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# Reduced serum levels of autoantibodies against monomeric C-reactive protein (CRP) in patients with acute coronary syndrome

Jonas Wetterö, Lennart Nilsson, Lena Jonasson and Christopher Sjöwall

## Abstract

## Introduction

Inflammation is pivotal in atherosclerosis. Minor C-reactive protein (CRP) response reflects low-grade vascular inflammation and the high-sensitivity CRP test with levels  $\geq 3.0$  mg/l predicts coronary vascular events and survival in angina pectoris as well as in healthy subjects. We and others recently reported autoantibodies against monomeric CRP (anti-CRP) in rheumatic conditions, *e.g.* systemic lupus erythematosus (SLE), and a connection between anti-CRP and cardiovascular disease in SLE has been suggested.

## Patients and methods

Anti-CRP serum levels were determined with ELISA in 140 individuals; 50 healthy controls and 90 patients with angiographically verified coronary artery disease of which 40 presented with acute coronary syndrome (ACS) and 50 with stable angina pectoris (SA).

## Results

Significantly lower anti-CRP levels were observed in ACS compared to SA and controls ( $p = 0.019$ ). ACS patients, who had not been prescribed statins before their respective cardiovascular event, had lower anti-CRP ( $p = 0.049$ ). BMI correlated directly to anti-CRP levels in cross section analysis ( $p = 0.043$ ), but there was no association between anti-CRP and smoking or cholesterol.

## Discussion

In ACS, it is plausible that ruptured plaques and inflamed tissue may be more prone to opsonization by monomeric CRP leading to consumption of anti-CRP. Hypothetically, surface-bound anti-CRP could thereby enhance the local inflammation in plaques.

## 1. Introduction

Inflammation plays a key role in the pathogenesis of atherosclerosis [1]. It is detectable in the plaque as well as systemically with changes in peripheral blood mononuclear cells, *e.g.* expansion of CD4+ T cells. The disease process is further associated with local formation of modified self antigens, for instance oxidized low-density lipoproteins (LDL), that are targeted by both innate and adaptive immune responses [2, 3]. Some studies have demonstrated that high concentrations of autoantibodies against oxidized LDL epitopes predict the progression of carotid disease and the risk of myocardial infarction, but their association with coronary artery disease (CAD) is a matter of debate [3, 4].

A rapid onset of the acute-phase response is common in acute CAD while slighter elevations of acute phase reactants, such as the pentraxin protein family member C-reactive protein (CRP), are found in chronic or stable CAD [1, 5-7]. Numerous studies have established the high-sensitivity CRP test with levels  $\geq 3.0$  mg/l as an independent biochemical marker in the prediction of future coronary vascular events and survival both in patients with CAD and in healthy subjects [8].

Systemic lupus erythematosus (SLE) is a multi-organ inflammatory disorder mainly affecting women and is associated with high cardiovascular morbidity and mortality [9]. CRP is a useful marker of inflammation in many conditions; in SLE, however, CRP can be surprisingly low despite evident inflammatory activity [10]. We and others have demonstrated the presence of IgG class autoantibodies against CRP (anti-CRP) in SLE and in some other rheumatic conditions [11, 12]. The anti-CRP antibodies are targeted against the monomeric and tissue-bound form of CRP [13]. Disease activity in SLE is associated with raised anti-CRP and high levels may also indicate renal involvement [10, 14].

It was recently suggested that anti-CRP antibodies could play a role in the atherosclerotic process, at least in SLE [15]. However, after having studied patient data based on medical records (history of vascular disease etc.), Rosenau et al. recently reported no apparent association between history of atherosclerotic cardiovascular disease and anti-CRP in patients without rheumatic disease [16]. Hence, the aim of this study was instead to investigate the presence of anti-CRP antibodies in a well-characterized patient material with sera from patients suffering from acute coronary syndrome (ACS) or stable angina pectoris (SA).

## **2. Patients and methods**

### **2.1. Subjects**

We studied a total of 90 patients with angiographically verified CAD, 40 with ACS (8 women, 32 men) and 50 (11 women, 39 men) with SA. The SA patients had effort-related angina in accordance with the Canadian Cardiovascular Society functional classes I and II without any worsening of symptoms for the latest 6 months. ACS patients were included if they had a diagnosis of unstable angina/non-ST elevation myocardial infarction on the basis of typical ECG-changes (ST-T segment depression and/or T-wave inversion) and/or elevated troponin T. Patients were excluded if they were older than 70 years, had diabetes, severe heart failure, immunologic disorders, neoplastic disease, evidence of acute or recent (< 2 months) infection, recent major trauma, surgery or revascularization procedure or treatment with immunosuppressive or anti-inflammatory agents (except low-dose aspirin). Fifty individuals (8 women, 42 men) of equivalent age, randomly selected from the population register, served as controls. They had to be anamnestically healthy without taking any medication and with normal routine laboratory tests. Blood was obtained by vein

puncture in the morning after a 12 h fast and serum was separated. In ACS patients, blood samples were drawn within 2–7 days (median of 6) after onset of symptoms and always prior to coronary intervention. All sera were kept frozen at – 20 °C until analyzed.

## **2.2. Flow cytometry analysis**

Leukocyte subpopulations were measured by two- or three-colour flow cytometry using FACS Calibur (BD Biosciences, San José, CA). Monoclonal antibodies against CD3, CD4, CD8, CD19, CD16 and CD56 were purchased from BD Biosciences. The antibodies were marked with one of three fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP). The cells were identified by combinations as follows: CD3 (FITC)/CD4 (PE)/CD8 (PerCP) (T helper cells and cytotoxic T cells), CD19 (PE) (B cells) and CD3 (FITC)/CD16 (PE)/CD56 (PerCP) (NK cells). A sample of 10 µl of each monoclonal reagent triple was added to 50 µl of whole blood in 12 × 75 test tubes, centrifuged gently and incubated at room temperature for 15 min in the dark. Red blood cells were lysed using 100 µl of Optilyse® B lysing solution (Immunotech, Beckman Coulter AB, Bromma, Sweden), containing less than 5% formaldehyde and incubated for 15 min at room temperature in the dark. The cells were washed twice with phosphate-buffered saline with 0.4% human serum albumin, resuspended in wash buffer and analyzed immediately. Data were analyzed using CELL Quest Pro software, Version 4.0.2 (BD Biosciences). NK cells were subgrouped into CD56<sup>bright</sup> or CD56<sup>dim</sup> cells according to the level of CD56 expression and further defined by CD16+ (mainly in the CD56<sup>dim</sup> population) and CD16– (mainly in the CD56<sup>bright</sup> population). Control of the instrument settings was performed daily with CaliBRITE™ 3 Beads (BD Biosciences) and FACSCOMP™ software according to the standard procedure. The laboratory is certified for leukocyte immunophenotyping and has participated in external quality programs, such as the United Kingdom National External Quality Assessment Scheme.

## **2.3. Measurement of anti-CRP antibodies and CRP**

IgG anti-CRP antibodies were measured with ELISA exactly as described previously [14]. To avoid systematic errors, samples from patients and from controls were always randomly mixed on the microtitre plates and analyzed at the same occasion. A positive reference sample from an SLE patient at flare was used as positive control.

Serum samples were assayed for C-reactive protein (CRP) by a highly sensitive latex-enhanced turbidimetric immunoassay with a lower detection limit of 0.03 mg/l (Roche Diagnostics GmbH, Vienna, Austria). The overall interassay coefficient of variation was 1.7%.

## **2.4. Cytokine detection**

Serum samples were also assayed for interleukin (IL)-6 using a highly sensitive ELISA immunoassay (Quantikine HS, R&D Systems Europe Ltd., Abingdon, Oxon, UK). The lower limit of detection for IL-6 was 0.039 pg/ml. The overall interassay coefficient of variation was 8.5%.

## 2.5. Statistical analysis

Data were analyzed using the SPSS for Windows version 15.0.0 (SPSS Inc.) and KaleidaGraph version 4.03 (Synergy Software). Differences between the three patient groups were examined using Kruskal–Wallis rank sum test. Correlational analysis was performed using Pearson  $r$ -correlation coefficient. For all base characteristics, the significance of any difference in means between patients and controls was tested by Student's  $t$ -test. Two-tailed  $p$ -values  $< 0.05$  were considered significant.

## 2.6. Ethics

Informed consent was obtained from all subjects. The research protocol was approved by the locally appointed ethical committee.

## 3. Results

The study population (ACS patients, SA patients and controls) is characterized in Table 1. Ninety percent of the SA patients and 93% of the ACS patients received  $\beta$ 1-receptor blockers. Ninety-six percent of the SA patients and 35% of the ACS patients were on long-term treatment, *i.e.*  $> 2$  months with the statin (3-hydroxy-3-ethylglutaryl coenzyme A reductase inhibitor) simvastatin (20–40 mg daily). In all ACS patients, therapy with aspirin, clopidogrel and low-molecular weight heparin was introduced on admission; 76% of the SA patients had low-dose aspirin, 6% had clopidogrel and 16% received a combination of low-dose aspirin and clopidogrel. Controls were all free from medication.

Anti-CRP antibody levels were determined in serum samples from all 140 individuals. As shown in Fig. 1, significantly lower anti-CRP antibody levels were observed in the ACS group compared to both the SA group and the controls ( $p = 0.019$ ). Two outliers were found, one with ACS and one control. When these two samples were excluded the significance was even stronger ( $p = 0.0089$ ). Within the ACS group, anti-CRP levels were equally decreased among unstable angina and non-ST elevation myocardial infarction patients ( $p = 0.24$ ).

The 32 male patients with ACS had significantly lower anti-CRP antibody levels than the 39 male patients in the SA group and the 42 male controls ( $p < 0.02$ ). No significant differences were found in females, but the groups here were small. Overall, no difference was found in anti-CRP antibody levels between men and women; this is in line with our previous findings [11, 14].

There were no significant differences in anti-CRP antibody levels between smokers and non-smokers in any group or when all samples were analyzed in total. In the ACS group, however, there was a tendency towards lower anti-CRP antibody levels in smokers ( $p = 0.091$ ). Anti-CRP antibody levels were not associated with age. Body mass index (BMI) correlated directly with anti-CRP antibody levels when analyzed in cross section ( $r = 0.18$ ,  $p = 0.043$ ); separate analyses for each group gave tendencies in the ACS group ( $p < 0.06$ ) and in the controls ( $p < 0.08$ ), but not in the SA group.

Table 1: Characteristics of patients and controls

	ACS patients	SA patients	Controls
Age (years)	59.2 (6.7)	59.3 (5.3)	60.8 (4.4)
Female/male (n)	8/32	11/39	8/42
BMI (kg/m <sup>2</sup> )	27.1 * (3.6)	27.1 * (3.9)	25.3 (2.9)
Blood pressure			
Systolic (mm Hg)	137.0 (18.6)	132.2 (17.7)	132.3 (15.9)
Diastolic (mm Hg)	80.1 (11.1)	80.9 (6.8)	81.1 (7.9)
Current smokers (%)	40	22	20
Laboratory variables			
Total cholesterol (mmol/l)	5.1 * * * (0.8)	4.7 * * * (0.83)	6.1 (1.0)
LDL cholesterol	3.2 * * * * * (0.8)	2.6 * * * (0.6)	4.0 (0.9)
HDL cholesterol	1.2 * * * (0.3)	1.2 * * (0.3)	1.5 (0.5)
Triglycerides	1.9 (0.9)	1.8 * * (1.1)	1.3 (0.5)
Blood glucose	4.8 (0.9)	4.9 (0.7)	4.9 (0.7)
C-reactive protein (mg/l)	4.6 * * * * * (4.5)	1.7 (1.9)	2.3 (1.7)
IL-6 (pg/ml)	5.8 * * * * * (6.2)	2.5 (1.5)	2.9 (2.7)
Prior history of CAD			
Percutaneous coronary intervention (%)	18	72	–
Coronary artery bypass (%)	3	32	–
Prior ACS (%)	15	76	–
> 2 significant stenoses	68	76	–
Elevated troponin T ( $\geq 0.05 \mu\text{g/l}$ ) (%)	70	–	–

Values are given as mean (S.D.).

\*  $p < 0.05$  compared with controls.

\* \*  $p < 0.01$  compared with controls.

\* \* \*  $p < 0.001$  compared with controls.

\* \* \* \*  $p < 0.001$  compared with SA.

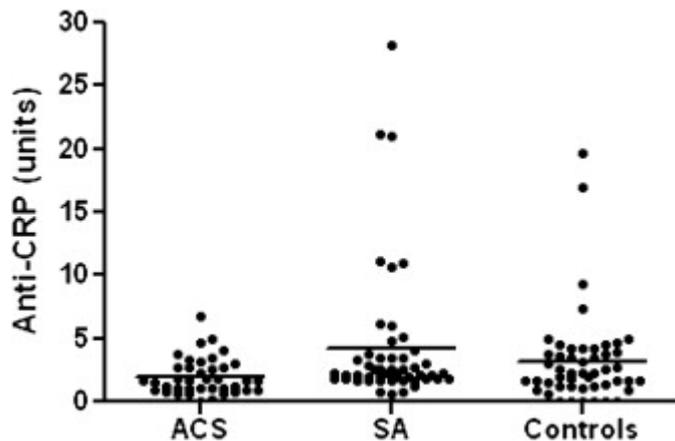


Fig. 1. Distribution with mean values of anti-CRP antibodies in acute coronary syndrome (ACS), stable angina pectoris (SA) and controls. Two outliers were excluded from the figure; one in the ACS group (69 units) and one in the control group (120 units). Comparing all samples ( $n = 140$ ), significantly lower anti-CRP antibody levels were seen in ACS than in SA and controls ( $p = 0.019$ ).

In the ACS group, a significant direct correlation between levels of triglycerides and anti-CRP ( $r = 0.43$ ,  $p = 0.006$ ) was recorded. However, when analyzing all samples in cross section this correlation disappeared. No associations between anti-CRP and total, LDL or HDL cholesterol were seen. In the controls, direct correlations between anti-CRP antibody levels and diastolic ( $r = 0.34$ ,  $p = 0.017$ ), as well as systolic ( $r = 0.33$ ,  $p = 0.018$ ), blood pressure were observed.

Patients in the ACS group, which had not been prescribed simvastatin before their respective cardiovascular event, had significantly lower anti-CRP antibody levels than corresponding patients in the SA group and controls ( $p = 0.049$ ). When comparing anti-CRP antibody levels in patients with long-term use of simvastatin, a tendency towards lower anti-CRP levels in ACS compared to SA was found ( $p = 0.067$ ). However, in cross section analysis with all 140 samples, individuals taking simvastatin regularly did not have different anti-CRP levels than individuals without use of statins.

Correlational analyses between anti-CRP antibody levels and leukocyte cell subsets (CD3+, CD4+, CD8+, CD4/CD8 quotient, CD3-CD56+, B cells, granulocytes, lymphocytes, monocytes) and IL-6 levels yielded no significant correlations. Neither did the levels of anti-CRP and native CRP correlate, which also is in line with previous findings [11, 12, 14].

#### 4. Discussion

In this study we show lower anti-CRP antibody levels in ACS than in SA and controls. CRP and its connection to atherosclerosis have met considerable interest over the last decade. The reason for this is not only that CRP levels correlate well with the occurrence of cardiovascular disease, but also that the biological functions of CRP are being better understood. CRP binds only to Fc $\gamma$  receptor-bearing cells and, in general, to apoptotic and damaged cells expressing phosphorylcholine. Furthermore, CRP activates complement via C1q, binds to modified forms of LDL, and is deposited together with LDL, complement and damaged cells at the atherosclerotic lesions and myocardial infarcts [1, 10].

SLE is an autoimmune disease associated with autoantibodies to antinuclear antibodies, antibodies against cytoplasmic and extracellular antigens, including plasma proteins [9]. These patients have a high risk of developing cardiovascular events that cannot be explained by traditional risk factors such as smoking, diabetes or hyperlipemia [17]. O'Neill et al. recently suggested that autoantibodies against CRP could be the missing link between increased cardiovascular risk and SLE [15]. In line with this, Rosenau et al. investigated 150 sera from patients with or without a history of atherosclerotic cardiovascular disease and found no association with anti-CRP [16]. We have identified important differences between the study by Rosenau and our study that could explain the different results. In contrast to the present study where diagnosis was confirmed by coronary angiogram, Rosenau et al. relied solely on medical records for the diagnosis of 'clinical atherosclerosis'. Most obviously, the authors did not study patients with ACS, but rather patients with previous cardiovascular events and/or peripheral vascular disease and/or SA. In addition, Rosenau et al. used bovine serum albumin (BSA) to block their anti-CRP antibody assay from unspecific binding [12, 16]. This could in fact introduce a specificity bias since anti-BSA antibodies, which commonly occur in healthy individuals, will also be detected and could give rise to falsely positive results [18]. Thus, at our laboratory we do not use any agent to block the anti-CRP assay, but instead we use lower concentration of the conjugated secondary antibody.

To our best knowledge, anti-CRP has not previously been studied in well-characterized patients with CAD. The main finding is that anti-CRP antibody levels were significantly lower in ACS than in SA and controls. Whether or not this is a difference of pathogenic importance is open for speculation. Lowered anti-CRP levels in the ACS group could be secondary to tissue breakdown and inflammation caused by the myocardial infarction *per se*. However, we did find equally decreased anti-CRP levels in unstable angina patients and patients with non-ST elevation myocardial infarction. This indicates that changes in anti-CRP seen in the ACS group might be viewed as part of the plaque destabilisation process rather than a phenomenon secondary to tissue damage. Furthermore, decreased antibody levels in ACS were not related to systemic inflammatory activity (such as CRP and IL-6). Other correlational analyses were not clear-cut and are somewhat difficult to interpret in the relatively small subgroups. Long-term treatment with statins (simvastatin) seemed to eliminate the statistically significant reduction in anti-CRP antibody levels comparing ACS and SA which is interesting regarding the proposed immunomodulatory effects mediated by statins [19].

Elevated anti-oxLDL levels have been reported in ACS [3, 20]. However, interestingly Schumacher et al. reported reduced levels of autoantibodies against oxidized LDL in individuals with severe myocardial infarction (CK-MB-mass values > 70 ng/ml) compared to milder myocardial infarction [21]. Ruptured plaques and inflamed vascular tissue may be more prone to expose monomeric CRP on their tissue surfaces which then would result in consumption of anti-CRP autoantibodies leading to reduced circulating levels. Furthermore, one intriguing speculation is that surface-bound anti-CRP antibodies could further enhance the local inflammation with activation of the complement cascade. We believe that the role of anti-CRP in ACS deserves further exploration.

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