African Journal of Biotechnology Vol. 10(34), pp. 6544-6548, 11 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1982
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Association of the Glu^{298}→Asp polymorphism in the endothelial nitric oxide synthase gene with risk of coronary artery disease

Nora A. Al-Faris[1], Zeid A. Al Othman[2] and Dilshad Ahmad[3]*

[1]Princess Nora Bint Abdulrahman University, P. O. Box 84428, Riyadh, Saudi Arabia.
[2]Department of Chemistry, College of Science, P. O. Box 2455, Riyadh, Saudi Arabia.
[3]Department of Food Science and Nutrition, College of Food and Agricultural Sciences, King Saud University, P. O. Box 2460 Riyadh, Saudi Arabia.

Accepted 8 April, 2011

Genetic variants of endothelial nitric oxide synthase (eNOS) could influence individual susceptibility to coronary artery disease (CAD) with or without associated demographic factors. The aim of this study was to assess whether Glu^{298}/Asp polymorphism of the eNOS gene is associated with the occurrence and severity of angiographycially defined coronary artery disease. Polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) analyses were carried out to detect the Glu^{298}/Asp variant of the eNOS gene in 279 patients with CAD as compared to controls (250). The prevalence of the Asp^{298} variant of eNOS was not found to be significantly and independently associated with the risk of CAD (OR = 1.08, 95% CI = 0.77 to 1.51, P = 0.663), extent of CAD on angiography (OR = 1.18, 95% CI = 0.63 to 2.23, P = 0.605) and in-hospital mortality (OR = 1.08, 95% CI = 0.29 to 4.04, P = 0.908). This investigation examined whether the Glu^{298}/Asp polymorphism of the eNOS could represent a useful genetic marker to identify individuals prone to the development of atherosclerotic diseases. More studies are needed to confirm whether the Glu^{298}/Asp polymorphism of the eNOS gene could represent a useful genetic marker to identify individuals of the study population prone to the development of atherosclerotic disease.

Key words: eNOS gene, polymorphisms, coronary artery disease (CAD), risk factors, genetic markers.

INTRODUCTION

Coronary atherosclerosis is a common disease that causes ischemic heart diseases, such as angina pectoris and myocardial infarction. Many patients have no symptom, despite the presence of coronary artery disease (CAD). They may have silent ischemia or are unaware of potentially dangerous abnormal heart rhythms (arrhythmias). The absence of chest pain or other common symptoms can also set the stage for a heart attack that occurs without warning. Hence, it is important to look at biomarkers for early detection of CAD. Coronary risk factors, such as hypertension, hypercholesterolemia and diabetes mellitus, are known to cause this disorder. Recently, the gene polymorphisms of angiotensin-converting enzyme (ACE)1 and apolipoprotein eNOS gene have been reported as independent risk factors for myocardial infarction, although the genetic cause of this disorder has not been proved completely. Epidemiological studies also indicate that hyperlipidmeia, hypertension, cigarette smoking, diabetes and obesity are risk factors for coronary artery disease. These environmental risk factors have, however, been ineffective and completely predicting development of the atherosclerotic process, suggesting that specific genetic predisposition should be taken into account as well. Vascular endothelium modulates blood vessel wall homeostasis through the production of factors regulating vessel tone coagulation state, cell growth, cell death and leukocyte trafficking. One of the most important

*Corresponding author. E-mail: drdilshadahmad@gmail.com.

endothelial cell products is nitric oxide (NO), which is synthesized from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS). Endothelial nitric oxide synthase (eNOS; NOS3) produces nitric oxide (NO) from L-arginine. NO has diverse physiologic regulatory functions and is involved in smooth muscle relaxation, inhibition of platelet aggregation, immune regulation, neurotransmission and blood pressure regulation (Moncada and Higgs, 1993; Schimidt and Walter, 1994; Forte et al., 1994). Moreover, it has been shown that eNOS inhibition accelerates atherosclerosis in animal models, and that abnormalities of the endothelial NO pathway is present in humans with atherosclerosis. This evidence suggests that NO may inhibit several key steps in the atherosclerotic process and that an alteration of NO production within the vascular endothelium could contribute to the pathogenesis of atherosclerosis. Thus eNOS could be a candidate gene for atherosclerosis.

A single base exchange ($G^{894}\rightarrow T$) in exon 7 of the human endothelial nitric oxide synthase (eNOS) gene results in a Glu→Asp substitution at residue 298 of the eNOS gene. The functional significance of this single nucleotide polymorphism remains an issue of controversy since homozygosity for the Asp^{298} variant has been related to reduced enzyme activity (Veldman et al., 2002) and basal NO production (Wang et al., 2000), possibly due to increased susceptibility to proteolytic cleavage, although more recent reports have convincingly demonstrated that this preferential cleavage could be a methodological artefact (Tesauro et al., 2000; Fairchild et al., 2001; Mc Donald et al., 2004).

In accordance with the hypothesis that this polymorphism may have an unfavorable effect on NO bioavailability, homozygosity for the Asp^{298} variant has been shown to influence vascular coronary reactivity as reported by Chang et al. (2001), responsiveness to a-adrenergic stimulation and event free survival in patients with nonischemic cardiomyopathy (Philip et a., 1999; McNamara et a., 2003). Based on these reports, a number of association studies have positively associated the presence of the Asp^{298} variant with risk of acute myocardial infarction (AMI) (Hingorani et al., 1999; Antoniades et al., 2005), carotid atherosclerosis (Lembo et al., 2001), early atherogenesis and coronary in-stent restenosis (Paradossi et al., 2004; Gomma et al., 2002) while several studies have found no evidence for an association between the $Glu^{298}\rightarrow Asp$ polymorphism and premature CAD (Granath et al., 2001; Yamada et al., 2002; Spence et al., 2004; Zhang et al., 2006).

Several polymorphisms have been identified in the eNOS gene, among which is one located in exon 7 (G894T) which modifies its coding sequence (Glu^{298}/Asp). Associations between this variant and coronary spasm, coronary artery disease and acute myocardial infarction have been reported, but data on its relation with disease severity are lacking. In this paper, we examined the associations between theGlu^{298}/Asp polymorphism of the eNOS gene and the occurrence of angiographically defined coronary artery disease in the ethnically variant population.

MATERIALS AND METHODS

Selection criteria

We included 279 patients with angiographically diagnosed CAD, consecutively admitted to our institution with angiographically proven coronary artery disease (more than 50% stenosis affecting at least one vessel) and 250 healthy controls, in whom angiographic examination excluded the presence of coronary artery disease. The controls were those who came to the hospital with pain in the chest but did not have a history of angina pectoris or AMI, and they showed a normal electrocardiogram.

Patient's characteristics

All the cases and age matched healthy controls were interviewed and epidemiological data/demographic data on smoking habits, hypertension, diabetes dyslipidaemia and family history of coronary artery disease were recorded. Informed consent was obtained from all the patients and the healthy controls, as required by our Hospital ethics committee.

Data on risk factors

For CAD risk factors, the following were used: subjects were defined as hypertensive if their blood pressure was >140/90 mmHg or if they were receiving any antihypertensive treatment; those with a history of diabetes or who were receiving any antidiabetic drug were considered to be diabetic; those with a total plasma cholesterol concentration of >200 mgs/dl or a triglyceride concentration of >180 mgs/dl, or who were receiving lipid lowering drugs, were considered dyslipidmic. Smoking history was recorded as either none or current smokers. A positive family history was the presence of a first degree relative with coronary artery disease at the age of <55 years for men and <60 years for women.

Angiographic study

All patients underwent coronary angiography. Coronary stenosis was considered significant in the presence of a luminal diameter narrowing of >50% of at least one pericardial coronary artery. The severity of coronary artery disease was expressed by the number of affected vessels (one, two, or three vessel disease) and also by means of the Duke scoring system13: a prognostic index that includes the number of diseased major vessels, the presence of left main coronary artery disease, the percentage of narrowing of the major vessels, and involvement of the left anterior descending coronary artery, particularly when the proximal segment shows severe stenosis (>95%). The Duke score ranges from 0 to 100 (0 = no disease, 100 = the most severe disease)

Biochemical analysis

Blood samples were collected from all the subjects after 12 h fasting and placed in EDTA tubes and stored at -80°C until the time of assay: for DNA extraction and for biochemical assays. The serum concentrations of triglyceride (TG), total cholesterol, LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C), urea, createnine and random blood sugar (separate blood sample was collected for

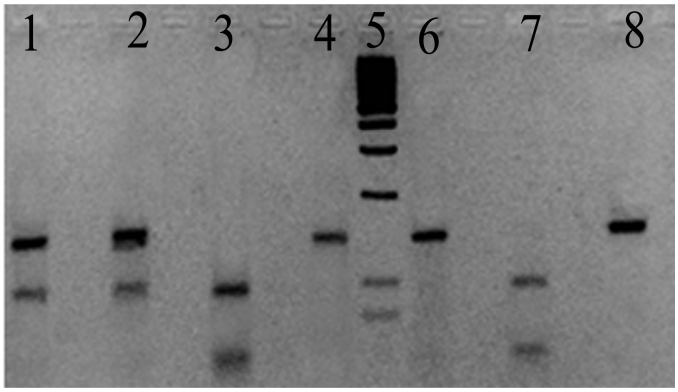

Figure 1. Restriction fragment polymorphism of eNOS gene: Bi-allelic polymorphism in exon 7 of the *eNOS 3* gene detected by *Mbo*I restriction endonuclease digestion of the 139-bp PCR product. Lanes 1 and 2, restriction digested samples showing restriction patterns corresponding to heterozygocity for Asp/Glu; Lanes 3 and 7, restricted digested samples showing restriction patterns corresponding to homozygosity for Asp^{298} ; Lanes 4, 6 and 8, samples subjected to restriction digestion samples showing restriction patterns corresponding to homozygosity for Glu^{298}; Lane 9, negative control.

doing random blood sugar) were measured by the standard methods (Auto-analyzer) used in the clinical laboratory of the hospital at the time of diagnosis of the patients.

Genotyping of Glu^{298}/Asp polymorphism for exon 7 of eNOS gene

Genomic DNA was extracted from samples of whole blood by standard methods (Shi et al., 2008) .The coding sequence variant was a G→T substitution at position 894 in exon 7 which determines the Glu to Asp amino acid substitution (in codon 298) in the mature eNOS protein. According to previously described procedure (Munshi et al., 2010), genotyping of all the subjects was performed by polymerase chain reaction amplification of exon 7 with the primers 5'-CATGAGGCTCAGCCCCAGAAC -3'(sense) and 5'-AGTCAATCCCTTTGGTGCTCAC -3'(antisense) followed by *MboI* restriction enzyme digestion for 16 h at 37°C. In the presence of a T at nucleotide 894 which corresponds to Asp^{298}, the139 base pair (bp) polymerase chain reaction product was cleaved into two fragments of 119 and 20 bp. The products of the digestion process were highlighted by electrophoresis on 3% agarose gel (Figure 1).

Statistical analysis

Genotype distributions in the cases and controls were examined for significant deviation ($P < 0.05$) from Hardy-Weinberg equilibrium. The frequencies of risk-associated variants were compared in the cases and controls by using the χ^2 test (SPSS 13.0 Chicago). Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. The differences between groups were examined by χ^2 test. The frequencies of the alleles and genotypes were compared between the patient and control groups by the χ^2. We performed multivariate logistic regression analysis to adjust risk factors, in which CAD was a dependent variable and independent variables were hypertension, smoking, diabetes, TG, total cholesterol level, LDL-C, HDL-C, CHO/HDL-C, LDL-C/HDL-C, urea, createnine and eNOS genotype. All the data was shown as mean ± SD.

RESULTS

The main baseline characteristics of the patients with CAD (cases) and of the subjects from the general population (controls) are presented in Table 1. The frequencies of the studied genotypes of the eNOS gene are shown in Table 2. The observed frequencies of the studied alleles were in Hardy-Weinberg equilibrium in both the cases ($\chi^2 = 0.20$, $P > 0.1$) and controls ($\chi^2 = 1.65$, $P > 0.1$).

The frequency of the Asp/Asp genotype was not found to differ significantly between the cases and controls in relation to the major coronary risk factors (gender, smoking status, diabetes mellitus, hypercholesterolemia, hypertension, obesity and family history of CAD). Furthermore, the average number of the earlier mentioned risk factors did not differ significantly in the carriers versus non-carriers of the Asp/Asp genotype, in both cases (2.36 vs. 2.32, P = 0.771) and controls (1.60 vs. 1.81, P = 0.137).

The frequency of the mutated genotype (Asp/Asp) did not differ significantly between cases and controls (11.2 vs. 10.7%, P = NS). Similarly, the Glu/Asp (43.8 vs. 40.9, P = NS) and the Glu/Glu (45.1 vs. 48.4, P = NS) genotypes did not differ significantly between the study groups in the univariate analysis. In backward stepwise logistic regression analysis with age, gender, smoking status, diabetes mellitus, hypercholesterolemia, hypertension, obesity and family history of CAD included as covariates, the presence of the Asp/Asp genotype was not found to be independently associated with CAD (OR = 1.08, 95% CI = 0.77–1.51, P = 0.663) (Figure 2).

The frequencies of the Asp/Asp genotype in relation to the number of diseased vessels were 13.3, 8.1, and 9.3% in patients with one-, two- and three-vessel disease, respectively (P = 0.192). In multivariate analysis adjusted for age, gender, diabetes mellitus, smoking status, hypertension, hypercholesterolemia and obesity, the Asp/Asp variant was not found to be independently associated with the number of diseased vessels on coronary angiography (OR = 1.18, 95% CI = 0.63 to 2.23, P = 0.605).

DISCUSSION

The eNOS gene has a number of polymorphic sites, including SNPs, di-nucleotide repeats and variable number tandem repeat sequences, and the opportunity exists to investigate polymorphic functional correlation as well as disease-specific associations, especially in cardiovascular disease, including coronary artery disease, and its most severe consequence, myocardial infarction. Several polymorphisms have been identified in

Table 1. Baseline characteristics of the study population.

Parameter	Case (n = 280)	Control (n = 250)	P value
Age (years)	62 ± 13	58 ± 15	< 0.001
Male gender	78.9% (221)	42.8% (107)	< 0.001
Diabetes	28.9% (81)	10.8% (27)	< 0.001
Cigarette smoking	63.9% (179)	34% (82)	< 0.001
Hypercholesterolaemia	50% (140)	34% (82)	< 0.001
Hypertension	45% (126)	32% (80)	< 0.001
Heredity for CAD	26% (73)	22% (55)	0.040

Table 2. Allele frequencies of the Glu^{298} —›Asp polymorphism in CAD patients and controls.

Parameter	Glu (%)	Asp (%)
Patients	60.02	37.97
Controls	64.55	23.41

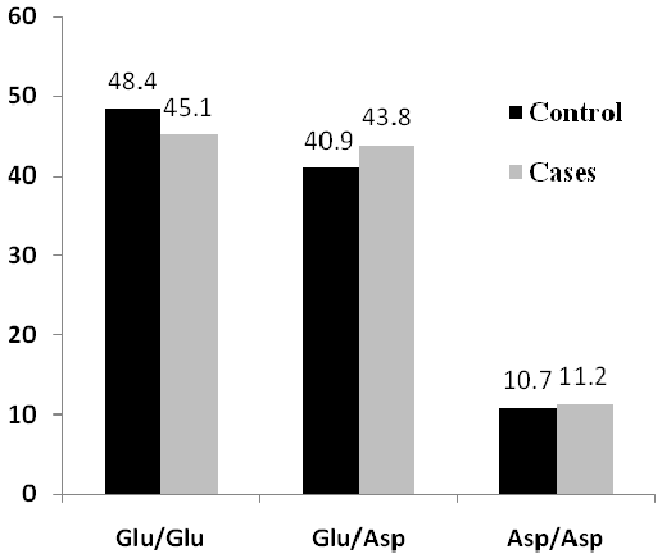

Figure 2. Synopsis of the results of genotype analyses. The distributions of genotypes in relation to the disease are shown (data shown in %). Detailed description of the variables included in each multivariate analysis is presented in the results section of the manuscript.

the eNOS gene, among which is one located in exon 7 (G894T) which modifies its coding sequence (Glu^{298} /Asp).

A case-control study evaluating the potential association between Glu^{298}→Asp eNOS polymorphism and the risk of CAD reached the conclusion that homozygosity for eNOS Asp^{298} allele was associated with a moderately, though significantly increased risk of CAD (OR = 1.31;95% CI = 1.13–1.51) (Casas et al., 2004). The reported results are amenable to criticism due to the significant heterogeneity of the individual odds ratios incorporated in the calculation of the summary odds ratio. After extracting the most influential odds ratio from the analysis of the study, the authors abrogated the methodological limitation of heterogeneity, but the calculated risk of CAD was largely blunted and bordered on significance (OR = 1.17; 95% CI = 1.00–1.36; P = 0.05).

Antoniades et al. (2005*)* in an elegantly designed study with 229 consecutive patients with premature CAD demonstrated that homozygosity for this polymorphism is associated with a significantly increased risk for premature CAD. Although discordant at a first glance with our results, the conclusions of this study refer to a different, younger than our patient population, where the relative contribution of the Glu^{298}→Asp polymorphism to the susceptibility for CAD might be enhanced. A large scale study on 5061 individuals of Japanese origin demonstrated no association of the Glu^{298}→Asp polymorphism with the risk of CAD (Yamada et al., 2002; Spence et al., 2004), using family-based association tests specifically designed for the study of the genetic basis of multifactorial diseases. There was no evidence that the Glu^{298}→Asp eNOS gene polymorphism was related to the development of CAD in a total of 1023 Caucasian individuals.

In a recent study of 861 diabetic men, no significant association was observed between Glu^{298}→Asp eNOS polymorphism and risk of CAD (Zhang et al., 2006). There is also discrepancy in the literature regarding the association between the Glu^{298}→Asp polymorphism and premature CAD. Granath and colleagues (Granatha et al., 2001) studying 573 patients younger than 50 years reported the absence of the association between Glu^{298}→Asp eNOS polymorphism and premature CAD, while in a recent smaller trial, the TT genotype was significantly and independently associated with premature CAD (Cam et al., 2005; Gardemann et al., 2002) under the studied young individuals with high risk of atherosclerotic profile and they reported an association between Glu^{298}→Asp eNOS polymorphism and CAD in this cohort. To our knowledge, this study is the first specifically designed study conducted in a Saudi poplation to test the possible association of this polymorphism with

risk of CAD. Our results have not validated the association between $G^{894}\rightarrow T$ polymorphism, in the eNOS gene and increased risk of CAD.

Identifying a correlation among genes and risk for coronary heart disease is a first step in a long path to potentially important clinical implications. What we are looking for, ultimately, are novel therapeutics and/or life-style modifications that can be recommended to individuals to help manage and reduce the risk of heart diseases.

REFERENCES

Antoniades C, Tousoulis D, Vasiliadou C, Pitsavos C, Chrysochoou C, Panagiotakos D, Tentolouris C, Marinou K, Koumallos N, Stefanadis C (2005). Genetic polymorphism on endothelial nitric oxide synthase affects endothelial activation and inflammatory response during the acute phase of myocardial infarction. J. Am. Coll. Cardiol,. 46:1101-1109.

Cam SF, Sekuri C, Tengiz I, Ercan E, Sagcan A, Akinm M, Berdeli A (2005). (The G894T polymorphism on endothelial nitric oxide synthase gene is associated with premature coronary artery disease in a Turkish population. Thromb. Res., 116: 287-292.

Casas JP, Bautista LE, Humphries SE, Hingorani AD (2004). Endothelial nitric oxide synthase genotype and ischemic heart disease: meta-analysis of 26 studies involving 23 028 subjects. Circulation, 109: 1359-1365.

Chang K, Baek SH, Seung KB, Kim PJ, Ihm SH, Chae JS, Kim JH, Hong SJ, Choi KB(2001). The Glu^{298}Asp polymorphism in the endothelial nitric oxide synthase gene is strongly associated with coronary spasm. Coron. Artery Dis., 14(4): 293-299.

Fairchild TA, Fulton D, Fontana JT, Gratton JP, McCabe TJ, Sessa WC (2001). Acidic hydrolysis as a mechanism for the cleavage of the Glu(298)3Asp variant of human endothelial nitric-oxide synthase. J. Biol. Chem., 276: 26674-26679.

Forte P, Copland M, Smith LM, Milne E, Sutherland J, Benjamin N (1997). Basal nitric oxide synthesis in essential hypertension. Lancet 1994, 349: 837–842.

Gardemann A, Lohre J, Cayci S, Katz N, Tillmanns H, Haberbosch W (2002). The T allele of the missense Glu(298)Asp endothelial nitric oxide synthase gene polymorphism is associated with coronary heart disease in younger individuals with high atherosclerotic risk profile. Atherosclerosis, 160: 167-175.

Gomma AH, Elrayess MA, Knight CJ, Hawe E, Fox KM, Humphries SE (2002). The endothelial nitric oxide synthase (Glu^{298}Asp and -786T>C) gene polymorphisms are associated with coronary in-stent restenosis. Eur. Heart J., 23: 1955-1962.

Granath B, Taylor RR, van Bockxmeer FM, Mamotte CD (2001). Lack of evidence for association between endothelial nitric oxide synthase gene polymorphisms and coronary artery disease in the Australian Caucasian population. J. Cardiovasc. Risk, 8: 235-241.

Hingorani AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A (1999). A common variant of the endothelial nitric oxide synthase (Glu^{298} Asp) is a major risk for coronary artery disease in the UK. Circulation, 100: 1515-1520

Lembo G, De Luca N, Battagli C, Iovino G, Aretini A, Musicco M, Frati G, Pompeo F, Vecchione C, Trimarco B (2001). (A common variant of endothelial nitric oxide synthase (Glu^{298}Asp) is an independent risk factor for carotid atherosclerosis. Stroke, 32: 735-740.

Mc Donald DM, Alp NJ, Channon KM (2004). Functional comparison of the endothelial nitric oxide synthase Glu^{298}Asp polymorphic variants in human endothelial cells. Pharmacogenetics, 14: 831-839.

Munshi AK, Rajeshwar SK, Chandana E, Gowhar S, Anila AN, Balakrishna N, Suvarna A, Jyothy A (2010). VNTR polymorphism in intron 4 of the eNOS gene and the risk of ischemic stroke in a South Indian population Brain Res. Bull., 82: 5-6.

McNamara DM, Holubkov R, Postava L, Ramani R, Janosko K, Mathier M, MacGowan GA, Murali S, Feldman AM, London B (2003). Effect of the Asp^{298} variant of endothelial nitric oxide synthase on survival for patients with congestive heart failure. Circulation, 107: 1598-1602.

Moncada S, Higgs A (1993). The L-arginine-nitric oxide pathway. N. Engl. J. Med., 329: 2002–2012.

Paradossi U, Ciofini E, Clerico A, Botto N, Biagini A, Colombo MG (2004). Endothelial function and carotid intima-media thickness in young healthy subjects among endothelial nitric oxide synthase Glu^{298}-->Asp and T-786-->C polymorphisms. Stroke, 35(6): 1305-1309.

Philip I, Plantefeve G, Vuillaumier-Barrot S, Vicaut E, LeMarie C, Henrion , Poirier O, Levy BI, Desmonts JM, Durant G, Benessiano (1999). G894T polymorphism in the endothelial nitric oxide synthase gene is associated with an enhanced vascular responsiveness to phenylephrine. Circulation, 99: 3096-3098.

Shi C, Kang X, Wang Y, Zhou Y (2008). The coagulation factor V Leiden, MTHFRC677T variant and eNOS 4ab polymorphism in young Chinese population with ischemic stroke. Clin. Chim. Acta, 396 (1-2): 7-9.

Schimidt HH, Walter U (1994). NO at work. Cell, 78(6): 919-925.

Spence MS, McGlinchey PG, Patterson CC, Allen AR, Murphy G, Bayraktutan U, Fogarty DG, Evans AE, McKeown PP (2004).. Endothelial nitric oxide synthase gene polymorphism and ischemic heart disease. Am. Heart J., 148:847-851.

Tesauro M, Thompson WC, Rogliani P, Qi, L, Chaudhary PP, Moss J (2000). Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: cleavage of proteins with aspartate vs. glutamate at position 298. Proc. Natl. Acad. Sci., 97: 2832-2835.

Veldman BA, Spiering W, Doevendans PA, Vervoort G, Kroon AA, de Leeuw PW, Smits P (2002). The Glu^{298}Asp polymorphism of the NOS3 gene as a determinant of the baseline production of nitric oxide. J. Hypertens., 20: 2023-2027.

Wang XL, Sim AS, Wang MX, Murrell GA, Trudinger B, Wang J (2000). Genotype dependent and cigarette specific effects on endothelial nitric oxide synthase gene expression and enzyme activity. FEBS Lett., 471:45-50.

Yamada Y, Izawa H, Ichihara S, Takatsu F, Ishihara H, Hirayama H, Sone T, Tanaka M, Yokota M (2002). Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. N. Engl. J. Med., 347:1916-23.

Zhang C, Lopez-Ridaura R, Hunter D, Rifai N, Hu FB (2006). Common variants of the endothelial nitric oxide synthase gene and the risk of coronary heart disease among U.S. diabetic men. Diabetes, 55: 2140-2147.