ENDOTHELIAL NITRIC OXIDE SYNTHASE INTRON 4 POLYMORPHISM IS A MARKER FOR CORONARY ARTERY DISEASE IN AFRICAN-AMERICAN AND CAUCASIAN MEN

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Objectives: We investigated the association of the intron 4 polymorphism of the endothelial nitric oxide synthase (eNOS) gene with coronary artery disease (CAD).

Background: Genetic alterations in the gene encoding for eNOS could contribute to the development and progression of CAD.

Methods: We genotyped for the eNOS intron 4 polymorphism in 194 subjects undergoing coronary angiography. Genotyping was performed with polymerase chain reaction-restriction fragment length polymorphism for the variable number of tandem repeats in intron 4. Coronary artery disease (CAD) was assessed by quantitative coronary angiography, and endothelial function was measured by brachial ultrasonography. We performed logistic regression analysis for the effect of eNOS intron 4 polymorphism and other coronary risk factors on multi-vessel CAD and endothelial function.

Results: The 4a-allele frequency in African Americans was 0.31, while the 4a-allele frequency in Caucasians was 0.15 ($P<.001$). The prevalence of the 4a-allele was highest among subjects with multi-vessel disease both for African Americans and for Caucasians. A race-adjusted comparison of the prevalence of the 4a-allele among subjects with multi-vessel disease vs those without was statistically significant ($P=.03$). No correlation was found between the eNOS intron 4 polymorphism and endothelial function.

Conclusions: The eNOS intron 4 polymorphism may be a marker of multi-vessel CAD in African Americans and Caucasians. (*Ethn Dis*. 2005;15:191–197)

Key Words: Coronary Artery Disease, Endothelium, Genetic Polymorphisms, Nitric Oxide Synthase

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INTRODUCTION

Nitric oxide (NO) is a highly diffusible, short-lived molecule that affects many biological pathways. It is produced enzymatically from the amino acid, L-arginine, by a family of three nitric oxide synthase (NOS) isoforms: endothelial NOS (eNOS or NOS-3), inducible NOS (iNOS or NOS-2), and neuronal NOS (nNOS or NOS-1).[1] Physiologically, NO diffuses from the endothelium to the vascular smooth muscle cell (VSMC) where it binds to the heme moiety of soluble guanylate cyclase and activates the conversion of guanosine 5′-monophosphate (GMP) to cyclic GMP (cGMP).[2] This increase in cGMP results in relaxation of vascular smooth muscle, mediating endothelium-dependent vasodilation, inhibiting platelet aggregation and monocyte adhesion to the endothelium, inhibiting VSMC growth and migration, and finally, inhibiting the oxidation of low-density lipoprotein (LDL) cholesterol.[3]

Recent studies have found much evidence for the role of oxidative stress in the degradation of NO. This reduction in vascular NO bioavailability has been shown to contribute to altered vasomotor tone, hypertension, endothelial dysfunction, and development and progression of atherosclerosis.[4,5] The human eNOS gene is located on chromosome 7q35–36 and comprises 26 exons that span 21 kb and encode a 135 kD protein.[6] Ten polymorphisms have been reported in the eNOS gene: three in the 5′-flanking region, two in the coding sequence, and five in intronic regions.[3] Clinical association studies have investigated the association of various polymorphisms of the eNOS gene to various cardiovascular diseases such as CAD, hypertension, stroke, and deep venous thrombosis (DVT), with conflicting results.[3] Different ethnic populations have been studied worldwide for the detection and frequency of the 27-bp variable number of tandem repeat (VNTR) polymorphism in the intron 4 of eNOS in relation to CAD.[7–19] Wang et al have reported that the eNOS intron 4 polymorphism has a major locus contribution to circulating nitrite and nitrate levels, a surrogate measure of NO.[20] Thus, we hypothesized that the eNOS intron 4a-allele may affect severity of CAD as measured by coronary angiography. In addition, since endothelial dysfunction plays an important role in atherosclerosis, we also studied endothelium-dependent and -independent vascular reactivity as an indicator of the functional consequence of the eNOS intron 4 polymorphism.

METHODS

Patient Population

Our study sample consisted of a well-characterized group of 194 Caucasian and African-American patients who were referred for coronary angiography between March 1999 and August 2000 at the Atlanta Veterans Affairs Medical Center. The study was approved by the institutional review board, and all sub-

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jects provided informed consent. We had no exclusion criteria. All patients who agreed to participate in the study also provided information about coronary risk factors and medications.[21] Smokers were defined as both current and past smokers. Participants were considered to have hypertension if they met the criteria of the World Health Organization or had been treated with antihypertensive drugs. They were hypercholesterolemic if their fasting total cholesterol was >220 mg/dL or they were taking lipid-lowering agents, and participants had diabetes mellitus if they met the diagniostic criteria of the World Health Organization or had been treated for diabetes. Family history of CAD was ascertained by interview. A history of peripheral vascular disease was determined by chart review.

Blood Sampling, Lymphocyte Immortalization, and Genotyping

Blood samples were collected during cardiac catheterization. Lymphocytes were isolated and immortalized as previously described.[21] Genomic DNA was isolated from whole blood by using a DNA extraction kit (QIAamp DNA mini kit, Qiagen, Valencia, Calif). The DNA fragment containing the eNOS intron 4 polymorphism was amplified from genomic DNA by a modified polymerase chain reaction (PCR) described by Ichihara et al.[8] The resulting PCR products were separated by 2% agarose gel electrophoresis and identified as 4a/4b heterozygote with fragments at 393 bp and 420 bp, 4a/4a homozygote with a 393-bp fragment, and 4b/4b homozygote with a 420-bp fragment by ethidium bromide staining.

Coronary Angiography

Coronary artery disease (CAD) was assessed by coronary angiography as described previously.[21] Major epicardial coronary arteries with at least 50% stenosis were defined as diseased. The study population was divided into three groups: 1) subjects without angiographically detectable CAD or with coronary arterial stenosis of <50%; 2) subjects with single-vessel disease; and 3) subjects with multi-vessel disease.

Brachial Ultrasonography

For our sub-study to assess endothelial function, subjects were contacted to return to the AVAMC, and a total of 33 patients volunteered. Endothelial function was assessed as described previously and per the guidelines published for the ultrasound assessment of endothelium-dependent (flow-mediated) and endothelium-independent (nitroglycerin-induced) vasodilation of the brachial artery.[21–23] Assessment of endothelium-dependent response in the brachial artery was performed by using high-resolution vascular ultrasonography (Toshiba SSH-140A). Participants were studied in a temperature-controlled room. The forearm was rested in a foam cradle, elevated slightly above the level of the right atrium. Using a 7.5 MHz ultrasound transducer, the optimal longitudinal image of the brachial artery immediately above the antecubital fossa on the right arm was obtained. Location was marked by ultrasound attenuator bands to facilitate subsequent measurements. A blood pressure cuff was placed around the widest part of the forearm. Brachial artery images at end diastole and resting flow velocity were recorded on a sVHS tape. Four diameters in three separate end-diastolic frames were measured to give a mean resting brachial artery diameter. In our experience, given the present resolution of the ultrasound probe, a minimum brachial artery diameter of 2.5 mm is required for accurate measurement of a vasodilator response. All 33 participants expressing interest in the protocol were thus prescreened for resting dimensions to determine eligibility for participation in the study. Thirty patients were eligible for our study. Four were African Americans.

To measure endothelium-dependent vasodilator responses, the forearm blood pressure cuff was inflated to >200 mm Hg for five minutes and then deflated rapidly. Continuous measurement of flow velocity was performed from the onset of cuff deflation to one minute post-cuff deflation. One minute after cuff deflation, four diameters on three separate end-diastolic frames were measured to give a mean brachial artery diameter at maximal flow stimulation. This measurement reflects the flow-mediated vasodilation, which is a direct measurement of the endothelium-dependent vasodilator response. To measure the endothelium-independent vasodilator response, the subject was given a standard dose of sublingual nitroglycerin (0.4 mg) with close monitoring of blood pressure and heart rate. After three minutes, four diameters on three separate end diastolic frames were again measured to give the mean brachial artery diameter. This protocol documented the maximal vasodilation of the vessel as an estimate of the maximum vessel diameter.

STATISTICAL ANALYSIS

A goodness-of-fit model using the chi-squared distribution was used to assess adherence of the genotypes to Hardy-Weinberg equilibrium. Logistic regression was used to contrast the prevalence of the 4a-allele across categories of extent of vessel disease and race. These are allelic analyses in which the number of alleles is twice the number of subjects. We modeled the prevalence

Table 1. Demographic characteristics and risk profile of study subjects

Variable	Nonsignificant CAD (*N*=50)	Single-vessel CAD (*N*=31)	Multi-vessel CAD (*N*=113)	*P**
Age	56.4 ± 9.3	59.3 ± 10.9	62.3 ± 9.9	<.01
Male	47 (94.0)	29 (93.5)	113 (100)	.03
Smoking status	33 (66.0)	23 (74.2)	95 (84.1)	.03
Hypercholesterolemia	28 (56.0)	21 (67.7)	93 (82.3)	<.01
Diabetes	13 (26.0)	9 (29.0)	49 (43.4)	.07
Hypertension	13 (66.0)	22 (71.0)	79 (69.0)	>.20
Family history of CAD	17 (34.0)	9 (29.0)	41 (36.3)	>.20
History of PVD	2 (4.0)	3 (9.7)	19 (16.8)	.06

Age is presented as mean ± SD. The other variables are presented as number (or percentage in parentheses) of subjects with the given characteristics.
* *P* value contrasting the 3 categories.
CAD = coronary artery disease; PVD = peripheral vascular disease.

of the 4a-allele with two indicator variables for the three categories of vessel disease, controlling for race. The difference of the means of the percentage change in brachial artery diameter for endothelium-dependent and endothelium-independent function for subjects with the 4b/4b genotype compared to those with the 4a/4b genotype was evaluated by standard regression methods and by the *t* test.

RESULTS

The characteristics and risk profile for the study patients are displayed in Table 1. Smoking and hypercholesterolemia were significantly related to the extent of vessel disease in our study, whereas a family history of CAD and a personal history of hypertension were not. The associations between diabetes and personal history of peripheral vascular disease with the extent of vessel disease were nearly statistically significant. The prevalence of a history of diabetes was highest among subjects with multi-vessel disease.

Severity of CAD and eNOS Intron 4 Polymorphism

Table 2 summarizes the distribution of the eNOS intron 4 genotypes within the three categories of severity of CAD for Caucasians and African Americans.

Table 2. Genotype distribution of eNOS intron 4 polymorphism and severity of CAD by coronary angiography according to race

eNOS Intron 4 Polymorphism	Nonsignificant CAD (*N*=29)	Caucasian Single-vessel CAD (*N*=25)	Multi-vessel CAD (*N*=97)
4a/4a	1 (3.4)	1 (4.0)	2 (2.1)
4a/4b	6 (20.7)	2 (8.0)	28 (28.9)
4b/4b	22 (75.9)	22 (88.0)	67 (69.1)
4a-allele frequency	0.14	0.08	0.16
eNOS intron 4 Polymorphism	**Nonsignificant CAD (*N*=21)**	**African American Single-vessel CAD (*N*=6)**	**Multi-vessel CAD (*N*=16)**
4a/4a	0 (0)	0 (0)	3 (18.8)
4a/4b	11 (52.4)	2 (33.3)	8 (50.0)
4b/4b	10 (47.6)	4 (66.7)	5 (31.2)
4a-allele frequency	0.26	0.17	0.44

Data are presented as number (percentage in parentheses) of subjects with the given genotypes.

The genotypic frequencies for the eNOS intron 4 polymorphism were in Hardy-Weinberg equilibrium both for Caucasians and for African Americans. The 4a-allele frequency in African Americans was 0.31, while the 4a-allele frequency in Caucasians was 0.15. This difference is highly statistically significant ($P<.001$), even after adjustment for extent of CAD. The difference in the race-adjusted prevalence of the 4a-allele across the three categories of extent of vessel disease nearly attained statistical significance ($P=.056$). The prevalence of the 4a-allele was highest among subjects with multi-vessel disease both for Caucasians and for African Americans. A race-adjusted comparison of the prevalence of the 4a-allele among subjects with multi-vessel disease vs those without multi-vessel disease was statistically significant ($P=.03$). Furthermore, the difference in the prevalence of the 4a-allele for those with and without multi-vessel disease persisted in a multi-variable logistic regression model after adjustment for age, race, smoking, hypercholesterolemia, diabetes, hypertension, family history of CAD, and peripheral vascular disease ($P=.018$).

Interactive Effect of eNOS Intron 4 Polymorphism and Smoking

Because smoking is a strong predictor of CAD severity, we explored the possible interaction between smoking

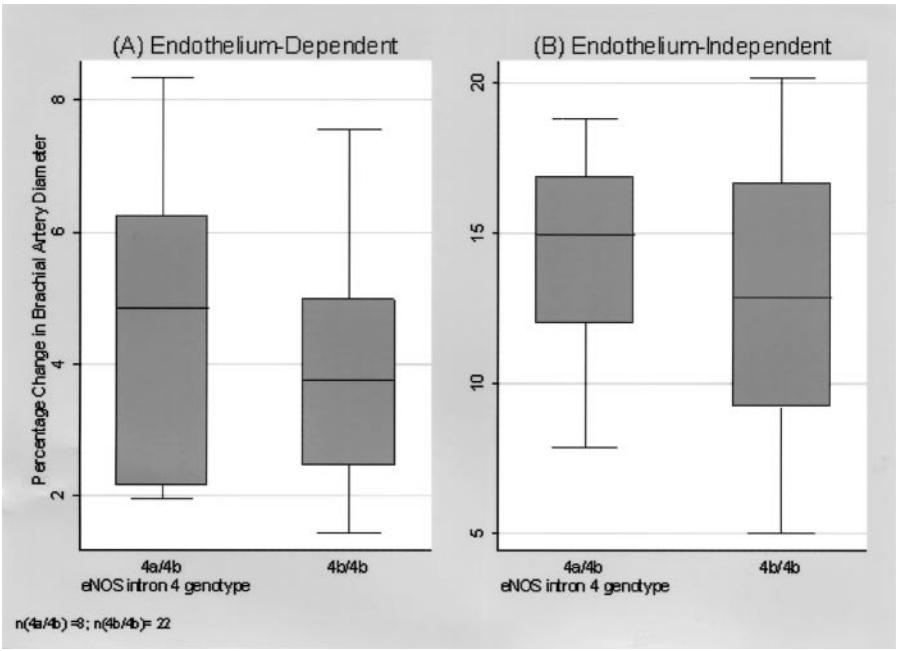

Fig 1A. Box-and-whiskers plot of effects of reactive hyperemia (endothelium dependent) on percent change in brachial artery diameter in patients with and without 4a-allele. Differences between the groups did not reach statistical significance (*P*>.20)

Fig 1B. Box-and-whiskers plot of effects of sublingual nitroglycerin (endothelium independent) on percent change in brachial artery diameter in patients with and without 4a-allele. Differences between the groups did not reach statistical significance (*P*>.20)

status, eNOS intron 4 polymorphism, and severity of CAD. Among 43 non-smokers, the odds of a 4a-allele for subjects with multi-vessel disease compared to those without multi-vessel disease is 0.95. Thus, no 4a-allele effect is seen among non-smokers. The corresponding odds ratio for 151 smokers is 2.0 (*P*=.04). Although this difference in odds ratios is large, it is not statistically significant (*P*>.20).

Endothelial Function and eNOS Intron 4 Polymorphism

In order to study endothelial function, we examined the effect of the eNOS intron 4 polymorphism on flow-mediated and nitroglycerin-induced vasodilation in a subgroup of the study population. Figure 1A and 1B illustrate the endothelium-dependent and -independent vascular reactivity, respectively, for subjects with the heterozygous 4a/4b and homozygous 4b/4b genotypes. No subjects had the homozygous 4a/4a genotype in the subgroup. The change in flow-mediated brachial artery diameter was slightly higher for subjects with the 4a/4b genotype (median=4.85, *N*=8) than subjects with the 4b/4b genotype (median=3.8, *N*=22). However, the crude mean difference (−0.71) was not statistically significant (*P*>.20), nor was the mean difference (*P*>.20) across genotypes after adjustment for race and extent and severity of CAD (Fig. 1A). Furthermore, the change in brachial artery diameter with sublingual nitroglycerin was slightly higher in subjects with the 4a/4b genotype (median=15.0, *N*=8) than subjects with the 4b/4b genotype (median=12.9, *N*=22; Fig.1B). However, the crude mean difference of −1.64 was not statistically significant (*P*>.20), nor was the mean difference (*P*>.20) adjusted for race and extent and severity of CAD (Fig. 1B).

The results of our study suggest that in predominantly older men referred for coronary angiography, a significant correlation is seen between the eNOS intron 4 polymorphism and multi-vessel CAD.

DISCUSSION

Both experimental and clinical research has established a link between a dysfunctional eNOS enzyme and cardiovascular disease.[24] Although intronic polymorphisms are less likely to have a functional role per se than either the promoter or coding region variants, they may have functional relevance to transcriptional or post-transcriptional regulation. Furthermore, intron 4 variants may act as markers for unidentified, potentially functional variants elsewhere in the gene.

We investigated the association of the eNOS intron 4 polymorphism with extent and severity of CAD and brachial endothelial function. The results of our study suggest that in predominantly older men referred for coronary angiography, a significant correlation is seen between the eNOS intron 4 polymorphism and multi-vessel CAD. Moreover, the association is stronger among African Americans compared with Caucasians. Among African Americans, the odds of having a 4a-allele among subjects with multi-vessel disease compared with subjects without multi-vessel disease is 2.45 (*P*=.06). The corresponding odds ratio for Caucasians is 1.58

Table 3. Clinical association studies of eNOS intron 4 polymorphism in patients with and without CAD

Author (year)	Ethnic Population	Age	4a-allele Frequency/eNOS Intron 4 Genotype	Clinical Association
Wang et al[7] (1996)	Caucasian CAD: 54 Control: 153	54.8 ± 0.9 42.0 ± 10.8	0.14 0.17	4a/4a genotype is associated with CAD in smokers
Ichihara et al[8] (1998)	Japanese MI: 455 Control: 550	 58 ± 8 58 ± 7	 0.14 0.10	4a-allele is associated with MI only in low risk patients
Hibi et al[9] (1998)	Japanese CAD: 226 Control: 357	 63.3 ± 0.7 62.8 ± 0.7	 0.12 0.11	4a/4a genotype is not associated with MI or severity of CAD
Odawara et al[10] (1998)	Japanese CAD: 42 Control: 122	 63.7 ± 8.8 61.8 ± 7.7	 0.13 0.08	4a-allele is not associated with CAD in patients with type 2 diabetes
Hooper et al[11] (1999)	African American MI: 110 Control: 185	 55 (29–84) 56 (21–93)	 0.36 0.26	4a-allele is associated with MI
Hingorani et al[12] (1999)	Caucasian CAD: 122 Control: 321	 58.9 ± 0.3 58.1 ± 0.3	 0.17 0.16	4a-allele is not associated with MI
Nakagami et al[13] (1999)	Japanese CAD: 40 Control: 34	 64 ± 10 62 ± 13	 4a/4a + 4a/4b = 48% 4a/4a + 4a/4b = 29%	4a/4a genotype is not associated with CAD
Fowkes et al[14] (2000)	Caucasian CAD: 137 Control: 300	 65.5 ± 0.5 63.2 ± 0.3	 0.16 0.12	4a-allele is associated with CAD in nonsmokers
Pulkkinen et al[15] (2000)	Caucasian CAD: 308 CAD + D.m.: 251 Control: 82	 58 ± 1 64 ± 1 51 ± 1	 0.19 0.21 0.15	4a-allele is not associated with CAD
Park et al[16] (2000)	Korean MI: 121 Control: 206	 53.5 ± 11.6 51.0 ± 9.5	 0.14 0.05	4a-allele is associated with MI in CAD patients < 51 years
Yoon et al[17] (2000)	Korean CAD: 110 Control: 128	 60.9 ± 8.6 59.3 ± 7	 0.16 0.17	4a-allele is not associated with CAD
Granath et al[18] (2001)	Caucasian CAD: 573 Control: 624	 43.9 ± 4.5 40.9 ± 5.8	 0.15 0.14	4a/4a genotype is not associated with CAD
Hwang et al[19] (2002)	Taiwanese CAD: 149 Control: 70	 62.8 ± 10.6 53.8 ± 14.3	 0.11 0.10	4a-allele is not associated with CAD, MI, or severity of CAD

Age is presented as mean ± SD (range). All other values are presented as numbers.
CAD: coronary artery disease; MI: myocardial infarction; DM = diabetes mellitus.

(P=.21). However, this difference in odds ratios is not statistically significant (P>.20) because our study is small. The pooled odds ratio for African Americans and Caucasians is 1.85 (P=.035). In contrast, results from endothelium-dependent and -independent vascular reactivity demonstrate that the polymorphism has no effect in altering endothelial function.

The frequency of the eNOS intron 4a-allele is different in various ethnic populations. Hooper et al first reported that the eNOS 4a-allele is more prevalent in African Americans.[11] Shortly thereafter, Tanus-Santos et al studied the effects of ethnicity on the distribution of various eNOS polymorphisms and found that the eNOS 4a-allele is more prevalent in the African-American population (q=0.265) than in the Caucasian and Asian populations (q=0.16 and q=0.13, respectively).[25] Similarly, in our study, the eNOS 4a-allele frequency was significantly higher in African Americans (q=0.31) than in Caucasians (q=0.15). The allele frequencies in our study within the ethnic subgroups were comparable to those found by other investigators.

Our study supported and extended the observation of Hooper et al regarding a positive association between CAD and myocardial infarction and the eNOS intron 4 polymorphism in African Americans. Previously, Hibi et al studied the association of the eNOS 4a-allele with severity of CAD in a Japanese population and found no correlation.[9] In a more recent study, Hwang et al investigated the same association in a hospital-based Taiwanese population and found no association.[19] Our findings differ from those of both investigators. We found a positive association between the 4a-allele and multi-vessel disease that persisted even after adjusting for CAD risk factors. Nevertheless, we cannot discard the possibility that the eNOS intron 4 polymorphism could be in linkage disequilibrium with another functional polymorphism.

In 1996, Wang et al initially reported a smoking-dependent risk of CAD in Australian subjects with the homozygous 4a/4a genotype.[7] While cigarette smoking decreased eNOS activity in the 4a-allele carrier, it increased eNOS activity in the 4b/4b genotype. Subsequently, other investigators have studied the effect of the 27-bp repeat polymorphism in intron 4 with cardiovascular endpoints (Table 3).[7–19] However, results have often been conflicting. For example, in contrast to the study by Wang et al, Fowkes et al found that the eNOS 4a-allele was associated with CAD in non-smokers.[14] Our study provides some support for a smoking-dependent eNOS 4a-allele effect, in that the odds ratio for the 4a-allele was elevated among smokers but not among non-smokers. These inconsistent findings from the studies cited above may be due to inadequate sample size in some of the studies and from differences in criteria used for phenotypic definitions.

Notwithstanding these findings, however, functional studies of the effects of the eNOS intron 4 polymorphism on endothelial function are lacking. Previous studies have shown that a deficiency in vascular NO might contribute to the impairment of endothelial function and development of atherosclerosis.[2] Consequently, we investigated the effect of the eNOS 4a-allele on brachial endothelial function in a subgroup of our study population. We found no statistically significant differences in either endothelium-dependent or -independent dilation between patients with or without the eNOS 4a-allele. Different relationships between the eNOS intron 4 polymorphism and circulating nitrite and nitrate levels were observed among healthy populations, whereas Yoon et al reported higher plasma nitrate and nitrite levels in CAD patients with hypertension as compared to CAD patients without hypertension and in controls.[17,20,26,27] Our endothelial function study subgroup is composed of patients with established CAD and multiple coronary risk factors, including hypertension and smoking, which may have confounded the results of our study. Furthermore, lack of the 4a/4a genotype in the endothelial function subgroup is a limitation of our study.

In summary, our study suggests a possible link between the presence of the eNOS intron 4a-allele and severity of CAD but not with endothelial function. Moreover, the association between the polymorphism and multivessel CAD appears stronger among African Americans compared with Caucasians. Since even small changes in the function of eNOS can affect the atherosclerotic process, further prospective studies in large populations of genetically-characterized subjects will be required to delineate precisely the effects of eNOS variants on endothelial function and their interaction with clinical coronary risk factors. Ultimately, the results of such studies may lead to design of therapies aimed at restoring endothelial NO production targeted to individuals with particular eNOS genotypes.

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REFERENCES

1. Cooke JP, Dzau VJ. Nitric oxide synthase: role in the genesis of vascular disease. *Annu Rev Med.* 1997;48:489–509.
2. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev.* 1991;43:109–142.
3. Wang XL, Wang J. Endothelial nitric oxide synthase gene sequence variations and vascular disease. *Mol Genet Metab.* 2000;70:241–251.
4. Aexander RW. Hypertension and the pathogenesis of atherosclerosis; oxidative stress and the mediation of arterial inflammatory response. *Hypertension.* 1995;7:447–451.
5. Hingorani AD. Polymorphisms in endothelial nitric oxide synthase and atherogenesis: John French lecture 2000. *Atherosclerosis.* 2000; 154:521–527.
6. Naduad S, Bonnardeaux A, Lathrop GM, et al. Gene structure, polymorphism, and mapping of the human endothelial nitric oxide synthase gene. *Biochem Biophys Res Commun.* 1994;198:1027–1033.
7. Wang XL, Sim AS, Badenhop RF, et al. A smoking-dependent risk of coronary artery disease associated with a polymorphism of the endothelial nitric oxide synthase gene. *Nat Med.* 1996;2:41–245.
8. Ichihara S, Yamada Y, Fujimura T, et al. Association of a polymorphism of the endothelial constitutive nitric oxide synthase gene with myocardial infarction in the Japanese population. *Am J Cardiol.* 1998;81:83–86.
9. Hibi K, Ishigami T, Tamura K, et al. Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. *Hypertension.* 1998;32:521–526.
10. Odawara M, Sasaki K, Tachi Y, et al. Endothelial nitric oxide synthase gene polymorphism and coronary heart disease in Japanese NIDDM. *Diabetologia.* 1998;41:365–367.
11. Hooper WC, Lally C, Austin H, et al. The relationship between polymorphism in the endothelial cell nitric oxide synthase gene and the platelet GPIIIa gene with myocardial infarction and venous thromboembolism in African Americans. *Chest.* 1999;116:880–886.
12. Hingorani AD, Liang CF, Fatibene J, et al. A common variant of the endothelial nitric oxide synthase (Glu298→Asp) is a major risk factor for coronary artery disease in the UK. *Circulation.* 1999;100:1515–1520.
13. Nakagami H, Ikeda U, Maeda Y, et al. *J Thromb Thrombolysis.* 1999;8:191–195.
14. Fowkes FGR, Lee AJ, Hau CM, et al. Meth-

ylene tetrahydrofolate reductase (MTHFR) and nitric oxide synthase (ecNOS) genes and risks of peripheral arterial disease and coronary heart disease: Edinburgh artery study. *Atherosclerosis.* 2000;150:179–185.
15. Pulkkinen A, Viitanen L, Kareinen A, et al. Intron 4 polymorphism of the endothelial nitric oxide synthase gene is associated with elevated blood pressure in type 2 diabetic patients with coronary heart disease. *J Mol Med.* 2000;78:372–379.
16. Park JE, Lee WH, Hwang TH, et al. Aging affects the association between endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction in the Korean male population. *Korean J Intern Med.* 2000; 15:65–70.
17. Yoon Y, Song J, Hong SH, et al. Plasma nitric oxide concentrations and nitric oxide synthase gene polymorphisms in coronary artery disease. *Clin Chem.* 2000;46:1626–1630.
18. Granath B, Taylor RR, van Bockxymeer FM, et al. Lack of evidence for association between endothelial nitric oxide synthase gene polymorphisms and coronary artery disease in the Australian Caucasian population. *J Card Risk.* 2001;8:235–241.
19. Hwang JJ, Tsai CT, Yeh HM, et al. The 27-bp tandem repeat polymorphism in intron 4 of the endothelial nitric oxide synthase gene is not associated with coronary artery disease in a hospital-based Taiwanese population. *Cardiology.* 2002;97:67–72.
20. Wang XL, Mahaney MC, Sim AS, et al. Genetic contribution of the endothelial constitutive nitric oxide gene to plasma nitric oxide levels. *Arterioscler Thromb Vasc Biol.* 1997;17: 3147–3153.
21. Zafari AM, Davidoff MN, Austin H, et al. The A640G and C242T p22phox polymorphisms in patients with coronary artery disease. *Antioxid Redox Signal.* 2002;4:675–680.
22. Celermajer DS, Sorensen KE, Gooch VM, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk for atherosclerosis. *Lancet.* 1992;340:1111–1115.
23. Corretti MC, Anderson TJ, Benjamin EJ, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery. *J Am Coll Cardiol.* 2002;39:257–265.
24. Loscalzo J. Functional polymorphisms in a candidate gene for atherothrombosis. *J Am Coll Cardiol.* 2003;41:946–948.
25. Tanus-Santos JE, Desai M, Flockhart DA. Effects of ethnicity on the distribution of clinically relevant endothelial nitric oxide variants. *Pharmacogenetics.* 2001;11:719–725.
26. Tsukada T, Yokoyama K, Arai T, et al. Evidence of association of the ecNOS gene polymorphism with plasma NO metabolite levels in humans. *Biochem Biophys Res Commun.* 1998;245:190–193.
27. Thomas PS, Bruce C, Birkhead A, et al. Effect of ecNOS polymorphisms and coronary artery disease upon exhaled nitric oxide. *J Mol Med.* 2002;80:181–186.

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