

# Endothelial Nitric Oxide Synthase Polymorphisms Are Associated With Type 2 Diabetes and the Insulin Resistance Syndrome

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Endothelial nitric oxide synthase (eNOS) variants were previously demonstrated in cardiovascular disease. To evaluate whether eNOS gene variants are associated with insulin resistance and type 2 diabetes, we evaluated polymorphisms in Exon7 (E298D), intron 18 (IVS18 + 27A→C), and intron 23 (IVS23 + 10G→T) in 159 type 2 diabetic patients without macrovascular complications and in 207 healthy control subjects. Samples for all hormonal and metabolic variables were obtained after an overnight fast. The D298 and IVS18 + 27C alleles, but not the IVS23 + 10G→T variant, were significantly more frequent in type 2 diabetic patients than in control subjects. The two- and three-loci haplotype analysis showed that there is a statistically significant association between the eNOS variants and type 2 diabetes. No significant differences were observed in the clinical characteristics of type 2 diabetic patients according to genotypes (except for visceral obesity [waist-to-hip ratio], which was significantly more present in D298 homozygotes). Healthy control subjects homozygous for both D298 and IVS18 + 27C presented higher insulin, C-peptide, and nitric oxide levels, as well as higher HOMA (homeostasis model assessment) values than the double wild-type homozygotes, with values superimposable on those found in type 2 diabetic patients. In conclusion, we described a significant association between eNOS gene polymorphisms and type 2 diabetes, suggesting a new genetic susceptibility factor for hyperinsulinemia, insulin resistance, and type 2 diabetes. *Diabetes* 52:1270–1275, 2003

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CHD, coronary heart disease; eNOS, endothelial nitric oxide synthase; HOMA, homeostasis model assessment; nNOS, neuronal nitric oxide synthase; SNP, single nucleotide polymorphism.

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**T**ype 2 diabetes is associated with a marked increase in coronary heart disease (CHD) (1), and increased risk factors for CHD before the onset of type 2 diabetes have been shown in several populations (2,3). In addition, type 2 diabetic patients with clinical cardiovascular disease sustain a worse prognosis for survival than cardiovascular disease patients without diabetes (4). This seems correlated to the high atherogenic state already present in a prediabetic state, which might be partly related to insulin resistance (5).

Recently, endothelial dysfunction, such as nitric oxide (NO) impairment, is regarded as an early step in the development of insulin resistance, atherosclerosis, and type 2 diabetes (6–8). Studies evaluating the relation between CHD and endothelial dysfunction have clearly demonstrated that reduced NO-dependent endothelial vasodilation is an early functional disturbance in the development of atherosclerotic lesions (6–8). In addition, among the many activities of NO, it has demonstrated its ability to modulate peripheral and hepatic glucose metabolism and insulin secretion, according to Pieper (9), who suggested that NO alterations play an important role in the evolution of insulin resistance and type 2 diabetes. Moreover, our group has found high NO levels and endothelial dysfunction in type 2 diabetic patients and first-degree relatives of subjects with type 2 diabetes (10), as well as in subjects with the insulin resistance syndrome (11).

NO is synthesized from L-arginine by a family of enzymes, called NO synthases (NOSs). The constitutively expressed NOS isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), are likely to be the major contributors to whole-body NO production. In particular, the eNOS gene, which maps on 7q35–36, is mainly expressed in endothelial cells. Recently, many studies strongly associated polymorphisms of the eNOS gene with an increased risk of hypertension, cardiovascular disease, coronary spastic angina, myocardial infarction, and stroke, but the results were not always conclusive (12).

In an attempt to evaluate whether eNOS variants, previously demonstrated in cardiovascular disease and atherosclerosis, may be associated with insulin resistance and type 2 diabetes, we evaluated three single nucleotide polymorphisms (SNPs): E298D, IVS18 + 27A→C, and IVS23 + 10G→T to look for associations. In support of our

TABLE 1  
Number of case and control subjects and OR and 95% CI for the Exon7, IVS18+, and IVS23+ genotypes

Genotype	Cases	Control subjects	OR	95% CI
Exon7				
0	52 (32.7)	86 (46.4)	1	—
1	63 (39.6)	82 (39.6)	1.63	(1.23–2.20)
2	44 (27.7)	29 (14.0)	2.66	(1.51–4.8)
Total	159 (100)	207 (100)		
$\chi^2$ (df = 1), $P = 0.0005$				
D298	151 (47.5)	140 (33.8)		
IVS18+				
0	76 (47.8)	125 (60.4)	1	—
1	66 (41.5)	74 (35.7)	1.65	(1.18–2.32)
2	17 (10.7)	8 (3.9)	2.72	(1.39–5.38)
Total	159 (100)	207 (100)		
$\chi^2$ (df = 1), $P = 0.003$				
27C	100 (31.4)	90 (21.7)		
IVS23+				
0	70 (44.0)	88 (42.5)	1	—
1	72 (45.3)	95 (45.9)	0.95	(0.69–1.29)
2	17 (10.7)	24 (11.6)	0.90	(0.48–1.66)
Total	159 (100)	207 (100)		
$\chi^2$ (df = 1), $P = 0.73$				
10T	106 (33.3)	143 (34.5)		

Data are  $n$  (%). 0 = 0/0, 1 = 0/1, 2 = 1/1. For each eNOS variant, the allele frequency of the mutant allele is also reported.

hypothesis, it has recently been shown that a region of chromosome 7q seems to influence both insulin resistance and blood pressure (13), suggesting that this locus may broadly influence traits associated with insulin resistance. In a second study, eNOS<sup>-/-</sup> mice were hypertensive and had fasting hyperinsulinemia, hyperlipidemia, and a 40% reduction of insulin-stimulated glucose uptake after clamp (14).

In the present study, E298D has been subsequently reported as Exon7, IVS18 + 27A→C as IVS18+, and IVS23 + 10G→T as IVS23+. Furthermore, in the tables and figures, for each SNP, we defined the wild-type homozygotes as 0, the heterozygotes as 1, and the variant homozygotes as 2.

The clinical characteristics of the type 2 diabetic patients and the healthy control subjects groups are shown in the online appendix (<http://diabetes.diabetesjournals.org>). The type 2 diabetic patients presented all the metabolic characteristics of the insulin resistance syndrome, i.e., hyperinsulinemia, insulin resistance, low HDL choles-

terol, visceral obesity, increased BMI, systolic blood pressure, and plasma NO levels.

Assuming a multiplicative penetrance model, a statistically significant association was found between type 2 diabetes and the Exon7 and IVS18+ polymorphisms, while no evidence of an association with IVS23+ was found. Table 1 reports the number of case and control subjects and odds ratio (OR) and 95% CI for the Exon7, IVS18+, and IVS23+ genotypes. (Since the nonmultiplicative model did not provide a significantly better fit to the data than the multiplicative model for each of the eNOS variants, the ORs reported in Table 1 were estimated under the multiplicative model.) The OR for ED298 homozygotes, as compared with the wild-type homozygotes, was 2.66 (95% CI 1.51–4.8), and the OR for the IVS18 + 27C homozygotes was 2.72 (95% CI 1.39–5.38).

The test for interaction between the two Exon7 and IVS18+ polymorphisms was statistically significant ( $P < 0.04$ ). In Table 2, the number of case and control subjects and the OR and 95% CI corresponding to all the possible

TABLE 2  
Number of case and control subjects and OR and 95% CI for each combination of the Exon7 and IVS18+ genotypes

Genotype	Cases	Control subjects	OR	95% CI
Exon7				
0				
1				
2				
0				
1				
2				
0				
1				
2				
Total	159	207		
$\chi^2$ (df = 3), $P = 0.04$				

Data are  $n$  (%).

TABLE 3

Number of estimated Exon7,IVS18+ and Exon7,IVS18+,IVS23+ haplotypes in case and control subjects and OR and 95% CI, as well as the likelihood ratio test of association between the haplotypes and the disease locus and the corresponding P value

	Cases	Control subjects	OR	95% CI
Exon7,IVS18+ haplotype				
0.0	152.11	261.15	1	—
0.1	14.89	12.85	1.98	(0.84–4.75)
1.0	65.89	62.85	1.79	(1.19–2.72)
1.1	85.11	77.15	1.89	(1.31–2.74)
Total	318	414		
$\chi^2 = 16.63$ (3 df), $P = 0.00084$				
IVS18+,IVS23+ haplotype				
0.0	115.23	181.00	1	
0.1	102.77	143.00	1.13	(0.8–1.60)
1.0	96.77	90	1.69	(1.16–2.46)
1.1	3.23	0	—	—
Total	318	414		
$\chi^2 = 11.54$ , (3 df) $P = 0.009$				
Exon7,IVS18+,IVS23+ haplotype				
0.0.0	77.57	132.38	1	—
0.0.1	74.70	128.95	0.99	(0.64–1.51)
0.1.0	13.11	12.66	1.77	(0.66–4.49)
0.1.1	1.62	0	—	—
1.0.0	37.85	48.62	1.33	(0.76–2.33)
1.0.1	27.88	14.05	3.39	(1.60–7.80)
1.1.0	83.47	77.34	1.84	(1.2–2.83)
1.1.1	1.8	0	—	—
Total	318	414		
$\chi^2 = 23.28$ (7 df), $P = 0.0015$				

combinations of the alleles at the two loci are shown. Only those combinations with at least one variant allele at the Exon7 locus were statistically different from 1, as can be seen from their respective CIs. Homozygotes for both D298 and IVS18 + 27C were significantly more frequent in type 2 diabetic patients than in control subjects, showing an OR of 6.28 (95% CI 2.14–18.45), which tended to be higher than the others since its CI is shifted toward higher values. Henceforth, it seems that the presence of IVS18 + 27C does not increase the baseline risk unless in combination with the mutant allele D298.

Table 3 reports the analysis by haplotype at the eNOS locus. There is a significant association between the haplotypes with two markers (Exon7,IVS18+ and IVS18+, IVS23+) or three markers (Exon7,IVS18+,IVS23+) and the disease locus. As suggested by the highly significant value for the Exon7,IVS18+ haplotype, there is a significant heterogeneity in the ORs across the different haplotypes; the E298D and IVS18 + 27A→C alleles are more frequently in *cis* in cases than in control subjects, showing a higher OR than that observed for the haplotype when the SNPs were in *trans*.

Table 4 shows the linkage disequilibrium values among the eNOs markers in case and control subjects, calculated by an expectation-maximization algorithm for maximum-likelihood estimates of haplotype frequencies (15,16). The disequilibrium values resemble the reciprocal physical distance.

The analysis of the clinical characteristics of type 2 diabetic patients, according to different genotypes, did not demonstrate any significant differences, except for visceral obesity (waist-to-hip ratio), which was significantly more present in D298 homozygotes (data not shown).

In type 2 diabetic patients, because metabolic events

related to glucose toxicity or lipotoxicity may supersede the genetic effect, playing a confounding role (17), we evaluated the metabolic parameters in diabetic patients as a whole group. Conversely, in healthy control subjects, metabolic variables were classified according to the genotype for both the Exon7 and IVS18+ loci. More specifically, we considered the double wild-type and variant homozygotes and the double heterozygotes.

Healthy control subjects homozygous for both D298 and IVS18 + 27C presented higher insulin, C-peptide, and NO levels and increased homeostasis model assessment (HOMA) values compared with the double wild-type homozygotes, but very similar levels and values compared with those observed in type 2 diabetic patients (Fig. 1). We therefore hypothesized a significant association between eNOS gene polymorphisms and metabolic traits of insulin resistance. However, no significant differences according

TABLE 4  
Linkage disequilibrium test between eNOs polymorphisms

Markers	Physical distance (kb)	$\delta^2$	D'
Exon7,IVS18+	10.3	0.334	0.784
		<u>0.260</u>	<u>0.716</u>
		<b>0.307</b>	<b>0.760</b>
IVS18+,IVS23+	3.1	0.147	1.000
		<u>0.187</u>	<u>0.903</u>
		<b>0.161</b>	<b>0.943</b>
Exon7,IVS23+	13.4	0.133	0.702
		<u>0.059</u>	<u>0.361</u>
		<b>0.090</b>	<b>0.513</b>

Values referring to control subjects, cases, and all individuals appear underlined, italicized, and bold, respectively.

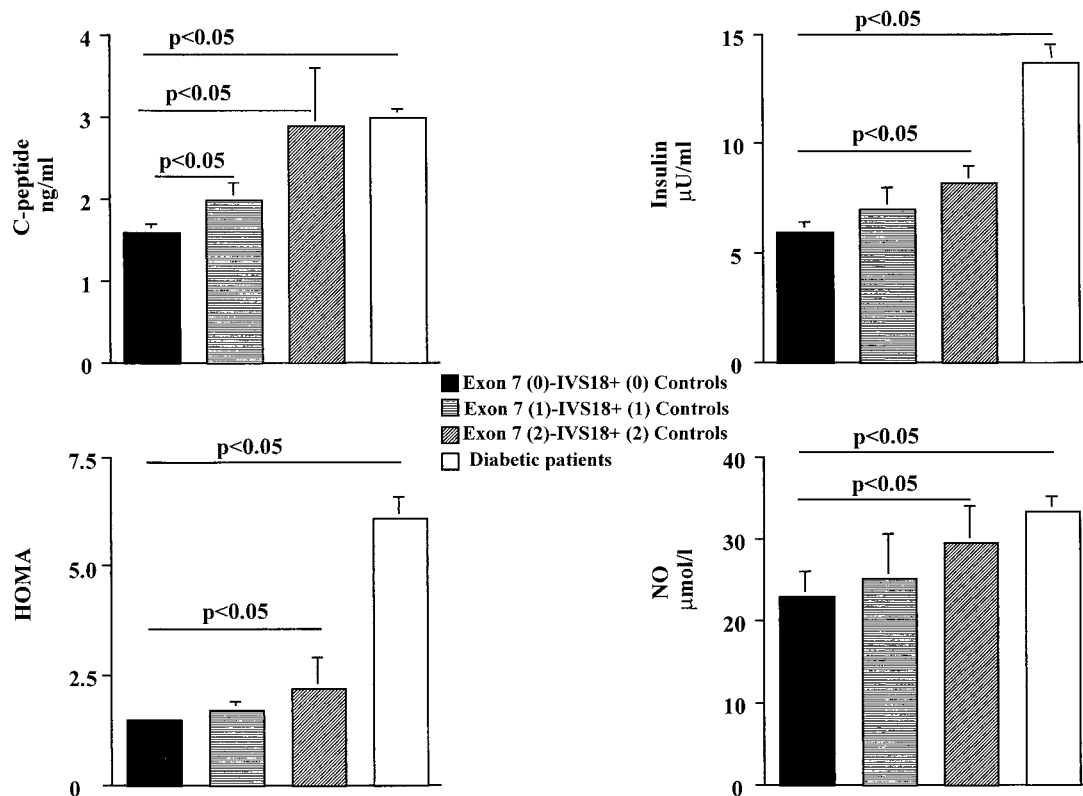


FIG. 1. C-peptide, insulin, HOMA, and NO levels in healthy control subjects according to the Exon7 and IVS18+ variants, as well as in type 2 diabetic patients as a whole group. In particular, for each SNP, we considered 0 = 0/0 as wild-type homozygotes, 1 = 0/1 as heterozygotes, and 2 = 1/1 as variant homozygotes.

to genotype were observed for other metabolic variables, such as plasma glucose, BMI, waist-to-hip ratio, plasma triglycerides, and systolic and diastolic blood pressure, as reported in the online appendix (<http://diabetes.diabetesjournals.org>). On the contrary, type 2 diabetic patients had increased levels of all these parameters compared with control subjects, except for diastolic blood pressure.

In the present study, a significant association of the Exon7 and IVS18+ loci with type 2 diabetes was found in a Caucasian population. In addition, our work supports the role of a genetic variation of the eNOS gene in triggering some of the metabolic abnormalities characteristic of the insulin-resistant states, which may predispose to cardiovascular disease. In fact, in healthy control subjects, a new genotype-phenotype association was observed. In particular, metabolic alterations typical of the insulin resistance syndrome, i.e., an increased insulin secretion and a marked degree of insulin resistance, were associated with polymorphic variants of the eNOS gene.

These characteristics highly resemble a state of "preglycemic diabetes" in which hyperinsulinemia and/or insulin resistance precedes the development of type 2 diabetes (18,19). Moreover, in healthy control subjects homozygous for the D298 and IVS18 + 27C eNOS variants, we demonstrated increased NO levels (Fig. 1). This finding allows an association between a genotype variant and a previously reported phenotype in which increased NO levels, reduced NO activity, hyperinsulinemia, and insulin resistance were reported in type 2 diabetic patients and their nondiabetic first-degree relatives, as well as in insulin-resistant subjects (10,11).

In the last decade, a great effort has been made to understand of the role of NO in insulin secretion and insulin resistance. In particular, the role of NO as a modulator of physiological insulin secretion has been extensively evaluated and recently revised by Spinass (20). In addition, it has been described that eNOS activation, specifically localized to skeletal muscle mitochondria (21,22), increases muscle blood flow, with increased delivery of insulin's major substrate, glucose, to the muscle cell (23). Baron et al. (23) suggest that ~30% of insulin's effect on glucose uptake can be accounted for by increased muscle perfusion. Thus, our data seem to suggest that a genetic defect in eNOS gene might be a new player in the evolution of hyperinsulinemia and insulin resistance.

The presence of an association between the D298 polymorphism and type 2 diabetes demonstrated in the present study parallels previous studies on myocardial infarction (24) and hypertension (25). eNOS allelic association suggests that these diseases, typical of the insulin resistance syndrome, might share a common genetic origin, causing different clinical aspects in a different period of life. Moreover, our results are consistent with those by Lembo et al. (26), who found that D298 homozygosity is an independent risk factor for carotid atherosclerosis in hypertensive patients, taking into account the early manifestations of atherosclerotic disease. In addition, the allelic frequency and OR of Exon7 locus in our diabetic patients were not different from those found in patients with cardiovascular disease (24). However, both eNOS variants seem to increase the risk of developing type 2

diabetes, since subjects homozygous for D298 and IVS18 + 27C eNOS variants have a OR that tended to be higher, as shown by its 95% CI.

Recently, Ukkola et al. (27) evaluated the presence of the D298 polymorphism in a population of type 2 diabetic patients with a high prevalence of macroangiopathy, but they did not find any difference in the allelic frequency between diabetic patients and control subjects. The main difference between this report and that of Ukkola et al. is due to the phenotypic state of the disease; their patients had already developed a quite severe macroangiopathy that might have caused a higher mortality rate for macroangiopathic complications in the diabetic group carrying the D298 polymorphism. The evidence for this could be that the allelic frequency of D298 was superimposable between our study and the healthy population in theirs, whereas it was reduced by 70% in their case population (29 vs. 47%, respectively).

It has been described that the D298 eNOS variant generates protein products with different susceptibility to cleavage, i.e., amino terminal 35-kDa and carboxy terminal 100-kDa fragments (28), suggesting that this polymorphism has a functional effect on the eNOS protein. In addition, it has been demonstrated that this polymorphism involves protein-protein interactions with chaperone proteins that control subcellular trafficking of the enzyme, as well as with proteins involved in its degradative processing. However, it is unknown whether the presence of both the D298 and IVS18 + 27C eNOS variants has different functional effects, and this important issue deserves further investigation.

In conclusion, we have described for the first time a significant association between eNOS gene polymorphisms and type 2 diabetes, suggesting a new genetic susceptibility factor for hyperinsulinemia, insulin resistance, and type 2 diabetes. Our data help to explain the finding that macroangiopathic complications sometimes begin 10–20 years before diagnosis of overt hyperglycemia and type 2 diabetes (29). In our opinion, the possibility of developing first diabetes or cardiovascular disease depends on a possible tissue specificity of eNOS variants, which culminates in localized vascular damage. Further studies using different ethnic population or perspective studies will clarify the relationship between type 2 diabetes and these variants.

## RESEARCH DESIGN AND METHODS

**Subjects.** We studied 159 type 2 diabetic patients and 207 healthy control subjects. Both case and control subjects were unrelated individuals of Caucasian ancestry who lived in Northern Italy and came from the same villages and cities.

Type 2 diabetic patients were defined by the absence of islet cell antibody and anti-GAD antibodies and a positive C-peptide release after intravenous glucagon test. Of patients, 60% were treated with diet only, while oral therapy was given to 40%. None were treated with insulin. They did not present proliferative retinopathy (evidenced by fluorangiography), peripheral vascular disease (determined by intermittent claudication and doppler ultrasound echography), or neuropathy (evaluated by electromyography). Their resting electrocardiogram and mono- and bidimensional echocardiography were normal in the absence of any history of cardiovascular or ischemic heart disease.

Healthy control subjects were without a family history of type 2 diabetes and were normal glucose tolerant after oral glucose tolerance test (75 g) according to World Health Organization criteria. Participants had a normal resting electrocardiogram in the absence of ischemic heart disease.

Subjects in the two groups were matched for age and sex and were

comparable for history of cigarette smoking, alcohol intake, and physical activity. History of cigarette smoking was obtained by questionnaire at interview. Subjects were classified as smokers if they reported smoking one or more cigarettes a day for the previous 6 months. Smokers were asked to interrupt smoking for a week before blood sample to reduce the influence of smoking on eNOS expression and enzyme activity to a minimum, as previously demonstrated. Moreover, since nitrogen intake could influence the measurement of  $\text{NO}_2^-/\text{NO}_3^-$  levels, the amount of nitrogen consumed was calculated in the 3 days before blood sample. (This precaution was taken despite the fact that the half-life of nitrate, a major component of the end product of NO, is 3 h; a 12-h overnight fast should be sufficient to result in total clearance of nitrogen dietary intake.) We observed that animal and vegetable nitrogen daily intakes were identical in the two subject groups, making it unlikely that variations in any of these variables would influence the measurement of  $\text{NO}_2^-/\text{NO}_3^-$  levels (10). All subjects provided informed consent, and local institutional ethical approvals were obtained.

**Protocol and laboratory methods.** Samples for all hormonal and metabolic variables were obtained in the morning after an overnight fast after resting for at least 15 min in the supine position. Blood pressure was taken in the recumbent position after 10 min of rest, using a random zero sphygmomanometer. Insulin resistance was calculated from fasting blood glucose and insulin levels with HOMA. Plasma glucose, triglycerides, and free fatty acids and serum total and HDL cholesterol levels were measured using automated enzymatic spectrophotometric techniques adapted to Cobas Fara II (Roche, Basel, Switzerland).

Serum insulin levels were assayed with a microparticle enzyme immunoassay (MEIA, IMX; Abbott Laboratories, North Chicago, IL), in which the lowest insulin sensitivity was  $1 \mu\text{U/ml}$ . Serum C-peptide levels (intra-assay coefficient of variation 3.0%, interassay coefficient of variation 3.0%) were assayed by radioimmunoassay using commercial kits (Dako, Cambridgeshire, U.K.) and NO levels by measurements of the end products of their metabolism, i.e., nitrite and nitrate levels ( $\text{NO}_2^-/\text{NO}_3^-$ ), using enzymatic catalysis coupled with Griess reaction, as previously reported (30).

**DNA extraction and genotyping.** Genomic DNA was prepared from blood leukocytes by established methods. E298D polymorphism was studied with PCR amplification followed by enzymatic digestion with *Ban*II. PCR primers were as follows: forward primer 5'-CCCTGGAGATGAAGGCAGGA-3' and reverse primer 5'-TGAGAGGCTCAGGGATGCCC-3'. The PCR product was 293 bp, which is cleaved into 169- and 124-bp fragments in the presence of E298.

The IVS18 + 27A→C polymorphism was studied with PCR amplification followed by allele-specific oligonucleotide hybridization. PCR primers were as follows: reverse primer 5'-TCCAGGCTACAATCCGTCTCAG-3' and forward primer 5'-TTGGGCGGGCAGACACCTA-3'. The size of PCR products was 270 bp. Hybridization probes were as follows: 5'-GCCCCCAACCCTG-3' and 5'-GCCCCCAACCCTG-3'. They were labeled with bacteriophage T4-poly-nucleotide kinase.

The IVS23 + 10G→T polymorphism was studied with PCR amplification followed by enzymatic digestion with *Mae* III. PCR primers were as follows: reverse primer 5'-GGGACCTGATGGAGTCTCTC-3' and forward primer 5'-CCTCTGTCCCTAGATTGTGTGA-3'. PCR products were 208 bp. (Overnight digestion with *Mae* III at 55°C gave fragments of 186 and 22 bp for the GG genotype; 186, 162, 22, and 24 bp for the heterozygous genotype; and 162, 22, and 24 bp for the TT genotype.)

**Statistical analysis.** Unpaired Student's *t* test was used to compare the clinical characteristics in case and control subjects, as whole groups.

**Genotypic association.** Dependence of disease risk on the eNOS variants was analyzed by a logistic regression model, in which the response variable was defined to take the value of 1 in cases (type 2 diabetic patients) and the value of 0 in control subjects.

Significance tests for model comparisons were performed using the likelihood ratio test (31). In the logistic model, each eNOS variants was modeled a biallelic: wild type was denoted as 1 and the mutant allele as 2. For each SNP, we defined a genetic factor *X* to take a value  $X = 0$  when the genotype was 0/0,  $X = 1$  when it was 0/1, and  $X = 2$  when it was 1/1.

In the logistic model, we compared the model under the nonmultiplicative penetrance model (where *X* is modeled as a categorical variable with three levels) with the model under a multiplicative penetrance model (where *X* is modeled as a continuous variable). In the latter, 1/1 genotype confers the greatest risk, 0/1 genotype an intermediate risk, and 0/0 genotype the lowest risk. To obtain more precise OR estimates, we estimated the OR under the multiplicative model. (If the nonmultiplicative model did not provide a significantly better fit to the data than the multiplicative model.)

Moreover, we investigated the interaction between the Exon7 and IVS18+ polymorphisms considered and type 2 diabetes via the likelihood ratio test. When the test was statistically significant, in order to interpret the nature of

the interaction, we created a new variable (X) that takes values between 0 and 8, with each value corresponding to a combination of the genotypes at the two loci, and we reported the OR for each genotype combination by taking the double wild-type homozygote as the baseline category.

In addition, the relationship between the metabolic variables typical of the insulin resistance syndrome and the eNOS variants at the genotypic level was studied by one-way ANOVA. A Scheffe's *F* test was used to perform comparison by allowing for multiple testing. In case of a statistically significant departure from normality of the metabolic variables, a Box-Cox transformation was applied before performing ANOVA.

**Aploypic association.** In a study of cases and unrelated control subjects, we can only reconstruct the haplotypes via a statistical model because, due to the absence of family data, the gametic phase cannot be observed. Dependence of disease risk on haplotypes was investigated via a log-linear model that allows for the indeterminacy of the haplotypes in individual heterozygous for two or more loci by using the EM algorithm to estimate haplotype frequencies. More specifically, each observed ambiguous genotype is expanded into all possible phases, and, at the E-step, its total observed frequency is divided between the possible phases according to their posterior probability, assuming Hardy-Weinberg equilibrium and current estimates of haplotype probabilities. A high dimensional contingency table of imputed haplotypes by disease status can then be calculated and the log-linear model fitted to this table (M-step). Particular ORs can be calculated from the cells of expected frequencies. CIs can be obtained with a profile likelihood approach (32). A detailed description of this method, as well as an application, has been previously reported (33).

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