Effects of *PNPLA3* on Liver Fat and Metabolic Profile in Hispanic Children and Adolescents

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Objective: A genome-wide study in adults identified a variant of *PNPLA3* (rs738409) associated with ~2-fold higher liver fat. The purpose of this study was to examine the influence of *PNPLA3* genotype on liver fat and other related metabolic outcomes in obese Hispanic children and adolescents.

Research Design & Methods: 327 Hispanics aged 8-18 years were genotyped for rs738409. 188 subjects had measures of visceral (VAT) and subcutaneous adipose tissue (SAT) volume and hepatic (HFF) and pancreatic fat fraction (PFF) by MRI. 139 subjects did not have HFF measures but had extensive measures of insulin sensitivity and fasting lipids.

Results: Liver fat in GG subjects was 1.7 and 2.4 times higher than GC and CC (11.1 $\pm$ 0.8% in GG vs 6.6 $\pm$ 0.7% in GC and 4.7 $\pm$ 0.9% in CC; p<0.0001), and this effect was observed even in the youngest children (8-10 years). The variant was not associated with VAT, SAT, PFF, or insulin sensitivity or other glucose/insulin indices. However, Hispanic children carrying the GG genotype had significantly lower HDL-cholesterol (40.9 $\pm$ 10.9 in CC vs 37.0 $\pm$ 8.3 in CG vs 35.7 $\pm$ 7.4 in GG; p=0.03) and a tendency towards lower free fatty acid levels (p=0.06).

Conclusions: These results provide new evidence that the effect of the *PNPLA3* variant is apparent in Hispanic children and adolescents, is unique fat deposition in liver as compared to other ectopic depots examined, and is associated with lower HDL-cholesterol.

The prevalence of childhood obesity has risen dramatically over the past 20 years and is associated with increased risk of pre-diabetes, type 2 diabetes, metabolic syndrome, and fatty liver disease (1). Visceral fat (adipose tissue inside the abdominal cavity) has long been hypothesized to be one of the major factors linking obesity to increased disease risks (2), and is certainly an important factor in childhood. In addition, ectopic fat deposition through spillover of triglycerides into peripheral tissues and organs, such as muscle and liver, is also an important factor linking obesity to increased metabolic disease risk (3).

Previous studies have suggested that hepatic triglyceride accumulation varies between populations and may thus lead, in part, to different mechanisms by which metabolic abnormalities arise. For example, a large study of hepatic fat accumulation (measured by NMR spectroscopy) in 2,287 adult participants of the Dallas Heart Study (4) found that fatty liver disease was highest in Hispanics and lowest in African Americans.

Interestingly, obesity, insulin resistance, or alcohol intake did not explain this striking ethnic difference. This disparity has also been reported in the pediatric population where a study of liver autopsy data from 742 children aged 2-19 years showed that 13% of all subjects had fatty liver disease (defined as $\geq$ 5% of hepatocytes with macrovesicular fat), with the prevalence increasing with age and obesity status (5). Moreover the prevalence of fatty liver (after adjusting for age and obesity) was highest in Hispanics (11.8%) and lowest in African Americans (1.5%), reflecting the trend observed in adults. These observations are supported by another recent study in adolescents which did not find detectable levels of hepatic fat in obese African American compared to Whites and Hispanics (6). Thus, fatty liver disease appears to be most problematic in Hispanics compared to other ethnic groups, and this phenomenon is evident by childhood.

A recent genome-wide association study in adults identified a novel genetic factor that may provide a potential explanation for this striking ethnic difference in liver fat accumulation (7). A C>G single

nucleotide polymorphism (rs738409) in *PNPLA3*, which encodes an amino acid substitution (I148M), was associated with over 2-fold higher liver fat content in adults, with the strongest effect observed in Hispanics in whom the frequency of the G allele was much higher (48%) than in other populations (~20%). The aim of the present study was to examine whether the effect of this PNPLA3 variant is manifested in Hispanic children and adolescents, and whether its effect was limited to an elevation in liver fat as opposed to other ectopic fat depots, insulin resistance, and /or fasting lipid levels.

METHODS

We pooled subjects from a variety of studies that used a common protocol for assessment of body fat distribution and metabolic phenotype as described below. These subjects included 327 Hispanic children and adolescents aged 8 – 18 years, all of whom were genotyped, with 188 subjects having complete measures of liver and pancreatic fat fraction as well as abdominal fat distribution (visceral versus subcutaneous) as described below. The remaining 139 subjects who were genotyped did not have liver fat measures but had extensive measures of insulin and glucose from an oral and an intravenous glucose tolerance test as well as fasting lipids. Data from some of these subjects has been previously reported (10-14), but this paper is the first to examine the relationship between genotype and these outcomes. Participants and parents were provided with a full description of the study and all participants signed an informed assent document while consent was obtained from their parents. All studies were approved by the USC Institutional Review Board.

Weight and height were measured to the nearest 0.1kg and 0.1cm, respectively, using a beam medical scale and wall-mounted stadiometer; BMI and BMI percentiles were then calculated. Total body fat was measured by DEXA using a Hologic QDR 4500 W (Hologic Inc, Bedford, MA). Abdominal fat distribution was measured by magnetic resonance imaging, on a General

Electric 1.5-Tesla magnet. Slices were acquired using a 420 mm field of view (FOV) and FOV phase of 75%. Three abdominal scans were performed consecutively and total acquisition time was 24 seconds per total abdominal scan. Each scan obtained 19 axial images of the abdomen with a thickness of 10 mm. After image acquisition, subcutaneous abdominal adipose tissue and visceral adipose tissue were segmented using image analysis software (SliceOmatic Tomovision, Montreal Canada) at Image Reading Center (New York City, New York). Hepatic and pancreatic fat fraction were assessed during the same MRI test using a modification of the Dixon 3-point technique. Abdominal scans were acquired contiguously using a breath-hold dual-echo spoiled gradient-recalled echo sequence with repetition time of 156 msec and echo time of 2.3 msec for out of phase (OP) images and 4.78 msec for in-phase (IP) images, acquired with flip angles of 70° and then 20° to provide T1-weighted and intermediate-weighted images, respectively. A third dual-echo gradient-echo breath-hold gradient-recalled echo sequence with two IP echoes (4.8 and 9.6 msec) was also performed to calculate T2* (15). The fat fraction in the liver was estimated from the signal intensity index obtained from in-phase and out-phase images. Quantitative corrections for the influence of T2* decay on the fat fraction estimates were taken into account by the third duel-echo sequence, where T2* for the liver was estimated on a pixel-by-pixel basis. Since the FF was estimated from low flip-angle images (20°), the effect of T1 relaxation on the quantification was minimized. Once the fat fraction images were calculated, three consecutive slices with maximum axial coverage of the liver were selected, avoiding any major blood vessels, to report the average liver fat fraction.

In all cases, subjects were genotyped for SNP rs738409 in PNPLA3 using the Applied Biosystems, Inc. (ABI) TaqMan system (16; 17) with an assay available through ABI's "Assays on Demand" database. Our overall genotype call rate was 97% and we obtained 100% genotype

concordance of four control CEPH DNA samples that were included on each DNA plate. Genotype frequencies did not deviate from that expected under Hardy-Weinberg equilibrium (p = 0.92).

Data were analyzed using General Linear Models in SPSS version 16.0 with genotype as the main factor, and age, gender and subcutaneous adipose tissue volume as co-variates (the overall results were similar when using no co-variates in the model). Logistic regression analysis was used to examine prevalence of fatty liver by genotype. In this analysis, likely fatty liver disease was defined as a liver fat fraction >5.5% which is based on previous studies in adults using NMR spectroscopy (18), a technique that we have previously validated against the MRI methodology used in the current study (19).

RESULTS

The overall frequency of the PNPLA3 variant was 51.5%, and the allele distribution was 28%GG/47%CG/24%CC. The clinical characteristics of the subjects with liver fat measures are shown in Table 1. As shown in Figure 1, there was a highly significant effect of genotype on liver fat fraction with GG carriers having almost double the amount of liver fat content (p<0.0001). In post hoc analysis, there was no significant difference in liver fat fraction in CC vs CG carriers. As also shown in Figure 1, this relationship remained significant even in a younger sub-group of Hispanic children (aged 8 – 10 years). Since liver fat data was skewed, we repeated these analysis after log transformation but the results were unchanged. The data also showed that overall, 38% of Hispanics had liver fat fraction >5.5%, indicating likely NAFLD. By comparison, 64% of GG carriers had liver fat fraction >5.5% (see Table 2), leading to an increased odds ratio of 4.7 for GG relative to GC/CC carriers having liver fat of >5.5% (95% CI = 2.4 − 9.3; p < 0.001). There was no significant effect of genotype on visceral adipose tissue volume, subcutaneous adipose tissue volume or pancreatic fat fraction, with or without adjustment for co-variates including age, gender, and overall adiposity.

To determine whether rs738409 was associated with other metabolic phenotypes, we analyzed data from a different group of 139 Hispanic subjects (mean age = 10.9 ± 1.7 years; 38.1 ± 6.2% body fat) who had extensive measures of insulin and glucose outcomes from both an oral and intravenous glucose tolerance tests, as well as fasting lipids, but liver fat measures were not available. There were no significant effects of genotype on any outcome related to insulin and glucose parameters or lipids, before or after adjusting for total fat, as measured by DEXA, with the exception of HDL-cholesterol which was significantly lower in GG subjects by 12.7% compared to the CC and CG groups (40.9 ± 10.9 in CC vs 37.0 ± 8.3 in CG vs 35.7 ± 7.4 in GG; p=0.03); in addition there was also a tendency towards lower free fatty acid levels in GG individuals (0.53 ± 0.21 in CC vs 0.55 ± 0.11 in CG vs 0.48 ± 0.1 in GG; p=0.06).

DISCUSSION

Since first reported in a genome wide association study (7), *PNPLA3* has been under investigation to determine its function and mechanism of action. Importantly, association of rs738409 has been replicated in several follow up studies with multiple liver-related phenotypes, including fat content, plasma enzyme levels, and fibrosis (8; 9; 20; 21). For the most part, these studies have all been carried out in adult populations. However, our present study now demonstrates that the effect of this *PNPLA3* variant: a) manifests early in life in obese Hispanic children; b) is specific to the deposition of triglycerides in liver as opposed to other ectopic fat depots, such as the pancreas and visceral or subcutaneous adipose tissue; and c) is also associated with significantly lower HDL-cholesterol levels in obese Hispanics.

Recent studies have shown that *PNPLA3* is up-regulated during adipocyte differentiation (22) and in response to positive energy balance, an atherogenic diet, insulin infusion, and nutritional status (23; 24). This

suggests that *PNPLA3* could have pleiotropic metabolic effects beyond its role in hepatic triglyceride accumulation. However, prior studies have shown that the *PNPLA3* variant is not associated with major alterations in glucose homeostasis or lipoprotein metabolism (7). Based on fasting levels of insulin and glucose in adults, the G allele was not associated with insulin resistance in lean individuals but interestingly was associated with increased insulin sensitivity in obese adults. In the current study we provide evidence from both an oral and intravenous glucose tolerance test to show that there is no significantly detectable association between any fasting or post challenge (oral and intravenous) glucose or insulin parameter in Hispanic children. These data support the idea, as previously proposed (25), that increased liver fat accumulation as a result of the G allele may be "metabolically benign", at least in terms of insulin resistance. Alternatively, the effect of rs738409 on glucose/insulin homeostasis may not manifest in children (unlike its effect on hepatic fat content) and thus take longer for it to develop. Lastly, the relatively small sample size of our study may have also precluded detecting association of rs738409 with such metabolic phenotypes.

Another interesting finding from our study is the observation that GG carriers have significantly lower HDL-cholesterol levels, which is consistent with a recent study reporting the same effect in Italian subjects (21). However, *PNPLA3* has not been uniformly associated with lipid levels in all studies. For example, associations between *PNPLA3* variation and serum lipids have either not been observed (7; 25) or only with apolipoprotein B-containing lipoproteins (22). Given these ambiguities, as well as those surrounding glucose/insulin measures, additional studies will be required to more fully understand the biological role of PNPLA3 in various processes related to metabolic homeostasis.

In conclusion, this brief report demonstrates that the rs738409 variant of *PNPLA3* influences liver fat deposition early in life in Hispanic children and adolescents. These results suggest that more effective therapeutic strategies for preventing and treating fatty liver need to be developed, especially for genetically predisposed pediatric populations.

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**Table 1: Clinical Characteristics of Subjects with Liver Fat Measurements.**

	n=188 (59 male/129 female)
Age (years)	13.6 $\pm$ 3.0
Body Mass Index	30.3 $\pm$ 8.9
Subcutaneous Abdominal Adipose Tissue Volume (L)	9.2 $\pm$ 5.9
Visceral Adipose Tissue Volume (L)	1.7 $\pm$ 1.1
Liver Fat Fraction (%)	7.1 $\pm$ 7.2; Median = 4.5; Interquartile range = 5.8
Pancreatic Fat Fraction (%)	5.6 $\pm$ 3.4

Values are mean $\pm$ standard deviation except where noted

Table 2: Distribution of Subjects by Genotype and by Liver Fat Fraction > 5.5%

Genotype	Liver fat <5.5	Liver fat > 5.5
GG (n=53)	36%	64%
CG (n=90)	67%	33%
CC (n=45)	84%	16%
Overall (n=188)	62%	38%

Figure 1: Liver fat fraction as a function of PNLPA3 genotype in Hispanic Children and Teenagers Aged 8 – 18 years and in a Sub-set aged 8 –10 years.
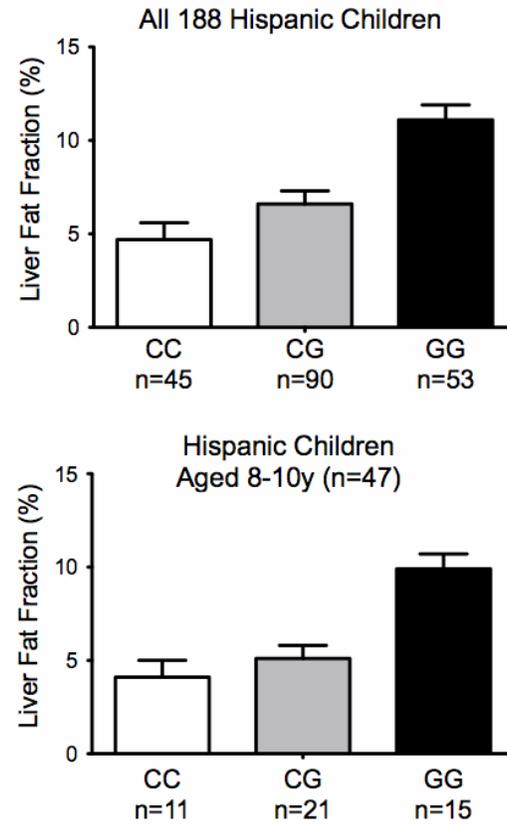
Note: Data are least square means $\pm$ standard error of the mean, after adjusting for gender, age and SAT; overall effect of genotype < 0.001.