Association Between *PNPLA3* rs738409 C>G Variant and Liver-Related Outcomes in Patients with Non-alcoholic Fatty Liver Disease

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BACKGROUND & AIMS: Patients with nonalcoholic fatty liver disease (NAFLD) have an increased risk for liver-related complications, such as decompensation, hepatocellular carcinoma (HCC), and death; the severity of liver fibrosis and metabolic comorbidities are the main risk factors. A single nucleotide polymorphism in patatin-like phospholipase domain-containing-3 (*PNPLA3*) gene is associated with higher prevalence of liver damage and HCC, but there are no data from prospective studies of outcomes of patients with this polymorphism. We investigated whether the common rs738409 variant in *PNPLA3* gene associates with the occurrence of liver-related events and death in a large cohort of patients with NAFLD.

METHODS: We followed 471 consecutive individuals at a hospital in Italy with a diagnosis of NAFLD based on histologic factors or a diagnosis of compensated NAFLD-related cirrhosis based on clinical factors for at least 6 months, from March 2004 through December 2018. We collected data on the occurrence of hepatic and extrahepatic outcomes, including decompensation and HCC, cardiovascular events and extrahepatic cancers, and overall and liver-related death. We detected the rs738409 G>C polymorphism in DNA from patient blood samples using the TaqMan assay.

RESULTS: During a median follow-up time of 64.6 months (range 6.1–175 months) 26 cases of decompensation, 13 HCCs, and 16 deaths (12 liver-related) were recorded. All liver-related events, including liver-related death, occurred in patients with F3 fibrosis or cirrhosis. The prevalence of *PNPLA3* rs738409 GG, GT, and TT genotypes was 31.8%, 45.6%, and 22.6%, respectively. After adjusting for clinical, metabolic, and histologic risk factors, *PNPLA3* C>G variant was associated with a higher risk of decompensation (hazard ratio [HR], 2.10; 95% CI, 1.03–4.29; *P* = .04), HCC (HR, 2.68; 95% CI, 1.01–7.26; *P* = .04), and liver-related death (HR, 3.64; 95% CI, 1.18–11.2; *P* = .02) by multivariate Cox regression analysis. In the subgroup of 162 patients with F3 fibrosis or cirrhosis, we confirmed the independent association between the *PNPLA3* variant and decompensation (HR, 2.00; 95% CI, 1.01–3.97; *P* = .04), HCC (HR, 2.66; 95% CI, 1.02–7.13; *P* = .04), and liver-related death (HR, 3.64, 95% CI, 1.02–7.13; *P* = .04), and liver-related death (HR, 3.64, 95% CI, 1.02–7.13; *P* = .04), and liver-related death (HR, 3.64, 95% CI, 1.02–7.13; *P* = .04), and liver-related death (HR, 3.64, 95% CI, 1.18–11.2; *P* = .02). We found no association between *PNPLA3* genotype and cardiovascular events, extrahepatic cancers, or overall mortality.

CONCLUSIONS: Patients with NAFLD carrying *PNPLA3* rs738409 G>C variant are at higher risk of liver-related events and death.

Keywords: Liver Cancer; Risk Factor; Prognostic Factor; Long-Term Outcome.

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Abbreviations used in this paper: CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio; LD, liver decompensation; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PLT, platelet count.

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2 Grimaudo et al

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Tonalcoholic fatty liver disease (NAFLD) affects N roughly 1 in 4 individuals, with prevalence rates higher than 50% in obese and/or diabetic patients. To date it represents the most common liver disease in Western countries,¹ accounting for the fastest growing cause of liver cirrhosis, hepatic decompensation, hepatocellular carcinoma (HCC), and liver transplantation.^{2,3} Furthermore, it is associated with an increased risk of both fatal and nonfatal cardiovascular events and extrahepatic cancers.⁴

127 Two retrospective cohort studies and a meta-analysis 128 of the natural history of NAFLD patients have clearly 129 shown that the severity of liver fibrosis estimated by 130 histology is the strongest predictor not only of liver-131 related complications but also of death due to extrahepatic diseases.^{5,6} 132

In this complex picture, there is also growing evi-133 134 dence that genetic background can affect the risk and 135 severity of NAFLD. The rs738409 C>G variant in PNPLA3 136 gene has been identified by genome-wide association 137 studies as an independent genetic risk factor for NAFLD.⁷ 138 This finding has been validated in cohorts from different 139 ethnic ancestry. Furthermore, in cross-sectional studies 140 the variant has been associated with the full spectrum of 141 severity of liver damage among NAFLD patients, as well as with the presence of HCC.^{8,9} However, prospective 142 143 data demonstrating the impact of the variant on the 144 occurrence of hepatic and extrahepatic events and death 145 are lacking. 146

To ascertain the clinical impact of the variant, we 147 tested the association of PNPLA3 rs738409 C>G variant 148 with the occurrence of hepatic and extrahepatic events. 149 including death, in a large single center cohort of patients 150 with histologic diagnosis of NAFLD or with clinical diagnosis of fully compensated hepatic cirrhosis due to 152 NAFLD.

Methods

Patient Selection

We analyzed data from 471 patients prospectively 159 160 recruited at the Gastrointestinal and Liver Unit of the 161 Palermo University Hospital from March 2004 to 162 December 2018 with histologic diagnosis of NAFLD or 163 clinical diagnosis of fully compensated Child-Pugh A5 164 cirrhosis due to NAFLD. Specifically, in patients without histology, cirrhosis was diagnosed by liver stiffness 165 measurement >11.5 KPa for M probe¹⁰ or >11 KPa for 166 XL probe,¹¹ and the diagnosis of NAFLD required the 167 168 presence of ultrasonography-assessed steatosis plus at 169 least 1 criterion of metabolic syndrome (obesity, dia-170 betes, arterial hypertension, dyslipidemia). Patients were 171 included if they had blood samples available for genetic 172 analysis and a follow-up of at least 6 months. The flow 173 chart of the study is depicted in Supplementary Figure 1. 174 Other causes of liver disease were ruled out, including

What You Need to Know

Background

A single nucleotide polymorphism in patatin-like phospholipase domain-containing-3 (PNPLA3) gene is associated with higher prevalence of liver damage and HCC. We investigated whether the common rs738409 variant in PNPLA3 gene associates with liver-related events and death in a large cohort of patients with NAFLD in Italy.

Findings

Patients with NAFLD carrying PNPLA3 rs738409 G>C variant are at higher risk of decompensation, liver cancer, and death.

Implications for patient care

Genotype analysis might be considered for patients with a diagnosis of NAFLD to identify those at greatest risk for liver complications or death.

alcohol intake (>20 g/day) as evaluated by a questionnaire, viral (hepatitis B surface antigen, anti-hepatitis C virus, and anti-human immunodeficiency virus negativity) and autoimmune hepatitis, hereditary hemochromatosis, and alpha-1 antitrypsin deficiency. Patients with decompensated cirrhosis, HCC, and current use of steatosis-inducing drugs were excluded.

The study was carried out in accordance with the principles of the Helsinki Declaration and with local and national laws. Approval was obtained from the AOUP "Giaccone" of Palermo.

Patient Evaluation

Clinical, anthropometric, and biochemical data were collected at the time of enrollment (Supplementary Material). Genotyping for PNPLA3 rs738409 C>G was carried out using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA).

The Kleiner classification¹² was used to grade steainflammation, and hepatocellular tosis, lobular ballooning and to stage fibrosis from 0 to 4. Nonalcoholic steatohepatitis (NASH) was considered to be present when steatosis, ballooning, and lobular inflammation were all present.

223 In patients with and without cirrhosis, as usually done in our center and not specifically for the present 224 study, follow-up visits and laboratory tests were done at 225 baseline and repeated at 6-month intervals. Ultrasound 226 examination was carried out yearly in patients with F0-227 F2 fibrosis and, as stricter surveillance for HCC, every 228 6 months in patients with F3 fibrosis or cirrhosis, ac-229 cording to international guidelines.¹³ In the presence of 230 cirrhosis, esophageal gastroscopy was performed at 231 baseline and repeated as recommended by clinical 232

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233 guidelines.¹⁴ Patients with progression to medium or 234 large (F2 or F3) esophageal varices were treated with β -235 blockers or underwent elastic banding, whereas no 236 prophylaxis was scheduled for patients with small (F1) 237 varices.¹⁴

238 During follow-up, hepatic and extrahepatic events 239 were recorded. Liver-related events were categorized as 240 either liver decompensation (LD) (occurrence of ascites 241 and/or bleeding varices and/or encephalopathy and/or 242 jaundice) or development of HCC. Patients who had a 243 diagnosis of HCC during follow-up were evaluated for 244 available therapies (surgical resection, radiofrequency 245 ablation, transarterial chemoembolization, or treatment 246 with sorafenib starting in 2007), as indicated in the 247 guidelines.¹³ They were also evaluated for liver trans-248 plantation, as were patients who experienced LD, when 249 indicated.¹⁵ Extrahepatic events were categorized as 250 either cardiovascular events (stroke, transient ischemic 251 attack, myocardial infarction, unstable angina) or extra-252 hepatic cancers. Evidence of extrahepatic events was 253 provided by clinical charts from emergency areas and/or 254 hospitalization. Death was also recorded and classified 255 according to associated events (liver-related, including 256 liver transplantation, or unrelated). The study was closed 257 in December 2018. Patients who did not provide clinical 258 data after June 2018 were considered dropouts and were 259 censored at the last available visit. 260

Statistics

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To evaluate the occurrence of LD, HCC, cardiovascular events, extrahepatic cancers, and death, we included all consecutive patients who had at least 6 months of followup.

268 Continuous variables were summarized as mean \pm 269 standard deviation and categorical variables as fre-270 quency and percentage. The proportion of patients who 271 experienced events was evaluated with Kaplan-Meier 272 curves. To account for competing risks, cause-specific 273 hazards were modeled via Cox regression to identify 274 baseline variables associated with the occurrence of LD, 275 HCC, cardiovascular events, extrahepatic cancer, overall 276 death, and liver-related death. Moreover, the propor-277 tional subdistribution hazard model by Fine and Gray¹⁶ 278 was fitted to estimate the effect of covariates on the 279 cumulative incidence of hepatic mortality, whereas 280 extrahepatic mortality was considered as a competing 281 risk (Supplementary Material). Covariates used for the 282 multivariate analyses were gender, age >57 years (sec-283 ond tertile in the population), PNPLA3 rs738409 geno-284 type (additive model), obesity, impaired fasting glucose/ 285 diabetes, arterial hypertension, platelet count (PLT) 286 <190,000 (second tertile in the population), albumin <4 287 g/dL (second tertile in the population), and F3-F4 288 fibrosis. They were chosen on the basis of their signifi-289 cance in univariate analysis (P < .10). Variables in the 290 final model with P value <.05 were considered

statistically significant. The results are expressed as adjusted hazard ratios (HRs) and their 95% confidence intervals (CIs).

The accuracy of models for predicting the occurrence of HCC, LD, and liver-related death in the subgroup of F3-F4 patients was assessed by using the area under the receiver operating characteristic curves.

Analyses were performed by using SPSS (SPSS Inc released 2009, PASW Statistics for Windows, Version 18.0; SPSS Inc, Chicago, IL), and the SAS macro PSHREG.¹⁷

Results

Time of Follow-up and Dropout Rate

From the start of the study until December 2018, the median time of follow-up was 64.6 months (range, 6.1–171.0 months). Of the 471 enrolled patients, 415 (88.1%) had a complete clinical follow-up, and 56 (11.9%) were lost to follow-up. This lost group had a median follow-up time (72.1 months; range, 6.8–151.1 months) not significantly different from the remaining cohort (63.3 months; range, 6.1–171.0 months) (P = .55 by log-rank test).

Baseline Clinical Features

Baseline characteristics of the 471 patients with NAFLD, overall and split according to advanced fibrosis, are shown in Table 1.

The diagnosis of NAFLD was supported by histology in 417 cases (88.5%). Cirrhosis was diagnosed clinically in 54 cases (11.5%). Among patients with cirrhosis, esophageal varices were found in 59 (60.8%), and specifically in 30.2% of those with histologic and 85.2% of those with clinical diagnosis.

Patients lost to follow-up compared with subjects remaining in the study were significantly younger (43.4 \pm 14.0 vs 50.2 \pm 13.9 years; *P* < .001), had a significantly lower prevalence of impaired fasting glucose/ diabetes (28.5% vs 48.4%; *P* = .005), and trended toward a lower prevalence of advanced fibrosis/cirrhosis (23.5% vs 35.9%; *P* = .06).

Hepatic and Extrahepatic Events/Death

During follow-up, 30 patients developed 39 liver-339 related events. In the total cohort of 471 patients, 13 340 (2.7%) developed HCC, and 26 (5.5%) developed LD (19 341 ascites, 5 encephalopathy, and 2 variceal bleeding; 3 342 cases developed after HCC occurrence) (Table 2). The 1-, 343 2-, 3-, 5-, and 10-year actuarial incidence rates of LD 344 were approximately twice as high as the rates of HCC 345 (Table 2). All liver-related events were observed in pa-346 tients with F3-F4 fibrosis, and in this subgroup the 347 actuarial incidence rates were again 2 times higher for 348

4 Grimaudo et al

Clinical Gastroenterology and Hepatology Vol. ■, No. ■

Table 1. Baseline Demographic, Laboratory, Metabolic, Genetic, and Histologic Features of 471 NAFLD Patients

Variables	All cases, $N = 471$	No advanced fibrosis/ cirrhosis, N = 309	Advanced fibrosis/ cirrhosis, $N = 162$	P value
Mean age, y	49.4 ± 14.1	44.4 ± 12.6	59.0 ± 11.6	<.001
Male gender	61.6%	66.1%	53.1%	.006
Mean BMI, <i>kg/m</i> ²	$\textbf{30.3} \pm \textbf{5.2}$	$\textbf{29.5} \pm \textbf{5.1}$	$\textbf{31.8} \pm \textbf{5.1}$	<.001
BMI ≥30	48.5%	40.3%	63.9%	<.001
ALT, <i>IU/L</i>	72.6 ± 51.0	77.4 ± 51.9	63.5 ± 48.0	.005
PLT, 10 ³ /mmc	$\textbf{219.9} \pm \textbf{77.7}$	$\textbf{241.9} \pm \textbf{66.3}$	177.5 ± 80.5	<.001
Albumin, <i>g/L</i>	4.4 ± 0.4	4.5 ± 0.4	4.3 ± 0.4	<.001
Total bilirubin, mg/dL	0.7 ± 0.5	0.7 ± 0.6	0.8 ± 0.4	.03
Blood glucose, mg/dL	104.2 ± 36.2	98.8 ± 34.9	114.9 ± 36.3	<.001
IFG/type 2 diabetes	46.1%	32.6%	71.6%	<.001
Arterial hypertension	35.9%	25.9%	54.93%	<.001
Total cholesterol, mg/dL	196.0 ± 45.8	205.7 ± 45.9	176.7 ± 39.1	<.001
Triglycerides, mg/dL	139.3 ± 77.3	141.2 ± 83.5	135.7 ± 63.2	.47
PNPLA3 rs				
CC	31.8	36.2	23.4	
CG	45.6	45.6	45.7	
GG	22.6	18.2	30.8	.001
Time of follow-up, <i>mo</i>	64.6 (6.1–175.0)	69.9 (6.1–175.0)	53.6 (8.6–171.0)	.003
Histology				
Steatosis grade 1-2-3	37.4/31.9/30.7ª	39.5/32.0/28.5	31.8/31.8/36.4 ^b	.23
NASH	76.2 ^a	71.5	89.7 ^b	<.001
Fibrosis stage 3-4	34.4	—	—	—

NOTE. Data are given as mean \pm standard deviation, or as percentage of cases (%).

BMI, body mass index; IFG, impaired fasting glucose; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

^aData referred to 417 patients.

^bData referred to 107 patients.

LD compared with HCC and up to 26.7% for LD and 13.5% for HCC, respectively (Table 2). As expected, patients with a clinical diagnosis of cirrhosis (n = 54) developed HCC, decompensation, and liver-related death (9, 19, and 8 events, respectively) more frequently than patients with a histologic diagnosis of cirrhosis (n = 43) (3, 6, and 2 events, respectively).

During follow-up, 8 patients experienced cardiovascular events (1.7%), and 17 had extrahepatic cancers (3.6%) (Table 2).

During the observation period, 16 patients (3.4%) died, 12 of liver causes (2 underwent liver trans-plantation) and 4 of extrahepatic causes (2 of cardio-vascular events and 2 of extrahepatic cancer). The actuarial incidence rates for overall death and liver-related death were 7.7% and 6.8% at 10 years, respec-tively (Table 2). All liver-related deaths were recorded in patients with baseline F3-F4 fibrosis, where the inci-dence rates were as high as 22.1% at 10 years, respec-tively (Table 2).

PNPLA3 rs738409 G Variant Predicts Liver Decompensation Occurrence in Nonalcoholic Fatty Liver Disease

In the entire cohort, a Cox univariate analysis showed
that *PNPLA3* G variant (LD, 2% in CC, 6.5% in CG, and
8.5% in GG) was associated with occurrence of LD (HR,

1.93; 95% CI, 1.13–3.30; P = .01) (Figure 1A). *PNPLA3* G Q5 variant (HR, 2.10; 95% CI, 1.03–4.29, P = .04), PLT <190,000/mmc (HR Inf; 95% CI, NA; P < .001), and F3-F4 fibrosis (HR Inf; 95% CI, NA; P < .001) were maintained as significant risk factors in Cox multivariate analysis (Table 3).

In the subgroup of patients with F3-F4 fibrosis, Cox multivariate analysis confirmed *PNPLA3* G variant (HR, 2.00; 95% CI, 1.01–3.97; P = .04) (Supplementary Figure 2A) and PLT <190,000/mmc (HR Inf; 95% CI, Q6 NA; P < .001) as independent predictors of LD (Table 3). The area under the receiver operating characteristics curve was 0.802. Cox multivariate analysis was used to assess the crude rate of LD at the end of follow-up among risk classes (Figure 2A). Q7

PNPLA3 rs738409 G Variant Predicts Hepatocellular Carcinoma Occurrence in Nonalcoholic Fatty Liver Disease

In the entire cohort of NAFLD patients, Cox univariate analysis showed that PNPLA3 G variant (HCC: 0.7% in CC, 3.2% in CG, and 4.7% in GG) was significantly associated with occurrence of HCC (HR, 2.26; 95% CI, 1.03-4.93; P = .04) (Figure 1B). PNPLA3 G variant (HR, 2.68; 95%) CI, 1.01–7.26; *P* = .04) and F3-F4 fibrosis (HR Inf; 95%) CI, NA; P < .001) were maintained as significant risk factors in the Cox multivariate analysis (Table 3).

Table 2. Hepatic and Extrahepatic Events Recorded During Follow-up in NAFLD Patients

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1 0.2% 0% 2 0.4% 1.3% 3 0.7% 1.3% 5 0.7% 1.3% 10 1.3% 6.7% Time to cardiovascular event, mo (median and range) 73.6 (13.2-104.4) 72.2 (13.2-96.5) Extrahepatic cancer 17 (3.6%) 5 (3.1%) Extrahepatic cancer rate (y) 1 0.6% 0% 1 0.6% 0% 3 3 2.3% 1.5% 3.5% 1 0.6% 3.5% 10 2 3.8% 3.5% 10 Overall death 16 (3.4%) 14 (8.6%) 0% 2 1.1% 2.0% 2.3% 5 2.3% 5.8% 10 14 (8.6%) Overall death rate (y) 16 (3.4%) 14 (8.6%) 0 14 (8.6%) Overall death rate (y) 12 (2.5%) 12 (7.4%) 12 (7.4%) Liver-related death rate (y) 12 (2.5%) 12 (7.4%) 12 (7.4%) Liver-related death rate (y) 0.6% 2.3% 1.3% 1.3% 1.3%			. (=:=,=,,
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Extrahepatic cancer rate (y) 0.6% 0% 1 0.6% 0% 2 1.3% 0% 3 2.3% 1.5% 5 3.8% 3.5% 10 4.9% 5.6% Time to extrahepatic cancer, mo (median and range) 34.4 (2.0–82.6) 36.1 (24.7–82.6) Death 16 (3.4%) 14 (8.6%) Overall death 16 (3.4%) 14 (8.6%) Overall death 16 (3.4%) 2.8% 2 1.1% 2.0% 3 1.4% 2.8% 5 2.3% 5.8% 10 7.7% 23.3% Time to overall death, mo (median and range) 53.6 (11.0–109.6) 64.6 (11.0–109.6) Liver-related death 12 (2.5%) 12 (7.4%) 1.3% 5 0.4% 1.3% 3.6% 1.3% 6.6% 22.1% 0.6% 2.1% 1.3% 3.6% 10 6.8% 22.1% 1.3% 3.6% 1.4% 4.3% 1.6%	Extrahepatic cancer		
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	Extrahepatic cancer rate (y)		
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1	0.6%	0%
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	2	1.3%	0%
104.9%5.6%Time to extrahepatic cancer, mo (median and range) 34.4 (2.0-82.6) 36.1 (24.7-82.6)Death16 (3.4%)14 (8.6%)Overall death rate (y) 16 0.4% 0.6% 1 0.4% 0.6% 2.0% 3 1.4% 2.8% 2.3% 5 2.3% 5.8% 10 7.7% 23.3% Time to overall death, mo (median and range) 53.6 (11.0-109.6) 64.6 (11.0-109.6)Liver-related death 12 (2.5%) 12 (7.4%)Liver-related death rate (y) 12 0.2% 0.6% 2 0.4% 1.3% 3.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0-109.6) 80.5 (11.0-109.6)Liver-related death rate (y) 1.4% 4.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0-109.6) 80.5 (11.0-109.6)Extrahepatic death rate (y) 4 (0.8%) 2 (1.2%)Extrahepatic death rate (y) 4 3.3% 2.1% 10 6.8% 22.1% 3.2% 5 1.8% 3.2% 3.2% 10 6.4% 3.2% 3.2% 11 0.4% 3.2% 3.2% 12 0.9% 2.2% 3.2% 13 3.4% 3.4% 3.4% 14 3.4% 3.4% 3.4% 15 3.4% 3.4% 3.4% 16 3.4% 3	3	2.3%	1.5%
Time to extrahepatic cancer, mo (median and range) 34.4 (2.0-82.6) 36.1 (24.7-82.6) Death 16 (3.4%) 14 (8.6%) Overall death rate (r) 0.4% 0.6% 1 0.4% 0.6% 2 1.1% 2.0% 3 1.4% 2.8% 5 2.3% 5.8% 10 7.7% 23.3% Time to overall death, mo (median and range) 53.6 (11.0-109.6) 64.6 (11.0-109.6) Liver-related death 12 (2.5%) 12 (7.4%) 12 (7.4%) Liver-related death rate (r) 0.2% 0.6% 1.3% 2 0.4% 1.3% 3 0.7% 1.3% 5 1.4% 4.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0-109.6) 80.5 (11.0-109.6) 80.5 (11.0-109.6) Extrahepatic death rate (r) 4 (0.8%) 2 (1.2%) 21.2% 3 0.4% 0.6% 2 (1.2%) Extrahepatic death rate (r) 0.4% 0.6% 2 (1.2%) <	5	3.8%	3.5%
Death 16 (3.4%) 14 (8.6%) Overall death rate (y) 0.4% 0.6% 1 0.4% 0.6% 2 1.1% 2.0% 3 1.4% 2.8% 5 2.3% 5.8% 10 7.7% 23.3% Time to overall death, mo (median and range) 53.6 (11.0–109.6) 64.6 (11.0–109.6) Liver-related death 12 (2.5%) 12 (7.4%) Liver-related death rate (y) 1 0.2% 0.6% 2 0.4% 1.3% 1.3% 5 1.4% 4.3% 10 10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0–109.6) 80.5 (11.0–109.6) Extrahepatic death 4 (0.8%) 2 (1.2%) Extrahepatic death rate (y) 1 0.4% 0.6% 2 0.9% 2.2% 3 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	10	4.9%	5.6%
Overall death 16 (3.4%) 14 (8.6%) Overall death rate (y) 0.4% 0.6% 1 0.4% 0.6% 2 1.1% 2.0% 3 1.4% 2.8% 5 2.3% 5.8% 10 7.7% 23.3% Time to overall death, mo (median and range) 53.6 (11.0–109.6) 64.6 (11.0–109.6) Liver-related death 12 (2.5%) 12 (7.4%) Liver-related death rate (y) 1 0.2% 0.6% 2 0.4% 1.3% 1.3% 5 1.4% 4.3% 1.3% 5 1.4% 4.3% 10 10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0–109.6) 80.5 (11.0–109.6) Extrahepatic death rate (y) 2 1.4% 4.3% 10 6.8% 22.1% 11.0–109.6) Extrahepatic death rate (y) 2 2.2% 3.2% 3 1.5% 3.2% 3.2%	Time to extrahepatic cancer, mo (median and range)	34.4 (2.0-82.6)	36.1 (24.7-82.6)
Overall death rate (y)1 0.4% 0.6% 2 1.1% 2.0% 3 1.4% 2.8% 5 2.3% 5.8% 10 7.7% 23.3% Time to overall death, mo (median and range) $53.6 (11.0-109.6)$ $64.6 (11.0-109.6)$ Liver-related death $12 (2.5\%)$ $12 (7.4\%)$ Liver-related death rate (y) 0.4% 0.3% 2 0.4% 1.3% 3 0.7% 1.3% 5 1.4% 4.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) $80.5 (11.0-109.6)$ $80.5 (11.0-109.6)$ Extrahepatic death $4 (0.8\%)$ $2 (1.2\%)$ Extrahepatic death rate (y) 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	Death		
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	Overall death	16 (3.4%)	14 (8.6%)
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	Overall death rate (y)		
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1	0.4%	0.6%
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	2	1.1%	2.0%
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	3	1.4%	2.8%
Time to overall death, mo (median and range) $53.6 (11.0-109.6)$ $12 (2.5%)$ $64.6 (11.0-109.6)$ $12 (7.4%)$ Liver-related death Liver-related death rate (y) 0.2% 0.6% 1 0.2% 0.6% 2 0.4% 1.3% 3 0.7% 1.3% 5 1.4% 4.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) $80.5 (11.0-109.6)$ $80.5 (11.0-109.6)$ Extrahepatic death $4 (0.8\%)$ $2 (1.2\%)$ Extrahepatic death $4 (0.8\%)$ $2 (2.5\%)$ 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	5	2.3%	5.8%
Liver-related death12 (2.5%)12 (7.4%)Liver-related death rate (y)0.2%0.6%20.4%1.3%30.7%1.3%51.4%4.3%10 6.8% 22.1%Time to liver-related death, mo (median and range) $80.5 (11.0-109.6)$ $80.5 (11.0-109.6)$ Extrahepatic death4 (0.8%)2 (1.2%)10.4%0.6%20.9%2.2%31.5%3.2%51.8%4.6%103.4%10%	10	7.7%	23.3%
Liver-related death rate (y) 0.2% 0.6% 2 0.4% 1.3% 3 0.7% 1.3% 5 1.4% 4.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) $80.5 (11.0-109.6)$ $80.5 (11.0-109.6)$ Extrahepatic death $4 (0.8\%)$ $2 (1.2\%)$ Extrahepatic death rate (y) 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	Time to overall death, mo (median and range)	53.6 (11.0–109.6)	64.6 (11.0–109.6)
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	Liver-related death	12 (2.5%)	12 (7.4%)
2 0.4% 1.3% 3 0.7% 1.3% 5 1.4% 4.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0–109.6) 80.5 (11.0–109.6) Extrahepatic death 4 (0.8%) 2 (1.2%) Extrahepatic death rate (y) 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	Liver-related death rate (y)		
3 0.7% 1.3% 5 1.4% 4.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0–109.6) 80.5 (11.0–109.6) Extrahepatic death 4 (0.8%) 2 (1.2%) Extrahepatic death rate (y) 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	1	0.2%	0.6%
5 1.4% 4.3% 10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0–109.6) 80.5 (11.0–109.6) Extrahepatic death 4 (0.8%) 2 (1.2%) Extrahepatic death rate (y) 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%		0.4%	1.3%
10 6.8% 22.1% Time to liver-related death, mo (median and range) 80.5 (11.0–109.6) 80.5 (11.0–109.6) Extrahepatic death 4 (0.8%) 2 (1.2%) Extrahepatic death rate (y) 0.4% 0.6% 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%		0.7%	1.3%
Time to liver-related death, mo (median and range) 80.5 (11.0–109.6) 80.5 (11.0–109.6) Extrahepatic death 4 (0.8%) 2 (1.2%) Extrahepatic death rate (y) 0.4% 0.6% 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	5	1.4%	4.3%
Extrahepatic death 4 (0.8%) 2 (1.2%) 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%		6.8%	22.1%
Extrahepatic death 4 (0.8%) 2 (1.2%) 1 0.4% 0.6% 2 0.9% 2.2% 3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	Time to liver-related death, mo (median and range)	80.5 (11.0–109.6)	80.5 (11.0–109.6)
10.4%0.6%20.9%2.2%31.5%3.2%51.8%4.6%103.4%10%	Extrahepatic death		2 (1.2%)
20.9%2.2%31.5%3.2%51.8%4.6%103.4%10%	Extrahepatic death rate (y)		
3 1.5% 3.2% 5 1.8% 4.6% 10 3.4% 10%	1	0.4%	
51.8%4.6%103.4%10%		0.9%	
10 3.4% 10%	3	1.5%	3.2%
		1.8%	
Time to extrahenatic death mo (median and range) $32.4.(4.2.99.4)$ $32.4.(4.2.99.4)$	10	3.4%	10%
32.4 (4.2-00.4)	Time to extrahepatic death, mo (median and range)	32.4 (4.2–88.4)	32.4 (4.2-88.4)

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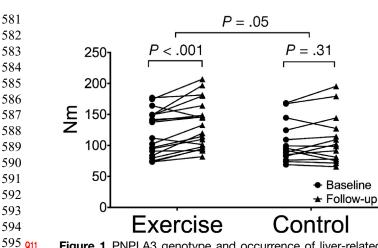


Figure 1. PNPLA3 genotype and occurrence of liver-related events in the entire cohort of NAFLD patients. (*A*) Liver decompensation; (*B*) hepatocellular carcinoma; (*C*) liverrelated death. *P* value by log-rank.

In the subgroup of patients with F3-F4 fibrosis, Cox multivariate analysis showed that *PNPLA3* G variant (HR, 2.66; 95% CI, 1.02–7.13; P = .04) (Supplementary Figure 2B) was the only independent predictor of HCC occurrence (Table 3). Cox multivariate analysis was used to assess the crude rate of HCC at the end of follow-up among PNPLA3 risk classes (Figure 2B).

607 PNPLA3 rs738409 G variant does not predict car608 diovascular events and extrahepatic cancers in NAFLD
609 (Supplementary Material). PNPLA3 rs738409 G variant
610 predicts liver-related death, not overall death, in NAFLD.
611 PNPLA3 G variant was not associated with overall death
612^{Q8} (Supplementary Table 1).

Otherwise, in the entire cohort of NAFLD patients, 613 Cox univariate analysis showed that PNPLA3 G variant 614 (liver death: 0.6% in CC, 2.8% in CG, and 4.7% in GG) 615 was significantly associated with liver-related death (HR, 616 2.42; 95% CI, 1.06–5.52; P = .03) (Figure 1C). PNPLA3 G 617 variant (HR, 3.64; 95% CI, 1.18–11.2; P = .02), PLT 618 <190,000/mmc (HR Inf; 95% CI, NA; P < .001), and F3-619 F4 fibrosis (HR Inf; 95% CI, NA; P < .001) were main-620 tained as significant risk factors in the Cox multivariate 621 analysis (Table 3). 622

In the subgroup of patients with F3-F4 fibrosis, Cox 623 multivariate analysis confirmed PNPLA3 G variant 624 (Supplementary Figure 2C) (HR, 3.64; 95% CI, 1.18–11.2; 625 P = .02) and PLT <190,000/mmc (HR Inf; 95% CI, NA; P 626 < .001) as independent predictors of liver-related death 627 (Table 3). The area under the receiver operating char-628 acteristics curve for this model was 0.833. Cox multi-629 variate analysis was used to assess the crude rate of 630 liver-related death at the end of follow-up among risk 631 classes (Figure 2C). 632

633 When considering the Fine and Gray model for the 634 subdistribution hazard of hepatic mortality and consid-635 ering extrahepatic mortality as a competing risk, we 636 confirmed that *PNPLA3* G variant (sub-HR, 3.16; 95% CI, 637 1.26–9.89; P = .02) significantly increases the cumulative 638 incidence of hepatic mortality, together with PLT

Clinical Gastroenterology and Hepatology Vol. ■, No. ■

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<190,000/mmc (sub-HR, 28.9; 95% CI, 3.23–3577; P = .03) and F3-F4 fibrosis (sub-HR, 32.3; 95% CI, 3.28–3491; P = .03).

Similar results were obtained when considering the subgroup of patients with F3-F4 fibrosis: *PNPLA3* G variant (sub-HR, 3.34; 95% CI, 1.27–9.97; P = .02) and PLT <190,000/mmc (sub-HR, 30.8; 95% CI, 3.22–3462; P = .03).

Discussion

In the present study carried out in a large cohort of European individuals with histologic diagnosis of NAFLD/NASH or clinical diagnosis of compensated cirrhosis due to NAFLD who were prospectively followed in a single center for a median follow-up time of 5 years, we found that *PNPLA3* rs738409 C>G variant predicts the occurrence of liver-related events including death. Notably, this feature was observed in the entire cohort, as well as in the at higher risk subgroup of patients with advanced fibrosis/cirrhosis and after adjusting for clinico-metabolic risk factors.

In our study, liver-related complications, all occurring 662 in patients with baseline advanced fibrosis/cirrhosis, 663 were the most frequently observed events (2.7% HCC 664 and 5.5% LD), followed by extrahepatic cancers (3.6%) 665 and cardiovascular events (1.7%). We observed an 666 overall death rate of 3.4%, mostly because of 667 liver-related causes (2.5%). Recent long-term studies 668 depicting the natural history of biopsy-proven NAFLD 669 patients showed that the relative risk of death was 670 particularly increased for liver-related causes when 671 compared with a control population, even if in terms of 672 absolute numbers the 2 most frequent causes of death 673 were cardiovascular events and extrahepatic cancers.^{7,8} 674 The occurrence rates of hepatic and extrahepatic out-675 comes reported here differ with respect to other studies, 676 perhaps because of the high prevalence of advanced 677 fibrosis and the inclusion in our study of patients with 678 clinical diagnosis of NAFLD cirrhosis, this last potentially 679 representing a selection bias.^{7,8} 680

The most relevant finding from our study is that 681 NAFLD patients carrying the PNPLA3 G allele had a 682 significantly higher risk of experiencing liver-related 683 events including death. Notably, these data were 684 observed in the entire cohort of NAFLD patients, were 685 replicated in those with advanced fibrosis/cirrhosis, 686 were maintained after adjusting for clinico-metabolic 687 risk factors and surrogates of liver function and portal 688 hypertension, and for liver-related death they were 689 confirmed even considering extrahepatic mortality as a 690 competing risk. Our study is a prospective longitudinal Q^9 691 report about the association between PNPLA3 gene 692 variants and liver-related outcomes in NAFLD. Until 693 now, evidence linking PNPLA3 G variant with HCC in 694 NAFLD has come only from case-control cross-sectional 695 studies.⁹ When considering the impact of PNPLA3 696

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 Table 3. Cox Regression Analysis of Factors Associated With Liver Events and Liver-Related Death in the Entire Cohort of

 NAFLD and in Patients With Advanced Fibrosis

$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	Group	Variable	Adjusted model HR (95% CI) P value
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$			Liver decompensation
$\label{eq:approximation} \begin{array}{llllllllllllllllllllllllllllllllllll$	Entire cohort (n = 471)		
$\label{eq:alpha} \begin{tabular}{lllllllllllllllllllllllllllllllllll$		BMI ≥30 kg/m²	0.48 (0.19–1.18) .11
$\label{eq:rescaled_rescale} \begin{tabular}{lllllllllllllllllllllllllllllllllll$		PLT <190*10 ³ /mmc	Inf (NA) <.001
$\label{eq:approximation} \begin{tabular}{ c c c c c c c c c c c c c c c c c c c$		Albumin <4 g/L	2.20 (0.96-5.05) .06
Advanced fibrosis/cirrhosis Inf (NA) < .001		IFG/type 2 diabetes	0.66 (0.27–1.63) .37
AFLD with advanced fibrosis/cirrhosis (n = 162) Age >57 y 7.02 (0.94-52.4) .06 BMI \geq 30 kg/m ² 0.46 (0.19-1.13) .10 Inf (NA) <.001		PNPLA3 CC vs CG vs GG	2.10 (1.03-4.29) .04
$ \begin{split} & \text{BMI} \ge 30 \ \text{kg/m}^2 & 0.46 \ (0.19-1.13) \ .10 \\ & \text{PLT} < 190^{-1}10^3/\text{mmc} & \text{Inf} \ (\text{NA}) < .001 \\ & \text{Albumin} < 4 \ gL \\ & \text{PNPLA3 CC vs CG vs GG} & 2.00 \ (1.01-3.97) \ .04 \\ \end{split} \\ & \text{trive cohort (n = 471)} & \text{Age} > 57 \ y & 4.23 \ (0.49-35.9) \ .18 \\ & \text{BMI} \ge 30 \ \text{kg/m}^2 & 0.87 \ (0.21-3.56) \ .85 \\ & \text{PLT} < 190^{+1}0^3/\text{mmc} & 5.22 \ (0.57-47.5) \ .14 \\ & \text{Albumin} < 4 \ gL & 1.22 \ (0.35-4.47) \ .72 \\ & \text{IFG/type 2 diabetes} & 0.90 \ (0.23-3.56) \ .88 \\ & \text{PNPLA3 CC vs CG vs GG} & 2.68 \ (1.01-7.26) \ .04 \\ & \text{Advanced fibrosis/cirrhosis (n = 162)} & \text{Age} > 57 \ y & 4.26 \ (0.50-35.8) \ .18 \\ & \text{BMI} \ge 30 \ \text{kg/m}^2 & 0.66 \ (0.21-3.51) \ .83 \\ & \text{PLT} < 190^{+1}0^3/\text{mmc} & 5.18 \ (0.57-46.8) \ .14 \\ & \text{Albumin} < 4 \ g/L & 1.24 \ (0.35-4.31) \ .73 \\ & \text{PLT} < 190^{+1}0^3/\text{mmc} & 5.18 \ (0.57-46.8) \ .14 \\ & \text{Albumin} < 4 \ g/L & 1.24 \ (0.35-4.31) \ .73 \\ & \text{PNPLA3 CC vs CG vs GG} & 2.66 \ (1.02-7.13) \ .04 \\ \end{split}$		Advanced fibrosis/cirrhosis	Inf (NA) <.001
$ \begin{split} & \text{BMI} \ge 30 \ \text{kg/m}^2 & 0.46 \ (0.19-1.13) \ .10 \\ & \text{PLT} < 190^{-1}10^3/\text{mmc} & \text{Inf} \ (\text{NA}) < .001 \\ & \text{Albumin} < 4 \ gL \\ & \text{PNPLA3 CC vs CG vs GG} & 2.00 \ (1.01-3.97) \ .04 \\ \end{split} \\ & \text{trive cohort (n = 471)} & \text{Age} > 57 \ y & 4.23 \ (0.49-35.9) \ .18 \\ & \text{BMI} \ge 30 \ \text{kg/m}^2 & 0.87 \ (0.21-3.56) \ .85 \\ & \text{PLT} < 190^{+1}0^3/\text{mmc} & 5.22 \ (0.57-47.5) \ .14 \\ & \text{Albumin} < 4 \ gL & 1.22 \ (0.35-4.47) \ .72 \\ & \text{IFG/type 2 diabetes} & 0.90 \ (0.23-3.56) \ .88 \\ & \text{PNPLA3 CC vs CG vs GG} & 2.68 \ (1.01-7.26) \ .04 \\ & \text{Advanced fibrosis/cirrhosis (n = 162)} & \text{Age} > 57 \ y & 4.26 \ (0.50-35.8) \ .18 \\ & \text{BMI} \ge 30 \ \text{kg/m}^2 & 0.66 \ (0.21-3.51) \ .83 \\ & \text{PLT} < 190^{+1}0^3/\text{mmc} & 5.18 \ (0.57-46.8) \ .14 \\ & \text{Albumin} < 4 \ g/L & 1.24 \ (0.35-4.31) \ .73 \\ & \text{PLT} < 190^{+1}0^3/\text{mmc} & 5.18 \ (0.57-46.8) \ .14 \\ & \text{Albumin} < 4 \ g/L & 1.24 \ (0.35-4.31) \ .73 \\ & \text{PNPLA3 CC vs CG vs GG} & 2.66 \ (1.02-7.13) \ .04 \\ \end{split}$	IAFLD with advanced fibrosis/cirrhosis (n = 162)	Age >57 v	7.02 (0.94–52.4) .06
$\label{eq:product} AFLD with advanced fibrosis/cirrhosis (n = 162) \\ AFLD with advanced fibros$	······································		
$\label{eq:approximation} Albumin <4 g/L 2.06 (0.91-4.68).08 \\ PNPLA3 CC vs CG vs GG 2.00 (1.01-3.97).04 \\ \\ Hepatocellular carcinoma \\ 4.23 (0.49-35.9).18 \\ BMI \ge 30 kg/m^2 0.87 (0.21-3.56).85 \\ PLT <190^{\circ}10^{3}/mmc 5.22 (0.57-47.5).14 \\ Albumin <4 g/L 1.26 (0.35-4.47).72 \\ IFG/type 2 diabetes 0.99 (0.23-3.56).88 \\ PNPLA3 CC vs CG vs GG 2.68 (1.01-7.26).04 \\ Advanced fibrosis/cirrhosis (n = 162) \\ AFLD with advanced fibrosis/cirrhosis (n = 162) \\ Age > 57 y \\ PLT <190^{\circ}10^{3}/mmc \\ Albumin <4 g/L \\ 1.22 (0.35-4.23).75 \\ PNPLA3 CC vs CG vs GG \\ 3.64 (1.18-11.2).02 \\ Advanced fibrosis/cirrhosis (n = 162) \\ Age > 57 y \\ Albumin <4 g/L \\ Advanced fibrosis/cirrhosis (n = 162) \\ Age > 57 y \\ Albumin <4 g/L \\ Advanced fibrosis/cirrhosis (n = 162) \\ Age > 57 y \\ Albumin <4 g/L \\ Albumin <4 $			
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there cohort (n = 471) Age >57 y 4.23 (0.49-35.9) .18 BMI \geq 30 kg/m ² 0.87 (0.21-3.56) .85 PLT < 190°10 ³ /mmc 5.22 (0.57-47.5) .14 Albumin <4 g/L			· · · · · ·
there cohort (n = 471) Age >57 y 4.23 (0.49-35.9) .18 BMI \geq 30 kg/m ² 0.87 (0.21-3.56) .85 PLT < 190°10 ³ /mmc 5.22 (0.57-47.5) .14 Albumin <4 g/L			Henatocellular carcinoma
$\label{eq:approximation} \begin{tabular}{lllllllllllllllllllllllllllllllllll$	Entire cohort (n $-$ 471)	$\Delta c = 57 v$	•
$\label{eq:product} AFLD with advanced fibrosis/cirrhosis (n = 162) \\ AFLD with advanced fibros$			
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$\label{eq:approx} \begin{tabular}{lllllllllllllllllllllllllllllllllll$. ,
$\label{eq:advanced} \begin{tabular}{lllllllllllllllllllllllllllllllllll$			
Advanced fibrosis/cirrhosis Inf (NA) .001 AFLD with advanced fibrosis/cirrhosis (n = 162) Age >57 y 4.26 (0.50–35.8) .18 BMI $\ge 30 \text{ kg/m}^2$ 0.86 (0.21–3.51) .83 PLT <190*10 ³ /mmc 5.18 (0.57–46.8) .14 Albumin <4 g/L			
AFLD with advanced fibrosis/cirrhosis (n = 162) Age >57 y 4.26 (0.50–35.8) .18 BMI \geq 30 kg/m² 0.86 (0.21–3.51) .83 PLT <190*10 ³ /mmc 5.18 (0.57–46.8) .14 Albumin <4 g/L			
$\begin{array}{llllllllllllllllllllllllllllllllllll$			
$\label{eq:plt_state} AFLD with advanced fibrosis/cirrhosis (n = 162) \\ AFLD with advanced fibr$	NAFLD with advanced fibrosis/cirrhosis (n = 162)		4.26 (0.50–35.8) .18
$\label{eq:AFLD} \begin{tabular}{lllllllllllllllllllllllllllllllllll$			0.86 (0.21–3.51) .83
$\label{eq:product} \begin{tabular}{lllllllllllllllllllllllllllllllllll$			
		PNPLA3 CC vs CG vs GG	2.66 (1.02-7.13) .04
$\label{eq:approx} \begin{tabular}{lllllllllllllllllllllllllllllllllll$			
$\begin{tabular}{lllllllllllllllllllllllllllllllllll$	Entire cohort (n = 471)		
PNPLA3 CC vs CG vs GG $3.64 (1.18-11.2) .02$ Advanced fibrosis/cirrhosis Inf (NA) <.001			
Advanced fibrosis/cirrhosis Inf (NA) <.001 AFLD with advanced fibrosis/cirrhosis (n = 162) Age >57 y 4.38 (0.55–34.7) .16 PLT <190*10 ³ /mmc Inf (NA) <.001		Albumin <4 g/L	
AFLD with advanced fibrosis/cirrhosis (n = 162) Age >57 y 4.38 (0.55–34.7) .16 PLT <190*10 ³ /mmc Inf (NA) <.001			· ,
PLT <190*10 ³ /mmc Inf (NA) <.001 Albumin <4 g/L 1.22 (0.35–4.23) .75		Advanced fibrosis/cirrhosis	Inf (NA) <.001
Albumin <4 g/L 1.22 (0.35–4.23) .75	IAFLD with advanced fibrosis/cirrhosis (n = 162)	Age >57 y	
			Inf (NA) <.001
PNPLA3 CC vs CG vs CG 3 64 (1 18–11 2) 02			
		PNPLA3 CC vs CG vs GG	3.64 (1.18–11.2) .02

BMI, body mass index; CI, confidence interval; HR, hazard ratio; IFG, impaired fasting glucose; NAFLD, nonalcoholic fatty liver disease; PLT, platelet count.

variants on LD and death, a recent study reported that *PNPLA3* GG homozygous patients are at increased risk of both LD and death.¹⁸ However, this study was performed in a more advanced cohort of patients with cirrhosis complicated by portal hypertension as assessed by hepatic venous pressure gradient and mostly due to viral infections or alcohol, with only a minority of cases (37 of 372) with a liver disease secondary to NAFLD.¹⁸

In our study we found that all patients who developed hepatic events and liver-related death had F3 fibrosis or cirrhosis at baseline. Our data completely agree with natural history studies and a recent metaanalysis that identified the severity of liver fibrosis as the main predictor of prognosis in NAFLD.^{6–8} In our cohort, HCC developed only in patients with advanced liver fibrosis, not in those with milder liver disease (F0-F2 fibrosis). Nevertheless, clinical studies and a metaanalysis support the possibility that HCC may develop in non-cirrhotic NASH and suggest that this is more frequent in NASH as compared with liver diseases due to other etiologies¹⁹ where the risk is low, as recently reported in 2 Asian and Western large cohort studies.^{20,21} In keeping with those findings, the young mean age of our population and a moderately long follow-up can explain why we did not observe HCC occurrence in NAFLD patients with F0-F2 fibrosis.

Finally, we also found that the presence of cirrhosis is associated with a lower risk of developing extrahepatic cancers. These data agree with evidence that in NAFLD 813

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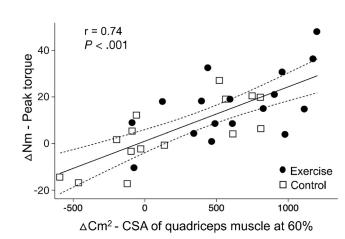


Figure 2. Crude rate of liver-related events at end of followup among risk classes in subgroup of NAFLD patients with F3-F4 fibrosis. (*A*) Liver decompensation; (*B*) hepatocellular carcinoma; (*C*) liver-related death.

cirrhotic patients, as compared with NAFLD with F3 fibrosis, the risk of liver-related complications is higher and the risk for extrahepatic cancers is lower.⁶

835 Although this study was not designed to fully 836 clarify the pathogenic link between PNPLA3 variants 837 and the occurrence of liver-related events in NAFLD, 838 several hypotheses may be put forward to mechanis-839 tically explain this association. The wild-type PNPLA3 840 protein hydrolyzes triglycerides and retinyl esters, 841 whereas the rs738409 C>G variant results in a loss of function, leading to the accumulation of triglycerides 842 843 and retinyl esters in lipid droplets within both hepatocytes and hepatic stellate cells.²²⁻²⁴ This may favor 844 845 liver damage and prevent the release of extracellular 846 proteins, which, especially in hepatic stellate cells, 847 could otherwise counteract the process of fibrosis 848 progression, portal hypertension, and tumorigenesis.²⁵ 849 The *PNPLA3* variant also exerts these prosteatogenic and profibrogenic effects in patients with alcoholic 850 851 liver disease, where alcohol abuse can lead to 852 increased fatty acid disposal by reduced beta oxida-853 tion, reduced assembly/secretion of very low density 854 lipoprotein, and increased fatty acid synthesis and to retention of retinoids.²⁶ The genetic similarities in 855 856 lipid metabolism between alcoholic liver disease and 857 NALFD account for studies also reporting a link be-858 tween PNPLA3 genotype and liver damage in alcoholic liver disease,²⁷ as well as association with HCC,²⁸ 859 LD,¹⁸ and liver-related death.¹⁸ 860

861 From a clinical point of view our study confirms 862 that the assessment of severe liver fibrosis is critical 863 to the evaluation of NAFLD because patients with F3-F4 fibrosis are at risk for liver-related events 864 865 including death. In these patients, PNPLA3 genotype 866 together with classic markers of liver function and 867 portal hypertension/fibrosis such as PLT can help 868 stratify the risk for HCC, LD, and liver-related death. These data, worthy of further validation in indepen-869 870 dent cohorts, could help to personalize follow-up,

Clinical Gastroenterology and Hepatology Vol. ■, No. ■

leading to optimization of healthcare and economic 871 resources. 872

The main limitation of this study lies in the potentially 873 limited external validity of the results for different pop-874 ulations and settings. Another limitation is the relatively 875 low number of observed events and the relatively short 876 follow-up. Our results are consistent with a lower rate of 877 liver-related events observed in advanced liver disease of 878 other etiologies compared with NAFLD.²⁹ Finally, we did 879 not include in our analysis the assessment of progression 880 in some patients from F0-F2 to F3-F4 fibrosis; this issue 881 potentially limits the interpretation of our results. 882

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In conclusion, this study in a cohort of European patients with histologic diagnosis of NAFLD or clinical diagnosis of compensated NAFLD-related cirrhosis showed that HCC and LD mostly occur in patients with advanced fibrosis/cirrhosis and that *PNPLA3* G variant is able to lead to a significant increase in the risk of liver-related events including death. If further validated, these data could help to personalize prognosis and follow-up in NAFLD.

Supplementary Data

Note: to access the supplementary materials accompanying this article, visit the online version of *Clinical Gastroenterology* and *Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2019.08.011.

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Clinical Gastroenterology and Hepatology Vol. ■, No. ■

Supplementary Material

1047 Body mass index was calculated on the basis of 1048 weight in kilograms and height in meters. Obesity was 1049 defined as body mass index \geq 30 kg/m². The diagnosis of 1050 impaired fasting glucose and type 2 diabetes was based 1051 on the revised criteria of the American Diabetes Associ-1052 ation, using values of fasting blood glucose from 110 to 1053 125 and \geq 126 mg/dL, respectively.¹ In patients with a 1054 previous diagnosis of type 2 diabetes, current therapy 1055 with insulin or oral hypoglycemic agents was docu-1056 mented. The same day a 12-hour overnight fasting blood 1057 sample was drawn to determine serum levels of aspar-1058 tate aminotransferase, alanine aminotransferase, PLT, 1059 albumin, total bilirubin, total cholesterol, triglycerides, 1060 and plasma glucose concentration.

1061 The proportional subdistribution hazard model by 1062 Fine and Gray¹⁶ was fitted to estimate the effect of 1063 covariates on the cumulative incidence of hepatic mor-1064 tality, while extrahepatic mortality was considered as a 1065 competing risk. The subdistribution hazard for a hepatic 1066 mortality is defined as the instantaneous rate of occur-1067 rence of hepatic mortality in patients who have not yet 1068 experienced such an event, that is by maintaining in the 1069 risks set all those patients who are either currently 1070 event-free or who have previously experienced the 1071

1103 competing risk. One main advantage of the approach by Fine and Gray on the classic cause-specific hazard 1104 regression model is that the former is also a model for 1105 the cumulative incidence function, a basic quantity used 1106 to estimate the cumulative incidence of occurrence of an 1107 event while taking competing risks into account. This 1108 means there is concordance between the sign of the 1109 subdistribution hazard regression parameters and the 1110 cumulative incidence function; positive regression co-1111 efficients indicate higher cumulative incidence functions, 1112 and negative coefficients mean lower cumulative inci-1113 dence functions. Note this property does not hold for 1114 cause-specific hazard models (Austin and Fine, 2017). Q12 1115 SAS macros pshreg¹⁷ and phreg were used to estimate 1116 the Fine and Gray model according to the method pro-1117 posed by Geskus (2011), and the Firth (1993) correction 1118 was used to stabilize parameter estimation due to rare 1119 events. Proportional hazard assumption was verified by 1120 formal tests on the correlation between Schoenfeld-type 1121 residuals and the log of time. 1122 1123

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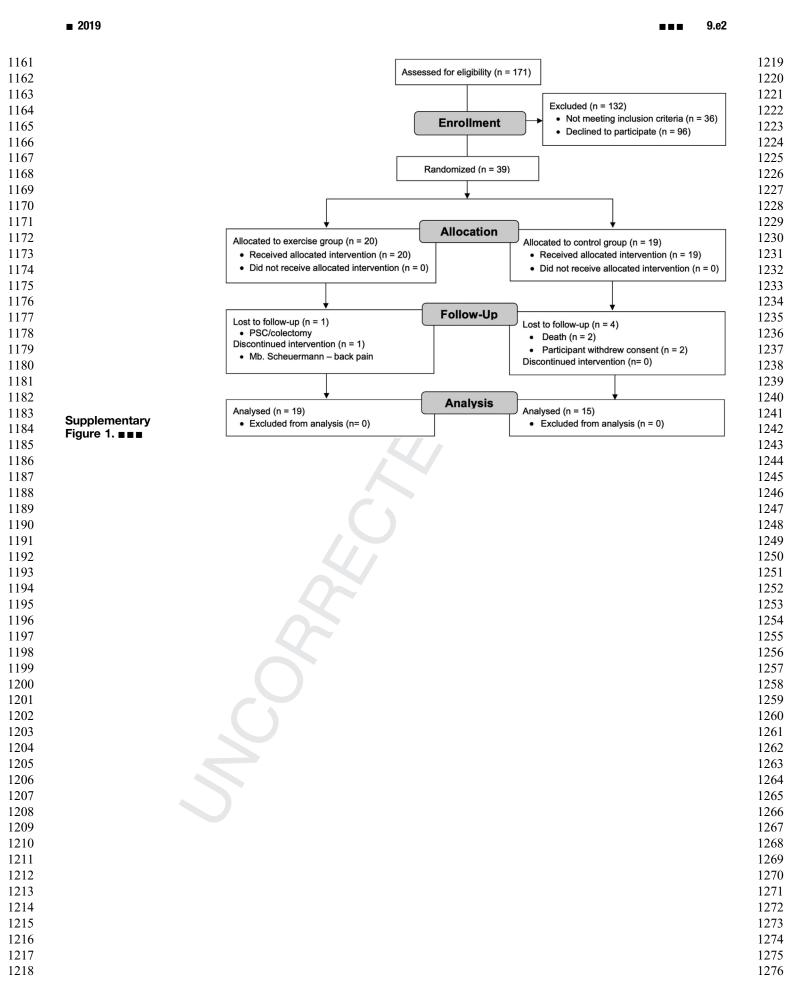
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1084 Supplementary Table 1. Multivariate Cox Regression Analyses of Factors Associated With Extrahepatic Events and Overall 1085 Death in the Entire Cohort of NAFLD Patients

Group	Variable	Adjusted model HR (95% Cl) <i>P</i> value
		Cardiovascular events
Entire cohort (n = 471)	Age >57 y	8.01 (1.48–43.3) .01
	Total cholesterol	1.01 (0.99–1.02) .09
	IFG/type 2 diabetes	2.64 (0.51–13.5) .24
		Extrahepatic cancers
Entire cohort (n = 471)	Age >57 y	2.53 (0.97–6.56) .05
		Overall death
Entire cohort (n $=$ 471)	Age >57 y	3.10 (0.76–12.5) .11
	PLT <190*10 ³ /mmc	5.45 (1.08–27.3) .03
	Albumin <4 g/L	1.75 (0.58–5.24) .31
	Advanced fibrosis/cirrhosis	4.12 (0.75–22.7) .10





9.e3 Grimaudo et al

Clinical Gastroenterology and Hepatology Vol. ■, No. ■

