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Association between rs738409 and rs139051 SNPs of the PNPLA3 gene and the presence of NAFLD

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Abstract

Genetics and epi-genetic alterations trigger the development of non-alcoholic fatty liver disease (NAFLD). Patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene is known to be a key regulator of lipid metabolism in the liver and is expressed in both adipocytes and hepatocytes. The aim is to evaluate the association between two single nucleotide polymorphism (SNPs) of PNPLA3 gene and NAFLD, and their relation to the degree of hepatic steatosis and fibrosis. Eighty individuals were selected and divided into two groups according to ultrasound finding of steatosis; a test group of 40 patients with bright liver and a group of 40 healthy subjects with normal liver. Steatosis was quantified by controlled attenuation parameter (CAP). Fibrosis assessment was done by fibrosis-4 (FIB-4), NAFLD fibrosis score, Fast score and Fibroscan. Complete blood count, liver enzymes, fasting blood glucose, and lipid profile were performed. Genomic DNA was isolated from blood. rs738409 C>G SNP of the PNPLA3 gene was determined via PCR-RFLP while rs139051 (A>G) polymorphism of the PNPLA3 gene was detected using TaqMan genotyping assay. GG genotype was significantly related to the presence of NAFLD (P=0.043) while the homozygous wild type (CC) was the common genotype among healthy controls. There was a significant association between the presence of the G allele (46.3%) in the PNPLA3 (I148 M) polymorphism and the presence of NAFLD (P <0.001). However, rs139051 PNPLA3 AG genotype was the most frequent genotype in both patients with NAFLD (82.5%) and healthy controls (85%). No significant correlation was found between any of the variants of both genotypes and the degrees of both steatosis and fibrosis detected by CAP and Fibroscan respectively. In conclusion, PNPLA3 gene polymorphism rs738409 but not rs139051 is significantly associated with NAFLD patients with simple steatosis. This blood test could serve as a screening tool for simple steatosis which warrants earlier follow-up and further intensive therapies.

Keywords: NAFLD; SNPs; PNPLA3; Gene polymorphism

1. Introduction

NAFLD is a clinico-pathologic syndrome that encompasses various medical entities including simple fatty liver or simple steatosis, nonalcoholic steatohepatitis (NASH), cirrhosis, and its complications [1]. NAFLD now affects up to 25% of people around the world. The highest prevalence rate is in the Middle East (32%) followed by South America (30%) while the lowest is in Africa (13%). It also accounts for 2% of total deaths [2]. The increase in NAFLD prevalence parallels the rise in obesity and is tightly associated with metabolic comorbidities (diabetes, hypertension, insulin resistance, and dyslipidemia). It also places patients at higher risk for progressive liver disease [3]. It became clear that like different complex multisystem disorders, NAFLD is triggered by a variety of underlying mechanisms; the most important one of them is the alterations in hepatic and extra-hepatic lipid metabolism [4]. The study of genetic factors in NAFLD is a rapidly growing field as they determine not only the response of different individuals to excess caloric consumption but also the resulting metabolic derangements [5].

Patatin-like phospholipase domain-containing protein 3 (PNPLA3), a membrane protein encodes 481-amino acid which is localized on the surface of lipid droplets and in the endoplasmic reticulum, is expressed in both adipocytes and hepatocytes [6, 7]. Adiponutrin, an enzyme encoded by PNPLA3 gene, is a key regulator of lipid metabolism in the liver through its actions on both triacylglycerol lipase and acyl glycerol O-acyltransferase. Loss-of-function of PNPLA3 is suggested to predispose individuals to NAFLD, by dysregulation of hepatic and serum lipid profile [8]. Recently, numerous reports have identified a strong association not only between polymorphisms in PNPLA3 and hepatic triglyceride accumulation (steatosis) but also between

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polymorphisms in PNPLA3 and histological severity of NAFLD [6, 9]. Furthermore, it has been shown that a single nucleotide polymorphism (SNP) in residue 148(rs738409), which exhibits a C-to-G transition leading to isoleucine-to-methionine substitution (I148 M), was strongly associated with expanded fat gathering and susceptibility to steatosis, steatohepatitis and hepatic inflammation and fibrosis.

Another SNP, PNPLA3 rs139051, has been reported to trigger the inflammatory progress of NAFLD by its modulation of the phospholipid metabolite profile [8].

Therefore, the present study aimed to confirm the association between rs738409 and rs139051 SNPs of the PNPLA3 gene and the presence of NAFLD, and evaluate their association with the degree of hepatic steatosis and fibrosis as well as the impact of multiple SNP interaction on NAFLD risk factors.

2. Methods:

The present study is a prospective study that was carried out on 80 subjects who attended outpatient clinics of the Internal Medicine department of Kasr Al Ainy Hospital Cairo, Egypt during the period from May 2019 to December 2020.

The selected subjects were divided into two groups according to the sonographic findings of steatosis: 40 NAFLD patients with bright liver echogenicity and 40 healthy subjects with normal liver echogenicity. All cases have age ranging between 18 to 60 years old.

Those with clinical, biochemical, or histological evidence of cirrhosis, those with known causes of liver disease [viral hepatitis B and C , autoimmune hepatitis, primary biliary cirrhosis, haemochromatosis or Wilson disease], those with history of current or past excessive alcohol drinking as defined by an average daily consumption of more than 20 g alcohol, drug-induced liver disease , pregnant women and patients on hormonal contraceptive drugs (oral, parenteral), hormone replacement therapy were excluded from the study. The study was approved by Medical Research Ethical Committee of the National Research Center, Cairo, Egypt (Approval No.19-001), and informed consent was obtained from all patients.

All patients were evaluated by history and clinical examination and measurement of anthropometric parameters as weight (kg), height (m), body mass index (BMI; kg/m^2), waist circumference (cm), and mid-arm circumference (cm).

Complete blood count was determined using the automated hematology analyzer SF-300 (Sysmex Corporation, Japan). Also, liver enzymes (ALT, AST, ALP, GGT), serum albumin, prothrombin time, INR, serum creatinine, lipid profile, fasting blood sugar were measured to all individuals according to the manufacture instructions. The reagents were purchased from Spectrum Company, Cairo, Egypt.

NAFLD fibrosis score (NFS), FIB-4 and Fast score were calculated as mentioned previously by Angulo et al., (2007) and Calès et al., (2009) [10, 11] to assess fibrosis of the NAFLD patients group.

NFS score = -1.675 + 0.037 × age [y] +0.094× BMI [kg/m^2] +1.13 × IFG/ diabetes [yes =1, no = 0] + 0.99 × AST/ALT ratio - 0.013 × platelet count [× 10^9/L] -0.66 × albumin [g/dL]

FIB-4 score = Age [y] × AST [U/L] / platelet [× 10^9/L] × ALT [U/L]

FAST score was calculated according to Newsome et al., (2020) [12] as:

$$FAST = \frac{e^{-1.65+1.07\times \ln(LSM)+2.66*10^{-8}\times CAP^3-63.3\times AST^{-1}}}{1+e^{-1.65+1.07\times \ln(LSM)+2.66*10^{-8}\times CAP^3-63.3\times AST^{-1}}}$$

Abdominal ultrasonography was performed to all individuals using the 3.5 MHz probe of Logic 6 of general electric machine

Liver stiffness measurement (LSM) and controlled attenuation parameter (CAP)

Fibroscan (M probe, Echosens, Paris) was carried out by an experienced examiner in all patients (with at least 6 h of fasting) in left lateral position and the median liver stiffness of the 10 successful measurements fulfilling the criteria (success rate of greater than 60% and interquartile range /median ratio of <30%) were noted (in kPa).

The final CAP value, which ranges from 100 to 400 (dB/m), is the median of individual measurements. As an indicator of variability, the ratio of the IQR of CAP values to the median (IQR/ MCAP) was calculated. The operator was blinded to the patients' clinical data

According to the manufacturer's instructions, in addition to previous studies, the stages of fibrosis (F0: 1–6, F1: 6.1–7, F2: 7–9, F3: 9.1–10.3, and F4: ≥ 10.4) were defined in kPa [11]. Moreover, steatosis stages (S0: < 215, S1: 216– 252, S2: 253–296, S3: > 296) were defined in dB/m [13].

Sample collection:

10 ml venous blood were drawn from all study participants in the morning after a 12 h fast; a portion of the blood was collected on EDTA tube for the extraction of DNA, and for determination of routine blood pictures (CBC) by Sysmex the automated hematology analyzer SF-300, which produced by Sysmex Corporation, Japan. The other portion is left to clot at room temperature. Serum was separated by centrifuging for 10 minutes at 3000 rpm. Sera were used immediately for other biochemical investigations including aspartate aminotransferase

(AST), alanine aminotransferase (ALT), bilirubin, serum albumin, fasting blood glucose, cholesterol, triglycerides, HDL-C, LDL-C according to the manufacturer's instructions. The reagents were purchased from Spectrum Company, Cairo, Egypt.

Genotyping:

DNA extraction and polymorphism determination:

Genomic DNA was isolated from blood using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific, Lithuania) following the manufacturer's suggestions.

The genotyping for this study was done using either digestion of PCR product by restriction enzyme (PCR-RFLP) or TaqMan SNP genotyping analysis based on the individual polymorphism being studied. The rs738409 C>G SNP, encoding I148M, of the PNPLA3 gene was genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

PCR-RFLP:

In this technique, a 333-bp region of the PNPLA3 gene incorporating the desired SNP was amplified using specific primers (forward primer: 5'-TGG GCC TGAAGT CCG AGG GT-3' and reverse primer: 5'-CCG ACA CCA GTG CCC TGC AG-3') [14].

The reaction was set up in a total volume of 25 µL including 12.5 µL dreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Lithuania), 9.5 µL nuclease-free water, 1µL forward primer (10 µM), 1 µL reverse primer (10 µM) and 1 µL of extracted DNA. The PCR conditions were: 94 ˚C for 2 min, and then 35 cycles of 94˚C for 30 sec, 66˚C for 30 sec, and 72 ˚C for 30 sec and a final extension step of 72˚C for 5 minutes.

PCR products were then processed at 50˚C with BtsCI restriction enzymes at least for four hours. Digested PCR fragments were separated by electrophoresis in a 2% Agarose gels with 2µL Ethidium bromide in a horizontal electrophoretic tank containing 1X TBE buffer at 110 V for 1 hr and the resulting bands were pictured utilizing gel documentation system.

TaqMan SNP genotyping analysis:

Regarding the rs139051 polymorphism (A>G) of the PNPLA3 gene, samples were subjected to a real-time PCR using the system "TaqMan genotyping assay", dbSNP rs139051 assay C_176091868_10 (Applied Biosystems, Lithuania) on a Rotor-Gene Q instrument, (QIAGEN).

The assay was standardized in a final volume of 20 µL: 10 µL of TaqMan MasterMix (Thermo Scientific, Lithuania), 0.5 µL of C_176091868_10 Genotyping Assay 40X (Thermo Scientific, Lithuania), 4.5 µL of ultrapure nuclease-free water (Thermo Scientific, Lithuania), and 5 µL of genomic DNA. The cycling was as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Statistical analysis:

SPSS version 16.0 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis with a two-side significant criterion at $P<0.05$. The clinical data were expressed as mean±SD (continuous, normally distributed variables). Categorical data were summarized as percentages. The significance for the difference between groups was determined by using a two-tailed Student's t-test. Also, qualitative variables were assessed by chi-squared $\chi 2$-test.

3. Results:

The present study is a case-control study recruited 80 adult subjects, (28 males and 52 females). Their age ranged from 19 to 56 years. The demographic, anthropometric, clinical, and biochemical characteristics of both groups (NAFLD and controls) are summarized in Table 1. Patients with NAFLD were significantly older than controls (mean age 42.18±11.1 4 y *vs.* 29.65±6.63 y, $p <0.0001$). There were more males in the control group (45%), but the majority was females in the NAFLD group (75%). NAFLD patients exhibited a higher mean BMI (31.8 ± 2.9 kg/m^2) than the control group (23.76 ± 1.4 kg/m^2) ($P<0.001$). Patients with NAFLD had a higher prevalence of hypertension and diabetes mellitus in comparison to healthy controls ($P<0.001$) (Table 1).

Table 1: Characteristics of Study Participants According to NAFLD Status

Variable / Groups	NAFLD group (N=40)	Control group (N=40)	P-value
Age (Yrs.)	42.18±11.14	29.65±6.63	**<0.001****
Gender[a]			
Male/Female	10/30	18/22	0.061
Percentage of Male	(25%)	(45%)	
Hypertension			
Absent	33(82.5%)	40(100%)	**0.006***
Present	7(17.5%)	0(0%)	
Diabetes			**<0.001****

Absent	20(50%)	40(100%)	
Oral hypoglycemic	9(22.5%)	0(0%)	
on insulin	11(27.5%)	0(0%)	
Family history of diabetes			
No	31(77.5%)	31(77.5%)	1.000
Yes	9(22.5%)	9(22.5%)	
Family history of liver diseases			
No	28(70%)	29(72.5%)	0.805
Yes	12(30%)	11(27.5%/)	
BMI (Kg/m2)	32.42 ± 3.59	23.66 ± 1.3	**<0.001****
Waist circumference (Cm)	117.9±9.4	74.3±6.8	**<0.001****
Mid-arm circumference (Cm)	31.8±4.3	25.5±1.5	**<0.001****
Ultrasound finding			
Enlarged liver size	25(62.5%)	0(0%)	<0.001**
Laboratory variables			
Fasting blood glucose (mg/dl)	122.6 ± 40.97	96.03 ± 7.77	**<0.001****
HB (g/dL)	11.56 ± 1.4	12.81 ± 1.06	**<0.001****
Platelets count(10^3/µL)	236 ± 72.78	260.63 ± 56.79	0.096
Total leucocytic count (10^3/µL)	6.79 ± 1.91	7.24 ± 1.7	0.273
ALT (U/L)	47.9 ± 18.3	30.6± 4.9	**<0.001****
AST (U/L)	33.55 ± 14.9	23.6 ± 4.6	**<0.001****
Total bilirubin (mg/dL)	0.76 ± 0.24	0.68 ± 0.2	0.132
ALP (U/L)	155.7± 37.5	115.02± 15.2	**<0.001****
GGT (U/L)	91.1± 64.2	34.5± 9.2	**<0.001****
Total Protein (g/dL)	8.1± 0.2	7.9± 0.24	**0.007****
INR	1.05 ± 0.1	1.0 ± 0.0	**0.003****
Serum Albumin (g/dL)	3.8 ± 0.38	3.8 ± 0.3	0.939
Cholesterol (mg/dL)	150.88±32.4	101.65 ± 19.76	**<0.001****
Triglycerides (mg/dL)	168.5 ± 44.15	143.9 ± 29.7	**0.005****
LDL (mg/dL)	133.7 ± 34.6	104.02 ± 16.1	**<0.001****
HDL (mg/dL)	48.4 ± 15.5	55 ± 12.7	**0.04***

BMI, body mass index; Hb, haemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; HDL, high-density lipoproteins; LDL, low-density lipoproteins.

Among studied NAFLD patients, 22.5% had a family history of diabetes, and 30% had family history of liver disease, 62.5 % of NAFLD cases (n=25) have enlarged liver size on ultrasound. The mean serum fasting blood glucose was significantly higher in NAFLD patients than that in controls (122.15±39.9 vs. 96.88±7.4); (P<0.001). In addition, hemoglobin levels were lower in NAFLD cases (11.55± 1.4 (g/dL) than in healthy controls (12.64 ± 1.12 (g/dL), (P<0.001). No significant difference was observed in total leucocytic count (TLC) and platelet count between the NAFLD and control groups (P > 0.05). NAFLD patients had significantly higher serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) compared to healthy controls (P<0.001). On the other hand, the mean albumin level was almost normal (3.8 ± 0.38 g/dL) in the NAFLD group. There was a significant elevation in total cholesterol, triglycerides, and LDL-cholesterol among NAFLD patients compared to controls, while there was significant decrement in HDL in the NAFLD group as opposed to controls (P < 0.05). The mean Fibroscan value in all NAFLD patients was 5.1 ±0.99 (kPa) indicating that all patients had mild fibrosis with a stage less than 3. Thirty patients had fibrosis belonging to stage 0, while the rest had fibrosis stage 1. Mean Fibroscan values for cases with fibrosis stages 0 and 1 were 4.7±0.67 and 6.5±0.3 (kPa) respectively. There was a statistically significant difference in liver stiffness measurements in patients with stage 0 fibrosis as compared to stage 1 fibrosis (P<0.001). In addition, there was a stepwise increase in Cap score parallel to the increase in severity of liver fibrosis (P<0.001) (Table 2).

Table 2: Results of Fibroscan, CAP, NAFLD Fibrosis Score, FIB 4, and FAST Score in NAFLD Group.

	Group I Total NAFLD patients N=40			Fibrosis stage (F0) N=30	Fibrosis stage (F1) N=10	P-value
Fibroscan (kPa**)**						
Mean±SD	5.1	±	0.99	4.7±0.67	6.5±0.3	<0.001**
Range	3.8	-	6.9	3.8-5.9	6.2-6.9	
CAP (dB/m)						
Mean±SD	263.9	±	11.61	260.4±10.9	274.2±6.44	<0.001**
Range	242	-	286	242-286	265-286	
FAST score						
Mean±SD	0.203	±	0.139	0.2377±0.14	0.099±0.09	0.001**
Range	0.04	-	0.47	0.05-0.47	0.04-0.25	
NAFLD fibrosis score						
Mean±SD	-1.37	±	1.38	-1.19±1.4	-1.92±1.01	0.147
Range	-4.1	-	1.22	-4.1-1.22	-3.2-0.33	
FIB 4						
Mean±SD	0.919	±	0.46	0.96±0.5	0.81±0.28	0.376
Range	0.33	-	2.39	0.33-2.39	0.36-1.14	

This study showed that both NFS and FIB-4 score were similar in patients with fibrosis stages 0 and those with fibrosis stages 1(P>0.05). This may be due to that all cases included in our study have mild fibrosis. Also, performances of FIB-4 and NFS to rule in advanced fibrosis are rather inadequate, meaning that further assessment with another test is needed in case of positive results.

Genetic analysis:

The pattern of genotypes for the two different polymorphisms (rs738409 and rs139051) in the PNPLA3 gene was studied using PCR–RFLP results and TaqMan SNP genotyping analysis (Table 3 and Figure 1&2).

Table 3: Distribution of PNPLA3 rs738409 and rs139051 Genotypes among NAFLD Patients and Controls

Genotypes	NAFLD group (N=40)		Control group (N=40)		$\chi 2$	P
PNPLA3 rs738409 C>G SNP						
CC	19	47.5%	27	67.5%	**6.270**	**0.043***
CG	5	12.5%	7	17.5%		
GG	16	40%	6	15%		
C allele	43	(53.8%)	61	(76.3%)	**8.901**	**0.003****
G allele	37	(46.3%)	19	(23.7%)		
PNPLA3 ***rs139051***						
AA	2	5%	5	12.5%	**3.967**	**0.138**
AG	33	82.5%	34	85%		
GG	5	12.5%	1	2.5%		
A allele	37	(46.3%)	44	(55%)	**1.225**	**0.268**
G allele	43	(53.8%)	36	(45%)		

The genotypes distribution of the PNPLA3 rs738409 in NAFLD and control groups showed that the GG genotype was significantly related to the presence of NAFLD (P=0.043). In the current study, the homozygous wild type (CC) was the common genotype among subjects in the control group compared to GG genotype that was the most frequent in NAFLD patients. The results also revealed a significant association between the presence of the G allele (46.3%) in the PNPLA3 (I148 M)

polymorphism and the incidence of NAFLD (P <0.001). However, rs139051 PNPLA3 A and G alleles showed that AG genotype was the most frequent genotype in both patients with NAFLD (82.5%) and controls (85%).

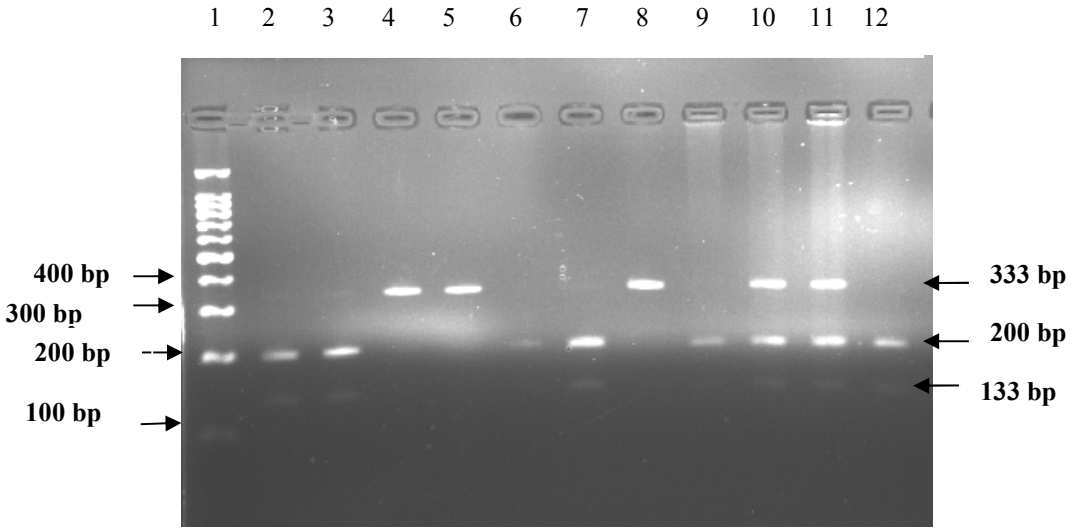

Fig. (1): Analysis of PNPLA3 gene (rs738409) C>G polymorphism: the Agarose gel picture showing PCR-RFLP analysis of PNPLA3 gene (rs738409) C>G genotypes of study subjects with restriction endonuclease enzyme BtsCI**.** M: (100 bp Plus) DNA ladder, Lane 1, 2, 5, 6, 8 and 11: CC homozygous (200 bp and 133 bp), Lane 3.4.and 7: GG homozygous (333 bp) and Lane 9 and 10: CG heterozygous (333 bp, 200 bp and 133bp).

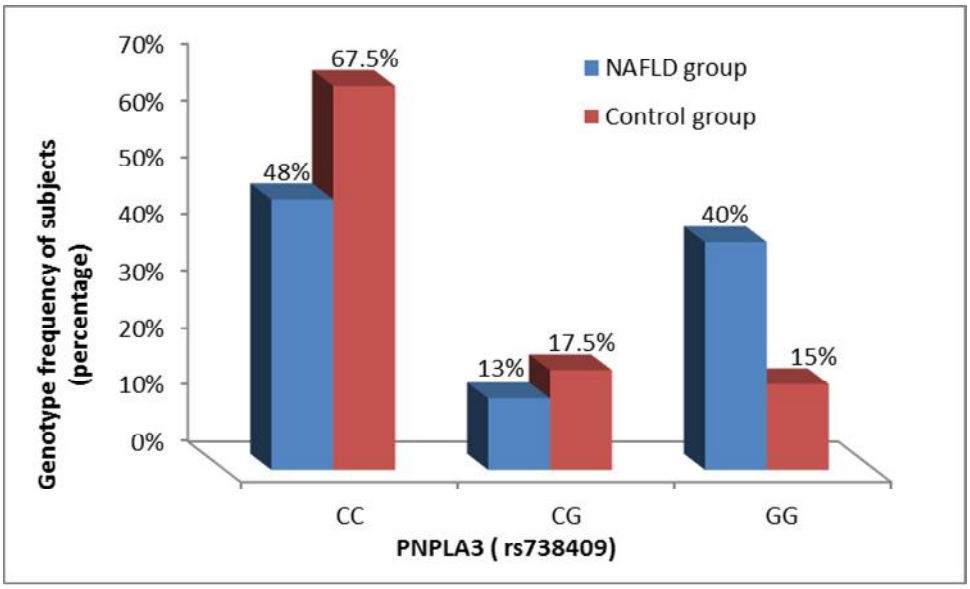

Fig. (2):The genotypes distribution of the PNPLA3 r s738409 in NAFLD and control groups

Additionally, the AA genotype was detected in 5% of patients with NAFLD and 12.5% in controls. There was no association between rs139051 PNPLA3 and the prevalence of NAFLD (P=0.138). Allele frequencies of A and G were almost the same in controls and NAFLD patients. Patients with NAFLD with liver fibrosis stage 1 had a higher percentage GG genotype of PNPLA3 (rs738409) compared to those with fibrosis stage 0 (60% versus 40% in non-NAFLD); but without significant difference (P>0.05). In addition, PNPLA3 (rs139051) had no effect alone or synergistically with PNPLA3 (rs738409) polymorphism for the detection of early stages of fibrosis and steatosis (Table 4).

Table 4: Relationship between Different Variants of both Genotypes and the Degrees of both Steatosis and Fibrosis

	PNPLA3 (rs738409)			P	*PNPLA3 (rs139051)*			P
	CC	CG	GG		*AA*	*AG*	*GG*	
Steatosis								
S1(N=6)	3(50%)	1(16.7%)	2(33.3%)	**0.911**	0(0%)	6(100%)	0(0%)	**0.473**
S2(N=34)	16(47.1%)	4(11.8%)	14(41.2%)		2(5.9%)	27(79.4%)	5(14.7%)	
Fibrosis								
F0 (N=30)	15(50%)	5(16.7%)	10(33.3%)	**0.206**	2(6.7%)	25(83.3%)	3(10%)	**0.528**
F1 (N=10)	4(40%)	0(0%)	6(60%)		0(0%)	8(80%)	2(20%)	

As shown in Table 5, we analyzed the influence of PNPLA3 genotype rs738409 on clinical and laboratory parameters; GGT and cholesterol levels of the PNPLA3 GG carriers were significantly higher than those of CC or CG carriers (P=0.015 and P=0.003 for GGT and P=0.003 and P=0. 012 for cholesterol) among all studied cases. Also, platelets count, and triglyceride levels of the PNPLA3 GG carriers were significantly higher than those of CC carriers. Additionally, LDL was considerably higher in GG carriers than CG carriers.

Table 5: PNPLA3 rs738409 and rs139051 Genotypes in Relation to The Clinical and Lab Parameter

	PNPLA3 rs738409			P	*rs139051*			P
	CC	CG	GG		*AA*	*AG*	*GG*	
Age	**34.6±10.51**	**34.83±11.5**	**39.18±11.8**	0.269	**33.14±14.2**	**35.85±10.7**	**39.8±13.01**	**0.557**
BMI	**27.7±5..6**	**26.6±3.9**	**29.5±4.6**	0.229	**24.84±3.3**	**28.05±5.24**	**31.6±3.7**^{a*}	**0.060**
TLC	**7.19±1.7**	**6.43±1.6**	**7.17±1.7**	0.373	**6.91±1.8**	**7.02±1.7**	**7.8±2.1**	**0.535**
HB	**12.28±1.3**	**12.09±1.5**	**12.04±1.4**	0.771	**12.44±1.01**	**12.13±1.4**	**12.48±1.6**	**0.736**
PLT	**233.37±56.6**	**255.75±50.7**	**275.5±83.1**^{a*}	0.042**	**216.14±27.6**	**254.93±67.3**	**212±67.7**	**0.125**
FBS	**109.15±31.4**	**113.17±31.3**	**107.55±35.53**	0.890	**97.71±9.013**	**109.3±31.7**	**123±51.35**	**0.374**
T.BIL	**0.71±0.24**	**0.64±0.3**	**0.77±0.18**	0.281	**0.85±0.15**	**0.71±0.2**	**0.725±0.26**	**0.314**
AST	**27.7±10.19**	**25.67±5.7**	**31.96±16.9**	0.266	**31.3±13.1**	**27.3±11.3**	**39.7±14.2**^{b*}	**0.043***
ALT	**38.09±14.8**	**34.67±12.2**	**44.2±19.1**	0.186	**41.6±16.7**	**37.9±15.4**	**51.7±17.7**^{b*}	**0.118**
ALP	**135.4±34.9**	**134.67±27.6**	**135.6±40.1**	0.997	**127.3±29.8**	**134.01±34.6**	**160±40.5**	**0.180**
GGT	**57.2±49.6**	**34±8.8**	**90.05±64.9** ^{a*,b**}	0.007**	**35±8.7**	**64.6±55.7**	**74.7±56.9**	**0.330**
T.PTN	**7.9±0.27**	**7.95±0.24**	**8.01±0.26**	0.775	**7.9±0.2**	**7.9±0.2**	**7.9±0.4**	**0.742**
Albumin	**3.8±0.3**	**3.81±0.32**	**3.8±0.36**	0.997	**3.81±0.4**	**3.8±0.3**	**3.7±0.4**	**0.704**
INR	**1.02±0.05**	**1.06±0.11**	**1.02±0.08**	0.163	**1.04±0.11**	**1.01±0.06**	**1.1±0.1**	**0.197**
Cholesterol	**119.5±38.8**	**114.67±19.7**	**146.7±30.4** ^{a**,b*}	0.006**	**105.86±27.7**	**125.6±36.18**	**157.8±30.8**^{a**,b*}	**0.032**
TG	**149.5±35.7**	**154.4±28.3**	**171.09±48.5**^{a*}	0.104	**126±44.9**	**159.2±37.19**^{a*}	**157.3±49.1**	**0.104**
HDL	**53.5±17.5**	**54.5±3.9**	**46.3±8.8**	0.120	**44.7±13.9**	**51.8±12.8**	**58.3±27.6**	**0.237**
LDL	**119.02±31.1**	**103.3±26.3**	**127.8±29.6**^{b*}	0.083	**102.6±8.9**	**117.94±29.9**	**151±35.4**^{ab**}	**0.012***
CAP (dB/m)	**263±13.18**	**258.8±6.57**	**266.4±10.7**	0.408	**265.5±0.7**	**263.2±11.7**	**267.8±13.9**	**0.702**
Fibroscan (kPa)	**4.9±1.04**	**4.84±0.6**	**5.3±0.9**	0.416	**5.3±0.4**	**5.02±0.9**	**5.7±1.1**	**0.402**
NFS	**-2.31±1.54**	**-2.78±0.96**	**-2.47±2.02**	0.666	**-2.33±1.7**	**-2.56±1.5**	**-1.06±1.5**^{b*}	**0.082**
FIB-4 score	**0.7±0.32**	**0.65±0.36**	**0.8±0.57**	0.516	**0.8±0.6**	**0.68±0.4**	**1.05±0.45** ^{b*}	**0.090**

Also, the influence of PNPLA3 genotype rs139051 on clinical and laboratory parameters showed that people with GG genotype had higher BMI than those with AA genotype (P=0.018). Also, patients with GG genotypes have elevated ALT and AST liver enzymes than those with AG genotype. Furthermore, the influence of PNPLA3 genotypes rs139051 on lipid profile showed that both cholesterol and LDL levels of the PNPLA3 GG carriers were significantly higher than those of AA and AG carriers (P<0.05 for each). Moreover, we found a remarkable difference between GG variant carriers and AG-carriers in terms of NFS, and FIB-4 values, as GG carriers have significantly higher NFS and FIB-4 values than AG carriers (P= 0.026 and P=0.034; respectively). Correlation between different non-invasive fibrosis markers and other parameters was mentioned in table 6.

Table 6: Correlation between Different Non-Invasive Fibrosis Markers and Other Parameters

Parameters	kpa(kPa)		CAP(dB/m)		FAST		NFS		FIB-4	
	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value
Kpa	1	-	0.838**	<0.001	-0.569**	<0.001	0.151	0.351	0.229	0.155
CAP	0.838**	<0.001	1	-	-0.470**	0.002	0.367*	0.02	0.288	0.072
FAST	-0.569**	<0.001	-0.470**	0.002	1	-	-0.390*	0.013	-0.514**	0.001
NFS	0.151	0.351	0.367*	0.02	-0.390*	0.013	1	-	0.757**	<0.001
FIB-4	0.229	0.155	0.288	0.072	-0.514**	0.001	0.757**	<0.001	1	-
Age	-0.04	0.806	-0.043	0.794	0.217	0.179	0.432**	<0.001	0.628**	<0.001
BMI	0.268	0.095	0.281	0.079	-0.275	0.086	0.635**	<0.001	0.411**	<0.001
TLC	0.143	0.380	0.114	0.483	-0.396*	0.011	0.195	0.083	0.271*	0.015
HB	0.371*	0.019	0.203	0.208	-0.190	0.798	-0.389**	<0.001	-0.255*	0.022
PLT	0.113	0.487	-0.050	0.760	0.171**	<0.001	-0.740**	<0.001	-0.502**	<0.001
FBS	-0.097	0.553	-0.063	0.699	-0.019	0.704	0.476**	<0.001	0.113	0.320
T.BIL	-0.027	0.867	0.055	0.737	-0.021**	<0.001	0.175	0.12	0.101	0.370
AST	0.262	0.103	0.095	0.561	-0.616**	<0.001	0.381**	<0.001	0.310**	0.005
ALT	0.477**	0.002	0.366*	0.020	-0.858**	<0.001	0.389**	<0.001	0.551**	<0.001
ALP	-0.020	0.903	-0.059	0.718	0.322*	0.043	0.317**	0.004	0.013	0.910
GGT	0.251	0.118	0.163	0.316	-0.553**	<0.001	0.441**	<0.001	0.403**	<0.001
T.PTN	-0.001	0.994	-0.025	0.876	-0.034	0.835	-0.030	0.790	0.135	0.233
Albumin	-0.156	0.338	-0.227	0.160	0.228	0.157	-0.299**	0.007	-0.110	0.329
INR	-0.096	0.558	-0.116	-0.475	0.089	0.583	0.205	0.068	0.186	0.099
Cholesterol	0.204	0.206	0.164	0.312	-0.353	0.026	0.417**	<0.001	0.340**	0.002
TG	-0.079	0.628.	0.160	0.324	0.180	0.268	0.1	0.377	0.075	0.511
HDL	0.057	0.729	0.052	0.750	-0.075	0.646	-0.020	0.858	-0.045	0.691
LDL	-0.235	0.144	-0.164	0.313	0.034	0.833	0.297**	0.007	0.108	0.338
PNPLA3 (rs738409)	0.184	0.257	0.136	0.404	-0.134	0.411	-0.051	0.655	-0.02	0.862
PNPLA3 (rs139051)	0.128	0.432	0.077	0.637	-0.117	0.470	0.177	0.116	0.157	0.165

Discussion:

NAFLD is known nowadays as the most common liver disorder in the 21st century. It is diagnosed by the presence of more than 5% fat accumulation in liver cells without excess alcohol consumption or secondary causes of fat accumulation in the background. Approximately 25% of the world's adults population has NAFLD, and the prevalence is still increasing [13]. NAFLD may eventually deteriorate to HCC as a result of excessive lipid accumulation, liver cell damage, immune system dysfunction which leads to scarring, and permanent liver damage [15]. In light of increasing NAFLD prevalence, early detection and diagnosis are needed for decision-making in clinical practice and could be helpful in the management of patients with NAFLD.

PNPLA3 is the liver ¬enriched member of the PNPLA family, which is located on the membrane of lipid droplets and endoplasmic reticulum in hepatocytes. PNPLA3 was recently identified to promote the transfer of very long chain polyunsaturated fatty acids from TAG to phospholipids. Also, it has an important regulatory role in lipid homeostasis [8].

Several studies suggested that the PNPLA3 genotype influences hepatic steatosis and liver fibrosis; however, this is not clarified for Egyptian patients. Thus, this study aims to examine the association between two SNPs of PNPLA3 genes (rs738409 and rs139051) and NAFLD and it seeks to evaluate the relation of these SNPs and the degree of hepatic steatosis and fibrosis as assessed by Fibroscan (Indices of noninvasive markers).

The present study which was conducted on forty patients with NAFLD and forty healthy controls showed a significant trend of elder age with the progression of non-alcoholic fatty liver disease. This finding substantiates previous findings in the literature which suggested that the prevalence of NAFLD increases with increasing age [16].

In the current study, the incidence of NAFLD has been increasing in concert with (the presence of multiple metabolic disorders) increased number of metabolic diseases detected such as dyslipidemia, diabetes, hypertension, and visceral obesity. As

expected, the incidence of diabetes and hypertension was significantly higher in patients suffering from NAFLD. This is in good agreement with previous studies that mentioned impaired glucose tolerance as an independent risk factor for the progression of NAFLD [17, 18].

Lonardo et al., declared that patients with T2DM have 80% higher liver fat contents compared to non-diabetic patients. They also reported that T2DM patients have a particularly high risk of developing NASH along with a 2-4 fold increased risk of fatty liver-related complications [19].

Also our NAFLD group had higher BMI and thus they are expected to be more liable to hepatic steatosis and our findings are compatible with the previously published studies which indicated that both BMI and waist circumference (WC) were significantly higher in NAFLD patients than in healthy controls. It was previously reported that obesity is found in 30-100% of subjects with NAFLD. Also, steatosis is 4.6 fold higher in obese cases than in normal-weight people [20, 21].

Liver enzymes, ALT, AST, ALP, and GGT were significantly higher in NAFLD cases compared to non-NAFLD participants. The elevation of both ALT and AST levels in NAFLD cases can be attributed to the role of these markers in necrosis and hepatocellular injury and thus their levels may be elevated in severe cases. Also, the elevation of liver enzymes, especially serum aminotransferases, and increased GGT indicate oxidative stress and inflammatory activity in NAFLD [22-24]. However, the elevations in ALT and (AST) are usually mild and are usually not more than four times the upper limit of normal [25].

Additionally, the current study is consistent with prior studies which showed an association between the elevated serum total cholesterol, LDL, triglyceride levels, as well as reduced HDL levels and the presence of NAFLD [26, 27].

This may be attributed to hepatic overproduction of the very low-density lipoprotein (VLDL) particles and dysregulated clearance of various lipoproteins from the circulation [28].

There are few numbers of studies that discussed the association between PNPLA3 polymorphism and NAFLD in the Egyptian population. The finding of this study implied that the GG-genotype of rs738409 is significantly associated with NAFLD; suggesting that it may be an essential causative factor for the incidence and progression of NAFLD. Indeed, different distribution patterns of rs738409 genotype between the NAFLD and control groups were detected as homozygous wild type (CC) was the commonest genotype among subjects in the control group, while the GG genotype was the most frequent in patients with NAFLD. The results also revealed that there was an association between the presence of the G allele (46.3%) in the PNPLA3 and the incidence of NAFLD.

Our finding is in agreement with the former studies which show that the risk gene [G] carriers are more prone to suffer from NAFLD [29-33].

It was reported in a meta-analysis study that the gene PNPLA3 rs738409 polymorphism is closely related to the occurrence and development of NAFLD. It has been identified that PNPLA3 is an important regulating factor for triglyceride, which could act as both phospholipase and acyl transfer enzyme (30). Although the exact physiological role of PNPLA3 on the liver is still unclear, the rs738409 gene polymorphism affects not only the triglyceride deposition in the liver but also the progression of fatty liver disease in patients [34]. The rs738409 C>G mutation induce NAFLD by allowing the accumulation of PNPLA3 protein at the surface of hepatic lipid droplets, thus inhibiting the activity of local lipases and limiting the binding of the substrate to the catalytic site, and therefore impairing the triglycerides mobilization and hydrolysis in liver cells, resulting in the accumulation of triglycerides in the liver and causing the occurrence of fatty liver. The transcriptional up-regulation of PNPLA3 that occurs during carbohydrate loading may exacerbate the effect of the variant by increasing PNPLA3 protein production and consequently, rising triglyceride accumulation in the liver [35].

No association between PNPLA3 gene polymorphism rs738409 and degrees of steatosis or fibrosis was noted in our study. This may be due to the lack of NAFLD patients with advanced fibrosis in this study. Further studies should be conducted to better address this issue as previous study by Caroline Manchiero found a significant association between the rs738409 polymorphism of the PNPLA3 gene genotype GG and hepatic steatosis and advanced fibrosis in patients with chronic hepatitis C [14].

Additionally, PNPLA3 rs139051 is suggested to have a protective influence against NAFLD-related impairment via its up-regulatory effect on both Lysophosphatidyl-choline and Lysophosphatidylcholine plasmalogen. This Lysophosphatidyl-choline is then catabolized into phosphatidylcholines which play a crucial role in maintaining the integrity of cellular and organelle membranes [36, 37].

The current study revealed that there was no association between rs139051 PNPLA3 and the prevalence and pathogenesis of NAFLD as rs139051

PNPLA3 AG genotype was the most frequent genotype in cases with and without NAFLD.

This finding was in contrast to the former study by Luo, et al. which showed that PNPLA3 rs139051 is suggested to act in hepatic inflammation of NAFLD by targeting, at least to a large extent, Lysophosphatidylcholine, and Lysophosphatidylcholine plasmalogen [8].

In addition, Peng et al published study also indicated that SNP rs139051 was relevant to NAFLD susceptibility [38].

Furthermore, as previously mentioned by Luo, et al the current study demonstrated that PNPLA3 rs139051 did not seem to exert any significant impact on NAFLD specific steatosis, ballooning, and fibrosis [8].

Conclusion:

PNPLA3 gene polymorphism rs738409 but not rs139051 could be used as a screening tool in NAFLD patients with simple steatosis, which warrants earlier follow-up and further intensive therapies.

Competing interests

The authors declare that they have no competing interests.

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