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Endothelial Nitric Oxide Synthase Gene Polymorphisms and Risk of Coronary Artery Disease

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Background: **Endothelial nitric oxide synthase (eNOS) could be a candidate gene for coronary artery disease (CAD). This study investigated the relationship of the eNOS Glu^{298}→Asp and T^{786}→C polymorphisms with the presence and severity of CAD in the Italian population.**

Methods: **We enrolled 415 unrelated individuals who underwent coronary angiography. The severity of CAD was expressed by means of the Duke score. The eNOS Glu^{298}→Asp and T^{786}→C variants were analyzed by PCR.**

Results: **There was significant linkage disequilibrium between the two eNOS polymorphisms (P <0.0001). Both variants were significantly associated with the occurrence and severity of CAD (P = 0.01 and 0.004 for Glu^{298}→Asp and T^{786}→C, respectively). The risk of CAD was increased among individuals homozygous for the C allele of the T^{786}→C polymorphism compared with individuals homozygous for the T allele (odds ratio = 2.5; P <0.01) and was independent of the other common risk factors (P = 0.04). Moreover, individuals with both the Asp/Asp genotype of the Glu^{298}→Asp polymorphism and at least one C allele of the T^{786}→C variant in the promoter region of the eNOS gene had an increased risk of CAD (odds ratio = 4.0; P <0.001) and a significantly higher mean Duke score (26.2 ± 2.9 vs 45.2 ± 3.7; P = 0.002) compared with individuals with the TT genotype and the Glu allele.**

Conclusions: **The present study provides evidence that the Glu^{298}→Asp and T^{786}→C polymorphisms of the eNOS gene are associated with the presence and severity of angiographically defined CAD in the Italian population and that those individuals carrying both eNOS variants simultaneously might have a higher risk of developing CAD.**

In the vascular endothelium, NO, a powerful short-lived vasoactive substance, is constitutively produced from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS)[1] *(1)*. NO plays a key role in the relaxation of vascular smooth muscle, inhibits adhesion of platelets and leukocytes to the endothelium, reduces vascular smooth muscle cells migration and proliferation, and limits the oxidation of atherogenic low-density lipoproteins *(2)*. Moreover, it has been shown that eNOS inhibition accelerates atherosclerosis in animal models and that abnormalities in the endothelial NO pathway are present in humans with atherosclerosis *(3, 4)*.

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. Therefore, functionally important variants of the eNOS gene could influence individual susceptibility to atherosclerosis by altering the amount of NO generated by the endothelium.

Several polymorphisms have been identified in the eNOS gene. Among them, a common variant located in exon 7 (G^{984}→T) of the eNOS gene that modifies its coding sequence (Glu^{298}→Asp) has been linked by several groups to the risk for coronary spasm, coronary artery disease (CAD), and acute myocardial infarction *(5–8)*. We have also previously reported that the

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[1] Nonstandard abbreviations: eNOS, endothelial nitric oxide synthase; CAD, coronary artery disease; and OR, odds ratio.

Glu^{298}→Asp polymorphism is associated with the occurrence and severity of CAD in the Italian population *(9)*. On the other hand, other studies failed to find any relationship between the Asp variant and the risk of atherosclerosis, suggesting that the Asp allele may act merely as a marker for a functional mutation in either eNOS or a nearby gene *(10–12)*.

Recently, a polymorphism in the 5′-flanking region of the eNOS gene (T^{786}→C) that affects eNOS expression has been associated with coronary spasm among Japanese *(13)*. Although this finding relates to a condition of low prevalence outside Japan, it has wide-ranging implications because it suggests a link between decreased endothelial NO production as a result of the eNOS T^{786}→C variant and vascular disease *(14, 15)*.

In the present study, we investigated the associations between the occurrence and severity of angiographically defined CAD and the T^{786}→C polymorphism in the 5′-flanking region of the eNOS gene alone and in combination with the Glu^{298}→Asp polymorphism.

Materials and Methods

STUDY POPULATION

We studied 268 patients consecutively admitted to our institute who had angiographically defined CAD (>50% stenosis affecting at least one coronary vessel; CAD cases) and 147 individuals recruited from patients admitted for valve replacement, in whom angiographic examination excluded the presence of CAD (controls). Controls were enrolled providing that they had neither a history nor clinical or instrumental evidence of atherosclerosis in peripheral vascular districts. Individuals with coronary spastic angina in the absence of luminal atheroma were not enrolled in the study. All patients and controls were from the Italian population.

All participants were interviewed, and data on smoking habits, hypertension, diabetes, dyslipidemia, and family history of CAD were recorded. Informed consent was obtained from all patients and controls according to the guidelines of our ethics committee. For coronary risk factors, the following definitions were used: individuals were defined as hypertensive if their blood pressure was >140/90 mmHg or if they were receiving any antihypertensive treatment; individuals with a history of diabetes or those receiving any antidiabetic medication were considered to be diabetic; individuals were deemed dyslipidemic when their total cholesterol concentration was ≥2200 mg/L, their triglyceride concentration was ≥2000 mg/L, or they were receiving lipid-lowering drugs. Smoking history was coded as never, ex-smoker (at least 6 months), and current smoker. The family history was considered positive for CAD if at least one first-degree male relative was diagnosed with CAD by the age of 55 or one first-degree female relative was diagnosed with CAD by the age of 65 years.

ANGIOGRAPHIC STUDY

All participants underwent coronary angiography. Coronary stenosis was considered significant if the luminal diameter of at least one epicardial coronary artery was narrowed by >50%. The extent and the severity of CAD were evaluated by means of the Duke scoring system *(16)*. This CAD prognostic index considers the number of diseased major vessels as well as 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%). Duke scores range from 0 to 100 (0 = no disease; 100 = most severe disease).

ANALYSIS OF Glu^{298}→Asp POLYMORPHISM IN EXON 7 OF THE eNOS GENE

Genomic DNA was extracted from samples of whole blood by standard methods *(17)*. The Glu^{298}→Asp polymorphism was a G→T substitution at nucleotide position 894 in exon 7 that encodes for replacement of glutamic acid by aspartic acid at residue 298 in the mature eNOS protein. Genotyping of all participants was by PCR amplification of exon 7 with the primers 5′-CATGAGGCTCAGCCCCAGAAC-3′ (sense) and 5′-AGTCAATCCCTTTGGTGCTCAC-3′ (antisense) followed by *Mbo*I restriction enzyme digestion for 16 h at 37 °C. In the presence of a T at nucleotide 894, which corresponds to Asp^{298}, the 206-bp PCR product is cleaved into two fragments of 119 and 87 bp. The products of the digestion process were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

ANALYSIS OF T^{786}→C POLYMORPHISM IN THE 5′-FLANKING REGION OF THE eNOS GENE

The presence of the T→C conversion at nucleotide position 786 in the 5′-flanking region of the eNOS gene was determined by PCR amplification with the primers 5′-ATGCTCCCACCAGGGCATCA-3′ (sense) and 5′-GTCCTTGAGTCTGACATTAGGG-3′ (antisense). The 236-bp PCR fragments were digested with *NgO*AIV restriction enzyme for 16 h at 37 °C. The wild-type allele (T) has no *NgO*AIV cleavage site, whereas the PCR product is cleaved into two fragments of 203 and 33 bp in the presence of the C^{786} allele.

STATISTICAL ANALYSIS

All statistical analyses were conducted with use of the Statview statistical package, Ver. 5.0.1 (SAS Institute). Data are expressed as the mean (SE). Differences between the means of the two continuous variables were evaluated by Student *t*-test. Differences between noncontinuous variables, genotype distribution, and Hardy–Weinberg equilibrium were tested by χ^2 analysis. Linkage disequilibrium between the two polymorphisms was examined by χ^2 analysis. The extent of disequilibrium was expressed as: $D' = D/D_{max}$ *(18)*. One-way ANOVA was used to analyze the relationships between genotypes and

the general characteristics and severity of CAD in terms of Duke score. Logistic regression analysis was used to assess the independent effect of each risk factor on the occurrence of CAD. The interaction between the eNOS $Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ genotypes on CAD risk was evaluated by adding the new product variable ($Glu^{298}\rightarrow Asp$ genotype) × ($T^{786}\rightarrow C$ genotype) in the logistic regression model. $P < 0.05$ was considered statistically significant.

Results

COMPARISON OF THE TWO STUDY GROUPS

The demographic and clinical characteristics of the study groups are shown in Table 1. The prevalence of atherogenic risk factors (age, sex, hypertension, diabetes, dyslipidemia, cigarette smoking, and family history of CAD) was significantly higher in the group of CAD patient.

RELATIONSHIP BETWEEN THE $Glu^{298}\rightarrow Asp$ AND $T^{786}\rightarrow C$ POLYMORPHISMS OF THE eNOS GENE

The relationship between the $Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ polymorphisms of the eNOS gene was assessed in 374 genotyped individuals. Comparison of allele and haplotype frequencies revealed that the eNOS Asp^{298} allele was weakly ($D' = 0.38$) but significantly linked to the $T^{786}\rightarrow C$ polymorphism in the 5′-flanking region of the eNOS gene because the Asp^{298} allele was preferentially found in a subgroup of individuals with at least one C^{786} allele (Table 2; $P < 0.0001$).

ASSOCIATION OF $Glu^{298}\rightarrow Asp$ AND $T^{786}\rightarrow C$ POLYMORPHISMS OF THE eNOS GENE WITH CAD

The distributions of the $Glu^{298}\rightarrow Asp$ genotypes in both CAD cases and controls satisfied the Hardy–Weinberg equilibrium. In agreement with our previous report *(9)*, we confirmed that the $Glu^{298}\rightarrow Asp$ polymorphism in exon 7 of the eNOS gene was significantly associated with the presence of CAD in this expanded sample size [total study population of 415 vs 315 in the previous report *(9)*; Table 3; $P = 0.03$].

As for the $Glu^{298}\rightarrow Asp$ variant, the $T^{786}\rightarrow C$ polymorphism in the 5′-flanking region of the eNOS gene was significantly associated with the presence of CAD in our patients as well (Table 3; $P = 0.02$). In fact, the proportion of C^{786} homozygotes was 24.6% in the CAD cases compared with 14.5% in controls. These frequencies were in agreement with those predicted by Hardy–Weinberg equilibrium.

Compared with T^{786} homozygotes, the odds ratio (OR) for CAD associated with the CC^{786} genotype was 2.5 ($P < 0.01$), whereas TC^{786} carriers had a slightly but not significantly increased risk of CAD (Table 4). Moreover, multivariate analysis showed that the CC^{786} genotype was an independent risk factor for CAD (Table 5).

We also investigated a possible synergistic effect between the $Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ polymorphisms of the eNOS gene (Table 6). When we evaluated the risk associated with the eNOS $T^{786}\rightarrow C$ variant alone, we found that among carriers of the Glu allele of the $Glu^{298}\rightarrow Asp$ polymorphism, individuals with at least one

Table 1. Demographic and clinical characteristics of the study population.

	CAD cases (n = 268)	Controls (n = 147)	*P*
Mean (SE) age, years	60.8 (0.6)	53.5 (1.2)	<0.0001
Male sex, n (%)	228 (85.1)	73 (49.7)	<0.0001
Hypertension, n (%)	162 (60.4)	37 (25.2)	<0.0001
Diabetes, n (%)	60 (22.5)	7 (4.8)	<0.0001
Dyslipidemia, n (%)	175 (65.3)	26 (17.7)	<0.0001
Smoking status			
Never smoked, n (%)	109 (40.7)	102 (69.4)	<0.0001
Ex-smokers, n (%)	107 (39.9)	29 (19.7)	
Current smokers, n (%)	52 (19.4)	16 (10.9)	
Family history of CAD, n (%)	117 (43.7)	28 (19)	<0.0001
Number of diseased vessels			
One vessel, n	102		
Two vessels, n	92		
Three vessels, n	74		

Table 2. Relationship between the $Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ polymorphisms of the eNOS gene.[a]

	$T^{786}\rightarrow C$ genotype			
$Glu^{298}\rightarrow Asp$ genotype	TT	TC	CC	Total
Glu/Glu	72	72	12	156
Glu/Asp	24	108	33	165
Asp/Asp	5	15	33	53
Total	101	195	78	374

[a] $P < 0.0001$ for all.

Table 3. Genotype and allele frequencies of $Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ polymorphisms of the eNOS gene in angiographically defined CAD cases and controls.

	CAD cases	Controls	*P*
$Glu^{298}\rightarrow Asp$ polymorphism			0.03
Glu/Glu, n (%)	109 (40.7)	65 (44.2)	
Glu/Asp, n (%)	116 (43.3)	72 (49)	
Asp/Asp, n (%)	43 (16.0)	10 (6.8)	
Total	268	147	
Allele			0.06
Glu, n (%)	334 (62.3)	202 (68.7)	
Asp, n (%)	202 (37.7)	92 (31.3)	
Total	536	294	
$T^{786}\rightarrow C$ polymorphism			0.02
TT, n (%)	54 (22.9)	47 (34.1)	
TC, n (%)	124 (52.5)	71 (51.4)	
CC, n (%)	58 (24.6)	20 (14.5)	
Total	236	138	
Allele			<0.01
T, n (%)	232 (49)	165 (60)	
C, n (%)	240 (51)	111 (40)	
Total	472	276	

Table 4. OR for CAD among carriers of the $T^{786}\rightarrow C$ and $Glu^{298}\rightarrow Asp$ variants.

Genotype	Reference group	OR (95% CI)[a]	P
CC	TT	2.5 (1.3–4.8)	<0.01
TC	TT	1.5 (0.9–2.4)	0.13
CC + TC	TT	1.7 (1.1–2.8)	0.02
Asp/Asp	Glu/Glu+Glu/Asp	2.6 (1.3–5.4)	<0.01

[a]CI, confidence interval.

C^{786} allele were at higher risk of CAD than carriers of the TT genotype in the promoter region of the eNOS gene (OR = 1.6; $P = 0.05$). Moreover, compared with individuals who were carriers of the Glu allele and TT genotype simultaneously, the OR for CAD associated with the presence of both the Asp/Asp genotype of the $Glu^{298}\rightarrow Asp$ polymorphism and at least one C allele of the $T^{786}\rightarrow C$ polymorphism was 4.0 ($P < 0.001$). Furthermore, we found that among individuals homozygous for the T^{786} allele of the promoter variant of the eNOS gene, the presence of Asp/Asp genotype conferred an increased risk for CAD (OR = 3.6), although it failed to reach statistical significance, probably because of the small number of carriers of Asp/Asp plus TT (n = 5; Table 2). In fact, for carriers of the C^{786} allele of the eNOS promoter polymorphism, the OR for CAD associated with the Asp/Asp genotype of the $Glu^{298}\rightarrow Asp$ polymorphism was significantly increased compared with those individuals with at least one Glu allele (OR = 2.2; $P = 0.03$).

$Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ polymorphisms of the eNOS gene and severity of CAD

The relationships between several variables and the $Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ genotypes were studied in all participants. We found no association between both eNOS polymorphisms and sex, hypertension, dyslipidemia, diabetes, smoking status, and family history of CAD (data not shown).

Of note was that the presence of the eNOS $T^{786}\rightarrow C$ allele was significantly associated with the extent and severity of CAD evaluated by means of the Duke scoring system (39.6 ± 2.9, 38.1 ± 2.2, and 27.3 ± 2.8 for CC^{786}, TC^{786}, and TT^{786}, respectively; $P = 0.004$; Fig. 1).

Moreover, as we have shown previously *(9)*, the mean Duke score was higher for individuals homozygous for the Asp/Asp genotype of the $Glu^{298}\rightarrow Asp$ polymorphism than for Glu/Glu and Glu/Asp carriers (44.7 ± 3.5 for Asp/Asp vs 34.5 ± 1.5 for Glu allele carriers; $P = 0.01$).

We also evaluated the potential interaction between both eNOS gene polymorphisms and the extent and severity of CAD in the total population (Fig. 2). Among carriers of the Glu allele for the $Glu^{298}\rightarrow Asp$ polymorphism, individuals with at least one C allele of the eNOS $T^{786}\rightarrow C$ variant had a significantly higher mean CAD Duke score than individuals with the TT genotype (37.2 ± 1.9 vs 26.2 ± 2.9; $P = 0.02$). Moreover, the severity of CAD was significantly associated with the presence of both the Asp/Asp genotype of the $Glu^{298}\rightarrow Asp$ polymorphism and at least one C allele of the $T^{786}\rightarrow C$ polymorphism (Duke score, 45.2 ± 3.7 vs 26.2 ± 2.9 for Asp/Asp genotype and C allele compared with Glu allele and TT genotype, respectively; $P = 0.002$). However, among carriers of the C allele of the $T^{786}\rightarrow C$ variant, those who were also homozygous for the Asp allele of the $Glu^{298}\rightarrow Asp$ polymorphism showed a slightly but not significant increase in the severity of CAD with respect to Glu allele carriers (Duke score, 45.2 ± 3.7 vs 37.2 ± 1.9; $P = 0.1$).

Table 5. Relative risk of CAD by coronary risk factors and by $T^{786}\rightarrow C$ polymorphism of the eNOS gene.

Risk factors	Relative risk (95% CI)[a]	P
Age	1.0 (1.0–1.1)	0.97
Male sex	4.0 (1.4–11.2)	<0.01
Smoke	3.3 (0.9–12)	0.06
Hypertension	4.5 (1.7–11.9)	<0.01
Diabetes	8.6 (1.5–47.8)	<0.01
Dyslipidemia	6.1 (2.4–15.6)	<0.0001
Family history of CAD	2.6 (1.0–7.1)	0.05
CC vs TT genotypes	2.7 (1.0–7.0)	0.04

[a]CI, confidence interval.

Discussion

This study investigated the relationship between the eNOS $Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ polymorphisms and the presence and severity of CAD and searched for potential interactions between these gene variations and the risk of CAD.

distributions of the $Glu^{298}\rightarrow Asp$ and $T^{786}\rightarrow C$ variants of the eNOS gene

According to our previous report, the $Glu^{298}\rightarrow Asp$ polymorphism in exon 7 of the eNOS gene is significantly associated with the presence of CAD *(9)*. Moreover, we reported the association between the common $T^{786}\rightarrow C$ polymorphism in the 5′-flanking region of the eNOS gene and the occurrence of CAD in the Italian population. Indeed, we found an excess of homozygosity for the C^{786} variant among CAD cases compared with controls (24.6% vs 14.5%), and these genotype frequencies were in agreement with those recently reported by Ghilardi et al. *(19)* among Italian individuals with moderate to severe internal carotid artery stenosis and healthy controls. The risk of developing CAD was 2.5-fold higher for C^{786} homozygotes with respect to individuals who were homozygous for the T^{786} allele in the eNOS gene promoter. Multivariate analysis demonstrated that this association was independent of other factors possibly related to CAD risk.

genetic linkage between the $T^{786}\rightarrow C$ and $Glu^{298}\rightarrow Asp$ polymorphisms of the eNOS gene

Among many polymorphisms of the eNOS gene, it has become clear that the intron 4b/a, the $Glu^{298}\rightarrow Asp$, and

Table 6. OR for CAD among combination of the Glu^{298}→Asp and T^{786}→C variants.

Genotypes	Reference group	OR (95% CI)[a]	*P*
(Glu/Glu or Glu/Asp)*(CC or TC)	(Glu/Glu or Glu/Asp)*TT	1.6 (1–2.6)	0.05
Asp/Asp*(CC or TC)	(Glu/Glu or Glu/Asp)*TT	4.0 (1.7–9.1)	<0.001
Asp/Asp*TT	(Glu/Glu or Glu/Asp)*TT	3.6 (0.4–33.4)	0.21
Asp/Asp*(CC or TC)	(Glu/Glu or Glu/Asp)*(CC or TC)	2.2 (1–4.7)	0.03

[a]CI, confidence interval.

the T^{786}→C variants have important implications in cardiovascular diseases *(5, 6, 13, 20, 21)*. The intronic polymorphism, which has been reported to be involved in a smoking-dependent risk for CAD, is less likely to have a functional role per se than either the promoter or coding region variants, but it may act as a marker for potentially functional variants elsewhere in the gene *(20)*. Indeed, Yoshimura et al. *(22)* first reported that the intron 4b/a polymorphism is in linkage disequilibrium with the eNOS T^{786}→C variant, suggesting that the T^{786}→C mutation underlies the functional characteristics of the intron 4a allele, whereas they found no relationship between the Glu^{298}→Asp and T^{786}→C variants. In contrast, Alvarez et al. *(23)* failed to confirm this previously described genetic linkage; they found that the CC^{786} genotype conferred an increased risk of an early episode of CAD in a Caucasian population but that the 4b/a polymorphism did not. Furthermore, another association study between genetic polymorphisms of the eNOS gene and hypertension in Japanese has recently shown that the T^{786}→C polymorphism is significantly linked to the Glu^{298}→Asp polymorphism with *trans* configuration (the T^{786} allele is linked to the Asp^{298} and the C^{786} allele to the Glu^{298}) *(24)*.

In this study we found that the Asp^{298} allele is weakly but significantly linked to the C^{786} allele of the T^{786}→C polymorphism in the 5′-flanking region of the eNOS gene; this evidence is in agreement with that recently reported in another European population in terms of both extent of disequilibrium and allele frequency *(25)*.

Functional significance of the T^{786}→C and Glu^{298}→Asp polymorphisms of the eNOS gene

We and others have previously reported that the risk for CAD is confined to individuals homozygous for the Asp allele of the Glu^{298}→Asp polymorphism, suggesting that homozygosity for aspartic acid in position 298 could produce a significant decrease in the amount of eNOS or its enzymatic activity *(6, 9)*. Indeed, it has recently been shown that the eNOS Asp^{298} protein has enhanced susceptibility to intracellular proteolytic cleavage compared with the eNOS Glu^{298} protein *(26)*. Another possible explanation for the association of the Glu^{298}→Asp polymorphism with the risk of CAD is that the Asp^{298} allele is in linkage disequilibrium with other functional variants within the eNOS or another gene.

In the present study, we found that the Asp^{298} allele is in linkage disequilibrium with the C^{786} variant in the promoter region of the eNOS gene; it is not known, however, whether the T^{786}→C polymorphism underlies the functional characteristic of the Asp^{298} allele. Indeed, in our population, carriers of both Asp/Asp and at least one C^{786} allele were at higher risk of CAD than carriers of single genotypes, suggesting the possibility of a synergistic effect between the Glu^{298}→Asp and T^{786}→C polymorphisms of the eNOS gene on CAD risk.

The T^{786}→C mutation in the 5′-flanking region of the eNOS gene was originally reported by Nakayama et al. *(13)* in patients with coronary vasospasm. Importantly, they found that the T^{786}→C mutation decreased promoter activity by ≈50%, suggesting that in many carriers of the mutant allele, the L-arginine/NO pathway does not function properly, leading to endothelial dysfunction.

It is well accepted that endothelial dysfunction occurs in response to cardiovascular risk factors and precedes the development of atherosclerosis *(27, 28)*. Thus, it is possi-

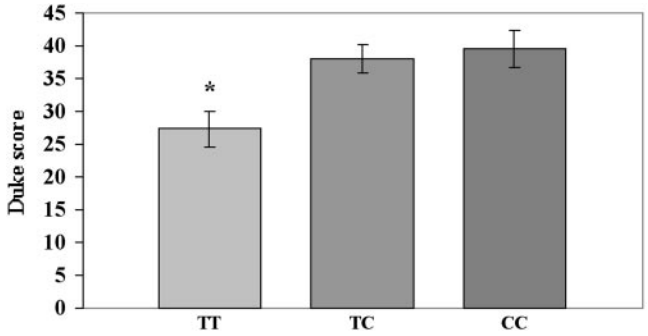

Fig. 1. T^{786}→C polymorphism of eNOS gene and severity of CAD.
*, *P* = 0.004, TT vs TC or CC genotype. *Error bars*, SE.

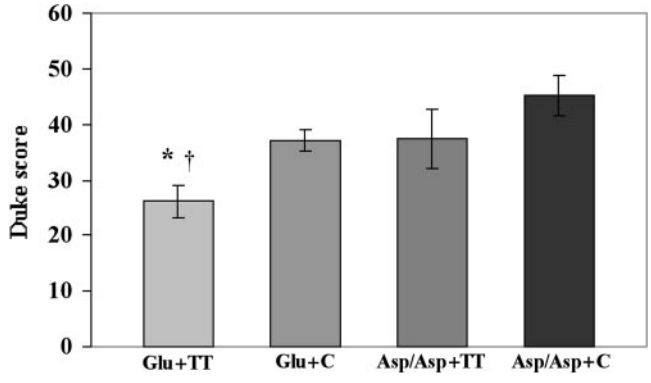

Fig. 2. Severity of CAD in individuals with different combinations of the eNOS T^{786}→C and Glu^{298}→Asp polymorphisms.
*, *P* = 0.02 vs simultaneous carriers of Glu and C alleles; †, *P* = 0.002 vs simultaneous carriers of Asp/Asp genotype and C allele. *Error bars*, SE.

ble that the T^{786}→C mutation in the 5′-flanking region of the eNOS gene, which affects promoter activity and endothelial synthesis of NO, and the Glu^{298}→Asp polymorphism may make carriers susceptible to the development of endothelial dysfunction and in turn to CAD *(29)*.

Moreover, it is noteworthy that we observed an association between the T^{786}→C polymorphism and the Duke scoring system, a prognostic index that considers not only the number of stenosed vessels but also the percentage of narrowing of the major vessels and the anatomical localization of the stenosis. Consequently, our data suggested the possibility that in the process of atherosclerotic remodeling of adult human vessels, alterations in NO production resulting from the C^{786} defect in the promoter region of the eNOS gene could have great impact on smooth muscle cell migration and proliferation. This hypothesis should be tested but is supported by in vivo evidence that eNOS-mutant mice displayed a paradoxical increase in wall thickness, accompanied by a hyperplastic response of the arterial wall in response to carotid artery ligation, indicating that a primary defect in the NOS/NO pathway may promote abnormal remodeling and pathologic changes in vessel wall morphology associated with atherosclerosis *(30)*.

In conclusion, the present study provides evidence that the Glu^{298}→Asp and T^{786}→C polymorphisms of the eNOS gene are associated with the presence, extent, and severity of angiographically defined CAD in the Italian population and that those individuals with both eNOS polymorphisms simultaneously might have a higher risk of developing CAD. However, it has recently been reported that neither Glu^{298}→Asp nor T^{786}→C polymorphisms significantly influenced plasma nitrate/nitrite concentrations and the risk of ischemic heart disease in a large cohort of middle-aged British men *(25)*. Therefore, further studies are needed to investigate whether the Glu^{298}→Asp and T^{786}→C polymorphisms of the eNOS gene could represent useful genetic markers for identifying individuals at risk for the development of CAD. In particular, it is necessary to explore and clarify the putative effects of the Glu^{298}→Asp and T^{786}→C polymorphisms both alone and in combination on the endothelial NO function and their significance in terms of risk for atherosclerosis-related disease in different populations. Finally, prospective studies are needed to determine how predictive the eNOS variants are for new onset of CAD.

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