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# Genetic Polymorphism on Endothelial Nitric Oxide Synthase Affects Endothelial Activation and Inflammatory Response During the Acute Phase of Myocardial Infarction

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**OBJECTIVES** This study sought to evaluate the effect of genetic polymorphism G894T on endothelial nitric oxide synthase (eNOS); on the risk for myocardial infarction (MI); and on the release of von Willebrand factor (vWF), interleukin (IL)-6, IL-1b, and oxidized low-density lipoprotein (ox-LDL) levels during the acute phase of MI and one year after the event.

**BACKGROUND** Genetic polymorphism G894T on eNOS has been associated with increased cardiovascular risk. However, its role during the acute phase of MI is unknown.

**METHODS** The study population consisted of 228 patients with a first event of premature MI and 519 matched control patients. One year after the event, 61 patients and 205 control patients were recalled for the follow-up study. Blood sampling was performed during the acute phase and after one year.

**RESULTS** The risk for MI in 894TT was 1.992 (95% confidence interval [CI], 1.131 to 3.485), $p < 0.05$ versus GG+GT; 2.038 (95% CI, 1.125 to 3.695), $p < 0.05$ versus GG; and 2.009 (95% CI, 1.106 to 3.651), $p < 0.05$ versus GT. During the acute phase, vWF was higher in GT+TT (121.02 ± 5.47%) versus GG (84.6 ± 7.1%, $p < 0.01$), an effect persisting after one year (90.4 ± 3.8 vs. 73.1 ± 4.6%, $p < 0.01$). During the acute phase, GT+TT had higher ox-LDL and IL-6 (131.2 ± 6.4 IU/l and 8.5 ± 0.7 pg/ml) compared with GG (101.7 ± 9.64 IU/l and 6.2 ± 0.8 pg/ml, $p < 0.05$ for both), but no difference was found at one year.

**CONCLUSIONS** G894T polymorphism on the eNOS gene increases the risk for premature MI and modifies the response of vascular endothelium during the acute phase of MI by affecting the release of vWF, IL-6, and oxidative stress status, an effect diminished one year after the event. (J Am Coll Cardiol 2005;46:1101–9) © 2005 by the American College of Cardiology Foundation

Endothelium plays an important role in vascular homeostasis by producing a number of biochemical mediators with vasodilatory or vasoconstrictive properties (1). Endothelium-derived nitric oxide (NO) produced by vascular endothelial cells is a key molecule regulating vascular tone that also has antiplatelet, anti-inflammatory, and antioxidant properties (1).

Previous studies have shown that the genetic polymorphism G894T on the endothelial nitric oxide synthase (eNOS) gene, which leads to a Glu298Asp substitution on eNOS, may affect the response of vascular endothelium to increased oxidative stress in healthy smokers (2), whereas it has also been associated with the development of myocardial infarction (MI) in several populations (3). However, the underlying molecular mechanisms of this association are controversial because the experimental models used until now have failed to explain these clinical observations (4).

In the present study, we evaluated the effect of the G894T polymorphism on the risk for premature MI, and we examined whether this polymorphism affects the release of von Willebrand factor (vWF), oxidative stress status, and proinflammatory cytokines levels under different conditions of vascular activation during the acute phase of premature MI and one year after the event in the same patients as well as in patients with no evidence of atherosclerosis.

## METHODS

**Study population.** This study enrolled a total of 747 patients: 228 patients of both sexes with a first event of premature MI, and 519 age- and gender-matched control patients. Patients 49 years old or less, consecutively admitted to our coronary care unit with a first event of ST-segment elevation MI were recruited. The diagnosis of ST-segment elevation MI was defined according to guidelines (5). All patients underwent thrombolysis and received the same standard therapy, including aspirin, low-molecular-weight heparin, statins, intravenous nitrates, and angiotensin-converting enzyme inhibitors, as appropriate.

Control patients had no clinical evidence of coronary heart disease, stroke, or any atherosclerotic disease (based on a detailed medical history and physical examination followed by electrocardiogram). All patients and control pa-

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**Abbreviations and Acronyms**

CABG = coronary artery bypass grafting
eNOS = endothelial nitric oxide synthase
HDL = high-density lipoprotein
IL = interleukin
MI = myocardial infarction
NO = nitric oxide
ox-LDL = oxidized low-density lipoprotein
PCI = percutaneous coronary intervention
vWF = von Willebrand factor

tients were Greek Caucasians, inhabitants of the province of Athens in Greece. Demographic characteristics are presented in Table 1.

**Protocol.** The study was approved by our Institutional Ethics Committee, and informed consent was given by all of the participants. Blood samples were taken at the 24th h after admission for genotyping and for evaluation of serum levels of interleukin (IL)-6, IL-1b, oxidized low-density lipoprotein (ox-LDL), and plasma levels of vWF. All of the patients were followed up prospectively for one year, and a well-defined group of patients with a stable clinical condition for at least the previous six months was recalled for the follow-up study one year after the event. Exclusion criteria from the follow-up study were the existence of any inflammatory or infective disease, liver or renal disease, malignancy, heart failure defined as ejection fraction <45%, or a history of deep vein thrombosis or pulmonary embolism, and patients receiving non-steroidal anti-inflammatory drugs or anticoagulants, as well as any dietary supplements or antioxidant vitamins, were also excluded. To avoid possible influences of percutaneous coronary intervention (PCI), catheter-related acute-phase reactions, or coronary artery bypass grafting (CABG) operation on the measured parameters during the acute phase of MI or at the follow-up, patients requiring urgent PCI, coronary angiographic investigation, or CABG within the first 24 h of the event or <6 mo before the follow-up study were also excluded. Of the 228 MI patients in the first part of the study, 72 fulfilled the inclusion criteria for the follow-up study, and 61 of them agreed to participate. All patients were receiving standard medication, as appropriate.

The control group in the follow-up study consisted of 205 individuals selected from the initial study cohort to be matched for age, gender, and the major risk factors with the patient group. This design allows safer comparisons between the groups because it diminishes any effects of the underlying risk factors on the expression of the examined markers of inflammation, oxidative stress, and endothelial activation. None of the control patients had evidence of cardiovascular disease (such as coronary artery disease or stroke), and they all had normal electrocardiogram and exercise stress test results. The same general exclusion criteria used for patients were also applied to the control group. All of the participants at the follow-up study were asked to abstain from tobacco, alcohol, and caffeine-containing beverages during the evening before blood sampling, and they discontinued their medications for 12 h before the study. Venous blood samples were centrifuged at 3,500 rpm at 4°C for 15 min, and plasma or serum was collected and stored at −80°C until assayed.

**Biochemical measurements.** Routine chemical methods were used to determine serum concentrations of total cholesterol, high-density lipoprotein (HDL), and triglycerides. Enzyme-linked immunosorbent assays were used for the determination of plasma levels of vWF (Asserachrom, Diagnostica Stago, Asnières sur Seine, France), ox-LDL

**Table 1.** Demographic Characteristics and Genotype Distribution

| | MI Cases | Control Cases | p Value |
|---|---|---|---|
| No. of subjects | 228 | 519 | |
| Age (yrs) | 46.96 ± 0.40 | 47.2 ± 0.66 | 0.198 |
| Gender (male/female) | 213/15 | 468/51 | 0.164 |
| Current smokers/ex-smokers | 200/4 | 301/13 | 0.001 |
| Hypercholesterolemia, n (%) | 126 (55.2) | 178 (34.3) | 0.001 |
| Hypertension, n (%) | 86 (37.7) | 156 (30.1) | 0.038 |
| Diabetes mellitus, n (%) | 25 (10.9) | 54 (10.4) | 0.898 |
| Body mass index (kg/m$^2$) | 27.7 ± 0.25 | 26.9 ± 0.18 | 0.013 |
| Cholesterol levels (mg/dl) | 249.22 ± 3.52 | 220.93 ± 2.26 | 0.001 |
| HDL cholesterol (mg/dl) | 35.83 ± 0.65 | 47.92 ± 1.29 | 0.001 |
| Triglycerides (mg/dl)* | 133 (99–190) | 112 (75–150) | 0.001 |
| Fasting glucose (mg/dl) | 120.18 ± 9.47 | 95.31 ± 1.32 | 0.001 |
| Genotype distribution | | | |
| 894GG, n (%) | 97 (42.5) | 255 (49.1) | |
| 894GT, n (%) | 99 (43.5) | 217 (41.8) | |
| 894TT, n (%) | 32 (14.0) | 47 (9.1) | |
| Recessive model† | | | |
| 894TT, n (%) | 32 (14.0) | 47 (9.1) | 0.016† |
| 894GG+894GT, n (%) | 196 (86.0) | 472 (90.9) | |

Values expressed as means ± standard error of the mean. *Value expressed as median (25th to 75th percentile values). †p by chi-square analysis after adjustment for age, gender, and classic risk factors.
HDL = high-density lipoprotein; MI = myocardial infarction.

**Table 2.** Odds Ratios as Estimates of Relative Risk for Premature Myocardial Infarction in Carriers of the 894T Allele

| Genotypes | Crude Odds Ratios (95% CI) | p Value | Odds Ratios (95% CI)* | p Value |
|---|---|---|---|---|
| TT vs. GG+GT | 1.640 (1.015–2.647) | 0.043 | 1.992 (1.139–3.485) | 0.016 |
| TT vs. GG | 1.790 (1.079–2.970) | 0.024 | 2.038 (1.125–3.699) | 0.019 |
| TT vs. GT | 1.492 (0.898–2.481) | 0.123 | 2.009 (1.106–3.651) | 0.022 |
| GT vs. GG | 1.199 (0.859–1.674) | 0.285 | 1.157 (0.843–1.654) | 0.423 |

p values by chi-square analysis. *Adjusted for age, gender, and classic risk factors.
CI = confidence interval.

(Mercodia AB, Uppsala, Sweden), and IL-1b and IL-6 (R&D Systems Inc., Minneapolis, Minnesota).

**DNA extraction and genotyping.** Genomic DNA was extracted from 2 to 5 ml of whole blood using standard methods (QIAamp DNA blood kit, Qiagen GmbH, Hilden, Germany). For the detection of G894T polymorphism on the eNOS gene, we used primer pairs to amplify a part of the eNOS gene containing exon 7, by polymerase chain reaction with the following flanking intronic primers: sense 5′-CAT GAG GCT CAG CCC CAG AAC-3′ and antisense 5′-AGT CAA TCC CTT TGG TGC TCA C-3′, followed by Mbo I restriction endonuclease digestion for 16 h at 37°C and resolution by electrophoresis on 3% agarose gel. The resulting 206 bp polymerase chain reaction product was cleaved into two smaller fragments of 119 and 87 bp in the presence of a T nucleotide at 894 (corresponding to Asp 298) but not in its absence. Digested fragments were visualized after ethidium bromide staining under ultraviolet light.

**Statistical analysis.** We tested that the allele frequencies conformed to Hardy-Weinberg equilibrium proportions by use of the chi-square test. According to the applied power analysis, a total sample size of 250 patients (case and control patients) was adequate to detect a 15% difference in the frequency of 894T allele between groups, achieving statistical power 90% at $p = 0.05$ probability level. The number of both cases and control patients was, however, increased ($n = 747$) to permit further analyses in subgroups. Qualitative variables are presented as absolute and relative frequencies. Genotype and allele frequencies were compared between groups by chi-square analysis, and conditional multiple logistic regression analysis was used to estimate odds ratios and 95% confidence intervals (CIs) of the development of MI as a function of G894T polymorphism. All odds ratios were adjusted for age, gender, and atherosclerosis risk factors such as hypertension, diabetes mellitus, hypercholesterolemia, obesity, and smoking. Continuous variables were tested for normal distribution by the Kolmokorov-Smirnov test. Normally distributed variables are presented as mean values ± SEM, whereas non-normally distributed data were log-transformed for analysis and are presented in the non-logarithmic format as median (25th to 75th percentile values). Comparisons of continuous variables between patients and control patients or between the genotypes were performed by unpaired Student *t* test if normally distributed, and by the Mann-Whitney *U* test if non-normally distributed. The changes of each biochemical variable from baseline (acute phase) to the one-year follow-up value and the comparisons of these changes between the different genotype groups were assessed by analysis of variance for repeated measurements. Comparisons between control patients and patients during the acute phase or patients at follow-up were performed using analysis of variance for multiple comparisons using the Bonferroni correction for post-hoc analysis to adjust for multiple testing.

Stepwise multivariate analysis was undertaken with the research indexes (vWF, IL-6, IL-1b, and ox-LDL) as dependent variables and clinical factors (age, gender, hypertension, diabetes, hypertension, smoking, hypercholesterolemia, body mass index, cholesterol levels, triglycerides levels, HDL levels) and G894T polymorphism as independent variables. We included as independent variables in each multivariate model the eNOS polymorphism (GG and GT+TT) as well as those that showed a significant association with the dependent variables in univariate analysis at 15% significance level. A backward elimination procedure was applied in all multivariate models (using $p < 0.05$ as the threshold for removing a variable from the model).

All reported p values are based on two-sided tests and compared with a significance level of 5%; SPSS version 12.0 (SPSS Inc., Chicago, Illinois) software was used for all the statistical calculations.

## RESULTS

**Clinical characteristics and genotype distribution.** Population characteristics, serum lipid levels at baseline, and genotype frequencies are presented in Table 1. There was a significant difference in the prevalence of TT genotype in cases (32 or 14.0%) compared with control patients (47 or 9.1%, $p < 0.05$) (Table 1). The risk for MI in TT was significantly increased compared with all of the other genotypes (Table 2), and remained unchanged after adjustment for age, gender, and classic risk factors (Table 2).

The angiographic extent of coronary artery disease in patients, as well as demographic characteristics, genotype distribution, lipid levels, and medication of patients and control patients in the follow-up study, are presented in Table 3. There were no significant differences in genotype distribution or any of the risk factors between patients and control patients in the follow-up study (Table 3). In the patient group, 13 had a CABG operation and 27 had PCI at least 6 months before the follow-up study, whereas there

**Table 3.** Demographic Characteristics of the Follow-Up Study Population

| | MI Cases | Control Cases | p Value |
|---|---|---|---|
| No. of subjects | 61 | 205 | |
| Genotype (GG/GT/TT) | 20/31/10 | 94/89/22 | 0.157† |
| Age (yrs) | 48.60 ± 0.92 | 49.7 ± 0.93 | 0.479† |
| Gender (male/female) | 180/18 | 58/3 | 0.423† |
| Hypercholesterolemia, n (%) | 28 (45.9) | 72 (35.1) | 0.135† |
| Active smokers, n (%) | 25 (40.9) | 100 (48.8) | 0.241† |
| Hypertension, n (%) | 28 (45.9) | 83 (40.5) | 0.658† |
| Diabetes mellitus, n (%) | 9 (14.7) | 32 (15.6) | 0.844† |
| BMI (kg/m$^2$) | 27.21 ± 0.48 | 26.48 ± 0.26 | 0.203 |
| Cholesterol levels (mg/dl) | 185.3 ± 6.0 | 178.5 ± 2.17 | 0.294 |
| HDL cholesterol (mg/dl) | 43.5 ± 1.7 | 49.3 ± 2.5 | 0.226 |
| Triglycerides (mg/dl)* | 129 (96–158) | 113 (82–153) | 0.051 |
| Fasting glucose (mg/dl)* | 129 (82–153) | 129 (97–157) | 0.984 |
| Medication | | | |
| Nitrates, n (%) | 2 (3.2) | 0 (0) | 0.055† |
| Angiotensin receptor antagonists, n (%) | 14 (23) | 15 (7.3) | 0.002† |
| Angiotensin-converting enzyme inhibitors, n (%) | 39 (64) | 66 (32) | 0.001† |
| Diuretics, n (%) | 21 (34) | 48 (23) | 0.136† |
| Calcium channel inhibitors, n (%) | 7 (11) | 8 (4) | 0.001† |
| Beta-blockers, n (%) | 40 (66) | 14 (7) | 0.001† |
| Statins, n (%) | 38 (62) | 72 (35) | 0.001† |
| Anti-diabetic medication, n (%) | 9 (15) | 27 (13) | 0.834† |
| Aspirin, n (%) | 60 (98) | 33 (16) | 0.001† |
| Extent of CAD at baseline (vessels with >50% stenosis) | | | |
| 1 vessel | 33 (54) | | |
| 2 vessels | 17 (28) | | |
| 3 vessels | 11 (18) | | |
| CABG operation after the event, n (%) | 13 (21) | | |
| PCI after the event, n (%) | 27 (44) | | |

Values expressed as mean ± standard error of the mean. *Value expressed as median (25th to 75th percentile values). †p by chi-square analysis.
BMI = body mass index; CABG = coronary artery bypass grafting operation; CAD = coronary artery disease; HDL = high-density lipoprotein; MI = myocardial infarction; PCI = percutaneous coronary intervention.

was no difference in genotype distribution among those patients.

**The role of G894T polymorphism during the acute phase of MI.** EFFECTS ON THE RELEASE OF VWF. Plasma levels of vWF were slightly higher in TT+GT compared with GG ($p < 0.05$) in patients one year after the event, although no difference was observed between the genotypes in matched control patients (Fig. 1, Table 4). During the acute phase of MI, patients with TT+GT genotype had significantly higher levels of vWF compared with GG ($p < 0.01$, Fig. 1, Table 4), and although vWF levels were increased in both TT+GT (by 30.60% [95% CI, 17.91 to 43.29] $p < 0.01$) and GG (by 11.6% [95% CI, 3.02 to 20.10] $p < 0.05$), the increase in TT+GT was significantly greater than the increase in the GG genotype group ($p < 0.05$). The vWF levels in patients one year after the event were significantly higher compared with control patients only among TT+GT ($p < 0.01$), whereas no significant difference was observed between patients and control patients in the presence of the GG genotype (after adjustment for multiple comparisons (p = NS, Fig. 1A, Table 4).

EFFECTS ON OX-LDL LEVELS. There was no significant difference in ox-LDL levels between TT+GT and GG either in patients one year after the event or in control patients (Fig. 1B, Table 4). However, during the acute phase of MI, serum levels of ox-LDL were significantly higher in TT+GT compared with GG ($p < 0.05$) (Fig. 1B, Table 4). Levels of ox-LDL were significantly increased in both TT+GT (by 32.27 IU/l [95% CI, 19.20 to 45.34] $p < 0.01$) and GG (by 17.02 IU/l [95% CI, 4.04 to 30.03] $p < 0.05$) compared with resting levels, and the increase in TT+GT was slightly but not significantly greater compared with the increase in GG (p = NS). Serum levels of ox-LDL were significantly higher in patients during the acute phase of MI (in GT+TT and GG) or one year after the event (in GT+TT) compared with matched control patients after adjustment for multiple comparisons (Fig. 1B, Table 4).

EFFECTS ON PROINFLAMMATORY CYTOKINES. Serum levels of IL-6 were not significantly different between genotypes in patients one year after the event or in matched control patients, but IL-6 was significantly higher in TT+GT compared with GG during the acute phase of MI ($p < 0.01$) (Fig. 2A, Table 4). Serum levels of IL-6 were significantly increased in both TT+GT (by 4.76 pg/ml [95% CI, 3.74 to 5.77] $p < 0.01$) and GG (by 3.06 pg/ml [95% CI, 1.52 to 4.61] $p < 0.01$) during the acute phase of MI; however, although the increase in TT+GT group was slightly greater than the increase in GG group, this difference did not reach statistical significance (p = NS). Serum levels of IL-6 were significantly higher in patients during

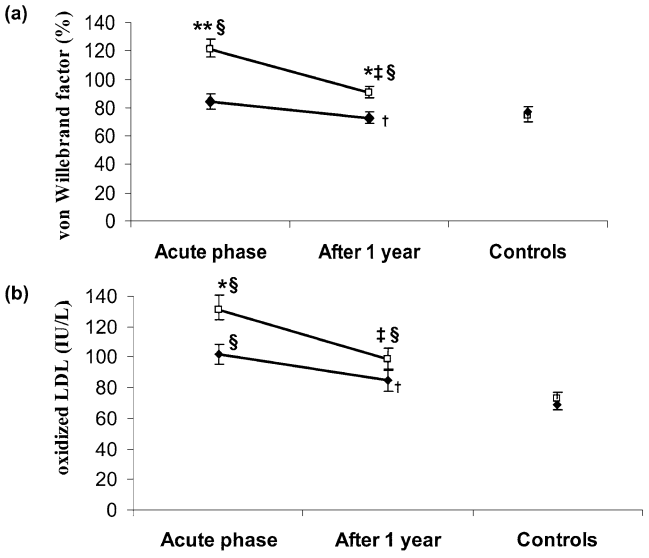


**Figure 1. (a)** Plasma levels of von Willebrand factor (vWF) were significantly higher during the acute phase of myocardial infarction in patients with GT+TT **(open squares)** compared with those with GG **(filled diamonds)** genotype ($p < 0.01$). One year after the event, plasma vWF was still higher in GT+TT compared with GG ($p < 0.05$), but there was no significant difference in vWF levels between genotypes in the control group ($p$ = NS). Values are expressed as mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. GG; †$p < 0.05$ and ‡$p < 0.01$ vs. acute phase; §$p < 0.05$ vs. matched control patients (adjusted according to Bonferoni). **(b)** Serum levels of oxidized LDL (ox-LDL) were significantly higher during the acute phase of myocardial infarction in patients with GT+TT **(open squares)** compared with those with GG **(filled diamonds)** genotype ($p < 0.05$). Serum oxidized low-density lipoprotein (LDL) was not significantly different in GT+TT compared with GG in patients one year after the event or in matched control patients ($p$ = NS). Values are expressed as mean ± SEM. $^{*}p < 0.05$ vs. GG; †$p < 0.05$ and ‡$p < 0.01$ vs. acute phase; $^{a}p < 0.05$ vs. matched control patients (adjusted according to Bonferroni).

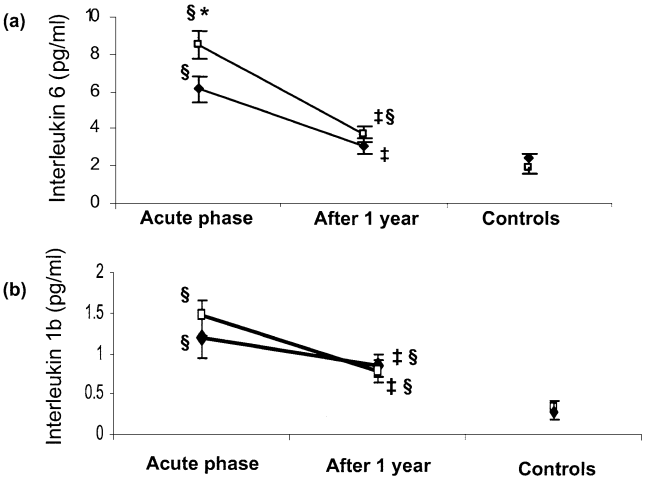


**Figure 2. (a)** Serum levels of interleukin (IL)-6 were significantly higher during the acute phase of myocardial infarction in patients with GT+TT **(open squares)** compared with those with GG **(filled diamonds)** genotype ($p < 0.05$). Serum IL-6 was not significantly different in GT+TT compared with GG in patients one year after the event or in control patients ($p$ = NS). Values are expressed as mean ± SEM. $^{*}p < 0.05$ vs. GG; ‡$p < 0.01$ vs. acute phase; §$p < 0.05$ vs. matched control patients (adjusted according to Bonferoni). **(b)** Serum levels of interleukin-1b (IL-1b) were significantly higher during the acute phase of myocardial infarction compared with one year later ($p < 0.01$ for all genotypes), but there was no significant difference between patients with GT+TT **(open squares)** and GG **(filled diamonds)** genotype ($p$ = NS). Serum IL-1b was not significantly different in GT+TT compared with GG in patients one year after the event or in matched control patients ($p$ = NS). Values are expressed as mean ± SEM. ‡$p < 0.01$ vs. acute phase; $^{a}p < 0.01$ vs. matched control patients (adjusted according to Bonferroni).

the acute phase of MI (in GT+TT and GG) or one year after the event (in GT+TT) compared with matched control patients after adjustment for multiple comparisons (Fig. 2A, Table 4).

Serum levels of IL-1b were not significantly different between genotypes in patients one year after the event or in matched control patients (Fig. 2B, Table 4). During the acute phase of MI, IL-1b levels were significantly increased in both TT+GT (by 0.673 pg/ml [95% CI, 0.342 to 1.003] $p < 0.01$) and GG (by 0.341 pg/ml [95% CI, 0.123 to 0.560] $p < 0.01$) compared with resting levels after one year, whereas the increase in TT+GT was slightly but not significantly greater compared with the increase in GG.

**Table 4.** Effects of G894T Polymorphism on eNOS Gene on the Inflammatory Process, Endothelial Cell Injury, and Oxidative Stress in the Acute Phase of Premature Myocardial Infarction, One Year After the Event and in Matched Control Patients in the Follow-Up Study

| | | Patients (n = 61) | | |
|---|---|---|---|---|
| | Genotype | Acute Phase of MI | Annual Follow-Up (Stable) | Control Patients (n = 205) |
| von Willebrand factor (%) | All | 109.09 ± 4.84‖ | 84.73 ± 3.13§‖ | 75.76 ± 2.22 |
| | GT+TT | 121.02 ± 5.47†‖ | 90.42 ± 3.79†§‖ | 74.30 ± 3.01 |
| | GG | 84.62 ± 7.05 | 73.06 ± 4.63‡ | 77.50 ± 3.21 |
| ox-LDL (IU/l) | All | 121.5 ± 5.58‖ | 94.2 ± 5.13§‖ | 71.5 ± 1.88 |
| | GT+TT | 131.21 ± 6.39*‖ | 98.90 ± 6.8§‖ | 72.36 ± 2.67 |
| | GG | 101.70 ± 9.64‖ | 84.64 ± 6.73‡ | 70.40 ± 2.60 |
| Interleukin-1b (pg/ml) | All | 1.37 ± 0.18‖ | 0.81 ± 0.10§‖ | 0.31 ± 0.026 |
| | GT+TT | 1.46 ± 0.25‖ | 0.79 ± 0.14§‖ | 0.32 ± 0.04 |
| | GG | 1.19 ± 0.20‖ | 0.85 ± 0.14§‖ | 0.28 ± 0.03 |
| Interleukin-6 (pg/ml) | All | 7.70 ± 0.55‖ | 3.49 ± 0.31§‖ | 2.16 ± 0.15 |
| | GT+TT | 8.46 ± 0.69*‖ | 3.70 ± 0.43§‖ | 2.07 ± 0.21 |
| | GG | 6.15 ± 0.83‖ | 3.07 ± 0.32§ | 2.27 ± 0.23 |

All values were expressed as mean ± SEM. *$p < 0.05$ vs GG. †$p < 0.01$ vs. GG. ‡$p < 0.05$ vs. acute phase. §$p < 0.01$ vs. acute phase. ‖$p < 0.05$ vs. controls after adjustment according to Bonferroni.

eNOS = endothelial nitric oxide synthase; MI = myocardial infarction; ox-LDL = oxidized low-density lipoprotein.

Serum levels of IL-1b were significantly lower in control patients compared with patients at both the acute phase and at follow-up after adjustment for multiple comparisons (Fig. 2B, Table 4).

**Correlations and multivariate analysis.** Univariate analysis in patients during the acute phase of MI showed that the presence of the T allele (GT+TT) was associated with levels of IL-6 ($r = 0.255$, $p < 0.05$), vWF ($r = 0.455$, $p < 0.001$), and ox-LDL ($r = 0.320$, $p < 0.05$). Similarly, the plasma level of vWF was associated with the existence of hypercholesterolemia ($r = 0.452$, $p < 0.05$), IL-6 with age ($r = 0.284$, $p < 0.05$), IL-1b with age ($r = 0.310$, $p < 0.05$), and negatively with HDL levels ($r = -0.365$, $p < 0.05$) and ox-LDL with age ($r = 0.230$, $p < 0.05$). After multivariate analysis, during the acute phase of MI, the existence of the T allele (GT+TT) was the only independent predictor of plasma vWF ($\beta = 35.139$ [SE, 9.213], $p = 0.0001$) and serum ox-LDL levels ($\beta = 32.088$ [SE, 12.878], $p = 0.016$), whereas there was a trend toward significance for IL-6 levels ($\beta = 1.4910$ [SE, 0.751], $p = 0.053$). The presence of the T allele was not an independent predictor for IL-1b levels (p = NS).

Univariate analysis in patients one year after the event showed that the presence of the T allele (GT+TT) was associated with higher levels of vWF ($r = 0.336$, $p < 0.01$), but it was not associated with IL-1b, IL-6, or ox-LDL (p = NS for all). Plasma vWF was associated with hypercholesterolemia ($r = 0.241$, $p < 0.05$) and smoking ($r = 0.233$, $p < 0.05$), whereas ox-LDL correlated with age ($r = 0.246$, $p < 0.05$) and IL-1b correlated with hypercholesterolemia ($r = 0.299$, $p < 0.05$) and negatively with serum levels of HDL ($r = -0.381$, $p < 0.01$). Serum levels of IL-6 correlated with age ($r = 0.355$, $p < 0.01$), diabetes mellitus ($r = 0.540$, $p < 0.05$), hypercholesterolemia ($r = 0.554$, $p < 0.05$), and hypertension ($r = 0.503$, $p < 0.05$). After multivariate analysis, in patients one year after MI, the presence of the T allele (GT+TT) was still the only independent predictor for vWF levels ($\beta = 15.460$ [SE, 7.522], $p = 0.046$), but not for IL-1b, IL-6, or ox-LDL (p = NS for all).

Univariate analysis in the control group showed that the IL-6 level was positively associated with diabetes mellitus ($r = 0.414$, $p < 0.01$), hypercholesterolemia ($r = 0.625$, $p < 0.01$), and hypertension ($r = 0.159$, $p < 0.05$). Similarly, IL-1b correlated with hypercholesterolemia ($r = 0.178$, $p < 0.05$) and ox-LDL correlated with age ($r = 0.177$, $p < 0.05$). Plasma levels of vWF correlated with hypercholesterolemia ($r = 0.407$, $p < 0.01$), smoking status ($r = 0.109$, $p < 0.05$), and diabetes mellitus ($r = 0.280$, $p < 0.01$). In multivariate analysis, in the control group, diabetes mellitus ($\beta = 1.133$ [SE:0.372], $p = 0.003$), hypercholesterolemia ($\beta = 2.438$ [SE, 0.276], $p = 0.0001$), and age ($\beta = 0.022$ [SE, 0.010], $p = 0.034$) were independent predictors for IL-6 levels. Furthermore, hypercholesterolemia ($\beta = 22.076$ [SE, 4.810], $p = 0.0001$) and diabetes mellitus ($\beta = 15.091$ [SE, 6.491], $p = 0.021$) were independent predictors for vWF levels, and age for ox-LDL levels ($\beta = 0.466$ [SE, 0.161], $p = 0.004$). In multivariate analysis, the presence of the T allele was unable to predict levels of IL-6, IL-1b, vWF, or ox-LDL (p = NS for all) in the control group.

## DISCUSSION

Our results provide evidence that G894T polymorphism on the eNOS gene is associated with a higher risk for premature MI. Carriers of the T allele appear to have higher levels of ox-LDL and IL-6 during the acute phase of MI, but not one year after the event. Similarly, carriers of the T allele appear to have higher levels of vWF during the acute phase of MI, and the effect is still observed one year after the event. However, this polymorphism had no effect on any of the examined parameters in the control group. These findings suggest that the 894T allele may predispose to higher endothelial cell activation, increased oxidative stress status, and enhanced proinflammatory cytokine expression during the acute phase on premature MI, whereas this effect is almost diminished in MI survivors one year after the event and is not observed in patients without coronary artery disease.

**G894T polymorphism and risk for premature MI.** Nitric oxide is produced in endothelial cells by eNOS; it plays a pivotal role in the pathogenesis of MI by regulating vascular tone, endothelial-leukocyte interactions, endothelial integrity, and permeability and by decreasing platelets adhesion and aggregation, and it also has antioxidant properties (1). Genetic polymorphism G894T on the eNOS gene leads to a Glu298Asp substitution on the eNOS molecule (6,7). Clinical evidence suggests that G894T may affect the development of MI because it has been associated with coronary spasm (8) and a higher risk for MI in the United Kingdom and in Japan (9,10), affecting also the extent and severity of coronary artery disease in the Italian population (11). However, compelling data from other studies performed in France and Northern Ireland (Etude Cas-Temoin de l'Infarctus du Myocarde [ECTIM] study) (12), Taiwan (13), Australia (14), and recently in Japan (15), indicate no association of G894T polymorphism with either coronary artery disease or MI. A recent meta-analysis showed that homozygosity for the 894T allele is associated with an increased risk for ischemic heart disease in 23,028 patients (3).

Evidence suggests that G894T may be associated with coronary artery disease mainly in the presence of atherosclerosis risk factors (16), implying that this genetic marker or another locus closely linked to it may be associated with a decreased NO bioavailability only under high endothelial cell stimulation. Indeed, recent studies suggest that G894T is associated with increased cardiovascular risk after coronary angioplasty (17) and endothelial dysfunction in smokers (2). However, its effect on vascular endothelium in patients without any risk factors for atherosclerosis is rather

controversial (18–20) because the initial reports that G894T polymorphism does not affect endothelial function in healthy individuals with no risk factors for atherosclerosis (2) have recently been questioned (20). Furthermore, no association was observed between G894T and endothelial function, in hypertensive patients (19) or in patients with stable coronary artery disease (21).

In the present study, we focused on relatively young patients who had multiple risk factors and were admitted with acute MI. We have shown that in our population, homozygosity for the T allele is related to an increased risk for premature MI.

**Effects of G894T during the acute phase of MI.** Endothelial dysfunction has a crucial role in the development of MI because decreased NO bioavailability may lead to vasoconstriction, atherosclerotic plaque rupture, and thrombus formation (21–23). The G894T polymorphism on eNOS gene has been associated with enhanced responsiveness to alpha-adrenergic stimulation (24), whereas it has been proposed that the eNOS 298Asp may be more susceptible to proteolytic cleavage than eNOS 298Glu (25). However, a recent instrumental work by McDonald et al. (4) showed by the use of a human microvascular endothelial cells model that eNOS 298Asp enhanced susceptibility to proteolytic cleavage may be caused by an in vitro artifact. These controversial results from basic research failed to explain the clinical evidence for an association between G894T and the risk for MI, as described by a recent meta-analysis on a number of 23,028 cases (3). Therefore, we hypothesized that G894T polymorphism may lead to a different response of eNOS to different endothelial stimulation, leading to a reduction in its capacity for NO production under conditions of higher activation. Conditions associated with increased vascular oxidative stress and higher endothelial activation may reduce bioavailability of a limited NO resource, partly explaining the stronger effect of the eNOS genotype on endothelial function (2) and on the risk for MI (16,26) in patients with multiple risk factors for atherosclerosis. Therefore, we hypothesized that the activity of eNOS may be modified by G894T polymorphism only under conditions of increased endothelial cell stimulation. We investigated the potential role of G894T on endothelial cell injury and activation, oxidative stress status, and inflammatory response during the acute phase of premature MI and in the same patients one year after the event, and we compared these results with a population of control patients, matched with patients for age, gender, and major cardiovascular risk factors.

EFFECTS ON VWF. The vWF is produced in both endothelial cells and megakaryocytes, but its increased plasma concentration is considered to be a marker of endothelial cell injury or activation (27). Conditions associated with high oxidative stress status are accompanied by increased release of vWF, mainly from the injured vascular endothelium (27), whereas inflammatory mediators such as proinflammatory cytokines stimulate the release of vWF (28). Increased plasma levels of vWF during the acute phase of MI (29) reflect the degree of endothelial cell injury and activation during MI (30). Recent evidence suggests that vWF levels reach their peak value 24 to 48 h after the onset of MI, and they seem to have a predictive value for long-term outcome in these patients (31,32). It is believed that oxidative stress mediates vWF increase during MI, because free radicals produced during MI damage endothelial cells, leading to increased release of vWF (30,33). Endothelium-derived NO has an important cytoprotective role during acute MI, but it is unknown whether genetic polymorphisms on eNOS can modify the enzymes' response to MI, affecting the release of vWF.

In the present study, we found that the presence of the T allele was associated with significantly higher levels of vWF during the acute phase of MI, an effect still observed to a lesser degree one year after the event. However, no significant difference in vWF levels was found between genotypes in the control group. Multivariate analysis showed that the presence of the T allele is an independent predictor for the increase of vWF levels, both during the acute phase of MI and at the follow-up, having no effect in the control group. This finding implies that G894T polymorphism may affect the response of eNOS to acute-phase stimulation, modifying endothelial cell injury and activation during MI. These findings are compatible with previous reports from experimental models suggesting that although G894T polymorphism does not affect eNOS activity in cell cultures (representing an in vitro model of resting clinical conditions) (4), it affects the responsiveness of eNOS to alpha-adrenergic stimulation (24), and it is associated with endothelial dysfunction in humans (2,19,20).

EFFECTS ON OXIDATIVE STRESS. During the acute phase of MI, reactive oxygen species, especially superoxide anion, are produced in large amounts by the activated immune system, whereas several enzymes, including xanthine oxidase, nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate oxidase, lipoxygenase, and uncoupled eNOS, are additional sources (22,32,34). Beyond the direct cytotoxic effect of oxidative stress on endothelial cells, free radicals also decrease NO bioavailability by decreasing its production and increasing its oxidative inactivation (35,36). In addition, reactive oxygen species peroxidize lipid components, leading to formation of ox-LDL, which stimulates production of cytokines from the vascular wall (22). These cytokines induce endothelial cell activation and depress eNOS expression and NO production (37). Endothelium-derived NO has antioxidant and anti-inflammatory properties (37), and the ability of endothelial cells to preserve NO production under conditions of increased oxidative stress may affect the development of MI. Recent epidemiologic evidence (38) suggests that genetic polymorphism G894T on the eNOS gene may modify oxidative stress status because the presence of the T allele has been associated with

higher levels of ox-LDL in the general population. However, its effect on the variations of oxidative stress during the acute phase of MI are unknown.

In the present study we have shown that G894T polymorphism modifies oxidized LDL production during the acute phase of MI. Serum levels of ox-LDL were higher in carriers of the T allele during the acute phase of MI. This effect was diminished in the same population one year after the event, whereas it was not observed in the control group. Multivariate analysis showed that the presence of the T allele is an independent predictor of the increase in ox-LDL only during the acute phase of MI. This finding suggests that G894T polymorphism affects oxidative stress during the acute phase of MI, possibly by affecting the ability of eNOS to maintain sufficient NO levels. However, it is unclear whether the presence of the T allele results in lower NO production because of eNOS instability or higher superoxide production because of increased eNOS uncoupling during MI.

**EFFECTS ON PROINFLAMMATORY CYTOKINES.** Proinflammatory cytokines (such as IL-6 and IL-1b) are produced by a variety of tissues, such as immune cells, myocardium, and even the vascular wall during the acute phase of MI, and they reach their peak values 24 h after the onset of symptoms (31,39). Serum levels of proinflammatory cytokines during the acute phase of MI have a prognostic value (40,41), and they are closely related to the stability of atheromatous plaque (42). Interleukins-1b and -6 trigger an acute-phase reaction in the liver, regulating the release of acute-phase proteins such as C-reactive protein during MI, whereas they up-regulate the expression of adhesion molecules in endothelial cells and depress NO production by inhibiting eNOS (43). Endothelium-derived NO has anti-inflammatory properties because it depresses proinflammatory cytokine release from the vascular wall and immune system, modifying the inflammatory response during unstable coronary syndromes (39,44).

In the present study we found that the G894T polymorphism on eNOS affects serum levels of the proinflammatory cytokines IL-1b and IL-6 during the acute phase of premature MI. The presence of the T allele was associated with higher levels of proinflammatory cytokines, an effect diminished one year after the event. Our findings are compatible with previous reports showing that G894T is associated with higher levels of fibrinogen in the general population (38), but this is the first study examining the role of this polymorphism during the acute phase of MI and one year later. It is unclear whether this effect disappears one year after the event as a result of the patients' stable clinical state or because of a modifying effect of the pharmaceutical treatment applied after the first event of premature MI. In either case, our findings suggest that G894T modifies the regulatory role of eNOS during MI, an effect not present one year after the event.

**Study limitations.** A limitation of this study is that the control group in the follow-up study consists of patients selected from the total cohort of control patients to be matched with patients according to age, gender, and classic risk factors for atherosclerosis, and they do not represent the whole population of healthy patients, because risk factors are over-represented. However, the second part of the study was designed to test the hypothesis that the G894T polymorphism may lead to different phenotypes under different conditions of vascular stimulation, observed in patients with coronary artery disease during the acute phase of MI and one year after the event, and to compare these effects with control patients with the same risk factors in the absence of coronary atherosclerosis.

**Conclusions.** In the present study we have shown that the G894T polymorphism on the eNOS gene is associated with a higher risk for premature MI. Furthermore, the presence of the T allele may be associated with a greater increase in vWF, oxidizing LDL, and proinflammatory cytokine levels during the acute phase of MI, an effect that is diminished one year after the event. These findings imply that the G894T polymorphism on the eNOS gene may modify endothelial injury, oxidative stress status, and inflammatory response during the acute phase of MI.

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