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Could inherited predisposition drive non-obese fatty liver disease? Results from German tertiary referral centers

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is frequent among obese individuals with metabolic syndrome. Variants *PNPLA3* p.I148M, *TM6SF2* p.E167K and *MBOAT7* rs641738 are associated with higher liver fat contents. Here we analyzed 63 biopsied non-obese, non-diabetic patients with NAFLD (39 men, age: 20–72 years) recruited within the German NAFLD CSG program. The frequencies of the *PNPLA3*, *TM6SF2* and *MBOAT7* polymorphisms were compared with the remaining patients in the NAFLD CSG cohort and with a control population ($n = 174$). Serum CK18-M30 was measured by ELISA. In non-obese NAFLD patients, the frequency of the *PNPLA3* p.I148M allele (74.6%), but not of the *TM6SF2* or *MBOAT7* polymorphisms, was significantly ($P < 0.05$) higher as compared to the other patients in the NAFLD CSG cohort (54.9%) or controls (40.2%). The presence of the minor *PNPLA3* p.I148M risk allele increased the risk of developing NAFLD (OR = 3.29, $P < 0.001$) and was associated with higher steatosis, fibrosis, and serum CK18-M30 levels (all $P < 0.05$). According to the population attributable fraction (PAF), 49.8% of NAFLD cases could be eliminated if the *PNPLA3* mutation was absent. The *MBOAT7* polymorphism was more frequent ($P = 0.019$) in patients with severe hepatic steatosis. In conclusion, *PNPLA3*, and to a lesser extent, *MBOAT7* variants are associated with NAFLD risk and modulate liver injury in non-obese patients without diabetes.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by increased hepatic triglyceride contents [1]. Obesity, diabetes mellitus and metabolic syndrome are regarded to represent the major risk factors for NAFLD [2]. Non-obese individuals are, in turn, thought to only rarely suffer from NAFLD. However, a growing body of information indicates that hepatic steatosis and steatohepatitis might develop in the absence of obesity or metabolic disturbances. For example, as much as 23.5% of individuals included in a cohort of Asian patients with NAFLD were non-obese [3] and the prevalence of NAFLD in a large population of lean North-American individuals was reported at 7.39% [4].

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Thus, NAFLD is prevalent and relevant even in non-obese individuals, which emphasizes the need to identify risk factors and pathomechanisms of increased hepatic fat contents in this population. The pathogenesis of NAFLD in non-obese patients is not fully understood [5] but it is straightforward to speculate that genetic predisposition could play a significant role in this setting. Of note, a recent albeit small twin-based study [6] indicated that, as much as 52% of the entire NAFLD risk might be heritable due to prosteatotic gene variants. To date, the variants *PNPLA3* p. I148M [7], *TM6SF2* p.E167K [8] and *MBOAT7* rs641738 [9] are thought to be the most prominent genetic risk factors for steatosis and steatohepatitis. As shown by others [10, 11] and us [12, 13], carriers of these variants develop NAFLD more frequently and are characterized by more severe hepatic phenotypes. Hepatocyte apoptosis represents a pathomechanism contributing to NAFLD severity and progression. During apoptosis, caspase-cleaved keratin-18 fragments are released into the blood and can be detected by M30-ELISA. This assay allows the detection of NAFLD severity as demonstrated by multiple studies [14].

So far, only very few studies have investigated the role of genetics in non-obese fatty liver disease, and available results are somewhat inconclusive. For example, Leung et al. [3] analyzed the biopsy-based cohort of Chinese patients with NAFLD but did not detect significant differences in the frequencies of either *PNPLA3* or *TM6SF2* variants between non-obese and obese patients. Of note, that study [3] used the Asian-specific BMI cutoffs for overweight and obesity, i.e., 23–25 kg/m^2 and ≥25 kg/m^2, respectively, which are lower in comparison to the cutoffs recommended for Caucasians. More precisely, current WHO recommendations [15] and data concerning the all-cause mortality [16] point to the cutoff of 30 kg/m^2 to be most appropriate for defining obesity, even if slightly lower cutoffs might apply for Asian and Pacific populations [15]. Down the same line, Feldman et al. [17] applied the BMI cutoff of 25 kg/m^2 in a cohort of 55 lean Caucasian NAFLD patients and detected a significant association between the common *PNPLA3* variant p.I148M,, but not *TM6SF2* polymorphisms, and the frequency of NAFLD in lean patients. Of note, in the above study [17] fatty liver was diagnosed by abdominal sonography and not quantified further, and 31% of the patients suffered from diabetes. To expand on the above mentioned observations, here we explore the association of genetic variants with histological and clinical features of fatty liver disease in a multicenter cohort of non-obese, non-diabetic patients with biopsy-proven NAFLD.

Materials and methods

Patients and genotyping

The analysis was based on the cohort of NAFLD patients recruited within the framework of the German NAFLD Clinical Study Group (NAFLD CSG) as described previously [13]. In brief, the studied cohort encompasses patients with fatty liver recruited in eight tertiary referral German academic medical centers. Acute and chronic liver diseases other than NAFLD were excluded in all patients. For the current study, we included only patients who fulfilled all of the following criteria: (i) BMI <30 kg/m^2; (ii) absence of diabetes mellitus type 2; (iii) available results of the liver biopsy; and (iv) hepatic steatosis grade S1 or higher in liver biopsy. All patients underwent a careful clinical examination, and fasted blood specimens were obtained for liver function tests and genotyping. In addition, we measured the apoptosis-associated CK18 by the M30-Apoptosense ELISA CK18-M30 fragment as marker of liver injury [18]. Hepatic steatosis (grades from S1 to S3) and fibrosis (stages from F0 to F4) were quantified according to the Kleiner score. The variants in the *PNPLA3* (rs738409), *TM6SF2* (rs58542926) and *MBOAT7* (rs641738) genes were genotyped using TaqMan assays as described [13]. Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Statistical analysis

Genotype frequencies were compared in contingency tables (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). The distribution of quantitative traits was analyzed using Kolmogorow–Smirnow tests. Differences in clinical traits between carriers of distinct *PNPLA3, TM6SF2* and *MBOAT7* genotypes were compared using ANOVA with Bonferroni post-hoc tests or Mann–Whitney U tests, as appropriate. Frequencies of qualitative phenotypes were analyzed using contingency table statistics. The population attributable fraction (PAF) was calculated using PARC software (http://www.miner.rochester.edu/cpm/education/match/productspubs.html). All other statistical analyses were performed with SPSS 20.0 (SPSS; Munich, Germany) or GraphPad Prism 5.0 (GraphPad Software; La Jolla, CA, USA).

Table 1 Baseline characteristics and genotype frequencies in non-obese non-diabetic patients with fatty liver recruited within the NAFLD CSG cohort

Variables	Results
N (female / male)	63 (24 / 39)
Age (years)	47 (20–72)
BMI (kg/m^2)	26.6 (17.6–29.9)
ALT (U/L)	75 (14–265)
AST (U/L)	46 (20–278)
γ-GT (U/L)	103 (11–693)
Statin use	5 (7.9%)
PNPLA3 p.I148M:	
[II]	16
[IM]	34
[MM]	13
TM6SF2 p.E167K:	
[EE]	45
[EK]	17
[KK]	1
MBOAT7 rs641738:	
[CC]	17
[CT]	32
[TT]	14

ALT alanine aminotransferase, AST aspartate transaminase, BMI body mass index, γ-GT gamma-glutamyl transpeptidase, E glutamic acidm I isoleucine, K lysine, M methionine, MBOAT7 membrane-bound O-acyltransferase domain containing 7, p protein (amino acid number), NAFLD CSG NAFLD Clinical Study Group, PNPLA3 patatin-like phospholipase-domain-containing protein 3, TM6SF2, transmembrane 6 superfamily member 2

Results

In total, 63 patients out of the total of 515 with NAFLD fulfilled the inclusion criteria. The median age in the non-obese group was 47 years and 61.9% were men (Table 1). Overall, 34.9% of patients presented with steatosis grade S3 (Table 2). Fibrosis stage F2 and higher was detected in 30.6% of individuals, and a single patient presented with liver cirrhosis (stage F4, Table 2). In total, 47 (74.6%) individuals carried at least one copy of the PNPLA3 p.148M risk allele. As illustrated in Fig. 1, this frequency was substantially and significantly higher as compared to the results in the remaining individuals in the NAFLD CSG cohort (54.9%) [13] and in German controls from the general population (40.2%) [12] presented in our previous analyses. Overall, the presence of the PNPLA3 p.148M allele was associated with a significantly increased risk of developing NAFLD in non-obese individuals as compared to controls (common OR = 3.29, $P < 0.001$). Figure 2a, b shows that carriers of the risk allele (p.148M) presented more frequently with S3 steatosis ($P = 0.006$) and were at

Table 2 Distribution of steatosis and fibrosis in studied patients with NAFLD

Steatosis:	
S1	21
S2	20
S3	22
Fibrosis[a]:	
F0	24
F1	19
F2	9
F3	9
F4	1

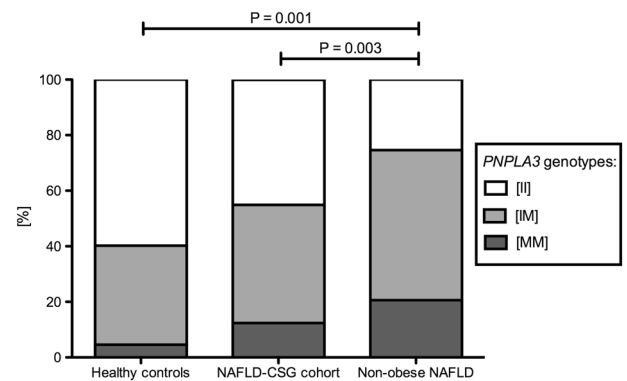[a] Data available in 62 patients

Fig. 1 Frequencies of the PNPLA3 p.I148M genotypes non-obese, non-diabetic patients with fatty liver ($n = 63$; [MM] $n = 13$, [IM] $n = 34$, [II] $n = 16$) in the remaining patients in the NAFLD CSG cohort ($n = 452$, [MM] $n = 65$, [IM] $n = 188$, [II] $n = 199$) [13] and in healthy controls ($n = 174$; [MM] $n = 8$ [IM] $n = 62$, [II] $n = 104$) [12]. Genotype frequencies were compared in contingency tables

increased risk of developing fibrosis stages above F1 ($P = 0.012$). Moreover, as shown in Fig. 3, we detected significantly ($P = 0.011$) higher serum CK18-M30 concentrations in non-obese patients with NAFLD who carried the PNPLA3 p.148M allele. Based on the results presented in Fig. 1, we calculated the population attributable fraction (PAF), i.e., the proportion of fatty liver disease due to the PNPLA3 p.I148M allele. PAF for homo- and heterozygous carriers of the risk allele is 0.498, indicating that the p.I148M variant contributes almost 50% of the total NAFLD risk. In other words, every second NAFLD case in our non-obese cohort would not have developed if the variant of the PNPLA3 gene was absent. On the other hand, the PNPLA3 risk variant did not significantly affect serum ALT, AST or γ-GT activities (all $P > 0.05$).

The TM6SF2 minor allele was carried by 18 (28.6%) and the MBOAT7 risk genotype by 14 (22.2 %) patients; these frequencies did not differ from the frequencies in the NAFLD CSG cohort (19.4% and 22.1%, respectively; both $P > 0.05$, Table 1) [13]. Neither TM6SF2 p.E167K nor the
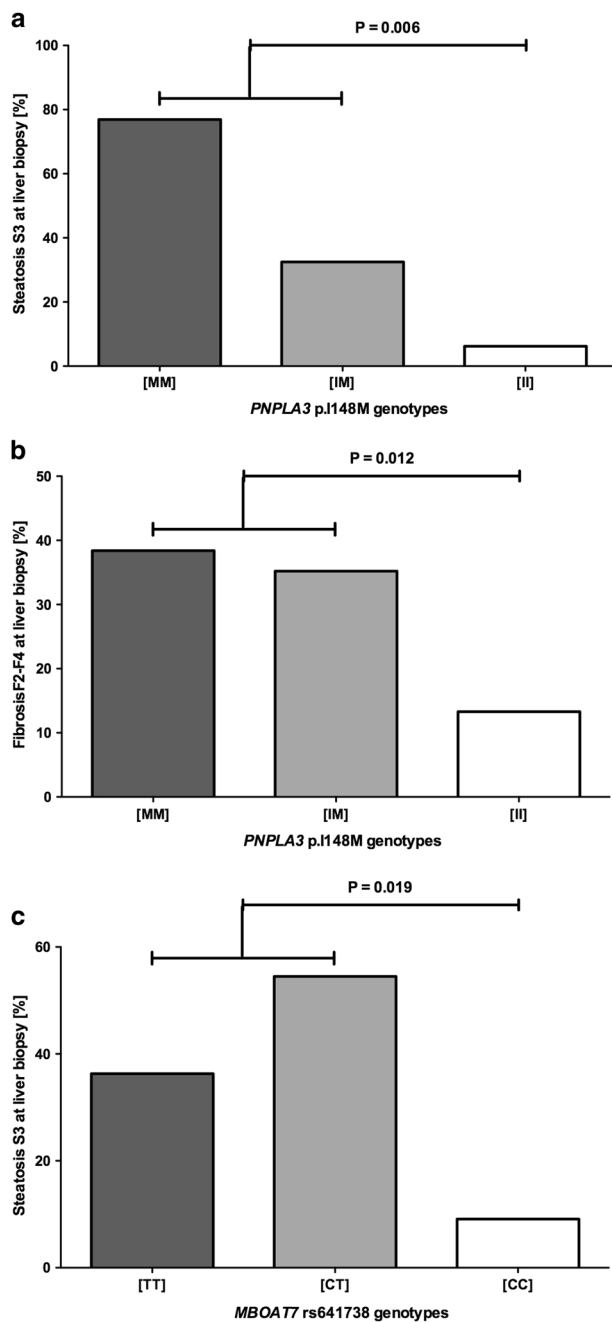
Fig. 2 a Frequency of non-obese, non-diabetic NAFLD patients presenting with S3 grade of hepatic steatosis in liver biopsy in relation to the *PNPLA3* p.I148M polymorphism. Genotype frequencies were compared with individuals with steatosis grades S1–S2 in contingency tables. **b** Frequency of non-obese, non-diabetic NAFLD patients presenting with F2–F4 hepatic fibrosis in liver biopsy in relation to the *PNPLA3* p.I148M polymorphism. Genotype frequencies were compared with individuals with fibrosis stages F0–F1 in contingency tables. **c** Frequency of non-obese, non-diabetic NAFLD patients presenting with hepatic steatosis grade S3 in liver biopsy in relation to the *MBOAT7* rs641738 polymorphism. Genotype frequencies were compared with individuals with steatosis grades S1–S2 in contingency tables
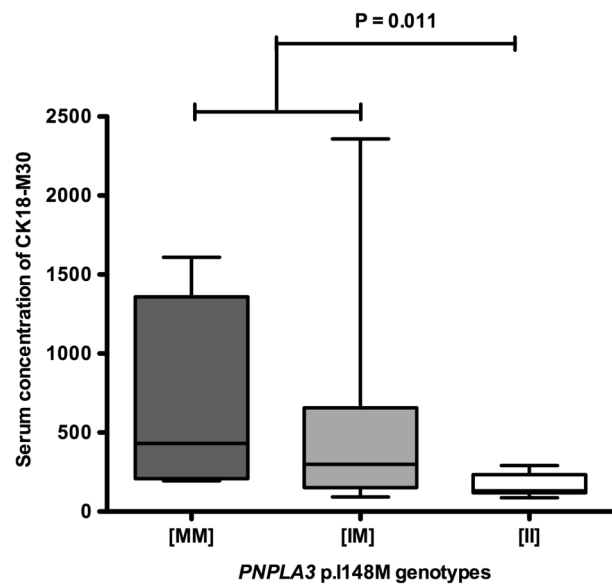

Fig. 3 Serum CK18-M30 in non-obese, non-diabetic NAFLD patients in relation to the *PNPLA3* p.I148M polymorphism (data available in 33 individuals)

MBOAT7 rs641738 polymorphism were associated with increased CK18-M30 concentrations or with fibrosis stage at liver biopsy (all $P > 0.05$). Of note, as presented in Fig. 2c, the *MBOAT7* polymorphism was significantly ($P = 0.019$) associated with the risk of developing severe (i.e., grade S3) steatosis: Only two individuals in this group carried the wild-type genotype.

Discussion

Non-obese fatty liver disease is receiving increasing attention [5]. Indeed, in contrast to common belief, obesity does not seem to be *conditio sine qua non* for the development of fatty liver. NAFLD in non-obese patients might even represent a distinct subtype of fatty liver with currently unclear pathogenesis and prognosis. In our study, we focused on the genetics as a determinant of NAFLD in non-obese patients. To our surprise, we documented a very high frequency of the *PNPLA3* variant in this group. The other two studied variants, in turn, did not seem to play a crucial role in the development of non-obese fatty liver disease. These results are in line with the concept of *PNPLA3*-associated steatohepatitis (PASH) [19], which we introduced previously as new predominantly gene-driven entity within the NAFLD spectrum. Indeed, patients with PASH might develop NAFLD and all its consequences mainly due to variant *PNPLA3* and even in the absence of other triggers of liver disease [19]. The mechanisms causing hepatic fat accumulation in the carriers of the *PNPLA3* p.148M variant remain controversial. In brief, *PNPLA3* encodes for

adiponutrin, a triglyceride hydrolase expressed at lipid droplets. The p.I148M mutation might lead to loss of the enzymatic function, resulting also in reduced VLDL secretion [20]. Recently, it has been appreciated that the *PNPLA3* risk allele disrupts the degradation of adiponutrin on the surface of lipid droplets, which might result in increased hepatic accumulation of lipids [21]. Of note, in the herein studied cohort, the presence of the *PNLPA3* risk allele was not only associated with the development of fatty liver but also increased hepatic injury. This is in line with the increased CK18-M30 serum concentrations in carriers of the *PNPLA3* variant. The role of genetic modifiers of fatty liver phenotypes is further underscored by the high frequency of the *MBOAT7* risk allele among individuals with the highest degree of hepatic steatosis. On the other hand, the low MAF of the *TM6SF2* polymorphism has to be kept in mind. Indeed, although we detected higher frequency of the minor *TM6SF2* allele in the non-obese cohort as compared to the remaining patients in the NAFLG CSG cohort, this difference was not significant. Furthermore, the number of patients carrying this allele might be too small to detect potential associations with hepatic injury.

In contrast to several published studies, which focused on the lean NAFLD, i.e., patients with by BMI <25 kg/m^2, here we set a slightly higher inclusion BMI but also added the lack of diabetes mellitus as important second inclusion criterion. By coupling these two prerequisites, we were able to define a subgroup of biopsy-proven NAFLD patients in whom genetic predisposition might play the central role in the development of fatty liver disease. Given that the *PNLPA3* p.I148M variant is known to be associated not only with severe steatosis but also with the risk of hepatocellular cancer [22–24], we reckon that further studies are required to investigate the pathogenesis of fat accumulation in non-obese patients carrying variants of *PNPLA3* and *MBOAT7*, and to define strategies to halt disease progression in this setting.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Neuschwander-Tetri BA. Non-alcoholic fatty liver disease. BMC Med. 2017;15:45.
2. Bellentani S. The epidemiology of non-alcoholic fatty liver disease. Liver Int. 2017;37(Suppl 1):81–4.
3. Leung JC, Loong TC, Wei JL, Wong GL, Chan AW, Choi PC, et al. Histological severity and clinical outcomes of nonalcoholic fatty liver disease in nonobese patients. Hepatology. 2017;65:54–64.
4. Younossi ZM, Stepanova M, Negro F, Hallaji S, Younossi Y, Lam B, et al. Nonalcoholic fatty liver disease in lean individuals in the United States. Medicine. 2012;91:319–27.
5. Kim D, Kim WR. Nonobese fatty liver disease. Clin Gastroenterol Hepatol. 2017;15:474–85.
6. Loomba R, Schork N, Chen CH, Bettencourt R, Bhatt A, Ang B, et al. Heritability of hepatic fibrosis and steatosis based on a prospective twin study. Gastroenterology. 2015;149:1784–93.
7. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2008;40:1461–5.
8. Kozlitina J, Smagris E, Stender S, Nordestgaard BG, Zhou HH, Tybjærg-Hansen A, et al. Exome-wide association study identifies a TM6SF2 variant that confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2014;46:352–6.
9. Mancina RM, Dongiovanni P, Petta S, Pingitore P, Meroni M, Rametta R, Borén J, et al. The MBOAT7-TMC4 Variant rs641738 Increases risk of nonalcoholic fatty liver disease in individuals of European descent. Gastroenterology. 2016;150:1219–30.
10. Anstee QM, Day CP. The genetics of nonalcoholic fatty liver disease: spotlight on PNPLA3 and TM6SF2. Semin Liver Dis. 2015;35:270–90.
11. Luukkonen PK, Zhou Y, Nidhina Haridas PA, Dwivedi OP, Hyötyläinen T, Ali A, et al. Impaired hepatic lipid synthesis from polyunsaturated fatty acids in TM6SF2 E167K variant carriers with NAFLD. J Hepatol. 2017;67:128–36.
12. Arslanow A, Stokes CS, Weber SN, Grünhage F, Lammert F, Krawczyk M. The common PNPLA3 variant p.I148M is associated with liver fat contents as quantified by controlled attenuation parameter (CAP). Liver Int. 2016;36:418–26.
13. Krawczyk M, Rau M, Schattenberg JM, Bantel H, Pathil A, Demir M, et al. Combined effects of the PNPLA3rs738409, TM6SF2 rs58542926, and MBOAT7 rs641738 variants on NAFLD severity: a multicenter biopsy-based study. J Lipid Res. 2017;58:247–55.
14. Ku NO, Strnad P, Bantel H, Omary MB. Keratins: Biomarkers and modulators of apoptotic and necrotic cell death in the liver. Hepatology. 2016;64:966–76.
15. Consultation WHOE. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. Lancet. 2004;363:157–63.
16. Flegal KM, Kit BK, Orpana H, Graubard BI. Association of all-cause mortality with overweight and obesity using standard body mass index categories: a systematic review and meta-analysis. JAMA. 2013;309:71–82.
17. Feldman A, Eder SK, Felder TK, Kedenko L, Paulweber B, Stadlmayr A, et al. Clinical and metabolic characterization of lean caucasian subjects with non-alcoholic fatty liver. Am J Gastroenterol. 2017;112:102–10.
18. Joka D, Wahl K, Moeller S, Schlue J, Vaske B, Bahr MJ, et al. Prospective biopsy-controlled evaluation of cell death biomarkers for prediction of liver fibrosis and nonalcoholic steatohepatitis. Hepatology. 2012;55:455–64.
19. Krawczyk M, Portincasa P, Lammert F. PNPLA3-associated steatohepatitis: toward a gene-based classification of fatty liver disease. Semin Liver Dis. 2013;33:369–79.
20. Pirazzi C, Adiels M, Burza MA, Mancina RM, Levin M, Ståhlman M, et al. Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro. J Hepatol. 2012;57:1276–82.
21. BasuRay S, Smagris E, Cohen JC, Hobbs HH. The PNPLA3 variant associated with fatty liver disease (I148M) accumulates on lipid droplets by evading ubiquitylation. Hepatology. 2017;66:1111–24.

22. Casper M, Krawczyk M, Behrmann I, Glanemann M, Lammert F. Variant PNPLA3 increases the HCCrisk: prospective study in patients treated at the Saarland University Medical Center. Z Gastroenterol. 2016;54:585–6.

23. Valenti L, Motta BM, Soardo G, Iavarone M, Donati B, Sangiovanni A, et al. PNPLA3 I148M polymorphism, clinical presentation, and survival in patients with hepatocellular carcinoma. PLoS ONE. 2013;8:e75982.

24. Krawczyk M, Stokes CS, Romeo S, Lammert F. HCC and liver disease risks in homozygous PNPLA3p.I148M carriers approach monogenic inheritance. J Hepatol. 2015;62:980–1.