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Major Article

IgG1 and IgG4 antibodies against Core and NS3 antigens of hepatitis C virus

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Abstract

Introduction: IgG subclasses involved in the immune response to hepatitis C virus (HCV) antigens have been rarely studied. We investigated the immune response mediated by IgG1 and IgG4 antibodies against the recombinant core and NS3 antigens in patients with chronic hepatitis C. **Methods:** Sixty patients infected with HCV genotype 1 without antiviral treatment and 60 healthy subjects participated in the study. Serum levels of alanine aminotransferase, HCV viremia, and the presence of cryoglobulinemia and liver fibrosis were determined. We investigated the serum IgG1 and IgG4 antibodies against recombinant HCV core and NS3 non-structural protein antigens using amplified indirect ELISA. **Results:** Anti-core and anti-NS3 IgG1 antibodies were detected in 33/60 (55%) and 46/60 (77%) patients, respectively, whereas only two healthy control samples reacted with an antigen (NS3). Anti-core IgG4 antibodies were not detected in either group, while 30/60 (50%) patients had anti-NS3 IgG4 antibodies. Even though there were higher levels of anti-NS3 IgG4 antibodies in patients with low viremia ($< 8 \times 10^5$ IU/mL), IgG1 and IgG4 antibody levels did not correlate with ALT levels, the presence of cryoglobulinemia, or degree of hepatic fibrosis. High production of anti-core and anti-NS3 IgG1 antibodies was observed in chronic hepatitis C patients. In contrast, IgG4 antibodies seemed to only be produced against the NS3 non-structural antigen and appeared to be involved in viremia control. **Conclusions:** IgG1 antibodies against structural and non-structural antigens can be detected in chronic hepatitis C, while IgG4 antibodies seem to be selectively stimulated by non-structural HCV proteins, such as the NS3 antigen.

Keywords: Hepatitis C. IgG1 antibodies. IgG4 antibodies. Core antigen. NS3 antigen. Viremia.

INTRODUCTION

Viral hepatitis C is the leading cause of liver disease worldwide, with a high prevalence in populations across several geographic regions. The overall prevalence of seropositivity for anti-hepatitis C virus (HCV) antibodies has been estimated to be 1.6% (1.3%–2.1%), corresponding to 115 million infected individuals worldwide[1,2].

During HCV infection, the appearance of biomarkers is observed in the following sequence: viral RNA, antigenemia, and anti-HCV antibodies. Total IgG antibodies are routinely investigated, as IgM antibodies are irregularly produced in acute HCV infection and intermittently produced during the chronic phase of hepatitis C[3]. The kinetics of the humoral (antibody-based) immune response consists initially of antibodies against epitopes of the non-structural protein NS3 and viral capsid. As infection progresses, antibodies against NS4 epitopes and against E1 and E2 envelope glycoproteins are detected. Neutralizing antibodies directed toward the hypervariable region of the E2 structural glycoprotein have been documented; however, their importance regarding viral replication control is controversial. Nevertheless, studies on the conformational epitopes of E1 and E2 glycoproteins suggest a protective role of antibodies with such specificities in HCV infection[4].

The identification of such antibodies is straight-forward in patients with the classical form of chronic HCV infection but not in cases of occult hepatitis C. Furthermore, standard IgG antibody and viral RNA detection in the blood may be negative in patients with altered liver function laboratory tests and in patients undergoing hemodialysis[5,6].

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Despite the current knowledge on the adaptive immune response mediated by total anti-HCV IgG antibodies, investigation of immune responses mediated by isotypes of IgG antibodies has been neglected, with only rare reports available on their antigen specificity[7,8]. One study investigated the immune response of IgG subclasses against structural and non-structural antigens in 10 untreated patients, and an effective response of IgG1 antibodies against the core antigen and NS3 was observed, which dropped after a sustained virological response[8].

The role of different IgG subclasses in the immune response to HCV antigens has been rarely studied, limiting a better knowledge of their participation in the adaptive immune response of patients infected with hepatitis C virus. Within this context, the objective of this work was to investigate the adaptive immune response specific to IgG1 and IgG4 antibodies against recombinant antigens of the core and non-structural NS3 protein in patients with chronic hepatitis C.

METHODS

Patients and healthy controls

In this study, 60 patients with chronic C virus infection of type 1 genotype were included. No patients were receiving treatment, both sexes were included, patients were aged between 21 and 71 years, and all were recruited from the Hepatitis Clinic of the Hospital Prof. Edgard Santos, Federal University of Bahia, Brazil. To be included in the study, patients were required to have a clinical diagnosis of chronic viral hepatitis C, which had been documented through clinical examination, positive serology for the presence of anti-HCV IgG antibodies, identification of the viral genotype, and viral load. For patients who had undergone a previous liver biopsy, histology results were retrieved from their medical records, and information about the level of fibrosis was evaluated according to the METAVIR system[9]. Patients with autoimmune diseases, viral co-infections (HCV/HBV, HCV/HIV, HCV/HTLV), or tuberculosis were not included. As controls, we included 60 healthy volunteers, matched by sex and age, with IgG-negative serology for HCV, and without any of the above-mentioned study exclusion criteria.

Laboratory methods

Laboratory exams were performed on sera from blood collected in a vacuum system, using 10 mL tubes without anticoagulant. After coagulation and separation, serum aliquots were stored at -80°C until use, except for cryoglobulin, which was immediately tested.

Serum cryoglobulin was investigated by cryoprecipitation testing, using a Wintrobe tube and incubation at 4°C for up to seven days. The dissolution of the cryoprecipitate after 1 h incubation at 37°C confirmed its presence[10]. ALT activity was determined in serum using a Kinetic-UV assay and a Labmax 240 analyzer (Labtest Diagnostica S. A, Lagoa Santa, MG, Brazil). An ALT level ≤ 41 U/L was used as a reference.

The data for HCV genotyping, liver fibrosis, and viral load in patients were collected from medical records. HCV genotyping was performed using the Versant HCV genotype assay LiPA 2.0 system (SIEMENS Healthcare Diagnósticos S. A., São Paulo, SP, Brazil), while the viral load was determined by real-time polymerase chain reaction (RealTi*m*e HCV, Abbott Laboratories, Abbott Park, Illinois, USA). Histological examination of the liver was performed according to the METAVIR system, using the following fibrosis scores: F0 = absence of fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with rare septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis[9].

The investigation of IgG1 and IgG4 antibodies against recombinant HCV antigens was performed using an indirect ELISA. In this immunoassay, the internal surface of flat bottom polystyrene microwells (Nunc-Immuno module, ThermoFisher, Waltham, MA, USA) was covered with the following HCV recombinant antigens (ABCAM, Cambridge, MA, USA): core (aa 2 to 192) and NS3 (aa 1450 to 1643). One hundred microliters of the solution containing 10 µg/mL of each antigen in carbonate buffer (0.1 M, pH 9.6) were added to separate microwells and incubated at 4°C for 18 h. The microwells were washed with PBS-T (150 mM NaCl/10 mM sodium phosphate, plus 0.05% Tween 20; pH 7.4), and their free sites were blocked with 0.5% bovine serum albumin (BSA; Sigma-Aldrich Brazil Ltda, São Paulo, SP, Brazil) in PBS-T for 1 h at 22°C.

IgG1 and IgG4 antibodies were tested using serum diluted at 1:100 and 1:25 in PBS-T plus 0.5% BSA, respectively. The primary antibody reactions were carried out by incubating 100 µL diluted serum samples in the antigen microwells for 1 h at 22°C. Following washing with PBS-T, the microwells were incubated with 100 µL mouse anti-IgG1 or anti-IgG4 monoclonal antibody solution (Sigma-Aldrich), diluted at 1:3,000 in PBS-T-BSA, for 1 h at 22°C.

After further washing, 100 µL of a biotinylated conjugate of goat anti-mouse IgG, diluted at 1:50,000 in PBS-T-BSA, was added to the microwells for 30 min at the same temperature. New wash cycles were performed, followed by addition of 100µL avidin-peroxidase conjugate (Sigma-Aldrich), diluted at 1:100,000 in PBS-T-BSA, for 15 min at 22°C. After washing, the reactions were incubated with a chromogenic substrate of hydrogen peroxide/tetramethylbenzidine for 15 min, at 22°C in the dark. Enzyme reactions were blocked with HCl 0.2 N, and their absorbances were read at 450–620 nm using an ELISA reader. The cut-off points of the ELISAs were statistically determined with 95% CI using sera from 10 healthy individuals that had both negative IgG serology and HCV RNA[11].

Statistical analysis

The D'Agostino–Pearson test was used to analyze the distribution of variables. According to the distribution, values were expressed as mean ± SD or median and interquartile range (Q1–Q3). Differences between groups were assessed using the unpaired *t*-test or non-parametric Mann–Whitney test, depending on the distribution. The correlation analyses were performed using either the Pearson or Spearman test, according to the distribution. Exact Fisher and Chi-square tests were applied to analyze associations between categorical groups. Diagnostic test evaluation was performed using a 2 x 2 table. Further, an estimated 1.01% prevalence of chronic hepatitis C in Bahia was used to calculate the sensitivity, specificity, and positive and negative predictive value

of the ELISAs with the HCV recombinant antigens. A p-value < 0.05 indicated a statistically significant difference. For descriptive statistical analyses of the results, the GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) and MedCalc Statistical Software version 18.10 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018) programs were used.

Ethical considerations

All participants were informed about the study and signed the Free and Informed Consent Form. The study was approved by the Ethics Committee of the Nursing School of Bahia Federal University.

RESULTS

Characterization of the study cohort

This study evaluated 60 individuals chronically infected with HCV (genotype 1). The demographic, clinical, and virological data of the study cohort are presented in **Table 1**. As shown, there was a similar proportion of infected men and women, with non-significant differences in age. A similar percentage of individuals with low and high viral loads was observed. A slight increase in ALT was observed in most HCV patients, although not statistically significant. Likewise, the percentage of individuals with no fibrosis, or mild to moderate fibrosis, was similar to patients with advanced degrees of fibrosis. The same was observed for the absence or presence of cryoglobulinemia.

IgG1 and IgG4 antibodies

All healthy controls and patients were tested for the presence of IgG1 and IgG4 antibodies against recombinant core antigens and the HCV NS3 non-structural protein, using the indirect ELISA technique and a statistically calculated cut-off (confidence level, 1-α = 95%; Anti-core IgG1 Ab, 0.207; Anti-core IgG4 Ab, 0.135; Anti-NS3 IgG1 and Anti-NS3 IgG4 antibodies, 0.083 and 0.115, respectively). We observed that 33/60 (55%) patients were seropositive for IgG1 antibodies against the core antigen, while none had IgG4 antibodies with this immunological reactivity (**Figure 1**).

IgG1 antibodies against the NS3 recombinant antigen were detected in 46/60 (77%) individuals, while 30/60 (50%) reacted with IgG4 antibodies of this antigen specificity. Healthy controls did not react with IgG1 or IgG4 antibodies against the core antigen, but two subjects exhibited a weak reaction with the NS3 non-structural protein (**Figure 1**). Anti-NS3 IgG1 antibodies were more prevalent than anti-core IgG1 antibodies (77% and 55%, respectively; p = 0.011). Additionally, the proportion of seropositive individuals for anti-NS3 IgG1 was higher than anti-NS3 IgG4 antibodies (77% and 50%, respectively, p = 0.002). The diagnostic characteristics of the immunoassays are presented in **Table 2.**

Anti-core and anti-NS3 antibodies and virological and clinical parameters

Anti-core and anti-NS3 IgG1 antibody levels did not differ when comparing patients according to viremia, presence or absence of cryoglobulinemia, ALT levels, and different degrees of fibrosis (**Figures 2 and 3**). Even though there were higher levels of anti-NS3 IgG4 antibodies in patients with low HCV viral load, the levels of these antibodies did not correlate with any of the other parameters analyzed (**Figure 4**).

TABLE 1: Demographic, clinical, and virological data for 60 hepatitis C patients.

Characteristics	n (%)	p-value
Gender		
Men	28/60 (47%)	
Women	32/60 (53%)	0.513
Age (years)		
Men	52 ± 09	
Women	56 ± 08	0.031
HCV genotype 1	60/60 (100%)	ND
Blood HCV load		
Low (< 8 × 10^5 UI/mL)	31/60 (52%)	
High (> 8 × 10^5 UI/mL)	29/60 (48%)	0.759
ALT (U/L)		
< 41 (U/L)	22/60 (37%)	
> 41 (U/L)	38/60 (63%)	0.054
Liver histology		
F0–F2	37/60 (62%)	
F3–F4	23/60 (38%)	0.073
Cryoglobulinemia		
Negative	36/60 (60%)	
Positive	24/60 (40%)	0.132

Results are presented as means ± SD or proportions (%). The means were compared using Student *t*-tests, whereas the proportions were compared using Chi-square tests. **F0–F2:** no fibrosis or mild to moderate fibrosis, **F3–F4:** advanced fibrosis. **ALT:** alanine aminotransferase; **HCV:** hepatitis C virus; **ND:** not done. A p-value < 0.05 was considered significant.

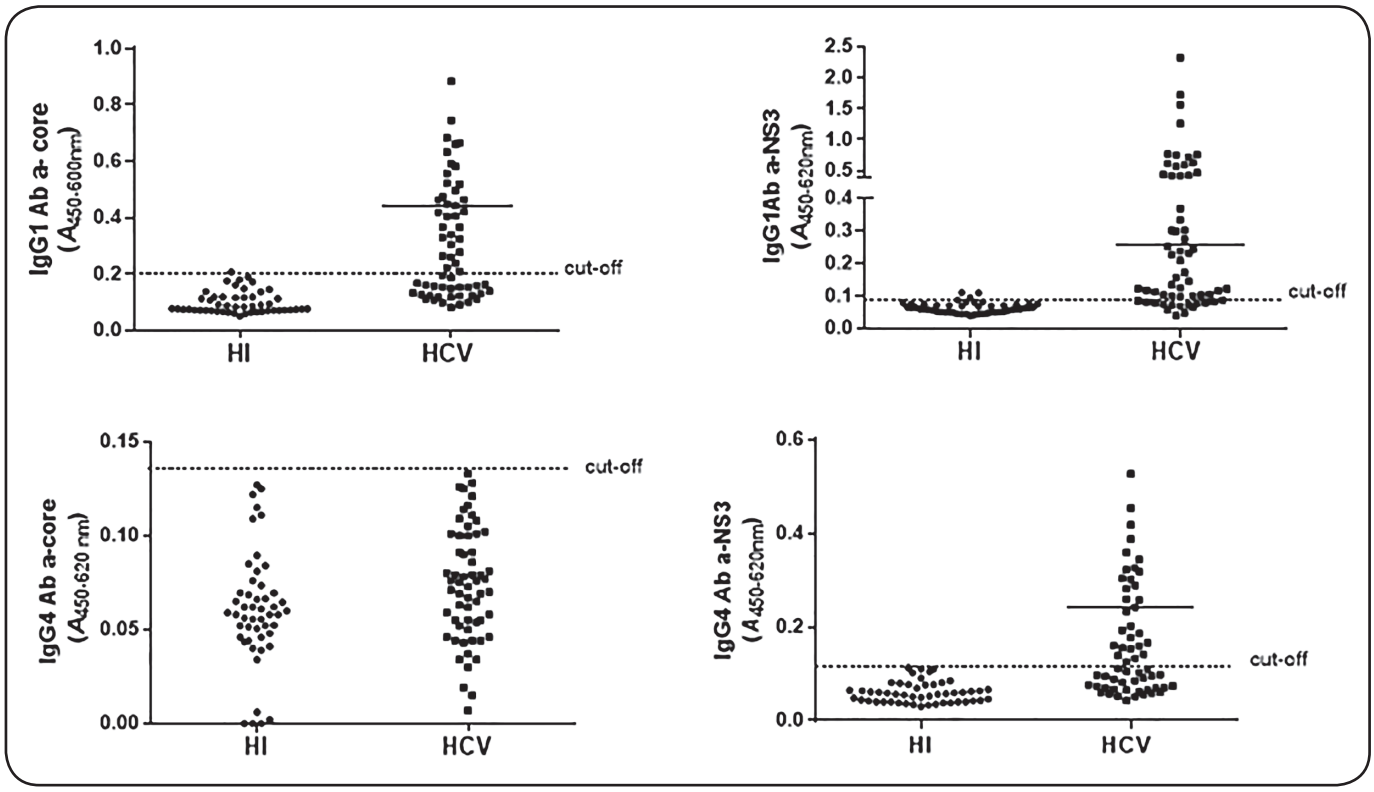

FIGURE 1: IgG1 and IgG4 antibodies against HCV recombinant antigens of core and NS3 proteins in sera from healthy individuals (HI) and subjects chronically infected with hepatitis C virus (HCV). Antibodies were detected using indirect ELISA tests. Solid horizontal bars represent the median levels of positive sera.

TABLE 2: Diagnostic characteristics of indirect ELISAs with HCV core and NS3 recombinant antigens.

Test	Sensitivity (%)	Specificity (%)	AUC	Positive Predictive Value (%)	Negative Predictive Value (%)
IgG1 anti-Core	55	100	0.77	100	99
IgG1 anti-NS3	77	97	0.87	19	100
IgG4 anti-NS3	50	100	0.75	100	99

The immunoassays tested for 60 chronically infected HCV Gen1 patients and 60 healthy individuals with negative tests for hepatitis C.

Correlation between antibody levels, ALT levels, and HCV viral load

The levels of anti-core and anti-NS3 antibodies did not correlate with ALT levels nor HCV viremia (data not shown). Although there was a statistically significant correlation between anti-NS3 IgG4 and viral load levels ($r = -0.26$, $P = 0.048$), the number of samples was insufficient to biologically validate the obtained coefficient (type I error, $\alpha = 0.05$, type II error, $\beta = 0.2$; required samples, $n = 113$).

DISCUSSION

In the present study, we investigated the presence of two subclasses of IgG antibodies, IgG1 and IgG4, with antigen specificity for epitopes of two recombinant HCV antigens: core protein and NS3 non-structural protein. Such recombinant antigens stimulate a strong immune response in peripheral Th17 cells of patients with chronic hepatitis C not receiving treatment[12]. Both antigens are important for the HCV cycle, actively participating in its replication and evasion of the host's immune response. The core protein exerts a suppressive effect on TCD4+ and TCD8+ lymphocyte activation, inhibiting IL-2 expression and increasing IL-10 production, in addition to promoting B lymphocyte proliferation and stimulating production of IgG and IgM antibodies[13]. In addition to cleaving the viral polyprotein, the NS3 non-structural protein participates in HCV replication and forms a complex with its cofactor, the non-structural protein NS4, which contributes to viral escape from the interferon-type I innate response[14].

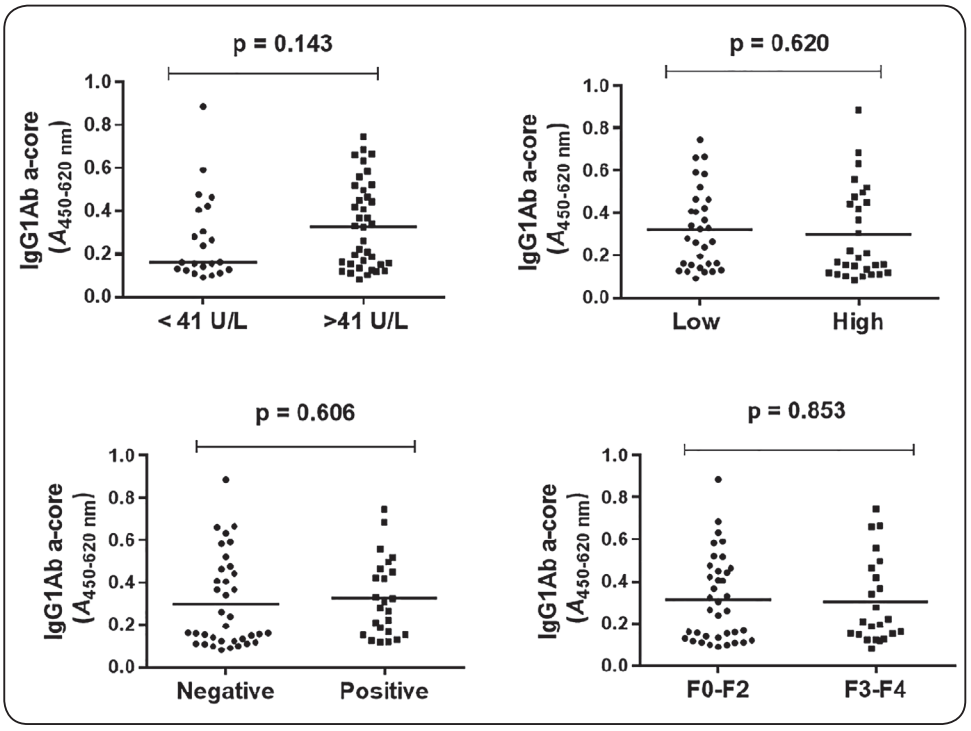

FIGURE 2: IgG1 antibodies against anti-HCV core in patients according to their ALT levels, presence of cryoglobulinemia, levels of blood HCV load (low, < 8 × 10^5 IU/mL; and high, > 8 × 10^5 IU/mL), and liver fibrosis stage (F0–F2, absent or mild to moderate; F3–F4, advanced fibrosis). Horizontal bars represent the medians, which were compared using Mann–Whitney tests. A p-value < 0.05 was considered significant. **ALT:** alanine aminotransferase; **HCV:** hepatitis C virus.

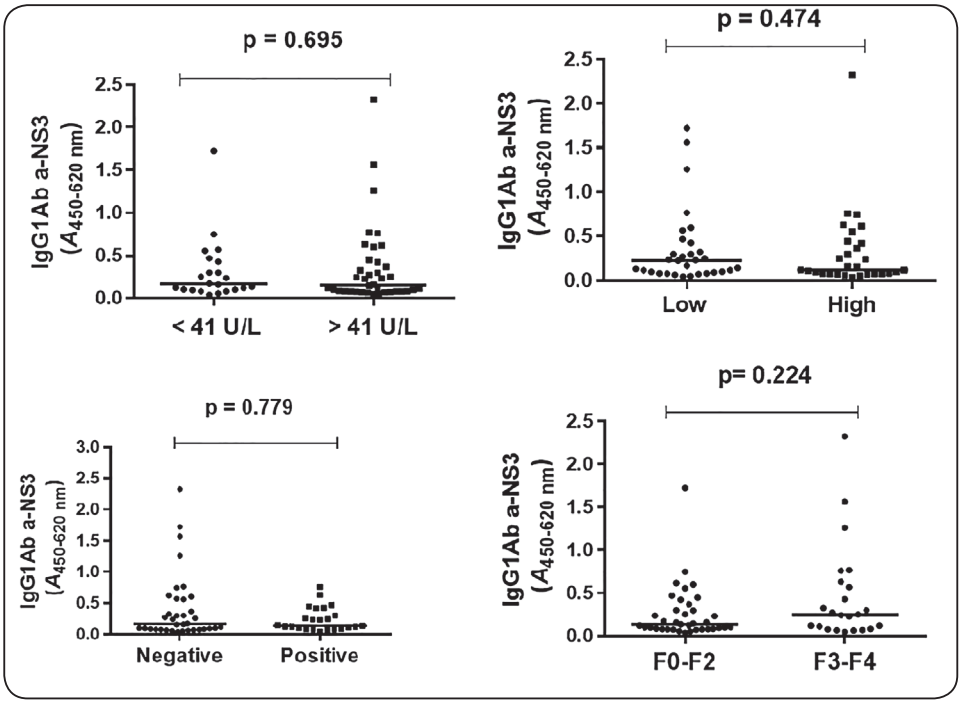

FIGURE 3: IgG1 antibodies against anti-HCV NS3 protein in patients according to their ALT levels, presence of cryoglobulinemia, levels of blood HCV load (low, < 8 × 10^5 IU/mL; and high, > 8 × 10^5 IU/mL), and liver fibrosis stage (F0–F2, absent or mild to moderate; F3–F4, advanced fibrosis). Horizontal bars represent the medians, which were compared using Mann–Whitney tests. A p-value < 0.05 was considered significant. **ALT:** alanine aminotransferase; **HCV:** hepatitis C virus.

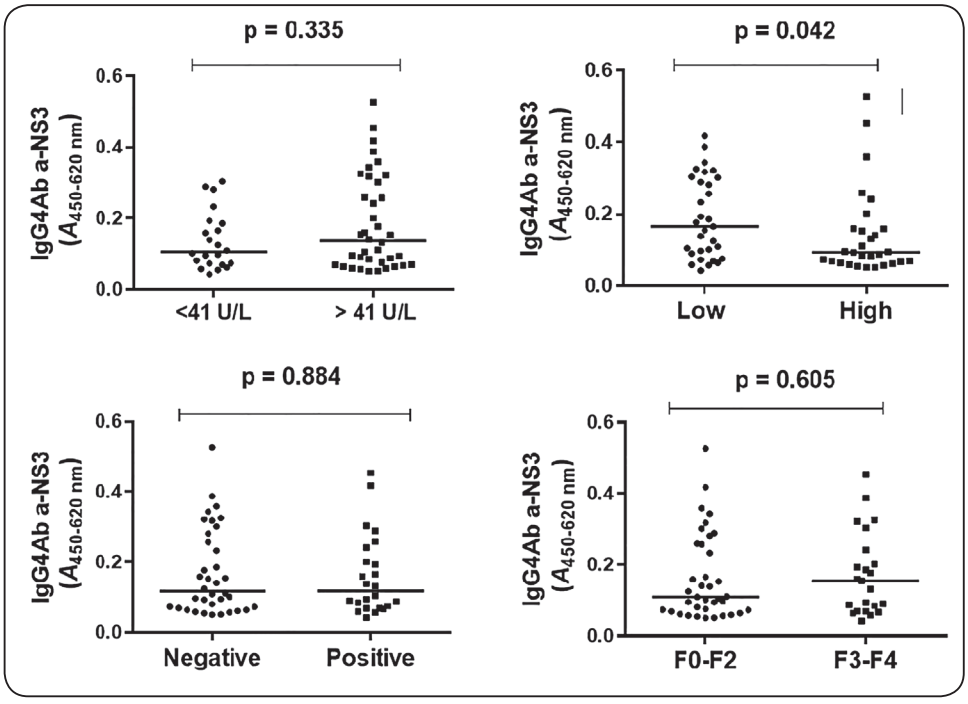

FIGURE 4: IgG4 antibodies against anti-HCV NS3 protein in patients according to their ALT levels, presence of cryoglobulinemia, levels of blood HCV load (low, < 8 × 10^5 IU/mL; and high, > 8 × 10^5 IU/mL), and liver fibrosis stage (F0–F2, absent or mild to moderate; F3–F4, advanced fibrosis). Horizontal bars represent the medians, which were compared using Mann–Whitney tests. A p-value < 0.05 was considered significant. **ALT:** alanine aminotransferase; **HCV**: hepatitis C virus.

Here, we demonstrate that untreated patients chronically infected with HCV genotype 1 may present serologic reactivity to these antigens. However, significant differences were shown for the two subclasses of antibodies detected. Thus, while IgG1 antibodies could be detected in patients against core and NS3 antigens, the same did not occur with IgG4 antibodies, whose serological reactivity was only against the NS3 antigen. Further, a higher proportion of seropositive patients was observed with anti-NS3 IgG1 antibodies than that with anti-core IgG1 and anti-NS3 IgG4 antibodies. The antibody response against core and NS3 antigens in this study were consistent with a previous report using ten hepatitis C patients residing outside of Brazil[15]. Further, they are in agreement with pioneering observations obtained using Chiron RIBA HCV-titering Strip Immunoblot Assay that showed a strong antibody response to the NS3 antigen in patients with persistent HCV viremia[8]. Subsequently, similar results were observed using sera from 68 chronic hepatitis C patients infected with different viral genotypes (1, 2, 3, and 4), determined by Western Blotting. There were no differences between immune responses mediated by the various HCV genotypes, and the main immunogens were the capsid and non-structural proteins NS3, NS4B, and NS5B[16].

Levels of anti-core and anti-NS3 IgG1 antibodies were not related to the viral load in patients nor to the presence of cryoglobulinemia, ALT levels, or degree of liver fibrosis. In contrast, anti-NS3 IgG4 antibody levels were higher in patients with lower viremia, suggesting the involvement of these immunoglobulins in the control of HCV replication, since the NS3 protein is directly involved in this viral process. Although there was a statistically significant correlation between anti-NS3 IgG4 antibody levels and viremia, it could not be biologically validated using our study's patient sample size (n = 60; error type I = 0.05, error type II = 0.2).

IgG1 immunoglobulin is the highest produced IgG subclass in the body, being the most abundant immunoglobulin of this isotype detected in secondary immune responses. IgG1 activates the classical complement pathway and possesses a precipitating capacity. The production of IgG1 antibodies is regulated by IL-21/IL-4/IL-10, and its Fcγ region confers the ability of these immunoglobulins to interact strongly with different Fcγ receptors, which are present in several cell types [17,18].

In contrast, IgG4 antibodies are present at lower serum concentrations. IgG4 antibodies interact weakly with Fcγ cell receptors and are unable to activate complement or cross-link epitopes of the same antigen, which is related to their property of recombining their combinatorial sites (Fab-Arm Exchange), forming antibodies with dual-antigenic specificity[19]. Induction of IgG4 antibodies depends on chronic or repeated exposure to antigens, the presence of IL-4, IL-13, and IL-10, and regulatory T cells[20]. IgG4 antibodies have been found mainly in autoimmune diseases, such as pemphigus and myasthenia gravis[21,22]. They have also been described in parasitic infections, such as schistosomiasis and toxoplasmosis, and in some viral diseases such as dengue[23]. IgG4 antibodies compete with other Ig subclasses for the same antigen. In allergic reactions, they behave as blocking antibodies, competing with IgE

to prevent basophil and mast cell activation and histamine release. Furthermore, IgG4 antibodies may interfere with T cell activation mediated by IgE antibody-regulated antigen presentation[24,25]. Thus, production of anti-NS3 IgG4 antibodies could result from the persistence of HCV infection, along with Th2 profile cytokines, IL-10, and regulatory T cells.

Although the results presented here are promising, the present study had some limitations, as mentioned above. Thus, it is necessary to test the presence of anti-NS3 IgG1 and IgG4 antibodies in a larger cohort of patients with chronic hepatitis C, including subjects infected with other HCV genotypes. Further, the impact of SVR on production of these antibodies deserves future investigation to elucidate the relationship between anti-NS3 IgG4 antibodies and HCV viremia. In conclusion, the immune response of IgG1 and IgG4 isotypes to structural and non-structural HCV antigens seems to differ in antigen specificity. Thus, while IgG1 antibodies can be produced against both antigen types, non-structural HCV proteins, such as the NS3 antigen, can selectively stimulate the immune response of IgG4 antibodies.

Conflict of Interest: The authors declare that there is no conflict of interest.

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