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Variants of endothelial nitric oxide synthase gene are associated with components of metabolic syndrome in an Arab population

Khalid M. Alkharfy[1), 2)], Nasser M. Al-Daghri[2),3)], Omar S. Al-Attas[2), 3)], Majed S. Alokail[2), 3)], Abdul Khader Mohammed[2)], Benjamin Vinodson[2)], Mario Clerici[2), 4)], Usamah Kazmi[2)], Tajamul Hussain[3)] and Hossam M. Draz[2), 5)]

[1)]*Department of Clinical Pharmacy, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*
[2)]*Biomarkers Research Program, Biochemistry Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*
[3)]*Center of Excellence in Biotechnology Research, King Saud University, Riyadh 11451, Saudi Arabia*
[4)]*Don C. Gnocchi Foundation and Department of Biomedical Sciences and Technology, Milano University Medical School, Milano 20100, Italy*
[5)]*Department of Biochemistry, National Research Centre, Dokki, Cairo 12311, Egypt*

Abstract. Genetics plays a crucial role in the development of metabolic syndrome (MetS). Here we examined the association between endothelial nitric oxide synthase (*eNOS*) gene polymorphisms and MetS in a Saudi Arabian cohort to extend the understanding of the genetic basis of MetS in diverse ethnic populations. Anthropometric, clinical and biochemical parameters as well as genotyping for 894G>T, -786T>C variants of *eNOS* gene by PCR-RFLP and 4a/b by direct PCR were performed in 886 Saudi Arabians (477 MetS and 409 Non-MetS). The genotype distribution (TT, p=0.001; TC, p=0.001; TC+CC, p=0.001) and allele (T, p=0.007; C, p=0.007) frequency of the -786T>C SNP were significantly different between Non-MetS and MetS subjects which remained significant after Bonferroni correction. Moreover: 1) the GT and GT+TT genotypes of the 894G>T SNP were associated with elevated blood pressure (p=0.017, and p=0.022, respectively); 2) the ab variant of 4a/b polymorphism was associated with decreased HDL levels (p= 0.044); and 3) the TC+CC genotype and C allele of the -786T>C SNP were associated with increased fasting glucose levels (p=0.039, and p=0.028, respectively). Also, G-a-C was identified as the risk haplotype for MetS susceptibility (p=0.034). The results suggest a significant association of 894G>T, 4a/b and -786T>C polymorphisms with MetS and its components is present in an Arab population. A genetic predisposition to develop abnormal metabolic phenotypes, consistent with an increased prevalence of metabolic phenotypes can be detected in this ethnic group.

Key words: Nitric oxide synthase, Gene polymorphism, Metabolic syndrome, Saudi Arabians

IT HAS BEEN SHOWN that components of the metabolic syndrome (MetS) (dyslipidemia, hyperglycemia, hypertension and obesity), individually and cumulatively, increase the risk of developing diabetes mellitus type 2 (DMT2) and cardiovascular diseases (CVD) [1-5]. Furthermore, endothelial dysfunction is a common feature of MetS components [6, 7], and can result from multiple mechanisms, including the reduced bioavailability of nitric oxide (NO) in the vasculature [8, 9]. NO is synthesized by a catalytic action of endothelial nitric oxide synthase (eNOS), and the acquired defects in its synthesis are associated with the components of MetS [3, 10, 11]. Consistently, mice lacking eNOS are affected by insulin resistance, hyperlipidemia, and hypertension [12, 13].

eNOS is encoded by the *eNOS* gene, which is mapped to chromosome 7q36; this gene has been extensively screened for genetic polymorphisms [14, 15]. G to T substitution at nucleotide 894 in exon 7 (894G>T), an insertion-deletion at 7q35-q36 region in intron 4 (4a/b) consisting of two alleles of *eNOS* gene, and a single nucleotide polymorphism (SNP) in the promoter region (-786T>C) are commonly studied because of their relevance to eNOS activity. 894G>T

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Correspondence to: Khalid M. Alkharfy, Pharm.D., Ph.D., Department of Clinical Pharmacy, College of Pharmacy, King Saud University, Riyadh, 11451, Saudi Arabia.
E-mail: alkharfy@ksu.edu.sa

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SNP results from an amino acid change from glutamate to aspartate at codon 298 (Glu298Asp) and causes a structural change of the eNOS protein that down regulates eNOS activity [16-19]. A 27 bp variable tandem repeats polymorphism in intron 4 (also called *eNOS* 4a/b) has been associated with variations in NO, nitrite, and nitrate plasma levels that may reflect the activity of eNOS, and -786T>C SNP reduces the promoter activity and affects *eNOS* protein expression and eNOS activity [16, 17].

Only very limited and contrasting informations are available on the possible association of 894G>T, 4a/b and -786T>C SNPs of *eNOS* gene with MetS, [20-25], and, in particular, no data are present in the literature on the likelihood that these genetic factors could play a role in the development of MetS in the Arab nations. These data are urgently needed considering that the incidence of MetS and its components is rising dramatically in many Arab countries including Saudi Arabia. We verified possible links between selected SNPs in *eNOS* gene and the features of MetS in a Saudi population in an attempt to shed light on the genetic basis of MetS in such population.

Research Design and Methods

Subjects

Eight-hundred-eighty-six native Saudi Arabians (477 MetS and 409 non-MetS) that are part of the Biomarker Screening Project in Riyadh (RIYADH COHORT), a capital-wide epidemiologic study taken from consenting subjects coming from different Primary Health Care Centers (PHCCs). The features of MetS include waist circumference ≥ 102 cm for men and ≥ 88 cm for women, triglycerides ≥ 1.7 mmol/L, HDL-cholesterol < 40 mg/dL for men and < 50 mg/dL for women, blood pressure ≥ 130/85 and fasting plasma glucose levels ≥ 5.6 mmol/L. Diagnosis was based on the International Diabetes Federation (IDF), which defines MetS as central obesity plus 2 other factors. Non-MetS subjects were those who did not match the criteria employed for the selection of MetS subjects. A standard questionnaire was also obtained from all subjects, collecting the information on demographics, medical and family histories, and current medications. Study was approved and 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 anthropometric parameters including blood pressure, weight, height, hip and waist circumferences were measured following the standard procedures. Body mass index (BMI) was calculated as weight/height^2 (Kg/m^2). Fasting blood samples were collected and the plasma glucose, triglyceride, total and HDL-cholesterol levels were measured by chemistry auto-analyzer (Konelab, Espoo, Finland) and concentrations of LDL-cholesterol were calculated using Friedwald's formula.

Genetic analysis

Genomic DNA was extracted from the peripheral blood using Blood genomic prep mini spin kit (GE Health Care, Buckinghamshire, UK) following the manufacturer's instruction. *eNOS* 4a/b polymorphism was screened by direct polymerase chain reaction (PCR)-amplification using KAPA Taq ready mix DNA Polymerase (Kapa Biosystems, Cambridge, MA, USA), while the 894G>T and -786T>C variants were studied by PCR followed by restriction enzyme digestion of the amplified products.

eNOS gene 894G>T and -786T>C polymorphisms

For 894G>T polymorphism, primers 5′- CAT GAG GCT CAG CCC CAG AAC -3′ (sense) and 5′- AGT CAA TCC CTT TGG TGC TCA C -3′ (antisense) were used to a amplify 206 bp DNA fragment where as for -786T>C primers 5′- TGG AGA GTG CTG GTG TAC CCC A -3′ (sense) and 5′- GCC TCC ACC CCC ACC CTG TC -3′ (antisense) were used to amplify 180 bp DNA fragment. PCRs were performed in a thermal cycler with initial denaturation at 95°C for 5 min followed by 35 cycles with each cycle containing denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. For 894G>T SNP, the PCR product (10 µL) was digested with 2 U MboI restriction endonuclease (New England Biolabs, Hertfordshire, UK) for 5 hours at 37°C and resolved by electrophoresis on a 2.5% agarose gel, The 206 bp PCR product was cleaved into 119 bp and 87 bp fragments in the presence of a T at nucleotide 894, which corresponds to Asp298 but not in its absence. Whereas for -786T>C polymorphisms, 10 µL of PCR product was digested with 4U MspI (New England Biolabs, Hertfordshire, UK) for 5h at 37°C, producing fragments of 140 and 40 bp for the wild-type allele (allele "T"), or 90, 50, and 40 bp in the case of a polymorphic variant (allele "C"). The digested fragments resolved

by electrophoresis on a 2.5% agarose gel.

eNOS gene 4a/b polymorphism

DNA fragment was amplified by PCR using primer pair 5′-AGG CCC TAT GGTAGT GCC TTT-3′ (sense) and 5′-TCT CTTAGT GCT GTG GTC AC-3′ (antisense) in a final volume of 20 µL at annealing temperature of 55 °C. The amplified products were analyzed by electrophoresis on a 3% agarose gel. The large allele, *eNOS4b*, contains 5 tandem 27 bp repeats and the smaller allele, *eNOS4a*, contains 4 repeats. The sizes of the PCR products were 393 bp and 420 bp respectively for the *eNOS4a* and *eNOS4b* alleles.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences for Windows (SPSS version 16.0, Chicago, IL, USA) and are expressed by mean ± standard deviation (SD). Independent sample t-test was used to test control and metabolic syndrome groups. Significance was set at $p < 0.05$. Allele and Genotype frequency differences between patients and controls were tested for each SNP using a chi-square test. Odds ratios (ORs) and 95% confidence intervals are calculated by multinomial logistic regression for the allele, genotype and haplotype frequencies. The most common haplotype was used as the reference and rare haplotypes were dropped from the analysis. We applied a Bonferroni correction testing at a significance level of α (0.05)/3 (number of tested SNPs) to lower the chance of a type 1 error. Haplotype frequencies were estimated by the Expectation-Maximization algorithm (EM algorithm) implemented in PROC Haplotype in SAS Genetics statistical software package (SAS institute, Cary, NC, USA). The standardized measure of linkage disequilibrium (LD), termed D' and R^2, was computed at pairs of SNP loci. Tests of departures from LD were performed by using the likelihood ratio test (LR test) of linkage disequilibrium as used in PROC Allele of SAS Genetics. Pairwise LD estimations were performed using Haploview 4.2 [26]. Power analysis was performed using G Power 3.1.2 [27].

Results

The anthropometric, clinical and biochemical features of individuals enrolled in the study are presented in Table 1. Subjects with MetS were significantly older, and had increased BMI, waist and hip circumferences, sagittal abdominal diameter (SAD), systolic and diastolic blood pressure, cholesterol, triglycerides, glucose, LDL-cholesterol and decreased HDL-cholesterol compared to non-MetS Subjects.

Genotype and allele distribution

The genotype and allele distribution of 894G>T, 4a/b, and -786T>C variants is summarized in Table 2. Distribution of genotypes and allele frequencies of the three polymorphisms in patients and controls satisfied the Hardy-Weinberg equilibrium. The genotype frequencies for the 4a/b and -786T>C polymor-

Table 1 Epidemiologic, anthropometric and metabolic characterization of the individuals enrolled in the study

	Non-MetS (N =409)	MetS (N =477)	*p*-value
Male/Female	193/216	226/251	
Age (years)	40.5 ± 12.4	50.1 ± 12.3	<0.001
Body Mass Index (kg/m^2)	29.6 ± 5.9	32.5 ± 5.3	<0.001
Waist (cm)	90.5 ± 19.3	103.9 ± 15.2	<0.001
Hips (cm)	101.4 ± 21.2	109.8 ± 17.3	<0.001
WHR	0.88 ± 0.16	0.95 ± 0.12	<0.001
SAD (cm)	22.2 ± 6.6	25.4 ± 6.9	<0.001
Systolic BP (mmHg)	117.9 ± 12.8	129.3 ± 14.5	<0.001
Diastolic BP (mmHg)	76.5 ± 7.8	82.1 ± 9.1	<0.001
Total Cholesterol (mmol/L)	5.2 ± 1.2	5.9 ± 1.5	<0.001
Triglycerides (mmol/L)	1.4 ± 0.15	2.5 ± 0.14	<0.001
Glucose (mmol/L)	5.9 ± 0.20	9.5 ± 0.50	<0.001
HDL-Cholesterol (mmol/L)	0.93 ± 0.41	0.84 ± 0.39	0.002
LDL-Cholesterol (mmol/L)	4.02 ± 1.2	4.52 ± 1.3	0.001

MetS: Individuals with metabolic syndrome defined by IDF, Non-MetS: Individuals free from metabolic syndrome WHR: Waist-Hip Ratio; SAD: Sagittal abdominal diameter.
Data are presented as mean ± standard deviation; statistical significance is shown.

Table 2 Genotype distribution of 894G>T, 4a/b and -786T>C polymorphisms in the individuals enrolled in the study.

Genotype/ Allele	Non-MetS N (%)	MetS N (%)	Odds ratio (95% CI)	*p*-value
894G>T				
GG	279 (68.2)	311 (65.2)	Reference	
GT	109 (26.7)	148 (31.0)	1.21 (0.906, 1.63)	0.190
TT	21 (5.1)	18 (3.8)	0.77 (0.401, 1.47)	0.427
GT+TT	130 (31.8)	166 (34.8)	1.14 (0.861, 1.50)	0.343
G	667 (81.5)	770 (80.7)	Reference	
T	151 (18.5)	184 (19.3)	1.05 (0.83, 1.34)	0.657
4a/b				
bb	251 (61.5)	281 (59.0)	Reference	
ab	146 (35.8)	169 (35.6)	1.03 (0.782, 1.36)	0.815
aa	11 (2.7)	26 (5.4)	2.11 (1.02, 4.36)	0.039*
aa+ab	157 (38.5)	195 (41.0)	1.11 (0.84, 1.45)	0.452
b	648 (79.5)	731 (76.8)	Reference	
a	168 (20.5)	221 (23.2)	1.16 (0.92, 1.46)	0.184
-786T>C				
TT	248 (60.7)	236 (49.6)	Reference	
TC	136 (33.2)	210 (44.2)	1.62 (1.22, 2.14)	0.001* $^{\beta}$
CC	25 (6.1)	30 (6.3)	1.26 (0.72, 2.20)	0.416
TC+CC	161 (39.4)	240 (50.4)	1.56 (1.20, 2.04)	0.001* $^{\beta}$
T	632 (77.3)	682 (71.6)	Reference	
C	186 (22.7)	270 (28.4)	1.34 (1.1, 1.70)	0.007* $^{\beta}$

MetS: Individuals with metabolic syndrome defined by IDF, Non-MetS: Individuals free from metabolic syndrome
Data are presented as mean ± standard deviation; *$p< 0.05$. $^{\beta}$ $p< 0.016$ (after Bonferroni correction (α=0.05/3 tested SNPs)
CI, Confidential interval

phisms were significantly different between MetS and Non-MetS subjects. Thus, the aa (p=0.039) genotype of 4a/b polymorphisms, the TC (p=0.001) and CC+TC (p=0.001) genotypes, and the C (p=0.007) allele of the -786T>C polymorphisms were significantly more common in MetS subjects whereas the TT (0.001) genotype and the T (0.007) allele of the -786T>C polymorphisms were significantly more prevalent in Non-MetS individuals. The aa genotype of 4a/b polymorphism was associated with MetS [Odds ratio(OR) 2.11 (95% Confidential interval (CI) 1.02, 4.36); p=0.039], however this significant association disappeared after Bonferroni correction. The TC, the CC+TC genotypes, and the C allele of -786T>C were associated with MetS [OR 1.62 (1.22, 2.14); p=0.001], [OR 1.56 (1.2, 2.04); p=0.001], and [OR 1.34 (1.1, 1.7); p=0.007], respectively. This effect for the TC and the CC+TC genotypes remained significant after Bonferroni correction. Finally, no differences were observed between MetS and Non-MetS groups in the genotypic and allelic distribution of the other analyzed *eNOS* variant (i.e., 894G>T SNP). Table 3 shows the genotype and allele distribution of 894G>T, 4a/b, and -786T>C variants based on gender. In male subjects, the aa genotype of 4a/b, and the TC, the CC+TC genotype, and the C allele of -786T>C of polymorphisms were associated with MetS [OR 3.3(1.1, 10.2); p=0.04], [OR 1.9(1.2, 2.8); p=0.004], [OR 1.8(1.2, 2.6); p=0.005], and [OR 1.46(1.0, 2.0); p=0.02], respectively, while in female subjects only the TC genotype of -786T>C polymorphism was associated with MetS [OR 1.2(1.0, 2.2); p=0.04]. Power analysis revealed that a power of 80% can be achieved by genotyping only 20% of the total number of samples used in our study.

Association of eNOS gene variants with metabolic parameters

The prevalence of the different components of MetS was subsequently analyzed based on the genotypes of 894G>T (GG *vs.* GT+TT), 4a/b, and -786T>C (TT *vs.* TC+CC) variants. Results showed that the GT+TT genotype of 894G>T SNP was significantly associated with higher systolic (p=0.005) and diastolic blood pressure (p=0.038) whereas the TC+CC genotype -786T>C was significantly associated with augmented fasting glucose levels (p=0.014), and lower HDL-cholesterol concentrations (p=0.038) (Table 4).

Possible relationship between *eNOS* gene variants

Table 3 Genotype distribution of 894G>T, 4a/b and -786T>C polymorphisms in males and females individuals

Genotype/ allelle	Non-MetS N (%)		MetS N (%)		Odds ratio (95% CI)		*p*-value	
	Males	Females	Males	Females	Males	Females	Males	Females
894G>T								
GG	114(66.3)	131(68.6)	149(64.7)	167(65.4)	Reference			
GT	46(26.7)	54(28.3)	72(31.3)	79(31.1)	1.2 (0.76, 1.3)	1.1(0.75, 1.7)	0.42	0.51
TT	12(7.0)	6(3.1)	9(4.0)	9(3.5)	0.57 (0.23, 1.4)	1.2(0.40, 3.4)	0.22	0.76
GT+TT	58(33.7)	60(31.4)	81(35.3)	88(34.6)	1.06(0.70, 1.6)	1.2(0.77, 1.7)	0.83	0.54
G	274(79.6)	316(82.7)	370(80.4)	413(81.0)	Reference			
T	70(20.4)	66(17.3)	90(19.5)	97(19.0)	0.95(0.67, 1.3)	1.1(0.79, 1.6)	0.78	0.50
4a/b								
bb	107(62.2)	113(59.5)	136(59.1)	149(58.6)	Reference			
ab	61(35.5)	70(36.8)	77(33.5)	95(37.4)	0.99(0.65, 1.5)	1.0(0.69, 1.5)	0.97	0.88
aa	4(2.3)	7(2.7)	17(7.4)	10(4.0)	3.3(1.1, 10.2)	1.1(0.40, 2.9)	0.04*†	0.87
ab+aa	65(37.8)	77(40.5)	94(40.9)	105(41.4)	1.1(0.75, 1.7)	1.0(0.70, 1.5)	0.53	0.92
b	275(80.0)	296(77.9)	349(75.9)	393(77.4)	Reference			
a	69(20.0)	84(22.1)	111(24.1)	115(22.6)	1.2(0.90,1.7)	1.0(0.74, 1.4)	0.09	0.87
-786T>C								
TT	106(61.6)	114(59.7)	109(47.4)	130(51.1)	Reference			
TC	53(30.8)	65(34.0)	102(44.3)	111(43.7)	1.9(1.2, 2.8)	1.2(1.0, 2.2)	0.004*	0.04*
CC	13(7.6)	12(6.2)	19(8.3)	13(5.2)	1.4(0.66, 3.0)	0.95(0.41,2.2)	0.35	0.90
TC+CC	66(38.4)	77(40.3)	121(52.6)	124(48.9)	1.8(1.2, 2.6)	1.4(0.96, 2.0)	0.005*	0.07
T	265(77.0)	293(76.7)	320(69.6)	371(73.0)	Reference			
C	79(23.0)	89(23.3)	140(30.4)	137(27.0)	1.46(1.0, 2.0)	1.2(0.89, 1.6)	0.02*	0.24

MetS: Individuals with metabolic syndrome defined by IDF, Non-MetS: Individuals free from metabolic syndrome
* $p< 0.05$, † with continuity correction CI, Confidential interval

Table 4 Anthropometric and metabolic parameters according to genotypes of 894G>T, 4a/b, -786T>C polymorphisms.

Parameter	894G>T			4a/b			-786T>C		
	GG	GT+TT	*p*-value	bb	ab + aa	*p*-value	TT	TC+CC	*p*-value
N	590	296		532	352		484	401	
Age (years)	45.6 ± 13.2	46.7 ± 12.8	0.23	46.0 ± 12.8	45.7 ± 13.5	0.75	45.8 ± 13.1	46.0 ± 13.2	0.88
Body Mass Index (kg/m^2)	31.0 ± 5.7	31.2 ± 6.5	0.54	31.1 ± 6.1	31.1 ± 5.8	0.97	30.8 ± 5.9	31.4 ± 6.1	0.15
Waist (cm)	97.5 ± 18.6	97.9 ± 18.6	0.77	97.9 ± 18.5	97.3 ± 18.8	0.60	96.8 ± 18.7	98.5 ± 18.5	0.19
Hips (cm)	106.9 ± 19.0	105.8 ± 19.4	0.41	107.0 ± 19.2	105.7 ± 18.9	0.34	106.2 ± 19.6	107.0 ± 18.6	0.54
WHR	0.91 ± 0.14	0.93 ± 0.15	0.12	0.92 ± 0.15	0.92 ± 0.14	0.86	0.92 ± 0.16	0.93 ± 0.13	0.59
SAD (cm)	23.8 ± 7.2	24.5 ± 6.4	0.20	24.2 ± 6.3	24.0 ± 7.7	0.68	23.9 ± 7.5	24.3 ± 6.2	0.35
Systolic BP (mmHg)	123.4 ± 15.0	126.5 ± 15.1	0.005*	124.5 ± 15.3	124.4 ± 14.8	0.99	124.2 ± 15.8	124.9 ± 14.4	0.46
Diastolic BP (mmHg)	79.3 ± 9.0	80.7 ± 9.5	0.038*	79.7 ± 9.6	80.0 ± 9.1	0.72	79.8 ± 9.4	79.8 ± 9.3	0.98
Total Cholesterol (mmol/L)	5.5 ± 1.4	5.6 ± 1.5	0.51	5.5 ± 1.4	5.6 ± 1.5	0.61	5.5 ± 1.4	5.6 ± 1.5	0.54
Triglycerides (mmol/L)	1.9 ± 0.16	1.8 ± 0.13	0.25	1.9 ± 0.15	1.9 ± 0.15	0.62	1.9 ± 0.15	2.0 ± 0.15	0.14
Glucose (mmol/L)	7.7 ± 0.48	7.7 ± 0.45	0.97	7.7 ± 0.47	7.6 ± 0.47	0.73	7.4 ± 0.41	8.0 ± 0.53	0.014*
HDL-Cholesterol (mmol/L)	0.91 ± 0.41	0.92 ± 0.38	0.74	0.93 ± 0.41	0.89 ± 0.38	0.09	0.94 ± 0.41	0.88 ± 0.38	0.038*
LDL-Cholesterol (mmol/L)	3.7 ± 1.2	3.8 ± 1.4	0.12	3.7 ± 1.2	3.8 ± 1.4	0.21	3.7 ± 1.1	3.7 ± 1.3	0.98

WHR: Waist-Hip Ratio; SAD: Sagittal abdominal diameter. Data are presented as mean ± standard deviation; * $p< 0.05$.

and each of the features of the metabolic syndrome separately (elevated blood pressure, elevated glucose and decreased HDL-cholesterol concentrations) as defined by IDF cutoffs were analyzed next. Results showed that the GT, GT+TT genotypes of 894G>T variant are more prevalent among the subjects with elevated blood pressure, and are associated with risk of hypertension [OR 1.45 (1.06, 1.97), p=0.017; 1.4 (1.1, 1.8) p=0.022] (Table 5). Additionally, the ab genotype of 4a/b variants was more frequent in subjects with decreased HDL-cholesterol concentrations [OR 1.33 (1.2, 1.90), p=0.044] (Table 5). Notably, whereas no significant association were observed between eNOS gene polymorphism and blood glucose using IDF cutoff (data not

Table 5 Genotype and allele distribution of eNOS polymorphisms in individuals with normal *vs*. elevated blood pressure (blood pressure ≥ 130/85) subjects; and in normal *vs*. reduced HDL (< 40 mg/dL for men and < 50 mg/dL for women); as well as in normal *vs*. elevated glucose (≥6.1 mmol/L) subjects.

Genotype/ Allele	Normotensive	Elevated blood pressure	Odds ratio (95% CI)	*p*-value	Normal HDL N (%)	Reduced HDL N (%)	Odds ratio (95% CI)	*p*-value	Normal N (%)	Elevated glucose N (%)	Odds ratio (95% CI)	*p*-value
894G>T												
GG	459 (68.9)	167 (61.1)	Reference		192 (69.6)	402 (66.4)	Reference		268 (67.0)	353 (66.7)	Reference	
GT	178 (26.7)	94 (34.5)	1.45 (1.06, 1.97)	0.01*	73 (26.4)	176 (29.1)	1.15 (0.83, 1.59)	0.31	112 (28.0)	156 (29.4)	1.05 (0.791, 1.41)	0.70
TT	29 (4.4)	12 (4.4)	1.13 (0.56, 2.28)	0.71	11 (4.0)	27 (4.5)	1.17 (0.57, 2.41)	0.66	20 (5.0)	20 (3.9)	0.75 (0.40, 1.40)	0.39
GT+TT	207 (31.1)	106 (38.9)	1.4 (1.1, 1.8)	0.02*	84 (30.4)	203 (33.6)	1.2 (0.84, 1.56)	0.36	132 (33.0)	176 (33.3)	1.01 (0.768, 1.34)	0.93
G	1096 (82.3)	428 (78.4)	Reference		457 (82.8)	980 (81.0)	Reference		648 (81.0)	862 (81.5)	Reference	
T	236 (17.7)	118 (21.6)	1.28 (1.0, 1.6)	0.05	95 (17.2)	230 (19.0)	1.12 (0.86, 1.5)	0.36	152 (19.0)	196 (18.5)	0.969 (0.766, 1.22)	0.79
4a/b												
bb	398 (59.8)	164 (60.3)	Reference		177 (64.6)	348 (57.6)	Reference		239 (59.8)	317 (60.0)	Reference	
ab	243 (36.5)	91 (33.5)	0.91 (0.672, 1.22)	0.53	85 (31.0)	229 (37.9)	1.33 (1.2, 1.90)	0.04*	144 (36.1)	186 (35.2)	0.97 (0.7401, 1.28)	0.85
aa	24 (3.7)	17 (6.2)	1.72 (0.90, 3.2)	0.09	12 (4.4)	27 (4.5)	1.14 (0.566, 2.31)	0.7	16 (4.1)	25 (4.8)	1.17 (0.615, 2.25)	0.62
ab+aa	267 (40.2)	108 (39.7)	0.982 (0.736, 1.30)	0.9	97 (35.4)	256 (42.4)	1.3 (1.0, 1.8)	0.05	160 (40.1)	211 (40.0)	0.994 (0.763, 1.29)	0.96
b	1039 (78.2)	419 (77.0)	Reference		439 (80.1)	925 (76.6)	Reference		622 (78.0)	820 (77.6)	Reference	
a	291 (21.8)	125 (23.0)	1.06 (0.83,1.35)	0.6	109 (19.9)	283 (23.4)	1.23 (0.961, 1.58)	0.09	176 (22.0)	236 (22.4)	1.02 (0.815, 1.26)	0.88
-786T>C												
TT	368 (55.4)	146 (53.4)	Reference	0.6	161 (58.5)	314 (51.8)	Reference		234 (58.5)	273 (51.7)	Reference	
TC	256 (38.4)	109 (40.0)	1.1 (0.78, 1.4)	0.63	97 (35.2)	253 (41.8)	1.35 (1.0, 1.83)	0.05	146 (36.5)	217 (41.1)	1.27 (0.970, 1.67)	0.08
CC	41 (6.2)	18 (6.6)	1.1 (0.616, 1.98)	0.73	17 (6.3)	39 (6.4)	1.2 (0.645, 2.14)	0.59	20 (5.0)	38 (7.2)	1.62 (0.922, 2.87)	0.09
TC+CC	297 (44.6)	127 (46.6)	1.07 (0.812, 1.43)	0.6	114 (41.5)	291 (48.0)	1.30 (0.981,1.74)	0.06	166 (41.5)	255 (48.3)	1.32 (1.1, 1.71)	0.03*
T	992 (74.6)	401 (73.4)	Reference		419 (76.2)	881 (72.7)	Reference		614 (76.7)	763 (72.2)	Reference	
C	338 (25.4)	145 (26.6)	1.06 (0.846, 1.3)	0.6	131 (23.8)	331 (27.3)	1.2 (0.950, 1.51)	0.12	186 (23.3)	293 (27.8)	1.26 (1.02, 1.56)	0.02

* $p < 0.05$ CI, Confidential interval

shown), the TC+CC genotype and the C allele of the -786T>C variant correlated with elevated glucose level [OR 1.32 (1.1, 1.71), p=0.039; OR 1.26 (1.02, 1.56), p=0.028, respectively] after using world health organization (WHO) cutoff for impaired fasting glucose (Table 5).

Haplotype frequency and linkage disequilibrium

The distribution of the estimated frequencies of 896G, 4a and -786C haplotype was significantly different when MetS were compared to Non-MetS individuals; notably, this haplotype significantly increased the risk of MetS [OR 1.54 (1.1, 2.3); p= 0.034]. This effect was not detected in any other common haplotypes (Table 6).

Linkage disequilibria values were subsequently generated to look for association among the three studied polymorphisms. All the three variants were in weak linkage disequilibrium with each other. Thus, 894G>T was in weak linkage disequilibrium with the 4a/b (R^2 = 0.052, D'=0.4401; p <0.001), and the -786T>C variants (R^2= 0.012, D' = 0.0679; p=0.0007); finally, 4a/b

Table 6 Haplotypes frequency in MetS versus Non-MetS individulas

Haplotype	Non-MetS	MetS	Odds ratio (95% CI)	*p*-value
G-b-T	232	253	0.84 (0.630, 1.13)	0.251
G-a-C	47	79	1.54 (1.1, 2.3)	0.034*
T-b-T	46	55	1.1(0.713, 1.68)	0.675
G-a-T	34	30	0.809 (0.480, 1.36)	0.426

MetS: Individuals with metabolic syndrome defined by IDF, Non-MetS: Individuals free from metabolic syndrome, * $p< 0.05$ CI, Confidential interval

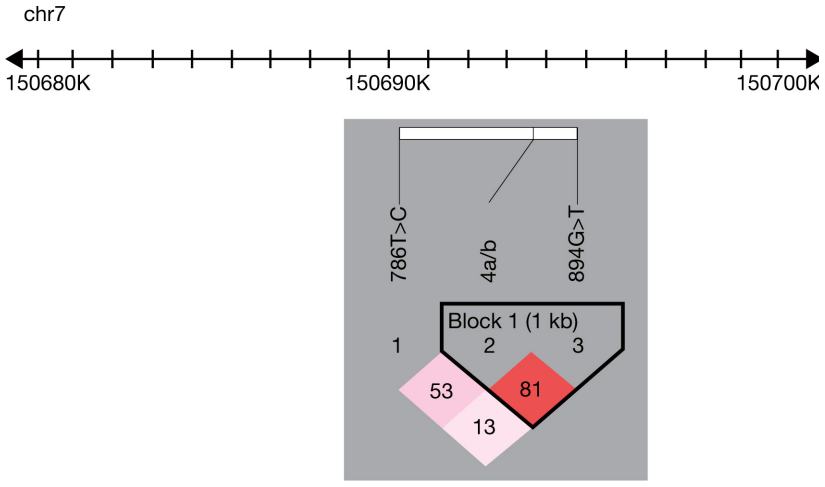

Fig. 1 Pairwise linkage disequilibrium (LD) for eNOS gene SNPs. The plot shows the pairwise correlation for the studied SNPs (894G>T, -786T>C and 4a/b).

was in weak linkage disequilibrium with -786T>C (R^2=0.182, D' = 0.2357; p <0.001). A strong pairwise LD (R^2=0.81) was detected between 4a/b and 894G>T polymorphisms using haploview software (Fig. 1).

Discussion

Studies describing the association of *eNOS* gene with MetS are few and inconsistent. There have been no data reporting this association from the Arab nations, particularly from Saudi Arabia despite an increased prevalence and incidence of metabolic phenotypes including CVD and DMT2 [28]. Results herein provide the first information on the eNOS gene polymorphism and the risk of MetS in this population. Thus, two of the three studied polymorphisms of the eNOS gene: 4a/b and, -786T>C were significantly associated with the risk of MetS. The marginal significant association between 4a/b polymorphism and MetS disappeared after Bonferroni correction while association for -786T>C remained significant. Additionally, the GT+TT genotype of 894G>T polymorphism was significantly associated with higher systolic and diastolic blood pressure, while the -786T>C SNP was significantly associated with higher glucose and lower HDL-cholesterol serum concentrations. Finally, significantly higher blood pressure was detected in carriers of the T allele (rare allele) in 894G>T SNP.

In a Spanish population, the SNP -786T>C is a risk factor for MetS among hypertensive patients [20]. In the same study, SNP 894G>T in linkage disequilibrium with -786T>C was found to be associated with MetS. Similarly, the 894G>T SNP, albeit in a haplotypic association with other SNPs, is linked to the features of MetS [21]. In our study, genotype distribution of 894G>T SNP was not associated with MetS which was in agreement with pervious study in Caucasian [25] but not with data from Chinese [22] and Brazilian [24] populations. This inconsistency could possibly be due to the difference in genetic background and sample

Table 7 Genotype distributions of 894G>T polymorphism of eNOS gene in control vs. MetS populations in relation to ethnicity

Genotype/ allelle	Saudi N= 886				Chinese[a] N=397				Caucasian[b] N=98				Latin American[c] N=613			
	MetS N (%)	Non-MetS N (%)	ORs	*p*-value	MetS N (%)	Non-MetS N (%)	ORs	*p*-value	MetS N (%)	Non-MetS N (%)	ORs	*p*-value	MetS N (%)	Non-MetS N (%)	ORs	*p*-value
GG	311 (65.2)	279 (68.2)	Reference		28 (43.08)	203 (61.2)	Reference		31 (54.4)	19 (46.3)	Reference		134 (34.9)	93 (40.4)	Reference	
GT	148 (31.0)	109 (26.7)	1.2 (0.90,1.3)	0.190	31 (47.69)	103 (31.0)	2.2 (1.2,3.8)	0.008	20 (35.1)	19 (46.3)	0.64 (0.27, 1.5)	0.38	208 (54.3)	122 (53.0)	1.2 (0.83, 1.6)	0.37
TT	18 (3.8)	21 (5.1)	0.77 (0.401,47)	0.427	6 (9.2)	26 (7.8)	1.6 (0.63, 4.4)	0.27	6 (10.5)	3 (7.3)	1.2 (0.27, 5.4)	0.90	41 (10.7)	15 (6.5)	1.9 (0.99, 3.6)	0.06
GT+TT	166 (34.8)	130 (31.8)	1.14 (0.86,1.50)	0.343	37 (56.9)	129 (38.8)	2.1 (1.2, 3.5)	0.009	26 (45.6)	22 (53.7)	0.72 (0.32, 1.6)	0.54	249 (65.1)	137 (59.6)	1.3 (0.90, 1.7)	0.19
G	770 (80.7)	667 (81.5)	Reference		87 (66.9)	509 (76.6)	Reference		82 (72.0)	57 (69.5)	Reference		476 (62.1)	308 (67.0)	Reference	
T	184 (19.3)	151 (18.5)	1.05 (0.83,1.34)	0.657	43 (33.1)	155 (23.3)	1.62 (1.1, 2.4)	0.02	32 (28.0)	25 (30.5)	0.89 (0.471.6)	0.75	290 (37.9)	152 (33.0)	1.2 (0.96, 1.5)	0.09

MetS: Individuals with metabolic syndrome, Non-MetS: Individuals free from metabolic syndrome, ORs: Odds ratio (95% CI), *$p < 0.05$ [a] Hsieh *et al*., 2008 [22], [b] Zeman *et al*., 2010 [25], [c] Piccoli *et al*., 2008 [24]

size (Table 7).

Our previous work showed that coronary artery disease patients with hypertension had a significantly high ORs of 894GT/894TT genotypes combined and -786CC/786TC combined [29]. Therefore, the current findings reveal that polymorphism in 894G>T SNP is associated with pre-hypertension status in Saudis. The correlation between the T allele of 894G>T SNP and elevated blood pressure confirms previous findings stemming from analyses performed in a Japanese cohort [30], whereas the G allele of this gene seems to be associated with hypertension in Caucasians [31]. Studies in Singapore and Serbian population did not detect any association of 894G>T polymorphisms with hypertension [32, 33]. Several studies support *eNOS* gene as a genetic biomarker for hypertension. Huang *et al.* reported that the transgenic mice lacking *eNOS* gene were hypertensive [34]. NO production is reduced in individuals with hypertension compared with that in healthy subjects [35]. Indeed, results show that the Asp298 variant (T894 allele) is more prone to cleavage by naturally occurring proteases, resulting in the generation of 100 and 35 kDa fragments that lower the endothelium-dependent vascular dilation [18, 36].

Several studies have described an association between *eNOS* gene variants and the dyslipidemic components. In our study, the ab genotype of 4a/b was significantly associated with decreased HDL-cholesterol but the significance of this association was marginal. In a Chinese population the GT+TT genotype of 894G>T SNP is significantly associated with total cholesterol, triglycerides and LDL-cholesterol [22], whereas the same genotype is a risk factor for HDL- and LDL-cholesterol and LDL particle size but not for total cholesterol and triglyceride in a Japanese population [23]. Likewise, the GT+TT genotype has been linked to dyslipidemia in Brazilian subjects with MetS [24], and to triglyceride levels in healthy African Americans [16]. In agreement with our result, the 4a/b polymorphism in Mexican Americans is associated with decreased HDL-cholesterol and the same polymorphism is associated decreased HDL- and increased LDL-cholesterol in Anglo-Celtic Caucasians [11, 37]. HDL maintains the concentration of caveolae-associated cholesterol, thereby preventing the negative impact of oxidized LDL[38]. It is thus tempting to speculate that the activity of eNOS, owing to 4a/b polymorphism may significantly differ from that of the normally produced eNOS, and therefore, may be leading to altered plasma lipid profiles.

Increased fasting glucose level is another essential trait of MetS and an indicator of the presence of insulin resistance, which is a well known risk factor for DMT2 and other metabolic phenotypes. Using WHO

cutoff for impaired fasting glucose (i.e., 6.1 mmol/L), we found that the TC+CC genotype and C allelle of the -786T>C SNP is significantly associated with increased fasting glucose concentrations. Several studies have examined the effects of *eNOS* gene variants on plasma glucose levels in different conditions. Fasting plasma glucose levels were found to be significantly elevated in the carriers of TT+GT genotype as compared to those of GG genotype of 894G>T SNP in Chinese subjects with MetS [22]. In accordance to our results, -786T>C SNP, but not 894G>T SNP, is linked to increased plasma glucose levels in healthy Japanese subjects [23], and in a prospective study, this SNP was found to be a significant predictor of glycemic status in Chinese subjects with impaired glucose tolerance [39]. NO facilitates the uptake of glucose into skeletal muscle and aids its subsequent metabolism [40, 41]. Studies in rodents and humans have also demonstrated that *in vivo* inhibition of all eNOS isoforms attenuates insulin-stimulated glucose uptake by skeletal muscle and other peripheral insulin-sensitive tissue, indicating that glucose uptake *via* insulin signaling pathways is NO dependent [42, 43]. Thus, impaired NO production secondarily to a genetically-based defective eNOS activity could explain the association of -786T>C SNPs with increased plasma glucose levels. Collectively, the inconsistencies in the findings about the relationship between *eNOS* polymorphism and the features of MetS could reflect either genetic heterogeneity or differences in environmental factors that influence phenotypic expression of the gene variants. Importantly, these determinants can be significantly affected by ethnic diversity.

Haplotype-based association analysis revealed that the distribution of G-a-C haplotypes was significantly different between MetS and Non-MetS subjects. A significant association between e*NOS* gene haplotypes and features of MetS was described in a Spanish population [21]. Another study on Spanish population identified the -786C/894G as the risk haplotype for metabolic syndrome susceptibility in hypertensive patients [20]. *eNOS* haplotypes were found be associated with hypertension in hypertensive patients [44], and in Brazilian patients with and without DMT2 [45]. In Asian Indians, T-a-C haplotype was associated with nearly 2.5 fold increase in the risk of diabetic nephropathy [46]. The haplotype analysis approach is expected to be more powerful than single marker analysis and the interaction of multiple genetic markers within a haplotype could be a key determinant of disease susceptibility rather than the individual polymorphism. Hence, testing of haplotypes may overcome some of the problems encountered with using single polymorphisms in genetic association studies [44-46].

Taken together, our results show for the first time in an Arab population that SNPs of the *eNOS* gene are significantly associated with components of MetS and MetS itself. These findings underscore the genetic susceptibility to develop metabolic phenotypes and are consistent with an increased prevalence and incidence of CVD and DMT2 in Saudi Arabia.

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Conflict of Interests

The authors have nothing to disclose.

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