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Hyperinsulinemia and impaired leptin-adiponectin ratio associate with endothelial nitric oxide synthase polymorphisms in subjects with in-stent restenosis

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Galluccio E, Piatti P, Citterio L, Lucotti PC, Setola E, Cassina L, Oldani M, Zavaroni I, Bosi E, Colombo A, Alfieri O, Casari G, Reaven GM, Monti LD. Hyperinsulinemia and impaired leptin-adiponectin ratio associate with endothelial nitric oxide synthase polymorphisms in subjects with in-stent restenosis. *Am J Physiol Endocrinol Metab* 294: E978–E986, 2008. First published March 18, 2008; doi:10.1152/ajpendo.00003.2008.—Little is known about the association of endothelial nitric oxide synthase (NOS3) gene polymorphisms and the presence of insulin resistance and the early evolution of atherosclerosis in nondiabetic subjects with cardiovascular disease (CAD) and stent implantation. The present study was performed in an attempt to better understand whether metabolic, endothelial, and angiographic findings characteristic of subjects with cardiovascular disease and in-stent restenosis are related to NOS3 variants. This is a case-control study performed from 2002 to 2006. All subjects admitted to the study were recruited in the Nord-Centre of Italy, most from Milan and its surrounding towns. Measures of glucose tolerance, insulin sensitivity, markers of endothelial dysfunction, forearm vasodilation, and adipokine levels were determined and associated to the frequency of two single-nucleotide polymorphisms of NOS3, i.e., Glu^{298}Asp (rs1799983, G/T) and rs753482 (intron 18 A/C). A total of 747 subjects, not known to have diabetes, were evaluated: 333 subjects had asymptomatic CAD, 106 subjects had unstable angina and were evaluated for in-stent restenosis 6 mo after stent placement, and 308 were control subjects. The presence of TT and CC minor alleles was significantly greater in case groups compared with control subjects. At phenotypic level, subjects with the polymorphisms were characterized by hyperinsulinemia and reduced reactive hyperemia, whereas increased leptin and decreased adiponectin levels were present in subjects with restenosis in the presence of reduced minimal lumen diameter and length of stenosis almost doubled. Hyperinsulinemia, endothelial dysfunction, and a more atherogenic profile seem to be peculiar features of subjects with asymptomatic CAD and restenosis carrying NOS3 gene variants.

NOS3 gene variants; coronary artery disease; type 2 diabetes

THE PROCESS LEADING TO CARDIOVASCULAR DISEASE (CAD) and in-stent restenosis is quite complex and strictly relates to impaired insulin sensitivity and endothelial dysfunction (31). Furthermore, a more extensive and diffuse coronary atherosclerosis, as well as increased neointimal hyperplasia (3), seems to be related to an increased risk of in-stent restenosis in subjects submitted to percutaneous coronary intervention (PCI) and stent implantation. Many studies have shown that hyperinsulinemia and insulin resistance increase neointimal index measured 6 mo after coronary stenting (32, 41, 42). In addition, neointimal hyperplasia is also dependent on reduction in nitric oxide activity that determines endothelial dysfunction and oxidative stress. A blunted endothelium-dependent vasodilation was found to predict CAD events independently of common risk factors (1, 21). Recently, leptin and adiponectin, concentrations, which have been found increased and reduced, respectively, in insulin-resistant states, were shown to be involved in the atherogenic process. Several clinical studies have shown that elevated leptin levels predict acute cardiovascular events, restenosis after coronary angioplasty, and cerebral stroke independently of traditional risk factors (4, 10, 31, 32). Conversely, reduction or lack of adiponectin resulted in accelerated atherosclerotic progression (17, 19). More recently, the evaluation of the leptin-adiponectin ratio (L/A ratio) has been suggested as an atherosclerotic index in healthy subjects and in subjects with type 2 diabetes mellitus (18, 26).

Nitric oxide (NO) has a key role in the endothelial function protecting the arterial wall from developing atherosclerotic lesions. This metabolite acts in regulating the vascular tone (15, 28) and blunting the activity of the nuclear factor-κB family of transcription factors (16, 36), thereby preventing the endothelial expression of adhesion molecules and inflammatory cytokines, which are instrumental for the triggering of atherogenesis (35).

NO is synthesized by endothelial cells and platelets from L-arginine through the action of the homodimeric enzyme endothelial nitric oxide synthase (NOS3). The association between several polymorphisms of the NOS3 gene and CAD and restenosis risks has been previously studied (2, 5, 6, 8, 25, 45). Zhang et al. (47) did observe a potential involvement of 786T→C, Glu^{298}Asp, and intron 8 single-nucleotide polymorphism (SNP) variants in the atherogenic process in diabetic subjects with CAD. Other studies showed that homozygosity of Glu^{298}Asp and −786T→C polymorphisms of the NOS3 gene represented an independent risk factor for in-stent restenosis (12, 40), and the 894G→T polymorphism of NOS3 gene

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was associated with an increased risk of death and/or myocardial infarction within 1 yr after stent placement (13).

Previously, we presented data that provide a potential link between polymorphisms in the NOS3 gene and insulin resistance and endothelial dysfunction (23). Specifically, we documented an association between two SNPs of the NOS3 gene: rs1799983 at position 150327044 ($Glu^{298}Asp$, G/T) and rs753482 at position 150337316 (intron 18 A/C) with type 2 diabetes and insulin resistance, suggesting a possible common genetic origin for both the cardiovascular heart disease and type 2 diabetes mellitus. The present study extended the results of the previous study with the specific aims *1*) to evaluate the association between the two previously reported SNPs of the NOS3 gene with the risk of CAD and restenosis and *2*) to define whether the presence of both polymorphisms is associated with insulin resistance/hyperinsulinemia as assessed by the release of insulin in response to a standard oral glucose challenge, with endothelial dysfunction as assessed by the evaluation of forearm blood flow (FBF) and reactive hyperemia and with circulating levels of adipokines, leptin, and adiponectin and leptin-adiponectin ratio (L/A ratio) as an index of the atherosclerotic process. Since a link was previously documented between type 2 diabetes mellitus and the two SNPs (23), the evaluation of the endothelial NOS variants and their interaction with CAD and the degree of restenosis 6 mo after successful coronary stenting was characterized only in subjects showing an impaired or normal glucose tolerance after an oral glucose load.

EXPERIMENTAL PROCEDURES

Case and control populations. A total of 747 Caucasian subjects were studied. All subjects were recruited in the Nord-Centre of Italy, most from Milan and its surrounding towns. Two groups of subjects were studied: 333 asymptomatic CAD subjects submitted to a prior PCI or coronary artery bypass grafting (CABG) for a previous positive coronagraphy, in the absence of angina episodes at rest or after exercise and with no evidence of restenosis at a control coronary angiography during the last 6 mo before study (*group 1*), and 106 subjects affected by unstable angina defined as an experienced chest pain at rest within the preceding 48 h (i.e., Braunwald's class III B), with the presence of a single, de novo native coronary artery lesion, successfully treated with customized stents from balloon-expandable Phitis Diamond Plus stent (*group 2*). In the last group, subjects were scheduled for repeated angiographic examination 6 mo after stent placement. If repeat angiography was required earlier than 6 mo for clinical reasons and restenosis was present, it was used as the poststenting observation. All subjects of *group 2* completed the follow-up period.

Control subjects were 308 unrelated individuals randomly selected from a population of free-living individuals screened for CAD risk factors. Participants had a normal resting electrocardiogram and exercise testing in the absence of a history of ischemic heart disease. A complete medical history of all subjects also was obtained about smoking habits, history of hypertension and type 2 diabetes, and current medication used.

The study was accepted by the local Ethical Committee, and all subjects gave their informed consent. The present study is part of a clinical trial having the registration number NCT 00520962.

Angiographic analysis. In *group 2*, quantitative coronary angiographic analysis was performed using a validated edge-detection program (CMS version 5.2; MEDIS, Leiden, The Netherlands). Follow-up restenosis was analyzed by measuring minimal lumen diameter (MLD) and length of stenosis after 6 mo. In addition, the following variables were assessed: "acute gain," defined as the MLD after the procedure minus the MLD before the procedure; "late loss," defined as the MLD after the procedure minus the MLD at follow-up; and "loss index," defined as the average ratio of late loss to acute gain. "Restenosis" was defined as a degree of stenosis >50% at follow-up.

Metabolic testing. None of the 747 subjects were known to have diabetes or were taking antihyperglycemic drugs, and all were submitted to a 75-g oral glucose load after an overnight fast at the Cardio-Diabetes Outsubjects Clinic. In particular, in *group 1*, the oral glucose load was performed at the first visit to the center, whereas in *group 2*, the oral glucose load was performed when they returned for the follow-up evaluation to reduce stress-induced insulin resistance. Blood samples were withdrawn before and 30, 60, and 120 min later for measurement of plasma glucose and insulin concentrations. In addition, blood samples were obtained before the oral glucose load for measurement of glycated hemoglobin (HbA1c), total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), free fatty acids (FFA), total leptin, adiponectin (all isoforms), and nitrite and nitrate (NOx) concentrations. Insulin resistance was calculated with the homeostasis model assessment (HOMA-IR) using the following equation: fasting insulin (IU/ml) × fasting glucose (mmol/l)/22.5. The trapezoidal rule was used to calculate total integrated area under the curve for insulin (total insulin AUC).

Forearm blood flow. FBF was measured by strain-gauge venous occlusion plethysmography. Before any measurement was taken, the hand circulation was occluded using a wrist cuff inflated to 240 mmHg. Baseline flow was calculated as the mean of at least three values. Reactive hyperemia (endothelium-dependent vasodilation) was measured after the release of a 5-min arterial occlusion, produced by inflating a standard sphygmomanometer cuff on the upper arm to 100 mmHg above systolic blood pressure (SBP).

After 15 min of rest, a new baseline blood flow was calculated and repeated after administration of 0.5 mg of sublingual nitroglycerin (nitroglycerin-mediated dilation, NMD) to evaluate endothelium-independent vasodilation.

Assays. Plasma glucose, HbA1c, HDL-C, total cholesterol, FFA, and TG were measured with spectrophotometric methods adapted to Cobas MIRA using commercial kits. NOx concentrations were estimated by measurement of metabolic end products, i.e., nitrite and nitrate, using enzymatic catalysis coupled with the Griess reaction (44). Serum insulin levels were assayed with a microparticle enzyme immunoassay (IMX; Abbott Laboratories, Rome, Italy) with a sensitivity of 1 μU/ml and intra- and interassay coefficients of variation (CVs) of 3.0 and 5.0%, respectively. Human leptin (sensitivity of 0.125 ng/ml and intra- and interassay CVs of 4.5 and 7.8%, respectively) and adiponectin levels (sensitivity of 0.78 ng/ml and intra- and interassay CVs of 3.0 and 6.0%, respectively) were assayed with an ELISA kit and a RIA kit (Linco Research, St. Charles, MO), respectively.

DNA extraction and genotyping. Genomic DNA was obtained from all subjects participating into the study by established methods (37). Allelic discrimination of two SNPs was performed, one rs1799983 at position 150327044 ($Glu^{298}Asp$) and the other rs753482 at position 150337316 (intron 18 A/C) using the TaqMan chemistry with the ABI Prism 7700 apparatus (Applied Biosystems, Foster City, CA). Primer and probe sets were designed and manufactured by Applied Biosystems as Custom TaqMan SNP genotyping assays. The forward and reverse primers and probes employed for the rs1799983 discrimination were 5′-GCTGCCCCTGCTGCT-3′, 5′-GCACCTCAAGGACCAGCTC-3′, 5′-VIC-CCAGATGAGCCCCCA-3′, and 5′-FAM-CCCAGATGATCCCCCA-3′, respectively, whereas the forward and reverse primers and probes employed for the rs753482 discrimination were 5′-TGAGGACGACGGCTTTACC-3′, 5′-CCAGGGTCAGGGTGTTCAG-3′, 5′-VIC-CCCCCAACCCCTG-3′, and 5′-FAM-CCCCCCACCCCTG-3′, respectively.

A final volume of 12 μl of polymerase chain reaction (PCR) fluid contained 50 ng of DNA, 6 μl of Master Mix, and 4 μl of H_2O. The

amplification conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 92°C for 15 s and 60°C for 1 min. Controls for each SNP were included in the assay, ~50% of samples were replicated with a concordance of 95%, and laboratory staff were blinded to case-control status.

Statistical analysis. Statistical analysis was focused on evaluating the association between two NOS3 SNPs and the risk of CAD and in-stent restenosis in subjects without known diabetes mellitus. The association of these NOS3 SNPs and metabolic variables, indexes of endothelial dysfunction, and angiographic findings were examined.

All values for clinical, metabolic, and angiographic measurements are expressed as means ± SD. Frequency distribution of characteristics of study participants were examined according to the case-control status. Comparisons among groups were performed using one-way ANOVA followed by Scheffé's post hoc test. χ^2 tests were used to determine differences in genotype frequencies between case and control subjects. Unconditional logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between SNPs and CAD or restenosis risks adjusted for age, sex, body mass index (BMI), and smoking status (never, past, or current smoker).

The SNPs' Hardy-Weinberg equilibrium and pairwise linkage disequilibrium (LD) were calculated using the HaploView program (http://www.broad.mit.edu/mpg/haploview/) (16); LD was expressed by the D' and r^2 statistics. Both haplotype frequencies, estimated with the expectation-maximization (EM) algorithm, and association tests were implemented using the HaploView program.

A two-tailed probability level of 0.05 was considered statistically significant. All analyses were performed using SPSS version 15.0 software (SPSS, Chicago, IL).

RESULTS

Comparison of clinical characteristics and CAD risk factors in *group 1*, *group 2*, and control subjects is shown in Table 1. The two classes of subjects had higher values for fasting insulin, SBP, insulin AUC, and TG concentration and lower values for HDL-C concentration compared with the control population. Reactive hyperemia and NMD were significantly impaired in both patient groups. Both groups of subjects had comparable antilipidemic and antihypertensive therapy, and women were postmenopausal in the absence of hormonal replacement therapy.

None of the 439 subjects had diabetes on the basis of their fasting plasma glucose concentration (43) or showed a diabetic answer to the 75-g oral glucose challenge. In particular, the answer to the 75-g oral glucose challenge showed that 152 subjects in *group 1* and 50 subjects in *group 2* had normal glucose tolerance, with the remainder having impaired glucose tolerance (181 subjects in *group 1* and 56 subjects in *group 2*, respectively). In contrast, by selection, all 308 control subjects had normal oral glucose tolerance.

In *group 2*, the results of the postangioplasty angiographic evaluation indicated that 25 of the 106 subjects (24%) had evidence of significant restenosis, as defined by a reduction in lumen diameter of the treated segment by >50%. Genotype frequency distributions of the two SNPs ($Glu^{298}Asp$ and intron 18 SNPs) did not deviate from the Hardy-Weinberg equilibrium among study participants (combined case and control subjects: $D' = 0.900$ and $r^2 = 0.490$).

Table 1. *Clinical and metabolic characteristics of group 1, group 2, and control subjects*

	Group 1	*Group 2*	Control Subjects
No. of subjects	333	106	308
Age, yr	59±10	58±10	58±3
Male/Female subjects	295/38	102/4	188/120
Subjects taking oral antiplatelet drugs	333 (100%)	106 (100%)	
Subjects taking β-blockers	199 (60%)	74 (70%)	
Subjects taking ACE inhibitors	183 (55%)	64 (60%)	
Subjects taking statins	216 (65%)	71 (67%)	
BMI, kg/m^2	26.8±4.3	26.6±3.6	25.8±2.5
SBP, mmHg	128±15†	133±15*	126±8
DBP, mmHg	80±9	82±8	80±4
Glucose, mg/dl	97±15	98±14	98±6
Insulin, μU/ml	10.7±6.1†	11.5±6.3*	7.4±3.8
HOMA-IR	2.64±1.67†	2.79±1.68*	1.71±0.79
Total insulin AUC, μU/ml at 120 min	8,218±4,640†	8,793±2,447*	5,025±604
HbA1c, %	5.7±0.7	5.6±0.5	5.6±0.4
NOx, μmol/l	21.2±14.1	21.6±15.0	21.6±2.2
Leptin, ng/ml	9.6±5.8	7.3±4.9	6.0±3.8
Adiponectin, ng/ml	12.0±4.6	8.9±4.7*	13.7±3.3
Cholesterol, mg/dl	177±46	193±48	209±10
HDL-C, mg/dl	40±11†	43±3*	60±6
LDL-C, mg/dl	103±38	120±49	126±39
TG, mg/dl	140±66†	154±65*	100±8
FFA, mmol/l	0.53±0.21	0.53±0.20	0.53±0.16
Basal FBF, ml·100 ml forearm^{-1}·min^{-1}	2.78±0.98	2.82±0.86	2.70±0.66
Reactive hyperemia, ml·100 ml forearm^{-1}·min^{-1}	13.99±7.84†	14.97±8.35*	22.21±7.03
NMD, ml·100 ml forearm^{-1}·min^{-1}	15.36±5.55†	15.39±4.89*	20.49±7.44

Values are means ± SD. ACE, angiotensin-converting enzyme; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; total insulin AUC, total integrated area under the curve for insulin; NOx, nitric oxide metabolic end products (nitrite and nitrate); HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; TG, triglyceride; FFA, free fatty acids; FBF, forearm blood flow; NMD, nitroglycerin-mediated dilation. †$P < 0.05$, coronary artery disease (CAD) case subjects vs. control subjects. *$P < 0.05$ CAD and coronary revascularization case subjects vs. control subjects.

Haplotype frequencies (Table 2) demonstrated that the two-allele risk haplotype composed by the two minor alleles (TC) was significantly increased in the two case populations. After adjustment for age, sex, BMI and smoking status, subjects carrying the two-allele risk haplotype had 1.74-fold increased risk in *group 1* (95% CI: 1.43–2.12) and 1.63-fold increased risk in *group 2* (95% CI: 1.34–1.98).

Evaluation of the metabolic, vascular, and atherosclerotic indexes after subjects of *group 1* (asymptomatic CAD) were subdivided on the basis of their $Glu^{298}Asp$ and intron 18 genotypes allowed us to demonstrate that carriers of the minor allele for both SNPs had fasting insulin levels increased by 42% in TT carriers and 62% in CC carriers. HOMA-IR increased by 39% in both groups, and total Insulin AUC increased by 24% in TT carriers and by 50% in CC carriers, whereas reactive hyperemia decreased by 53% in TT carriers and by 60% in CC carriers. No significant differences were observed for glucose, HbA1c, leptin, adiponectin, L/A ratio, basal FBF, and NMD (Table 3).

In subjects of *group 2* presenting restenosis at quantitative angiography, haplotype frequencies demonstrated that after adjustment for age, sex, BMI, and smoking status, subjects carrying the two-allele risk haplotype had 3.64-fold increased risk of restenosis (95% CI: 3.04–4.36; Table 2). After subjects of *group 2* were subdivided on the basis of their $Glu^{298}Asp$ and intron 18 genotypes, it was possible to demonstrate that HOMA-IR was almost doubled in both carriers of the minor alleles, whereas fasting glucose, HbA1c, NOx, basal FBF, and NMD were comparable (Table 4). Figure 1 depicts the results of *group 2* regarding fasting insulin, total insulin AUC, leptin, adiponectin, L/A ratio, and reactive hyperemia according to the genotypes in exon 7 ($Glu^{298}Asp$) and intron 18 (rs753482). Subjects carrying $Glu^{298}Asp$ and intron 18 minor alleles were characterized by hyperinsulinemia both in the fasting state and after the glucose load, as well as increased leptin and decreased adiponectin with a concomitant higher L/A ratio, an index of increased atherogenesis, whereas forearm vasodilation after reactive hyperemia was decreased. In particular, fasting insulin levels nearly doubled (TT: 19.3 ± 9.8 vs. GG: 9.6 ± 4.2 μU/ml, $P < 0.0001$; and CC: 19.2 ± 9.9 vs. AA: 10.0 ± 3.8 μU/ml, $P < 0.0001$) and total insulin AUC increased in both groups (TT: 9,699 ± 1,790 vs. GG: 7,828 ± 1,149 μU/ml, 120 min, $P < 0.001$; and CC: 13,149 ± 2,089 vs. AA: 7,893 ± 1,436 μU/ml, 120 min, $P < 0.001$). At a difference from *group 1*, leptin levels in subjects of *group 2* were significantly increased (TT: 10.0 ± 3.3 vs. GG: 4.8 ± 2.5 ng/ml, $P < 0.01$; and CC: 10.8 ± 3.8 vs. AA: 5.7 ± 2.8 ng/ml, $P < 0.01$). Conversely, adiponectin levels were significantly decreased (TT: 6.6 ± 1.5 vs. GG: 9.3 ± 1.9 ng/ml, $P < 0.05$; and CC: 6.2 ± 2.2 vs. AA: 10.2 ± 2.1 ng/ml, $P < 0.05$). L/A ratio significantly increased in subjects carrying Asp^{298} polymorphism (TT: 2.49 ± 0.50 vs. GG: 0.61 ± 0.41, $P < 0.01$) and in patients carrying intron 18 minor allele (CC: 2.56 ± 0.37 vs. AA: 0.78 ± 0.53, $P < 0.01$). FBF after reactive hyperemia was significantly reduced in the homozygous carriers of the minor allele compared with the wild-type and heterozygous subjects (TT: 7.48 ± 4.33 vs. GG: 20.22 ± 5.01 ml·100 ml $forearm^{-1}\cdot min^{-1}$, $P < 0.001$; and CC: 7.47 ± 4.33 vs. AA: 19.26 ± 6.36 ml·100 ml $forearm^{-1}\cdot min^{-1}$, $P < 0.001$). Figure 2 depicts angiographic parameters according to the genotypic characterization of the subjects of *group 2*. MLD at follow-up was significantly reduced in subjects carrying the two polymorphisms (TT: 1.09 ± 0.21 vs. GG: 2.57 ± 0.43 mm, $P < 0.02$; and CC: 1.15 ± 0.22 vs. AA: 2.30 ± 0.31 mm, $P < 0.05$). Evaluation of the loss index demonstrated a 100% increase in both groups (TT: 0.81 ± 0.36 vs. GG: 0.41 ± 0.26, $P < 0.05$; and CC: 0.77 ± 0.38 vs. AA: 0.46 ± 0.29, $P < 0.05$). Interestingly, length of stenosis at follow-up increased threefold in the homozygous carriers of the minor allele for the two SNPs (TT: 19.2 ± 6.2 vs. GG: 6.1 ± 0.7 mm, $P < 0.005$; and CC: 17.9 ± 6.3 vs. AA: 8.3 ± 0.9 mm, $P < 0.04$).

Table 2. *Haplotype frequencies according to genotypes*

Haplotype	*Group 1*	Control Subjects	χ^2	*P*	OR	95% CI
GA	0.545	0.648	14.107	0.0002	1	
GC	0.014	0.020	0.644	0.422	0.83	(0.42–1.65)
TA	0.130	0.121	0.247	0.619	1.28	(0.98–1.66)
TC	0.311	0.212	16.375	0.00005	1.74	(1.43–2.12)
Haplotype	*Group 2*	Control Subjects	χ^2	*P*	OR	95% CI
GA	0.564	0.648	4.736	0.0295	1	
GC	0.011	0.020	0.64	0.4237	0.63	(0.31–1.30)
TA	0.125	0.121	0.021	0.8835	1.19	(0.91–1.55)
TC	0.300	0.211	6.879	0.0087	1.63	(1.34–1.98)
	Group 2					
Haplotype	Restenosis	No restenosis	χ^2	*P*	OR	95% CI
GA	0.359	0.629	11.284	0.0008	1	
GC	0.001	0.013	0.58	0.4465	0.14	(0.03–0.79)
TA	0.141	0.118	0.178	0.6727	2.09	(1.61–2.72)
TC	0.499	0.240	12.23	0.0005	3.64	(3.04–4.36)

Haplotype frequencies according to genotypes in exon 7 (Glu^{298} Asp, rs1799983) and intron 18 (rs753482, position 150337316) in *group 1* and control subjects, in *group 2* and control subjects, and in *group 2* with or without restenosis. G is the major allele of Glu^{298} Asp polymorphism, whereas T is the minor allele; A is the major allele for intron 18 (rs753482) polymorphism, whereas C are is the minor allele. 95% Confidence intervals (CI) are adjusted for age, sex, BMI, and smoking status.

Table 3. *Clinical and metabolic characteristics of group 1 subjects as a function of Glu^{298} Asp and rs753482 variants*

Glu^{298} Asp	GG	GT	TT	*P*
n	111	151	71	
Glucose, mg/dl	98±15	97±15	97±17	NS
Insulin, μU/ml	9.8±5.2	9.7±4.7	13.9±8.5	0.005
HOMA-IR	2.4±1.4	2.4±1.3	3.4±2.3	0.005
Total insulin AUC, μU/ml at 120 min	7,828±4,149	7,941±4,431	9,699±5,790	0.05
HbA1c, %	5.7±0.9	5.6±0.5	5.8±0.6	NS
NOx, μmol/l	18.9±11.3	20.5±16.7	21.7±12.5	NS
Leptin, ng/ml	10.0±6.1	9.6±5.1	9.2±6.8	NS
Adiponectin, ng/ml	11.9±4.4	11.8±4.8	11.5±3.5	NS
L/A ratio	0.94±0.57	0.92±0.77	0.86±0.32	NS
Basal FBF, ml·100 ml forearm^{-1}·min^{-1}	2.79±1.08	2.84±0.93	2.72±1.08	NS
Reactive hyperemia, ml·100 ml forearm^{-1}·min^{-1}	18.76±6.73	14.47±4.71	8.74±5.63	0.01
NMD, ml·100 ml forearm^{-1}·min^{-1}	16.01±4.41	14.90±4.72	15.18±6.42	NS
rs753482	AA	AC	CC	*P*
n	164	122	47	
Glucose, mg/dl	97.1±15.3	97.4±15.4	97.6±17.4	NS
Insulin, μU/ml	9.7±4.9	10.0±5.2	15.7±8.9	0.005
HOMA-IR	2.3±1.3	2.5±1.5	3.9±2.4	0.005
Total insulin AUC, μU/ml at 120 min	7,660±3,558	7,970±4,954	11,460±6,366	0.001
HbA1c, %	5.7±0.8	5.6±0.6	5.8±0.6	NS
NOx, μmol/l	18.9±11.3	20.3±16.7	21.7±11.6	NS
Leptin, ng/ml	9.7±5.7	9.5±5.2	9.8±5.8	NS
Adiponectin, ng/ml	11.8±4.5	11.7±4.4	12.0±4.3	NS
L/A ratio	0.98±0.70	0.87±0.68	0.86±0.32	NS
Basal FBF, ml·100 ml forearm^{-1}·min^{-1}	2.82±0.86	2.77±0.88	2.75±0.94	NS
Reactive hyperemia, ml·100 ml forearm^{-1}·min^{-1}	19.08±4.65	15.32±5.62	7.57±4.53	0.01
NMD, ml·100 ml forearm^{-1}·min^{-1}	15.44±5.16	15.60±3.34	14.04±5.12	NS

Values are means ± SD. GG are the homozygous subjects for the major allele of Glu^{298} Asp polymorphism, GT are the heterozygous subjects, and TT are the homozygous subjects for the minor allele; AA are the homozygous subjects for the major allele for intron 18 (rs753482) polymorphism, AC are the heterozygous subjects, and CC are the homozygous subjects for the minor allele. NS, not significant.

DISCUSSION

Based on the results of the present study, it appears that fasting hyperinsulinemia, the increased total integrated plasma insulin to the oral glucose challenge, and an impaired forearm vascular reactivity highly associated with the two SNPs of the NOS3 gene were the physiological changes that most distinguished subjects with CAD from the control population. In a subgroup of subjects with CAD affected by unstable angina and differentiated for the presence (or not) of restenosis after quantitative angiography, in addition to the previously described metabolic and vascular alterations, a different profile of adipokines and an altered L/A ratio was associated with the presence of NOS3 gene variants, suggesting a more incipient and pronounced atherosclerotic profile in these subjects.

The new finding of the present study relates to the fact that CAD subjects homozygous for the two NOS3 SNPs have metabolic changes that certainly contribute to the adverse vascular outcome. The main metabolic change is the fact that

Table 4. *Clinical and metabolic characteristics of group 2 subjects as a function of Glu^{298} Asp and rs753482 variants*

Glu^{298} Asp	GG	GT	TT	*P*
n	35	52	19	
Glucose, mg/dl	94±17	99±12	98±15	NS
HOMA-IR	2.3±1.2	2.4±0.8	4.7±2.8	0.0005
HbA1c, %	5.5±0.6	5.6±0.5	5.8±0.5	NS
NOx, μmol/l	18.2±10.4	22.4±18.3	25.4±12.4	NS
Basal FBF, ml·100 ml forearm^{-1}·min^{-1}	3.00±0.76	2.70±0.82	2.70±1.17	NS
NMD, ml·100 ml forearm^{-1}·min^{-1}	15.86±6.16	15.10±4.08	15.32±4.72	NS
rs753482	AA	AC	CC	*P*
n	56	34	16	
Glucose, mg/dl	98.2±15.0	96.4±12.5	98.2±15.3	NS
HOMA-IR	2.5±1.1	2.3±0.8	4.9±1.8	0.005
HbA1c, %	5.6±0.6	5.6±0.4	5.9±0.5	NS
NOx, μmol/l	21.2±17.9	21.9±12.8	21.9±10.9	NS
Basal FBF, ml·100 ml forearm^{-1}·min^{-1}	2.83±0.79	2.87±0.84	2.70±1.07	NS
NMD, ml·100 ml forearm^{-1}·min^{-1}	16.40±5.51	13.60±3.27	15.52±4.72	NS

Values are means ± SD. Genotypes are as defined in Table 3.

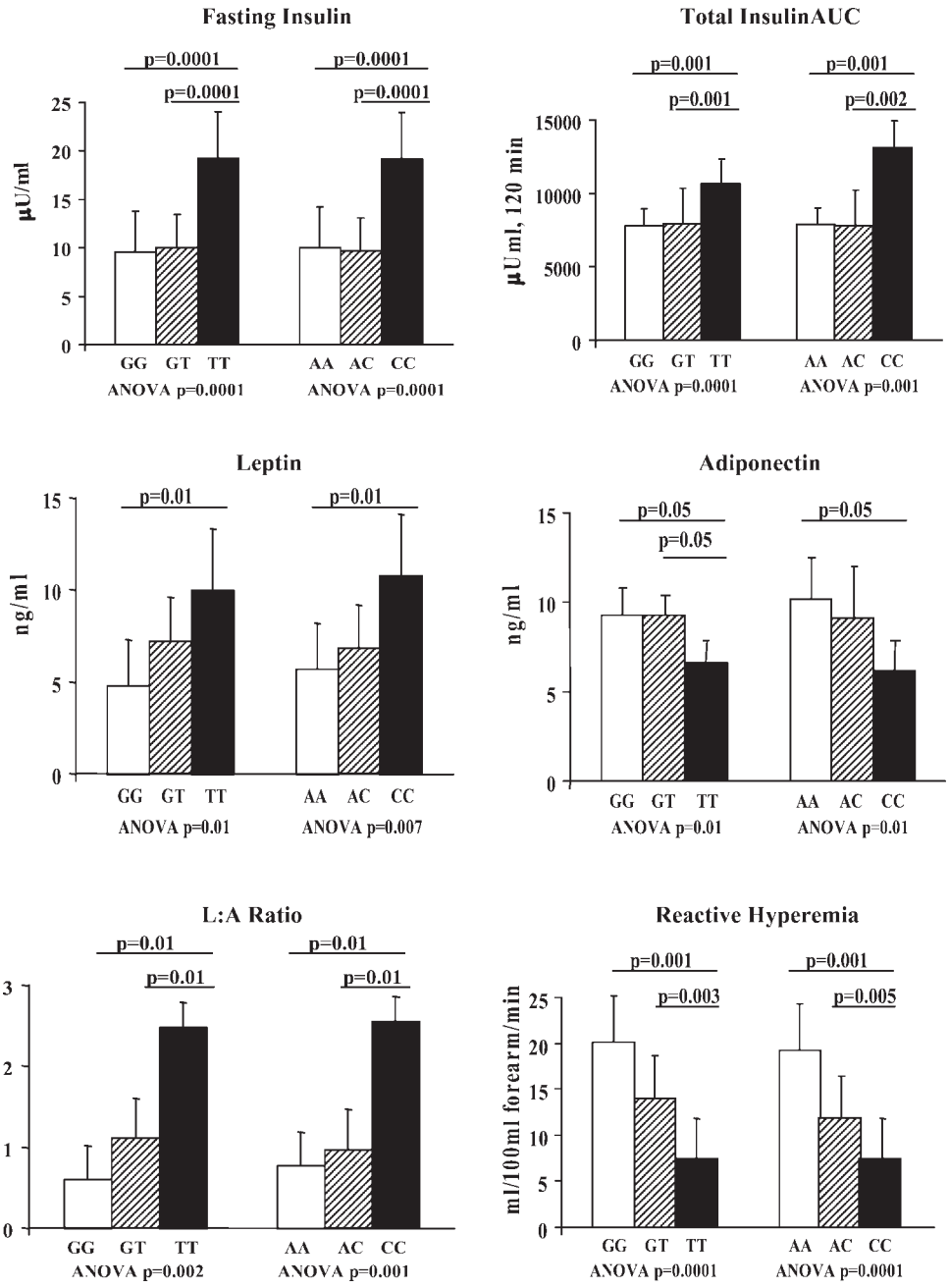

Fig. 1. Results of *group 2* regarding fasting insulin, total integrated area under the curve for insulin (total insulin AUC), leptin, adiponectin, leptin-adiponectin ratio (L/A ratio), and reactive hyperemia according to the genotypes in exon 7 ($Glu^{298}Asp$, rs1799983) and intron 18 (rs753482, position 150337316). GG, homozygous subjects for the major allele of $Glu^{298}Asp$ polymorphism (n = 35); GT, heterozygous subjects (n = 52); TT, homozygous subjects for the minor allele (n = 19); AA, homozygous subjects for the major allele of intron 18 (rs753482) polymorphism (n = 56); AC, heterozygous subjects (n = 34); CC, homozygous subjects for the minor allele (n = 16). All data are means ± SD.

homozygous subjects for the two SNPs are more insulin resistant/hyperinsulinemic/glucose intolerant. None of the subjects with CAD enrolled in this study had known diabetes, and subjects with a diabetic answer after the oral glucose challenge were excluded from the study, since it is known that type 2 diabetes mellitus has well-known detrimental effects on insulin resistance and endothelial dysfunction. From the CAD population admitted to the study, almost 50% had IGT, based on their response to an oral glucose challenge.

These data are in line with previous reports on the increased incidence of glucose intolerance in subjects with CAD. In fact, in a large cohort of Italian subjects with a myocardial infarction within the previous 3 mo but free of diabetes, the annual incidence rate of diabetes or IGT was threefold higher than in population-based cohorts (24). Furthermore, subjects that were homozygous for the two SNPs had higher fasting and post-glucose challenge insulin concentrations than either the wild-type or heterozygous individuals. As such, these findings are consistent with our previous demonstration of an association between hyperinsulinemia/insulin resistance and NOS3 polymorphisms in $Glu^{298}Asp$ and intron 18 in nondiabetic individuals homozygous for both mutations, compared with double wild-type homozygous individuals (23). In addition, reactive hyperemia, an index of vascular reactivity, was decreased in subjects homozygous for the two SNPs. The present study reinforces previous evidence demonstrating that the presence of hyperinsulinemia (32, 33, 42) and peripheral endothelial dysfunction are predictors of cardiovascular events (14, 27), linking these findings with a specific genetic background, i.e., NOS3 gene variants.

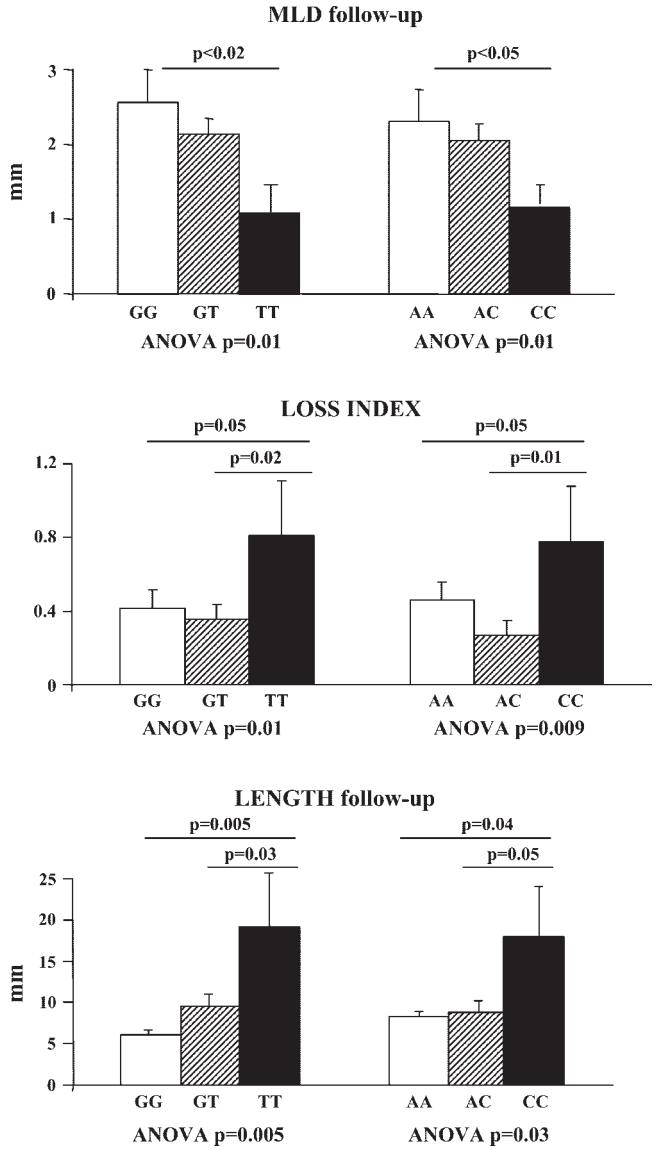

Fig. 2. Results of *group 2* regarding minimal lumen diameter (MLD) at follow-up, length of stenosis at follow-up, and loss index according to the genotypes in exon 7 ($Glu^{298}Asp$, rs1799983) and intron 18 (rs753482, position 150337316). Genotypes are as defined in Fig. 1 legend, and data were obtained from the same number of subjects. All data are means ± SD.

The results of the current study provided new insight into the metabolic and vascular characterization of subjects who evidenced restenosis and the presence of polymorphisms of the NOS3 gene, in addition to insulin resistance and endothelial dysfunction also demonstrated in asymptomatic CAD subjects. Previously, Wu et al. (46) demonstrated that subjects with in-stent restenosis 6 mo after coronary stenting had impairment in NO-dependent endothelial vasodilation compared with subjects without restenosis submitted to a similar procedure. Patti et al. (30) found a direct involvement of endothelial function in the process of restenosis, as represented by a significant decrease of reactive hyperemia. The results of the present study, even if confirmatory of the previous studies, give a new light in the process of restenosis, showing a relationship between NOS3 SNPs and the reduction of reactive hyperemia.

The novelty of the present study is the direct correlation between NOS3 polymorphisms and the altered adipokine pattern, suggesting a new trait of the subjects carrying NOS3 gene variants, characterized by a more aggressive vascular atherosclerosis that could help to explain their increased degree of restenosis. In fact, in the present study, subjects homozygous for the two SNPs evidenced increased leptin and decreased adiponectin levels, an impaired L/A ratio accompanied by lower MLD at follow-up, and, conversely, length of stenosis nearly doubled.

Leptin has emerged as a metabolic hormone that contributes importantly to regulation of vascular biology, and a leptin modulation of endothelial NO synthesis has been reported (9, 34). Our group previously demonstrated increased leptin levels in the presence of in-stent restenosis (32). Moreover, it is well known that adiponectin has an important role in the atherosclerosis process. Adiponectin-null mice appeared to have a proinflammatory state and showed profound neointimal formation in mechanically injured arteries (19, 22), whereas adiponectin supplement attenuated neointimal thickening, suggesting important antiatherogenic properties of adiponectin and a role of adiponectin in preventing restenosis after vascular intervention, as reported previously (29).

L/A ratio is a new atherosclerotic index previously demonstrated in obese and type 2 diabetic subjects (18) and recently related to carotid intima-media thickness (26). In the present study, L/A ratio appears to be a better marker for the progression of arterial sclerosis than the measurements of leptin and adiponectin alone, since L/A ratio showed a staircase increase with a significant difference not only between those homozygotes for the two SNPs and the wild-type individuals but also between the homozygous and the heterozygous individuals. These data seem to reinforce the clinical significance of L/A ratio in CAD and restenosis.

Based on the data of the present study, it seems reasonable to suggest that these two polymorphisms in the NOS3 gene represent a common genetic link to insulin resistance, endothelial dysfunction, CAD, and restenosis. An analysis of Framingham data demonstrated that insulin resistance was independently associated with incident CAD over 7 yr of follow-up (38). Since adipokines have been found to have a significant role in atherosclerosis, the demonstration of an altered adipokines pattern, related to NOS3 variants, on the restenosis process seems particularly interesting.

The results of this study provide evidence of the importance of NOS3 $Glu^{298}Asp$ and intron 18 polymorphisms in CAD, and to some extent, a stronger association with NOS3 polymorphisms and the development of in-stent restenosis 6 mo after coronary stenting are confirmatory of previous studies in CAD with or without restenosis (8, 12, 13, 40), and the per allele ORs found in the present study were superimposable to those reported in a large meta analysis (23). On the contrary, our results differed from those of Zhang et al. (47), who observed no association of $Glu^{298}Asp$ polymorphism and CAD risk in diabetic men. To try to reconcile the latter and our present results, it must be considered that in type 2 diabetes mellitus, other risk factors are more prevalent and could mask the influence of NOS3 variants on CAD.

Regarding the association between intronic variants and CAD risks, previously Zhang et al. (47) demonstrated a significant association between an intron 8 polymorphism and

CAD risk. In addition, it was demonstrated that high numbers of CA repeats in intron 13 of NOS3 gene were associated with CAD risk (39) and were accompanied by truncated, dominant negative splice variants of NOS3 gene with a potential functional effect (20). Also in the present study, even if not yet demonstrated, it is possible to postulate that intron 18 allele has a functional significance affecting mRNA stability and enzyme levels by affecting splicing. Because of the specific position, this polymorphism might have an influence on the regulatory region Ser^{1177} in the reductase domain, determining an alteration of NOS3 activity (11). It is also possible that this intronic variant could act as a marker for another functional polymorphism in NOS3 gene or be in linkage disequilibrium with certain SNPs in genes within this area implicated in both insulin resistance and the atherosclerotic process, i.e., leptin gene, insulin-induced gene-1. However, all these issues were beyond the scope of the present study and need further investigation.

In conclusion, our data suggest that insulin resistance/hyperinsulinemia and endothelial dysfunction are strongly associated with two polymorphisms of the NOS3 gene (i.e., $Glu^{298}Asp$ and intron 18 polymorphisms) in subjects with coronary artery disease. In the presence of restenosis, in addition to the previously described metabolic and vascular alterations, a more atherogenic profile is linked to the two polymorphisms of the NOS3 gene. The genetic basis represented by NOS3 variants might help to explain the relationship among insulin resistance, hyperinsulinemia, endothelial dysfunction, coronary artery disease, and accentuated restenosis following coronary stenting.

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