Hyperhomocysteinemia, Endothelial Nitric Oxide Synthase Polymorphism, and Risk of Coronary Artery Disease

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Background: Hyperhomocysteinemia is an independent, graded risk factor for coronary artery disease (CAD). The G894T variant of endothelial nitric oxide synthase (eNOS) was postulated to be associated with hyperhomocysteinemia and could influence individual susceptibility to CAD. The aims of this study were to investigate (a) the relationship of the eNOS G894T polymorphism with the presence and the severity of CAD and (b) the possible relationship between hyperhomocysteinemia and the eNOS G894T variant for the risk of CAD severity in a Tunisian population.

Methods: We used PCR with restriction fragment length polymorphism analysis to detect the G894T variant of the eNOS gene in 100 patients with CAD and 120 healthy controls. The severity of CAD was expressed by the number of affected vessels. Total plasma homocysteine concentrations were determined by direct chemiluminescence assay.

Results: The frequencies of the eNOS GG, GT, and TT genotypes in the CAD group were significantly different from those in the control group (45%, 44%, and 11% vs 60%, 35.8% and 4.2%, respectively; P = 0.035). There was no association between the eNOS G894T genotype frequencies and the number of stenosed vessels (P = 0.149). In the CAD group, the coexistence of the 894 GT or TT genotypes and hyperhomocysteinemia led to an increased risk of CAD severity.

Conclusion: The G894T polymorphism of the eNOS gene is associated with the presence of CAD, and in conjunction with hyperhomocysteinemia, increased the risk of CAD severity in a Tunisian population.

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Epidemiologic studies have shown that dyslipidemia, diabetes mellitus, obesity, hypertension, and cigarette smoking are risk factors for coronary artery disease (CAD)6 (1–3). Assessment of these metabolic or lifestyle risk factors has, however, been ineffective in completely predicting the development of the atherosclerotic process, suggesting that specific genetic predisposition should also be taken into account (4, 5).

The vascular endothelium modulates blood vessel wall homeostasis through the production of factors regulating vessel tone, coagulation state, cell growth, cell death, and leukocyte trafficking (6). One of the most important endothelial cell products is nitric oxide (NO), which is synthesized from l-arginine by the enzyme endothelial nitric oxide synthase (eNOS) (7). 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 oxidation of the atherogenic LDLs (8). NO may modulate homocysteine (Hcy) concentration directly by inhibiting methionine synthase, the enzyme that synthesizes methionine from homocysteine and 5-methyltetrahydrofolate (9). Alternatively, NO may modulate Hcy concentrations indirectly via folate catabolism by inhibiting the synthesis of ferritin (10), a protein that promotes the irreversible oxidative cleavage of folate (11). Although

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Nonstandard abbreviations: CAD, coronary artery disease; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; and Hcy, homocysteine.
low folate concentrations are associated with hyperhomocysteinemia (12), which is a risk factor for atherosclerosis (13, 14), the relative contributions of these potential mechanisms to Hcy modulation in vivo remain unclear.

eNOS inhibition has also been shown to accelerate atherosclerosis in animal models, and abnormalities of the endothelial NO pathway are present in humans with atherosclerosis (15, 16). This evidence suggests that NO may inhibit several key steps in the atherosclerotic process and that alteration of NO production within the vascular endothelium could contribute to the pathogenesis of atherosclerosis. Thus, eNOS could be a candidate gene for atherosclerosis.

Several polymorphisms have been identified in the eNOS gene, among which is one located in exon 7 (G984T), which modifies its coding sequence (Glu298Asp). Associations between this variant and coronary spasm, CAD, and acute myocardial infarction have been reported, but data on its relationship with disease severity are lacking (17, 18). Here we describe the association between the G984T polymorphism of the eNOS gene and the presence and severity of CAD; we also evaluate the relationship between hyperhomocysteinemia, the eNOS G894T variant, and the risk of CAD severity in the Tunisian population.

Materials and Methods

STUDY POPULATION

The study population consisted of homogeneous Tunisian Arab descendents who resided in Tunisia and had no known Negroid or Mongoloid ancestry. The control group included 120 healthy volunteers (87 males) with no history of CAD, diabetes mellitus, or cerebrovascular diseases. Their mean (SD) age was 54 (10) years.

One hundred consecutive patients (74 males) with angiographically documented CAD were enrolled from University Hospital Fattouma Bourguiba in Monastir (Cardiovascular Department). The mean age of this group was 59 (10) years. The number of significantly stenosed coronary arteries and lesions determined the severity of CAD (>50% luminal stenosis). The angiograms were assessed by 2 cardiologists who were unaware that the patients were to be included in the study and enabled patient subclassification as follows: group G0 (10%) had no stenosed vessels, group G1 (40%) had 1 stenosed vessel, group G2 (35%) had 2 stenosed vessels, and group G3 (15%) had severe CAD involving all 3 major coronary arteries.

All participants were interviewed, and data on dyslipidemia, diabetes mellitus, hypertension, and smoking habits were recorded. Informed consent was obtained from each patient and control 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 mellitus or those receiving any antidiabetic medication were considered to have diabetes; individuals were deemed dyslipidemic when their total cholesterol concentration was ≥5.68 mmol/L, their triglyceride concentration was ≥2.28 mmol/L, or they were receiving lipid-lowering drugs. Smoking history was coded as never or current smoker.

MEASUREMENT OF TOTAL HCY

Plasma concentrations of total Hcy were measured by direct chemiluminescence using reagents and an ACS:180 automated analyzer from Bayer Vital GmbH.

ANALYSIS OF G894T POLYMORPHISM OF eNOS GENE

Genomic DNA was extracted from whole blood samples by rapid methods (19). The coding sequence variant was a G→T substitution at position 894 in exon 7, which determines the Glu-to-Asp amino acid substitution (in codon 298) in the mature eNOS protein. Using a previously described procedure (18), we genotyped all participants by PCR amplification of exon 7 with the primers 5′-CATGAGGCTCAGGGCCAGAAC-3′ (sense) and 5′-AGTCAATTCCCTTGTGCTCAC-3′ (antisense) followed by MboI restriction enzyme digestion for 16 h at 37 °C. In the presence of a T at nucleotide 894, which corresponds to Asp298, the 206-bp PCR product is cleaved into 2 fragments of 119 and 87 bp. The products of the digestion process were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7%).

STATISTICAL ANALYSES

Statistical analyses were performed with Statistical Package for Social Sciences for Windows, Ver. 10.0 (SPSS). Differences between the means of the 2 continuous variables were evaluated by Student t-test. Differences between noncontinuous variables, genotype distribution, and Hardy–Weinberg equilibrium were tested by χ2 analysis. One-way ANOVA was used to analyze the relationships between genotypes and the general characteristic and severity of CAD. Logistic regression analysis was used to assess the independent effect of each risk factor on the presence of CAD. Hyperhomocysteinemia was defined as a mildly increased Hcy (fasting total Hcy >15 μmol/L). Results are reported as the median and 25th and 75th percentiles, and P <0.05 was considered statistically significant.

Results

COMPARISON OF THE 2 STUDY GROUPS

The demographic and clinical characteristics of the CAD and control groups are given in Table 1. The prevalence of atherogenic risk factors (including age, sex, hypertension, diabetes mellitus, and dyslipidemia) was significantly higher in the CAD group. Total Hcy was significantly higher in the CAD group than in the control group.
distribution of the G894T polymorphism of the eNOS gene
Although the distribution of genotypes in both the CAD and control groups satisfied the Hardy–Weinberg equilibrium, the G894T polymorphism of the eNOS gene was significantly associated with the presence of CAD in our patients (Table 2). The proportion of TT homozygotes was 11% in the CAD group and 4.2% in the control group ($P/0.035$).

G894T polymorphism of the eNOS gene and severity of CAD
Patients with CAD ($n = 100$) were subclassified into 4 subgroups (G0, G1, G2, and G3) according to the number of affected coronary arteries (Table 3). Statistically, the G894T polymorphism did not correlate with the extent of CAD ($P = 0.149$), but it tended to suggest a strong association with CAD severity. We found increased proportions of patients with the 894T allele presenting with 0- to 3-vessel stenosis ($P = 0.056$).

ASSOCIATION BETWEEN eNOS G894T GENOTYPE AND Hcy CONCENTRATIONS IN STUDY POPULATION
The eNOS G894T genotype was significantly associated with Hcy in the CAD group (Table 4). The eNOS 984TT genotype was associated with increased Hcy in our patients ($P < 0.001$). However, in the control group, the eNOS G894T genotype was not significantly associated with Hcy concentrations, probably because of the small number of individuals with the TT genotype ($n = 5$). Table 4 also shows the Hcy concentrations in patients presenting with 0- to 3-vessel stenosis according to eNOS gene polymorphism. We observed that patients with GT or TT genotypes and increased Hcy had more severe CAD. Interestingly, the coexistence of the 894GT or 894TT genotype and increased Hcy led to increased risk of CAD severity.

RESULTS OF MULTIVARIATE ANALYSIS
We used multiple logistic regression to test for independent correlates of the presence of CAD. Included in the model were age, sex, smoking, hypertension, diabetes mellitus, dyslipidemia, and eNOS G894T polymorphism. Age ($P < 0.001$), sex ($P < 0.001$), smoking ($P = 0.031$), hypertension ($P = 0.018$), diabetes mellitus ($P = 0.01$), and dyslipidemia ($P = 0.026$) were independent correlates of the presence of CAD, whereas the eNOS G894T polymorphism ($P = 0.209$) was not an independent predictor of CAD.

Discussion
We report an association between the common G894T polymorphism of the eNOS gene and the presence of CAD in the Tunisian population. We found an excess of homozygosity for the T894 variant among CAD cases compared with controls. Multivariate analysis showed that this association was not independent of other factors related to artery disease risk.

Methylenetetrahydrofolate reductase and apolipoprotein E polymorphisms have been investigated as CAD risk

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### Table 2. Genotype and allele frequencies of the G894T polymorphism of the eNOS gene in CAD and control groups.

<table>
<thead>
<tr>
<th></th>
<th>CAD group</th>
<th>Control group</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>100</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>G894T polymorphism, n (%)</td>
<td></td>
<td></td>
<td>0.035</td>
</tr>
<tr>
<td>GG</td>
<td>45 (45)</td>
<td>72 (60)</td>
<td></td>
</tr>
<tr>
<td>GT</td>
<td>44 (44)</td>
<td>43 (35.8)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>11 (11)</td>
<td>5 (4.2)</td>
<td></td>
</tr>
<tr>
<td>Allele, n (%)</td>
<td></td>
<td></td>
<td>0.026</td>
</tr>
<tr>
<td>G</td>
<td>134 (67)</td>
<td>187 (78)</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>66 (33)</td>
<td>53 (22)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Genotype and allele frequencies of G894T polymorphism of the eNOS gene and CAD severity.

<table>
<thead>
<tr>
<th></th>
<th>CAD group ($n = 100$)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>G894T polymorphism, n (%)</td>
<td>$G_0$ ($n = 10$)</td>
<td>$G_1$ ($n = 40$)</td>
<td>$G_2$ ($n = 35$)</td>
<td>$G_3$ ($n = 15$)</td>
<td>$P$</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>7 (70)</td>
<td>21 (52.5)</td>
<td>14 (40)</td>
<td>3 (20)</td>
<td>0.149</td>
<td></td>
</tr>
<tr>
<td>GT</td>
<td>2 (20)</td>
<td>15 (37.5)</td>
<td>16 (45.7)</td>
<td>11 (73.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>1 (10)</td>
<td>4 (10)</td>
<td>5 (14.3)</td>
<td>1 (6.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.056</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>16 (80)</td>
<td>57 (71.3)</td>
<td>44 (62.8)</td>
<td>17 (56.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>4 (20)</td>
<td>23 (28.7)</td>
<td>26 (37.2)</td>
<td>13 (43.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
factors (20). Gardemann et al. (21) showed that the TT genotype of the methylenetetrahydrofolate reductase C677T gene polymorphism is associated with extent of coronary atherosclerosis in patients at high risk for CAD.

To date, the G894T polymorphism of the eNOS gene has been linked to increased risk of stroke, myocardial infarction, coronary atherosclerosis, and venous thrombosis (22–25). Previous studies from Japan and the United Kingdom have already suggested a role for the G894T polymorphism in the development of coronary atherosclerosis, with the excess risk being confined to TT894 homozygosity (18, 26, 27), as in our study. These studies, however, also showed that the genotype frequency of the G894T polymorphism can vary considerably among different populations. Nevertheless, it is important to emphasize that some authors have failed to find any relationship between the G894T polymorphism and the risk of atherosclerosis (28–31).

In our study we investigated the association between the common G894T polymorphism of the eNOS gene and the severity of CAD. We found a strong, but not statistically significant, association. Controversial results regarding this association have been reported. Colombo and coworkers (32, 33) showed that the G894T polymorphism of the eNOS gene is associated with the severity of CAD, but authors of other studies found no association and no differences in the progression of atherosclerosis (34, 35).

In our study we investigated whether a relationship between the eNOS G894T variant and increased Hcy concentrations increased the risk of CAD severity. This relationship had not been described previously. Heil et al. (25) showed that the G894T variant of eNOS increases the risk of recurrent venous thrombosis through interaction with increased Hcy concentrations. Brown et al. (36) postulated that the eNOS G894T variant influences plasma Hcy concentrations via folate catabolism. Our data suggest that the coexistence of the 894GT or 894TT genotype and hyperhomocysteinemia is associated with the number of vessels affected and increases the risk of CAD severity. Although this study was a preliminary study, these results are very interesting and could stimulate larger studies.

Several studies have demonstrated that the bioavailability of NO is decreased in hyperhomocysteinemia (37, 38), which might be attributable to decreased NO production or to alternative mechanisms such oxidative stress or nitrosylation (37–39). NO can react with thiols such as Hcy to form S-nitrosothiols, which have vasodilatory and antiplatelet effects and are more stable than NO (40). Stamler et al. (40) hypothesized that under physiologic conditions, endothelial cells may modulate the deleterious effects of Hcy by releasing NO, which facilitates the formation of S-nitrosohomocysteine. When less NO is available, decreased amounts of S-nitrosohomocysteine will be formed, which would lead to exposure

| Table 4. Association between eNOS G894T genotype and Hcy concentrations in control and CAD groups. |
|---|---|---|---|
| Controls | GG | GT | TT |
| n | 72 | 43 | 5 |
| Hcy, μmol/L | 9.2 (8–12.5) | 0.9 (3.2–14) | 11.6 (9–15.3) |
| CAD | 44 | 45 | 11 |
| n | 11.47 (10–14.1) | 14.13 (10.7–20) | 20.3 (14.5–32) |
| Severity of CAD | 0 vessels | 1 vessel | 2 vessels | 3 vessels |
| n | 7 | 2 | 1 |
| Hcy, μmol/L | 10 (8.7–11) | 12.8 (10–15.8) | 10.55 |
| n | 21 | 15 | 4 |
| Hcy, μmol/L | 11.6 (9.9–14) | 13.6 (9.6–22.3) | 14.13 (11.6–18) |
| Severity of CAD | 3 vessels | 0 vessels | 1 vessel | 2 vessels |
| n | 3 | 11 | 1 |
| Hcy, μmol/L | 13.5 (10.3–13.9) | 19.2 (16.8–39.8) | 35.65 |

a Hcy concentrations are the median (25th–75th percentile).

b Significantly different from patients or controls with GG genotype (P < 0.05).

c Significantly different from patients with GT genotype presenting with 1-vessel stenosis (P < 0.05).

d Significantly different from patients with TT genotype presenting with 1-vessel stenosis (P < 0.01).

e Significantly different from patients with GT genotype presenting with 2-vessel stenosis (P < 0.05) and patients with GT genotype presenting with 1-vessel stenosis (P < 0.01).
of the homeostatic system to the detrimental effects of Hcy (40).

The eNOS G894T variant leads to an amino acid substitution of aspartate for glutamate at position 298 of the protein. An in vitro study showed that the eNOS protein with aspartate, but not glutamate, at position 298 is subject to cleavage by endogenous protease and produces 35-kDa amino-terminal and 100-kDa carboxyl-terminal fragments (41). The “degraded” 100-kDa eNOS is only a small fraction of the total. Nevertheless, Tesauro et al. (41) found significant potential structural changes in the Chou–Fasman secondary structure and pointed out that the coding region polymorphism has functional consequences.

We therefore speculate that, in vivo, the eNOS 894TT genotype leads to altered NO production. In combination with the presence of hyperhomocysteinemia, this might lead to less capturing of Hcy in S-nitrosohomocysteine, with subsequent exposure of the homeostatic system to the toxic effects of Hcy. We hypothesize that interaction of the eNOS G894T variant and hyperhomocysteinemia induces less S-nitrosylation and could be one mechanism determining the extent of obstruction in CAD.

In conclusion, the present study provides evidence that the G894T polymorphism of the eNOS gene is associated with the presence of CAD in the Tunisian population. The G894T polymorphism could represent a useful genetic marker to identify individuals prone to the development of atherosclerotic diseases. We found a relationship between hyperhomocysteinemia, the eNOS G894T variant, and the risk of CAD severity, which can be increased by the number of arteries stenosed. Further studies are needed to investigate the relationship of hyperhomocysteinemia associated with the eNOS polymorphism and the extent of obstructive CAD.

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References


