

High prevalence of autoantibodies to C-reactive protein in patients with chronic hepatitis C infection: association with liver fibrosis and portal inflammation

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Full-length article

Title: High prevalence of autoantibodies to C-reactive protein in patients with chronic hepatitis C infection: Association with liver fibrosis and portal inflammation

Abbreviated title: Anti-CRP and histopathology in hepatitis C

Authors: Christopher Sjöwall ^{a,*}, Kristina Cardell ^b, Elisabeth A Boström ^c, Maria I Bokarewa ^c, Helena Enocsson ^a, Mattias Ekstedt ^d, Liselott Lindvall ^b, Aril Frydén ^b, Sven Almer ^d

^a Rheumatology/AIR, Department of Clinical and Experimental Medicine, Linköping University,

^b Infectious Diseases, Department of Clinical and Experimental Medicine, Linköping University,

^c Department of Rheumatology and Inflammation Research, University of Gothenburg,

^d Gastroenterology and Hepatology, Department of Clinical and Experimental Medicine, Linköping University, Sweden.

*** Corresponding author at:**

Rheumatology Unit, University Hospital, SE-581 85 Linköping, Sweden.

E-mail address: christopher.sjowall@liu.se

Telephone +46 10 1032416

Fax: +46 10 1031844

Abbreviations: AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMA, anti-mitochondrial antibodies; ANA; anti-nuclear antibodies; AST, aspartate aminotransferase; BSA, bovine serum albumin; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; GGT, gamma-glutamyl transferase; HC, healthy controls; HCV, hepatitis C virus; IFN, interferon; LD, lactate dehydrogenase; NAFLD, non-alcoholic fatty liver disease; OD, optical density; PK-INR, prothrombin complex international normalized ratio; RF, rheumatoid factor; SLE, systemic lupus erythematosus; SMA, smooth muscle antibodies; SVR, sustained virological response

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Abstract (200 words)

The presence of autoantibodies against C-reactive protein (anti-CRP) has been reported in association with autoimmunity and histopathology in chronic hepatitis C virus (HCV) infection. Resistin could play a role in the pathogenesis of hepatitis, although results on HCV infection are ambiguous. Here we retrospectively analyzed anti-CRP and resistin levels in sera of 38 untreated and well-characterized HCV patients at the time-point of their first liver biopsy. HCV activity and general health were assessed by a physician at least yearly until follow-up ended. Anti-CRP and resistin were also measured in patients with autoimmune hepatitis (AIH) and non-alcoholic fatty liver disease (NAFLD). Anti-CRP antibodies were registered in all HCV patients whereas only a few of AIH (11%) and NAFLD (12%) sera were positive. Anti-CRP levels were related to histopathological severity being highest in patients with cirrhosis at baseline. Resistin levels were similar in HCV, AIH and NAFLD patients, but high levels of resistin were associated with early mortality in HCV patients. Neither anti-CRP nor resistin predicted response to interferon-based therapy, cirrhosis development or were associated with liver-related mortality. We conclude that anti-CRP antibodies are frequently found in chronic HCV infection and could be a useful marker of advanced fibrosis and portal inflammation.

Keywords: Hepatitis C; Autoantibodies; C-reactive protein; Resistin; Interferon- α

1. INTRODUCTION

The hepatitis C virus (HCV) is a linear, single-stranded RNA virus with extensive genomic variability that infects both the liver and lymphoid cells [1, 2]. Globally, an estimated number of 180 million people are chronically infected with HCV and 3–4 million are newly infected each year [1]. Persistent infection occurs in 50–80% of those infected and may lead to the development of cirrhosis and subsequent hepatocellular carcinoma, whereas successful combined therapy with interferon (IFN) α and ribavirin significantly reduces the risk for long-term complications [1]. The overall sustained virological response (SVR) has increased from 15–20% with IFN- α alone to 75–80% with modern combination therapy, although differences in response rate with regard to HCV genotype are considerable [3].

In a prospective study of HCV patients, liver fibrosis was shown to be an independent predictor of liver-related mortality [4]. Deposition of fibrillar extracellular matrix, altered hepatocyte regeneration, deranged microvascular architecture ultimately leads to cirrhosis, which is the major determinant of morbidity and mortality in patients with liver disease, predisposing to liver failure and hepatocellular carcinoma [5]. Although liver biopsy still remains the gold standard to assess hepatic fibrosis, it would be desirable to enable monitoring and prediction of the fibrotic process with non-invasive methods. Transient elastography may possibly be an important instrument in the future, but presently its precise role in the evaluation of patients with viral hepatitis remains to be established [6, 7].

The prevalence of immune-mediated extra-hepatic manifestations, such as Sjögren-like syndrome, mixed cryoglobulinemic vasculitis, non-erosive arthritis and myalgia is significant in patients with chronic HCV infection, but may not be associated with liver disease severity or gender [2, 8]. Autoantibodies like rheumatoid factor (RF), anti-nuclear (ANA), anti-mitochondrial antibodies (AMA), anti-liver-kidney-microsomal and smooth muscle antibodies (SMA) are commonplace and can be found both in conjunction with and without autoimmune manifestations [8, 9]. In addition, the only conceivable treatment used for HCV infection, *i.e.* interferon-based therapy, can in itself

be associated with side effects such as autoimmune thyroiditis, cytopenia, fever, alopecia and lupus-like syndromes [10].

The presence of autoantibodies directed against the acute-phase reactant C-reactive protein (CRP) was first described in a patient with systemic lupus erythematosus (SLE) [11]. Since then, several groups have confirmed the finding of IgG class autoantibodies to CRP (anti-CRP) in SLE [12–14]. Analyses of antigen specificity of the anti-CRP assay used have clearly revealed that anti-CRP in SLE are directed against monomeric CRP, which is assumed to be the tissue-deposited form of the acute-phase reactant. Hypothetically, anti-CRP could have nephritogenic properties in lupus by interfering with the physiological CRP-mediated removal of immune complexes and/or nuclear constituents [15]. One study has addressed the presence of anti-CRP antibodies in patients with chronic HCV infection [16]. Interestingly, this group found raised anti-CRP antibodies in 17% of the HCV patients, and the levels were associated with presence of RF and cryoglobulinemia, but not with ANA or anti-cardiolipin antibodies. In addition, liver biopsies from anti-CRP positive individuals were shown to have a greater liver disease severity, whereas the presence of anti-CRP did not discriminate between patients with and without cirrhosis [16].

The adipokine family member resistin, a 12.5 kDa cysteine-rich peptide of 108 amino acids with inter-cellular signalling activity, is secreted by mononuclear cells and neutrophils following proinflammatory stimuli, *e.g.* endotoxins, IL-6 or TNF [17]. Low levels of resistin are expressed in hepatocytes [18] and the production of resistin can be induced through a NF- κ B-dependent cascade in human stellate cells and in a Hep G2 cell line [19]. The observation of the regulatory effect of resistin on carbohydrate metabolism in mice [20] initiated attempts to identify resistin-dependent mechanisms of fat deposition and insulin resistance in humans [18]. In fact, resistin has been described as a biomarker of insulin resistance and cirrhosis related to metabolic liver disease [21], but this has also been a matter of debate [22, 23]. Studies on resistin in chronic HCV infection and its potential association with fibrosis have partially shown conflicting results [24–26].

The aims of the present study were (i) to investigate anti-CRP and resistin levels in patients with chronic HCV infection and compare it to patients with non-infectious liver diseases and healthy controls; (ii) to evaluate an impact of anti-CRP and resistin in histological signs of inflammation and fibrosis, inflammatory activity, production of other autoantibodies, HCV genotypes and virus levels; and (iii) to examine the predictive potentials of anti-CRP and resistin for the response to interferon-based therapy, cirrhosis development and mortality.

2. MATERIAL AND METHODS

2.1. Subjects

Between October 1987 and November 2000, 38 patients diagnosed with chronic hepatitis C infection based on clinical, serological, biochemical, histological and genotypical findings were followed in a clinical control program at the Clinic of Infectious Diseases, Linköping University Hospital. Venous blood was drawn when patients underwent their first liver biopsy and were considered for treatment with IFN- α in monotherapy or in combination with ribavirin. Sera were kept frozen at -70°C until analyzed.

Approximately 50% of the subjects had been infected through blood transfusions. 25/38 (66%) patients received therapy that was recommended by national and local guidelines at the time. HCV-RNA was analyzed at baseline, at 12 weeks of treatment and at 6 months after completed treatment. SVR was defined as negative HCV-RNA 6 months post treatment. None of the treated patients used corticosteroids or other immunomodulators whereas 2 of the non-treated patients used subcutaneous immunoglobulins for hypogammaglobulinemia. Regardless of whether active treatment with IFN- α \pm ribavirin was initiated or not, all patients were followed yearly by a physician.

A systematic review of medical records was carried out in January 2011, particularly with regard to cirrhosis and liver-related mortality. All patients that had developed clinical symptoms of cirrhosis underwent a second liver biopsy. The mean time from blood sampling to follow-up was 17.0 years (range 1.4–23.1); in fact, all but 3 patients (92%) had a follow-up time of >10 years. Further characteristics of the HCV patients are given in Table 1.

For comparative analyses, sera from patients with non-infectious liver diseases were collected at the Clinic of Gastroenterology and Hepatology, Linköping University Hospital. Thus, samples from 46 patients (11 men, 35 women; mean age 59.7 years; range 23–83) with a diagnosis of autoimmune hepatitis (AIH) based on the revised AIH-score were enrolled [27]. In addition, sera from 51 patients (38 men, 13 women; mean age 61.0 years; range 39–80) diagnosed with non-alcoholic fatty liver disease (NAFLD) based on the presence of biopsy-proven hepatic steatosis, self-reported alcohol consumption ≤ 140 g/week and no evidence of any other liver disease or medication

associated with fatty infiltration of the liver were included [28].

Finally, 2 different groups of apparently healthy controls without ongoing medication served as reference; 100 for the anti-CRP antibody analyses (50 men, 50 women; mean age 45.8 years; range 22–70) and 40 individuals for the resistin analysis (10 men, 30 women; mean age 56.3 years; range 24–67).

Table 1. Baseline characteristics of the 38 HCV patients. HCV genotypes were available in 32 individuals. Data in brackets are given as standard deviations or percent (where indicated).

Characteristic	
Female/male	14 (37%) / 24 (63%)
Age (years), mean	44.0 (17.2)
HCV disease duration (years), mean	17.0 (7.0)
* Previous coronary bypass surgery	5 (13%)
Previous myocardial infarction	3 (8%)
Type-2 diabetes	1 (3%)
Co-infection with hepatitis B	1 (3%)
Serological signs of cleared hepatitis B infection	8 (21%)
Patients treated with interferon- α \pm ribavirin	25 (66%)
Cirrhosis (fibrosis stage 4) at baseline	5 (14%)
HCV genotype 1a	8 (25%)
HCV genotype 1b	3 (9%)
HCV genotype 2b	9 (28%)
HCV genotype 3a	12 (38%)
HCV virus levels ($\times 10^6$ IU/mL), mean	2.7 (3.8)

* Whereof one patient with previous myocardial infarction.

2.2. Biochemical parameters

Biochemical assessment included albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), CRP, lactate dehydrogenase (LD), gamma-glutamyl transferase (GGT), bilirubin, prothrombin complex international normalized ratio (PK-INR) and platelet count.

2.3. Serotyping, RNA detection/quantification and genotyping

HCV serology. The first generation anti-HCV enzyme-linked immunosorbent assay (ELISA; Ortho, Raritan, NJ, USA) was used 1990–1991 and then replaced by the second-generation anti-HCV ELISA test (Ortho). The third generation ELISAs were introduced in 1994 (Ortho) and from 1997 anti-HCV Microparticle Enzyme Immuno Assay (Abbott, Abbott Park, Chicago, IL, USA) were used. Confirmation was performed with a RIBA assay.

HCV-RNA detection. RNA was extracted from plasma and initially analyzed with an in-house nested PCR, which was succeeded by Cobas Amplicor Hepatitis C Virus Test version 2.0 (Roche Diagnostics, Branchburg, NJ, USA).

HCV-RNA quantification. HCV-RNA was quantified by the branched DNA technique (Quantiplex HCV RNA version 2.0; Chiron Corp, Emeryville, CA, USA).

HCV genotyping. HCV genotypes were determined after hybridisation of biotin-labelled PCR products to oligonucleotide probes bound in strips on nitrocellulose membranes (Inno-Lipa, Innogenetics, Brussels, Belgium).

2.4. Histopathology

36/38 (95%) HCV patients underwent liver biopsy performed by percutaneous puncture according to a standard protocol. Global inflammation grade (0–4) and fibrosis stage (0–4) was assessed according to the standardized semiquantitative histological scoring system developed by Batts and Ludwig [29]. Portal inflammation was reported separately (0–4), as described by Knodell *et al.* [30].

2.5. Indirect immunofluorescence microscopy

ANA were analyzed with indirect immunofluorescence technique using multispot slides with fixed HEP-2 cells (ImmunoConcepts, Sacramento, CA, USA) as substrate. AMA and

SMA were detected with indirect immunofluorescence technique using rat kidney, stomach and liver slides (Inova Diagnostics, San Diego, CA, USA) as substrate. Briefly, sera serially diluted 1:200 in phosphate-buffered saline (PBS, pH 7.4) were applied to each slide and kept incubated in a moist chamber for 30 min at room temperature. After 10 min washing in PBS, fluorescein-isothiocyanate (FITC) conjugated gamma-chain-specific rabbit antihuman-IgG (Dako, Glostrup, Denmark) was applied (at an optimal dilution decided by checkerboard titration) for 30 min. The slides were washed with PBS, mounted with PBS-buffered glycerine, and inspected under a Nikon fluorescence microscope with mercury lamp (EXFO X-Cite® 120 PC) epi-illumination and filters for FITC activation/emission. The results were judged as positive or negative, and positive results were categorized into staining patterns. Samples with immunofluorescent staining by the initial dilution were subjected to further analysis using serial dilutions (endpoint titration). Cut-off was set at dilutions $\geq 1:200$ based on the 95th percentile of the laboratory's control material from 150 healthy female blood donors [31].

2.6. Anti-nucleosome, anti-cardiolipin and anti-CRP assays

The HCV sera were analyzed with a commercial anti-nucleosome antibody assay (Quanta Lite Chromatin, Inova Diagnostics, San Diego, CA, USA) using histone-H1-stripped calf-thymocyte chromatin as antigen. Reference serum was provided with the kit, and the procedure was performed according to instructions from the manufacturer. Patient sera were diluted 1:100 and run in duplicates. The results were obtained by reading optical density in an automatic plate reader at 450 nm with 620 nm as a reference wavelength.

Detection of IgG class cardiolipin antibodies was performed using a commercial standardized ELISA (Orgentec, Mainz, Germany).

IgG anti-CRP antibodies were measured with an ELISA as previously described [32]. To avoid systematic errors, samples from all patients and controls were randomly mixed on the microtitre plates and analyzed simultaneously at one occasion. Anti-CRP antibody levels were expressed as the percentage of a positive reference sample from a SLE patient at flare representing 100 arbitrary units. The cut-off limit for positive result was calculated from the 95th percentile obtained in the controls (12 units) and results between the 95th and 98th percentile (30 units) were termed "moderately positive". The

SLE reference was always included. To exclude the possibility of non-specific binding, each serum was also tested in the same way on uncoated plates.

9 HCV sera with reactivity regarding anti-CRP antibodies were pre-incubated overnight with PBS-Tween or native CRP (Sigma, St Louis, MO, USA) at a concentration of 30 µg/mL in order to evaluate potential ability to block antibody binding to solid-phase CRP. All samples were diluted 1:50 and analyzed in quadruplicate but were otherwise treated as previously described [32].

2.7. Resistin measurements

Serum resistin concentrations were determined using quantitative sandwich ELISA (R&D Systems, Abingdon, UK) as previously described [23].

2.8. Statistics

Figures were prepared in GraphPad Prism (version 4.0; GraphPad Software Inc., San Diego, CA, USA). Correlation analyses were performed using Spearman's rank correlation and differences between groups were calculated with either Wilcoxon signed rank test, Mann-Whitney *U* test or Kruskal-Wallis test (SPSS for Windows version 15.0.0; SPSS Inc. (IBM), Chicago, IL, USA). Two-tailed $p < 0.05$ was considered significant.

2.9. Ethics

The study protocol was approved by the Ethical Committee of the Linköping University Hospital (2010/406–31).

3. RESULTS

3.1. *Increased levels of anti-CRP antibodies in HCV compared to non-infectious liver disease*

No differences in anti-CRP antibody and resistin levels were apparent considering age and gender in the healthy controls (HC). As demonstrated in Figure 1a, anti-CRP levels were significantly raised in HCV patients compared to HC (in which mean value corresponded to 5% of HCV), AIH (12%) and NAFLD (14%). All HCV sera tested achieved signals above the cut-off limit, but 8 sera (21%) were merely moderately positive (12–30 units). 5/46 (11%) of AIH sera and 6/51 (12%) of NAFLD sera were anti-CRP positive; 8/11 positive AIH/NAFLD sera were moderately positive. Anti-CRP levels were not significantly different in AIH and NAFLD, but both groups had higher levels than HC. In contrast, Figure 1b demonstrates that resistin levels were not significantly elevated in HCV compared to AIH, NAFLD or HC. AIH sera presented significantly higher resistin levels than NAFLD sera ($p < 0.01$).

3.2. *Resistin and anti-CRP in relation to biochemical parameters, autoantibodies and HCV genotypes*

Resistin levels were not associated with any biochemical parameter whereas significant correlations for anti-CRP levels were found with conjugated bilirubin ($r = 0.51$, $p = 0.03$) and CRP ($r = 0.35$, $p = 0.04$). Anti-CRP and resistin levels were not correlated ($r = 0.17$, $p = 0.31$). The capacity of native CRP to block antibody binding to microtitre plate-bound CRP was studied in anti-CRP positive HCV sera. As shown in Figure 1c, pre-incubation of these sera with soluble native CRP overnight moderately inhibited the anti-CRP antibody signal ($p = 0.004$) with a reduction ranging from 2.4–32.3%.

Autoantibody profiles of the HCV patients were as follows: 3/37 (8%) sera were ANA positive at titre 1:200; 2 with homogenous and 1 with centromere staining pattern. 2/37 (5%) sera were SMA positive at titre 1:200, whereas none were AMA positive. 9/36 (25%) sera were anti-nucleosome antibody positive; 8 with moderate and 1 with high levels. 6/35 (17%) sera were anti-cardiolipin antibody positive. Autoimmune serology, defined as the presence of at least one autoantibody (ANA, SMA, anti-cardiolipin or anti-nucleosome), were not significantly associated with anti-CRP positivity (>30 units) or resistin levels above 7 ng/mL (corresponding to median value of HC).

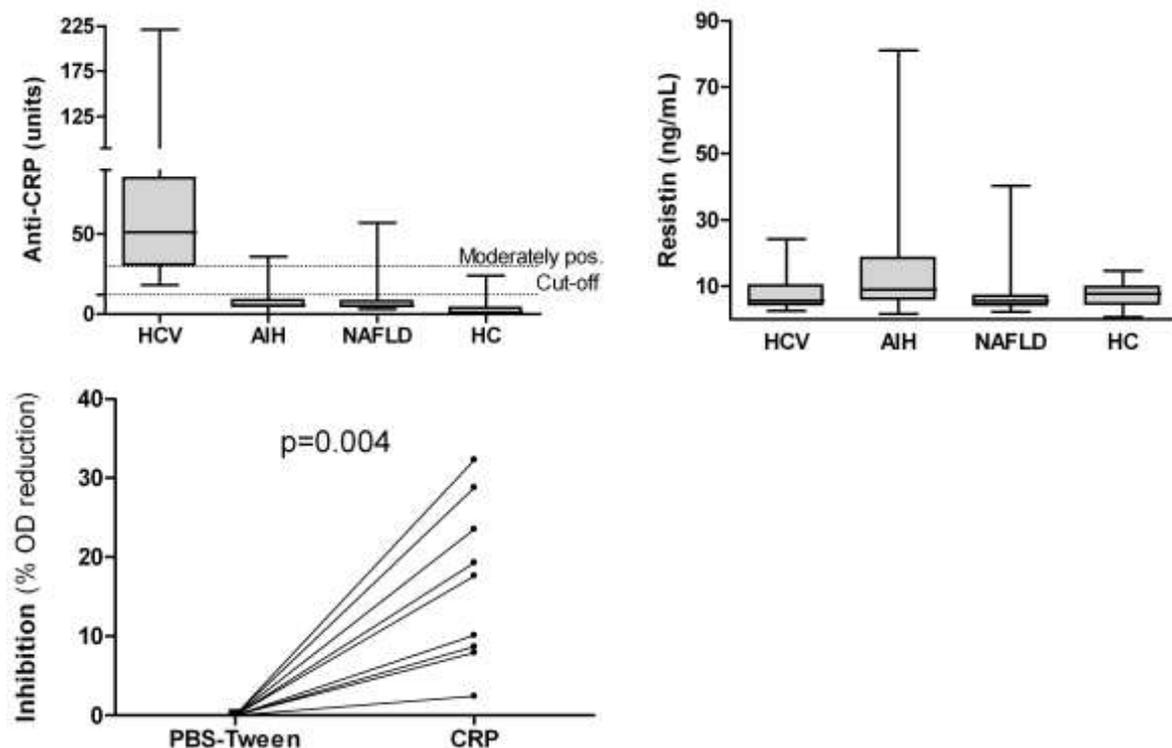


Fig. 1. Anti-C-reactive protein (anti-CRP) antibody (a) and resistin levels (b) demonstrated in patients with chronic hepatitis C virus (HCV) infection, autoimmune hepatitis (AIH) and non-alcoholic fatty liver disease (NAFLD), as well as in healthy controls (HC). Pre-incubation of 9 anti-CRP positive HCV sera with buffer or native CRP resulted in a significant reduction (Wilcoxon signed rank test) of optical density (OD) in samples pre-incubated with soluble CRP (c).

Viral genotypes were available in 32 individuals; HCV genotype 3a was the commonest and present in most of the patients that presented with cirrhosis at baseline (Table 1). None of the genotypes were associated with the levels of resistin or anti-CRP. Resistin was, however, inversely correlated with virus levels ($r=-0.50$, $p=0.017$).

3.3. Anti-CRP antibody levels are associated with liver fibrosis and portal inflammation, but do not predict development of cirrhosis or treatment response

At baseline, 5/36 (14%) patients showed histological signs of cirrhosis (fibrosis stage 4). These 5 cirrhotic patients had higher anti-CRP levels compared to non-cirrhotics (median 115 [range 32–221] vs. 45 [range 18–108] units; $p=0.04$); a similar trend was seen for resistin ($p=0.08$). In addition, anti-CRP levels correlated with the stage of fibrosis in liver biopsies (Figure 2a). Apart from anti-CRP, low platelets, raised AST and resistin were associated with advanced fibrosis at baseline (Table 2).

Table 2. Laboratory items given with mean values \pm standard deviations of 36 HCV patients that underwent liver biopsy at baseline.

	Fibrosis stage 0–2	Fibrosis stage 3–4	Mann-Whitney
Female/male	11/17	3/5	
Age (years)	41.7 (16.0)	50.0 (19.4)	n.s.
HCV disease duration (years)	8.5 (7.1)	5.4 (6.8)	n.s.
Virus levels ($\times 10^6$ IU/mL)	3.0 (4.0)	0.57 (0.5)	n.s.
Platelet count ($\times 10^9$ /L)	246 (53)	201 (71)	p=0.02
CRP (mg/L)	1.5 (1.6)	2.8 (4.8)	n.s.
AST (μ kat/L)	1.2 (0.7)	2.0 (1.3)	p=0.05
ALT (μ kat/L)	2.1 (1.8)	3.2 (2.6)	n.s.
LD (μ kat/L)	5.8 (1.4)	6.2 (1.6)	n.s.
GGT (μ kat/L)	1.1 (1.1)	2.2 (2.3)	n.s.
Unconjugated bilirubin (μ mol/L)	10.7 (6.7)	12.3 (6.3)	n.s.
Conjugated bilirubin (μ mol/L)	3.1 (2.1)	4.8 (4.7)	n.s.
Albumin (g/L)	41 (4)	40 (3)	n.s.
ALP (μ kat/L)	2.6 (0.7)	3.6 (2.3)	n.s.
PK-INR	1.0 (0.1)	1.1 (0.1)	n.s.
Anti-CRP (units)	50 (21)	98 (62)	p=0.03
Resistin, ng/mL	6.9 (4.6)	9.3 (3.4)	p=0.05
ANA \geq titre 1:200	1	2	n.s.
Anti-nucleosome (units)	12 (19)	15 (19)	n.s.
Anti-cardiolipin (units)	3.9 (0.8)	6.0 (4.4)	n.s.
Global inflammation *			
Grade 0/1/2/3/4	5/16/6/1/0	0/5/1/2/0	n.s.
Portal inflammation **			
Grade 0/1/2/3/4	0/6/16/6/0	0/0/2/4/2	p=0.006
Fibrosis stage (0–4)	11/8/9	3/5	

* According to the inflammatory component of the Batts–Ludwig scoring system [29].

** According to the Knodell scoring system [30].

n.s. = not significant

During the follow-up period, another 4 patients developed cirrhotic symptoms and the diagnosis of cirrhosis was confirmed on a second biopsy in these individuals. Although the number of patients was low, anti-CRP did not predict further development onto cirrhosis in patients with fibrosis stage 0–3 at baseline (data not shown). No significant association with any laboratory item was found with the global inflammation component of the Batts–Ludwig scoring system. Histological signs of portal inflammation were correlated with high levels of anti-CRP (Figure 2b). Portal inflammation was also associated with fibrosis stage (Table 2).

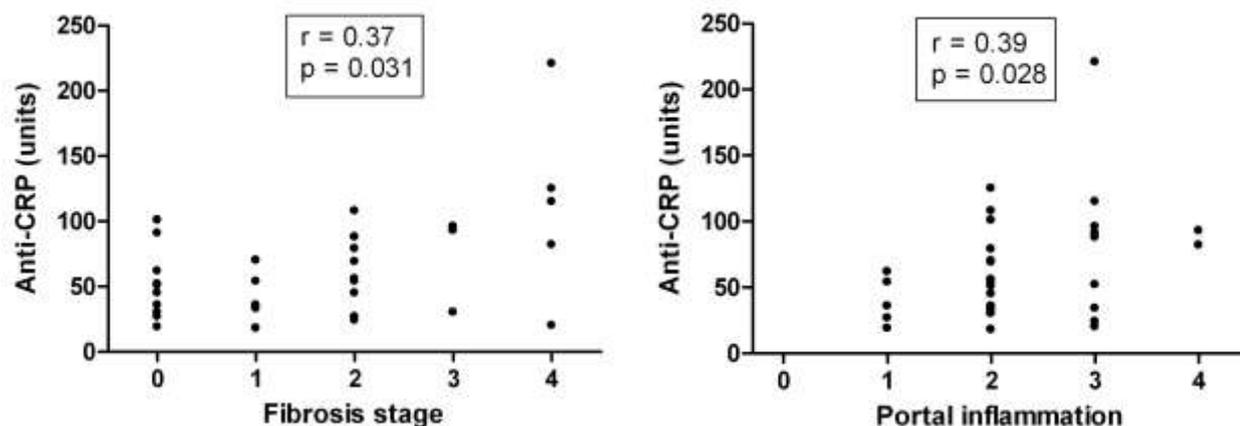


Fig. 2. Anti-C-reactive protein (anti-CRP) antibody levels were significantly correlated with stage of fibrosis (a) as well as with portal inflammation (b) using Spearman's rank correlation.

25 patients were treated with interferon-based treatment, 15 in monotherapy and 10 in combination with ribavirin. In the monotherapy group, 8 achieved SVR, 2 individuals were classified as response-relapse and 5 as non-responders. In the combination group, 8 achieved SVR, 1 individual was classified as response-relapse and 1 as non-responder. Neither anti-CRP, nor resistin, levels could predict the response to therapy (Figure 3ab). At follow-up in January 2011, 9 (24%) individuals were deceased. Mean time of estimated HCV disease duration in these patients was 14.8 years (range 4–23); the cause of death was not classified as liver-related in any case. All 9 patients had received interferon-based therapy, but only 1 in combination with ribavirin; 4 had achieved SVR. 3 of the deceased individuals had cirrhosis at baseline. Resistin levels were significantly higher ($p=0.05$) in deceased patients compared to others whereas anti-CRP levels were similar.

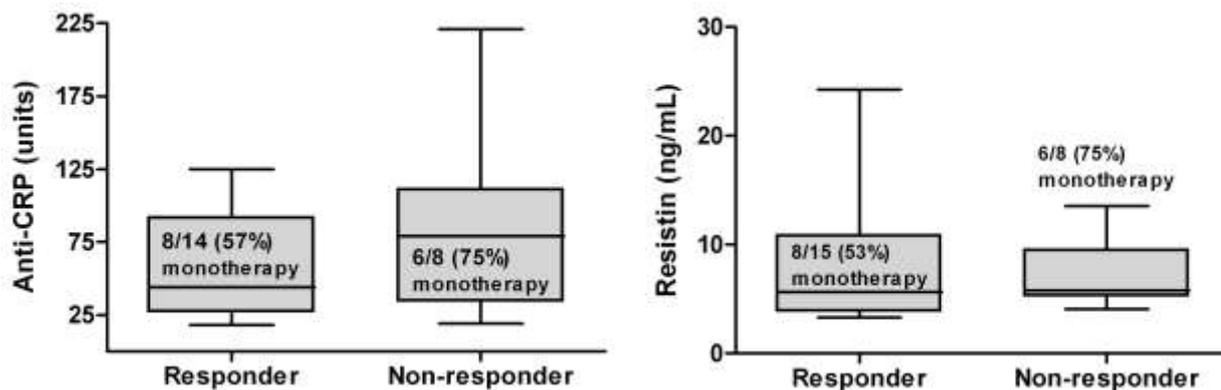


Fig. 3. Baseline anti-C-reactive protein (anti-CRP) antibody and resistin levels demonstrated in HCV patients with/without sustained viral response. Median anti-CRP value of responders was 44 vs. 79 units in non-responders (a). Median resistin value of responders was 5.7 vs. 5.8 ng/mL in non-responders (b). The limitations extend down from the lowest value and up to the highest. Boxes show the 25th to 75th percentile, with median values marked inside.

4. DISCUSSION

The assessment of hepatic inflammation and fibrosis is a major issue in the management of patients with chronic hepatitis C, and consequently, there is a need for reliable biochemical markers. Such candidate biomarker could not only aid in monitoring and staging of fibrosis, but also help understanding the underlying pathology driving the development of cirrhosis in viral hepatitis. Numerous serum biomarkers have been proposed to be associated with liver fibrosis with varying reliability [33]. This study, which comprises a limited number of well-characterized hepatitis C patients with long-term follow-up, was undertaken to evaluate 2 biomarkers and their potential associations with liver disease, autoimmunity, histopathology and treatment outcome.

The main finding in this study is the unexpectedly high prevalence of anti-CRP positive HCV sera compared to AIH and NAFLD. The only previous observation of anti-CRP antibodies in HCV infection is that made by Kessel *et al.* [16], reporting 17% anti-CRP positive HCV patients compared to 6% in healthy matched controls. Although the prevalence of anti-CRP in the present study differs considerably, it confirms previous correlations with histopathology. Kessel *et al.* preferred to use the histology activity index, which include dimensions of global inflammation and fibrosis as well as portal changes [16]. In our hands, anti-CRP levels were primarily associated with the stage of fibrosis and portal inflammation. Furthermore, anti-CRP, together with well-known predictors (low platelets and raised AST), was associated with advanced fibrosis (Table 2). We did not have access to data on cryoglobulins and RF, but found no evidence of association between anti-CRP (or resistin) and other autoantibodies tested.

The significant discrepancy between our and the study by Kessel *et al.* concerning the prevalence of anti-CRP might have several reasons. Apart from differences in the selection of patients in which disease duration, HCV genotype and disease severity may play important roles, we also identified important differences in the methodology of anti-CRP detection. Kessel and co-workers used bovine serum albumin (BSA) to block their anti-CRP assay from unspecific binding [16]. This procedure could in fact introduce a specificity bias since anti-BSA antibodies, which commonly occur in healthy individuals, will also be detected and confound the results in patients as well as in

controls with serious impact on the cut-off level [34]. Therefore, at our laboratory we avoid the use of BSA as “blocking agent” or carrier protein in immunoassays [35].

Our interest for anti-CRP antibodies in liver disease arose when we screened anti-dsDNA antibody positive sera for the presence of antibodies directed against CRP. We have previously regarded anti-CRP antibodies as rather specific for SLE, but this study and others clearly show that this does not hold true [16, 36, 37]. On the other hand, SLE and chronic HCV infection share several disease characteristics. In addition to the fact that musculoskeletal symptoms and the presence of autoantibodies are commonplace, IFN- α plays a significant role in both conditions since lupus patients have an increased expression of type I IFN regulated genes and a continuous production of IFN- α [8]. The IFN- α levels correlate with disease activity in SLE, and therapeutic options with monoclonal antibodies inhibiting IFN- α are under way [38]. In chronic HCV infection, treatment with IFN- α has long been used successfully, but (although rare) lupus development is also a known side effect [10].

Although most of the HCV samples contained low concentrations of CRP we observed a direct correlation with anti-CRP levels, contrasting to our previous findings in SLE and acute coronary syndrome [32, 37]. This urged us to perform an inhibition assay with a high concentration of native (pentameric) CRP in solution. The achieved data showed a modest, but significant, reduction in the anti-CRP signal. This could indicate that anti-CRP antibodies in HCV patients may partially be targeted against native epitopes of CRP and not to hidden neo-epitopes (monomeric) CRP as is the case in lupus. Potential pathogenic roles for anti-CRP include impaired clearance of immune complexes and apoptotic debris as well as loss of complement-inhibitory effects of CRP in solution [14, 39–42]; both scenarios could result in advanced damage in targeted organs [16, 37, 43–45].

Resistin levels were equal in HCV patients compared to healthy controls, as well as to patients with immune-mediated and metabolic liver disease. Resistin levels if anything were higher in patients with advanced fibrosis (Table 2), but no association with HCV genotypes was found. No correlations between resistin and histological inflammation were either found. Several other observations have been made concerning the

importance of resistin in chronic HCV infection. Tsochatzis and colleagues found an inverse association between resistin levels and the severity of fibrosis (according to Ishak) in chronic viral hepatitis; and a nearly significant trend towards higher resistin levels in patients with HCV genotype-3 compared to other genotypes [24, 46]. Tiftikci *et al.* confirmed the finding of higher resistin levels in HCV patients with limited fibrosis (METAVIR scoring system), but found no association to HCV genotype [25, 47]. More recently, Baranova and co-workers reported a clear association between HCV genotype-3 and raised resistin levels compared to patients with non-genotype-3 infection [26]. Apart from the fact that our cohort is slightly smaller than in earlier studies, the reason for the deviating findings could also be a result of the selection of different study subjects (*e.g.* disease duration, host and viral factors, HCV genotypes) and the use of different scoring systems for fibrosis.

To conclude, the occurrence of anti-CRP is common in chronic HCV infection, but rarer in NAFLD and AIH. In line with the previous finding by Kessel *et al.* [16], anti-CRP levels appear to be associated with histopathological severity. Resistin levels are weakly associated with advanced fibrosis, but not with viral genotype or autoimmunity. Anti-CRP and resistin levels seem to reflect ongoing fibrosis rather than indicating a risk for future cirrhosis. Analysis of anti-CRP antibodies could possibly be of use as a surrogate marker for fibrosis/cirrhosis or portal inflammation in chronic HCV infection, but further studies of prospective character are needed.

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CONFLICT OF INTERESTS

None declared. All authors have approved the final version of the manuscript.

AUTHORS' CONTRIBUTIONS

CS contributed to the original idea, laboratory work, interpretation of data and manuscript writing. **KC** contributed to patient characterization, acquisition of data and draft of manuscript. **EAB** contributed to the laboratory work, interpretation of data and draft of manuscript. **MIB** contributed to the original idea, interpretation of data and draft of manuscript. **HE** contributed to laboratory work and draft of manuscript. **ME** contributed to acquisition and interpretation of data and draft of manuscript. **LL** contributed to acquisition and draft of manuscript. **AF** contributed to patient characterization, acquisition and interpretation of data and draft of manuscript. **SA** contributed to the original idea, patient characterization, acquisition and interpretation of data and draft of manuscript.

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