

Increased hepatic fat in overweight Hispanic youth influenced by interaction between genetic variation in *PNPLA3* and high dietary carbohydrate and sugar consumption^{1–4}

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ABSTRACT

Background: Recently, a genetic variant (rs738409; C→G) of the *PNPLA3* gene was identified to be associated with increased hepatic fat deposition, and the effect was more pronounced in Hispanics. Animal models have also shown that *PNPLA3* expression can be regulated by dietary carbohydrate.

Objective: The aim of this study was to examine whether the influence of *PNPLA3* genotype on hepatic fat is modulated by dietary factors in Hispanic children.

Design: *PNPLA3* was genotyped in 153 Hispanic children (75% female, ages 8–18 y) by using the TaqMan method. Dietary intake was assessed by using three 24-h dietary recalls or diet records. Visceral adipose tissue (VAT), subcutaneous abdominal adipose tissue (SAAT), and hepatic fat fraction (HFF) were assessed in multiple abdominal slices by magnetic resonance imaging. Analysis of covariance was used to assess the diet × genotype interaction in liver fat, with the following a priori covariates: sex, age, energy, VAT, and SAAT.

Results: HFF was influenced by a significant interaction between genotype and diet (genotype × carbohydrate, $P = 0.04$; genotype × total sugar, $P = 0.01$). HFF was positively related to carbohydrate ($r = 0.31$, $P = 0.04$) and total sugar ($r = 0.34$, $P = 0.02$) intakes but only in the *GG* group, independent of covariates. Dietary variables were not related to HFF in the *CC* or *CG* group or to other fat depots in all genotype groups.

Conclusions: These findings suggest that Hispanic children carrying the *GG* genotype are susceptible to increased hepatic fat when dietary carbohydrate intake, specifically sugar, is high. Specific dietary interventions based on genetic predisposition in this population may lead to more effective therapeutic outcomes for fatty liver. This trial was registered at clinicaltrials.gov as NCT00697580, 195-1642394A1, and NCT00693511. *Am J Clin Nutr* doi: 10.3945/ajcn.2010.30185.

INTRODUCTION

Hispanics are particularly susceptible to the accumulation of fat in the liver, in part because of genetic predisposition. Recent reports suggest that nearly 4 of 10 obese Hispanic children and adolescents have nonalcoholic fatty liver disease (NAFLD)—a condition that can lead to cirrhosis, liver disease, and eventually liver cancer (1). A recent genome-wide association study in adults identified a novel genetic factor that explains some of the striking ethnic differences in liver fat accumulation (2). An amino

acid substitution (I148M; C→G; rs738409) in the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene was associated with a >2-fold higher liver fat content in adults, with the strongest effect observed in Hispanics, in whom the frequency of the variant was much higher (49%) than in whites (23%) or African Americans (17%). Importantly, these associations were not confounded by genetic admixture, which supports the consistent effects observed across populations. We recently validated the prevalence and effect of this genotype on liver fat accumulation in Hispanic children and adolescents (3). Genotype distributions were significantly different between Hispanics and African Americans, which is consistent with previous reports (4). For example, the liver fat fraction in Hispanic subjects with the *GG* genotype was 1.68 and 2.36 times higher than that in those with the *GC* or *CC* genotype, respectively, and this effect was observed even in the youngest children (age 8–10 y).

The function of the *PNPLA3* gene is not entirely known but has been reported to have lipase-like activity and to promote triglyceride hydrolysis in the liver (5). A recent animal study has also shown that expression levels of hepatic *PNPLA3* mRNA is low during fasting and increases ≈90 fold in response to carbohydrate feeding (6). This effect occurs as a secondary effect of insulin-mediated up-regulation of sterol regulatory element

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binding protein 1 (SREBP-1) and liver X receptor, which are major transcription factors responsible for fat metabolism in the liver (6). Whereas this effect would presumably not differ across genotypes, recent studies have shown that the *G* allele, which encodes a methionine substitution at position 148, abolishes *PNPLA3* activity. As a consequence, *GG* subjects could be more susceptible to the effects of dietary sugar because transcriptional up-regulation of *PNPLA3* would still result in a protein with severely reduced function and therefore reduced hydrolysis of hepatic triglycerides (6). Thus, the purpose of this study was to examine whether the effects of *PNPLA3* genotype on liver fat in Hispanic children might be exacerbated under conditions of a high carbohydrate/sugar intake. On the basis of our previous observations, we hypothesized that sugar intake will be more related to liver fat accumulation in subjects with the *GG* genotype than in those with the *GC* and *CC* genotype.

SUBJECTS AND METHODS

Participants

For this study we pooled subjects from a variety of studies using essentially identical protocols and measures conducted by the University of Southern California Childhood Obesity Research Center over the past 6 y. In brief, subjects were recruited from schools, community centers, health clinics, health fairs via word of mouth, flyers/brochures, and in-person contact. These subjects include 153 Hispanic children and adolescents (ages 8–18 y) for whom complete genotype, visceral, subcutaneous, and liver fat, and dietary data were available. All subjects were of Hispanic ancestry (all 4 grandparents of Hispanic origin as determined by parental self-report). Subjects were ineligible if they were taking medications known to affect body composition, had syndromes or diseases known to affect body composition or fat distribution, had any major illness since birth, or had participated in structured exercise, nutrition, or a weight-loss program within the 6 mo before recruitment. Data from some of these subjects was previously reported (7–9), but this study is the first to examine the relation between diet and fat depots among *PNPLA3* genotypes. The Institutional Review Board of the University of Southern California, Health Sciences Campus, approved this study. Informed written consent and assent were obtained from both parents and child before testing commenced.

Adiposity measures

Weight and height were measured to the nearest 0.1 kg and 0.1 cm, respectively, by using a beam medical scale and wall-mounted stadiometer; body mass index (BMI) and BMI percentiles for age and sex were determined by using EpiInfo 2000 (version 1.1, 2001; Centers for Disease Control and Prevention, Atlanta, GA) (10). Total fat mass and total lean mass were measured by dual-energy X-ray absorptiometry by using a Hologic QDR 4500W (Hologic, Bedford, MA). Abdominal fat distribution was measured by magnetic resonance imaging (MRI) on a Siemens (Malvern, PA) 1.5-Tesla magnet. Slices were acquired by using a 420-mm field of view (FOV) and FOV phase of 75%. Three abdominal scans were performed consecutively, and the total acquisition time was 24 s 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 (SAAT) and visceral adipose tissue (VAT) were segmented by using image analysis software (SliceOmatic Tomovision, Montreal, Canada) at the Image Reading Center (New York, NY). SAAT and VAT volumes were calculated from these images as previously described (11). Hepatic fat fraction (HFF) was assessed during the same MRI test by using a modification of the Dixon 3-point technique. Quantitative corrections for the influence of T2 decay on the fat fraction estimates are taken into account by the third dual-echo sequence, where T2 is estimated on a pixel-by-pixel basis. Because the fat fraction was estimated from low flip-angle images (20°), the effect of T1 relaxation on the quantification was minimized.

Genotyping

A fasting blood sample was obtained after an overnight fast (nothing to eat or drink after 2000) for DNA isolation. Genotyping of SNP rs738409 in *PNPLA3* was performed on all subjects at the same time by using the Applied Biosystems Inc (ABI) TaqMan system (12, 13) 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 4 control DNA samples that were included on each DNA plate. Genotype frequencies did not deviate from that expected under Hardy-Weinberg equilibrium ($P = 0.92$).

Dietary intake

Dietary intake was assessed by either three 24-h dietary recalls ($n = 30$) by using the multiple-pass technique or by 3-d diet records (2 weekdays, 1 weekend day; $n = 123$). No difference in energy or macronutrients was observed between subjects who had dietary recalls and those with diet records. All recalls or records were administered or clarified by a bilingual dietary technician in person or via phone. Nutrition data were analyzed by using the Nutrition Data System for Research (NDS-R version 5.0_35), a software program developed by the University of Minnesota. The NDS-R program calculated key dietary variables for this analysis, including total sugars, dietary fiber, added sugar, glycemic index, and glycemic load (by using both the standard glucose and the white bread reference). Added sugars were defined as only those sugars/syrups added to foods during food preparation or commercial food processing (eg, high-fructose corn syrup) but not naturally occurring sugars such as lactose and fructose (14). The dietary data were carefully screened for plausibility through a multistep process. Data were first screened by evaluating the participants' comments; 4 subjects were excluded because they reported an illness. The dietary data were also examined for plausibility of caloric intake by assessing the distribution of the residuals of the linear regression of caloric intake by body weight at baseline. None of the participants had a residual that was >2 SDs from the mean; thus, 153 participants were included in the present analyses.

Statistical procedures

Data were examined for normality, and transformations were made if data were found to be significantly different from normal. The following outcome variables were nonnormally distributed

and log-transformed before analysis: total dual-energy X-ray absorptiometry fat, VAT, SAAT, and HFF. However, we present nontransformed values in the tables and figures for ease of interpretation. For the preliminary analyses, analysis of variance with post hoc pairwise comparisons with Bonferroni adjustments and chi-square tests (for sex only) were used to assess differences in physical and adiposity measures and dietary variables between genotypes. Several subjects had extremely high amounts of liver fat (>20%), and analyses were run with and without these outliers. A linear regression was used to assess whether there was a diet \times genotype interaction on the various fat depots (VAT, SAAT, and HFF) as continuous variables. We first computed the diet \times genotype (*GG*, *CG*, and *CC*) interaction for all dietary variables of interest (energy, macronutrients, sugar, fiber, glycemic index, and glycemic load) by simply multiplying the genotype group by the dietary variable. The dependent variable was the fat depot, and the independent factor was the diet \times genotype interaction. In all models, the following a priori covariates were included: sex, age, energy, BMI, the diet variable of interest, VAT (when SAAT and HFF were dependent variables), and SAAT (when VAT was a dependent variable). If there was a significant diet \times gene interaction, partial correlations were performed to assess the relation between the diet variable and the adiposity measures. Scatter plots were then produced to display these significant relations. All analyses were performed by using SPSS version 16.0 (SPSS, Chicago, IL), and the significance level was set at $P < 0.05$.

RESULTS

The physical and adiposity measures between genotype groups are shown in **Table 1**. Aside from increased hepatic fat in the *GG* group that we reported recently (3), no significant differences were observed between genotype groups for any of the other variables. Dietary variables between genotypes are shown in **Table 2**. The *GG* group consumed 20% less protein (g/d) than did the *CG* group ($P = 0.03$). The *CG* group also consumed

16%, 16%, and 18% less total, insoluble, and soluble fiber (g/1000 kcal), respectively, than did the *CC* group ($P < 0.05$). A trend for the *GG* group to consume 11% and 14% less energy than the *CC* and *CG* groups was observed. Carbohydrate and sugar intake was similar between genotype groups.

A significant carbohydrate (% of energy/d) \times genotype interaction on HFF ($P = 0.04$) was observed, independent of sex, age, BMI, and VAT. The relation between hepatic fat and total carbohydrate for each genotype is shown in **Figure 1**. Hepatic fat was positively related to carbohydrate ($r = 0.38$, $P = 0.02$) only in the *GG* group, independent of covariates. Carbohydrate intake was not related to hepatic fat in the *CC* or *CG* group. The results were essentially the same when carbohydrate was expressed as g/d and energy intake was included as a covariate. The results were also similar after exclusion of subjects who were outliers for hepatic fat or carbohydrate intake.

A significant total sugar (% of energy/d) \times genotype interaction on HFF was observed ($P = 0.01$), independent of the covariates listed above. The relation between hepatic fat and total sugar for each genotype is shown in **Figure 2**. Hepatic fat was positively related to total sugar intake ($r = 0.33$, $P = 0.04$) only in the *GG* group, independent of covariates and unrelated to liver fat in the *CC* or *CG* group. A trend for an added sugar (% of energy/d) \times genotype interaction on HFF was also observed ($P = 0.06$). However, when relation within each genotype group was examined, hepatic fat was positively related to added sugar in the *GG* group, but the trend was attenuated ($r = 0.25$, $P = 0.12$) and negatively related in the *CC* group ($r = -0.28$, $P = 0.12$). The results were essentially the same when total and added sugar intake was expressed as g/d and energy intake was included as a covariate. In addition, exclusion of outliers for hepatic fat and dietary sugar did not change the results. No other diet \times genotype interactions were significant for hepatic fat or for any of the other adiposity measures (ie, total fat, VAT, or SAAT), and no dietary variable was related to adiposity measures overall, when not split by genotype group.

TABLE 1
Physical and metabolic characteristics of subjects by genotype ($n = 153$)¹

	<i>CC</i> ($n = 38$)	<i>CG</i> ($n = 70$)	<i>GG</i> ($n = 45$)	P value ²
Sex (n)				0.42
Male	6	22	11	
Female	32	48	34	
Age (y)	13.3 \pm 3.0 ³	14.1 \pm 2.9	14.0 \pm 2.9	0.32
Weight (kg)	75.9 \pm 33.8	77.9 \pm 28.7	81.5 \pm 31.8	0.74
BMI (kg/m ²)	30.7 \pm 8.6	31.4 \pm 7.5	32.5 \pm 8.6	0.61
BMI percentile	91.7 \pm 14.7	94.7 \pm 9.4	95.7 \pm 6.4	0.50
BMI z score	1.8 \pm 0.8	2.0 \pm 0.6	2.0 \pm 0.6	0.22
Total fat mass (kg)	30.3 \pm 15.1	30.4 \pm 11.6	31.8 \pm 14.9	0.87
Total lean mass (kg) ⁴	45.0 \pm 15.1	44.7 \pm 14.7	45.5 \pm 15.3	0.98
VAT (L)	1.6 \pm 1.0	1.7 \pm 1.3	1.8 \pm 1.0	0.89
SAAT (L)	9.1 \pm 5.2	10.0 \pm 6.2	10.3 \pm 6.8	0.63
Hepatic fat (%)	4.5 \pm 4.1 ^a	5.9 \pm 5.8 ^a	9.7 \pm 8.10 ^b	<0.001

¹ VAT, visceral adipose tissue; SAAT, subcutaneous abdominal adipose tissue. Values with different superscript letter are significantly different, $P < 0.001$ (Bonferroni multiple comparisons).

² ANOVAs with Bonferroni adjustments and chi-square tests (for sex only) were used to determine significant differences between genotype groups. The P values represent the overall significance between genotype groups.

³ Mean \pm SD (all such values).

⁴ Does not include bone mineral content.

TABLE 2
Dietary characteristics of subjects by genotype ($n = 153$)¹

Nutrients	CC ($n = 38$)	CG ($n = 70$)	GG ($n = 45$)	P value ²
Energy (kcal)	1801.2 ± 575.4	1850.6 ± 544.8	1599.4 ± 463.9	0.06
Protein (g/d)	69.5 ± 22.6 ^{ab}	74.8 ± 25.5 ^a	62.3 ± 20.1 ^b	0.03
Protein (% of energy)	15.6 ± 3.0	16.4 ± 3.6	15.8 ± 3.3	0.45
Fat (g/d)	67.0 ± 27.9	69.6 ± 26.3	58.3 ± 21.7	0.09
Fat (% of energy)	31.8 ± 6.0	32.5 ± 6.1	31.3 ± 6.7	0.57
Carbohydrates (g/d)	236.6 ± 76.4	235.6 ± 74.2	211.4 ± 71.2	0.21
Carbohydrates (% of energy)	53.0 ± 7.1	51.3 ± 6.8	53.3 ± 8.5	0.29
Total sugar (g/d)	112.0 ± 48.0	104.3 ± 42.8	96.1 ± 44.8	0.28
Total sugar (% of energy)	24.7 ± 6.6	22.7 ± 6.7	23.7 ± 7.5	0.34
Added sugar (g/d)	73.8 ± 44.6	67.5 ± 38.9	69.5 ± 47.5	0.75
Added sugar (% of energy)	15.9 ± 7.4	14.6 ± 6.8	16.5 ± 8.9	0.41
Dietary fiber (g/1000 kcal)	9.1 ± 3.5 ^a	7.6 ± 2.4 ^b	8.1 ± 3.4 ^{ab}	0.03
Insoluble fiber (g/1000 kcal)	6.2 ± 2.5 ^a	5.1 ± 1.9 ^b	5.5 ± 2.7 ^{ab}	0.05
Soluble fiber (g/1000 kcal)	2.8 ± 1.1 ^a	2.3 ± 0.7 ^b	2.4 ± 1.0 ^{ab}	0.02
Glycemic index ³	59.2 ± 3.7	59.0 ± 3.9	59.7 ± 4.7	0.45
Glycemic load ³	131.6 ± 45.8	131.5 ± 41.9	119.2 ± 45.5	0.33

¹ All values are means ± SDs. Values with different superscript letters are significantly different, $P < 0.05$ (Bonferroni multiple comparisons).

² ANOVAs with Bonferroni adjustments were used to determine significant differences between genotype groups. The P values represent the overall significance between genotype groups.

³ Calculated by using glucose as the reference.

DISCUSSION

To our knowledge, this was the first study to assess the effect of interactions between dietary intake and *PNPLA3* gene variants on hepatic fat accumulation, particularly in a Hispanic pediatric population. We showed a positive association between carbohydrate intake, specifically total and added sugars, and hepatic fat in the *GG* group only. No other diet × genotype interactions were present, nor were any dietary variables related to hepatic fat overall. These novel findings highlight the importance of examining this data by genotype and suggest that obese Hispanic children with the *GG* genotype have an increased capacity for fat storage and decreased hepatic lipid mobilization in the context of diets high in sugar.

In the past decade, the decline in dietary carbohydrate quality has been identified as a key factor leading to increased adiposity and metabolic disorders among children and adults. In numerous studies conducted in Hispanic youth (10–17 y of age) by our group, we showed that increased sugar intake is associated with increased adiposity and decreased insulin secretion and β cell function (15, 16). We have consistently shown that sugar intake is relatively high in Hispanic youth living in Los Angeles (>100 g/d) and accounts for nearly 50% of the overall daily carbohydrate intake and 25% of the energy intake. Whereas a link between high sugar intake and excessive liver fat in Hispanic children is not unexpected, our observation that this relation was only found in participants with the *GG* genotype is of particular interest and has important implications for strategies to treat NAFLD.

Human and animal studies have consistently shown that the *PNPLA3* gene is highly responsive to changes in energy balance (17), ie, it is down-regulated by fasting and dramatically up-regulated by refeeding (18, 19). One study found that mice fed a high-fat diet had a 23-fold increase in the expression level of *PNPLA3*, which was reversed under fasting conditions (19). Another recent animal study showed that hepatic *PNPLA3*

mRNA levels increased ≈90 fold in response to carbohydrate feeding through insulin-mediated stimulation of transcriptional regulatory networks involving SREBP-1 and liver X receptor (6). Carbohydrate, specifically fructose, is a key player in liver fat deposition because it activates SREBP-1, which stimulates hepatic de novo lipogenesis and inhibits hepatic fatty acid oxidation (20, 21). Therefore, our results are consistent with the notion that the reduced capacity of subjects with the *GG* genotype to hydrolyze triglycerides in the liver would be exacerbated in the context of high dietary sugar because carbohydrate-mediated up-regulation of *PNPLA3* with a methionine substitution at position 148 would still lead to an enzyme with severely reduced activity.

Although, this study reports on the relation between nutrient intake and hepatic fat among genotype groups, we also assessed the relation between hepatic fat and different types of sugar (ie, glucose, fructose, sucrose, lactose, and maltose) and food and beverage servings per day (ie, meat, dairy products, fruit, vegetable, fat, grains, refined grains/breads, whole-wheat grains/breads, sugar-sweetened beverages, and sweets). Whereas total carbohydrate and total and added sugar intake were positively related to hepatic fat among *GG* carriers, specific types of sugar or food or beverages (servings/d) were not. One explanation for these findings may be that the intake of carbohydrate and total and added sugar made up a larger percentage of daily calories (≈50% and 25%) compared with specific types of sugars and/or food and beverage groups, which were present in much smaller proportions. Thus, detection of significant nutrigenetic relations with these specific dietary variables and hepatic fat accumulation would be more difficult and require larger sample sizes. In addition, determination of the fructose intake, specifically that coming from high-fructose corn syrup, is very difficult to estimate with the use of dietary recalls/records and available nutrition software programs. Nonetheless, our results suggest that high intakes of any type of dietary sugar lead to excessive liver fat accumulation in carriers of the *GG* genotype.

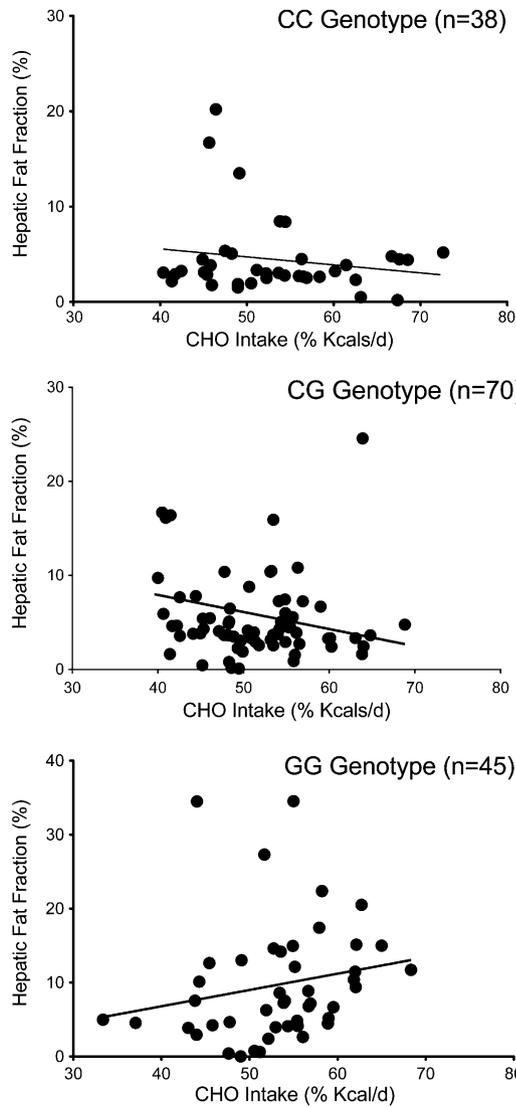


FIGURE 1. Relation between liver fat and carbohydrate (CHO) intakes for each of the *PNPLA3* genotypes. There was a significant CHO \times genotype interaction on the hepatic fat fraction ($P = 0.04$), independent of sex, age, BMI, and visceral adipose tissue. Liver fat was positively related to CHO ($r = 0.38$, $P = 0.02$) only in the *GG* group, independent of sex, age, BMI, and visceral adipose tissue. CHO intake was not related to liver fat in the *CC* or *CG* group.

Several limitations of our study need to be noted. Because this study was cross-sectional, a cause and effect relation between dietary intake and hepatic fat between the genotypes cannot be made. An efficacy intervention trial testing the effects of reducing carbohydrates, specifically sugars, in subjects with different variants of the *PNPLA3* gene may thus help to determine causality. The current study is also limited by the use of either dietary recalls or records, which rely solely on the participants' self-report and are often prone to errors. However, several steps were taken to ensure the accuracy of dietary data, such as using well-trained diet technicians to clarify records or collect recalls, screening for participants' comments, and assessing the plausibility of caloric intake by body weight. In addition, the literature suggests that overweight children underreport dietary intake to a greater extent than do lean children (22). However, because most of our subjects were overweight ($n = 133$, or 87%), we would expect a similar degree of underreporting across the whole sample. A review

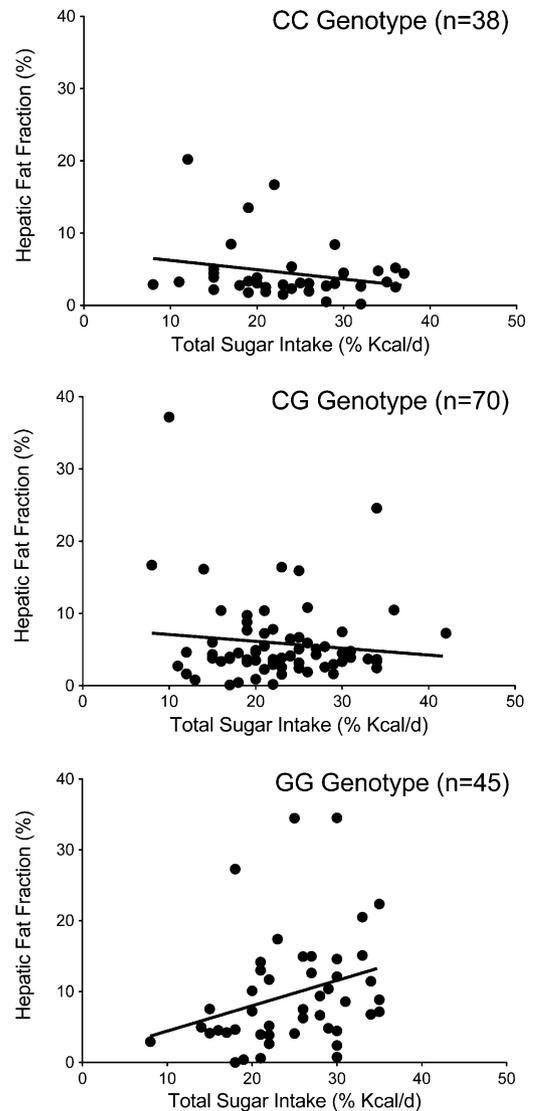


FIGURE 2. Relation between liver fat and total sugar intake for each of the *PNPLA3* genotypes. There was a significant total sugar \times genotype interaction on the hepatic fat fraction ($P = 0.01$), independent of sex, age, BMI, and visceral adipose tissue. Liver fat was positively related to total sugar intake ($r = 0.33$, $P = 0.04$) only in the *GG* group, independent of sex, age, BMI, and visceral adipose tissue. Total sugar intake was not related to liver fat in the *CC* or *CG* group.

study by McPherson et al (23) on dietary assessment methods among school-age children, including Hispanic children, showed that multiple recalls and records agreed more with the validation standards than did the FFQ or dietary screens. Several studies also showed that dietary recalls and records had excellent agreement on both food and nutrition information (24, 25). Another potential limitation was that most of our study subjects were female ($n = 114$, or 75%), which we addressed by controlling for sex in all analyses. Additional studies in larger samples that include more males, leaner subjects and/or other ethnicities are warranted to further understand the relation between *PNPLA3*, dietary nutrients, and liver fat accumulation.

These findings have potentially important clinical implications. Given that our data show that 38% of obese Hispanic children and adolescents have a liver fat content $>5.5\%$ by MRI, which likely indicates NAFLD (3), it is crucial to understand the

causes of hepatic triglyceride accumulation and how to prevent or reverse it in children. Numerous studies have shown that reducing weight by $\geq 5\%$, primarily through caloric restriction and exercise, has beneficial effects on NAFLD, as evidenced by a concomitant 30–60% reduction of liver fat (26–28). Thus, aggressive strategies aimed at weight loss would perhaps be the obvious approach for addressing NAFLD. However, in our ongoing experience with interventions in Hispanic children and adolescents, this weight loss goal is difficult to achieve (11, 29) and even more difficult to maintain for extended periods of time. There is therefore a great need to identify novel and more sustainable strategies that can address this problem based on the mechanism of liver fat accumulation, especially in individuals with a genetic predisposition.

In conclusion, these findings suggest that personalized dietary interventions based on reductions in dietary sugar intake in genetically predisposed individuals with the *GG* genotype may lead to more effective therapeutic outcomes for fatty liver. Interventions focused on reducing sugar intake could have immediate potential for clinical translation and long-term sustainability, particularly for genetically predisposed individuals in the Hispanic population.

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The authors' responsibilities were as follows—JND: was responsible for the study design, statistical analysis, and manuscript preparation; K-AL: assisted with the data evaluation and manuscript preparation; RWW and SV: assisted with data processing, analysis, and manuscript preparation; DS-M and MJW: assisted with evaluation and interpretation of the data and manuscript preparation; HA (Director of the Molecular Genetics Laboratory, where the genetic analyses were performed): assisted with the study design, statistical analyses, interpretation of the results, and manuscript preparation; MIG (Director of the Childhood Obesity Research Center): assisted with the study design, statistical analyses, interpretation of the results, and manuscript preparation; and JND, DS-M, MJW, and MIG: were principal investigators on studies whose subjects were used in this analysis. None of the authors had any financial or personal conflicts of interest.

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