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Original article

A nutrigenetic precision approach for the management of non-alcoholic fatty liver disease

Nuria Perez-Diaz-del-Campo [a], Eleonora Dileo [a], Gabriele Castelnuovo [a], Aurora Nicolosi [a], Marta Guariglia [a], Gian Paolo Caviglia [a], Chiara Rosso [a], Angelo Armandi [a, b], Elisabetta Bugianesi [a, c, *]

[a] Department of Medical Sciences, University of Turin, 10126 Turin, Italy
[b] Metabolic Liver Disease Research Program, I. Department of Medicine, University Medical Center of the Johannes Gutenberg-University, 55131 Mainz, Germany
[c] Gastroenterology Unit, Città della Salute e della Scienza—Molinette Hospital, 10126 Turin, Italy

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ABSTRACT

Background & aims: The Patatin-like phospholipase domain–containing 3 (*PNPLA3*) rs738409 single nucleotide polymorphism (SNP) is one of the major genetic determinant of non-alcoholic fatty liver disease (NAFLD) and is strongly regulated by changes in energy balance and dietary factors. We aimed to investigate the association between the *PNPLA3* rs738409 SNP, nutrient intake and NAFLD severity.

Method: *PNPLA3*-rs738409 SNP was genotyped in 181 patients with NAFLD who completed the EPIC Food Frequency Questionnaire. Liver steatosis was evaluated by Controlled Attenuation Parameter (CAP) (Fibroscan®530, Echosens). According to the established cut-off, a CAP value ≥ 300 dB/m was used to identify severe steatosis (S3). An independent group of 46 biopsy-proven NAFLD subjects was used as validation cohort.

Results: Overall, median age was 53 years (range 44; 62) and 60.2% of patients were male. Most subjects (56.3%) had S3 and showed increased liver stiffness ($p < 0.001$), AST ($p = 0.003$) and ALT levels ($p < 0.001$) compared to those with CAP < 300 dB/m. At logistic regression analyses we found that the interaction between carbohydrates intake and the carriers of the *PNPLA3* G risk allele was significantly associated with S3 ($p = 0.001$). The same result was confirmed in the validation cohort, were the interaction between high carbohydrate intake (48%) and *PNPLA3* SNP was significantly associated with steatosis ≥33% ($p = 0.038$).

Conclusion: The intake of greater than or equal to 48% carbohydrate in NAFLD patients carriers of the CG/GG allele of *PNPLA3* rs738409 may increase the risk of significant steatosis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most prominent cause of chronic liver disease in the world, with a prevalence that is rising parallel to the obesity burden [1] as it is tightly linked to insulin resistance and metabolic syndrome features, including obesity, type 2 diabetes mellitus (T2DM), dyslipidemia and hypertension [2]. NAFLD may present as simple steatosis or with a potentially progressive inflammatory form (non-alcoholic steatohepatitis - NASH), leading to fibrosis and ultimately to cirrhosis and/or hepatocellular cancer (HCC) [3]. The underlying mechanisms for the development of NAFLD are complex and multiple environmental and genetic factors are involved [4]. In this regard, the main association uncovered is with the Patatin-like phospholipase domain–containing 3 (*PNPLA3*) rs738409 single nucleotide polymorphism (SNP), which has also been shown to interact with the environment [5]. Of note, the dietary components of an obesogenic environment may unveil or amplify the risk associated with genetic variants [6].

Since no pharmacological drug has yet been approved specifically for the treatment of NAFLD, lifestyle modification that leads to weight loss (hypocaloric diets and/or increased physical activity) is the cornerstone approaches so far [7]. Factors such as a high calorie intake and the excessive consumption of saturated fats or refined carbohydrates have been related to NAFLD developments [8]. However, there is little agreement regarding whether diet composition or following a particular dietary pattern or strategy provides greater benefit [9]. Precision nutrition is an emerging therapeutic approach that takes into account the individual's genetic and epigenetic information, as well as age, sex or particular pathophysiological state, with the aim of establishing nutritional guidelines for specific subgroups [10]. In this approach, the identification of people who carry a specific genetic variant predisposing to NASH allows targeting of the same specific gene or molecular

* Corresponding author. Department of Medical Sciences, University of Turin, 10126 Turin, Italy.
E-mail address: elisabetta.bugianesi@unito.it (E. Bugianesi).

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pathway to halt or reverse their hepatic steatosis, inflammation and fibrosis [11,12].

In this context, the aim of this study was first, to investigate the association of dietary components and liver damage in patients with NAFLD. Next, we tested the impact of interactions in dietary factors and the *PNPLA3* rs738409 genotype on the risk of NAFLD.

2. Materials & methods

2.1. Study design and participants

The current study included 181 Caucasian men and women aged 18–65 years with NAFLD diagnosed by ultrasound consecutively enrolled at the University Hospital of Turin, Italy. The exclusion criteria included refusal or inability to provide informed consent or an average alcohol consumption of more than 21/14 units/week (male/female) in the previous 6 months or history of excessive alcohol consumption in the last 5 years, among others. Subsequently, a validation analysis was carried out in 46 Caucasian subjects with biopsy-proven NAFLD. The presence of the Metabolic Syndrome (MetS) was detected according to the presence of any three out of five components (waist circumference >102 cm for men and >88 cm for women; fasting blood glucose ≥100 mg/dL; triglycerides ≥150 mg/dL or under treatment; blood pressure ≥130/85 mm Hg or under treatment; and high density lipoprotein-cholesterol cholesterol <40 mg/dL in men or <50 mg/dL in women) [13]. Subjects aged ≥18 years signed the informed consent for participation in the study.

2.2. Anthropometric, biochemical and dietary assessment

The assessment of anthropometrical measurements and blood samples was carried out after overnight fasting at the time of enrolment. Body Mass Index (BMI) was calculated as the body weight divided by the squared height (kg/m^2). Blood glucose, total cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) concentrations were retrieved from patients' medical records.

The dietary intake of participants was assessed by the European Prospective Investigation into Cancer and Nutrition Food Frequency Questionnaire (EPIC) [14]. The following variables were analysed: total energy (kcal/d), carbohydrates (% of energy), fat (% of energy), protein (% of energy), alcohol (% of alcohol), fiber, monounsaturated fatty acids (MUFA) (g/d), polyunsaturated fatty acids (g/d) and saturated fatty acids intake (SFA) (g/d).

2.3. 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 [15]. Hardy–Weinberg equilibrium, linkage disequilibrium and haplotype inferences were estimated using the Convert program (Version 1.31) and the Arlequin software (Version 3.0). Hardy–Weinberg equilibrium was calculated with a statistical test (Chi-square). The variant of *PNPLA3* gene was in Hardy Weinberg equilibrium ($p > 0.05$).

2.4. Evaluation of liver status

The presence/entity of liver stiffness (LS) and steatosis was indirectly evaluated by transient elastography (FibroScan®,Echosens) and Controlled Attenuation Parameter (CAP) in fasting condition with the subject in the supine position and the right arm in maximum abduction. Depending on the obesity status, M and XL probes at 50 Hz between the 6th and the 9th intercostal space were selected under the professional criteria. Repeated shots were performed until obtaining 10 valid values of which the median was the selected value. All measurements were considered technically reliable according to the interquartile range (IQR)/median range below 30%.

In the subset of patients with histological diagnosis of NAFLD, liver biopsies were centrally read at the University of Torino. Liver specimens were stained with haematoxylin and eosin, Masson's trichrome, and special stains for iron and copper and examined by a local expert liver pathologist blinded to patient clinical information. The average length of liver tissue was 25 mm (range 14–45 mm), with a minimum of 11 portal tracts. Histological features of NAFLD, i.e. steatosis, inflammation, hepatocyte ballooning, and fibrosis were assessed and scored as described elsewhere [16]. Diagnosis of NASH was established according to the joint presence of steatosis, hepatocyte ballooning, and lobular inflammation with or without fibrosis.

2.5. Statistical analysis

Continuous variables are expressed as means and standard deviation (SD) or as medians and interquartile ranges (IQR) depending on their distribution, while qualitative categorical variables were analyzed with the X^2 test and reported as absolute (n) and relative frequencies (%). Distribution of variables was assessed through the Shapiro-Wilk test. Data normality and outliers were also checked using boxplots. Those variables following a normal distribution were analyzed using parametric statistical tests while for those variables with a non-normal distribution, non-parametric statistics were applied. Descriptive statistics were used to compare baseline data of participants. For continuous variables, Student's t-tests (for parametric) of independent samples and Mann-Whitney U tests (for non-parametric) were applied. In the whole study population, liver steatosis was classified in two different groups CAP <300 vs. CAP≥300 according to established cut-off for severe steatosis, while advanced liver fibrosis was classified in two different groups LS < 8.7 vs. LS ≥ 8.8 [17]. In the subgroup with liver biopsy, significant liver steatosis was classified as steatosis ≥33% and advanced fibrosis as ≥ F3 according to Kleiner score [16].

Diagnostic tests of the regression assumption for linearity and equal variance of residuals, and the variance inflation factor (VIF) for testing collinearity between independent variables, were conducted. Logistic regression models were set up to evaluate the association of both liver steatosis and liver fibrosis risk (dependent variables) with carbohydrate intake (%E) (independent variable). To carry out the analyses, the carbohydrates intake was split into tertiles, evaluating the effect of tertile one plus tertile two *versus* tertile three. Moreover, we stratifies the study population under a dominant genetic model of the rs738409 *PNPLA3* genetic variant (CC vs. CG + GG). Regression models were adjusted for potential confounders showing at least a marginal statistical trend ($p < 0.10$) at the univariate logistic analysis, as well as potential interaction introducing the corresponding interaction terms to the models. Data were expressed in Odds Ratio (OR) and Confidence Interval (CI, 95%).

Analyses were performed using STATA 12.0 software (Stata Corp College Station, TX, USA). All calculated *p*-values were two-tailed. Values of $p < 0.05$ were considered to be statistically significant in the analyses.

3. Results

3.1. Clinical, biochemical and hepatic features of the study population

A total of 181 participants with US-diagnosed or histologically confirmed NAFLD were included in the study. The *PNPLA3* rs738409 genotype was CC in 64 (35.4%), CG in 86 (47.5%), and GG in 31 (17.1%) pa-

tients. Main body composition and biochemical features are reported in Table 1. Median age was 53 years (range 44–62) and most of the individuals were males (60.2%). Overall, the median BMI was 29.4 (range 26.1; 32.5), 57.46% had of the patients had MetS while T2DM was found in 27.6% of the participants. In addition, among patients with type 2 diabetes mellitus (N = 50), 22 (44%) were treated with metformin, 8 (16%) were treated with glucagon-like peptide-1 receptor agonists (GLP-1 agonists), 2 (4%) with pioglitazone and 4 (8%) with sodium-glucose cotransporter 2 inhibitors (SGLT-2 inhibitors), while 14 (28%) were under dietary treatment. Moreover, 33 (18.23%) patients with dyslipidaemia received statins.

Concerning indirect assessment of liver damage, liver stiffness had a median value of 5.1 (range 4.4; 6.1), while 102 (56.3%) of the participants had a CAP≥300 dB/m, indicative of severe steatosis. In terms of dietary intake (median values), energy intake was 1694 kcal (range 1275; 2134), while macronutrient values were as follows: percentage of carbohydrates 45.0 (range 41.0; 49.7), percentage of protein intake 18.7 (range 15.8; 21.5) and percentage of fat intake 36.5 (range 33.0; 40.7).

Characteristics of the study cohort according to liver steatosis (mild or moderate vs severe) and liver stiffness (mild/moderate vs advanced)

For the purpose of the analysis, participants were split into those with mild or moderate steatosis (CAP < 300 dB/m, n = 79) versus those with severe steatosis (CAP≥300 dB/m, n = 102). Baseline anthropometric, biochemical and hepatic data as well as dietary intake characteristics of the study cohort according to hepatic steatosis groups are presented in Table 2. No sex and age differences were found between groups. However, patients with severe steatosis had a higher rate of diabetes ($p < 0.001$), higher BMI as well as increased AST ($p = 0.003$), ALT ($p < 0.001$), glucose ($p = 0.017$) and triglycerides levels ($p = 0.017$). Of note, the prevalence of the *PNPLA3* G-allele (68.6%) and liver stiffness were significantly increased in patients with a CAP≥300 compared to the mild or moderate steatosis group. Regarding dietary intake, no significant differences were found in any of the variables.

Moreover, patients were also classified into two different groups LS < 8.7 (n = 164) versus LS ≥ 8.8 (n = 17) for the assessment of advanced liver stiffness (Table 3). Significant differences were observed among groups in age ($p = 0.002$), as well as in the presence of diabetes ($p < 0.001$). Regarding biochemical variables, patients with advanced stiffness had higher levels of AST ($p = 0.011$), GGT ($p = 0.001$), glucose ($p < 0.001$) and triglycerides ($p = 0.027$). Importantly, a higher CAP value was observed in the advanced fibrosis group, while the distribution of PNPLA3 genotype was not different. In terms of dietary assessment, patients with advanced stiffness had lower levels of MUFA ($p = 0.029$) and SFA ($p = 0.025$).

Table 1
General characteristics of participants.

Variables	n = 181
Sex (Male/Female)	109/72
Age (years)	53.0 (44; 62)
BMI (kg/m^2)	29.4 (26.1; 32.5)
Metabolic syndrome, n (%)	104 (57.46)
T2DM n (%)	50 (27.62)
PNPLA3 CG + GG n (%)	117 (64.64)
Liver and biomarkers assessment	
Liver stiffness kPa, n (%)	
≤7 kPa (F0–F1)	151 (83.43)
7.1–8.7 kPa (F2)	13 (7.18)
8.8–10.3 Kpa (F3)	6 (3.31)
≥ 10.4 (F4)	11 (6.08)
CAP dB/m, n (%)	
≤ 234	22 (12.15)
234-269	28 (15.47)
270-300	29 (16.02)
≥ 300	102 (56.35)
AST (UI)	29.5 (24; 38)
ALT (UI)	40 (28; 60)
GGT (UI)	39 (26; 75)
Glucose (mg/dl)	96 (87; 113)
Triglycerides (mg/dl)	129 (96; 178)
Cholesterol (mg/dL)	196 (170; 222)
HDL-cholesterol (mg/dL)	47.5 (42; 57)
Dietary intake	
Energy intake (kcal/d)	1694 (1275; 2134)
Alcohol intake (% of energy)	0.4 (0; 1.4)
Carbohydrates intake (% of energy)	45.0 (41.0; 49.7)
Fiber (g/d)	14.3 (10.7; 20.4)
Protein intake (% of energy)	18.7 (15.8; 21.5)
Fat intake (% of energy)	36.5 (33.0; 40.7)
MUFA (g/d)	24.3 (17.4; 30.7)
PUFA (g/d)	13.4 (9.9; 20.8)
SFA (g/d)	22.7 (16.3; 32.3)

Data are shown as n (%) or median (IQR) according its distribution. ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BMI, Body Mass Index; CAP, Controlled Attenuation Parameter; GGT, Gamma-Glutamyl Transferase; HDL-c, High density lipoprotein-cholesterol; MUFA, Monounsaturated Fatty Acids; *PNPLA3*, Patatin-like phospholipase domain–containing; PUFA, Polyunsaturated Fatty Acids; SFA, Saturated Fatty Acids; T2DM, Type 2 Diabetes Mellitus.

Table 2
Characteristics of participants according to the steatosis degree.

	CAP < 300 dB/m	CAP≥300 dB/m	*p*-value
n	79	102	
Sex n (%)			
Male	50 (63.29)	59 (57.84)	0.458
Female	29 (36.71)	43 (42.169	
Age (y)	52 (41; 60)	55 (46; 63)	0.139
Diabetes n (%)	12 (15.19)	38 (37.25)	**<0.001**
BMI (kg/m^2)	27.7 (24.1; 30.4)	30.8 (27.8; 35.1)	**<0.001**
PNPLA3 n (%)			
CC	32 (40.51)	32 (31.37)	0.202
CG + GG	47 (59.49)	70 (68.63)	
Liver and biomarkers assessment			
Liver stiffness (kPa)	4.6 (4; 5.4)	5.7 (4.9; 7)	**<0.001**
AST (UI)	27 (22; 32)	32 (26; 41)	**0.003**
ALT (UI)	34 (24; 51)	47 (33; 67)	**<0.001**
GGT (UI)	40 (24; 81)	38 (27; 71)	0.889
Glucose mg/dl	93.5 (84; 104)	101 (89; 117)	**0.017**
Triglycerides (mg/dl)	108 (85; 167)	142 (108; 180.5)	**0.017**
Cholesterol (mg/dL)	199.1 (37.5)	195.6 (46.7)	0.621
HDL-c (mg/dL)	49.5 (43; 56)	47 (41; 58)	0.409
Dietary intake			
Energy intake (kcal/d)	1688 (1235; 2177)	1709 (1294; 2104)	0.965
Alcohol intake (% of energy)	0.5 (0; 1.8)	0.3 (0; 1.4)	0.259
Carbohydrates intake (% of energy)	45.0 (40.7; 50.9)	44.6 (41.0; 49.2)	0.549
Fiber (g/d)	13.8 (10.5; 22.0)	14.5 (10.7; 20.4)	0.770
Protein intake (% of energy)	18.6 (15.0; 21.2)	18.7 (16.1; 21.8)	0.267
Fat intake (% of energy)	36.9 (6.5)	36.5 (6.0)	0.700
MUFA (g/d)	23.9 (17.4; 33.4)	24.3 (17.0; 30.5)	0.845
PUFA (g/d)	13.3 (9.9; 21.8)	13.4 (9.8; 20.6)	0.904
SFA (g/d)	22.1 (16.2; 33.3)	23.4 (16.3; 31.2)	0.845

Data are shown as n (%), mean (SD) or median (IQR) according its distribution. ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BMI, Body Mass Index; CAP, Controlled Attenuation Parameter; GGT, Gamma-Glutamyl Transferase; HDL-c. High density lipoprotein-cholesterol; MUFA, Monounsaturated Fatty Acids; *PNPLA3*, Patatin-like phospholipase domain–containing; PUFA, Polyunsaturated Fatty Acids; SFA, Saturated Fatty Acids.

Table 3
Characteristics of participants according to liver stiffness degree.

	LS < 8.7 kPa	LS ≥ 8.8 kPa	*p*-value
n	164	17	
Sex n (%)			
Male	101 (61.5)	8 (47.06)	0.244
Female	63 (38.4)	9 (52.94)	
Age (y)	52 (44; 61)	63 (58; 68)	**0.002**
Diabetes n (%)	36 (21.95)	14 (82.35)	**<0.001**
BMI (kg/m^2)	29.3 (25.9; 32.5)	30.6 (26.3; 36.4)	0.346
PNPLA3 n (%)			
CC	59 (35.98)	5 (29.41)	0.590
CG + GG	105 (64.02)	12 (70.59)	
CAP (dB/m)	301.5 (263; 335)	348 (312; 360)	**0.002**
AST (UI)	29 (23.5; 37)	38.5 (29; 46)	**0.011**
ALT (UI)	39 (28; 62)	47 (38; 55)	0.502
GGT (UI)	38 (25; 65)	89 (42; 161)	**0.001**
Glucose mg/dl	94 (87; 108)	125.5 (105; 131)	**<0.001**
Triglycerides (mg/dl)	126 (93; 172)	159 (125.5; 215.5)	**0.027**
Cholesterol (mg/dL)	197.4 (42.7)	194.2 (47.2)	0.780
HDL-c (mg/dL)	48.5 (34; 55)	42.5 (34; 55)	0.126
Dietary intake			
Energy intake (kcal/d)	1709 (1285; 2141)	1451 (1048; 1901)	0.096
Alcohol intake (% of energy)	0.4 (0; 1.7)	0 (0; 0.5)	0.250
Carbohydrates intake (% of energy)	45.0 (41.0; 49.5)	43.0 (39.2; 51.9)	0.785
Fiber (g/d)	14.4 (10.6; 20.5)	13.9 (11.1; 19.0)	0.895
Protein intake (% of energy)	18.7 (15.7; 21.3)	18.5 (17.2; 24.6)	0.298
Fat intake (% of energy)	36.9 (6.2)	34.8 (6.2)	0.199
MUFA (g/d)	24.5 (17.4; 32.0)	18.4 (13.5; 27.1)	**0.029**
PUFA (g/d)	13.8 (10.1; 21.5)	11.7 (9.5; 14.4)	0.176
SFA (g/d)	23.2 (16.7; 32.6)	19.1 (15.1; 21.5)	**0.025**

Data are shown as n (%), mean (SD) or median (IQR) according its distribution. ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BMI, Body Mass Index; CAP, Controlled Attenuation Parameter; GGT, Gamma-Glutamyl Transferase; HDL-c. High density lipoprotein-cholesterol; MUFA, Monounsaturated Fatty Acids; *PNPLA3*, Patatin-like phospholipase domain–containing; PUFA, Polyunsaturated Fatty Acids; SFA, Saturated Fatty Acids.

Logistic regression analysis: prediction of severe hepatic steatosis and fibrosis based on the interaction between PNPLA3 CG + GG genotype x carbohydrates intake (E%)

In order to evaluate the risk of liver steatosis according on the interaction between genetics and dietary intake a logistic regression model was constructed. After adjusting for sex, age, protein intake, carbohydrate intake, energy intake and presence of diabetes, we found a significant interaction between carriers of the risk genotype (CG or GG) of the *PNPLA3* genetic variant and carbohydrate intake (*p-value for interaction* = 0.001) (Table 4). Specifically, the risk of developing hepatic steatosis is higher in subjects with a carbohydrate intake ≥48% carrying the risk allele PNPLA3 CG or GG, when compared to a wild type *PNPLA3* carriers who ingests the same amount of carbohydrates (Fig. 1). When we repeated the same analysis according to LS threshold of 8.8 kPa, we did not observe any statistically significant interactions between dietary components and *PNPLA3* (*p-value for interaction* = 0.591).

3.2. Subgroup analysis in NAFLD patients with liver biopsy: Clinical, biochemical and histological features of the study population

A total of 46 biopsy-proven cases of NAFLD were included in the study (Table 5). The median age was 49 years (range 41–60) and 54.3% were male. The *PNPLA3* rs738409 genotype was CC in 15 (32.61%), CG in 22 (47.83%) and GG in 9 (19.57%) patients. The prevalence of T2DM was 36.96% while 67.39% of the patients had MetS. Moreover, among patients with T2DM (N = 17) 4 (23.5%) were treated with metformin, 2 (11.8%) with GLP-1 agonists and 5 (29.4%) with SGLT-2 inhibitors, while 6 (35.3%) patients were being treated with diet. Also, among patients with dyslipidaemia, 4 (8.70%) took statins.

As for the indirect assessment of liver damage, CAP values correlated with the amount of steatosis at liver biopsy (r = 0.413, $p = 0.025$) and LS with fibrosis stage (r = 0.653, $p < 0.001$). Overall, 15/46 patients (32.6%) had advanced fibrosis (F ≥ 3) on liver histology and 30/46 (65.2%) had at least 33% of hepatic fat at histology. The median energy intake was 1824 kcal (range 1468; 2166) while the percentage of macronutrients was carbohydrates 42.6% (range 37.4; 48.5), protein 18.1% (range 14.7; 20.6) and fat intake 40.5% (range 35.4; 44.9).

Table 4
Univariate and multivariate logistic regression analysis for risk of severe steatosis (CAP≥300 dB/m) by carbohydrates intake.

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
CH intake (≥48%) x *PNPLA3* (CG + GG)			11.5 (2.6; 49.2)	0.001
Sex (Women)	1.2 (0.6; 2.2)	0.458	–	–
Age	1.0 (0.9; 1.0)	0.164	–	–
Energy (kcal)	1.0 (0.9; 1.0)	0.662	–	–
Fat intake (%)	0.9 (0.94; 1.0)	0.698	–	–
Protein intake (%)	1.0 (0.9; 1.1)	0.178	–	–
T2DM	3.3 (1.5; 6.9)	0.001	3.4 (1.6; 7.4)	0.001

CH, carbohydrates; T2DM, type 2 diabetes mellitus.

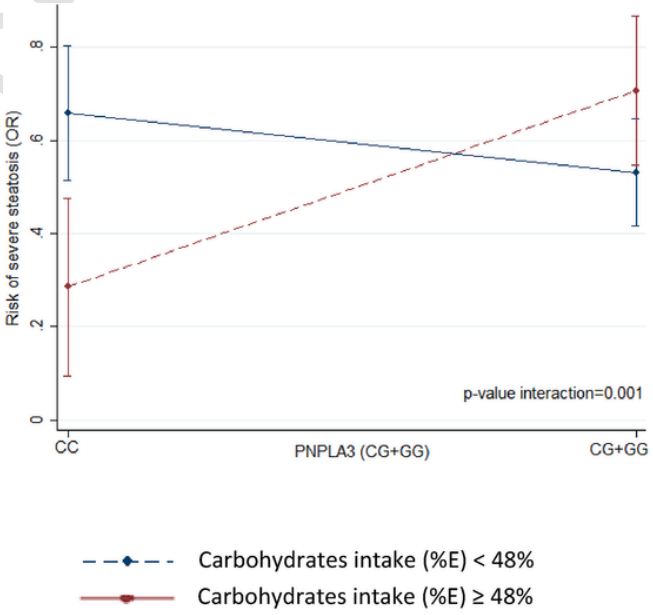

Fig. 1. Impact of the *PNPLA3* rs738409 polymorphism on carbohydrates intake on the risk for severe steatosis (CAP≥300 dB/m). The model was adjusted by T2DM. Abbreviations: *PNPLA3*, Patatin-like phospholipase domain–containing.

Table 5
General characteristics of participants.

Variables	n = 46
Sex (Male/Female)	25/21
Age (years)	49 (41; 60)
Weight (kg)	90.5 (83; 98)
BMI (kg/m^2)	30.5 (28.5; 34.1)
T2DM, n (%)	17 (36.96)
Metabolic syndrome, n (%)	31 (67.39)
PNPLA3 CG + GG, n (%)	31 (67.39)
Liver stiffness (kPa)	7.1 (5.3; 9)
CAP (dB/m)	323 (302; 350)
AST (UI)	29.5 (24; 42)
ALT (UI)	49.5 (34; 83)
GGT (UI)	44.5 (28; 67)
Glucose mg/dl	94.5 (85.5; 111)
Triglycerides (mg/dl)	135.5 (100; 197)
Cholesterol (mg/dL)	184.5 (153; 230.5)
HDL-c (mg/dL)	42 (39; 49)
Histopathological report	
Fibrosis stages, n (%)	
0	7 (15.22)
1	20 (43.48)
2	4 (8.70)
3	11 (23.91)
4	4 (8.70)
Steatosis, n (%)	
5–33%	16 (34.78)
33–66%	22 (47.83)
>66%	8 (17.39)
Lobular inflammation, n (%)	
No foci	6 (12.77)
<2 foci/200x	32 (69.57)
>2 foci/200x	8 (17.39)
Ballooning, n (%)	
None	8 (17.39)
Few	31 (67.39)
Many	7 (15.22)
NAS Kleiner, n (%)	
NAS<4	17 (36.96)
NAS≥4	29 (63.04)
Dietary intake	
Energy intake (kcal/d)	1824 (1468; 2166)
Alcohol intake (% of energy)	0 (0; 0.63)
Carbohydrates intake (% of energy)	42.6 (37.4; 48.5)
Fiber (g/d)	15.1 (12.5; 18.4)
Protein intake (% of energy)	18.1 (14.7; 20.6)
Fat intake (% of energy)	40.5 (35.4; 44.9)
MUFA (g/d)	27.9 (18.3; 33.7)
PUFA (g/d)	21.6 (12.8; 27.1)
SFA (g/d)	27.6 (21.0; 31.4)

Data are shown as n (percent) or median (IQR). ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BMI, Body Mass Index; CAP, Controlled Attenuation Parameter; GGT, Gamma-Glutamyl Transferase; HDL-c. High density lipoprotein-cholesterol; MUFA, Monounsaturated Fatty Acids; NAS Kleiner, NAFLD activity score; *PNPLA3*, Patatin-like phospholipase domain–containing; PUFA, Polyunsaturated Fatty Acids; SFA, Saturated Fatty Acids; T2DM, Type 2 Diabetes Mellitus.

Logistic regression analysis: prediction of histological significant steatosis and advanced fibrosis based on an interaction between PNPLA3 CG + GG x carbohydrates intake (E%)

To assess the risk of steatosis and liver fibrosis according to genetic and dietary intake, a logistic regression model was constructed (Table S1). First, we observed a significant interaction between the *PNPLA3* CG + GG genetic variants and carbohydrate intake (*p-value for interaction* = 0.038) for the risk of significant steatosis (≥33%) (Fig. 2). This model was adjusted for fat intake (%). Second, a logistic regression analysis adjusted by sex, age was performed to evaluate the risk of advanced fibrosis (≥F3). In this case, the results showed only a trend towards a significant interaction between the *PNPLA3* risk genotype and carbohydrates intake (*p-value for interaction* = 0.105) (Fig. S1).

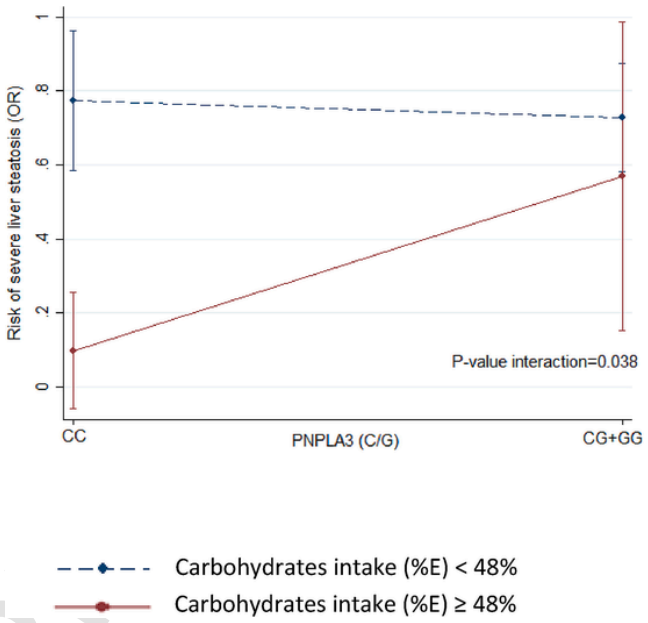

Fig. 2. Impact of the *PNPLA3* rs738409 polymorphism on carbohydrates intake on the risk for significant liver steatosis (≥33%) Abbreviations: *PNPLA3*, Patatin-like phospholipase domain–containing.

4. Discussion

In this study, we demonstrated an interaction of genetic and dietary factors in the development of the main hallmarks of liver damage in NAFLD. Specifically, we demonstrated that, carrying the *PNPLA3* CG + GG alleles together with a high carbohydrate intake (≥48%) was associated with an increased risk of severe hepatic steatosis (≥300 dB/m) by CAP. This finding has been confirmed by a subsequent analysis in 46 patients with biopsy-proven NAFLD: *PNPLA3* rs738409 G-allele carriers have an altered susceptibility to high carbohydrate intake (≥48%) leading to an increased risk of significant (>33%) steatosis. In this specific subgroup of PNPLA3 risk allele with high carbohydrate intake we also noticed a clear trend for an increased risk of advanced fibrosis at liver biopsy, that was borderline significant because of the limited number of subjects.

One of the biggest challenges in the field of NAFLD is the identification and management of high-risk individuals [18]. From this point of view, medical treatment of NAFLD has long focused on weight reduction, mainly through low-energy dietary intervention with or without modified macronutrient distribution [19]. In our results, participants carrying the CG/GG *PNPLA3* genotype along with an intake of carbohydrates ≥48% showed a higher risk of developing steatosis compared to carriers of the CC *PNPLA3* genotype. Human and animal studies have strongly demonstrates that the *PNPLA3* gene is related to changes in energy balance, being it expression strongly suppressed by fasting and stimulated by refeeding [20]. Carbohydrates, in particular fructose, are key factors in liver fat deposition because they activate sterol regulatory element-binding protein-1, which stimulates hepatic de novo lipogenesis and inhibits hepatic fatty acid oxidation [21]. In accordance with our results, a previous study carried out in Hispanic children, observed that carriers of the GG *PNPLA3* genotype have reduced capacity to hydrolyze triglycerides in the liver, being therefore more susceptible to increased hepatic fat when dietary carbohydrate intake, specifically sugar, was high. Moreover, recent evidence from *in vivo* studies demonstrated the role of downregulation of the *PNPLA3* mutant allele in improving all features of NAFLD [22], implying that factors targeting this

gene variant may be considered as part of the management for treating fatty liver. Our findings extend prior studies observing interactions between *PNPLA3* variant and adiposity [23,24], *PNPLA3* and body weight [25] and *PNPLA3* variant and *PNPLA3* variant and alcohol [26]. Altogether, these results imply that the effect of *PNPLA3* genetic variant on liver disease may be modulated by environmental factors.

On the other hand, we also found an increased risk of significant liver fibrosis in individuals carrying the CG/GG *PNPLA3* genotype and a carbohydrate intake ≥48%. This result is in line with a previous study carried out in 452 non-Hispanic whites with histologically confirmed NAFLD [27]. In this study, authors speculated that *PNPLA3* rs738409 G-allele might modulate the effect of specific dietary nutrients on risk of fibrosis in patients with NAFLD. Also, it has been recently published a moderate effect of PNPLA3 rs738409 G allele on the association between visceral fat area and risk of significant liver fibrosis ($F \geq 2$) in adult subjects. However, although *PNPLA3* gene expression is known to be regulated by some nutrients, the relationships between rs738409, nutrient intake and liver histology in NAFLD are not well understood [28].

One of the main strengths of this research is the conceptual modelling for personalized diet prescription using genetic information. In this regard, we showed for the first time a differential response of *PNPLA3* rs738409 genetic variant to carbohydrate intake on the risk of severe steatosis and significant fibrosis. It is also important to highlight that our findings by non-invasive tools were corroborated by similar results in the subgroup with liver biopsy, which is considered the gold standard for the diagnosis of NAFLD. Our study has some drawbacks: firstly, the mutation rates for *PNPLA3* rs738409 polymorphism vary among different ethnic populations, thus, the findings of our study might not be generalizable to other ethnic group. Second, due to the cross-sectional design of the study, causal inferences cannot be made. However, since *PNPLA3* rs738409 polymorphism is inherited, reverse causation does not apply. Thirdly, dietary and data including information about competing causes of liver disease were collected either using self-reported information or based on a clinical interview. Thus, results may be susceptible to some biases. Lastly, we lack physical activity assessment in these patients as well as a non-NAFLD control group, so further studies including a larger number of individuals and different cohorts could confirm whether the PNPLA3 rs738409-diet interactions observed in patients with NAFLD are reproducible in the context of other diseases.

5. Conclusion

In this study, we showed that a carbohydrate intake greater than or equal to 48% in NAFLD patients carriers of the CG/GG allele of *PNPLA3* rs738409 may increase the risk of significant steatosis. Future nutritional and lifestyle interventions will benefit greatly from personalized and tailored treatment strategies (i.e. “precision nutrition”) rather than aiming for weight loss *per se*.

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Author contributions

Conceptualization, N.P.D.D.C. and E.B.; data curation, N.P.D.D.C.; formal analysis, N.P.D.D.C.; funding acquisition, E.B; investigation, N.P.D.D.C.; methodology, N.P.D.D.C., E.D., G.P.C. and C.R..; project administration, E.B; resources, E.B.; software, N.P.D.D.C.; supervision, E.B; validation N.P.D.D.C. and E.B.; visualization, G.C., G.P.C., C.R., A.A and E.B; Roles/Writing—original draft preparation, N.P.D.D.C.; writing—review and editing, G.P.C., C.R., and E.B. All authors have read and agreed to the published version of the manuscript.

Data availability statement

Data available on request due to privacy/ethical restrictions.

Conflict of Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2023.09.022.

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