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PNPLA3 rs738409 I748M is associated with steatohepatitis in 434 non-obese subjects with hepatitis C

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Title: *PNPLA3* rs738409 I748M Variant and Histological Features of Nonalcoholic Steatohepatitis in Patients with Genotype 1 Chronic Hepatitis C

SHORT TITLE: *PNPLA3* AND NASH IN CHRONIC HEPATITIS C

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ABBREVIATIONS: CHC: chronic hepatitis C; G1: genotype 1;.

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Abstract

Background and Aims: The PNPLA3/Adiponutrin rs738409 C/G single nucleotide polymorphism(SNP) is associated with the severity of steatosis, non-alcoholic steatohepatitis (NASH) and fibrosis in patients with non-alcoholic fatty liver disease, as well as with the severity of steatosis and fibrosis in patients with chronic hepatitis C(CHC). We tested in genotype 1(G1) CHC patients the putative association between the *PNPLA3* variant and histological features of NASH, as well as their impact on the severity of fibrosis.

Methods: Four hundred and thirty-four consecutively biopsied Caucasian G1-CHC patients were genotyped for the *PNPLA3* rs738409. Their metabolic profile included assessment of lipid levels and insulin resistance by the homeostasis model assessment(HOMA). Histological features of NASH in CHC were assessed using the Bedossa classification. Hepatic expression of PNPLA3 mRNA was evaluated in a subgroup of 63 patients.

Results: The prevalence of NASH progressively increased from 16.5% in patients with PNPLA3 CC, to 23.2% in CG and 29.2% in GG genotype(p=0.02). By multiple logistic regression PNPLA3 genotype(OR 1.54,95%CI 1.03-2.30,p=0.03) together with age(OR 1.03,95%CI 1.00-1.05,p=0.02), BMI≥30(OR 2.06,95%CI 1.04-4.10,p=0.03) and HOMA (OR 1.18,95%CI 1.04-1.32,p=0.006) was independently linked to histological features of NASH. When stratifying for obesity, PNPLA3 was associated with NASH in nonobese patients only(12.0% in CC vs 18.3% in CG vs 27.3% in GG, p=0.01), including after correction for metabolic confounders(OR 2.06,95%CI 1.26-3.36,p=0.004). We confirmed the independent association of rs738409 with the severity of steatosis(OR 1.71,95%CI 1.20-2.45,p=0.003), and indirectly by promoting steatohepatitis (OR 2.05,95%CI 1.05-4.02,p=0.003) with severe fibrosis. Higher liver PNPLA3 mRNA was associated both with the severity of steatosis(adjusted p=0.03) and steatohepatitis after adjusting for gender, age, BMI and HOMA (p=0.002).

Conclusions: In Caucasian G1-CHC patients, the *PNPLA3* rs738409 G variant is associated with a higher risk of both steatosis severity and steatohepatitis in chronic hepatitis C, particularly nonobese subjects at lower metabolic risk.

Introduction

The natural history of any chronic liver disease is now known to be modulated by a number of emerging risk factors including but not limited to gender, menopausal status, age, alcohol intake, fructose consumption, obesity, insulin resistance (IR) and hyperuricemia [1,2]. Recent evidence also suggests a key role for the host genetic architecture in prompting liver disease occurrence and its severity. In particular, the C→G rs738409 variant in the PNPLA3 gene is a risk factor for non-alcoholic fatty liver disease (NAFLD), with the G allele connoting a greater genetic risk [3,4]. The G allele is also associated with a higher prevalence of non-alcoholic steatohepatitis (NASH) and significant liver fibrosis [5], as well as a greater risk of hepatocellular carcinoma (HCC) in NAFLD [5], as validated by two recent meta-analyses [6,7].

When considering the role of PNPLA3 in CHC, similar results were observed. Of note some studies on cohorts of mostly genotype 1 (G1) CHC patients reported an association between the PNPLA3 rs738409 G variant and the severity of both steatosis and fibrosis as well as the risk of HCC occurrence [8-10].Bedossa and colleagues, in liver biopsies from CHC patients, recently characterized the presence of histological features of steatohepatitis, showing that the occurrence of NASH in this clinical setting not only was related to a poor at-risk metabolic profile, but was also a risk factor for the severity of liver damage [11]. This data however did not investigate the impact of genetic background on the presence of steatohepatitis in the context of CHC.

Hence, the aims of the present study in GH1 CHC patients were to assess the putative association between *PNPLA3* polymorphism and histological features of steatohepatitis, as well as their impact on the severity of liver fibrosis. We are confident that the use of the accurate scoring system of Bedossa for steatohepatitis diagnosis in CHC, will allow to clarify, first in CHC, the complex interplay among PNPLA3 genotype, steatohepatitis, and fibrosis severity.

Patients and Methods

This cohort study comprised 434 patients with CHC consecutively enrolled in three centers (Gastro-hepatology Division of the University Hospital Torino, Italy (n=70), Westmead Millenium Institute, Sydney, Australia (n=110) and the Gastrointestinal and Liver Unit of the University Hospital Palermo, Italy (n=254) and fulfilling the following inclusion criteria: (a) diagnosis of CHC genotype 1 infection based on hepatitis C serology and viral RNA (b)

histological diagnosis on liver biopsy, and (c) alcohol consumption <20 g/day in the last 12 months. Patients were excluded from the study if they were co-infected with either the hepatitis B virus or HIV, or if they were not of northern European descent. All participants who met the eligibility criteria were recruited after they provided written informed consent.

Anthropometric and Laboratory Evaluation

Clinical and anthropometric data were collected at the time of liver biopsy. BMI was calculated on the basis of weight in kilograms and height (in meters). Subjects were classified as normal weight (BMI, 18.5–24.9 kg/m2), overweight (BMI, 25–29.9) and obese (BMI≥30). The diagnosis of type 2 diabetes was based on the revised criteria of the American Diabetes Association using a value of fasting blood glucose of ≥126 mg/dl on at least two occasions. In patients with a previous diagnosis of type 2 diabetes, current therapy with oral hypoglycemic agents was documented.

Serum levels of total cholesterol, triglycerides and LFTs were determined by routine laboratory techniques on the day of liver biopsy. Plasma glucose levels were measured by the glucose oxidase method (Beckman Instruments, Fullerton CA; interassay CV 4%). Quantitative measurement of insulin in serum was performed using a immunoradiometric assay kit (Radim, Pomezia, Italy; interassay CV 13%). IR was determined by the homeostasis model assessment (HOMA) method, using the following equation: Insulin resistance (HOMA)=Fasting insulin (μU/ml) × Fasting glucose (mmol/L)/22.5 [12].

Genotyping

DNA was extracted from peripheral blood collected at the time of enrollment in all patients.Genotyping for PNPLA3 (rs738409) was carried out using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA). Commercial genotyping assays were available for the following SNPs: rs738409 (cat. C_7241_10. A custom assay was created by Applied Biosystem for rs12979860. The genotyping call was done with SDS software v.1.3.0 (ABI Prism 7500, Foster City, CA, USA).Genotyping was conducted in a blinded fashion relative to patient characteristics.

Histopathology

Liver biopsy was performed in all patients. Liver histology was used to determine the extent of steatosis, hepatic necroinflammation and fibrosis. Steatosis was graded as absent/mild (0-10%), moderate (11-29%) or severe (≥30%). Liver fibrosis was classified as absent/mild/moderate or severe (bridging fibrosis/cirrhosis), while necroinflammatory activity was classified as absent/mild/moderate or severe (based on the degree of piecemeal necrosis and lobular necrosis) according to Metavir [13]. According to the classification of Bedossa et al [11], histological features of steatohepatitis in CHC were also assessed. In particular steatohepatitis was defined according to a score made by the addition of clarification/ballooning (0 absent, 1 mild, 2 significant) and perisinusoidal fibrosis semiquantitation (0 absent, 1 mild, 2 significant); a score of ≥3 was considered diagnostic for steatohepatitis [11].

Hepatic expression of PNPLA3 mRNA

Liver samples were stored at-80°C and homogenized using The TissueRuptor aparatus (Qiagen) immediately before nucleic acid extraction. DNA, RNA and proteins from biopsy or from cells cultures were extracted using the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacture instructions. A quantity of 0.5µg of RNA was used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (Qiagen). The levels of PNPLA3 mRNA in the samples were determined using a real-time reverse transcription quantitative PCR (RT-qPCR) (ABI PRISM 7500 Fast Real Time System, Applied Biosystems). The detection was performed using SYBR Green and Quantitect Primer Assays (Qiagen), which provide a PCR reaction with high efficiency and specificity. rRNA 18S was used as the endogenous control gene. Results were expressed as N-fold difference in target gene expression relative to the rRNA 18S gene compared with the expression in the HepG2 cell line used as calibrator. Data analysis was performed using Sequence Detection Software Version 1.3-7500 from Applied Biosystems.

Statistical Analysis.

Continuous variables were summarized as mean ± standard deviation, and categorical variables as frequency and percentage. The t-test, Chi-square test and ANOVA tests were used where appropriate. Multiple logistic regression models were used to assess the factors associated with steatohepatitis and severe fibrosis, while multiple ordinal regression model was used to assess variables associated with the severity of steatosis.

In the first model, the dependent variable was steatohepatitis coded as 0 if absent, and as 1 if present; in the second model, the dependent variable was severe fibrosis, coded as 0 if absent/mild/moderate, and 1 if bridging fibrosis or cirrhosis; in the third model the dependent variable was steatosis classified as 0 if ≤10% (absent/mild), as 1 if from 11% to 29% (moderate), and as 3 if ≥30% (severe). Input variables included in the models were age, gender, BMI, BMI≥30, triglycerides, total- and HDL cholesterol, blood glucose, insulin, HOMA score, diabetes, *PNPLA3* rs738409 genotype, steatosis, lobular inflammation, steatohepatitis and fibrosis. The effect of *PNPLA3* rs738409 was evaluated according to the literature by using an additive model [5]. Variables associated with the dependent variable on simple logistic/ordinal regression (probability threshold, $P < 0.10$) were included in multiple logistic/ordinal regression. To avoid the effect of co-linearity, HOMA score, blood glucose, insulin levels and diabetes, as well as total- and HDL-cholesterol were not included in the same model. Regression analyses were done using Proc Logistic, Proc Ordinal, Proc t-test, and Proc freq in SAS (SAS Institute, Inc., Cary, North Carolina, U.S.A.) [14].

Patient Features and Histology

The baseline characteristics of the 434 patients are shown in Supplemental Table 1. The mean age was 52 yrs and 46% were female. Obesity and type 2 diabetes were observed in about 20% and 10% of patients, respectively. Mean values for total cholesterol, HDL cholesterol and triglycerides were within the normal range, while mean HOMA value was 3.25. One patient in four had severe fibrosis, and one in five had histological features of steatohepatitis.

A minority (9.4%) of patients had the PNPLA3 rs738409 GG polymorphism, compared to 34.8% and 55.8% with CG and CC variants, respectively. Interestingly, the clinical, biochemical and metabolic profile did not differed according to the *PNPLA3* genotype (Table 1). Instead, the severity of hepatic steatosis ($p<0.001$) as well as the prevalence of histological features of steatohepatitis ($p=0.02$) progressively increased according to the number of PNPLA3 G alleles (Table 1).

PNPLA3 rs738409 genotype is associated with histological features of steatohepatitis and steatosis in G1 CHC patients

The prevalence of histological features of steatohepatitis progressively increased from 16.5% in patients with PNPLA3 CC genotype, to 23.2% in CG and further to 29.2% in GG (p=0.02) (Figure 1). By simple logistic regression, older age, BMI≥30, higher blood glucose, insulin and HOMAHOMA, higher triglycerides, and PNPLA3 G variant were associated with histological features of steatohepatitis ($p < 0.10$) (Table 2). After correction for all the above variables, older age (OR 1.03, 95% CI 1.00-1.05,p=0.02), BMI≥30 (OR 2.06, 95% CI 1.04-4.10,p=0.03), higher HOMA (OR 1.18, 95% CI 1.04-1.32,p=0.006) and PNPLA3 G variant (OR 1.54, 95% CI 1.03-2.30,p=0.03) remained significantly associated with histological features of steatohepatitis by multivariate logistic regression analysis.

Considering that both PNPLA3 G variant and BMI≥30 were independently linked to histological features of steatohepatitis, we stratified the population according to obesity. As expected we found that obese patients had higher metabolic alterations, and a higher severity of liver damage in terms of steatosis, fibrosis and histological features of steatohepatitis, respect to non obese subjects (Supplemental table 2). Of note, we observed that the association between steatohepatitis and the PNPLA3 G variant, was lost in obese patients (34.8% in CC vs 38.4% in CG vs 37.5% in GG, p=0.79), while it was maintained in non-obese subjects (12.0% in CC vs 18.3% in CG vs 27.3% in GG, p=0.01) (Figure 1). Consistent with this data, in non obese subjects steatohepatitis was associated with higher HOMA (OR 1.36, 95% CI 1.12-1.66,p=0.002), higher HDL (OR 1.02, 95% CI 1.00-1.04,p=0.02) and PNPLA3 G variant (OR 2.06, 95% CI 1.26-3.36,p=0.004) by multivariate logistic regression analysis. Along this line we observed that while obese patients carrying the at high (GG) or at low (CC) risk PNPLA3 genotypes had a similar prevalence of steatohepatitis (33.3% vs. 34.9%, p=0.95), different issues were observed in nonobese ones. Specifically nonobese patients with PNPLA3 GG genotype had a higher prevalence of steatohepatitis compared to their counterpart with PNPLA3 CC (27.2% vs. 12.0%, p=0.05), that was similar to that of obese PNPLA3 CC or GG patients (p=0.60, and p=0.79, respectively), but was indepentend of insulin resistance assessed by HOMA-R. (table 3).

As previously reported, by ordinal regression analysis we confirmed the association of PNPLA3 G variant (OR 1.71, 95% CI 1.20-2.45,p=0.003) with the severity of steatosis,

together with BMI≥30 (OR 2.58, 95% CI 1.41-4.72,p=0.002), and higher HOMA (OR 1.22, 95% CI 1.10-1.35,p<0.001).

Histological features of steatohepatitis are associated with severe hepatic fibrosis in CHC

By simple logistic regression, older age, higher BMI, higher blood glucose, insulin and HOMA, lower total and HDL cholesterol, higher triglycerides, severity of steatosis, severe necroinflammatory activity and steatohepatitis were associated with severe fibrosis ($p < 0.10$) (Table 4). Multiple logistic regression confirmed only older age (OR 1.03, 95% CI 1.01-1.06,p=0.002), lower total cholesterol (OR 0.98, 95% CI 0.97-0.99, p<0.001), higher HOMA (OR 1.18, 95% CI 1.07-1.31,p<0.001), severe necroinflammatory activity (OR 2.16, 95% CI 1.23-3.79,p=0.007), and steatohepatitis (OR 2.05, 95% CI 1.05-4.02,p=0.003) as independent predictors of severe fibrosis (Table 3).

Notably, after stratifying for BMI, in the subgroup of nonobese patients 47.1% of those with steatohepatitis had F3-F4 fibrosis compared with 21.2% of those without (p<0.001). Along this line steatohepatitis (OR 2.28, 95% CI 1.07-4.85,p=0.03) remained significantly associated with F3-F4 fibrosis after correction for metabolic and liver confounders. Similar results were observed in the subgroup of obese patients: 57.1% of those with steatohepatitis had severe fibrosis compared with 26.5% of those without (adjusted OR 3.77, 95% CI 1.23-11.5,p=0.02)

Hepatic expression of PNPLA3 mRNA

In a subgroup of 63 patients with available frozen liver samples and with characteristics similar to the entire cohort (male 50%, mean age 53.5±9.2 yrs, mean BMI 27.9±5.5 Kg/m^2, PNPLA3 CC 54.8%, CG 30.7%, GG 14.5%, 25.8% with NASH, and 30.6% with severe fibrosis), we assessed the hepatic expression of PNPLA3 mRNA. The expression of PNPLA3 was not influenced by PNPLA3 genotype, and was also not associated with clinical or biochemical variables (p>0.10 for all) except for older age (p=0.006). A significant association was found between liver PNPLA3 expression and the severity of steatosis (1.74±0.50 in steatosis absent-mild vs 1.64±0.28 in moderate vs 2.13±0.39 in

severe, p=0.04 by ANOVA), steatohepatitis (1.67±0.46 in no steatohepatitis vs 2.16±0.38 in steatohepatitis, p<0.001) (Figure 2), and severe fibrosis (1.71±0.51 in F0-F2 vs 2.00±0.37 in F3-F4, p=0.02), even if after correction gender, age, BMI, HOMA, PNPLA3 G variant, and steatohepatitis (for fibrosis only), the association was maintained for severe steatosis (p=0.03) and steatohepatitis (p=0.002) only, but not for severe fibrosis (0.75).

Discussion

In this study we demonstrated that in Caucasian patients with G1 CHC the PNPLA3 rs738409 G variant was associated not only with the severity of steatosis, but also with the presence of histological features of steatohepatitis. Of particular interest the association with steatohepatitis was principally evident in non obese patients that have a lower metabolic risk than obese individuals. Hepatic PNPLA3 expression was also correlated with both steatosis and steatohepatitis.

Different lines of evidence have demonstrated that the PNPLA3 rs738409 genotype leads to a higher risk of hepatic steatosis [3,4], and among NAFLD patients with a greater risk of steatohepatitis and severe fibrosis [5,6]. In CHC, the G allele is also associated with a higher risk of steatosis and severe fibrosis [8-10].

This study is unique in that we have been able to determine the association between the PNPLA3 rs738409 G allele and histological steatohepatitis. This has not been previously possible, because of the lack of an objective histological scoring system for steatohepatitis in the context of hepatitis C infection. This was achieved in the present study by using the well validated score from Bedossa and colleagues [11]. Attesting to the robust nature of the score, the association with steatohepatitis was maintained after correction for well-known metabolic risk factors for NASH like age, obesity, and IR. Furthermore the association with histological steatohepatitis persisted after stratification for obesity. Of note this association was lost in obese individuals peraphs because metabolic risk factors per se overcame the genetic effect of PNPLA3 variant. Instead, the association between PNPLA3 variant and steatohepatitis was observed in nonobese patients only, i.e. in those

at lower metabolic risk, where the presence of the PNPLA3 GG prompted a risk of steatohepatitis similar to that generated by metabolic factors in obese ones but independent of insulin resistance. The stronger effect of PNPLA3 in non obese patients fits with a recent study showing that the amount of liver fat is similar in obese NAFLD compared to PNPLA3 NAFLD [15],

Our data confirmed the link reported in CHC between the severity of steatosis and PNPLA3 rs738409 variant [8-10], but we were unable to confirm a direct association with the severity of fibrosis [8-10]. Differences in clinical, metabolic and histological characteristics of studied populations, as well as in the analysis of data could explain the different results. Of note our data suggests that PNPLA3 is able to indirectly modulate the severity of liver fibrosis by prompting steatohepatitis development. Along this line the studies reporting an association between PNPLA3 gene variant and fibrosis in CHC did not adjust for steatohepatitis [9], and in some cases neither for steatosis [8,10].

Our study was unable to demonstrate any association between PNPLA3 rs738409 and other metabolic alterations, including IR. These results agree with that already reported in G1 CHC patients [8-10], but not with the paper of Rembeck and colleagues [16] observing a direct link between PNPLA3 gene variant and both IR and viral load in a small group of 102 patients with genotype 2 CHC. Instead our data strongly agree with the lack of association between PNPLA3 SNP and metabolic variables as showed in large cohort studies among NAFLD patients, these data adding strength to the robustness of our results [4,17].

Our cross sectional study was not designed to assess the pathogenic mechanisms linking the *PNPLA3* genotype to NASH and liver damage in CHC, but a few hypotheses may be entertained according to the experimental literature and the data we produced. Wild-type PNPLA3 is expressed in hepatocytes and has a lipolytic activity towards triglycerides, while the I148M mutation leads to a loss of function [18,19]. Also, the PNPLA3 variant has been associated with a hepatocyte gain of lipogenic activity leading to the synthesis of phosphatidic acid [20], as well as to a loss of retinyl-palmitate lipase activity in stellate cells [21]. When translating this evidence to humans, contrasting and few data exist on the link between PNPLA3 liver expression and liver damage. Some study reported no association with liver damage in NAFLD [5], some other an inverse link with severity of fibrosis in alcoholic liver disease [22], and some other a direct association with severity of steatosis

in obese nondiabetic NAFLD [23]. In our study we firstly observed in CHC patients a direct link between PNPLA3 mRNA liver expression and both the severity of steatosis and steatohepatitis, these associations being maintained after adjusting for metabolic and liver confounders. All in all the above quoted data supports clinical evidence about the association of PNPLA3 with both steatosis and steatohepatitis, further suggesting that the increase of liver PNPLA3 expression could be the response to fatty accumulation – due or not due to PNPLA3 variant – and could lead to an increase in pathological functions of PNPLA3 – especially in I148M variant-.

From a clinical point of view our data confirm the role of PNPLA3 rs738409gene variant as a major disease modifying factor in CHC that promotes histological steatohepatitis, and in turn contributing to the progression of the liver disease. Interestingly our data also identified in the non-obese CHC population, i.e. those potentially at lower risk of disease progression, the clinical setting in which the hepatic phenotypic effect of PNPLA3 variant is strongest and not dominated by metabolic confounders including insulin resistance. All in all these data, if prospectically validated, suggest to consider PNPLA3 genotype as a new tool for the identification of nonobese CHC patients worthy of a more intensive follow-up while awaiting for the new antiviral drugs.

The main limitation of this study lies in the potential limited external validity of the results for different populations and settings. Thus, replicating our results in other cohorts, is important. Lack of data on serum levels of adipocytokines, and on other polymorphisms that could conceivably confound the data may have also affected the results.

In conclusion, we found that in a large cohort of G1 CHC patients, the *PNPLA3* rs738409 G variant is associated with a higher risk of both steatosis severity and steatohepatitis especially in non-obese subjects at lower metabolic risk. Hence, these data, if prospectically confirmed, could lead to the identification of patients worthy of a careful follow-up even after HCV eradication by new direct antiviral agents. We also added new pathogenic insides about liver PNPLA3 expression and liver damage, worthy to be confirmed and explored by functional studies.

Figure Legends

Figure 1. Prevalence of histological features of nonalcoholic steatohepatitis according to PNPLA3 genotype in the entire cohort of genotype 1 chronic hepatitis patients and in subgroups discriminated according to presence or absence of obesity.

Figure 2. Liver PNPLA3 mRNA expression in a subgroup of 63 genotype 1 chronic hepatitis C patients with or without histological features of nonalcoholic steatohepatitis. P values was adjusted for gender, age, BMI, HOMA and PNPLA3 G variant.

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