ORIGINAL PAPER

DOI: http://dx.doi.org/10.15403/jgld-506

PNPLA3 I148M Polymorphism in Patients with Nonalcoholic Fatty Liver Disease, Obesity and Prediabetes

Vera Karamfilova[1], Antoaneta Gateva[1], Yavor Assyov[1], Asen Alexiev[2], Alexey Savov[3], Nadejda Yaneva[3], Irena Ivanova[4], Radina Ivanova-Boyanova[5], Raya Ivanova[6], Zlatina Vlahova[7], Ludmila Mateva[2], Zdravko Kamenov[1]

1) Clinic of Endocrinology and Metabolic Diseases, Alexandrovska University Hospital, Department of Internal Medicine, Medical University Sofia; 2) Clinic of Gastroenterology, St. Ivan Rilski University Hospital, Department of Internal Medicine, Medical University Sofia; 3) National Genetic Laboratory, University Hospital of Obstetrics and Gynecology, Department of Obstetrics and Gynecology, Medical University Sofia; 4) Clinical Laboratory Department, St. Ivan Rilski University Hospital, Medical University Sofia; 5) Laboratory of Clinical Pathology, Department of Endocrinology, Medical University Sofia, St. Ivan Rilski University Hospital, Sofia; 6) Clinic of Cardiology, Alexandrovska University Hospital, Department of Internal Medicine, Medical University Sofia; 7) Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Address for correspondence:
Vera Karamfilova
Clinic of Endocrinol and Metabol Dis, Alexandrovska University Hospital Dept of Internal Medicine, Medical University Sofia, 1, Georgi Sofiiski Str., 1431 Sofia, Bulgaria
verakaramfilova@abv.bg

Received: 15.10.2019
Accepted: 29.11.2019

ABSTRACT

Background & Aims: Nonalcoholic fatty liver disease (NAFLD) is closely associated with obesity and insulin resistance, and therefore predisposes to type 2 diabetes and cardiovascular diseases. Lipid deposition in the liver seems to be critical in the pathogenesis of NAFLD. A common genetic variant, the patatin-like phospholipase domain-containing protein 3 (PNPLA3) has been associated with NAFLD. The aim of the present study was to evaluate the association between PNPLA3, key gene of lipid metabolism and the metabolic traits in obesity NAFLD patients with and without prediabetes.
Methods: A total of 208 obese NAFLD patients without (n=125) and with prediabetes (n=83) were included. The genotyping of *PNPLA3* I148M variant (rs738409) was performed by restriction analysis.
Results: Regarding rs738409 (I148M) polymorphism, CG genotype was positively correlated with prediabetes, insulin resistance, dyslipidemia and metabolic syndrome compared to the wild CC genotype. The carriers of the *PNPLA3* I148M variant have 9.6-fold higher risk of glucose disturbances compared to wild genotype (OR 9.649, 95%CI 2.100-44.328, p=0.004). The carriers of the *PNPLA3* I148M variant also have a 3 times higher risk for the presence of metabolic syndrome (OR 2.939, 95% CI: 1.590-5.434, p=0.001) and a 2.1-fold higher risk for the presence of insulin resistance (OR 2.127, 95% CI: 1.078-4.194, p=0.029).
Conclusions: *PNPLA3* I148M is associated with increased risk of prediabetes, metabolic syndrome and insulin resistance in obese patients with NAFLD.

Key words: PNPLA3 – prediabetes – nonalcoholic fatty liver disease – obesity – metabolic syndrome.

Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; BIA: Body Impedance; ELF: enhanced liver fibrosis; FIB-4: fibrosis stage 4 score; GGT: gamma glutamyl transferase; HCC: hepatocellular carcinoma; HDL: high-density lipoproteins; IR: insulin resistance; LDL: low-density lipoproteins; MetS: metabolic syndrome; NAFLD: nonalcoholic fatty liver disease; NASH: steatohepatitis; NFS: NAFLD fibrosis score; OGTT: oral glucose tolerance test; PNPLA3: patatin-like phospholipase domain-containing protein 3; SNP: single nucleotide polymorphism; TG: triglycerides; VAI: visceral adiposity index; VLDL: very low-density lipoprotein; WHR: waist-to-hip ratio; WSR: waist-to-stature ratio.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide, affecting up to 25% of the global population and this prevalence is particularly high in obese adults (80%–90%), patients with type 2 diabetes (70-80%), and up to 90% in patients with hyperlipidemia [1]. NAFLD comprises a spectrum of liver diseases ranging from simple steatosis (hepatic triglyceride content > 5%), through steatohepatitis (fat plus inflammation and hepatocellular ballooning degeneration; NASH), to fibrosis, cirrhosis and hepatocellular carcinoma (HCC), in the absence of excessive alcohol consumption (a threshold of < 20g/d for women and < 30g/d for men) [2]. NAFLD is closely associated with features of the metabolic syndrome (MetS), but common genetic causes also exist [3, 4]. The role of genetic variation in NAFLD, specifically single nucleotide polymorphisms (SNPs), has been the focus of extensive research in the last decade. The nonsynonymous rs738409 CG variant in patatin-like phospholipase domain containing protein 3 (*PNPLA3*) is considered the major genetic determinant of NAFLD [5]. SNPs rs738409 C>G in the *PNPLA3* gene encodes for the

isoleucine to methionine substitution at position 148 (I148M), and is involved in hepatocellular lipid droplets remodeling and very low-density lipoprotein (VLDL) secretion, as a major determinant of inter individual and ethnicity-related differences in hepatic fat content [6]. In humans, *PNPLA3*, also known as adiponutrin, has the highest expression in the hepatic stellate cells, retina, and hepatocytes [6]. The mechanism underlying the progression of liver disease is still an area of active research but it has been shown that *PNPLA3* has a triglyceride and retinyl palmitate esterase activity [7]. The isoleucine to methionine substitution leads to a loss of function of the enzymes activities, leading to impairment of the lipid catabolism, lipid droplets remodeling, and VLDL secretions in hepatocytes [8, 9]. It is very important to note that the promoter activity of *PNPLA3* is upregulated by glucose concentrations in a dose dependent manner [10]. In a meta-analysis carriers of the I148M variant had 73% more liver fat, a 3.2-fold higher risk of necro-inflammation, and a 3.2-fold greater risk of developing fibrosis than the non-carriers [11]. More recent research has also shown that this gene variant increases the risk of cirrhosis 1.9-fold and the risk of HCC by a factor of 1.8 [12, 13]. In the era of global obesity and MetS prevalence, both the genetic and metabolic causes of NAFLD may exist in the same individual, and there are also many with the so-called "double trouble NAFLD" [14, 15]. Lipid deposition in the liver seems to be critical in the pathogenesis of NAFLD, so its regulatory processes need to be further elucidated [16, 17]. Furthermore, these potential regulatory mechanisms are complicated by the interaction of genetic and metabolic factors.

The aim of the present study was to evaluate the association between *PNPLA3* I148M variant and various metabolic traits in obese NAFLD patients with and without prediabetes in the Bulgarian population.

METHODS

The protocol of the study was in accordance with the declaration of Helsinki [18] and was approved by the Ethics Committee of the Medical University Sofia (Protocol № 4557/01.12.2017). All participating subjects signed a written informed consent.

Study population

A total of 208 patients with NAFLD (32 male, 176 female; mean age 50.22±10.86 years, from 31 to 73 years old), recruited in a Clinic of Endocrinology and Metabolic Diseases, Alexandrovska University Hospital, Sofia, participated in the study. Inclusion criteria were: ultrasound based diagnosis of NAFLD [19] with enhanced liver fibrosis (ELF) score < 9.8 [20]; fibrosis stage 4 score (FIB-4) <1.30 [21]; NAFLD fibrosis score (NFS) < 0.675 [22]; obesity (BMI ≥ 30 kg/m^2); impaired glucose tolerance (oral glucose tolerance test (OGTT) venous plasma glucose on 120 min between 7.8 and 11.0 mmol/l) and/or impaired fasting glucose (between 6.1 and 6.9 mmol/l) [23] ; age above 18 years. Study participants were not included if any of the following criteria were present: secondary cause of hepatic steatosis and absolute alcohol consumption > 20 g daily for women and > 30 g daily for men (2), diabetes mellitus, proven neoplasia, chronic kidney disease (eGFR calculated by CKD-EPI formula <60 ml/min/1.73m^2); Heart Failure NYHA class III and IV; Cushing's syndrome; acromegaly; hypothyroidism. All subjects were divided into two groups: a group without prediabetes (included 125 obese NAFLD patients) and a group with prediabetes (83 obese NAFLD patients with impaired fasting glucose and/or impaired glucose tolerance).

Anthropometric parameters as weight (kg), height (m), body mass index (BMI; kg/m^2), waist circumference (WC) and hip circumference (cm), and arterial blood pressure (mmHg) were measured by standard criteria. Waist-to-hip ratio (WHR) and waist-to-stature ratio (WSR) were calculated. Visceral adiposity index (VAI) was performed using the following formulas: VAI=(WC/(36.85+(1.89xBMI))x(TG/0.81)x(1.52/HDL) for females VAI=(WC/(39.68+(1.88xBMI))x(TG/1.03)x(1.31/HDL) for males [24]. Percentage Body Fat (%) was measured by means of Body Impedance (BIA) by a TANITA™ TBF-215 GS Body Composition Analyzer in fasting state. MetS was diagnosed according to the International Diabetes Federation criteria [25]. A standard OGTT with measurement of glucose and insulin on 0 min (glucose 0, insulin 0), 60 min (glucose 60, insulin 60) and 120 min (glucose120, insulin120), as well as other laboratory tests were performed in the Central Laboratory of the Alexandrovska University Hospital, which is referent for Bulgaria. Homeostatic model assessment for insulin resistance (HOMA – IR) was calculated, using the following formula: HOMA-IR = fasting plasma glucose (mmol/l) x fasting serum insulin (μIU/ml) / 22.5. Insulin resistance (IR) was defined as a value of HOMA-IR > 2.5. Dyslipidemia was classified as a total cholesterol > 5.2 mmol/l, and/or low-density lipoproteins (LDL) > 2.6 mmol/l and/or high-density lipoproteins (HDL) < 1.3 mmol/l for female and <1.0 mmol/l for male and/or triglycerides (TG) > 1.7 mmol/l/ and/or ongoing treatment. The ELF score was measured using an ADIVA Centaur automated system. The ELF score was calculated using the published algorithm combining tissue inhibitor of metallo-proteinases-1 (TIMP-1), amino-terminal propeptide of type III procollagen (PIIINP) and hyaluronic acid (HA) values. ELF, NFS and FIB-4 was calculated to exclude advanced fibrosis. Abdominal ultrasonography was performed in all patients.

Genotyping

DNA was extracted from the peripheral blood. All subjects were genotyped for rs738409 polymorphism, using polymerase chain reaction (PCR) and Restriction fragment length polymorphism analysis (RFLP) according to Islek et al. [26].

Sanger sequencing was performed in some samples as confirmatory analysis. PCR was performed with Sigma-aldrih Taq Polymerase. 14 μl of master mix was dispensed into each PCR tube and then were added the patient's DNA.

The Primers were: forward primer (5'-TCAGGAAAATTAAAAGGGTGCT -3') and reverse primer (5'- GACTCAGCGCTAGCAGAGAAA -3'). The PCR cycling conditions for the rs738409 variant were as follows: pre denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 60°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 5 min. The digestion of the amplified 154 bp fragment with the restriction endonuclease NlaIII was carried out at 37°C overnight. The digested PCR products were

resolved on 3% agarose gels stained with ethidium bromide. PCR method resulted in a 154 bp product of CC genotype and the GG genotype showed two fragments of 81 and 73 bp. In heterozygous genotype samples (G/C) showed three fragments of 154, 81, and 73 bp.

Statistical analysis

For statistical analysis the data were processed using the statistical package IBM SPSS Statistics 25.0. The variant was first tested for Hardy-Weinberg equilibrium (HWE) using a $\chi 2$ test prior to analysis in two groups. The following statistical methods were applied: descriptive analysis, variation analysis, graphic analysis, Kolmogorov-Smirnov's one sample non-parametric test, and Shapiro Wilk test. χ^2 tests and Fischer's exact test - to look for dependency between category variables. Correlation analysis was used - for linear dependence between quantitative signs and binary logistics regression for the evaluation of the impact of researched factors.The association between the I148M variant and the presence of metabolic abnormalities was evaluated by multivariate logistic regression analysis. The level of significance for rejecting the null hypothesis was $p < 0.05$.

RESULTS

A significant higher levels of VAI, VLDL, TG, blood glucose, insulin from OGTT, HOMA-IR, as well as lower levels of HDL were found in obese NAFLD patients with prediabetes rather than in the group without prediabetes (Tables I and II). Statistical analyses were adjusted for age and gender. All studied patients were homogeneous by BMI (p=0.341), WC (p=0.115), hip circumference (p=0.660) and percent fat mass (p=0.217).

Genotypes did not differ significantly from the expected by the Hardy-Weinberg equilibrium in two groups (without prediabetes: p= 0.865 and with prediabetes: p=0.687). Among all patients the frequencies of *PNPLA3* I148M CC, CG and GG alleles were 121 (58.17%); 77 (37.01%) and 10 (4.80%), respectively. Analysis of the relationship between the *PNPLA3* rs738409 C>G genotype (encoding for I148M) in the groups, is shown in Table III. The frequency of the mutant G allele was significantly higher in adipose NAFLD patients with prediabetes, compared to patients without glucose disturbances ($p < 0.001$; Table III). In the group without prediabetes, the normal homozygotes CC were statistically more prevalent. There was no significant difference between the GG homozygote variant in the two main groups. Furthermore, the carriers of the *PNPLA3* I148M CG+GG variant had a 9.6-fold higher risk of glucose disturbances compared to the wild genotype CC (OR 9.649, 95%CI: 2.100-44.328, p=0.004).

The effect of the rs738409 adiponutrin genotype, encoding for the I148M protein variant, on MetS in the patients is shown in Table IV. In patients with and without MetS, we evaluated the *PNPLA3*/adiponutrin and we looked at the association of *PNPLA3* I148M genotype with the presence of MetS. In the patients with MetS, CG heterozygotes were significantly more common, compared to CC homozygotes (p=0.003). There was no significant difference between GG homozygotes variant in the patients with or without MetS (Table IV). Binary logistics

Table I. Comparative analysis between the groups according to age and anthropometric parameters

Parameters	Without Prediabetes n=125		With Prediabetes n=83		p
	Mean	SD	Mean	SD	
Age (years)	48.29	10.82	53.36	10.25	0.001
BMI (kg/m^2)	37.33	6.63	37.74	5.84	0.341
Waist (cm)	110.36	14.02	113.20	12.88	0.115
Hip (cm)	118.70	13.07	117.99	11.91	0.660
WHR	0.93	0.12	0.95	0.14	0.033
WSR	0.67	0.09	0.69	0.08	0.103
VAI	48.29	10.82	53.36	10.25	<0.001
Fat tissue (%)	46.08	4.58	46.73	6.93	0.217

BMI: body mass index; WHR: Waist-to-hip ratio; WSR: waist-to-stature ratio; VAI: visceral adiposity tissue.

Table II. Comparative analysis between the groups according to arterial pressure and metabolic parameters

Parameters	Without Prediabetes n= 125		With Prediabetes n=83		P
	Mean	SD	Mean	SD	
SBP (mmHg)	132.07	16.22	135.21	15.80	0.149
DBP (mmHg)	81.63	10.02	85.18	10.25	0.021
TC (mmol/l)	5.18	1.02	5.28	0.98	0.329
HDL (mmol/l)	1.30	0.33	1.17	0.27	0.007
LDL (mmol/l)	3.22	0.89	3.17	0.93	0.699
VLDL(mmol/l)	0.65	0.28	0.85	0.31	<0.001
TG (mmol/l)	1.51	0.81	2.10	1.00	<0.001
Glu 0	5.33	0.52	6.90	2.69	<0,001
Glu 60	8.36	2.31	11.10	2.50	<0.001
Glu 120	5.55	1.48	8.63	2.31	<0.001
Insulin 0	15.03	8.67	25.24	29.26	<0.001
Insulin 60	107.03	67.83	136.66	105.68	0.024
Insulin 120	53.40	42.81	123.93	136.34	<0.001
HOMA-IR	3.53	2.20	7.04	8.40	<0.001

SBP: systolic blood preassure; DBP: diastolic blood preassure; HDL: high density lipoproteins; LDL: low density lipoproteins; VLDL: very low density lipoproteins; TG: triglycerides; Glu0: glucose at the zero minute; Glu60: glucose at 60 minutes; Glu120: glucose at 120 minutes; Insulin0: insulin at the zero minute; Insulin60: insulin at 60 minutes; Insulin120: insulin at 120 minutes; HOMA-IR: Homeostatic model assessment of insulin resistance;

Table III. Analysis of the relationship between the PNPLA3 rs738409 C→G genotype (encoding for I148M) in the groups

		Frequency	Without Prediabetes	With Prediabetes	p
PNPLA3	CC	n	87	34	<0.001
		%	71.9a	39.5b	
	CG	n	30	46	
		%	24.8a	53.5b	
	GG	n	4	6	
		%	3.3a	7.0a	

* - the equal letters on the horizontal mean a lack of significant difference, and the different - the presence of significant difference (p <0.05)

regression has shown that *PNPLA3* I148M both with the CG and GG genotypes were associated with a 3- times higher risk of development of MetS, compared to the wild CC genotype (OR 2.939, 95%CI: 1.590-5.434, p=0.001).

Table IV. Analysis of the relationship between the PNPLA3 rs738409 C→G genotype (encoding for I148M) in the patients with and without MetS

		Frequency	Without MetS	With MetS	p
PNPLA3	CC	n	57	64	0.003
		%	73.1a	49.2b	
	CG	n	19	58	
		%	24.4a	44.6b	
	GG	n	2	8	
		%	2.6a	6.2a	

* - the equal letters on the horizontal mean a lack of significant difference, and the different - the presence of significant difference (p <0.05)

Broadly similar data was obtained in respect to IR and dyslipidemia. A significantly higher percentage of CG and GG genotype was found in subjects with IR (45.6% vs 28.3%, p=0.034). Moreover, the carriers of the *PNPLA3* I148M allele had a 2.1-fold higher risk of being insulin resistant (OR 2.127, 95% CI 1.078-4.197, p=0.029). In patients with dyslipidemia, CG heterozygotes were significantly more prevalent compared to CC homozygotes (p=0.021, Tabel V). There was no significant difference between the GG homozygotes variant and the patients with or without dyslipidemia. Finally, a positive correlation of serum concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) with rs738409 G allele was observed in our study population. The carriers of the *PNPLA3* I148M GG variant had higher mean levels of liver enzymes compared to those with the wild allele and heterozygotic variant (Fig. 1). GG and CG genotype was associated with significantly higher levels of AST compared to CC homozygotes (30.27±14.47; 21.95±10.95; 18.35±6.18, p<0.05, respectively). With regard to ALT and GGT, statistically significantly higher values were observed in the GG carriers in comparison to the CC wild type carriers, while in the CG variant, they did not differ significantly.

Table V. Analysis of the relationship between the PNPLA3 rs738409 C→G genotype (encoding for I148M) in the patients with and without dyslipidemia

		Frequency	Without dyslipidemia	With dyslipidemia	p
PNPLA3	CC	n	71	50	0.021
		%	65.1a	50.5b	
	CG	n	31	46	
		%	28.4a	46.5b	
	GG	n	7	3	
		%	6.4a	3.0a	

* - the equal letters on the horizontal mean a lack of significant difference, and the different - the presence of significant difference (p <0.05)

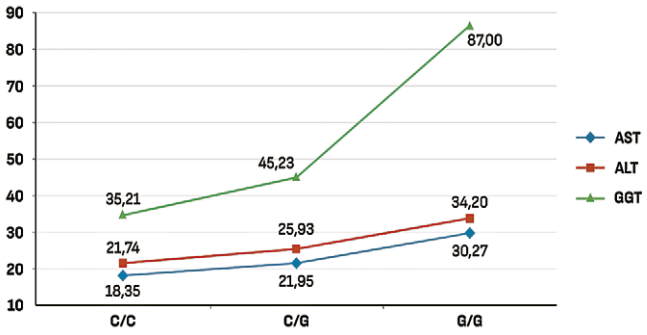

Fig. 1. Analysis of the relationship between the PNPLA3 rs738409 C→G genotype (encoding for I148M) and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT).

DISCUSSION

This is the first study in Bulgarian obese NAFLD patients with and without prediabetes, evaluating the association between *PNPLA3* polymorphism and metabolic traits. Abdominal ultrasonography was performed in all patients and non-invasive scoring systems (ELF score, the NAFLD fibrosis score, and FIB-4 indexes) were used to exclude advance fibrosis. In our study, the frequency of the mutant G allele of *PNPLA3* was significantly higher in obese NAFLD patients with prediabetes, compared to patients without glycemia disturdances. Furthermore, the carriers of the *PNPLA3* I148M variant had a 9.6- fold higher risk of prediabetes compared to the wild CC genotype. Palmer at al. [27] showed that the *PNPLA3* I148M allele was associated with insulin resistance and increased type 2 diabetes risk in a large obese cohort, despite relatively lower serum triglycerides, for the first time in two large studies: the SOS study (n =3,473) and the Go-DARTS Study (n=15,448) [27]. The authors concluded that the gene/obesity interaction where the association is only seen in a fairly extreme subgroup, might explain why the *PNPLA3* I148M allele has not been widely associated with serum triglyceride levels, insulin resistance and type 2 diabetes in other studies. In our research, all patients were obese and homogeneous by BMI, but with a different glucose status (with or without prediabetes).

We demonstrated a relationship between IR and the *PNPLA3* I148M allele. Significantly higher percentage of CG and GG genotype was found in patients with IR, and carriers of the *PNPLA3* I148M allele were found to have a 2.1-fold higher risk of being insulin resistant.

Insulin resistance is a key factor in NAFLD pathogenesis and is deeply involved in the progression of liver disease, but the causal relationship between IR and fibrogenesis remains unclear. Functional common SNPs of genes included in the insulin signaling pathway influence IR and the susceptibility to type 2 diabetes [28]. Hepatic IR has a causal role in the progression of liver damage in NASH and therefore, the amelioration of IR may improve the long-term progression of the disease and decrease the risk of type 2 diabetes. Broad knowledge of the role of *PNPLA3* in the regulation of liver metabolic functioning was recently gained by using different strategies, including functional in vitro studies and experimental animals [29]. Studies in mice have shown the

potential of treating NAFLD by inhibiting the *PNPLA3* 148M [30]. The results of the present study support such potential option: downregulating the *PNPLA3* might improve liver disease and the metabolic syndrome.

Some authors observed that obese patients with NAFLD and features of the MetS have "Obese/Metabolic NAFLD" irrespective of genetic risk factors. On the other hand, they defined "*PNPLA3* NAFLD", in which the increase of liver fat in the carriers of the *PNPLA3* I148M gene variant was due to polyunsaturated triglycerides [14, 15]. In "Obese/Metabolic NAFLD" the concentration of saturated triglycerides and insulin resistance-inducing ceramides is increased. Petäjä et al. [15] claimed that while "Obese/Metabolic NAFLD" was associated with NAFLD and features of the MetS and an increased risk of type 2 diabetes and cardiovascular disease, NAFLD caused by I148M variant in *PNPLA3* and the E167K variant in TM6SF2 was not accompanied by IR. Thus, the lack of IR does not exclude NAFLD and not all patients with NAFLD are at an increased risk of type 2 diabetes and cardiovascular disease [15].

A recent meta-analysis of 65 studies established that the *PNPLA3* rs738409 polymorphism was not only significantly associated with the susceptibility of NAFLD, but was also related to the susceptibility of aggressive disease [31]. The association of the I148M variant with hepatic lipid content is more pronounced in the presence of other risk factors, such as severe obesity, visceral adiposity, increased intake of carbohydrates or omega-6 poly-unsaturated fatty acids, and other genetic factor [32, 33]. In this study, we established a correlation of the *PNPLA3* I148M genotype with the presence of MetS. In patients with MetS, rs738409 in CG heterozygotes were significantly higher compared to CC homozygotes. *PNPLA3* I148M CG and GG genotypes were associated with a 3-times higher risk of development of MetS compared to the wild CC genotype. In the patients with dyslipidemia, CG heterozygotes were also significantly more frequent compared to CC homozygotes. *PNPLA3* has not been equally associated with lipid levels in all studies. A study of Italian and UK NAFLD patients demonstrated that the GG genotype was associated with higher LDL, and both the CG and GG genotypes were associated with lower HDL cholesterol [34].

Cui et al. [35] observed significantly shared gene effects between steatosis and MetS components, and these results were supported by the fact that 44% of the genes associated with NAFLD were shared amongst 14% of the subjects with type 2 diabetes, obesity, and hypertension combined [35]. Many studies so far strongly suggest the presence of genetic commonality between NAFLD and MetS, but they depend not just on genetics but also on environmental factors as well as the interaction between the two [36].

All these findings suggest that *PNPLA3* I148M could have pleotropic metabolic effects beyond its role in hepatic triglyceride accumulation, and its role in the development of prediabetes and metabolic syndrome should be taken in consideration.

This study has several limitations. Firstly, the relatively small number of patients for genetic screening. Secondly, our study included more women than men because the participation rate was higher among women. Strengths of this study are that all subjects had NAFLD with or without prediabetes, and did not use any potentially interfering. The whole group was homogeneous with respect to BMI and other anthropometric parameters.

CONCLUSIONS

Our results showed for the first time that *PNPLA3* I148M was associated with an increased risk of prediabetes, metabolic syndrome and insulin resistance in Bulgarian obese NAFLD patients. The rs738409 G variant was associated with higher blood glucose, dyslipidemia and higher levels of liver enzymes. Further studies with a larger group of NAFLD patients are required required to validate these findings.

Conflicts of interests: None to declare.

Authors' contribution: V.K. conceived the study, V.K, A.G., Y.A., A.A., R.I.B., R.I., L.M., Z.K.: recruited the patients, V.K, A.G., Y.A., A.A., R.I.B., R.I., L.M., Z.K. wrote the manuscript. A.S., N.Y., I.I. performed the genetic evaluations. Z.K. designed the design. All the authors approved the final version of the manuscript.

Acknowledgements: The study was funded by the Medical University of Sofia and performed at the University Hospital Alexandrovska, Sofia, Bulgaria. Project № 4728/19.07.2017. Contract № D-233/2017.

REFERENCES

1. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta analytic assessment of prevalence, incidence, and outcomes. Hepatology 2016;64:73–84. doi:10.1002/hep.28431
2. European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. J Hepatol 2016;64:1388-1402. doi:10.1016/j.jhep.2015.11.004
3. Anstee QM, Targher G, Day CP. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. Nat Rev Gastroenterol Hepatol 2013;10:330–344. doi:10.1038/nrgastro.2013.41
4. Yki-Järvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. Lancet Diabetes Endocrinol 2014;2:901–910. doi:10.1016/S2213-8587(14)70032-4
5. Eslam M, Valenti L, Romeo S. Genetics and epigenetics of NAFLD and NASH: Clinical impact. J Hepatol 2018;68:268-279. doi:10.1016/j.jhep.2017.09.003
6. Romeo S, Kozlitina J, Xing C, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461-1465. doi:10.1038/ng.257
7. Pingitore P, Pirazzi C, Mancina RM, et al. Recombinant PNPLA3 protein shows triglyceride hydrolase activity and its I148M mutation results in loss of function. Biochim Biophys Acta 2014;1841:574-580. doi:10.1016/j.bbalip.2013.12.006
8. He S, McPhaul C, Li JZ, et al. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. J Biol Chem 2010;285:6706-6715. doi:10.1074/jbc.M109.064501

9. Ruhanen H, Perttilä J, Hölttä-Vuori M, et al. PNPLA3 mediates hepatocyte triacylglycerol remodeling. J Lipid Res 2014;55:739–746. doi:10.1194/jlr.M046607
10. Rae-Whitcombe SM, Kennedy D, Voyles M, Thompson MP. Regulation of the promoter region of the human adiponutrin/PNPLA3 gene by glucose and insulin. Biochem Biophys Res Commun 2010;402:767-772. doi:10.1016/j.bbrc.2010.10.106
11. Sookoian S, Pirola CJ. Meta-analysis of the influence of I148M variant of patatin-like phospholipase domain containing 3 gene (PNPLA3) on the susceptibility and histological severity of nonalcoholic fatty liver disease. Hepatology 2011;53:1883–1894. doi:10.1002/hep.24283
12. Shen JH, Li YL, Li D, Wang NN, Jing L, Huang YH. The rs738409 (I148M) variant of the PNPLA3 gene and cirrhosis: A meta-analysis. J Lipid Res 2015;56:167–175. doi:10.1194/jlr.M048777
13. Trépo E, Nahon P, Bontempi G, et al. Association between the PNPLA3 (rs738409 C>G) variant and hepatocellular carcinoma: Evidence from a meta-analysis of individual participant data. Hepatology 2014;59:2170–2177. doi:10.1002/hep.26767
14. Luukkonen PK, Zhou Y, Sädevirta S, et al. Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. J Hepatol 2016;64:1167–1175. doi:10.1016/j.jhep.2016.01.002
15. Petäjä EM, Yki-Järvinen H. Definitions of Normal Liver Fat and the Association of Insulin Sensitivity with Acquired and Genetic NAFLD—A Systematic Review. Int J Mol Sci 2016;17:E633. doi:10.3390/ijms17050633
16. Dongiovanni P, Stender S, Pietrelli A, et al. Causal relationship of hepatic fat with liver damage and insulin resistance in nonalcoholic fatty liver. J Intern Med 2018;283:356-370. doi:10.1111/joim.12719
17. Pingitore P, Sasidharan K, Ekstrand M, Prill S, Lindén D, Romeo S. Human Multilineage 3D Spheroids as a Model of Liver Steatosis and Fibrosis. Int J Mol Sci 2019;20:E1629. doi:10.3390/ijms20071629
18. World Medical Association.World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA 2013;310:2191-2194. doi:10.1001/jama.2013.281053
19. Yoo J, Lee JM, Joo I, et al. Reproducibility of ultrasound attenuation imaging for the noninvasive evaluation of hepatic steatosis. Ultrasonography 2019 Aug 12. doi:10.14366/usg.19034
20. Xie O, Zhou X, Huang P, Wei J, Wang W, Shusen Zheng. The performance of enhanced liver fibrosis (ELF) test for the staging of liver fibrosis: a meta-analysis. PLoS One 2014;9:e92772. doi:10.1371/journal.pone.0092772
21. Sterling RK, Lissen E, Clumeck N, et al. Development of a simple noninvasive index to predict significant fibrosis patients with HIV/HCV co-infection. Hepatology 2006;43:1317-1325. doi:10.1002/hep.21178
22. Angulo P, Hui JM, Marchesini G, at al. The NAFLD fibrosis score:a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology 2007;45:846-854. doi:10.1002/hep.21496
23. American Diabetes Association. Standards of Medical Care in Diabetes-2019 Abridged for Primary Care Providers. Clin Diabetes 2019;37:11-34. doi:10.2337/cd18-0105
24. Amato MC, Giordano C, Galia M, et al.Visceral Adiposity Index:a reliable indicator of visceral fat function associated with cardiometabolic risk. Diabetes Care 2010;33:920-922. doi:10.2337/dc09-1825
25. Alberti KG, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation 2009;120:1640-1645. doi:10.1161/CIRCULATIONAHA.109.192644
26. Islek EE, Sazci A, Deniz MD, Aygun C. Genetic variants in the PNPLA3 gene are associated with nonalcoholic steatohepatitis. Genet Test Mol Biomarkers 2014;18:489–496. doi:10.1089/gtmb.2014.0019
27. Palmer CNA, Maglio C, Pirazzi C, et al. Paradoxical Lower Serum Triglyceride Levels and Higher Type 2 Diabetes Mellitus Susceptibility in Obese Individuals with the PNPLA3 148M Variant. PLoS One 2012;7:e39362. doi:10.1371/journal.pone.0039362
28. Dongiovanni P, Anstee QM, Valenti L. Genetic predisposition in NAFLD and NASH: impact on severity of liver disease and response to treatment. Curr Pharm Des 2013;19:5219-5238. doi:10.2174/13816128113199990381
29. BasuRay S, Wang Y, Smagris E, Cohen JC, Hobbs HH. Accumulation of PNPLA3 on lipid droplets is the basis of associated hepatic steatosis. Proc Natl Acad Sci U S A 2019;116:9521-9526. doi:10.1073/pnas.1901974116
30. Lindén D, Ahnmark A, Pingitore P, et al. Pnpla3 silencing with antisense oligonucleotides ameliorates non-alcoholic steatohepatitis and fibrosis in Pnpla3 I148M knock-in mice. Mol Metab 2019;22:49-61. doi:10.1016/j.molmet.2019.01.013
31. Dai G, Liu P, Li X, Zhou X, He S. Association between PNPLA3 rs738409 polymorphism and nonalcoholic fatty liver disease (NAFLD) susceptibility and severity: A meta-analysis. Medicine (Baltimore) 2019;98:e14324. doi:10.1097/MD.0000000000014324
32. Romeo S, Sentinelli F, Dash S, et al. Morbid obesity exposes the association between PNPLA3 I148M (rs738409) and indices of hepatic injury in individuals of European descent. Int J Obes (Lond) 2010;34:190-194. doi:10.1038/ijo.2009.216
33. Davis JN, Lê KA, Walker RW, et al. Increased hepatic fat in overweight Hispanic youth influenced by interaction between genetic variation in PNPLA3 and high dietary carbohydrate and sugar consumption. Am J Clin Nutr 2010;92:1522-1527. doi:10.3945/ajcn.2010.30185
34. Valenti L, Al-Serri A, Daly AK, et al. Homozygosity for the patatin-like phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. Hepatology 2010;51:1209 –1217. doi:10.1002/hep.23622
35. Cui J, Chen CH, Lo MT, et al. Shared genetic effects between hepatic steatosis and fibrosis:a prospective twin study. Hepatology 2016;64:1547- 1558. doi:10.1002/hep.28674
36. Sookoian S, Pirola CJ. Nonalcoholic fatty liver disease and metabolic syndrome: Shared genetic basis of pathogenesis. Hepatology 2016;64:1417-1420. doi:10.1002/hep.28746