The T−786C and Glu298Asp Polymorphisms of the Endothelial Nitric Oxide Gene Affect the Forearm Blood Flow Responses of Caucasian Hypertensive Patients

Gian Paolo Rossi, MD, FACC, FAHA,* Stefano Taddei, MD,† Agostino Virdis, MD,† Martina Cavallin, BIOLD,* Lorenzo Ghiadoni, MD,† Stefania Favilla, BIOLD,† Daniele Versari, MD,† Isabella Sudano, MD,† Achille C. Pessina, MD, PhD,* Antonio Salvetti, MD†

Padova and Pisa, Italy

OBJECTIVES

We sought to investigate whether two polymorphisms located in the promoter (T−786C) and exon 7 (Glu298Asp) of the endothelial nitric oxide (NO) synthase (eNOS) gene affected agonists-mediated NO release.

BACKGROUND

Endothelial dysfunction can be genetically determined. Therefore, we investigated whether two polymorphisms located in the eNOS gene affected agonists-mediated NO release.

METHODS

We compared endothelial-dependent and -independent vasodilation of the different eNOS genotypes in a cross-sectional study on 187 subjects, of whom 137 were uncomplicated essential hypertensive patients (PH) (49 ± 9 years, 151 ± 11/99 ± 5 mm Hg) and 50 healthy normotensive subjects (NT) (45 ± 16 years, 123 ± 10/78 ± 7 mm Hg). Endothelial-dependent and -independent vasodilation was assessed as the forearm blood flow response to incrementally increasing doses of acetylcholine (0.15, 0.45, 1.5, 4.5, 15 μg/100 ml/min) and sodium nitroprusside (1, 2, 4 μg/100 ml/min), respectively. Genotyping was performed with melting curve analysis (Lightcycler) of polymerase chain reaction products from acceptor (5′ end-labeled with LCRed 640) and donor probes (3′ end-labeled with fluorescein) specific for each polymorphism.

RESULTS

The genotype distribution of T−786C (CC = 21.9%, CT = 48.7%, TT = 29.4%) and Glu298Asp (GG = 39.0%, GT =51.9%, TT = 9.1%) was similar in PH and NT. A repeated measure analysis of variance showed a blunting of endothelium-dependent vasodilation in PH compared with NT (p < 0.001). A significant effect of the T−786C (p = 0.002) but not of the Glu298Asp (p = NS) eNOS polymorphism on endothelial-dependent vasodilation was found. However, we also detected a significant interaction between the T−786C and Glu298Asp polymorphism (p < 0.001). No effect on either polymorphism on endothelial-independent vasodilation was seen.

CONCLUSIONS

The T−786C promoter polymorphism and its interaction with exon 7 Glu298Asp affect endothelium-dependent vasodilation in mild-to-moderate PH patients and NT Caucasian subjects. (J Am Coll Cardiol 2003;41:938–45) © 2003 by the American College of Cardiology Foundation

Primary (essential) hypertension (PH) is a highly prevalent heterogeneous disease that is genetically determined, albeit in a complex fashion. Genetic factors may account for 30% to 40% of blood pressure (BP) variance, but the identification of genetic determinants of PH has not been accomplished thus far (1), despite intensive investigative efforts. The development of the concept of the “intermediate phenotype,” a physiological feature that allows stratifying the heterogeneous population of PH patients into homogeneous subsets, has been a major advancement in this field. However, the identification of intermediate phenotypes proved to be substantially more difficult than are the genetic studies and, therefore, the progress has been slow.

A substantial proportion of PH patients show an impaired endothelium-dependent vasodilation (EDV) (2), which occurs also in other conditions associated with accelerated atherosclerosis, such as cigarette smoking, diabetes mellitus, hypercholesterolemia, hyperhomocyst(e) nemia, and aging. Furthermore, a blunted EDV can predict cardiovascular events independently of the common risk factors (3) and, therefore, might be an intermediate phenotype of PH and by at large of cardiovascular disease. Of interest, a blunted EDV was found also in normotensive offspring of hypertensive parents, therefore, it appears to be genetically determined, albeit through unknown mechanisms (4).

Nitric oxide (NO), a major mediator of EDV, is formed...
in endothelial cells from l-arginine through the action of the homodimeric enzyme endothelial nitric oxide synthase (eNOS). The eNOS gene (5) is a likely candidate of endothelial dysfunction because it undergoes both expression and functional regulation through multiple regulatory steps, including the Ca\(^{2+}\)/calmodulin regulation and tyrosine phosphorylation (6) and comprises several polymorphisms (7) that have been linked to cardiovascular phenotypes (8–14). The latter includes PH and its complications (11,12,14–16), although the association with PH remains controversial (8,9). One of these polymorphisms was found to be associated with coronary spasm (10), but none has been linked to an impaired agonists-stimulated availability of NO so far (7).

The promoter region that is important for the regulation of the eNOS gene transcription rate harbors three novel polymorphisms, which were always linked with each other (17). Of note, the T\(^{–786}\)→C allele implied a blunted transcription rate of the gene in response to hypoxia and was seen in 15% of Japanese patients with vasospastic angina and in 3% of control subjects (p < 0.0001) (17). Thus, it was suggested to be a major determinant of an altered coronary vasomotor reactivity and possibly of an impaired EDV (17). However, this hypothesis has not been prospectively investigated so far, and, although the mutant allele was found to be far more common in Caucasian than in Japanese individuals (18) (Rossi et al. study in this issue of the Journal), there is no information on its impact on EDV in humans.

Another polymorphism, the G→T transversion at nucleotide position 894 (G\(^{894}\)T), results in a GAG to GAT substitution in exon 7 and, thus, in the replacement of glutamate by aspartate (Glu298Asp). This might impair the function of eNOS and, thereby, account for the association of this polymorphism with coronary spasm in Japanese patients (10).

Thus, within a large prospective collaborative project aimed at identifying the genetic determinants of endothelial dysfunction, we investigated if the T\(^{–786}\)C and the Glu298Asp polymorphisms affected EDV in uncomplicated PH patients and normotensive (NT) subjects.

**METHODS**

**Design and methods.** The criteria for enrolment of the PH patients and NT subjects were reported in detail elsewhere (19). A total of 137 PH and 48 healthy NT volunteers consented to participate in this study, which was approved by the medical ethics committees of our universities. Exclusion criteria were hypercholesterolemia, diabetes mellitus, cardiac and/or cerebral ischemic vascular disease, impaired renal function, and other major pathologies. Subjects were defined as NT if they had no family history of PH and BP values consistently <140/90 mm Hg. The vast majority (>80%) of PH patients had never been treated, or reported a history of discontinued (for at least six months) or ineffective pharmacological treatment. Secondary forms of hypertension were excluded by routine diagnostic procedures. Pharmacologic treatment was withdrawn at least two weeks before performing the study.

**Experimental procedure.** Endothelial function was assessed by the perfused forearm blood flow (FBF) measurements, as described (19). Briefly, the brachial artery was cannulated for drug infusion at systemically ineffective rates, while intra-arterial BP and heart rate were monitored; FBF was measured in both forearms by strain-gauge venous plethysmography (19). Circulation to the hand was excluded 1 min before FBF measurement by inflating a pediatric cuff around the wrist at suprasystolic BP. All details of our methodology have already been published (19).

Endothelium-dependent vasodilation was estimated by performing a dose-response curve to intra-arterial acetylcholine (ACH) (cumulative increase of the infusion rates: 0.15, 0.45, 1.5, 4.5, 15 μg/100 ml forearm tissue/min, for 5 min at each dose) while endothelium-independent vasodilation (EIV) was assessed with intra-arterial infusion of sodium nitroprusside (SNP) (cumulative increase by 1, 2, and 4 μg/100 ml forearm tissue/min, for 5 min at each dose). These rates were selected to induce vasodilation comparable to that obtained with ACH. The ACH or SNP infusion sequence was randomized; 30-min washout was allowed between each dose-response curve. These procedures were carried out in the Department of Internal Medicine of the University of Pisa.

**Extraction of deoxyribonucleic acid (DNA) and eNOS genotyping.** The blood was collected in ethylenediaminetetraacetic acid and stored at –20°C until DNA was extracted according to standard procedures, and quantified by spectrophotometer. For each polymorphism we designed four oligonucleotides: two serving as amplification primers and two as fluorescence resonance energy transfer probes. These probes contiguously hybridize to an internal sequence of the amplified fragment during the annealing phase of polymerase chain reaction (PCR). When this occurs, energy fluorescence transfer (by resonance) from one (donor) to the other (acceptor) probe generates fluorescence. The acceptor probe was labeled at the 5’-end with a red fluorophore (LCRed640), while the donor probe at the 3’-end with

---

**Abbreviations and Acronyms**

- ACH = acetylcholine
- ANOVA = analysis of variance
- BP = blood pressure
- DNA = deoxyribonucleic acid
- EDV = endothelium-dependent vasodilation
- EIV = endothelium-independent vasodilation
- eNOS = endothelial nitric oxide synthase
- FBF = forearm blood flow
- NO = nitric oxide
- NT = normotensive
- PCR = polymerase chain reaction
- PH = primary (essential) hypertension
- SNP = sodium nitroprusside

---
Figure 1. Representative results of melting curve analysis of the polymerase chain reaction products from subjects who were genotyped for the T-786C (upper) and the Glu298Asp (lower) polymorphism. An unequivocal identification of the different genotypes was consistently accomplished with a 100% accuracy versus sequencing (see text for details).
Table 1. Primer Pairs and Probes for T-786C and Glu298Asp Polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer Pairs Sense/Antisense</th>
<th>Probes Donor/Acceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-786C</td>
<td>5'-gCCgTCgTAACgACgACgAC</td>
<td>CCATgCTCCCCACCAaggCATgACgAC X</td>
</tr>
<tr>
<td></td>
<td>5'-gCCgTCAATgTCgCAggACgAC</td>
<td>LC Red640-5TCCCCACCAaggCATgACgAC p</td>
</tr>
<tr>
<td></td>
<td>5'-gCCgTCACggACgACgACgACgAC</td>
<td>CCAaggCATgACgACgACgACgACgAC</td>
</tr>
<tr>
<td>Glu298Asp</td>
<td>5'-CCATgACgATgTCgCAggACgAC</td>
<td>LC Red640-5TCCCCACCAaggCATgACgAC</td>
</tr>
<tr>
<td></td>
<td>5'-CCATgACgATgTCgCAggACgAC</td>
<td>LC Red640-5TCCCCACCAaggCATgACgAC</td>
</tr>
</tbody>
</table>

Fluorescein. Probe/DNA hybrids that contain a mismatch melt at lower temperatures than perfectly matched hybrids, thus resulting in a different melting temperature (Tm) during melting curve analysis (see the following text). Hence, distinction of wild type, mutant, and heterozygous genotype can be easily accomplished by differences in their respective melting temperature (Fig. 1).

Amplification primers and fluorescent probes are shown in Table 1. The PCR reaction mixture contained of Lightcycler DNA Master Hybridization Probes 10× (Roche Diagnostics, Milan, Italy) to which primers (0.3 and 0.5 μM) and probes (0.2 and 0.4 μM) for the Glu298Asp and T-786C polymorphisms, respectively, were added.

The cycling program entailed a denaturation step (95°C for 2 min) followed by 50 cycles for both the Glu298Asp (95°C, 0 s; 63°C, 12 s; 72°C, 10 s) and the T-786C (95°C, 2 s; 60°C, 10 s; 72°C, 10 s) polymorphism, respectively. For the analysis of the melting curves at the end of PCR, temperature was raised to 95°C, lowered to 45°C, and then slowly raised to 85°C to allow monitoring of the decline of fluorescence generated by melting of the hybrids, as a function of temperature. Melting curves were automatically converted to fluorescence peaks, thus allowing distinction of genotypes.

Statistical analysis. Results are expressed as mean ± SD; SEM was used in Figure 2 for visual clarity. A regression analysis was used to identify predictors of FBF responses to ACH or SNP; useful predictors were then entered in a model for repeated measures analysis of variance (ANOVA) to adjust the FBF responses for comparison of genotypes. The use of a multivariate test (Hotelling’s trace and Roy’s largest root) to detect significant effects was decided a priori because it does not require the sphericity assumption, that is, the variance-covariance matrix of the dependent variables should be circular in form. Interaction terms between eNOS genotypes, gender, and hypertension were also used in this model. One-way ANOVA followed by Bonferroni post-hoc test was used to locate the origin for any significant difference in the adjusted maximal FBF response to ACH and SNP between groups and genotypes. Statistical significance was set at p < 0.05. All analyses were carried out with the SPSS for Windows statistical package (version 10.0, SPSS Inc., Chicago, Illinois).

RESULTS

Demographic characteristics and eNOS genotype distribution. The demographic and clinical characteristics of the PH patients and NT subjects are shown in Table 2. There were significantly more nonsmokers and smokers and less ex-smokers in PH patients than in NT subjects; however, most cigarette smokers reported to smoke <5 cigarettes per day. Besides the obvious differences of BP and left ventricular mass index, the PH patients were older; more commonly of male gender; and had significantly higher heart rate, body mass index, uric acid, glucose, triglycerides, and total cholesterol and lower high-density lipoprotein cholesterol compared with the NT subjects. They did not differ for serum creatinine, Na+, K+, supine plasma renin activity, and aldosterone and for resting baseline FBF.

Figure 1 shows exemplary results of the genotyping for the T-786C and Glu298Asp polymorphisms with melting curve analysis. We compared this technique with sequencing and found it to be 100% accurate versus for the genotyping of these and other polymorphisms.

The genotype distribution was CC = 21.9%, CT = 48.7%, and TT = 29.4% for the T-786C polymorphism; it was similar in PH and NT and consistent with the Hardy-Weinberg equilibrium, the overall proportion of the T-786C T and C allele of 0.54 and 0.46, respectively. For the Glu298Asp polymorphism, the genotype distribution was GG = 39.0%, GT = 51.9%, and TT = 9.1%; the overall proportion of the G and T allele was 0.65 and 0.35, respectively, and, therefore, it was skewed toward the G allele. However, the genotype distribution was similar in PH and NT. For both polymorphisms, there were no significant differences between genotypes for all the biometric variables.

EDV and EIV. There were no significant differences in baseline FBF of the ACH and of the SNP study between PH and NT (Table 2), possibly because of the larger number of smokers among control than hypertensive patients; likewise, there were no significant differences between different genotypes. Acetylcholine induced a significant increase of FBF in both PH patients and NT subjects (Fig. 2), but this was blunted in the former, compared with the latter. At the maximum dosage of ACH, the FBF was 15.04 ± 5.29 ml/min/100 ml versus 19.54 ± 6.36 (p < 0.001) in PH and NT, respectively. We found a significant effect of age (p = 0.013), hypertension (p = 0.019), and the T-786C eNOS genotype (p = 0.002) on FBF responses to ACH. A significant effect of an interaction between T-786C and Glu298Asp eNOS genotypes (p < 0.001) on EDV was also evident. These genotype effects remained significant after adjusting FBF responses for age and hypertension.

Sodium nitroprusside significantly increased FBF in both PH patients and NT subjects, but there was no difference...
between them at any dosages of SNP. No significant effect of either eNOS genotypes and of their interaction on EIV was detected at repeated measures ANOVA (not shown).

When the maximal FBF responses were analyzed in the PH and NT subgroups, there was a highly significant higher EDV, but not EIV, in PH with the T-786C TT genotype as compared with both CC (+10%, p = 0.003) and CT (+13%, p < 0.0001) (Table 3). No such difference among subjects with the different Glu298Asp genotypes was found.

**DISCUSSION**

We assessed the EDV and EIV in a relatively large population of uncomplicated mild-to-moderate PH, most of whom had never been previously treated, and in a control group of healthy NT subjects. All were carefully genotyped for two eNOS polymorphisms (Fig. 1) chosen, among others, because they were found to have functional consequences. Our results confirmed previous reports of signifi-
showed that the T-786C affects the response to ACH in human beings. We identified some genetic determinants of the impaired EDV showing that these eNOS polymorphisms affected EDV, we compared with NT subjects (2). More importantly, by significantly blunted FBF responses to ACH in PH patients –JACC Vol. 41, No. 6, 2003
Rossi et al.
Results are expressed as mean ± SD.
ACH = acetylcholine; BMI = body mass index; BP = blood pressure; HDL = high-density lipoprotein; LDL = low-density lipoprotein; LVMI = left ventricular mass index; PRA = plasma renin activity; SNP = sodium nitroprusside.

Effect of the T-786C polymorphism. Reporter gene studies showed that the T-786C substitution in the promoter region of eNOS reduced by 50% the rate of transcription of eNOS, both under baseline conditions and in response to hypoxia (17), and was associated with decreased serum levels of nitrite/nitrate (20). These effects might depend upon the fact the mutant allele can bind the replication protein A1, which acts as a gene repressor protein (20). This substitution was shown to be associated with coronary spasm (17) and with enhanced coronary vasostriction to ACH and impaired EIV (21) in Japanese patients. However, there was no information on the relevance of this polymorphism in non-Japanese populations and its impact on EDV. Our results filled this gap by showing that the T-786C affects the FBF response to ACH in Caucasian patients with mild-to-moderate PH and in NT controls. Subjects who were homozygous for the T allele of the T-786C polymorphism exhibited a significantly higher increase of FBF in response to ACH than CT and CC subjects, although we could not detect a clear-cut allele-dose effect. We found no evidence for an effect of this polymorphism on donated (SNP) NO responses, thus suggesting that the T-786C substitution has either a modest or a negligible impact on structural remodeling and/or on cyclic guanosine monophosphate-mediated pathways in this population.

**Effect of the Glu298Asp (G394T) polymorphism.** Glutamate and aspartate are deemed to be conservative replacements. Accordingly, this polymorphism could be a marker for a functional locus elsewhere in the gene. In keeping with this contention, enzymatic studies of recombinant eNOS showed no discernible difference in the Michaelis constant or the limiting velocity between the Asp298 and Glu298 form of the enzyme (7). Thus, no effect of the Glu298Asp polymorphism on hand veins and FBF responses to ACH was found in healthy subjects with and

### Table 2. Clinical Characteristics of Essential Hypertensive Patients and Control Subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Primary Hypertensives (n = 137)</th>
<th>Controls (n = 48)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>49 ± 9</td>
<td>43 ± 16</td>
<td>0.001</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>115 (84%)/22 (16%)</td>
<td>31 (62%)/19 (38%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Nonsmokers/smokers/ex-smokers (%)</td>
<td>61/20/19</td>
<td>15/85/0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.4 ± 2.7</td>
<td>23.8 ± 3.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>87 ± 15</td>
<td>85 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>Uricemia (mmol/l)</td>
<td>5.5 ± 1.1</td>
<td>4.9 ± 1.1</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum K⁺ (mmol/l)</td>
<td>4.0 ± 0.3</td>
<td>4.0 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Na⁺ (mmol/l)</td>
<td>141 ± 2</td>
<td>141 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Supine PRA (ng Ang I/ml/h)</td>
<td>1.43 ± 1.30</td>
<td>1.51 ± 1.32</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma aldosterone (pmol/l)</td>
<td>24.5 ± 17.0</td>
<td>27.5 ± 12.5</td>
<td>NS</td>
</tr>
<tr>
<td>Na⁺ × V (mmol/24 h)</td>
<td>121 ± 67</td>
<td>115 ± 79</td>
<td>NS</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>67 ± 5</td>
<td>65 ± 4</td>
<td>0.032</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>151 ± 11</td>
<td>123 ± 10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>99 ± 5</td>
<td>78 ± 7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean BP (mm Hg)</td>
<td>116 ± 6</td>
<td>93 ± 7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Glycemia (mmol/l)</td>
<td>5.16 ± 0.58</td>
<td>4.94 ± 0.63</td>
<td>0.044</td>
</tr>
<tr>
<td>LVMI (g/m² BSA)</td>
<td>119.3 ± 19.4</td>
<td>92.7 ± 10.6</td>
<td>0.001</td>
</tr>
<tr>
<td>T-cholesterol (mg/dl)</td>
<td>215.4 ± 28.7</td>
<td>192.6 ± 32.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>47.6 ± 11.4</td>
<td>54.9 ± 14.0</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>139.1 ± 30.0</td>
<td>118.7 ± 29.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>147.3 ± 60.6</td>
<td>111.7 ± 58.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Baseline FBF (ACH, ml/100 g/min)</td>
<td>3.07 ± 0.60</td>
<td>3.13 ± 0.623</td>
<td>NS</td>
</tr>
<tr>
<td>Baseline FBF (SNP, ml/100 g/min)</td>
<td>3.14 ± 0.61</td>
<td>3.24 ± 0.58</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.
ACH = acetylcholine; BMI = body mass index; BP = blood pressure; HDL = high-density lipoprotein; LDL = low-density lipoprotein; LVMI = left ventricular mass index; PRA = plasma renin activity; SNP = sodium nitroprusside.

### Table 3. Maximal FBF Responses to ACH and SNP in the PH and NT Control Subjects Classified by T-786C Genotype

<table>
<thead>
<tr>
<th>Maximal FBF Response (% of Baseline)</th>
<th>CC (n = 30)</th>
<th>CT (n = 69)</th>
<th>TT (n = 38)</th>
<th>CC (n = 10)</th>
<th>CT (n = 22)</th>
<th>TT (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACH</td>
<td>392 ± 63</td>
<td>378 ± 52</td>
<td>437 ± 50†</td>
<td>572 ± 44‡</td>
<td>524 ± 32‡</td>
<td>516 ± 36‡</td>
</tr>
<tr>
<td>SNP</td>
<td>373 ± 59</td>
<td>405 ± 38</td>
<td>383 ± 38</td>
<td>456 ± 68‡</td>
<td>439 ± 23‡</td>
<td>474 ± 56‡</td>
</tr>
</tbody>
</table>

*p < 0.0001 vs. CT; †p = 0.003 vs. CC; ‡p < 0.0001 vs. PH patients with the same genotype.
ACH = acetylcholine; FBF = forearm blood flow; NT = normotensive; PH = essential hypertensive patients; SNP = sodium nitroprusside.
without hypercholesterolemia (22,23). However, it was also reported that the pressor response to phenylephrine was significantly higher in humans carrying the T allele (TT and TG, Asp298) than in the GG homozygous, thus suggesting an effect of this polymorphism on vascular reactivity (24).

Furthermore, a vasomotor dysfunction related to an increased microvascular resting tone in the left anterior descending coronary artery territory in subjects carrying the Asp298 allele was described (25). Of further note, pregnant women homozygous for this allele showed a blunted flow-mediated vasodilation of the brachial artery at 12-week gestation, as compared with Glu298 homozygous (26). This might be due to the fact that eNOS with Asp298, but not with Glu298, is susceptible to inactivation by cleavage (27), possibly because of a tighter turn of the alpha-helix. Previous studies on the impact of this polymorphism on EDV were equally distributed between those yielding negative (22,23) or positive results (25,26). Our study, which had a larger sample size and, therefore, a higher statistical power to detect a significant effect of this polymorphism on EDV, showed that the effect of Glu298Asp on EDV was weak and by itself did not reach statistical significance. In addition, we could not detect any effect of this polymorphism on EIV. Nonetheless, we found evidence for an interaction, in statistical terms, of the Glu298Asp with the T-786C polymorphism. This interaction, which is unlikely to be due to a linkage disequilibrium of the two polymorphisms (28), might occur at the transcriptional or post-transcriptional level, but its precise nature remains to be investigated.

**Differential effect of the T-786C and Glu298Asp polymorphisms in PH patients and NT subjects.** A subgroup analysis of the PH patients and the NT subjects revealed that the impact of the T allele of the T-786C polymorphism was due to the PH patients, who exhibited a blunted EDV compared with the NT subjects. Thus, it would appear that a favorable or unfavorable T-786C genotype might reveal itself mainly under conditions of impaired NO bioavailability. However, as we could not investigate a larger number of healthy NT subjects, we cannot rule out the alternative explanation that our study was underpowered to detect a genotype effect in this latter population. It has to be acknowledged also that the significant effect of the TT genotype on EDV entailed an 11% increase of the vasodilatory response to the maximal dose of ACH, that is, was rather small. However, it has been shown that, in PH patients, the NO-mediated EDV is blunted because of an impaired bioavailability of NO caused by oxidative stress, and that an alternative ouabain-sensitive pathway, possibly involving activation of an endothelium–derived hyperpolarizing factor, mainly accounts for the EDV response of the forearm (29). Therefore, the small contribution of eNOS gene variation to EDV variance is not unexpected in these patients.

**Study limitations.** This study comprised exclusively Caucasian individuals who had neither hypercholesterolemia nor diabetes mellitus; furthermore, only a minority of them were cigarette smokers. Additionally, only the forearm circulation with only one EDV stimulus (ACH) was investigated, and, therefore, our findings might apply neither to other race and risk groups nor to other vascular districts and EDV stimuli. However, it has to be acknowledged that the same (T-786C) polymorphism associated with an impaired EDV in this study was linked to altered coronary responses to ACH in Japanese patients (21). Furthermore, estrogens are known to enhance NO availability, a fact that might partially account for protection of fertile women from cardiovascular events. Because the vast majority of our study population was men, this study does not clarify if the T-786C and Glu298Asp eNOS polymorphisms similarly affect EDV in both genders. Finally, controversy exists concerning the association of each eNOS variant with hypertension (30). Our study was not designed to address this issue and, therefore, its statistical power does not warrant any conclusion in this regard.

**Conclusions.** The present results indicate that the T-786C polymorphism of the promoter region of the eNOS gene affects EDV in PH patients and NT subjects. Thus, they support the contention that variations in the eNOS gene affect NO-mediated endothelial pathways and might, in the long run, affect atherogenesis and cardiovascular damage.

Reprint requests and correspondence: Prof. Gian Paolo Rossi, Department of Clinical and Experimental Medicine, University Hospital, via Giustiniani, 2, 35126 Padova, Italy. E-mail: gianpaolo.rossi@unipd.it.

**REFERENCES**

2. Linder L, Kiowski W, Bühler FR, Luscher TF. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation with only one EDV stimulus (ACH) was investigated, and, therefore, our findings might apply neither to other race and risk groups nor to other vascular districts and EDV stimuli. However, it has to be acknowledged that the same (T-786C) polymorphism associated with an impaired EDV in this study was linked to altered coronary responses to ACH in Japanese patients (21). Furthermore, estrogens are known to enhance NO availability, a fact that might partially account for protection of fertile women from cardiovascular events. Because the vast majority of our study population was men, this study does not clarify if the T-786C and Glu298Asp eNOS polymorphisms similarly affect EDV in both genders. Finally, controversy exists concerning the association of each eNOS variant with hypertension (30). Our study was not designed to address this issue and, therefore, its statistical power does not warrant any conclusion in this regard.

**Conclusions.** The present results indicate that the T-786C polymorphism of the promoter region of the eNOS gene affects EDV in PH patients and NT subjects. Thus, they support the contention that variations in the eNOS gene affect NO-mediated endothelial pathways and might, in the long run, affect atherogenesis and cardiovascular damage.

Reprint requests and correspondence: Prof. Gian Paolo Rossi, Department of Clinical and Experimental Medicine, University Hospital, via Giustiniani, 2, 35126 Padova, Italy. E-mail: gianpaolo.rossi@unipd.it.

**REFERENCES**

2. Linder L, Kiowski W, Bühler FR, Luscher TF. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation with only one EDV stimulus (ACH) was investigated, and, therefore, our findings might apply neither to other race and risk groups nor to other vascular districts and EDV stimuli. However, it has to be acknowledged that the same (T-786C) polymorphism associated with an impaired EDV in this study was linked to altered coronary responses to ACH in Japanese patients (21). Furthermore, estrogens are known to enhance NO availability, a fact that might partially account for protection of fertile women from cardiovascular events. Because the vast majority of our study population was men, this study does not clarify if the T-786C and Glu298Asp eNOS polymorphisms similarly affect EDV in both genders. Finally, controversy exists concerning the association of each eNOS variant with hypertension (30). Our study was not designed to address this issue and, therefore, its statistical power does not warrant any conclusion in this regard.

**Conclusions.** The present results indicate that the T-786C polymorphism of the promoter region of the eNOS gene affects EDV in PH patients and NT subjects. Thus, they support the contention that variations in the eNOS gene affect NO-mediated endothelial pathways and might, in the long run, affect atherogenesis and cardiovascular damage.

Reprint requests and correspondence: Prof. Gian Paolo Rossi, Department of Clinical and Experimental Medicine, University Hospital, via Giustiniani, 2, 35126 Padova, Italy. E-mail: gianpaolo.rossi@unipd.it.


23. Grossmann M, Dobrev D, Sifert W, Kirch W. Heterogeneity in hand vein responses to acetylcholine is not associated with polymorphisms in the G-protein β1 subunit (C825T) and endothelial nitric oxide synthase (G894T) genes but with serum low density lipoprotein cholesterol. Pharmacogenetics 2001;11:307–16.


