

# The Association Between Hepatic Fat Content and Liver Injury in Obese Children and Adolescents

Effects of ethnicity, insulin resistance, and common gene variants

NICOLA SANTORO, MD, PHD<sup>1</sup>  
 ARIEL E. FELDSTEIN, MD, PHD<sup>2</sup>  
 ERIK ENOKSSON, MS<sup>1,3</sup>  
 BRIDGET PIERPONT, MA<sup>1</sup>

ROMY KURSAWE, PHD<sup>1</sup>  
 GRACE KIM, MD<sup>1</sup>  
 SONIA CAPRIO, MD<sup>1</sup>

**OBJECTIVE**—Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (NASH) are highly prevalent in obese youth. Herein, we aimed to study the association between hepatic fat accumulation as assessed by magnetic resonance imaging and circulating levels of cytokeratin-18 (CK-18) fragments, a robust NASH biomarker, and to explore the impact on this association of ethnicity, insulin resistance, and single nucleotide polymorphisms (SNPs) associated with steatosis (rs738409 in the *PNPLA3*, rs1260326 in the *GCKR*) or NASH severity (rs2645424 in the *FDFT1*).

**RESEARCH DESIGN AND METHODS**—Two-hundred twenty-nine obese youths (87 Caucasians, 61 African Americans, and 81 Hispanics; mean age,  $12.8 \pm 2.9$  years; mean BMI,  $31.4 \pm 7.4$ ) underwent magnetic resonance imaging, oral glucose tolerance test, and CK-18 levels measurement; 12 subjects underwent liver biopsy.

**RESULTS**—African Americans showed lower CK-18 levels than Hispanics ( $P < 0.001$ ) and Caucasians ( $P = 0.004$ ). Hepatic fat content (HFF%) and whole body insulin sensitivity index (WBISI) modulated CK-18 levels in Caucasians and Hispanics ( $P = 0.02$  and  $P = 0.011$ ), but not in African Americans; in fact, CK-18 was associated with HFF% and WBISI in Caucasians ( $P = 0.0018$  and  $P < 0.0001$ ) and Hispanics ( $P < 0.0001$  and  $P = 0.02$ ), but not in African Americans (both  $P = 0.5$ ). The *PNPLA3* SNP showed association in Caucasians ( $P = 0.02$ ) and Hispanics ( $P = 0.05$ ), and *FDFT1* SNP showed an association in Caucasians ( $P = 0.05$ ) and Hispanics ( $P = 0.02$ ), with the same trend in African Americans ( $P = 0.07$ ).

**CONCLUSIONS**—African Americans have lower levels of CK-18 than Caucasians and Hispanics irrespective of HFF% and insulin resistance. Moreover, SNPs in the *PNPLA3* and *FDFT1* may drive the individual predisposition to development of hepatic injury.

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**N**onalcoholic fatty liver disease (NAFLD) is a clinicopathological diagnosis in which  $>5\%$  of hepatocytes demonstrate macrovesicular steatosis in an individual without significant history of alcohol intake (1). NAFLD encompasses a range of disease severity spanning from simple steatosis to

nonalcoholic steatohepatitis (NASH), which in turn can progress to cirrhosis (1). Paralleling the worldwide epidemic of childhood obesity, NAFLD has become the most common cause of liver disease in pediatrics (2). Furthermore, it is now clear that it represents not only a risk for liver failure and liver carcinoma but also a

strong cardiovascular risk factor closely related to insulin resistance. In particular, recent studies in obese children and adolescents demonstrated that the prevalence of metabolic syndrome and prediabetes increases with the increase in hepatic fat content (3), that subjects showing hepatic steatosis have a pronounced dyslipidemic profile characterized by high levels of large VLDL, small dense LDL, and decreased large HDL concentrations (4), and that fatty liver, independent of visceral and intramyocellular lipid content, plays a central role in the impairment of liver, muscle, and adipose insulin sensitivity (5).

Ethnic differences in the prevalence of NAFLD have been pointed out in a landmark article by Browning et al. in 2004 (6). In that study, the authors observed that there is a clear difference in hepatic steatosis prevalence across ethnicities, with African Americans showing the lowest rate, Hispanics showing the highest rate, and Caucasians having a rate in the middle (6). Interestingly, the same group also observed dissociation between fatty liver and insulin resistance in African Americans (7), which suggests that African Americans are protected from hepatic fat accumulation even in presence of insulin resistance. Furthermore, it has been shown that along with the lowest prevalence of steatosis, African Americans also show the lowest prevalence of NASH and less severe fibrosis than Caucasian and Hispanic patients, despite a similar degree of obesity and insulin resistance (8).

A better understanding of the influence of ethnicity on the development of hepatic damage may provide additional insight into the pathophysiology of NAFLD. Given the paucity of data regarding interethnic differences in NAFLD and NASH in obese youths, in the current study we aimed to assess in a multiethnic cohort of obese children and adolescents: whether ethnicity affects liver damage as evaluated by circulating levels of a biomarker of apoptosis cytokeratin-18 (CK-18), a systemic noninvasive marker of NASH; whether the degree of insulin resistance

From the <sup>1</sup>Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut; the <sup>2</sup>Division of Pediatric Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, University of California, San Diego, San Diego, California; and the <sup>3</sup>Karolinska Institutet, School of Medicine, Stockholm, Sweden.

Corresponding authors: Sonia Caprio, [sonia.caprio@yale.edu](mailto:sonia.caprio@yale.edu), or Nicola Santoro, [nicola.santoro@yale.edu](mailto:nicola.santoro@yale.edu).

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A slide set summarizing this article is available online.

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is associated with the degree of liver damage; and genetic underpinnings that might drive susceptibility to steatohepatitis. Moreover, given the growing evidence of the role of adiponectin in the pathogenesis of NAFLD and NASH (9,10) and the known association between adiponectin levels and insulin resistance (9), herein we also explored the putative association between adiponectin levels and liver injury according to ethnicity.

To pursue our aims, we studied a multiethnic cohort of obese youths in whom hepatic steatosis was assessed by fast magnetic resonance imaging (MRI), and hepatic damage was assessed non-invasively by measuring the caspase-cleaved CK-18 fragment levels. CK-18 is the major intermediate filament protein in the liver; during activation of cell death pathways, it is cleaved by the caspases (mainly caspase-3) and its fragments are released into the circulation (11–14). CK-18 levels have been shown to correlate with the magnitude of hepatocyte apoptosis and to predict the presence of NASH in adults as well as in children (11–14). In particular, CK-18 levels represent a robust marker of steatohepatitis being able to detect the presence of NASH with a specificity of 90% and a sensitivity of 80% (11).

To explore the genetic basis predisposing to liver damage in youths, we genotyped three single nucleotide polymorphisms (SNPs): two of them previously associated with hepatic steatosis (rs738409 in the patatin-like phospholipase domain-containing protein 3, *PNPLA3*, gene and rs1260326 in the glucokinase regulatory protein, *GCKR*, gene) (15–17) and one previously associated with the severity of NASH, rs2645424, in the farnesyl diphosphate farnesyl transferase 1 (*FDFT1*) gene (18).

## RESEARCH DESIGN AND METHODS

### The Yale Pediatric NAFLD/NASH cohort

In an effort to understand the role of NAFLD/NASH in the pathophysiology of youth-onset type 2 diabetes, we began in 2008 to form a multiethnic cohort of obese children and adolescents. As of the time of writing this article, the cohort consists of 229 obese children and adolescents (87 Caucasians, 61 African Americans, and 81 Hispanics; mean age, 12.8 ± 2.9 years; mean BMI, 31.4 ± 7.4) from the New Haven area (New Haven, CT)

recruited through the Yale Pediatric Obesity Clinic. Caucasians (15.1 ± 4.0 years) and African Americans (15.1 ± 3.3 years) tended to be older than Hispanics (13.5 ± 2.7 years;  $P = 0.005$ ), whereas the BMI was similar among ethnicities ( $P = 0.16$ ). Seventeen Caucasians (13 girls), 17 African Americans (13 girls), and 30 Hispanics (14 girls) showed impaired glucose tolerance, whereas 1 Caucasian (girl), 1 African American (girl), and 2 Hispanics (1 girl) showed type 2 diabetes ( $P = 0.15$ ). The cohort was carefully phenotyped with respect to quantification of hepatic fat content and abdominal fat distribution using MRI, systemic biomarkers of apoptosis such as noninvasive indicators of NASH, fasting lipid and lipoprotein profiles, glucose homeostasis, and genetic markers of NAFLD and NASH.

The study subjects underwent metabolic and imaging studies, biochemical analyses, and genotyping. Detailed information about these studies is provided as Supplementary Material.

The study was approved by the Yale University Human Investigation Committee. Written parental informed consent and written child assent were obtained from all participants.

**Liver biopsy.** The liver biopsy was performed in 12 subjects (4 girls) because of persistent elevation in alanine transaminase (ALT) (mean ALT, 133.0 ± 64.4). Biopsy specimens were formalin-fixed, paraffin-embedded, stained with hematoxylin and eosin and trichrome, and underwent Gordon reticulin techniques. All biopsy samples were 2 cm or more in length and were reviewed by a pediatric pathologist according to the Brunt approach (19). The specimens were analyzed and steatosis, ballooning, inflammation, and fibrosis were scored according to Kleiner et al. (20). The NAFLD activity score was calculated by adding the scores of steatosis, inflammation, and ballooning, whereas the stage of fibrosis was determined by using a 4-point scale (20).

**Statistics.** Before analyzing the data, all the variables were tested for normality, with non-normally distributed variables log-transformed to be better approximated by normality, except for hepatic fat content (HFF%), for which a square-root transformation was used. All continuous variables were compared among the groups using the ANOVA. Adjusted comparisons were performed using a general linear model, adjusting for age, sex, and percent of total body fat. Prevalence among groups was compared using the

$\chi^2$  statistic. A Pearson correlation was used to test correlations between CK-18 and HFF%, whole body insulin sensitivity index (WBISI), or adiponectin in each ethnic group. A Spearman correlation was used to test the correlation between the CK-18 and the NAFLD activity score and liver fibrosis. To evaluate the interaction between ethnicity and HFF% or WBISI, a general linear model including the single terms and an interaction term (e.g., HFF% × ethnicity or WBISI × ethnicity) was performed. To evaluate the interaction between ethnicity and HFF%, age, sex, total body fat, and WBISI were used as covariates; to evaluate the interaction between ethnicity and WBISI, the HFF% was included in the model.

Within each ethnic group, the association between the genotypes and quantitative traits was evaluated by coding the genotype with an additive model of inheritance, i.e., the genotype is coded with 0, 1, or 2, corresponding to the number of minor alleles carried by each individual; age, sex, and total body fat were used as covariates when appropriate. The partial correlation coefficients ( $r^2$ ) were used to evaluate the degree of variance of CK-18 explained by the genotype. The  $\chi^2$  test was used to assess whether the genotypes were in Hardy-Weinberg equilibrium and to test differences in genotype distribution among different ethnic groups. Unless otherwise specified, for all the data raw means and SD are shown.

## RESULTS

### Correlation between CK-18 and NAFLD activity score in obese children and adolescents

Twelve subjects (2 Caucasians and 10 Hispanics) had NASH proven by liver biopsy. When compared with the entire cohort, these subjects showed higher HFF% ( $P = 0.0008$ ), higher CK-18 ( $P < 0.0001$ ), ALT ( $P < 0.0001$ ), and triglyceride levels ( $P = 0.02$ ), and lower WBISI ( $P = 0.02$ ). Consistent with previous reports (12) in this subgroup, the CK-18 showed a strong correlation with the NAFLD activity score ( $r = 0.70$ ;  $P = 0.01$ ) and fibrosis ( $r = 0.68$ ;  $P = 0.03$ ).

### CK-18 levels according to ethnicity and liver features

Characteristics of the studied population irrespective of degree of HFF% and presence of NASH are shown in Supplementary Table 1. Hispanics tended to be younger than African Americans and

Caucasians ( $P = 0.006$ ), but there was no difference among ethnicities in terms of sex prevalence, BMI, total body fat, and glucose tolerance. Whereas Caucasians and African Americans did not differ in terms of insulin resistance ( $P = 0.44$ ), Hispanics showed a WBISI lower than the other two ethnic groups, independent of age, sex, and total body fat ( $P = 0.001$ ). As expected, African Americans showed lower hepatic fat content than Caucasians and Hispanics, independent of age, sex, total body fat ( $P < 0.001$ ), and visceral fat ( $P = 0.001$ ); similarly, they also showed lower triglycerides ( $P = 0.0002$ ) and ALT ( $P = 0.05$ ) independent of age, sex, total body fat, and HFF%. Of particular note, CK-18 levels were different between Caucasians and African Americans ( $P = 0.004$ ) and between Hispanics and African Americans ( $P < 0.0001$ ), but not between Caucasians and Hispanics, although Hispanics tended to show higher levels than Caucasians ( $P = 0.09$ ).

Clinical and laboratory characteristics of patients according to presence of steatosis and NASH are shown in Table 1. Among both Caucasians and Hispanics, subjects with biopsy-proven NASH showed higher BMI ( $P = 0.03$  and  $P < 0.001$ , respectively), total body fat ( $P = 0.001$  and  $P < 0.001$ , respectively),

higher HFF% (both  $P < 0.001$ ), and ALT levels (both  $P < 0.001$ ) than subjects with and without steatosis (Table 1). CK-18 levels were higher in subjects with biopsy-proven NASH in both Caucasians and Hispanics (Fig. 1); furthermore, in Caucasians and Hispanics, subjects with and without hepatic steatosis differed for the CK-18 levels of  $\sim 20\%$  (both  $P = 0.04$ ), whereas in African Americans the CK-18 levels were similar between subjects with and without hepatic steatosis ( $P = 0.50$ ) (Fig. 1).

### Ethnicity modulates the effect of fatty liver and insulin resistance on liver damage

To answer our first question and to assess whether the relationship between CK-18 and HFF% is modulated by ethnicity, we performed a regression model including an interaction term between ethnicity and HFF% as an independent variable and CK-18 as the dependent variable. We observed an interaction between ethnicity and HFF% ( $P = 0.022$ ) that remained significant even after adjusting for age, sex, WBISI, and total body fat ( $P = 0.011$ ). In fact, although the CK-18 was associated with HFF% in Caucasians and Hispanics, there was no association in African Americans (Fig. 2A). CK-18 levels were directly

correlated with HFF% in Caucasians ( $r = 0.43$ ;  $P < 0.0001$ ) and Hispanics ( $r = 0.34$ ;  $P = 0.0018$ ), but not in African Americans ( $r = -0.08$ ;  $P = 0.50$ ).

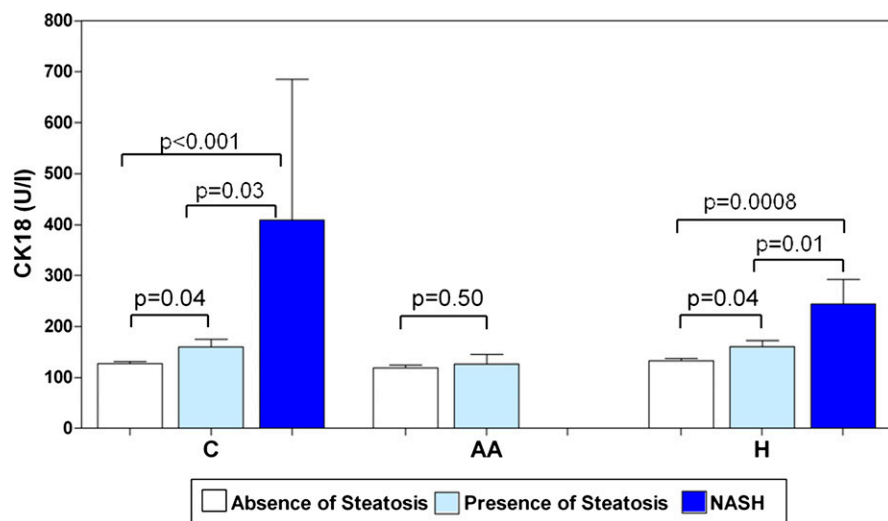
We also asked whether the degree of insulin resistance might be associated to the degree of liver damage. To answer this question we performed a regression model including an interaction term between ethnicity and WBISI as an independent variable and CK-18 as a dependent variable. Interestingly, also in this case, we observed an interaction between ethnicity and WBISI ( $P = 0.004$ ), independent of age, sex, total body fat ( $P = 0.018$ ), and HFF% ( $P = 0.002$ ) (Fig. 2B). CK-18 levels, in fact, were inversely correlated with WBISI in Caucasians ( $r = -0.40$ ;  $P < 0.0001$ ) and Hispanics ( $r = -0.24$ ;  $P = 0.024$ ), but not in African Americans ( $r = 0.08$ ;  $P = 0.52$ ). Similarly, we observed the same trend for an interaction between ethnicity and adiponectin ( $P = 0.05$ ), independent of age, sex, total body fat ( $P = 0.03$ ), and HFF% ( $P = 0.06$ ) (Fig. 2C). CK-18 levels were inversely correlated with adiponectin in Caucasians ( $r = -0.26$ ;  $P = 0.01$ ) and Hispanics ( $r = -0.27$ ;  $P = 0.01$ ), whereas there was no correlation in African Americans ( $r = 0.12$ ;  $P = 0.35$ ).

### Association between GCKR, PNPLA3, and FDFT1 gene variants and CK-18 levels

The third aim of this study was to explore whether three gene variants previously associated with HFF% (the rs1260326 in the GCKR and the rs738409 in the PNPLA3) (15–17) or with NAFLD activity score (the rs2645424 in the FDFT1) (18) might be associated with the degree of liver damage in obese youths as determined by a noninvasive biomarker.

The GCKR SNP rs1260326 minor allele (T) frequency was 0.366 in Caucasians, 0.152 in African Americans, and 0.390 in Hispanics. The frequency of the PNPLA3 rs738409 minor allele (G) was 0.305 in Caucasians, 0.186 in African Americans, and 0.460 in Hispanics. The frequency of the FDFT1 rs2645424 A allele was 0.458 in Caucasians, 0.466 in African Americans, and 0.350 in Hispanics. Within each ethnic group there was no evidence against the null hypothesis that the genotype distribution was in Hardy-Weinberg equilibrium for all of the variants (all  $P > 0.05$ ).

Patient features data according to the genotype are provided in Supplementary Table 1 (Caucasians), Supplementary



**Figure 1**—CK-18 levels according to the degree of hepatic damage in the three ethnic groups. The figure displays the differences in CK-18 levels according to the degree of hepatic damage in three ethnic groups of obese children and adolescents. Subjects with biopsy-proven NASH showed higher CK-18 levels than subjects with and without steatosis ( $P = 0.0008$  in Caucasians and  $P = 0.01$  in Hispanics). In Caucasians and Hispanics, the levels of CK-18 were different by  $\sim 20\%$  between subjects with and without hepatic steatosis (both  $P = 0.04$ ), independent of age, sex, and total body fat, whereas in the groups of African Americans there was no difference in CK18 levels between subjects with and without steatosis ( $P = 0.50$ ). AA, African Americans; C, Caucasians; H, Hispanics.

Table 1—Clinical features of the study population according to the presence or absence of hepatic steatosis and biopsy-proven nonalcoholic steatohepatitis

	Caucasians			African Americans			Hispanics		
	MRI-measured NAFL	Biopsy-proven	P	MRI-measured NAFL	Biopsy-proven	P	MRI-measured NAFL	Biopsy-proven	P
Hepatic phenotype	≤5.5% (58)	NASH (2)	0.02	≤5.5% (52)	0.001	≤5.5% (32)	0.001	≤5.5% (39)	0.001
Age, years	15.9 ± 4.3	15.5 ± 0.7	0.02	15.1 ± 3.2	15.5 ± 3.8	0.77	13.7 ± 2.8	13.6 ± 2.7	0.90
Sex, male/female, %	35/65	0/100	0.09	38/62	40/60	0.90	34/66	67/43	0.03
NGT/IGT/T2D, %	85/15/0	0/50/50	<0.001	79/19/2	20/80/0	0.001	63/34/3	64/33/3	0.63
BMI, kg/m <sup>2</sup>	29.9 ± 7.6	37.8 ± 5.3	0.03	32.0 ± 7.2	39.1 ± 3.7	0.002	27.3 ± 8.1	33.3 ± 5.6	<0.001
Total body fat, %	36.2 ± 11.3	46.2 ± 14.3	0.001	39.2 ± 12.4	48.2 ± 4.6	0.03	32.2 ± 13.7	43.6 ± 9.30	<0.001
HFF%	1.14 ± 1.57	20.7 ± 11.6	<0.001	0.71 ± 1.34	14.5 ± 9.5	<0.001	0.78 ± 1.29	20.5 ± 10.9	<0.001
Visceral fat, cm <sup>2</sup>	47.0 ± 26.8	76.5 ± 21.0	0.05	36.9 ± 20.4	65.5 ± 37.5	0.10	38.5 ± 26.1	75.3 ± 30.3	0.01
Subcutaneous fat, cm <sup>2</sup>	407 ± 209.7	480 ± 144	0.61	446 ± 244	560 ± 130	0.44	337 ± 232	513 ± 174	0.88
ALT, IU/L	18.5 ± 16.4	39.8 ± 34.1	<0.001	13.7 ± 7.1	32.4 ± 36.6	0.01	14.3 ± 6.7	34.9 ± 23.5	<0.001
Triglycerides, mg/dL	88.2 ± 43.7	141.9 ± 84.9	0.01	67.3 ± 47.0	99.2 ± 55.2	0.03	91.2 ± 48.6	122.7 ± 51	0.23
Fasting glucose, mg/dL	79.9 ± 7.7	95.4 ± 6.7	0.004	92.6 ± 9.6	100.6 ± 11.9	<0.001	95.9 ± 9.9	96.0 ± 8.4	0.13
2-h glucose, mg/dL	112.5 ± 28.6	130 ± 21	0.16	118.9 ± 28.6	149 ± 18	<0.001	126.9 ± 29.1	131 ± 26	0.73
WBISI	2.67 ± 1.47	1.47 ± 0.54	0.013	2.73 ± 1.89	0.98 ± 0.54	<0.001	2.43 ± 1.40	1.27 ± 0.69	0.16
Adiponectin, μg/mL	10.2 ± 4.4	8.2 ± 3.3	0.44	9.1 ± 4.7	5.4 ± 2.7	0.04	10.3 ± 4.0	7.2 ± 3.3	0.04
IGI	2.90 ± 2.10	3.7 ± 1.8	0.64	3.87 ± 3.46	4.7 ± 2.1	0.82	2.98 ± 1.86	5.1 ± 3.2	0.07
DI	6.4 ± 3.9	5.0 ± 2.3	0.02	7.4 ± 4.1	4.4 ± 2.1	0.001	6.3 ± 5.1	6.1 ± 5.7	0.72

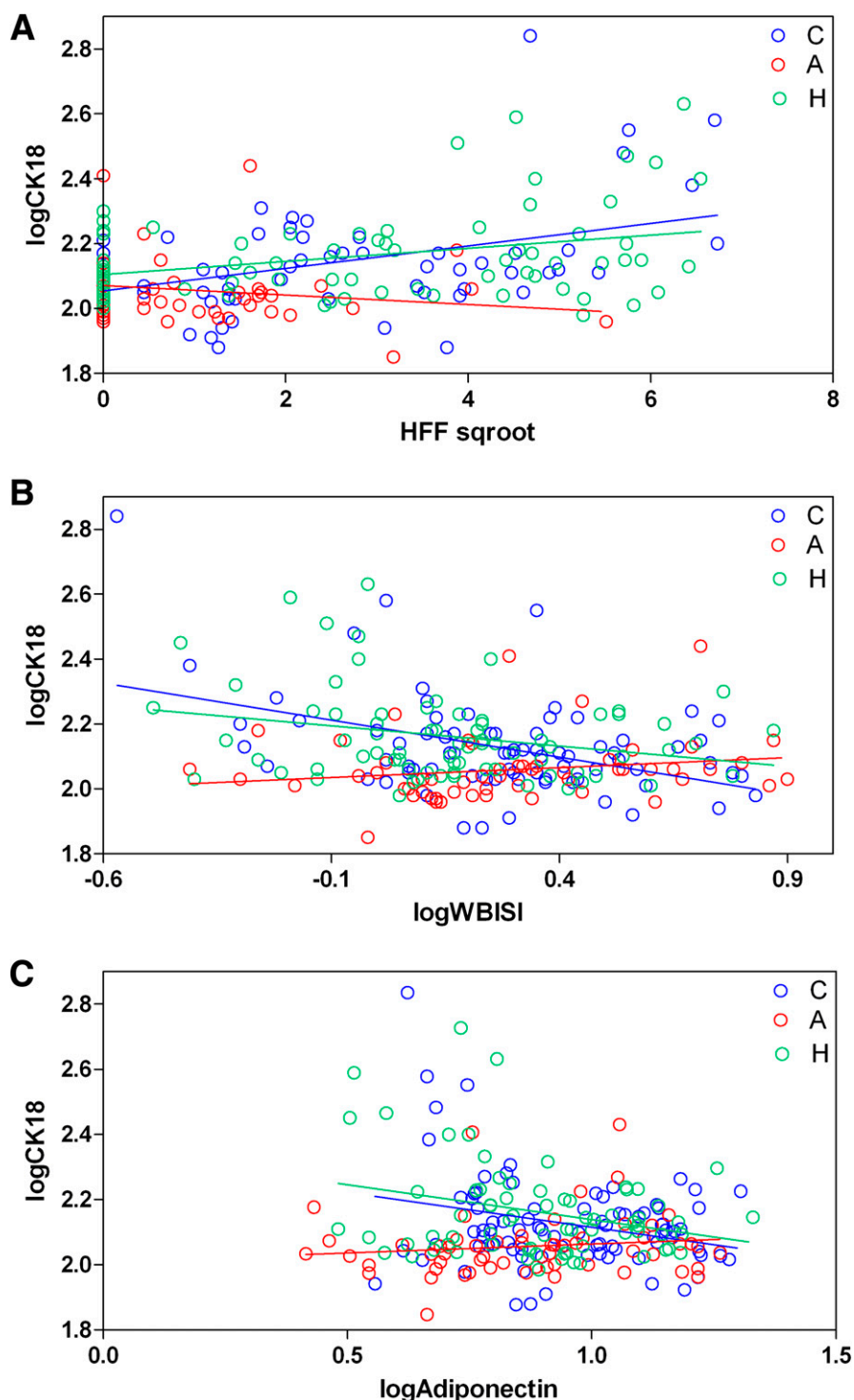
DI, disposition index; IGI, insulinogenic index; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; T2D, type 2 diabetes. ANOVA was used to test the differences among the groups. Presence of HFF% indicated by >5.5. Absence of HFF% indicated by ≤5.5.

Table 2 (African Americans), and Supplementary Table 3 (Hispanics). Whereas the *FDFT1* rs2645424 was not associated with the HFF% (Supplementary Tables 1, 2, and 3), we observed an association between the *FDFT1* rs2645424 and CK-18 in Caucasians and Hispanics, independent of age, sex, and total body fat ( $P = 0.05$  and  $P = 0.02$ , respectively); although not statistically significant, the same trend was observed in African Americans ( $P = 0.07$ ) (Fig. 3A). The association between *FDFT1* rs2645424 and CK-18 was independent of HFF% (Caucasians,  $P = 0.01$ ; Hispanics,  $P = 0.02$ ). We also observed an association between the CK-18 and *PNPLA3* rs738409 in Caucasians and Hispanics, independent of age, sex, and total body fat ( $P = 0.02$  and  $P = 0.05$ , respectively), whereas we were not able to detect the association in African Americans ( $P = 0.26$ ) (Fig. 3B). This association was independent of HFF% in Caucasians ( $P = 0.01$ ) and Hispanics ( $P = 0.05$ ). The *GCKR* rs1260326 was not associated with the CK-18 levels in any ethnicities (Fig. 3C).

**CONCLUSIONS**—The main finding of this study relates to the relationship between hepatic fat accumulation and liver injury in obese youth and the factors that influence this relationship. Our results demonstrate that the association between hepatic fat content as measured by fast MRI and liver injury as determined by levels of CK-18 fragments is strongly affected by ethnicity. In particular, we observed that for the same degree of HFF%, African American obese youths show lower levels of CK-18 than Caucasians and Hispanics, meaning that African Americans tend to experience a lower degree of hepatic damage irrespective of the degree of hepatic fat accumulation.

In addition, we found that the CK-18 levels were associated with insulin resistance in Caucasians and Hispanics, but not in African Americans, meaning that in this latter population the effect of insulin resistance in the development of NASH is probably marginal.

Although previously some authors have attributed the lower propensity to development of NAFLD or NASH in African Americans to the lower degree of insulin resistance (21), a report from the Dallas Heart Study clearly has excluded the different rate of insulin resistance as the major cause for the lower rate of NAFLD in African Americans and suggested that such a diversity is more likely to



**Figure 2**—Hepatic fat content and insulin resistance modulate CK-18 levels according to ethnicity. A: The interaction between ethnicity and HFF% in modulating CK-18 levels. There was an association between the HFF% (square-root-transformed) and CK-18 (log<sub>10</sub>-transformed) in Caucasians ( $r^2 = 0.12$ ;  $P = 0.0018$ ) and Hispanics ( $r^2 = 0.18$ ;  $P < 0.0001$ ), but not African Americans ( $r^2 = 0.007$ ;  $P = 0.50$ ). The interaction between ethnicity and HFF% was statistically significant ( $P = 0.022$ ) and remained significant after adjusting for age, sex, WBISI, and total body fat ( $P = 0.011$ ). B: The interaction between ethnicity and WBISI in modulating CK-18 levels. There was association between the WBISI (log<sub>10</sub>-transformed) and CK-18 (log<sub>10</sub>-transformed) in Caucasians ( $r^2 = 0.15$ ;  $P < 0.0001$ ) and Hispanics ( $r^2 = 0.06$ ;  $P = 0.02$ ), but not African Americans ( $r^2 = 0.007$ ;  $P = 0.50$ ). The interaction between ethnicity and WBISI was statistically significant ( $P = 0.004$ ), independent of age, sex, total body fat, and HFF% ( $P = 0.018$ ). C: The interaction between ethnicity and adiponectin in modulating CK-18 levels. There was association

reflect biological and genetic differences in lipid metabolism rather than differences in insulin resistance, obesity, or alcohol intake (6,7). In agreement with these studies, our observations suggest that obese African American youth tend to show lower rates of hepatic fat accretion and a lower degree of hepatic fat damage, independent of the degree of insulin resistance. In fact, whereas insulin resistance is associated with liver damage in Caucasians and Hispanics, this association was lacking in African Americans, meaning that although insulin resistance may play a role in the progression of NAFLD in Caucasians and Hispanics, it may not be associated with the progression of the disease in African Americans.

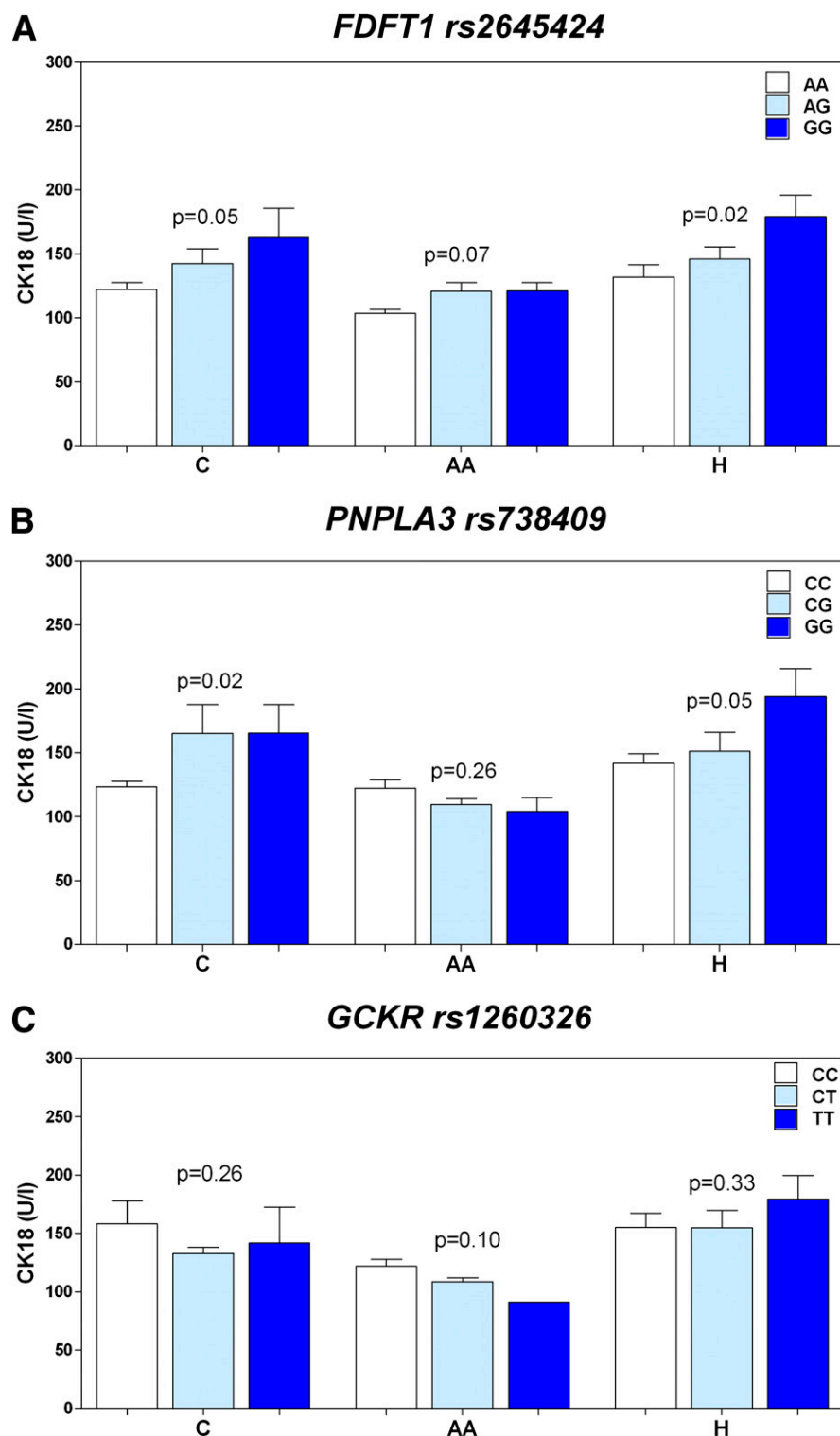
Thus, two key questions arise. Why is there no association between hepatic steatosis or insulin resistance and liver damage in African Americans? What protects African Americans from development of NASH, even in presence of severe obesity and insulin resistance?

Whereas the pathogenetic mechanisms responsible for the progression from simple steatosis to NASH are largely still unknown, it has been clearly shown that the excessive release of free fatty acids plays a key role in the development of hepatic “lipotoxicity” in NAFLD (22–24). Because the first source of free fatty acids for hepatic triglycerides synthesis (~70%) is the adipose tissue (25), and because African Americans tend to show low plasma triglycerides and hepatic triglycerides content even in presence of severe obesity and insulin resistance, the possibility exists that they may physiologically have a lower release of free fatty acids from adipose tissue. This hypothesis would explain both the lower rate of NAFLD as well as the lower propensity for development of NASH.

It also can be hypothesized that in African Americans, adiponectin still can play a protective role against liver injury even in presence of hepatic fat accumulation;

between the adiponectin (log<sub>10</sub>-transformed) and CK-18 (log<sub>10</sub>-transformed) in Caucasians ( $r^2 = 0.06$ ;  $P = 0.01$ ) and Hispanics ( $r^2 = 0.06$ ;  $P = 0.01$ ), but not in African Americans ( $r^2 = 0.002$ ;  $P = 0.35$ ). The interaction between ethnicity and adiponectin was statistically significant ( $P = 0.05$ ), independent of age, sex, total body fat ( $P = 0.03$ ), and HFF% ( $P = 0.06$ ). Blue line and circles = Caucasians; red line and circles = African Americans; green line and circles = Hispanics.





**Figure 3**—Association between CK-18 levels and *FDFT1* rs2645424, *PNPLA3* rs738409, and *GCKR* rs1260326. A: CK-18 levels according to the *FDFT1* rs2645424 genotypes (AA, AG, GG, CC, CG, CT and TT) in the three ethnic groups (Caucasians,  $P = 0.05$ ; African Americans,  $P = 0.08$ ; Hispanics,  $P = 0.02$ ). The white bars represent the AA, the light blue bars represent the AG, and the dark blue bars represent the GG. B: CK-18 levels according to the *PNPLA3* rs738409 genotypes in the three ethnic groups (Caucasians,  $P = 0.02$ ; African Americans,  $P = 0.26$ ; Hispanics,  $P = 0.05$ ). The white bars represent the CC, the light blue bars represent the CG, and the dark blue bars represent the GG. C: CK-18 levels according to the *GCKR* rs1260326 genotypes in the three ethnic groups (Caucasians,  $P = 0.26$ ; African Americans,  $P = 0.10$ ; Hispanics,  $P = 0.33$ ). The white bars represent the CC, the light blue bars represent the CT, and the dark blue bars represent the TT. AA, African Americans; C, Caucasians; H, Hispanics.

in fact, whereas in Caucasians and Hispanics low levels of adiponectin were associated with an increase of CK-18 levels, we did not observe any association between adiponectin levels and CK-18 in African Americans. These data would be consistent with recent animal and human studies showing that adiponectin may have a hepato-protective effect (10,26,27).

Nevertheless, it is likely that these differences are genetically driven and that different variants in genes involved in several pathways (such as lipid metabolism in the liver and adipose cells, inflammation, and others) might be responsible for it.

**The rs738409 in the *PNPLA3* gene and the rs2645424 in the *FDFT1* gene are associated with CK-18 levels**

In an effort to unravel some of the genetic determinants responsible for the hepatic damage in obese children and adolescents, we studied three gene variants, the rs738409 in the *PNPLA3* gene and the rs1260326 in the *GCKR* gene, both previously associated with hepatic steatosis (15–17), and the rs2645424 in the *FDFT1*, previously associated with the NAFLD activity score (18).

Here, we show that the rs1260326 variant in the *GCKR* is not associated with CK-18 levels. We previously have shown that it is associated with hepatic fat content, triglycerides levels, and large VLDL levels (17), and we have suggested, along with other investigators (28), that the mechanism by which it leads to hepatic steatosis is via an increased rate of hepatic *de novo* lipogenesis. Nevertheless, this variant seems to predispose to development of intrahepatic fat accumulation, without contributing to the progression of NAFLD.

We observed that the rs738409 in the *PNPLA3* gene is associated with CK-18; this observation is consistent with previous reports showing that the *PNPLA3* rs738409 variant plays a role not only in predisposing to liver fat accumulation but also in the progression to NASH (29,30). Recent studies have shown that the rs738409 variant in the *PNPLA3* leads to hepatic steatosis and steatohepatitis by enhancing the lipogenic activity and by impairing the lipolytic activity of the *PNPLA3* in the liver (31,32).

Here, we also show for the first time an association between the rs2645424 in the *FDFT1* gene and CK-18 levels in our

multiethnic cohort of obese youths. This variant, in fact, has been found to be associated with NAFLD activity score in a cohort of adult women by a genome-wide association study performed in sample of 236 non-Hispanic white women with NAFLD (15). The *FDFT1* gene, located on chromosome 8, is a key regulator of cholesterol biosynthesis (33,34). It encodes the squalene synthase, an enzyme involved in sterol synthesis; in particular, it converts two molecules of farnesyl pyrophosphate into squalene, which is a precursor to cholesterol. Because the rs2645424 is an intronic variant, it is difficult to speculate on how it may affect the enzyme activity. It is possible that this SNP is in linkage disequilibrium with a variant in the promoter so that enhancing its expression leads to an increased activation of the squalene synthase and to the intrahepatic accumulation of cholesterol. Animal studies have, in fact, shown that transient overexpression of the *FDFT1* gene in the liver of both wild-type and *LDLR* knockout mice resulted in increased de novo cholesterol biosynthesis, oversecretion of cholesterol-rich LDL, higher cholesterol levels, and a 37% increase in liver weight compared with controls attributable to hepatocyte proliferation (35). This hypothesis also would be consistent with recent studies showing the role of intrahepatic cholesterol accumulation in the pathogenesis of NASH (36).

Interestingly, none of the studied SNPs was associated with insulin resistance (Supplementary Tables 1, 2, and 3). Dissociation between insulin resistance and fatty liver in subjects carrying the *PNPLA3* rs738409 variant has been previously observed (16,37). In particular, as first suggested by Romeo et al. (16), the dissociation between fatty liver and insulin resistance in subjects carrying the rs738409 risk allele was demonstrated by Stefan et al. (37) using a hyperinsulinemic–euglycemic clamp. The authors suggested that the *PNPLA3* variant might be involved in the generation of a metabolically benign fatty liver (37). Taken together with our findings, those data corroborate the hypothesis that the *PNPLA3* rs738409 may contribute to the dissociation between NAFLD and insulin resistance observed in some individuals (38).

We did not observe any association between insulin resistance and *FDFT1* variant; although this may suggest that this gene variant predisposes to liver damage independent of insulin resistance,

studies using state-of-the-art techniques are needed to highlight this point.

### Strengths and limitations

This study has several strengths, such as the following: the young age of the patients; the absence of risk factors linked to alcohol consumption and aging; the use of MRI measurement to assess hepatic fat content; and the use of a strongly validated marker of NASH (CK-18).

We acknowledge that the major limitation of this study is the lack of liver biopsies performed in the entire cohort of subjects. Although this is a weakness, it should be pointed out that these studies can be performed only in cohorts showing a wide spectrum of the disease, and thus only enrolling subjects with and without NASH, and that liver biopsy performed in healthy children is unethical. We also acknowledge that CK-18 might not be specific for NASH because it can be elevated in a number of other conditions and thus sometimes may give spurious results (39). In fact, it has been shown that one possible limitation of the use of CK-18 for the prediction of NASH is its intrinsic inability to discriminate between NASH and other chronic diseases that involve apoptosis, such as cholangitis and cholestasis, chronic hepatitis, cancer, and trauma (39).

Another limitation of the study is the relatively small sample size of the overall population. Although this is not a large sample, to our best knowledge, so far this is the largest study dealing with liver injury in a multiethnic cohort of obese children and adolescents carefully phenotyped for liver (e.g., liver fat accumulation, CK-18) and metabolic parameters (oral glucose tolerance test–derived measures).

### Conclusion

This study provides the evidence that African American obese children and adolescents show a lower degree of liver damage than Caucasians and Hispanics, independent of the degree of hepatic fat accumulation and insulin resistance. These data suggest that African Americans are protected from hepatic damage even in presence of a high degree of hepatic fat accumulation and insulin resistance. Our observation of a different interethnic propensity to development of liver injury is clinically relevant. This suggests that Hispanic and Caucasian obese children and adolescents require closer follow-up of their liver metabolic status. Also, the observation that genes involved in the lipid

metabolism (de novo lipogenesis for *PNPLA3*, *GCKR*, and cholesterol synthesis for *FDFT1*) are associated with liver injury provide indirect evidence that common gene variants in genes involved in this pathway may play a major role in the pathogenesis of NAFLD and NASH, and that studying functionally relevant gene variants of genes modulating lipid metabolism may provide more insights in the pathophysiology and the treatment of fatty liver disease in obese children and adolescents.

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N.S. analyzed the data and wrote the manuscript. A.E.F., E.E., B.P., R.K., and G.K. researched the data. S.C. reviewed the data and wrote and edited the manuscript. S.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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### References

1. Mencin AA, Lavine JE. Advances in pediatric nonalcoholic fatty liver disease. *Pediatr Clin North Am* 2011;58:1375–1392, x
2. Schwimmer JB, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. *Pediatrics* 2006;118:1388–1393
3. Cali AM, De Oliveira AM, Kim H, et al. Glucose dysregulation and hepatic steatosis in obese adolescents: is there a link? *Hepatology* 2009;49:1896–1903
4. Cali AM, Zern TL, Taksali SE, et al. Intrahepatic fat accumulation and alterations in lipoprotein composition in obese adolescents: a perfect proatherogenic state. *Diabetes Care* 2007;30:3093–3098
5. D'Adamo E, Cali AM, Weiss R, et al. Central role of fatty liver in the pathogenesis

- of insulin resistance in obese adolescents. *Diabetes Care* 2010;33:1817–1822
6. Browning JD, Szczepaniak LS, Dobbins R, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004;40:1387–1395
  7. Guerrero R, Vega GL, Grundy SM, Browning JD. Ethnic differences in hepatic steatosis: an insulin resistance paradox? *Hepatology* 2009;49:791–801
  8. Kallwitz ER, Guzman G, TenCate V, et al. The histologic spectrum of liver disease in African-American, non-Hispanic white, and Hispanic obesity surgery patients. *Am J Gastroenterol* 2009;104:64–69
  9. Bertolani C, Marra F. The role of adipokines in liver fibrosis. *Pathophysiology* 2008;15:91–101
  10. Gastaldelli A, Harrison S, Belfort-Aguilar R, et al. Pioglitazone in the treatment of NASH: the role of adiponectin. *Aliment Pharmacol Ther* 2010;32:769–775
  11. Tamimi TI, Elgouhari HM, Alkhoury N, et al. An apoptosis panel for nonalcoholic steatohepatitis diagnosis. *J Hepatol* 2011;54:1224–1229
  12. Feldstein AE, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology* 2009;50:1072–1078
  13. Diab DL, Yerian L, Schauer P, et al. Cytokeratin 18 fragment levels as a noninvasive biomarker for nonalcoholic steatohepatitis in bariatric surgery patients. *Clin Gastroenterol Hepatol* 2008;6:1249–1254
  14. Lebensztejn DM, Wierzbicka A, Socha P, et al. Cytokeratin-18 and hyaluronic acid levels predict liver fibrosis in children with non-alcoholic fatty liver disease. *Acta Biochim Pol* 2011;58:563–566
  15. Romeo S, Kozlitina J, Xing C, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2008;40:1461–1465
  16. Santoro N, Kursawe R, D'Adamo E, et al. A common variant in the patatin-like phospholipase 3 gene (PNPLA3) is associated with fatty liver disease in obese children and adolescents. *Hepatology* 2010;52:1281–1290
  17. Santoro N, Zhang CK, Zhao H, et al. Variant in the glucokinase regulatory protein (GCKR) gene is associated with fatty liver in obese children and adolescents. *Hepatology* 2012;55:781–789
  18. Chalasani N, Guo X, Loomba R, et al; Nonalcoholic Steatohepatitis Clinical Research Network. Genome-wide association study identifies variants associated with histologic features of nonalcoholic Fatty liver disease. *Gastroenterology* 2010;139:1567–1576, 1576, e1–e6
  19. Yeh MM, Brunt EM. Pathology of non-alcoholic fatty liver disease. *Am J Clin Pathol* 2007;128:837–847
  20. Kleiner DE, Brunt EM, Van Natta M, et al; Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for non-alcoholic fatty liver disease. *Hepatology* 2005;41:1313–1321
  21. Mohanty SR, Troy TN, Huo D, O'Brien BL, Jensen DM, Hart J. Influence of ethnicity on histological differences in non-alcoholic fatty liver disease. *J Hepatol* 2009;50:797–804
  22. Vanni E, Bugianesi E, Kotronen A, De Minicis S, Yki-Järvinen H, Svegliati-Baroni G. From the metabolic syndrome to NAFLD or vice versa? *Dig Liver Dis* 2010;42:320–330
  23. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of non-alcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010;52:774–788
  24. Lomonaco R, Ortiz-Lopez C, Orsak B, et al. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with non-alcoholic fatty liver disease. *Hepatology* 2012;55:1389–1397
  25. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with non-alcoholic fatty liver disease. *J Clin Invest* 2005;115:1343–1351
  26. Xu A, Wang Y, Keshaw H, Xu LY, Lam KS, Cooper GJ. The fat-derived hormone adiponectin alleviates alcoholic and non-alcoholic fatty liver diseases in mice. *J Clin Invest* 2003;112:91–100
  27. Ding X, Saxena NK, Lin S, Xu A, Srinivasan S, Anania FA. The roles of leptin and adiponectin: a novel paradigm in adipocytokine regulation of liver fibrosis and stellate cell biology. *Am J Pathol* 2005;166:1655–1669
  28. Rees MG, Wincovitch S, Schultz J, et al. Cellular characterisation of the GCKR P446L variant associated with type 2 diabetes risk. *Diabetologia* 2012;55:114–122
  29. Valenti L, Al-Serri A, Daly AK, et al. Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology* 2010;51:1209–1217
  30. Valenti L, Alisi A, Galmozzi E, et al. I148M patatin-like phospholipase domain-containing 3 gene variant and severity of pediatric nonalcoholic fatty liver disease. *Hepatology* 2010;52:1274–1280
  31. Kumari M, Schoiswohl G, Chitrapu C, et al. Adiponutrin functions as a nutritionally regulated lysophosphatidic acid acyltransferase. *Cell Metab* 2012;15:691–702
  32. Li JZ, Huang Y, Karaman R, et al. Chronic overexpression of PNPLA3I148M in mouse liver causes hepatic steatosis. *J Clin Invest* (in press)
  33. Ness GC, Zhao Z, Keller RK. Effect of squalene synthase inhibition on the expression of hepatic cholesterol biosynthetic enzymes, LDL receptor, and cholesterol 7 alpha hydroxylase. *Arch Biochem Biophys* 1994;311:277–285
  34. Schechter I, Conrad DG, Hart I, et al. Localization of the squalene synthase gene (FDFT1) to human chromosome 8p22-p23.1. *Genomics* 1994;20:116–118
  35. Okazaki H, Tazoe F, Okazaki S, et al. Increased cholesterol biosynthesis and hypercholesterolemia in mice overexpressing squalene synthase in the liver. *J Lipid Res* 2006;47:1950–1958
  36. Farrell GC, van Rooyen DM. Liver cholesterol: is it playing possum in NASH? *Am J Physiol Gastrointest Liver Physiol* 2012;303:G9–G11
  37. Kantartzis K, Peter A, Machicao F, et al. Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin-like phospholipase 3 gene. *Diabetes* 2009;58:2616–2623
  38. Stefan N, Häring HU. The metabolically benign and malignant fatty liver. *Diabetes* 2011;60:2011–2017
  39. Younossi ZM, Page S, Rafiq N, et al. A biomarker panel for non-alcoholic steatohepatitis (NASH) and NASH-related fibrosis. *Obes Surg* 2011;21:431–439