Hasan et al. Egyptian Liver Journal (2021) 11:37
https://doi.org/10.1186/s43066-021-00103-w

Egyptian Liver Journal

ORIGINAL RESEARCH ARTICLE Open Access

The association of adiponectin gene polymorphisms with susceptibility and progression of NAFLD in a cohort of Egyptian patients

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Abstract

Background: Several genetic polymorphisms have been proven to play a key role in the progression of non-alcoholic fatty liver disease (NAFLD) from simple steatosis to NASH with fibrosis. Our aim was to study the effect of single nucleotide polymorphisms (SNPs) in the adiponectin gene, namely rs266729 and rs3774261, on susceptibility to NAFLD and disease progression.

Results: There was a definitive association between polymorphisms of the studied SNPs and NAFLD. Among rs266729, CG was significantly higher among patients than controls showing increased risk for NAFLD ($P<0.05$). AA genotype of the rs3774261 variant was significantly lower in patients than in controls (P value< 0.001) while AG and GG genotypes were significantly higher in patients than in controls (P value<0.05); A allele was significantly higher among controls ($P=0.019$) which might have a protective effect. None of the variants correlated significantly with the degree of steatosis. Using multivariate regression analysis, there was no significant correlation with any of the independent risk factors to the degree of steatosis.

Conclusions: There was an association between polymorphisms of the studied SNPs of rs266729 and rs3774261 of the adiponectin gene and NAFLD.

Keywords: NAFLD, NASH, SNPs, Adiponectin gene, CAP

Background

Non-alcoholic fatty liver disease (NAFLD) is rapidly becoming the most common liver disease worldwide [1]. The prognosis and outcome of these patients are mainly determined by the liver fibrosis severity. Accordingly, precise differentiation between NASH and NAFL so as to define the best treatment is needed [2]. Excess visceral fat and waist circumference are considered the main risk factors for NAFLD and are closely related to disease severity [3].

Considering that liver biopsy is the diagnostic gold standard of NAFLD, however, it is an invasive procedure with potential risks or complications. Thus, different non-invasive diagnostic tests have been suggested as alternatives to liver biopsy [4]. Among these tests, the controlled attenuation parameter (CAP) has been developed to assess liver steatosis. CAP is measured using the FibroScan (Echosens®, Paris, France), which is based on vibration-controlled transient elastography (VCTE), a technique initially developed to assess liver stiffness (LS), which is highly correlated with liver fibrosis [5].

The study of genetic factors in NAFLD is a rapidly growing field [6]. Salman et al. and Jamali et al. have demonstrated the association of NAFLD and its severity

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with the levels of serum adipokines, a group of bioactive proteins secreted by adipose tissue and having actions on inflammation and insulin resistance [7, 8]. Several studies have indicated that adiponectin is protective against NAFLD [9]. The aim of this study was to investigate the association between two SNPs of adiponectin gene, namely rs266729 and rs3774261, and NAFLD as well as the impact of multiple SNP interaction on NAFL D risk based on an Egyptian population study, and its relation to the degree of hepatic steatosis as assessed by controlled attenuation parameter.

Methods

The present study included 86 Egyptian patients, prospectively selected from the hepatology outpatient clinic of Kasr Al-Ainy Hospital over a period of 12 months (from February 2017 to February 2018), who were suspected to have NAFLD according to the following inclusion and exclusion criteria, in addition to 86 age-matched healthy subjects serving as a control group. The patients were further subdivided into (1) the biopsy group (17 patients) and (2) the non-biopsy group (69 patients). All participants aged above 18 years, and all patients were suspected to have NAFLD on the basis of increased hepatic brightness by abdominal ultrasound with the exclusion of other causes of secondary steatosis such as negative viral markers (HCV Ab, HBsAg, HBc total), negative ANA, transferrin saturation <45% coupled with normal ferritin levels, normal serum levels of ceruloplasmin, and negative history for significant alcohol consumption (>20 g/d for females and 30 g/d for males) and for use of medications that can cause fatty liver.

All participants were interviewed for their medical history, and their residence was classified into urban or rural areas based on the infrastructure and the available services [10]. The BMI was calculated as body weight in kilograms divided by height in square meters (kg/m^2). Zheng et al. evaluated waist to hip ratio (WHR) as a predictor of NAFLD with a cutoff point of 0.89 [11], whereas in the present study, we applied the waist circumference that was measured midway between the last rib margin and the iliac crest in a standing position. In order to diagnose metabolic syndrome [12], blood samples were obtained from each participant after a fasting period of at least 12–14 h for lipid profile. The blood glucose level was measured using the glucose oxidase method. A fasting blood sugar level from 100 to 125 mg/dl is considered impaired fasting glucose and 126 mg/dl or higher is considered diabetes [12]. Serum total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride levels were measured on an auto-analyzer using enzymatic calorimetry, and hypertriglyceridemia is considered when triglycerides ≥150 mg/dL [12]. Serum levels of ALT, AST, GGT, alkaline phosphatase, and albumin, as well as serum ferritin and ceruloplasmin, were measured. HBsAg, HBc total, HCV Ab, ANA were measured using ELISA. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) was applied to detect the distribution of genetic polymorphisms of the adiponectin gene. Transabdominal ultrasonography used a Toshiba SSA-340A machine with a 3.5-MHz convex linear transducer. Ultrasonography-guided liver biopsy was done for only 17 of the patients (the biopsy group), and scoring used the NAS scoring system according to the Histological Scoring System for NAFLD and used Masson trichrome stain for assessing the degree of fibrosis [13]. Fibrosis staging was evaluated (separately from NASH) from 0 to 4 scales. The hepatic steatosis was graded from 0 to 3 based on the number of hepatocytes with steatosis in the biopsy: S0, <5%; S1, 5–33%; S2, 34–66%; and S3, >66% [14]. Controlled attenuated parameter (CAP) was performed for all patients and controls using the 3.5-MHz M probe of Echosens FibroScan®. Liver stiffness measurement (LSM) was performed for all patients using Echosens FibroScan

Measurement of LSM and CAP

The LSM was expressed as kPa, and the examination was considered reliable if 10 valid LSMs were obtained, the success rate exceeded 60%, and the ratio of the interquartile range (IQR) to the median of 10 measurements (IQR/M) was 0.3.

CAP measures the ultrasound attenuation (go and return path) using signals acquired by either the 3.5-MHz or 2.5-MHz FibroScan probe. 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 and histological data.

Detection of adiponectin gene polymorphisms

Blood samples collection

Five milliliters of whole blood sample was collected from each patient. The whole blood was subjected to DNA extraction and followed by further assessment of adiponectin polymorphisms rs266729 and rs3774261 by restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR).

RFLP-PCR procedure for adiponectin polymorphism diagnosis

DNA extraction

All samples were in the form of frozen whole blood and were processed using the Zymoresearch Quick-gDNA™ MiniPrep kit, Catalog No. D3024 (available online at

http://www.zymoresearch.com/downloads/dl/file/id/18/d3006i.pdf), through Epigenetics Company.

The following steps were applied on all blood samples. (1) For the preparation of the Genomic Lysis Buffer, 250 μl of beta-mercaptoethanol was added to 50 ml of the buffer. (2) One hundred microliters of blood was added to 400 μl of Genomic Lysis Buffer. (3) They were mixed together completely by vortexing for 4–6 s. (4) The mixture was let stand 5–10 min at room temperature. (5) The mixture was transferred to a Zymo-Spin™ Column in a collection tube and centrifuged at 10,000×*g* for 1 min, and the collection tube was discarded with the flow-through. (6) The Zymo-Spin™ Column was transferred to a new collection tube, and 200 μl of DNA Pre-Wash Buffer was added to the spin column and centrifuged at 10,000 x g for 1 min. 7. 500 μl of g-DNA Wash Buffer was added to the spin column and then centrifuged at 10,000×*g* for 1 min. (8) The spin column was transferred to a clean microcentrifuge tube, then 60 μl of DNA Elution Buffer was added to the spin column. (9) It was incubated 2–5 min at room temperature and then centrifuged at top speed for 30 s to elute the DNA. (10) The eluted DNA was stored at −20°C for future use.

Real-time PCR

This step involved DNA amplification and detection of the studied SNPs using Real MODTM Real-time PCR Master Mix (Intron Biotechnology, Catalog no. 25341; available online at http://eshop.intronbio.com/product/detail04.asp?pIdx=89#).

Quantification of DNA

The concentration of DNA was determined by measuring the absorbance at 260 nm (A260) using the Nanodrop ND-1000* spectrophotometer. Distilled water was used to zero the spectrophotometer. The concentration is displayed in ng/μl. The recommended DNA template concentration for TaqMan SNP Genotyping Assay is 1–20 ng/μl. During the present assay, 20 ng was the goal, and the volume was adjusted accordingly.

Steps

The reaction mixture was prepared according to the following: 10 μl for 2x RealMODTM Real-time PCR Master Mix Solution, 2 μl for primers (forward+ reverse) + TaqMan® probe, 1 μl for template, and DNA 7 μl for RNAse-free water. The mixture was mixed thoroughly and appropriate volumes were aliquoted into the real-time PCR plates. The real-time PCR instrument (Applied Biosystems (StepOne System. SDS software v2.1 and RQ Manager 1.2)) was programmed. The real-time PCR plate was placed in the real-time PCR instrument, and the cycling program was started. After the reaction has been completed, the amplification curve was verified.

Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 23. The data was summarized using median, minimum and maximum in quantitative data, and frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests [15].

For comparing the categorical data, the chi-square test was performed. The exact test was used instead when the expected frequency is less than 5. Genotype and allele frequencies were compared between every 2 groups using chi-square tests [16].

Odds ratio (OR) with 95% confidence intervals was calculated. Multivariate logistic regression analysis was done to detect the independent predictors of NAFLD. *P* values less than 0.05 were considered as statistically significant.

Tests of normality of distribution of genetics (Hardy Winderberg) in controls (86 samples) were performed. The likelihoods of calculated X^2 value are in Table 1. For the rs266729, SNP X^2 = 2.6, p allele frequency = 0.732, and q allele frequency = 0.267. For the rs3774261, SNP X^2 = 43, p allele frequency = 0.674, and q allele frequency = 0.326.

Results

The age of the studied patients ranged from 25 to 67years (44.3±9.45 years), out of which 65.2% were females and 34.8% were males. BMI ranged from 23.3 to 50.3 kg/m^2(33.76±5.436kg/m^2), whereas the waist circumference (WC) was about 107.3±11.66cm. On the other hand, among the control group, ages ranged from 20 to 63 years (38.67±8.854years), 33.7% were females and 66.3% were males, BMI was 25.65±1.623kg/m^2, and WC was 81.38±5.363cm.

According to the BMI, patients and controls were categorized into normal (18.5–24.9kg/m^2), overweight (25.0–29.9 kg/m^2), obese (30.0–40.0 kg/m^2), and

Table 1 Likelihood of calculated X^2 value for test of normality of distribution of genetics in controls

		Expected	Observed
rs266729	CC	46.1	44
	CG	33.6	38
	GG	6.1	4
rs3774261	AA	78.14	39
	AG	75.59	38
	GG	18.28	9

morbidly obese (>40.0 kg/m^2). Among patients, 17 (19.8%) were overweight, 57 (66.3%) were obese, and 10 (11.6%) were morbidly obese, whereas among controls, 32 (37.2%) were within normal BMI, 53 (62.6%) were overweight, and 1 (1.2%) was obese, while none was morbidly obese.

BMI distribution was determined in patients according to their age as follows: in young adults (25–30 years), 33.3% were overweight, 33.3% were obese, and 33.3% were morbidly obese; in adults (31–40 years), 0% were overweight, 25.9% were obese, and 74.1% were morbidly obese; in middle-aged patients (40–50 years), 0% were overweight, 17.2% were obese, and 58.7% were morbidly obese; in the older age (50-60 years and 60–66 years) patients, none were overweight, 13.6% and 0% were obese, and 72.5% and 100% were morbidly obese, respectively.

Among patients and controls, 47.7% and 24.4% were housewives, 2.3% and 3.5% were unemployed, while 14% and 46.5% had labor-intensive occupations (workers, mechanics, and farmers), respectively. The rest occupied jobs with a more clerical nature. According to the governorate of residence, the largest number of patients and controls were living in urban areas accounting for 74.4% and 81.4% among patients and controls, respectively

Among known risk factors for the development of NAFLD, increased BMI was found in almost all except two patients (2.3%) with NAFLD, 19.8% were overweight, 66.3% were obese, and 11.6% were morbidly obese, whereas in the control group, 37.2% were normal, 61.6% were overweight, only one subject (1.2%) was obese, and none were morbidly obese (P<0.01).

As regards the presence of diabetes mellitus among patients versus controls, 27.9% had DM, as opposed to 2.3% of the controls (P<0.01), whereas systemic hypertension was found in 15.1% in the patient group and in 0% (none) of the control group. None of the control subjects had a family history of NAFLD; however, 25.6% of the patient group had a family history of NAFLD in at least one first-degree relative.

Metabolic syndrome is defined by the presence of three of the following five criteria: fasting glucose ≥100 mg/dL (or receiving drug therapy for hyperglycemia), blood pressure ≥130/85 mmHg (or receiving drug therapy for hypertension), triglycerides ≥150 mg/dL (or receiving drug therapy for hypertriglyceridemia), HDL-C < 40 mg/dL in men or < 50 mg/dL in women (or receiving drug therapy for reduced HDL-C), and waist circumference ≥102 cm in men or ≥88 cm in women. Metabolic syndrome was identifiable in 19.8% as opposed to 0% of the controls (P<0.01).

Smoking was present among 13.9% and 11.6% of patients and controls, respectively, whereas 1.16% (1 patient) was identified as an ex-smoker. None of the patients or the controls gave a history of heavy alcohol consumption or of drug abuse.

Serum lipid abnormalities in the form of elevated total cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-c) levels, and reduced serum high-density lipoprotein cholesterol (HDL-c) levels were significantly detected in the patient group as opposed to the control group (P< 0.0001). Moreover, serum fasting and post-prandial blood sugar were significantly higher in the patient group as opposed to the control group (P < 0.0001). Hemoglobin A1C results, however, showed no significant difference among patients versus controls (P= 0.85).

None of the patients had abnormal thyroid function profiles; however, ten gave a history of hypothyroidism for which they receive ongoing treatment in the form of L-thyroxin tablets. None of the patients had abnormal serum ferritin levels.

Among patients, the most common complaints were right upper quadrant pain, occurring in 41 patients (47.7%), and abdominal discomfort, occurring in 21 patients (24.4%), followed by fatigue in 20 patients (23.3%), and lower limb edema and history of IHD in 2 patients (2.3%) and 3 patients (3.5%), respectively. Among the control group, no complaints were reported.

By physical examination, mild hepatomegaly was detected in 6 patients (7%), out of which 3 had tender hepatomegaly (3.5%). In all of these patients, the liver was felt soft in consistency, with a round edge. Only one of the patients had a palpable spleen (1.2%). Otherwise, physical examination among both patients and controls was unremarkable. None of the patients or the controls showed signs suggestive of liver cell failure in the form of jaundice, ascites associated with lower limb edema, or disturbed level of consciousness.

As regards laboratory data of the studied groups, serum transaminases, were found to be higher in the patients' group as opposed to the control group (P< 0.0001). Noteworthy, 48.8% (42/86) of the patients had serum hepatic transaminases below the upper limit of normal.

Using trans-abdominal ultrasonography in assessing the hepatic echogenicity, control subjects showed normal hepatic echogenicity. Whereas liver brightness was graded into grades I, II, and III among patients as follows: grade I brightness was diagnosed in 18 patients (20.9%), grade II brightness was diagnosed in 37 patients (43.0%), and grade III brightness was diagnosed in 31 patients (36.1%).

Among the 17 patients who underwent liver biopsy, according to histopathological findings (degree of steatosis, degree of inflammation, presence of ballooning) and NAS score, NAFLD was classified into 2 subgroups (simple steatosis and NASH) with their histopathological features using the NAS Score, with a cutoff value of ≥5, 4 patients were identified as having NAFL, whereas 13 had biopsy-proven steatohepatitis (Table 2).

No significant relation was found between grades of steatosis by liver biopsy and presence or absence of NASH by (NAS) score where between 13 biopsy-proven NASH patients, 5 (38.5%) and 8 (61.5%) had histological evidence of moderate and marked hepatic steatosis. On the other hand, among 4 patients with simple steatosis, 2 (50%) had mild steatosis, and 2 (50%) had moderate steatosis.

As regards the level of steatosis detected by CAP and biopsy results, among the biopsy group, two patients (11.8%) who had biopsy-proven mild steatosis (S1), when further assessed by controlled attenuation parameter, showed marked steatosis (S3) (16.6%). Out of the 7 patients (41.2%) with biopsy-proven moderate (S2), 6 (85.7%) showed S2, and only one (14.3%) showed S3 upon further assessment by CAP, and out of the 8 (47.0%) who biopsy-proven severe (S3) steatosis, 7 (87.5%) showed S3, and 1 (12.5%) S2 when further assessed by CAP (Table 3).

Upon further evaluation of the biopsy group, the degree of fibrosis detected by biopsy was not significantly related to the fibrosis stage (F0–4) detected by FibroScan (Echosenes®). There was no significant relation between the degree of steatosis detected by CAP and the biopsy-proven fibrosis stage (Table 4).

Among all studied patient groups, the degree of steatosis when measured using CAP was mild (S1) in 19.8% (17 patients), moderate (S2) in 39.5% (34 patients), and severe in 40.7% (35 patients), whereas the degree of fibrosis was mild (F0, F1) in 80.2% (61 patients with F0, 8 patients F1), moderate (F2) in 10.5% (9 patients), and marked (F3–4) in 7% (6 patients) and 2.3% (2 patients) using FibroScan (Echosens®), respectively.

The degree of steatosis by CAP when compared to the grades of brightness by trans-abdominal US, we found that the different grades of brightness by trans-abdominal ultrasonography showed significant relation with the degree of steatosis measured utilizing CAP with a *P* value <0.001.

As regards the relation between the degree of steatosis detected by CAP and different clinical parameters, the degree of moderate and advanced steatosis was higher among females than among males in advanced steatosis (68.6% vs 31.4% and 68.8% vs. 31.4%, respectively), which was not significant statistically (*P*=0.318). WC and BMI significantly related with the different degrees of steatosis measured by CAP. The degree of steatosis detected by CAP was associated with higher serum TGs, but it was not a significant relation (Table 5).

The distribution of rs266729 adiponectin C and G allele genotype among patients and controls showed that CG genotype was significantly related to the presence of NAFLD (*P*=0.032), while CC genotype was significantly related to controls (*P*=0.003), whereas rs3774261 adiponectin A and G alleles showed that AG genotype was the most frequent genotype in patients with NAFLD/963.9%, while AA genotype was the most frequent genotype in controls (45.3%) (Table 6).

No significant relation was found between any of the variants of both genotypes and the degrees of both steatosis and fibrosis detected by CAP and FibroScan Echosens respectively, nor with any of the significant clinical and lab parameters (Table 7).

No significant correlation was found between ALT and AST relative to BMI or waist circumference (Table 8).

By studying the adiponectin genotype, CAP, and different metabolic parameters in relation to obesity and its degree, we found that the severity of steatosis, BMI, waist circumference, fasting blood glucose, and AST was significantly higher among obese and related to the degree of obesity at *P*<0.0001 (Table 9).

The relationship between the studied SNPs showed no statistically significant relation between rs266729 and rs3774261. Multivariate regression analysis showed no significant relation between the degree of steatosis detected by CAP and any of the independent risk factors including BMI, waist circumference, TGs, sex, rs266729, and rs3774261.

Table 3 The relation between biopsy-proven steatosis and CAP

		Biopsy proven steatosis						*P* value
		S1		S2		S3		
		Count	%	Count	Count	%	Count	
CAP (S0–S3)	S0	0	0	0	0	0	0	*<0.001*
	S1	0	0	0	0	0	0	
	S2	0	0	6	85.7	1	12.5	
	S3	2	100	1	14.29	7	87.5	

Discussion

Previous studies have shown the association of NAFLD and its severity with the levels of serum adipokines, considering adiponectin a protective protein against NAFLD [7–9]. Moreover, the recent genetic study of steatosis showed associations between single-nucleotide polymorphisms (SNPs) and disease phenotypes, which could be helpful in the management and prognosis of patients with NAFLD [17].

The aim of this study was to investigate the association between two SNPs of adiponectin genes rs266729 and rs3774261, and NAFLD as well as the impact of multiple SNP interaction on NAFLD risk based on an Egyptian population study and its relation to the degree of hepatic steatosis as assessed by controlled attenuation parameter.

In our study, gender distribution showed a female predominance (65.2%). Similar findings were found by [18, 19]. While many studies have indicated that NAFLD is more common in men [20, 21], in our study, the higher

Table 2 Liver biopsy findings among the biopsy group of patients

		NASH		Simple steatosis		*P* value
		%	Count	%	Count	
Steatosis	No grade 0	*0*	*.0*	*0*	*.0*	*0.5751*
	Mild grade 1	*0*	*.0*	*2*	*50*	
	Moderate grade 2	*5*	*38.5*	*2*	*50*	
	Marked grade 3	*8*	*61.5*	*0*	*.0*	
Inflammation	Mild	*4*	*30.8*	*4*	*100.0*	*0.9296*
	Moderate	*9*	*69.2*	*0*	*.0*	
Ballooning	Focal	0	*.0*	*1*	*25*	*0.074*
	Diffuse	13	*100*	*3*	*75*	
Fibrosis	Minimal	0	*4.5*	*0*	*.0*	*0.152*
	Mild	4	*30.8*	*2*	*50*	
	Moderate	5	*38.4*	*2*	*50*	
	Severe	4	*30.8*	*0*	*.0*	

prevalence among females could be attributed to the fact that the prevalence of central obesity is higher among Egyptian females than males [22, 23].

As regards age, the prevalence of NAFLD was significantly increased with age (P <0.0001). The degree of steatosis detected by CAP was higher among older patients, although it did not show a statistical significance. These results were supported by previous studies [1].

Obesity is closely associated with the increased prevalence and severity of NAFLD. Obesity induced steatosis causing insulin resistance by activating NF-kB that induces proinflammatory cytokines resulting in insulin resistance [24]. The body mass index and waist circumference in our study were proved to be significantly higher in NAFLD cases than in normal controls. Moreover, the degree of obesity significantly related to the presence of hepatic steatosis. These findings were compatible with the findings of previous studies [25]. Other studies, however, concluded that NAFLD can occur in non-obese subjects who are physically inactive [26].

In this study, the prevalence of diabetes mellitus was significantly higher in NAFLD patients (27.9%) compared to normal controls (2.3%). This finding was in agreement with previous studies, which concluded that diabetes mellitus is an independent risk factor for the progression of NAFLD [1, 25].

As regards metabolic syndrome, in the present study, the prevalence of MS was significantly higher among NAFLD patients (19.8%) compared to the control group (0.0%) at a P value <0.001. Previous studies reached similar conclusions, whereas NAFLD is presently considered the hepatic manifestation of MS [1, 27].

The present study validated the documented facts whereas elevated serum total cholesterol, LDL-c, and triglyceride levels, as well as reduced HDL-c levels, were found to be significantly related to the presence of NAFLD ($P \leq 0.001$). Furthermore, higher serum levels of triglycerides and LDL-c were shown to have a relation with the different degrees of steatosis detected by CAP, but it was not a significant relation (P=0.088 and 0.79, respectively).

Table 4 Relation between biopsy-proven fibrosis stage and TE and CAP results

		Fibrosis stage								*P* value
		4/4		¾		2/4		¼		
		Count	%	Count	%	Count	%	Count	%	
FibroScan (Echosens)	**F0**	*3*	*50.0*	*0*	*.0*	*0*	*.0*	*0*	*.0*	***0.5884***
	F1	*3*	*50.0*	*1*	*14.3*	*0*	*.0*	*0*	*.0*	
	F3	*0*	*.0*	*6*	*85.7*	*2*	*100*	*0*	*.0*	
	F4	*0*	*.0*	*0*	*.0*	*0*	*.0*	*2*	*100*	
CAP S(0–3)	**S0**	0	*.0*	*0*	*.0*	*0*	*.0*	*0*	*.0*	***0.6751***
	S1	0	*.0*	*5*	*71.4*	*0*	*.0*	*0*	*.0*	
	S2	1	*16.7*	*2*	*28.6*	*1*	*50.0*	*0*	*.0*	
	S3	5	*83.3*	*0*	*.0*	*1*	*50.0*	*2*	*100.0*	

Table 5 Relation between degree of steatosis detected by CAP and various clinical and laboratory parameters

	S1		S2		S3		*P* value
	Mean	SD	Mean	SD	Mean	SD	
Age	39.75	7.452	45.63	9.353	45.06	9.956	0.1004
Waist circumference	100.6	11.69	107.8	11.29	109.9	11.13	0.0282*
BMI	30.55	4.839	33.92	4.939	35.07	5.701	0.0200*
Blood pressure	118-72	8.6	121-7	12.10	119-74	12.11	0.5
Fasting glucose	93	22	92	19	104	36	0.16
ALT	39.43	26.22	48.06	36.16	38.71	24.28	0.5445
HDL	41.69	9.192	52.59	36.26	44.19	13.28	0.5974
LDL	127.3	56.9	130	56.21	125.1	48.78	0.9488
TGs	154.3	88.79	168.4	101.1	205.7	103.8	0.1538

Fasting blood glucose levels were found to be significantly higher in our NAFLD cases ($P< 0.001$). Recent studies also concluded that impaired glucose tolerance is an independent risk factor for the development of NAFL D and its progression to NASH [28].

ALT and AST are two of the most reliable markers of hepatocellular injury or necrosis. Previous studies concluded that serum ALT level alone is not predictive of NASH or fibrosis level as ALT can be increased in up to 53% of NAFLD patients without NASH [29, 30]. In the present study, both serum ALT and AST levels were found to be significantly higher among NAFLD patients versus normal controls ($P \leq 0.0001$). However, 48.8% of our patients had serum hepatic transaminases below the upper limit of normal.

In the present study, 17 patients for whom liver biopsy was performed were further divided by NAS score into two groups, the NASH group (13 patients) and the simple steatosis group (4 patients). No significant relation was found between grades of steatosis and presence or absence of NASH; 38.5% and 61.5% of the NASH group had histological evidence of moderate and marked hepatic steatosis, respectively. These results matched with those of previous studies, which identified the degree of steatosis as an independent risk factor for the progression of simple steatosis to NASH [31]. The lack of a statistical significance in our study could be explained by the small number of patients in the biopsy group.

When comparing the results obtained from liver biopsy to CAP in the biopsy group, the degree of steatosis appeared to be the only histological parameter with significant relation. CAP does not seem to be influenced by fibrosis or inflammation which was consistent with the results from previous studies showing that ultrasound attenuation was similar in healthy and cirrhotic patients and much lower than in fatty liver patients [32].

On the other hand, the degree of fibrosis detected histologically in the biopsy group did not appear to be significantly related to that measured using elastography techniques, with comparable results using both Echosens® FibroScan and ARFI. However, this could be explained by the insufficiently small number of patients in the biopsy group. Moreover, the discrepancy appeared among early stages of fibrosis, but not in the more advanced F3 and F4 patients. Previous

Table 6 Distribution of adiponectin rs266729 and rs3774261 genotypes among NAFLD patients and controls

Adiponectin variant		Patients (86)		Controls (86)		OR (95%CI)	*P* value
		%	Count	%	Count		
rs266729	GG	*5*	*5.8*	*4*	*4.6*	0.732	1.265 (0.362 to 4.239)
	CG	*53*	*61.7*	*38*	*44.2*	0.032*	2.029 (1.097 to 3.665)
	CC	*28*	*32.5*	*44*	*51.2*	0.003*	0.382 (0.205 to 0.719)
	Allele G	*63*	*36.6*	*46*	*26.7*	0.0635	1.58 (1 to 2.47)
	Allele C	*109*	*63.4*	*126*	*73.3*	0.0635	0.632 (0.404 to 1)
rs3774261	AA	11	12.80%	39	45.30%	*< 0.001*	0.177 (0.0815 to 0.373)
	AG	55	63.90%	38	44.20%	0.0093*	2.24 (1.21 to 4.09)
	GG	20	23.30%	9	10.50%	0.0404*	2.59 (1.09 to 6.42)
	Allele A	77	44.80%	116	67.40%	*< 0.001*	0.391 (0.253 to 0.608)
	Allele G	95	55.20%	56	32.60%	*< 0.001*	2.56 (1.64 to 3.95)

Table 7 Adiponectin rs266729 CG and rs3774261 AG genotypes in relation to the degree of steatosis by CAP, degree of fibrosis by TE, waist circumference, BMI, TGs, and ALT

	rs266729 CG			rs3774261 AG		
	Yes (53), *N* (%)	No (33), *N* (%)	*P* value	Yes (55), *N* (%)	No (31), *N* (%)	*P* value
Steatosis						
S1 (16)	8 (15.1)	8 (24.2)	0.434	2 (10)	14 (21.21)	
S2 (35)	24 (45.3)	11 (33.3)		12 (60)	23 (34.85)	
S3 (35)	21 (39.6)	14 (42.5)		6 (30)	29 (43.9)	
FibroScan						
F0 (61)	36 (67.9)	25 (75.8)	0.469	14 (70)	47 (71.2)	0.9144
F1 (8)	*7* (13.2)	1 (3)		2 (10)	6 (9.1)	
F (9)	6 (11.3)	3 (9.1)		2 (10)	7 (10.6)	
F3 (6)	2 (3.8)	3 (9.1)		2 (10)	4 (6.1)	
F4 (2)	2 (3.8)	1 (3)		0	2 (3)	
	Mean±SD	**Mean±SD**		**Mean±SD**	**Mean±SD**	
Waist circumference	108.1±12.14	106±10.9	0.4171	106.3±12.24	107.6±11.5	0.6635
BMI	34.33±5.8	32.85±4.7	0.2237	32.68±4.16	34.09±5.7	0.3141
Blood pressure	119 ±13 73±11	120±12 73± 9.4	0.71	120±12 73 ± 10	120 ±13 72 ±10	0.78
Fasting glucose	94 ± 20	103 ± 12	0.13	100 ±32	100 ± 18	0.2
TGs	181±102.9	180.9±99.9	0.9957	171.7±95.5	183.8±103.4	0.6433
ALT	42.85±31.92	42.33±27.1	0.9381	47.4±41.5	41.21±25.76	0.4222

studies have shown that the specificity and sensitivity of FibroScan® were highest at ≥F4 [33]. Other studies have validated TE as a non-invasive mean with high diagnostic accuracy in both identifying and staging of fibrosis in NAFLD patients [34].

In comparison with the gold standard biopsy, CAP can be used for steatosis detection and semi-quantification as it offers several advantages over it, being easy to perform, non-invasive, and inexpensive with immediate results, and has less sampling error [32].

Trans-abdominal ultrasonography is considered an inexpensive non-invasive easy technique for the initial screening of NAFLD with acceptable sensitivity and specificity [35, 36]. CAP had the benefits of being non-operator-dependent and efficient in the quantification of hepatic steatosis from 10% and up, and simultaneous assessment of liver fibrosis makes the CAP an alternative method for assessing hepatic steatosis [32]. In the present study, the different grades of brightness detected by trans-abdominal ultrasonography showed a significant correlation with the degree of steatosis measured utilizing CAP (P<0.001).

As regards age and gender, the degree of steatosis detected by CAP showed no significant statistical difference between males and females in our study group and between different age groups. These findings were contradictory with previous studies [37]; the reason could be due to the higher prevalence of females among our study group and that our study group was not representative of all age groups owing to the relatively small number of our study patients.

The gene coding for adiponectin is located on chromosome 3q27 and consists of three exons and two introns, spanning a total of 16 kb of the genomic sequence. Growing evidence demonstrates the association of single nucleotide polymorphisms (SNPs) of the adiponectin gene with varying levels of circulating adiponectin [9].

SNPs of rs266729 (−11377 C>G) in the proximal promoter of the adiponectin gene have been widely studied by epidemiological studies. Variant alleles at rs266729, which is associated with lower adiponectin levels, have been shown to be related to obesity, type 2 diabetes (T2DM), and insulin resistance [38, 39].

Table 8 Correlation between liver transaminases and BMI and waist circumference

	BMI, *P* value	Correlation coef. (*r*)	Waist circumference, *P* value	Correlation coef. (*r*)
ALT	0.38	0.095	0.086	0.18
AST	0.42	0.088	0.06	0.2

Table 9 Adiponectin genotype and polymorphism, CAP, and metabolic parameters in relation to the degree of obesity

	Obese (67), *N* (%)	Non obese (19), *N* (%)	*P* value	Mild (30-34.9), N=38, *N* (%)	Moderate (35–39.9), *N*= 19, *N* (%)	Morbid (>40), *N*=10, *N* (%)	*P* value
rs266729 CG							
Yes	44 (65.67)	9 (47.37)	0.18	25 (65.79)	13 (68.42)	4 (40)	0.9
No	23 (34.33)	10 (52.63)		13 (34.2)	6 (31.58)	6 (60)	
rs3774261 AG							
Yes	44 (65.67)	11 (57.9)	0.59	24 (63.16)	14 (73.68)	6 (60)	0.67
No	23 (34.33)	8 (42.1)		14 (36.84)	5 (26.32)	4 (40)	
Steatosis							
S1	8 (11.9)	8 (42.1)	0.011	5 (13.16)	3 (15.79)	0 (0)	0.43
S2	29 (43.3)	6 (31.6)		19 (50)	6 (31.58)	4 (40)	
S3	30 (44.8)	5 (26.3)		14 (36.84)	10 (52.63)	6 (60)	
	Mean±SD	**Mean±SD**		**Mean±SD**	**Mean±SD**	**Mean±SD**	
Waist circumference	111 ±10	96 ±8	< 0.0001	105 ±7.5	114 ± 8.2	125 ±4.6	< 0.0001
BMI	36 ±4.7	27 ±1.9	< 0.0001	32 ±1.5	37 ±1.4	45 ±3.6	< 0.0001
ALT	44 ± 33	38 ±19	0.97	38 ±33 9–180 30	53 ± 31 18–121 45	47 ±30 8–89 37	0.05
TGS	188 ±105	157 ±82	0.36	167 ±100	230 +118	185 + 83	0.16
Fasting glucose level	98 ±30	95 ±23	0.72	93 ±18	92 ±25	127 ±53	0.0048
AST	41 ±25	35 ±13	0.84	37 ±26	49 ± 23	42 ± 25	0.044

However, to our knowledge, the variants of rs3774261 were not well reported in the Egyptian population. In addition, although studies have examined the association between rs266729 polymorphisms and the risk of NAFLD [40], no study on the interactions among multiple SNPs in relation to NAFLD risk in the Egyptian population has yet been published.

Studying the prevalence of SNPs at adiponectin rs3774261 in all studied patients and controls showed that all genotypes were statistically significant, where the G allele was the most frequent among patients (55.2%) compared to 32.6% among controls (P value < 0.001). This is consistent with the results obtained by [41, 42] who found that the GG genotype in rs3774261 is a risk factor of NAFLD. However, unlike the results obtained from this present study, which showed no significant correlation between any of the rs3774261 variants and the degrees of steatosis or fibrosis detected by CAP and FibroScan Echosens, respectively, nor with any of the significant clinical parameters, their study showed that these variants significantly affected blood glucose and lipid metabolism.

Moreover, analysis of the variant rs266729 showed that the presence of the CG genotype (P value=0.032) and the CC genotype (P value=0.003) was significantly related with the presence of NAFLD. None of them however was significantly related to the degree of steatosis nor fibrosis, or with different clinical parameters such as WC, BMI, TGs, and ALT levels. This is consistent with the results obtained in previous studies where Hsieh et al. [43] found that there was a significant difference in the frequency of adiponectin rs266729 gene polymorphisms between patients with and those without NAFLD.

The relationship between the SNPs of the most significant genotypes was studied and showed no significant relation between the genotypes of rs266729 and those of rs3774261 in the studied NAFLD patients.

Multivariate regression analysis showed no significant correlation with any of the independent risk factors to the degree of steatosis detected by CAP.

Conclusions

There was an association between polymorphisms of the studied SNPs of rs266729 rs3774261 of the adiponectin gene and NAFLD.

Abbreviations

NAFLD: Non-alcoholic fatty liver disease; CAP: Controlled attenuation parameter; TE: Transient elastography; LSM: Liver stiffness measurement; SNPs: Single nucleotide polymorphisms; HCV: Hepatitis C virus; HBV: Hepatitis B virus; BMI: Body mass index; IQR: Interquartile range

Acknowledgements
Not applicable.

Authors' contributions
EM and AY did the study design and administrative support. HA did the data collection, assembly, and analysis. RA and ZZ did the literature searches. DS did the SNP analysis. HA and YG did the FibroScan and CAP. ZZ wrote the manuscript. All authors read and approved the final manuscript.

Funding
No financial or any other support was supplied.

Availability of data and materials
We have an Excel sheet including the raw data.

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Received: 20 November 2020 Accepted: 28 April 2021
Published online: 13 May 2021

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