Infection with hepatitis C virus (HCV) may result in different clinical outcomes, ranging from viral elimination to the development of end-stage liver disease. Hepatitis C treatment generally consists of a combination of pegylated interferon (PEG-IFN) alpha and the antiviral drug ribavirin (RBV) (Fried et al. 2002). Analysis of HCV isolates has shown substantial heterogeneity of nucleotide sequences, leading to the classification of HCV into six main genotypes with numerous subtypes. Because HCV genotype 1, the most prevalent genotype in Brazil, is less sensitive to therapy, patients have to be treated for 48 weeks vs. 24 weeks for the other genotypes (Hadziyannis et al. 2004). Sustained virological response (SVR), which is defined as having no detectable HCV RNA six months after stopping treatment, occurs in less than 50% of patients infected with genotype 1 isolates. Moreover, interindividual variations in therapeutic response have been observed.

Single nucleotide polymorphisms (SNPs) in genes encoding for cytokines involved in pro and anti-inflammatory effects may modulate, directly or indirectly, the benefits of antiviral therapy (Ge et al. 2009, Dogra et al. 2011). Genome-wide association studies have suggested that common SNPs located on a linkage disequilibrium (D) block in the vicinity of three genes on chromosome 28A, namely interferons λ1 ([interleukin (IL)-29], λ2 (IL-28A) and λ3 (IL-28B)), are strongly associated with the therapeutic response of HCV genotype 1-infected patients treated with PEG-IFN/RBV (Suppiah et al. 2009, Tanaka et al. 2009, Romero-Gomez et al. 2011).

Tumour necrosis factor (TNF) and IL-10 participate in the regulation of the cellular immune response to HCV infection (Larrea et al. 1996, Napoli et al. 1996). Heterogeneity in the promoter region of the IL-10 gene has been reported to play a role in determining the initial and sustained IFN-α treatment response in chronic hepatitis C (Yee et al. 2001, Persico et al. 2006). TNF is a potent pro-inflammatory cytokine and an antagonist of IL-10. Well-characterised TNF polymorphisms at posi-

**Key words:** TNF - polymorphisms - HCV - virological response - Brazil
Gene polymorphisms and the response to PEG-IFN/RBV therapy - The distribution of genotypes was consistent with the proportions expected under Hardy-Weinberg equilibrium. The associations between polymorphisms and treatment response are shown in Table II. No significant differences were detected in the distribution of the IL-10 -1082 genotypes between patients with SVR, Serum HCV RNA levels were classified as low (<600,000 IU/mL) or high (≥600,000 IU/mL) viral load for analysis.

Analysis of polymorphisms - Polymorphisms in the IL-10 (-1082 A/G, rs1800896), TNF (-308 G/A, rs1800629 and -238 G/A, rs361525) and Mx1 (-123 C/A, rs1700900 and -88 G/T, rs2071430) gene promoters were assessed. Among the different IL-10 gene polymorphisms, the SNP at position -1082 was prioritised as the most cited in the literature (Persico et al. 2006). For this purpose, genomic DNA was extracted from dried blood samples preserved in Whatman FTA elute cards (GE Healthcare, Uppsala, Sweden), following the manufacturer’s instructions and amplified by PCR. Table I shows the sequences of the oligonucleotide primers and PCR conditions used in this study. PCR mixes contained 10-100 ng of genomic DNA, 2.5 mM MgCl₂, 500 mM dNTPs, 12.5 pmol of each primer and 1 U of Taq polymerase in a final volume of 25 μL. Direct sequencing of the PCR products was performed in both directions using a BigDye Terminator v.1.1 cycle sequencing kit (Life Technologies, Carlsbad, CA, USA). These procedures resulted in a success rate of genotyping calls ranging from 97.5-99% in the different files. For quality control purposes, 5% of the samples were selected randomly to be genotyped twice independently, which resulted in a concordance rate of 99.9%. Statistical analysis - Allele frequencies were calculated using the gene counting method. Both deviation from Hardy-Weinberg equilibrium and allelic distributions between groups were assessed by χ² tests or, when appropriate, by Fisher’s exact test using GraphPad InStat software v.2.04a (GraphPad Software, La Jolla, CA, USA). Haplotypes and linkage D were estimated using ARLEQUIN software, v.3.1 (Excoffier et al. 2007). D theoretical maximum (D₀) and D/Dmax (D’) values were calculated as described previously (Lewontin 1998). Univariate logistic regression analyses were used to determine the predictors of treatment success. Age, gender, baseline viral load and the presence of liver cirrhosis as well as the previously genotyped IL-28B SNP rs12979870 (Grandi et al. 2013) were included as covariates in a multivariate logistic regression model to estimate adjusted odds ratios (OR) and 95% confidence intervals (CI). Mean adjusted variables were compared among TNF genotypes and haplotypes using ANOVA or Student’s t test.

RESULTS

Gene polymorphisms and the response to PEG-IFN/RBV therapy - The distribution of genotypes was consistent with the proportions expected under Hardy-Weinberg equilibrium. The associations between polymorphisms and treatment response are shown in Table II. No significant differences were detected in the distribution of the IL-10 -1082 genotypes between patients with SVR,
relapsers and non-responders. Similarly, the analyses of two biallelic polymorphisms in the Mx1 gene promoter (-123 C/A and -88 G/T) did not show any significant association between the genotype and the response to PEG-IFN/RBV treatment. The genotypes GG, GA and AA at position -308 in the TNF promoter were found in 183 (69.6%), 73 (27.6%) and seven (2.7%) patients, respectively. At position -238, the corresponding distribution was 205 (77.9%), 54 (20.5%) and four (1.5%), respectively. Both TNF gene polymorphisms -308 G/A and -238 G/A were significantly associated with a null virological response to therapy with PEG-IFN/RBV (p < 0.001), with higher SVR frequencies among patients with the GG genotype. A cumulative effect was observed since 80.6% of the individuals showing two-four A alleles were non-responders, compared to 35.4% and 20.9% of the subjects with one and no A allele, respectively (Table II).

**TNF polymorphisms -308 and -238 are predictors of a null virological response** - A logistic regression analysis was performed to determine whether TNF genotypes and diplotypes are predictors of a null virological response or relapsing. A multivariate model was designed with age, sex, baseline viral load, presence of liver cirrhosis and IL-28B SNP rs12979870 as covariates because they might constitute confounding variables. Comparisons were performed between patients with ETR and non-responders and comparisons were performed between patients with SVR and relapers. The results are shown in Table III. After adjusting for eventual confounding effects, it was observed that carriers of one or two A allele(s) at position -308 exhibited a significantly increased risk of having a null virological response (OR = 2.58, CI = 1.44-4.63, p = 0.001) and relapsing (OR = 2.87, CI = 1.51-5.44, p = 0.001). Similar increasing risks were observed for patients with A allele(s) at position -238 (OR = 7.33, CI = 3.59-14.93, p < 0.001 for null virological response and OR = 4.20, CI = 1.93-9.10, p < 0.001 for relapsing). An analysis performed with a non-adjusted logistic model also revealed increased risks (see footnote of Table III).

Because each TNF polymorphism (-308 and -238) was associated with a treatment outcome, a possible combined effect when both polymorphisms were present was investigated. The haplotype frequencies for two TNF gene polymorphisms were estimated using a maximum likelihood method. The polymorphisms were in low linkage D (D' = 0.31 and r² = 0.06) and the haplotype frequencies were 76.8%, 7.6%, 11.8% and 4.9% for G/G, G/A, A/G and A/A, respectively (data not shown). The haplotype combinations (diplotypes) were assessed in terms of the association with (i) ETR (after 48 weeks of treatment) and (ii) SVR within the group of patients who had achieved ETR (72 weeks). A multivariate model showed a cumulative effect of -308/-238 polymorphisms on the treatment outcome. Individuals with two-four copies of the A allele at positions -308 and -238 exhibited a significantly increased risk of having a null virological response (OR = 16.43, CI = 5.70-47.34, p < 0.001) and relapsing (OR = 6.71, CI = 2.18-20.66, p = 0.001) (Table III).

### TABLE I

| Oligonucleotide primers and polymerase chain reaction (PCR) conditions used in this study | References |
|------------------------------------------|-------------|
| **Gene** | **Polymorphisms** | **Oligonucleotide primers** | **PCR conditions** | **References** |
| IL-10 | -308 G/A and -238 G/A | Sense | ATCCAAGAGCACACTACTA | 95ºC 5 min; 95ºC 40 s, 66ºC 45 s, 72ºC 30 s (35x); 72ºC 7 min | Wu et al. (2002) |
|       |                         | Antisense | TAAATATCCTAAAGTTCC | 95ºC 5 min; 94ºC 30 s, 54ºC 30 s, 72ºC 30 s (35x); 72ºC 7 min | - |
| TNF   | -308 G/A and -238 G/A | Sense | CAAACACAGGCCTCAGGACTC | 94ºC 5 min; 94ºC 30 s, 54ºC 45 s, 72ºC 30 s (35x); 72ºC 7 min | - |
|       |                         | Antisense | AGGGAGCGTCTGCTGGCTG | 94ºC 5 min; 94ºC 30 s, 60ºC 30 s, 72ºC 1 min (40x); 72ºC 7 min | Spriewald et al. (2005) |
| Mx1   | -123 C/A and -88 G/T | Sense | TGAAGACCCCCAATTACCAA | 94ºC 5 min; 94ºC 30 s, 60ºC 30 s, 72ºC 30 s (35x); 72ºC 7 min | - |
|       |                         | Antisense | AGGGAGCGTCTGCTGGCTG | 94ºC 5 min; 94ºC 30 s, 60ºC 30 s, 72ºC 30 s (35x); 72ºC 7 min | Knapp et al. (2003b) |
|       |                         | Antisense | CTGCTGGCTGCTTTCAC | 94ºC 5 min; 94ºC 30 s, 60ºC 30 s, 72ºC 30 s (35x); 72ºC 7 min | - |

IL: interleukin; Mx1: myxovirus resistance protein 1; TNF: tumour necrosis factor.
TABLE II
Genotypes and alleles frequencies of IL-10, Mx1 and TNF polymorphisms in patients with sustained virological response (SVR), relapsers and non-responders

| Genotypes and alleles | Number of patients | SVR vs. relapsers | SVR vs. non-responders |
|-----------------------|--------------------|-------------------|-----------------------|
|                       | n (%)              |                   |                       |
| All (n = 263)         |                    |                   |                       |
| With SVR (n = 114)    |                    |                   |                       |
| Relapsers (n = 64)    |                    |                   |                       |
| Non-responders (n = 85) |                  |                   |                       |
| IL-10 -1082 A/G       |                    |                   |                       |
| AA                    | 116 (44.1)         | 45 (39.5)         | 36 (56.2)             | NS | NS |
| AG                    | 107 (40.7)         | 49 (43)           | 20 (31.3)             | 38 (44.7) | - | - |
| GG                    | 40 (15.2)          | 20 (17.5)         | 8 (12.5)              | 12 (14.1) | - | - |
| A                     | 339 (64.4)         | 139 (61)          | 92 (71.9)             | 108 (63.5) | NS | NS |
| G                     | 187 (35.6)         | 89 (39)           | 36 (28.1)             | 62 (36.5) | - | - |
| Mx1 -123 C/A          |                    |                   |                       |
| CC                    | 211 (80.5)         | 94 (82.5)         | 51 (79.7)             | 66 (78.6) | NS | NS |
| CA                    | 47 (17.9)          | 18 (15.8)         | 11 (17.2)             | 18 (21.4) | - | - |
| AA                    | 4 (1.5)            | 2 (1.7)           | 2 (3.1)               | 0 (0) | - | - |
| C                     | 469 (89.5)         | 206 (90.3)        | 113 (88)              | 150 (89) | NS | NS |
| A                     | 55 (10.5)          | 22 (9.6)          | 15 (12)               | 18 (11) | - | - |
| Mx1 -88 G/T           |                    |                   |                       |
| GG                    | 202 (76.8)         | 91 (79.8)         | 47 (73.4)             | 64 (75.3) | NS | NS |
| GT                    | 55 (20.9)          | 21 (18.4)         | 15 (23.4)             | 19 (22.3) | - | - |
| TT                    | 6 (2.3)            | 2 (1.8)           | 2 (3.1)               | 2 (2.4) | - | - |
| GG                    | 459 (87.3)         | 203 (89)          | 109 (85.2)            | 147 (86.5) | NS | NS |
| G                     | 67 (12.7)          | 25 (11)           | 19 (14.8)             | 23 (13.5) | - | - |
| TNF -308 G/A          |                    |                   |                       |
| GG                    | 183 (69.6)         | 91 (79.8)         | 48 (75)               | 44 (51.8) | NS | < 0.001 |
| GA                    | 73 (27.6)          | 21 (18.4)         | 16 (25)               | 36 (42.3) | - | - |
| AA                    | 7 (2.7)            | 2 (1.8)           | 0 (0)                 | 5 (5.9) | - | - |
| G                     | 439 (83.5)         | 203 (89)          | 112 (87.5)            | 124 (72.9) | NS | < 0.001 |
| A                     | 87 (16.5)          | 25 (11)           | 16 (12.5)             | 46 (27.1) | - | - |
| TNF -238 G/A          |                    |                   |                       |
| GG                    | 205 (77.9)         | 101 (88.6)        | 55 (85.9)             | 49 (57.6) | NS | < 0.001 |
| GA                    | 54 (20.5)          | 13 (11.4)         | 9 (14.1)              | 32 (37.6) | - | - |
| AA                    | 4 (1.5)            | 0 (0)             | 0 (0)                 | 4 (4.7) | - | - |
| G                     | 464 (88.2)         | 215 (94.3)        | 119 (93)              | 130 (76.5) | NS | < 0.001 |
| A                     | 62 (11.8)          | 13 (5.7)          | 9 (7)                 | 40 (23.5) | - | - |
| TNF -308/-238 diplotypes$^a$ |          |                   |                       |
| No A allele           | 153                | 81 (52.9)         | 40 (26.1)             | 32 (20.9) | NS | < 0.001 |
| 1 A allele            | 79                 | 28 (35.4)         | 23 (29.1)             | 28 (35.4) | - | - |
| 2-4 A alleles         | 31                 | 5 (16.1)          | 1 (3.2)               | 25 (80.6) | - | - |

a: combinations are as follows. No A allele: G/G + G/G; one A allele: G/G + G/A and G/G + A/G; two A alleles: G/G + A/A, G/G + G/A, G/A + A/G and A/G + A/G; three A alleles: G/A + A/A and A/G + A/A; four A alleles: A/A + A/A. Assuming that the larger the number of copies of allele A, the greater the risk, the samples were divided into categories, according to the number of A copies. However, none of the samples showed three A alleles and only four showed four A alleles. For this reason, all the samples with two-four A alleles were grouped in a unique category. In the genotypic model, dominant model was used because having one allele increases the chance of not responding to therapy. The statistical power of the sample to detect an association for the non-significant single nucleotide polymorphisms (SNPs) [interleukin (IL)-10 and myxovirus resistance protein 1 (Mx1)] with an odds ratio of 3 ranged from 61-91%. NS: not significant; TNF: tumour necrosis factor.
DISCUSSION

Host immune response as a consequence of genetic background has been shown to play a crucial role in HCV infection pathogenesis and interindividual heterogeneity of disease outcomes (Amini & Poustchi 2012). Cytokine production varies among individuals and these variations are associated with SNPs located in the coding and promoter regions of cytokine genes (Ollier 2004). TNF, in particular, has been reported to play a critical role in the host immune response to HCV infection (Hohler et al. 1998, Dai et al. 2006).

The TNF gene promoter has been shown to contain numerous binding sites for transcription factors. Therefore, the presence of SNPs in the TNF gene promoter might influence the transcriptional regulation of the gene. However, whereas some studies have reported a significant association between TNF polymorphisms and response to hepatitis C therapy, others have not (Rosen et al. 2002, Barrett et al. 2003, Dai et al. 2006, Kusumoto et al. 2006). Currently, the G/A genotypes associated with polymorphisms at positions -308 and -238 are the best characterised. Hohler et al. (1998) reported an association between the A allele of the polymorphism G/A at position -238 and chronic hepatitis C, suggesting that this polymorphism may contribute to viral persistence. Dai et al. (2006) suggested that the TNF polymorphism at position -308 may be a predictor of treatment failure in patients treated with a combination of IFN-α and RBV. However, studies conducted in the United States of America, Ireland and Japan have been unable to identify any association between TNF genetic polymorphisms and histological severity or response to antiviral therapy (Hohler et al. 1998, Ollier 2004, Amini & Poustchi 2012).

| Features | Patients with ETR vs. non-responders | Patients with SVR vs. non-responders + relapsers |
|----------|-------------------------------------|-----------------------------------------------|
|          | Adjusted OR (95% CI) | p | Adjusted OR (95% CI) | p |
| **TNF -308** | | | | |
| GA+AA | 2.58 (1.44-4.63) | 0.001 | 2.87 (1.51-5.44) | 0.001 |
| Age | 0.99 (0.97-1.02) | 0.929 | 1.01 (0.99-1.04) | 0.208 |
| Sex | 0.74 (0.41-1.34) | 0.323 | 0.55 (0.30-0.99) | 0.047 |
| Viral load ≥ 600,000 IU/mL | 2.59 (1.20-5.60) | 0.015 | 3.71 (1.86-7.39) | < 0.001 |
| Liver cirrhosis | 2.32 (1.11-4.84) | 0.024 | 2.12 (0.94-4.76) | 0.069 |
| IL-28B | 6.94 (2.01-23.91) | 0.002 | 6.29 (2.67-14.82) | < 0.001 |
| **TNF -238** | | | | |
| GA+AA | 7.33 (3.59-14.93) | < 0.001 | 4.20 (1.93-9.10) | < 0.001 |
| Age | 0.99 (0.96-1.01) | 0.636 | 1.01 (0.98-1.04) | 0.259 |
| Sex | 0.91 (0.49-1.71) | 0.788 | 0.64 (0.35-1.16) | 0.146 |
| Viral load ≥ 600,000 IU/mL | 2.85 (1.26-6.44) | 0.012 | 3.55 (1.78-7.10) | < 0.001 |
| Liver cirrhosis | 2.84 (1.30-6.17) | 0.008 | 2.32 (1.02-5.26) | 0.043 |
| IL-28B | 9.22 (2.47-34.43) | 0.001 | 6.75 (2.77-16.42) | < 0.001 |
| **TNF -308/-238 diplotypes** | | | | |
| 1 A allele | 1.88 (0.99-3.56) | 0.052 | 2.68 (1.41-5.11) | 0.003 |
| 2-4 A alleles | 16.43 (5.70-47.34) | < 0.001 | 6.71 (2.18-20.66) | 0.001 |
| Age | 0.99 (0.97-1.02) | 0.948 | 1.01 (0.98-1.04) | 0.235 |
| Sex | 0.75 (0.40-1.41) | 0.385 | 0.58 (0.31-1.05) | 0.075 |
| Viral load ≥ 600,000 IU/mL | 2.79 (1.22-6.36) | 0.014 | 3.88 (1.92-7.86) | < 0.001 |
| Liver cirrhosis | 2.60 (1.20-5.61) | 0.015 | 2.24 (0.97-5.15) | 0.056 |
| IL-28B | 7.10 (1.98-25.34) | 0.003 | 6.53 (2.72-15.68) | < 0.001 |

a: non-adjusted odds ratios (OR) = 3.36, p < 0.001; b: non-adjusted OR = 2.45, p = 0.002; c: non-adjusted OR = 5.71, p < 0.001; d: non-adjusted OR = 2.69, p < 0.001. OR were calculated by logistic regression, taking GG genotype as a reference. To calculate unadjusted OR, genotype was the only parameter considered. For adjusted OR, all other variables were included. CI: confidence interval; ETR: expected treatment response; SVR: sustained virological response.
In the present study, a significant association was found between each of the two TNF promoter polymorphisms and SVR rates after combination therapy with PEG-IFN and RBV. Serum HCV RNA levels and the presence of A alleles at positions -308 and -238 were predictors of SVR in patients infected with HCV genotype 1 (Table III). The disadvantage for a patient of having A alleles at those positions was even more evident when all diplotype combinations were considered, indicating a cumulative effect. Therefore, carrying two or more copies of the A allele may be a valuable indicator for predicting treatment difficulties. Furthermore, this study also confirmed that the IL-28B polymorphism was an independent predictor of SVR in the study participants (Table III). These results, corroborating those of a recently published paper (Pasha et al. 2013), suggested that polymorphisms could be used as tools of clinical utility to categorise patients before starting hepatitis C treatment.

SNPs in the IL-10 promoter region have been associated with a beneficial treatment response and to a lesser extent with the spontaneous resolution of HCV infection (Yee et al. 2001, Vidigal et al. 2002, Knapp et al. 2003a, Mangia et al. 2004). However, other investigations have reported that the GG genotype does not influence HCV infection outcomes or the response to PEG-IFN/RBV therapy (Barrett et al. 2003, Chuang et al. 2009). Here, no significant association was found between the IL-10 -1082 A/G polymorphism and the response to PEG-IFN/RBV therapy.

Infection with HCV leads to a rapid type I IFN response within the liver. Antiviral proteins involved in the type I IFN pathway, such as the MxA protein, together with pro-inflammatory cytokines, have been associated with treatment responses in patients with chronic hepatitis due to HCV genotype 1 infection (Cheong et al. 2006, Persico et al. 2006). Previous studies have reported that MxA polymorphisms are important in predicting the IFN therapy response among patients with chronic hepatitis C (Hijikata et al. 2001, Knapp et al. 2003b, Suzuki et al. 2004), although another study did not confirm these results (Vidal et al. 2012). However, in the present study, no correlation was established between the polymorphisms -123 C/A and -88 G/T in the MxA gene promoter and the response to PEG-IFN/RBV therapy.

In a general manner, the discrepancies between studies may be due not only to the type of therapy (monotherapy with IFN alone vs. combination therapy with RBV), but also to differences between ethnic groups (Layden-Almer et al. 2003, Conjeevaram et al. 2006). In this respect, it is noteworthy that the genetic structure of the Brazilian population is one of the most heterogeneous in the world, due to the ethnic mix of the population resulting from five centuries of massive interethnic crosses between people from different continents (Alves-Silva et al. 2000, Pena et al. 2011). Different from what occurs in other countries, the majority of Brazilians cannot be classified in a determined ethnic group based only on skin colour. However, in an attempt to allow comparisons with other studies, it may be interesting to mention that the genomic proportions of European, African and Amerindian ancestry in the South Brazil, where this study was conducted, have been reported to be 79.5%, 10.3% and 9.4%, respectively (Pena et al. 2011). Therefore, the genetic admixture of the Brazilian population constitutes a limitation of this study, as it was not possible to determine whether the three different genetic backgrounds were equally distributed among the patients with SVR, ETR, relapse or non-responders.

In conclusion, the results from this study corroborate that the PEG-IFN/RBV therapy response to chronic hepatitis C may be associated, at least in part, with host genetic factors, particularly TNF promoter polymorphisms. A replication study with a larger, well-characterised independent cohort will be necessary to confirm these associations.

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