Egypt. J. Chem. **Vol. 67**, No. 2. pp. 295 - 310 (2024)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

The Influence of Resistin (rs1862513) Gene Single Nucleotide Polymorphism in Egyptian Patients with Nonalcoholic Fatty Liver Disease

Mervat M. El-Sayed [a*], Soha M. Hamdy [a], Sara H. A. Agwa [b], Ola N. Sayed [a]

[a] *Chemistry Department, Biochemistry division, Faculty of Science, El Fayoum University, El- Fayoum, Egypt.*

[b] *Clinical Pathology and Molecular Genomics Unit, Medical Ain Shams Research Institute (MASRI), Ain Shams University, Cairo, Egypt.*

Abstract

Liver diseases are emerging global health issues, non-alcoholic fatty liver disease (NAFLD) represents the most prevalent severe liver disease worldwide in the 21st century. Besides environmental factors, genetic predisposition triggers NAFLD development. This study investigated the sensitivity of Resistin (rs1862513) gene polymorphism in NAFLD and hepatic fibrosis in NAFLD Egyptian patients. The study was performed on 126 subjects as 63 healthy control subjects and 63 NAFLD patients. Gene polymorphism for NAFLD patients and healthy controls were done by PCR method. The biochemical parameters in NAFLD group showed a significantly elevated WBCs count; ALT; AST; TG; FBS; NFS; serum insulin; HOMA-IR; Total and direct bilirubin when compare with control and showed a significant decrease in both albumin level and PLT count. There were no significant differences regarding genotypic and allelic frequencies among NAFLD subjects and control individuals. Fibrosis progression estimated by NFS may accompanied with higher age. PLTs, WBCs count, Hb and TG significantly decreased while INR, AST, total and direct bilirubin, FBS and HOMA-IR increased in correlation to level of fibrosis. Genotype frequencies differed significantly among the different fibrosis stages groups. CG+GG genotype carriers showed significantly higher age and ALT while lower WBCs count with fibrosis progression. Our study stated that the best diagnostic tool was FBS followed by direct bilirubin that also proved as a good tool in detecting NAFLD patients with AUC 1.000 and 0.781, sensitivity of 100% and 60% for FBS and direct bilirubin respectively and 100% specificity for both. AST displayed the greatest AUC, sensitivity and also specificity values (1.000, 100%, 100% respectively) for detecting all G allele possessing patients.

Keywords: Non-alcoholic fatty liver disease, gene polymorphism, AST, ALT, PLT

1. Introduction

Liver diseases are emerging global health issues. Excessive hepatic fat accumulation results in NAFLD and alcoholic liver disease (AFLD) development, the two most prevalent causes of liver disease [1, 2]. NAFLD is regarded as the hepatic component of metabolic disorder commonly related to obesity and diabetes. It is among the largest global health threats of the twenty-first century [3, 4].

NAFLD prevalence varies greatly depending on the geographic regions of the world. In a meta-analysis covering data between 1989 and 2015 estimated that NAFLD affects globally about 25% of general population [5] with higher prevalence in Middle East and South America (around 30%), intermediate in Asia (27%), United States (24%), and Europe (23%), while lower rates in Africa (14%)[6]. A meta-analysis recently has suggested the pooled worldwide NAFLD prevalence to be 30.9% using 1989 to 2020 data with highest prevalence in 2019 reported from North Africa and Middle East regions (42.6%) followed by Latin America (34.5%) and Asia at 30.8% [7].

In Africa while Sudan recorded highest prevalence (20%), Nigeria reported the lowest one (9%) [5]. More recent meta-analysis studies by [8] and [9] reported NAFLD prevalence at 28.2% and

*Corresponding author e-mail: mms@fayoum.edu.eg.; (Mervat M. El-Sayed).
Received date 10 July 2023; revised date 25 July 2023; accepted date 10 August 2023
DOI: 10.21608/EJCHEM.2023.222135.8237

56.8% respectively. NAFLD prevalence estimates vary widely in Africa because of the scarcity of trustworthy data [10, 11].

There are scarce studies about NAFLD epidemiology in Egypt. A study by [12] in Alexandria revealed NAFLD prevalence in schoolchildren to be 15.8% and to increase significantly with age. In addition, NAFLD prevalence in healthy college Egyptian students was 31.8% by transient elastography [13]. NAFLD prevalence in Egypt is increasing owing to rising obesity prevalence. In addition, NAFLD was noted in more than 50% of female obese individuals [14]. Moreover, 52% of young women suffered from polycystic ovary syndrome using US were identified to have NAFLD [15].

NAFLD incidence rate is high in patients having central obesity, T2DM, dyslipidemia and MS, and it is additionally the main etiology of chronic hepatic disease worldwide [16-18].Concerning diabetes and NAFLD, a bidirectional interaction has been noticed between them while T2DM exists in about a quarter of NAFLD cases, NAFLD is found in about three quarters of those patients having T2DM with mortality increased in NAFLD-T2DM patients [6, 19].

The human gene encoding resistin (RETN) mapped on chromosome no.19 (19p13.2) consists of 4 exons and 3 introns. The length of its mRNA is 476 bp with a 326 bp coding region that encodes for 108 amino acids. Several SNPs in resistin gene have been identified including -420C/G, -638G/A and 299G/A with RETN –420C>G (rs1862513) being the most cruical of these SNPs due to its reported association with regulating RETN gene expression and resistin serum levels. This polymorphism has 3 genotypes, CC, CG and also GG which correlate with metabolic syndrome [20-22]. The aim of the study is investigation of the sensitivity of Resistin (rs1862513) gene polymorphism in NAFLD and hepatic fibrosis in NAFLD Egyptian patients.

2. Subjects and Methods

Sixty three patients and sixty three healthy volunteers representing the control were involved in our present study from Internal Medicine and the Outpatient Clinic Department of Ain Shams University hospital, Cairo, Egypt. The Research Ethical committee of Faculty of Medicine, Ain Shams University, Egypt approved the study protocol (Number code: FWA 000017585, FMASU R 38/2021) and each participant signed an informed consent for participating in the study.

126 individuals were participated in present study which were two groups:

Group (1): NAFLD group: composed of 63 patients newly sonography diagnosed and had not received any treatment.

Group (2): Control group: composed of 63 healthy volunteers with normal transaminases, hepatic ultrasound and negative HBsAg and HCV-Ab; 41.3% and 71.4% were males; 58.7% and 28.6% were females for patient and healthy control groups respectively.

- **Exclusion Criteria:**

Exclusion of other chronic hepatic disease and liver cirrhosis causes including hepatitis B or C virus infection, self-reported alcohol intake in the preceding six months, or autoimmune hepatitis, with ruling out the history of previous NAFLD treatment and with exclusion of hepatic malignancies presence.

All patients were subjected to full history taking; Fasting blood sugar was measured colorimetrically using an enzymatic method [23]. Quantitative measurement of insulin was carried out using an ELISA kit purchased from Monobind Company, Accubind, USA. Serum ALT and AST activities, total, direct bilirubin and albumin concentrations were determined using Human Gesellschaft für Biochemica und Diagnostica mbH (Germany) laboratories diagnostic kits. Serum triglyceride concentrations were determined using kits provided from Spinreact company [24]. Quantitative determination of prothrombin time was carried out using STA® - NeoPTimal kits for determination of INR according to Holbrook et al [25]**.** Complete blood count was performed using the Sysmex KX-21N analyzer. The insulin resistance index was assessed by the homoeostasis model assessment insulin resistance (HOMA-IR), calculated with Matthews et al [26] equation. Genotyping of Resistin rs1862513 SNP was performed by Taqman method using TaqMan® SNP Genotyping Assay, human (rs1862513) kit. In NAFLD, NFS was created to evaluate fibrosis and calculated as mentioned previously by **Angulo *et al*** [27] formula. NAFLD patients were divided into four groups according to NFS as follows : F0 stage group (NFS < 0, n=16), F1 stage group (0≤ NFS <0.6 , n= 19), F2 stage group (0.6≤ NFS <1 , n=13) and F3 stage group (1 ≤ NFS, n=15).

2.1. Blood sample collection and storage:

5 ml of venous blood sample was withdrawn from each participant under complete aseptic conditions and divided into: 1ml of venous blood was added to tube containing potassium-ethylene diamine tetra acetic acid (K-EDTA) for complete blood counting (CBC) and DNA extraction; 1ml was incorporated into a tube containing sodium fluoride aims to obtain plasma for blood sugar testing; 2ml of venous blood was immediately centrifuged at 3000 rpm to obtain serum for estimation of fasting insulin (Fins), ALT, AST, total and direct bilirubin, serum albumin and triglyceride (TG) and finally 1ml was added to a graduated vacuum plastic tube containing 0.2 ml tri-sodium citrate (3.2 %) for prothrombin time (PT) which after centrifugation at 2500 rpm/15 minutes, plasma was then separated and PT was estimated immediately. All samples were stored at -80 °C until use.

2.2. Genotyping of Resistin rs1862513 single nucleotide polymorphism:

DNA extraction from blood samples: Genomic DNA was extracted from EDTA treated whole blood samples using Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit (catalog no. K0781)[28, 29].

Resistin SNP rs1862513 genotyping was performed using 7500 HT Fast Real Time PCR System (Applied Biosystems,USA) by TaqMan SNP Genotyping Assay [30]. The used primers were:

FW 5´-CCACCTCCTGACCAGTCTCT-3´ REV 5´-AGCCTTCCCACTTCCAACAG-3.

The used probes were: VIC 5-CATGAAGACGGAGGCC-3 for 420C and FAM 5-ATGAAGAGGGAGGCC-3 for 420G. The assay took place in 10 µl of reaction mixture that contains 5 µl Genotyping Master Mix, 0.5 µl specific TaqMan genotyping assays and 4.5 µl of gDNA upon manufacturer's instructions. The following cycling conditions had been adopted, 95 °C/10 minutes hold then denaturation at 95 °C/15 seconds and primer annealing and extension for 1 minute at 60 °C in 40 cycles.

2.3. Statistical data analysis:

Data analysis was operated using IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 25 (2017) for Windows. Quantitative variables were presented by mean ± SD, while categorical ones were described as count and percentages. Also, chisquared test and independent sample T-test were applied for comparison of all parameters between controls and cases and between different genotype groups in healthy and patient groups. One way ANOVA test followed by post-hoc test were applied to compare the different parameters among different genotypes in NAFLD group and among genotype groups with severity of fibrosis. Chi-square and Pearson correlation tests were also used. ROC curve was applied for evaluating the predictive value for different studied serum biochemical parameters in NAFLD participants and those patients with different genotype groups. All the statistical tests were 2-tailed and a *P*-value *≤0.05* were statistically significant [31].

3. Results

3.1 Demographic, anthropometric data and other biochemical parameters in control and patient groups:

Regarding sex among NAFLD group and control group, 41.3% and 71.4% were males and 58.7% and 28.6% were females respectively. Also, our patients were significantly older (55.57±11.29 and 37.63±15.21 respectively) comparing to healthy participants with *p<0.001*. Regarding BMI, our results demonstrated a significant variation among patient and control group (31.27±3.44 and 22.35±1.89 respectively) with *p< 0.001* (table 1).

Our data revealed a significant increase in ALT and also AST activities in NAFLD group compared to control volunteers (65.83±8.77 *vs.* 19.57±8.94) respectively with percentage change 236.38% and *P* value < *0.001* for ALT and 67.51±14.78 *vs.* 21.98±8.54, percentage change 207.09% and *P* value < *0.001* for AST activity. When compared with healthy group, TG concentrations in patients were considerably higher (141.96±20.07and 95.81±14.97 respectively) with *p< 0.001*. While, Albumin level significantly decreased in patient group versus healthy volunteers (3.31±0.22 and 4.13±0.21 respectively and p< 0.001) (table 1).

Form current study, direct bilirubin levels were statistically significant high in NAFLD group 0.32±0.2 and 0.15±0.04 respectively with percentage change 106.67% and p< 0.001. However, total bilirubin concentrations didn't differ significantly among cases and healthy participants (table 1).

Table 1 show FBS and serum insulin concentrations significantly increased (216.49±32.59 and 5.37±1.25) in patient subjects comparing to

healthy individuals (99.28±7.99 and 3.22±0.65) with percentage changes (118.06% and 66.77% and p <0.001) respectively. In comparison with control group, HOMA-IR displayed a significant increase in patient group (3.42±1.2 vs. 0.80±0.19 with percentage change 327.5% and p < 0.001).

Moreover, platelets count significantly declined in NAFLD cases versus control group (201.98±44.02 *vs.* 247.76±73.29 respectively with percentage change 18.48% and *p< 0.001*) for PLTs count. NAFLD group demonstrated a significantly increased WBCs count (9.1±2.65) comparing to control group (5.92±1.56) with percentage change 53.72% and *p< 0.001*. There were also no statistical significant differences for either haemoglobin concentrations or INR between control persons and patients. When compared with healthy persons, NFS significantly raised in patient group (0.56±0.51 *vs.* -2.6±0.72 with percentage change 78.46%) Table 1.

Table 1. Represented mean ±S.D and % changes of demographic, anthropometric data and other biochemical parameters in control and patient groups:

Parameter		Control group (n=63)	Patient group (n=63)	% of changes from control	*P* value
Demographic and anthropometric data					
Sex (n,%)	**Male**	45 (71.4%)	26 (41.3%)	$x^2 = 11.65$	
	Female	18 (28.6%)	37 (58.7%)		
Age (years)		37.63±15.21	55.57±11.29	↑ 47.67%	***< 0.001***
BMI (kg/m^2)		22.35±1.89	31.27±3.44	↑ 39.91%	***< 0.001***
Hematological data					
PLT (x10^3/ µL)		247.76±73.29	201.98±44.02	↓ 18.48%	***< 0.001***
Hb (g/dL)		11.09±1.82	10.68±1.94	↓ 3.69%	*0.261*
WBC (x10^3/ µL)		5.92±1.56	9.1±2.65	↑ 53.72%	***< 0.001***
INR		1.09±0.08	1.11±0.12	↑1.83%	*0.182*
Fibrosis score (NFS)		-2.6±0.72	0.56±0.51	↑78.46%	***< 0.001***
Biochemical data					
ALT (U/L)		19.57±8.94	65.83±8.77	↑236.38%	***< 0.001***
AST (U/L)		21.98±8.54	67.51±14.78	↑207.09%	***< 0.001***
TG (mg/dL)		95.81±14.97	141.96±20.07	↑48.17%	***< 0.001***
Alb (g/dL)		4.13±0.21	3.31±0.22	↓19.85%	***< 0.001***
Total bilirubin (mg/dL)		0.82±0.22	0.90±0.49	↑9.76%	*0.328*
Direct bilirubin (mg/dL)		0.15±0.04	0.32±0.2	↑106.67%	***< 0.001***
FBS (mg/dL)		99.28±7.99	216.49±32.59	↑118.06%	***< 0.001***
Fasting insulin (µIU/mL)		3.22±0.65	5.37±1.25	↑66.77%	***< 0.001***
HOMA-IR		0.80±0.19	3.42±1.2	↑327.5%	***< 0.001***

- *P value* versus control group; x^2: Chisquare
- Significance at *P value ≤ 0.05*
- BMI, Body mass index; PLT, Platelet count; Hb, hemoglobin concentrations; WBCs, White blood cell count; INR, International normalized ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, Triglyceride; Alb, albmin; FBS, Fasting Blood Sugar; HOMA-IR, homeostasis model assessment-insulin resistance

3.2 RETN SNP rs1862513 C/G genotyping in cases and controls:

Our patients and also controls'DNA samples were further genotyped for SNP rs1862513 C/G using 7500 HT Fast Real Time PCR system (Applied Biosystems). Real Time PCR system displays clusters for the three possible genotypes (Allele 1 homozygous (Wild type; CC) (red), allele 2 homozygous (GG) (blue), and allele 1/2 heterozygous (CG) and a cluster for the negative controls (black). The allelic discrimination plot shows a crossmark (X-undetermined) for each sample if the autocaller is disabled as in fig.1.

SNP rs1862513 C/G genotyping in both patient and control individuals had been performed with the exclusion of 2 subjects from this study (undetermined genotype) one from each group. The 62 control group subjects; (15) of them gave CC-genotype, (35) gave CG-genotype and (12) gave GG-genotype with percentages (24.2%, 56.4% and 19.4% respectively). From the 62 patients, (22) subjects were CC-genotype, (34) were CG-genotype and (6) were GG-

genotype with percentages (35.5%, 54.8% and 9.7% respectively) (Fig. 2.and Table (2).

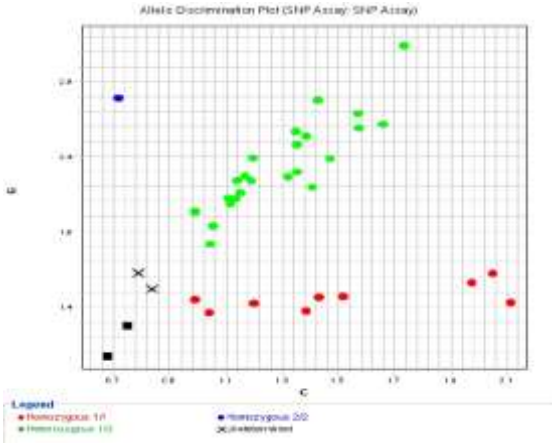

Fig. 1. Allelic discrimination blot for SNP rs 1862513 C/G in patients

Control and patient groups didn't differ significantly in the genotype frequency of RETN gene (-420 C/G) based upon the additive inheritance model (*P value > 0.05*). For further comparisons, we divided studied individuals depending upon the dominant inheritant model into the CG+GG and CC genotype bearing individuals. Similarly, no noticed significant difference for CG+GG vs. CC genotypes distribution between patient subjects and the healthy controls. Furthermore, not much a difference was seen between patient and control individuals regarding allele frequencies.

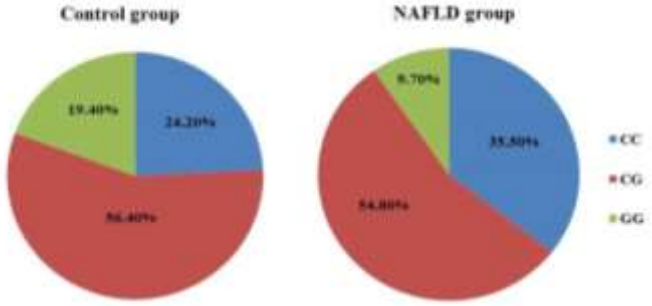

Fig. 2. Genotype frequencies (n,%) in healthy group and patient group

Table 2. Genotypic and allelic frequencies of SNP -420 C>G in control and NAFLD group

Parameter		Control group (n= 62)	NAFLD group (n=62)	OR (95%CI)	*P value*
Genotype frequencies	**CC, n (%)**	15 (24.2%)	22 (35.5%)	Reference	
	CG, n (%)	35 (56.4%)	34 (54.8%)	0.66 (0.29-1.49)	*0.318*
	GG, n (%)	12 (19.4%)	6 (9.7%)	0.34 (0.10-1.11)	*0.069*
Dominant model	**CC, n (%)**	15 (24.2%)	22 (35.5%)	Reference	
	CG+GG , n (%)	47 (75.8%)	40 (64.5%)	0.58 (0.27-1.27)	*0.169*
Allele frequencies	**C–allele , n (%)**	65 (52.4%)	78 (62.9 %)	Reference	
	G–allele , n (%)	59 (47.6%)	46 (37.1 %)	0.65 (0.39-1.08)	*0.095*

P: statistical significance; OR: odds ratio

3.3 Demographic, anthropometric, hematological and biochemical data of patient group among different Resistin gene -420C/G (rs1862513) genotypes and genotype groups:

Table (3) showed demographic, anthropometric and hematological data among different genotypes and CG+GG *vs.* CC subjects in patients. Significant differences among the studied genotypes regarding age (*P=0.032*), BMI (*P=0.001*), Hb (*P=0.028*), WBCs (*P=0.001*), AST (*P<0.001*), Alb (*P=0.004*), total bilirubin (*P=0.05*), direct bilirubin (*P=0.035*) and HOMA-IR (*P=0.001*) were noticed.

G allele harboring patients reported significantly higher fibrosis score (*P=0.033*), higher AST levels (*P=0.002*), lower Alb (*P=0.001*), higher total bilirubin (*P=0.035*) and higher insulin resistance (*P=0.022*) than CC wild type bearing patients. However, no reported significant differences

regarding sex, PLT, INR, ALT, TG, FBS and fasting insulin concentrations were reported comparing different genotypes and genotype group.

Table 3. Demographic, anthropometric, hematological and biochemical data of patients among different genotypes and genotype groups of Resistin gene -420C/G (rs1862513):

Parameter	CC	CG	GG	*P* value[a]	*P* value[b]
Demographic and anthropometric data					
Sex (M/F,%)	45.5/54.5%	35.3/64.7%	35.3/64.7%	*0.333*	*0.677*
Age (years)	54.18±11.58[a]	54.44±11.28[a]	67.00±1.41[b]	***0.032***	*0.483*
BMI (kg/m^2)	32.26±3.02[a]	31.39±3.44[a]	26.68±0.15[b]	***0.001***	*0.087*
Hematological data					
PLT (x10^3/ µL)	210.04±32.38	192.54±51.44	221±3.85	*0.188*	*0.280*
Hb (g/dL)	10.07±1.52[a]	11.75±2.88[b]	9.78±0.11[ab]	***0.028***	*0.051*
WBC (x10^3/ µL)	8.27±1.17[a]	8.30±3.88[a]	2.86±0.12[b]	***0.001***	*0.418*
INR	1.12±0.12	1.13±0.12	1.02±0.02	*0.162*	*0.914*
Fibrosis score (NFS)	0.36±0.34	0.63±0.58	0.67±0.01	*0.102*	***0.033***
Biochemical data					
ALT (U/L)	65.58±10.21	66.32±8.64	66±2.09	*0.957*	*0.776*
AST (U/L)	58.47±9.52[a]	67.81±14.77[b]	85.5±1.87[c]	***< 0.001***	***0.002***
TG (mg/dL)	143.41±40.18	128.71±33.50	129.17±1.72	*0.211*	*0.077*
Alb (g/dL)	3.42±0.16[a]	3.23±0.23[b]	3.3±0.09[ab]	***0.004***	***0.001***
Total bilirubin (mg/dL)	0.73±0.51[a]	0.99±0.53[ab]	1.25±0.05[b]	***0.05***	***0.035***
Direct bilirubin (mg/dL)	0.22±0.21[a]	0.34±0.18[b]	0.19±0.03[ab]	***0.035***	*0.063*
FBS (mg/dL)	197.37±34.3	196.24±28.33	222.5±1.87	*0.141*	*0.655*
Fasting insulin (µIU/mL)	6.44±0.54[a]	6.51±3.05[a]	4.28±0.07[b]	*0.099*	*0.669*
HOMA-IR	2.91±0.68[a]	3.92±1.3[b]	2.35±0.02[a]	***0.001***	***0.022***

- Sex was expressed as number and percentage
- Other values were presented as mean ±S.D ;Significance at *P value ≤ 0.05*
- The dissimilar superscript litters within the same parameter are significantly different
- *P* value[a] : CC/CG/GG
- *P* value[b] : CG+GG/CC

3.4 Demographic, anthropometric, haematological and biochemical data of different fibrosis groups

Our study indicated that fibrosis progression may have accompanied with age with fibrosis level and NFS increased with age (*P=0.002*). Conversely, hematological parameters reduced significantly in count of PLTs, WBCs and Hb levels (*P<0.001*) while INR (*P=0.022*) increased significantly in correlation to fibrosis level. Biochemical parameters showed significant increase regarding AST (*P=0.003*), total, direct bilirubin, FBS (*P<0.001*) and HOMA-IR (*P=0.011*) associating with fibrosis severity while TG significantly decreased (*P=0.003*)(Table 4).

Table 4. Demographics, anthropometric, hematological and biochemical characteristics of different fibrosis groups :

Parameter		F0 group (n=16)	F1 group (n=19)	F2 group (n=13)	F3 group (n=15)	*P* value
Demographic and anthropometric data						
Sex (n,%)	**Male**	7 (43.8%)	6 (31.6%)	10 (76.9%)	3 (20%)	x^2=10.39
	Female	9 (56.3%)	13 (68.4%)	3 (23.1%)	12 (80%)	
Age (years)		48.94±6.69[a]	56.58±13.98[b]	61.46±6.02[b]	62.09±7.45[b]	***0.002***
BMI (kg/m^2)		31.03±2.14	31.69±3.13	29.21±2.79	30.91±2.63	*0.102*
Hematological data						
PLT (x10^3/ µL)		239.46±30.38[a]	222.26±37.79[ab]	203.3±24.06[b]	154.46±13.05[c]	***<0.001***
Hb (g/dL)		13.91±2.32[a]	11.73±1.46[b]	10.3±0.74[c]	9.34±1.08[c]	***<0.001***
WBC (x10^3/ µL)		10.88±2.95[a]	7.86±1.55[b]	6.07±3.43[bd]	4.61±1.89[cd]	***<0.001***

INR	1.09±0.07[a]	1.09±0.08[a]	1.12±0.14[a]	1.21±0.14[b]	***0.022***
Fibrosis score (NFS)	-0.19±0.04[a]	0.49±0.06[b]	0.80±0.13[c]	1.21±0.12[d]	***<0.001***
Biochemical data					
ALT (U/L)	71.69±8.79	67.05±14.57	65.45±7	68.47±10.42	*0.519*
AST (U/L)	59.77±15.73[a]	69.44±9.67[b]	75.7±12.79[bc]	76.28±8.82[bc]	***0.003***
TG (mg/dL)	157.69±15.18[a]	145.75±10.07[ab]	130.31±12.52[bc]	122.67±45.57[c]	***0.003***
Alb (g/dL)	3.45±0.19	3.41±0.13	3.35±0.19	3.31±0.19	*0.160*
Total bilirubin (mg/dL)	0.51±0.18[a]	0.81±0.39[a]	1.37±0.24[b]	1.5±0.77[b]	***<0.001***
Direct bilirubin (mg/dL)	0.16±0.09[a]	0.31±0.22[b]	0.53±0.07[c]	0.52±0.14[c]	***<0.001***
FBS (mg/dL)	190.12±28.49[a]	204.56±21.95[a]	214.31±28.52[a]	338.36±72.9[b]	***<0.001***
Fasting insulin (µIU/mL)	5.98±0.76	6.51±0.87	6.89±3.75	6.13±4.91	*0.885*
HOMA-IR	2.86±0.72[a]	2.99±0.59[a]	3.84±1.77[b]	4.11±0.92[b]	***0.011***

- Sex was expressed as number and percentage
- All other values were showed as mean ±S.D
- *P* value *vs.*control group; x^2: Chisquare
- The dissimilar superscript litters within the same parameter are significantly different
- Significance at *P value ≤ 0.05*

3.5 Genotypic and allelic frequencies of SNP -420 C>G in NAFLD persons with different fibrosis grades:

To evaluate RETN -420 C>G polymorphism association within NAFLD individuals with liver fibrosis, genotypes and alleles frequencies were compared among different grades of NAFLD-fibrosis score. Statistically significant variation was seen in the genotype frequency upon the additive inheritance model (*P<0.001*). Regarding CG+GG *vs.* CC individuals, the distribution also differed between different stages groups. CG+GG genotypes appeared significantly more frequently within F3 stage compared to F0 stage (OR=2.56 (95% CI; 1.53-4.25); *P=0.007*). F3 stage had CG+GG genotypes more frequently than F1 stage (OR=3.00 (95% CI; 1.64-5.49); *P<0.001*). No significant change was noticed in CG+GG genotypes distribution among F3 and F2 stages (Table 5).

Table 5. Genotype frequencies of RETN -420 C>G polymorphism within NAFLD subjects with different fibrosis grades:

Genotype	**F0**	**F1**	**F2**	**F3**
CC	7 (43.8%)	12 (63.2%)	3 (23.1%)	0 (0.0%)
CG	9 (56.3%)	7 (36.8%)	4 (30.8%)	14 (100%)
GG	0 (0.0%)	0 (0.0%)	6 (46.2%)	0 (0.0%)
CG+GG	9 (56.3%)	7 (36.8%)	10 (76.9%)	14(100%)
Comparison	**X^2**	***P*-value**	**OR**	**(95%CI)**
CC/CG/GG	40.26	***<0.001***	-	-
CG+GG/CC	15.41 [a]	***0.001***	-	-
	7.99 [b]	***0.007***	2.56	(1.53-4.25)
	13.89 [c]	***<0.001***	3.00	(1.64-5.49)
	3.63 [d]	*0.098*	2.40	(1.49-3.85)

P: statistical significance; X^2 :Chisquare; OR: odds ratio
[a] Difference between F0,F1,F2 and F3.
[b] Difference between F0 and F3
[c] Difference between F1and F3
[d] Difference between F2 and F3

3.6 Demographic, anthropometric, hematological and also biochemical data within particular groups carrying CG+GG or CC genotypes among different fibrosis grades:

PLTs count, Hb conc. and TG concentrations significantly decreased either among individuals carrying CG or GG genotype (*P<0.001, P<0.001* and *P=0.040* respectively) or among those having CC genotype (*P=0.002, P=0.006* and *P=0.038* respectively) in correlation to fibrosis severity. Conversely, INR, NFS, total, direct bilirubin, FBS and also HOMA-IR showed significant rise in CG or GG genotype harboring patients (*P=0.010, P<0.001, P=0.018, P=0.003, P<0.001* and *P=0.029* respectively) and in CC genotype possessing subjects (*P<0.010, P<0.001, P<0.001, P<0.001, P=0.047* and *P=0.028* respectively) relating to fibrosis level.

CG+GG genotype carriers showed significantly elevated age (*P<0.001*) and ALT (*P=0.016*) while lower WBCs (*P<0.001*) count with fibrosis progression. With increasing fibrosis severity, no significant differences were seen regarding BMI, Alb

and fasting insulin in CC genotype and in those from CG+GG genotype group (Table 6).

Table 6. Demographic, anthropometric, hematological and biochemical data within particular groups carrying CG+GG or CC genotypes among different fibrosis grades:

Parameters	Genotype	F0 group	F1 group	F2 group	F3 group	P value
Demographic and anthropometric data						
Sex (M/F,%)	**CG+GG**	33/67%	43/57%	70/30%	21/79%	*0.121*
	CC	57/43%	25/75%	100/0%	----	***0.047***
Age (years)	**CG+GG**	44.44±3.16^{a}	63.85±8.7^{b}	64.12±5.46^{b}	64.4±6.17^{b}	***<0.001***
	CC	54.7±5.41	56.2±13.26	60.33±1.53	----	*0.730*
BMI (kg/m^2)	**CG+GG**	30.26±1.93	30.54±0.86	28.42±2.52	30.76±2.67	*0.099*
	CC	31.68±2.22	32.37±3.78	31.83±2.19	----	*0.897*
Hematological data						
PLT (x10^3/ µL)	**CG+GG**	315.33±121.61^{a}	254±8.69^{ab}	204.12±31.55^{bc}	155.14±13.26^{c}	***<0.001***
	CC	240.86±11.94^{a}	197.91±30.96^{b}	182.67±3.05^{b}	----	***0.002***
Hb (g/dL)	**CG+GG**	14.4±2.09^{a}	12.57±1.78^{b}	10.28±1.33^{c}	9.4±1.09^{c}	***<0.001***
	CC	14.02±2.25^{a}	11.34±0.11^{b}	11.33±0.21^{b}	----	***0.006***
WBC (x10^3/ µL)	**CG+GG**	13.33±1.83^{a}	7.84±2.43^{b}	6.16±4.36^{b}	4.75±1.9^{b}	***<0.001***
	CC	8.78±1.86	8.72±1.55	8.27±0.25	----	*0.885*
INR	**CG+GG**	1.06±0.07^{a}	1.08±0.1^{a}	1.09±0.07^{a}	1.21±0.14^{b}	***0.010***
	CC	1.13±0.04^{a}	1.04±0.04^{b}	1.4±0.02^{c}	----	***<0.001***
NFS	**CG+GG**	-0.21±0.03^{a}	0.45±0.07^{b}	0.76±0.14^{c}	1.18±0.08^{d}	***<0.001***
	CC	-0.14±0.06^{a}	0.51±0.05^{b}	0.85±0.02^{c}	----	***<0.001***
Biochemical data						
ALT (U/L)	**CG+GG**	60.71±6.32^{a}	60.71±4.42^{a}	68.5±4.52^{b}	69.57±9.85^{b}	***0.016***
	CC	75.43±10.42	70.75±17.21	55.33±1.53	----	*0.152*
AST (U/L)	**CG+GG**	65±21.98	78.28±17.58	79.75±10.77	80±7.3	*0.200*
	CC	55.28±6.42^{a}	71.17±10.67^{b}	44.0±1.0^{a}	----	***<0.001***
TG (mg/dL)	**CG+GG**	148±16.89^{a}	139.43±2.57^{a}	128.5±13.88^{ab}	108.54±45.52^{b}	***0.040***
	CC	166±6.95^{a}	143.5±24.96^{b}	136.33±1.53^{b}	----	***0.038***
Alb (g/dL)	**CG+GG**	3.4±0.30	3.34±0.09	3.33±0.17	3.29±0.18	*0.726*
	CC	3.41±0.19	3.41±0.10	3.4±0.26	----	*0.992*
Total bilirubin (mg/dL)	**CG+GG**	0.68±0.24^{a}	1.08±0.22^{ab}	1.24±0.05^{b}	1.53±0.81^{b}	***0.018***
	CC	0.43±0.11^{a}	0.45±00.14^{a}	1.77±0.06^{b}	----	***<0.001***
Direct bilirubin (mg/dL)	**CG+GG**	0.24±0.09^{a}	0.48±0.17^{b}	0.5±0.02^{b}	0.5±0.15^{b}	***0.003***
	CC	0.1±0.02^{a}	0.20±0.18^{a}	0.6±0.1^{b}	----	***<0.001***
FBS (mg/dL)	**CG+GG**	194.78±14.18^{a}	218.71±8.26^{a}	219.57±7.93^{a}	364.33±49.52^{b}	***<0.001***
	CC	184.14±41.07^{a}	193.55±23.25^{a}	239.67±2.52^{b}	----	***0.047***
Fasting insulin (µIU/mL)	**CG+GG**	5.37±0.19	6.47±1.24	6.43±3.95	6.24±5.07	*0.950*
	CC	6.51±0.65	6.53±0.49	6±0.2	----	*0.319*
HOMA-IR	**CG+GG**	2.68±0.22^{a}	3.04±0.07^{a}	3.29±1.75^{a}	4.5±0.87^{b}	***0.029***
	CC	2.59±0.8^{a}	2.62±0.31^{a}	3.56±0.06^{b}	----	***0.028***

- Sex was expressed as percentage; All other values were expressed as mean ±S.D ; Significance at *P value ≤ 0.05*
- The dissimilar superscript litters within the same parameter are significantly different

3.7 Correlations between NFS and TG, FBS, fasting insulin and HOMA-IR:

Multiple correlations between NFS and TG, FBS, fasting insulin and HOMA-IR were reported in table (7). In all our patients, NFS was showed to be correlated positively with FBS whereas negatively correlated with fasting insulin (*r=0.428, P=0.003* and *r=-0.484, P=0.000* respectively). In F0 group, positive correlation among NFS and F.ins. (*r=0.825, P=0.003*). A positive correlation existed between NFS and TG (*r=0.556, P=0.025)* and negative correlations with fasting insulin and HOMA-IR (*r=-0.731, P=0.001* and *r=-0.862, P=0.000*) respectively in F1 group was also detected. In F2 group, NFS associated positively with fasting insulin (*r=0.889, P=0.000*) and HOMA-IR (*r=0.937, P=0.000*).

In patients carrying CC genotype, negative correlations between NFS and fasting insulin (*r=-0.648, P=0.023*) and positive correlations with FBS (*r=0.567, P=0.027*) and HOMA-IR (*r=0.701, P=0.011)* were demonstrated. CC genotype subjects in F0 stage showed positive associations between NFS and TG (*r=0.936, P=0.019*), FBS (*r=0.971,*

P=0.006) and fasting insulin (*r=0.905, P=0.034*). NFS was demonstrated to be negatively correlated with fasting insulin and HOMA-IR (*r=-0.670, P=0.048* and *r=-0.677, P=0.045* respectively) in CC genotype harbors in F1 group. In F2 CC genotype group, correlations between NFS and FBS (*r=1, P=0.000*) and with HOMA-IR (*r=-0.998, P=0.045*) were confirmed.

In patients with CG+GG genotype, NFS was negatively correlated with FBS (*r=-0.526, P=0.012*) and fasting insulin (*r=-0.706, P=0.000*). F0 individuals with CG+GG genotype indicated positive correlations among NFS and fasting insulin (*r=0.965, P=0.002*) and HOMA-IR (*r=0.832, P=0.04*). In CG or GG harboring individuals in F1 stage, positive associations between NFS and FBS (*r=0.919, P=0.003*) and negative correlations with fasting insulin (*r=-0.952, P=0.001*). Regarding CG or GG bearing patients in F2 stage, our results also revealed significant positive correlations between NFS and fasting insulin (*r=0.995, P=0.000*) and HOMA-IR (*r=0.997, P=0.000*) and negative correlations with TG (*r=-0.965, P=0.000*) and FBS (*r=-0.974, P=0.000*).

Table 7. Correlations between NFS and TG, FBS, fasting insulin and HOMA-IR:

Parameter	Groups	All patients	F0 stage	F1 stage	F2 stage	F3 stage
TG	**Both**	*r=-0.167* N.S.	*r=-0.119* N.S.	***r=0.556 P=0.025***	*r=0.058* N.S.	*r=0.412* N.S.
	CG+GG	*r=0.03* N.S.	*r=-0.794* N.S.	*r=-0.591* N.S.	***r=-0.965 P=0.000***	*r=-0.048* N.S.
	CC	*r=-0.341* N.S.	***r=0.936 P=0.019***	*r=0.098* N.S.	*r=0.954* N.S.	---
FBS	**Both**	***r=0.428 P=0.003***	*r=-0.490* N.S.	*r=0.002* N.S.	*r=-0.543* N.S.	*r=-0.335* N.S.
	CG+GG	***r=-0.526 P=0.012***	*r=-0.197* N.S.	***r=0.919 P=0.003***	***r=-0.974 P=0.000***	*r=0.437* N.S.
	CC	***r=0.567 P=0.027***	***r=0.971 P=0.006***	*r=0.163* N.S.	***r=1.000 P=0.000***	---
Fasting insulin	**Both**	***r=-0.484 P=0.000***	***r=0.825 P=0.003***	***r=-0.731 P=0.001***	***r=0.889 P=0.000***	*r=-0.206* N.S.
	CG+GG	***r=-0.706 P=0.000***	***r=0.965 P=0.002***	***r=-0.952 P=0.001***	***r=0.995 P=0.000***	*r=-0.214* N.S.
	CC	***r=-0.648 P=0.023***	***r=0.905 P=0.034***	***r=-0.670 P=0.048***	*r=-0.993* N.S.	---
HOMA-IR	**Both**	*r=0.200* N.S.	*r=-0.240* N.S.	***r=-0.862 P=0.000***	***r=0.937 P=0.000***	*r=-0.050* N.S.
	CG+GG	*r=0.071* N.S.	***r=0.832 P=0.04***	*r=-0.345* N.S.	***r=0.997 P=0.000***	*r=0.075* N.S.
	CC	***r=0.701 P=0.011***	*r=0.000* N.S.	***r=-0.677 P=0.045***	***r=-0.998 P=0.045***	---

r= Correlation coefficient , N.S. = Non significant with *P value > 0.05*

3.8 ROC curve for some biochemical parameters to give the evidence as potential diagnostic biomarkers for NAFLD and G allele NAFLD carrying subjects:

Fig.3. illustrates ROC curve of direct bilirubin and FBS concentrations to discriminate NAFLD patients from healthy controls. FBS has the strongest diagnostic value for NAFLD with an area under curve (AUC) 1.000 (95% CI: 1.000-1.000, *P=0.000*) with an optimal cut-off point of 141 associated with sensitivity of 100% and specificity of 100%. Direct bilirubin has a fair AUC 0.781 (95% CI: 0.675-0.887, *P=0.000*) associated with an optimal cut-off point, sensitivity and specificity of 0.23, 60% and 100% respectively.

ROC curve for discriminating G allele carrying patients showed AST as being the most accurate for G allele carriers diagnosis (AUC 1.000 , 95% CI: 1.000-1.000, *P=0.000*, optimal cut-off point 39, sensitivity 100% and specificity 100%)(Fig.4.).

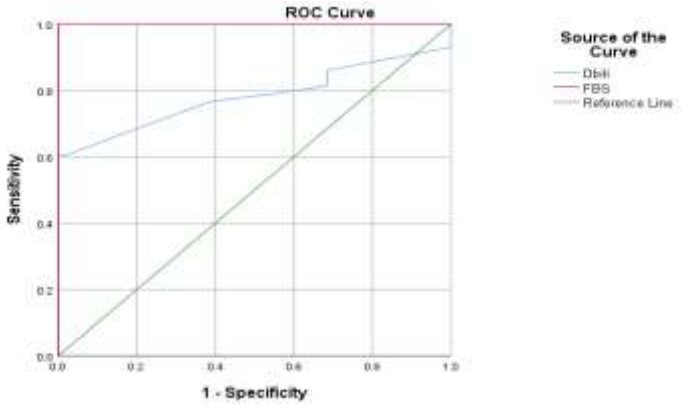

Fig. 3. ROC curve of the diagnostic accuracy of direct bilirubin and FBS concentrations between NAFLD patients and controls

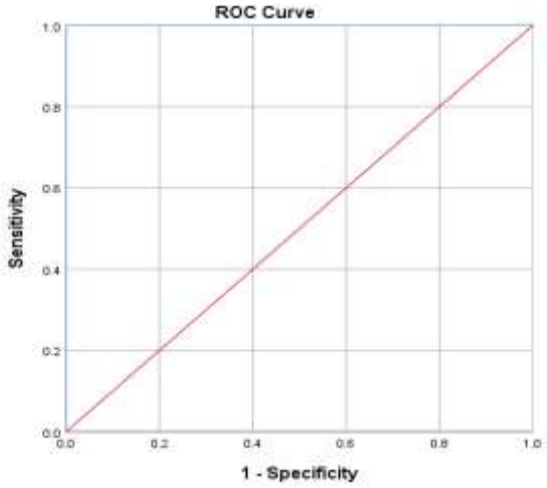

Fig. 4. ROC curve of the diagnostic accuracy of AST for diagnosing G allele carrying patients.

4. Discussion

NAFLD is globally the most prominent chronic hepatic disorder in 21st century [24, 25]. NAFLD includes a wide spectrum of progressive hepatic disorders varing from simple steatosis to steatohepatitis (NASH), fibrosis then cirrhosis and even HCC [34-37].

NAFLD had been verified to aggravate other extrahepatic disorders as CVD [38], T2DM [39] and kidney disease [40]. NAFLD pathogenesis is multifactorial involving dynamic interactions among IR, lipid metabolism, genetic predisposition and other environmental factors [41, 42].

Demographic, anthropometric and the biochemical characteristics among NAFLD participants and healthy persons were first studied. Regarding gender role in NAFLD occurrence, results are conflicting. While a lot of studies have confirmed NAFLD prevalence in men [43, 44], [45-47] with our study have reported the higher NAFLD prevalence among female gender.

Regarding gender distribution among control and NAFLD groups, our NAFLD group included (41.3%) males and (58.7%) females. Conversely, [48-52] noticed no significant variation regarding gender distribution among patient and control group.

NAFLD persons were older than control group participants (55.57±11.29 *vs.* 37.63±15.21 respectively). Our results agreed also with [53-56]. Opposite to previous mentioned results, [47, 49, 57-59] reported no notable difference for age between NAFLD participants and control group.

Multiple studies confirmed the relation between NAFLD and increased BMI [60-62]. Higher BMI patients have more adipose tissue and fatty acids amount reaching liver is rising. Additionally, patients with higher BMI frequently follow a high-fat diet for a prolonged time, which increases exogenous fat absorption and causes arise in fatty acids and their lipidation in the liver [63, 64]. These data along with [55, 56, 65, 66] went also with our results.

During NAFLD onset and progression, excessive lipid deposition and also oxidative stress could impair the mitochondrial functions through inflammatory mediators and then affected thrombopoietin synthesis [67]. PLTs counts reduction could occur within this process.

In addition, our study displayed significantly lower PLTs count in our patients comparing with controls (201.98±44.02 *vs.* 247.76±73.29 respectively). Several studies including those by [68, 69] agreed with our work .Whereas [47, 48, 55, 57] demonstrated no significant differences in PLTs count between patient and healthy participants. [44] disagreed with us and reported significant higher PLTs count in cases comparing to controls.

Current study showed no statistical significant difference among NAFLD studied patients and those healthy people in Hb concentration and INR. Our data were also with [47, 48] who didn't reveal significant differences for INR and Hb conc. between controls and cases. NAFLD patients had higher WBCs count than those included in controls (9.1±2.65 *vs.* 5.92±1.56 respectively). Similar results by [55, 70]. While [47] reported the opposite, [48] revealed no significant change within study groups in WBCs count.

Our studied NAFLD patients recorded higher NFS than healthy individuals (0.56±0.51 *vs.* -2.6±0.72 respectively). An Egyptian study conducted by [47] confirmed our findings.

ALT and even AST activities of patients were significantly elevated than in those not having NAFLD (for ALT 65.83±8.77 *vs.* 19.57±8.94 respectively and for AST 67.51±14.78 *vs.* 21.98±8.54 respectively). Similar study results were reported by [71, 72] in Asian populations. Those results were also corroborated with [54, 58, 66, 73] findings.

As opposed to healthy persons, patients had significantly raised TG concentrations (141.96±20.07 *vs.* 95.81±14.97 respectively). This result agrees with [51, 54, 55, 56, 66] and with [74] who reported hyperlipidemia as a distinct predictor of NAFLD onset. Our results also demonstrated subjects with NAFLD to have significant lower albumin levels comparing with control group (3.31±0.22 *vs.* 4.13±0.21 respectively). Our results were comparable to findings from [44, 68].

When compared with healthy group, while total bilirubin levels didn't differ significantly between our group of patients and controls, levels of direct bilirubin were higher significantly among patients than healthy individuals (0.32±0.2 *vs.* 0.15±0.04, $P < 0.001$). However, [47, 48, 55, 57] demonstrated no difference among cases and our controls for both direct and total bilirubin ($P > 0.05$).

When comparing NAFLD individuals to normal controls, a significant high FBS concentrations (216.49±32.59 *vs.* 99.28±7.99 respectively) was reported agreeing with [51, 57]. Regarding concentrations of insulin and HOMA-IR, patients having NAFLD had a statistically significantly increased insulin concentrations and HOMA-IR than controls (5.37±1.25 *vs.* 3.22±0.65 for insulin and 3.42±1.2 *vs.* 0.80±0.19, with $P<0.001$ for both). Similarly, previous findings have been stated by [54].

Resistin often referred as adipose tissue-specific secretory factor is a cysteine-rich peptide adipocytokine. It belongs to resistin-like molecules gene family (RELM) and known also as "found in inflammatory zone" (FIIZ), it acts as a pro-inflammatory adipokine [75, 76]. Resistin exhibited profibrogenic effects via triggering HSCs [77,78]. NAFLD patients G allele relation with higher fibrosis score, higher AST activities, lower Alb, higher total bilirubin and higher insulin resistance than CC wild genotype was also showed.

In addition, our study indicated that fibrosis level and NFS increased with advancing age. While INR, AST, Total, direct bilirubin, FBS and HOMA-IR increased, PLTs, WBCs count, Hb and TG decreased significantly in correlation to fibrosis level. Partially going with our results, [79] noticed that higher AST, FBS, insulin and also HOMA-IR, but lower albumin concentrations were linked to more severe fibrosis however, no significant alterations regarding age, TG, ALT, BMI and PLT was found.

Moreover, advanced fibrosis association with elevated BMI, HOMA-IR, activities of ALT, of AST, INR, NFS and reduced PLTs count and also albumin levels [80] was showed. [81] also revealed higher age and HOMA-IR association as liver fibrosis progressed. Our results along with those by [82, 83] noticed that PLT count [84] correlated inversely with hepatic fibrosis stage in NAFLD persons.

PLTs count, Hb and TG conc.s significantly decreased while INR, NFS, total, direct bilirubin, FBS and IR significantly increased either among

CG+GG genotype bearing individuals or among CC genotype possessing patients in correlation to fibrosis severity. Additionally, CG+GG genotype carriers had statistically significant higher age and ALT whereas lower WBCs count with fibrosis progression.

Parallel with our results, several studies reported G allele association with higher age [85]. In disagreement with us, [86] reported no significant relation regarding age and ALT levels with G allele presence. Controversial results were presented by [86] showing higher WBCs count and lower age by [87] study with existence of G allele.

Significant correlations noticed within our study were also confirmed by the positive correlations among NFS and FBS among Egyptian patients in Ragab *et al*., [88] study and TG, FBS, fasting insulin and HOMA-IR in [89] study. However, no genotyping in those previously mentioned studies was made.

Our study stated that the best diagnostic tool was FBS (AUC 1.000) followed by direct bilirubin (AUC 0.781) that also proved as a good tool in detecting NAFLD patients with100% and 60% sensitivity for FBS and direct bilirubin respectively and 100% specificity for both. AST displayed the greatest AUC, sensitivity and also specificity values (1.000, 100%, 100% respectively) for detecting all G allele possessing patients.

5. Conclusion

In conclusion, this study provided evidence about absence of association among RETN -420C>G SNP and NAFLD susceptibility within Egyptian population, but proved association with increased NAFLD associated fibrosis risk. G allele harboring patients reported significantly higher fibrosis score, AST levels, total bilirubin and IR whereas lower Alb than CC wild type bearing patients. Moreover, G allele associations with higher age and ALT activities whereas lower WBCs count with fibrosis progression was also indicated.

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; PCR, polymerase Chain Reaction; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, Triglyceride; WBCs, White Blood Cells count; PLT, Platelet count; FBS, Fasting Blood Sugar; NFS, Non-alcoholic fatty liver Fibrosis Score; HOMA-IR, homeostasis model assessment-insulin resistance; INR, International normalized ratio; BMI, Body mass index; PT, prothrombin time.

6. Conflict of interest

None

7. Funding

This research did not receive any specific grant.

8. References

[1] Mokdad, A. H., Forouzanfar, M. H., Daoud, F., Mokdad, A. A., El Bcheraoui, C., Moradi-Lakeh, M., ... & Murray, C. J. (2016). Global burden of diseases, injuries, and risk factors for young people's health during 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*, *387*(10036), 2383-2401.

[2] Asrani, S. K., Devarbhavi, H., Eaton, J., & Kamath, P. S. (2019). Burden of liver diseases in the world. *Journal of hepatology*, *70*(1), 151-171.

[3] Marjot, T., Moolla, A., Cobbold, J. F., Hodson, L., & Tomlinson, J. W. (2020). Nonalcoholic fatty liver disease in adults: current concepts in etiology, outcomes, and management. *Endocrine Reviews*, *41*(1), 66-117.

[4] Ding, L., Oligschlaeger, Y., Shiri-Sverdlov, R., & Houben, T. (2022). Nonalcoholic fatty liver disease. *Handbook of Experimental Pharmacology*, 270:233-269.

[5] Younossi, Z., Anstee, Q. M., Marietti, M., Hardy, T., Henry, L., Eslam, M., ... & Bugianesi, E. (2018). Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nature reviews Gastroenterology & hepatology*, *15*(1), 11-20.

[6] Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., & Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease—meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*, *64*(1), 73-84.

[7] Younossi, Z. M., Golabi, P., Paik, J. M., Henry, A., Van Dongen, C., & Henry, L. (2022). The most recent and in-depth meta-analytic assessment of the global epidemiology of nonalcoholic fatty liver disease (NAFLD). *South Asia*, *4*(5,475), 2-016.

[8] Le, M. H., Yeo, Y. H., Li, X., Li, J., Zou, B., Wu, Y., ... & Nguyen, M. H. (2022). 2019 global NAFLD prevalence: a systematic review and meta-analysis. *Clinical Gastroenterology and Hepatology*, *20*(12), 2809-2817.

[9] Riazi, K., Azhari, H., Charette, J. H., Underwood, F. E., King, J. A., Afshar, E. E., ... & Shaheen, A. A. (2022). The prevalence and incidence of NAFLD worldwide: a systematic review and meta-analysis. *The Lancet Gastroenterology & Hepatology*, 7, 851-861.

[10] Ge, X., Zheng, L., Wang, M., Du, Y., & Jiang, J. (2020). Prevalence trends in non-alcoholic fatty liver disease at the global, regional and national levels, 1990–2017: a population-based observational study. *BMJ open*, *10*(8), e036663.

[11] Spearman, C. W., Afihene, M., Betiku, O., Bobat, B., Cunha, L., Kassianides, C., ... & Sonderup, M. W. (2021). Epidemiology, risk factors, social determinants of health, and current management for non-alcoholic fatty liver disease in sub-Saharan Africa. *The Lancet Gastroenterology & Hepatology*, *6*(12), 1036-1046.

[12] Alkassabany, Y. M., Farghaly, A. G., & El-Ghitany, E. M. (2014). Prevalence, risk factors, and predictors of nonalcoholic fatty liver disease among schoolchildren: a hospital-based study in Alexandria, Egypt. *Arab Journal of Gastroenterology*, *15*(2), 76-81.

[13] Tomah, S., Hamdy, O., Abuelmagd, M. M., Hassan, A. H., Alkhouri, N., Al-Badri, M. R., ... & Eid, E. A. (2021). Prevalence of and risk factors for non-alcoholic fatty liver disease (NAFLD) and fibrosis among young adults in Egypt. *BMJ open gastroenterology*, *8*(1), e000780.

[14] Zaki, M., Ezzat, W., Elhosary, Y., & Saleh, O. (2014). Predictors of non-alcoholic fatty liver disease in Egyptian obese adolescents. *International Scholarly and Scientific Research & Innovation*, *8*(9):657–660.

[15] Eldesoky, A. E. E., Gad, Y. Z., & Ahmed, N. (2013). Nonalcoholic fatty liver disease in young adult Egyptian women with polycystic ovary syndrome. *Egyptian Liver Journal*, *3*(1), 15-19.

[16] Su, Q., Kim, S. Y., Adewale, F., Zhou, Y., Aldler, C., Ni, M., ... & Cheng, X. (2021). Single-cell RNA transcriptome landscape of hepatocytes and non-parenchymal cells in healthy and NAFLD mouse liver. *Iscience*, *24*(11), 103233.

[17] Chen, T., Dalton, G., Oh, S. H., Maeso-Diaz, R., Du, K., Meyers, R. A., ... & Diehl, A. M. (2023). Hepatocyte smoothened activity controls susceptibility to insulin resistance and nonalcoholic fatty liver disease. *Cellular and Molecular Gastroenterology and Hepatology*, *15*(4), 949-970.

[18] Cheu, J. W. S., Lee, D., Li, Q., Goh, C. C., Bao, M. H. R., Yuen, V. W. H., ... & Wong, C. C. L. (2023). Ferroptosis suppressor protein 1 inhibition promotes tumor ferroptosis and anti-tumor immune responses in liver cancer. *Cellular and Molecular Gastroenterology and Hepatology*, *16*(1), 133-159.

[19] Kwok, R., Choi, K. C., Wong, G. L. H., Zhang, Y., Chan, H. L. Y., Luk, A. O. Y., ... & Wong, V. W. S. (2016). Screening diabetic patients for non-alcoholic fatty liver disease with controlled attenuation parameter and liver stiffness measurements: a prospective cohort study. *Gut*, *65*(8), 1359-1368.

[20] Azuma, K., Oguchi, S., Matsubara, Y., Mamizuka, T., Murata, M., Kikuchi, H., ... & Saruta, T. (2004). Novel resistin promoter polymorphisms: association with serum resistin level in Japanese obese individuals. *Hormone and metabolic research*, *36*(08), 564-570.

[21] Bik, W., Ostrowski, J., Baranowska-Bik, A., Wolinska-Witort, E., Bialkowska, M., Martynska, L., & Baranowska, B. (2010). Adipokines and genetic factors in overweight or obese but metabolically healthy Polish women. *Neuroendocrinology Letters*, *31*(4), 497-506.

[22] Boumaiza, I., Omezzine, A., Rejeb, J., Rebhi, L., Ben Rejeb, N., Nabli, N., ... & Bouslama, A. (2012). Association between four resistin polymorphisms, obesity, and metabolic syndrome parameters in Tunisian volunteers. *Genetic Testing and Molecular Biomarkers*, *16*(12), 1356-1362.

[23] Trinder, P. (1969). Enzymatic determination of glucose in blood serum. *Ann. Clin. Biochem*, *6*, 24.

[24] Fossati, P., & Prencipe, L. (1982). Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clinical chemistry*, *28*(10), 2077-2080.

[25] Holbrook, A., Schulman, S., Witt, D. M., Vandvik, P. O., Fish, J., Kovacs, M. J., ... & Guyatt, G. H. (2012). Evidence-based management of anticoagulant therapy: antithrombotic therapy and prevention of thrombosis: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest*, *141*(2), e152S-e184S.

[26] Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., & Turner, R. C. (1985). Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *diabetologia*, *28*, 412-419.

[27] Angulo, P., Hui, J. M., Marchesini, G., Bugianesi, E., George, J., Farrell, G. C., ... & Day, C. P. (2007). The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology*, *45*(4), 846-854.

[28] Mullis, K. B., & Faloona, F. A. (1987). [21] Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. In *Methods in enzymology* (Vol. 155, pp. 335-350). Academic Press.

[29] Afonina, I., Zivarts, M., Kutyavin, I., Lukhtanov, E., Gamper, H., & Meyer, R. B. (1997). Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic acids research*, *25*(13), 2657-2660.

[30] Kutyavin, I. V., Lukhtanov, E. A., Gamper, H. B., & Meyer, R. B. (1997). Oligonucleotides with conjugated dihydropyrroloindole tripeptides: base composition and backbone effects on

hybridization. *Nucleic acids research*, *25*(18), 3718-3723.

[31] Razali, N. M., & Wah, Y. B. (2011). Power comparisons of shapiro-wilk, kolmogorov-smirnov, lilliefors and anderson-darling tests. *Journal of statistical modeling and analytics*, *2*(1), 21-33.

[32] Zheng, K. I., Fan, J. G., Shi, J. P., Wong, V. W. S., Eslam, M., George, J., & Zheng, M. H. (2020). From NAFLD to MAFLD: a" redefining" moment for fatty liver disease. *Chinese medical journal*, *133*(19), 2271-2273.

[33] Eslam, M., Newsome, P. N., Sarin, S. K., Anstee, Q. M., Targher, G., Romero-Gomez, M., ... & George, J. (2020). A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *Journal of hepatology*, *73*(1), 202-209.

[34] Morandi, A., Di Sessa, A., Zusi, C., Umano, G. R., El Mazloum, D., Fornari, E., ... & Maffeis, C. (2020). Nonalcoholic fatty liver disease and estimated insulin resistance in obese youth: a mendelian randomization analysis. *The Journal of Clinical Endocrinology & Metabolism*, *105*(11), e4046-e4054.

[35] Lazarus, J. V., Mark, H. E., Anstee, Q. M., Arab, J. P., Batterham, R. L., Castera, L., ... & Zelber-Sagi, S. (2022). Advancing the global public health agenda for NAFLD: a consensus statement. *Nature Reviews Gastroenterology & Hepatology*, *19*(1), 60-78.

[36] Song, K., Park, G., Lee, H. S., Lee, M., Lee, H. I., Ahn, J., ... & Chae, H. W. (2022a). Trends in prediabetes and non-alcoholic fatty liver disease associated with abdominal obesity among korean children and adolescents: Based on the korea national health and nutrition examination survey between 2009 and 2018. *Biomedicines*, *10*(3), 584.

[37] Song, K., Park, G., Lee, H. S., Lee, M., Lee, H. I., Choi, H. S., ... & Chae, H. W. (2022b). Comparison of the triglyceride glucose index and modified triglyceride glucose indices to predict nonalcoholic fatty liver disease in youths. *The Journal of Pediatrics*, *242*, 79-85.

[38] Targher, G., Day, C. P., & Bonora, E. (2010). Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *New England Journal of Medicine*, *363*(14), 1341-1350.

[39] Tilg, H., Moschen, A. R., & Roden, M. (2017). NAFLD and diabetes mellitus. *Nature reviews Gastroenterology & hepatology*, *14*(1), 32-42.

[40] Byrne, C. D., & Targher, G. (2020). NAFLD as a driver of chronic kidney disease. *Journal of hepatology*, *72*(4), 785-801.

[41] Buzzetti, E., Pinzani, M., & Tsochatzis, E. A. (2016). The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metabolism*, *65*(8), 1038-1048.

[42] Eslam, M., & George, J. (2016). Genetic and epigenetic mechanisms of NASH. *Hepatology international*, *10*, 394-406.

[43] Duseja, A., Das, A., Das, R., Dhiman, R. K., Chawla, Y., Bhansali, A., & Kalra, N. (2007). The clinicopathological profile of Indian patients with nonalcoholic fatty liver disease (NAFLD) is different from that in the West. *Digestive diseases and sciences*, *52*, 2368-2374.

[44] Ciardullo, S., Monti, T., Sala, I., Grassi, G., Mancia, G., & Perseghin, G. (2020). Nonalcoholic fatty liver disease and advanced fibrosis in US adults across blood pressure categories. *Hypertension*, *76*(2), 562-568.

[45] El Saeed, K. M., Ahmed, O. A., Khalifa, M. O., & Fahmy, E. M. (2019). Detection of patatin-like phospholipase domain-containing protein 3 in nonalcoholic fatty liver disease among egyptian patients. *The Egyptian Journal of Internal Medicine*, *31*(3), 273-280.

[46] Liu, S., Weng, R., Gu, X., Li, L., & Zhong, Z. (2021). Association between apolipoprotein E gene polymorphism and nonalcoholic fatty liver disease in Southern China: A case-control study. *Journal of Clinical Laboratory Analysis*, *35*(12), e24061.

[47] Amin, M. A., Ragab, H. M., El Maksoud, N. A. & Elaziz, W. A. (2022). Clinical Significance of Serum CD14 and Its Polymorphism as a Biomarker of Non-Alcoholic Fatty Liver Disease. *J Clin Biomed Invest*, *2*(1), 15-23.

[48] Gad, A. I., Elmedames, M. R., Abdelhai, A. R., & Marei, A. M. (2020). The association between vitamin D status and non-alcoholic fatty liver disease in adults: a hospital-based study. *Egyptian Liver Journal*, *10*(1), 1-8.

[49] Tan, H. L., Zain, S. M., Eng, H. S., Mohamed, Z., Mahadeva, S., Chan, W. K., ... & Mohamed, R. (2020). Allele HLA-DQB1* 06 reduces fibrosis score in patients with non-alcoholic fatty liver disease. *Hepatology Research*, *50*(8), 947-954.

[50] Masroor, M., & Haque, Z. (2021). HbA1C as a biomarker of non-alcoholic fatty liver disease: comparison with anthropometric parameters. *Journal of Clinical and Translational Hepatology*, *9*(1), 15.

[51] Wang, M., Zhang, R., Wang, M., Zhang, L., Ding, Y., Tang, Z., ... & Wang, J. (2021). Genetic Polymorphism of Vitamin D Family Genes CYP2R1, CYP24A1, and CYP27B1 Are Associated With a High Risk of Non-alcoholic Fatty Liver Disease: A Case-Control Study. *Frontiers in genetics*, *12*.

[52] Zhang, R., Wang, M., Wang, M., Zhang, L., Ding, Y., Tang, Z., ... & Wang, J. (2021). Vitamin D level and vitamin D receptor genetic variation were involved in the risk of non-alcoholic fatty liver disease: a case-control study. *Frontiers in endocrinology*, *12*, 648844.

[53] Akkiz, H., Taskin, E., Karaogullarindan, U., Delik, A., Kuran, S., & Kutlu, O. (2021). The influence of RS738409 I148M polymorphism of patatin-like phospholipase domain containing 3 gene on the susceptibility of non-alcoholic fatty liver disease. *Medicine*, *100*(19).

[54] Marjani, S., Nezhadali, M., Hekmat, A., & Yeganeh, M. Z. (2022). Investigating Visfatin gene Polymorphism rs4730153 with Insulin Resistance and Non-Alcoholic Fatty Liver Diseases in Iranian Population. *Iranian Journal of Public Health*, *51*(5), 1143-1151.

[55] Ali, M. A. E.,Galal, G. M., Younis, M. A. A., & Zaghloul, A. M. (2022). Anthropometric Measures as Predictors of Non-Alcoholic Fatty Liver Disease in Adult Asymptomatic Egyptians. *The Egyptian Journal of Hospital Medicine*, *87*(1), 1400-1406.

[56] Lu, C. W., Chou, T. J., Wu, T. Y., Lee, Y. H., Yang, H. J., & Huang, K. C. (2023). Association of Genetic Variations in Lean Adults with Nonalcoholic Fatty Liver Disease in Asian Populations.

[57] Lisboa, Q. C., Nardelli, M. J., de Araújo Pereira, P., Miranda, D. M., Ribeiro, S. N., Costa, R. S. N., ... & Couto, C. A. (2020). PNPLA3 and TM6SF2 polymorphisms in Brazilian patients with nonalcoholic fatty liver disease. *World Journal of Hepatology*, *12*(10), 792.

[58] Rezapour, S., khosroshahi, S.A., Farajnia, H., Mohseni, F., Khoshbaten, M., & Farajnia, S. (2021). Association of 45-bp ins/del polymorphism of uncoupling protein 2 (UCP2) and susceptibility to nonalcoholic fatty liver and type 2 diabetes mellitus in North-west of Iran. *BMC Research Notes*, *14*(1), 1-7.

[59] Damavandi, N., & Zeinali, S. (2021). Association of xenobiotic-metabolizing enzymes (GSTM1 and GSTT 1), and pro-inflammatory cytokines (TNF-α and IL-6) genetic polymorphisms with non-alcoholic fatty liver disease. *Molecular Biology Reports*, *48*(2), 1225-1231.

[60] Borai, I. H., Shaker, Y., Kamal, M. M., Ezzat, W. M., Ashour, E., Afify, M., ... & Elbrashy, M. M. (2017). Evaluation of biomarkers in egyptian patients with different grades of nonalcoholic fatty liver disease. *Journal of Clinical and Translational Hepatology*, *5*(2), 109-118.

[61] Dai, H., Wang, W., Chen, R., Chen, Z., Lu, Y., & Yuan, H. (2017). Lipid accumulation product is a powerful tool to predict non-alcoholic fatty liver disease in Chinese adults. *Nutrition & metabolism*, *14*, 1-9.

[62] Chen, L. D., Huang, J. F., Chen, Q. S., Lin, G. F., Zeng, H. X., Lin, X. F., ... & Lin, Q. C. (2019a). Validation of fatty liver index and hepatic steatosis index for screening of non-alcoholic fatty liver disease in adults with obstructive sleep apnea hypopnea syndrome. *Chinese Medical Journal*, *132*(22), 2670-2676.

[63] Stefan, N., Kantartzis, K., & Häring, H. U. (2008). Causes and metabolic consequences of fatty liver. *Endocrine reviews*, *29*(7), 939-960.

[64] Musso, G., Gambino, R., & Cassader, M. (2009). Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Progress in lipid research*, *48*(1), 1-26.

[65] Tabaeian, S. P., Mahmoudi, T., Sabzikarian, M., Rezamand, G., Dabiri, R., Nobakht, H., ... & Zali, M. R. (2021). The Leu72Met (rs696217 G> T) Polymorphism of the Ghrelin Gene Might Be a Protective Factor for Nonalcoholic Fatty Liver Disease. *Journal of Gastrointestinal & Liver Diseases*, *30*(2).

[66] Song, H., Zhao, Z., Liu, S., Li, C., Zhou, Y., & Xin, Y. (2023). Methylenetetrahydrofolate Reductase Gene rs1801131 and rs1801133 Polymorphisms were Associated with Susceptibility to Coronary Artery Disease and Nonalcoholic Fatty Liver Disease. *Gene Expression*, (000), 0-0.

[67] Adams, L. A., Lymp, J. F., Sauver, J. S., Sanderson, S. O., Lindor, K. D., Feldstein, A., & Angulo, P. (2005). The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology*, *129*(1), 113-121.

[68] Zakarya, Z. M., & Gaafar, A. (2019). Vitamin D and parathyroid hormone levels in Egyptian patients with non-alcoholic fatty liver disease-a case control study. *Am J Biochem*, *9*(1), 3-6.

[69] Milaciu, M. V., Vesa, Ș. C., Bocșan, I. C., Ciumărnean, L., Sâmpelean, D., Negrean, V., ... & Acalovschi, M. (2019). Paraoxonase-1 serum concentration and PON1 gene polymorphisms: relationship with non-alcoholic fatty liver disease. *Journal of clinical medicine*, *8*(12), 2200.

[70] Yu, Y. Y., Cai, J. T., Song, Z. Y., Tong, Y. L., & Wang, J. H. (2018). The associations among Helicobacter pylori infection, white blood cell count and nonalcoholic fatty liver disease in a large Chinese population. *Medicine*, *97*(46).

[71] Hotta, K., Yoneda, M., Hyogo, H., Ochi, H., Mizusawa, S., Ueno, T., ... & Sekine, A. (2010). Association of the rs738409 polymorphism in PNPLA3 with liver damage and the development of nonalcoholic fatty liver disease. *BMC medical genetics*, *11*, 1-10.

[72] Bhatt, S. P., Nigam, P., Misra, A., Guleria, R., Pandey, R. M., & Pasha, M. Q. (2013). Genetic variation in the patatin-like phospholipase domain-containing protein-3 (PNPLA-3) gene in Asian Indians with nonalcoholic fatty liver disease. *Metabolic syndrome and related disorders*, *11*(5), 329-335.

[73] Mohammadi, S., Farajnia, S., Shadmand, M., Mohseni, F., & Baghban, R. (2020). Association of rs780094 polymorphism of glucokinase regulatory

protein with non-alcoholic fatty liver disease. *BMC Research Notes*, *13*(1), 1-5.

[74] Mahmoud, H., Helal, M., Hassan, M., & Sherif, M. (2015). Correlation between anthropometric measures, lipid profile and serum adiponectin and steatosis in nondiabetic nonalcoholic fatty liver disease. *British Journal of Medicine and Medical Research*, *7*(9), 771-778.

[75] Polyzos, S. A., Kountouras, J., & Mantzoros, C. S. (2016). Adipokines in nonalcoholic fatty liver disease. *Metabolism*, *65*(8), 1062-1079.

[76] Chen, S. S., Tang, C. H., Chie, M. J., Tsai, C. H., Fong, Y. C., Lu, Y. C., ... & Wang, S. W. (2019b). Resistin facilitates VEGF-A-dependent angiogenesis by inhibiting miR-16-5p in human chondrosarcoma cells. *Cell Death & Disease*, *10*(1), 31.

[77] Dong, Z. X., Su, L., Brymora, J., Bird, C., Xie, Q., George, J., & Wang, J. H. (2013). Resistin mediates the hepatic stellate cell phenotype. *World journal of gastroenterology: WJG*, *19*(28), 4475-4485.

[78] Saxena, N. K., & Anania, F. A. (2015). Adipocytokines and hepatic fibrosis. *Trends in Endocrinology & Metabolism*, *26*(3), 153-161.

[79] Souza, M. J., Pereira, J. G., de Souza Mangrich, A. C., Pasinato, A. P. B. F., Dantas-Corrêa, E. B., de Lucca Schiavon, L., & Narciso-Schiavon, J. L. (2023). Revista ACTA Revista ACTA Órgano Oficial de SAGE. *Acta Gastroenterol Latinoam*, *53*(1), 39-48.

[80] Ajmera, V., Cepin, S., Tesfai, K., Hofflich, H., Cadman, K., Lopez, S., ... & Loomba, R. (2023). A prospective study on the prevalence of NAFLD, advanced fibrosis, cirrhosis and hepatocellular carcinoma in people with type 2 diabetes. *Journal of Hepatology*, *78*(3), 471-478.

[81] Cetin, E. G., Demir, N., & Sen, I. (2020). The relationship between insulin resistance and liver damage in non-alcoholic fatty liver patients. *The Medical Bulletin of Sisli Etfal Hospital*, *54*(4), 411.

[82] Mohamed, M. S., Youssef, T. M., Abdullah, E. E., & Ahmed, A. E. (2021). Correlation between adiponectin level and the degree of fibrosis in patients with non-alcoholic fatty liver disease. *Egyptian Liver Journal*, *11*(1), 1-10.

[83] Moran-Lev, H., Cohen, S., Webb, M., Yerushalmy-Feler, A., Amir, A., Gal, D. L., & Lubetzky, R. (2021). Higher BMI predicts liver fibrosis among obese children and adolescents with NAFLD-an interventional pilot study. *BMC pediatrics*, *21*, 1-8.

[84] Cao, W., Zhao, C., Shen, C., & Wang, Y. (2013). Cytokeratin 18, alanine aminotransferase, platelets and triglycerides predict the presence of nonalcoholic steatohepatitis. *PloS one*, *8*(12), e82092.

[85] Amal, S., Pasha, H. F., & Rashad, N. M. (2013). Association of resistin gene polymorphisms with insulin resistance in Egyptian obese patients. *Gene*, *515*(1), 233-238.

[86] Suriyaprom, K., Tungtrongchitr, R., & Namjuntra, P. (2015). Associations of resistin levels with resistin gene polymorphism and metabolic syndrome in Thais. Journal of Medical Biochemistry, 34(2), 170.

[87] Miyamoto, Y., Morisaki, H., Kokubo, Y., Yamanaka, I., Tomoike, H., Okayama, A., ... & Morisaki, T. (2009). Resistin gene variations are associated with the metabolic syndrome in Japanese men. *Obesity Research & Clinical Practice*, *3*(2), 65-74.

[88] Ragab, H. M., Attaby, F. A., Abd El Maksoud, N., Amin, M. A., A Elhakim, H. K., & Abd Elaziz, W. (2022). Association between rs738409 and rs139051 SNPs of the PNPLA3 gene and the presence of NAFLD. *Egyptian Journal of Chemistry*, *65*(9), 127-137.

[89] Kassem, A., & Aboelenin, M. M. (2015). STUDY OF THE PLASMA LEVELS OF LEPTIN, INTERLEUKIN-6, AND TUMOR NECROSIS FACTOR-A IN EGYPTIAN PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE.