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Research paper

Genetic variants upstream of TNFAIP3 in the 6q23 region are associated with liver disease severity in HIV/HCV-coinfected patients: A cross-sectional study

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ABSTRACT

Background: TNFAIP3 is a crucial hepatoprotective factor due to its anti-inflammatory, anti-apoptotic, antioxidant and pro-regenerative functions. The aim of this study was to analyze the associations between genetic variants upstream of *TNFAIP3* (rs675520, rs9376293 and rs6920220) and liver fibrosis severity and inflammation in HIV/HCV-coinfected patients.

Methods: A cross-sectional study was carried out in 215 HIV/HCV-coinfected patients, who underwent a liver biopsy. *TNFAIP3* polymorphisms were genotyped using GoldenGate® assay. Outcome variables were: a) liver fibrosis (Metavir score) [fibrosis stage (F0, F1, F2, F3 and F4) and advanced fibrosis and cirrhosis (F ≥ 3 and F4, respectively)]; b) non-invasive indexes [FIB-4, APRI, and their cut-offs (FIB-4 ≥ 3.25 and APRI ≥ 1.5)]; c) inflammation-related biomarkers (leptin, HGF, NGF, sFasL, sFas, MIF, HA, Ang-2, TIMP1, MMP1 and MMP2).

Results: Patients with rs675520 AG/GG genotypes had decreased odds of having cirrhosis (F4) and advanced fibrosis (FIB-4 ≥ 3.25 and APRI ≥ 1.5) [adjusted Odd Ratio (aOR) = 0.30 (p = 0.025), aOR = 0.20 (p = 0.014), and aOR = 0.34 (p = 0.017), respectively] and lower levels of FIB-4 and APRI [adjusted arithmetic mean ratio (aAMR) = 0.76 (p = 0.003) and aAMR = 0.72 (p = 0.006), respectively]. Patients with rs9376293 CT/CC genotypes had decreased odds of APRI ≥ 1.5 [aOR = 0.39 (p = 0.030)] and lower levels of APRI [aAMR = 0.77 (p = 0.018)]. Patients with rs6920220 AG/AA genotypes had higher odds of having FIB-4 ≥ 3.25 [aOR = 3.72 (p = 0.043)]. Moreover, rs675520 AG/GG genotypes, compared to AA genotype, were associated with lower levels of leptin and NGF (p = 0.002 and p = 0.001, respectively) and higher levels of sFas, MIF, TIMP1 and MMP2 (p = 0.004, p = 0.007, p = 0.020 and p = 0.036, respectively). Also, rs9376293 CT/CC genotypes were related to lower leptin levels (p = 0.026) and higher sFas, MIF, TIMP1 and MMP2 levels (p = 0.029, p = 0.040, p = 0.022 and p = 0.024, respectively).

Conclusions: Genetic variants upstream of *TNFAIP3* were associated with the liver fibrosis severity and inflammation in HIV/HCV-coinfected patients.

1. Introduction

Chronic hepatitis C (CHC) is linked to an important morbidity and mortality, mainly due to the development of liver fibrosis and its evolution towards cirrhosis and hepatocarcinoma (Mengshol et al., 2007; Heim and Thimme, 2014). Several factors may accelerate the risk of these complications such as age at infection, sex, route of infection, hepatitis C virus (HCV) genotype and obesity, among others (Ortiz et al., 2002; Rotman and Liang, 2009; Rueger et al., 2015). Additionally, human immunodeficiency virus (HIV) infection has a negative impact on the natural history of CHC in HIV/HCV coinfected patients, promoting the risk of fibrosis and cirrhosis, and being an

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significant cause of morbidity and death in this group of patients (Lopez-Dieguez et al., 2011; Macias et al., 2009).

HCV infection modulates host antiviral immune response leading to chronic inflammation and thus promotes liver fibrogenesis (Friedman, 2010). Both antiviral and inflammatory responses are necessary for the control of HCV infection, but they have to be tightly regulated to avoid excessive inflammation and host liver damage. These responses are regulated to a great extent by processes of ubiquitination/deubiquitination of different proteins. Tumor necrosis factor alpha induced protein 3 (TNFAIP3) is a key player in the negative regulation of these pathways by its dual function as both a deubiquitinase and a ubiquitin ligase (Arguello et al., 2014). HCV core protein has been shown to induce *TNFAIP3* expression in macrophages, which results in inefficient M1 macrophage polarization and cytokine balance dysregulation leading to low-grade chronic inflammation during HCV infection (Song et al., 2016; Fan et al., 2015). Overexpression of *TNFAIP3* may also promote immune suppression via dendritic cells (DCs) (Breckpot et al., 2009; Song et al., 2008), and negatively regulate myeloid DC function in patients with CHC (Ma et al., 2014).

In addition, TNFAIP3 has been described as a crucial hepatoprotective factor due to multiple functions: a) anti-inflammatory effects, via a blockade of nuclear factor-kB (NF-kB) activation in response to several pro-inflammatory mediators; b) anti-apoptotic effects, by inhibiting pro-caspase 8 cleavage and preserving mitochondrial integrity; c) anti-oxidant effects, by leading to higher expression of Peroxisome Proliferator Activated Receptor alpha and improved energy production, as well as limiting reactive oxygen species formation; and d) pro-regenerative effects, by decreasing the expression of cell cycle inhibitors and increasing IL6/STAT3 proliferative signals (da Silva et al., 2014).

Several single nucleotide polymorphisms (SNPs) around the *TNFAIP3* gene have an important role in susceptibility to inflammatory and autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, juvenile idiopathic arthritis, coeliac disease, psoriasis, coronary artery disease in type 2 diabetes, systemic sclerosis, type I diabetes and Sjogren's syndrome (Mele et al., 2014; Coenen et al., 2009; Stuart et al., 2015; Wang et al., 2016; Zhang et al., 2016a; Zhang et al., 2016b). Also, some articles have described a significant association between *TNFAIP3* polymorphisms and changes in *TNFAIP3* mRNA expression (Ungerback et al., 2012). Some of these polymorphisms, including those reported in the present study, are located in a non-coding region upstream of *TNFAIP3* and oligodendrocyte transcripcion factor 3 *(OLIG3)* in 6q23. OLIG3 is a basic helix-loop-helix transcription factor with known implications in the development of the nervous system (Liu et al., 2008). However, these SNPs have been related to *TNFAIP3* rather than the *OLIG3* gene due to its implication in inflammation and immunity (Zhu et al., 2015).

The objective of this study was to investigate the associations between genetic variants upstream of *TNFAIP3* (rs675520, rs9376293 and rs6920220) and liver fibrosis severity and inflammation in HIV/HCV-coinfected patients.

2. Material and methods

2.1. Patients and study design

A cross-sectional study was carried out in 215 HIV/HCV-coinfected patients, in whom liver disease was evaluated by liver biopsy from September 2000 to November 2008. The study was performed in accordance with the Declaration of Helsinki and all involved patients signed the consent. The Research Ethic Committee and the Institutional Review Board of the Instituto de Salud Carlos III (ISCIII) gave their approval to our study.

Liver biopsies were performed on naïve patients who were potential candidates for anti-HCV therapy. Selection criteria were: detectable HCV RNA by polymerase chain reaction (PCR), no clinical evidence of hepatic decompensation and availability of a DNA sample, CD4+ lymphocyte count > 200 cells/μL, and stable combination antiretroviral therapy (cART) or no need for cART for at least 6 months before study entry according to treatment guidelines (Panel de expertos de Gesida Plan Nacional sobre el Sida y Asociación Española para el Estudio del Hígado, 2010; Ghany et al., 2009). Patients with positive hepatitis B surface antigen, active opportunistic infections, active drug addiction, and other concomitant severe diseases were excluded. All patients included in this study were white of European descent.

2.2. Epidemiological and clinical data

Clinical and epidemiological data were obtained from medical records. Alcohol intake of > 50 g of alcohol per day for at least 12 months was considered as high intake. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. The duration of HCV infection for patients with a history of intravenous drug use was estimated as starting from the first year they shared needles and other injection paraphernalia, which are the most relevant risk practices for HCV transmission.

2.3. Liver biopsy

Liver biopsies were carried out as previously described by our gruop (Resino et al., 2011). Fibrosis and necroinflammatory activity stage were estimated according to Metavir score as follows: F0, no fibrosis; F1, mild fibrosis; F2, significant fibrosis; F3, advanced fibrosis; and F4, definite cirrhosis. The degree of necroinflammation (activity grade) was scored as follows: A0, no activity; A1, mild activity; A2, moderate activity; A3, severe activity. Liver steatosis was determined according to the existence of hepatocytes containing visible macrovesicular fat droplets, which was clinically significant when fatty hepatocytes exceeded 10% of hepatic parenchyma.

2.4. Biochemistry analysis

Biochemistry panel was measured from serum samples using a clinical chemistry analyzer (Hitachi 912, Boehringer Mannheim, Germany) in fasting patients.

Non-invasive liver fibrosis indexes were calculated according to the formula originally described for the Aspartate aminotransferase to platelet ratio index (APRI): [aspartate aminotransferase (AST)(U/L)/upper limit of normal for AST (ULN)]/platelet [10^9/L] × 100; and the fibrosis 4 index (FIB-4): [age (years) × AST (U/L)]/[platelets (10^9/L) × [alanine aminotransferase (ALT) (U/L)]$^{1/2}$)]. These non-invasive markers are robust tools to assess liver fibrosis with a potential role in disease management and clinical decision-making on patients (Resino et al., 2012).

The insulin resistance degree was determined for each patient using the homeostatic model assessment (HOMA): [fasting plasma glucose (mmol/L) × fasting serum insulin (mU/L)]/22.5.

2.5. DNA genotyping

Total DNA was extracted from 200 μL of peripheral blood with Qiagen kit (QIAamp DNA Blood Midi/Maxi; Qiagen, Hilden, Germany). Genetic variants upstream of *TNFAIP3* (rs675520, rs9376293 and rs6920220) were genotyped at the Spanish National Genotyping Center (CeGen). Genotyping was performed by Agena Bioscience's MassARRAY platform (San Diego, CA, USA) using the iPLEX® Gold assay design system. The genotyping process consists of two reactions. First, DNA fragments are amplified by multiplex PCR and second, the allelic discrimination reaction and first extension are carried out. In the iPLEX® Gold technology, all reactions end after a single base extension that generates a mass difference in the extension product according to the added base. This difference in mass is detected by MALDI-TOF mass spectrometry.

Frequencies of alleles and genotypes from healthy subjects for the studied polymorphisms were obtained using the 1000 Genomes Project website (http://www.1000genomes.org/home). This database contains a broad representation of common human genetic variations from multiple populations (Auton et al., 2015). We selected the Iberian populations in Spain (IBS) that included 107 individuals.

2.6. Plasma inflammation-related biomarkers

Multiplex kits (LINCOplext; LINCO Research, St Charles, MO, USA) were used to specifically evaluate six plasma biomarkers with the Luminex 100TM analyzer (Luminex Corporation, Austin, TX, United States). The plasma biomarkers analyzed were leptin, hepatocyte growth factor (HGF), nerve growth factor (NGF), soluble Fas-associated death domain protein ligand (sFasL), soluble Fas-associated death domain protein (sFas), and macrophage migration inhibitory factor (MIF).

Enzyme-linked immunosorbent assay (ELISA) kits were used to test the following six plasma biomarkers: hyaluronic acid (HA, HA-ELISA; Echelon Biosciences Inc., Salt Lake City, UT, USA), angiopoietin-II (Ang-2, R&D Systems, Minneapolis, MN, USA), tissue inhibitor of metalloproteinase-1 (TIMP1), matrix metalloproteinase-1 (MMP1), matrix metalloproteinase-2 (MMP2) (GE Healthcare UK Limited, Buckinghamshire, UK), and chitinase 3 like 1 (CHI3L1) (Quidel Corporation, San Diego, CA, USA).

2.7. Outcome variables

We analyzed several outcome variables related to severity of liver disease, after a minimum follow-up time of 10 years with HCV infection, by using:

a) Liver biopsy (Metavir score): fibrosis stage as ordinal variable (F0, F1, F2, F3 and F4) and advanced fibrosis and cirrhosis (F ≥ 3 and F4, respectively) as dichotomous variable;
b) Non-invasive fibrosis indexes: FIB-4 and APRI as quantitative variables and their cut-off points for advanced fibrosis (FIB-4 ≥ 3.25 and APRI ≥ 1.5) as dichotomous variable;
c) Twelve Inflammation-related biomarkers: leptin, HGF, NGF, sFasL, sFas, MIF, HA, Ang-2, TIMP1, CHI3L1, MMP1, and MMP2.

2.8. Statistics

Chi-square test was conducted to analyze the differences in allelic and genotypic frequencies. For the genetic association study, statistical analysis was carried out according to additive, recessive and dominant models, selecting the model that best fit the outcome variable analyzed in each case. Generalized Linear Model (GLM) was used to investigate the relationship among genetic variants upstream of *TNFAIP3* and severity of liver disease (unadjusted and adjusted models), but depending on the outcome variable the used model was different: a) For continuous variables (FIB-4 and APRI): a GLM with a gamma distribution (log-link) was used. This test provides the differences between groups and the arithmetic mean ratio (AMR) or the ratio by which the arithmetic mean of the original outcome is multiplied. b) For categorical variables: a GLM with ordinal distribution (cumlogit link) and GLM with binomial distribution (logit-link) were used to investigate the association with ordinal (fibrosis stage) and dichotomous (F3, F4, FIB-4 ≥ 3.25 and APRI ≥ 1.5) outcome variables, respectively. This test provides the differences between groups and the odds ratio (OR) for categorical outcome variables. Each regression test was adjusted by gender, age, nadir CD4+ T-cells, time on cART, BMI, alcohol intake, AIDS, undetectable HIV-RNA (< 50 copies/ml), HCV genotype, HCV-RNA ≥ 500,000 IU/ml, HOMA, and interleukin 28B (*IL28B*) rs12980275 and patatin like phospholipase domain containing 3 (*PNPLA3*) rs738409 polymorphisms. Additionally, GLM with a gamma distribution (log-link) was also used to investigate the association between genetic variants near *TNFAIP3* and inflammation-related biomarkers. The co-variables used in the multivariate model were nadir CD4+ T-cells, CD4+ T-cells, time on cART, HIV-RNA (< 50 copies/ml) and HCV-RNA ≥ 500,000 IU/ml; since these co-variables may very directly influence the plasma markers of inflammation.

All statistical tests were carried out with the Statistical Package for the Social Sciences (SPSS) 21.0 software (IBM Corp., Chicago, USA). All p-values were two-sided and statistical significance was defined as p value < 0.05.

In addition, Hardy-Weinberg equilibrium (HWE) analyses and pairwise linkage disequilibrium (LD) analysis were computed by Haploview 4.2 software, considering equilibrium when p > 0.05. Haplotype-based association test was performed with Plink software (http://pngu.mgh.harvard.edu/~purcell/plink/) and adjusted by the same variables as for association with individual SNPs. All p-values were two-tailed and statistical significance was defined as p < 0.05.

3. Results

3.1. Patients and TNFAIP3 polymorphisms

The summary of epidemiological and clinical characteristics of 215 HIV/HCV-coinfected patients at the time of liver biopsy is shown in Table 1.

All SNPs had a minimum allele frequency (MAF) > 5%, displayed missing values < 5%, and were in HWE (p > 0.05) (Table 2). The frequencies of rs675520, rs6920220 and rs9376293 were similar to those described for healthy IBS subjects and were in accordance with the data listed on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/).

A high LD, defined as non-random association of alleles at different loci, among genetic variants upstream of *TNFAIP3* was found (Fig. 1), meaning that there is no evidence for recombination between these SNPs. However, the r-square among SNPs was low (r-square < 0.60; Fig. 1), meaning that genetic variants upstream of *TNFAIP3* do not provide the same information and cannot be substituted for each other.

3.2. Genetic variants upstream of TNFAIP3 and liver disease

The dominant model of inheritance was the model that best fitted our data. Patients carrying rs675520 AG/GG had lower values of FIB-4 (p = 0.027; Fig. 2a1) and lower percentages of cirrhosis (F4) and advanced fibrosis (FIB-4 ≥ 3.25 and APRI ≥ 1.5) (p = 0.037, p = 0.039 and p = 0.039 respectively; Fig. 2a2) than carriers of AA genotype. Patients with rs9376293 CT/CC genotypes had lower values of APRI (p = 0.030; Fig. 2b1) and lower percentages of APRI ≥ 1.5 (p = 0.033; Fig. 2b2) than carriers of TT genotype. Furthermore, patients with rs6920220 AG/AA genotypes had higher values of APRI (p = 0.047; Fig. 2c1) and higher percentages of F4, FIB-4 ≥ 3.25 and APRI ≥ 1.5 (p = 0.047, p = 0.013 and p = 0.041 respectively; Fig. 2c2) than carriers of GG genotype.

In multivariate analysis, rs675520 AG/GG was associated with decreased odds of having F4, FIB-4 ≥ 3.25 and APRI ≥ 1.5 [adjusted OR (aOR) = 0.30 (p = 0.025), aOR = 0.20 (p = 0.014), and aOR = 0.34 (p = 0.017); respectively] (Fig. 3A). In addition, the rs675520 AG/GG genotypes were significantly related to lower levels of FIB-4 and APRI [adjusted AMR (aAMR) = 0.76 (p = 0.003) and aAMR = 0.72 (p = 0.006); respectively] (Fig. 3A). When patients were stratified by HCV genotype, rs675520 AG/GG was related with decreased odds of having FIB-4 ≥ 3.25 [aOR = 0.19 (p = 0.035)] and reduced levels of FIB-4 [aAMR = 0.77 (p = 0.026)] in patients with HCV genotype 1/4 (Fig. 3B). Regarding patients with HCV genotype 2/3, rs675520 AG/GG was associated with decreased odds of having F ≥ 3 [aOR = 0.08 (p = 0.028)] and advanced fibrosis stage according to Metavir score [aOR = 0.25 (p = 0.044)], and lower levels of FIB-4 and APRI [aAMR = 0.75 (p = 0.031), and aAMR = 0.67 (p = 0.013),

Table 1
Clinical and epidemiological characteristics of HIV/HCV-coinfected patients.

Characteristics	All patients
Total No. or patients	215
Male	161 (74.9%)
Age, years	39.8 (37.5; 44.1)
Epidemiological history	
HIV acquired by IVDU	190 (88.4%)
Years since HCV infection (n = 200)	21.4 (17.1; 24.4)
High alcohol intake (> 50 g per day)	116 (54.2%)
CDC category C	59 (27.4%)
Metabolic markers	
BMI, kg/m^2	22.3 (20.8; 24.5)
BMI ≥25 kg/m^2	46 (21.6%)
HOMA	2.10 (1.27; 3.68)
HOMA ≥ 3	68 (32.9%)
Antiretroviral therapy	
cART,	178 (82.8%)
Time on cART, years	4.5 (2.6; 6.6)
Current cART protocols	
Non-treated	33 (15.3%)
PI-based	45 (20.9%)
NNRTI-based	115 (53.5%)
NRTI-based	22 (10.2%)
HIV markers	
Nadir CD4+, T cells/μL	195 (84; 325)
CD4+, T cells/μL	472 (324; 682)
HIV-RNA < 50 copies/mL,	158 (73.5%)
HCV markers	
HCV genotype	
1	119 (56.1%)
2	5 (2.4%)
3	48 (22.6%)
4	40 (18.9%)
HCV RNA ≥ 500,000 IU/mL	159 (75.0%)
Liver biopsy	
Hepatic fibrosis	
F0	25 (11.6%)
F1	86 (40.0%)
F2	58 (27.0%)
F3	25 (11.6%)
F4	21 (9.8%)
Activity grade	
A ≤ 1	101 (47.0%)
A2	86 (40.8%)
A3	23 (10.9%)
A4	1 (0.5%)
Steatosis	114 (60.6%)
FIB-4	1.46 (1.03; 2.03)
FIB-4 ≥ 3.25	18 (8.9%)
APRI	0.75 (0.45; 1.30)
APRI≥1.5	35 (17.2%)

Values are expressed as absolute numbers (%) and median (percentile 25; percentile 75).
Abbreviations: IVDU, intravenous drug user; HCV, hepatitis C virus; CDC, Centers for Disease Control and Prevention; BMI, body mass index; HOMA, homeostatic model assessment; cART, combined antiretroviral therapy; PI-based, protein inhibitor-based therapy; NNRTI-based, non-nucleoside reverse transcriptase inhibitor -based therapy; NRTI-based, nucleoside reverse transcriptase inhibitor-based therapy; HIV; human immunodeficiency virus; HIV-RNA, HIV plasma viral load; HCV-RNA, HCV plasma viral load; FIB-4, Fibrosis-4; APRI, aspartate aminotransferase to platelet ratio index.

respectively] (Fig. 3C). Regarding rs9376293 polymorphism, the significant outcomes were the same as those obtained by the unadjusted model. Patients with CT/CC genotypes had lower levels of APRI [aAMR = 0.77 (p = 0.018)] and decreased odds of having values of APRI ≥ 1.5 [aOR = 0.39 (p = 0.030)] (Fig. 3D), being the significance only remained for patients with HCV genotype 1/4 (Fig. 3E and Fig. 3F). Also, patients with rs6920220 AG/AA genotypes had higher odds of having FIB-4 ≥ 3.25 [aOR = 3.72 (p = 0.043)] (Fig. 3G). This association was only remained for patients with HCV genotype 1/4 (Fig. 3H and Fig. 3I).

As long as the r^2 between polymorphisms is very low, we performed a haplotype analysis (comprised of rs675520, rs9376293 and rs6920220) in order to capture more genetic variability in the region. (Supplemental Table 1). The ATA haplotype carriers had higher odds of having FIB-4 ≥ 3.25 (aOR = 2.80; p = 0.021). The remaining haplotypes did not show any significant association.

3.3. Genetic variants upstream of TNFAIP3 and inflammation-related plasma biomarkers

In using unadjusted regression models (Table 3), patients with rs675520 AG/GG genotypes had lower levels of leptin and NGF (p = 0.002 and p = 0.001, respectively) and higher levels of sFas, MIF, TIMP1 and MMP2 (p = 0.008, p = 0.004, p = 0.016 and p = 0.026, respectively), compared to AA carriers. Furthermore, patients with rs9376293 CT/CC genotypes had lower leptin levels (p = 0.018) and higher sFas, MIF, TIMP1 and MMP2 levels (p = 0.025, p = 0.016, p = 0.028 and p = 0.012, respectively) than patients with TT genotype. Patients with rs6920220 AG/AA genotypes only had lower MMP1 levels than rs6920220 GG genotype (p = 0.021).

When adjusted regression models were used (Table 3), the rs675520 AG/GG genotypes were associated with lower levels of leptin and NGF [aAMR = 0.44 (p = 0.002) and aAMR = 0.39 (p = 0.002); respectively], and higher levels of sFas, MIF, TIMP1 and MMP2 [aAMR = 1.55 (p = 0.004), aAMR = 1.53 (p = 0.007), aAMR = 1.45 (p = 0.020), and aAMR = 1.65 (p = 0.033); respectively]. The rs9376293 CT/CC genotypes were related to lower levels of leptin [aAMR = 0.56 (p = 0.026)] and higher levels of sFas, MIF, TIMP1 and MMP2 [aAMR = 1.37 (p = 0.029), aAMR = 1.36 (p = 0.040), aAMR = 1.40 (p = 0.022), and aAMR = 1.62 (p = 0.022); respectively]. The rs6920220 AG/GG genotypes were only associated with lower levels of MMP1 [aAMR = 0.79 (p = 0.006)].

4. Discussion

To our knowledge, this study is the first description of the association of genetic variants upstream of *TNFAIP3* with severity of CHC in HIV/HCV-coinfected patients. The major findings were: i) the rs675520 AG/GG genotypes and the rs9376293 CT/CC genotypes were associated with lower odds of severe liver disease, and a favorable profile of plasma biomarkers of inflammation and fibrosis; ii) the rs6920220 AG/AA (risk) genotypes were linked to advanced liver disease by using FIB-4 and APRI.

The polymorphisms analyzed in this study map to an intergenic region of 6q23, between the genes *OLIG3* and *TNFAIP3*. OLIG3 appears to be involved in neuronal development, and therefore is not likely to be associated with immune-mediated diseases. By contrast, TNFAIP3 is a negative regulator of NF-kappaB activation and a powerful suppressor of cytokine signaling (Song et al., 2016), preventing chronic liver inflammation (Catrysse et al., 2016) and liver fibrosis (Yang et al., 2012), and it may modulate the inflammatory response to HCV in the liver (Song et al., 2016; Fan et al., 2015; Ma et al., 2014). Additionally, TNFAIP3 has a key role in HCV infection. For instance, *TNFAIP3* expression is induced by HCV core protein and contributes to viral persistence by protecting hepatocytes from cell death due to an anti-apoptotic effect (Nguyen et al., 2006). TNFAIP3 might also promote the chronification of HCV infection by maintaining low-grade chronic inflammation, which could lead to the long-term development of liver fibrosis (Song et al., 2016; Fan et al., 2015). With regard to *TNFAIP3* polymorphisms, significant implications have been previously described in numerous inflammatory and autoimmune diseases (Coenen et al., 2009; Stuart et al., 2015; Wang et al., 2016; Zhang et al., 2016a; Zhang et al., 2016b). Our data suggest an important role of certain genetic variants upstream of *TNFAIP3* on the severity of liver disease,

Table 2
Allele and genotype frequencies and Hardy Weinberg Equilibrium (HWE) polymorphisms in the 6q23 region upstream of *TNFAIP3* in HIV/HCV coinfected patients compared to healthy Iberian populations in Spain from 1000 genomes data (http://browser.1000genomes.org/index.html).

	Our HIV/HCV group					IBS group (n = 107)					χ^2 test(a)	χ^2 test (b)
SNPs	Genotypes		Alleles		HWE	Genotypes		Alleles		HWE	p-value	p-value
rs675520	AA	27.7%	A	51.1%	0.547	AA	19.6%	A	44.9%	0.758	0.380	0.389
	AG	46.9%	G	48.9%		AG	50.5%	G	55.1%			
	GG	25.4%				GG	29.9%					
rs9376293	CC	16.3%	C	40.9%	0.813	CC	18.7%	C	46.7%	0.173	0.408	0.364
	CT	49.3%	T	59.1%		CT	56.1%	T	53.3%			
	TT	34.4%				TT	25.2%					
rs6920220	GG	67.4%	G	82.1%	0.948	GG	69.2%	G	83.2%	0.664	0.837	0.954
	AG	29.3%	A	17.9%		AG	28.0%	A	16.8%			
	AA	3.3%				AA	2.8%					

Statistical: P-values were calculated by Chi-squared test; (a), differences in allelic frequencies; (b), differences in genotypic frequencies.
Abbreviations: HWE, Hardy Weinberg Equilibrium; HIV, human immunodeficiency virus; HCV, hepatitis C virus; IBS, Iberian populations in Spain.

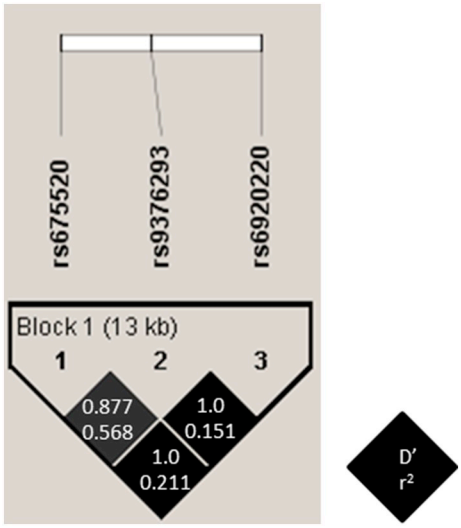

Fig. 1. Linkage disequilibrium among polymorphisms in the 6q23 region upstream of *TNFAIP3*. Each square represents a pairwise comparison between two SNPs.

found by using different markers of fibrosis (Metavir score from liver biopsy and qualitative and quantitative non-invasive markers), which provides robustness to our findings. Besides, *TNFAIP3* polymorphisms were associated with liver fibrosis independently of other relevant polymorphisms such as *IL28B* rs12980275 and *PNPLA3* rs738409 polymorphisms.

In order to investigate the possible mechanisms by which these genetic variants upstream of *TNFAIP3* are involved in the development of advanced fibrosis, we performed an *in-silico* analysis. By using rVarBase (Guo et al., 2016), we found that the polymorphism rs675520 is located in a genomic region that overlaps with the location of two long non-coding RNAs (lncRNAs), *lnc-TNFAIP3-3* and *lnc-OLIG3-1*. LncRNAs are large RNA transcripts that do not encode for proteins and have been implicated in a wide range of processes and diseases (Kung et al., 2013) by targeting different components of the transcription process. By using lncRNASNP software (Gong et al., 2015), we found that rs675520 implies a change in the structure of *lnc-OLIG3-1* and causes an increase in interaction between *lnc-OLIG3-1* and the microRNA hsa-miR-4645-3p. Thus, it is plausible that changes in gene expression of not only *TNFAIP3* but also *OLIG3* could be implicated in the susceptibility to inflammatory and immune diseases as well as in the severity of liver disease. Changes in *TNFAIP3* expression have been associated with rs6920220 polymorphism (Ungerback et al., 2012; Elsby et al., 2010). Additionally, rs9376293 and rs6920220 seem to have influence on the chromatin state of the region surrounding the variant, based on experimental information from the Roadmap epigenomics project, as it was annotated in rVarBase. Chromatin state is a crucial contributor to all genomic processes, controlling and defining gene expression profiles and contributing to disease (Li et al., 2012). All these data could explain the association observed between genetic variants upstream of *TNFAIP3* and liver fibrosis in our study. However, it is also necessary to consider that the three SNPs are in strong LD with other regulatory SNPs, which could be the causal variant implicated in the association to liver fibrosis.

Haplotypes (formed by rs675520, rs9376293 and rs6920220) were also investigated to analyze the association with advanced fibrosis. We found that only ATA haplotype was associated with advanced fibrosis, specifically FIB-4 ≥ 3.25. This indicates that haplotypes do not improve the prediction of advanced fibrosis compared to individual SNPs.

Moreover, our study for the first time describes an association between genetic variants upstream of *TNFAIP3* and several inflammation-related biomarkers in plasma. On the one hand, rs675520 AG/GG and rs9376293 CT/CC genotypes were linked to lower levels of leptin, and rs675520 AG/GG genotypes were also associated with lower levels of NGF. Leptin is an adipokine that mediates hepatic stellate cell (HSC) activation and liver fibrosis through indirect effects on Kupffer cells, partially mediated by TGF-β1 (Sacchi et al., 2015). NGF is a neurotrophic factor synthesized by immune cells (Lambiase et al., 1997), which has also been involved in inflammatory and fibrotic disorders (Bonini et al., 1999). The decreased levels of these profibrogenic and proinflammatory molecules in rs675520 AG/GG and rs9376293 CT/CC genotypes are consistent with the protective effect of genetic variants upstream of *TNFAIP3* on the severity of liver disease. On the other hand, rs675520 AG/GG and rs9376293 CT/CC genotypes were associated with higher levels of sFas, MIF, TIMP1 and MMP2; and rs6920220 AA genotype with higher values of MMP1. Firstly, the Fas molecule is a member of the TNF superfamily characterized by initiating apoptosis in Fas-bearing cells and inducing the release of proinflammatory cytokines, enhancing the immune response and self-tissue damage (Caulfield and Lathem, 2014). However, sFas is generated by alternative splicing and may block Fas-signaling (Hughes and Crispe, 1995), leading to apoptosis resistance (Liu et al., 2002), and consequently, it could ameliorate innate host defense and inflammation. MIF is a pleiotropic inflammatory cytokine that has been implicated in various inflammatory diseases (Morrison and Kleemann, 2015). However, an antifibrotic effect of MIF has also been described through the use of C57BL/6 *Mif*$^{-/-}$ mice (Heinrichs et al., 2011), which showed strong induced liver fibrosis in the absence of MIF. This finding supports our observation that genotypes related to higher MIF levels were associated with lower odds of advanced liver disease.

Regarding the increased levels of TIMP1, MMP1, and MMP2 observed in patients carrying the protective genotypes, it could be explained by the existence of dynamic changes in TIMP1, MMP1, and

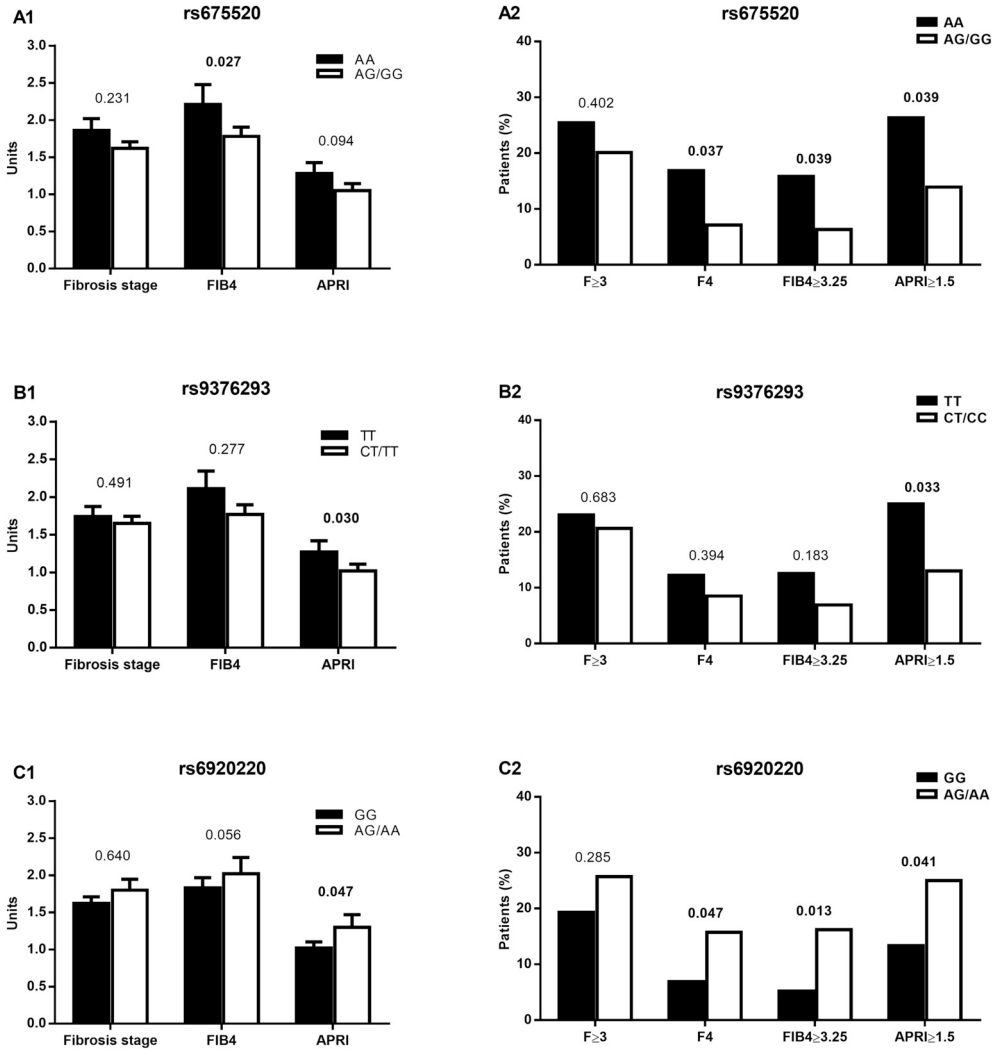

Fig. 2. Unadjusted association between polymorphisms in the 6q23 region upstream of *TNFAIP3* and liver disease severity in HIV/HCV-coinfected patients.
Statistical: Data are expressed as estimated marginal means from unadjusted generalized linear model for quantitative variables and percentages for dichotomous variables. P-values were calculated by unadjusted generalized linear model. Statistically significant differences are shown in bold.
Abbreviations: FIB-4, Fibrosis 4 index; APRI, aspartate aminotransferase to platelet ratio index.

MMP2 levels during liver fibrosis. These molecules are involved in extracellular matrix (ECM) remodeling (Mormone et al., 2011). Serum MMP1 is decreased in patients with advanced fibrosis stage (Trocme et al., 2006) and necroinflammation (Murawaki et al., 1999; Leroy et al., 2004), and increases after liver fibrosis improvement (Trocme et al., 2006; Murawaki et al., 1999). Conversely, MMP2 is activated in fibrotic liver and remains elevated during fibrotic progression (Trocme et al., 2006; Leroy et al., 2004; Latronico et al., 2016; Huang et al., 2015), but has also been related to accelerated matrix degradation during fibrosis resolution (Kantari-Mimoun et al., 2017). In fact, the treatment of HSCs with potential antifibrotic agents has resulted in increased MMP2 levels (Sakaida et al., 2004). On the other hand, TIMP1 blocks MMP activity (Mormone et al., 2011) and plays an important role in promoting liver fibrosis (Mormone et al., 2011; Parsons et al., 2004). The plasma level of TIMP1 is also elevated in CHC patients with advanced fibrosis stage (Latronico et al., 2016; Resino et al., 2010). However, TIMP1 has also been reported as a protector molecule against acute and chronic liver injury and liver fibrosis induced by hepatotoxic agents (Wang et al., 2011). Thus, plasma levels of TIMP1 and MMP2 could reflect the fibrotic process in the liver in such a way that the increase of TIMP1 (pro-fibrotic) levels would be compatible with the increase of MMP2 (anti-fibrotic) to compensate its effects.

Finally, several aspects must be considered for the correct interpretation of our results. Firstly, this report has a cross-sectional design with a limited number of patients, which could limit the finding of significant values in some groups. Secondly, all selected patients met a set of criteria for starting HCV treatment (e.g., no alcohol abuse, high CD4 cell counts, controlled HIV replication, and good treatment adherence), and this may have introduced a selection bias. Thirdly, this study was performed on patients with European descent, and it would also be required to carry out these analyses on different ethnic groups. Finally, our study only included HIV/HCV-coinfected patients and it would be interesting to know the role of the studied polymorphisms in HCV monoinfected patients, but we did not have access to a cohort of HCV monoinfected patients with biopsy data.

5. Conclusions

In conclusion, the rs675520, rs9376293 and rs6920220 polymorphisms upstream of *TNFAIP3* were associated with the severity of liver fibrosis, suggesting that these polymorphisms might play a

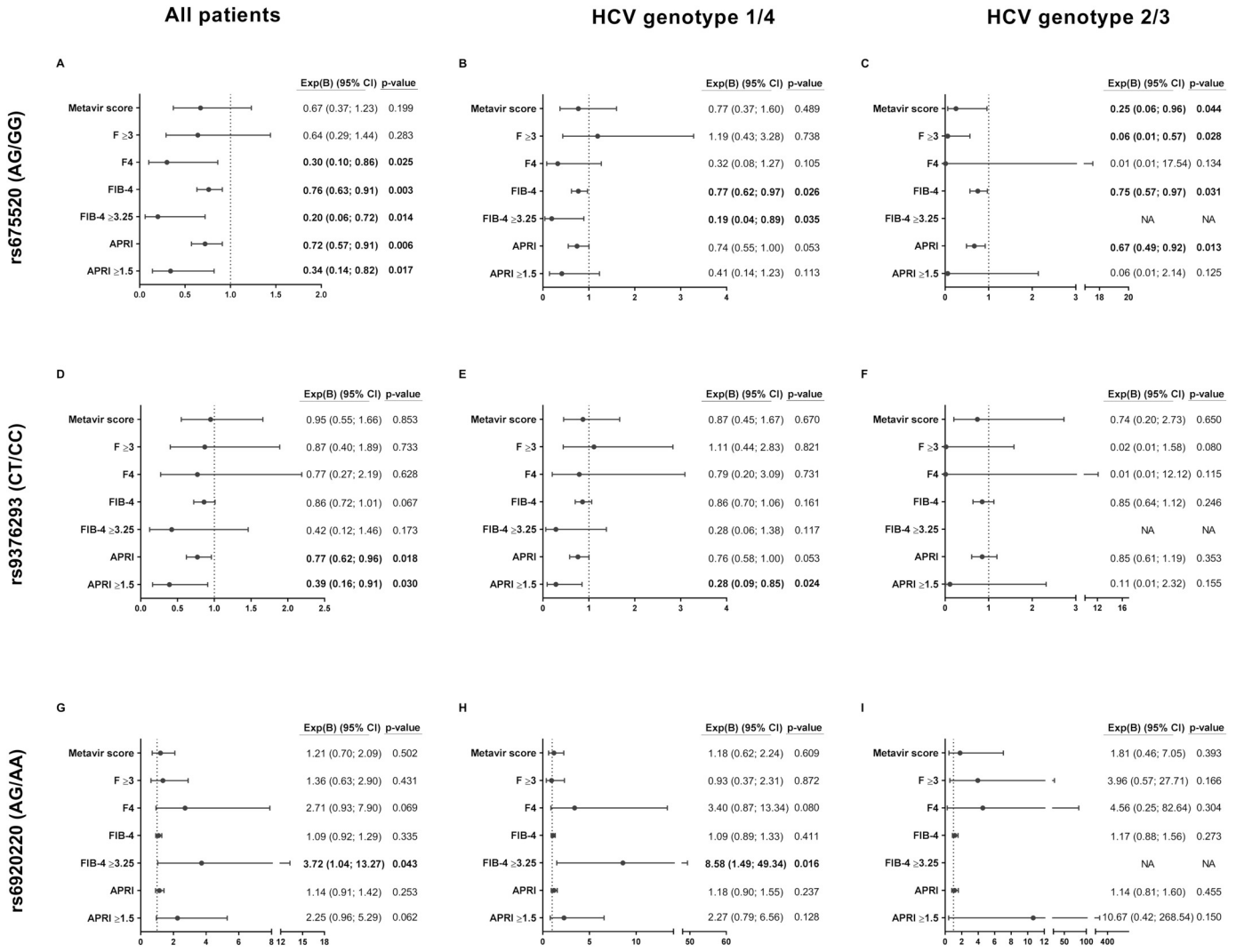

Fig. 3. Association between polymorphisms in the 6q23 region upstream of *TNFAIP3* and liver disease severity in HIV/HCV-coinfected patients, for all patients and after stratifying by HCV genotype.

Statistical: P-values were calculated by generalized linear model adjusted by the most important clinical and epidemiological characteristics (see **statistical analysis** section). Statistically significant differences are shown in bold.

Abbreviations: HIV, human immunodeficiency virus; HCV, hepatitis C virus; Exp(B) is arithmetic mean ratio (AMR) for continuous variables or odds ratio (OR) for categorical variables; 95%CI, 95% of confidence interval; p-value, level of significance; HCV-GT, HCV genotype; F ≥ 3, advance fibrosis; F4, cirrhosis; FIB-4, Fibrosis 4 index; APRI, aspartate aminotransferase to platelet ratio index; NA, not available results by small sample size in some comparison group.

significant role in CHC progression, as well as decisions for the optimal management of infected patients with HIV and HCV. Further analyses are needed to corroborate these conclusions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.10.008.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study may be made available by the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Table 3
Relationship between polymorphisms in the 6q23 region upstream of *TNFAIP3* and liver disease severity in HIV/HCV coinfected patients according to HCV genotype.

	Unadjusted			Adjusted	
A) rs675520 (AG/GG)	AA	AG/GG	p-value [(a)]	aAMR (95% CI)	p-value [(b)]
Leptin (pg/ml)	13,457.03	6263.34	**0.002**	0.44 (0.26; 0.75)	**0.002**
NGF (pg/ml)	28.43	11.73	**0.001**	0.39 (0.21; 0.71)	**0.002**
sFas (pg/ml)	8817.86	13,327.13	**0.008**	1.55 (1.15; 2.09)	**0.004**
MIF (pg/ml)	1538.40	2416.44	**0.004**	1.53 (1.12; 2.08)	**0.007**
TIMP1 (pg/ml)	213.37	310.00	**0.016**	1.45 (1.06; 1.98)	**0.020**
MMP1 (pg/ml)	204.92	211.13	0.751	1.02 (0.84; 1.22)	0.869
MMP2 (pg/ml)	197.30	321.50	**0.026**	1.65 (1.04; 2.61)	**0.033**
B) rs9376293 (CT/CC)	**TT**	**CT/CC**	**p-value[(a)]**	**aAMR (95% CI)**	**p-value[(b)]**
Leptin (pg/ml)	12,057.91	6823.02	**0.018**	0.56 (0.34; 0.93)	**0.026**
NGF (pg/ml)	19.50	14.32	0.240	0.91 (0.54; 1.74)	0.914
sFas (pg/ml)	9636.61	13,324.15	**0.025**	1.37 (1.03; 1.81)	**0.029**
MIF (pg/ml)	1706.74	2419.89	**0.016**	1.36 (1.01; 1.82)	**0.040**
TIMP1 (pg/ml)	225.24	309.94	**0.028**	1.40 (1.05; 1.87)	**0.022**
MMP1 (pg/ml)	190.13	217.07	0.123	1.11 (0.93; 1.31)	0.241
MMP2 (pg/ml)	203.93	337.86	**0.012**	1.62 (1.07; 2.45)	**0.022**
C) rs6920220 (AG/AA)	**GG**	**AG/AA**	**p-value[(a)]**	**aAMR (95% CI)**	**p-value[(b)]**
Leptin (pg/ml)	8029.07	9694.80	0.444	1.15 (0.71; 1.87)	0.565
NGF (pg/ml)	18.44	11.04	0.052	0.63 (0.37; 1.08)	0.095
sFas (pg/ml)	12,754.30	10,716.38	0.238	0.81 (0.61; 1.07)	0.140
MIF (pg/ml)	2308.04	1919.00	0.214	0.83 (0.62; 1.10)	0.195
TIMP1 (pg/ml)	281.98	281.16	0.984	0.92 (0.68; 1.24)	0.577
MMP1 (pg/ml)	220.60	180.83	**0.021**	0.79 (0.67; 0.94)	**0.006**
MMP2 (pg/ml)	308.31	259.29	0.406	0.83 (0.55; 1.26)	0.375

Data are expressed as estimated marginal means from unadjusted generalized linear model.
Statistical: (a), p-values were calculated by univariate generalized linear model; (b), p-values were calculated by multivariate generalized linear model adjusted by the most important clinical and epidemiological characteristics (see **statistical analysis** section). Statistically significant differences are shown in bold.
Abbreviations: HIV, human immunodeficiency virus; HCV, hepatitis C virus; aAMR, adjusted arithmetic mean ratio; 95%CI, 95% of confidence interval; p-value, level of significance; NGF, nerve growth factor; sFas, soluble Fas cell surface death receptor; MIF, macrophage migration inhibitory factor; TIMP1, TIMP metallopeptidase inhibitor 1; MMP1, matrix metallopeptidase 1; MMP2, matrix metallopeptidase 2.

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Investigation: MAJS, IMG, JB, and SR.
Formal analysis: MAJS and SR.
Writing – original draft preparation: MAJS, IMG, and SR.
Writing – Review & Editing: JB, AFR, and LMM.
Visualization, supervision and funding acquisition: SR.

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