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Association of*PNPLA3* with non-alcoholic fatty liver disease in a minority cohort: the Insulin Resistance Atherosclerosis Family Study

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Abstract

Background—Non-alcoholic fatty liver disease (NAFLD) is a highly prevalent condition, particularly among Hispanic Americans. A genetic variant in *PNPLA3* (rs738409) has been identified as a strong predictor of hepatic fat content.

Aims—To confirm the association of this variant with NAFLD in two minority cohorts, Hispanic Americans and African Americans, in whom liver density was quantified by computed tomography (CT).

Methods—This analysis was conducted in the Insulin Resistance Atherosclerosis (IRAS) Family Study. Participants were recruited from the general community and included 843 Hispanic American and 371 African American adults aged 18–81 years. A single variant in *PNPLA3* (rs738409) was genotyped. Liver density was calculated in Hounsfield Units from abdominal CT scans.

Results—Single nucleotide polymorphism (SNP) rs738409 was strongly associated with reduced liver density (i.e. NAFLD) in Hispanic Americans (1.18×10^{-9}) and in African Americans ($P = 4.99 \times 10^{-6}$). The association followed an additive genetic model with the G allele conferring risk. The allele was two times more common in Hispanic Americans than in African Americans (40 vs 19%), consistent with the greater prevalence of NAFLD in Hispanic Americans (24 vs 9%). The SNP explained 4.4 and 5.6% of the variance of the adjusted liver density outcome in Hispanic Americans and African Americans, respectively.

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Conclusions—We confirmed the association of a *PNPLA3* variant with NAFLD in Hispanic Americans and African Americans, suggesting that *PNPLA3* contributes to the variation in NAFLD across multiple ethnicities. This study adds to the growing evidence that some of the ethnic variation in NAFLD is genetic.

Keywords

African Americans; computed tomography; genetic epidemiology; hepatic steatosis; Hispanic Americans; non-alcoholic fatty liver disease; *PNPLA3*

Non-alcoholic fatty liver disease (NAFLD) is a common condition associated with insulin resistance, diabetes, inflammation and obesity. The condition has an unusual degree of ethnic variation. Hispanic Americans are at extremely high risk of both hepatic steatosis and cirrhosis, while African Americans are at considerably lower risk relative to Caucasians, which is not explained by differences in conventional risk factors (1, 2). A single nucleotide polymorphism (SNP) in adiponutrin/patatin-like phospholipase domain-containing protein 3 gene (*PNPLA3*) has been implicated in the variation of NAFLD (3, 4) and in the severity of histologically confirmed NAFLD in both adults and children (5–8). Specifically, a non-synonymous variant (rs738409) resulting in an isoleucine to methionine substitution is strongly associated, in an additive fashion, with hepatic fat content. The purpose of this study is to test for an association between rs738409 and quantitative measures of NAFLD as measured by abdominal computed tomography (CT) in a large well-phenotyped collection of Hispanic American and African American pedigrees.

Research design and methods

Study design, recruitment and phenotyping in the Insulin Resistance Atherosclerosis Study (IRAS) Family Study have been described in detail (9). Briefly, this multicentre study was designed to identify genetic determinants of insulin resistance and adiposity. This report examines 843 individuals of self-reported Hispanic American ethnicity from San Antonio, TX, and San Luis Valley, CA, and 371 individuals of self-reported African American ethnicity from Los Angeles, CA. The IRAS Family Study was an extension of the non-family-based IRAS (10). Family members of participants in the IRAS who had reported a large family structure on a family medical history questionnaire were recruited to participate in the IRAS Family Study. Two clinical examinations of this family-based cohort were conducted at a 5-year interval. For this report, we present data from the second examination, which occurred between 2005 and 2006. The examination included an interview for health behaviours and medical history, anthropometric measurements, abdominal CT scanning, resting blood pressure and blood drawing for plasma and DNA. Alanine transaminase (ALT) and aspartate transaminase (AST) were determined by enzymatic colourimetric assays using a Chemistry Analyzer Model ATAC 8000 (Elan Diagnostic Co., Lakewood, NJ, USA). Height and weight were measured to the nearest 0.5 cm and 0.1 kg respectively. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$. Usual consumption of beer, wine and liquor in the past year was assessed by self-report. Exclusions were made for usual alcohol consumption, which exceeded two drinks/day in men and one drink/day in women. History of liver disease was not collected. Diabetes was defined as a single fasting glucose measure exceeding 126 mg/dl or use of hypoglycaemic medications. A measure of insulin sensitivity was obtained at the baseline examination using the frequently sampled intravenous glucose tolerance test (11). This is the only measure utilized in this report that was not obtained concurrently with the CT measure of liver density. Approval for the study was granted by all appropriate institutional review committees and all participants provided written informed consent.

Computed tomographic imaging for abdominal fat distribution was obtained under a standardized protocol and scans were read centrally at the University of Color-ado School of Medicine, Department of Radiology, Bio-Imaging Research Laboratory. Participants received a scout view of the abdomen and pelvis followed by three axial images all during suspended respiration. The three 10-mm-thick images were obtained through the L2–L3, L4–L5 and T11–T12 disc spaces. If the T11–T12 image did not include liver and spleen, a fourth image was obtained by using the scout to determine an appropriate intervertebral disc location. Liver and spleen density were then quantified in Hounsfield Units in the entire liver and spleen as visualized in the slice, excluding any visible vasculature (12). The image obtained at the L4–L5 disc space was used for the determination of visceral adipose tissue (VAT) area; bowel fat is excluded from measurement. All CT images were coded for pathology and image quality; poor-quality studies were excluded from analysis.

The ratio of liver density-to-spleen density (liver:spleen ratio) <1.0 has become an accepted cutpoint for a discrete outcome of NAFLD (13). Liver density unadjusted for spleen density has also been proposed as an accurate measure of fat infiltration of the liver (14) with lower density measures indicating greater fat content. We have previously reported greater heritability estimates for liver density than for liver:spleen ratio (1). Both measures are used in these analyses.

DNA extraction was performed using the PureGene system (Gentra Systems, Minneapolis, MN, USA). SNP rs738409 corresponding to the cytosine to guanine substitution that changes codon 148 from isoleucine to methionine in the *PNPLA3* gene was genotyped using an iPLEX Gold SBE assay on a MassARRAY genotyping system (Sequenom, San Diego, CA, USA). Locus-specific primers were designed using the system's software (MASSARRAY ASSAY DESIGN *3.0). Mass spectrograms were analysed using MASSARRAY TYPER software. Genotyping efficiency was >98.5% and 70 blind duplicate samples included to evaluate genotyping accuracy were 100% concordant.*

Statistical analysis

The rs738409 polymorphism was examined for Mendelian inconsistencies using PEDCHECK (15), resulting in two genotypes converted to missing. Estimates of heritability of liver density and liver:spleen ratio as well as tests of association between the rs738409 polymorphism and each phenotype were computed under a variance component model as implemented in SOLAR (16). Consistent with previous reports, an additive genetic model was tested. Liver density was transformed using the square transformation to best approximate the distributional assumptions of the test and to minimize heterogeneity of the variance. Models adjusted for age, sex, recruitment center (in Hispanic models only), BMI and admixture. The covariates for admixture were estimated, separately for each ethnic group, using a principal components analysis of ancestry informative markers. Models were further adjusted for insulin sensitivity index, resulting in minimal impact on the estimates (not shown). To explore the impact of this SNP on known ethnic differences in liver density, we added the SNP and an SNP × ethnicity interaction term to a base model that included ethnicity, age, sex, insulin sensitivity, VAT and BMI.

Adjustments for multiple comparison tests were not performed for the primary analysis because SNP selection was based on an *a priori* hypothesis. For our secondary analyses, testing the association of rs738409 with a large panel of metabolic phenotypes, we set our threshold at $P < 0.001$.

Results

The average age of the cohort was 43 years (range: 18–81 years) (Table 1). Prevalence of NAFLD was higher in Hispanic Americans than in African Americans (24 vs 9%, P <0.0001), similarly expressed as lower values of both liver density and liver:spleen ratio in Hispanic Americans. Hispanic Americans had greater VAT areas but lower BMI and greater insulin sensitivity than African Americans. The prevalence of diabetes was approximately 11% in both ethnic groups.

The frequency of the rs738409 G allele was 40% in the Hispanic American sample and 19% in the African American sample. The G allele of rs738409 was consistently associated with decreased liver density in Hispanic Americans ($P = 1.18 \times 10^{-9}$) and African Americans ($P = 4.99 \times 10^{-6}$), with adjustment for age, sex, centre, BMI and admixture (Table 2). In each ethnic group, the association with liver density followed an additive genetic model with a step-wise decrease in phenotypic mean corresponding to the number of G alleles present. Prevalence of NAFLD increased as the number of G alleles increased. For 0, 1 and 2 G alleles, the prevalence of NAFLD in Hispanic Americans was 16, 27 and 36% (P <0.0001), respectively, and in African Americans it was 7, 11 and 42% ($P = 0.001$). Within African Americans, the increase in prevalence was also consistent with a recessive model. The association between the variant and liver fat was persistent (and significant) when the trait was examined as liver:spleen ratio and within the individual Hispanic American cohorts (San Antonio and San Luis Valley). The SNP explained 4.4% of the variation in liver density in Hispanic Americans and 5.6% in African Americans. Finally, an elevation of serum concentrations of ALT and AST were associated with rs738409 G allele in Hispanic Americans (additive model $P = 0.07$ and 0.0009 respectively) and in African Americans (recessive model $P = 6.4 \times 10^{-4}$ and 0.04 respectively).

The association between ethnic-specific admixture and liver density was examined with and without adjustment for the SNP. In Hispanic Americans, the correlation between liver density and the Hispanic ancestry admixture variable was −0.18 (P <0.0001); adjustment for the SNP reduced this correlation only modestly ($r = -0.15$, P <0.0001). In African Americans, the correlation between liver density and the African ancestry admixture variable was −0.07 ($P = 0.20$). Adjustment for the SNP strengthened the correlation ($r = -0.12$, $P = 0.02$).

Heritability was examined with and without adjustment for the SNP. In Hispanic Americans, the heritability of liver density, adjusted for age, sex, BMI, VAT, insulin sensitivity, admixture and centre was 0.31; further adjustment for the SNP reduced the heritability to 0.25. In African Americans, the heritability of liver density was 0.24; further adjustment for the SNP increased the heritability to 0.27.

The coefficient for ethnicity on liver density, adjusted for age, sex, insulin sensitivity, VAT and BMI was highly significant (β= 359, standard error = 86; $P = 3.3 \times 10^{-5}$). Further adjustment for the SNP had considerable impact on the coefficient (β= 232, standard error = 83; $P = 0.005$), yet ethnicity remained a significant factor in explaining the variation in liver density. The SNP × ethnicity interaction term was not significant.

We examined the association of rs738409 with a number of metabolic phenotypes; none reached statistical significance (P <0.001) including insulin sensitivity index, acute insulin response, metabolic clearance rate of insulin, disposition index, homeostasis model assessment estimated insulin resistance, fasting insulin, fasting glucose, adiponectin, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, fibrinogen, plasminogen activator inhibitor-1, BMI, waist, VAT and subcutaneous adipose tissue.

Discussion

Our data corroborate the association between a single variant in *PNPLA3* (rs738409) and reduced liver density in Hispanic Americans and African Americans. The associations were strong ($1.18 \times 10^{-9} < P < 4.99 \times 10^{-6}$), consistent across the two ethnic groups and across the two regions in which we recruited Hispanic Americans, and consistent in direction with previous reports (3, 4). The SNP appears to explain a large proportion of the variance in liver density (4.4–5.6%). Furthermore, the variant is specific for liver density; none of the other extensive list of metabolic phenotypes was associated with the *PNPLA3* variant. This is the first confirmation of this association in Hispanic American and African American adults, as initially reported from the tri-ethnic Dallas Heart Study cohort (3). The only other report from a US minority population describes 85 children and adolescents attending a paediatric obesity clinic (17). The rs738409 *PNPLA3* G allele conferred susceptibility to hepatic steatosis in African Americans and Caucasians but not in Hispanic Americans (17). Together, these studies point to a consistent association between this *PNPLA3* variant and hepatic steatosis across multiple ethnicities and age groups.

The significant inverse correlation between liver density and the Hispanic American ancestry admixture variable, which persisted even after accounting for the SNP ($r = -0.15$, $P < 0.0001$), is important and suggests that other components of ancestry beyond *PNPLA3* contribute to the variation in liver density. African American ancestry was modestly associated with liver density, albeit after adjustment for the SNP ($r = -0.12$, $P = 0.02$). Thus, admixture accounts for approximately 1.4–2.3% of the variation in liver density after accounting for important covariates and this SNP.

In our previous work, we were unable to explain the large difference in liver density between Hispanic Americans and African Americans through adjustment for multiple metabolic risk factors (1). In this report, we examined whether the *PNPLA3* variant would explain this difference, and although the SNP explained a significant proportion of the variance in liver density, it does not explain the ethnic disparity.

There are several mechanisms whereby this variant could affect the fat content of the liver. *In vitro* rodent studies have shown that the variant promotes triglyceride accumulation by limiting triglyceride hydrolysis, thereby promoting hepatic steatosis (18). *PNPLA3* appears to play a major role in the liver under conditions of high lipid exposure, as demonstrated in studies of mice fed a western-type diet (19). We examined dietary fat × gene interactions in our data but were unable to replicate this effect (data not shown).

In conclusion, in this first large study in a US minority cohort, we confirmed that a SNP in *PNPLA3* (adiponutrin) explains a significant portion of the variation of NAFLD in Hispanic Americans and African Americans. The rs738409 G variant is associated with lower liver density, i.e. increased infiltration of fat in the liver, and elevated liver enzymes. The frequency of the variant (40 and 19% in Hispanic Americans and African Americans respectively) closely parallels the prevalence of NAFLD (24 and 9% respectively). However, a significant ethnic disparity in liver density exists even with adjustment for this variant. *PNPLA3* has an important role in the variation of liver density in US minority populations, yet it does not explain the unusually high prevalence of the NAFLD in Hispanic Americans.

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Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BMI	body mass index
CT	computed tomography
IRAS	Insulin Resistance Atherosclerosis Study
NAFLD	non-alcoholic fatty liver disease
PNPLA3	patatin-like phospholipase domain-containing protein 3 gene
SNP	single nucleotide polymorphism
VAT	visceral adipose tissue

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Table 1

Summary statistics for Insulin Resistance Atherosclerosis Family Study participants (mean and standard deviation, or per cent)

	Hispanic Americans (n = 843)	African Americans (n = 371)	*P*-value
Gender (female, %)	62.6	61.7	0.38
Age (years)	48.0 ± 14.1	50.3 ± 13.9	0.07
Liver:spleen ratio	1.13 ± 0.26	1.18 ± 0.18	0.0001
Non-alcoholic fatty liver disease, % (liver:spleen ratio <1.0)	24.4	8.9	<0.0001
Liver density (HU)	51.9 ± 12.3	55.9 ± 8.3	<0.0001
Alanine aminotransferase (ALT) (IU/l)	26.4 ± 16.1	22.3 ± 10.8	<0.0001
Aspartate aminotransferase (AST) (IU/l)	24.7 ± 10.2	23.9 ± 7.0	0.20
Diabetes (%)	10.8	11.1	0.45
Insulin sensitivity index ($\times 10^{-4}$/min/UU/ml)	2.13 ± 1.83	1.57 ± 1.19	<0.0001
Visceral adipose tissue area (cm^2)	113.0 ± 58.7	96.2 ± 57.6	<0.0001
Body mass index (BMI) (kg/m^2)	29.0 ± 6.1	30.1 ± 6.7	0.006

Table 2

Association results with non-alcoholic fatty liver disease traits in the Insulin Resistance Atherosclerosis Family Study adjusted for age, sex, centre, body mass index and admixture

		Genotype, means ± SD			P-value	
Trait	**Population**	**C/C (n)**	**C/G (n)**	**G/G (n)**	**Additive model**	**R^2**
Liver density (HU)	African American	57 ± 7 (248)	55 ± 9 (111)	46 ± 15 (12)	4.99 ± 10^{-6} *	0.056
	Hispanic American	55 ± 11 (324)	51 ± 12 (367)	47 ± 14 (152)	1.18 ± 10^{-9}	0.044
Liver:spleen ratio	African American	1.19 ± 0.15 (248)	1.18 ± 0.20 (111)	0.97 ± 0.32 (12)	6.42 ± 10^{-4} *	0.027
	Hispanic American	1.18 ± 0.23 (324)	1.11 ± 0.26 (367)	1.05 ± 0.32 (152)	9.24 ± 10^{-7}	0.025

* In African Americans, the recessive model *P*-values are for liver density, $P = 2.03 \times 10^{-6}$; for liver:spleen ratio, $P = 5.23 \times 10^{-6}$.