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Authors

Barata, Llilda

Feitosa, Mary F

Bielak, Lawrence F

et al.

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Insulin Resistance Exacerbates Genetic Predisposition to Nonalcoholic Fatty Liver Disease in Individuals Without Diabetes

Llilda Barata,[1] Mary F. Feitosa,[1] Lawrence F. Bielak,[2] Brian Halligan,[3] Abigail S. Baldridge,[4] Xiuqing Guo,[5] Laura M. Yerges-Armstrong,[6] Albert V. Smith,[7] Jie Yao,[5] Nicholette D. Palmer,[8] Lisa B. VanWagner ,[4,9] J. Jeffrey Carr,[10] Yii-Der I. Chen,[5] Matthew Allison,[11] Matthew J. Budoff,[12] Samuel K. Handelman,[3] Sharon L.R. Kardia,[2] Thomas H. Mosley Jr.,[13] Kathleen Ryan,[6] Tamara B. Harris,[14] Lenore J. Launer,[14] Vilmundur Gudnason,[15,16] Jerome I. Rotter,[5] Myriam Fornage,[17] Laura J. Rasmussen-Torvik,[4] Ingrid B. Borecki,[1] Jeffrey R. O'Connell,[6] Patricia A. Peyser,[2] Elizabeth K. Speliotes,[3]* and Michael A. Province[1]*

The accumulation of excess fat in the liver (hepatic steatosis) in the absence of heavy alcohol consumption causes nonalcoholic fatty liver disease (NAFLD), which has become a global epidemic. Identifying metabolic risk factors that interact with the genetic risk of NAFLD is important for reducing disease burden. We tested whether serum glucose, insulin, insulin resistance, triglyceride (TG), low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, body mass index (BMI), and waist-to-hip ratio adjusted for BMI interact with genetic variants in or near the patatin-like phospholipase domain containing 3 (*PNPLA3*) gene, the glucokinase regulatory protein (*GCKR*) gene, the neurocan/transmembrane 6 superfamily member 2 (*NCAN/TM6SF2*) gene, and the lysophospholipase-like 1 (*LYPLAL1*) gene to exacerbate hepatic steatosis, estimated by liver attenuation. We performed association analyses in 10 population-based cohorts separately and then meta-analyzed results in up to 14,751 individuals (11,870 of European ancestry and 2,881 of African ancestry). We found that *PNPLA3*-rs738409 significantly interacted with insulin, insulin resistance, BMI, glucose, and TG to increase hepatic steatosis in nondiabetic individuals carrying the G allele. Additionally, *GCKR*-rs780094 significantly interacted with insulin, insulin resistance, and TG. Conditional analyses using the two largest European ancestry cohorts in the study showed that insulin levels accounted for most of the interaction of *PNPLA3*-rs738409 with BMI, glucose, and TG in nondiabetic individuals. Insulin, *PNPLA3*-rs738409, and their interaction accounted for at least 8% of the variance in hepatic steatosis in these two cohorts. *Conclusion:* Insulin resistance, either directly or through the resultant elevated insulin levels, more than other metabolic traits, appears to amplify the *PNPLA3*-rs738409-G genetic risk for hepatic steatosis. Improving insulin resistance in nondiabetic individuals carrying *PNPLA3*-rs738409-G may preferentially decrease hepatic steatosis. (*Hepatology Communications* 2019;3:894-907).

Nonalcoholic fatty liver disease (NAFLD) is a result of the excess accumulation of lipids in hepatocytes (hepatic steatosis) in the absence of heavy alcohol consumption.[1] Hepatic steatosis is also associated with the risk of developing dyslipidemia or dysglycemia[2] as well as cardiovascular

Abbreviations: AA, African ancestry; AGES, Age, Gene/Environment Susceptibility-Reykjavik; Amish, Old Order Amish; BMI, body mass index; CARDIA, Coronary Artery Risk Development in Young Adults; CI, confidence interval; EA, European ancestry; FamHS, Family Heart Study; FHS, Framingham Heart Study; GCKR, glucokinase regulatory protein; GENOA, Genetic Epidemiology Network of Arteriopathy; HDL, high-density lipoprotein; HOMA-IR, homeostatic model of insulin resistance; HU, Hounsfield units; LA, liver attenuation; LA_{ivn}, inverse normal-transformed residuals of liver attenuation; LDL, low-density lipoprotein; LYPLAL1, lysophospholipase-like 1; MBOAT7/TMCA, membrane bound O-acyltransferase domain containing 7/Transmembrane channel-like 4; MESA, Multi-Ethnic Study of Atherosclerosis; MT_{ivn}, inverse normal-transformed residuals of metabolic trait; NAFLD, nonalcoholic fatty liver disease; NCAN, neurocan; OR, odds ratio; PNPLA3, patatin-like phospholipase domain-containing protein 3; SD, standard deviation; SE, standard errors; SNP, single nucleotide polymorphism; TG, triglyceride; TM6SF2, transmembrane 6 superfamily member 2; WHR_{adj}BMI, waist-to-hip ratio adjusted for body mass index.

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disease, which is the number one cause of death in individuals with NAFLD.[3,4] Hepatic steatosis may progress to advanced liver disease in the form of nonalcoholic steatohepatitis, fibrosis (cirrhosis), and cancer (hepatocellular carcinoma).[5-7] In the United States, the prevalence of hepatic steatosis in the adult population is between 10% and 30%; worldwide it is 25%-45%.[8] While the pathogenesis of NAFLD is not entirely understood, both genetic factors and metabolic traits increase the risk of hepatic steatosis.

Heritability of hepatic steatosis ranges from 22% to 38% across all ancestries, suggesting that specific genotypes may predispose individuals to NAFLD.[1] Previously, the Genetics of Obesity-Related Liver

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**These authors contributed equally to this work.*

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ARTICLE INFORMATION:

From the [1]Division of Statistical Genomics, Department of Genetics, Washington University School of Medicine, St. Louis, MO; [2]Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, MI; [3]Division of Gastroenterology, Department of Internal Medicine, Department of Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, MI; [4]Department of Preventive Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL; [5]Institute for Translational Genomics and Population Sciences, LABioMed and the Department of Pediatrics, Harbor-University of California Los Angeles Medical Center, Torrance, CA; [6]Department of Medicine, University of Maryland School of Medicine, Baltimore, MD; [7]Department of Biostatistics, School of Public Health, University of Michigan, Ann Arbor, MI; [8]Department of Biochemistry, Wake Forest School of Medicine, Winston-Salem, NC; [9]Division of Gastroenterology and Hepatology, Northwestern University Feinberg School of Medicine, Chicago, IL; [10]Department of Radiology, Vanderbilt University School of Medicine, Nashville, TN; [11]Department of Family Medicine and Public Health, University of California San Diego, San Diego, CA; [12]Division of Cardiology, Los Angeles Biomedical Research Institute, Torrance, CA; [13]Department of Medicine, Division of Geriatrics, University of Mississippi Medical Center, Jackson, MS; [14]Laboratory of Epidemiology and Population Sciences, National Institute of Aging, Bethesda, MD; [15]Icelandic Heart Association, Kopavogur, Iceland; [16]Faculty of Medicine, University of Iceland, Reykjavik, Iceland; [17]University of Texas Health Science Center, Houston, TX.

Disease Consortium conducted a genome-wide association study in 7,176 individuals of European ancestry (EA), with replication in histology-based samples.[9] This study identified that rs738409 (in patatin-like phospholipase domain containing 3 [*PNPLA3*]), a missense single nucleotide polymorphism (SNP) first associated with hepatic fat content a decade ago[10]; the missense variant rs2228603 (in neurocan/transmembrane 6 superfamily member 2 [*NCAN/TM6SF2*]); and intronic variants rs12137855 (in lysophospholipase-like 1 [*LYPLAL1*]) and rs780094 (in glucokinase regulatory protein [*GCKR*]) were significantly associated with hepatic steatosis.[9] We and others have replicated the association of these common variants with hepatic steatosis in other populations and ethnicities,[11-13] and the associations are consistent between those of EA and African ancestry (AA) (direction of effect is similar).[11] Further, the G allele for rs738409 was associated with susceptibility to nonalcoholic steatohepatitis (odds ratio [OR], 2.64; 95% confidence interval [CI], 1.85-3.75; $P < 1.0E–04$), nonalcoholic steatohepatitis severity (OR, 1.85; 95% CI, 1.05-3.26; $P < 3.5E–02$), and fibrosis (OR, 1.95; 95% CI, 1.17-3.26; $P < 1.3E–02$) in EA individuals.[14]

Traits that predispose to metabolic syndrome, i.e., higher body mass index (BMI),[15] dyslipidemia, hyperglycemia, and insulin resistance, are associated with hepatic steatosis.[2,3,16] Approximately 80%-90% of adults with obesity (BMI ≥ 30 kg/m^2) have hepatic steatosis,[17] while 20%-80% of individuals with hepatic steatosis also have higher levels of triglyceride (TG) and low-density lipoprotein (LDL) cholesterol but lower levels of high-density lipoprotein (HDL) cholesterol.[18] Diabetes is also commonly associated with hepatic steatosis.[19] How these modifiable metabolic traits interact with genetic variation to influence the risk for hepatic steatosis is not known.

In this cross-sectional study, we tested whether several metabolic traits interact with the four genetic variants associated with hepatic steatosis[9] to affect liver attenuation (LA), a computed tomographic quantitative measure that is inversely related to histologically measured liver fat.[20] The metabolic traits tested were insulin resistance (as homeostatic model of insulin resistance [HOMA-IR]), fasting insulin, fasting glucose, BMI, centralized fat deposition measured by waist-to-hip ratio adjusted for BMI ($WHR_{adj}BMI$), fasting TG, fasting HDL, and fasting LDL. We first carried out interaction analyses between each of these traits and each of the genetic variants in 10 separate population-based cohorts from seven different studies. We then meta-analyzed results by ancestry (EA, n = 11,870; AA, n = 2,881) and across cohorts in up to 14,751 individuals. We then carried out conditional analyses in the two largest EA cohorts in the study to determine the driving metabolic factor.

Participants and Methods

ETHICS STATEMENT

This study was approved by the Icelandic National Bioethics Committee (VSN 00-063) and the institutional review boards or equivalent committees of all participating studies. The principal investigator of each institution obtained written consent from participants.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Llilda Barata, Ph.D., M.P.H.
Department of Genetics, Division of Statistical Genomics
Washington University School of Medicine
4523 Clayton Ave
Campus Box 8506-98-601
St. Louis, MO, 63110
E-mail: barata@wustl.edu
Tel.: +1-314-747-4133
or
Elizabeth K. Speliotes, M.D., Ph.D., M.P.H.
Division of Gastroenterology
Department of Internal Medicine
University of Michigan
Medical Sciences Research Building I
Room 6520
1150 West Medical Center Drive
Ann Arbor, MI, 48109-5682
E-mail: espeliot@med.umich.edu
Tel.: +1-617-642-5497
or
Michael A. Province, Ph.D.
Department of Genetics, Division of Statistical Genomics
Washington University School of Medicine
4523 Clayton Ave
Campus Box 8506-98-601
St. Louis, MO, 63110
E-mail: mprovince@wustl.edu
Tel.: +1-314-362-3616

STUDY DESCRIPTION

The study was comprised of up to 14,751 individuals (EA, n = 11,870; AA, n = 2,881), and 56% of participants were women. The sample derived from seven population-based studies participating in the Genetics of Obesity-Related Liver Disease Consortium: Age, Gene/Environment Susceptibility-Reykjavik (AGES), Old Order Amish (Amish), Coronary Artery Risk Development in Young Adults (CARDIA), Family Heart Study (FamHS), Framingham Heart Study (FHS), Genetic Epidemiology Network of Arteriopathy (GENOA), and Multi-Ethnic Study of Atherosclerosis (MESA). In total, 10 cohorts were included in the analysis as three studies contributed two ancestry groups (AA, EA). Each ancestry group was analyzed separately. CARDIA, MESA, and AGES have unrelated individuals, while FHS, Amish, GENOA, and FamHS are family-based studies. Detailed information about the characteristics and design of each study is provided in Supporting Table S1.

OUTCOME VARIABLE AND METABOLIC TRAITS

The outcome variable was LA, measured noninvasively with computed tomography in Hounsfield units (HU).[21] LA is inversely proportional to liver fat, i.e., lower LA values indicate a higher fat content in the liver (more hepatic steatosis).[2] The procedures followed by each cohort to measure LA are described in Supporting Table S2. Individuals with active malignancies, focal lesions, or other incidental findings on computed tomography were excluded from the studies.

Metabolic traits of interest were harmonized across cohorts following standard clinical definitions. Overall adiposity was characterized by BMI (kg/m^2) and abdominal adiposity by $WHR_{adj}BMI$ (cm). Because waist-to-hip ratio is correlated with both BMI and visceral fat, we chose to use $WHR_{adj}BMI$ to have a measure that is independent of overall fatness (i.e., BMI) but reflects visceral adiposity and is easily measured in the clinic. Fasting insulin (mU/L) and fasting glucose (mmol/L) were measured from plasma or serum using standard laboratory techniques detailed in Supporting Table S2. When fasting glucose was measured from whole blood, it was converted to plasma glucose using a correction factor of 1.13.[22] HOMA-IR was assessed using fasting glucose (mmol/L) × fasting insulin (mU/L) divided by 22.5.[23] Each cohort assayed fasting TG (mg/dL) and fasting HDL (mg/dL), using methods described in Supporting Table S2. If fasting LDL (mg/dL) was assayed, it was used; otherwise, LDL was calculated using the Friedewald formula LDL_F = (total cholesterol [mg/dL] – HDL [mg/dL] – TG [mg/dL]/5.0), only if TG <400 mg/dL.[24]

Alcohol consumption, history of diabetes, and use of lipid-lowering medications were acquired by questionnaire. Total alcohol consumption, defined in drinks per week, was calculated from daily intake of beer, wine, and spirits. One drink was defined as a serving of 14 g ethanol, the same as a 12-ounce (354.88 mL) bottle or can of beer, 5-ounce (147.87 mL) glass of wine, or 1.5-ounce (44.36 mL) shot of 80-proof spirits, such as gin, vodka, or whiskey.[25] Heavy drinking was defined as ≥8 drinks per week for women and ≥15 drinks per week for men.[26] Diabetes (type 1, type 2) was defined as having fasting plasma glucose levels ≥7 mmol/L (126 mg/dL), or self-reporting the use of insulin or oral antidiabetic medications, or having a physician's diagnosis of diabetes. The use of statins was assessed from medication questionnaires.

GENOTYPING AND IMPUTATION

Four common variants were included in the analyses: rs738409, a missense variant in the *PNPLA3* gene; rs780094, an intronic variant within the *GCKR* gene that is in high linkage disequilibrium (r^2 = 0.93) with rs1260326, a likely functional missense variant in this gene; rs2228603, a missense variant in the *NCAN* gene that is in high linkage disequilibrium (r^2 = 0.798) with rs585422926, a likely functional missense variant in the *TM6SF2* gene; and rs12137855, an intronic variant in the *LYPLAL1* gene. These variants were either directly genotyped (allele counts were coded 0, 1, or 2) or dosages were imputed from HapMap II or 1000G. Genotype calling algorithms and imputation methods are detailed in Supporting Table S3.

STATISTICAL ANALYSIS

Cohort-Specific Analyses

Analyses were performed separately in each ancestry group (EA, AA). LA and metabolic traits, used as continuous variables in all analyses, were adjusted for sex, age, principal component estimates of ancestry,

and study-specific covariates, using linear regression as detailed in Supporting Table S2. LA was also adjusted for alcohol consumption, a continuous variable (drinks/week), and for scan penetrance using phantom or spleen density. Residuals from adjusted LA and metabolic traits were transformed using inverse normal transformation to reduce the influence of outliers and to standardize the phenotypes across cohorts. Inverse normal-transformed residuals of LA (LA_{ivn}) and each metabolic trait (MT_{ivn}) were used to fit the interaction models.

Each cohort tested for statistical interactions between each variant and each metabolic trait, using multivariable linear regression or mixed linear modeling. LA_{ivn} was the dependent variable. The independent variables were each SNP and MT_{ivn}, plus their interaction: $LA_{ivn} = \alpha + \beta_1(SNP) + \beta_2(MT_{ivn}) + \beta_3(SNP \times MT_{ivn}) + \epsilon$. An additive model of inheritance was assumed. Studies with family data (FHS, GENOA, Amish, and FamHS) used linear mixed models to account for family relatedness among participants and computed robust standard errors (SE). Participants with diabetes (type1 and type 2) were excluded from the insulin, glucose and HOMA-IR models, and those taking statins were excluded from the LDL model. As a secondary analysis, BMI was included as a covariate in the models to investigate whether the effect of the interaction between each SNP and each metabolic trait on LA_{ivn} occurred independent of overall adiposity. Associations were carried out using MMAP,[27] R,[28] and SAS[29] software.

Meta-Analyses

We conducted fixed-effects meta-analyses by ancestry and overall on the parameter estimates (β coefficient and SE) for the main effects and interaction effects. We used the inverse variance weighting method implemented in METAL.[30] Using Cochran's Q test,[31] we tested for heterogeneity of effects across all analyses. Within ancestries and focusing on interactions, we found evidence of heterogeneity only for the interaction between TG and *GCKR* in the EA cohorts. We did not find any heterogeneity for the interaction in the meta-analyses between the two ancestry groups (EA versus AA); thus, we report the combined ancestry meta-analyses. To determine the level of statistical significance while accounting for multiple testing, we applied a Bonferroni correction that consisted of grouping correlated traits into three metabolic domains: insulin-glucose, adiposity, and lipids. The critical P value $\alpha = 0.05$ was divided by 12 (4 variants × 3 metabolic domains) to obtain a corrected P value. Meta-analyses results and heterogeneity tests were considered significant if $P \leq 4.17E{-}03$ (two-tailed). As a secondary analysis to investigate whether the statistically significant interactions were consistent between sexes, we fit the interaction models in men and women separately and meta-analyzed results within each sex.

Conditional Analyses in FamHS and FHS

To determine whether the interaction of BMI, glucose, or TG with *PNPLA3*-rs738409 was independent of insulin, we analyzed each trait's interaction effect before and after including insulin in the model. The analyses were performed with EA individuals in FamHS and replicated in FHS. We chose these two cohorts because they are the two largest cohorts in the study; together they represent more than one third of our total sample. Individuals with diabetes and/or missing information for the metabolic traits of interest were excluded, resulting in a sample of 2,280 individuals in FamHS and 2,581 in FHS. After adjusting LA for phantom in both cohorts and for field centers in FamHS, LA residuals were transformed using inverse normal transformation to approximate normality. LA_{ivn} were used as the dependent variable. Using linear mixed models, we first regressed LA_{ivn} on either BMI, glucose, or TG and their interaction with *PNPLA3*-rs738409 (Supporting Text). We then added insulin to the models and its interaction with *PNPLA3*-rs738409 and the metabolic trait (either BMI, glucose, or TG). Insulin and TG were log-transformed due to the presence of influential outliers. Models were adjusted for age, sex, and alcohol consumption (drinks/week) and for genotype batch effects in FamHS. Results from conditional analyses in each cohort were then meta-analyzed.

The conditional models included principal components to adjust for population stratification. Because the principal components were not associated with LA_{ivn} in either cohort and their inclusion in the conditional models did not change the inferences, we present the models without them. We also performed

conditional analyses after excluding individuals from FamHS (n = 231) and FHS (n = 371) who reported heavy alcohol use (≥8 drinks per week for women and ≥15 drinks per week for men) (Supporting Tables S10-S12).[26] Because the inferences were unchanged, we included all individuals to increase power and adjusted for alcohol as a covariate. Additionally, we conducted the conditional analyses with log-transformed HOMA-IR instead of log-transformed insulin (Supporting Tables S13-S15). Insulin and HOMA-IR provided similar inferences. Because glucose explained significantly less of the variation in LA_{inv}, we focused on insulin over HOMA-IR because there was no added benefit of measuring glucose on variance explained by HOMA-IR than with just measuring insulin.

Illustration in FamHS of the Interaction Between Insulin and *PNPLA3*-rs738409 in Individuals Without Diabetes

To assess the interaction effect of insulin with *PNPLA3*-rs738409 on hepatic steatosis prevalence in FamHS, we plotted the percentage of individuals with LA ≤60 HU per *PNPLA3*-rs738409 genotype by the lowest and highest quartile of insulin. Individuals with diabetes and/or missing information for insulin were excluded and ancestries were combined to obtain a sample of n = 2,725, which was divided into quartiles of insulin. LA and insulin were not adjusted or transformed. The LA cut-off point of ≤60 HU, which corresponds to a liver:spleen ratio of 1.1, has been shown to identify individuals with moderate to severe macrovesicular steatosis (≥30% of the liver parenchyma with fat) at histology with a high diagnostic accuracy.[32] In the literature, ≥30% liver fat suggests moderate to severe hepatic steatosis.[33]

Results

Demographics and clinical characteristics across the study cohorts are presented in Table 1. The mean age ± standard deviation (SD) across cohorts ranged from 49.47 ± 3.86 to 76.38 ± 5.46 years old. All cohorts included more women than men. The mean ± SD of LA across cohorts ranged from 55.05 ± 12.28 HU to 65.40 ± 9.83 HU. In those without diabetes, mean ± SD of fasting insulin levels ranged from 8.30 ± 5.73 to 13.02 ± 10.22 mU/L and fasting blood glucose levels ranged from 4.90 ± 0.58 to 5.49 ± 0.50 mmol/L. The lowest mean ± SD for HOMA-IR in those without diabetes was 1.99 ± 1.27 and the highest was 3.14 ± 2.69. The mean ± SD of BMI ranged from 27.00 ± 4.49 to 32.71 ± 7.37 kg/m^2. Several cohorts reported mean fasting TG >100 mg/dL. Mean ± SD for fasting LDL cholesterol in nonstatin users was borderline high in Amish (141.31 ± 38.66 mg/dL) and AGES (146.84 ± 35.73 mg/dL). Across cohorts, the range of fasting HDL cholesterol was within the recommended limit of ≥40 mg/dL. Heavy drinking varied among studies, with GENOA having the lowest percentage (0%) and CARDIA the highest (37%).

PNPLA3-rs738409 AND *GCKR*-rs780094 INTERACT WITH SEVERAL METABOLIC TRAITS

We found significant interactions for *PNPLA3*-rs738409 and *GCKR*-rs780094 with several metabolic traits in combined ancestries after adjusting for multiple comparisons (Table 2; Supporting Table S4). *PNPLA3*-rs738409 interacted with insulin (P = 4.79E–14), HOMA-IR (P = 4.68E–15), glucose (P = 1.26E–03), BMI (P = 8.13E–08), and TG (P = 2.95E–03). As each of these metabolic traits increased, a decrease in LA_{ivn} (i.e., higher fat content in the liver) became more pronounced in the presence of the G allele at *PNPLA3*-rs738409 compared to the presence of the C allele. Additionally, *GCKR*-rs780094 interacted with insulin (P = 4.57E–04), HOMA-IR (P = 1.32E–03), and TG (P = 4.17E–03). As levels of insulin, HOMA-IR, and TG increased, a decrease in LA_{ivn} (i.e., higher fat content in the liver) became more pronounced in the presence of the T allele at *GCKR*-rs780094 compared to the C allele. All interactions remained significant after adjusting for BMI (Supporting Table S5), suggesting that overall adiposity did not alter these effects. We did not find evidence of significant interactions between any of the four genetic variants and WHR_{adj}BMI, LDL, or HDL. Although the interaction between WHR_{adj}BMI and *PNPLA3*-rs*738409* did not reach the Bonferroni significance level, it was borderline significant. This

TABLE 1. DEMOGRAPHIC AND CHARACTERISTICS OF STUDY PARTICIPANTS IN EACH COHORT BY ANCESTRY

	AGES	Amish	CARDIA	FamHS	FHS	MESA	CARDIA	FamHS	GENOA	MESA
Demographic	European Ancestry (11,870)						African Ancestry (2,881)			
N = 14,751	2,865	541	1,282	2,684	2,966	1,532	642	620	560	1,059
Age	76.38 ± 5.46	56.84 ± 12.81	50.74 ± 3.33	57.14 ± 13.28	50.54 ± 10.14	63.05 ± 10.49	49.47 ± 3.86	53.35 ± 10.82	68.86 ± 8.01	63.17 ± 10.00
Men (6,444)	1,139 (40%)	252 (47%)	595 (46%)	1,207 (45%)	1,454 (49%)	746 (49%)	233 (36%)	212 (34%)	141 (25%)	465 (44%)
Women (8,307)	1,726 (60%)	289 (53%)	687 (54%)	1,477 (55%)	1,512 (51%)	786 (51%)	409 (64%)	408 (66%)	419 (75%)	594 (56%)
Characteristics										
Liver attenuation (HU)[||]	59.22 ± 8.64	63.05 ± 7.76	55.05 ± 12.28	59.14 ± 11.19	65.40 ± 9.83	59.33 ± 12.43	56.38 ± 10.86	59.52 ± 9.23	60.10 ± 9.39	61.18 ± 9.06
Insulin (mU/L)	9.22 ± 6.39	11.75 ± 6.22	9.26 ± 6.77	9.88 ± 7.16	9.05 ± 7.38	8.90 ± 4.94	11.60 ± 8.29	13.02 ± 10.22	8.30 ± 5.73	9.61 ± 5.53
HOMA-IR[‡]	2.31 ± 1.76	2.69 ± 1.63	2.20 ± 1.77	2.37 ± 1.87	2.32 ± 2.31	1.99 ± 1.27	2.75 ± 2.14	3.14 ± 2.69	2.02 ± 1.46	2.19 ± 1.41
Glucose (mmol/L)	5.49 ± 0.50	4.94 ± 0.52	5.18 ± 0.50	5.25 ± 0.53	5.47 ± 1.12	4.90 ± 0.58	5.17 ± 0.54	5.25 ± 0.57	5.37 ± 0.50	5.03 ± 0.59
BMI (kg/m^2)	27.00 ± 4.49	27.72 ± 4.85	28.50 ± 6.18	28.86 ± 5.69	27.51 ± 5.22	28.06 ± 5.05	31.94 ± 7.48	32.71 ± 7.37	32.71 ± 7.27	29.95 ± 5.77
Obese[†]	618 (22%)	155 (29%)	425 (33%)	972 (36%)	769 (26%)	449 (29%)	352 (55%)	377 (61%)	332 (59%)	465 (44%)
WHR (cm)[§]	nval	0.87 ± 0.07	0.85 ± 0.10	0.91 ± 0.10	0.94 ± 0.08	0.93 ± 0.09	0.85 ± 0.08	0.92 ± 0.07	0.89 ± 0.08	0.92 ± 0.08
TG (mg/dL)	106.48 ± 59.06	90.42 ± 57.45	121.64 ± 85.07	144.03 ± 94.05	126.11 ± 88.07	136.55 ± 99.31	101.55 ± 73.24	111.82 ± 80.09	100.28 ± 62.67	103.82 ± 60.61
LDL (mg/dL)	146.84 ± 35.73	141.31 ± 38.66	116.27 ± 30.15	112.90 ± 34.22	117.70 ± 31.71	120.24 ± 30.42	112.59 ± 33.83	115.39 ± 36.05	123.85 ± 33.59	118.39 ± 32.87
HDL (mg/dL)	61.75 ± 17.31	57.05 ± 15.37	58.43 ± 18.42	48.82 ± 14.37	54.16 ± 16.77	51.68 ± 15.59	57.59 ± 16.70	53.55 ± 15.41	57.31 ± 16.52	52.39 ± 15.14
Alcohol (drinks/week)	1.09 ± 2.37	nval	5.73 ± 10.07	2.98 ± 7.10	5.39 ± 7.88	5.06 ± 8.40	3.86 ± 10.60	3.24 ± 9.45	0.28 ± 1.18	3.86 ± 8.89
Heavy drinkers*	17 (0.59%)	nval	470 (37%)	152 (6%)	424 (14.3%)	335 (22%)	144 (22%)	69 (11%)	0	139 (13%)

Statistics are presented as mean ± SD or as n (%). The table shows summary statistics for individuals with LA and genetic information from each cohort who were included in analyses. LA and metabolic traits were not adjusted for covariates. The sample size for each metabolic trait varied from N depending on the data available. Summary statistics for fasting insulin, HOMA-IR, and fasting glucose excludes individuals with diabetes; fasting LDL excludes statin users.
*Defined as ≥8 drinks per week for women and ≥15 drinks per week for men; the Amish do not consume alcohol; [†]Defined as BMI ≥30 kg/m^2; [‡]calculated as (fasting insulin [mU/L] × fasting glucose [mmol/L]/22.5); [§]not adjusted for BMI; [||]Raw LA measured in Hounsfield units.
Abbreviations: nval, not available in cohort; SD, standard deviation.

TABLE 2. META-ANALYSES RESULTS FOR INTERACTIONS BETWEEN FOUR SNPs AND INVERSE NORMAL-TRANSFORMED RESIDUALS OF METABOLIC TRAITS ON LA_{ivn} IN COMBINED ANCESTRIES

	rs738409				rs780094*				rs2228603†				rs12137855			
	Gene	Chr	Alleles (Ref/O)	Ref AF	Gene	Chr	Alleles (Ref/O)	Ref AF	Gene	Chr	Alleles (Ref/O)	Ref AF	Gene	Chr	Alleles (Ref/O)	Ref AF
	PNPLA3	22	G/C	0.22	*GCKR*	2	T/C	0.38	*NCAN/ TM6SF2*	19	T/C	0.09	*LYPLAL1*	8	C/T	0.79
	(SNP × Metabolic Traits)															
Metabolic traits	β_{int}	SE	*P* Value	n	β_{int}	SE	*P* Value	n	β_{int}	SE	*P* Value	n	β_{int}	SE	*P* Value	n
Insulin	−0.11	0.02	4.79E−14	12,651	−0.04	0.01	4.57E−04	12,651	−0.06	0.03	4.37E−02	12,651	−0.02	0.02	1.55E−01	12,651
HOMA-IR	−0.12	0.02	4.68E−15	12,554	−0.04	0.01	1.32E−03	12,554	−0.06	0.03	3.63E−02	12,554	−0.02	0.02	1.38E−01	12,554
Glucose	−0.05	0.02	1.26E−03	12,742	−0.01	0.01	4.37E−01	12,742	−0.06	0.03	7.41E−02	12,742	−0.02	0.02	1.91E−01	12,742
BMI	−0.08	0.01	8.13E−08	14,693	−0.03	0.01	6.31E−03	14,693	−0.05	0.03	5.89E−02	14,693	−0.02	0.01	8.18E−01	14,693
$WHR_{adj}BMI$	−0.05	0.02	7.59E−03	10,051	−0.04	0.02	1.32E−02	10,051	−0.08	0.03	1.26E−02	10,051	0.01	0.02	7.76E−01	10,051
TG	−0.05	0.02	2.95E−03	14,551	−0.04	0.01	4.17E−03	14,551	0.00	0.03	9.77E−01	14,551	−0.03	0.02	5.75E−02	14,551
LDL	0.00	0.02	7.94E−01	12,123	0.00	0.01	9.33E−01	12,123	−0.06	0.03	5.50E−02	12,123	0.02	0.02	2.29E−01	12,123
HDL	0.04	0.02	1.41E−02	14,543	0.03	0.01	3.72E−02	14,543	0.00	0.03	9.55E−01	14,543	−0.01	0.02	3.72E−01	14,543

$P \leq 4.17E{-}03$ is considered significant; n is the highest sample size in meta-analyses.
*rs780094 is in LD ($r^2 = 0.93$) with rs1260326, a functional missense variant in *GCKR*; †rs2228603 is in LD ($r^2 = 0.79$) with rs58542926, a functional missense variant in *TM6SF2*.
Abbreviations: β_{int}, interaction effect size; Chr, chromosome; LD, linkage disequilibrium; Ref AF, reference allele frequency; Ref/O, reference/other allele (reference allele is the effect allele of each SNP); SE, standard error; SNP, single nucleotide polymorphism.

suggests that a larger sample size may be needed to detect an interaction. Alternatively, the lack of statistical significance could be because $WHR_{adj}BMI$ does not represent overall fatness to the extent that BMI or other anthropometric measurements do.

We also carried out meta-analyses in men and women separately to investigate possible sex differences, focusing only on the statistically significant interactions with *PNPLA3*-rs738409 and *GCKR*-rs780094 (Supporting Table S6). Women made up 56% of our study sample. The interaction effects of insulin and HOMA-IR with *PNPLA3*-rs738409 did not differ between men and women, and both reached statistical significance (for insulin, women, $P = 3.24E{-}11$; men, 7.24E–05; for HOMA-IR, women, $P = 1.62E{-}11$; men, $P = 2.88E{-}05$). For glucose, the interaction effect was slightly less in men than in women (smaller beta) and did not reach significance in men. These results suggest that sex did not alter the interactions between *PNPLA3*-rs738409 and insulin/HOMA-IR and that the interaction effect of glucose was still present only in women in the present study. Further, the interaction effects of BMI with *PNPLA3*-rs738409 were similar between men and women and reached significance in both ($P = 1.20E{-}03$ and $P = 3.39E{-}05$, respectively). The interaction effect of TG with *PNPLA3*-rs738409 did not reach statistical significance in either sex. Moreover, the interaction effects of both insulin and HOMA-IR with *GCKR*-rs780094 reached significance only in women ($P = 1.02E{-}03$ and $P = 6.46E{-}04$, respectively). Similarly, the interaction of TG with *GCKR*-rs780094 was significant only in women ($P = 8.71E{-}04$). Stratifying by sex substantially reduced our sample size and as a result, power.

CONDITIONAL ANALYSES SUGGEST THAT INSULIN MAY MEDIATE THE INTERACTION EFFECT OF BMI, TG, AND GLUCOSE ON LA_{ivn} IN INDIVIDUALS WITHOUT DIABETES

We observed that the interaction of insulin with *PNPLA3*-rs738409 had a greater effect on LA_{ivn} (hepatic steatosis) than that of BMI, TG, or glucose. To determine if the interaction of BMI, TG, or glucose with *PNPLA3*-rs738409 was independent of insulin, we carried out conditional analyses in FamHS and FHS and meta-analyzed results. We found that the interaction of BMI ($P = 7.57E{-}02$), TG ($P = 3.49E{-}01$), or glucose ($P = 9.09E{-}01$) with *PNPLA3*-rs738409 was no longer statistically significant after including insulin as a main effect and interactor with *PNPLA3*-rs738409 and the respective metabolic trait in the models (Supporting Tables S7-S9). In contrast, the interaction of insulin with *PNPLA3*-rs738409 remained significant after controlling for BMI, TG, or glucose ($P_{insulin–BMI} = 4.04E{-}04$; $P_{insulin–TG} = 3.24E{-}06$; $P_{insulin–glucose} = 8.40E{-}08$), although the effect sizes and *P* values were attenuated. These results suggest that insulin may account for most of the interaction effect of BMI, glucose, and TG with *PNPLA3*-rs738409 on LA_{ivn}. Previously, we reported that *PNPLA3*-rs738409 explained 2.4% of the variance in hepatic steatosis, estimated by LA, in EA individuals.[11] In the present study, *PNPLA3*-rs738409, insulin, and their interaction together explain as much as 8% of the variance in hepatic steatosis in the two largest EA cohorts, excluding individuals with diagnosed diabetes. This suggests that insulin levels/insulin resistance may be a key contributor to NAFLD. Excluding heavy drinkers from the conditional analyses did not change our inferences regarding *PNPLA3*-rs738409 (Supporting Tables S10-S12). We were not powered to carry out these analyses for *GCKR*-rs780094.

INTERACTION EFFECT OF INSULIN WITH *PNPLA3* ON HEPATIC STEATOSIS PREVALENCE IN FamHS

We assessed the interaction effect of insulin with *PNPLA3*-rs738409 on hepatic steatosis prevalence in individuals without diabetes (Fig. 1). In the lowest quartile of insulin levels (≤5.20 mU/L), the percentage of individuals with ≥30% liver fat (i.e., moderate to severe hepatic steatosis) was 23.42%, 35.81%, and 39.47% for CC, CG, and GG individuals, respectively. In the highest quartile of insulin levels (≥13.06 mU/L), the percentage of individuals with ≥30% liver fat was 54.44%, 76.32%, and 94.29% for CC, CG, and GG individuals, respectively. The data show that as insulin levels increase, the percentage of individuals with moderate to severe hepatic steatosis increases. However, among those with the GG genotype, this effect is magnified. The difference in the percentage of individuals with moderate to severe hepatic steatosis increases by 55 percentage points between the lowest

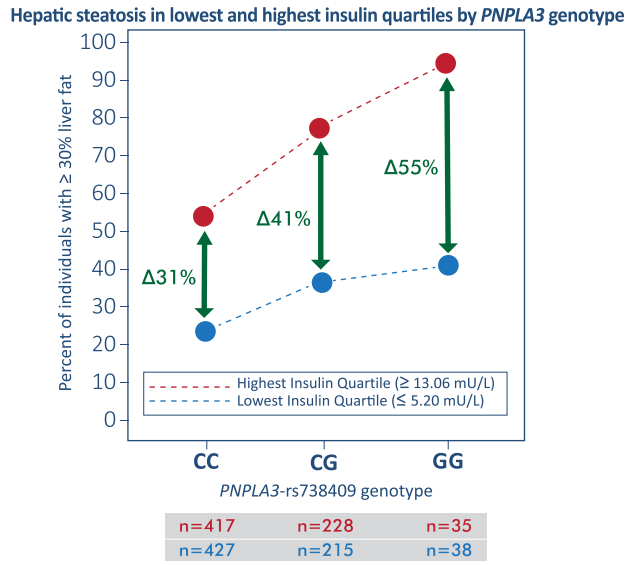

FIG. 1. Percentage of individuals without diabetes in FamHS with ≥30% fat in the liver (moderate to severe hepatic steatosis) is shown per *PNPLA3*-rs738409 genotype in the lowest and highest quartile of insulin levels. The number of individuals (n) in the lowest (blue circle) and highest (red circle) insulin quartiles are shown by genotype. As the level of insulin increases, the percentage of individuals with ≥30% fat in the liver increases more markedly with increasing copies of the G risk allele (nonparallel lines show interaction). Among those with the GG genotype, the difference (Δ) in the percentage of individuals with moderate to severe liver fat increases by 55 percentage points between the lowest and highest insulin quartiles. In contrast, this difference is lower among those with the CG genotype (41%) and CC genotype (31%).

and highest insulin quartiles among those with the GG genotype and increases by 41 percentage points among heterozygotes, while that difference increases only by 31 percentage points among those with the CC genotype. These data suggest that insulin has a strong effect on exacerbating the accumulation of liver fat in individuals without diabetes who have one or two G alleles at *PNPLA3*-rs738409.

Discussion

In a sample of 14,751 EA and AA individuals, we found interactions between *PNPLA3*-rs738409 and insulin, HOMA-IR, BMI, glucose, and TG on LA_{inv} (hepatic steatosis) after adjusting for differences in age, sex, and alcohol consumption. We also found interactions between *GCKR*-rs780094 and insulin, HOMA-IR, and TG on LA_{inv}. Conditional analyses in more than 5,000 EA individuals suggest that insulin, more than glucose, BMI, or TG, drives the interaction with *PNPLA3*-rs738409 to affect LA_{inv} in those without diabetes. We did not see significant interactions between *PNPLA3*-rs738409 and BMI, TG, or glucose once insulin was accounted for, whereas the reverse was not true. That is, there was still evidence for an interaction between *PNPLA3*-rs738409 and insulin even after accounting for the other metabolic traits. These results persist after accounting for alcohol intake, sex, and overall adiposity. We estimated in FamHS and FHS that as much as 8% of the variance in hepatic steatosis is explained by *PNLPA3*-rs738409, insulin, and their interaction in EA individuals without diabetes. In our previous study, *PNPLA3*-rs738409 alone explained only 2.4% of hepatic steatosis variance in EA individuals.[11]

Our findings suggest that individuals without diabetes with *PNPLA3*-rs738409-G and high insulin levels may have a particularly high risk for hepatic steatosis. The *PNPLA3* gene encodes adiponutrin, an enzyme found on the membrane of lipid droplets within hepatocytes.[34] Its function may be to break down TG stored in the droplets, helping regulate hepatic TG content.[34,35] The missense polymorphism rs738409 (C>G) in *PNPLA3* substitutes the amino acid isoleucine for methionine at residue 148 (I148M), changing the configuration of adiponutrin's catalytic site and rendering the enzyme inactive.[10,36] The accumulation of the inactive enzyme on lipid droplets is associated with TG buildup in hepatocytes.[36] Humans and mice carrying one or two copies of the I148M mutation (rs738409 CG or GG genotype) accumulate excess TG in lipid droplets and show more pronounced hepatic steatosis and NAFLD than those without the mutation.[35,36]

It is possible that having high insulin levels in addition to the *PNPLA3*-rs738409-G allele may result in a strong synergistic effect that exacerbates the accumulation of fat in the liver of individuals without diabetes, predisposing them to NAFLD. Insulin resistance stimulates the hydrolysis of TG in adipose tissue, releasing fatty acids in the bloodstream, which are taken up by the liver in an unregulated manner, promoting the accumulation of TG in hepatocytes.[37] Higher insulin levels also activate fatty acid synthesis in the liver, further driving the formation and storage of TG.[34] In addition, insulin resistance elevates plasma glucose,

which is sequestered by the liver, phosphorylated, and metabolized to make glycerol and acetyl-coenzyme A, the building blocks for the synthesis of TG.[34,38] In this context, it is possible that increased lipid synthesis and fatty acid delivery to the liver may combine with the inability of hepatocytes to dispose of TG from lipid droplets due to the presence of *PNPLA3*-rs738408-G and lead to increased hepatic steatosis. High insulin levels and *PNPLA3*-rs738409-G may also be involved in molecular feedback loops that increase hepatic steatosis. Insulin resistance and increased insulin levels augment the activity of transcription factors, such as sterol regulatory element binding protein 1c.[39] These transcription factors may promote TG synthesis in the liver and up-regulate the expression of *PNPLA3* I148M by binding to its promoter in a positive-feedback loop.[39] In this way, insulin and *PNPLA3* I148M may synergize to promote hepatic steatosis. This conjecture is also consistent with the enhanced risk of steatosis and liver damage, as evident by elevated liver enzymes and liver fat content seen with liver-directed long-acting insulin analogues in type 2 diabetics carrying the *PNPLA3* variant.[40]

When taken together, results show evidence that insulin and *PNPLA3*-rs738409 interact to have an important role in hepatic steatosis and as a result, NAFLD. Consequently, lowering the risk of hepatic steatosis and its liver complications in individuals with *PNPLA3*-rs738409-G may be achieved by reducing insulin resistance and concomitant high levels of insulin. One way to accomplish this could be through lifestyle changes that include increased exercise, weight loss, and better nutrition.[41] For example, decreasing exposure to carbohydrate-rich diets, which adversely increase insulin levels, may mitigate risk.[42,43] Also, treatments that target insulin resistance may be of greater benefit for preventing or treating hepatic steatosis than drugs that simply lower glucose. For example, insulin-sensitizing medications, such as pioglitazone, may be an option; it has already been shown to improve NAFLD, although at the expense of weight gain.[44] More studies are warranted to better understand the effect of the relationship between insulin levels and *PNPLA3*-rs738409-G on hepatic steatosis in different populations.

We also observed significant interactions of *PNPLA3*-rs738409 with BMI, glucose, and TG. Our results support the findings of Stender et al.[45] who reported that high BMI augmented the effect of *PNPLA3*-rs738409-G on hepatic steatosis, conferring susceptibility to NAFLD. Graff et al.[46] also showed an interaction effect between *PNPLA3*-rs738409 and visceral fat content, a measure of metabolic dysfunction. However, we found that the effect of BMI in exacerbating hepatic steatosis in the presence of *PNPLA3*-rs738409-G is attenuated by controlling for insulin levels in the model. We made the same observation for glucose and TG, suggesting that insulin/insulin resistance in the presence of *PNPLA3*-rs738409-G may confer most of the risk for hepatic steatosis on its own or through other metabolic intermediates.

Studies have reported an association between LDL and hepatic steatosis.[47,48] However, our study did not find an interaction between any of the genetic variants considered and LDL. This suggests that for individuals carrying *PNPLA3*-rs738409-G, reducing insulin levels or insulin resistance may have a greater effect on reducing the risk of hepatic steatosis than reducing LDL.

In addition to *PNPLA3*, we found that *GCKR* interacts with insulin resistance to increase susceptibility to hepatic steatosis. *GCKR* encodes the glucokinase regulatory protein, which has an important role in glucose metabolism.[49] The glucokinase regulatory protein binds to the glucose metabolizing enzyme glucokinase to inhibit its role in the uptake and storage of dietary glucose through stimulating *de novo* lipogenesis.[49] The variant rs780094/rs12060326 in the glucokinase regulatory protein reduces its ability to inhibit glucokinase.[49] This results in increased activity of glucokinase in the liver, which promotes *de novo* lipogenesis. When this mutation is combined with insulin resistance, it may amplify *de novo* lipogenesis to promote hepatic steatosis. We did not replicate the interaction between *TM6SF2* and BMI reported by Stender et al.[45]; however, our results show a similar trend. The interaction was borderline nonsignificant in the combined ancestry meta-analyses (interaction effect size, $\beta_{int} = -0.05$; $P = 5.89E{-}02$). Some differences between Stender et al. and this study may explain why we did not detect a statistically significant interaction. First, Stender et al. used proton magnetic resonance spectrometry to measure steatosis, which is a more sensitive measure than computed tomography. Second, they used the genotyped missense variant rs58542926; we used the proxy imputed variant rs2228603. The two variants are in high-linkage disequilibrium (D' = 0.926, $r^2 = 0.798$). Third, Stender

et al. combined the heterozygotes (EK) and homozygotes (KK) and compared them to those without the risk allele (EE). These three differences may have increased their power to see the weak effect they reported.

Our study has several limitations. It is a cross-sectional design that cannot prove temporal causality of insulin exposure on increasing hepatic steatosis. Because we used population-based cohorts that lacked biopsy information, we do not know whether we included individuals with advanced stages of NAFLD, such as nonalcoholic steatohepatitis, fibrosis, or cirrhosis. We also could not differentiate peripheral insulin resistance from hepatic insulin resistance with our data. Moreover, even though HOMA-IR was highly correlated to a single value of insulin (r^2 = 0.98) in individuals who were euglycemic, we do not have direct measures of dynamic glucose regulation. Therefore, functional studies are needed to gain more insight into the biological processes driving our observations. Finally, our study did not include the missense variant, rs641738, at the membrane bound O-acyltransferase domain containing 7/Transmembrane channel-like 4 (*MBOAT7/TMC4*) locus. It has been associated with hepatic fat accumulation.[50] In our prior association analyses,[11] we did not see an association between *MBOAT7/TMC4* and LA (β = –0.03; *P* = 0.15). Because our inclusion criterion for variants was that they needed to be associated with LA and we could not substantiate the association of *MBOAT7/TMC4* in our sample, we excluded it.

In conclusion, to our knowledge, this is the largest study examining the interaction between multiple metabolic traits and four genetic variants on hepatic steatosis in multiple cohorts representing two different ancestry groups. Our findings suggest that insulin levels/insulin resistance more than other correlated metabolic traits, including glucose, TG, and BMI, interact with genetic variants in *PNPLA3* to promote hepatic steatosis. Through conditional analyses, we show that insulin levels explain the interactions observed between *PNPLA3*-rs738409 and BMI as well as the interactions between *PNPLA3*-rs738409 and glucose and TG in almost 5,000 EA individuals without diabetes. Our work suggests that improving insulin resistance and reducing insulin levels in individuals who are prediabetic and who carry fatty liver-promoting alleles at *PNPLA3*-rs738409 may offer preferential benefit and mitigate their risk of developing NAFLD. Although *PNPLA3* genotype information is not currently used to make clinical decisions, it may be helpful in the future not only to risk stratify individuals but also to tailor their treatment. Our work contributes to the understanding of the pathophysiology of NAFLD and informs further interventional research to better diagnose and/or treat individuals with increased risk of NAFLD.

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Author names in bold designate shared co-first authorship.

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