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Investigation of Gene Variation among Non-Alcoholic Fatty Liver Disease Patients in Gaza Strip: A Preliminary Study

(Kajian Variasi Gen dalam Kalangan Pesakit Hati Berlemak Bukan Alkohol di Semenanjung Gaza: Suatu Kajian Awal)

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ABSTRACT

The prevalent hepatic presentation of the metabolic syndrome is a non-alcoholic fatty liver disease (NAFLD), one of the most common types of chronic liver illnesses. Patients with NAFLD may develop liver damage depending on their genetic heritage. In this preliminary study, our main aim was to detect the genetic association of p85α (Met326Ile), PNPLA3 (C>G), IL28B275 (A>G), and IL28B860 (C>T) single nucleotide polymorphisms (SNPs) with steatosis and NASH in patients from Gaza Strip. We performed an SNP analysis by RFLP-PCR in 33 cases of steatosis and 28 cases of non-alcoholic steatohepatitis (NASH), in addition to 29 age- and sex-matched controls. We found that only the mutant T allele of IL28B860 was significantly associated with an increased risk of steatosis (P = 0.04). The other studied alleles and genotypes were not significantly associated with increased or decreased risk of steatosis, NASH, or combined steatosis or NASH groups. Among all of the studied variables (age, sex, diabetes, and BMI), only BMI was significantly associated with an increased risk of steatosis as well as NASH. A linkage disequilibrium analysis showed that the association between the two SNPs of IL28B860 and IL28B275 was significant. Having the TG haplotype increased the risk of steatosis by 2.97 fold and the risk of combined steatosis or NASH by 2.44 fold. This haplotype increased the risk of NASH, but the effect was not significant.

Keywords: NAFLD; NASH; preliminary study; single nucleotide polymorphism; steatosis

ABSTRAK

Persembahan hati yang lazim bagi sindrom metabolik ialah penyakit hati berlemak bukan alkohol (NAFLD), salah satu jenis penyakit hati kronik yang paling biasa. Pesakit dengan NAFLD mungkin mengalami kerosakan hati bergantung kepada warisan genetik mereka. Dalam kajian awal ini, matlamat utama kami adalah untuk mengesan perkaitan genetik p85α (Met326Ile), PNPLA3 (C> G), IL28B275 (A> G) dan IL28B860 (C> T) polimorfisme nukleotida tunggal (SNP) dengan steatosis dan NASH pada pesakit dari Semenanjung Gaza. Kami menjalankan analisis SNP dengan RFLP-PCR dalam 33 kes steatosis dan 28 kes steatohepatitis bukan alkohol (NASH), sebagai tambahan kepada 29 kawalan padanan umur dan jantina. Kami mendapati bahawa hanya alel T mutan IL28B860 dikaitkan dengan peningkatan risiko steatosis (P = 0.04). Kajian lain Alel dan genotip tidak dikaitkan dengan peningkatan atau penurunan risiko steatosis, NASH, atau gabungan steatosis atau kumpulan NASH. Antara semua pemboleh ubah yang dikaji (umur, jantina, diabetes dan BMI), hanya BMI dikaitkan dengan peningkatan risiko steatosis serta NASH. Analisis ketidakseimbangan kaitan menunjukkan bahawa perkaitan antara dua SNP IL28B860 dan IL28B275 adalah signifikan. Mempunyai haplotip TG meningkatkan risiko steatosis sebanyak 2.97 kali ganda dan risiko gabungan steatosis atau NASH sebanyak 2.44 kali ganda. Haplotip ini meningkatkan risiko NASH, tetapi kesannya tidak ketara.

Kata kunci: Kajian awal; NAFLD; NASHl; polimorfisme nukleotida tunggal; steatosis

INTRODUCTION

In 1980, Ludwig et al. first used the term NAFLD to refer to fatty liver disease in individuals without a history of drinking too much alcohol. NAFLD is considered a risk factor for developing cirrhosis, fibrosis, NASH, and hepatocellular cancer (Schreuder et al. 2008). Previous

studies showed that 0.77 deaths per 1,000 persons per year were attributable to liver disease in patients with NAFLD (Xia et al. 2019). It was estimated that the frequency of the disease in East Asian countries is 15-45% and 20-30% in developed Western countries (Zhang et al. 2015). Obesity, insulin-resistant diabetes, and metabolic syndrome are all connected to the prevalence of NAFLD (Tai et al. 2015). A cohort study confirmed that NAFLD is a strong risk factor for developing diabetes mellitus in middle-aged healthy Japanese men (Shibata et al. 2007). Furthermore, others found that the highest prevalence of NAFLD in Iranian adults was among patients with type 2 diabetes mellitus (T2DM), which was as high as 55.8% (Lankarani et al. 2013). Inactivity and an unhealthy diet are other causes of NAFLD.

In addition to the traditional risk factors, emerging contradictory evidence points to the potential importance of hereditary variables, particularly SNPs in genes related to inflammation, oxidative stress, and fibrogenesis, in the susceptibility to NAFLD and the severity of liver disease. There are around 7 groups of genes that have been linked to NAFLD, and they are organized as follows: (1) hepatic lipid export/oxidation in steatosis (PNPLA3, TM6SF2, NR1I2, PPARalpha, PEMT, MTTP, APOC3, and APOE); (2) glucose metabolism and insulin resistance (ENPP1/IRS1, GCKR, SLC2A1, GOAT, TCF7L2, and PPARG); (3) steatosis-hepatic lipid import or synthesis (SLC27A5, FADS1, and LPIN1); (4) steatohepatitis-oxidative stress (HFE, GCLC/ GCLM, ABCC2, and SOD2); (5) steatohepatitis-endotoxin response (TLR4 and CD14); (6) cytokines (TNF and IL6); and (7) fibrosis (AGTR1 and KLF6) (Anstee & Day 2015; Dutta 2011; Engwa et al. 2018; Rüstemoğlu et al. 2016).

The SNP (rs738409) in the patatin-like phospholipase domain-containing protein 3 (PNPLA3) is one of the most significant genetic factors for NAFLD. Chromosome 22's long arm is where PNPLA3 is found in humans. The uniqueness of the specific SNP rs738409 (rs738409 C>G) is that it encodes an amino acid change from isoleucine to methionine at position 148 (I148M). Triacylglycerol lipase, also known as adiponutrin, is encoded by PNPLA3 and is involved in the hydrolysis of triacylglycerol in adipocytes. This amino acid alteration decreases enzymatic activity and encourages the onset of steatosis (Pirazzi et al. 2012).

It was shown that in individuals with chronic hepatitis C (CHC), SNPs around the gene encoding interleukin 28B (IL28B) (rs12979860 CC and rs8099917 TT) highly predict the accomplishment of a sustained virologic response after standard antiviral therapy and the extent of liver disease (Abe et al. 2010; Ge et al. 2009; Tillmann et al. 2011). On the other hand, Petta et al. (2012) found that IL28B (rs12979860 and rs8099917 SNPs), together with PNPLA3 (rs738409 C>G SNP), is associated with fibrosis and lobular inflammation in NAFLD patients.

Phosphoinositide 3-Kinase (PI3K) causes phosphorylation of phosphoinositides. It is crucial for insulin activity, particularly in the transfer of glucose, the production of glycogen, and the inhibition of lipolysis. It consists of a p85-regulatory subunit (p85α) and a p110 catalytic subunit (Fruman, Meyers & Cantley 1998). Chen et al. (2005) found that the Met326Ile variation in the gene encoding the p85α protein might contribute to the increased risk of T2DM and hypertension in Chinese. Furthermore, a common molecular event that is associated with metabolic syndrome, obesity, and NAFLD is the deregulation of the PI3K pathway in hepatocytes (Matsuda, Kobayashi & Kitagishi 2013). Because of this close association, we aimed to examine the relationship between Met326Ile variation of p85α, steatosis, and NASH.

The severity of NAFLD differs among different populations; these variances could be due to fluctuating frequencies of genetic variants in diverse ethnic groups. With this in mind, we decided to assess the association of p85α (Met326Ile), PNPLA3 (C>G), IL28B275 (A>G), and IL28B860 (C>T) SNPs with steatosis and NASH in patients from Gaza Strip.

Materials and Methods

Ethical Approval

The Helsinki Committee provided the required ethical approval for the study to be conducted in Gaza City. All the participants were provided with sufficient knowledge regarding the purpose of the study. All participants agreed to participate. An official letter was obtained from the Palestinian Ministry of Health to facilitate the research.

Study Population

A retrospective case-control study was conducted on a target population aged 12-79 years, made up of NAFLD patients (case group) and apparently healthy persons (control group). The patients were diagnosed based on symptoms, biopsy, CT, or Ultrasound. The cases were 33 cases of steatosis and 28 cases of NASH, which were registered at the Department of Internal Medicine at Al-Shifa hospital and AL-Quds hospital in Gaza City. The

control group included 29 persons who were randomly chosen. Cases and controls were matched for age and gender. Patients with HBV or HCV, any other liver disease, and any acute or chronic illness (severe kidney disease requiring dialysis, thalassemia, hemochromatosis, or malignancy) were excluded from the study. A face-to-face interview was conducted to fill in a structural questionnaire designated for cases and controls to meet the study requirements. The questionnaire included questions on personal information (age, height, and weight), socioeconomic character, and medical history. Height and weight were measured for each subject; then, the BMI was calculated for each subject as follows: BMI = body weight in kg/height in square meters (unit kg/m^2).

DNA EXTRACTION AND GENOTYPING

After twelve hours of overnight fasting, venous blood samples (5 mL) were collected from all participants. One mL was placed in an EDTA tube, and the remaining was placed into a plain tube for further analysis. Genomic DNA was isolated from a 200 µL whole blood sample by using a Jena Bioscience DNA Extraction Kit (Cat# PP-237S, Jena Bioscience, Germany) and according to the manufacturer's instructions. Each target gene was amplified by PCR, using 5X FIREPol® Master Mix Ready to Load (Solis BioDyne, Estonia) and primers (Macrogen, Korea, Table S1). The conditions used for thermal cycling (MultiGene machine, Labnet, USA) were as follows: denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 50 s, annealing at a specified temperature, according to Table S1, for 40 s, and extension at 72 °C for 50 s. At the end of the cycles, the reaction mixture was kept at 72 °C for 7 minutes and then cooled to 4 °C. The PCR product was separated by 2% ethidium bromide-stained agarose gel (Expedeon, USA) and visualized on a UV transilluminator (Cleaver Scientific/UK). To perform the RFLP assay, the amplicon of each target was digested with a restriction endonuclease enzyme (Table S2) according to the manufacturer's instructions (New England Biolabs, USA) for 3 hours. Restriction digestion products for each gene target (Table S2) were separated by 2.5% ethidium bromide-stained agarose gel (Expedeon, USA) and visualized on a UV transilluminator (Cleaver Scientific/UK).

STATISTICAL ANALYSIS

Age was expressed as median and interquartile range, whereas categorical variables were summarized with frequencies and proportions. The Hardy-Weinberg equilibrium was tested for each SNP in the control group, using the function 'HWExact' in the 'Hard-Weinberg' package in R, which performed the Haldane Exact test. Odds ratios (OR) were calculated for carrying the heterozygous or homozygous mutant genotypes relative to the normal homozygous in the NASH or Steatosis groups relative to the control. Similarly, ORs were calculated for carrying the mutant allele relative to the normal allele in the disease groups relative to controls. ORs were calculated using the function 'oddsratio' in package 'epitools' in R. ORs were also adjusted for demographic variables, using binary logistic regression. The outcome (disease) was treated as a binary variable (either presence or absence). Possible confounders were entered into the model as predictive variables including age, sex, diabetes, and BMI. Binary logistic regression was carried out using the 'glm' function with family 'binomial' in R. Linkage disequilibrium was calculated for the SNPs IL28B860 and IL28B275, using the function 'LD' in package 'genetics' in R. The different allele combinations for these two SNPs were then tested in binary logistic regression for association with NASH or Steatosis. Haplotype frequencies were calculated using the web-based implementation of the R package 'SNPStats'.

RESULTS

Both PPARγ and p85αIVS were homozygous normal (NN) for individuals in the overall sample (n = 90). Thus, they were not included in the analyses.

COMPARISON OF THE PATIENT GROUP'S AND THE CONTROL GROUP'S DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

In this case-control study, a total of 90 individuals were included, including 33 steatosis patients, 28 NASH patients, and 29 healthy controls. It was shown that 86.9% of the cases were smokers compared to 62.1% of the controls; the difference was statistically significant ($P = 0.007$). On the other hand, 72.4% of the controls were physically active compared to 27.9% of cases; the difference was also statistically significant ($P < 0.001$). The majority of the cases (93.4%) had hyperlipidemia compared to 10.3% of the controls; the difference was statistically significant ($P < 0.001$). Moreover, 42.6% of the cases had hypertension compared to 10.3% of the controls, and the difference was statistically significant ($P = 0.004$) (data not shown). The BMI of the disease groups and the control groups were significantly different, with the NASH groups having the highest median BMI values. The median age of the steatosis group was significantly higher than the control group (Table 1).

TABLE 1. Descriptive statistics of covariates for the dataset

	All subjects (n=90)	Control (n=29)	Steatosis (n=33)		NASH (n=28)		NASH/Steatosis (n=61)	
			Median (IQR) or Count (Prop.)	P value	Median (IQR) or Count (Prop.)	P value	Median (IQR) or Count (Prop.)	P value
Age	44 (35-55)	39 (33-46)	48 (41-57)	0.011	44 (35-57.5)	0.184	47 (38-57)	0.023
Sex								
Male	59 (65.6)	18 (62.1)	19 (57.6)	0.92	22 (78.6)	0.284	41 (67.2)	0.808
Female	31 (34.4)	11 (37.9)	14 (42.4)		6 (21.4)		20 (32.8)	
BMI	30.8 (27.2-34.6)	26.0 (22.8-28.4)	31.9 (29.1-34.7)	1.13×10^{-6}	35.0 (32.4-40.9)	7.18×10^{-9}	33.2 (30.3-38.3)	9.8×10^{-10}
Diabetes								
Yes	16 (17.8)	2 (6.9)	7 (2.1)	0.155	7 (25.0)	0.079	14 (23.0)	0.080
No	74 (82.2)	27 (93.1)	26 (78.8)		21 (75.0)		47 (77.0)	

IQR: Interquartile range, Prop.: Proportion from the total of the relevant group. For age and BMI, P value is calculated from Wilcoxon rank sum test, for sex, it is from Chi square test, and for diabetes P value is from Fisher's exact test. All P values are based on tests between each disease group and the control

GENOTYPE AND ALLELE FREQUENCIES

Table 2 shows the genotype and allele frequency of the different studied SNPs. SNP p85αMet had the lowest minor allele frequency between 3-7% in the different subgroups of the sample. For all four SNPs in Table 2, the control group had the highest proportion of the normal homozygous genotype except for p85αMet in steatosis. On the other hand, steatosis had the highest proportion of homozygous mutant genotypes for both PNPLA3 and IL28B860. The NASH group had the highest proportion of the heterozygous genotype for all SNPs except for IL28B275. In the case of all four SNPs, the mutant allele was present at a higher proportion in either steatosis or NASH relative to the control (Table 2). All four of the SNPs were in Hardy-Weinberg equilibrium ($P > 0.05$) in the control group.

ASSOCIATION BETWEEN SNP POLYMORPHISM AND STEATOSIS/NASH

Binary logistic regression was used to test the relationship between Steatosis, or NASH, or the combined group of either Steatosis or NASH, with the different genotypes for each SNP. As shown in Table 2, carrying the mutant 'T' allele at IL28B860 was found to be significantly associated with an increased risk of steatosis. Specifically, carrying the T allele increased the risk in the steatosis group by 2.10 fold (CI: 1.02-4.38, $P = 0.040$). Indeed, carrying the genotype CT was associated with a 1.13 (0.32-4.01) fold increase in the risk of steatosis, and having two T alleles (TT) was associated with a 2.87 (0.84-10.73) fold increase in the risk of this disease. So far, both of these ORs were not significant. The mutant allele and genotypes containing the mutant allele of p85αMet were associated with lower or no risk in the steatosis group and a higher risk in the NASH group; these associations were not significant. For PNPLA3, only the heterozygous genotype was associated with an increased risk of NASH, whereas both the heterozygous and mutant homozygous genotypes increased the risk of steatosis, yet these associations were not significant. Both heterozygous and mutant homozygous genotypes of IL28B275 and IL28B860 were associated with an increased risk of both steatosis and NASH, but these associations were not significant.

GENOTYPE AND ALLELE ODDS RATIOS FOR DISEASE WITH ADJUSTMENT FOR AGE, SEX, DIABETES, AND BMI

A binary logistic regression was used to obtain adjusted odds ratios for SNP genotypes by inserting demographic characteristics into the model. Table 3 shows that only BMI was significantly associated with an increased risk of steatosis. Specifically, every one-unit increase in BMI was associated with a 53-59% increased risk of steatosis as opposed to the control group, depending on the SNP entered in the model. The heterozygous genotypes of p85αMet and PNPLA3 were protective of Steatosis (OR < 1), but these results were not significant. All the mutant homozygous genotypes increased the risk of steatosis. Males were less likely to be in the steatosis group than females when they both carried the same genotypes of p85αMet, IL28B275, and IL28B860, but these results were also not significant.

TABLE 2. Genotype, allele frequencies and Odds ratios for being in the Steatosis, NASH, or Steatosis/NASH groups for patients carrying the indicated genotypes or alleles relative to the normal homozygous genotype or normal allele

Gene	Genotype/ Allele	Control	Steatosis			NASH			Steatosis/NASH		
		Count (Prop.)	Count (Prop.)	OR (CI)	P value	Count (Prop.)	OR (CI)	P value	Count (Prop.)	OR (CI)	P value
p85αMet	NN	24 (0.83)	28 (0.85)	Ref.	-	18 (0.64)	Ref.	-	46 (0.75)	Ref.	-
	NM	5(0.17)	4 (0.12)	0.69 (0.15-3.02)	0.624	8 (0.29)	2.08 (0.58-8.17)	0.262	12 (0.20)	1.23 (0.40-4.36)	0.726
	MM	0(0.0)	1 (0.03)	NA	NA	2 (0.07)	NA	NA	3 (0.05)	NA	NA
	N	53 (0.91)	60 (0.91)	Ref.	-	44 (0.79)	Ref.	-	104 (0.85)	Ref.	-
	M	5 (0.09)	6 (0.09)	1.05 (0.29-3.97)	0.935	12 (0.21)	2.82 (0.95-9.67)	0.062	18 (0.15)	1.79 (0.67-5.79)	0.260
PNPLA3	CC	13 (0.45)	12 (0.36)	Ref.	-	11 (0.39)	Ref.	-	23 (0.38)	Ref.	-
	CG	9 (0.31)	11 (0.33)	1.31 (0.40-4.43)	0.656	11 (0.39)	1.43 (0.43-4.90)	0.563	22 (0.36)	1.37 (0.49-3.99)	0.553
	GG	7 (0.24)	10 (0.30)	1.53 (0.43-5.58)	0.512	6 (0.21)	1.01 (0.25-4.08)	0.984	16 (0.26)	1.28 (0.42-4.14)	0.670
	C	35 (0.60)	35 (0.53)	Ref.	-	33 (0.59)	Ref.	-	68 (0.56)	Ref.	-
	G	23 (0.40)	31 (0.47)	1.34 (0.66-2.77)	0.420	23 (0.41)	1.06 (0.50-2.26)	0.880	54 (0.44)	1.21 (0.64-2.30)	0.566
IL28B275	AA	15 (0.52)	9 (0.27)	Ref.	-	12 (0.43)	Ref.	-	21 (0.34)	Ref.	-
	AG	9 (0.31)	16 (0.48)	2.87 (0.91-9.72)	0.074	9 (0.32)	1.24 (0.37-4.25)	0.726	25 (0.41)	1.95 (0.71-5.60)	0.194
	GG	5 (0.17)	8 (0.24)	2.57 (0.64-11.32)	0.186	7 (0.25)	1.71 (0.42-7.38)	0.451	15 (0.25)	2.09 (0.64-7.77)	0.230
	A	39 (0.67)	34 (0.52)	Ref.	-	33 (0.59)	Ref.	-	67 (0.55)	Ref.	-
	G	19 (0.33)	32 (0.48)	1.92 (0.93-4.05)	0.080	23 (0.41)	1.42 (0.66-3.10)	0.367	55 (0.45)	1.68 (0.876-3.28)	0.120
IL28B860	CC	14 (0.48)	11 (0.33)	Ref.	-	9 (0.32)	Ref.	-	20 (0.33)	Ref.	-
	CT	9 (0.31)	8 (0.24)	1.13 (0.32-4.01)	0.851	13 (0.46)	2.19 (0.66-7.62)	0.199	21 (0.34)	1.61 (0.57-4.74)	0.369
	TT	6 (0.21)	14 (0.42)	2.87 (0.84-10.73)	0.094	6 (0.21)	1.53 (0.36-6.64)	0.561	20 (0.33)	2.28 (0.74-7.71)	0.154
	C	37 (0.64)	30 (0.45)	Ref.	-	31 (0.55)	Ref.	-	61 (0.5)	Ref.	-
	T	21 (0.36)	36 (0.55)	2.10 (1.02-4.38)	0.040	25 (0.45)	1.41 (0.67-3.04)	0.367	61 (0.5)	1.75 (0.93-3.38)	0.085

CI: 95% Confidence interval for OR, NASH: Nonalcoholic steatohepatitis, Prop.: Proportion relative to the total in each group, Ref.: Reference genotype or allele against which disease odds are compared. P values from mid-p exact method

In relation to NASH, only BMI was significantly associated with an increased risk of NASH regardless of the SNP that was included in the model. The heterozygous genotypes of PNPLA3, IL28B275, and the mutant homozygous of IL28B860 were slightly protective in the NASH group, yet these odds ratios were not significant. Diabetes was associated with a nonsignificant decreased risk of NASH group for any two individuals with the same genotype (Table 4). A crude model with diabetes alone in the model had an odds ratio of 4.5 for NASH, but when the model was adjusted for age, sex, BMI, and genotype, it lost its contribution to being in the NASH group (data not shown).

When NASH and Steatosis were combined, the heterozygous genotype of p85αMet was protective in the disease group, but this effect was not significant. The mutant homozygous genotypes of PNPLA3, IL28B275, and IL28B860, in addition to the heterozygous genotype of IL28B275 and IL28B860, non-significantly increased the risk of the disease group. Diabetes decreased the risk of the disease group for the same genotype at any SNP, but this effect was not significant. BMI was again the only covariate significantly associated with increased risk of the disease group (data not shown).

TABLE 3. Binary logistic regression of steatosis as the outcome variable and the variables in the first column as the predictive variables

	OR	Wald statistic	P value
P85αMet			
Age	1.03 (0.98 - 1.09)	1.111	0.267
Sex (male vs. female)	0.73 (0.15 - 3.56)	-0.383	0.702
Diabetes	0.89 (0.094 - 8.36)	-0.105	0.916
BMI	1.56 (1.22 - 1.99)	3.539	0.00040
p85αMet (NM vs NN)	0.73 (0.081 - 6.53)	-0.283	0.777
p85αMet (MM vs. NN)	NA	NA	NA
PNPLA3			
Age	1.04 (0.99 - 1.09)	1.432	0.152
Sex (male vs. female)	0.95 (0.20 - 4.49)	-0.065	0.948
Diabetes	1.08 (0.097 - 11.9)	0.062	0.951
BMI	1.59 (1.24 - 2.05)	3.608	0.00031
PNPLA3 (CG vs. CC)	0.86 (0.16 - 4.62)	-0.181	0.856
PNPLA3 (GG vs. CC)	3.21 (0.53 - 19.6)	1.261	0.207
IL28B275			
Age	1.04 (0.98 - 1.09)	1.347	0.178
Sex (male vs. female)	0.74 (0.16 - 3.51)	-0.382	0.703
Diabetes	0.82 (0.070 - 9.57)	-0.162	0.872
BMI	1.53 (1.21 - 1.93)	3.530	0.00042
IL28B275 (AG vs. AA)	3.32 (0.65 - 16.9)	1.445	0.149
IL28B275 (GG vs. AA)	2.25 (0.35 - 14.6)	0.851	0.395
IL28B860			
Age	1.03 (0.97 - 1.08)	0.939	0.348
Sex (male vs. female)	0.53 (0.10 - 2.78)	-0.749	0.454
Diabetes	0.79 (0.068 - 9.08)	-0.193	0.847
BMI	1.59 (1.23 - 2.04)	3.605	0.00031
IL28B860 (CT vs. CC)	1.53 (0.28 - 8.34)	0.488	0.626
IL28B860 (TT vs. CC)	5.76 (0.89 - 37.5)	1.832	0.067

BMI: Body mass index, OR: Odds ratio of being in the disease group relative to the control, NA: The calculations could not be done because the control had no individuals with the mutant homozygous genotype

TABLE 4. Binary logistic regression of NASH as the outcome variable and the variables in the first column as the predictive variables

	OR	Wald statistic	P value
p85αMet			
Age	1 (0.93 - 1.06)	-0.105	0.916
Sex (male vs. female)	1 (0.11 - 9.41)	0.00266	0.998
Diabetes	0.77 (0.041 - 14.3)	-0.177	0.860
BMI	1.75 (1.25 - 2.46)	3.235	0.00122
p85αMet (NM vs NN)	1.11 (0.096 - 12.8)	0.0816	0.935
p85αMet (MM vs. NN)	NA	NA	NA
PNPLA3			
Age	1.01 (0.94 - 1.08)	0.224	0.823
Sex (male vs. female)	0.95 (0.12 - 7.27)	-0.0539	0.957
Diabetes	0.93 (0.039 - 21.9)	-0.0462	0.963
BMI	1.82 (1.3 - 2.55)	3.470	0.00052
PNPLA3 (CG vs. CC)	0.86 (0.11 - 6.87)	-0.139	0.890
PNPLA3 (GG vs. CC)	5.67 (0.40 - 81)	1.279	0.201
IL28B275			
Age	1 (0.94 - 1.07)	0.048	0.962
Sex (male vs. female)	0.84 (0.11 - 6.38)	-0.171	0.864
Diabetes	0.64 (0.038 - 11)	-0.305	0.760
BMI	1.84 (1.27 - 2.66)	3.223	0.0013
IL28B275 (AG vs. AA)	0.83 (0.11 - 6.47)	-0.178	0.859
IL28B275 (GG vs. AA)	2.43 (0.23 - 25.5)	0.741	0.458
IL28B860			
Age	1.01 (0.94 - 1.09)	0.201	0.841
Sex (male vs. female)	0.81 (0.11 - 6.17)	-0.201	0.840
Diabetes	0.65 (0.034 - 12.3)	-0.288	0.773
BMI	1.82 (1.28 - 2.59)	3.312	0.0012
IL28B860 (CT vs. CC)	3.26 (0.40 - 26.9)	1.098	0.272
IL28B860 (TT vs. CC)	0.83 (0.059 - 11.8)	-0.136	0.892

BMI: Body mass index, OR: Odds ratio of being in the disease group relative to the control, NA: The calculations could not be done because the control had no individuals with the mutant homozygous genotype

LINKAGE DISEQUILIBRIUM BETWEEN IL28B860 AND IL28B275 AND ASSOCIATION BETWEEN HAPLOTYPES AND DISEASE

Since IL28B860 and IL28B275 are located close to each other on the same chromosome, we analyzed their linkage disequilibrium. The measures of disequilibrium were D = 0.12, D' = 0.53, and $r^2 = 0.49$. This means that the two SNPs are inherited together 53% of the time (based on d'), and the allele at one is fairly predictive of the other (based on the correlation r^2). The linkage disequilibrium between the two SNPs was significant, χ^2=42.4 and P value = 7.304×10^{-11}. The haplotype with the highest frequency in the control group was CA, and it was used as the reference, and the odd ratios were calculated relative

to this haplotype. The frequency of the TG haplotype was found to be significantly associated with an increased risk of steatosis, or the combined group of steatosis or NASH. With this haplotype, there was a 2.97-fold increase in the risk of having steatosis and a 2.44-fold increase in the likelihood of having both NASH and steatosis. This haplotype also increased the risk of NASH, but the effect was not significant. The TA haplotype was protective of NASH, but the effect was not significant. Similarly, the haplotype CG was protective for all disease groups, but the effect was not significant (Table 5).

DISCUSSION

NAFLD is a complex metabolic condition. It has been linked to other metabolic illnesses including obesity and T2DM. NAFLD can be best described as a metabolic dysfunction that stems from insulin resistance-induced hepatic lipogenesis. This lipogenesis increases oxidative stress and hepatic inflammation and is often potentiated by genetic and gut microbiome dysfunction. A recent clinical investigation unequivocally demonstrated that environmental factors and genetic background play a significant role in triggering the development of NAFLD (Schwimmer et al. 2009). The prevalence and severity of NAFLD may be affected by SNPs in genes implicated in inflammatory and metabolic balance (Tilg & Moschen 2010). The rs738409 C>G polymorphism of PNPLA3 is linked to more extensive histological injury in patients with biopsy-proven NAFLD and an increased proportion of NAFLD (Sookoian & Pirola 2011).

TABLE 5. Odds ratios for being in the Steatosis, NASH, or Steatosis/NASH groups for patients carrying the indicated haplotypes relative to the most frequent haplotype in the control 'CA'

IL28B-860	IL28B-275	Control	Steatosis			NASH			Steatosis/NASH		
		Count (Prop.)	Count (Prop.)	OR (CI)	P value	Count (Prop.)	OR (CI)	P value	Count (Prop.)	OR (CI)	P value
C	**A**	28.2 (0.487)	24.4 (0.369)	Ref.	-	26.6 (0.476)	Ref.	-	51 (0.418)	Ref.	-
T	**G**	10.2 (0.177)	26.4 (0.400)	2.97 (1.21-7.72)	0.017	18.6 (0.333)	1.94 (0.77-5.13)	0.160	45 (0.369)	2.44 (1.09-5.83)	0.030
T	**A**	10.8 (0.185)	9.6 (0.146)	1.06 (0.37-2.98)	0.911	6.4 (0.114)	0.58 (0.17-1.76)	0.337	16 (0.131)	0.80 (0.32-2.00)	0.627
C	**G**	8.8 (0.151)	5.6 (0.085)	0.79 (0.23-2.55)	0.690	4.4 (0.078)	0.47 (0.11-1.68)	0.254	10 (0.082)	0.61 (0.22-1.74)	0.351

CI: 95% Confidence interval for OR, NASH: Nonalcoholic steatohepatitis, Prop.: Proportion relative to the total in each group, Ref.: Reference haplotype P values from mid-p exact method. the indicated haplotypes relative to the most frequent haplotype in the control 'CA'

In this study, we demonstrated that only the CG genotype of PNPLA3 was associated with an increased risk of NASH, whereas both CG and the mutant GG genotypes increased the risk of Steatosis in patients from Gaza Strip. Yet, these associations are not statistically different. In contrast, Petta et al. (2012) demonstrated that PNPLA3 GG homozygosis in NAFLD patients was independently linked to a more severe degree of steatosis and a NAFLD Activity Score (NAS) ≥ P5, suggesting a diagnosis of NASH. The rs738409 GG genotype of PNPLA3 was shown to be associated with a 73% higher liver fat content compared to the CC genotype as well as a 3.24-fold increased risk of more extreme necro-inflammatory scores and a 3.2-fold increased chance of having fibrosis, according to a separate meta-analysis that included 16 studies (Sookoian & Pirola 2011). Additionally, in a meta-analysis of 23 case-control studies, Xu et al. (2015) discovered a strong connection between the PNPLA3 rs738409 polymorphism and a high cross-ethnicity risk for both NAFLD and NASH. We proposed that discrepancies in the examined population's demographic, epidemiological, and histopathological

characteristics as well as variations in the liver disease's cause may potentially contribute to the observed variances. Also, genetic diversity among racial and ethnic groups could substantially account for the disparity between these findings. Finally, a significant aspect of these differences is the variation in sample size.

In our study, we were able to find a significant association of the mutant T allele at IL28B860 with an increased risk of steatosis (P = 0.04). So, our data are in line with those described by Tillmann et al. (2011), who found a linkage between IL28B rs12979860, serum lipids, and steatosis (Li et al. 2010; Tillmann et al. 2011). Furthermore, we could not find any significant association of CT and mutant TT genotypes of IL28B275 and IL28B860 with increased risk of steatosis and NASH. These data do not agree with some studies which show that steatosis was concordantly found to be associated with the less favorable IL28B genotypes (CT and TT) in patients with hepatitis C, while a relation to inflammation was not reported (Agundez et al. 2012; Clark et al. 2012a, 2012b). In our work, we considered the CC genotype of IL28B860 as a reference against which disease odd ratios were compared because this genotype is the most frequent. It was shown that the severity of the histological signs of liver disease is related to the IL28B rs12979860 CC genotype, independently of HOMA and hyperuricemia, two well-known predisposing factors for hepatic injury in NAFLD (Petta et al. 2012). In addition, Thompson et al. (2010) reported a powerful relation between the genotype CC of IL28B at rs12979860 and liver necro-inflammatory activity in a significant number of Western G1 Chronic hepatitis C patients. Given that IL28 increases the expression of interferon-stimulated genes, including several inflammatory cytokines, it makes sense that inflammation is worse in patients with high IL28 production (Honda et al. 2010).

To the best of our knowledge, we are the first group that has studied the association of NAFLD disease with p85αMet polymorphism. The mutant allele and genotypes containing the mutant allele of p85αMet were associated with lower or no risk of steatosis and a higher risk of NASH in Gaza Strip patients. These associations were not significant. Studies that have examined the connections between p85α polymorphisms and diabetes have resulted in contradictory findings. For example, Hansen et al. (1997) found no association between the Met326Ile polymorphism and the risk of diabetes in Danish Caucasians; however, they discovered that the Ile326Ile genotype was linked to a propensity toward decreased insulin sensitivity in a later analysis of 380 young, healthy individuals. In contrast, Baier et al. (1998) showed that Pima women with the Ile326Ile genotype had a lower incidence of diabetes and greater acute insulin response. There is no evidence to support this polymorphism as a susceptibility factor for T2DM, according to studies in Japanese and Swedish Caucasian populations (Hansen et al. 2001; Kawanishi et al. 1997). In our study, we could also not find any significant association between diabetes mellitus and p85αMet polymorphism. However, it is unclear what mechanism underlies this connection. Whether this variant is in linkage disequilibrium with an undiscovered underlying genetic variant or is the causal variant is still unknown. Additional functional studies will be required to support such hypotheses.

Obesity is an independent risk factor for NAFLD. The obesity-mediated NAFLD risk is caused by increased insulin resistance (IR) and inflammation. The multiplication of M1 macrophages, which secrete pro-inflammatory biomarkers such as IL-6 and TNF-α, is one mechanism by which increasing adipose tissue in the liver causes increased inflammation and IR (Tanase et al. 2020). This study detects a significant association between BMI and steatosis, in which every one-unit increase in BMI was associated with a 53-59% increased risk of steatosis compared to the control group, regardless of the SNP studied. Moreover, we were able to observe a strong linkage between BMI and NASH in our patients. Our results were in agreement with those of Li et al. (2016) who found that obesity increased the risk of NAFLD by 3.5 fold. It is worth noting that in spite of the close association between NAFLD and obesity, it can occur in lean patients as well. About 10-20% of non-obese Americans have NAFLD, and between 7-18% of the lean patients in Asia may have NAFLD, and in Japan, 15.2% of the non-obese patients developed NAFLD (Younes & Bugianesi 2019). Increased lipolysis overcomes the body's ability to store lipids subcutaneously, resulting in a free fatty acid buildup in visceral parts of the body, including the liver, which is the clear explanation for the metabolic development of 'lean' NAFLD (Ficarella, Laviola & Giorgino 2015).

Conclusions

In conclusion, the rising frequency of NAFLD and its links to a variety of illnesses present serious concerns for clinicians today. Genetic factors are known to influence NAFLD. Many SNPs affect the pathophysiology in patients with chronic liver disease. Many studies

have highlighted the role of genetic variables in the development of steatosis, fibrosis, cirrhosis, and HCC in NAFLD over the previous years. We found that only the mutant T allele of IL28B860 was significantly associated with an increased risk of steatosis. The other studied alleles and genotypes were not significantly associated with increased or decreased risk of steatosis, NASH, or combined steatosis or NASH groups.

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TABLE S1. The target genes, the Primers' sequences and the annealing temperatures used for PCR

No.	Gene target	Primers' Sequences	Annealing temp.	Final Conc. (μM)	Reference
1	PNPLA3	F: 5'-TGG GCC TGA AGT CCG AGG GT-3' R: 5'-CCG ACA CCA GTG CCC TGC AG-3'	66	1	(Dutta 2011)
2	PPARγ	F: 5'-GCC AAT TCA AGC CCA GTC-3' R: 5'-GAT ATG TTT GCA GAC AGT GTA TC-3'	56	1	(Engwa et al. 2018)
3	p85αMet	F: 5'-TAG CCA ACA ACG GTA TGA ATA ACC A-3′ R: 5′-ATC CAG CAC CCA CAG TTC TC-3′	58	0.25	(Chen et al. 2005)
4	p85αIVS	F: 5′-TAG ATA CAC CCT CCG TGG ACT TG-3′ R: 5′-GGG ACA GGT TAT TAC TAC TAT TTT CTA ACC AGT A-3′	60	0.25	(Chen et al. 2005)
5	IL28B860	F: 5′-AGG GCC CCT AAC CTC TGC ACA GTC T-3′ R: 5′-GCT GAG GGA CCG CTA CGT AAG TCA CC-3′	58	0.5	(Rüstemoğlu et al. 2016)
6	IL28B275	F:5′-GAG AGC AAG AGG AGG GAA GGA A-3′ R: 5′-GTG TGC CAT TAG CCA GTC AGA T-3′	58	0.5	(Rüstemoğlu et al. 2016)

TABLE S2. Details of the target genes, SNPs, PCR product size, restriction enzymes used and digestion products

No.	Target gene	SNP	Product (bp)	RE	Digestion product size (bp)
1	PNPLA3	rs738409, C>G	333	BtsCI	N Homo (CC): 200, 133 M Heter (CG): 333, 200, 133 M Homo (GG): 333
2	PPARγ	Pro12Ala, C>G	267	Bst UI	N Homo (Pro/Pro): 270 M Heter (Pro/Ala): 270, 227, 43 M Homo (Ala/Ala): 227, 43
3	p85αMet	Met326Ile, G>A	249	NdeI	N Homo (Met/Met): 220, 25 M Heter (Met/Ile): 245, 220, 25 M Homo (Ile/Ile): 245
4	p85αIVS	IVS4+82, A>G	255	RsaI	N Homo (AA): 251 M Heter (AG): 251, 171, 80 M Homo (GG): 171, 80
5	IL28B860	rs12979860, C>T	403	BstUI	M Homo (TT): 184, 130, 89 M Heter (CT): 184, 130, 105, 89, 25 N Homo (CC): 184, 105, 89, 25
6	IL28B275	rs12980275, G>A	441	BslI	Homo (AA): 320, 121 Heter (AG): 320, 290, 121, 30 Homo (GG): 290, 121, 30

Bp: base pair, RE: Restriction enzyme, SNP: Single nucleotide polymorphism