

## Lack of association between endothelial nitric oxide synthase glu298Asp variation, visceral obesity and insulin related phenotypes in Turkish type 2 diabetic patients

Burcu Bayoglu<sup>1</sup>, Melike Ersoz<sup>2</sup>, Penbe Cagatay<sup>3</sup>, Cavlan Ciftci<sup>4</sup>, Belgin Súsleyici Duman<sup>5</sup>

<sup>1</sup> Istanbul University, Cerrahpasa Faculty of Medicine, Department of Medical Biology, Istanbul, Turkey.

<sup>2</sup> Istanbul Bilim University, Faculty of Medicine, Basic Sciences Laboratory, Istanbul, Turkey.

<sup>3</sup> Istanbul University, Cerrahpasa Faculty of Medicine, Department of Biostatistics, Istanbul, Turkey.

<sup>4</sup> Istanbul Bilim University, Faculty of Medicine, Department of Cardiology, Istanbul, Turkey.

<sup>5</sup> Marmara University, Science and Art Faculty, Biology Division, Molecular Biology Department, Goztepe-Istanbul, Turkey

Corresponding author:

Belgin Súsleyici Duman  
Assoc. Prof. Dr.

Marmara University, Science and Art Faculty,  
Biology Division,

Molecular Biology Department,  
34722 Goztepe-Istanbul, Turkey

[belgin.susleyici@marmara.edu.tr](mailto:belgin.susleyici@marmara.edu.tr)

Nitric oxide (NO) is an endothelium derived relaxing factor (EDRF) important in regulating heart-vessel physiology. The objective of this study was to investigate whether the eNOS gene Glu298Asp variation influenced the lipid parameters, visceral obesity, insulin related phenotypes and type 2 diabetes mellitus (T2DM) development, for the first time in a Turkish study group. We analyzed the the eNOS gene Glu298Asp genotype frequencies in 115 type 2 diabetic and 68 healthy control subjects. Serum lipids and insulin-related phenotypes were also analyzed. No significant difference for genotypic frequencies was observed for the Ban II (Eco241) restriction site in T2DM patients as compared to controls. eNOS Glu298Asp polymorphism was not found to affect visceral obesity and insulin related phenotypes. However, T2DM patients with Asp/Asp genotype were found to have lower hepatic insulin sensitivity (HIS) in comparison to Glu/Glu. In healthy controls, the insulin and HOMA levels were found to be lower in Glu/Asp genotype with respect to Glu/Glu genotype carriers ( $p > 0.05$ ). In T2DM patients, visceral obesity was observed in higher frequencies with Asp/Asp genotype, in comparison to Glu/Glu genotype. eNOS Glu298Asp polymorphism was not found to affect serum lipid levels in the T2DM group. However in the control group, lower serum apoB levels were observed in Asp/Asp genotype carriers in comparison to Glu/Glu genotype ( $p \leq 0.05$ ). The eNOS gene Glu298Asp polymorphism was not found to be associated with T2DM in the present study group. Although not significant, since the eNOS Glu298Asp genotypes were found to be related to HIS, insulin, HOMA and visceral obesity in the present study, further studies on larger samples are needed to explore the exact role of eNOS Glu298Asp polymorphism in insulin related phenotypes and visceral obesity.

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## Introduction

Type 2 diabetes mellitus (T2DM) is a heterogeneous disorder that develops in response to both genetic and environmental factors (1). The predisposition to T2DM is thought to be conferred by a number of different genes that in isolation may have minor effects, but in combination lead to the characteristic pathophysiological condition (2).

Nitric oxide (NO) is synthesized from L-Arginine by nitric oxide synthase (NOS). NOS has three isoforms; neuronal (nNOS), induced (iNOS), and endothelial (eNOS). eNOS is the only isoform constitutively synthesized both in vivo and in vitro (3). Arginine deficiency is a rare occurrence, however, there can be competitive inhibition by the endogenously produced asymmetrical dimethylarginine (ADMA) and nitroarginine. ADMA is emerging as an important cause of endothelial cell dysfunction. The relative deficiency of L-arginine due to elevation of ADMA levels contributes to oxidative stress and results in the atheroscleropathy associated with insulin resistance, metabolic syndrome, prediabetes and overt T2DM (4).

Genetic polymorphisms of eNOS have been shown to have a significant effect on NO levels, plasma lipids and have been associated with T2DM (5), heart failure (6), coronary spasm (7), atherosclerosis (8), myocardial infarction (9) and hypertension (9) in some studies. Several studies have reported restriction fragment length polymorphisms (RFLP) of eNOS Glu298Asp to be associated with type 2 diabetes while others did not find such an association (5, 10, 11). Glu298Asp polymorphism of the eNOS gene is caused by a base substitution (G→T) in the position 894 of the exon 7, changing Glutamic acid to Aspartic acid (12).

Since the contribution of eNOS gene polymorphisms to the development of type 2 diabetes differ among populations, the aim of the present study was to evaluate the frequency distributions of eNOS Glu298Asp

genotypes in Turkish patients with T2DM as compared to controls. Also the influence of Glu298Asp polymorphism over lipid parameters, visceral obesity, insulin related phenotypes together with their association with type 2 diabetes was evaluated.

## Methods

### *Population sample*

We studied 115 unrelated type 2 diabetic patients (67 men and 48 women; age:  $58.24 \pm 0.94$  years). The patients were recruited from Caglayan Florence Nightingale Hospital (Istanbul, Turkey). Age at diabetes onset was  $45.05 \pm 11.85$  years. Type 2 diabetic patients were selected according to WHO criteria (13). Of the 115 type 2 diabetic patients, 64 were treated with sulphonylurea drugs, 38 with metformin and 13 with sulphonylurea drugs in combination with metformin. The study protocol was approved by the Ethics Committee of the Kadir Has University, Faculty of Medicine, and informed consent was obtained from each participant. The control group consisted of 68 unrelated healthy individuals (47 men and 21 women; age:  $55.31 \pm 1.47$  years) without medication, who attended a routine health check at a general practice in Caglayan Florence Nightingale Hospital (Istanbul, Turkey). The hepatic and endocrine functions of the patients were normal and all were relatively well controlled with glycosylated hemoglobin (HbA1c)  $\leq 7\%$  (normal range  $\leq 8\%$ ). Patients with macro- and microangiopathic complications were excluded from the study. No member of the sample populations admitted to alcohol intake and none had a history of smoking.

### *Clinical and biochemical evaluation*

Blood samples were collected after overnight (>12h) fasting. The biochemical analysis included determination of fasting plasma glucose, insulin, HbA1c, hepatic insulin sensi-

tivity (HIS), index of  $\beta$ -cell secretory force (HOMA), total cholesterol (T-Chol), triacylglycerol (TAG), apolipoprotein E (apo E), apolipoprotein A1 (apo A1) and apolipoprotein B (apo B). Serum TAG and T-Chol levels were measured using standard enzymatic methods (Merck, Darmstadt, Germany), automated on an AU5021 (Olympus, Merck). Serum apo E was determined by turbidimetry automated on a Cobas-Mira analyzer (Roche, Meylan, France); serum apo A1 and apo B were determined by immunonephelometry on a Behring Nephelometer analyzer with Behring reagents (Behringwerke, Marburg, Germany). Sera were analyzed without pretreatment and diluted in double-distilled water when lipid or apolipoprotein levels exceeded reference values.

#### *Anthropometric measurements*

##### *Body mass index (BMI)*

The body mass index (BMI) was calculated and overweight (obese) was defined as a value  $\geq 25 \text{ kg/m}^2$  (14).

##### *Waist to hip circumference ratio (WHCR)*

Waist circumference was measured at the level of the umbilicus while the subject was standing and breathing normally. Hip circumference was measured at the level of greatest hip girth. All participants were accepted as abdominal (visceral) obese, since their WHCR were greater than 0.95 and 1.0 for females and males, respectively.

##### *Pancreatic $\beta$ -cell secretory capacity*

Pancreatic  $\beta$ -cell secretory capacity was estimated by  $\beta$ -index (index of  $\beta$ -cell secretory force; HOMA  $\beta$ -cell index) by the formula proposed by Hosker et al. (15), HOMA  $\beta$ -cell index =  $20 \times \text{insulin}^F / (\text{glucose}^F - 3.5)$ .

##### *Hepatic insulin sensitivity*

Hepatic insulin sensitivity was assessed by the following formulas realized by Matsuda

and De Fronzo (16): Hepatic insulin sensitivity (HIS) =  $k / (G^F \times I^F)$  ( $k = 22.5 \times 18 = 405$ ;  $G^F$  and  $I^F$ , fasting plasma glucose (mg/dl) and insulin ( $\mu\text{U/ml}$ ), respectively).

#### *Molecular analysis*

Genomic DNA was extracted from leukocytes by a salting out procedure (17). The desired segments were amplified by PCR (18) using the eNOS Ban II (Eco241) protocol with primers (Integrated DNA Technologies, IDT, USA): 298F: 5'-GAC CCT GGA GAT GAA GGC AGG AGA-3' and 298R: 5'-ACC ACC AGG ATG TTG TAG CGG TGA-3'. The final 248 bp amplification products produce 163 bp and 85 bp products for the Glu298 allele, but fails to cleave the 248 bp fragment containing the Asp298 allele after digestion with Ban II. Restricted products were visualized on 2% agarose gel.

#### *Statistical analysis*

Statistical analyses were conducted using the Unistat 5.1 software program. Data were expressed as means  $\pm$  SE. Baseline differences between patients and controls were examined by Student t-test. Hardy-Weinberg equilibrium for genotype frequencies was estimated by the Chi-square test. The variables across the various genotypes and groups were estimated by two way ANOVA with an interaction term to test the influence of eNOS Glu298Asp genotypes on analyzed parameters. The Bonferroni correction for multiple testing was applied to T2DM and control groups separately as required. P values less than 0.05 were considered significant.

## **Results**

The genotype frequency distributions of the 115 type 2 diabetic and 68 control subjects with respect to Glu298Asp polymorphism were compared (Table 1).

Table 1 eNOS Glu298Asp genotype frequencies in type 2 diabetic patients and control subjects

	eNOS Glu298Asp Genotype frequencies		
	Glu/Glu; n (%)	Glu/Asp; n (%)	Asp/Asp; n (%)
Diabetic	15 (13.0)	43 (37.4)	57 (49.6)
Control	11 (16.2)	29 (42.6)	28 (41.2)

Results are expressed as numbers (percentage). The eNOS genotype frequencies of the control and diabetic groups were compared with Chi-square test and no significance was found ( $\chi^2 = 0.243$ ,  $p = 0.537$ ).

The eNOS gene Glu298Asp polymorphism frequencies for Glu/Glu, Glu/Asp and Asp/Asp genotypes were respectively 13%, 37.4%, 49.6% in subjects with type 2 diabetes and 16.2%, 42.6%, 41.2% in the control group. No significant difference was observed in genotype frequencies between the type 2 diabetic and control groups ( $\chi^2 = 1.243$ ,  $p = 0.537$ ). When the demographic characteristics of the study subjects, together with the eNOS Glu298Asp genotype effect and group genotype interaction were examined no significant difference was observed for any analyzed characteristic when the diabetic and control groups were compared (data not included). Also no significant effect of eNOS genotypes was found over the demographic parameters. The clinical characteristics of the study subjects are compared as a function of groups and eNOS genotypes. In detail, insulin ( $p \leq 0.001$ ), HbA1c ( $p \leq 0.001$ ), fasting glucose ( $p \leq 0.001$ ) and  $\beta$ -cell index ( $p \leq 0.001$ ) were significantly higher in diabetic patients compared to controls, whereas HIS ( $p \leq 0.001$ ) was higher in controls. No significant difference was observed ( $p \geq 0.05$ ) for the T-Chol, TAG, apo E, apo A1 and apo B levels when the diabetic and control groups were compared (data not included). The effects of the eNOS polymorphism on clinical or biochemical characteristics were analyzed and not found to be significantly effective (data

not included). The demographic and clinical parameters were compared between eNOS Glu298Asp genotypes separately for type 2 diabetic and control groups (Table 2).

Although no significant difference was observed, T2DM patients with Asp/Asp genotype were found to have lower HIS levels in comparison to Glu/Glu. In healthy controls, the insulin and HOMA levels were found to be lower in Glu/Asp genotype with respect to Glu/Glu genotype carriers ( $p > 0.05$ ). Also, in T2DM patients with Asp/Asp genotype, visceral obesity was found to be higher in comparison to the Glu/Glu genotype. None of the analyzed serum lipids were found to differ between the eNOS Glu298Asp genotypes in patients with T2DM (Table 2). In the control group, the demographic, clinical and biochemical parameters were not found to be different among eNOS Glu298Asp genotypes except for apo B levels. In detail, lower serum apo B levels were observed in the Asp/Asp genotype when compared to Glu/Glu genotype carriers ( $p \leq 0.05$ ).

## Discussion

Multiple studies provide evidence that genetic factors are important contributors to the inter-individual variation in diabetes susceptibility (19-21). The eNOS gene Glu298Asp polymorphism has been reported to decrease the basal NO production in healthy subjects (22). eNOS Glu298Asp polymorphism may interact with other gene polymorphisms of other endogenous antioxidant enzymes and especially environmental conditions such as smoking, obesity, toxicities of insulin resistance, metabolic syndrome and T2DM as we know their antioxidant reserve is compromised (23-24). eNOS Glu298Asp polymorphism causes endothelial dysfunction, and thus oxidative stress, may be responsible for insulin resistance and T2DM (4). For this reason we thought that

Table 2 Clinical characteristics of type 2 diabetic and control subjects with respect to eNOS gene Glu298Asp genotypes

Parameter	eNOS gene Glu298Asp Genotype					
	T2DM			Control		
	Glu/Glu (n = 15)	Glu/Asp (n = 43)	Asp/Asp (n = 57)	Glu/Glu (n = 11)	Glu/Asp (n = 29)	Asp/Asp (n = 28)
Weight (kg)	69.00 ± 2.38	76.90 ± 2.12	72.35 ± 1.57	72.72 ± 4.82	71.78 ± 2.08	73.14 ± 2.47
Height (m)	1.61 ± 0.02	1.65 ± 0.01	1.64 ± 0.01	1.63 ± 0.03	1.63 ± 0.01	1.63 ± 0.01
BMI (kg/m <sup>2</sup> )	26.48 ± 1.02	28.22 ± 0.71	26.99 ± 0.56	26.94 ± 1.21	27.01 ± 0.75	27.47 ± 0.84
Waist (cm)	88.13 ± 5.94	98.62 ± 2.80	97.78 ± 2.47	90.81 ± 7.78	96.82 ± 3.89	96.92 ± 3.93
Hip (cm)	101.20 ± 2.53	104.97 ± 1.32	103.37 ± 1.14	100.45 ± 2.95	104.39 ± 1.43	104.71 ± 1.40
Waist to hip ratio (cm)	0.86 ± 0.04	0.93 ± 0.02	0.94 ± 0.02	0.90 ± 0.06	0.92 ± 0.03	0.92 ± 0.03
Fasting glucose (mmol/l)	9.43 ± 1.36	9.66 ± 0.56	9.61 ± 0.50	3.52 ± 0.17	3.74 ± 0.16	3.51 ± 0.13
HbA1c (%)	7.43 ± 0.65	7.63 ± 0.35	7.83 ± 0.30	4.46 ± 0.14	4.66 ± 0.09	4.54 ± 0.08
Insulin (μU/ml)	11.64 ± 1.62	15.43 ± 0.94	13.88 ± 0.79	4.00 ± 0.61	3.78 ± 0.27	3.87 ± 0.33
Hepatic insulin sensitivity	0.37 ± 0.07	0.22 ± 0.03	0.27 ± 0.03	1.98 ± 0.29	1.93 ± 0.19	2.00 ± 0.19
β- cell index (HOMA)	1.54 ± 0.24	1.95 ± 0.13	1.78 ± 0.10	1.36 ± 0.22	1.22 ± 0.09	1.34 ± 0.13
Total-cholesterol (mmol/l)	5.64 ± 0.41	5.37 ± 0.16	5.39 ± 0.16	6.33 ± 0.37	5.65 ± 0.24	5.30 ± 0.27
Triacylglycerol (mg/dl)	1.40 ± 0.17	2.17 ± 0.27	2.03 ± 0.35	1.69 ± 0.16	2.02 ± 0.19	1.67 ± 0.20
Apolipoprotein E (mg/l)	41.29 ± 3.31	47.28 ± 3.65	45.01 ± 4.19	43.44 ± 4.29	58.04 ± 8.61	49.56 ± 4.87
Apolipoprotein A1 (g/l)	1.45 ± 0.09	1.39 ± 0.03	1.43 ± 0.03	1.41 ± 0.07	1.37 ± 0.04	1.43 ± 0.05
Apolipoprotein B (g/l)	1.14 ± 0.09	1.11 ± 0.03	1.19 ± 0.06	1.33 ± 0.08 <sup>A</sup>	1.16 ± 0.06	1.08 ± 0.04

Values are represented as mean ± SE. BMI: body mass index. HbA1c: glycosylated hemoglobin. HIS: hepatic insulin sensitivity (=  $k / \text{fasting insulin} \times \text{fasting plasma glucose}$  (where  $k = 22.5 \times 18 = 405$ ). HOMA: homeostasis model assessment (=  $20 \times \text{fasting insulin} / (\text{fasting plasma glucose} - 3.5)$ ). <sup>A</sup> $p \leq 0.05$  Glu/Glu versus genotype in controls.

Glu298Asp variation may be responsible for T2DM.

A limited number of studies have examined eNOS gene Glu298Asp polymorphism (5, 10, 11, 25) in patients with T2DM. According to our knowledge, only one study (5) has found an association between eNOS Glu298Asp polymorphism and T2DM. In detail, Monti et al. (5) evaluated Glu298Asp

polymorphism in exon 7 in 159 type 2 diabetic patients without macrovascular complications and in 207 healthy control subjects and described a significant association between eNOS gene Glu298Asp polymorphism and T2DM. Monti et al. (5) suggested eNOS Glu298Asp polymorphism as a new genetic susceptibility factor for hyperinsulinemia, insulin resistance and T2DM. In a study

from the United Kingdom (10), 152 SNPs in 71 candidate genes were examined in 2134 Caucasians, where no association was found between eNOS Glu298Asp polymorphism and T2DM. Thameem et al. (25) investigated whether the T-786C, Glu298Asp and 27bp-VNTR variants of the eNOS gene are associated with T2DM and its related traits in Mexican Americans and did not find Glu298Asp polymorphism as a significant contributor to disease. Similar to Barroso et al. (10) and Thameem (25), we were not able to demonstrate any association between eNOS Glu298Asp polymorphism and T2DM in the present study. A possible explanation for the lack of relationship of polymorphism with disease in the present study may be that patients with gene polymorphism of the eNOS enzyme may be capable of withstanding many years of redox stress before the defect in eNOS becomes evident.

The Glu298Asp allelic variation of the eNOS gene shows variations in different ethnic groups. Ukkola et al. (11) evaluated the presence of the Glu298Asp polymorphism in 239 Caucasian patients with T2DM with a high prevalence of macroangiopathy and 245 control subjects, but did not find any significant difference in the allelic frequency between the T2DM and the control groups. In 159 Caucasian T2DM patients without macrovascular complications Monti et al. (5) reported Glu298Asp Glu/Glu, Glu/Asp and Asp/Asp genotype frequencies to be 32.7%, 39.6% and 27.7%; whereas in 207 healthy control subjects they were 46.4%, 39.6% and 14%. Monti et al. (5) found Glu298Asp genotype frequencies significantly different among their study groups ( $\chi^2 = 1$ ,  $p = 0.0005$ ). In detail, Asp/Asp genotype frequency was higher in type 2 diabetic patients in comparison to controls. Thadeem et al. (25) evaluated 670 low-income Mexican Americans with T2DM, and all first-, second- and third degree relatives. They reported Glu298Asp Glu/Glu, Glu/Asp and

Asp/Asp genotype frequencies to be 65%, 30% and 5% (25). In another study, Srivastava et al. (26) reported Glu298Asp Glu/Glu, Glu/Asp, Asp/Asp genotype frequencies respectively as, 71.22%, 28.06%, 0.72% in 139 healthy Indians, and did not find any significant difference between the groups with respect to Glu298Asp genotypes. In our study the Glu/Glu, Glu/Asp and Asp/Asp genotype frequencies were respectively as 13.0%, 37.4% 49.6 %for the diabetic group; and 16.2%, 42.6% and 41.2% for the control group. We did not find any significant difference between the T2DM and control groups, when the frequency of eNOS genotypes were compared ( $\chi^2 = 1.243$ ,  $p = 0.537$ ).

Monti et al. (5) were not able to find any difference in metabolic parameters (plasma glucose, BMI, TAG, systolic and diastolic blood pressure) except visceral obesity (waist to hip ratio). They found visceral obesity much higher in Asp/Asp genotype carriers in comparison to Glu/Glu and Glu/Asp (5). In agreement with the results of Monti et al. (5), although not statistically significant, visceral obesity was found to be higher in type 2 diabetic patients with Asp/Asp genotype when compared to Glu/Glu in our study. Furthermore, Yoshimura et al. (27) showed that Asp/Asp genotype lowered HIS levels in coronary artery disease. Interestingly, in our study we observed that in type 2 diabetic patients the Asp/Asp genotype carriers had lower HIS levels when compared to Glu/Glu genotype carriers. Exercise induced skeletal muscle glucose transport (GLUT4) is eNOS dependent. If the production of eNO were defective due to gene polymorphism and environmental interaction there would be increasing peripheral insulin resistance (28-30). Monti et al. (5) showed that in healthy controls, Asp/Asp genotype carriers had higher insulin, C-peptide, NO, and HOMA levels compared to Glu/Glu genotype. In the present study, in healthy controls, the insulin and HOMA levels were found to be lower in

Glu/Asp genotype with respect to Glu/Glu genotype carriers.

Paradossi et al. (31) evaluated Glu298Asp polymorphism in 118 healthy control subjects and found no influence on lipid parameters. In the present study the Glu298Asp variant of the eNOS gene was not found to be associated with the lipid parameters in the T2DM group. However, Ukkola et al. (11) showed that male diabetic patients with Asp/Asp genotype had higher plasma very-low density lipoprotein (VLDL) cholesterol and VLDL-triacylglycerol concentrations than those with the genotypes Glu/Glu or Glu/Asp. In the present study we found an association between the presence of the Glu-298Asp polymorphism of the eNOS gene and apo B levels in the control group. But we did not find any association between Glu298Asp variation and the clinical parameters in the T2DM group.

## Conclusion

There was no significant difference in genotypic frequencies of the Glu298Asp polymorphism of the eNOS gene between the T2DM and control groups. In the present study, the Glu298Asp polymorphism of the eNOS gene is not associated either with visceral obesity or with insulin related phenotypes in Turkish samples with T2DM, but is related to apo B levels in the control group. Since the limitation of this study was the relatively small sample size, the study should be replicated with a larger sample. Increasing the sample size would improve the statistical power of the study to detect significant changes.

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