TNFA -308G>A and *IL10* -1082A>G Polymorphisms as Predictive Biomarkers of Chronic HCV Infection

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Research Article

Keywords: HCV, TNF-α, IL-10, polymorphism

Posted Date: June 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-537692/v1

Abstract

Genetic changes may induce dysregulated cytokine production and affect the progression of the chronic disease caused by the hepacivirus C (HCV) because the balance of pro- and anti-inflammatory cytokines determines the outcome of infection. This study evaluated the *TNFA* − 308G > A and *IL10* -1082A > G polymorphisms in the susceptibility and progress of chronic hepatitis C. The study included 101 samples from patients with chronic hepatitis C and 300 samples from healthy donors. Polymorphisms were typed by real-time PCR and were analyzed for associations with histopathological parameters (according to METAVIR classification) and HCV viral load. The polymorphic genotype for the *TNFA* − 308G > A variant was not present in the group of patients with chronic hepatitis C and was associated with protection against HCV infection ($p = 0.0477$). Patients with the polymorphic genotype of the *IL10* -1082A > G polymorphism had higher HCV viral load than wild-type patients ($p = 0.0428$). Neither polymorphism was associated with different levels of necroinflammatory activity or fibrosis scores. The polymorphic genotype at *TNFA* − 308G > A protected against chronic HCV infection, and the polymorphic genotype at the *IL10* -1082A > G variant was associated with viral persistence.

Introduction

Hepacivirus C (HCV) is the causative agent of hepatitis C and is considered the main cause of liver cancer. It is estimated that over 71 million people are chronically infected by the virus and approximately 399 000 people died from hepatitis C, mostly from cirrhosis and hepatocellular carcinoma [1]. Persistent HCV infection leads to chronic hepatitis, which is mainly due to the inability of the immune system to eliminate the virus [2]. Dysregulated cytokine production is related to the chronicity of hepatitis C; however, no profile of cytokines involved in the development of liver injury has been identified [3].

Tumor necrosis factor (TNF-α) is a pro-inflammatory cytokine that acts both as a mediator of innate immunity and in the cellular immune response. Abnormal TNF-α levels have been associated with chronic HCV infection [4]. Some polymorphisms in the *TNFA* gene are associated with the regulation of cytokine production and coincide with the binding regions of transcription factors [5]. The *TNFA* − 308G > A polymorphism has higher transcriptional activity than the wild-type allele [6] and has been associated with different infectious diseases [7–11].

Interleukin (IL)-10 is a potent suppressor of the effector function of T cells, natural killer (NK) cells and, mainly, activated macrophages [8]. Several functional polymorphisms have been described in the promoter region of the *IL10* gene 10 (12), among which the *IL10* -1082A > G polymorphism promotes changes in cytokine levels, with the A allele being related to lower levels and the G allele with higher levels of IL-10 [12]. This polymorphism has been associated with chronic and infectious diseases [13, 14].

Because the liver is a highly immunotolerant organ, an imbalance of the components related to its suppressor and effector functions may contribute to the persistence of HCV and the progression of chronic cases of hepatitis C. On this background, the present study investigated the influence of the *TNFA*

− 308G > A and *IL10* -1082A > G polymorphisms on the susceptibility to chronic HCV infection, the progression to different disease stages, and viral persistence. The findings in this study may help to understand the physiology of the biomarkers analyzed and their response to chronic HCV infection as well as the progression to the hepatic diseases.

Methods

Study population

The study included patients, both sexes, treated at the liver disease outpatient clinics of the Santa Casa de Misericórdia do Pará Foundation and the João de Barros Barreto University Hospital. Consecutive patients with chronic HCV were included. The HCV group consisted of 101 patients with chronic hepatitis C, characterized by clinical changes, abnormal liver tests and HCV RNA positivity. For diagram of patient flow chart see Fig. 1.

The inclusion criteria adopted for the individuals were as follows: age 18 or older, and positivity for HBsAg for more than 6 months or positivity for HCV RNA, as criteria for chronic HCV infection, and without antiviral therapy. Individuals coinfected with hepatitis B virus (HBV), hepatitis delta virus, or human immunodeficiency virus (HIV) and patients who used or were using specific antiviral therapy against HCV were excluded from the study.

A control group was formed to compare the genotypic and allelic frequencies of the investigated polymorphisms, which included 300 blood samples from volunteer donors from the Foundation Center for Hemotherapy and Hematology of Pará (Fundação Centro de Hemoterapia e Hematologia do Pará). These volunteers were matched with the patient group for age and sex; were seronegative for HCV, HBV, HIV-1, human T-lymphotropic virus 1/2.

The present project was submitted to and approved by the research ethics committees of Santa Casa de Misericórdia do Pará (opinion 772,782/2014) and João de Barros Barreto University Hospital (opinions 962,537/2015 and 2,165,948/2017), in accordance with the principles of the Declaration of Helsinki. All participants were informed about the objectives of the study, and those who agreed to participate signed an informed consent form and answered an epidemiological questionnaire.

DNA extraction

DNA was extracted from peripheral-blood leukocytes using the Puregene kit (Gentra Systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol. The procedure included the steps of cell lysis, protein precipitation, DNA precipitation, and DNA hydration.

Genotyping TNF − 308G > A (rs1800629) e IL10 − 1082A > G (rs1800896)

Polymorphisms were genotyped by quantitative real-time polymerase chain reaction in the StepOne PLUS Sequence Detector (Applied Biosystems, Foster City, CA, USA). The assay used for each polymorphism contained a pair of primers and a pair of VIC- and FAM-labeled probes for the respective alleles. For both polymorphisms, predesigned and customized TaqMan® SNP Genotyping Assays were used: C_7514879_10 for *TNFA* − 308G > A and C_1747360_10 for *IL10* -1082A > G (Thermo Fisher, Carlsbad, California, USA). For each reaction, 2X TaqMan® Universal PCR Master Mix, 1X TaqMan® Assay (diluted from 20X), and 20 ng of DNA was used in a final reaction volume of 10 μL. The following temperature cycle was used for the amplification: 60°C for 30 seconds, 95°C for 10 minutes, and 50 cycles of 92°C for 30 seconds and 60°C for 1 minute and 30 seconds.

Complementary exams

All selected patients were clinically evaluated and subjected to a complimentary investigation consisting of biochemical (liver enzyme levels: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT), serological (HBV surface antigen (HBsAg), HBV e antigen (HBeAg), anti-HBeAg, total anti-HBc and anti-HCV), virological (HBV DNA and hepacivirus C RNA), ultrasound, endoscopic tests and liver biopsies. These data were transcribed from the medical records into a form developed specifically for this study.

Histopathological procedures

Liver biopsy specimens were obtained only from patients with medical indications for the investigation of liver parenchyma changes, in compliance with the clinical care protocol. The liver biopsies were performed by a medical professional from one of the study hospitals using a Tru-Cut needle under ultrasound guidance. The sample was sent to the Department of Pathological Anatomy of Federal University of Pará, where they were examined following the department's routines, which included hematoxylin–eosin (HE), chromotrope aniline blue (CAB), Gomori's reticulin, and Shikata's orcein staining.

The histopathological diagnosis followed the METAVIR classification [15], which classifies the activity of the portal and periportal inflammatory infiltrate from 0 to 3 (A0-A3), A0-A1 indicating absent to mild inflammation and A2-A3 indicating moderate to severe inflammation. The structural changes in the liver parenchyma (degree of fibrosis) were classified from 0 to 4 (F0-F4), F0-F1 indicating absent to mild liver fibrosis, F2 indicating moderate liver fibrosis, and F3-F4 indicating liver fibrosis that has progressed to cirrhosis. All data regarding the histopathological profile were obtained from the patients' medical records.

Statistical analysis

Hardy-Weinberg equilibrium analysis was performed in all samples through the chi-squared test. Comparative analyses of the allelic and genotypic frequencies were done using the G-test and chi-squared test. Comparisons of viral load levels (HCV RNA) were done using the Kruskal-Wallis test and the Mann-Whitney test. Statistical analyses were done with BioEstat software version 5.3, adopting a significance level of $p < 0.05$.

Results

Most patients with chronic HCV were male (n = 52; 51%). The median alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyltransferase concentrations were 77.9 UI/L, 69.9 UI/L, and 99.6 UI/L, respectively. The mean viral load of 5.4 log (Table 1).

Table 1
Characterization of patients with chronic HCV.

Variable	HCV
Sex (F/M), n (%)	49 (48.5)/52 (51.5)
ALT (IU/L), median/IQR	77.9 ± 58.4
AST (IU/L), median/IQR	69.9 ± 48.6
GGT (IU/L), median/IQR	99.6 ± 95.2
Viral load (log_{10}), median/IQR	5.4 ± 0.9
Fibrosis score, n (%)	
0 to 2	67 (66.3%)
3 to 4	34 (33.7%)
Inflammatory activity, n (%)	*
0 to 1	54 (60%)
2 to 3	36 (40%)

n = individuals number; **ALT**: alanine aminotransferase (reference value: 16–40 IU/L); **AST**: aspartate aminotransferase (reference value: 8–54 IU/L); **GGT**: gamma-glutamyltransferase (reference value: 8–63 IU/L). *Inflammatory activity n = 90.

According to the METAVIR classification (Table 1), most patients with HCV had absent to moderate fibrosis, F0-F2 (n = 67; 66.3%). Inflammatory activity was evaluated only in 90 patients because 11 were diagnosed with liver cirrhosis by imaging tests and therefore did not meet the medical indications for biopsy. The majority of the evaluated patients presented with absent or mild inflammatory activity, A0-A1 (n = 54; 60%).

All the genotype frequencies were in Hardy-Weinberg equilibrium. Evaluation of the *TNFA* − 308G > A polymorphism (rs1800629) showed that no patient with chronic HCV had the homozygous polymorphic genotype (AA). The comparison of genotypic frequencies showed a significant difference between the HCV group and control group (p = 0.0477). However, no significant differences were found in allelic frequencies (Table 2).

Table 2
genotypic and allelic frequencies of the *TNFA* − 308G > A and *IL10* -1082A > G polymorphisms in the study groups.

Allelic or Genotypic Profile	**HCV** **n (%)**	**CG** **n (%)**	***p***
TNFA **− 308G > A**			
GG	82 (81.2)	222 (74)	0.0477*
GA	19 (18.8)	69 (23)	
AA	0 (0)	9 (3)	
*G	0.91	0.86	0.3753**
*A	0.09	0.14	
IL10 **-1082A > G**			
AA	62 (61.4)	164 (54.7)	0.4847**
AG	32 (31.7)	109 (36.3)	
GG	7 (6.9)	27 (9)	
*A	0.77	0.73	0.6242**
*G	0.23	0.27	
HCV: patients with chronic hepatitis C; **CG**: control group; *****G-test; **chi-squared test.			

At the *IL10* -1082A > G polymorphism (rs1800896), the wild-type genotype and allele (AA and A, respectively) were the most frequent in the HCV and control groups, with no significant differences between the groups (Table 2).

The evaluation of the frequencies of genotypes and alleles of *TNFA* − 308G > A and *IL10* -1082A > G in relation to histopathological markers of inflammatory activity and fibrosis score showed no significant differences (Table 3).

Table 3
Genotypic and allelic frequencies of the *TNFA* − 308G > A and *IL10* -1082A > G polymorphisms in relation to the histopathological aspects of chronic HCV carriers.

Genetic profile	Inflammatory activity*			Fibrosis score*		
	0 to 1	2 to 3	p	0 to 2	3 to 4	p
	n (%)	n (%)		n (%)	n (%)	
TNFA **− 308G > A**						
GG	43 (79.6)	30 (83.3)	0.8688	54 (80.6)	28 (82.4)	0.9553
GG	11 (20.4)	6 (16.7)		13 (19.4)	6 (17.6)	
AA	0	0		0	0	
*G	0.90	0.92	0.8048	0.90	0.91	1.000
*A	0.10	0.08		0.10	0.09	
IL10 **-1082A > G**						
AA	31 (56.4)	22 (62.9)	0.7530	40 (59.7)	22 (64.7)	0.6839
AG	20 (36.4)	10 (28.6)		23 (34.3)	9 (26.5)	
GG	4 (7.2)	3 (8.5)		4 (6.0)	3 (8.8)	
*A	0.75	0.77	0.8685	0.77	0.78	1.000
*G	0.25	0.23		0.23	0.22	
*G-test.						

The analysis of viral load showed no significant difference between patients with different *TNFA* − 308G > A genotypes. However, patients with the polymorphic genotype of *IL10* -1082A > G had higher HCV viral load than those with the wild-type genotype (p = 0.0428; Fig. 2).

Discussion

The course and outcome of HCV infection are determined by its virological characteristics and the immune responses triggered by the virus [16]. HCV is a hepatotropic virus that induces the development of acute and chronic necroinflammatory disease, escaping the immune system in up to 85% of cases [3]. Several cytokines play dual roles in viral infection and are responsible for viral clearance and tissue damage [17].

TNF-α is an important cytokine in the immune response, mediating the inflammatory process through innate immunity pathways and activation of the cellular response, which induces apoptosis or necrosis

[18]. Thus, genetic variations in the *TNFA* gene that alter cytokine production levels may contribute to the progression of HCV infection.

In the present study, the polymorphic genotype for the *TNFA* − 308G > A variant was not present in the group of patients with HCV. This genotype is correlated with increased expression of the cytokine [19]. In this case, the presence of the homozygous allele may contribute to better immune control, preventing the progression of HCV infection. High levels of TNF-α increase the expression of vascular endothelial adhesion molecules and increase the stimulation of endothelial cells and macrophages, which may lead to better infection resolution [17].

The high frequency of the wild-type allele (G) in the group of patients with chronic HCV infection suggests that in addition to having a higher risk of developing the infection, these patients seem to have a greater chance of developing the chronic form of the disease. The inadequate production of TNF-α by dendritic cells favors the differentiation of $CD4^+$ T cells into IL-10- and non-IFN-γ-producing cells [20]. As the IL-10 cytokine is not effective in resolving the infection, the infection progressed to the chronic form.

Studies on the *TNFA* − 308G > A polymorphism performed in other ethnic groups also observed different frequencies of the polymorphic genotype in patients with HCV than without, showing that although the presence of the homozygous polymorphism was not observed in patients from France [21], in India the prevalence of the polymorphism was higher in the group of patients with HCV [22]. As the population evaluated in this study is trihybrid, formed from the genetic contributions of whites, blacks, and indigenous people [23], the association of the polymorphism with the prevention or risk of HCV infection needs to be better investigated in other ethnic groups.

IL-10 is an anti-inflammatory cytokine produced by Th2 cells that inhibits the activity of Th1, NK, and macrophage cells, the main cells responsible for pathogen elimination. The cytokine acts by limiting the marked pro-inflammatory response and damage caused by inflammation [8]. In infectious processes, there is a direct correlation between lower IL-10 production and greater disease severity [24].

The *IL10* -1082A > G polymorphism is associated with changes in IL-10 level, the wild-type genotype being associated with lower levels [12]. In the present study, no differences in genotype frequencies were found between the groups with and without HCV infection. The evaluation of this polymorphism in HCV infection by other studies has shown different results. Although the present results were similar to those of another study, which also did not find an association between the frequency of the *IL10* -1082A > G polymorphism and susceptibility to HCV infection [25], Ramos *et al*. [26] observed that the GG (polymorphic) genotype was associated with increased chances of viral infection resolution. The combined analysis of these results shows that the polymorphism does not influence the protection from or susceptibility to HCV infection but can influence the disease resolution, reducing the chances of progression to the chronic form among those who develop hepatitis C.

The polymorphic genotype (GG) of the *IL10* -1082A > G variant was associated with higher HCV viral load than the wild-type genotype. Most studies that investigated this polymorphism in HCV infection did not

assess viral load levels. In the study by Abbas *et al*. [27], no difference in viral load was observed between genotypes in patients from Pakistan. Viral load has been associated with the frequency of the homozygous genotype (AA) and that of the wild-type (A) allele [28]. The divergence between the results of that study and the present study may be related to the type of analysis performed, Gao *et al*. [28] evaluated the frequencies of genotypes in relation to the presence or absence of HCV RNA, while the present study evaluated the absolute plasma levels, which were converted to their base-10 logarithm. In addition, the differences may also be related to the ethnicity of the populations assessed between the different studies. The population evaluated in this work is originally from the Brazilian Amazon and has a genetic contribution from Europeans, native Indians and Africans [23], which could contribute to the result found. Some studies have shown that the genetic influence of ethnicity is associated with variations in genes related to the individual's response to diseases [29, 30].

The polymorphic genotype (GG) for *IL10* -1082A > G is associated with higher IL-10 expression. This cytokine inhibits the activation of Th1, NK, and macrophage cells, which are the main cells responsible for the elimination of HCV; higher levels of IL-10 reduce inflammatory activity at the infection site, favoring the persistence of the virus in the tissue, the main characteristic of chronic infection [2]. In this sense, our findings raise the hypothesis that the polymorphic genotype may favor the persistence of HCV in the liver tissue. Follow-up studies are needed to confirm this hypothesis.

The *TNFA* − 308G > A and *IL10* -1082A > G polymorphisms were not associated with different levels of necroinflammatory activity or with fibrosis score. Several studies have also found no relationship between these polymorphisms in the *TNF* and *IL10* genes and different stages of the disease [21, 25, 28, 31, 32). Thus, these polymorphisms seem not to influence the progression of the histopathological processes of chronic HCV infection because in this disease, in addition to the host immunological factors, others factors inherent to the virus act directly on the inflammatory process, causing tissue damage.

In conclusion, the polymorphic genotype at *TNFA* − 308G > A was not present in the group of patients with chronic hepatitis C, but we do not know if it could represent a protective action of this SNP against HCV infection. In the same way, considering that this was a cross-sectional study, the polymorphic genotype for variant *IL10* -1082A > G need to be better analyzed in a follow-up study in order to confirm its association with viral persistence.

Declarations

Acknowledgments

We thank all the participants in this study.

Author Disclosure Statement

The authors have no relevant financial or non-financial interests to disclose.

Funding Information

This study was funded by the National Council for Scientific and Technological Development (CNPQ #480128/2013-8) and by the Federal University of Pará (PROPESP/PAPQ/2020).

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Figures

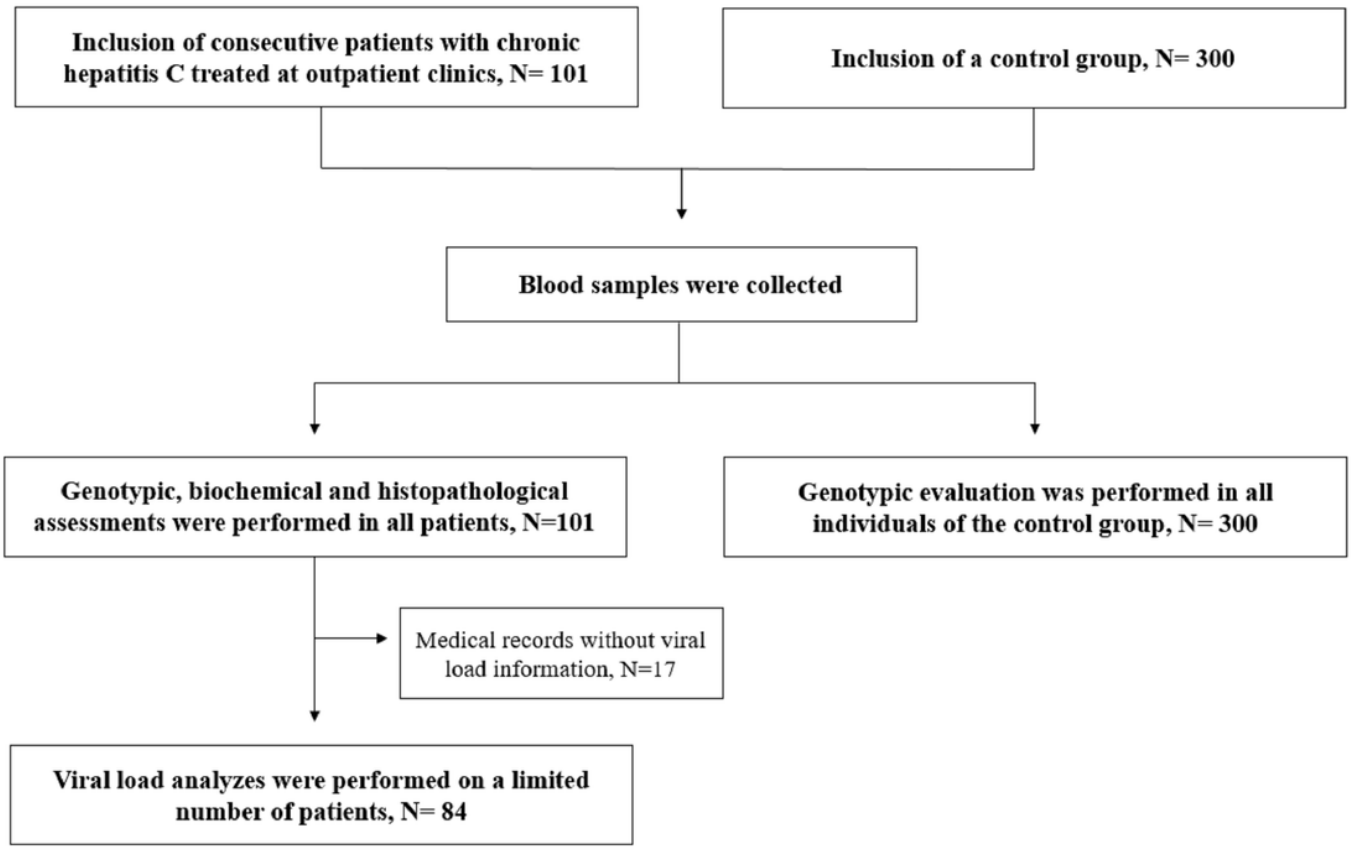

Figure 1

Diagram illustrating the flow of participating patients during the study.

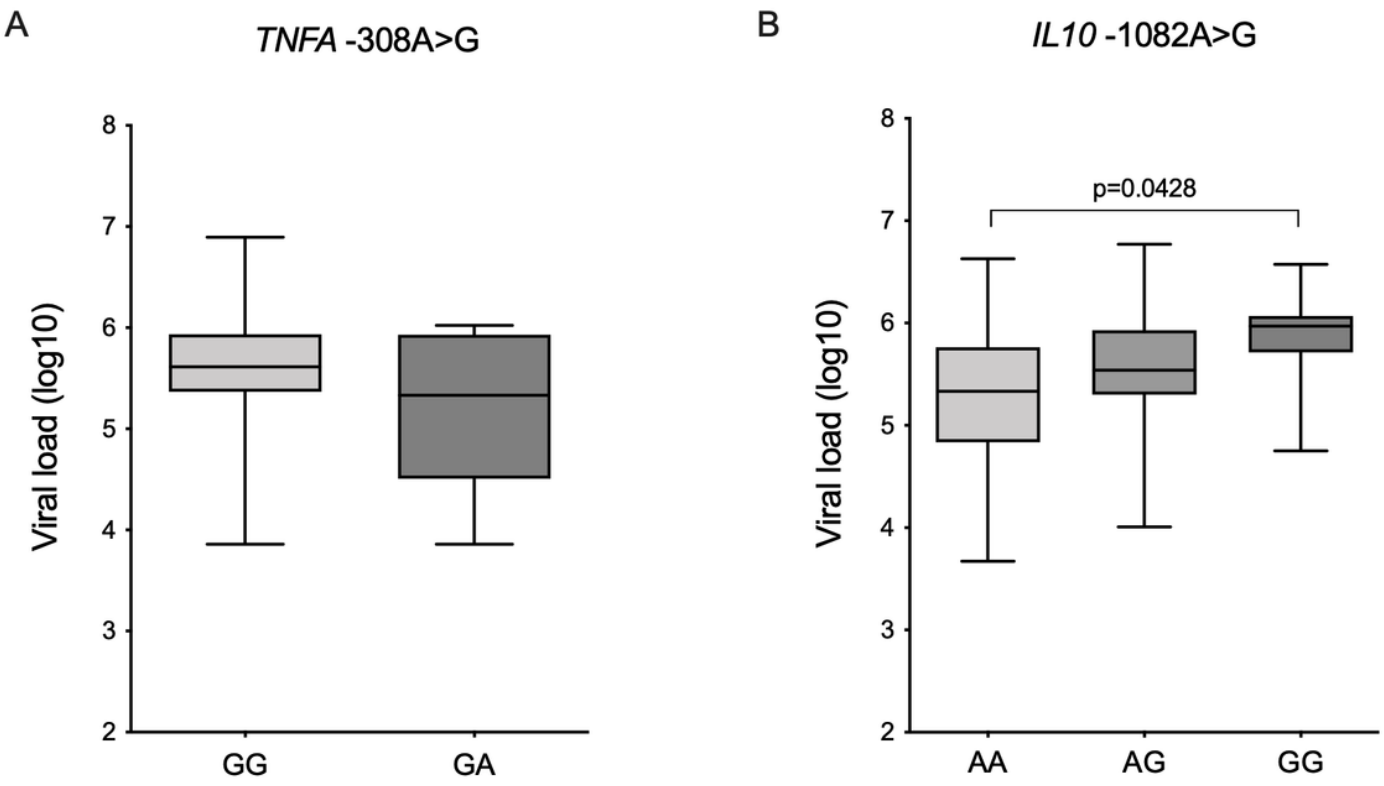

Figure 2

HCV viral load according the genotype for each polymorphism: (A) TNFA -308G>A and (B) IL10 -1082A>G.