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

Stefania Grimaudo,[*,a] Rosaria Maria Pipitone,[*,a] Grazia Pennisi,[*] Ciro Celsa,[*] Calogero Cammà,[*] Vito Di Marco,[*] Maria Rosa Barcellona,[*] Roberta Boemi,[*] Marco Enea,[‡] Aurora Giannetti,[*] Federica Spatola,[*] Giulio Marchesini,[§] Antonio Craxì,[*] and Salvatore Petta[*]

*Sezione di Gastroenterologia e Epatologia, PROMISE, University of Palermo, Palermo, Italy; [‡]Dipartimento di Scienze Economiche, Aziendali e Statistiche, University of Palermo, Palermo, Italy; and [§]SSD Malattie del Metabolismo e Dietetica Clinica, Università "Alma Mater", Bologna, Italy

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, 1.18–11.2; $P = .02$). 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.

[a]Authors share co-first authorship.

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.

1542-3565/$36.00
https://doi.org/10.1016/j.cgh.2019.08.011

Nonalcoholic fatty liver disease (NAFLD) affects 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]

Two retrospective cohort studies and a meta-analysis of the natural history of NAFLD patients have clearly shown that the severity of liver fibrosis estimated by histology is the strongest predictor not only of liver-related complications but also of death due to extrahepatic diseases.[5,6]

In this complex picture, there is also growing evidence that genetic background can affect the risk and severity of NAFLD. The rs738409 C>G variant in *PNPLA3* gene has been identified by genome-wide association studies as an independent genetic risk factor for NAFLD.[7] This finding has been validated in cohorts from different ethnic ancestry. Furthermore, in cross-sectional studies the variant has been associated with the full spectrum of severity of liver damage among NAFLD patients, as well as with the presence of HCC.[8,9] However, prospective data demonstrating the impact of the variant on the occurrence of hepatic and extrahepatic events and death are lacking.

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

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.

Methods

Patient Selection

We analyzed data from 471 patients prospectively recruited at the Gastrointestinal and Liver Unit of the Palermo University Hospital from March 2004 to December 2018 with histologic diagnosis of NAFLD or clinical diagnosis of fully compensated Child-Pugh A5 cirrhosis due to NAFLD. Specifically, in patients without histology, cirrhosis was diagnosed by liver stiffness measurement >11.5 KPa for M probe[10] or >11 KPa for XL probe,[11] and the diagnosis of NAFLD required the presence of ultrasonography-assessed steatosis plus at least 1 criterion of metabolic syndrome (obesity, diabetes, arterial hypertension, dyslipidemia). Patients were included if they had blood samples available for genetic analysis and a follow-up of at least 6 months. The flow chart of the study is depicted in Supplementary Figure 1. Other causes of liver disease were ruled out, including 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 classification[12] was used to grade steatosis, lobular inflammation, and hepatocellular 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.

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

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

During follow-up, hepatic and extrahepatic events were recorded. Liver-related events were categorized as either liver decompensation (LD) (occurrence of ascites and/or bleeding varices and/or encephalopathy and/or jaundice) or development of HCC. Patients who had a diagnosis of HCC during follow-up were evaluated for available therapies (surgical resection, radiofrequency ablation, transarterial chemoembolization, or treatment with sorafenib starting in 2007), as indicated in the guidelines.[13] They were also evaluated for liver transplantation, as were patients who experienced LD, when indicated.[15] Extrahepatic events were categorized as either cardiovascular events (stroke, transient ischemic attack, myocardial infarction, unstable angina) or extrahepatic cancers. Evidence of extrahepatic events was provided by clinical charts from emergency areas and/or hospitalization. Death was also recorded and classified according to associated events (liver-related, including liver transplantation, or unrelated). The study was closed in December 2018. Patients who did not provide clinical data after June 2018 were considered dropouts and were censored at the last available visit.

Statistics

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 follow-up.

Continuous variables were summarized as mean ± standard deviation and categorical variables as frequency and percentage. The proportion of patients who experienced events was evaluated with Kaplan-Meier curves. To account for competing risks, cause-specific hazards were modeled via Cox regression to identify baseline variables associated with the occurrence of LD, HCC, cardiovascular events, extrahepatic cancer, overall death, and liver-related death. Moreover, the proportional subdistribution hazard model by Fine and Gray[16] was fitted to estimate the effect of covariates on the cumulative incidence of hepatic mortality, whereas extrahepatic mortality was considered as a competing risk (Supplementary Material). Covariates used for the multivariate analyses were gender, age ≥57 years (second tertile in the population), *PNPLA3* rs738409 genotype (additive model), obesity, impaired fasting glucose/diabetes, arterial hypertension, platelet count (PLT) <190,000 (second tertile in the population), albumin <4 g/dL (second tertile in the population), and F3-F4 fibrosis. They were chosen on the basis of their significance in univariate analysis ($P < .10$). Variables in the 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 ± 14.0 vs 50.2 ± 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-related events. In the total cohort of 471 patients, 13 (2.7%) developed HCC, and 26 (5.5%) developed LD (19 ascites, 5 encephalopathy, and 2 variceal bleeding; 3 cases developed after HCC occurrence) (Table 2). The 1-, 2-, 3-, 5-, and 10-year actuarial incidence rates of LD were approximately twice as high as the rates of HCC (Table 2). All liver-related events were observed in patients with F3-F4 fibrosis, and in this subgroup the actuarial incidence rates were again 2 times higher for

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, kg/m^2	30.3 ± 5.2	29.5 ± 5.1	31.8 ± 5.1	<.001
BMI ≥30	48.5%	40.3%	63.9%	<.001
ALT, *IU/L*	72.6 ± 51.0	77.4 ± 51.9	63.5 ± 48.0	.005
PLT, $10^3/mmc$	219.9 ± 77.7	241.9 ± 66.3	177.5 ± 80.5	<.001
Albumin, *g/L*	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, *mo*	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	37.4/31.9/30.7[a]	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 ± standard deviation, or as percentage of cases (%).
BMI, body mass index; IFG, impaired fasting glucose; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.
[a]Data referred to 417 patients.
[b]Data 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 transplantation) and 4 of extrahepatic causes (2 of cardiovascular 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, respectively (Table 2). All liver-related deaths were recorded in patients with baseline F3-F4 fibrosis, where the incidence rates were as high as 22.1% at 10 years, respectively (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 1*A*). *PNPLA3* G 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 2*A*) and PLT <190,000/mmc (HR Inf; 95% CI, 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 2*A*).

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 1*B*). *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

	Entire cohort of NAFLD, N = 471	NAFLD with advanced fibrosis/cirrhosis, N = 162
Liver events		
Liver decompensation	26 (5.5%)	26 (16.1%)
Liver decompensation rate (*y*)		
1	0.4%	3.1%
2	2.0%	5.8%
3	3.5%	10.5%
5	4.4%	13.5%
10	8.2%	26.7%
Time to liver decompensation, *mo* (median and range)	34.8 (6.6–92.6)	34.8 (6.6–92.6)
Hepatocellular carcinoma	13 (2.7%)	13 (8.0%)
Hepatocellular carcinoma rate (*y*)		
1	0.2%	1.3%
2	0.9%	2.7%
3	1.1%	3.4%
5	3.0%	9.3%
10	4.2%	13.5%
Time to hepatocellular carcinoma, *mo* (median and range)	40.6 (5.8–90.3)	40.6 (5.8–90.3)
Extrahepatic events		
Cardiovascular events	8 (1.7%)	4 (2.5%)
Cardiovascular event rate (*y*)		
1	0.2%	0%
2	0.4%	1.3%
3	0.7%	1.3%
5	0.7%	1.3%
10	4.3%	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%
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		
Overall death	16 (3.4%)	14 (8.6%)
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*)		
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 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%
Time to extrahepatic death, *mo* (median and range)	32.4 (4.2–88.4)	32.4 (4.2–88.4)

NAFLD, nonalcoholic fatty liver disease.

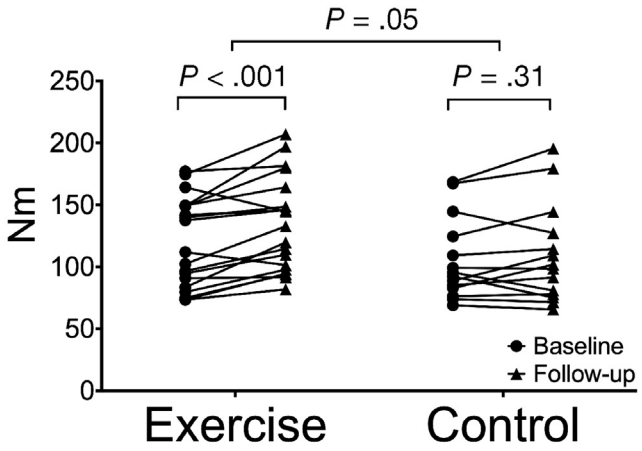


Figure 1. PNPLA3 genotype and occurrence of liver-related events in the entire cohort of NAFLD patients. (*A*) Liver decompensation; (*B*) hepatocellular carcinoma; (*C*) liver-related death. *P* value by log-rank.

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 2*B*) 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 2*B*).

PNPLA3 rs738409 G variant does not predict cardiovascular events and extrahepatic cancers in NAFLD (Supplementary Material). *PNPLA3* rs738409 G variant predicts liver-related death, not overall death, in NAFLD. *PNPLA3* G variant was not associated with overall death (Supplementary Table 1).

Otherwise, in the entire cohort of NAFLD patients, Cox univariate analysis showed that *PNPLA3* G variant (liver death: 0.6% in CC, 2.8% in CG, and 4.7% in GG) was significantly associated with liver-related death (HR, 2.42; 95% CI, 1.06–5.52; $P = .03$) (Figure 1*C*). *PNPLA3* G variant (HR, 3.64; 95% CI, 1.18–11.2; $P = .02$), 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 the Cox multivariate analysis (Table 3).

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

When considering the Fine and Gray model for the subdistribution hazard of hepatic mortality and considering extrahepatic mortality as a competing risk, we confirmed that *PNPLA3* G variant (sub-HR, 3.16; 95% CI, 1.26–9.89; $P = .02$) significantly increases the cumulative incidence of hepatic mortality, together with PLT <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 in patients with baseline advanced fibrosis/cirrhosis, were the most frequently observed events (2.7% HCC and 5.5% LD), followed by extrahepatic cancers (3.6%) and cardiovascular events (1.7%). We observed an overall death rate of 3.4%, mostly because of liver-related causes (2.5%). Recent long-term studies depicting the natural history of biopsy-proven NAFLD patients showed that the relative risk of death was particularly increased for liver-related causes when compared with a control population, even if in terms of absolute numbers the 2 most frequent causes of death were cardiovascular events and extrahepatic cancers.[7,8] The occurrence rates of hepatic and extrahepatic outcomes reported here differ with respect to other studies, perhaps because of the high prevalence of advanced fibrosis and the inclusion in our study of patients with clinical diagnosis of NAFLD cirrhosis, this last potentially representing a selection bias.[7,8]

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

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

Group	Variable	Adjusted model HR (95% CI) *P* value
		Liver decompensation
Entire cohort (n = 471)	Age >57 y	6.33 (0.83–48.0) .07
	BMI ≥30 kg/m^2	0.48 (0.19–1.18) .11
	PLT <190*10^3/mmc	Inf (NA) <.001
	Albumin <4 g/L	2.20 (0.96–5.05) .06
	IFG/type 2 diabetes	0.66 (0.27–1.63) .37
	PNPLA3 CC vs CG vs GG	2.10 (1.03–4.29) .04
	Advanced fibrosis/cirrhosis	Inf (NA) <.001
NAFLD with advanced fibrosis/cirrhosis (n = 162)	Age >57 y	7.02 (0.94–52.4) .06
	BMI ≥30 kg/m^2	0.46 (0.19–1.13) .10
	PLT <190*10^3/mmc	Inf (NA) <.001
	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
Entire cohort (n = 471)	Age >57 y	4.23 (0.49–35.9) .18
	BMI ≥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	1.26 (0.35–4.47) .72
	IFG/type 2 diabetes	0.90 (0.23–3.56) .88
	PNPLA3 CC vs CG vs GG	2.68 (1.01–7.26) .04
	Advanced fibrosis/cirrhosis	Inf (NA) .001
NAFLD with advanced fibrosis/cirrhosis (n = 162)	Age >57 y	4.26 (0.50–35.8) .18
	BMI ≥30 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	1.24 (0.35–4.31) .73
	PNPLA3 CC vs CG vs GG	2.66 (1.02–7.13) .04
		Liver-related death
Entire cohort (n = 471)	Age >57 y	4.38 (0.55–34.7) .16
	PLT <190*10^3/mmc	Inf (NA) <.001
	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	Inf (NA) <.001
NAFLD 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	1.22 (0.35–4.23) .75
	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 meta-analysis 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 meta-analysis 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 etiologies[19] 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

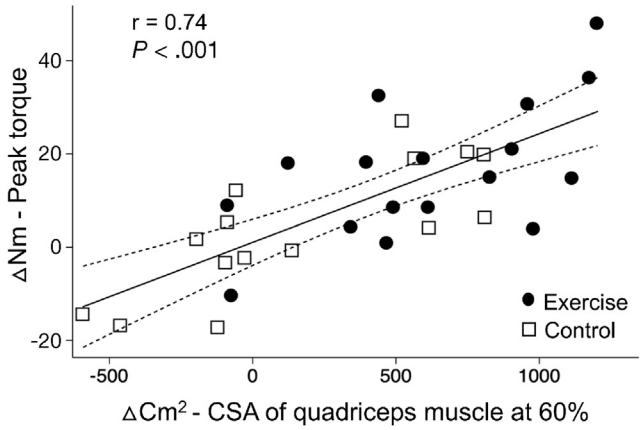


Figure 2. Crude rate of liver-related events at end of follow-up among risk classes in subgroup of NAFLD patients with F3-F4 fibrosis. (*A*) Liver decompensation; (*B*) hepatocellular carcinoma; (*C*) liver-related death.

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]

Although this study was not designed to fully clarify the pathogenic link between *PNPLA3* variants and the occurrence of liver-related events in NAFLD, several hypotheses may be put forward to mechanistically explain this association. The wild-type PNPLA3 protein hydrolyzes triglycerides and retinyl esters, whereas the rs738409 C>G variant results in a loss of function, leading to the accumulation of triglycerides and retinyl esters in lipid droplets within both hepatocytes and hepatic stellate cells.[22–24] This may favor liver damage and prevent the release of extracellular proteins, which, especially in hepatic stellate cells, could otherwise counteract the process of fibrosis progression, portal hypertension, and tumorigenesis.[25] The *PNPLA3* variant also exerts these prosteatogenic and profibrogenic effects in patients with alcoholic liver disease, where alcohol abuse can lead to increased fatty acid disposal by reduced beta oxidation, reduced assembly/secretion of very low density lipoprotein, and increased fatty acid synthesis and to retention of retinoids.[26] The genetic similarities in lipid metabolism between alcoholic liver disease and NALFD account for studies also reporting a link between *PNPLA3* genotype and liver damage in alcoholic liver disease,[27] as well as association with HCC,[28] LD,[18] and liver-related death.[18]

From a clinical point of view our study confirms that the assessment of severe liver fibrosis is critical to the evaluation of NAFLD because patients with F3-F4 fibrosis are at risk for liver-related events including death. In these patients, *PNPLA3* genotype together with classic markers of liver function and portal hypertension/fibrosis such as PLT can help stratify the risk for HCC, LD, and liver-related death. These data, worthy of further validation in independent cohorts, could help to personalize follow-up, leading to optimization of healthcare and economic resources.

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

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. Q13

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.

References

1. Younossi ZM, et al. Global epidemiology of non-alcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence and outcomes. Hepatology 2016;64:73–84. Q10
2. Dyson J, et al. Hepatocellular cancer: the impact of obesity, type 2 diabetes and a multidisciplinary team. J Hepatol 2014; 60:110–117.
3. Wong RJ, et al. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. Gastroenterology 2015; 148:547–555.
4. Adams LA, et al. Non-alcoholic fatty liver disease and its relationship with cardiovascular disease and other extrahepatic diseases. Gut 2017;66:1138–1153.
5. Dulai PS, et al. Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: systematic review and meta-analysis. Hepatology 2017;65:1557–1565.
6. Vilar-Gomez E, et al. Fibrosis severity as a determinant of cause-specific mortality in patients with advanced nonalcoholic fatty liver disease: a multi-national cohort study. Gastroenterology 2018;155:443–457.e17.
7. Romeo S, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008; 40:1461–1465.
8. Valenti L, et al. Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. Hepatology 2010;51:1209–1217.
9. Liu YL, et al. Carriage of the PNPLA3 rs738409 C >G polymorphism confers an increased risk of non-alcoholic fatty liver

disease associated hepatocellular carcinoma. J Hepatol 2014; 61:75–81.
10. Wong VW, et al. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. Hepatology 2010;51:454–462.
11. Wong VW, et al. Liver stiffness measurement using XL probe in patients with nonalcoholic fatty liver disease. Am J Gastroenterol 2012;107:1862–1871.
12. Kleiner DE, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005; 411:313–321.
13. EASL clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2018;69:182–236.
14. de Franchis R, Baveno VI Faculty. Expanding consensus in portal hypertension: report of the Baveno VI Consensus Workshop—stratifying risk and individualizing care for portal hypertension. J Hepatol 2015;63:743–752.
15. Cillo U, et al. A multistep, consensus-based approach to organ allocation in liver transplantation: toward a "blended principle model. Am J Transplant 2015;15:2552–2561.
16. Fine JP, et al. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc 1999; 94:496–509.
17. Kohl M, et al. PSHREG: a SAS macro for proportional and nonproportional subdistribution hazards regression. Comput Methods Programs Biomed 2015;118:218–233.
18. Mandorfer M, et al. Impact of patatin-like phospholipase domain containing 3 rs738409 G/G genotype on hepatic decompensation and mortality in patients with portal hypertension. Aliment Pharmacol Ther 2018;48:451–459.
19. Stine JG, et al. Systematic review with meta-analysis: risk of hepatocellular carcinoma in non-alcoholic steatohepatitis without cirrhosis compared to other liver diseases. Aliment Pharmacol Ther 2018;48:696–703.
20. Kawamura Y, et al. Large-scale long-term follow-up study of Japanese patients with non-alcoholic fatty liver disease for the onset of hepatocellular carcinoma. Am J Gastroenterol 2012; 107:253–261.
21. Kanwal F, et al. Risk of hepatocellular cancer in patients with non-alcoholic fatty liver disease. Gastroenterology 2018; 155:1828–1837.e2.
22. Huang Y, et al. Expression and characterization of a PNPLA3 protein isoform (I148M) associated with nonalcoholic fatty liver disease. J Biol Chem 2011;286:37085–37093.
23. Pingitore P, et al. Recombinant PNPLA3 protein shows triglyceride hydrolase activity and its I148M mutation results in loss of function. Biochim Biophys Acta 2014;1841:574–580.
24. Pirazzi C, et al. PNPLA3 has retinyl-palmitate lipase activity in human hepatic stellate cells. Hum Mol Genet 2014;23:4077–4085.
25. Pingitore P, et al. PNPLA3 overexpression results in reduction of proteins predisposing to fibrosis. Hum Mol Genet 2016; 25:5212–5222.
26. Stickel F, et al. The genetics of alcohol dependence and alcohol-related liver disease. J Hepatol 2017;66:195–211.
27. Buch S, et al. A twostage genome-wide association study confirms PNPLA3 and identifies TM6SF2 and MBOAT7 as novel risk loci for alcohol-related cirrhosis. Nat Genet 2015; 47:1443–1448.
28. Trépo E, et al. PNPLA3 (rs738409 C>G) is a common risk variant associated with hepatocellular carcinoma in alcoholic cirrhosis. Hepatology 2012;55:1307–1308.
29. Bhala N, et al. The natural history of nonalcoholic fatty liver disease with advanced fibrosis or cirrhosis: an international collaborative study. Hepatology 2011;54:1208–1216.

Reprint requests
Address requests for reprints to: Salvatore Petta, Section of Gastroenterology and Hepatology, PROMISE, Policlinico Universitario Paolo Giaccone, Piazza delle Cliniche, 2, 90127 Palermo, Italy. e-mail: salvatore.petta@unipa.it; fax: +39-091-655-2156. Q2Q3

Conflicts of interest Q4
The authors disclose no conflicts.

Supplementary Material

Body mass index was calculated on the basis of weight in kilograms and height in meters. Obesity was defined as body mass index $\geq$30 kg/m^2. The diagnosis of impaired fasting glucose and type 2 diabetes was based on the revised criteria of the American Diabetes Association, using values of fasting blood glucose from 110 to 125 and $\geq$126 mg/dL, respectively.[1] In patients with a previous diagnosis of type 2 diabetes, current therapy with insulin or oral hypoglycemic agents was documented. The same day a 12-hour overnight fasting blood sample was drawn to determine serum levels of aspartate aminotransferase, alanine aminotransferase, PLT, albumin, total bilirubin, total cholesterol, triglycerides, and plasma glucose concentration.

The proportional subdistribution hazard model by Fine and Gray[16] was fitted to estimate the effect of covariates on the cumulative incidence of hepatic mortality, while extrahepatic mortality was considered as a competing risk. The subdistribution hazard for a hepatic mortality is defined as the instantaneous rate of occurrence of hepatic mortality in patients who have not yet experienced such an event, that is by maintaining in the risks set all those patients who are either currently event-free or who have previously experienced the competing risk. One main advantage of the approach by Fine and Gray on the classic cause-specific hazard regression model is that the former is also a model for the cumulative incidence function, a basic quantity used to estimate the cumulative incidence of occurrence of an event while taking competing risks into account. This means there is concordance between the sign of the subdistribution hazard regression parameters and the cumulative incidence function; positive regression coefficients indicate higher cumulative incidence functions, and negative coefficients mean lower cumulative incidence functions. Note this property does not hold for cause-specific hazard models (Austin and Fine, 2017). Q12 SAS macros pshreg[17] and phreg were used to estimate the Fine and Gray model according to the method proposed by Geskus (2011), and the Firth (1993) correction was used to stabilize parameter estimation due to rare events. Proportional hazard assumption was verified by formal tests on the correlation between Schoenfeld-type residuals and the log of time.

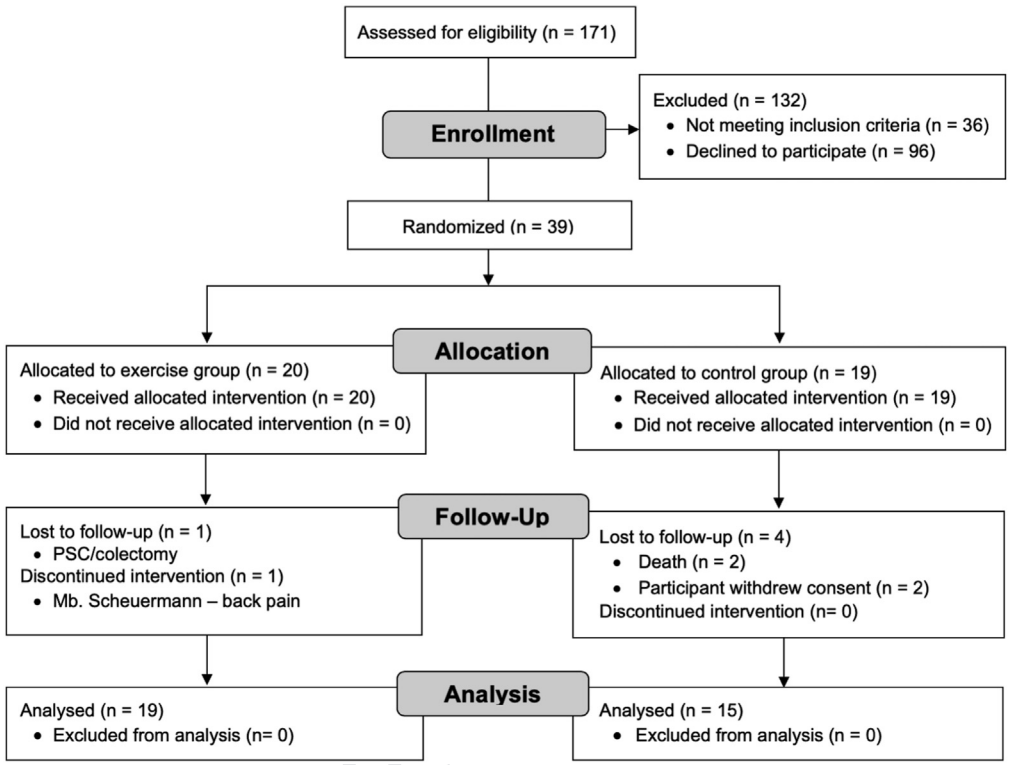
Reference

1. American Diabetes Association. Report of the expert committee on the diagnosis and classification of diabetes mellitus. American Diabetes Association: Clinical Practice Recommendations 2000 Committee Report. Diabetes Care 2000;23:S4–S19.

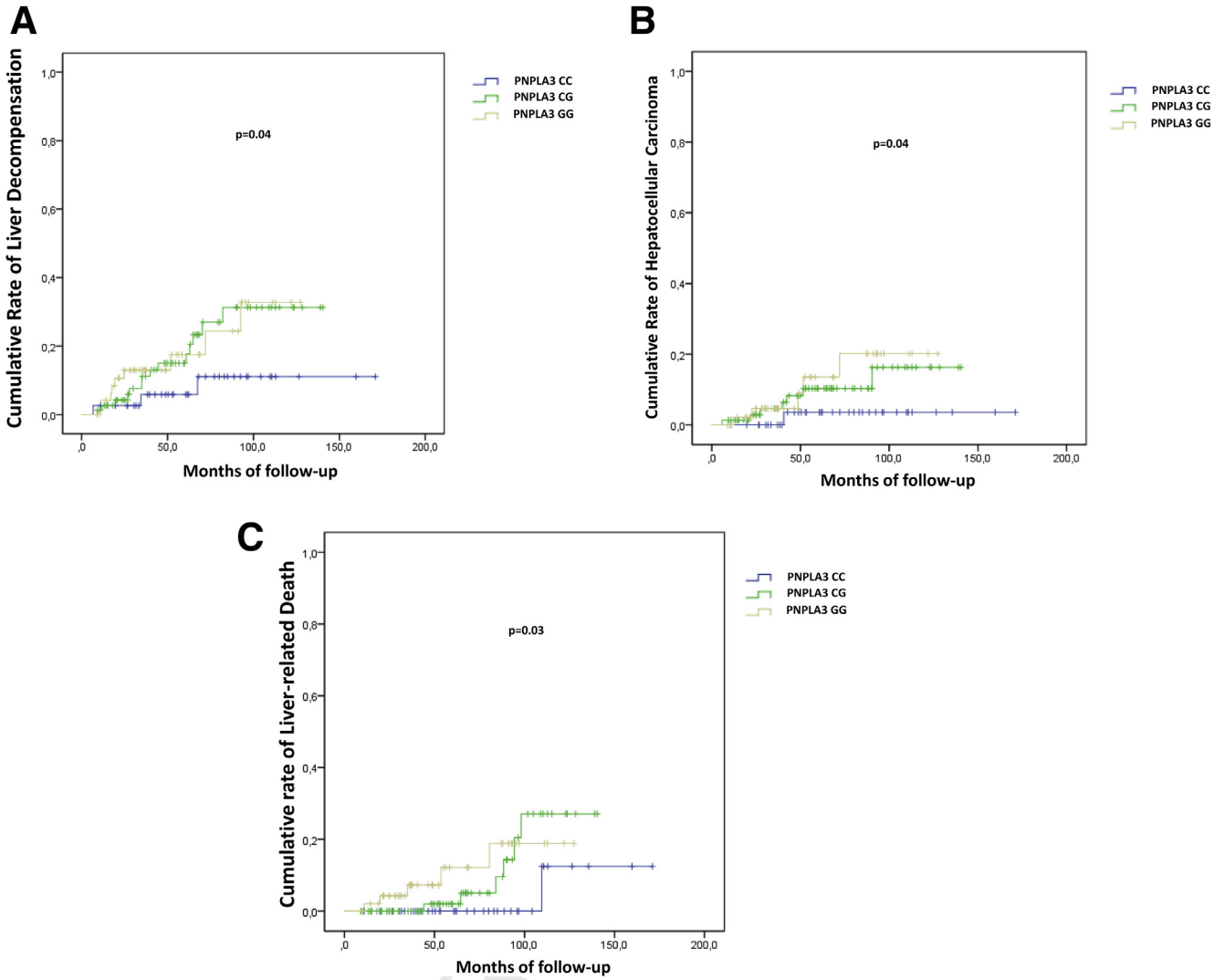
Supplementary Table 1. Multivariate Cox Regression Analyses of Factors Associated With Extrahepatic Events and Overall Death in the Entire Cohort of NAFLD Patients

Group	Variable	Adjusted model HR (95% CI) *P* 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

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



Supplementary Figure 1. ■■■



Supplementary Figure 2. PNPLA3 genotype and occurrence of liver-related events in the subgroup of nonalcoholic fatty liver disease patients with F3-F4 fibrosis. (*A*) Liver decompensation; (*B*) hepatocellular carcinoma; (*C*) liver-related death. *P* value by log-rank.