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Inflammatory Predictors of Mortality in the Scandinavian Simvastatin Survival Study

FILIPPO CREA, M.D., CLAUDIA MONACO, M.D., GAETANO A. LANZA, M.D., ELENA MAGGI, M.D.,* FRANCESCA GINNETTI, B.SC., DOMENICO CIANFLONE, M.D., GIAMPAOLO NICCOLI, M.D., THOMAS COOK, M.D.,† GIORGIO BELLOMO, M.D.,* JOHN KJEKSHUS, M.D.‡

Istituto di Cardiologia, Università Cattolica, Rome; *Department of Medical Sciences, University of Torino, Novara, Italy; †Merck Research Laboratories, Rahway, New Jersey, USA; ‡Department of Cardiology, Rikshospitalitet, University of Oslo, Oslo, Norway

Summary

Background and hypothesis: The predictive value of specific markers of infection and autoimmunity for coronary events, such as the effects of statins on inflammation, is still controversial.

Methods: A case-control design was used to compare C-reactive protein (CRP) levels, seropositivity for *Chlamydia pneumoniae* and *Helicobacter pylori*, and anti-oxidized low-density lipoprotein (oxLDL) antibody levels in prerandomization blood samples from 129 participants in the Scandinavian Simvastatin Survival Study who died (cases), and from 129 matched participants who were alive during 5-year follow-up (controls).

Results: Patients with CRP levels in the highest quartile had an increased risk of death compared with those in the first through third quartile (odds ratio [OR] = 2.51, 95% confidence interval [CI] 1.3–4.8). Seropositivity for *Chlamydia pneumoniae* or *Helicobacter pylori* and anti-oxLDL antibody levels were similar in cases and controls (p = NS). At a 4-month control, simvastatin reduced CRP levels (p = 0.009) while placebo did not (p = NS). However, the risk of death associated with high baseline CRP levels was similar in patients randomized to placebo (OR = 2.36, 95% CI 1.06–5.26) or simvastatin (OR = 3.13, 95% CI 1.06–9.21).

Conclusions: Elevated CRP levels, but not seropositivity for *Chlamydia pneumoniae* or *Helicobacter pylori*, nor levels of anti-oxLDL antibodies, predict the risk of death in patients with stable ischemic heart disease. Simvastatin treatment reduces CRP levels, but without affecting the increased risk conferred by higher CRP levels at baseline.

Key words: lipids, mortality, infection, inflammation, coronary disease

Introduction

Low-grade inflammation, as assessed by C-reactive protein (CRP) serum levels, predicts the risk of myocardial infarction (MI) and other atherosclerotic events in apparently healthy middle-aged men.[1, 2] In addition, CRP appears to predict the risk of MI and coronary death in individuals with risk factors for coronary disease,[3, 4] as well as in those with stable or unstable angina.[5–7] Statins are powerful lipid-lowering agents that have been shown to have beneficial effects in primary[8] and secondary[9] prevention of coronary mortality, but their favorable effect might, at least partially, be mediated by inhibition of inflammatory cell activation in coronary atherosclerotic plaques, as recently suggested by Ridker *et al.* in patients post MI.[10] Indeed, statin-induced inhibition of mevalonate synthesis might blunt inflammatory cell activation[11–13] regardless of the causes of the low-grade inflammation. However, the question of whether statins affect CRP levels and whether this possible effect influences prognosis has not adequately been explored. Finally, the causes responsible for elevated levels of CRP in patients with a higher risk of clinical events are still elusive, although infectious agents such as *Chlamydia pneumoniae*[14–17] and *Helicobacter pylori*,[17, 18] and autoantigens, such as oxidized low-density lipoproteins (oxLDL), are potential candidates.[19] To address all these issues, we measured serum levels of CRP and of antibodies anti-*Chlamydia pneumoniae*, anti-*Helicobacter pylori*, and anti-oxLDL among patients enrolled in the Scandinavian Simvastatin Survival Study (4S) who were prospectively followed up for incident occurrence of death.[9]

Address for reprints:

Filippo Crea, M.D.
Istituto di Cardiologia
Università Cattolica del Sacro Cuore
L.go A. Gemelli, 8
00168 Rome, Italy
e-mail: f.crea@flashnet.it

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Methods

Patients

The 4S study was a randomized, double-blind, placebo-controlled trial of a dose of simvastatin able to reduce total serum cholesterol to 3.0–5.2 mmol/l in the secondary prevention of clinical events. A total of 4,444 patients with a prior history of ischemic heart disease (angina pectoris or acute MI) who had total serum cholesterol > 5.5 mmol/l (after 8 weeks of dietary advice) were enrolled. Exclusion criteria included total serum cholesterol > 8 mmol/l, premenopausal women of childbearing potential, secondary hypercholesterolemia, unstable or Prinzmetal angina, tendon xanthomata, planned coronary artery surgery or angioplasty, MI during the preceding 6 months, antiarrhythmic therapy, congestive heart failure, chronic atrial fibrillation, cardiomegaly, hemodynamically important valvular heart disease, history of completed stroke, impaired hepatic function, partial ileal bypass, history of drug or alcohol abuse, poor mental function, other serious disease, current treatment with another investigational drug, or hypersensitivity to 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. The primary endpoint of the 4S was total mortality. Blood samples collected at baseline and after randomization were shipped to a central collection site on dry ice and frozen at −80˚C for future analyses.

For the present study, baseline blood samples were assayed for CRP and for anti-*Chlamydia pneumoniae*, anti-*Helicobacter pylori*, and anti-oxLDL antibodies in the 129 patients, in whom serum was still available, out of the 438 who subsequently died during 5-year follow-up (cases), and in an equal number of age-, gender-, and treatment-matched study participants who remained alive during 5-year follow-up (controls). A second sample, obtained about 4 months after the baseline sample, was available for 249 subjects and was assayed for the same substances. Case and control blood samples were assayed in blinded pairs. Laboratory investigators were blinded to case or control status.

Laboratory Assays

C-reactive protein was measured in an ultrasensitive CRP assay by nephelometry (Dade-Behring, BN-100, Marburg, Germany), with a range of detection from 0.2 to 1000 mg/l and a coefficient of variation < 3% at a range of 2–4 mg/l.

Antibody levels anti-*Chlamydia pneumoniae* were measured using a microimmunofluorescence method (MRL Diagnostics, Cypress, Calif., USA). Samples were diluted from 1:8 to 1:64. Seropositivity for *Chlamydia pneumoniae* was considered in the presence of specific antibodies at a dilution of ≥ 1:32.

Antibody levels anti-*Helicobacter pylori* were measured using a commercial enzyme-linked immunosorbent assay (ELISA) for specific IgG (Behring Diagnostics, Marburg, Germany). Seropositivity for *Helicobacter pylori* was considered in the presence of specific antibody serum levels > 8 U/ml.

Levels of anti-oxLDL IgG antibodies were measured according to methods previously described.[19–22] Briefly, three different antigens were employed to coat multiwell ELISA plates, namely air-oxidized, minimally-modified LDL (mm-oxLDL), LDL oxidized with CuSO4, (Cu-oxLDL), and LDL oxidized with the combination of peroxidase and hydrogen peroxide (HRP-oxLDL). This choice was made in order to mimic the formation of some antigenic epitopes formed in vivo during LDL oxidation. The quantitation of anti-oxLDL antibody levels was performed using ELISA techniques and serum diluted 1:20. Results were expressed as arbitrary units (AU) of the spectrophotometric readings of the corresponding wells in microtitre plates.

Statistical Analysis

The comparisons between groups of continuous variables were made by Student's *t*-test for unpaired data. However, as CRP and antibodies serum levels showed no normal distribution, median concentrations of these parameters were computed and differences between cases and controls were assessed by Mann-Whitney U-test. Differences in proportions were tested by chi-square statistic with Yates' correction.

After dividing patients into quartiles of CRP values, chi-square analysis was applied to assess the association of CRP levels with mortality. Univariate and multivariate logistic regression analyses were also used to evaluate the risk of death associated with top quartile CRP serum levels. In the multivariate analysis, the risk of death associated with top quartile CRP serum levels was adjusted for those variables which showed a p value ≤ 0.1 on univariate analysis.

Two-way analysis of variance with a repeated measure design was applied on baseline and follow-up serum levels of CRP, total cholesterol, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) to assess the effect of simvastatin, compared with that of placebo, on these parameters. For this analysis, CRP concentrations were transformed in natural logarithmic values to obtain a nearly normal distribution of the variable.

Values are reported as mean ± standard deviation or median with interquartile interval, as appropriate. P values were considered significant if < 0.05. Confidence intervals (CI) were calculated at the 95% level.

Results

Predictors of Mortality

The baseline clinical characteristics of the study groups are shown in Table I. By matching, cases and controls were similar in age, gender, and randomized treatment. There was no difference in aspirin use between the two groups. Among the other baseline variables, smoker prevalence, systolic blood pressure levels, total cholesterol, and LDL-C serum levels were significantly higher in cases than in controls.

Baseline serum levels of CRP were higher in cases than in controls (p = 0.005, Table II). At chi-square analysis, serum CRP levels in the upper quartile (> 4.0 mg/l) were associated

TABLE I Baseline clinical features of the two groups of patients

	Cases (n =129)	Controls (n =129)	p Value
Age, years	61.9 ± 6.0	61.9 ± 5.9	Matching criteria
Male sex (%)	115 (89.15)	115 (89.15)	Matching criteria
Treatment, No			Matching criteria
Placebo	78	78	
Simvastatin	51	51	
Smoking (%)	31.7	20.1	0.046
Blood pressure (mmHg)			
Systolic	146.3 ± 19.7	141.6 ± 18.4	0.045
Diastolic	85.0 ± 9.7	83.6 ± 9.5	0.24
Diabetes (%)	7.7	5.4	0.60
Body mass index (kg/m^2)	24.8 ± 7.0	25.1 ± 4.3	0.67
TC (mmol/l)	6.78 ± 0.68	6.61 ± 0.66	0.047
LDL-C (mmol/l)	4.95 ± 0.65	4.79 ± 0.66	0.045
HDL-C (mmol/l)	3.11 ± 1.98	2.80 ± 1.88	0.20
ApoB (mmol/l)	1.18 ± 0.21	1.14 ± 0.22	0.12
Triglycerides (mmol/l)	1.51 ± 0.51	1.45 ± 0.48	0.27
Aspirin use (%)	30.2	34.1	0.59

Abbreviations: ApoB = apolipoprotein B, TC = total cholesterol, LDL-C = low-density lipoprotein cholesterol, HDL-C = high-density lipoprotein cholesterol.

with an increased risk of death compared with CRP levels in the bottom quartile (odds ratio [OR] 3.05, 95% CI 1.43–6.56, p < 0.0001). The risk of death was similar in patients with baseline CRP levels in the three lowest quartiles (Fig. 1).

Univariate analysis confirmed that patients with CRP levels in the upper quartile had a significant increase in the incidence of death compared with patients in the lowest three quartiles (OR 2.63, 95% CI 1.38–5.03). After adjustment for smoking, systolic blood pressure, total cholesterol, and LDL-C serum levels, CRP levels in the upper quartile were still an independent predictor of death (OR 2.51, 95% CI 1.3–4.8, p = 0.005), with only smoking and systolic blood pressure being of borderline significance (Table III).

Seropositivity for *Chlamydia pneumoniae* or *Helicobacter pylori* and anti-oxLDL antibody levels were similar in cases and controls (Table II).

TABLE II Baseline C-reactive protein serum levels, seropositivity for *Chlamydia pneumoniae* and *Helicobacter pylori*, and anti-oxidized low-density lipoprotein antibodies in the two groups of patients

	Cases (n = 129)	Controls (n = 129)	p Value
C-reactive protein (mg/l) (median, range)	2.5 (0–48)	1.9 (0–51)	0.0053
Seropositivity for CP (%)	46	45	0.90
Seropositivity for HP (%)	70	79	0.11
Anti-mm-oxLDL (AU) (median, range)	0.23 (0.09–1.09)	0.25 (0.08–0.62)	0.12
Anti-Cu-oxLDL (AU) (median, range)	0.40 (0.18–2.19)	0.41 (0.13–1.51)	0.38
Anti-HRP-oxLDL (AU) (median, range)	0.41 (0.11–2.29)	0.43 (0.13–1.60)	0.62

Abbreviations: CP = *Chlamydia pneumoniae*, HP = *Helicobacter pylori*, Cu-oxLDL = low density lipoproteins oxidized with $CuSO^4$, HRP-oxLDL = LDL oxidized with peroxidase and hydrogen peroxide, mm-oxLD = minimally oxidized LDL, AU = arbitrary units.

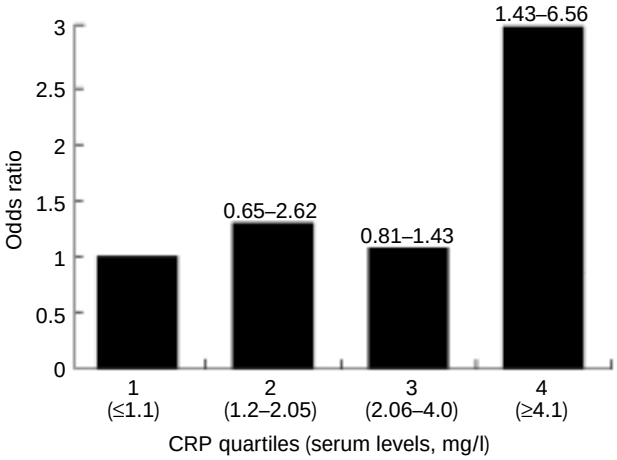

FIG. 1 Odds ratios for overall mortality according to quartiles of baseline C-reactive protein (CRP) serum levels; figures on the top of the histograms represent 95% confidence intervals. Compared with the bottom quartile, the risk of death significantly increased in patients with CRP levels in the top quartile (p < 0.0001) but not in those with CRP levels in the second or third quartile.

TABLE III Independent predictors of mortality at multivariate analysis. Odds ratio for C-reactive protein refers to top quartile levels (≥ 4.1 mg/l) versus lower three quartiles. Systolic blood pressure was inserted as a continuous variable

	Odds ratio	95% Confidence interval	p Value
C-reactive protein	2.51	1.3–4.8	0.005
Smoking	1.84	1.0–3.3	0.046
Systolic blood pressure	1.01	0.99–1.03	0.055

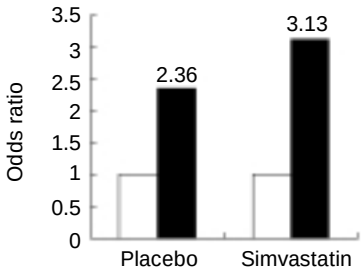

FIG. 2 Odds ratios for overall mortality according to quartiles (QRT) of baseline C-reactive protein (CRP) serum levels in patients randomized to placebo (n = 156) or simvastatin (n = 102). The risk of death was significantly increased in patients with CRP levels in the top quartile compared with those in the first to third quartiles randomized both to placebo (p = 0.034) and to simvastatin (p = 0.035), with no statistical difference in the odds ratio between the two groups (p = 0.68). □ = Quartiles 1–3, ■ = quartile 4.

Interaction between C-Reactive Protein Levels at Baseline and Treatment

The association of CRP serum levels in the top quartile with death was assessed separately in the two treatment groups (simvastatin or placebo). Serum CRP levels in the upper quartile were associated with an increased risk of death, compared with CRP levels in the first to third quartile, both in patients randomized to placebo (OR 2.36, 95% CI 1.06–5.26, p = 0.034) and in those randomized to simvastatin (OR 3.13, 95% CI 1.06–9.21, p = 0.035). There was no significant statistical difference between the two treatment groups in the ORs for death associated with top quartile CRP serum levels (p = 0.68) (Fig. 2).

Effects of Treatment on C-Reactive Protein Levels

A follow-up blood sample, obtained about 4 months after enrollment, was available for 154 patients randomized to placebo and 95 patients randomized to simvastatin. Total cholesterol, LDL-C, HDL-C, and triglycerides levels were all reduced significantly in patients randomized to simvastatin but not in those randomized to placebo (Table IV). Patients randomized to simvastatin showed a significant reduction in CRP serum levels (−20.9%, 95% CI −3.1, −38.7%, p = 0.009), whereas no difference was observed in patients randomized to placebo (p = 0.64) (Fig. 3). Simvastatin did not affect anti-oxLDL antibody levels (data not shown).

Discussion

This study is based on the analysis of blood samples obtained from stable patients with coronary artery disease, with or without a prior history of MI. It extends recent observations in which composite or nonfatal endpoints were utilized[10] by showing that, in this population, CRP predicts the single hard endpoint of overall mortality. The reasons that high CRP levels predict death are probably twofold. First, high levels of CRP are associated with an increased risk of developing plaque instability leading to occlusive thrombosis and MI.[1] Second, in

TABLE IV Serum levels of C-reactive protein and blood lipids in patients randomized to simvastatin (n = 95) or placebo (n = 154) at baseline and at 4-month follow-up

	Simvastatin (n = 95)			Placebo (n = 154)		
	Baseline	4-month FU	p Value	Baseline	4-month FU	p Value
CRP (mg/l) (median, range)	1.95 (0–51)	1.5 (0–45)	0.009	2.25 (0–48)	2.2 (0–51)	NS
TC (mmol/l) (mean, SD)	6.68 ± 0.66	4.76 ± 0.90	< 0.0001	6.70 ± 0.69	6.66 ± 0.95	NS
LDL-C (mmol/l) (mean, SD)	4.88 ± 0.67	2.98 ± 0.78	< 0.0001	4.87 ± 0.66	4.87 ± 0.89	NS
HDL-C (mmol/l) (mean, SD)	1.13 ± 0.27	1.22 ± 0.32	< 0.0001	1.16 ± 0.27	1.13 ± 0.28	NS
Triglycerides (mmol/l) (mean, SD)	1.48 ± 0.49	1.24 ± 0.61	< 0.0001	1.48 ± 0.51	1.45 ± 0.61	NS

P < 0.002 difference between follow-up and baseline in patients randomized to simvastatin versus difference in patients randomized to placebo.
Abbreviations: CRP = C-reactive protein, FU = follow-up, LDL-C = low-density lipoprotein cholesterol, TC = total cholesterol, HDL-C = high-density lipoprotein cholesterol, NS = not significant.

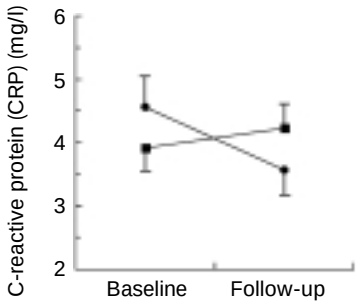

FIG. 3 Serum levels of C-reactive protein in patients randomized to simvastatin (n = 95) or placebo (n = 154) at baseline and at 4-month follow-up. Values are expressed mean ± standard error of the mean. Patients randomized to simvastatin, but not those randomized to placebo, exhibited a significant reduction (p = 0.001) of CRP serum levels. —●— = Simvastatin, —■— = placebo.

patients with acute MI, higher levels of CRP are associated with an increased mortality due to cardiac rupture, independent of infarct size.[23]

The reasons that high levels of CRP are associated with an increased risk of developing plaque instability are complex. C-reactive protein in humans is synthesized by hepatocytes mainly under stimulation of interleukin-6 and, to a lesser extent, of interleukin-1,[24, 25] predominantly released by activated inflammatory cells. Thus, elevated levels of CRP indicate enhanced cytokine production which, in turn, has the potential for activating the endothelium, transforming its antiadhesive and anticoagulant properties into adhesive and procoagulant properties.[26] Furthermore, cytokines may reduce matrix synthesis and increase its degradation thus favoring plaque rupture.[27] Finally, in endothelial cells and macrophages, cytokines may increase synthesis of the powerful vasoconstrictor endothelin, which may also enhance smooth muscle reactivity to other vasoconstrictor agents.[28]

The reason that higher levels of CRP are an independent predictor of cardiac rupture following MI is probably due again to a greater degree of activation of inflammatory cells, resulting in a more intense infiltration of leukocytes in the infarcted myocardial region which is known to be correlated with an increased risk of cardiac rupture.[29]

The causes responsible for the higher levels of CRP in patients who undergo coronary events at follow-up are still elusive. In this study, seropositivity for *Chlamydia pneumoniae* and *Helicobacter pylori* were similar in cases and controls, suggesting that these microorganisms are unlikely to be major determinants of the higher levels of CRP in high-risk patients. Our results do not confirm previous findings by Gupta *et al.*,[15] who showed an association between seropositivity for *Chlamydia pneumoniae* and major cardiac events in patients with a history of previous MI. However, there are some major differences between the two studies, including patient selection and endpoints (major cardiac events vs. overall mortality, respectively). Of note, Ridker *et al.*[30] recently failed to find any association between seropositivity for *Chlamydia pneumoniae* and risk of future MI in a prospective cohort of 15,000 healthy men. Regarding *Helicobacter pylori*, to the best of our knowledge this is the first prospective study aimed at evaluating the association between this agent and clinical outcome. Our data do not support a prognostic role for *Helicobacter* infection in patients with stable coronary artery disease. It is worth noting, however, that we are aware that seropositivity for IgG does not necessarily reflect active infection, but is only a marker of previous exposure to the infectious agent.

Furthermore, to the best of our knowledge this is the first study to assess the predictive value of anti-oxLDL antibody in patients with stable ischemic heart disease. The lack of differences in anti-oxLDL antibody levels (assessed using three different oxidative agents) between cases and controls suggests that the humoral autoimmune response triggered by oxLDL also is unlikely to be responsible for the higher levels of CRP in cases and is not associated with a worse prognosis. Accordingly, we have shown in a recent study[31] that anti-oxLDL antibody titers reflect the atherosclerotic burden, rather than disease activity. Taken together, our findings suggest that an autoimmune response to oxLDL is associated with atherosclerosis, but it is unlikely to be a prevalent trigger of acute coronary syndromes.

In this study, simvastatin treatment resulted in a significant, albeit small reduction of CRP serum levels; the reduction was similar to that observed with pravastatin in the Cholesterol And Recurrent Events (CARE) study[32] (about 20% in both studies). Simvastatin might directly inhibit the hepatic synthesis of CRP. Alternatively, experimental studies suggest that statins, by inhibiting endogenous cholesterol synthesis in macrophages, may have the potential for reducing lymphocytes and macrophage activation, inhibit the expression of proinflammatory cytokines, and modulate the immune function in vitro.[11, 12] Accordingly, in a recent clinical study statins were found to reduce the expression of adhesion molecules on circulating monocytes in humans;[13] yet, simvastatin failed to reduce the enhanced risk of mortality conferred by higher CRP levels at baseline. Therefore, CRP levels at baseline do not allow us to identify which patients are susceptible to the beneficial effects of simvastatin on mortality observed in the 4S study.[9] The reasons that the reduction of CRP levels caused by simvastatin did not translate into a reduction of the risk of mortality in patients with higher CRP levels at baseline cannot be ascertained by the results of our study. Of note, in a case-control study, Ridker *et al.* compared prerandomization serum CRP levels in 391 patients with previous MI enrolled in the CARE trial, who had cardiac events, and in matched controls;[10] the authors found that the beneficial effects of treatment with pravastatin were predominantly present in patients with raised CRP levels at baseline. More recently, in a trial of primary prevention, Ridker *et al.* found a beneficial effect of lovastatin treatment in patients with high CRP levels even in the absence of hyperlipidemia.[33] The differences between our findings and those of Ridker *et al.* might be related to different endpoints (death and nonfatal MI vs. death, respectively) or, possibly, to the use of different statins (pravastatin or lovastatin vs. simvastatin, respectively).

Conclusion

C-reactive protein serum levels, but neither seropositivity for *Chlamydia pneumoniae* or *Helicobacter pylori*, nor anti-oxLDL antibody levels, predict the risk of death in stable ischemic heart disease. Simvastatin treatment reduces CRP serum levels, but without affecting the increased risk conferred by higher CRP levels at baseline.

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