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**Genetic variants regulating insulin receptor signalling are associated with the severity of liver damage in patients with non-alcoholic fatty liver disease**

P Dongiovanni, L Valenti, R Rametta, A K Daly, V Nobili, E Mozzi, J B S Leathart, A Pietrobattista, A D Burt, M Maggioni, A L Fracanzani, E Lattuada, M A Zappa, G Roviaro, G Marchesini, C P Day, S Fargion

**ABSTRACT**

**Background/aims** The aim of this study was to assess the effect of functional ENPP1 (ectoenzyme nucleotide pyrophosphate phosphodiesterase 1)/PC-1 (plasma cell antigen-1) and IRS-1 (insulin receptor substrate-1) polymorphisms influencing insulin receptor activity on liver damage in non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome, whose progression is associated with the severity of insulin resistance.

**Patients and methods** 702 patients with biopsy-proven NAFLD from Italy and the UK, and 310 healthy controls. The Lys121Gln ENPP1/PC-1 and the Gly972Arg IRS-1 polymorphisms were evaluated by restriction analysis. Fibrosis was evaluated according to Kleiner. Insulin signalling activity was evaluated by measuring phosphoAKT levels by western blotting in a subset of obese non-diabetic patients.

**Results** The ENPP1 121Gln and IRS-1 972Arg polymorphisms were detected in 28.7% and 18.1% of patients and associated with increased body weight/dyslipidaemia and diabetes risk, respectively. The ENPP1 121Gln allele was significantly associated with increased prevalence of fibrosis stage >1 and >2, which was higher in subjects also positive for the 972Arg IRS-1 polymorphism. At multivariate analysis, the presence of the 972Arg IRS-1 polymorphism was independently associated with fibrosis >1 (OR 1.55, 95% CI 1.24 to 1.97; and OR 1.57, 95% CI 1.12 to 2.23, respectively). Both polymorphisms were associated with a marked reduction of ~70% of AKT activation status, reflecting insulin resistance and disease severity, in obese patients with NAFLD.

**Conclusions** The ENPP1 121Gln and IRS-1 972Arg polymorphisms affecting insulin receptor activity predispose to liver damage and decrease hepatic insulin signalling in patients with NAFLD. Defective insulin signalling may play a causal role in the progression of liver damage in NAFLD.
The ENPP1 glycoprotein and INSR, inhibiting INSR activity, and is associated with an increased risk of type 2 diabetes.\textsuperscript{19} Downstream, the IRS-1 Gly972Arg SNP decreases IRS-1 activity and inhibits INSR autophosphorylation and activity,\textsuperscript{20} and has repeatedly been associated with increased risk of insulin resistance and diabetes.\textsuperscript{21}

With the hypothesis that insulin resistance plays a causal role in the progression of liver damage in NAFLD, the aim of this study was to assess the effect of these well-characterised common functional SNPs on the severity of liver disease in a large series of patients with biopsy-proven NAFLD, and to determine their influence on hepatic insulin signalling in a subset of cases.

**METHODS**

**Subjects**

We considered 702 unrelated patients from Italy and the UK with biopsy-proven NAFLD diagnosed between January 1999 and January 2008, whose DNA samples and complete clinical data were available (out of a larger group of 940 subjects with incomplete information or unavailable DNA samples). These included 240 adult patients from Milan, 325 from Newcastle and 71 children from the Rome centre (tertiary referral centres), submitted to liver biopsy because of persistently abnormal liver enzymes/serum ferritin or a long-lasting history of steatosis (cirrhosis). The minimum biopsy size was 1.7 cm and the number of portal areas was 10.

We chose as main outcome a fibrosis stage $>1$ according to Kleiner et al.,\textsuperscript{23} in an attempt to identify patients with potentially progressive disease at an early stage. However, we also provided a separate analysis for fibrosis $>2$.

**Genetic analysis**

DNA was extracted from peripheral blood by the phenol–chlooroform method. The success rate in extracting DNA was 100% for each study group. The ENPP1 Lys121Gln (rs1044498) and IRS-1 Gly972Arg (rs1801278) SNPs were determined by restriction analysis, as previously described\textsuperscript{24} by personnel unaware of the subjects' clinical status. Samples from both patients with NAFLD and controls were included in all batches analysed, and quality controls were performed to verify the reproducibility of the results. Valid genotypic data were obtained for $>99\%$ of subjects analysed. Subjects for whom incomplete genetic data were available were excluded from the analyses.

**Evaluation of AKT phosphorylation status**

The activation status of the insulin signalling pathway was estimated by measuring the AKT activation status in a subgroup of 24 patients with severe obesity without diabetes, who were not taking drugs influencing glucose levels and insulin sensitivity, and for whom sufficient frozen biological samples were available. These subjects were younger and had a higher BMI than the rest of the cohort ($p<0.05$), but their clinical features were not significantly different from those of the other subjects submitted to bariatric surgery included in the study.

Tissues (10 mg) were lysed in RIPA buffer. Equal amounts of proteins (25 μg) were separated by SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred electrophoretically to nitrocellulose membrane (Biorad, Hercules, California, USA). The blot was incubated with anti-phosphoAKT (pAKT, Thr308 and Ser473) (Cell Signaling Technology, Danvers, Massachusetts, USA) and β-actin antibodies (from Santa Cruz Biotechnology, Santa Cruz, California, USA). The gels were densitometrically scanned and images were analysed with the ImageJ analysis software provided by the NIH.\textsuperscript{16}

**Statistical analysis**

The manuscript was prepared in accordance with the STROBE guidelines.\textsuperscript{26} The sample size to assess the effect of genetic factors on liver fibrosis was calculated on the basis of the expected relative risk of the mutant allele versus the wild-type allele, the desired power and significance. Our sample had a $70−99\%$ power of detecting an odds ratio (OR) of 2.5 for fibrosis $>1$ in patients with NAFLD, according to genotype frequencies, with a significance of $5\%$. Results are expressed as means (SD) and considered significant when $p<0.05$ (two-tailed). Mean values were compared by $t$ test and frequencies by Fisher exact test.

The association between the ENPP1 and IRS-1 SNPs and the presence of moderate/severe fibrosis (Kleiner stage $>1$) was evaluated by logistic regression analysis adjusted for confounders (age, BMI, ALT, glucose and ferritin levels, considered as continuous variables). Analyses were carried out with JMP 6.0 statistical analysis software (SAS Institute, Cary, North Carolina, USA).
RESULTS

The genotype distribution of all polymorphic alleles was in Hardy–Weinberg equilibrium in both patients and controls. The frequency distributions of the evaluated SNPs were not significantly different between Italian patients with NAFLD and controls (Supplementary table 1 online), but we observed a trend for a higher prevalence of the ENPP1/PC-1 SNP and a lower prevalence of the IRS-1 SNP in patients from the UK than in those from Italy, which was consistent with the reported differences in the prevalence of genetic factors across European populations.27 In the case of ENPP1, genotype frequencies for the patients from the UK with NAFLD were not significantly different from those of a large British control cohort.28 For IRS-1, patients with NAFLD showed a borderline significant decrease in frequency of carriage of the variant compared with a British control group (p = 0.043).28

Effect of the ENPP1/IRS-1 SNPs on metabolic features

To check whether the genetic variants considered were associated with insulin resistance in patients with NAFLD, we first evaluated the association between the ENPP1 and IRS-1 SNPs with parameters of the metabolic syndrome (table 2). The ENPP1 121Gln allele was significantly associated with higher BMI, waist circumference in both males and females, and with lower high-density lipoprotein (HDL) levels in males. At logistic regression analysis considering sex, BMI, and the presence of the ENPP1 SNP as independent variables (ie, variables associated with insulin resistance in patients with NAFLD), we reported in figure 1 and table 4 the OR of fibrosis for the ENPP1 SNP considering these genotype status, the prevalence of both fibrosis >1 and fibrosis >2 was significantly higher in patients positive for the ENPP1 121Gln SNP than in patients negative for both the ENPP1/IRS-1 SNPs and than in those positive for the IRS-1 972Arg SNP alone.

The prevalence of fibrosis >1 in patients subdivided according to the geographical origin is shown in table 3. The results in the two major groups of adult patients from Italy and the UK confirmed the overall effect of the ENPP1 121Gln SNP on the severity of liver disease. In Italian patients, the severity of liver disease was significantly more marked in patients positive for the ENPP1 and IRS-1 SNPs than in those positive for the ENPP1 SNP alone, and the presence of the IRS-1 SNP was associated with a higher prevalence of fibrosis >1 in Italian children. Independent predictors of fibrosis >1, after adjustment for age, BMI, ALT, glucose and ferritin levels (the clinical variables associated with fibrosis risk at multivariate analysis), and considering the ENPP1 and IRS-1 genotypes as independent variables are shown in table 4. Since the risk of fibrosis for the presence of the ENPP1 SNP appeared to be higher in patients positive than in those negative for the IRS-1 SNP, we reported in table 4 the OR of fibrosis for the ENPP1 SNP considering these

Effect of the ENPP1/IRS-1 SNPs on the severity of liver disease

The prevalence of NASH, and of fibrosis stage >1 and >2 in the whole series of 702 patients is shown in figure 1. Whereas the prevalence of NASH was not influenced by the ENPP1/IRS-1

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic and clinical features of patients with non-alcoholic fatty liver disease (NAFLD) and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Italian controls</td>
</tr>
<tr>
<td>Number</td>
<td>310</td>
</tr>
<tr>
<td>Female sex</td>
<td>55 (17.7%)</td>
</tr>
<tr>
<td>Age, years</td>
<td>47.3 (13)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.1 (2.6)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>194.1 (33)</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>56.3 (13)</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>98.9 (42)</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>88.6 (10)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.9 (1.6)</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>22.7 (8)</td>
</tr>
<tr>
<td>GGT, U/l</td>
<td>23 (18)</td>
</tr>
<tr>
<td>NASH</td>
<td>–</td>
</tr>
<tr>
<td>Fibrosis stage F0/F1/F2/F3/F4†</td>
<td>–</td>
</tr>
</tbody>
</table>

Values are given as mean (SD), unless indicated otherwise. *p<0.0001 between patients and controls. †Values in parentheses are percentages.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, y-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment—insulin resistance index; NA, not available; NASH, non-alcoholic steatohepatitis.
two groups separately. The risk of fibrosis >1 was higher in patients carrying both the ENPP1 and the IRS-1 SNPs compared with those who were negative (OR 1.55 95% CI 1.24 and 1.97, p=0.0002), but the effect of the ENPP1 SNP was higher in patients who were also positive for the IRS-1 SNP.

**Functional effect of ENPP1/IRS-1 SNPs on hepatic insulin signalling**

To check whether the effect of ENPP1/IRS-1 SNPs on liver damage may be mediated by increased hepatic insulin resistance, we evaluated the activation status of the hepatic insulin signalling pathway by measuring pAKT levels in a subgroup of non-diabetic patients with severe obesity who underwent bariatric surgery in Milan. Results are shown in figure 2.

AKT activity, as detected by the phosphorylation status at position Thr308, was significantly decreased in patients with fatty liver compared with those with normal liver, and to a larger extent in those affected by NASH than in those with pure fatty liver (figure 2A). AKT activity, as detected by the phosphorylation status at both position Thr308 and Ser473, was significantly decreased in patients positive for the ENPP1 121Gln or the IRS-1 972Arg alleles compared with patients negative for both SNPs (figure 2B). AKT phosphorylation was ~30% of that of controls in patients mutated at both Thr308 and Ser473: 27% and 51% in the presence of the IRS-1 and ENPP1 SNPs, respectively, at Thr308, and 21% and 46%, respectively, at Ser473. A representative western blot is shown in figure 2C.

**DISCUSSION**

In this study we evaluated the effect of SNPs of genes involved in the regulation of INSR signalling on liver injury in patients with NAFLD, and found that both the ENPP1 121Gln and IRS-1 972Arg alleles affect insulin resistance and are associated with fibrosis severity.

Since NAFLD is the hepatic expression of metabolic syndrome, having a strong genetic component, and insulin resistance is associated with disease progression,9 we hypothesised that genetic factors influencing insulin signalling might predispose to liver damage in patients with NAFLD.

The results indicate that the PC-1/ENPP1 121Gln and IRS-1 972Arg alleles, present in 28.7% and 18.1% of patients, respectively, influence the severity of hepatic fibrosis independently of confounders. Both ENPP1 and IRS-1 interact directly with INSR. ENPP1 is a membrane glycoprotein, which inhibits insulin signalling and when overexpressed causes insulin resistance and related abnormalities. The 121Gln polymorphism is a gain-of-function allele causing stronger interaction with INSR and inhibition of its kinase activity.30 IRS-1 transduces INSR signalling to downstream kinases regulating glucose and lipid metabolism, cell survival and proliferation. The 972Arg polymorphism is a loss-of-function allele characterised by increased association

*Table 3 Effect of combined ENPP1 and IRS-1 genotype on the prevalence of fibrosis stage >1 in patients with NAFLD subdivided according to geographic origin and clinical characteristics*

<table>
<thead>
<tr>
<th>ENPP1 121Gln</th>
<th>IRS-1 972Arg</th>
<th>Prevalence %</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(reference)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Italy, adults</td>
<td>23/150 (15.3%)</td>
<td>5/38 (13.2%)</td>
<td>9/40 (22.5%)</td>
</tr>
<tr>
<td>Italy, children</td>
<td>0/44</td>
<td>1/11 # (9.1%)</td>
<td>1/14 (7.1%)</td>
</tr>
<tr>
<td>Italy, obese</td>
<td>6/31 (19.3%)</td>
<td>4/14 (28.6)</td>
<td>1/14 (7.1)</td>
</tr>
<tr>
<td>UK, adults</td>
<td>87/219 (39.7%)</td>
<td>10/20 (50%)</td>
<td>41/78 # (53%)</td>
</tr>
<tr>
<td>Combined</td>
<td>116/444 (26.1%)</td>
<td>20/83 (24.1%)</td>
<td>52/146 (35.6%)</td>
</tr>
</tbody>
</table>

* p<0.05 vs patients negative for both IRS-1 and ENPP1 single nucleotide polymorphisms (SNPs; reference group).

**p<0.05 vs patients negative for the IRS-1 SNP.

Enpp1, ectoenzyme nucleotide pyrophosphate phosphodiesterase 1; INSR, insulin receptor substrate-1; NAFLD, non-alcoholic fatty liver disease.
with INSR resulting in decreased autophosphorylation activity, and, similarly to what was observed in this study, transgenic mice expressing a mutated human 972Arg allele show increased insulin resistance and decreased \( \beta \)-cell function leading to hyperglycaemia. Both genetic variants have consistently been associated with an increased risk of diabetes and metabolic disease. The ENPP1 and IRS-1 SNPs were also associated with insulin resistance-related abnormalities in patients with NAFLD, confirming that their effect may be mediated by insulin resistance. The risk of hyperglycaemia was 32% higher in carriers of the IRS-1 polymorphism, independently of age and BMI, which are well-known risk factors. On the other hand, the

**Table 4** Clinical and genetic predictors of fibrosis >1 at logistic regression analysis in 702 patients with NAFLD

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted OR</th>
<th>p Value</th>
<th>Adjusted OR</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age years</td>
<td>1.05 (1.04 to 1.07)</td>
<td>&lt;0.0001</td>
<td>1.06 (1.04 to 1.08)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ALT, U/ml</td>
<td>1.007 (1.004 to 1.011)</td>
<td>&lt;0.0001</td>
<td>1.009 (1.004 to 1.012)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>1.07 (1.04 to 1.09)</td>
<td>&lt;0.0001</td>
<td>1.11 (1.07 to 1.16)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>1.014 (1.010 to 1.019)</td>
<td>&lt;0.0001</td>
<td>1.008 (1.004 to 1.12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ferritin, ng/ml</td>
<td>1.00 (1 to 1.001)</td>
<td>0.5</td>
<td>1.001 (1 to 1.002)</td>
<td>0.05</td>
</tr>
<tr>
<td>ENPP1, 121Gln+, IRS1 972Arg+</td>
<td>1.73 (1.11 to 2.71)</td>
<td>0.01</td>
<td>2.92 (1.55 to 5.72)</td>
<td>0.001</td>
</tr>
<tr>
<td>ENPP1 121Gln+, IRS1 972Arg−</td>
<td>1.26 (1.03 to 1.53)</td>
<td>0.02</td>
<td>1.30 (1.01 to 1.67)</td>
<td>0.03</td>
</tr>
<tr>
<td>IRS-1 972Arg+</td>
<td>1.12 (0.88 to 1.42)</td>
<td>0.3</td>
<td>1.57 (1.12 to 2.23)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Model \( \chi^2 \) 172.3, \( p<0.0001 \).

ALT, alanine aminotransferase; BMI, body mass index; ENPP1, ectoenzyme nucleotide pyrophosphate phosphodiesterase 1; IRS-1, insulin receptor substrate-1; NAFLD, non-alcoholic fatty liver disease.

**Figure 2** (A) Hepatic AKT activity status (phospho-Thr308 (PT308) and phospho-Ser473 (PS473)) in 24 patients with non-alcoholic steatohepatitis (NASH) subdivided according to histological findings. Data were normalised for \( \beta \)-actin. *\( p<0.05 \) vs patients with normal liver (reference group). (B) Hepatic AKT activity status (PT308 and PS473) in 24 patients with NASH subdivided according to the presence or not of the ENPP1 (ectoenzyme nucleotide pyrophosphate phosphodiesterase 1) 121Gln and IRS-1 (insulin receptor substrate-1) 972Arg single nucleotide polymorphisms (SNPs). Data were normalised for \( \beta \)-actin. *\( p<0.05 \) vs patients negative for both IRS-1 and ENPP1 SNPs (reference group). White bars, PT308 AKT; black bars, PS473 AKT. (C) Hepatic AKT activity status (PT308 and PS473) as detected by western blotting in 14 representative obese subjects subdivided according to the hepatic pathological findings and the presence of the ENPP1 and IRS-1 SNPs. Total AKT and \( \beta \)-actin levels are shown as a control.
ENPP1 121Gln polymorphism seems to determine insulin resistance by a different mechanism, as it was associated with lower HDL values, a sensitive marker of hepatic insulin resistance, and with higher BMI and waist circumference. Interestingly, the ENPP1 121Gln allele has previously been reported to predispose to obesity, possibly by altering appetite control via insulin resistance in the hypothalamus.

To provide further support for the effects of these SNPs on hepatic insulin resistance, we were also able to show that they were associated with decreased hepatic PAKT levels, reflecting reduced insulin signalling activity, although a limitation of this analysis is that data were available only in a subgroup of obese non-diabetic patients requiring bariatric surgery. Interestingly, the effect of these inherited factors was more marked than that exerted by liver histology, as the presence of fatty liver affected only Thr308 AKT phosphorylation, whereas the presence of ENPP1/IRS-1 SNPs decreased by ~70% both Thr308 and Ser473 AKT phosphorylation, with a potentially higher effect on insulin signalling.

Importantly, the effect of the ENPP1 and IRS-1 SNPs on the severity of liver fibrosis was independent of the ethnic background, as it was observed in patients from both Italy and the UK. The higher prevalence of fibrosis >1 in the UK compared with Italy is possibly related to the higher BMI, to the different diet and other as yet unknown genetic factors.

It is noteworthy that the ENPP1 and IRS-1 SNPs conferred an increased risk of fibrosis in patients with biochemical abnormalities resistant to lifestyle modification, independently of all other clinical predictors identified, including glucose levels. Therefore, the mechanism leading to hepatocellular damage and fibrogenesis involves not only hyperglycaemia, but also decreased cell survival and altered lipogenesis due to an altered INSR/ENPP1/IRS-1 pathway. Increased metabolic abnormalities and susceptibility to cell death would thus lead to accelerated fibrogenesis in patients carrying these inherited variants.

Given that in the whole cohort the risk of fibrosis was higher in subjects positive for both the ENPP1 and IRS-1 SNPs compared with those positive for either ENPP1 or IRS-1, a synergic role for the two genetic variants in determining liver damage in patients with NAFLD could be hypothesised. The contemporaneous presence of two defects at the level of INSR may overcome cellular escape mechanisms. In particular, the IRS-1 SNP had no effect when present alone, but increased the risk of fibrosis by almost threefold in the presence of the ENPP1 SNP.

Although the prevalence of combined heterozygosity for the ENPP1 and IRS-1 SNPs may seem low, from our data and from the prevalence of fatty liver in the general population, it could be roughly estimated that at least 1% of the Western population has NAFLD and carries both the ENPP1 and IRS-1 SNPs, and half of roughly estimated that at least 1% of the Western population has NAFLD and carries both the ENPP1 and IRS-1 SNPs, and half of

REFERENCES


