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## Genetic determinants of healthy longevity

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# Chapter 5

## APOE PLASMA LEVELS AND RISK OF CARDIOVASCULAR MORTALITY IN OLD AGE

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## Abstract

The  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$  alleles of the apolipoprotein E gene (*APOE*) encode three isoforms, apoE2, E3, and E4, respectively. The apoE isoforms circulate in different plasma concentrations, but plasma concentrations of the same isoform also differ between individuals. Whereas the isoforms have been associated with cardiovascular disease, the relation between plasma apoE levels and cardiovascular disease is unknown.

We assessed *APOE* genotypes, plasma levels of apoE, cardiovascular risk factors, and mortality in a population-based sample of 546 individuals aged 85 y who participated in the Leiden 85-plus Study and were prospectively followed for specific causes of death for 5 y. Participants in the highest tertile of apoE levels suffered a twofold-increased risk of cardiovascular mortality (hazard ratio compared to lowest tertile, 2.08; 95% confidence interval [CI], 1.30 to 3.33). Among the 324 participants with the  $\epsilon 3\epsilon 3$  genotype, the hazard from cardiovascular disease was threefold increased (highest versus lowest tertile 3.01; 95% CI 1.60 to 5.66), with similar estimates for men and women. Other causes of death were not increased significantly. Plasma levels of apoE in  $\epsilon 3\epsilon 3$  participants were positively correlated with total cholesterol ( $p < 0.001$ ), low-density lipoprotein cholesterol ( $p < 0.001$ ) and triglycerides ( $p < 0.001$ ) and negatively with high-density lipoprotein cholesterol levels ( $p = 0.010$ ). Adjustment for plasma lipids did not change the hazard ratios, whereas interaction was absent. The risk associated with high levels of apoE, however, was strongest in participants from the lowest tertile of C-reactive protein (CRP) levels and absent in those from the highest tertile ( $p_{\text{interaction}} < 0.001$ ). Among participants from the lowest tertile of CRP levels, those with a high apoE levels had a significantly steeper increase in CRP than those with low apoE levels ( $p = 0.020$ ). Similar cardiovascular mortality risks as in  $\epsilon 3\epsilon 3$  participants were found in  $\epsilon 2$  and  $\epsilon 4$  carriers.

In old age, high plasma apoE levels precede an increase of circulating CRP and strongly associates with cardiovascular mortality, independent of *APOE* genotype and plasma lipids.

## INTRODUCTION

The apolipoprotein E (*APOE*) gene influences lipid metabolism and disease risk. The encoded 299-residue plasma protein apoE is a surface component of primarily triglyceride-rich lipoproteins, such as very low-density lipoproteins (VLDLs), their remnants, chylomicron remnants, and high-density lipoproteins (HDLs). ApoE is the main ligand for clearance of VLDLs and chylomicron remnants, and as such affects circulating concentrations of lipoproteins and plasma levels of cholesterol and triglycerides (Mahley and Ji, 1999).

The biological activity of apoE can be influenced by modification of its structure and/or quantity. A structural alteration arises from the two common  $\epsilon 2/\epsilon 3/\epsilon 4$  polymorphisms (Weisgraber et al., 1981), encoding apoE2, apoE3, and apoE4, respectively. ApoE2 exhibits lower affinity for the LDL receptor, resulting in slower clearance of apoE and higher plasma apoE levels (Smit et al., 1988; Utermann et al., 1979b). In response, the liver up-regulates the LDL receptor, resulting in lower cholesterol levels. Conversely, apoE4 is cleared more efficiently, resulting in lower apoE levels and higher cholesterol levels (Smit et al., 1988; Utermann et al., 1979). The genetic variations thus affect lipid metabolism (Schaefer, 2002) and have been shown to alter risk of cardiovascular disease (Eichner et al., 2002) and dementia (Herz and Beffert, 2000). Plasma apoE levels are only partially explained by the  $\epsilon 2/\epsilon 3/\epsilon 4$  polymorphism (Neale et al., 2000), and plasma apoE levels vary between individuals with the same *APOE* genotype. Irrespective of *APOE* genotype, plasma apoE levels are also associated with cholesterol levels (Boerwinkle and Utermann, 1988; Haddy et al., 2002). Moreover, it was shown recently that apoE mediates the presentation of lipid antigens to the immune system and in this way influences the inflammatory process (van den Elzen et al., 2005). Both lipids and inflammation are involved in the pathogenesis of atherosclerosis, but the relation of plasma apoE levels and cardiovascular disease risk has not yet been reported.

Here, we studied the association of apoE plasma levels with cardiovascular mortality, independent of *APOE* genotype. To this end, within the Leiden 85-plus Study, a population-based prospective follow-up study, we determined the plasma levels of apoE, lipids, and C-reactive protein (CRP); *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotypes; and mortality from specific causes in old age.

## METHODS

### PARTICIPANTS

Between 1 September 1997 and 1 September 1 1999, a total of 705 inhabitants of the community of Leiden, the Netherlands, reached the age of 85 y. Among these 85-y-old persons, we initiated a follow-up study to investigate determinants of successful aging (Weverling-Rijnsburger et al., 2003). There were no selection criteria on health or demographic characteristics. Fourteen inhabitants died before they could be enrolled. The response rate was 87%; a total of 599 persons (397 women and 202 men) participated (Bootsma van der Wiel et al., 2002). There were no significant differences in various demographic characteristics between the 599 respondents and the source population. Of the 599 participants in the cohort, 38 refused to provide a blood sample, yielding a total number of 561 participants for the present study. The Medical Ethical Committee of the Leiden University Medical Center approved the study, and informed consent was obtained from all participants.

### CARDIOVASCULAR DISEASE HISTORY

History of myocardial infarction was obtained from the participants and from the treating physician. Information on use of medication was obtained from the participants' pharmacy records.

### APOE GENOTYPES

For genotyping, two TaqMan assays (Applied Biosystems, Foster City, California, United States) were used. For the single nucleotide polymorphism (SNP) in codon 112, primers were sense 5'-GCTGGGCGCGGACAT-3' and antisense 5'-CACCTCGCCGCGGTACT-3', and probes were 5'-CGGCCGCGCAGTCC-3' labeled with FAM and 5'-AGGCGGCCGACACGTC-3' labeled with VIC. For the SNP in codon 158, an Assay-On-Demand (Applied Biosystems) was used (assay ID C\_\_\_\_904973\_10). The assays were run on a 7900HT (Applied Biosystems) according to manufacturer's specifications, with the following modifications: a Eurogentec qPCR core kit (Eurogentec, Seraing, Belgium) was used according to standard specifications; half the concentrations of primers and probes were used; the number of PCR cycles was 50. Fluorescence intensities were measured after the PCR, and genotypes were indicated by the Sequence Detection Software version 2.0 (Applied Biosystems).

## PLASMA MEASUREMENTS

At baseline, participants were visited twice at their places of residence within 1 mo after their 85th birthday. All blood samples were collected early in the morning, although not fasting. Within the 5 y after inclusion, participants were visited each year within a month of their birthday. Blood samples were drawn each year, and CRP and lipid levels were measured directly in fresh samples as described. ApoE levels were determined in 2005 in one batch of plasma samples that were collected at age 85 y at baseline of the study and stored frozen. Plasma apoE levels were determined using a human apoE-specific sandwich ELISA essentially as described (van Vlijmen et al., 1994); an affinity-purified polyclonal sheep antihuman apoE antibody (Sh $\alpha$ E/E, obtained by genetic immunization of sheep followed by boosting of animals with human apoE) was coated overnight onto MaxiSorp immune plates (Nunc Intermed, Roskilde, Denmark) (dilution 1:10<sup>3</sup> in PBS [pH 7.4]; 100  $\mu$ l/well) at 4 °C. Plates were washed three times with PBS containing 0.05% Tween-20 (v/v) (PBS-T), and unspecific binding sites were blocked for 1 h at 37 °C with blocking buffer (PBS containing 0.1% casein). Plates were washed three times with PBS-T, and 100  $\mu$ L of the samples, reference sera (both dilution 1:8,000), and standards (plasma from C57Bl/6 mice spiked with 0–0.32  $\mu$ g/l of human apoE) were added (all diluted in blocking buffer). The plates were incubated overnight at 37 °C. Plates were washed five times with PBS-T to remove unbound and/or nonspecifically bound proteins, and captured antigen was detected by adding 100  $\mu$ L of horseradish peroxidase (HRP)-conjugated polyclonal sheep antihuman apoE antibody (dilution 1:10<sup>3</sup> in blocking-buffer containing 0.05% Tween-20). After a 2 h incubation at 37 °C, plates were washed five times with PBS-T and 100  $\mu$ l/well of freshly prepared tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) was added, and the plates were put in the dark. After 20 min at room temperature the product formation was ended by addition of 100  $\mu$ l/well of 2.5 M sulfuric acid. Following brief mixing, absorbance at 450 nm was measured. The inter-assay coefficient of variance was typically less than 10%, while the intra-assay coefficient of variance was typically less than 4%. Correlation coefficients of the calibration curves were typically better than 0.99. Reagent blanks had a typical absorbance of 0.06 (A450).

Plasma levels of total cholesterol, HDL cholesterol, triglycerides, and CRP were analyzed on fully automated computerized analyzers (Hitachi 747 and 911; Hitachi, Tokyo, Japan). The level of LDL cholesterol was estimated by the Friedewald equation (LDL cholesterol [mmol/l] = total cholesterol – HDL cholesterol – [triglycerides/2.2]), whereby participants with a triglyceride concentration higher than 443 mg/dl (5 mmol/l) were excluded ( $n = 5$ ).

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#### OTHER CARDIOVASCULAR RISK FACTORS

Participants were classified as having diabetes when they met at least one of the following criteria: (1) history of type 2 diabetes obtained from the general practitioner or treating physician; (2) use of sulfonylureas, biguanides, or insulin, based on information obtained from the participant's pharmacist; or (3) nonfasting glucose of 11.1 mmol/l or higher. Participants were classified as having hypertension when they met at least one of the following criteria: (1) history of hypertension obtained from the general practitioner or treating physician; or (2) mean systolic blood pressure of 165 mmHg or greater, or diastolic blood pressure of 95 mmHg or greater. Height and weight were measured at baseline, and body mass index (BMI) was calculated from these measurements. Self-reported history of smoking was categorized in three categories: never smoked, did smoke but not anymore, and current smokers.

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#### CAUSES OF DEATH

For the analyses presented in this research, all participants were followed for mortality until 1 April 2004. During this period, of a total of 546 participants, 274 (50%) died. The date of death was obtained from the civic registries. Shortly after the civic registries reported the death of a participant, the general practitioner or nursing home physician was interviewed to determine the cause of death by means of a standardized questionnaire. Two senior specialists of internal medicine, unaware of the outcomes of the analyses, reviewed the causes of death and classified each death into primary causes of death according to the International Classification of Diseases and Related Health Problems, 10th Revision, as cardiovascular mortality (ICD codes I00–I99), infectious disease (ICD codes A00–B99 or J11–J18), cancer (ICD codes C00–D48), or other causes (all other ICD codes). Specific causes of death were not available for six participants.

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#### STATISTICAL ANALYSES

Levels of total, LDL, and HDL cholesterol were normally distributed. Levels of plasma apoE, triglycerides, and CRP were not normally distributed, and are reported as geometric means. To allow transformation, undetectable levels of CRP were assigned the mean value 0.25. Differences in variables between groups were compared with a sex-adjusted linear regression. Sex-adjusted (geometrical) means and 95% confidence intervals (95% CIs), and differences between groups were calculated using a linear mixed model. Mortality risks were estimated using Cox proportional hazards model, and all were adjusted for gender. Prospective analysis of CRP levels was performed using a linear mixed model, adjusting for

gender. All calculations were performed using SPSS 12.0.1 (SPSS, Chicago, Illinois, United States), and Kaplan-Meier curves were generated using STATA 8 SE (Stata, College Station, Texas, United States) and replicated by two researchers independently (SPM, DvH).

## RESULTS

### BASELINE STUDY POPULATION CHARACTERISTICS

From the 561 eligible participants, measurement of plasma apoE failed in two and *APOE* genotyping failed in 13. The remaining 546 participants were included in the present analysis, of which the baseline characteristics are listed in table 5.1. The *APOE* allele frequencies in our population were:  $\epsilon 2$ , 0.10;  $\epsilon 3$ , 0.77; and  $\epsilon 4$ , 0.13. The genotypes were in Hardy-Weinberg equilibrium and the allele frequencies were in line with comparable populations (Haddy et al., 2002).

**Table 5.1.** Baseline Characteristics of Study Participants

Characteristic	Category	Value
Total number		546
Sex	Female, number (%)	365 (67)
Living status	Independent, number (%)	449 (82)
Cardiovascular disease	History of myocardial infarction, number (%)	56 (10)
	Use of lipid-lowering medication, number (%)	6 (1)
Cardiovascular risk factors	Diabetes mellitus, number (%)	89 (16)
	Hypertension, number (%)	255 (53)
	History of smoking, number (%)	204 (37)
	BMI, mean (SD)	27.2 (4.9)
<i>APOE</i> genotype, number (%)	$\epsilon 2\epsilon 2$ , number (%)	4 (1)
	$\epsilon 2\epsilon 3$ , number (%)	90 (17)
	$\epsilon 2\epsilon 4$ , number (%)	13 (2)
	$\epsilon 3\epsilon 3$ , number (%)	324 (59)
	$\epsilon 3\epsilon 4$ , number (%)	100 (18)
	$\epsilon 4\epsilon 4$ , number (%)	15 (3)
Plasma apolipoprotein levels	ApoE levels, median (IQR), mg/l	0.50 (0.35–0.72)
CRP level	Plasma CRP level, median (IQR), mg/l	4.0 (1.0–8.0)
Plasma lipid levels	Total cholesterol, mean (SD), mmol/l	5.71 (1.13)
	LDL cholesterol, mean (SD), mmol/l	3.68 (0.98)
	HDL cholesterol, mean (SD), mmol/l	1.32 (0.40)
	Triglycerides, median (IQR), mmol/l	1.33 (1.00–1.95)

IQR, interquartile range; SD, standard deviation



### ASSOCIATION OF APOE GENOTYPE WITH PLASMA APOE, CRP, AND LIPID LEVELS AT BASELINE AND CARDIOVASCULAR MORTALITY DURING FOLLOW-UP

When assessing the relation of *APOE* genotype with plasma apoE levels, lipids, CRP levels, and cardiovascular mortality we found associations similar to those already described (Boerwinkle and Utermann, 1988; Haddy et al., 2002; Smit et al., 1988; Utermann et al., 1979). In brief, participants who were carriers of the  $\epsilon 2$  allele had significantly higher plasma levels of apoE and lower levels of LDL cholesterol and total cholesterol, whereas carriers of the  $\epsilon 4$  allele had lower levels of plasma apoE and higher levels of LDL and total cholesterol (table 5.2). Levels of HDL cholesterol, triglycerides, and circulating CRP did not differ between the various genotypes. Compared to  $\epsilon 3\epsilon 3$  participants, cardiovascular mortality was lower in  $\epsilon 2\epsilon 3$  and  $\epsilon 2\epsilon 4$  carriers, and higher in  $\epsilon 2\epsilon 2$ ,  $\epsilon 3\epsilon 4$ , and  $\epsilon 4\epsilon 4$  carriers, although none of the differences are statistically significant, possibly due to small numbers.

**Table 5.2.** Plasma Levels of ApoE, Lipids, CRP, and Cardiovascular Mortality Risk Dependent on *APOE* Genotype

Category	<i>APOE</i> Genotype					
	$\epsilon 2\epsilon 2$	$\epsilon 2\epsilon 3$	$\epsilon 2\epsilon 4$	$\epsilon 3\epsilon 3$	$\epsilon 3\epsilon 4$	$\epsilon 4\epsilon 4$
Number	4	90	13	324	100	15
ApoE <sup>a</sup> , mean (95% CI), mg/l	80.6 (49.0–133)	67.5 *** (61.1–75.2)	64.9 (49.3–85.5)	50.0 (47.2–52.7)	44.1 ** (39.9–48.7)	31.4 *** (24.3–40.6)
Total cholesterol, mean (95% CI), mmol/l	3.91 *** (2.88–4.95)	5.36 *** (5.14–5.58)	5.50 (4.93–6.08)	5.70 (5.59–5.82)	6.15 *** (5.94–6.36)	5.84 (5.31–6.38)
LDL cholesterol, mean (95% CI), mmol/l	2.07 *** (1.17–2.97)	3.23 *** (3.04–3.42)	3.20 (2.68–3.72)	3.70 (3.60–3.80)	4.14 *** (3.96–4.32)	3.77 (3.31–4.24)
HDL cholesterol, mean (95% CI), mmol/l	1.31 (0.92–1.70)	1.36 (1.28–1.44)	1.45 (1.23–1.66)	1.31 (1.27–1.36)	1.27 (1.19–1.35)	1.41 (1.20–1.61)
Triglycerides <sup>a</sup> , mean (95% CI), mmol/l	1.16 (0.74–1.82)	1.44 (1.31–1.59)	1.33 (1.04–1.71)	1.38 (1.32–1.45)	1.46 (1.33–1.59)	1.36 (1.08–1.71)
CRP <sup>a</sup> , mean (95% CI), mg/l	4.06 (0.97–17.0)	3.46 (2.55–4.67)	2.54 (1.15–5.62)	2.69 (2.30–3.16)	2.73 (2.05–3.64)	2.12 (1.01–4.45)
Cardiovascular mortality, HR (95%CI) <sup>b</sup>	2.93 (0.72–12.0)	0.63 (0.35–1.14)	0.77 (0.19–3.15)	1 (ref)	1.36 (0.86–2.13)	2.07 (0.75–5.96)

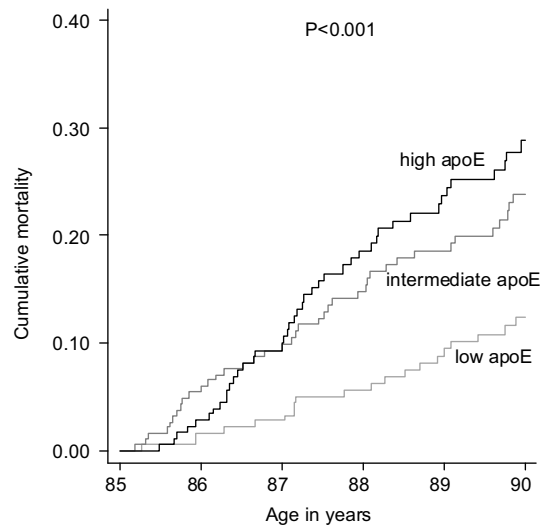
Mean levels and 95% CIs were sex adjusted and all participants were aged 85 y. Statistical significance: \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; for each comparison, the  $\epsilon 3\epsilon 3$  genotype served as the reference group.

Geometrical mean. Cardiovascular mortality risk from Cox proportional hazard model adjusted for sex.

### ASSOCIATION OF PLASMA APOE LEVELS AND MORTALITY DURING FOLLOW-UP

Mean follow-up time was 4.2 y. During follow-up, of a total of 546 participants, 274 (50%) died, of which 115 (42%) died of cardiovascular causes, 47 (17%) of infectious causes, 44 (16%) of cancer, and 62 (23%) of other causes. In the total study population, high apoE levels were associated with a 2.08-fold (95% CI, 1.30 to 3.33) increased risk, and intermediate levels with a 1.74-fold (95% CI, 1.09 to 2.79) increased risk, of cardiovascular mortality compared to those with low apoE levels (figure 5.1). This observation seems to

contradict the associations described above, where the  $\epsilon 2\epsilon 3$  genotype was associated with higher levels of apoE, lower LDL cholesterol, and a lower mortality risk, and  $\epsilon 3\epsilon 4$  and  $\epsilon 4\epsilon 4$  genotypes had lower levels of apoE, higher LDL cholesterol, and a higher mortality risk, albeit nonsignificantly.



**Figure 5.1.** Kaplan-Meier Mortality Curve for Tertiles of ApoE Levels  
Plasma apoE is indicated at high (black), intermediate (dark grey) and low (light grey) levels, showing that high plasma apoE levels associate with increased cardiovascular mortality. The  $p$ -value indicates the statistical significance of the association of plasma apoE level with cardiovascular mortality in a sex-adjusted Cox proportional hazards model.

To further investigate the effect of plasma apoE levels only and to eliminate the potentially distorting effect of structural changes in the apoE proteins, the relations between plasma levels of apoE and mortality were studied in  $\epsilon 3\epsilon 3$  participants only. First, we assessed whether the effect of levels of apoE was specific for cardiovascular mortality as compared with other causes of death. Estimates of mortality risk dependent on plasma levels of apoE in  $\epsilon 3\epsilon 3$  participants are listed in table 5.3.

**Table 5.3.** Mortality Risk According to Tertiles of Plasma Levels of ApoE in Participants with the  $\epsilon 3\epsilon 3$  Genotype

Cause of Death	ApoE Plasma Level in $\epsilon 3\epsilon 3$ Participants			<i>p</i> -Value for Trend
	Low ( <i>n</i> = 108)	Intermediate ( <i>n</i> = 108)	High ( <i>n</i> = 108)	
All causes ( <i>n</i> = 159)	1 (ref)	1.47 (0.98–2.19)	2.05 (1.39–3.02)	<0.001
Cardiovascular disease ( <i>n</i> = 68)	1 (ref)	2.12 (1.11–4.06)	3.01 (1.60–5.66)	0.001
Infectious disease ( <i>n</i> = 27)	1 (ref)	1.36 (0.52–3.55)	1.80 (0.70–4.61)	0.218
Cancer ( <i>n</i> = 31)	1 (ref)	2.26 (0.90–5.71)	2.26 (0.87–5.90)	0.095
Other causes ( <i>n</i> = 31)	1 (ref)	0.54 (0.21–1.42)	1.21 (0.54–2.70)	0.740

Participants were categorized in tertiles of low, intermediate or high plasma apoE level. Hazard ratios were calculated using a sex-adjusted Cox proportional hazards model for high levels of apoE compared with low levels of apoE as reference (ref) category. *p*-Value for trend was calculated with the tertiles as continuous variables into the model. Specific causes of death were not available for two individuals.

Participants with high levels of plasma apoE had a twofold increased all-cause mortality risk (hazard ratio, 2.05; 95% CI, 1.39 to 3.02) as compared to those with low levels. This was predominantly due to a threefold increase in cardiovascular mortality risk (hazard ratio, 3.01; 95% CI, 1.60 to 5.66), whereas other causes of death were nonsignificantly increased. A similar increase in cardiovascular mortality was found in men (hazard ratio, 2.89; 95% CI 1.13 to 7.36) and women (hazard ratio, 2.96; 95% CI 1.27 to 6.90). Other causes of death were not increased significantly. When combined, mortality from all causes except cardiovascular disease was not significantly increased for participants with intermediate apoE levels (hazard ratio, 1.19; 95% CI 0.71 to 2.01) and high apoE levels (hazard ratio, 1.63; 95% CI 0.98 to 2.71), compared to participants with low levels. Second, we assessed whether the relation between levels of apoE and cardiovascular mortality risk was linear. When analyzing cardiovascular mortality risk over ten strata of plasma apoE level, we observed increasing cardiovascular mortality associated with increasing plasma apoE level in a gradual fashion (unpublished data).

#### ASSOCIATION OF PLASMA APOE LEVELS WITH LIPID LEVELS AT BASELINE AND RELATIONSHIP OF CARDIOVASCULAR MORTALITY RISK ASSOCIATED WITH PLASMA APOE AND LIPID LEVELS

As expected, plasma levels of apoE strongly associated with levels of LDL and HDL cholesterol and triglycerides (table 5.4).

**Table 5.4.** Plasma Levels of Total, LDL, and HDL Cholesterol and Triglycerides According to Tertiles Plasma Levels of ApoE in Participants with the  $\epsilon 3\epsilon 3$  Genotype

Plasma Component	ApoE Plasma Levels in $\epsilon 3\epsilon 3$ Participants			<i>p</i> -Value for Trend <sup>a</sup>
	Low ( <i>n</i> = 108)	Intermediate ( <i>n</i> = 108)	High ( <i>n</i> = 108)	
ApoE, mean (95% CI), mg/l <sup>b</sup>	29.1 (27.9–30.3)	48.5 (46.5–50.5)	88.1 (84.4–91.9)	ND
Total cholesterol, mean (95% CI), mmol/l	5.47 (5.28–5.66)	5.59 (5.41–5.78)	6.05 (5.86–6.24)	<0.001
LDL cholesterol, mean (95% CI), mmol/l	3.51 (3.34–3.68)	3.66 (3.49–3.82)	3.93 (3.76–4.10)	0.001
HDL cholesterol, mean (95% CI), mmol/l	1.39 (1.32–1.46)	1.29 (1.22–1.36)	1.26 (1.19–1.33)	0.010
Triglycerides, mean (95% CI), mmol/l <sup>b</sup>	1.16 (1.08–1.25)	1.32 (1.23–1.43)	1.72 (1.60–1.86)	<0.001

Mean levels and 95% CIs of the mean were sex-adjusted. All subjects were aged 85 y.<sup>a</sup>*p*-Value for trend was calculated with the tertiles as continuous variable into the model.<sup>b</sup>Geometrical means. ND, not determined.

Among  $\epsilon 3\epsilon 3$  participants, those with high levels of apoE had significantly higher levels of total cholesterol ( $p < 0.001$ ), LDL cholesterol ( $p < 0.001$ ), and triglycerides ( $p < 0.001$ ) and lower HDL cholesterol ( $p = 0.010$ ). High levels of HDL cholesterol associated with decreased cardiovascular mortality compared to low levels (hazard ratio, 0.58; 95% CI, 0.36 to 0.92). No association was found with cardiovascular mortality for high levels of LDL cholesterol (hazard ratio, 0.89; 95% CI, 0.58 to 1.39) or triglycerides (hazard ratio, 1.00; 95% CI 0.65–1.56). To investigate whether the association of apoE levels with cardiovascular mortality was explained by an adverse lipid profile, we first analyzed the association of apoE with cardiovascular mortality in strata of low, middle, and high LDL and HDL cholesterol and triglycerides. We found that the risk associated with high level of apoE was similar in all tertiles of LDL and HDL cholesterol and triglycerides. Formal testing for interactions showed that there was no significant interaction between the risk of cardiovascular mortality associated with apoE and these lipids (all  $p_{\text{interaction}} > 0.6$ ). We then adjusted the mortality analysis for levels of LDL and HDL cholesterol and triglycerides. Participants with high levels of apoE had mortality risks similar to those in the crude model (hazard ratio, 3.09; 95% CI, 1.55 to 6.15). Further adjustment for the cardiovascular risk factors hypertension, diabetes, BMI, and smoking did not change the estimate (hazard ratio, 3.20; 95% CI, 1.56 to 6.59). Finally, we repeated the cardiovascular mortality analysis within the  $\epsilon 3\epsilon 3$  group, excluding individuals with a positive history of myocardial infarction ( $n = 28$ ) and those on lipid-lowering medication ( $n = 4$ ). In the fully adjusted model the risk associated with high levels of apoE was 4.14-fold higher than that of participants with low levels (95% CI, 1.75 to 9.84).

### ASSOCIATION OF PLASMA APOE LEVEL AND INCREASE IN CRP LEVEL DURING FOLLOW-UP

We also studied the risk of apoE in strata of CRP, another cardiovascular risk factor. In the total population, high levels of CRP are associated with increased cardiovascular mortality compared to low levels (hazard ratio, 2.42; 95% CI, 1.58 to 3.70). At baseline,  $\epsilon 3\epsilon 3$  participants with high levels of apoE had significantly higher levels of CRP than those with intermediate and low levels ( $p_{\text{trend}} < 0.001$ ), and adjustment for sex and lipids did not alter the association ( $p_{\text{trend}} < 0.001$ ). The cardiovascular mortality risk associated with high levels of apoE was dependent on level of CRP (table 5.5), as it was highest in participants with low levels of CRP and absent in those with high levels of CRP ( $p_{\text{interaction}} < 0.001$ ). Adjustment for lipids ( $p_{\text{interaction}} < 0.001$ ) and other cardiovascular risk factors ( $p_{\text{interaction}} < 0.001$ ) did not alter the interaction. When participants with extreme levels of CRP ( $> 10$  mg/l) were excluded from the analysis, the results remained similar. One possible interpretation of this interaction is that high levels of apoE precede an increase of CRP and in this way contribute to the pathogenesis of atherosclerosis. To test this hypothesis we investigated the association of baseline levels of apoE with levels of CRP during follow-up. We performed this analysis in the 91 participants in the lowest CRP stratum, because their risk of cardiovascular mortality associated with apoE was highest (table 5.5).

**Table 5.5.** Cardiovascular Mortality Risk Associated with High Levels of ApoE in Strata of CRP in  $\epsilon 3\epsilon 3$  Carriers

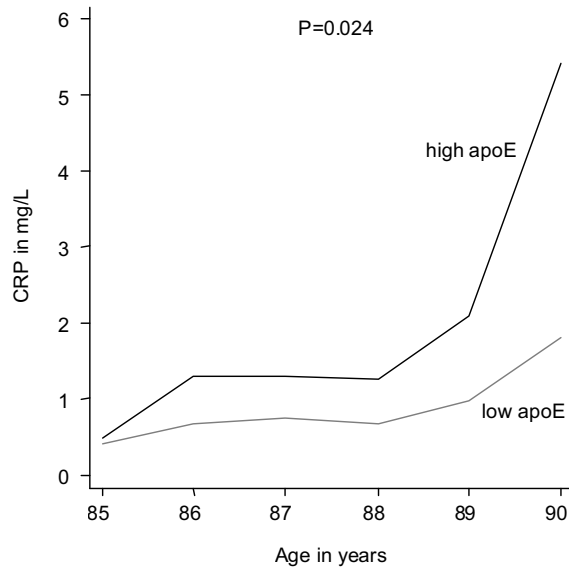
ApoE Level	Stratum of CRP Level			$p_{\text{interaction}}$ -Value
	Low (0–1 mg/l) ( <i>n</i> = 91)	Intermediate (2–5 mg/l) ( <i>n</i> = 131)	High (>5 mg/l) ( <i>n</i> = 102)	
Low	1 (ref)	1 (ref)	1 (ref)	–
High, crude	3.99 (0.96–16.6)	2.38 (1.02–5.54)	1.16 (0.58–2.30)	<0.001
High, adjusted <sup>a</sup>	5.76 (1.20–26.7)	2.10 (0.81–5.45)	1.07 (0.49–2.31)	<0.001
High, adjusted <sup>b</sup>	6.08 (0.99–37.3)	2.19 (0.75–6.39)	1.18 (0.50–2.76)	<0.001

Levels of apoE were dichotomized on the median in each stratum of CRP. Number of deaths from cardiovascular cause was ten in low CRP, 24 in intermediate CRP, and 34 in high CRP. Hazard ratios were calculated using a sex-adjusted Cox proportional hazards model for high levels of apoE compared with low levels of apoE as the reference (ref) category. Interaction was calculated with apoE and CRP and the interaction term as continuous variables in a Cox proportional hazards model.

<sup>a</sup>Adjusted for levels of lipids.

<sup>b</sup>Adjusted for levels of lipids, BMI, history of diabetes, hypertension, and smoking.

Figure 5.2 shows the association of baseline levels of apoE with increases in CRP during follow-up. We found that high levels of apoE at baseline correlate with higher increases in levels of CRP during follow-up ( $p = 0.024$ ). Adjusting for classical cardiovascular risk factors did not change the observed association ( $p = 0.020$ ). A similar association was observed in  $\epsilon 4$  carriers ( $p = 0.041$ ), but was absent in  $\epsilon 2$  carriers ( $p = 0.76$ ).



**Figure 5.2.** High Versus Low Levels of Plasma ApoE

High (red) and low (green) levels of the protein are depicted. Plasma apoE level was dichotomized on the median, showing that participants with high plasma apoE develop higher CRP levels during follow-up. Estimates of the geometric mean of circulating CRP levels for all years were calculated using a sex-adjusted linear mixed model. The  $p$ -value indicates the statistical significance of additional annual increases of CRP between high and low levels of apoE.

#### ASSOCIATION OF PLASMA APOE LEVELS AND CARDIOVASCULAR MORTALITY IN DIFFERENT APOE GENOTYPES

To investigate whether the association of plasma apoE with cardiovascular mortality was also present in carriers of other genotypes, we repeated the mortality analysis in  $\epsilon 2$  and  $\epsilon 4$  carriers separately (table 5.6). We found a similar increase in cardiovascular mortality for participants in the highest apoE tertile as compared to the lowest tertile in  $\epsilon 2$  carriers (hazard ratio, 2.43; 95% CI, 0.44 to 13.5) and  $\epsilon 4$  carriers (hazard ratio, 4.74; 95% CI, 1.54 to 41.6). When the entire cohort was analyzed with *APOE* genotype accounted for, participants with high levels of apoE had a 3.45-fold increased risk of cardiovascular mortality (95% CI, 1.98 to 6.02).

**Table 5.6.** Cardiovascular Mortality Risk Dependent on Plasma ApoE Level in Subjects with Different *APOE* Genotypes

Genotype	Plasma ApoE Level			p-Value
	Low	Intermediate	High	
ε2 carriers (n = 94)	1 (ref)	0.82 (0.15–4.38)	2.35 (0.44–12.6)	0.314
ε3ε3 (n = 324)	1 (ref)	1.94 (0.96–3.93)	3.20 (1.56–6.59)	0.001
ε4 carriers (n = 115)	1 (ref)	2.81 (1.02–7.74)	4.25 (1.38–13.0)	0.009
All (n = 546) <sup>a</sup>	1 (ref)	2.10 (1.25–3.53)	3.34 (1.92–5.81)	<0.001

Within each genotype group, subjects were categorized in tertiles of low, intermediate, or high plasma apoE level. For ε2 carriers, low is 51 mg/l or lower, and high is 83 mg/l or higher; for ε3ε3 carriers, low is 39 mg/l or lower, and high is 61 mg/l or higher; for ε4 carriers, low is 33 mg/l or lower, and high is 57 mg/l or higher; for the total cohort, low is 40 mg/l or lower, and high is 63 mg/l or higher. Number of deaths from cardiovascular causes was 15 in ε2 carriers, 68 in ε3ε3 carriers, 30 in ε4 carriers, and 115 in the total population. To allow for comparison between the effect of the ε2 and ε4 alleles, ε2ε4 genotypes were not included in this table. Hazard ratios were calculated with a Cox proportional model for intermediate and high levels of apoE compared with low levels of apoE as reference (ref) category, adjusted for sex, lipids, BMI, and a history of smoking, diabetes, and hypertension.

<sup>a</sup>Additionally adjusted for *APOE* genotype.

## DISCUSSION

The main finding of our study is that in old age, individuals with high plasma levels of apoE are at an increased risk of cardiovascular mortality, independent of their *APOE* genotype, lipid levels, and other cardiovascular risk factors. In the prospective analysis, we also found that high plasma apoE levels precede a rise in CRP levels. Similar mortality risks were observed among carriers of the ε2 or ε4 allele.

### PLASMA APOE LEVELS MARK PROATHEROGENESIS

ApoE is often referred to as an antiatherosclerotic protein (reviewed in (Larkin et al., 2000)). This notion has its basis in observations in both mice and humans. Mice normally do not develop high LDL cholesterol levels or atherosclerosis, presumably because of higher VLDL turnover and the lack of cholesteryl ester transfer protein. Mice with genetically induced or diet-induced hyperlipidemia develop accelerated atherosclerosis that closely resembles all stages of human atherosclerosis, up to the stage of plaque formation. Although in mice plaque rupture does not occur and therefore no cardiovascular events occur, mouse models have proven valuable to study the biological mechanisms underlying the development of atherosclerosis in humans. In mice, complete knockout of the *apoe* gene causes hypercholesterolemia and early atherosclerosis (Plump et al., 1992). Stem cell transplantation that enables these mice to produce small amounts of murine (Linton et al., 1995) or human (Bellosta et al., 1995) apoE in macrophages rescues them from atherosclerosis, whereas mice overexpressing rat apoE have a reduction in plasma lipoproteins and are resistant to diet-induced hypercholesterolemia

(Shimano et al., 1992). In humans, a rare heritable deficiency of apoE also leads to the development of hyperlipoproteinemia and cardiovascular disease (Schaefer et al., 1986). Within atherosclerotic lesions macrophages accumulate cholesterol and become foam cells, which play an important role in the initiation and progression of atherosclerosis (Larkin et al., 2000). ApoE is expressed by macrophages in atherosclerotic lesions in vessel walls (O'Brien et al., 1994) and up-regulates cholesterol efflux from macrophages (Zhang et al., 1996). In this way apoE is involved in decreasing the atherogenic effect of cholesterol. However, the main portion of the apoE plasma pool is produced by the liver and only a small fraction by macrophages (Newman et al., 1985), and plasma apoE levels are under tight genetic control (Beekman et al., 2002; Siest et al., 1995; Smit et al., 1988). This contrasts with apoE expression by macrophages, which is dependent on local factors, such as cholesterol loading and cytokines (Larkin et al., 2000). These findings suggest that plasma apoE levels may not correlate with macrophage apoE expression levels. Moreover, the antiatherosclerotic effect of apoE appears to be specific for macrophages, as transplantation of apoE-deficient macrophages into wild-type mice increases development of atherosclerosis, without affecting plasma levels of lipids and apoE (Fazio et al., 1997). Furthermore, we cannot exclude the possibility that expression of apoE in other tissues also leads to a reduction in atherosclerosis (Thorngate et al., 2000; Tsukamoto et al., 2000). These observations suggest that apoE plasma levels may have little relation to the antiatherosclerotic effect of apoE in macrophages.

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**PROPOSED MECHANISMS: PLASMA APOE LEVELS ASSOCIATE WITH A DETRIMENTAL LIPOPROTEIN PROFILE AND/OR WITH A PROINFLAMMATION RESPONSE**

Here we show that high plasma apoE levels in fact do associate with an increased cardiovascular mortality risk. High plasma apoE levels may contribute to cardiovascular disease via one or both of two hypothesized mechanisms. First, high levels of apoE may reflect a detrimental lipoprotein profile. We speculate that high apoE levels reflect the presence of higher levels of lipoprotein classes such as small, dense LDL, or VLDL and chylomicron remnants, which are proatherogenic (Carmena et al., 2004; Lada and Rudel, 2004). However, because we found in our analysis that the effect of apoE on cardiovascular mortality is independent of lipid levels, the effects of high apoE may not be mediated by LDL or HDL cholesterol or triglycerides per se. Levels of triglycerides are closely correlated with remnant concentrations (Twickler et al., 2004), but did not associate with cardiovascular disease in our study (Weverling-Rijnsburger et al., 1997; Weverling-Rijnsburger et al., 2003). Furthermore, triglyceride levels do not contribute to



the association of levels of apoE with cardiovascular disease mortality, as stratification and correction for triglyceride levels did not alter the effect of apoE levels on cardiovascular disease mortality. Changes in lipoprotein particle sizes have also been associated with longevity (Barzilai et al., 2003), suggesting that the association of apoE levels with cardiovascular mortality might be explained by changes in particle sizes. The standard fractionation method, which was used in the present study, is unable to distinguish between these subclasses. Alternative measurements of specific subclasses may help to elucidate the underlying detrimental lipid profile. Furthermore, measuring apoE concentrations in these subclasses could yield insight into which lipoprotein fraction contributes to the detrimental effect of high plasma apoE levels, or whether the detrimental effect of apoE is, for instance, specific for HDL-apoE or non-HDL apoE. However, since storage of plasma samples results in a shift of apoE between the different lipoproteins (Cohn et al., 2004), and because a large portion of our study population has died, we are unable to perform such analysis on this cohort. We suggest that a separate study is initiated, including fractionation of fresh plasma, and measuring a series of apolipoproteins, including at least apoAI, apoB, and apoE within in these subclasses.

The second possible mechanism by which apoE contributes to cardiovascular disease may be related to the finding that apoE has proinflammatory properties. Plasma apoE avidly binds lipid antigens and appears to be critical for the presentation of lipid antigens by binding to antigen-presenting cells through the LDL receptor, which is followed by endocytosis (van den Elzen et al., 2005). The concomitant inflammatory response adequately eliminates the lipid antigen from the circulation. In this model, high plasma levels of apoE in combination with increased lipid-antigen presentation lead to chronic inflammation and thus may contribute to atherosclerosis. In line with these experimental data, we now show that high plasma apoE levels precede chronic inflammation (of which circulating levels of CRP are a marker (Ridker et al., 2005)) and cardiovascular mortality risk. Taken together, we interpret these findings to suggest that, in old age, high plasma apoE levels associate better with a proinflammatory response and/or detrimental lipoprotein profile than with the antiatherosclerotic effect of apoE expression in macrophages.

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#### PLASMA APOE LEVELS ARE A CAUSE, NOT A CONSEQUENCE, OF ATHEROSCLEROSIS

The association of apoE level and cardiovascular mortality provides new insight into mechanisms contributing to atherosclerosis. As the cardiovascular mortality risk associated with high levels of apoE was even higher in participants who did not have a

history of myocardial infarction compared to those who did, high levels of apoE are unlikely to be the consequence of cardiovascular disease. In our study, the association of apoE levels with cardiovascular disease was strongest in participants with low levels of CRP, and in these persons high levels of apoE preceded an increase in CRP. However, in participants with high levels of CRP these associations were not observed. We interpret these results to indicate that high plasma apoE levels are an early and specific indicator of cardiovascular disease risk and drive a proinflammatory response. However, once there is a proinflammatory state—through whichever mechanism—high plasma apoE levels do not add to an increased risk. Taken together, our data may suggest that plasma apoE levels are a causal factor in cardiovascular mortality risk, and that at least part of the mechanism involves a proinflammatory response.

The observed association of high plasma apoE levels with increased inflammation seems to contradict earlier reports on specific anti-inflammatory properties of apoE. For instance, apoE was involved in the direct binding of lipopolysaccharide, the main toxic component of gram-negative bacteria (Rensen et al., 1997), thereby decreasing the inflammatory response to lipopolysaccharide (Van Oosten et al., 2001). However, the pathways that neutralize endogenous and exogenous stimuli may be different. It is logical to assume such a pleiotropic effect, since genes regulating the inflammatory host response are under balanced evolutionary selection pressure.

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#### LITERATURE CONTEXT

In our study population, as in other (younger) populations, apoE level associates with levels of total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides. In youth and middle age, high LDL cholesterol and low HDL cholesterol are strong risk factors of cardiovascular disease. In contrast, in old age, only levels of HDL cholesterol, but not of total cholesterol, LDL cholesterol, and triglycerides, affect cardiovascular disease risk (Packard et al., 2005; Shepherd et al., 2002; Weverling-Rijnsburger et al., 1997; Weverling-Rijnsburger et al., 2003). It is tempting to speculate that a high apoE plasma level is an independent risk factor of cardiovascular disease in middle age as it is in old age.

We found, as in other (younger) study populations, that *APOE* genotypes associate with plasma levels of apoE. Plasma apoE levels are highly dependent on heritable factors (Beekman et al., 2002; Siest et al., 1995; Smit et al., 1988), but the *APOE*  $\epsilon 2/\epsilon 3/\epsilon 4$  genotype is only a minor determinant of these levels (Beekman et al., 2004; Neale et al., 2000), and accordingly the plasma levels of apoE varied markedly in persons with  $\epsilon 3\epsilon 3$  genotypes. ApoE2 has lower receptor binding affinity than apoE3 and apoE4 (Lund-Katz et

al., 2001). ApoE4 binds to lipid particles with higher affinity than does apoE3 and shows preference for VLDL, whereas apoE2 and apoE3 mainly bind to HDL (Saito et al., 2003). The results of individual studies on the association of the  $\epsilon$ 2 allele and cardiovascular disease have been conflicting. Some studies report significant, both harmful and protective associations, whereas others have reported no associations (Wilson et al., 1996). On the other hand, the  $\epsilon$ 4 allele has been associated with an increased risk of cardiovascular disease in a meta-analysis of studies up to 1996 (Wilson et al., 1996). Here we show that the effect of the plasma levels of apoE masks the effect of the common *APOE*  $\epsilon$ 2/ $\epsilon$ 3/ $\epsilon$ 4 genotypes and may explain why the reported associations of the *APOE* genotype have been contradictory; the effect of genotype should be adjusted for plasma apoE levels.

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#### STRENGTHS AND WEAKNESSES OF THE PRESENT STUDY

Blood samples were not drawn fasting, which could add to the random error of the measurement of triglycerides, apoE, and CRP. However, all samples were drawn in the early morning, and the observed associations of *APOE* genotype with plasma apoE level, and of apoE plasma level with triglyceride level, were similar to those found in other studies using fasting samples, which suggests that the magnitude of random error is relatively small. The low number of participants on lipid-lowering medication may result from the restrictive medication policy by Dutch general practitioners at the time of the enrollment of the Leiden 85-plus Study (1997–1999). This restrictive policy has been one of the motivations to initiate a clinical trial. The PROSPER study, in part carried out in the same region as the current study, showed that lipid-lowering medication (in this case pravastatin) protects individuals from coronary artery disease at least up to the age of 82 (Shepherd et al., 2002). However, the results of this study were published several years after the start of the Leiden 85-plus Study (1997). CRP levels were not measured with high-sensitivity methods. The use of high-sensitivity CRP measurement is especially informative in discriminating between samples with none or very little CRP. However, in this age group the number of participants with levels of CRP below the detection limit of the assay was relatively small (102 out of 563 participants), and the variation in CRP levels was considerable. Furthermore, most of the analyses performed with these CRP levels involved stratification for CRP, and it is unlikely that high-sensitivity measurements would have systematically changed the attrition of participants to these strata. Therefore we believe that the measurement that we used resulted in adequate discriminative power.

A limitation of our study is that this finding cannot directly be extrapolated to the incidence of nonfatal cardiovascular disease, and not beyond this age group. The current

study was performed in a population-based sample of 85-y-olds. The participants of this study were the survivors of their birth cohort, and earlier studies in people of over 70 y of age have shown that biology in old age may be different from that in other age groups, which may prevent extrapolation of the results to the population at large. For instance, LDL cholesterol is no longer a cardiovascular risk factor (Shepherd et al., 2002). Reaching the age of 85 y is becoming less unusual than is generally expected. The proportion of the original birth cohort that has reached this age is 36% for women and 15% for men, and this proportion will further increase in the future. Furthermore, to our knowledge we are the first to report an association of plasma apoE levels with cardiovascular mortality in any age group, and it is tempting to speculate that this finding holds in other age groups. A strength of our study is the specificity and sensitivity of the analyses due to the high incidence of fatal events during follow-up. Moreover, the data come from a population-based study without inclusion criteria based on health and demographic characteristics. Another strong point is that we could restrict our analyses to *APOE* ε3ε3 carriers, thereby eliminating the potentially distorting effect of structural changes in the apoE protein.

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#### GENERAL CONCLUSION

The present data show that a high apoE plasma level is a new predictor of cardiovascular mortality in elderly people. We conclude that levels of apoE may indicate a detrimental lipoprotein profile and/or a proinflammatory response, which both contribute to cardiovascular mortality.

