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Inflammation biomarkers in chronic hepatitis C: association with liver histopathology, HCV genotype and cryoglobulinemia

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Abstract
Objective This work investigated the profile of inflammation biomarkers in patients with chronic hepatitis C and its association with liver fibrosis, hepatic necroinflammatory activity, viral genotypes and cryoglobulinemia.
Subjects and methods Seventy-eight untreated patients were studied. Biomarker levels were determined by immunoassays, cryoglobulinemia by cryoprecipitation and liver histopathology investigated using METAVIR scores.
Results Decreased levels of α_1-acid glycoprotein (AGP), C3 and haptoglobin (Hp) were observed in the patients ($P < 0.0001$). Increased α_1-antitrypsin ($P < 0.01$) and ferritin ($P < 0.0001$) levels were found in this group, but C-reactive protein (CRP) and C4 levels were unaltered. Alanine aminotransferase inversely correlated with Hp ($P < 0.01$) and AGP ($P = 0.01$), whereas it was directly correlated with ferritin ($P < 0.05$) and AGP ($P < 0.0001$). The levels of CRP, C3 and C4 were lower in the patients with hepatic necroinflammatory activity ($P < 0.05$). Patients with advanced fibrosis had low levels of Hp and AGP ($P < 0.05$ and $P < 0.01$, respectively). Neither infection with different viral genotypes nor cryoglobulinemia caused an alteration in biomarker levels.
Conclusion Chronic hepatitis C virus infection alters the levels of some biomarkers, which are mainly observed in patients with liver fibrosis and hepatic necroinflammatory activity.

Keywords Hepatitis C · Inflammation biomarkers · Liver histopathology · Viral genotype · Cryoglobulinemia

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Introduction

Hepatitis C virus (HCV) infection is a worldwide health problem that affects around 170 million people. Rapid viral replication of HCV and inefficient antiviral cell-mediated immunity against this virus have been associated with the high prevalence of the chronic form of hepatitis C, which can evolve to liver cirrhosis or hepatocellular carcinoma [1–3]. Additionally, a high prevalence of extra-hepatic manifestations of autoimmunity, mainly including cryoglobulinaemia and non-organ-specific autoantibody (NOSA) production, may be observed in HCV carriers [3–5].

Although several studies have demonstrated that HCV infection induces the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6 and IL-1β, they have rarely showed changes in the serum levels of proteins involved with inflammation, also known as acute phase proteins or APP [6, 7]. In this work we investigated the serum profile of the most important of these biomarkers in Brazilian patients chronically infected with HCV, looking for its association with liver histopathology, cryoglobulinemia and HCV genotype.

Subjects and methods

Subjects

Seventy-eight untreated HCV carriers [46 men, median age 47.5 years, interquartile range (IQR) = 42.7–51.2 years, and 32 women, median age 53 years, IQR = 40.7–58.0 years], from two hepatitis centers in Salvador (Bahia, Brazil) were included in the study. Patients co-infected with human immunodeficiency virus, hepatitis B virus, human T-lymphotropic virus types I/II or presenting systemic autoimmune diseases such as rheumatoid arthritis or lupus were excluded. A control group represented by 40 healthy blood donors (22 men and 18 women; mean age 34 ± 12 years, 95 % confidence interval = 31–39) was used as a reference. An informed and written consent was obtained from all participants of the study, which was approved by a local Ethics Committee in Human Research.

Methods

Laboratory analysis

The laboratory diagnosis of chronic hepatitis C was made by a third generation enzyme-linked immunosorbent assay (AXSYM System; Abbott Laboratories, Chicago, IL, USA) and RNA-polymerase chain reaction (Amplicor® HCV Detection KIT v2.0; Roche Molecular Systems Inc., Somerville, NJ, USA). The genotyping of HCV was performed with the Inno-LiPA test (HCV LineProbe Assay; Innogenetics, Zwijndrecht, Belgium).

Alanine aminotransferase (ALT) levels were determined by automated analysis. Cryoglobulins were investigated by tube cryoprecipitation and gel-diffusion. Except for ferritin, which was quantified by an immunometric chemiluminescent immunoassay (Access 2; Beckman Coulter, USA), the serum levels of α_1-antitrypsin (A1AT), α_1-acid glycoprotein (AGP), complement C3 and C4, haptoglobin (Hp) and C-reactive protein (CRP) were determined by nephelometry, using the Immage System (Beckman Coulter, USA). The following reference values were used in the biochemical analyses: ALT 31 and 41 U/L for woman and man, respectively, A1AT 90–200 mg/dL, AGP 50–120 mg/dL, ferritin 36–262 and 24–155 mg/dL for man and woman, respectively, and CRP <5 mg/dL.

The METAVIR score was used to classify the stage of hepatic fibrosis and the intensity of necroinflammatory lesions observed in the liver histology examination carried out with hematoxylin–eosin, Picrosirius red, and Perl's stains. Fibrosis score were F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with few septa, F3 = numerous septa without cirrhosis and F4 = cirrhosis, whereas the necroinflammatory lesions were classified as A0 = no activity, A1 = mild activity, A2 = moderate activity and A3 = severe activity [8].

Statistical analysis

The results were expressed as median and interquartile range (IQR = Q1–Q3) or mean ± standard deviation (SD) according their distribution in the D'Agostino and Pearson "omnibus" normality test. Depending on this distribution, the groups of HCV carriers and healthy controls were compared using the unpaired *t* test or the non-parametric Mann–Whitney test, whereas the difference between three or more groups was analyzed by ANOVA or the Kruskal–Wallis test. The Fisher exact test was employed to analyze the association between two categorical groups. Correlation analysis was performed with the Spearman test. Multivariate cluster analysis (average linkage, squared Euclidean distance) was performed using MultiVariate Statistical Package software (Kovach Computing Services, USA), whereas the descriptive analyses were performed with GraphPad Prism statistical software (version 5.01, GraphPad Software, Inc., USA). The significance level was set at $P < 0.05$.

Results

Clinical features of the patients

Forty-nine individuals (49/78, 62.8 %) were infected with HCV genotype 1, 24 (30.8 %) with HCV genotype 3 and five (6.4 %) with HCV genotype 2. The following liver fibrosis scores were observed in the histopathology: F0 ($n = 4$), F1 ($n = 21$), F2 ($n = 26$), F3 ($n = 13$) and F4 ($n = 14$), whereas the necroinflammatory activity was A0

Table 1 Serum levels of acute phase proteins in HCV carrier and healthy control groups

Protein	HCV carriers ($n = 78$)	Healthy controls ($n = 40$)	*P* value
A1AT (mg/dL)	141.6 ± 37.4	115 (90.1–155.5)	<0.05
AGP (mg/dL)	59.9 (47.4–75.9)	85.2 (72.3–101.5)	<0.0001
C3 (mg/dL)	123.6 ± 31.60	164.1 ± 44.06	<0.0001
C4 (mg/dL)	29.8 ± 12.4	32.9 ± 10.3	>0.05
CRP (mg/L)	3.8 (2.3–5.4)	3.6 (2.8–4.8)	>0.05
Ferritin (ng/mL)	206.0 (97.4–370.5)	85.4 (33.2–133.0)	<0.0001
Hp (mg/dL)	75.2 (38.8–109.0)	124.5 (71.8–190.5)	<0.0001

The results are expressed as mean ± SD or median and interquartile range (IQR = Q1–Q3) in accordance with their distribution in the D'Agostino and Pearson test. The means and the medians were compared with the unpaired *t* test and the Mann–Whitney test, respectively

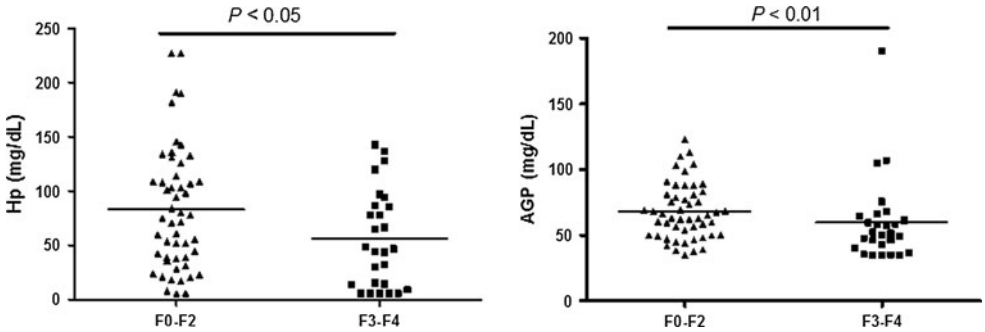

Fig. 1 Serum levels of haptoglobin (*Hp*) and α_1-acid glycoprotein (*AGP*) in HCV carriers with absent or mild liver fibrosis (F0–F2) or advanced (F3–F4) liver fibrosis. The means of Hp levels (83.3 ± 56.9 and 55.9 ± 44.3 mg/dL) and the medians of AGP levels (64.9 mg/dL, IQR = 49.8–81 and 51.5 mg/dL, IQR = 40.0–64.3 mg/dL) are represented by *horizontal lines* and were compared using the unpaired *t* test and the Mann–Whitney test, respectively

($n = 19$), A1 ($n = 30$), A2 ($n = 22$) and A3 ($n = 3$). The levels of ALT were higher in HCV carriers than in healthy controls (45.0 U/L, IQR = 24.0–80.0 and 11 U/L, IQR = 9–14.5 U/L, respectively) ($P < 0.0001$). Cryoglobulinemia was detected in 43 out of 78 (55.1 %) HCV-infected patients.

Serum levels of APP

The serum levels of A1AT and ferritin were higher in HCV carriers, whereas their levels of Hp, AGP and C3 were lower than those from the healthy controls. There was no difference in either C4 or CRP levels when HCV and control groups were compared (Table 1).

Patients with advanced liver fibrosis (F3–F4) had lower serum levels of Hp and AGP ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 1). HCV carriers presenting hepatic necro-inflammatory lesions when compared with patients without these lesions had lower C3 (110.6 ± 36.6 vs. 129.0 ± 29.3 mg/dL; $P < 0.05$), C4 (24.1 ± 7.8 vs. 32.5 ± 13.1 mg/dL; $P < 0.05$), AGP (56.2 mg/dL, IQR = 43.8–66.2 mg/dL vs. 64.3, IQR = 50.1–81.0; $P < 0.05$) and CRP levels (1.7 mg/L, IQR = 1.0–3.1 mg/L vs. 3.4, IQR = 1.9–5.4 mg/L; $P < 0.05$). However, their levels of Hp (75.6 ± 46.5 vs. 77.5 ± 56.4 mg/dL), A1AT (145.0 ± 27.0 vs. 140.6 ± 41.7 mg/dL) and ferritin (155 ng/mL, IQR = 93.2–357.2 vs. 210 ng/mL, IQR = 98.8–397.9 ng/mL) were unaltered ($P > 0.05$).

The serum levels of the acute phase proteins were similar in cryoglobulinemic patients and in patients without cryoglobulins (Table 2).

There was an inverse correlation between serum levels of Hp and ALT and also between the serum levels of AGP and ALT ($r = -0.3049$, $P < 0.01$ and $r = -0.3664$, $P = 0.001$, respectively). In contrast, ferritin and A1AT levels were positively correlated with the levels of ALT ($r = 0.2431$, $P < 0.05$ and $r = 0.4806$, $P < 0.0001$, respectively) (Fig. 2). Cryoglobulinemia was not associated with advanced fibrosis (F3–F4) nor was more severe hepatic inflammation associated with the presence of this cryoprecipitate ($P > 0.05$).

Table 2 Serum levels of acute phase proteins in HCV carriers with and without cryoglobulinaemia

Protein	With cryoglobulinemia ($n = 43$)	Without cryoglobulinemia ($n = 35$)	*P* value
A1AT (mg/dL)	143.9 ± 40.0	139.7 ± 35.5	>0.05
AGP (mg/dL)	64.3 (49.8–81.0)	58.4 (44.7–75.2)	>0.05
C3 (mg/dL)	124.7 ± 29.4	122.7 ± 33.6	>0.05
C4 (mg/dL)	31.4 ± 12.5	28.4 ± 12.3	>0.05
CRP (mg/L)	2.3 (1.1–4.7)	3.4 (1.9–5.3)	>0.05
Ferritin (ng/mL)	210.4 (92.9–443.2)	184.1 (100.7–300.7)	>0.05
Hp (mg/dL)	75.6 ± 46.5	77.5 ± 56.4	>0.05

The results are expressed as mean ± SD or median and interquartile range (IQR = Q1–Q3) in accordance with their distribution in the D'Agostino and Pearson test. The means and the medians were compared with the unpaired *t* test and the Mann–Whitney test, respectively

Viral genotype

Two clusters of acute-phase proteins were observed in multivariate analysis, in both HCV genotype 1 and HCV genotype 3 infections. The first was formed by C3/A1AT and the second by C4/CRP/ferritin/Hp/AGP. However, in HCV genotype 1 infection Hp/AGP was the second node, whereas in HCV genotype 3 infection it was represented by A1AT/C3 (Fig. 3).

Discussion

This work investigated the serum profile of proteins that are regulated by pro-inflammatory cytokines and are routinely used as important biomarkers of inflammation in

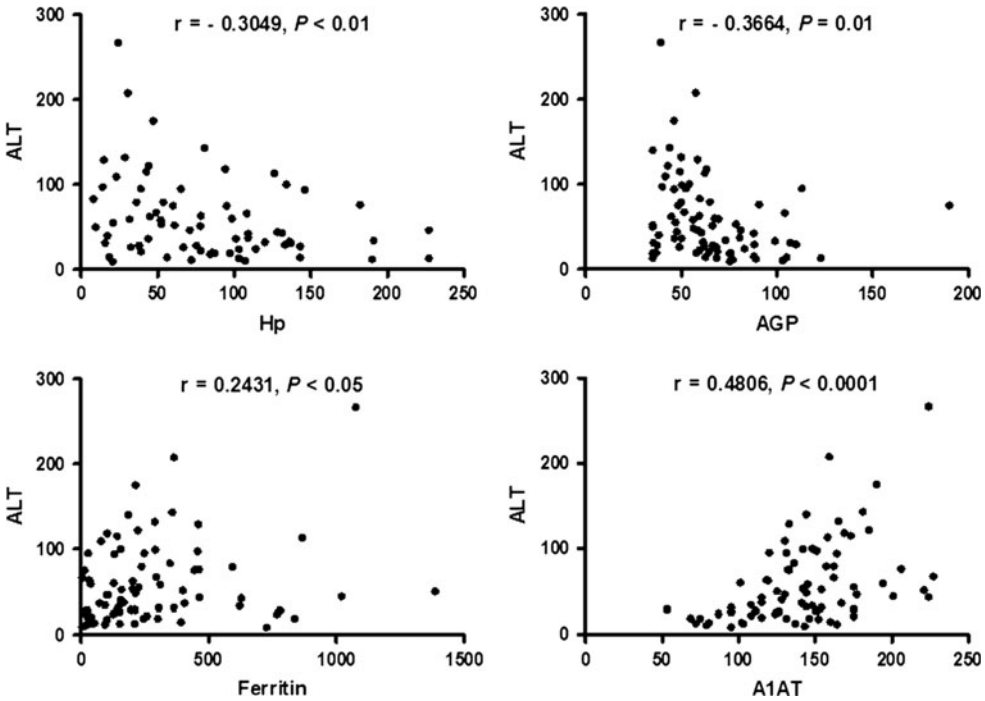

Fig. 2 Inverse correlation between alanine aminotransferase (*ALT*) and haptoglobin (*Hp*) and ALT and AGP levels (*upper*), and direct correlation between ALT and ferritin and A1AT levels (*lower*) in HCV carriers. Analyses were carried out with the Spearman test

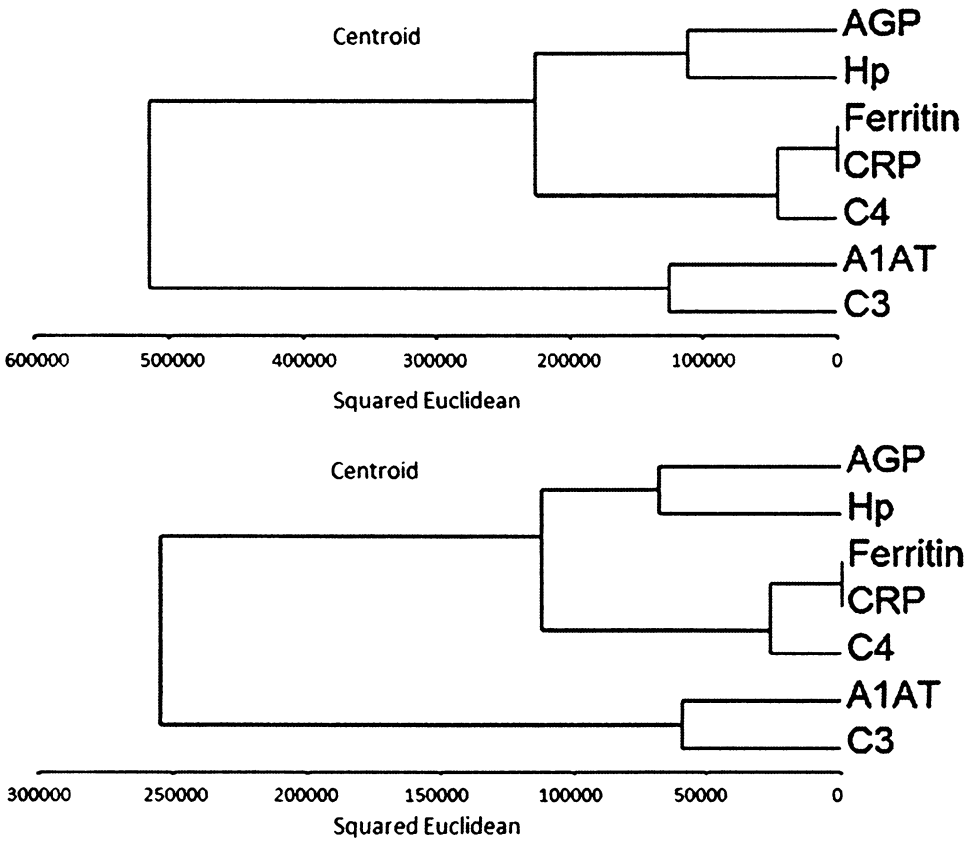

Fig. 3 Multivariate cluster analysis of acute phase proteins in patients chronically infected with HCV genotype 1 (*upper*) and HCV genotype 3 (*lower*)

individuals chronically infected with HCV. Hp and AGP were drastically reduced in the HCV carriers with advanced liver fibrosis (METAVIR stages F3, F4). Interestingly, both Hp and AGP levels were inversely correlated with the levels of ALT, an enzyme whose increase that has been associated with liver injury in hepatitis C. Nevertheless, we did not find an association between the presence of liver necroinflammatory activity and low levels of either

Hp or AGP. Although several studies have demonstrated low serum levels of Hp in HCV patients presenting liver fibrosis, a decrease in the serum level of AGP in the individuals with this hepatic pathology has not yet been reported. Low Hp levels were first observed in French HCV carriers presenting advanced fibrosis, which were negatively correlated with the histology activity index of Knodel for fibrosis and periportal necrosis. Although lower Hp serum level has been reported as a genetic characteristic of African descents, the healthy controls included in this study had normal levels of this protein, therefore confirming the association between liver fibrosis and decrease in the serum level of this APP in chronic hepatitis C [9–12].

The observation that AGP level was lower in HCV carriers is in accordance with a previous study demonstrating the decrease in the concentration of this APP in chronic liver diseases, which has been associated with impaired synthesis caused by hepatic injury. AGP is a highly glycosylated protein from the lipocalin family whose main function is the transport of small hydrophobic molecules. This glycoprotein is an immunocalin that has immunomodulatory effects on inflammation, inducing the expression of IL-1β, IL-6, IL-12 and TNF-α by human monocytes. On the other hand, AGP inhibits neutrophil activities such as chemotaxis and superoxide generation, promotes cell aggregation and inhibits the proliferation of a human T-cell subset. Alteration in AGP glycosylation, represented by the presence of *N*-acetylgalactosamine (GalNAc) on the *N*-linked chains of AGP and mainly by hyperfucosylation, has been described in hepatitis C, being more prevalent in patients with cirrhosis [13–15].

In our study, we verified that the serum levels of A1AT and ferritin were higher in HCV carriers than in control individuals. Despite the demonstration that the levels of these two proteins were not influenced by either advanced liver fibrosis or hepatic necroinflammatory activity, their levels were positively correlated with ALT levels. Our A1AT findings warrant future studies mainly because the involvement of A1AT in chronic liver diseases has been mostly demonstrated in patients with A1AT deficiency. Although a high prevalence of either virus B or C infection was observed in these subjects, there was no over-representation of these viral diseases in heterozygous A1AT deficiency [16, 17]. On the other hand, ferritin is an iron storage protein whose increased serum levels in chronic hepatitis C have been reported by others and us and which is able to exert suppressive effects on effector function of either T or B cells and can reduce granulocyte phagocytosis. A high level of ferritin has been associated with an inefficient antiviral response to the combined therapy of interferon-α plus ribavirin [18–25], justifying its laboratory use in the follow-up of chronic hepatitis C treatment.

In accordance with a previous report, we did not verify alterations in the serum level of CRP when HCV carriers were compared with healthy controls, but CRP levels were lower in patients with liver necroinflammation. However, this finding can be caused by the presence of anti-CRP antibodies, already detected and correlated with both autoimmunity and hepatitis C severity, which could interfere in the immunoassay used in this work to quantify this protein [26, 27]. Although low levels of C3, C4 and AGP in patients with liver lesions could be caused by a decreased liver secretion of these proteins, an inhibitory effect of HCV proteins on C3 synthesis has been recently demonstrated by reduction of C3 mRNA in hepatocytes upon infection with cell-culture-grown HCV genotype 1a or 2a in vitro [28]. However, the effects of these viral proteins on the synthesis of different inflammation biomarkers such as AGP and C4 still need investigation. On the other hand, HCV chronic infection causes extra-hepatic clinical manifestations, which could also be involved in the changes observed here in the levels of some inflammation biomarkers.

The presence of cryoglobulinemia did not have any effect on the levels of either complement C3 or C4, but they were decreased when patients with hepatic necroinflammatory lesions were compared with HCV-infected subjects without such a liver pathology. Thus, our findings are partially in agreement with those of a previous study reporting a decrease in CH50 and C4 levels, but normal C3 levels, when HCV carriers were compared with healthy controls [29]. Nonetheless, in contrast to that study, the involvement of rheumatoid factor in complement activation and C4 decrease was excluded from our study since this autoantibody is a natural component of hepatitis C mixed cryoglobulinemia [30].

The chronic inflammation caused by persistent HCV infection provokes a serum profile of acute phase proteins that is independent of the genotype of the infecting HCV, as demonstrated by similar dendrograms in the multivariate cluster analysis carried out. Moreover, the presence of cryoglobulinemia did not exert any influence on the levels of the APP investigated.

We concluded that the inflammation caused by persistent HCV infection is characterized by changes in the levels of some acute phase proteins, which are mainly observed in both liver fibrosis and hepatic necroinflammatory activity. However, the involvement of systemic manifestations of chronic HCV infection on the serum levels of some acute phase proteins studied here cannot be excluded and needs to be investigated.

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