
Significance of the influence of patatin-like phospholipase domain-containing 3 (PNPLA3) rs738409 polymorphism on non-alcoholic fatty liver disease

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is characterized by diffuse hepatocyte steatosis. The pathogenesis of NAFLD is not recognized. It has become apparent that genetic, environmental, and metabolic factors drive NAFLD. Moreover, earlier studies have observed that some polymorphisms raise the likelihood of NAFLD. Among these, the most robustly observed was the relationship between NAFLD risk and patatin-like phospholipase domain-containing 3 (PNPLA3). **Aim:** We aimed to assess the effect of the rs738409 polymorphism of the PNPLA3 gene (encoding I148M) on NAFLD. **Patients and Methods:** This study, which included 30 participants, was conducted with meticulous attention to detail and thoroughness: 20 patients with NAFLD and 10 healthy participants as a control group. Participants were diagnosed according to a liver biopsy taken during surgery from patients who were candidates for bariatric surgery and as part of a predonation assessment for candidates for donation in liver transplantation. Genotyping of the PNPLA3 gene variant (rs738409 C/G) was assessed using the TaqMan assay quantitative polymerase chain reaction in blood cells. **Results:** Considering the distribution of the PNPLA3 gene variant (rs738409 C/G), a greater prevalence of heterozygous rs738409 CG and homozygous rs738409 GG variants occurred in the NAFLD patients in contrast to the control group ($p = 0.048$). In addition, NAFLD patients with the homozygous GG variant had a higher incidence of hepatic fibrosis ($p = 0.018$). Furthermore, PNPLA3 gene polymorphism significantly predicted hepatic steatosis and fibrosis in NAFLD patients ($p = 0.033$ and $p < 0.001$, respectively). **Conclusion:** The PNPLA3 rs738409 polymorphism is significantly associated with the susceptibility and severity of non-alcoholic fatty liver disease.

Keywords: *Non-alcoholic Fatty Liver Disease; Patatin-Like Phospholipase Domain-Containing Protein 3; rs738409; Mean Platelet Volume; Neutrophil to Lymphocyte Ratio.*

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by disseminated hepatocyte steatosis, excluding extreme alcohol consumption and other hepatic diseases [1]. It affects approximately 32.4% of the general population [2,3]. NAFLD has a broad spectrum extending from non-alcoholic fatty liver, which does not cause significant health risks, to non-alcoholic steatohepatitis (NASH), hepatic cirrhosis, and finally hepatocellular carcinoma (HCC) [4,5]. Further, NAFLD is implicated in 36% of liver-related mortality, especially with progressive clinical forms of the disease [6].

The pathogenesis of NAFLD is not fully recognized [7]. Recently, NAFLD has been considered a multifactorial disease driven by genetic, environmental, and metabolic factors [8]. Substantial inter-individual variations exist in the progression and disease severity in NAFLD cases, and

they share the same environmental and metabolic risk factors [9]. In addition, inter-ethnic variations and familial clustering point to the substantial impact of genetic factors in the pathophysiology of NAFLD [10]. Moreover, earlier studies have detected that some polymorphisms raise the risk of NAFLD [11-13]. Among these, the most robustly observed is the relationship between NAFLD risk and the patatin-like phospholipase domain-containing 3 (PNPLA3) gene [11]. The PNPLA3 is a transmembrane protein of 481 amino acids, primarily expressed in hepatocytes, skin, and adipocytes [14,15]. In addition, it is a lipotrophic protein that influences hepatic fat metabolism through triacylglycerol lipase and acylglycerol O-acyltransferase activities. The nonsynonymous gene variant (C > G, rs738409), where isoleucine is replaced at position 148 with methionine (PNPLA3-I148M), was regarded as a significant genetic predisposing factor for NAFLD and NASH [16].

The exact mechanism and outcomes of PNPLA3-I148M are not yet comprehensively known. Nevertheless, this variant can produce both a loss and gain of function. The loss of function occurs by impairing lipolytic activity, which enhances triglyceride deposition [17]. Additionally, it was suggested that this variant promotes intracellular lipid accumulation through its effects on the excretion of ApoB-containing lipoproteins and esterification of very low-density lipoproteins [18]. Moreover, this variant impaired triglyceride hydrolysis in hepatocytes [19]. In addition, PNPLA3 encodes adipo-nutrients found on the lipid droplets and endoplasmic reticulum in hepatocytes, which may be another process by which the PNPLA3-I148M mutation results in increased hepatic triglyceride content [20]. Evidence also indicates increased hepatic triglyceride synthesis by promoting the function of lysophosphatidic acid acyltransferase through the gain of function caused by this variant [21]. Moreover, this mutation enhances the development of hepatic fibrosis through impaired retinyl-palmitate lipase activity, upregulating proinflammatory cytokines, c-Jun N-terminal kinase (JNK), and activator protein-1 (AP-1) and enhancing matrix metalloproteinase expression [22]. Other authors have further replicated these findings, as the PNPLA3-I148M variant caused mitochondrial failure *via* the deposition of cholesterol by decreasing the ABCG1 protein expression and preventing cholesterol efflux, causing the progression of hepatic fibrosis [23]. Hence, we aim to assess the effect of the PNPLA3 (rs738409) gene polymorphism on NAFLD.

Patients and Methods

This study involved 30 participants, 20 NAFLD cases, and 10 normal individuals as a control group enrolled from the Internal Medicine and Surgical departments at Ain Shams University Hospitals from December 2021 to February 2023. Participants were diagnosed according to a liver biopsy taken during surgery from candidates for bariatric surgery (NAFLD cases) and as part of a predonation assessment for candidates for donation for liver transplantation as a control.

Patients 18 years and older were involved in the research, excluding those with hepatic diseases of other known aetiologies, such as autoimmune hepatitis, viral hepatitis, and Wilson's disease. In addition, those with alcoholic liver disease, medication use known to induce hepatic steatosis or

immunosuppression, and a history of malignancy or hematological diseases or current infection were also excluded. All participants underwent the following:

- A complete blood count and calculation of the neutrophil to lymphocyte ratio (NLR) and mean platelet volume (MPV) [24],
- Genotyping of the PNPLA3 gene variant (rs738409 C/G) using the TaqMan assay qPCR in blood cells. Per the manufacturer's instructions, DNA was extracted from all participants using a Genomic whole-blood extraction kit (Puregene, USA). The DNA was dissolved in a TE buffer, and the DNA purity and concentration were determined using spectrophotometry. Real-time PCR was applied to detect single nucleotide polymorphisms in the PNPLA3 gene (rs738409), and the allelic discrimination of the PNPLA3 polymorphisms was investigated using the TaqMan assay,
- Abdominal ultrasonography,
- Liver biopsy specimens were analyzed using the scoring system in Table 1.

Tab 1. The clinical research network system for scoring activity and fibrosis in NAFLD [25]

The grade of steatosis	Hepatic lobular inflammation	Hepatocellular ballooning
0 = < 5%	0 = None	0 = None
1 = 5-33%	1 = < 2	1 = Few ballooned cells
2 = 34-66%	2 = 2-4	2 = Many ballooned cells
3 = > 66%	3 = > 4	

Statistical analysis

Statistical analysis was conducted using the student t-test, Chi-square test, Person's correlation coefficient, regression coefficient, and Analysis of variance [ANOVA] test by SPSS V25. Data were presented as mean \pm standard deviation, median and range, or number and percentage. P-value ≤ 0.05 was considered significant.

Results

The present research was performed on 30 participants. They were categorized into two groups following histopathology. The NAFLD group comprised 20 patients, and the control group comprised 10 healthy participants. Table 2 lists the clinical data of the study participants. NAFLD patients had higher BMI and MPV than the control group ($p < 0.001$ and $p = 0.012$, Table 2). In addition, table 2 demonstrates the differences in ultrasound and liver biopsy findings between the study groups ($p \leq 0.05$, Table 2). No statistically significant correlation was noted between the NLR and MPV, liver enzymes, lipid panel, body mass index (BMI), the existence of fatty liver on ultrasound, and hepatic steatosis and fibrosis in liver biopsy ($p \geq 0.05$, Supp. table 1). A significant correlation existed between the BMI and the existence of fatty liver on ultrasound and steatosis and fibrosis in liver biopsy ($p = 0.04, 0.022, \text{ and } 0.035$, respectively, Supp. table 2).

Tab 2. Study participant characteristics

Variable	NAFLD (n=20)	Control (n=10)	P value
Sex	Male	12 (60%)	0.602
	Female	8 (40%)	
	Mean ± SD	Mean ± SD	
Age (years)	28.75±5.26	28.50±6.55	0.911
Body mass index	33.50±5.30	22.90±2.18	<0.001
Total leucocytes count (x10 ³)/μL	6.77±2.23	6.51±1.95	0.753
Hemoglobin (g/dL)	13.51±1.33	13.83±1.91	0.596
Platelets count (x10 ³)/μL	269.45±80.81	267±48.78	0.931
Aspartate aminotransferase (U/mL)	21 (10.6 – 63.0)	15 (13.0 – 28.0)	0.42
Alanine aminotransferase (U/mL)	21 (8 – 90)	15 (9.0 – 33.0)	0.28

Serum albumin (mg/dL)		4.49±0.45	4.41±0.42	0.641
Total bilirubin (mg/mL)		0.62 (0.3 – 1.6)	0.5 (0.37 – 1.09)	0.603
Direct bilirubin (mg/mL)		0.2 (0.1 – 0.4)	0.2 (0.1 – 0.4)	0.81
International normalized ratio		1.0 (0.9 – 1.2)	1.0 (0.8 – 1.04)	0.14
Blood urea nitrogen (mg/mL)		14.50±4.75	14.70±4.21	0.911
Creatinine (mg/mL)		0.89±0.14	0.78±0.13	0.050
Total cholesterol (mg/mL)		196.50 ±37.68	174.60±22.32	0.103
High-density lipoprotein (mg/mL)		38.90±9.06	45.10±9.04	0.088
Triglycerides (mg/mL)		160.05±66.38	112.60±52.41	0.059
LDL-Cholesterol (mg/mL)		111.35±20.64	106.30±21.56	0.539
Fasting blood glucose (mg/mL)		82.95±10.68	86.20±8.32	0.408
2 hours PP blood glucose (mg/mL)		100.35±15.70	107.60±16.72	0.253
HbA1C		5.39±0.39	5.45±0.37	0.693
Mean platelet volume (fL)		10.39±1.10	9.14±1.39	0.012
Neutrophil to lymphocyte ratio		1.47±0.28	1.49±0.38	0.863
Fatty liver in ultrasound	Grade 0	3 (15%)	10 (100%)	<0.001
	Grade I	12 (60)	0	
	Grade II	4 (20%)	0	
	Grade III	1 (5%)	0	
Hepatic steatosis	Grade I	18 (90%)	0	-

	Grade II	2 (10%)	0	
	No	3 (15%)	9 (90%)	
Lobular inflammation	Mild	12 (60%)	1 (10%)	0.002
	Moderate	1 (5%)	0	
	Severe	4 (20%)	0	
	No	3 (15%)	10 (100%)	
Hepatocyte ballooning	Mild	7 (35%)	0	<0.001
	Severe	10 (50%)	0	
	No	3 (15%)	10 (100%)	
Hepatic fibrosis	No	3 (15%)	10 (100%)	<0.001
	Yes	17 (85%)	0	

Considering the distribution of the PNPLA3 gene variant (rs738409 C/G), a greater prevalence of heterozygous rs738409 CG and homozygous rs738409 GG variants occurred in the NAFLD patients in contrast to the control group ($p = 0.048$, Table 3). Table 4 shows the grades of hepatic steatosis, hepatic fibrosis, and hepatocyte ballooning in NAFLD cases according to the PNPLA3 gene variant status. Patients with NAFLD with the homozygous GG variant had a higher incidence of hepatic fibrosis ($p = 0.018$). However, no significant variation was found regarding hepatocyte ballooning and hepatic steatosis ($p \geq 0.05$). However, those with the homozygous rs738409 GG variant had grade II steatosis with a higher prevalence of hepatocyte ballooning. Further, no significant variations occurred in the NLR, ALT, AST, total cholesterol, LDL cholesterol, and triglyceride values in NAFLD cases according to the PNPLA3 gene rs738409C/G variant status ($p \geq 0.05$, Supp. table 3). At the same time, MPV was significantly different between the groups ($p = 0.023$, Supp. Table 3).

Tab 3. Distribution of the PNPLA3 polymorphism among NAFLD and control groups

PNPLA3 polymorphism	Total participants (n=30)	NAFLD (n=20)	Control (n=10)	P value
	<i>n (%)</i>	<i>n (%)</i>	<i>n (%)</i>	
Homozygous CC	10 (33.3%)	4 (20%)	6 (60%)	
Heterozygous CG	9 (30%)	7 (35%)	2 (20%)	0.048
Homozygous GG	11 (36.7%)	9 (45%)	2 (20%)	

Tab 4. The hepatic steatosis, hepatocellular ballooning, and hepatic fibrosis in NAFLD patients according to the PNPLA3 polymorphism

Variable		Homozygous CC	Heterozygous CG	Homozygous GG	<i>P</i> value
Hepatic steatosis	Grade I	4 (100%)	7 (100%)	7 (77.78%)	0.155
	Grade II	0	0	2 (22.22%)	
Hepatocyte ballooning	No	0	3 (42.86%)	0	0.126
	Mild	1 (25%)	2 (28.57%)	4 (44.44%)	
	Severe	3 (75%)	2 (28.57%)	5 (55.56%)	
Hepatic fibrosis	No	4 (100%)	3 (42.86%)	0	0.018
	Yes	0	4 (57.14%)	9 (100%)	

Table 5 shows that hepatic steatosis correlated positively with PNPLA3 gene polymorphism and BMI ($p = 0.033$ and $p < 0.001$, respectively). Additionally, they were predictors for hepatic steatosis ($p = 0.033$ and $p < 0.001$, respectively, Supp. Table 4). Table 6 shows that hepatic fibrosis correlated positively with PNPLA3 gene polymorphism, BMI, and total cholesterol ($p < 0.001$, $p < 0.001$, and $p = 0.049$, respectively). However, PNPLA3 gene polymorphism was the only predictor for hepatic fibrosis ($p < 0.001$, Supp. Table 5).

Tab 5. Correlations between hepatic steatosis and other studied parameters

	Hepatic steatosis	
	<i>r</i>	<i>P</i> value

Neutrophil to lymphocyte ratio	0.209	0.377
Mean platelet volume	0.168	0.479
Total cholesterol	0.436	0.055
LDL-Cholesterol	0.246	0.295
Body mass index	0.863	<0.001
PNPLA3 gene polymorphism	0.391	0.033

Tab 6. Correlations between hepatic fibrosis and other studied parameters

	Hepatic fibrosis	
	r	P value
Neutrophil to lymphocyte ratio	0.094	0.623
Mean platelet volume	0.127	0.503
Total cholesterol	0.363	0.049
LDL-Cholesterol	0.067	0.724
Body mass index	0.642	<0.001
PNPLA3 gene polymorphism	0.689	<0.001

Discussion

The first genome-wide association study (GWAS) regarding NAFLD found that the allele of PNPLA3 rs738409 is significantly correlated with hepatic steatosis and inflammation [26]. This finding gave new insight into the pathogenesis of NAFLD. Since then, knowledge regarding the genetic constituents of NAFLD has substantially increased. Several studies have reported that this variant leads to the development of NAFLD by increasing triglyceride production and deposition in hepatocytes, increasing the risk of hepatic hepatocyte ballooning, steatosis, and lobular inflammation [27,28]. Furthermore, PNPLA3 rs738409 polymorphism is strongly linked to NASH, cirrhosis, HCC, and death [29-31]. The impact of this polymorphism is observed even in individuals with lean NAFLD [32].

The current study detected a greater prevalence of heterozygous rs738409 CG and homozygous rs738409 GG PNPLA3 gene variants in the NAFLD group compared to the controls. Additionally, NAFLD cases with heterozygous rs738409 CG and homozygous rs738409 GG PNPLA3 gene variants had a higher incidence of hepatic fibrosis compared to the homozygous rs738409 CC gene variant. Furthermore, PNPLA3 rs738409 polymorphism was a significant predictor for both hepatic steatosis and fibrosis. The results align with a meta-analysis consisting of 16 studies that examined 2,124 patients and detected that the homozygous PNPLA3 rs738409 GG had a 73% higher hepatic fat content in comparison to the homozygous rs738409 CC polymorphism. In addition, rs738409 GG had a greater risk of developing fibrosis than homozygous CC [16].

In agreement with the current study, case-control research, including the genomic data of 2,950 NAFLD patients and 12,907 healthy controls, detected that the frequency of the PNPLA3 rs738409 GG allele was higher in NAFLD patients than in the control group. Furthermore, the PNPLA3 rs738409 GG and GC variants had more NAFLD patients than the PNPLA3 rs738409 CC variant (19.9% vs 16%). In addition, the PNPLA3 rs738409 GG allele was an independent risk factor for NAFLD [33].

In agreement with the current results, a GWAS by Anstee *et al.* reported that the PNPLA3 rs738409 variant is a risk factor for the full pathological spectrum of NAFLD [28]. Another study found that the BMI, serum triglyceride, and AST levels were risk factors for NAFLD [34]. In addition, a meta-analysis consisting of 20 studies recorded a significant correlation between NAFLD susceptibility and PNPLA3 rs738409 polymorphism [20]. However, in contrast to our results, Li *et al.* [35] detected no correlation between hepatic steatosis and PNPLA3 rs738409 gene polymorphism.

Many studies have confirmed a strong relationship between metabolic syndrome and the grade of fibrosis and steatosis in NAFLD [36,37]. In agreement with earlier studies [33,34,38], NAFLD patients had higher BMI values than the control group. Moreover, a strong association was detected between the BMI and hepatic steatosis and fibrosis in liver biopsy.

Substantial evidence suggests that stimulating the immune system with proinflammatory mediators secreted by visceral adipose tissue is crucial to disease severity and progression. In addition, intrahepatic lipids are implicated in inducing oxidative stress, triggering portal and lobular

inflammation, and characterizing the severe histological forms of the disease [39]. Further, accumulating data supports that the NLR is a simple marker in estimating the NAFLD severity [40]. In contrast to our results, Lesmana *et al.* [38] observed that cases with moderate to severe steatosis had a greater NLR than patients with mild steatosis. Additionally, Yilmaz *et al.* [41] noted that cases with NASH had a greater NLR than controls. However, in agreement with the present results, a large cohort study found that the NLR is unrelated to fibrosis and inflammation in NAFLD [42].

In agreement with an earlier study [43], NAFLD patients had a greater MPV than the control group. However, MPV was not statistically different between the NAFLD group and controls, nor was it a predictive factor for NAFLD in another study [34].

A significant strength of the present research is evaluating hepatic disease with biopsy-proven NAFLD, the golden standard for assessing liver disease severity. However, the present study is restricted by the small sample size. According to the current results, PNPLA3 genotyping may improve risk stratification and prognostication and allow for the prioritization of intensive interventions in NAFLD patients [44]. Additionally, with this knowledge, it has become possible to apply its effects on NAFLD into clinical applications, such as innovative drugs, and discover potential targets to treat the disease [45,46]. In this context, antisense oligonucleotide-mediated silencing of the PNPLA3 gene was investigated in a mouse model. Notably, there was a decline in the NAFLD activity score, hepatic inflammation, steatosis, and fibrosis grade [47].

Conclusions

The PNPLA3 rs738409 polymorphism is significantly associated with the susceptibility and severity of non-alcoholic fatty liver disease.

Footnotes.

The peer reviewers were Amr Shaban Hanafy (professor of internal medicine, gastroenterology, hepatology unit), Marwa Shabana (assistant professor of clinical pathology), and Amany Mohamed (Assistant professor of family medicine).

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Ethical approval: All procedures involving human participants followed the institutional and national research committee's moral standards, the 1964 Helsinki Declaration, and its later amendments or comparable ethical standards. All authors declare that consent was obtained from the patients (or other approved parties) to publish this study.

The Clinical Research Ethics Committee of the Faculty of Medicine, Ain Shams University (**FMASU MD 284/2018**) authorized the study protocol. All contributors signed a written informed agreement.

Data and materials availability: The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

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Authors' contributions

Enas Mahmoud Foda and Shereen Abu Bakr Saleh conceived the research concept. At the same time, Moheb Shoraby Eskandaros, Nashwa Nagy El-Khazragy, and Heba Mohamed Abu Bakr conducted the clinical examinations and monitored the patients. Yasmin Mohamed Massoud and Ghada Abdelrahman Mohamed collaborated to gather laboratory data. All authors actively participated in analyzing and interpreting the patient information and composing the manuscript. All authors thoroughly reviewed and approved the final version of the manuscript.

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