

## A nonsynonymous gene variant in the adiponutrin gene is associated with nonalcoholic fatty liver disease severity

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**Abstract** We explored the role of the adiponutrin (*PNPLA3*) nonsynonymous-rs738409 single nucleotide polymorphism (SNP) in genetic susceptibility to nonalcoholic fatty liver disease (NAFLD) and whether this SNP contributes to the severity of histological disease. Two hundred sixty-six individuals were evaluated in a case-control association study, which included 172 patients with features of NAFLD and 94 control subjects. The rs738409 G allele was significantly associated with NAFLD ( $P < 0.001$ ; OR 2.8 95%, CI 1.5–5.2), independent of age, sex, body mass index (BMI), and Homeostasis Model Assessment (HOMA) index. When we tested the hypothesis of a relation between the SNP and the histological spectrum of NAFLD, a significant association was observed [ $\chi^2$  19.9, degree of freedom (df): 2,  $P < 5 \times 10^{-5}$ , adjusted for HOMA and BMI]. The degree of liver steatosis, as evaluated by liver biopsy, was significantly associated with the rs738409 G allele. Patients with CC genotype showed a lower steatosis score ( $14.9\% \pm 3.9$ ) in comparison with the CG genotype ( $26.3\% \pm 3.5$ ) and GG genotype ( $33.3\% \pm 4.0$ ) ( $P < 0.005$ ). The proportion of the total variation attributed to rs738409 genotypes was 5.3% ( $\beta$  0.23  $\pm$  0.07;  $P < 0.002$ ). Our data suggest that the rs738409 G allele is associated not only with fat accumulation in the liver but also with liver injury, possibly triggered by lipotoxicity.—Sookoian, S., G. O. Castaño, A. L. Burgueño, T. F. Gianotti, M. S. Rosselli, and C. J. Pirola. A nonsynonymous gene variant in the adiponutrin gene is associated with nonalcoholic fatty liver disease severity. *J. Lipid Res.* 2009. 50: 2111–2116.

**Supplementary key words** SNP • genetics • replication study • PNPLA3 • fatty liver • nonalcoholic steatohepatitis • NASH

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Nonalcoholic fatty liver disease (NAFLD) is an emerging epidemic disease with increasing prevalence worldwide. NAFLD not only affects the adult population (an estimated 20–40% of adults in Western countries have excess fat accumulation in the liver) (1), but also is the most common cause of pediatric liver disease (2).

The pathogenesis of NAFLD is multifactorial; as a complex disease, the disorder develops from the interplay between genetic susceptibility and the environment.

NAFLD refers to a wide spectrum of liver diseases, ranging from fatty liver alone to nonalcoholic steatohepatitis (NASH) with evidence of liver cell injury, a mixed inflammatory lobular infiltrate, and variable fibrosis (3). Patients with simple steatosis usually have a benign prognosis for liver disease. In contrast, up to 20% of patients with NASH can progress to cirrhosis (4).

While it is well known that environmental risk factors influence the development and progression of NAFLD (5, 6), the contribution of the individual genetic variation to disease predisposition is still uncertain, despite the fact that several genes in isolated studies have been suggested as potential candidates either for NAFLD susceptibility or disease progression (7–14).

Advances in genome analysis (including the development of comprehensive sets of informative genetic markers, improved physical mapping methods, and improvements in high throughput genotyping technology)

Abbreviations: ADPN, adiponutrin; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; df, degree of freedom; GGT, glutamyl-transferase; GWAS, genome-wide association studies; HOMA, Homeostasis Model Assessment; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; *PNPLA3*, patatin-like phospholipase domain containing 3 gene; SABP, systolic arterial blood pressure; SNP, single nucleotide polymorphism; US, ultrasonographic.

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have strongly contributed to the understanding of the pathogenesis of complex diseases. In fact, genome-wide association studies (GWAS) using a dense map of single nucleotide polymorphisms (SNP) enable scientists to detect common genetic variants that influence susceptibility to complex diseases, illuminating both disease mechanisms and the translation of this knowledge for diagnosis, prognosis, and therapy. A remarkable example of the impact of the use of GWAS in the understanding of the genetic architecture of human diseases is given by the recent three type 2 diabetes GWAS as meta-analyzed by Zeggini et al. (15).

Although factors promoting NAFLD include obesity and type 2 diabetes, and NAFLD is now considered the hepatic manifestation of the metabolic syndrome (16), there have been no studies using this approach until recently when researchers of the Dallas Heart Study performed a GWAS for liver fat content in 2,111 individuals from different ancestry groups (17). Interestingly, this study examined 9,222 nonsynonymous variants and showed strong evidence of association of NAFLD, evaluated by proton magnetic resonance spectroscopy, with the rs738409 G allele of the patatin-like phospholipase domain containing 3 gene (*PNPLA3*), also known as adiponutrin (*ADPN* gene) (17). In addition, a significant association was also observed between the G allele and the elevation of alanine aminotransferase (ALT) levels, at least in the Hispanic population. Another population-based GWAS of plasma liver-enzyme levels in the Caucasian population also reported that the *PNPLA3* locus is strongly associated with ALT levels (18), supporting an inflammatory effect of this gene on the liver.

While the functional impact of the reported SNP is still unknown, data from these two studies are very consistent and point out the role of the *PNPLA3* rs738409 [G/C], also known as Met148Ile, in the susceptibility to NAFLD. None of the above-mentioned studies examined the relation between *PNPLA3* SNP and NAFLD severity. The main reason seems to be the population-based nature of both studies; none of them included patients with NAFLD diagnosed by liver biopsy.

We performed a hospital-based adult case-control association study to replicate for the first time the association between the rs738409 and NAFLD susceptibility and to further evaluate the association between the SNP and the histological disease severity.

## PATIENTS AND METHODS

We performed a cross-sectional study on NAFLD in a county hospital of the city of Buenos Aires. The study involved a total of 266 unrelated individuals (80 males and 186 females), of which 172 patients had features of NAFLD and 94 were control subjects.

The screening criteria was liver ultrasonographic (US) examination indicative of fatty infiltration (19), which was carried out by the same operator and performed in all the participants.

Secondary causes of steatosis, including alcohol abuse ( $\geq 30$  g/d alcohol for men and  $\geq 20$  g for women), total parenteral nutrition, hepatitis B and hepatitis C virus infection, and the use of

drugs known to precipitate steatosis were excluded. In addition, patients with any of the following diseases were excluded from participation in this study: autoimmune liver disease, metabolic liver disease, Wilson's disease, and  $\alpha$ -1-antitrypsin deficiency.

For the purpose of exploring the hypothesis of a relation between the rs738409 and NAFLD, we included in the analysis all the NAFLD patients ( $n = 172$ ), and NAFLD was considered as a discrete trait. For the purpose of testing the hypothesis of a relation between the rs738409 and the histological severity of NAFLD, we included in the analysis only those patients that had histopathologic evidence of fatty liver disease, either simple steatosis or NASH, on liver biopsy performed within this study ( $n = 103$ ) (details in the liver biopsy description).

Control subjects were selected from patients attending our hospital for check-up purposes whose sex matched the NAFLD patients (63 females and 31 males).

In addition to the standard health examination, all the control individuals were subjected to liver US. They were included in the study if they did not have evidence of fatty change or biochemical abnormalities. Furthermore, control subjects were confirmed not to have any of the features of the metabolic syndrome as defined by the National Cholesterol Education Program Adult Treatment Panel III (20) and did not abuse alcohol.

The case participants and the controls were selected during the same study period from the same population of patients attending our institution, and all of them shared the same demographic characteristics (occupation, educational level, place of residence and ethnicity).

## Physical, anthropometric, and biochemical evaluation

Health examination included anthropometric measurements, a questionnaire on health-related behaviors, and biochemical determinations.

Body mass index (BMI) was calculated as weight/height<sup>2</sup> (kg/m<sup>2</sup>) and used as the index for relative weight. Waist and hip circumference were also assessed. Blood was drawn from fasting subjects who had been supine for at least 30 min. Serum insulin, total cholesterol, HDL and LDL cholesterol, triglycerides, plasma glucose, and liver function tests were measured by standard clinical laboratory techniques. Homeostasis Model Assessment (HOMA) was used to evaluate an insulin resistance index and was calculated as fasting serum insulin ( $\mu$ U/ml)  $\times$  fasting plasma glucose (mmol/l)/22.5.

Glucose metabolism was evaluated only in NAFLD patients using the oral glucose tolerance test performed with 75 g of glucose according to World Health Organization criteria.

Elevated blood pressure was defined as systolic arterial blood pressure (SABP)  $\geq 130$  mm Hg and/or DABP  $\geq 85$  mm Hg or being prescribed antihypertensive medications.

Measurement of body fat content was performed using a bioelectrical impedance method at 50 kHz and 500  $\mu$ A (OMRON Body Fat Analyzer, model HBF-306, OMRON Healthcare). The body fat content was calculated by a formula that includes five factors: electric resistance, height, weight, age, and gender. Body fat percentage was calculated as body fat mass/body weight (lbs.)  $\times 100$  as indicated by the manufacturer.

Patients were defined to have abnormal liver enzymes (LFT) in the presence of at least one of: 1) elevated serum ALT and/or aspartate aminotransferase (AST), defined as  $>41$  U/l, 2)  $\gamma$ -glutamyl-transferase (GGT)  $> 50$  U/l, and 3) alkaline phosphatase (AP)  $> 250$  UI/l.

All the investigations performed in this study were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki. Written consent from individuals was obtained in accordance with the procedures approved by the ethical committee of our institution.

## Liver biopsy and histopathological evaluation

Indication of liver biopsy was based on previous recommendations: benefits of obtaining liver for histology should be weighed against the possible morbidity and mortality of the procedure (21). Hence, NAFLD patients were offered a percutaneous liver biopsy if they showed either abnormal liver enzymes (AST, ALT, AP, or GGT), or severe insulin resistance (HOMA value > 3) (4, 22).

Sixty-nine patients that showed US features of mild liver steatosis as well as persistently normal ALT, AST, AP, and GGT during 12 months of follow-up were not included in the histological evaluation.

Liver biopsy was performed with US guidance and modified 1.4-mm diameter Menghini needles (Hepafix) on an outpatient basis. Liver biopsy specimens were routinely fixed in 40 g/L formaldehyde (pH 7.4) embedded in paraffin and stained with hematoxylin and eosin, Masson trichrome, and silver impregnation for reticular fibers. The same liver pathologist, who was blinded to patient details, read all the biopsies. All the biopsies were at least 2 cm in length and contained a minimum of 8 portal tracts. The degree of steatosis was assessed according to the system developed by Brunt et al. (23) recently modified by Kleiner et al. (24) based on the percentage of hepatocytes containing macrovesicular fat droplets, as follows: grade 0, no steatosis; grade 1, <33% of hepatocytes containing macrovesicular fat droplets; grade 2, 33–66% of hepatocytes containing macrovesicular fat droplets; and grade 3, >66% of hepatocytes containing macrovesicular fat droplets. NASH was defined as steatosis plus mixed inflammatory cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory's hyaline, and any stage of fibrosis, including absent fibrosis (3). The severity of necroinflammatory activity was expressed on a 3-point scale, as follows: grade 1 (mild), grade 2 (moderate), and grade 3 (severe) as described by Brunt et al. (23). The severity of fibrosis was expressed on a 5-point scale, as follows: 0 = none, 1 = perivenular and/or perisinusoidal fibrosis in zone 3, 2 = combined pericellular portal fibrosis, 3 = septal/bridging fibrosis, and 4 = cirrhosis.

## Genotype analysis

The genetic analyses were done on genomic DNA extracted from white blood cells by a standard method as previously described (25).

Genotyping of the *PNPLA3* rs738409 was performed by a high-throughput genotyping method involving PCR amplification of genomic DNA with two-tailed allele-specific primers that introduce priming sites for universal energy transfer-labeled primers as previously described (26).

To ensure genotyping quality, we included DNA samples as internal controls, hidden samples of known genotype, and negative controls (water). Genotypes with a signal below a negative control were not scored. The analysis error was estimated by replicating a blinded sample (always belonging to the same individual) eight times across the templates of the project. Of the 216 genotypes for the blinded sample, we had only one not-matched genotype (0.46% error); the observed error rate is estimated to be <0.5%. Overall genotype completion rate was 100%.

To explore possible stratification in the population, we used a collection of 13 SNPs (rs6830727, rs12639788, rs1282807, rs1947745, rs7162312, rs12951674, rs7212346, rs1934869, rs9542666, rs11843545, rs9725124, rs2798659, and rs2199940) at different loci (located in chromosome 4, 15, 17, 13, 1, and 3) and then analyzed the data with the Structure program Version 2 (27). We found no evidence of stratification in our sample, because the cases and the controls showed similar Q values and were assigned with a similar distance to clusters by the program Structure with

no further improvement in the fitting model by adding up to four clusters (the ln of likelihood was maximum for K = 1).

## Statistical analysis

Unless otherwise indicated, phenotypic quantitative data were expressed as mean  $\pm$  SD. For univariate analysis, and to avoid any assumption about variable distribution and homoscedasticity, differences between groups were assessed by the nonparametric Mann-Whitney test or Kruskal-Wallis for two or more groups, respectively. To test the association between genotypes and disease severity, we used chi-square test and a regression analysis for an ordinal multinomial distribution (Probit as the Link function) with disease severity as the dependent (response) variable coding controls, simple steatosis, and NASH subjects as 0, 1 and 2, respectively; HOMA and BMI as continuous predictor variables; and, sex and genotypes as grouping variables. Moreover, logistic regression analysis was included for the evaluation of the association between genotypes and histological disease severity.

To assess the association between genotypes with NAFLD or quantitative traits such as ALT and AST, we used chi-square test and logistic regression or multiple regression, adjusting for covariables such as age, HOMA, BMI, etc., respectively.

We used the CSS/Statistica program package, StatSoft V 6.0 (Tulsa, OK) for the analyses.

## RESULTS

Clinical features, anthropometric variables, and laboratory findings at diagnosis in NAFLD patients and controls are shown in **Table 1**. NAFLD patients were older and showed most of the risk factors of the metabolic syndrome: elevated BMI, waist-hip ratio, fasting glucose, and insulin and HOMA index; 46 individuals had type 2 diabetes, 22 of them received metformin. The rest of the patients were assigned to the conventional management of type 2 diabetes with a program of diet, weight loss, and physical activity.

Based on histological findings, 40 patients were assigned to the simple steatosis group and 63 were included in the NASH group. The histological features of patients who underwent liver biopsy are shown in Table 1.

In the controls, the frequencies of the G allele (Met148) and the C allele (Ile148) were 33.6% and 66.4%, respectively, and the distribution of the genotypes was in Hardy-Weinberg equilibrium (data not shown).

Discrete trait analysis of NAFLD in the whole population showed that the rs738409 G allele was associated with a 2-fold increase in the risk for NAFLD (OR: 2.04, 95% CI: 1.43–2.89;  $P < 0.00007$ ). The association persisted even after adjusting for covariates, because in the multivariate logistic regression analysis, the rs738409 was strongly associated with NAFLD ( $P < 0.001$ ; OR 2.80 per G allele; 95% CI 1.50–5.20) independent of age, sex, BMI, and HOMA index. The association was still significant after excluding the type 2 diabetic patients ( $P < 0.004$ ; OR 2.75; 95% CI: 1.38–5.48), independent of age, sex, BMI, and HOMA index.

In the analysis of the association of rs738409 and NAFLD progression from simple steatosis to NASH, we observed a significant association between the rs738409 genotypes and histological disease severity (**Table 2**). In fact, we observed significantly higher scores of disease



TABLE 1. Clinical and biochemical characteristics of the whole population according to disease status and histological features of patients with NAFLD

Variables	Control Subjects	NAFLD Patients	Nominal PValue
Number of subjects	94	172	—
Female/male	63/31	123/49	NS
Age (years)	46.6 ± 11.3	55.3 ± 11.9	2 × 10 <sup>-5</sup>
BMI (kg/m <sup>2</sup> )	24.9 ± 4.4	32.5 ± 5.7	1 × 10 <sup>-7</sup>
Waist circumference (cm)	83.4 ± 16.0	103.7 ± 14.6	1 × 10 <sup>-7</sup>
SABP (mmHg)	116.1 ± 13.8	122.5 ± 14.7	0.02
DABP (mmHg)	72.0 ± 9.0	76.3 ± 10.6	0.01
Body fat content (%)	29.6 ± 6.8	37.8 ± 7.3	1 × 10 <sup>-6</sup>
Fasting plasma glucose (mmol/L)	4.5 ± 0.6	5.7 ± 2.0	1 × 10 <sup>-11</sup>
Fasting plasma insulin (pmol/L)	48.1 ± 36.1	94.6 ± 72.4	1 × 10 <sup>-11</sup>
HOMA index	1.4 ± 1.2	3.5 ± 3.1	1 × 10 <sup>-14</sup>
Total cholesterol (mmol/L)	5.4 ± 1.0	5.5 ± 1.4	NS
HDL cholesterol (mmol/L)	1.4 ± 0.3	1.2 ± 0.5	0.02
LDL-cholesterol (mmol/L)	3.3 ± 0.9	3.2 ± 1.4	NS
Triglycerides (mmol/L)	1.3 ± 0.8	1.9 ± 1.3	0.0001
Uric acid (μmol/L)	174 ± 7	258 ± 246	1 × 10 <sup>-4</sup>
ALT (U/L)	16.7 ± 9.4	46.5 ± 51.4	1 × 10 <sup>-11</sup>
AST (U/L)	18.5 ± 9.2	35.8 ± 25.4	1 × 10 <sup>-11</sup>
GGT (U/L)	24.3 ± 23.1	54.1 ± 55.3	1 × 10 <sup>-6</sup>
AP (U/L)	138.5 ± 56.7	225.5 ± 115.4	1 × 10 <sup>-7</sup>
Histological features of NAFLD patients	Simple steatosis patients	NASH patients	—
Number of subjects	40	63	—
Degree of steatosis			—
1	11	6	—
2	18	22	—
3	11	35	—
Necroinflammatory activity			—
1	NA	26	—
2		35	—
3		2	—
Fibrosis stage			—
0	NA	31	—
1		19	—
2		1	—
3		11	—
4		1	—

DABP: diastolic arterial blood pressure. Results are expressed as mean ± SD. Nominal P-value stands for statistical significance using Mann-Whitney Test. NS: non significant. All measurements are in SI units. NA: not applicable. Degree of steatosis: grade 1, <33% of hepatocytes containing macrovesicular fat droplets; grade 2, 33%–66% of hepatocytes containing macrovesicular fat droplets; and grade 3, >66% of hepatocytes containing macrovesicular fat droplets. Necroinflammatory activity: grade 1 (mild), grade 2 (moderate) and grade 3 (severe). Fibrosis stage: 0 = none, 1 = perivenular and/or perisinusoidal fibrosis in zone 3, 2 = combined pericellular portal fibrosis, 3 = septal/bridging fibrosis, and 4 = cirrhosis.

severity in individuals with the GG genotype (2.12 ± 0.15) in comparison with those with the GC genotype (1.32 ± 0.15) and the CC genotype (0.70 ± 0.09;  $P < 0.00001$ ).

To adjust for potential confounders, we used a regression analysis for an ordinal multinomial distribution with Probit function by coding the histological grade that ranged from healthy subjects to NASH patients: control subjects, simple steatosis patients proven by liver biopsy and NASH patients proven by liver biopsy as 0, 1 and 2, respectively. Following this analysis, the association [chi-square 19.9, degree of freedom (df): 2;  $P < 5 \times 10^{-5}$ ]

TABLE 2. *PNPLA3*-rs738409 genotypes according to histological features of NAFLD patients who underwent a percutaneous liver biopsy

Population	CC Genotype	CG Genotype	GG Genotype	P	Allele Frequency Difference OR (95%CI)
Patients with simple steatosis (n = 40)	10	18	12	0.02	2.095 (1.172–3.745)
Patients with NASH (n = 63)	8	22	33		

P-value stands for statistical significance using Pearson's goodness-of-fit chi-square (degree of freedom = 1).

persisted after adjusting for HOMA and BMI as independent continuous predictor variables. As this result may merely represent the association between NAFLD and the SNP, we performed a logistic regression analysis considering the NASH group versus the simple steatosis one (including only the 103 patients that had liver biopsy). We still observed a significant association between the rs738409 genotypes and the histological disease severity (OR 1.88 per G allele; 95% CI 1.03–3.43;  $P < 0.04$ ). As both the groups included type 2 diabetic patients, after adjusting for type 2 diabetes, we observed that the association still persisted (OR 1.79; 95% CI 1.05–3.20;  $P < 0.05$ ). Furthermore, after excluding from the analysis the type 2 diabetic patients, the association was significant and persisted after adjusting for HOMA index (data not shown).

The degree of liver steatosis (as evaluated by liver biopsy) was significantly associated with the rs738409 G allele, as subjects with CC genotype showed lower steatosis scores (14.9% ± 3.9) in comparison with CG genotype (26.3% ± 3.5) and GG genotype (33.3% ± 4.0) ( $P < 0.005$ ). The proportion of the total variation accounted for the rs738409 genotypes, after correcting for age, sex, BMI, and HOMA, was 5.3% ( $\beta \pm SE$ : 0.23 ± 0.07;  $P < 0.002$ ).

Finally, the rs738409 G allele at *PNPLA3* was significantly associated with serum ALT and AST levels. These associations persisted after adjusting for age, BMI, HOMA, and even plasma triglycerides (Table 3).

## DISCUSSION

A large association study from the multiethnic population-based Dallas Heart Study revealed an association between a nonsynonymous variant (rs738409) in *PNPLA3* gene and liver fat content, as measured and quantified by proton magnetic resonance (17). To our knowledge, we have performed for the first time a replication study in a hospital-based population to evaluate not only the relationship between the SNP and the presence of fatty liver but also a possible association with NAFLD severity, as determined by liver biopsy. In the multivariate logistic regression analysis, we observed that the rs738409 was significantly associated with fatty liver, showing that carriers of the G allele (Met148 variant) are, at least, 1.5-fold per G allele (OR 2.8; 95% CI 1.5–5.2) more likely to have NAFLD in comparison with noncarriers, independent of

TABLE 3. Multiple regression analysis of serum liver enzymes (ALT and AST) as dependent variable and *PNPLA3* rs738409 genotypes, age, BMI, HOMA, and plasma triglycerides as independent ones

Variables	ALT			AST		
	$\beta \pm SE$	B $\pm$ SE	Plevel	$\beta \pm SE$	B $\pm$ SE	Plevel
rs738409	0.19 $\pm$ 0.09	13.99 $\pm$ 6.39	0.030	0.24 $\pm$ 0.09	8.60 $\pm$ 3.09	0.006
Age	-0.06 $\pm$ 0.09	-0.25 $\pm$ 0.41	0.53	-0.05 $\pm$ 0.09	-0.11 $\pm$ 0.20	0.58
BMI	-0.08 $\pm$ 0.11	-0.71 $\pm$ 1.03	0.49	-0.05 $\pm$ 0.11	-0.25 $\pm$ 0.50	0.62
HOMA	-0.01 $\pm$ 0.10	-0.11 $\pm$ 1.88	0.95	0.03 $\pm$ 0.10	0.24 $\pm$ 0.90	0.79
Plasma triglycerides	0.04 $\pm$ 0.09	0.02 $\pm$ 0.04	0.62	0.09 $\pm$ 0.09	0.02 $\pm$ 0.02	0.29

age, sex, BMI, and HOMA index. This association was also observed after removing type 2 diabetic patients from the analysis. In addition, we observed a significant association between the histological spectrum of NAFLD (simple steatosis and NASH) and the rs738409 G allele, independent of the effect of the BMI or insulin resistance. Furthermore, carriers of G allele showed higher degrees of histological severity and higher liver steatosis scores; the presence of each G allele increased the degree of liver steatosis by 10%. This association with histological disease severity persisted even after adjusting for type 2 diabetes. The lack of association between the variant and type 2 diabetes observed in our study suggests that the underlying mechanisms by which the rs738409 operate are independent of type 2 diabetes.


Moreover, the rs738409 G allele was significantly and positively associated with serum levels of both ALT and AST, a finding that also replicates the results of a recently published GWAS on several loci influencing plasma levels of liver enzymes (18).

Our results on patients carrying the 738409 G allele having higher scores of liver steatosis on liver biopsy may be explained by a possible direct relation between a loss-of-function of the *PNPLA3* and an increase in hepatic triglyceride content. Earlier data has shown that the greater the liver fat accumulation, the more liver necrosis is induced by intracellular lipid toxicity (28). This may additionally explain the significant association we have observed between the SNP and the histological disease severity and serum liver enzymes.

Notwithstanding the difficulties in replicating findings in genetic association studies, our results support the importance of the rs738409 in modifying not only hepatic triglyceride content-explaining population differences in disease susceptibility, but also explaining interindividual differences in inflammatory response to liver lipotoxicity.

While no functional studies about the role of the variant have been conducted, the fact that rs738409 is a nonsynonymous SNP supports a possible functional impact leading, for instance, to a loss-of-function of the protein, particularly as the amino acid switch (Met148Ile) is located in the lipolytic and lipogenic patatin domain of the protein. Although rs738409 SNP deserves biochemical investigation, the Function Analysis and Selection Tool for Single Nucleotide Polymorphisms resource (29) showed that it has a high score risk for splicing regulation with protein structural damage, consequently abolishing the protein domain. Similar results were observed using another pre-

dictor of the impact of amino acid substitutions, PolyPhen (<http://genetics.bwh.harvard.edu/pph/>).

In conclusion, based on the knowledge about human genetic variation information provided by the HapMap Project, GWAS are opening powerful insights on the pathophysiology of the common diseases. In particular, one of the major outcomes of these studies is the elucidation of important aspects of disease pathogenesis through the discovery of novel genes or genomic regions previously unrelated to a disease. Such is the case of the *PNPLA3* associated with the risk of NAFLD. However, while GWAS are becoming increasingly affordable, they are still costly and laborious and require optimal multistage designs that include genotyping hundreds of thousands of markers in thousands of subjects. Thus, while other genes influencing fatty liver disease predisposition and progression remain to be identified, the replication of the data generated by exploratory research strategies in small but well-characterized populations should be encouraged. In this setting, studies of new outcomes such as disease severity are recommended. 

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