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SHORT COMMUNICATION

A common variant in *PNPLA3*, which encodes adiponutrin, is associated with liver fat content in humans

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Abstract
Aims/hypothesis It has recently been suggested that the rs738409 G allele in *PNPLA3*, which encodes adiponutrin, is strongly associated with increased liver fat content in three different ethnic groups. The aims of the present study were as follows: (1) to try to replicate these findings in European individuals with quantitative measures of hepatic fat content; (2) to study whether the polymorphism influences hepatic and adipose tissue insulin sensitivity; and (3) to investigate whether *PNPLA3* expression is altered in the human fatty liver.
Methods We genotyped 291 Finnish individuals in whom liver fat had been measured using proton magnetic resonance spectroscopy. Hepatic *PNPLA3* expression was measured in 32 participants. Hepatic and adipose tissue insulin sensitivities were measured using a euglycaemic–hyperinsulinaemic (insulin infusion 0.3 mU kg^{-1} min^{-1}) clamp technique combined with infusion of [3-^{3}H]glucose in 109 participants.
Results The rs738409 G allele in *PNPLA3* was associated with increased quantitative measures of liver fat content (p=0.011) and serum aspartate aminotransferase concentrations (p=0.002) independently of age, sex and BMI. Fasting serum insulin and hepatic and adipose tissue insulin sensitivity were related to liver fat content independently of genotype status. *PNPLA3* mRNA expression in the liver was positively related to obesity (r=0.62, p<0.0001) and to liver fat content (r=0.58, p=0.025) in participants who were not morbidly obese (BMI <40 kg/m^2).
Conclusions/interpretation A common variant in *PNPLA3* increases the risk of hepatic steatosis in humans.

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Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
^{1}H-MRS	Proton magnetic resonance spectroscopy
LSD	Least square difference
SNP	Single nucleotide polymorphism
TG	Triacylglycerol

Introduction

Hepatic fat accumulation due to non-alcoholic causes is related to the metabolic syndrome, obesity and type 2 diabetes [1]. It is estimated that approximately 30% of the adult population in the USA has a fatty liver [2]. Non-alcoholic fatty liver may progress to non-alcoholic steatohepatitis (NASH), which significantly increases the risk of developing end-stage liver disease [1]. NASH has been predicted to become the leading cause of liver transplantation by the year 2020 [3]. Little is known about the influence of genetic factors on hepatic fat content and inflammation.

A genome-wide association scan in African-Americans, European-Americans, and Hispanic individuals participating in the Dallas Heart Study has suggested that the rs738409 single nucleotide polymorphism (SNP) in *PNPLA3* (encoding the protein adiponutrin) is associated with increased liver fat content ($p=3.4\times10^{-4}$ in European-Americans, $p=7.5\times10^{-9}$ in African-Americans and $p=2.0\times10^{-10}$ in Hispanics [4]). To determine whether this finding can be reproduced in Europeans, we genotyped this SNP in 291 Finnish individuals in whom liver fat content was measured using quantitative proton magnetic resonance spectroscopy (^{1}H-MRS) as previously described [5]. In the Dallas Heart Study, the rs738409 SNP was not associated with insulin sensitivity measured indirectly using the HOMA-IR index [4]. It has been suggested that adiponutrin has both lipogenic and lipolytic properties [6], but its exact function is unknown. Nor is it known whether *PNPLA3* expression is altered in the human fatty liver. We therefore examined whether expression of *PNPLA3* in human liver samples correlates with liver fat content, or, as has been shown in adipose tissue [7], with obesity. We also determined whether the *PNPLA3* genotype is associated with alterations of hepatic or adipose tissue insulin sensitivity in a subset of 109 participants.

Methods

Participants A total of 291 Finnish individuals were recruited for metabolic studies using the following inclusion criteria: (1) age 20–75 years; (2) no known acute or chronic disease, based on history and physical examination and standard laboratory tests (blood counts, serum creatinine, thyroid-stimulating hormone and electrolyte concentrations) and ECG; and (3) alcohol consumption <20 g/day. Elevated liver enzymes (serum alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) were not exclusion criteria. However, individuals with evidence of hepatitis B or C, autoimmune hepatitis, clinical signs or symptoms of inborn errors of metabolism or a history of use of toxins or drugs associated with liver steatosis were excluded. Eighty-three participants had type 2 diabetes.

The participants from whom a liver biopsy was available ($n=32$) were either recruited from patients undergoing laparoscopic gastric bypass surgery ($n=24$, three with type 2 diabetes: nine had the CC, eight the CG and five the GG genotype; genotypes of two individuals could not be analysed due to lack of ethical permission for genetic analyses) or were among those referred to a gastroenterologist due to elevated liver function tests ($n=8$, two with type 2 diabetes; all with the CC genotype), which were all due to non-alcoholic fatty liver disease. However, three out of eight participants turned out to have normal liver fat content (≤5% by histology, vide infra). All participants consumed <20 g alcohol per day. Approximately one half of the liver sample was sent to a pathologist for routine histopathological assessment, while the rest was immediately frozen and stored in liquid nitrogen. The fat content of the liver biopsy specimens (per cent of hepatocytes with macrovesicular steatosis) was determined by a liver pathologist (J. Arola) in a blinded fashion. All protocols were approved by the ethics committee of the Helsinki University Central Hospital, and each participant provided written informed consent.

Liver fat content, hepatic and adipose tissue insulin sensitivity and biochemical measurements Liver fat content was measured by ^{1}H-MRS as previously described [8]. Insulin sensitivity was assessed by a euglycaemic–hyperinsulinaemic clamp technique combined with an infusion of [3-^{3}H]glucose as previously described [9]. Because hepatic glucose production is more sensitive to suppression by insulin than stimulation of muscle glucose uptake, we used a low insulin infusion rate (0.3 mU kg^{-1} min^{-1}) to accurately quantify interindividual variation in hepatic insulin sensitivity. These measurements were available from 109 individuals. Rates of glucose appearance and disappearance were calculated using Steele's non-steady-

state equations. Since insulin clearance is altered by a fatty liver, hepatic insulin sensitivity was calculated by dividing the per cent suppression of hepatic glucose production by the mean serum insulin concentration (pmol/l) [9]. Adipose tissue insulin sensitivity was calculated by dividing the per cent suppression of serum NEFA by insulin by the mean serum insulin concentration (pmol/l) [9]. Body composition and circulating variables were measured as previously described for these individuals [8].

Genotyping Genomic DNA was extracted from whole blood. Approximately 10 ng DNA was used for genotyping with the TaqMan PCR method (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Post-PCR allelic discrimination was carried out measuring allele-specific fluorescence on an ABI Prism Sequence Detection System ABI 7900HT (Applied Biosystems). This assay is designed for the reverse strand so that the G allele corresponds to the 148Met phenotype while the gene is transcribed from the forward strand. The success rate for genotyping was >95%. The genotype was in Hardy–Weinberg equilibrium.

Hepatic mRNA expression analyses RNA was converted to cDNA using a Quantitect Reverse Transcription kit according to the manufacturer's recommendations (Qiagen, Düsseldorf, Germany). Levels of mRNA expression were analysed using TaqMan *PNPLA3* Assay on demand (Hs00228747_m1) and the endogenous control cyclophilin A (4326316E) on the ABI 7900HT according to manufacturer's recommendation (Applied Biosystems). Ten nanograms of cDNA was used in each reaction and all samples were run in triplicate. Data were analysed using the Δ^{Ct} method.

Statistical analyses Non-normally distributed data were used after $\log_{10}$ transformation. If distributed normally, data are shown as means±SEM, whereas non-normally distributed data are shown as median (25% percentile, 75% percentile). Hardy–Weinberg equilibrium was assessed using the χ^2 test. One-way ANOVA was used to assess effects of the genotype on different variables. The least square difference (LSD) test was used for post hoc analyses. Analysis of covariance was used to adjust liver fat for age, sex and BMI. Analysis of covariance was used to compare slopes and intercepts of regression lines of the associations between insulin sensitivities and liver fat content in different genotype carriers.

Calculations were made using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA) and figures using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered statistically significant.

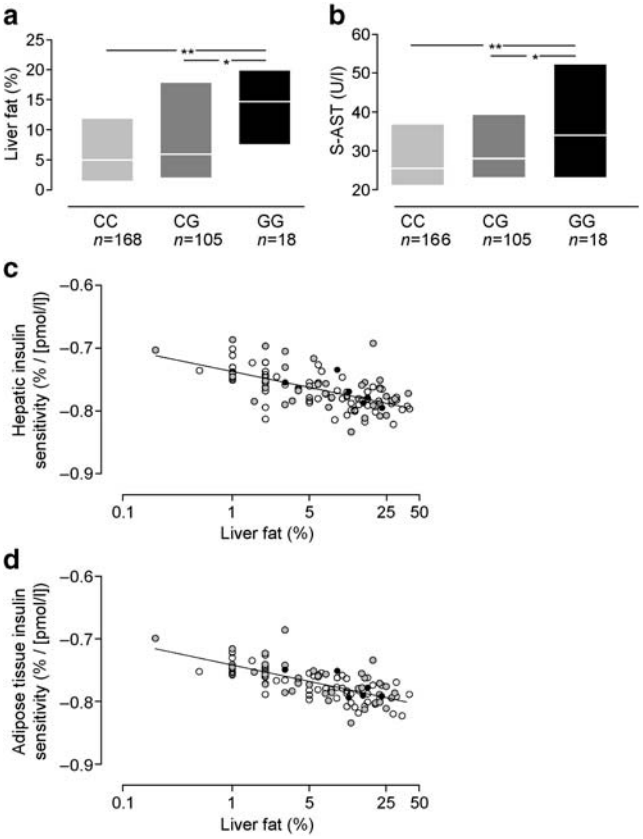

Fig. 1 Medians and interquartile ranges of liver fat content measured by ^{1}H-MRS ($p=0.011$) (**a**) and serum AST (S-AST) ($p=0.002$) (**b**) in *PNPLA3* rs738409 genotypes. *$p<0.05$, **$p<0.01$ for LSD post hoc test. Relationships between liver fat content ($\log_{10}$ scale) and hepatic (**c**, $r=-0.62$, $p<0.0001$) and adipose tissue (**d**, $r=-0.71$, $p<0.0001$) insulin sensitivity. There were no differences in slopes and intercepts of these associations between CC (white circles, $n=62$ [**c**] and $n=64$ [**d**]), CG (grey circles, $n=41$) and GG (black circles, $n=6$ [**c**] and $n=7$ [**d**]) *PNPLA3* rs738409 genotype carriers

Results

Liver fat Median liver fat content of the whole cohort was 5.95% (interquartile range 2.00–15.55%). The rs738409 G allele was associated with increased liver fat content ($p=0.011$, Fig. 1a). The difference became even more significant after adjustment for age, sex and BMI ($p=0.002$), and after additional adjustment for glucose tolerance status and medications ($p=0.004$). There were no differences in fasting plasma glucose, fasting serum triacylglycerol (TG), HDL- and LDL-cholesterol, or total serum cholesterol concentrations between different *PNPLA3* genotype carriers (Table 1).

The rs738409 G allele was associated with increased serum AST concentrations ($p=0.002$, Fig. 1b) also after adjustment for age, sex and BMI ($p=0.002$). Serum ALT concentrations also showed a tendency to be higher in

Table 1 Associations between *PNPLA3* rs738409 and clinical variables

Variable	*PNPLA3* rs738409		
	CC	CG	GG
n	168	105	18
Age (years)	42±1	44±1	37±2
Sex (female)	86 (51)	63 (60)	11 (61)
Type 2 diabetes	39 (23)	33 (31)	4 (22)
BMI (kg/m^2)	30.5±0.5	30.0±0.6	32.2±1.9
Fasting plasma glucose (mmol/l)	6.4±0.2	6.5±0.2	6.6±0.7
Fasting serum insulin (pmol/l)	72±4	70±5	74±14
Fasting serum TG (mmol/l)	1.82±0.13	1.60±0.09	1.52±0.25
Fasting serum HDL-cholesterol (mmol/l)	1.35±0.03	1.41±0.04	1.27±0.09
Fasting serum LDL-cholesterol (mmol/l)	2.93±0.07	2.83±0.07	3.00±0.25
Total serum cholesterol (mmol/l)	5.05±0.08	4.94±0.08	4.82±0.28
Fasting serum ALT (U/l)	41±3	43±3	67±18
Fasting serum AST (U/l)	32±2	37±2*	48±9**,†
Liver fat (%)	9.0±0.8	10.4±1.1*	14.1±1.9**,†

Data are *n* (%) or means±SEM

† $p<0.05$ for one-way ANOVA; *$p<0.05$, **$p<0.01$ for LSD post hoc test compared with CC genotype

rs738409 G allele carriers ($p=0.068$; $p=0.056$ after adjustment for age, sex and BMI).

Insulin sensitivity There were no differences in fasting serum insulin concentrations between different *PNPLA3* genotype carriers (Table 1). The slopes and intercepts of the associations between liver fat content and directly measured hepatic insulin sensitivity also did not differ significantly between different genotype carriers (Fig. 1c). This was also true for the association between adipose tissue insulin sensitivity and liver fat content (Fig. 1d).

PNPLA3 gene expression in the liver We next analysed *PNPLA3* mRNA expression in the liver of 32 individuals. BMI was closely related to *PNPLA3* expression in the liver ($r=0.62$, $p<0.0001$). We therefore divided the participants into two groups of individuals using a cut-off of morbid obesity (BMI=40 kg/m^2). Hepatic *PNPLA3* expression was positively related to liver fat content in participants with BMI <40 kg/m^2 ($r=0.58$, $p=0.025$, $n=15$), but not in those with BMI >40 kg/m^2 ($r=-0.30$, NS, $n=17$). This relationship remained significant after adjustment for age, sex and BMI ($r=0.59$, $p=0.021$).

Discussion

Here we report that the *PNPLA3* rs738409 G allele, encoding I148M, is associated with increased liver fat content independently of obesity in Europeans. Our results are similar to the very recent findings in African-Americans, European-Americans and Hispanic individuals participating in the Dallas Heart Study [4], and with a recent report showing an association between genetic variation in the same region and liver enzyme concentrations [10]. In these, as well as in the present study, liver fat content was measured using state-of-the-art methodology, which allows precise quantification of hepatic fat content. Although liver fat content is related to features of insulin resistance [5], no relationships between the rs738409 SNP and insulin resistance were observed. The result is consistent with data from the Dallas Heart Study, where liver fat but not fasting insulin was associated with the rs738409 G allele.

We found that serum AST concentrations were significantly related to the rs738409 SNP ($p=0.002$), suggesting that this SNP indeed influences risk of non-alcoholic fatty liver disease. In the present study, serum ALT concentrations were almost significantly related to the rs738409 SNP ($p=0.056$). In the Dallas Heart Study, the rs738409 G allele was associated with increased serum ALT concentrations in Hispanics [4].

Increased liver fat content associates with increased hepatic and adipose tissue insulin resistance in both non-diabetic individuals and type 2 diabetic patients [9]. In the present study, we found that the relationships between liver fat and directly measured hepatic and adipose tissue insulin sensitivity were uninfluenced by the rs738409 genotype (Fig. 1c, d), although these data should be interpreted with caution because the number of individuals homozygous for the rare allele was low. These results suggest that both hepatic and adipose tissue insulin sensitivities decrease with increasing liver fat content independently of the *PNPLA3* rs738409 genotype. However, although the rare GG *PNPLA3* genotype does not influence insulin sensitivity, it appears to increase liver fat content.

In animals, adiponutrin is primarily expressed in white and brown adipose tissues [11]. Adiponutrin levels are low in the fasting state and increase especially after high-carbohydrate feeding [12]. In humans, expression of adiponutrin in both subcutaneous and intra-abdominal adipose tissue is positively related to obesity [7] and reduced by weight loss [13]. In the present study we found, to the best of our knowledge for the first time in humans, that expression of adiponutrin is positively correlated with obesity also in the liver. Given that adiponutrin has transacetylase activity, which catalyses TG synthesis in adipocytes [6], and is upregulated by insulin [7] and carbohydrate refeeding [13], adiponutrin could have a lipogenic function in the liver. If so, one might expect expression of the gene encoding adiponutrin to correlate positively with liver fat content. This was observed in individuals who were not morbidly obese. Because it is not ethically possible to sample normal human liver, the liver biopsies were obtained from either patients referred to a gastroenterologist because of suspected non-alcoholic fatty liver disease or from obese patients undergoing laparoscopic gastric bypass operation, which is a limitation. However, 47% (15 out of 24) of the individuals had a normal liver fat content.

In conclusion, the common variant in the *PNPLA3* gene increases risk of hepatic steatosis. It will be important to delineate the mechanisms by which these genetic variants influence the adiponutrin protein to exert these effects.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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