Impact of *PNPLA3* rs738409 Polymorphism on the Development of Liver-Related Events in Patients With Nonalcoholic Fatty Liver Disease

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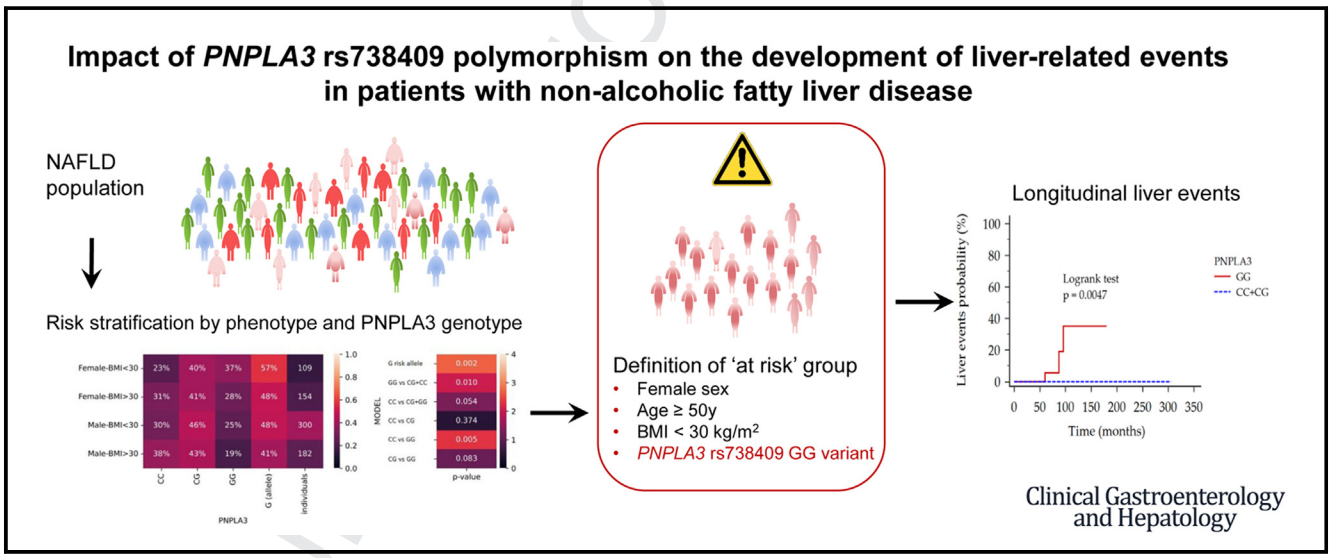

BACKGROUND AND AIMS: **Nonalcoholic fatty liver disease (NAFLD) is a complex disease, resulting from the interplay between environmental determinants and genetic variations. Single nucleotide polymorphism rs738409 C>G in the *PNPLA3* gene is associated with hepatic fibrosis and with higher risk of developing hepatocellular carcinoma. Here, we analyzed a longitudinal cohort of biopsy-proven NAFLD subjects with the aim to identify individuals in whom genetics may have a stronger impact on disease progression.**

Abbreviations used in this paper: BMI, body mass index; HCC, hepatocellular carcinoma; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SNP, single nucleotide polymorphism.

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METHODS: We retrospectively analyzed 756 consecutive, prospectively enrolled biopsy-proven NAFLD subjects from Italy, United Kingdom, and Spain who were followed for a median of 84 months (interquartile range, 65–109 months). We stratified the study cohort according to sex, body mass index (BMI) </≥30 kg/m^2) and age (</≥50 years). Liver-related events (hepatic decompensation, hepatic encephalopathy, esophageal variceal bleeding, and hepatocellular carcinoma) were recorded during the follow-up and the log-rank test was used to compare groups.

RESULTS: Overall, the median age was 48 years and most individuals were men (64.7%). The *PNPLA3* rs738409 genotype was CC in 235 (31.1%), CG in 328 (43.4%), and GG in 193 (25.5%) patients. At univariate analysis, the *PNPLA3* GG risk genotype was associated with female sex and inversely related to BMI (odds ratio, 1.6; 95% confidence interval, 1.1–2.2; $P = .006$; and odds ratio, 0.97; 95% confidence interval, 0.94–0.99; $P = .043$, respectively). Specifically, *PNPLA3* GG risk homozygosis was more prevalent in female vs male individuals (31.5% vs 22.3%; $P = .006$) and in nonobese compared with obese NAFLD subjects (50.0% vs 44.2%; $P = .011$). Following stratification for age, sex, and BMI, we observed an increased incidence of liver-related events in the subgroup of nonobese women older than 50 years of age carrying the *PNPLA3* GG risk genotype (log-rank test, $P = .0047$).

CONCLUSIONS: Nonobese female patients with NAFLD 50 years of age and older, and carrying the *PNPLA3* GG risk genotype, are at higher risk of developing liver-related events compared with those with the wild-type allele (CC/CG). This finding may have implications in clinical practice for risk stratification and personalized medicine.

Keywords: NAFLD; NASH; *PNPLA3*; Liver-Related Outcomes.

Nonalcoholic fatty liver disease (NAFLD) is a complex and multifactorial disease, resulting from the interplay between environmental determinants, metabolic derangements, and genetic predisposition. NAFLD encompasses a wide spectrum of liver damage, ranging from simple fatty liver (nonalcoholic fatty liver) to nonalcoholic steatohepatitis (NASH), that in turn can progress to hepatic fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC).[1,2] The progression from nonalcoholic fatty liver to NASH and hepatic fibrosis varies among individuals, with only a subset of subjects developing severe liver disease. However, the early identification of individuals at high risk of progressive disease remains elusive. It is recognized that the onset of hepatic steatosis and its progression to NASH and fibrosis is influenced by inherited factors.[3] For example, the rs738409 single nucleotide polymorphism (SNP) C>G in the *PNPLA3* gene accounts for the largest fraction of genetic variability of hepatic fat accumulation in the general population.[4] Homozygosis for the GG variant is associated with hepatic fibrosis and with higher risk of developing NAFLD/NASH-related HCC than in carriers of the wild-type allele.[5–8] However, longitudinal studies evaluating the impact of this genetic variant on the occurrence of clinical events in heterogeneous groups of NAFLD patients are sparse.[9] In this study, we analyzed a longitudinal cohort of biopsy-proven NAFLD subjects to assess the impact of the *PNPLA3* rs738409 polymorphism on the development of liver-related events and to identify subgroups of individuals in which this genetic variant has a stronger impact on disease progression.

Patients and Methods

The study population included 756 subjects with biopsy-proven NAFLD, selected from 1173 patients according to the availability of genetic data. All patients had been consecutively enrolled from 1995 to 2015 and prospectively followed up in tertiary centers in Italy (Turin, Milan, Rome, Palermo), the United Kingdom (Newcastle Upon-Tyne), and Spain (Seville). Inclusion criteria were ≥18 years of age and absence of other causes of liver disease such as drug-induced liver disease, viral hepatitis, autoimmune hepatitis, cholestatic and metabolic or genetic diseases, and alcoholic hepatitis. Alcohol-induced liver disease was excluded based on weekly ethanol consumption (<140 g in women and <210 g in men); moreover, past and current ethanol intake was confirmed through direct questioning of patients and their close relatives. Clinical, anthropometric, and biochemical data were collected at the time of liver biopsy. Patients were followed by a clinician every year or 6 months as appropriate. During the follow-up visits, medical history was reviewed, and the following liver-related outcomes were collected: liver decompensation, jaundice, variceal bleeding, encephalopathy, HCC occurrence (defined by imaging/histology criteria according to the current clinical guidelines),[10] and patient deaths. Baseline and longitudinal data were collected in the European NAFLD Registry according to established common criteria.[11] Outcomes were retrieved from patients' medical records and by phone interviews in the case of loss to follow-up. The study was approved by the ethics committee of each center, and all the patients signed an informed consent for participation in the study.

Genotyping

Genomic DNA was isolated from the whole blood sample according to the specific procedures in each center. Genotyping for *PNPLA3* SNP rs738409 was performed by real-time allelic discrimination assay (TaqMan SNP Genotyping Assay; Applied Biosystems, Foster City, CA) using TaqMan SNP Genotyping Master Mix (Applied Biosystems) on a real-time polymerase chain reaction instrument.

Liver Histology

Liver biopsies were stained with hematoxylin and eosin, Masson's trichrome, and special stains for iron and copper. Biopsies were scored by a total of seven expert liver pathologists, blinded to patient clinical characteristics, using the Kleiner classification.[12] All the pathologists participated in previous pathology consortia in which the strength of their overall agreement was above 75%.[13] The average size of liver biopsies was 25 mm, and they had a minimum of 11 portal tracts; inadequate biopsies were excluded. NASH was defined according to the joint presence of steatosis, hepatocyte ballooning, and lobular inflammation with or without fibrosis.[12]

Statistical Analysis

Continuous variables were reported as median (interquartile range), while categorical variables were reported as frequency and percentage. To assess differences between the *PNPLA3* genotypes, we used both the additive (CC vs CG + GG) and recessive (CC + CG vs GG) models. The Mann-Whitney nonparametric test was used to assess differences between groups. Subgroup analyses were performed by splitting patients by sex, age, and BMI, with a threshold of 50 years for age and 30 kg/m^2 for BMI, thereby yielding 8 subgroups. Tests that were repeated for each group were considered significant by the Bonferroni correction for multiple comparisons, that is, when their P value was $<.00625$ (.05/8). Differences in allele frequency between subgroups were tested with Pearson's chi-square test. Differences in survival and time to events in the follow-up for a recessive genetic model (*PNPLA3* GG vs CG/CC) were tested by the log-rank test. The chi-square and log-rank tests and subgroup analysis were performed in Python 3.8.12 using the packages scipy 1.7.3 and lifelines 0.26.4.[14]

What You Need to Know

Background

Nonalcoholic fatty liver disease is a complex disease, resulting from the interplay between environmental determinants, metabolic derangements, and genetic predisposition. It is important to identify subgroups of individuals at high risk of disease progression.

Findings

Nonobese women with nonalcoholic fatty liver disease older than 50 years of age carrying the *PNPLA3* GG risk genotype are at higher risk of developing liver-related events compared with those carrying the wild type allele.

Implications for patient care

PNPLA3 polymorphism may be a useful tool for risk stratification in the context of personalized medicine.

Results

Clinical, Biochemical, and Histological Features of the Study Population

The study cohort comprised 756 patients with biopsy-proven NAFLD genotyped for the rs738409 SNP in the *PNPLA3* gene. The flowchart of the study is reported in Figure 1. The *PNPLA3* rs738409 genotype was CC in 235 (31.1%), CG in 328 (43.4%), and GG in 193 (25.5%) patients. Baseline anthropometric, biochemical, and histological characteristics of the study cohort are reported in Table 1. The median age was 48 (range, 15–77) years and most of the individuals were male

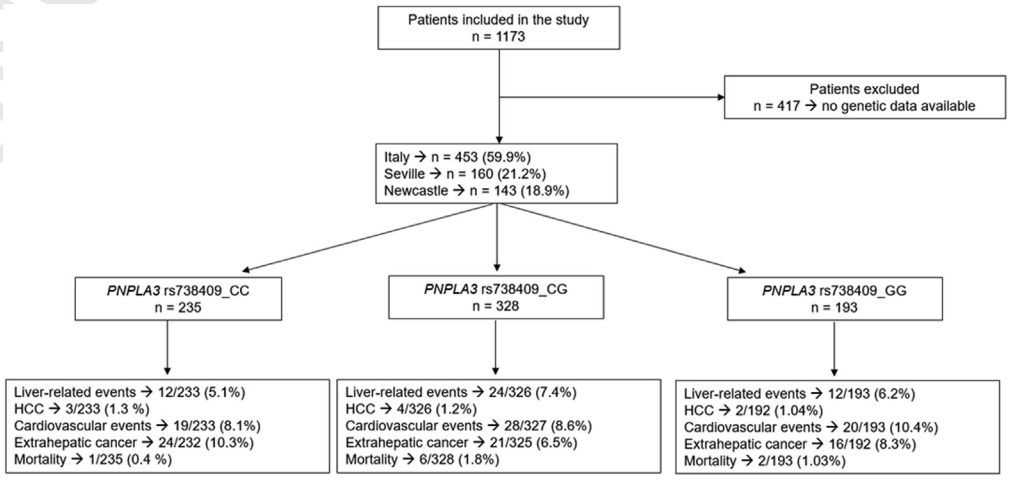

Figure 1. •••

Table 1. Clinical and Demographic Characteristics of the Whole Cohort (N = 756)

Age, y	48 (38–57)
Sex	
Male	489 (64.7)
Female	267 (35.3)
Body mass index (n = 748), kg/m^2	30 (27–34)
Waist circumference (n = 633), cm	102 (94–110)
ALT, UI	59 (40–87)
AST, UI	37 (28–54)
Total bilirubin (n = 744), mg/dL	0.6 (0.5–0.9)
Albumin (n = 705), g/dL	4.6 (4.3–4.8)
Alkaline phosphatase (n = 739), IU/L	79 (62–110)
Platelet (×10^9) (n = 751)	231 (191–276)
Glucose (n = 725), mg/dL	95 (85–113)
Triglycerides (n = 729), mg/dL	139 (99–195)
Total cholesterol (n = 735), mg/dL	197 (169–227)
HDL cholesterol (n = 694), mg/dL	48 (40–58)
LDL cholesterol (n = 486), mg/dL	124 (97–156)
Ferritin (n = 664), ng/mL	184 (92–326)
Diabetes at baseline	205 (27.1)
PNPLA3 rs738409	
CC	235 (31.1)
CG	328 (43.4)
GG	193 (25.5)
Histological features	
Steatosis grade	
0	1 (0.1)[a]
1	287 (38)
2	274 (36.2)
3	194 (25.7)
Fibrosis stage	
0	193 (25.5)
1	231 (30.6)
2	161 (21.3)
3	116 (15.3)
4	55 (7.3)
Lobular inflammation (n = 754)	
0	114 (15.1)
1	408 (54.1)
2	232 (30.8)
Ballooning (n = 755)	
0	202 (26.8)
1	387 (51.2)
2	166 (22)
NASH (n = 754)	501 (66.4)

Values are median (interquartile range) or n (%).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NASH, nonalcoholic steatohepatitis.
[a]This patient underwent liver biopsy for suspicion of NASH and showed F4 fibrosis at histology, with steatosis <5%.

(64.7%). Overall, 51.9% of the patients were obese, and type 2 diabetes mellitus was found in 27.1% of the population. At liver biopsy, NASH was diagnosed in 501 (66.4%) patients. Specifically, hepatocyte ballooning and lobular inflammation were found in 73.2% and 84.9% of the cases, respectively; hepatic steatosis was mild (<33%), moderate (≥33% to <66%), and severe (≥66%) in 38%, 36.2%, and 25.7% of the cases, respectively. Cirrhosis was found at liver biopsy in 55 (7.3%) patients, while 277 (36.6%) of 756 subjects had severe fibrosis (F3/F4).

Characteristics of the Study Cohort According to the PNPLA3 Polymorphism

Clinical, biochemical, and histological characteristics of the study cohort according to the *PNPLA3* genotypes are reported in Supplementary Table 1. Patients who carried the *PNPLA3* G risk allele (additive model) or the GG homozygosis (recessive model) had a lower BMI and showed higher levels of aspartate aminotransferase and alanine aminotransferase and lower levels of glucose and triglycerides compared with those carrying the CC genotype or the C wild-type allele, respectively (Supplementary Table 1). *PNPLA3* G risk allele frequency was significantly associated with sex (52% in female patients vs 45% in male patients; $P = .008$) and BMI (50% in lean vs 44% in obese individuals), but not with age when comparing subjects younger or older than 50 years.

Regarding histology, the rate of cirrhosis increased from the *PNPLA3* CC to CG to GG genotypes (5.1%, 6.7%, and 10.9%, respectively) (Supplementary Table 1), and conversely, the prevalence of subjects without hepatic fibrosis decreased (28.2%, 26.5%, and 20.2%, respectively) (Supplementary Table 1). The prevalence of NASH was significantly higher in patients carrying the *PNPLA3* G risk allele compared with those with the wild-type (69.3 vs 43.1; $P = .009$) (Supplementary Table 1).

PNPLA3 Genetic Variants According to Specific Subgroup of Patients

More pronounced differences in the *PNPLA3* G risk allele frequency (dominant model) were found stratifying individuals by sex and BMI: 57% in lean female subjects vs 41% in obese male subjects ($P = .001$). On the other hand, obese female subjects and lean male subjects had intermediate and not significantly different frequencies with 48% for both (Figure 2). We found similar results by analyzing the recessive model; specifically, the frequency of the *PNPLA3* GG risk genotype was different according to sex (31% in female vs 22% in male subjects; $P = .006$) and BMI (50% in lean vs 44% in obese individuals; $P = .011$). In the additive model, more pronounced differences were found after splitting individuals by both sex and BMI: *PNPLA3* GG risk genotype frequency was 37% in lean female vs 19% in obese male individuals ($P = .003$). Again, obese female and lean male individuals did not show significantly different frequencies (28% and 25%, respectively), as shown in

Figure 2. At univariate logistic regression analysis, the *PNPLA3* at risk variant was positively associated with female sex and inversely related to BMI in both the additive and recessive models, while the association with cirrhosis was confirmed in the recessive model only (Table 2).

Longitudinal Analysis: Prediction of Long-Term Outcomes Based on PNPLA3 *GG Genotype, Age, Sex, and BMI*

After a median follow-up of 84 (interquartile range, 65–109) months, 9 (1.2%) patients died, while 48 (6.3%) of 756 patients had liver-related events and 9 (1.2%) patients developed HCC. Concerning nonhepatic events, 61 (8.1%) of 756 patients developed extrahepatic cancers, 67 (8.9%) patients with cardiovascular events and 55 (7.3%) with type 2 diabetes mellitus. Overall, the presence of the *PNPLA3* polymorphism did not affect the occurrence of clinical outcomes (Table 3 and Supplementary Figure 1). In the subgroup of 55 nonobese female individuals older than 50 years of age, we performed a log-rank test to check for differences in liver-related events according to *PNPLA3* genotype variants: 3 of 19 patients carrying the GG variant developed liver-related events, compared with none of the 36 patients carrying the CG/CC (15.8% vs 0%; $P = .0047$) (Figure 3). To further explore this concept, we grouped the entire cohort according to the *PNPLA3* genotype and the stage of hepatic fibrosis as follows: group 1 was *PNPLA3* CC+CG and F0–F2, group 2 was *PNPLA3* CC+CG and F3–F4, group 3 was *PNPLA3* GG and F0–F2, and group 4 was *PNPLA3* GG and F3–F4. Overall, the incidence of liver events in group 2 vs group 4 was 18.5% vs 19.6% ($P = .871$) (Supplementary Figure 2*A*), showing a small, nonsignificant effect of genetic on liver outcomes at Kaplan-Meier survival analysis (log-rank test, $P = .105$) (Supplementary Figure 2*B*). However, in the subgroup of nonobese female individuals ≥50 years of age, we found a statistically significant difference in the incidence rate of liver events in group 2 vs group 4 (0% vs 40%; $P = .038$) (Supplementary Figure 2*C*) as well as at Kaplan-Meier survival analysis (log-rank test, $P = .011$) (Supplementary Figure 2*D*). This result confirms that the carriage of the *PNPLA3* GG variant in selected subgroups may affect the occurrence of liver events over time independent from advanced hepatic fibrosis.

Discussion

In this study performed in a large European cohort of patients with NAFLD at liver biopsy, we found that the presence of the *PNPLA3* GG variant impacts the development of liver-related events during follow-up in the subgroup of nonobese female individuals 50 years of age and older.

The *PNPLA3* risk variant is considered to be responsible for NAFLD/NASH, particularly in lean subjects (BMI <25 kg/m^2), but in this category the occurrence of metabolic comorbidities and liver-related events over time is independent from the carriage of the *PNPLA3* polymorphism.[15] Thus, we further analyzed the impact of this risk variant after stratification in different subgroups, and we found that nonobese (ie, both lean and overweight) women with NAFLD ≥50 years of age carrying the *PNPLA3* GG variant are at highest risk of developing liver-related outcomes compared with the other subgroups. The significant effect of the *PNPLA3* polymorphism in this subgroup of was independent of age. Further, while severe fibrosis was confirmed to be the strongest predictor of liver events in the entire study cohort, carriage of the *PNPLA3* GG risk homozygosis in this selected subgroup seems to affect liver-related outcomes over time independent from advanced hepatic fibrosis. Conversely, the *PNPLA3* rs738409 variant had no clear impact on the development of HCC, cardiovascular events, and extrahepatic cancers.

Despite that nonobese women ≥50 years of age had a prevalence of NASH and severe fibrosis at histology that was higher (72.7% and 69.1%, respectively), the diagnostic accuracy of *PNPLA3* polymorphism in identifying a more severe liver disease (NASH patients with fibrosis F3/F4) showed poor accuracy (data not shown). From the statistical point of view, the most appropriate method to evaluate the utility of genetic variants for risk stratification is still under debate. The diagnostic accuracy of genetic markers is usually assessed by the area under the receiver-operating characteristic curve, but to

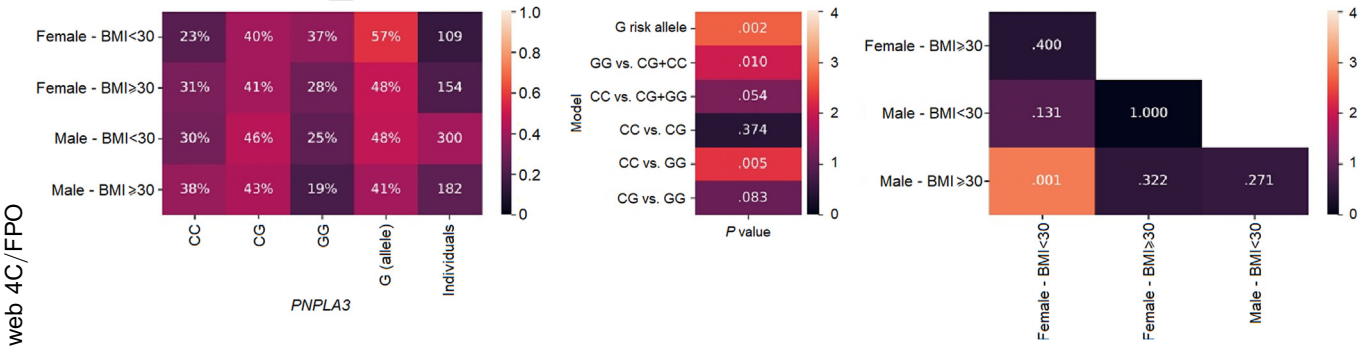

Figure 2. •••

Table 2. Univariate Logistic Regression Analysis for the Association With the *PNPLA3* Genotypes

	Recessive Model (CC+CG vs GG)		Additive Model (CC vs CG+GG)	
Variables	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Age	1.00 (0.99–1.01)	.9164	0.99 (0.98–1.01)	.3739
Female	1.60 (1.10–2.20)	.0059	1.32 (0.95–1.83)	.1008
BMI	0.97 (0.94–0.99)	.0013	0.96 (0.94–0.98)	.0007
Type 2 diabetes	0.86 (0.59–1.24)	.4163	0.88 (0.62–1.23)	.4499

BMI, body mass index; CI, confidence interval; OR, odds ratio.

maintain the rate of false positives under 10%, a sensitivity of 80% and an odds ratio higher than 50 are required.[16] The most relevant advantage of using genetic markers for risk stratification is that once determined, they do not change over time.[17] Furthermore, they can be combined to build polygenic risk scores, emerging tools able to quantify genetic predisposition and to predict the risk of NAFLD progression, and helping clinicians with disease risk stratification.[18,19] For example, in a recent study by Pennisi et al,[20] a composite score based on clinical, metabolic, and genetic variables showed good accuracy for predicting liver-related event occurrence in a selected cohort of NAFLD patients with advanced fibrosis according to the Fibrosis-4 score.[20] Diverse genetic variants can have different cumulative effects on the fate of NAFLD patients.[21] For example, in a cohort genotyped for both *PNPLA3* C>G and *HSD17B13* T>TA variants, the latter seems to mitigate the negative effect of the *PNPLA3* SNP.[22] Unfortunately, we did not assess other genetic polymorphisms in this cohort, and further studies are necessary to explore genetic interactions in their complexity.

Other limitations of this study are its retrospective design (although in cohorts generated with homogeneous criteria) and the lack of menopausal status at enrolment, for which an age threshold of 50 years was used as a surrogate. The low rate of liver-related events that limited the power of our analysis in this cohort is due to the very low prevalence of cirrhosis at enrolment (n = 55 of 756 [7.3%]), in agreement with what recently observed in a large U.S. longitudinal study (n = 167 of 1733 [9.4%]).[23] Moreover, no previous history of hepatic decompensation was reported in our cohort of histological-proven cirrhotic patients, while in the study by Sanyal et al,[23] 23 patients had experienced liver-related outcomes (ascites or encephalopathy) before enrollment.

In conclusion, we showed that nonobese NAFLD women ≥50 years old carrying the *PNPLA3* GG risk genotype are at higher risk of developing liver-related events compared with those carrying the wild-type allele both in homozygosis and in heterozygosis (CC/CG), suggesting a careful follow-up in this specific subgroup. *PNPLA3* polymorphism may be a useful tool for risk stratification in the context of personalized medicine, although external replication in independent cohorts is necessary to validate the clinical applicability of our findings in specific subsets of patients with NAFLD.

Table 3. Cumulative Incidence of Outcomes by *PNPLA3* Genotypes in the Entire Cohort (N = 756)

	PNPLA3 CC (n = 235)	*PNPLA3* CG (n = 328)	*PNPLA3* GG (n = 193)	*P* Value[a]	*P* Value[b]
Liver-related events	12 (0.75)	24 (1.03)	12 (0.90)	.7945	.1651
HCC	3 (0.19)	4 (0.65)	2 (0.15)	.8657	.9551
Extrahepatic cancers	24 (1.57)	21 (0.91)	16 (1.21)	.7641	.2800
Cardiovascular events	19 (1.19)	28 (1.20)	20 (1.50)	.7031	.3992
Type 2 diabetes	22 (1.37)	26 (1.60)	7 (0.68)	.7307	.4040
Mortality	1 (0.05)	6 (0.26)	2 (0.15)	.8683	.1491

Values are n (cumulative incidence rate per 1000 patient-years). The cumulative incidence rate per 100 patient-years was derived by the ratio of the number of events to the patient-years multiplied by 100. *P* values were calculated by the log-rank test.
HCC, hepatocellular carcinoma.
[a]Refers to the differences in the recessive model (*PNPLA3* CC/CG vs GG).
[b]Refers to the difference in the additive model (*PNPLA3* CC vs CG/GG).

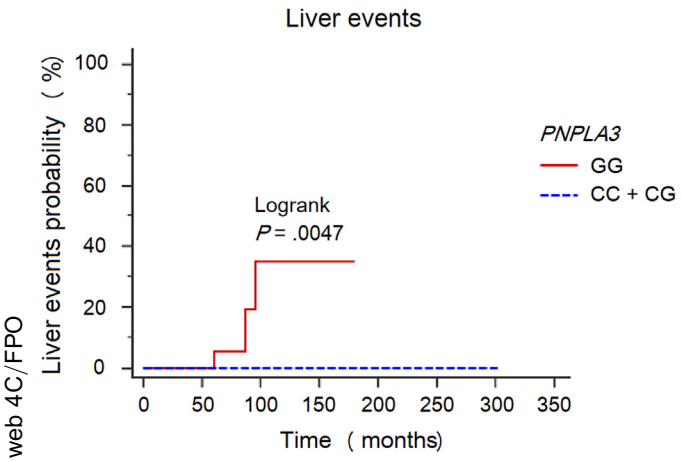

Figure 3. •••

Supplementary Material

Note: To access the supplementary material 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.2023.04.024.

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Conflicts of Interest
The authors disclose no conflicts. Q4

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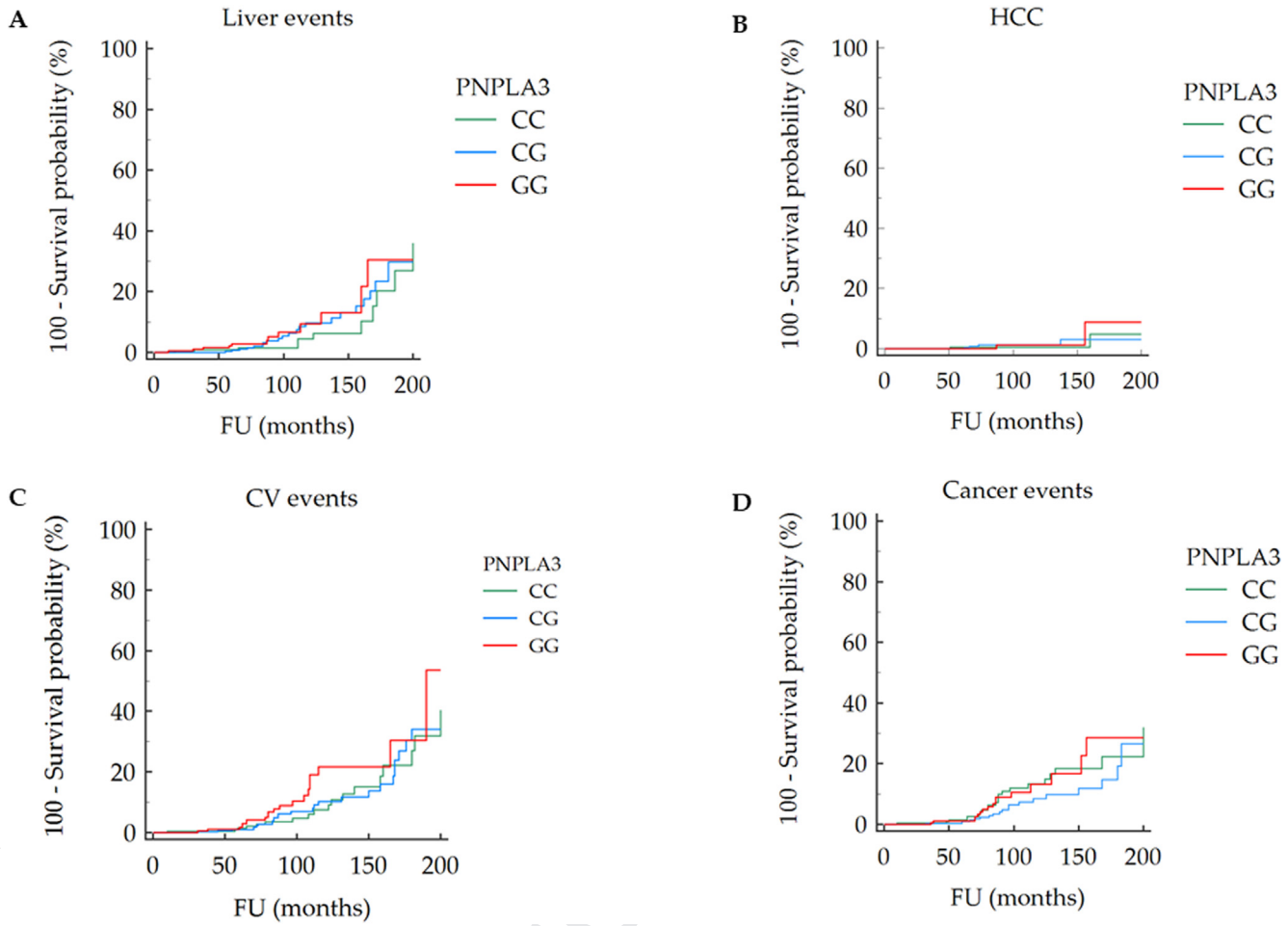
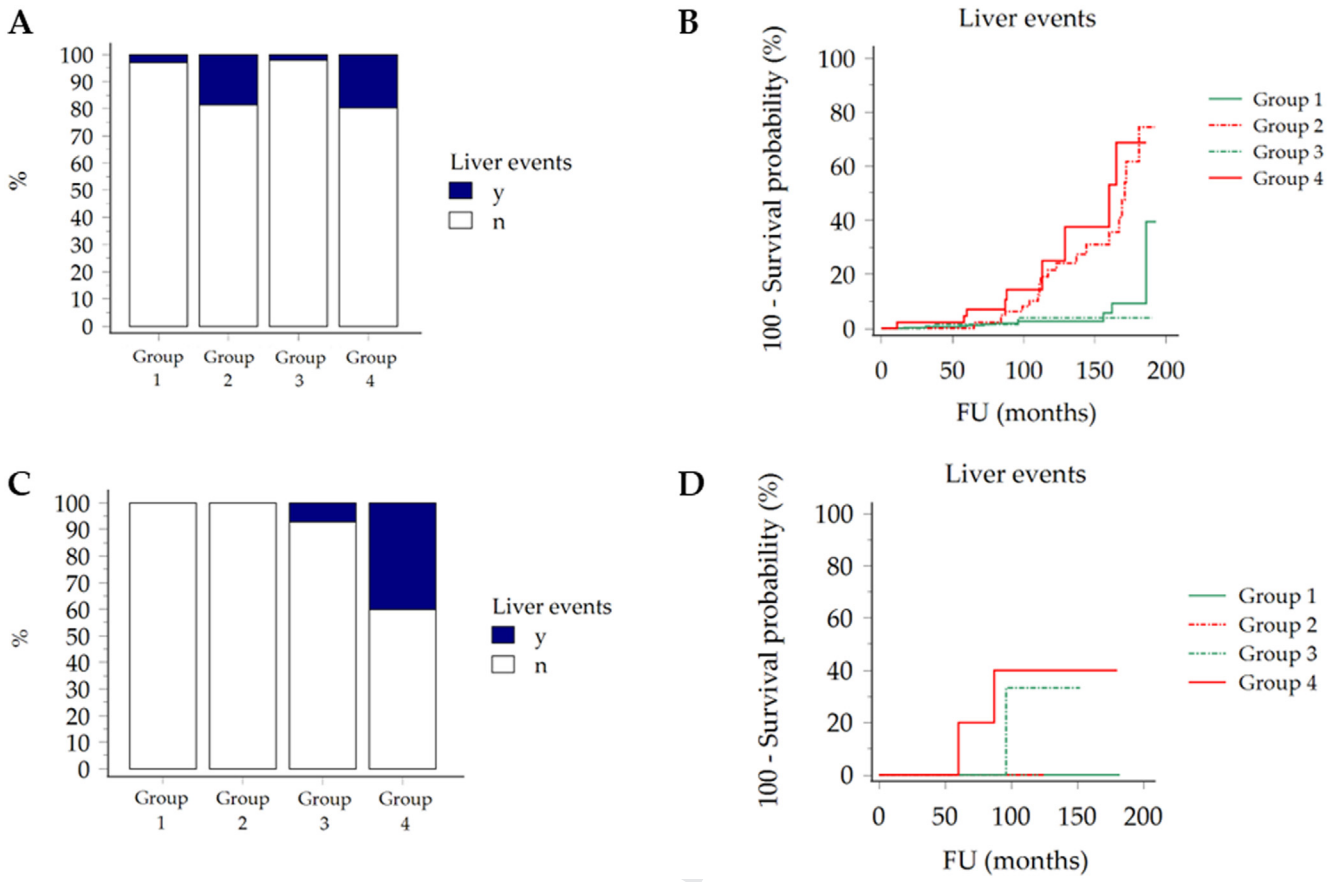


Supplementary Figure 1. •••



Supplementary Figure 2. •••

Supplementary Table 1. Clinical, Anthropometric, and Biochemical Characteristics of the Study Cohort According to the *PNPLA3* Genotypes

	PNPLA3 CC (n = 235)	*PNPLA3* CG (n = 328)	*PNPLA3* GG (n = 193)	*P* Value[a]	*P* Value[b]
Age, y	49 (39–57)	48 (37–57)	47 (40–57)	.6058	.8259
Sex				.1005	.0088
Female	73 (31.1)	110 (33.5)	84 (43.5)		
Male	162 (68.9)	218 (66.5)	109 (56.5)		
Body mass index, kg/m^2	31 (27.7–36)	29 (26–34)	29 (26–33)	.0036	.0107
Waist circumference, cm	102 (96–112)	102 (93–110)	102 (93–110)	.0197	.0656
Diabetes at baseline	68 (28.9)	89 (27.1)	48 (24.9)	.4500	.3478
ALT, UI	55 (36–76)	60 (42–89)	65 (42–98)	.0138	.0256
AST, UI	35 (26–50)	37 (28–56)	39 (28–55)	.0231	.0574
Albumin, g/dL	4.6 (4.3–4.8)	4.6 (4.3–4.8)	4.5 (4.3–4.8)	.4329	.7204
Platelet ($\times 10^9$)	236 (195–287)	222 (191–269)	232 (188–276)	.1484	.3397
Glucose, mg/dL	98 (87–119)	94 (85–112)	95 (85–109)	.0099	.0358
Triglycerides, mg/dL	158 (109–229)	133 (95–181)	134 (97–184)	.0002	.0008
Total cholesterol, mg/dL	195 (166–225)	198 (170–227)	198 (171–234)	.3071	.5841
HDL cholesterol, mg/dL	47 (41–56)	49 (41–58)	48 (38–59)	.8998	.5055
NASH	140 (43.1)	224 (68.3)	137 (71)	.0090	.1087
Hepatic fibrosis				.0164	.0585
F0	67 (28.5)	87 (26.5)	39 (20.2)		
F1	84 (35.7)	86 (26.2)	61 (31.6)		
F2	37 (15.7)	77 (23.5)	47 (24.3)		
F3	35 (15)	56 (17.1)	25 (13)		
F4	12 (5.1)	22 (6.7)	21 (10.9)		

Values are median (interquartile range) or n (%).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; NASH, nonalcoholic steatohepatitis.
[a]Refers to the difference in the additive model (*PNPLA3* CC vs CG/GG).
[b]Refers to the differences in the recessive model (*PNPLA3* CC/CG vs GG).