Title page

Type of manuscript: Concise communication

Title: Impact of patatin-like phospholipase domain-containing 3 gene polymorphism (rs738409) on severity of liver disease in HIV/hepatitis C virus-coinfected patients

Running head: *PNPLA3* and HIV/HCV coinfection

Authors: María A JIMÉNEZ-SOUSA, PhD. [1(*)]; Juan BERENGUER, M.D., PhD. [2,3]; Mónica GARCÍA-ÁLVAREZ, PhD. [1]; Mónica GUTIERREZ-RIVAS, PhD.[1]; Teresa ALDÁMIZ-ECHEVARRIA, M.D. [2,3], Francisco TEJERINA, M.D. [2,3], Cristina DIEZ, M.D. [2,3], Sonia VÁZQUEZ-MORÓN, PhD. [1]; Salvador RESINO, PhD. [1(*)]; (*) Corresponding authors.

Current affiliations: (1) Unidad de Infección Viral e Inmunidad, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain. (2) Unidad de Enfermedades Infecciosas/VIH; Hospital General Universitario "Gregorio Marañón", Madrid, Spain. (3) Instituto de Investigación Sanitaria Gregorio Marañón (IiSGM), Madrid, Spain.

Correspondence and requests for reprints: Salvador Resino; Centro Nacional de Microbiología, Instituto de Salud Carlos III (Campus Majadahonda); Carretera Majadahonda- Pozuelo, Km 2.2; 28220 Majadahonda (Madrid); Telf.: +34 918 223 266; Fax: +34 918 223 269; e-mail: sresino@isciii.es

ABSTRACT

Objective: To analyze the association between *PNPLA3* rs738409 polymorphism and severity of liver disease in human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfected patients.

Methods: We performed a cross-sectional study of 215 patients who underwent a liver biopsy. *PNPLA3* rs738409 polymorphism was genotyped using GoldenGate® assay. The outcome variables were: i) advanced fibrosis (F≥3 and FIB-4≥3.25), ii) rapid fibrosis progression (FPR≥0.10 fibrosis units/year), iii) severe activity grade (A≥3), and iv) steatosis (fatty hepatocytes ≥10%). T genetic association analysis was carried out according to an additive genetic model through logistic regressions adjusted by the most significant covariables.

Results: Overall, 21.4% had F≥3, 8.9% had FIB-4≥3.25, 11.4% had A≥3, 60.6% had steatosis, and 32.5% had FPR≥0.10. For each rs738409 G allele, we found an increased frequency of patients with advanced fibrosis (F≥3) (0% CC, 18.5% CG, and 25.2% GG; p=0.049) and FIB-4≥3.25 (0% CC, 3.8% CG, and 13.2% GG; p=0.016). Furthermore, for each rs738409 G allele, the odds of having F≥3 increased 2.15 times (95% of confidence interval (95%CI)=1.07; 4.35; p=0.029) and having FIB-4≥3.25 increased 8.77 times (95%CI=1.11; 69.0; p=0.039). Note that rs738409 G allele carriers tended to higher likelihood of having FPR≥0.10, but statistical significance was not reached (p=0.054). Finally, we did not find any association for A≥3 and liver steatosis.

Conclusion: *PNPLA3* rs738409 polymorphism was associated with the severity of liver fibrosis in patients coinfected with HIV and HCV, suggesting that this polymorphism might also play a significant role in the progression of hepatic fibrosis in this group of patients.

Key words: AIDS; chronic hepatitis C; *PNPLA3*; SNPs; liver fibrosis

INTRODUCTION

Chronic hepatitis C (CHC) may cause liver fibrosis and steatosis, leading to cirrhosis and hepatocellular carcinoma [1, 2]. The natural course of CHC varies widely among individuals and several factors have been associated with liver fibrosis progression, including age at infection, sex, route of infection, hepatitis C virus (HCV) genotype, obesity and human immunodeficiency virus (HIV) coinfection, among others [3-5]. Regarding HCV/HIV coinfection, the natural history of CHC is accelerated in presence of HIV infection [6-8]. Thus, HCV infection constitutes an important cause of morbidity and mortality among HCV/HIV coinfected patients [9, 10].

However, liver fibrosis progression remains variable between individuals with similar environmental risk factors. Thus, genetic background could be another important factor to take into account [11]. In this setting, rs738409 C>G polymorphism, which encodes for the 148 Isoleucine to Methionine protein variant (I148M) of patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene, has been recognized as a risk factor for liver disease severity [5]. The *PNPLA3* rs738409 polymorphism has been widely related to nonalcoholic steatohepatitis (NASH) and progressive liver fibrosis in HCV-infected patients [12-16]. However, to our knowledge, there is no report related to severity of liver disease in HIV/HCV coinfected patients.

The aim of this study was to analyze the association between *PNPLA3* rs738409 polymorphism and severity of liver disease in HIV/HCV coinfected patients.

PATIENTS AND METHODS

Study population

We performed a cross-sectional study in 215 HIV/HCV-coinfected patients who underwent a liver biopsy between September 2000 and November 2008. The study was conducted in accordance with the Declaration of Helsinki and patients gave their written consent for the study. The Institutional Review Board and the Research Ethic Committee of the Instituto de Salud Carlos III (ISCIII) approved the study. Liver biopsies were performed on patients who were potential candidates for anti-HCV therapy and had not received previous interferon therapy. Selection criteria were: no clinical evidence of hepatic decompensation, detectable HCV RNA by polymerase chain reaction (PCR), negative hepatitis B surface antigen, availability of DNA sample, CD4+ lymphocyte count higher than 200 cells/µL, and stable combination antiretroviral therapy (cART) for at least 6 months before study entry or no need for cART according to treatment guidelines used in the study period [17]. Patients with active opportunistic infections, active drug addiction, and other concomitant severe diseases were excluded. High alcohol intake was not a contraindication to initiate anti-HCV therapy, unless the clinician felt that it could compromise treatment adherence. All subjects included in our study were European white.

Clinical and laboratory assessment

Clinical and epidemiological data were obtained from medical records. Alcohol intake of more than 50g 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.
Biochemistry panel was measured using a clinical chemistry analyzer (Hitachi 912, Boehringer Mannheim, Germany) in fasting patients. The degree of insulin resistance (IR) was estimated for each patient using the homeostatic model assessment (HOMA): fasting plasma glucose (mmol/L) times fasting serum insulin (mU/L) divided by 22.5. FIB-4 index, a non-invasive test of liver fibrosis in HIV/HCV-coinfected patients, was calculated as age ([yr] x AST [U/L]) / ((PLT [10(9)/L]) x (ALT [U/L])(1⁄2)) [18].
Liver biopsies were performed as we described previously [19]. Liver fibrosis and necroinflammatory activity were estimated according to Metavir score as follows [20]: 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 evaluated according to the existence of hepatocytes containing visible macrovesicular fat droplets, which was clinically significant when fatty hepatocytes exceeded 10% of parenchyma hepatic.

The duration of HCV infection for patients with a history of intravenous drug use (IDU) was estimated using the first year they shared needles and other injection paraphernalia, which are the most relevant risk practices for HCV transmission. The duration of HCV infection was not calculated when the date of initiation of their HCV infection could not be determined with certainty (n=15). The fibrosis progression rate (FPR) was calculated dividing the fibrosis stage (0 to 4) by the estimated duration of HCV infection in years (fibrosis units per year) [21].

Genomic DNA was extracted from peripheral blood with Qiagen columns (QIAamp DNA Blood Midi/Maxi; Qiagen, Hilden, Germany). DNA samples were sent to the Spanish National Genotyping Center (CeGen; http://www.cegen.org/) in order to genotype *PNPLA3* rs738409 and *IL28B* rs12980275 polymorphisms. Genotyping was performed by using Sequenom's MassARRAY platform (San Diego, CA, USA) using the iPLEX® Gold assay design system.

Data analysis

The outcome variables were: i) advanced fibrosis (F≥3 and FIB4≥3.25), ii) rapid fibrosis progression (FPR≥0.10 fibrosis units/year), iii) severe activity grade (A≥3), and iv) liver steatosis (fatty hepatocytes ≥10%)[22]. These outcomes were developed after a minimum follow-up time of 10 years with HCV infection.
The genetic association analysis was carried out according to an additive genetic model, which was the model that best fit to our data. Chi-square test and logistic regression analysis were used to investigate the relationship among *PNPLA3* rs738409 polymorphism and severe liver disease. Each

regression analysis was always adjusted by the most significant co-variables associated with each one of the outcome variables, avoiding the over-fitting of the regression. The co-variables were selected by "Stepwise" algorithm (p-value of entry and exit of 0.15 and 0.20, respectively), including gender, age, alcohol intake, BMI, HOMA, nadir CD4+ T-cells, AIDS, undetectable HIV-RNA (<50 copies/ml), CD4+ T-cells, time on cART, HCV-RNA ≥500,000 IU/ml, HCV genotype, and *IL28B* rs12980275 polymorphism (see **Supplemental table 1**).

These analyses were performed by using the IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp, Chicago, Armonk, NY, USA). All p-values were two-tailed and statistical significance was defined as $p<0.05$.

RESULTS

Table 1 shows the epidemiological and clinical characteristics of 215 HIV/HCV-coinfected patients at the time of liver biopsy. Regarding liver biopsy, 21.4% had advanced fibrosis (F≥3), 8.9% had FIB-4≥3.25, 11.4% had severe necroinflammatory activity (A≥3), 60.6% had steatosis, and 32.5% had FPR≥0.10.

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

Characteristics	All Patients
No., (%)	215
Male, n (%)	161 (74.9%)
Age, years	38.8 (37.4; 44.1)
Epidemiological history	
HIV acquired by IVDU, n (%)	190 (88.4%)
Years since HCV infection (n=200)	21.4 (17.1; 24.4)
High alcohol intake	116 (54.2%)
High alcohol intake at biopsy study	32 (14.8%)
CDC category C, n (%)	59 (27.4%)
Metabolic markers	
BMI, kg/m2(n=213)	22.3 (20.7; 24.6)
BMI ≥25 kg/m2	46 (21.4%)
HOMA	2.10 (1.27; 3.68)
HOMA ≥3	68 (32.9%)
Antiretroviral therapy	
cART, n (%)	178 (82.8%)
Time on cART, years	4.4 (2.5; 6.6)
Current cART protocols, n (%)	
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, n (%)	162 (74%)
HCV markers, n (%)	
HCV genotype	
1	119 (56.1%)
2	5 (2.4%)
3	48 (22.6%)
4	40 (18.9%)
HCV RNA >500,000 UI/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	102 (48.2%)

A2	86 (40.8%)
A3	24 (11.4%)
Steatosis	114 (60.6%)
Fibrosis progression rate (FPR)	0.07 (0.04; 0.12)
FPR≥0.10	65 (32.5%)
FIB-4	1.46 (1.03; 2.03)
FIB-4≥3.25	18 (8.9%)

Values are expressed as absolute numbers (%) and median (percentile 25; percentile 75).
Abbreviations: BMI, body mass index; HOMA, homeostatic model assessment; HCV, Hepatitis C virus; CDC category C, centers for disease control and prevention classification system, clinical category C which includes any condition listed in the CDC´s 1897 surveillance case definition of AIDS; PI-based, protein inhibitor-based therapy; NNRTI-based, non-nucleoside reverse transcriptase inhibitor -based therapy; 3NRTI-based, triple nucleoside regimen; HIV; Human immunodeficiency virus; HIV-RNA, HIV plasma viral load; HCV-RNA, HCV plasma viral load.

Allele frequencies for *PNPLA3* rs738409 polymorphism were 0.76 for G allele and 0.24 for T allele. Genotype frequencies were 0.57, 0.38 and 0.05 for GG, CG and CC genotypes, respectively. These frequencies in our dataset were in accordance with the data listed on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=rs738409). The *PNPLA3* rs738409 polymorphism fulfilled the minimum allele frequency >0.05 and had missing values <5%. Furthermore, rs738409 polymorphism was in Hardy–Weinberg equilibrium (p=0.710).
Table 2 shows the relationship of *PNPLA3* rs738409 polymorphism with severity of liver disease under a model of additive inheritance. For each rs738409 G allele, we found an increased frequency of patients with advanced fibrosis (F≥3) (0% CC, 18.5% CG, and 25.2% GG; p=0.049) and FIB-4≥3.25 (0% CC, 3.8% CG, and 13.2% GG; p=0.016). Furthermore, for each rs738409 G allele, the probability of having values of F≥3 increased 2.15 times (p=0.029) and having FIB-4 ≥3.25 increased 8.77 times (p=0.039). Note that rs738409 G allele tended to higher likelihood of having FPR ≥0.10, but statistical significance was not reached (p=0.054). Finally, we did not find any association for severe activity grade (A≥3) and liver steatosis (fatty hepatocytes ≥10%). **Supplemental Table 1** shows the significant covariates maintained in each logistic regression model by using Stepwise algorithm.

Table 2. Relationship between *PNPLA3* rs738409 polymorphism and severity of liver disease in HIV/HCV coinfected patients.

Liver disease	No.	CC	CG	GG	p [(a)]	OR (95%CI)	p [(b)]
Significant fibrosis (F ≥3)	215	0/11 (0%)	15/81 (18.5%)	31/123 (25.2%)	**0.049**	2.15 (1.07; 4.35)	**0.029**
Significant fibrosis (FIB4 ≥3.25)	203	0/11 (0%)	3/78 (3.8%)	15/114 (13.2%)	**0.016**	8.77 (1.11; 69.0)	**0.039**
High fibrosis progression (FPR ≥0.10)	200	0/10 (0%)	24/74 (32.4%)	41/116 (35.2%)	0.095	1.81 (0.99; 3.30)	0.054
Severe activity grade (A ≥3)	211	0/11 (0%)	8/80 (10.0%)	16/120 (13.3%)	0.190	1.69 (0.64; 4.42)	0.286
Liver steatosis (FH ≥10%)	188	7/10 (70%)	45/71 (63.4%)	62/107 (57.9%)	0.341	0.72 (0.41; 1.27)	0.260

95%CI, 95% of confidence interval; FPR, fibrosis progression rate; OR, odds ratio; P-value, level of significance.
Statistically significant differences are shown in bold.
[a]P-values were calculated by linear-by-linear association x^2 test.
[b]P-values were calculated by multivariate logistic regression adjusted by the most important clinical and epidemiological characteristics (see statistical analysis section).

DISCUSSION

The major finding of the present study was that the presence of *PNPLA3* rs738409 G allele increased the odds of having advanced liver fibrosis in HIV/HCV-coinfected patients. To our knowledge, this study is the first description of the relationship between *PNPLA3* rs738409 polymorphism and severity of liver fibrosis in HIV/HCV-coinfected patients.

PNPLA3 is a protein with lipase activity which is highly expressed in the liver and adipose tissue. The nonsynonymous rs738409 (G allele; I148M substitution) seems not to affect *PNPLA3* mRNA levels; however it results in a loss of PNPLA3 function, influencing individual susceptibility to hepatocyte lipid accumulation [23, 24]. Emerging evidence has linked *PNPLA3* rs738409 polymorphism with fibrosis progression in patients with HCV infection [5, 13, 14]. The mechanism implicated in liver fibrosis could be related to lipotoxicity because lipid accumulation may lead to inflammatory mediators release, oxidative stress, and hepatocyte apoptosis [25]. Thus, liver steatosis would be responsible for liver fibrosis development and progression [26]. However, the effect of *PNPLA3* rs738409 polymorphism on HCV-related liver disease severity remains largely unknown in HIV/HCV coinfected patients [27]. In our study, rs738409 G allele was associated with significant fibrosis, which is in concordance with those results found for HCV monoinfected patients. Thus, rs738409 genotyping could be useful in both HCV monoinfected and HIV/HCV coinfected patients.

Apart from liver biopsy, we used FIB-4 as an outcome to evaluate the influence of rs738409 on liver fibrosis. FIB-4 was chosen because it is a non-invasive test widely validated for HIV/HCV coinfected patients [28]; unlike others more commonly used for HCV monoinfected patients. In our study, the association between rs738409 and FIB-4 was even stronger than that noted with liver biopsy, being the probability of developing advanced fibrosis eight times higher for G carriers.

Regarding fibrosis progression, rs738409 G allele has been described as a risk factor for accelerated FPR in HCV monoinfected patients [5]. In our study, the association was close to statistical significance and we cannot discard that the lack of association may be due to a limited sample population. Thus, note that further studies with a longer sample size would be interesting.

As mentioned above, *PNPLA3* rs738409 polymorphism has been widely associated with steatosis [12, 15, 16, 29]. However, we found no significant association between *PNPLA3* rs738409 polymorphism and steatosis. This inconsistence with previous reports could be explained by our unavailability of continuous values to define steatosis. Steatosis was defined as a dichotomous variable (fatty hepatocytes ≥10%) according to the procedure established in our hospital, and data collection was performed by retrospective review of medical records. However, the large majority of the articles that found association between rs738409 polymorphism and steatosis used steatosis in an ordinal scale [12, 15, 16, 29]. Thus, it may have limited the finding of statistically significant results in our study, not ruling out the association between rs738409 polymorphism and steatosis.

The treatment regimens with new direct-acting antivirals (DAAs) are characterized by shorter duration, simplified dosing, improved safety profile and effectivity, and > 90% of sustained virological response. These HCV regimens are the HCV therapies recommended by international guidelines. However, DAAs are being prioritized in patients at high risk for liver-related complications because of cost [30]. In this setting, the PNPLA3 rs738409 genotyping could be a valuable tool to identify patients at high risk for developing advanced fibrosis, who could be prioritized. So, these patients could benefit from the predictive value of rs738409 polymorphism.

Finally, several aspects have to be taken into account for the correct interpretation of the results. Firstly, this is a cross-sectional study with a limited number of patients, which could limit achieving statistically significant values. Besides, the cross-sectional design of the analyses may be a confounding factor. Secondly, data were collected retrospectively, which entails a lack of uniformity. Thirdly, 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. Fourthly, this study was performed on patients with European ancestry, and it would also be interesting to perform these analyses on different ethnic groups.

In conclusion, *PNPLA3* rs738409 polymorphism was associated with severity of liver fibrosis in patients coinfected with HIV and HCV, suggesting that this polymorphism might also play a significant role in the progression of hepatic fibrosis in this group of patients. Further studies with bigger cohorts should be performed to confirm the trend of our results.

ACKNOWLEDGEMENTS

The authors wish to thank the Spanish National Genotyping Center (CeGen) for providing the SNP genotyping services (http://www.cegen.org).

Potential conflicts of interest: The authors do not have a commercial or other association that might pose a conflict of interest.

Funding/Support: This work has been supported by grants given by Fondo de Investigación de Sanidad en España (FIS) [Spanish Health Founds for Research] [grant numbers PI11/01556, PI14/01094, PI11/00245, PI14CIII/00011], and "Fundación para la Investigación y la Prevención del Sida en España" (FIPSE) [grant number 361020/10]. This work has been (partially) funded by the RD12/0017/0024 and RD12/0017/0004 projects as part of the Plan Nacional R + D + I and cofinanced by ISCIII- Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER).
JB is an investigator from the Programa de Intensificación de la Actividad Investigadora en el Sistema Nacional de Salud (I3SNS), Refs INT10/009 and INT12/154. MGA, DPT, MAJS and MGF are supported by "Instituto de Salud Carlos III" [grant numbers CD12/00442, CM12/00043, CD13/00013 and RD12/0017/0024, respectively].
This work has been (partially) funded by the RD12/0017 project as part of the Plan Nacional R + D + I and cofinanced by ISCIII- Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER).

AUTHORS´ CONTRIBUTIONS

MAJS and SR performed all statistical analysis, interpretation of the data and wrote the manuscript.
JB and SR participated in the study concept and design.
JB, TAE, CD, and FT, participated in patient selection, collection of samples and acquisition of data.
MGA, SVM and MGR participated in sample preparation, DNA isolation and genotyping pre-procedure, and contributed with critical revision of the manuscript.
SR supervised the study.
All authors revised the manuscript from a draft by SR.

REFERENCES

1. Mengshol JA, Golden-Mason L, Rosen HR. Mechanisms of Disease: HCV-induced liver injury. *Nat Clin Pract Gastroenterol Hepatol* 2007,**4**:622-634.
2. Heim MH, Thimme R. Innate and adaptive immune responses in HCV infections. *J Hepatol* 2014,**61**:S14-S25.
3. Ortiz V, Berenguer M, Rayon JM, Carrasco D, Berenguer J. Contribution of obesity to hepatitis C-related fibrosis progression. *Am J Gastroenterol* 2002,**97**:2408-2414.
4. Rotman Y, Liang TJ. Coinfection with hepatitis C virus and human immunodeficiency virus: virological, immunological, and clinical outcomes. *J Virol* 2009,**83**:7366-7374.
5. Rueger S, Bochud PY, Dufour JF, Mullhaupt B, Semela D, Heim MH*, et al.* Impact of common risk factors of fibrosis progression in chronic hepatitis C. *Gut* 2014.
6. Lopez-Dieguez M, Montes ML, Pascual-Pareja JF, Quereda C, Von Wichmann MA, Berenguer J*, et al.* The natural history of liver cirrhosis in HIV-hepatitis C virus-coinfected patients. *AIDS* 2011,**25**:899-904.
7. Macias J, Berenguer J, Japon MA, Giron JA, Rivero A, Lopez-Cortes LF*, et al.* Fast fibrosis progression between repeated liver biopsies in patients coinfected with human immunodeficiency virus/hepatitis C virus. *Hepatology* 2009,**50**:1056-1063.
8. Lo Re V, 3rd, Kallan MJ, Tate JP, Localio AR, Lim JK, Goetz MB*, et al.* Hepatic decompensation in antiretroviral-treated patients co-infected with HIV and hepatitis C virus compared with hepatitis C virus-monoinfected patients: a cohort study. *Ann Intern Med* 2014,**160**:369-379.
9. Lewden C, May T, Rosenthal E, Burty C, Bonnet F, Costagliola D*, et al.* Changes in causes of death among adults infected by HIV between 2000 and 2005: The "Mortalite 2000 and 2005" surveys (ANRS EN19 and Mortavic). *J Acquir Immune Defic Syndr* 2008,**48**:590-598.
10. Graham CS, Baden LR, Yu E, Mrus JM, Carnie J, Heeren T*, et al.* Influence of human immunodeficiency virus infection on the course of hepatitis C virus infection: a meta-analysis. *Clinical Infectious Diseases* 2001,**33**:562-569.
11. Westbrook RH, Dusheiko G. Natural history of hepatitis C. *Journal of Hepatology* 2014,**61**:S58-68.
12. Petta S, Vanni E, Bugianesi E, Rosso C, Cabibi D, Camma C*, et al.* PNPLA3 rs738409 I748M is associated with steatohepatitis in 434 non-obese subjects with hepatitis C. *Aliment Pharmacol Ther* 2015,**41**:939-948.
13. Yasui K, Kawaguchi T, Shima T, Mitsuyoshi H, Seki K, Sendo R*, et al.* Effect of PNPLA3 rs738409 variant (I148 M) on hepatic steatosis, necroinflammation, and fibrosis in Japanese patients with chronic hepatitis C. *J Gastroenterol* 2014.
14. Huang CF, Dai CY, Yeh ML, Huang CI, Tai CM, Hsieh MH*, et al.* Association of diabetes and PNPLA3 genetic variants with disease severity of patients with chronic hepatitis C virus infection. *J Hepatol* 2015,**62**:512-518.
15. Trepo E, Pradat P, Potthoff A, Momozawa Y, Quertinmont E, Gustot T*, et al.* Impact of patatin-like phospholipase-3 (rs738409 C>G) polymorphism on fibrosis progression and steatosis in chronic hepatitis C. *Hepatology* 2011,**54**:60-69.
16. Valenti L, Rumi M, Galmozzi E, Aghemo A, Del Menico B, De Nicola S*, et al.* Patatin-like phospholipase domain-containing 3 I148M polymorphism, steatosis, and liver damage in chronic hepatitis C. *Hepatology* 2011,**53**:791-799.
17. Panel de expertos de Gesida Plan Nacional sobre el Sida y Asociación Española para el Estudio del Hígado. [Recommendations of Gesida/PNS/AEEH for the management and treatment of the adult patient co-infected with HIV and hepatitis A, B and C virus]. *Enferm Infecc Microbiol Clin* 2010,**28**:31 e31-31.
18. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J*, et al.* Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006,**43**:1317-1325.
19. Resino S, Seoane JA, Bellon JM, Dorado J, Martin-Sanchez F, Alvarez E*, et al.* An artificial neural network improves the non-invasive diagnosis of significant fibrosis in HIV/HCV

coinfected patients. *J Infect* 2011,**62**:77-86.
20. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996,**24**:289-293.
21. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997,**349**:825-832.
22. Obika M, Noguchi H. Diagnosis and evaluation of nonalcoholic fatty liver disease. *Exp Diabetes Res* 2012,**2012**:145754.
23. Anstee QM, Day CP. The genetics of NAFLD. *Nat Rev Gastroenterol Hepatol* 2013,**10**:645-655.
24. Romeo S, Huang-Doran I, Baroni MG, Kotronen A. Unravelling the pathogenesis of fatty liver disease: patatin-like phospholipase domain-containing 3 protein. *Curr Opin Lipidol* 2010,**21**:247-252.
25. Marra F, Lotersztajn S. Pathophysiology of NASH: perspectives for a targeted treatment. *Curr Pharm Des* 2013,**19**:5250-5269.
26. Leandro G, Mangia A, Hui J, Fabris P, Rubbia-Brandt L, Colloredo G*, et al.* Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. *Gastroenterology* 2006,**130**:1636-1642.
27. Singal AG, Manjunath H, Yopp AC, Beg MS, Marrero JA, Gopal P*, et al.* The effect of PNPLA3 on fibrosis progression and development of hepatocellular carcinoma: a meta-analysis. *Am J Gastroenterol* 2014,**109**:325-334.
28. Berenguer J, Zamora FX, Aldamiz-Echevarria T, Von Wichmann MA, Crespo M, Lopez-Aldeguer J*, et al.* Comparison of the prognostic value of liver biopsy and FIB-4 index in patients coinfected with HIV and hepatitis C virus. *Clin Infect Dis* 2015,**60**:950-958.
29. Zhang L, You W, Zhang H, Peng R, Zhu Q, Yao A*, et al.* PNPLA3 polymorphisms (rs738409) and non-alcoholic fatty liver disease risk and related phenotypes: a meta-analysis. *J Gastroenterol Hepatol* 2015,**30**:821-829.
30. EASL Recommendations on Treatment of Hepatitis C 2015. *J Hepatol* 2015,**63**:199-236.

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Adjusted likelihood between *PNPLA3* rs738409 polymorphism and severity of liver disease in HIV/HCV coinfected patients.

	Hosmer-Lemeshow		Adjusted model	
Severity of liver disease	**Chi-square**	**p-value**	**aOR (95%CI)**	**p [(a)]**
Significant fibrosis (F ≥3)	**7.38**	**0.497**	**2.15 (1.07; 4.35)**	**0.029**
HOMA			1.06 (0.99; 1.13)	0.061
High alcohol intake			1.38 (0.93; 2.05)	0.082
Significant fibrosis (FIB4 ≥3.25)	**9.28**	**0.319**	**8.77 (1.11; 69.0)**	**0.039**
Age (years)			1.12 (1.03; 1.23)	0.007
Time on cART (years)			0.79 (0.64; 0.99)	0.048
BMI			0.78 (0.61; 0.98)	0.036
High fibrosis progression (FPR ≥0.10)	**5.19**	**0.737**	**1.81 (0.99; 3.30)**	**0.054**
HOMA			1.09 (1.01; 1.17)	0.038
Time on cART (years)			0.88 (0.78; 0.99)	0.036
Severe activity grade (A ≥3)	**5.65**	**0.686**	**1.69 (0.64; 4.42)**	**0.286**
Male			3.25 (0.70; 15.03)	0.132
CD4 nadir			1.01 (1.00; 1.01)	0.058
Time on cART (years)			0.85 (0.70; 1.03)	0.105
Liver steatosis (FH ≥10%)	**2.89**	**0.941**	**0.72 (0.41; 1.27)**	**0.260**
Age (years)			1.08 (1.02; 1.15)	0.008
IL28B rs12980275 AA			0.35 (0.18; 0.72)	0.004
HCV GT3			5.30 (1.99; 14.06)	0.001
HCV RNA >500,000 UI/mL			0.39 (0.17; 0.92)	0.031

Statistically significant differences are shown in bold. (a), P-values were calculated by multivariate logistic regression adjusted by the most important clinical and epidemiological characteristics (see **statistical analysis** section). The Hosmer–Lemeshow test evaluates the goodness of fit for logistic regression models. **Abbreviations:** 95%CI, 95% of confidence interval; aOR, adjusted odds ratio; p-value, level of significance; FPR, fibrosis progression rate; FH, fatty hepatocytes.

Title page

Type of manuscript: Concise communication

Title: Impact of *PNPLA3* polymorphism (rs738409) on severity of liver disease in HIV/HCV-coinfected patients
Running head: *PNPLA3* and HIV/HCV coinfection

Authors: María A JIMÉNEZ-SOUSA, PhD. [1(*)]; Juan BERENGUER, M.D., PhD. [2,3]; Mónica GARCÍA-ÁLVAREZ, PhD. [1]; Mónica GUTIERREZ-RIVAS, PhD.[1]; Teresa ALDÁMIZ-ECHEVARRIA, M.D. [2,3], Francisco TEJERINA, M.D. [2,3], Cristina DIEZ, M.D. [2,3], Sonia VÁZQUEZ-MORÓN, PhD. [1]; Salvador RESINO, PhD. [1(*)]; (*) Corresponding authors.

Current affiliations: (1) Unidad de Infección Viral e Inmunidad, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain. (2) Unidad de Enfermedades Infecciosas/VIH; Hospital General Universitario "Gregorio Marañón", Madrid, Spain. (3) Instituto de Investigación Sanitaria Gregorio Marañón (IiSGM), Madrid, Spain.

Correspondence and requests for reprints: Salvador Resino; Centro Nacional de Microbiología, Instituto de Salud Carlos III (Campus Majadahonda); Carretera Majadahonda- Pozuelo, Km 2.2; 28220 Majadahonda (Madrid); Telf.: +34 918 223 266; Fax: +34 918 223 269; e-mail: sresino@isciii.es
Alternative corresponding author: María A Jiménez-Sousa; Centro Nacional de Microbiología, Instituto de Salud Carlos III (Campus Majadahonda); Carretera Majadahonda- Pozuelo, Km 2.2; 28220 Majadahonda (Madrid); Telf.: +34 918 223 266; Fax: +34 918 223 269; e-mail: majimenezsousa@yahoo.es

ABSTRACT

Objective: To analyze the association between *PNPLA3* rs738409 polymorphism and severity of liver disease in human immunodeficiency virus (HIV)/hepatitis C virus (HCV) coinfected patients.

Methods: We performed a cross-sectional study of 215 patients who underwent a liver biopsy. *PNPLA3* rs738409 polymorphism was genotyped using GoldenGate® assay. The outcome variables were: i) advanced fibrosis (F≥3 and FIB-4≥3.25), ii) rapid fibrosis progression (FPR≥0.10 fibrosis units/year), iii) severe activity grade (A≥3), and iv) steatosis (fatty hepatocytes ≥10%). T genetic association analysis was carried out according to an additive genetic model through logistic regressions adjusted by the most significant covariables.

Results: Overall, 21.4% had F≥3, 8.9% had FIB-4≥3.25, 11.4% had A≥3, 60.6% had steatosis, and 32.5% had FPR≥0.10. For each rs738409 G allele, we found an increased frequency of patients with advanced fibrosis (F≥3) (0% CC, 18.5% CG, and 25.2% GG; p=0.049) and FIB-4≥3.25 (0% CC, 3.8% CG, and 13.2% GG; p=0.016). Furthermore, for each rs738409 G allele, the odds of having F≥3 increased 2.15 times (95% of confidence interval (95%CI)=1.07; 4.35; p=0.029) and having FIB-4≥3.25 increased 8.77 times (95%CI=1.11; 69.0; p=0.039). Note that rs738409 G allele carriers tended to higher likelihood of having FPR≥0.10, but statistical significance was not reached (p=0.054). Finally, we did not find any association for A≥3 and liver steatosis.

Conclusion: *PNPLA3* rs738409 polymorphism was associated with the severity of liver fibrosis in patients coinfected with HIV and HCV, suggesting that this polymorphism might also play a significant role in the progression of hepatic fibrosis in this group of patients.

Key words: AIDS; chronic hepatitis C; *PNPLA3*; SNPs; liver fibrosis

INTRODUCTION

Chronic hepatitis C (CHC) may cause liver fibrosis and steatosis, leading to cirrhosis and hepatocellular carcinoma [1, 2]. The natural course of CHC varies widely among individuals and several factors have been associated with liver fibrosis progression, including age at infection, sex, route of infection, hepatitis C virus (HCV) genotype, obesity and human immunodeficiency virus (HIV) coinfection, among others [3-5]. Regarding HCV/HIV coinfection, the natural history of CHC is accelerated in presence of HIV infection [6-8]. Thus, HCV infection constitutes an important cause of morbidity and mortality among HCV/HIV coinfected patients [9, 10].

However, liver fibrosis progression remains variable between individuals with similar environmental risk factors. Thus, genetic background could be another important factor to take into account [11]. In this setting, rs738409 C>G polymorphism, which encodes for the 148 Isoleucine to Methionine protein variant (I148M) of patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene, has been recognized as a risk factor for liver disease severity [5]. The *PNPLA3* rs738409 polymorphism has been widely related to nonalcoholic steatohepatitis (NASH) and progressive liver fibrosis in HCV-infected patients [12-16]. However, to our knowledge, there is no report related to severity of liver disease in HIV/HCV coinfected patients.

The aim of this study was to analyze the association between *PNPLA3* rs738409 polymorphism and severity of liver disease in HIV/HCV coinfected patients.

PATIENTS AND METHODS

Study population

We performed a cross-sectional study in 215 HIV/HCV-coinfected patients who underwent a liver biopsy between September 2000 and November 2008. The study was conducted in accordance with the Declaration of Helsinki and patients gave their written consent for the study. The Institutional Review Board and the Research Ethic Committee of the Instituto de Salud Carlos III (ISCIII) approved the study. Liver biopsies were performed on patients who were potential candidates for anti-HCV therapy and had not received previous interferon therapy. Selection criteria were: no clinical evidence of hepatic decompensation, detectable HCV RNA by polymerase chain reaction (PCR), negative hepatitis B surface antigen, availability of DNA sample, CD4+ lymphocyte count higher than 200 cells/µL, and stable combination antiretroviral therapy (cART) for at least 6 months before study entry or no need for cART according to treatment guidelines used in the study period [17]. Patients with active opportunistic infections, active drug addiction, and other concomitant severe diseases were excluded. High alcohol intake was not a contraindication to initiate anti-HCV therapy, unless the clinician felt that it could compromise treatment adherence. All subjects included in our study were European white.

Clinical and laboratory assessment

Clinical and epidemiological data were obtained from medical records. Alcohol intake of more than 50g 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.
Biochemistry panel was measured using a clinical chemistry analyzer (Hitachi 912, Boehringer Mannheim, Germany) in fasting patients. The degree of insulin resistance (IR) was estimated for each patient using the homeostatic model assessment (HOMA): fasting plasma glucose (mmol/L) times fasting serum insulin (mU/L) divided by 22.5. FIB-4 index, a non-invasive test of liver fibrosis in HIV/HCV-coinfected patients, was calculated as age ([yr] x AST [U/L]) / ((PLT [10(9)/L]) x (ALT [U/L])(1⁄2)) [18].
Liver biopsies were performed as we described previously [19]. Liver fibrosis and necroinflammatory activity were estimated according to Metavir score as follows [20]: 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 evaluated according to the existence of hepatocytes containing visible macrovesicular fat droplets, which was clinically significant when fatty hepatocytes exceeded 10% of parenchyma hepatic.

The duration of HCV infection for patients with a history of intravenous drug use (IDU) was estimated using the first year they shared needles and other injection paraphernalia, which are the most relevant risk practices for HCV transmission. The duration of HCV infection was not calculated when the date of initiation of their HCV infection could not be determined with certainty (n=15). The fibrosis progression rate (FPR) was calculated dividing the fibrosis stage (0 to 4) by the estimated duration of HCV infection in years (fibrosis units per year) [21].

Genomic DNA was extracted from peripheral blood with Qiagen columns (QIAamp DNA Blood Midi/Maxi; Qiagen, Hilden, Germany). DNA samples were sent to the Spanish National Genotyping Center (CeGen; http://www.cegen.org/) in order to genotype *PNPLA3* rs738409 and *IL28B* rs12980275 polymorphisms. Genotyping was performed by using Sequenom's MassARRAY platform (San Diego, CA, USA) using the iPLEX® Gold assay design system.

Data analysis

The outcome variables were: i) advanced fibrosis (F≥3 and FIB4≥3.25), ii) rapid fibrosis progression (FPR≥0.10 fibrosis units/year), iii) severe activity grade (A≥3), and iv) liver steatosis (fatty hepatocytes ≥10%)[22]. These outcomes were developed after a minimum follow-up time of 10 years with HCV infection.
The genetic association analysis was carried out according to an additive genetic model, which was the model that best fit to our data. Chi-square test and logistic regression analysis were used to investigate the relationship among *PNPLA3* rs738409 polymorphism and severe liver disease. Each

regression analysis was always adjusted by the most significant co-variables associated with each one of the outcome variables, avoiding the over-fitting of the regression. The co-variables were selected by "Stepwise" algorithm (p-value of entry and exit of 0.15 and 0.20, respectively), including gender, age, alcohol intake, BMI, HOMA, nadir CD4+ T-cells, AIDS, undetectable HIV-RNA (<50 copies/ml), CD4+ T-cells, time on cART, HCV-RNA ≥500,000 IU/ml, HCV genotype, and *IL28B* rs12980275 polymorphism (see **Supplemental table 1**).

These analyses were performed by using the IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp, Chicago, Armonk, NY, USA). All p-values were two-tailed and statistical significance was defined as $p<0.05$.

RESULTS

Table 1 shows the epidemiological and clinical characteristics of 215 HIV/HCV-coinfected patients at the time of liver biopsy. Regarding liver biopsy, 21.4% had advanced fibrosis (F≥3), 8.9% had FIB-4≥3.25, 11.4% had severe necroinflammatory activity (A≥3), 60.6% had steatosis, and 32.5% had FPR≥0.10.

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

Characteristics	All Patients
No., (%)	215
Male, n (%)	161 (74.9%)
Age, years	38.8 (37.4; 44.1)
Epidemiological history	
HIV acquired by IVDU, n (%)	190 (88.4%)
Years since HCV infection (n=200)	21.4 (17.1; 24.4)
High alcohol intake	116 (54.2%)
High alcohol intake at biopsy study	32 (14.8%)
CDC category C, n (%)	59 (27.4%)
Metabolic markers	
BMI, kg/m2(n=213)	22.3 (20.7; 24.6)
BMI ≥25 kg/m2	46 (21.4%)
HOMA	2.10 (1.27; 3.68)
HOMA ≥3	68 (32.9%)
Antiretroviral therapy	
cART, n (%)	178 (82.8%)
Time on cART, years	4.4 (2.5; 6.6)
Current cART protocols, n (%)	
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, n (%)	162 (74%)
HCV markers, n (%)	
HCV genotype	
1	119 (56.1%)
2	5 (2.4%)
3	48 (22.6%)
4	40 (18.9%)
HCV RNA >500,000 UI/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	102 (48.2%)

A2	86 (40.8%)
A3	24 (11.4%)
Steatosis	114 (60.6%)
Fibrosis progression rate (FPR)	0.07 (0.04; 0.12)
FPR≥0.10	65 (32.5%)
FIB-4	1.46 (1.03; 2.03)
FIB-4≥3.25	18 (8.9%)

Values are expressed as absolute numbers (%) and median (percentile 25; percentile 75).
Abbreviations: BMI, body mass index; HOMA, homeostatic model assessment; HCV, Hepatitis C virus; CDC category C, centers for disease control and prevention classification system, clinical category C which includes any condition listed in the CDC´s 1897 surveillance case definition of AIDS; PI-based, protein inhibitor-based therapy; NNRTI-based, non-nucleoside reverse transcriptase inhibitor -based therapy; 3NRTI-based, triple nucleoside regimen; HIV; Human immunodeficiency virus; HIV-RNA, HIV plasma viral load; HCV-RNA, HCV plasma viral load.

Allele frequencies for *PNPLA3* rs738409 polymorphism were 0.76 for G allele and 0.24 for T allele. Genotype frequencies were 0.57, 0.38 and 0.05 for GG, CG and CC genotypes, respectively. These frequencies in our dataset were in accordance with the data listed on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=rs738409). The *PNPLA3* rs738409 polymorphism fulfilled the minimum allele frequency >0.05 and had missing values <5%. Furthermore, rs738409 polymorphism was in Hardy–Weinberg equilibrium (p=0.710).
Table 2 shows the relationship of *PNPLA3* rs738409 polymorphism with severity of liver disease under a model of additive inheritance. For each rs738409 G allele, we found an increased frequency of patients with advanced fibrosis (F≥3) (0% CC, 18.5% CG, and 25.2% GG; p=0.049) and FIB-4≥3.25 (0% CC, 3.8% CG, and 13.2% GG; p=0.016). Furthermore, for each rs738409 G allele, the probability of having values of F≥3 increased 2.15 times (p=0.029) and having FIB-4 ≥3.25 increased 8.77 times (p=0.039). Note that rs738409 G allele tended to higher likelihood of having FPR ≥0.10, but statistical significance was not reached (p=0.054). Finally, we did not find any association for severe activity grade (A≥3) and liver steatosis (fatty hepatocytes ≥10%). **Supplemental Table 1** shows the significant covariates maintained in each logistic regression model by using Stepwise algorithm.

Table 2. Relationship between *PNPLA3* rs738409 polymorphism and severity of liver disease in HIV/HCV coinfected patients.

Liver disease	No.	CC	CG	GG	p [(a)]	OR (95%CI)	p [(b)]
Significant fibrosis (F ≥3)	215	0/11 (0%)	15/81 (18.5%)	31/123 (25.2%)	**0.049**	2.15 (1.07; 4.35)	**0.029**
Significant fibrosis (FIB4 ≥3.25)	203	0/11 (0%)	3/78 (3.8%)	15/114 (13.2%)	**0.016**	8.77 (1.11; 69.0)	**0.039**
High fibrosis progression (FPR ≥0.10)	200	0/10 (0%)	24/74 (32.4%)	41/116 (35.2%)	0.095	1.81 (0.99; 3.30)	0.054
Severe activity grade (A ≥3)	211	0/11 (0%)	8/80 (10.0%)	16/120 (13.3%)	0.190	1.69 (0.64; 4.42)	0.286
Liver steatosis (FH ≥10%)	188	7/10 (70%)	45/71 (63.4%)	62/107 (57.9%)	0.341	0.72 (0.41; 1.27)	0.260

Statistically significant differences are shown in bold. (a), P-values were calculated by linear-by-linear association Chi-squared test; (b), P-values were calculated by multivariate logistic regression adjusted by the most important clinical and epidemiological characteristics (see **statistical analysis** section).
Abbreviations: 95%CI, 95% of confidence interval; OR, odds ratio; p-value, level of significance; FPR, fibrosis progression rate; FH, fatty hepatocytes

DISCUSSION

The major finding of the present study was that the presence of *PNPLA3* rs738409 G allele increased the odds of having advanced liver fibrosis in HIV/HCV-coinfected patients. To our knowledge, this study is the first description of the relationship between *PNPLA3* rs738409 polymorphism and severity of liver fibrosis in HIV/HCV-coinfected patients.

PNPLA3 is a protein with lipase activity which is highly expressed in the liver and adipose tissue. The nonsynonymous rs738409 (G allele; I148M substitution) seems not to affect *PNPLA3* mRNA levels; however it results in a loss of PNPLA3 function, influencing individual susceptibility to hepatocyte lipid accumulation [23, 24]. Emerging evidence has linked *PNPLA3* rs738409 polymorphism with fibrosis progression in patients with HCV infection [5, 13, 14]. The mechanism implicated in liver fibrosis could be related to lipotoxicity because lipid accumulation may lead to inflammatory mediators release, oxidative stress, and hepatocyte apoptosis [25]. Thus, liver steatosis would be responsible for liver fibrosis development and progression [26]. However, the effect of *PNPLA3* rs738409 polymorphism on HCV-related liver disease severity remains largely unknown in HIV/HCV coinfected patients [27]. In our study, rs738409 G allele was associated with significant fibrosis, which is in concordance with those results found for HCV monoinfected patients. Thus, rs738409 genotyping could be useful in both HCV monoinfected and HIV/HCV coinfected patients.

Apart from liver biopsy, we used FIB-4 as an outcome to evaluate the influence of rs738409 on liver fibrosis. FIB-4 was chosen because it is a non-invasive test widely validated for HIV/HCV coinfected patients [28]; unlike others more commonly used for HCV monoinfected patients. In our study, the association between rs738409 and FIB-4 was even stronger than that noted with liver biopsy, being the probability of developing advanced fibrosis eight times higher for G carriers.

Regarding fibrosis progression, rs738409 G allele has been described as a risk factor for accelerated FPR in HCV monoinfected patients [5]. In our study, the association was close to statistical significance and we cannot discard that the lack of association may be due to a limited sample population. Thus, note that further studies with a longer sample size would be interesting.

As mentioned above, *PNPLA3* rs738409 polymorphism has been widely associated with steatosis [12, 15, 16, 29]. However, we found no significant association between *PNPLA3* rs738409 polymorphism and steatosis. This inconsistence with previous reports could be explained by our unavailability of continuous values to define steatosis. Steatosis was defined as a dichotomous variable (fatty hepatocytes ≥10%) according to the procedure established in our hospital, and data collection was performed by retrospective review of medical records. However, the large majority of the articles that found association between rs738409 polymorphism and steatosis used steatosis in an ordinal scale [12, 15, 16, 29]. Thus, it may have limited the finding of statistically significant results in our study, not ruling out the association between rs738409 polymorphism and steatosis.

The treatment regimens with new direct-acting antivirals (DAAs) are characterized by shorter duration, simplified dosing, improved safety profile and effectivity, and > 90% of sustained virological response. These HCV regimens are the HCV therapies recommended by international guidelines. However, DAAs are being prioritized in patients at high risk for liver-related complications because of cost [30]. In this setting, the PNPLA3 rs738409 genotyping could be a valuable tool to identify patients at high risk for developing advanced fibrosis, who could be prioritized. So, these patients could benefit from the predictive value of rs738409 polymorphism.

Finally, several aspects have to be taken into account for the correct interpretation of the results. Firstly, this is a cross-sectional study with a limited number of patients, which could limit achieving statistically significant values. Besides, the cross-sectional design of the analyses may be a confounding factor. Secondly, data were collected retrospectively, which entails a lack of uniformity. Thirdly, 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. Fourthly, this study was performed on patients with European ancestry, and it would also be interesting to perform these analyses on different ethnic groups.

In conclusion, *PNPLA3* rs738409 polymorphism was associated with severity of liver fibrosis in patients coinfected with HIV and HCV, suggesting that this polymorphism might also play a significant

role in the progression of hepatic fibrosis in this group of patients. Further studies with bigger cohorts should be performed to confirm the trend of our results.

ACKNOWLEDGEMENTS

The authors wish to thank the Spanish National Genotyping Center (CeGen) for providing the SNP genotyping services (http://www.cegen.org).

Potential conflicts of interest: The authors do not have a commercial or other association that might pose a conflict of interest.

Funding/Support: This work has been supported by grants given by Fondo de Investigación de Sanidad en España (FIS) [Spanish Health Founds for Research] [grant numbers PI11/01556, PI14/01094, PI11/00245, PI14CIII/00011], and "Fundación para la Investigación y la Prevención del Sida en España" (FIPSE) [grant number 361020/10]. This work has been (partially) funded by the RD12/0017/0024 and RD12/0017/0004 projects as part of the Plan Nacional R + D + I and cofinanced by ISCIII- Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER).
JB is an investigator from the Programa de Intensificación de la Actividad Investigadora en el Sistema Nacional de Salud (I3SNS), Refs INT10/009 and INT12/154. MGA, DPT, MAJS and MGF are supported by "Instituto de Salud Carlos III" [grant numbers CD12/00442, CM12/00043, CD13/00013 and RD12/0017/0024, respectively].
This work has been (partially) funded by the RD12/0017 project as part of the Plan Nacional R + D + I and cofinanced by ISCIII- Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER).

AUTHORS´ CONTRIBUTIONS

MAJS and SR performed all statistical analysis, interpretation of the data and wrote the manuscript.
JB and SR participated in the study concept and design.
JB, TAE, CD, and FT, participated in patient selection, collection of samples and acquisition of data.
MGA, SVM and MGR participated in sample preparation, DNA isolation and genotyping pre-procedure, and contributed with critical revision of the manuscript.
SR supervised the study.
All authors revised the manuscript from a draft by SR.

REFERENCES

1. Mengshol JA, Golden-Mason L, Rosen HR. Mechanisms of Disease: HCV-induced liver injury. *Nat Clin Pract Gastroenterol Hepatol* 2007,**4**:622-634.
2. Heim MH, Thimme R. Innate and adaptive immune responses in HCV infections. *J Hepatol* 2014,**61**:S14-S25.
3. Ortiz V, Berenguer M, Rayon JM, Carrasco D, Berenguer J. Contribution of obesity to hepatitis C-related fibrosis progression. *Am J Gastroenterol* 2002,**97**:2408-2414.
4. Rotman Y, Liang TJ. Coinfection with hepatitis C virus and human immunodeficiency virus: virological, immunological, and clinical outcomes. *J Virol* 2009,**83**:7366-7374.
5. Rueger S, Bochud PY, Dufour JF, Mullhaupt B, Semela D, Heim MH*, et al.* Impact of common risk factors of fibrosis progression in chronic hepatitis C. *Gut* 2014.
6. Lopez-Dieguez M, Montes ML, Pascual-Pareja JF, Quereda C, Von Wichmann MA, Berenguer J*, et al.* The natural history of liver cirrhosis in HIV-hepatitis C virus-coinfected patients. *AIDS* 2011,**25**:899-904.
7. Macias J, Berenguer J, Japon MA, Giron JA, Rivero A, Lopez-Cortes LF*, et al.* Fast fibrosis progression between repeated liver biopsies in patients coinfected with human immunodeficiency virus/hepatitis C virus. *Hepatology* 2009,**50**:1056-1063.
8. Lo Re V, 3rd, Kallan MJ, Tate JP, Localio AR, Lim JK, Goetz MB*, et al.* Hepatic decompensation in antiretroviral-treated patients co-infected with HIV and hepatitis C virus compared with hepatitis C virus-monoinfected patients: a cohort study. *Ann Intern Med* 2014,**160**:369-379.
9. Lewden C, May T, Rosenthal E, Burty C, Bonnet F, Costagliola D*, et al.* Changes in causes of death among adults infected by HIV between 2000 and 2005: The "Mortalite 2000 and 2005" surveys (ANRS EN19 and Mortavic). *J Acquir Immune Defic Syndr* 2008,**48**:590-598.
10. Graham CS, Baden LR, Yu E, Mrus JM, Carnie J, Heeren T*, et al.* Influence of human immunodeficiency virus infection on the course of hepatitis C virus infection: a meta-analysis. *Clinical Infectious Diseases* 2001,**33**:562-569.
11. Westbrook RH, Dusheiko G. Natural history of hepatitis C. *Journal of Hepatology* 2014,**61**:S58-68.
12. Petta S, Vanni E, Bugianesi E, Rosso C, Cabibi D, Camma C*, et al.* PNPLA3 rs738409 I748M is associated with steatohepatitis in 434 non-obese subjects with hepatitis C. *Aliment Pharmacol Ther* 2015,**41**:939-948.
13. Yasui K, Kawaguchi T, Shima T, Mitsuyoshi H, Seki K, Sendo R*, et al.* Effect of PNPLA3 rs738409 variant (I148 M) on hepatic steatosis, necroinflammation, and fibrosis in Japanese patients with chronic hepatitis C. *J Gastroenterol* 2014.
14. Huang CF, Dai CY, Yeh ML, Huang CI, Tai CM, Hsieh MH*, et al.* Association of diabetes and PNPLA3 genetic variants with disease severity of patients with chronic hepatitis C virus infection. *J Hepatol* 2015,**62**:512-518.
15. Trepo E, Pradat P, Potthoff A, Momozawa Y, Quertinmont E, Gustot T*, et al.* Impact of patatin-like phospholipase-3 (rs738409 C>G) polymorphism on fibrosis progression and steatosis in chronic hepatitis C. *Hepatology* 2011,**54**:60-69.
16. Valenti L, Rumi M, Galmozzi E, Aghemo A, Del Menico B, De Nicola S*, et al.* Patatin-like phospholipase domain-containing 3 I148M polymorphism, steatosis, and liver damage in chronic hepatitis C. *Hepatology* 2011,**53**:791-799.
17. Panel de expertos de Gesida Plan Nacional sobre el Sida y Asociación Española para el Estudio del Hígado. [Recommendations of Gesida/PNS/AEEH for the management and treatment of the adult patient co-infected with HIV and hepatitis A, B and C virus]. *Enferm Infecc Microbiol Clin* 2010,**28**:31 e31-31.
18. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J*, et al.* Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 2006,**43**:1317-1325.
19. Resino S, Seoane JA, Bellon JM, Dorado J, Martin-Sanchez F, Alvarez E*, et al.* An artificial neural network improves the non-invasive diagnosis of significant fibrosis in HIV/HCV

coinfected patients. *J Infect* 2011,**62**:77-86.
20. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996,**24**:289-293.
21. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997,**349**:825-832.
22. Obika M, Noguchi H. Diagnosis and evaluation of nonalcoholic fatty liver disease. *Exp Diabetes Res* 2012,**2012**:145754.
23. Anstee QM, Day CP. The genetics of NAFLD. *Nat Rev Gastroenterol Hepatol* 2013,**10**:645-655.
24. Romeo S, Huang-Doran I, Baroni MG, Kotronen A. Unravelling the pathogenesis of fatty liver disease: patatin-like phospholipase domain-containing 3 protein. *Curr Opin Lipidol* 2010,**21**:247-252.
25. Marra F, Lotersztajn S. Pathophysiology of NASH: perspectives for a targeted treatment. *Curr Pharm Des* 2013,**19**:5250-5269.
26. Leandro G, Mangia A, Hui J, Fabris P, Rubbia-Brandt L, Colloredo G*, et al.* Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. *Gastroenterology* 2006,**130**:1636-1642.
27. Singal AG, Manjunath H, Yopp AC, Beg MS, Marrero JA, Gopal P*, et al.* The effect of PNPLA3 on fibrosis progression and development of hepatocellular carcinoma: a meta-analysis. *Am J Gastroenterol* 2014,**109**:325-334.
28. Berenguer J, Zamora FX, Aldamiz-Echevarria T, Von Wichmann MA, Crespo M, Lopez-Aldeguer J*, et al.* Comparison of the prognostic value of liver biopsy and FIB-4 index in patients coinfected with HIV and hepatitis C virus. *Clin Infect Dis* 2015,**60**:950-958.
29. Zhang L, You W, Zhang H, Peng R, Zhu Q, Yao A*, et al.* PNPLA3 polymorphisms (rs738409) and non-alcoholic fatty liver disease risk and related phenotypes: a meta-analysis. *J Gastroenterol Hepatol* 2015,**30**:821-829.
30. EASL Recommendations on Treatment of Hepatitis C 2015. *J Hepatol* 2015,**63**:199-236.

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Adjusted likelihood between *PNPLA3* rs738409 polymorphism and severity of liver disease in HIV/HCV coinfected patients.

	Hosmer-Lemeshow		Adjusted model	
Severity of liver disease	**Chi-square**	**p-value**	**aOR (95%CI)**	**p [(a)]**
Significant fibrosis (F ≥3)	**7.38**	**0.497**	**2.15 (1.07; 4.35)**	**0.029**
HOMA			1.06 (0.99; 1.13)	0.061
High alcohol intake			1.38 (0.93; 2.05)	0.082
Significant fibrosis (FIB4 ≥3.25)	**9.28**	**0.319**	**8.77 (1.11; 69.0)**	**0.039**
Age (years)			1.12 (1.03; 1.23)	0.007
Time on cART (years)			0.79 (0.64; 0.99)	0.048
BMI			0.78 (0.61; 0.98)	0.036
High fibrosis progression (FPR ≥0.10)	**5.19**	**0.737**	**1.81 (0.99; 3.30)**	**0.054**
HOMA			1.09 (1.01; 1.17)	0.038
Time on cART (years)			0.88 (0.78; 0.99)	0.036
Severe activity grade (A ≥3)	**5.65**	**0.686**	**1.69 (0.64; 4.42)**	**0.286**
Male			3.25 (0.70; 15.03)	0.132
CD4 nadir			1.01 (1.00; 1.01)	0.058
Time on cART (years)			0.85 (0.70; 1.03)	0.105
Liver steatosis (FH ≥10%)	**2.89**	**0.941**	**0.72 (0.41; 1.27)**	**0.260**
Age (years)			1.08 (1.02; 1.15)	0.008
IL28B rs12980275 AA			0.35 (0.18; 0.72)	0.004
HCV GT3			5.30 (1.99; 14.06)	0.001
HCV RNA >500,000 UI/mL			0.39 (0.17; 0.92)	0.031

Statistically significant differences are shown in bold. (a), P-values were calculated by multivariate logistic regression adjusted by the most important clinical and epidemiological characteristics (see **statistical analysis** section). The Hosmer–Lemeshow test evaluates the goodness of fit for logistic regression models. **Abbreviations:** 95%CI, 95% of confidence interval; aOR, adjusted odds ratio; p-value, level of significance; FPR, fibrosis progression rate; FH, fatty hepatocytes.