HHS Public Access

Author manuscript

Liver Int. Author manuscript; available in PMC 2023 June 01.

Published in final edited form as:

Liver Int. 2022 June ; 42(6): 1470–1474. doi:10.1111/liv.15268.

Frequency of the *PNPLA3* rs738409 polymorphism and other genetic loci for liver disease in a Guatemalan adult population

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Keywords

Genetics; NAFLD; *PNPLA3*; Guatemala; Latinos

Introduction

Spurred by the epidemics of diabetes and obesity, nonalcoholic fatty liver disease (NAFLD) has become one of the most common chronic liver conditions in the world.[1] In the Americas, individuals of Latino ancestry experience disproportionate rates of NAFLD and other liver disorders.[2] Genetic susceptibility to NAFLD has been identified, with the I149M polymorphism (rs738409) in the patatin-like phospholipase gene *PNPLA3* found to be most consistently associated. A meta-analysis found that individuals homozygous for the risk allele (G) have a more than 3.3-fold greater risk of NAFLD.[3] Associations of the *PNPLA3* rs738409 polymorphism have also been reported with elevated liver enzymes,

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liver inflammation, fibrosis, and liver cancer.[4] In the U.S., the frequency of the *PNPLA3* rs738409 risk allele mirrors the prevalence of NAFLD, with Latinos having the highest prevalence. Variants in other loci previously associated with NAFLD include *NCAN* rs2228603, *GCKR* rs780094 and L *YPLAL1* rs12137855, as well as others.[5] Most genetic susceptibility studies, however, have been conducted among European or Asian populations, with relatively few conducted among Latino populations.[3]

Guatemala has very high rates of liver disease, obesity, diabetes, and elevated liver enzymes (suggestive of NAFLD).[6] However, to our knowledge, genetic susceptibility to liver disease among Guatemalans has not been previously examined. The main objective of this study was to examine the prevalence of genetic variants previously associated with liver disease, and the associations of these variants with subclinical liver disease.

Methods

Study population

The current study, conducted in 2016 in five Guatemalan communities, has been described in detail previously.[6] In brief, 461 adults (≥ 40 years old) were recruited through household visits, of whom 444 providing blood samples. Participants were excluded if they were hepatitis B virus (HBV) or hepatitis C virus (HCV) seropositive (n=5); they reported high alcohol consumption (≥ 7 drinks per week among women and ≥14 drinks per week among men) (n=14); or they were missing alanine aminotransferase (ALT), aspartate aminotransferase (AST) or gamma-glutamyl transferase (GGT) determinations (n=2). The final analytical sample consisted of 403 individuals. The study was approved by institutional review boards of the Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) (IRB #6877) and the Institute of Nutrition of Central America and Panama (INCAP) (Guatemala City, Guatemala) (IRB #053–2015).

Data collection

Study participants were interviewed by trained staff using structured, validated questionnaires. Height, weight and waist circumference were measured using standard protocols.[6] Body mass index (BMI) was calculated as weight in kg divided by height in meters squared (kg/m^2). Fasting blood samples were obtained by trained phlebotomists.

Laboratory assessments

Glucose was measured in plasma, while triglycerides, ALT, AST and GGT were measured in serum. All analytes were assayed on a Cobas c111 clinical chemistry analyzer (Roche Diagnostics, Basel, Switzerland). Seropositivity for HBV was determined by the presence of HBV surface antigen (HBsAg) and seropositivity for HCV was determined by the presence of HCV antibodies (anti-HCV). HBsAg and anti-HCV were assessed in the Hepatitis Diagnostic Laboratory of Hannover Medical School (Hannover, Germany).

DNA extraction

Genomic DNA extractions from 400 uL of frozen whole blood were performed following standard operating procedures for the QIASymphony Midi kit (Qiagen, Germantown, MD).

MassARRAY genotyping

Genotyping of *PNPLA3* rs738409, *NCAN* rs2228603, *GCKR* rs780094, *LPRLAL1* rs12137855 and 59 ancestry informative SNPs was performed using single base extension (Sequenom, San Diego, CA). Assay Design Suite, V2.0 (Agena Bioscience, San Diego, CA) was used to design amplification and extension primers. PCR, neutralization of unincorporated dNTPs and single base extension were performed using the iPLEX Gold Reagent kit (Agena Bioscience, San Diego, CA). Data were generated with Spectro ACQUIRE software version 3.0 and analyzed using Typer software, version 4.0.22 (Sequenom). Genotyping was performed at the Johns Hopkins Institute for Clinical & Translational Research (ICTR) laboratory.

Definition of liver disease

The primary measure of liver disease was an elevated level of ALT or AST, hereafter referred to as elevated ALT/AST. Elevated ALT was defined as >41 U/L among men and >33 IU/L among women. Elevated AST was defined as >40 U/L among men and >32 U/L among women. In the absence of excessive alcohol consumption and HBV or HCV infection, elevated ALT/AST would be clinically classified as NAFLD. Secondary liver measures included the fatty liver index (FLI) and the hepatic steatosis index (HSI).

Statistical analysis

Descriptive statistics were calculated overall and by elevated ALT/AST status. Generalized linear regression with a quasibinomial link function was used to predict missing SNP calls. Logistic and linear regression models were used to examine the associations between each SNP and elevated ALT/AST, the other liver measures and metabolic conditions (diabetes, obesity, central obesity and metabolic syndrome). The models were adjusted for age, sex, BMI (except for the liver indices, central obesity and obesity), alcohol consumption, and the first 5 principal components generated from the ancestry informative SNPs. For genetic variants significantly associated with elevated ALT/AST, the attributable fraction of elevated ALT/AST was calculated using Levin's formula.[7] The risk ratio was derived from the odds ratio of the logistic model using the formula by Zhang et al.[8] All tests of significance were 2-sided and p-values less than 0.05 were considered statistically significant. Statistical analyses were conducted in SAS v9.4 (SAS Institute, Cary, NC) and R statistical software v4.0.5.

Results

The study population was composed of 60.3% women and 54.6% individuals who self-identified as indigenous. The overall prevalence of elevated ALT/AST was 31.3% but was significantly more common among women (39.5%) than men (18.8%) ($p<0.001$). There was no significant difference in elevated ALT/AST by self-reported ethnicity. Persons with elevated ALT/AST had a significantly higher mean BMI ($p<0.001$), and waist circumference ($p<.001$) and were more likely to have metabolic syndrome ($p<.001$).

As shown in Table 2, the frequencies of the previously reported risk allele at each loci were: *PNPLA3* rs738409 G allele = 0.69, *NCAN* rs2228603 T allele = 0.01, *GCKR*

rs780094 T allele = 0.39 and *LYPLAL1* rs12137855 C allele=0.94. The rs738409 G allele frequency was particularly high (0.79) among persons who self-identified as indigenous compared to the persons who did not (0.64). After adjusting for potential confounders, the *PNPLA3* rs738409 G allele was significantly associated with elevated ALT/AST (OR=2.04, 95%CI 1.38, 3.03). In contrast, none of the remaining SNP's was associated with elevated ALT/AST. In addition, there were no associations between any of the polymorphisms and the other liver measures or the metabolic conditions. The attributable fraction of elevated ALT/AST due to the *PNPLA3* rs738409 G allele was estimated to be 33%.

Discussion

To our knowledge, the current study demonstrates for the first time the association between the *PNPLA3* rs738409 polymorphism and elevated ALT/AST among a Guatemalan population. These results are important given the disproportionate burden of liver disease morbidity and mortality among Guatemalans and other Latino populations, and the limited data available on genetic susceptibility among this group. Further, the results demonstrate an exceptionally high prevalence of the risk variant (0.69) among Guatemalan adults. In contrast, the frequency of the rs738409 G allele in the U.S. general population is 0.25,[5] and in the U.S. Mexican-American population is 0.54,[5] while the 1000 Genome project, in examining other Latino groups, reported frequencies of 0.55 among Mexicans, and 0.41 among Colombians.

The *PNPLA3* rs738409 variant has been consistently found to be significantly associated with many liver-related outcomes. Persons heterozygous for the risk allele have a two-fold higher risk of liver disease, while persons who are homozygous have a greater than threefold risk.[3] In Guatemala, we estimate that >30% of NAFLD among adults may be related to rs738409. Associations between rs738409 and the two fatty liver indices, FLI and HSI, were not evident, perhaps because the mechanisms by which the *PNPLA3* polymorphism predisposes to liver fat accumulation and subsequent injury are related to accumulation of ubiquitylation resistant PNPLA3 protein in lipid droplets.[9] Significant associations between *PNPLA3* rs738409 and liver enzymes or hepatic steatosis, and modest effects on other metabolic traits have been previously reported,[10] suggesting genetic heterogeneity in both fatty liver disease and other metabolic traits.

Strengths of the current study include that the participants were broadly representative of the Guatemalan population, were recruited from different departments of Guatemala, and from both urban and rural areas. Limitations include that the testing of interactions between the genetic variants and obesity could not be done due to a relatively small sample size. In addition, the study relied on surrogate markers of liver disease, although the markers have been widely used in population studies. In addition, since the study was undertaken, more liver disease related SNPs have been reported.

The Latino population in the Americas is composed of persons from different ancestral groups which vary by country. These findings highlight the necessity of examining genetic susceptibility among individual Latino ancestries. In addition, studies are needed to examine the role of genetic testing to inform prevention and management of persons at risk of,

or with, NAFLD, and to understand whether environmental factors modulate genetic predisposition.

Acknowledgments

This work was supported by National Institutes of Health grants P30CA006973–52S3, 1UL1RR025005, ML was supported also by the Linda Kao Award from the Johns Hopkins School of Public Health. This study was also funded in part by the Intramural Research Program of the National Cancer Institute.

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Table 1.

Study participant characteristics overall and by elevated ALT/AST status

	Overall (n=403)		Elevated ALT or AST				
			No (n=277)		Yes (n=126)		*P-value* [1]
	mean	**(SD)**	**mean**	**(SD)**	**mean**	**(SD)**	
Age (years), mean (SD)	55.4	10.6	56.1	11.2	53.9	9.1	0.13
BMI (kg/m^2)	28.0	5.5	27.4	5.6	29.4	5.0	*<0.001*
ALT (U/L), mean (SD)	30.0	19.2	21.6	6.5	48.5	24.3	*<0.001*
AST (U/L), mean (SD)	31.8	17.4	24.0	5.2	49.0	21.9	*<0.001*
GGT (U/L), mean (SD)	49.4	43.2	36.8	27.6	77.3	56.4	*<0.001*
Waist circumference (cm)	94.3	12.5	92.9	12.8	97.4	11.4	*<0.001*
Triglycerides	226.5	125.5	221.12	119.8	238.2	136.8	*0.17*
	N	**(%)**	**N**	**(%)**	**N**	**(%)**	***P-value*** [2]
Sex							
Men	160	39.7	130	46.9	30	23.8	*<0.001*
Women	243	60.3	147	53.1	96	76.2	
Ethnicity (self-reported)							
Indigenous	220	54.6	155	56.0	65	52.0	0.46
Non-Indigenous	182	45.2	122	44.0	60	48.0	
Unknown	1						
Alcohol (drinks/week)							
Never	117	29.0	72	26.0	45	35.7	0.08
Former	206	51.1	144	52.0	62	49.2	
Current [3]	80	19.9	61	22.0	19	15.1	
Smoking							
Never	236	58.6	146	52.7	90	71.4	*<0.001*
Former	138	34.2	103	37.2	35	27.8	
Current	29	7.2	28	10.1	1	0.8	
Elevated FLI [4]							
No	155	39.0	130	48.0	25	19.8	*<0.001*
Yes	242	61.0	141	52.0	101	80.2	
Unknown	6						
Elevated HSI [5]							
No	149	37.3	126	46.2	23	18.3	*<0.001*
Yes	250	62.6	147	53.9	103	81.8	
Unknown	4						
Diabetes [6]							
No	318	78.9	218	78.7	100	79.4	0.88
Yes	85	21.1	59	21.3	26	20.6	

	Overall (n=403)		Elevated ALT or AST				*P-value* [1]
			No (n=277)		Yes (n=126)		
	mean	**(SD)**	**mean**	**(SD)**	**mean**	**(SD)**	
Metabolic Syndrome [7]							
No	145	36.0	121	43.7	24	19.1	*<0.001*
Yes	258	64.0	156	56.3	102	81.0	

[1] P-value for No vs Yes elevated ALT/AST calculated using the Wilcoxon test

[2] P-value for No vs Yes elevated ALT/AST calculated using the Chi-square test

[3] Current drinkers: women <7 drinks/week; men <14 drinks/week

[4] FLI was calculated as: (e0.953 × loge(triglycerides) + 0.139 × BMI + 0.718 × loge(GGT) + 0.053 × waist circumference − 15.745) / (1 + e0.953 × loge(triglycerides) + 0.139 × BMI + 0.718 × loge(GGT) + 0.053 × waist circumference − 15.745) × 100

[5] HSI was calculated as 8 ×(ALT/AST ratio) + BMI (+2, if female; +2, if diabetes mellitus)

[6] Diabetes defined as self-reported diagnosis by a physician, a fasting glucose level >=126 mg/dL or self-reported use of diabetic medications

[7] Metabolic syndrome defined as the presence of central obesity + 2 or more of the following measured abnormalities: serum triglycerides =150 mg/dl, HDL cholesterol <40 mg/dl in men or <50 mg/dl in women, blood pressure >130/85 mmHg, or serum glucose >100 mg/dl

Table 2.

Allele frequencies, odds ratios (OR) and 95% confidence intervals (95%CI) comparing the genetic loci and elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST) levels.

	Overall[1]		Elevated ALT/AST					
			No		Yes		OR[2]	(95%CI)
	N	%	N	%	N	%		
PNPLA3 rs738409								
GG	196	(48.6)	115	(41.5)	81	(64.3)		
CG	163	(40.5)	126	(45.5)	37	(29.4)		
CC	44	(10.9)	36	(13.0)	8	(6.4)	2.04	(1.38, 3.03)
G allele freq	0.69		0.64		0.79			
NCAN rs2228603								
CC	397	(98.5)	273	(98.6)	124	(98.4)		
TC	6	(1.5)	4	(1.4)	2	(1.6)	0.70	(0.11, 4.59)
T allele freq	0.01		0.01		0.02			
GCKR rs780094								
CC	151	(39.2)	108	(40.6)	43	(36.1)		
TC	171	(44.4)	115	(43.2)	56	(47.1)		
TT	63	(16.4)	43	(16.2)	20	(16.8)	0.87	(0.62, 1.20)
T allele freq	0.39		0.38		0.40			
LYPLAL1 rs12137855								
CC	356	(88.3)	240	(86.6)	116	(92.1)		
CT	47	(11.7)	37	(13.4)	10	(7.9)	1.94	(0.87, 4.30)
C allele freq	0.94		0.93		0.96			

[1] Frequencies may not represent total number of study participants due to missing covariates

[2] Adjusted for age, sex, low/moderate alcohol consumption, ancestry informative markers and BMI (except for the liver indices, central obesity and obesity)