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THE COMMON ADIPONUTRIN VARIANT P.I148M DOES NOT CONFER GALLSTONE RISK BUT AFFECTS FASTING GLUCOSE AND TRIGLYCERIDE LEVELS

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Recently the common adiponutrin (*PNPLA3*) polymorphism p.I148M has been identified as a genetic determinant of severe forms of non-alcoholic fatty liver disease and alcoholic liver disease. Additionally, insulin resistance - linked to the development of non-alcoholic steatohepatitis - increases the risk of developing gallstones. Here we assessed whether the *PNPLA3* p.I148M (c.444 C>G) polymorphism affects glucose and lipid levels and increases gallstone risk. We analysed 229 individuals with gallstones from 108 families (age 24-80 years, BMI 17-55 kg/m^2) and 258 gallstone-free controls (age 20-70 years, BMI 14-43 kg/m^2). Fasting glucose, triglyceride and cholesterol serum levels were determined. The p.I148M polymorphism was genotyped using a PCR-based assay with 5'-nuclease and fluorescence detection. Case-control association tests and nonparametric linkage (NPL) analysis in sib-pairs were performed. Individuals carrying the [GG] genotype had significantly ($P<0.0001$) higher median fasting glucose levels as compared to [GC] and [CC] carriers. After adjustment for multiple testing, we detected a trend for an association between triglyceride levels and variant adiponutrin in gallstone patients ($P=0.032$), and gallstone cases carrying the genotype [CC] presented with significantly higher triglyceride levels than the corresponding controls ($P<0.003$). No significant effects on cholesterol metabolism were detected. Neither genotype distributions nor NPL scores provided evidence for association or linkage between the *PNPLA3* variant and gallstones. In conclusion, homozygous carriers of the *PNPLA3* risk allele display higher fasting glucose. Although this adiponutrin variant may affect triglyceride homeostasis, it does not increase the risk of cholelithiasis.

Key words: *adiponutrin, insulin resistance, metabolic syndrome, PNPLA3, single nucleotide polymorphism, triglyceride*

INTRODUCTION

Gallstones are one of the most prevalent gastrointestinal conditions affecting more than 20% of Europeans (1). As the costs of the disease in the USA are estimated to reach 6.5 billion dollars yearly, it is one of the most costly disorders as well. Interaction between environmental and genetic risk factors has for long been regarded to underlie the formation of gallstones in humans. Nevertheless, only lately the analysis of Swedish twins by Katsika *et al*. (2) provided evidence that the genetic background accounts for over 25% of the total gallstone risk. Since then genetic polymorphisms in the hepatobiliary cholesterol transporter *ABCG8* (3-5), the nuclear bile acid receptor *FXR* (6), the apical sodium-dependent bile acid transporter *SLC10A2* (7) and the Gilbert syndrome promoter variant of UDP glucuronosyltransferase 1A1 (8) have been identified as potential genetic determinants of gallstone disease. These studies point to genetic variants of hepatobiliary and intestinal transport system as causative factors in cholelithiasis. On the other hand, the previously identified polymorphisms taken together do not account for the total hereditary gallstone risk (2), hence other currently unknown variants are being sought for (1, 9, 10).

Age, obesity and parity are the major non-genetic risk factors for gallstone formation (11). Lately, this notion has been extended by the link between metabolic syndrome and gallstone disease in humans. In fact, patients with insulin resistance and diabetes mellitus are at higher risk of developing stones as compared to the general population (12, 13). Moreover, increased serum triglycerides, reduced HDL cholesterol, raised fasting plasma glucose levels, hypertension and central obesity (*i.e.* increased waist circumference), in other words the criteria of the so-called metabolic syndrome (14, 15), all represent risk factors for cholelithiasis (16). Individuals with metabolic syndrome are at risk of developing fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH) (17-19). Both these entities are known to further increase the prevalence of gallstone disease (20), and currently non-invasive markers for the detection of NAFLD and associated conditions are being evaluated (21).

A recent genome-wide association study (GWAS) has identified an increased frequency of a specific single nucleotide polymorphism (SNP) in the adiponutrin gene (*PNPLA3* p.I148M) in individuals with severe forms of NAFLD (22). Subsequent analyses also demonstrated an association of this variant with severe forms of alcoholic liver disease (ALD) (23-

25) and hepatocellular carcinoma (HCC) in patients with chronic hepatitis C virus infection (26, 27). In particular, the minor allele is associated with higher hepatic fat contents. Interestingly, this adiponutrin variant might influence insulin sensitivity in selected populations as well (28, 29). Adiponutrin is an enzyme localized in hepatic lipid droplets where it hydrolyzes emulsified triglycerides (30). As a result of the substitution of isoleucine with methionine at the amino acid position 148, patients carrying the risk variant present with increased hepatic fat contents. Lately we have shown that this phenomenon might overwhelm the detoxification capacity of hepatocytes and contribute to hepatic injury resulting in liver fibrosis and cirrhosis among patients with a wide spectrum of chronic liver diseases (31). Of note, previous ultrasound and autopsy studies have shown that gallstones are more common among cirrhotic patients as compared to the general population (32, 33).

Taking into account the previously reported association between the adiponutrin variant and non-genetic risk factors for gallstones (*e.g.* hepatic fat accumulation, liver injury, distorted glucose metabolism), we now aimed to dissect the possible role of the *PNPLA3* SNP in gallstone formation. In this respect we genotyped a cohort of sibs with gallstones and unrelated gallstone-free controls. To investigate the role of variant adiponutrin in other metabolic traits, we related *PNPLA3* genotypes to serum lipid and glucose levels.

MATERIAL AND METHODS

Patients

Only individuals with documented Caucasian ethnicity were included in the study. As shown in *Table 1* and *2*, two distinctive Romanian cohorts were analysed: a group of 258 prospectively recruited gallstone-free controls (20-70 years old, 88% females, BMI 14-43 kg/m^2) and 229 individuals with gallstones recruited from 108 families (age 24-80 years, 87% women, BMI 17-55 kg/m^2). The cohort of individuals with gallstones was composed of 223 gallstone-affected sib-pairs (ASPs) (*Table 1*) and 6 mothers of sibs also suffering from gallstones (*Table 2*). The presence of gallstones was confirmed by abdominal ultrasonography during inclusion into the study or by prior history of cholecystecomy. Individuals with neither gallstones nor gallbladder sludge as confirmed by abdominal ultrasonography were included in the control cohort.

In all study participants glucose, TG and cholesterol levels in serum (mg/dL) were determined by standard assays after an overnight fasting period.

The study was conducted according to a study design approved by the local ethical committee, and all participants signed an informed consent form.

Genotyping

Genomic DNA was isolated from EDTA anticoagulated blood according to the membrane-based QIAamp DNA extraction protocol (Qiagen, Hilden, Germany). The *PNPLA3* coding SNP p.I148M (*rs738409*) was genotyped using solution-phase hybridization reactions with 5'-nuclease and fluorescence detection (*TaqMan* assays) in a 7300 real-time PCR system (Applera, Norwalk, CT). Primer and probe sequences were: forward primer 5'-AACTTCTCTCTCCTTTGCTTTCACA-3'; reverse primer 5'-TCTACAGTGGCCTTATCCCTCC-3'; VIC 5'-TTCCTGCTTCATGCC-3'; FAM 5'-CCTGCTTCATCCC-3'. To ensure genotyping quality, we included negative controls and DNA samples with known *PNPLA3* genotypes as internal controls. PCR reactions contained 20 ng DNA, 900 nM of each primer, 1× *TaqMan* Universal Master Mix, and 200 nM of VIC-labelled and FAM-labelled probes in 25 µL-reactions. Amplification conditions were 95°C for 10 min, 40 cycles of 92°C for 15 s, and 60°C for 1 min.

Statistics

Unless stated otherwise, statistical analysis was performed with SPSS 18.0 (SPSS, Munich, Germany). All phenotypic quantitative data were expressed as medians and ranges, unless stated specifically. Because we performed multiple tests (n=17), the significance threshold was corrected for multiple testing and two-sided P values <0.003 were considered as significant.

The effects of the adiponutrin SNP and of other potential lithogenic factors (age, BMI, gender, serum glucose and lipid levels) (10, 11, 34) on the development of gallstones were estimated by logistic regression analysis. Kolmogorov-Smirnof's test was used to determine whether data sets had a normal distribution. The comparison of age, BMI and metabolic traits between controls and ASPs was performed with Student's t-test or Mann-Whitney rank sum test as appropriate, whereas one-way analysis of variance (ANOVA) or the Kruskall-Wallis nonparametric ANOVA on ranks were used to assess these traits between carriers of different variants of the adiponutrin polymorphism.

Exact tests were performed to check the consistency of genotyping results with Hardy-Weinberg equilibrium (HWE) (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). We performed power calculations using PS: Power and Sample Size Calculation v.3.0 (http://biostat. mc.vanderbilt.edu/wiki/Main/PowerSampleSize) (35). Association case-control analysis and non-parametric linkage (NPL) tests were performed to investigate the role of the *PNPLA3* p.I148M variant in the development of gallstones. For the association analysis, all gallstone-free controls and a single randomly selected member (these individuals are denoted cases throughout this report) of each sib-pair family were included. The association between the adiponutrin variant and cholelithiasis was tested in contingency tables (genotypes: Armitrage's trend test; alleles: chi^2 test). NPL scores were calculated using GENEHUNTER-MODSCORE v2.0.1 (www.staff.uni-marburg.de/_strauch/software.html) (36) both for the risk (minor) allele frequencies (MAF) in our ASP cohort and for alleles frequencies provided in the *Entrez* SNP database (http://www.ncbi.nlm.nih.gov/snp). In short, the NPL score allows estimation of the significance of a given allele shared among the family members in the development of the disease; for this the allele frequencies at the analysed genetic locus are compared with the null hypothesis of no linkage (4). Thus, if gallstone disease is linked to the *PNPLA3* polymorphism, affected sibs are more likely to share the same allele.

RESULTS

Obesity enhances gallstone risk

Table 1 shows that controls were significantly younger and leaner than ASPs (both P<0.003). As shown in *Table 3*, the analysis of known lithogenic risk factors (see Methods) by univariate regression analysis provided significant results only for BMI (OR=1.11; 95% CI=1.06-1.16; P<0.001) and fasting glucose levels (OR=1.02, 95% CI=1.01-1.02; P=0.001), whereas total serum cholesterol levels were slightly lower in gallstone carriers (P=0.002). The inclusion of these factors in a multivariate analysis (*Table 3B*) demonstrated that in our cohort only BMI (OR=1.10; 95% CI=1.05-1.16; P<0.001) represented a strong risk factor for gallstones, whereas higher total

Table 1. Clinical characteristics of gallstone-free controls, gallstone-affected sib-pairs (ASP) and cases. ASPs - all individuals with gallstones included in the study. From each pair of sibs one individual (denoted 'case') was chosen for the case-control analysis. Age was calculated at the date of inclusion in the study. All values are given as medians and ranges. * P<0.003 *vs*. gallstone-free controls. *Abbreviations:* ASPs, affected sib-pairs; BMI, body mass index, HDL, high density lipoproteins; LDL, low density lipoproteins.

		Affected	
	Controls	**ASPs**	**Cases**
Total (n)	258	223	108
Gender (n)			
Female	227 (88%)	193 (87%)	94 (87%)
Male	31 (12%)	30 (13%)	14 (13%)
Age (years)	50 (20–70)	56 (24–80)*	56 (24–80)
BMI (kg/m^2)	25 (14–43)	28 (17–55)*	28 (18–55)*
Glucose (mg/dl)	91 (57–279)	98 (56–361)*	98 (71–361)*
Triglycerides (mg/dl)	122 (33–662)	137 (29–604)*	136 (47–485)
Total cholesterol (mg/dl)	221 (122–456)	207 (109–369)*	205 (109–349)*
HDL cholesterol (mg/dl)	54 (28–99)	50 (16–86)	50 (16–86)
LDL cholesterol (mg/dl)	171 (72–395)	153 (53–347)	151 (53–294)

Table 2. Summary of gallstone-affected sib-pairs.
Abbreviation: ASPs; affected sib-pairs.

Affected siblings per pedigree (n)	**Pedigrees (n)**	**Independent ASPs (n) per pedigree**	**Pedigrees (n) with available data for mothers**	**Total number of affected individuals**
2	99	1	5	203
3	7	2	1	22
4	1	3	0	4
	108			229

Table 3. Risk factors for developing gallstones.
Abbreviations: BMI, body mass index; CI, confidence interval; I, isoleucine; HDL, high density lipoproteins; LDL, low density lipoproteins; M, methionine; OR, odds ratio; p, protein (amino acid number); *PNPLA3*, adiponutrin.

(A) Univariate analysis			
Factor	**OR**	**95% CI**	**P**
***PNPLA3* p.I148M**	0.945	0.669–1.335	0.748
Age	1.030	1.007–1.053	0.009
BMI	1.109	1.061–1.159	<0.001
Gender	0.534	0.257–1.108	0.092
Glucose	1.015	1.006–1.023	0.001
Triglycerides	1.004	1.001–1.007	0.016
Total cholesterol	0.992	0.986–0.997	0.002
LDL cholesterol	0.992	0.987–0.998	0.008
HDL cholesterol	0.986	0.969–1.003	0.098
(B) Multivariate analysis			
Factor	**OR**	**95% CI**	**P**
BMI	1.104	1.052–1.158	<0.001
Serum glucose	1.012	1.003–1.021	0.012
Serum total cholesterol	0.990	0.984–0.995	<0.001

cholesterol levels were protective against cholelithiasis (P<0.001; *Table 3*). On the other hand the regression analysis demonstrated that neither gender nor the adiponutrin polymorphism increased stone risk in our cohort significantly (all P>0.003).

PNPLA3 p.I148M variant and gallstone risk: case-control association and sib-pairs analyses

The adiponutrin p.I148M variant was successfully genotyped in all individuals with gallstones (n=229, *Table 2*) and controls (n=258). The genotyping frequencies (*Table 4*) were in line with

Table 4. Allele and genotype distributions of the adiponutrin *rs738409* SNP in cases and controls and association tests. Results were calculated using contingency table statistics. *Abbreviations:* CI, confidence interval; HCV, hepatitis C virus; I, isoleucine; M, methionine; OR, odds ratio; p, protein (amino acid number); *PNPLA3*, adiponutrin.

***PNPLA3* p.I148M allele / genotype**	**Counts of alleles/genotypes**	
	Controls (2N=516)	**Cases (2N=216)**
C	376 (0.73)	160 (0.74)
G	140 (0.27)	56 (0.26)
CC	141 (0.55)	62 (0.58)
GC	94 (0.36)	36 (0.33)
GG	23 (0.09)	10 (0.09)
Association tests	**χ^2**	**P**
Allele frequency difference test	0.11	0.73
Armitrage's trend test	0.10	0.75
OR statistics	**OR**	**95% CI**
[G] ↔ [C]	0.871	0.535–1.417
[GG] ↔ [GC]	0.871	0.535–1.417
[GG] ↔ [GC + CC]	1.043	0.478–1.043
[GC + GG] ↔ [CC]	0.894	0.568–1.407

Table 5. Non-parametric linkage analysis. NPL scores were calculated according to the frequencies in our cohort or the Entrez SNP database, using GENEHUNTER-MODSCORE v2.0.1 (www.staff.uni-marburg.de/_strauch/ software.html) (36). [G] - risk allele. *Abbreviations:* ASPs, affected sib-pairs; LOD, logarithm of the odds ratio; MAF, minor allele frequency; NPL, non-parametric linkage score; SNP, single nucleotide polymorphism.

MAF source	**[G] allele frequency**	**NPL**	**LOD**	**P**
Study cohort	0.26	0.613	-1.232	0.319
Entrez SNP database	0.23	0.534	-0.969	0.295

Table 6. Plasma lipid levels in relation to the adiponutrin variant in cases and controls; Cases - unrelated patients with gallstones confirmed at abdominal ultrasonography or by positive history of cholecystecomy. Controls - individuals with gallstones and gallbladder sludge excluded at abdominal ultrasonography. Results are given as medians and ranges. *Values differ significantly (P<0.003) different between cases and controls. *Abbreviations:* ANOVA, analysis of variance; HDL, high density lipoprotein; LDL, low desity lipoprotein; PNPLA3, adiponutrin.

(A) Cases

***PNPLA3* genotype**	**Triglycerides (mg/dl)**	**Total cholesterol (mg/dl)**	**HDL cholesterol (mg/dl)**	**LDL cholesterol (mg/dl)**
[CC]	155 (60–485)*	212 (119–349)	55 (16–84)	154 (66–266)
[CG]	122 (47–257)	198 (109–308)	50 (16–86)	137 (53–231)
[GG]	105 (50–240)	199 (151–253)	54 (33–86)	146 (126–294)
ANOVA P	0.032	>0.05	>0.05	>0.05

(B) Controls

***PNPLA3* genotype**	**Triglycerides (mg/dl)**	**Total cholesterol (mg/dl)**	**HDL cholesterol (mg/dl)**	**LDL cholesterol (mg/dl)**
[CC]	112 (33–499)*	226 (131–359)	55(28–99)	172(78–308)
[CG]	136 (52–334)	218 (122–456)	52(32–78)	164 (72–395)
[GG]	126 (64–237)	223 (138–293)	52(33–91)	174 (98–256)
ANOVA P	>0.05	>0.05	>0.05	>0.05

the frequencies reported in the *Entrez* SNP database as well as previous studies (22, 23). Both allele and genotypes frequencies did not deviate from HWE (P>0.05), which indicates robust genotyping.

Nevertheless, the association tests showed lack of evidence for the involvement of the *PNPLA3* SNP in gallstone formation (P>0.05). However, this analysis is underpowered (power=0.47 after correction for multiple testing), as choosing only one sib from each family reduces number of cases. Hence we subsequently analyzed sib-pairs from the 108 distinctive families (*Table 2*). In line with the association analysis (*Table 4*), the NPL scores (*Table 5*) did not support a causative role of the

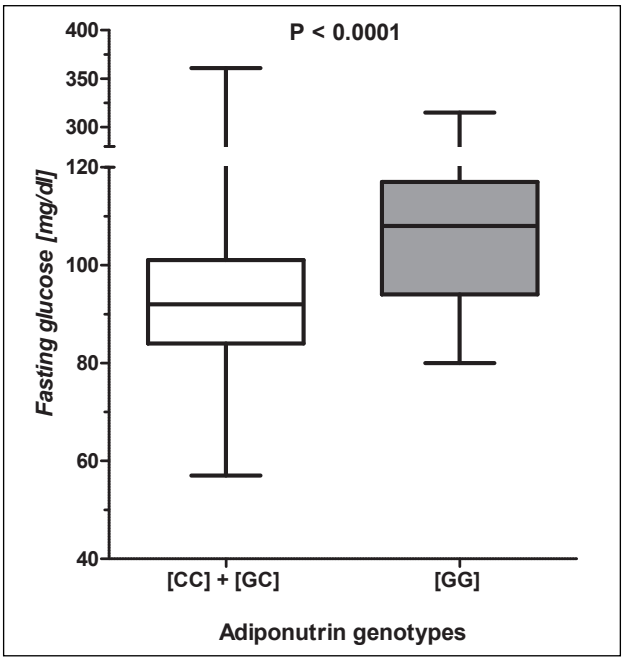

Fig. 1. Box-and-Whisker plots illustrating median glucose levels stratified according to adiponutrin genotypes. Serum glucose levels differ significantly (P<0.0001) between carriers of distinct *PNPLA3* variants. In particular, patients carrying the [GG] genotype (N=33) display increased glucose levels as compared to individuals with genotypes [GC] and [GG] (N=333).

PNPLA3 polymorphism in gallstone formation (P>0.05). Calculation of NPL scores with respect to other minor allele frequencies reported in the *Entrez* SNP database did not reveal an association of the adiponutrin polymorphism with cholelithiasis either (P>0.05).

Association between variant adiponutrin and metabolic traits

For this analysis we included only unrelated cases (n=108) and all controls (n=258). *Fig. 1* shows that the homozygous carriers of the *PNPLA3* risk allele [G] demonstrated significantly (P<0.0001) higher median fasting glucose levels (108 mg/dl, range 80-315 mg/dl) as compared to individuals with genotypes [GC] and [CC] (92 mg/dl, range 57-361 mg/dl). *Table 6* summarizes serum triglyceride and cholesterol levels. In gallstone patients, we observed a trend (P=0.03) for an association between triglyceride levels and the *PNPLA3* variant ([CC] 155, [CG] 122, [GG] 105 mg/dl). Cases carrying the genotype [CC] had significantly P<0.003 higher TG levels as compared to [CC] controls (155 *vs*. 112 mg/dl; *Table 6*). On the other hand, regression analysis did not provide evidence for a significant association between triglyceride levels and adiponutrin genotype in the entire cohort without stratification for gallstone disease (all P>0.003). After correction for multiple testing, we did not detect any association between the *PNPLA3* polymorphism and cholesterol levels either (*Table 6*). Of note, although controls were younger than ASPs (see *Table 1*), we did not detect any significant effect of age on the tested metabolic traits.

DISCUSSION

This study demonstrates that the adiponutrin p.I148M variant influences glucose and triglyceride levels in our study population. On the other hand, although it has been previously shown that fatty liver disease and enhanced liver fibrosis are both risk factors for cholelithiasis, the variant does not increase gallstone risk *per se*. Since our case-control study investigating the effect of the adiponutrin variant on gallstone formation is underpowered, we also performed a non-parametric linkage analysis. Indeed, the study cohort let us previously identify the *ABCG8* p.D19H variant as the first genetic risk factor for gallstone formation in humans (4). In this study the analysis of sib-pairs showed that this variant was strongly associated with cholelithiasis (NPL score =7.1, P=4.6 x 10^{-13}), which was in line with results of a large GWAS in gallstone patients (3). Hence, the cohort of sib-pairs can be regarded as robust framework for identifying genes associated with gallstone formation. Additionally, sib-pair analysis omits the bias that is encountered in case-control analysis as controls could develop gallbladder stones later in life. Hence, the present analysis of sib-pairs, which did not reveal an association between gallstones and the *PNPLA3* variant p.I148M, excludes this SNP as a major risk factor for cholelithiasis.

BMI and serum glucose levels are known risk factors for gallstone formation (11). Our results show that each of these factors increases the disease risk, which is in line with the notion that cholelithiasis is a complex multifactorial disorder. Interestingly, we observed that increased serum cholesterol levels lowered the chance of developing gallstones. In contrast, previous studies have demonstrated that patients carrying the *ABCG8* (35) and *SLC10A2* (7) cholelithiasis risk variants present with lower total serum cholesterol concentrations. It can be hypothesised that the lower risk of developing gallstones in patients with increased serum cholesterol levels might be primarily due to decreased transport of cholesterol into bile. This might lead to increased serum cholesterol but lower biliary cholesterol concentrations. On the other hand, the use of cholesterol lowering drugs (*e.g.* statins) may significantly lower the risk of developing gallstones (37-39). Hence a functional link between cholesterol levels, hepatobiliary transporters and gallstone formation has not yet been thoroughly investigated and future studies are warranted.

It has been previously shown that the *PNPLA3* risk allele is associated with severe forms of hepatic fat accumulation (22, 23, 29, 31). However, the results concerning the effect of the *PNPLA3* polymorphism on glucose and lipid metabolism remain controversial. Kantartzis *et al*. (29) have previously analysed a cohort of 220 individuals for association between the p.I148M SNP and insulin sensitivity as well as serum glucose and lipid levels. Interestingly, in this study variant adiponutrin did neither affect insulin sensitivity nor lipid or glucose levels. On the other hand, Johansson *et al*. (28) demonstrated that carriers of the common allele are more insulin-resistant at lower BMI, whereas carriers of the risk allele display decreased insulin secretion after oral glucose challenge. Notwithstanding, these findings have not been replicated in larger cohorts. In our cohort we were not able to obtain data on fasting insulin levels, but we observed differences in fasting glucose levels in patients with distinct genotypes (*Fig. 1*), supporting a potential role of adiponutrin in glucose metabolism. Of note, none of the studied individuals was under glucose lowering therapy at inclusion. In this setting, the risk allele carriers, to date known to be prone to fatty liver disease (22), may also be at risk of increased fasting glucose serum levels. On the other hand, previous publications and a meta-analysis did not find any association between the *PNPLA3* variant and indices of insulin sensitivity such as fasting glucose and insulin concentrations, or HOMA index (22, 29, 40-44), thus association between the adiponutrin variant and glucose levels may be restricted to selected populations. With respect to lipid homoeostasis, a previous analysis of eight large cohorts by

Kollerits *et al*. (45) revealed an association between variant adiponutrin and total cholesterol as well as LDL cholesterol concentrations (45). In contrast to our results, Kollerits *et al*. (45) and Speliotes *et al*. (42) did not observe an association between the SNP and triglyceride levels. Higher triglyceride levels in [CC] homozygotes with gallstones in our cohort are consistent with the putative role of adiponutrin in lipid metabolism in the liver. Indeed, He *et al*. (30) showed that the amino acid substitution of isoleucine for methionine abolishes the lipid emulsifying function of recombinant adiponutrin *in vitro*. Hence, the decrease of serum lipid levels in carriers of the *PNPLA3* risk allele might represent an adaptive response of the lipid metabolism. On the other hand, we did not detect an effect of adiponutrin variant on triglyceride levels in the whole cohort, indicating that the effects might dependon specific phenotypes, *e.g.* gallstone disease.

In summary, our current study underscores the possible metabolic role of the adiponutrin p.I148M polymorphism. Nevertheless, given the negative results from the previous large studies this effect might be apparent only in selected individuals, for example in those who have gallstones as an additional phenotype. Although the variant does not increase the risk of developing gallstones *per se*, additional functional studies are warranted to define the molecular link between adiponutrin and metabolic traits.

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