

IL28B SNPs rs12979860 and rs8099917 Are Associated with Inflammatory Response in Argentine Chronic HCV Patients

Andrés Machicote¹, Diego Flichmann², Eloisa Arana^{1,3}, Silvia Paz⁴, Hugo Fainboim⁴, Leonardo Fainboim^{1,3}, Pablo M. Fernández^{1,3*}

¹Instituto de Inmunología, Genética y Metabolismo (INIGEM), Hospital de Clínicas, Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina

²Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

³Departamento de Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

⁴Unidad de Hepatología, Hospital "Francisco J. Muñiz," Buenos Aires, Argentina

Email: *pfernandez1120@gmail.com

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Abstract

Background: Hepatitis C virus (HCV) is a major cause of chronic liver disease, including cirrhosis and liver cancer. The aim of our study was to determine whether IL28B single nucleotide polymorphisms (SNPs) rs12979860 and rs8099917 can be considered a prognostic host factor in untreated chronic HCV patients. **Methods:** We set up a real-time Allele Specific PCR amplification to determine the allele present in each polymorphic site, and statistically grouped and compared this result with clinical data. **Results:** We determined rs12979860 and rs8099917 genotype and allele frequencies in a single cohort of untreated chronically HCV-infected patients. We found significant associations between higher inflammatory activity, measured as ALT levels or METAVIR scores and rs12979860 CC ($P = 0.0013$ and $P = 0.0033$, respectively) and rs8099917 TT ($P = 0.0005$ and $P = 0.0264$, respectively) genotypes. Interestingly, considering both genotypes together, we also found association with ALT levels ($P = 0.0003$; OR = 5.125) or METAVIR scores ($P = 0.0038$; OR = 5.179), suggesting an additive effect on liver inflammation in these patients. **Conclusion:** we show association between hepatic inflammatory activity in a single Argentinean untreated chronically HCV cohort and SNPs located in the interferon lambda gene region. The studied polymorphisms, together with further innate and adaptive immune responses, clearly play a role in modulating the HCV infected patients outcome, contributing to hepatic inflammation and possible fibrosis/cirrhosis.

Keywords

Hepatic Inflammation, Viral Hepatitis, Chronic Hepatitis C, IL28B, Latin American Patients

1. Introduction

Chronic hepatitis C is a liver disease caused by the hepatitis C virus (HCV), a blood-borne virus mostly transmitted through unsafe injection practices, but also from inadequate sterilization of medical equipment, or unscreened blood and blood products. HCV can cause both, self limited and chronic infections, ranging in severity from a mild, few weeks lasting illness, to a serious, lifelong illness. Based on World Health Organization reports, 150 million people are infected with chronic hepatitis C (approximately 600,000 in Argentina), and around 500,000 people die each year from hepatitis C-related liver diseases [1]. Unfortunately a vaccine for hepatitis C is not currently available. Early diagnosis of the HCV infection is rare. Those people who go on to develop chronic HCV infection remain undiagnosed, often until serious liver damage has developed. Although many direct acting antivirals have been developed in recent years, these are not widely available. The most widespread standard treatment at the moment is interferon alpha plus ribavirin. The success of such therapy depends on host and virus factors [2]. The degree of liver damage, measured by liver biopsies or through a variety of non-invasive tests, together with the determination of HCV genotype, is used to guide therapeutic decisions and management of the disease, to determine the most appropriate approach for each patient. In order to contribute to decisions related to the course of treatment, several groups working in Genome Wide Association Studies identified Single Nucleotide Polymorphisms (SNPs) associated to the outcome of antiviral therapy in chronic HCV patients [3] [4] [5] [6] [7]. Among them the SNPs rs12979860 and rs8099917 showed the most significant statistical relevance. These SNPs that were also associated with spontaneous virus clearance in acute infection [8] [9] [10], and appeared to modify the natural course of disease [11], are located close to the IL28B locus, containing a gene coding for interferon lambda 3 (IFN- λ 3) that belongs to the type III interferon-family [12]. However, it is unclear how these SNPs affect transcription or protein expression. Regarding IFN- λ s, these are known to be able to inhibit virus replication [13] [14], including HCV [15], and share with type I interferon a similar anti-viral effect. The role of these SNPs in the host inflammatory response and evolution of chronic infection in untreated chronic HCV patients is not well understood. Recent studies yielded contradictory results and have shown rs12979860 CC (or rs8099917 TT) association with more advanced fibrosis or cirrhosis [16] [17] and worse clinical outcomes [18], while others have reported rs12979860 TT (or rs8099917 GG) to be associated with more advanced fibrosis or cirrhosis [19] [20] [21] or even no association of

IL28B genotype with fibrosis [22]. The aim of our study was to determine whether SNPs rs12979860 and rs8099917 can be considered a prognostic host factor in untreated chronically HCV-infected patients from an Argentine cohort, by analyzing host haplotypes involved in modulation of patient's immune responses.

2. Materials and Methods

2.1. Study Design

The study was designed and performed (years 2015-2017) using samples stocked during routine medical practice. The study has been approved by the Research and Ethics Committee at both involved centers and performed in accordance with the ethical standards adopted in the Declaration of Helsinki and revised forms. Informed consent was obtained from all donors.

2.2. Subjects

Samples from 150 HCV chronically infected patients were included. All individuals were aged ≥ 18 years, and patients were not undergoing any kind of HCV antiviral therapy. Exclusion criteria included alcohol intake greater than 20 g day⁻¹, history of organ transplantation, creatinine clearance < 50 mL min⁻¹, co-infection with hepatitis B virus or human immunodeficiency virus and African American or Asian ethnicity. Patients were also excluded if they presented evidence of other liver disease, such as autoimmune hepatitis, primary biliary cholangitis, sclerosing cholangitis, Wilson's disease or alpha-1-antitrypsin deficiency.

2.3. Genotype Determination

Genomic DNA was obtained from blood samples using the standard method of phenol:chloroform extraction. Genotypes were determined by allele specific amplification on a real-time PCR detection system (Mx3000P, Stratagene) using SYBR Green as fluorescent DNA binding dye, complementary primer sets in separate tubes, and Taq Platinum polymerase (Invitrogen).

Primers sequences were:

rs12979860:

Forward: 5'-CGCTTATCGCATACGGCTAG-3',

Reverse C: 5'-GCAATTCAACCCTGGTTTCGC-3',

Reverse T: 5'-GCAATTCAACCCTGGTTCAC-3'.

rs8099917:

Forward: 5'-CCTCATCCCCTTCTGGAAC-3',

Reverse T: 5'-CATGGTTCCAATTTGGGTGAAA-3',

Reverse G: 5'-CATGGTTCCAATTTGGGTGACA-3'.

Primer sets yielded a single product of the correct size with their specific DNA templates and no or extremely retarded amplification with the unspecific alleles.

2.4. Histological Parameters

Liver biopsies were obtained from patients, simultaneously with peripheral blood

samples, and before any treatment against HCV infection. Tissue was fixed in formaldehyde (10%) and included in paraffin. After Masson's trichromic stain, inflammatory activity was determined by microscopic analysis attributing METAVIR scores for each patient based on histopathologic features [23]. Patients being attributed METAVIR scores A2 or A3 were considered as having a high inflammatory activity. This activity was also measured indirectly by Alanine aminotransferase (ALT) determination in blood samples, considering as high those values over twice the normal value (N, men: 41 UI mL⁻¹, women: 31 UI mL⁻¹). Patients being attributed METAVIR scores F3 and F4 were considered as presenting advanced fibrosis. For those patients without liver biopsies, ultrasonic transient elastography (Fibroscan 502) study was performed, considering scores F3 and F4 as indicator of advanced fibrosis.

2.5. Statistics

Association between liver inflammatory activity and SNPs was studied with Fisher exact and Chi square tests. Analysis was performed with GraphPad Prism 3.0 (GraphPad Software). *P* values were considered significant when lower than 0.05. Odds ratios (OR) were determined using the following formula: $OR = \frac{A \times D}{B \times C}$, being A the number of patients harboring the studied allele and B the number of patients not harboring the studied allele, both with high inflammatory activity, C, the number of patients harboring the studied allele and D the number of patients not harboring the studied allele, both with low inflammatory activity.

3. Results

3.1. Clinical Features of Patients

A total of 150 untreated chronic HCV-infected patients were included. Clinical features are summarized in **Table 1**. Sixty-two percent were males, with a mean age of 48.9 ± 9.8 years (range 19 - 76 years old). The date of infection could be estimated for 37 patients (25%) being the use of unsafe injections the main infection route (34 patients, 23%). Viral genotype was established for 135 patients. Most of them were infected with genotype 1 (n = 93, 62%), where genotype 1a was found in 43 patients, and genotype 1b in 30 patients. Viral load (n = 113) ranged from 1978 IU mL⁻¹ to 30,800,000 IU mL⁻¹ (Mean: $1,668,031.79 \pm 2,657,526.68$ IU mL⁻¹), Serum ALT levels were determined in 137 patients, obtaining normal values in 71 patients (47%). Hepatic biopsies were available for 91 patients. Among them, 38 patients presented a METAVIR score A2 - A3, considered as advanced inflammatory activity. Fibrosis level was determined in 136 patients, and an advanced level of fibrosis was detected in 49 of them (33%).

3.2. rs12979860 and rs8099917

For rs12979860 the most frequent allele presented a base C at the polymorphic position (60%), being the heterozygous CT genotype the most frequent (45%).

Table 1. Clinical features of patients.

<i>Mean age</i>	48.9 ± 9.8 years
<i>Gender</i>	62% men/38% women
<i>rs8099917 TT genotype</i>	42%
<i>rs12979860 CC genotype</i>	38%
<i>Cause of infection:</i>	
<i>Transfusions</i>	2%
<i>Unsafe injections</i>	23%
<i>Unknown</i>	75%
<i>HCV genotype 1</i>	62%
<i>Mean HCV RNA</i>	1,668,031 ± 2,657,526 IU/mL
<i>Elevated ALT (>2xN)</i>	44%
<i>Biopsy A2 - A3</i>	25%
<i>Metavir F3 - F4 score</i>	33%

Table 2. Genotypes and alleles representation in samples (a) and distribution of coincident alleles (b).

(a)					
		rs12979860		rs8099917	
<u>Genotype:</u>	CC	38%	TT	42%	
	CT	45%	TG	42%	
	TT	17%	GG	16%	
<u>Allele:</u>	C	60%	T	63%	
	T	40%	G	37%	

(b)			
rs12979860	rs8099917	n	
C	T	136	
C	G	18	
T	T	25	
T	G	75	

For rs8099917, allele T was the most represented in samples (63%). Both TT and TG genotypes were equally represented (42%), (**Table 2(a)**). Alleles C (rs12979860) and T (rs8099917) were coincident in 88% of cases, whereas alleles T (rs12979860) and G (rs8099917) were present together in 75% of cases (**Table 2(b)**).

3.3. SNPs rs12979860 and rs8099917 and Inflammatory Activity

The ALT levels were abnormal in 67% of HCV patients harboring rs12979860 genotype CC, whereas this was observed in only 38% of non-CC patients ($P =$

0.0013; OR = 3.200; **Figure 1(a)**). This association was also found when the analysis of infected patients included only the HCV genotype 1, where 75% of

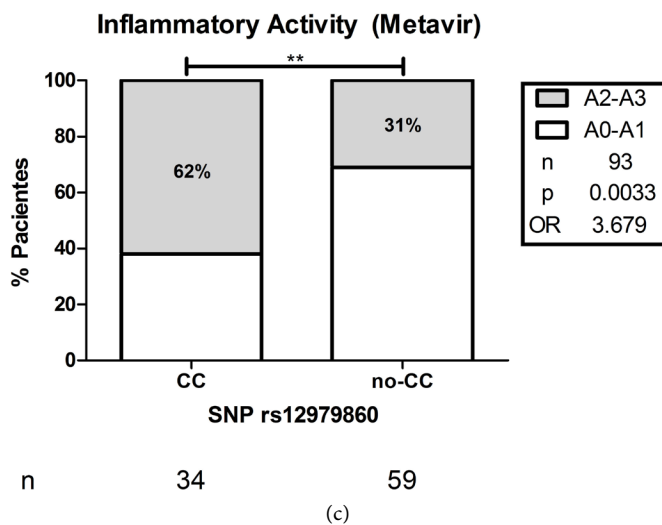
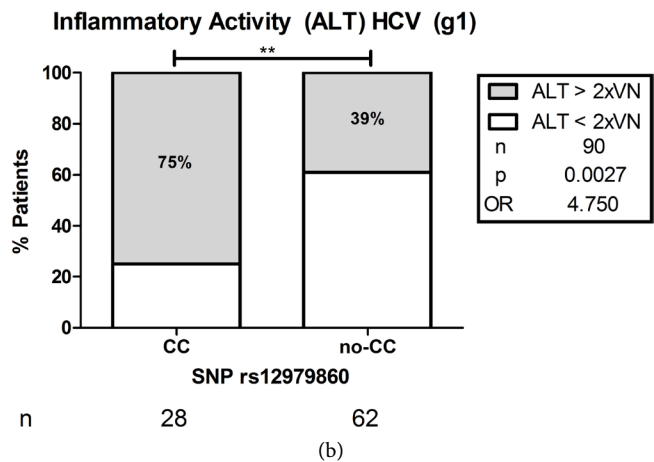
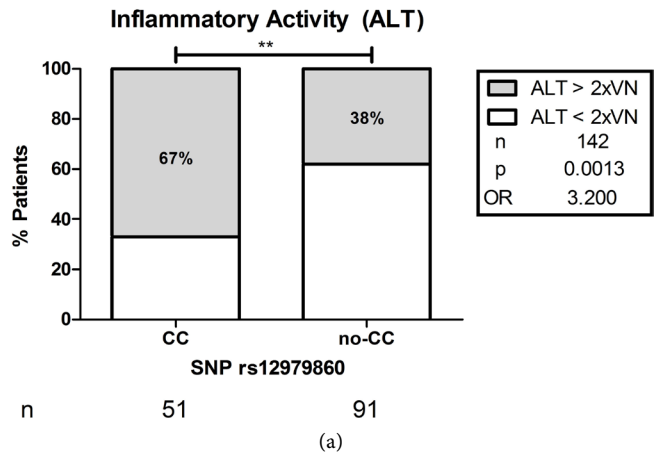
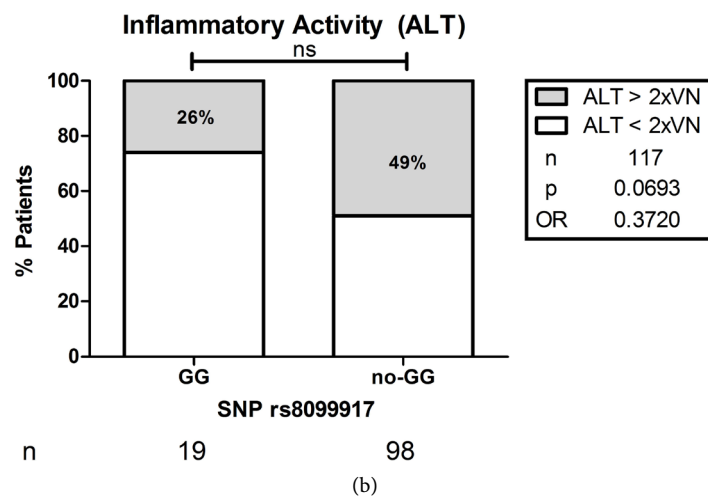
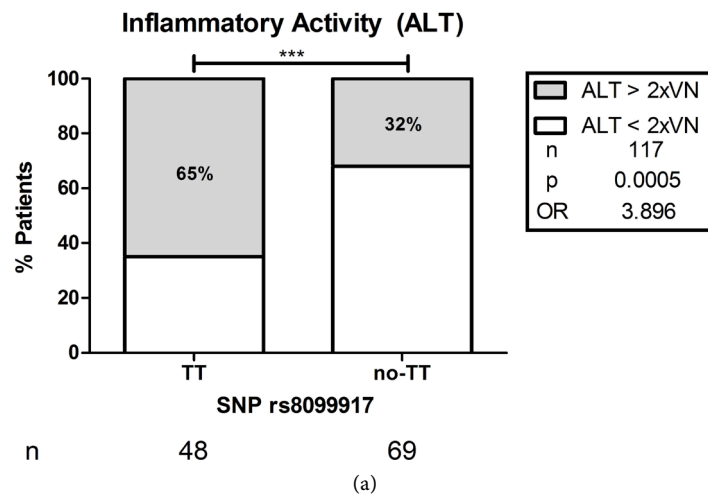


Figure 1. Association of rs12979860 and inflammatory activity. SNP rs12979860 is associated with higher ALT levels in CC compared to no-CC patients, independently of HCV genotype (a) or considering only HCV genotype 1 (b). Similar association is found when using METAVIR score as inflammation indicator (c). Data were analyzed by Chi square test. n, P and odd ratios (OR) are indicated in each panel. Asterisks denote significance level.

CC individuals showed high ALT levels in contrast to 39% of non-CC individuals ($P = 0.0027$; OR = 4.750; **Figure 1(b)**), and when considering genotypes 1a and 1b independently ($P = 0.039$, $P = 0.044$, respectively, data not shown). Using METAVIR score, a direct measure of inflammatory activity, 62% of CC patients showed a high score (A2 - A3) in comparison with 31% of non-CC individuals ($P = 0.0033$; OR = 3.801; **Figure 1(c)**). On the other hand, 65% of HCV patients harboring rs8099917 genotype TT presented high ALT levels, in contrast to 32% of non-TT individuals ($P = 0.0005$; OR = 3.896; **Figure 2(a)**), and this association was also found when we analyzed separately HCV genotype 1 infected patients ($P = 0.03$, data not shown). Analyzing METAVIR scores, 57% of TT patients presented a high score (A2 - A3) compared to 31% of non-TT individuals ($P = 0.0264$; OR = 2.877; **Figure 2(c)**). When analysis was performed considering rs8099917 genotype GG *versus* non-GG, only 26% of rs8099917 GG patients showed high ALT levels, in contrast to 49% of non-GG individuals ($P = 0.0693$; OR = 0.3720; **Figure 2(b)**). Association was found when using METAVIR scores for comparison: 91% of rs8099917 GG individual had lower inflammatory activity compared to 46% of non-GG patients ($P = 0.0202$; OR = 8.611; **Figure 2(d)**). Considering the association between CC (rs12979860) and TT (rs8099917)



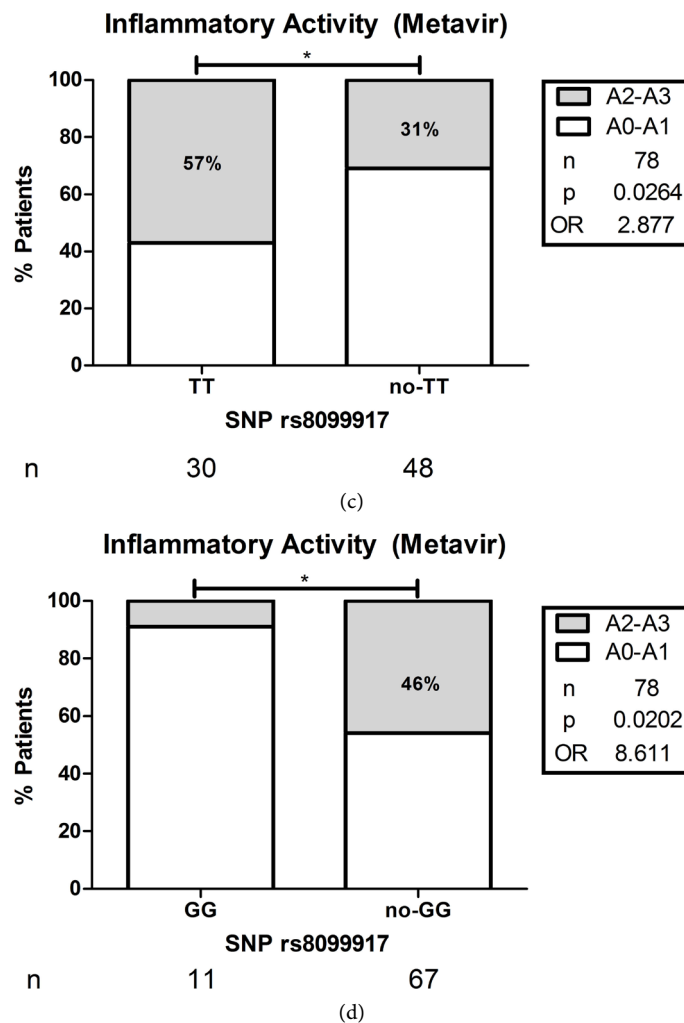


Figure 2. Association of rs8099917 and inflammatory activity. SNP rs8099917 is associated with higher ALT levels in TT patients (a), but not when comparing GG vs no-GG patients (b). When using METAVIR score as inflammation indicator, statistically significant association was found in both comparisons, TT vs no-TT (c), and GG vs no-GG (d). Data were analyzed by Fisher exact test. n, P and odd ratios (OR) are indicated in each panel. Asterisks denote significance level.

genotypes and higher ALT levels, we analyzed the possible association of both polymorphisms together. 71% of HCV patients harboring genotypes CC and TT (rs12979860 and rs8099917, respectively) showed high ALT levels, in contrast to 33% of non-CC/non-TT individuals ($P = 0.0003$; OR = 5.125, **Figure 3(a)**). When using METAVIR scores for comparison, 68% of CC/TT patients showed higher inflammatory activity compared to 29% of non-CC/non-TT patients ($P = 0.0038$; OR = 5.179; **Figure 3(b)**). Finally, to explore whether these relationships between studied SNPs and increased inflammatory activity could be confounded by HCV RNA levels, we compared viral load in the different rs12979860 and rs8099917 genotypes. No association was found.

In summary, SNPs rs12979860 and rs8099917 are associated with higher inflammatory activity as measured by alanine transaminase (ALT) levels and

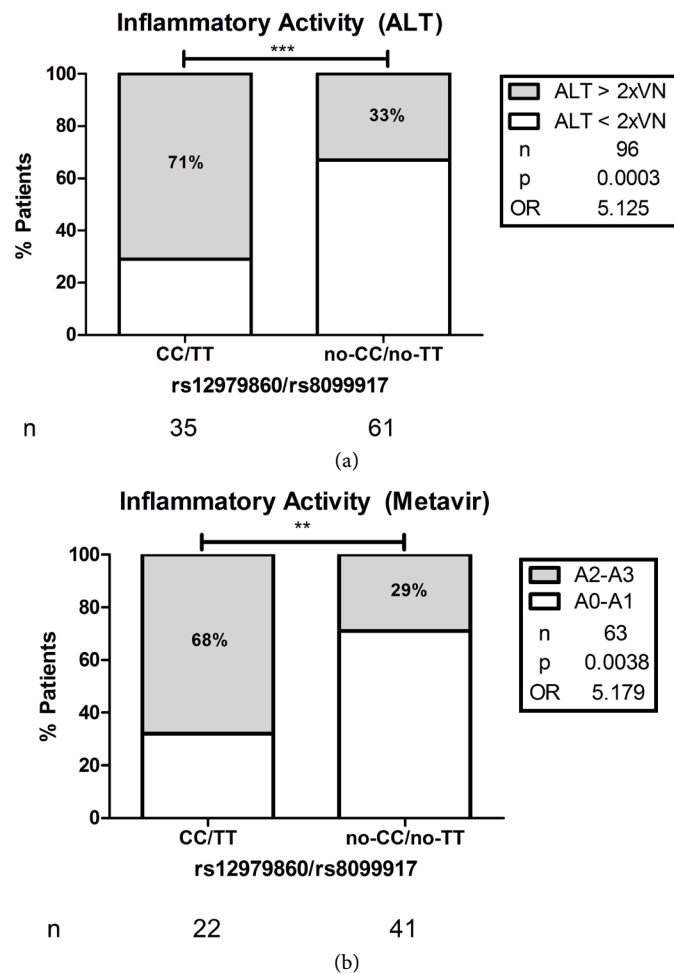


Figure 3. Association of CC (rs12979860) and TT (rs8099917) genotypes and high inflammatory activity. Genotypes CC (rs12979860) and TT (rs8099917) are associated with high inflammatory activity as measured by ALT levels. Data were analyzed by Chi square test (a) and Fisher exact test (b). n, P and odd ratio (OR) are indicated in the panel. Asterisks denote significance level.

METAVIR score.

3.4. rs12979860 and rs8099917 and Fibrosis

As a high liver inflammatory activity can lead to fibrosis/cirrhosis, we analyzed the association of specific patient alleles with advanced fibrosis scores (F3 - F4). The rs12979860 CC genotype was present in 33% of patients with early stage fibrosis, compared to 45% of patients with advanced fibrosis, a difference that was not statistically significant. Similar result was obtained when analyzing rs8099917 genotypes: 34% of patients harboring rs8099917 TT genotype presented early stage fibrosis, compared to 48% of patients with advanced fibrosis (Data not shown). These results suggest that SNPs are not significantly associated with fibrosis.

4. Discussion

HCV faces, during chronic infection, complex mechanisms of host innate and

adaptive immunity. As previously mentioned, host genetics plays an important role in the outcome of antiviral therapy [3] [4] [5] [6] [7]. We found that C and T were the most represented alleles for rs12979860 and rs8099917, respectively. Considering the possible genotypes, we found that CT was the most represented for rs12979860 and TT and TG for rs8099917. C/T and T/G alleles (rs12979860/rs8099917) were found together in approximately 85% of individuals, suggesting a strong linkage and inheritance as a haplotype, as has been previously reported [3]. However, the allele frequency of rs8099917 differs between populations worldwide, so this linkage may vary between diverse cohorts [24]. A study carried out in a Spanish cohort showed similar results for rs12979860 allele C frequency [8]. Our results are consistent with a previous report [7] in Argentine patients of European ancestry, and to our knowledge, the first report with such a genetic analysis on a single Latin-American cohort in untreated HCV-infected patients. Furthermore, we show for the first time an association between hepatic inflammatory activity in a single Argentine cohort of untreated chronically HCV-infected patients and single nucleotide polymorphisms located close to IFN- λ 3. The association we described here was neither dependent on HCV genotype nor confounded by viral load. The immune response to HCV infection is established and modulated by liver-infiltrated immune cells [25]. The hepatic inflammatory activity is a clinically useful tool to follow such response, through the measurement of ALT levels as well as through the analysis of liver biopsies. In the untreated chronically HCV infected patients we found a statistically significant association between a higher inflammatory activity grade and the rs12979860 CC genotype. Previous studies have shown that this genotype was also associated with a better response to pegylated interferon-alpha and ribavirin treatment [3] [4] [5] [6] [7] [9] [12] and with spontaneous virus clearance [9]. In our group of untreated patients we found a significant association with a higher inflammatory activity grade for the rs8099917 TT genotype. Further, considering both genotypes together (rs12979860 CC and rs8099917 TT), we also found this association to be significant, suggesting an additive effect on liver inflammation in patients harboring both genotypes. As these polymorphisms are close to IFN- λ 3, which has been involved in the modulation of antiviral responses [13] [26] [27] [28], our results, in agreement with a recent report concluding that IFN- λ 3 rather than IFN- λ 4 likely mediates haplotype-dependent hepatic inflammation and fibrosis [29], support the hypothesis that this interferon is contributing to a stronger immune response to HCV during the acute infection phase, favoring the spontaneous clearance. Moreover, they also suggest a role for this cytokine in the chronic infection inducing a favorable outcome to the antiviral therapy. For those untreated patients who entered into a chronic phase the presence of these genotypes clearly favor an inflammatory liver state. While other groups have reported similar findings [11] [18], our study further confirms and extends the knowledge on the field through the analysis of a single cohort recruited in a specialized unit where all parameters were evaluated by a single pan-

el of experts, limiting the effects of sampling errors, and with simple clinical tools routinely used in public health facilities from poor countries. Hepatic inflammatory activity can lead, on time, to the appearance of fibrosis and lately to cirrhosis. When we analyzed the fibrotic liver stage in patients, we found that rs12979860 CC and rs8099917 TT genotypes were not significantly associated with advanced fibrosis, albeit they were more represented at these stages. This was in agreement with previous studies [18] [22], but opposed to other results reporting an association either for the major genotypes [16] [17] or the minor ones (rs12979860 TT and rs8099917 GG) [19] [20] [21]. There might be multiple reasons contributing to such conflicting results, including study design, sample size, known (*i.e.* infection lasting) and unknown mechanisms contributing to fibrosis progression, etc. Further studies on chronically untreated HCV-infected patients with known time from infection are required to elucidate whether the studied polymorphisms can contribute to fibrosis/cirrhosis through a prolonged inflammatory context. A full understanding of the implications of harboring specific rs12979860 and rs8099917 genotypes will require considerably larger patient group size, that would allow individual analysis considering other factors (like alcohol consumption, gender, age, body mass index, etc.) influencing the immune response to HCV. The applicability of our findings for the actual direct-acting antiviral therapies, where there are no data available on long term clinical effects, mainly in terms of chronic infection and inflammation, has to be demonstrated in future studies. Our study adds a piece of knowledge to the implications of genetic polymorphisms in the evolution of untreated chronic HCV infection. The studied polymorphisms, together with further innate and adaptive immune responses, clearly play a role in modulating the HCV infected patient outcome. In some of such patients, rs12979860 CC and rs8099917 TT genotypes will contribute to a spontaneous or treatment-induced clearance of the virus. In other cases, particularly on those that will develop chronic infection, the same genotypes will contribute to hepatic inflammation and possibly fibrosis/cirrhosis.

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