Annals of Gastroenterology (2022) **35,** 297-306

Association between rs738409 and rs2896019 single-nucleotide polymorphisms of phospholipase domain-containing protein 3 and susceptibility to nonalcoholic fatty liver disease in Greek children and adolescents

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Abstract

Background Several studies have detected a strong association linking rs738409 and rs2896019 polymorphisms in the patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene with hepatic steatosis and steatohepatitis. In the present study we aimed to determine the association of those PNPLA3 variants with nonalcoholic fatty liver disease (NAFLD) susceptibility in obese and nonobese Greek children and adolescents.

Methods The study recruited 91 children and adolescents of Greek descent with NAFLD or biopsy-proven nonalcoholic steatohepatitis, and 91 healthy subjects of normal weight (control group) with sex distribution similar to the patient group. DNA samples were amplified using polymerase chain reaction with specifically designed primers. Data were analyzed using the statistical software SPSS version 24.0.

Results A significant correlation was shown between the rs738409 polymorphism (CG and GG genotypes) and the rs2896019 polymorphism (TG genotype) with the development of hepatic steatosis ($P<0.001$). The incidences of rs738409 GG, rs738409 CG and rs2896019 TG genotypes were found to be increased in patients with hepatic steatosis (obese and nonobese), but not in obese patients without liver disease. The combined expression of the 2 polymorphisms was associated with a lower age of diagnosis of hepatic steatosis in nonobese patients.

Conclusions We confirmed a strong association between the 2 polymorphisms and hepatic steatosis. The association of the rs2896019 single-nucleotide polymorphism with hepatic steatosis in obese and nonobese pediatric patients, and the combined study of both polymorphisms in a pediatric population of Greek origin are described for the first time.

Keywords Nonalcoholic fatty liver disease, children, adolescents, single-nucleotide polymorphism, PNPLA3

Ann Gastroenterol 2022; 35 (3): 297-306

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Conflict of Interest: None

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Received 19 December 2020; accepted 13 February 2021; published online 25 March 2022

DOI: https://doi.org/10.20524/aog.2022.0706

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in children and a significant cause of liver-related morbidity and mortality [1]. The prevalence of childhood obesity is increasing, whereas the incidence of hepatic steatosis is estimated to be almost 10% in the pediatric population and may be as high as 38% in obese children [2]. Children with NAFLD are at higher risk of progressive liver disease and cirrhosis, with a consequent need for liver transplantation [3-6]. Environmental risk factors are widely known to influence the development and progression of NAFLD [7]. Genetic variability plays a critical role in NAFLD predisposition and is characterized by gene polymorphisms. The major genetic determinants of the pathogenesis and severity of liver steatosis are presented in Table 1 [8-25].

Adiponutrin/patatin-like phospholipase domain-containing protein 3 (PNPLA3), or adiponutrin, belongs

to a group of lipid-metabolizing enzymes first identified in potato tubers. Most members of the PNPLA family are soluble proteins and show nonspecific lipid acyl hydrolase activity [26]. Adiponutrin has been shown to possess both triacylglycerol lipase and acylglycerol transacylase properties [27]. Among the 9 members of the PNPLA family, PNPLA3 plays a critical role in triglyceride metabolism by mediating a rate-limiting step in triglyceride hydrolysis [28,29]. Genome-wide association studies link the nonsynonymous genetic polymorphisms rs738409 and rs2896019 in the *PNPLA3* gene to hepatic triglyceride accumulation (steatosis), inflammation, fibrosis, cirrhosis, and even hepatocellular carcinoma [30-32]. Considering the morbidity associated with NAFLD in the pediatric population, it is important to identify children with hepatic steatosis who have the highest risk of developing progressive liver disease [33].

In the present study we aimed to determine: 1) the associations of the PNPLA3 rs738409 and rs2896019 single-nucleotide polymorphisms (SNPs) with NAFLD susceptibility in obese and nonobese Greek children and adolescents with hepatic steatosis; 2) the associations between the SNPs, anthropometric variables, and the laboratory findings; and 3) the associations of polymorphisms with either early-onset of steatosis or steatohepatitis in a subgroup of patients undergoing a liver biopsy.

Patients and methods

Study population and data collection

This was a cross-sectional case-control study in a population of Greek origin. We studied 182 children and adolescents monitored in the Pediatric Gastroenterology Unit of the Fourth Department of Pediatrics of the Aristotle University of Thessaloniki.

The patients were divided into 3 groups (A, B, and C) according to clinical, laboratory and imaging findings. Group A included 31 overweight or obese patients (body mass index, BMI ≥85th percentile) with NAFDL or nonalcoholic steatohepatitis (NASH), of whom 21 were boys and 10 were girls (mean age 10.45±2.39 years). Group B included 33 overweight or obese subjects (BMI ≥85th percentile) without liver disease, of whom 17 were boys and 16 were girls (mean age 10.18±2.5 years). Group C included 27 non-overweight,

Table 1 Genetic associations implicated in the pathogenesis of NAFLD

Gene	Protein function	Association with NAFLD
PNPLA3/adiponutrin	Lipolytic and lipogenic function *in vitro* gene expression enhanced by SREBP-1c	I148M: significantly associated with hepatic fat content, fibrosis, NASH, ALT-AST activities, and HCC
TM6SF2	VLDL secretion	TM6SF2: increases risk of NAFLD and NASH rs58542926: risk of NAFLD and low levels of VLDL
PPARα	Activates fatty acid oxidation and hepatic lipid hydrolysis	Ala227 PPARα variant is associated with lower fat indices in NAFLD patients
MTTP	VLDL synthesis and secretion	493G/T SNP: associated with NAFLD GG homozygous: increased risk of severe steatosis and more atherogenic postprandial lipid profile in NASH patients, independent of insulin resistance
APOC-III	Inhibition of LPL	C482T and T455C variant carriers: increased fasting plasma APOC-III and 60% increased fasting plasma triglycerides 38% of the carriers displayed NAFLD
APOE	Plasma lipid transport protein	APOE - ε3 allele: high incidence in NASH patients APOE - e2 allele: may be protective against NAFLD
Adiponectin	Insulin-sensitizing, anti-inflammatory adipokine	rs767870 ADIPOR2: associated with a 50% decrease in hepatic fat accumulation and decrease in GGT & triglycerides in a Finnish cohort
IRS1	Downstream regulator of insulin action	Gly172Arg polymorphism affects insulin receptor activity and is significantly associated with NAFLD disease severity in Caucasian NAFLD patients
GCKR	Inhibits glycogen synthesis	rs780094 SNP: associated with NAFLD and serum lipids
IL-6	Proinflammatory cytokine	IL-6-174C: correlation with higher NASH incidence & increased insulin resistance in Caucasian race
TNFα	Proinflammatory cytokine	TNFα-238: high prevalence in NAFLD patients

ADIPOR2, adiponectin receptor 2; ALT, alanine aminotransferase; APOC-III, apolipoprotein C-III; APOE, apolipoprotein E; AST, aspartate aminotransferase; GCKR, glucokinase regulatory protein; GGT, γ-glutamyl transferase; HCC, hepatocellular carcinoma; IL-6, interleukin-6; IRS1, insulin receptor substrate 1; LPL, lipoprotein lipase; MTTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PNPLA3, patatin-like phospholipase domain containing 3; PPARα, peroxisome proliferator-activated receptor α; SNP, single nucleotide polymorphisms; SREBP-1c, sterol regulatory element-binding protein-1c; TM6SF2, transmembrane 6 superfamily 2; TNFα, tumor necrosis factor-α; VLDL, very-low-density lipoprotein

nonobese patients (BMI <85th percentile) with NAFLD or NASH, of whom 14 were boys and 13 were girls (mean age 9.15±3.86 years). The patients in this group were selected on the basis of hepatic steatosis detected on liver ultrasonography performed as part of a diagnostic investigation. In addition, a control group (Group D) included 91 age- and sex-matched, phenotypically healthy children and adolescents with normal liver ultrasonography and normal laboratory findings.

All the subjects were tested for secondary causes of steatosis, such as the use of drugs known to precipitate steatosis, whereas viruses were ruled out using the appropriate tests. In all cases, autoimmune liver disease, metabolic liver diseases, Wilson's disease, celiac disease, and alpha-1-antitrypsin deficiency were eliminated by standard clinical, and laboratory evaluations and liver biopsy. The study was performed in accordance with the principles outlined in the 1975 Declaration of Helsinki. Informed consent was obtained from each responsible guardian.

Anthropometric and biochemical measures

Detailed records of previous medical and family histories were reviewed. All the participants underwent a systematic physical examination by the same pediatrician, and a blood sample was collected for determination of biochemical and immunological parameters. Waist circumference (WC) was also assessed. BMI was calculated using the formula, body weight (kg)/height^2 (m^2). Blood pressure was measured using the appropriate cuff size, with the participant in the sitting position. The lipid profile (total cholesterol [TC], low-density lipoprotein-cholesterol [LDL-C], high-density lipoprotein-cholesterol [HDL-C], and triglycerides) was assessed in the morning blood sample obtained after a 12-h fast, using the Abbott Architect c16000 Automatic Biochemistry Analyzer. The homeostasis model assessment of insulin resistance (HOMA-IR) index was used to evaluate insulin resistance. Normal lipid levels were considered as TC values <200 mg/dL, LDL-C levels <130 mg/dL, HDL-C levels >40 mg/dL, triglycerides levels <100 mg/dL for children up to 10 years of age and <130 mg/dL for children >10 years of age.

Genotyping

DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Venlo, Limburg, The Netherlands), and DNA samples were amplified by polymerase chain reaction as previously described (Dutta AK, 2012), using the sense primer 5′-TGGGCCTGAAGTCCGAGGGT-3′ and antisense primer 5′-CCGACACCAGTGCCCTGCAG-3′ for the rs738409 polymorphism and the sense primer 5′-CCTTCCCCTAAACCCACAAT-3′ and the antisense primer 5′-CATGACAGCCCTTTCCTCAT-3′ for the rs2896019 polymorphism [34,35].

Statistical analysis

A descriptive statistical analysis was performed for all the study data. Phenotypic quantitative data were expressed as mean ± standard deviation. The Kolmogorov-Smirnov test was used to test the normality of the distributions of the quantitative variables. For the qualitative variables, frequencies were expressed in absolute values and percentages. Categorical variables were presented as frequencies and percentages (n, [%]). The parametric method of the Student's *t* test was used when the data of the variables followed a normal curve, and the non-parametric method of the Mann-Whitney *U* test was used otherwise. The independence test between the categorical variables was performed using the Fisher χ^2 test for dichotomous variables and with the Pearson χ^2 test for variables characterized by more than 2 levels (3 genotypes to polymorphism). To assess the association between the genotypes and NAFLD or the quantitative traits, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), we used the χ^2 test and logistic regression or multiple regression, adjusting for covariables such as age, HOMA-IR index and BMI. Finally, we used operating characteristic (receiver-operating characteristic, [ROC]) analyses to assess the usefulness of the laboratory values, clinical measures, and the PNPLA3 rs738409 and rs2896019 SNPs as predictors for discriminating between the NAFLD cases and controls. P-values <0.05 were considered statistically significant and, where appropriate, were adjusted for multiple comparisons. For this purpose, the criterion χ^2 was used as a goodness-of-fit test. Data were analyzed using the statistical software Statistical Package for Social Science version 24.0 software for Windows (SPSS Inc., Chicago, Illinois, USA). The HOMA-IR index was calculated as the product of the fasting plasma insulin concentration and the fasting plasma glucose (mg/dL) concentration divided by 405 (HOMA-IR = [insulin × glucose]/405).

Results

Case control association study

The frequency distribution of PNPLA3 rs738409 and rs2896019 SNPs was in Hardy-Weinberg equilibrium. In groups A and C, 58 patients were found to have hepatic steatosis; 11 of them underwent a liver biopsy and were diagnosed as having steatohepatitis. The criteria for liver biopsy in patients with hepatic steatosis were a positive family history for NAFLD, NASH, or diabetes mellitus; abnormal ALT values (>80 IU/mL); and hepatosplenomegaly during clinical examination [36].

rs738409 and rs2896019 polymorphisms in the study population

The frequencies of the PNPLA3 rs738409 and the rs2896019 SNPs in the study population are shown in Table 2. The frequency

of normal homozygotes (rs738409 CC) was found to be higher in the obese patients without liver disease (75.8% in group B and 73.6% in the control group) but was lower in the patients with hepatic steatosis (groups A and C: 35.5% and 7.4%, respectively). The incidence of rs738409 polymorphism (CG+GG) was found to be 58.2% in the patient population (groups A, B, and C), higher than that in the control subjects (27.5%). The frequency of the rs738409 GG genotype was found to be higher (33%) in the patient groups and much lower in the control group (2.2%). In the patients with hepatic steatosis (groups A and C), the frequency of rs738409 polymorphism (CG+GG) was 77.6%, and the frequency of G allele was 46.6% (Table 2).

Hepatic steatosis and steatohepatitis were diagnosed in 90% of the rs738409 GG carriers and in 78.3% of the rs738409 CG carriers. The carriers of the PNPLA3 risk allele (rs378409 GG) showed the highest probability for developing liver disease (odds ratio [OR] 20.73, 95% confidence interval [CI] 7.38-58.23, P<0.001; and OR 7.4, 95%CI 0.4-125.8, P<0.001 after logistic regression), in contrast to the carriers of the wild allele (CC: OR 0.1, 95%CI 0.05-0.21; P<0.001, and OR 0.01, 95%CI 0.001-0.29; P<0.001 after logistic regression; Table 3).

Regarding the rs2896019 polymorphism, the incidence of normal homozygotes (TT) was found to be greater in the obese patients (groups A and B) and in the control group (71%, 72.7% and 80.2%, respectively), and smaller in the normal-weight patients with NAFLD (33.3%). Correspondingly, the incidence of the rs2896019 polymorphism (TG+GG) was 39.6% in the patient population (group A, B, and C), greater than in control subjects (19.8%). In the patients with hepatic steatosis (groups A and C), the frequency of the rs2896019 polymorphism (TG+GG) was 46.6% (Table 2).

NAFLD was diagnosed in 45.5% of the normal homozygotes (TT) and 64.7% of the heterozygotes (TG). The TG genotype was strongly associated with NAFLD (OR 3.06, 95%CI 1.56-6.01; P<0.001, and OR 2.07, 95%CI 0.1-38.7; P<0.001 after logistic regression; Table 4). The combined presence of the 2 polymorphisms, as found in 27 patients, regardless of the combination of the alleles, increased the probability for developing liver disease from 2 to 8 times higher (OR 4.27, 95%Cl 2.12-8.58; P<0.001).

The mean ages at NAFLD and NASH diagnosis were 9.85±3.25 years (n=47) and 9.82±3.13 years (n=11), respectively. The mean age at NAFLD diagnosis in the carriers of both risk alleles of rs738409 SNP was 1 year younger than that in the non-carriers, whereas the mean age at NASH diagnosis was 2.7 years (P<0.001). Regarding the rs2896019 polymorphism, comparisons between the carriers and non-carriers of both risk alleles showed an earlier age at diagnosis of liver disease (1.5 years earlier for NAFLD and 1.6 years for NASH, P=0.754) The patients with a combination of genotypes of the 2 polymorphisms (heterozygous or homozygous form) had a younger age at NAFLD and NASH diagnosis, at the statistical significance level of P=0.096. The age difference in the diagnosis of hepatic steatosis and steatohepatitis was more pronounced in the group of patients with lean NAFLD (group C).

Association of rs738409 and rs2896019 with the clinical and anthropometric parameters

The patients with the rs738409 CG and rs738409 GG genotype showed a statistically significantly lower mean BMI than the patients with the rs738409 CC genotype (21.99±6.1 kg/m^2 and 21.33±7.7 kg/m^2 vs. 26.97±4.2 kg/m^2, P<0.001). The mean BMI for the carriers of rs2896019 polymorphism was 22.4±9.4 kg/m^2 for those carrying the rs2896019 GG genotype and 21.96±8.2 kg/m^2 for those carrying the rs2896019 TG genotype. A higher mean BMI (25.1±4.99 kg/m2) was found in the patients with genotype rs2896019 TT (Tables 3 and 4).

No significant correlation was detected between the rs738409 and rs2896019 polymorphisms and the mean WC, systolic blood pressure (SBP) or diastolic blood pressure (Tables 3 and 4).

Association of rs738409 and rs2896019 with the laboratory findings

Children homozygous for the PNPLA3 minor allele (rs738409 GG) showed a stronger correlation between AST,

Table 2 Frequency of the rs738409 and rs2896019 SNPs in the study population

SNP	Group A	/31	Group B	/33	Group C	/27	Control Group	/91	Study Population	/182
CC	35.5%	11	75.8%	25	7.4%	2	73.6%	67	57.7%	105
CG	29%	9	15.2%	5	33.3%	9	24.2%	22	24.7%	45
GG	35.5%	11	9%	3	59.3%	16	2.2%	2	17.6%	32
CG+GG	64.5%	20	24.2%	8	92.6%	25	27.5%	25	42.3%	77
TT	71%	22	72.7%	24	33.3%	9	80.2%	73	70.3%	128
TG	29%	9	24.3%	8	63%	17	19.8%	18	28.6%	52
GG	0%	0	3%	1	3.7%	1	0%	0	1.1%	2
TG & GG	29%	9	27.3%	9	66.7%	18	19.8%	18	29.7%	54

SNP, single-nucleotide polymorphism

Table 3 Correlations of the rs738409 genotypes with the clinical and laboratory findings

Characteristics	CC	P-value	OR 95%CI	CG	P-value	OR 95%CI	GG	P-value	OR 95%CI
NAFLD/ NASH (%)	13 (34)	<0.001	0.1 0.05-0.21	18 (78)	0.199		27 (90)	<0.001	20.73 7.38-58.23
Age (years)	10.39±2.7	0.245		10±3.4	0.433		9.4±3	0.313	
Male (%)	23 (60.5)	0.669		13 (56.5)	>0.99		16 (53.3)	0.656	
BMI (kg/m^2)	26.97±4.2	0.663		21.99±6.1	<0.001	4.98 1.09-8.87	21.33±7.7	<0.001	5.63 2.04-9.23
WC (cm)	74.3±12.7	0.195		69.8±13.2	0.426		68.2±16.8	0.857	
SABP (mmHg)	106.4±4.3	0.997		106.5±13.4	0.997		106.7±15.9	0.997	
DABP (mmHg)	61.8±12.2	0.667		63.5±11.1	0.667		60.8±9.3	0.667	
ALT (IU/mL)	35.6±47.3	<0.001	0.14 0.06-0.35	49.78±49	0.503		59.1±52.2	<0.001	7.82 3.32-18.44
AST (IU/mL)	30.8±26	<0.001	0.11 0.03-0.40	36.34±21	0.574		43.4±29.43	<0.001	7.12 2.60-19.48
TC (mg/dL)	174.5±30.8	0.473		151.43±41.25	0.301		165.83±31.49	0.032	2.61 1.15-5.97
Triglycerides (mg/dL)	109.1±79.7	0.113		84.7±35.7	0.764		105±61.5	0.010	4.39 1.5-12.86
HDL-C (mg/dL)	49.8±18.2	0.109		40.3±10.6	0.031	2.98 1.19-7.46	44.8±12.22	0.770	
LDL-C (mg/dL)	107.1±23.3	<0.001	0.32 0.17-0.61	92±34.75	>0.99		97.3±24.7	<0.001	6.26 2.73-14.39
HOMA-IR index	1.86±2	0.008	0.35 0.17-0.76	1.4±1.6	0.284		2.15±2.7	<0.001	6.89 2.97-16.03

Data are represented as the mean ± SD (25th, 50th, and 75th percentile). P-values were obtained by comparing the quantitative phenotype between 2 groups, CC–CG and CC–GG (Mann-Whitney *U* test).

*Male/female ratio was analyzed by Fisher's exact test

AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; DABP, diastolic arterial blood pressure; HDL-C, high-density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OR, odds ratio; SABP, systolic arterial blood pressure; SD, standard deviation; TC, total cholesterol; WC, waist circumference

ALT, TC, triglycerides, and LDL-C levels. The carriers of the rs738409 G allele were found to have moderate-to-severe insulin resistance as compared with the carriers of the wild allele (OR 6.89, 95%CI 2.97-16.03; P<0.001 vs. OR 0.35, 95%CI 0.17-0.76; P=0.008; Table 3). The rs2896019 TG allele was observed to increase the probability of elevated ALT values (OR 2.95, 95%CI 1.36-6.44; P=0.010). A statistically significant correlation was found between abnormal HOMA-IR index values and the TG genotype (OR 2.59, 95%CI 1.21-5.57; P=0.021). No statistically significant correlation was found between high AST, TC, triglycerides or HDL-C levels and the 3 genotypes of the rs2896019 polymorphism (Table 4).

Patients with NAFLD and lean NAFLD

Comparison of the clinical and laboratory parameters of groups A and C (obese and nonobese children with hepatic steatosis or steatohepatitis) revealed several differences, some of which were assessed as statistically significant. The frequencies of the rs738409 CC and rs2896019 TT genotypes were higher in the obese group (P<0.001), whereas that of the rs2896019 TG genotype was higher in the normal-weight group (P<0.001). No significant statistical difference was found between the mean age at diagnosis and the sex of patients in the 2 groups (P=0.123). We found no significant differences between the AST and ALT levels in both groups (P=0.723 and P=0.526 respectively; Table 5). The lowest HOMA-IR index values were observed in the patients with lean NAFLD (P=0.067; Table 5).

ROC curve for the rs738409 and rs2896019 SNPs

To identify the children at higher risk for developing liver disease, we performed a ROC curve analysis. The first

Table 4 Correlations of the rs2896019 genotypes with the clinical and laboratory findings

Characteristics	TT	P-value	OR 95%CI	TG	P-value	OR 95%CI	GG	P-value
NAFLD/NASH (%)	31 (56.4)	<0.001	0.32 0.16-0.62	26 (76.5)	<0.001	3.06 1.56-6.01	1 (50)	0.537
Age (years)	10.38±2.7	0.754		9.32±3.3	0.245		9.5±4.95	0.125
Male (%)	34 (61.8)	>0.99		17 (50)	0.876		1 (50)	0.765
BMI (kg/m^2)	25.1±4.99	0.085		21,96±8.2	0.825		22.4±9.4	0,799
WC (cm)	71.5±12.3	0.478		69.99±16.8	0.405		82.5±28.8	0.183
SABP (mmHg)	106.2±13.8	0.620		106.4±15.3	0.620		116.5±26.2	0.620
DABP (mmHg)	61.4±10.8	0.411		63.2±11.5	0.411		53.5±2.1	0.411
ALT (IU/mL)	45.6±52.2	0.012	0.36 0.17-0.79	50.6±47.7	0.010	2.95 1.36-6.44	21±8.5	>0.99
AST (IU/mL)	34.2±25.8	0.109		40.8±27.8	0.064		21.5±2.1	>0.99
TC (mg/dL)	165±39	0.697		167.9±27.9	0.556		152.5±13.4	>0.99
Triglycerides (mg/dL)	98±72	0.781		108.4±53.8	>0.99		83.5±57.3	>0.99
HDL-C (mg/dL)	47.4±16.7	0.464		42.6±11.8	0.451		53.5±16.3	>0.99
LDL-C (mg/dL)	100±30.8	0.025	0.45 0.24-0.88	101±21.8	0.058	1.92 0.99-3.73	82±18.4	0.111
HOMA-IR index	1.6±1.8	0.012	2.8 1.31-6.01	2.2±2.7	0.021	2.59 1.21-5.57	1.3±1.3	0.348

Data are represented as the mean ± SD (25th, 50th, and 75th percentile). P-values were obtained by comparing the quantitative phenotype between 2 groups, CC–CG and CC–GG (Mann-Whitney *U* test)

*Male/female ratio was analyzed by Fisher's exact test

AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; DABP, diastolic arterial blood pressure; HDL-C, high-density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OR, odds ratio; SABP, systolic arterial blood pressure; SD, standard deviation; TC, total cholesterol; WC, waist circumference

approach concerned the assessment of prognostic capacity based individually on gene testing (genotypes CG and GG of the rs738409 polymorphism and genotypes TG and GG for the rs2896019 polymorphism). The area under the curve (AUC) index was 0.804, with a minimum value of 0.5 and an excellent value of 1.00. Predictiveness assessment was performed with a χ^2 test (OR 24.37, 95%CI 7.9-74.9; P<0.001; Fig. 1).

The second approach assessed the prognostic ability by combining genetic testing with laboratory findings (ALT and HOMA-IR index). The AUC was 0.998 (OR 3477, 95%CI 308.9-39139.6; P<0.001; Fig. 2).

Discussion

In this study, we evaluated the effects of PNPLA3 rs738409 and rs2896019 SNPs on the development of hepatic steatosis in children and adolescents of Greek origin and the probability of early detection of patients at higher risk for developing liver disease. To our knowledge, this is the first report of the association of rs2896019 polymorphism with hepatic steatosis in obese and nonobese pediatric patients, and the combined study of rs738409 and rs2896019 polymorphisms exclusively in the pediatric population.

The total frequencies of the rs738409 (CG+GG) and rs2896019 (TG+GG) genotypes in the control group (27.5% and 19.8%, respectively) are in line with the frequencies reported for the European population (23.3% and 20.4%, respectively) by the National Centre for Biotechnology Information [37,38]. The frequency of the rs738409 G allele in our study agrees with other studies conducted on children worldwide (Table 6) [39-43]. The frequencies of the rs738409 polymorphism genotypes and their association with hepatic steatosis and steatohepatitis are consistent with studies involving other white populations from Italy [33], Finland [10], Germany [44], England and Switzerland [45], as well in populations of Spanish [46] and Asian origins [47,48]. There are no relevant literature data for the comparison of the frequency of the rs2896019 polymorphism in European pediatric populations. Most references relate mainly to adult patients of Asian descent, such as the studies by Islek *et al*, Kitamoto *et al*, and Kawaguchi *et al* [35,49,50].

In the multivariate logistic regression analysis, we observed that both rs738409 and rs2896019 were significantly associated with fatty liver, which indicates that the carriers of the G allele (OR 7.4, 95%CI 0.4-125.8, P<0.001; and OR 2.07, 95%CI 0.1-38.7, P<0.001, respectively) were more

Table 5 Comparison of various quantitative phenotypes among the different genotypes at rs738409 and rs2896019 in PNPLA3 in the obese and nonobese patients with NAFLD

Characteristics	BMI>85th percentile (n=31)	BMI <85th percentile (n=27)	P (lean to non-lean)
Age of diagnosis (years)	10.45±2.4	9.15±3.9	0.123
Male (%)	21 (67.7)	14 (51.9)	0.285
rs738409 CC (%)	11 (35.5)	2 (7.4)	0.013
rs738409 CG (%)	9 (29)	9 (33.3)	0.113
rs738409 GG (%)	11 (35.5)	16 (59.3)	0.781
rs2896019 TT (%)	22 (71)	9 (33.3)	0.008
rs2896019 TG (%)	9 (29)	17 (63)	0.017
rs2896019 GG (%)	0 (0)	1 (3.7)	0.466
BMI (kg/m^2)	27.3±5	15.5±1.5	<0.001
WC (cm)	75.9±13.9	58.2±8.1	<0.001
SABP (mmHg)	112.6±13.9	97.6±6.3	<0.001
DABP (mmHg)	65.5±10.9	57.1±5.4	0.001
AST (IU/mL)	41.1±32.1	44±30.5	0.723
ALT (IU/mL)	65.6±62.9	55.8±52.2	0.526
TC (mg/dL)	161.9±39.9	160.7±38	0.905
Triglycerides (mg/dL)	108.4±83.4	90.9±50.9	0.347
HDL-C (mg/dL)	44.6±17.2	45±10.8	0.927
LDL-C (mg/dL)	98.5±31.56	93.9±28.3	0.574
HOMA-IR	2.5±3	1.4±1.2	0.067

Data are represented as the mean ± SD (25th, 50th, and 75th percentile). P-values were obtained by comparing the quantitative phenotype between 2 groups, (Mann-Whitney *U* test)

*Male/female ratio was analyzed by Fisher's exact test

AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; DABP, diastolic arterial blood pressure; HDL-C, high-density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-C, low-density lipoprotein; SABP, systolic arterial blood pressure; TC, total cholesterol; WC, waist circumference

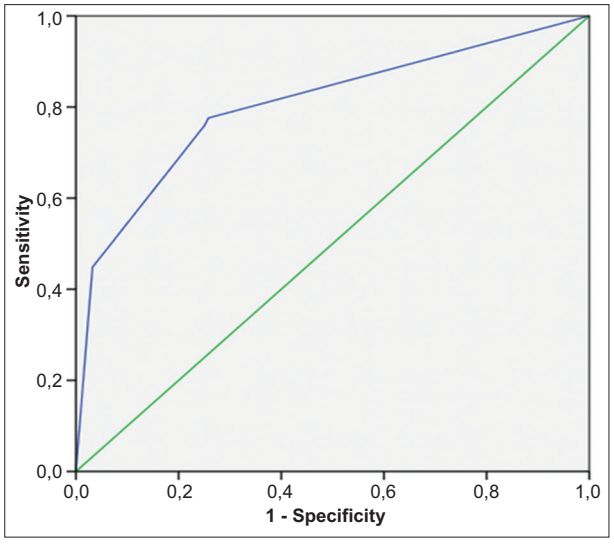

Figure 1 Receiver operating characteristic curve for predictive ability of the rs738409 and rs2896019 single-nucleotide polymorphisms

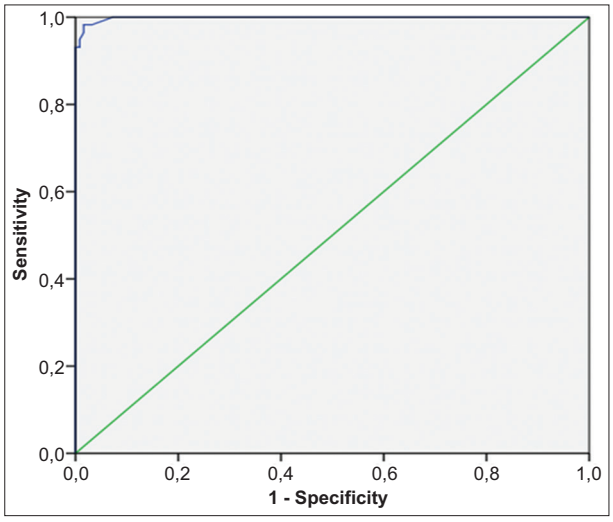

Figure 2 Receiver operating characteristic curve for predictive ability of the rs738409 and rs2896019 single-nucleotide polymorphisms combined with alanine aminotransferase level and homeostatic model assessment for insulin resistance

Table 6 The frequency of the rs738409 G allele in the pediatric population

Study [ref.]	Year	Population	N	Location	Age	rs738409 SNP risk allele (G)
Valenti *et al* [67]	2010	NAFLD Clinic	149	Italy	6-13	37.5%
Rossi *et al* [39]	2012	NAFLD Clinic	118	Italy	mean 10.2	48.3%
Romeo *et al* [33]	2010	Obesity Clinic	475	Italy	mean 10	45.9%
Goran *et al* [46]	2010	Hispanic children in General Clinical Research Centre	188	USA	8-18	51.5%
Santoro *et al* [52]	2010	Pediatric Obesity Clinic	85	USA	8-18	32%
Giudice *et al* [40]	2011	Childhood obesity service	1048	Italy	2-16	29%
Lin *et al* [41]	2011	Obese children recruited from school	520	Taiwan	6-18	39.2%
Carrasco *et al* [66]	2013	Summer camp	1037	Mexico	6-12	61%
Viitasalo *et al* [64]	2015	Children enrolled in school	481	Finland	6-8	41.2%
Mangge *et al* [42]	2015	Overweight/obese Caucasians	510	Austria	10-20	41.8%
Wang *et al* [43]	2016	Overweight and obese children from school	1093	China	7-18	32%

NAFLD, nonalcoholic fatty liver disease; SNP, single nucleotide polymorphisms; USA, United States of America

likely to have NAFLD than the non-carriers, independently of age, sex, BMI and HOMA-IR index. The conclusions of the present study agree with the literature on the association of the rs738409 polymorphism with hepatic steatosis, as found in several studies in both adult and pediatric patients [10,33,44,51-53]. Although the literature data on the rs2896019 polymorphism are limited, studies in Caucasian, Hispanic, and Asian populations also correlate the TG and GG genotypes of the rs2896019 polymorphism with hepatic steatosis [49,50,54].

In the present study, we also focused on the role of adiponutrin gene polymorphisms in both obese and nonobese patients with hepatic steatosis. The incidence of normal homozygotes of the 2 polymorphisms was greater in the obese group without liver disease (75.8% and 72.7%), whereas higher incidences of the rs738409 CG+GG and rs2896019 TG+GG genotypes were observed in the patients with normal BMI values (BMI <85th percentile) and hepatic steatosis or steatohepatitis (92.6% and 66.7%, respectively). Adiponutrin gene polymorphisms were not correlated with the clinical features of metabolic syndrome (BMI, WC, SBP, DBP). Although obesity continues to be a major risk factor for hepatic steatosis, the genetic background appears to burden both obese and nonobese patients in terms of both the early onset and the severity of the disease.

The incidence of hepatic steatosis in the normal-weight patients in this study population was 14.8%, consistent with the incidences of hepatic steatosis reported in other studies of normal-weight patients (lean NAFLD) belonging to white or Asian populations [55-61]. The presence of the rs738409 CG and rs738409 GG genotypes could be related to the increased morbidity observed in the patients with normal BMI and hepatic steatosis, considering the role of the polymorphism in the process of lipogenesis, in the accumulation of triglycerides in the hepatic cells, and in the inhibition of lipophagy, which occurs regardless of total body fat.

Regarding the patients' biochemical test results, the rs738409 G allele was significantly associated with the serum levels of both AST and ALT, whereas the rs2896019 G allele was associated only with the ALT levels. Several studies have correlated elevated aminotransferase levels, particularly ALT, with the presence of the 2 polymorphisms, although significant population variations have been observed [33,47,62-65]. In studies in the pediatric population, Goran *et al* [46] and Carrasco *et al* [66] also found a significant correlation between liver enzyme levels, especially ALT, and the rs738409 GG genotype, in both obese and normal-weight children ($P<0.001$).

Drawing conclusions from the present study, we can say that the sample of the present study was quantitatively satisfactory for conducting a monocentric study in children and adolescents of Greek descent who have hepatic steatosis. However, indications of the low frequency of the rs2896019 GG genotype in the Greek population certainly exist, and a multicenter study may be required to draw safe conclusions about this particular genotype.

The clinical usefulness of our findings lies in the investigation of the role of adiponutrin polymorphisms, a powerful genetic factor in liver steatosis, in children and adolescents of Greek origin. Such a study is needed in the Greek population to determine the frequency of the polymorphisms of the adiponutrin gene, given its large geographical variation. Understanding the contribution of the adiponutrin gene to the natural history of hepatic steatosis could be crucial for developing future preventive or therapeutic strategies aimed at preventing the progression of hepatic steatosis to more severe forms of liver disease. The high prevalence of hepatic steatosis in the general population and the progression of the disease to steatohepatitis, cirrhosis, and hepatocellular carcinoma, which increases morbidity and mortality in adulthood, are a major challenge for the existing health infrastructure.

Summary Box

What is already known:

- Children with obesity have at least a 38% chance of developing fatty liver disease; variations in several genes contribute to the risk of developing hepatic steatosis
- Data indicate that the rs738409 patatin-like phospholipase domain-containing protein 3 (PNPLA3) single nucleotide polymorphism (SNP) is associated with hepatic steatosis
- The prevalence of PNPLA3 polymorphisms differs between geographic areas
- The rs2896019 polymorphism may also be involved in the pathogenesis of hepatic steatosis

What the new findings are:

- The frequencies of the rs738409 and rs2896019 PNPLA3 variants were greater in obese and nonobese pediatric patients of Greek origin with hepatic steatosis
- Earlier diagnosis of hepatic steatosis and steatohepatitis was necessary in nonobese carriers of both SNPs
- Obese children with a normal genotype were less likely to develop hepatic steatosis early in life
- Nonobese pediatric patients with hepatic steatosis had predominantly abnormal genotypes

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