

Carriage of the *PNPLA3* rs738409 C >G polymorphism confers an increased risk of non-alcoholic fatty liver disease associated hepatocellular carcinoma

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Background & Aims: Subtle inter-patient genetic variation and environmental factors combine to determine disease progression in non-alcoholic fatty liver disease (NAFLD). Carriage of the *PNPLA3* rs738409 c.444C >G minor allele (encoding the I148M variant) has been robustly associated with advanced NAFLD. Although most hepatocellular carcinoma (HCC) is related to chronic viral hepatitis or alcoholic liver disease, the incidence of NAFLD-related HCC is increasing. We examined whether rs738409 C >G was associated with HCC-risk in patients with NAFLD.

Methods: *PNPLA3* rs738409 genotype was determined by allelic discrimination in 100 European Caucasians with NAFLD-related HCC and 275 controls with histologically characterised NAFLD.

Results: Genotype frequencies were significantly different between NAFLD-HCC cases (CC = 28, CG = 43, GG = 29) and NAFLD-controls (CC = 125, CG = 117, GG = 33) ($p = 0.0001$). In multivariate analysis adjusted for age, gender, diabetes, BMI, and presence of cirrhosis, carriage of each copy of the rs738409 minor (G) allele conferred an additive risk for HCC (adjusted OR 2.26 [95% CI 1.23–4.14], $p = 0.0082$), with GG homozygotes exhibiting a 5-fold [1.47–17.29], $p = 0.01$ increased risk over CC. When compared to the UK general population (1958 British Birth Cohort, $n = 1476$), the risk-effect was more pronounced (GC vs. CC: unadjusted OR 2.52 [1.55–4.10], $p = 0.0002$; GG vs. CC: OR 12.19 [6.89–21.58], $p < 0.0001$).

Conclusions: Carriage of the *PNPLA3* rs738409 C >G polymorphism is not only associated with greater risk of progressive steatohepatitis and fibrosis but also of HCC. If validated, these findings suggest that *PNPLA3* genotyping has the potential to contribute to multi-factorial patient-risk stratification, identifying those to whom HCC surveillance may be targeted.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) describes a spectrum ranging from simple steatosis, through steatohepatitis (NASH) to fibrosis, in the absence of excessive alcohol consumption [1]. NAFLD is strongly associated with features of the metabolic syndrome, including obesity, insulin resistance or type 2 diabetes mellitus (T2DM) and dyslipidaemia [1]. As lifestyles have become increasingly sedentary, NAFLD has rapidly become one of the most common causes of liver disease worldwide [1–3].

The rise in the burden of NAFLD coincides with a marked increase in the incidence of HCC in many countries [4–7]. Worldwide, most HCC are related to chronic viral hepatitis; however, more than half of all HCC cases in developed countries occur in the viral hepatitis negative population [8,9]. Features of the metabolic syndrome including obesity and T2DM (with gender, age, and ethnicity) are independent risk factors for HCC [10,11] whilst the pathogenic processes that favour progression from steatosis to steatohepatitis are also pro-carcinogenic [4]. Longitudinal studies indicate HCC prevalence to be ~0.5% in steatosis and ~2.8% in NASH and so, whilst HCC remains an infrequent complication, the high prevalence of NAFLD means that NAFLD-related HCC contribute significantly to the disease burden [10]. The majority of NAFLD-related HCC are thought to develop on a background of cirrhosis with an estimated incidence of 2.6%/year, although recent studies suggest that in this context a substantial proportion of HCC occur in the absence of advanced fibrosis [12–14].

Keywords: NAFLD; NASH; Steatohepatitis; *PNPLA3*; Hepatocellular carcinoma; HCC; Gene.

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Despite its high prevalence, only a minority of NAFLD patients exhibit steatohepatitis, progress to significant fibrosis or experience associated morbidity and mortality [1]. The reasons for the apparent variation in individual susceptibility to progressive NAFLD in general and NAFLD-related HCC in particular are incompletely understood [9,15]. NAFLD-related HCC develops through the complex interplay of environmental and genetic factors that determine individual risk [6,16]. The role of the non-synonymous *patatin-like phospholipase domain-containing 3* (*PNPLA3*) c.444C >G single nucleotide polymorphism (rs738409) that encodes the p.I148M (isoleucine-to-methionine substitution at residue 148) variant is well recognized as a modifier of hepatic triacylglycerol (TAG) accumulation and NAFLD progression [15,17,18]. This variant has been associated with increased HCC risk in alcohol-related liver disease [19–22] and, more variably, in chronic viral hepatitis [19,20,22–24]. Data has also been presented showing an association with HCC in morbidly obese patients [25] and a mixed-aetiology cohort [26]. Although it may be hypothesised that these latter associations are related to underlying NAFLD, to date no studies have specifically addressed the effect of *PNPLA3* rs738409 C >G minor allele carriage on HCC risk in a NAFLD cohort.

The aim of the current study was to determine whether carriage of the *PNPLA3* rs738409 C >G polymorphism confers an increased risk of NAFLD-associated HCC, and whether that effect is independent of presence of cirrhosis. To address this, we performed a case-control associated study in a well-characterised Northern European cohort with established NAFLD.

Materials and methods

Patients

Patients were recruited from hepatology clinics at two European specialist centres, the Freeman Hospital, Newcastle-upon-Tyne, UK and Inselspital Hospital, Bern, Switzerland. The study had all the necessary ethical approvals in both the countries and all participants gave informed consent.

A cohort of 100 consecutive Northern European Caucasian patients with primary HCC arising on a background of NAFLD was identified (UK 82, Switzerland 18 patients). The diagnosis of HCC was established histologically or through non-invasive assessment according to the EASL-EORTC clinical practice guidelines [6]. The presence of NAFLD was determined through histological assessment of non-tumour liver tissue or, when biopsy was not clinically appropriate, through radiological evidence of hepatic steatosis. As a comparator, a cohort of 275 consecutive UK patients with histologically characterised NAFLD of different stages of disease but without clinical evidence of HCC was assembled. These were unrelated patients with histologically characterised NAFLD, derived from a patient popula-

tion originally identified as having ultrasonographically detected bright liver and abnormal biochemical tests (ALT and/or GGT). In all cases, alternative diagnoses were excluded, including excess alcohol intake (alcohol intake <20 g/day for women; <30 g/day for men), chronic viral hepatitis (hepatitis B and hepatitis C), autoimmune liver diseases, hereditary hemochromatosis, α 1-antitrypsin deficiency, Wilson's disease, and drug induced liver injury.

Clinical and laboratory data were collected at the time of diagnosis. These included basic anthropometrics so that body mass index (BMI) could be calculated. Relevant co-morbidity including the presence of diabetes mellitus (fasting glucose ≥ 7.1 mmol/L [≥ 128 mg/dl]) or treatment with anti-diabetic drugs) and evidence of underlying cirrhosis was recorded. Laboratory evaluation included routine liver biochemistry (alanine and aspartate aminotransferase, total bilirubin, albumin, alkaline phosphatase, and gamma glutamyl transpeptidase); full blood count; total- and HDL-cholesterol and total triglycerides; viral serology for hepatitis B and C infection and autoantibodies. Demographic details of the cohorts are shown in Table 1.

Liver biopsy

Where conducted, liver biopsy was performed under radiological guidance. Specimens (at least 1.6 cm length and 5 μ m thick) were fixed in formalin for evaluation. Tissue sections were stained with hematoxylin and eosin, impregnated with silver for visualizing reticulin framework and stained with Pico Sirius Red for visualizing collagen. Liver biopsies were reviewed by a single expert liver pathologist at each participating centre, unaware of clinical or genetic data. The severity of steatosis, necroinflammatory grade, and stage of fibrosis were scored according to the validated Kleiner criteria [27]. This assessment allowed confirmation of a NAFLD diagnosis and assessment of fibrosis so that patients were classified as cirrhotic or non-cirrhotic. In 25 HCC patients, the diagnosis of HCC was confirmed histologically and staged according to Edmondson [28], adapted for needle biopsy specimens.

DNA preparation

Venous blood was collected from each patient and DNA was prepared from peripheral blood lymphocytes using a perchlorate-chloroform isolation method as described previously [29]. Genotyping was performed by personnel unaware of clinical status or histology of patients.

PNPLA3 rs738409 genotyping

PNPLA3 rs738409 genotype was determined by allelic discrimination using TaqMan reagents (Assay #4351379, Applied Biosystems Inc., USA) according to the manufacturer's protocol. Control samples of known genotype were also included in every 96-well plate (blank, homozygous wild-type, homozygous mutant, and heterozygous).

Statistical analysis

Statistical analyses were performed using SPSS v19.0 (IBM, USA) to collate and analyse cohort phenotype data. Continuous variables were tested using Student's *t* test/one-way ANOVA and categorical variables by χ^2 test unless otherwise sta-

Table 1. Details of NAFLD-HCC and NAFLD cohorts.

Phenotype	NAFLD-HCC cohort n = 100	NAFLD cohort n = 275	<i>p</i> value
<i>PNPLA3</i> rs738409 G-allele frequency	0.505	0.333	<0.0001
Age (Mean \pm SD)	70.3 \pm 8.0	50.9 \pm 12.4	<0.0001
Sex, male (%)	82 (0.82)	161 (0.59)	<0.0001
BMI (Mean \pm SD)	32.0 \pm 6.6	34.4 \pm 5.2	0.0003
Diabetes (%)	68 (0.68)	117 (0.43)	<0.0001
Cirrhosis (%)	67 (0.67)	26 (0.09)*	<0.0001

Categorical values are shown as n (%). Continuous variables are shown as mean \pm SD.

*Fibrosis stage distribution in NAFLD cohort: F0 89 (32.4%), F1 97 (35.3%), F2 37 (13.5%), F3 26 (9.5%), F4 26 (9.5%).

ted. PLINK v1.07 [30] (via the gPLINK v2.050 GUI) was used to conduct the genetic analysis. An initial univariate χ^2 analysis was performed to determine whether *PNPLA3* rs738409 C >G carriage differed between the NAFLD-HCC and control NAFLD-Cohort (Table 1). Subsequently multivariate logistic regression analysis was conducted incorporating rs738409 genotype and those biologically relevant covariates that were associated with risk of disease progression to HCC (age, gender, coexisting T2DM, BMI and presence of cirrhosis) to test the genetic association. Consistent with previous studies [17,31,32], an additive genetic model best fitted the data and is reported. Results are expressed as odds ratio (OR) with 95% confidence intervals (CI). Significance was taken as $p < 0.05$ throughout.

Results

Cohort characteristics

One hundred NAFLD-HCC patients and 275 histologically characterised NAFLD control patients were recruited. Characteristics of the NAFLD-HCC and NAFLD control populations are provided in Table 1. Patients with NAFLD-HCC were significantly older (mean age 70.3 ± 8.0 vs. 50.9 ± 12.4 years, $p < 0.0001$) than a general NAFLD cohort. NAFLD-HCC patients were also significantly more likely to be male (82% vs. 59%, $p < 0.0001$) and to be diabetic (68% vs. 43%, $p < 0.0001$). The presence of NAFLD-HCC was also significantly associated with underlying advanced fibrosis/cirrhosis (Kleiner F4 67% vs. 9%, $p < 0.0001$). NAFLD-HCC patients exhibited a lower mean BMI than the NAFLD control population.

PNPLA3 rs738409 C >G polymorphism carriage is associated with increased risk of HCC relative to a tertiary centre NAFLD cohort

To determine whether carriage of the *PNPLA3* rs738409 C >G polymorphism influenced susceptibility to NAFLD-related HCC, we studied a cohort of Northern European adult patients with established NAFLD. The total study population of 375 individuals (100 NAFLD-HCC, 275 NAFLD only) was genotyped for *PNPLA3* rs738409. *PNPLA3* rs738409 genotypes were confirmed to be in Hardy-Weinberg equilibrium. Reflecting the known association with NAFLD, the minor allele (G) frequency observed in the present study (G617: 0.38) was slightly higher than that observed in a population of North-Western European descent by the International HapMap project (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=738409) but similar to that reported in previous NAFLD studies [18,33]. Genotype frequencies are summarised in Table 2.

Compared to a cohort of NAFLD patients with varying severity, carriage of the *PNPLA3* rs738409 minor (G) allele (I148M variant) was strongly associated with the presence of NAFLD-related HCC

(unadjusted OR 2.046 [95% CI 1.47–2.84]; χ^2 18.50, $p < 0.0001$) and exhibited a gene-dosage effect with the incidence of HCC increasing with the number of G alleles present (Cochran-Armitage χ^2 for trend 16.92, $p < 0.0001$). Adopting an additive model relative to the NAFLD patient cohort, an approximate doubling of HCC risk was observed for each copy of the minor (G) allele carried (unadjusted OR 1.95 [95% CI 1.40–2.70], $p < 0.0001$). The unadjusted odds ratio increased to 3.92 (95% CI 2.06–7.48, $p = 0.0001$) when only homozygotes were considered (Table 2). The relationship between *PNPLA3* genotype and a number of relevant patient-specific and clinical parameters is summarised in Table 3. Amongst those samples where HCC was histologically characterised, carriage of the rs738409 C >G polymorphism was associated with the presence of more poorly differentiated tumour (Fisher's Exact Test $p = 0.018$) (Table 3).

PNPLA3 rs738409 C >G polymorphism carriage in NAFLD-HCC relative to an unselected population cohort

The published *PNPLA3* rs738409 C >G minor allele frequency in a UK general population sample (the MRC/Wellcome Trust UK 1958 Birth Cohort, <http://www.b58cgenome.sgul.ac.uk/>) is 0.23 (95% CI 0.21–0.24) with genotype frequencies of CC 0.59, CG 0.36, GG 0.05. Carriage of the rs738409 C >G polymorphism was increased in the NAFLD-HCC cohort relative to this unselected population (Table 2). As limited phenotype data is available on the population cohort only unadjusted odds ratios could be calculated however, adopting an additive model relative to the UK general population, a greater than 3-fold increased HCC risk was observed for each copy of the minor (G) allele carried (OR 3.43 [95% CI 2.54–4.62], $p < 0.0001$). When only homozygotes were considered an odds ratio of 12.19 (95% CI 6.89–21.58, $p < 0.0001$) for GG over CC was observed.

Contribution of the *PNPLA3* rs738409 C >G polymorphism to NAFLD-HCC risk is independent of presence of cirrhosis

Given the previous reports demonstrating a role for rs738409 C >G as a modifier of disease progression and fibrosis in NAFLD, and the association between presence of age, T2DM, obesity and cirrhosis with HCC, a multivariate logistic regression analysis using an additive model was performed to control for any potential confounding effects. Along with genotype, gender, age at diagnosis, presence of advanced fibrosis/cirrhosis, T2DM and BMI were included in the analysis. *PNPLA3* genotype (OR 2.26 [95% CI 1.23–4.14], $p = 0.0082$), gender, age, and presence of cirrhosis were independent predictors of NAFLD-HCC, Table 4. Of

Table 2. *PNPLA3* rs738409 genotype frequencies and their relationship to risk of HCC development.

Genotype	NAFLD-HCC n (%)	NAFLD cohort n(%)	Unadjusted OR (95% CI)	<i>p</i> value	Adjusted OR (95% CI)*	<i>p</i> value	UK Pop ⁿ n (%)†	Unadjusted OR (95% CI)	<i>p</i> value
CC	28 (0.28)	125 (0.46)	-	-	-	-	871 (0.59)	-	-
CG	43 (0.43)	117 (0.42)	1.64 (0.96-2.81)	0.072	2.35 (0.90-6.13)	0.082	531 (0.36)	2.52 (1.55-4.10)	0.0002
GG	29 (0.29)	33 (0.12)	3.92 (2.06-7.48)	<0.0001	5.05 (1.47-17.29)	0.01	74 (0.05)	12.19 (6.89-21.58)	<0.0001

*Odds ratio for HCC relative to CC genotype adjusted for age, gender, diabetes, BMI, and cirrhosis.

†The MRC/Wellcome Trust UK 1958 Birth Cohort.

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Table 3. Comparison of selected characteristics according to *PNPLA3* rs738409 genotype within NAFLD-HCC and NAFLD cohorts.

Genotype n (%)	NAFLD-HCC cohort				NAFLD cohort			
	CC n = 28 (0.28)	CG n = 43 (0.43)	GG n = 29 (0.29)	<i>p</i> value [†]	CC n = 125 (0.46)	CG n = 117 (0.42)	GG n = 33 (0.12)	<i>p</i> value [†]
Age (Mean ± SD)	73.6 ± 8.1	68.9 ± 7.9	69.2 ± 7.4	0.034	50.9 ± 12	51 ± 12	50 ± 15.2	0.906
Sex, male (%)	23 (0.82)	36 (0.84)	23 (0.79)	0.892	75 (0.60)	74 (0.63)	12 (0.36)	0.020
BMI (Mean ± SD)	30.4 ± 6.7	31.9 ± 4.5	33.6 ± 8.9	0.201	34.4 ± 4.8	34.6 ± 5.7	33.9 ± 4.8	0.765
Diabetes (%)	18 (0.64)	31 (0.72)	19 (0.66)	0.744	51 (0.41)	56 (0.48)	10 (0.30)	0.171
Cirrhosis (%)	13 (0.46)	30 (0.70)	24 (0.83)	0.012	11 (0.01)	9 (0.01)	6 (0.18)	0.181
Histological grade of HCC*	5 (0.45)/ 6 (0.54)	8 (0.80)/ 2 (0.20)	0 (0.00)/ 4 (1.00)	0.018	-	-	-	-

Categorical values are shown as n (%). Continuous variables are shown as mean ± SD.

[†]Comparison between *PNPLA3* genotypes within each study cohort (χ^2 test used for categorical variables and one-way ANOVA for continuous variables unless otherwise stated).

*25 cases had histologically characterised HCC. Results expressed as n (%) of cases with 'well differentiated'/'moderate-poorly differentiated' tumour. *p* value calculated by Fisher's Exact Test.

Table 4. Multivariate analysis of the effect of *PNPLA3* genotype on NAFLD-related HCC risk.

Variables	OR (95% CI)	<i>p</i> value
<i>PNPLA3</i> rs738409 genotype	2.26 (1.23-4.14)	0.0082
Age	1.24 (1.17-1.32)	<0.0001
Sex (Male)	11.11 (4.17-33.33)	<0.0001
BMI	0.94 (0.87-1.02)	0.148
Diabetes	2.33 (0.93-5.81)	0.070
Cirrhosis	9.37 (3.82-23.00)	<0.0001

Additive model including age, gender, BMI, diabetes, and cirrhosis as covariates.

these factors, male gender (OR 11.11 [95% CI 4.17–33.33], $p < 0.0001$) and the presence of underlying cirrhosis (OR 9.37 [95% CI 3.82–23.00], $p < 0.0001$) conferred the greatest risk. The presence of T2DM or raised BMI were not significant predictors of HCC risk in this analysis, although a trend towards significance was observed for T2DM (OR 2.33 [95% CI 0.93–5.81], $p = 0.070$). No significant interactions were identified within the regression model. The results of multivariate analysis correcting for age, gender, T2DM, and BMI but not cirrhosis are shown in [Supplementary Tables 1 and 2](#).

NAFLD-HCC patients carrying the G allele on average developed HCC at a younger age than those that do not ([Table 3](#)). When NAFLD-HCC patients were stratified by median age (≤ 70 years) a significant enrichment in G allele carriage was present in the younger HCC patients over the older ones (MAF Young 0.59 vs. Old 0.42, χ^2 5.76, $p = 0.016$). Similarly, when compared to the NAFLD-Cohort control group, the effect of rs738409 carriage was statistically significant in those younger than the median age (OR 2.85 [95% CI 1.85–4.37], χ^2 24.09, $p < 0.0001$) but not in those older.

Consistent with previous reports [18], carriage of the rs738409 C > G polymorphism was also significantly associated with the presence of NAFLD-related cirrhosis (OR 2.33 [95% CI 1.66–3.27]; χ^2 24.8, $p < 0.0001$) across the entire 375 patient NAFLD cohort and exhibited a gene-dosage effect with the incidence of cirrhosis increasing with the number of G alleles present (Cochran-Armitage χ^2 for trend 22.68, $p < 0.0001$). To further

examine the effect of *PNPLA3* on HCC risk independent of degree of fibrosis, only those patients with coexistent cirrhosis were studied (NAFLD-HCC $n = 67$, NAFLD-Cohort $n = 26$). Study-group sizes were relatively small in this sub-analysis however carriage of the rs739409 C > G polymorphism remained significantly associated with NAFLD-HCC (OR 2.06 [95% CI 1.07–3.94]; χ^2 4.78, $p = 0.029$) in patients with cirrhosis. Adopting an additive model incorporating genotype, gender, age at diagnosis, T2DM, and BMI, *PNPLA3* remained significantly associated with HCC (OR 3.41 [95% CI 1.39–8.37], $p = 0.0074$) amongst patients with cirrhosis. The effect did not reach statistical significance in the non-cirrhotic cohort, but for HCC this group included only 23 cases.

Discussion

Using a large, well-characterised Northern European cohort with biopsy-proven NAFLD we show a strong association between *PNPLA3* rs738409 genotype and HCC risk. This highly significant effect was independent of potentially confounding factors including age, gender, co-existent diabetes, obesity, and the presence of cirrhosis. Although *PNPLA3* rs738409 is principally recognised as a disease modifier in NAFLD [15], studies thus far reporting associations with HCC have been in individuals with viral hepatitis [19,20,23,24], alcoholic liver disease [19–21] or obesity [25] (reviewed [16,22]). We report the first NAFLD study in which multivariate analysis relative to a well-characterised NAFLD population demonstrates that carriage of each G allele is associated with a doubling of HCC risk (adjusted OR 2.26 [95% CI 1.23–4.14], $p = 0.0082$).

As a tertiary centre the NAFLD cohort represents a more severe spectrum of disease than is present in an unselected population cohort, it is likely to be enriched for rs738409 C > G minor allele carriage. Therefore, we also compared the NAFLD-HCC cohort with a UK general population sample (the MRC/Wellcome Trust funded UK 1958 Birth Cohort) to provide a measure of effect relative to an unselected background population. Although additional phenotype data for this secondary analysis was limited, with only univariate comparisons possible, the results were striking. Carriage of the G allele was strongly associated with HCC, with the homozygote GG genotype being associated with an unadjusted odds ratio for HCC of 12.19 (95% CI 6.89–21.58) over CC. Consequent to the nature of the cohort in this second analysis,

potential confounders such as age, gender, co-existent diabetes, obesity and the presence of cirrhosis were not controlled for – which should be remembered when regarding the estimates of effect-size in this comparison. However, in combination with the results of our primary analysis comparing NAFLD-HCC with histologically characterised NAFLD, the data consistently demonstrate significantly increased HCC risk with *PNPLA3* rs738409 C >G minor allele carriage. These results have implications for our understanding of HCC pathogenesis in NAFLD and, if supported by further validation, are also potentially of clinical relevance.

In keeping with established predictors of progression from steatosis to NASH and fibrosis, and the recognised independent associations of HCC with both cirrhosis and T2DM [1,34], we show that NAFLD-HCC patients are likely to be older males with T2DM and underlying cirrhosis. In contrast, although NAFLD-HCC patients were generally obese, at the time of HCC diagnosis the mean BMI was lower than that of the reference NAFLD population. The reason for this is not clear however this observation is possibly attributable to HCC induced cachexia.

A key finding in the current study is that the influence of rs738409 C >G on HCC risk was greater than can be accounted for by the associated increased risk of progression to cirrhosis. This was demonstrated both in the multivariate analysis, where the effect of cirrhosis was controlled by inclusion as a covariate, and also in a sub-analysis with the cohort stratified according to presence of cirrhosis. Amongst cirrhotics, rs738409 C >G remained a highly significant factor even after the other covariates were included in the model (OR 3.41 [95% CI 1.39–8.37], $p = 0.0074$), indeed the adjusted odds ratio increased. Thus, supporting the conclusions of the multivariate regression analysis, HCC risk conferred by *PNPLA3* genotype is not mediated solely through progression to advanced fibrosis. The effect of *PNPLA3* did not reach significance in the non-cirrhotic sub-group and so it is tempting to speculate that the effect of rs738409 on HCC risk is largely confined to those with cirrhosis; however, only 23 HCC patients were included in that analysis and overall G allele carriage was low, severely limiting statistical power and so the negative result should be interpreted with caution. Furthermore, no significant interaction between genotype and cirrhosis was identified in the logistic regression model making this interpretation less likely. Validation in a larger NAFLD-HCC cohort will be required to clarify this point; irrespective of this our data support the view that HCC promotion by *PNPLA3* in NAFLD is independent of its role in fibrosis progression.

It is noteworthy rs738409 C >G polymorphism carriage was associated with a mean 4-year younger age at tumour presentation than the CC genotype (Table 3). Consistent with a recent report that *PNPLA3* genotype may have a greater impact on hepatic fibrosis progression at younger onset of alcohol consumption [35], our observation that minor allele carriers were younger at HCC diagnosis possibly suggests the influence of genetic factors on HCC risk diminishes with age, whilst acquired exposure to environmental factors exerts a greater effect. Stratification by median age showed the greatest effect of *PNPLA3* in those aged less than seventy and so would support this view however, as discussed earlier, such an interaction is not supported by the multivariate analysis and so further studies in larger cohorts will be needed to address this.

The *PNPLA3* gene encodes a protein that is closely related to adipose triglyceride lipase (*ATGL/PNPLA2*), the major TAG hydrolase in adipose tissue [36,37]. *PNPLA3* represents one of a small

number of genes that has been consistently identified as a modifier of NAFLD severity (reviewed [15]). Notably, the variant has also been associated with inflammation and fibrosis independent of TAG accumulation [18,31,32,38]. The pathogenesis of HCC in NAFLD has been the subject of recent reviews, highlighting the likely contributions of lipotoxicity [9], metabolic or stress response pathways [39], gut microbiota, bile acid receptors, vitamin D, senescence, and autophagy in hepatic stellate cells as well as progenitor cell dysregulation [40]. Our findings suggest that the role of *PNPLA3*, probably in the context of these proposed mechanisms, warrants further investigation. Taken together with the observation that carriage of the rs738409 GG genotype was associated with more poorly differentiated tumour amongst those with histologically characterised NAFLD-HCC, these findings suggest that *PNPLA3* genotype may directly influence tumour biology. Additional studies will be required to establish how these effects of *PNPLA3* are mediated. However, it may be that accumulating fat promoted by *PNPLA3* I148M, oxidative stress, and the low-grade inflammatory response present in NAFLD, favour a pro-carcinogenic milieu within the liver [9]. This interpretation would be consistent with previous studies that suggest a greater effect of the *PNPLA3* variant on HCC risk in steatotic liver diseases (e.g., ALD, and here NAFLD) than that observed in non-steatotic conditions such as viral hepatitis [22,41].

The high prevalence of NAFLD within the general population and the potential for disease progression both to cirrhosis and HCC poses a major challenge to existing healthcare infrastructure [3]. Understanding the contribution of *PNPLA3* to this process may theoretically have relevance to future preventive or therapeutic strategies targeting NAFLD-HCC. Ultrasonography and serological examinations (alpha-fetoprotein, AFP) [6] are presently used surveillance strategies, but even when targeting the cirrhotic population, their cost-effectiveness is debatable [42,43]. Like most common diseases, NAFLD-related HCC is a complex disease trait with risk influenced by a combination of genetic and environmental factors. Whilst the odds ratios reported here are substantial and highly statistically significant, the *PNPLA3* rs738409 variant is not be regarded as the sole driver for HCC and it cannot in isolation be used to stratify individual HCC risk [44]. However, if the results of the current study are corroborated, *PNPLA3* rs738409 genotype could potentially be included in a broader multi-factorial risk assessment to help physicians to identify those amongst the expanding population of obese individuals with NAFLD at greatest risk of HCC, both in the presence and absence of cirrhosis [7,9]. If the current findings are validated, a prospective economic evaluation of strategies incorporating assessment of *PNPLA3* rs738409 C >G status to target surveillance to those at greatest HCC risk may be warranted.

In summary, we report a striking association between *PNPLA3* rs738409 and the development of NAFLD-HCC, which is independent of other known risk factors. These data highlight the importance of understanding the contribution of *PNPLA3* I148M to NAFLD-HCC pathogenesis, and if validated, may contribute to a tailored approach to the cost effective surveillance and detection of NAFLD-HCC in those at greatest risk.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2014.02.030>.

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