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D. CRISAN[1,2], M. GRIGORESCU[3,4], N. CRISAN[2,5], R. CRACIUN[4], M. LUPSOR[4,6], C. RADU[3,4], M.D. GRIGORESCU[4], A. SUCIU[4], F. EPURE[6], L. AVRAM[1,2], N. LEACH[7]

ASSOCIATION BETWEEN *PNPLA3*[G]/I148M VARIANT, STEATOSIS AND FIBROSIS STAGE IN HEPATITIS C VIRUS - GENETICS MATTERS

[1]Fifth Medical Clinic, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; [2]Municipal Clinical Hospital, Cluj-Napoca, Romania; [3]Third Medical Clinic, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; [4]"Prof. Dr. Octavian Fodor" Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania; [5]Department of Surgical Specialties, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; [6]Department of Medical Imaging, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; [7]Fourth Medical Clinic, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania

There is an established correlation between the *PNPLA3* rs738409 C > G single nucleotide polymorphism (SNP) and hepatic steatosis and fibrosis in hepatitis C virus (HCV) infected patients. However not all data is convergent regarding the exact impact of this SNP on the pattern of disease progression in different clinical settings. In this study, we aimed to further bridge the knowledge gap on this topic by investigating the role of the G allele in promoting steatosis, fibrosis and disease progression in relation to other metabolic and anthropometric host factors. Two hundred and fifty consecutive patients, previously diagnosed with chronic hepatitis C (CHC) underwent liver biopsy. Histology was assessed using the Metavir scoring system. Transient elastography was used for follow-up. Ninety-eight patients were genotyped for *PNPLA3* rs738409 and followed up for fibrosis progression. *PNPLA3* rs738409[G] allele was significantly correlated with severe steatosis (P = 0.04), severe fibrosis at the time of enrollment (P = 0.0005) and fibrosis progression with an OR of 10.31 (95% CI 1.06 – 99.59, P = 0.04), after a mean follow-up time of 62.85 (95%CI: 52.21 – 76.15) months. Severe steatosis at the time of enrollment had an OR of 11.02 (95% CI 1.48 – 82.09, P = 0.01) for the association with fibrosis progression. The HOMA-IR index was also positively correlated with severe fibrosis (P = 0.03) and fibrosis progression on univariate analysis (P = 0.02). *PNPLA3* rs738409[G] allele is a reliable predictor for steatosis and fibrosis in CHC. The presence of G allele, along with severe steatosis and insulin resistance are significant predictors for fibrosis progression.

Key words: *chronic hepatitis C, hepatitis C virus, PNPLA3, fibrosis, steatosis, insulin resistance, single nucleotide polymorphism*

INTRODUCTION

Chronic hepatitis C (CHC) infection continues to be a major healthcare issue, in spite of the recent introduction of highly efficient direct acting antivirals (DAAs) in the treatment arsenal. This is largely due to the high cost and low accessibility of the DAAs in developing countries, with some insurance policies covering treatment only for advanced disease. Therefore, by studying the promoters of disease progression in CHC, clinicians can better predict the window of opportunity for a cost-efficient treatment strategy.

The usual sequence of disease progression in CHC infection is chronic hepatitis, with gradually increasing liver inflammation leading to progressive fibrosis, cirrhosis and hepatocellular carcinoma (1, 2). Studies have shown that hepatic steatosis is more common in CHC patients compared to general population (3). Steatosis is linked to a quicker progression of fibrosis (4-6). The factors which contribute to steatosis in CHC can be virus-related (some genotypes being more steatogenic than others) or host related (insulin resistance/diabetes, metabolic imbalances, alcohol consumption, genetic susceptibility) (4-6). In our research we focus mainly on genetic susceptibility.

There are several genetic polymorphisms linked to different patterns of disease progression, which have been discovered *via* genome wide association studies. Most data comes from studying a single nucleotide polymorphism (SNP) in rs738409 in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene (also known as adiponutrin). This SNP leads to isoleucine being substituted by methionin at position 148 (I148M). Previous comparisons between G and C alleles of the *PNPLA3* gene have shown different steatosis and fibrosis progression trends in CHC. The data is heterogeneous, suggesting there are differences between populations in allele frequency and steatogenic impact of *PNPLA3* variants (7-15). Furthermore, in the era of precision medicine, targeting *PNPLA3* variants might have therapeutic valences in addition to its prognostic value. Recently published data have shown that silencing PNPLA3 using antisense oligonucleotides might

improve features of non-alcoholic fatty liver disease (NAFLD), including fibrosis progression, in mice models (16).

The main objective of our current study was to investigate the role of *PNPLA3* gene polymorphisms (G/C alleles) in promoting steatosis, fibrosis and disease progression, assessed both *via* liver biopsy and noninvasively (using transient elastography - Fibroscan) at the time of enrollment and followed-up using transient elastography. A secondary aim was to assess other host factors (mostly related to the metabolic profile) in relation to *PNPLA3* variants, steatosis and fibrosis.

MATERIALS AND METHODS

Patients

The study was approved by the local Ethical Committee of the Regional Institute of Gastroenterology and Hepatology 'Prof. Dr. Octavian Fodor' in Cluj-Napoca. Written informed consents were obtained from all patients.

This was a prospective study, including 250 consecutive patients, previously diagnosed with chronic hepatitis C. All patients underwent liver biopsy at 3rd Medical Clinic, Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca, Romania, starting in 2008. Ninety eight patients were genotyped for *PNPLA3* rs738409 and followed up for fibrosis progression. The current study is an extension of previously published research on the same cohort (17, 18).

We set two criteria for defining CHC: the presence of anti-HCV for at least six months and detectable HCV-RNA. Patients with other etiologies of chronic liver disease were excluded: hepatitis B, autoimmune liver disease, Wilson's disease, haemochromatosis, α1-antitripsin deficiency, and those with a history of hepatotoxic or steatosis-inducing drug use or alcohol consumption (more than 20 g/day for women and 30 g/day for men). No genetically related patients were included. All patients were naive for antiviral treatment at study enrollment.

Anthropometric data

We evaluated height, weight and waist circumference (WC). BMI was calculated based on height and weight (kg/m^2). The assessment of the metabolic syndrome (MS) was performed according to the Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention 2009 (19). It implies the presence of any three of the following five features: elevated WC $\geq$ 94 cm for men and $\geq$ 80 cm for women and elevated triglyceride (TG) levels $\geq$ 150 mg/dl; reduced high-density lipoprotein (HDL)-cholesterol < 40 mg/dl in males and < 50 mg/dl in females; raised blood pressure: systolic $\geq$ 130 or diastolic $\geq$ 85 mmHg; raised fasting plasma glucose $\geq$ 100 mg/dl or previously diagnosed type 2 diabetes.

Laboratory investigations

All patients underwent haematological, biochemical and viral load assessments on the day of enrollment. Abdominal ultrasonography was performed on all patients at the time of enrollment in order to exclude biliary obstruction and the presence of focal liver lesions. A blood sample (3 ml) was obtained after eight hours of overnight fasting for routine investigations (aspartate transaminase (AST), alanine transaminase (ALT), platelet count, gamma-glutamyl transpeptidase (GGT), bilirubin, urea, prothrombin time, cholesterol, fasting plasma glucose, HDL-cholesterol and triglycerides (TGs)).

All assessments were made on an automatic analyzer (Konelab 30 I - Thermo Electron Corp, Finland). Fasting insulin was measured by ELISA (Mercodia AB, Sweden). The degree of insulin resistance (IR) was calculated according to the homeostasis model assessment for IR (HOMA-IR) using the following formula: fasting insulin level (mUI/l) × fasting glucose level (mg/dl)/40516 (20).

Serum HCV-RNA was measured by PCR (Cobas Amplicor HCV 2.0 version, Roche, Germany). Serum samples from each patient were stored at –70°C for further biochemical analysis. Since more than 99% of HCV carriers in our country have genotype 1, out of which more than 93% carry subtype 1b (21), no real time-PCR viral genotype analysis was performed.

All samples were stored at –70°C until assayed.

Genetic analysis: DNA was extracted from whole blood samples, which were collected into EDTA tubes and preserved at la –70°C, using the Roche High Pure PCR Template Preparation Kit. The primers were provided by TIB Molbiol.

The DNA probes had the following sequence: GGAGGGATAAGGCCACTGTAGAAGGG [C/G] ATGAAGCAGGAACATACCAAGGCCT (antisense strand). Reagents from the LightCycler FastStart DNA Master HybProbe, Roche kit were used to prepare the master mix for real time PCR. The analysis of the *PNPLA3* (adiponutrin) gene polymorphism, located on chromosome 22, was performed. The PCR reaction took place under the following protocol: 95*C for 10 minutes, followed by 45 reaction cycles at 95*C - for 10 seconds, then 10 seconds at 60*C and 15 seconds at 72g*C. Subsequently, the DNA heterduplex dissociation curve (allelic discrimination) analysis was performed. The dominant homozygous genotype is CC, the heterozygous genotype is CG, while the mutant/recessive homozygous genotype is GG. The *PNPLA3* rs738409 G > C polymorphism was detected using the Roche LightCycler Nano device, utilising the LightSNiP technique. The differences between the dissociation temperatures of the three DNA duplexes were thus analyzed. After the genetic analysis, the genotype distribution was tested against the Hardy-Weinberg law and the results were not within the Hardy-Weinberg equilibrium, potentially because of the small sample size. We will address this issue at discussions.

Pathological study

Ultrasound guided liver biopsies were stained with haematoxylin-eosin and Masson's trichrome and were blindly assessed according to the METAVIR scoring system (22, 23). Fibrosis was staged on a scale from F0 to F4, as follows: F0: no fibrosis; F1: portal fibrosis, without septa; F2: few septa; F3: many septa without cirrhosis and F4: cirrhosis. The patients were divided depending on the presence or absence of advanced fibrosis in two groups: F0 – F2 stages were considered as non-severe fibrosis and F3 – F4 as severe fibrosis. Steatosis was staged on a scale from S0 to S3, as the classification of Kleiner recommends (S0 (< 5%), S1 (5 – 33%), S2 (33 – 66%), S3 (> 66%) (24). Liver biopsies were obtained from all patients within 12 months before inclusion in the study. Only biopsies with a length exceeding 1.2 cm and containing more than 6 portal tracts were considered to be eligible for the study.

Antiviral treatment

Of the patients included, 132 patients received standard antiviral treatment (standard of care, SOC at that time): Peg-interferon (Peg-IFN) alpha 2a or 2b and ribavirin, 48 wk. There were 98 patients who did not receive treatment or who were non-responders to antiviral treatment who were then followed up for the evolution of fibrosis and factors associated with it, until the end of the observation or until the initiation of a new generation of antivirals (DAA). Fibrosis was assessed during the follow up time

using transient elastography (TE) from FibroScan, Echosens, Paris, France. We followed the technical background and examination procedure previously described. TE was considered valid if 10 measurements were obtained with a success rate of > 60% and an interquartile range < 30% of the median (25-27). The success rate was calculated as the number of validated liver stiffness measurements (LSMs) divided by the number of total measurements. All LSMs were performed by two experienced operators. Significant fibrosis progression at follow-up was defined as an increase in liver stiffness that determines a reclassification from low grade fibrosis (F0 – F2) to advanced fibrosis (F3 – F4).

Statistical analysis

Comparison between groups was performed using Student's t-test for continuous variables with normal distribution and Chi-square test for categorical variables. The continuous variables with non-normal distribution were expressed as median and 95% confidence interval (CI) and the differences were analyzed with Mann-Whitney test. The variables that were found to be significantly associated in univariate analysis were included in a multivariate analysis, using logistic regression. MedCalc® 13.3.9.0 software and SPSS software version 15.0 (SPSS Inc. Chicago, IL, USA) were used for the statistical analysis.

RESULTS

The baseline characteristics of our cohort, including clinical, biochemical, histological and genetic data, are summarized in *Table 1*. We present here the general data resulted from the whole study group analysis, as well as the smaller group included later in the follow up. It is worth mentioning that there were no significant differences between the two groups.

The differences between groups according to adiponutrin genotype are summarized in *Table 2*. *PNPLA3* rs738409[G] allele was significantly correlated with severe steatosis (P = 0.04) and severe fibrosis assessed via liver biopsy at the time of enrollment (P = 0.0005). AST level was significantly influenced by the adiponutrin polymorphism (P = 0.04). No significant differences were found regarding other biochemical liver tests such as HOMA index (P = 0.25) or GGT (P = 0.23). Furthermore, we found no significant correlation between allele G and metabolic variables such as waist circumference, total cholesterol levels, HDL, triglycerides. We found a borderline significant association of BMI and diastolic blood pressure with the genetic allele G of *PNPLA3* (P = 0.06).

The comparison between groups according to the severity of steatosis is summarized in *Table 3*. The presence of the G allele was a significant predictor for severe steatosis (S2-S3), P = 0.04. Patients in the severe steatosis group had significantly higher GGT levels (P = 0.02), blood sugar levels (P = 0.03), weight, waist circumference and BMI (P < 0.001). There were no statistically significant differences in viral load, ALT, AST, cholesterol levels. On multivariate analysis, the presence of the G allele and BMI retained statistical significance, the former being the strongest predictor for severe steatosis with an OR of 3.21 (1.01 – 11.28 95%CI, P = 0.05).

Patients were also compared according to the severity of fibrosis (*Table 4*). Severe fibrosis was significantly correlated with

Table 1. Baseline characteristics of patients at the time of enrollment.

Variable	Median (95% CI)/Mean ± SD* Analysis on the group of 98 patients included in follow up	Median(95% CI)/Mean ± SD* Analysis of the whole group at the time of enrollment
Age	50 (47.00 – 53.07)	51 (49.00 – 52.00)
Gender (M/F)	32/66	95/155
HCV-RNA (IU×10^{-3}/mm^3)	893 (938.28 – 1100)	1190 (794.20 – 1339.48)
ALT (U/L)	76.5 (70 – 86)	73.74 ± 49.87*
AST (U/L)	50 (41 – 56)	36 (30.90 – 43.28)
GGT (U/L)	57(49 – 63.35)	55 (44.77 – 67.46)
Cholesterol (mg/dl)	193 (182 – 201.03)	195.50 (177.29 – 221.57)
HDL-cholesterol (mg/dl)	54.05 ± 16.97*	55.84 ± 15.74*
TGL (mg/dl)	119 (107.50 –124.49)	114 (97.54 – 157.86)
Waist circumference (cm)	90 (88.44 – 92)	90 (85 – 91.68)
BMI (kg/m^2)	26.3 (25.35 – 27.06)	26 (24.88 – 26.69)
HOMA-IR score	2.72 (1.99 – 3.55)	2.63 (1.85 – 3.83)
Blood sugar level (mg/dl)	99 (96.55 – 100)	95.84 ± 18.43*
Insulin* (μU/ml)	13.13 ± 9.91*	15.74 ± 12.44*
Weight (kg)	75.01 (71.44 – 77.55)	72 (68 – 75.68)
Platelets count (×10^{-3}/mm^3)	231.50 (200 – 250)	240 (200 – 250)
Fibroscan (kPa)	6.7 (6.1 – 7.1)	6.2 (5.60 – 7.10)
SBP (mmHg)	120 (120 – 120)	115.65 ± 15.32*
DBP (mmHg)	80 (70 – 80)	72.39 ± 8.77*
PNPLA3variant **CC/CG/GG**	44/35/19	–

Continuous variables are shown as mean ± standard deviation (SD)* (normally distributed) or median (95% confidence interval (CI)), for skewed variables.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; GGT, gamma-glutamyl-transpeptidase; HCV-RNA, hepatitis C virus RNA; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; SBP, systolic blood pressure; TGL, triglycerides.

Table 2. Univariate analysis of the biological and morphological features associated with PNPLA3 rs738409[G] allele.

Variable	CC (N = 44)	CG, GG (N = 54)	P
Age	49.00 (43.48 – 53.51)	53.00 (50.31 – 55.68)	0.14
Gender (male)	18 (40.90%)	14 (25.92%)	0.31
Steatosis (S2-3)	15 (34.09%)	30 (55.55%)	**0.04**
Fibrosis (F3-4)	6 (13.63%)	28 (51.85%)	**0.0005**
Fibroscan T0 (kPa)	6.7 (5.50 – 7.6)	6.8 (5.77 – 9.06)	0.39
HCV-RNA ($IU \times 10^{-3}/mm^3$)	1000.00 (522.60 – 1306.50)	1000.00 (638.06 – 1390.72)	0.49
ALT (U/L)	54 (36.56 – 79.52)	74 (51.45 – 106.85)	0.11
AST (U/L)	34 (25.78 – 40.65)	51 (37.24 – 60.51)	**0.04**
GGT (U/L)	51 (39.13 – 66.93)	58 (46.76 – 74.23)	0.23
Cholesterol (mg/dl)*	284.52 ± 418.32	199.61 ± 44.41	0.24
HDL-cholesterol (mg/dl)*	56 (44.78 – 70.21)	54 (45 – 58.17)	0.47
TGL (mg/dl)*	126.26 ± 59.24	141.29 ± 71.98	0.44
Waist circumference (cm)	88.5 (83.33 – 90.66)	90.5 (86 – 93.66)	0.24
BMI (kg/m^2)	25.77 (23.35 – 26.50)	27 (25.42 – 27.8)	**0.06**
HOMA-IR score	2.63 (1.75 – 5.26)	3.03 (1.88 – 5.56)	0.25
Blood sugar level (mg/dl)	97 (89.81 – 99)	94 (89.12 – 99.87)	0.91
Insulin (μU/ml)	14.43 (6.00 – 28.93)	11.23 (4.85 – 13.90)	0.59
Weight (kg)	73 (65.33 – 78.66)	70 (67.33- 76.33)	0.85
Platelets count ($\times 10^{-3}/mm^3$)	235 (190.00-261.39)	240.00 (189.75 – 256.00)	0.94
SBP* (mmHg)	117.77 ± 15.92	121.36 ± 15.11	0.43
DBP* (mmHg)	71.66 ± 9.85	76.66 ± 8.35	**0.06**
Previously treated with Peg Interferon and NR	37 (84%)	27 (50%)	**0.66**

Continuous variables are shown as mean ± standard deviation (SD)* (normally distributed) or median (95% confidence interval (CI)), for skewed variables.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase, BMI, body mass index; DBP, diastolic blood pressure; Fibroscan T0, Fibroscan at the time of enrollment; GGT, gamma-glutamyl-transpeptidase; HCV-RNA, hepatitis C virus RNA; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; NR, non-responder; SBP, systolic blood pressure; TGL, triglycerides.

the presence of *PNPLA3* [G] allele (P = 0.005), higher HOMA-IR scores (P = 0.03), lower HDL- cholesterol levels (P = 0.04) and lower platelets count (P = 0.018). As expected, the non-invasive assessment of liver fibrosis using transient elastography closely matched the data obtained from the liver biopsies (P < 0.001). We found no significant differences in ALT, AST, GGT or viral load between the groups, although the latter fell just short of statistical significance (P = 0.07). On multivariate analysis, only the presence of *PNPLA3* [G] allele came close to reaching statistical significance, with an OR of 3.15 (P = 0.09).

After a follow up time of 62.85 (52.21 – 76.15) months, fibrosis was reevaluated using transient elastography, setting up a 9.5 kPa threshold for severe fibrosis. On univariate analysis, steatosis (P = 0.04), the HOMA-IR score (P = 0.02), age (P = 0.01) and GGT (P = 0.0001) were significant predictors for severe fibrosis, while PNPLA3 rs738409[C] allele was a protective factor against advanced fibrosis at the EOFU (P = 0.03) (*Tables 5* and *6*). In multivariate analysis, the presence of the G allele was associated with advanced fibrosis at the EOFU with an OR of 10.31 (95% CI 1.06 – 99.59), while severe steatosis at the time of enrollment had an OR of 11.02 (95% CI 1.48 – 82.09). HOMA-IR was close to reaching the statistical significance for influencing advanced fibrosis (P = 0.06).

Furthermore, we have singled out patients exhibiting a progressive pattern of fibrosis by advancing from mild fibrosis (F0 – F2) to severe fibrosis (F3 – F4). Thus, we divided the patients in follow-up into two groups: those with stationary (or regressive) fibrosis staging and those with fibrosis progression. On univariate analysis, the presence of G allele (P = 0.01) and advanced steatosis (S2 – S3) at enrolment (P = 0.05) were the only significant predictors for progression (*Table 7*).

DISCUSSION

In the current study we assessed the impact of the PNPLA3 rs738409 gene polymorphism on liver steatosis, fibrosis and fibrosis progression on a study group diagnosed with CHC and followed up for a median time of 5 years (maximum time of follow up being 9 years). Our data suggest that allele G of the PNPLA3 gene (either homozygous GG or heterozygous CG) is strongly correlated with steatosis and fibrosis, while allele C appears to be inversely correlated with these variables.

This specific polymorphism has been first studied in correlation with non-alcoholic fat liver disease (NAFLD), both in experimental *in vitro* studies and in large scale clinical situations (28). One of the first major studies to prove a significant link between adiponutrin allele G and NAFLD progression was published more than 10 years ago by Romeo *et al*. (11), showing a more than twofold increase in hepatic fat content in homozygous PNPLA3 rs738409 [G] when compared to non-carriers. Further studies, including a large scale meta-analysis by Sookoian S *et al*. (12), reinforced the role of this polymorphism in NAFLD progression. Consequently, the

Table 3. Comparison between study subgroups regarding the presence of severe steatosis (univariate analysis).

Variable	**S0-S1** N = 53	**S2-S3** N = 45	**P value**
Age	47.00 (41.25 – 52.74)	52.00 (50.00 – 55.01)	0.006
Gender (male)	21 (39.62%)	21 (46.66%)	0.72
PNPLA3 (GG,GC)	24 (45.28%)	30 (66.66%)	**0.04**
Fibrosis (F3-4)	15 (28.30%)	19 (42.22%)	0.8
Fibroscan T0 (kPa)	6.5 (5.70 – 7.09)	7.2 (5.81 – 8.5)	0.48
HCV-RNA ($IU \times 10^{-3}/mm^3$)	913.00 (535.14 – 1410.31)	904.50 (615.87 – 1163.96)	0.48
ALT (U/L)	75.5 (70.72 – 98.51)	71 (57.25 – 84.00)	0.57
AST (U/L)	49.5 (38.74 – 63.02)	45 (40.00 – 55.74)	0.56
GGT (U/L)	48.5 (37.89 – 63.55)	60.5 (51 – 75.12)	**0.02**
Cholesterol (mg/dl)	194.5 (172.97 – 215.51)	189 (177.50 – 205.24)	0.93
HDL-cholesterol (mg/dl)*	50.71 ± 17.30	52.76 ± 14.99	0.51
TGL (mg/dl)	112 (108.57 – 150)	119 (103.75 – 129.49)	0.62
Waist circumference (cm)*	84.37 ± 13.74	97.00 ± 15.06	**<0.0001**
BMI (kg/m^2)	24.22 (23.36 – 24.89)	29.30 (27.77 – 30.47)	**<0.0001**
HOMA-IR score	2.07 (1.53 – 3.83)	2.85 (2.10 – 4.08)	0.21
Blood sugar level (mg/dl)	96.5 (90 – 99.12)	101 (98.09 – 103)	**0.03**
Insulin (μU/ml)	9.57 (6.15 – 17.63)	12.33 (3.44 – 21.14)	0.63
Weight (kg)*	66.89 ± 15.47	82.11 ± 15.85	**<0.0001**
Platelets count ($\times 10^{-3}/mm^3$)*	241.25 ± 82.15	215.78 ± 798.06	0.22
SBP (mmHg)	120 (110 – 120)	122.5 (120 – 130)	**0.007**
DBP (mmHg)	70 (70 – 80)	80 (70 – 80)	0.12

Continuous variables are shown as mean ± standard deviation (SD)* (normally distributed) or median (95% confidence interval (CI)), for skewed variables.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase, BMI, body mass index; DBP, diastolic blood pressure; Fibroscan T0, Fibroscan at the time of enrollment; GGT, gamma-glutamyl-transpeptidase; HCV-RNA, hepatitis C virus RNA; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; SBP, systolic blood pressure; TGL, triglycerides.

Table 4. Univariate analysis of features associated with severe fibrosis.

Variable	**F0-F2** N = 58	**F3-F4** N = 4	**P**
Age	49.00 (46.00 – 51.00)	54.00 (51.00 – 56.00)	**0.007**
Gender (M, no, %)	19 (32.75%)	11 (27.5%)	0.58
PNPLA3 [G], no, (%)	26 (44.82%)	28 (70%)	**0.005**
Steatosis (S2-3), no, %	34 (58.62%)	19 (47.5%)	0.80
Fibroscan T0	5.9 (5.55 – 6.64)	10.9 (8.11 – 13.81)	< **0.0001**
HCV-RNA ($IU \times 10^{-3}/mm^3$)	690.50 (513.74 – 936.44)	1135.00 (813.86 – 1312.60)	0.0716
ALT (U/L)	78 (70 – 95.74)	73 (57.48 – 98.02)	0.85
AST (U/L)	54 (44.62 – 59)	43.5 (37.74 – 56.51)	0.26
GGT (U/L)	51 (47.71 – 64.28)	63 (56.06 – 75)	0.10
Cholesterol* (mg/l)	208.93 ± 185.92	197.22 ± 42.00	0.66
HDL-cholesterol (mg/dl)*	53.64 ± 18.15	53.55 ± 14.48	**0.04**
TGL (mg/l)	113 (102 – 125.55)	121.5 (103.74 – 133.32)	0.70
Waist circumference (cm)	90 (88 – 92)	90 (87.06 – 96.93)	0.57
BMI (kg/m^2)	26.3 (24.98 – 27.00)	26.57 (24.90 – 29.34)	0.49
HOMA-IR score	2 (1.38 – 3.1)	3.38 (2.65 – 5.59)	**0.03**
Blood sugar levels (mg/dl)	99 (96 – 101)	101 (97 – 106)	0.24
Insulin (μU/ml)	9.21 (3.84 – 13.31)	12.23 (8.22 – 19.64)	0.16
Weight (kg)	74 (70 – 78)	75 (71.06 – 80)	0.51
Platelets count* ($\times 10^{-3}/mm^3$)	299.01 ± 273.29	172.55 ± 57.80	**0.01**
SBP (mmHg)	120 (120 – 125)	120 (120 – 120)	0.74
DBP (mmHg)	80 (70 – 80)	75 (70 – 80)	0.95

Continuous variables are shown as mean ± standard deviation (SD)* (normally distributed) or median (95% confidence interval (CI)), for skewed variables.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; Fibroscan T0, Fibroscan at the time of enrollment; GGT, gamma-glutamyl-transpeptidase; HCV-RNA, hepatitis C virus RNA; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; SBP, systolic blood pressure; TGL, triglycerides.

Table 5. Factors associated with advanced fibrosis at the end of follow-up (assessed *via* Fibroscan).

Variable	F0-F2 EOFU N = 29	F3-F4 EOFU N = 67	P
Age	49.50 (45.62 – 52.00)	52.00 (50.76 – 55.00)	**0.01**
Gender (M, no, %)	10 (34.48%)	22 (32.83%)	0.87
***PNPLA3*[C], (no, %)**	25 (86.2%)	36 (53.73%)	**0.03**
Steatosis S2-3 (no, %)	19 (65.51%)	34 (50.74%)	**0.04**
HCV-RNA (IU×10^{-3}/mm^3)	750.5 (530.45 – 1058.66)	1000.00 (612.63 – 1249.50)	0.66
ALT (U/L)*	85.79 ± 49.82	88.41 ± 48.74	0.77
AST (U/L)	45 (39 – 54.25)	51 (39.88 – 61.11)	0.79
GGT (U/L)	50 (41.42 – 58)	76 (58.59 – 87.80)	**0.0001**
Cholesterol (mg/dl)*	213.73 ± 209.39	200 ± 46.34	0.66
HDL-cholesterol (mg/dl)*	53.97 ± 18.81	56.02 ± 15.76	0.54
TGL (mg/dl) *	126.97 ± 62.01	125.88 ± 50.89	0.92
Waist circumference (cm)	90 (87 – 92)	90 (86 – 94)	0.84
BMI (kg/m^2)	26.3 (25.14 – 27.01)	25.23 (24.14 – 27.63)	0.32
HOMA-IR score	1.89 (1.47 – 2.60)	3.69 (3.02 – 5.66)	**0.0027**
Blood sugar level (mg/dl)	98.5 (94 – 100)	98.5 (96-105.31)	0.34
Insulin (μU/ml)	8.51 (4.57 – 13.94)	12.9 (11.18-18.34)	0.11
Platelets count (×10^{-3}/mm^3)*	282.44 ± 269.92	200.36 ± 85.07	0.11
SBP (mmHg)*	123.48 ± 19.07	119.20 ± 13.59	0.19
DBP (mmHg)	80 (76.57 – 80)	72.5 (70 – 80)	0.14

Continuous variables are shown as mean ± standard deviation (SD)* (normally distributed) or median (95% confidence interval (CI), for skewed variables.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase, BMI, body mass index; DBP, diastolic blood pressure; EOFU, end of follow-up; GGT, gamma-glutamyl-transpeptidase; HCV-RNA, hepatitis C virus RNA; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; SBP, systolic blood pressure; TGL, triglycerides.

Table 6. Multivariate analysis of factors independently associated in with advanced fibrosis at the end of follow-up (assessed *via* Fibroscan).

Variable	Odds ratio	95% CI	P
Age (years)	1.19	0.98 – 1.44	0.06
HOMA-IR index	1.68	0.97 – 2.91	0.06
S2-S3 versus **S0-S1**	11.02	1.48 – 82.09	**0.0191**
***PNPLA3* G allele**	10.31	1.06 – 99.59	**0.0437**
GGT	1.00	0.99 – 1.02	0.27

Abbreviations: CI, confidence interval; GGT, gamma-glutamyl-transpeptidase; HOMA-IR, homeostasis model assessment for insulin resistance;

PNLPA3 gene has been studied in relation to other chronic liver diseases such as alcoholic liver disease (29), hepatocellular carcinoma (15) and CHC, with similar steatogenetic and fibrogenetic patterns being found.

Our findings are consistent with previously published studies regarding the impact of PNPLA3 rs738409 [G] allele on HCV infected patients. In our study we found that carrying the G allele is an independent predictor for severe (S2-S3) steatosis. The PNPLA3 [G] allele was significantly associated with advanced (F3 – F4) fibrosis on univariate analysis, only to fall slightly short of statistical significance on multivariate analysis. Petta *et al*. (30) found a significant correlation between steatosis severity and the G variant, while also accounting for steato-hepatitis. Two studies on large Asian cohorts (7, 13) also showed association between the G allele and hepatic steatosis. A meta-analysis comprising five studies and 2037 patients concluded that the G allele was a predictor for severe steatosis and fibrosis, especially in Caucasian populations. The fact that PNPLA3[G] is promoting fibrosis was also shown in a study on Italian patients, where not only fibrosis was favored by adiponutrin polymorphism, but also steatosis. From a geographical and population standpoint, our dataset has similarities with the cohort studied by the Lithuanian team led by Kupcinskas J *et al*. (14), confirming the impact of PNPLA 3 G allele on patients with CHC in Eastern European countries. Other polymorphisms such as RNF7 rs16851720, MERTK and PCSK7 SNPs were also assessed in relation with liver fibrosis in the previously mentioned paper, however only the former was positively associated with fibrosis (14). While each of the aforementioned studies had slightly different methods and endpoints, the general trend was towards a positive correlation between PNPLA3 rs738409 [G] allele, liver steatosis and fibrosis. Our data were in line with this trend.

On the other hand, in our study, the PNPLA 3 [G] was a significant predictor for fibrosis progression, along with severe steatosis and the HOMA-IR index (the latter having only borderline statistical significance). Gene polymorphism and age at the time of HCV infection diagnosis significantly determined fibrosis progression in other studies (31). As an extension of HALT - C study, some important data were published showing

Table 7. Univariate analysis of factors associated with fibrosis progression.

Variables	**Stationary fibrosis** (N = 56)	**Progressive fibrosis** (N = 42)	**P**
Age (years)	50 (44.74 – 54.00)	50 (47.00 – 56.00)	0.40
BMI (kg/m^2)*	25.61 ± 3.43	25.16 ± 7.73	0.78
AST (U/L)*	53.21 ± 30.88	42.09 ± 14.52	0.46
HDL-cholesterol (mg/dl)*	60.00 ± 16.80	52.63 ± 15.14	0.27
Adiponutrin G	16 (28.57%)	29 (69.04%)	**0.01**
Platelets (x10^{-3}/mm^3)*	240.94 ± 84.59	294.00 ± 373.50	0.36
HOMA-IR	2.37 (1.78 – 3.84)	3.55 (1.42 – 5.55)	0.38
HCV-RNA (IUx10^{-3}/mm^3)	870.00 (524.78 – 1282.10)	1190.00(571.19 – 1870.99)	0.47
S2-S3	18 (32.14%)	27 (64.28%)	**0.05**

Continuous variables are shown as mean ± standard deviation (SD)* (normally distributed) or median (95% confidence interval (CI)), for skewed variables.
Abbreviations: AST, aspartate aminotransferase, BMI, body mass index; HCV, hepatitis C virus; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance.

that, in multivariate analysis, the progression of fibrosis was associated with allele G and obesity (32). In our study, in spite of insulin resistance and steatosis being strong predictors for fibrosis progression, we found no significant impact of BMI on this variable. Previously published research (33), including a large scale meta-analysis (5), have also demonstrated a strong correlation between steatosis and increased fibrosis.

Similar results regarding fibrosis progression and cirrhosis occurrence were reported also on HCV/HIV co-infected patients (34), however we did not account for HIV co-infection in our research since it is not included in standard diagnosis protocol in our country.

Coming as secondary results of our study, we identified several other factors promoting steatosis and fibrosis. We found that waist circumference, BMI, blood sugar levels, GGT and systolic blood pressure, shortly, the main components of metabolic syndrome, were positively correlated with steatosis, while low HDL cholesterol levels, the HOMA score and low platelets count were predictors of fibrosis. Our findings are in accordance to literature data. There are many studies that showed, among other findings, a strong correlation between insulin resistance (assessed using the HOMA-IR index, insulin levels) and fibrosis (35, 36). As expected, BMI was also correlated with steatosis in HCV patients (7).

In our current research, we did not aim to study the pathophysiology of liver steatosis on a molecular basis. However, in this particular clinical setting, the results of the study are reflecting previous basic scientific claims regarding PNPLA 3 gene activity. Increased liver triglyceride load by reducing hydrolysis (37), and decreased hepatic VLDL cholesterol secretion (38) might translate to overall increased steatosis. The impact of mutant PNPLA3 allele on fibrogenesis appears to be tied to retinoid metabolism within the hepatic stellate cell (HSC). As first shown by Pirazzi *et al*. (39), wild-type PNPLA3 plays a key role in hydrolyzing retinyl palmitate (stored in the HSC) to retinol and palmitic acid, the former being released in response to insulin. The PNPLA3 [G] allele leads to the loss of this function, therefore increasing retinyl palmitate storage and retinyl palmitate to retinol ratio within the HSCs (39, 40). As a consequence, the mutant allele appears to generate an imbalance between the secretion of matrix metallopeptidase 2 (MMP2) and tissue inhibitor of metalloproteinase 1 and 2 (TIMP1 and TIMP2) in favor of fibrogenesis, since the equilibrium is maintained *via* retinoid metabolism (41). There are also several papers discussing the relationship between HCV infection, alterations of the metabolic profile and increased insulin resistance (42-46). In an article published on this topic (42), the authors have shown that women older than 49 and males irrespective of age exhibited HCV-associated hypolipidemia (expressed as low triglyceride, total cholesterol and HDL cholesterol levels). While an association with low HDL levels is arguably expected, an overall hypolipidemic profile is counter-intuitive and might suggest a more intricate, non-linear relationship between HCV, lipid metabolism, obesity and the metabolic syndrome. This hypothesis was further reinforced by multiple studies (43, 46), which have found that the MS was highly prevalent in non-obese, non-diabetic patients. We have also published our data on this topic, in response to a study by Gonzales *et al*. (46), reaching a similar conclusion (44).

In the era of precision medicine, future directions of study should aim to better understand the subtle impact of different genes and molecules on liver disease progression. Recent studies have hinted towards a link between different molecules and liver disease progression in various clinical settings. A paper published in 2019 by Waluga *et al*. (47) studied the role of two adipokines, omentin and vaspin and a myokin - irisin in the development of liver pathology. Significant correlations were found between these molecules and patterns of disease progression or severity in patients with alcoholic cirrhosis, NAFLD and primary biliary colangitis. Studying these cytokines in the setting of viral hepatitis could further enhance our knowledge in the field. Furthermore, clinical practice might be improved by finding sensitive biomarkers for complications of liver disease, such as the established link between endocan and infection-induced cirrhosis decompensation (48). Not least, since PNPLA3 and steatosis are tightly linked in patients with CHC, further correlations with other established molecules in NAFLD could emerge. Both fibroblast growth factor 21 (FGF21) and omentin-1 mRNA appear to have an increased expression in obese patients with NAFLD, but correlations regarding their expression in patients with lean steatosis or PNPLA3 induced steatosis have yet to be proven (49), and could be particularly important when assessing patients with CHC and metabolic syndrome.

However, our study had several limitations. A significant caveat in our study was the deviation of the genotype frequencies from the Hardy-Weinberg Equilibrium, potentially pointing towards a selection bias in our sample. Since our sample size is relatively small, and the genetic analysis was performed by an experienced geneticist, the probability of genotyping errors to affect our results is relatively low. Therefore, this issue might have been caused by the small sample size, along with our

research being performed in a tertiary care hospital, skewing our sample towards a certain category of patients (advanced disease, co-morbidities, long-standing disease). Another limitation of our research was the predominance of genotype 1b in our cohort, closely tied with the geographical distribution of viral strains. A meta-analysis including studies with similar designs, performed in different areas should partially correct for the shortcomings of single-centered studies in topics where populations and geography are relevant. Finally, a higher number of patients followed up during this clinical observation would have been probably giving more relevant information on the problem of fibrosis evolution in HCV patients. Nevertheless, in spite of our limitations, our results confirm previous findings on this topic, further strengthening the case for genetic input on advanced liver disease.

While some studies on PNPLA3 and CHC focused on correlations with SVR during the interferon-based regimen era and in the early days of DAAs (8, 10), we have chosen to approach disease progression regardless of pre-DAA treatment outcomes. Further data are needed in order to perform an accurate pre and post-DAA treatment assessment for the CHC patients. It might prove useful to take into account other factors, such as genetic testing or features of the metabolic syndrome, besides fibrosis, when deciding who should start treatment.

In summary, PNPLA3 rs738409[G] allele is a reliable predictor for steatosis and fibrosis in patients with CHC, and therefore it might be linked to faster disease progression. For the progression of fibrosis, not only genetic factors are important, but also other morphologic factors like steatosis itself and biological markers related to steatosis. While we cannot fight our genes, we can improve outcomes by correcting metabolic imbalances, fighting against obesity and the metabolic syndrome and therefore halting the progression of steatosis and fibrosis.

D. Crisan and M. Grigorescu equally contributed to this paper.

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Authors' address: Dr. Nicolae Crisan, Municipal Clinical Hospital, 11 Tabacarilor Str., Cluj-Napoca 400139, Romania.
E-mail: drnicolaecrisan@gmail.com

Dr. Rares Craciun, "Prof. Dr. Octavian Fodor" Regional Institute of Gastroenterology and Hepatology, 19-21 Croitorilor Str., Cluj-Napoca 400162, Romania.
E-mail: rarescraciun@ymail.com