Association between endothelial nitric oxide synthase polymorphisms and risk of metabolic syndrome

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Abstract.

BACKGROUND: Previous studies inferring that the *NOS3* gene was associated with the pathogenesis of metabolic syndrome (MetS) had inconsistent findings. We investigated the role of three *NOS3* polymorphisms (T-786C, intron 4b/a, and G894T) in the risk of MetS using a hospital-based case-control study.

METHODS: We recruited 339 MetS cases and 783 non-MetS controls at a central Taiwanese hospital. Information on sociodemographic and lifestyle factors was obtained using a self-administered questionnaire. Genotypes of *NOS3* polymorphisms were compared between cases and controls. Effects of interactions between gene polymorphisms and smoking and between gene polymorphisms and drinking on the risk of MetS were also determined.

RESULTS: The T-786C TC+CC genotype was significantly associated with a decreased risk of MetS (odds ratio (OR), 0.63; 95% confidence interval (CI), 0.43–0.91), compared to the T-786C TT genotype, according to a logistic regression analysis. This beneficial effect was much greater for those who had ever smoked cigarettes (OR, 0.47; 95% CI, 0.26–0.87) or those who had not consumed alcohol (OR, 0.45; 95% CI, 0.26–0.77). In addition, the intron 4b/a variant genotype was marginally associated with a reduced risk of MetS (OR, 0.68; 95% CI, 0.47–1.00), compared to the intron 4b/a bb genotype, particularly for never alcohol consumers (OR, 0.56; 95% CI, 0.33–0.95). In the haplotype analysis, there was a 53% decrease in the MetS risk among C4bG haplotype carriers (OR, 0.47; 95% CI, 0.25–0.90), compared to those with the most common T4bG haplotype.

CONCLUSIONS: Our results suggest that the *NOS3* T-786C and intron 4b/a polymorphisms may contribute to the risk of MetS. Further studies are needed to confirm the findings.

Keywords: Metabolic syndrome, endothelial nitric oxide synthase, polymorphisms, smoking, drinking

1. Introduction

Metabolic syndrome (MetS) is a cluster of manifestations including abdominal obesity, an increased serum

concentration of triglycerides (TGs), a decreased serum concentration of high-density lipoprotein cholesterol (HDL-C), high blood pressure, and an increased fasting blood glucose level [1]. The prevalence of MetS varies widely among ethnic groups, being high among Western populations but lower among Asians [2,3]. However, a recent population-based survey in Taiwan reported a MetS prevalence rate of 33% among 2357 adults ≥ 40 years of age in a metropolitan area [4]. In-

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dividuals with MetS are at high risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) [5].

In addition to environmental factors such as a poor diet and physical inactivity [6], several studies suggested a genetic etiology of MetS [7,8]. Animal models indicated that a lack of endothelial nitric oxide synthase (eNOS) is related to hypertension, hypertriglyceridemia, and insulin resistance [9]. eNOS involves the production of nitric oxide (NO), which is a ubiquitous molecule responsible for maintaining normal endothelial function. NO is an important molecule involved in vasodilatation, neuronal transmission, smooth muscle relaxation, and immunity [10]. Studies clearly demonstrated that reduced NO-dependent endothelial vasodilatation is an early functional disturbance in the development of atherosclerotic lesions [11,12]. In addition, NO modulation of peripheral and hepatic glucose metabolism and insulin secretion suggests that NO alterations play important roles in the evolution of insulin resistance and T2DM [13]. Moreover, studies reported high NO levels and endothelial dysfunction in T2DM patients, their first-degree relatives [14], and subjects with insulin-resistance syndrome [15].

In humans, eNOS is encoded by the *NOS3* gene located on chromosome 7q35-36 [16], and the eNOS protein constitutively synthesizes NO via the conversion of L-arginine into L-citrulline, involving the transfer of five electrons provided by NADPH [17]. Several polymorphisms were identified in the *NOS3* gene. Much attention was focused on putatively functional variants: T-786C (rs2070744), G894T (Glu298Asp) (rs1799983), and the intron 4b/a VNTR (27-bp variable number of tandem repeats) [18]. A large meta-analysis with 71 studies concluded that these three *NOS3* polymorphisms are associated with coronary heart disease [19]. In addition, variants of the *NOS3* gene were related to the development of T2DM and insulin resistance [20–22], and effects on plasma lipids and the body-mass index (BMI) [23,24]. Therefore, *NOS3* might be a good candidate gene for evaluating MetS. Although evidence from epidemiological studies implied that the *NOS3* gene was related to the pathogenesis of MetS, findings are inconsistent and controversial [25–37].

The aim of the present study was to test the hypothesis that the three *NOS3* polymorphisms may play important roles in the risk of MetS in an ethnic Chinese population (i.e., Taiwanese) in Taiwan. In addition, because tobacco smoke and ethanol are involved in modulating eNOS activity [38–40], we also investi-

gated how cigarette smoking and alcohol consumption interact with the relationship between *NOS3* polymorphisms and MetS risk.

2. Materials and methods

2.1. Participants

We recruited 1269 eligible volunteer adult participants who underwent a comprehensive health check-up at China Medical University Hospital in 2006. The study was approved by the hospital Human Research Ethics Committee, and written informed consent was obtained from each participant. Cases of MetS were defined according to the modified Third Report of the National Cholesterol Education Program's Adult Treatment Panel (NCEP ATP III). The NCEP ATP III defines MetS as the presence at least 3 of the following: (1) a fasting plasma glucose of ≥ 110 mg/dL; (2) serum TGs of ≥ 150 mg/dL; (3) serum HDL-C of < 40 mg/dL in men and < 50 mg/dL in women; (4) a blood pressure of $\geq 130/85$ mmHg; and (5) a waist circumference (WC) of > 90 cm in men and > 80 cm in women. Finally, we recruited 400 MetS cases and 869 non-MetS controls in the study.

2.2. Data scope and collection

Anthropometric measurements were obtained during a complete physical examination. Weight and height were measured on an autoanthropometer (Super-view, HW-666, Taipei, Taiwan), with the subjects shoeless and wearing light clothing. The BMI was derived from the formula of weight/height²(kg/m²). With the participant standing, the WC was measured at the midway point between the inferior margin of the last rib and the crest of the ilium in a horizontal plane. Blood pressure was measured at the right brachial artery using a random-zero sphygmomanometer with the participant in a sitting position.

Data on sociodemographic characteristics, including gender, age, educational attainment, marital status, household income, smoking, drinking, physical activity, occupational activity, menopausal status, dietary habits, family history of cardiovascular-related diseases, physician-diagnosed diseases, and medication history were collected using a standard questionnaire when the participant underwent the complete physical exam [41]. Smoking status and alcohol consumption history were classified into 3 groups: current users, nonusers, and ex-users.

2.3. Laboratory examination

Five mL blood was drawn with minimal trauma from the antecubital vein in tubes without anticoagulants in the morning, after a 12-h overnight fast, and was kept at room temperature and sent for analysis within 4 h of collection. Serum biochemical markers such as HDL-C, TGs, urine albumin, uric acid, and creatinine were analyzed using a biochemical autoanalyzer (Synchro LX-20; Beckman Coulter, Brea, CA, USA) at the Clinical Laboratory Department of China Medical University Hospital. The fasting plasma glucose level was determined by a glucose oxidase method (Astra-8, Beckman Instruments, Fullerton, CA, USA), and the serum insulin level was measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic Products, Los Angeles, CA, USA).

2.4. Genotyping

Genomic DNA was extracted from another 5 mL blood samples collected in tubes containing the anticoagulant EDTA using a Gentra Puregene Blood Kit (Gentra Systems, Minneapolis, MN, USA). Quantitation of DNA and the purity index were evaluated by NanoDrop ND-1000 spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) at an absorbance ratio of 260/280 nm. The extracted DNA was dissolved in Tris-EDTA buffer to a concentration of 500 µg/mL and kept in a -80°C freezer for further genotyping assays.

Polymorphisms of T-786C (rs2070744) and G894T (rs1799983) in the *NOS3* gene were determined by a 5'-nuclease assay with allele-specific TaqMan probes. TaqMan® single-nucleotide polymorphism (SNP) genotyping assays were purchased from Applied Biosystems (Foster City, CA, USA) with assay IDs of AHVI30Z and C_3219460_20. Genotyping was performed using an allelic discrimination assay in the StepOne Real-Time polymerase chain reaction (PCR) System (Applied Biosystems), and genotypes were distinguished using automated software (SDS 2.3, Applied Biosystems). Reactions were run in 5-µL volumes using an amplification protocol of 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.

The 27-bp intron 4b/a VNTR polymorphism was analyzed using a primer pair (forward, 5'-AGG CCC TAT GGT AGT GCC TT-3'; reverse, 5'-TCT CTT AGT GCT GTG GTC AC-3') [42]. A 25-µL reaction volume was used for the PCR, including 0.5 µL (5 pM)

of each primer, 0.2 µL (2.0 mM) dNTPs, 1.0 U of Taq polymerase, and 2.5 µL 10 × PCR buffer together with 25 ng DNA. The thermocycling procedure consisted of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 45 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCR products were separated by 6% acrylamide gel electrophoresis and visualized by ethidium bromide staining. In addition to the wild-type allele (4b), which has five 27-bp repeats, we detected two variants for the intron 4b/a polymorphism. Variants 4a and 4c, which respectively corresponded to four and six 27-bp repeats, were previously observed [42]. For quality control, 10% random samples were repeated and showed 100% concordance for all polymorphisms.

2.5. Statistical analysis

In total, 147 subjects were excluded because of few blood samples or loss of DNA extraction ($N = 105$) and genotyping failure for any polymorphisms of the *NOS3* gene ($N = 42$), leaving 339 MetS cases and 783 non-MetS controls for the final analyses. The demographic characteristics of excluded subjects did not differ from those of included subjects. Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) (termed D' and r^2) of the SNPs were assessed using THESIAS among the non-MetS group [43]. A two-sample *t*-test was used to compare differences in quantitative traits between the MetS and non-MetS groups, while Pearson's Chi-square test was used to compare discrete traits. Due to the relatively low allelic frequency of the variant alleles for all *NOS3* polymorphisms, participants were classified as carriers vs. non-carriers of the variant alleles. A simple logistic regression model was used to calculate the odds ratios (ORs) and their 95% confidence intervals (CIs) to assess the univariate association between each polymorphisms and MetS risk. A multiple logistic regression model was applied to estimate the age- and gender-adjusted ORs and their 95% CIs. Because tobacco smoke and ethanol play important roles in modulating eNOS activity [38–40], cigarette smoking and alcohol consumption (never and had ever) were included in the multiple logistic regression models.

Based on the multiplicative scale, a likelihood ratio test was used to evaluate the effect of the gene-environment interaction on MetS risk. Interactions between polymorphisms and cigarette smoking or alcohol consumption were assessed by including cross-product

Table 1
Demographic and biochemical characteristics of the study population

Variable ^a	Non-MetS, n(%) N = 783	MetS, n(%) N = 339	P value
Sex			0.32 ^b
Male	453 (57.9)	207 (61.1)	
Female	330 (42.1)	132 (38.9)	
Age (mean $\pm$ SD), years	48.3 $\pm$ 11.5	55.2 $\pm$ 11.4	< 0.001 ^c
Education			< 0.001 ^b
Below senior high school	170 (23.7)	145 (48.2)	
Senior high school	197 (27.5)	63 (20.9)	
Above senior high school	350 (48.8)	93 (30.9)	
Married	653 (91.1)	288 (96.0)	0.006 ^b
Physically active	452 (60.1)	187 (58.1)	0.534 ^b
Cigarette smoking			0.023 ^b
None	522 (68.3)	205 (62.7)	
Current smoker	172 (22.5)	74 (22.6)	
Ex-smoker	70 (9.2)	48 (14.7)	
Alcohol drinking			0.006 ^b
None	507 (66.4)	192 (58.4)	
Current drinker	232 (30.4)	115 (35.0)	
Ex-drinker	25 (3.3)	22 (6.7)	
BMI (mean $\pm$ SD), kg/m ²	22.9 $\pm$ 3.0	27.4 $\pm$ 3.5	< 0.001 ^c
Waist (mean $\pm$ SD), cm	78.4 $\pm$ 9.0	91.6 $\pm$ 8.8	< 0.001 ^c
Serum cholesterol(mean $\pm$ SD), mg/dL	192.5 $\pm$ 36.3	205.0 $\pm$ 41.0	< 0.001 ^c
Serum HDL-C (mean $\pm$ SD), mg/dL	47.6 $\pm$ 14.8	34.4 $\pm$ 6.8	< 0.001 ^c
Serum LDL-C (mean $\pm$ SD), mg/dL	132.9 $\pm$ 36.3	142.8 $\pm$ 40.7	< 0.001 ^c
Serum triglyceride (mean $\pm$ SD), mg/dL	90.0 $\pm$ 62.5	187.1 $\pm$ 115.2	< 0.001 ^c
SBP (mean $\pm$ SD), mm Hg	114.2 $\pm$ 13.3	130.1 $\pm$ 16.8	< 0.001 ^c
DBP (mean $\pm$ SD), mm Hg	73.7 $\pm$ 8.6	81.1 $\pm$ 10.0	< 0.001 ^c
Plasma glucose (mean $\pm$ SD), mg/dL	90.2 $\pm$ 14.4	114.1 $\pm$ 34.9	< 0.001 ^c

MetS: metabolic syndrome; SD: standard deviation; BMI: body mass index; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure.

^aNumbers not being equal to the total number were due to missing data. ^bChi-square test. ^cStudent's *t*-test.

terms and the main effect term in the multiple logistic regression models. For example, the gene-smoking interaction model included the following covariates: interaction term between genotypes and cigarette smoking, genotypes, alcohol consumption, age, and gender. ORs for differences in MetS risk due to environmental factors (i.e., cigarette smoking and alcohol consumption) were estimated using stratified analyses with the wild-type genotypes as the reference in each stratum. All statistical analyses were performed using the Statistical Analysis Software (SAS) package (vers. 9.1.3 for windows; SAS Institute, Cary, NC, USA), and two-sided *P* values of < 0.05 were considered significant.

3. Results

Demographic characteristics of participants in the study are summarized in Table 1. Compared to the non-MetS controls, MetS cases were more likely to be older, less educated, married, cigarette smokers, and alcohol drinkers (*P* < 0.05). The study MetS cases

also had a much larger mean BMI (27.4 $\pm$ 3.56 vs. 22.9 $\pm$ 2.97 kg/m²).

Table 2 shows the genotypic distributions of the three *NOS3* polymorphisms for both the non-MetS and MetS groups. Frequencies of the T-786C C allele, intron 4b/a 4a allele and 4c allele, and G894T T allele among the non-MetS subjects were 10.1%, 8.4%, 0.89%, and 10.3%, respectively. These allele frequencies were in HWE. After adjusting for age, gender, cigarette smoking, and alcohol consumption, the T-786C TC+CC genotype was associated with a significantly reduced risk of MetS, compared to the T-786C TT genotype (OR, 0.63; 95% CI, 0.43–0.91). In addition, the intron 4b/a variant genotype was marginally associated with a reduced risk of MetS, compared to the intron 4b/a bb genotype (OR, 0.68; 95% CI, 0.47–1.00). However, the G894T polymorphism was not significantly associated with MetS risk.

Six haplotypes were observed among twelve possible haplotypes defined by the three *NOS3* polymorphisms in our study population (Table 3). The most common T4bG haplotype, which served as the reference haplo-

Table 2
Association between polymorphisms in the *NOS3* gene and the risk of metabolic syndrome (MetS)

<i>NOS3</i> genotype ^a	Non-MetS, n(%) N = 783	MetS, n(%) N = 339	OR (95% CI) ^b	P value
T-786C				
TT	630 (80.5)	288 (85.0)	1.00	
TC	146 (18.7)	50 (14.8)	0.65 (0.45–0.95)	0.023*
CC	6 (0.9)	1 (0.3)	0.23 (0.03–1.92)	0.175
TT	630 (80.5)	288 (85.0)	1.00	
TC+CC	152 (19.4)	51 (15.0)	0.63 (0.43–0.91)	0.015*
Intron 4b/a				
bb	643 (82.1)	293 (86.4)	1.00	
ba	120 (15.3)	40 (11.8)	0.70 (0.46–1.04)	0.079
aa	6 (0.8)	1 (0.3)	0.27 (0.03–2.42)	0.241
bc	14 (1.8)	5 (1.5)	0.80 (0.27–2.37)	0.684
bb	643 (82.1)	293 (86.4)	1.00	
ba+aa+bc	140 (17.9)	46 (13.6)	0.68 (0.47–1.00)	0.051
G894T				
GG	628 (80.2)	263 (77.6)	1.00	
GT	149 (19.0)	75 (22.1)	1.10 (0.78–1.53)	0.598
TT	6 (0.8)	1 (0.3)	0.37 (0.04–3.20)	0.370
GG	628 (80.2)	263 (77.6)	1.00	
GT+TT	155 (19.8)	76 (22.4)	1.06 (0.76–1.48)	0.716

OR: odds ratio; CI: confidence interval. ^aNumbers not being equal to the total number were due to missing data. ^bAdjusted for age, gender, cigarette smoking, and alcohol consumption. * $P < 0.05$.

Table 3
Estimated *NOS3* haplotype frequencies in the non-metabolic syndrome (MetS) and MetS groups and their effects on the risk of MetS

Haplotype ^a			Non-MetS, n(%)	MetS, n(%)	OR (95% CI) ^c	P
T-786C	Intron 4b/a ^b	G894T				
T	4b	G	1162 (76.84)	522 (80.03)	1.00	
C	4b	G	54 (3.54)	12 (1.88)	0.47 (0.25–0.90)	0.023*
T	4b	T	154 (10.17)	72 (11.03)	0.99 (0.71–1.37)	0.95
T	4a	G	36 (2.40)	8 (1.25)	0.57 (0.25–1.31)	0.18
C	4a	G	101 (6.71)	37 (5.64)	0.70 (0.45–1.08)	0.11
T	4a	T	5 (0.35)	1 (0.16)	0.62 (0.04–9.78)	0.76

OR: odds ratio; CI: confidence interval. ^aNumbers not being equal to the total number were due to missing data. ^b4b allele included 4c allele. ^cAdjusted for age, gender, cigarette smoking, and alcohol consumption. * $P < 0.05$.

type in our analyses, was present in 77.8% of the study population. We found that the C4bG haplotype was significantly associated with a 53% decrease in MetS risk (OR, 0.47; 95% CI, 0.25–0.90). In non-MetS subjects, all the three variants were in weak LD with each other. Thus, G894T was in weak LD with the 4b/a ($r^2 = 0.01$, $D' = -0.66$; $P = 0.0045$), and the T-786C variants ($r^2 = 0.01$, $D' = -1.0$; $P < 0.001$); finally, 4b/a was in weak LD with T-786C ($r^2 = 0.42$, $D' = 0.68$; $P < 0.001$).

Furthermore, we evaluated the association between *NOS3* polymorphisms and the risk of MetS stratified by the cigarette smoking status and alcohol consumption status. Table 4 shows the gene-smoking interaction on the risk of MetS. Although the association between the three *NOS3* polymorphisms and MetS risk was not significantly modified by the cigarette smoking status, we

found that the protective effect of the T-786C TC+CC genotype was significantly stronger among those who had ever smoked cigarettes (OR, 0.47; 95% CI, 0.26–0.87).

A moderate gene-environment interaction between the T-786C polymorphism and alcohol consumption on the risk of MetS was observed as shown in Table 5 (P for interaction = 0.07). Among those who had never consumed alcohol, individuals carrying the T-786C TC+CC genotype had a decreased risk of 55% for MetS (OR, 0.45; 95% CI, 0.26–0.77), compared to those carrying the TT genotype. Although there was no significant association between the intron 4b/a and G894T polymorphisms and MetS risk measured for either drinkers or non-drinkers, among never alcohol consumers, the intron 4b/a variant (ba+aa+bc) genotype was significantly associated with a decreased risk

Table 4
Association between polymorphisms in the *NOS3* gene and the risk of metabolic syndrome (MetS) stratified by the cigarette smoking status

<i>NOS3</i> genotype ^a	Cigarette smoking ^b	Non-MetS, n(%) N = 783	MetS, n(%) N = 339	OR (95% CI) ^c	P
T-786C					
TT	Never	433 (56.7)	174 (53.2)	1.00	0.21
TC+CC	Never	89 (11.7)	31 (9.5)	0.73 (0.46–1.18)	
TT	Had ever	181 (23.7)	105 (32.1)	1.00	
TC+CC	Had ever	61 (8.0)	17 (5.2)	0.47 (0.26–0.87)	
P for interaction ^d				0.22	0.015*
Intron 4b/a					
bb	Never	431 (56.4)	178 (54.4)	1.00	0.068
ba+aa+bc	Never	91 (11.9)	27 (8.3)	0.63 (0.38–1.03)	
bb	Had ever	194 (25.4)	104 (31.8)	1.00	
ba+aa+bc	Had ever	48 (6.3)	18 (5.5)	0.75 (0.41–1.37)	
P for interaction ^d				0.75	0.35
G894T					
GG	Never	409 (53.5)	161 (49.2)	1.00	0.67
GT+TT	Never	113 (14.8)	44 (13.5)	0.91 (0.60–1.39)	
GG	Had ever	202 (26.4)	93 (28.4)	1.00	
GT+TT	Had ever	40 (5.2)	29 (8.9)	1.47 (0.84–2.58)	
P for interaction ^d				0.28	0.18

OR: odds ratio; CI: confidence interval. ^aNumbers not being equal to the total number were due to missing data. ^b“Never” indicates non-smokers and “Had ever” is a combination of current smokers and ex-smokers. ^cAdjusted for age, gender, and alcohol consumption. ^dBased on the likelihood ratio test and adjusted for age, gender, and alcohol consumption. **P* < 0.05.

Table 5
Association between polymorphisms in the *NOS3* gene and the risk of metabolic syndrome (MetS) stratified by the alcohol consumption

<i>NOS3</i> genotype ^a	Alcohol drinking ^b	Non-MetS, n(%) N = 783	MetS, n(%) N = 339	OR (95% CI) ^c	P
T-786C					
TT	Never	414 (54.2)	173 (52.6)	1.00	0.004*
TC+CC	Never	93 (12.2)	19 (5.8)	0.45 (0.26–0.77)	
TT	Had ever	202 (26.4)	108 (32.8)	1.00	
TC+CC	Had ever	55 (7.2)	29 (8.8)	0.92 (0.54–1.55)	
P for interaction ^d				0.07	0.74
Intron 4b/a					
bb	Never	420 (55.0)	171 (52.0)	1.00	0.032*
ba+aa+bc	Never	87 (11.4)	21 (6.4)	0.56 (0.33–0.95)	
bb	Had ever	207 (27.1)	113 (34.4)	1.00	
ba+aa+bc	Had ever	50 (6.5)	24 (7.3)	0.87 (0.50–1.53)	
P for interaction ^d				0.28	0.63
G894T					
GG	Never	399 (52.2)	150 (45.6)	1.00	0.88
GT+TT	Never	108 (14.1)	42 (12.8)	1.03 (0.67–1.58)	
GG	Had ever	211 (27.6)	107 (32.5)	1.00	
GT+TT	Had ever	46 (6.0)	30 (9.1)	1.20 (0.70–2.07)	
P for interaction ^d				0.87	0.51

OR: odds ratio; CI: confidence interval. ^aNumbers not being equal to the total number were due to missing data. ^b“Never” indicates non-drinkers and “Had ever” is a combination of current and ex-drinkers. ^cAdjusted for age, gender, and cigarette smoking. ^dBased on the likelihood ratio test and adjusted for age, gender, and cigarette smoking. **P* < 0.05.

of MetS, compared to the intron 4b/a bb genotype (OR, 0.56; 95% CI, 0.33–0.95).

To investigate the pathophysiological mechanisms of how polymorphisms in the *NOS3* gene influence the risk of MetS, we tested both *NOS3* polymorphisms for associations with quantitative traits of MetS in our

study subjects (Table 6). Subjects with the T-786C TC+CC and intron 4b/a ba+aa+bc genotypes had a significantly lower BMI (*P* = 0.009 and *P* = 0.02), compared to those with the T-786C TT and intron 4b/a bb genotypes, respectively. Additionally, the variant intron 4b/a genotypes were marginally associated with

Table 6
Anthropometric and metabolic components in relation to the three *NOS3* polymorphisms of the study population

Variable (mean $\pm$ SD)	T-786C			Intron 4b/a			G894T		
	TT N = 918	TC+CC N = 204	P ^a	bb N = 936	ba+aa+bc N = 186	P ^a	GG N = 891	GT+TT N = 231	P ^a
Waist circumference, cm	82.6 $\pm$ 10.9	81.3 $\pm$ 10.1	0.11*	82.7 $\pm$ 10.9	81.1 $\pm$ 10.4	0.07*	82.5 $\pm$ 10.7	82.2 $\pm$ 11.1	0.72
WHR	0.87 $\pm$ 0.08	0.87 $\pm$ 0.07	0.77	0.87 $\pm$ 0.08	0.87 $\pm$ 0.07	0.73	0.87 $\pm$ 0.07	0.87 $\pm$ 0.08	0.60
BMI, kg/m ²	24.4 $\pm$ 3.8	23.6 $\pm$ 3.4	0.009*	24.4 $\pm$ 3.8	23.7 $\pm$ 3.6	0.02*	24.3 $\pm$ 3.7	24.2 $\pm$ 3.9	0.83
Serum cholesterol, mg/dL	196.5 $\pm$ 39.1	195.6 $\pm$ 34.2	0.78	196.8 $\pm$ 38.3	193.8 $\pm$ 37.9	0.34	195.9 $\pm$ 39.1	197.7 $\pm$ 34.6	0.53
Serum HDL-C, mg/dL	43.5 $\pm$ 14.5	44.1 $\pm$ 13.6	0.62	43.4 $\pm$ 14.3	45.0 $\pm$ 14.3	0.16	43.6 $\pm$ 14.6	43.8 $\pm$ 13.0	0.88
Serum LDL-C, mg/dL	136.1 $\pm$ 37.9	134.8 $\pm$ 38.2	0.64	136.6 $\pm$ 37.6	132.5 $\pm$ 39.7	0.18	134.8 $\pm$ 38.1	140.0 $\pm$ 37.3	0.06
Serum triglyceride, mg/dL	120.3 $\pm$ 95.2	114.9 $\pm$ 84.7	0.45	120.6 $\pm$ 95.1	113.1 $\pm$ 84.1	0.32	121.7 $\pm$ 97.6	110.1 $\pm$ 74.2	0.09
SBP, mm Hg	119.2 $\pm$ 16.3	117.9 $\pm$ 15.9	0.30	119.1 $\pm$ 16.3	118.1 $\pm$ 15.6	0.41	118.7 $\pm$ 16.3	120.0 $\pm$ 15.8	0.26
DBP, mm Hg	76.0 $\pm$ 9.8	75.6 $\pm$ 9.3	0.59	76.0 $\pm$ 9.7	75.7 $\pm$ 9.3	0.71	75.8 $\pm$ 9.6	76.4 $\pm$ 9.9	0.41
Plasma glucose, mg/dL	97.4 $\pm$ 24.7	97.4 $\pm$ 27.0	0.98	97.2 $\pm$ 23.2	98.3 $\pm$ 33.3	0.58	97.0 $\pm$ 25.1	98.8 $\pm$ 25.5	0.35

WHR, waist-to-hip ratio; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure. ^aStudent's *t*-test. **P* < 0.05 (after adjusting for age, gender, cigarette smoking, and alcohol consumption correction).

lower waist circumference (*P* = 0.07). However, the T-786C and intron 4b/a polymorphisms were not associated with other components of MetS. For the G894T polymorphism, the GT+TT genotype (compared to the GG genotype) showed borderline significance for the association with higher LDL-C but lower TGs (*P* = 0.06 and *P* = 0.09, respectively). No other metabolic components differed with G894T genotypes. Nevertheless, after adjusting for age, gender, cigarette smoking, and alcohol consumption, both the BMI and waist circumference remained significantly different according to the T-786C genotypes (both *P* = 0.001) and the intron 4b/a genotypes (*P* = 0.011 and *P* = 0.014, respectively).

4. Discussion

In this large hospital-based case-control study, we found a significantly negative relationship between the T-786C TC+CC genotype of the *NOS3* gene and the risk of MetS (OR = 0.63). This protective genetic effect was prominent for cigarette smokers (OR = 0.47) and those who had never consumed alcohol (OR = 0.45). Similarly, the *NOS3* intron 4b/a variant genotype was marginally associated with a reduced risk of MetS (OR = 0.68), particularly for never alcohol consumers (OR = 0.56). In addition, the C4bG haplotype for the *NOS3* T-786C, intron 4b/a, and G894T polymorphisms was significantly associated with a 53% decreased risk of MetS (OR = 0.47), compared to the most common T4bG haplotype.

To the best of our knowledge, our study is the first to evaluate three *NOS3* polymorphisms and their constructed haplotypes in relation to the risk of MetS in an

ethnic Chinese (i.e., Taiwanese) population. The variant *NOS3* G894T polymorphism was associated with a risk of MetS in five studies [28–32], but not in six other studies [25,27,33–36]. In addition, a positive association between the T-786C polymorphism and MetS was shown in five studies [26–28,34,36], but not in three other studies [33,35,37]. However, three studies were unable to demonstrate an association between the intron 4b/a polymorphisms and MetS risk [34–36].

Discrepancies across those studies might have occurred because of the relatively small sample size in previous studies or because of ethnic differences in allelic frequencies of these polymorphisms. Frequencies of the T-786C C allele, intron 4b/a a allele, and G894T T allele among non-MetS controls in this study (10.1%, 8.4%, and 10.3%, respectively) were similar to controls in a previous study conducted in Taiwan [42]. The frequency of the *NOS3* gene in our controls was also comparable to populations in Japan [28,44], but much lower than those reported for Caucasian populations (39.7%–45.3%, 18.1%, and 30.5%–33.6%, respectively) [25,27,35].

In the present study, we observed that the variant T-786C C allele (TC+CC genotype) was significantly associated with a 37% decrease in the risk of MetS, which was only apparent for the specific haplotype harboring the variant C allele (C4bG vs. T4bG OR, 0.47; 95% CI, 0.25–0.90). In contrast to our study, Gonzalez-Sanchez et al. [26] recently reported that only the haplotype containing the variant T-786C C allele was associated with an increased risk of MetS (OR, 1.81; 95% CI, 1.15–2.84). In addition, Fernandez et al. [27] also reported a positive association between the –786C/894G haplotype and MetS in hypertensive patients (*P* = 0.011). Alkharfy et al. [36] also identified G-a-C as the risk

haplotype for MetS susceptibility (OR, 1.54; 95% CI, 1.1–2.3). However, two studies failed to find significant differences in the distribution of *NOS3* haplotypes [34, 35]. The T-786C polymorphism in the 5'-flanking region of the *NOS3* gene was associated with reduced eNOS activity in an *in vitro* assay [45]. Lower eNOS mRNA and serum nitrite/nitrate levels were found in individuals with the T-786C C variant in one [46] but not in another study [47]. Although our results suggest a protective effect of the T-786C C allele, the underlying mechanism is not clear. We hypothesize that the reduced eNOS level has a protective role in MetS development. Additional research is needed to confirm our observation.

We also observed that the intron 4b/a variant (ba+aa+bc) genotype was slightly associated with decreased risk of MetS (OR = 0.68, $P = 0.051$). Three studies evaluated the association of MetS with the intron 4b/a polymorphism, but they found no significant association [34–36]. Although earlier study suggested that individuals with the 'a' allele of the intron 4b/a polymorphism have decreased plasma NO metabolites than the subjects with the 'b' allele [48], recent studies have shown no significant difference observed in plasma NO concentrations between the intron 4b/a genotypes [49,50]. This may be because the intron 4b/a 4a allele has been reported in linkage disequilibrium with the T-786C C allele [51], and they regulates transcription efficiency in a haplotype-specific way [52]. In a pGL3 reporter system, Wang et al. indicated that the –786C promoter with the 5x27-bp (intron 4b/a 4b allele) insertion had the highest transcription efficiency [52]. Consistent with this, in the present study significant reduced MetS risks were observed for individuals who carrying the *NOS3* intron 4b/a variant genotype and C4bG haplotype.

The present study is the first to report that the MetS risk is reduced for individuals with the variant genotypes in the T-786C and intron 4b/a polymorphisms and those who had never consumed alcohol. Endothelial cell experimental studies demonstrated that both acute and chronic ethanol exposure may increase *NOS3* gene transcription and increase NO production [38,39]. Alcohol consumption may thus overwhelm the genetic advantage of the T-786C and intron 4b/a substitutions. Therefore, the protective effects of the T-786C TC+CC genotype and the intron 4b/a ba+aa+bc genotype on the risk of MetS were prominent among those who had never consumed alcohol. In contrast, because tobacco smoke causes an irreversible inhibition of eNOS activity and reductions in eNOS protein and mRNA lev-

els [40,53], smokers carrying the T-786C C allele may acquire a lower risk of MetS.

This study further demonstrated a significant relationship between the variant genotypes in the *NOS3* T-786C and intron 4b/a polymorphisms and a lower BMI and waist circumference. Although previous studies were unable to find any significant association between the T-786C genotype and BMI [28,54], a recent study reported that some specific *NOS3* haplotypes were associated with three obesity measures (BMI, waist circumference, and percent body fat) [55]. Studies have shown that increased NO production, preferably by eNOS, may cause decreased subcutaneous adipose tissue lipolysis in obesity [56,57]. Our hypothesis could be further speculated that the T-786C and intron 4b/a polymorphisms might prevent MetS by decreasing the obesity.

There are some limitations inherent in this hospital-based study. Participants were recruited from one hospital; therefore, the results may mainly refer to local populations in central Taiwan. Because this quite-sized hospital is a prominent choice for medical services in the local area, our findings may reflect the situation of a good portion of the general population in central Taiwan. The other potential limitation is the absence of functional assays. Future research is needed to determine how *NOS3* polymorphisms functionally underlie a mechanism leading to MetS. In addition, recall bias is another source of information bias in this study. However, the recall bias may be similar between cases and controls using the standardized study procedure. Finally, this study is a cross-sectional study, whether *NOS3* T-786C and intron 4 b/a polymorphisms indeed reduces risk of MetS requires longitudinal follow-up study.

5. Conclusions

We found a significant negative association between MetS and the *NOS3* C4bG haplotype, which harbors only the variant allele of T-786C for the studied *NOS3* polymorphisms. In addition, the T-786C TC+CC genotype was associated with a decreased risk of MetS in those who had never consumed alcohol as well as in those who had ever smoked cigarettes. Similarly, the intron 4b/a variant genotype was significant associated with a reduced risk of MetS in never alcohol consumers. Our results suggest that *NOS3* polymorphisms may contribute to a risk of metabolic syndrome, but further studies are needed to confirm the findings.

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References

- [1] E. Expert Panel on Detection, and Treatment of High Blood Cholesterol in Adults, Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III), *JAMA* **285** (2001), 2486-2497.
- [2] E.S. Ford, W.H. Giles and W.H. Dietz, Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey, *JAMA* **287** (2002), 356-359.
- [3] K. Shiwaku, A. Nogi, K. Kitajima, E. Anuurad, B. Enkhmaa, M. Yamasaki et al., Prevalence of the metabolic syndrome using the modified ATP III definitions for workers in Japan, Korea and Mongolia, *J Occup Health* **47** (2005), 126-135.
- [4] C.C. Lin, C.S. Liu, M.M. Lai, C.I. Li, C.C. Chen, P.C. Chang et al., Metabolic syndrome in a Taiwanese metropolitan adult population, *BMC Public Health* **7** (2007), 239.
- [5] R.H. Eckel, S.M. Grundy and P.Z. Zimmet, The metabolic syndrome, *Lancet* **365** (2005), 1415-1428.
- [6] Y.W. Park, S. Zhu, L. Palaniappan, S. Heshka, M.R. Carnethon and S.B. Heymsfield, The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988-1994, *Arch Intern Med* **163** (2003), 427-436.
- [7] L. Groop, Genetics of the metabolic syndrome, *Br J Nutr* **83**(Suppl 1) (2000), S39-48.
- [8] A.H. Kissebah, G.E. Sonnenberg, J. Myklebust, M. Goldstein, K. Broman, R.G. James et al., Quantitative trait loci on chromosomes 3 and 17 influence phenotypes of the metabolic syndrome, *Proc Natl Acad Sci USA* **97** (2000), 14478-14483.
- [9] H. Duplain, R. Burcelin, C. Sartori, S. Cook, M. Egli, M. Lepori et al., Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase, *Circulation* **104** (2001), 342-345.
- [10] D.S. Brecht, Endogenous nitric oxide synthesis: biological functions and pathophysiology, *Free Radic Res* **31** (1999), 577-596.
- [11] D.A. Cox, J.A. Vita, C.B. Treasure, R.D. Fish, R.W. Alexander, P. Ganz et al., Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans, *Circulation* **80** (1989), 458-465.
- [12] H. Drexler, A.M. Zeiher, H. Wollschlaeger, T. Meinertz, H. Just and T. Bonzel, Flow-dependent coronary artery dilatation in humans, *Circulation* **80** (1989), 466-474.
- [13] G.M. Pieper, Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration, *Diabetologia* **42** (1999), 204-213.
- [14] P.M. Piatti, L.D. Monti, I. Zavaroni, G. Valsecchi, C. Van Phan, S. Costa et al., Alterations in nitric oxide/cyclic-GMP pathway in nondiabetic siblings of patients with type 2 diabetes, *J Clin Endocrinol Metab* **85** (2000), 2416-2420.
- [15] I. Zavaroni, P.M. Piatti, L.D. Monti, P. Gasparini, L.A. Barilli, P. Massironi et al., Plasma nitric oxide concentrations are elevated in insulin-resistant healthy subjects, *Metabolism* **49** (2000), 959-961.
- [16] P.A. Marsden, H.H. Heng, S.W. Scherer, R.J. Stewart, A.V. Hall, X.M. Shi et al., Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene, *J Biol Chem* **268** (1993), 17478-17488.
- [17] B. Mayer and B. Hemmens, Biosynthesis and action of nitric oxide in mammalian cells, *Trends Biochem Sci* **22** (1997), 477-481.
- [18] D.J. Stuehr, Structure-function aspects in the nitric oxide synthases, *Annu Rev Pharmacol Toxicol* **37** (1997), 339-359.
- [19] J.P. Casas, G.L. Cavalleri, L.E. Bautista, L. Smeeth, S.E. Humphries and A.D. Hingorani, Endothelial nitric oxide synthase gene polymorphisms and cardiovascular disease: a HuGE review, *Am J Epidemiol* **164** (2006), 921-935.
- [20] L.D. Monti, C. Barlassina, L. Citterio, E. Galluccio, C. Berzuni, E. Setola et al., Endothelial nitric oxide synthase polymorphisms are associated with type 2 diabetes and the insulin resistance syndrome, *Diabetes* **52** (2003), 1270-1275.
- [21] K. Ohtoshi, Y. Yamasaki, S. Gorogawa, R. Hayaishi-Okano, K. Node, M. Matsuhisa et al., Association of (-)786T-C mutation of endothelial nitric oxide synthase gene with insulin resistance, *Diabetologia* **45** (2002), 1594-1601.
- [22] A.W. Tso, K.C. Tan, N.M. Wat, E.D. Janus, T.H. Lam and S.L.L. K, Endothelial nitric oxide synthase G894T (Glu298Asp) polymorphism was predictive of glycemic status in a 5-year prospective study of Chinese subjects with impaired glucose tolerance, *Metabolism* **55** (2006), 1155-1158.
- [23] A.V. Benjafield and B.J. Morris, Association analyses of endothelial nitric oxide synthase gene polymorphisms in essential hypertension, *Am J Hypertens* **13** (2000), 994-998.
- [24] O. Ukkola, P.H. Erkkila, M.J. Savolainen and Y.A. Kesäniemi, Lack of association between polymorphisms of catalase, copper-zinc superoxide dismutase (SOD), extracellular SOD and endothelial nitric oxide synthase genes and macroangiopathy in patients with type 2 diabetes mellitus, *J Intern Med* **249** (2001), 451-459.
- [25] M. Zeman, M. Jachymova, R. Jirak, M. Veccka, E. Tvřizcka, B. Stankova et al., Polymorphisms of genes for brain-derived neurotrophic factor, methylenetetrahydrofolate reductase, tyrosine hydroxylase, and endothelial nitric oxide synthase in depression and metabolic syndrome, *Folia Biol (Praha)* **56** (2010), 19-26.
- [26] J.L. Gonzalez-Sanchez, M.T. Martinez-Larrad, M.E. Saez, C. Zabena, M.J. Martinez-Calatrava and M. Serrano-Rios, Endothelial nitric oxide synthase haplotypes are associated with features of metabolic syndrome, *Clin Chem* **53** (2007), 91-97.
- [27] M.L. Fernandez, R. Ruiz, M.A. Gonzalez, R. Ramirez-Lorca, C. Couto, A. Ramos et al., Association of NOS3 gene with metabolic syndrome in hypertensive patients, *Thromb Haemost* **92** (2004), 413-418.
- [28] A. Imamura, R. Takahashi, R. Murakami, H. Kataoka, X.W. Cheng, Y. Numaguchi et al., The effects of endothelial nitric oxide synthase gene polymorphisms on endothelial function

- and metabolic risk factors in healthy subjects: the significance of plasma adiponectin levels, *Eur J Endocrinol* **158** (2008), 189-195.
- [29] M.C. Hsieh, J.Y. Hsiao, K.J. Tien, S.J. Chang, P.C. Lin, S.C. Hsu et al., The association of endothelial nitric oxide synthase G894T polymorphism with C-reactive protein level and metabolic syndrome in a Chinese study group, *Metabolism* **57** (2008), 1125-1129.
- [30] J.C. Piccoli, M.G. Gottlieb, L. Castro, L.C. Bodanese, E.R. Manenti, M.R. Bogo et al., Association between 894G>T endothelial nitric oxide synthase gene polymorphisms and metabolic syndrome, *Arq Bras Endocrinol Metabol* **52** (2008), 1367-1373.
- [31] D. Pons, P.S. Monraats, A.H. Zwinderman, M.P. de Maat, P.A. Doevendans, R.J. de Winter et al., Metabolic background determines the importance of NOS3 polymorphisms in restenosis after percutaneous coronary intervention: A study in patients with and without the metabolic syndrome, *Dis Markers* **26** (2009), 75-83.
- [32] Y.C. Lee, S.P. Huang, C.C. Liu, Y.H. Yang, H.C. Yeh, W.M. Li et al., The association of eNOS G894T polymorphism with metabolic syndrome and erectile dysfunction, *J Sex Med* **9** (2012), 837-843.
- [33] K. Kotaska, J. Kolarova, K. Kotrcova, J. Cepova and R. Prusa, Correlation between common genetic variants and risk factors associated with prediction of cardiovascular diseases in dyslipidemic patients, *Genet Test Mol Biomarkers* **16** (2012), 210-214.
- [34] J.A. Miranda, V.A. Belo, D.C. Souza-Costa, C.M. Lanna and J.E. Tanus-Santos, eNOS polymorphism associated with metabolic syndrome in children and adolescents, *Mol Cell Biochem* **372** (2013), 155-160.
- [35] G. Dell'Omo, G. Penno, L. Pucci, C. Fotino, D. Lucchesi, S. Del Prato et al., Lack of association between endothelial nitric oxide synthase gene polymorphisms, microalbuminuria and endothelial dysfunction in hypertensive men, *J Hypertens* **25** (2007), 1389-1395.
- [36] K.M. Alkharfy, N.M. Al-Daghri, O.S. Al-Attas, M.S. Alokail, A.K. Mohammed, B. Vinodson et al., Variants of endothelial nitric oxide synthase gene are associated with components of metabolic syndrome in an Arab population, *Endocr J* **59** (2012), 253-263.
- [37] B. Misiak, M. Krolak, A. Kukowka, A. Lewera, P. Leszczynski, J. Stankiewicz-Olczyk et al., The Role of -786T/C Polymorphism in the Endothelial Nitric Oxide Synthase Gene in Males with Clinical and Biochemical Features of the Metabolic Syndrome, *Int J Endocrinol* **2011** (2011), 458750.
- [38] C.G. Acevedo, G. Carrasco, M. Burotto, S. Rojas and I. Bravo, Ethanol inhibits L-arginine uptake and enhances NO formation in human placenta, *Life Sci* **68** (2001), 2893-2903.
- [39] J.A. Polikandriotis, H.L. Rupnow and C.M. Hart, Chronic ethanol exposure stimulates endothelial cell nitric oxide production through PI-3 kinase-and hsp90-dependent mechanisms, *Alcohol Clin Exp Res* **29** (2005), 1932-1938.
- [40] Y. Su, W. Han, C. Giraldo, Y. De Li and E.R. Block, Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells, *Am J Respir Cell Mol Biol* **19** (1998), 819-825.
- [41] C.S. Liu, H.S. Hsu, C.I. Li, C.I. Jan, T.C. Li, W.Y. Lin et al., Central obesity and atherogenic dyslipidemia in metabolic syndrome are associated with increased risk for colorectal adenoma in a Chinese population, *BMC Gastroenterol* **10** (2010), 51.
- [42] C.C. Yeh, R.M. Santella, L.L. Hsieh, F.C. Sung and R. Tang, An intron 4 VNTR polymorphism of the endothelial nitric oxide synthase gene is associated with early-onset colorectal cancer, *Int J Cancer* **124** (2009), 1565-1571.
- [43] D.A. Tregouet and V. Garelle, A new JAVA interface implementation of THESIAS: testing haplotype effects in association studies, *Bioinformatics* **23** (2007), 1038-1039.
- [44] T. Awata, T. Neda, H. Iizuka, S. Kurihara, T. Ohkubo, N. Takata et al., Endothelial nitric oxide synthase gene is associated with diabetic macular edema in type 2 diabetes, *Diabetes Care* **27** (2004), 2184-2190.
- [45] M. Nakayama, H. Yasue, M. Yoshimura, Y. Shimasaki, K. Kugiyama, H. Ogawa et al., T-786->C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm, *Circulation* **99** (1999), 2864-2870.
- [46] Y. Miyamoto, Y. Saito, M. Nakayama, Y. Shimasaki, T. Yoshimura, M. Yoshimura et al., Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a -786T->C mutation associated with coronary spastic angina, *Hum Mol Genet* **9** (2000), 2629-2637.
- [47] N. Jeerooburkhan, L.C. Jones, S. Bujac, J.A. Cooper, G.J. Miller, P. Vallance et al., Genetic and environmental determinants of plasma nitrogen oxides and risk of ischemic heart disease, *Hypertension* **38** (2001), 1054-1061.
- [48] T. Tsukada, K. Yokoyama, T. Arai, F. Takemoto, S. Hara, A. Yamada et al., Evidence of association of the eNOS gene polymorphism with plasma NO metabolite levels in humans, *Biochem Biophys Res Commun* **245** (1998), 190-193.
- [49] S. Salimi, M. Firoozrai, I. Nourmohammadi, M. Shabani and A. Mohebbi, Endothelial nitric oxide synthase gene intron4 VNTR polymorphism in patients with coronary artery disease in Iran, *Indian J Med Res* **124** (2006), 683-688.
- [50] T. Angeline, W. Isabel and G.J. Tsongalis, Endothelial nitric oxide gene polymorphisms, nitric oxide production and coronary artery disease risk in a South Indian population, *Exp Mol Pathol* **89** (2010), 205-208.
- [51] M. Yoshimura, H. Yasue, M. Nakayama, Y. Shimasaki, H. Ogawa, K. Kugiyama et al., Genetic risk factors for coronary artery spasm: significance of endothelial nitric oxide synthase gene T-786->C and missense Glu298Asp variants, *J Investig Med* **48** (2000), 367-374.
- [52] J. Wang, D. Dudley and X.L. Wang, Haplotype-specific effects on endothelial NO synthase promoter efficiency: modifiable by cigarette smoking, *Arterioscler Thromb Vasc Biol* **22** (2002), e1-4.
- [53] X.L. Wang, A.S. Sim, M.X. Wang, G.A. Murrell, B. Trudinger and J. Wang, Genotype dependent and cigarette specific effects on endothelial nitric oxide synthase gene expression and enzyme activity, *FEBS Lett* **471** (2000), 45-50.
- [54] S.D. Nath, X. He, V.S. Voruganti, J. Blangero, J.W. McCluer, A.G. Comuzzie et al., The 27-bp repeat polymorphism in intron 4 (27 bp-VNTR) of endothelial nitric oxide synthase (eNOS) gene is associated with albumin to creatinine ratio in Mexican Americans, *Mol Cell Biochem* **331** (2009), 201-205.
- [55] V. Lagou, G. Liu, H. Zhu, I.S. Stallmann-Jorgensen, B. Gutin, Y. Dong et al., Lifestyle and socioeconomic-status modify the effects of ADRB2 and NOS3 on adiposity in European-American and African-American adolescents, *Obesity (Silver Spring)* **19** (2011), 595-603.
- [56] M. Elizalde, M. Ryden, V. van Harmelen, P. Eneroth, H. Gyllenhammar, C. Holm et al., Expression of nitric oxide synthases in subcutaneous adipose tissue of nonobese and obese humans, *J Lipid Res* **41** (2000), 1244-1251.

- [57] M. Ryden, M. Elizalde, V. van Harmelen, A. Ohlund, J. Hoffstedt, S. Bringman et al., Increased expression of eNOS protein in omental versus subcutaneous adipose tissue in obese human subjects, *Int J Obes Relat Metab Disord* **25** (2001), 811-815.