–9.06)	2.35 (1.14–4.88)[a]
Dyslipidemia (D)	0.29 (0.05–6.37)	1.26 (0.25–6.37)	3.53 (1.55–8.08)[b]
Smoking (S)	1.04 (0.31–3.53)	2.92 (0.89–9.58)	2.81 (1.56–5.08)[b]
A + G + H	1.25 (0.23–6.72)	3.30 (0.65–16.80)	2.74 (1.21–6.17)[a]
A + G + D	0.40 (0.06–2.80)	1.95 (0.30–12.56)	4.29 (1.73–10.62)[b]
A + G + S	0.99 (0.24–4.05)	3.86 (1.0–15.01)[a]	3.91 (1.88–8.12)[b]
A + G + H + D + S	0.44 (0.05–4.13)	1.75 (0.21–14.80)	3.56 (1.34–9.47)[a]

Data are expressed as OR (95% CI).
OR, odds ratio; CI, confidence interval; T2DM, type 2 diabetes mellitus.
[a]Denotes significance at <0.05.
[b]Denotes significance at <0.01.

Table 4. ORs for cardiovascular risk factors by eNOS −786T >C genotype distribution in patients (n = 142) and controls (n = 145)

	TC	CC	CC + TC
Crude OR	0.95 (0.53−1.70)	3.31 (1.73−6.32)[b]	3.41 (1.98−5.87)[b]
Age years (A)	1.30 (0.59−2.87)	2.54 (1.04−6.20)[a]	2.17 (1.02−4.61)[a]
Males (G)	1.17 (0.58−2.34)	3.77 (1.70−8.34)[b]	3.43 (1.75−6.72)[b]
T2DM	1.18 (0.55−2.54)	3.26 (1.36−7.85)[b]	2.95 (1.40−6.22)[b]
Hypertension (H)	1.41 (0.59−3.35)	4.42 (1.62−12.11)[b]	3.60 (1.53−8.44)[b]
Dyslipidemia (D)	1.25 (0.49−3.18)	3.52 (1.22−10.19)[a]	3.07 (1.25−7.54)[a]
Smoking (S)	1.17 (0.58−2.34)	3.80 (1.71−8.46)[b]	3.46 (1.76−6.81)[b]
A + G + H	1.62 (0.62−4.23)	3.50 (1.19−10.31)[a]	2.59 (1.06−6.32)[a]
A + G + D	1.45 (0.52−4.06)	2.65 (0.85−8.31)	2.11 (0.82−5.43)
A + G + S	1.30 (0.41−1.68)	2.53 (1.03−6.19)[a]	2.16 (1.01−4.61)[a]
A + G + H + D + S	2.23 (0.69−16.26)	4.30 (1.14−16.26)[a]	2.51 (0.88−7.17)

Data are expressed as OR (95% CI).
OR, odds ratio; T2DM, type 2 diabetes mellitus.
[a]Denotes significance at <0.05.
[b]Denotes significance at <0.01.

polymorphisms with CAD are independent for the studied risk factors, there may however be additional unaccounted risk factors existing in the individuals influencing the disease risk, possibly explaining why the control subjects are disease free despite the presence of these polymorphisms. Therefore, these SNPs must be considered independent risk factors for CAD only for the adjusted risk factors.

We also examined the genotype distribution of 894G>T and −786T>C *eNOS* gene polymorphisms in control population in order to assess the prevalence of genetic predisposition for CAD in the Saudi population in comparison to other ethnic populations. Genotype distribution of 894G>T and −786T>C polymorphisms in the control population was found to be comparable with Caucasian but not with Asian populations (Table 5). It will be of interest to follow-up this observed genetic susceptibility in the control population and possible interaction with the potential risk factors with respect to the development of CAD.

Together we showed for the first time that 894G>T and −786T>C polymorphisms of *eNOS* gene were independently associated with CAD in a Saudi population and in combination with potential risk factors can influence the risk of developing the CAD. Additionally, distributions of 894G>T and −786T>C genotypes in CAD patients and control population were inclined towards Caucasians as compared to Asians. Our study, however, has several limitations. The first is the lack of functional studies. Whether the 894G>T and −786T>C polymorphisms functionally underlies a mechanism leading to premature CAD should be determined. Second, due to relatively small sample size, the observations need to be followed-up with a much larger sample size to make a causal inference.

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Table 5. Genotype distributions of 894G>T and −786T>C polymorphisms of eNOS gene in control populations in relation to ethnicity

Genotype	British[a] n = 600 (%)	Italian[b] n = 537 (%)	Japanese[c] n = 119 (%)	Korean[d] n = 222 (%)	Saudi Arabia[e] n = 145 (%)
894GG	242 (40.5)	236 (43.9)	106 (89.1)	181 (81.5)	98 (67.6)
894GT	294 (49.2)	243 (45.2)	13 (10.9)	38 (17.1)	40 (27.6)
894TT	62 (10.4)	58 (10.8)	0 (0)	0 (0)	7 (4.8)
−786TT	220 (36.7)	199 (37.0)	97 (81.5)	182 (82.0)	62 (42.7)
−786TC	283 (47.2)	260 (48.3)	22 (18.5)	38 (17.3)	50 (34.5)
−786CC	96 (16.0)	78 (14.5)	0 (0)	0 (0)	33 (22.8)

[a]Hassan et al. (19).
[b]Fatini et al. (21).
[c]Asakimori et al. (20).
[d]Kim et al. (13).
[e]Present study.

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