

Genetic Variation at *NCAN* Locus Is Associated with Inflammation and Fibrosis in Non-Alcoholic Fatty Liver Disease in Morbid Obesity

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Key Words

Obesity · Dyslipidemia · Steatohepatitis · Cirrhosis · Steatosis

Abstract

Objective: Obesity-associated non-alcoholic fatty liver disease (NAFLD) may cause liver dysfunction and failure. In a previously reported genome-wide association meta-analysis, single nucleotide polymorphisms (SNPs) near *PNPLA3*, *NCAN*, *GCKR*, *LYPLAL1* and *PPP1R3B* were associated with NAFLD and with distinctive serum lipid profiles. The present study examined the relevance of these variants to NAFLD in extreme obesity. **Methods:** In 1,092 bariatric surgery patients, the candidate SNPs were genotyped and association analyses with liver histology and serum lipids were performed. **Results:** We replicated the association of hepatosteatosis with *PNPLA3* rs738409[G] and with *NCAN* rs2228603[T]. We also replicated the association of rs2228603[T] with hepatic inflammation and fibrosis. rs2228603[T] was associated

with lower serum low-density lipoprotein, total cholesterol and triglycerides. After stratification by the presence or absence of NAFLD, these associations were present predominantly in the subgroup with NAFLD. **Conclusion:** *NCAN* rs2228603[T] is a risk factor for liver inflammation and fibrosis, suggesting that this locus is responsible for progression from steatosis to steatohepatitis. In this bariatric cohort, rs2228603[T] was associated with low serum lipids only in patients with NAFLD. This supports a NAFLD model in which the liver may sequester triglycerides as a result of either increased triglyceride uptake and/or decreased lipolysis.

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Background

Obesity is a driving force for the metabolic syndrome, which is a combination of cardiovascular risk factors that include visceral obesity, hypertension, high serum triglycerides, low serum high-density lipoprotein (HDL)-cholesterol, and glucose intolerance or diabetes [1]. In a substantial number of obese individuals with metabolic syndrome, fatty infiltration of the liver (hepatosteatosis) is observed. Non-alcoholic fatty liver disease (NAFLD) is often associated with inflammation (non-alcoholic steatohepatitis or NASH) which may progress to fibrosis and cirrhosis [2, 3]. With the current epidemic of obesity, NAFLD and its progression is the leading cause of liver dysfunction and failure [4–7].

It is unknown why only a fraction of obese individuals develop hepatic steatosis and only a subset of those patients progress to NASH, fibrosis, and cirrhosis. Recently, genetic factors have been implicated. In a genome-wide association study by Hobbs and colleagues [8], a nonsynonymous variation I148M (rs738409) in the *PNPLA3* gene was found to be associated with NAFLD. This variant has also been associated with increased serum aspartate transaminase and alanine transaminase levels and histological evidence of NASH [9, 10]. More recently, the Genetics of Obesity-Related Liver Disease (GOLD) Consortium performed a genome-wide association meta-analysis of NAFLD in 7,176 subjects of European descent [11]. In this study, 3 novel loci (*NCAN*, *GCKR*, and *LYPLAL1*) and the previously reported *PNPLA3* locus were found to be associated with liver fat content as measured by both electron beam CT as well as liver biopsy. Another locus, *PPP1R3B*, was associated with liver content, as measured by electron beam CT, but not with histologically defined NAFLD. Interestingly, some of these variants were associated with distinct changes in serum lipid levels. In particular, the T allele of rs2228603 in *NCAN* was associated with increased liver fat and, seemingly paradoxically, lower serum triglyceride and low-density lipoprotein (LDL)-cholesterol levels [11]. These differences suggest that each locus may affect lipid metabolism and NAFLD through a distinct mechanism.

To further examine the role of these loci in NAFLD, we performed association studies of these variants in an independent cohort of severely obese patients who underwent bariatric surgery and from whom histologically well-characterized liver samples were obtained. Our aim was not simply to replicate the findings of the GOLD Consortium, but also to determine the relevance of these

loci in extreme obesity, a condition in which both strong genetic and environmental factors contribute to the phenotype and its metabolic complications.

Methods

Study Population and Phenotyping

The study population consisted of 1,092 bariatric surgery patients from the Geisinger Medical Center in Danville, Pa., USA, with normal liver tissue or varying degrees of NAFLD, ranging from simple steatosis to steatohepatitis to cirrhosis (fig. 1 – these subjects were not included in the original GOLD Consortium meta-analysis). The protocol was approved by the Geisinger Clinic's Institutional Review Board, and all subjects provided written informed consent. Prior to surgery, the patients were extensively phenotyped, including a comprehensive medical history and physical examination, anthropometry, fasting serum glucose and lipid levels, liver and kidney function, and medication usage [12].

Intra-operative liver biopsy specimens were formalin-fixed and stained with hematoxylin and eosin for routine histology and Masson's trichrome for assessment of fibrosis [13]. All specimens were read by experienced pathologists and graded according to standard NAFLD criteria [14]. Steatosis was graded in severity from 0 (no steatosis) to 3 (severe steatosis). Scoring for lobular inflammation, hepatocyte ballooning, and perivenular fibrosis was dichotomized, with a score of 0 indicating the absence of the feature and a score of 1 indicating any presence of the feature (ranging from mild to severe).

SNP Genotyping

Genomic DNA was isolated from peripheral blood leukocytes [15]. The 5 hepatic steatosis-associated SNPs from the GOLD Consortium meta-analysis were genotyped using TaqMan® SNP Genotyping Assays (Life Technologies, Carlsbad, Calif., USA). These included: rs12137855 (C/T) on chromosome 1 near *LYPLAL1*, rs780094 (C/T) on chromosome 2 in *GCKR*, rs4240624 (A/G) on chromosome 8 near *PPP1R3B*, rs2228603 (C/T) on chromosome 19 in *NCAN*, and rs738409 (G/C) on chromosome 22 in *PNPLA3*. PCR amplification was performed on the GeneAMP PCR System 9700 thermal cycler (Applied Biosystems, Grand Island, N.Y.) under the following conditions: 10 min at 95°C, then 40 cycles of 15 s at 92°C and 1 min at 60°C; and allelic discrimination was performed on the ABI Prism 7900 HT Sequence Detection System (SDS; Applied Biosystems, Grand Island, N.Y.) and SDS software according to the manufacturer's directions. All 5 SNPs passed genotyping quality control. The average genotype call rate was 96.7%. The genotype concordance rate of blind replicates was 99.2%. None of the SNP allele frequencies deviated significantly from the Hardy-Weinberg equilibrium.

Statistical Analysis

Statistical analyses were carried out using SAS version 9.2 (SAS Institute Inc., Cary, N.C., USA). For continuous traits (steatosis grade, serum total, LDL- and HDL-cholesterol, and triglycerides), associations between SNPs and phenotypes were assessed by linear regression; and dichotomous traits (lobular inflammation, ballooning, perivenular fibrosis and cirrhosis) were analyzed using logistic regression. Analyses were adjusted for age, sex, and lipid-lowering

Table 1. Characteristics of the study population

	NAFLD n = (748)	No NAFLD n = (344)	p value*
Females, n (%)	579 (77.4)	296.0 (86.0)	0.001
Mean age ± SD, years	47±10.6	46±11.8	0.36
Mean BMI ± SD	50.4±8.9	49.6±8.6	0.22
Mean waist circumference ± SD, inches	54.2±6.7	52.4±6.2	<0.0001
Diabetes, n (%)	292 (39)	93 (27)	0.0007
Mean total cholesterol ± SD, mg/dl	191.7±40.8	185.4±40.8	0.006
Mean HDL-cholesterol ± SD, mg/dl	46.3±10.6	50.9±12.1	<0.0001
Mean LDL-cholesterol ± SD, mg/dl	107.6±34.5	106.4±36.0	0.39
Mean triglycerides ± SD, mg/dl	197.4±141.2	140.3±69.4	<0.0001
Statin therapy, n (%)	228 (30)	117 (34)	0.07
Fibrate therapy, n (%)	41 (5)	20 (6)	0.38
Statin or fibrate therapy, n (%)	250 (33)	122 (35)	0.14

* Adjusted for age and sex (sex is only adjusted for age and age is only adjusted for sex). NAFLD was graded using the criteria of Brunt et al. [14]. NAFLD = grade ≥1; No NAFLD = grade 0.

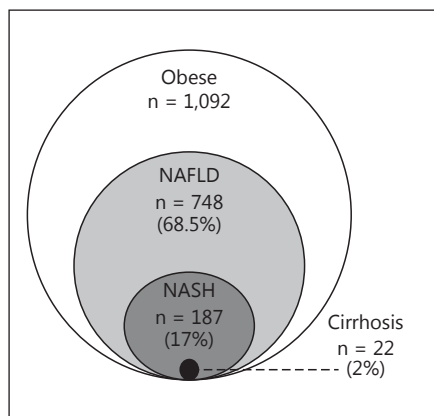


Fig. 1. Spectrum of NAFLD represented in the bariatric surgery cohort (n = 1,092).

medication. Adjustment for lipid-lowering therapy was performed using as covariates whether the subject was or was not on statin therapy and whether the subject was or was not on fibrate therapy (the vast majority of subjects on lipid-lowering therapy were taking statins). Regression analyses tested for an additive SNP association between the number of copies (0, 1 or 2) of the NAFLD-associated allele and the trait of interest. Due to the relatively low minor allele frequency of the T allele of rs2228603 in *NCAN* and the G allele of rs4240624 in *PPP1R3B* (7.4 and 8.3%, respectively), the rare homozygous and heterozygous genotype groups were combined into a single group and compared to the common homozygous genotype for analysis. Since a small number of SNPs were genotyped, each with a high posterior probability of being true positive, a two-sided p value <0.05 was used as the threshold for statistical significance.

Results

Like most bariatric surgery cohorts [16–18], the majority (80%) of the cohort was female (table 1). The average age was 46 years and the mean pre-operative BMI was 50. Although the BMI did not differ between patients with and those without NAFLD ($p = 0.22$), those with NAFLD had greater waist circumferences ($p < 0.0001$), lower serum HDL-cholesterol levels ($p < 0.0001$) and, as shown in other studies [17, 19], higher serum triglyceride levels ($p < 0.0001$). In addition, more patients in the NAFLD group had diabetes ($p = 0.0007$). About one third of all subjects were on statin or fibrate therapy. However, the percentage of subjects on lipid-regulating medication was not significantly different between subjects with and those without NAFLD or among any of the genotypes examined.

Of all 1,092 obese subjects, 32% had no evidence of hepatic steatosis, 39% had grade 1 steatosis, 19% had grade 2, and 10% had grade 3 (fig. 2). Of the 748 patients with evidence of hepatic steatosis, 187 (25%) had at least one histological feature of steatohepatitis. The biopsies from these 187 NASH patients revealed lobular inflammation (mild: 24%, moderate: 7%, and severe: 1%), hepatocyte ballooning (mild: 17%, moderate: 8%, and severe: 0%) and perivenular fibrosis (mild: 11%, moderate: 5%, and severe: 3%) (fig. 3). Of the 187 NASH patients, 12% had cirrhosis.

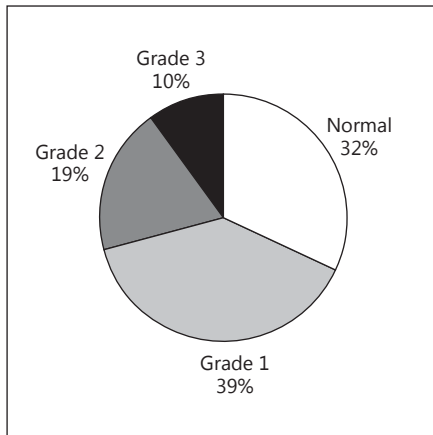


Fig. 2. Proportions of bariatric surgery patients with varying degrees of hepatic steatosis (n = 1,092). Grading was performed by experienced pathologists using the criteria of Brunt et al. [14].

Of the 5 SNPs associated with NAFLD in the GOLD Consortium meta-analysis, only rs780094[C] in *GCKR* was associated with higher weight ($p = 0.001$) and BMI ($p = 0.001$) in this bariatric cohort (online suppl. table; for all online supplementary material, see www.karger.com/doi/10.1159/000346195). SNPs rs780094[C] in *GCKR* and rs2228603[T] in *NCAN* were associated with increased waist circumference ($p = 0.02$ and 0.03 , respectively; online suppl. table).

Except for rs12137855[C] near *LYPLAL1*, all of the SNPs were significantly associated with (or trended toward) increased steatosis in the same direction and with similar effect sizes as reported in the GOLD Consortium meta-analysis (table 2). Consistent with the results from the GOLD Consortium [11], our data showed an association of *PNPLA3* SNP rs738409[G] with both hepatic steatosis ($p = 4.8 \times 10^{-8}$) and hepatocyte ballooning ($p = 0.006$; fig. 4; table 2). Similarly, as previously reported [11], we found an association of the T allele of SNP rs2228603 in *NCAN* with liver steatosis ($p = 0.03$) as well as with lobular inflammation ($p = 0.02$) and perivenular fibrosis ($p = 0.002$) (fig. 4, 5; table 2). Though not statistically significant, this allele also was associated with a trend toward increased hepatocyte ballooning ($p = 0.06$; fig. 5; table 2). These findings show that in extreme obesity, *PNPLA3* rs738409[G] and *NCAN* rs2228603[T] are associated with liver steatosis and an increased risk for progression from simple steatosis to NASH. By contrast, variants in *LYPLAL1*, *GCKR* and *PPP1R3B* did not appear to show an increased risk for lobular inflammation,

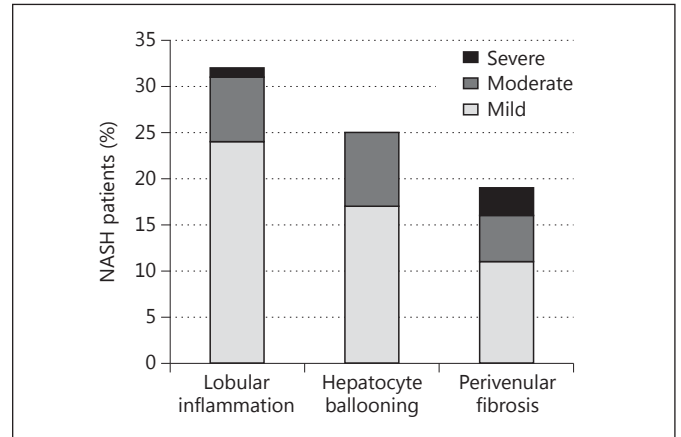


Fig. 3. Liver histology features (lobular inflammation, hepatocyte ballooning and perivenular fibrosis) in bariatric surgery patients with NASH (n = 187). Grading was performed by experienced pathologists using the criteria of Brunt et al. [14].

hepatocyte ballooning or perivenular fibrosis. Although there was only a trend toward statistical significance, the *PPP1R3B* SNP rs4240624[A] was the only variant associated with cirrhosis ($p = 0.07$).

As observed in the GOLD Consortium study, the data from this bariatric surgery cohort revealed that some NAFLD-associated SNPs were associated with a distinct pattern of serum lipid levels (table 3). These effects were most apparent in subjects with NAFLD. In NAFLD subjects, the NAFLD-associated G allele of rs738409 in *PNPLA3* was associated with lower total cholesterol ($p = 0.03$). Similarly, in NAFLD subjects, the NAFLD-associated T allele of rs2228603 in *NCAN* was associated with decreased serum total cholesterol ($p = 0.0002$), LDL-cholesterol ($p = 0.009$) and triglycerides ($p = 0.004$). By contrast, as in the GOLD Consortium meta-analysis, the NAFLD-associated T allele of *GCKR* SNP rs780094 was associated with increased serum triglyceride levels; this effect was most evident in subjects with NAFLD ($p = 0.04$). Also consistent with the GOLD Consortium's findings, we did not find any significant association between either *LYPLAL1* SNP rs12137855 or *PPP1R3B* SNP rs4240624 and serum lipids.

Discussion

Using an independent sample of 1,092 well-phenotyped morbidly obese subjects who underwent bariatric surgery and in whom liver histology was determined, we found that

Fig. 4. Association between SNP genotype and liver steatosis in all bariatric surgery patients (n = 1,092).

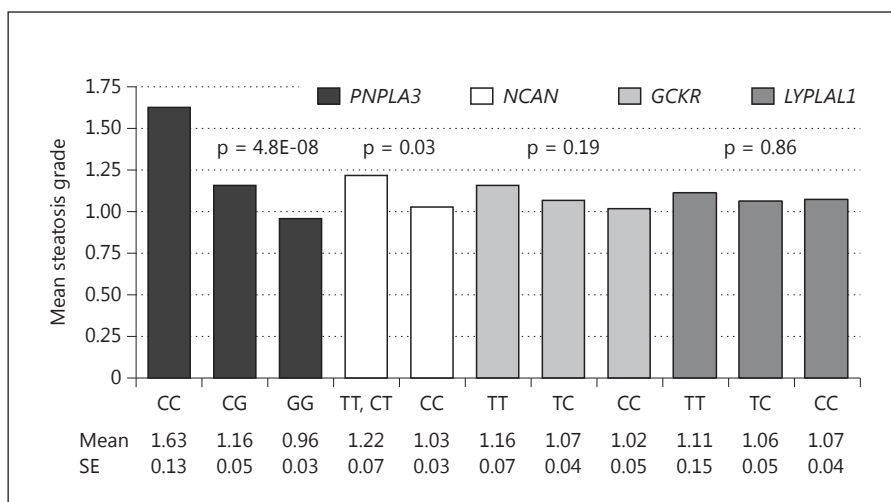


Table 2. Association of SNPs with NAFLD characteristics

	rs780094			rs2228603		rs12137855			rs4240624		rs738409		
	TT (n =)	CT	CC	-T	CC	CC	CT	TT	AA	-G	GG	GC	CC
Effect allele	T			T		C			A		G		
Other allele	C			C		T			G		C		
Effect allele frequency, %	40.9			7.4		73.5			8.3		22.0		
Nearest gene	<i>GCKR</i>			<i>NCAN</i>		<i>LYPLAL1</i>			<i>PPP1R3B</i>		<i>PNPLA3</i>		
Mean steatosis ^a	1.16	1.07	1.02	1.22	1.03	1.07	1.06	1.11	1.08	1.02	1.63	1.16	0.96
SE	0.07	0.04	0.05	0.07	0.03	0.04	0.05	0.15	0.03	0.07	0.13	0.05	0.03
p value		0.19		0.03			0.86		0.49		4.80E-08		
Lobular inflammation (grade >0), % ^b	34.4	30.9	31.1	39.7	30.1	31.4	32.2	32.4	31.4	32.7	32.7	34.8	29.6
p value		0.68		0.02			0.98		0.68		0.11		
Hepatocyte ballooning (grade >0), % ^b	23.4	29.1	21.6	32.6	23.7	24.2	28.0	23.8	24.9	27.6	31.4	31.3	21.5
p value		0.48		0.06			0.49		0.48		0.006		
Perivenular fibrosis (grade >0), % ^b	20.0	18.8	18.8	27.8	17.2	19.2	18.0	21.6	19.1	17.3	21.2	22.0	17.1
p value		0.98		0.002			0.74		0.65		0.06		
Cirrhosis (grade >0), % ^b	2.8	2.5	1.6	4.0	2.1	2.5	1.9	2.7	2.0	4.2	1.9	3.3	1.7
p value		0.47		0.21			0.61		0.07		0.21		

Adjusted for age, sex, and lipid-lowering medication: mean and standard error (SE) are presented for each genotype group.

^a Steatosis was examined histologically and graded in severity from 0 (no steatosis) to 3 (severe steatosis).

^b Dichotomized scoring for inflammation, ballooning, fibrosis and cirrhosis: grade 0 versus grade >0.

despite similar BMI, subjects with NAFLD had a higher prevalence of diabetes, hyperlipidemia and a greater waist circumference than those without NAFLD. These data support that NAFLD is associated with insulin resistance and other features of the metabolic syndrome largely independent of obesity. Association analyses of 5 NAFLD-associated genetic variants, identified by the GOLD Consortium

genome-wide association meta-analysis, revealed a non-synonymous variant in *PLPLA3* to be associated not only with the presence of steatosis but also with NASH, a more progressive and clinically ominous manifestation of NAFLD. These findings extend previously reported studies to patients with extreme obesity. They are also consistent with other studies in which the G allele of *PLPLA3* rs738409

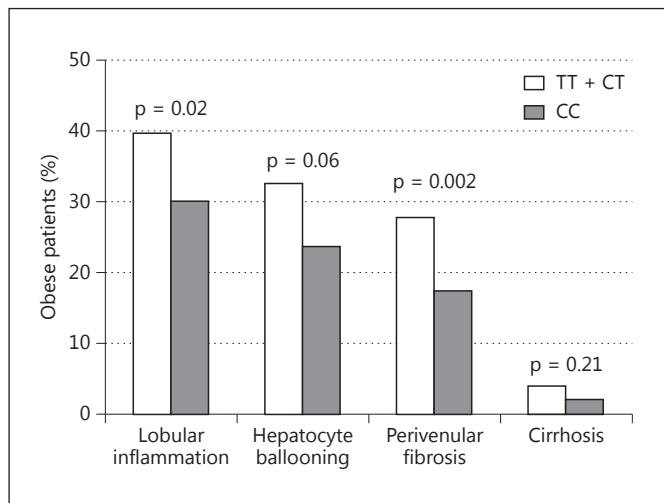


Fig. 5. Association of *NCAN* rs2228603 genotype with features of NASH and cirrhosis in all bariatric surgery patients (n = 1,092).

was not only associated with simple fat accumulation, but also with the degree of liver steatosis as evaluated by liver biopsy [20–22]. In other studies, the same allele was associated with steatosis, portal inflammation, lobular inflammation, Mallory–Denk bodies, NAFLD activity score, and fibrosis [8, 10].

Furthermore, we replicated the GOLD Consortium’s association of the T allele of rs2228603 in *NCAN* with the degree of liver steatosis as well as with signs of lobular inflammation and perivenular fibrosis, which are consistent with NASH, a more advanced stage of NAFLD. For *GCKR* rs780094 and *PPP1R3B* rs4240624, although not statistically significantly associated with NAFLD, the direction of effect and the effect size showed similar trends to the GOLD Consortium’s results. It is likely that the inability to achieve statistical significance is due to a lack of power to discern the effect of variants of modest effect size. The absence of even a trend for association of *LYPLAL1* rs12137855 with NAFLD may be due to differences among populations, a false negative finding, or a false positive finding in the initial GOLD Consortium study. Assuming a 24% population prevalence of NAFLD, our sample had 80% power to detect an OR of 1.48 for presence of steatosis for a variant with allele frequency of at least 0.05 at $p < 0.05$. Using a higher population prevalence of 50%, which may be more suitable for a bariatric surgery population, our sample had 80% power to detect an OR of 1.29 for a variant with an allele frequency of at least 0.05 at $p < 0.05$.

Our data revealed that the *PPP1R3B* SNP rs4240624 was not significantly associated with liver steatosis, lobular inflammation, or hepatocyte ballooning. However, this variant was the only SNP in our study that tended to be associated with cirrhosis ($p = 0.07$). In the GOLD Consortium meta-analysis, *PPP1R3B* was associated with fatty liver identified on CT scan, but it was not associated with histological features of NAFLD. As hypothesized by Speliotes et al. [11], this may suggest that *PPP1R3B* is related to liver steatosis but not hepatocyte inflammation and fibrosis. Alternatively, the SNP may be associated with the intrahepatic accumulation of a substance that resembles steatosis on abdominal radiographic imaging and causes cirrhosis (i.e. glycogen, which accumulates in glycogen storage disease type IV is characterized by glycogen precipitation in the liver that results in cirrhosis).

Our data also replicate distinct patterns of serum lipids for some NAFLD-associated variants. The GOLD Consortium meta-analysis found no association between lipid levels and *PNPLA3* rs738409[G]. Our data suggest a relationship between the NAFLD-associated G allele and lower total and HDL-cholesterol levels in the full cohort ($p = 0.03$). This may be a type I error or alternatively due to a difference in ascertainment; all subjects in our study were selected for extreme obesity. With regard to serum triglycerides, there was no association between this SNP and serum triglyceride levels in the overall cohort, but there was a trend toward an association with lower triglyceride levels in patients with NAFLD ($p = 0.08$). In support of this finding, the relationship between this SNP and serum triglyceride levels has been reported by others [23, 24].

As in the GOLD Consortium meta-analysis, we found a relationship between the NAFLD-associated *NCAN* rs2228603[T] allele and lower serum LDL-cholesterol, total cholesterol and triglyceride levels, particularly in those with NAFLD. Interestingly, this association was not found in equally obese patients without NAFLD, suggesting that NAFLD and lower serum lipids may be mechanistically linked in rs2228603[T] carriers. Since NAFLD is clinically associated with higher serum lipids, this seemingly paradoxical finding suggests that more than one NAFLD subtype may exist. Elucidation of the causative gene/variant(s) at this locus may uncover a novel disease mechanism in which sequestration of triglycerides in the liver, either as a result of increased triglyceride uptake and/or decreased lipolysis, results in lower serum lipid levels.

By contrast, the NAFLD-associated *GCKR* rs780094[T] allele was associated with higher serum triglycerides.

Table 3. Association of SNPs with fasting serum lipid levels

	rs780094			rs2228603		rs12137855			rs4240624		rs738409		
	TT	CT	CC	-T	CC	CC	CT	TT	AA	-G	GG	GC	CC
Effect allele	T			T		C			A		G		
Other allele	C			C		T			G		C		
Nearest gene	GCKR			NCAN		LYPLAL1			PPP1R3B		PNPLA3		
<i>Patients with NAFLD, n</i>													
Mean total cholesterol, mg/dl	129	352	247	114	583	449	207	27	575	107	47	244	411
SE	197.6	189.4	191.0	178.4	194.7	192.8	189.6	191.5	192.4	192.7	180.6	190.1	194.7
p value	3.9	2.1	2.6	3.6	1.7	1.9	2.7	6.9	1.7	3.8	5.9	2.5	2.0
		0.11		0.0002			0.58		0.96			0.03	
Mean LDL-cholesterol, mg/dl	109.0	106.2	108.2	100.2	109.2	108.0	105.8	108.7	107.9	108.2	98.5	107.8	108.8
SE	3.5	1.8	2.2	3.1	1.5	1.7	2.3	5.8	1.5	3.2	5.3	2.2	1.7
p value		0.68		0.009			0.80		0.96			0.16	
Mean HDL-cholesterol, mg/dl	46.8	45.9	46.0	45.3	46.3	46.6	44.9	48.3	46.2	47.4	46.1	45.8	46.5
SE	0.9	0.6	0.7	0.9	0.4	0.5	0.7	1.9	0.5	1.0	1.5	0.6	0.6
p value		0.13		0.47			0.73		0.41			0.38	
Mean triglycerides, mg/dl ^a	213.3	198.8	188.6	166.6	205.4	198.3	202.4	193.0	201.0	187.7	175.7	186.1	209.3
SE	11.6	7.6	9.8	8.2	6.3	6.1	11.4	36.7	6.5	11.9	14.7	6.3	8.2
p value		0.04		0.004			0.64		0.78			0.08	
<i>Patients without NAFLD, n</i>													
Mean total cholesterol, mg/dl	49	156	122	31	295	208	103	8	262	55	5	103	218
SE	189.0	183.8	184.7	189.1	184.6	186.0	183.4	192.8	185.5	183.7	192.0	178.8	187.8
p value	6.2	3.2	3.7	6.1	2.4	2.9	3.6	14.6	2.6	5.1	26.4	3.7	2.8
		0.62		0.62			0.70		0.72			0.15	
Mean LDL-cholesterol, mg/dl	106.1	106.2	105.9	106.5	106.0	107.2	104.3	111.5	106.3	106.3	111.6	100.4	108.5
SE	5.1	2.9	3.3	6.1	2.1	2.6	3.1	13.4	2.3	4.6	26.4	3.3	2.5
p value		0.90		0.92			0.56		0.89			0.15	
Mean HDL-cholesterol, mg/dl	52.9	50.2	50.9	53.1	50.5	50.6	51.4	53.3	51.1	49.6	56.8	48.7	51.6
SE	1.6	0.9	1.1	2.0	0.7	0.9	1.1	5.4	0.8	1.4	4.9	1.1	0.8
p value		0.52		0.44			0.53		0.44			0.13	
Mean triglycerides, mg/dl ^a	145.5	138.3	139.7	147.6	140.4	140.0	140.5	139.6	140.5	139.3	116.8	148.1	138.3
SE	12.2	5.8	5.4	13.2	4.0	4.5	7.0	36.5	4.4	7.4	16.7	7.4	4.6
p value		0.74		0.61			0.83		0.59			0.39	
<i>All Patients with and without NAFLD, n</i>													
Mean total cholesterol, mg/dl	186	522	384	155	928	696	325	39	883	172	54	370	664
SE	195.1	187.1	188.8	180.1	191.0	190.2	187.4	191.0	189.8	190.3	181.1	186.8	191.8
p value	3.3	1.8	2.1	3.1	1.4	1.6	2.2	6.0	1.4	3.0	5.7	2.1	1.6
		0.12		0.003			0.58		0.92			0.03	
Mean LDL-cholesterol, mg/d	108.1	105.6	107.3	101.2	107.9	107.4	105.2	108.6	107.0	108.0	99.9	105.8	108.2
SE	2.8	1.5	1.8	2.7	1.2	1.4	1.8	5.1	1.2	2.6	5.2	1.8	1.4
p value		0.75		0.02			0.61		0.85			0.12	
Mean HDL-cholesterol, mg/dl	48.6	47.3	47.6	46.7	47.8	47.9	47.1	49.2	47.7	48.3	46.9	46.7	48.3
SE	0.8	0.5	0.5	0.9	0.4	0.4	0.6	1.9	0.4	0.8	1.5	0.5	0.5
p value		0.21		0.24			0.97		0.66			0.03	
Mean triglycerides, mg/dl ^a	193.5	178.5	172.1	162.4	182.7	179.0	181.0	180.6	181.1	171.3	167.3	173.7	184.2
SE	9.0	5.5	6.7	6.9	4.4	4.5	7.9	28.1	4.4	8.2	13.2	4.9	5.6
p value		0.08		0.18			0.78		0.98			0.63	

Adjusted for age, sex, and lipid-lowering medication: mean and standard error (SE) are presented for each genotype group.

^a log-transformed for analysis and back-transformed for presentation.

These findings are similar to those of the GOLD Consortium meta-analysis as well as other studies [25–27]. Finally, we did not detect any association between the *LYPLAL1* or *PPP1R3B* SNPs and serum lipids. It is possible that the lack of association is due to an inadequate sample size and power, especially for *PPP1R3B*, in which the GOLD Consortium reported increased LDL- and HDL-cholesterol.

The *NCAN* locus contains at least 20 genes in a 500-kb region on chromosome 19p13 [28–30]. None of these genes are particularly compelling positional candidates for NAFLD or lipid homeostasis. Additional studies are needed to fine map this locus and perform functional studies. Neurocan, the protein product of *NCAN*, is a chondroitin sulfate proteoglycan, primarily expressed in the nervous system, that is thought to be involved in cell adhesion and migration [31–34]. rs2228603 is located in exon 3 of *NCAN* and encodes a nonconservative nonsynonymous mutation (Pro92Ser), which is predicted by the software tool PolyPhen-2 to alter protein structure and function [35]. While neurocan itself has not yet been shown to be expressed in the liver or to directly affect lipid metabolism or hepatic steatosis, it is becoming increasingly recognized that the central nervous system (CNS) is an important regulator of peripheral glucose and triglyceride metabolism [36–38]. The liver is highly innervated by both sympathetic and parasympathetic nerves [39]. Bruinstroop et al. [40] have shown that postprandial serum triglyceride levels were significantly elevated in parasympathetic or sympathetic denervated rats compared to sham-operated animals. Robertson et al. [41] demonstrated that vagal stimulation increases hepatic secretion of very low-density lipoprotein (VLDL) triglyceride. Since *NCAN* rs2228603 has been associated with increased liver fat and decreased serum triglyceride [11, 29], it is plausible that this variant is also associated with a brain-liver axis that, when deregulated, increases the risk for NAFLD. Furthermore, this axis may be modulated by increased dietary fat intake, as suggested by a 50% increase in hepatic VLDL synthesis after fat intake [42].

Further evidence for a central control of the liver lipid metabolism comes from studies of neuropeptide Y (NPY), a 36-amino acid peptide neurotransmitter secreted by the hypothalamus that has glucose and lipid regulatory effects in the liver. Fasting rats treated with intracerebroventricular injections of NPY had a 2.5-fold increased VLDL secretion into the bloodstream [43]. Intracerebroventricular administrations of an NPY-Y5 receptor agonist reproduced this effect, while an NPY-Y1

receptor antagonist decreased VLDL secretion. These findings demonstrate that the CNS can control VLDL secretion.

In summary, in light of the increased risk for NAFLD conferred by obesity, we have extended the findings by the GOLD Consortium to include a large population of bariatric surgery patients in whom liver steatosis, inflammation, and fibrosis had been documented histologically. Specifically, we have shown that variants in *PNPLA3* and *NCAN* are associated not only with liver steatosis, but also NASH, a more progressive and clinically ominous manifestation of NAFLD. *NCAN* is a gene expressed in brain and thus may identify an untapped potential role for the CNS in the mechanism of fatty liver. Alternatively variants in other genes at this locus may be responsible for the NAFLD phenotype. Fine mapping and functional studies will be required to identify the causative genes/variants, which may provide insight into a novel pathway for NAFLD development, leading to new strategies for the prevention and treatment of this disease. In addition, follow-up analyses of the effects of gastric bypass surgery on liver steatosis, inflammation and dyslipidemia may provide additional insight into the potential role of these loci as determinants of metabolic responses to weight loss.

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