Genetic variation in the ADIPOR2 gene is associated with liver fat content and its surrogate markers in three independent cohorts

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Abstract

Aims: We investigated whether polymorphisms in candidate genes involved in lipid metabolism and type 2 diabetes are related to liver fat content.

Methods: Liver fat content was measured using proton magnetic resonance spectroscopy (1H-MRS) in 302 Finns, in whom single nucleotide polymorphisms (SNPs) in acyl-CoA synthetase long-chain family member 4 (ACSL4), adiponectin receptors 1 and 2 (ADIPOR1 and ADIPOR2), and the three peroxisome proliferator-activated receptors (PPARA, PPARD, and PPARG) were analyzed. To validate our findings, SNPs significantly associated with liver fat content were studied in two independent cohorts and related to surrogate markers of liver fat content.

Results: In the Finnish subjects, polymorphisms in ACSL4 (rs7887981), ADIPOR2 (rs767870), and PPARG (rs3856806) were significantly associated with liver fat content measured with 1H-MRS after adjusting for age, gender, and BMI. Anthropometric and circulating parameters were comparable between genotypes. In the first validation cohort of ~ 600 Swedish men, ACSL4 rs7887981 was related to fasting insulin and triglyceride concentrations, and ADIPOR2 rs767870 to serum γ glutamyltransferase concentrations after adjusting for BMI. The SNP in PPARG (rs3856806) was not significantly associated with any relevant metabolic parameter in this cohort. In the second validation cohort of ~ 3000 subjects from Western Finland, ADIPOR2 rs767870, but not ACSL4 rs7887981 was related to fasting triglyceride concentrations.

Conclusions: Genetic variation, particularly in the ADIPOR2 gene, contributes to variation in hepatic fat accumulation in humans.

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Introduction

Accumulation of fat in the liver impairs the ability of insulin to inhibit the production of both glucose (1,2) and VLDL (3,4), which leads to hyperglycemia (1,2), hypertriglyceridemia, hyperinsulinemia, and a low HDL-cholesterol concentration (5). These data would explain why fat accumulation in the liver characterizes those subjects who develop the insulin resistance syndrome independent of obesity (5). Regarding factors that regulate liver fat, studies addressing weight loss (6), and those performed in monozygous twins discordant for obesity (7) have shown that acquired obesity is one such factor. On the other hand, the correlation between body mass index (BMI) or waist circumference and liver fat is weak (5) and sometimes non-existent (8), implying that hepatic fat accumulation is regulated by other factors as well.

Little is known about whether and which genetic factors influence liver fat content. This lack of information may be due in part to an absence of simple tools for reliable measurement of liver fat content. Fasting insulin concentration has been proposed as the best marker of hepatic fat content.
(5), but circulating concentrations of liver enzymes such as ALT and gamma glutamyltransferase (γGT) are also used (9). However, these are indirect measurements and inevitably provide only an approximation of the actual liver fat content. To date, only a limited number of studies, the majority with a rather small number of study subjects, have investigated relationships between specific SNPs and liver fat content (10–14). In the present study, we investigated, whether polymorphisms in selected candidate genes involved in lipid metabolism and type 2 diabetes are related to hepatic fat content measured by proton magnetic resonance spectroscopy in 302 Finns. As an initial effort to identify genes associated with hepatic fat accumulation, we chose 15 single nucleotide polymorphisms (SNPs) in six candidate genes that have been demonstrated to play important roles in relevant metabolic pathways.

For adiponectin receptors 1 and 2 (ADIPOR1 and ADIPOR2) and the three peroxisome proliferator-activated receptors (α, δ and γ: PPARA, PPARD, and PPARG), we selected SNPs that have been previously associated with relevant phenotypes. ADIPOR1 and ADIPOR2 play central roles in mediating the insulin-sensitizing effects of the adipose tissue-derived hormone adiponectin. Since adiponectin suppresses hepatic gluconeogenesis and stimulates fatty acid oxidation in the liver, this suggests that the expression and function of ADIPOR1 and ADIPOR2 may influence hepatic lipid accumulation. Polymorphisms in both genes have been associated with insulin resistance and type 2 diabetes (11,15). The PPARs are ligand-binding nuclear factors, activated by fatty acids, regulating genes involved in lipid and glucose metabolism. PPAR activation results in important lipid-lowering effects in vivo, thus restricting the progress of lipid-induced insulin resistance. Genetic studies have revealed associations between polymorphisms in these genes and diabetes-related traits (16–18).

The rationale for performing genetic studies of acyl-CoA synthetase long-chain family member 4 (ACSL4) was based on previous expression studies showing hepatic ACSL4 mRNA expression levels to increase with increasing liver fat content (19), combined with the fact that no studies have previously investigated genetic variation within ACSL4 in relation to liver fat content or related phenotypes.

In order to validate our findings from the discovery cohort with quantitative hepatic fat determination, SNPs significantly associated with liver fat content were investigated in a larger surrogate replication cohort, comprising ~ 600 healthy individuals, to investigate their relationships to phenotypes of relevance (surrogate markers) for fatty liver. SNPs that remained significantly associated with surrogate markers of liver steatosis were then genotyped in a large population-based cohort consisting of ~ 3000 individuals in order to further confirm the results.

**Methods**

**Subjects**

A total of 302 Finnish subjects were recruited for metabolic studies using the following inclusion criteria: i) age between 20 and 75 years; ii) no known acute or chronic disease based on history and physical examination and standard laboratory tests (blood counts, serum creatinine, TSH, and electrolyte concentrations) and ECG; iii) alcohol consumption less than 20 g per day. Elevated liver enzymes (serum alanine aminotransferase (ALT), and aspartate aminotransferase (AST)) were not exclusion criteria. However, subjects with evidence of hepatitis B or C, autoimmune hepatitis, clinical signs or symptoms of inborn errors of metabolism or a history of the use of toxins or drugs associated with liver steatosis were excluded. A total of 15% of the subjects were receiving medications for dyslipidemia (statins), and 22% were receiving medications for hypertension (ACE-inhibitors or Ca-channel blockers). A total of 83 subjects had type 2 diabetes. Data on the non-diabetic subjects (5) and the type 2 diabetic patients (20) have previously been reported. All protocols were approved by the ethics committee of the Helsinki University Central Hospital, and each subject provided written informed consent for the study of polymorphisms of genes regulating liver fat.

The first surrogate replication cohort consisted of a total of 619 50-year old men living in the county of Stockholm who were selected at random from a registry of permanent residents (21). Exclusion criteria were non-Caucasian descent, chronic disease, history of cardiovascular disease, familial hypercholesterolemia, alcohol abuse, psychiatric disorders, and participation in other ongoing studies. Studies were approved by the Karolinska Hospital local ethics committee and all subjects gave their informed consent.

The second surrogate replication cohort was the Botnia – Prevalence, Prediction, and Prevention of Diabetes Study (PPP-Botnia), a population-based study from the Botnia region of Western Finland. The present study was initiated in 2004 in a population comprising around 135 000 individuals. Using a population registry, a random sample of subjects aged between 18 and 75 years was selected. Altogether 6075 individuals were invited to participate in the study and 3621 took part. Of these individuals, 3394 have data and DNA available for the present study. Individuals on lipid lowering medication (n=362) were excluded from the analysis, due to their influence on the parameters of interest, leaving a total of 3050 subjects. All protocols were approved by the ethics committee of the Helsinki University Central Hospital, and all subjects gave their informed consent.

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Liver fat content

Liver fat content was measured by proton magnetic resonance spectroscopy as previously described (2). This measurement has been validated against histologically determined lipid content (22, 23), and against estimates of fatty degeneration or infiltration by X-ray computer-assisted tomography (2). All spectra were analyzed by a physicist who was unaware of the clinical data. The reproducibility of repeated measurements of liver fat in non-diabetic subjects studied on two occasions in our laboratory is 11% (24).

Selection of polymorphisms

To identify genes associated with hepatic fat accumulation, we selected the following 15 SNPs in six candidate genes, based on previous reports (Table 1): ADIPOR1 (rs6666089); ADIPOR2 (rs929434, rs767870); PPARA (rs135539, rs1800206, rs4253778); PPARD (rs6902123, rs2016520, rs2076167); and PPARG (rs1801282, rs10865710, rs3856806). No polymorphisms in ACSL4 have previously been analyzed in relation to metabolic parameters. Therefore, we used the HapMap data (www.hapmap.org) and the HaploView programme (minimum minor allele frequency of 0.1, $r^2$ threshold = 0.8) to choose SNPs covering the genetic variability of the whole ACSL4 gene including 5 kb of the 5’ and 3’ regions. The tagSNPs selected were rs1324805, rs5985403, and rs7887981. According to the HapMap data, these SNPs cover more than 95% of the genetic variability in this locus.

Genotyping

Genomic DNA was extracted from whole blood (17,25). Approximately, 20 ng DNA were used for genotyping with the TaqMan PCR method (Applied Biosystems) according to manufacturer’s instructions. Post-PCR allelic discrimination was carried out measuring allele-specific fluorescence on the ABI Prism Sequence Detection System (Applied Biosystems). The success rate for genotyping was >95% in all cases and therefore there is variation in the number of samples for which genotyping data are available.

Analytical procedures and other measurements

All blood samples were taken after an overnight fast. The analyses of anthropometric measurements and circulating parameters of the Finnish subjects were analyzed as previously described (5). In Swedish men, blood samples were analyzed as previously described (21). Serum γGT was measured in the routine Clinical Chemistry laboratory at Karolinska Hospital. In the PPP-Botnia cohort, serum ALT concentrations were measured using modified IFCC (26). Occasional data points for some parameters are unavailable. Glucose tolerance was classified according to the WHO 1999 criteria (27).

Statistical analyses

Non-normally distributed data were used after logarithmic (base 10) transformation. If distributed normally, data are shown as mean ± S.E.M., whereas non-normally distributed data are shown as median (25% percentile, 75% percentile). Hardy–Weinberg equilibrium was assessed using χ² test. ACSL4 is located on the X chromosome and therefore women with a heterozygous genotype were excluded from the analyses in Finnish subjects. An unpaired Student’s t-test was used to compare mean values between two groups. One-way ANOVA was used to test the effects of genotypes on different parameters. The LSD test was used for post hoc analysis. Analysis of covariance was used to adjust liver fat and its surrogate markers for age, gender, BMI, use of lipid lowering medication, and glucose tolerance status.

Calculations were made using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA), SysStatStatistical Package (SysStat version 10; SysStat, Evanston,IL, USA), and SPSS 14.0 for Windows (SPSS, Chicago, IL, USA). The StatView (SAS Institute Inc., Cary, NC, USA) software was used for the statistical analysis of the Swedish data. A P-value of less than 0.05 was considered statistically significant.

Results

Association of candidate polymorphisms with liver fat content

The discovery cohort of Finnish subjects, in which liver fat content was determined using quantitative ¹H-MRS spectroscopy, consisted of 160 females and 142 males, age 43 ± 1 and 43 ± 1 years (P = 0.60), BMI 31.7 ± 0.5 and 28.9 ± 0.5 kg/m² (P < 0.0001), and liver fat content 5.0% (2.0–12.0%) and 6.0% (2.0–17.5%, P = 0.26) respectively. All polymorphisms were in Hardy–Weinberg equilibrium. Of the six genes analyzed, polymorphisms in ACSL4, ADIPOR2, and PPARG were significantly associated with liver fat content after adjusting for age, gender, and BMI, while there was no association for any of the SNPs in ADIPOR1, PPARA or PPARD (Table 1).

Subjects carrying only the A allele in intron 15 of ACSL4 (rs7887981) had significantly higher liver fat content adjusted for age, gender, and BMI than the subjects carrying only the G allele (P = 0.024) (Table 1, Fig. 1A). The groups were comparable with respect to anthropometric measurements and circulating parameters (Supplementary Table 1, which can be viewed online at http://www.eje-online.org/supplemental/).
Table 1  Associations between selected single nucleotide polymorphisms and liver fat content in the Finnish discovery cohort.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP rs-number</th>
<th>Genotype frequencies</th>
<th>LFAT (%)</th>
<th>P</th>
<th>P adjusted for BMI, age, and gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSL4</td>
<td>Intron 1 A&gt;G</td>
<td>A(A): 186 G(G): 49</td>
<td>6.0 (2.0–16.0) 7.0 (2.0–18.5)</td>
<td>1.00</td>
<td>0.59*</td>
</tr>
<tr>
<td></td>
<td>Intron 1 C&gt;G</td>
<td>C(C): 119 G(G): 99</td>
<td>5.0 (1.7–17.0) 5.5 (2.0–16.0)</td>
<td>0.66</td>
<td>0.17*</td>
</tr>
<tr>
<td></td>
<td>Intron 15 G&gt;A</td>
<td>G(G): 160 A(A): 68</td>
<td>5.0 (1.7–16.2) 6.5 (2.5–17.0)</td>
<td>0.21</td>
<td>0.024*</td>
</tr>
<tr>
<td>ADIPOR1</td>
<td>−8503 G&gt;A</td>
<td>GG: 161 GA: 115 AA: 18</td>
<td>6.0 (2.0–17.0) 5.0 (2.0–13.0) 6.7 (2.0–19.5)</td>
<td>0.55</td>
<td>0.53</td>
</tr>
<tr>
<td>ADIPOR2</td>
<td>Intron 1 C&gt;T</td>
<td>CC: 232 CT: 61 TT: 4</td>
<td>6.0 (2.0–15.3) 5.2 (1.4–18.0) 16.5 (11.1–27.8)</td>
<td>0.15</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Intron 5 T&gt;C</td>
<td>TT: 114 TC: 133 CC: 40</td>
<td>5.7 (2.0–16.4) 7.0 (2.25–17.0) 3.0 (1.55–10.0)</td>
<td>0.08†</td>
<td>0.024‡</td>
</tr>
<tr>
<td>PPARA</td>
<td>Intron 2 T&gt;G</td>
<td>TT: 85 TG: 138 GG: 69</td>
<td>6.0 (2.0–14.3) 5.0 (2.0–14.5) 6.0 (2.0–19.0)</td>
<td>0.95</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>L126V (C&gt;G)</td>
<td>CC: 278 CG: 21 GG: 2</td>
<td>6.0 (2.0–16.6) 2.0 (1.3–6.0) 6.5; 7.9</td>
<td>0.13</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Intron 7 C&gt;G</td>
<td>GG: 234 GC: 56 CC: 9</td>
<td>6.0 (2.0–16.2) 5.0 (1.6–12.5) 6.3 (2.0–12.3)</td>
<td>0.60</td>
<td>0.38</td>
</tr>
<tr>
<td>PPARD</td>
<td>Exon 4 294T&gt;C</td>
<td>TT: 235 TC: 61 CC: 5</td>
<td>5.0 (2.0–14.0) 7.0 (1.8–21.0) 9.0 (4.0–22.5)</td>
<td>0.24</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Exon 7 798T&gt;C</td>
<td>TT: 236 TC: 61 CC: 2</td>
<td>5.0 (2.0–14.5) 7.0 (1.5–20.0) 2.0; 9.0</td>
<td>0.49</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Intron 2 T&gt;C</td>
<td>TT: 287 TC: 13 CC: 0</td>
<td>6.9 (2.0–16.0) 3.5 (1.0–14.3)</td>
<td>0.46</td>
<td>0.23</td>
</tr>
<tr>
<td>PPARG</td>
<td>Pro12Ala C&gt;G</td>
<td>CC: 198 CG: 98 GG: 12</td>
<td>5.4 (2.0–16.0) 6.0 (2.0–15.0) 6.5 (1.3–16.3)</td>
<td>0.88</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Intron 1 C&gt;G</td>
<td>CC: 133 CG: 137 GG: 30</td>
<td>6.0 (2.0–15.6) 5.5 (2.0–16.3) 5.3 (2.0–16.0)</td>
<td>0.99</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>His449His C&gt;T</td>
<td>CC: 187 CT: 99 TT: 15</td>
<td>6.0 (2.0–16.0) 5.0 (2.0–16.0) 3.0 (1.0–11.0)</td>
<td>0.32§</td>
<td>0.033‖</td>
</tr>
</tbody>
</table>

Median (IQR) with statistical analysis performed on log-transformed data. *For analysis of SNPs in ACSL4, women heterozygous for each SNP were excluded from analysis. Therefore, LFAT is only adjusted for age and BMI; †Post hoc analyses: P=0.47 for TT versus TC, P=0.026 for TC versus CC, P=0.09 for TT versus CC; ‡Post hoc analyses: P=0.29 for TT versus TC, P=0.006 for TC versus CC, P=0.050 for TT versus CC; §Post hoc analyses: P=0.69 for CC versus CT, P=0.20 for CT versus TT, P=0.13 for CC versus TT; ‖Post hoc analyses: P=0.27 for CC versus CT, P=0.051 for CT versus TT, P=0.021 for CC versus TT.

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Subjects homozygous for the C allele of the ADIPOR2 intron five polymorphism (rs767870) had 50% less liver fat than subjects with either TT or TC genotypes (CC versus TT + TC, 3.0% (1.6–10.0%) versus 6.0% (2.0–17.0%), P=0.025). Differences in liver fat content between subjects with different genotypes became more prominent after adjusting for age, gender, and BMI (P=0.024 for ANOVA, P=0.012 for CC versus TT + TC) (Table 1, Fig. 1B). Anthropometric and circulating parameters were comparable between genotypes (Supplementary Table 1).

There was a significant association of the PPARG exon 7 His449His C>T polymorphism (rs3856806) with liver fat content adjusted for age, gender, and BMI (Table 1, Fig. 1C). Subjects homozygous for the T allele had significantly less liver fat than individuals heterozygous or homozygous for the C allele (P=0.017, TT versus CT + CC). The genotype groups were comparable with respect to anthropometric measurements and circulating parameters (Supplementary Table 1).

Associations between genotype and liver fat content remained essentially unchanged after additional adjustment for the use of lipid-lowering medication (P values of 0.051, 0.031, and 0.032 for ACSL4 rs7887981, ADIPOR2 rs767870, and PPARG rs3856806 respectively).

Confirmation of associations with surrogate markers of liver fat content in independent cohorts

In order to validate our findings in independent cohorts, we first genotyped the three SNPs that were significantly associated with liver fat content in the discovery cohort (rs7887981, rs767870 and rs3856806) in a cohort of 50-year old Swedish men. Analysis of ACSL4 rs7887981 revealed an interaction between genotype and BMI in relation to the variables analyzed. Therefore, subjects were categorized either above or below median BMI (25.7 kg/m²). In the more obese subjects (BMI ≥ 25.7 kg/m²), the rare A allele (associated with increased hepatic fat content) was associated with higher insulin (P=0.049) and TG (P=0.022) concentrations, but no such association was seen in the leaner subjects (BMI < 25.7 kg/m²) (Table 2). Concentrations

Table 2 Associations between ACSL4 rs7887981 and surrogate markers of liver fat content in the Swedish cohort.

<table>
<thead>
<tr>
<th>ACSL4 rs7887981</th>
<th>BMI &lt;25.74 kg/m²</th>
<th>BMI ≥25.74 kg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>209</td>
<td>99</td>
</tr>
<tr>
<td>23.5±0.1</td>
<td>23.7±0.1</td>
<td>0.23</td>
</tr>
<tr>
<td>f-Insulin (pmol/l)</td>
<td>31.3 (24.3–42.1)</td>
<td>30.4 (23.4–40.5)</td>
</tr>
<tr>
<td>f-yGT (ukat/l)</td>
<td>0.4 (0.4–0.6)</td>
<td>0.5 (0.4–0.7)</td>
</tr>
<tr>
<td>f-TG (mmol/l)</td>
<td>1.03 (0.79–1.48)</td>
<td>1.09 (0.78–1.63)</td>
</tr>
<tr>
<td>f-HDL-chol (mmol/l)</td>
<td>1.36±0.03</td>
<td>1.28±0.03</td>
</tr>
</tbody>
</table>

Confirmations of associations with surrogate markers of liver fat content in independent cohorts

In order to validate our findings in independent cohorts, we first genotyped the three SNPs that were significantly associated with liver fat content in the discovery cohort (rs7887981, rs767870 and rs3856806) in a cohort of 50-year old Swedish men. Analysis of ACSL4 rs7887981 revealed an interaction between genotype and BMI in relation to the variables analyzed. Therefore, subjects were categorized either above or below median BMI (25.7 kg/m²). In the more obese subjects (BMI ≥ 25.7 kg/m²), the rare A allele (associated with increased hepatic fat content) was associated with higher insulin (P=0.049) and TG (P=0.022) concentrations, but no such association was seen in the leaner subjects (BMI < 25.7 kg/m²) (Table 2). Concentrations
of γGT (adjusted for BMI) differed significantly according to ADIPOR2 rs767870 genotype (Table 3), with the lowest concentrations in subjects homozygous for the rare C allele (associated with a lower hepatic fat content). The SNP in PPARG (rs3856806) was not significantly associated with any relevant metabolic parameter in this cohort (Table 3).

In a final step, the SNPs in ACSL4 and ADIPOR2 were genotyped in a second independent surrogate replication cohort, comprising 3032 subjects from Western Finland (PPP-Bohtnia cohort), mean age 47±1 years. No consistent patterns emerged for ACSL4 rs7887981 (Table 4). However, ADIPOR2 rs767870 was associated with lower TG concentrations in men after correction for BMI (P=0.028), with the lowest concentrations in those homozygous for the rare C allele (Table 4). The results remained unchanged after additional adjustment for glucose tolerance status (Table 4).

The distribution of genotypes for all polymorphisms investigated in the surrogate replication cohorts were in Hardy–Weinberg equilibrium.

**Table 3** Associations between ADIPOR2 rs767870 and PPARG rs3856806 and surrogate markers of liver fat content in the Swedish cohort.

<table>
<thead>
<tr>
<th>ADIPOR2 rs767870</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>TC</td>
</tr>
<tr>
<td>N</td>
<td>425</td>
<td>180</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.1±0.2</td>
<td>25.8±0.3</td>
</tr>
<tr>
<td>f-Insulin (pmol/l)</td>
<td>37.7 (27.5–54.5)</td>
<td>39.1 (27.5–54.5)</td>
</tr>
<tr>
<td>f-γGT (μkat/l)</td>
<td>0.50 (0.40–0.80)</td>
<td>0.60 (0.40–0.95)</td>
</tr>
<tr>
<td>f-TG (mmol/l)</td>
<td>1.29 (0.89–1.89)</td>
<td>1.23 (0.85–1.87)</td>
</tr>
<tr>
<td>f-HDL-chol (mmol/l)</td>
<td>1.22±0.02</td>
<td>1.25±0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PPARG rs3856806</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>N</td>
<td>472</td>
<td>138</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.0±0.2</td>
<td>25.9±0.3</td>
</tr>
<tr>
<td>f-Insulin (pmol/l)</td>
<td>38.5 (26.9–52.6)</td>
<td>37.3 (27.3–53.8)</td>
</tr>
<tr>
<td>f-γGT (μkat/l)</td>
<td>0.50 (0.40–0.80)</td>
<td>0.60 (0.40–0.90)</td>
</tr>
<tr>
<td>f-TG (mmol/l)</td>
<td>1.28 (0.89–1.94)</td>
<td>1.24 (0.89–1.79)</td>
</tr>
<tr>
<td>f-HDL-chol (mmol/l)</td>
<td>1.22±0.02</td>
<td>1.26±0.03</td>
</tr>
</tbody>
</table>

Mean ± S.E.M., or median (IQR) with statistical analysis performed on log-transformed data. *Post hoc analyses: P=0.042 for TT versus TC, P=0.14 for TC versus CC, P=0.34 for TT versus CC; †Post hoc analyses: P=0.019 for TT versus TC, P=0.08 for TC versus CC, P=0.27 for TT versus CC.

Discussion

We have used a candidate gene approach to investigate association between genetic variation and accumulation of fat in the liver due to non-alcoholic causes. Liver fat content was determined using state-of-the-art 1H-MRS methodology in 302 Finnish subjects. SNPs significantly associated with liver fat content were investigated in metabolically well-characterized surrogate replication cohorts to investigate relationships between SNPs and surrogate markers of liver fat content, namely circulating γGT/ALT, TG, HDL cholesterol and insulin concentrations. Surrogate replication cohorts were population-based, comprising ~600 Swedish men, all aged 50-years, and around 3000 Finnish men and women, aged 18–75 years, ensuring our results are applicable to different ethnicities (Finnish and Swedish) and to the general population. Using this strategy, we identified ADIPOR2 as a gene of potential importance for accumulation of fat in the liver.

The role of genetic factors in non-alcoholic fatty liver disease has long remained unknown. SNPs in genes encoding peroxisome proliferator-activated receptor γ coactivator 1α (28), adiponutrin (29), CLOK transcription factor (30), PPARD (10), and upstream transcription factor 1 (13) have recently been shown to be related to variation in liver fat content in humans. Two polymorphisms in ADIPOR2 (−64,241 T/G and +33447 C/T) were shown to be associated with serum ALT and AST concentrations in patients with type 2 diabetes, but not in non-diabetic subjects (31). In addition, loci on chromosomes 10 (CPN1-ERLIN1-CHUK) and 22 (PNPLA3-SAMM50) were associated with ALT concentrations, and a locus on chromosome 12 (HNF1A) with γGT concentrations (32) in individuals from the general population.

In the present study, SNPs in ACSL4 (rs7887981), ADIPOR2 (rs767870), and PPARG (rs3856806) were significantly associated with liver fat content after accounting for the influence of acquired obesity. In the initial surrogate replication cohort, the rare allele of ACSL4 rs7887981 (associated with increased hepatic fat content) was associated with increased insulin and TG concentrations in the more obese subjects. Concentrations of γGT (adjusted for BMI) differed significantly according to ADIPOR2 rs767870 genotype, with the lowest concentrations in subjects homozygous for the rare C allele (associated with a lower hepatic fat content). However, ADIPOR2 rs767870 was associated with lower TG concentrations in men after correction for BMI (P=0.028), with the lowest concentrations in those homozygous for the rare C allele (Table 4). The results remained unchanged after additional adjustment for glucose tolerance status (Table 4).

The distribution of genotypes for all polymorphisms investigated in the surrogate replication cohorts were in Hardy–Weinberg equilibrium.

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Genetic variation in ADIPOR2 and liver fat content

Table 4

<table>
<thead>
<tr>
<th>rs767870</th>
<th>N</th>
<th>Men</th>
<th>Women</th>
<th>P</th>
<th>P adjusted*</th>
</tr>
</thead>
<tbody>
<tr>
<td>G(G)</td>
<td></td>
<td>1002</td>
<td>1047</td>
<td>26</td>
<td>0.51</td>
</tr>
<tr>
<td>GA</td>
<td>703</td>
<td>1128</td>
<td>0.91</td>
<td>0.70</td>
<td>0.94</td>
</tr>
<tr>
<td>AA</td>
<td>398</td>
<td>336</td>
<td>0.25</td>
<td>0.26</td>
<td>0.38</td>
</tr>
</tbody>
</table>

P-values for both HDL-C (P = 0.017) and TG (P = 0.086) in genome wide analysis of a case-control study for type 2 diabetes (data available at http://www.broad.mit.edu/diabetes/), which does provide some support for a role of the ADIPOR2 locus in these traits. This discrepancy highlights the difficulties of identifying specific SNPs with only relatively modest impact on complex phenotypic traits such as hepatic fat content, which are of polygenic nature and influenced by lifestyle factors. For example, genes initially identified in animal models, confirmed and replicated in human studies as conferring risk for myocardial infarction (39,40) have not been identified in genome-wide association studies for myocardial infarction (41–43).

In the present study, some differences were noted between the cohorts for ADIPOR2 rs767870 and PPARG rs3856806 allelic frequencies. In both cases, frequencies were similar in the two surrogate replication cohorts, but the rare alleles were less common in the discovery cohort. However, the highly selected nature of the study subjects within the discovery cohort may underlie this difference, compared with the population-based surrogate replication cohorts. All genotypes, in all cohorts, were in Hardy–Weinberg equilibrium, indicating that the frequency differences were not due to methodological issues.

The anti-diabetic and insulin-sensitizing effects of adiponectin (33) are mediated through adiponectin receptors 1 and 2 (34). ADIPOR2 is an intermediate-affinity receptor for both globular and full-length adiponectin (34), and is the receptor predominantly responsible for mediating the effects of adiponectin in the liver (34). In mice, adenosine inhibition of ADIPOR2 causes hepatic steatosis, whereas adenosine overexpression of ADIPOR2 protects against the development of a fatty liver (35). In a recent study of 2876 French patients with type 2 diabetes and non-diabetic subjects, the rare allele of the ADIPOR2 intron 5 T>G polymorphism (rs767870) was associated with significantly higher incidence of type 2 diabetes (15). By contrast, this SNP was not associated with metabolic parameters, anthropometric measurements or liver fat content (determined in 85 subjects) in a German cohort (11). However, Mexican–Americans either heterozygous or homozygous for the C allele had lower serum TG concentrations than non-carriers (36), an observation consistent with the present findings. Although, recent genome wide association analyses have not identified the ADIPOR2 locus as a regulator of TG or HDL cholesterol concentrations (37,38), one SNP in ADIPOR2, rs2058032, gave borderline significant nominal P-values for both HDL-C (P = 0.017) and TG (P = 0.086) in genome wide analysis of a case-control study for type 2 diabetes (data available at http://www.broad.mit.edu/diabetes/), which does provide some support for a role of the ADIPOR2 locus in these traits. This discrepancy highlights the difficulties of identifying specific SNPs with only relatively modest impact on complex phenotypic traits such as hepatic fat content, which are of polygenic nature and influenced by lifestyle factors. For example, genes initially identified in animal models, confirmed and replicated in human studies as conferring risk for myocardial infarction (39,40) have not been identified in genome-wide association studies for myocardial infarction (41–43).

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We found the minor allele of ADIPOR2 rs767870 to be associated with reduced liver fat content, when measured directly using $^1$H-MRS in Finnish subjects, and with lower circulating concentrations of $\gamma$G1 and TG in independent cohorts of men from Sweden and men and women from Finland respectively. Moreover, although weaker, these relationships were still seen when no adjustment was made for BMI, a strong confounding factor for liver fat content (44). Additional adjustment for glucose tolerance status in the second replication cohort did not influence this relationship, implying that this association cannot be attributed to the effect of type 2 diabetes on the degree of hepatic steatosis (20). We have used concentrations of $\gamma$G1 and TG as surrogate markers of liver fat content, markers which provide only an estimate of the actual amount of hepatic fat. Indeed, serum concentrations of ALT and $\gamma$G1 are normal in $\sim 80\%$ of subjects with a fatty liver due to non-alcoholic causes (45,46) and fasting TG concentrations were shown to explain less than 20% of the variation of liver fat content determined by proton spectroscopy (5). Despite the limited ability of surrogate markers to reflect hepatic fat content, the relationships between ADIPOR2 rs767870 and both direct and indirect measurements of hepatic fat were consistent.

ADIPOR2 may play an important role in the regulation of liver fat content and associated metabolic phenotype, presumably through mediation of the effects of adiponectin in the liver. This is supported by a recent study in mice addressing the importance of ADIPOR2 in the development of both non-alcoholic fatty liver and non-alcoholic steatohepatits (35). Genetic variation within the ADIPOR2 locus may influence ADIPOR2 expression levels and/or function. Disturbances to either of these could modulate the actions of adiponectin in the liver, resulting in hepatic fat accumulation and the onset of metabolic perturbations. However, relationships between ADIPOR2 mRNA levels and hepatic fat content are ambiguous, with some (47,48) but not all studies (19,49) reporting increased ADIPOR2 expression in human fatty liver. ADIPOR2 rs767870 lies within an intron, in a region poorly conserved between species, which suggests that this SNP may be a marker for (an)other functional SNP(s). The observation that the association between ADIPOR2 rs767870 and TG concentrations was seen only in men, not in women, in the second surrogate replication cohort is intriguing. Whether this can be explained by gender differences in hepatic ADIPOR2 expression is unknown. There are no gender differences in ADIPOR2 expression in s.c. adipose tissue (50), but circulating adiponectin concentrations are lower in men than women (51), suggesting that men might be more susceptible to modest modulations in ADIPOR2 expression or function.

In conclusion, we have employed a genetic strategy involving three complementary cohorts of different ethnicities. First, we investigated polymorphisms in candidate genes in relation to liver fat content due to non-alcoholic causes in a discovery cohort where hepatic fat accumulation was determined accurately using $^1$H-MRS methodology. Significantly associated SNPs were analyzed in population-based surrogate replication cohorts to explore relationships with surrogate markers of hepatic steatosis. Using this strategy, we identified a SNP in ADIPOR2 (rs767870) as being significantly related to hepatic fat accumulation. Furthermore, we confirmed our findings using surrogate markers of fatty liver in two independent cohorts comprising over 3500 individuals. These data support the concept that genetic factors contribute to variation in hepatic fat accumulation in humans, and ascribe a potentially important role to ADIPOR2.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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