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The *adiponectin* gene *SNP+276G>T* associates with early-onset coronary artery disease and with lower levels of adiponectin in younger coronary artery disease patients (age ≤ 50 years)

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Abstract Adiponectin, an adipocyte-derived protein, is an essential modulator of insulin sensitivity and several studies suggest an important role of adiponectin in the processes leading to atherosclerosis, thus indicating the *adiponectin* gene as a potential candidate for coronary artery disease (CAD). In the present study we have studied the association between two single nucleotide polymorphisms (SNPs) ($+45T>G$ and $+276G>T$) of the *adiponectin* gene and CAD, looking also into the possible influence of these SNPs on adiponectin plasma levels. The SNPs were analysed in a first cohort of 595 subjects, 325 with CAD and 270 matched controls. We observed a significant association ($p<0.001$) between the SNP $+276G>T$ in the *adiponectin* gene and CAD. In multivariate analysis, carriers of



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the $+276G>T$ SNP had an odds ratio (OR) for CAD of 4.99 ($p<0.0007$). A strong interaction between the $+276G>T$ SNP and age was also present (OR, 1.03; $p<0.0001$). The increase in CAD risk was most evident among individuals with early-onset CAD (age ≤ 50 years), whereas in older CAD subjects other factors, and not the adiponectin SNP, were the major determinants. Furthermore, in CAD subjects with early-onset disease this SNP was also a significant determinant of lower levels of serum adiponectin levels. This association resulted independent from the other variables known to be associated with CAD in our pop-

ulation, including sex, body mass index, high-density lipoprotein and Homeostasis Model Assessment for insulin resistance. To confirm the results the +276G>T SNP was analysed in a second cohort of CAD and controls. The difference between CAD and controls in the +276G>T SNP frequencies showed a similar trend as before, although not significant. The combination of the two cohorts (1,046 subjects: 580 CAD and 466 controls) showed a statistically significant association, particularly in CAD subjects with early-onset of disease. In addition, we confirmed that in younger CAD subjects the SNP was a significant determinant of lower levels of adiponectin. In view of these results, it could be speculated that the *adiponectin* gene variant, or a mutation in linkage with it, determines lower *adiponectin* gene expression, causing in turn an increased risk to develop insulin resistance, atherosclerosis and cardiovascular disease. The significant association of the *adiponectin* gene in subjects with early-onset CAD also suggests that that genetic factors for late-onset diseases may exert a greater influence in younger persons, when other risk factors are not as prevalent as in older age groups.

Keywords *Adiponectin* gene · CAD · HOMA_{IR} · Body mass index (BMI) · Real-time PCR · Single nucleotide polymorphisms (SNPs)

Introduction

Coronary artery disease (CAD) accounts for roughly one-half of all cardiovascular deaths and is a major cause of morbidity and mortality. Genetic and epidemiological studies strongly suggest that CAD is, at least in part, genetically determined. Between the genetic determinants of CAD, an important role is played by genes determining susceptibility to insulin resistance, which is considered the core factor in the pathogenesis of common disorders such as type 2 diabetes, the metabolic syndrome and atherosclerosis, all leading to CAD.

Adiponectin, an adipocyte-derived protein, is an important modulator of insulin sensitivity and it has been shown to play a role in both human and animal models of insulin resistance. The protein is abundant in human plasma, accounting for ~0.01% of total plasma proteins [1]. In patients with diabetes, obesity and coronary artery disease (CAD), adiponectin levels have been found consistently to be lower than in control subjects [2–5]. Several experimental studies suggest an important role of adiponectin in the atherosclerotic process. In knockout mice, lack of adiponectin expression determines severe neointimal thickening and increased proliferation of vascular smooth cells in injured arteries [6]. Adiponectin has also been shown to accumulate in the subendothelial space of an injured human artery [1]. These results are confirmed by the demonstration that adiponectin acts as a modulator of vascular remodelling [7], inhibiting proliferation and migration of human aortic smooth cells. Furthermore, it has been shown that human recombinant adiponectin suppresses the expression of endothelial adhesion molecules, the production

of TNF- α by macrophages, and macrophages-to-foam cell transformation [7–9].

Together with these direct effects on the atherosclerotic and inflammatory processes, adiponectin has been shown to modulate insulin sensitivity, acting in the homeostatic control of glucose, lipid, and energy metabolism. Adiponectin stimulates fatty acid oxidation, decreases plasma triglycerides, and improves glucose metabolism by increasing insulin sensitivity [10]. Also, treatment with adiponectin improves insulin resistance in diabetic mice [11, 12], and administration of thiazolidinediones (TZD), an insulin-sensitising class of drugs, to insulin-resistant subjects significantly increases plasma adiponectin levels, and this effect is correlated with the amelioration of insulin resistance in these subjects [12].

Genetic studies suggest a possible role for the *adiponectin* gene in the susceptibility to insulin resistance, type 2 diabetes and obesity: several SNPs have been associated to an increased risk to develop insulin resistance and diabetes [13–18]. A few studies [14, 17] have also demonstrated that the intronic +276 SNP of the *adiponectin* gene is associated with insulin resistance in the Italian population. This SNP also associates with lower adiponectin levels in different populations [16, 17, 19]. Moreover, in the study of Menzaghi et al. [14] a haplotype of the *adiponectin* gene (which includes the two SNPs +45T>G in exon 2 and +276 G>T in intron 2), was associated in Italian non-diabetic individuals with several features of insulin resistance, including low serum adiponectin levels. As a possible candidate gene for CAD, a very recent study on the *adiponectin* gene I164T mutation reported association with the metabolic syndrome and with CAD in a Japanese population [20].

All together, these data strongly indicate the *adiponectin* gene as a potential candidate for CAD. In the present study we have studied in a cohort of subjects with CAD and in matched controls, all from the Centre-West Coast of Italy, the association between two SNPs (+45T>G and +276 G>T) of the *adiponectin* gene and CAD, looking also into the possible influence of these SNPs on adiponectin levels. These SNPs were chosen because of the previous associations reported in the Italian population [14, 17]. The +276 G>T SNP was also analysed in a second independent population of CAD and control subjects to confirm the results, and in the whole cohort of 1,046 subjects.

Research design and methods

Subjects A total of 595 Caucasian subjects were studied (denominated group 1). All subjects were recruited in the Centre-West Coast of Italy, most from Rome and its surrounding towns. The 325 coronary subjects were consecutively recruited among subjects undergoing coronary angioplasty or presented with clear evidence of CAD (one or more stenoses greater than 50% in at least one major coronary artery after coronary catheterisation and clinical symptoms of angina). Subjects with concurrent liver or renal disease were not included. Control subjects were 270

unrelated individuals randomly selected from a population of free-living individuals screened for CAD risk factors. Exclusion criteria were the following: (1) the presence of type 2 diabetes or of a first-degree relative with type 2 diabetes, (2) the presence of hypertension, (3) the presence of CAD, (4) the presence of the metabolic syndrome. CAD was excluded by use of the Rose questionnaire [21] and ECG (Minnesota coding). A complete medical history of all subjects was obtained; history taking included questions about smoking habits, history of hypertension and type 2 diabetes and current medication used. Diagnosis of type 2 diabetes was based on history of hypoglycaemic treatment and/or confirmed fasting blood glucose >126 mg/dl (7.0 mmol/l) [22]. The metabolic syndrome was diagnosed according to WHO criteria [23]. Diagnosis of hypertension was based on the presence of elevated systolic (>140 mmHg) and/or diastolic (>90 mmHg) blood pressure and/or the current use of antihypertensive medications. The Homeostasis Model Assessment for insulin resistance (HOMA_{IR}) was calculated as described by Matthews et al. [24].

To confirm the results, a second cohort of 451 subjects (denominated group 2) was recruited from the same geographical area. The 255 CAD subjects and 196 control subjects were selected with the same criteria as for group 1.

All subjects gave their written informed consent to their participation to the study. The study was approved by the University of Rome "La Sapienza" Ethical Committee.

Laboratory measurements Cholesterol and triglyceride concentrations in total plasma and lipoprotein fractions were measured with a Technicon RA-1000 Autoanalyzer. High-density lipoprotein (HDL) cholesterol was determined in the whole plasma after precipitation of apoB-containing lipoproteins with phosphotungstic acid/MgCl₂. Plasma glucose was determined by the glucose oxidase method [Autoanalyzer, Beckman Coulter, USA; coefficient of variation (CV), 1.9±0.2%]. Plasma insulin concentration was measured on frozen samples using a radioimmunoassay (Biodata Insulin Kit, Milan, Italy) with an interassay CV of 7.5%.

Serum adiponectin levels were measured by commercial radioimmunoassay (LINCO Research Inc., USA), according to manufacturer instructions.

Mutation detection using fluorescence resonance energy transfer The SNPs +45T>G in exon 2 and the intronic SNP +276G>T of the human *adiponectin* gene were detected in real-time PCR with LightCycler hybridisation probes, using fluorescent-labelled nucleotides as previously described [17].

Statistical analysis Categorical variables were compared by chi-square or Fisher's exact test. Differences between continuous variables were evaluated by two-tailed Student's *t* test and by ANOVA with age correction [22]. Logarithmic transformation was used to normalise distributions of BMI, HOMA_{IR}, plasma insulin, total and HDL cholesterol, and triglycerides. Genotype distributions and allele frequencies

between the study groups were compared by 2×2 and 2×3 contingency tables and chi-square analysis.

Linkage disequilibrium between the +45T>G and +276G>T SNPs was assessed by calculating the disequilibrium statistic *D'* [25]. The sign of *D'* (positive or negative) depends on the arbitrary choice of the alleles paired at the two loci and indicates whether the same or opposite allelic association is present.

Analysis of covariance (ANCOVA) and multiple regression analysis were applied in all associations between the genetic variants and clinical or biochemical parameters that were significant in the univariate analysis, controlling each significant association for the other variables.

To estimate the association of CAD with the *adiponectin* gene SNPs, odds ratios (i.e. odds of CAD given the presence of the variant genotype) were calculated by logistic regression analysis, after adjustment for other modulators known to affect this condition (including sex, age, BMI, HDL), and that resulted independently associated with CAD in our population. Furthermore, in the regression analysis we tested the interaction between genotypes and age on CAD. For each odds ratio we estimated two-tailed *p* values and 95% confidence intervals (CI). General linear model (GLM) was employed to elucidate the proportion of variance of adiponectin values (dependent variables) explained by the gene variant independently from other variables (including sex, age, BMI, HDL). *p* values <0.05 or less were taken as statistically significant. All statistical analyses were performed with SPSS statistical package.

Results

Analysis in group 1

Clinical and metabolic characteristics of group 1

The clinical characteristics of the study subjects are shown in Table 1. As expected, patients with CAD were older, with a higher prevalence of male sex, hypertension, diabetes and number of smokers. The CAD patients had significantly higher levels of fasting blood glucose, insulin, HOMA_{IR} and triglycerides, and showed significantly lower BMI and HDL cholesterol. To clarify if the difference in HDL cholesterol was due to the difference in gender between CAD and controls, we analysed this data by ANCOVA, with status and gender as covariates on the dependent variables HDL cholesterol. ANCOVA demonstrated that both CAD status (*p*<0.001) and gender (*p*<0.001) were significant determinant of lower levels of HDL. Total and LDL cholesterol were not different between cases and controls, possibly because of lipid-lowering treatment in CAD patients. Circulating adiponectin concentration was measured in a subgroup of 185 subjects (out of the total 595 subjects studied). These subjects included all available carriers of the TT genotype of the +276G>T SNP (47 in total from CAD and controls), 58 heterozygote subjects (27 CAD and 31 controls) and 80 wild-type carriers (25 CAD and 55

Table 1 Clinical characteristics of study subjects CAD and controls

	Group 1		Group 2		All subjects	
	CAD subjects (n=325)	Control subjects (n=270)	CAD subjects (n=255)	Control subjects (n=196)	CAD subjects (n=580)	Control subjects (n=466)
Age (years)	59.1±9.4	53.6±14.8*	61.7±10.4	47.1±12.8*	60.3±10.0	50.9±14.4*
Gender (M/F)	272/53	105/165*	174/81	126/70	446/134	231/235*
Hypertension (%)	44	14*	64	36*	53	23*
Diabetes (%)	13	0*	29	0*	20	0*
Smoking (%)	49	12*	25	26	39	18*
Body mass index (kg/m ²)	26.8±3.6	28.8±6.8*	27.4±3.8	28.4±8.8	27.0±3.7	28.6±7.7*
Blood glucose (mg/dl)	87.9±25.4	80.7±18.0*	118.3±59.5	90.8±17.4*	100.9±45.9	84.9±18.4*
Fasting plasma insulin (μU/ml)	13.3 ±8.0	11.1±8.6**	–	–	–	–
HOMA _{IR}	2.99±2.50	2.35±2.15**	–	–	–	–
Total cholesterol (mg/dl)	218.2±44.6	218.5±40.1	167.6±48.9	198.3±38.2*	197.7±52.6	210.4±40.6*
Total triglycerides (mg/dl)	187.2±118.3	139.8±78.8*	129.3±78.3	114.6±43.8***	163.8±107.7	130.7±69.3*
HDL cholesterol (mg/dl)	41±11.1	57.3±15.0*	40.8±14.1	53.9±18.7*	40.9±12.4	55.9±16.7*
LDL cholesterol (mg/dl)	141.2±40.0	138.0±34.5	101.4±38.7	110.4±38.7	124.7±44.0	125.6±38.9
Adiponectin level (μg/ml) ^a	7.9±4.4	23.8±22.8*	9.2±6.1	11.6±6.7 [†]	8.6±5.5	17.3±13.7*

Fasting plasma insulin and, consequently, HOMA_{IR} were not available for subjects of group 2. Data are given as means±SDM.

Transformation was used to normalise the distributions of BMI, HOMA_{IR}, plasma insulin, total and HDL, cholesterol and triglycerides, but the untransformed values are given in the table. Continuous variables were compared by *t* test and categorical variables by χ^2 test.

**p*<0.0001

***p*=0.002

****p*=0.03

[†]*p*=0.02

^aMeasured in a subgroup of patients selected by genotype, matched by age, sex and BMI

controls), all matched by sex, age and BMI (data not shown). Mean serum adiponectin levels were significantly different (*p*<0.001) between CAD and controls (Table 1). Analysis of covariance showed that both CAD status (*p*<0.0001) and male sex (*p*<0.001) were significantly associated with lower adiponectin levels. This result is in agreement with previous reports, showing low adiponectinemia in patients affected by metabolic and atherosclerotic diseases [2, 20]. All these variables were analysed by logistic regression to determine which factors were independently associated with CAD in this population. Sex, age, BMI, HDL and HOMA_{IR} all resulted independently associated with the disease (data not shown) and were used as covariates in the logistic analysis of the *adiponectin* gene +276G>T SNP.

Genotype and allele frequencies of the *adiponectin* gene+45T>G and+276G>T SNPs

The +45T>G and +276G>T SNPs were detected in real-time PCR with LightCycler hybridisation probes. In each amplification, two controls (one homozygous and one het-

erozygous subject) were added to ensure correct genotyping. Furthermore, some of the DNAs were sequenced to confirm genotypes, resulting always correct. Allele frequencies for both SNPs were in Hardy–Weinberg equilibrium. Genotype and allele frequencies differed significantly between CAD patients and control subjects for the +276G>T SNP of the *adiponectin* gene (Table 2), whereas genotype and allele frequencies were not significantly different for the +45T>G SNP (data not shown). Estimation of linkage disequilibrium between the two SNPs by the disequilibrium statistic *D'* showed that the two SNPs are in strong linkage disequilibrium (*D'*=-1, *p*<0.01). The +45/+276 haplotypes showed identical associations as the +276G>T SNP, thus, results are presented for this single SNP.

Logistic regression analysis of +276G>T SNP and CAD

To estimate the independent contribution of the +276G>T SNP of the *adiponectin* gene to CAD, we performed multivariate analysis to control for the confounding effect of

Table 2 Genotype distributions and allele frequencies for *adiponectin* gene SNPs in CAD subjects and control subjects

	Number	+276G>T SNP genotypes			Allele frequencies	
		GG	TG	TT	G	T
Group 1^a						
CAD subjects	325	152 (46.8)	145 (44.6)	28 (8.6)	0.69	0.31
Control subjects	270	155 (57.4)	96 (35.6)	19 (7)	0.75	0.25
Group 2^b						
CAD subjects	255	135 (53)	96 (37.6)	24 (9.4)	0.72	0.28
Control subjects	196	111 (57)	71 (36)	14 (7)	0.75	0.25
All subjects^c						
CAD subjects	580	287 (49.0)	241 (42.0)	52 (9.0)	0.70	0.30
Control subjects	466	266 (57)	167 (36)	33 (7)	0.75	0.25

Values in parentheses are percentages. Genotype distributions and allele frequencies were compared by chi-square analysis

^aCAD vs control subjects: genotypes, $\chi^2=6.7$, $df=2$, $p=0.035$; allele frequencies, $\chi^2=5.14$, $df=1$, $p=0.023$

^bCAD vs control subjects: genotypes, $\chi^2=0.93$, $df=2$, $p=NS$ (not significant); allele frequencies, $\chi^2=0.85$, $df=1$, $p=NS$

^cCAD vs control subjects: genotypes, $\chi^2=6.1$, $df=2$, $p=0.047$; allele frequencies, $\chi^2=5.6$, $df=1$, $p=0.018$

other modulators previously shown in the logistic analysis to be independently associated with CAD (sex, age, BMI, HDL and HOMA_{IR} were all included). As shown in Table 3 the +276G>T SNP was also independently associated with CAD. Furthermore, since the strongest effect on CAD was most evident for age, in the regression model we included the interaction between age and the +276G>T SNP (Table 3). The interaction between age and the +276G>T SNP was even a stronger determinant of CAD ($p<0.0001$).

Assuming that a genetic effect might be more evident in younger subjects, we analysed the possible influence of the +276G>T SNP according to age, stratifying the CAD population in subjects ≤ 50 years of age (the early-onset CAD group), and ≥ 51 years of age (the older CAD group). These age limit was based on the incidence curves of CAD and the estimates of genetic effect at various ages [26, 27]. Logistic regression analysis (Table 4) demonstrated that sex, BMI, HDL and the +276G>T SNP had the strongest effect in the young CAD group, whereas in the older CAD group the *adiponectin* gene variant was not significantly associated. In this group HOMA_{IR} was instead a significant determinant.

Table 3 Logistic regression analysis of determinants of CAD in group 1

Variables	OR	95% CI	<i>p</i>
Sex	4.11	2.3–7.1	<0.00001
Age	1.06	1.04–1.08	<0.00001
BMI	1.12	1.07–1.18	<0.0003
HDL	1.09	1.06–1.11	<0.0001
HOMA _{IR}	1.41	1.18–1.69	<0.0001
+276G>T carriers ^a	4.99	1.53–16.2	<0.0007
Age +276G>T	1.03	1.00–1.05	<0.0001

For sex, age and HDL, ORs indicate the increase in the relative risk of CAD for each unitary increase in the variable

^aThe relative risk associated with carriers of T allele of +276G>T SNP has been estimated vs GG genotype

Serum adiponectin level and +276G>T SNP

To test for the possible effect of the +276G>T SNP in the CAD group with age ≤ 50 years on adiponectin levels, circulating adiponectin concentrations were compared between TT + TG carriers (assuming a codominant model of inheritance) and GG carriers.

A significant difference between the two groups was observed, with TT + TG carriers showing lower levels of serum adiponectin (15.5 ± 13 ng/ml) compared to GG carriers (22.6 ± 15 ng/ml, $p<0.03$). When the effect of the +276G>T SNP of the *adiponectin* gene on serum adiponectinemia was analysed by GLM together with the other variables shown to be associated with CAD (sex, BMI, HDL) in the younger CAD group, it resulted independently associated ($F=0.582$, $p<0.02$) together only with BMI ($F=7.19$, $p<0.01$), thus suggesting a possible influence of the +276G>T SNP on adiponectin levels in these younger CAD subjects. This association was not significant in subjects with age ≥ 51 years.

Confirmation of the results in group 2 and in the whole population

Clinical and metabolic characteristics of group 2 and all subjects

In Table 1 are shown the clinical characteristics of group 2. CAD and control subjects were significantly different in age, hypertension and diabetes. In this group sex distribution was not different between CAD and controls, whilst an imbalance of male and female individuals was present in group 1. The CAD subjects showed higher levels of fasting blood glucose and triglycerides, and lower levels of HDL. Again HDL levels were determined (ANCOVA) by gender ($p<0.0001$) and CAD status ($p<0.0001$). Circulating adiponectin concentration was measured in a subgroup of 175 subjects (91 CAD and 84 controls) all matched by

Table 4 Logistic regression analysis of determinants of CAD in subjects of group 1 with age ≤ 50 and in subjects with age ≥ 51

Variables	Subjects with age ≤ 50 ($n=175$)			Subjects with age ≥ 51 ($n=420$)		
	OR	95% CI	<i>p</i>	OR	95% CI	<i>p</i>
Sex	2.95	1.54–5.64	<0.0011	2.08	1.51–2.88	<0.0001
BMI	1.17	1.05–1.31	<0.006	1.09	1.00–1.18	<0.051
HDL	1.10	1.04–1.15	<0.0002	1.09	1.06–1.11	<0.0001
HOMA _{IR}	0.84	0.64–1.11	NS	1.75	1.33–2.3	<0.0001
+276G>T carriers ^a	2.05	1.19–3.55	<0.0098	0.93	0.51–1.70	NS

For sex and HOMA_{IR} ORs indicate the increase in the relative risk of CAD for each unitary increase in the variable
NS not significant

^aThe relative risk associated with carriers of T allele of +276G>T SNP has been estimated vs GG genotype

sex, age and BMI. This subgroup was selected according to genotypes of the +276G>T SNP (CAD: T carriers=50, GG=41; controls: T carriers=40, GG=44). ANCOVA confirmed that serum adiponectin levels were significantly different ($p < 0.02$) between CAD and controls, and they were also different between ($p < 0.001$) males and females (data not shown).

The clinical characteristics of the all 1,046 subjects are summarized in Table 1 and the differences between 580 CAD and 466 controls subjects were significant for age, sex, hypertension, diabetes and smoke. Also, BMI, blood glucose, triglycerides were significantly higher in CAD subjects whereas in these patients HDL and serum adiponectin were significantly lower, with both CAD status and gender being significant determinants.

As for group 1 all these variables were analysed by logistic regression to determine which factors were independently associated with CAD in the whole population. Again sex, age, BMI and HDL resulted independently associated with the disease (data not shown) and were used as covariates in the logistic analysis of the *adiponectin* gene +276G>T SNP.

Genotype and allele frequencies for the +276G>T SNP of group 2 and all subjects

Genotype distribution and allele frequencies for group 2 and all subjects are shown in Table 2. In group 2, genotype and allele frequencies showed a similar trend as in group 1, although it did not reach significance. When the two groups were analysed together, the genotype and allele

frequencies differed significantly ($p=0.047$ and $p=0.018$, respectively) between CAD patients and control subjects for the +276G>T SNP.

Logistic regression analysis of +276G>T SNP and CAD in all subjects

To confirm that the +276G>T SNP was independently associated with CAD we then repeated the logistic analysis in all subjects. We confirmed that +276G>T SNP carrier status (OR, 5.96; CI, 1.14–31.04; $p=0.034$), sex (OR, 1.65; CI, 1.00–2.75; $p=0.05$), BMI (OR, 1.05; CI, 1.01–1.08; $p=0.01$), HDL (OR, 1.09; CI, 1.07–1.11; $p < 0.0001$) and the interaction between age and +276G>T SNP (OR, 1.03; CI, 1.00–1.06; $p=0.049$) were all significant determinants of CAD disease. As for group 1, we performed logistic regression analysis in all subjects stratifying CAD patients in subjects ≤ 50 years of age and ≥ 51 years of age (Table 5). We confirmed that the +276G>T SNP was significantly associated only in CAD subjects with early-onset disease, together with sex, BMI and HDL.

Serum adiponectin level and +276G>T SNP in the whole CAD population

Figure 1 shows adiponectin levels in younger CAD patients (≤ 50 years) divided in TT + TG carriers and GG carriers. T allele carriers had significantly ($p < 0.018$) lower levels of serum adiponectin (13.3 ± 11.2 ng/ml) compared with GG carriers (19.1 ± 13.8 ng/ml) confirming the signif-

Table 5 Logistic regression analysis of determinants of CAD in all 1046 subjects, divided by age ≤ 50 and by age ≥ 51

Variables	Subject with age ≤ 50 ($n=339$)			Subject with age ≥ 51 ($n=707$)		
	OR	95% CI	<i>p</i>	OR	95% CI	<i>p</i>
Sex	11.47	3.29–39.96	<0.0001	2.08	1.51–2.88	<0.0001
BMI	1.13	1.06–1.20	<0.0002	1.02	0.98–1.07	NS
HDL	1.11	1.07–1.15	<0.0001	1.09	1.07–1.10	<0.0001
+276G>T carriers ^a	2.07	1.05–4.07	<0.035	1.04	0.70–1.54	NS

For BMI and HDL, ORs indicate the increase in the relative risk of CAD for each unitary increase in the variable
NS not significant

^aThe relative risk associated with carriers of T allele of +276G>T SNP has been estimated vs. GG genotype

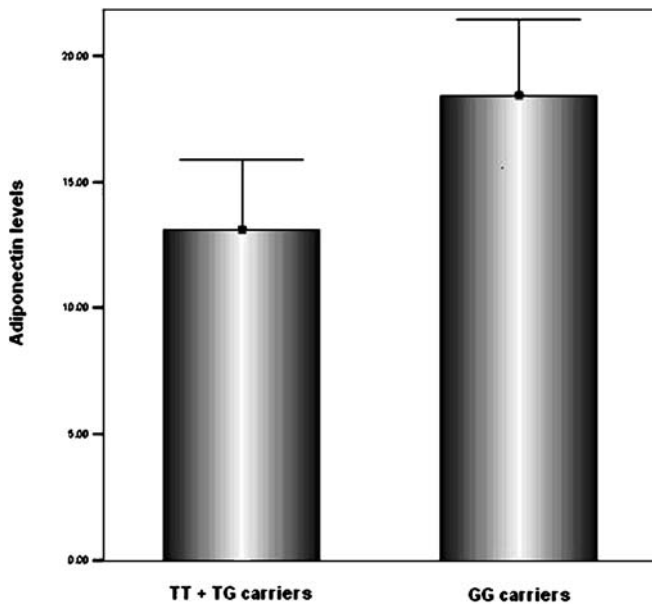


Fig. 1 Adiponectin levels in T allele carriers and GG carriers in younger CAD patients (<50 years). The adiponectin levels in TT + TG carriers were significantly lower (13.3 ± 11.2 ng/ml) compared with GG carriers (19.1 ± 13.8 ng/ml, $p=0.018$). Data are presented as mean \pm SDM

icant difference between the two groups. GLM analysis again confirmed that the $+276G>T$ was independently associated with lower serum adiponectinemia in the younger CAD group ($F=4.68$, $p=0.033$), together with BMI ($F=60.4$, $p<0.0001$) and HDL ($F=10.4$, $p=0.002$). This association was not significant in subjects with age ≥ 51 years.

Discussion

The present study provides evidence of association between the $+276G>T$ SNP of the *adiponectin* gene and CAD in this Italian population. This association was first observed in a relatively small population of 595 subjects. To confirm the results, a second population of CAD and controls was analysed. In this second study group the difference between CAD and controls in the genotype and allele frequencies of the $+276G>T$ SNP showed a similar trend as before, although not significant. However, the combination of the two populations, which has brought the total number of subjects to 1,046 (580 CAD and 466 controls), again showed a statistically significant association. In particular, we observed that the increase in association with CAD was most evident in subjects with early-onset CAD (with age ≤ 50 years) carrying the variant, suggesting that the genetic susceptibility determined by the *adiponectin* gene background may be more pronounced when other risk factors are not as prevalent as in older age groups. In fact, in the first cohort of subjects with age ≥ 51 years one of the strongest risk factor for CAD was the insulin resistance index $HOMA_{IR}$, which was not significant in younger subjects. Thus, it is possible that genetic factors for late-onset diseases may exert

a greater influence in younger persons than in older persons, whereas environmental factors may exert a greater influence in older persons.

Also serum adiponectin levels were associated with the *adiponectin* gene $+276G>T$ SNP, and again this association was significant in subjects with age ≤ 50 years. In accordance with a previous report [20] adiponectin levels were not dependent on BMI, pointing once more toward a stronger genetic effect in these subjects.

To the best of our knowledge this is the first report of such a relationship between *adiponectin* gene and age in the modulation of CAD risk. Furthermore, an interaction between age and *adiponectin* gene was evident also for serum adiponectin levels.

Adiponectin serum levels have been demonstrated consistently to be reduced in patients with diabetes, obesity and coronary artery disease [2–4], suggesting a central role in the mechanisms leading to the metabolic abnormalities present in these disorders. A strong inverse relationship between serum adiponectin and insulin resistance index, lipids and blood pressure has been demonstrated in Japanese subjects [28]. Tschritter et al. [5] have demonstrated that adiponectin levels affect glucose and lipid metabolism independently from body mass, and that interindividual variability is probably genetically determined. Very recently, a large prospective case-control study has demonstrated that high adiponectin concentrations are associated with a lower risk of myocardial infarction [29], suggesting that low adiponectin is not only a marker of cardiovascular risk, but it could also be a causal risk factor. In favour of this hypothesis is the demonstration that adenovirus-mediated increase of adiponectin significantly suppresses the progression of atherosclerotic lesions in apoE-deficient mice [30], an animal model that develops hyperlipidemia and vascular lesions similar to human atherosclerosis.

Our findings support the hypothesis that the $+276G>T$ SNP of the *adiponectin* gene, or another variant in linkage disequilibrium with it, may determine a reduced expression of the protein, which could lead to a reduction of the anti-atherosclerotic actions of adiponectin. Adiponectin has been shown to exert several effects on vascular structure and function, including inhibition of endothelial thickening [31], induction of arterial vasodilation [32], inhibition of macrophage-to-foam cell transformation and suppression of the expression of adhesion molecules and of TNF- α by macrophages [7–9]. It is interesting to note that some of these effects are independent from insulin sensitivity [32], as is suggested also from our results in CAD subjects with age ≤ 50 years, in which the $HOMA_{IR}$ index was not a risk factor for CAD.

How the intronic $+276G>T$ SNP affects *adiponectin* gene function is an open question. SNPs with no apparently biological significance have been shown to affect gene expression, and an effect on mRNA expression has been recently reported for the *adiponectin* gene [33]. This possibility is also suggested by the observation that two independent studies have shown decreased adiponectin levels associated with genetic variants of the *adiponectin* gene [14, 16], including the $+276G>T$ SNP. More likely, another

er possibility is that this SNP is in linkage disequilibrium with another mutation either within, or in other genes close to, the *adiponectin* gene that determines its negative effects.

In summary, we have observed association between the +276G>T SNP in the *adiponectin* gene and CAD, particularly among individuals with early-onset CAD. Furthermore, carriers of the at-risk T allele had significantly lower serum adiponectin levels. In view of these results, it could be speculated that the *adiponectin* gene variant, or a mutation in linkage with it, determines lower *adiponectin* gene expression, causing in turn an increased risk to develop insulin resistance, atherosclerosis and cardiovascular disease. The significant association of the *adiponectin* gene in subjects with early-onset CAD also suggests that genetic factors for late-onset diseases may exert a greater influence in younger persons, when other risk factors are not as prevalent as in older age groups.

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