Jiménez-Sousa, María Ángeles; Gómez-Moreno, Ana Zaida; Pineda-Tenor, Daniel; Sánchez-Ruano, Juan José; Fernández-Rodríguez, Amanda;
Artaza-Varasa, Tomas; Gómez-Sanz, Alicia; Martín-Vicente, María; Vázquez-Morón, Sonia; Resino, Salvador. **PNPLA3 rs738409 polymorphism is associated with liver fibrosis progression in patients with chronic hepatitis C: A repeated measures study**. J Clin Virol. 2018 Jun:103:71-74.

https://doi.org/10.1016/j.jcv.2018.04.008

Short communication

Title: *PNPLA3* rs738409 polymorphism is associated with liver fibrosis progression in patients with chronic hepatitis C: a repeated measures study

Running head: rs738409 is related to liver fibrosis progression

Authors: María Ángeles JIMÉNEZ-SOUSA [1, ¥], Ph.D.; Ana Zaida GÓMEZ-MORENO [2, ¥] M.D.; Daniel PINEDA-TENOR [3], Ph.D.; Juan José SÁNCHEZ-RUANO [2], M.D., Ph.D.; Amanda FERNÁNDEZ-RODRÍGUEZ, PhD. [1]; Tomas ARTAZA-VARASA [2], M.D., Ph.D.; **Alicia GÓMEZ-SANZ [1]**; María MARTIN-VICENTE [1], Bs.C.; Sonia VÁZQUEZ-MORÓN [1]; Ph.D.; Salvador RESINO [1], Ph.D. [(*)]

(¥), Both authors contributed equally to this study; (*) Corresponding author.

Current affiliations: (1) Unidad de Infección Viral e Inmunidad, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Spain. (2) Servicio de Digestivo, Hospital Virgen de la Salud, Toledo, Spain. (3) Servicio de Laboratorio Clínico, Hospital Universitario de Fuenlabrada, Madrid, Spain.

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

Word Count: 1364

Abstract

Background: Host genetic background has been associated with liver fibrosis progression.

Objective: To analyze the association between the patatin-like phospholipase domain-containing 3 (*PNPLA3*) rs738409 polymorphism and liver fibrosis progression in hepatitis C virus (HCV)-infected patients.

Study design: In this retrospective cohort study, 187 patients with chronic HCV infection were included, who had at least two liver stiffness measurements (LSM) by transient elastography during the follow-up. Results were expressed in kilopascals (kPa). The analysis of genetic association was carried out according to additive model by using Generalized Linear Models.

Results: No patients had advanced fibrosis/cirrhosis at baseline. During a median follow-up time of 47.9 months, 15 patients developed advanced fibrosis and 17 cirrhosis. In multivariate analysis adjusted by the main clinical and epidemiological covariates, the rs738409 G allele was related to higher increase of LSM values during the follow-up (adjusted arithmetic mean ratio (aAMR)=1.16 (95%CI=1.04; 1.29); p=0.006) and higher odds of having progression to advanced fibrosis [aOR=2.03 (95%CI=1.01; 4.06); p=0.045], and progression to cirrhosis [aOR=3.03 (95%CI=1.26; 7.30); p=0.014].

Conclusions: *PNPLA3* rs738409 polymorphism appears to be related to the increased progression of liver fibrosis in HCV infected patients.

Keywords

Liver stiffness; chronic hepatitis C; cirrhosis; hepatic fibrosis; PNPLA3; SNPs

Background

The natural course of chronic hepatitis C (CHC) varies widely among individuals [1], and the early recognition of patients at risk for developing liver fibrosis and cirrhosis is essential to take preventive measures that may affect the course of CHC [2]. Several risk factors have been associated with liver fibrosis progression, including age at infection, sex, route of infection, HCV genotype, and obesity among others [3]. In this regard, single nucleotide polymorphisms have been also associated with liver disease progression[3, 4]. Among them, the patatin-like phospholipase domain-containing 3 (*PNPLA3*) rs738409 polymorphism (G allele) promotes nonalcoholic steatohepatitis, liver fibrosis progression and possibly other outcomes, such as hepatocellular carcinoma [5-8] in Caucasian CHC patients. However, the vast majority of these studies had a cross-sectional or case-control design [5, 6], which may have introduced inherent biases.

Objectives

In this study, we aimed to analyze the association between *PNPLA3* rs738409 polymorphism and liver fibrosis progression in HCV-infected patients.

Study design

We carried out a retrospective cohort study with a longitudinal design of repeated measures and follow-up over a prolonged period in 187 HCV-infected patients from Hospital Virgen de la Salud (Toledo, Spain) between 2008 and 2015. The study ran from the day of the first LSM recorded to the last follow-up visit with LSM data, or the initiation of antiviral treatment for HCV in responder patients, who clearance HCV infection.

This work was conducted in accordance with the 1975 Declaration of Helsinki. The Institutional Review Board of the Instituto de Salud Carlos III approved the study, and all patients signed the consent.

Patient selection criteria were: 1) detectable plasma HCV RNA during the follow-up; 2) availability of DNA sample; 3) availability of baseline liver stiffness measurement (LSM) and final LSM with a separation of at least 12 months. The exclusion criteria were: 1) advanced fibrosis/cirrhosis at baseline (F≥3 (LSM≥9.5)); 2) co-infection with hepatitis B virus or human immunodeficiency virus.

Patients received conventional CHC management during the follow-up and consequently, patients could have been treated before or after entering the study with HCV therapy according to clinical guidelines [9, 10]. However, in the case of patients who were treated before, we only included those patients who were non-responders; and in the case of patients who were treated after entering the study and achieved sustained virological response (SVR), their follow-ups were truncated at the time of starting HCV therapy. High alcohol intake was considered as >20 g/day in women and ≥60 g/day in men. Time since HCV diagnosis was defined as the time between HCV diagnosis and the first LSM (LSM1, baseline of study). Time of follow-up was defined as the time between the last LSM (LSM2, the end of study) and the first LSM (LSM1).

DNA samples were genotyped for *PNPLA3* rs738409 polymorphism at the Spanish National Genotyping Center (CeGen; http://www.cegen.org/). Genotyping was performed by using Agena Bioscience's MassARRAY platform (San Diego, CA, USA) using the iPLEX® Gold assay design system [11].

LSM was assessed by transient elastography (FibroScan®, Echosens, Paris, France) using a single machine. Results were expressed in kilopascals (kPa) with a range of 2.5 to 75 kPa [12]. Transient elastography was performed in our unit by a trained hepatologist, and measurements were

considered reliable when the interquartile-range-to-median ratio for at least ten successful measurements was lower than 0.30. All LSM measurements were performed with at least four hours of fasting. Advanced obese patients were not included in this study because we did not have access to XL probe. The following cut-offs of LSM were used to stratify patients [13]: <7.1 kPa (F0-F1 - no or portal/periportal fibrosis), 7.1-9.4 kPa (F2 - septal fibrosis), 9.5-12.4 kPa (F3 (bridging fibrosis)), and ≥12.5 kPa (F4 (cirrhosis)).

The primary outcome variable was the change in LSM values during follow-up (continuous variable). We calculated the LSM variation during the follow-up (ratio LSM2/LSM1) and whether the LSM increase (ΔLSM=LSM2-LSM1) was higher than 5 kPa (ΔLSM≥5 kPa). Furthermore, we evaluated the progression to F≥3, which is a dichotomous variable that may have values of +1 [if F≤2 (F0, F1 or F2) change to F≥3 (F3 or F4)] or 0 [if F ≤2 (F0, F1 or F2) remains)]. The progression to cirrhosis (F4) may also have values of +1 [if F≤2 (F0, F1, or F2) change to F4] or 0 (if F≤2 (F0, F1 or F2) remains), since none patients had advanced fibrosis/cirrhosis at baseline (F≥3 (LSM≥9.5)).

Generalized Linear Model (GLM) was used to analyze the genetic association of *PNPLA3* rs738409 polymorphism with the outcome variables. GLM with a gamma distribution (log-link) was used for continuous variables. This test gives the differences between groups and the arithmetic mean ratio (AMR). Moreover, GLM with binomial distribution (logit-link) was used to investigate the association with dichotomous variables (ΔLSM≥5 kPa, progression to F≥3, and progression to F4). This test gives the differences between groups and the odds ratio (OR). Each regression test was adjusted by age, gender, time since HCV diagnosis, HCV genotype, injection drug use, high alcohol intake, diabetes, HCV antiviral therapy prior to baseline and during follow-up (patients who failed therapy), baseline of LSM, and time of follow-up. All statistical tests were performed with the Statistical Package for the Social Sciences (SPSS) 21.0 software (IBM Corp., Chicago, USA). All p-values were two-tailed and statistical significance was defined as $p<0.05$.

Results

Table 1 shows the baseline characteristics of 187 HCV-infected patients without advanced fibrosis/cirrhosis. We did not find any significant differences at baseline between patients with different *PNPLA3* rs738409 genotypes. Moreover, during a median follow-up time of 47.9 months, 15 patients developed advanced fibrosis and 17 cirrhosis.

During the follow-up, the rs738409 G allele had significant positive relationship with the increase of LSM (AMR=1.13; p=0.024; **Figure 1A**) and with the frequency of patients with ΔLSM≥5 kPa (OR=1.97; p=0.052; **Figure 1B**), progression to F≥3 (OR=1.84; p=0.037; **Figure 1C**), and progression to F4 (OR=2.72; p=0.008; **Figure 1D**). Additionally, these trends were maintained in the multivariate analysis adjusted by the main clinical and epidemiological covariates (**Figure 1E**). Thus, the rs738409 G allele was independently associated with a higher increase of LSM values during the follow-up (adjusted AMR=1.16; p=0.006) and higher odds of having progression to F≥3 [aOR=2.03; p=0.045], and progression to F4 [aOR=3.03; p=0.014], after controlling confounders by including them in the model.

Table 1. Clinical and epidemiological characteristics of HCV-infected patients stratified by *PNPLA3* rs738409 genotypes.

		***PNPLA3* rs738409 polymorphism**			
Characteristic	**All Patients**	**CC**	**CG**	**GG**	**p-value**
No.	187	101	72	14	
Male sex	102 (54.5%)	53 (52.5%)	40 (55.6%)	9 (64.3%)	0.691
Age (years)	46.4 (40.9; 55.8)	47.8 (42.1; 56.1)	46.1 (40.6; 55.9)	44.9 (40.9; 49.2)	0.255
Time of HCV infection (years)	7.5 (2.9; 12.9)	6.5 (2.7; 12.9)	8.7 (2.4; 12.5)	11.4 (4.2; 13.2)	0.401
High alcohol intake	25 (13.4%)	13 (12.9%)	9 (12.5%)	3 (21.4%)	0.653
Prior injection drug use	20 (10.7%)	7 (6.9%)	10 (13.9%)	3 (21.4%)	0.138
HCV genotype (n=216)					
1	154 (83.7%)	84 (84.8%)	59 (83.1%)	11 (78.6%)	0.645
3	14 (7.6%)	9 (9.1%)	4 (5.6%)	1 (7.1%)	-
4	15 (8.2%)	5 (5.1%)	8 (11.3%)	2 (14.3%)	-
5	1 (0.5%)	1 (1%)	0 (0%)	0 (0%)	-
Prior peg-IFN-α/RBV therapy failed	42 (22.5%)	21 (20.8%)	18 (25%)	3 (21.4%)	0.804
Baseline LSM (kPa)	6.1 (5.3; 8.4)	5.8 (4.9; 6.8)	6 (5; 7.3)	6.2 (5.6; 7.3)	0.394
F0-F1 (<7.1 kPa)	149 (79.7%)	85 (84.2%)	54 (75.0%)	10 (71.4%)	0.245
F2 (7.1-9.4 kPa)	38 (20.3%)	16 (15.8%)	18 (25.0%)	4 (28.6%)	-
Follow-up time (months)	47.9 (29.2; 62.7)	50.5 (30.5; 63.8)	45.8 (27.4; 61.5)	44 (34.4; 60.6)	0.503
Final LSM (kPa)	6.7 (5.3; 8.6)	6.5 (5.3; 7.9)	7.1 (5.5; 8.7)	7.4 (5.9; 14.6)	0.190
F0-F1 (<7.1 kPa)	108 (57.8%)	66 (65.3%)	36 (50.0%)	6 (42.9%)	0.043
F2 (7.1-9.4 kPa)	47 (25.1%)	23 (22.8%)	20 (27.8%)	4 (28.6%)	-
F3 (9.5-12.4 kPa)	15 (8.0%)	7 (6.9%)	8 (11.1%)	0 (0%)	
F4 (≥12.5 kPa)	17 (9.1%)	5 (5.0%)	8 (11.1%)	4 (28.6%)	-

Values expressed as absolute numbers (%) and median (percentile 25; percentile 75). p-values were estimated with nonparametric Mann-Whitney U test for continuous variables and Chi-square test for categorical variables.

Abbreviations: Abbreviations: HCV, hepatitis C virus; LSM, liver stiffness measure; kPa, kilopascal; peg-IFN-α/RBV, peg-interferon-alpha/ribavirin; PNPLA3, patatin-like phospholipase domain-containing protein 3

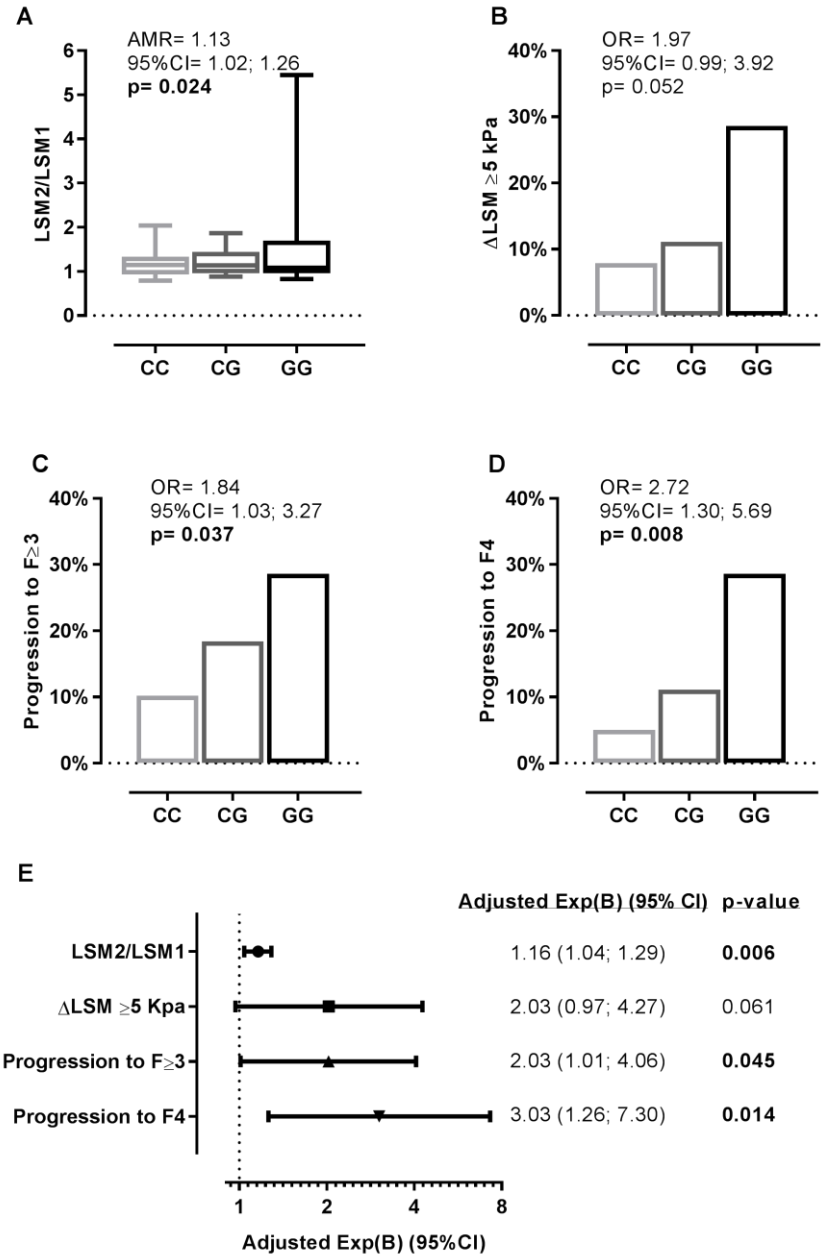

Figure 1. Relationship between *PNPLA3* polymorphism and variation of LSM values and fibrosis stages in HCV-infected patients with an additive inheritance model.
Statistical: p-values were calculated by univariate and multivariate regression adjusted by the most important clinical and epidemiological characteristics (see statistical analysis section). Statistically significant differences are shown in bold.
Abbreviations: LSM, liver stiffness measure; kPa, kilopascal; LSM1, baseline LSM; LSM2, final LSM; Δ or delta, change in one variable [Δx (x2-x1)]; Exp(B), arithmetic mean ratio (AMR) for continuous variable and odds ratio (OR) for categorical variables; 95%CI, 95% of confidence interval; p-value, level of significance; F≥3, advanced fibrosis; F4, cirrhosis; PNPLA3, patatin-like phospholipase domain-containing protein 3.

Discussion

The major finding of this longitudinal study of repeated measures was the *PNPLA3* rs738409 G allele was associated with higher liver fibrosis progression in HCV-infected patients. Our results are in concordance with previous studies, where rs738409 polymorphism was related to progressive liver fibrosis in both Caucasian HCV-infected patients [6] and HIV/HCV coinfected patients [14, 15]. Thus, rs738409 polymorphism seems to play a crucial role in the physiopathology of CHC and could be used to stratify the risk of progression successfully.

The pathophysiological mechanisms of PNPLA3 are still unclear [16]. PNPLA3 is a protein with lipase activity which is highly expressed in the liver and adipose tissue. The nonsynonymous rs738409 polymorphism (G allele; I148M substitution) seems to disrupt ubiquitylation and proteasomal degradation of PNPLA3, resulting in accumulation of PNPLA3 and impaired mobilization of triglycerides from hepatic lipid droplets [17]. 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 [18], promoting liver steatosis and subsequent fibrosis [19]. Additionally, *PNPLA3* rs738409 polymorphism could produce a shift to anaerobic metabolism and mitochondrial dysfunction in hepatocytes [20].

Several aspects must be considered for the correct interpretation of the results. Firstly, we performed a retrospective study in patients were selected among subjects who came to the Hospital and had a sufficient follow-up time. However, the design of repeated measures provides robustness to this study. Additionally, we did not have access to some key variables since were not evaluated in the clinical routine for all patients, such as obesity, metabolic syndrome, and hepatic steatosis. Secondly, our study has a limited sample size, which may impair the ability to detect stronger associations. Thirdly, about 23% of patients were non-responders to previous interferon therapy, but these patients were not excluded because interferon treatment failure does not appear to protect against the natural course of CHC during long-term follow-up [21].

In conclusion, *PNPLA3* rs738409 polymorphism appears to be related to an increased progression of liver fibrosis in HCV infected patients.

CONFLICT OF INTEREST DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Financial support:

This work has been supported by grants given by Instituto de Salud Carlos III (ISCIII) [grant numbers PI14CIII/00011 and PI15CIII/00024]. MAJS and AFR are also supported by ISCIII [grant numbers CD13/00013 and CP14/0010].

Author's contributions

Conceptualization: MAJS, AZG, and SR.

Resources and data curation: AZG, DPT, MAJS, AGS, MMV, JJSR, and TAV.

Investigation: MAJS, AZG, and DPT.

Formal analysis: MAJS and SR.

Writing – original draft preparation: MAJS, AZG, DPT, and SR.

Writing – Review & Editing: AFR and SVM.

Visualization, supervision and funding acquisition: SR.

Acknowledgements:

The authors thank the Spanish National Genotyping Center (CEGEN-PRB2-ISCIII) for providing SNP genotyping services (http://www.cegen.org). CEGEN is supported by grant PT13/0001, ISCIII-SGEFI / FEDER.

References

1. Westbrook RH, Dusheiko G. Natural history of hepatitis C. J Hepatol. 2014;61:S58-68.

2. Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. Lancet. 2014;383:1749-61.

3. 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. 2015;64:1605-15.

4. Heim MH, Bochud PY, George J. Host - hepatitis C viral interactions: The role of genetics. J Hepatol. 2016;65:S22-32.

5. Ali M, Yopp A, Gopal P, Beg MS, Zhu H, Lee W, et al. A Variant in PNPLA3 Associated With Fibrosis Progression but not Hepatocellular Carcinoma in Patients With Hepatitis C Virus Infection. Clin Gastroenterol Hepatol. 2016;14:295-300.

6. Fan JH, Xiang MQ, Li QL, Shi HT, Guo JJ. PNPLA3 rs738409 Polymorphism Associated with Hepatic Steatosis and Advanced Fibrosis in Patients with Chronic Hepatitis C Virus: A Meta-Analysis. Gut Liver. 2016;10:456-63.

7. 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;54:60-9.

8. 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;53:791-9.

9. Calvaruso V, Craxì A. 2011 European Association of the Study of the Liver hepatitis C virus clinical practice guidelines. Liver International. 2012;32:2-8.

10. EASL Clinical Practice Guidelines: management of hepatitis C virus infection. J Hepatol. 2014;60:392-420.

11. Gabriel S, Ziaugra L, Tabbaa D. SNP genotyping using the Sequenom MassARRAY iPLEX platform. Current protocols in human genetics. 2009;Chapter 2:Unit 2 12.

12. Sandrin L, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. Ultrasound Med Biol. 2003;29:1705-13.

13. Castera L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology. 2005;128:343-50.

14. Nunez-Torres R, Macias J, Mancebo M, Frias M, Dolci G, Tellez F, et al. The PNPLA3 Genetic Variant rs738409 Influences the Progression to Cirrhosis in HIV/Hepatitis C Virus Coinfected Patients. PLoS One. 2016;11:e0168265.

15. Jimenez-Sousa MA, Berenguer J, Garcia-Alvarez M, Gutierrez-Rivas M, Aldamiz-Echevarria T, Tejerina F, et al. Impact of patatin-like phospholipase domain-containing 3 gene polymorphism (rs738409) on severity of liver disease in HIV/hepatitis C virus-coinfected patients. AIDS. 2016;30:465-70.

16. 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;109:325-34.

17. BasuRay S, Smagris E, Cohen JC, Hobbs HH. The PNPLA3 variant associated with fatty liver disease (I148M) accumulates on lipid droplets by evading ubiquitylation. Hepatology. 2017;66:1111-24.

18. Marra F, Lotersztajn S. Pathophysiology of NASH: perspectives for a targeted treatment. Curr Pharm Des. 2013;19:5250-69.

19. 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;130:1636-42.

20. Min HK, Sookoian S, Pirola CJ, Cheng J, Mirshahi F, Sanyal AJ. Metabolic profiling reveals that PNPLA3 induces widespread effects on metabolism beyond triacylglycerol remodeling in Huh-7 hepatoma cells. Am J Physiol Gastrointest Liver Physiol. 2014;307:G66-76.

21. Carmona I, Cordero P, Ampuero J, Rojas A, Romero-Gomez M. Role of assessing liver fibrosis in management of chronic hepatitis C virus infection. Clin Microbiol Infect. 2016;22:839-45.