# Genetic determinants of healthy longevity

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CHAPTER 8

# PLASMA APOLIPOPROTEIN CI PROTECTS AGAINST MORTALITY FROM INFECTION IN OLD AGE

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## ABSTRACT

The high-density lipoprotein (HDL)-constituent apolipoprotein-CI (apoCI) protects mice against mortality in bacterial sepsis. Therefore, we assessed whether high plasma apoCI levels protect against mortality from infection in humans.

We determined plasma levels of apoCI, lipids, and C-reactive protein in 85-year-old participants of the prospective population-based Leiden 85-plus Study (n=561). Participants were followed for specific causes of death.

High apoCI levels associated with 40% reduced risk of mortality from infection (HR, 0.60 [95% CI, 0.42-0.86]; *P*=0.005) for every increase of one standard deviation in apoCI level. A similar association was observed for high HDL-cholesterol levels (HR, 0.65 [0.46-0.94]; *P*=0.022), but not for LDL-cholesterol, triglycerides, and C-reactive protein levels. Importantly, the association of apoCI level was independent of HDL-cholesterol, as multivariate analysis did not alter the association for apoCI (HR, 0.63 [0.44-0.90]; *P*=0.013), while for HDL-cholesterol significance was lost.

We conclude that high apoCI levels associate with reduced mortality from infection in humans, in line with experimental evidence in rodents.

## INTRODUCTION

Apart from regulating lipid metabolism, evidence accumulates that lipoproteins are also involved in the outcome of infectious disease (Canturk et al., 2002; Delgado-Rodriguez et al., 1997; van Leeuwen et al., 2001; Weverling-Rijnsburger et al., 2003; Wu et al., 2004). In particular high high-density lipoprotein (HDL)-cholesterol levels have been associated with increased protection against infection-related mortality (Delgado-Rodriguez et al., 1997; Weverling-Rijnsburger et al., 2003; Wu et al., 2004). Experimental studies in rodents suggest that not the lipid-content of the lipoproteins, but rather the associated surface apolipoproteins, are responsible for the protective effect against infection (Berbee et al., 2005; Berbee et al., 2006; de Bont et al., 1999; Feingold and Grunfeld, 1997; Ma et al., 2004; Rensen et al., 1997; Van der Poll et al., 1995; Van Oosten et al., 2001; Vowinkel et al., 2004). Recently, we showed that apolipoprotein-CI (apoCI) protected against mortality in bacterial sepsis, by using genetically engineered mice that either lack apoCI or overexpress apoCI (Berbee et al., 2006). The relation between plasma levels of apoCI and infectious disease mortality in humans has not yet been studied.

ApoCI predominantly circulates as a surface component of HDL at a relatively high plasma concentration of about 6-10 mg/dL (Cohn et al., 2003; Curry et al., 1981; Shachter et al., 2005). With 6.6 kDa it is the smallest apolipoprotein known to date. Studies *in vitro* (Conde-Knape et al., 2002; Gautier et al., 2000; Kinnunen and Ehnolm, 1976; Soutar et al., 1975) and *in vivo* (Gautier et al., 2002) show that apoCI modulates the activity of plasma factors involved in HDL metabolism such as cholesteryl ester transfer protein (CETP) (Gautier et al., 2000; Gautier et al., 2002), lecithin cholesterol acyltransferase (LCAT) (Soutar et al., 1975), and hepatic lipase (HL)(Conde-Knape et al., 2002; Kinnunen and Ehnolm, 1976). Studies with apoCI-deficient mice indeed showed that apoCI expression correlated positively with HDL-cholesterol levels (Gautier et al., 2007). However, the fact that administration of lipid-free apoCI enhanced a beneficial proinflammatory host response towards bacterial products *in vivo*, without altering HDL-cholesterol levels (Berbee et al., 2006), strongly suggests that the effect of apoCI on infection-related outcome is independent of HDL-cholesterol levels.

Here we analyzed whether in humans, high plasma apoCI levels protect against mortality from infection. To this end, we determined the plasma apoCI, lipid, and C-reactive protein (CRP) levels, and mortality from infection within the Leiden 85-plus Study, a prospective population based follow-up study of elderly aged 85 years. Within this age category, 17% of deaths occur due to infection-related causes. Our findings reveal that in the population at large, high apoCI levels indeed associate with reduced mortality from infection.

## METHODS

### PARTICIPANTS.

Between September 1, 1997, and September 1, 1999, a total of 705 inhabitants of the community of Leiden, the Netherlands, reached the age of 85 years. Among these 85-year-old persons, we initiated a follow-up study to investigate determinants of successful aging. There were no selection criteria on health or demographic characteristics. The response rate was 87%; a total of 599 individuals 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 subjects.

### PLASMA PARAMETERS.

At baseline, subjects were visited twice at their place of residence within one month after the subjects' 85$^{th}$ birthday. All blood samples were collected before 11.00 a.m., although not fasting. Plasma apoCI levels were determined using a human apoCI-specific sandwich ELISA as described previously (Berbee et al., 2005). In short, a polyclonal goat anti-human apoCI antibody (Academy Biomedical Co., Houston, TX) was coated onto Costar medium binding plates (Corning, Inc., New York, NY) and incubated with diluted human plasma (dilution 1:150,000). Subsequently, the wells were incubated with horse radish peroxidase (HRP)-conjugated polyclonal goat anti-human apoCI antibody (Academy Biomedical Co.), and finally HRP was detected by incubation with tetramethylbezidine (Organon Teknika, Boxtel, The Netherlands). Plasma from C57Bl/6 mice spiked with human apoCI (Labconsult, Brussels, Belgium) was used as a standard.

Plasma levels of total cholesterol, HDL-cholesterol, triglycerides (TG), and CRP were analyzed on fully automated computerized analyzers (Hitachi 747 and 911; Hitachi, Ltd, Tokyo, Japan). The level of low-density lipoprotein (LDL)-cholesterol was estimated by the Friedewald equation (LDL-cholesterol [mmol/L] = total cholesterol - HDL-cholesterol - [TG/2.2]), whereby subjects with a TG concentration higher than 443 mg/dL (5 mmol/L) were excluded (n = 5).

## CAUSES OF DEATH

For the analyses presented in this research, all subjects were followed for mortality until April 1st, 2004. The date of death was obtained from the civic registries. Shortly after civic registries reported the death of a subject, 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, in 2004 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 (ICD-10)*. Cardiovascular mortality was classified as ICD-codes I00-I99, mortality from infection as ICD-codes A00-B99 and J10-J18, and cancer as C00-D48. All other ICD-codes were grouped as other cause mortality. Of 6 subjects the cause of death could not be established.

### STATISTICAL ANALYSES

Plasma levels of apoCI and total-, LDL- and HDL-cholesterol were normally distributed and are presented as means. Plasma levels of TG and CRP were not normally distributed and are presented as medians with interquartile range to assess distribution in the total population and as geometric means to compare means between groups. Individuals with undetectable CRP levels were attributed half of the minimal detection limit (0.25 mg/L) to allow log-transformation. Differences in levels of these lipids were calculated using sex-adjusted linear regression. Mortality risks were estimated using Cox Proportional Hazards models, which were all adjusted for gender. All calculations were performed using SPSS 12.0.1, Kaplan Meier curves were generated using STATA 9 SE.

## RESULTS

### BASELINE CHARACTERISTICS AND LIPID CORRELATIONS

The baseline characteristics of the study population are listed in table 8.1. The mean plasma apoCI concentration was 6.68 ± 2.07 mg/dL, comparable with concentrations reported in other populations (Cohn et al., 2003; Curry et al., 1981; Shachter et al., 2005).

Correlation of plasma apoCI levels with all classical lipid parameters revealed a strong and positive association between plasma apoCI levels and levels of total-, LDL- and HDL-cholesterol, and TG (table 8.2; all $P<0.001$). In addition, we found a negative association between plasma apoCI levels and plasma CRP levels (table 8.2; $P<0.01$).

**Table 8.1**. Baseline characteristics of study participants.

| Characteristic | Value |
|---|---|
| Total number (n) | 561 |
| Female (number, %) | 374 (67%) |
| Plasma apolipoprotein levels in mg/dL | |
| ApoCI, mean (SD) | 6.68 (2.07) |
| Plasma lipid levels in mmol/L | |
| Total cholesterol, mean (SD) | 5.71 (1.13) |
| LDL-cholesterol, mean (SD) | 3.68 (0.97) |
| HDL-cholesterol, mean (SD) | 1.31 (0.40) |
| Triglycerides, median (IQR) | 1.34 (1.00-1.95) |
| Plasma cytokine levels | |
| CRP, median (IQR) in mg/L | 4.0 (1.0-8.0) |

Abbreviations: SD, Standard Deviation; IQR, Inter Quartile Range; CRP, C-reactive protein.

**Table 8.2**. Plasma levels of lipid parameters and CRP according to quartiles of plasma apoCI levels.

| Lipid level | Quartiles of plasma apoCI levels | | | | *P* for trend* |
|---|---|---|---|---|---|
| | very low (n=140) | low (n=141) | high (n=138) | very high (n=142) | |
| ApoCI (mg/dL) | 4.30 (4.17-4.45) | 5.86 (5.72-6.00) | 7.16 (7.02-7.31) | 9.38 (9.24-9.52) | n.d. |
| Total cholesterol (mmol/L) | 5.09 (4.92-5.26) | 5.54 (5.37-5.70) | 5.88 (5.72-6.05) | 6.34 (6.17-6.50) | <0.001 |
| LDL-cholesterol (mmol/L) | 3.35 (3.20-3.51) | 3.55 (3.40-3.70) | 3.78 (3.63-3.94) | 4.03 (3.87-4.19) | <0.001 |
| HDL-cholesterol (mmol/L) | 1.14 (1.07-1.20) | 1.31 (1.25-1.37) | 1.41 (1.35-1.47) | 1.40 (1.34-1.47) | <0.001 |
| Triglycerides (mmol/L)† | 1.22 (1.13-1.32) | 1.35 (1.26-1.45) | 1.40 (1.30-1.51) | 1.70 (1.58-1.83) | <0.001 |
| CRP (mg/L) † | 4.39 (3.45-5.64) | 2.36 (1.86-3.00) | 2.64 (2.08-3.39) | 2.32 (1.82-2.94) | 0.002 |

Mean levels (and 95% confidence interval of the mean (95% CI)) were sex-adjusted. All subjects were aged 85 years. Abbreviations: n.d., not determined; CRP, C-reactive protein. * *P* for trend calculated using sex-adjusted linear regression † geometrical means

## MORTALITY

Since our previous experimental studies in mice showed that high plasma apoCI levels are associated with reduced mortality from infection (Berbee et al., 2006), we calculated the risk of mortality from infection dependent on the plasma levels of apoCI. Of the 561 participants, 48 subjects (17%) died due to infection-related cause. As shown in figure 8.1, subjects with high plasma levels of apoCI (above the median) had a significant reduced cumulative risk of mortality from infection as compared with subjects with low plasma apoCI levels (below the median). In a sex-adjusted Cox proportional hazards model the risk

of mortality decreased by a factor of 0.60 (95% confidence interval 0.42-0.86; *P*=0.005) for every increase of one standard deviation in apoCI level.

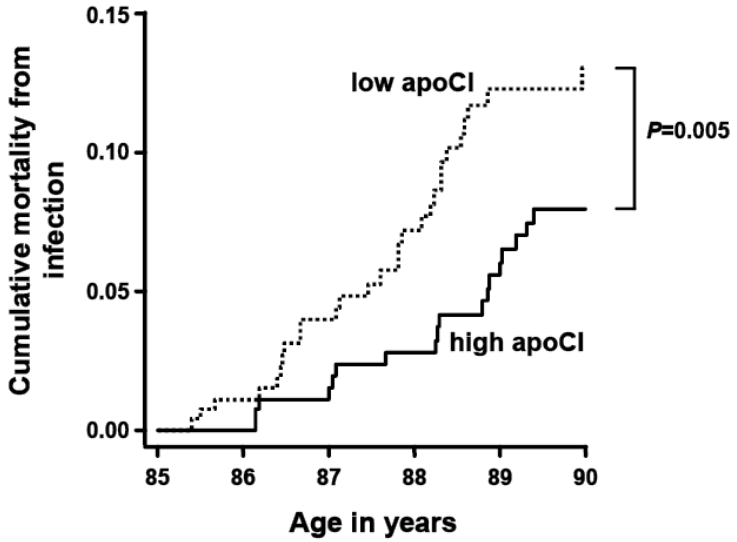


**Figure 8.1.** Cumulative mortality from infection dependent on plasma apoCI levels.
Kaplan-Meier curves for mortality from infection for subjects with high (above median) and low (below median) plasma apoCI level. The *P*-value indicates statistical significant in a sex-adjusted Cox proportional hazards model.

To determine whether high levels of apoCI specifically associated with reduced mortality from infection, and were not a general reflection of good health, we calculated risk of mortality from different causes dependent on plasma apoCI levels (table 8.3). The risk of all cause mortality decreased by a factor of 0.79 (95% confidence interval 0.69-0.91; *P*=0.001) for every increase of one standard deviation in plasma apoCI level. In contrast to the strong association of plasma apoCI levels with mortality from infection, the association with cardiovascular disease mortality was weaker (0.79; 95% confidence interval 0.64-0.99; *P*=0.036). No significant associations between plasma apoCI levels and mortality from cancer (0.88; 95% confidence interval 0.62-1.27) or from 'other causes' (0.88; 95% confidence interval 0.67-1.16) were found.

**Table 8.3**. Risk of all cause mortality and mortality from specific causes dependent on plasma apoCI levels.

| Cause (cases) | Hazard ratio per SD increase in apoCI level | *P*-value * |
|---|---|---|
| All causes (n=282) | 0.79 (0.69-0.91) | 0.001 |
| Infection (n=48) | 0.60 (0.42-0.86) | 0.005 |
| Cardiovascular (n=117) | 0.79 (0.64-0.99) | 0.036 |
| Cancer (n=45) | 0.88 (0.62-1.27) | 0.427 |
| Other (n=66) | 0.88 (0.67-1.16) | 0.376 |

Hazard ratios for all cause mortality and mortality from specific causes represent the relative risk of mortality associated with 1 standard deviation (SD) increase of plasma apoCI level, calculated using a sex-adjusted Cox proportional hazards model.
* *P*-values indicate statistical significance of difference of the reported hazard ratio from unity.

When analyzing the association of other plasma parameters with mortality from infection, we found that high levels of CRP were not significantly associated with an increased risk of mortality from infection (1.23; 95% confidence interval 0.99-1.53). When entered into a multivariate model, levels of CRP did not affect the observed association of high levels of apoCI with reduced risk of mortality from infection (0.61; 95% confidence interval 0.42-0.87). Of the classical lipid parameters in particular HDL-cholesterol has been associated with reduced mortality from infection (Delgado-Rodrigues et al., 1997; Weverling-Rijnsburger et al., 2003; Wu et al., 2004). In our population, apart from plasma apoCI, only HDL-cholesterol level was significantly associated with mortality from infection (0.65; 95% confidence interval 0.46-0.94; P=0.022), and not LDL-cholesterol (0.77; 95% confidence interval 0.57-1.05) or TG (0.90; 95% confidence interval 0.67-1.20). In addition, since most of the circulating apoCI can be found on the surface of HDL in normolipidemic subjects (i.e. 90-95%) (Cohn et al., 2003), and, although relatively weak, we show here that plasma apoCI levels were positively correlated with HDL-cholesterol levels (table 8.2), we investigated whether the reduced risk of mortality from infection with high apoCI levels was dependent on the association of apoCI with HDL-cholesterol. When apoCI and HDL-cholesterol were simultaneously entered in the model, the protective effect of apoCI on mortality from infection remained similar (0.63; 95% confidence interval 0.44-0.90; P=0.013), while for HDL-cholesterol the protective effect slightly decreased and significance was lost (0.72; confidence interval 0.49-1.04; P=0.074). Moreover, there was no significant interaction of the risk of mortality from infection associated with high levels of apoCI and the risk of mortality from infection associated with high levels of HDL-cholesterol (not shown; P=0.681).

Previously, we found that in elderly only high HDL-cholesterol levels are associated with decreased mortality form infection, and not LDL-cholesterol or TG levels (Weverling-Rijnsburger et al., 2003). Although relatively weak, we show here that plasma apoCI levels were positively correlated with HDL-cholesterol levels (table 8.2). Therefore, we

investigated whether the reduced risk of mortality from infection with high apoCI levels was dependent on the association of apoCI with HDL-cholesterol. To this end, we repeated the mortality analysis for HDL-cholesterol. For every increase of one standard deviation in plasma HDL-cholesterol level the risk of mortality from infection decreased by a factor of 0.65 (95% confidence interval 0.46-0.94; *P*=0.022). When apoCI and HDL-cholesterol were simultaneously entered in the model, the protective effect of apoCI on mortality from infection remained similar (0.63; 95% confidence interval 0.44-0.90; *P*=0.013), while for HDL-cholesterol the protective effect slightly decreased and significance was lost (0.72; confidence interval 0.49-1.04; *P*=0.074). Moreover, there was no significant interaction of the risk of mortality from infection associated with high levels of apoCI and the risk of mortality from infection associated with high levels of HDL-cholesterol (not shown; *P*=0.681).

## DISCUSSION

The results of the present prospective population-based study show that in old age high plasma apoCI levels are strongly associated with lower risk of mortality from infection. The protective effect of apoCI was specific for mortality from infection and independent of HDL-cholesterol levels. Our previous experimental studies in which we showed that apoCI was protective in a murine bacterial sepsis model (Berbee et al., 2006) can thus be extrapolated to humans.

Our results show that, within the Leiden 85-plus Study, subjects with high plasma apoCI levels at baseline are less prone to mortality from infection during a five-year follow-up period. To our knowledge there is only one report published on the relation between apoCI and infection in humans, which showed that HDL was virtually depleted from apoCI during human sepsis (Barlage et al., 2001), supportive for a role of apoCI in human infection. We also found total plasma levels of apoCI to be decreased in septic patients, and that this decrease was selective as compared with levels of lipoprotein lipids (Berbée JFP, Havekes LM, and Rensen PCN, unpublished data).

From our previous study (Berbee et al., 2006), we have concluded that high plasma levels of apoCI effectuate a more efficient killing of invading microorganisms, by virtue of an increased sensitivity to respond to microorganisms and a concomitant increased proinflammatory host response. This results in a low bacterial load, and consequently a reduced mortality risk. This host defense mechanism has been outstandingly described by Netea *et al.* (Netea et al., 2003), who stated that a proinflammatory cytokine response is crucial to surmount early phase bacterial infection, whereas in a late phase a high

proinflammatory response is often harmful and may lead to tissue damage and organ failure. The mechanism behind the decrease in apoCI levels during sepsis is not yet understood. It may well be that the decrease in apoCI levels is a natural response of the host in an attempt to protect itself against an overwhelming cytokine response in a latter phase of infection, a speculation which is subject of ongoing investigation.

Based on our current findings, the association of plasma apoCI levels with reduced mortality appears to be specific for mortality from infection, since apoCI levels are not significantly associated with cancer mortality and 'other cause' mortality, and we only found a weak association between apoCI levels and cardiovascular disease mortality. This weak association between reduced cardiovascular disease mortality and high apoCI levels is in line with the observed negative association between plasma CRP and apoCI levels in these subjects. High circulating levels of CRP are a marker of chronic inflammation and cardiovascular disease (Ridker et al., 2005; Tuomisto et al., 2006). This intriguing association between high apoCI and low CRP levels is not yet understood. It may be related to an apoCI-mediated protection against the development of infections in general, thereby reducing the incidence of chronic inflammation during life-time and improving the general well-being as reflected by low plasma CRP levels. Another explanation for the observation that in our population high levels of apoCI are associated with decreased cardiovascular mortality is that plasma apoCI levels positively associate with HDL-cholesterol levels as we show in this study. The role of HDL in preventing cardiovascular disease has indisputably been established (2002; Gordon et al., 1989; Packard et al., 2005). ApoCI is able to directly increase HDL levels by modulation of several enzymes involved in HDL metabolism. ApoCI is the main endogenous inhibitor of CETP (Gautier et al., 2000; Gautier et al., 2002), an inhibitor of HL (Conde-Knape et al., 2002; Kinnunen and Ehnolm, 1976), and an activator of LCAT (Soutar et al., 1975). These are all mechanisms shown to increase HDL-cholesterol, indicating that high plasma apoCI levels could improve cardiovascular outcome via increasing HDL-cholesterol levels.

The observed association of high levels of apoCI with decreased mortality from infection was independent of HDL-cholesterol levels. A possible explanation for this finding is that a dual protective mechanism is exerted by apoCI and HDL. ApoCI acts proinflammatory and is involved in a more effective elimination of the pathogens in the early phase of infection, as discussed above, which may result in a reduced incidence of developing both nonfatal and fatal infections, and thereby may reduce the incidence of mortality from infection. On the other hand, HDL as a whole acts primarily anti-inflammatory and may improve neutralization of microbial products such as LPS. This most likely does not result in a reduced incidence of ApoCI and mortality from infection developing (non)fatal infections,

but HDL may be protective against mortality in the latter phase of infection by dampening the host's exaggerated inflammatory response towards the microbial products. Unfortunately, the design of the study only allowed us to study the relation between plasma apoCI and HDL levels and mortality from infection, and not with incidence of developing (non)fatal infections during follow-up. Another possibility is that this knowledge 'explains' why high cholesterol levels, and in particular high HDL-cholesterol levels, have previously been associated with a beneficial outcome of infection-related mortality in humans, a finding that is not yet been properly understood (Canturk et al., 2002; Delgado-Rodriguez et al., 1997; van Leeuwen et al., 2001; Weverling-Rijnsburger et al., 2003; Wu et al., 2004). Accumulating evidence from primarily experimental studies shows that not the lipids(Berbee et al., 2006; Rensen et al., 1997; Van der Poll et al., 1995), but rather the apolipoproteins located on the surface of lipoproteins determine the effect of the lipoproteins on the host response to infectious agents(Feingold and Grunfeld, 1997; Rensen et al., 1997; van den Elzen et al., 2005) and subsequent survival(de Bont et al., 1999; Ma et al., 2004; Rensen et al., 1997; Van Oosten et al., 2001; Vowinkel et al., 2004). Van der Poll *et al.*(Van der Poll et al., 1995) showed that continuous infusion of Intralipid, a protein-free lipid emulsion, in humans did not affect inflammatory responses to LPS, the toxic component of Gram-negative bacteria. Likewise, LDL-receptor (LDLr) and LDLr-related protein double-deficient mice showed no altered inflammatory response after LPS-stimulation as compared to wild-type mice (Berbee et al., 2006), despite the severe hyperlipidemic phenotype in these mice (Espirito Santo et al., 2005). These findings indicate that lipids per se do not alter the infection-related host responses.

Our previous experiments in mice mainly focused on the role of apoCI in Gram-negative bacterial sepsis (Berbee et al., 2006), since we found that the C-terminus of apoCI contains a highly conserved lysine-rich motif (i.e. *KVKEKLK*) involved in the avid binding of apoCI to LPS. We demonstrated that apoCI avidly bound LPS and stimulated the inflammatory response to LPS, thereby improving the antibacterial attack. However in the population at large, in addition to Gram-negative bacteria as one of the major contributors, also Gram-positive bacteria and other infectious microorganisms such as fungi are responsible for infectious disease mortality (Martin et al., 2003). Since we show here that high plasma levels of apoCI protect against mortality from infection in the population at large, one may speculate that apoCI has a role in the host defense against microorganisms beyond that of Gram-negative bacteria only.

A limitation of our study is that this finding cannot be directly extrapolated beyond this age group. The risk of high plasma apoCI levels for mortality from infection in middle age has yet to be determined. On the basis of official data from the Dutch Bureau of Statistics,

15% of men and 36% of women born between 1912 and 1914 actually survived until the age of 85 years. On the one hand, this may be regarded as a minority of the total birth cohort and does not allow for extrapolation of our findings to other age groups. On the other hand, apparently a substantial portion of the total population reaches this age-category and an even larger portion will reach it in the near future. Blood samples were not drawn in a fasted state, which could have added random error to plasma apoCI and triglycerides levels.However, all samples were drawn in the early morning, and it has been shown that apoCI levels in postprandial plasma do not differ from those in fasting samples (Hamsten et al., 2005). In addition, the triglyceride levels were similarly low as observed under fasting conditions (Cohn et al., 2003), suggesting that the magnitude of random error is relatively small. 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 on health and demographic characteristics.

We conclude that high plasma apoCI levels protect against mortality from infection independent of HDL-cholesterol. Our results support our previously proposed mechanism, in which apoCI functions as part of the innate host defense mechanism against invading microorganisms.