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## Genetic study of common variants at the Apo E, Apo AI, Apo CIII, Apo B, lipoprotein lipase (LPL) and hepatic lipase (LIPC) genes and coronary artery disease (CAD): variation in LIPC gene associates with clinical outcomes in patients with established CAD

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### Abstract

**Background:** Current evidence demonstrates that positive family history and several alterations in lipid metabolism are all important risk factors for coronary artery disease (CAD). All lipid abnormalities themselves have genetic determinants. Thus, objective of this study was to determine whether 6 genetic variants potentially related to altered lipid metabolism were associated with CAD and with lipid abnormalities in an Italian population. These genetic variables were: *apolipoprotein E (Apo E)*, *Apo AI*, *Apo CIII*, *Apo B*, *lipoprotein lipase (LPL)* and the *hepatic lipase (LIPC)* genes. Furthermore, an 8 years prospective analysis of clinical cardiovascular events was related to the various genetic markers.

**Methods:** 102 subjects with established coronary artery disease and 104 unrelated normal subjects were studied. CAD Patients were followed up for 8 years, and clinical CAD outcomes (a second coronary angioplasty (PTCA), myocardial infarction, coronary artery by-pass graft (CABG), cardiovascular deaths), available from 60 subjects, were related to the genetic variants by multiple regression analysis. Results. Of the six lipid loci studied (for a total of 11 polymorphisms) only the *apolipoprotein E*, *Apo B* and *LIPC* polymorphisms distinguished between case and controls. However, multivariate analysis accounting for clinical and metabolic predictors of CAD showed that only the *ApoB XbaI* and *ApoE4* polymorphism associated with CAD in this Italian population. When lipid parameters were related to genotypes, the *ApoE*, *ApoB*, and *LIPC* gene polymorphisms were associated to various markers of dyslipidaemia in the CAD patients, confirming previous reports. When the occurrence of a second cardiovascular event was related to genotypes, an independent role was observed for the *LIPC gene T202T* variant.

**Conclusions:** variation in *LIPC (hepatic lipase) gene* associates with clinical outcomes in Italian patients with established CAD. Further studies on the *LIPC* gene in CAD patients are warranted, in particular looking at the possible influences on clinical outcomes.

## Background

Coronary artery disease (CAD) accounts for roughly one-half of all cardiovascular deaths and is a major cause of morbidity and mortality. Twin studies [1,2] have demonstrated that the concordance rates for monozygotic twins are higher than those for dizygotic twins and familial aggregation of CAD has long been known [1,3,4]. Current evidence demonstrates that positive family history and several alterations in lipid metabolism, including high LDL (low density lipoprotein) and low HDL (high density lipoprotein) cholesterol levels (separately as well as jointly), high triglycerides levels, high apoB levels, high lipoprotein (a) (Lp(a) levels, are all important risk factors for CAD. All these lipid abnormalities themselves have genetic determinants [5,7]. Lipoprotein levels are partly determined by genes that code for proteins that regulate lipoprotein synthesis, interconversions and catabolism. Mutations in these genes may cause disturbances in one or more of the pathways in lipoprotein metabolism resulting in hyperlipoproteinemia, and some of these disorders lead to premature atherosclerosis.

Marked differences in mortality rates for CAD have been observed within Europe [8], with northern Europeans having the highest incidence. Although environmental factors and dietary habits may account for this difference, a different genetic predisposition may also be involved. Furthermore, genetic loci conferring susceptibility or protection to CAD may differ between populations. Association study designs provide statistical power to reveal the modest contributions of weak alleles, and evidence is mounting that common genetic polymorphisms play a role in complex diseases.

Thus, the associations of 6 genetic variants potentially related to coronary artery disease (CAD) were evaluated in univariate and multivariate models in an Italian CAD population. These variables included: variants at the *apolipoprotein E* (*Apo E*) [9], *Apo AI* and *Apo CIII* [10,11], *Apo B*, *lipoprotein lipase* (*LPL*) [12], and *LIPC* (the gene encoding hepatic lipase) genes [13].

Furthermore, a prospective analysis was performed in our CAD population, evaluating after 8 years from recruitment the occurrence of a second cardiovascular event (a second coronary angioplasty (PTCA), myocardial infarction, coronary artery by-pass graft (CABG), cardiovascular death) in those who had a PTCA at the time of recruitment as sign of progression of the atherosclerotic disease, and these events were related to the various genetic markers.

## Methods

### Subjects

A total number of 206 Caucasian subjects were studied and all subjects were recruited in the Lazio region of Italy.

The 102 coronary subjects (96 men and 6 women) were consecutively recruited among subjects undergoing coronary angioplasty (60% of the sample) or presented with clear evidence of CAD (one or more stenoses greater than 50% in at least one major coronary artery using the Sones and Judkins scoring technique [14] after coronary catheterisation and clinical symptoms of angina). All subjects were recruited in the years 1993–1994. Subjects with concomitant liver or renal disease were not included. Also excluded were those who were on lipid lowering diet and medications.

As controls, we considered 104 age-matched unrelated Caucasian individuals consecutively recruited from a population of individuals screened for CAD risk factors. Exclusion criteria were: 1) age below 45 years, 2) the presence of type 2 diabetes, and 3) the presence of CAD. In these subjects, CAD was excluded by use of the Rose questionnaire and ECG (Minnesota coding) [15]. In both patients and controls a complete medical history was obtained by questionnaire. Diagnosis of type 2 diabetes was based on history of hypoglycaemic treatment and/or fasting blood glucose >126 mg/dl (7 mmol/L) [16]; that of hypertension was based on the presence of elevated systolic (>160 mmHg) and/or diastolic (>95 mmHg) blood pressure and/or the current use of anti-hypertensive medications. This study complies with the Declaration of Helsinki and was authorized by the local ethical committee.

### Lipoprotein analyses

Plasma cholesterol, triglyceride, and lipoproteins were determined by enzymatic methods with commercially available kits (Boehringer Mannheim and Beckman Array Protein System, Beckman Instruments Brea, CA.). HDL-cholesterol was measured after precipitation of apolipoprotein B containing lipoproteins and LDL-cholesterol was calculated according to the Friedewald formula. Apolipoproteins were determined by immunonephelometry using monospecific polyclonal antisera Greiner Biochemica (Flacht, Germany).

### Genetic analyses

DNA was isolated from fresh or frozen EDTA whole blood cells using a spin column method (Nucleon, Scotlab U.K.). Variants at six loci previously shown to be associated with CAD [17–19] were studied, and the 11 variants analysed were: *apolipoprotein A1*: *Xmn1* 5' to the *Apo A1* gene, *Msp1* in intron 3, *Pst1* 3' of the gene; *apolipoprotein B*: *Xba1*; *apolipoprotein CIII*: *Sst1* for C3175G, exon 4, *apolipoprotein E*: *Hha1* for *e2*, *e3*, *e4*; *LIPC*: *Msp1* for Thr202Thr (C to G); and *lipoprotein lipase*: *Hind* 111 in intron 8, *Taq1* for D9N (G to A), *Rsa1* for N291S (A to G), and *Mnl1* for S447X (C to G).

Primers for the polymerase chain reaction were obtained from Genosys, Cambridge, U.K. The primer sequences were derived from published data [17–19].

Genomic DNA (0.2–0.5 ug) was amplified with specific primers for the 11 polymorphisms in 25 ul reaction mixtures according to previously published methods. Five microliters of digestion mixture containing the manufacturer's recommended restriction buffer and 5 U of the above mentioned restriction enzymes were added to the amplification product and incubated as described [17–19].

Genotypes were scored by two independent investigators who did not know whether the samples were from a case patient or from controls.

### 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. Logarithmic transformation was used to normalise distributions of BMI, plasma total and HDL cholesterol, triglycerides and Lp(a) values. Genotype distributions and allele frequencies between the study groups were compared by construction of 2 × 2 and 2 × 3 contingency tables and chi-square analysis. The study was powered to allow detection with 80% and an error rate of 5% for differences in allele frequencies of 11%. The relation between genotypes and concomitant variables were evaluated by ANOVA after age-standardisation.

To estimate the risk of CAD and the progression of CAD associated with gene variants, odds ratios (i.e. odds of CAD given the presence of the variant genotype) were calculated by multiple regression analysis, after adjustment for other modulators known to affect both conditions (including sex, age, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, BMI). For each odds ratio we estimated two-tailed P values and 95% confidence intervals (CI). P values < 0.05 or less were taken as statistically significant.

### Results

#### Clinical and biochemical characteristics of study groups

Table 1 summarises the clinical features and biochemical characteristics of the study groups. The two groups were slightly different in age, and this is the reason why all statistics were age-corrected. As expected, the mean levels of LDL-cholesterol, total triglycerides, Apo AI, Apo AII, Apo CIII, Apo E and Apo B were significantly different in the arterial disease group compared to controls. HDL-cholesterol levels were significantly lower in CAD subjects, but the total plasma cholesterol did not differ between the groups.

Multiple regression analysis of known predictors of CAD confirmed the independent role of age (OR 1.08, 95% CI 1.01–1.15,  $p < 0.02$ ), sex (OR 26.4, 95% CI 4.7–146.5,  $p < 0.01$ ), LDL-cholesterol (OR 1.12, 95% CI 1.05–1.27,  $p < 0.001$ ) and HDL-cholesterol (OR 1.14, 95% CI 1.09–1.23,  $p < 0.01$ ) as risk factors for coronary artery disease in this Italian population.

**Table 1: Clinical and biochemical characteristics of study subjects**

	CAD subjects (n = 102)	Control subjects (n = 104)	p-value
Age (years)	50.1 ± 5.3	47.5 ± 3.7	<0.02
Sex (M/F)	96/6	89/15	NS
Body Mass Index (kg/m <sup>2</sup> )	26.9 ± 3.2	26.4 ± 3.7	NS
Smokers (%)	85.2%	52.5%	<0.002
Hypertensive (%)	22.5	14.5	NS
Total cholesterol (mg/dl)	197.3 ± 33.7	199.8 ± 41.7	NS
HDL-cholesterol (mg/dl)	27.6 ± 8.2	38.1 ± 10.2	<0.0001
LDL-cholesterol (mg/dl)	145.6 ± 36.9	119.5 ± 32.9	<0.0001
Total triglycerides (mg/dl)	211.3 ± 63.6	188.1 ± 97.4	<0.0006
APO AI (mg/dl)	107.2 ± 17.5	129.1 ± 23.1	<0.0001
APO B (mg/dl)	117.7 ± 25.5	101.1 ± 24.9	<0.01
APO AII (mg/dl)	38.7 ± 6.4	43.9 ± 9.0	<0.001
APO CII (mg/dl)	4.03 ± 1.4	3.99 ± 1.6	NS
APO CIII (mg/dl)	12.1 ± 2.3	13.1 ± 3.2	<0.008
APO E (mg/dl)	8.6 ± 2.1	10.2 ± 3.3	<0.0001

Data are given as means ± SD. Smokers were those currently smoking at baseline. The statistical analysis of total triglycerides and Lp(a) were performed on log-transformed values, but the untransformed values are given in table. After age-standardisation, continuous variables were compared by t-test and ANOVA, and categorical variables by  $\chi^2$  test

**Table 2: Frequencies of rare alleles in CAD and controls subjects at each gene locus**

Gene polymorphism	Allele frequencies		p-value
	CAD subjects n = 102	Control subjects n = 104	
<b>Apo A1</b>			
Xmn I	0.154	0.228	NS
Msp I	0.081	0.094	NS
Pst I	0.091	0.094	NS
<b>Apo B</b>			
Xba I	0.330	0.440	<0.03§
<b>Apo CIII</b>			
C3175G	0.112	0.099	NS
<b>Apo E</b>			
e2	0.080	0.026	P < 0.05#
e3	0.840	0.914	
e4	0.080	0.060	
<b>LIPC</b>			
T202T (C to G)	0.362	0.526	<0.02¶
<b>LPL</b>			
HindIII	0.280	0.278	NS
S447X (C to G)	0.112	0.133	NS
N291S (A to G)	0.012	0.017	NS
D9N (G to A)	0.031	0.011	NS

§ CAD subjects vs controls: allele frequencies  $\chi^2 = 5.02$ , df = 1, p < 0.03. # CAD subjects vs controls: allele frequencies  $\chi^2 = 7.02$ , df = 2, p < 0.05. ¶ CAD subjects vs controls: allele frequencies  $\chi^2 = 5.45$ , df = 1, p < 0.02.

**Genotypes in Arterial disease**

Case-control analyses of genotype distributions and allele frequencies between the two groups with or without arterial disease showed significant differences only for the apolipoprotein B Xba1 polymorphism, for the Apo E variants and for the T202T variant of the LIPC gene (Table 2). All the other gene polymorphisms were not associated with CAD in our population. Multivariate analysis of all the six genes, accounting for clinical (age, sex, BMI) and lipid parameters, showed that the ApoB Xba1 polymorphism and apolipoprotein E4 allele were independently associated with arterial disease (p < 0.01, data not shown).

**Apolipoprotein, Lipoprotein lipase and LIPC genotypes and lipid levels**

Associations with lipid parameters were analysed in those genes that were associated with CAD in univariate analysis. In the CAD group, significant comparisons of lipid, lipoprotein and apolipoprotein levels between alleles are presented in Table 3. Plasma cholesterol (p = 0.03), LDL-cholesterol (p = 0.005) and Apo B (p = 0.002) levels were associated to Apo E variants. Also Apo E level were related to the Apo E alleles. Alleles at the Apo B Xba1 polymorphisms associated with plasma HDL-cholesterol and Apo B levels (p < 0.01).

Finally, lower HDL-cholesterol and higher triglycerides levels were associated with the T202T variant of LIPC gene (p = 0.01). (Table 3).

**Evaluation of clinical outcomes of CAD and gene variants**

In our CAD population we investigated from clinical records and from interviews the occurrence of a second cardiovascular event (a second coronary angioplasty (PTCA), myocardial infarction, coronary artery by-pass graft (CABG), cardiovascular deaths) in those who had a PTCA at the time of recruitment (1993–94). Data on clinical outcomes after 8 years were available for 60 subjects: 45 (72.5%) had a cardiovascular event, of which 22 had a CABG and 23 underwent a second PTCA (6 within one year); 4 of these 45 patients were reported dead. Because of the relatively small size of this sample, we were unable to analyse subsets of these patients, and considered the second CAD event as evidence of the progression of the atherosclerotic disease. Baseline characteristics of these patients were not significantly different from the other CAD patients (data not shown). When the occurrence of a second cardiovascular event was related to genotypes, a strong association was observed with the LIPC gene T202T variant (p < 0.02). This association was confirmed by multiple logistic regression analysis, were a significant independent association was demonstrated in the presence of other known risk factors. (table 4)

**Table 3: Comparison of plasma lipids and apoproteins levels according to alleles in CAD subjects (n. 102)**

	<i>Apo E</i>		<i>Apo B XbaI</i>		<i>LIPC T202T</i>		
	-/2 (14)	3/3 (74)	-/4 (14)	X1 (35)	X2 (67)	C carriers (39)	G carriers (65)
Total cholesterol (mg/dl)	<b>156 ± 20</b>	<b>199.29</b>	<b>204 ± 34*</b>	191 ± 34	206 ± 35	199 ± 41	198 ± 33
HDL-cholesterol (mg/dl)	26.8.4 ± 5.2	25.9 ± 7.1	26.76 ± 6.7	<b>29 ± 9.6</b>	<b>26 ± 2.0*</b>	<b>34 ± 10</b>	<b>31.7 ± 10*</b>
LDL-cholesterol (mg/dl)	<b>98 ± 24</b>	<b>134 ± 31</b>	<b>141 ± 11*</b>	138 ± 35	149 ± 36	130 ± 34	133 ± 39
Total triglycerides (mg/dl)	189.7 ± 45	178.3 ± 101	178.3 ± 80	210 ± 57	212 ± 67	<b>170 ± 83</b>	<b>203 ± 81*</b>
APO AI (mg/dl)	108.3 ± 14	105.4 ± 15	106.0 ± 10	107 ± 17	106 ± 15	119 ± 23	117 ± 22
APO AII (mg/dl)	37 ± 5.2	38 ± 6.1	38 ± 9.2	38.7 ± 6.6	38.5 ± 6.3	41 ± 8.2	41 ± 6.1
APO B (mg/dl)	<b>77 ± 16</b>	<b>110 ± 19</b>	<b>117 ± 21*</b>	<b>100 ± 30</b>	<b>112 ± 22¶</b>	104 ± 27	105 ± 23
APO CII (mg/dl)	4.4 ± 1.8	3.8 ± 1.6	3.7 ± 1.4	4.0 ± 1.3	3.9 ± 1.9	4.0 ± 1.6	3.9 ± 1.7
APO CIII (mg/dl)	11.6 ± 2.3	11.3 ± 2.6	12.7 ± 2.4	13.6 ± 2.1	12.3 ± 2.3	12.8 ± 2.8	12.4 ± 2.9
APO E (mg/dl)	<b>11.0 ± 3.2</b>	<b>7.9 ± 1.7</b>	<b>8.7 ± 1.7¶</b>	9.4 ± 2.7	9.0 ± 2.6	9.4 ± 2.6	9.3 ± 3.1

Data are given as means ± SDM. Significant differences are in bold **Apo E**: -/2 = carriers of allele ε 2; 3/3 = homozygous carriers of allele ε 3; -/4 carriers allele ε 4. \*p < 0.01, ¶ p < 0.03 **Apo B**: X1X1 v. X1X2 + X2X2. \*p < 0.01 ¶ p < 0.05 **LIPC** (Hepatic lipase): C allele carriers (wild type) v. G allele carriers (CG + GG) \* p < 0.02; † p < 0.01 The statistical analysis of total triglycerides and LP(a) were performed on log-transformed values, but the untransformed values are given in table. After age-standardisation, continuous variables were compared by t-test and ANOVA, and categorical variables by χ<sup>2</sup> test

**Table 4: Relative risk of clinical outcomes (a second PTCA, myocardial infarction, CABG, cardiovascular death) according to LIPC gene genotypes**

<b>LIPC status</b>	<b>CAD subjects with clinical outcomes</b>		<b>CAD subjects without clinical outcomes</b>		<b>Odds ratio (95% CI)</b>
	<b>n. (%)</b>	<b>n. (%)</b>	<b>n. (%)</b>	<b>n. (%)</b>	
<b>CC (wild type) carriers</b>	6 (13.4%)	9 (60%)	9 (60%)	6 (40%)	1
<b>GG + GC carriers</b>	39 (86.6%)	6 (40%)	6 (40%)	39 (86.6%)	7.6 (1.01–57.2)*

Odds ratios were adjusted for age, sex, plasma triglycerides, LDL-cholesterol, HDL-cholesterol, smoking habits (yes/no). Triglycerides were also independent predictors of clinical outcomes (OR 1.01 (95% CI: 1.00–1.02), p < 0.04) \* p < 0.03

**Discussion**

Italy ranks as one of the lowest in mortality rates for coronary artery disease in Europe [8], yet many of the conventional risk factors showed significant differences in our patients with coronary artery disease compared to healthy Italian controls. The frequency of smokers was higher in the arterial disease group; and plasma LDL cholesterol, triglycerides, and Lp(a) were also raised in the disease group whereas HDL-cholesterol was reduced. Interestingly total plasma cholesterol did not discriminate between cases and controls as in other European countries [17]. Multiple regression analysis confirmed the independent predictive role for all of the above mentioned lipid parameters.

Of the six lipid loci studied (for a total of 11 polymorphisms) the *apolipoprotein E*, *ApoB* and *LIPC* polymorphisms distinguished between case and controls in univariate analysis. However, multivariate analysis

accounting for clinical and metabolic predictors of CAD showed that only *Apo E* and *ApoB XbaI* polymorphisms were independently associated with CAD in this Italian population. This latter result confirms previous association studies where the allele possessing the *XbaI* site was found to be associated with higher levels of total cholesterol, LDL, apoB and triglycerides, and it was concluded that this genotype confers increased risk of myocardial infarction [5,20]. Also results on the *Apo E* gene association confirm previous observations [9]. When lipid parameters were related to genotypes, the *ApoE*, *ApoB* and, *LIPC* gene polymorphisms were associated with various markers of dyslipidaemia in this CAD population, confirming previous reports of the influence of these gene polymorphisms on lipid parameters (reviewed in [7]).

The most interesting result in our study was the observation that the *LIPC (hepatic lipase) gene T202T* polymorphisms was independently associated with clinical

outcomes in patients with established CAD. Thus, when the occurrence of a second cardiovascular event was related to genotypes, a strong association by multiple logistic regression analysis was observed with the *LIPC* gene *T202T* variant. Also this variant was associated with reduced HDL-cholesterol levels in our population. Low hepatic lipase activity has been shown to be associated with an increased risk for CAD [21]. Hepatic lipase is a lipolytic enzyme synthesised in liver cells, and has a dual role in lipid metabolism. It is involved in chylomicron remnant catabolism and in HDL metabolism, influencing hepatic HDL interconversion [22] from large, cholesterol ester-rich HDL particles to smaller particles that are ready to accept cholesterol from cell membranes. Furthermore, LDL buoyancy and size appear to be inversely associated with hepatic lipase activity levels [23], and hepatic lipase appears to influence LDL lipid composition by affecting the surface lipid components.

Studies on hepatic lipase enzyme activity show that hepatic lipase deficiency in most cases leads to triglyceride enrichment in LDL and HDL lipoprotein fractions, presence of circulating  $\beta$ -VLDL and abnormal chylomicron catabolism [24].

A role for the *LIPC* gene in atherosclerosis and CAD has been proposed by genetic and functional studies [25,26]. Guerra et al. [25] examined the relationship between polymorphism in the gene coding for hepatic lipase, using a sequential approach comprising linkage analysis, DNA sequencing and association studies. This study concluded that allelic variation at, or closely linked to, the *LIPC* gene accounts for about 25% of the plasma HDL-cholesterol variation in concentrations. Moennig et al. [26] sequenced the *LIPC* gene in patients with low HDL/high triglycerides and CAD, and their results suggested that mutations in *LIPC* may play a role in the pathogenesis of atherosclerosis. The polymorphism that we have studied (*T202T*) has been shown to be in linkage disequilibrium with other *LIPC* gene polymorphisms: *L334F*, *T457T* (linkage disequilibrium coefficients were -1.00 and -0.96, respectively) and weakly to *C480T* [27], also described as *C-514T*. This latter polymorphism was reported associated with a lower hepatic lipase activity in CAD patients [28]. This observation has been subsequently confirmed [29], suggesting that the common *LIPC* promoter variant-*C480T* is functional and associated with an impaired hepatic lipase activity influencing lipoproteins metabolism. Furthermore, hepatic lipase enzyme with the *L334F* variant was shown in *in vitro* expression studies to have only about 30% of the enzymatic activity of the wild-type enzyme [30]. It is thus possible that the *T202T* variant, which is one of the more common polymorphisms of *LIPC* gene, is a simple marker of one of the other *LIPC* variants, considering also that it is in linkage disequilibrium

with these other polymorphisms and in particular with the *L334F* variant. We acknowledge that our sample was small, and these results should be considered preliminary, although an 8 years follow up is long enough to determine clinical outcomes. Moreover, several reports have demonstrated an important role for the *LIPC* gene in the risk of CAD and its related lipid abnormalities. Low HDL-cholesterol and LDL subclass distribution, both influenced by hepatic lipase activity, have been shown to be strong risk factors for early atherosclerosis [23,31]. Thus, further studies on sequence variations of the *LIPC* gene in CAD patients are warranted, in particular looking at the possible influences on clinical outcomes. The possibility to predict by genetic analysis the risk of progression of coronary atherosclerotic lesions would be of great importance for the management of CAD patients.

### Competing interests

None declared.

### Authors' contributions

MGB carried out the recruitment of subjects, the genetic studies, performed the statistical analysis and drafted the manuscript. AB participated in the CAD patients recruitment and in the drafting of the manuscript, SR carried out the 8 year follow up study and participated in the genetic analysis. MA participated in the statistical analysis. TT participated in the recruitment of subjects and in the follow up study GS participated in the recruitment of subjects and in the follow up study. UDM participated in the design of the study and DJG conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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