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ARTICLE

The complex interaction between APOE promoter and AD: an Italian case–control study

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The single nucleotide polymorphisms (SNPs) rs449647, rs769446 and rs405509 in the promoter region of the *APOE* gene have been variously suggested to be ε4-independent risk factors for Alzheimer's disease (AD). A previous Italian study found that the rs449647 was significantly associated with late-onset AD. The aim of this study was to verify whether these *APOE* promoter SNPs are genetic risk factors for AD and to investigate their interaction with the common *APOE* polymorphism. A total of 169 clinically diagnosed AD patients and 99 cognitively intact age-matched controls were included in the study. Significant associations with AD independent from sex, age and APOE/ε4 status were found for rs449647 A/A and rs405509 G/G genotypes (positive), and rs449647 A/T and rs405509 T/T genotypes (negative). Haplotype frequency estimation at the *APOE* locus showed significant associations for the ATG4, ATT4 and ACG3 (positive) and ATT2, ATT3 and TCG3 (negative) haplotypes. Therefore this study confirms the role of the rs449647 A/A genotype as risk factor for AD in Italy and suggests that promoter genotypes and *APOE* haplotypes might have a complex function in AD-associated genetic risk factors.
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Introduction

Alzheimer's disease (AD), in its sporadic form, is a paradigmatic model of complex diseases resulting from the interaction between genetic and nongenetic factors such as environmental ones.[1,2] Mendelian inheritance of pathogenic mutations has been described only for a small amount of AD cases (less than 5%).[1] To find an association with sporadic AD more than 100 biological or genetic factors have been analyzed.[1,2] In spite of several studies carried out in large AD samples of ethnically different subgroups, the ε4 allele of the *APOE* gene remains the only established genetic risk factor for AD.[1] The pathogenetic mechanism by which ε4 might promote the development of AD is still unclear. Several evidences indicate a possible effect of the ε4 allele on β-amyloid (Aβ) formation and deposition and a consequent role in the 'amyloid cascade'.[1] This effect, however, could explain only less than half the cases of AD because of the presence of AD patients ε4 noncarriers; in fact, there is a general agreement about the ε4 allele as a variable neither necessary nor sufficient to develop AD.[3,4] In search for other genetic risk factors potentially associated with AD pathogenesis, three common single nucleotide polymorphisms (SNPs) located in the promoter region of the *APOE* gene have been described.[5] This region, spanning from position −1017 to +406 of the *APOE* genetic locus (19q13.31), showed three major SNPs, rs449647 ($A^{-491}\rightarrow T$), rs769446 ($C^{-427}\rightarrow T$) and

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rs405509 ($G^{-219}\rightarrow T$) with a potential functional role.[1,6–8] The promoter region could modulate the transcriptional activity of *APOE* coding region,[7,8,9–11] and this modified expression of *APOE* may be the pathogenetic trigger depending on the promoter polymorphisms in AD.[11] However, comprehensive analyses of these studies reported contradictory results[1,8] and data on this polymorphic region regarding an association with AD in the Italian population are rare.[12] The aim of this study was to assess whether the association among AD and the three SNPs of the promoter region of *APOE* gene might be considered specific risk factors *per se* independently of *APOE*/ε4 status.

Materials and methods

Patients and controls

The study was designed as a hospital-based case–control study. It was conducted according to the Declaration of Helsinki Principles and the guidelines for Good Clinical Practice, and was approved by the respective local ethics committee. Written informed consent was obtained from the patients or from their legal guardians before participation into the study. A total of 268 unrelated Caucasians (enrolled from central and southern Italy) were consecutively evaluated at the Neuropsychological Unit of the Catholic University School of Medicine in Rome, Italy. Among these subjects, 169 were diagnosed as probable AD according to NINDS-ADRDA criteria and 99 as cognitively intact (MMSE score >28/30). Clinical diagnosis of sporadic probable AD was confirmed at the follow up after 12 months. In cognitively intact subjects no personal or familial psychiatric or cognitive impairment history, and no alcohol or drug abuse were reported. We considered these subjects as controls (CTRL). Moreover, all subjects affected by cerebrovascular diseases were excluded from the study because they were considered at risk to develop a vascular form of dementia. Clinical and demographic characteristics of patients and controls are reported in Table 1. No extra European as well as Jewish subjects were included in the study.

Table 1 Subjects characteristics

	AD	*Controls*
Number of subjects	169	99
Sex		
Male/Female	53/116	50/49
Male (%)	31.36	50.51
Mean age (years ± SD)	63.38 ± 7.39	66.21 ± 7.31
Early onset[a]		
Number of subjects	53	43
Mean age (years ± SD)	59.81 ± 3.73	59.72 ± 3.32
Late onset[b]		
Number of subjects	116	56
Mean age (years ± SD)	72.29 ± 4.93	71.20 ± 5.35

Abbreviation: AD, Alzheimer's disease.
[a]Age < 65 years for both cases and controls.
[b]Age ≥ 65 years for both cases and controls.

Genetic analyses

Genomic DNA was manually purified from 4 ml of frozen blood samples by organic protein extraction and ethanol precipitation according to standard methods. The *APOE* promoter genotypes were determined by PCR and agarose gel electrophoresis. The rs449647 ($A^{-491}\rightarrow T$) and rs769446 ($T^{-427}\rightarrow C$) genotypes were determined by a previously described method based on a nested PCR.[5] A first amplification of 374 bp fragment was obtained using a two-base mismatched forward primer 5′-CATGTTGGC CAGGCTGGTtTtAA-3 and the reverse primer 5′-GGAAG GAGGTGGGGCATAGA-3′ at the following reaction conditions: 94°C for 5 min followed by 30 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 30 s, with an 8 min of final extension. The second amplification of the 374 bp PCR product, producing a 304 bp fragment, was performed with the same forward mismatched primer and the reverse primer 5′-CCCAGTAATACAGACACCCTCC-3′ at the following conditions: 94°C for 5 min followed by 30 cycles at 95°C for 30 s, 63°C for 30 s and 72°C for 30 s with an 8 min of final extension. Restriction analysis with *Dra*I or *Alu*I permits to identify the rs449647 and rs769446 genotypes on 3% agarose gel. The rs405509 (−219 GT) genotype was determined by a previously described method,[13] by an amplification of a 220 bp fragment using the following forward primer 5′-AGAATGGAGGAGGGTGTCCG-3′ and reverse primer 5′-ACTTGTCCAATTATAGGGCTCC-3′. PCR conditions were 94°C for 5 min followed by 35 cycles at 95°C for 30 1 min, 58°C for 1 min and 72°C for 1 min, with an 8 min of final extension. Restriction analysis with *Hpa*II allows to determine the three possible genotypes on 3% standard agarose gel. The rs429358 ($C^{3937}\rightarrow T$) and rs7412 ($C^{4075}\rightarrow T$) genotypes forming the APOE coding region polymorphism were analyzed as recently described.[14]

Statistical analysis

Agreement of the observed genotype frequencies with the expected Hardy–Weinberg (HW) frequencies was verified for each SNP in both study groups, including the two SNPs generating the common *APOE* polymorphism. Relative allele frequencies were estimated by the gene-counting method.[15] Genotype frequencies in 3×2 cross tables were compared by means of Pearson's χ^2-test. Comparison of genotype and allele frequencies in 2×2 cross tables was made by means of Fisher's exact test according to the two-way contingency table analysis of the Interactive Statistical Calculation Pages (available at URL: http://statpages.org/ctab2x2.html). The odds ratios (ORs) and relative risk (RR) were also calculated. Correlations between the promoter polymorphisms were tested by using the Spearman's

correlation rank-sum test. Binary logistic regression analysis using sex and age at onset as covariates was also used to estimate the adjusted significances. Both these analyses were made with the SPSS version 10.1.3 (SPSS Inc., Chicago, IL, USA) statistical software package. The synergy between the investigated SNPs was estimated according to Leandro *et al*[16] assuming an additive model. Briefly, the exceeding of relative excess risk was verified according to the formula RR(AB)−1 > RR(A)−1 + RR(B)−1, with A and B indicating the genotype to be evaluated. In the stratification of the *APOE* promoter polymorphism, according to the common *APOE* polymorphism, we assumed as $\varepsilon 2^+$ genotypes all genotypes containing at least one $\varepsilon 2$ allele, that is genotypes $\varepsilon 2/\varepsilon 2$, $\varepsilon 2/\varepsilon 3$ and $\varepsilon 2/\varepsilon 4$. Similarly, $\varepsilon 4^+$ genotypes were considered the genotypes $\varepsilon 2/\varepsilon 4$, $\varepsilon 3/\varepsilon 4$ and $\varepsilon 4/\varepsilon 4$. The power of the study, including an estimation of the effect size (h), was also calculated. The haplotype analysis was made with the PHASE software version 2.1.1 assuming default parameters.[17,18] The overall phase probability at the APOE locus was calculated as the product of the probability at each polymorphism. The linkage disequilibrium (LD) analysis was made with the Haploview software version 4.1 (downloaded from URL: http://www.broad.mit.edu/haploview/haploview). If not already specified, all the statistical analyses were made with the R software for statistical computing, version 2.7.2 (downloaded from URL: http://www.r-project.org/). In all statistical analyses a two-tail *P*-values, setting statistical significance at $P<0.05$, were considered. ORs and the 95% confidence intervals (95% CI) were also reported.

Results

The APOE promoter polymorphisms

Genotypes and estimated allele frequencies of the three promoter SNPs are reported in Table 2. No differences to the expected HW frequencies were found in both AD and controls for rs449647 ($P=0.093$ and 0.787, respectively), rs769446 ($P=0.208$ and 0.182, respectively) and rs405509 ($P=0.111$ and 0.412, respectively). In the analysis of rs449647 a significant difference was found between AD and controls ($P=0.005$). The frequency of the wild-type A/A was higher in AD than in controls (76.33 *vs* 57.58%; OR = 2.376, 95% CI 1.397–4.043), whereas the frequency of the A/T heterozygote was lower in AD than in controls (20.12 *vs* 37.37%; OR = 0.422, 95% CI 0.243–0.733). Thus, the frequency of the A allele resulted higher in AD than in controls (0.864 *vs* 0.763%; OR = 1.976, 95% CI 1.261–3.097), whereas the frequency of the T allele resulted lower in AD than in controls (0.136 *vs* 0.237%; OR = 0.506, 95% CI 0.323–0.793). In the analysis of rs769446 no difference was found between AD and controls ($P=0.345$). In the analysis of rs405509 a significant difference was found between AD and controls ($P<0.001$). The frequency of the wild-type G/G was higher in AD than in controls (42.60 *vs* 29.29%; OR = 1.792, 95% CI 1.058–3.034), whereas the frequency of the T/T homozygotes was lower in AD than in controls (7.69 *vs* 25.25%; OR = 0.247, 95% CI 0.121–0.505). Thus, the frequency of the G allele was higher in AD than in controls (0.675 *vs* 0.520%; OR = 1.912, 95% CI 1.335–2.738), whereas the frequency of the T allele was lower in AD than in controls (0.325 *vs* 0.480%; OR = 0.523,

Table 2 Major SNPs in the promoter region of *APOE* gene

		AD (n = 169)		*Controls (n = 99)*			
DNA change (SNP ID)	*Genotype/allele*	*N*	*Frequency*	*N*	*Frequency*	*OR*	*95% CI*
$A^{-491}\rightarrow T$ (rs449647)	A/A	129	76.33%	57	57.58%	**2.376**	**1.397–4.043**
	A/T	34	20.12%	37	37.37%	**0.422**	**0.243–0.733**
	T/T	6	3.55%	5	5.05%	0.692	0.218–2.196
	A	292	0.864	151	0.763	**1.976**	**1.261–3.097**
	T	46	0.136	47	0.237	**0.506**	**0.323–0.793**
$T^{-427}\rightarrow C$ (rs769446)	T/T	129	76.33%	83	83.84%	0.622	0.330–1.174
	T/C	35	20.71%	14	14.14%	1.586	0.812–3.092
	C/C	5	2.96%	2	2.02%	1.479	0.323–6.721
	T	293	0.867	180	0.909	0.651	0.368–1.153
	C	45	0.133	18	0.133	1.536	0.867–2.718
$G^{-219}\rightarrow T$ (rs405509)	G/G	72	42.60%	29	29.29%	**1.792**	**1.058–3.034**
	G/T	84	49.70%	45	45.45%	1.186	0.722–1.947
	T/T	13	7.69%	25	25.25%	**0.247**	**0.121–0.505**
	G	228	0.675	103	0.520	**1.912**	**1.335–2.738**
	T	110	0.325	95	0.480	**0.523**	**0.365–0.749**

Abbreviations: AD, Alzheimer's disease; SNP, single nucleotide polymorphisms.
Significant values are in boldface.
Spearman's correlation rank-sum test: rs449647 and rs769446, $P=0.096$; rs449647 and rs405509, $P=0.946$; rs769446 and rs405509; $P=0.308$.
Synergistic effect: A/A (rs449647) and G/G (rs405509), relative excess risk 0.365 vs −0.074; A/T (rs449647) and T/T (rs405509), relative excess risk −0.783 vs −0.797.
Genotypes and estimated allele frequencies.

95% CI 0.365–0.749). Sex- and age-adjusted estimates for the association of the *APOE* promoter and *APOE* polymorphism genotypes with AD were estimated; this analysis of the adjustment for sex and age at onset did not change the strength of the associations (data not shown).

The APOE promoter polymorphisms according to the common APOE alleles

As expected, significant differences were found in the *APOE* genotype distribution between AD and controls. The frequency of the $\varepsilon 2^+$ genotypes was lower in AD than in controls (7.56 *vs* 17.00 %; OR = 0.399, 95% CI 0.187–0.851). Similarly, the frequency of the $\varepsilon 3/\varepsilon 3$ genotype was lower in AD than in controls (44.19 *vs* 73.00%; OR = 0.293; 95% CI 0.172–0.498). Conversely, the frequency of the $\varepsilon 4^+$ genotypes was higher in AD than in controls (48.26 *vs* 10.00%; OR = 8.389, 95% CI 4.133–17.009).

Genotype frequencies of the *APOE* promoter polymorphisms according to the $\varepsilon 2$, the $\varepsilon 3/\varepsilon 3$ and $\varepsilon 4$ status are reported in Table 3. Genotype frequencies of rs449647 according to the *APOE* status are reported in Table 3a. In $\varepsilon 2^+$ subjects the frequency of A/A genotype was lower in AD than in controls (4.14 *vs* 12.12%; OR = 0.215, 95% CI 0.082–0.566). No differences were found for the A/T and T/T genotypes. In $\varepsilon 3/\varepsilon 3$ subjects the frequency of the A/A genotype was lower in AD than in controls (34.32 *vs* 40.40%; OR = 0.347, 95% CI 0.180–0.672). Similarly, the frequency of the A/T genotype was lower in AD than in controls (80.88 *vs* 29.29%; OR = 0.218, 95% CI 0.079–0.605). No differences were found for the T/T genotype. In $\varepsilon 4^+$ subjects the frequency of the A/A genotype was higher in AD than in controls (38.46 *vs* 6.06%; OR = 8.633, 95% CI 3.536–20.978). Similarly, the frequency of the A/T genotype was higher in AD than in controls (9.47 *vs* 4.04%; OR = 7.333, 95% CI 2.204–24.046). Notably, the T/T genotype was absent in controls.

Genotype frequencies of rs769446 according to the APOE status are reported in Table 3b. In $\varepsilon 2^+$ subjects no differences were found in the frequency distribution of the A/T or the T/C genotypes between cases and controls. No C/C genotypes were found in AD. In $\varepsilon 3/\varepsilon 3$ subjects, the frequency of the T/T genotype was lower in AD than in controls (34.91 *vs* 62.63%; OR = 0.285, 95% CI 0.157–0.521). Similarly, the frequency of the T/C genotype was lower in AD than in controls (8.28 *vs* 10.10%; OR = 0.267, 95% CI 0.073–0.982). In $\varepsilon 4^+$ subjects the frequency of the T/T genotype was higher in AD than in controls (36.09 *vs* 7.07%; OR = 9.739, 95% CI 4.243–22.227). Similarly, the frequency of the T/C genotype was higher in AD than in controls (11.83 *vs* 2.02%; OR = 8.000, 95% CI 1.696–36.325).

Genotype frequencies of rs44405509 according to the *APOE* status are reported in Table 3c. In $\varepsilon 2^+$ subjects a lower frequency of the G/T genotype was found in AD than in controls (4.14 *vs* 13.13%; OR = 0.224, 95% CI 0.084–0.599), whereas no differences were found for the G/G or T/T genotypes. In $\varepsilon 3/\varepsilon 3$ subjects, a lower frequency of the G/G genotype was found in AD than in controls (21.30 *vs* 24.24; OR = 0.208, 95% CI 0.074–0.590). Similarly, a lower frequency was found for both G/T and T/T genotypes in AD than in controls (20.71 *vs* 27.27%; OR = 0.476, 95% CI 0.229–0.990 and 2.96 *vs* 22.22%; OR = 0.085, 95% CI 0.018–0.418, respectively). In $\varepsilon 4^+$ subjects a higher frequency in AD than in controls was

Table 3 Genotype frequencies of the most common rs449647 (A), rs769446 (B) and rs44405509 (C) according to the *APOE* genotypes

	AD (n = *169*) N *(%)*		*Controls* (n = *99*) N *(%)*		*OR*	*95% CI*
(a)						
$\varepsilon 2^+$						
A/A	7	(4.14)	12	(12.12)	**0.215**	**0.082–0.566**
A/T	5	(2.96)	4	(4.04)	1.422	0.374–5.390
T/T	1	(0.59)	1	(1.01)	0.800	0.062–10.286
$\varepsilon 3/\varepsilon 3$						
A/A	58	(34.32)	40	(40.40)	**0.347**	**0.180–0.672**
A/T	15	(8.88)	29	(29.29)	**0.218**	**0.079–0.605**
T/T	3	(1.78)	4	(4.04)	0.250	0.024–3.035
$\varepsilon 4^+$						
A/A	65	(38.46)	6	(6.06)	**8.633**	**3.536–20.978**
A/T	16	(9.47)	4	(4.04)	**7.333**	**2.204–24.046**
T/T	2	(1.18)	—	—	—	—
(b)						
$\varepsilon 2^+$						
T/T	11	(7.69)	14	(14.14)	0.459	0.201–1.051
T/C	2	(6.51)	2	(2.02)	0.364	0.057–2.309
C/C	—	—	1	(1.01)	—	—
$\varepsilon 3/\varepsilon 3$						
T/T	59	(34.91)	62	(62.63)	**0.285**	**0.157–0.521**
T/C	14	(8.28)	10	(10.10)	**0.267**	**0.073–0.982**
C/C	3	(1.78)	1	(1.01)	1.500	0.093–25.586
$\varepsilon 4^+$						
T/T	61	36.09)	7	(7.07)	**9.739**	**4.243–22.227**
T/C	20	(11.83)	2	(2.02)	**8.000**	**1.696–36.325**
C/C	2	(1.18)	1	(1.01)	0.667	0.039–10.802
(c)						
$\varepsilon 2^+$						
G/G	5	(2.96)	3	(3.03)	0.647	0.157–2.626
G/T	7	(4.14)	13	(13.13)	**0.224**	**0.084–0.599**
T/T	1	(0.59)	1	(1.01)	2.000	0.192–20.852
$\varepsilon 3/\varepsilon 3$						
G/G	36	(21.30)	24	(924.24)	**0.208**	**0.074–0.590**
G/T	35	(20.71)	27	(27.27)	**0.476**	**0.229–0.990**
T/T	5	(2.96)	22	(22.22)	**0.085**	**0.018–0.418**
$\varepsilon 4^+$						
G/G	33	(19.53)	2	(2.02)	**15.654**	**3.851–62.639**
G/T	42	(24.85)	6	(6.06)	**6.500**	**2.540–16.527**
T/T	8	(4.73)	2	(2.02)	**18.400**	**3.217–101.166**

Abbreviation: AD, Alzheimer's disease.
AD group: $\varepsilon 2/\varepsilon 3$, 5.92% ($n = 10$); $\varepsilon 2/\varepsilon 4$, 1.78% ($n = 3$); $\varepsilon 3/\varepsilon 3$, 44.97% ($n = 76$); $\varepsilon 3/\varepsilon 4$, 40.83% ($n = 69$); $\varepsilon 4/\varepsilon 4$, 6.51% ($n = 11$). HWE $P = 0.931$. Control group: $\varepsilon 2/\varepsilon 3$, 16.16% ($n = 16$); $\varepsilon 2/\varepsilon 4$, 1.01% ($n = 1$); $\varepsilon 3/\varepsilon 3$, 73.74% ($n = 73$); $\varepsilon 3/\varepsilon 4$, 9.09% ($n = 9$). HWE $P = 0.999$. Significant values are in boldface.

found for G/G (19.53 *vs* 2.02%; OR = 15.654, 95% CI 3.851–62.639), G/T (24.85 *vs* 6.06%; OR = 6.500, 95% CI 2.540–16.257) and T/T (4.73 *vs* 2.02%; OR = 18.400, 95% CI 3.217–101.166).

Haplotype analysis

Estimation of the haplotype frequencies in the 3 kb block spanning the *APOE* gene locus is summarized in Table 4. The ATG3 haplotype resulted the most represented in both AD and controls (37.87 and 34.85%, respectively), followed in decreasing order by the ATT3 haplotype (11.24 *vs* 26.26%, respectively). In AD or in controls all the other haplotypes showed a frequency less than 10%. Notably, the total frequency of the haplotypes showing both the −491 A and the −427 T alleles, that is, the first six haplotypes in Table 4 (AT− haplotypes), represented more than 70% of the total frequency of the estimated haplotypes (73.66 and 72.23%, respectively for AD and controls). In the analysis, the frequency of the ATG4 haplotype (10.36 *vs* 1.01%; OR = 11.320, 95% CI 2.971–43.025) as well as ATT4 haplotype (11.83 *vs* 3.03%; OR = 4.295, 95% CI 1.829–10.069) was higher in AD than in controls. Notably, the frequency of the ACG3 haplotype was also higher in AD than in controls (10.95 *vs* 1.01%; OR = 12.047, 95% CI 3.167–45.709). Conversely, the frequency of the ATT2 haplotype (1.18 *vs* 5.56%; OR = 0.204, 95% CI 0.068–0.615), as well as the ATT3 haplotype (11.24% *vs* 26.26%; OR = 0.356, 95% CI 0.224–0.564) and the TTG3 haplotype (3.55 *vs* 12.12%; OR = 0.267, 95% CI 0.132–0.540) was lower in AD than in controls. A schematic representation of the LD coefficient D' among the five SNPs, that is, the three SNPs in the promoter region and the two in the coding region of the *APOE* gene, is shown in Figure 1. As expected, according to the genetics of the *APOE* polymorphism,[19] a strong LD was observed in AD between markers rs429358 and rs7412 ($D' = 1.0$). Similarly, an LD between the same markers was suggested in controls ($D' = 0.9$). We also observed in AD, and not in controls, a strong LD between markers rs769446 and rs7412 ($D' = 1.0$). Conversely, the presence of LD was suggested in controls and not in AD between the markers rs449647 and rs429358 ($D' = 0.5$).

The phase probability, for each marker and for the 3 kb block spanning the *APOE* locus, for the ε2- and ε4-containing haplotypes common to both AD and controls is summarized in Table 5. No difference in phase probability estimation was found between AD and controls for the ε2- and ε4-containing haplotypes (data not shown).

An estimation of the power of the analysis of this study based on the association of the rs449647 A/A genotype

Table 4 Haplotype frequencies in the 3 kb block spanning the APOE locus

	AD (n = *338*)		*Controls* (n = *198*)			
Haplotype	N	*(%)*	N	*(%)*	*OR*	*95% CI*
ATG2	4	(1.18)	3	(1.52)	0.778	0.193–3.140
ATG3	128	(37.87)	69	(34.85)	0.140	0.791–1.641
ATG4	35	(10.36)	2	(1.01)	**11.320**	**2.971–43.025**
ATT2	4	(1.18)	11	(5.56)	**0.204**	**0.068–0.615**
ATT3	38	(11.24)	52	(26.26)	**0.356**	**0.224–0.564**
ATT4	40	(11.83)	6	(3.03)	**4.295**	**1.829–10.069**
ACG3	37	(10.95)	2	(1.01)	**12.047**	**3.167–45.709**
ACG4	—	—	2	(1.01)	—	—
ACT2	—	—	1	(0.51)	—	—
ACT3	1	(0.30)	3	(1.52)	0.193	0.027–1.359
ACT4	5	(1.48)	—	—	—	—
TTG2	4	(1.18)	1	(0.51)	2.359	0.351–15.780
TTG3	12	(3.55)	24	(12.12)	**0.267**	**0.132–0.540**
TTG4	6	(1.78)	—	—	—	—
TTT2	1	(0.30)	—	—	—	—
TTT3	13	(3.85)	12	(6.06)	0.620	0.282–1.362
TTT4	8	(2.37)	—	—	—	—
TCG3	2	(0.59)	—	—	—	—
TCT2	—	—	1	(0.51)	—	—
TCT3	—	—	9	(4.55)	—	—

Abbreviation: AD, Alzheimer's disease.
Significant values are in boldface.

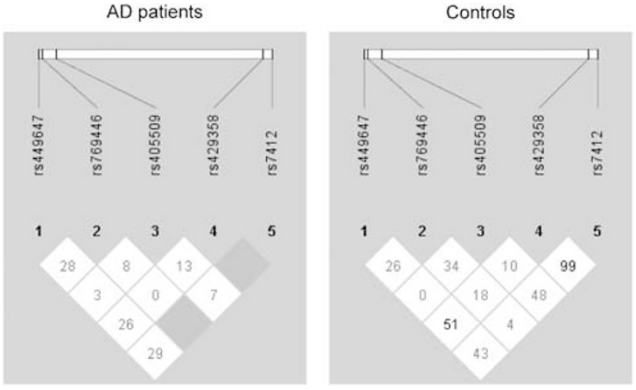

Figure 1 Schematic representation of linkage disequilibrium (D' coefficient) among the markers spanning a 3 kb block at the *APOE* gene locus.

Table 5 Phase probability at the APOE locus

AD	*CTRS*	*Single phase probability*[a]	*Overall phase probability*
ATG2/ ATG3	ATG2/ ATG3	k k k 1.00	1.00
ATG3/ ATG4	ATG3/ ATG4	k k k 1.00	1.00
ATG3/ ATT2	ATG3/ ATT2	k k 0.50 1.00	0.50
ATG3/ ATT4	ATG3/ ATT4	k k 0.50 1.00	0.50
ATT3/ ATT4	ATT3/ ATT4	k k k 1.00	1.00

Abbreviation: AD, Alzheimer's disease.
[a]k indicates a known phase.

showed an $h = 0.402$ with a power of 0.889. To be even more accurate in the estimate of the power of the study it would be possible to make the calculation on the less common genotype where an association resulted (T/T of the rs405509). As a consequence the estimate would be even more relevant than the previous one resulting an $h = 0.491$ and a power of 0.972.

Discussion

The main purpose of this study was to analyze the role of the major *APOE* promoter polymorphisms in AD susceptibility and their interactions with the APOE polymorphism.

This study shows a statistically significant increased frequency of the A/A genotype and A allele of the rs449647 in AD. This result is consistent with that of a previous Italian study,[12] reporting an increased A/A genotype and A allele frequencies in AD. We confirmed this association also when the estimated rs449647 frequencies were adjusted for sex and age at onset. This association has been reported by previous studies[9,12,20–25] and resulted in agreement with similar data collected from several independent samples genotyped for the apoE coding region and *APOE* promoter polymorphisms.[26] Whereas our data support these reported positive associations between rs449647 and AD, other studies failed to show similar results.[27–29] It is not still clear how rs449647 exerts its effect on AD risk. A possible explanation might be its potential role to modify the *APOE* transcriptional activity.[10,30] Several studies reported that *APOE* expression is important for brain amyloid loading and deposition in AD and in elderly healthy individuals, also independently from *APOE* genotype.[11,31,32] It might be possible that the risk for AD may be modulated by the apoE protein or apoE mRNA levels as well as by the common apoE protein isoforms.[33,34] Recent studies[30,31] supported the notion that rs449647 might be related to variations in apoE brain levels and reported an increased level of apoE in the brains of AD carrying the A/A genotype as compared to controls with the same genotype. Significantly higher plasma apoE levels, independent from the $\varepsilon 4$ status, in AD subjects carrying the A/A genotype have been reported.[32] On the contrary, another recent study, performed as a part of the Rotterdam study,[24] suggested a lowering effect of the rs449647 A/A genotype on plasma apoE levels in AD. Thus, the biological effect of the rs449647 polymorphism remains controversial.

The analysis of the distribution of genotypic and estimated allelic frequencies of rs769446 did not show any difference between AD and controls. These data are in agreement with previous studies.[9,13] One of the earlier investigations on the possible functional effects of the ApoE promoter polymorphisms[5] showed that only the −491 and the −219 polymorphic sites produce variations on the transcriptional activity of the APOE coding region, whereas the T to C substitution at the rs769446 site had no significant effect on APOE promoter activity. On the other hand, a study carried out on a Spanish population reported an association between rs769446 polymorphism and AD showing an increased risk for AD in subjects bearing the A^{-491} and C^{-427} haplotypes.[11] The results of this study suggested a possible effect of the C^{-427} allele on the transcriptional activity of the *APOE* gene with a consequent higher level of apoE in plasma and brain. At variance, a recent study on a French population[35] showed a positive association between T^{-427} allele and AD risk; in the same study the −427 allele has been found in the haplotype conferring an increased risk for AD. However, as observed in these two studies, the positive association between rs769446 and AD shows contrasting results on the possible allele (T or C) conferring a higher AD risk. The analysis of the distribution of the genotypic and estimated allelic frequencies of rs405509 showed an overrepresentation of G/G genotype and the G allele in AD patients according to Myllykangas *et al*.[36] At variance, our results are in contrast with those carried out on French population[9,22] reporting an increased frequency of the T^{-219} allele in AD patients. The same results were obtained in elderly AD subjects as reported in a recent meta-analysis[26] and in other studies on population-based cohort.[37,38] In line with these conflicting results, it is not clear yet how the $G^{-219} \rightarrow$ T polymorphism exerts its possible biological effect in AD.[5,40]

To evaluate the possible interaction between the promoter variants and the common *APOE* polymorphism, we also classified the promoter polymorphisms according to the $\varepsilon 2^+$, $\varepsilon 3/\varepsilon 3$ and $\varepsilon 4^+$ *APOE* genotypes. For rs449647 the overrepresentation of the A/A genotype and for rs405509 the overrepresentation of the G/G genotype observed in AD were confirmed only in presence of the $\varepsilon 4^+$ genotypes. For rs769446 significant differences were observed, meanly in the presence of $\varepsilon 3/\varepsilon 3$ and $\varepsilon 4^+$ genotypes. However, these associations may not be explained by LD (Figure 1).

The haplotype analysis showed an overrepresentation of the ATG family haplotype in AD ($P = 0.007$) and confirmed previous results describing the haplotypes of the ATG family as the most frequent haplotypes, and one ATG-haplotype at risk for AD.[35] At variance, we did not confirm an overrepresentation of the ATT- haplotype in AD,[35] ($P = 0.010$). Haplotype analysis also showed a significant increased frequencies of the two major haplotypes of the -$\varepsilon 4$ family (ATG4 and ATT4) in AD. The strongest association to AD observed for the ATG4 haplotype, compared to the ATT4, might be explained by the presence of the G^{-219} allele. The overall association of these two haplotypes of the $\varepsilon 4$ family, however, slightly changed the association of the $\varepsilon 4$ allele with AD. On the contrary, two haplotypes of the -$\varepsilon 3$ family (ATT3 and TTG3) showed a significant decreased frequency in AD. The overall association of these

haplotypes of the -ε3 family, however, did not significantly change the association of the ε3 allele. Notably, we observed an -ε3 haplotype (ACG3) that was overrepresented in AD than in controls. This result might suggest a possible interaction between A^{-491} and C^{-427} alleles, independently from *APOE*4. A similar observation was previously reported by Artiga *et al*[11] on Spanish population.

Despite several studies on the *APOE* promoter polymorphisms in AD, the haplotype analysis is not common and, when estimated, it has been often restricted to one or two promoter polymorphisms.[27,39] A Finnish study,[27] estimating a two-point haplotype (rs449647 and the common exon 4 polymorphism) indicated the A4 as the higher-risk haplotype for AD and this haplotype frequency was the same of the ε4 allele in AD group confirming an LD between the two alleles. This result is in agreement with another earlier study,[39] which found the highest AD risk for the A4 haplotype and the lowest for the T3 ones. On the contrary, a recent study on Colombian population using an haplogroup analysis of the APOE promoter polymorphisms confirmed their independent contribution as genetic risk factors for AD.[40] Different possible explanations of the contrasting results on allelic and haplotype distribution of the *APOE* promoter polymorphisms have been reported. The first might be the presence of an LD among the different alleles of the *APOE* promoter polymorphisms and the *APOE* coding region. The real risk-conferring allele such as that reported for the A^{-491} might bear persistently another allele in the haplogroup or haplotype. A second possible explanation, considering the absence of an LD in our sample, might be that for a specific combination of alleles or genotypes of the *APOE* promoter polymorphisms it is necessary to perform the complex interaction between these biological factors that led to a modified transcriptional activity of the *APOE* coding region.

To investigate the possible *cis*-acting interaction of quantitative promoter effects on qualitative effects of the *APOE* coding variants we estimated the phase probability for the investigated haplotypes. The phase probabilities of the genotypes common to both AD and controls are summarized in Table 5. In fact, it has been reported that the T^{-491} allele caused a decrease in *APOE* promoter activity, whereas the T^{-219} caused an increased promoter activity.[11,31] Thus, haplotypes containing these alleles may have a *cis*-acting effects, in particular on the expression of the ε4 allele.

The contrasting findings reported by previous studies on *APOE* promoter polymorphisms and AD probably depend on background heterogeneity and sample selection criteria, both important parameters for the *APOE* polymorphisms evaluation. These parameters include ethnic origins and the age range of the investigated samples. Therefore our study confirms the conclusions reached by other studies on Caucasians, especially on Spanish population samples, about a function of the promoter polymorphisms in AD, suggesting that the same genetic background might be the more plausible explanation for these similar results. However, future studies on larger cohort of AD might further increase the power of the analysis as well as confirm the possible risk/protective effects associated with these polymorphisms/haplotypes and their complex interaction.

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