Title

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Journal

Journal of Pediatric Gastroenterology and Nutrition, 66(5)

ISSN

0277-2116

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Publication Date

2018-05-01

DOI

10.1097/mpg.0000000000001926

Peer reviewed

HHS Public Access

Author manuscript

J Pediatr Gastroenterol Nutr. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:

J Pediatr Gastroenterol Nutr. 2018 May ; 66(5): 789–796. doi:10.1097/MPG.0000000000001926.

Genetic Variants Associated with Obesity and Insulin Resistance in Hispanic Boys with Nonalcoholic Fatty Liver Disease

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Abstract

Background and Objectives—Nonalcoholic fatty liver disease (NAFLD) disproportionately affects Hispanic boys. Further, obesity and insulin resistance are major risk factors for NAFLD. No gene localization studies had been performed on children with biopsy-proven NAFLD. This study aims to identify genomic variants associated with increased adiposity and insulin resistance in a population of children with varying histologic severity of NAFLD.

Methods—We conducted a genome-wide association scan (GWAS) including 624,297 single nucleotide polymorphisms (SNPs) distributed among all 22 autosomal chromosomes in 234 Hispanic boys (up to 18 years of age) who were consecutively recruited in a prospective cohort

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Conflicts of Interest:
John C Rausch, Joel E Lavine, Xiuqing Guo, Soonil Kwon, Jeffrey Schwimmer, Jean Molleston, Rohit Loomba, Elizabeth M Brunt, Yii-Der Ida Chen, Mark O Goodarzi, Kent D Taylor, Katherine P Yates, Aynur Unalp-Arida, and Jerome I Rotter: none
Naga P Chalasani: Dr. Chalasani has consulting agreements and research grants with pharmaceutical industry but none of them represent a conflict for this particular paper.

ClinicalTrials.gov Identifier: NCT01061684 <https://clinicaltrials.gov/show/NCT01061684>

History of Changes <https://clinicaltrials.gov/ct2/archive/NCT01061684>

study in the Nonalcoholic Steatohepatitis Clinical Research Network Studies. Traits were examined quantitatively using linear regression. SNPs with p-value $<10^{-5}$ and a minor allele frequency > 5% were considered potentially significant.

Results—Evaluated subjects had a median age of 12.0 years, BMI of 31.4, and hemoglobin A1C (Hgb A1C) of 5.3. The prevalence of NAFL, borderline NASH and definite NASH were 23%, 53%, and 22%, respectively. The GWAS identified 10 SNPs that were associated with BMI z-score, 6 within chromosome 2, and 1 within CAMK1D, which has a potential role in liver gluconeogenesis. In addition, the GWAS identified 9 novel variants associated with insulin resistance: HOMA-IR (6) and HbA1c (3).

Conclusions—This study of Hispanic boys with biopsy-proven NAFLD with increased risk for the metabolic syndrome revealed novel genetic variants that are associated with obesity and insulin resistance.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease in children and its prevalence is rising in relation to the increasing number of overweight and obese children. Prevalence of NAFLD may approach 11% of all children in some areas (1). NAFLD is often accompanied by insulin resistance (2) and is considered by some to be the hepatic manifestation of the metabolic syndrome (3). Hispanic boys are at increased risk for NAFLD as well as for obesity and its comorbidities, including the metabolic syndrome, making them a particularly vulnerable group (4–6). While the prevalence of obesity has stabilized in certain groups, it continues to increase in Hispanic boys (7). Further, severe obesity continues to increase in childhood with an associated increase in the prevalence of cardiometabolic risk factors, particularly in boys. Thus, understanding the genetic underpinnings of obesity and its comorbidities in Hispanic boys is particularly important.

There is a significant genetic predilection to obesity, with estimates of heritability up to 70% (8). Genome-wide association studies (GWAS) offer a powerful hypothesis-free approach to identify the location of potential genetic contributions to obesity and its metabolic consequences (9). Such studies have identified over 100 single nucleotide polymorphisms (SNPs) that are associated with obesity in adults (10, 11). Limited studies in children have validated some of the more significant genetic loci, including the fat mass and obesity-associated (FTO) gene (12). There are at least 15 candidate genes that have been associated with elevated hemoglobin A1C in adults, and a number of other potential loci (13, 14). To our knowledge, no GWAS studies have been reported on solely obese children to examine loci associated with elevated hemoglobin A1C.

No genetic variant studies have been performed on children with biopsy-proven NAFLD to determine associated genetic risk factors for adiposity and insulin resistance. The aim of the current study is to identify genetic loci associated with adiposity as measured by BMI z-score and markers of insulin resistance by homeostasis model of assessment-insulin resistance (HOMA-IR) and hemoglobin A1C (Hgb A1C) in a population of Hispanic boys with biopsy-confirmed NAFLD. Due to the high prevalence of co-morbidities in this at risk population, this group of boys serves as an appropriate discovery cohort.

Methods

Subjects

As described previously, this discovery cohort included all of the self-identified Hispanic boys with liver biopsies who were enrolled in the Nonalcoholic Steatohepatitis (NASH) Clinical Research Network (CRN) in the NAFLD Database I Study (n=234) (15, 16). This prospective longitudinal cohort with over 4,400 subjects began in 2002. These subjects all met exclusion criteria to rule out any other potential contributors to fatty liver disease. Biopsy specimens were reviewed and scored centrally by the NASH CRN Pathology Committee according to the histology scoring system established by the NASH CRN (17). We focused on Hispanic male adolescents in this analysis because this minimizes the potential heterogeneity of characteristics that could be identified in a more diverse population. This approach has been utilized in similar analyses in the past (15). The protocol was approved by the Institutional Review Boards at each participating center. All parents provided consent and all children older than 7 years of age provided assent.

Genotyping and Quality Control

As described previously, genotyping was performed using Illumina OmniExpress chips that included 624,297 single nucleotide polymorphisms (SNPs) spanning all 22 autosomal chromosomes (16). Quality controls (QCs) were performed on the 234 samples and 657,675 single-nucleotide polymorphisms (SNPs) using PLINK (18).

Filtering criteria that were applied included a genotype missing rate > 0.02, minor allele frequency (MAF) <0.01, Hardy-Weinberg equilibrium (HWE) p-value < 10^{-6}, and heterozygosity > 0.53. This led to a total of 624,297 SNPs available for genome-wide association analysis. We also performed QCs at an individual level to check for missing rate and cryptic relatedness ($\hat{\pi}$). We observed no sample with missing rate > 0.02, but found 22 pairs of samples with $\hat{\pi} \geq 0.125$. Principal component analysis (PCA) was then carried out using EIGENSTRAT (19) to examine potential population stratification among our study samples. Four samples were identified as population outliers. We thereafter excluded 26 samples (22 cryptic relatedness and 4 PCA outliers) from further association analysis. To adjust for potential population stratification, we included the first 2 PCs as covariates in the model of association analysis. The final data set for the association analysis after QCs had 624,297 SNPs and 208 samples.

Data Analysis

Genome-wide single SNP association analysis was done using linear regression for BMI z-score, HOMA-IR, and Hgb A1C. HOMA-IR is an assessment of insulin sensitivity and resistance and was computed from the clinical data: HOMA-IR = (insulin*glucose)/22.5, where fasting insulin is in microU/mL and fasting glucose is in mmol/L(20). For each SNP, association analyses were run using both an additive and a dominant genetic model using PLINK software. Additive and dominant models assume different methods of inheritance, as the underlying method of inheritance is unknown (21). The assumption of different methods of inheritance produce different results, as our results show.

With the association results obtained from additive and dominant genetic models, we generated Manhattan plots showing the $-\log_{10}$(p-value) along with the 22 autosomal chromosomes for BMI z-score, HOMA-IR, and Hgb A1C. Given the sample size of the population only those traits that had a p-value of $<10^{-5}$ and had a mean minor allele frequency of at least 5% were considered potentially significant. The p-value was set at 10^{-5} to account in part for multiple comparisons. We calculated quantile–quantile (QQ) plots for each model and these are shown in Figure 2. Quantile-Quantile plots are a graphical tool to assess the normality of the data.

Results

Subjects

The baseline demographic characteristics, laboratory values, and histologic characteristics of the patient population were previously reported and the study subjects remain the same, as described in Table 1 (16). The subjects were overweight/obese Hispanic boys with a median BMI of 31.4 kg/m^2 and BMI z-score of 2.4. They were young adolescents with a median age of 12 years. Serum aminotransferases were elevated with a median ALT of 83 U/L and AST of 51 U/L. While only 4 boys had type 2 diabetes the subjects demonstrated signs of potential insulin resistance with a median insulin of 26 U/ml and Hgb A1C level of 5.3%. Lipid analysis revealed an abnormally low HDL with a median level of 38 mg/dL. The median levels for VLDL and LDL were within normal limits. Per the NASH CRN histology scoring system (17), the majority demonstrated significant steatosis with 71% having at least a steatosis grade of 2 (34–66%) according to NAS scoring, meaning at least 34% of hepatocytes demonstrated macrovesicular steatosis. As often found in pediatric populations, 59% of samples demonstrated no evidence of hepatocellular ballooning, a sign of cell injury necessary for the diagnosis of NASH. In terms of diagnostic pattern, 53% of subjects had evidence of borderline NASH and another 22% had definite NASH.

Adiposity

Manhattan plots from both the dominant and additive models for BMI z-score are shown in Figure 1. The dominant model resulted in 4 significant SNPs, one of which showed a previous association with adiposity, specifically CAMK1D (Table 2). All were located within introns. The allele on chromosome 10 in rs17583338 in the calcium/calmodulin-dependent protein kinase ID gene (CAMK1D) which codes for a calcium/calmodulin-dependent protein kinase was of special interest. A qualitative model using linear regression was also found in association with BMI z-score ($p=5 \times 10^{-6}$, data not shown). Another allele was located in chromosome 2 in rs295120 in the spermatogenesis associated, serine-rich 2-like gene (SPATS2L) which codes for the uncharacterized protein, spermatogenesis associated, serine-rich 2-like. There was also an association with an allele on chromosome 5 in SNP rs2303752 in the sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A gene (SEMA6A) which codes for the cell surface receptor semaporin-6A. The final association was with an allele on chromosome 11 in rs11026723 located in the growth arrest-specific 2 gene (GAS2) which codes for the growth arrest-specific protein 2, which may play a role in apoptosis.

The additive model resulted in 7 SNPs of interest, none showing a previous association with adiposity in other populations (Table 2). Six were located within introns and another was located within an uncharacterized region. There was an association with 4 alleles on chromosome 2 in SNPs rs12619898, rs17397163, rs11687204 and rs17397380 all located within non coding regions of NCK-associated protein 5 gene (NCKAP5) which codes for the protein NCK–associated protein 5. Another 2 alleles were located in chromosome 2 in SNPs rs99521 and rs295120 in the spermatogenesis associated, serine-rich 2-like gene (SPATS2L) which codes for the uncharacterized spermatogenesis associated, serine-rich 2-like protein. This gene, and in fact one of the exact SNPs rs295120, was also identified in the dominant model. The final association was with an allele on chromosome 14 in SNP rs8005339 which is of unknown significance.

Insulin Resistance

The dominant models for HOMA-IR and Hgb A1C did not produce any significant results. Each of the additive models, on the other hand had a number of potentially intriguing findings. Manhattan plots for each of these models are in Figure 1(c) and (d). There were 6 significant SNPs associated with HOMA-IR, none of which has shown a previous association with insulin resistance in other populations (Table 2). Five were located within introns and another was located within an uncharacterized region. There was significance with an allele on chromosome 17 on rs11773571 in the Williams-Beuren syndrome chromosome region gene (WBSCR17) which codes for the putative polypeptide N-acetylgalactosaminyltransferase-like protein 3 which may catalyze the initial reaction in oligosaccharide biosynthesis. Another association was with an allele on chromosome 3 on rs9846667 in the noncoding region of the family with sequence similarity 19 (chemokine [C-C motif-like], member A1) gene (FAM19A1) which codes for an uncharacterized protein. A third association was with an allele on chromosome 2 on rs10865041 in the intron of RFX family member 8, lacking RFX DNA binding domain gene (RFX8) which codes for the DNA-binding protein RFX8. A further association was on chromosome 20 in rs4361192 located in the intron of double zinc ribbon and ankyrin repeat domains gene (RFX8) which codes for double zinc ribbon and ankyrin repeat-containing protein 1. Another association on chromosome 20 was rs2295067 in the intron of LINC00851 which has unknown significance. The final association was with an allele on chromosome 16 in SNP rs8046133 which is of unknown significance.

There were 3 significant SNPs associated with Hgb A1C where the minor allele frequency was greater than 0.05, none of which has shown a previous association with insulin resistance (Table 2). Two of the significant alleles were on chromosome 11, rs3923850 and rs11727927, both located in the introns of the opioid binding protein/cell adhesion molecule-like gene (OPCML) coding for opioid-binding cell adhesion molecule. The final association was with an allele on chromosome 16 in SNP rs11644684 which is of unknown significance.

Discussion

This GWAS study was undertaken to investigate potential genetic influences relating to pediatric adiposity and insulin resistance in a discovery cohort of Hispanic boys with

biopsy-proven NAFLD. Hispanic boys with NAFLD were chosen because of their higher propensity to have obesity and diabetes and other aspects of the metabolic syndrome and because of the increased risk of metabolic syndrome features in NAFLD independent of obesity (4–6). The genetic homogeneity of this population, primarily Mexican-Americans, increases power to detect associations of interest that would require greater sample sizes in a more heterogeneous sample broadening ethnicity, gender and age (22). With this population, we report 19 genetic variant associations with adiposity and glucose metabolism, one previously recognized as having an association with adiposity and insulin resistance, but the majority representing novel loci. Of note, alleles of patatin like phospholipase domain containing 3 (PNPLA3) which have been associated with NAFLD in numerous other studies were not associated with NAS or fibrosis in this study. Thus, perhaps it is not surprising that in this cohort it did not associate with BMI z-score or HOMA.

Adiposity

There were several variants associated with BMI z-score. One previously identified association is within CAMK1D. This gene encodes a member of the Ca2+/calmodulin-dependent protein kinase 1 subfamily of serine/threonine kinase cell cycle regulators. This has been recognized as being associated with fat mass deposition and fat mass change in Hispanic children (23). CAMK1D also associates with risk of type II diabetes in a number of ethnic groups, including Mexican Mestizos, a comparable background to the children described here (24). This validates the role of this gene in the development of obesity and insulin resistance in young Mexican American boys. There is a biologically plausible pathway for the function of CAMK1D in obesity. In an *in vitro* model of primary human hepatocytes, down regulation of CAMK1D reduces expression of phosphoenolpyruvate carboxykinase 1 (PCK1) gene in an insulin-independent signaling pathway (25). The PCK1 gene encodes the cytosolic isozyme of phosphoenolpyruvate carboxykinase (PEPCK-C), which is a gluconeogenic enzyme in liver and kidney (26). The role of adipocyte PEPCK identified in rodent models is to regulate fatty acid storage and release via the production of glycerol-3-phosphate (26). In a mouse model where the PEPCK gene is overexpressed the mice are obese (26). It is unknown if it serves similar functions in humans and deserves further study in *in vitro* and *in vivo* models.

Another genetic variant not previously recognized with adiposity is the SPATS2L gene. The gene was found to be associated with bronchodilator response in asthmatics (27). This function implies that SPASTL2 may be a regulator of beta adrenergic function. In human smooth muscle cells, knockdown of SPATS2L mRNA leads to increased levels of beta-2 adrenoceptor proteins (27). The beta 2 adrenoceptor is a lipolytic receptor in human fat cells and different polymorphisms associate with adiposity in women (28). Homozygotes with the Glu27 polymorphism have significant excess body fat, 50% increase in fat cell size and evidence of insulin resistance (28). This offers a yet to be characterized, but plausible relationship between SPATS2L and the association with BMI in this current study.

A polymorphism within the NCKAP5 gene was found to be associated with BMI z-score. Variants within this gene are associated with height (29), essential hypersomnia, bipolar disorder, attention deficit hyperactivity disorder, and schizophrenia (30), but has not been

with reported with adiposity. A final notable association was related to GAS2. This gene associates with length of survival on dialysis in African-American patients (31). What its relationship with adiposity in Hispanic adolescent boys may be is unknown at this time.

Insulin Resistance

In terms of measures of insulin resistance, there were several novel locations found. One was an association between HOMA-IR and WBSCR17. This is an opioid binding protein/cell adhesion molecule that has been associated with antihypertensive response to an angiotensin II receptor blocker (32). Another association relates to hemoglobin A1C and OPCML. This gene encodes a protein in the IgLON subfamily of cell adhesion molecules that acts as a tumor suppressor (33). OPCML has a documented association with the development of coronary artery calcified plaque in African Americans with type 2 diabetes (34) and visceral adipose tissue/subcutaneous adipose tissue ratio in women (35).

Limitations of this study include the lack of testing of generalizability of the findings to pediatric girls, and to other pediatric racial and ethnic groups. Whether the findings are germane to other populations remains to be studied. Also, while GWAS studies indicate possible association, they do not specify any underlying mechanism or directly map to any particular gene. While several of the genes suspected have a plausible biologic mechanism, whether or not these pathways are in fact responsible for the outcomes observed remains to be determined in functional studies. Finally, while most of the patients were of Mexican Mestizo ancestry, this was self-reported and future studies should include genetic markers that more precisely determine ancestry.

In conclusion, in this group of Hispanic boys with biopsy-proven NAFLD we found suggestive evidence for the association of a number of both known and novel loci with obesity and insulin resistance. Validation studies are needed to confirm the contributions of these genes to the increased likelihood of obesity and insulin resistance. If any of these loci are replicated and possibly validated in other cohorts this will provide potential targets for understanding the underlying processes relating obesity and insulin resistance in individuals with fatty liver disease. Understanding the genetic influences relating obesity and insulin resistance in this young and potentially vulnerable population will give us greater understanding of the mechanisms associated with NAFLD and its numerous metabolic correlates.

Acknowledgments

Members of the Nonalcoholic Steatohepatitis Clinical Research Network Pediatric Clinical Centers

Baylor College of Medicine, Houston, TX: Stephanie H. Abrams, MD, MS (2007–2013); Sarah Barlow, MD; Ryan Himes, MD; Rajesh Krisnamurthy, MD; Leanel Maldonado, RN (2007–2012); Rory Mahabir, BS

Cincinnati Children’s Hospital Medical Center, Cincinnati, OH: April Carr, BS, CCRP; Kimberlee Bernstein, BS, CCRP; Kristin Bramlage, MD; Kim Cecil, PhD; Stephanie DeVore, MSPH (2009–2011); Rohit Kohli, MD; Kathleen Lake, MSW (2009–2012); Daniel Podberesky, MD (2009–2014); Alex Towbin, MD; Stavra Xanthakos, MD

Columbia University, New York, NY: Gerald Behr, MD; Joel E. Lavine, MD, PhD; Jay H. Lefkowitch, MD; Ali Mencin, MD; Elena Reynoso, MD

Emory University, Atlanta, GA: Adina Alazraki, MD; Rebecca Cleeton, MPH, CCRP; Maria Cordero; Albert Hernandez; Saul Karpen, MD, PhD; Jessica Cruz Munos (2013–2015); Nicholas Raviele (2012–2014); Miriam Vos, MD, MSPH, FAHA

Indiana University School of Medicine, Indianapolis, IN: Molly Bozic, MD; Oscar W. Cummings, MD; Ann Klipsch, RN; Jean P. Molleston, MD; Emily Ragozzino; Kumar Sandrasegaran, MD; Girish Subbarao, MD; Laura Walker, RN

Johns Hopkins Hospital, Baltimore, MD: Kimberly Kafka, RN; Ann Scheimann, MD

Northwestern University Feinberg School of Medicine/Ann & Robert H. Lurie Children's Hospital of Chicago: Joy Ito, RN; Mark H. Fishbein, MD; Saeed Mohammad, MD; Cynthia Rigsby, MD; Lisa Sharda, RD; Peter F. Whitington, MD

Saint Louis University, St Louis, MO: Sarah Barlow, MD (2002–2007); Theresa Cattoor, RN; Jose Derdoy, MD (2007–2011); Janet Freebersyser, RN; Ajay Jain MD; Debra King, RN (2004–2015); Jinping Lai, MD; Pat Osmack; Joan Siegner, RN (2004–2015); Susan Stewart, RN (2004–2015); Susan Torretta; Kristina Wriston, RN (2015)

University at Buffalo, Buffalo, NY: Susan S. Baker, MD, PhD; Diana Lopez-Graham; Sonja Williams; Lixin Zhu, PhD

University of California San Diego, San Diego, CA: Jonathan Africa, MD; Hannah Awai, MD; Cynthia Behling, MD, PhD; Craig Bross; Jennifer Collins; Janis Durelle; Kathryn Harlow, MD; Michael Middleton, MD, PhD; Kimberly Newton, MD; Melissa Paiz; Jeffrey B. Schwimmer, MD; Claude Sirlin, MD; Patricia Ugalde-Nicalo, MD; Mariana Dominguez Villarreal

University of California San Francisco, San Francisco, CA: Bradley Aouizerat, PhD; Jesse Courtier, MD; Linda D. Ferrell, MD; Natasha Feier, MS; Ryan Gill, MD, PhD; Camille Langlois, MS; Emily Rothbaum Perito, MD; Philip Rosenthal, MD; Patrika Tsai, MD

University of Washington Medical Center and Seattle Children's Hospital, Seattle, WA: Kara Cooper; Simon Horslen, MB, ChB; Evelyn Hsu, MD; Karen Murray, MD; Randolph Otto, MD; Matthew Yeh, MD, PhD; Melissa Young

Washington University, St. Louis, MO: Elizabeth M. Brunt, MD (2002–2015); Kathryn Fowler, MD (2012–2015)

Resource Centers

National Cancer Institute, Bethesda, MD: David E. Kleiner, MD, PhD

National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD: Sherry Brown, MS; Edward C. Doo, MD; Jay H. Hoofnagle, MD; Patricia R. Robuck, PhD, MPH (2002–2011); Averell Sherker, MD; Rebecca Torrance, RN, MS

Johns Hopkins University, Bloomberg School of Public Health (Data Coordinating Center), Baltimore, MD: Patricia Belt, BS; Jeanne M. Clark, MD, MPH; Michele Donithan, MHS; Erin Hallinan, MHS; Milana Isaacson, BS; Kevin P. May, MS; Laura Miriel, BS; Alice Sternberg, ScM; James Tonascia, PhD; Mark Van Natta, MHS; Laura Wilson, ScM; Katherine Yates, ScM

Support:

The study was supported by NIDDK (U01DK061734, U01DK061718, U01DK061728, U01DK061731, U01DK061732, U01DK061737, U01DK061738, U01DK061730, U01DK061713) and NICHD. It was also supported by NIH CTSA awards (UL1TR000040, UL1RR024989, UL1RR025761, M01RR00188, UL1RR024131, UL1RR025014, UL1RR031990, UL1RR025741, UL1RR029887, UL1RR24156, UL1RR025055, UL1RR031980), and DRC HDK063491.

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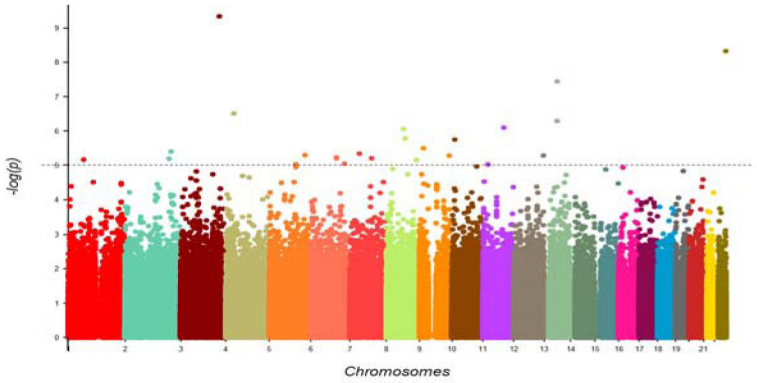
What is Known

- Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease in children.
- NAFLD is associated with increased rates of pediatric obesity and with insulin resistance.
- No genetic variant studies have been performed on children with biopsy-proven NAFLD to determine associated genetic risk factors for adiposity and insulin resistance.

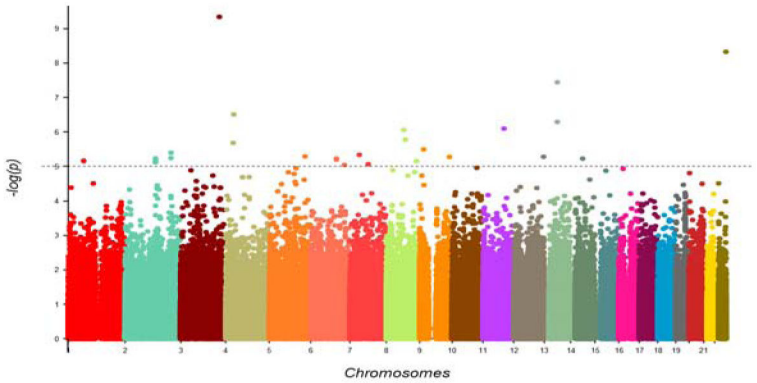
What is New

- This genome-wide association scan (GWAS) identified 10 SNPs that were associated with BMI z-score, 6 within chromosome 2, and 1 within CAMK1D, which has a potential role in liver gluconeogenesis.
- The GWAS ALSO identified 9 novel variants associated with insulin resistance: 6 with HOMA-IR and 3 with Hemoglobin A1C.

A) BMI z-score using dominant model



B) BMI z-score using additive model



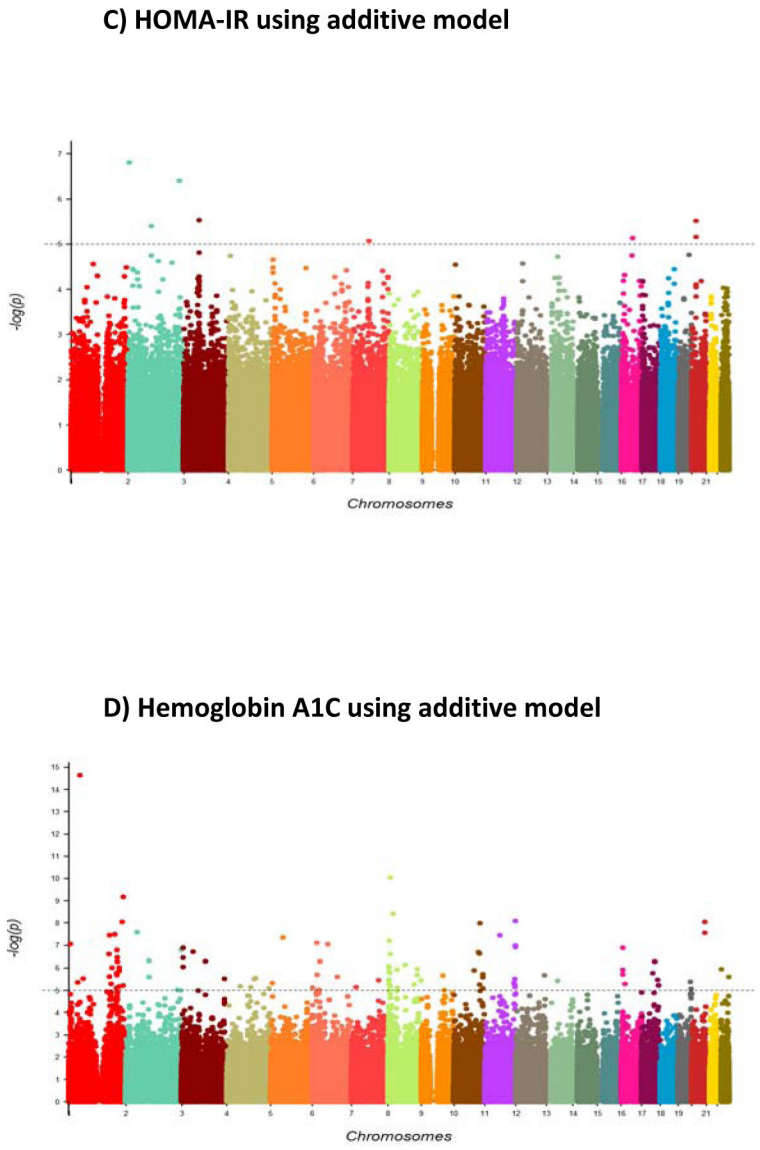
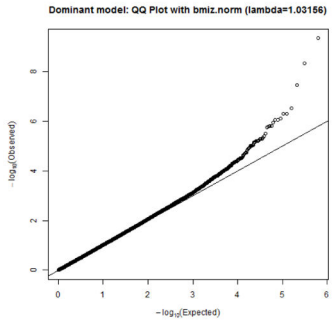


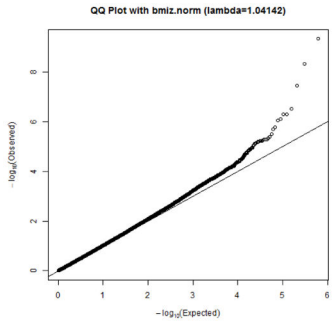
Figure 1. Manhattan Plots

Manhattan plots of BMI z-score, HOMA-IR, and hemoglobin A1C using linear regression genome-wide association studies. (A) represents BMI z-score using a dominant model, (B) represents BMI z-score using an additive model, (C) represents HOMA-IR using an additive model, and (D) represents hemoglobin A1C using an additive model. The y-axis is the $-\log_{10}$(p-value). The x-axis is the position on the 22 autosomal chromosomes. The different colored circles each represent individual single nucleotide polymorphisms (SNPs). The dotted line indicates significance at p-value of $<10^{-6}$.

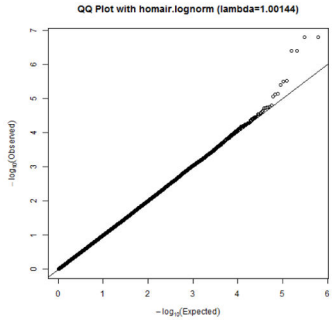
A) BMI z-score (dominant model)



B) BMI z-score (additive model)



C) HOMA-IR



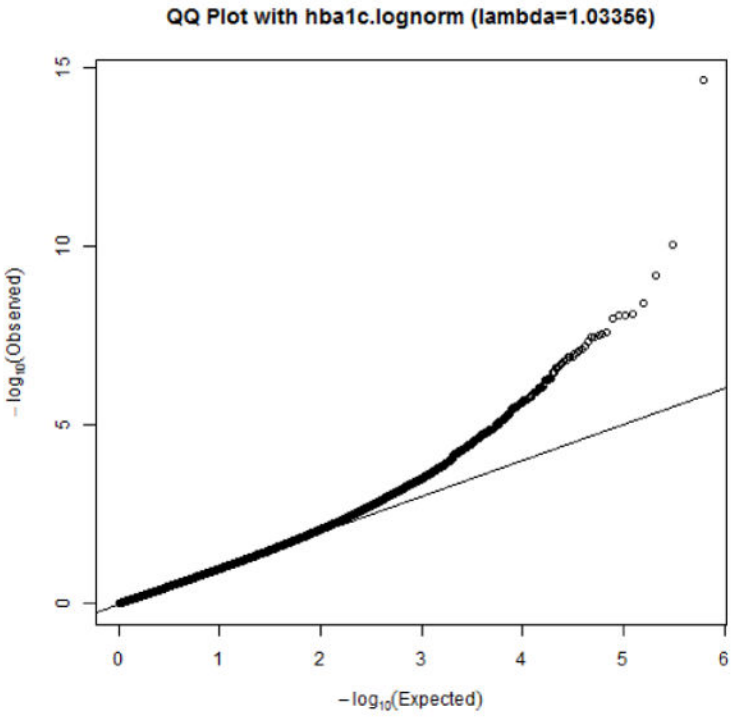


Figure 2. Quantile-Quantile Plots

Quantile–Quantile (QQ) plots for BMI z-score, HOMA-IR, and hemoglobin A1C in genome-wide association studies. (A) represents BMI z-score using a dominant model, (B) represents BMI z-score using an additive model, (C) represents HOMA-IR using an additive model, and (D) represents hemoglobin A1C using an additive model.

Table 1

Baseline Subject Values (N=208)

Characteristic	Median (25th–75th%)	Actual # of subjects
Demographic Characteristics		
Gender (male), n (%)	208 (100%)	208
Ethnicity (Hispanic), n (%)	208 (100%)	208
Age (years)	12.0 (11.0–14.0)	208
BMI (kg/m^2)	31.4 (27.8–35.5)	207
BMI Z-Score	2.4 (2.1–2.6)	208
Waist/Hip Ratio	1.00 (0.96–1.04)	207
Diabetes mellitus, n (%)	4 (2)	4
Laboratory Measures		
ALT (U/L)	83 (61–138)	208
AST (U/L)	51 (38–72)	208
Total cholesterol, mg/dL	167 (142.5–187)	207
HDL cholesterol, mg/dL	38 (32–43)	207
LDL cholesterol, mg/dL	101 (83.3–118.8)	206
Triglycerides, mg/dL	118 (82–157.5)	207
Serum glucose (mg/dl)	86 (82–92)	207
Serum insulin(U/ml)	26 (17–42)	201
HBA1c (%)	5.3 (5.1–5.5)	205
Histologic characteristics		
Steatosis, n (%)	**NASH CRN Score**	**Number of Subjects (Percentage of total)**
<5%	0	8 (4)
5%–33%	1	52 (25)
34%–66%	2	60 (29)
>66%	3	88 (42)
Lobular inflammation, n (%)	**NASH CRN Score**	
0	0	0 (0)
<2 under 20x magnification	1	113 (54)
2–4 under 20x magnification	2	81 (39)
>4 under 20_xmagnification	3	14 (7)
Ballooning, n (%)	**NASH CRN Score**	
None	0	123 (59)
Few	1	54 (26)
Many	2	31 (15)
NASH diagnosis, n (%)		
No NASH		48 (23)
Borderline zone 3 pattern		26 (13)
Borderline zone 1 pattern		84 (40)
Yes, definite		46 (22)
Missing		4 (2)

Table 2

Significant SNP relations to obesity and insulin resistance using linear regression modeling.

Trait	SNP	Chromosome	BP	Gene	Minor	Major	Minor Allele Frequency	P-value
BMI z-score	rs295120	2	201241967	SPATS2L	A	C	0.07933	4.0×10^{-6}
(Dominant Model)	rs2303752	5	115785512	SEMA6A	T	C	0.1178	9.4×10^{-6}
	rs17583338	10	12849721	CAMK1D	T	C	0.271	1.8×10^{-6}
	rs11026723	11	22689884	GAS2	A	G	0.4303	9.5×10^{-6}
BMI z-score	rs12619898	2	133492286	NCKAP5	G	A	0.1498	6.3×10^{-6}
(Additive Model)	rs17397163	2	133491553	NCKAP5	G	A	0.149	5.9×10^{-6}
	rs11687204	2	133513838	NCKAP5	C	T	0.1442	7.5×10^{-6}
	rs17397380	2	133516599	NCKAP5	A	C	0.1442	7.5×10^{-6}
	rs99521	2	201201190	SPATS2L	T	G	0.1178	5.7×10^{-6}
	rs295120	2	201241967	SPATS2L	A	C	0.07938	4.0×10^{-6}
	rs8005339	14	52215324	Unknown	A	G	0.5	5.9×10^{-6}
HOMA-IR	rs10865041	2	102019801	RFX8	G	T	0.2415	4.0×10^{-6}
(Additive Model)	rs9846667	3	68565824	FAM19A1	A	G	0.262	2.9×10^{-6}
	rs11773571	7	71037932	WBSCR17	C	T	0.3534	8.5×10^{-6}
	rs8046133	16	49502003	Unknown	G	A	0.05288	7.4×10^{-6}
	rs4361192	20	18375581	DZANK1	C	T	0.2428	3.0×10^{-6}
	rs2295067	20	18362386	LINC00851	A	G	0.2284	7.0×10^{-6}
Hgb A1C	rs3923850	11	133197686	OPCML	A	G	0.05529	1.2×10^{-7}
(Additive Model)	rs11727927	11	133214582	OPMCL	G	A	0.07971	1.0×10^{-7}
	rs11644684	16	7934611	Unknown	C	A	0.06522	1.3×10^{-7}