Impact of PNPLA3 rs738409 polymorphism on the development of liver-related events in patients with non-alcoholic fatty liver disease

Impact of *PNPLA3* polymorphism on the development of liver-related events in patients with non-alcoholic fatty liver disease

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Abstract

Background & Aims. Non-alcoholic fatty liver disease (NAFLD) is a complex trait, resulting from the interplay between environmental determinants and genetic variations. Single nucleotide polymorphism (SNP) rs738409 C>G in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene is associated with hepatic fibrosis and with higher risk of developing hepatocellular carcinoma. Here, we analysed a longitudinal cohort of biopsy-proven NAFLD subjects with the aim to identify individuals in which genetics may have a stronger impact on disease progression.

Methods. We retrospectively included in the study 756 consecutive, prospectively enrolled Caucasian biopsy-proven NAFLD subjects from Italy, UK and Spain who were followed for a median follow-up of 84 months (interquartile range 65 - 109 months). We stratified the study cohort according to gender, body mass index (BMI) </≥30 kg/m2) and age (</≥50 years). Liver-related events (hepatic decompensation, hepatic encephalopathy, esophageal bleeding and hepatocellular carcinoma) were recorded during the follow-up and the log-rank test was used to compare groups.

Results. Overall, median age was 48 years and most of individuals were males (64.7 %). The *PNPLA3* rs738409 genotype was CC in 235, CG in 328 and GG in 193 patients (31.1%, 43.4% and 25.5%, respectively). At univariate analysis, the *PNPLA3* GG risk genotype was associated with female gender and inversely related to BMI (OR = 1.6, 95% CI = 1.1 - 2.2, p = 0.006 and OR = 0.97, 95% CI = 0.94 - 0.99, P = 0.043, respectively). Specifically, *PNPLA3* GG risk homozygosis was more prevalent in females *vs*. males (31.5% *vs.* 22.3%, P = 0.006) and in non-obese compared to obese NAFLD subjects (50.0% *vs*. 44.2%, P = 0.011). Following stratification for age, gender and BMI, we observed an increased incidence of liver-related events in the sub-group of non-obese women older than 50 years carrying the *PNPLA3* GG risk genotype (Log-rank test, p = 0.0047).

Conclusions. Non-obese female patients with NAFLD older than 50 years carrying the *PNPLA3* GG risk genotype are at higher risk of developing liver-related events compared to those with the wild type allele (CC/CG). This finding may have implications in clinical practice for risk stratification and personalised medicine.

Keywords: NAFLD; NASH; *PNPLA3*; liver-relate outcomes

Introduction

Non-alcoholic 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 (NAFL) to non-alcoholic steatohepatitis (NASH), that in turn can progress to hepatic fibrosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (1,2). The progression from NAFL 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 accounts for by inherited factors (3). For example, the rs738409 single nucleotide polymorphism (SNP) C>G in the patatin-like phospholipase domain-containing 3 (*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 scanty (9). In this study, we analysed 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 includes 756 Caucasian 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 centres in Italy (Turin, Milan, Rome, Palermo), the United Kingdom (Newcastle Upon-Tyne) and Spain (Seville). Inclusion criteria were age ≥ 18 years, 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 had been excluded based on weekly ethanol consumption (<140 g in women and <210 g in men); moreover, past and current ethanol intake had been confirmed through direct questioning of patients and their close relatives. Clinical, anthropometric, and biochemical data had been collected at the time of liver biopsy. Patients were followed by the 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 had been retrieved by patients' medical records and by phone interviews whenever the patient had been lost at follow up. The study was approved by the ethics committee of each centre 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 centre. 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 PCR instrument.

Liver histology

Liver biopsies were stained with haematoxylin 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 consortiums where 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 and 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 non-parametric test was used to assess differences between groups. Subgroup analyses were performed by splitting patients by gender, age and BMI, with a threshold of 50 years for age and 30 kg/m2 for BMI, thereby yielding eight 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 less than 0.00625 (0.05/8). 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).

Results

Clinical, biochemical, and histological features of the study population

The study cohort comprises 756 patients with biopsy-proven NAFLD genotyped for the rs738409 SNP in the *PNPLA3* gene. Flow-chart of the study is reported in Figure 1. The *PNPLA3* rs738409 genotype was CC in 235, CG in 328 and GG in 193 patients (31.1%, 43.4% and 25.5%, respectively). Baseline anthropometric, biochemical, and histological characteristics of the study cohort are reported in Table 1. Median age was 48 years (range: 15-77) and most of the individuals were males (64.7 %). Overall, 51.9% of the patients were obese and T2DM was found in 27.1% of the population. At liver biopsy, NASH was diagnosed in 501 patients (66.4%). 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% < 66%) and severe (≥ 66%) in 38%, 36.2% and 25.7% of the cases, respectively. Cirrhosis was found at liver biopsy in 55 patients (7.3%), while 277/756 subjects (36.6%) 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 AST and ALT and lower levels of glucose and triglycerides compared to those carrying the CC genotype or the C wild type allele, respectively (Supplementary Table 1). *PNPLA3* G risk allele frequency was significantly associated to gender (52% in females versus 45% in males, P = 0.008) and BMI (50% in lean versus 44% in obese individuals), but not to age when comparing subjects younger or older than 50 years.

Concerning histology, the rate of cirrhosis increased from the *PNPLA3* CC to CG to GG genotypes (5.1%, 6.7%, 10.9%, Supplementary Table 1) and conversely the prevalence of subjects without hepatic fibrosis decreased (28.2%, 26.5%, 20.2%, Supplementary Table 1). The prevalence of NASH was significantly higher in patients carrying the *PNPLA3* G risk allele compared to those with the wild type (69.3 *vs*. 43.1, P = 0.009, 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 splitting individuals by gender and BMI: 57% in lean females versus 41% in obese males (P = 0.001). On the other hand, obese females and lean males had intermediate and not significantly different frequencies (48% for both), Figure 2. We found similar results by analysing the recessive model; specifically, the frequency of the *PNPLA3* GG risk genotype was different according to sex (31% in females versus 22% in males, P = 0.006) and BMI (50% in lean versus 44% in obese individuals P = 0.011). In the additive model, more pronounced differences were found after splitting individuals by both gender and BMI: *PNPLA3* GG risk genotype frequency was 37% in lean females versus 19% in obese males (P = 0.003). Again, obese females and lean males did not show significantly different frequencies (28% and 25%, respectively), 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 model, 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, gender and BMI

After a median follow up of 84 months (interquartile range 65-109 months), 9 patients (1.2%) died, while 48 out of 756 patients (6.3%) had liver-related events and 9 patients (1.2%) developed HCC. Concerning non-hepatic events, 61 out of 756 patients (8.1%) developed extra-hepatic cancers, 67 patients (8.9%) cardiovascular events and 55 patients (7.3%) T2DM. 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 non-obese female older than 50 years, we performed a Logrank test to check for differences in liver-related events according to *PNPLA3* genotype variants: 3/19 patients carrying the GG variant developed liver-related events, compared to none of the 36 patients carrying the CG/CC (15.8% vs 0%, P = 0.0047), Figure 3. To further explore this concept, we grouped the whole cohort according to the *PNPLA3* genotype and the stage of hepatic fibrosis as follow: group 1) *PNPLA3* CC+CG and F0-F2; group 2) *PNPLA3* CC+CG and F3-F4; group 3) *PNPLA3* GG and F0-F2; group 4) *PNPLA3* GG and F3-F4. Overall, the incidence of liver events in group 2 vs group 4 was 18.5% vs 19.6% (Supplementary Figure 2A, P=0.871) showing a small, non-significant effect of genetic on liver outcomes at Kaplan-Meier survival analysis (Supplementary Figure 2B, Long-rank test, P=0.105). However, only in the subgroup of non-obese female older than 50 years, we found a statistically significant difference in the incidence rate of liver events in group 2 vs group 4 (0% vs 40%, P=0.038; Supplementary Figure 2C) as well as at Kaplan-Meier survival analysis (Supplementary Figure 2D, Logrank test, P=0.011). This result

confirms that the carriage of the PNPLA3 GG risk homozygosis in selected sub-groups 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 risk impacts on the development of liver-related events during follow-up in the subgroup of non-obese female older than 50 years.

The *PNPLA3* risk variant is considered to be responsible for NAFLD/NASH particularly in lean subjects (BMI < 25kg/m2), 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 analysed the impact of this risk variant after stratification in different subgroups, and we found that non-obese (i.e. both lean and overweight) NAFLD women older than 50 years carrying the *PNPLA3* GG variant are at highest risk of developing liver-related outcomes compared with the other subgroups analysed. 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 whole study cohort, carriage of the *PNPLA3* GG risk homozygosis in this selected sub-groups seems to affect liver-related outcomes over time independent from advanced hepatic fibrosis. On the contrary, the *PNPLA3* rs738409 variant had no clear impact on the development of HCC, cardiovascular events and extrahepatic cancers.

Despite in the subgroup of non-obese female older than 50 years, the prevalence of NASH and severe fibrosis at histology 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 (AUROC), but to 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 helping clinicians with disease risk stratification (18,19). For example, in a recent study by Pennisi G et al., a composite score based on clinical, metabolic, and genetic variables showed good accuracy for predicting liver related events 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, which is surrogated by an age threshold of 50 years. The low rate of liver-related events that limited the strength of our analysis in this cohort is due to the very low prevalence of cirrhosis at enrolment, in agreement with what recently observed in a large US longitudinal study (23).

In conclusion, we showed that non-obese NAFLD females older than 50 years carrying the *PNPLA3* GG risk genotype are at higher risk of developing liver-related events compared to those carrying the wild type allele both in homozygosis and in heterozygosis (CC/CG), suggesting a careful follow up in clinical practice in this specific subgroup. Although externally replication in independent cohorts is necessary before clinical application, our finding suggest that the *PNPLA3* polymorphism may be useful for risk stratification in the context of personalised medicine.

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Tables

Table 1. Clinical and demographic characteristics of the whole cohort (n = 756).

Footnote: Data are reported as median (interquartile range), or number (proportion) of patients with a condition. Number in brackets after each variable indicates the number of patients who had that variable measured. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; NASH, non-alcoholic steatohepatitis.

*This patient underwent liver biopsy for suspicion of NASH and showed F4 fibrosis at histology, with steatosis less than 5%.

Table 2. Univariate logistic regression analysis for the association with the *PNPLA3* genotypes.

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

Table 3. Number of events and the 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 x 100. P values were calculated by the log-rank test. HCC, hepatocellular carcinoma; PNPLA3, patatin-like phospholipase domain-containing 3.

*P value refers to the differences in the recessive model (*PNPLA3* CC/CG vs GG).

#P value refers to the difference in the additive model (*PNPLA3* CC vs CG/GG).

Figures

Figure 1. Flow-chart of the study.

Footnote. HCC, hepatocellular carcinoma; *PNPLA3*, patatin-like phospholipase domain containing 3.

Figure 2. PNPLA3 rs738409 genotypes frequency according to gender and obesity.

Footnote. BMI, body mass index; *PNPLA3*, patatin-like phospholipase domain containing 3.

Figure 3. Survival curves for the incidence of liver-related events according to *PNPLA3* genotypes in non-obese females older than 50 years (N = 55).

Supplementary Tables

Supplementary Table 1. Clinical, anthropometric and biochemical characteristics of the study cohort according to the *PNPLA3* genotypes.

Footnote. Data are reported as median and interquartile range). ALT, alanine aminotransferase; AST, aspartate aminotransferase; F, hepatic fibrosis; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; NASH, non-alcoholic steatohepatitis; *PNPLA3*, patatin like phospholipase domain containing 3.

*P value refers to the difference in the additive model (*PNPLA3* CC vs CG/GG).

#P value refers to the differences in the recessive model (*PNPLA3* CC/CG vs GG).

Supplementary Figures

Supplementary Figure 1. Clinical events occurrence according to the presence of the *PNPLA3* genotype.

Footnote. Kaplan-Meier analysis according to the *PNPLA3* genotypes was performed for liver events (A), HCC (B), CV events (C) and cancers (D). CV, cardiovascular events; FU, follow-up; HCC, hepatocellular carcinoma; *PNPLA3*, patatin like phospholipase domain containing 3.

Supplementary Figure 2. Liver related events occurrence according to the presence of the *PNPLA3* genotype and hepatic fibrosis.

Footnote. The whole cohort has been grouped according to the *PNPLA3* genotype and the stage of hepatic fibrosis as follow: group 1) *PNPLA3* CC+CG and F0-F2; group 2) *PNPLA3* CC+CG and F3-F4; group 3) *PNPLA3* GG and F0-F2; group 4) *PNPLA3* GG and F3-F4. The incidence of liver related events and the relative Kaplan-Meier analysis have been performed in the whole cohort (a-b) and in the sub-group of non-obese women younger than 50 y (c-d). FU, follow-up; *PNPLA3*, patatin like phospholipase domain containing 3.