Inflammation underlying cardiovascular mortality is a late consequence of evolutionary programming

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ABSTRACT

With the increase in life expectancy, death from cardiovascular disease has risen greatly. There is increasing evidence that inflammation plays an important role in cardiovascular disease. We postulate that the development of cardiovascular disease in old age is a late consequence of evolutionary programming for a pro-inflammatory response to resist infections in early age. In 311 women, aged 85 yr old, the production of the pro- and anti-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-10 was determined in lipopolysaccharide-stimulated whole blood samples and studied prospectively in association with cardiovascular mortality. High TNF-α was a risk factor for death from cardiovascular disease (relative risk [RR] = 1.56; 95% confidence interval [CI]: 1–2.40), whereas high IL-10 was protective (RR = 0.58; 95% CI: 0.40–0.85). A genetic variant of the IL-10 gene promoter, which is associated with lower IL-10 production, was found to predispose to a 2.8-fold higher cardiovascular mortality risk (95% CI: 1.17–6.60). Reproductive success, which was studied as a measure of evolutionary programming because it trades off with early survival by pro-inflammatory resistance genes, was negatively associated with an increasing production of TNF-α (RR = 0.77; 95% CI: 0.68–0.88), while a positive association with IL-10 was found (RR = 1.22; 95% CI: 1.05–1.41). We suggest that cardiovascular mortality is a late consequence of evolutionary programming for a pro-inflammatory response.

Key words: ageing • pleiotropy • pro-inflammatory • anti-inflammatory

Over the past two centuries, life expectancy in affluent countries has more than doubled, mainly due to the control of infectious diseases (1). Consequently, in these parts of the world, the burden of disease has shifted away from infections toward chronic diseases that are typically expressed in old age. Accordingly, whereas fatal infections still account for the majority of deaths in less-developed parts of the world, especially at younger ages,
cardiovascular disease has become the leading cause of mortality in aging populations, accounting for 30% of all deaths worldwide each year (2, 3).

There is increasing evidence that inflammatory processes contribute to the development of cardiovascular disease. For example, levels of C-reactive protein, a marker of inflammation, have been associated with coronary disease, myocardial ischemia, and infarction (4–6). Moreover, results from population-based studies have demonstrated that increased levels of markers of inflammation such as cytokines, adhesion molecules, and acute-phase reactants are associated with cardiovascular events (7, 8). Because our immune system has evolved in the abundant presence of pathogens, pro-inflammatory responses are likely to be evolutionarily programmed to resist fatal infections, whereas anti-inflammatory responses developed to control hyperreactivity. Because genetic variations leading to differences in the production capacity of both pro- and anti-inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin (IL)-10, have been associated with the susceptibility and outcome of infection (9–13), it can be hypothesized that evolutionary programming acts via selection on these genetic traits.

Despite their protective role, inflammatory processes are potentially harmful. For example, it has been shown that tissue damage at the site of infection is associated with strong pro-inflammatory and low anti-inflammatory responses (10, 14–16). Moreover, elevated pro-inflammatory but reduced anti-inflammatory responses, measured by levels of TNF-α and IL-10, respectively, have been associated with recurrent spontaneous abortions (17–19) and thus reproductive success (20). Hence, selection for resistance to infection may be traded against selection for fertility (21–24), resulting in a compromise that is optimal for the fitness of the species in a specific environment (25).

Here, we present a study in which we tested the hypothesis that cardiovascular mortality in old age is the late consequence of evolutionary programming by means of pro- and anti-inflammatory responsiveness. A group of 85-yr-old Dutch women, derived from the general population, were followed for death from cardiovascular disease, and details regarding their reproductive history were obtained as a measure for evolutionary programming. The capacity of innate pro- and anti-inflammatory responsiveness was assessed by measuring levels of TNF-α and IL-10 in whole blood samples stimulated with lipopolysaccharide (LPS). Associations with both cardiovascular mortality and reproductive success were studied. Because evolutionary programming is assumed to act via genetic selection, associations with the IL-10 gene promoter were investigated.

MATERIALS AND METHODS

Subject recruitment

The study was conducted within the framework of the Leiden 85-plus study, which is a population-based study of inhabitants in Leiden, The Netherlands, in which no selection criteria for health or demographic characteristics are used. Between September 1997 and September 1999, all inhabitants who were born between 1912 and 1914 (n=705) were contacted within a month after their 85th birthday. A total of 599 people (397 women and 202 men) agreed to participate, whereas 92 refused and 14 had died before they could be enrolled. There were no significant differences for various demographic characteristics between the 599 respondents and
During two home visit interviews, physical examinations were performed, and during a third visit, a venous blood sample was drawn from 563 subjects (375 women and 188 men): Twenty-nine refused and another seven had died before the third visit could be made. Because reproductive success was one of the primary outcomes of the current study, only women were included who married before their 45th birthday and from whom a venous blood sample was available (n=311). There were no women participating in the 85-plus study that had children but did not get married. Notably, 22% of the women who had children were pregnant before their marriage.

Informed consent was obtained from all subjects. For cognitively impaired subjects, informed consent was obtained from a guardian. The Medical Ethical Committee of the Leiden University Medical Center in Leiden, The Netherlands, approved the study.

**Innate inflammatory responsiveness**

Cytokine production was assessed by stimulating ex vivo whole blood samples with LPS as described elsewhere (27). In brief, venous blood samples were collected in heparinized tubes, diluted twofold with RPMI-1640, and incubated in the absence or presence of 10 ng/ml *Escherichia coli*-derived LPS (Difco Laboratories, Detroit, MI) at 37°C and 5% CO2. After 4 and 24 h, supernatants were collected and stored at –80°C for later measurement of TNF-α and IL-10, respectively. Standard ELISA techniques were performed according to the manufacturer’s guidelines (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). For most participants, cytokine levels in unstimulated control samples were below the detection limits. All participants produced detectable levels of TNF-α and IL-10 upon LPS stimulation.

**IL-10 promoter gene polymorphisms**

Participants were genotyped for being carriers of the IL-10 promoter gene –2849 AA allele or –2849 AG/GG alleles. DNA was extracted from peripheral blood mononuclear cells (PBMC) that were sodium dodecyl sulfate-lysed and treated with proteinase-K by protein precipitation using potassium acetate and chloroform extraction. Genotyping was performed with a real-time polymerase chain reaction (PCR) using molecular beacons (28). The nucleotide sequence of the molecular beacon for detection of the –2849A allele was TET-5′-CCCCACACCAAGGCAGGCAGATCA-TTGCTGGG-3′-DABCYL, and the nucleotide sequence of the molecular beacon for detection of the –2849G allele was FAM-5′-CCTGACCAAGGCAGGCAGATCA-CGTCAGG-3′-DABCYL (arm sequences are underlined, and the allele-specific nucleotide is in bold; TET and FAM are fluorescent labels, and DABCYL is the quencher). The –2849 AG polymorphism is located in an *Alu*-repeat. To ensure the specificity of the PCR, the upstream primer was designed outside the repeat (TGAGATAATTAGCTACATTTTCAGAACA, position –2954), whereas the reverse primer (AGAGACGGGTTTACTGTGTTA) started at position –2792 within the *Alu*-repeat. Real-time PCR was performed in an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Reaction mixtures (30 µl) contained 20 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2, 200 µM of each dNTP, 150 nM of each primer, 200 nM of each beacon, and 0.75 U AmpliTaq Gold. The following cycling conditions were used: 40 cycles at 95°C for 20 s and 59°C for 40 s. The genotypes of the samples were determined by ascertaining the presence of a
sigmoidal fluorescence curve with a threshold cycle value of $<34$. All genotypes were independently assessed by two observers. A randomly chosen 10% of the samples were reamplified and genotyped by digesting the PCR product with the restriction endonuclease AlwI followed by electrophoresis on a 7.5% polyacrylamide MADGE gels (29). Cycling and reaction conditions were the same as for genotyping using molecular beacons except that the MgCl$_2$ concentration was 1.5 mM. All previously assigned genotypes were confirmed.

In line with earlier findings (20), we found in the present study that carriers of the $-2849$ AA allele ($n=21$) produced lower levels of IL-10 (geometric mean (GM) = 495 pg/ml; 95% CI: 349–704) than $-2849$ AG carriers ($n=139$) (GM = 640 pg/ml; 95% CI: 573–715), who on their turn produced lower levels than $-2849$ GG carriers ($n=151$) (GM = 743 pg/ml; 95% CI: 684–807) ($p_{\text{trend}} = 0.009$).

Primary outcomes

All subjects were followed up for mortality until September 1, 2002. Depending on the time of recruitment of the participant, follow-up periods therefore ranged from 3 to 5 years. The date of death was obtained from the civic registries. Shortly after a civic registry 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 independent senior specialists of internal medicine reviewed the causes of death and classified each death into primary and, if applicable secondary, causes of death, according to the *International Classification of Diseases and Related Health Problems*, 10th revision (ICD-10) (30). Disagreements between the two specialists were solved by discussion and consensus. Cardiovascular disease, coronary artery disease, and stroke were classified as ICD-10 codes I00-I29, I20-I25, and I60-I69, respectively. Infection, pneumonia, sepsis, and cancer were classified as A00-B99, J12-J18, A40-A41, and C00-D48, respectively. A secondary cause of death was defined as a disease that also directly contributed to the death of the subject. Because primary and secondary causes of death were often strongly interlinked, causes of death presented here are based on both the primary and secondary cause of death.

Data with respect to the number of children women had, as well as the date of their marriage(s), were obtained from the Central Bureau of Genealogy (CBG), which is the major documentation and information center for family history and heraldry in The Netherlands. Women were considered fertile if they had successfully given birth at least once.

Secondary outcomes

Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, hemoglobin A1c (HbA$_{1c}$), and glucose concentrations were determined in serum, using a fully automated Hitachi (Tokyo, Japan) 747 and Hitachi 911. Low-density lipoprotein (LDL) cholesterol was estimated using the Friedenwald equation: LDL cholesterol = total cholesterol–HDL cholesterol–(triglycerides/2.2).

Participants were extensively interviewed for lifestyle and clinical characteristics. Clinical data were obtained from their general practitioner or from their nursing home physician. Information about drug use was obtained from computerized pharmacy registries. Criteria for cardiovascular
disease included a positive history of myocardial infarction, angina pectoris, arterial surgery, stroke, intermittent claudication, or signs of myocardial infarction or myocardial ischemia on the electrocardiogram (ECG). Subjects were classified as having type 2 diabetes when they met at least one of the following criteria: 1) history of type 2 diabetes obtained from the general practitioner; 2) use of sulfonylureas, biguanides, or insulin; or 3) nonfasting glucose of ≥11.1 mmol/l. Cognition was measured by the Mini-Mental State Examination (31).

**Data analysis**

Significance differences prevalent between groups was tested using the Pearson chi-square test, unless a table included a cell with an expected frequency of less than five, for which the Fisher’s exact test was used instead.

Normally distributed and not normally distributed continuous data (TNF-α, IL-10, their ratio, HDL cholesterol, and triglycerides) are presented as means and geometric means with 95% CI, respectively, the latter after having been log_{10}-transformed. Differences between two groups were tested by using Student’s t test and the nonparametric Mann-Whitney test, respectively. When more than two groups were compared, the hypothesis that groups were equal was tested by calculating a p-for-trend by linear regression, which allows adjustment for possible confounders.

Survival analysis (Cox regression), which has the advantage of taking into account the time until the event, was applied to model the effect of the pro- and anti-inflammatory cytokines TNF-α and IL-10 on both age at death from cardiovascular disease and age at giving birth for the first time. Models predicting reproductive success were adjusted for the confounding effect of the age that women married. Because pro- and anti-inflammatory cytokines act antagonistically, the covariates TNF-α and IL-10 were entered simultaneously in all models, concordant with their biological activity. Outcomes of Cox regression models, that is, hazard ratios, are presented in the text as relative risks (RR), with RR > 1 indicating an increased risk and RR < 1 indicating a reduced risk. An association was considered to be significant at \( P < 0.05 \).

To make the estimates of the various cytokines in different groups comparable, levels of TNF-α and IL-10 were entered in the models as differences in standard deviation (SD) in relation to the geometric mean: This way, an outcome represents a shift in risk concordant to an SD increase in cytokine production. Because four SDs cover the range in cytokine production of 95% of the study population, the variance in risk between study participants as a function of their cytokine production could be calculated by raising the relative risk of one SD increase to the fourth. To calculate the difference in SD for each individual, we transformed levels of TNF-α and IL-10 by the following equation: \[ \log_{10} (\text{IL-10 or TNF-}\alpha) - \text{mean of } \log_{10} (\text{IL-10 or TNF-}\alpha) / \text{SD of } \log_{10} (\text{IL-10 or TNF-}\alpha). \]

**RESULTS**

**Baseline characteristics**

On average, the 311 women included in the study married at the age of 25.1 years (95% CI: 24.6–25.6). Lifestyle and clinical characteristics of the participants are described in Table 1. The innate capacity of pro- and anti-inflammatory responsiveness was determined in Table 1. The innate capacity of pro- and anti-inflammatory responsiveness was determined by measuring levels of TNF-α and IL-10 in supernatants of whole blood samples stimulated with LPS. All
participants produced detectable levels of both cytokines. Geometric means were 9141 pg/ml for TNF-α (95% CI: 8652–9658) and 676 pg/ml for IL-10 (95% CI: 631–725).

**Cardiovascular mortality**

During the follow-up period (on average, 3 yr and 5 months) 93 women died (30%), 42 from cardiovascular causes. Women who died of a cardiovascular event produced significantly higher levels of TNF-α to IL-10 (ratio is 16.3; 95% CI: 14.0–18.9) compared with the rest of the cohort (ratio is 13.1; 95% CI: 12.4–13.9) \( P=0.018 \), including both women who died of other mortality causes (ratio is 12.9; 95% CI: 10.7–15.6) as well as women who did not die during the follow-up (ratio is 13.2; 95% CI: 12.4–14.0) (Fig. 1). Accordingly, when pro- and anti-inflammatory responses were studied as predictors of cardiovascular mortality after the age of 85 yr old, a high TNF-α production capacity was found to be associated with a greatly elevated risk of death from a cardiovascular event, whereas this risk was markedly reduced by an increased capacity to produce IL-10 (Table 2). The effect of TNF-α and IL-10 on cardiovascular mortality was found to be independent of risk factors, including total cholesterol, HDL cholesterol, triglycerides, and type 2 diabetes, because the effect of the production capacity of these cytokines on cardiovascular mortality remained unchanged after adjustment for these risk factors (Table 2).

When genetic variation of the IL-10 gene promoter was studied in association with cardiovascular mortality, 29% (6/21) of the –2849 AA carriers, which is the genetic variant associated with the lowest IL-10 production (see Materials and Methods), were found to die from a cardiovascular disease compared with 12% (16/139) of the –2849 AG carriers and 13% (20/151) of the –2849 GG carriers. In a Cox regression model, this corresponded with –2849 AA carriers of 85 yr old having an almost threefold higher cardiovascular mortality risk (RR=2.77; 95% CI: 1.17–6.60, \( P=0.021 \)) than –2849 AG and –2849 GG carriers (Fig. 2).

**Death from fatal infections and overall mortality**

During the follow-up, 26 women died of fatal infections. An increased production of TNF-α was found to have a reducing effect on the risk of death from a fatal infection (RR=0.58; 95% CI: 0.38–0.58, \( P=0.012 \)), whereas no effect of IL-10 was seen (RR=0.78; 95% CI: 0.50–1.23, \( P=0.288 \)). At age 85, the TNF-α to IL-10 production of women dying of a fatal infection (ratio is 12.5; 95% CI: 10.1–15.5) was significantly lower than that of the 42 women who died of a cardiovascular event (\( P=0.049 \)). However, produced levels did not differ between women who died of fatal infections versus those who either survived or died from causes other than cardiovascular mortality and fatal infections (ratio is 13.1; 95% CI: 9.8–17.5).

When overall mortality was studied, a protective effect of IL-10 was found (RR=0.74; 95% CI: 0.57–0.96, \( P=0.023 \)), with no additional effect of TNF-α (RR=0.46, 95% CI: 0.14–1.55, \( P=0.213 \)).

**Reproductive success as a read-out of evolutionary programming**

A total of 32 married women (10%) were found to have remained childless. Among the 279 women that had children, the mean number of live births (parity) was 3 (3.3; 95% CI: 3.1–3.5), with a maximum of 11. Women with children married at a significantly younger age (mean 24.6
The ratio of TNF-α to IL-10 was found to decline with the number of progeny women had ($p_{\text{trend}}=0.031$) (Fig. 3), indicating that parity is associated with lower pro-inflammatory and higher anti-inflammatory production capacity. Accordingly, in a Cox regression model that takes into account the age of the mother when her first child was born, this inverse effect of IL-10 and TNF-α on reproductive success was confirmed: A higher IL-10 production capacity increased the chance of reproductive success, whereas the reverse was found for TNF-α (Table 3).

In accordance with findings of an earlier study, 12.5% of the childless women (4/32) were found to carry the –2849 AA allele compared with 6% of the women with children (17/269), whereas 53% (17/32) and 34% (11/32) of the childless women and 44% (122/279) and 50% (140/279) of the women with children were carriers of the AG and GG allele, respectively. In Cox regression, this corresponded with carriers of the –2849 AA allele having half the chance on reproductive success ($RR=0.54$; 95% CI: 0.33–0.90, $P=0.018$) compared with carriers of the –2849 GG allele, whereas this was reduced by 23% for carriers of the –2849 AG allele ($RR=0.77$; 95% CI: 0.61–0.99, $P=0.040$).

**DISCUSSION**

Cardiovascular and other inflammatory diseases have become prominent causes of mortality in affluent countries where, following improved health care, life expectancy started to increase (2, 3). Although many studies have tried to identify causes for the rise in these diseases, results have so far been ambiguous. Because cardiovascular disease is typically late-onset, we postulated it to be a consequence of aging. We here provide evidence in line with the antagonistic pleiotropy theory on the evolution of aging, which postulates that senescence is due to the late deleterious effect of genes that are beneficial in early life (32, 33), that cardiovascular death is the late consequence of an evolutionary selection for pro-inflammatory resistance genes. Evolutionary programming in our ancestral environment is postulated to have been associated with a positive selection for resistance genes coding for strong pro-inflammatory responses but low anti-inflammatory responses (11, 12). We show here, by means of a high TNF-α but low IL-10 production capacity, that such a predisposition puts people at an increased risk of cardiovascular death in old age. The hypothesis that a genetic predisposition for strong pro-inflammatory responses underlies cardiovascular disease in old age is in line with indications that genetic risk factors (34) and inflammatory processes (4–8) play a role in the onset of cardiovascular disease.

Experiments with *Drosophila melanogaster* have revealed trade-offs between fertility and longevity (21, 22). Evidence that infertile women live longer than fertile women (23, 24) suggests that the same holds true for humans. We previously proposed an immunogenetic explanation for this trade-off in humans, by showing that high pro-inflammatory (TNF-α) but low anti-inflammatory responses (IL-10) have a reducing effect on reproductive success (20), plausibly by increasing the risk of spontaneous abortions (17–19). Based on this rationale, we decided to study reproductive success instead of early survival as a read-out of evolutionary programming, because the latter option was not feasible in our study population of older women. Here, we reconfirm our previous finding that women with a strong TNF-α but low IL-10 response have a reduced chance of reproductive success. The consistency in these data, but also
that of a recent study reporting that low concentrations of macrophage inhibitory factor-1 (MIC-
1) strongly predict miscarriage (35), strongly support the hypothesis that pro-inflammatory
responses play an important role in mechanisms underlying pregnancy loss. The finding of a
genetic component that associates both with this immunological risk profile and the outcome of
reproductive success emphasizes that a pathological pathway and not an epiphenomenon is
studied. Moreover, in our opinion, this genetic association provides clear evidence for our
hypothesis that reproductive success is a suitable measure of evolutionary programming, as the
latter is assumed to act via genetic selection.

Despite the old age of our study population and the control of infectious diseases in Western
societies over the past few decades, we found to our surprise that the protective role of a high
production capacity of TNF-α and low IL-10, which is assumed to be associated with an
increased resistance to pathogens and survival at young age (refs 9–13), remained at old age.
This indicates that selection for strong pro-inflammatory responders is putatively not limited to
young age but may last up to old age. However, as shown here, at old age, the protective effect
of such inflammatory responses trades-off with the increasing risk of death from cardiovascular
events, thereby reducing life expectancy. This is confirmed by our finding that at old age, a high
IL-10 production capacity is protective against overall mortality, that is, is associated with
survival. We therefore suggest that our study participants, who survived up to the age of 85,
represent the fittest and strongest pro-inflammatory responders of their birth cohort, but during
the ageing process, their advantageous pro-inflammatory risk profiles turns against them by
putting them at an elevated risk to die of a cardiovascular disease.

Despite the clear association with inflammatory responses, other risk factors are likely to
contribute to the risk of cardiovascular death. We studied whether cholesterol and diabetes type
2, which are well-known risk factors of cardiovascular mortality (36), might confound our
results, but the effect of the inflammatory responses on death from cardiovascular disease was
found to be independent of these risk factors. Nonetheless, in contrast to cholesterol, type 2
diabetes was found to contribute independently to cardiovascular death. In line with an earlier
finding that a low production capacity of IL-10 is a predictor for type 2 diabetes (37), this
indicates that a reduced anti-inflammatory response increases both the risk of cardiovascular
disease and of type 2 diabetes, while the latter has an additional effect on cardiovascular
mortality. It may therefore be hypothesized that a genetic predisposition to produce high pro-
inflammatory but low anti-inflammatory responses elevates the risk of cardiovascular mortality
at old age in a multifactorial manner.

In conclusion, here, we provide evidence that the rise in death from cardiovascular disease in
progressing aging populations is, in part, a deleterious late effect of genes that have been
evolutionary programmed to have beneficial early-life effects. Moreover, we demonstrate that
genes encoding for inflammatory responses fulfill the criteria of pleiotropy: Protection against
fatal infections goes with an increased cardiovascular mortality risk. In view of the still-
increasing life expectancy in the world, this association suggests a need for increased wariness
on the risk of cardiovascular disease, especially in populations in developing countries where the
selection for strong pro-inflammatory responsiveness is likely still present.
REFERENCES


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Table 1

Lifestyle and clinical characteristics of the 311 85-year-old women

<table>
<thead>
<tr>
<th>Demographic/lifestyle</th>
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</tr>
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<tbody>
<tr>
<td>Living of minimum income</td>
<td>61/304 (20%)</td>
</tr>
<tr>
<td>Primary school only</td>
<td>228/310 (74%)</td>
</tr>
<tr>
<td>Smoking (past)</td>
<td>88/291 (30%)</td>
</tr>
<tr>
<td>Smoking (present)</td>
<td>35/295 (12%)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.8 (27.3–28.4)</td>
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</table>

<table>
<thead>
<tr>
<th>Medical history</th>
<th></th>
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<tbody>
<tr>
<td>Diabetes</td>
<td>60/311 (19%)</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>191/311 (61%)</td>
</tr>
<tr>
<td>Poor cognition (MMSE &lt;19)</td>
<td>59/311 (19%)</td>
</tr>
<tr>
<td>Poor subjective well-being</td>
<td>51/306 (17%)</td>
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<table>
<thead>
<tr>
<th>Blood measures</th>
<th></th>
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<tbody>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.84 (3.72–3.95)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.32 (1.28–1.36)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.49 (1.42–1.57)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.99 (6.68–7.31)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.87 (5.73–6.00)</td>
</tr>
</tbody>
</table>

*BMI, body mass index; MMSE, mini-mental state examination; LDL, low-density lipoprotein; HDL, high-density lipoprotein; and HbA1c, hemoglobin A1c.

bContinuous data are expressed as means with 95% confidence intervals.

cContinuous data are expressed as geometric means when data were not normally distributed.
Table 2

Association between cytokine production and cardiovascular mortality\textsuperscript{a}

<table>
<thead>
<tr>
<th>LPS-induced cytokine response</th>
<th>Relative risk\textsuperscript{b} (95% CI)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unadjusted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>1.56 (1.01–2.40)</td>
<td>=0.045</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.58 (0.40–0.85)</td>
<td>=0.005</td>
</tr>
<tr>
<td><strong>Adjusted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>1.53 (1.01–2.31)</td>
<td>=0.043</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.61 (0.41–0.89)</td>
<td>=0.011</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The association between the production of the antagonistic pro- and anti-inflammatory cytokines TNF-\(\alpha\) and IL-10 on the risk of cardiovascular mortality was studied by simultaneously entering the cytokine levels in multiple Cox regression models that were unadjusted and adjusted for the confounding effect of serum levels of total cholesterol, high-density lipoprotein cholesterol, triglycerides, and a history of diabetes type 2.

\textsuperscript{b}Estimates represent risks per SD difference in TNF-\(\alpha\) and IL-10 production. Because four SDs cover the range in cytokine production of 95\% of the study population, the risk of cardiovascular death that is calculated by raising the relative risk of one SD increase to the fourth, varies six- to ninefold depending on the production capacity of TNF-\(\alpha\) and IL-10, respectively, according to the unadjusted model.
Table 3

Association between cytokine production and reproductive success<sup>a</sup>

<table>
<thead>
<tr>
<th>LPS-induced cytokine response</th>
<th>Relative risk&lt;sup&gt;b&lt;/sup&gt; (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0.77 (0.68–0.88)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.22 (1.05–1.41)</td>
<td>=0.008</td>
</tr>
</tbody>
</table>

<sup>a</sup>The association between the production of the antagonistic pro- and anti-inflammatory cytokines TNF-α and IL-10 and reproductive success was studied by simultaneously entering the cytokine levels in a Cox regression model that measures the age at which women gave birth for the first time, while adjusting for the confounding effect of age of marriage.

<sup>b</sup>Estimates represent risks per SD difference in TNF-α and IL-10 production. Because four SDs cover the range in cytokine production of 95% of the study population, the chance of reproductive success, which is calculated by raising the relative risk of one SD increase to the fourth, varies two- to threefold depending on the production capacity of IL-10 and TNF-α, respectively.
Figure 1. TNF-α to IL-10 production according to death from cardiovascular disease. The ratio of the antagonistic pro- and anti-inflammatory cytokines TNF-α to IL-10 was plotted for women that after their 85th year of life died of a cardiovascular disease (CVD) (n=42) against those that died of other mortality causes (other cause) (n=51) or did not die during the follow up period (survivors) (n=218). Bars represent geometric means of the ratio with geometric-standard errors of the means. Significance between two groups was tested by using the nonparametric Mann-Whitney test.
Figure 2. Proportional cardiovascular mortality curves according to variation of IL-10 promoter gene. The (Kaplan-Meier) curves represent the proportional cardiovascular mortality of the 85-year-old women according to their IL-10 promoter genotype. In line with earlier findings (20), carriers of the –2849 AA allele produced significantly lower levels of the anti-inflammatory cytokine IL-10 than carriers of the –2849 AG or –2849 GG alleles (material and methods).
Figure 3. TNF-α to IL-10 production according to reproductive success. The ratio of the antagonistic pro- and anti-inflammatory cytokines TNF-α to IL-10 was plotted against the number of children women had. Bars represent geometric means with geometric-standard errors of the means. To test the hypothesis that the TNF-α to IL-10 production is equal for the different groups, we calculated a p-for-trend by linear regression by measuring the association between number of progeny and the ratio of TNF-α/IL-10. Because this outcome of reproductive success depends on the age at which women married, the calculated association was adjusted for this confounding variable.