

# Endothelial Nitric Oxide Synthase (eNOS) 4a/b and G894T Polymorphisms and Susceptibility to Preeclampsia

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## Abstract

**Background:** Preeclampsia is a pregnancy complication with unknown etiology and its incidence is associated with genetic and environmental factors. There are inconsistent reports related to the role of endothelial nitric oxide synthase (eNOS) 4a/b polymorphism on the risk of preeclampsia development. The aim of the present study was to investigate the possible influence of eNOS 4a/b and its synergism with eNOS G894T polymorphism on the risk of preeclampsia.

**Methods:** The present case-control study consisted of 179 unrelated women with preeclampsia including 118 with mild and 61 with severe preeclampsia and 96 unrelated women with normal pregnancy as controls. All studied women were from Kermanshah Province of Iran. eNOS 4a/b and G894T genotypes were detected using polymerase chain reaction (PCR), and PCR-restriction fragment length polymorphism (RFLP) methods, respectively. The categorical variables between groups were compared using  $\chi^2$  test and the Odds ratios (OR) were obtained by SPSS logistic regression. Statistical significance was assumed at  $p < 0.05$  level.

**Results:** The frequency of eNOS a allele was slightly higher in both mild (16.5%) and severe (17.2%) preeclamptic women than controls (15.1%). Also, no significant difference was found between early- and late-onset preeclamptic women regarding the distribution of eNOS 4a/b genotypes. The presence of each allele of eNOS a or T was not associated with the risk of preeclampsia. However, the concomitant presence of both eNOS a and T alleles was associated with a non significant increased risk of severe preeclampsia by 1.77-fold ( $p = 0.35$ ).

**Conclusion:** The present study indicates the lack of association between eNOS a and T alleles with the risk of mild preeclampsia and a non significant increased risk of severe preeclampsia in the presence of both alleles which needs to be investigated in a study with larger samples.

**Keywords:** eNOS 4a/b, eNOS G894T, Mild preeclampsia, Polymorphism, Severe preeclampsia.

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## Introduction

Preeclampsia is manifested with hypertension, proteinuria, and edema. The incidence of this pregnancy complication with unknown etiology is related to both genetic and environmental factors. Abnormal utero-placental circulation and

endothelial cell dysfunction are involved in the pathogenesis of preeclampsia (1, 2). Previously, we have reported a frequency of 7.5% for preeclampsia among pregnant women at a referral hospital in Kermanshah (3).

The vasodilator molecule of nitric oxide (NO) is produced from L-arginine by endothelial nitric oxide synthase (eNOS). NO regulates endothelial function and is an important factor in the maintenance of homeostasis. The presence of eNOS variants attenuates NO production and might contribute to endothelial dysfunction (4). A decreased eNOS activity and a reduced plasma level of NO have been reported in the presence of both polymorphisms of eNOS 4a/b and G894T (5, 6).

A variable number of tandem repeats (VNTR) in intron 4 of eNOS (NOS3) has been reported which consisted of two alleles of eNOS 4a with 4 tandem 27-repeats and eNOS 4b with 5 repeats. The other most clinically important polymorphism of NOS3 is G894T which results in a substitution of aspartate for glutamate at amino acid position 298 of the NOS3 protein (6).

Due to differences in the distributions of eNOS variants among different ethnic groups, association of the variants of this gene with preeclampsia might be ethnically dependent. There are inconsistent reports related to the role of eNOS 4a/b genotypes and haplotypes on the risk of developing hypertensive disorders of pregnancy including preeclampsia (7–11).

We have recently reported the absence of association between eNOS G894T polymorphism and the risk of preeclampsia among a population in Kermanshah Province of Iran (2).

The aim of the present study was to investigate the possible association between eNOS 4a/b genotypes and alleles with the risk of preeclampsia. Also, the interaction between eNOS 4a/b with eNOS G894T polymorphism and its influence on the risk of preeclampsia was examined.

### Methods

The studied individuals consisted of 179 unrelated women with preeclampsia including 118 with mild and 61 with severe preeclampsia and 96 unrelated women with normal pregnancy as controls. The mean ages of mild and severe preeclamptic and control women were  $29.3 \pm 5.8$ ,  $29.1 \pm 6.5$  and  $27.5 \pm 6.4$  years ( $p=0.12$ ), respectively. The subjects had been admitted to the obstetric clinic of Imam Reza Hospital of Kermanshah University of Medical Sciences. All preeclamptic women who referred to the hospital were included in the study except those with multiple-birth pregnancies, previous hypertension, diabetes, cardiac and renal diseases.

All studied individuals were from Kermanshah with Kurdish ethnic background. There were 19 patients with early-onset preeclampsia, before 34 weeks of gestation, (15 severe and 4 mild preeclampsia) and 160 with late onset-preeclampsia.

The diagnosis criteria for defining preeclampsia were systolic blood pressure equal or higher than 140 mmHg, diastolic blood pressure equal or higher than 90 mmHg, presence of proteinuria by 24 hr urinary excretion exceeding 300 mg, a urine protein: creatinine ratio of  $>0.3$ , equal or higher than 30 mg/dl protein in random urine sample (1+reaction on a standard urine dipstick). Severe preeclampsia was defined as blood pressure equal or more than 160/110 mmHg on 2 occasions at least 6 hr apart while patient is on bed rest, proteinuria  $>3+$  on 2 random urine samples collected at least 4 hr apart, headache, visual disturbances, upper abdominal pain, serum creatinine and transaminase elevation, thrombocytopenia, fetal-growth retardation (12).

Informed written consent was obtained from each individual before participation in the study. The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences and was in accordance with the principles of the Declaration of Helsinki II.

**Genotype analysis:** eNOS 4a/b polymorphism was detected by polymerase chain reaction (PCR) method using the forward primer of 5' AGG CCC TAT GGT AGT GCC TTT 3' and the reverse primer of 5' TCT CTT AGT GCT GTG GTC AC 3' flanking the 27 bp repeat in intron 4 of the gene. The PCR products were electrophoresed on a 3% agarose gel. In the presence of eNOS a allele a fragment with 393 bp was produced while the eNOS b allele produced a fragment with 420 bp (4).

eNOS G894T polymorphism was identified using the forward primer of 5'-AAG GCA GGA GAC AGT GGA TGG A-3' and the reverse primer of 5'- CCC AGT CAA TCC CTT TGG TGC TCA-3'. Using the primers, a region in exon 7 of eNOS gene containing this polymorphism was amplified. After amplification, PCR products were digested overnight by 5 units of MboI restriction endonuclease at 37°C. The genotyping of the eNOS gene was determined by fragment separation on a 3.0% agarose gel. The three genotypes of eNOS G894T polymorphism obtained from restriction enzyme of MboI were GG, GT, and TT genotypes with band sizes of 248 bp, 248 bp/158 bp/90 bp, and 158 bp/90 bp, respectively (6).

**Biochemical analysis:** Using an automated RA-1000 (Technicon, CA, USA) and standard enzymatic method (Pars Azmon kit, Tehran, Iran), the levels of serum total cholesterol (TC) and triglycerides (TG) were measured. Serum low density lipoprotein-cholesterol (LDL-C) and high density lipoprotein-cholesterol (HDL-C) levels were measured using commercially available enzyme assay kits (Pars Azmon kit, Tehran, Iran).

**Statistical analysis:** The allelic frequencies were calculated by the chromosome counting method. The degrees of significance of differences in genotype and allele frequencies of eNOS between patients and controls were calculated using  $\chi^2$  test. Odds ratios (OR) were calculated as estimates of relative risk for the disease and a 95% confidence interval (CI) was obtained by SPSS logistic regression. The interaction between the two polymorphisms of eNOS 4a/b and eNOS G894T was determined using logistic regression model. The correlation values of biochemical and clinical data with the eNOS polymorphism between studied groups were calculated using linear regression and an unpaired t test. Two-tailed Student's t-test and ANOVA analysis were also used to compare quantitative data. The categorical variables among groups were compared using  $\chi^2$  test. Statistical significance was assumed at  $p < 0.05$  level. The SPSS statistical software package version 16.0 was used for the statistical analysis.

## Results

The gestational ages in preeclamptic women and controls were 30–39 and 36–39 weeks, respectively. Using T test and ANOVA analysis the quantitative hematological and biochemical pa-

rameters were compared between studied groups. The hematological and biochemical characteristics of studied women are demonstrated in table 1. The level of TG was significantly higher in both mild ( $177.2 \pm 28.9$  mg/dl) and severe ( $181.2 \pm 36.4$  mg/dl) preeclamptic women compared to controls ( $165.7 \pm 30.2$  mg/dl,  $p = 0.005$ ). However, the total cholesterol level was significantly lower in mild preeclamptic women ( $233.4 \pm 54.6$  mg/dl) than controls ( $249.2 \pm 45.4$  mg/dl,  $p = 0.025$ ).

Using  $\chi^2$  test the differences in the distribution of genotypes and alleles between studied groups were calculated. The distribution of eNOS a/b genotypes and alleles in women with preeclampsia and healthy pregnant women are shown in table 2. As demonstrated in table 2, the frequency of minor allele of eNOS a was higher in both mild (16.5%) and severe (17.2%) preeclamptic women than controls (15.1%). However, the difference did not reach to a statistically significant level ( $p > 0.05$ ). Also, no significant difference was found between early- and late-onset preeclamptic women regarding the distribution of eNOS 4a/b genotypes.

There were no significant differences between the levels of total cholesterol, LDL-C, HDL-C, TG, systolic and diastolic blood pressure in wild and mutant genotypes among total preeclamptic patients.

The interaction of eNOS a with eNOS 894 T allele in mild and severe preeclamptic women were calculated using logistic regression. While the presence of each allele of eNOS a or T alone did not affect the risk of developing preeclampsia, the concomitant presence of both eNOS a and T alleles increased the risk of severe preeclampsia by

**Table 1.** The hematological and biochemical characteristics of preeclamptic and control women

Variables	Mild preeclampsia (n=118)	Severe preeclampsia (n=61)	Healthy pregnant women (n=96)
<b>Blood pressure (mmHg)</b>			
Systolic	$140.5 \pm 11.9$ $p < 0.001$	$160.3 \pm 27.8$ $p < 0.001$	$113.2 \pm 9.4$
Diastolic	$87.5 \pm 7.8$ $p < 0.001$	$100.9 \pm 16.2$ $p < 0.001$	$74.3 \pm 7.4$
Total cholesterol (mg/dl)	$233.4 \pm 54.6$ $p = 0.025$	$250.9 \pm 74.6$ $p = 0.85$	$249.2 \pm 45.4$
TG (mg/dl)	$177.2 \pm 28.9$ $p = 0.005$	$181.2 \pm 36.4$ $p = 0.005$	$165.7 \pm 30.2$
HDL-C (mg/dl)	$55.9 \pm 11.7$ $p = 0.85$	$54.8 \pm 12.1$ $p = 0.41$	$56.1 \pm 9.1$
LDL-C (mg/dl)	$133.1 \pm 35.6$ $p = 0.07$	$139.2 \pm 38.4$ $p = 0.75$	$140.8 \pm 25.9$

Comparison with healthy pregnant women has been made

**Table 2.** The distribution of eNOS a/b genotypes and alleles in preeclamptic patients and controls

	Mild preeclampsia (n=118)	Severe preeclampsia (n=61)	Total preeclampsia (n=179)	Controls (n=96)
<b>eNOS genotypes</b>				
aa	2 (1.7%)	0 (0%)	2 (1.1%)	2 (2.1%)
ab	35 (29.7%)	21 (34.4%)	56 (31.3%)	25 (26%)
bb	81 (68.6%)	40 (65.6%)	121 (67.6%)	69 (71.9%)
	( $\chi^2=0.36$ , p=0.83)	( $\chi^2=2.37$ , p=0.3)	( $\chi^2=1.15$ , p=0.56)	
<b>eNOS alleles</b>				
a	39 (16.5%)	21 (17.2%)	60 (16.8%)	29 (15.1%)
b	197 (83.5%)	101 (82.8%)	298 (83.2%)	163 (84.9%)
	( $\chi^2=0.16$ , p=0.68)	( $\chi^2=0.24$ , p=0.61)	( $\chi^2=0.25$ , p=0.61)	
	OR=1.11 (0.65-1.87, p=0.68)	OR=1.16 (0.63-2.15, p=0.61)	OR=1.13 (0.69-1.83, p=0.61)	

1.77-fold [OR=1.77 (95% CI: 0.53- 5.9, p=0.35)] that did not reach to a statistically significant level which might be due to a small sample size.

### Discussion

Preeclampsia, a placenta-dependent pregnancy, is the most common pregnancy-specific complication and one of the major obstetric problems (13). Vasoconstriction, dysfunction of the vascular endothelium, and hypertension are characteristics of preeclampsia. It has been suggested that NO level in hypertensive disorders of pregnancy is reduced (14).

There are some studies related to the role of eNOS genotypes and haplotypes in hypertensive disorders of pregnancy. In the study of Sandrim et al. (1), three polymorphisms of eNOS T-786C, Glu298Asp and intron 4 a/b were not independently associated with the risk of preeclampsia. However, they found that the haplotype "C Glu a" was more common among women with hypertensive disorders of pregnancy including preeclampsia compared to women with normal pregnancy (7). They suggested the contribution of eNOS haplotypes to the development of hypertensive disorders of pregnancy that is obscured when specific eNOS genotypes alone are considered (7). Among Balooch preeclamptic patients from Southeastern Iran, eNOS a/b polymorphism was not associated with the risk of preeclampsia (10). Also, in north Indian women with hypertensive disorders of pregnancy, no association was found between the presence of NOS3 gene polymor-

phism with these disorders (11). However, in a population of preeclamptic women from China, the frequency of both T allele of eNOS G894T and a allele of eNOS 4a/b polymorphism was significantly lower in preeclamptic women than controls. They suggested that both polymorphisms may have a protective role against preeclampsia in Chinese populations (9). Meta-analysis of 18 genetic association studies indicated that intron 4a allele, homozygosity for intron 4a of eNOS a/b are positively associated with preeclampsia (8). This meta-analysis was confirmed by a recent meta-analysis which demonstrated eNOS 4a/b but not G894T polymorphism is associated with a significant risk of preeclampsia (15). However, recently a systematic review of 33 studies including 10671 individuals indicated that eNOS G894T variant significantly increased the risk of preeclampsia and no significant increased risk of preeclampsia was observed in the presence of eNOS 4 a/b (16). It has been reported that there is no significant difference in the distribution of genotype or allele of eNOS polymorphisms of T-786C, Glu298Asp and intron 4a/b between responsive and non responsive preeclamptic women or women with gestational hypertension in antihypertensive therapy. However, eNOS haplotypes affected the responsiveness to antihypertensive therapy in preeclamptic women (17).

In the present study, a slightly higher frequency of a allele of eNOS a/b polymorphism was detected in preeclamptic women compared to controls. However, no association was found between this



polymorphism with susceptibility to preeclampsia in our population. It seems that the variants of eNOS a/b in patients with Kurdish and Balooch (10) ethnic backgrounds are not involved in the susceptibility to preeclampsia.

Previously, we indicated the lack of association between eNOS G894T polymorphism and the risk of preeclampsia (2). In the present study, we examined if the concomitant presence of both alleles of eNOS a and T are associated with the risk of preeclampsia. We found in the presence of each allele of a or T alone there was no increased risk of preeclampsia. However, the presence of both alleles was associated with a 1.77-fold increased risk of severe preeclampsia that did not reach to a statistically significant level.

In summary, the present study demonstrated the lack of association between eNOS a/b genotypes and alleles with the risk of preeclampsia in our population. However, a non significant increased risk of preeclampsia in the presence of both minor alleles of eNOS a/b and G894T was observed. The results of the present study need to be confirmed in larger samples.

One of the limitations of this study is the small number of controls compared with patients. The small number of patients and controls available for the analysis of the concomitant presence of both polymorphisms may also be considered as another limitation.

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#### Conflict of Interest

The authors declare no conflict of interest.

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