Original Article

Evaluation of Biomarkers in Egyptian Patients with Different Grades of Nonalcoholic Fatty Liver Disease

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Abstract

Background and Aims: Nonalcoholic fatty liver disease (NAFLD) is a silent disease; its spectrum includes simple steatosis, nonalcoholic steatohepatitis and fibrosis. Pro- and anti-inflammatory cytokines play roles in the pathogenesis of NAFLD and insulin resistance (IR). Moreover, plasma cell antigen-1 (PC-1) is related to IR and associated with NAFLD progression. Therefore, we aimed to detect biomarkers, ultrasonographic and anthropometric findings capable of differentiating NAFLD grades, since most previous investigators were concerned more with NAFLD patients without classifying them into grades. ***Methods:*** A total of 87 NAFLD patients (31 with grade 1 (mild NAFLD), 26 with grade 2 (moderate NAFLD) and 30 with grade 3 (severe NAFLD) were included in the study, in addition to 47 controls (grade 0). All subjects underwent ultrasonographic examination for NAFLD diagnosis. Serum interleukin-10 (IL-10), plasma interleukin-18 (IL-18) and plasma PC-1 levels were determined using enzyme-linked immunosorbent assay. ***Results:*** Homoeostasis model assessment (HOMA)-IR was higher in different NAFLD grades than in controls. Ultrasonographic and anthropometric findings and lipid profile indices (except for high-density lipoprotein cholesterol, which was decreased) were increased with NAFLD progression. Grade 3 patients showed significant increase in levels of IL-18 and significant decrease in IL-10 and PC-1 levels when compared to grade 1 patients. ***Conclusion:*** Anthropometric and ultrasonographic findings were valuable in differentiating NAFLD grades. IR is very important in NAFLD pathogenesis. IL-18, HOMA-index and PC-1 levels could be used to differentiate between NAFLD grades, together with other measurements.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by the presence of extra fat in the liver, exceeding 5–10% of liver weight. Most patients with NAFLD have increased liver fat only (simple steatosis). Some of the patients develop hepatic inflammation, a condition known as nonalcoholic steatohepatitis (NASH), and up to 20% of patients experience progressive hepatic fibrosis and may eventually progress to liver cirrhosis or failure and even hepatocellular carcinoma.[1] Precautionary procedures for NAFLD screening and diagnosis should be taken due to the disturbing increase in the NAFLD worldwide frequency and the moderate development in finding successful medicinal treatment. Primary assessment of early stages of fatty liver requires abdominal ultrasonographic examination, measurement of lipid profile and liver functions, exclusion of hepatitis B and C and alcohol toxicity, and screening for insulin resistance (IR).[2]

The gold standard for NASH diagnosis is liver biopsy. This procedure, however, is invasive, overpriced, and associated with rare but potentially risky complications and sampling errors; hence, it is not appropriate as a screening tool.[3] One of the imaging techniques which is used as noninvasive diagnostic test for NAFLD is ultrasonography, by which the incidence and severity of fatty liver are measured by grading of fatty liver (Grade 1, 2 and 3) according to the hyperechogenicity of the liver tissue, the divergence between liver and diaphragm and the visibility of vascular structures.[1,4,5]

NAFLD pathogenesis involves a multi-hit process. Steatosis which is believed to be initiated by IR is considered as the first hit, while changes in cytokines and oxidative stress are considered as the second hit, resulting in disease progression.[6] Cytokines are produced by T helper cells, which are categorized as T helper 1 cells, secreting pro-inflammatory cytokines such as interleukin (IL)-18,[7] and T helper 2 cells, secreting anti-inflammatory cytokines such as IL-10.[8] Several lines of evidence support a role for IL-18 in the pathogenesis of IR and NAFLD,[6] while only a few studies have examined the role of IL-10 in NAFLD pathogenesis.[3] Beside the insulin-sensitizing effects of IL-10,[9] an imbalance between pro- and anti- inflammatory cytokines has been found in the context of NASH in the liver.[10]

Keywords: NAFLD; Interleukin-18(IL-18); Interleukin-10(IL-10); Plasma cell antigen-1(PC-1).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; HDL, high-density lipoprotein; HOMA-IR, homoeostasis model assessment-insulin resistance; IL, interleukin; IR, insulin resistance; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PC-1, plasma cell antigen-1; ROC, receiver operating characteristic; SCF, subcutaneous fat; SFT, subcutaneous fat thickness; US, ultrasound; VF, visceral fat; VFT, visceral fat thickness; VLDL, very low-density lipoprotein; WC, waist circumference.

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The plasma cell membrane glycoprotein, plasma cell antigen-1 (PC-1), is a type II transmembrane glycoprotein associated with the insulin receptor on the cell surface and inhibits insulin signaling.[11] It has been reported that PC-1 is significantly associated with progression of NAFLD.[12]

Therefore, we aimed to evaluate the significance of some biomarkers as well as ultrasonographic and anthropometric findings in differentiating between NAFLD grades, since most previous studies[13–17] have been more concerned with studying NAFLD patients without classifying them ultrasonographically into grades.

Methods

Subjects

From January 2015 to October 2015, a total of 87 non-diabetic (blood sugar < 126 mg/dL) obese patients with NAFLD, who have never taken any medication for diabetes, and 47 control subjects were recruited from the Liver Clinic, Medical Service Unit at the National Research Center. All patients and controls were examined ultrasonographically. Written informed consent was obtained from all subjects and this study was approved by the Human Ethics Committee of the National Research Center (Code No. 14075).

Exclusion criteria

Patients with hepatitis B or hepatitis C infection, diabetes, splenectomy, cholestasis, coronary artery disease or pregnancy were excluded. Patients with alcohol consumption, cigarette smoking and use of amiodarone, corticosteroids, tamoxifen or methotrexate were also excluded.

Ultrasound (US) examination

US examinations were performed by the same physician using SonoAce R5 (6 MHz; Samsung). Examination of all the patients was done for diagnosing and classifying grades of NAFLD according to Kakrani *et al*.[4] as follows:

Grade-0 (control group): This group included 20 females and 27 males with normal findings on US; their ages ranged from 25 to 57 years, and they were age- and sex-matched with patients (Fig. 1a).
Grade-I (mild NAFLD): This group included 16 females and 15 males, in which the US showed fine diffuse increase in echogenicity of liver texture; their ages ranged from 25 to 58 years (Fig. 1b).
Grade-II (moderate NAFLD): This group included 20 females and 6 males with diffuse increased coarse echogenicity of liver texture and with mild attenuation of US sound beams; their ages ranged from 25 to 60 years (Fig. 1c).
Grade-III (severe NAFLD): This group included 22 females and 8 males with diffuse increased coarse echogenicity of liver texture, resulting in poor visibility of portal vein radicle walls and right hemi diaphragm; their ages ranged from 25 to 60 years (Fig. 1d).

Liver size was demonstrated by measuring distance between upper and lower borders in the mid-clavicular line. Liver parenchyma was examined with sagittal as well as longitudinal guidance of the probe and completed by lateral and intercostal views.[18] Transverse scanning was performed to assess the maximum subcutaneous fat thickness (SFT), which was defined as the distance between the external face of the recto-abdominal muscle and the internal layer of the skin, and visceral fat thickness (VFT), which was defined as the distance between the anterior wall of the aorta and the internal layer of the recto-abdominal muscle perpendicular to the aorta.[19]

Anthropometric measurements

Body mass index (BMI) was determined by dividing weight by squared height (kg/m^2). Waist circumference (WC) was obtained from each subject by measuring at the midpoint between the lower rib margin and the iliac crest using a conventional tape graduated in centimeters (cm). Hip was measured as the greatest abdominal circumference at the level of greater trochanters. Waist-to-hip ratio was calculated by dividing the waist by the hip circumference.

Samples collection

Blood samples (4 mL) were drawn in the morning after 12 hours fasting then divided into three portions. The first portion (2 mL) was left to clot for 30 min at room temperature and then centrifuged at 3000 rpm for serum separation to determine the levels of insulin, IL-10, AST, ALT, albumin, total protein and lipid profile parameters. The second portion (1 mL) was collected in EDTA-containing tubes and centrifuged at 3000 rpm for plasma separation to determine the levels of IL-18 and PC-1. The third portion (1 mL) was collected in a mixture of EDTA and fluoride and centrifuged at 3000 rpm for plasma separation to determine fasting plasma glucose.

Biochemical analyses

Fasting plasma glucose test was performed according to Heinz and Beushausen[20] using the kit supplied by Stanbio Laboratory (USA). Plasma IL-18 levels were assayed by an enzyme-linked immunosorbent assay (ELISA) kit purchased from Wuhan EIAab Science Company (China). Plasma PC-1 level was determined quantitatively by an ELISA kit purchased from Glory Science Company (USA). Serum insulin concentration was determined by an ELISA kit purchased from Monobind Company (USA). Serum interleukin-10 concentration was determined quantitatively by an ELISA kit obtained from R&D Systems Company (USA). Serum AST and ALT activities were determined according to Bermeyer and Horder[21] using a kit obtained from Human Company (Germany). Serum albumin concentration was determined colorimetrically by the BCG-method[22] using a kit obtained from Human Company. Serum total protein concentration was determined colorimetrically by the Biuret method[23] using a kit obtained from Human Company. Lipid profile (total cholesterol, triglycerides, high-density lipoprotein (HDL)-cholesterol) kits were obtained from Stanbio Laboratory. Serum total cholesterol was determined by an enzymatic colorimetric method,[24] while serum triglycerides were determined by an enzymatic colorimetric method according to Fredrickson *et al*.[25] HDL-cholesterol was determined according to Finley *et al*.,[26] while low-density lipoprotein (LDL)-cholesterol was calculated according to the Friedewald *et al*.[27] equation:

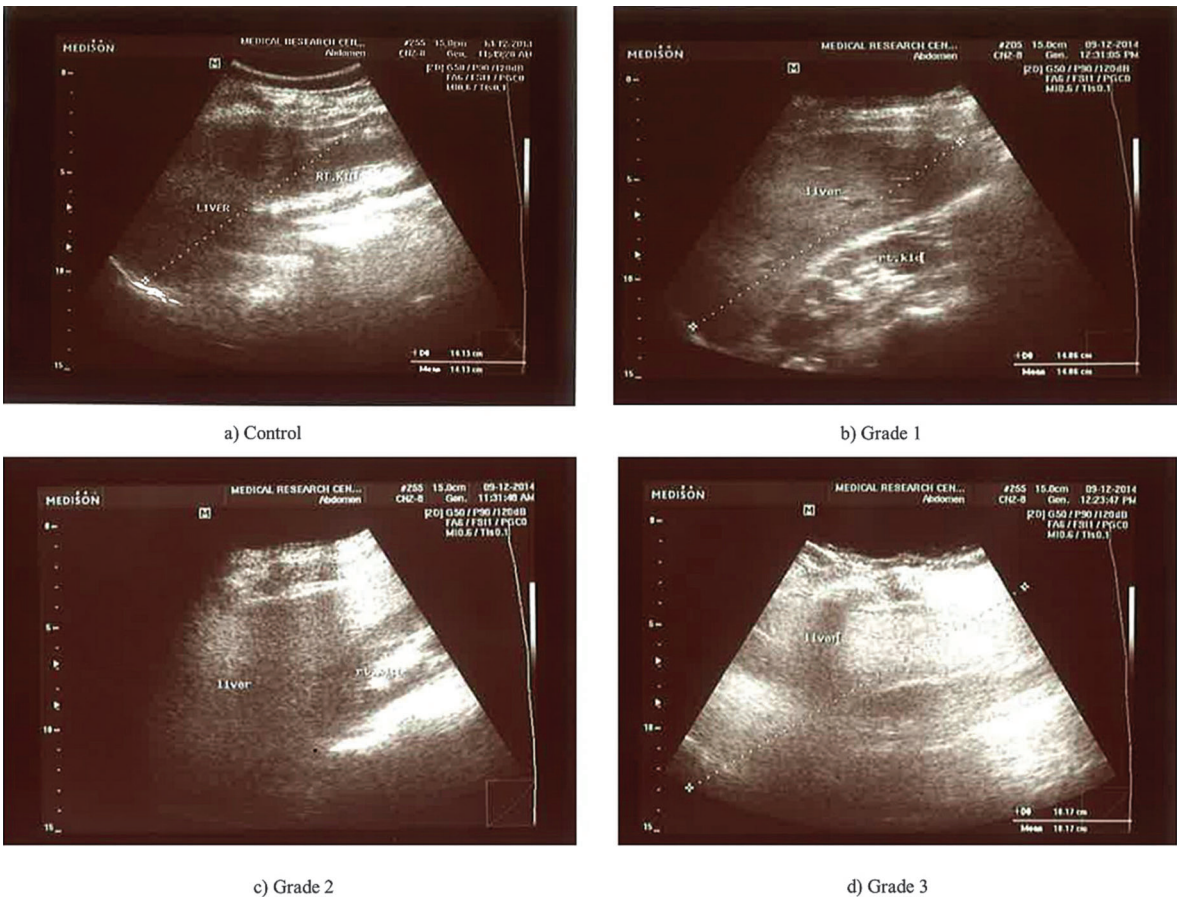

Fig. 1. Grades of fatty liver on visual analysis. Ultrasound image shows (a) normal liver echogenicity, (b) grade 1 fatty liver with increased liver echogenicity, (c) grade 2 fatty liver with the echogenic liver obscuring the echogenic walls of the portal venous branches, and (d) grade 3 fatty liver in which the diaphragmatic outline is obscured.

$$\text{LDL-cholesterol (mg/dL)} = \text{total cholesterol-HDL-triglycerides/5}$$

The IR index was assessed by the homoeostasis model assessment (HOMA)-IR, calculated by the following equation by Matthews *et al*.[28]

$$\text{HOMA-IR} = \text{glucose (mg/dL)} \times \text{insulin (mIU/mL)/22.5}$$

Statistical analyses

The Statistical Package for the Social Sciences software [version 17.0; SPSS, USA] was used. Normally distributed continuous variables were expressed as mean (± SE); qualitative variables were presented as proportions. Quantitative variables were compared using one-way ANOVA; the least significant difference test was used for multiple post-hoc comparisons. On the other hand, qualitative variables were compared using the chi-square [X^2] test or Fischer's exact test. Variables which were significant on univariate analysis were included in the multivariate analysis and independent variables with $p > 0.1$ were excluded sequentially from the models. The odds ratios and associated p-values of the remaining variables are reported. Two-sided p-values ≤ 0.05 were considered statistically significant.

Results

There were significant differences in sex distribution (males) among grade 2 and grade 3 when compared to grade 0 (Table 1). There were significant differences in age, BMI, waist and hip circumference, diastolic and systolic blood pressure, size of liver and spleen, subcutaneous fat (SCF) and visceral fat (VF) in different NAFLD grades when compared to controls (Table 1).

Age and BMI were significantly higher in grade 2 and grade 3 than in grade 1. It is worth noting that age and BMI were significantly higher in grade 3 than in grade 2. Concerning systolic blood pressure and diastolic blood pressure, there were no significant differences among the NAFLD grades. WC and hip circumference were significantly higher among grade 2 and grade 3 compared to grade 1. Liver size and SCF were significantly higher among grade 2 and grade 3 patients compared to grade 1 patients, while grade 3 patients showing more highly significant differences than grade 2 (Table 1).

Insulin, HOMA-IR and fasting plasma glucose were significantly higher in NAFLD grades than in grade 0. In addition, insulin and HOMA-IR were significantly higher in grade 3 than grade 1. On the other hand, no significant differences ($p > 0.05$) were found in AST, ALT albumin or total protein levels between the NAFLD grades ($p > 0.05$) (Table 2).

Table 1. Demographic characteristics of NAFLD patients and control subjects

Variable / Groups		Grade 0 (n = 47)	Grade 1 (n = 31)	Grade 2 (n = 26)	Grade 3 (n = 30)
Age (years)		35.80 ± 1.45	44.51 ± 1.81^{a***}	51.12 ± 2^{a***,b**}	59.4 ± 1.86$^{abc:***}$
Sex	Male	27 (57.4%)	15 (48.4%)	6 (23.1%)^{a**}	9 (30.0%)^{a*}
	Female	20 (42.6%)	16 (51.6%)	20 (76.9%)	21 (70.0%)
BMI (kg/dL)		22.70 ± 0.24	32.49 ± 0.7^{a***}	39.05 ± 1.13$^{ab:***}$	43.90 ± 1.15$^{abc:***}$
DBP (mmHg)		111.49 ± 1.79	126.77 ± 2.71^{a***}	125.96 ± 2.80^{a***}	126.4 ± 2.35^{a***}
SBP (mmHg)		72.98 ± 1.65	84.03 ± 2.34^{a***}	85.38 ± 1.61 ^{a***}	81.1 ± 1.84^{a**}
WC (cm)		81.55 ± 0.81	102.58 ± 1.57^{a***}	108.88 ± 2.06^{a***,b*}	112.13 ± 2.46$^{ab:***}$
HC (cm)		100.74 ± 1.02	114.22 ± 0.96^{a***}	123.76 ± 2.36$^{ab:***}$	124.53 ± 1.63$^{ab:***}$
W/H ratio		0.81 ± .006	0.89 ± .013^{a***}	0.88 ± .013^{a***}	0.89 ± .012^{a***}
Liver size (cm)		13.52 ± 0.203	14.79 ± 0.179^{a***}	16.27 ± 0.175$^{ab:***}$	18.04 ± 0.178$^{abc:***}$
Spleen size (cm)		9.14 ± 0.201	10.51 ± 0.388^{a***}	11.05 ± 0.194^{a***}	10.99 ± 0.223^{a***}
SCF (cm)		1.20 ± 0.05	1.72 ± 0.105^{a**}	2.29 ± 0.167^{a***,b**}	2.69 ± 0.203$^{ab:***,c*}$
VF (cm)		3.09 ± 0.203	5.71 ± 0.342^{a***}	6.04 ± 0.355^{a***}	8.22 ± 0.260$^{abc:***}$

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; WC, waist circumference; HC, hip circumference; W/H, waist/hip; SCF, subcutaneous fat; VF, visceral fat.

Data are presented as mean ± SE, a: significant difference from grade 0, b: significant difference from grade 1, c: significant difference from grade 2.

*p < 0.05, **p < 0.01, ***p < 0.001.

In different NAFLD grades, lipid profile parameters (total cholesterol, triacylglycerol, LDL-cholesterol and very low-density lipoprotein (VLDL)-cholesterol) were significantly higher when compared to grade 0, while there was significant decrement in HDL between NAFLD grades and grade 0. However, comparing the different NAFLD grades to each other, there was significant differences in the lipid profile parameters (Table 2).

IL-18 was significantly higher in grade 3 compared to the other grades. On the other hand, IL-10 was significantly higher in grade 1 compared to grades 0, 2 and 3. PC-1 level was significantly higher in grade 1 compared to both grade 0 and grade 3 (Table 2)

Receiver operating characteristic (ROC) curve was constructed to determine the threshold value for optimal

Table 2. Biochemical data among NAFLD grades and controls

Variable / Groups	Grade 0 (n = 47)	Grade 1 (n = 31)	Grade 2 (n = 26)	Grade 3 (n = 30)
FBG (mg/dL)	83.90 ± 1.13	95.14 ± 2.37^{a**}	98.13 ± 5.38^{a**}	103.66 ± 4.33^{a***}
Insulin (mIU/mL)	6.56 ± 0.24	8.74 ± 0.67^{a*}	10.55 ± 0.94^{a***}	11.16 ± 1.01^{a***,b*}
HOMA-IR index	1.33 ± 0.05	1.99 ± 0.14^{a**}	2.5 ± 0.24^{a***}	2.82 ± 0.28^{a***,b**}
Albumin (g/dL)	4.24 ± 0.11	4.3 ± 0.11	4.38 ± 0.18	4.41 ± 0.12
Total protein (g/dL)	7.69 ± 0.14	8.01 ± 0.17	7.80 ± 0.24	7.87 ± 0.23
AST (IU/L)	14.11 ± 1.16	15.3 ± 0.70	15.9 ± 1.81	17.4 ± 1.07
ALT (IU/L)	13.36 ± 1.60	14.1 ± 0.86	15.3 ± 1.34	16.04 ± 0.68
Total cholesterol (mg/dL)	179.37 ± 4.11	250.01 ± 10.41^{a***}	261.97 ± 9.25^{a***}	289.14 ± 10.36^{a***,b**,c*}
Triacylglycerol (mg/dL)	106.81 ± 3.05	186.93 ± 12.63^{a***}	213.23 ± 11.71^{a***}	255.43 ± 10.99$^{ab:***,c**}$
HDL-cholesterol (mg/dL)	72.10 ± 3.25	53.81 ± 2.37^{a***}	50.15 ± 4.61^{a***}	40.66 ± 1.37^{a***,b**,c*}
LDL-cholesterol (mg/dL)	85.90 ± 5.40	158.81 ± 9.97^{a***}	169.16 ± 9.67^{a***}	197.40 ± 10.48^{a***,b**,c*}
VLDL (mg/dL)	21.36 ± 0.61	37.38 ± 2.52^{a***}	42.65 ± 2.34^{a***}	51.07 ± 2.19$^{ab:***,c**}$
IL-18 (pg/mL)	10.54 ± 0.55	11.47 ± 0.61	10.55 ± 0.94	14.81 ± 1.62^{a**,b*,c**}
IL-10 (pg/mL)	4.87 ± 0.205	7 ± 0.688^{a***}	5.38 ± 0.412^{b*}	5.19 ± 0.411^{b**}
PC-1 (pg/mL)	5 ± 0.27	8.45 ± 1.51^{a**}	6.52 ± 1.18	5.45 ± 0.77^{b*}

Abbreviations: FPG, fasting plasma glucose; HOMA-IR, homoeostasis model assessment-insulin resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IL-18, interleukin-18; IL-10, interleukin-10; PC-1, plasma cell antigen-1.

Data are presented as mean ± SE, a: significant difference from grade 0, b: significant difference from grade 1, c: significant difference from grade 2.

*p < 0.05, **p < 0.01, ***p < 0.001.

Table 3. ROC results for WC, hip circumference and BMI

	Grade 0 & Grade 1			Grade 0 & Grade 2			Grade 0 & Grade 3			Grade 1 & Grade 2			Grade 1 & Grade 3		
	WC	HC	BMI	WC	HC	BMI	WC	HC	BMI	WC	HC	BMI	W/H ratio	BMI	
AUC	0.98	0.94	0.99	0.99	0.96	0.99	0.98	0.99	1	0.68	0.81	0.83	0.51	0.94	
Cut-off value	93	106	24.9	94	109	24.9	91	109	26.2	100	116	35.2	0.84	38.5	
Sensitivity	90.3	96.8	100	92.3	96.2	100	93.3	100	100	88.5	80.8	80.8	83.3	83.3	
Specificity	97.9	82.9	97.9	100	91.5	97.9	93.6	91.5	100	45.2	74.2	77.4	29.03	93.6	
PLR	42.5	5.7	47		11.3	47	14.6	11.8		1.6	3.13	3.6	1.17	12.9	
NLR	0.099	0.039	0.00	0.077	0.042	0.00	0.071	0.00	0.00	0.26	0.26	0.25	0.57	0.18	
PPV	96.6	78.9	96.9	100	86.2	96.3	90.3	88.2	100	57.5	72.4	75	53.2	92.6	
NPV	93.9	97.5	100	95.9	97.7	100	95.7	100	100	82.4	82.1	82.8	64.3	85.3	
Accuracy	94.9	88.5	98.7	97.3	93.1	98.6	93.5	94.8	100	64.9	77.2	78.9	55.7	88.5	
p-value					<0.0001					0.012		<0.0001	0.88	<0.0001	
95% confidence interval	0.92–0.99	0.86–0.98	0.95–1	0.93–1	0.88–0.99	0.95–1	0.92–0.99	0.93–1	0.95–1	0.544–0.789	0.683–0.901	0.712–0.919	0.380–0.642	0.851–0.986	

Abbreviations: WC, waist circumference; HC, hip circumference; BMI, body mass index; W/H, waist to hip; AUC, area under the curve; PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.

sensitivity (≥80%) and best values for area under the curve (AUC ≥0.5) in order to be able to differentiate between the different NAFLD grades. Different studied parameters, like the anthropometric (WC, hip circumference and BMI) and ultrasonographic parameters, lipid profile, IL-18, PC-1 and HOMA-IR have both optimal sensitivity (≥80%) and best values for AUC ≥0.5, and could differentiate between the different NAFLD grades as shown in Tables 3, 4, 5 and 6.

Table 7 presents the results of logistic regression, by which WC, hip circumference, waist to hip ratio, IL-10, PC-1, HOMA-IR, ultrasonographic findings and lipid profile were associated with grade 1 due to their significant *p*-values (≤0.05). Moreover, WC, hip circumference, waist to hip ratio, HOMA-IR, ultrasonographic findings and lipid profile were found to be associated with grade 2 due to their significant *p*-values (<0.01). WC, hip circumference, waist to hip ratio, IL-18, HOMA-IR, ultrasonographic findings (except for liver size) and lipid profile (except for triglycerides) were found to be associated with grade 3 due to their significant *p*-values (<0.01). Furthermore, by multivariate analysis, HOMA-IR showed a significant *p*-value (<0.01) when combined with IL-10, IL-18 or PC-1, or combined all together. Thus, there were associations between these parameters in the different NAFLD grades (grade 1, grade 2 and grade 3).

Discussion

NAFLD is a silent disease influencing the Egyptian population. Numerous risk factors have been suggested in NAFLD pathogenesis, including advanced age, obesity, IR and hyperlipidemia, beside the roles of pro- and anti-inflammatory cytokines.[3,10]

The results from the current study revealed that the risk of NAFLD development rises with increasing age. These results confirm the finding of Mahmoud *et al*.,[1] who reported that age is an independent risk factor for developing more severe NAFLD. This finding may be attributed to increased fat accumulation that occurs in liver with advancing age.

In a previous study, Ezzat and colleagues[18] found that abdominal adipose tissue comprises SCF and VF, which are considered as distinct anatomic depots. SCF varies from VF in that venous drainage from SCF is directed into the systemic circulation, while venous drainage from VF is directed into the portal vein directly to the liver; thus, the metabolic products reach the liver directly and exercise a first-pass influence on liver metabolism. Multivariate analysis in the current study showed that SCF and VF were significantly associated (p <0.01) with grades 1, 2 and 3 (Table 7), this is due to the significant increase in SCF and VF in parallel with NAFLD grades; besides, SCF and VF showed high sensitivity and specificity in differentiating between grade 3, grade 2 and grade 0 (controls) and in differentiating between grade 2, grade 1 and grade 3. Moreover, VF showed high sensitivity and specificity in differentiating between grade 2 and grade 1.

In agreement with previous findings, it has been suggested that visceral fat releases adipokines and free fatty acids, leading to fat accumulation inside liver.[18] Our results showed that size of the liver was significantly increased in parallel with NAFLD grades, also spleen size was significantly higher in all NAFLD grades than in grade 0, but stayed within the average (11 cm).[29] So, our NAFLD patients did not have splenomegaly or non-cirrhotic portal hypertension. Furthermore, our study showed that liver size and spleen size have high sensitivity and specificity in differentiating between

Table 4. ROC results for IL-18, PC-1 and HOMA-IR index

	Grade 0 & Grade 1	Grade 0 & Grade 2	Grade 0 & Grade 3		Grade 1 & Grade 2	Grade 1 & Grade 3	Grade 2 & Grade 3	
	IL-18	PC-1	PC-1	HOMA-index	PC-1	PC-1	PC-1	IL-18
AUC	0.59	0.58	0.53	0.89	0.51	0.56	0.57	0.69
Cut-off value	7.5	3.8	3.8	1.7	7	7	6	9.2
Sensitivity	100	92.3	90	83.3	92.3	96.7	90	80
Specificity	23.4	27.7	27.7	91.5	29.03	29.03	23.08	61.5
PLR	1.3	1.28	1.24	9.8	1.3	1.4	1.17	2.08
NLR	0.000	0.28	0.36	0.18	0.26	0.11	0.43	0.33
PPV	46.3	41.4	44.3	86.2	52.2	56.9	57.4	70.6
NPV	100	86.7	81.2	89.6	81.8	90	66.7	72.7
Accuracy	53.8	50.7	51.9	88.3	57.9	62.3	58.9	71.4
p-value	0.145	0.201	0.637	<0.0001	0.896	0.386	0.354	0.009
95% confidence interval	0.476–0.704	0.463–0.699	0.413–0.645	0.8–0.951	0.374–0.645	0.430–0.690	0.430–0.701	0.553–0.807

Abbreviations: ROC, receiver operating characteristic; IL-18, interleukin-18; PC-1, plasma cell antigen-1; HOMA-IR, homoeostasis model assessment-insulin resistance; AUC, area under the curve; PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.

grade 3, grade 2 and grade 0 and in differentiating between grade 2 and grade 1, but only liver size could differentiate between grade 2, grade 1 and grade 3. To differentiate between grade 1 and grade 0, only spleen size could be used due to its having the highest sensitivity and specificity. This is in agreement with the report by Mahmoud *et al*.,[1] which stated that the sizes of liver and spleen were significantly higher in patients with steatosis than in non-steatosis patients.

Lipid profile parameters were significantly increased (except for HDL-cholesterol, which was decreased) in parallel with NAFLD grades. Moreover, cholesterol and LDL have high sensitivity and specificity in differentiating between grade 2, grade 1 and grade 0, but only cholesterol has high sensitivity and specificity in differentiating between grade 2 and grade 1. Triglycerides have high sensitivity and specificity in differentiating between all studied NAFLD grades except between grade 1 and grade 0. Furthermore, HDL has high sensitivity and specificity in differentiating between all studied NAFLD grades, except between grade 2 and grade 1. Multivariate analysis showed that cholesterol, HDL and LDL were significantly associated (p =0.001) with grades 1, 2 and 3, but triglycerides were significantly associated (p=0.001) with only grades 1 and 2. This is in agreement with the report by Mahmoud *et al*.,[1] which stated that hyperlipidemia was an independent predictor of NAFLD development. In contrast, Paredes-Turrubiarte *et al*.[30] reported no significant differences in the values of lipid profile when comparing all different NAFLD grades.

NAFLD is related to central obesity indices, one of which is WC. Central obesity contributes in causing IR, and increased visceral adiposity might be significant in NAFLD pathogenesis.[31] In our study, hip circumference showed gradual increase with increase of NAFLD grades. In addition, BMI and WC were significantly increased in parallel with NAFLD grades. This is in agreement with the report by Tominaga *et al*.,[5] which stated that BMI and WC were independent risk factors for NAFLD. Kim *et al*.[32] have also reported a significant association between the occurrence of fatty liver and its severity with an increase in BMI and WC. Multivariate analysis in our study showed that WC, hip circumference and waist to hip ratio were significantly associated with grades 1, 2 and 3, but surprisingly BMI was not associated ($p > 0.05$) with all NAFLD grades (Table 7). Furthermore, WC, hip circumference and BMI showed high sensitivity and specificity in differentiating between NAFLD grades and grade 0 and in differentiating between grade 2 and grade 1. Only the waist to hip ratio and BMI showed high sensitivity and specificity for differentiating between grade 3 and grade 1.

The HOMA-IR index has been approved as an indicator of the insulin-resistant condition.[5] An upper boundary of normal HOMA-IR index is 1.5.[33] HOMA-IR index and insulin showed significant differences between NAFLD grades and controls in our study. All NAFLD patients in different grades showed HOMA-IR index value >1.5, indicating that they are in an IR state, and this was confirmed by the multivariate analysis that showed the HOMA-IR index as being significantly associated (p= 0.001) with all NAFLD grades. However, the highest sensitivity and specificity of HOMA-IR index was found in differentiating between grade 3 and grade 0. This is in agreement with a report by Hegazy *et al*.,[31] which showed a direct association between insulin and HOMA-IR with NAFLD grades.

Obesity, defined as a condition of chronic low-grade inflammation caused by over-nutrition, is a main cause of NAFLD. Obesity causes lipid accumulation in adipocytes, which activates signaling pathways, thereby increasing the production of pro-inflammatory cytokines,[34] such as IL-18.[35] Meanwhile, anti-inflammatory protein expression (e.g. IL-10) decreases during weight gain and therefore causes fat mass expansion.[36] IL-18 was associated only with grade 3 in our study (OR = 1.1, 95%CI: 1.02-1.26, $p < 0.05$). Furthermore, IL-18 has high sensitivity and specificity in differentiating between grade 1 and grade 0 and in differentiating between grade 3 and grade 2. The results of the IL-18 mean value in grade 3 showed significant increase in comparison with other

Table 5. ROC results for ultrasonography findings

	Grade 0 & Grade 1	Grade 0 & Grade 2				Grade 0 & Grade 3				Grade 1 & Grade 2			Grade 1 & Grade 3			Grade 2 & Grade 3		
	SS	LS	SS	SCF	VF	LS	SS	SCF	VF	LS	SS	VF	LS	SCF	VF	LS	VF	SCF
AUC	0.72	0.97	0.87	0.9	0.9	1	0.84	0.98	0.99	0.87	0.72	0.53	0.99	0.82	0.86	0.92	0.83	0.59
Cut-off value	9.3	15.2	9.7	1.5	3.4	15.7	9.3	1.5	5	15.7	10	3	16.3	1.6	6.7	16.5	6.7	1.6
Sensitivity	80.7	88.5	92.3	80.8	96.2	100	90	100	100	80.8	92.3	100	100	100	90	100	90	100
Specificity	63.8	95.7	72.3	85.1	72.3	100	63.8	85.1	95.7	83.9	58.1	12.9	96.8	58.1	70.97	69.2	73.1	26.9
PLR	2.23	20.8	3.3	5.4	3.5		2.5	6.7	23.5	5.01	2.2	1.15	31	2.4	3.1	3.3	3.3	1.4
NLR	0.3	0.12	0.11	0.23	0.05	0.00	0.16	0.00	0.00	0.23	0.13	0.00	0.00	0.00	0.14	0.00	0.14	0.00
PPV	59.5	92	64.9	75	65.8	100	61.4	81.1	93.8	80.8	64.9	49.1	96.8	69.8	75	78.9	79.4	61.2
NPV	83.3	93.7	94.4	88.9	97.1	100	90.9	100	100	83.9	90	`100	100	100	88	100	86.4	100
Accuracy	70.5	93.1	79.4	83.6	80.8	100	74.04	90.9	97.4	82.5	73.7	52.6	98.4	78.7	80.3	85.7	82.1	66.1
p-value	<0.001					<0.0001					0.002	0.68			<0.0001			0.27
95% confidence interval	0.61–0.81	0.89–0.99	0.76–0.93	0.81–0.96	0.81–0.96	0.95–1	0.73–0.91	0.92–0.99	0.94–1	0.76–0.96	0.58–0.83	0.39–0.67	0.93–1	0.70–0.91	0.75–0.94	0.82–0.98	0.70–0.92	0.45–0.72

Abbreviations: ROC, receiver operating characteristic; SS, spleen size; LS, liver size; SCF, subcutaneous fat; VF, visceral fat; AUC, area under the curve; PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.

Table 6. ROC results for lipid profile

	Grade 0 & Grade 1			Grade 0 & Grade 2				Grade 0 & Grade 3		Grade 1 & Grade 2		Grade 1 & Grade 3		Grade 2 & Grade 3	
	TC	HDL	LDL	TC	TG	HDL	LDL	TG	HDL	TC	TG	TG	HDL	TG	HDL
AUC	0.89	0.74	0.87	0.94	0.96	0.77	0.92	1	0.89	0.58	0.62	0.79	0.82	0.68	0.60
Cut-off value	200.6	61.8	91.4	223.5	136	59.03	116.3	157.8	55	219.8	171.3	189.6	47.2	188.8	51.7
Sensitivity	83.9	87.1	90.3	80.8	88.5	80.8	88.5	100	96.7	84.6	80.8	90	86.7	90	93.3
Specificity	82.9	70.2	70.2	95.7	95.7	70.2	78.7	100	76.6	35.5	48.4	54.8	67.7	42.3	42.3
PLR	4.9	2.9	3.03	18.9	20.8	2.7	4.2		4.13	1.3	1.6	1.99	2.7	1.6	1.6
NLR	0.19	0.18	0.14	0.2	0.12	0.27	0.15	0.00	0.044	0.43	0.4	0.18	0.2	0.24	0.16
PPV	76.5	65.9	66.7	91.3	92	60	69.7	100	72.5	52.4	56.8	65.9	72.2	64.3	65.1
NPV	88.6	89.2	91.7	90	93.7	86.8	92.5	100	97.3	73.3	75	85	84	78.6	84.6
Accuracy	83.3	76.9	78.2	90.4	93.1	73.9	82.2	100	84.4	57.9	63.2	72.14	77.05	67.9	69.7
p-value					<0.0001					0.295	0.102	<0.0001		0.01	0.212
95% confidence interval	0.79–0.95	0.63–0.84	0.77–0.93	0.86–0.98	0.89–0.99	0.66–0.86	0.83–0.97	0.95–1	0.79–0.95	0.44–0.71	0.49–0.75	0.67–0.88	0.69–0.90	0.55–0.80	0.46–0.73

Abbreviations: ROC, receiver operating characteristic; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; AUC, area under the curve; PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.

Table 7. Variables associated with different NAFLD grades (multivariate analysis)

	Grade 1				Grade 2				Grade 3			
			95%CI				95%CI				95%CI	
Parameter	*p*-value	OR	Lower	Upper	*p*-value	OR	Lower	Upper	*p*-value	OR	Lower	Upper
WC	0.001	1.494	1.217	1.834	0.002	1.513	1.168	1.960	0.001	1.384	1.141	1.679
HC	0.001	1.407	1.213	1.633	0.001	1.315	1.156	1.497	0.001	1.592	1.213	2.088
W/H ratio	0.001	3.19E +13	9.81E +06	1.04E +20	0.001	1.70E +11	2.60E +05	1.11E +17	0.001	7.38E +13	1.69E +07	3.22E +20
BMI	0.068	18.514	0.805	425.530	0.150	12.249	0.404	371.450	0.993	181.597	0.001	.
Liver size	0.001	2.486	1.502	4.116	0.001	14.204	3.738	53.974	0.990	1.46E +14	0.00E +00	.
Spleen size	0.003	1.865	1.233	2.820	0.001	4.465	2.160	9.230	0.001	2.978	1.793	4.949
SCF	0.001	12.853	3.283	50.322	0.001	44.494	6.368	310.899	0.005	1.43E +05	3.89E +01	5.25E +08
VF	0.001	2.410	1.647	3.528	0.001	3.034	1.843	4.995	0.001	9.233	2.535	33.634
Cholesterol	0.001	1.051	1.027	1.075	0.001	1.073	1.037	1.110	0.001	1.056	1.027	1.086
Triglycerides	0.001	1.050	1.026	1.075	0.001	1.081	1.037	1.128	0.957	1.32E +04	0.00E +00	1.7E +153
HDL	0.001	0.951	0.924	0.978	0.001	0.957	0.933	0.982	0.001	0.889	0.841	0.940
LDL	0.001	1.035	1.020	1.051	0.001	1.047	1.025	1.069	0.001	1.039	1.023	1.056
VLDL	0.001	1.278	1.137	1.436	0.001	1.476	1.197	1.821	0.952	1.48E +24	0.00E +00	.
IL-10	0.005	1.478	1.125	1.942	0.218	1.195	0.900	1.588	0.434	1.109	0.856	1.435
IL-18	0.268	1.073	0.947	1.217	0.992	1.001	0.891	1.124	0.016	1.139	1.024	1.267
PC-1	0.046	1.164	1.003	1.350	0.183	1.117	0.949	1.314	0.540	1.049	0.899	1.225
HOMA-IR index	0.001	8.751	2.792	27.428	0.001	20.894	3.667	119.068	0.001	25.485	5.264	123.376
IL-10, IL-18	0.007	1.019	1.005	1.033	0.452	1.005	0.992	1.019	0.020	1.017	1.003	1.031
IL-10, PC-1	0.002	1.045	1.016	1.075	0.106	1.023	0.995	1.051	0.579	1.009	0.979	1.039
IL-18, PC-1	0.015	1.017	1.003	1.032	0.352	1.005	0.995	1.015	0.059	1.015	0.999	1.030
IL-10, HOMA-IR index	0.001	1.419	1.193	1.687	0.001	1.321	1.125	1.552	0.001	1.334	1.137	1.565
IL-18, HOMA-IR index	0.001	1.107	1.041	1.177	0.003	1.089	1.029	1.153	0.001	1.150	1.079	1.226
PC-1, HOMA-IR index	0.004	1.225	1.066	1.407	0.001	1.308	1.131	1.513	0.001	1.359	1.159	1.593
IL-10, IL-18, PC-1	0.001	1.004	1.001	1.006	0.257	1.001	0.999	1.003	0.055	1.002	1.000	1.005
IL-10, IL-18, PC-1, HOMA-IR index	0.002	1.003	1.001	1.004	0.013	1.002	1.000	1.003	0.001	1.003	1.001	1.005

Abbreviations: CI, confidence interval; OR, odds ratio.

grades due to the fact that grade 3 patients had more IR than the other NAFLD grades. This is in accordance with a report by Wang *et al*.,[37] which stated that IL-18 may contribute to the development of NAFLD by causing IR. In an insulin-resistant state, the incapability of insulin to inhibit lipolysis leads to raised fluctuation of free fatty acids to the liver from adipose tissue. Enlarged de novo lipogenesis and augmented consumption of dietary fat contribute to the development of NAFLD.[38] Li *et al*.[15] found that IL-18 was significantly higher in NAFLD patients than in controls, while Vecchiet *et al*.[14] reported that IL-18 plasma levels were not significantly increased in NAFLD patients compared to controls. However, Tapan *et al*.[16] did not find any significant differences regarding the IL-18 plasma concentrations between patients with NASH and simple steatosis.

In our study, IL-10 was associated only with grade 1 (OR = 1.5, 95%CI: 1.1-1.9, $p < 0.01$);this is due to the fact that IL-10 mean value in grade 1 showed significant increase when compared to grade 0. The increase itself may be due to an IL-10 compensatory reaction for pro-inflammatory activation as found in healthy subjects.[39] Since HOMA-IR values in grade 2 and grade 3 were significantly higher than in grade 1, IL-10 was decreased in grade 2 and grade 3 compared to grade 1; this finding confirmed the insulin-sensitizing effects of IL-10.[9] The current results are in agreement with those reported by Paredes-Turrubiarte *et al*.,[30] namely the pronounced reduction in IL-10 that was demonstrated in severe NAFLD when compared to mild NAFLD, supporting a role for inflammatory mediators in promoting steatosis progression. Unfortunately, IL-10 failed in differentiating between NAFLD grades due to its low sensitivity (≤80%).

In our study, PC-1 levels were significantly higher in grade 1 patients; with low IR (HOMA-IR index =1.99) compared to grade 3 patients, who showed high IR (HOMA-IR index=2.82). This finding is in agreement with those reported by Frittitta *et al*.,[40] namely the decreased PC-1 levels demonstrated in insulin-resistant subjects compared to insulin-sensitive non-diabetic subjects. The reason for PC-1 decrease in grade 3 is that in insulin-resistant subjects, circulating PC-1 is cleared at a higher rate from plasma (either bound or degraded).[40] PC-1 levels were also higher in grade 1 patients than grade 0 patients, who were in an insulin-sensitive state, this may be due to the HOMA-IR index value not being significantly higher in grade 1 (1.99) compared to grade 0 (1.3). Furthermore, PC-1 has high sensitivity and specificity in differentiating between all studied NAFLD grades, except between grade 1 and grade 0.

Multivariate analysis showed that IL-10, IL-18 and PC-1 when combined with HOMA-IR index were associated with different NAFLD grades (grade 1, grade 2, grade 3) (Table 7). This is attributed to their important effects on the insulin signaling pathway.[9,11,37]

Conclusions

- BMI could differentiate between all different studied NAFLD grades, except between grade 3 and grade 2. WC and hip circumference could differentiate between all different studied NAFLD grades, except between grade 3 and grades 1 and 2, but only waist to hip ratio could differentiate between grade 3 and grade 1.
- Ultrasonographic findings and lipid profile could differentiate between grade 2 and grade 0. Ultrasonographic findings, triglycerides and HDL could differentiate between grade 3 and grade 0. Ultrasonographic findings (except for SCF), cholesterol and triglycerides could differentiate between grade 2 and grade 1. Ultrasonographic findings (except for spleen size), triglycerides and HDL could differentiate between grade 3 and grades 1 and 2. Spleen size and lipid profile (except for triglycerides) could differentiate between grade 1 and grade 0.
- IL-18 might differentiate between grade 1 and grade 0 and differentiate between grade 3 and grade 2. PC-1 could differentiate between all different studied NAFLD grades, except between grade 1 and grade 0.
- HOMA-IR index could differentiate between grade 3 and grade 0.
- Finally, the studied parameters could differentiate between the different grades to a certain extent. It is better to use these findings together with ultrasonography.

Limitations of the study

1. The small size of this study was due to the cost of kits for the biochemical investigations; we recommend to increase the number of patients in future studies.
2. In our study, we searched for cytokines that are related to the wide spectrum of NAFLD, which ranges from simple steatosis to NASH. We did not aim to focus on NASH cases in our study, since diagnosis for liver fibrosis or hepatocellular injury is invasive and very expensive.
3. Although abdominal ultrasonography has low sensitivity for detecting mild NAFLD, as reported in previous literature, it is the best low-cost method available that is also a non-invasive technique for detecting NAFLD. Because of ethical considerations, we did not carry out a liver biopsy (none of our patients had clinical presentation or significant elevation in liver enzymes). Moreover, our studied patients considered themselves healthy and refused to undergo further invasive techniques, such as pathological examinations for liver biopsy to detect fibrosis, and our enrolled controls were selected carefully and did not show any risk factor for NAFLD development.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Contributed equally to the work and have approved the final version submitted for publication (IHB, YS, MMK, WME, EA, MA, WG, MME).

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