The nuclear receptor *PXR* gene variants are associated with liver injury in nonalcoholic fatty liver disease

Silvia Sookoian[a,c,d], Gustavo O. Castaño[c,d], Adriana L. Burgueño[b], Tomas Fernández Gianotti[b], María Soledad Rosselli[a,b] and Carlos Jose Pirola[b]

Objective **To explore the contribution of gene variants and derived haplotypes of the pregnane X receptor (*NR1I2*) to the severity of nonalcoholic fatty liver disease (NAFLD).**

Methods **A total of 290 individuals were evaluated in a case-control association study, including 188 NAFLD patients with different stages of disease severity and 102 healthy individuals. Four tag single nucleotide polymorphisms (SNPs; rs12488820 C/T, rs2472671 C/T, rs2461823 A/G, and rs1054191 A/G) encompassing 36 kb in chromosome 3 and representing 33 polymorphic sites ($r^2 > 0.8$) were genotyped. Four additional SNPs (rs3814055, rs3814057, rs6785049, and rs7643645) were also included because they showed earlier evidence of functionality.**

Results **Genotypic tests for single SNPs showed that rs7643645 and rs2461823 were significantly associated with disease severity by ordinal multinomial analysis ($P < 0.0015$ and 0.039, respectively). A significant association was also observed under the additive model for both variants ($P < 0.00038$ and 0.012, respectively). Consistent with the analysis of individual markers, we observed that the multimarker composed of rs2461823/A-rs7643645/G was significantly associated with disease severity ($P < 6.9 \times 10^{-5}$, β: 0.45). In addition, the rs7643645/G variant was significantly associated with ALT level ($P < 0.026$), a surrogate marker of severe liver injury. Finally, in univariate analysis rs7643645/G was significantly associated with fatty liver disease ($P < 0.04$), with an odds ratio of 1.457 (95% confidence interval: 1.018–2.086).**

Conclusion **Our study suggests that pregnane X receptor polymorphisms and related haplotypes may contribute to disease severity in NAFLD by influencing the individual susceptibility to progress to more severe stages of the disease. *Pharmacogenetics and Genomics* 00:000–000 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]Clinical and Molecular Hepatology Laboratory, [b]Molecular Genetics and Biology of the Metabolic Syndrome Laboratory, Department of Molecular Genetics and Biology of Complex Diseases, Institute of Medical Research A Lanari-IDIM, University of Buenos Aires-National Council of Scientific and Technological Research (CONICET), [c]Research Council of GCBA and [d]Department of Medicine and Surgery, Hospital Abel Zubizarreta, Ciudad Autónoma de Buenos Aires, Argentina

Correspondence to Dr Silvia Sookoian, MD, PhD and Carlos J Pirola, PhD, FAHA, Instituto de Investigaciones Médicas, UBA-CONICET, Combatiente de Malvinas 3150, Buenos Aires (1427), Argentina
Tel: +54 11 4514 8701; fax: +54 11 4523 8947;
e-mail: ssookoian@lanari.fmed.uba.ar; pirola.carlos@lanari.fmed.uba.ar

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Introduction

Nonalcoholic fatty liver disease (NAFLD), a disease characterized by accumulation of fat in the liver without chronic alcohol consumption, is an emerging epidemic disease affecting 10–40% of the general population in Western countries [1], and up to 80% of obese and diabetic individuals [2].

In a subset of affected patients, fat in the liver triggers a local inflammatory response, with evidence of liver cell injury, mixed inflammatory infiltrates, and variable fibrosis – a disease stage also known as nonalcoholic steatohepatitis (NASH). The most important aspect of the clinical picture of NAFLD is that, whereas fatty liver alone is mostly a benign disorder, NASH carries a risk of progressive liver disease, leading to cirrhosis and end-stage liver disease in 20% of individuals [3,4].

Despite several epidemiological studies in NAFLD patients showing that central obesity and insulin resistance are the most frequent risk factors associated with severe liver injury [5,6], the physiopathological mechanisms involved in disease progression are still unknown.

The pathogenesis of NAFLD is multifactorial, and there is evidence of the contribution of genetic variation to disease predisposition. For instance, the influence of individual genetic variation on disease susceptibility has been explored by a number of candidate gene association studies [7–10]. In addition, a recent genome-wide association study on NAFLD in the multiethnic population-based Dallas Heart Study [11] and a replication study performed by our group [12] have revealed an association between a nonsynonymous variant in the *PNPLA3* (adiponutrin) gene and liver fat content.

Current evidence, elegantly reviewed by Cave *et al.* [13], has shown that altered dietary macronutrient composition (for instance, high fructose syrup consumption in soft drinks) may modulate NAFLD, even without body weight modification, by affecting the liver capacity to process xenobiotics. Consumption of refined carbohydrates

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has also been reported to be a key factor in the pro-inflammatory response associated with liver injury in NAFLD [14].

An earlier animal study showed that dietary manipulation, such as high lard and high sucrose diets, produces hepatic steatosis associated with xenobiotic-induced liver injury [15]. In addition, experimental models of NASH induced by diets deficient in choline and methionine are associated with liver injury through the alteration of hepatic expression of drug-metabolizing enzymes, such as CYP2E1, CYP4A, and CYP2C11 [16].

Elevated circulating and/or hepatic saturated free fatty acids may also promote not only hepatic steatosis but also the progression of liver disease, causing hepatocyte apoptosis and disruption in hepatic endoplasmic reticulum homeostasis [17,18].

In support of these findings, it has been observed that patients with NAFLD have an impaired detoxification/neutralization capacity, showing a down-regulation of the liver conjugation enzymes (glutathione *S* transferases) and cytochromes P450 [13]. Thus, NAFLD increases the hepatic sensitivity to xenobiotics [13].

Pregnane X receptor (*PXR*), also known as nuclear receptor subfamily 1, group I, member 2 (*NR1I2*), steroid and xenobiotic receptor (*SXR*), or pregnane-activated receptor, is a member of the nuclear receptor superfamily, whose primary function is the regulation of an entire network of genes involved in the detoxification and elimination of xenobiotics from the body, including their oxidation, conjugation, and transport [19]. Emerging evidence has also pointed to an equally important role of *PXR* as an endobiotic receptor [20], in particular, by impacting on lipid homeostasis. Interestingly, it was reported that liver *PXR* activation in mice causes hepatic steatosis, which is likely the result of a combined effect of increased hepatic free fatty acid uptake, lipogenesis, and suppression of β-oxidation [21,22].

Together, we speculate that *PXR* gene variants and their predicted haplotypes may contribute to disease severity in NAFLD by influencing individual susceptibility to progress to more severe stages of the disease.

Patients and methods

We performed a cross-sectional, hospital-based case-control association study on NAFLD in a county hospital of the city of Buenos Aires. The study involved a total of 290 unrelated individuals of 'self-reported' European ancestry (90 males and 200 females), including 188 consecutive patients with features of NAFLD at different stages of disease. The screening criterion was liver ultrasonographic (US) examination indicative of fatty infiltration [23], which was carried out by the same operator and performed in all the participants. Secondary causes of steatosis, including alcohol abuse ($\geq$ 30 g alcohol daily 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 always excluded. By using standard clinical and laboratory evaluation as well as liver biopsy features when applicable, autoimmune liver disease, metabolic liver disease, Wilson's disease, and α-1-antitrypsin deficiency were likewise ruled out in all patients.

For the evaluation of disease severity, NAFLD cases were classified as follows: fatty liver with persistently normal liver function test (LFT) and the absence of insulin resistance during 12 months of follow-up (FL-NLFT), simple steatosis, and NASH, with the last two groups proven through biopsy diagnosis, as described below.

Included in the study were 102 healthy individuals with the same demographic background and who attended our hospital for check-up purposes during the same study period.

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 and did not abuse alcohol.

Physical, anthropometric, and biochemical evaluation

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

Body mass index (BMI) was calculated as weight/height^2 (kg/m^2) and was used as the index for relative weight. Additionally, waist and hip circumference were also assessed. Blood was drawn from fasting subjects who had lain in a supine resting position for at least 30 min. Serum insulin, total cholesterol, HDL-cholesterol and LDL-cholesterol, triglycerides, plasma glucose, and LFTs were measured by standard clinical laboratory techniques. Homeostasis Model Assessment was used to evaluate an insulin resistance index and was calculated as fasting serum insulin (μU/ml) × fasting plasma glucose (mmol/l)/22.5.

Glucose metabolism was evaluated by 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 $\geq$ 130 mmHg and/or diastolic arterial blood pressure $\geq$ 85 mmHg or receipt of anti-hypertensive medications.

Measurement of body fat content was performed by using a bioelectrical impedance method at 50 kHz and 500 μA (OMRON Body Fat Analyzer, model HBF-306, OMRON Healthcare, Inc., Illinois, USA). The body fat content is calculated by a formula that includes five

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factors: electric resistance, height, weight, age, and sex. Body fat percentage (%) was calculated as body fat mass (lbs)/body weight (lbs) $\times$ 100.

Patients were defined to have abnormal LFT in the presence of at least one of the following biochemical criteria: (i) elevated serum alanine (ALT) and/or aspartate aminotransferase (AST), defined as >41 U/l; (ii) gamma-glutamyl-transferase (γGT) >50 U/l; and (iii) 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 biopsies and histopathological evaluation

According to our Institutional Review Board, NAFLD patients are offered a percutaneous liver biopsy if they show either abnormal liver enzymes (AST, ALT, AP, and γGT) or severe insulin resistance. Thus, a liver biopsy was performed in 113 patients who, after being informed about the disease, gave written consent.

The liver biopsy was carried out with ultrasound guidance and modified 1.4 mm diameter Menghini needles (Hepafix, Braun, Germany) 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 biopsies. All biopsies were at least 2 cm in length and contained a minimum of eight portal tracts. The degree of steatosis was assessed according to the system developed by Brunt *et al.* [24] on the basis of the percentage of hepatocytes containing macrovesicular fat droplets, as follows: grade 0, no steatosis; grade 1, less than 33% of hepatocytes containing macrovesicular fat droplets; grade 2, 33–66% of hepatocytes containing macrovesicular fat droplets; and grade 3, more than 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 [25]. The severity of necroinflammatory activity was expressed on a 3-point scale, as follows: grade 1, mild; grade 2, moderate; and grade 3, severe [24]. 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 and haplotype analysis

The genetic analyses were done on genomic DNA extracted from white blood cells by a standard method as previously described [26].

To assess the contribution of *PXR* gene variants to NAFLD, we selected tag single nucleotide polymorphisms (SNPs) using the Tagger computer program, which is a tool for the selection and evaluation of tag SNPs from data of the International HapMap Project (HapMap) [27]. A tag SNP is a representative SNP in a region of the genome with high linkage disequilibrium (LD). We used the aggressive tagging method to construct a single marker or multimarkers to capture alleles of interest based on the computed correlation r^2 between them and the phase II genotyping data from the HapMap for Whites from the CEU dataset (Utah residents with ancestry from northern and western Europe) with a minor allele frequency (MAF) of ≥ 0.10 and a minimum r^2 of 0.8 [27].

Moreover, we included four additional SNPs (rs3814055, rs3814057, rs6785049, and rs7643645) because earlier reports showed evidence of functionally association with either altered regulation of critical downstream effectors genes involved in xenobiotic removal (*CYP3A4* and *MDR1*) [28], or loss of consensus transcription factor binding sites for nuclear factor kappa B (*NFκB*) and hepatocyte nuclear factor 1 alpha (*HNF-1α*) [29,30].

Genotyping of the *PXR* gene variants 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 [31].

PLINK software was used to assess the association between SNPs and affection status and quantitative traits, and to test the Hardy–Weinberg equilibrium [32]. We tested crude associations of each polymorphism with the disease trait under assumptions of dominant, additive, and recessive models of inheritance. SNP haplotype or multimarker analysis was performed by PLINK (Boston, Massachusetts, USA), Haploview (Boston, Massachusetts, USA) [33], and SimHap software (Nedlands, Washington, Australia) [34].

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 8 times a blinded sample (always belonging to the same individual) across the templates of the project. On 216 genotypes for the 'blinded sample', we had only one not-matched genotype (0.46% error); then, the observed error rate was estimated to be less than 0.5%. Overall genotype completion rate was near 100%.

To explore a possible stratification in the population we used a collection of 13 SNPs at different loci (located in chromosomes 4, 15, 17, 13, 1, and 3) and then analyzed the data with the Structure program Version 2 [35]. We found no evidence of stratification in our sample, because cases and controls showed similar Q values and were

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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 log of likelihood was maximum for $K=1$).

A priori power estimation for the utilized sample was performed for single-point allelic effects [36], odds ratio (OR) of 1.5, at a nominal significance level of 0.05 for HapMap-predicted MAF (considering the higher and lower MAF among the variants) of 0.40 (rs3814055) to 0.10 (rs2472671) of a potential susceptibility marker and a 40% prevalence of the disease [1]. This analysis gave us an estimated power of 98 and 94% under both the multiplicative and the additive models, respectively, for rs3814055, and 83 and 80% for the multiplicative and the additive models, respectively, for rs2472671.

Statistical analysis

Unless otherwise indicated, phenotypic quantitative data are expressed as mean ± 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 than two groups, respectively. The association between NAFLD and gene variants was initially tested by Cochran-Armitage test and genotypic linear regression.

To test the association between genotypes and disease severity, we used Kruskal–Wallis and a generalized nonlinear regression analysis (analysis of covariance for an ordinal multinomial distribution, Probit as the Link function) with disease severity as the dependent (response) variable coding control, fatty liver with persistently normal LFT and the absence of insulin resistance, simple steatosis, and NASH participants as 0, 1, 2, and 3, respectively; HOMA and BMI as continuous predictor variables and sex and genotypes (coded as 0, 1, 2 reflecting the number of copies of the risk allele) as categorical factors. To assess the association between genotypes with NAFLD or quantitative traits such as ALT, AST, and γGT, we used logistic regression or multiple regression adjusting for co-variables such as age, sex, HOMA, BMI, etc., respectively.

As a secondary analysis, we tested the genetic model that seemed to best fit the data after visual inspection. We performed a permutation test on the basis of the most significant result of allelic, dominant and recessive models. This procedure controls for the fact that we have selected the best out of three correlated tests for each SNP. We used the CSS/Statistical program package, StatSoft V 6.0 (Tulsa, Oklahoma) to perform these analyses.

Results

Clinical features, anthropometric variables, and laboratory findings at diagnosis in NAFLD patients and healthy individuals are shown in Table 1. NAFLD patients were older and showed most of the risk factors of the metabolic syndrome: elevated blood pressure, BMI, waist-hip ratio, fasting glucose and insulin and HOMA index in addition to decreased HDL cholesterol and elevated triglycerides and uric acid.

Table 1 **Clinical and biochemical characteristics of the studied individuals**

Variables	Control participants	NAFLD patients	*P* value
Number of participants	102	188	
Female/male	66/36	134/54	NS
Age (years)	47.00 ± 11.06	54.89 ± 11.81	3.3×10^{-5}
BMI (kg/m^2)	25.00 ± 4.34	32.37 ± 5.72	1×10^{-17}
Waist circumference (cm)	83.66 ± 15.75	103.53 ± 14.44	1×10^{-17}
SABP (mmHg)	115.68 ± 13.89	124.41 ± 15.84	5.1×10^{-5}
DABP (mmHg)	71.58 ± 9.31	77.29 ± 11.16	3.4×10^{-5}
Body fat content (%)	29.42 ± 6.55	37.79 ± 7.52	1×10^{-7}
Fasting plasma glucose (mmol/l)	4.50 ± 0.60	5.66 ± 2.03	2.5×10^{-12}
Fasting plasma insulin (pmol/l)	48.3 ± 35.3	92.9 ± 70.4	1.1×10^{-12}
HOMA index	1.41 ± 1.09	3.41 ± 3.04	1×10^{-15}
Total cholesterol (mmol/l)	5.41 ± 1.07	5.50 ± 1.42	0.55
HDL-cholesterol (mmol/l)	1.40 ± 0.34	1.25 ± 0.50	0.0018
LDL-cholesterol (mmol/l)	3.29 ± 0.97	3.21 ± 1.39	0.83
Triglycerides (mmol/l)	1.27 ± 0.79	1.99 ± 1.28	5.2×10^{-6}
Uric acid (μmol/l)	174 ± 74	285 ± 422	1.5×10^{-5}
ALT (U/l)	17.04 ± 9.18	47.12 ± 49.75	1.6×10^{-13}
AST (U/l)	18.78 ± 9.03	36.13 ± 24.81	2.8×10^{-13}
γGT (U/l)	24.46 ± 22.25	55.21 ± 55.22	9.1×10^{-8}
AP (U/l)	139.68 ± 59.07	226.00 ± 112.09	5.8×10^{-9}

Results are expressed as mean ± SD. All measurements are in SI units.
ALT and AST, serum alanine and aspartate aminotransferase; AP, alkaline phosphatase; BMI, body mass index; γGT, gamma-glutamyl-transferase; HOMA, homeostatic model assessment; NAFLD, nonalcoholic fatty liver disease; NS, non-significant; SABP and DABP, systolic and diastolic arterial blood pressure.
P value stands for statistical significance using Mann–Whitney Test.

In the patient group, 75 of 188 showed liver US features of mild liver steatosis and persistently normal ALT, AST, AF, and γGT during 12 months of follow-up. In addition, they showed normal glucose metabolism. In the remaining group of 113 patients who underwent liver biopsy and based on the histological findings, 46 patients were assigned to the simple steatosis group and 67 to the NASH group. The histological features of patients are shown in Table 2.

PXR gene variants

The human *PXR*, encoded by the nuclear receptor subfamily 1, group I, member 2 (*NR1I2*) gene, is located at 3q12–q13.3 and consists of 9 exons; exons 2–9 contain the coding region for a 434 amino acid protein [29].

To minimize the number of markers selected for genotyping (the HapMap B35 full set database includes 62 polymorphic sites with a MAF ≥ 0.05), we selected four tag SNPs showing a MAF greater than 10% (rs12488820 C/T; rs2472671 C/T; rs2461823 A/G; rs1054191 A/G) encompassing 36 kb in chromosome.3 (120984247–121020021) and representing 33 polymorphic sites ($r^2 > 0.8$) considering the HapMap data. Table 3 illustrates the tag SNPs characteristics, along with the four additional SNPs added to the analysis as described above.

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Test results from the *Tagger* algorithm used to select the marker tests to capture SNPs of MAF $\geq 10\%$ and $r^2 \geq 0.8$ are shown in Table 4. The basic LD plot among the studied SNPs in our population is shown in Fig. 1.

The genotyping success rate was 98% for rs3814055, rs12488820, and rs3814057; 94% for rs2472671 and rs7643645; 95% for rs2461823; and 96% for rs6785049 and rs1054191.

We found that tests for associations between NAFLD as a disease trait and the eight tag SNPs showed significant differences in univariate allelic analysis only for rs7643645 (Cochran-Armitage test for trend showed $P < 0.02$, G being the risk allele), with OR of 1.457 and 95% confidence interval (95% CI) of 1.018–2.086, $P < 0.04$. A similar result was obtained by genotypic linear regression ($P < 0.019$). The calculated risk for NAFLD in homozygous GG subjects for the rs7643645 variant was around 3-fold (OR: 3.48, 95% CI: 1.25–10.62, $P < 0.008$) in comparison with AA homozygous ones taken as the reference group; the OR for heterozygous AG individuals was 1.12 (95% CI: 95, $P < 0.71$). Then, the association was better explained under the recessive model (GG vs. AG + AA, OR: 3.292; 95% CI: 1.398–7.752, $P < 0.0062$).

Table 2 Histological features of patients with simple steatosis and NASH

Histological features	Simple steatosis patients	NASH patients
Number of participants	46	67
Degree of steatosis		
1	15	9
2	20	24
3	11	34
Necroinflammatory activity		
1	NA	29
2	NA	36
3	NA	2
Fibrosis stage		
0	NA	34
1	NA	19
2	NA	2
3	NA	11
4	NA	1

Degree of steatosis: grade 1, less than 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. NA, not applicable; NASH, nonalcoholic steatohepatitis.

Even though we tested the best model after visual inspection, we performed a permutation test based on the most significant result of allelic, dominant and recessive models. That is, for the rs7643645, the best original result was compared against the best of these three tests for every replicate doing 100 000 permutations. A still significant result was obtained (empirical $P < 0.01$).

We next tested the hypothesis of a relation between the gene variants and the clinical and histological spectrum of NAFLD (disease severity by using the variable coding of disease as a grade ranging from healthy individuals to NASH patients as follows: controls, FL-NLFT, simple steatosis, and NASH subjects as 0, 1, 2, and 3, respectively). Single SNP genotypic tests showed that rs7643645 and rs2461823 were significantly associated with disease severity ($P < 0.0015$ and 0.039, respectively). In addition, a significant association was also observed under the additive model for both variants ($P < 0.00038$ and 0.012, respectively).

Table 3 Characteristics of the single nucleotide polymorphisms of the *PXR* gene genotyped in the study

NCBI SNP reference[a]	Location in the *PXR* gene	Chromosome position	Previous knowledge about functionality	dsSNP allele	MAF
rs3814055	5′ UTR	120982725	Regulates expression of *CYP3A4* Shows a consensus transcription factor binding site for *NF-κB*	C/T	0.394
rs12488820	Intronic	120984759	–	C/T	0.419
rs2472671	Intronic	120985735	–	C/T	0.141
rs2461823	Intronic	121002815	–	A/G	0.437
rs7643645[b]	Near-promoter/Intronic	121008187	Regulates expression of *CYP3A4* and *MDR1* Transcription factor binding site for *HNF4* lost in G allele [28]	A/G	0.468
rs6785049[c]	Intronic	121016423	Regulates expression of *CYP3A4*: 7635A–G transition in intron 5 was associated with higher magnitude of induction of intestinal CYP3A by inductors of *PXR* [29]	A/G	0.368
rs1054191	3′ UTR	121019587	–	A/G	0.124
rs3814057[d]	3′ UTR	121019944	Regulates expression of *MDR1*: people heterozygous with at least one 11156C had 1.45-fold lower P-glycoprotein levels in gut biopsies compared with those with homozygous 11156A [29]	A/C	0.174

CYP3A4, cytochrome P450, family 3, subfamily A, polypeptide 4; MAF[b], minor allele frequency within controls in the study; *MDR1*, ATP-binding cassette, sub-family B (MDR/TAP), member 1; *NF-κB*, nuclear factor Kappa B; *PXR*, pregnane X receptor.
[a]Single nucleotide polymorphisms on NCBI Reference Assembly.
[b]Position from translation start site in AF364606: −601.
[c]Position from translation start site in AF364606: 7635.
[d]Position from translation start site in AF364606: 11156.

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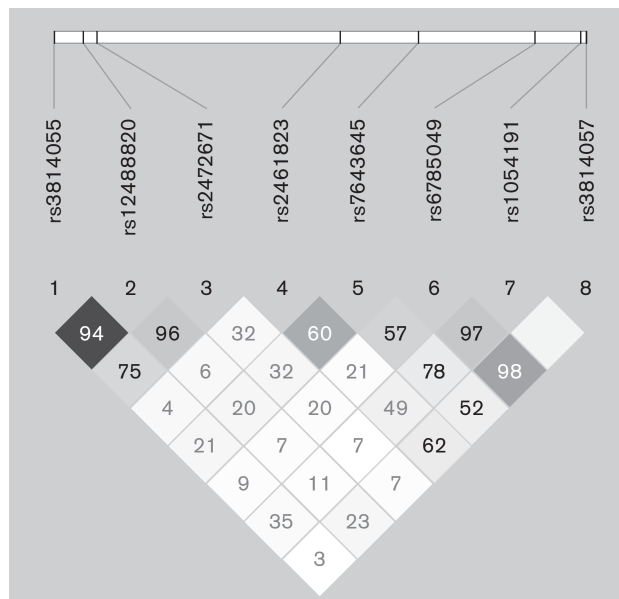
Table 4 Test results from the *Tagger* algorithm used to select marker tests to capture SNPs of MAF ≥ 10 and $r^2 \geq 0.8$ using the HapMap-CEU (Utah residents with ancestry from northern and western Europe) data (*http://www.hapmap.org*)

SNP ID	MB Position	Location in the *PXR* gene[a]	Tagging test	r^2
rs1581451	120978189	NL	rs3814055	1.000
rs7643038	120979735	NL	rs3814055	1.000
rs1523127	12098729	5′ UTR	rs3814055	0.966
rs2276706	120983997	NL	rs3814055	1.000
rs1523130	120982197	5′ UTR	rs12488820	1.000
rs3814055	120982725	5′ UTR	rs12488820	0.966
rs1523127	120983729	5′ UTR	rs12488820	0.932
rs10934498	120987071	Intronic	rs12488820	1.000
rs2276706	120983997	5′ near gene	rs12488820	0.966
rs10934498	120987071	Intronic	rs12488820	1.000
rs2472670	120985695	Intronic	rs2472671	1.000
rs13071341	120986661	Intronic	rs2472671	1.000
rs11712211	120986830	Intronic	rs2472671	1.000
rs2472672	120987453	Intronic	rs2472671	1.000
rs4234666	120988007	Intronic	rs2472671	1.000
rs2056530	120989152	Intronic	rs2472671	1.000
rs2873951	120989434	Intronic	rs2472671	1.000
rs1403527	120989570	Intronic	rs2472671	1.000
rs2472670	120985695	Intronic	rs2472671	0.892
rs2472677	121001107	Intronic	rs2461823	0.930
rs6438546	121001588	Intronic	rs2461823	1.000
rs2461822	121002855	Intronic	rs2461823	1.000
rs2461821	121003248	Intronic	rs2461823	1.000
rs13059232	121003745	Intronic	rs2461823	0.964
rs2461817	121007341	Intronic	rs2461823	0.930
rs11929668	121018485	Intronic	rs1054191	1.000
rs10511395	121019249	3′ UTR	rs1054191	1.000
rs11926554	120978246	NL	rs3814055–rs2472671	1.000
rs16830505	120997758	Intronic	rs2461823–rs7643645	0.861
rs6772976	121002154	Intronic	rs2461823–rs7643645	0.904
rs6438549	121006436	Intronic	rs2461823–rs7643645	0.872
rs2461818	121006562	Intronic	rs2461823–rs7643645	0.872
rs6771638	121006856	Intronic	rs2461823–rs7643645	0.904

MB position, mapped chromosome position (International HapMap Project); NL, No link established by analysis of contig annotation or BLAST analysis of mRNA sequences; SNP ID, single nucleotide polymorphisms on NCBI reference assembly. [a]Location in the *PXR* gene, according to the single nucleotide polymorphisms on NCBI Reference Assembly.

Fig. 1



Basic linkage disequilibrium (LD) plot between the studied single nucleotide polymorphisms (SNPs) across the pregnane X receptor gene in our studied population. The horizontal white line depicts the 36 kb DNA segment of chromosome 3q12–q13.3 analyzed in our sample. The location of the eight SNPs genotyped in this study are indicated by hatch marks. A linkage disequilibrium plot is depicted in the bottom part of the figure. Each diamond represents the magnitude of LD for a single pair of markers, with colors indicating strong LD (black $r^2 = 1.0$) and no LD (white, $r^2 = 0$) as the extremes (different gray tones indicate intermediate LD). Numbers inside the diamonds stand for D′ values × 100.

Furthermore, significantly higher scores of disease severity were observed in individuals carrying the rs7643645 GG genotype (1.79 ± 0.17, mean ± SE) in comparison with the AG genotype (1.19 ± 0.08) and the AA genotype (0.98 ± 0.14), $P < 0.002$. By using an analysis of covariance with an ordinal multinomial distribution with probit link we observed that the association still persisted (Wald test: 7.7, $P < 0.023$, and likelihood type 1 test, χ^2: 7.28, d.f.: 2, $P < 0.03$) after adjusting for age, sex, BMI, and HOMA index, which is more significant for the recessive model (GG vs. AG + AA, χ^2: 7.08, d.f.: 1, $P < 0.008$) after adjusting for the same co-variables.

We further evaluated the LD pattern of the previously mentioned SNPs. The estimation of haplotypes from genotype data showed 18 possible combinations covering more than 88% of the haplotypes with a frequency higher than 0.01 from 64 possible combinations. Among all the haplotypes, both the GCTAGAGA and GCTCAAGA composed of the combinations of rs3814055, rs12488820, rs2472671, rs2461823, rs7643645, rs6785049, rs1054191, and rs3814057, respectively in that order, were significantly associated with NAFLD disease severity ($P < 0.005$ and 0.0024, respectively). As an exploratory test and to dissect the association signal, we performed the analysis using the PLINK software by dropping individual markers one by one and analyzing paired multimarkers. We observed that the multimarker composed of rs2461823/A–rs7643645/G was significantly associated with disease severity ($P < 6.9 \times 10^{-5}$, β: 0.45), explaining much of the global effect and 6.0% of the total variance of the trait. These two markers are in low/moderate LD in our population (r^2: 0.27) and capture several untyped SNPs (see Table 4). A note of caution should be added, as we did not cross-validate the multimarker selection.

We also assessed the association between the *PXR* SNPs and the quantitative traits related to disease severity such as serum liver enzyme activities. Genotypic tests showed that ALT value was significantly associated with rs7643645 ($P < 0.026$); this association was also observed

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under the recessive model (β: 8.48, $P < 0.016$, linear regression analysis). Individuals with the rs7643645 GG genotype showed significantly higher level of ALT (50.64 ± 5.30 IU/l) than those with AA (33.68 ± 4.51 IU/l) and AG (35.56 ± 2.83 IU/l). Interestingly, the multi-marker composed of the rs2461823/A and rs7643645/G variants was significantly associated with ALT levels (β: 10.07, $P < 0.005$ explaining 3.8% of the variance). No association was observed with other LFT.

Discussion

NAFLD is the most common cause of chronic liver disease in Western countries. Current evidence suggests that the disease may progress to end-stage liver disease, and although the natural history of NAFLD is not well defined, it seems to be determined by the severity of the histologic damage [2]. Nevertheless, injury to the steatotic liver and progression of liver disease are observed only in a minority of participants, suggesting an important interindividual variability. Hence, in addition to the known risk factors associated with disease severity, such as insulin resistance and oxidative stress [37], a certain genetic predisposition has been suggested.

In this study, we explored the potential contribution of *PXR* gene variants to the severity and progression of the liver injury in patients with NAFLD and observed that among eight studied SNPs (four tagging SNPs, which represent 33 polymorphic sites encompassing 36 kb in chromosome 3, and four functional SNPs), rs7643645 and rs2461823 were significantly associated with disease severity, independently of potential confounders such as BMI and HOMA index. In addition, the test of haplotypes on the basis of multi-marker predictors showed that the one composed of the rs2461823/A and rs7643645/G variants was significantly associated with disease severity ($P < 6.9 \times 10^{-5}$, β: 0.45). Reinforcing the biological plausibility of this association, the same haplotype was also significantly associated with ALT levels, a surrogate marker of liver injury.

To our knowledge, the potential contribution of *PXR* to the severity of NAFLD has not been described in humans, and our study is the first to provide evidence of this association. Although the molecular mechanisms related to the contribution of the *PXR* gene variation to NAFLD are unclear, several lines of evidence support our findings. For instance, it has been reported that expression of activated *PXR* in the livers of transgenic mice resulted in an increased hepatic deposit of triglycerides [21]. Interestingly, *PXR*-mediated lipid accumulation was the result of increased hepatic free fatty acid uptake induced by the activation of the fatty acid transporter CD36 [22]. CD36 has been proposed as a surrogate of macrophage activation and inflammation [38] and recently, circulating concentration of soluble CD36 was associated with markers of liver injury [39]. Finally, *PXR* not only regulates drug metabolism but also hepatic energy metabolism, gluconeogenesis, glycogenolysis, fatty acid β-oxidation, ketogenesis, lipogenesis, thyroid hormone activity, and inflammation as recently reviewed [40,41].

In addition to the above-mentioned role in regulating metabolic pathways, *PXR* functions as a sensor of exogenous chemicals and of toxic byproducts derived from the endogenous metabolism. In this context, we may speculate that altered function of *PXR* variants renders fatty livers more vulnerable against toxic substances. Thus, *PXR* is a novel but interesting gene influencing the 'second hit' needed to trigger the hepatocyte injury and inflammation observed in NASH patients [42].

It was previously shown that the G allele of rs7643645 disrupts the transcription factor-binding site for *HNF4α* [20,29]. This is a worthy observation as *HNF4α* is as an important regulator of the physiological transcriptional response of *PXR* [43]. In our study, carriers of the G allele showed more severe scores of disease severity, supporting a potential role of *PXR* polymorphisms in the susceptibility to liver injury in patients with fatty liver.

As a final point, although our observations are based on a moderate sample of individuals when compared with the sample size of other genetic association studies of common diseases, a power estimation of our sample size gave us an estimated power of 94% under the additive model, or greater under the multiplicative model (98%). Moreover, some criticisms may be expected because we limited liver biopsy to patients with abnormal LFT. To perform liver biopsies on asymptomatic patients without evidence of abnormality during a long follow-up period in none of the LFT (ALT, AST, AP, and γ-GT) is, at least, questionable, particularly because no intervention besides lifestyle measures are recommended. In our study we preclassified the patients for the evaluation of clinical disease severity according to a panel of four LFT monitored during a 12-month follow-up period as it was shown that liver biopsy is invasive, costly, and prone to severe complications [44]. This issue could be a drawback in our study when making conclusions about histological disease severity. It is worthy to note, however, that we did not preclassify patients taking into account an isolated value of ALT and AST. On the contrary, we stratified and selected for biopsy those patients who showed a combination of abnormal LFT that were earlier shown to be strongly associated with disease severity and its complications [45,46].

In conclusion, although we cannot rule out the possibility that the association observed in our study was due to another functional polymorphism in LD with the reported variants, our results may indicate that the *PXR* is worthy of consideration as a candidate gene influencing the severity and progression of NAFLD.

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References

1 Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**:1387–1395.
2 Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221–1231.
3 Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, *et al.* Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006; **44**:865–873.
4 Adams LA, Sanderson S, Lindor KD, Angulo P. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 2005; **42**:132–138.
5 Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* 1999; **30**:1356–1362.
6 Fracanzani AL, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, *et al.* Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. *Hepatology* 2008; **48**:792–798.
7 Merriman RB, Aouizerat BE, Bass NM. Genetic influences in nonalcoholic fatty liver disease. *J Clin Gastroenterol* 2006; **40**:S30–S33.
8 Sookoian S, Castano G, Gianotti TF, Gemma C, Pirola CJ. Polymorphisms of MRP2 (ABCC2) are associated with susceptibility to nonalcoholic fatty liver disease. *J Nutr Biochem* 2009; **20**:765–770.
9 Sookoian S, Gemma C, Gianotti TF, Burgueno A, Castano G, Pirola CJ. Genetic variants of Clock transcription factor are associated with individual susceptibility to obesity. *Am J Clin Nutr* 2008; **87**:1606–1615.
10 Sookoian S, Castano G, Gianotti TF, Gemma C, Rosselli MS, Pirola CJ. Genetic variants in STAT3 are associated with nonalcoholic fatty liver disease. *Cytokine* 2008; **44**:201–206.
11 Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, *et al.* Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2008; **40**:1461–1465.
12 Sookoian S, Castano GO, Burgueno AL, Fernandez GT, Rosselli MS, Pirola CJ. A nonsynonymous gene variant in adiponutrin gene is associated with nonalcoholic fatty liver disease severity. *J Lipid Res* 2009; **50**:2111–2116.
13 Cave M, Deaciuc I, Mendez C, Song Z, Joshi-Barve S, Barve S, *et al.* Nonalcoholic fatty liver disease: predisposing factors and the role of nutrition. *J Nutr Biochem* 2007; **18**:184–195.
14 Bergheim I, Weber S, Vos M, Kramer S, Volynets V, Kaserouni S, *et al.* Antibiotics protect against fructose-induced hepatic lipid accumulation in mice: role of endotoxin. *J Hepatol* 2008; **48**:983–992.
15 Sugatani J, Wada T, Osabe M, Yamakawa K, Yoshinari K, Miwa M. Dietary inulin alleviates hepatic steatosis and xenobiotics-induced liver injury in rats fed a high-fat and high-sucrose diet: association with the suppression of hepatic cytochrome P450 and hepatocyte nuclear factor 4alpha expression. *Drug Metab Dispos* 2006; **34**:1677–1687.
16 Weltman MD, Farrell GC, Liddle C. Increased hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation. *Gastroenterology* 1996; **111**:1645–1653.
17 Wei Y, Wang D, Topczewski F, Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am J Physiol Endocrinol Metab* 2006; **291**:E275–E281.
18 Malhi H, Barreyro FJ, Isomoto H, Bronk SF, Gores GJ. Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. *Gut* 2007; **56**:1124–1131.
19 Kliewer SA, Goodwin B, Willson TM. The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev* 2002; **23**:687–702.
20 Zhang B, Xie W, Krasowski MD. PXR: a xenobiotic receptor of diverse function implicated in pharmacogenetics. *Pharmacogenomics* 2008; **9**:1695–1709.
21 Zhou J, Zhai Y, Mu Y, Gong H, Uppal H, Toma D, *et al.* A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J Biol Chem* 2006; **281**:15013–15020.
22 Zhou J, Febbraio M, Wada T, Zhai Y, Kuruba R, He J, *et al.* Hepatic fatty acid transporter Cd36 is a common target of LXR, PXR, and PPAR gamma in promoting steatosis. *Gastroenterology* 2008; **134**:556–567.
23 Mendler MH, Bouillet P, Le Sidaner A, Lavoine E, Labrousse F, Sautereau D, *et al.* Dual-energy CT in the diagnosis and quantification of fatty liver: limited clinical value in comparison to ultrasound scan and single-energy CT, with special reference to iron overload. *J Hepatol* 1998; **28**:785–794.
24 Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999; **94**:2467–2474.
25 Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003; **37**:1202–1219.
26 Kawasaki ES. Sample preparation from blood, cells, and other fluids. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. *PCR Protocols. A guide to Methods and Applications*. San Diego: Academic Press, INC.; 1990 pp. 146–152.
27 De Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet* 2005; **37**:1217–1223.
28 Lamba J, Lamba V, Strom S, Venkataramanan R, Schuetz E. Novel single nucleotide polymorphisms in the promoter and intron 1 of human pregnane X receptor/NR1I2 and their association with CYP3A4 expression. *Drug Metab Dispos* 2008; **36**:169–181.
29 Zhang J, Kuehl P, Green ED, Touchman JW, Watkins PB, Daly A, *et al.* The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics* 2001; **11**:555–572.
30 Hustert E, Zibat A, Presecan-Siedel E, Eiselt R, Mueller R, Fuss C, *et al.* Natural protein variants of pregnane X receptor with altered transactivation activity toward CYP3A4. *Drug Metab Dispos* 2001; **29**:1454–1459.
31 Myakishev MV, Khripin Y, Hu S, Hamer DH. High-throughput SNP genotyping by allele-specific PCR with universal energy-transfer-labeled primers. *Genome Res* 2001; **11**:163–169.
32 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81**:559–575.
33 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**:263–265.
34 Carter KW, McCaskie PA, Palmer LJ. SimHap GUI: An intuitive graphical user interface for genetic association analysis. *BMC Bioinformatics* 2008; **9**:557.
35 Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. *Genetics* 2000; **155**:945–959.
36 Skol AD, Scott LJ, Abecasis GR, Boehnke M. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. *Nat Genet* 2006; **38**:209–213.
37 Greenfield V, Cheung O, Sanyal AJ. Recent advances in nonalcoholic fatty liver disease. *Curr Opin Gastroenterol* 2008; **24**:320–327.
38 Tuomisto TT, Riekkinen MS, Viita H, Levonen AL, Yla-Herttuala S. Analysis of gene and protein expression during monocyte-macrophage differentiation and cholesterol loading–cDNA and protein array study. *Atherosclerosis* 2005; **180**:283–291.
39 Fernandez-Real JM, Handberg A, Ortega F, Hoejlund K, Vendrell J, Ricart W. Circulating soluble CD36 is a novel marker of liver injury in subjects with altered glucose tolerance. *J Nutr Biochem* 2008; **20**:477–484.
40 Konno Y, Negishi M, Kodama S. The roles of nuclear receptors CAR and PXR in hepatic energy metabolism. *Drug Metab Pharmacokinet* 2008; **23**:8–13.
41 Kakizaki S, Yamazaki Y, Takizawa D, Negishi M. New insights on the xenobiotic-sensing nuclear receptors in liver diseases–CAR and PXR–. *Curr Drug Metab* 2008; **9**:614–621.
42 Day CP, James OF. Steatohepatitis: a tale of two hits? *Gastroenterology* 1998; **114**:842–845.
43 Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, *et al.* The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* 2003; **9**:220–224.
44 Cadranel JF, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the study of the liver (AFEF). *Hepatology* 2000; **32**:477–481.
45 Nannipieri M, Gonzales C, Baldi S, Posadas R, Williams K, Haffner SM, *et al.* Liver enzymes, the metabolic syndrome, and incident diabetes: the Mexico City Diabetes Study. *Diabetes Care* 2005; **28**:1757–1762.
46 Lee DS, Evans JC, Robins SJ, Wilson PW, Albano I, Fox CS, *et al.* Gamma glutamyl transferase and metabolic syndrome, cardiovascular disease, and mortality risk: the Framingham Heart Study. *Arterioscler Thromb Vasc Biol* 2007; **27**:127–133.

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