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Endothelial Function and Carotid Intima-Media Thickness in Young Healthy Subjects Among Endothelial Nitric Oxide Synthase $Glu^{298}\rightarrow Asp$ and $T^{-786}\rightarrow C$ Polymorphisms

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Background and Purpose—To assess the role of the endothelial nitric oxide synthase (eNOS) gene variants as risk factors for early atherosclerosis, we sought to investigate whether two polymorphisms located in the exon 7 ($Glu^{298}\rightarrow Asp$) and in the promoter region ($T^{-786}\rightarrow C$) of the eNOS gene were associated with functional changes in the endothelium and carotid intima-media thickness (IMT).

Methods—Endothelium-dependent flow-mediated brachial artery dilation (FMD), endothelium-independent dilation response to glyceryl trinitrate (GTN), and carotid IMT were assessed by high-resolution ultrasound in 118 healthy young nonsmoker subjects (30.1±0.5 years) genotyped for the eNOS $Glu^{298}\rightarrow Asp$ and $T^{-786}\rightarrow C$ polymorphisms.

Results—Carotid IMT was inversely related to FMD by univariate analysis ($r=-0.28$, $P=0.002$) and after adjustment for possible confounders in all the subjects ($P<0.01$). Asp homozygotes had a significantly lower FMD than Glu carriers (Glu/Glu: 15.0%±1.0%, Glu/Asp: 13.3%±0.7%, Asp/Asp: 9.6%±1.6%; $P=0.005$), whereas FMD was unaffected by the $T^{-786}\rightarrow C$ variant. Neither the $Glu^{298}\rightarrow Asp$ nor the $T^{-786}\rightarrow C$ polymorphisms influenced the GTN-mediated dilation. With respect to Glu carriers, Asp/Asp genotype displayed a significantly greater carotid IMT (Glu/Glu: 0.37±0.01 mm, Glu/Asp: 0.35±0.01 mm, Asp/Asp: 0.45±0.03 mm; $P=0.0002$) and significant correlations between carotid IMT and FMD ($r=-0.48$, $P=0.04$) and between carotid IMT and resting brachial artery diameter ($r=0.70$, $P=0.001$). No difference in IMT was found across the $T^{-786}\rightarrow C$ genotypes. By multivariate regression analysis, Asp/Asp genotype was the only significant and independent predictor of flow-mediated brachial artery dilation (FMD) (P=0.04) and carotid intima-media thickness (IMT) (P=0.006).

Conclusions—The eNOS $Glu^{298}\rightarrow Asp$ polymorphism may be related to early atherogenesis. **(*Stroke*. 2004;35:1305-1309.)**

Key Words: nitric oxide synthase ■ atherosclerosis ■ genetics

In the vascular endothelium, nitric oxide (NO) produced from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS) is a principal mediator of normal endothelial function.[1] NO plays a key role in the relaxation of vascular smooth muscle, inhibits platelet and leukocyte adhesion to the endothelium, reduces vascular smooth muscle cell migration and proliferation, and limits the oxidation of atherogenic low-density lipoproteins.[2] Because of these multiple actions, endothelial NO plays a central role in maintaining normal vascular homeostasis and is considered to be atheroprotective. Therefore, eNOS could be a potential candidate gene for atherosclerosis.

Consequently, clinical research has focused on the association of eNOS genetic variants to late cardiovascular outcome, whereas less attention has been paid to genetic influences on the vascular biology of atherosclerosis during the long preclinical phase that begins in childhood.

A common variant of the eNOS gene, located in exon 7 ($G^{984}\rightarrow T$) that modifies its coding sequence ($Glu^{298}\rightarrow Asp$), has been linked to an increased risk for carotid atherosclerosis, coronary spasm, coronary artery disease (CAD), and myocardial infarction.[3–5]

Recently, a polymorphism in the 5′-flanking region of the eNOS gene ($T^{-786}\rightarrow C$) has been associated with coronary spasm among Japanese and with angiographic CAD in white patients.[6–8]

However, the influence of the eNOS $Glu^{298}\rightarrow Asp$ and $T^{-786}\rightarrow C$ variants at an early stage in atherogenesis is not currently known.

Therefore, we undertook the current study in a group of young healthy subjects free of conventional cardiovascular risk factors to investigate whether the eNOS $Glu^{298}\rightarrow Asp$ and $T^{-786}\rightarrow C$ polymorphisms were related to brachial artery reactivity and carotid intima-media thickness (IMT), two markers of early atherosclerosis.

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Subjects and Methods

Study Population

A total of 118 healthy subjects from the Italian population (46 male) aged 21 to 45 years consented to participate in this study. Each subject gave informed written consent, which was approved by our ethical committee. All individuals had never smoked, were free of cardiovascular risk factors, and were not using any medication.

In all subjects, fasting venous blood samples were analyzed for glucose, total cholesterol, high-density lipoprotein, low-density lipoprotein, and triglyceride concentrations by routine methods.

Rest supine systolic and diastolic blood pressure were measured in each subject using a standard sphygmomanometer before the beginning of the vascular measurements.

Measurement of Endothelium-Dependent and Endothelium-Independent Dilation of the Brachial Artery

Endothelium-dependent, flow-mediated dilation (FMD) and endothelium-independent dilation response to glyceryltrinitrate (GTND) of the right brachial artery were measured by echo Doppler technique, as previously described.[9]

All individuals were studied at least 4 hours after last eating and lay supine for 10 minutes before the beginning of the study. The diameter of the brachial artery was measured from 2-dimensional ultrasound images using a 7.5-MHz probe (Hewlett-Packard Sonos 5500). Images were digitized and calibrated electronic calipers were used to measure brachial artery diameter as the distance from the anterior to posterior intimal interfaces along a line perpendicular to the long axis of the artery. Arterial diameter was measured at rest, after reactive hyperemia, at rest again, and after sublingual GTN. The brachial artery was scanned in longitudinal section, approximately 2 cm above the antecubital fossa. Using ECG gating during image acquisition, the arterial diameter was measured at the onset of the R-wave, which is used to identify end diastole, at a fixed distance from an anatomical marker, such as a bifurcation. Five cardiac cycles were analyzed and averaged for each scan. After the baseline measurements, a pneumatic cuff, placed proximal to the imaged brachial artery segments on the upper arm, was inflated to a pressure of 300 mm Hg for 4.5 minutes. The cuff was then rapidly deflated and brachial artery diameter was recorded from 1 minute after cuff deflation. After a 10-minute rest period, a further baseline measurement of the brachial artery was recorded, then GTN (400 μg) was administered and the brachial artery recording was made after 3 minutes. The vascular responses were expressed as percentage and absolute changes in the brachial artery diameter, using baseline diameter as the reference. All tests were performed by the same operator, who was blinded to the eNOS genotypes in the subjects. The coefficients of variation in measured brachial artery diameters and measured brachial artery reactivity were 1.8% and 2.1%, respectively.

Measurement of Carotid IMT

Longitudinal ultrasonographic scans of the carotid artery were obtained on the same day as the studies of the brachial artery reactivity and included the evaluation of the right and left common carotid arteries 1 cm proximal to the carotid bulb. In each examination, the same operator used different scanning angles to identify the greatest IMT, defined as the distance between the junction of the lumen and intima and that of the media and adventitia. Three measurements of IMT were obtained from the right and left carotid arteries, respectively, and were averaged to determine the mean IMT for both sides combined. The coefficient of variation was 2.5%.

Analysis of $Glu^{298}\rightarrow Asp$ and $T^{-786}\rightarrow C$ Polymorphisms of eNOS Gene

Genotyping of all subjects was performed by polymerase chain reaction amplification, as described previously.[7] The quality and reliability of the restriction enzyme genotyping were checked by sequence analysis of randomly selected samples.

TABLE 1. General Characteristics of the Study Group

Variables	Male n=46	Female n=72	*P*
Age, y	30.8±0.8	29.7±0.6	0.29
Smoke	—	—	—
Glucose, mg/dL	93.6±1.4	90.2±1.4	0.10
Total cholesterol, mg/dL	175.0±5.5	178.5±4.5	0.62
LDL cholesterol, mg/dL	105.4±4.5	103.8±4.2	0.81
HDL cholesterol, mg/dL	51.3±3.0	63.5±2.0	<0.001
Triglyceride, mg/dL	77.0±10.6	58.5±4.4	0.10
Systolic blood pressure, mm Hg	123.1±1.7	112.8±1.3	<0.0001
Diastolic blood pressure, mm Hg	78.7±0.8	74.4±0.7	<0.001
Resting vessel size, mm	3.92±0.06	3.03±0.03	<0.0001
FMD, %	11.3±0.7	14.6±0.8	<0.01
GTND, %	16.1±0.7	21.1±0.7	<0.0001
FMD, mm	0.44±0.03	0.43±0.02	0.91
GTND, mm	0.62±0.02	0.63±0.02	0.64
Carotid IMT, mm	0.39±0.02	0.36±0.01	0.13

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein.

Statistical Analysis

All statistical analyses were conducted with the Statview statistical package, version 5.0.1 (SAS Institute). Data are expressed as mean±SEM. Differences between the means of 2 continuous variables were evaluated by Student *t* test. Differences in noncontinuous variables were tested by χ^2 analysis. One-way ANOVA followed by Scheffe test was used to analyze the relations between genotypes and the general characteristics, vascular function, and carotid IMT. Continuous relationships between variables, vascular function, carotid IMT, and genotypes were analyzed by univariate regression analysis. Multiple regression analysis was then performed to identify the variables that independently predicted the relationships. Statistical significance was defined as $P<0.05$.

Results

General Characteristics of the Study Group

The characteristics of the study group are summarized in Table 1. Males displayed lower high-density lipoprotein levels and higher systolic and diastolic blood pressures than female subjects, even if within normal limits. Females had smaller brachial artery resting diameters and, consequently, higher percent FMD and GTN compared with males because baseline diameter influences percent change in an inversely proportional manner. The absolute changes in brachial artery diameter after reactive hyperemia and GTN did not differ between sexes.

Percent FMD was significantly related to resting diameter ($r=-0.43$, $P<0.0001$), systolic ($r=-0.27$, $P<0.01$), and diastolic ($r=-0.20$, $P=0.03$) blood pressure by simple regression analysis in all the subjects. On multivariate analysis, only brachial artery resting diameter was significantly and independently related to percent FMD ($r=-0.39$, $P<0.0001$) and GTND ($r=-0.60$, $P<0.0001$).

Carotid IMT was significantly associated with percent FMD ($r=-0.28$, $P=0.002$) and diastolic blood pressure ($r=0.21$, $P=0.02$) by simple regression analysis for all the subjects. After multivariate analysis, IMT was inversely related to FMD only ($r=-0.24$, $P<0.01$).

TABLE 2. General Characteristics of the Study Group Among Glu^{298}→Asp and T^{-786}→C Genotypes

	Glu^{298}→Asp Polymorphism				T^{-786}→C Polymorphism			
Variables	Glu/Glu n=43	Glu/Asp n=57	Asp/Asp n=18	*P*	TT n=43	TC n=58	CC n=17	*P*
Age, y	30.4±0.8	29.8±0.6	30.8±1.1	0.71	30.0±0.8	30.3±0.6	30.1±1.2	0.97
Male sex, %	32.6	40.3	50	0.43	37.2	43.1	29.4	0.57
Glucose, mg/dL	93.3±1.8	90.5±1.3	90.0±3.4	0.40	90.4±1.7	92.2±1.3	91.4±4.2	0.71
Total cholesterol, mg/dL	180.7±5.4	172.4±4.5	185.8±12.5	0.34	172.3±5.4	179.0±5.0	183.8±9.5	0.52
LDL cholesterol, mg/dL	108.0±4.6	102.3±4.0	102.2±13.2	0.67	96.8±4.9	108.7±4.3	107.6±8.7	0.20
HDL cholesterol, mg/dL	58.6±2.4	57.9±2.6	63.4±7.8	0.61	60.5±3.6	56.3±2.1	64.2±4.8	0.30
Triglyceride, mg/dL	70.6±8.9	63.4±7.1	60.4±8.6	0.74	61.3±8.6	70.3±7.4	58.4±5.9	0.61
Systolic blood pressure, mm Hg	117.9±2.0	116.2±1.5	120.4±2.7	0.40	117.2±2.0	116.3±1.6	118.0±2.5	0.86
Diastolic blood pressure, mm Hg	75.9±1.1	75.9±0.7	77.2±1.4	0.71	75.1±1.0	76.9±0.8	76.0±1.7	0.36
Resting vessel size, mm	3.38±0.09	3.36±0.07	3.44±0.14	0.87	3.38±0.10	3.40±0.07	3.30±0.10	0.82

Glu^{298}→Asp and T^{-786}→C Polymorphisms of the eNOS Gene, Endothelial Function, and Carotid IMT

Both polymorphisms were in Hardy-Weinberg equilibrium (Glu^{298}→Asp: P=0.93; T^{-786}→C: P=0.69).

There were no significant differences in the general characteristic of the study group on the basis of the eNOS Glu^{298}→Asp and T^{-786}→C genotypes (Table 2).

With respect to Glu carriers, Asp homozygotes exhibited a significantly lower FMD (Glu/Glu: 15.0%±1.0%, Glu/Asp: 13.3±0.7, Asp/Asp: 9.6%±1.6%; *P*=0.005), whereas FMD was unaffected by the T^{-786}→C variant (TT: 14.5%±1.0%, TC: 12.5%±0.8%, CC: 13.2%±1.6%; *P*=0.31) (Figure 1).

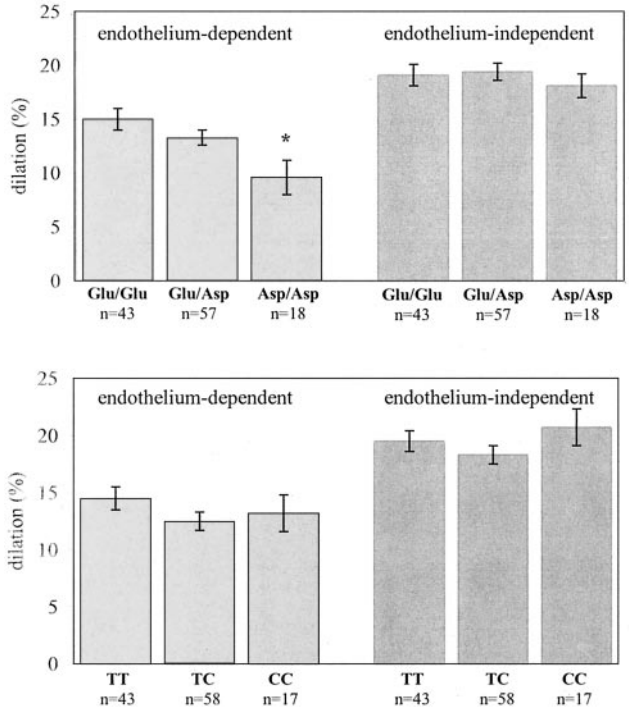

Figure 1. Glu^{298}→Asp and T^{-786}→C polymorphisms and brachial artery reactivity. **P*=0.005 and *P*=0.007 for Asp/Asp vs Glu allele and Glu/Glu carriers, respectively, for endothelium-dependent vasodilation.

Neither the Glu^{298}→Asp nor the T^{-786}→C polymorphisms influenced the GTN-mediated dilation of the brachial artery (*P*=0.72 and *P*=0.30, respectively).

The same results for both eNOS gene variants were obtained when the absolute changes in the brachial artery diameter after reactive hyperemia and GTN were considered (data not shown).

Furthermore, even if all of the subjects were young and no carotid plaques were observed in this study group, Asp homozygotes displayed a significantly greater carotid IMT as compared with Glu carriers (Glu/Glu: 0.37±0.01 mm, Glu/Asp: 0.35±0.01 mm, Asp/Asp: 0.45±0.03 mm, *P*=0.0002; Figure 2). No difference in carotid IMT was found across the T^{-786}→C genotypes (TT: 0.38±0.02 mm, TC: 0.37±0.01 mm, CC: 0.36±0.02 mm; *P*=0.90).

By multivariate regression analysis, only FMD (r=−0.19, P=0.04) and carotid IMT (r=0.27, P=0.006) were independently related to Asp/Asp genotype in our study group.

After stratification for the Glu^{298}→Asp polymorphism, an inverse correlation between carotid IMT and FMD was found only among Asp/Asp (Glu/Glu: *r*=−0.13, *P*=0.40; Glu/Asp: *r*=−0.21, *P*=0.11; Asp/Asp: *r*=−0.48, *P*=0.04). Moreover, carotid IMT was significantly associated with resting brachial artery diameter among Asp/Asp but not Glu carriers (Glu/Glu: *r*=−0.01, *P*=0.97; Glu/Asp: *r*=0.03, *P*=0.82; Asp/Asp: *r*=0.70, *P*=0.001).

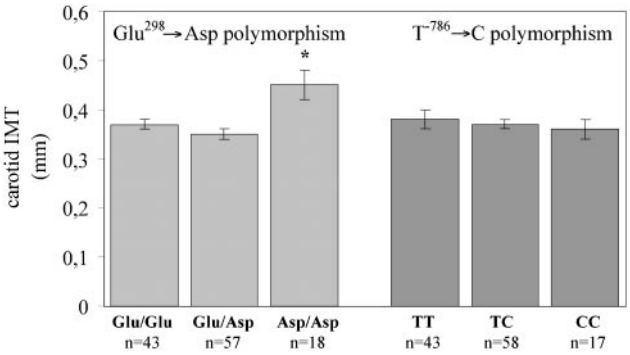

Figure 2. eNOS polymorphisms and carotid IMT. **P*=0.0002 and *P*=0.01 for Asp/Asp vs Glu allele and Glu/Glu carriers, respectively, of the Glu^{298}→Asp polymorphism.

Discussion

The present study was designed to assess whether the eNOS Glu^{298}→Asp and T^{-786}→C polymorphisms were associated with initial functional and structural changes in the arterial wall of young healthy subjects. These vascular changes can be assessed noninvasively by measuring brachial artery FMD, an NO-dependent endothelial response,[10] and carotid IMT by high-resolution ultrasound, and may be used as indices of the atherosclerotic vascular process.[11,12]

Previous reports in young people and even in children have shown that smoking, diabetes, hypercholesterolemia, and hypertension are associated with brachial artery endothelial dysfunction and increased carotid IMT.[13–18] Consequently, subjects with such risk factors were intentionally excluded, as were those who were using medications that might alter their endothelium or smooth muscle-dependent responses.

eNOS Glu^{298}→Asp and T^{-786}→C Polymorphisms, Endothelial Function, and Carotid IMT

The present study showed that Asp/Asp genotype of the eNOS Glu^{298}→Asp polymorphism is significantly and independently associated with decreased brachial artery FMD and increased carotid IMT in a group of young healthy individuals free of traditional cardiovascular risk factors.

In addition, a strong correlation between carotid IMT and resting brachial artery diameter was present only among Asp homozygotes, suggesting also that vessel size could be related to asymptomatic vascular changes. Indeed, large brachial artery diameter has been reported to be an independent predictor of significant angiographic CAD in women with chest pain and, interestingly, in a subanalysis of women without CAD, mean resting diameter tended to increase with number of risk factors.[19] Therefore, because atherosclerosis disrupts the arterial architecture that regulates vessel size, resting brachial artery diameter could be an indicator of atherosclerosis. However, its physiological importance remains unclear.

So far, the Glu^{298}→Asp polymorphism has been reported to be an independent risk factor for carotid atherosclerosis,[3] but was not related to carotid IMT and other cardiovascular alterations in the white population.[3,20] Moreover, Leeson et al[21] found no overall influence of this genetic variant on endothelial function in a group of young preclinical subjects with a representative range of environmental risk factors. However, it should be considered that the effect of one gene on complex traits, such as atherosclerosis and endothelial function, might be difficult to observe in population-based cohorts exposed to environmental confounding factors. On the contrary, there are subgroups of subjects, as those selected in our study, in which the effect of a single gene may be more evident. To our knowledge, this is the first study that has examined the relation between eNOS genetic variants and measurements of vascular structure and function in a group of young people free of traditional risk factors.

Previously, Gaeta et al[22] have shown that alterations in brachial artery reactivity and carotid IMT are present in young offspring of patients with premature myocardial infarction and occurred independently of several traditional cardiovascular risk factors.[23] Moreover, Zannad et al[24] have recently suggested that 30% of the variation in carotid IMT may be explained by genetic factors. Accordingly, we found a negative correlation between carotid IMT and FMD in all the subjects, but this association was most prominent among Asp/Asp carriers.

Therefore, our data support the hypothesis that the eNOS Glu^{298}→Asp polymorphism may be related to early atherogenesis, probably by affecting the activity of the endothelial NO system.

Functional Significance of the eNOS Glu^{298}→Asp and T^{-786}→C Polymorphisms

The eNOS Asp^{298} protein has been reported to have an enhanced susceptibility to intracellular proteolytic cleavage compared with the eNOS Glu^{298}.[25] Accordingly, the Asp^{298} allele has been associated with enhanced vascular responsiveness to phenylephrine,[26] and with differences in endothelial responses to smoking and ω-3 fatty acid levels,[21] even if other studies excluded a relevant effect of this polymorphism.[27–29]

The T^{-786}→C variation in the 5′ flanking region of the eNOS gene was originally reported by Nakayama et al[6] to be associated with coronary vasospasm by affecting eNOS expression, supporting the hypothesis that in many carriers of the mutant allele, the L-arginine-NO pathway does not function properly leading to endothelial dysfunction. However, we found no evidence for an effect of the T^{-786}→C variant on endothelial function and carotid thickening in this study group. Our results are in agreement with those of Rossi et al[29] who showed that the T^{-786}→C polymorphism affected the forearm blood flow among hypertensive patients but not among normotensive controls, suggesting that this variant alone is insufficient to account for enhanced susceptibility to vascular dysfunction, and that it is a disease-modifying allele.

However, we cannot rule out the alternative explanation that our study was underpowered to detect a relation between the eNOS T^{-786}→C polymorphism and brachial artery FMD and carotid IMT.

Study Limitations

This study exclusively comprised Italian individuals who were free of conventional cardiovascular risk factors. Consequently, our findings might apply neither to other populations nor to subjects at risk for coronary disease. Moreover, because our findings are based on relatively few individuals because of the careful selection of this study group, the replication of our observations in larger cohorts of different populations is needed. Finally, the lack of standardization of the methodology for the assessment of brachial artery FMD is another limitation of our study.[30] Indeed, even if brachial artery ultrasound imaging during reactive hyperemia is a widely used tool for quantifying endothelium-dependent responses, at the moment, however, there is no uniformly accepted technique for performing this test. This is based on certain technical aspects of the procedure, such as cuff position and duration of vessel occlusion, which can alter the degree of FMD. We chose to perform upper arm occlusion because of the larger percent FMD that can be achieved and, thus, the larger differences between groups being studied.

Conclusions

The present study showed that the eNOS Glu^{298}→Asp polymorphism affects brachial artery FMD and carotid IMT, two markers of early atherosclerosis, suggesting a genetically determined modulation of early changes in arterial structure and function related to atherogenesis. Therefore, the eNOS Glu^{298}→Asp polymorphism might, in the long-term, play a significant role in atherogenesis and cardiovascular damage, raising the possibility of genotype prevention strategies.

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