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Genetic variations in host IL28B links to the detection of peripheral blood mononuclear cells–associated hepatitis C virus RNA in chronically infected patients

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SUMMARY. Hepatitis C virus (HCV) is mainly hepatotropic; however, several reports document the presence of genomic viral RNA in extrahepatic sites including peripheral blood mononuclear cells (PBMCs). In this study, the presence of HCV RNA was initially evaluated in the plasma and peripheral blood mononuclear cells (PBMCs) of 53 HCV-infected patients who were treated *per protocol*. PBMC-associated HCV RNA was detectable in 79% of patients. Early virological response to combined pegylated interferon-α (PegIFN) and ribavirin (RBV) therapy in patients with undetectable levels of PBMCs-associated HCV RNA was 100%, while it was 60% ($P = 0.003$) in those who had detectable levels of PBMC-associated HCV RNA. A sustained virological response was observed in 35% of patients with detectable PBMC-associated HCV RNA, but was 70% in patients with undetectable levels of PBMC-associated HCV RNA ($P = 0.07$). In a multivariate analysis incorporating parameters such as HCV genotype, viral load, presence of cirrhosis and absence of PBMC-associated HCV RNA, a significant relationship was observed between the detection of PBMC-associated HCV RNA and the sustained virological response (OR 19.4, 95% CI: 2.1–486.2, $P = 0.0061$). The association between single nucleotide polymorphism (SNP) in IL28B, known predictor of antiviral therapy outcome, and the occurrence of HCV RNA in PBMC in 84 chronically infected patients was then evaluated. Results suggest that the presence of a G allele in rs8099917, known to associate to a poor response to PegIFN/RBV therapy, also predicts an increased association of HCV RNA with PBMC (OR: 3.564; 95% CI: 1.114–11.40, $P = 0.0437$).

Keywords: anti-HCV therapy, HCV, IL28B, PBMC, PBMC-associated HCV RNA.

INTRODUCTION

Hepatitis C virus (HCV), the main aetiological agent of post-transfusion and sporadic non-A, non-B hepatitis, afflicts more than 170 million people worldwide. In the large majority of acutely infected patients, HCV persists and leads to chronic hepatitis, cirrhosis and hepatocellular carcinoma. To date, there is no vaccine against HCV, and therapeutic options are limited. Current HCV treatment is based on the antiviral action of pegylated interferon-α in combination with ribavirin (PegIFN/RBV) [1,2]. PegIFN/RBV-based therapy is effective at reducing HCV to undetectable levels and does improve prognosis, yet it does exhibit a number of shortcomings and limitations. For instance, sustained virological response (SVR), defined as undetectable serum HCV RNA levels 24 weeks post-therapy, is attained in only 55% of PegIFN/RBV-treated patients [1–3]. Although new compounds (compounds such as viral protease or polymerase inhibitors) and novel therapeutic avenues that are designed to block HCV or host dependency factors are changing this paradigm, PegIFN/RBV still remains at the backbone of anti-HCV therapy [2,4].

The success of antiviral therapy is dependent on a number of indeterminate host- and virus-related factors [1,2]. Comorbidities such as diabetes mellitus, insulin resistance and HIV-1 co-infection negatively impact upon therapeutic response rates [1,2]. The presence of specific

Abbreviations: EVR, Early virological response; GWAS, genome-wide association studies; HBV, hepatitis B virus; HCV, Hepatitis C virus; IFN, Interferon; ISG, IFN-stimulated genes; PBMCs, peripheral blood mononuclear cells; RT-PCR, reverse transcription polymerase chain reaction; SNP, single nucleotide polymorphism; SVR, sustained virological response.

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polymorphisms within the interleukin (IL)28B locus is also a strong predictor of patient response to antiviral therapy [5–9]. Recent genome-wide association studies (GWAS) of individuals chronically infected with HCV participating in clinical treatment trials with PegIFN/RBV revealed that single nucleotide polymorphisms (SNPs) in linkage disequilibrium that map upstream of the IL28B gene, rs12979860 and rs8099917 are strong predictors of SVR [5–8,10]. At position rs12979860, the C allele homozygosity is associated with SVR when compared to heterozygosity or homozygosity for the minor T allele, whereas at the rs8099917 position when compared with the T allele, heterozygosity or homozygosity for the minor G allele associated with an increase risk for not responding to therapy [5–8,10]. Among the viral determinants, the viral genotype and the pretreatment viral load are the two most important predictors of SVR [11]. Another consideration is that PegIFN/RBV therapy frequently induces severe side effects that require treatment discontinuation or dose modification [1,2,12].

HCV is an enveloped RNA virus, classified in the *Hepacivirus* genus within the *Flaviviridae* family. The virus is primarily hepatotropic, but there is evidence suggesting the possibility of extra-hepatic HCV replication [13,14]. Extra-hepatic replication was initially suspected because plasma-free leucocytes from patients with post-transfusional non-A, non-B hepatitis were able to transmit the disease to chimpanzees [15]. HCV genomic RNA has been detected not only in liver of chronically infected patients but also in peripheral blood mononuclear cells (PBMCs), and in other tissues (reviewed in [13,14]). Interestingly, PBMCs were found to harbour HCV variants that differed from that found circulating in serum [16–18], supporting the possibility of autonomous HCV replication in these cells. Replication in these extrahepatic sites is further supported by reports claiming the development of B-cell-based continuous HCV cultures [19–21]. However, these studies sharply contrast with others that show that serum-derived HCV virions associate with the cell surface but do not infect or replicate in PBMCs [22–24]. The significance of extra-hepatic replication of HCV or the presence of PBMC-associated HCV RNA in the clinical progression of the viral-induced diseases remains a matter of strong debate. Some reports, however, do suggest that HCV persists, albeit at very low levels, in circulating lymphoid cells for several years after apparently complete clinical and virological resolution of chronic hepatitis C, suggesting that PBMCs represent a natural reservoir for HCV [25–28]. Recent reports suggest that HCV-infected lymphoid cells are resistant to IFN and RBV [29,30], suggesting a link between the presence of HCV RNA in PBMCs with a poor response to IFN therapy [31]. Yet, again these observations remain highly controversial [24].

In this new study, the relationship between the detection of PBMC-associated viral RNA and the response to PegIFN/RBV treatment in individuals infected chronically with HCV was evaluated. Results suggest that the detection of PBMC-associated HCV RNA prior to the initiation of PegIFN/RBV treatment can be linked to the antiviral therapy outcome. Next, and based on these finding, the possible relationship between the PBMC-associated viral RNA and IL28B polymorphisms was assessed in a cohort of HCV chronically infected patients. Results suggest that SNP rs8099917, previously associated with an increase risk for not responding to PegIFN/RBV therapy, is also linked with the detection of PBMC-associated viral RNA. The biological significance of this finding is discussed.

MATERIAL AND METHODS

Study population

Patients infected chronically with HCV were recruited from 6 hospitals in the Metropolitan Area of Santiago, all of whom are part of a Pilot Program for hepatitis C treatment in the Chilean public health system. Protocols and consent forms used in this study were approved by the Ethical Review Board of the Faculty of Medicine, Pontificia Universidad Católica de Chile. Donors were informed about the study and completed a written consent form before donating blood. Patient inclusion criteria were the following: over 18 years of age with chronic hepatitis C, defined by positive for serum anti-HCV antibodies and positive for plasma HCV RNA for more than 6 months. HCV RNA levels in plasma were determined using the COBAS Amplicor HCV Monitor V2.0 (Roche Diagnostics AG, Rotkreuz, Switzerland), with a lower limit of quantification of 25 IU/mL (1 IU/mL is equivalent to 2.5 RNA copies/mL; [32]). Patients included in the study were scheduled to undergo a medical visit and to have a blood sample taken for biochemical and virological assessment prior to the initiation of antiviral therapy (week 0: baseline) in plasma and in PBMCs. Patients with contraindications for hepatitis C treatment, patients with anaemia (haematocrit less than 20%), pregnant women, HIV-1-positive or hepatitis B (HBV)-positive patients, subjects who had received an organ transplantation, patients undergoing chemotherapy, or patients having previously received antiviral treatment were excluded from the study [18,33]. Normal healthy controls were screened with an analogous battery of tests to confirm their serum status for HCV, HIV-1 and HBV.

Liver fibrosis

For most patients, liver fibrosis was assessed by liver biopsy. Liver biopsy was not conducted in patients with a clinical diagnosis of cirrhosis based on signs, imaging studies, endoscopy (oesophageal varices) and laboratory analysis (such as low platelet counts).

Antiviral therapy

Patients infected chronically with HCV were treated for 24–48 weeks with 180 μg per week of PegIFN α-2a given subcutaneously, combined with a body weight–adjusted daily dose of RBV, namely 1000 mg (body weight ≤ 75 kg) or 1200 mg (body weight ≥ 75 kg). Patients were monitored by a physician, with the weekly PegIFN α-2a, dose self-administered under direct observation of a nurse. Blood samples were collected periodically (for biochemical and virological assessments) and at 24 weeks following treatment.

PBMC isolation from donor samples

Peripheral blood was collected into EDTA-Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA), and plasma samples recovered from donors were stored at −80 °C until needed. PBMCs were isolated by centrifugation over a density gradient (Lymphocyte separation medium; Cellgro®, Mediatech Inc., Herndon, VA, USA) as previously described [33].

RNA purification and RT-PCR

RNA was extracted from plasma (200 μL) and PBMCs (1×10^6 cells) using the High Pure Viral Nucleic Acid kit (Roche Diagnostics AG) following the manufacture's protocol. HCV RNA was detected by a one-step reverse transcription (RT) polymerase chain reaction (PCR) using the SuperScript™ III one-step RT-PCR system with Platinum® Taq DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA) kit with a lower limit of detection of 1×10^3 HCV genomic copies. Therefore, HCV RNA concentrations lower than 1×10^3 genomic copies were reported as negative. The amplification of the HCV 5′ UTR was achieved using the sense primer: P1-XhoI 5′-GCCGCTCGAGTTGGGGGCGACACTCCACCATAGATC-3′ and the antisense primer: P4-EcoRI 5′-CGGGGAATTCGTTACGTTTGGTTTTTCTTTGAGG-3′, as previously described [34]. *In vitro* transcribed RNA (T7 RNA polymerase; Fermentas) generated from plasmid DL HCV 1b [34] and RNA extracted from a HCV-positive serum (as determined by COBAS Amplicor HCV Monitor; Roche Diagnostics AG) were used as a positive control, while water, RNA extracted from an HCV-negative serum and from PBMCs from a negative donor (confirmed by COBAS Amplicor HCV Monitor) were used as negative controls.

HCV genotyping

Initial HCV genotyping of virus present in plasma and PBMCs was performed using the reverse-hybridization line probe assay, INNO-LiPA HCV II kit (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions [18,33].

Genomic DNA extraction

Genomic DNA was extracted from 200 μL of plasma using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturers protocol. The genomic DNA was eluted in 50 μL of free nuclease water and quantified by spectrophotometry (ND-1000 NanoDrop®). The recovered DNA was diluted to a final concentration 1 ng/μL.

SNP genotyping

The allelic discrimination was carried out using a Stratagene Mx3000P thermal cycler (Agilent Technologies, Inc., Santa Clara, CA, USA). The rs12979860 SNP genotyping was performed using 3 ng of DNA, 1X Taqman Genotyping Master Mix (Applied Biosystems, Life Technologies), 1X Custom Taq Man SNP Assay (Applied Biosystems, Life Technologies), containing 900 nM of each primer (RealSNP860F: 5′-GCCTGTCGTGTACTGAACCA-3′; RealSNP860R: 5′-GCGCGGAGTGCAATTCAAC-3′) and 200 nM of each TaqMan MGB probe (MGBSNP860: 5′-TGGTTCGCGCCTTC-3′, [VIC]; MGBSNP860: 5′- CTGGTTCACGCCTTC-3′, [FAM]) (Applied Biosystems), in a total volume of 10 μL. The rs8099917 SNP genotyping was determined by a TaqMan® Pre-designed SNP Assay (Applied Biosystems) (AB) reference: C_11710096_10, using 3 ng of DNA. The amplification protocol in both cases was as follows: 2 min of incubation at 50 °C and 10 min of denaturation at 95 °C, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing and extension for 1 min at 60 °C as previously described [35]. Genotyping of each sample was attributed automatically by the MXPro software (Agilent Technologies). Positive and negative controls were used in each genotyping assay.

The genomic DNA from 405 nonrelated and noninfected individuals used in this study to estimate the allele frequencies within the Chilean population were recovered from a well-characterized DNA library considered to be representative of the Chilean population [36–38]. The use of these samples for this study was authorized by the Ethical Review Board of the Faculty of Medicine, Pontificia Universidad Católica de Chile.

Statistical analysis

Comparisons between the two groups were made using the Wilcoxon test for nonnormal continuous variables. For categorical variables, the Pearson or Fisher exact tests were used as appropriate. Significance was set at a P value of less than 0.05. Multivariable logistic regression analysis with backward elimination was performed to identify baseline factors that were associated with sustained virological response. Variables with a P value of less than 0.1 in the univariate analysis were considered for constructing the

model. All analyses were performed using JMP, Version 9, SAS Institute Inc., Cary, NC, 1989–2011. Associations between the two IL28B polymorphisms and sustained virological response or PBMCs-associated HCV RNA were analysed by a two-tailed Fisher's exact test, using the GraphPad v5.03 program (La Jolla, CA 92037, USA).

RESULTS

Treatment response of chronic HCV-infected patients with detectable amounts of PBMC-associated HCV RNA

The demographic and clinical characteristics of patients at baseline are described in Table 1. The mean age of the study cohort was 50 years, predominantly female (62%) and with a history of blood transfusion (65%) and a long duration of infection (mean 27 years). HCV genotypic analysis confirmed the prevalence of genotype 1b (73%) in the cohort. Most patients (55%) exhibited a high baseline viral load (>400 000 IU/mL), and a considerable proportion of patients were overweight (mean body mass index 28) and cirrhotic (44%). Patients requiring dose modification or therapy discontinuation due to intolerance or side effects were excluded from further analysis ($n = 19$), whereas those who stopped treatment because of futility (not achieving a 2 log drop in viral load at week 12 or maintaining detectable HCV RNA in plasma at week 24) were included in the analysis as treatment failures. Among the remaining 88 patients, an available baseline sample, that is, before starting antiviral therapy, for simultaneous plasma and PBMCs HCV RNA determination was available in only 53 patients. In these 53 patients, total RNA isolated from plasma and PBMCs, as indicated in Material and Methods, was used as template in a specific single-step RT-PCR, targeting the highly conserved HCV 5′untranslated region (5′ UTR) [19,33,34]. The 5′UTR was successfully amplified from all plasma samples (100%), whereas 11 of 53 (20.7%) HCV-infected patients had no detectable viral RNA associated with PBMCs (Table 2). To confirm the absence of RT-PCR inhibitors within the RNA extracted from PBMCs obtained from HCV-positive donors (as determined by COBAS Amplicor HCV Monitor) that showed to be negative for viral RNA, random samples were spiked with *in vitro* transcribed HCV RNA. Consistently, an amplicon was observed in preparations spiked with HCV RNA (data not shown).

The differences in treatment outcomes between patients who harboured detectable PBMC-associated HCV RNA (42 patients; 79.2%) at baseline and those in whom viral RNA was undetectable (11 patients; 20.8%) are summarized in Table 2. Results show that there was no significant difference in HCV viral load, HCV genotype or cirrhosis between these two groups of patients. Nevertheless, treatment response differed significantly between individuals who had detectable HCV RNA associated with PBMCs with those that did not. Early virological response (EVR), defined as either undetectable or a $\geq 2\text{-log}_{10}$ decrease in serum HCV RNA by week 12 following therapy initiation, was 100% in patients with undetectable levels of PBMC-associated HCV RNA, while it was achieved in only 60% of patients who exhibited detectable PBMC-associated HCV RNA ($P = 0.003$). Similarly, sustained virological response (SVR) was significantly greater in patients with undetectable HCV RNA in PBMCs compared with those showing detectable HCV RNA in these cells (70% *vs* 35%, $P = 0.07$). Based on this observation, a possible relationship was sought between the presence of PBMC-associated HCV RNA at baseline and SVR. A multivariate analysis of predictors of response (Table 3) confirmed that detection of PBMCs-associated HCV RNA at baseline correlates with SVR even when correcting for HCV genotype and presence of cirrhosis (OR 19.4, 95% CI: 2.1–489.2,

Table 1 Baseline characteristics of patients

Variable	$N = 107$
Male gender [*n* (%)]	41 (38.3)
Age (years, mean ± SD)	50.0 ± 12.3
Body mass index (kg/m^2, mean ± SD)	28.3 ± 4.2
Duration of infection (years, mean ± SD) *	26.6 ± 11.4
HCV genotype [*n* (%)]	
1a	3 (2.8)
1b	78 (72.9)
2a	3 (2.8)
3a	22 (20.6)
4	1 (0.9)
Route of transmission [*n* (%)]	
Transfusion	70 (65.4)
Intravenous drug use	15 (14.0)
Vertical	1 (0.9)
Healthcare exposure	1 (0.9)
Unknown	20 (18.7)
ALT (U/mL mean ± SD)	109 ± 97
AST (U/mL, mean ± SD)	89 ± 67
HCV RNA (log IU/mL, mean ± SD)	5.57 ± 0.6
HCV RNA > 400 000 IU/mL [*n* (%)]	59 (55)
Liver fibrosis [*n* (%)**]	
Mild fibrosis (F0-F2)	56 (53.3)
Advanced fibrosis (F3-F4)	49 (46.7)
Cirrhosis (F4)	46 (43.8)
Platelet count (10^3/mL)	169 ± 72
Bilirrubin (mg/dL, mean ± SD)	1.04 ± 0.6
Albumin (g/dL, mean ± SD)	4.2 ± 0.6
Creatinine (mg/dL, mean ± SD)	0.81 ± 0.1
INR (mean ± SD)	1.22 ± 0.4
MELD score (mean ± SD)	8.9 ± 2.4

SD, Standard deviation. *Only patients with a known risk factor for infection were included in this analysis. **105 patients with available information.

Table 2 Comparison of variables and treatment outcomes among patients with detectable versus undetectable HCV RNA in peripheral blood mononuclear cell (PBMC) at baseline

Variable	Total group ($N = 53$)	PBMC-associated HCV RNA		P
		Detectable ($N = 42$)	Undetectable ($n = 11$)	
Male gender [n (%)]	21 (39.6)	14 (33)	7 (64)	*0.08
Age (years, mean ± SD)	50.8 ± 12.1	50.5 ± 12.9	51.8 ± 9.2	0.7
Duration of infection (years, mean ± SD)	29.1 ± 12.2	27.9 ± 9.6	32.6 ± 18.0	0.3
HCV genotype 1 or 4 [n (%)]	43 (81)	35 (83)	8 (73)	0.4
HCV genotype 2 or 3 [n (%)]	10 (19)	7 (17)	3 (27)	0.4
HCV viral load (log IU/mL, mean ± SD)	5.65 ± 0.5	5.69 ± 0.49	5.52 ± 0.51	0.3
Cirrhosis [n (%)]	19 (35.8)	14 (33)	5 (45)	0.7
ALT (U/mL mean ± SD)	116 ± 102	127 ± 111	83 ± 60	0.2
Early virological response [n (%)]	35/51 (69)	24/40 (60)	11/11 (100)	**0.003
End of treatment response [n (%)]	28/39 (72)	19/29 (66)	9/10 (90)	**0.02
Sustained virological response [n (%)]	21/50 (42)	14/40 (35)	7/10 (70)	*0.07
Relapse [n (%)]	6/26 (23)	4/18 (22)	2/8 (25)	0.8

*$P < 0.1$; **$P < 0.05$.

Table 3 Multivariable analysis of sustained virological response

Variable	OR	95% CI	P
Genotype 2 or 3 *vs* genotype 1 or 4	16.39	2.1–377.4	**0.0055
Cirrhosis	0.41	0.05–2.18	0.3121
Undetectable peripheral blood mononuclear cell-associated HCV RNA at baseline	19.4	2.1–486.2	**0.0061
rs12979860 *CC vs* CT/TT	4.95	0.7–42.1	*0.0972
rs8099917 TT *vs* TG/GG	2.67	0.3–32.5	0.3862

OR, odds ratio; CI, confidence interval; *$P < 0.1$; **$P < 0.05$.

$P = 0.0061$). Thus, data suggest the detection of PBMC-associated HCV RNA emerges as a negative predictor of SVR.

IL28B polymorphisms and the detection of PBMC-associated HCV RNA

A strong link between SNPs rs12979860 and rs8099917 that tag a haplotype block on chromosome 19 spanning IL28B, with the response to PegIFN/RBV treatment for HCV, spontaneous clearance of HCV, and progression to chronic hepatitis, has been described [5–8,10]. Nonetheless, no study exists that shows the distribution of these polymorphisms within the Chilean population. Taking into account that studies have shown that IL28B polymorphisms differ according to ethnic background [5], we sought to determine the allele frequencies of rs12979860 and rs8099917 within the Chilean population. For this, the genomic DNA from 405 noninfected individuals obtained from a well-characterized DNA library considered to be representative of the Chilean population [36–38] was analysed. The frequencies for the rs12979860 CC, CT and TT genotypes were 0.37, 0.44 and 0.19, while frequencies for the rs8099917 TT, GT and GG genotypes were 0.47, 0.43 and 0.10 (Fig. 1a). Both SNPs genotypes were shown to be in Hardy–Weinberg equilibrium (chi-square tests $P \geq 0.1$).

Even though a significant link between IL28B polymorphisms and SVR was not clearly established by the multivariate analysis of predictors of response (Table 3) conducted in 53 patients, a recent study that used a higher number of patients confirms the association between IL28B polymorphisms and SVR to therapy in Chilean patients [39]. Based on these findings, we next evaluated whether the detection of PBMCs-associated HCV RNA at baseline could be linked to other known predictors of antiviral treatment response such as SNPs rs12979860 and rs8099917. For this analysis, the initial 53 treated patients (described in Table 2) plus an additional 31 patients who did not received treatment, but data regarding simultaneous plasma and PBMCs HCV RNA determination were available, were genotyped for SNPs rs12979860 and rs8099917. Among the 84 patients, the frequencies for the rs12979860 CC, CT and TT genotypes were 0.35, 0.51 and 0.14, while frequencies for the rs8099917 TT, GT and GG genotypes were 0.21, 0.69 and 0.10. Patients

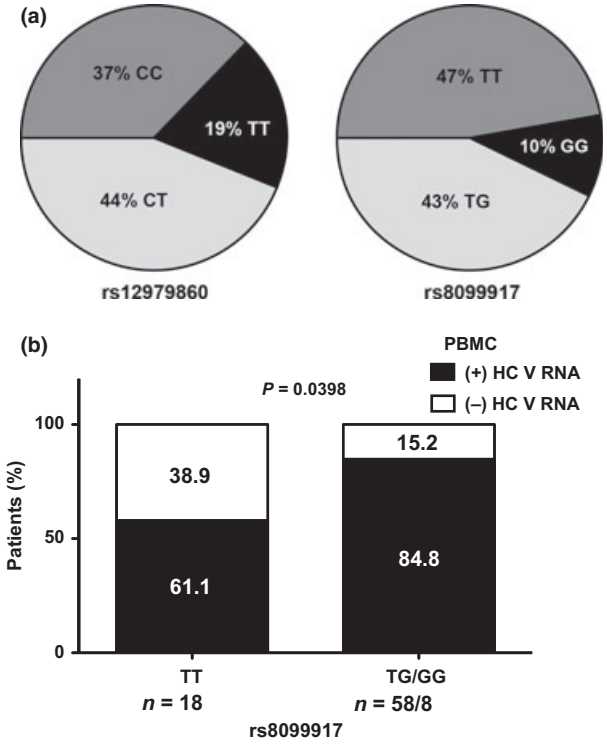

Fig. 1 IL28B polymorphism analysis. (a) Distribution of IL28B SNP rs12979860 and SNP rs8099917 among 405 noninfected individuals obtained from a well-characterized DNA library considered to be representative of the Chilean population [36–38]. (b) Peripheral blood mononuclear cellS (PBMCs)-associated HCV RNA and IL28B SNP genotype. Patients were grouped in those carrying the protective SNP rs8099917 TT genotype and those showing heterozygosity or homozygosity for the minor G allele. The total number of patients (*n*) in each group of genotypes was set as 100%, and the presence (+) or the absence (−) of HCV RNA associated with PBMCs was analysed as a dichotomous variable as indicated in the text. The numbers within each box corresponds to the percentage of patients with (black) or without (white) PBMCs-associated HCV RNA.

were then grouped in those with (+) or without (−) detectable amounts of PBMC-associated HCV RNA at baseline, the total number of patients (*n*) in each group of genotypes was set as 100%, and data were analysed as a dichotomous variable as indicated in Material and Methods. For SNP rs12979860, results showed no association between SNP rs12979860 and the detection of PBMCs-associated HCV RNA (OR: 1.333; 95% CI: 0.4727–3.761, $P = 0.6001$). Analysis of SNP rs8099917 revealed a link between the genotypes that carry the minor G allele, heterozygosity (58 patients) or homozygosity (8 patients), and the detection of PBMCs-associated HCV RNA (OR: 3.564; 95% CI: 1.114–11.40, $P = 0.0437$) (Fig. 1b). Results suggest that the presence of a G allele in rs8099917 somehow predicts the likelihood of detecting HCV RNA associated with PBMC.

DISCUSSION

Hepatitis C remains one of the most significant causes of liver-related mortality and morbidity in Chile and worldwide [40,41]. The HCV 1b genotype, which is associated with a poor antiviral response to PegIFN/RBV treatment and a greater risk of hepatocellular carcinoma [42], is predominant among the infected Chilean population [40,41]. This motivated us to search for new prognostic and treatment indicators of HCV infection.

Several reports indicate that HCV can efficiently infect extra-hepatic tissue, including PBMCs [13,14]. This and other observations [25–28,30,43] collectively suggest that PBMCs may represent a privileged reservoir for HCV. Notably, a recent study demonstrated that host-based resistance to RBV may occur in HCV patients undergoing combination PegIFN/RBV therapy [29]. The study showed that RBV uptake decreases in PBMCs over time, a phenomenon that could at least in part explain why PBMCs could become a safe haven for HCV. Consistent with this possibility, Inokuchi *et al.* (2012) showed that HCV-infected B cells exhibit an IFN-resistant phenotype. A degree of viral adaptation to the PBMC or lymphoid cell environment may also be a factor impacting on the efficiency of viral clearance by the host, resulting in the selection of distinct viral variants that may persist in cells upon treatment [17,18,44]. Consequently, HCV infection and replication in PBMCs may have important implications in the natural history of the disease.

A number of reports have focused on the discovery of HCV RNA in PBMCs upon termination of antiviral treatment. However, the reported results have been highly controversial to show how PBMCs are implicated in HCV disease progression [25–28,30,33,45–50]. Additionally, other studies suggest that PBMCs are only passive carriers of the virus; therefore, detection of PBMC-associated RNA does not necessarily mean HCV infection of these cells [22–24]. It should be clearly stated the design of the herein study did not consider experiments to validate or disregard HCV infection or replication in PBMCs. Therefore, conclusions are restricted to the detection or not of HCV RNA associated with PBMCs. One of the general findings outlined in the present study, however, established that the detection of PBMC-associated HCV RNA is not restricted to immunosuppressed individuals or HIV-1 co-infected patients [18,33,51]. The herein study, in agreement with previous reports [52,53], shows that the presence of PBMC-associated HCV RNA is not linked with elevated viral load, a particular HCV genotype, with the age of the patient, the duration of the infection or with liver cirrhosis (Table 2). A multivariate analysis of predictors of response showed, however, that the detection of PBMC-associated HCV RNA

correlated significantly with a lower EVR and SVR in patients infected chronically with HCV who underwent PegIFN/RBV treatment (Table 2). Results show that detection of PBMC-associated HCV RNA is a strong predictor of poor SVR (OR 19.4). These findings are consistent with previous reports [30,31,45,49,54,55] and strongly suggest that PBMC-associated HCV RNA may be an additional factor that somehow affects the likelihood of SVR upon treatment with PegIFN/RBV. The cell population known as PBMCs include, among others cells, lymphocytes such as T and B cells. Several reports show that B cells can be infected by HCV. The work of Inokuchi *et al.* (2012) reports the utility of the detection of HCV RNA in B cells as a predictive factor for the outcome of IFN-based therapy. Furthermore, HCV productive B-cell lines with continuous HCV replication have been generated by immortalizing naturally infected B cells [19–21]. Interestingly, HCV isolates that have infected and/or are associated with B cells show an IFN-resistant phenotype [30,55]. Thus, it is plausible that HCV resistance or poor response to treatment as well as its persistence after PegIFN/RBV might be associated with viruses generated in these extrahepatic compartments [25–28].

Host *IL28B* genotype is the most important baseline predictor of SVR after treatment with PegIFN/RBV in patients with chronic HCV infection [5–8]. Having established a link between PBMC-associated HCV RNA and SVR, we were interested in evaluating whether SNPs rs12979860 and rs8099917 and the detection of PBMC-associated HCV RNA were also associated. For this, 84 well-characterized HCV chronically infected patients were studied. As expected, the frequency of the protective rs8099917 TT genotype, which strongly predicted spontaneous clearance of HCV infection [10], was less prevalent among the studied HCV-infected patients when compared to the noninfected population. However, no differences between the frequencies of the rs12979860 CC genotype, also expected to predict spontaneous clearance of HCV infection [8,56], in the general population and the studied HCV-infected patients were observed. This apparent discrepancy is most probably due to the high viral load observed in the studied patients (Table 1). Several reports describe an association between high viral loads and the rs12979860 CC genotype [5,11,57], others suggest that patients with the favourable rs12979860 CC allele might be able to spontaneously clear the virus only when the viral load is low [58]. When data are analysed considering the SNP IL28B polymorphisms and the detection of PBMC-associated HCV RNA, results showed a relationship between the presence of the minor G allele of rs8099917 and the detection of PBMC-associated HCV RNA (Fig. 1b). This observation contrast to what was previously reported by Inokuchi *et al.* (2012), who did not find a link between IL28B genotype and the detection of B-cell-associated HCV infection. The apparent disparity between the herein reported findings and the report of Inokuchi *et al.* (2012) cannot be fully explained at the time. Nonetheless, one possibility is that HCV RNA might also associate with other cell populations that are present within PBMC and not exclusively to B cell [23,24,59]. If so, the possibility that the difference in PBMC-associated HCV RNA detection between patients may be a consequence of different distribution of PBMC subsets between patients cannot be discarded.

The functional consequences and mechanism by which *IL28B* variation influences viral decline and treatment outcomes remain unclear. Neither rs12979860 nor rs8099917 are located within known regulatory elements or the IL28B gene coding region thus, which are unlikely to be the direct cause of the observed phenomenon. Their location upstream from the IL28B coding region would suggest that rs12979860 and rs8099917 are most probable tag-SNPs that are linked to other factors that directly regulate IL28B gene expression. IL28B gene encodes for IFN-λ3, a member of the type III IFN family [60]. The type III IFN family shares downstream signalling pathways with type I INF; they signal via Jak/signal transducers and activators of transcription (STAT) intracellular pathways and up-regulate transcription of IFN-stimulated genes (ISGs), leading to an antiviral responses [60]. Nonetheless, although type I and type III INF induced similar cell signalling, the intensity of cell signalling as measured by STAT1 activation appeared to be significantly lower for type III IFNs [61]. Honda *et al.* (2010) described a strong association of hepatic ISGs expression and the IL28B rs8099917 genotype. The proposed model posits that an increased expression of IFN-λ3 in patients with the minor allele would result in higher ISG expression in the liver, which would then lead to a nonresponsive state to PegIFN/RBV therapy [62]. However, data supporting this possibility are contradicted by the report of Dill *et al.* (2010) that fails to show a direct link between IL28B rs8099917 genotype and hepatic ISG induction [63]. Additionally in the study by Dill *et al.* (2010), the expression of IFN-λ3 in the liver is decreased rather than increased in patients with the minor allele. These later results concur with others studies that using PBMCs showing that the presence of the minor G allele correlates with a lower expression of IFN-λ3 [6,7].

HCV infection induces IFN-λ3 expression in cells [64]. But HCV appears to have the ability to persist in quiescent hepatic cells under conditions of an interferon-induced antiviral response [65]. As mentioned above, Rauch *et al.* (2012) when searching the human genome for determinants of spontaneous HCV clearance using a genome-wide association study found that rs8099917 strongly predicted clearance of HCV infection, effect that was similar in HCV-monoinfected and HIV/HCV co-infected individuals [10]. The study shows that rs8099917 homozygous (GG) and heterozygous (GT) patients had a higher risk of chronicity compared with patients carrying the common genotype (TT). Here, we show that HCV-infected patients carrying

an rs8099917 homozygous (GG) and heterozygous (GT) genotype have a higher risk of extrahepatic association of viral RNA (Fig. 1b), suggesting that both events might be also linked. Although tempting to speculate, the evident limitations of the herein study does not allow this link to be established. Ideally, the analysis of a larger cohort of patients is required to confirm these findings and establish with greater reliability if the detection of PBMC-associated HCV RNA is indeed to be considered as a baseline predictor of treatment outcome. Even though the number of individual analysed in the herein study is relatively low, the knowledge gained by the study is high and its value resides in the fact that it clearly suggests that rs8099917 genotype might be associated with extrahepatic localization of HCV RNA. Furthermore, establishing a link between rs8099917 genotype and extrahepatic localization of HCV RNA strongly suggests that the association of HCV RNA with PBMCs is more than just a random association event.

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