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ARTICLE

Independent effects of the −219 G > T and ε2/ε3/ε4 polymorphisms in the apolipoprotein E gene on coronary artery disease: The Southampton Atherosclerosis Study

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A number of studies have shown that coronary artery disease severity is associated with the ε2/ε3/ε4 polymorphism in the coding region of the apolipoprotein E gene. In this study, we investigated whether the severity of the disease was also influenced by a functional polymorphism (−219 G > T) in the promoter of the gene, and if so, whether the effects of the two polymorphisms were independent. A cohort of 1170 patients with angiographically documented coronary artery disease were genotyped for the two polymorphisms. The frequency of the ε4 allele of the ε2/ε3/ε4 polymorphism increased linearly with increasing number of diseased vessels, so did the −219T allele of the −219 G > T polymorphism. In the sample as a whole, logistic regression analyses indicated that compared with the G/G genotype, the T/T genotype conferred an odds ratio of 1.598 (95% CI = 1.161–2.201, $P = 0.004$) in favor of increased disease severity, and the relationship remained significant after adjustment for ε2/ε3/ε4 polymorphism genotypes, plasma cholesterol and triglyceride levels, and other risk factors. The effect of the T/T genotype on disease severity was more significant in patients who did not carry the ε4 allele (OR = 1.510, 95% CI = 1.028–2.221) than in ε4 allele carriers (OR = 1.303, 95% CI = 0.619–2.742). There was considerable linkage disequilibrium between the two polymorphisms ($\rho = 0.9$, $P < 0.001$). Logistic regression analysis showed that the −219T-ε4 haplotype conferred an odds ratio of 1.488 (95% CI = 1.133–1.954). These findings suggest that the −219 G > T and ε2/ε3/ε4 polymorphisms, which may affect respectively the quantity and quality of apoE, have independent and possibly additive effects on coronary artery disease severity.
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Introduction

Apolipoprotein E (ApoE) serves as a ligand mediating the binding of chylomicron remnants and very-low-density lipoprotein remnants to lipoprotein receptors on liver cells, thus facilitating the clearance of these atherogenic lipoproteins from the plasma.[1] In addition to this important role, apoE has other antiatherogenic properties.[2] Particularly noteworthy is that apoE is expressed in macrophage-derived lipid-laden foam cells in atherosclerotic lesions and facilitates cholesterol efflux from such cells.[2–5] ApoE-deficient mice develop severe hypercholesterolemia and diffuse atherosclerotic lesions.[6] Expressing apoE in macrophages in such apoE-null mice can markedly attenuate the development of atherosclerosis, even in the

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presence of high levels of circulating atherogenic lipoproteins.[7]

There is compelling evidence of an association between variation in the apoE gene and risk and severity of coronary artery disease (CAD) and Alzheimer's disease. The $\varepsilon2/\varepsilon3/\varepsilon4$ polymorphism in the coding region of the gene has been studied extensively, with the $\varepsilon4$ allele consistently found to be associated with increased risk of these diseases.[8,9] The $\varepsilon2$, $\varepsilon3$ and $\varepsilon4$ alleles, respectively, encode the apoE2, apoE3 and apoE4 isoforms that differ in amino-acid sequence at residues 112 and 158, with the E2 isoform having a cysteine at both sites, the E3 isoform having a cysteine and an arginine, respectively, and the E4 isoform possessing an arginine at both residues. Several polymorphisms in the promoter of the gene have also been identified, and it has been shown that the $-219\,G>T$ polymorphism exerts an allele-specific effect on apoE expression such that the T allelic promoter has a lower transcriptional activity than the G allelic promoter.[10–13] Recently, it has been reported that individuals carrying the T allele have lower apoE concentrations in the plasma and are at increased risk of myocardial infarction.[14]

Genetic factors for multifactorial disorders such as CAD may be classified into disease susceptibility genes and disease-modifying genes. The former may contribute to the initiation of the disease process, whereas the latter may influence the progression and outcome of the disease after it has been initiated. Previous studies have indicated that the $\varepsilon2/\varepsilon3/\varepsilon4$ polymorphism is associated with the severity of CAD.[15–17] In this study, we investigated whether the $-219\,G>T$ polymorphism also had an impact on disease severity and if so, whether the effect of this promoter polymorphism was independent of the $\varepsilon2/\varepsilon3/\varepsilon4$ polymorphism and plasma cholesterol levels. This might provide a better estimate of the contribution of the apoE gene to CAD and insights into the underlying mechanisms. It is plausible that the genetic basis of CAD is related not only to the qualitative change in the apoE protein resulting from the $\varepsilon2/\varepsilon3/\varepsilon4$ polymorphism but also to quantitative differences in the apoE expression resulting from variation in the apoE gene promoter.

Subjects and methods

Subjects

We recruited 1501 consecutive Caucasian patients undergoing interventional or diagnostic coronary angiography in the Wessex Cardiothoracic Unit, Southampton General Hospital. Among the 1501 subjects, 1170 had angiographically documented CAD as having $>50\%$ diameter stenosis in at least one major epicardial coronary artery and the remaining 331 had normal angiogram. The study was approved by the South and West Local Research Ethics Committee (number 298/99), and all subjects gave written consent. We recorded demographic and clinical data including age, gender, weight, height, occupation, smoking habit and number of cigarettes consumed per day by each smoker, the presence or absence of hyperlipidemia (defined as cholesterol >5.2 mmol/l and/or triglyceride >3 mmol/l), current medications particularly the use of lipid-lowering drugs, the presence or absence of hypertension (defined as diastolic blood pressure >95 mmHg and/or systolic blood pressure >160 mmHg), the presence or absence of type 1 or type 2 diabetes, the presence or absence of previous myocardial infarction, and the presence or absence of coronary heart disease in first-degree relatives under 65 years of age. The main demographic and clinical characteristics of patients with angiographically documented CAD are summarized in Table 1. In all, 54% of the patients had a previous history of myocardial infarction or unstable angina. Total cholesterol and triglyceride levels were measured by the clinical chemistry department of the Southampton General Hospital using standard quality controlled enzymatic methods. All coronary angiograms were assessed by one consultant cardiologist. A 10 ml blood sample was taken from each subject and DNA was extracted using a salt precipitation method as previously described.[18]

Genotyping

Genotypes for the $\varepsilon2/\varepsilon3/\varepsilon4$ and $-219\,C>T$ polymorphisms were determined using previously described methods.[11,19] In brief, a sequence containing the $\varepsilon2/\varepsilon3/\varepsilon4$ polymorphic site was amplified by PCR using primers 5′-AGAATTC GCCCCGGCCTGGTACAC-3′ and 5′-TAAGCTTGGCACGG CTGTCCAAGGA-3′, and the amplicon was subjected to digestion with restriction endonuclease *Hha* I and gel electrophoresis. Similarly, a sequence containing the $-219\,G>T$ polymorphic site was amplified using PCR primers 5′-AGAATGGAGGAGGGTGCCTG-3′ and 5′-ACT CAAGGATCCCAGACTTG-3′, followed by digestion with restriction enzyme *Bst*N I and gel electrophoresis.

Statistical analysis

One-way ANOVA and χ^2 tests were carried out to examine differences in quantitative and categorical variables between patient groups who had one, two or three coronary arteries with $>50\%$ stenosis, and between apoE genotypes. In these analyses, triglyceride values were log transformed to normalize the distribution. Logistic regression analysis was used to evaluate the effects (expressed as odds ratios) of genotypes on severity of CAD. In these analyses, the number of diseased vessels was entered as ordinal variable and the genotypes as independent variable. These were first carried out separately for the $-219\,G>T$ and $\varepsilon2/\varepsilon3/\varepsilon4$ polymorphisms, and analyses were performed firstly without and then with adjustment for covariates including age, gender, body mass index, smoking habit, diabetes, hypertension and cholesterol and triglyceride levels. A subsequent logistic regression analysis was performed with

−219 G>T genotypes and ε2/ε3/ε4 genotypes both entered in the equation, to evaluate independent contributions of these polymorphisms to severity of CAD.

Linkage disequilibrium between the −219 G>T and ε2/ε3/ε4 polymorphisms was examined by χ^2 analysis. The association metric ρ between the two polymorphisms was obtained using the ALLASS program (http://cedar.genetics.soton.ac.uk/public_html/). Haplotype frequencies were estimated using the SNPHAP program (Clayton V0.2 2002, http://www-gene.cimr.ac.uk/clayton/software) that employs an EM algorithm. The relationship between alleles and disease was assessed by logistic regression analyses in which the independent variables were scores (as 1 or 0) for the presence/absence of an allele in an individual. The relation between haplotypes and disease was examined by scoring the two haplotypes in an individual in probability using the haplotype frequency estimates from the SNPHAP program. Haplotypes could be scored without error except where an individual was heterozygous at more than one locus. In these cases, the score was allocated as a probability at each of the possible haplotypes. The table of scores for each individual at each of the six possible haplotypes was used in stepwise logistic regression in which we, firstly, included and, secondly, corrected for covariates (age, gender, BMI and genotypes). This allowed the testing of a haplotype effect over and above an allele-specific effect on the trait.

Results

Demographic and clinical characteristics of the subjects

We studied a cohort of 1170 patients with CAD documented angiographically as having >50% diameter stenosis in at least one major coronary artery. The characteristics of the subjects are summarized in Table 1. There was a positive correlation between age and the number of coronary arteries with >50% stenosis. The severity of CAD was also associated with the male gender and types I and II diabetes. There was no statistically significant difference between patient groups in lipid levels, body mass index, smoking habit, and the rates of hypertension and family history of CAD.

The ε2/ε3/ε4 polymorphism and CAD severity

Allele frequencies in the SAS sample were fully consistent with previous studies in other Caucasian samples, including unselected population studies. In the SAS sample as a whole, the frequencies of the ε2/ε2, ε2/ε3, ε3/ε3, ε3/ε4, ε4/ε4 and ε2/ε4 genotypes were 0.005 ($n=6$), 0.118 ($n=138$), 0.614 ($n=718$), 0.225 ($n=263$), 0.021 ($n=24$) and 0.018 ($n=21$), respectively. Expected numbers for each genotype were, respectively, 6.2, 134.1, 721.0, 260.8, 23.6 and 24.3, giving a χ^2 value of 0.61 and confirming extremely close fit to Hardy–Weinberg equilibrium. Applying the overall allele frequencies ($\varepsilon2=0.073$, $\varepsilon3=0.785$ and $\varepsilon4=0.142$) to estimate the number of subjects expected in each genotype category for subgroups reflecting 1, 2 or 3 vessel disease, identified obvious deviation in the ε3/ε4 genotype group. Here, the predicted numbers in 1, 2 and 3 vessel disease groups were 105.5, 80.3 and 67.1, whereas the observed numbers were 94, 84 and 85, representing a 21% over-representation of ε3/ε4 subjects in the group with 3 vessel disease.

Since the ε2/ε2, ε4/ε4 and ε2/ε4 genotypes are rare, to facilitate valid statistical analyses, we adopted a strategy used in many previous studies[16,20] such that the subjects were grouped into those carrying the ε2 allele (ie ε2/ε2+ε2/ε3), those with the ε3/ε3 genotype, and those carrying the ε4 allele (ε3/ε4+ε4/ε4), and the patients with the ε2/ε4 genotype were excluded as it would be difficult to assign them to any of the above groups. The frequency of ε4 allele carriers increased linearly with increasing number of diseased vessels ($P=0.045$ by χ^2 analysis, Table 2). Logistic regression analysis indicated that compared with the ε3/ε3 genotype, the genotypes with the ε4 allele were associated with an odds ratio of 1.347 (95% CI = 1.047–1.735, $P=0.021$) in favor of a more severe CAD, and this relationship remained significant after adjusting for covariates including cholesterol and triglyceride levels (Table 2).

Table 1 Demographic and clinical data

	All (n=1170)	*1 vessel (n=473)*	*2 vessel (n=396)*	*3 vessel (n=301)*	*P-value*
Age (years)	63.291 (9.967)	61.780 (10.227)	63.564 (9.264)	65.389 (10.056)	<0.001
Male gender	76.6%	73.0%	75.4%	83.6%	0.003
Current and ex-smokers	74.5%	75.3%	76.0%	91.9%	NS
Body mass index (kg/m^2)	27.499 (4.257)	27.456 (4.410)	27.203 (3.709)	27.951 (4.640)	NS
Plasma cholesterol (mmol/l)	5.109 (1.023)	5.124 (1.000)	5.097 (1.069)	5.101 (0.999)	NS
Plasma triglyceride (mmol/l)	1.859 (1.219)	1.873 (1.060)	1.886 (1.470)	1.800 (1.088)	NS
Hyperlipidemia	81.8%	81.9%	81.6%	81.9%	NS
Hypertension	44.8%	42.3%	43.7%	50.2%	NS
Type I diabetes	3.2%	1.5%	3.1%	6.0%	<0.001
Type II diabetes	10.2%	6.6%	9.8%	16.4%	<0.001
Family history of CAD	48.4%	46.4%	50.6%	48.5%	NS

Quantitative data are presented as mean (standard deviation).

Table 2 Relationship between apoE genotypes and extent of coronary artery disease

		Number (%)				
Polymorphism	*Genotype*	*1 vessel*	*2 vessel*	*3 vessel*	*Odds ratio (95% CI)*	*Adjusted odds ratio (95% CI)*[a]
ε2/ε3/ε4	ε4/ε4+ε3/ε4	102 (22.1%)	94 (24.2%)	91 (30.4%)	1.347 (1.047, 1.735), *P*=0.021	1.446 (1.095, 1.912), *P*=0.009
	ε3/ε3	296 (64.1%)	248 (63.9%)	174 (58.2%)	1	1
	ε2/ε2+ε2/ε3	64 (13.9%)	46 (11.9%)	34 (11.4%)	0.908 (0.651, 1.266), *P*=0.569	1.140 (0.781, 1.664), *P*=0.497
−219 G>T	T/T	81 (17.2%)	72 (18.4%)	74 (24.7%)	1.598 (1.161, 2.201), *P*=0.004	1.514 (1.065, 2.156), *P*=0.021
	T/G	259 (55.1%)	209 (53.5%)	167 (55.9%)	1.230 (0.952, 1.589), *P*=0.114	1.248 (0.556, 1.652), *P*=0.121
	G/G	130 (27.7%)	110 (28.1%)	58 (19.4%)	1	1

[a]Adjusted for age, gender, body mass index, smoking, diabetes, hypertension and cholesterol and triglyceride levels.

The −219 G>T polymorphism and CAD severity

There was also a significant difference in the distribution of genotypes for the −219 G>T polymorphism between patients with either one, two or three diseased vessels, that is the frequency of the T/T genotype increased with increasing number of diseased vessels ($P=0.017$ by χ^2 analysis, Table 2). Logistic regression analysis indicated that compared with the G/G genotype, the T/T genotype was associated with an odds ratio of 1.598 (95% CI = 1.161–2.201, $P=0.004$) and the T/G genotype associated with an odds ratio of 1.230 (95% CI = 0.952–1.589, $P=0.114$). This relationship remained after adjustment for plasma cholesterol and triglyceride levels and other risk factors (Table 2).

Independent effects of the −219 G>T polymorphism and the ε2/ε3/ε4 polymorphism on severity of CAD

To test whether the effect of the −219 G>T polymorphism on CAD severity was independent of the ε2/ε3/ε4 polymorphism, a logistic regression analysis was carried out with the genotypes for the −219 G>T and the genotypes for the ε2/ε3/ε4 polymorphism all entered in the equation. There was a significant interaction between the two polymorphisms (Table 3). The association between the T/T genotype of the −219 G>T polymorphism and increased CAD severity remained significant (odds ratio = 1.459, 95% CI = 1.040–2.048, $P=0.028$, Table 3) after adjusting for the ε2/ε3/ε4 polymorphism, indicating that the effect of the −219 G>T polymorphism was independent of the ε2/ε3/ε4 polymorphism. To further assess the independence of the −219 G>T polymorphism, we stratified the sample into two groups according to the presence/absence of the ε4 allele, and performed logistic regression analyses to test the effect of the −219 G>T polymorphism on CAD severity. The analyses indicated that the T/T genotype was significantly associated with greater CAD severity (odds ratio = 1.510, 95% CI = 1.028–2.221, $P=0.036$) in patients who did not carry the ε4 allele, and had a similar but smaller effect in ε4 allele carriers, again suggesting that the effect of the −219 G>T polymorphism was independent of the ε2/ε3/ε4 polymorphism. Similarly, effects of the ε4 allele on CAD severity were consistent in the three −219 G>T genotype groups (Table 3).

Linkage disequilibrium and haplotypic effects on CAD

There was considerable linkage disequilibrium between the −219 G>T and ε2/ε3/ε4 polymorphisms, with the −219T allele being associated with the ε4 allele ($\rho=0.9$, $P<0.001$). Since there could be additive effects from the two polymorphisms, we examined whether haplotypes harboring the deleterious alleles at both polymorphic sites had a greater effect on CAD than those carrying the deleterious allele at only one site. To address this question, the effects of all genotypes and haplotypes were assessed in a stepwise logistic regression analysis that allowed the testing of a haplotype effect over and above an allele-specific effect on the trait. In this analysis, only the −219T-ε4 and −219T-ε3 haplotypes remained significant, indicating that these haplotypes had greater effects than any single polymorphism alone. The −219T-ε4 haplotype gave an odds ratio of 1.488 (95% CI = 1.133–1.954) in this analysis, compared with an odds ratio of 1.328 for the ε4 allele and an odds ratio of 1.230 for the −219T allele when the two polymorphisms were examined individually (Table 4).

ApoE genotypes and lipid levels

There was an association between the ε2/ε3/ε4 polymorphism and plasma levels of cholesterol, with the mean cholesterol level being 3.5% higher in patients carrying the ε4 allele and 1% lower in patients carrying the ε2 allele, compared with those with the ε3/ε3 genotype ($P=0.033$, Table 5). Plasma levels of triglyceride were also different in these three genotype groups such that the mean triglyceride level was 17% higher in patients with ε2 allele and 6% higher in patients with the ε4 allele, than in the ε3/ε3 homozygotes ($P=0.033$, Table 5). These associations remained significant after adjustment for age, gender and lipid-lowering medication.

In contrast, there was no significant difference in plasma levels of cholesterol and triglyceride between patients with different genotypes for the −219 G>T polymorphism.

Table 3 Independent effects of the −219 G>T and ε2/ε3/ε4 polymorphisms on CAD severity

	Genotype	*Odds ratio (95% CI)*	*Adjusted odds ratio (95% CI)*[a]
Interaction effects in all subjects	T/T and $\varepsilon4^+$	1.733 (1.086, 2.768), *P*=0.021	1.867 (1.114, 3.124), *P*=0.018
	T/G and $\varepsilon4^+$	1.388 (1.064, 2.050), *P*=0.099	1.624 (1.062, 2.487), *P*=0.025
Main effects in all subjects			
Adjusted for the ε2/ε3/ε4 polymorphism	T/T	1.459 (1.040, 2.048), *P*=0.028	1.406 (1.035, 2.046), *P*=0.075
	T/G	1.155 (0.990, 1.659), *P*=0.288	1.145 (0.825, 1.536), *P*=0.368
	G/G	1	1
Adjusted for −219 G>T polymorphism	ε4/ε4+ε3/ε4	1.281 (0.990, 1.659), *P*=0.059	1.348 (0.838, 1.795), *P*=0.041
	ε3/ε3	1	1
	ε2/ε2+ε2/ε3	0.981 (0.696, 1.383), *P*=0.914	1.241 (0.838, 1.838), *P*=0.281
Main effects of −219 G>T polymorphism in $\varepsilon4^-$ subjects (*n*=849)	T/T	1.510 (1.028, 2.221), *P*=0.036	1.333 (0.868, 2.046), *P*=0.188
	T/G	1.177 (0.885, 1.565), *P*=0.263	1.137 (0.830, 1.558), *P*=0.424
	G/G	1	1
Main effects of −219 G>T polymorphism in $\varepsilon4^+$ subjects (*n*=285)	T/T	1.303 (0.619, 2.742), *P*=0.485	1.757 (0.815, 3.784), *P*=0.150
	T/G	1.051 (0.523, 2.110), *P*=0.889	1.329 (0.815, 3.784), *P*=0.423
	G/G	1	1
Main effects of the ε2/ε3/ε4 polymorphism in −219 T/T subjects (*n*=225)	ε4/ε4+ε3/ε4	1.231 (0.752, 2.0157), *P*=0.408	1.308 (0.723, 2.370), *P*=0.374
	ε3/ε3	1	1
	ε2/ε2+ε2/ε3	1.143 (0.087, 14.864), *P*=0.919	(only 1 subject)
Main effects of the ε2/ε3/ε4 polymorphism in −219 T/G subjects (*n*=621)	ε4/ε4+ε3/ε4	1.306 (0.9333, 1.829), *P*=0.120	1.440 (0.980, 2.117), *P*=0.063
	ε3/ε3	1	1
	ε2/ε2+ε2/ε3	1.231 (0.7641, 1.985), *P*=0.393	1.502 (0.845, 2.667), *P*=0.165
Main effects of the ε2/ε3/ε4 polymorphism in −219 G/G subjects (*n*=288)	ε4/ε4+ε3/ε4	1.347 (0.672, 2.699), *P*=0.401	1.294 (0.6107, 2.742), *P*=0.500
	ε3/ε3	1	1
	ε2/ε2+ε2/ε3	0.749 (0.449, 1.251), *P*=0.271	1.021 (0.544, 1.761), *P*=0.944

[a]Adjusted for age, gender, body mass index, smoking, diabetes, hypertension and cholesterol and triglyceride levels.

Table 4 Relationship between apoE haplotypes and extent of coronary artery disease

	Frequency				
	1 vessel	*2 vessel*	*3 vessel*	*Odds ratio (95% CI)*	*Adjusted odds ratio (95% CI)*[a]
ε4 allele	0.128	0.141	0.164	1.328 (1.026, 1.717), *P*=0.031	1.358 (1.020, 1.808), *P*=0.036
ε3 allele	0.791	0.787	0.774	—	—
ε2 allele	0.081	0.072	0.062	—	—
Total	1	1	1		
T allele	0.448	0.451	0.527	1.230 (0.952, 1.589), *P*=0.114	1.214 (1.091, 1.610), *P*=0.176
G allele	0.552	0.549	0.473	—	—
Total	1	1	1		
T-ε4 haplotype	0.094	0.098	0.133	1.488 (1.133, 1.954), *P*=0.039	1.404 (1.073, 1.838), *P*=0.015
T-ε3 haplotype	0.352	0.353	0.390	1.199 (1.032, 1.424), *P*=0.016	—
T-ε2 haplotype	0.002	0.001	0.004	—	—
G-ε4 haplotype	0.035	0.044	0.033	—	—
G-ε3 haplotype	0.437	0.434	0.383	—	—
G-ε2 haplotype	0.080	0.071	0.058	—	—
Total	1	1	1		

[a]Adjusted for age, gender, body mass index, smoking, diabetes, hypertension and cholesterol and triglyceride levels.

ApoE genotypes and other variables

Both polymorphisms had similar genotype and allele frequencies in males and females. Neither polymorphism was associated with body mass index, smoking, diabetes, hypertension, personal history of myocardial infarction or family history of coronary artery disease. There was no difference in mean age between the genotype groups.

Discussion

In a large cohort of patients with angiographically documented CAD, we examined the relationship between severity of the disease and the −219 G>T and ε2/ε3/ε4 polymorphisms in the apoE gene. The key findings of the study are that the two polymorphisms have independent effects on CAD severity and that haplotypes harboring the

Table 5 Plasma cholesterol and triglyceride levels according to apoE genotype

Polymorphism	*Genotype*	*Cholesterol*	*Triglyceride*
−219 G>T	T/T	5.079±0.974 (*n*=210)	1.884±1.636 (*n*=190)
	G/T	5.101±1.043 (*n*=596)	1.844±1.153 (*n*=554)
	G/G	5.150±1.018 (*n*=279)	1.873±0.977 (*n*=260)
	P-value	0.719	0.908
ε2/ε3/ε4	ε4/ε4+ε3/ε4	5.253±1.027 (*n*=266)	1.905±1.524 (*n*=244)
	ε3/ε3	5.075±1.023 (*n*=675)	1.792±1.051 (*n*=630)
	ε2/ε2+ε2/ε3	5.027±1.014 (*n*=130)	2.098±1.241 (*n*=116)
	P-value	0.033	0.033
	R^2	0.006	0.007

Cholesterol and triglyceride levels are shown as mean±standard deviation in mmol/l.

deleterious alleles in both polymorphic sites are associated with a further increase in CAD severity. This suggests that the progression of the disease may be influenced by both the qualitative change in the apoE protein arising from the ε2/ε3/ε4 polymorphism and quantitative differences in the levels of apoE expression because of variation in the promoter of the gene. The results also indicate that the effect of these polymorphisms on CAD is not entirely accounted for by differences in plasma levels of cholesterol and triglyceride, but is likely also attributable to other possible mechanisms. For example, it is plausible that the efficiency of cholesterol efflux from macrophages is reduced in individuals carrying the deleterious alleles of the apoE gene, resulting in a more abundant accumulation of lipid-laden foam cells and more extensive atherosclerotic lesions.

The results of this angiography-based study are consistent with the findings of two previous studies of the ε2/ε3/ε4 polymorphism based on pathological examinations. In the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) cohort consisting of 720 Caucasian and African-American males who died of external causes at the age of 15–34 years and subjected to autopsy, it was found that those carrying the ε4 allele had significantly higher mean percent surface area involvement with atherosclerotic lesions in the aorta, compared with those with the ε3/ε3 genotype.[15] Similarly, in an autopsy study (Helsinki Sudden Death Study) of 700 men from Finland, the ε4 allele was associated with a larger atherosclerotic lesion area in the right and left anterior descending coronary arteries in those <53 years old.[17]

Three polymorphisms (−219 G>T, −427 C>T and −491 A>T) in the promoter of the apoE gene have been reported.[10–13] A systematic scanning for variation in a 5.5 kb sequence spanning the entire ApoE genomic region did not detect any other common polymorphism in the promoter region of this gene in two Caucasian samples respectively consisting of 884 European-Americans and 452 Europeans.[21] Of the three promoter polymorphisms, the −219 G>T has the strongest effect on apoE gene expression as demonstrated by *in vitro* reporter assays and measurements of apoE mRNA levels in *ex vivo* brain tissues from patients with Alzheimer's disease.[12,13] It has been shown that plasma apoE concentration is lowest in individuals who are homozygous for the T allele and highest in homozygotes for the G allele.[14] It has also been reported that the −219 G>T polymorphism, but not the −427 C>T or −491 A>T variants, is associated with risk of myocardial infarction in a study of 567 myocardial infarction patients and 678 healthy control subjects.[14] The present study suggests that this functional promoter polymorphism can also affect CAD severity.

Neither the study by Lambert *et al*[14] nor the present study detects a significant association between the −219 G>T polymorphism and plasma cholesterol and triglyceride levels, although the polymorphism has been shown to influence plasma apoE concentration. In contrast, the ε2/ε3/ε4 polymorphism is thought to be one of the most important genetic factors on the regulation of cholesterol metabolism at the population level, with the ε4 allele being associated with elevation and the ε2 allele with a reduction of cholesterol levels. In this study, the ε2/ε3/ε4 polymorphism was found to account for about 0.6% of the variance of total cholesterol levels in plasma, which is consistent with the findings from other studies.[22] Although the precise mechanism underlying the effect of the ε2/ε3/ε4 polymorphism on lipid levels has not been defined, several hypothesis has been proposed. It has been demonstrated that compared with the E3 isoform, the E4 isoform has increased affinity and the E2 isoform has reduced affinity to lipoprotein receptors on liver cells.[23] Thus, it has been suggested that compared with E2 and E3, apoE4 competes more effectively with LDL for binding to receptors on liver cells for clearance, leading to increased LDL cholesterol levels.[23]

Many studies have shown an association between the ε4 allele and CAD susceptibility and severity. In contrast, the relationship of the ε2 allele with CAD remains controversial. Although some studies have suggested that the ε2 allele is associated with a reduced risk of CAD, other studies

have not found such a protective effect.[8,20,24] The present study and many others have found that individuals carrying the ε2 allele have lower cholesterol but increased triglyceride levels.[25–27] This disparate effect of the ε2 allele on the two lipids might explain why it may have a protective or unfavorable effect on CAD development.

The effects of apoE genotype on CAD may be modulated by other factors, for example, the strength of association between the apoE ε2/ε3/ε4 polymorphism and CAD severity is reduced after taking other risk factors into account, including high-density lipoprotein and apolipoprotein B.[16] However, the apoE genotypic effects on CAD remain significant after adjustment for such covariates, [14,16,20] suggesting that apoE genotype is an independent risk factor for CAD.

Although the relationship between the apoE gene and CAD is well established, most studies to date have been focused on the ε2/ε3/ε4 polymorphism. The present study indicates that the −219 G>T polymorphism influences CAD severity, which is independent of and possibly additive to the effect of the ε2/ε3/ε4 polymorphism. This allelic heterogeneity of the apoE gene in CAD may represent a paradigm for other disease genes for this and other common disorders.

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References

1 Mahley RW: Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 1988; **240**: 622–630.
2 Mazzone T: Apolipoprotein E secretion by macrophages: its potential physiological functions. *Curr Opin Lipidol* 1996; **7**: 303–307.
3 Rosenfeld ME, Butler S, Ord VA *et al*: Abundant expression of apoprotein E by macrophages in human and rabbit atherosclerotic lesions. *Arterioscler Thromb* 1993; **13**: 1382–1389.
4 Huang Y, von Eckardstein A, Wu S, Maeda N, Assmann G: A plasma lipoprotein containing only apolipoprotein E and with gamma mobility on electrophoresis releases cholesterol from cells. *Proc Natl Acad Sci USA* 1994; **91**: 1834–1838.
5 Zhang WY, Gaynor PM, Kruth HS: Apolipoprotein E produced by human monocyte-derived macrophages mediates cholesterol efflux that occurs in the absence of added cholesterol acceptors. *J Biol Chem* 1996; **271**: 28641-–28646.
6 Zhang SH, Reddick RL, Piedrahita JA, Maeda N: Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 1992; **258**: 468–471.
7 Bellosta S, Mahley RW, Sanan DA *et al*: Macrophage-specific expression of human apolipoprotein E reduces atherosclerosis in hypercholesterolemic apolipoprotein E-null mice. *J Clin Invest* 1995; **96**: 2170–2179.
8 Wilson PW, Schaefer EJ, Larson MG, Ordovas JM: Apolipoprotein E alleles and risk of coronary disease. A meta-analysis. *Arterioscler Thromb Vasc Biol* 1996; **16**: 1250–1255.
9 Eichner JE, Dunn ST, Perveen G, Thompson DM, Stewart KE, Stroehla BC: Apolipoprotein E polymorphism and cardiovascular disease: a HuGE review. *Am J Epidemiol* 2002; **155**: 487–495.
10 Bullido MJ, Artiga MJ, Recuero M *et al*: A polymorphism in the regulatory region of APOE associated with risk for Alzheimer's dementia. *Nat Genet* 1998; **18**: 69–71.
11 Lambert JC *et al*: A new polymorphism in the APOE promoter associated with risk of developing Alzheimer's disease. *Hum Mol Genet* 1998; **7**: 533–540.
12 Lambert JC, Berr C, Pasquier F *et al*: Pronounced impact of Th1/E47cs mutation compared with −491 AT mutation on neural APOE gene expression and risk of developing Alzheimer's disease. *Hum Mol Genet* 1998; **7**: 1511-–1516.
13 Artiga MJ, Bullido MJ, Sastre I *et al*: Allelic polymorphisms in the transcriptional regulatory region of apolipoprotein E gene. *FEBS Lett* 1998; **421**: 105–108.
14 Lambert JC, Brousseau T, Defosse V *et al*: Independent association of an APOE gene promoter polymorphism with increased risk of myocardial infarction and decreased APOE plasma concentrations–the ECTIM study. *Hum Mol Genet* 2000; **9**: 57–61.
15 Hixson JE: Apolipoprotein E polymorphisms affect atherosclerosis in young males. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. *Arterioscler Thromb* 1991; **11**: 1237–1244.
16 Wang XL, McCredie RM, Wilcken DE: Polymorphisms of the apolipoprotein E gene and severity of coronary artery disease defined by angiography. *Arterioscler Thromb Vasc Biol* 1995; **15**: 1030–1034.
17 Ilveskoski E, Perola M, Lehtimaki T *et al*: Age-dependent association of apolipoprotein E genotype with coronary and aortic atherosclerosis in middle-aged men: an autopsy study. *Circulation* 1999; **100**: 608–613.
18 Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**: 1215.
19 Dallinga-Thie GM, Linde-Sibenius TM, Kock LA, De Bruin TW: Apolipoprotein E2/E3/E4 genotyping with agarose gels. *Clin Chem* 1995; **41**: 73–75.
20 Humphries SE, Talmud PJ, Hawe E, Bolla M, Day IN, Miller GJ: Apolipoprotein E4 and coronary heart disease in middle-aged men who smoke: a prospective study. *Lancet* 2001; **358**: 115–119.
21 Nickerson DA *et al*: Sequence diversity and large-scale typing of SNPs in the human apolipoprotein E gene. *Genome Res* 2000; **10**: 1532–1545.
22 Sing CF, Davignon J: Role of the apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. *Am J Hum Genet* 1985; **37**: 268–285.
23 Mamotte CD, Sturm M, Foo JI, van Bockxmeer FM, Taylor RR: Comparison of the LDL-receptor binding of VLDL and LDL from apoE4 and apoE3 homozygotes. *Am J Physiol* 1999; **276**: E553–E557.
24 Lahoz C, Schaefer EJ, Cupples LA *et al*: Apolipoprotein E genotype and cardiovascular disease in the Framingham Heart Study. *Atherosclerosis* 2001; **154**: 529–537.
25 Wilson PW, Myers RH, Larson MG, Ordovas JM, Wolf PA, Schaefer EJ: Apolipoprotein E alleles, dyslipidemia, and coronary heart disease. The Framingham Offspring Study. *JAMA* 1994; **272**: 1666–1671.
26 Luc G, Bard JM, Arveiler D *et al*: Impact of apolipoprotein E polymorphism on lipoproteins and risk of myocardial infarction. The ECTIM Study. *Arterioscler Thromb* 1994; **14**: 1412–1419.
27 Tiret L, de Knijff P, Menzel HJ, Ehnholm C, Nicaud V, Havekes LM: ApoE polymorphism and predisposition to coronary heart disease in youths of different European populations. The EARS Study. European Atherosclerosis Research Study. *Arterioscler Thromb* 1994; **14**: 1617–1624.