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A genetic risk score is associated with hepatic triglyceride content and non-alcoholic steatohepatitis in Mexicans with morbid obesity

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ABSTRACT

Background and aims: Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) near/in *PNPLA3*, *NCAN*, *LYPLAL1*, *PPP1R3B*, and *GCKR* genes associated with non-alcoholic fatty liver disease (NAFLD) mainly in individuals of European ancestry. The aim of the study was to test whether these genetic variants and a genetic risk score (GRS) are associated with elevated liver fat content and non-alcoholic steatohepatitis (NASH) in Mexicans with morbid obesity.
Methods: 130 morbidly obese Mexican individuals were genotyped for six SNPs in/near *PNPLA3*, *NCAN*, *LYPLAL1*, *PPP1R3B*, and *GCKR* genes. Hepatic fat content [triglyceride (HTG) and total cholesterol (HTC)] was quantified directly in liver biopsies and NASH was diagnosed by histology. A GRS was tested for association with liver fat content and NASH using logistic regression models. In addition, 95 ancestry-informative markers were genotyped to estimate population admixture proportions.
Results: After adjusting for age, sex and admixture, *PNPLA3*, *LYPLAL1*, *GCKR* and *PPP1R3B* polymorphisms were associated with higher HTG content ($P < 0.05$ for *PNPLA3*, *LYPLAL1*, *GCKR* polymorphisms and $P = 0.086$ for *PPP1R3B*). The GRS was significantly associated with higher HTG and HTC content ($P = 1.0 \times 10^{-4}$ and 0.048, respectively), steatosis stage ($P = 0.029$), and higher ALT levels ($P = 0.002$). Subjects with GRS ≥6 showed a significantly increased risk of NASH (OR = 2.55, $P = 0.045$) compared to those with GRS ≤5. However, the GRS did not predict NASH status, as AUC of ROC curves was 0.56 ($P = 0.219$).
Conclusion: NAFLD associated loci in Europeans and a GRS based on these loci contribute to the accumulation of hepatic lipids and NASH in morbidly obese Mexican individuals.

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1. Introduction

The prevalence of obesity is rapidly increasing worldwide (Kelly et al., 2008). Obesity is a primary risk factor for non-alcoholic fatty liver disease (NAFLD) and approximately 95% of morbidly obese individuals develop NAFLD (Dixon et al., 2001). NAFLD is predicted to become the leading cause of liver disease in the world by 2020 (Bellentani and Marino, 2009), and includes a spectrum of diseases ranging from fatty infiltration (steatosis), fat and inflammation (non-alcoholic steatohepatitis/NASH) to fibrosis/cirrhosis (Farell and Larter, 2006). Nowadays the prevalence of hepatic steatosis is over 50% in some populations and is significantly higher in Hispanics than in black and white Americans (Browning et al., 2004; López-Velázquez et al., 2014). The prevalence of NAFLD is particularly high in morbidly obese subjects of Hispanic origin (Guajardo-Salinas and Hilmy, 2010).

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There is increasing evidence that genetic factors are involved in the development of NAFLD. Recently, large-scale genome-wide association (GWA) studies have identified novel genetic variants associated with NAFLD (Romeo et al., 2008; Speliotes et al., 2011). A single variant (rs738409; I148M) in *PNPLA3* (patatin like phospholipase domain containing protein 3) was strongly associated with increased hepatic fat content, inflammation, fibrosis and hepatic enzyme levels, suggesting that the variant increases the risk of NASH (Romeo et al., 2008; Rotman et al., 2010; Sookoian et al., 2009; Speliotes et al., 2010; Valenti et al., 2010). Additionally, the Genetics of Obesity-related Liver Disease (GOLD) Consortium identified four additional genetic variants that were associated with hepatic steatosis (HS) in or near genes *LYPLAL1* (lysophospholipase like 1), *PPP1R3B* (protein phosphatase 1, regulatory subunit 3b), *NCAN* (neurocan), and *GCKR* (glucokinase regulatory protein) (Speliotes et al., 2011). To date, the association of *PNPLA3*/I148M with NAFLD or transaminase levels has been widely replicated in various populations (Kotronen et al., 2009; Larrieta-Carrasco et al., 2013, 2014), however associations with other variants have not been consistent (Gorden et al., 2013; Hernaez et al., 2013; Lin et al., 2014; Palmer et al., 2013).

In the present study, we investigated whether the NAFLD-associated polymorphisms identified in GWA studies and a genetic risk score (GRS) based on these SNPs are associated with hepatic lipid content (triglycerides and cholesterol) and NAFLD in Mexican subjects with morbid obesity.

2. Materials and methods

2.1. Subjects

We studied 130 consecutive Mexican Mestizo subjects aged 18 to 59 years with severe obesity (BMI $\geq$ 40 kg/m^2) who underwent bariatric surgery, recruited from the Hospital General Ruben Leñero in Mexico City. Subjects with a history of viral hepatitis, evidence of other concomitant chronic liver disease, or with alcohol consumption >20 g/day were excluded from the study. The protocol was approved by the INMEGEN Ethics Committee. All participants provided written informed consent prior to their inclusion. The study was performed according to the principles of the Declaration of Helsinki.

2.2. Anthropometric and biochemical parameters

Clinical and anthropometric data were collected from all participants. Anthropometric measurements were determined as previously described (León-Mimila et al., 2013). BMI was calculated as weight in kilograms divided by the square of height in meters. Morbid obesity status was determined according to WHO (World Health Organization) criteria with a BMI value $\geq$40 kg/m^2 (WHO, 2000).

Blood samples were taken after a 10-h overnight fast for biochemical measurements. Serum glucose, insulin, triglycerides, total and HDL cholesterol (HDL-C), were measured as previously described (Villarreal-Molina et al., 2007), and insulin sensitivity was calculated using the homeostatic model assessment (HOMA-IR) index (Matthews et al., 1985). Serum concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with commercially available standardized methods (Beckman Coulter, Fullerton, CA, USA). Serum adiponectin and leptin levels were determined using ELISA methods (Linco, St. Louis, MO). Type 2 diabetes (T2D) was defined as either self-reported use of glucose-lowering medication or fasting plasma glucose levels $\geq$126 mg/dL (ADA, 2010).

2.3. Liver histology

Liver biopsies were collected in RNAlater (Ambion/Applied Biosystems) at the time of surgery and stored at −80 °C. Liver biopsy specimens were fixed in 10% formaldehyde embedded in paraffin, stained with hematoxylin–eosin and evaluated by an experienced pathologist. Histological characteristics were determined according to the Kleiner scoring system (Kleiner et al., 2005). Steatosis was scored as 0–3, inflammation 0–3, and hepatocellular ballooning 0–2. The sum of these pathological features was used to estimate NAFLD activity score (NAS). All participants were classified as controls (subjects with normal liver histology), individuals with simple steatosis (steatosis grade ranging from 1 to 2 without the presence of inflammation), individuals with possible or borderline NASH (NAS ranging from 3 to 4), and NASH-definite (NAS $\geq$ 5).

2.4. Measurement of hepatic triglyceride (HTG) and total cholesterol (HTC) content

Liver tissue (50 mg) was homogenized in saline solution (0.9%) and total lipids were extracted according to the method of Folch et al. (1957). Total lipids were extracted and homogenized in chloroform/methanol (2:1 vol/vol). The organic phase was dried under nitrogen stream, lipids were re-suspended in isopropanol and 10% Triton X-100. Triglycerides and total cholesterol concentrations were determined by spectrophotometry (Beckman DU 640, Beckman Instruments, Fullerton, CA), using commercial colorimetric assay kits (Diagnostic Systems, Holzheim, Germany) and normalized to total protein concentration.

2.5. Genotyping

Genomic DNA was isolated from peripheral white blood cells using a commercial kit based on the salt fractionation method (QIAamp 96 DNA Blood Kit, Quiagen, Hilden, Germany). Genotyping was performed using predesigned TaqMan Probes (Applied Biosystems, Foster City, CA) in a LightCycler 480 Instrument II, Roche Applied Science, for 6 candidate SNPs: rs738409 (*PNPLA3*), rs12137855 (*LYPLAL1*), rs780094 and rs1260326 (*GCKR*), rs2228603 (*NCAN*) and rs4240624 (*PPP1R3B*). Call rate exceeded 95% for all SNPs tested, with no discordant genotypes in 10% of duplicate samples.

Because the Mexican-Mestizo population is admixed, ancestry informative markers (AIMs) were used to assess whether any association could be confounded by population stratification. A set of 95 AIMs distributed across the genome was genotyped using a GoldenGate BeadArray (Illumina). The AIMs were selected from a published report and distinguish mainly between three continental populations (American, European and African) (Kosoy et al., 2009).

2.6. Construction of the genetic risk score (GRS)

Four SNPs (*PNPLA3*, *LYPLAL1*, *PPP1R3B* and *GCKR*) were used to calculate the combined GRS, which was constructed by summing the number of risk alleles from these 4 SNPs in each individual. rs2228603 (*NCAN*) and rs780094 (*GCKR*) were not included in the GRS estimate because rs2228603 showed a very low allelic frequency (0.02) and rs780094 was in high linkage disequilibrium (LD) with rs1260326 ($D' = 0.92$). A weighted GRS (wGRS) was calculated multiplying the number of risk alleles for each SNP by its estimated effect (beta) obtained from the association analysis for HTG content. Genotypes for each SNP were scored using an additive model (0 for homozygous for the non-risk allele, 1 for heterozygous, and 2 for homozygous for the risk allele).

2.7. Statistical analysis

The chi-square test was used to assess whether the genotypes were in Hardy–Weinberg equilibrium (HWE) and to compare differences in categorical variables. All SNPs were in HWE. General linear models were used to compare mean values of quantitative traits across groups, with adjustment for age and sex. Population admixture proportions were determined using a maximum likelihood method implemented

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