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Anthropometry, laboratory, and PNPLA3 polymorphisms in a novel model for early identification and evaluation of nonalcoholic fatty liver disease

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ABSTRACT

Background: Some anthropometric, laboratory, and genetic variations, such as patatin-like phospholipase domain-containing protein 3 (PNPLA3) genetic variants, have been associated with nonalcoholic fatty liver disease (NAFLD). Liver biopsy is the most accurate NAFLD diagnostic method, but it is invasive; hence, noninvasive diagnostics are required for the early diagnosis and assessment of NAFLD.

Patient and methods: This prospective case-control study included 107 NAFLD patients and 107 healthy controls. All individuals underwent anthropometric measurements, abdominal ultrasonography, laboratory tests, and evaluation for PNPLA3 polymorphisms.

Results: Patients with NAFLD had higher levels of C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) than healthy individuals ($p = 0.03$, $p < 0.0001$). Additionally, patients with NAFLD had substantially lower albumin ($P = 0.01$) and leptin ($P < 0.0001$) levels than healthy individuals. BMI leptin and

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CRP levels were independent indicators of NAFLD severity (p = 0.05–0.004). GG is the most prevalent genotype in patients with moderate to severe NAFLD. A novel model based on four markers (leptin, CRP, BMI, and PNPLA3 polymorphism) was developed. The AUC values for distinguishing between the healthy subjects and those with varying degrees of NAFLD severity (mild, moderate, and severe) were 0.99, 0.99, and 1.0, respectively.
Conclusion: Anthropometric measurements, such as BMI and laboratory results, including liver enzymes, CRP, inflammatory markers, lipid parameters, and genetic markers, especially PNPLA3 polymorphisms, can provide an accurate, sensitive, and specific noninvasive approach for the early identification and assessment of NAFLD and can guide its management. This may minimize the need for liver biopsy to assess NAFLD. Further large-scale studies are needed to confirm these findings and verify the model in larger studies.

1. Introduction

The term “nonalcoholic fatty liver disease" (NAFLD) is used to describe a wide range of conditions characterized by the presence of macrovesicular steatosis or hepatic steatosis on imaging or histology and the lack of secondary causes of hepatic steatosis, such as significant alcohol consumption and long-term use of medications that can exacerbate hepatic steatosis or inherited disorders. Non-alcoholic fatty liver disease is mostly discovered accidently during imaging or when complications arise [1]. NAFLD is a hepatic manifestation of metabolic syndrome. Approximately 50–70 % of individuals with diabetes also have nonalcoholic fatty liver disease [2]. In Western nations, NAFLD affects 20–30 % of the population [3]. Some of the most plausible causes are rising obesity rates, increased childhood obesity, sedentary lifestyles, consumption of bad fast food, and longer lifespans. As fatty liver disease is frequently detected using ultrasonography, the incidence and prevalence of NAFLD remain underreported. NAFLD is more common in obese adults (80–90 %), diabetes mellitus (30–50 %), hyperlipidemia (90–100 %), children (3–10 %), and obese children (40–70 %) [4]. The onset and progression of non-alcoholic fatty liver disease (NAFLD) are influenced by both hereditary and environmental factors. Patients with NAFLD in their first-degree relatives are more susceptible than the general population.

[5,6]. Day and James (1998) presented a two-hit model of pathogenesis. Insulin resistance is the initial strike, causing triglyceride-filled fat droplets to accumulate in the cytoplasm of hepatocytes, eventually resulting in steatosis [7]. The development of non-alcoholic steatohepatitis (NASH) and hepatocellular damage is the result of a multifactorial second blow. The liver is more susceptible to damage in the presence of excessive fatty acids. The damage may be caused by peroxisomal fatty acid oxidation, formation of reactive oxygen species (ROS) from the mitochondrial respiratory chain, metabolism of fatty acids by cytochrome P450, or hepatic metabolism of alcohol supplied from the gut. The second impact is caused by obesity, as fat tissue releases inflammatory mediators that harm hepatocytes, including leptin, interleukin (IL)-6, and tumor necrosis factor (TNF)-alpha. Hepatocytes undergo necrosis, apoptosis, cytoskeletal aggregation, and ballooning [8,9]. Although most individuals with non-alcoholic fatty liver disease (NAFLD) are asymptomatic, others may exhibit a wide range of nonspecific symptoms long before the illness is diagnosed. Fatigue is one of the most prevalent symptoms. Upper stomach discomfort can be mild or sharp, thirst, bloating, or disturbed sleep [10]. The following symptoms are common in patients with end-stage liver disease, hepatocellular carcinoma (HCC), and non-alcoholic steatohepatitis (NASH)-associated cirrhosis: nausea, vomiting, jaundice, pruritus, ascites, memory loss, bleeding, and appetite loss. Hepatomegaly, ranging from mild to robust, was the most prevalent clinical symptom. Signs of end-stage liver disease include jaundice, spider angiomas, palmar erythema, caput medusae, gynecomastia, dupuytren contracture, ascites, and petechiae in advanced stages of the disease [11–13].

While most individuals with non-alcoholic fatty liver disease (NAFLD) have normal liver enzyme levels, the main abnormality in their blood is mildly increased serum aminotransferase levels. The alanine aminotransferase (ALT) to aspartate aminotransferase (AST) ratio was < one. As nonalcoholic fatty liver disease (NAFLD) progresses, hepatic synthetic dysfunction-related factors, such as hypoalbuminemia, hyperbilirubinemia, thrombocytopenia, and prolonged prothrombin time, may be present [14]. Liver biopsy is generally accepted as the gold standard for diagnosing nonalcoholic fatty liver disease (NAFLD). However, there are several serious drawbacks to this procedure, such as its invasiveness and high cost, and other issues, including discomfort, complication risk, and sample mistake risk [15]. Inflammatory mediators such as CRP, IL-1β, IL-6, TNF-α, and ICAM-1 may function as biomarkers for individuals with nonalcoholic fatty liver disease (NAFLD) and offer fresh perspectives on the genesis of this condition, in addition to early detection and treatment [16]. The peptide hormone Leptin, an adipokine derived from adipocytes, plays a critical role in the central regulation of obesity and energy metabolism. However, they also play important regulatory roles in various physiological systems and diseases, including autoimmunity, cancer, reproduction, and bone physiology. Furthermore, over the past few decades, observational and interventional studies and experimental models have demonstrated the role of leptin in nonalcoholic fatty liver disease (NAFLD) [17]. NAFLD is a clinicopathological entity that develops in the absence of excessive alcohol consumption, and is usually defined as <20 g/day for women and <30 g/day for men. NAFLD comprises a variety of diseases such as cirrhosis, hepatic fibrosis, hepatic fibrosis, and hepatocellular carcinoma [18]. Barbara et al. reviewed a list of NAFLD genetic variations that are constantly expanding with an emphasis on major mutations. Among all the genetic variations, PNPLA-3 has become the most prominent determinant of NAFLD and may be included in future personalized care plans [19,20]. PNPLA3 is an essential lipid regulator found in the stellate cells and hepatocytes. In stellate cells, PNAPL3 is controlled by TGF-β to release retinol from retinyl esters, which are involved in fibrogenesis and stellate-cell activation. PNPLA3 is found in lipid droplets inside hepatocytes, where it hydrolyzes triglycerides (TGs) and facilitates the conversion of polyunsaturated fatty acids (PUFAs) from di- and triacylglycerols to phosphocholines. It plays a critical role in the remodeling of lipid droplet phospholipids [21]. The Dallas Heart Study cohort revealed that, regardless of BMI, diabetes status, or alcohol consumption, there was a strong correlation between NAFLD and rs738409C > G, which encodes Ile148Met (I148 M) [22]. The extent to which PNPLA3 genetics influence liver-related outcomes is significant. According to a meta-analysis involving 14,266 NAFLD participants, individuals with M-variants (GG and GC) had 2.14- and 3.24-fold greater risks of HCC, respectively, than those who were homozygous for the wild-type (CC) [23].

The goal of this study was to develop a new prediction model for diagnosing NAFLD using blood-based biomarkers, clinical data, and genetic variations in the PNPLA3.

2. Methods and patients

2.1. patients and ethical committee

This prospective case-control study was conducted in the outpatient clinic of the Hepatology and Gastrointestinal Unit at the Faculty of Medicine, Minia University (Minia, Egypt) between 2021 and 2022. The

study included a cohort of patients with confirmed nonalcoholic fatty liver disease drawn from individuals referred to outpatient clinics. The cohort of patients with NAFLD was juxtaposed with that of healthy individuals. A comprehensive clinical assessment of the anthropometric measurements was conducted for all participants. To ensure a statistical power of at least 80 %, a sample of 107 individuals diagnosed with NAFLD was recruited for this investigation. Concurrently, 107 healthy participants were randomly chosen from a control group consisting of healthy individuals from the same community, socioeconomic, cultural, and geographic backgrounds who visited the hospital within the same timeframe and voluntarily agreed to participate in the study. The control group underwent standard laboratory tests and abdominal ultrasound assessments performed by the same radiologist. In the control group, 43 individuals (40.2 %) were male and 64 (59.8 %) were female; strict ethical considerations were meticulously upheld throughout the study. The Institutional Ethics Committee of the School of Medicine at Minia University (Cairo, Egypt) approved all facets of the study. Adherence to the fundamental principles outlined in the 1975 Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice was maintained throughout the course of the study. Written informed consent was obtained from all participants involved in the study with the utmost diligence.

2.2. Inclusion criteria

Nonalcoholic fatty liver disease was ascertained using both abdominal ultrasonography and laboratory assessments in accordance with the methodology outlined by Bedogni et al. [24]. The steatosis hepatitis classification was based on a comparative evaluation of the hepatic parenchyma. Based on the established diagnostic criteria, the extent of hepatic lipid infiltration was categorized as follows: “mild" (I°), characterized by more echogenic liver parenchyma and absence of dorsal sound reduction; “moderate" (II°), denoting additional dorsal sound reduction with the diaphragm still discernible; and “severe" (III°), indicating dorsal sound reduction to the extent that the diaphragm is no longer visually identifiable.

2.3. Exclusion criteria

Individuals with a medical background of viral hepatitis (hepatitis B and hepatitis C viruses), autoimmune hepatitis, hepatocellular carcinoma, alcohol use, liver cirrhosis, diabetes mellitus, Wilson’s disease, secondary causes of hepatitis, drug-induced hepatitis, or obstructive biliary disease were not considered eligible for participation in this study and were therefore excluded.

2.4. biochemical analysis

2.4.1. Liver function tests, complete blood picture, INR, and lipid profile

Upon hospital admission, participants underwent venous blood sample collection following an 8- to 12-h fasting period. Venous blood (10 ml of venous blood was collected for analysis. From this volume, a 3-mL portion was carefully placed into sterile dry vacutainer tubes. Aliquots were clotted at room temperature before centrifugation at 3000*g* for 10 min at 4 °C. This meticulous procedure facilitates the separation of serum, which is a pivotal step in enabling the subsequent biochemical analysis of various liver function parameters.

Liver function was evaluated using a comprehensive array of assessments including alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, and albumin. Additionally, a comprehensive assessment of lipid profiles was conducted, encompassing measurements of triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol levels. Alpha-fetoprotein (AFP) levels were meticulously determined. Serum samples were assayed using a Cobas c 311 platform (Roche Diagnostics GmbH, Mannheim, Germany). Simultaneously, a parallel process involved the collection of 2 ml of blood into tubes containing ethylenediaminetetraacetic acid (EDTA), facilitating the subsequent execution of a comprehensive complete blood count (CBC) using the Phoenix 3300 instrument. Furthermore, 2 ml of blood was collected in tubes containing sodium citrate, a crucial component of the international normalized ratio (INR) detection.

2.5. Virological assays

Anti-hepatitis C virus (HCV) antibodies were detected using a second-generation enzyme-linked immunosorbent assay kit (Ortho-Clinical Diagnostics Co.). Inc., Tokyo, Japan. Assessment of hepatitis B surface antigen was performed using radioimmunoassay kits, specifically Lumipulse II hepatitis B surface antigen kits provided by Fujirebio Co., Inc. (Tokyo, Japan).

2.6. leptin and inflammatory biomarkers

Serum levels of leptin, tumor necrosis factor-alpha, interleukin 6, and C-reactive protein were determined using an enzyme-linked immunosorbent assay (ELISA). These assays were performed in strict accordance with the manufacturer’s instructions using kits from R&D Systems (Minneapolis, MN, USA). Measurements were performed using a Stat Fax Microstrip enzyme-linked immunosorbent assay (ELISA) reader.

2.7. PNPLA3 polymorphism determination

Blood mononuclear cells were obtained from whole ethylenediaminetetraacetic acid (EDTA) blood samples (3 ml) to determine the presence of PNPLA3 polymorphisms. Genomic DNA was extracted from these cells using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s protocol. The extracted DNA was preserved at −20 °C until sequencing procedures were conducted.

The quality, quantity, and purity of DNA were assessed using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The specific polymorphism examined was PNPLA3 rs738409C > G, located on chromosome 22 at position 43928847 in the GRCh38 genome assembly. This polymorphism was chosen because of its established significant association with nonalcoholic fatty liver disease (NAFLD). Single nucleotide polymorphisms (SNPs) were selected based on a global minor allele frequency (MAF) of >0.1. SNPs were sourced from databases, such as SNPedia and NCBI dbSNP.

For genotyping rs738409, predesigned primer/probe sets (Assay ID: C_7241_10, catalog number: 4351379) were used. TaqMan allelic discrimination was used for real-time DNA amplification. TaqMan Master Mix, comprising 12.5 μL TaqMan, 1.25 μL TaqMan probe/primer solution, 1 μL DNA, and 10.25 μL H2O in a total volume of 25 μL.

3. Statistical analysis

The collected data were subjected to thorough analysis using the IBM SPSS software (version 21, USA) and GraphPad Prism software. The Kolmogorov-Smirnov test was used to assess the normal distribution of parameters in both study groups. Descriptive statistics were used to summarize the data, with parametric data characterized by the mean and standard deviation (SD), whereas nonparametric data were summarized using the median or interquartile range.

The analytical process involved the utilization of various statistical tests, including the Student’s t-test, Mann–Whitney *U* test, and chi-square test. Univariate analysis was conducted using these tests, both individually and in conjunction with multivariate analysis, to identify the autonomous risk factors associated with NAFLD.

Furthermore, diagnostic precision evaluation included estimations of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). For statistical comparisons, a significance

threshold of $p < 0.05$ was used. Additionally, receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was computed. These outcomes were subsequently integrated into stepwise logistic regression analysis to enhance the depth of inquiry.

4. Results

4.1. patient characteristics

This study included a cohort of individuals diagnosed with NAFLD consisting of 45 (42.1 %) males and 62 (57.9 %) females. The mean age was 52.8 ± 10.6 years. This group was juxtaposed with a healthy control group. Table 1 presents the characteristics of the study population. No statistically significant differences in sex, age, or hemoglobin levels were observed between patients with NAFLD and healthy individuals ($p > 0.05$). In contrast, notable differences were observed in various parameters, as outlined below.

BMI, liver enzyme concentrations, lipid profile metrics, INR-prothrombin time, alpha-fetoprotein, CRP, TNF-α, and IL-6 levels were significantly greater ($p = 0.03$, $p < 0.0001$, respectively) in patients with NAFLD than in healthy individuals. Conversely, albumin ($p = 0.01$) and leptin ($p < 0.0001$) levels were markedly lower in patients with NAFLD than in their healthy counterparts.

Furthermore, PNPLA3 polymorphisms were analyzed in both patients with NAFLD and healthy subjects. The GG genotype was more prevalent in the NAFLD group (11.2 %) than in the control group (4.7 %). Similarly, the CG genotype was more prevalent in the NAFLD cohort (41.1 %) than in the control group (33.6 %) (Table 1).

The severity of steatosis in the patients with NAFLD was also examined (Table 2). Notably, BMI ($p = 0.05$), leptin ($p = 0.04$), and CRP ($p = 0.004$) levels significantly increased with increasing NAFLD severity. The GG genotype is predominantly observed in individuals with moderate NAFLD severity.

The diagnostic effectiveness of individual markers, specifically leptin, CRP, BMI, and the PNPLA3 polymorphism, in identifying nonalcoholic fatty liver disease (NAFLD) was evaluated through the calculation of the area under the receiver operating characteristic curve (ROC-AUC). The results are summarized in (Table 3).

Among the clinical and laboratory variables examined, leptin level exhibited the highest discriminatory capacity. Moreover, when considering combinations of variables, leptin emerged as the most effective component for distinguishing patients with NAFLD. Consequently, each variable independently contributes unique information, thereby fostering the anticipation of enhanced diagnostic performance through amalgamation.

4.2. Diagnostic performance of the novel model

The optimal linear combinations of blood markers were selected using stepwise multidiscriminant analysis to establish a novel model

Table 1
Characteristics of the study populations (NAFLD patients and healthy controls).

Variables	Healthy (n = 107)	All NAFLD (n = 107)	P value
Gender			
Male (no, %)	43 (40.2 %)	45 (42.1 %)	0.44
Female (no, %)	64 (59.8 %)	62 (57.9 %)	
Lifestyle			
Low fat (no, %)	74 (69.2 %)	50 (46.7 %)	0.002
Moderate fat	29 (27.1 %)	45 (42.1 %)	
High fat	4 (3.7 %)	12 (11.2 %)	
Age (years)	50.1 ± 12.3	52.8 ± 10.6	0.15
BMI(kg/m^2)	25.3 ± 4.5	33.4 ± 4.6	<0.0001
Hemoglobin (g/L)	113.4 ± 19.1	112.3 ± 16.5	0.64
Glucose (mm/L)	5.2 ± 0.62	6.3 ± 1.1	<0.0001
Alanine aminotransferase (U/L)	30.2 ± 5.5	38.7 ± 7.5	<0.0001
Aspartate aminotransferase (U/L)	33.3 ± 7.4	40.0 ± 7.4	<0.0001
Gamma-glutamyl transferase (U/L)	35.0 ± 9.5	50.8 ± 18.9	<0.0001
Albumin (mg/dL)	38.1 ± 2.1	38 ± 2.5	0.01
Total Bilirubin (mg/dl)	12.9 ± 3.2	16.6 ± 4.9	<0.0001
C-reactive protein (mg/dL)	5.7 ± 1.3	16.1 ± 5.1	<0.0001
Alpha fetoprotein (ng/mL)	6.3 ± 3.3	10.7 ± 5.9	<0.0001
International Normalized Ratio-PT	0.98 ± 0.1	1.2 ± 0.21	<0.0001
Cholesterol (mmol/L)	4.2 ± 0.76	4.9 ± 0.78	<0.0001
Triglycerides (mmol/L)	1.8 ± 0.38	1.9 ± 0.45	0.038
High-density lipoprotein (mmol/L)	1.1 ± 0.2	0.92 ± 0.25	<0.0001
Low-density lipoprotein (mmol/L)	2.9 ± 0.35	3.3 ± 0.71	<0.0001
Leptin (ng/ml)	12.9 ± 5.6	3.6 ± 1.8	<0.0001
TNF (pg/mL)	26.8 ± 4.4	31.7 ± 12.5	<0.0001
IL-6 (pg/mL)	21.1 ± 5.8	28.3 ± 9.6	<0.0001
PNPLA3 Gene (No; %)			0.05
CC	66 (61.7 %)	51 (47.7 %)	
CG	36 (33.6 %)	44 (41.1 %)	
GG	5 (4.7 %)	12 (11.2 %)	

ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; TNF, tumor necrosis factor; IL-6, interleukin 6; PNPLA3, patatin-like phospholipase domain-containing protein 3 (PNPLA3).

Table 2
Characteristics of the NAFLD patients in relation to steatosis severity.

Variables	Degree of steatosis			P value
	Mild	Moderate	Severe	
No (%)	43 (40.2 %)	51 (47.7 %)	13 (12.1 %)	
Gender				
Male (no, %)	16 (35.6 %)	23 (51.1 %)	6 (13.3 %)	0.84
Female (no, %)	27 (43.5 %)	28 (45.2 %)	7 (11.3 %)	
Age (years)	52.7 ± 10.6	53.5 ± 10.2	50.5 ± 11.5	0.7
BMI(kg/m^2)	32.1 ± 4.7	34.6 ± 4.5	35.0 ± 4.5	**0.05**
Hemoglobin (g/L)	115.2 ± 17.2	110 ± 16.2	105.5 ± 12.5	0.31
Glucose (mm/L)	6.4 ± 1.6	6.3 ± 1.2	5.9 ± 1.1	0.92
ALT (U/L)	40.7 ± 8.0	37.6 ± 6.9	36.6 ± 8.1	0.12
AST (U/L)	40.0 ± 7.2	40.2 ± 8.2	39.0 ± 5.0	0.9
GGT (U/L)	50.6 ± 20.2	51.9 ± 17.3	52.6 ± 21.4	0.6
Albumin (mg/dL)	37.5 ±0.2.2	38.4 ± 2.1	38.3 ± 3.1	0.15
Total Bilirubin (mg/dl)	16.2 ± 2.6	16 ± 2.9	17.2 ± 3.0	0.4
C-reactive protein (mg/dl)	14.1 ± 4.0	16.1 ± 3.0	22.5 ± 2.6	**0.004**
Alpha fetoprotein (ng/mL)	10.7 ± 2.3	10.7 ± 2.2	10.2 ± 1.7	0.99
INR	1.2 ± 0.1	1.29 ± 0.23	1.2 ± 0.3	0.15
Cholesterol (mmol/l)	5.1 ± 0.63	4.8 ± 0.55	4.7 ± 0.96	0.1
Triglycerides (mmol/l)	1.8 ± 0.42	1.9 ± 0.45	2.2 ± 0.6	0.16
HDL (mmol/l)	0.98 ± 0.27	0.88 ± 0.2	0.83 ± 0.32	0.16
LDL (mmol/)	3.3 ± 0.8	3.1 ± 0.63	3.3 ± 0.79	0.55
Leptin (ng/ml)	3.0 ± 1.6	3.9 ± 1.9	4.4 ± 1.5	**0.04**
TNF (pg/mL)	31.7 ± 13.5	31.9 ± 11.7	36.9 ± 14.7	0.27
IL-6 (pg/mL)	29.5 ± 11.2	28.3 ± 8.9	25.6 ± 7.6	0.4
PNPLA3 Gene (No; %)				<0.0001
CC	39 (90.7 %)	12 (23.5 %)	0 (0.0 %)	
CG	3 (7 %)	38 (74.5 %)	3 (37.5 %)	
GG	1 (2.3 %)	1 (2 %)	10 (62.5 %)	

ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; TNF, tumor necrosis factor; IL-6, interleukin 6; PNPLA3, patatin-like phospholipase domain-containing protein 3 (PNPLA3).

Table 3
The diagnostic power of single and combined candidate markers (leptin + CRP + BMI + PNP3 gene) for diagnosing NAFLD.

Variables	AUC (95 % CI)	P value	Cutoff	Sensitivity	Specificity	PPV	NPV	Youden index
Leptin	0.96 (0.93–0.98)	<0.0001	6	89	87	87	87	0.82
CRP	0.91 (0.87–0.96)	<0.0001	6	70	94	91	75	0.77
BMI	0.88 (0.84–0.92)	<0.0001	25	65	91	82	71	0.61
Leptin + CRP + BMI + PNPLA3 Gene	0.99 (0.99–1.0)	<0.0001	1.4	91	99	99	91	0.94

BMI, body mass index; CRP, C-reactive protein; patatin-like phospholipase domain-containing protein 3 (PNPLA3).

based on four markers: leptin, CRP, BMI, and PNPLA3 polymorphism. The model equation is as follows:

$$\text{Model} = [(\text{BMI (kg/m2)} \times 0.026 + \text{CRP (mg/L)} \times 0.021) + (\text{PNPLA3} \times 0.036) + 0.774) - (\text{leptin} \times 0.033)].$$

In this equation, the PNPLA3 gene is encoded as follows: 1- CC polymorphism, 2- CG polymorphism, and 3-GG polymorphism.

The levels in this model were compared between healthy individuals and those with NAFLD (Fig. 1). The mean [SD] model scores were 1.1 [0.23] for healthy individuals and 1.8 [0.19] for NAFLD patients, showing highly significant differences ($p < 0.0001$). Similarly, among the NAFLD severity groups, the mean [SD] model scores were 1.8 [0.21] for mild NAFLD, 1.8 [0.17] for moderate NAFLD, and 2.0 [0.14] for severe NAFLD, all of which exhibited highly significant differences ($p < 0.0001$).

The diagnostic efficacy of the model was assessed by calculating the area under the curve (AUC) and various diagnostic indices (Table 3). Notably, NAFLD model performance did not exhibit a significant difference based on sex ($p > 0.05$). The AUC values for distinguishing between the healthy subjects and those with varying degrees of NAFLD severity (mild, moderate, and severe) were 0.99, 0.99, and 1.0, respectively. Moreover, to distinguish between mild and moderate NAFLD severity, and between moderate and severe NAFLD severity, the AUC values were 0.54, 0.82, and 1.0, respectively, as illustrated in Fig. 1. A flowchart of the developed model is shown in Fig. 2.

5. Discussion

Liver biopsy is the gold standard for diagnosing NAFLD and NASH; however, it is not always necessary for all individuals with suspected disease [25]. It comes with risks, is costly, and is invasive. Therefore, our study revealed other non-invasive measurements for early NAFLD diagnosis and assessment.

In the present study, we documented the associations between BMI, lipid parameters (LDL and HDL), and NAFLD in the present study [17,26–36]. Our results revealed that the greater the BMI and lipid parameters, the greater was the risk of developing NAFLD and NASH. These results are in agreement with the findings of several previous studies that elevated body mass index (BMI) is an independent risk factor for fatty liver according to several investigations [29,37–39].

A higher BMI may be associated with increased adipose tissue and fatty acid flow to the liver. Additionally, individuals with a higher body mass index frequently follow a long-term high-fat diet, which enhances the absorption of external fat and increases the levels of fatty acids and their lipidation in the liver. Triglycerides eventually accumulate in the liver and cause fatty liver, although the production of phospholipids and apolipoprotein B is comparatively low. To put it simply, fatty liver disease is independently and dose-dependently associated with higher BMIs (overweight/obesity); hence, treating obesity is critical [39–41].

Regarding inflammatory markers, especially CRP, there was a significant positive correlation between nonalcoholic fatty liver grade and serum CRP level. Several investigations have shown that serum CRP levels correlate with non-alcoholic fatty liver disease (NAFLD) [42–45]. However, no link was found between CRP levels and fatty liver grade in a Norwegian study conducted by Haukeland et al. [46].

Increased CRP levels have been associated with metabolic syndrome and its components in clinical studies. While the liver is the primary source of CRP, visceral fat contributes considerably to CRP synthesis [45,47]. The concept of metabolic syndrome is based on increased BMI-related insulin resistance and hepatic function. In our investigation, the fatty liver grade increased as BMI increased. Increased inflammatory indices, such as CRP, TNF-α, and IL-6, are linked to worse insulin sensitivity and higher BMI [48].

According to our study, an increase in blood CRP levels may be the sole indication for NAFLD. Thus, sequential CRP readings may be useful for follow-up the clinical treatment of patients with NAFLD.

Our study demonstrated a positive correlation between serum leptin level and NAFLD severity. These findings support previous studies' results [49]. Leptin is known to exacerbate fibrosis, inflammation, and hepatic steatosis. Leptin has a pro-inflammatory effect that leads to the development of fibrosis and increases insulin resistance [50]. This can be partially attributed to the increased expression of α2 (I) collagen and its excessive accumulation in the liver [51]. Triglyceride synthesis and hepatic steatosis are exacerbated by insulin resistance and hyperinsulinemia, which increase blood free fatty acid levels [52]. It has been suggested that profibrotic cytokines are stimulated by chronic insulin resistance, which also leads to hepatic lipogenesis [52,53]. It is plausible that leptin plays a role in the development of nonalcoholic fatty liver disease (NAFLD) in overweight individuals who are more susceptible to metabolic syndrome via increased insulin resistance, inflammation, and collagen deposition [49]. Generally, the trend of leptin levels varies among studies, and further research is necessary to understand this pattern and its significance.

The fourth marker was the PNPLA3 polymorphism. Our results highlight the frequency of the GG and GC genotypes (mutation alleles) in the NAFLD group and their correlation with NAFLD severity. The extent to which PNPLA3 genetics influence liver-related outcomes is significant. According to a previous meta-analysis, those with the CC genotype had a 52 % lower risk of developing nonalcoholic fatty liver disease, whereas those with the CG genotype had a 19 % higher risk. Additionally, the GG genotype group had a 105 % increased risk of nonalcoholic fatty liver disease developing NAFLD. Furthermore, an 88 % greater disease risk was observed in populations with CG + GG genotypes, indicating the role of the G allele in nonalcoholic fatty liver disease [54]. Additionally, a 2020 study conducted in India revealed that the G allele is crucial for the development of NAFLD [55].

Two genes, PNPLA3 I148 M and TM6SF2 E167K, have been found to be the most likely genetic contributors to the development of nonalcoholic fatty liver disease (NAFLD) in previous genome studies [56]. Based on a meta-analysis conducted by Zhang et al. (2015) in a few studies conducted in Asian countries, the probability of nonalcoholic fatty liver disease was found to be 1.92 when individuals with the G allele were compared to a population with the C allele. This means that the G allele is likely to increase the risk of developing nonalcoholic fatty liver disease to the liver in people with the G allele by 92 %, and it may increase serum alanine transaminase (ALT) levels and the risk of renal fibrosis. Individuals with the dominant phenotype (CG + GG) developed NAFLD at a rate 110 % higher than that of individuals with the recessive phenotype. However, it was shown that the homozygous GG group had a greater risk of nonalcoholic fatty liver disease (NAFLD) than the other populations when CG + GG populations with the CG genotype were compared [57].

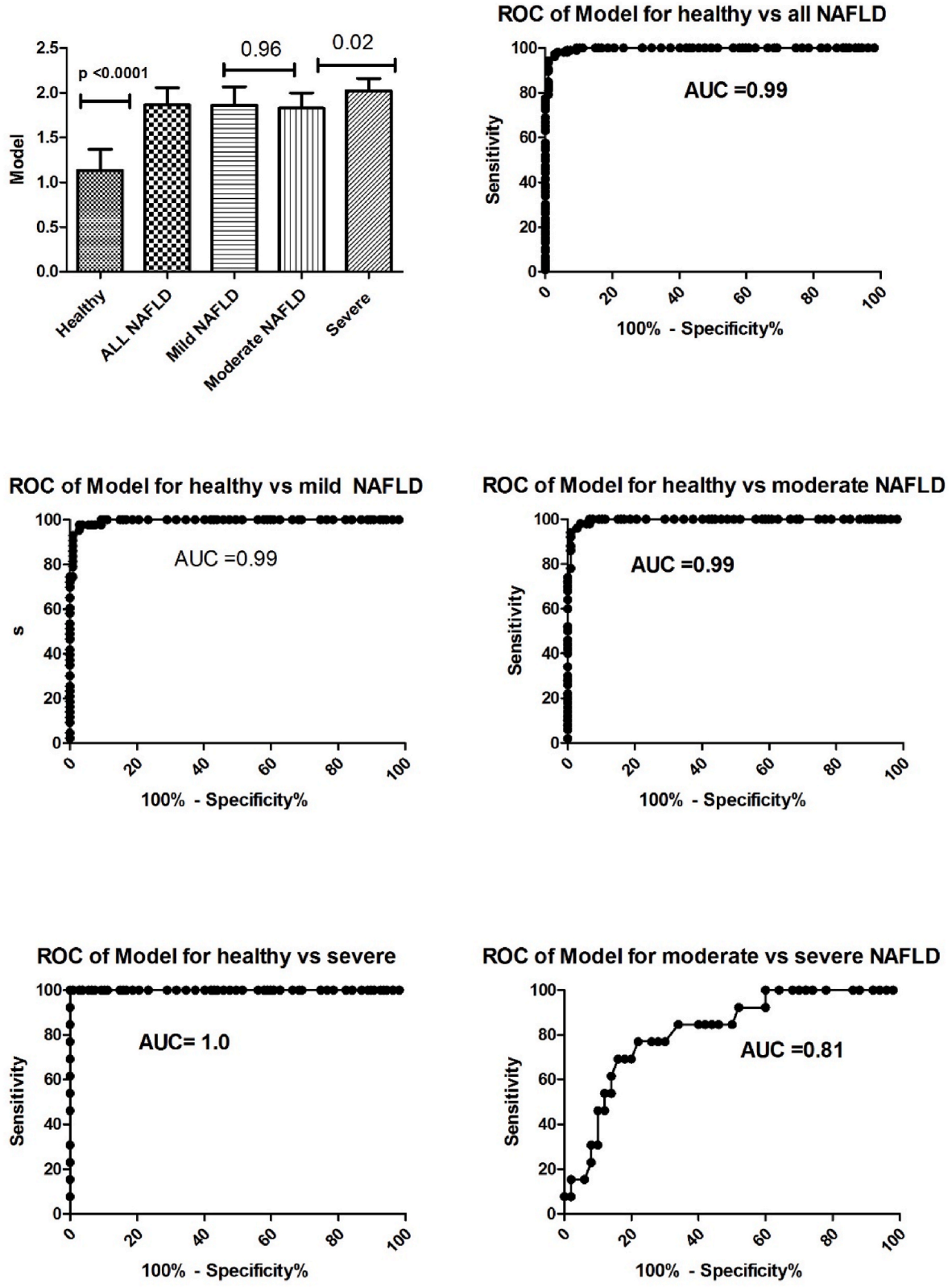

Fig. 1. Different ROC models for the identification and evaluation of NAFLD.

The G allele was thought to be a risk factor for NAFLD, as evidenced by another meta-analysis carried out in 2019 that revealed that this polymorphism had a significant effect on the development of tissue damage in the liver and that the ratio of disease development in those with one G allele to those without it was 1.88 and 4.01 in those where both alleles were G. Additionally, it has been proposed that this gene increases blood alanine aminotransferase levels [23]. Jiaying et al. (2020) conducted a meta-analysis and revealed that this gene is associated with factors such as serum alanine transaminase, aspartate transaminase, and gamma-glutamyl transferase, which are markers of

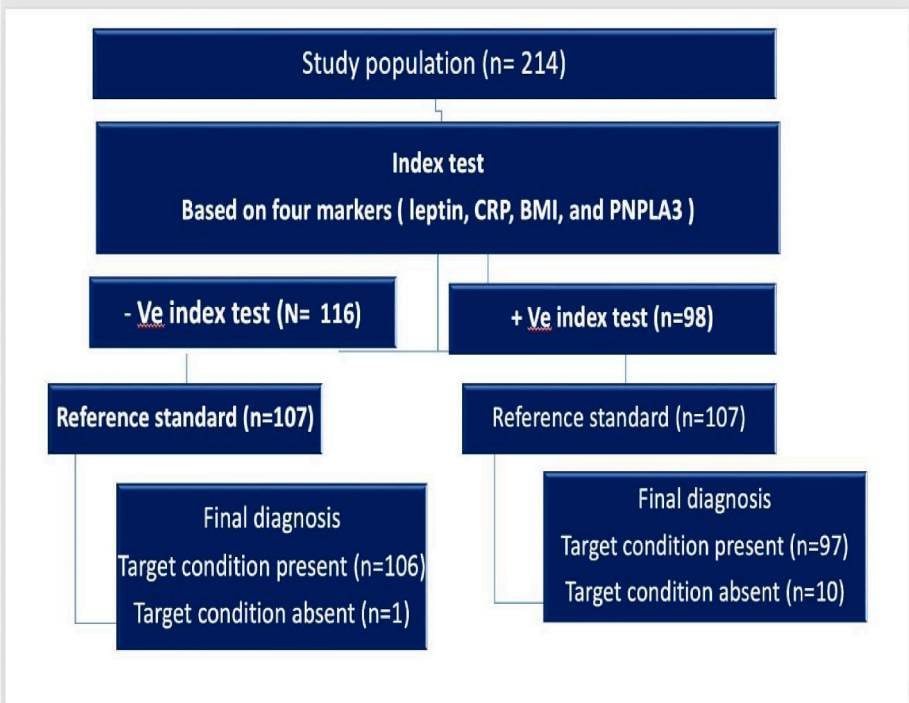

Fig. 2. The following chart of the developed model.

liver damage and are implicated in the development of nonalcoholic osteopathy (NASH) in children and adolescents [58].

A meta-analysis published in 2015 revealed a substantial correlation between the occurrence of NAFLD and NASH, particularly in Asian and Spanish populations, and all genetic variants in the rs738409 polymorphism in the PNPLA3 gene. However, no correlation between rs738409 polymorphism and hepatic steatosis was observed in this study. Renal fibrosis and NAFLD development were significantly influenced by the GG genotype. According to previous reports, the ratio of this genotype to the incidence of inflammation is 3.13 [59]. Research conducted by Chobin et al. revealed that the CG genotype predisposes an individual to a 2.63-fold increase in the risk of contracting an illness. Furthermore, the GG genotype had a protective effect, indicating that having such a genotype reduces the risk of NAFLD by 59 %. Moreover, 1.78 % of those with the CC genotype were likely to contract the illness [60].

In a different study conducted in Japan, the odds ratio of the GG genotype was 36.5 % in obese individuals and 47.8 % in non-obese individuals with fatty liver. Additionally, the adjusted odds ratio for nonalcoholic fatty liver disease in people with the GG genotype was found to be 2.76 in the obese population and 4.15 in the non-obese population. Additionally, there is an increased risk of steatosis and liver fibrosis with this genotype [61]. Children with a family history of nonalcoholic fatty liver disease (NAFLD) may have elevated alanine transaminase (ALT) and cholesterol levels. Furthermore, it has been shown that children's risk of developing NAFLD increases by approximately 1.5 times for every 10-unit increase in alanine transaminase (ALT) (measured in IU/L) and by approximately 2 times for every 20-unit (measured in mg/L) increase in body cholesterol [62,63].

The membrane protein encoded by PNPLA3 is found on the surface of lipid droplets within the endoplasmic reticulum. Hydrolase activity is exhibited by this highly expressed protein in human hepatocytes and hepatic stellate cells (HSCs) [64]. When methionine replaces isoleucine at position 148 of the PNPLA gene, a loss-of-function protein (I148 M) is produced instead of a functioning enzyme responsible for the breakdown of retinyl ester in HSCs and triglycerides in hepatocytes [65–67].

Finally, the altered protein is retained on lipid droplets, preventing proteasomal destruction and hindering the ability of hepatocytes to mobilize fatty acids [68]. Continued retention of the aberrant protein in the fat droplet may prevent other lipases from accessing the droplet, ultimately leading to the NAFLD phenotype [69].

6. Clinical significance

Our study has significant clinical significance because it demonstrates multiple inexpensive, applicable, and noninvasive measurements that can be used for NAFLD screening to protect asymptomatic patients from complications including liver cirrhosis and liver cell failure. Noninvasive diagnostic methods for NAFLD can be categorized into blood-based tests, techniques that evaluate the physical attributes of liver tissue, and imaging modalities. Various biomarkers and indices are currently used to diagnose or grade steatosis, either independently or in conjunction with anthropometric parameters, in order to construct models. Within this context, serum biomarkers stand out as parameters that are measured and analyzed to serve as indicators of underlying biological processes [70]. The initial biochemical indication for the diagnosis of NAFLD, as suggested by the clinical care pathway established by the American Gastroenterology Association (AGA), is the presence of abnormal blood-liver biochemistry [71].

7. Limitations

The limitations of this study are that it was a single-center study and NAFLD markers should be validated in multicenter studies. In addition, ultrasound is not an ideal technique for the assessment of fatty liver disease as it may miss mild steatosis, which is why some controls may have NAFLD. Further research is required to validate these results.

8. Conclusion

Anthropometric measurements, such as BMI and laboratory results, including liver enzymes, CRP, inflammatory markers, lipid parameters, and genetic markers, especially PNPLA3 polymorphisms, can provide an accurate, sensitive, and specific noninvasive approach for the early

identification and assessment of NAFLD and can guide its management. This may minimize the need for liver biopsy to assess NAFLD. Further large-scale studies are needed to confirm these findings and verify the model in larger studies.

9. Ethics consideration

The Institutional Ethics Committee of the School of Medicine at Minia University (Cairo, Egypt) approved all facets of the study. Adherence to the fundamental principles outlined in the 1975 Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice was maintained throughout the course of the study. Written informed consent was obtained from all participants involved in the study with the utmost diligence.

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CRediT authorship contribution statement

Amal A. Mohamed: Writing – original draft, Project administration, Formal analysis, Conceptualization. **Rania Al Dweik:** Writing – original draft, Visualization, Investigation, Conceptualization. **Reem A. Abdelghafour:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Ahmed Ramadan:** Writing – original draft, Validation, Formal analysis, Conceptualization. **Abbas M. Abbas:** Writing – original draft, Resources, Formal analysis, Data curation. **Hussein H. Samir:** Writing – review & editing, Methodology, Data curation. **Nashwa M. Muharram:** Supervision, Resources, Formal analysis, Data curation. **Randa Ibrahim Ahmed Elshiha:** Writing – original draft, Software, Investigation, Data curation. **Naglaa El-Salawy:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Data curation. **Doaa Ghaith:** Writing – original draft, Resources, Methodology, Funding acquisition, Data curation. **Marwa K. Darwish:** Writing – review & editing, Methodology, Funding acquisition. **Soha M. Abd El Salam:** Writing – review & editing, Validation, Investigation, Data curation. **Eman A. Sultan:** Writing – review & editing, Resources, Funding acquisition. **Amina S. Soliman:** Writing – original draft, Resources, Investigation, Formal analysis. **Mohamed Ezz AL Arab:** Supervision, Methodology, Funding acquisition, Formal analysis, Data curation. **Ahmed Yosri Elamir:** Writing – original draft, Methodology, Funding acquisition, Data curation. **Ahmed Ali Mohamed:** Writing – review & editing, Resources, Funding acquisition, Data curation. **Al-Shaymaa A. Hassanin:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Alaa Ali Mohamed Abouaggour:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Wael Hafez:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mohamed M. Omran:** Writing – original draft, Supervision, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

NAFLD	Nonalcoholic fatty liver disease
PNPLA3	Patatin-like phospholipase domain-containing protein 3
GWAS	Genome-wide association studies
ALT	Alanine transaminase
AST	aspartate transaminase
GGT	Gamma-glutamyl transferase
ALP	Alkaline phosphatase
TG	triglyceride
BMI	Body mass index
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
INR	International normalizing ratio
TNF-α	Tumor necrosis factor-alpha
CRP	C-reactive protein
PPV	Positive predictive value
NPV	Negative predictive value
AUC	Area under the curve
ROC	Receiver operating characteristic curve

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