Basuni *et al. Egyptian Liver Journal* (2024) 14:70
https://doi.org/10.1186/s43066-024-00378-9

Egyptian Liver Journal

RESEARCH

Open Access

Exploring serum bile acids as potential noninvasive biomarkers for nonalcoholic fatty liver disease

Ashraf Abbass Basuni[1], Dina Sweed[2], Mohammed Fathey Elgazzar[3] and Ashraf Khalil[1*]

Abstract

Background Bile acids are vital regulators of liver metabolism, and their dysregulation is closely linked with the progression of nonalcoholic fatty liver disease (NAFLD). Profiling these bile acids may provide valuable diagnostic and prognostic markers for these conditions. This study aimed to evaluate bile acid profiles in NAFLD patients and assess their potential as biomarkers for diagnosing and predicting disease progression. Serum levels of 14 bile acids were measured in 25 normal healthy controls (NHC), 35patients with metabolic dysfunction–associated steatotic liver disease (MASLD), and 40 patients with NASH, categorized by the NAFLD Activity Score (NAS). Quantification was performed using high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS).

Results Primary unconjugated bile acids, CA and CDCA, along with conjugated acids GCA, GCDCA, TCA, and TCDCA, were significantly elevated in both MASLD and NASH compared to NHC (all $p < 0.05$). While levels increased progressively from NHC to MASLD to NASH, no significant differences were observed between MASLD and NASH except for GCA and TCA ($P < 0.05$). Similarly, secondary bile acids LCA, TLCA, GUDCA, and TUDCA were higher in MASLD and NASH compared to NHC (all $p < 0.05$).

Logistic regression identified CA (odds ratio = 2.05, $p = 0.02$), CDCA (odds ratio = 1.58, $p = 0.04$), GCA (odds ratio = 1.92, $p = 0.03$) and DCA (odds ratio = 2.06, $p = 0.04$) as significant predictors of fibrosis. For active inflammation, GCA (odds ratio = 2.04, $p = 0.04$), and TCA (odds ratio = 1.94, $p = 0.04$) were significant predictors. In steatosis, CA, CDCA, GCA, DCA, TDCA, TLCA, and UDCA were notable predictors, with high odds ratios.

Conclusion The study highlights significant alterations in bile acid profiles associated with NAFLD progression. Specific bile acids, such as CA, GCA, TCA, and TCDCA are strong predictors of disease severity, indicating their potential as biomarkers for NAFLD treatment and prognosis.

Keywords Bile acids, NAFLD, Nonalcoholic fatty liver disease, NASH, Nonalcoholic steatohepatitis, Liquid chromatography-mass spectrometry

Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of liver disorders, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). The mildest form, nonalcoholic fatty liver (NAFL), is characterized by lipid accumulation in hepatocytes, also known as hepatic steatosis. However, 20%–30% of NAFL cases may progress to NASH, marked by significant lobular inflammation and hepatocyte ballooning, potentially leading to fibrosis and cirrhosis [1, 2]. NAFLD has emerged as the

*Correspondence:
Ashraf Khalil
ashkalil2010@gmail.com
[1] Department of Biochemistry and Molecular Diagnostics, National Liver Institute, Menoufia University, Shebin El Kom, Egypt
[2] Department of Pathology, National Liver Institute, Menoufia University, Shebin El Kom, Egypt
[3] Department of Hepatology and Gastroenterology, National Liver Institute, Menoufia University, Shebin El Kom, Egypt

leading cause of chronic liver disease worldwide, with a prevalence estimated at 25% [3–5]. NASH, a severe form of NAFLD, affects about 1.5% to 6.45% of the population and can lead to serious complications such as cirrhosis, hepatocellular carcinoma, and liver-related mortality. The prevalence of NASH is projected to increase by 63% between 2015 and 2030, posing significant challenges to global healthcare [5–7].

The pathogenesis of NASH is complex and involves multiple mechanisms, including abnormal lipid accumulation and inflammation within hepatocytes or extrahepatic tissues, leading to hepatotoxic injuries [8, 9]. NAFLD is strongly associated with metabolic dysfunctions, such as insulin resistance, obesity, and type 2 diabetes, particularly in Western populations. To better describe the metabolic basis of NAFLD, the term metabolic dysfunction–associated fatty liver disease (MAFLD) has been proposed [10, 11].

Bile acids are increasingly recognized as critical signaling molecules in NASH pathogenesis, facilitating communication between the liver, intestine, and other organs. These molecules, synthesized from cholesterol in the liver, play a vital role in emulsifying fats and in the digestion and absorption of lipids and fat-soluble vitamins. Primary bile acids, such as cholic acid and chenodeoxycholic acid, are transformed by intestinal bacteria into secondary bile acids, including lithocholic acid and deoxycholic acid [12, 13]. Besides their traditional roles, bile acids act as signaling molecules that regulate metabolic homeostasis and immune responses, primarily through receptors such as FXR and G protein-coupled bile acid receptor 1 (TGR5) [14]. Dysregulation of bile acid homeostasis is implicated in several metabolic diseases, including NASH, making bile acids and their receptors potential therapeutic targets [14, 15].

Despite advances in understanding NAFLD, predicting which patients are at risk for disease progression and complications remains challenging [16]. Liver biopsy is the gold standard for assessing inflammation and fibrosis in NAFLD but is invasive and carries risks such as bleeding and infection. Efforts to identify noninvasive biomarkers, particularly for inflammation and fibrosis, have produced mixed results, limiting their clinical utility [17–19]. Bile acids have emerged as promising biomarkers due to their roles in lipid absorption and regulation of hepatic glucose and lipid metabolism. High serum insulin levels can inhibit bile acid synthesis by suppressing CYP7A1, a key enzyme in bile acid biosynthesis, while elevated bile acids can reduce insulin secretion via glucagon-like peptide 1. This complex interplay highlights the connections between NAFLD, metabolic syndrome, and gut health [20–23].

The aim of this study is to comprehensively evaluate bile acid profiles as noninvasive biomarkers in NAFLD, with the goal of establishing these profiles as reliable diagnostic tools for assessing liver disease severity and predicting progression to NASH.

Patients

The study was conducted between October 2022 and April 2024 at the Department of Clinical Biochemistry and Molecular Diagnostics and the Department of Hepatobiliary and Gastroenterology. A total of 75 patients with liver ultrasound and biopsy-proven NAFLD were enrolled. Biopsy confirmation was essential to ensure accurate differentiation between simple steatosis and nonalcoholic steatohepatitis (NASH), thus enabling a precise evaluation of bile acid metabolism across different stages of liver disease. A control group of 25 individuals without any liver impairment, verified through clinical assessment and abdominal ultrasound, was also included to provide a baseline for comparison.

NAFLD patients were further categorized into two subgroups based on histological assessment: 35 patients were classified as having metabolic dysfunction–associated steatotic Liver Disease (MASLD) with minimal or no fibrosis (F0-F1), no significant inflammation (A0-A1), and varying degrees of steatosis (G0-G3). The remaining 40 patients were diagnosed with NASH, characterized by the presence of steatosis, inflammation of grade $\geq$ A2, and/or fibrosis. The severity of liver disease was quantified using the NAFLD Activity Score (NAS) [24], with a score of ≥ 5 strongly indicative of NASH and a score of ≤ 3 associated with MASLD.

Patients with other liver conditions, such as viral hepatitis, autoimmune liver diseases, hepatotoxic drug use, iron overload, Wilson's disease, chronic cholestasis, and extrahepatic obstructive gallbladder diseases, were excluded from the study. Additionally, individuals with severe renal or systemic conditions affecting liver function were excluded to ensure that bile acid alterations could be attributed specifically to NAFLD. The study protocol was approved by the Ethics Committee of the National Liver Institute (IRB 00570/2024), and written informed consent was obtained from all participants. Figure 1 represents different histological stages of patients with Metabolic Dysfunction–Associated Steatosis Liver Disease.

Serum sample collection and bile acid measurement

Blood samples were obtained from both patients and controls following an overnight fast (8-12h). Three milliliters of blood were collected using sterile venipuncture techniques, and the extracted serum was stored at -80°C until analysis. Laboratory measurements, encompassing

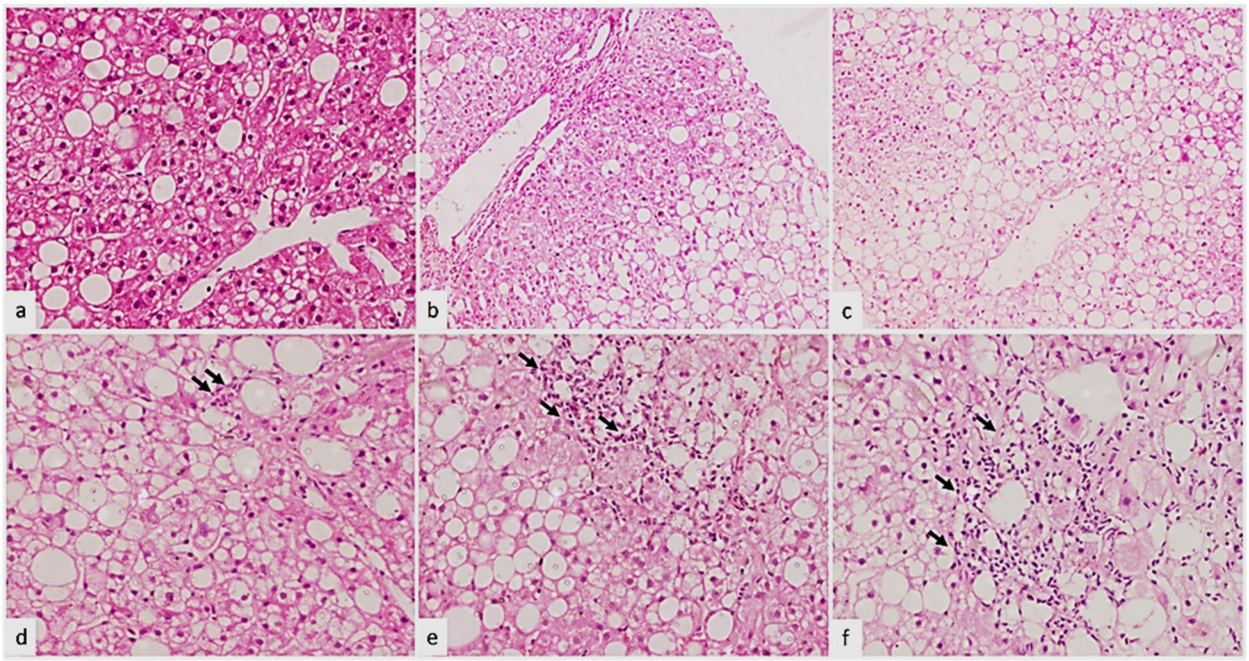


Fig. 1 Activity grade of the metabolic dysfunction–associated steatotic liver disease (MASLD) cases: **a** shows mild steatosis limited to zone 3, Steatosis 1 (H&E 100x), **b** shows moderate steatosis extending to zone 2, Steatosis 2 (H&E 100x), **c** shows severe steatosis involving zone 3, 2 and 1, Steatosis 3 (H&E 100x), **d** shows mild lobular necroinflammation (arrows) without associated hepatocytes ballooning degeneration, Activity 2 (H&E 200x), **e** shows moderate lobular necroinflammation (arrows) with mild hepatocytes ballooning degeneration, Activity 3 (H&E 200x), **f** shows marked lobular necroinflammation (arrows) with prominent hepatocytes ballooning degeneration, Activity 4 (H&E 200x)

fasting blood glucose, HbA1c, lipid profile including total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, triglycerides; liver function tests AST, ALT, gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), direct and total bilirubin, albumin, and total proteins, AFP; kidney function tests as BUN, creatinine were conducted through standardized laboratory methods (Cobas 8000, Roche Diagnostics GmbH, Mannheim, Germany). Hematological parameters were measured by Sysmex XT-1800i, and CS-1600 automated hematology analyzers (Sysmex Corporation, Kobe – Japan). Serum bile acids concentrations were measured in serum using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS), employing a reversed-phase (C18) column (1.7 µm, 100 mm × 2.1 mm internal dimensions) (Waters ACQUITY, Milford, MA) and a methanol/water gradient. The assay included a total of 14 bile acids, categorized into six primary and eight secondary bile acids. The primary bile acids consisted of unconjugated forms, including CA (cholic acid) and CDCA (chenodeoxycholic acid), and conjugated forms, including GCA (glycocholic acid), GCDCA (glycochenodeoxycholic acid), TCA (taurocholic acid), and TCDCA (taurochenodeoxycholic acid). The secondary bile acids included unconjugated forms, including DCA (deoxycholic acid) and LCA (lithocholic acid), and conjugated forms, including GDCA (glycodeoxycholic acid), TDCA (taurodeoxycholic acid), TLCA (taurolithocholic acid), UDCA (ursodeoxycholic acid), GUDCA (glycoursodeoxycholic acid), and TUDCA (tauroursodeoxycholic acid). All these chemical standards were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). HPLC grade water was obtained from Millipore pure water purification system (Diamond TII, USA). HPLC grade methanol, acetonitrile, and formic acid were purchased from Fisher Scientific (Daytona plus, Randox laboratories limited, UK).

Serum sample preparation and bile acid detection

Serum bile acids underwent preparation for UPLC/MS/MS as previously described [13, 14]. Briefly, 100 µl of serum samples were treated with 400 µl of ice-cold 100% methanol, followed by centrifugation at 12,000 rpm for 20 min. Fifty microliters of the supernatant were mixed with 100 µl formic acid (1:1000), and 5 µl were injected into a C18 column at 50°C. Bile acids were eluted by gradient at a flow rate of 0.5 ml/min, with the mass spectrometer operating in the negative ion mode via Multiple Reactions Monitoring (MRM). UPLC-MS data were analyzed using MassLynx software version 4.1 (Waters Corp., Milford, MA, USA) to generate calibration equations and

calculate the quantitative concentration of each bile acid in the sample. The UPLC-MS/MS analysis demonstrated robust performance, including precision and accuracy at concentrations of 0.02 μMol/L, 0.2 μMol/L, and 2 μMol/L, with low relative standard deviation (RSD%) values indicating excellent precision. The relative error (RE%) values were close to 0%, underscoring the high accuracy of quantitation. All 14 individual bile acids were effectively resolved and quantified. The assay exhibited strong linearity over a wide concentration range (0.012 to 5×10^3 μMol/L), highlighting its suitability for various analytical needs. With a lower quantitation limit of 2 ng/mL, the UPLC-MS/MS assay demonstrated exceptional sensitivity, making it highly reliable for quantitative assessments in biomedical research.

Statistical analysis

Data were analyzed using SPSS 23 (SPSS Inc., CA, USA). The Kolmogorov–Smirnov test was employed to verify the normality of distribution. Numerical variables were presented as mean ± standard deviation or as medians and interquartile ranges (IQR), while categorical variables were expressed as numbers (percentages). Differences between groups were analyzed using the Kruskal–Wallis H test and the Mann–Whitney U test as appropriate. A *P*-value of <0.05 was considered statistically significant for all analyses. Non-parametric Spearman correlation was used to assess the relationships between bile acids and other numerical variables, such as lipid profiles and blood chemistry parameters. Principal Component Analysis (PCA) was conducted to explore patterns in bile acid concentrations among the study cohort, which included individuals with NAFLD and control subjects. The receiver operating characteristic (ROC) curve was used to evaluate the discriminatory power of bile acids in distinguishing controls from NAFLD cases. Binary logistic regression was applied to assess the predictive value of bile acids for fibrosis, lobular inflammation, and steatosis in NAFLD patients.

Results

Demographic and clinical characteristics in normal and NAFLD groups

The data presented in Table 1 summarize the clinical characteristics of the study population, divided into three groups: Control, NASH, and MASLD. NASH and MASLD groups had a significantly higher proportion of hyperlipidemic patients compared to the control group ($\chi^2=14.23$, $p=0.001$). The NASH and MASLD groups demonstrated higher mean body mass index (BMI) values compared to the control group ($p<0.001$), indicating a greater prevalence of obesity among patients with NASH and MASLD. Furthermore, the presence of diabetes mellitus was more common in the NASH and MASLD groups, with a higher percentage of patients being diagnosed with DM ($\chi^2=5.42$, $p=0.067$).

Age differences across the groups were not statistically significant ($t=1.02$, $p=0.313$), suggesting that age is not a primary differentiating factor in the progression of NAFLD. Similarly, gender distribution did not differ significantly between the groups ($\chi^2=0.73$, $p=0.695$).

Hypertension prevalence was assessed across the groups, but no significant differences were observed ($\chi^2=1.44$, $p=0.487$). However, the trend indicated a higher occurrence of hypertension in the NASH group compared to the control and MASLD groups.

There were significant differences in fibrosis and inflammation activity scores between the groups, with NASH patients having more advanced fibrosis (F2-F3) and higher activity (A2-A3) compared to the control group ($p<0.001$). The distribution of steatosis grades and ultrasound findings also differed significantly among the groups, with the NASH and MASLD groups showing more severe liver changes. These results highlight the progressive nature of liver disease in NAFLD and underscore the importance of early diagnosis and management.

Biochemical and hematological parameters across control, NASH, and MASLD groups

Table 2 details the biochemical and hematological parameters, showing significantly higher median FBS levels in NASH and MASLD groups, indicating a tendency towards hyperglycemia. Direct bilirubin and GGT levels were elevated in NASH and MASLD groups, suggesting liver dysfunction. Lipid profile analysis revealed higher total cholesterol levels in NASH and MASLD groups, reflecting dyslipidemia. Hematological markers such as hemoglobin and platelet count remained stable across groups.

Alterations in bile acid across NAFLD groups

Table 3 summarizes the bile acid profiles among NHC, MASLD, and nonalcoholic steatohepatitis (NASH). Primary unconjugated bile acids, CA and CDCA, were significantly elevated in both MASLD and NASH compared to NHC (all $p<0.05$), with no significant differences between MASLD and NASH (all $p>0.05$). Similarly, primary conjugated bile acids, including GCA, GCDCA, TCA, and TCDCA, were significantly higher in NASH and MASLD compared to NHC (all $p<0.05$). GCA and TCA levels were notably higher in NASH compared to MASLD (all $p<0.05$), whereas GCDCA and TCDCA did not differ significantly between MASLD and NASH (all $p>0.05$).

Secondary bile acids, LCA, TLCA, GUDCA, and TUDCA, were significantly elevated in NASH and

Table 1 Clinical characteristics of the studied groups

	NHC ($n=25$)	MASLD ($n=35$)	NASH ($n=40$)	*P*-value
Gender -Female	16 (64%)	23 (65.7%)	23 (57.5%)	$\chi^2=0.73$, $p=0.695$
-Male	9 (36%)	12 (34.3%)	17 (42.5%)	
Age (years)	50.32 ± 9.45	50.31 ± 8.56	48.13 ± 8.77	t = 1.02, $p=0.313$
Weight (kg)	72.92 ± 5.90	81.26 ± 10.27	77.00 ± 10.71	t = 3.34, $p=0.001$
Height (cm)	169.20 ± 8.97	162.69 ± 8.39	163.25 ± 9.81	t = 2.49, $p=0.015$
BMI (kg/m^2)	25.73 ± 3.88	30.91 ± 4.77	29.22 ± 5.64	t = 4.50, $p<0.001$
Diabetes Mellitus				
-DM	4 (16%)	13 (37.1%)	12 (30%)	$\chi^2=5.42$, $p=0.067$
-No DM	14 (56%)	13 (37.1%)	17 (42.5%)	
-Prediabetes (pDM)	7 (28%)	9 (25.7%)	11 (27.5%)	
Hypertension				
-Yes	7 (28%)	10 (28.6%)	16 (40%)	$\chi^2=1.44$, $p=0.487$
-No	18 (72%)	25 (71.4%)	24 (60%)	
Lipid Status				
-Hyperlipidemic	6 (24%)	20 (57.1%)	26 (65%)	$\chi^2=14.23$, $p=0.001$
-Normolipidemic	19 (76%)	15 (42.9%)	14 (35%)	
Fibrosis Stage				
-F0	25 (100%)	25 (71.4%)	0 (0%)	$\chi^2=58.75$, $p<0.001$
-F1	0 (0%)	10 (28.6%)	0 (0%)	
-F2	0 (0%)	0 (0%)	16 (40%)	
-F3	0 (0%)	0 (0%)	24 (60%)	
Activity Grade				
-A0	25 (100%)	22 (62.9%)	0 (0%)	$\chi^2=54.43$, $p<0.001$
-A1	0 (0%)	13 (37.1%)	0 (0%)	
-A2	0 (0%)	0 (0%)	16 (40%)	
-A3	0 (0%)	0 (0%)	24 (60%)	
Steatosis Grade				
-G0	25 (100%)	0 (0%)	0 (0%)	$\chi^2=75.00$, $p<0.001$
-G1	0 (0%)	18 (51.4%)	0 (0%)	
-G2	0 (0%)	11 (31.4%)	16 (40%)	
-G3	0 (0%)	6 (17.1%)	24 (60%)	
Ultrasound Findings				
-Normal homogenous liver	25 (100%)	0 (0%)	0 (0%)	$\chi^2=88.75$, $p<0.001$
-Fatty liver	0 (0%)	17 (48.6%)	0 (0%)	
-Mildly enlarged fatty liver	0 (0%)	8 (22.9%)	9 (22.5%)	
-Moderately enlarged fatty liver	0 (0%)	10 (28.6%)	31 (77.5%)	

Clinical characteristics of NHC, MASLD, and NASH groups with statistical significance for demographic, anthropometric, metabolic, and histological variables

NHC Normal healthy control, *MASLD* Metabolic dysfunction–associated steatotic liver disease, *NASH* Nonalcoholic steatohepatitis, *F0* No fibrosis F1: Mild fibrosis F2: Moderate fibrosis F3: Advanced fibrosis. Lobular inflammation Grade: A0: No lobular inflammation A1: Mild lobular inflammation A2: Moderate lobular inflammation, A3: Severe lobular inflammation. Steatosis Grade: G0: No steatosis (no fat accumulation in the liver), G1: Mild steatosis G2: Moderate steatosis G3: Severe steatosis. Ultrasound Findings: Normal homogeneous liver: Liver with normal echotexture. Fatty liver: Liver with increased echogenicity indicating fat accumulation. Mildly enlarged fatty liver: Slight increase in liver size with fatty infiltration. Moderately enlarged fatty liver: Moderate increase in liver size with more pronounced fatty infiltration

MASLD compared to NHC (all $p<0.05$), with no significant differences between MASLD and NASH (all $p>0.05$). DCA, GDCA, TDCA, and UDCA did not show significant differences between either MASLD or NASH and NHC (all $p>0.05$).

Bile acids in discriminating NAFLD patients and control

ROC curves (Fig. 2) and Principal Component Analysis (PCA) (Fig. 3) were used to examine the capacity of bile acids in discriminating healthy from NAFLD patients. Figure 2a, b summarize the ROC curve

Table 2 Descriptive Statistics and Kruskal–Wallis Test Results for Biochemical and Hematological Parameters Across NHC, NASH, and MASLD Groups

Parameter	NHC ($n=25$)	MASLD ($n=35$)	NASH ($n=40$)	*P*-value
Blood Glucose & Hemoglobin				
FBS (mg/dL)	112 (21.5)	130 (71)[a]	143 (88.25)[b]	0.011
HA1C (%)	5.6 (0.35)	5.8 (0.9)	5.8 (1.475)	0.467
Renal Function				
Creatinine (mg/dL)	0.77 (0.205)	0.78 (0.15)	0.77 (0.255)	0.358
Urea (mg/dL)	23 (6.5)	23 (11)	23.5 (12.75)	0.364
UA (mg/dL)	5.3 (2.0)	5.3 (0.8)	5.3 (2.0)	0.668
Liver Function				
AST (U/L)	16 (8.5)	20 (31)	23 (41)	0.071
ALT (U/L)	21 (18.5)	24 (15)	30.5 (40.25)	0.136
TBIL (mg/dL)	0.9 (0.4)	0.98 (0.11)	0.9 (0.1)	0.215
DBIL (mg/dL)	0.11 (0.035)	0.13 (0.07)[a]	0.15(0.035)[b]	0.038
ALB (g/dL)	3.8 (0.5)	4.2 (1.1)	3.8 (0.775)	0.141
Glob (g/dL)	3.9 (0.9)	3.1 (1.3)[a]	3.7 (1.05)	0.016
TP (g/dL)	7.5 (0.45)	7.5 (0.7)	7.6 (0.55)	0.963
GGT (U/L)	12.4 (8.5)	23 (41)[a]	20 (12)[b]	0.002
Lipid Profile				
Chol (mg/dL)	188 (47)	214 (62)[a]	207 (45.5)[b]	0.014
LDL (mg/dL)	105 (8.5)	99 (45)	102 (28)	0.269
HDL (mg/dL)	56 (14)	65 (31)	64 (28.5)	0.174
TG (mg/dL)	123 (25)	116 (27)	123 (35.75)	0.294
Hematological Parameters				
Hb (g/dL)	11.7 (2.8)	12.0 (2.4)	12.8 (2.575)	0.489
RBC (10^6/μL)	4.71 (1.125)	4.71 (0.83)	4.71 (0.98)	0.945
Platelets (10^3/μL)	250 (40)	240 (41)	235 (53.75)	0.103
WBCs (10^3/μL)	6.1 (2.7)	5.8 (2.7)	5.9 (4.15)	0.602

Comparative analysis of bile acid profiles using Kruskal–Wallis and Pairwise comparisons across NHC, MASLD, and NASH Groups, *IQR* Interquartile rang, *K* Kruskal–Wallis test comparison among all groups

NHC Normal healthy control, *MASLD* Metabolic dysfunction–associated steatotic liver disease, *NASH* Nonalcoholic steatohepatitis, *AST* Aspartate transaminase, *ALT* Alanine transaminase, *GGT* Gamma-Glutamyl Transferase, *ALP* Alkaline phosphatase, *TBil* Total bilirubin, *DBIL* Direct bilirubin, *TP* Total protein, *Alb* Albumin, *UA* Uric acid, *Chol* Cholesterol, *LDL* Low density lipoprotein, *HDL* high density lipoprotein. *TG* Triglyceride, *Hb* Hemoglobin, *WBCs* White blood cells. *RBCs* Red blood cells

P-value < 0.05 indicates significant. Mann–Whitney U test comparison between two groups. *P*-value < 0.05 indicates significant

[a] Comparing between NHC vs. MASLD group

[b] Comparing between NHC vs. NASH group

[c] Comparing between MASLD vs. NASH group

analysis for primary and secondary bile acids in differentiating healthy controls from NAFLD patients (MASLD + NASH). The primary unconjugated bile acid CDCA showed high diagnostic potential with an AUC of 0.937, 100% specificity, and 74.7% sensitivity at a cutoff of 0.002. The primary conjugated bile acid GCDCA achieved an AUC of 0.877, 100% specificity, and 80% sensitivity at a cutoff of 2.48.

The secondary bile acids collectively demonstrated moderate diagnostic performance. LCA had an AUC of 0.864, with 96% specificity and 74.7% sensitivity at a cutoff of 0.002, while TLCA had an AUC of 0.840, with 12% specificity and 80% sensitivity at a cutoff of 0.002. Other secondary bile acids, including TUDCA and GDCA, displayed varying degrees of diagnostic accuracy, with AUCs ranging from 0.784 to 0.420, reflecting different levels of specificity and sensitivity.

Overall, primary bile acids exhibited superior diagnostic accuracy compared to secondary bile acids, underscoring their potential utility as biomarkers for distinguishing NAFLD patients from healthy controls. In the subsequent analysis comparing MASLD and NASH, the ROC curves for both primary and secondary bile acids showed limited diagnostic utility, with AUC values generally indicating weak discrimination. Primary bile acids like CDCA and CA had AUCs of 0.596 and 0.575,

Table 3 Comparative Analysis of Bile Acid Profiles Across NHC, MASLD, and NASH Groups

Bile Acid	Class	NHC Median (IQR)	MASLD Median (IQR)	NASH Median (IQR)	K-Wallis *P*-value
CA	1ryU	0.130 (0.12)	0.230 (1.55)[a]	0.410 (1.41)[b]	0.000
CDCA	1ryU	0.001 (0.000)	0.050 (3.298)[a]	0.830 (3.29)[b]	0.000
GCA	1rygc	0.190 (0.165)	0.400 (1.76)[a,c]	1.770 (4.83)[b,c]	0.031
GCDCA	1rygc	2.120 (0.3)	3.730 (1.97)[a]	4.220 (9.14)[b]	0.000
TCA	1rytc	0.002 (0.015)	0.130 (0.028)[a,c]	0.310 (0.019)[b,c]	0.004
TCDCA	1rytc	0.002 (0.059)	3.205 (4.32)[a]	4.390 (4.40)[b]	0.000
DCA	2ryU	0.160 (0.06)	0.280 (0.51)	0.335 (0.56)	0.588
LCA	2ryU	0.001 (0.000)	0.030 (0.218)[a]	0.006 (0.206)[b]	0.000
GDCA	2rygc	0.060 (0.13)	0.110 (0.168)	0.105 (0.143)	0.447
TDCA	2rytc	0.002 (0.001)	0.002 (0.049)	0.002 (0.002)	0.218
TLCA	2rytc	0.001 (0.000)	0.003 (0.008)[a]	0.010 (0.468)[b]	0.000
UDCA	2ryU	0.030 (0.034)	0.002 (0.009)	0.002 (0.059)	0.506
GUDCA	2rygc	0.003 (0.001)	1.200 (1.698)[a]	1.300 (1.698)[b]	0.025
TUDCA	2rytg	0.001 (0.001)	0.005 (0.259)[a]	0.005 (0.039)[b]	0.000

Comparative analysis of bile acid profiles using Kruskal–Wallis and Pairwise comparisons across NHC, MASLD, and NASH Groups: *IQR* Interquartile rang, *K* Kruskal–Wallis test comparison among all groups

NHC Normal healthy control, *MASLD* Metabolic dysfunction–associated steatotic liver disease, *NASH* Nonalcoholic steatohepatitis. *CA* Cholic acid, *CDCA* Chenodeoxycholic acid, *DCA* Deoxycholic acid, *LCA* Lithocholic acid, *UDCA* Ursodeoxycholic acid, *GCA* Glycholic acid, *GCDCA* Glycochenodeoxycholic acid, *GDCA* Glycodeoxycholic acid, *GUDCA* Glycoursodeoxycholic acid, *TCA* Taurocholic acid, *TCDCA* Taurochenodeoxycholic acid, *TDCA* Taurodeoxycholic acid, *TLCA* Taurolithocholic acid, *TUDCA* Tauroursodeoxycholic acid. *1ryU* primary unconjugated, 1rygc: primary glycoconjugated, *1rytc* primary tauroconjugated, *2ryU* secondary unconjugated, *2rygc* secondary glycoconjugated, *2rytc* secondary tauroconjugated

[*] *P*-value < 0.05 indicates significant. Mann–Whitney U test comparison between two groups. *P*-value < 0.05 indicates significant

[a] Comparing between NHC vs. MASLD group

[b] Comparing between NHC vs. NASH group

[c] Comparing between MASLD vs. NASH group

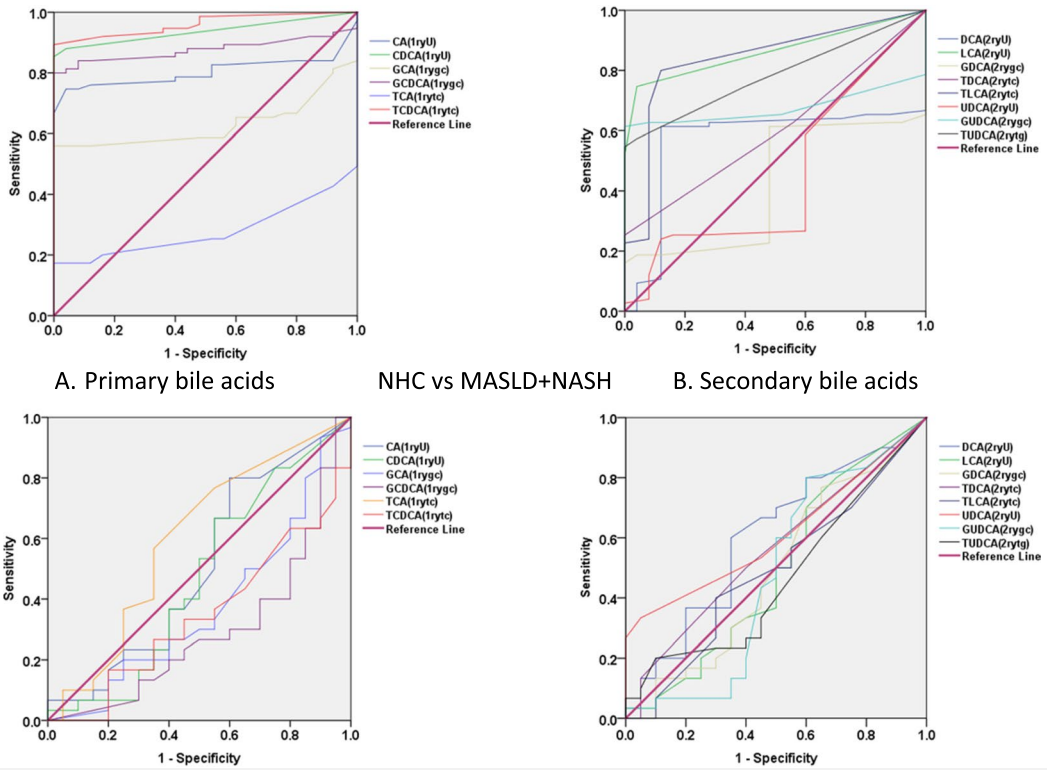
respectively, demonstrating moderate accuracy. Sensitivity and specificity were inconsistent, with significant variability. Secondary bile acids exhibited similarly low AUC values, ranging from 0.466 for LCA to 0.571 for TLCA, indicating limited differentiation between the conditions (Fig. 2c, d). These findings, suggest that the bile acids assessed are not robust biomarkers for distinguishing MASLD from NASH. PCA of these bile acids across the studied groups. PCA demonstrated that the first two principal components (PC1 and PC2) accounted for 55.82% of the total variance, with eigenvalues of 5.27 and 2.54, respectively. Key bile acids, including GCDCA, CA, and CDCA, emerged as significant contributors, underscoring their potential as biomarkers for differentiating NAFLD from the control group (Fig. 3).

Impact of gender, diabetes status, and lipid profile on bile acid levels in MASLD and NASH patients

Table 4 presents the analysis of bile acid levels in NAFLD (MASLD and NASH) patients across gender, diabetes status, and lipid profile, highlighting several key differences. Significant differences were observed in TDCA and TUDCA levels between genders, with females showing higher levels ($p=0.033$ and $p=0.037$, respectively). For diabetes status, CA, CDCA, GCA, and TCDCA levels were significantly different, with the highest levels found in the DM group ($p<0.05$ for all). Hyperlipidemic patients exhibit higher significant differences in bile acids CA, LCA, GCA and GUDCA than normolipidemic with $p<0.05$ for all these bile acids, suggesting that lipid profile substantially impact bile acid metabolism in NAFLD. Further correlation analysis between bile acids and lipid profile in MASLD and NASH revealed several significant associations between some bile acids and lipid profile parameters (Table 5).

In the MASLD group, CA and CDCA had strong negative correlations with LDL (CA: r = -0.57**, $p<0.01$; CDCA: $r=$ -0.53**, $p<0.01$) and positive correlations with HDL (CA: $r=0.57$**, $p<0.01$; CDCA: $r=0.56$**, $p<0.01$). LCA and GDCA were also negatively correlated with LDL (LCA: $r=$ -0.69**, $p<0.01$; GDCA: $r=$ -0.41*, $p<0.05$) and positively correlated with HDL (LCA: $r=0.70$**, $p<0.01$); GDCA: $r=0.39$*, $p<0.05$). Conversely, other bile acids did not show significant correlations with lipid components.

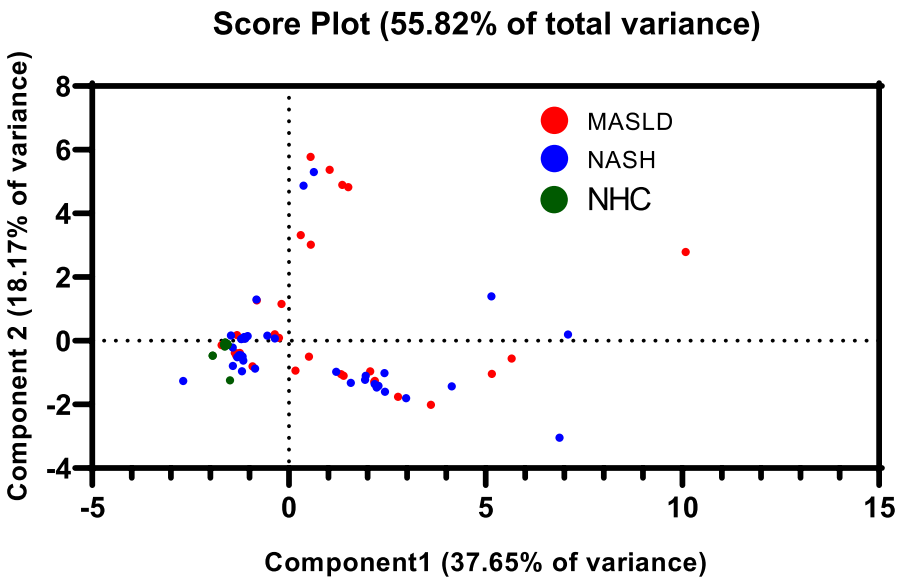
In the NASH group, unconjugated bile acids, CA was positively correlated with HDL (CA: $r=0.42$**, $p<0.01$) and negatively correlated with LDL (CA: $r=$ -0.31*,



		Con vs MASLD+NASH				MASLD vs NASH			
Bile Acid	Class	AUC	Optimal Cutoff	Sensitivity	Specificity	AUC	Optimal Cutoff	Sensitivity	Specificity
CA	1ryU	0.804	0.055	84	80	0.575	0.006	97.5	2.9
CDCA	1ryU	0.937	0.002	74.7	100	0.596	0.002	97.5	22.9
GCA	1rygc	0.63	0.145	81.3	92	0.385	0.002	97.5	0
GCDCA	1rygc	0.877	2.480	80	100	0.411	2.085	90	8.6
TCA	1rytc	0.279	0.023	25.3	56	0.516	0.006	47.5	51.4
TCDCA	1rytc	0.961	0.070	92	16	0.422	0.006	97.5	2.9
DCA	2ryU	0.568	0.125	65.3	80	0.509	0.002	77.5	8.6
LCA	2ryU	0.864	0.002	74.7	96	0.466	0.002	70	20
GDCA	2rygc	0.42	0.085	60	48	0.48	0.002	92.5	14.3
TDCA	2rytc	0.604	0.002	62.7	56	0.525	0.002	67.5	42.9
TLCA	2rytc	0.84	0.002	80	12	0.571	0.002	80	20
UDCA	2ryU	0.451	0.035	25.3	24	0.558	0.002	62.5	54.3
GUDCA	2rygc	0.676	0.245	54.7	0	0.528	0.002	80	22.9
TUDCA	2rytg	0.784	0.004	57.3	4	0.513	0.002	75	25.7

Fig. 2 ROC Curves for Primary and Secondary Bile Acids in distinguishing healthy controls from MASLD and NASH groups (top) and MASLD from NASH bottom). The AUC values indicate the diagnostic accuracy, with sensitivity and specificity providing additional details on the effectiveness of each bile acid as a biomarker. The optimal cutoffs are the thresholds that maximize the sum of sensitivity and specificity, indicating the most effective point for differentiating the conditions. NHC: Normal healthy control, MASLD: Metabolic dysfunction–associated steatotic liver disease, NASH: Nonalcoholic steatohepatitis. CA: Cholic acid, CDCA: Chenodeoxycholic acid, DCA: Deoxycholic acid, LCA: Lithocholic acid, UDCA: Ursodeoxycholic acid, GCA: Glycholic acid, GCDCA: Glycochenodeoxycholic acid, GDCA: Glycodeoxycholic acid, GUDCA: Glycoursodeoxycholic acid, TCA: Taurocholic acid, TCDCA: Taurochenodeoxycholic acid, TDCA: Taurodeoxycholic acid, TLCA: Taurolithocholic acid, TUDCA: Tauroursodeoxycholic acid. 1ryU: primary unconjugated, 1rygc: primary glycoconjugated, 1rytc: primary tauroconjugated, 2ryU: secondary unconjugated, 2rygc: secondary glycoconjugated, 2rytc: secondary tauroconjugated

$p<0.05$). Primary conjugated bile acids GCA, GCDCA and TCDCA were positively correlated with cholesterol (GCA: $r=0.49^{**}$, $p<0.01$; GCDCA: $r=0.38^{*}$, $p<0.05$; TCDCA: $r=0.31^{*}$, $p<0.05$). Secondary bile acids, GDCA, and GDUCA were positively correlated with cholesterol (GDCA: $r=0.39^{*}$, $p<0.01$; GDUCA: $r=0.38^{*}$, $p<0.05$) and HDL (GDCA: $r=0.46^{**}$, $p<0.01$; GUDCA: $r=0.50^{**}$, $p<0.01$) and negatively correlated with LDL (GDCA:



Component	Eigenvalue	Proportion of Variance	Cumulative Proportion of Variance	Key Bile Acids Contributing to Separation
PC1	5.27	37.65%	37.65%	GCDCA, CA, CDCA
PC2	2.54	18.17%	55.82%	TCA, GUDCA, GCA

Fig. 3 Score plot from Principal Component Analysis (PCA) illustrating the separation of bile acid profiles among NHC, MASLD, and NASH groups. The x-axis represents the first principal component (PC1), which explains 37.65% of the variance, while the y-axis represents the second principal component (PC2), accounting for 18.17% of the variance. The plot shows the distinct clustering of the groups, highlighting the differential bile acid signatures that distinguish the groups. The percentage values indicate the proportion of total variance captured by each component. NHC: Normal healthy control, MASLD: Metabolic dysfunction–associated steatotic liver disease, NASH, Nonalcoholic steatohepatitis

r = -0.44**, $p < 0.01$; GDUCA: r = -0.41*, $p < 0.05$). TUDCA positively correlated with cholesterol ($r = 0.37$*, $p < 0.05$) and HDL ($r = 0.38$*, $p < 0.05$). Other bile acids did not show significant correlations with any lipid components.

Bile acid levels across fibrosis, inflammation, and steatosis grades in NAFLD patients

Table 6 presents a detailed comparison of bile acid levels across various stages of fibrosis, inflammation, and steatosis in patients with either MASLD or NASH.

In the context of fibrosis: CA, CDCA and GCA levels were significantly higher in the F2-F3 group compared to the F0-F1 group (all $p < 0.05$). No significant differences were found for, in the primary conjugated GCDCA, TCA, TCDCA, and all the secondaries uncogitated and conjugated bile acid DCA, LCA, GDCA, TDCA, TLCA, UDCA, GUDCA, and TUDCA (all $p > 0.05$).

In the context of inflammation GCA, TCA and TLCA levels were significantly higher in the A2-A3 group compared to A0-A1 ($p < 0.05$). In contrast, LCA levels were notably higher in A0-A1 compared to A2-A3 but did not reach the level of significant ($p > 0.05$). No significant differences were found for CDCA, GCDCA, TCDCA, DCA, GDCA, TDCA, UDCA, GUDCA, and TUDCA (all $p > 0.05$).

In the context of steatosis, CA, GCA, TCA, TCDCA, and TLCA levels were significantly elevated in the G2-G3 group compared to G0-G1 (CA: $p = 0.003$; GCA: $p = 0.032$). DCA levels were significantly higher in G0-G1 compared to G2-G3 ($p < 0.05$). No significant differences were observed for CDCA, GCDCA, LCA, GDCA, TDCA, UDCA, GUDCA, and TUDCA (all $p > 0.05$).

Predictive value of bile acids for fibrosis, inflammation, and steatosis in NAFLD: logistic regression analysis

Table 7 reveals the logistic regression analysis results for bile acids as predictors of fibrosis, active inflammation, and steatosis in NAFLD patients (MASLD and NASH). For fibrosis the analysis identified several bile acids with significant associations with fibrosis, CA, CDCA and DCA were notable predictors, with CA showing an odds ratio of 2.05 ($p = 0.02$); CDCA had an odds ratio of (Exp(B) = 1.58, $p = 0.04$) and DCA an odds ratio of 2.06 ($p = 0.04$).

Table 4 Bile Acid Levels Across Gender, Diabetes Status, and Lipid Profile in NAFLD Patients

Bile Acid	Class	Female (Median [IQR])	Male (Median [IQR])	*P*-value	DM (Median (IQR)	Pre-DM (Median (IQR)	No DM Median (IQR)	*P*-value	Normolipidemic (Median [IQR])	Hyperlipidemic (Median [IQR])	Mann–Whitney U	*P*-value
CA	1ryU	0.900 (1.53)	0.320 (1.41)	0.952	1.06 (0.39)[a]	0.34 (0.44)	0.32 (0.66)	0.034	0.93 (1.38)	0.29 (1.59)	795.5	0.001
CDCA	1ryU	1.100 (3.32)	0.650 (3.33)	0.719	2.50 (0.75)[a]	1.98 (0.65)[b]	1.38 (3.32)	0.045	1.35 (3.53)	0.08 (3.25)	802.5	0.167
GCA	1rygc	1.765 (2.26)	0.280 (2.02)	0.116	2.80 (0.80)[a]	1.60 (0.59)[b]	1.09 (0.74)	0.039	1.23 (1.85)	1.78 (3.52)	582.5	0.016
GCDCA	1rygc	4.175 (4.21)	3.850 (1.94)	0.918	4.24 (9.36)	3.98 (3.43)	3.41 (2.04)	0.146	4.05 (1.75)	4.17 (6.73)	628.5	0.615
TCA	1rytc	0.006 (0.04)	0.002 (0.02)	0.991	0.002 (0.029)	0.01 (0.018)	0.002 (1.5)	0.892	0.00 (0.02)	0.01 (0.04)	541.0	0.139
TCDCA	1rytc	4.390 (4.36)	2.980 (4.39)	0.723	4.40 (4.16) [a]	3.65 (4.35)[b]	2.37 (4.32)	0.020*	4.06 (4.13)	4.22 (4.58)	667.5	0.935
DCA	2ryU	0.280 (0.50)	0.420 (0.61)	0.682	0.18 (0.495)	0.46 (0.495)	0.27 (0.54)	0.376	0.28 (0.52)	0.33 (0.55)	657.0	0.845
LCA	2ryU	0.030 (0.21)	0.002 (0.19)	0.235	0.002 (0.079)	0.007 (0.218)	0.11 (0.274)	0.616	0.04 (0.22)	0.08 (0.21)	713.5	0.023
GDCA	2rygc	0.110 (0.15)	0.110 (0.16)	0.934	0.01 (0.148)	0.12 (0.212)	0.11 (0.118)	0.115	0.11 (0.17)	0.11 (0.13)	753.5	0.392
TDCA	2rytc	0.004 (0.01)	0.002 (0.08)	0.033*	0.001 (0.001)	0.002 (0.079)	0.002 (0.001)	0.104	0.00 (0.00)	0.00 (0.04)	678.0	0.973
TLCA	2rytc	0.005 (0.18)	0.010 (0.01)	0.490	0.01 (0.008)	0.005 (0.177)	0.01 (0.458)	0.943	0.01 (0.02)	0.01 (0.01)	626.0	0.591
UDCA	2ryU	0.001 (0.01)	0.002 (0.06)	0.170	0.001 (0.030)	0.002 (0.059)	0.002 (0.009)	0.535	0.00 (0.04)	0.00 (0.05)	669.5	0.950
GUDCA	2rygc	0.245 (1.70)	1.200 (1.70)	0.755	0.10 (1.648)	1.20 (1.697)	1.20 (1.698)	0.439	0.77 (1.70)	1.20 (1.71)	644.0	0.036
TUDCA	2rytg	0.012 (0.24)	0.001 (0.17)	0.037*	0.003 (0.164)	0.005 (0.093)	0.006 (1.499)	0.871	0.01 (0.29)	0.01 (0.07)	683.5	0.926

Comparative analysis of bile acid profiles using Kruskal–Wallis and Pairwise comparisons across diabetic groups: IQR: Interquartile rang

DM Diabetes mellitus, *p-DM* pre-diabetic, *No DM* No diabetes mellitus, *CA* Cholic acid, *CDCA* Chenodeoxycholic acid, *DCA* Deoxycholic acid, *LCA* Lithocholic acid, *UDCA* Ursodeoxycholic acid, *GCA* Glycholic acid, *GCDCA* Glycochenodeoxycholic acid, *GDCA* Glycodeoxycholic acid, *GUDCA* Glycoursodeoxycholic acid, *TCA* Taurocholic acid, *TCDCA* Taurochenodeoxycholic acid, *TDCA* Taurodeoxycholic acid, *TLCA* Taurolithocholic acid, *TUDCA* Tauroursodeoxycholic acid. *1ryU* primary unconjugated, *1rygc* primary glycoconjugated, *1rytc* primary tauroconjugated, *2ryU* secondary unconjugated, *2rygc* secondary glycoconjugated, *2rytc* secondary tauroconjugated

[*] *P*-value < 0.05 indicates significant. Pairwise comparisons

[a] Comparing between No DM vs. DM group

[b] Comparing between No DM vs. pre-DM group

[c] Comparing between pre-DM vs. DM group. Mann–Whitney U test comparison between two groups (gender, lipidemic state). *P*-value < 0.05 indicates significant

Table 5 Correlation Analysis Between Bile Acids and Lipid Profile in MASLD and NASH

		MASLD, $n=35$				NASH, $n=40$			
Bile Acid	**Class**	**Chol**	**LDL**	**HDL**	**TG**	**Chol**	**LDL**	**HDL**	**TG**
CA	1ryU	0.22	-0.57**	0.57**	-0.12	0.18	-0.31*	0.42**	-0.11
CDCA	1ryU	0.15	-0.53**	0.56**	-0.12	0.29	-0.29	0.25	0.01
GCA	1rygc	0.14	0.06	-0.07	-0.08	0.49**	-0.19	0.28	-0.03
GCDCA	1rygc	-0.08	0.00	-0.02	-0.07	0.38*	-0.11	0.20	0.15
TCA	1rytc	-0.18	0.21	-0.20	-0.29	0.16	-0.01	0.29	0.05
TCDCA	1rytc	-0.32	0.32	-0.14	-0.13	0.31*	-0.14	0.29	0.13
DCA	2ryU	-0.13	-0.24	-0.24	-0.14	0.30	-0.22	0.20	-0.04
LCA	2ryU	0.14	-0.69**	0.70**	-0.17	0.29	-0.14	0.32	0.11
GDCA	2rygc	-0.09	-0.41*	0.39*	-0.16	0.39*	-0.44**	0.46**	-0.13
TDCA	2rytc	0.27	-0.27	0.29	-0.05	-0.26	0.29	-0.18	-0.04
TLCA	2rytc	-0.15	-0.15	0.07	-0.23	0.09	-0.09	0.07	0.05
UDCA	2ryU	0.00	0.28	-0.12	0.03	-0.08	0.08	-0.04	0.03
GUDCA	2rygc	-0.13	-0.09	0.28	-0.11	0.38*	-0.41*	0.50**	-0.09
TUDCA	2rytg	0.15	-0.20	0.01	0.02	0.37*	-0.23	0.38*	0.01

Spearman Rank Correlation Coefficients (r) Among Variables Across NHC, MASLD, and NASH Groups

NHC Normal healthy control, *MASLD* Metabolic dysfunction–associated steatotic liver disease, *NASH* Nonalcoholic steatohepatitis, *CA* Cholic acid, *CDCA* Chenodeoxycholic acid, *DCA* Deoxycholic acid, *LCA* Lithocholic acid, *UDCA* Ursodeoxycholic acid, *GCA* Glycholic acid, *GCDCA* Glycochenodeoxycholic acid, *GDCA* Glycodeoxycholic acid, *GUDCA* Glycoursodeoxycholic acid, *TCA* Taurocholic acid, *TCDCA* Taurochenodeoxycholic acid, *TDCA* Taurodeoxycholic acid, *TLCA* Taurolithocholic acid, *TUDCA* Tauroursodeoxycholic acid. *1ryU* primary unconjugated, *1rygc* primary glycoconjugated, *1rytc* primary tauroconjugated, *2ryU* secondary unconjugated, *2rygc* secondary glycoconjugated, *2rytc* secondary tauroconjugated

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

Table 6 Comparison of bile acid levels in NAFLD patients categorized by Fibrosis, Inflammation, and Steatosis Grades

		Fibrosis			Inflammation			Steatosis		
Bile Acid	**Class**	**F0-F1**	**F2-F3**	***P*-value**	**A0-A1**	**A2-A3**	***P*-value**	**G0-G1**	**G2-G3**	***P*-value**
CA	1ryU	0.12 (1.05)	0.41 (1.40)	0.024	0.23 (1.55)	0.41 (1.40)	0.264	0.21 (0.54)	0.41 (0.42)	0.003
CDCA	1ryU	0.06 (3.30)	0.83 (0.29)	0.011	0.05 (3.30)	0.83 (3.29)	0.154	0.05 (3.14)	0.81 (3.32)	0.332
GCA	1rygc	1.57 (3.63)	0.40 (1.35)	0.041	1.77 (4.83)	0.40 (1.76)	0.048	1.27 (1.14)	2.76 (1.06)	0.032
GCDCA	1rygc	4.22 (9.14)	3.73 (1.97)	0.184	4.22 (9.14)	3.73 (1.97)	0.184	3.90 (3.20)	4.13 (3.20)	0.794
TCA	1rytc	0.01 (0.01)	0.00 (0.03)	0.803	0.01 (0.01)	0.04 (0.03)	0.033	0.02 (0.03)	0.04 (0.03)	0.025
TCDCA	1rytc	2.39 (4.11)	3.51 (4.32)	0.247	4.39 (4.30)	3.21 (4.32)	0.247	2.05 (4.35)	4.23 (4.38)	0.034
DCA	2ryU	0.21 (0.31)	0.24 (0.26)	0.890	0.28 (0.51)	0.34 (0.23)	0.890	0.40 (0.57)	0.28 (0.15)	0.047
LCA	2ryU	0.02 (0.29)	0.02 (0.23)	0.609	0.03 (0.19)	0.01 (0.20)	0.609	0.00 (0.19)	0.02 (0.20)	0.840
GDCA	2rygc	0.11 (0.04)	0.11 (0.05)	0.765	0.11 (0.06)	0.11 (0.04)	0.765	0.11 (0.15)	0.10 (0.15)	0.990
TDCA	2rytc	0.01 (0.00)	0.01 (0.00)	0.698	0.00 (0.00)	0.00 (0.00)	0.698	0.00 (0.08)	0.00 (0.00)	0.386
TLCA	2rytc	0.00 (0.42)	0.01 (0.46)	0.286	0.00 (0.02)	0.02 (0.46)	0.026	0.00 (0.01)	0.01 (0.60)	0.049
UDCA	2ryU	0.00 (0.04)	0.00 (0.06)	0.263	0.00 (0.01)	0.00 (0.06)	0.363	0.00 (0.06)	0.00 (0.02)	0.974
GUDCA	2rygc	1.28 (0.39)	1.34 (0.52)	0.673	1.20 (0.50)	1.30 (0.40)	0.673	1.20 (1.74)	0.34 (1.70)	0.950
TUDCA	2rytg	0.01 (0.37)	0.01 (0.14)	0.842	0.01 (0.26)	0.01 (0.04)	0.842	0.01 (0.13)	0.01 (0.24)	0.568

Comparison of bile acid levels in NAFLD patients categorized by fibrosis (F0-F1 vs. F2-F3), inflammation (A0-A1 vs. A2-A3), and steatosis (G0-G1 vs. G2-G3) grades. Mann–Whitney U test comparison between two groups. indicates significant, data are presented as median (IQR), with P-value < 0.05 indicates the statistical significance of differences between groups

CA Cholic acid, *CDCA* Chenodeoxycholic acid, *DCA* Deoxycholic acid, *LCA* Lithocholic acid, *UDCA* Ursodeoxycholic acid, *GCA* Glycholic acid, *GCDCA* Glycochenodeoxycholic acid, *GDCA* Glycodeoxycholic acid, *GUDCA* Glycoursodeoxycholic acid, *TCA* Taurocholic acid, *TCDCA* Taurochenodeoxycholic acid, *TDCA* Taurodeoxycholic acid, *TLCA* Taurolithocholic acid, *TUDCA* Tauroursodeoxycholic acid. *1ryU* primary unconjugated, *1rygc* primary glycoconjugated, *1rytc* primary tauroconjugated, *2ryU* secondary unconjugated, *2rygc* secondary glycoconjugated, *2rytc* secondary tauroconjugated

Table 7 Binary Logistic Regression Analysis for Predicting Fibrosis, Lobular Inflammation, and Steatosis in NAFLD

Bile Acid	Class	Fibrosis F0-1 vs F2-3				Active inflammation A0-1 vs A2-3				Steatosis G0-1 vs G2-3			
		B	Wald	Sig	Exp(B)	B	Wald	Sig	Exp(B)	B	Wald	Sig	Exp(B)
CA	1ryU	0.72	1.29	0.02	2.05	0.09	0.02	0.90	1.10	0.96	0.50	0.048	2.62
CDCA	1ryU	-0.55	0.93	0.04	1.58	-0.41	0.48	0.49	0.66	0.23	0.09	0.017	1.25
GCA	1rygc	-0.08	0.45	0.03	1.92	0.04	0.02	0.04	2.04	-0.09	0.47	0.041	2.92
GCDCA	1rygc	-0.20	1.67	0.20	0.82	-0.19	0.89	0.35	0.82	0.14	0.45	0.50	1.15
TCA	1rytc	0.18	1.11	0.29	1.20	-0.06	0.13	0.02	1.94	0.12	0.54	0.46	1.12
TCDCA	1rytc	0.12	0.54	0.47	1.12	0.16	0.98	0.32	1.17	-0.12	0.55	0.46	0.89
DCA	2ryU	0.72	0.31	0.04	2.06	0.37	0.16	0.69	1.45	-1.42	0.71	0.042	3.24
LCA	2ryU	-3.63	0.51	0.48	0.03	-0.67	0.40	0.53	0.51	-4.42	0.34	0.56	0.01
GDCA	2rygc	-1.66	0.63	0.43	0.19	-0.08	0.00	0.95	0.93	-4.11	3.81	0.05	0.02
TDCA	2rytc	-28.9	2.54	0.11	0.00	-1.03	1.24	0.27	0.36	1.47	1.21	0.04	4.35
TLCA	2rytc	2.91	2.70	0.10	18.39	2.77	1.76	0.03	15.95	14.45	2.45	0.03	20
UDCA	2ryU	19.17	0.93	0.34	20	16.48	1.12	0.03	20	17.13	1.19	0.02	20
GUDCA	2rygc	0.31	0.54	0.47	1.37	-0.02	0.00	0.96	0.98	-0.60	1.22	0.27	0.55
TUDCA	2rytg	0.59	1.16	0.28	1.80	0.16	0.14	0.71	1.18	0.85	0.96	0.33	2.34

The binary logistic regression analysis results are presented for three comparisons: fibrosis (F0-1 vs. F2-3), active inflammation (A0-1 vs. A2-3), and steatosis (G0-1 vs. G2-3) in NAFLD patients, using various bile acids as predictors

CA Cholic acid, *CDCA* Chenodeoxycholic acid, *DCA* Deoxycholic acid, *LCA* Lithocholic acid, *UDCA* Ursodeoxycholic acid, *GCA* Glycholic acid, *GCDCA* Glycochenodeoxycholic acid, *GDCA* Glycodeoxycholic acid, *GUDCA* Glycoursodeoxycholic acid, *TCA* Taurocholic acid, *TCDCA* Taurochenodeoxycholic acid, *TDCA* Taurodeoxycholic acid, *TLCA* Taurolithocholic acid, *TUDCA* Tauroursodeoxycholic acid. *1ryU* primary unconjugated, *1rygc* primary glycoconjugated, *1rytc* primary tauroconjugated, *2ryU* secondary unconjugated, *2rygc* secondary glycoconjugated, *2rytc* secondary tauroconjugated

In terms of active inflammation, GCA was a significant predictor (Exp(B) = 1.92, $p = 0.03$), along with TCA (Exp(B) = 1.94, $p = 0.02$, and TLCA (Exp(B) = 15.95, $p = 0.03$). CDCA did not show a significant association with inflammation (Exp(B) = 0.49, $p = 0.66$).

In the context of steatosis, 1ry bile acids CA, CDCA, and GCA, were significant predictors (CA: Exp(B) = 2.62, $p = 0.048$; CDCA: Exp(B) = 1.25, $p = 0.017$; GCA: Exp(B) = 2.92, $p = 0.041$. Additionally,2ry Bile acid, DCA, TDCA, TLCA, UDCA showed a significant association and predictor of steatosis DCA: Exp(B) = 3.24, $p = 0.042$; TDCA: Exp(B) = 4.35, $p = 0.04$; TLCA: Exp(B) = 20, $p = 0.03$); and UDCA (Exp(B) = 20, $p = 0.02$).

These results highlight the role of specific bile acids, such as CA, CDCA and GCA, in predicting NAFLD severity, with implications for both fibrosis and steatosis. The significant associations of DCA, TDCA, and TLCA with steatosis suggest their potential importance in hepatic fat accumulation. Additionally, the significant correlations of GCA, TCA, and TLCA with inflammation suggest their potential utility in predicting liver inflammation activity.

Discussion

This study provides a comprehensive analysis of bile acid profiles across the spectrum of NAFLD, from simple MASLD to the more advanced hepatic inflammation in nonalcoholic steatohepatitis (NASH) and fibrosis. The findings underscore the critical role of bile acids as potential biomarkers in the progression of NAFLD, offering insights into the metabolic disturbances underlying the disease. Significant differences in clinical parameters, such as BMI, gender, and the prevalence of dyslipidemia and diabetes mellitus, were observed between the NASH and MASLD groups compared to the NHC group. These differences align with existing literature that links metabolic disturbances, including obesity, dyslipidemia, and diabetes mellitus, to the severity of NAFLD, further emphasizing the role of these factors in NAFLD progression [4, 6].

The main finding of the current study is that bile acid profiles are significantly altered in NAFLD compared to healthy controls, with specific bile acids potentially serving as biomarkers for distinguishing between healthy individuals and NAFLD patients. Additionally, these bile acids may have predictive value for the progression of the disease, particularly in relation to fibrosis, inflammation, and steatosis.

Both primary unconjugated and conjugated bile acids were significantly elevated in MASLD and NASH compared to healthy controls, with a particular increase in CA, CDCA, GCA, and TCA levels. The minimal differences between MASLD and NASH in many bile acids suggest that these metabolic alterations may occur early

in the disease process and persist as NAFLD progresses. These results are in line with previous research indicating that bile acid metabolism is disrupted in NAFLD, likely due to impaired hepatic bile acid synthesis and secretion, as well as altered gut microbiota [25–29].

Secondary bile acids also showed significant changes across NAFLD groups. Elevated levels of secondary bile acids such as LCA, TLCA, GUDCA, and TUDCA were observed in both NASH and MASLD compared to NHC, reflecting the disruption in bile acid metabolism. However, no significant differences were found between MASLD and NASH, indicating that secondary bile acids might not provide robust differentiation between these subtypes. These findings align with Cassey et al., who reported higher total primary bile acids and lower secondary bile acids in NAFLD patients compared to controls [30]. Gillard et al. also noted variability in bile acid levels across NAFLD studies, with some reports showing elevated levels and others unchanged [30, 31]. Chen et al. (2020) emphasized the role of gut microbiota in altering bile acid profiles, which is consistent with the observed increase in primary bile acids and decrease in secondary bile acids in NASH [27]. The superior diagnostic accuracy of primary bile acids compared to secondary bile acids likely stems from their central role in bile acid synthesis and regulation within the liver. Primary bile acids, such as CA and CDCA, are directly synthesized in the liver and are more closely tied to hepatic function and metabolic processes that are disrupted early in NAFLD [2]. These disruptions manifest as elevated levels of primary bile acids, making them more reliable biomarkers for distinguishing between healthy and diseased states. Secondary bile acids, formed through the action of gut microbiota, may reflect later or more complex alterations in bile acid metabolism that do not differentiate as clearly between NAFLD subtypes. Thus, primary bile acids, being more indicative of hepatic dysfunction, exhibit better diagnostic accuracy in identifying NAFLD [2, 32].

Diagnostic assessments of the discriminative power of bile acids through ROC curve analysis and PCA further highlight the potential of certain bile acids, especially primary bile acid CA, CDCA, GCDCA, and TCDCA and secondary bile acids LCA and TLCA in distinguishing NAFLD patients from healthy individuals with moderate sensitivity and high specificity, however, the limited ability of these bile acids to differentiate between MASLD and NASH, for both primary and secondary bile acids, suggests that while bile acids are useful in identifying the presence of NAFLD, they may not be as effective in distinguishing its subtypes. PCA also identified significant contributors, including GCDCA, CA, and CDCA, indicating their relevance in distinguishing NAFLD from healthy states. These results mirror findings from other studies that emphasize the metabolic alterations in bile acids during NAFLD progression [33]. Notably, both ROC analysis and PCA demonstrated limited effectiveness in differentiating between MASLD and NASH, suggesting these bile acids alone are insufficient for precise NAFLD subtyping. This limitation is consistent with broader challenges in the field, where a need for more specific biomarkers has been frequently noted [34, 35].

The study demonstrated significant bile acid dysregulation in diabetic and pre-diabetic patients, particularly elevated levels of CA, CDCA, GCA, and TCDCA, with the highest levels observed in diabetic patients. These elevations suggest a progressive impairment in bile acid metabolism linked to worsening glucose regulation. Additionally, hyperlipidemic patients exhibited higher levels of CA, LCA, GCA, and GUDCA, highlighting the interplay between lipid metabolism and bile acid profiles in NAFLD. The presence of elevated bile acids in pre-diabetic individuals indicates early metabolic changes, underscoring their potential as early biomarkers for disease progression. These findings align with previous research, which has reported similar increases in unconjugated bile acids, particularly CA, in diabetic and pre-diabetic patients [36], and suggest a compensatory increase in conjugated bile acids as an adaptive response [28, 29, 37–40].

The study also observed gender differences, with female patients exhibiting higher levels of TDCA and TUDCA. These findings align with the findings of Puri et al., who suggested that sex hormones significantly influence bile acid metabolism by impacting the enzymes involved in bile acid synthesis and clearance. These results underscore the complexity of bile acid metabolism and highlight the importance of considering factors such as diabetes status, lipid profile, and gender when interpreting bile acid levels in clinical practice [41].

Exploring the relationship between bile acid levels and the severity of steatosis, inflammation, and fibrosis in NAFLD patients revealed that high levels of CA, GCA, TCA, TCDCA, DCA and TLCA, were found to be significantly associated with of steatosis. Higher levels of GCA, TCA, and TLCA were associated with liver inflammation, however, CA, CDCA, and GCA were significantly elevated in patients with advanced fibrosis. The logistic regression analysis also identified several bile acids as significant predictors of either steatosis, inflammation or fibrosis. Elevated CA, CDCA, GCA, TDCA, DCA TLCA, and UDCA were found to be significant predictors of steatosis. GCA, TCA and TLCA were identified as significant predictors of inflammation. Similarly, CA, CDCA, GCA and DCA were identified as significant predictors of fibrosis, emphasizing their potential utility in monitoring disease progression and severity.

Previous research partially or fully supports such associations [41–43]. For instance, Aranha et al. (2008) observed that elevated levels of CA, CDCA, and DCA in liver tissue correlated with steatosis and fibrosis in NASH patients [44]. Similarly, Puri et al. (2018) reported that increased serum levels of these bile acids were associated with steatosis and fibrosis in NAFLD patients, suggesting a role for these bile acids in the early detection and progression of the disease [41]. Furthermore, the elevated levels of GCA and DCA observed in this study align with earlier findings, indicating that these bile acids are involved in the pathogenesis of NAFLD and may reflect alterations in bile acid metabolism and liver function [27, 45, 46].

Chen et al. (2020) highlighted the importance of bile acids CA and CDCA in the progression from simple steatosis to more advanced stages of liver disease, emphasizing their role in the inflammatory and fibrotic processes [27]. TLCA, a secondary bile acid, has also been identified in previous studies as a marker of disease severity. Caussy et al. (2019) demonstrated significant changes in TLCA levels corresponding with advanced liver fibrosis, suggesting that TLCA could be a valuable marker for assessing the extent of liver damage [30]. Elevated levels of CA, CDCA, and TLCA in this study are consistent with observations from Khalil et al. (2022), who reported that elevated serum levels of these bile acids are associated with liver fibrosis and cirrhosis [47]. Additionally, Gottlieb and Canbay (2019) noted that increased conjugated bile acids such as TLCA are indicative of heightened inflammatory activity, which aligns with our findings that these bile acids are significant predictors of inflammation [45].

In summary, the study's findings that CA, CDCA, GCA, and DCA are predictors of steatosis, and CA, CDCA, TCA, and TLCA are predictors of fibrosis and inflammation, underscore the potential utility of these bile acids in monitoring the progression and severity of NAFLD. These observations are supported by existing literature, which highlights the role of these bile acids in the pathogenesis and progression of liver disease [32, 48, 49]. The identification of these bile acids as significant predictors provides valuable insights into their potential use as biomarkers for early diagnosis, disease monitoring, and therapeutic targeting in NAFLD [50].

Strengths and limitations

The study's strengths include its comprehensive analysis of bile acid profiles across the spectrum of NAFLD, from simple metabolic-associated steatotic liver disease (MASLD) to advanced nonalcoholic steatohepatitis (NASH) and fibrosis. By identifying significant alterations in primary and secondary bile acids, the study highlights their potential as biomarkers for distinguishing NAFLD from healthy controls and predicting disease progression [32, 50]. Additionally, the exploration of clinical parameters such as BMI, gender, and comorbidities like diabetes and dyslipidemia enriches the findings, providing insights into the relationship between bile acid metabolism and NAFLD severity. However, this study has several limitations. The cross-sectional design limits the ability to establish causality between bile acid alterations and NAFLD progression. Additionally, while the sample size is sufficient for detecting significant differences, it may limit the generalizability of the findings. The study also did not account for dietary intake or genetic factors, which could influence bile acid metabolism [33, 51–53].

In conclusion, this study highlights the significant alterations in bile acid profiles in NAFLD and their potential utility as biomarkers for disease presence and progression. The associations identified between specific bile acids such as (CA, GCA, TCA, TCDCA, DCA and TLCA), and key pathological features like steatosis, fibrosis, and inflammation, underscore their relevance as indicators of disease severity. The predictive value of CA, CDCA, and TLCA for fibrosis and inflammation further emphasizes their potential role in monitoring NAFLD progression. These findings are consistent with existing literature, which underscores the involvement of these bile acids in the pathogenesis and advancement of liver disease [42, 51]. The identification of these bile acids as significant predictors offers valuable insights into their potential application in early diagnosis, disease monitoring, and therapeutic targeting in NAFLD. Future research should focus on longitudinal studies to track changes in bile acid profiles over time and assess their relationship with NAFLD progression, as well as evaluate the impact of therapeutic interventions on these biomarkers [54].

Abbreviations

NHC	Normal healthy control
MASLD	Metabolic dysfunction–associated steatotic liver disease
NASH	Nonalcoholic steatohepatitis
NAFLD	Nonalcoholic fatty liver disease
CA	Cholic acid
CDCA	Chenodeoxycholic acid
GCA	Glycholic acid
GCDCA	Glycochenodeoxycholic acid
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
DCA	Deoxycholic acid
LCA	Lithocholic acid
GDCA	Glycodeoxycholic acid
TDCA	Taurodeoxycholic acid
TLCA	Taurolithocholic acid
UDCA	Ursodeoxycholic acid
GUDCA	Glycoursodeoxycholic acid
TUDCA	Tauroursodeoxycholic acid
1ryU	Primary unconjugated
1rygc	Primary glycoconjugated
1rytc	Primary tauroconjugated
2ryU	Secondary unconjugated

2rygc	Secondary glycoconjugated
2rytc	Secondary tauroconjugated
AST	Aspartate transaminase
ALT	Alanine transaminase
GGT	Gamma-glutamyl transferase
ALP	Alkaline phosphatase
TBil	Total bilirubin
DBIL	Direct bilirubin
TP	Total protein
Alb	Albumin
UA	Uric acid
Chol	Cholesterol
LDL	Low density lipoprotein
HDL	High density lipoprotein
TG	Triglyceride
Hb	Hemoglobin
WBCs	White blood cells
RBCs	Red blood cells

Acknowledgements
The authors would like to express their gratitude to Dr. Samah Elsheaty, Dr. Ghada Salah, Dr. Azza Elsheashaey, and Ms. Maryam Nagah for their dedicated efforts in patient care during sample collection and their outstanding work with HPLC at the National Liver Institute, Menoufia University.

Authors' contributions
AAB: Conducted the UPLC/MS/MS analysis method. Contributed to the study concept and design, manuscript preparation. MFE: Patient recruitment and evaluation, collection of clinical data. DA: Conducted the pathological examination of the liver biopsy, staged and confirmed the diagnosis, and contributed to the concept and protocol development. AK: Corresponding author, study concept and design, analyzed the data, generated the result, wrote and edited the manuscript. All authors reviewed and approved the final version of the manuscript.

Funding
The authors declare they do not have any financial disclosure.

Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The research ethics committees of the National Liver Institute (IRB005700 -2024), Menoufia University, approved the research proposal and the protocols to comply with national research guidelines. Patients provided informed written consent for the use of tissue for research purposes.

Consent for publication
Obtained from all participants.

Competing interests
The authors declare no competing interests.

Received: 11 August 2024 Accepted: 30 September 2024
Published online: 09 October 2024

References

1. Diehl AM, Day C (2017) Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis. N Engl J Med 377(21):2063–2072
2. Boldys A, Buldak L (2024) Metabolic dysfunction-associated steatotic liver disease: Navigating terminological evolution, diagnostic frontiers and therapeutic horizon-an editorial exploration. World J Gastroenterol 30(18):2387–2390
3. Paik JM, Golabi P, Younossi Y, Mishra A, Younossi ZM (2020) Changes in the Global Burden of Chronic Liver Diseases From 2012 to 2017: The Growing Impact of NAFLD. Hepatology 72(5):1605–1616
4. Younossi Z, Tacke F, Arrese M, Chander Sharma B, Mostafa I, Bugianesi E, Wai-Sun Wong V, Yilmaz Y, George J, Fan J, Vos MB (2019) Global Perspectives on Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. Hepatology 69(6):2672–2682
5. Dulai PS, Singh S, Patel J, Soni M, Prokop LJ, Younossi Z, Loomba R (2017) Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: Systematic review and meta-analysis. Hepatology 65(5):1557–1565
6. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, Sanyal AJ (2018) The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology 67(1):328–357
7. Estes C, Razavi H, Loomba R, Younossi Z, Sanyal AJ (2018) Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. Hepatology 67(1):123–133
8. Bessone F, Razoriand MV, Roma MG (2019) Molecular pathways of nonalcoholic fatty liver disease development and progression. Cell Mol Life Sci 76(1):99–128
9. Takahashi Y, Dungubat E, Kusano H, Fukusato T (2023) Pathology and pathogenesis of metabolic dysfunction-associated Steatotic Liver Disease-Associated Hepatic Tumors. Biomedicines 11(10):2761
10 Juanola O, Martinez-Lopez S, Frances R, Gomez-Hurtado I (2021) Non-Alcoholic Fatty Liver Disease: Metabolic, Genetic, Epigenetic and Environmental Risk Factors. Int J Environ Res Public Health 18(10):5227
11 Eslam M, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-Gomez M, George J (2020) A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. J Hepatol 73(1):202–209
12. Xue H, Fang S, Zheng M, Wu J, Li H, Zhang M, Ma Y (2021) Da-Huang-Xiao-Shi decoction protects against3, 5-diethoxycarbonyl-1,4-dihydroxychollidine-induced chronic cholestasis by upregulating bile acid metabolic enzymes and efflux transporters. J Ethnopharmacol 269:113706
13 Dawson PA, Lanand T, Rao A (2009) Bile acid transporters. J Lipid Res 50(12):2340–57
14 Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fujino M (2003) A G protein-coupled receptor responsive to bile acids. J Biol Chem 278(11):9435–40
15 Li T, Chiang JYL (2020) Bile acid-based therapies for non-alcoholic steatohepatitis and alcoholic liver disease. Hepatobiliary Surg Nutr 9(2):152–169
16. Wegermann K, Howe C, Henao R, Wang Y, Guy CD, Abdelmalek MF, Moylan CA (2021) Serum Bile Acid, Vitamin E, and Serotonin Metabolites Are Associated With Future Liver-Related Events in Nonalcoholic Fatty Liver Disease. Hepatol Commun 5(4):608–617
17 Pearce SG, Thosaniand NC, Pan JJ (2013) Noninvasive biomarkers for the diagnosis of steatohepatitis and advanced fibrosis in NAFLD. Biomark Res 1(1):7
18. Ajmera V, Loomba R (2021) Imaging biomarkers of NAFLD, NASH, and fibrosis. Mol Metab 50:101167
19. Andersson A, Kelly M, Imajo K, Nakajima A, Fallowfield JA, Hirschfield G, Harrison S (2022) Clinical utility of magnetic resonance imaging biomarkers for identifying nonalcoholic Steatohepatitis patients at high risk of progression: a multicenter pooled data and meta-analysis. Clin Gastroenterol Hepatol 20(11):2451-2461 e3
20. Chow MD, Leeand YH, Guo GL (2017) The role of bile acids in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Mol Aspects Med 56:34–44
21 Park WH, Pak YK (2011) Insulin-dependent suppression of cholesterol 7alpha-hydroxylase is a possible link between glucose and cholesterol metabolisms. Exp Mol Med 43(10):571–9
22 Fuchs C, Claudeland T, Trauner M (2013) Bile acid-mediated control of liver triglycerides. Semin Liver Dis 33(4):330–42
23 Trauner M, Claudel T, Fickert P, Moustafa T, Wagner M (2010) Bile acids as regulators of hepatic lipid and glucose metabolism. Dig Dis 28(1):220–4
24. Brunt EM, Kleiner DE, Wilson LA, Belt P, BA Neuschwander-Tetriand NCR Network (2011) Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. Hepatology 53(3):810–20

25. Zhou Y, Liu Z, Lynch EC, He L, Cheng H, Liu L, Xie L (2021) Osr1 regulates hepatic inflammation and cell survival in the progression of non-alcoholic fatty liver disease. Lab Invest 101(4):477–489
26 Ferslew BC, Xie G, Johnston CK, Su M, Stewart PW, Jia W (2015) ASt Barritt, Altered Bile Acid Metabolome in Patients with Nonalcoholic Steatohepatitis. Dig Dis Sci 60(11):3318–28
27. Chen W, Wei Y, Xiong A, Li Y, Guan H, Wang Q, Ma X (2020) Comprehensive Analysis of Serum and Fecal Bile Acid Profiles and Interaction with Gut Microbiota in Primary Biliary Cholangitis. Clin Rev Allergy Immunol 58(1):25–38
28. Cai J, Rimal B, Jiang C, Chiangand JYL, Patterson AD (2022) Bile acid metabolism and signaling, the microbiota, and metabolic disease. Pharmacol Ther 237:108238
29 Jia W, Xieand G, Jia W (2018) Bile acid-microbiota crosstalk in gastrointestinal inflammation and carcinogenesis. Nat Rev Gastroenterol Hepatol 15(2):111–128
30. Caussy C, Hsu C, Singh S, Bassirian S, Kolar J, Faulkner C, Loomba R (2019) Serum bile acid patterns are associated with the presence of NAFLD in twins, and dose-dependent changes with increase in fibrosis stage in patients with biopsy-proven NAFLD. Aliment Pharmacol Ther 49(2):183–193
31. Gillard J, Leclercq IA (2023) Biological tuners to reshape the bile acid pool for therapeutic purposes in non-alcoholic fatty liver disease. Clin Sci (Lond) 137(1):65–85
32 Lai J, Luo L, Zhou T, Feng X, Ye J, Zhong B (2023) Alterations in circulating bile acids in metabolic dysfunction-associated steatotic liver disease: a systematic review and meta-analysis. Biomolecules 13(9):1356
33 Powell EE, Wong VW, Rinella M (2021) Non-alcoholic fatty liver disease. Lancet 397(10290):2212–2224
34. Kim HY (2021) Recent advances in nonalcoholic fatty liver disease metabolomics. Clin Mol Hepatol 27(4):553–559
35 Paroutoglou K, Papadavid E, Christodoulatosa GS, Dalamaga M (2020) Deciphering the association between psoriasis and obesity: current evidence and treatment considerations. Curr Obes Rep 9(3):165–178
36. Yang L, Wang F, Liu S, Xian Z, Yang S, Xu Y, Li Y (2024) Unique metabolomics characteristics for distinguishing cirrhosis related to different liver diseases: A systematic review and meta-analysis. Diabetes Metab Syndr 18(6):103068
37. Haeusler RA, Astiarraga B, Camastra S, Accili D, Ferrannini E (2013) Human insulin resistance is associated with increased plasma levels of 12alpha-hydroxylated bile acids. Diabetes 62(12):4184–91
38 Puri P, Wiest MM, Cheung O, Mirshahi F, Sargeant C, Min HK, Sanyal AJ (2009) The plasma lipidomic signature of nonalcoholic steatohepatitis. Hepatology 50(6):1827–38
39. Wang M, Wang C, Hanand RH, Han X (2016) Novel advances in shotgun lipidomics for biology and medicine. Prog Lipid Res 61:83–108
40. Al-Jebawi AF, YoussefAgha AH, Al Suwaidi HS, Albadwawi MS, Al Marzooqi AS, Banihammad AH, Alkaabi MK (2017) Attenuated PTH responsiveness to vitamin D deficiency among patients with type 2 diabetes and chronic hyperglycemia. Diabetes Res Clin Pract 128:119–126
41. Puri P, Daita K, Joyce A, Mirshahi F, Santhekadur PK, Cazanave S, Sanyal AJ (2018) The presence and severity of nonalcoholic steatohepatitis is associated with specific changes in circulating bile acids. Hepatology 67(2):534–548
42 Bertolini A, Fiorottoand R, Strazzabosco M (2022) Bile acids and their receptors: modulators and therapeutic targets in liver inflammation. Semin Immunopathol 44(4):547–564
43 Evangelakos I, Heeren J, Verkade E, Kuipers F (2021) Role of bile acids in inflammatory liver diseases. Semin Immunopathol 43(4):577–590
44. Aranha MM, Cortez-Pinto H, Costa A, da Silva IB, Camilo ME, de Mouraand MC, Rodrigues CM (2008) Bile acid levels are increased in the liver of patients with steatohepatitis. Eur J Gastroenterol Hepatol 20(6):519–25
45 Gottlieb A, Canbay A (2019) Why Bile Acids Are So Important in Non-Alcoholic Fatty Liver Disease (NAFLD) Progression. Cells 8(11):1358
46. Xue R, Su L, Lai S, Wang Y, Zhao D, Fan J, Zhou H (2021) Bile acid receptors and the gut-liver axis in nonalcoholic fatty liver disease. Cells 10(11):2806
47. Khalil A, ElSheashaey A, Abdelsameea E, Obada M, Bayomy FFM, El-Said H (2022) Value of bile acids in diagnosing hepatitis C virus-induced liver cirrhosis and hepatocellular carcinoma. Br J Biomed Sci 79:10191
48. Zhang G, Chen H, Ren W, Huang J (2023) Efficacy of bile acid profiles in diagnosing and staging of alcoholic liver disease. Scand J Clin Lab Invest 83(1):8–17
49. Liu AN, Xu CF, Liu YR, Sun DQ, Jiang L, Tang LJ, Ni Y (2023) Secondary bile acids improve risk prediction for non-invasive identification of mild liver fibrosis in nonalcoholic fatty liver disease. Aliment Pharmacol Ther 57(8):872–885
50 Portincasa P, Khalil M, Mahdi L, Perniola V, Idone V, Graziani A, Di Ciaula A (2024) Metabolic dysfunction-associated steatotic liver disease: from pathogenesis to current therapeutic options. Int J Mol Sci 25(11):5640
51. Dong S, Zhan ZY, Cao HY, Wu C, Bian YQ, Li JY, Sun MY (2017) Urinary metabolomics analysis identifies key biomarkers of different stages of nonalcoholic fatty liver disease. World J Gastroenterol 23(15):2771–2784
52. Appleby RN, Moghul I, Khan S, Yee M, Manousou P, Neal TD, Walters JRF (2019) Non-alcoholic fatty liver disease is associated with dysregulated bile acid synthesis and diarrhea: A prospective observational study. PLoS One 14(1):e0211348
53. Deng Y, Pan M, Nie H, Zheng C, Tang K, Zhang Y, Yang Q (2019) Lipidomic Analysis of the Protective Effects of Shenling Baizhu San on Non-Alcoholic Fatty Liver Disease in Rats. Molecules 24(21):3943
54 Thibaut MM, Bindels LB (2022) Crosstalk between bile acid-activated receptors and microbiome in entero-hepatic inflammation. Trends Mol Med 28(3):223–236

Publisher's Note