



Atherogenic cytokines and chemokines in chronic hepatitis C are not associated with the presence of cardiovascular diseases



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ABSTRACT

There appears to be an associative link between chronic hepatitis C (CHC) and cardiovascular diseases (CVDs). However, the exact nature of the relationship between CHC and CVDs has not been elucidated. We investigated the presence of CVDs and the clinical and laboratory alterations associated with these diseases in CHC patients. Twenty-six CHC patients, 35 individuals with atherosclerosis (Athero) and 27 healthy individuals were examined for risk factors for CVD, lipid profile, atherogenic risk indexes, and insulin resistance (IR). Cardiac biomarkers and the chemokines and cytokines involved in atherosclerosis were also evaluated. A higher prevalence of prior acute myocardial infarction was found in the Athero group. Most CHC patients were infected with the hepatitis C virus genotype 1 and exhibited either no hepatic fibrosis or a mild to moderate liver fibrosis. The apolipoprotein B/apolipoprotein A-I and triglyceride/high-density lipoprotein cholesterol ratios and C-reactive protein levels were lower in CHC patients than in the Athero group. Further, IR was elevated in the CHC group and associated with the waist circumference. High GDF-15 levels were observed in the CHC group, which were inversely correlated with APOB levels. Peripheral blood mononuclear cells from CHC patients produced more IFN- γ , TNF- α and IL-6 than CAD PBMC but the production of IL-10 and IL-1 β was similar. CHC and CAD groups presented similar levels of IL-8, MCP-1 and LAP-TGF- β 1. Increased IR, elevated levels of GDF-15, and high production of atherogenic cytokines can be observed in Brazilian CHC patients without association with diabetes and clinical manifestation of cardiovascular diseases.

1. Introduction

Chronic hepatitis C is a significant public health problem, especially in developing countries. Despite the therapeutic success obtained by the introduction of the new direct-acting antiviral agents, 177 million people are still infected with the hepatitis C virus (HCV) [1,2]. In addition to the hepatic manifestations, such as advanced fibrosis, cirrhosis, and hepatocellular carcinoma, commonly associated with this infection, extrahepatic disorders have been described in patients infected with HCV [3].

The association between HCV infection and the development of lymphoproliferative and autoimmune diseases is well established and is the subject of consensus in the scientific literature. However, the association of HCV with certain cardiovascular diseases (CVDs), whose pathogenesis is attributed to the atherosclerotic process, is still the

subject of considerable controversy [4–7].

Metabolic changes associated with HCV infection may contribute to atherogenesis, since CHC has been linked to the development of insulin resistance (IR) and type 2 diabetes mellitus (DM2) [8–11]. In addition to the deficiency in insulin signaling, HCV infection can result in changes in the hepatic lipid metabolism associated with the development of steatosis and can increase oxidative stress contributing to chronic inflammatory response in the liver [12–15].

In recent decades, new cardiovascular risk biomarkers have been identified to improve the diagnosis and prognosis of cardiovascular diseases. Among these are the type B natriuretic peptide (BNP), whose usefulness in cardiology has been established in recent years [16], and the growth differentiation factor 15 (GDF-15), a promising marker for the prevention of complications associated with chronic liver disease [17]. However, while there are reports on the potential use of these

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biomarkers, the mechanisms underlying their effects and their clinical utility of these biomarkers need to be better explored.

In the present study, we investigated the presence of IR, CVD risk factors and cardiac biomarkers, and cytokines and chemokines involved in atherogenesis in Brazilian CHC patients. Assays were performed to analyze the liver histology, HCV viremia, and the presence of cryoglobulinemia and non-organ specific autoantibodies (NOSA). Subjects with CHC were compared with patients classified as having a high cardiovascular risk, with those diagnosed with coronary artery disease (CAD), and with healthy volunteers.

2. Material and methods

2.1. Subjects

Twenty-six individuals, 15 women and 11 men, infected with chronic HCV, without previous antiviral treatment, formed the CHC group in this study. They were diagnosed with CHC in the Gastro-Hepatology Service of the Professor Edgard Santos Hospital at Federal Bahia University (Brazil). Laboratory diagnosis of hepatitis C was based on the presence of anti-HCV antibodies (using third generation ELISA), detectable HCV-RNA (using PCR), and alanine aminotransferase determination. Information about HCV genotyping, blood viral load, and liver histology or liver elastography (Fibro Scan™) was obtained from their medical records.

Thirty-five dyslipidemic individuals with atherosclerotic disease, diagnosed by coronary angiography (median stenosis 75%) at the Hemodynamic Service of the Ana Nery Hospital (Salvador-Bahia), formed the Athero group; while, 27 healthy volunteers from the local population, without any evidence of metabolic, infectious, or autoimmune diseases, formed the HI control group. Individuals co-infected with HIV, HTLV, hepatitis B virus, or with known history of neoplasia, chronic autoimmune or inflammatory conditions were not included in the study. All participants were informed about the research and signed a written consent before their participation. The study was approved by the Research Ethics Committee of the Nursing School of the Bahia Federal University and was carried out in accordance with The Code of Ethics of The World Medical Association (Declaration of Helsinki).

2.2. Clinical evaluation

Patients and healthy controls were interviewed regarding the presence of major cardiovascular and associated conditions as diabetes, family history of CVD, smoking, and alcohol intake. The use of regular medication and practice of physical activity was also enquired during the interview. The anthropometric measures of weight, height, and abdominal circumference (ABD) were performed in all participants to determine their body mass index (BMI). The participants were classified as normal ($BMI < 25 \text{ kg/m}^2$), overweight ($BMI > 25 \text{ kg/m}^2$) or obese ($BMI > 30 \text{ kg/m}^2$) based on the BMI. Arterial systolic and diastolic pressures were measured, and the presence of hypertension was confirmed when they were > 140 and > 90 mm Hg, respectively. The diagnosis of metabolic syndrome (MS) was assessed according to the International Diabetes Federation (IDF), which establishes alterations in at least three of the following criteria: abdominal obesity ($ABD > 90$ cm for men and > 80 cm for women); blood pressure $> 130 \times 85$ mm Hg; high-density lipoprotein cholesterol (HDL-C) level < 40 mg/dL and < 50 mg/dL for men and women, respectively; serum triglyceride (TG) level > 150 mg/dL and fasting glucose > 100 mg/dL [18].

2.3. Laboratory exams

Blood samples were collected in the morning, after 8–12 h of fasting, in EDTA for hematology analysis using the CELL DYN-RUBY system (Abbott Diagnostics, Illinois, USA) and in heparin for isolation of

peripheral blood mononuclear cells (PBMCs). Serum obtained from blood collected without anticoagulant was used for biochemical and immunological analyses or stored at -80°C for the measurement of cytokines, chemokines, and cardiac biomarkers.

The lipid profile included the determination of the serum levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), HDL-C, TG, and non-HDL cholesterol. Alanine aminotransferase (ALT) levels were measured by an UV-kinetic method using the B3000 PLUS analyzer (Winner Lab Group, Buenos Aires, Argentina). LDL-C levels were calculated using the Friedewald formula for TG values < 400 mg/dL [19]. The reference values for the lipid profile were those recommended for Brazilian adults: $CT < 200$ mg/dL, $LDL-C < 130$ mg/dL, $HDL-C > 60$ mg/dL, $non-HDL-C < 160$ mg/dL, and $TG < 150$ mg/dL [20].

Serum levels of apolipoprotein A-I (APOA-I), apolipoprotein B (APOB) and C-reactive protein (CRP) were determined by nephelometry using the IMMAGE system (Beckman Coulter, California, USA). The following reference values were used in this study for APOA-I (female, 90–170 mg/dL and male, 107–214 mg/dL), APOB (female, 56–162 mg/dL and male, 51–171 mg/dL) and $CRP < 3$ mg/L [20,21]. Atherogenic indexes were APOB: APOA-I ratio (low risk, 0.30–0.59; medium risk, 0.60–0.79; and high risk, 0.80–1.0), TG: HDL-C ratio or Castelli index I (reference value < 3.0), and neutrophil: lymphocyte ratio (N: L, reference value < 2.0) [22–24].

Serum insulin levels were determined using a chemiluminescent immunoassay (ACCESS2, Beckman Coulter), while glucose levels were determined using an enzymatic assay (B3000 PLUS, Wiener Lab Group, Buenos Aires, Argentina). Insulin resistance was measured with the homeostatic model assessment (HOMA)-IR [fasting glycemia (mmol/L) \times insulin ($\mu\text{IU/mL}$)/22.5] [25]. As previously recommended, a $HOMA-IR < 2.71$ was used as a reference for the non-diabetic population [26].

Serum levels of GDF-15 and proBNP were determined using commercial antigen-capture enzyme immunoassays (Invitrogen, California, USA) according to the manufacturer's recommendations. The lower detection limits of these immunoassays were $GDF-15 = 2$ pg/mL and $proBNP = 0.14$ ng/mL.

2.4. Evaluation of autoimmunity

Indirect immunofluorescence was used to investigate the presence of antinuclear antibodies (ANA) and smooth muscle antibodies (SMA) using HEP-2000® cells (Immuno Concepts, California, USA; cutoff sample dilution 1:40) and tissue sections of the kidney, liver, and stomach (Viro-Immun Labor-Diagnostika GmbH, Oberursel, Germany; cutoff sample dilution 1:40), respectively. Cryoglobulinemia was investigated by serum cryoprecipitation at 4°C for 7 days followed by cryoprecipitate dissolution after 1 h incubation, at 37°C . Rheumatoid factor was detected by nephelometry using the IMMAGE apparatus (Beckman-Coulter; cutoff < 20 IU/mL).

2.5. Mitogenic stimulation of PBMCs

PBMCs were isolated from blood diluted 1:2 in saline (150 mM NaCl), using density gradient centrifugation with Ficoll-Paque Premium™ (GE Healthcare Bio-Sciences Ab, Uppsala, Sweden). After washing, the cell suspension was adjusted to a cell density of 1.0×10^6 cells/mL in RPMI 1640 medium (Gibco, New York, USA) containing 10% fetal bovine serum, 2 mM glutamine, 25 mM HEPES (pH 7.2), and 10 mg/mL gentamicin (Sigma-Aldrich, USA). Two hundred microliters of the cell suspension were added to the microwells of a polystyrene culture plate (KASVI, China) and the PBMCs from each patient and healthy control were stimulated in triplicate with 10 μL of phytohemagglutinin solution (PHA, 5 $\mu\text{g/mL}$; Sigma-Aldrich). For the negative control, PBMCs were challenged with 10 μL of the mitogen diluent. After 72 h of incubation at 37°C , in 5% CO_2 atmosphere, the culture

supernatants were collected after centrifugation at 4 °C (1900 rpm, 10 min), and stored at –80 °C until analysis of cytokine levels.

2.6. Cytokine and chemokine immunoassays

The levels of interferon-gamma (IFN- γ), tumor necrosis factor- α (TNF- α), IL-6 and IL-10 in the culture supernatant were determined by a Th1/Th2 cytometric bead assay (CBA) using the FACS Canto II cytometer as per manufacturer's instructions (BD Biosciences, California, USA). The lower limits of detection of the cytokine immunoassay were IFN- γ = 3.7 pg/mL, TNF- α = 3.8 pg/mL, IL-6 = 2.4 pg/mL, and IL-10 = 4.5 pg/mL. Serum levels of IL-8, MCP-1, and LAP-TGF- β 1 were determined using capture enzyme immunoassays (Invitrogen, Thermo Fisher Scientific, Illinois, USA). The analytical sensitivities of these assays were IL-1 β = 2 pg/mL, MCP-1 = 7 pg/mL, and LAP-TGF- β 1 = 156.3 pg/mL.

2.7. Statistical analysis

Continuous variables were tested for distribution with the D'Agostino-Pearson test. Categorical variables and proportions were compared using the chi-square and the Fisher's exact tests. Means and medians of three or more groups were compared using one-way ANOVA or the Kruskal-Wallis test, followed by Tukey's and Dunn's multiple comparisons tests, respectively. The Spearman test was used to analyze correlations. A significance level of $p < 0.05$ was considered statistically significant. Statistical analysis was performed with the Prism software version 6.0 (GraphPad Software Inc., USA).

3. Results

3.1. Demographic and clinical characteristics of individuals

Patients with CHC had a median age of 59.0 years (interquartile range (IQR): 55.8–66.3), ranging from 41 to 78 years. The infection with HCV genotype 1 was prevalent, followed by those caused by HCV Gen3, and very rarely HCV Gen2. There was no difference between the proportions of individuals presenting high and low HCV viremia. Most individuals studied in this group (80%) had no fibrosis or exhibited mild to moderate stages of liver fibrosis (F0, F1-F2), while 20% had advanced fibrosis (F3-F4). Among the NOSAs, there was a higher proportion of anti-IgG antibodies (rheumatoid factor), followed by the smooth muscle and antinuclear antibodies, respectively. The presence of cryoglobulinemia was observed in about one-fifth of these individuals (Table 1).

There was a difference in the BMI of the three groups, being observed more obese subjects in the CHC group. On the other hand, patients with CAD had a higher prevalence of CVD associated conditions, such as hypertension, metabolic syndrome, diabetes, angina, previous myocardial infarction, and family history of CVD; however, the proportion of CHC subjects with hypertension was higher than in healthy individuals. The proportion of individuals from the three groups practicing regular physical exercise, smoking, and alcohol intake was similar (Table 2).

3.2. Laboratory findings

CT, HDL-C and APOA-I levels differed among the lipid profiles of the three groups studied. Patients with CHC who presented with high viral load of HCV ($> 8 \times 10^5$ IU/mL) had lower serum levels of TC than patients with low HCV viremia ($157.9 \text{ mg/dL} \pm 47.1$ vs. 199.9 ± 38.2 ; $p = 0.022$).

Patients with CHC had lower levels of serum CRP, and only 22.7% (5/26) had serum CRP levels higher than above 3 mg/L. In contrast, individuals with CAD exhibited higher levels of CRP, and 57.1% (20/35) had a CRP level $> 3 \text{ mg/L}$ ($p = 0.014$). The N:L ratio was increased

Table 1

Virological findings and hepatic and extra-hepatic manifestations in patients with chronic hepatitis C (CHC group).

| Findings | Proportion (%) | p value |
|--------------------------------------|----------------|----------|
| HCV genotyping | | |
| GEN1 | 21/26 (81%) | < 0.0001 |
| GEN3 | 4/26 (15%) | |
| GEN2 | 1/26 (4%) | |
| Blood HCV load | | |
| $< 8 \times 10^5$ IU/ML | 14/26 (54%) | 0.567 |
| $> 8 \times 10^5$ IU/ML | 12/26 (46%) | |
| ALT | | |
| > 41 U/L | 15/26 (58%) | 0.253 |
| < 41 U/L | 11/26 (42%) | |
| Fibrosis* | | |
| Absent, mild to moderate (F0, F1-F2) | 16/20 (80%) | 0.0002 |
| Advanced (F3-F4) | 4/20 (20%) | |
| Not examined | 6/26 (23%) | |
| Non-organ-specific antibody | | |
| Rheumatoid factor | 11/26 (42%) | 0.0001 |
| ANA | 6/26 (23%) | |
| SMA | 5/26 (19%) | |
| Cryoglobulinemia | | |
| Negative | 20/26 (77%) | 0.0001 |
| Positive | 6/26 (23%) | |

* Liver fibrosis was diagnosed by liver histology exam (METAVIR score) or using the Fibro Scan®. HCV, hepatitis C virus; GEN1, HCV genotype 1; GEN2, HCV genotype 2; GEN3, HCV genotype 3; ALT, alanine aminotransferase; ANA, antinuclear antibody; and SMA, smooth-muscle antibodies.

Table 2

Demographic and clinical findings in healthy individuals (HI), in atherosclerosis patients (Athero) and in subjects with chronic hepatitis C (CHC).

| | HI (n = 27) | Athero (n = 35) | CHC (n = 26) | p value |
|--------------------------|---------------|-----------------|---------------|----------|
| Gender (male/female) | 10/17 | 18/17 | 11/15 | 0.618 |
| Age (years) | 55 (46–61) | 59.0 (53–64) | 58.0 (56–66) | 0.095 |
| BMI (Kg/m ²) | | | | |
| Normal (< 25) | 12 (44.5%) | 16 (46%) | 3 (11%) | 0.011 |
| Overweight (25–29.9) | 9 (33.3%) | 7 (20%) | 8 (31%) | 0.451 |
| Obesity (≥ 30) | 6 (22.2%) | 12 (34%) | 15 (58%) | 0.025 |
| SYS (mm Hg) | 126 (106–141) | 148 (122–160) | 127 (118–142) | 0.005 |
| Dias (mm Hg) | 77 (70–90) | 80 (70–90) | 80 (71–87) | 0.838 |
| Risk factors | | | | |
| Hypertension | 0 (0%) | 32 (91%) | 12 (46%) | < 0.0001 |
| Diabetes | 0 (0%) | 12 (34%) | 2 (8%) | 0.0003 |
| Metabolic syndrome | 0 (0%) | 19 (56%) | 6 (23%) | < 0.0001 |
| Sedentarism | 14 (52%) | 30 (86%) | 17 (64%) | 0.031 |
| Family history of CVD | 19 (70%) | 31 (89%) | 18 (69%) | 0.120 |
| Smoking | 0 (0%) | 5 (14%) | 2 (8%) | 0.098 |
| Previous smoking | 3 (11%) | 18 (51%) | 7 (27%) | 0.003 |
| Angina | 0 (0%) | 13 (37%) | 1 (4%) | < 0.0001 |
| Prior AMI | 0 (0%) | 21 (60%) | 1 (4%) | < 0.0001 |
| Statin medication | 0 (0%) | 29 (83%) | 2 (8%) | < 0.0001 |

Results are presented as median and interquartile range or proportion (%). The medians were compared using the Kruskal-Wallis test, and the proportions were compared using the chi-square test. BMI, body mass index; SYS, systolic pressure; DIAS, diastolic pressure; CVD, cardiovascular disease; and AMI, acute myocardial infarction.

in the CAD group as compared with the other groups analyzed. However, the N:L ratio was not associated with an increased occurrence of cardiovascular events. The mean APOB: APOA-I ratio was similar between subjects with CHC and healthy controls, but CHC patients

Table 3

Laboratory findings in healthy individuals (HI), atherosclerosis patients (Athero) and in subjects with chronic hepatitis C (CHC).

| Groups | HI (n = 27) | Athero (n = 35) | CHC (n = 26) | p value |
|-----------------------------|------------------|------------------|------------------|----------|
| Lipid profile | | | | |
| Total cholesterol (mg/dL) | 203 [180–213] | 172 [135–196] | 173 [134–218] | 0.016 |
| LDL-C (mg/dL) | 111 [91–128] | 107 [74–132] | 95 [64–121] | 0.321 |
| HDL-C (mg/dL) | 67 [53–77] | 45 [38–57] | 50 [43–65] | 0.0003 |
| Triglycerides (mg/dL) | 103 [91–143] | 110 [88–162] | 112 [79–138] | 0.550 |
| Non-HDL cholesterol (mg/dL) | 133 [112–151] | 127 [92–152] | 123 [85–146] | 0.445 |
| APOA-I (mg/dL) | 164 [143–191] | 128 [108–156] | 166 [136–207] | 0.0002 |
| APOB (mg/dL) | 122 [103–156] | 114 [98–163] | 107 [90–159] | 0.731 |
| Atherogenic risk | | | | |
| APOB: APOA-I ratio | 0.73 [0.63–0.92] | 0.91 [0.74–1.11] | 0.63 [0.47–0.86] | 0.009 |
| TG: HDL-C ratio | 1.56 [1.22–2.19] | 2.68 [1.70–3.64] | 1.97 [1.42–2.88] | 0.006 |
| N:L ratio | 1.44 [1.07–1.81] | 1.93 [1.43–2.63] | 1.51 [0.97–1.8] | 0.006 |
| CRP (mg/L) | 2.58 [1.49–3.71] | 3.77 [1.75–7.38] | 1.63 [0.54–3.0] | 0.012 |
| HOMA-IR | 1.12 [0.83–1.79] | 2.07 [1.35–2.90] | 2.34 [1.58–3.85] | < 0.0001 |
| ALT (U/L) | 20 [15–28] | 19 [12–30] | 58 [22–126] | < 0.0001 |

Results are presented as medians and interquartile range or proportions (%). The medians were compared using the Kruskal-Wallis test, and the proportions were compared using the chi-square test. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; APOA, apolipoprotein A; APOB, apolipoprotein B; TG; triglyceride; N, neutrophil; L, lymphocyte; CRP, C-reactive protein; HOMA; homeostatic model assessment; IR, insulin resistance; and ALT, alanine aminotransferase.

differed from CAD patients, who had a high atherogenic index. Further, the Castelli I index (TG: HDL) was higher in patients with CAD when compared to healthy individuals. However, the medians of TG: HDL ratio in the three groups were unaltered (< 3) (Table 3).

3.3. Insulin resistance (IR)

Around a half (46%) of the CHC individuals presented a HOMA-IR higher than 2.71, demonstrating a state of IR. Interestingly, only two subjects had diabetes in this group, but with normal HOMA-IR. The presence of IR in the CHC group was not correlated with age or associated with fasting hyperglycemia, hypertension, obesity, or the presence of metabolic syndrome and DM2. There was no significant association between IR and HCV viremia or liver damage in CHC patients, but a positive correlation was found between HOMA-IR and WC ($r = 0.66$, $p = 0.0003$) in CHC individuals (Fig. 1). In the group of patients with atherosclerosis, only 8.7% (2/23) individuals exhibited IR.

3.4. Cardiac markers

Serum levels of GDF-15 differed among the three groups (HI = 1079 ± 323 pg/mL, Athero = 1846 ± 580 pg/mL, and CHC = 2322 ± 738 pg/mL; $p < 0.0001$). GDF-15 levels were the highest in individuals with CHC (Fig. 2). The levels of GDF-15 in CHC patients did not correlate with HCV viremia, fibrosis stage, and serum

levels of ALT. In addition, the GDF-15 levels were not correlated with the levels of CRP and HOMA-IR, and with the N: L, APOB: APOA-I and TG: HDL ratios. GDF-15 levels were not influenced by the presence of NOSA or cryoglobulinemia. However, there was a negative correlation between GDF-15 levels and APOB in CHC group (Fig. 3).

There was a difference in the proBNP levels among the three groups studied (HI = 1.40 ng/mL, IQR: 1.0–1.68; Athero = 1.91 ng/mL, IQR: 1.49–2.17; and CHC = 1.89 ng/mL, IQR: 1.53–2.16; $p = 0.0008$) (Fig. 2). The levels of proBNP were not associated with virological findings, ALT levels, fibrosis stage, NOSA, or atherogenic indexes.

3.5. Cytokines and chemokines

When PBMCs from the three groups were stimulated with PHA, they differed in the production of IFN- γ ($p = 0.031$), TNF- α ($p = 0.031$), IL-6 ($p < 0.0001$), IL-10 ($p = 0.0032$), and IL-1 β ($p = 0.012$). PBMCs from the CHC group produced more IFN- γ ($p = 0.0071$), TNF- α ($p = 0.013$), and IL-6 ($p = 0.0049$) than the mononuclear cells from the CAD group; but, the production of IL-10 ($p = 0.858$) and IL-1 β ($p = 0.073$) were similar between these groups (Fig. 4). Although serum IL-8 levels were higher in the CHC group in comparison to the HI group ($p = 0.0012$), the IL-8 levels in the CHC group were similar to those of the CAD group ($p = 0.216$). No differences in serum levels of MCP-1 and LAP-TGF- β 1 were observed among the three groups ($p = 0.198$ and $p = 0.140$, respectively). There were no correlations between the levels of IFN- γ , TNF- α , IL-6, IL-10, and IL-1 β produced by

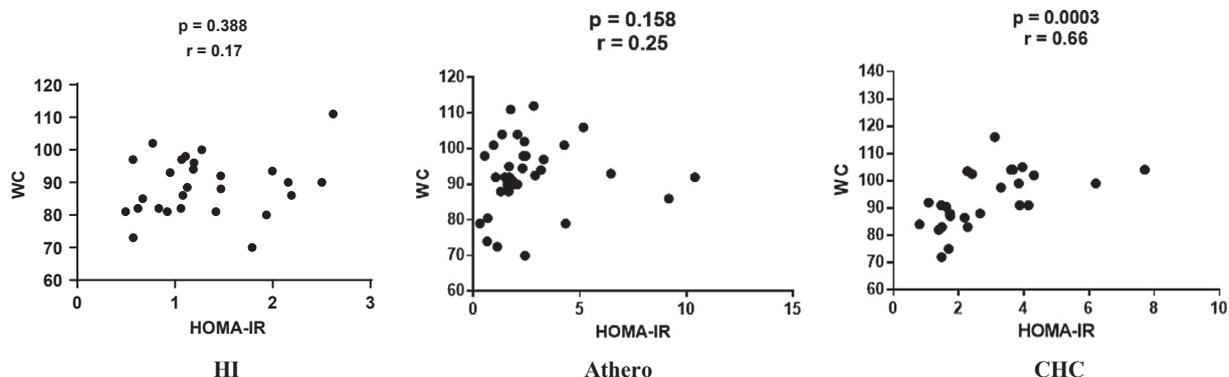


Fig. 1. Correlation between waist circumference (WC) and insulin resistance (HOMA-IR) in healthy individuals (HI) and in patients with atherosclerosis and chronic hepatitis C (Athero and CHC, respectively). Spearman's tests were used for statistical analysis.

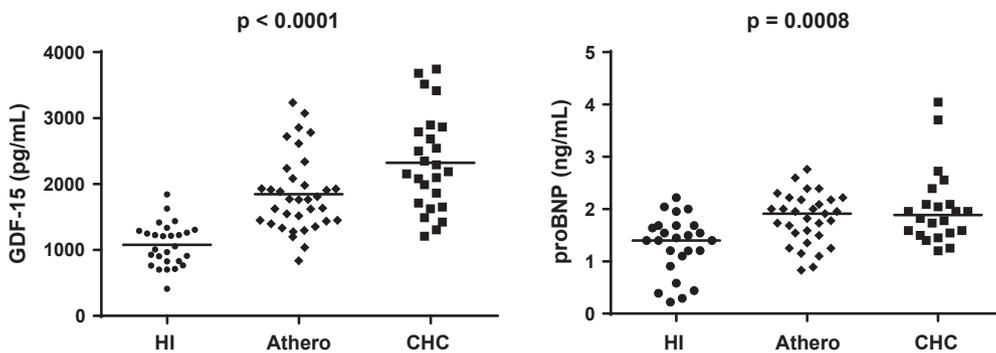


Fig. 2. Serum levels of GDF-15 and proBNP in healthy individuals (HI), in patients with atherosclerosis (Athero) and in subjects with chronic hepatitis C (CHC). The levels of these cardiac markers were determined using ELISA. GDF-15 levels were compared using One-Way ANOVA and the Tukey's post hoc test, while proBNP levels were compared with the Kruskal-Wallis test and the Dunn's post hoc test.

the PBMCs with the CRP levels or with the atherogenic indexes observed in the CHC subjects (N: L, APOB: APOA and TG: HDL-C ratios). Similarly, there were no correlations between the levels of the cytokines produced by the PBMCs and the serum levels of IL-8, MCP-1, and LAP-TGF- β (Fig. 5).

4. Discussion

The present study investigated clinical conditions and bioactive molecules associated with cardiovascular diseases in patients chronically infected with HCV using individuals with atherosclerosis and history of cardiovascular disease as reference. Similar to results from previous reports, we found a prevalence of hypertension in the individuals with atherosclerosis [27,28]. Cardiovascular events, such as acute myocardial infarction and stroke, were rarely reported. Interestingly, an unaltered lipid profile was observed in the HCV-infected individuals, with normal HDL, TC, LDL, and TG levels. However, individuals with high viral load had lower serum levels of TC.

Patients with CHC and healthy controls presented similar APOB: APOA-I ratio that differed from that of CAD individuals. Although the TG: HDL-C ratio of the CHC group was like that of the CAD group, it differed from the TG: HDL-C ratio presented by healthy subjects. Nevertheless, in the three groups, this atherogenic index was within the reference limits.

Low CRP levels and N:L ratio were observed in Brazilian CHC patients. These results corroborate similar results previously reported outside Brazil [29–31]. Although liver injury could cause a decrease in CRP synthesis, we did not demonstrate a correlation between CRP levels and ALT levels or N:L ratio. We also did not find an association between CRP levels and advanced liver fibrosis.

Insulin resistance has frequently been observed in patients with CHC and was initially associated with advanced liver fibrosis, as well as with an inadequate viral response to the treatment [32]. In our study, 50% of the CHC patients had IR, but not a DM2 diagnosis, validating

the findings of a previous study performed by our group [33]. The primary causal factors for the development of IR included obesity and augmented waist circumference. However, we only found a correlation of IR with the last [34]. Although some evidence suggests a positive relationship between HOMA-IR and viral load, fibrosis grade and hepatic steatosis, we and others did not find such correlations [35,36]. Thus, we suggest that chronic HCV infection induced IR regardless of the occurrence of liver injury.

Serum levels of GDF-15 were higher in the CHC patients, confirming the results from previous studies [17,37–39]. In patients with CAD, there was an increase in the levels of GDF-15, but it was lower than that observed in the CHC group. GDF-15 is a transforming growth factor β (TGF- β) associated with infection, fibrosis, and apoptosis that up-regulates the HCV replication and alters cell signaling pathways and growth, being related to the development of hepatocellular carcinoma [17]. Here, we did not find a relation between GDF-15 levels and blood HCV load or liver fibrosis. Nevertheless, the small number of patients with advanced fibrosis and the absence of individuals with hepatocellular carcinoma in the present study could explain our results. Thus, the inverse correlation between serum levels of apolipoprotein B and GDF-15 seems to be associated with the positive regulation of GDF-15 on HCV replication, which involves the interaction between HCV envelope proteins E1-E2 and APOB and elimination of misfolded APOB by autophagy, lowering the serum level of this apolipoprotein [40].

The serum levels of proBNP were higher in patients with CHC and CAD, when compared with healthy individuals, confirming a few reports on the increase of the serum level of this biomarker in hepatitis C [29,41,42]. Unlike for NT-proBNP and BNP, there are no established reference values for proBNP; however, a previous study showed a strong correlation between these forms of BNP in subjects with heart failure [43]. Thus, the use of proBNP as a biomarker of cardiac injury in patients with CHC still needs to be fully investigated.

In the present study, we compared the ability of blood mononuclear cells from CHC patients and CAD individuals to produce atherogenic

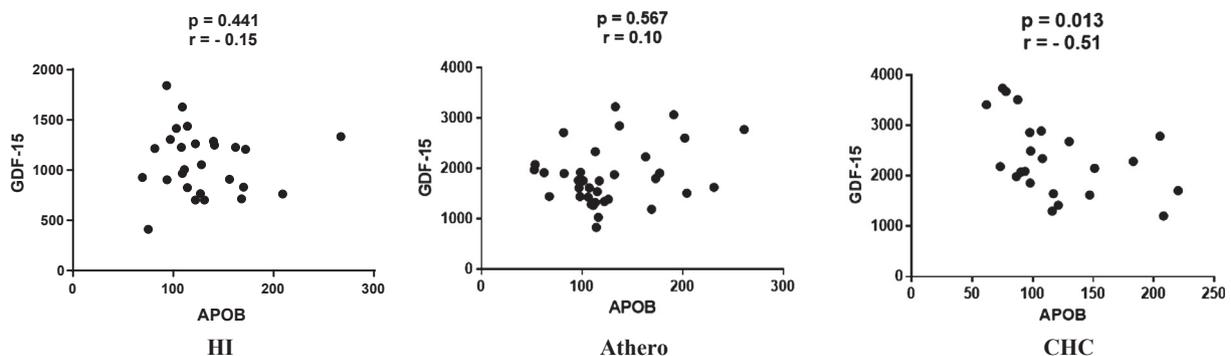


Fig. 3. Correlation between GDF-15 and APOB levels in healthy individuals (HI) and in patients with atherosclerosis and chronic hepatitis C (Athero and CHC, respectively). The serum levels of GDF-15 were determined using ELISA, while APOB levels were measured by nephelometry. Spearman's tests were used for statistical analysis.

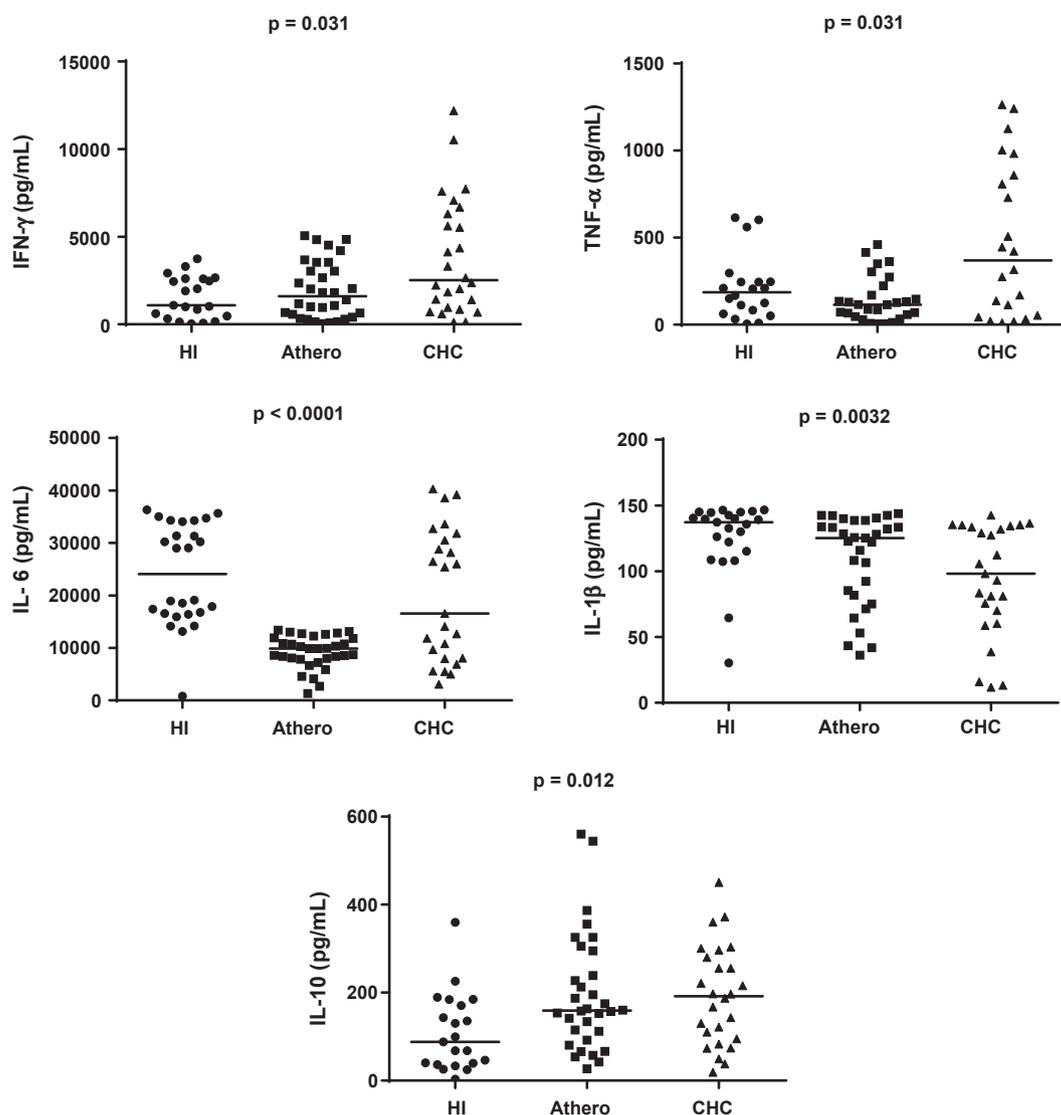


Fig. 4. Levels of cytokines in the culture supernatants from peripheral blood mononuclear cells from healthy individuals (HI), atherosclerosis patients (Athero) and subjects with chronic hepatitis C (CHC) stimulated with phytohemagglutinin (5 $\mu\text{g}/\text{mL}$, for 72 h). Cytokine levels were determined using a Th1/Th2 cytokine bead array in the FACS Canto II cytometer, and their medians were compared using Kruskal-Wallis tests followed by Dunn's multiple comparisons tests.

and anti-atherogenic cytokines and chemokines and verified that PBMC from CHC patients produced more IFN- γ , TNF- α and IL-6 after stimulation with PHA. However, the production of IL-1 β , IL-10 and the serum levels of IL-8, MCP-1 and TGF- β 1 (LAP TGF- β 1) in CHC and CAD patients were similar.

The high levels of pro-atherogenic cytokines (IFN- γ , TNF- α , IL-6) and IL-8 in the CHC group, in comparison to those in patients with proven atherosclerosis, may be explained in our study by the anti-inflammatory effect of hydroxymethylglutaryl CoA reductase inhibitors used in the lipid-lowering treatment in the individuals with CAD [44]. Previous results from our group corroborated this information, showing significant reduction of IL-1 β and IL-8 in individuals treated with statins who exhibit different degrees of coronary stenosis [45].

In this study, the production of IL-10 by PBMCs or the serum level of TGF- β 1, both immunoregulatory cytokines that down-regulate atherogenesis, were different from the classical findings suggesting a decrease in the expression of these immune mediators in atherosclerosis and related diseases [46]. However, some studies have shown unaltered levels of serum IL-10 and the production of IL-10 by PBMCs of HCV infected patients, as well as unaltered serum levels of TGF- β [47,48].

Based on the analysis of our findings, we concluded that the patients

chronically infected with HCV exhibited significant IR, elevated serum levels of GDF-15 and increased production of pro-inflammatory cytokines. However, the presence of IR or increased biomarkers of inflammation in these individuals did not represent an increased risk of cardiovascular disease, nor was it associated with viral load, or severity of liver injury. However, it is important to mention that this study has some limitations, among which are the small number of participants and the small number of individuals with more advanced stages of liver fibrosis and those evaluated for hepatic steatosis. Thus, the disagreements between some of our results and previously reported findings can be explained by the differences in population characteristics, genetic variability of the HCV, and by the different methodological approaches used to study cytokine profiles and CVD biomarkers in patients with CHC and CAD.

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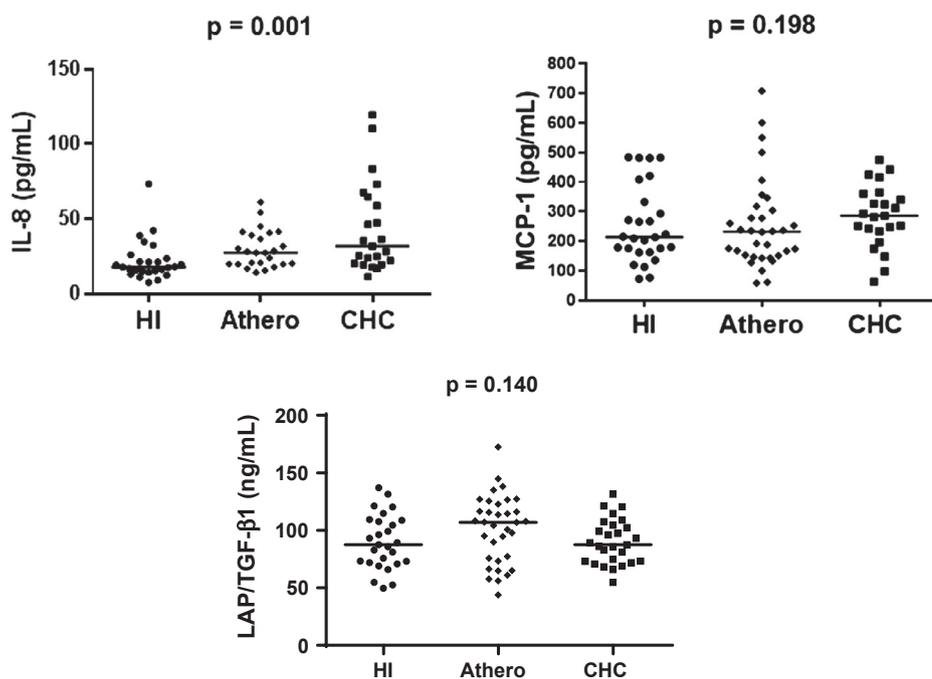


Fig. 5. Serum levels of chemokines (IL-8 and MCP-1) and of LAP-TGF-β1 in healthy individuals and in patients with atherosclerosis and chronic hepatitis C (Athero and CHC, respectively). The serum levels of the immune mediators were determined using ELISA and their medians were compared using Kruskal-Wallis tests followed by Dunn's multiple comparisons tests.

study's design, data collection and analysis, writing of the report, or the decision to submit the report for publication.

Conflict of interest

The authors declare that they have no conflicts of interest associated with this study.

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