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Influence of cytokine and cytokine receptor gene polymorphisms on the degree of liver damage in patients with chronic hepatitis C

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ABSTRACT

Hepatic fibrosis may be the result of repetitive injury to hepatocytes caused by HCV infection and the immune response to it. Cytokines regulate the inflammatory response to injury and modulate hepatic fibrogenesis. Single nucleotide polymorphisms (SNPs) located in cytokine genes may influence the cytokine expression and secretion that may contribute to hepatic fibrogenesis in HCV infection. The aim of this study was to determine the genotype of 22 SNPs found in the genes of 13 cytokines/cytokine receptors to assess the influence of polymorphic variants on the stage of liver damage in Brazilian patients chronically infected with HCV genotype 1 only. 141 unrelated patients were grouped according to their stage of fibrosis: absence of fibrosis or patients in the initial stages of fibrosis (F0-F2, n = 84), patients with advanced stages of fibrosis or cirrhosis (F3-F4, n = 57), without cirrhosis (F0-F3, n = 103), and with cirrhosis (F4, n = 38). The comparison of frequencies in each sub-sample was performed by 2 × 2 contingency tables using the chi-square or Fisher's exact test. Stepwise logistic regression was also used to assess independent associations between cirrhosis or fibrosis with polymorphic variants. The *TNFA*-308G:A genotype conferred increased risk of fibrosis and cirrhosis. The *TNFA*-238G:G genotype was associated with protection from cirrhosis. The *IL10*-819C:T genotype conferred protection from fibrosis and the *IL1B*-511C:T genotype conferred increased risk of cirrhosis. Some of these genotypes showed results on the borderline of statistical significance in the bivariate analysis. We conclude that gene variants of cytokines/receptors may influence liver damage in patients chronically infected by HCV genotype 1.

1. Introduction

The hepatitis C virus (HCV) is a pathogen responsible for the chronic infection in around two thirds of infected individuals, due to its ability to evade both innate and acquired immunity (Dustin and Rice, 2007). According to the latest population-based prevalence study of infections by hepatitis virus, realized between 2005 and 2009, with regard to hepatitis C, the prevalence in the age group between 10 and 69 years was 1,38% (Ministério da Saúde, 2012). Chronic infection may be evident by histopathological changes in the liver, beginning with an inflammatory process often associated with fibrosis which may progress to cirrhosis and, in some cases, to hepatocellular carcinoma (Thomas and Seeff, 2005). Fibrosis is the result of repetitive injuries to the hepatocytes by HCV infection and the immune response to it, leading to a failure in the regenerative process and deposition of an abundant amount of extracellular matrix. The progressive accumulation of matrix generates nodules, causing injury and hepatic cirrhosis, which are responsible for the high rate of morbidity and mortality worldwide (Bataller and Brenner, 2005; Zimmer and Lammert, 2011). Although an inflammatory process precedes fibrogenesis, studies have shown that fibrogenesis is not always characterized by persistent inflammation. Therefore, the mechanisms controlling fibrogenesis are partially different from those regulating inflammation. There is strong evidence that cytokines regulate the inflammatory response to injury and modulate hepatic fibrogenesis both *in vivo* and *in vitro* (Czaja, 2014).

The intensity of liver damage is highly variable among individuals and may be influenced by viral, environmental, and host-related factors. The diversity of genes involved in the immune response could partly explain the variability in the response to infection by the same etiological

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agent (Rau et al., 2012). Single nucleotide polymorphisms (SNPs), located in regulatory/coding regions of cytokine genes, might influence the expression and secretion of cytokines, resulting in the production of different phenotypes (Perrey et al., 1998; Tambur et al., 2001). Furthermore, changes in the levels of different cytokines seem to contribute to hepatic fibrogenesis in HCV infection (Guo et al., 1999; Andersen et al., 2011).

Although there are studies relating polymorphic variants in cytokine genes to the severity of liver damage in chronic hepatitis C (Romero-Gomez et al., 2011), some studies have yielded contradictory results due to poor study design. Most studies apply heterogeneous HCV genotype samples, with or without concurrent hepatitis B virus (HBV) and human immunodeficiency virus (HIV) viral infections. Therefore, further research is required to clarify the current role of genetic variants in liver fibrosis (Bataller et al., 2003). Moreover, no studies using this approach were previously carried out in Brazil. Therefore, the objective of this study was to evaluate the influence of polymorphic variants in cytokine and cytokine receptor genes, in some way associated with the development of fibrosis or cirrhosis (Xu et al., 2012), on the stages of liver damage in Brazilian patients chronically infected with HCV genotype 1 only, the more frequent one in Brazil (Campiotto et al., 2005).

2. Materials and methods

2.1. Casuistry

Seven hundred and sixty one patients seen at the Division of Gastroenterology, University Hospital at Botucatu's Medicine School, Brazil, between September 2004 and January 2009, were diagnosed as infected by HCV. Of these, 141 unrelated patients were included in this study, according to the following criteria: infection only by HCV genotype 1, diagnosis based on the presence of viral RNA confirmed by molecular tests, chronicity characterized by liver biopsy and persistence of viral RNA in serum, liver biopsy performed prior to the beginning of antiviral treatment, and signing the consent form. Patients with positive serology for hepatitis B and/or human immunodeficiency virus, and hemophiliac patients with other liver diseases were excluded. Patients that used alcohol or recreational drugs during treatment were also excluded. Clinical data were obtained from medical records. Patients were regarded as a mixed ethnic group (excluding Oriental ancestry), given that phenotypic evaluations based on physical characteristics such as skin color are not good predictors of genomic African ancestry in Brazilian populations (Parra et al., 2003). The study protocol was approved by the Ethics Committee in Human Research, Botucatu's Medicine School, Universidade Estadual Paulista and have been performed according to the World Medical Association Declaration of Helsinki.

2.2. Liver biopsy

The stage of fibrosis was determined by histological liver assessment. Percutaneous biopsies were performed by a pathologist with the use of Tru Cut or Menghini needles. The fragment analysis was only performed when at least eight portal areas could be seen. Tissues were stained with hematoxylin–eosin, Masson's trichrome, and reticulin stains and analyzed by the METAVIR scale system (Asselah et al., 2009), which classifies the damage of the liver sample from zero to four (F0 - no fibrosis, F1 - portal fibrosis without septa, F2 - portal fibrosis with few septa, F3 - portal fibrosis with many septa, F4 - cirrhosis).

Patients were grouped according to their stage of fibrosis: the absence of fibrosis or patients in the initial stages of fibrosis (F0-F2, $n = 84$), patients with advanced stages of fibrosis or cirrhosis (F3-F4, $n = 57$), without cirrhosis (F0-F3, $n = 103$), and with cirrhosis (F4, $n = 38$). Subsequently, the allele, genotype, and haplotype frequencies were compared between the first and second group of patients, to evaluate if the studied polymorphic variants influenced the development of hepatic fibrosis. The frequencies were also compared between the last two groups of patients to evaluate their influence on the development of hepatic cirrhosis.

2.3. Viral genotyping

HCV genotyping was defined through the reverse line probe assay technique (INNOLIPA® v.1.0, Innogenetics, Ghent, Belgium), according to the manufacturer's instructions. This genotyping was preceded by the extraction of total RNA present in the patient's plasma, followed by a reverse-transcription-polymerase chain reaction (RT-PCR), using the Amplicor HCV test version 2.0 kit (Roche Diagnostic System, Branchburg, NJ, USA).

2.4. Genomic DNA extraction

Genomic DNA was extracted from whole blood obtained from an initial volume of 10 ml, collected into tubes containing EDTA. The extraction was performed through the Salting-out technique (Lahiri and Nurnberger, 1991) or a BioPur commercial kit (Biometrix Diagnóstica, Curitiba, Pr, Brazil).

2.5. Genotyping of polymorphic variants in cytokine/cytokine receptors genes

The genotyping of the polymorphic variants of cytokine genes was performed with 75–125 ng/μl of DNA, by PCR-SSP (polymerase chain reaction with sequence-specific primers) using the Cytokine Genotyping kit (Dynal Biotech, Invitrogen® Corporation, Brown Deer, WI, USA) according to the manufacturer's instructions. It was determined that the alleles, genotypes and haplotypes for 22 SNPs were located in 13 cytokine/cytokine receptors genes (Table 1). The amplified fragments were separated in a 2% agarose gel in a horizontal electrophoresis system. The interpretation of the results was performed according to standard forms provided by the manufacturer of the Cytokine Genotyping kit. Ten patients had not all SNPs typed because typing problems in kit used.

2.6. Statistical analysis

The allelic and genotypic frequencies were obtained by direct counting. Haplotype frequencies were estimated based on the genotype frequencies observed through the likelihood method using the EM algorithm (expectation maximization), which is part of an integrated software package available in Arlequin version 3.5 (Excoffier et al., 2005). Convert software was used to prepare the input file for the Arlequin package (Glaubitz, 2004). The Hardy–Weinberg equilibrium of the genotype frequencies was evaluated through Arlequin version 3.5 (Excoffier et al., 2005).

The comparison of frequencies in each sub-sample was performed by 2×2 contingency tables using the chi-square or Fisher's exact test, when $n \leq 5$ in any cell. Differences were considered statistically significant when $P \leq 0.05$. The association strength was assessed by Odds Ratio (OR) obtained with a confidence interval (CI) of 95% (Woolf, 1955). Haldane correction was employed when $n \leq 5$ in any cell (Svejgaard and Ryder, 1994). Statistical analyzes were performed using the Vassar Stats software (http://faculty.vassar.edu/lowry/VassarStats.html). Stepwise logistic regression was also used to assess independent associations between cirrhosis or fibrosis with polymorphic variants, besides some other categorical explanatory variables, such as age and gender. The analysis was conducted using the SPSS 20.0 statistical package (SPSS, Inc., Chicago, IL, USA).

Table 1
List of the investigated cytokine SNPs.

Cytokine gene	Gene chromosome location	SNP designation in the kit	dbSNP-ID	SNP chromosome position (reference)	Location
IL1A	2q14	−889C>T	rs1800587	113259431	5′-UTR
IL1B	2q14	−511 T>C	rs16944	113311338	Promoter
		+3962C>T	rs1143634	113306861	Coding/synonymous
IL1R1	2q12	pst1 1970C>T	rs2234650	102124759	Distal promoter
IL1RN	2q14.2	mspa1 11100C>T	rs315952	113606775	Coding/synonymous
IL4RA	16p12.1–p11.2	+1902G>A	rs1801275	27281901	Coding/missense
IL12B	5q31.1–33.1	−1188 A>C	rs3212227	158675528	3′-UTR
IFNG	12q14	+874 T>A	rs2430561	66838787	Intron
TGFB1	19q13.1	codon 10C>T	rs1800470	46550761	Coding/missense
		codon 25G>C	rs1800471	46550716	Coding/missense
TNF	6p21.3	−308G>A	rs1800629	31651010	Promoter
		−238G>A	rs361525	31651080	Promoter
IL2	4q26–27	−330 T>G	rs2069762	123597430	Promoter
		+166G>T	rs2069763	123596932	Coding/synonymous
IL4	5q31.1	−1098 T>G	rs2243248	132036543	Promoter
		−590C>T	rs2243250	132037053	Promoter
		−33 T>C	rs2070874	132037609	5′-UTR
IL6	7p21	−174G>C	rs1800795	22733170	Promoter
		nt565G>A	rs1800797	22732746	Promoter
IL10	1q31–q32	−1082 A>G	rs1800896	205013520	Promoter
		−819C>T	rs1800871	205013257	Promoter
		−592C>A	rs1800872	205013030	Promoter

3. Results

3.1. Demographic and clinical information of patients

Demographic and clinical information of patients is shown in Table 2. According to METAVIR score, 59.6% of patients presented no or mild fibrosis (F0-F2) and 73.1% presented no cirrhosis (F0-F3) (5.0% were F0, 31.2% F1, 23.4% F2, 13.5% F3 and 26.9% F4). The mean age and duration of infection of patients with advanced fibrosis or cirrhosis (F3-F4) was higher than for the patients with no or mild fibrosis (F0-F2) (49.0 ± 9.2 years *vs* 40.1 ± 9.6 years, $P < 0.05$ and 26.0 ± 9.0 years *vs* 19.0 ± 6.9 years, $P < 0.05$, respectively). The same trend was observed for patients with cirrhosis (F4), when compared to non-cirrhosis patients (F0-F3) (50.1 ± 9.5 years *vs* 41.3 ± 9.7 years, $P < 0.05$ and 27.9 ± 9.7 years *vs* 19.8 ± 7.1 years, $P < 0.05$, respectively).

3.2. Allele frequencies

Allele frequencies of polymorphic variants in the assessed cytokine genes are shown in Table 3. For technical reasons, some patients did not have all their SNPs typed. The frequency of the *IL4RA* + 1902/A allele was higher in patients with advanced stages of hepatic fibrosis (80.7% *vs* 69.6%, $P = 0.0374$; OR = 1.8228; IC = 1.0312–3.2222). The *TNFA*-308/A allele was also more frequent in patients with advanced stages of hepatic fibrosis (20.0% *vs* 10.1% $P = 0.0203$; OR = 2.2206; IC = 1.1190–4.4066), as well as in patients with cirrhosis (22.2% *vs* 11.2% $P = 0.0200$; OR = 2.2733; IC = 1.1235–4.5998). Accordingly, reciprocal association (protection) was also observed for the *TNFA*-308/G allele.

3.3. Genotype frequencies

The genotype frequencies of polymorphic variants in cytokine genes are shown in Table 4. The following genotypes were more frequent in patients with less severe stages of fibrosis (F0-F2): *TNFA*-308/G:G (82.1% *vs* 60.0%; $P = 0.0038$; OR = 0.326; IC = 0.15–0.7088) and *IL6*-174/G:G (58.0% *vs* 40.4%; $P = 0.0409$; OR = 0.489; IC = 0.2457–0.9747). Genotypes *TNFA*-308/G:A (15.5% *vs* 40.0%; $P = 0.0011$; OR = 3.641; IC = 1.6354–8.1065), *IL6*-174/G:C (33.3% *vs* 52.6%; $P = 0.0233$; OR = 2.222; IC = 1.1085–4.455) and *IL6*nt565/G:A (30.9% *vs* 50.9%; $P = 0.0176$; OR = 2.32; IC = 1.1505–4.6784) were more frequent in patients with more severe stages of liver fibrosis (F3-F4).

Genotypes *IL4RA* + 1902/G:G (15.5% *vs* 2.6%; $P = 0.0414$; OR = 0.147; IC = 0.0188–1.1491) and *TNFA*-308/G:G (79.7% *vs* 55.6%; $P = 0.0049$; OR = 0.3201; IC = 0.1419–0.7222) were more frequent in patients without liver cirrhosis (F0-F3), while genotype *TNFA*-308/G:A (18.4% *vs* 44.4%; $P = 0.0019$; OR = 3.5368; IC = 1.5505–8.0681) was more frequent in the cirrhosis patients (F4).

Table 2
Demographic and clinical information of patients of a Brazilian population with chronic hepatitis C classified according to the degree of fibrosis by the Metavir scale.

Characteristics	Patients (n = 141)		F0-F2 (n = 84)		F3-F4 (n = 57)		F0-F3 (n = 103)		F4 (n = 38)	
Age (years, mean ± SD)*	43.7	±10.4	40.1	±9.6	49.0	±9.2	41.3	±9.7	50.1	±9.5
Gender n (%)										
Male	108	(76.6)	65	(77.4)	43	(75.4)	81	(78.6)	27	(71.0)
Female	33	(23.4)	19	(22.6)	14	(24.6)	22	(21.4)	11	(28.0)
Duration of infection (years, mean ± SD)*,[a]	21.8	±8.5	19.0	±6.9	26.0	±9.0	19.8	±7.1	27.9	±9.7
METAVIR stage n (%)[b]										
F0	7	(5.0)	7	(8.3)	–	–	7	(6.8)	–	–
F1	44	(31.2)	44	(52.4)	–	–	44	(42.7)	–	–
F2	33	(23.4)	33	(39.3)	–	–	33	(32.0)	–	–
F3	19	(13.5)	–	–	19	(33.3)	19	(18.5)	–	–
F4	38	(26.9)	–	–	38	(66.7)	–	–	38	(100.0)

* $P < 0,05$ when comparing F0-F2 *vs* F3-F4 and F0-F3 *vs* F4.
[a] METAVIR score: F0 – no fibrosis, F1 – portal fibrosis without septa, F2 – portal fibrosis and few septa, F3 – numerous septa without cirrhosis, F4 – cirrhosis.
[b] Duration of infection was calculated only for 87 patients (F0 = 4, F1 = 25, F2 = 23, F3 = 14, F4 = 21). The duration of infection for 54 patients is unknown.

Table 3
Distribution of allele frequencies of polymorphisms in cytokine genes in patients of a Brazilian population with chronic hepatitis C, classified according to their degree of fibrosis by the Metavir scale.

Polymorphism	Alleles	F0-F2 (n = 84)		F3-F4 (n = 57)		F0-F3 (n = 103)		F4 (n = 38)	
		n	(F%)	n	(F%)	n	(F%)	n	(F%)
IL1A-889	C	123	(74.1)	79	(74.5)	151	(74.0)	51	(75.0)
	T	43	(25.9)	27	(25.5)	53	(26.0)	17	(25.0)
IL1B-511	C	99	(61.1)	74	(64.9)	123	(61.5)	50	(65.8)
	T	63	(38.9)	40	(35.1)	77	(38.5)	26	(34.2)
IL1B + 3962	C	131	(79.9)	88	(77.2)	160	(79.2)	59	(77.6)
	T	33	(20.1)	26	(22.8)	42	(20.8)	17	(22.4)
IL1R1 pst1 1970	T	57	(34.3)	28	(24.6)	64	(31.4)	21	(27.6)
	C	109	(65.7)	86	(75.4)	140	(68.6)	55	(72.4)
IL1RA mspa1 11100	T	121	(72.0)	87	(76.3)	150	(72.8)	58	(76.3)
	C	47	(28.0)	27	(23.7)	56	(27.2)	18	(23.7)
IL4RA + 1902	**A**[a]	**117**	**(69.6)**	**92**	**(80.7)**	147	(71.4)	62	(81.6)
	G	51	(30.4)	22	(19.3)	59	(28.6)	14	(18.4)
IL12B-1188	A	120	(75.0)	76	(70.4)	146	(73.7)	50	(71.4)
	C	40	(25.0)	32	(29.6)	52	(26.3)	20	(28.6)
IFNG + 874	A	91	(54.2)	56	(50.9)	109	(52.9)	38	(52.8)
	T	77	(45.8)	54	(49.1)	97	(47.1)	34	(47.2)
TGFB1 cdn10	C	80	(47.6)	51	(45.5)	96	(46.6)	35	(47.3)
	T	88	(52.4)	61	(54.5)	110	(53.4)	39	(52.7)
TGFB1 cdn25	C	9	(5.4)	8	(7.1)	12	(5.8)	5	(6.7)
	G	159	(94.6)	104	(92.9)	194	(94.2)	69	(93.3)
TNFA-308	G	151	(89.9)	88	(80.0)	183	(88.8)	56	(77.8)
	A[b]	**17**	**(10.1)**	**22**	**(20.0)**	**23**	**(11.2)**	**16**	**(22.2)**
TNFA-238	G	160	(95.2)	104	(94.6)	197	(95.6)	67	(93.0)
	A	8	(4.8)	6	(5.5)	9	(4.4)	5	(7.0)
IL2-330	G	51	(31.1)	30	(26.8)	62	(30.7)	19	(25.7)
	T	113	(68.9)	82	(73.2)	140	(69.3)	55	(74.3)
IL2 + 166	G	110	(67.1)	72	(64.3)	134	(66.3)	48	(64.9)
	T	54	(32.9)	40	(35.7)	68	(33.7)	26	(35.1)
IL4-1098	T	121	(76.6)	86	(79.6)	152	(78.4)	55	(76.4)
	G	37	(23.4)	22	(20.4)	42	(21.6)	17	(23.6)
IL4-590	C	120	(76.0)	87	(80.5)	148	(76.3)	59	(81.9)
	T	38	(24.0)	21	(19.5)	46	(23.7)	13	(18.1)
IL4-33	T	35	(22.2)	18	(16.7)	41	(21.1)	12	(16.7)
	C	123	(77.8)	90	(83.3)	153	(78.9)	60	(83.3)
IL6-174	G	121	(74.7)	76	(66.7)	146	(73.0)	51	(67.1)
	C	41	(25.3)	38	(33.3)	54	(27.0)	25	(32.9)
*IL6*nt565	G	123	(75.9)	79	(69.3)	149	(74.5)	53	(69.7)
	A	39	(24.1)	35	(30.7)	51	(25.5)	23	(30.3)
IL10-1082	G	67	(39.9)	41	(37.3)	81	(39.7)	27	(36.5)
	A	101	(60.1)	69	(62.7)	123	(60.3)	47	(63.5)
IL10-819	C	114	(67.9)	79	(71.8)	143	(70.1)	50	(67.6)
	T	54	(32.1)	31	(28.2)	61	(29.9)	24	(32.4)
IL10-592	C	114	(67.9)	79	(71.8)	143	(70.1)	50	(67.6)
	A	54	(32.1)	31	(28.2)	61	(29.9)	24	(32.4)

For technical reasons, some patients did not have all their SNPs typed, so N is variable depending on the SNP.
P obtained through chi-square test or Fisher's test.
n = number of alleles; F% = allele relative frequency.
F0-F2, early degree of liver fibrosis; F3-F4, advanced degrees of liver fibrosis or cirrhosis; F0-F3, no cirrhosis; F4, cirrhosis.
[a] F3-F4 *vs* F0-F2: 92 (80.7%) *vs* 117 (69.6%). $P = 0.0374$; OR = 1.8228; IC = 1.0312–3.2222[b] F3-F4 *vs* F0-F2: 22 (20.0%) *vs* 17 (10.1%) $P = 0.0203$; OR = 2.2206; IC = 1.1190–4.4066.
[b] F4 *vs* F0-F3: 16 (22.2%) *vs* 23 (11.2%) $P = 0.0200$; OR = 2.2733; IC = 1.1235–4.5998.

3.4. Haplotype frequencies

The haplotype frequency of polymorphic variants in cytokine genes is shown in Table 5. The haplotype *TNFA*-308; -238/GG was more frequent in patients with less severe stages of liver fibrosis as well as in the no cirrhosis group (F0-F2 *vs* F3-F4: 85.1% *vs* 74.5%; $P = 0.0281$; OR = 0.512; IC = 0.2799–0.9365 and F0-F3 *vs* F4: 84.5% *vs* 70.8%; $P = 0.0112$; OR = 0.4466; IC = 0.2372–0.8409, respectively). The haplotype TNFA-308; −238/AG was more frequent in patients with a more severe stage of fibrosis, as well as in the group with cirrhosis (F0-F2 *vs* F3-F4: 10.1% *vs* 20.0%; $P = 0.0203$; OR = 2.2206; IC = 1.119–4.4066 and F0-F3 *vs* F4: 11.1% *vs* 22.2%; $P = 0.0200$; OR = 2.2733; IC = 1.1235–4.5998).

3.5. Multivariate analysis

Multivariate logistic regression, adjusting for the simultaneous contributions of independent variables (gender, age, and polymorphic variants), indicated that age (F0-F2 *vs* F3-F4: $P = 0.0001$; OR = 1.101 and F0-F3 *vs* F4: $P = 0.0001$; OR = 1.162) and the *TNFA*-308/G:A genotype (F0-F2 *vs* F3-F4: $P = 0.006$; OR = 3.784 and F0-F3 *vs* F4: $P = 0.008$; OR = 4.495) conferred increased risk of fibrosis and cirrhosis. Moreover, the genotype *TNFA*-238G:G was associated with protection from cirrhosis (F0-F3 *vs* F4: $P = 0.005$; OR = 0.078). The *IL10*-819C:T genotype (F0-F2 *vs* F3-F4: $P = 0.014$; OR = 0.334) also conferred protection from fibrosis and the *IL1B*-511C:T genotype (F0-F3 *vs* F4: $P = 0.011$; OR = 3.871) conferred increased risk of cirrhosis. These results are shown in Table 6.

Some of these genotypes showed results on the borderline of statistical significance in the bivariate analysis; the *IL10*-819C:T (F0-F2 *vs* F3-F4: 50.0% *vs* 34.5%; $P = 0.0727$; OR = 0.5278; IC = 0.2617–1.0642) and the *IL1B*-511C:T (F0-F3 *vs* F4: 41.0% *vs* 57.9%; $P = 0.0750$; OR = 1.9787; IC = 0.9278–4.2196) genotypes. See Table 4.

4. Discussion

The genotype frequencies for all analyzed SNPs except the *IL4RA* + 1902 position ($P = 0.0017$) are in Hardy–Weinberg equilibrium. It is not uncommon to find SNP frequencies not in Hardy–Weinberg equilibrium in patient samples (control free). Esser and Tomluk (2005) comment that if the deviation from Hardy–Weinberg equilibrium occurs only in the patient group, this provides further evidence of a real association with the disease observed for the marker in question (Esser and Tomluk, 2005).

Bivariate analysis work with two paired data sets studying whether a relationship exists between them; not taking in consideration the influence of the other interleukin genotypes that were also analyzed. On the other hand, the multivariate analysis allows to explore the joint performance of the genotypes, and to test for the effect of each one in the presence of the effect the other genotypes. We believe that this analysis better reflects what happens "*in vivo*", where different genotype products can interact to produce a certain phenotype (Warner, 2012). So, we decided to include in the Discussion section only the genotypes that presented associations with the multivariate statistical analysis. We decided to kept the results with the bivariate analysis in the Results section of the paper because this is the statistical method most applied by researchers, so our data can be compared to others that use bivariate statistical analysis.

An association between the *TNFA*-308/A allele and more severe stages of liver fibrosis/cirrhosis has been observed in this work; individuals carrying this allele are about twice as likely to develop advanced stages of liver fibrosis/cirrhosis as non-carriers. Our results are in agreement with the literature (Yee et al., 2000; Yu et al., 2003; Dai et al., 2006; Kusumoto et al., 2006; Jeng et al., 2007). Nevertheless, other authors did not observe this association, or observed an inverse one. Goyal et al. (Goyal et al., 2004), when studying an Indian population chronically infected by HCV of different genotypes, found no association between the polymorphic variants of the *TNFA*-308 SNP and liver damage. Bouzgarrou et al. (Bouzgarrou et al., 2010), Barret et al. (Barrett et al., 2003), and Powell et al. (Powell et al., 2000) also found no association between alleles, genotypes, and phenotypes of cytokine production and fibrosis when studying populations of Tunisia, Ireland, and Australia, respectively. Similarly, Bahr et al. (Bahr et al., 2003) found no association between the *TNFA*-308 SNP and liver cirrhosis in a German population. Goncharova et al. (Goncharova et al., 2008), on the other hand, reported a higher frequency of the *TNFA*-308/A allele

in Russian patients with a lower stage of liver fibrosis/cirrhosis. The conflict between the results could be partially explained by ethnic differences among patients. Furthermore, most studies show sample group heterogeneity, which is formed, for example, by individuals infected with different viral genotypes, and in some cases, with an unrepresentative sample size.

Table 4
Distribution of genotype frequencies of polymorphisms in cytokine genes in patients of a Brazilian population with chronic hepatitis C, classified according with the fibrosis degree by the Metavir scale.

Polymorphism	Genotypes	F0-F2 (n = 84)		F3-F4 (n = 57)		F0-F3 (n = 103)		F4 (n = 38)	
		n	(F%)	n	(F%)	n	(F%)	n	(F%)
IL1A-889	C:C	45	(54.2)	31	(58.5)	56	(54.9)	20	(58.8)
	C:T	33	(39.8)	17	(32.1)	39	(38.2)	11	(32.4)
	T:T	5	(6.0)	5	(9.4)	7	(6.9)	3	(8.8)
IL1B-511	C:C	32	(39.5)	23	(40.4)	41	(41.0)	14	(36.8)
	C:T	35	(43.2)	28	(49.1)	41	(41.0)	22	(57.9)
	T:T	14	(17.3)	6	(10.5)	18	(18.0)	2	(5.3)
IL1B + 3962	C:C	52	(63.4)	34	(59.7)	63	(62.3)	23	(60.5)
	T:C	27	(32.9)	20	(35.1)	34	(33.7)	13	(34.2)
	T:T	3	(3.7)	3	(5.3)	4	(4.0)	2	(5.3)
IL1R1 pst1 1970	T:T	8	(9.6)	3	(5.3)	8	(7.8)	3	(7.9)
	C:T	41	(49.4)	22	(38.6)	48	(47.1)	15	(39.5)
	C:C	34	(41.0)	32	(56.1)	46	(45.1)	20	(52.6)
IL1RA mspa1 11100	T:T	44	(52.4)	33	(57.9)	56	(54.4)	21	(55.3)
	T:C	33	(39.3)	21	(36.8)	38	(36.9)	16	(42.1)
	C:C	7	(8.3)	3	(5.3)	9	(8.7)	1	(2.6)
IL4RA + 1902	A:A	46	(54.8)	39	(68.4)	60	(58.3)	25	(65.8)
	G:A	25	(29.8)	14	(24.6)	27	(26.2)	12	(31.6)
	G:G[a]	**13**	**(15.4)**	**4**	**(7.0)**	**16**	**(15.5)**	**1**	**(2.6)**
IL12B-1188	A:A	44	(55.0)	26	(48.2)	53	(53.5)	17	(48.6)
	C:A	32	(40.0)	24	(44.4)	40	(40.4)	16	(45.7)
	C:C	4	(5.0)	4	(7.4)	6	(6.1)	2	(5.7)
IFNG + 874	A:A	22	(26.2)	17	(30.9)	28	(27.2)	11	(30.6)
	A:T	47	(56.0)	22	(40.0)	53	(51.5)	16	(44.4)
	T:T	15	(17.8)	16	(29.1)	22	(21.3)	9	(25.0)
TGFB1 cdn10	C:C	22	(26.1)	11	(19.6)	25	(24.3)	8	(21.6)
	C:T	36	(42.9)	29	(51.8)	46	(44.7)	19	(51.4)
	T:T	26	(31.0)	16	(28.6)	32	(31.0)	10	(27.0)
TGFB1 cdn25	C:C	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
	C:G	9	(10.7)	8	(14.3)	12	(11.7)	5	(13.5)
	G:G	75	(89.3)	48	(85.7)	91	(88.3)	32	(86.5)
TNFA-308	**G:G**[b,c]	**69**	**(82.1)**	**33**	**(60.0)**	**82**	**(79.7)**	**20**	**(55.6)**
	G:A[d,e]	**13**	**(15.5)**	**22**	**(40.0)**	**19**	**(18.4)**	**16**	**(44.4)**
	A:A	2	(2.4)	0	(0.0)	2	(1.9)	0	(0.0)
TNFA-238	G:G	76	(90.5)	49	(89.1)	94	(91.3)	31	(86.1)
	G:A	8	(9.5)	6	(10.9)	9	(8.7)	5	(13.9)
	A:A	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
IL2-330	G:G	9	(11.0)	4	(7.1)	11	(10.9)	2	(5.4)
	G:T	33	(40.2)	22	(39.3)	40	(39.6)	15	(40.5)
	T:T	40	(48.8)	30	(53.6)	50	(49.5)	20	(54.1)
IL2 + 166	G:G	38	(46.3)	24	(42.9)	46	(45.5)	16	(43.2)
	G:T	34	(41.5)	24	(42.9)	42	(41.6)	16	(43.2)
	T:T	10	(12.2)	8	(14.2)	13	(12.9)	5	(13.6)
IL4-1098	T:T	44	(55.7)	34	(63.0)	58	(59.8)	20	(55.5)
	T:G	33	(41.8)	18	(33.3)	36	(37.1)	15	(41.7)
	G:G	2	(2.5)	2	(3.7)	3	(3.1)	1	(2.8)
IL4-590	C:C	46	(58.2)	36	(66.7)	56	(57.7)	26	(72.3)
	C:T	28	(35.4)	15	(27.8)	36	(37.1)	7	(19.4)
	T:T	5	(6.4)	3	(5.5)	5	(5.2)	3	(8.3)
IL4-33	T:T	4	(5.1)	2	(3.7)	4	(4.1)	2	(5.6)
	T:C	27	(34.2)	14	(26.0)	33	(34.0)	8	(22.2)
	C:C	48	(60.7)	38	(70.3)	60	(61.9)	26	(72.2)
IL6-174	**G:G**[f]	**47**	**(58.0)**	**23**	**(40.4)**	**54**	**(54.0)**	**16**	**(42.1)**
	G:C[g]	**27**	**(33.3)**	**30**	**(52.6)**	**38**	**(38.0)**	**19**	**(50.0)**
	C:C	7	(8.7)	4	(7.0)	8	(8.0)	3	(7.9)
*IL6*nt565	G:G	49	(60.5)	25	(43.9)	56	(56.0)	18	(47.4)
	G:A[h]	**25**	**(30.9)**	**29**	**(50.9)**	**37**	**(37.0)**	**17**	**(44.7)**
	A:A	7	(8.6)	3	(5.2)	7	(7.0)	3	(7.9)
IL10-1082	G:G	14	(16.7)	9	(16.4)	16	(15.7)	7	(18.9)
	G:A	39	(46.4)	23	(41.8)	49	(48.0)	13	(35.2)
	A:A	31	(36.9)	23	(41.8)	37	(36.3)	17	(45.9)
IL10-819	C:C	36	(42.9)	30	(54.6)	48	(47.0)	18	(48.7)
	C:T	42	(50.0)	19	(34.5)	47	(46.1)	14	(37.8)
	T:T	6	(7.1)	6	(10.9)	7	(6.9)	5	(13.5)
IL10-592	C:C	36	(42.9)	30	(54.6)	48	(47.0)	18	(48.7)
	C:A	42	(50.0)	19	(34.5)	47	(46.1)	14	(37.8)
	A:A	6	(7.1)	6	(10.9)	7	(6.9)	5	(13.5)

Notes to Table 4:
For technical reasons, some patients did not have all their SNPs typed, so N is variable depending on the SNP.
P obtained through the chi-square test or Fisher's test.
n = number of genotype; F% = relative frequency of genotypes.
F0-F2: early stages of liver fibrosis; F3-F4, advanced stages of liver fibrosis or cirrhosis; F0-F3, no cirrhosis; F4, cirrhosis.
[a] F0-F3 *vs* F4: 16 (15.5%) *vs* 1 (2.6%) $P = 0.0414$; OR = 0.147; IC = 0.0188–1.1491.
[b] F0-F2 *vs* F3-F4: 47 (58.0%) *vs* 23 (40.4%) $P = 0.0409$; OR = 0.489; IC = 0.2457–0.9747.
[c] F0-F2 *vs* F3-F4: 27 (33.3%) *vs* 30 (52.6%) $P = 0.0233$; OR = 2.222; IC = 1.1085–4.455.
[d] F0-F2 *vs* F3-F4: 25 (30.9%) *vs* 29 (50.9%) $P = 0.0176$; OR = 2.32; IC = 1.1505–4.6784.
[e] F0-F2 *vs* F3-F4: 69 (82.1%) *vs* 33 (60.0%) $P = 0.0038$; OR = 0.326; IC = 0.15–0.7088.
[f] F0-F3 *vs* F4: 82 (79.7%) *vs* 20 (55.6%) $P = 0.0049$; OR = 0.3201; IC = 0.1419–0.7222.
[g] F0-F2 *vs* F3-F4: 13 (15.5%) *vs* 22 (40.0%) $P = 0.0011$; OR = 3.641; IC = 1.6354–8.1065.
[h] F0-F3 *vs* F4: 19 (18.4%) *vs* 16 (44.4%) $P = 0.0019$; OR = 3.5368; IC = 1.5505–8.0681.

Bivariate and multivariate analysis revealed *TNFA*-308G>A genotypic associations. The *TNFA*-308/G:G genotype showed a negative association with liver damage, while the *TNFA*-308/G:A genotype was positively associated with it. Radwan et al. (2012) also observed association between the G:A genotype and the development of liver cirrhosis, while Bahr et al. (Bahr et al., 2003) did not observe this association. Corchado et al. (Corchado et al., 2013), studying HIV co-infected patients, also found no associations for the *TNFA*-308G>A position. However, they observed an association of *TNFA*-238/G:G genotype with cirrhosis. In the present study, this genotype showed a protective role. Our result is in agreement with the literature, since other authors have observed an association between *TNFA*-238/A and development of chronic active hepatitis C, advanced fibrosis progression, or high risk of cirrhosis (Hohler et al., 1998; Yee et al., 2000). Furthermore, an association between the *TNFA*-238/A allele and more intense inflammatory activity was observed (Pociot et al., 1995) but not with fibrosis/cirrhosis.

A possible biological explanation for the associations of the *TNFA*-308G>A position found in this study is the influence of the *TNFA*-308/A allele in gene transcription. Despite some controversy (Smith and Humphries, 2009), there is evidence that *TNF* transcription is highly influenced by the -308G>A polymorphism. The *TNFA*-308/A allele has been shown to almost double the *TNFA* gene expression and, thus, the plasma levels of the TNF-α cytokine, possibly due to generating a different nuclear protein binding site (Kroeger et al., 1997; Minton et al., 2005). The number of −308/A alleles that an individual possess also plays a role in the plasma levels of TNF-α as genotypes −308/A:A and −308/G:A are associated with high production of the cytokine (Perrey et al., 1998; Tambur et al., 2001; Minton et al., 2005). Although we did not measure TNF-α plasma levels, it would be plausible to assume that the high plasma (Crespo et al., 2002; Neuman et al., 2002; Andersen et al., 2011) and intrahepatic levels (Llorent et al., 1996; Mahmood et al., 2002) of this cytokine seen in patients with high stages of fibrosis/cirrhosis could be the result of a higher expression of the *TNFA*-308/A allele in these patients. The high TNF-α levels would be likely to intensify its pro-inflammatory action in the fibrotic process and would increase its stimulation of hepatic stellate cells, protagonists of the fibrogenesis process (Ruuls and Sedgwick, 1999; Albanis and Friedman, 2001; Wang et al., 2013). Connolly et al. (Connolly et al., 2009), using mouse models, investigated the contribution of dendritic cells in the fibrotic environment and reported that TNF-α was the means by which dendritic cells control liver inflammation and fibrogenesis. It has been found that dendritic cells doubled the production of TNF-α and IL-6 after hepatic fibrosis induction, and the secretion of TNF-α allowed them to stimulate natural killer cells, T lymphocytes, and hepatic stellate cells.

Our results showed that the haplotype GG (*TNFA*-308G/−238G) was negatively associated with liver damage. However, haplotype AG

Table 5
Distribution of haplotype frequencies of polymorphisms in cytokine genes in patients of a Brazilian population with chronic hepatitis C, classified according with the fibrosis degree by the Metavir scale.

Polymorphism	Haplotypes	F0-F2 (n = 84)		F3-F4 (n = 57)		F0-F3 (n = 103)		F4 (n = 38)	
		n	(F%)	n	(F%)	n	(F%)	n	(F%)
TGFBβ1	CG	71	(42.2)	43	(38.3)	84	(40.8)	30	(40.5)
(cdn10;	TG	88	(52.4)	61	(54.5)	110	(53.4)	39	(52.7)
cdn25)	CC	9	(5.4)	8	(7.2)	12	(5.8)	5	(6.8)
TNFA	**GG**[a,b]	**143**	**(85.1)**	**82**	**(74.5)**	**174**	**(84.5)**	**51**	**(70.8)**
(−308;	GA	8	(4.8)	6	(5.5)	9	(4.4)	5	(6.9)
−238)	**AG**[c,d]	**17**	**(10.1)**	**22**	**(20.0)**	**23**	**(11.1)**	**16**	**(22.2)**
IL2 (−330;	TG	59	(36.0)	42	(37.5)	72	(35.6)	29	(39.2)
+166)	GG	51	(31.1)	30	(26.8)	62	(30.7)	19	(25.7)
	TT	54	(32.9)	40	(35.7)	68	(33.7)	26	(35.1)
IL4 (−1098;	GCC	35	(22.2)	21	(19.4)	40	(20.6)	16	(22.2)
−590;	TCC	85	(53.8)	66	(61.1)	108	(55.7)	43	(59.7)
−33)	TTT	34	(21.5)	18	(16.7)	40	(20.6)	12	(16.7)
	TTC	2	(1.3)	2	(1.9)	4	(2.1)	0	(0.0)
	GTT	1	(0.6)	0	(0.0)	1	(0.5)	0	(0.0)
	GTC	1	(0.6)	1	(0.9)	1	(0.5)	1	(1.4)
IL6 (−174;	GG	121	(74.7)	76	(66.7)	146	(73.0)	51	(67.1)
nt565)	*CA*	39	(24.1)	35	(30.7)	51	(25.5)	23	(30.3)
	CG	2	(1.2)	3	(2.6)	3	(1.5)	2	(2.6)
IL10 (−1082;	ATA	54	(32.1)	31	(27.7)	61	(29.6)	24	(32.4)
−819;	ACC	47	(28.0)	39	(34.8)	63	(30.6)	23	(31.1)
−592)	GCC	67	(39.9)	42	(37.5)	82	(39.8)	27	(36.5)

For technical reasons, some patients did not have all their SNPs typed, so N is variable depending on the SNP.
P obtained through the chi-square test or Fisher's test.
n = number of haplotype; F% = relative frequency of haplotype.
F0-F2: early stages of liver fibrosis; F3-F4, advanced stages of liver fibrosis or cirrhosis; F0-F3, no cirrhosis; F4, cirrhosis.
[a] F0-F2 *vs* F3-F4: 143 (85.1%) *vs* 82 (74.5%) $P = 0.0281$; OR = 0.512; IC = 0.2799–0.9365.
[b] F0-F3 *vs* F4: 174 (84.5%) *vs* 51 (70.8%) $P = 0.0112$; OR = 0.4466; IC = 0.2372–0.8409.
[c] F0-F2 *vs* F3-F4: 17 (10.1%) *vs* 22 (20.0%) $P = 0.0203$; OR = 2.2206; IC = 1.119–4.4066.
[d] F0-F3 *vs* F4: 23 (11.1%) *vs* 16 (22.2%) $P = 0.0200$; OR = 2.2733; IC = 1.1235–4.5998.

Table 6
Multivariate analysis of predictors of fibrosis or cirrhosis among patients of a Brazilian population with chronic hepatitis C classified according to the degree of fibrosis by the Metavir scale.

Response variable	Independent variable	*P*	OR	−2 Log likelihood
Degree of fibrosis (F0-F2 *vs* F3-F4)	Age (years)	0.000	1.101	139.957
	TNFA-308G:A	0.006	3.784	
	IL10-819C:T	0.014	0.334	
Presence or absence of cirrhosis	Age (years)	0.000	1.162	104.678
(F0-F3 *vs* F4)	*IL1B*-511C:T	0.011	3.871	
	TNFA-308G:A	0.008	4.495	
	TNFA-238G:G	0.005	0.078	

METAVIR score: F0 – no fibrosis, F1 – portal fibrosis without septa, F2 – portal fibrosis and few septa, F3 – numerous septa without cirrhosis, F4 – cirrhosis.

(*TNFA*-308 A/−238G) was positively associated with it, increasing by 2 times the chance of developing more advanced stages of liver fibrosis/cirrhosis. Since there was no change of the allele in the −238 position in both haplotypes, these haplotype associations seem to be influenced by allelic variants in the −308 position only.

In our study it was observed, by multivariate analysis, that the *IL10*-819C:T genotype conferred fibrosis protection. However, no other author has observed this association when studying this polymorphism in the context of fibrosis/cirrhosis (Abbas et al., 2009). Interleukin-10 is an anti-inflammatory cytokine and an immune response modulator with anti-fibrotic properties (Swiatek, 2012). It has been shown that about 75% of the variation in IL-10 production is genetically determined (Westendorp et al., 1997). Three SNPs located in the promoter region of the *IL10* gene, −1082G>A, −819C>T and -592C>A (Alamartine et al., 2003) seem to be associated with different levels of gene expression, since they possibly alter specific transcription factor recognition sites, thus affecting the levels of cytokine production (Powell et al., 2000). The alleles −1082/G, −819/C, and −592/C could be associated with high production of IL-10 *in vitro*, while their respective alternate alleles would be related to low production (Rosenwasser and Borish, 1997; Turner et al., 1997). Zeng et al. (Zeng et al., 2009) observed that the functional association of these polymorphisms with IL-10 production were allele-dose dependent; therefore, it is expected that the *IL10*-819C:T genotype leads to intermediate production of this cytokine. Abbas et al. (Abbas et al., 2005) observed that the fibrosis score is also allele-dose dependent. An intermediate production of IL-10 by *IL10*-819C carriers, probably associated with other factors, could down-regulate the synthesis of pro-inflammatory cytokines, including TNF-α, and thus play a role in controlling liver disease progression (Swiatek, 2012). It is reasonable to assume that hepatitis C patients who produce high levels of IL-10 have less hepatocellular injury.

There are several polymorphisms in the *IL1B* gene, one being at position −511C>T (Wilson et al., 1993; Tseng et al., 2002). Bahr et al. (Bahr et al., 2003) found an association between -511/T:T genotype and liver cirrhosis. In the present study, multivariate analysis revealed the *IL1B*-511/C:T genotype associated with development of liver cirrhosis. Other authors found no association for this position (Abbas et al., 2005). Findings on the biological functionality of this polymorphism have not been consistent across studies. The −511/C:C genotype showed an increased release of IL-1β (Iacoviello et al., 2005), while the −511/T:T genotype also has been associated with higher levels of IL-1β (Hwang et al., 2002). There isn't information on the level of IL-1β related to −511/C:T genotype. Some studies, however, indicate that multiple polymorphic loci may have combined effects on *IL1B* gene expression (Hall et al., 2004; Chen et al., 2006). So far, the association of the *IL1B*-511/C:T genotype with cirrhosis in the present study can't be explained by IL-1 release level.

5. Conclusions

Our results show that polymorphic variants for the *TNFA*-308G>A, *TNFA*-238G>A, *IL10*-819C>T, and *IL1B*-511 T>C positions are associated with the stage of liver damage during chronic infection with HCV genotype 1. Some of our data confirmed the results of previous studies conducted in other populations, while others were novel and require replication to confirm. In this study, patients were thoroughly characterized with respect to the stage of liver damage and the time of infection, among other possible non-genetic interfering factors, forming a homogeneous group. These efforts may have more clearly characterized the host's genetic interfering factors leading to liver damage of chronically HCV-1 infected patients. We are aware, however, that polymorphisms in cytokine/cytokine receptor genes are obviously not the only factors that influence the stage of liver damage and that polymorphisms in other genes certainly contribute to the process. Therefore, the conclusion is that the hepatic damage in chronically HCV-1 infected patients seems to be under the influence of gene polymorphisms for both cytokines and cytokine receptors; the knowledge of these markers may have prognostic significance in patients chronically infected with HCV, allowing a more aggressive therapy for those with increased risk of evolving to more severe forms of the disease.

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