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Genetic Variants in Nicotinamide-N-Methyltransferase (NNMT) Gene are Related to the Stage of Non-Alcoholic Fatty Liver Disease Diagnosed by Controlled Attenuation Parameter (CAP)-FibroScan

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ABSTRACT

Background & Aims: Various genetic polymorphisms play a key-role in the pathogenesis of NAFLD and progression to NASH with fibrosis to cirrhosis. We aimed to study the association between single-nucleotide polymorphisms (SNPs) in NNMT gene, namely rs694539 and the development of different stages of NAFLD diagnosed by controlled attenuation parameter (CAP) of FibroScan Echosens®.

Methods: Transient elastography (FibroScan®) with controlled attenuation parameter (CAP) measurement was performed in 81 NAFLD patients (35 of them with liver biopsy) and 80 non-NAFLD controls. The accuracy of CAP and FibroScan for the detection and quantification of hepatic steatosis/fibrosis, respectively, was assessed based on liver biopsy aspect. Genetic variants of NNMT gene rs694539 were analyzed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: According to BMI (kg/m^2), among the patients, 17 (21%) were overweight, 56 (69.1%) obese, and 8 (9.9%) morbidly obese. CAP and FibroScan diagnosed steatosis/fibrosis correlated significantly with liver biopsy. There was a significant association between polymorphisms of rs694539-NNMT gene and NAFLD presence and stages. The mutant type (AA-genotype) was found in 33% NAFLD patients versus 1.2% controls (P<0.001), whereas the wild type (GG-genotype) was present in 21% versus 63.8% controls (P<0.001). Moreover, the AA-genotype significantly correlated with the steatosis degree by CAP but not the fibrosis degree by FibroScan. Multivariate regression analysis of all the independent risk factors showed non-significant correlations with the degree of steatosis on CAP. However, by using a stepwise approach, waist circumference showed significance as an independent predictor of NAFLD.

Conclusions: Polymorphisms in rs694539-NNMT gene (mutant AA-genotype) could be a genetic risk factor for developing NAFLD and NASH (indicating susceptibility for progression and complications). Individuals with wild type (GG-genotype) are at less risk of NAFLD development. CAP and FibroScan efficiently diagnosed steatosis and fibrosis.

Key words: Non-alcoholic Fatty Liver Disease (NAFLD) – Non-alcoholic Steatohepatitis (NASH) – Nicotinamide-N-methyltransferase (NNMT) gene – Single Nucleotide Polymorphisms (SNPs) – Controlled attenuation parameter (CAP) – FibroScan.

Abbreviations: CAP: controlled attenuation parameter; FBG: fasting blood glucose; LSM: Liver stiffness measurement; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic Steatohepatitis; NNMT: Nicotinamide-N-methyltransferase; PCR-RFLP: Polymerase chain reaction-Restriction fragment length polymorphism; SNPs: Single Nucleotide Polymorphisms; TE: transient elastography; TG: triglycerides; T2DM: type 2 dabetes mellitus.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of obesity and is rapidly becoming the most common liver disease worldwide with a prevalence of 21.3%. About 2–3% of the general population is estimated to have non-alcoholic steatohepatitis (NASH), which may progress to liver cirrhosis and hepatocellular carcinoma (HCC) [1].

NAFLD prognosis is predominantly determined by the liver fibrosis degree and steatohepatitis. This emphasizes the need for accurate differentiation between NASH and NAFL to determine prognosis and optimal treatment [2].

NAFLD develops from a complex process including genetic susceptibility and environmental insults. Susceptibility to high-prevalence diseases such as obesity, type 2 diabetes mellitus (T2DM), cardiovascular disease, and NAFLD comprises a heritable component variously accounting for up to 30 to 50% of the relative risk [3].

Genome-wide association studies identified patatin-like phospholipase domain-containing 3 (PNPLA3) I148M gene and transmembrane 6 superfamily member 2 (TM6SF2) E167K gene variants, as major determinants of liver steatosis and susceptibility to progressive NASH. Both can determine liver fat accumulation through lipid droplets and very low-density lipoprotein (VLDL) modifications. Several other genetic variants, including rare mutations, involved in the regulation of hepatocellular lipid metabolism, are being scrutinized [4].

The possible role of nicotinamide-N-methyltransferase (NNMT) gene rs694539 variant in the development of NASH was strongly associated with increased risk for NASH. Individuals with the GG genotype appeared to have protection against NASH, whereas the AA genotype showed statistically significant increased risk for NASH [5].

The prognosis and outcome of the patients with NAFLD is predominantly determined by the degree of liver fibrosis and steatohepatitis. This emphasizes the need for accurate differentiation between NASH and NAFLD in order to determine the prognosis and optimal treatment [2]. Currently, the routinely used modalities (laboratory tests and ultrasonography) cannot adequately determine the degrees of steatosis and fibrosis or cannot be applied as a screening procedure.

Among the non-invasive tests, transient elastography (TE) (FibroScan®) with controlled attenuation parameter (CAP) has demonstrated a good accuracy in quantifying the levels of liver steatosis and fibrosis in patients with NAFLD. CAP is measured using FibroScan (Echosens®) based on vibration-controlled transient elastography (VCTE), a technique initially developed to assess liver stiffness (LS) which correlates with fibrosis. CAP measures ultrasound (US) attenuation at 3.5-MHz using the postulate that fat affects US propagation [6].

On the other hand, the study of genetic factors in NAFLD is a rapidly growing field. To-date, the PNPLA3-I148M and TM6SF2-E167K gene variants are the major determinants of inter-individual differences in liver steatosis and susceptibility to progressive NASH. Both of these genes determine liver fat retention through lipid droplets and VLDL modifications. PNPLA3 affects directly hepatic stellate cells and retinol metabolism. These findings suggest that hepatocellular accumulation of neutral lipids is harmful. Several other genetic variants, including rare mutations, involved in the regulation of hepatocellular lipid metabolism, are being scrutinized [4].

The primary objective of this work was to evaluate the possible role of NNMT rs694539 genetic polymorphism as a risk factor for the development of NAFLD and its relation to different stages of steatosis (diagnosed by CAP and FibroScan measurement) among a cohort of patients with NAFLD.

PATIENTS AND METHODS

Study population

This cross-sectional study included 81 patients attending the Hepatology outpatient clinic and Cairo University Centre for Hepatic fibrosis (CUC-HF) of Kasr Al-Ainy Hospital, Faculty of Medicine, Cairo University over a period of 12 months (2/2015 to 2/2016), who had NAFLD. In addition, 80 age-matched healthy persons served as a control group. All included patients and controls are Egyptians with North African ethnicity.

Inclusion criteria

Patients with a body mass index (BMI) higher than 25.0 kg/m^2 were suspected to have NAFLD on basis of increased hepatic brightness by abdominal ultrasonography with exclusion of other causes of secondary steatosis: negative viral markers (HCV-Ab, HBsAg, HBc-total), negative Anti-nuclear antibody (ANA), normal serum ferritin and ceruloplasmin, 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.

Controls were selected from healthy persons with negative viral markers, normal blood tests [liver function tests, INR, CBC, fasting blood glucose (FBG), glycated hemoglobin (HbA1C), kidney function tests], normal BMI and normal liver appearance by ultrasonography.

Patients' eligibility

We excluded patients who were younger than 18 years old, those with decompensated cirrhosis, those having other metabolic disorders than DM, obesity and/or dyslipidemia, those with autoimmune disorders, viral hepatitis (positive HCVAb, HBsAg or HBcAb total) or current or past alcohol or hepatotoxic drugs consumption, and those with contraindication(s) for liver biopsy.

This study was approved by the department committee (IRB) and the institution Ethics Committee based on the 1975 (as revised in 1983) Declaration of Helsinki. Informed consent was obtained from all participants.

Study course

The patients were subjected to: history taking with emphasis on hepatotoxic drugs, BMI calculation, waist circumference (WC), serum ALT, AST, GGT, ALP, albumin, serum ferritin and ceruloplasmin, serum total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides (TGs), FBG, HBsAg, HBcAb total, HCV Ab, ANA, abdominal ultrasonography especially grade of liver brightness.

CAP was performed in all patients and controls using the 3.5-MHz M-probe of Echosens Fibroscan®. Both TE and CAP were done at Cairo University Centre for Hepatic fibrosis (CUC-HF).

Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) was applied to detect the distribution of genetic polymorphisms of the rs694539 NNMT gene in blood regarding the wild type (G-genotype) and the mutant type (A-genotype).

Percutaneous ultrasound-guided liver biopsy was performed in 35 out of 81 patients. Two experienced hepatic pathologists blinded to the clinical data independently reviewed the histological findings. 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 %; S3: >66 % [7]. In our study, NASH was defined as NAS score ≥5 in liver biopsy. FibroScan and CAP were evaluated in reference to liver biopsy and data showed that fibrosis detected by FibroScan and steatosis detected by CAP significantly correlated to the liver biopsy findings.

Liver biopsy was performed in 35 patients only due to many reasons, since we have compared biopsy findings with FibroScan and CAP and found significant match in the fibrosis and steatosis stages between biopsy and FibroScan and CAP, respectively (results in publication). In addition, owing to the risks associated with the more invasive liver biopsy and also that some of the patients did not give consent for biopsy, we were satisfied with the CAP and FibroScan findings and decided to assess the degree of steatosis and fibrosis using only non-invasive techniques in the rest of the patients.

Measurement of CAP

All TE and CAP measurements were done as per-the-manufacturer's recommendations by experienced operators blinded to the patients' clinical and histological data.

Assisted by the US image, a portion of the liver at least 6 cm thick and free of large vessels was identified using a portable 3.5-MHz M-probe of Echosens FibroScan®, which measured the liver stiffness 25–65 mm underneath the skin surface. The 3.5-MHz ultrasound transducer mounted on the axis of an electromechanical transducer transmitted a vibration of 50-Hz and induced an elastic shear wave that propagates through the underlying tissues.

The LS measurement (LSM) was expressed as kPa, and the examination was considered reliable if 10 valid measurements were obtained, the success rate exceeded 60%, and the ratio of the interquartile range (IQR) to the median of 10 measurements (IQR/M) was <30%. For this study, only LSMs using the M-probe were considered because the CAP algorithm is specific to this device. TE cutoffs used were those previously discussed [8]. CAP is a proprietary algorithm performed simultaneously with LSM using the US signals acquired by the FibroScan to detect hepatic steatosis. CAP measures the ultrasound attenuation using signals acquired by the 3.5-MHz FibroScan probe.

The CAP is measured only on validated measurements according to the same criteria used for LSM and on the same signals, ensuring that one obtains a liver ultrasonic attenuation simultaneously and in the same volume of liver parenchyma as LSM. The final CAP value, which ranges from 100 to 400 decibels per meter (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 steatosis cutoffs diagnosed by CAP are those of de Lédinghen et al., considering S0= 180-217, S1=223-268, S2=268-301, and S3=301-346 [9].

RFLP-PCR procedure for NNMT polymorphism diagnosis

DNA extraction

Total DNA was isolated from whole blood mononuclear cells using the Qiagene cells/tissue extraction kit (Qiagene, USA) according to instructions of manufacturer.

The isolation of intact DNA requires four essential steps: effective disruption of cells, denaturation of nucleoprotein complexes, inactivation of endogenous deoxyribonuclease (DNase) activity and removal of contaminating RNA and proteins.

The Total DNA Isolation System combines the disruptive and protective properties of guanidine thiocyanate (GTC) and β- Mercaptoethanol to inactivate the deoxyribonucleases present in cell extracts. GTC acts to disrupt nucleoprotein complexes, allowing the DNA to be released into solution and isolated free of protein.

Lysis of cells

175 µl of AVL lysis buffer, 25 µl of protease and 4µl RNase were added to the cells, the mixture was dispersed and mixed by pipetting. The mixture was incubated for 60 minutes at 55°C.

DNA purification

200 µl of 95% ethanol was added to the cleared lysate, mixed in a fresh microcentrifuge tube then transferred to the Spin Column Assembly and centrifuged at 8000 rpm for one minute.

600 µl of AW1 DNA wash solution was added and centrifuged at 8000 rpm for one minute. Then, the addition of 600 µl of AW2 DNA wash solution with ethanol (100 ml of 95% ethanol to a bottle containing 58.8ml concentrated AW2 DNA wash solution) which was centrifuged at 8000 rpm one minute. The collection tube was emptied and 250 µl of AW2 DNA wash solution with ethanol were added and centrifuged at 14,000 rpm for 2 minutes.

The spin basket was transferred from the collection tube to the elution tube, 100 µl of nuclease-free water were added to the membrane and centrifuged at 12,000-14,000 rpm for one minute. The spin basket was discarded and the elution tube containing the purified DNA obtained.

The purity (A260/A280 ratio) and the concentration of DNA were obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA). The extracted and purified DNA samples were stored at –80°C for further use.

RFLP-PCR

NNMT gene polymorphism (rs694539) was detected by RFLP-PCR method. A 187 base-pair (bp) product was obtained. EzWayTM Direct Taq PCR Master mix (Koma Biotech Inc., Seoul, Korea) in 25 µL reaction volume was used. The amplification was carried out in a PCR thermal cycler Biosmetra, Germany. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide (0.5 mg/L). Ten µl of the PCR products were digested with 1 unit of the NlaIII restriction endonuclease (New England Biolabs, Hitchin, UK) in a total volume of 20 µl at 37°C overnight.

Applied biosystems reagents in reaction volume conducted in the RFLP-PCR analyses using PCR Master Mix were performed in duplicate (all steps) to verify the accuracy of the detected genotypes. Both homozygous (GG and AA) and

heterozygous genotypes (GA) were estimated on 4% agarose gel. The primers for the studied gene [5]:

Forward primer sequence (5‘-3‘)
ACGTTGGATGGTCCTAGAGTCCTA
Reverse primer sequence (5‘-3‘)
ACGTTGGATGCAGCCATCTCAAATG

Statistical analysis

Data were coded and entered using the statistical package SPSS version 22. Data was summarized using mean and standard deviation (SD) for quantitative variables and frequencies (numbers) and relative frequencies (percentages) for categorical variables. unpaired *t* test was used when comparing two groups and analysis of variance (ANOVA) with multiple comparisons post hoc test when comparing more than two groups.

For comparing categorical data, the chi square (χ^2) test was performed. Exact test was used instead when the expected frequency was less than 5. Correlations between quantitative variables were made using Spearman correlation coefficient. Genotype and allele frequencies were compared between the disease and the control groups using χ^2 tests. Odds ratio (OR) with 95% confidence intervals was calculated. P-values less than 0.05 were considered as statistically significant.

The study of NNMT genetic SNPs in NAFLD had been performed before in only one research with a sample size of 80 patients, so we used the same sample size estimated before (81 patients in our study).

Tests of normality of distribution of genetics (Hardy Weinberg) in controls (80 samples counted) were done, where $\chi^2 = 1.77$, for likelihoods of calculated χ^2 value (Table I), the p allele frequency = 0.81; the q allele frequency = 0.19, the P value was >0.05 so it was not deviated from equilibrium [10].

Table I. Likelihood of calculated χ^2 value in the test of normality of distribution of genetics in controls

Genotype	Expected	Observed
Common homozygotes	52.81	51
Heterozygotes	24.38	28
Rare homozygotes	2.81	1

RESULTS

Basic characteristics

Among patients, ages ranged from 19 to 66 years (44.58±9.44), with 49 (60.5%) females and 32 (39.5%) males. BMI ranged from 27.8 kg/m^2 to 46.9 kg/m^2 (33.69± 4.45), whereas the WC was 108.33±11.29 cm.

In the control group, ages ranged from 21 to 63 years (38.84±8.19), with 48 (60%) females and 32 (40%) males, BMI was 25.68±1.48 kg/m^2, WC was 81.31±5.49 cm.

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

Among patients, 21% (17 patients) were overweight, 69.1% (56 patients) were obese, and 9.9% (8 patients) were morbidly obese, whereas among controls, 36.2% (29 subjects) had normal BMI, 63.8% (51 subjects) were overweight, and none were obese.

Distribution of risk factors for NAFLD among patients and controls

The known risk factors for the development of NAFLD as obesity, DM, hyperlipidemia and metabolic syndrome (MS) showed significant difference between patients and controls (Table II). All patients had negative history of alcohol or drug abuse. ALT, AST and GGT were significantly higher in patients than controls. All patients and controls had normal INR and renal function (Table II).

Table II. Distribution of clinical risks and laboratory findings among NAFLD patients and controls

Item	Patients		Controls		P value
	Number	%	Number	%	
BMI					
Overweight	17	21	51	63.8	**< 0.001**
≥Obese	64	79	--	--	
T2DM	22	27.2	2	2.5	**< 0.001**
HTN	13	16.0	0	0.0	-----
Family history	26	38.8	0	0.0	-----
MS	12	14.8	0	0.0	**<0.001**
Smoking	14	17.3	9	11.2	0.317
Laboratory findings (mean, SD)					
T. Cholesterol	221.94	58.78	130.22	27.84	**< 0.001**
HDL-C	45.02	20.33	64.39	3.73	**< 0.001**
LDL-C	135.69	54.88	91.56	16.07	**< 0.001**
TGs	194.15	96.61	85.89	17.46	**< 0.001**
FBS	104.30	38.58	84.84	9.24	**< 0.001**
PP-BG	176.16	48.62	150.19	19.47	**< 0.001**
HGB	13.12	1.87	13.86	1.52	0.505
PLT	277.47	70.77	337.58	67.19	0.317
WBCs	6.10	2.07	6.04	1.94	0.611
T. Bilirubin	0.74	0.28	0.62	0.23	0.324
T. Protein	7.12	0.52	8.04	0.44	**< 0.001**
ALB	4.26	0.45	4.43	0.52	0.451
AST	46.67	40.14	18.19	4.94	**< 0.001**
ALT	50.02	35.09	16.08	5.81	**< 0.001**
GGT	35.89	69.05	17.79	5.36	**0.021**
ALP	48.59	21.24	48.42	22.11	0.505
INR	1.02	0.08	1.02	0.05	0.6

BMI: Body mass index; HDL-c: High Density Lipoprotein Cholesterol; HTN: hypertension; LDL-c: Low Density Lipoprotein Cholesterol; MS: metabolic syndrome; TGs: Triglycerides; FBS: Fasting Blood Sugar; PP-BG: Postprandial Blood Glucose; WBCs: White Blood Cells.

All controls showed normal hepatic echogenicity by US (mostly excluding NAFLD presence in controls). Among patients, liver brightness grade I was diagnosed in 22 (27.2%), grade II in 40 (49.4%) and grade III in 19 (23.5%) patients.

The degree of steatosis using CAP was mild (S1) in 28.4% (23 patients), moderate (S2) in 23.5% (19 patients) and severe in 48.1% (39 patients), whereas the degree of fibrosis using

Table III. Correlation between degree (P1) and presence (P2, CC) of steatosis by CAP and various parameters

Item	S1		S2		S3		P1	P2	Correlation coefficient
	Mean	SD	Mean	SD	Mean	SD			
Age	41.52	7.70	49.21	9.30	44.13	9.77	**0.027**	0.866	0.019
WC	107.22	10.72	111.58	11.88	118.74	6.44	**<0.001**	**0.001**	0.373
BMI	33.00	4.61	33.76	4.10	36.29	4.16	**0.009**	**0.001**	0.356
ALT	40.90	31.17	64.26	46.69	48.46	29.02	0.091	0.504	0.075
HDL	42.35	8.97	48.57	33.04	44.86	17.19	0.618	0.492	-0.077
LDL	149.70	62.96	139.37	48.20	125.65	52.11	0.238	**0.035**	-0.235
TGs	170.91	89.68	203.68	99.29	313.87	63.30	**<0.001**	**<0.001**	0.500

WC: waist circumference

FibroScan (Echosens®) was mild (F0, F1) in 91.4% (57 patients F0, 17 patients F1), moderate (F2) in 0% and marked in 8.6% (F3 in 4 and F4 in 3 patients).

Correlation between the degree of steatosis by CAP and different parameters

The degree of steatosis was higher among females than males, with 39.1% of males and 60.9% of females having values corresponding to S1, 42.1% versus 57.9% having S2, and 38.5% versus 61.5% having S3 among males and females respectively. However, there was no significant difference (P = 0.964).

By univariate analysis, among parameters shown in Table III, age, WC, BMI and higher TGs were significantly correlated with the degree of steatosis measured by CAP. While WC, BMI, LDL and TGs correlated with the mere presence of steatosis by CAP.

When multivariate regression analysis was conducted, all the independent risk factors showed non-significant correlations with the degree of steatosis on CAP. However, when using a stepwise approach, only the WC showed significance as an independent predictor of NAFLD (Table IV).

The different grades of brightness by US showed significant correlation with the degree of steatosis by CAP (P value <0.001). Grade I brightness had 12 (S1), 7 (S2) and 3 (S3) patients according to CAP, grade II brightness had 9 (S1), 12 (S2) and 19 (S3) according to CAP and grade III brightness had 2 (S1), 0 (S2) and 17 (S3) patients according to CAP.

Table IV. Multivariate analysis of steatosis presence in relation to different factors

Item	OR	Standard Error	z	P	95% CI	
Age	0.98	0.12	-0.16	0.87	0.77	1.24
Sex	26.41	92.17	0.94	0.35	0.03	24703.97
Waist C	5.025	2.01	0.15	0.997	0.88	1.09
BMI	24.76	48.88	1.63	0.10	0.52	1146.84
WBC	0.82	0.59	-0.27	0.79	0.20	3.38
AST	1.28	0.29	1.08	0.28	0.82	2.01
ALT	1.03	0.18	0.14	0.89	0.73	1.45
ALP	1.08	0.05	1.55	0.12	0.98	1.19
GGT	0.90	0.10	-0.96	0.34	0.73	1.11
AA mutation	0.26	0.52	-0.67	0.50	0.01	13.02
Stepwise Multivariate analysis						
WAIST C	OR: 1.655 (1.327-2.063)			P <0.001		

Cholesterol was set as low/high at a cutoff 200, TG were set as low/high at a cut of 150. (All patients without NASH had low cholesterol and low TG, so they could not be introduced in the multivariate analysis).

NNMT gene rs694539-variant among patients with NAFLD and its relation to CAP, FibroScan and clinical parameters

The comparison of rs694539-NNMT A and G alleles genotype distribution between NAFLD patients and controls showed that A allele was significantly higher in patients (P <0.001), G allele was significantly higher in controls (P <0.001). The mutant AA-genotype was found in 33.3% of patients and 1.2% controls with high significance (P <0.001) and 21% of patients had the wild GG-genotype versus 64% controls with high significance (P <0.001) (Table V, Fig. 1).

The presence of the mutant AA-genotype showed a significant correlation with the degree of steatosis detected by CAP and a non-significant correlation with the degree of fibrosis (by FibroScan) (Table VI).

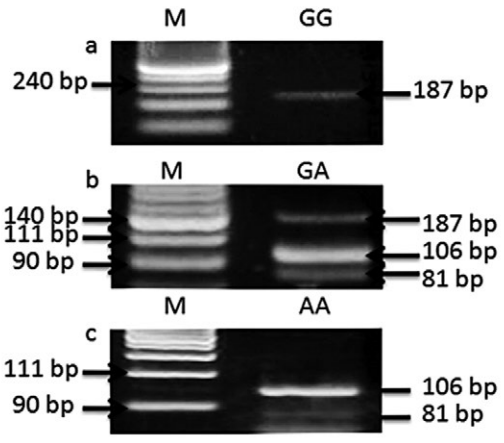

Fig. 1. The NNMT rs694539 genetic polymorphism using PCR-RFLP. The amplified 187 bp fragment was cut with the restriction endonuclease NlaIII and analyzed on a 4% agarose gel electrophoresis. Lane M showing the marker (M). (a) lane GG showing the GG-genotype with one fragment of 187 bp; (b) lane GA showing the GA-genotype with three fragments of 187, 106, and 81 bp; and (c) lane AA showing the AA-genotype with two fragments of 106 and 81 bp.

Table V. NNMT rs694539 variant among NAFLD patients versus controls and in obese versus non-obese individuals

NNMT rs694539 variant	Patients (81)		Controls (80)		P value	OR (95%CI)
	No	%	No	%		
Wild GG	17	21.0	51	63.8	< 0.001	0.151 (0.075-0.305)
GA	37	45.7	28	35.0	0.167	1.562 (0.828-2.945)
Mutant AA	27	33.3	1	1.2	< 0.001	39.5 (5.210-299.469)
G Allele	71	43.8	130	81.2	< 0.001	0.180 (0.109-0.298)
A Allele	91	56.2	30	18.8	< 0.001	5.552 (3.356-9.193)
According to the BMI						
BMI	Obese (64)		Non obese (97)		P value	OR (95%CI)
Wild GG	14	21.9	54	55.7	<0.001	0.223 (0.109-0.456
GA	30	46.9	35	36.1	0.172	1.563 (0.822-2.972)
Mutant AA	20	31.2	8	8.2	<0.001	5.057 (2.046-12.387)
G Allele	58	45.3	143	73.7	<0.001	0.296 (0.184-0.474)
A Allele	70	54.7	51	26.3	<0.001	3.384 (2.110-5.428)

Table VI. Correlation between NNMT rs694539 AA genotype and the degree of steatosis by CAP (S) and fibrosis by FibroScan (F)

CAP & FibroScan	rs694539variant of NNMT=AA				P value
	Yes		No		
	Count	%	Count	%	
S1	4	14.8	19	35.2	0.05
S2	10	37.0	9	16.7	
S3	13	48.2	26	48.1	
F0	15	55.6	42	77.8	0.135
F1	9	33.3	8	14.8	
F3	2	7.4	2	3.7	
F4	1	3.7	2	3.7	

The relation between the mutant AA-genotype of NNMT-rs694539 and different clinical parameters showed that in patients with the mutant AA genotype, WC was 114.30± 12.50, BMI was 35.05 ±4.70, TGs level was 248.63 ± 94.50 and ALT was 54.52± 33.70. While in patients without the mutant AA genotype, WC was 113.54 ±9.32, BMI was 34.62± 4.41, TGs was 246.83 ±107.95, and ALT was 47.77 ±35.86. These correlations showed no statistical significance.

The mutant AA genotype and the A allele were significantly higher in obese vs. non-obese subjects (P <0.001), on the contrary, the wild GG genotype and the G allele were significantly higher in non-obese subjects (P <0.001) (Table V).

DISCUSSION

NALFD is considered the hepatic manifestation of obesity and MS. Progression from NALFD to NASH increases the risk of liver-related outcomes [11]. So, the knowledge of whether a patient has simple steatosis or NASH is important prognostically [12].

Several non-invasive modalities have demonstrated promise for aiding in the diagnostic approach of NASH. These include serum markers, both direct and indirect, clinical scoring systems, and novel imaging modalities, such as shear wave elastography.

In the last years, the genetic determinants of NAFLD are being unrevealed using genome-wide association studies (GWAS), which offer a powerful technique for discovering novel associations between SNPs and disease phenotypes. Newly-identified genetic risk variants could provide a useful tool for the clinical management and prognosis of obese patients with NAFLD [4].

The rs694539-variant of the NNMT gene is a SNP that is found to be significantly associated with hyperhomocysteinemia which causes steatosis [13]. Sazci et al. found that individuals with the GG-genotype of NNMT gene rs694539-variant appeared to have protection against NASH, whereas persons with the AA-genotype showed significant increased risk for NASH, thereby reporting for the first time the rs694539-variant of NNMT gene as a genetic risk factor for developing NASH [5].

The BMI and WC were significantly higher in our NAFLD cases than in controls. Previous studies concluded that the prevalence of NAFLD increases with obesity, ranging between 34.2% and 60.9% as recently reported in a meta-analysis [1]. Other studies, however, concluded that NAFLD can occur in non-obese patients who are physically inactive [14, 15]. In addition, some studies reported that WC served as a reliable predictor for the risk of NAFLD among obese patients [16].

In our study, the prevalence of DM and MS was significantly higher in NAFLD patients compared to controls. Many studies showed that the prevalence of NAFLD and NASH is higher among diabetics, with a prevalence of 22.51% and 43.63%, respectively and considered NAFLD and NASH as the hepatic manifestation of MS, with an overall pooled prevalence of 42.54% and 70.65%, respectively (1). Other studies also identified DM as an independent risk factor for the progression of NAFL [17].

In one study, the prevalence estimates of hyperlipidemia/dyslipidemia among NAFLD and NASH obese patients were 69.16% and 72.13%, respectively, and those of hypertriglyceridemia were 40.74% and 83.33% for NAFLD and

NASH, respectively (1). Similarly, among our patients, elevated cholesterol, LDL-c and TG levels, as well as reduced HDL-c levels correlated to NAFLD presence. Furthermore, higher TGs and lower LDL-c correlated with steatosis degree detected by CAP.

Previous studies showed that, in up to nearly 60% of NAFLD obese patients with NASH, ALT could be normal [18, 19]. Nevertheless, ALT can be increased in up to 53% of NAFLD patients without NASH [18, 20]. Those studies thereby concluded that ALT level alone is not predictive of NASH or fibrosis level [20].

In our study, both ALT and AST levels were found to be significantly higher among NAFLD patients versus controls. Moreover, GGT was significantly higher among NAFLD patients as well. Pulzi et al. found that GGT ≥ 30 IU/L is an adequate predictive marker of NASH, others considered GGT as a marker of increased mortality [21]. On the other hand, Tahan et al. divided obese NAFLD patients into normal GGT and high GGT groups and found that differences in reference to histological steatosis grade and inflammation were not significant [22].

In comparison with abdominal US, in addition to being non-ionizing and inexpensive, CAP presents the added benefits of being machine-independent and non-subject to operator interpretation. Furthermore, CAP was shown to be efficient to offer quantification of hepatic steatosis from 10% and up. All this combined with the simultaneous assessment of liver fibrosis using liver stiffness makes CAP a more appealing novel method for assessing hepatic steatosis [23].

The degree of steatosis, in our study, correlated with the increase in age but not the gender difference. Previous studies identified age and male gender as independent risk factors for progression of steatosis [24, 25]; the reason why the later finding could not be reproduced in our study could be due to the higher prevalence of females.

The increased BMI and WC and the worsening lipid profiles correlated with the degree of steatosis detected by CAP in our study. Previous studies demonstrated the same correlations in BMI and WC [23, 26] and serum TGs and LDL [26, 27].

SNPs of different genes were evaluated for prediction and differentiating simple steatosis from NASH. Our study showed consistent results to those demonstrated by Sazci et al. [5]. The mutant AA-genotype was found in 33.3% of NAFLD patients and in only 1.2% of controls, the wild GG was found in 21% of NAFLD patients versus 63.8% controls. Moreover, the mutant AA genotype and the A allele were significantly more frequent in obese subjects and on the contrary, the wild GG genotype and the G allele were significantly more frequent in non-obese subjects. From these results, we can conclude that the NNMT gene rs694539-variant mutant AA-genotype significantly correlates with the presence of NAFLD in obese Egyptians, while the wild GG-genotype appeared to offer protection against its development.

Moreover, the degree of steatosis detected by CAP appeared to significantly correlate with the presence of the mutant AA-genotype, where the percentage of patients with the mutant AA-genotype increased in correlation to the degree of steatosis.

Although the mutant AA genotype significantly correlated with steatosis presence and degree, it did not correlate with the degree of fibrosis (which is the major prognostic determinant in NAFLD). The NNMT gene (and its expressions) were not considered in this study and the previous one study as non-invasive fibrosis markers. Nevertheless, marked steatosis degrees have been proved by this study and other studies that they will mostly and eventually progress (with a second hit) to inflammation and fibrosis.

Therefore, the major outcome from the clinical point of view elaborates that the mutant AA genotype could be a good prognostic marker as it can diagnose marked steatosis (higher susceptibility of NASH progression) and most importantly can predict clinically significant fibrosis and its complications years before its development.

Further studies should be performed to combine the NNMT gene with the other major genetic risk factors for NAFLD, e.g. PNPLA3, TM6SF2, MBOAT7 and GCKR variants.

Also, studies with larger sample size are required and results should be replicated in an independent cohort with a different ethnicity to ascertain this study conclusions.

CONCLUSIONS

As found in our study, polymorphisms in rs694539-NNMT gene (mutant AA-genotype) could be a genetic risk factor for developing NAFLD and NASH (indicating susceptibility for progression and complications). Individuals with wild type (GG-genotype) are at less risk from NAFLD development. CAP and FibroScan efficiently diagnosed steatosis and fibrosis, respectively. The degree of steatosis by CAP correlated significantly with age, BMI, WC, and high TG levels.

Conflicts of interest: None declared.

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