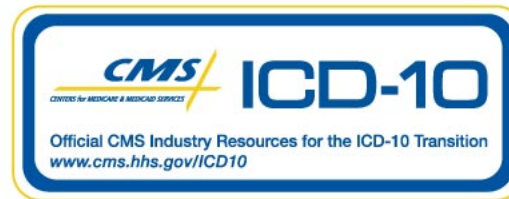


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With Stable and Unstable Coronary Artery Disease**

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Apolipoprotein E Genotype and Circulating Interleukin-10 Levels in Patients With Stable and Unstable Coronary Artery Disease

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OBJECTIVES	This study was designed to assess the relation between apolipoprotein E (apoE) genotype and serum interleukin (IL)-10 levels in patients with acute coronary syndrome (ACS) and chronic stable angina (CSA).
BACKGROUND	Genetic variations in the apoE gene affect the risk for coronary artery disease (i.e., carriers of the e4 allele have an increased risk). Increased levels of C-reactive protein (CRP), an inflammatory marker, correlate with an increased risk of acute coronary events, whereas increased IL-10 concentrations have an atheroprotective role. Studies have reported a negative association between the apoE e4 allele and CRP levels.
METHODS	Apolipoprotein E genotypes were assessed in 166 consecutive ACS patients (119 men, mean age 68 years, interquartile range [IQR] 60 to 74 years) and 70 CSA patients (54 men, mean age 65 years, IQR 62 to 68 years). Serum IL-10 and CRP were assessed at study entry.
RESULTS	Analysis of covariance showed that genetic variation in the apoE gene locus significantly influences serum IL-10 levels in both ACS ($p = 0.009$) and CSA patients ($p = 0.013$). Among ACS patients, IL-10 levels were lower in E3/E4 carriers compared with E3/E3 carriers ($p = 0.01$) and marginally lower compared with E2/E3 carriers ($p = 0.065$). Among CSA patients, IL-10 levels were lower in E3/E4 carriers compared with E2/E3 carriers ($p = 0.004$) and marginally lower compared with E3/E3 carriers ($p = 0.086$).
CONCLUSIONS	The IL-10 concentrations differ in ACS and in CSA patients with different apoE genotypes. The e4 allele was associated with a trend toward lower IL-10 serum levels. Our results may provide an explanation of findings in previous studies that cardiovascular risk is higher in e4 carriers despite the presence of low CRP levels. (J Am Coll Cardiol 2006;48:2471–81) © 2006 by the American College of Cardiology Foundation

Apolipoprotein E (apoE) is a structural component of both chylomicrons and very low-density lipoprotein (LDL) remnants. Apolipoprotein E is thought to play a central role in atherosclerosis by participating in overall plasma cholesterol homeostasis, mainly via the regulation of hepatic uptake of remnant lipoproteins, by facilitating cholesterol efflux from macrophage foam cells within atherosclerotic lesions, and by modifying inflammatory responses (1). Apolipoprotein E displays genetic polymorphism with three common alleles, e2, e3, and e4, in a single-gene locus in chromosome 19 that give rise to 3 homozygous (apoE2/2, apoE3/3, apoE4/4) and 3 heterozygous genotypes (apoE2/3, apoE2/4, apoE3/4) (2). The apoE allele e4 is associated with increased LDL cholesterol levels and decreased apoE plasma concentra-

tions (2,3). Conversely, the e2 allele is associated with reduced LDL cholesterol levels and higher apoE plasma concentrations (2,3). There is evidence that the e4 allele is associated with increased risk of coronary artery disease (CAD), whereas the e2 allele is considered to confer protection against CAD. The mechanisms responsible for these associations have not been elucidated (4–6).

Inflammation plays a central role in the pathogenesis of atherosclerosis (7). Increased levels of the acute-phase reactant C-reactive protein (CRP) predict future cardiovascular events (8–10), whereas elevated serum levels of interleukin (IL)-10, an anti-inflammatory cytokine with atheroprotective actions (11), are known to be associated with a more favorable prognosis (12). Recent studies investigating the association between apoE and inflammatory responses showed that genetic variation in apoE gene locus influences CRP levels (13–15). Indeed, carriers of the e4 allele were found to have reduced CRP levels compared with carriers of e3 or e2 alleles (13–15).

These findings contrast with the fact that the e4 allele is most strongly associated with CAD; therefore, we sought to assess the impact of the apoE genotypic variation on serum IL-10 levels.

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Abbreviations and Acronyms

ANCOVA	= analysis of covariance
apoE	= apolipoprotein E
CAD	= coronary artery disease
CRP	= C-reactive protein
CSA	= chronic stable angina
HDL	= high-density lipoprotein
IL	= interleukin
IQR	= interquartile range
LDL	= low-density lipoprotein
MI	= myocardial infarction
NSTEMI	= non-ST-segment elevation myocardial infarction
STEMI	= ST-segment elevation myocardial infarction
UA	= unstable angina

METHODS

Patients. We assessed 166 consecutive white patients who were admitted to the coronary care unit with a diagnosis of acute coronary syndrome (ACS), including acute myocardial infarction (MI) (both ST-segment elevation [STEMI] and non-ST-segment elevation [NSTEMI]) and unstable angina (UA). In addition, we studied 70 white chronic stable angina (CSA) patients undergoing coronary arteriography for the assessment of angina chest pain.

Myocardial infarction and UA were diagnosed using the joint European Society of Cardiology/American College of Cardiology criteria (16–18) as follows: MI was diagnosed in the presence of prolonged (>20 min) chest discomfort in the past 24 h, with ST-segment changes suggestive of myocardial ischemia or necrosis on the standard 12-lead electrocardiogram, associated with increased serum markers of myocardial damage measured on at least 2 occasions during the first 24 h after the index event (>2-fold increase over the upper normal range required for troponin T –0.1 ng/ml). Myocardial infarction patients were considered to have STEMI in the presence of ≥ 0.2 mV ST-segment elevation at the J point in 2 or more contiguous electrocardiogram leads. The NSTEMI was diagnosed in the presence of new ST-segment depression (≥ 0.1 mV) or T-wave inversion (≥ 0.3 mV) in 2 or more contiguous leads (16–18). Unstable angina was defined as anginal pain at rest fulfilling Braunwald's IIIb criteria with transient significant ischemic ST-segment or T-wave changes, or both, without evidence of myocardial damage (18). Stable CAD was defined as typical exertional chest pain relieved by rest and/or nitrates, without a change in frequency or pattern for 3 months before study entry, and with a positive response (>1 mm ST-segment depression) to exercise stress testing or reversible perfusion defects during myocardial perfusion scintigraphy. All CSA patients had >50% coronary artery stenoses on coronary angiography.

Whole blood and serum samples were obtained from every patient at study entry for the assessment of apoE genotype and inflammatory markers, respectively. The use of concomitant medication at study entry was recorded.

The study was approved by the hospital's ethics committee, and all patients gave written informed consent before study entry. We did not include patients with a history of excessive alcohol intake (>8 U/week) or hematologic, neoplastic, renal, liver, or thyroid disease. Furthermore, patients who had infectious or autoimmune diseases, familial hyperlipidemia, or triglycerides >400 mg/dl or who were undergoing surgical procedures in the preceding 3 months were also excluded. None of the patients included in the study was receiving treatment with anti-inflammatory drugs or hormone replacement therapy.

Laboratory methods. In all patients, 2 peripheral blood samples were drawn within 1 h from admission. Whole-blood specimens for apoE genotype measurement were collected in standard vacutainer tubes containing ethylenediaminetetraacetic acid as anticoagulant and immediately frozen and stored at -20°C . Blood samples intended for biochemical measurements were centrifuged at 3,000 rpm for 10 min, and the serum thus obtained was frozen and stored at -70°C until assessment.

Genomic deoxyribonucleic acid was extracted from whole blood samples by salting out as described by Miller et al. (19). We used semi-nested polymerase chain reaction following a modification of the procedure described by Hixson et al. (20) to amplify a 218-bp-long deoxyribonucleic acid fragment containing 2 polymorphic sites at positions 112 (Arg \rightarrow Cys) and 158 (Arg \rightarrow Cys). The following primers were used for amplification: 1EF2: 5' ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A 3', 2AER: 5' TAA GCT TGG CAC GGC TGT CCA A 3', and 2ERC: 5' TCC AAG GAG CTG CAG GCG GCG CA 3'. Alleles were defined as follows: e2 by T and Arg at position 112, by T at position 158, and no HhaI recognition site at either; allele e3 by T at position 112, C and HhaI recognition site at position 158; and allele e4 by C at position 112, C at position 158, and HhaI recognition sites at both positions. The polymerase chain reaction product was subsequently digested with HhaI and the produced fragments were assessed in a 4% metafor agarose gel.

Sandwich enzyme immunoassay was performed for measuring concentrations of serum IL-10 using Quantikine HS human IL-10 (R&D Systems Inc., Minneapolis, Minnesota) (21) commercial kits with monoclonal antibodies, with a minimum detectable concentration <0.5 pg/ml. The intra-assay precision and inter-assay precision of the method were 8.5% and 15.6%, respectively.

Troponin T measurement was performed using commercially available assays (Cardiac Troponin T, Roche Diagnostics, Basel, Switzerland) with a minimum detectable concentration of 0.01 ng/ml on the Elecsys 1010 (Roche Diagnostics) based on electrochemiluminescence method. The intra-assay precision and inter-assay precision of the method were <1.4% and <5.1%, respectively.

High-sensitivity CRP was measured by immunonephelometry using Dade Behring (Marburg, Germany) commercial kits (22), with a minimum detectable concentration

of 0.17 mg/l. Total, LDL, and high-density lipoprotein (HDL) cholesterol measurements, as well as triglycerides and creatinine measurements, were carried out by our biochemistry department using standard methods.

Statistical analysis. Values are expressed as median values with interquartile ranges (IQRs). The Kolmogorov-Smirnov test was used to examine the distribution of the variables under assessment. Differences in continuous variables between 2 groups were assessed using Mann-Whitney *U* test, whereas differences between >2 groups were assessed using Kruskal-Wallis test. Comparisons between categorical variables were performed by chi-square test or Fisher exact test when required. Although the E3/E3, E3/E4 and E2/E3 genotypes were present in both the ACS and CSA patient groups, the other apoE genotypes (E2/E4 and E2/E2) were rarely found in these patients; therefore, comparisons for those specific genotypes were not performed.

Before carrying out parametric statistical procedures, all variables that were not normally distributed were transformed to ensure a more normal distribution and equal variance before further parametric analysis and to fulfill statistical requirements for multiple regression analysis and analysis of covariance. Specifically, total, HDL, and LDL cholesterol as well as triglyceride levels were logarithmically transformed, and IL-10 and CRP levels were square-root transformed.

Significant covariates for IL-10 and CRP were identified using stepwise linear regression models where age, gender, body mass index, disease on admission (presence of STEMI vs. NSTEMI/UA), smoking status, presence of diabetes mellitus, hypertension, dyslipidemia, family history of CAD, previous history of CAD, lipid levels, and medication use were entered into the analyses. Significance levels of 0.05 and 0.1 were chosen to exclude and include terms, respectively. After identification of significant covariates, we tested differences in IL-10 and CRP levels among apoE genotypes using analysis of covariance (ANCOVA) adjusting for appropriate covariates. Subsequent post-hoc least significant difference tests were used to evaluate differences in IL-10 and CRP levels among the 3 groups in a pairwise fashion.

All analyses were performed separately for ACS and CSA patients.

Correlation analysis between variables of the study was carried out using Spearman's correlation coefficient *r*. All statistical calculations were performed using SPSS 12.0 statistical software package (SPSS Inc., Chicago, Illinois). A *p* value <0.05 was considered statistically significant.

RESULTS

Study population—baseline characteristics. Apolipoprotein E genotype was measured in 166 ACS patients (119 [72%] men, median age 68 [IQR 60 to 74] years) and 70 CSA patients (54 [77%] men, median age 65 [IQR 62 to 68] years). Of the 236 study participants, 164 (69.5%) were E3/E3 carriers, 52 (22%) E3/E4 carriers, and 18 (7.6%) E2/E3 carriers. One patient (0.4%) had the E2/E4 geno-

Table 1. Distribution of the ApoE Polymorphism in Acute Coronary Syndrome Patients (n = 166) and Chronic Stable Angina Patients (n = 70)

ApoE Genotype	ACS, n (%)	CSA, n (%)
22	1 (0.6%)	—
23	14 (8.4%)	4 (5.7%)
24	1 (0.6%)	—
33	116 (70%)	48 (68.6%)
34	34 (20.4%)	18 (25.7%)
44	—	—
Total	166	70

Chi-square = 1.94,
p = 0.746

Allele Frequency*	<i>p</i> Value		
e2	0.0512	0.0285	0.401
e3	0.8433	0.8428	0.988
e4	0.1054	0.1285	0.987

*Allele frequency was calculated by allele counting, *p* for chi-square contingency test. ACS = acute coronary syndrome; ApoE = apolipoprotein E; CSA = chronic stable angina; e = e allele.

type, 1 patient (0.4%) had the E2/E2 genotype, and no patients had the E4/E4 genotype. There were no significant differences in the distribution of apoE genotype and allele frequency between ACS and CSA patient groups (Table 1).

Baseline characteristics of patients with CSA and ACS are presented in Table 2. A higher proportion of ACS patients had a history of previous CAD. Established risk factors for CAD were similar in the 2 groups, with the exception of family history of CAD, which was more common in stable CAD patients than in ACS patients. The medications taken by both groups at study entry were similar, with the exception of beta-blockers, calcium channel blockers, and aspirin, which, as expected, were significantly more common in the stable CAD group. Total cholesterol, LDL cholesterol, and CRP levels were significantly higher in ACS patients. In contrast, HDL cholesterol and IL-10 levels were higher in the stable CAD group.

ApoE polymorphism in the ACS study population. Of the 166 ACS study participants, 116 (70%) were E3/E3 carriers, 34 (20.4%) were E3/E4 carriers, and 14 (8.4%) were E2/E3 carriers. One patient (0.6%) had the E2/E4 genotype, 1 patient (0.6%) had the E2/E2 genotype, and none of the patients had the E4/E4 genotype.

The LDL cholesterol levels were similar among the 3 apoE genotype groups; however, LDL cholesterol levels were non-significantly higher among the E3/E4 carriers. Table 3 summarizes baseline characteristics and biochemistry results among the 3 different apoE genotype groups at study entry.

Association of the apoE polymorphism and IL-10 levels in ACS patients. Nonparametric analysis of variance showed that genetic variance in apoE gene locus significantly influences serum IL-10 levels among ACS patients (*p* = 0.008) (Fig. 1). As multiple a priori pairwise comparisons of IL-10 levels in different apoE genotype subgroups (Table 3) may be subject to type I error risk, parametric

Table 2. Baseline Characteristics of 166 Patients With Acute Coronary Syndrome (ACS) and 70 Patients With Chronic Stable Angina (CSA)

	ACS (n = 166)	CSA (n = 70)	p Value
Age (yrs)	68 (60–74)	65 (62–68)	0.167
Men, n (%)	119 (72%)	54 (77%)	0.424
BMI (kg/m ²)	29 (28–30)	29 (27–30)	0.750
Disease, n (%)			
STEMI	69 (42%)	—	n/a
NSTEMI	47 (28%)	—	n/a
UA	50 (30%)	—	n/a
Stable angina	—	70 (100%)	n/a
Risk factors, n (%)			
Smoking	52 (31%)	24 (34%)	0.651
Diabetes mellitus	56 (34%)	22 (31%)	0.764
Hypertension	110 (66%)	46 (66%)	1
Dyslipidaemia	107 (65%)	36 (51%)	0.08
Family history	20 (12%)	18 (26%)	0.012*
Prior CAD	69 (42%)	12 (17%)	<0.001*
Previous MI	46 (28%)	7 (10%)	<0.001*
Prior CVD disease	16 (10%)	6 (9%)	1
Prior PCI	18 (11%)	2 (3%)	0.078
Prior CABG	14 (8%)	4 (6%)	0.597
Medications, n (%)			
ACE inhibitor	53 (32%)	26 (37%)	0.453
Beta-blocker	49 (29%)	34 (49%)	0.007*
Calcium channel blocker	55 (33%)	34 (49%)	0.028*
Nitrate	50 (30%)	24 (34%)	0.542
ARB	14 (8%)	12 (17%)	0.068
Statin	53 (32%)	30 (43%)	0.135
Aspirin	60 (36%)	44 (63%)	<0.001*
Clopidogrel	22 (13%)	16 (23%)	0.081
Biochemistry			
Creatinine (mg/dl)	1 (1–1.2)	1 (1–1.2)	0.848
Total cholesterol (mg/dl)	214 (189–230)	198 (160–223)	0.011†
LDL cholesterol (mg/dl)	126 (115–146)	112 (83–137)	<0.001†
HDL cholesterol (mg/dl)	46 (43–51)	52 (46–60)	<0.001†
Triglycerides (mg/dl)	128 (108–171)	147 (98–193)	0.560
CRP (mg/l)	9 (5.5–10)	4 (2–12)	<0.001†
IL-10 (pg/ml)	6.7 (5.1–7.5)	16.2 (12.8–21.3)	<0.001†

*p for chi-square test. †p for Mann-Whitney U nonparametric test.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; BMI = body mass index; CABG = coronary artery bypass grafting; CAD = coronary artery disease; CVD = cerebrovascular disease; CRP = C-reactive protein; HDL = high-density lipoprotein; IL = interleukin; LDL = low-density lipoprotein; MI = myocardial infarction; n/a = not applicable; NSTEMI = non-ST-segment elevation myocardial infarction; PCI = percutaneous coronary intervention; STEMI = ST-Segment elevation myocardial infarction; UA = unstable angina.

analysis of covariance with subsequent post-hoc tests were performed.

Using stepwise linear regression analysis, HDL cholesterol (for log HDL cholesterol beta = 0.168, p = 0.029) and LDL cholesterol levels (for log LDL cholesterol beta = -0.194, p = 0.015) were identified as significant covariates for IL-10 levels. The independent contribution of the genetic variation in apoE locus on IL-10 levels was preserved (p = 0.009) in an ANCOVA model after adjustment for HDL and LDL cholesterol levels (Table 4). Post-hoc analysis showed that E3/E4 carriers had significantly lower IL-10 levels compared with E3/E3 carriers (p = 0.01). Furthermore, E3/E4 carriers had lower, yet marginally not significant, IL-10 levels compared with E2/E3 carriers (p = 0.065). In contrast, post-hoc tests did not show any difference in IL-10 levels between E3/E3 and E2/E3 carriers (p = 0.775).

As the presence of myocardial necrosis may influence cytokine levels, as well as inflammatory and counter-regulatory mechanisms, we assessed the effect of e4 allele on serum IL-10 levels in a subgroup of unstable angina patients without evidence of myocardial damage as assessed by troponin T measurements. Troponin T-negative patients (n = 50) who were carriers of the e4 allele had significantly (p = 0.006) lower IL-10 levels (n = 9, IL-10: 4.5 pg/ml, IQR 4.1 to 5.6) compared with noncarriers of the e4 allele (n = 41, IL-10: 6.7 pg/ml, IQR 5.4 to 7.7).

Association of the apoE polymorphism and CRP levels in ACS patients. Kruskal-Wallis test showed that CRP levels differed significantly in the different apoE gene polymorphism groups (p < 0.001). To minimize type I error risk associated with multiple a priori pairwise comparisons (Table 3), parametric analyses of covariance with

Table 3. Baseline Characteristics Among the Most Common ApoE Genotypes in the Acute Coronary Syndrome Study Population (n = 164)

	ApoE Gene Polymorphism			Overall p Value	E3/E3 vs. E2/E3 p Value	E3/E3 vs. E3/E4 p Value	E2/E3 vs. E3/E4 p Value
	E3/E3	E2/E3	E3/E4				
Patients, n (%)	116 (70%)	14 (8.4%)	34 (20.4%)	n/a	n/a	n/a	n/a
Age (yrs)	68 (61–74)	67 (59–76)	67 (58–71)	0.789	0.786	0.375	0.828
Men, n (%)	86 (74%)	9 (64%)	24 (71%)	0.707	0.524	0.666	0.738
BMI (kg/m ²)	29 (28–30)	28.5 (28–30)	29 (28–30)	0.612	0.334	0.906	0.383
Disease, n (%)				0.632	0.333	0.731	0.656
STEMI	45 (41%)	7 (50%)	15 (44%)				
NSTEMI	30 (26%)	5 (36%)	10 (30%)				
UA	39 (34%)	2 (14%)	9 (27%)				
Risk factors, n (%)							
Smoking	37 (32%)	3 (21%)	11 (32%)	0.715	0.548	1	0.510
Diabetes mellitus	39 (34%)	3 (21%)	13 (38%)	0.533	0.547	0.683	0.328
Hypertension	78 (67%)	10 (71%)	20 (59%)	0.594	1	0.414	0.521
Dyslipidemia	80 (69%)	8 (57%)	19 (56%)	0.297	0.379	0.216	1
Family history	12 (10%)	3 (21%)	5 (15%)	0.430	0.206	0.539	0.676
Prior CAD disease	51 (44%)	3 (21%)	15 (44%)	0.262	0.152	1	0.196
Previous MI	34 (29%)	2 (14%)	10 (29%)	0.488	0.347	1	0.465
Prior CVD disease	10 (9%)	0 (0%)	5 (15%)	0.258	0.599	0.332	0.303
Prior PCI	15 (13%)	1 (7%)	2 (6%)	0.457	1	0.362	1
Prior CABG	9 (8%)	2 (14%)	3 (9%)	0.710	0.337	0.735	0.621
Medications, n (%)							
ACE inhibitor	34 (29%)	6 (43%)	12 (35%)	0.519	0.360	0.530	0.746
Beta-blocker	39 (34%)	3 (21%)	7 (21%)	0.265	0.547	0.204	1
Calcium channel blocker	44 (38%)	2 (14%)	8 (23%)	0.087	0.136	0.153	0.701
Nitrate	38 (33%)	2 (14%)	9 (27%)	0.321	0.224	0.535	0.469
ARB	12 (10%)	1 (7%)	1 (3%)	0.390	1	0.299	0.503
Statin	37 (32%)	4 (29%)	12 (35%)	0.888	1	0.836	0.746
Aspirin	43 (37%)	4 (29%)	12 (35%)	0.819	0.769	1	0.746
Clopidogrel	19 (16%)	1 (7%)	2 (6%)	0.222	0.694	0.163	1
Biochemistry							
Creatinine (mg/dl)	1.1 (1–1.2)	1.1 (1–1.1)	1.1 (1–1.2)	0.667	0.607	0.420	0.917
Total cholesterol (mg/dl)	214 (194–227)	198 (171–227)	212 (182–246)	0.459	0.232	0.677	0.334
LDL cholesterol (mg/dl)	126 (117–139)	135 (109–149)	144 (112–155)	0.190	0.476	0.081	0.426
HDL cholesterol (mg/dl)	46 (43–50)	43 (42–50)	44 (41–51)	0.272	0.268	0.185	0.658
Triglycerides (mg/dl)	128 (111–167)	129 (80–204)	138 (104–185)	0.651	0.499	0.543	0.454
CRP (mg/l)	9 (8–10)	9 (8–10)	4 (3–5)	<0.001*	0.913	<0.001†	<0.001†
IL-10 (pg/ml)	6.7 (5.4–7.7)	7 (5.2–7.8)	5.6 (4.2–6.7)	0.008*	0.620	0.003†	0.049†

One patient (0.6%) had E2/E2 apoE gene polymorphism and 1 patient (0.6%) had E2/E4 apoE gene polymorphism, whereas none of the patients had the E4/E4 apoE gene polymorphism. *p for Kruskal-Wallis nonparametric test. †p for Mann-Whitney U nonparametric test.

Abbreviations as in Table 2.

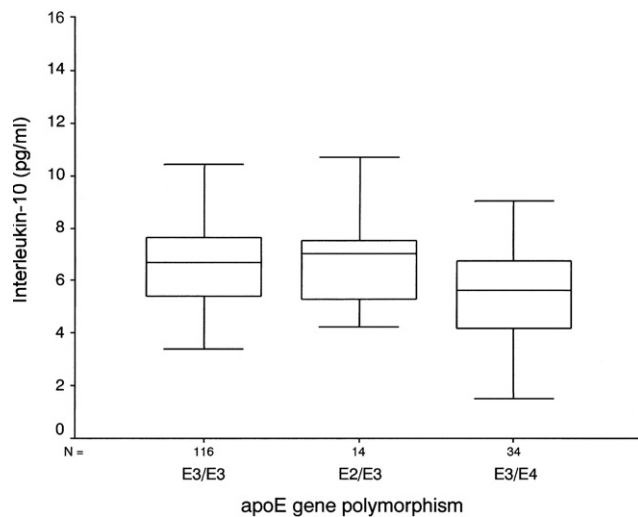


Figure 1. Interleukin-10 levels among apolipoprotein (apo)E genotype groups in acute coronary syndrome patients group. Box plots represent median levels with 25th and 75th percentiles of observed data; whiskers represent the 5th and 95th percentiles in each group.

subsequent post-hoc tests were used to compare CRP levels between different apoE genotype subgroups.

Stepwise linear regression analysis identified the presence of STEMI versus NSTEMI/UA (beta = 0.168, p = 0.014), HDL-cholesterol levels (for log HDL cholesterol beta = -0.171, p = 0.014), triglyceride levels (for log triglyceride beta = 0.239, p = 0.001), and smoking status (beta = 0.143, p = 0.039) as significant covariates for CRP levels. C-reactive protein levels remained significantly different (p < 0.001) among the different apoE gene polymorphism groups in an ANCOVA model after adjustment for all the aforementioned covariates (Table 4). Post-hoc analysis showed that E3/E4 carriers had significantly lower CRP levels compared with E3/E3 carriers (p < 0.001) and E2/E3 carriers (p < 0.001). In contrast, post-hoc tests did not show any difference in CRP levels between E3/E3 and E2/E3 carriers (p = 0.729).

Correlation analysis in ACS patients. We did not observe a significant association between serum IL-10 levels and CRP levels in the ACS study group (n = 164, r = 0.138, p = 0.078). Furthermore, no association was found between

the aforementioned variables among E3/E3 carriers (n = 116, r = 0.068, p = 0.467), E2/E3 carriers (n = 14, r = -0.256, p = 0.377), and E3/E4 carriers (n = 34, r = 0.030, p = 0.865).

ApoE polymorphism in the CSA study population. Of the 70 CSA study participants, 48 (68.6%) were E3/E3 carriers, 18 (25.7%) were E3/E4 carriers, and 4 (5.7%) were E2/E3 carriers; none of the patients had the E4/E4, E2/E2, or E2/E4 genotype. Total cholesterol (p = 0.049), LDL cholesterol (p = 0.003), and HDL cholesterol (p = 0.007) levels were significantly different among the 3 apoE genotype groups. In particular, E3/E4 carriers had significantly higher total (p = 0.031) and LDL cholesterol (p = 0.003) levels compared with E3/E3 carriers. In addition, E2/E3 carriers had higher HDL-cholesterol levels compared to E3/E3 (p < 0.001) and E3/E4 carriers (p = 0.042). Table 5 summarizes baseline characteristics and biochemistry results among the 3 different apoE genotype groups at study entry.

Association regarding the apoE polymorphism and IL-10 levels in CSA patients. Nonparametric analysis of variance showed that genetic variance in the apoE gene locus significantly influences serum IL-10 levels (p < 0.001) among CSA patients (Fig. 2). As multiple a priori pairwise comparisons regarding IL-10 levels between different apoE genotype subgroups (Table 5) are subject to type I error risk, parametric analysis of covariance with subsequent post-hoc tests were performed.

Using stepwise linear regression analysis, HDL cholesterol (for log HDL cholesterol beta = 0.528, p < 0.001), LDL cholesterol (for log LDL cholesterol beta = -0.263, p = 0.022), triglyceride levels (for log triglycerides beta = 0.456, p = 0.001), and calcium channel blocker use (beta = 0.292, p = 0.011) were identified as significant covariates for IL-10 levels. The independent contribution of the genetic variation in apoE locus on IL-10 levels was preserved (p = 0.013) in an ANCOVA model after adjustment for calcium channel blocker use, HDL and LDL cholesterol, and triglyceride levels (Table 6). Post-hoc analysis showed that E3/E4 carriers had significantly lower IL-10 levels compared with E2/E3 carriers (p = 0.004). Further-

Table 4. Estimated Adjusted Means of Interleukin-10 (IL-10) and C-Reactive Protein (CRP) in Different ApoE Genotypes in the Acute Coronary Syndrome Patient Group

ApoE Genotype	IL-10 (pg/ml)		CRP (mg/l)	
	Estimated Mean*	95% CI	Estimated Mean†	95% CI
E3/E3	6.7	6.3-7.1	9.1	8.3-9.8
E2/E3	6.9	5.7-8.1	8.7	6.7-10.9
E3/E4	5.6	4.9-6.3	4.5	3.6-5.5

ANCOVA for IL-10 model: R² = 0.293. ApoE genotype (F = 13.26, p = 0.009). Evaluated at covariates in the model log LDL cholesterol (F = 5.44, p = 0.021) and log HDL cholesterol (F = 3.93, p = 0.049). *Because IL-10 was square root transformed before entering the model, we present the squared estimated means. ANCOVA for CRP model: R² = 0.306. ApoE genotype (F = 23.48, p < 0.001). Evaluated at covariates in the model log HDL cholesterol (F = 5.53, p = 0.020), disease on presentation (STEMI vs. NSTEMI) (F = 5.78, p = 0.017), log triglycerides (F = 13.53, p < 0.001), and smoking status (F = 3.9, p = 0.05). †Because CRP was square root transformed before entering the model, we present the squared estimated means.

ApoE = apolipoprotein E; CI = confidence interval; F = f statistic for each variable included in the model; other abbreviations as in Table 2.

Table 5. Baseline Characteristics Among the Most Common ApoE Genotypes in the Chronic Stable Angina Study Population (n = 70)

	ApoE Gene Polymorphism			Overall p Value	E3/E3 vs. E2/E3 p Value	E3/E3 vs. E3/E4 p Value	E2/E3 vs. E3/E4 p Value
	E3/E3	E2/E3	E3/E4				
Patients, n (%)	48 (68.6%)	4 (5.7%)	18 (25.7%)	n/a	n/a	n/a	n/a
Age (yrs)	63 (61–69)	65 (64–66)	65 (64–67)	0.857	0.830	0.618	0.652
Men, n (%)	36 (75%)	4 (100%)	14 (78%)	0.518	0.562	1	0.554
BMI (kg/m ²)	29 (28–31)	30 (28–31)	28 (27–30)	0.156	0.435	0.117	0.098
Risk factors, n (%)							
Smoking	20 (42%)	—	4 (22%)	0.110	0.151	0.165	0.554
Diabetes mellitus	18 (37%)	2 (50%)	2 (11%)	0.086	0.634	0.069	0.135
Hypertension	28 (58%)	4 (100%)	17 (78%)	0.110	0.151	0.165	0.554
Dyslipidemia	24 (50%)	4 (100%)	8 (44%)	0.115	0.115	0.785	0.096
Family history	12 (25%)	—	6 (33%)	0.378	0.562	0.543	0.541
Prior CAD disease	6 (12%)	—	6 (33%)	0.087	1	0.073	0.541
Previous MI	4 (8%)	—	2 (11%)	0.769	1	0.661	1
Prior CVD disease	4 (8%)	—	2 (11%)	0.769	1	0.661	1
Prior PCI	2 (4%)	—	—	0.624	1	1	n/a
Prior CABG	2 (4%)	—	2 (11%)	0.489	1	0.298	1
Medications, n (%)							
ACE inhibitor	18 (37%)	2 (50%)	6 (33%)	0.820	0.634	1	0.602
Beta-blocker	26 (54%)	2 (50%)	6 (33%)	0.320	1	0.171	0.602
Calcium channel blocker	22 (46%)	2 (50%)	10 (56%)	0.779	1	0.584	1
Nitrate	18 (37%)	—	6 (33%)	0.314	0.285	1	0.541
ARB	10 (21%)	—	2 (11%)	0.417	0.576	0.487	1
Statin	24 (50%)	2 (50%)	4 (22%)	0.122	1	0.153	0.292
Aspirin	28 (58%)	4 (100%)	12 (67%)	0.235	0.151	0.585	0.541
Clopidogrel	12 (25%)	2 (50%)	2 (11%)	0.201	0.291	0.318	0.135
Biochemistry							
Creatinine (mg/dl)	1 (1–1.2)	1.1 (1.1–1.3)	1.1 (1–1.3)	0.114	0.206	0.076	0.652
Total cholesterol (mg/dl)	190 (154–219)	178 (175–182)	217 (186–253)	0.049*	0.359	0.031†	0.098
LDL cholesterol (mg/dl)	111 (81–131)	84 (79–90)	136 (112–163)	0.003*	0.139	0.003†	0.014†
HDL cholesterol (mg/dl)	51 (44–59)	68 (66–71)	48 (48–56)	0.007*	<0.001†	0.488	0.042†
Triglycerides (mg/dl)	151 (116–215)	126 (94–158)	147 (76–164)	0.269	0.359	0.150	0.774
CRP (mg/l)	5 (2–16)	10 (4–17)	1 (1–3)	<0.001*	0.607	<0.001†	<0.001†
IL-10 (pg/ml)	16.2 (13–21.9)	35.6 (20.1–51.1)	14.2 (8–18.8)	<0.001*	0.055	0.039†	0.014†

None of the patients had E2/E2, E2/E4, or E4/E4 apoE gene polymorphism. *p for Kruskal-Wallis nonparametric test. †p for Mann-Whitney *U* nonparametric test. Abbreviations as in Table 2.

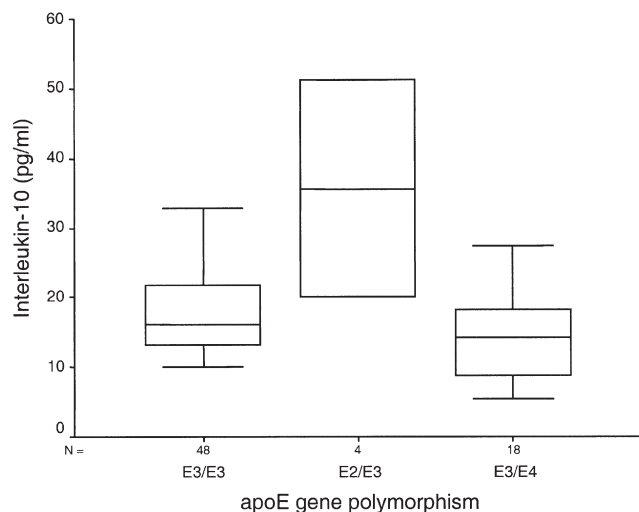


Figure 2. Interleukin-10 levels among apolipoprotein (apo)E genotype groups in chronic stable angina patients group. Box plots represent median levels with 25th and 75th percentiles of observed data; whiskers represent the 5th and 95th percentiles in each group.

more, E3/E4 carriers had lower, yet marginally not significant, IL-10 levels compared with E3/E3 carriers ($p = 0.086$). Post-hoc tests also showed that E2/E3 carriers had significantly higher IL-10 levels compared with E3/E3 carriers ($p = 0.02$).

Associations between the apoE polymorphism and CRP levels in CSA patients. Kruskal-Wallis test showed that CRP levels differed significantly in the different apoE gene polymorphism groups ($p < 0.001$). Similarly, because of the type I error risk associated with multiple a priori pairwise comparisons (Table 5), parametric ANCOVA with subsequent post-hoc tests were used to compare CRP levels between different apoE genotype subgroups.

Stepwise linear regression analysis identified HDL cholesterol levels (for log HDL cholesterol $\beta = -0.253$, $p = 0.025$), and beta-blocker use ($\beta = -0.349$, $p = 0.002$) as significant covariates for CRP levels. C-reactive protein levels remained to be significantly different ($p < 0.001$) among the different apoE gene polymorphism groups in an ANCOVA model after adjustment for all the aforementioned covariates (Table 6). Post-hoc analysis showed that E3/E4 carriers had significantly lower CRP levels compared

with E3/E3 carriers ($p < 0.001$). Furthermore, E3/E4 carriers had lower, yet marginally not significant CRP levels compared with E2/E3 carriers ($p = 0.069$). In contrast, post-hoc tests did not show any difference in CRP levels between E3/E3 and E2/E3 carriers ($p = 0.809$).

Correlation analysis in CSA patients. We did not observe any significant association between serum IL-10 levels and CRP levels in CSA study population ($n = 70$, $r = 0.209$, $p = 0.083$). Furthermore, no association was found between the aforementioned variables among E3/E3 carriers ($n = 48$, $r = -0.053$, $p = 0.718$), and E3/E4 carriers ($n = 18$, $r = 0.045$, $p = 0.823$).

DISCUSSION

Our study is among the first to evaluate the association between apoE genotypes and anti-inflammatory markers. Overall, we found that genetic variation in apoE gene locus significantly influences serum IL-10 levels both in ACS and CSA patients and, specifically, that e4 allele carriers are associated with a trend toward lower circulating IL-10 levels. In particular, among ACS patients, IL-10 levels were lower in E3/E4 carriers compared with E3/E3 and marginally lower compared with E2/E3 carriers. Similarly, among CSA patients IL-10 levels were lower in E3/E4 carriers compared to E2/E3 carriers and marginally lower compared to E3/E3 carriers. In addition, this association was independent of LDL cholesterol levels, which are known to differ in different apoE genotypes and could influence IL-10 serum concentration. This finding is of clinical relevance as it may provide an explanation to findings in previous reports that cardiovascular risk is higher in e4 carriers even in the presence of low CRP levels, probably as a result of reduced protection against the damaging effect of inflammatory mechanisms (4,23).

It is established that a major physiologic role for apoE is that of receptor-binding ligand, mediating the binding and hepatic uptake of chylomicron remnants and apoE-containing lipoproteins by both the LDL receptor and LDL receptor-related protein (24). The apoE polymorphisms, which presumably arose as mutations of the e3 allele, are the result of variation in arginine and cysteine composition at

Table 6. Estimated Adjusted Means of Interleukin-10 (IL-10) and C-Reactive Protein (CRP) in Different ApoE Genotypes in Chronic Stable Angina Patient Group

ApoE Genotype	IL-10 (pg/ml)		CRP (mg/l)	
	Estimated Mean*	95% CI	Estimated Mean†	95% CI
E3/E3	17.8	15.8–19.9	7.1	5.6–8.8
E2/E3	28.6	19.6–39.2	6.3	2–13
E3/E4	14.3	11.4–17.7	2	0.9–3.6

ANCOVA for IL-10 model: $R^2 = 0.354$. ApoE genotype ($F = 4.63$, $p = 0.013$). Evaluated at covariates in the model log HDL cholesterol ($F = 5.95$, $p = 0.018$), log LDL cholesterol ($F = 0.94$, $p = 0.336$), log triglyceride levels ($F = 5.91$, $p = 0.018$), and calcium channel blocker use ($F = 6.47$, $p = 0.013$). *Because IL-10 was square root transformed before entering the model, we present the squared estimated means. ANCOVA for CRP model: $R^2 = 0.368$. ApoE genotype ($F = 9.36$, $p < 0.001$). Evaluated at covariates in the model log HDL cholesterol levels ($F = 7.29$, $p = 0.009$) and b-blocker use ($F = 7.28$, $p = 0.009$). †Because CRP was square root transformed before entering the model, we present the squared estimated means.

ApoE = apolipoprotein E; CI = confidence interval; F = f statistic for each variable included in the model; other abbreviations as in Table 2.

residues 112 and 158. Allele e2 has a cysteine at both 112 and 158 residues, whereas the e4 allele has arginine instead (24). The e3 allele has cysteine at 112 and arginine at 158 and is the most common isoform observed in all populations (24).

It has been suggested that the presence of the e4 allele may lead to enhanced uptake of apoE containing lipoproteins and chylomicron remnants and, consequently, to a higher concentration of cholesterol within the hepatocytes. This may result in the down-regulation of the LDL receptor, which, in turn, might reduce LDL catabolism, accounting for the higher serum LDL concentrations observed in e4 carriers (24). Carriers of e2 alleles are less efficient in the removal of very low-density lipoprotein and chylomicron remnants because of the e2 allele's lower receptor binding ability. Slower rates of hepatic clearance of remnants might lead to lower hepatic cholesterol levels, more expression of LDL receptors, increase in LDL metabolism, and thus lower serum LDL cholesterol levels (24). The present study showed significantly higher LDL cholesterol levels in E3/E4 carriers among CSA patients, and a similar, albeit not significant, trend among apoE genotypes in ACS patients. In keeping with our results, other studies in the literature have reported nonsignificant differences in LDL cholesterol levels among different apoE genotypes (5,14,25). Despite conflicting evidence there appears to be some modulation of the relation between apoE genotype and LDL cholesterol levels by amount and type of fat/cholesterol intake (23). Because the majority of our patients were on a Mediterranean diet characterized by low levels of cholesterol and high intake of unsaturated fat, it is possible that this type of diet could have attenuated the LDL-modifying effect of apoE. However, in the ACS patient group ($n = 166$) carriers of the e4 allele ($n = 35$, LDL cholesterol: 144 mg/dl, IQR 112 to 166) had significantly higher LDL cholesterol levels ($p = 0.046$) compared with non-e4 carriers ($n=131$, LDL cholesterol: 126 mg/dl, IQR 116 to 143).

Furthermore, apoE regulates the inflammatory response. Apolipoprotein E expression modifies both T-lymphocyte and macrophage cytokine production and thus appears to directly influence both the innate and the acquired immune responses (1). Our findings that E3/E4 carriers are associated with lower CRP levels are in agreement with previous studies suggesting an inverse relationship between CRP levels and carriers of the e4 allele (13-15). This is a surprising finding given the association of the apoE e4 allele with higher concentrations of LDL cholesterol (2,3) and increased risk of CAD (4-6). Different and varying explanations have been given for this awkward observation. März et al. (15) suggested a "non-inflammatory" hypothesis, according to which the CRP down-regulating effect of the e4 allele is not related to inflammation, suggesting that in e4 allele carriers the mevalonate pathway is down-regulated, and thus the CRP is low. Furthermore, in the same study März et al. (15) proposed a "true anti-inflammatory" hypothesis in which the remnant lipoproteins accumulating in

carriers of e2 acted as pro-inflammatory agents at the site of the vessel wall and initiated or maintained a low-grade systemic inflammation, whereas their fast removal in e4 carriers led to an anti-inflammatory effect. Judson et al. (14) and Bach-Ngohou et al. (26) suggested that these anti-inflammatory-related protective effects may be outweighed by the lipid-related deleterious effects of the e4 allele. However, the contribution of the apoE allele to risk of CAD via its effects on plasma cholesterol levels seems to be minimal (23,25). The novel finding in our study that E3/E4 carriers are associated with a trend toward reduced levels of the anti-inflammatory cytokine IL-10 is of particular importance. It has been hypothesized that e4 allele carriers may show an "inflammatory imbalance" between pro- and anti-inflammatory mediators rather than a true "anti-inflammatory" profile. Previous studies (27) have shown, in agreement with our results, that e4 carriers are associated with attenuated anti-inflammatory activity. Human apoE has antioxidant activity (1). Miyata and Smith (27) quantified apoE allele-specific antioxidant activity by means of measuring allele-specific protection of cultured cells from oxidative cell death (27). In this regard, the e2 allele was found to be most effective in protecting from oxidative damage, whereas the e4 allele was least effective.

Although the isoform-specific effects of apoE4 on IL-10 levels could be related to reduced antioxidant activity of apoE4 relative to apoE3, the mechanisms by which apoE exerts its immunomodulatory effects remain incompletely understood. A possibility exists, as suggested by recent neurology studies (28,29), that cellular Ca^{++} homeostasis may be affected by apoE4, which in an isoform-specific fashion could stimulate the influx of extracellular Ca^{++} into neuronal cells. Furthermore, it has been shown (30) that apoE is capable of initiating a calcium-dependent signaling response in immunocompetent cells, also in an isoform-specific manner. Accordingly it is conceivable that apoE could attenuate basal IL-10 production by immune cells (i.e., T lymphocytes, macrophages, and monocytes) through an increased influx of Ca^{++} and a consequent dysregulation of Ca^{++} homeostasis. However, the possibility that the effects of the e4 allele on serum IL-10 levels are mediated by other mechanisms cannot be excluded.

Interleukin-10 is capable of down-regulating numerous inflammatory pathways that play an important role in the progression and stability of atherosclerotic plaque, including inhibition of the pro-inflammatory transcription factor nuclear factor kappa B leading to suppressed cytokine production (31). Furthermore, IL-10 inhibits matrix metalloproteinase production (32), tissue factor (33), and adhesion molecule expression (34), as well as the release of potent chemoattractants (35).

Recent *in vitro* and animal studies have demonstrated that IL-10 expression in advanced human atherosclerotic plaques is associated with anti-atherogenic effects and atheromatous plaque stability (36). Clinical studies showed that elevated IL-10 serum levels are associated with a better

prognosis during a 6-month follow-up in patients with ACS, compared with patients with lower IL-10 levels (12). Of importance, Heeschen et al. (12) reported that the beneficial effects of elevated serum IL-10 were present also in patients with elevated serum CRP levels. Therefore, we speculate that in e4 carriers, the deleterious effects of low IL-10 levels may outweigh the protective effects of low CRP levels found in these patients, and that an imbalance between anti- and pro-inflammatory forces may be responsible for the increased CAD risk associated with the e4 apoE genotype. Theoretically, low IL-10 levels along with low CRP levels in e4 carriers could simply reflect the presence of a counter-regulatory response to inflammatory stimuli. However, this is unlikely to be the case in the present study, as no association was found between serum IL-10 levels and CRP levels either in the ACS or CSA patients, nor among the 3 different apoE genotypes.

Despite the novelty and importance of our findings, our study may have limitations. First, we have not provided data regarding clinical outcome in our patients, which would have further strengthened our hypothesis that low IL-10 concentrations could have accounted for the increased cardiovascular risk encountered in e4 carriers in the presence of low CRP levels. However, the design of the present study did not allow us to specifically investigate this issue. Larger, ad hoc prospective studies are required to establish the prognostic value of our findings.

Second, in agreement with previous studies, CSA patients in our study had higher IL-10 levels (21) and lower CRP levels (37), but we did not see differences in e4 allele frequency between CAD patients with clinical stable disease versus those with unstable disease. The lack of an association between differences in e4 frequency and IL-10 levels among CSA and ACS patients is likely to be due to the relatively small patient sample, as these associations largely depend on the prevalence of the e4 allele in the study population. However, the finding in the present study that the e4 allele is associated with a trend toward lower IL-10 levels in CSA patients, independent of the possible confounding effects of acute clinical events or the presence of myocardial necrosis, further strengthens the hypothesis of an association between apoE genetic polymorphism and circulating IL-10 levels. We may also have included CAD patients with a higher prevalence of cardiovascular risk factors. However, even if such a bias existed, it is unlikely that it would have substantially influenced our results, as we accounted for factors potentially affecting CRP and IL-10 concentrations using multivariable models. Moreover, apoE genotype frequencies found in our study are similar to those reported previously in other CAD series (14,15,23,25).

Another limitation of the study is that post-hoc analysis addressing the type I error risk associated with multiple a priori pairwise comparisons showed marginally significant differences in certain pairwise comparisons regarding IL-10 levels. The inability of the present study to show significant differences in all pairwise comparisons probably reflects the

small study sample size. However, even with this relatively small patient number, our study showed that apoE gene locus polymorphism significantly correlates with circulating IL-10 levels. Moreover, our study also showed a trend toward lower IL-10 levels among e4 carriers. Larger studies are needed to validate our preliminary results in geographically diverse study populations and among the whole spectrum of apoE gene polymorphisms. Finally, reporting results with more than 1 inflammatory marker could have provided more information regarding the immunomodulatory effects of apoE4. However, we assessed only CRP, as it is the only inflammatory marker that has been suggested to have a role for risk stratification in cardiovascular disease (38,39).

In conclusion, our study demonstrated that a common polymorphism of apoE is associated with lower circulating concentrations of IL-10 in ACS and CSA patients. The present study provides the first indication for the existence of such a relationship and may provide a possible explanation for the previously reported findings of an increased cardiovascular risk in e4 carriers despite low CRP levels. Our findings are also likely to stimulate further research on the impact of apoE polymorphism on the various inflammatory pathways related to CAD risk.

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Apolipoprotein E Genotype and Circulating Interleukin-10 Levels in Patients With Stable and Unstable Coronary Artery Disease

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