Implications of PNPLA3 polymorphism in chronic hepatitis C patients receiving peginterferon plus ribavirin

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SUMMARY

Background
Homozygosity for the PNPLA3 p.I148M polymorphism influences steatosis and fibrogenesis in chronic hepatitis C (CHC).

Aim
To evaluate the effect of p.148M/M on sustained virological response (SVR) and viral kinetics in patients who underwent antiviral therapy with peginterferon and ribavirin, stratified according to viral genotype and fibrosis severity, and secondarily, the interaction with interleukin-28B (IL28B) genotype on liver damage.

Methods
In this observational study, we considered 602 treatment-naïve consecutive patients from tertiary referral centres in Milan and Vienna [61% genotype 1 (G1), 30% advanced fibrosis, 33% IL28B rs12979860 CC].

Results
The p.148M/M genotype, detected in 8% of patients, did not influence SVR in the overall series (P = 0.29), but it was associated with SVR (3/17, 17% vs. 56/121, 46%; P = 0.034) and complete early viral response (4/17, 23% vs. 68/121, 56%; P = 0.018) in G1/4 patients with advanced fibrosis. After adjustment for age, viral load, IL28B CC genotype, treatment dose, and steatosis, p.148M/M remained a predictor of SVR in G1/4 patients with advanced fibrosis (OR 0.23, 95% CI 0.04–0.87). The p.148M/M genotype was associated with more advanced fibrosis in the overall series (P = 0.049), whereas the rs12979860 IL28B CC genotype only in patients negative for p.148M/M (P = 0.017), independently of age, BMI and alanine transaminase levels (OR 1.51, 95% CI 1.01–2.27).

Conclusions
PNPLA3 p.148M/M genotype was negatively associated with SVR and early viral kinetics independently of steatosis, albeit only in difficult-to-cure G1/4 patients with advanced fibrosis, whereas stratification for the p.148M/M PNPLA3 genotype unmasked an association between IL28B CC genotype and more severe liver fibrosis.

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INTRODUCTION
Chronic hepatitis C (CHC), a leading cause of liver-related mortality in Western countries, is frequently associated with hepatic steatosis, which accelerates disease progression and either be due to direct cytopathic effects of the viral infection or to metabolic factors.\textsuperscript{1, 2} The Patatin-like phospholipase domain-containing-3 (PNPLA3) rs738409 polymorphism, encoding for the I148M protein variant (p.I148M), is a strong genetic determinant of hepatic fat accumulation and progressive non-alcoholic as well as alcoholic steatohepatitis in adults and children,\textsuperscript{3-6} explaining almost a quarter of cirrhosis variability in alcoholic liver disease.\textsuperscript{7} We and others\textsuperscript{8-11} have shown that in CHC patients, the p.I148M PNPLA3 polymorphism influences steatosis development in nongenotype 3 (G3) patients, the progression of liver damage and the susceptibility to develop cirrhosis and its complications, including hepatocellular carcinoma.\textsuperscript{12, 13}

Steatosis also negatively influences the outcome of treatment with peg-interferon and ribavirin,\textsuperscript{14} which still represents the standard of care (SOC) for hepatitis C virus (HCV) infection in most countries. Recently, a major role of host genetic factors, namely interleukin-28B (IL28B) genotype, has been recognised in the prediction of the outcome of SOC therapy in CHC,\textsuperscript{15-19} and interestingly, favourable IL28B genotypes have also been associated with protection from hepatic steatosis,\textsuperscript{8, 20-22} whereas the association with fibrosis progression is controversial.\textsuperscript{23-25} Furthermore, scant data (and limited to steatosis) are available on the interaction between IL28B and PNPLA3 genotypes and liver damage in CHC.\textsuperscript{21}

We previously reported that homozygosity for the p.148M PNPLA3 variant at risk may influence the response to antiviral treatment in 470 Italian CHC patients; however, this information was derived from a cohort that was rather heterogeneous in terms of viral features, treatment regimens and schedules, and also included a subset of re-treated patients.\textsuperscript{9} Later, Trepo \textit{et al.} confirmed the association between p.148M homozygosity and treatment outcome in 229 European non-G3 patients who underwent SOC, but the effect of PNPLA3 genotype was not independent of the severity of liver fibrosis. Furthermore, in both studies, viral kinetics and the interaction with viral genotype, IL28B genotype, and other risk factors for treatment outcome were not analysed.\textsuperscript{9, 11} Therefore, the aim of this study was to evaluate the association of p.148M/M with the rate of SVR and viral kinetics in a large multicentre series of naïve CHC patients who underwent SOC antiviral therapy, stratified according to viral genotype (G 1/4 vs. 2/3), and the severity of fibrosis (presence of bridging fibrosis or cirrhosis), after adjustment for rs12979860 IL28B CC genotype and other risk factors. A secondary goal was to explore the interaction between PNPLA3 and IL28B host genotypes and liver damage (fibrosis severity) in this quite large cross-sectional series of naïve CHC patients with histological evaluation.

MATERIALS AND METHODS

Patients
We considered 602 naïve consecutive patients from two tertiary referral centres in Milan and one in Vienna, for whom DNA samples and liver biopsy were available. Patients from Milan were from the Metabolic Liver Disease Unit, Department of Internal Medicine (\(n = 121\)), and the A.M. Migliavacca Center for Liver Disease, at the First Division of Gastroenterology (\(n = 217\)). These subjects were naïve CHC patients who underwent SOC antiviral therapy with peg-interferon and ribavirin, who were included in a previously described series.\textsuperscript{9} Patients with coexistent causes of liver disease, including excessive alcohol intake (>60/40 g/day for more than 5 years for M/F), HBsAg positivity, HIV infection, autoimmune hepatitis, hereditary haemochromatosis and alpha1-antitrypsin deficiency, schistosomiasis, or lack of or inadequate histological evaluation were already excluded from the previous study.\textsuperscript{9} Patients with alcohol intake >30/20 g/day in M/F were also excluded from treatment.

Patients from Vienna (\(n = 264\)) met the same inclusion criteria. Most of these subjects were part of a recently described series of patients, who participated in a randomised controlled trial on response-guided therapy and were randomised to SOC therapy.\textsuperscript{26}

Demographic, clinical and genetic data of the patients are shown in Table 1. Compared with Italian patients, Austrian patients were younger, more frequently infected with HCV G1/4, and were all treated by peginterferon z2a (\(P < 0.05\)). Data on the effect of PNPLA3 and IL28B genotypes on steatosis in the non-G3 patients included in this cohort have previously been reported.\textsuperscript{21}

The study protocol was approved by the Institutional Review Board of the Ospedale Policlinico Ca’ Granda IRCI-CCS, Milan, Italy, and by the Medical University of Vienna. Informed written consent was obtained from each patient and control subject, and the study conforms to the ethical guidelines of the 1975 declaration of Helsinki.
Treatment

Patients received ribavirin (Rebetol; Schering Plough Corp, Kenilworth, NJ, USA, or Copegus; Roche, Basel, Switzerland) combined with either peginterferon (Peg-IFN) α2a (Pegasys; Roche) 180 lg/week or PegIFN α2b 1.5 lg/kg/week (PegIntron; Schering Plough Corp) for 48 weeks. PegIFN α2a was administered together with ribavirin (Rbv) 1000–1200 mg/day (<75 kg; ≥75 kg) while PegIFNα2b was given with Rbv 800 mg for patients of less than 65 kg body weight, 1000 mg for 65–85 kg and 1200 mg for ≥85 kg. Therapy was discontinued if quantitative HCV-RNA testing at week 12 dropped by less than 2 log_{10}s compared with baseline values, and at week 24 if HCV-RNA was still detectable. All patients were evaluated for safety and tolerance of treatment every 4 weeks during the treatment period. PegIFNα2a was reduced to 135 μg and PegIFNα2b to 1.0 μg/kg/week in patients with absolute neutrophils count (ANC) <0.50 × 10^9/L. The same dose reductions were applied if platelets fell under 50 × 10^3 cells/mm^3 with PegIFN being discontinued when reaching the 25 × 10^3 cells/mm^3 threshold. In both treatment arms, Rbv dose was tapered by 200 mg/day in patients with haemoglobin <10 g/dL, whereas it was discontinued in patients with haemoglobin levels below 8.5 g/dL. Adequate drug exposure, which could not be achieved in all patients due to lack of compliance or more often side effects, was defined by >80% of Peg-IFN dose and >80% of ribavirin dose for >80% of treatment duration. Information on rapid virological response (RVR), complete early virological response (cEVR), partial early virological response (pEVR), end of treatment response (EOT) and relapse rates were available for each patient included.

Histological assessment

Tissue sections were stained with haematoxylin and eosin, impregnated with silver for reticulin framework, and stained with trichrome for collagen. One expert,

**Table 1 | Demographic, clinical and genetic data of the 602 chronic hepatitis C naïve patients who underwent antiviral treatment with peginterferon and ribavirin**

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Milan, IM</th>
<th>Milan, GE</th>
<th>Vienna</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>602</td>
<td>121</td>
<td>217</td>
<td>264</td>
</tr>
<tr>
<td>Gender F</td>
<td>235 (39)</td>
<td>53 (44)</td>
<td>95 (44)</td>
<td>87 (33)</td>
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<tr>
<td>Age years</td>
<td>51 ± 12</td>
<td>53 ± 12</td>
<td>57 ± 12</td>
<td>45 ± 10</td>
</tr>
<tr>
<td>BMI</td>
<td>25.1 ± 3.8</td>
<td>25.2 ± 4.3</td>
<td>24.4 ± 3.2</td>
<td>25.5 ± 3.8</td>
</tr>
<tr>
<td>ALT IU/mL</td>
<td>77 ± 73</td>
<td>56 ± 53</td>
<td>70 ± 82</td>
<td>92 ± 71</td>
</tr>
<tr>
<td>HCV genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>368 (61)</td>
<td>67 (55)</td>
<td>95 (44)</td>
<td>206 (78)</td>
</tr>
<tr>
<td>2</td>
<td>105 (17)</td>
<td>31 (26)</td>
<td>74 (34)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>35 (6)</td>
<td>11 (9)</td>
<td>18 (8)</td>
<td>6 (2)</td>
</tr>
<tr>
<td>4</td>
<td>94 (16)</td>
<td>12 (10)</td>
<td>30 (14)</td>
<td>52 (20)</td>
</tr>
<tr>
<td>Viral load log_{10} IU/mL</td>
<td>5.9 ± 0.8</td>
<td>5.7 ± 0.9</td>
<td>5.9 ± 0.8</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>Steatosis</td>
<td>406 (67)</td>
<td>80 (66)</td>
<td>149 (69)</td>
<td>177 (67)</td>
</tr>
<tr>
<td>Advanced fibrosis</td>
<td>179 (30)</td>
<td>29 (24)</td>
<td>64 (30)</td>
<td>86 (32)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>122 (20)</td>
<td>19 (16)</td>
<td>38 (17)</td>
<td>65 (25)</td>
</tr>
<tr>
<td>Peginterferon α2a</td>
<td>477 (79)</td>
<td>64 (53)</td>
<td>149 (67)</td>
<td>264 (100)</td>
</tr>
<tr>
<td>RVR</td>
<td>223 (37)</td>
<td>54 (45)</td>
<td>106 (49)</td>
<td>64 (24)</td>
</tr>
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<td>cEVR</td>
<td>413 (69)</td>
<td>88 (73)</td>
<td>156 (72)</td>
<td>169 (64)</td>
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<tr>
<td>SVR</td>
<td>351 (58)</td>
<td>68 (56)</td>
<td>131 (60)</td>
<td>152 (57)</td>
</tr>
<tr>
<td>Relapse</td>
<td>106 (18)</td>
<td>19 (16)</td>
<td>43 (20)</td>
<td>44 (17)</td>
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<tr>
<td>IL28B rs12979860 CC</td>
<td>198 (33)</td>
<td>32 (26)</td>
<td>74 (34)</td>
<td>92 (35)</td>
</tr>
<tr>
<td>PNPLA3 rs738409 CC</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CC (p.148I/I)</td>
<td>321 (53)</td>
<td>63 (52)</td>
<td>110 (51)</td>
<td>148 (56)</td>
</tr>
<tr>
<td>CG (p.148I/M)</td>
<td>232 (39)</td>
<td>50 (41)</td>
<td>85 (39)</td>
<td>97 (37)</td>
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<tr>
<td>GG (p.148M/M)</td>
<td>49 (8)</td>
<td>8 (7)</td>
<td>22 (10)</td>
<td>19 (7)</td>
</tr>
</tbody>
</table>

BMI, body mass index; cEVR, complete early virological response; F, females; GE, gastroenterology; IM, internal medicine; RVR, rapid virological response; SVR, sustained virological response.

N, number; (): % values.

Advanced fibrosis: Metavir >2.
board-certified pathologist at each centre, who was unaware of clinical and genetic data, reviewed all biopsies for steatosis grade and fibrosis stage, according to Ishak and METAVIR. The minimum biopsy size was 1.7 cm and the number of portal areas 10. Steatosis was graded as 0: absent or <5%, 1: 5–33%, 2: 34–66%, 3: >66% of hepatocytes affected. The extent of steatosis was evaluated by calculating the percentage of lipid droplets counting hepatocytes of the total number of hepatocytes in the biopsy section at a 40× magnification. Advanced fibrosis was defined in the presence of bridging fibrosis or cirrhosis.

Genetic analysis
The rs738409 C>G SNP, encoding p.I148M, was genotyped by a 5′nuclease Taqman assay (assay on demand for rs738409; Applied Biosystems, Foster City, CA, USA) by personnel unaware of patients and controls clinical status as previously described. We could genotype 100% of patients evaluated. The IL28B rs12979860 genotype was also determined by sequence allele specific polymerase chain reaction in Italian patients28 and by real-time polymerase chain reaction in Austrian patients29 in all patients included.

Statistical analysis
Our sample had a >90% power of detecting an OR of 2.0 for SVR, representing the primary objective of the study, according to genotype frequencies, with a significance of 5% (presupposing a recessive mode of inheritance). Results are expressed as means ± standard deviation and considered significant when P ≤ 0.05 (two-tailed). Mean values were compared by ANOVA, and frequencies by Chi-squared test.

The association between the PNPLA3 rs738409 SNP and SVR and fibrosis was evaluated by logistic regression analysis adjusted for confounding variables, which included those selected a priori for their biological relevance plus those which were found associated with the outcome of interest at univariate analysis (specified in the Result section), under a recessive inheritance model, which was chosen based on previous results on the association between PNPLA3 genotype and fibrosis in CHC patients. We investigated potential effect modifications between the two genetic factors considered using interaction terms. Analyses were carried out with JMP 9.0 statistical analysis software (SAS Institute Inc, Cary, NC, USA) and with the PASW 18.0 (SPSS-IBM, Chicago, IL, USA).

RESULTS
Frequency distribution of PNPLA3 p.I148M and IL28B rs12979860 polymorphisms
The frequency distribution of the rs738409 genotype was in Hardy-Weinberg equilibrium and not significantly different between patients from different centres (Table 1). Homozygosity for the PNPLA3 p.148M variant was detected in 49 patients (8%), whereas the IL28B rs12979860 CC genotype was observed in 198 patients (33%).

As expected, the presence of the PNPLA3 p.148M variant was not associated with demographic, anthropometric and virological parameters, whereas IL28B rs12979860 CC genotype was associated with higher viral load (6.0 ± 0.8 vs. 5.8 ± 0.7 log10 IU/mL; P = 0.018).

Effect of PNPLA3 genotype on treatment response
In the overall series (n = 602), the p.148M/M genotype had no impact on SVR (25/49, 51% vs. 326/553, 59%; P = 0.29), as well as on RVR, cEVR, pEVR, EOT and relapse rates (not shown). In G1/4 patients with advanced fibrosis (n = 138), p.148M/M was associated with a lower SVR rate (3/17, 17% vs. 56/121, 46%; P = 0.034, Figure 1a), and cEVR rate (4/17, 23% vs. 68/121, 56%; P = 0.018, Figure 2a). The effect of PNPLA3 genotype was similar in G1 and G4 patients with advanced fibrosis: in G1 patients, SVR rates were 43/96, 45% in those negative vs. 2/11 (18%) in those positive for p.148M/M (P = 0.1), whereas in G4 patients, SVR rates were 13/25, 52% in those negative vs. 1/6 (17%) in those positive for p.148M/M (P = 0.1). The low rates of SVR in G1/4 patients with advanced fibrosis carrying the p.148M/M genotype were also consistently low across centres: 1/9, 11% vs. 17/47, 36% in patients negative in Milan, and 2/8, 25% vs. 35/74, 47% in patients negative in Vienna.

The p.148M/M genotype was associated with SVR also in G1/4 patients with bridging fibrosis, but without cirrhosis: SVR rates were 23/40, 58% in those negative vs. 0/6 in those positive for p.148M/M (P = 0.02). The p.148M/M genotype had no impact on SVR in G2/3 patients, as well as in G1/4 patients without advanced fibrosis (not shown).

As expected in line with previous studies, the IL28B rs12979860CC genotype was strongly associated with SVR in the overall series (163/198, 82% vs. 188/404, 47%; P < 0.0001), as well as in G1/4 (115/145, 79% vs. 121/317, 38%; P < 0.0001), but not in G2/3 patients (48/
53, 91% vs. 67/87, 77%; \( P = 0.07 \), or in G3 only patients (11/14, 78% vs. 11/21, 52%; \( P = 0.16 \)).

In an exploratory analysis, stratification for the presence of the rs12979860 CC genotype did not significantly influence SVR according to \( PNPLA3 \) status in G1/4 patients (7/27, 26% vs. 114/290, 39%; \( P = 0.2 \) in patients without rs12979860 CC, 6/8, 75% vs. 109/137, 79%; \( P = 0.7 \) in patients without rs12979860 CC), even when the patients were stratified for the degree of fibrosis (Figure 1b).

**Figure 1** | Effect of \( PNPLA3 \) genotype on treatment outcome in 138 patients with G1/4 CHC. (a) Effect of \( PNPLA3 \) p.148M/M on treatment outcome in patients with advanced fibrosis (bridging fibrosis and cirrhosis). (b) Combined effect of \( PNPLA3 \) and \( IL28B \) genotypes on SVR in G1/4 patients. cEVR, complete early virological response; EOT, end-of-treatment response; pEVR, partial early virological response; RVR, rapid virological response; SVR, sustained virological response.

**Figure 2** | Effect of \( PNPLA3 \) genotype on steatosis and fibrosis in 602 patients with CHC. (a) Effect of p.148M/M on the presence of steatosis and of advanced fibrosis (Metavir >2). (b) Effect of the combined p.148M/M \( PNPLA3 \) and rs12979860 CC \( IL28B \) genotypes on the presence of steatosis and of advanced fibrosis (Metavir >2).
In this nonrandomised study, there was no significant difference in SVR rates between patients treated with Peg-IFNα2a and PegIFNα2b, and no significant difference in the association between PNPLA3 genotype and treatment outcome according to PegIFN type (not shown in details).

In G1/4 patients with advanced fibrosis (n = 138), logistic regression multivariate analysis identified p.148M/M as an independent negative predictor of SVR (OR 0.23, 95% CI 0.04–0.87; P = 0.031) after adjustment for age, baseline viral load, IL28B genotype, completion of adequate dose treatment and the presence of steatosis (Table 2). Indeed, treatment dose reduction related to side effects negatively influenced SVR in this group (4/25, 16% vs. 55/113, 49%; P = 0.003). However, the negative association between p.148M/M and SVR remained unchanged when post-treatment variables (completion of adequate dose treatment) were excluded from the model (OR 0.20, 95% CI 0.04–0.87; P = 0.020). On the other hand, although this cohort might not be sufficiently powered to address this issue, the effect p.148M/M on SVR may also be independent of strong on-treatment predictors of viral response such as RVR (OR 0.18, 95% CI 0.04–1.02; P = 0.056, in the model presented in Table 2 plus RVR).

Effect of PNPLA3 and IL28B genotypes on steatosis and fibrosis

At univariate analysis, homozygosity for the PNPLA3 p.148M variant was associated (Figure 2a) with steatosis (42/49, 86% vs. 364/553, 65%; P = 0.0037), and with advanced fibrosis (21/49, 43% vs. 158/553, 28%; P = 0.0001) (bridging fibrosis and cirrhosis). The PNPLA3 rs12979860 CC genotype tended to be associated, although non-significantly, with absence of steatosis (74/198, 37% vs. 122/404, 30%; P = 0.079), and with advanced fibrosis (129/198, 65% vs. 294/404, 73%; P = 0.058). Both homozygosity for the PNPLA3 p.148M (20/49, 41% vs. 81/553, 15%; P < 0.0001) and IL28B rs12979860 CC genotype (24/198, 12% vs. 77/404, 19%; P = 0.036) were significantly associated with moderate-severe steatosis grade (grade 2–3, involving >33% of hepatocytes), although in opposite directions. Results were not affected by exclusion of G3 patients (18/47, 38% vs. 69/520, 13%; P < 0.0001 for PNPLA3 and 17/184, 9% vs. 70/383 18%; P = 0.0059 for IL28B). Evaluation of the interaction between IL28B and PNPLA3 genotypes in the pathogenesis of steatosis in non-G3 patients of this cohort has been previously presented elsewhere.21

The prevalence of steatosis and advanced fibrosis according to the combined PNPLA3 and IL28B genotypes is shown in Figure 2b. Interestingly, the IL28B rs12979860 CC genotype was associated with advanced fibrosis only in patients without the PNPLA3 GG genotype encoding for the p.148M/M variant (65/185, 35% vs. 93/368, 25%; P = 0.0168). This was also true in G1 patients (40/109, 37% vs. 56/236, 24%; P = 0.0143). Independent predictors of advanced fibrosis are shown in Table 3, upper part. At multivariate logistic regression analysis including as independent variables clinical and genetic variables significant at univariate analysis, advanced fibrosis was associated with older age, higher BMI, ALT levels and homozygosity for the PNPLA3 p.148M variant (OR 1.89, 95% CI 1.00–3.61), but not

<p>| Table 3 | Independent predictors of advanced fibrosis (bridging fibrosis and cirrhosis) in 602 patients with chronic hepatitis C, and in 553 patients negative for the rs738490 GG PNPLA3 genotype (encoding for the p.148M/M variant) |</p>
<table>
<thead>
<tr>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall series (n = 602)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.03</td>
<td>1.04–1.06</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1.05</td>
<td>1.01–1.11</td>
</tr>
<tr>
<td>ALT (IU/mL)</td>
<td>1.006</td>
<td>1.003–1.008</td>
</tr>
<tr>
<td>IL28B rs12979860 CC</td>
<td>1.36</td>
<td>0.92–2.00</td>
</tr>
<tr>
<td>PNPLA3 rs738409 GG</td>
<td>1.89</td>
<td>1.00–3.61</td>
</tr>
<tr>
<td>Non-PNPLA3 rs738409 GG (n = 553)</td>
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<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.05</td>
<td>1.03–1.07</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1.07</td>
<td>1.01–1.13</td>
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<tr>
<td>ALT (IU/mL)</td>
<td>1.005</td>
<td>1.003–1.008</td>
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<tr>
<td>IL28B rs12979860 CC</td>
<td>1.51</td>
<td>1.01–2.27</td>
</tr>
</tbody>
</table>

BMI, body mass index.

Demographic, clinical and genetic variable included were those significant at univariate analysis (other histological features were not considered).
with IL28B genotype. However, there was a significant interaction between PNPLA3 and IL28B genotypes in this model \((P = 0.045)\). In non-p.148M/M patients (Table 3, bottom), the rs12979860 CC IL28B genotype was associated with an increased risk of severe fibrosis (OR 1.51, 95% CI 1.01–2.27).

**DISCUSSION**

In this study, we evaluated the impact of the PNPLA3 p.148M polymorphism on the response to peg-interferon plus ribavirin treatment in a large series of consecutive naïve CHC patients, stratified according to viral genotypes and the severity of fibrosis. Our results suggest that homozygosity for the PNPLA3 p.148M variant does not influence treatment response in unselected patients with CHC, but it may negatively affect SVR rates in a selected subgroup G1/4 patients with advanced fibrosis at baseline, i.e. in the subset of patients with lower SVR rates to SOC treatment.

Similarly, as expected, IL28B rs12979860 genotype was strongly associated with SVR, but, as previously reported,\(^3\) the effect was much less stronger and not statistically significant in G2/3 patients, already characterised by a high SVR rate. In line with previous findings,\(^3\) we observed a trend for better SVR rates in G3 patients carrying the rs12979860 CC genotype, but possibly due to the limited number of G3 patients included, it did not reach the statistical significance. Similarly, PNPLA3 genotype did not influence SVR rates in G2/3 patients. However, differently from IL28B genotype, PNPLA3 p.148M/M did not influence SVR in G1/4 patients without advanced fibrosis, and had a lower prevalence compared with the rs12979860 CC genotype, being detected in 12% of G1/4 patients with advanced fibrosis (23% of this series) vs. roughly 30% of patients for the IL28B CC genotype.

Notwithstanding, in G1/4 patients with advanced fibrosis, the association of PNPLA3 genotype with treatment outcome was independent of the other risk factors considered, including IL28B and the presence of histological steatosis, confirming previous results that suggested that the deleterious effect of PNPLA3 p.148M on hepatic fibrosis and on treatment response in CHC is not fully explained by increased hepatic triglyceride concentration.\(^4,\)\(^9\) Although we cannot exclude that patients homozygous for the p.148M PNPLA3 variant had a more severe liver disease, results seem to suggest that the effect of PNPLA3 on treatment outcome does not require the presence of cirrhosis, as it was also evident in patients with bridging fibrosis. It is, however, presently unknown whether the negative predictive power shown by the PNPLA3 p.148M variant on treatment outcome in HCV G1 patients with advanced fibrosis will still be clinically relevant when directly acting antivirals will become part of the SOC. Notwithstanding, provided that these results will be confirmed in independent cohorts, evaluation of PNPLA3 genotype may still prove helpful to select the most appropriate first-line treatment when cost-effectiveness is taken into consideration, and possibly to refine, together with IL28B genotype, treatment personalisation in G4 patients,\(^3\)\(^2\) which should be evaluated in future larger studies.

Concerning the interaction between the evaluated host genetic factors in the pathogenesis of liver damage, similar to previous reports,\(^8\)\(^–\)\(^11\) in this series, PNPLA3 genotype was not associated with virological parameters (genotype and viral load), but with the extent of steatosis and advanced fibrosis, in particular in patients not infected by G3,\(^2\)\(^1\) and with advanced fibrosis. It is noteworthy that the association between PNPLA3 genotype, steatosis and fibrosis was monotonous, whereas there was dissociation between the protective effect of IL28B rs12979860 CC genotype on steatosis\(^8\)\(^,\)\(^2\)\(^0\) and, on the other hand, the promoting effect on fibrosis. In a previous report, IL28B genotype did not influence fibrosis progression in a well-characterised series of CHC patients with known date of infection, whereas steatosis did so.\(^2\)\(^3\) In the present cross-sectional series of naïve patients, predominantly infected by HCV G1/4, similarly to that reported for fibrosis progression by Bochud et al.,\(^2\)\(^4\) we observed a higher risk of severe fibrosis in patients carrying the favourable rs12979860 CC genotype, but only in those who were not homozygous for the pro-fibrogenic PNPLA3 p.148M variant, which was confirmed at multivariate analysis. This association holds true also in G1 patients. Although our data require independent validation, it adds to the controversy about the relationship between IL28B genotype and liver fibrosis, suggesting that stratification for the PNPLA3 status might be useful to assess the effect of IL28B genotype on fibrosis and fibrogenesis and the progression of liver disease in future studies.\(^2\)\(^4\)\(^,\)\(^3\)\(^4\) Given the cross-sectional evaluation of fibrosis severity in the present study, we cannot rule out a major role of steatosis on fibrosis progression in this cohort, or that the loss of liver fat, which accompanies the development of severe fibrosis, may have negatively affected the association among PNPLA3 genotype, steatosis and fibrosis severity as previously reported in non-alcoholic fatty liver disease and in a larger series of CHC patients.\(^4\)\(^,\)\(^5\)\(^,\)\(^9\) However, data are also consistent with the hypothesis that PNPLA3
genotype might influence lipid metabolism, inflammatory mediators and fibrogenesis independently of histologically detectable steatosis.4, 5, 9

In conclusion, PNPLA3 genotype may represent a negative prognostic factor for antiviral treatment outcome, which is dependent on infection with G1/4 genotypes and on the presence of advanced fibrosis, i.e. it is evident in the most difficult-to-cure CHC patients, and stratification for the relationship p.148M/M unmasked an association between IL28B CC genotype and more severe liver fibrosis. Whether PNPLA3 genotype predicts the outcome of triple therapies with direct antiviral agents or could be useful to personalise treatment in G4 patients needs to be evaluated in future studies.

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